ACTIONS OF PHARMACOLOGICALLY-DISTINCT FORMS OF PROTEIN KINASE C IN RAT ANTERIOR PITUITARY CELLS

by

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This Thesis is dedicated to my parents, who through their love and encouragement, made everything possible.

Thanks.

I declare that the studies presented in this Thesis are the result of my own independent investigation with the exception of the PKC activity assays using DOG as an activator, which were carried out with the assistance of Melanie S Johnson.

The work has not been and is not being currently submitted for candidature for any other degree.

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ABBREVIATIONS

5-iodo-C8	=	N-(8-aminooctyl)-5-iodonaphthalene-1-sulphonamide
8-Br-cyclic GMP	=	8-bromoguanosine-3':5'-cyclic monophosphate
AA	=	arachidonic acid
ACTH	=	adrenocorticotrophic hormone
ADMB	=	3-(N-acetylamino)-5-(N-decyl-N-methylamino)benzylalcohol
AMG-C16	=	1-0-hexadecyl-2-0-methyl-*rac*-glycerol
BAYK8644	=	methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate
cDNA	=	complementary deoxyribonucleic acid
CDP	=	cytidine diphosphate
CT	=	cytidine triphosphate:phosphocholine cytidyltransferase
CTP	=	cytidine triphosphate
DHI	=	6-(N-decylamino)-4-hydroxymethylindole
DNA	=	deoxyribonucleic acid
DOG	=	1,2-dioctanoyl-*sn*-glycerol
DOPPA	=	12-deoxyphorbol 13-phenylacetate 20-acetate
DPB	=	12-deoxyphorbol 13-isobutyrate
DTPA	=	diethylenetriamine pentaacetic acid
EDTA	=	ethylenediaminetetraacetic acid
EGTA	=	ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
ETYA	=	eicosa-5,8,11,14-tetraynoic acid
FSH	=	follicle-stimulating hormone
G protein	=	guanine nucleotide-binding protein
GDP	=	guanosine diphosphate
GH	=	growth hormone
GTP	=	guanosine triphosphate
H7	=	1-(5-isoquinolinesulfonyl)-2-methylpiperazine
HA1004	=	N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIP-70	=	hormone-induced protein -70 kD
$Ins(1,3,4,5)P_4$	=	inositol 1,3,4,5-tetrakisphosphate
$Ins(1,4,5)P_3$	=	inositol 1,4,5-trisphosphate
K252a	=	(8R,9S,11S)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a,-triazadibenzo(a,g)cycloocta(c,d,e)trinden-1-one
LH	=	luteinising hormone
LHRH	=	luteinising hormone-releasing hormone (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2)
MPMA	=	phorbol 12-myristate 13-acetate 4-0-methyl ether
mRNA	=	messenger ribonucleic acid
NDGA	=	nordihydroguiairetic acid
OAG	=	1-oleoyl-2-acetyl-*sn*-glycerol
PC	=	phosphatidylcholine

PDBu	=	phorbol 12,13-dibutyrate
PDD	=	phorbol 12,13-didecanoate
PI-PLC	=	phosphoinisitide-specific phospholipase C
PKC	=	protein kinase C
PLA_1	=	phospholipase A_1
PLA_2	=	phospholipase A_2
PLC	=	phospholipase C
PLD	=	phospholipase D
PMA	=	phorbol 12-myristate 13-acetate
PRA	=	phorbol 12-retinoate 13-acetate
PtdIns	=	phosphatidylinositol
PtdIns(3,4,5)P_3	=	phosphatidylinositol 3,4,5-trisphosphate
PtdIns(4)P	=	phosphatidylinositol 4-phosphate
PtdIns(4,5)P_2	=	phosphatidylinositol 4,5-bisphosphate
R59022	=	6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5*H*-thiazolo[3,2-α]pyrimidin-5-one
RHC80267	=	1,6-di(0-(carbamoyl)cyclohexaneoxime)hexane
SKF525A	=	N,N-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (Proadifen)
TRH	=	thyrotrophin-releasing hormone (pyroGlu-His-Pro-NH_2)
TSH	=	thyroid-stimulating hormone

ABSTRACT

The actions of protein kinase C (PKC) on several aspects of cellular control of Ca^{2+} movement were investigated in rat anterior pituitary cells. Depolarising concentrations of K^+ induced influx of $^{45}Ca^{2+}$ into rat anterior pituitary prisms and into cells of the GH_3 rat anterior pituitary cell line. These responses were used as models to investigate the effects of activators and inhibitors of PKC. Depolarisation-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms and GH_3 cells was inhibited by the 'L'-type Ca^{2+} channel blocker, nimodipine with equal potency in both tissues; suggesting that similar 'L'-type Ca^{2+} channels were being utilised in both preparations. Activators of PKC such as phorbol 12,13-dibutyrate (PDBu) and 4ß-phorbol 12,13-didecanoate (4ß-PDD) enhanced K^+-induced $^{45}Ca^{2+}$ influx in anterior pituitary pieces, but inhibited K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells. The modulation seen with these phorbol esters was stereo-specific and concentration-dependent and of a similar time course in both tissues. The phorboid, mezerein, and some related phorbol esters could mimic PDBu at enhancing K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary pieces, whereas the same compounds did not mimic the action of PDBu in GH_3 cells, but instead enhanced K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells. Furthermore, the PDBu-induced enhancement of K^+-evoked $^{45}Ca^{2+}$ influx into anterior pituitary pieces and the PDBu-induced inhibition of K^+-evoked $^{45}Ca^{2+}$ influx into GH_3 cells was reversed by the PKC inhibitors, staurosporine and H7, but not their less active congeners K252a and HA1004 respectively. However, the PDBu-induced response in anterior pituitary was distinguished by the greatly reduced potency of H7 but not the other antagonists. Examples of two classes of endogenous activators of PKC, the diacylglycerol, 1,2-dioctanoyl-*sn*-glycerol (DOG) and the fatty acid, arachidonic acid (AA) each selectively mimicked PDBu on only one of the two PKC-mediated responses. In anterior pituitary, DOG (but not AA) could mimic PDBu; whereas in GH_3 cells, only AA (but not DOG) could mimic PDBu. The maximal inhibitory effect of AA in GH_3 cells was greater than that which could be achieved by phorbol esters. Evidence was obtained in

support of a dual action of AA: (i) enhancement of PKC (perhaps α-isoform) activity (which also occurs with PDBu) and (ii) the raising of cytosolic Ca^{2+} concentrations (by AA but not PDBu). The data thus indicated that 'L'-type Ca^{2+} channels may be subject to dual and reciprocal modulation *in vivo* by at least two distinct forms of PKC. The working hypothesis proposed was that these phenomena may represent actions of sequence isoforms of PKC. A programme of experiments was therefore carried out to elucidate the biochemical pharmacology of particular PKC isoforms with the aim of assigning an isoform identity to the PKCs regulating Ca^{2+} channels here. In specific [^{3}H]-PDBu binding studies using cytosol preparations from tissues with known content of PKC isoforms, AA, at concentrations > 50 μM, was seen to enhance specific [^{3}H]-PDBu binding only to preparations with very high content of α-isoform of PKC, whereas short, saturated acyl chain diglycerides (particularly DOG) displaced [^{3}H]-PDBu binding with distinctly lower affinity for the α-isoform of PKC, compared to the other PKC isoforms investigated. Furthermore, in kinase enzyme activity studies *in vitro,* preparations from tissue with a mixture of all known isoforms of PKC were activated more potently by DOG than were those highly enriched in the α-isoform of PKC. Therefore, DOG may have reduced affinity for and be poorly-active at α-PKC. This may underly disparities in the effects of phorbol esters and diglycerides observed at certain PKC-mediated responses in whole cells/tissues. Enzyme activity studies on partially-purified PKCs also revealed an H7-insensitive, PKC activity. This was seen in the Ca^{2+}-independent activity of cytosol preparations from male and female rat anterior pituitary, female rat midbrain and rat lung. This H7-resistant PKC may be that which mediates the facilitatory modulation of K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary cells and certain other PKC-mediated events in these cells. The pharmacological properties of this PKC, especially H7-resistance, do not match those described for any of the isoforms so far investigated in detail (α, ß, γ, ε). Furthermore, the tissue-distribution of this H7-resistant PKC does not match the distribution of any other known PKC isoform, and may represent a novel form (perhaps sequence isoform) of PKC or an unknown PKC-like kinase.

Some of the results presented in this Thesis have been published as follows:

Johnson, M.S., MacEwan, D.J., Mitchell, R. & Thomson, F.J. (1989) "Lithium enhances the effect of protein kinase C on LHRH-induced $^{45}Ca^{2+}$ influx into rat anterior pitiutary tissue *in vitro*." J. Physiol. **418**, 185P

Johnson, M.S., MacEwan, D.J. & Mitchell, R (1989) "Depolarisation-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary cells *in vitro* can be modulated in two ways by protein kinase C." J. Physiol. **418**, 186P

MacEwan, D. & Mitchell, R. (1990) "Arachidonic acid acts through protein kinase C to inhibit depolarisation-induced $^{45}Ca^{2+}$ influx" Biochem. Soc. Trans. **18**, 432-433

Mitchell, R., MacEwan, D., Johnson, M., Dougan, L. & Bladon, C. (1990) "Biological activity of some TRH analogues substituted at the 2-position." Biochem. Soc. Trans. **18**, 433-434

MacEwan, D.J. & Mitchell, R. (1990) "Inhibition of depolarisation-induced calcium influx into GH_3 cells by arachidonic acid may involve direct activation of protein kinase C." J. Physiol. **424**, 42P

Johnson, M.S., MacEwan, D.J., Mitchell, R. & Thomson, F.J. (1990) "Effects of calmodulin antagonists on neurohormone-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary tissue *in vitro*." J. Physiol. **424**, 43P

Mitchell, R., Johnson, M.S., MacEwan, D. & Thomson, F. (1990) "Studies on the mechanism of LHRH-induced $^{45}Ca^{2+}$ influx in rat anterior pituitary cells." J. Endocrinol. (suppl) **124**, 183

MacEwan, D.J., Mitchell, R., Johnson, M.S. & Thomson, F.J. (1990) "Selectivity of protein kinase C activators in the regulation of Ca^{2+}-channels in rat anterior pituitary cells." J. Endocrinol. (suppl) **124**, 198

Mitchell, R., MacEwan, D., Johnson, M. & Thomson, F. (1990) "Pharmacology and cellular actions of protein kinase C isoforms." Eur. J. Pharmacol. **183**, 750-751

MacEwan, D., Mitchell, R., Johnson, M. & Thomson, F. (1990) "Pharmacology of protein kinase C isoforms and their cellular actions in anterior pituitary cells." Proceedings of 6th Int. Symp. Cellular Endocrinol. p106

MacEwan, D.J., Mitchell, R., Johnson, M.S. & Thomson, F.J. (1991) "Protein kinase C-dependent and -independent actions of arachidonic acid in GH_3 cells." Biochem. Soc. Trans. **19**, 115S

Mitchell, R., MacEwan, D.J., Dougan, L., Johnson, M.S. & Thomson, F.J. (1991) "Does inositol hexakisphosphate induce Ca^{2+} entry into GH_3 cells?" Biochem. Soc. Trans. **19**, 116S

Thomson, F.J., Mitchell, R., Johnson, F.J. & MacEwan, D.J. (1991) "Differential activation of phospholipase A_2 by protein kinase C in pituitary cells." Biochem. Soc. Trans. **19**, 117S

Johnson, M.S., Mitchell, R., MacEwan D.J. & Thomson, F.J. (1991) "Modulation by phorbol 12,13-dibutyrate of dihydropyridine-sensitive hormone release from rat anterior pituitary tissue *in vitro*." J. Physiol. **434**, 98P

Thomson, F.J., Mitchell, R., Johnson, M.S. & MacEwan, D.J. (1991) "A possible role of phospholipase A_2 in phorbol ester-induced release of hormones from rat anterior pituitary tissue *in vitro*." J. Physiol. **434**, 99P

Evans, A.M., MacEwan, D.J. & Palmer, S. (1991) "The D_1-like dopamine receptor of the cockroach salivary gland is coupled to phospholipase C in addition to adenylyl cyclase." Brit. J. Pharmacol. **102**, 212P

MacEwan, D.J., Mitchell, R., Johnson, M.S., & Thomson, F.J. (1991) "Heterogeneous profiles of protein kinase C activation." Brit. J. Pharmacol. **102**, 258P

Thomson, F.J., Mitchell, R., Johnson, M.S. & MacEwan, D.J. (1991) "Differences in the effect of putative activators of protein kinase C on secretion of pituitary hormones." Brit. J. Pharmacol. **102**, 271P

MacEwan, D.J., Mitchell, R., Thomson, F.J. & Johnson, M.S. (1991) "Inhibition of depolarisation-induced calcium influx into GH_3 cells: the involvement of protein kinase C." Biochim. Biophys. Acta **1043**, 346-354.

MacEwan, D.J. & Mitchell, R. (1991) "Calcium influx through 'L'-type channels into rat anterior pituitary cells can be modulated in two ways by protein kinase C (PKC-isoform selectivity of 1,2-dioctanoyl *sn*-glycerol?)" FEBS Lett. **291**, 79-83.

Mitchell, R., MacEwan, D., Johnson, M. & Thomson, F. (1991) "Selective pharmacology of protein kinase C." Proceedings 8th U.K. Discussion Meeting on Inositides and Cell Signalling.

Thomson, F.J., Johnson, M.S., MacEwan, D.J., Fink, G. & Mitchell, R. (1991) "Cellular actions of pharmacologically distinct forms of protein kinase C." Proceedings 21st Ann. Meeting Soc. Neurosci.

Johnson, M.S., Thomson, F.J., Avery, J.L., MacEwan, D.J. & Mitchell, R. (1991) "Differential actions of protein kinase C modulators on the release of hormones from rat anterior pituitary tissue *in vitro*." J. Physiol. (in press).

MacEwan, D.J., Johnson, M.S., Thomson, F.J. & Mitchell, R. (1991) "Protein kinase C may be required to maintain activity of nimodipine-sensitive Ca^{2+} channels in rat anterior pituitary cells *in vitro*." J. Physiol. (in press).

Thomson, F.J., Mitchell, R., MacEwan, D.J., Harvey, J. & Johnson, M.S. (1992) "Characterisation of [^{3}H]-dimethylstaurosporine binding sites by displacement studies using protein kinase C inhibitors." Brit. J. Pharmacol. (in press).

MacEwan, D.J. & Guild, S. (1992) "Cholecystokinin-octapeptide stimulates ACTH release from AtT-20 tumour cells through a mechanism independent of polyphosphoinositol hydrolysis." Brit. J. Pharmacol. (in press).

MacEwan, D.J., Mitchell, R., Johnson, M.S. & Thomson, F.J. (1992) "Regional differences in the affinity of diacylglycerol analogues for [^{3}H]-phorbol 12,13-dibutyrate binding sites." Brit. J. Pharmacol. (in press).

Mitchell, R., Johnson, M.S., Avery J.L., MacEwan, D.J. & Thomson, F.J. (1992) "The effects of different classes of activators of protein kinase C from rat midbrain." Brit. J. Pharmacol. (in press).

MacEwan, D.J., Simpson, J., Mitchell, R., Johnson, M.S. & Thomson, F.J. (1992) "Properties and [^{32}P] phosphorylation targets of a novel form of protein kinase C in pituitary." Biochem Soc Trans. (in press).

MacEwan, D.J., Mitchell, R., Johnson, M.S., Thomson, F.J. & Lutz, E.M. (1991) "Regional selectivity in the interaction of lipid activators with cytosolic protein kinase C." FEBS Lett. (submitted).

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CHAPTER 1

INTRODUCTION

1.0. RATIONALE FOR ANTERIOR PITUITARY CELL CA^{2+} CHANNELS AS A MODEL FOR PROTEIN KINASE C ISOFORM ACTION

Current investigation within our laboratory has revealed various pharmacologically distinct profiles of rat anterior pituitary function. For example, in studies measuring the secretion of growth hormone and luteinising hormone, and in the priming phenomenon of luteinising hormone releasing hormone, various disparities between the pharmacological profiles of protein kinase C function have been observed using the PKC modulators, H7, 1,2-dioctanoyl-*sn*-glycerol and mezerein.

Voltage-dependent Ca^{2+} channels are present in both rat anterior pituitary and GH_3 cells (see section 1.3). Anterior pituitaries contain a mixed population of cells including mainly (approximately 50%) somatotrophs and some gonadotrophs, which are both known to possess marked 'L'-type Ca^{2+} channel activity (see Mason *et al,* 1988; Thorner *et al,* 1988). Similarly in GH_3 cells, an 'L'-type Ca^{2+} channel activity is present (Marchetti and Brown, 1988). The exact composition of voltage-dependent Ca^{2+} channels in both preparations is at present uncertain, especially since the existence of the newly discovered 'P'- and 'BI'-type Ca^{2+} channels remains to be characterised in both tissues, however the presence of 'T'- a nd possibly 'N'-type Ca^{2+} channels in GH_3 cells has been reported (Marchetti and Brown, 1988; Suzuki and Yoshioka, 1987). The major component of Ca^{2+} channel activity in both preparations still remains of the 'L'-type due to the favourable conductance and poor inactivation of the channel (see Table 1.2, in section 1.3).

Activation of PKC can either lead to an increase or decrease in 'L'-type Ca^{2+} channel activity (see section 1.3) and the two distinct PKC-phosphorylation sites on the 'L'-type Ca^{2+} channel (α_1 and ß subunits) may represent the sites of either the inhibitory or facilitatory influence on the channel caused by PKC. It is not unreasonable to assume that the dual modulation of the 'L'-type Ca^{2+} channel in these preparations may be due to the opposing actions of distinct isoforms of PKC. It is also

hoped that the observation of any similar pharmacological profiles of protein kinase C between 'L'-type Ca^{2+} channel function in anterior pituitary cells and anterior pituitary functional studies (eg, secretion studies carried out in parallel) may represent separate ways of investigating the same forms of PKC.

It is for these reasons, that the effect of PKC modulators on depolarisation-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms and GH_3 cells was investigated, with a hope that the models may reveal differing actions of distinct forms (perhaps sequence isoforms) of PKC. Such models would enable any pharmacological differences to be characterised and to provide supplementary investigations into the neuroendocrine effects of PKC modulators on anterior pituitary which ran concurrently with the investigations here. Ultimately, any pharmacological distinctions between differing forms of PKC could be exploited in an array of tests in an attempt to reveal any PKC isoform-specific sites or functions that may operate in the rat anterior pituitary gland *in vitro*.

The following sections outline those fields, knowledge of which was essential to the investigations carried out within this thesis.

1.1. SIGNALLING THROUGH PHOSPHOINOSITIDE-HYDROLYSIS

In the 1950s it was first recognised that the stimulation of cell surface receptors could lead to an increased metabolic turnover in the inositol phospholipids of cells (Hokin and Hokin, 1953). It was demonstrated in pigeon pancreatic slices that carbachol induced a rapid incorporation of ^{32}P orthophosphate into the phospholipids, phosphatidylinositol (PtdIns) and phosphatidic acid. It later became evident that this incorporation of ^{32}P resulted from the enhanced breakdown and re-synthesis of inositol phospholipids, which also occurs in many different cells to extracellular signals such as certain hormones, neurotransmitters, antigens, growth factors and many other biologically active substances (Michell, 1975; Fisher *et al,* 1984; Downes and Michell, 1985). It was proposed by Durrell *et al* (1969) that the increased phosphoinositide

metabolism may be part of the function of the receptor. Although originally thought to be a consequence of the Ca^{2+} signal that was seen, it was not until 1975 that Michell postulated that this inositol phospholipid breakdown (caused by receptors that raised intracellular Ca^{2+} levels) is what actually causes the gating of Ca^{2+}; while having previously shown that the phospholipids phosphatidylinositol 4-phosphate (PtdIns(4)P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) are produced from PtdIns by sequential phosphorylation of the myo-inositol moiety (Michell and Hawthorne, 1965; Kai *et al,* 1966). In 1983, Streb *et al* demonstrated that the water-soluble molecule inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3), one of the products of PtdIns(4,5)P_2 hydrolysis by phosphoinositide-specific phospholipase C (PI-PLC) (see Figure 1.1), acts as a messenger to mobilize Ca^{2+} from intracellular stores, probably the endoplasmic reticulum. The other product of PtdIns(4,5)P_2 hydrolysis, 1,2-diacylglycerol being a lipid is presumed to remain in membranes, where it causes the activation of protein kinase C (PKC) (Nishizuka, 1983; 1984a; 1984b; 1984c; 1986).

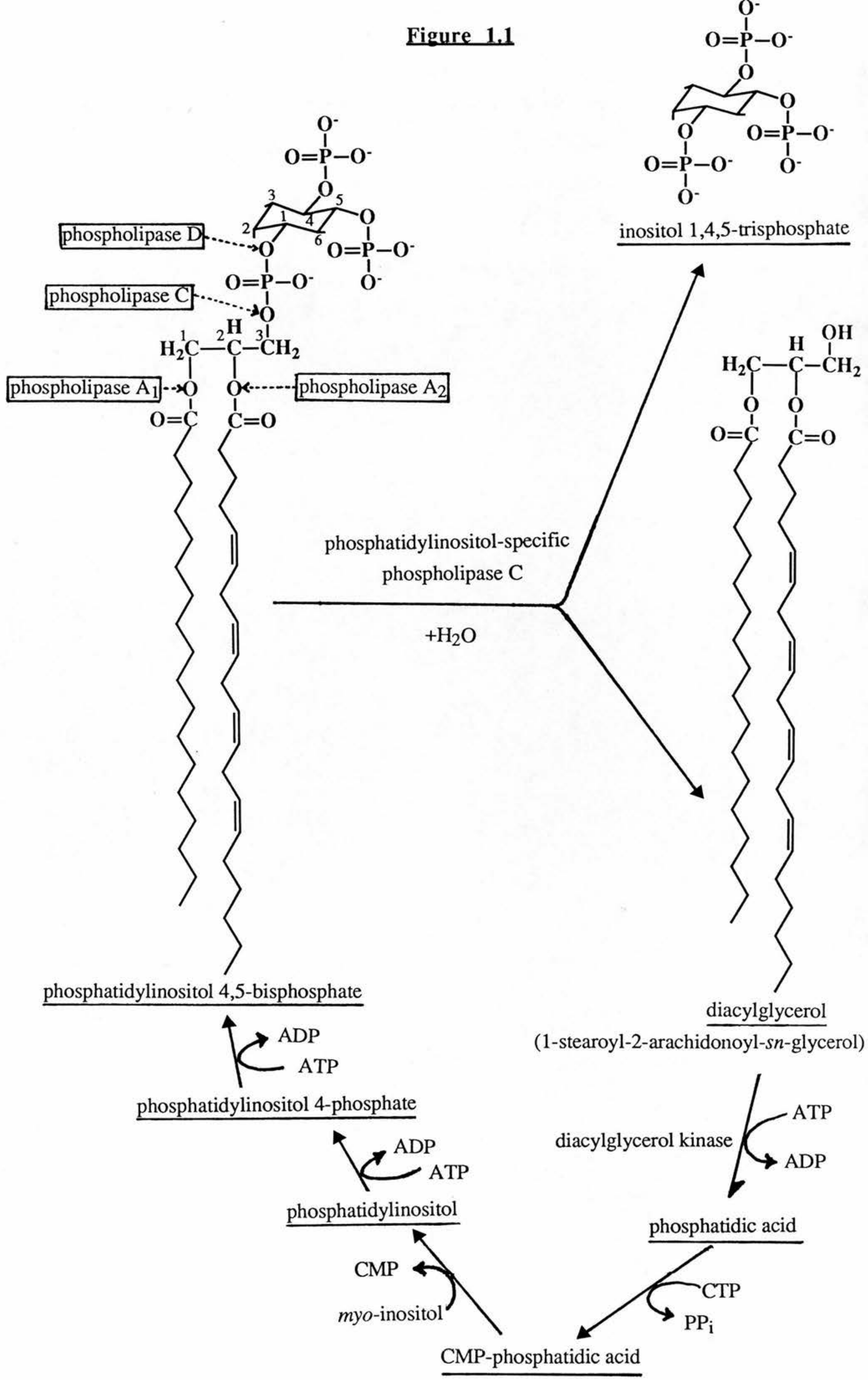
The formation of the substrate for PI-PLC, PtdIns(4,5)P_2, occurs initially by phosphorylation of the inositol structure PtdIns at its 4-position to produce PtdIns(4)P. This phospholipid can then by phosphorylated at the 5-position to produce PtdIns(4,5)P_2, which is the parent molecule for Ins(1,4,5)P_3 and diacylglycerol production. Both phosphorylation reactions occur through specific kinases (Michell, 1975). PtdIns(4,5)P_2 can be either dephosphorylated by specific phosphomonoesterases back to PtdIns(4)P and PtdIns, which has become known as the "futile cycle" which occurs mainly in the absence of receptor stimulation of PtdIns(4,5)P_2 hydrolysis (Berridge and Irvine, 1984).

The existence of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) has been detected in response to N-formylmethionylleucylphenylalanine (f-met-leu-phe), and the production of this novel phosphoinositide was suggested to occur by phosphorylation of PtdIns(4,5)P_2 by a 3-kinase (Carpenter and Cantley, 1990; Stevens *et al,* 1991) although PtdIns(3,4,5)P_3 could also occur by a 5-kinase acting on

Figure 1.1. **Diagram of the PI-PLC generation of Ins(1,4,5)P_3 and 1,2-diacyl-*sn*-glycerol from PtdIns(4,5)P_2.**

The site of potential hydrolysis by phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), phospholipase C (PLC) and phospholipase D (PLD) is indicated. The polyphosphoinositide shown here contains stearic and arachidonic acid as acyl chains and is known to occur physiologically and is a preferred substrate for PI-PLC (Downes and Michell, 1985), however, other combinations of acyl chains occur, which are other possible substrates for the enzymes shown. The figure is adapted from Downes and Michell (1985) and Meldrum *et al* (1991).

Figure 1.1



$PtdIns(3,4)P_2$, as was proposed for platelets (Majerus *et al,* 1990). The function of $PtdIns(3,4,5)P_3$ is unknown (especially as it is metabolically 'expensive' to make) as this novel phosphoinositide is not a good substrate for PI-PLC (Meldrum *et al,* 1991).

The formation of various inositol phosphates and diacylglycerol from phosphatidylinositols is catalysed by the Ca^{2+}-dependent enzyme, PI-PLC. However, PI-PLC is not a single entity, but exists as a large family of isoenzymes (α, ß, γ and δ families) with varying requirements for Ca^{2+} to achieve maximal activity (Crooke and Bennett, 1989; Rhee *et al,* 1989; Meldrum *et al,* 1991). The PI-PLC isoenzymes are localized throughout the body in both cytosol and membranes and generally have a greater substrate preference for $PtdIns(4,5)P_2$ than for PtdIns(4)P or PtdIns. Some of the PI-PLC isoenzymes are activated by GTP analogues and it is generally accepted that PI-PLC activity (at least for ß1 isoform (Taylor *et al,* 1991)) is controlled by a specific guanine nucleotide-binding (G) protein(s), which can be pertussis toxin-blocked or -insensitive (Medrum *et al,* 1991; Simon *et al,* 1991). In general, those receptors which are considered to interact with the second messenger-producing enzymes are thought to do so not directly, but by activating a specific G protein first, which then transduces the signal to the second messenger-producing enzymes (Neer and Clapham, 1988; Simon *et al,* 1991). Thus extracellular signals can remain outside the cell and influence intracellular events by utilising transduction processes which control the intracellular concentrations of a variety of second messenger molecules, which function to achieve the signal-specific cellular response. G proteins also exist as a family of subtypes (Simon *et al,* 1991) which can interact with ion channels as well as second messenger-producing enzymes (Rosenthal and Schultz, 1987; Neer and Clapham, 1988). G proteins are heterotrimers containing an α-, ß- and γ-subunit. The ßγ-subunits do not readily dissociate *in vitro* and have an uncertain function. Once receptor activated, the α-subunit of a G protein dissociates from the ßγ-subunits and binds GTP. The α-subunit is now active and carries out its regulatory function until its intrinsic GTPase activity cleaves the terminal phosphate of GTP to leave GDP. The α-subunit now has

high affinity for the ßγ-subunits again and once they are bound, the holoenzyme is now inactive and ready to restart its cycle of receptor-induced activity again (Graziano and Gilman, 1987). Cholera and pertussis toxins can also ADP-ribosylate specific amino acid residues on the α-subunits of certain G proteins, producing either inactive or continually active G protein α-subunits (Graziano and Gilman, 1987). The identity of the G protein(s) which activate PI-PLC enzymes has been unclear until more recently, a new class of G protein seems to be able to fulfil the role of activating PI-PLCs. The more convincing activators of PI-PLC appear to be the Gq class of G proteins (Strathman and Simon, 1990; Simon *et al,* 1991; Smrcka *et al,* 1991), and it was reported that α-subunits of these G proteins activated the ß1-isozyme, but not the γ1- or δ1-isozymes of PI-PLC (Taylor *et al,* 1991). It is possible that each PI-PLC isoenzyme has a specific G protein activator (if indeed one is needed) and the continued discovery of more classes of G proteins may see further types which play a role in phosphoinositide-signalling processes (Simon *et al,* 1991).

The PI-PLC catalysed hydrolysis of PtdIns(4,5)P_2 results in the formation of Ins(1,4,5)P_3, which binds to its own specific receptor (Snyder and Supattopone, 1989). The Ins(1,4,5)P_3 receptor has high selectivity for Ins(1,4,5)P_3 (K_D = 40 nM) over other inositol phosphate metabolites and Ins(1,4,5)P_3 binding is reduced by physiological concentrations of Ca^{2+} ions (K_i = 500 nM) (Worley *et al,* 1987). It has been proposed by Irvine (1991), that the Ins(1,4,5)P_3 receptor could contain an allosteric site for Ca^{2+} on its lumenal surface, which potentiates the action of Ins(1,4,5)P_3. Such an interaction could account for Ca^{2+}-dependent Ca^{2+} release from these calcium stores (Berridge and Irvine, 1990), and was proposed to be the mechanism of the spontaneous Ca^{2+}-release in Ca^{2+}-overloaded hepatocytes (Missiaen *et al,* 1991). This Ins(1,4,5)P_3 receptor appears to be localised on the rough endoplasmic reticulum (a store of Ca^{2+} ions) and consists of four identical 260 kD subunits, which form a Ca^{2+}-channel complex (Snyder and Supattapone, 1989). The Ins(1,4,5)P_3 receptor is also selectively phosphorylated by cyclic AMP-dependent

protein kinase, which reduces 10 fold the potency of Ins(1,4,5)P_3 as a calcium-releaser. The Ins(1,4,5)P_3 second messenger molecule is metabolised to dephosphorylated *myo*-inositol (Berridge and Irvine, 1989). The enzymes which dephosphorylate inositol phosphate (inositol phosphate phosphatases) and in particular, the enzyme, inositol polyphosphate-1-phosphatase, are sensitive to non-competitive inhibition by Li^+ ions which is activity-dependent (Berridge and Irvine, 1989). Lithium treatment gradually results in the depletion of cellular inositol stores, indirectly causing a reduction in the ability of the cells to produce Ins(1,4,5)P_3 (Berridge *et al,* 1982), presumably most marked for formerly overactive responses. This function of Li^+ is interesting as it may explain the actions of lithium, which is presently used clinically to treat manic-depressive illness.

Ins(1,4,5)P_3 can be further phosphorylated to inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) which itself is reported to possess a second-messenger function in some systems, in that it may promote the transfer of calcium into Ins(1,4,5)P_3-sensitive Ca^{2+} pools (Berridge and Irvine, 1989). The formation of Ins(1,3,4,5)P_4 could occur by hydrolysis by PI-PLC of the more recently discovered phosphoinositide, PtdIns(3,4,5)P_3 (Stevens and Irvine, 1991; Carpenter and Cantley, 1990; Majerus *et al,* 1990; Omann and Saklar, 1989; Whitman *et al,* 1988). PtdIns(3,4,5)P_3 as well as PtdIns(3,4)P_2 are not normally present at significant levels in quiescent, unstimulated cells, however they can be generated upon cell stimulation (Traynor-Kaplan *et al,* 1988; Auger *et al,* 1989; Kucera and Rittenhouse, 1990). The activity of a PtdIns(3) kinase enzyme has been associated with a number of tyrosine kinase oncogene products and growth factor receptors (Kucera and Rittenhouse, 1990; Bjorge *et al,* 1990; Fukui *et al,* 1989); however the role of these novel phosphoinositides may well be important, but remains poorly understood, especially as they do not appear to be good substrates for any of the PI-PLC enzymes thus far investigated (Meldrum *et al,* 1991). It is possible that PtdIns(3,4,5)P_3 could be cleaved to produce Ins(1,3,4,5)P_4 without the production of Ins(1,4,5)P_3, from which

$Ins(1,3,4,5)P_4$ is normally derived. Inositol pentakisphosphates and inositol hexakisphosphates are produced within cells (Berridge and Irvine, 1989), but their function is unknown, although it has been suggested that they are extracellular signals (Vallejo *et al,* 1987).

Irrespective of which phosphoinositide is acted upon by PI-PLC, and irrespective of which inositol phosphate(s) are derived and which of their metabolites occur, the remaining portion of the phosphoinositide molecule after PI-PLC activity, is always diacylglycerol. Diacylglycerol is an endogenous activator of PKC, and completes the second of the two signalling processes which are utilised by receptors which act through polyphosphoinositide hydrolysis to access this "bifurcating pathway".

1.2. THE CONTROL OF PROTEIN KINASE C ACTIVITY

1.2.1. Protein kinase C

Protein kinase C was identified by Nishizuka and his co-workers in 1977 (Takai *et al,* 1977; Inoue *et al,* 1977) as a proteolytically-activated protein kinase which had no obvious role at that time. It was later shown to be a Ca^{2+}-activated, phospholipid- (particularly phosphatidylserine) dependent enzyme (Takai *et al,* 1979a) which was modulated by diacylglycerol, resulting in a greatly increased affinity for Ca^{2+} (Takai *et al,* 1979b; Kishimoto *et al,* 1980). Thereby, diacylglycerols can effectively activate protein kinase C without any change in Ca^{2+} levels (Kaibuchi *et al,* 1981). The enzyme is known to be ubiquitously distributed in tissues and organs (Kuo *et al,* 1980). In tissues other than brain, the enzyme, extracted in the presence of Ca^{2+} chelators, is recovered mainly from the soluble fraction as the inactive form (Kikkawa *et al,* 1982; Girard *et al,* 1986) and can be translocated to the membranous fraction in a Ca^{2+} dependent fashion when cells are stimulated (Kraft and Anderson, 1983; Hirota *et al,* 1985; Wolf *et al,* 1985). Activation of protein kinase C requires a diacylglycerol in a 1,2-*sn* configuration, with various fatty acid chain length compositions, but those

diacylglycerols containing unsaturated fatty acids are the most active (Mori *et al,* 1982). The 2,3-*sn*- and 1,3-diacylglycerol stereoisomers are incapable of modulating activity of the enzyme, whereas 1,2-*rac* or *sn* diacylglycerol can activate PKC (Rando and Young, 1984; Boni and Rando, 1985). Extensive analyses of the activation requirements for PKC have resulted in a two step model of activation (Bazzi and Nelsestuen, 1988; Hannun and Bell, 1990). Firstly, soluble PKC (which is inactive and readily interacts with MgATP) binds anionic phospholipids (especially phosphatidylserine) and Ca^{2+},which results in PKC becoming membrane-associated. This membrane-associated form of PKC is what is known as translocated, but has relatively low kinase activity. Diacylglycerols or phorbol esters can then induce greater PKC activity. High levels of diacylglycerol/phorbol ester and Ca^{2+} cause the PKC to become membrane-inserted and lose most of its co-factor requirements for activity. Mg^{2+} ions are necessary for enzyme activity, and the stoichiometry of the interaction of the co-factors *in vitro* is 4 - 10 phosphatidylserine molecules to one diacylglycerol/phorbol ester molecule to one Ca^{2+} ion for every monomeric PKC molecule (Hannun *et al,* 1986a; Ganong *et al,* 1986; Hannun and Bell, 1990).

The earlier biochemical characterisation of protein kinase C indicated that it consisted of a single polypeptide chain with molecular weight of approximately 77 kD with two functionally different domains, which could be separated by Ca^{2+}-dependent thiol proteases (Kikkawa *et al,* 1982; Kishimoto *et al,* 1983). One fragment was hydrophobic in nature and could bind to membranes, whereas the other smaller fragment was the hydrophilic catalytic region which was fully active without either Ca^{2+}, phospholipids or diacylglycerol. This limited proteolysis to produce a regulatory and catalytic subunit respectively, could be performed by calpain and was known as proleolytic 'activation', as it created an unregulated constitutively-active kinase molecule which later became known as PKM (Kishimoto *et al,* 1983). It is still unclear as to the physiological role of this proteolysis, but it may contribute to sustained kinase activity which is necessary in situations of maximal stimulation. Membrane-bound

PKC was more susceptible to this proteolytic activation (Kishimoto *et al,* 1983). Both the holoenzyme and the catalytic-fragment of the proteolytically-cleaved kinase can phosphorylate *in vitro* serine and threonine residues (but not tyrosine residues) on a vast number of proteins from most tissues (Nishizuka *et al,* 1984; Nishizuka, 1986; and see Table 1.1). In addition to these other proteins, PKC can also phosphorylate itself at several sites (Huang *et al,* 1986; Mochly-Rosen and Koshland, 1987; Newton and Koshland, 1990; Flint *et al,* 1990) which leads to the enhanced activity of the enzyme.

The characterisation of PKC, notably by Nishizuka's group, and the separate characterisation of the receptor for the phorbol ester tumour promoters (Niedel *et al,* 1983; Sando and Young, 1983; Ashendel *et al,* 1983; Leach *et al,* 1983) revealed that protein kinase C enzymatic activity and phorbol ester binding activity co-purified, ultimately to homogeneity. Steric similarities exist between the 12- and 13-position side-groups in many phorbol ester molecules and the 2- and 3-acyl chains in diacylglycerols, one of the endogenous activators of PKC (Nishizuka, 1984a). Indeed, 1,2-diacylglycerols displace the binding of 12,13-phorbol ester ligands to PKC (Leach *et al,* 1983) providing evidence that phorbol esters mimic diacylglycerols and activate PKC at its diacylglycerol binding site. The identification of protein kinase C as the major target for the phorbol ester tumour promoters implicated the importance of protein kinase C in cell growth, differentiation and neoplasia; typical actions of the phorbol ester tumour promotors. The oncogene v-*raf* is distantly related to PKC and other serine and threonine kinases (Moelling *et al,* 1984; Sutrare *et al,* 1984). This fact, in addition to the high specificity of phorbol ester tumour promotors for activating PKC, strongly furthermore implicates a role for PKC in normal and disordered cell signalling states.

Table 1.1. **Table of the known and possible substrates of protein kinase C *in vivo*.**

The data for this table were compiled from information supplied by Nishizuka (1986), Campbell *et al* (1988), Shearman *et al* (1990), Huang (1989), Bushfield *et al,* (1990) and Katada *et al* (1985).

Table 1.1

Receptor proteins

epidermal growth factor receptor
insulin receptor
somatomedin C receptor
transferrin receptor
interleukin-2 receptor
nicotinic acetylcholine receptor
ß-adrenergic receptor
immunoglobulin E receptor

Membrane proteins

Ca^{2+}-transport ATPase
Na^+/K^+ ATPase
voltage-dependent Na^+-channel
K^+-channels (voltage-dependent , Ca^{2+}-activated, M, S and Type III)
Na^+/H^+ exchange system
'L'-type Ca^{2+} channel (α_1- and ß-subunits)
'N'-type Ca^{2+} channel
'T'-type Ca^{2+} channel
Cl^--channels (outwardly rectifying and $GABA_A$ complex)
glucose transporter
GTP-binding protein (at least G_{i2} and G_o)
HLA antigen
chromaffin granule-binding protein
synaptic B50 (F1) protein

Contractile and cytoskeletal proteins

myosin light chain
troponin T and I
vinculin
filamin
caldesmon
cardiac C-protein
microtubule-associated proteins (MAP-2 and tau)

Enzymes

glycogen phosphorylase kinase
glycogen synthase
phosphofructokinase
ß-hydroxy-ß-methylglutaryl-coenzyme A reductase
tyrosine hydroxylase
NADPH oxidase
cytochrome P450
guanylate cyclase
DNA methylase
myosin light chain kinase
initiation factor 2

Other proteins

neuromodulin (also designated P-57, GAP(growth associated protein)-43, pp46, B-50 or F-1)
fibrinogen
retinoid-binding proteins
vitamin D-binding protein
ribosomal S6 protein
GABA modulin
stress proteins
myelin basic protein
high-mobility group proteins
middle T antigen
$pp60^{src}$ protein
p87, p17
myristolated alanine-rich C kinase substrate (MARCKS)

1.2.2. Protein kinase C isoenzymes

The discovery by Nishizuka's group in 1977 of protein kinase C led to its original classification as a Ca^{2+}-activated, phospholipid-dependent protein kinase. Since that time, it has become evident that protein kinase C consists of a family of more than one subspecies (Nishizuka, 1988). Initial screening of various complementary DNA (cDNA) libraries revealed four subspecies of a calcium-dependent nature (Parker *et al,* 1986; Coussens *et al,* 1986; Knopf *et al,* 1986; Ono *et al,* 1986; Kikkawa *et al,* 1987; Ohno *et al,* 1987; Kubo *et al,* 1987; Ohno *et al,* 1988a). This group of PKC isozymes (known as the A-series (see Figure 1.2)) all required Ca^{2+} for activation and contained isoforms which were designated as α, ßI, ßII and γ (ßI and ßII being alternative-splice variants from the same gene (Ono *et al,* 1987a)). The coding sequences for this group of PKC cDNAs contain four conserved (C1 - C4) and five variable (V1 - V5) regions. Using a mixture of α, ßII and γ cDNA probes under lower stringency conditions, three further subspecies of PKC were identified and were designated as the δ, ε and ζ isoforms of protein kinase C (Ono *et al,* 1987b; Ono *et al,* 1988a; Ohno *et al,* 1988b; Schaap *et al,* 1989). This group of PKC-isoenzymes was collectively known as the B-series PKC-isoforms which were of considerable interest as they did not require Ca^{2+} for their kinase activation. The coding sequences for the B-group cDNAs revealed broadly equivalent conserved and variable regions, but the C2 region which is highly conserved among the A-group PKCs, is absent from group B-PKCs and may be the region responsible for conferring Ca^{2+}-dependency on the enzymes. The C1 region consistently contains a pseudosubstrate sequence (in α-PKC, for example, Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys), which may be responsible for maintaining the enzyme's kinase region in an inactive state in the absence of activators (House and Kemp, 1987). Substitution of the alanine residue with serine converts the inhibitory substrate peptide equivalent to this sequence into an excellent substrate for phosphorylation. The C1 region of the enzyme also contains a tandem repeat cysteine-

rich Zn^{2+}-finger sequence, Cys-X_2-Cys-$X_{13(14)}$-Cys-X_2-Cys-X_7-Cys-X_7-Cys (where X represents any amino acid), found in many metalloproteins and DNA-binding proteins that are involved in transcriptional regulation (Berg, 1986). In the case of PKC however, the sequence is considered to be the site of phorbol ester binding to PKC (Ono *et al,* 1989). Interestingly, it has also been reported that PKC also has the ability to bind to DNA (Testori *et al,* 1988) and is present in rat liver nuclei (Masmoudi *et al,* 1989). The V3 region of the PKC family appears to join the catalytic and regulatory domains (Parker *et al,* 1986; Coussens *et al,* 1986) and encompasses calpain- and trypsin-sensitive sites of the enzymes which appear to be critical in the proteolytic degradation of the PKCs (Kishimoto *et al,* 1989; Huang *et al,* 1989). There is evidence that different subspecies may be degraded at different rates (Kishimoto *et al,* 1989). The C3 region of the enzymes contains a sequence which is typical of a consensus sequence in ATP-binding proteins. Interestingly, a second consensus ATP-binding site sequence is also present in the C4 region of the α- and ß, but not the γ isoforms (Huang, 1989).

It appears (at least for the Ca^{2+}-dependent PKCs), that it is necessary for them to translocate to and bind to membraneous phospholipids (notably phosphatidylserine, phosphatidylinositol or phosphatidylglycerol prior to their activation (Huang, 1989; Schaap and Parker, 1990)). It is in this membrane-bound state, that diacylglycerols or phorbol esters are considered to stabilize the membrane-associated PKC and activate the enzyme fully (Bazzi and Nelsestuen, 1988). It is less clear however, how the more recently discovered Ca^{2+}-independent PKCs will be encouraged to translocate and activate (should this be necessary), according to this oversimplified scheme of PKC translocation and activation.

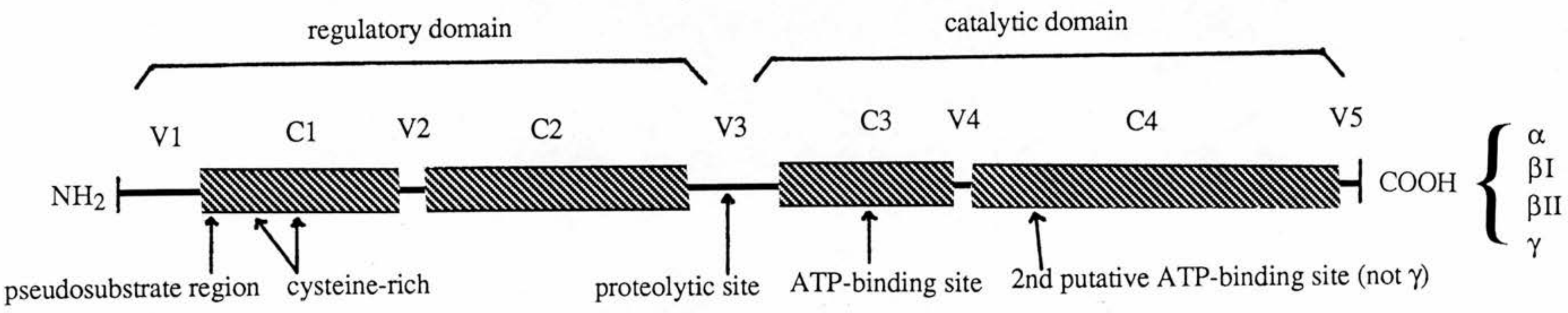
All PKC isoforms thus far discovered, show differential and distinctive tissue and cellular distribution (Nishizuka, 1988a). Protein kinase C-α and PKC-ß are widely distributed throughout the body, with highest levels being found in the central nervous system and in spleen. Naor *et al* (1989) demonstrated that α- and ß-PKC, but

Figure 1.2. **Simplified diagram of the sequences of each of the seven sequenced PKC isoforms.**

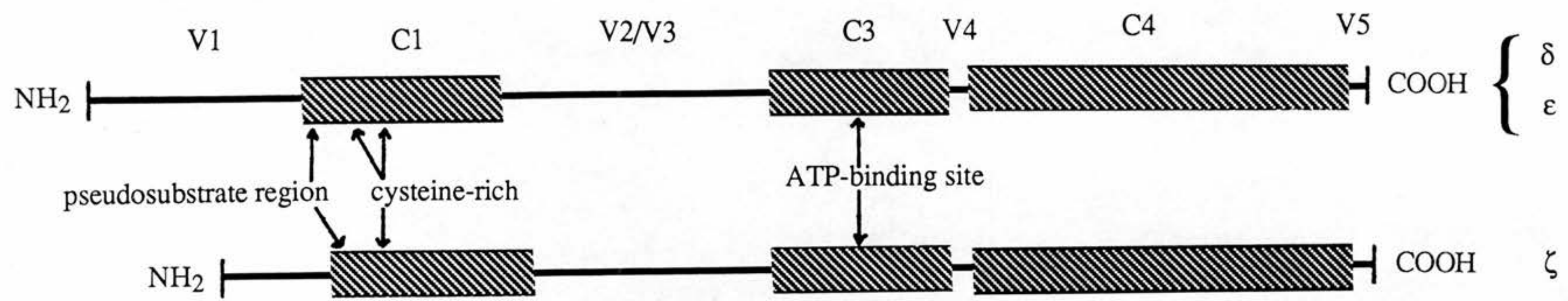
These isoenzymes of PKC have been grouped into two classes, those considered to require Ca^{2+} for their activation (α, ßI, ßII and γ isoforms, termed the A series), and those isoenzymes considered to be calcium-independent with respect to their activation kinetics (δ, ε and ζ isoforms, termed the B series). The shaded regions indicate the regions with marked homology between the isoenzyme sequences (termed conserved, C) and the unshaded regions indicate sequences with variable (V) homology amongst the isoenzyme sequences. This figure is adapted from Huang (1989).

Figure 1.2

A series (α, β and γ)



B series (δ, ε and ζ)



not γ-PKC, would induce luteinising hormone release from permeabilised gonadotrophs. The γ-isoform of PKC is found in high levels in the central nervous system (Shearman *et al,* 1988) but is absent from peripheral tissue. However, in the U937 promonocytic (non-neuronal) cell line, γ-PKC is absent, but it has been shown to be induced by TPA treatment (Strulovici *et al,* 1989). Similar results were found in the related cell line, HL-60, in which PKC-γ was expressed using either dimethylsulphoxide or retinoic acid (Makowske *et al,* 1988). The B-group series of PKCs are found to be differentially distributed throughout the body, but are most highly expressed in the central nervous system (Nishizuka, 1988) and it is possible that they can be distinguished from the A series isoforms by their down regulation behaviour. Treatment of thymocytes with phorbol ester, down-regulates PKC-ß, without any down-regulation of PKC-ε, however, thrombin induced the down-regulation of both isoforms (Strulovici *et al,* 1991). This result indicates that differential regulation of isoforms can occur *in vivo.* PKC-isoforms may also have specific localisations at the subcellular level (Nishizuka, 1988) which raises the question as to whether isoform-specific phosphorylation targets exist in cells or whether PKC isoform-dependent cell-functions occur, and indeed whether pharmacological tools can be developed by which to selectively interfere with these PKC isoform-selective actions, and give us a fuller understanding of PKC isoform-specific functions?

1.2.3. Protein kinase C pharmacology

The idea that the isoforms of protein kinase C (PKC) may display distinct pharmacological properties is of considerable interest, as pharmacological agents with sufficiently selective action would be useful for characterisation of the cellular actions of PKC isoforms and may provide novel therapeutic opportunities. Due to PKC being involved in cellular processes such as cell growth/differentiation and tumourogenesis, drugs which can successfully modulate PKC function may be of benefit clinically, for

example in the treatment of cancer, memory loss and neuronal damage (see Nishizuka, 1984a; Nishizuka, 1988; Blumberg, 1988).

Protein kinase C was originally classified by Nishizuka and his co-workers in 1977 as a calcium-activated, phospholipid-dependent protein kinase. Since that time, Ca^{2+}-activated, phospholipid-dependent PKC has been discovered to represent part of a family of at least seven isoforms (Nishizuka, 1988), four (α, ßI and ßII and γ) being calcium-dependent (termed A series), and three (δ, ε and ζ) being calcium-independent (termed B series) with regard to their activation. Thus, one simple pharmacological distinction between PKC isoforms is already evident, and agents which activate PKC without raising cytosolic calcium levels (for example diacylglycerols) if they are generated by means outwith phosphoinositide hydrolysis (or applied exogenously), should preferentially activate the Ca^{2+}-independent (B-series) isoforms of PKC, over those isoforms which additionally require Ca^{2+} for their activation (A-series) and may lead to a quite distinct array of PKC-mediated actions (proposed by Parker *et al,* 1989). Phosphoinositide hydrolysis in response to receptor activation would thus be likely to result in the activation of both A and B series PKC isoforms. There are, however, already evidence which conflicts with these logical predictions. In HeLa cells, which possess α-, ß- and ε-PKC isoforms, interferon-α does not cause the hydrolysis of phosphoinositides but induces phosphatidylcholine (PC) hydrolysis (Pfeffer *et al,* 1990). Phosphatidylcholine hydrolysis induced by interferon-α selectively induces the activation/translocation of the ß (calcium-dependent)-isoform of PKC (Pfeffer *et al,* 1990). It would be expected that the ε (calcium-independent)-isoform of PKC should selectively be activated as PC hydrolysis would produce diacylglycerol (which activates PKC) and the apparently inert choline phosphate. Thus there may well be further aspects of the endogenous regulation of different PKC isoforms yet to be elucidated.

Isoform differences are also evident when considering the phospholipid-dependence of PKC. Huang *et al* (1988) showed that γ-PKC was more susceptible than α- or ß-PKC to activation by a variety of phospholipids including

phosphatidylserine, phosphatidylglycerol, phosphatidic acid and cardiolipin. The purified γ-isoform of PKC was also more susceptible than the α- or ß-isoforms to an inactivation process elicited by the same phospholipids which occurs in the absence of Ca^{2+} (Huang and Huang, 1990). Activation of PKC isoforms by fatty acids which show some discrimination between different isoforms has also been described. Arachidonic acid (AA), as well as some other unsaturated fatty acids, have been shown to activate PKC in the absence (McPhail *et al,* 1984; Murakami and Routtenberg, 1985; Leach and Blumberg, 1985; Murakami *et al,* 1986; Hanson *et al,* 1986; Sekiguchi *et al,* 1987; Sekiguchi *et al,* 1988; Seifert *et al,* 1988; Naor *et al,* 1988a; Shearman *et al,* 1989a; Burns *et al,* 1990) or the additional presence (Shinomura *et al,* 1991) of diacylglycerol. Naor *et al* (1988a) have shown that purified hypothalamic PKC isoforms responded differently in their activation by AA. In the absence of $CaCl_2$ (0.3 mM), neither the α- nor the ß-isoform of PKC was stimulated significantly by AA, whereas the γ-isoform displayed a biphasic stimulation to increasing concentrations of AA, with highest activity at 12 μM AA. In the cell free system in the presence of calcium, γ-PKC activity to AA was unchanged, but α-PKC was activated in a concentration-dependent manner with increasing doses of AA (up to 100 μM AA, which induced 70% of the maximal phorbol-elicited α-PKC activity). The ß-isoform of PKC is also activated by fatty acids, but to a lesser extent than α-PKC (Sekiguchi *et al,* 1987; Sekiguchi *et al,* 1988; Naor *et al,* 1988a). Linoleic acid is also a good activator of γ-PKC and oleic acid shows moderate ability to activate γ-PKC, but lipoxin A4 is as potent (if not more so) as AA in the activation of the PKCs (Hanson *et al,* 1986; Shearman *et al,* 1989a).

Some PKC isoform differences are also apparent in activation via the site recognised endogenously by diacylglycerols (Nishizuka, 1984a) and exogenously by phorbol esters (Blumberg, 1988), although the basis for such discrepancies and the PKC isoforms responsible have rarely been identified. The diacylglycerol analogue, 1,2-dioctanoyl-*sn*-glycerol (DOG), has been shown to mimic selectively only one

response of the phorbol ester-induced changes in activity of cardiac 'L'-type Ca^{2+}-channels (Lacerda *et al,* 1988). Similarly, phorbol ester-stimulated interleukin 1ß messenger ribonucleic acid (mRNA) induction and protein phosphorylation patterns could be only partially mimicked by DOG (Strulovici *et al,* 1989). It has been reported that ß-PKC(s) are more sensitive to activation by diacylglycerols in the absence of Ca^{2+} than α- or γ-PKC (Nishizuka, 1988; Sekiguchi *et al,* 1988), however, other reports which used expressed ßII-PKC state that this isoform is less sensitive to activation by diacylglycerol in either the absence or presence of Ca^{2+} (Burns *et al,* 1990). The diacylglycerols DOG and 1,2-didecanoyl-*sn*-glycerol were able to stimulate mouse epidermal PKC activity to maximal levels similar to those seen with the phorbol ester, phorbol 12-myristate 13-acetate (PMA) and a structurally related analogue of PMA, mezerein (Smart *et al,* 1989). Mezerein is an activator of PKC, but its range of biological actions do not fully mimic that of other biologically active phorbol esters such as PMA (Blumberg, 1988; Jaken *et al,* 1983a). The phorbol esters, of which PMA is typical, act as full tumour promoters, produce inflammation and induce ornithine decarboxylase activity amongst many other actions. In contrast, mezerein and indeed certain phorbol ester analogues such as 12-deoxyphorbol 13-isobutyrate (DPB), phorbol 12-myristate 13-acetate 4-0-methyl ether (MPMA) and phorbol 12-retinoate 13-acetate (PRA), display some of of the PMA-induced responses, but not the full, broad spectrum, activity of the latter (Blumberg, 1988; Dunn and Blumberg, 1983). The selective biological nature of mezerein was lost in an unsaturated analogue of mezerein, octahydromezerein (Sharkey *et al,* 1989) as assessed by murine skin tumour promotion and [^{3}H]-PDBu binding studies. The selective nature of mezerein could possibly be due to its preferential association of a 'cryptic' state of down-modulated PKC (with reduced affinity to phorbol esters) described by Jaken *et al* (1983b). However, the postulate by Jaken *et al* (1983b) was before the knowledge that various forms (including sequence isoforms) of PKC existed, and mezerein to this date remains to be a curious compound of current investigation, with the precise mechanism of its

selective nature being poorly understood. Mezerein, DPB, MPMA and PRA show selectivity in binding to one or more of the multiple phorbol ester binding sites previously identified using [^{3}H]-DPB and [^{3}H]-phorbol 12,13-dibutyrate (PDBu) binding in mouse skin (Delctos *et al,* 1980; Driedger and Blumberg, 1980; Dunn and Blumberg, 1983), which may represent different forms/isoforms of PKC.

It has also been reported that phorbol esters have different affinities towards purified PKC isoforms (Sekiguchi *et al,* 1988; Naor, 1990; Marais and Parker, 1989) where PMA was a more potent activator of ß-PKC than of α-PKC and γ-PKC, being less well activated by PMA. The Km values for the purified α, $ß_1$ and γ isoforms of PKC showed only modest differences in affinity towards PMA, and Vmax values calculated per mole of purified protein gave no suggestion of reduced efficacy at any of the PKC subspecies tested (Marais and Parker, 1989). Resiniferatoxin (RTX) is a naturally occurring diterpene which is structurally related to phorbol esters and is known to be an analogue of capsaicin (the irritant in red pepper, which causes release of neuropeptides (including substance P) from small primary afferent neurones) (Szallasi and Blumberg, 1989a; de Vries and Blumberg, 1989). Although much weaker than PMA, RTX (and certain analogues) can act as weak activators of PKC (Driedger and Blumberg, 1980; Ellis *et al,* 1987; Ryves *et al,* 1989). Like mezerein, RTX shows only a narrow portion of the total spectrum of biological activity seen with the conventional phorbol ester PMA (Szallasi and Blumberg, 1990) and may selectively act on one or more of the PKC isoforms. A kinase activity similar to the PKCs but selectively activated by RTX and only very poorly by phorbol esters has been described but not fully characterised (Ryves *et al,* 1989; Evans *et al,* 1991).

It has been suggested that phorbol esters such as PMA could have actions which are PKC-independent and may in part involve activation of phospholipases A_2 and D (Kolesnick and Paley, 1987; Kiss *et al,* 1987; Billah and Anthes, 1990). It may be therefore that the excess actions of phorbols over mezerein and other analogues are spurious with respect to interactions with PKC subtypes. Nevertheless, it remains

possible that some of the apparently selective agents such as mezerein or RTX may act on a protein kinase which is either one of the PKC isoforms or is related to the PKC family and is highly sensitive to mezerein and RTX, but rather less sensitive to PMA. The same could also be true of the PKC-activating compound, bryostatin, which is structurally unrelated to phorbol esters but can fully mimic some, but not all, of the activities of PMA (Kiss *et al,* 1987; Zwelling *et al,* 1991). In GH_4C_5 cells, bryostatin and DOG were shown to have differing specificities on a number of actions (including cell stretching, prolactin release and prolactin synthesis) induced by these PKC activators; however, bryostatin could displace [^{3}H]-PDBu binding and activate PKC with the same ability as PMA (Ramsdell *et al,* 1986). The authors concluded that these activators of PKC (bryostatin and DOG) could be selectively activating multiple forms of the kinase.

Some less-well investigated activators of PKC include aplysiatoxin and teleocidin, with its derivative (-)-indolactam V and its less active enantiomer (+)-indolactam V (Fujiki *et al,* 1984) which show differences in affinity between models of PMA-induced tumour promotion and were suggested to be possibly acting differentially towards different PKC isoforms. Jeffrey and Liskamp (1986) used computer molecular modelling techniques to study a range of phorbol ester-related compounds with known carcinogenic properties, including PMA, PRA, mezerein, teleocidin and 4ß-PDD. It was from these initial studies and from their own structural analyses that Wender *et al* (1988) predicted that the newly-synthesised compounds ADMB (3-(N-acetylamino)-5-(N-decyl-N-methylamino)benzylalcohol) and DHI (6-(N-decylamino)-4-hydroxymethylindole) should act as activators of PKC. The prediction proved correct in that both ADMB and DHI bound to PKC in competition with PDBu and acted to exert PKC-mediated effects, but with reduced affinity compared to diacylglycerols (Wender *et al,* 1988). These compounds have been used little since because of their low potency, but have helped provide evidence for the key atomic structure necessary to generate an active phorbol ester pharmacophore. In addition to an hydroxyl group in

the ß-configuration at position 4 of the phorbol structure (see structures section), Gschwendt *et al* (1991) concluded that a carbonyl (C=0) group at position 3, and hydroxyl (OH) groups at positions 9 and 20 of phorbol esters are essential for binding to the putative phorbol ester binding site within PKC(s), however, the authors admit that other positions may well also be crucial and that their interpretation could well turn out to be simplistic.

Other activators of PKC with interesting profiles of activation include the phorbol esters, 12-deoxyphorbol 13-phenylacetate 20-acetate (DOPPA); Sapintoxin A and α-sapanin acetate (Brooks *et al,* 1987; Brooks *et al,* 1990) which vary in their dependence on Ca^{2+} for activation of PKC, and somewhat on their activation of hydroxyapatite-separated PKC subspecies. Brooks *et al* (1990) concluded that the actions of DOPPA were selectively on an as yet unidentified isoform of PKC, which is not only present in platelets, but also in GH_3 cells (Brooks *et al,* 1987). More recently, Evans *et al* (1991) concluded that DOPPA was a selective activator of ß-PKC although ε and ζ isoforms were untested as such, and the purified enzyme fractions affirmed to represent α, ß and γ isoforms were in fact admitted to be contaminated by varying proportions of B series PKC isoforms.

A functional inhibition of PKC activity has been observed by a variety of agents which interfere with the interaction of PKC with Ca^{2+}/phospholipids, diacylglycerols/phorbol esters, ATP or by inhibiting interaction of PKC with its protein substrate. There are limitations in the specificity of the aforementioned interactions, in that not only PKC, but other protein kinases, utilise some of these mechanisms of activation, and in many cases pharmacological agents with selectivity for PKC over other protein kinases would seem difficult to derive. Inhibition of the interaction of Ca^{2+} and phospholipids with PKC can be achieved by agents such as trifluoperazine, dibucane, adriamycin, polymixin B and melittin which are thought to disrupt the binding of Ca^{2+} to the PKC/phospholipid complex (Hidaka and Hagiwara, 1987; Huang, 1989), but these are also as effective at inhibiting Ca^{2+}/calmodulin-dependent

protein kinases. Due to their poor selectivity, these compounds are rarely used as inhibitors of PKC.

Sphingosine is an 18 carbon sphingolipid which is a natural component of membranes. Sphingosine competes with the Ca^{2+}/phospholipid and the diacylglycerol interaction with PKC and is a potent inhibitor of PKC activity (Hannun *et al,* 1986) but is also reported to be equally effective at inhibiting Ca^{2+}/calmodulin-dependent kinases (Jefferson and Schulman, 1988) and has been termed a non-specific inhibitor of PKC (Huang, 1989). Agents which have been cited as being inhibitors of PKC acting by interfering with the interaction of diacylglycerol/phorbol esters are cremophor EL (Zhao *et al,* 1989), 1-0-hexadecyl-2-0-methyl-*rac*-glycerol (AMG-C16) (Kramer *et al,* 1989) and calphostin C (Kobayashi *et al,* 1989), and they are purported to exhibit reasonable selectivity for PKC over other kinases (in particular calphostin C, which had 1,000 times greater an affinity for inhibition of PKC than for inhibition of cyclic AMP-dependent and tyrosine-specific protein kinases).

Competition with ATP for the nucleotide binding site in PKC is the mechanism of inhibition that is thought to be used by H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) and related isoquinolines (Ohta *et al,* 1988). As the ATP-binding site is highly conserved between the kinases, it would be expected that inhibitors using the ATP binding region as a site of action would not be able to exert any selectivity for PKC. However, H7 does have some selectivity towards PKC over a variety of other kinases (Hidaka *et al,* 1984) but only when comparing the actions of H7 to that of a control analogue (HA1004) which has similar inhibitory activity to H7 for myosin light chain kinase, cyclic GMP- and cyclic AMP-dependent protein kinases, but a reduced inhibitory activity for PKC when compared to H7. It is interesting to note, that α- and ß-PKC (but not γ-PKC) have an additional region within the sequence representing a second putative ATP-binding site as well as the characterised ATP-binding site sequence found on all seven PKC isoforms (see Figure 1.2; Huang, 1989).

Like H7, the microbial products staurosporine and K252a inhibit PKC apparently in a manner competitive with ATP (Kase *et al,* 1987; Nakadate *et al,* 1988), but are thought to interact not solely at the ATP-binding region but also by interacting directly with the catalytic domain of PKC to exert its inhibitory actions (Huang, 1989). Staurosporine, although a potent kinase inhibitor, can in no way be considered a specific inhibitor of PKC and, when assessing PKC activity, needs to be used in comparison to its control analogues such as K252a, which have reduced inhibitory actions at PKC but similar potencies to kinases other than PKC (Rüegg and Burgess, 1989). Recently, more selective analogues of staurosporine have been synthesised which display over 100 times greater selectivity than staurosporine for inhibition of PKC compared to cyclic AMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases (such as the compound termed Ro318220; Davis *et al,* 1989), but, as yet, these compounds are not commercially available.

Staurosporine and H7 are generally regarded as being unable to inhibit particular PKC-isoforms selectively (Schaap and Parker, 1990); however, H7 has been shown to inhibit selectively certain aspects of phorbol ester-stimulated actions, without any effect on other phorbol ester-induced processes. In mouse epidermal cells, H7 inhibited selectively PMA-stimulated ornithine decarboxylase induction, but failed to inhibit (at the same concentrations) PMA-induced reduction in epidermal growth factor-binding, whereas sphingosine inhibited both PMA-stimulated responses with similar affinity (Nakadate *et al,* 1989). Phorbol ester-stimulated ornithine decarboxylase induction and oedema production in mouse skin could not be inhibited by staurosporine, but phorbol ester-stimulated protein phosphorylation was inhibited by staurosporine and H7 (Yamamoto *et al,* 1989). The staurosporine analogue, K252a was found to inhibit a mixture of partially-purified, unidentified (probably α, ß and γ isoforms (Gschwendt *et al,* 1989a)) PKC from mouse brain. In contrast, a purified PMA-stimulated, calcium-independent protein kinase from porcine spleen (termed p76-kinase) was found to be less sensitive to inhibition with K252a, this inhibitor having

over 100 fold less affinity at inhibiting p76-kinase than other PKC(s) from mouse brain (Gschwendt *et al,* 1989a; Gschwendt *et al,* 1989b). These apparently selective actions of the inhibitors H7 or staurosporine and its derivatives may be due to a pharmacologically-selective nature of these compounds between PKC isoforms (or related kinases). Another explanation for these selective effects of these PKC inhibitors may be due to phorbol esters having actions in cells which are independent of PKC activation (see section 1.2.5) and consequently unresponsive to kinase inhibitor.

It has been reported that differences in potency of certain PKC inhibitors may be due to the ineffectiveness of certain types of PKC inhibitors towards PKM, the catalytically-cleaved kinase domain of PKC which lacks regulation from Ca^{2+}, diacylglycerol/phorbol esters or phosphatidylserine (see section 1.2.1). It was reported (Junco *et al,* 1990; Boscá *et al,* 1990) that agents which inhibit PKC by interacting with its kinase region (eg, H7 and staurosporine) had markedly reduced potency at inhibiting PKM in contrast to another PKC inhibitor, quercetin, which is widely considered to act also by competing with ATP for its binding site on PKC and other kinases (Horn *et al,* 1985; Nakadate *et al,* 1985; Ferriola *et al,* 1989). The authors also reported that quercetin while potently inhibiting PKM, only poorly inhibited PKC activity. These findings are at total variance with previous, more comprehensive reports that quercetin (and its analogues) are relatively potent inhibitors of PKC (Horn *et al,* 1985; Nakadate *et al,* 1988; Ferriola *et al,* 1989) and thus must be viewed with some caution.

Other inhibitors of PKC have been reported (for reviews, see Huang, 1989; Epand and Lester, 1990) which include suramin (Hensey *et al,* 1989), aminoacridines (Hannun and Bell, 1988) and chelerythrine which is purported to be a moderately selective inhibitor of PKC compared to other kinases (Herbert *et al,* 1990). Many of these agents mentioned however inhibit PKC activity only at very high concentrations, where non-specific effects are considered to be a distinct possibility. Their modes of inhibitory action are at present poorly investigated and any consideration of PKC-isoform selectivity of the compounds remains to be addressed.

An interaction with the protein substrate binding region is suggested to be the mechanism of action of certain peptides which resemble the pseudosubstrate sequences of the PKCs (House and Kemp, 1987; see also section 1.2.1). In elegant work by House *et al* (1989), a 29 amino acid peptide was synthesised which corresponded to part of the putative substrate binding region of PKC which interacts with the pseudosubstrate sequence. They found that this peptide enhanced PKC activity presumably by binding to and 'masking' the pseudosubstrate region of the enzyme and removing the inhibitory influence that the endogenous pseudosubstrate region exerts on PKC activity (House *et al,* 1989). Peptides which resemble the putative pseudosubstrate region in PKC can act as inhibitors of the enzyme activity (Alexander *et al,* 1990; Eichholtz *et al,* 1990). Pseudosubstrate peptides with the appropriate replacement of serine for alanine will convert an inhibitory peptide into a substrate peptide for PKC (House and Kemp, 1987). Although the pseudosubstrate region for PKC varies among the known PKC isoforms, no significant isoform-selective inhibition of particular isoforms by specific pseudosubstrate peptides has yet been shown.

Magainin-2 acts as a substrate for PKC and an analogue, magainin B, can inhibit α- and ß-PKC slightly more selectively than γ-PKC (Nakabayashi *et al,* 1990). However, the inhibitory effect of magainin B is thought not to be mediated solely at the PKC substrate site, but also to involve interference with phospholipid and diacylglycerol effects on the PKC isoforms. Modest isoform differences are also evident when comparing the substrate affinities of α-, ß- and γ-isoforms of PKC (Marais and Parker, 1989; Burns *et al,* 1990). Differences are most evident when using poly (lysine-serine), histone and pseudosubstrate-derived peptides as the acceptor substrates, with γ-PKC tending to have greater affinity for these substrates compared to the α, ßI or ßII isoforms. However, the serine-substituted pseudosubstrate peptide of ε-PKC (ε-peptide) showed more marked differences in affinity as a substrate with isoform-affinities for the substrate being $\alpha > \beta 1 > \gamma$ (Marais and Parker, 1989).

Although the selectivity of PKC-isoforms for these substrates is not large, *in vivo* the differences may be amplified by means of the existence of very highly specific target proteins and their regulated subcellular availability to various PKC isoforms. Differences in the requirements of particular PKC-isoforms for diacylglycerols and other auxiliary endogenous activators (such as AA) and the question of Ca^{2+}-dependence, may also contribute in providing a large specificity for endogenous target proteins in cells (Nishizuka, 1988; Huang, 1989; Parker *et al,* 1989). The contribution of such factors may result in a physiological 'fine-tuning' of PKC function and a possibility of selective pharmacological intervention.

1.2.4. Arachidonic acid: its production, metabolism and actions

Arachidonic acid is a 20-carbon lipid, containing four *cis* carbon double bonds in the 5, 8, 11 and 14 positions and which can be released from the esterified stores of cellular membrane phospholipids by the action of the enzyme phospholipase A_2 (PLA_2) (Needleman *et al,* 1986), (see Figure 1.1). Phospholipase A_2 can act on a wide variety of phospholipids as substrates, and is generally regarded as a Ca^{2+}-dependent enzyme (Shimizu and Wolfe, 1990), although Ca^{2+}-independent forms of PLA_2 have been proposed (Loeb and Gross, 1986) and may represent a Ca^{2+}-independent route of arachidonate production (Axelrod *et al,* 1988). A number of distinct forms of PLA_2 are present in mammalian cells which may serve either signalling or other functions (Clark *et al,* 1991a). Phospholipids (in particular phosphoinositides) may contain arachidonate at the 2-position acyl chain (Meldrum *et al,* 1991), so arachidonate may be released in any response involving PLA_2 activation. There is also some evidence for forms of PLA_2 with significant preference for phospholipids with 2-position arachidonate chains (Clark *et al,* 1991a). Consequently, diglycerides may contain an arachidonic acid acyl chain and phospholipase C-mediated production of diglycerides from phosphoinositides or phosphatidylcholine can occur. Arachidonic acid present in these arachidonyldiglycerides could be liberated by the action of diglyceride lipase

(Irvine, 1982; Loeb and Gross, 1986) although the amount of arachidonate generated through this route in any physiological situation is uncertain. Physiological inhibition of PLA_2 is reportedly achieved by the lipocortin family of proteins which can be induced by glucocorticoids (Flower, 1988); however, a direct action of lipocortin (and related peptides) on PLA_2 has been questioned (Whitehouse, 1989) and it is possible that lipocortins may simply limit availability of substrate for PLA_2. Activation of PLA_2 may be achieved physiologically by a protein known as PLA_2-activating protein (Clark *et al,* 1987; Crooke *et al,* 1989) which has been cloned and may be the endogenous factor which is mimicked by the wasp venom peptide, mellitin in its activation of PLA_2 (Clark *et al,* 1987; Clark *et al,* 1991b). PLA_2-activating protein is also a substrate for PKC, which induces phosphorylation and increased activity of PLA_2-activating protein and thus increases PLA_2 activity (Calignano *et al,* 1991).

As well as second messenger modulation of PLA_2, activation of the enzyme can be driven by receptor-mediated processes. Several receptors including α_1-adrenergic (Burch *et al,* 1986; Han *et al,* 1987; Schimmel, 1988), M_1- and M_3-muscarinic (Conklin *et al,* 1988), NMDA-glutamatergic (Dumuis *et al,* 1988), B_1-bradykinin (Slivka and Insel, 1987) and also the $GABA_B$, vasoactive intestinal peptide and H_1-histaminergic receptors (Axelrod *et al,* 1988) have been shown to stimulate release of AA, as has the light-activation of rod outer segments found in the bovine retina (Jelsema, 1987). As well as protein kinase C-mediated enhancement of ionomycin (a Ca^{2+} ionophore)-induced PLA_2 activity (Halenda and Rehm, 1987; Froissart *et al,* 1989), which may be due to the action of PKC via a G protein (Akiba *et al,* 1990), guanine nucleotide binding (G)-protein activation of PLA_2 has been shown (Nakashima *et al,* 1987). This G protein-mediated activation of PLA_2 was proposed to be caused by the $\beta\gamma$-subunits and not the α-subunit of the G protein-linked receptor-mediated response in rod outer segments, as assessed by adding purified G protein (transducin) subunits and measuring AA production (Jelsema, 1987; Jelsema and Axelrod, 1987; Axelrod *et al,* 1988). Activation of PLA_2 by $\beta\gamma$ subunits was also

implicated in rat atrial membranes, since addition of ßγ subunits induced the opening of a K^+-channel which was alternatively opened by a metabolite of AA (Kim *et al,* 1989). This opening of the K^+-channel by ßγ subunits did not occur in atrial patches incubated with an antibody which blocks PLA_2 activity. Thus receptor-mediated activation of PLA_2 is a mechanism by which various receptors can generate AA, but the exact G protein-mechanism of PLA_2 activation is still controversial (Axelrod *et al,* 1988; Shimizu and Wolfe, 1990), as indeed is the mechanism by which PKC causes activation of PLA_2 (Slivka and Insel, 1987; Akiba *et al,* 1990).

Arachidonic acid (AA) may be produced by mechanisms other than PLA_2 activity on phospholipids, for example *de novo* synthesis or triglyceride metabolism, but once generated, AA can be metabolised to a vast range of bioactive compounds (Shimizu and Wolfe, 1990). The main routes of metabolism of AA are: via the cyclo-oxygenase enzyme (prostaglandin endoperoxide synthase) to generate prostaglandin G_2, the precursor for all prostaglandins and thromboxanes; via the 5-, 12- or 13-lipoxygenase enzymes to generate leukotrienes, lipoxins and hydroperoxy acids; via the cytochrome P_{450} mono-oxygenase complex to epoxyeicosatrienoic acid or via auto-oxidation to hydroperoxy acids. The cyclo-oxygenase, lipoxygenase and cytochrome P_{450} enzymes are sensitive to blockade by a range of more or less selective pharmacological-inhibiting agents such as indomethacin (and other non-steroidal anti-inflammatory drugs), nordihydroguaiaretic acid and piperonyl butoxide respectively (Chang *et al,* 1987; Flower, 1988; Taylor and Clarke, 1986; Luini and Axelrod, 1985).

The range of biological activities of the AA metabolites even exceeds the vast number of AA metabolites thus far detected. AA metabolites have a crucial role in the control of the oestrous cycle in mammals, exerting their effects at the levels of hypothalamus, anterior pituitary, ovary and uterus (for reviews see Poyser, 1977; Poyser, 1978; Behrman, 1979). Arachidonic acid and its metabolites may be involved in the control of pituitary hormone release, since 8,9-EET induces secretion of oxytocin and 5,6-EET causes secretion of somatostatin from the median eminence and secretion

of thyroid stimulating hormone, prolactin and luteinizing hormone (LH) from pituitaries (Ojeda *et al,* 1989; Shimizu and Wolfe, 1990). The AA metabolites LTC_4 and PGE_2 evoke luteinizing hormone-releasing hormone (LHRH) release from the median eminence and LH release from anterior pituitary, whereas PGD_2 inhibits LHRH release as well as inhibiting release of prolactin and LH from pituitaries (Kinoshita *et al,* 1982; Ogeda *et al,* 1982; Hulting *et al,* 1985). Hedqvist (1977) was one of the first to introduce the concept that AA and its metabolites could leave cells and interact with extracellular eicosanoid receptors to act as local messengers for neighbouring cells. However, use of a range of inhibitors of AA metabolism have excluded the effect of eicosanoids in the present studies, and although it is well established that metabolites of AA may have a role in hypothalamic-pituitary function as well as a variety of physiological processes but presumably not in the present studies.

It has now become clear that AA does not always have to be metabolised to have a biological action. Both McPhail *et al* (1984) and Seifert *et al* (1987) showed that AA itself, as well as its metabolites, could activate protein kinase C extracted from whole brain. Nishizuka and his co-workers further characterised this AA-mediated activation of PKC in cell-free systems to show that the γ-isoform (and to a lesser extent the α-and ß-isoforms) of PKC was potently activated by AA and some of its metabolites, notably 12-HPETE and Lipoxin A4 (12(5)-hydroxy-5,8,10,14-eicosatetraenoic acid and 5(S),6(R),15(S)-trihydroxy-7,9,13-*trans*,11-*cis* eicosatetraenoic acid respectively) (Naor *et al,* 1988a; Shearman *et al,* 1989a). The AA-induced activation of α-PKC was greater than ß-PKC but both were concentration-dependent and apparent only in the presence of calcium, however AA activation of γ-PKC was biphasic with highest activation occurring at 12 μM AA and also occurred in the absence of free calcium ions (Naor *et al,* 1989a). Inhibition of Ca^{2+}/calmodulin-dependent protein kinase II can also occur with both AA and its metabolites in cell free systems, without any inhibition of type I or III Ca^{2+}/calmodulin kinase or cyclic AMP-dependent protein kinase (Piomelli *et al,* 1989).

Direct activation of smooth muscle K^+-channels by AA and its metabolites has been shown in reconstituted vesicles (Ordway *et al,* 1989), which does not require metabolic conversion of AA or its activation of G-proteins or kinases. Similar results were seen in atrial K^+-channels (Kim *et al,* 1989; Kurachi *et al,* 1989) although the action of AA to enhance the channel activity could be ascribed to a metabolite. Airway epithelial Cl^--channels are inhibited by both AA and its metabolites (Hwang *et al,* 1990; Anderson and Welsh, 1990) but this may be due to an indirect effect on the channel via kinase enzymes. Squid giant axon Na^+-currents are also inhibited by AA and its metabolites but only at very high concentrations, where these lipids may be affecting the membrane environment surrounding the channel and thus affecting its activity (Ordway *et al,* 1991). Likewise, AA inhibits glutamate uptake into glial cells possibly by increasing membrane fluidity (Barbour *et al,* 1989), a phenomenon which may perhaps contribute to anoxia-induced neuronal death and neuronal long term-potentiation mechanisms.

Arachidonic acid has been reported to both stimulate phosphoinositide hydrolysis (Murphy and Welk, 1989; Negishi *et al,* 1990) and to inhibit phosphoinositide hydrolysis (Chaudhry *et al,* 1989) through a mechanism which is unresponsive to the metabolising enzyme inhibitors, indomethacin and nordihydroguaiaretic acid. The exact mechanisms for these AA-mediated actions is uncertain, however as PI-PLC may be a Ca^{2+}-stimulated enzyme (Meldrum *et al,* 1991), a raised intracellular calcium concentration could lead to phosphoinositide hydrolysis. In a mechanism which is independent of phosphoinositide hydrolysis, AA itself (Chan and Turk, 1987; Beaumier *et al,* 1987) as well as its metabolites and linoleic acid, but not arachidic acid (Chow and Jondal, 1990) can release intracellularly stored Ca^{2+} from a pool(s) which includes the inositol 1,4,5-trisphosphate-sensitive pool, leading to a raised intracellular Ca^{2+} concentration. The Ca^{2+}-mobilizing effect of AA described in these reports was apparently not caused by compromising membrane fluidity/permeability (assessed using other lipid analogues of AA), or by

inhibiting the Ca^{2+}-ATPase Ca^{2+} extrusion mechanism (since AA did not affect Ca^{2+}-ATPase activity).

In summary, AA can be metabolised to a range of compounds which have major roles in cell function. Alternatively, AA can act itself (without being metabolised), on a range of signalling processes. Both AA and some of its metabolites have the ability to activate PKC(s) and thereby any cellular process which generates AA has the potential to activate PKC through a non-diacylglycerol-dependent route.

1.2.5. Alternative routes of diglyceride and fatty acid production

Classically, the routes of generation of diacylglycerol and free fatty acid (such as AA) were considered to be through PI-PLC and PLA_2 activity respectively (as described in sections 1.1 and 1.2.4). However, there is increasing evidence that diacylglycerols and fatty acids (including AA) can be produced through mechanisms other than those outlined previously. The next section will focus on the regulation and function of the pathways which could lead to the activation of protein kinase C by means other than PI-PLC-induced diacylglycerol production or PLA_2-induced arachidonic acid production.

In 1981, Mufson *et al* showed in mouse fibroblasts labelled with [^{3}H]-choline, that stimulation with phorbol esters caused a released of radioactive choline and phosphocholine. They concluded from this work that the source of this choline was labelled phosphatidylcholine (PC). Phorbol esters (as well as insulin, insulin-like growth factors I and II, vasopressin and thyrotropin-releasing hormone can stimulate the synthesis of PC (Warden and Friedkin, 1985; Kolesnick, 1987) by a mechanism which involves the translocation from cytosol to endoplasmic reticulum and activation of the rate-limiting enzyme for PC synthesis, cytidine triphosphate: phosphocholine cytidyltransferase (CT) (Pelech and Vance, 1984). However, the importance of this receptor-stimulated PC synthesis is unclear as phorbol esters have also been shown to stimulate the breakdown of PC and the production of lyso-PC,

phosphatidic acid, diacylglycerol, phosphocholine and choline (Ito and Klein, 1987; Cabot *et al,* 1988; Pelech and Vance, 1984; Grove and Schimmel, 1982; Daniel *et al,* 1986) and whether the PC synthesis is required just for replenishment or is secondary to its hydrolysis is unknown.

It is now clear that not only can phospholipase D (PLD)-induced hydrolysis of PC occur (to produce phosphatidic acid and choline) but also PLD-induced hydrolysis of phosphatidylethanolamine and phosphoinositides, as well as PLC-induced hydrolysis of PC and phosphatidyethanolamine can occur (Billah and Anthes, 1990) (see Figure 1.1). In addition to phorbol ester-stimulated PC hydrolysis, agonist-stimulated PC breakdown has also been shown. In 1985, Bocckino *et al* published evidence that, in vasopressin-stimulated hepatocytes, diacylglycerol formation was occurring through a pathway in addition to that through a phosphatidylinositol-specific phospholipase C (PI-PLC) pathway. The diacylglycerol produced from PI-PLC activity was enriched in arachidonate and stearate whereas the vasopressin-stimulated diacylglycerol was composed mainly of palmitic, oleic and linoeic acid side chains. The same agonist-stimulation of PC hydrolysis could also be shown by adrenaline and angiotensin-II. Furthermore, it was observed that the time course for formation of these non phosphoinositide-derived diacylglycerols was delayed; being markedly later than the production of diacylglycerols by stimulation with other Ca^{2+}-mobilising agents which primarily caused activation of PI-PLC and subsequently a peak-production of inositol 1,4,5-triphosphate (IP_3) (Charest *et al,* 1985), although the concentration-dependence of both vasopressin-induced diacylglycerol and IP_3 production was similar. It was concluded that diacylglycerol production caused by vasopressin was occurring via hydrolysis of some additional phospholipid other than phosphoinositide as substrate (perhaps PC) (Bocckino *et al,* 1985). Thus, receptor stimulation of PC hydrolysis was occurring, and it was later clarified that the resulting production of phosphatidic acid was not occurring through *de novo* incorporation of radioactivity into diacylglycerol and its conversion then to phosphatidic acid, but being instead

D (PLD) mechanism (Bocckino *et al,* 1987) which produces free choline and phosphatidic acid directly from PC (Pelech and Vance, 1989).

Phosphatidic acid can be readily dephosphorylated by the enzyme phosphatidic acid phosphohydrolase (which is actually activated by PKC) to produce diacylglycerol - another PKC activator (Billah and Anthes, 1990; and see Figure 1.3). The extent to which phosphatidic acid to diacylglycerol is a requirement for receptor-mediated responses and the extent to which PLD-generated diacylglycerols have the ability to activate PKC are issues of current controversy (Billah *et al,* 1991; Leach *et al,* 1991). Diacylglycerol may also be produced by mechanisms other than those outlined above. For example, by *de novo* synthesis or by triglyceride metabolism into diglycerides, albeit with a stereo-configuration which may have no influence on PKC activity.

Although Bocckino *et al* (1985) did not demonstrate an agonist which produces hydrolysis of phosphoinositides only (and not PC), cases of receptor-mediated PC-hydrolysis without phosphoinositide-hydrolysis are reported, for example by interleukin-1 and interleukin-3, which both apparently stimulate PC hydrolysis (Roscoff *et al,* 1988; Pelech and Vance, 1989) without hydrolysing phosphoinositides (Roscoff *et al,* 1988; Whetton *et al,* 1988) thereby producing diacylglycerol which may activate PKC without generating inositol phosphates, as occurs in PI-PLC activity. However, the agonists which generate PC hydrolysis (via a PLC or PLD activity) are generally those which can also activate PI-PLC activity (Pelech and Vance, 1989; Löffelholz, 1989; Billah and Anthes, 1990; Exton, 1990; Billah *et al,* 1991).

Although PC hydrolysis by PLC or PLD can be induced as a secondary consequence of the action of PKC presumably activated by PI-PLC activity (Löffelholz, 1989; Kiss and Anderson, 1989; Billah *et al,* 1989; Exton, 1990; Martin *et al,* 1990; van Blitterswijk *et al,* 1991a; van Blitterswijk *et al,* 1991b; Billah *et al,* 1991). Protein kinase C-independent (Billah *et al,* 1989; Billah *et al,* 1991) as well as 'partially' PKC-independent (Sandeman *et al,* 1991) hydrolysis of PC by PLC or PLD,

has been reported. Therefore, it is uncertain whether receptor-mediated PLC or PLD hydrolysis of PC (or phosphoinositides or phosphatidylethanolamine (Billah and Anthes, 1990)) occurs as a consequence of initial PI-PLC activity (which raises intracellular Ca^{2+} and activates PKC) in all systems. Indeed, expression of the ß1 isoform of PKC in fibroblasts enhanced PLD-mediated hydrolysis of PC (Pai *et al,* 1991), whereas α-thrombin stimulation of fibroblasts leads to phosphoinositide plus PC hydrolysis at higher concentration of α-thrombin, but only PC hydrolysis occurs at lower agonist concentrations of α-thrombin (Leach *et al,* 1991). Therefore, it seems that receptor-mediated PC hydrolysis (and generation of diacylglycerol to activate PKC) can occur separately from receptor-mediated phosphoinositide hydrolysis, and although PKC-induced PC-specific PLC or PLD activation may occur, PC hydrolysis can also occur independently to produce diacylglycerol without a concomitant production of Ca^{2+}-mobilizing inositol phosphates.

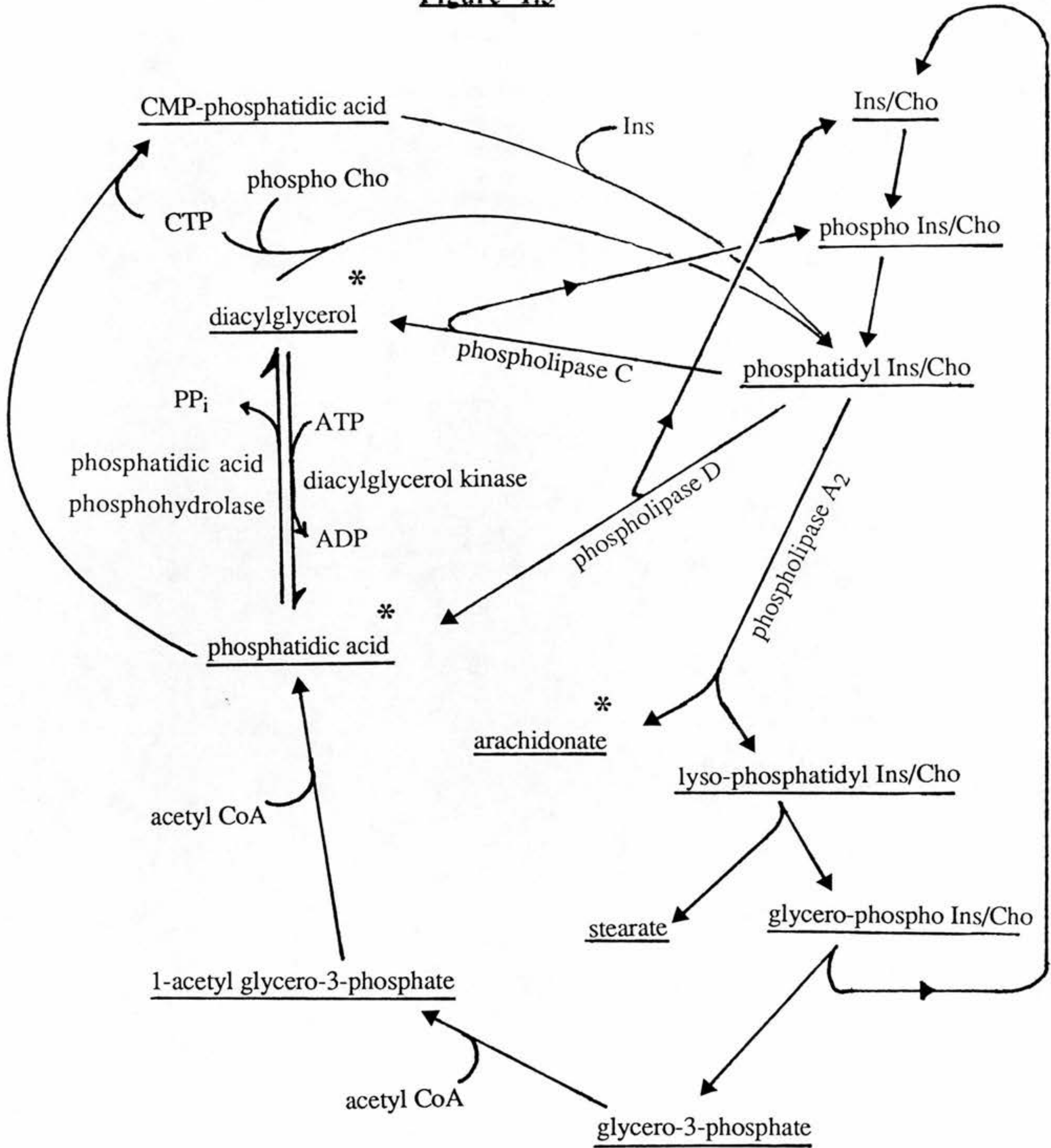
Billah and Anthes (1990) have proposed that multiple forms of PLD exist because of the variation in modulation of PLD found in different cell types and activated by a wide variety of hormones, growth factor, etc. It appears that Ca^{2+} influx, activation of G proteins with non hydrolysable GTP analogues and activation of PKC by phorbol esters or diacylglycerols can all play a role in enhancing PLD activity.

It has been proposed that phorbol esters may have some activity in activating PLD in a process which is independent of PKC (Billah and Anthes, 1990). Diacylglycerols have also been proposed to act independently of PKC to stimulate PLA_2 activity or to stimulate PC synthesis involving activation of phosphocholine cytidyltransferase (CT), but curiously, these actions of diacylglycerol were not matched using phorbol esters instead of diacylglycerol in GH_3 cells (Kolesnick and Paley, 1987; Kolesnick and Hemar, 1990) and the physiological significance as to this action of diacylglycerol can only be pondered. Phorbol ester can stimulate PLD activity which is independent of direct phosphorylation, or which is not inhibited by PKC inhibitors (Billah and Anthes, 1990), but a direct action of PKC on PLD is not necessary for

Figure 1.3. Diagram of the possible routes of diacylglycerol and free fatty acid production.

The production of diacylglycerol and arachidonic acid can lead to the activation of PKC, although which isoforms are preferentially activated by specific lipids is at present uncertain. The free fatty acids shown are arachidonic acid and stearic acid, but the species of fatty acid generated clearly depends on the acyl chain composition of the phospholipid from which the molecule is derived. The molecules which are indicated by an asterisk are those which have been previously shown to activate PKC. The figure is adapted from Pelech and Vance (1989).

Figure 1.3



PKC-mediated enhancement of PLD activity. However, the possibility that phorbol esters may directly activate PLD cannot be ignored. The PKC-independent effects of diacylglycerols mentioned above may be reflected in the observation that both phorbol esters and diacylglycerols can activate the CT enzyme, whereas only diacylglycerols (at > 58 μM) caused a characteristic translocation of the enzyme, but this may be due to a lipid-diluting effect of the diacylglycerol (Kolesnick and Hemar, 1990), leading to the conclusion that the diacylglycerol is acting independently of PKC. If the lipid-dilution effect of diacylglycerol accounts for its non-PKC activity which is not seen with phorbol ester, then it may be an action of the diacylglycerol which is in additional to its PKC-activation properties, which led to the inference that PLA_2 activity was PKC-independent too (Kolesnick and Paley, 1987). However, it is clear that diacylglycerol-activated PKC could alternatively cause a secondary activation of PLA_2 as has been seen in other systems (Halenda and Rehm, 1987; Froissart *et al,* 1989; Akiba *et al,* 1990).

The choline which is produced by PLD hydrolysis of PC has no known signalling function (except for acetylcholine resynthesis (Hattori and Kanfer, 1985; Pelech and Vance, 1989)), however, phosphatidic acid has been shown to partially activate PKC in cell free systems (Huang *et al,* 1988) which may explain several second messenger roles attributed to phosphatidic acid which include acting as a Ca^{2+}-ionophore or at least enhancing agonist-mediated Ca^{2+} entry (Serhan *et al,* 1981; Putney *et al,* 1980) or that it may have an involvement in secretory processes as a 'fusogen' (Simmonds and Halsey, 1985; Liscovitch and Amsterdam 1989).

There exist phospholipase C (PLC) enzymes which are not specific for phosphoinositides and can hydrolyse PC (Edgar and Freysz, 1982; Irving and Exton, 1987; Clark *et al,* 1986; Martin *et al,* 1987) as well as PLC enzymes which can hydrolyse phosphatidylethanolamine and phosphatidylglycerol in addition to phosphoinositides and PC (Matsuzawa and Hostetler, 1980). The PC-hydrolysing phospholipase C enzymes are not as extensively studied as the PI-PLC isoenzymes, but

initial studies indicate that PC-hydrolysing PLC may be similar to PLD in terms of the ways it can be modulated (ie, receptor-linked activation, possibly involving multiple factors including Ca^{2+}, diacylglycerol, protein kinase C and a G protein(s) (Billah and Anthes, 1990; Exton, 1990; Billah *et al,* 1991). Hydrolysis of PC by PC-hydrolysing PLC will produce phosphocholine (again with unknown function) and, like PI-PLC activity, diacylglycerol, but in this case of course with a fatty acid composition which could be dissimilar to the diacylglycerol preferentially produced by PI-PLC activity (Thomson, 1969; Bell *et al,* 1979). Phosphatidylcholine can compose up to 50% of total cellular phospholipid content and may be the source by which saturated diacylglycerol production can be achieved, as the phosphoinositide pool is more limited (Billah and Anthes, 1990). It also appears that the potential consequence of PC hydrolysis (that is PKC activation without concomitant Ins(1,4,5)P_3-induced rise in intracellular calcium), could be achieved, thus allowing a significantly different profile of intracellular consequences from those elicited by phosphoinositide hydrolysis (outlined in Parker *et al,* 1990).

The presence of diacylglycerol is thought to be the principal means by which activation of PKC can occur. A major route of diacylglycerol removal is its phosphorylation by the enzyme 1,2-diacylglycerol kinase to convert the second messenger molecule into phosphatidic acid (PA) (Hokin and Hokin, 1963; Lapetina and Hawthorne, 1971). An inhibitor of diacylglycerol kinase, R59022 (6-[2-[4-[(4-fluorophenyl) phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5*H*-thiazolo[3,2-α]pyrimidin-5-one) has been used to raise intracellular diacylglycerol levels (de Chaffoy de Courcelles *et al,* 1985; Nunn and Watson, 1987; Muid *et al,* 1987), however, serious concern as to the selectivity of this agent have been expressed by others (Mahadevappa and Sicilia, 1988; Nasmith and Grinstein, 1989; Joseph and Krishnamurthi, 1989). The generated PA can then be recycled into forming phosphoinositides via a cytidine monophosphate (CMP)-phosphatidic acid intermediate. The CMP moiety is replaced by inositol to then generate

phosphatidylinositol, the precursor for various phosphoinositides (see Figure 1.1; Michell, 1975; Downes and Michell, 1985). Diacylglycerol, in addition to activating PKC and being phosphorylated to phosphatidic acid, can also be a substrate for lipase activity, which would liberate the acyl chains which compose the diacylglycerol (Chau and Tai, 1981; Irvine, 1982; Downes and Michell, 1985; Axelrod *et al,* 1988).

Diacylglycerol lipase activity followed by monoglyceride lipase activity is able to deacylate the fatty acid-acyl chains form the glycerol backbone of a diacylglycerol (Bell *et al,* 1979; Chan and Tai, 1987), thus generating the free fatty acids which composed the diacylglycerol molecule. Diacylglycerol lipase is a poorly investigated enzyme and its substrate specificities are not fully understood (Bell *et al,* 1979) although diacylglycerol containing stearic acid in the 1-position plus arachidonic acid in the 2-position is the major diglyceride derived from phosphoinositide hydrolysis (Thomson, 1969; Bell *et al,* 1979), at least in brain and platelets. Thus, diacylglycerols can be degraded into their separate fatty acid from which they are composed (in the case of phosphoinositide-derived diacylglycerol, usually 1-steroyl-2-arachidonoyl-*sn*-glycerol). Clearly the most important fatty acid that might be liberated by this pathway would be arachidonic acid (AA) which, amongst other actions, serves as the precursor for eicosanoid synthesis (see section 1.2.4). Protein kinase C activation by AA can occur (MacPhail *et al,* 1985; Seifert *et al,* 1988; Naor *et al,* 1988a), but other fatty acids such as linoleic acid, oleic acid and palmitoleic acid are also capable of activating PKC (Seifert *et al,* 1988). Since the glyceride chain composition of diacylglycerol varies depending on the source of phospholipid it was derived from (for example vasopressin stimulates the accumulation of diglyceride species with various acyl complements in hepatocytes, presumably through PC hydrolysis (Bocckino *et al,* 1985)), then not only AA, but a range of potentially relevant fatty acids may be produced by the action of the diacylglycerol lipase pathway, although the relative contribution of this pathway in signalling events is difficult to assess. A useful tool in assessing the contribution of the diglyceride lipase pathway in cell systems is the diglyceride lipase inhibitor 1,6-di(0-

(carbamoyl)cyclohexaneoxime)hexane (RHC80267). Chang *et al* (1988) found that luteinizing hormone-releasing hormone-induced hormone release from pituitary was markedly inhibited by RHC80267, indicating a significant role of the diglyceride lipase pathway. However, concerns as to the specificity of RHC80267 were expressed and it seems most likely that the diacylglycerol kinase pathway may be the major pathway of diacylglycerol removal, as has been implicated earlier (Downes and Michell, 1985).

In conclusion, diacylglycerol production cannot occur only from phosphoinositide breakdown, but also from the hydrolysis of other phospholipids which are present in the membranes of cells. However, the metabolism of diacylglycerol does not definitely terminate its PKC-activating function, as several of the possible lipid metabolites of diacylglycerol (PA, AA and other free fatty acids) are also capable of activating PKC.

1.3. MECHANISMS OF CELLULAR Ca^{2+} ENTRY

Regulation of the intracellular calcium concentration is crucial for a variety of cellular actions such as contraction, secretion, energy metabolism, neurite outgrowth/retraction and gene transcription (Shearman *et al,* 1989b). Calcium is also a major signal for the activation of Ca^{2+}/calmodulin-dependent protein kinase and for the activation of protein kinase(s) C (A series at least). Changes in intracellular calcium concentrations can be achieved by mobilisation of intracellular calcium stores (Berridge and Irvine, 1984) or by increasing the permeability of the plasma membrane to allow calcium ions to flow down their concentration gradient into the cell (Meldolesi and Pozzan, 1987). A super family of ion channels exist, and molecular cloning analysis of their primary sequence enables comparison of all channels and their division into smaller groups. Voltage-operated (such as Na^{+}, K^{+}, and some Ca^{2+} channels) and ligand-operated channels (such as the nicotinic acetylcholine (a non-specific cation conductor) and $GABA_A$ receptor associated channels) show marked diversity between the two groups but both have conserved regions which are considered to form into

transmembrane α-helical segments, that form a ring structure to act as the channel pore (Maelicke, 1988; Catterall, 1988). Influx of Ca^{2+} is known to occur either through voltage-operated or ligand-operated calcium channels which can be further divided into two subgroups: receptor-operated channels such as the ATP-activated Ca^{2+} channels found in rabbit ear artery smooth muscle (Benham and Tsien, 1987); or second-messenger-operated channels like those altered by Ca^{2+} in neutrophils (Von Tscharner *et al,* 1986), thrombin-activated channels in platelets (Zschauer *et al,* 1988), and the inositol-1,4,5-trisphosphate-sensitive Ca^{2+} channels in T-lymphocytes and mast cells (Kuno and Gardner, 1987; Penner *et al,* 1988).

Voltage-sensitive calcium channels are known to be quite heterogeneous in nature (Miller, 1987; and see Table 1.2). Tsien, Fox and their colleagues defined at least three types of calcium currents in chick dorsal root ganglion neurones (Nowycky *et al,* 1985). These three different types of calcium currents were classified as T (transient), N (neuronal) and L (long-lasting). Each of these three voltage-dependent currents had characteristic conductances and activation/inactivation states as well as varying sensitivity to calcium-channel-blocking agents. Weak depolarisations of the dorsal root ganglionic neurone membrane potential to around or above -70 mV induces the T-type component of the calcium currents. This T-type current is known to have a conductance level of approximately 8 pS (defined in single channel patch-clamp recordings) which is resistant to dihydropyridines and is poorly and reversibly blocked by ω-conotoxin (Tsien *et al,* 1988). As the name suggests, T (transient)-Ca^{2+} channels have a rapid inactivation rate (tau approximately 20 - 50 ms) which is in a voltage-dependent manner (close faster at more depolarised potentials), but quickly reactivate following repolarisation of the cell. Nickel ions are more potent blockers of T-type Ca^{2+} conductances than Cd^{2+} ions. The L-type Ca^{2+} channels in dorsal root ganglion neurones have a much larger conductance (approximately 25 pS) than T-channels, and inactivate only very slowly (tau > 500 ms) and single-channel analysis on cardiac L-type Ca^{2+} channels shows that a depolarising prepulse can in fact increase the

probability of open time for the channels (Pietrobon and Hess, 1990). Thus, of any calcium influx caused in response to cell depolarisation, the majority of the current may be carried by L-channels, but only if the depolarisation is strong enough, as the L-type channel opens between -10 and +20 mV membrane potential. The L-type Ca^{2+} channel is sensitive to the dihydropyridine calcium channel modifiers of which there are antagonists, such as nimodipine, or agonists, such as the compound BAY K8644 as well as being blocked by the phenylalkylamine and benzothiazepine classes of compounds (Hoffman *et al,* 1987). L-type calcium channels are more sensitive to block by Cd^{2+} ions than Ni^{2+} ions, and are resistant to block by ω-conotoxin (Tsien *et al,* 1988). The 'N'-type current was defined as being neither 'T' or 'L' and channel opening occurred at relatively intermediate depolarisations of membrane potential to around -20 mV. The single channel analysis of the 'N'-current revealed a conductance of approximately 13 pS with a moderate inactivation rate (tau approximately 50 - 80 ms). Like 'T'-channels, 'N'-currents were resistant to dihydropyridine modulation, but like 'L'-, 'N'-channels were more sensitive to blockade by Cd^{2+} ions than Ni^{2+} ions and ω-conotoxin induced a persistent block of the channel. All three types of channel are activated by depolarisations greater than or equal to their individual thresholds for activation, but are strongly inactivated by membrane potentials more negative than the threshold levels. The 'N' and 'L' channels preferentially conduct Ba^{2+} rather than Ca^{2+}, whereas 'T' channels allow Ba^{2+} and Ca^{2+} to pass without preference.

However, as with almost all pharmacological tools, the dihydropyridines have non-specific sites of action. The effect of dihydropyridines on 'L'-type calcium channels display a very high affinity (Kd in the nanomolar range) but dihydropyridines are also known to interact at lower affinity with many other cellular entities including: nucleoside transporters; Na^{+}/K^{+} ATPase; Ca^{2+}-ATPase; Na^{+}-Ca^{2+} exchanger; calmodulin; cyclic AMP phosphodiesterase and the Ca^{2+}-H^{+} antiport enzyme (Zemig,

Table 1.2. Table of the pharmacological properties of the known voltage-activated calcium channels.

The data for this table were compiled from that of Tsien *et al* (1988; 1991).

Table 1.2

Voltage-activated Ca^{2+}-channel type	'L'	'N'	'T'	'P'	'BI'
Approximate single channel conductance (with Ba^{2+} as charge carrier)	25 pS	13 pS	8 pS	0.2 pA	1 pA
Relative conductance	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} = Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} > Ca^{2+}$
Activation range (mV)	-40 to positive	-20 to positive	-70 to positive	-60 to positive	-10 to positive
Approximate inactivation rate (tau)	very slow (> 500 ms)	moderate (50 - 80 ms)	rapid (20 - 50 ms)	very slow (> 500 ms)	slow (200 ms)
Dihydropyridine-sensitivity	sensitive	resistant	resistant	resistant	modest inhibition with Bay K 8644, no effect of nifedipine
ω-conotoxin-block	resistant	persistent	weak, reversible	resistant	resistant
Crude funnel web spider-sensitivity	sensitive	sensitive	sensitive	sensitive	sensitive

1990). Considering the affinity of dihydropyridines for a cationic channel, it may not be surprising that these agents also have some affinity for other ionic-interacting structures found within cells. Interestingly, dihydropyridines also have affinity for the non L-type Ca^{2+} channel found in osteoblasts (Guggino *et al,* 1988; Guggino *et al,* 1990) and various differences exist in the modulation of L-type channels (Hoffman *et al,* 1987) suggesting possibly more than one type of L-channel, which now seems certain due to the slight differences found between purified 'L'-type channels from brain, skeletal and cardiac muscle (Tsien *et al,* 1991) Thus care must be used if classifying dihydropyridine-sensitive Ca^{2+} channels as the 'L'-type Ca^{2+} channel described above.

In 1986, Flockererzi *et al* purified the dihydropyridine binding site from skeletal muscle t-tubules which consisted of three peptides with different relative molecular masses, and showed it to be a functional calcium channel when incorporated into phospholipid vesicles. Tanabe *et al* (1987) elucidated the primary amino acid sequence of part of the dihydropyridine-binding site (the ion channel portion) from the complementary DNA of its messenger RNA and later showed that expression of the complementary DNA for the complete dihydropyridine binding site could restore the excitation-contraction coupling and slow calcium current that was absent in cultured skeletal muscle cells from mice with muscular dysgenesis (Tanabe *et al,* 1988). The dihydropyridine-binding calcium channel is known to consist of at least four (perhaps five) subunits designated the α_1, α_2, ß, γ (and δ) (Campbell *et al,* 1988). The complete primary structure of all of these subunits has not been determined but the α_1 subunit is known to contain the dihydropyridine binding domain and may be the ion-conducting and voltage-sensing unit of the channel. The α_1 and ß subunits of this 'L'-type calcium channel contain sites for phosphorylation by cyclic AMP-dependent protein kinase and protein kinase C. The α_1 subunit can also be phosphorylated by a Ca^{2+}/calmodulin-dependent protein kinase and the γ subunit may also have a regulatory role in the channel activity (Campbell *et al,* 1988). From functional studies it appears

that phosphorylation of this channel by cyclic AMP-dependent protein kinase is necessary for normal responses of the channel to membrane depolarisation and that such phosphorylation by cyclic AMP-dependent protein kinase enhances and maintains the 'L'-type channel activity (Armstrong and Eckert, 1987; Hoffman *et al,* 1987). Calcineurin, a calcium-activated phosphatase (Armstrong, 1989) which is present in GH_3 cells (Faber *et al,* 1987), acts to reduce the dihydropyridine-sensitive channel activity by removal of phosphate from the α_1-subunit of the channel. 'L'- and 'T'-type Ca^{2+}-channels are present in GH_3 cells, and reduce their channel activities upon PKC phosphorylation (Marchetti and Brown, 1988). Whereas 'L'-type channels decrease activity to PKC phosphorylation in GH_3 cells, and in other tissues (DiVirgilio *et al,* 1986; Lewis and Weight, 1988; Rosenthal *et al,* 1988; Rane *et al,* 1989) phosphorylation of 'L'-channels by PKC in other systems leads to an increase in channel activity (DeRiemer *et al,* 1985; Strong *et al,* 1987; Fish *et al,* 1988; Velasco and Peterson, 1989).

Another regulatory mechanism apparent in dihydropyridine-sensitive calcium channel has been described by Dolphin and Scott (1988) and is the GTP-dependence of dihydropyridine-agonist enhancement of 'L'-type channel activity. Internal bathing of whole cell, voltage-clamped dorsal root ganglionic neurones with a non-hydrolysable analogue of GTP, GTP-γ-S (a G-protein activator (Graziano and Gilman, 1987)) massively enhanced Bay K8644 potentiation of barium currents. Curiously, pretreatment of the cells with pertussis toxin to inactivate certain G-proteins (Graziano and Gilman, 1987), causes Bay K8644 to reduce the channel currents. It was concluded that dihydropyridines can act as agonists or antagonist on 'L'-type Ca^{2+} channels depending on the inactivation state of the channel and that this channel inactivation state is governed by pertussis toxin-sensitive G-proteins as well as membrane potential.

These conclusions do not take into account more recently discovered Ca^{2+}-channels which has activation characteristic similar to the 'L'-type channel (Mori *et al,*

1991; Tsien *et al,* 1991; and see Table 1.2). These newly-discovered Ca^{2+}-channels could be found in brain and heart tissue and were designated as the 'P'-type and 'BI'-type channels. Like the 'L'-type Ca^{2+}-channel, these channels activated at high depolarisations with relatively slow inactivation kinetics. However, unlike the 'L'-channel, the 'P'- and 'BI'-channels are mostly insensitive to modulations by dihydropyridines and unlike the 'N'-type Ca^{2+}-channel, the 'P'- and 'BI'-channels are insensitive to ω-conotoxin and have slow inactivation kinetics. Therefore voltage-sensitive Ca^{2+}-channels cannot simply be characterised as 'N'-, 'L'- or 'T'-types and the more recently discovered 'P'-type and 'BI'-type Ca^{2+}-channels must also be considered, however functional studies on these channels (eg, effect of phosphorylation) have still to be published.

Along these lines, are other studies on the 'L'-type calcium channels in dorsal root ganglion neurones where neuropeptide Y and bradykinin can inhibit the voltage-activated Ca^{2+} currents (Ewald *et al,* 1988; Ewald *et al,* 1989). Pretreatment of the cells with pertussis toxin abolishes the hormonal inhibition of the calcium-currents and hormone-stimulated channel inhibition can only be restored to varying extents by reconstitution with one or more purified G-protein α-subunits (α_0, α_i1, and α_i2). G_0-like proteins have also been implicated in the regulation of Ca^{2+} currents by opioids in neuroblastoma x glioma hybrid cells (Hescheier *et al,* 1987) and by dopamine in snail neurons (Harris-Warrick *et al,* 1988). It is not clear whether these G-protein subunits interact directly with the channel or an associated structure or via some other secondary messenger system. Thus 'L'-type Ca2+ channels are heavily modulated ion channels.

Stimulation of phosphoinositide hydrolysis by receptors results in the production of two second messengers: a diacylglycerol messenger (for example 1-stearoyl-2-arachidonoyl-*sn*-glycerol) which stimulates protein kinase C activity (Nishizuka, 1984a); and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) which stimulated release of intracellular Ca^{2+} into the cytoplasm by binding to its integral receptor/ion channel complex found in the membranes of a fraction of the endoplasmic reticulum

(Berridge and Irvine, 1989). Emptying of these $Ins(1,4,5)P_3$-sensitive stores of their calcium was proposed by Putney (1986) to be intimately linked with the Ca^{2+} influx into the cell that was seen during receptor stimulated phosphoinositide hydrolysis. Controversy surrounds the exact mechanisms by which receptor stimulation leads to the influx of Ca^{2+} across the plasma membrane. The influx of extracellular calcium may or may not be a consequence of emptying of calcium stores by agonist-stimulated $Ins(1,4,5)P_3$ (Taylor, 1990).

Unlike ATP-stimulated Ca^{2+} currents in rabbit ear artery smooth muscle cells (Benham and Tsien, 1987), which is thought to be a receptor/calcium channel complex as its activation does not require the action of a soluble second messenger, other types of receptor-mediated calcium influx, such as thrombin or histamine-stimulated Ca^{2+} influx in human umbical-vein endothelial cells (Hallam *et al,* 1988), requires the preliminary action of receptor-produced $Ins(1,4,5)P_3$ to empty calcium stores. Indeed, thrombin-stimulated calcium channels have been isolated electrophysiologically (Zschauer *et al,* 1988). This latter, second-messenger-mediated type of calcium influx may occur through gap junctions (Taylor, 1990) and/or various calcium pools (eg, $Ins(1,4,5)P_3$-, caffeine- or thapsigargin-sensitive calcium stores) which may communicate or require modulation by GTP-binding proteins (Gill *et al,* 1986) in order to refill $Ins(1,4,5)P_3$-depleted stores, but the true route of calcium entry is unknown, and may depend on the agonist which stimulates the receptor-operated calcium entry (Rink, 1991). Morris *et al* (1987) showed that an inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$), a metabolite of $Ins(1,4,5)P_3$ by $Ins(1,4,5)P_3$-3-kinase (a Ca^{2+}/calmodulin-dependent enzyme) was necessary for receptor-stimulated calcium influx and acts in unison with $Ins(1,4,5)P_3$. Irvine (1991) has also proposed that the $Ins(1,4,5)P_3$ and the $Ins(1,3,4,5)P_4$ receptor sites communicate to control the amount of extracellular-calcium influx into $Ins(1,4,5)P_3$-sensitive calcium stores. However, $Ins(1,3,4,5)P_4$ decays with a half-life of 40 s after termination of a muscarinic stimulation in the parotid gland (Hughes *et al,* 1988) yet Ca^{2+} pools that are

emptied by stimulation in the absence of extracellular Ca^{2+} refill similarly whether Ca^{2+} is restored rapidly or 20 minutes after addition of atropine (Takemura and Putney, 1989). Furthermore, in acinar cells, application of Ins(2,4,5)P_3, an analogue which can release intracellular calcium stores but is poorly metabolised by the 3-kinase enzyme (resulting in little or no Ins(2,3,4,5)P_4 production), was sufficient to induce Ca^{2+} entry into the cells (Bird *et al,* 1991). However, only small amounts of Ins(2,3,4,5)P_4 may be necessary to facilitate Ins(2,4,5)P_3-induced calcium entry. Hence, although Ins(1,4,5)P_3 is responsible for emptying Ca^{2+} stores, neither it nor Ins(1,3,4,5)P_4 may be directly involved in controlling calcium entry but may have a modulatory role in receptor-stimulated calcium influx (Hallam and Rink, 1989; Berridge and Irvine, 1989).

The development of receptor-operated calcium entry inhibitors such as SKF 96365 (Rink, 1991) will contribute to a better understanding of the mechanisms involved. Unfortunately SKF 96365 has poor specificity, being able to block voltage-operated calcium channels also. So it seems that at least for the near future, the mechanisms of second-messenger-mediated Ca^{2+} influx will remain to be poorly understood and will continue to be a subject of active investigation.

1.4. ANTERIOR PITUITARY CELLS

The pituitary gland (or hypophysis) is attached to the base of the brain by the hypophysial stalk which is itself composed of pituitary tissue and which brings the gland into a close anatomical and functional relationship with the hypothalamic region of the central nervous system (Harris, 1955). The endocrine function of the posterior pituitary is essentially governed by innervating nerves which originate from the paraventricular and supraoptic nuclei regions of the hypothalamus. However, extensive earlier studies which involved directly electrically stimulating the hypothalamus and pituitary, and measuring the function of the anterior lobe of the gland, led to the conclusion that direct neural innervation into the anterior pituitary was

not the mechanism by which its function was controlled. Instead, it was postulated that neurohumoral substances were released from nerve endings in the median eminence and that these substances travelled to the anterior pituitary to increase or decrease the gland activity (Harris, 1955). Since that time, it has become evident that the hypothesis of Harris was correct (see Fink, 1986 for review) and factors which influence the control of anterior pituitary function are released from the median eminence and do indeed travel to the anterior pituitary gland via hypophysial portal blood vessels. The hypothalamus receives controlling innervation from many regions of the brain and translates environmental information (such as mental state, circadian rhythms, sunlight and diet) to the anterior pituitary, which in turn dictates the endocrinological progress of physiological processes such as growth, stress, water retention, fertility and metabolic rate (Murad and Haynes, 1980). Regions within the hypothalamus such as the preoptic area and the medio-basal hypothalamus communicate to the median eminence to govern the release of a wide range of factors (neuropeptides and certain of the classical neurotransmitters) into the hypophysial portal blood vessels. In addition to this direct hypothalamic-hypophysial system of communication via the portal blood vessels, pituitary control is also influenced by external factors such as circulating adrenocortical- and gonadal-steroids and thyroidal hormones (Schally, 1973; Murad and Haynes, 1980).

The anterior pituitary is known to consist mainly of some five or six different hormone-secreting cell types and to be the site of release of at least six different circulating hormones (Denef, 1982). The anterior pituitary hormones are: luteinising hormone (LH) and follicle-stimulating hormone (FSH, both of which are synthesised and released from gonadotrophs); growth hormone (GH, from somatotrophs); prolactin (from mammotrophs); adrenocorticotrophic hormone (ACTH, from corticotrophs); and thyroid-stimulating hormone (TSH, from thyrotrophs). There also exists a population of somato-mammotrophs which secrete both GH and prolactin (Herlant, 1964; Farquhar *et al,* 1975; Denef, 1982). Whether these should be

considered as a lineage distinct from somatotrophs and mammotrophs or just a central portion of a continuous spectrum of a family of similar cells is a problem that remains to be solved. The known hypothalamic factors which dictate the release of these hormones are mainly (but not entirely) peptidergic molecules. Two such peptidergic factors are the decapeptide luteinising hormone-releasing hormone (LHRH, pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) and (TRH, pyro-Glu-His-Pro-NH_2) which stimulate the release of LH/FSH and TSH/GH/PRL respectively. The receptors for both of these hormones are known to control hydrolysis of polyphosphoinositides as their mechanisms of signalling in the anterior pituitary (Schrey, 1985; Drummond, 1986).

Initially, LHRH was thought to stimulate LH release from gonadotrophs by signalling involving cyclic AMP, however this hypothesis was soon discredited and cyclic AMP is thought to have a modulatory function on hormone release. Instead, it became clear that Ca^{2+} had a crucial involvement in LHRH-induced LH release, as manipulation of calcium concentrations showed a crucial involvement of the ion for LHRH-induced hormone exocytosis from gonadotrophs (for reviews, see Conn *et al,* 1980; Conn, 1989). Metabolism of phosphoinositides stimulated by LHRH was reported in dispersed anterior pituitary cells (Snyder and Bleasdale, 1982) and in populations of enriched gonadotrophs (Raymond *et al,* 1984; Andrews and Conn, 1986). Indeed, it was later shown that in pituitary, LHRH stimulated a PI-PLC enzyme activity to hydrolyse PtdIns(4,5)P_2 into Ins(1,4,5)P_3 (Schrey, 1985; Mitchell *et al,* 1988) and that the cytosolic calcium concentrations of gonadotroph-enriched cell populations were raised as a consequence (Naor *et al,* 1988b; Limor *et al,* 1987). Further evidence in support of this mode of signalling came from results showing that LHRH stimulated an increase in diacylglycerol production in purified gonadotrophs (Andrews and Conn, 1986) and that PKC translocation occurred by LHRH stimulation (Hirota *et al,* 1985; McArdle and Conn, 1986). A role of raised Ca^{2+} and activated PKC has been implicated in LHRH-induced LH release; however, a crucial role for

PKC instead of a modulatory role in LH secretion is a controversial issue (Conn, 1989). Apart from this evidence that LHRH receptors in pituitary signal through phosphoinositide hydrolysis, it has been shown that LHRH can stimulate a phospholipase D activity to produce phosphatidic acid and subsequently diacylglycerol (Liscevitch and Amsterdam, 1989), but the physiological consequences of these events are uncertain at present.

The secretion of LH and FSH induced by LHRH may involve arachidonic acid (AA) and/or certain of its metabolites (Naor *et al,* 1983), but as with cyclic AMP, AA and its metabolites are thought to perhaps function as modulators, rather than mediators, of LHRH function in gonadotrophs, particularly in times of high secretory activity. In the GH_3 somato-mammotroph cells, LHRH has been reported to activate 'L'-type Ca^{2+}-channels and thereby raise cytosolic calcium levels, thereby providing a possible mechanism for LHRH-induced LH release (Rosenthal *et al,* 1988). However, more extensive studies by Mason and his co-workers revealed in gonadotrophs that LHRH does not induce a major depolarisation of the cell membrane (such as would be required to open 'L'-channels) nor increased 'L'-channel activity (reviewed in Mason *et al,* 1988). Instead, LHRH increased membrane 'noise' and induced a small depolarisation of 2 - 5 mV, suggesting the opening of non-voltage activated, second-messenger operated channels, however no second-messenger has been identified to mimic this LHRH action on gonadotrophs.

Curiously, endothelin was recently reported to be almost as good a secretagogue in purified gonadotrophs as LHRH (Stojilkovic *et al,* 1990) and also appeared to signal via phosphoinositide hydrolysis, but any physiological role of this secretagogue regulating gonadotroph function is as yet unproven.

The interests of the present project lie in the mechanism of action neuropeptides signalling through phosphoinositide hydrolysis; especially in the role of PKC in cellular responses and how they relate to physiological consequences in the anterior pituitary gland. One particular aspect of interest is the 'self-priming'

phenomenon of LHRH, which is the mechanism by which LHRH induces an increase in gonadotroph responsiveness (Aiyer *et al,* 1974). This is an essential factor in bringing about the LH 'surge' of release from the pituitary which triggers ovulation in both animals and humans (Fink, 1986). Protein kinase C has been implicated in the self-priming effect of LHRH (Johnson *et al,* 1988). In our laboratory, we are investigating the role of the PKC(s) mediating the LHRH-self-priming and comparing the properties and actions of those PKC(s) to the PKC(s) involved in unprimed, normal LHRH-induced LH release. Ovulation is the process by which ovarian follicles rupture to release an ovum which is subsequently taken through the fallopian tubes, where fertilisation may occur. Ovulation is triggered by the LH 'peak' or 'surge' (Fink, 1986; Fink, 1988). The high levels of LH released from the anterior pituitary during the LH 'surge' are preceded by high levels of circulating oestrogens (mainly oestradiol-17ß) and low levels of progesterone (Fink, 1986; Fink, 1988).

The hypothalamic release of LHRH is pulsatile in nature, and although the frequency of LHRH release is increased near the LH surge, the amount of LHRH released is far too small to account for the amount of LH secreted from the pituitary (Fink *et al,* 1982). The increase in pituitary responsiveness is initiated by circulating oestrogen, is further intensified by the ovarian secretion of progesterone during the early part of the LH surge, and also by the 'priming' effect of LHRH (Fink, 1988). The role of the priming effect of LHRH is to co-ordinate the increase in hypothalamic LHRH release and the increased responsiveness of the pituitary so that both events result in a massive surge of LH release form the pituitary, a consequence of maximal effects occurring simultaneously, and thus initiating ovulation in female mammals.

Oestrogen induces several proteins in the rat ventromedial hypothalamus, one of which is known as hormone-induced protein-70 kD (HIP-70), which is expressed in the midbrain central gray area of the brain, and is thought to facilitate female sexual behaviour, but only in the presence of oestrogen (Pfaff, 1980; Sakuma and Pfaff, 1980). Curtis *et al* (1985) reported that LHRH induced a protein with a

molecular size of 69 kD in female rat pituitary tissue (LHRH-70A). Further investigation showed that HIP-70 and LHRH-70A were the same protein (Mobbs *et al,* 1990a) and that this protein showed some structural homology to, and may indeed be the α-isoform of phosphoinositide-specific phospholipase C (Mobbs *et al,* 1990b). Interestingly, PLCα is structurally divergent from the other isoforms of PI-PLC (ie, ß, γ, δ and ε; Rhee *et al,* 1989) and doubts have been expressed by some groups as to whether the cloned cDNA for PLCα really encodes a functional PI-PLC enzyme (Meldrum *et al,* 1991). Nevertheless, these facts are consistent with earlier work in which Mitchell *et al* (1988) showed an increase in LHRH-induced inositol trisphosphate production (a product of phosphoinositide-specific phospholipase C) in LHRH-primed pituitaries, without a change in LHRH receptor number. One aspect of the LHRH-priming effect on pituitary may have been revealed but the precise mechanisms of LHRH self priming remains to be ascertained. Nevertheless, a form of PKC with some distinctive characteristics appears to be responsible (Fink *et al,* 1990). Certain aspects of the present project are thus concerned with investigating the properties and other cellular actions of that form of PKC which is responsible for bringing about LHRH priming.

Biochemical studies on pituitary cell types have been dogged with problems of cell-specificity, because of the heterogeneous nature of gland. One way of overcoming the problems of cell type-specificity is the use of clonal anterior pituitary cell lines. Until the late stages of the present project, no gonadotrophic cell line existed. However, by use of transgenic-animal technology, Mellon and her colleagues managed to produce several strains of murine clonal cell lines with the properties of gonadotrophs (Windle *et al,* 1990; Horn *et al,* 1991). However, as with all transformed cell lines, one is never sure of what extent the cell line function actually matches the physiology of the normal, untransformed cell type. For example, one of these cell lines, the αT3-1 line, express high affinity LHRH receptors, but however does not synthesise or secrete the ß subunits of LH or FSH (Windle *et al,* 1990). It is

anticipated that in the future, either these (or novel) lines of gonadotrophic cells will help in the biochemical characterisation of gonadotroph function.

Clonal lines of GH- and prolactin-producing tumour cells from anterior pituitary were established in 1965 (Tashjian *et al,* 1968) from a radiation-induced rat pituitary tumour termed MtT/W5 (Takemoto *et al,* 1962). Various strains of the tumour cells were generated (Buonassisi *et al,* 1962; Tashjian *et al,* 1970) and all were found to secrete prolactin, with most of the strains (including the GH_3 and GH_4C_1 strains) also secreting GH (Tashjian, 1979). The somato-mammotrophic clonal cell line, GH_3, possesses TRH, oestrogen, M_2-muscarinic and bombesin receptors among others (Hinkle and Tashjian, 1973; Tashjian, 1979; Yagisawa *et al,* 1989; Drummond, 1986). It was using the GH_3 cell line that the discovery was made that TRH was able to induce the release of prolactin (Tashjian *et al,* 1971) which was later shown to be present in a variety of species and be perhaps a physiologically relevant process (Leong *et al,* 1983).

It became evident mainly by use of the GH cell lines, that TRH-receptor signalling mechanisms were of the class which mobilised Ca^{2+} (for review, see Gershengorn, 1982). Despite considerable early efforts to show that TRH stimulated cyclic AMP production, any TRH-induced rise in cyclic AMP appears to be indirect and not result directly in hormone secretion. It soon became evident that TRH-stimulation of hormone release was a calcium-dependent process (Vale *et al,* 1977; Tashjian *et al,* 1978). Using $^{45}Ca^{2+}$-preradiolabelled GH_3 cells (Gershengorn *et al,* 1981; Moriarty and Leuschen, 1981), it was shown that lack of TRH response in somatotrophs incubated in low Ca^{2+} medium was due to loss of Ca^{2+} from a cell-associated pool(s) and that Ca^{2+} influx was necessary for TRH-induced hormone secretion. It soon became clear that TRH, which could stimulate the incorporation of ^{32}P into phosphoinositides in GH_3 cells (Rebecchi *et al,* 1981; Drummond and Macphee, 1981), was primarily signalling through the phosphatidylinositide hydrolysis pathway. Stimulation of GH_3 cells with TRH resulted in the production of diacylglycerol

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(Macphee and Drummond, 1984; Rebecchi *et al,* 1988) from the receptor-induced hydrolysis of PtdIns(4,5)P_2 as well as inositol trisphosphate (Rebecchi and Gershengorn, 1983; Martin, 1983; Drummond *et al,* 1984). It was furthermore clear that the PtdIns(4,5)P_2 hydrolysis was not secondary to a raised intracellular calcium concentration (Schlegel *et al,* 1984). Similar observations that TRH signals through phosphoinositide metabolism have been made in normal anterior pituitary cells (Hollingshead *et al,* 1985). It is now clear that TRH produces Ins(1,4,5)P_3 to release calcium stores and raise intracellular calcium (Drummond, 1984; Drummond, 1986; Mason *et al,* 1988; Thorner *et al,* 1988) and that the initial peak raised calcium is transient and due to Ins(1,4,5)P_3-induced release of intracellular calcium, whereas the more sustained secondary plateau of raised intracellular calcium is due to influx of extracellular calcium partly through dihydropyridine-sensitive Ca^{2+}-channels (Albert and Tashjian 1984a; Albert and Tashjian, 1984b; Schegel and Wollheim, 1984; Gershengorn and Thaw, 1985) with the remaining component of the 'plateau' raised calcium occurring either through non 'L'-type Ca^{2+}-channels or by a PKC-mediated reduction in extrusion processes (Mason *et al,* 1988; Drummond, 1986). The precise mechanism of the raised cytosolic calcium 'plateau' is unclear but may also involve an increase in spontaneous Ca^{2+}-action potential frequency (Mason *et al,* 1988; Drummond, 1986; Thorner *et al,* 1988 for reviews), but is probably not due to any one specific mechanism.

Thus, GH_3 cells have proved to be a useful tool in understanding the mechanisms of a Ca^{2+}-mobilising-receptor function and of TRH-induced biochemistry/electrophysiology in anterior pituitary cells. GH_3 cells are still used as an extremely useful model for investigating events in stimulus-secretion coupling and signal transduction.

The work in this thesis was performed with the following aims:

(i) To assess the effects of PKC on 'L'-type Ca^{2+}-channels in anterior pituitary and GH_3 cells.

(ii) To investigate the pharmacological properties of the distinct modes of modulation observed in (i) above.

(iii) To assess the possibility that AA may act in a similar fashion to phorbol esters to enhance PKC activity in GH_3 cells.

(iv) To characterise the pharmacological properties of distinct PKC isoforms which may be involved in 'L' channel regulation, by means of ligand binding and kinase activity assays on tissues enriched in various PKC isoforms.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

Standard laboratory chemicals were of Analar grade obtained from BDH Chemicals Ltd, Poole, Dorset, UK. COB-Wistar rats were purchased from Charles River UK Ltd, Margate, Kent, UK. GH_3 were obtained from Flow Laboratories Ltd, Irvine, Strathclyde, UK. I-10, LC-540 and R-2C Leydig cell lines and COS 7 cells were obtained from the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, UK.

$^{45}CaCl_2$ (specific activity = 17 mCi/mg) was supplied by Amersham International plc, Amersham, Bucks, UK. [20-^{3}H(N)]-phorbol 12,13-dibutyrate([^{3}H]-PDBu) (specific activity approximately 19 mCi/mmol), [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid([^{3}H]-AA) (specificity approximately 240 Ci/mmol) and adenosine triphosphate-γ-^{35}S(ATP-γ-^{35}S) (specific activity approximately 1256 Ci/mmol) were supplied by Du Pont, Dreieich, Germany. Foetal bovine serum was obtained from Flow Laboratories Ltd, Irvine, Strathclyde, UK. Ham's F-10 Medium with l-glutamine (Ham, 1963). Alpha modification of Minimal Essential Medium (α-MEM) (without ribonucleosides and without deoxyribonucleosides), Dulbecco's modification of Minimal Essential Medium (DMEM) (with l-glutamine and 4500 mg/l d-glucose, without sodium pyruvate) (Dulbecco and Freeman, 1959), Hank's Balanced Salt Solution (HBSS) (without phenol red) (Hank and Wright, 1949) and Trypsin (0.25% (v/v)) in a solution of (0.4 g/l KCl, 2.2 g/l $NaHCO_3$, 6.8 g/l NaCl and 1.0 g/l glucose) were supplied by Gibco-BRL, Paisley, Strathclyde, UK. Penicillin, streptomycin, l-glutamine, bovine serum albumin (essential fatty acid-free), arachidonic acid (AA) (sodium salt), AA-methyl ester, linoleic acid, PDBu, NDGA, 8-Br-cyclic GMP (sodium salt), N-methyl d-glucamine, 2-mercaptoethanol, E-64, leupeptin, lysine-rich histone-IIIS, myelin basic protein, sodium orthovanadate (Na_3VO_4), indomethacin and Indo-1-acetoxymethylester (Indo-1-AM), cremaphor EL, gossypol, sphingosine and phosphatidylserine were all purchased from the Sigma Chemical Company Ltd, Poole,

Dorset, UK. Piperonyl butoxide was supplied by the Aldrich Chemical Company Ltd, Gillingham, Dorset, UK. SKF-525A ('Proadifen') was obtained from Research Biochemicals Inc Natick, Massachusetts, USA. N-methylthyrotropin-releasing hormone (N-methyl TRH) and 'α-peptide' (Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val ([Ser^{25}]PKC-α)) were purchased from Peninsula Laboratories Inc., St Helens, Merseyside, UK. The (+) and (-) enantiomers of indolactam-V and DHI were purchased from LC Services Corporation, Woburn, MA, USA. AMG-C16 was supplied by Bachem, Bubendorf, Switzerland. H7 and HA1004 were obtained from Seikagaku America Inc., St Petersburg, Florida, USA. Staurosporine and K252a were supplied by Kyowa Medex Co Ltd, Tokyo, Japan. Ionomycin was purchased from Novabiochem (UK) Ltd, Nottingham, Notts, UK. The enzyme inhibitors, ETYA and Ro318220 were gifts from Roche Products Ltd, Welwyn Garden City, Herts, UK.

2.2. METHODS

2.2.1. Animals

Male COB-Wistar rats (>250 g) were maintained under controlled lighting (lights on from 05.00 to 19.00 h) and temperature (22°C) and allowed free access to diet 41B (Oxoid Ltd, Basingstoke, Hants, UK.) and tap water. Rats were killed by cervical dislocation and tissue was rapidly dissected out and homogenised as appropriate.

2.2.2. Cell Culture

All cell cultures were grown in a humidified atmosphere of 95% air/5% CO_2 at a constant temperature of 37°C, and received fresh medium every 3 - 4 days. GH_3 and I-10 Leydig cells were grown in Ham's F-10 medium supplemented with 15% foetal bovine serum (heat-inactivated), 1 mM l-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. LC-540 Leydig cells were grown in α-MEM medium supplemented with 10% foetal bovine serum (heat-inactivated), 1 mM l-glutamine,

100 U/ml penicillin and 0.1 mg/ml streptomycin. R-2C Leydig cells were cultured in Ham's F-10 medium supplemented with 15% foetal bovine serum (γ-irradiated), 2 mM l-glutamine, 100 u/ml penicillin and 0.1 mg/ml streptomycin. COS 7 cells were grown in DMEM supplemented with 10% foetal bovine serum (heat-inactivated), 100 U/ml penicillin and 0.1 mg/ml streptomycin. GH_3 cells were harvested by agitation (medium + cells replaced with fresh medium), washed once by resuspension centrifugation (1,000 g, 10 min, 25°C) and finally resuspended at the appropriate density in the required medium. Cell lines were used at > 90% confluency, except for GH_3 cells, which were used when maximally (50 - 70%) confluent. Except for GH_3 cells, which were continually harvested from the same flask, all cell lines were split when 100% confluent and seeded at low density in fresh flasks. I-10, LC540, and R-2C Leydig cells and COS 7 cells were harvested by trypsin-digestion: medium was removed from the flask and replaced with 5 ml of 0.25% (v/v) trypsin solution (37°C) which was allowed to cover the monolayer for 15 - 30 s before removal. Flasks were then incubated for 10 - 20 min at 37°C before cells were detached from the culture flask and neighbouring cells by agitation using the appropriate fresh growth medium. The cell suspension was washed and finally resuspended at the appropriate density in the required medium for assaying purposes, or split into new flasks for cell-splitting purposes.

Cell lines were frozen for storage in liquid N_2 by resuspending harvested cells at an estimated density of 10^7 cells/ml in 95% foetal bovine serum (heat-inactivated) + 5% dimethylsulphoxide. The cell suspension was aliquoted in a labelled cryostat tube and placed in a closed polystyrene box (room temperature). The closed box containing the sealed cryostat tubes was placed at -70°C for 3 - 5 h before being transferred into liquid N_2. Frozen cells were recovered from liquid N_2 by placing the frozen cryostat tube straight into a beaker of water (37°C) and thawed. The cryostat tube was then sterilized by wiping with 70% ethanol and the cell suspension was transferred using a sterile syringe and wide gauge needle into a pre-warmed (37°C)

flask of appropriate growth medium. Dimethylsulphoxide was removed after cells were allowed 24 h recovery, by total replacement of growth medium with fresh growth medium. From this stage, cells were grown as normal.

2.2.3. Calcium Influx Studies (see Figure 2.4)

GH_3 cells were diluted to a density of 5 x 10^6 cells/ml in 'calcium uptake medium' (concentrations in mM: NaCl, 154; KCl, 5.4; $CaCl_2$, 1.5; d-glucose, 11; HEPES, 6; pH adjusted to 7.4 with Tris base and supplemented with 0.05% BSA). Alternatively, one quartered hemipituitary was used per assay tube for studies of $^{45}Ca^{2+}$ influx into anterior pituitaries. Aliquots of this suspension (0.5 ml/tube) or one hemipituitary (in 0.5 ml calcium uptake medium) were preincubated (30 min, 37°C, O_2 atmosphere) before a 10 min incubation (37°C, O_2) with drugs or solvent alone. Tissue was then exposed to 1 ml of calcium uptake medium containing either low K^+-(5.4 mM final concentration) or high K^+-(60 mM) with 4 μM $^{45}CaCl_2$ (approximately 3 μCi/tube). After 30 s (37°C), $^{45}Ca^{2+}$ uptake was halted by quenching with 3 ml of ice-cold 2 mM EGTA (Ca^{2+}-free) calcium uptake medium and tissue was separated by vacuum-filtration through Millipore SCWP cellulose acetate/nitrate filters (8 μm pore size) underlain by GF/B filters on Millipore 1225 sampling manifolds (Millipore UK Ltd., Harrow, Middlx., UK). Samples were washed once immediately with 3 ml ice-cold EGTA calcium uptake medium and then washed a further three times for 2 min each in the same medium. The radioactivity associated with the cellulose filters and cells was determined by liquid scintillation counting. Preliminary experiments on GH_3 cells matched similar extensive studies on anterior pituitary prisms (Mitchell *et al,* 1988) revealing that these conditions gave the optimal signal to noise ratio (as shown in Figures 2.1 and 2.2). Typically, non-specific adsorbtion to cell surfaces and filters accounted for around 400 dpm as determined in zero-time blanks, in which cells were incubated as normal, but EGTA quench was added to the tube before the 30 s incubation with $^{45}Ca^{2+}$. The amount of non-specific adsorbtion remained constant

Figure 2.1. **Time-course of basal and high-potassium-stimulated accumulation of $^{45}Ca^{2+}$ in GH_3 cells.**

$^{45}Ca^{2+}$ accumulation into GH_3 cells was measured for the indicated time under basal (5.4 mM K^+) or high K^+ (60 mM K^+) bathing conditions. Cells were preincubated (37°C, O_2 atmosphere) for 30 min prior to exposure to $^{45}Ca^{2+}$ in either high or basal K^+-containing 'calcium uptake medium'. Influx of $^{45}Ca^{2+}$ into GH_3 cells was halted by quenching with 2 mM EGTA (no Ca^{2+})-containing 'calcium uptake medium', immediate filtration and then extensive washing as described in the text. The $^{45}Ca^{2+}$ presented to the cells here was 4 times greater than normally used, being 12 μM (~ 12 μCi/tube). The data represent the means of 2 separate determinations, with each value (dpm) varying by $< 20\%$. Stimulated $^{45}Ca^{2+}$ influx was maximal at 30 s, with 60 s showing no further increase in influx over basal rates of influx.

Figure 2.1

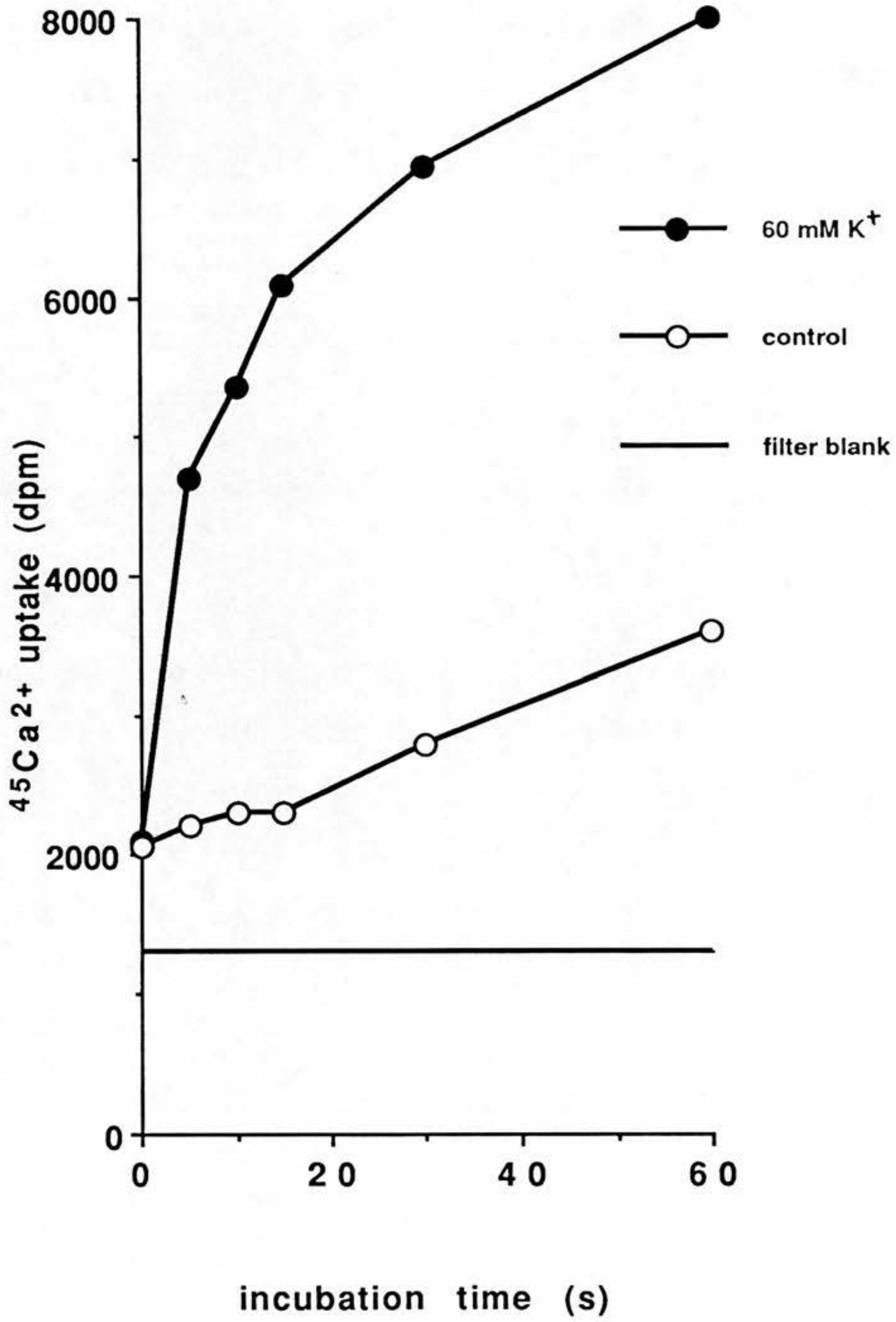


Figure 2.2. **Effect of the number of washes with EGTA-containing 'calcium uptake medium' on the amount of $^{45}Ca^{2+}$ accumulation in GH_3 cells.**

Cells were preincubated (30 min, 37°C, O_2 atmosphere) before exposure to basal (5.4 mM) or high (60 mM) K^+-containing 'calcium uptake medium' and $^{45}Ca^{2+}$ for 30 s. Each wash was for a 2 min period with 3 ml of EGTA-containing 'calcium uptake medium' (4°C) and is in addition to the initial 3 immediate washes with 3 ml of ice-cold EGTA-containing 'calcium uptake medium'. The values were calculated by collecting and counting the successive wash eluates and recalculating the initial value (100% at zero 2 min washes) from the final counts in the filtered cells plus those counts in the eluates. The data represent the means of 2 determinations.

Figure 2.2

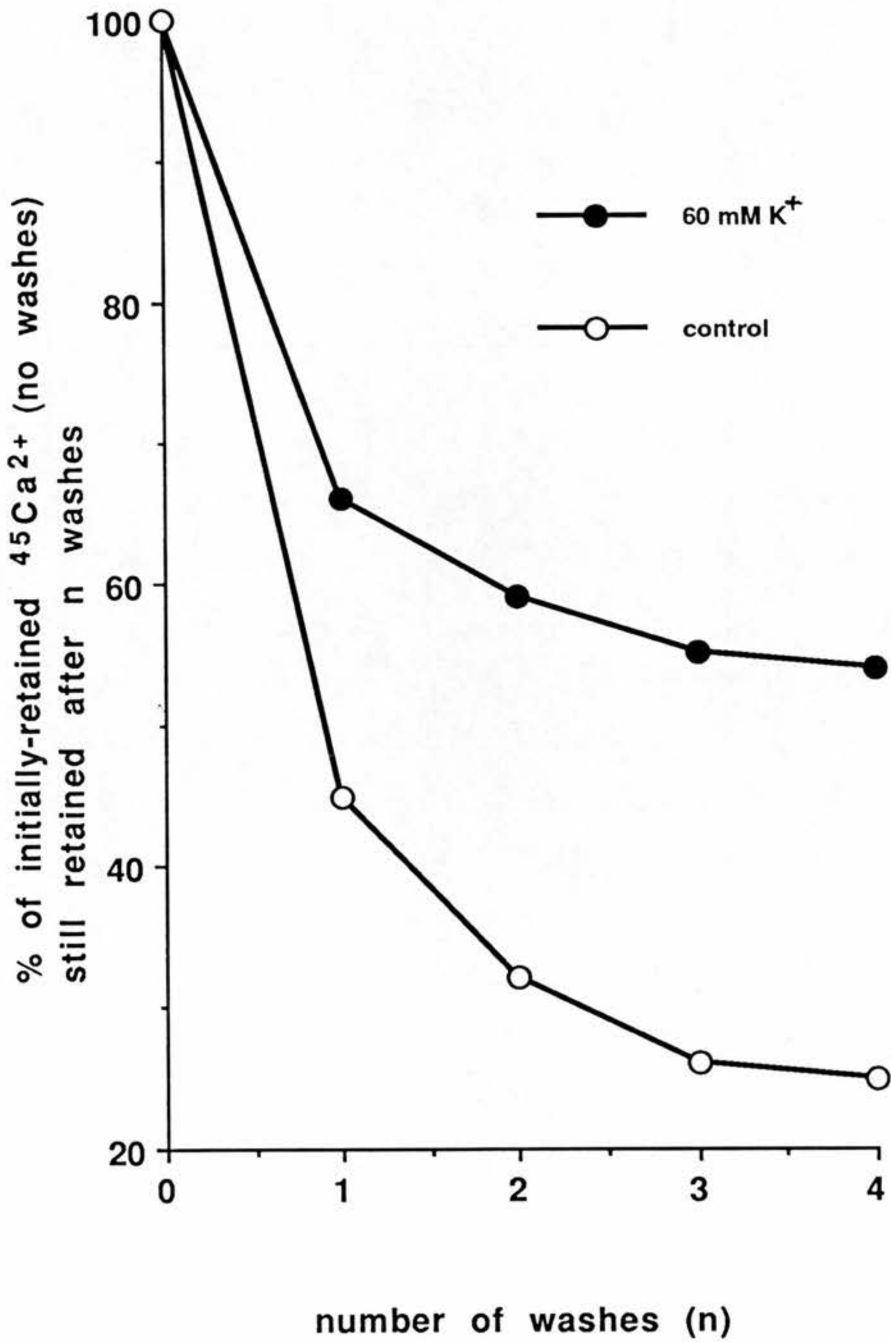


Figure 2.3. **Effect of various stimuli on $^{45}Ca^{2+}$ accumulation into GH_3 cells.**

The figure displays the $^{45}Ca^{2+}$ accumulation into GH_3 cells after a 30 min preincubation (37°C, O_2 atmosphere) under the conditions indicated: basal (control with 'calcium uptake medium' alone); N-methyl TRH (1 μM in 'calcium uptake medium')-; high K^+ (60 mM K^+-containing 'calcium uptake medium')- or ionomycin (30 μM dissolved in dimethyl formamide (0.3% final) in 'calcium uptake medium'). Influx of $^{45}Ca^{2+}$ was measured for a 30 s period before halting with ice-cold EGTA-containing 'calcium uptake medium' as described in the Methods section. The dotted line represents the mean accumulation to cell-surfaces and filters as determined in two zero-time blanks. Accumulation to filters alone in the absence of cells typically accounts for around 350 dpm (30 fmoles $^{45}Ca^{2+}$ per 10^6 cells per min) of $^{45}Ca^{2+}$. The data represent the mean ± SEM of 4 determinations for the basal, high K^+ and ionomycin assay conditions, and of 12 determinations for the N-methyl TRH assay condition. Each stimulus produed a statistically significant increase in $^{45}Ca^{2+}$ accumulation over basal levels ($P \leq 0.05$, Mann-Whitney U-test).

Figure 2.3

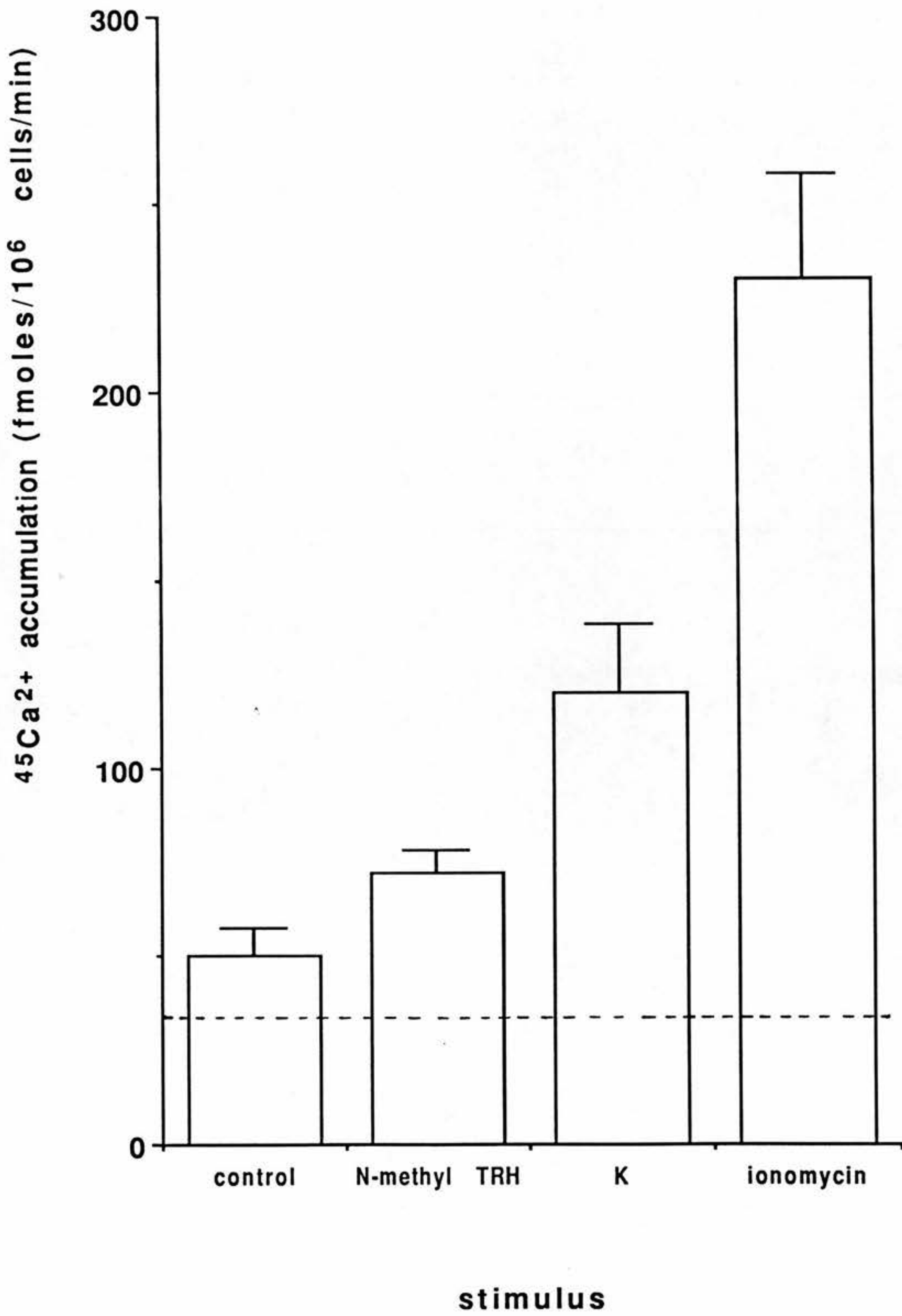
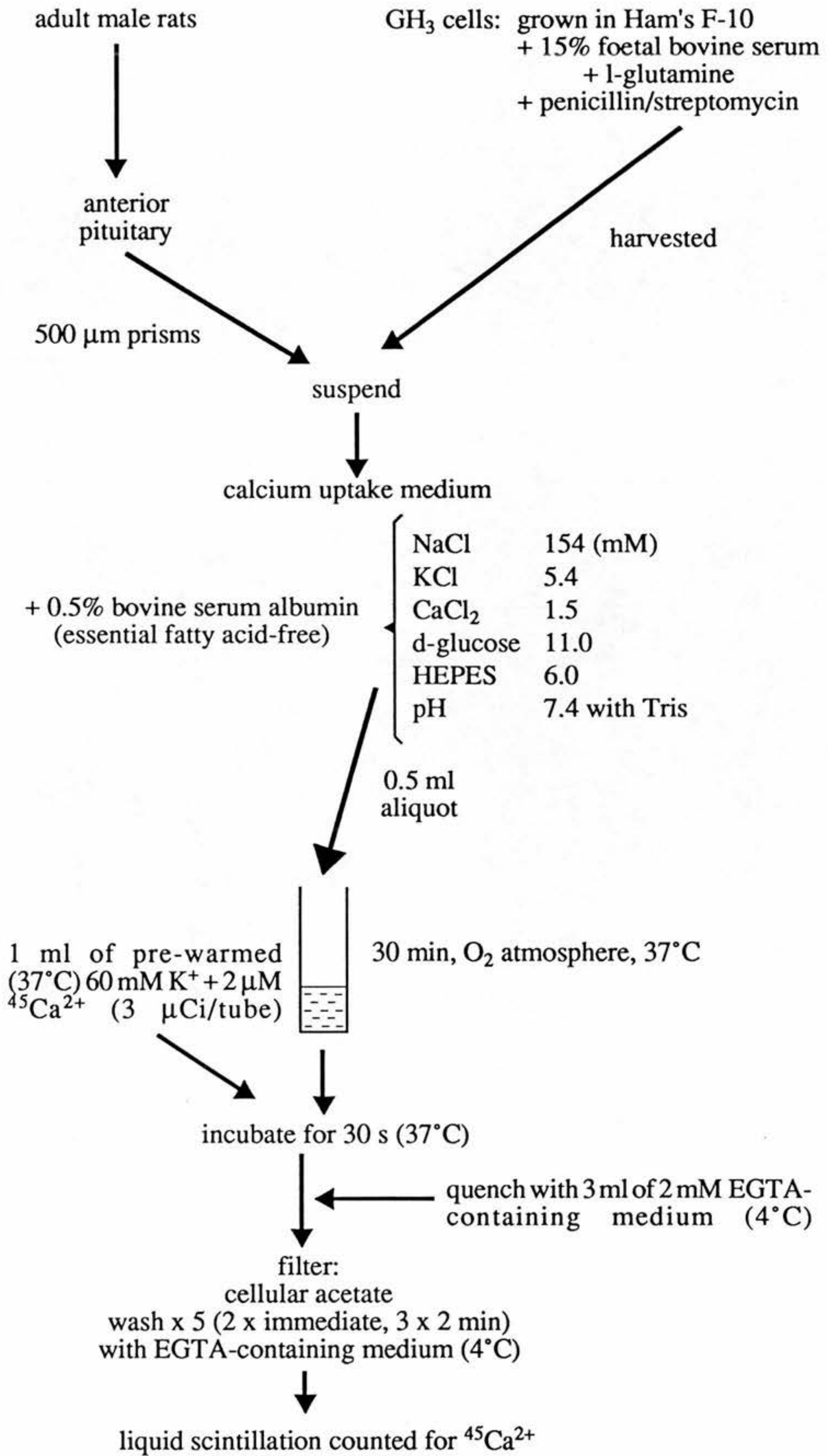


Figure 2.4. Flow diagram of the $^{45}Ca^{2+}$ influx methodology used in our studies.

Figure 2.4



from 5 to 60 s incubation with $^{45}Ca^{2+}$ at 37°C and was independent of a high K^+ stimulus being present (Figure 2.1). Accumulation in 30 s (37°C) of $^{45}Ca^{2+}$ to filters alone (ie, in the absence of cells) accounted for around 350 dpm. Basal $^{45}Ca^{2+}$ accumulation in GH_3 cells linearly increased with time from 5 to 60 s (Figure 2.1). High K^+-stimulation of GH_3 cells led to an accumulation of $^{45}Ca^{2+}$ above basal influx at 5, 10, 15 and 30 s incubation, however the increased rate of uptake was maximal at 30 s. Incubation with high K^+ for 60 s showed $^{45}Ca^{2+}$ accumulation which was greater than basal, but the rate of accumulation was similar to the basal rate of $^{45}Ca^{2+}$ accumulation, indicating that a maximal stimulation of $^{45}Ca^{2+}$ influx into GH_3 cells occurred with 30 s incubation of 60 mM K^+. In further experiments, 30 s incubation periods with stimulus and $^{45}Ca^{2+}$ were used, as this incubation time generated the maximum signal to noise ratio. The stimulus-induced influx of $^{45}Ca^{2+}$ in excess of basal controls was maximal within 30 s, suggesting that it represented a specific stimulus evoked flux rather than adsorption or steady accumulation by storage pools.

Figure 2.3 demonstrates the effects of various stimuli on $^{45}Ca^{2+}$ accumulation into GH_3 cells. Incubation for 30 s with 30 μM ionomycin showed the greatest $^{45}Ca^{2+}$ influx over basal accumulation, among the the stimuli tested. 60 mM K^+-stimulation and 1 μM N-methyl TRH-stimulation for 30 s accounted for 37 ± 15% and 14 ± 8 % of ionomycin-stimulated $^{45}Ca^{2+}$ influx respectively.

2.2.4. Calcium Efflux Studies (see Figure 2.6)

Harvested GH_3 cells were resuspended at a concentration of 1 x 10^7 cells/ml in calcium uptake medium containing 0.05% BSA and incubated for 20 min (37°C, O_2) before addition of 1 ml of medium containing 6 μM $^{45}Ca^{2+}$ (approximately 4.5 μCi/tube). Samples were incubated for 1 hr at 37°C under O_2 before loading onto separate GF/B filters which had been pre-washed with calcium uptake medium under gentle vacuum on a Millipore 1225 sampling manifold (eluate discarded). Cells were then washed three times for 2 min with 3 ml of pre-warmed (37°C), pre-oxygenated

Figure 2.5. **Effect of ionomycin on $^{45}Ca^{2+}$ efflux from preloaded GH_3 cells.**

Aliquots of GH_3 cells were loaded in 'calcium uptake medium' with 6 μM $^{45}Ca^{2+}$ (~ 4.5 μCi/tube) for 1 h (37°C) under O_2 before being loaded onto separate filters under vacuum. The filters were washed 3 times for 2 min with prewarmed (37°C) 'calcium uptake medium' and the eluates were discarded. The end of the 3rd 2 min wash is denoted as time = 0 min. The eluate of each successive 2 min wash with 'calcium uptake medium' was collected and counted for $^{45}Ca^{2+}$ radioactivity. The presence in the 'calcium uptake medium'-wash of 30 μM ionomycin after the 3rd collected fraction, is indicated by the solid line, which was present until the end of the experiment. Control efflux levels are measured in the presence of the solvent for ionomycin, dimethylformamide (1% final). The data represent the mean ± SEM of 3 determinations.

Figure 2.5

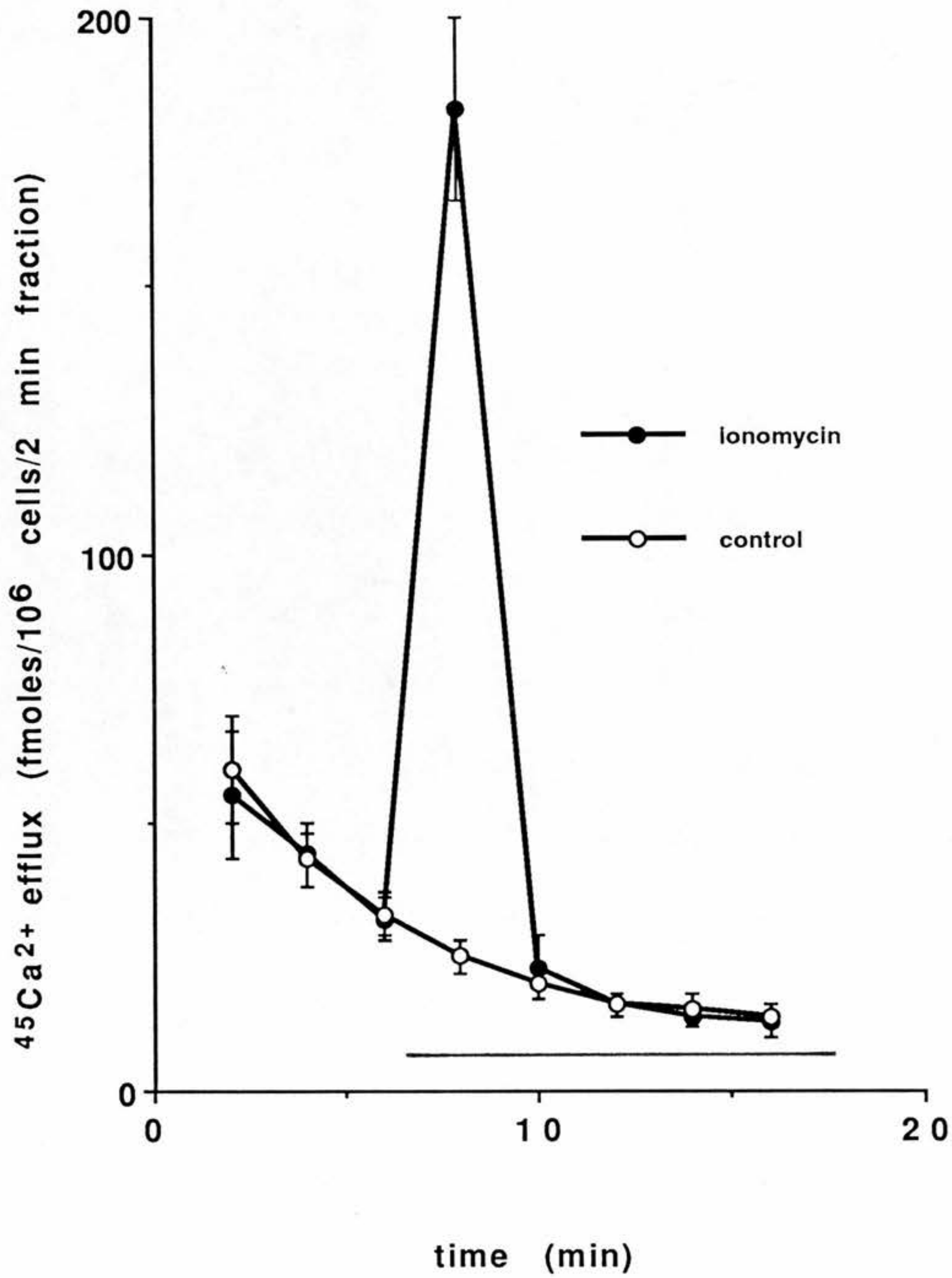


Figure 2.6. Flow diagram of the $^{45}Ca^{2+}$ efflux methodology used in our studies.

Figure 2.6

Harvested GH_3 cells at a density of
1×10^7 cells/ml in 'calcium uptake medium'
((mM) NaCl, 154; KCl, 5.4; $CaCl_2$, 1.5; d-glucose,
11.0; HEPES, 6.0 and pH to 7.4)
with Tris and containing 0.05% bovine
serum albumin (essential fatty acid-free)

⬇ 0.5 ml aliquot

20 min preincubation (37°C, O_2 atmosphere)

⬇

add 1 ml of 'calcium uptake medium'
containing 6 μM $^{45}Ca^{2+}$ (4.5 μCi/tube)

⬇

pre-incubate 1 h (37°C, O_2 atmosphere)

⬇

loaded onto separate, pre-washed GF/B filters
under vacuum

⬇

11 x wash for 2 min with 3 ml of pre-warmed
(37°C) 'calcium uptake medium'

⬇

first 3 washes discarded, following 8 washes collected and
counted for $^{45}Ca^{2+}$ by liquid scintillation counting

Inhibitors were present throughout all washes. Stimuli were present in the 7th and subsequent washes.

calcium uptake medium alone and the eluates discarded. There then followed a further 8 washes of 2 min each with pre-warmed calcium uptake medium containing the appropriate inhibitor or solvent for controls. Each successive 2 min wash with 3 ml of calcium uptake medium was collected and the radioactivity in each fraction measured by liquid scintillation counting. The 4th and subsequent washes contained the stimulus. Inhibitors (if investigated) were present in the washes from the start of each run.

In preliminary experiments, an ionomycin (100 μM) stimulus was applied during various washes to assess the best time of introducing a stimulus to the cells. It was found that when rate of extrusion from GH_3 cells was highest (ie, at the start of the washing procedure), the rapid loss of $^{45}Ca^{2+}$ was quite variable (even when expressed as a percentage of the total radioactivity collected), probably representing the removal of largely extracellularly bound $^{45}Ca^{2+}$ in the first several washes. Therefore, the first three washes were discarded and the ionomycin (or other stimulus) was introduced to the cells at the 7th (4th collected) wash (Figure 2.5) as the efflux of $^{45}Ca^{2+}$ had stabilised, and replicates of basal efflux and stimulated efflux levels were more similar. If the stimulus was introduced later, after even more extensive washing, the evoked response in excess of basal efflux was diminished.

2.2.5. Cytosolic Calcium Measurements (see Figure 2.8)

Cytosolic Ca^{2+} concentrations were measured by use of the calcium fluorescent dye Indo-1 (Grynkiewicz *et al,* 1985). Indo-1 is an indicator dye which, when excited with a wavelength of ~ 330 nm, will emit light with a characteristic spectrum of a maximum intensity at 490 nm. However, Indo-1 which binds Ca^{2+} is cell-impermeant. Thus the acetoxymethyl ester, Indo-1-AM, which is cell permeable, can load into cells and is trapped there by intracellular esterase action on Indo-1-AM to liberate Indo-1. GH_3 cells were loaded with 10 μM Indo-1-AM for 45 min in culture medium at 37°C, 95% air, 5% CO_2. Cells were then harvested, washed and resuspended in Hank's Balanced Salt Solution (HBSS). A 30 min incubation in the

dark at room temperature was then given to allow more complete hydrolysis of intracellular Indo-1-AM. To remove extracellular dye, the suspension was then centrifuged (1,000 g, 10 min, 25°C) and the cell pellet was again resuspended in HBSS at a concentration of 5 x 10^6 cells/ml. A sample (2.5 ml) of the cell suspension was loaded into a quartz cuvette which was stirred magnetically and maintained at a constant temperature of 37°C. Fluorescence was measured in a Shimadzu RF-5000 spectrofluorophotometer. Excitation was at 332 nm (band width = 5 nm) and emission was measured at 400 nm (band width = 5 nm). Emission wavelengths were not ratioed between 400 and 490 nm so that the possibility of introducing significant autofluorescence artefacts in the final signal (Luckhoff, 1986) would be reduced. A fluorescence value was taken every 2 s and at the end of each experiment, 10 μM ionomycin followed by 10 mM $MnCl_2$ (final concentrations) were added to give a measure of the maximum and minimum fluorescent values of the cuvette contents respectively. An assessment of extracellular fluorescence resulting from dye leakage was made according to the method proposed by Rink and Pozzan (1985). Thus, appropriate corrections were made for the small changes in basal fluorescence induced by the addition of 100 μM $MnCl_2$ which were rapidly and fully reversed by the Mn^{2+}-chelator DTPA (200 μM). The experimental values for cytosolic Ca^{2+} concentrations were calculated according to Grynkiewicz *et al* (1985) and Luckhoff (1986) as shown (Figure 2.7).

This method represents the treatment of GH_3 cells needed to obtain an optimal signal of cytosolic calcium concentration using Indo-1 as a Ca^{2+}-indicator. After loading with Indo-1-AM, the cells require to be washed sufficiently so as to remove extracellular dye ester and this manoeuvre is important to reduce autofluorescence since any Indo-1 indicator present extracellularly (in the presence of 1.26 mM Ca^{2+}), which would significantly reduce the signal to noise ratio for cell responses. The number of washes needed to be optimised so enough GH_3 cells were still present finally in the cuvette, and a reasonable fluorescence signal was measured.

Figure 2.7. **Calculation of cytosolic calcium concentration from Indo-1-loaded GH_3 cells.**

GH_3 cells were loaded with 10 μM Indo-1-AM for 45 min in their culture flasks (37°C, 5% CO_2/95% air/humidified atmosphere) and were harvested, washed and resuspended in Hank's balanced salt solution. After a 30 min incubation in the dark at room temperature, fluorescence measurements and calcium concentration calculations were performed as described in the Methods section. The fluorescence signal was allowed to stabilise before the experiment commenced. The trace is representative also of two others. The trace represents a typical result of a calibration procedure, including assessment of extracellular dye leakage, as conducted on cell sample aliquots in parallel. The addition of agents to the cuvette was as follows:

(a) Mn^{2+} (100 μM final)

(b) CaDTPA (200 μM)

(c) ionomycin (15 μM)

(d) Mn^{2+} (5 mM)

The abscissa scale bar represents 1 min. Cytosolic Ca^{2+} concentration was calculated as follows:

Grynkiewicz *et al* (1985)

$$[Ca^{2+}] = \frac{F - Fmin}{Fmax - F} \times K_d$$

where:

K_d = 250 nM for Indo-1: Ca^{2+}

F = sample fluorescence

F_{max} = value after ionomycin (at c) to permeabilise to external Ca^{2+}

F_{min} = value after ionomycin and then saturating Mn^{2+} to quench all Ca^{2+}-induced fluorescence.

Figure 2.7. (continued)

Rink & Pozzan (1985)

Correction procedure for dye leakage was calculated as follows:

Addition of a low concentration of extracellular Mn^{2+} (100 μM) will quench Ca^{2+} signal due to dye leakage and addition of the highly selective Mn^{2+} allowing further manipulations to proceed:

True = $F_{apparent}$ - leak
(where leak = reduction from $F_{apparent}$ after Mn^{2+} (100 μM) at (a) which should be reversed by Ca DTPA (200 μM) at (b)).

True F_{max} = $F_{max\ apparent}$ - leak

True F_{min} = $F_{min\ apparent}$ - correction factor (CF)
(where CF = $^{1}/_{11.22}$ x leak)

The constant for CF is derived from the relative fluorescence of Ca:Indo-1 complex and of Ca^{2+}-free:Indo-1 (or Mn^{2+}:Indo-1 complex) at the relevant wavelengths (Grynkiewicz *et al,* 1985).

In the present example:

$F_{apparent}$ ≅ 68 units

$F_{max\ apparent}$ ≅ 139 units

$F_{min\ apparent}$ ≅ 10 units

leak ≅ 24 units

Thus:

$$\text{basal } [Ca^{2+}]_i = \frac{F - Fmin}{Fmax - F} \times K_d$$

$$\cong \frac{(68 - 24) - (10 - {}^{1}/_{11.22} \times 24)}{139 - 24) - 68 - 24} \times 250$$

$$\cong \frac{44 - 8}{115 - 44} \times 250$$

$$= \frac{36}{71} \times 250$$

$$= 127 \text{ nM}$$

Figure 2.7

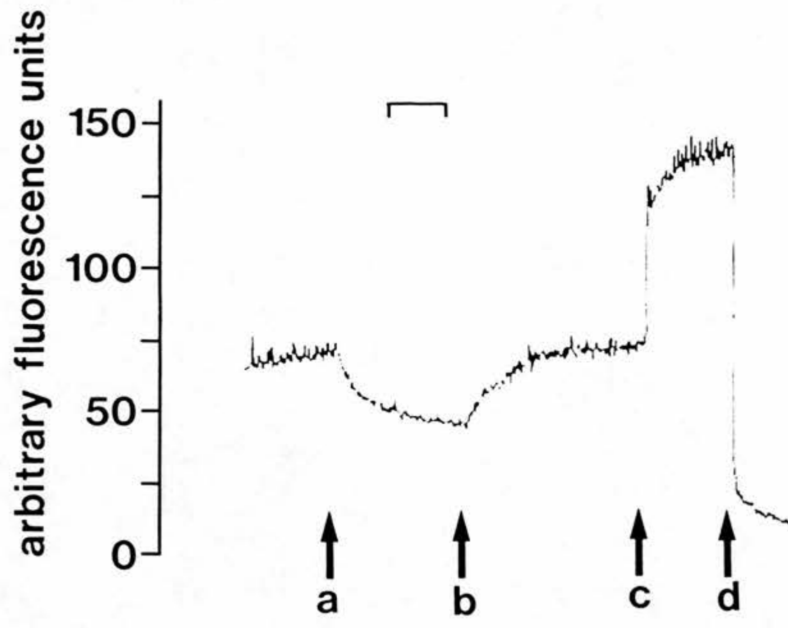
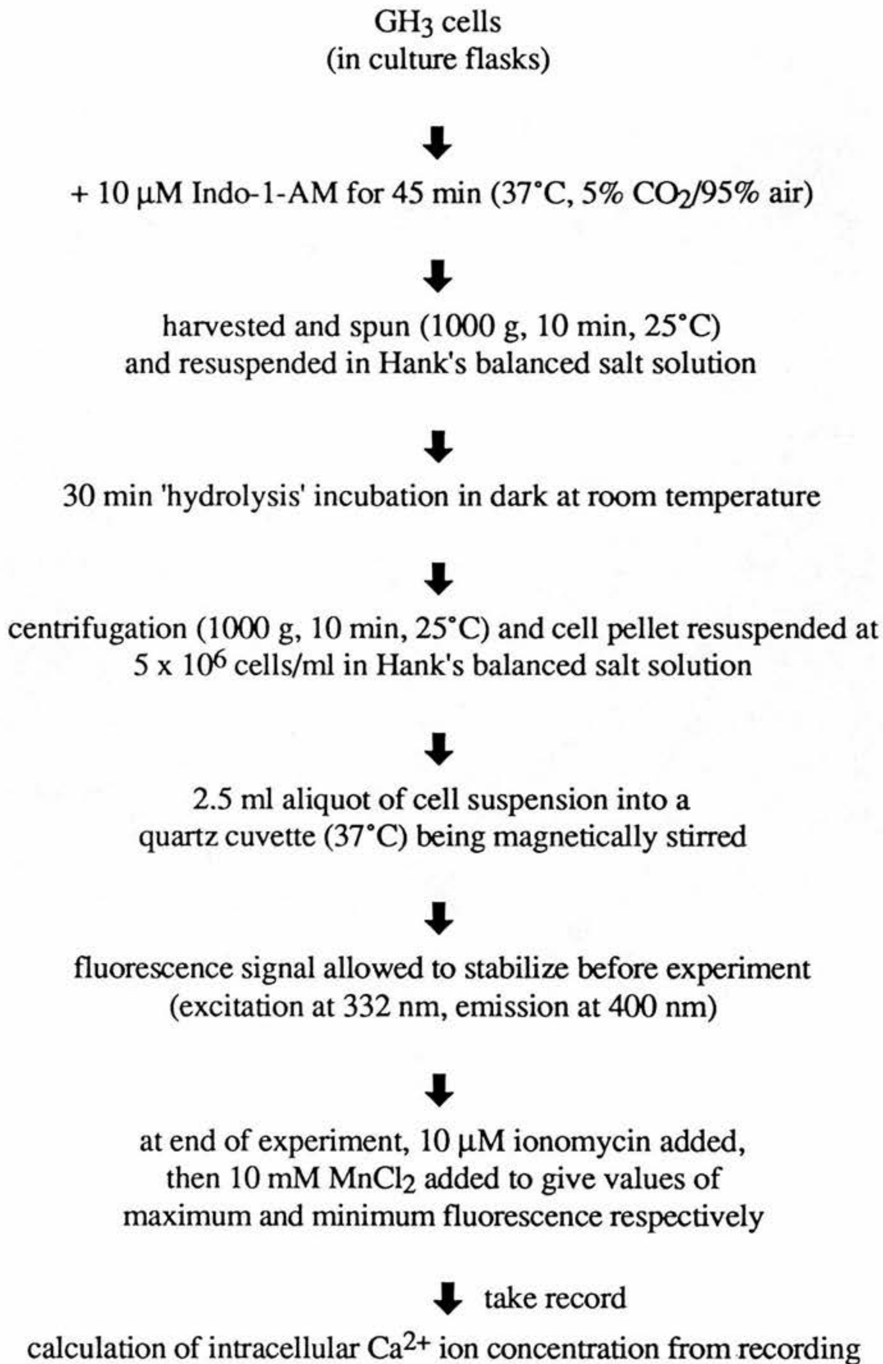


Figure 2.8. Flow diagram of the methodology used to assess cytosolic calcium concentration in GH_3 cell populations.

Figure 2.8



Loading of GH_3 cells needs to be reasonably quick so as the Indo-1 is mainly in the cytosol, and not sufficiently hydrolysed in organelles such as calcium stores, to which Indo-1-AM eventually permeates. Finally, the amount of Indo-1 within the cells should not be too high or else formaldehyde (a biproduct of its formation) may accumulate to a toxic concentration within the cytosol (product information from R P Haughland, Molecular Probes Catalogue, 1989).

The fluorescent Ca^{2+}-dye Quin-2 (Drummond, 1985) was used in earlier studies on GH_3 cells, but since the signal to noise ratio obtained was poor, the dye was replaced by trials with the improved Ca^{2+}-indicators Fura-2 and Indo-1, which have greater wavelength shifts upon Ca^{2+}-binding, show much stronger fluorescence and provide better information as to 'total' cytosolic calcium concentration and not 'relative' cytosolic calcium concentration. However, Fura-2-AM was poorly hydrolysed within GH_3 cells, whereas Indo-1-AM hydrolysis within GH_3 cells was sufficient to produce an effective signal of their cytosolic Ca^{2+} levels.

2.2.6. [^{3}H]-arachidonic acid release studies

Release of [^{3}H]-arachidonic acid ([^{3}H]-AA) into the incubation medium from preloaded GH_3 cells was measured by lipid extraction and reverse-phase chromatography on octadecylsilyl silica using the solvent system first described by Powell (1982). GH_3 cells were grown to maximal (approximately 70%) confluency in 35 mm-diameter culture wells in Ham's F-10 medium supplemented with 15% foetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Eighteen hours before the experiment, each multiwell of GH_3 cells had its medium replaced with 2 ml/well of minimal essential medium (MEM) with 100 U/ml penicillin, 0.1 mg/ml streptomycin and approximately 1 μCi of [^{3}H]-AA. Cells were then incubated in a radioactive-incubator for 18 h at 37°C (95% O_2/5%CO_2, humified atmosphere) in order to label cellular lipids with [^{3}H]-AA. Prelabelled cells then had their medium removed, were washed twice for 2 min with 2 ml of prewarmed (37°C) MEM plus 1% bovine serum

albumin (essential fatty acid-free) (BSA) while in a shaking water bath (37°C) and with an atmosphere of 95% O_2/5%CO_2. After these initial washes, the medium was replaced with 1 ml/well of MEM plus 0.5% BSA which was supplemented with nothing, 1 μM final N-methyl TRH (dissolved in distilled water (0.3% final)) or 30 μM final ionomycin (dissolved in dimethylformamide (0.3% final)) to assess basal, N-methyl TRH-stimulated or ionomycin-stimulated levels of [^{3}H]-AA release respectively. After a 15 min (37°C, 95% O_2/5%CO_2) incubation in a shaking water bath with the appropriate stimuli, the medium from each well was removed and stored on ice. The monolayer of cells was washed twice with 1 ml/well of MEM plus 1% BSA (both washes discarded). The monolayer of cells was then scraped using a silicated rubber plunger from a 1 ml syringe into 0.5 ml of MEM plus 0.5% BSA. This scraping procedure was repeated into another 0.5 ml of MEM plus 1% BSA and both aliquots were pooled and stored on ice until homogenisation with a 1 ml glass hand homogeniser. All syringes, centrifuge tubes and homogenisers had been previously coated with sigmacote (Sigma) and left to dry overnight in a fume hood. Both the 1 ml of cellular homogenate and the 1 ml of medium from the incubation with stimulus had 2 ml of ethanol added, vortexed, a further 3.7 ml of distilled water added before vortexing and centrifugation (5,000 g, 10 min, 4°C). The supernatant from each centrifuged sample had the pH adjusted to approximately 3 with 1 M HCl. A sample of 200 μl was retained for radioactive counting, and 4 ml of each sample was loaded onto separate Seppak C_{18} cartridges (Waters chromatography division, Millipore Corp, Milford, MA, USA) which had been previously primed by passing through 5 ml of absolute ethanol, then 5 ml of distilled water. 20 ml of 30% ethanol in distilled water followed by 20 ml of distilled water was passed through each loaded column and the elutions discarded. Elution of [^{3}H]-AA was then performed by passing 10 ml of petroleum ether through each column (eluate collected). Elution of [^{3}H]-AA metabolites occurs by passing 20 ml of petroleum ether/chloroform in a 1:1 mixture,

again the eluate was collected. The amount of ß-radioactivity in a 4 ml aliquot of each fraction was determined by liquid scintillation counting.

The separation of [^{3}H]-AA and its metabolites described was the same as that defined by Powell (1982). Further calibrations using fresh [^{3}H]-AA through the Seppak C_{18} cartridges, independently determined that 83% of the total [^{3}H]-AA was eluted at the appropriate step, and that no marked contamination of fresh [^{3}H]-AA occurred in any other fraction (F J Thomson and R Mitchell, unpublished observations). The values of [^{3}H]-AA and [^{3}H]-AA metabolites liberated into the incubation medium were expressed as a % of the total [^{3}H]-AA loaded onto columns for both medium plus cell homogenate. Due to the high variability from day to day in the absolute incorporation of [^{3}H]-AA into GH_3 cells, the values were expressed as a % of the basal release of [^{3}H]-AA from control determinations performed on the same multiwells, on the same day.

2.2.7. [^{3}H]-phorbol 12,13-dibutyrate binding studies (see Figure 2.11)

In binding studies, rats were killed by cervical dislocation and tissue was rapidly dissected over ice. Specific binding of [^{3}H]-PDBu ([^{3}H]-phorbol 12,13-dibutyrate) to membranes was performed as described by Declos *et al* (1980), on weighed tissue which had been homogenised in 100 vol of 50 mM Tris HCl (pH 7.4). Chopped tissue was homogenised using a Ystral high frequency homogeniser and was then ultracentrifuged (38,000 g, 1 h, 4°C) to separate membranous and cytosolic fractions. The supernatant from the spin was discarded and the pellet was resuspended using the Ystral homogeniser in 50 mM Tris HCl (pH 7.4) with 4 mg/ml bovine serum albumin (essential fatty acid-free) (BSA) to a concentration which gave total binding of approximately 5 - 10% of total radioactivity present. The membraneous suspension (50 µl) was aliquoted into tubes on ice containing: 400 µl 50 mM Tris HCl (pH 7.4) + 4 mg/ml BSA; 50 µl of [^{3}H]-PDBu (5 nM final concentration, approximately 0.03 µCi/tube); 2.5 µl of drug at 100 times the required final concentration and 2.5 µl

dimethylformamide (1% final (which had no effect on levels of total or non-specific binding)) or PDBu (20 μM final) in dimethylformamide for measurement of total or non-specific binding levels respectively. Tubes were capped, vortexed and incubated (30 min, 37°C) before being centrifuged (16,000 g, 5 min, 4·C) and the supernatant aspirated. The tip of the tube containing the pellet was cut off using a scalpel blade and left to dissolve overnight in 10 ml of scintillation fluid (Emulsifier-Safe, Packard Ltd, Groningen, Netherlands) before being counted for ß-radioactivity in a Beckman LS 1801 counter.

Cytosolic [^{3}H]-PDBu binding was performed in a method similar to that described by Leach *et al* (1983). Dissected tissue was homogenised in 2 volumes of 50 mM Tris HCl (pH 9.0) containing 1 mM phenylmethylsulfonyl fluoride and 1 mM $MnCl_2$ and ultracentrifuged (100,000 g, 1 h, 4°C). The pellet was discarded and the supernatant was again ultracentrifuged (120,000 g, 1 h, 4°C). The supernatant from the second spin was regarded as cytosol and stored at -40°C until use. Cytosol was diluted in 'assay buffer' (50 mM Tris HCl (pH 7.4), 4 mg/ml BSA, 1 mM $CaCl_2$ and 75 mM magnesium acetate) to a concentration which gave total binding of approximately 5 - 10% of total radioactivity present (assessement as shown in Figure 2.9), then 25 μl was incubated (30 min, 37°C) with 200 μl of phosphatidylserine (sodium salt) in assay buffer (1 mg/ml final concentration), 25 μl [^{3}H]-PDBu (5 nM final, approximately 0.03 μCi per tube), various concentrations of the investigated compound, dimethylformamide or 20 μM unlabelled PDBu final in dimethylformamide (1% final, which had no effect on the levels of total or non-specific binding) for total and non-specific binding respectively. Protein was precipitated on ice by addition of 100 μl of 12 mg/ml bovine gamma-globulin and 300 μl of 24% polyethyleneglycol 8,000 in 50 mM Tris HCl (pH 7.4) (1.8 mg/ml and 11% final concentrations respectively), capped and vortexed vigorously. After 20 min (4°C), assay tubes were spun (12,000 g, 5 min, 4°C), aspirated and the radioactivity in each pellet was determined by being dissolved overnight in 10 ml scintillation fluid, then liquid

scintillation counted for ß-radioactivity. Phosphatidylserine, diglycerides and arachidonic acid (AA) were dissolved in chloroform and dried under a stream of N_2 before sonication with a Ystral high frequency homogeniser (2 x 30 s, setting 4) followed by two full strength 30 s pulses in an MSE micro-tip sonicator. Various concentrations of diacylglycerols or AA were added to the assay in a mixed micellar method where the drug concentration was diluted by mixing with micelles which contained phosphatidylserine alone. High chain length diacylglycerols were unable to fully dissolve in dimethylformamide or other suitable solvents at convenient concentrations, and were therefore applied to binding studies in a mixed micellar method. However, 1,2-dioctanoyl-*sn*-glycerol (DOG) is more soluble in dimethylformamide and in some of the binding studies 0.5 - 500 μM DOG was applied dissolved in the solvent (1% final). Other investigated compounds were dissolved in dimethylformamide unless otherwise indicated. Differences in potency of DOG were observed between the mixed micellar and solvent-dissolved application of DOG, with the mixed micellar application of DOG being of lower potency. The potency of DOG when applied in solvent was thought to represent a more realistic estimate of the true potency of DOG for PKC as there was more possibility experimentally, for loss of phosphatidylserine and DOG in the mixed micelle method (for example, to glass tube surfaces). *In vivo* however, the lipid composition of membranes is likely to influence the value in a manner which may well be better investigated by a mixed micellar approach.

In binding studies where both the membraneous and cytosolic binding of [^{3}H]-PDBu was investigated from the same tissue, the tissue was homogenised in 10 vol of 50 mM Tris HCl (pH 9.0), 1 mM phenylmethylsulfonyl fluoride and 1 mM $MnCl_2$ and ultracentrifuged (120,000 g 1 h, 4°C). The supernatant was used for cytosolic binding as described above, and the pellet was washed three times with 2 ml of 50 mM Tris HCl (pH 7.4) + 4 mg/ml BSA before being resuspended in 50 mM Tris

Figure 2.9. **Tissue concentration-dependence of total and non-specific binding of [^{3}H]-PDBu to rat frontal cerebral cortex cytosol.**

The tissue-concentrations expressed relate to the wet weight of frontal cerebral cortex tissue from which the cytosol preparation was derived. The various concentrations of cytosol preparation are dilutions in 'homogenisation buffer' from the original cytosol preparation. Total binding of [^{3}H]-PDBu (dpm) was assessed in the presence of dimethylformamide (< 1% final) and non-specific binding (nsb) was defined in the presence of 20 μM unlabelled PDBu dissolved in dimethylformamide. Other assay conditions are the same as described in the Methods section. The total amount of radioactivity presented to each assay tube was in the order of 129,000 dpm. The data represent the means of 2 determinations, with values varying by < 20%.

Figure 2.9

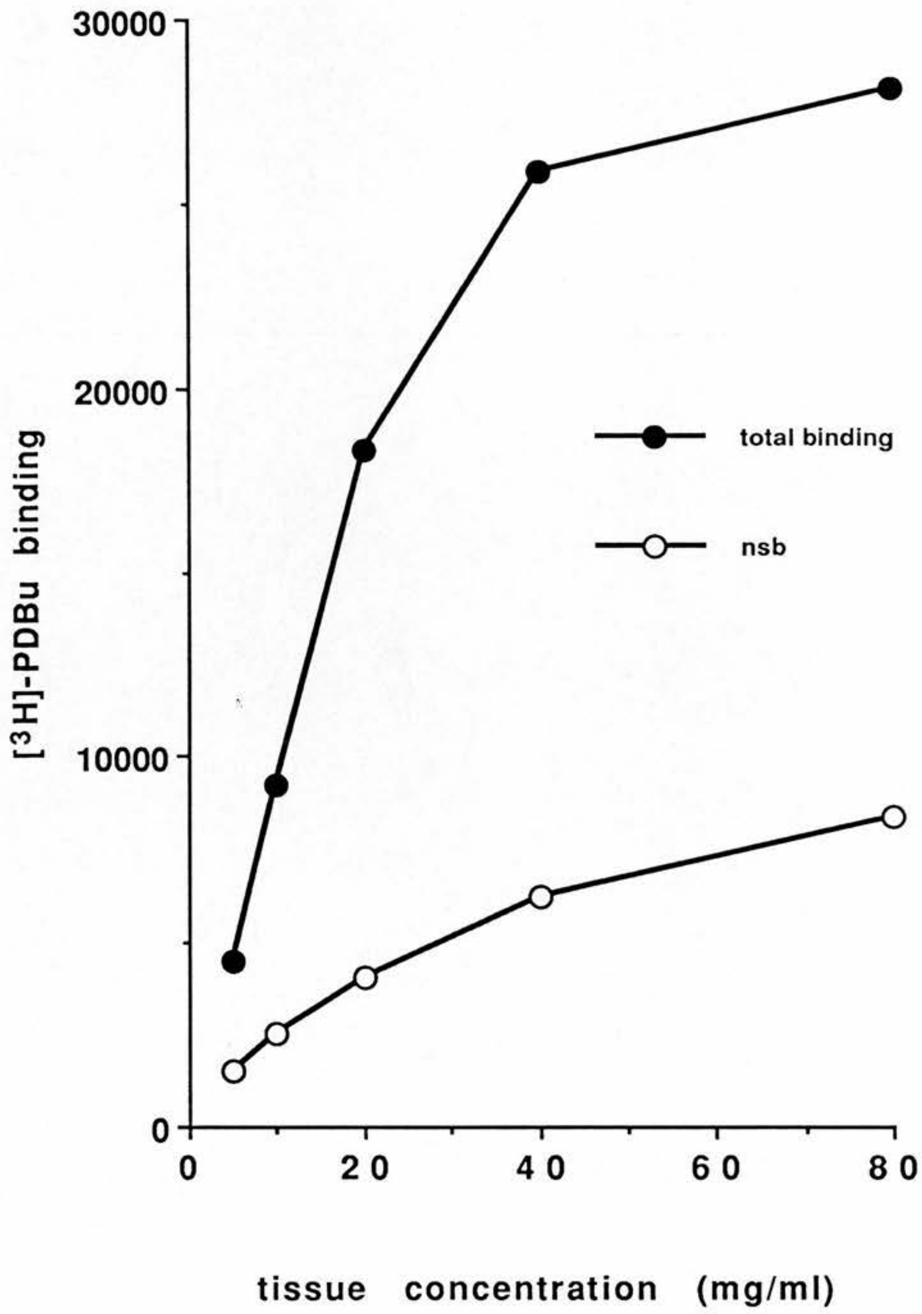


Figure 2.10. Concentration-response relationship for PDBu-displacement of [^{3}H]-PDBu binding to rat frontal cerebral cortex cytosol.

Total binding was determined in the presence of dimethylformamide (< 1% final) and non-specific binding was determined as the amount of [^{3}H]-PDBu binding remaining in the presence of 20 μM unlabelled PDBu dissolved in dimethylformamide (< 1% final). The data represent the mean ± SEM of 4 determinations. The insert shows the information provided from the non-linear curve-fitting program 'P-fit' using an asymmetric sigmoid (allosteric Hill kinetics) fit. The 'P' value in the insert is the Hill-slope value which tends towards 1, which is consistent with the involvement of a single binding site.

Figure 2.10

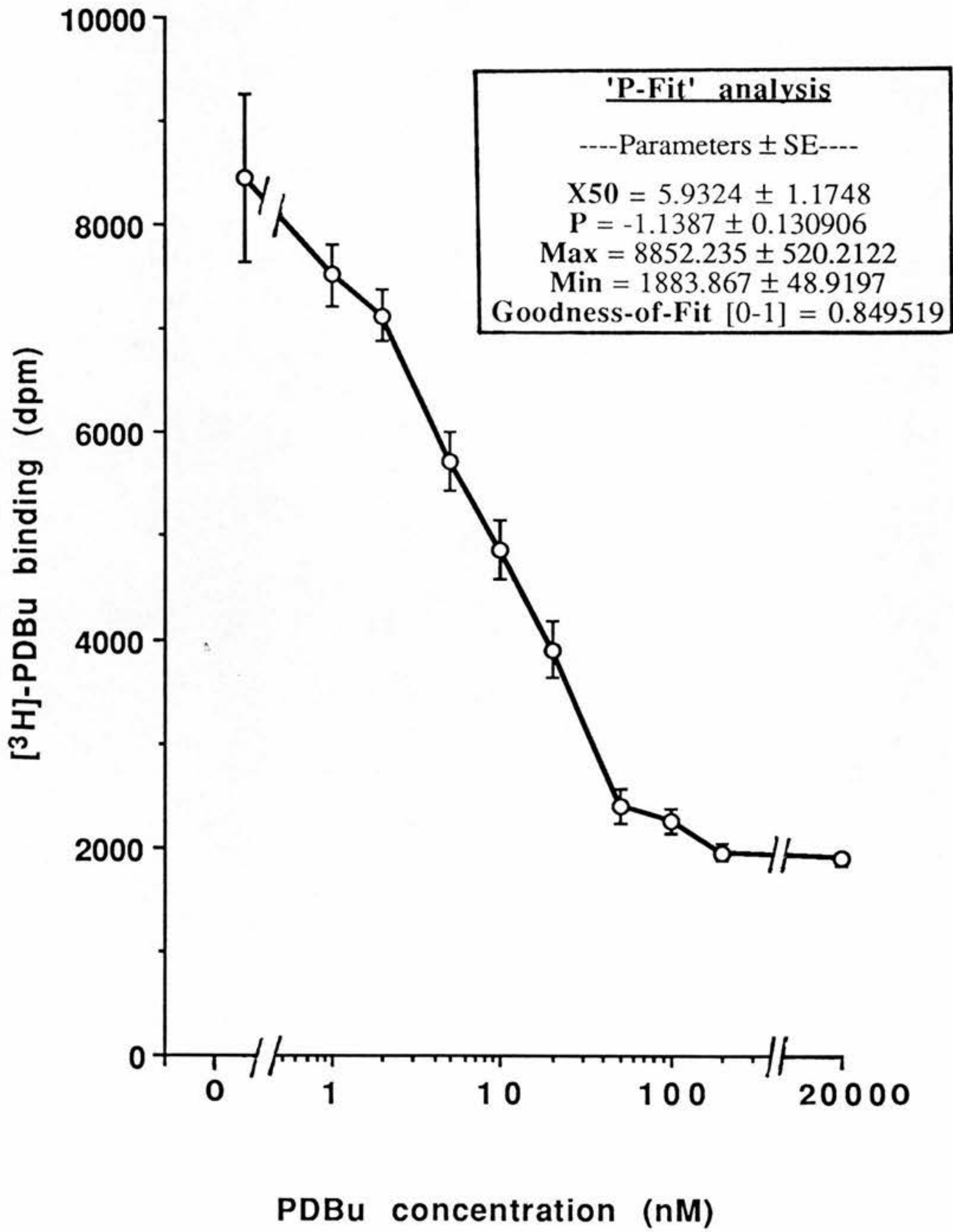
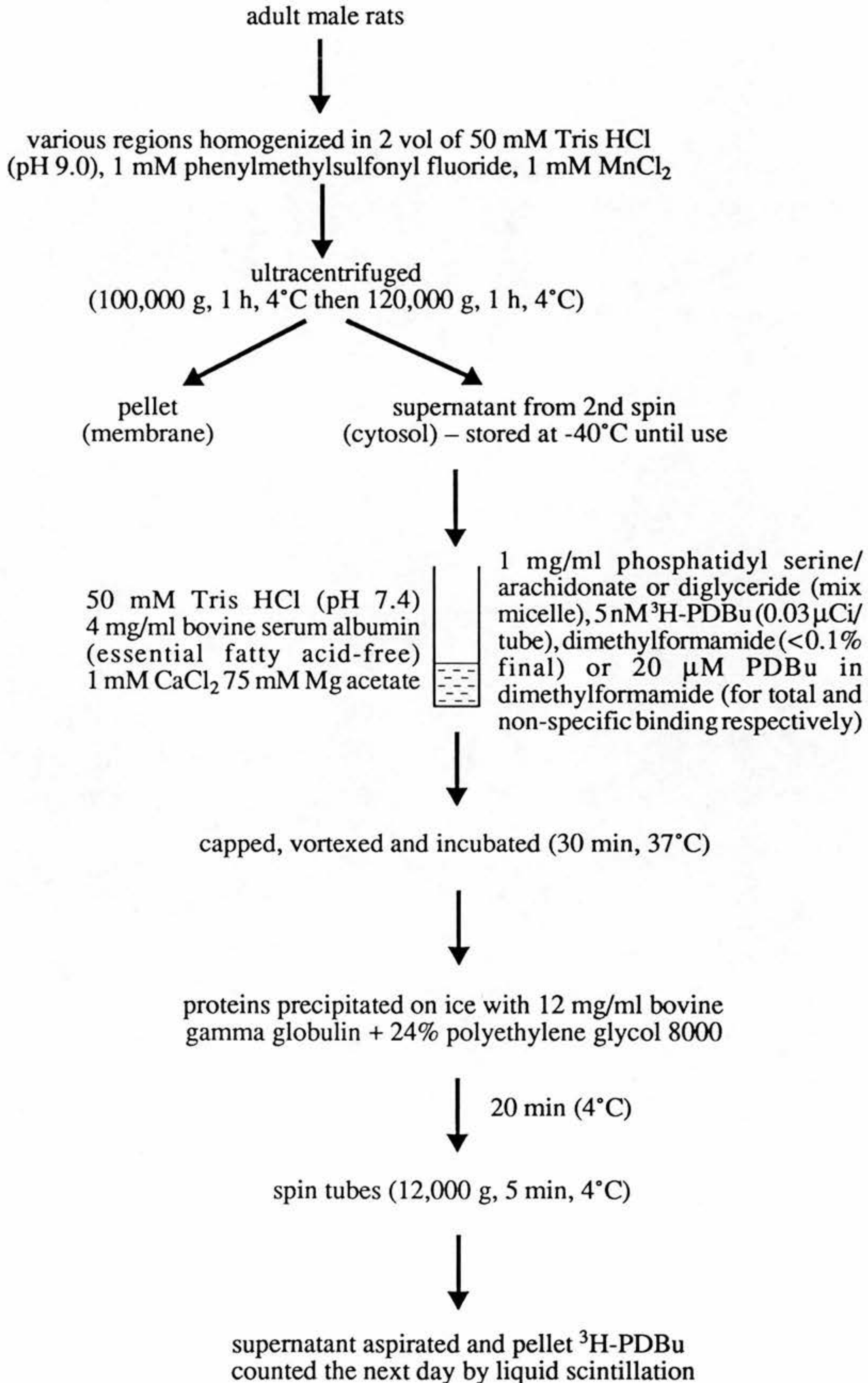


Figure 2.11. Flow diagram of [^{3}H]-PDBu binding methodology used in our studies.

Figure 2.11



HCl (pH 7.4) + 4 mg/ml BSA and assayed as described above for membraneous [^{3}H]-PDBu binding.

Analysis of binding data was performed as shown in Figure 2.10.

2.2.8. Protein kinase C activity assay (see Figure 2.19)

Rats were killed by cervical dislocation and dissected tissues were homogenised in 2 volumes of 'Kuo' Buffer (20 mM Tris HCl (pH 7.5) 50 mM 2-mercaptoethanol, 2 mM EDTA and 1 mM phenylmethylsulphonyl fluoride) containing 0.01% leupeptin and 20 μM E-64 and then centrifuged (16,000 g, 20 min, 4°C). The supernatant was collected (pellet discarded) and recentrifuged (16,000 g, 5 min, 4°C). The second supernatant was regarded as cytosol and was partially purified by loading onto 1.5 ml DE52 diethylaminoethyl cellulose (Whatman Biosystems Ltd, Maidstone, Kent, UK) (pH 7.5) held in a Bio-Rad Poly-Prep Chromatography column (Bio-Rad Laboratories, Richmond, CA, USA) in a cold-room and prewashed with 9 column volumes of 'Kuo' buffer + 0.01% leupeptin + 20 μM E-64. The cytosol was allowed to run through before washing with a further 9 column volumes of 'Kuo' buffer + 0.01% leupeptin + 20 μM E-64. The partially purified cytosol was eluted with 3 column volumes of 'Kuo' buffer + 0.01% leupeptin + 20 μM E-64 + 150 mM NaCl.

Phosphatidylserine vesicles were produced by dissolving phosphatidylserine in chloroform then blowing the glass tube dry under a stream of N_2. The phosphatidylserine 'film' was scraped into 'Tris-EGTA' buffer (20 mM Tris HCl (pH 7.5) with 0.5 mM EGTA) at a stock concentration of 400 μg/ml. The phosphatidylserine/Tris-EGTA mixture was kept on ice before sonication in a Ystral high frequency homogeniser (2 x 30 s, setting 6) followed by sonication in a microtip sonicator (1 min, full power). In mixed micelle experiments with detergent-defined micellar structure, the suspension had Nonidet P-40 added (usually 0.16% final in stock solution) and was vortexed for 2 min before allowing to settle at room temperature for a minimum of 15 min.

The PKC activity assay was similar to the methods of Huang *et al* (1988), Hannun *et al* (1985) and Marais and Parker (1989), on which the developed method here is based. All assay dilutions were performed in 'Tris-EGTA' buffer. Generally, 15 - 25 μl of partially purified cytosol (DE52, 0-150 mM NaCl fraction) was added finally to notched Sarstedt 1.5 ml Eppendorf tubes (Sarstedt Ltd, Beaumont Leys, Leics, UK) containing (final concentrations): 1.25 mM $MgCl_2$, 100 μg/ml phosphatidylserine with 0.04% Nonidet P-40, 1.25 mg/ml histone III-S or 1.25 mg/ml myelin basic protein or 10 μM α-peptide as substrate, and 100 μM ATP-γ-^{35}S (0.177 μCi/tube). The Ca^{2+}-independent PDBu-activated kinase activity was measured in the presence of PDBu (1 μM final) and EGTA (5.5 mM final). Total PKC activity, that is calcium-dependent activity superimposed on Ca^{2+}-independent activity, was measured in the presence of PDBu and either 100 nM or 100 μM-free Ca^{2+}. The appropriate calcium ion concentration to add in order to achieve the correct free-Ca^{2+} concentration was estimated using Ca^{2+}/EGTA buffering software (provided by B L Ginsborg). The actual free Ca^{2+} concentration present in an appropriate mix of the assay constituents was assessed by spectrofluorophotometric analysis using the Ca^{2+}-indicator Indo-1 and some adjustment from the standard Ca^{2+}:EGTA buffering values was required to compensate for other chelators and traces of divalents in the assay. Such a free Ca^{2+} concentration-activity response relationship is shown in Figure 2.15. The calcium concentrations were set to achieve 100 nM and 100 μM free-Ca^{2+} in the assay; chosen so as to represent typical cells of the basal cytosolic Ca^{2+} concentrations and a maximally-activating value respectively. The final assay volume was 100 μl when cytosol was added to pre-warmed (30°C) tubes containing the other assay constituents. Reactions were started by centrifugation in a bench-top centrifuge to allow all the assay ingredients which were separately 'spotted' to mix in the bottom of the tube, then incubated for 15 min at 30°C. Reactions were usually stopped by addition of 20 μl of ice-cold 0.1 M ATP in 0.1 M EDTA (pH 7.0) to cool the preparation, to chelate both Ca^{2+} and Mg^{2+}, and to dilute-out the ATP-γ-^{35}S in the reaction mixture (Mochley-

Rosen and Koshland, 1987). In experiments using α-peptide as substrate, reactions were stopped by addition of 20 μl ice-cold 24% trichloroacetic acid to precipitate proteins, followed by being placed on ice for 20 min and then spun (16,000 g, 15 min, 4°C). A 50 μl aliquot of each stopped sample (or 50 μl of supernatant from spin when using α-peptide as acceptor) was spotted onto a 4 cm^2 piece of Whatman P81 cellulose phosphate ion-exchange chromatography paper which strongly binds proteins and peptides with more than two basic amino acid residues (Kemp, 1979) (Whatman International Ltd, Maidstone, Kent, UK), and washed (3 x 2 min, room temperature) in 10 ml of 75 mM H_3PO_4 in order to wash away any unbound ^{35}S. Papers were dried overnight in a fume hood before counting in liquid scintillation fluid for ß-radioactivity.

The difference in kinase activity between tubes containing 5.5 mM EGTA/1 μM PDBu and those with 5.5 mM EGTA alone was considered as calcium-independent kinase activity. The additional increment in kinase activity in tubes containing $CaCl_2$/PDBu above those with EGTA/PDBu was considered as calcium-dependent kinase activity.

ATP-γ-^{35}S was used as the phosphate donor instead of ATP-γ-^{32}P, since ATP-γ-^{35}S is known to be a good substrate for many protein kinases (Eckstein, 1985). The thiophosphorylated substrates produced are more resistant to subsequent phosphatase activity (Coyne *et al,* 1987; Wagner and Vu, 1989) however, thiophosphorylation occurs at a slower rate than phosphorylation with ^{32}P (Wise *et al,* 1982b). The radio-isotope ^{35}S is a lower energy ß-particle emitter than ^{32}P and is therefore also safer to work with. Initial work on the development of the PKC activity assay was performed on partially purified midbrain cytosol which was partially purified by 'salting-out' the PKC with ammonium sulphate in order to remove endogenous inhibitors and phosphatases which would obscure any results (Rodriguez-Pena and Rozengurt, 1984). This was performed on cytosol preparations in 'Kuo' buffer (stirred on ice) by slowly adding solid ammonium sulphate to a concentration of 21% and allowing the crystals to dissolve. The suspension was then centrifuged (16,000 g,

20 min, 4°C). The pellet was discarded and the supernatant put back on ice, stirred and had another 24% (45% total) ammonium sulphate slowly added to it. The suspension was again centrifuged (16,000 g, 20 min, 4°C) and the pellet was weighed, resuspended in 2 vol of 'Kuo' buffer and added to the above kinase assay to measure the 21 - 45% ammonium sulphate-'cut' PKC activity. Although a reasonable PKC activity was obtained using the ammonium sulphate-'cut' purification methodology (Figure 2.12), it was abandoned in favour of the DE52-partial purification method which was much less time consuming and easier to standardise. Furthermore, any influence of high levels of ammonium sulphate on PKC activity would be absent. DE52 (diethylaminoethyl cellulose) is an ion-exchange resin which acidic proteins (including PKC) stick well to (Wise *et al*, 1982a). Other non-acidic proteins can be washed through the column (partially-purified) using an increasing NaCl concentration in the eluting buffer. PKC(s) consistently elutes from the column a characteristic NaCl concentration (Mochley-Rosen and Koshland, 1987). Using both DE52-purified (0 - 80 mM NaCl) and ammonium sulphate-'cut'-purified (21 - 45% ammonium sulphate) PKC, thiophosphorylation kinetics were known to be linear up to 30 min (Figure 2.12), therefore a 15 min incubation time at 30°C was used to ensure enough signal for analysis, but certainly to be within the linear range. It was estimated that under our assay conditions where the protease inhibitors leupeptin and E-64 were included not only in the homogenisation and purification steps, but also in the PKC activity assay, PKC degradation was minimal within 15 min, since no evidence for loss of regulated kinase activity or further appearance of kinase activity was apparent in the time course experiments (Figure 2.12). A major concern was the state of the PKC in the assay, as a marked amount of kinase activity was present with phosphatidylserine alone, indicating that an unregulated catalytic fragment of PKC could be present. As discussed by Hannun *et al* (1985) and Huang *et al* (1988), the physical interactions occurring between Ca^{2+} and acidic phospholipids (such as phosphatidylserine) which can result in the formation of undefined multilamellar complex micelles and the partial

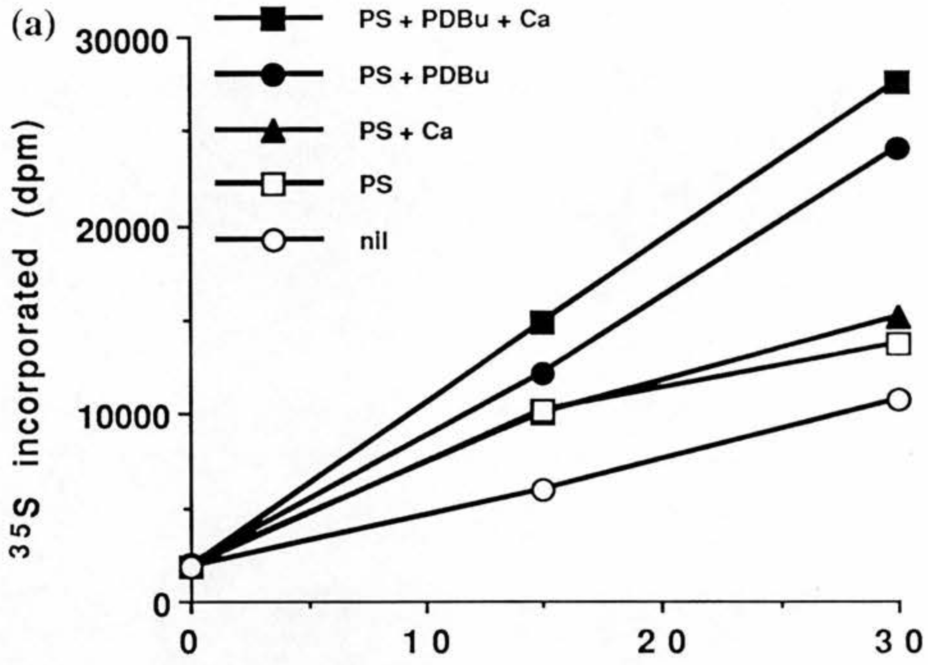
preactivation of PKC in a phorbol ester/diacylglycerol-independent manner. The presentation of phosphatidylserine in fixed composition micelles generated by certain detergents can overcome this problem without a marked deleterious effect of the detergent on the kinase (Hannun *et al,* 1985; Huang *et al,* 1988). Workers using sonicated phosphatidylserine alone (such as Nishizuka's group) thus usually have results with a high basal activity in the presence of phosphatidylserine/Ca^{2+} alone and see relatively less phorbol ester/diacylglycerol regulation than in the study or those of others (Hannun *et al,* 1985; Huang *et al,* 1988; Marais and Parker, 1989). Thus, the inclusion of either Triton X-100 (0.25%) or Nonidet P-40 (0.04%) detergents within the phosphatidylserine micelles provides a more uniform lipid environment for PKC and removed the unstimulated PKC activity in the presence of phosphatidylserine alone, with no detriment to total PKC activity until concentrations of Nonidet P-40 of 0.05% or greater (Figure 2.14). Increasing the micellar concentration of phosphatidylserine resulted in an increase in both Ca^{2+}-dependent and Ca^{2+}-independent PKC activity, despite the constant presence of 0.04% Nonidet P-40 (Figure 2.16).

On the basis of work in the literature (Fearon and Tashjian, 1985) it was originally thought that the 0 - 80 mM NaCl fraction of DE52-purified male rat midbrain cytosol may well contain all of the PKC extractable from the tissue. The specific [^{3}H]-PDBu binding in the prewash, the 0 - 80 mM NaCl and the 80 - 200 mM NaCl revealed that specific [^{3}H]-PDBu binding was present not only in the 0 - 80 but also in the 80 - 200 mM NaCl elution fractions with small amounts also present in the prewash. Further extensive studies were performed on the PKC activity of the prewash, 0 - 50, 50 - 100, 100 - 150 and the 150 - 300 mM NaCl elution fractions from the DE52 column loaded with male midbrain (Figure 2.13). No phorbol ester-stimulated kinase activity could be found in the prewash, with all other fractions containing kinase activity (for histone at least), however the 150 - 300 mM NaCl elution fraction kinase was not stimulated by either PDBu or Ca^{2+}, suggesting that it could be a proteolytically

Figure 2.12. Time-course of partially-purified male rat midbrain cytosol.

Male rat midbrain cytosolic PKC activity partially-purified by either a 21 - 45% ammonium sulphate 'cut' (a) or 0 - 80 mM NaCl elution from DE52 (b) was measured using α-peptide as a substrate. The α-peptide kinase activity was halted as described in the Methods section. The assay conditions represent either no additions (5.5 mM EGTA final, open circles), phosphatidylserine (PS, 8 μg/ml sonicated vesicles and 5.5 mM EGTA final), open squares, PS (8 μg/ml) + Ca (600 μM Ca^{2+}/500 μM EGTA final, triangles), PS (8 μg/ml) + PDBu (100 nM PDBu final) and 5.5 mM EGTA (closed circles), or PS (8 μg/ml final) + PDBu (100 μM final) + Ca (600 μM Ca^{2+}/500 μM EGTA final (closed squares)). The data shown in section (a) represent the mean of 2 determinations which varied by < 20%, whereas the data in (b) represent the mean ± SEM of 4 determinations. Enzyme kinetics were linear at 15 min. Note maximal enzyme activity was greater with DE52-purified PKC.

Figure 2.12



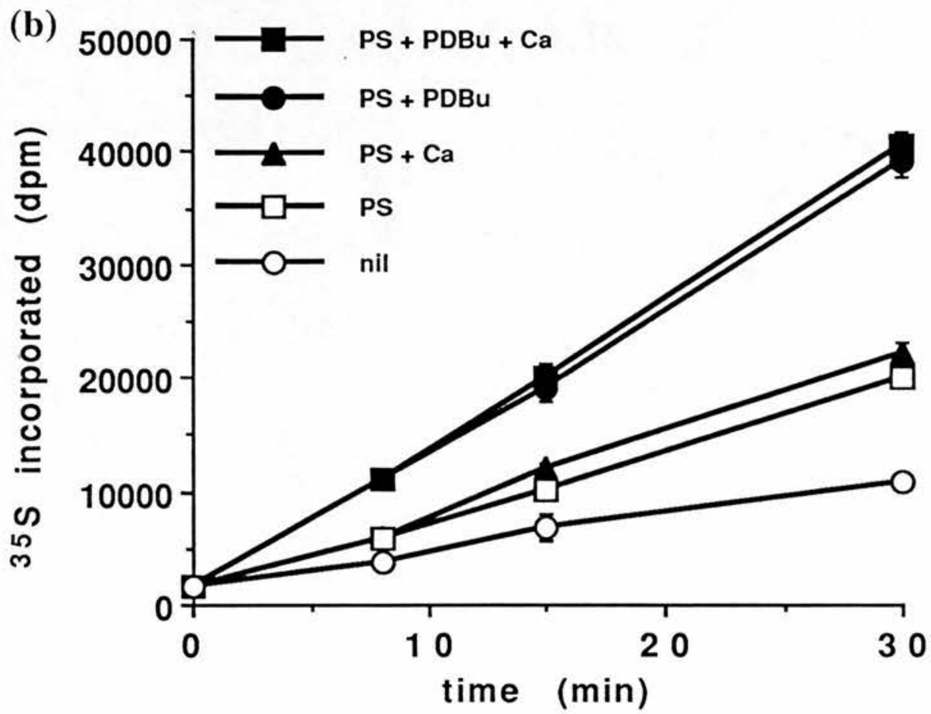


Figure 2.13. Kinase activity of, and [^{3}H]-PDBu binding to PKCs in various elution fractions from male rat midbrain cytosol partially-purified on DE52.

Kinase activity in the presence of PS (8 μg/ml phosphatidylserine final), PS + PDBu (100 nM final) or PS + PDBu + Ca (600 μM Ca^{2+}/500 μM EGTA final) using α-peptide (a) or histone (b) as substrates. The levels of [^{3}H]-PDBu binding (c) to the various DE52-elutions was also determined. Total binding was assessed in the presence of dimethylformamide (< 1% final) and non-specific binding (nsb) was assessed in the presence of 20 μM PDBu (final concentration) dissolved in dimethylformamide. The cytosolic [^{3}H]-PDBu binding assay was performed as described in section 5.2, in the presence of 300 μg/ml phosphatidylserine (final) reconstituted in 20 mM Tris HCl (pH 7.5) + 0.5 mg/ml bovine serum albumin buffer, and in the additional presence of 1 mM Ca^{2+} (+Ca^{2+}) or Ca^{2+}-free (-Ca^{2+}) conditions respectively. Non-specific binding was determined in Ca^{2+}-free conditions although the presence of Ca^{2+} produced no perceptible change to the levels of non-specific binding. The DE52 column elutions were performed with 'Kuo' buffer (20 mM Tris HCl (pH 7.5), 50 mM 2-mercaptoethanol, 2 mM EDTA and 1 mM phenylmethylsulphonyl fluoride) which was supplemented with the indicated concentration of NaCl. The elutions from zero salt to high salt were performed sequentially. Each of the determinations was performed from a separate DE52-column loaded with separate rat midbrain cytosol preparations (ie, each elution used in (a), (b) and (c) were from individual rats and not determinations of kinase activity and [^{3}H]-PDBu binding from the same elution). The data represent the means of 2 determinations, which generally varied by < 25%. Note that almost all the PDBu-stimulated kinase activity eluted from the column between 0 and 150 mM NaCl..

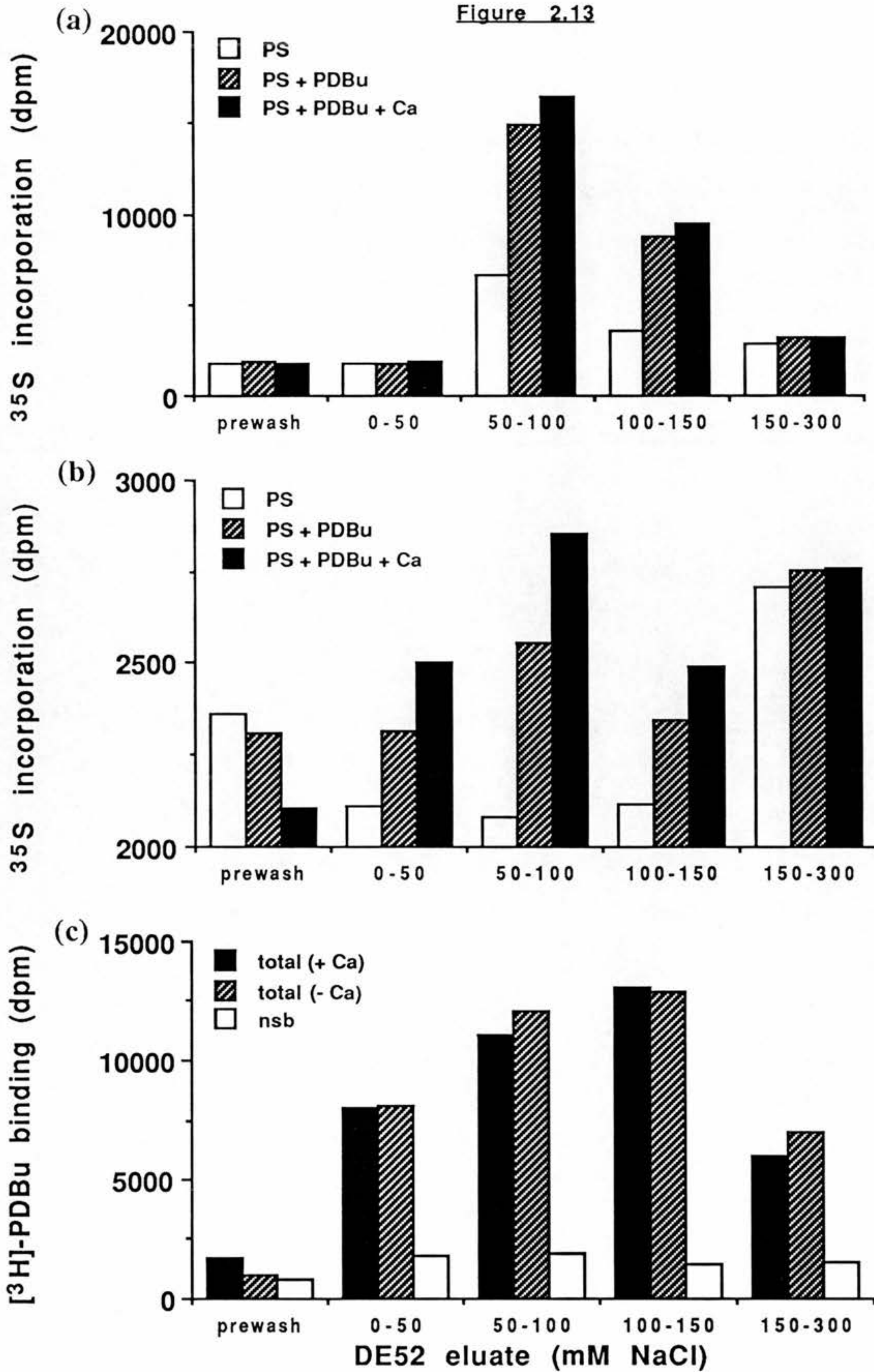


Figure 2.13
(a)
20000
10000
0
PS
PS + PDBu
PS + PDBu + Ca
35S incorporation (dpm)
prewash
0-50
50-100
100-150
150-300
(b)
3000
2500
2000
PS
PS + PDBu
PS + PDBu + Ca
35S incorporation (dpm)
prewash
0-50
50-100
100-150
150-300
(c)
15000
10000
5000
0
total (+ Ca)
total (- Ca)
nsb
[3H]-PDBu binding (dpm)
prewash
0-50
50-100
100-150
150-300
DE52 eluate (mM NaCl)

Figure 2.14. Effect of Nonidet-P40 concentration on the kinase activity of partially-purified male rat midbrain.

Partially-purified (0 - 150 mM NaCl, DE52) male rat midbrain kinase activity was determined in the presence of no additions (only 5.5 mM EGTA) (a), PS (100 μg/ml phosphatidylserine final (b)), PS + Ca^{2+} (600 μM Ca^{2+}/500 μM EGTA final (c)), PS + PDBu (1 μM final (d)) or PS + PDBu + Ca^{2+} (e). Note the increased micellar concentration of phosphatidylserine (8 μg/ml final in previous work) which is due to its dilution with the Nonidet-P40 and the increase is to maintain a concentration of the lipid great enough to support PKC activity. The markings on the columns indicate the concentration of Nonidet-P40 in the micelles (0.03% - 0.06%). Histone was used as a substrate. The data represent the means of 2 determinations, with values generally varying by < 20%. Note that PS alone no longer induces a substantial increase in kinase activity.

Figure 2.14

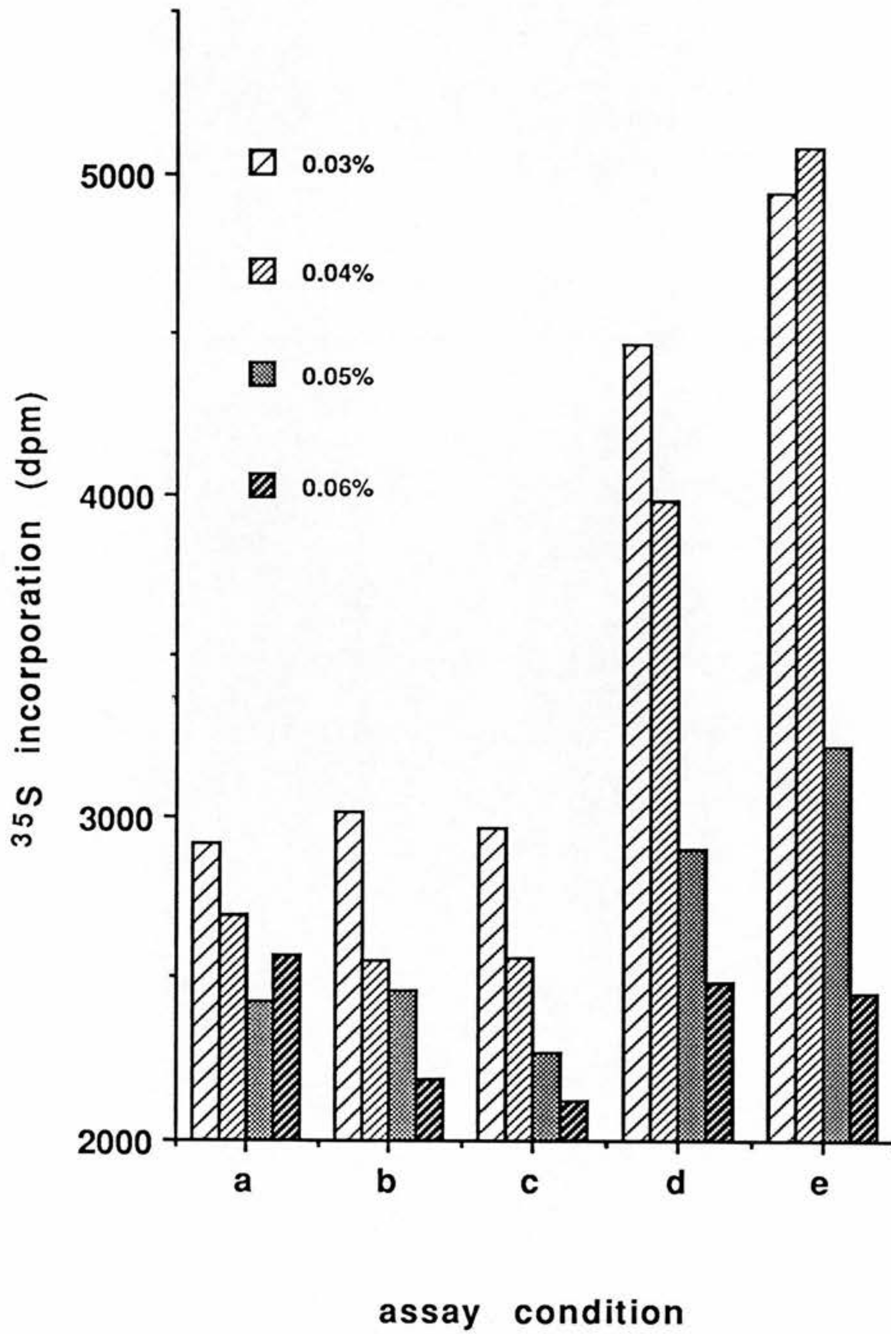


Figure 2.15. Effect of Ca^{2+} concentration on PKC activity of partialy-purified male rat midbrain.

Concentration-response relationship for free Ca^{2+} ion concentration and partially-purified (with 0 - 150 mM NaCl on DE52) male rat midbrain cytosolic PKC activity using histone as a substrate. The free Ca^{2+} concentration was calculated by a program to assess Ca/Mg/H/EGTA complex formation kindly provided by Professor Bernard L Ginsborg, and was confirmed independently by spectrofluorometry using Indo-1 salt as described in the text of section 2.2.9. The level of activity in the absence of 1 μM (final) PDBu but in the presence of phosphatidylserine (100 μg/ml) and 0.04% Nonidet-P40, is approximately at the levels of the abscissa (ie, slightly less than 3,000 dpm). Data show the PKC activity in the presence of 1 μM PDBu, with the additional presence (squares) or absence (circles) of the calmodulin inhibitor 5-iodo-C_8 (20 μM final) generously supplied by Dr Sheila McNeil. The data represent the mean of 2 determinations, which generally varied by $< 20\%$.

Figure 2.15

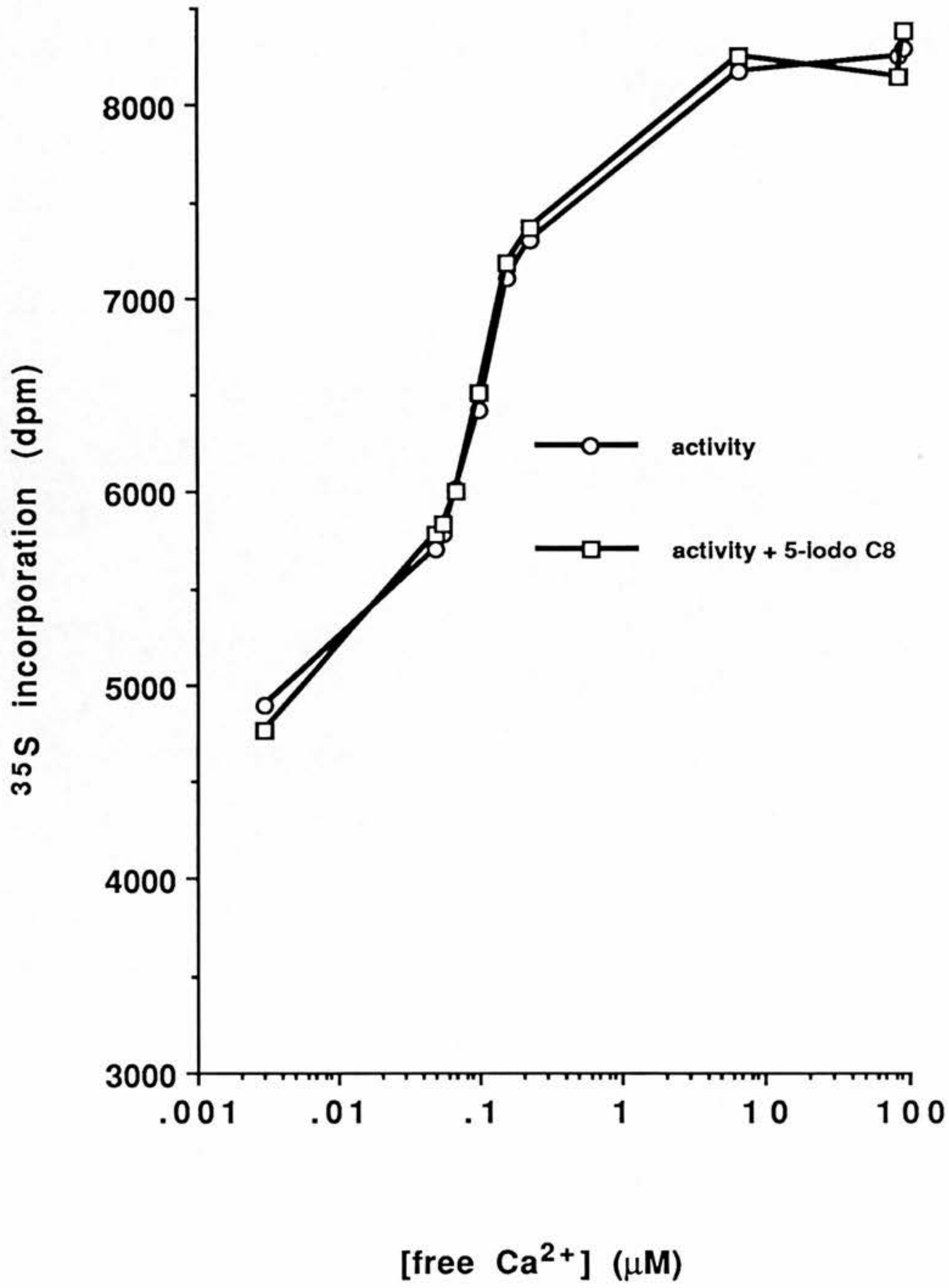


Figure 2.16. Effect of phosphatidylserine concentration on PDBu-stimulated kinase activity of partially-purified male rat midbrain.

Concentration-response relationships for PDBu-stimulated partially-purified (0 - 150 mM NaCl, DE52) midbrain kinase activity (using histone as a substrate), at various phosphatidylserine concentrations, as indicated in the insert. Kinase activity was determined in calcium-free (5.5 mM EGTA final, (a)) or in the presence of calcium (600 μM Ca^{2+}/500 μM EGTA final (b)). The indicated micellar phosphatidylserine concentrations are in the added presence of 0.04% Nonidet-P40 throughout. The data are the means of 2 determinations, with values varying by $< 20\%$.

Figure 2.16

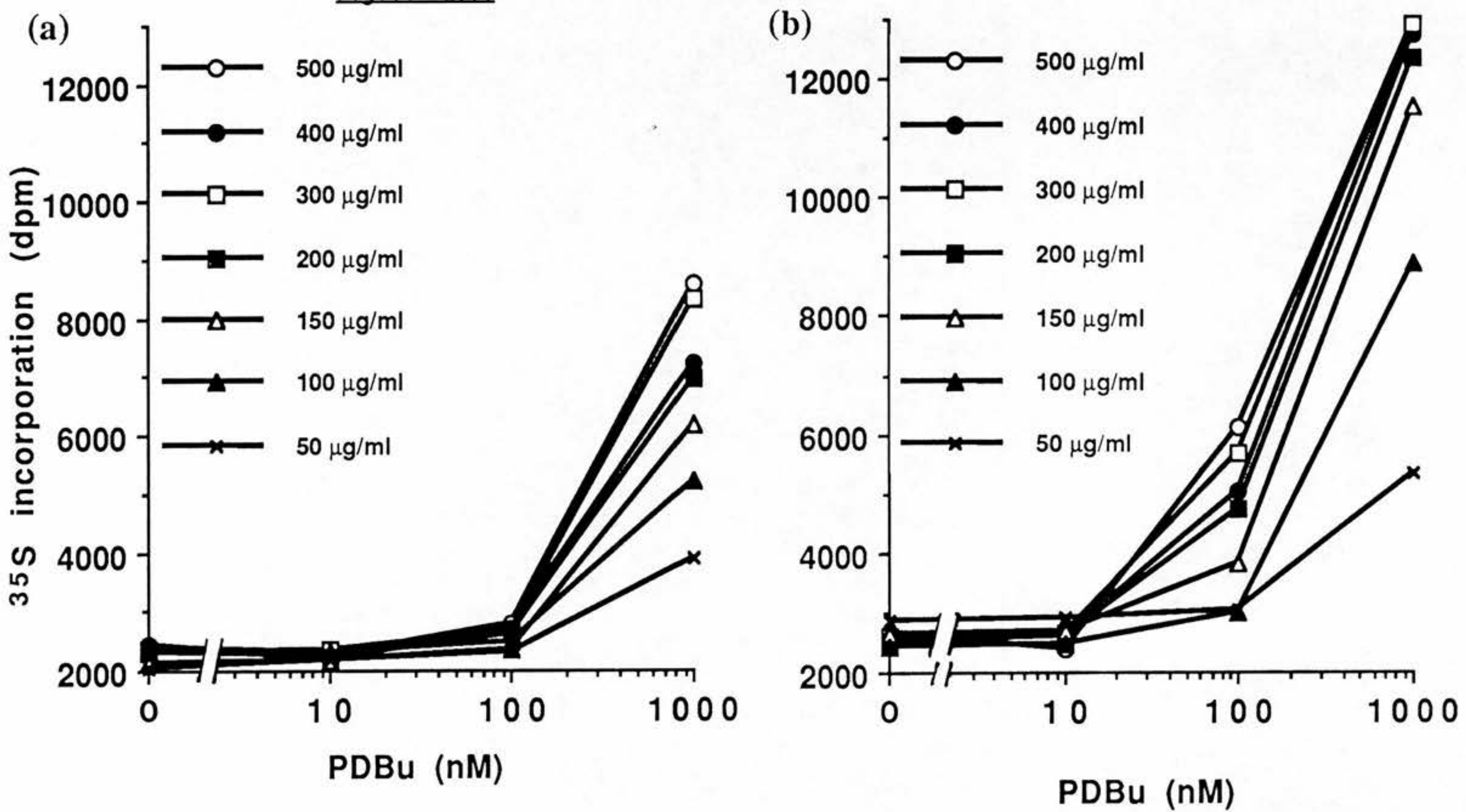


Figure 2.17. Effectiveness of the substrates histone-IIIS and α-peptide for PKC-mediated thiophosphorylation in a calcium-dependent fashion.

Partially-purified (0 - 150 mM NaCl. DE52) male rat midbrain kinase activity towards histone and α-peptide. The final concentrations of substrate used was 1.25 mg/ml for histone-IIIS and 10 μM for α-peptide. The assay conditions shown indicate no additions (5.5 mM EGTA) (a), PS (100 μg/ml phosphatidylserine + 0.04% Nonidet-P40 final + 5.5 mM EGTA) (b), PS + PDBu (1 μM final) (c), PS + PDBu + Ca^{2+} (600 μM Ca^{2+}/500 μM EGTA) (d) and PS + PDBu + Ca + 5-iodo-C8 (30 μM final of a selective calmodulin inhibitor generously provided by Dr Sheila McNeil) (e). The data represent the means of 2 determinations, with < 25% variation. Note that PKC activity towards α-peptide as a substrate appears to be markedly inhibited by 5-iodo-C8 and that PKC activity displays calcium-dependence with histone (but not α-peptide) as substrate, although ^{35}S incorporation into α-peptide is far greater than the incorporation into histone.

Figure 2.17

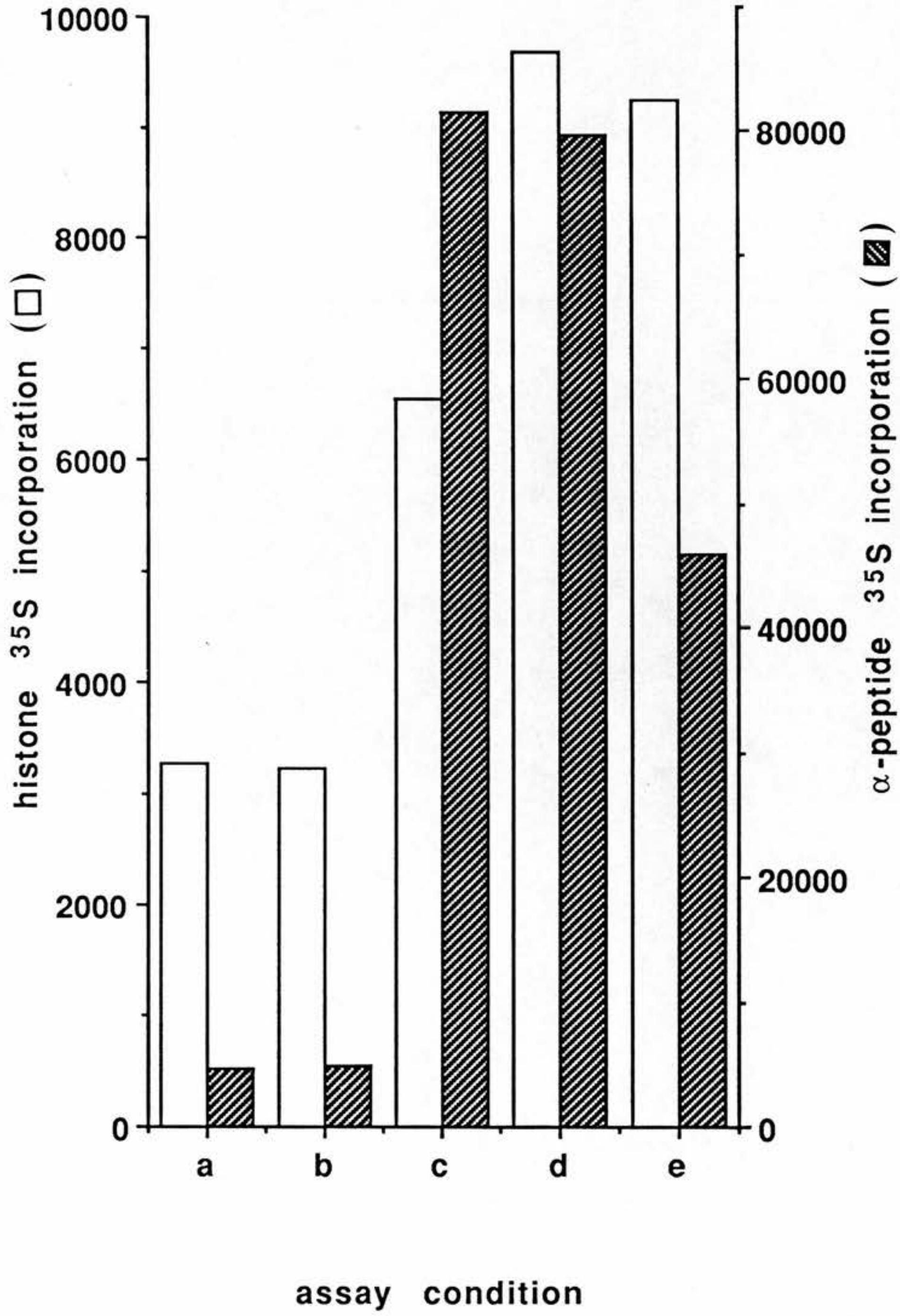


Figure 2.18. Effectiveness of histone-IIIS and myelin basic protein as thiophosphorylation substrates and the calcium-dependence of these events.

Partially-purified (0 - 150 mM NaCl, DE52) PKC from male rat midbrain cytosol was tested using histone-IIIS (a) and myelin basic protein (b) as a substrate. The concentration of substrate used was 1.25 mg/ml for both histone and myelin basic protein. Calcium-free conditions (-Ca^{2+}) were produced with excess EGTA (5.5 mM final) and activity with calcium (+Ca^{2+}) was determined with 600 μM Ca^{2+}/500 μM EGTA (approximately 100 μM free final Ca^{2+} ion concentration). The concentration-response relationships to PDBu indicate that histone is quite calcium-dependent in its substrate kinetics, unlike myelin basic protein which was again, able to incorporate more [^{35}S]-thiophosphate than histone. The data represent the means of 2 determinations, which generally varied by < 25%.

Figure 2.18

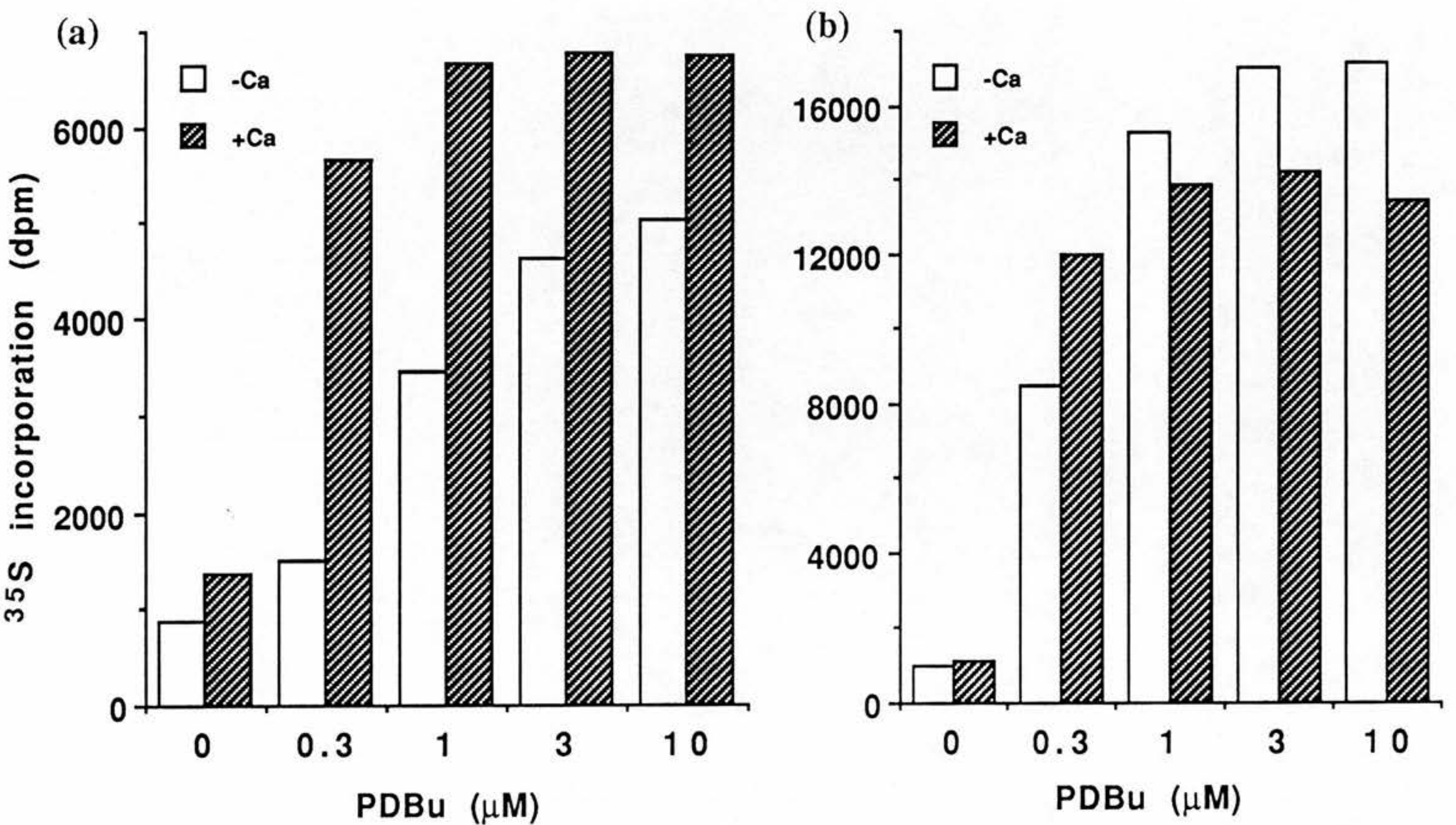
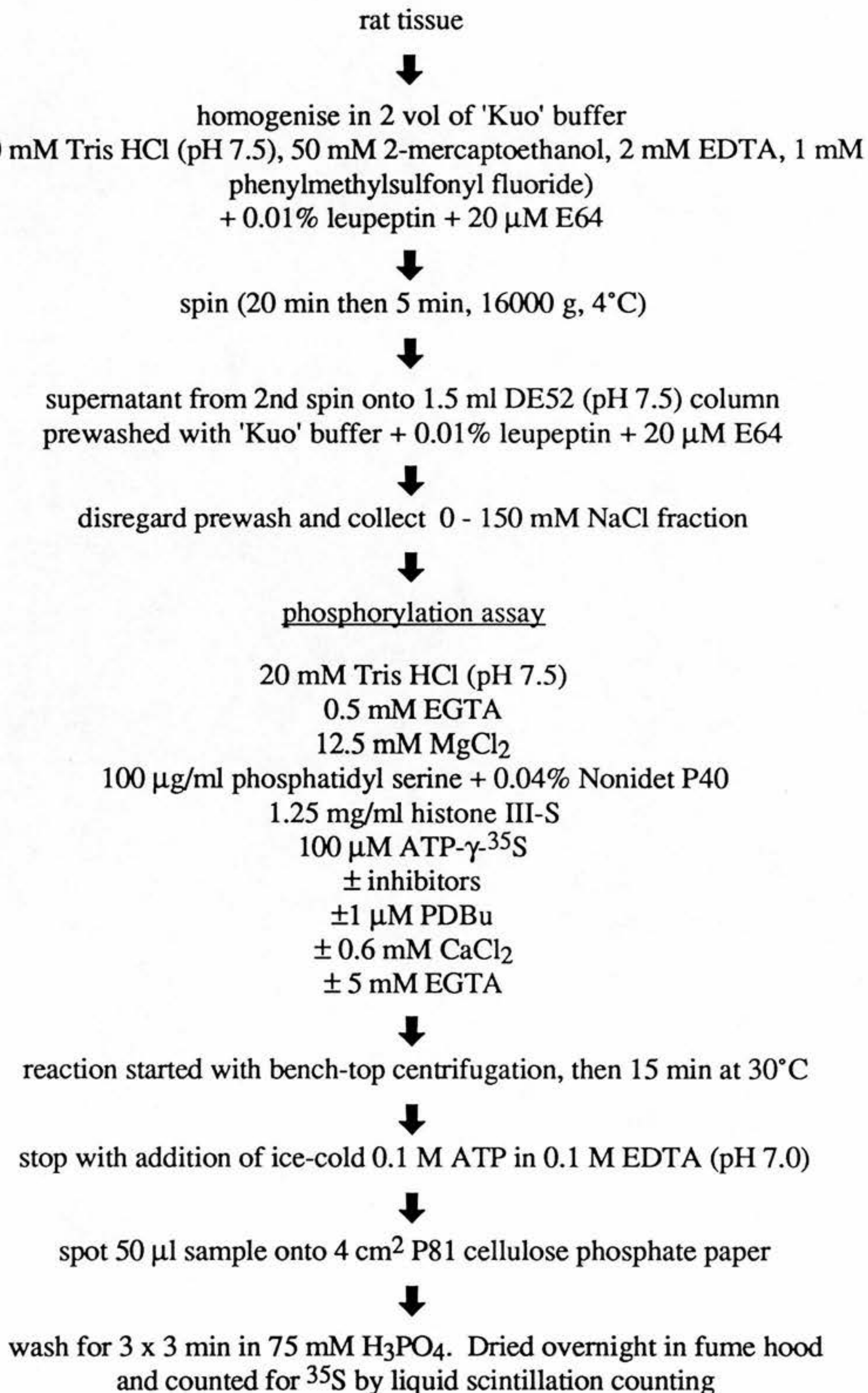


Figure 2.19. **Flow diagram of methodology used to assess partially-purified PKC activity used in our studies.**

Figure 2.19



cleaved catalytic fragment of PKC with unregulated kinase activity (also known as PKM, which is known to elute from DE52 at higher salt concentrations than native PKC (Mochley-Rosen and Koshland, 1987)). These results are consistent with cleaved regulatory and catalytic fragments of PKC being present. However, the prewash (which contains the cleaved regulatory fragments of PKC that can still bind PDBu) was discarded. Only the 0 - 150 mM NaCl elution fraction was taken from the DE52, ignoring the protein left on the column (which contains the catalytic fragment of PKC that shows unregulated kinase activity) (Mochley-Rosen and Koshland, 1987). In this way, our approach recovered the vast majority of native PKC in the cytosol while ensuring that the PKC breakdown products were not being assayed along with the PKC.

Figures 2.17 and 2.18 demonstrate the ability of the substrates histone III-S, α-peptide and myelin basic protein to be used as substrates for rat midbrain cytosolic PKC in a Ca^{2+}-dependent fashion. These data suggest that only histone III-S confers Ca^{2+}-dependency upon PKC activity, and the merits of using it as a substrate are discussed further in chapter 6.

2.2.9. Protein assay

Protein content was determined by use of the Pierce Coomassie protein assay kit (Pierce, Rockford, IL, USA) which is a ready-to-use Coomassie Blue-containing reagent solution which is based on the Bradford (1976) method of protein concentration determination. The protein standard used was bovine serum albumin (essential fatty acid-free) (BSA) diluted in the appropriate assay medium (usually 'Kuo' buffer; see section 2.2.8) to give standard concentrations of protein in the range 125 to 2,000 μg/ml. A 100 μl aliquot of each standard or unknown (or buffer alone for blank) had 5 ml of the Protein Assay Reagent added (which consists of Coomassie Blue G-250 dye, phosphoric acid, methanol, water and a solubilizing agent) and each sample was mixed well by repeated inversion. The absorbance at 595 nm of each sample was

determined using a Cecil Instruments CE 292 digital ultraviolet spectrophotometer after standardising the absorbance to deionized, distilled water. The absorbance values for blanks were subtracted from each sample absorbance. The protein concentrations for the unknown samples were determined against the standard curve, which was consistently linear with the standard protein concentrations used.

2.2.10. Data analysis

Concentration-response data were analysed, by a non-linear, iterative, individually-weighted curve-fitting program ('P-fit'; Biosoft, Cambridge, Cambs, UK). The values quoted represent the calculated means of either an EC_{50} (effective concentration which produces 50% of the maximal response) or IC_{50}(concentration which inhibits 50% of the maximal response), with errors representing the standard errors of the mean (SEM).

Where appropriate, the statistical significance of changes was assessed by Mann-Whitney U-test. This is a nonparametric statistical analysis test which makes no assumptions of a normal distribution of data and errors. It is therefore a convenient statistical test to analyse both raw data, and data which is transformed (eg, ratioed to some control value) as is mostly presented here. Although the Mann-Whitney U-test is not the most powerful test to detect small differences, it does not demand the constraints on data distribution of the Student's t test, which can be violated easily in practice, nor does it produce the false positives which can arise from the Wilcoxon test or the matched pair t-test (Kranth, 1983). To apply the test, all the data have to be assigned a rank order of magnitude (eg, number 1 for lowest value to number n for the highest value (n = total number of values in both data groups being compared)). The rank orders in both groups are summed, and the statistical significance for the appropriate number of values in each group, can be determined using a Mann-Whitney or Wilcoxon significance table. Significance was accepted at probability values of $P \leq 0.05$.

All averaged data presented throughout this thesis are expressed as the mean ± standard error of the mean (SEM) of greater than two values. The n values quoted are the number of independent, individual experiments from which the mean values and errors are calculated. In some of the method development studies, results are indicated as the mean from two experiments. The individual values generally differed by no more than 20% and are plotted directly in some figures.

CHAPTER 3

THE EFFECT OF ACTIVATORS AND INHIBITORS OF PROTEIN KINASE C ON DEPOLARISATION-INDUCED CALCIUM INFLUX INTO RAT ANTERIOR PITUITARY CELLS

3.1. INTRODUCTION

Since its initial discovery in 1977 by Nishizuka's group, Ca^{2+}-activated, phospholipid-dependent kinase (protein kinase C (PKC)) has been extremely well characterised both in terms of its molecular biology and particularly, its biochemistry (Nishizuka, 1984a; Nishizuka, 1988; Huang, 1989; Parker *et al*, 1989). In comparison with this, the pharmacology of PKC has been far less well characterised over the last 14 years. A major step in the study of the enzyme came in 1983, when Niedel *et al* published evidence almost simultaneously with other laboratories, that PKC was the major receptor for the phorbol ester class of tumour promoters. This fact spurred much research into the enzyme for two reasons: (i) that the enzyme now had a relatively specific tool for studying its activation and function, and (ii) the fact that PKC was the major receptor for a carcinogen, implicated its role in tumorigenesis. A major contribution to the understanding in PKC pharmacology has come from research by Blumberg and his colleagues (see Blumberg, 1988 for review). It soon became evident that various phorbol esters could interact differently with a number of different phorbol ester binding sites (Dunn and Blumberg, 1983; Jaken *et al*, 1983) and it was suspected that PKC was not a single enzyme entity but existed in multiple forms. In 1986, Parker *et al* and Coussens *et al* published the primary sequence of PKC and provided evidence that multiple isoforms of PKC existed, each being products from different genes. Soon after these reports, it was established that at least seven isoforms of PKC could be detected (for review see Nishizuka, 1988). Four of the isoforms (α, ßI, ßII and γ (the ßI and ßII isoforms being mRNA splice-variants)) contained a putative Ca^{2+}-binding domain, whereas three isoforms (δ, ε and ζ) did not. Use of PKC isoform-specific antibodies and mRNA *in situ* hybridisation technology, revealed the PKC isoforms to be differentially distributed through both the body and cells (Nishizuka, 1988) and it was suspected that PKC isoform-specific cellular substrates contributed to physiologic cell-specific actions of PKC. Although some interesting pharmacological

differences amongst some of the PKC isoforms have been described (Naor *et al,* 1988a; Naor, 1990; Evans *et al,* 1991), our knowledge of any selective pharmacology remains extremely basic. Most work has centred around purified enzymes or PKC isoforms generated from their cDNAs-cellular systems when investigating the pharmacology, co-factor requirements and substrate targets of PKC isoforms. Such studies have provided an invaluable basis as to further research on PKC, but further characterisation of the properties of PKC isoform, especially their modulation and actions in a cellular context, required their investigation in more physiological models.

In the present study, we have attempted to provide models of endogenous PKC isoform actions in functioning cells and to provide some pharmacological characterisation of the models of PKC isoform function. It has been reported that activation of PKC can lead to either an increase or decrease in 'L'-type Ca^{2+} channel activity which is dependent upon the cell type investigated (see section 1.3). Since the PKC isoform-content varies among cell types, it is our hypothesis that differences in PKC isoform among the cells may be the basis by which this cell-specific dual modulation of the 'L'-type Ca^{2+} channel occurs. Furthermore, 'L'-type Ca^{2+} channels are relatively easily accessible to biochemical investigation due to their voltage-activation, slow inactivation and dihydropyridine-sensitivity (see section 1.3). Opening of 'L'-type Ca^{2+} channels can be blocked by inclusion of the dihydropyridine, nimodipine. The chosen model for our initial studies was high K^+-depolarisation-induced influx of $^{45}Ca^{2+}$ into both rat anterior pituitary pieces and the rat anterior pituitary GH_3 clonal cell line. The effects of preincubation with various activators and/or inhibitors of PKC are investigated.

3.2. SPECIFIC METHODOLOGY

High K^+-induced $^{45}Ca^{2+}$ influx was as described in section 2.2.3. All of the agents investigated were added to the assay dissolved in dimethylformamide (1% final) with the exceptions of arachidonic acid, which was added to the assay dissolved

in ethanol (0.4% final). H7 and HA1004 were added to the assay dissolved in distilled water (5 μl per 500 μl of 'calcium uptake medium'). Parallel controls for the effects of vehicle were always carried out and the vehicles at the concentrations used had no detectable effect on any of the cellular parameters measured.

3.3. RESULTS

Calcium influx studies on anterior pituitaries prisms and GH_3 cells populations

The development of the $^{45}Ca^{2+}$ influx protocol and an account of the distribution of radioactivity has been discussed in section 2.2.3. Briefly, under the conditions employed, non-specific adsorbtion of $^{45}Ca^{2+}$ to the filters and cells (as measured in zero time blanks) typically accounted for around 400 dpm of the calcium accumulation. The concentration of GH_3 cells within the assay was adjusted to match the amount of calcium influx observed in anterior pituitary pieces for 30 s (a time which displayed a maximal signal-noise ratio). Thirty second incubation of quartered, hemisected anterior pituitary prisms or GH_3 cells (at a density of 2.5 x 10^6/0.5 ml) in the presence of $^{45}Ca^{2+}$-containing medium but in the absence of an influx stimulus, accounted for the accumulation of 550 - 600 dpm of $^{45}Ca^{2+}$. A 60 mM K^+ stimulus for 30 s induced both anterior pituitary pieces and GH_3 cells to typically accumulate 1400 - 1800 dpm of $^{45}Ca^{2+}$ (120 - 155 fmoles of $^{45}Ca^{2+}$/hemisected anterior pituitary/min or $^{45}Ca^{2+}/10^6$ GH_3 cells/min) in addition to control influx of $^{45}Ca^{2+}$ over the same period. The K^+-stimulated $^{45}Ca^{2+}$ movement into both anterior pituitaries and GH_3 cells was inhibited to a similar extent by the voltage-sensitive, 'L'-type Ca^{2+}-channel blocker, nimodipine (Tsien *et al,* 1988) with IC_{50}s = 3 ± 2 and 5 ± 2 nM respectively (n = 4) (Figure 3.1). A statistically significant ($P \leq 0.05$, Mann-Whitney U-test) inhibition of $^{45}Ca^{2+}$ influx into both preparations was achieved with concentrations of nimodipine of 10 nM and greater. Greater than 85% of K^+-induced $^{45}Ca^{2+}$ influx in both preparations was inhibited by 1,000 nM nimodipine, indicating

the route of calcium influx into both preparation was similar. The remaining 15% of the $^{45}Ca^{2+}$ influx into both anterior pituitary and GH_3 cells may be occurring through some other nimodipine-insensitive, voltage-activated Ca^{2+}-channel, which can contribute significantly to the influx of $^{45}Ca^{2+}$ within 30 s.

Effect of phorbol esters (PDD and PDBu) on K^+-induced Ca^{2+} influx into anterior pituitary and GH_3 cells

GH_3 cells were originally investigated in the hope that they were a convenient model to mimic rat anterior pituitary Ca^{2+}-influx, but a major difference in PKC-modulation of 'L'-type Ca^{2+}-channel function became evident. Activators of protein kinase C such as 4ß-phorbol 12,13-didecanoate 4ß-(PDD) and phorbol 12,13-dibutyrate (PDBu) (Blumberg, 1988) modulated the influx of K^+-induced $^{45}Ca^{2+}$ influx into both anterior pituitary prisms and into GH_3 cells. In anterior pituitary prisms, (Figure 3.2) 10 min preincubation with PDBu (3 - 1,000 nM) and 4ß-PDD (10 - 1,000 nM) enhanced K^+-induced $^{45}Ca^{2+}$ influx in a concentration-dependent manner. The maximal enhancement seen with 1,000 nM of either PDBu or 4ß-PDD was 280 ± 17 and 282 ± 26% of control K^+-induced $^{45}Ca^{2+}$ influx (n = 8 and 4 respectively) with EC_{50} values of 55 ± 22 and 75 ± 33 nM. The enhancement of K^+-induced $^{45}Ca^{2+}$ influx with 300 and 1,000 nM PDBu and 100 and 1,000 nM 4ß-PDD was statistically significantly different ($P \leq 0.05$) from control K^+-induced $^{45}Ca^{2+}$ influx levels. At concentrations of 100 and 1,000 nM, 4α-PDD (the less-active stereoisomer of 4ß-PDD) did not significantly enhance the K^+-induced calcium influx (104 ± 10 and 143 ± 22% of control K^+-induced calcium influx respectively, n = 4) but was significantly different ($P \leq 0.05$) from the enhancement of influx seen with 4ß-PDD at the same concentrations. In contrast, K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells (Figure 3.3) was inhibited in a concentration-dependent fashion by 10 min preincubation with PDBu (3 - 1,000 nM) and 4ß-PDD (3 - 300 nM) producing influx levels of 49 ± 4 and 56 ± 8% of control K^+-induced influx (n = 8 and 4) respectively at the highest concentration tested.

The calculated IC_{50} values for PDBu and 4ß-PDD were 17 ± 12 and 26 ± 18 nM respectively and the inhibition of influx by PDBu (300 and 1,000 nM) and 4ß-PDD (300 nM) was significantly different ($P \leq 0.05$) from control influx levels. Again, 4α-PDD (3 - 300 nM) poorly mimicked 4ß-PDD and did not significantly reduce calcium influx levels (90 ± 7% of control K^+-induced $^{45}Ca^{2+}$ influx at 300 nM 4α-PDD) and the levels of inhibition by 300 nM 4α-PDD were statistically different ($P \leq 0.05$) from the inhibition levels seen with 4ß-PDD at the same concentration. Neither PDBu nor PDD had any effect on basal $^{45}Ca^{2+}$ accumulation (in either pituitary tissue or GH_3 cells) throughout the concentration range used.

Although not markedly different between preparations, the EC_{50} and IC_{50} values for PDBu and 4ß-PDD were slightly higher in anterior pituitary pieces than in GH_3 cells, which could be accounted for by drug accessibility, but may also reflect a slightly less PDBu/4ß-PDD-sensitive PKC which is involved in the enhancement of K^+-induced $^{45}Ca^{2+}$ influx into pituitary, compared to the PKC involved in inhibiting depolarisation-induced calcium influx into GH_3. Nevertheless, in these two tissues PDBu and 4ß-PDD can enhance or inhibit nimodipine-sensitive calcium influx in a concentration-dependent and stereo-specific manner, thereby providing two convenient and distinct models for PKC function in anterior pituitary cells.

Since the effect of PDBu on 'L'-type calcium influx was different between the two preparations, and since Lacerda *et al* (1988) found that the phorbol ester-induced increase and decrease in 'L'-type channel activity in myocytes was time-dependent, the time-course of the PDBu-effect in both preparations was investigated (see Figure 3.4). Preincubation of both preparations for 20 min with 300 nM PDBu did not markedly alter the levels of channel activity from those levels seen with 300 nM PDBu pretreatment for 10 min (n = 2). In anterior pituitary, including 300 nM PDBu in the influx measurement period only (defined as 30 s preincubation with PDBu) enhancement of K^+-induced $^{45}Ca^{2+}$ influx (although to a lesser extent than with 10 min preincubation). Similarly, in GH_3 cells, 30 s preincubation with 300 nM PDBu

resulted in an inhibition of K^+-induced $^{45}Ca^{2+}$ influx, which again was to a lesser extent than the inhibition seen with 10 min preincubation with PDBu. Preincubation times of 30 s or greater with 300 nM PDBu in both tissues was significantly ($P \leq 0.05$) different from levels of influx of $^{45}Ca^{2+}$ at zero preincubation time. Clearly therefore, the 'L'-type channel activity in either preparation did not change from enhancement to inhibition, (or vice-versa) with PDBu within 30 s (the limit of reducing the preincubation time of drugs). The half-maximal effect of PDBu in anterior pituitary was estimated to occur at 38 ± 12 s preincubation (n = 6). The corresponding time for half-maximal effect of PDBu in GH_3 cells was 44 ± 16 s (n = 6), not markedly different from anterior pituitary pieces. Thus, although the characteristic effect of PDBu on both models is different, no temporal difference in action of PDBu could be seen.

Effect of 1,2-dioctanoyl-*sn*-glycerol on K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitaries and GH_3 cells

1,2-diacyl-*sn*-glycerols are endogenous activators of PKC (Nishizuka, 1984) and by nature, are highly lipophilic and therefore difficult to dissolve fully in aqueous solutions. The synthetic diacyglycerol analogues 1-oleoyl 2-acetyl-*sn*-glycerol (OAG) and the saturated acyl chain analogue 1,2-dioctanoyl-*sn*-glycerol (DOG) are more hydrophilic than naturally occurring diacylglycerols (Blumberg, 1988) and are still capable of activating PKC (Ebeling *et al,* 1982), however, OAG is known to have actions which may be independent of PKC (Hockberger *et al,* 1989). Through the concentration range 3 - 100 µM, DOG acted in anterior pituitary in a similar manner to PDBu and 4ß-PDD to enhance in a concentration-dependent fashion, K^+-induced $^{45}Ca^{2+}$ influx to a level of 210 ± 15% of control K^+-induced influx (n = 4) by 100 µM DOG. The EC_{50} concentration of DOG in anterior pituitary was 46 ± 8µM and the levels of influx were significantly ($P \leq 0.05$) different from control K^+-induced levels with 30 and 100 µM DOG (Figure 3.5). In contrast however, DOG (3 - 100 µM) was

ineffective at modulating K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells. No significant reduction in influx levels were seen up to 100 µM with a response of still 96 ± 4% of control K^+-induced influx levels at the highest concentration of DOG used (n = 4). Even DOG (3 - 100 µM) purchased from Novabiochem (UK) Ltd instead of the Sigma Chemical Company, failed to elicit an inhibition of K^+-induced calcium influx into GH_3 cells (n = 2). (The DOG from the new source was equally effective in anterior pituitary pieces (n = 1)).

Diacylglycerols of higher saturated acyl chain length such as 1,2-dilauroyl-*rac*-glycerol (12 carbons) (3 - 100 µM) at higher concentrations clearly reached their solubility limit once dispersed from stock into the 'calcium uptake medium'. This precipitation was reflected in an apparently increased accumulation of $^{45}Ca^{2+}$ in the filter blanks in the assay (112 - 141% of control K^+-induced influx at 100 µM) (n = 2), and caution must be exercised when performing the calcium influx studies to be aware of any such factors which may influence non-specific label retention by the filters. Unfortunately, 1,2-didecanoyl-*rac*-glycerol (10 carbon saturated acyl chain) just began to show turbidity in the 'calcium uptake medium' at 30 and 100 µM and thus gave slight but artefactual increases in the apparent Ca^{2+} influx with GH_3 cells of 106 - 111% of control K^+-induced influx (n = 2 in each case). Calcium influx studies performed in the absence of tissue, indicated that diacylglycerol analogues with saturated acyl chain lengths longer than 8 showed at higher concentrations, greater accumulation of $^{45}Ca^{2+}$ to filters alone than the control accumulation in the presence of solvent alone (n = 2). The saturated acyl chain diacylglycerol analogue, 1,2-dihexanoyl-*sn*-glycerol (100 µM), like DOG, was also unable to mimic PDBu and 4ß-PDD to reduce K^+-induced influx into GH_3 cells (90 ± 8% of control K^+-induced influx (n = 4)), but acted similarly to PDBu, 4ß-PDD and DOG in anterior pituitaries to enhance K^+-induced $^{45}Ca^{2+}$ influx to 189 ± 18% of control K^+-induced influx levels (n = 4). Neither DOG nor 1,2-dihexanoyl-*sn*-glycerol had any effect on basal $^{45}Ca^{2+}$

accumulation by either pituitary tissue or GH_3 cells throughout the concentration range used.

Effect of other putative protein kinase C activators on K^+-induced Ca^{2+} influx

The activity of a range of phorbol ester analogues on PKC and its function has been extensively assessed in Blumberg's laboratories. Phorbol esters are a class of tumour promoting compounds, and the major receptor for phorbol esters was found to be PKC (Niedel *et al,* 1983; Sando and Young, 1983; Ashendel *et al,* 1983; Leach *et al,* 1983). Further investigation into the binding characteristics of a range of phorboid analogues (eg, mezerein, phorbol 12-myristate 13-acetate 4-0-methyl ether (MPMA), phorbol 12-retinoate 13-acetate (PRA), deoxyphorbol 12,13-isobutyrate (DPB)) suggested that more than one [^{3}H]-PDBu binding site existed, and that some phorboid analogues were selective in their interaction with the multiple PKC-binding sites (Dunn and Blumberg, 1983). The same phorboid analogues could selectively elicit only certain actions of the PDBu-effect on a range of models, for example, inflammation, tumour promotion and ornithine decarboxylase induction. The effect of mezerein, MPMA, PRA and DPB was investigated on both anterior pituitary pieces and GH_3 cell K^+-induced $^{45}Ca^{2+}$ influx models (Figure 3.6). In anterior pituitary, mezerein (10 - 1,000 nM) mimicked PDBu and 4ß-PDD at enhancing K^+-induced $^{45}Ca^{2+}$ influx in a concentration-dependent manner (EC_{50} = 128 ± 26 nM) to give an enhancement of influx to 195 ± 18% of control K^+-induced $^{45}Ca^{2+}$ influx (n = 4) at the highest concentration of mezerein used. The enhancement by mezerein was significantly different ($P \leq 0.05$) from control K^+-induced influx into anterior pituitary at concentration of 50 nM and greater. Mezerein (10 - 1,000 nM) was unable to mimic PDBu and 4ß-PDD in GH_3 cells at reducing K^+-induced $^{45}Ca^{2+}$ influx, but to our surprise, enhanced K^+-induced $^{45}Ca^{2+}$ influx into GH_3 in a concentration-dependent fashion (EC_{50} = 26 ± 17 nM), increasing influx levels to 172 ± 4% of control at the

highest concentration used (n = 4). The enhancement of influx into GH_3 cells with 50 nM and greater was significantly different ($P \leq 0.05$) from control K^+-induced influx levels.

Preincubation of anterior pituitary with MPMA (10 - 1,000 nM) or PRA (10 - 1,000 nM) resulted in a concentration-dependent enhancement of K^+-induced $^{45}Ca^{2+}$ influx, with calculated EC_{50}s = 8 ± 5 nM and 3 ± 6 nM (n = 4) respectively. At 1,000 nM, MPMA and PRA enhanced K^+-induced influx into pituitary to 193 ± 9% and 216 ± 16% of control K^+-induced $^{45}Ca^{2+}$ influx levels respectively, with the difference in $^{45}Ca^{2+}$ influx levels from control K^+-induced levels being statistically significant ($P \leq 0.05$) at concentrations of MPMA and PRA of 10 nM and greater. In GH_3 cells, MPMA and PRA mimicked the effect of mezerein by concentration-dependently enhancing K^+-induced $^{45}Ca^{2+}$ influx but to a lesser extent than mezerein (123 ± 9% and 121 ± 3% of control K^+-induced influx at 1,000 nM, n = 4) with EC_{50} = 124 ± 162 nM and 32 ± 24 nM respectively. At 1,000 nM MPMA and PRA, the difference in influx levels in GH_3 cells from control K^+-induced influx levels was statistically significant ($P \leq 0.05$). Finally, DPB was less effective at modulating K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary and GH_3 cells, than the other phorbol ester analogues tested. Whereas DPB (10 - 1,000 nM) could modestly enhance K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary in a concentration-dependent manner (EC_{50} = 16 ± 26 nM, n = 4) to levels of enhancement (130 ± 5% of control K^+-induced influx at 1,000 nM DPB) markedly lower than those seen with MPMA and PRA at the same concentration. Furthermore, DPB (10 - 1,000 nM) was ineffective at modulating K^+-induced influx into GH_3 cells (n = 4), with no significant difference from control K^+-induced influx levels, even at 1,000 nM DPB. The enhancement by DPB at 200 and 1,000 nM in anterior pituitary pieces was significantly ($P \leq 0.05$) different from control K^+-induced $^{45}Ca^{2+}$ influx levels. None of the phorboid PKC activators tested here had any effect on basal $^{45}Ca^{2+}$ accumulation in either pituitary tissue or GH_3 cell at the concentrations used.

The analysis of phorboid analogue effects was not sufficiently extensive, nor some of the changes of sufficient magnitude to enable the curve-fitting program to generate accurate values for EC_{50}s, but a number of striking qualitative differences can be readily seen.

Not only can some modified phorbol esters such as DPB mimic the effect of DOG on pituitary and GH_3 cells to enhance and be ineffective on K^+-induced influx respectively, but some phorboids (mezerein, MPMA and PRA) are able to enhance influx into both anterior pituitary and GH_3 cells, unlike PDBu and 4ß-PDD which show an inverse modulation of 'L'-channel activity within the two preparations. The enhancement of K^+-induced $^{45}Ca^{2+}$ influx into both preparations by any of the phorbol ester analogues was not caused by precipitation of the drugs within the filters (as described before) as 1,000 nM concentrations of each drug did not alter $^{45}Ca^{2+}$ accumulation to filters in the absence of any tissue (n = 2).

Arachidonic acid (AA) had been shown to activate α, ß- and γ-PKC to varying extents in cell free systems (Naor *et al,* 1988a) as well as having an array of other cellular actions (Wolfe and Shimizu, 1990). Preincubation of anterior pituitary pieces with AA (3 - 100 μM) for 10 min before exposure to high K^+ and $^{45}Ca^{2+}$, had no significant effect on K^+-induced $^{45}Ca^{2+}$ influx levels (Figure 3.7), with maximal enhancement of 120 ± 15% of control K^+-induced $^{45}Ca^{2+}$ influx levels with 100 μM AA (n = 4). In contrast in GH_3 cells, AA (1 - 100 μM) fully inhibited K^+-induced $^{45}Ca^{2+}$ influx in a concentration-dependent manner (IC_{50} = 19 ± 3 μM) to inhibition levels of 3 ± 2% of control K^+-induced $^{45}Ca^{2+}$ influx at 100 μM AA (n = 4 - 12). The inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells by 30 and 100 μM AA was significantly ($P < 0.05$) different from control values. Activation of PKC with PDBu results in only approximately 50% maximal inhibition in GH_3 cells. The mechanisms for this greater inhibition of K^+-induced $^{45}Ca^{2+}$ influx by AA will be discussed in the following chapter.

Effect of staurosporine and H7 on the modulation by PDBu of K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary and GH_3 cells

Inhibition of PKC can be achieved by a number of agents, including staurosporine (Kase *et al,* 1987) and H7 (Hidaka *et al,* 1984) which not only inhibit PKC but indeed most protein kinases with varying degrees of potency. The hydrophilic properties of PDBu make it an ideal phorbol ester (Blumberg, 1988) which has sufficiently high lipophilicity to be cell permeable and active, and sufficiently high hydrophilicity to be of use in the laboratory. The actions of phorbol esters are quite selective towards PKC (Blumberg, 1988) and thereby inhibition by H7 and staurosporine of PDBu-induced actions should represent their inhibition of PKC activity (or PKC-mediated actions). The ability of PDBu to enhance anterior pituitary and reduce GH_3 cell K^+-induced $^{45}Ca^{2+}$ influx can be reversed in a concentration-dependent manner by staurosporine. In anterior pituitary prisms (Figure 3.8), staurosporine (1 - 1,000 nM) reversed, in a concentration-dependent fashion, the 300 nM PDBu-induced enhancement of K^+-evoked $^{45}Ca^{2+}$ influx, with an $IC_{50} = 19 \pm 8$ nM (n = 4 - 6). The reversal of PDBu-induced enhancement was statistically different ($P < 0.05$) for concentrations of 10 - 1,000 nM staurosporine, with $13 \pm 11\%$ of the PDBu-effect remaining at the highest concentration of staurosporine used. In contrast, K252a (300 - 10,000 nM), an analogue of staurosporine with reduced potency of inhibition towards PKC, but with similar inhibitory potency on other kinases (Kase *et al,* 1987) was unable to significantly reverse the effect of 300 nM PDBu on K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms until 10,000 nM, with a maximal inhibition of the PDBu-effect of $34 \pm 10\%$ of control influx at the highest concentration used (n = 6). The inhibition of the PDBu-induced enhancement of K^+-stimulated $^{45}Ca^{2+}$ influx in anterior pituitary pieces by staurosporine, was significantly ($P < 0.05$) different from the inhibition of the PDBu-response by K252a at 1,000 nM concentration of the drugs. Similarly, staurosporine was able to reverse the inhibition by 300 nM PDBu of K^+-

induced $^{45}Ca^{2+}$ influx into GH_3 cells. The reversal of the PDBu-effect by staurosporine (1 - 1,000 nM) was concentration-dependent, giving 92 ± 9% inhibition of the PDBu-effect at the highest concentration, with a calculated IC_{50} = 15 ± 10 nM (n = 6). The reversal by staurosporine of 300 nM PDBu-induced inhibition of K^+-evoked $^{45}Ca^{2+}$ influx into GH_3 cells was significantly different from control influx levels at concentrations of 10 nM and greater. Reversal by K252a (300 - 10,000 nM) was again poor, with 72 ± 8% inhibition at the highest concentration (n = 4). The difference in reversal by staurosporine and K252a in GH_3 cells was statistically significant ($P \leq 0.05$) at 300 and 1,000 nM concentrations of the drugs.

Thus, staurosporine reversed the response of PDBu in both anterior pituitary and GH_3 cells but was the PDBu-response was less sensitive to K252a. This indicated that PDBu-responses are indeed mediated by PKC, as the pharmacology of both responses was as would be expected for PKC-mediation. Investigation with a new PKC inhibitor, Ro318220 which unlike staurosporine, is reported to have over 100 times greater inhibitory activity towards PKC than the other kinases tested (Davis *et al*, 1989). Inclusion of Ro318220 (1 - 30 μM) reversed in a concentration-dependent manner (IC_{50} = 8 ± 4 μM) the effect of 300 nM PDBu on K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary pieces (Figure 3.9) resulting in an 85 ± 8% inhibition of control K^+-induced $^{45}Ca^{2+}$ influx levels at the highest concentration used (n = 4). Similarly, in GH_3 cells, the effect of 300 nM PDBu was reversed by Ro318220 (1 - 30 μM) in a concentration-dependent manner (IC_{50} = 7 ± 4 μM) resulting in a 92 ± 11% inhibition of control K^+-induced $^{45}Ca^{2+}$ influx levels at the highest concentration used (n = 4). Both in anterior pituitary pieces and GH_3 cells, the inhibition of control K^+-induced $^{45}Ca^{2+}$ influx was significantly ($P \leq 0.05$) for concentrations of Ro318220 of 5 μM and greater. At the concentrations used, Ro318220 had no effect on basal $^{45}Ca^{2+}$ accumulation in either pituitary or GH_3 cells.

The reversal of the PDBu effect by Ro318220 (which is generally regarded as a selective PKC inhibitor) further indicates that both the PDBu-induced enhancement

or inhibition of K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms and GH_3 cells respectively is indeed mediated by PKC.

Another protein kinase C inhibitor, H7, has a quite different structure to staurosporine, and has relatively lower potency (Hidaka *et al,* 1984). Reversal of the PDBu-induced effect on K^+-evoked $^{45}Ca^{2+}$ influx into both anterior pituitaries and GH_3 cells could be achieved by H7 (Figure 3.10). In anterior pituitary prisms, H7 (10 - 100 µm) reversed 300 nM PDBu-induced enhancement of K^+-evoked $^{45}Ca^{2+}$ influx in a concentration-dependent manner with maximal inhibition of the PDBu-effect of 86 ± 15% inhibition at the highest concentration used (n = 8). The IC_{50} was unexpectedly very high at 62 ± 6 µM. The reversal by H7 of the PDBu-response on K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitaries was statistically different ($P < 0.05$) from control levels with 100 µM H7. A structural analogue of H7, HA1004, has relatively reduced potency of inhibition of PKC, but with similar potencies toward other kinases (Hidaka *et al,* 1984). The effect of PDBu in anterior pituitary was poorly reversed by HA1004 (2 - 200 µM), with a 45 ± 11% inhibition of the PDBu-response at the highest concentration of HA1004 used (n = 4) which was significantly ($P \leq 0.05$) different from control K^+-induced influx. The difference in levels of inhibition of the PDBu-induced enhancement of K^+-stimulated $^{45}Ca^{2+}$ influx into anterior pituitary pieces by H7 and HA1004, was statistically significant ($P < 0.05$) at a concentration of 100 µM of the drugs. In GH_3 cells, the 300 nM PDBu-induced inhibition of K^+-evoked $^{45}Ca^{2+}$ influx was reversed by H7 more potently than reversal of the PDBu-response in anterior pituitary prisms. The reversal of the PDBu-induced inhibition of K^+-stimulated influx into GH_3 cells by H7 (5 - 50 µM) was concentration-dependent, giving a 88 ± 11% inhibition of the PDBu-effect at the highest concentration of H7 used, and a calculated IC_{50} value of 10 ± 2 µM (n = 4), markedly more potent than the corresponding IC_{50} in anterior pituitary. The reversal of the effect of PDBu on GH_3 cells by H7 was statistically significant ($P \leq 0.05$) with all the concentrations of H7 used. When using HA1004 (20 - 200 µM), the reversal of the PDBu-response in GH_3

cells was again very poor, with an inhibition of $52 \pm 12\%$ at the highest concentration ($n = 4$). The difference between the inhibition of the PDBu-response in GH_3 cells by H7 and HA1004, was statistically significantly at 20 and 50 μM concentration of the drugs.

Thereby, HA1004 does not potently reverse the PDBu-induced responses in anterior pituitary of GH_3 cell calcium influx, whereas H7 does reverse the responses. However, the potency of H7 varied between the two tissues, being of expected potency (Schaap and Parker, 1991) in GH_3 cells, but with a lower potency in anterior pituitary. In contrast with the selectivity shown by H7, neither staurosporine, Ro318220, K252a nor HA1004 showed any difference in IC_{50} on the two PDBu responses. Neither H7, staurosporine nor Ro318220 at their highest concentration used had any effect alone on basal or K^+-induced $^{45}Ca^{2+}$ accumulation.

Figure 3.1. **Concentration-dependent inhibition by nimodipine of K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms and GH_3 cells.** Typically, basal accumulation of $^{45}Ca^{2+}$ accounted for around 50 fmoles $^{45}Ca^{2+}$/hemi-pituitary or 10^6 GH_3 cells/min of which non-specific adsorbtion to filter and cell surfaces accounted for 34 fmoles $^{45}Ca^{2+}$/min. Stimulation with 60 mM K^+ increased accumulation to around 120 fmoles $^{45}Ca^{2+}$/hemipituitary or 10^6 GH_3 cells/min (approximately 1600 dpm per assay). $^{45}Ca^{2+}$ represented 1 part in 375 of the total Ca^{2+} concentration. Anterior pituitary prisms (circles) or GH_3 cells (squares) were preincubated with nimodipine (or solvent (dimethylformamide, 1% final) alone for control measurements) for 10 min before exposure to 60 mM K^+-containing medium with radioactive Ca^{2+}. After 30 s, $^{45}Ca^{2+}$ influx was halted as described in the Methods section. The data represent the means ± SEM of 4 determinations. Significant ($P \leq 0.05$) inhibition of K^+-induced $^{45}Ca^{2+}$ influx was achieved with concentrations of nimodipine of 10 nM and greater.

Figure 3.1

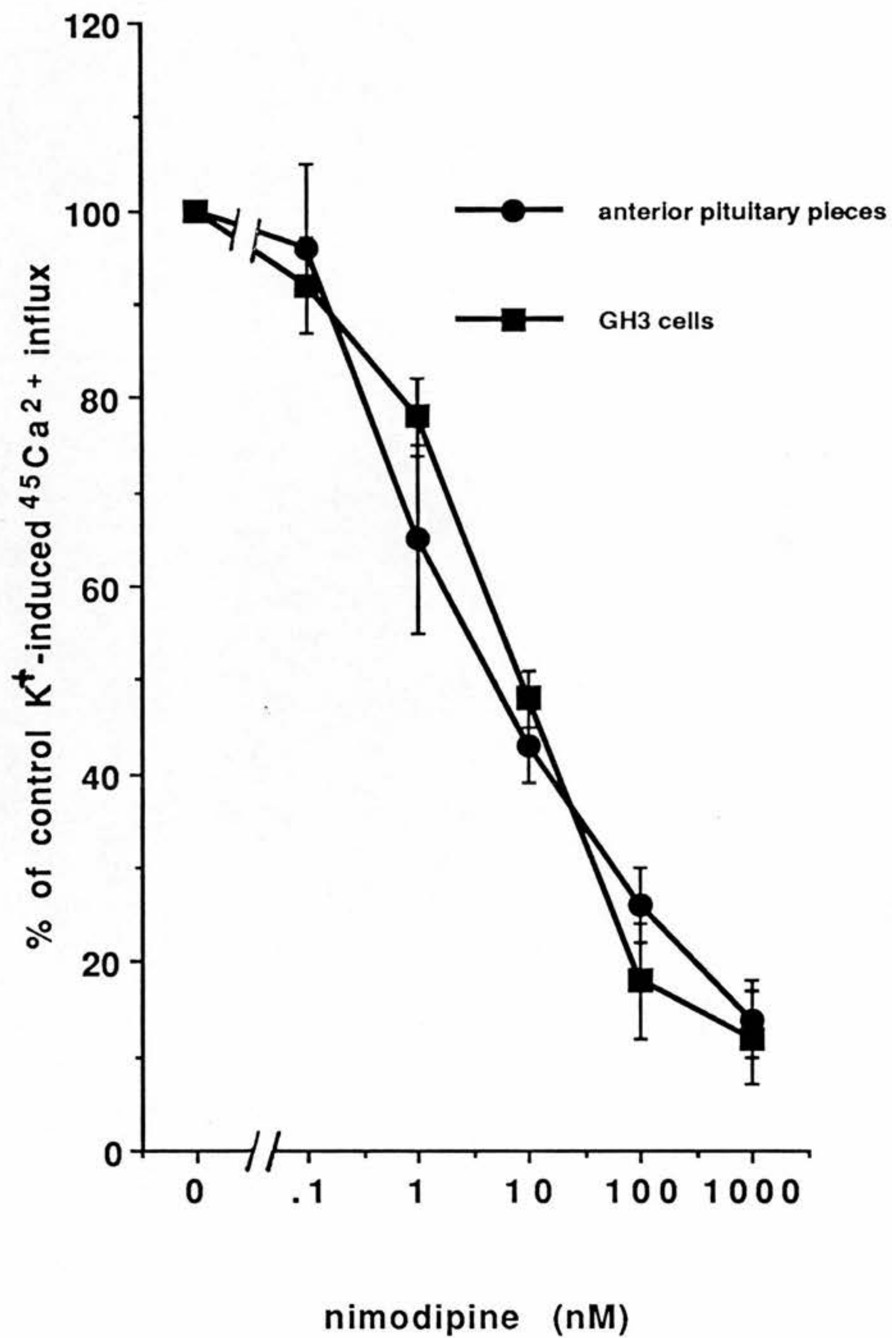


Figure 3.2. **Concentration-dependent enhancement by phorbol esters of K^+-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms.**

Rat anterior pituitary prisms were preincubated for 10 min with PDBu (squares), 4β-PDD (closed circles) or 4α-PDD (open circles) before exposure to 60 mM K^+-containing medium. $^{45}Ca^{2+}$ influx was measured as described in the Methods section. None of the compounds had any effects on basal $^{45}Ca^{2+}$ influx at the concentrations used. The data represent the means ± SEM from 4 - 8 determinations. Significant ($P < 0.05$) enhancement of K^+-induced $^{45}Ca^{2+}$ influx levels were produced by PDBu and 4ß-PDD at concentrations of 100 nM and greater.

Figure 3.2

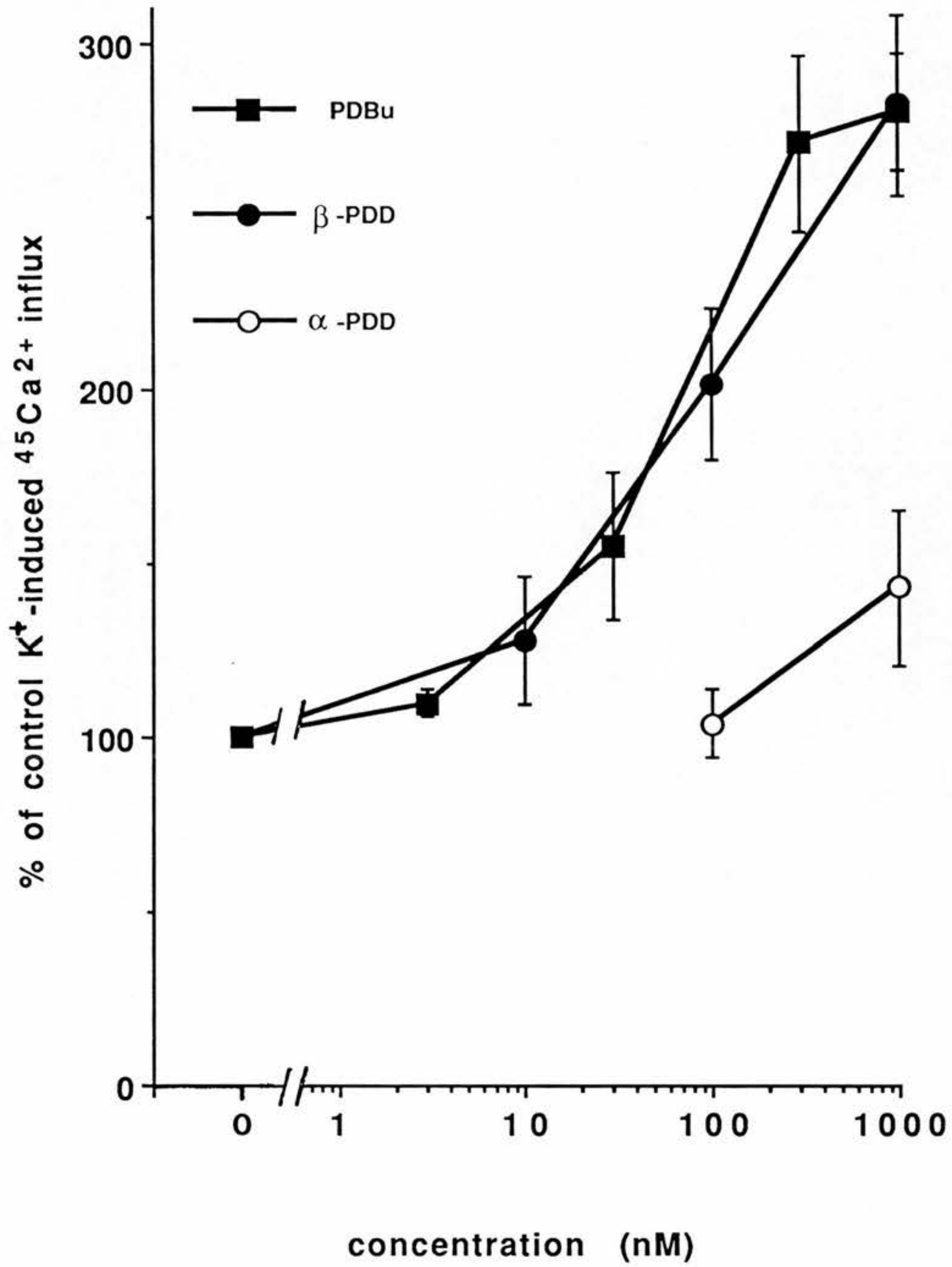


Figure 3.3. Concentration-dependent inhibition by phorbol esters of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells.

GH_3 cells were preincubated for 10 min with PDBu (squares), 4β-PDD (closed circles) or 4α-PDD (open circles) before exposure to 60 mM K^+-containing medium. $^{45}Ca^{2+}$ influx was measured as described in the Methods section. None of the compounds had any effects on basal $^{45}Ca^{2+}$ influx at the concentrations used. The data represent the means ± SEM from 4 - 8 determinations. Significant ($P < 0.05$) inhibition of K^+-induced $^{45}Ca^{2+}$ influx was achieved by PDBu and 4ß-PDD at concentrations of 300 nM and greater.

Figure 3.3

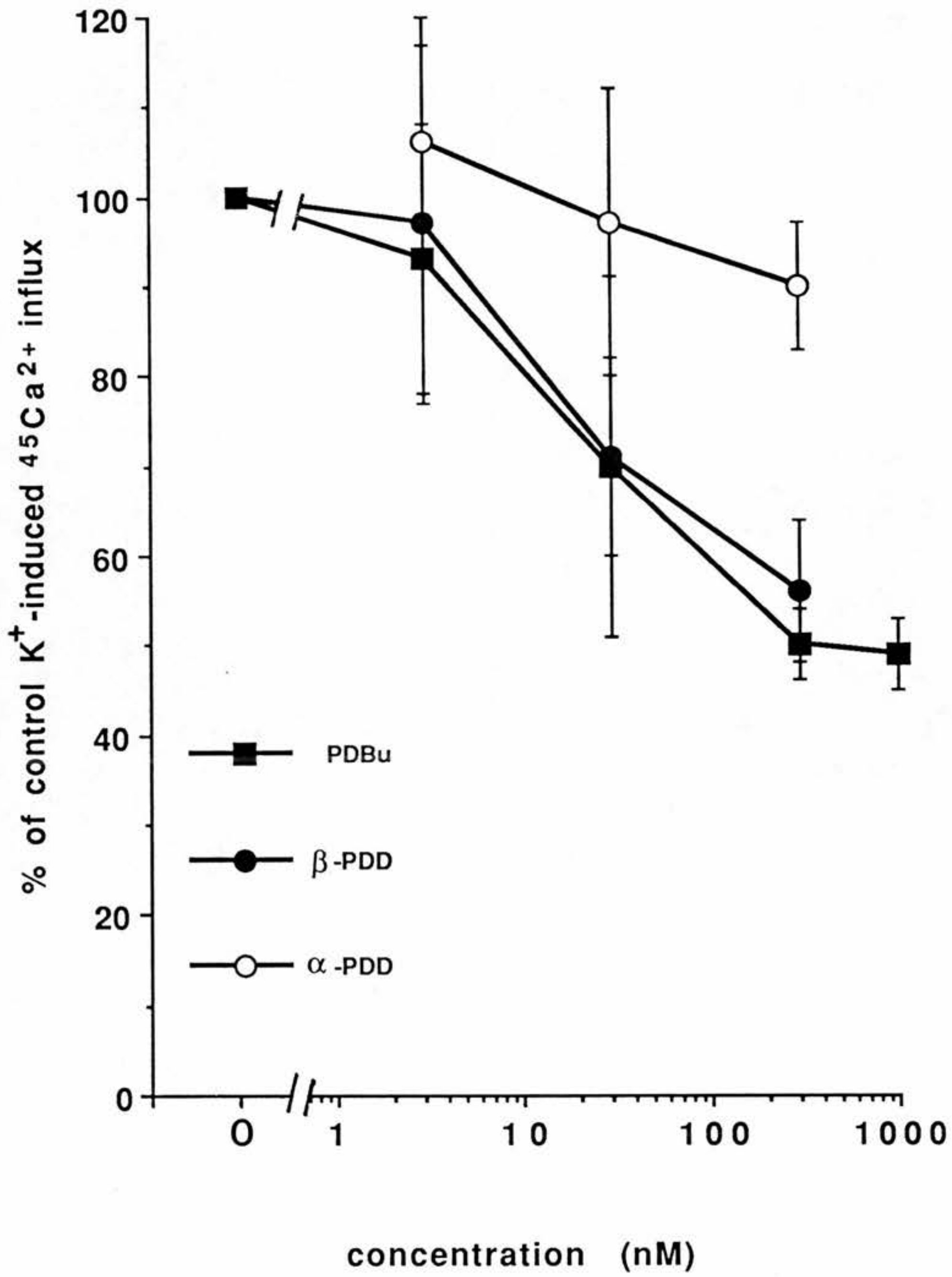


Figure 3.4. Time-course of the PDBu modulation of $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms and GH_3 cells.

Anterior pituitary prisms (circles) and GH_3 cells (squares) were preincubated with 300 nM PDBu for the indicated time before exposure to 60 mM K^+. The preincubation time with PDBu is inclusive of the 30 s $^{45}Ca^{2+}$ influx measurement period. The data represent the means ± SEM of 6 determinations. All of the data points are significantly ($P < 0.05$) different from zero time levels of K^+-induced $^{45}Ca^{2+}$ influx.

Figure 3.4

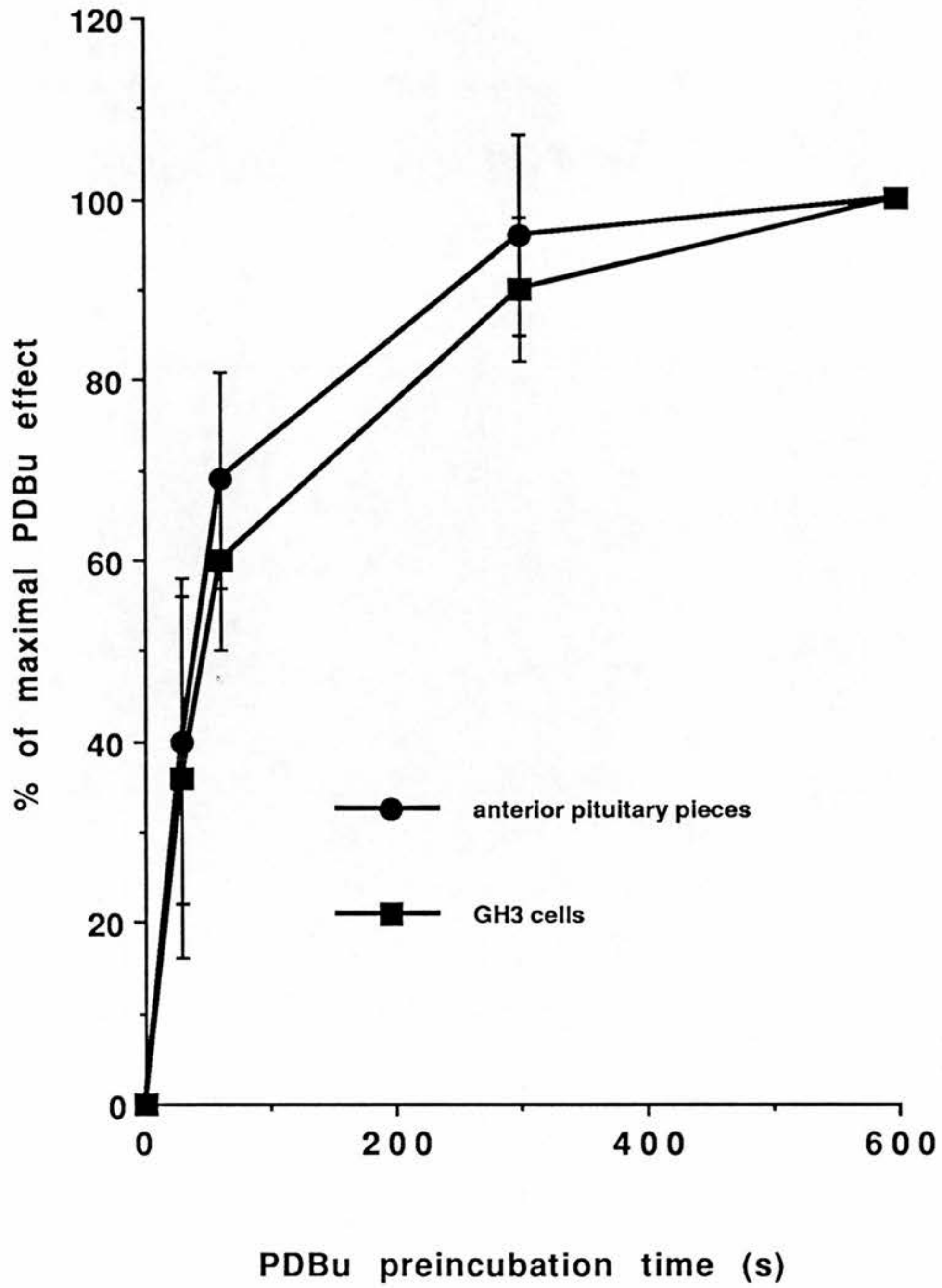


Figure 3.5. Concentration-dependent enhancement by DOG of K^+-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms and inability of DOG to modulate K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells.

Anterior pituitary prisms (circles) and GH_3 cells (squares) were preincubated for 10 min with the indicated concentration of DOG (dissolved in dimethylformamide, 1% final) before exposure to 60 mM K^+-containing medium. $^{45}Ca^{2+}$ influx was measured as described in the Methods section. There was no effect of 100 μM DOG on basal $^{45}Ca^{2+}$ influx, or on non-specific adsorbtion of $^{45}Ca^{2+}$ to filter blanks. The data represent the means ± SEM of 4 determinations. In anterior pituitary pieces, DOG at 30 and 100 μM eliciated a significant ($P \leq 0.05$) enhancement of K^+-induced $^{45}Ca^{2+}$ influx levels.

Figure 3.5

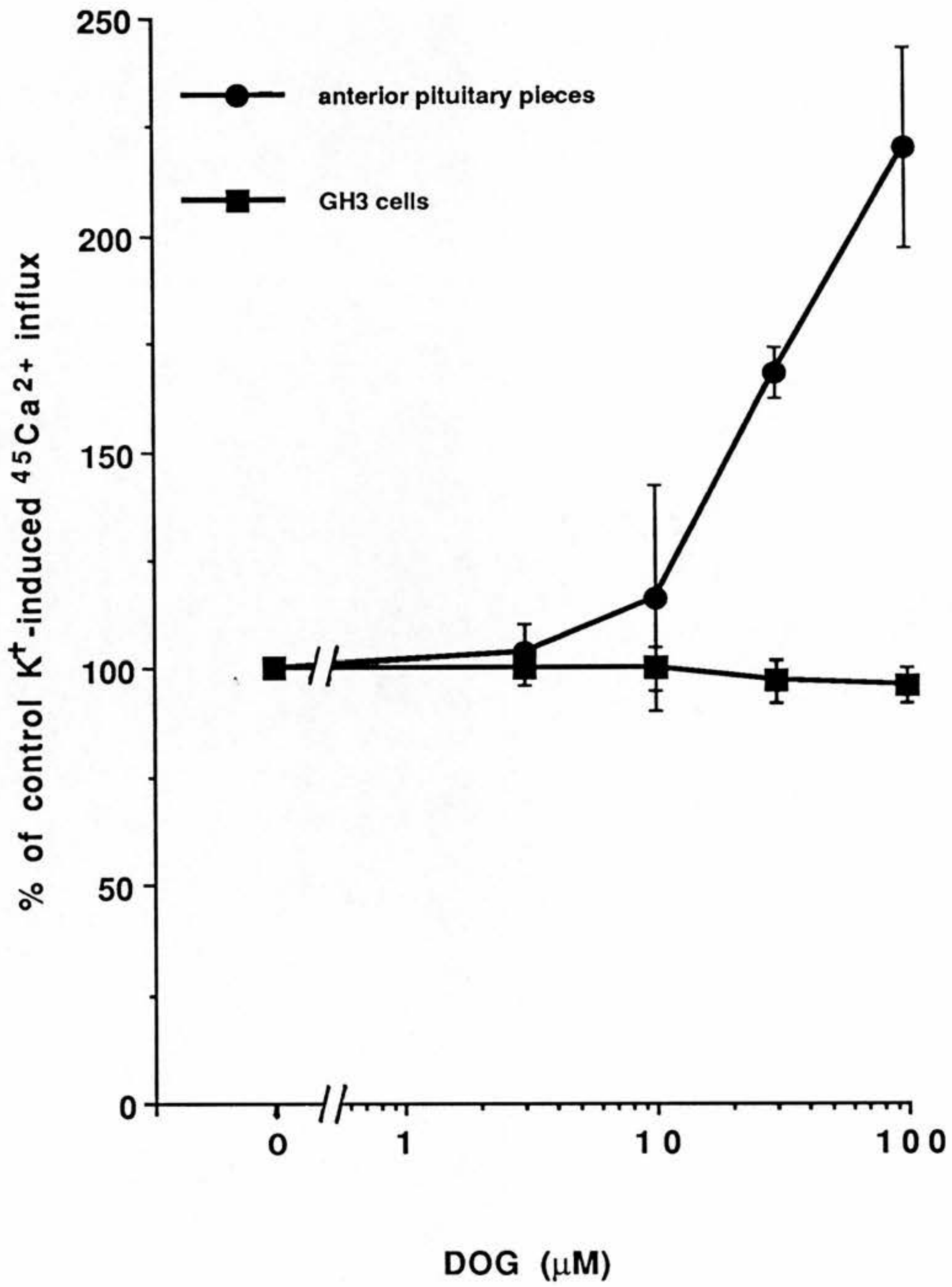


Figure 3.6. Effect of mezerein, MPMA, PRA and DPB on K^+-induced $^{45}Ca^{2+}$ influx rat anterior pituitary prisms and GH_3 cells.

Mezerein (a), MPMA (b), PRA (c) and DPB (d) were preincubated with the tissue for 10 min (37°C, O_2 atmosphere) before exposure of anterior pituitary pieces (circles) or GH_3 cells (squares) to 60 mM K^+-containing 'calcium uptake medium' and $^{45}Ca^{2+}$. Influx was halted as described in the Methods section. Each agent was dissolved in dimethylformamide (1% final) and at the concentrations used, had no effect on basal $^{45}Ca^{2+}$ accumulation or on accumulation of $^{45}Ca^{2+}$ by filters alone. The data represent the means ± SEM of 4 determinations. Significant ($P \leq 0.05$) enhancement of K^+-induced $^{45}Ca^{2+}$ influx was achieved by concentrations of mezerein of 50 nM and greater in both preparations, however a significant enhancement of influx occurred in anterior pituitary prisms only with the concentrations of MPMA and PRA used, and with DPB at 200 and 1000 nM.

Figure 3.6

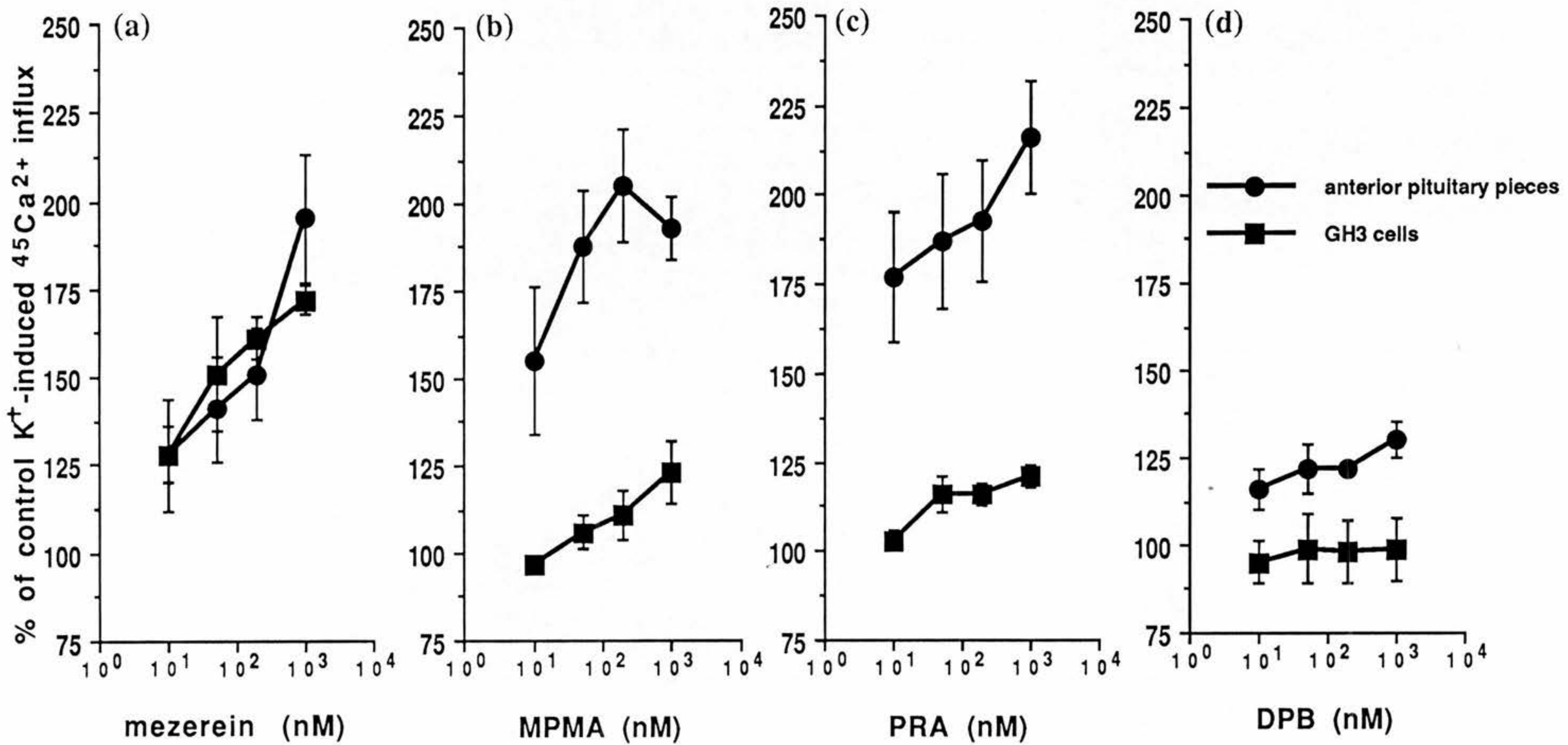


Figure 3.7. **Effect of arachidonic acid on K^+-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary pieces and GH_3 cells.**

Anterior pituitary prisms (circles) and GH_3 cells (squares) were preincubated for 10 min with AA (dissolved in dimethylformamide, $\leq 0.4\%$ final) before exposure to 60 mM K^+ and $^{45}Ca^{2+}$ as described in the Methods section. Arachidonic acid at the concentrations used had no effect on basal $^{45}Ca^{2+}$ accumulation or on $^{45}Ca^{2+}$ accumulation by filters alone. The data represent the means ± SEM of 4 - 12 determinations. The inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells by 30 and 100 μM AA was statistically significant ($P < 0.05$).

Figure 3.7

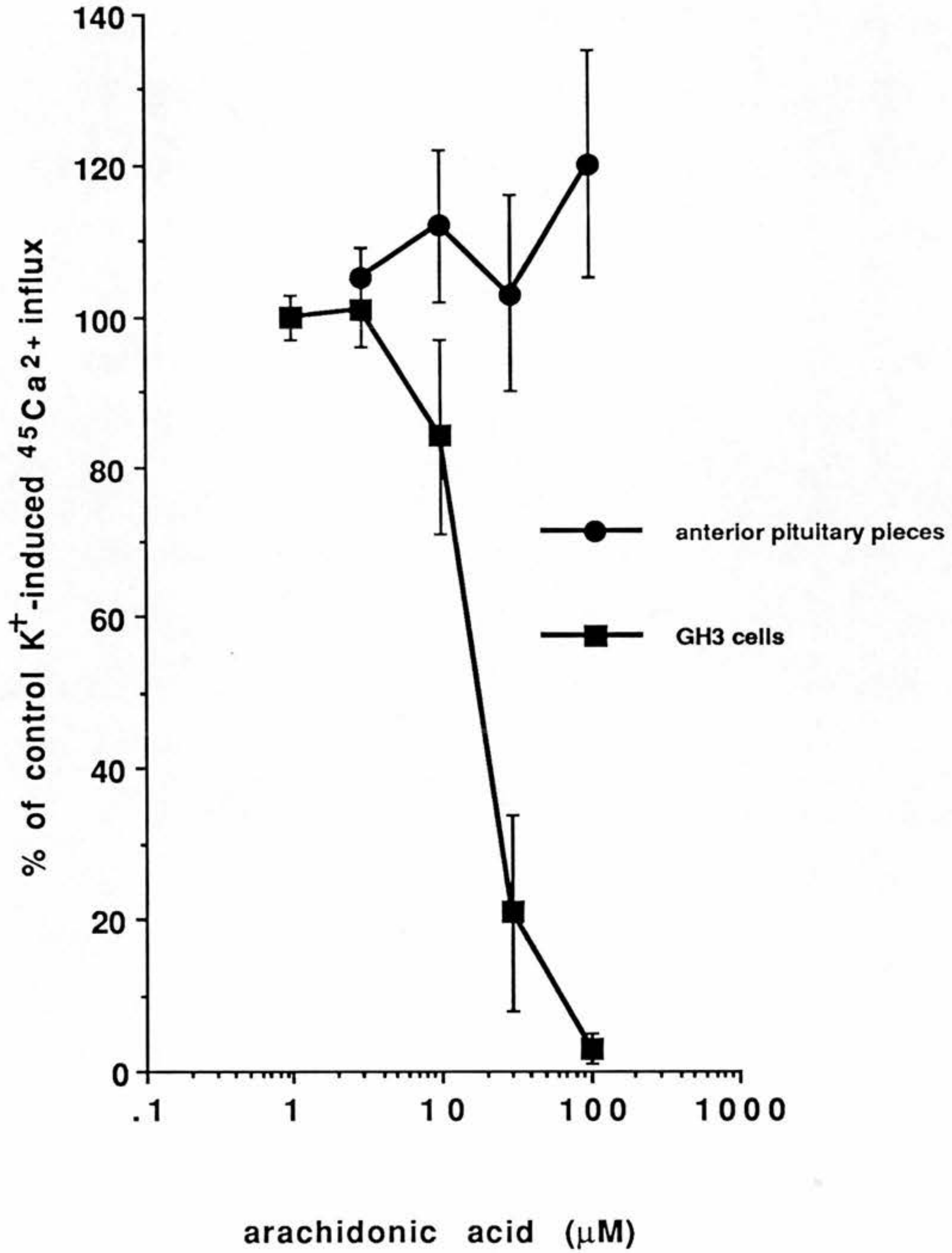


Figure 3.8. **Ability of staurosporine and K252a to inhibit the effect of PDBu on K^+-induced $^{45}Ca^{2+}$ influx in rat anterior pituitary and GH_3 cells.**

Concentration-response relationships for staurosporine (filled symbols) and K252a (open symbols) to reverse the effect of 300 nM PDBu to enhance or inhibit K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms (circles) or GH_3 cells (squares) respectively. The inhibition by 10,000 μM K252a and by concentrations of staurosporine of 10 nM and greater, were significantly ($P < 0.05$) different from control K^+-induced $^{45}Ca^{2+}$ influx levels in both anterior pituitary and GH_3 cells. The data represent the means ± SEM of 4 - 8 determinations.

Figure 3.8

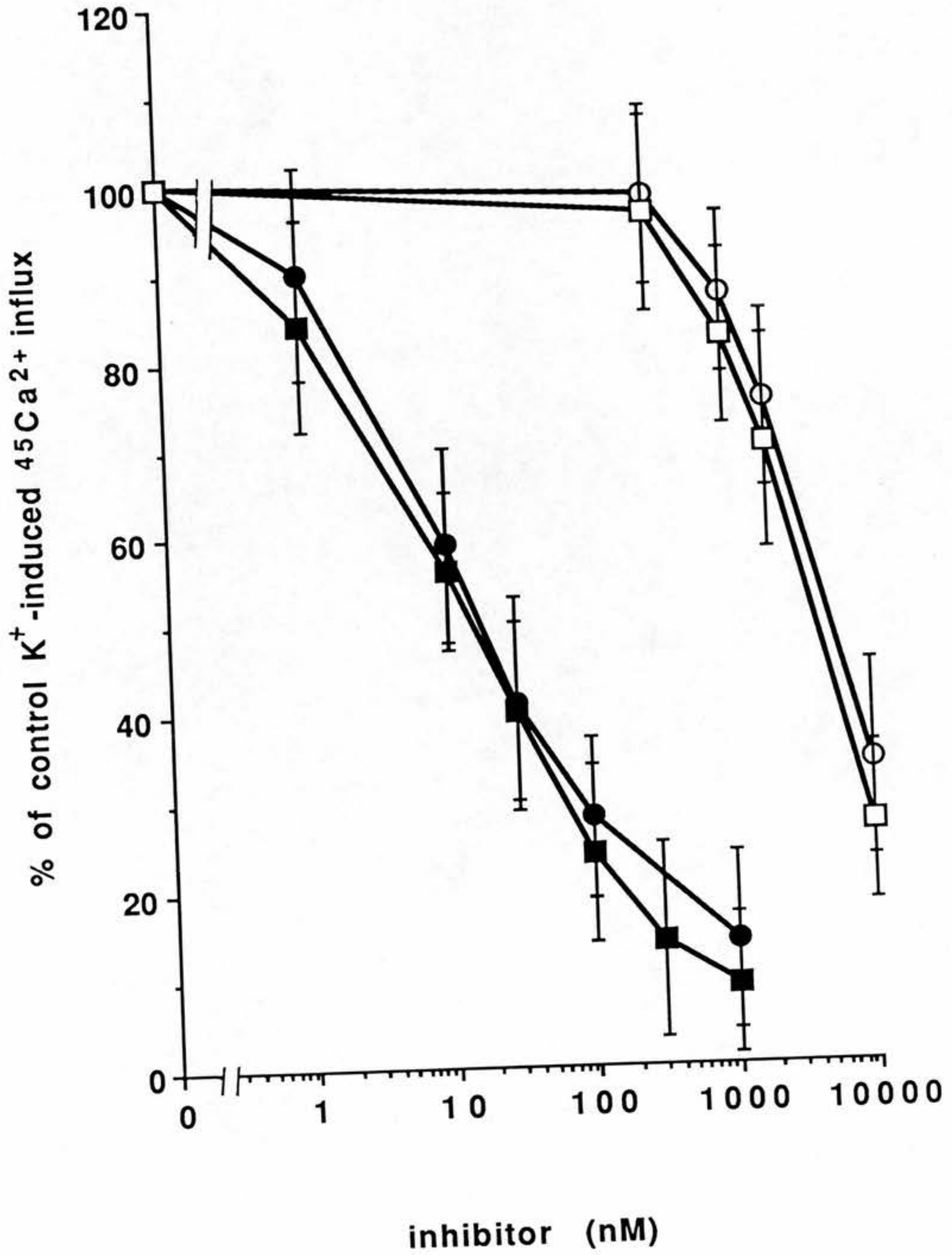


Figure 3.9. **Ability of Ro318220 to inhibit the effect of PDBu on K^+-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary and GH_3 cells.**

Concentration-response relationships for inhibition by Ro318220 of the effect of 300 nM on K^+-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms (circles) or GH_3 cells (squares). The inhibition by concentrations of Ro318220 of 5 μM and greater was significantly ($P \leq 0.05$) different from control K^+-induced $^{45}Ca^{2+}$ influx levels in both anterior pituitary pieces and GH_3 cells. The data represent the mean ± SEM of 4 determinations.

Figure 3.9

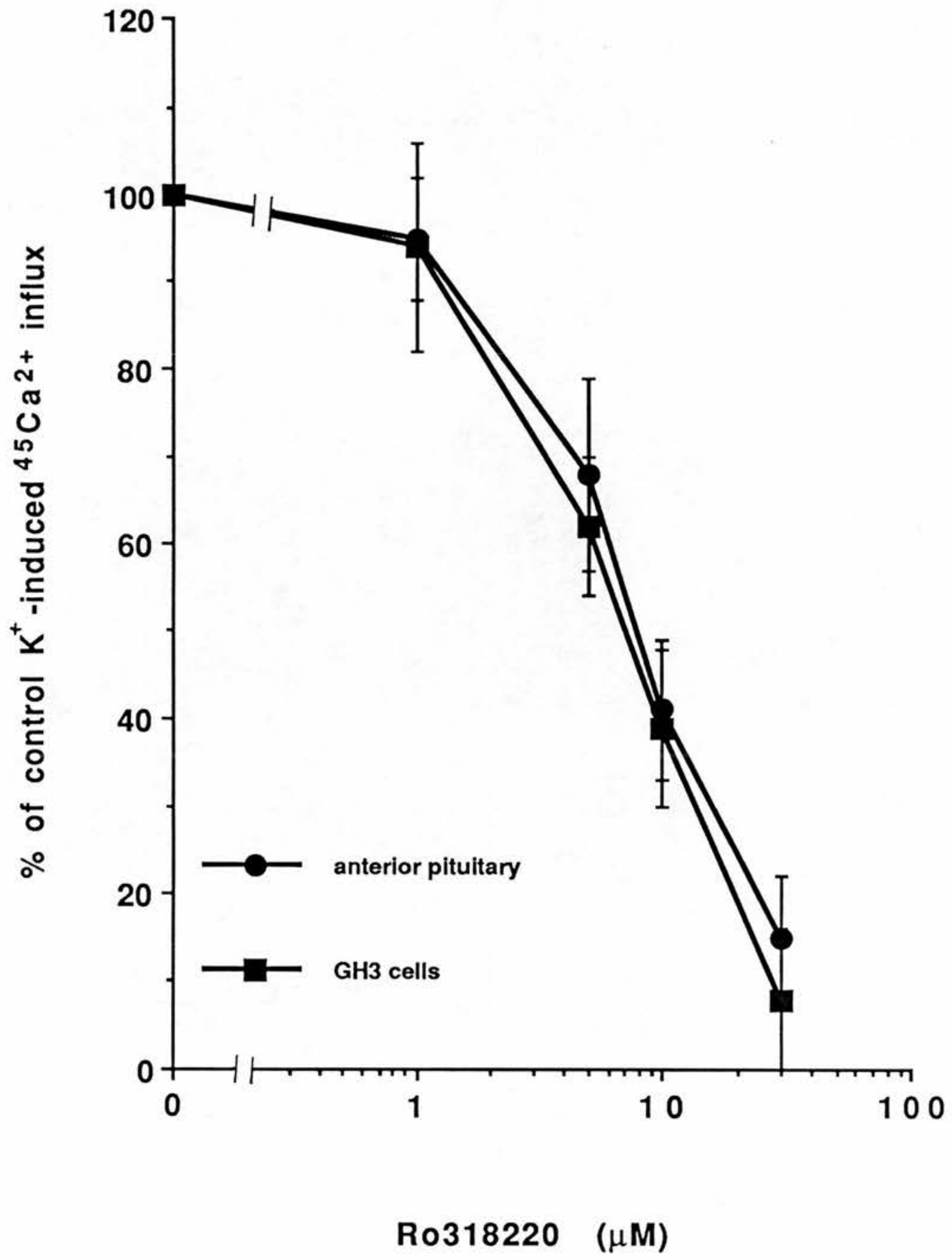
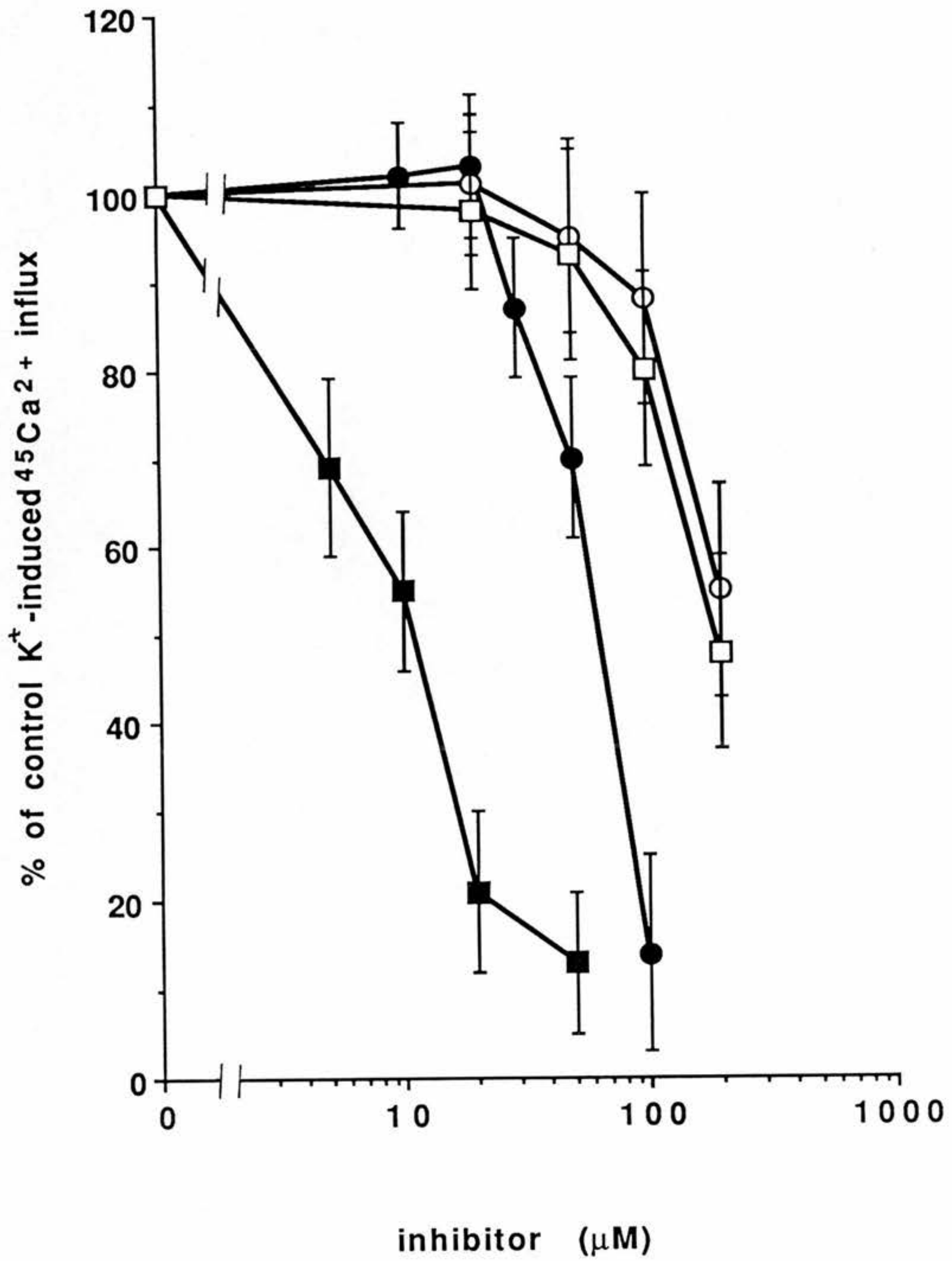


Figure 3.10. Ability of H7 and HA1004 to inhibit the effect of PDBu on K^+-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary and GH_3 cells. Concentration-response relationships for inhibition by H7 (filled symbols) or HA1004 (open symbols) of the effect of 300 nM PDBu on K^+-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms (circles) or GH_3 cells (squares). The inhibition by 200 μM HA1004 was significantly ($P \leq 0.05$) different from control K^+-induced $^{45}Ca^{2+}$ influx levels. The inhibition by concentration of H7 of 3 μM and greater in GH_3 cells, produced an inhibition which was significantly ($P < 0.05$) different from control influx levels, however only the level of inhibition by 100 μM H7 in anterior pituitaries was significantly ($P < 0.05$) different from control K^+-induced $^{45}Ca^{2+}$ influx levels. The data represent the mean ± SEM of 4 - 8 determinations.

Figure 3.10



3.4. DISCUSSION

Measurement of $^{45}Ca^{2+}$ movement into anterior pituitary prisms and GH_3 cell populations is a convenient method of investigating Ca^{2+} channel properties into both cell preparations. Since the general principle of the assay is to use $^{45}Ca^{2+}$ as a tracer to represent movements of $^{45}Ca^{2+}$ (present at mM levels in extracellular fluid), there is consequently a major dilution of the specific activity of the label. Apart from influx experiments where the external tracer may be expected to distribute rapidly into equilibrium with unlabelled calcium, it is not certain that the $^{45}Ca^{2+}$ will equilibrate rapidly and equally with all cellular pools without very extended incubation. Nevertheless, in influx experiments (Figure 2.1) where the signal above the noise is maximal within 30 s, such considerations of exchange rates into sequestered pools seem unlikely to bear major influence on the data. The experimental signal to noise ratio will depend partly on the technical reproducibility that can be achieved using reasonable levels of tracer in an experiment, clearly on the amount of tissue reasonably available per experiment and also on optimising the quench/wash procedure (Figure 2.2) and the time-course in order to obtain the greatest signal above basal. The steady linear accumulation of $^{45}Ca^{2+}$ over several minutes of basal conditions seems likely to represent movement into more slowly-exchanging, larger capacity pools than the cytosol. Depolarisation-induced entry is rapidly saturable (other rapid time-course experiments in the laboratory have shown that the signal actually approaches maximum by as little as 9 - 12 s) and then remains apparently constant for several minutes. It may, of course, be that the stimulus-responsive pool is a route of transit of $^{45}Ca^{2+}$ into the slowly sequestering pools during this time but its characteristics seem fully consistent with its representing largely the cytosol – the expected initial destination of Ca^{2+} entry in response to K^+, TRH and the other stimuli investigated. Successful measurement of $^{45}Ca^{2+}$ entry into anterior pituitary tissue can also be hindered by the use of lipophilic compounds close to the limit of their solubility in aqueous solution or,

more particularly, in the ice-cold EGTA wash (eg, with arachidic acid), leading to partial blocking of filters and non-specific retention of label. In such cases it is clearly not possible to investigate such compounds with the protocol used here. Thereby caution must always be used (Mitchell *et al,* 1991) to ensure that the agents used (at the appropriate concentrations) do not alter apparent $^{45}Ca^{2+}$ accumulation. Electrophysiological measurements of calcium channel activity can clearly provide a useful counterpart which is in many ways far superior to the present studies. Indeed, the effects of a range of PKC-related compounds is being investigated on 'L'-type Ca^{2+} channel activity in GH_3 cells in collaborative experiments with Drs R J Martin, L Patmore and A M Evans. However, these electrophysiological investigations have their limitations too. Whilst being extremely accurate and sensitive in comparison to the relatively more 'crude' measurement of $^{45}Ca^{2+}$ influx, electrophysiological measurements are technically harder to set up and are not as readily amenable to extensive pharmacological analysis. There may be technical difficulties such as obtaining cells dispersed from pituitary tissue without damaging receptor channels by the necessary enzymes, and the need for some caution in procedures to eliminate the much larger K^+ currents which would normally obscure Ca^{2+} currents. Electrophysiological measurements do not provide useful information on total calcium influx/efflux and do not inform us of the 'overall' picture of cellular calcium handling, which may be achieved if employing a range of techniques (eg, $^{45}Ca^{2+}$ influx/efflux, electrophysiology and intracellular free-calcium ion concentration (measurement using calcium indicator dyes)). The outward K^+ gradient sustained across the plasmalemma will be substantially decreased by an extracellular concentration of 60 mM K^+. This challenge would be expected to shift the cellular membrane potential from in the order of -60 mV to a more positive membrane potential, sufficiently depolarised to activate all of the known voltage-activated Ca^{2+} channels (van Breemen *et al,* 1973). Such a depolarisation is indeed sufficient to activate fully any voltage-activated channels which exist in anterior pituitary cells and which might be detectable in our protocol, since

$^{45}Ca^{2+}$ entry is maximal with a 60 mM K^+ stimulus showing no further increases at 90 or 120 mM (R Mitchell, unpublished observations) in both rat anterior pituitaries and GH_3 cells. Both preparations are known to contain 'T'- and 'L'-type Ca^{2+} channels (see section 1.5). In the present protocol, influx of $^{45}Ca^{2+}$ into anterior pituitary tissue is measured over a period of 30 s. 'T'-type Ca^{2+} channels open only very transiently (tau = 20 - 50 ms) whereas 'L'-type Ca^{2+} channels remain open for much longer periods of time (tau > 500 ms) in response to a continued depolarisation-stimuli (see Table 1.2). Thus the influx of $^{45}Ca^{2+}$ over 30 s into both preparations in response to high K^+ seems likely to represent influx which occurs mainly through 'L'-type Ca^{2+} channels. This contention is supported by the finding that high K^+-induced influx into both anterior pituitary pieces and GH_3 cells is inhibited in concentration-dependent fashion and with similar high potencies by the dihydropyridine, nimodipine (Figure 3.1). Dihydropyridines such as nimodipine can inhibit 'L'-type Ca^{2+} channel activity, however the influx of $^{45}Ca^{2+}$ into both preparations although being potently blocked, is not quite completely inhibited by nimodipine at 1 μM. The remaining small amount of K^+-induced $^{45}Ca^{2+}$ influx (approximately 15% in both preparations) may represent a small proportion of the 'L'-type Ca^{2+} channel activity in the tissues which is unblockable by nimodipine. Alternatively, the remaining nimodipine-insensitive $^{45}Ca^{2+}$ influx may occur through a distinct voltage-activated channel(s) which is present in both preparations. One candidate for such a channel is the 'T'-type Ca^{2+} channel, and in fact, 'T'-type Ca^{2+} channels have been reported to show some (modest) sensitivity to blockade by dihydropyridines (Bean, 1985; Bean *et al,* 1986). Other work however, indicates that in the related GH_4C_1 cell line, nimodipine has no effect on the 'T'-channel currents present, but totally blocks 'L'-currents (Cohen and McCarthy, 1985). The existence of 'N'-type Ca^{2+} channels in both preparations is uncertain, however 'N'-type Ca^{2+} channels are also inactivate rather quickly and their contribution to the total $^{45}Ca^{2+}$ influx over 30 s should also be minimal. What may be a more likely explanation for the dihydropyridine-insensitive K^+-induced $^{45}Ca^{2+}$ influx

into both preparations is the presence of other more-recently discovered types of voltage-activated Ca^{2+} channels (see section 1.3). High voltage-activated Ca^{2+} channels which are nimodipine-insensitive and slowly inactivate, have been characterised by Llinas and colleagues (who termed their channel the 'P'-type Ca^{2+} channel) and Mori *et al* (1990) (who termed their channel the 'BI'-type channel – which could possibly be the same channel). The original classification of voltage-activated Ca^{2+} channels into 'L', 'N' and 'T' now seems to be an oversimplification of the types of Ca^{2+} channels that exist. For example, differences exist in cardiac, skeletal muscle and brain 'L'-type Ca^{2+} channels (for review see Tsien *et al,* 1991). Although the presence of Ca^{2+} channel types other than 'L', 'N' and 'T' in anterior pituitary cells has yet to be investigated, it is possible that such a dihydropyridine-insensitive, slow-inactivating channel (as has been described or has yet to be discovered) may exist in rat anterior pituitary tissue.

The complete structure of the dihydropyridine-sensitive channel from skeletal muscle has been determined and it is known that two separate sites for PKC phosphorylation exist on different subunits (α_1 and ß) of this 'L'-channel. Activation of PKC by incubation with PDBu in anterior pituitary pieces leads to an enhancement of K^+-induced $^{45}Ca^{2+}$ influx, whereas, in GH_3 cells, PDBu reduced the K^+-induced influx of $^{45}Ca^{2+}$ (Figures 3.2 and 3.3) In both preparations PDBu has no effect on basal accumulation in the tissue. In anterior pituitary prisms, the enhancement by PDBu of K^+-induced $^{45}Ca^{2+}$ influx is also blocked by nimodipine with similar potency to that seen on control K^+-induced influx (R Mitchell, unpublished observations). Furthermore, ionomycin-induced $^{45}Ca^{2+}$ influx into anterior pituitary pieces of GH_3 cells is unaffected by phorbol ester preincubation. This suggests that the phorbol ester-response is to enhance 'L'-channel activity in anterior pituitary pieces, and not simply enhance general calcium uptake and sequestration mechanisms or inhibit Ca^{2+} extrusion mechanisms. The reason for this inverse modulation seen between the two tissues in response to PDBu could be that the dihydropyridine-sensitive, 'L'-type Ca^{2+} channels

in the two tissues may be in some way different and responds in opposing ways to PDBu activation of PKC. There is no evidence in direct support of this, in contrast to other possibilities (see below). Another possibility is that PKC-independent effects may be more prevalent in one tissue preparation, leading to apparent differences in Ca^{2+} channel modulation characteristics (eg, phorbol esters have been reported to modulate phosphatidylcholine metabolism in GH_3 cells, independently of PKC (Kolesnick and Hemer, 1990 and see section 1.2.5). However, the time-courses of the PDBu effects on GH_3 cells and anterior pituitary pieces are consistent with the action of a kinase, also that the pharmacology of both influences (ie, produced by PDBu and 4ß-PDD, but not by the PKC-inactive isomer 4α-PDD and blocked by PKC antagonists) is entirely consistent with both modulations being primarily mediated by phorbol-stimulated PKC activity, and not a non-specific influence of the phorbol esters tested. Alternatively, either tissue preparation may possess some PKC-induced regulatory mechanism which is not present in the other tissue preparation, which leads to the opposite PKC-mediated modulation becoming apparent, but no such mechanism has been reported. It is further theoretically possible along these lines that the characteristic history of the tissue types may have set a tonic influence on channel function prior to the experiments; by, for example, phosphorylating (by PKA, PKC or other kinases) or dephosphorylating the channel. There is no evidence to specifically support such a possibility, nor do we have evidence to indicate that PKC is directly phosphorylating the 'L'-channel, but it would clearly be of interest to investigate these points further. In fact, we do have evidence that both in pituitary tissue and GH_3 cells (with no apparent distinction between them) that the basal 'L'-channel function does indeed require tonic activity of a kinase (that is staurosporine and not H7-sensitive, and down regulated by prolonged PDBu-incubation; ie, presumably a PKC) (MacEwan *et al,* 1991). Work by Pelech *et al* (1990; 1991) showed that treatment of rabbit platelets with phorbol ester or platelet-activating factor leads to the covalent modification of membrane-associated PKC, leading to changes in its activation characteristics (eg, by Ca^{2+} and

phospholipids). These forms of PKC may be simply those forms found when PKC is autophosphorylated and tends to become membrane-inserted (see section 1.2.1). Although the various modified states of PKC (ß-isoform was investigated) were never more fully characterised as to their pharmacological properties, it is entirely possible that differences in pharmacological profiles seen on $^{45}Ca^{2+}$ influx studies in anterior pituitary cells could reflects the differential action of distinct forms (not sequence isoforms) of PKC as discussed by Pelech *et al.* However, this possibility seems unlikely as a spectrum of characteristic effects of different activators exists within the same cell type (eg, PDBu to mezerein in GH_3 cells) which presumably have their PKCs in the same 'modification' state from experiment to experiment. In various tissues, PKC activation can result in enhanced (DeRiemer *et al,* 1985; Strong *et al,* 1987; Fish *et al,* 1986; Velasco and Petersen, 1989) or reduced (DiVirgilio *et al,* 1986; Lewis and Weight, 1988; Marchetti and Brown, 1988; Rosenthal *et al,* 1988; Rane *et al,* 1989) 'L'-channel activity, and what seems a more likely explanation for the dual modulation here is that the same (or similar) 'L'-channel exists in rat anterior pituitary pieces and GH_3 cells, and that distinct forms of PKC (perhaps sequence isoforms) have opposing modulatory roles on the 'L'-channel function (with similar time-courses) by their action on the distinct PKC-phosphorylation sites on the channel subunits. The proportion of certain PKC-isoforms is markedly different between the two tissue preparations (see section 5.4) and it may be that it is the proportion of a certain isoform(s) (which may act to enhance or reduce 'L'-channel activity) and the net influence of the said proportion of PKC-isoform which governs the tissue-specific effect of PKC activation of 'L'-type Ca^{2+} channel activity. Supportive of this 'PKC-isoform/dual-modulation' theory is the fact that not all activators of PKC will produce the response seen with PDBu on 'L'-channel function in the models. The range of PKC activators used in this chapter give varying extents of modulation in anterior pituitary prisms and GH_3 cells, which fails to support the theories other than the last-mentioned above concerning the mechanism for dual modulation of 'L'-channel function.

The contention that it is the relative influence/proportion of distinct PKC isoforms which governs the 'direction' of PKC modulation of 'L'-channel function, also carries the prediction that in certain tissues which have the appropriate proportion of the necessary PKC-isoforms, activation of PKC by PDBu would lead to the inhibitory and facilitatory PKC-influences being balanced and cancelling each other out to give the appearance of PKC being ineffective at modulating 'L'-channel function. What can also be derived by interpreting the data in the context of our original theory is that certain PKC-activating agents seem very likely to be acting selectively to influence the PKC isoform(s) which is (are) responsible for either the enhancement or inhibition of the channel activity. Both the experiments reported here and further studies attempt to assign a PKC-isoform(s) identity to the inhibitory and facilitatory PKC-influences on 'L'-type Ca^{2+} channel activity in our anterior pituitary cell models.

The phorbol ester PDBu is acting in our two models to produce an inhibitory and facilitatory modulation of 'L'-type channel function in GH_3 cells and anterior pituitary pieces respectively. It is believed that PDBu and PMA (phorbol 12-myristate 13-acetate) do not display any PKC isoform selective nature as assessed by its similar affinity for the multiple [^{3}H]-PDBu/[^{3}H]-DPB binding sites found in mouse skin (Dunn and Blumberg, 1983). Furthermore, the affinity of PDBu and PMA on two models of PKC activity in osteosarcoma cells was identical, but the phorbol-analogue, mezerein, showed marked differences in potency at the two apparently PKC-mediated effects (Jaken *et al,* 1983). Mezerein also displayed differential affinity towards the two phorbol ester-binding sites in mouse skin (Dunn and Blumberg, 1983). In the present experiments, PDBu (and PMA; R Mitchell, unpublished observations) display both the inhibitory and facilitatory influence on Ca^{2+} channel function in our two models, whereas mezerein displays only a facilitatory profile in both GH_3 cells and anterior pituitary prisms (see Figure 3.6). Taken together, the most probable explanation of the differences in these phorbol ester actions is that PDBu and PMA are acting with similar affinity towards the PKC isoforms which are present in these cells and control the

activity of 'L'-type Ca^{2+} channel function. Mezerein, on the other hand, may have greater affinity towards the PKC isoform(s) which controls the facilitatory modulation of 'L'-channel function, and reduced activity towards the PKC isoform(s) which inhibits 'L'-channel function. Displacement of [^{3}H]-PDBu binding in lung and frontal cerebral cortex cytosols (regions enriched in α and ß isoforms respectively) by mezerein, displays similar affinities for displacement in both regions (see section 5.3). This would suggest that the PKC-isoform(s) which may be selectively activated by mezerein to promote pituitary 'L'-channel activity may not be prominently present in rat lung or frontal cerebral cortex cytosols, or that the compound has similar affinity towards the PKC-isoforms found in lung and frontal cerebral cortex, but could display different efficacy (ie, partial agonist) properties at some of the isoforms. It is thus possible that mezerein is a less efficacious activator of the PKC which is responsible for inhibiting 'L'-channel activity than at the PKC responsible for facilitation of the channels. Other unknown factors could potentially alter the effectiveness of mezerein. Clearly, more analysis of the selective actions of mezerein was required, and so its effects on PKC activity from various tissues is currently being investigated (Johnson *et al,* 1991; Mitchell *et al,* 1992). To some extent, DPB, MPMA and PRA mimicked the selective profile of mezerein on K^{+}-induced $^{45}Ca^{2+}$ influx into GH_3 cells and anterior pituitary pieces (see Figure 3.6). It is probable that these phorbol analogues show a similar affinity for the PKC isoform(s) which mezerein selects for, but are not as potent or selective as mezerein. Unlike mezerein and MPMA and PRA, DPB does not enhance K^{+}-stimulated $^{45}Ca^{2+}$ influx into GH_3 cells but modestly mimics the action of mezerein, MPMA and PRA in anterior pituitary $^{45}Ca^{2+}$ influx studies. The reason for DPB not enhancing K^{+}-induced $^{45}Ca^{2+}$ influx into GH_3 cells may simply reflect the less selective nature of DPB on the two PKC-forms (tending to be intermediary in its selectivity in comparison to PDBu and mezerein). The magnitude of the DPB-induced modulation of K^{+}-induced $^{45}Ca^{2+}$ influx into both preparations is also far less than the magnitude of enhancement of $^{45}Ca^{2+}$ influx seen in anterior pituitary tissue with other

agents such as PDBu and mezerein. In conclusion, our two systems which investigate K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary cells probably represent two separate models of differential function of distinct PKC isoforms, with PDBu and PMA showing no selective nature between the two PKC isoform-influences, and mezerein showing distinct selectivity for the form(s) of PKC which control the enhancement of 'L'-type Ca^{2+} channel function. The agents MPMA, PRA and DPB show intermediate activity in selecting for the PKC isoform(s) which is involved in facilitating 'L'-channel activity in both anterior pituitary prisms and GH_3 cells, however as yet, no definite identity can be ascribed to the form(s) of PKC which enhances 'L'-channel activity. In contrast, the isoforms of PKC which are probably involved in the reduction of 'L'-type Ca^{2+} channel activity are better understood. In GH_3 cells at least, AA can activate an isoform(s) of PKC resulting in a reduction of 'L'-channel activity (this will be discussed in greater detail in the following chapter). It is now clear that AA can potently activate the γ-isoform of PKC, with the α and ß isoforms of PKC being less potently activated by AA (the α isoform being better activated than the ß isoform: Sekiguchi *et al,* 1987; Naor *et al,* 1988a; Sekiguchi *et al,* 1988; Shearman *et al,* 1989a; Burns *et al,* 1990; Shinomura *et al,* 1991). However, it is also known that anterior pituitary and GH_3 cells do not possess γ-PKC but do contain α- and ß-PKC (Naor, 1990). The isoform which produces a facilitation for inhibition in 'L'-channel activity cannot be γ as it is absent from both tissues. Unfortunately, at present no comprehensive information exists as to the influence of AA on, or the existence in pituitary cells of the B series isoforms of PKC and thus any interpretation cannot include the influence of these isoforms. The inhibitory PKC(s) which is responsible for inhibiting 'L'-type Ca^{2+} channel activity could therefore be α- or ß-PKC (or both), and as AA only poorly enhances 'L'-channel activity in anterior pituitary pieces (see Figure 3.7), then it reasonable to assume that if in anterior pituitary pieces AA is selectively activating certain isoforms of PKC, then the isoform(s) with the greatest net influence in this tissue which is responsible for enhancing 'L'-channel activity, is

poorly responsive to AA. However, the direct influence of AA on PKC-mediated actions in anterior pituitary pieces has not been determined. The diacylglycerol, DOG has been reported only to selectively mimic certain properties of phorbol esters and other longer chain diglycerides (Sekiguchi *et al,* 1988; Lacerda *et al,* 1988; Strulovici *et al,* 1989) and in our studies too, DOG can mimic only PDBu at enhancing 'L'-channel function in anterior pituitary pieces without influencing 'L'-channel activity in GH_3 cells (see Figure 3.5). This is consistent with the findings of Lacerda *et al* (1988) who reported that cardiac 'L'-type Ca^{2+} channel activity is both inhibited and enhanced by phorbol ester (the two profiles being temporally separated), whereas DOG could mimic only the enhancement of 'L'-channel activity (as is seen here in anterior pituitary pieces) without influencing the inhibitory effect on 'L'-channel activity (as seen here in GH_3 cells with DOG). As assessed in [^{3}H]-PDBu binding studies here (see Chapter 5), DOG has reduced affinity towards α-PKC. Thereby, it may be th α-isoform of PKC which is responsible for the inhibitory influence on 'L'-type Ca^{2+} channels in pituitary cells. However, other work from our laboratory suggests that DOG is a relatively poor activator of both α- and ß-PKC, and that the phorbol ester, DOPPA (see section 1.2.3) which is reported to selectively activate ß-PKC (Evans *et al,* 1991), can also induce an inhibition of 'L'-channel activity in both GH_3 cells <u>and</u> anterior pituitary pieces (R Mitchell, unpublished observations). Thus it is probable that α- and perhaps also ß-PKC (at least), but not γ-PKC, are the isoforms of PKC which are responsible for the inhibition of 'L'-type Ca^{2+} channel function in anterior pituitary pieces and GH_3 cells.

Both the inhibitory influence on 'L'-type Ca^{2+} channel function in GH_3 cells and the facilitatory influence on 'L'-type Ca^{2+} channel function in anterior pituitary pieces induced by PDBu have similar time courses of onset (see Figure 3.4). Lacerda *et al* (1988) found that stimulation of myocytes with phorbol ester induced an inhibitory and facilitatory modulation of 'L'-type Ca^{2+} channel activity which were separated temporarily (enhancement seen at 5 s, inhibition viewed at 20 min) and that

DOG induced only an enhancement of 'L'-type Ca^{2+} channel activity. Our protocol here is too crude to see any significant change in $^{45}Ca^{2+}$ influx when measured after a 5 s stimulus, however both the inhibitory and facilitatory modulations seen in our two different tissues showed very similar temporal profiles of channel modulation.

The facilitatory and inhibitory profiles of 'L'-channel modulation in anterior pituitary pieces and GH_3 cells respectively, again both showed similar potencies with respect to inhibition of the PDBu responses with staurosporine, K252a (see Figure 3.8) and to the more PKC-selective inhibitor Ro318220 (see Figure 3.9) with a pharmacological profile which would suggest the involvement of PKC, as would be expected in a PDBu-induced response. The inhibition by PDBu of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells was inhibited more potently by the kinase inhibitor H7 than its control analogue HA1004 (see Figure 3.10) again suggesting the involvement of PKC; whereas the PDBu-induced enhancement of 'L'-type Ca^{2+} channel activity in anterior pituitary pieces was far less potently inhibited by H7 (Figure 3.10) These data would suggest that the PKC isoform(s) which is activated by PDBu treatment and is responsible for the facilitatory modulation of the 'L'-type Ca^{2+} channel function in anterior pituitary prisms is an isoform which displays in general the expected pharmacological profile for PKC-selective kinase inhibitors. However, unlike the PKC isoform which causes an inhibition of 'L'-channel activity in GH_3 cells (which displays the expected high sensitivity to H7), the facilitatory isoform which is present in anterior pituitary pieces shows a distinct and characteristic resistance to inhibition by H7. Curious mismatches in PKC pharmacology have been noted before, with PKC inhibitors such as H7, staurosporine and K252a having similar potency of inhibition on a range of PKC-mediated responses, but with some phorbol ester-induced responses showing differential sensitivity towards the inhibitor (see section 1.2.3). These previous reports did not mention the possibility that certain isoforms of PKC may have reduced sensitivity to inhibition to certain PKC inhibitors, such as is seen here with H7. Indeed, work by Schaap and Parker (1990) on purified ε-PKC and on a mixture

of α, ß and γ isoforms showed no difference in sensitivity to H7. In general, it has been assumed that H7 probably does not inhibit selectively any PKC isoforms due to the extensive sequence homology of the ATP-binding domain (at which H7 is thought to act) amongst the isozymes identified so far. However, α- and ß-PKC are known to contain an additional consensus ATP-binding sequence which may alter their sensitivity to H7 (Huang, 1989). The prediction that α- or ß-PKC may have altered sensitivity to H7 due to their additional ATP-binding sequence does not seem to be the case, as the inhibition of 'L'-type Ca^{2+} channel activity seems to be mediated by α- and ß-PKC, and this PDBu-induced response is potently inhibited by H7. Indeed, it is the enhancement of 'L'-type Ca^{2+} channel activity by PDBu which shows the insensitivity to reversal by H7 and thus the H7-insensitive isoform(s) of PKC does not appear to be α- and/or ß-PKC as these isoforms are responsible for the inhibition of 'L'-type channel activity. Another prediction which results from these data is that the isoforms(s) which mezerein selectively activates to enhance 'L'-channel activity could be the same H7-insensitive isoform, or alternatively, both the mezerein-sensitive and H7-insensitive isoforms are distinct entities but both are able to enhance 'L'-channel activity. Prelimenary data from our laboratory suggest that the mezerein-sensitive, H7-insensitive kinases(s) may be the same entity, as recent studies on K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells indicate that the enhancement of 'L'-type Ca^{2+} channel activity by mezerein shows low sensitivity to inhibition by H7 (R Mitchell, unpublished observations). The experiments described in detail in Chapter 6 indicate that an H7-insensitive (but staurosporine- and Ro318220-sensitive) form of PKC does exist, and that its distribution is limited to male and female anterior pituitary, female midbrain and lung and that this H7-insensitive form of PKC (or related kinase) is relatively Ca^{2+}-independent with respect to its activation characteristics (see section 6.3). The distribution of this H7-insensitive PKC does not match the distributions of the thus far reported isoforms of PKC (A and B series) and may in fact represent a novel PKC (or

related kinase) which has been discovered through pharmacological strategies (see Chapter 6).

CHAPTER 4

EFFECTS OF ARACHIDONIC ACID IN GH_3 CELLS

4.1. INTRODUCTION

Arachidonic acid (AA) has a wide variety of cellular actions, some of which are mediated by the fatty acid itself, and others which are mediated by products of its metabolism (see section 1.2.5). One such action of AA (and certain of its metabolites) is to activate protein kinase C (PKC) (McPhail *et al,* 1984; Murikami and Routtenberg, 1985; Murikami *et al,* 1986), however AA can activate certain isoforms of PKC better than others. The activation of PKC isoforms by AA has been restricted to studies on α, ßI, ßII and γ isoforms, with the general consensus being that γ-PKC is more potently activated by AA than the α- or ß-isoforms (α and ß activate only in the presence of Ca^{2+}). Furthermore, the consensus of opinion is that α-PKC is generally better activated by AA than ß-PKC (Sekiguchi *et al,* 1987; Naor *et al,* 1988a; Sekiguchi *et al,* 1988; Shearman *et al,* 1989a; Burns *et al,* 1990; Shinomura *et al,* 1991).

In chapter 3, our two models of PKC isoform-action showed some quite distinct pharmacological profiles for activators of PKC such as 1,2-dioctanoyl-*sn*-glycerol and mezerein. When tested on our two models of rat anterior pituitary cells K^+-induced $^{45}Ca^{2+}$ channel modulation by PKCs, AA showed a quite distinct profile (Figure 3.7). Whereas phorbol 12,13-dibutyrate (PDBu) could markedly enhance K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary pieces and reduce K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells, AA was able only to mimic the effect of PDBu on GH_3 cells. The selectivity of AA shown in our two models could indicate that AA is acting selectively on the PKC profile which reduces 'L'-type Ca^{2+} channel activity (if AA is activating PKC) and is indeed a PKC isoform-selective agent, as has been reported on studies with AA on purified PKC isoforms (Sekiguchi *et al,* 1987; Sekiguchi *et al,* 1988; Naor *et al,* 1988a; Shearman *et al,* 1989a; Shinomura *et al,* 1991) and PKC isoforms from their cDNA-expression systems (Burns *et al,* 1990). However, to our knowledge, no action of AA to activate PKC in a normal (more 'physiological') system has been reported and it may be that in our studies, AA is acting analogous to PDBu in

GH_3 cells to reduce 'L'-channel $^{45}Ca^{2+}$ influx by activating PKC. Such a possibility was investigated. Whereas PDBu will induce an inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells of around 50% maximum (Figure 3.3), AA can induce a full inhibition of K^+-evoked $^{45}Ca^{2+}$ influx into GH_3 cells (Figure 3.7). The mechanism by which the full inhibition of influx into GH_3 cells with AA (but not PDBu) was also studied.

4.2. SPECIFIC METHODOLOGY

Studies on K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells were performed as described in section 2.2.3 and $^{45}Ca^{2+}$ efflux studies on GH_3 cells were as described in section 2.2.4. Measurement of intracellular free-Ca^{2+} concentration was performed as outlined in section 2.2.5. The concentration of AA and AA-methyl ester used in the fluorimetric studies (17 μM) was a concentration added in the solvent ethanol (0.4% final concentration in cuvette) which produced no record of a precipitatory artefact (as described in section 4.3). The release of [^{3}H]-AA from prelabelled GH_3 cells was as mentioned in section 2.2.6. In studies in which GH_3 cells had their PKC down-regulated, the protocol followed was similar to that previously reported on GH_3 cells (Ballester and Rosen, 1985). GH_3 cell culture flasks (containing 20 ml of growth medium) were incubated for 24 h with 300 nM PDBu dissolved in dimethylformamide (0.01% final) or dimethylformamide alone for control-treated cells. Cells were then centrifuged (1,000 g 10 min, 25°C) and the cell pellet was washed three times by resuspension and centrifugation (1,000 g, 10 min, 25°C) in either 'calcium uptake medium' for $^{45}Ca^{2+}$ studies or Hank's balanced salt solution for fluorimetric studies, in order to remove the PDBu.

The solvents used were: dimethylformamide (≤ 1% final) for PDBu, staurosporine, K252a, NDGA, ETYA, indomethacin, SKF525A, piperonyl butoxide and ionomycin; ethanol (≤ 0.4% final) for AA, AA-methyl ester, linoleic acid; and distilled water for N-methyl TRH, H7, HA1004 and 8-Br-cyclic GMP. In experiments

involving N-methyl d-glucamine, GH_3 cells were washed and resuspended in the appropriate medium that was Na^+-free, which was replaced by N-methyl d-glucamine (154 mM). At the concentrations used, solvents alone had no apparent effect on GH_3 cells.

4.3. RESULTS

The effect of arachidonic acid on K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells

Preincubation of anterior pituitary pieces for 10 min with AA (3 - 100 μM) resulted in no significant alteration in K^+-induced $^{45}Ca^{2+}$ influx, with the greatest change being to 120 ± 15% of control K^+-induced $^{45}Ca^{2+}$ influx at 100 μM AA (n = 4). Arachidonic acid is known to selectively activate certain isoforms of PKC (Naor *et al,* 1988a), but is unable to mimic the effect of PDBu and 4ß-PDD on K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary (Figure 3.7). However, 10 min preincubation of GH_3 cells with AA (1 - 100 μM) inhibited K^+-induced $^{45}Ca^{2+}$ influx in a concentration-dependent manner (IC_{50} = 19 ± 3 μM, n = 4 - 12). In contrast to the maximal 50% inhibition induced by PDBu or 4ß-PDD on K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells (Figure 3.3), AA will fully inhibit K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells (3 ± 3% of control K^+-induced influx at 100 μM AA), with the inhibition of influx being statistically significant ($P < 0.05$) from control K^+-induced $^{45}Ca^{2+}$ influx levels with 30 and 100 μM AA. The reasons for the greater inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells produced by AA than that with PDBu alone, are investigated in this chapter.

Basal influx into GH_3 cells preincubated for 10 min with 30 or 100 μM AA was not significantly altered from control basal $^{45}Ca^{2+}$ influx, measured in the presence of the solvent ethanol (0.4% final) (104 ± 3 and 108 ± 6% of control basal influx respectively (n = 4)). The trend towards a marginal increase in basal accumulation at the highest AA concentrations was matched by a similar increase over control $^{45}Ca^{2+}$

accumulation to filters in the absence of GH_3 cells with 100 μM AA. Correspondingly, the assay tubes containing 100 μM AA were noted to have a slight oily 'film' which presumably caused the small increase in filter accumulation of $^{45}Ca^{2+}$. Measurements to assess the precipitation of AA at various concentrations were performed by measuring the turbidity of a series of concentrations of AA in 'calcium uptake medium', as assessed by light-scattering in a spectrofluorophotometer using excitation, and emission wavelengths at 520 nM. Under these conditions (calcium uptake buffer containing 0.4% ethanol vehicle), no detectable increase in light-scattering could be detected up to and including 32 μM AA. However, marked turbidity (and presumably precipitation) were noted with 100 μM AA. Thus with AA, the greater inhibition of $^{45}Ca^{2+}$ influx than PDBu could not be accounted for by precipitation of AA, as the inhibitory concentration response curve was nearly complete with 30 μM AA (Figure 4.1) (which had no precipitation in 'calcium uptake medium'). Furthermore, precipitation of compounds results in an apparent increase in $^{45}Ca^{2+}$ accumulation to filters and cells, not the reduction in $^{45}Ca^{2+}$ accumulation seen with high concentrations of AA.

Several structural analogues of AA were tested to ascertain the specificity of the AA-induced inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells. The analogue AA-methyl ester was reported to be unable to activate PKC (Seifert *et al,* 1988), as well as being poorly metabolised to AA by esterase enzymes (Chan and Turk, 1987). Concentrations of AA-methyl ester of 30 and 100 μM, preincubated with cells for 10 min, could not mimic the effect of AA on K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells (87 $\pm$ 3 and 78 $\pm$ 5% of control K^+-induced influx levels respectively, $n = 4$), although both reductions of K^+-induced $^{45}Ca^{2+}$ influx were statistically significant ($P \leq 0.05$). In light-scattering experiments with a spectrofluorophotometer, AA-methyl ester showed slight turbidity at 32 μM (with very significant precipitation at 100 μM AA-methyl ester), similar to the results with AA. Therefore, substantial precipitation was not a cause for the inability of AA-methyl ester to match the inhibition of K^+-induced

influx by AA. Interestingly, the combination of 30 μM AA methyl ester and 300 nM PDBu, almost completely inhibited K^+-induced $^{45}Ca^{2+}$ influx levels into GH_3 cells to 14 ± 2% of control K^+-induced influx (n = 4; Figure 4.1). Thus, AA-methyl ester and PDBu together can mimic the effect of AA alone on K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells. Arachidic acid is similar in structure to AA, but contains no unsaturated carbon-carbon bonds. Unfortunately, arachidic acid in the concentration range used (30 and 100 μM) could not be used to assess the specificity of the AA-induced inhibition of $^{45}Ca^{2+}$ influx, as it precipitated rapidly in the quench/wash procedure with the ice-cold EGTA. Precipitation of arachidic acid was so extensive that it caused the filters to clog and so produce marked retention of label (equivalent to 1,077 and 1,369% of control K^+-induced $^{45}Ca^{2+}$ accumulation, n = 2). The effect of arachidic acid to precipitate and clog the filter could also be seen in the absence of cells (n = 1). Linoleic acid is a fatty acid of 18 carbons with two unsaturated bonds (AA has 4 unsaturated bonds and 20 carbons), which was reported to activate PKC, but to an extent which was lesser than the activation by AA at the same concentrations (Seifert *et al,* 1988). Linoleic acid (30 μM) did not significantly` reduce K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells (79 ± 14% of control K^+-stimulated influx, n = 4). The effect of 100 μM linoleic acid on the assay could not be determined with any certainty as the linoleic acid could not be fully dissolved in ethanol at sufficient concentration to keep below the vehicle concentration limit needed in the assay tube. Thus AA-methyl ester and probably also linoleic acid could not fully mimic the effect of AA on K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells, although some small degree of inhibition was observed. The fact that this result does not correlate accurately with their reported activities on PKC and the expected synergy between AA-methyl ester and PDBu suggest that PKC activation by AA cannot fully account for its complete inhibition of $^{45}Ca^{2+}$ influx into GH_3 cells.

The effects of H7 and staurosporine on AA-induced inhibition of $^{45}Ca^{2+}$ influx into GH_3 cells

In GH_3 cells, the PDBu-induced inhibition of K^+-induced $^{45}Ca^{2+}$ influx is fully reversed by the PKC inhibitors H7 and staurosporine. The effect of these two kinase inhibitors was examined on the inhibition of $^{45}Ca^{2+}$ influx into GH_3 cells caused by AA. Inclusion of H7 (2 - 50 μM) in the 10 min preincubation of cells with AA (30 μM) before measurement of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells, (Figure 4.2) reversed the AA-induced inhibition in a concentration-dependent manner (IC_{50} = 14 ± 4 μM n = 12). The inhibition of K^+-induced $^{45}Ca^{2+}$ influx caused by 30 μM AA (23 ± 9% of control K^+-induced influx, n = 12) was significantly ($P \leq 0.05$) reversed by 50 μM H7, returning to levels of 88 ± 14% of control K^+-induced influx. This reversal of the response to 30 μM AA in GH_3 cells was not seen with a congener of H7 that has reduced affinity for PKC, HA 1004 (2 - 50 μM). This compound showed no significant reversal of the effect of AA at the highest concentration of HA 1004 tested (28 ± 8% of control K^+-induced influx levels at 50 μM, n = 8). The effect of 30 μM AA on K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells was reversed by staurosporine (5 - 300 nM) in a concentration-dependent fashion (Figure 4.3), resulting in an IC_{50} = 52 ± 15 nM, n = 8). The reversal of 30 μM AA-induced inhibition was significant ($P \leq 0.05$) at 100 and 300 nM staurosporine, which respectively gave levels of 78 ± 9 and 83 ± 14% of control K^+-induced $^{45}Ca^{2+}$ influx. A congener of staurosporine that has reduced affinity for PKC, K252a (5 - 300 nM) did not significantly reverse the AA-induced inhibition of influx, with the highest concentration used giving an influx still reduced to 31 ± 8% of control K^+-induced $^{45}Ca^{2+}$ influx (n = 4). Staurosporine or H7 alone had no effect on K^+-induced $^{45}Ca^{2+}$ influx or basal influx levels through the concentration ranges tested.

The effect of procedures to down-regulate PKC on AA-induced inhibition of K^+-evoked $^{45}Ca^{2+}$ influx into GH_3 cells

Incubation of GH_3 cells with 300 nM PDBu for 24 h is reported to result in a > 50% reduction of PKC levels and reduced PKC-mediated responses (Ballester and Rosen, 1985). Application of a similar protocol here resulted in the magnitude of the K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells being somewhat reduced (29 ± 9% of control influx), but it was very clear that the ability of AA (3 - 100 μM) to inhibit K^+-induced $^{45}Ca^{2+}$ influx was reduced (Figure 4.4). The potency of inhibition by AA was reduced to an IC_{50} of 98 ± 19 μM, (n = 6) when compared to that in control cells treated with dimethylformamide (0.01% final volume in culture flask) alone (IC_{50} = 27 ± 4 μM, n = 6). The percentage inhibition of K^+-induced $^{45}Ca^{2+}$ influx by 30 μM AA in PDBu-treated cells, was statistically different ($P \leq 0.05$) from that in control-treated cells at the same concentration of AA. However, at 100 μM AA, a significant reduction in K^+-induced $^{45}Ca^{2+}$ influx in PDBu-treated cells was apparent (47 ± 9% of control K^+-induced influx), indicating that some AA-induced inhibition of influx is still possible in PKC-down regulated GH_3 cells.

These data and the reversal of the AA-response by H7 and staurosporine (but not HA 1004 or K252a) indicate a role for PKC in the AA-induced inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells. The problem still remained to be explained that PDBu-activation of PKC will only result in around 50% inhibition of K^+-stimulated calcium influx into GH_3 cells, whereas AA will fully inhibit influx, although the AA-response is fully reversed by PKC inhibitors.

Time-course of AA-induced inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx into GH_3 cells

Testing the hypothesis that activation of PKC may be involved in the AA-induced inhibition of K^+-evoked $^{45}Ca^{2+}$ influx, the time-course of the AA-response into GH_3 cells was investigated. Incubation of cells for 30, 45 and 90 s (inclusive of

the 30 s $^{45}Ca^{2+}$ influx measurement period) with 30 μM AA, time-dependently reduced K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells (Table 4.1). The reduction in $^{45}Ca^{2+}$ influx was statistically significant ($P \leq 0.05$) from control K^+-induced $^{45}Ca^{2+}$ influx levels at each time-point measured. With 30 s AA, the K^+-induced influx was inhibited to 52 ± 6% of control K^+-induced calcium influx levels (n = 4). The half-time for the maximal response to occur to AA was of a similar half-time for the maximal response for PDBu to occur (Figure 3.4), further consistent with a PKC mediation of the effect of AA. An effect of AA (or a metabolite) to activate K^+-channels (Kurachi *et al,* 1989; Kim *et al,* 1989; Ordway *et al,* 1991) and thereby hyperpolarise the cell could account for the inhibition of depolarisation-induced $^{45}Ca^{2+}$ influx into GH_3 cells. Such a mechanism seems unlikely, since the effect of AA on K^+-channels is maximal within seconds (Ordway *et al,* 1989; Ordway *et al,* 1991) unlike the time-course of the response to AA in GH_3 cells (Table 4.1) which is half-maximal at around 30 s. Perhaps more consistent with our observations are the findings by Kim *et al* (1989) and Kurachi *et al* (1989) who observed that their activation of K^+-channels by a metabolite of AA occurred about 1 min after application of AA. However, as will be demonstrated later (Table 4.2), inhibitors of AA metabolism do not effect the response to AA in GH_3 cells, and the inhibition of K^+-induced $^{45}Ca^{2+}$ influx appears to be a direct action of AA itself. Furthermore, evidence here for the role of PKC in the AA action would suggest that such a mechanism on K^+ channels would have no more than a modest contribution to the effect of AA to inhibit $^{45}Ca^{2+}$ influx through 'L'-channels in GH_3 cells.

Effect of inhibitors of eicosanoid metabolism on the inhibition by AA of K^+-evoked $^{45}Ca^{2+}$ influx into GH_3 cells

Major routes of AA metabolism occur via the lipoxygenase, cyclo-oxygenase and cytochrome P_{450} enzyme pathways (Shimizu and Wolfe, 1990; also see section 1.2.4). As many of the metabolites of AA have biological activity, we

investigated the possibility that AA-metabolites may be responsible for the complete inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells. The cyclo-oxygenase and lipoxygenase inhibitor ETYA (10 μM) (Taylor and Clark, 1986), the lipoxygenase inhibitor NDGA (30 μM) (Taylor and Clark, 1986) and the cytochrome P_{450} inhibitors piperonyl butoxide (30 μM) and SKF525A (10 μM) (Luini and Axelrod, 1985) were included in the preincubation for 10 min with 30 μM AA (Table 4.2). None of these agents caused any significant alteration in the inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells with 30 μM AA (0 ± 10, 15 ± 12, 8 ± 13 and 4 ± 6% alteration of the AA response with ETYA, NDGA, piperonyl butoxide and SKF525A respectively (n = 6, 6, 4 and 4)). Thus the inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx into GH_3 cells by AA does not appear to be due to a metabolite of one of these pathways and probably is mostly due to AA itself.

Effects of arachidonic acid on general aspects of Ca^{2+}-handling in GH_3 cells

It was possible that AA may be influencing Ca^{2+}-extrusion processes as well as influx mechanisms. If AA enhanced the extrusion rate of $^{45}Ca^{2+}$ as well as inhibiting 'L'-type Ca^{2+}-channel influx, then this could be a possible mechanism underlying the complete inhibition of $^{45}Ca^{2+}$ influx into GH_3 cells caused by AA.

In order to assess the possible role of changes in the activity of the Na^+/Ca^{2+}-exchanger and Ca^{2+}-ATPase enzymes in the AA-response, we used the large inpermeant monovalent cation N-methyl d-glucamine (replacing Na^+ in the medium, 154 mM) and Na_3VO_4 (1 mM in normal 'calcium uptake medium') which are reported to block the respective calcium-transport mechanisms (Gill *et al,* 1981; Di Polo *et al,* 1979). In the presence of 100 μM AA, K^+-induced influx was reduced to 8 ± 6% of control K^+-induced $^{45}Ca^{2+}$ influx levels (n = 4). N-methyl d-glucamine and Na_3V0_4 had no clear effect on the AA-induced inhibition of influx: in the presence of each, AA (100 μM) inhibited K^+-induced influx to 30 ± 6 and 16 ± 2% of control K^+-induced

influx respectively (n = 4; Table 4.3). However, K^+-induced $^{45}Ca^{2+}$ influx in the presence of both Ca^{2+} extrusion inhibitors was raised to 130 ± 8% of control K^+-induced influx levels (n = 4). Therefore, the slight rises in $^{45}Ca^{2+}$ accumulation with N-methyl d-glucamine (and Na_3VO_4) in the presence of AA appear to reflect a general rise in $^{45}Ca^{2+}$ accumulation in GH_3 cells, and not in any way a specific reversal of the AA-induced by the agent. Thus AA appears to exert its effects on K^+-induced $^{45}Ca^{2+}$ influx in GH_3 cells at a step(s) prior to extrusion of $^{45}Ca^{2+}$ (at least through the Ca^{2+}-extrusion enzymes investigated).

Effects of AA on N-methyl TRH- and ionomycin-induced $^{45}Ca^{2+}$ influx into GH_3 cells

The effect of arachidonic acid of both N-methyl TRH- and ionomycin-induced $^{45}Ca^{2+}$ influx into GH_3 cells was investigated to see whether the AA-induced inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells was particular to a K^+-stimulus for calcium influx or was a general phenomenon of modifying calcium influx caused by any stimuli. Thyrotropin releasing hormone (TRH) receptors are present on GH_3 cells. Activation of these receptors leads to hydrolysis of phosphoinositides and calcium influx (Drummond, 1986). Incubation of GH_3 cells with potent TRH agonist [N-methyl]^2-TRH (N-methyl TRH) and $^{45}Ca^{2+}$ for 30 s, caused an influx of $^{45}Ca^{2+}$ (Figure 2.3 and Table 4.6). The amount of N-methyl TRH-induced $^{45}Ca^{2+}$ influx was 31 ± 9% of the influx of $^{45}Ca^{2+}$ seen with 60 mM K^+-containing medium (n = 12). The effect of preincubation with 50 μM AA for 10 min prior to exposure to the N-methyl TRH stimulus, was to reduce slightly the influx of $^{45}Ca^{2+}$ into GH_3 cells to 73 ± 12% of the control response to N-methyl TRH-induced $^{45}Ca^{2+}$ influx (n = 12), although this apparent reduction did not reach statistical significance. Ionomycin is a compound which implants itself into membranes and acts as Ca^{2+} ionophore, allowing Ca^{2+} to flow down its concentration gradient (into GH_3 cells under these conditions). Incubation of GH_3 cells with 30 μM ionomycin for 30 s caused influx of $^{45}Ca^{2+}$ into

GH_3 cells to levels which were significantly ($P \leq 0.05$) above basal or K^+-stimulated influx levels ($257 \pm 40\%$ of control K^+-induced $^{45}Ca^{2+}$ influx, n = 4). Preincubation of GH_3 cells with 50 μM AA for 10 min before exposure to ionomycin reduced $^{45}Ca^{2+}$ influx to $83 \pm 10\%$ of control ionomycin-induced $^{45}Ca^{2+}$ influx levels (n = 4), a reduction in influx which was not significantly different from control ionomycin-induced responses. However, in the same experiments 10 min preincubation of GH_3 cells with 50 μM AA before 30 s exposure to 60 mM K^+, reduced the K^+-induced $^{45}Ca^{2+}$ influx to levels of $3 \pm 14\%$ of control K^+-induced $^{45}Ca^{2+}$ influx levels (n = 4). The reduction by AA of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells was statistically significant ($P \leq 0.05$). Therefore, the AA-induced reduction of K^+-stimulated $^{45}Ca^{2+}$ influx into GH_3 cells is not matched by the AA-induced reductions in N-methyl TRH- and ionomycin-induced $^{45}Ca^{2+}$ influx. This suggests that the AA-inhibition of $^{45}Ca^{2+}$ influx is not just an AA-induced change in Ca^{2+} handling of the cells, but is relatively specific towards K^+-stimulated influx. Both this observation and the failure of N-methyl d-glucamine or Na_3VO_4 (Na^+/Ca^{2+} exchange and Ca^{2+}-ATPase inhibitors respectively) to modify the AA-induced inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx into GH_3 cells, suggest the effect of AA is not just occurring by a change in Ca^{2+}-extrusion processes (or Na^+/Ca^{2+} exchanger/Ca^{2+}-ATPase at least). The main route of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells is through a nimodipine-sensitive 'L'-type Ca^{2+} channel, and this channel is known to be modulated by PKC (Campbell *et al,* 1988). Thus, activation of PKC by AA could account for the decrease in K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells (similar to the reduction seen with PDBu) and could explain why the ionomycin-induced $^{45}Ca^{2+}$ is insensitive to modulation by AA, as PKC would not interact with the ionophore. The TRH-induced influx of Ca^{2+} into GH_3 cells within 30 s is largely dihydropyridine-insensitive and TRH-induced influx consists of both 'L'-type Ca^{2+}-channel and non 'L'-type Ca^{2+}-channel/receptor-operated Ca^{2+}-channel mechanisms (Mitchell *et al,* 1989). Therefore, any AA-induced reduction of N-methyl TRH-stimulated $^{45}Ca^{2+}$ influx here may reflect the minor component of the stimulated

influx occurring through an 'L'-type Ca^{2+}-channel; and an effect of AA on this minor 'L'-channel component which is similar to that seen for K^+-induced $^{45}Ca^{2+}$ influx but manifests itself as only a slight reduction of the total N-methyl TRH-induced $^{45}Ca^{2+}$ influx in 30 s.

Effect of 8-Bromo cyclic GMP on K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells

It was reported that AA could activate guanylate cyclase (Glass *et al,* 1977), the enzyme responsible for the production of cyclic-GMP. Cyclic-GMP activates cyclic-GMP-dependent protein kinase which could in principle be responsible for the full reduction in K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells. The cell-permeable activator of cyclic-GMP-dependent protein kinase, 8-Br-cyclic GMP (3 - 100 μM) was totally unable to mimic the effect of AA on K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells (Table 4.4). At no point throughout the concentration range used, did 8-Br-cyclic GMP significantly alter K^+-induced $^{45}Ca^{2+}$ calcium influx (n = 4). We therefore could find no evidence that presumed activation of cyclic GMP-dependent protein kinase in GH_3 cells reduces K^+-induced $^{45}Ca^{2+}$ influx and, therefore no support for the hypothesis that AA activation of guanylate cyclase could account for the effect of AA on K^+-induced calcium influx.

Effect of AA on $^{45}Ca^{2+}$ extrusion from GH_3 cells

The cytosolic free calcium concentration is governed by the rate of both calcium influx and extrusion from this cellular compartment (Blaustein, 1988). Changes in this dynamic equilibrium will alter the cellular cytosolic calcium concentration. Raised cytosolic calcium will result in the extrusion of more Ca^{2+} as the extrusion enzymes compensate to try to maintain a steady concentration of calcium in the cytosol. Our hypothesis was that AA could raise cytosolic Ca^{2+} concentrations, as was found to be the case in other cell types (Chan and Turk, 1987; Beaumier *et al,* 1987; Chow and Jondal, 1990). The continued presence of ionomycin (30 μM) in the

bathing medium increased the rate of $^{45}Ca^{2+}$ efflux from GH_3 cells to levels of 1148 ± 39 dpm greater than basal efflux levels (n = 3; Figure 2.5). The period of increased rate of efflux with ionomycin was complete within 4 min, as the efflux rate had returned to basal efflux levels by the next measurement. The continued presence of 50 μM AA in the bathing medium induced a rise in $^{45}Ca^{2+}$ efflux from GH_3 cells of 318 ± 56, 545 ± 34 and 168 ± 64 dpm above basal efflux levels (n = 3; Figure 4.5) in each consecutive efflux fraction-collection (2 min each) respectively, before returning to basal levels of efflux despite the continued presence of 50 μM AA. The total efflux of $^{45}Ca^{2+}$ induced by 50 μM AA from each collection accounted for 36 ± 3% of the total dpm recovered with 30 μM ionomycin (Table 4.5). This suggests an action of AA to release a limited capacity pool of accumulated $^{45}Ca^{2+}$ rather than an ongoing process. When a number of inhibitors of AA metabolism were present from the beginning of fraction collection, ETYA (10 μM), SKF 525A (10 μM) had no measurable effect on $^{45}Ca^{2+}$ efflux rates (96 ± 9 and 96 ± 12% of control AA-induced $^{45}Ca^{2+}$ efflux levels respectively (n = 3)), although 10 μM NDGA slightly reduced the effect of 50 μM AA to 69 ± 20% of the total rise in $^{45}Ca^{2+}$ extrusion by AA (n = 3). However, the effect of NDGA could not be considered definitive, being only apparent on the peak-fraction. Furthermore, another cyclo-oxygenase inhibitor, indomethacin (10 μM) was ineffective at altering the $^{45}Ca^{2+}$ efflux response of AA (98 ± 18% of control AA-induced $^{45}Ca^{2+}$ efflux (n = 3)). Thus, it seems that the raised $^{45}Ca^{2+}$ efflux from GH_3 cells induced by AA may well be due to AA itself and not a metabolite. Replacement of 154 mM Na^+ in the medium with N-methyl d-glucamine had no effect on the increased efflux of $^{45}Ca^{2+}$ induced by 50 μM AA (n = 3) whereas the presence of 1 mM Na_3VO_4 completely blocked the increase in $^{45}Ca^{2+}$ efflux from GH_3 cells by AA (1 ± 6% of control AA-induced $^{45}Ca^{2+}$ efflux, n = 3).

At a concentration of 300 nM, PDBu produced no measurable increase in $^{45}Ca^{2+}$ efflux from GH_3 cells (2 ± 4% of ionomycin-induced efflux, n = 3) and the presence of the PKC inhibitor H7 (30 μM) did not affect the increase in $^{45}Ca^{2+}$ efflux

in response to 50 μM AA (102 ± 8% of the control response to AA in the absence of H7, n = 3). This suggested that the effect of AA on $^{45}Ca^{2+}$ efflux did not involve a mechanism of PKC activation which is consistent with the lack of effect of H7. Since there have been reports that PKC can activate Ca^{2+}-ATPase (Drummond, 1984), it seems that the increase in $^{45}Ca^{2+}$ efflux here shown by AA but not PDBu, reflects something other than a change in the activity of the Ca^{2+}-ATPase. Entirely consistent with all these points is the evidence that AA can mobilise intracellular Ca^{2+}pools (Chan and Turk, 1987; Beaumier *et al,* 1987; Chow and Jondal, 1990). It seems quite likely that the AA-induced $^{45}Ca^{2+}$ efflux simply reflects clearance of the elevated cytosolic Ca^{2+} concentration caused by AA, rather than a particular change in extrusion processes *per se*. Further experiments would of course be required to establish this in detail. It is clear, however that the effect on efflux here of AA is not underlying the change in $^{45}Ca^{2+}$ influx since the two phenomena display differential sensitivity to H7 and Na_3VO_4, and the effect of AA on $^{45}Ca^{2+}$ influx is only partially mimicked by PDBu.

Effect of AA on cytosolic calcium levels in GH_3 cells

To further investigate the possible effects of AA on cellular Ca^{2+} stores, cytosolic Ca^{2+} concentrations were measured in GH_3 cells using the Ca^{2+}-indicator dye Indo-1. All experiments were performed separately at least three times (with the exception of the PKC down-regulated GH_3 cell studies which were performed twice). All results are representative for each experiment, with the data not varying outwith 30% of the value shown. Unfortunately, staurosporine showed large fluorescence artefacts at the wavelengths used and could not be investigated. High concentrations of AA also induced marked instantaneous rises in the apparent fluorescence signal and therefore we tested a range of concentrations of AA until reaching the limit of fluorescence artefact at the wavelengths used. A concentration of 17 μM AA with a solvent (ethanol) limit of 0.4% (v/v) did not change fluorescence in the absence of cells

or Indo-1 at the wavelength measured. This is in agreement with the turbidity experiments at which concentration of AA < 32 μM showed no light scattering artefact, measured at 520 nM. Ethanol at 0.4% concentration had no effect on basal cytosolic Ca^{2+} fluorescence on GH_3 cells, or on a response of the cells to 40 mM (final concentration) K^+ solution. Arachidonic acid (17 μM) induced a rise in cytosolic calcium concentrations from resting $[Ca^{2+}]_i$ levels of around 200 nM to approximately 250 - 300 nM (Figure 4.6). This AA-induced rise in $[Ca^{2+}]_i$ was apparent immediately after addition of the compound and took 1 to $2^1/_2$ min to plateau to its maximum level, where it was maintained. Addition of high K^+ (40 mM final) stimulus resulted in a transient rise in $[Ca^{2+}]_i$ to peak concentrations of approximately 1,000 nM, plateauing to approximately 500 nM in the presence of solvent alone (0.4% ethanol) applied 4 min previously. The rise in $[Ca^{2+}]_i$ seen with high K^+ was similar in magnitude and temporal pattern as that of Drummond (1984) who stimulated GH_3 cells with high K^+ while measuring $[Ca^{2+}]_i$ using Quin-2 as a Ca^{2+}-indicator. When a high K^+ challenge was applied to GH_3 cells at least 4 min after the addition of 15 μM AA, there was a much diminished transient rise in intracellular calcium (to levels of approximately 300 nM, in comparison of the control rise in $[Ca^{2+}]_i$ to 1,000 nM seen in the presence of ethanol alone). In the presence of the PKC inhibitor, H7 (30 μM, pre-incubated with the GH_3 cells in the cuvette for > 6 min), the rise in intracellular calcium induced by AA (17 μM) was still present and was comparable to the rise seen in the absence of H7. However, the response to K^+ in the presence of both AA and H7, was returned to levels comparable to those seen in response to K^+ in the presence of ethanol alone (Figure 4.6). The action of AA to raise intracellular Ca^{2+} concentrations was thought to be independent of PKC, as 300 nM PDBu did not induce any discernible rise in basal (approximately 200 nM) GH_3 cell cytosolic Ca^{2+} concentration (Figure 4.7). In these experiments, high K^+ induced a peak rise in cytosolic calcium concentration to approximately 600 nM, the peak then plateauing to concentrations of approximately 400 nM (in the presence of 0.1% dimethylformamide, included $1^1/_2$ min earlier (Figure

4.7(a)). The role of PKC was implicated in the reduction of high K^+-evoked raised $[Ca^{2+}]_i$, as the effect of PDBu (applied $2^1/_2$ min previously) on this K^+-induced rise in $[Ca^{2+}]_i$ was to reduce the magnitude (both peak and plateau levels) (Figure 4.7(b)) to levels (which were approximately half those in control experiment) which were similar to those seen in K^+-induced $^{45}Ca^{2+}$ influx studies with GH_3 cells (approximately 50%; Figure 3.3). This effect of PDBu to inhibit K^+-induced rises in cytosolic $[Ca^{2+}]$ was reversed by inclusion of 30 μM H7, 2 min prior to the start of the recording (Figure 4.5(c)). Furthermore, AA-methyl ester, which is a poor activator of PKC (Seifert *et al,* 1988) but can release stored Ca^{2+} pools (Chan and Turk, 1987), raised intracellular Ca^{2+} levels to similar levels (approximately 50 nM) seen with 17 μM AA, but could not induce any reduction in the high K^+-induced rise in cytosolic Ca^{2+} concentrations, which plateaued at approximately 800 nM (Figure 4.8(b)). Similarly, in GH_3 cells which had been pre-treated for a prolonged period with 300 nM PDBu to down-regulate PKC levels (Figure 4.8(c,d)), a rise in $[Ca^{2+}]_i$ in response to 17 μM AA was still apparent but showed only a small reduction in the response to high K^+ (approximately 200 nM rise in $[Ca^{2+}]_i$; Figure 4.8(d)) compared to the response to high K^+ with ethanol alone in PKC down-regulated cells (Figure 4.8(c)). The reasons for the markedly reduced effect of high K^+-concentrations to raise cytosolic calcium concentrations (and K^+-induced $^{45}Ca^{2+}$ influx levels) are uncertain. The AA analogue, AA-methyl ester (17 μM) in 0.4% ethanol also had no fluorescence artefact at the wavelength used. The magnitude and temporal pattern of the response to AA-methyl ester to raise cytosolic calcium levels, was similar to that seen for AA, and therefore unlike AA, AA-methyl ester is a convenient tool for raising $[Ca^{2+}]_i$ levels in GH_3 cells without activating PKC. Thus AA and AA-methyl ester will raise $[Ca^{2+}]_i$ in GH_3 cells (and PKC down-regulated GH_3 cells), but only AA will markedly reduce any rise in cytosolic calcium to a subsequent K^+-stimulus. The effect of AA to reduce the K^+-induced rise in $[Ca^{2+}]_i$ is reversed by H7 and is greatly diminished from PKC-down regulated GH_3 cells. These results are consistent with AA (and AA-methyl ester)

raising $[Ca^{2+}]_i$ independently of PKC activation. In contrast, the activation of PKC by AA (and PDBu) may be responsible for the inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells, with AA being able to fully inhibit K^+-induced $^{45}Ca^{2+}$ into GH_3 cells by utilising both mechanisms. Furthermore, the reduced ability of PKC-down regulated GH_3 cells to respond to a depolarisation stimulus may represent a reduced activity of the 'L'-type Ca^{2+} channel (similar to the effect found in the $^{45}Ca^{2+}$ influx studies).

Figure 4.1. Concentration-response data for the inhibition of depolarisation-induced calcium influx in GH_3 cells by fatty acids.

Cells were exposed to $^{45}Ca^{2+}$ for 30 s at 37°C and calcium influx was stopped as described in the Methods section. Cells were preincubated with arachidonic acid (AA, filled circles), arachidonic acid-methyl ester (AA-methyl ester, open circles), linoleic acid (square) 300 nM PDBu (open triangle) or with 300 nM PDBu plus 30 μM AA-methyl ester (closed triangle) for 10 min at 37°C before exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Values represent means ± SEM, n = 4 - 12. Statistically significant ($P < 0.05$) inhibition of K^+-induced $^{45}Ca^{2+}$ influx was achieved by 30 and 100 μM AA, PDBu alone or PDBu + AA-methyl ester. The concentration-response curve for AA here is the same as seen in Figure 3.7.

Figure 4.1

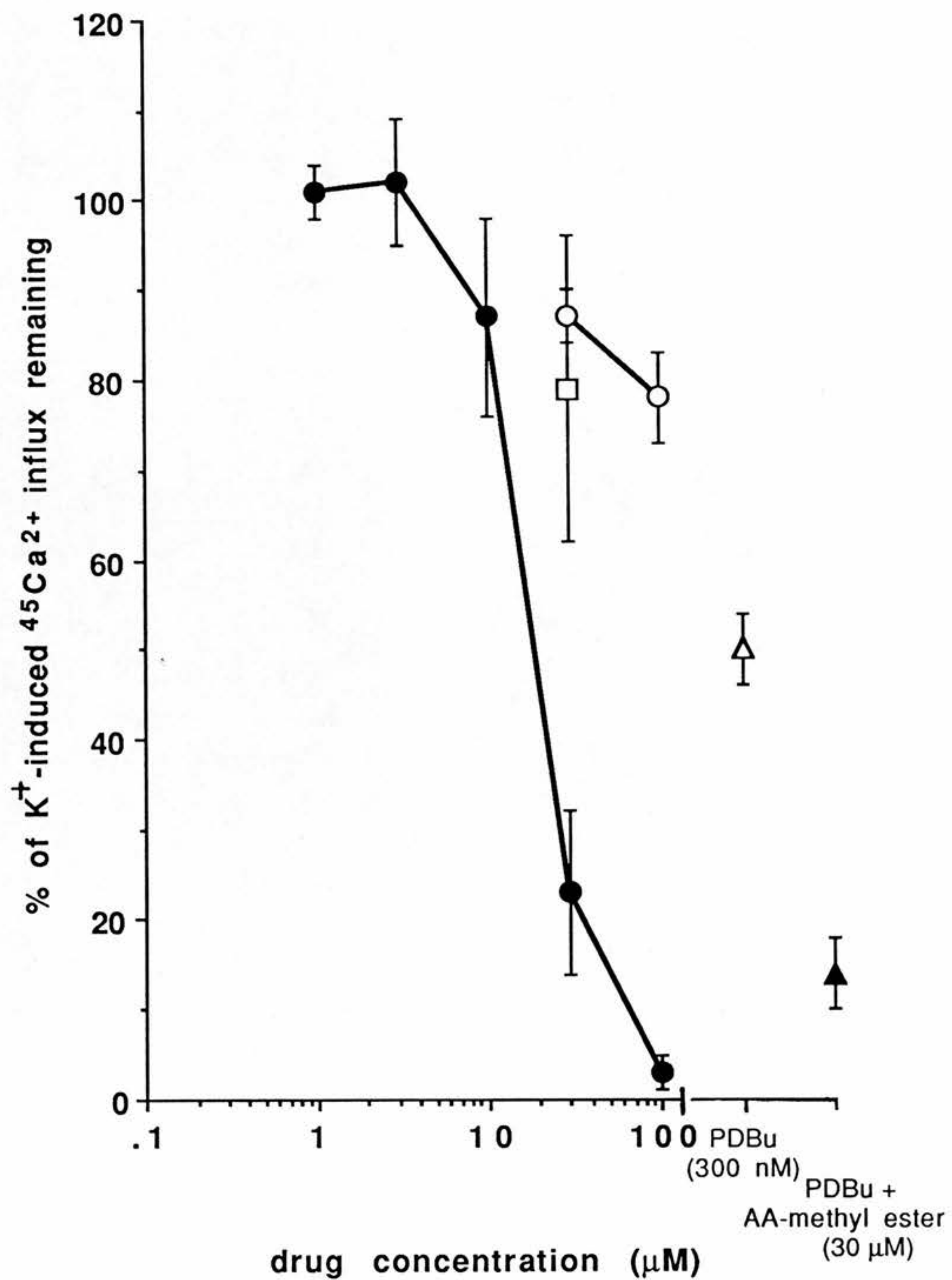


Figure 4.2. **Reversal by H7 but not HA1004 of the inhibition by AA of depolarisation-induced calcium influx into GH_3 cells.**

GH_3 cells were exposed to $^{45}Ca^{2+}$ for 30 s at 37°C and calcium influx was stopped as described in the Methods section. H7 (filled squares) and HA1004 (open squares) were added immediately prior to addition of AA (30 µM), then cells were preincubated for 10 min at 37°C before exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Control K^+-induced $^{45}Ca^{2+}$ influx data in the presence of AA is shown by the circle. Values represent means ± SEM, n = 8 - 12. Both H7 and HA1004 at 50 µM had no effect alone on basal or K^+-induced $^{45}Ca^{2+}$ influx. The inhibition of K^+-induced $^{45}Ca^{2+}$ influx due to AA was significantly reversed by 50 µM H7 ($P < 0.05$).

Figure 4.2

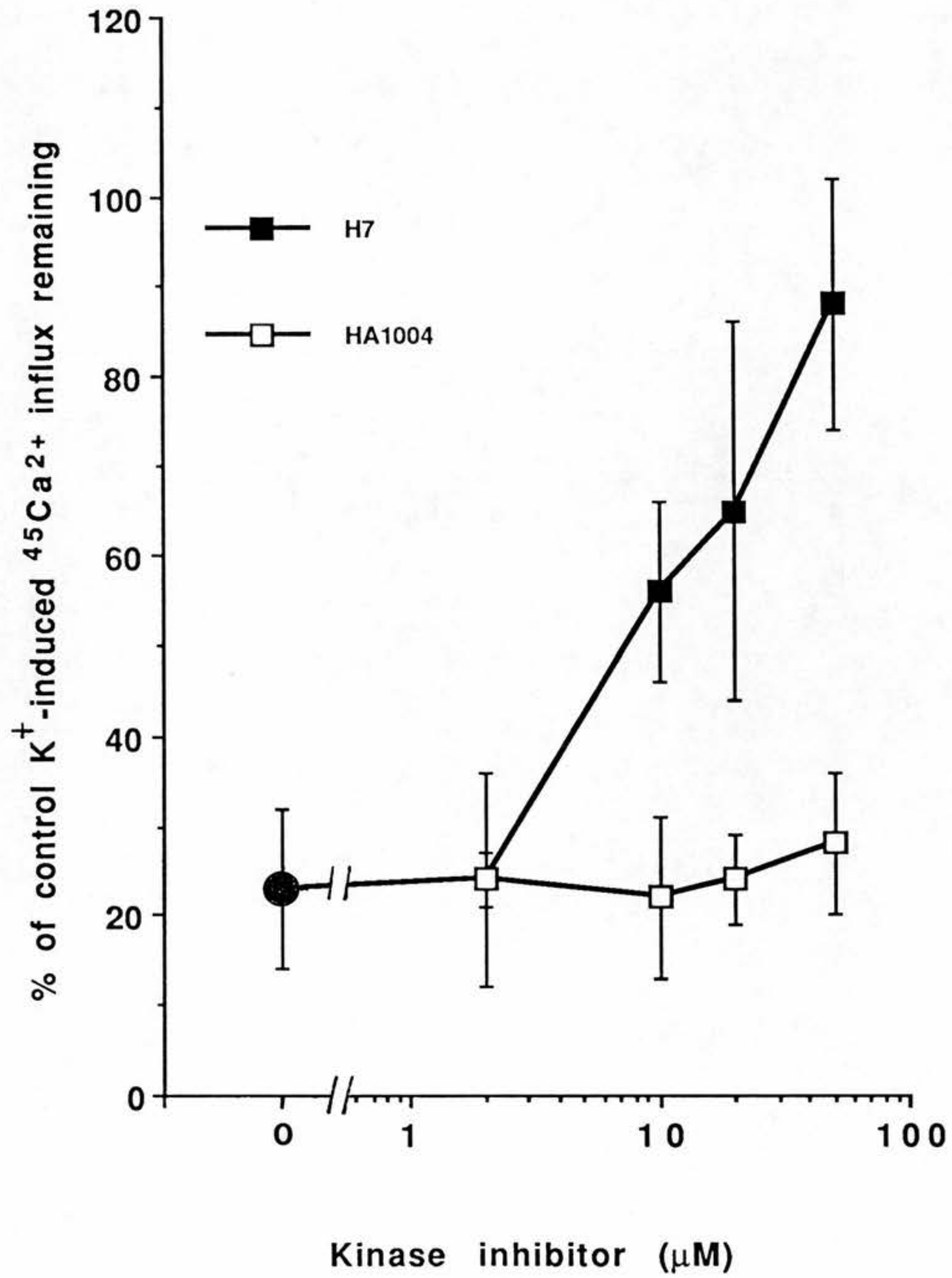


Figure 4.3. Reversal by staurosporine but not K252a of the inhibition by AA of depolarisation-induced calcium influx into GH_3 cells.

GH_3 cells were exposed to $^{45}Ca^{2+}$ for 30 s at 37°C and calcium influx was stopped as described in the Methods section. Staurosporine (filled squares) and K252a (open squares) were added immediately prior to addition of AA (30 μM) then cells were preincubated for 10 min at 37°C before exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Control K^+-induced $^{45}Ca^{2+}$ influx data in the presence of AA is shown by the circle. Values represent means ± SEM, n = 4 - 8. Both staurosporine and K252a at 300 nM had no effect alone on basal or K^+-induced $^{45}Ca^{2+}$ influx. The inhibition of K^+-induced $^{45}Ca^{2+}$ influx due to AA was significantly reversed by 100 and 300 nM staurosporine ($P < 0.05$).

Figure 4.3

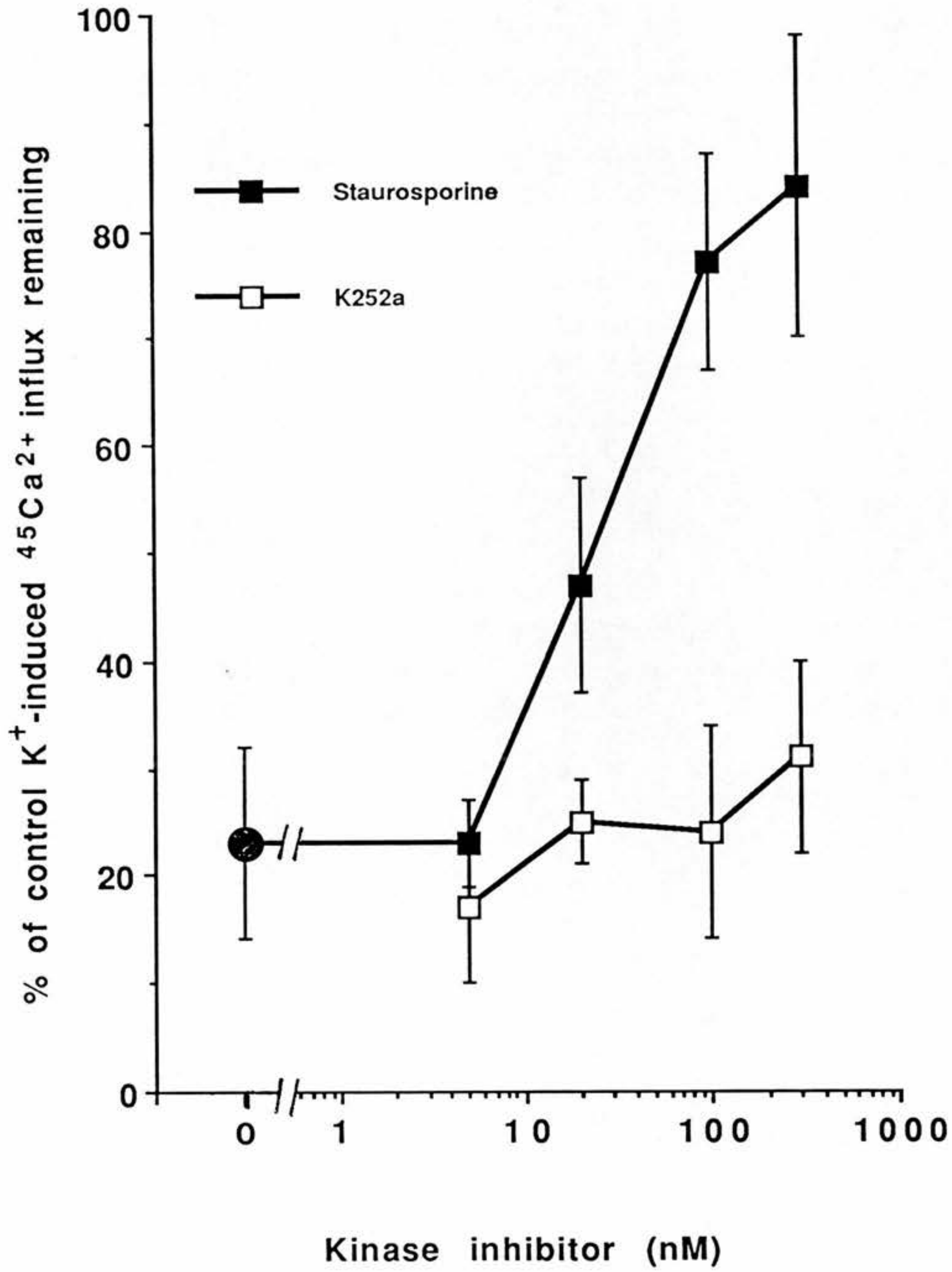


Figure 4.4. **Effect of PKC-down regulation of GH_3 cells on arachidonic acid inhibition of depolarisation-induced calcium influx.**

GH_3 cells were cultured for 24 h with 300 nM PDBu in dimethyl formamide (0.01% final volume, filled circles), dimethylformamide alone (open circles) or nothing (triangles) and then extensively washed by centrifugation and resuspension in 'calcium uptake medium' (3 x 10 min, 1000 g, 25°C). Arachidonic acid was added 10 min before a 30 s exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Values represent means ± SEM, n = 6. The reduction in $^{45}Ca^{2+}$ influx caused by AA (30 µM) was significantly attenuated by prolonged preincubation with PDBu compared to its solvent alone ($P < 0.05$).

Figure 4.4

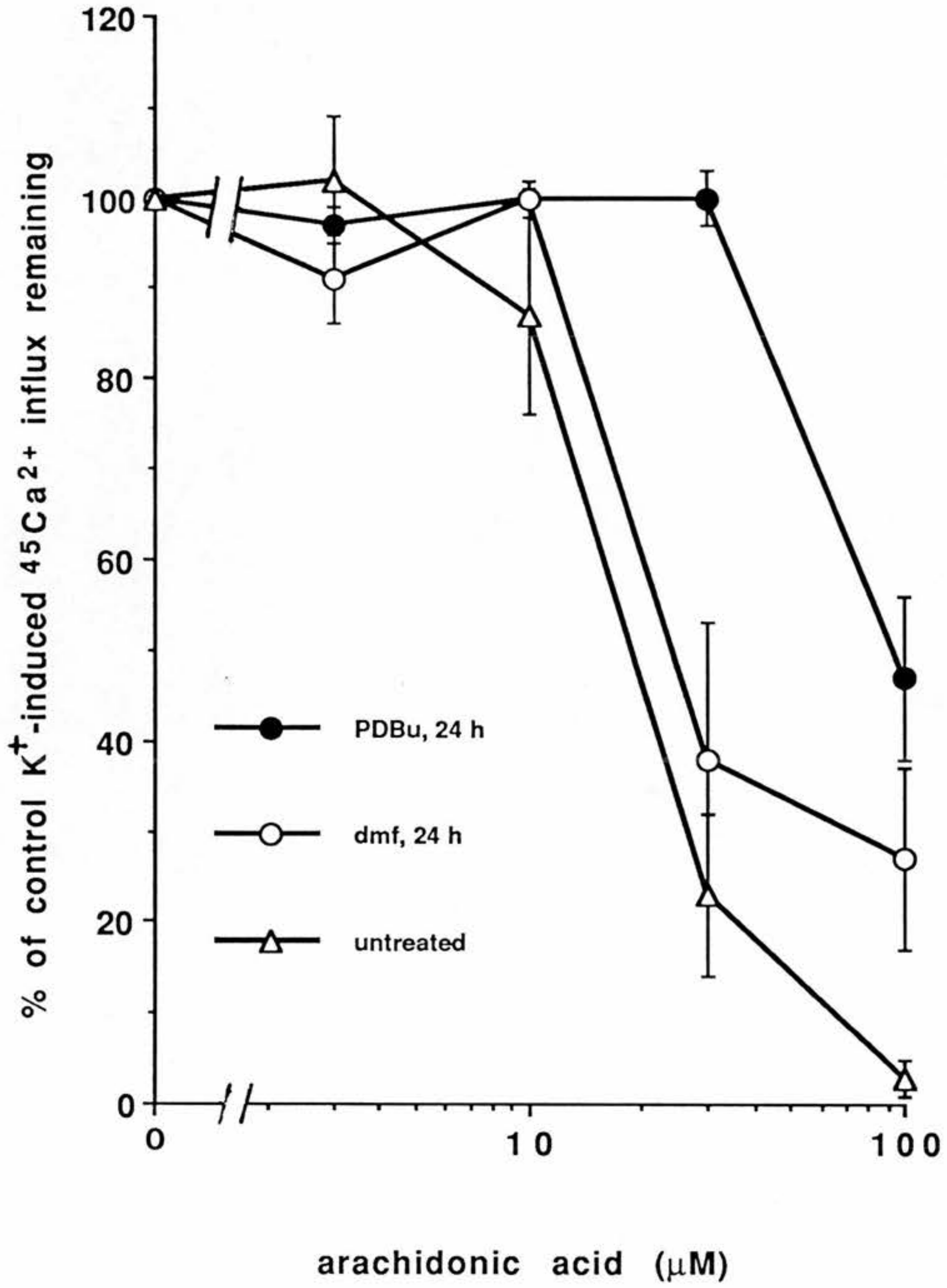


Figure 4.5. Effect of arachidonic acid on calcium efflux from GH_3 cells.

Measurement of $^{45}Ca^{2+}$ efflux was performed as described in the Methods section. Cells were batch-perfused in either the presence (triangles) or absence (filled circles) of 10 μM NDGA. Arachidonic acid (50 μM) in ethanol (or ethanol alone for control, 0.1% (open circles)) was added to the medium from 6 - 16 min where indicated by the line. Values represent the means ± SEM, n = 3.

Figure 4.5

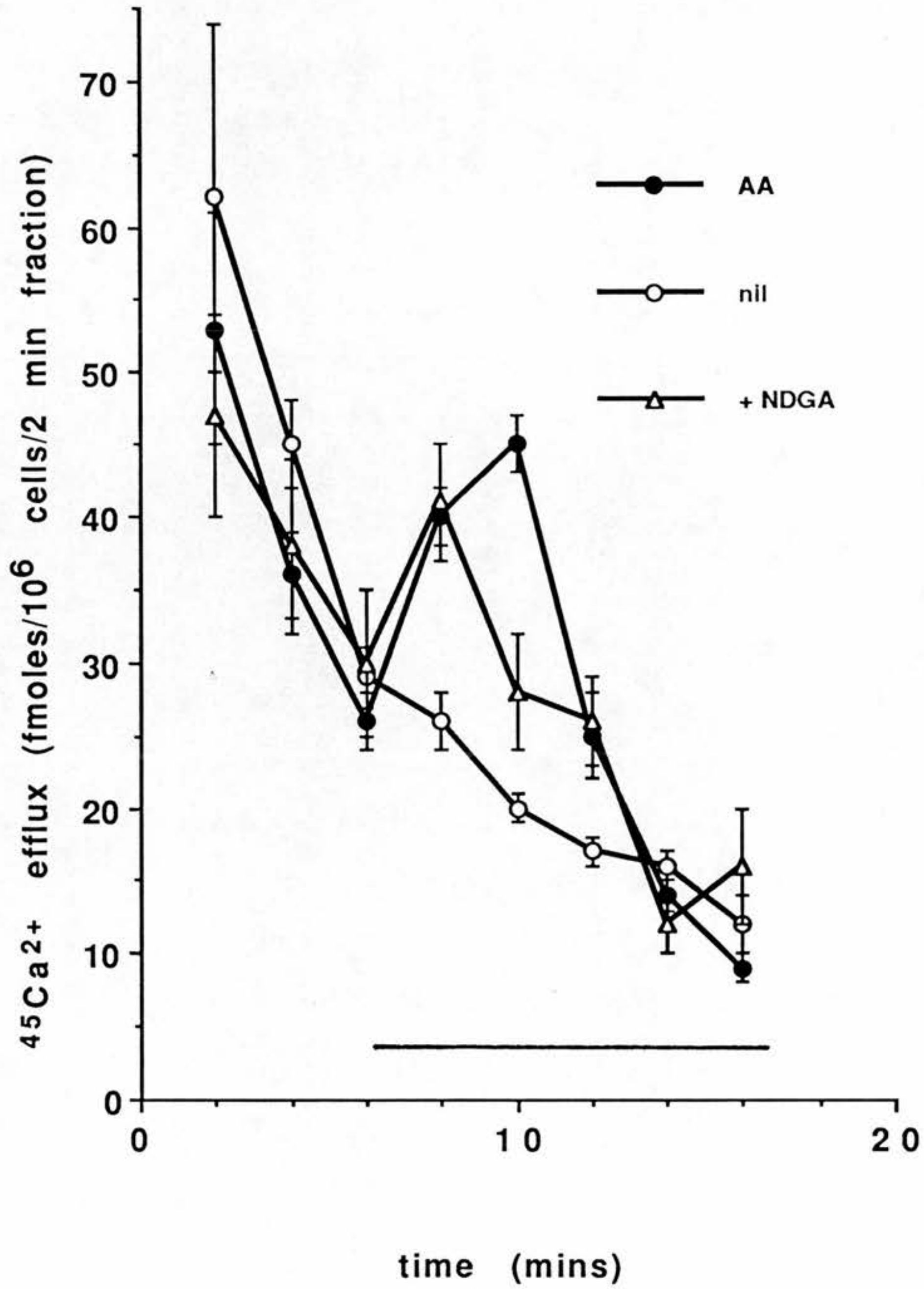
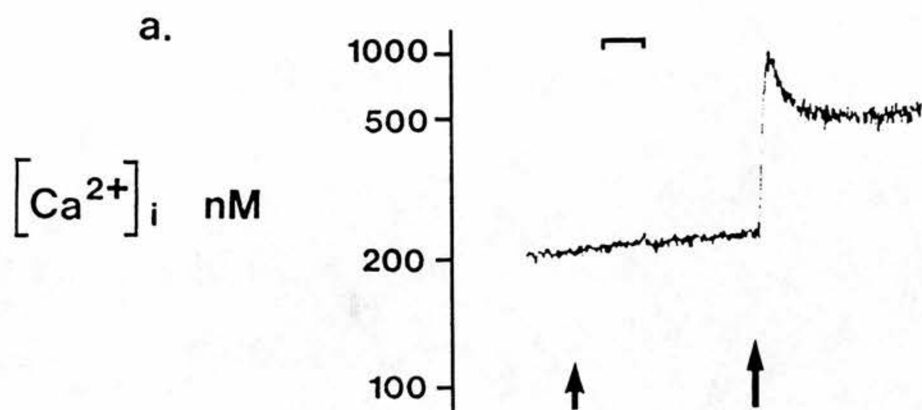


Figure 4.6. **Effect of arachidonic acid on the cytosolic calcium concentration in GH_3 cells.**

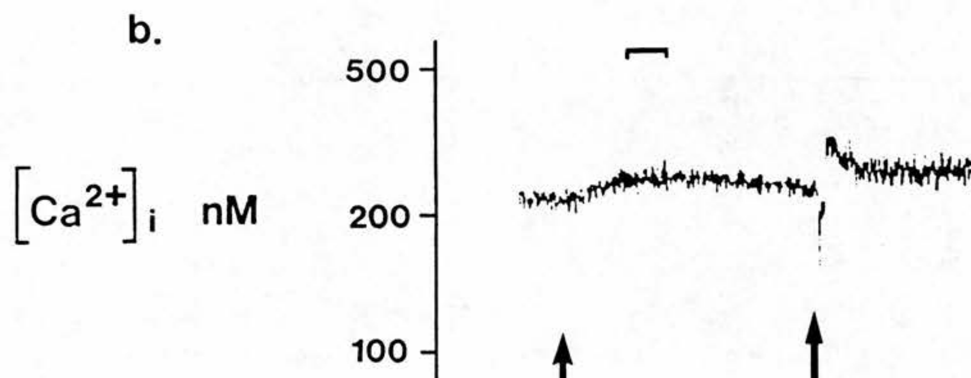
Cells were prepared and intracellular Ca^{2+} levels estimated by Indo-1 fluorescence as described in section 2.2.5. Arachidonic acid (AA, 17 μM) in ethanol (b and c) or ethanol alone (a) were added where indicated by the first (shorter) arrows. Ethanol at concentrations of up to 0.4% (as used) had no apparent effect on basal or K^+-induced increments in calcium concentrations. At the concentration used, AA produced no turbidity artefact in fluorescence records made in the absence of cells. At the second (longer) arrow, KCl was added to a final concentration of 40 mM. Addition of 40 mM NaCl rather than KCl had no effect. Part (a) illustrates a typical response to K^+ in the presence of ethanol, the vehicle for AA (0.4%) which had no effect alone at this concentration. In (b), AA induced a rise in basal Ca^{2+} levels and a diminution of the subsequent response to 40 mM K^+. In (c), when 30 μM H7 had been present from the start of the record, the AA-induced rise in basal Ca^{2+} levels was still present, but the diminution by AA of the response to K^+ was reversed. There was no apparent effect of H7 alone. Staurosporine could not be tested because of the unfavourable fluorescence spectrum of the compound. Each trace is representative of at least 3 similar results. The abscissa represents time with each scale bar indicating 1 min.

Figure 4.6

a.



b.



c.

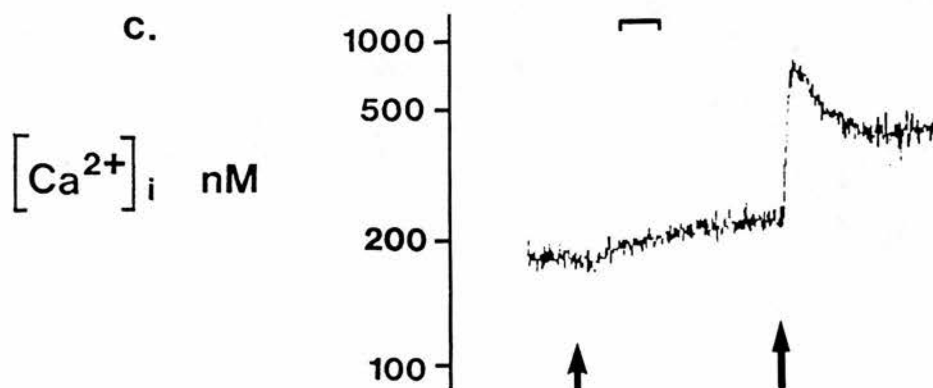
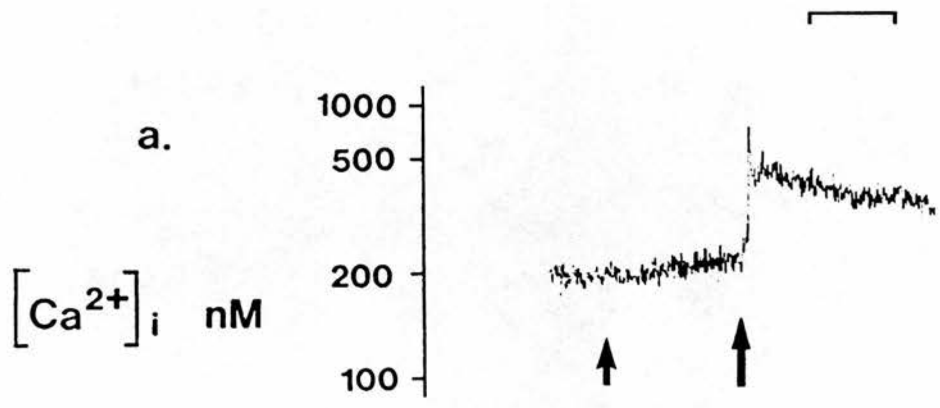
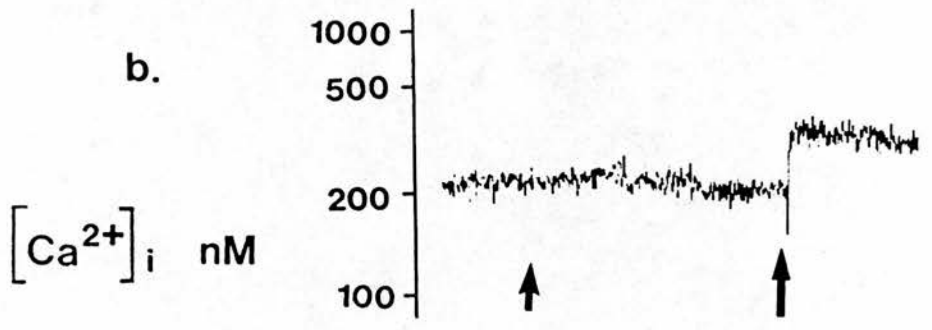


Figure 4.7. **Effect of PDBu on the cytosolic calcium concentration in GH_3 cells.**

Cells were prepared on intracellular Ca^{2+} levels estimated by Indo-1 fluorescence as described in section 2.2.5. The effect of 300 nM PDBu (b and c; shorter arrows) or 0.1% dimethylformamide (a; shorter arrow) was assessed on a subsequent stimulus of 40 mM K^+ (final concentration; longer arrows). The trace in (c) is similar to that in (b), except that 30 μM H7 was present in the cuvette, 2 min prior to the start of the trace. The scale bar indicates a time of 1 min. 300 nM PDBu dissolved in dimethylformamide (0.1% final) had no apparent fluorescent artefact in the absence of cells. The results are representative of three similar experiments.

Figure 4.7





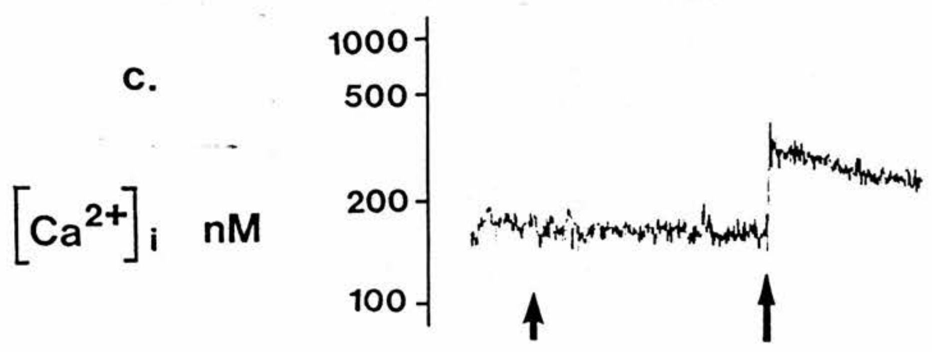
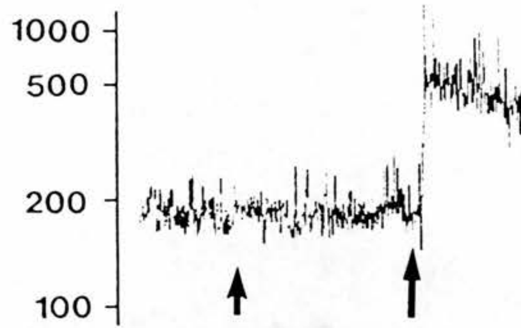


Figure 4.8. **Effect on the cytosolic calcium concentration of GH_3 cells of AA-methyl ester and of AA on PKC down-regulated GH_3 cells.** Cells were prepared and intracellular Ca^{2+} levels estimated by Indo-1 fluorescence as described in section 2.2.5. Treatment of GH_3 cells to down-regulate PKC levels was as described in section 5.2, with preparation of cells for fluorometric studies as normal from culture flasks of PKC down-regulated GH_3 cells. The effect of 17 μM AA-methyl ester (b; shorter arrow) or 0.4% ethanol (a; shorter arrow) on a subsequent 40 mM K^+ (a and b; longer arrows) stimulus was investigated on GH_3 cells cytosolic Ca^{2+} concentration. In GH_3 cells that had their PKCs down-regulated by 24 h, 300 nM PDBu (c and d), the effect of 17 μM AA (d; shorter arrow) or 0.4% ethanol (c; shorter arrow) on a subsequent 40 mM K^+ (c and d; longer arrows) stimulus was also investigated. The time scale is the same as Figure 4.8. Addition of 17 μM AA or AA-methyl ester dissolved in 0.4% ethanol (final concentration) had no apparent fluorescence artefact in the absence of cells. The traces in (a) and (b) are representative of three similar experiments, whereas the recordings in (c) and (d) are typical of one other experiment.

Figure 4.8

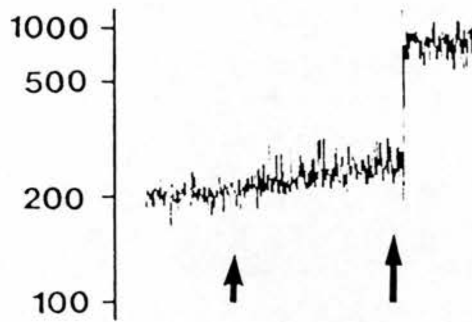
a.

$[Ca^{2+}]_i$ nM



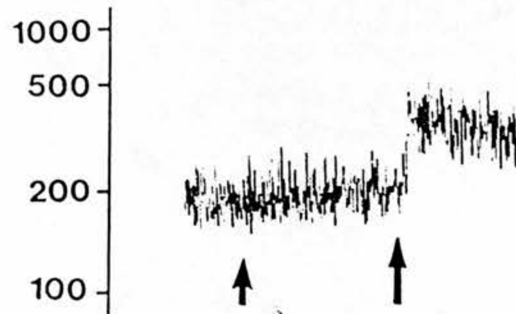
b.

$[Ca^{2+}]_i$ nM



c.

$[Ca^{2+}]_i$ nM



d.

$[Ca^{2+}]_i$ nM

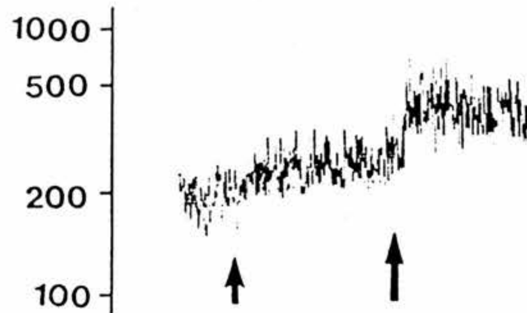


Table 4.1. Time course of inhibition of depolarisation-induced calcium influx by arachidonic acid (AA) into GH_3 cells.

Values represent means ± SEM, n = 4 - 6. The total $^{45}Ca^{2+}$ accumulated in the presence of 60 mM K^+ was typically around 1600 dpm per assay, whereas basal $^{45}Ca^{2+}$ accumulation was around 600 dpm, of which non-specific adsorbtion to the filter and cell surfaces (determined in zero-time blanks) was responsible for around 400 dpm. $^{45}Ca^{2+}$ accumulation was measured over 30 s and the incubation times with AA are inclusive of the 30 s influx measurement period. Incubations were at 37°C and $^{45}Ca^{2+}$ influx was stopped as described in the Methods section. Statistically significant inhibition of K^+-induced $^{45}Ca^{2+}$ influx occurred with 30, 45 and 90 s AA-incubation ($P < 0.05$).

Table 4.1

Conditions	$^{45}Ca^{2+}$ accumulation (fmoles/10^6cells/min)
non-specific adsorbtion (zero-time blank)	34 ± 2
basal	50 ± 4
basal, 100 μM AA (10 min)	54 ± 6
60 mM K^+	120 ± 8
60 mM K^+, 30 μM AA (30 s)	86 ± 6
60 mM K^+, 30 μM AA (45 s)	64 ± 3
60 mM K^+, 30 μM AA (90 s)	52 ± 3

Table 4.2. **Effect of inhibitors of arachidonic acid metabolism on its inhibition of depolarisation-induced calcium influx.**

GH_3 cells were exposed to 30 μM AA alone, or in combination with 10 μM ETYA, 30 μM NDGA, 10 μM SKF 525A or 30 μM piperonyl butoxide for 10 min before and during addition of 60 mM K^+ plus $^{45}Ca^{2+}$ medium. None of these drugs caused any significant alteration in the inhibition of K^+-induced $^{45}Ca^{2+}$ accumulation due to 30 μM AA or had any apparent effect on basal K^+-induced $^{45}Ca^{2+}$ influx alone. Values represent the means ± SEM, n = 4 - 8.

Table 4.2

Drug concentration	% of control K^+-induced $^{45}Ca^{2+}$ influx remaining
60 mM K^+ medium alone	100%
+ 30 μM arachidonic acid	23 ± 9%
+ 30 μM arachidonic acid + 10 μM ETYA	23 ± 4%
+ 30 μM arachidonic acid + 30 μM NDGA	35 ± 8%
+ 30 μM arachidonic acid + 10 μM SKF 525A	26 ± 3%
+ 30 μM arachidonic acid + 30 μM piperonyl butoxide	29 ± 5%

Table 4.3. **Effect of inhibitors of calcium extrusion processes on ability of arachidonic acid to reduce depolarisation-induced calcium influx into GH_3 cells.**

GH_3 cells were harvested and then resuspended in either normal 'calcium uptake medium' with or without 1 mM Na_3VO_4 or sodium-free 'calcium uptake medium' with N-methyl d-glucamine replacing sodium (154 mM). Cells were incubated with or without 100 μM arachidonic acid for 10 min before exposure to 60 mM K^+ and $^{45}Ca^{2+}$ medium as described in the Methods section. Values represent the means ± SEM, n = 4. Neither K^+-induced $^{45}Ca^{2+}$ nor the inhibition of that response by AA were significantly altered by Na_3VO_4 or N-methyl d-glucamine.

Table 4.3

Conditions	% of control K^+-induced $^{45}Ca^{2+}$ influx remaining
60 mM K^+	100%
60 mM K^+, N-methyl d-glucamine + Na_3VO_4	130 ± 8%
60 mM K^+, 100 μM AA	8 ± 6%
60 mM K^+, 100 μM AA, N-methyl d-glucamine	30 ± 6%
60 mM K^+, 100 μM AA, Na_3VO_4	16 ± 2%

Table 4.4. **Inability of 8-Br cyclic GMP to mimic arachidonic acid at inhibiting K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells.**

Measurement of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cell populations was performed as described in the Methods section. Various concentrations of 8-Br cyclic GMP were preincubated (37°C) for 10 min before exposure of the GH_3 cells to high (60 mM) K^+ and $^{45}Ca^{2+}$. The data represent the means ± SEM of 4 determinations.

Table 4.4

Treatment	% of control K^+-induced $^{45}Ca^{2+}$ influx
K^+ (60 mM)	100%
+ 8-Br cyclic GMP (3 μM)	110 ± 6%
+ 8-Br cyclic GMP (10 μM)	114 ± 4%
+ 8-Br cyclic GMP (30 μM)	112 ± 8%
+ 8-Br cyclic GMP (100 μM)	110 ± 11%

Table 4.5. **Effect of various agents on $^{45}Ca^{2+}$ efflux from GH_3 cells.**

The increase in rates of efflux over basal, induced by 30 μM ionomycin, is defined as 100%. Arachidonic acid was dissolved in ethanol, H7 and Na_3VO_4 in distilled water, and all other compounds in dimethylformamide (0.1% final), and 0.1% solvent alone showed no change in the rate of $^{45}Ca^{2+}$ efflux from GH_3 cells. Inhibitors were present for 6 min before exposure of the GH_3 cells to AA and the total rise in efflux rates over each of the remaining fractions (not at a single time point) is used to calculate the efflux as a % of ionomycin-induced efflux. The data represent means ± SEM of 3 determinations.

Table 4.5

Addition	$^{45}Ca^{2+}$ Efflux (% of ionomycin-induced efflux)
ionomycin (30 μM)	100%
basal	0%
PDBu (300 nM)	2 ± 4%
AA (50 μM)	36 ± 3%
AA + Na^+-free/N-methyl d-glucamine (154 mM)	37 ± 4%
AA + Na_3VO_4 (1 mM)	0 ± 4%
AA + NDGA (10 μM)	25 ± 5%
AA + indomethacin (10 μM)	35 ± 2%
AA + ETYA (10 μM)	33 ± 3%
AA + piperonyl butoxide (10 μM)	36 ± 6%
AA + SKF 525A (10 μM)	33 ± 4%
AA + H7 (30 μM)	38 ± 2%

Table 4.6. **Effect of arachidonic acid on calcium influx into GH_3 cells induced by 60 mM K^+, N-methyl TRH and ionomycin.**

GH_3 cell $^{45}Ca^{2+}$ influx was measured as described in the Methods section. Cells were preincubated with 50 μM arachidonic acid 10 min before exposure to either 60 mM K^+-, 1 μM N-methyl TRH- or 30 μM ionomycin-containing $^{45}Ca^{2+}$ 'calcium uptake medium' for 30 s at 37°C. Values represent the means ± SEM, n = 4 - 12. The effect of AA on the response to K^+, but not ionomycin or N-methyl TRH was statistically significant ($P \leq 0.05$).

Table 4.6

Stimulus	$^{45}Ca^{2+}$ accumulation (fmoles/10^6cells/min)		% inhibition of stimulus-evoked increment in $^{45}Ca^{2+}$ influx by 50 μM AA
	-AA	+50 μM AA	
nil	50 ± 10	49 ± 15	–
60 mM KCl	120 ± 18	52 ± 18	97 ± 16%
1 μM N-methyl TRH	72± 6	66 ± 4	23 ± 11%
30 μM ionomycin	230 ± 28	198 ± 19	17 ±10%

Table 4.7. **Effect of N-methyl TRH and ionomycin on the liberation of [^{3}H]-arachidonic acid from preloaded GH_3 cells.**

GH_3 cells grown on multiwell plates were loaded with [^{3}H]-AA and assayed for [^{3}H]-AA liberation as described in section 2.2. The amount of [^{3}H]-AA and its metabolites were detected under basal (< 1% dimethylformamide), N-methyl TRH (1 μM)-stimulated or ionomycin (30 μM, dissolved in dimethylformamide)-stimulated conditions. The levels of ionomycin-induced liberation of both [^{3}H]-AA and [^{3}H]-AA metabolites were significantly ($P < 0.05$) raised above basal liberation levels. The data represent the means ± SEM, with the number of determinations for each result in parentheses.

Table 4.7

	$[^3H]$-AA liberated (% of basal release)	
Treatment	**$[^3H]$-AA**	**$[^3H]$-AA metabolites**
basal	100% (12)	100% (12)
N-methyl TRH (1 μM)	138 ± 24% (11)	110 ± 15% (11)
ionomycin (30 μM)	195 ± 29% (8)	151 ± 21% (9)

4.4. DISCUSSION

Arachidonic acid and its metabolites have a wide range of biological actions (see section 1.2.4). One such action of arachidonic acid and some of its metabolites is to activate PKC. An initial report by McPhail *et al* (1985) showed that AA could activate PKC, and implied a function for AA in signal transduction mechanisms which involved PKC enzyme activity. This initial report was supported by others who found that AA and certain of its metabolites could also activate partially-purified PKC from brain (Murakami and Routtenberg, 1985; Murakami *et al,* 1986; Seifert *et al,* 1988). However more information has recently been provided with respect to highly-purified isoforms of PKC and the extent to which each of these isoforms (or at least the A series which are the only series investigated so far) is activated by AA (Sekiguchi *et al,* 1987; Naor *et al,* 1988a; Shearman *et al,* 1989a; Sekiguchi *et al,* 1988; Burns *et al,* 1990; Shinomura *et al,* 1991). Although slight differences exist between these reports, it is the general consensus that AA more potently activates γ-PKC than α- or ß-PKC and its activation of γ-PKC is biphasic (greatest activation at around 12 μM AA) in the absence or presence of Ca^{2+} ions. Activation of α- and ß-PKC by AA is reported to be concentration-dependent and occurs only in the presence of Ca^{2+} ions. The α-isoform of PKC is generally better activated by AA than the ß-isoform. All of these studies were carried out on partially purified PKCs and to our knowledge, no physiological correlate of AA activation of PKC has been shown, although the importance of such an event has been anticipated (Nishizuka, 1988; Shinomura *et al,* 1991).

Arachidonic acid has no clear effect on K-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary pieces but fully inhibited K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells (see Figure 3.7). Since AA is known to selectively activate certain PKC isoforms (as described above) and an enhancement and inhibition of 'L'-channel function in anterior pituitary prisms and GH_3 cells respectively could indicate the involvement of distinct forms of PKC, AA might be selectively activating those isoforms which predominate in

GH_3 cells, and are involved in an inhibition of 'L'-type Ca^{2+} channel activity rather than PKC-mediated enhancement of 'L'-channel activity, as is usually seen in anterior pituitary prisms. However, unlike PDBu which only produces a maximal inhibition of K^+-induced $^{45}Ca^{2+}$ influx of around 50% (see Figure 3.3), AA can fully inhibit K^+-induced $^{45}Ca^{2+}$ influx. The involvement of PKC in the AA-induced inhibition of $^{45}Ca^{2+}$ influx into GH_3 cells was investigated in this chapter as was the mechanism by which AA can modulate K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells in a manner which was greater than the maximal inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells produced by PDBu.

Arachidonic acid has been reported to activate guanylate cyclase and therefore increase cellular cyclic GMP levels (Glass *et al,* 1977). Raised cyclic GMP levels would activate cyclic GMP-dependent protein kinases which could enhance efflux mechanisms and therefore could account for the reduction in apparent $^{45}Ca^{2+}$ influx which is caused by AA. However, incubation of GH_3 cells with the cell-permeable analogue of cyclic GMP, 8-Bromo-cyclic GMP, had no effect up to 100 μM on the K^+-induced influx of $^{45}Ca^{2+}$ (Table 4.4). Therefore, the mechanism by which AA fully inhibits K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells is probably not activation of guanylate cyclase. It has been reported that AA (and some of its metabolites can inhibit Ca^{2+}/calmodulin-dependent kinase II (Piomelli *et al,* 1989) which perhaps accounts for the mechanism of AA in pituitary cells. However the lack of effect of calmodulin antagonists on K^+-induced $^{45}Ca^{2+}$ influx in anterior pituitary pieces (Johnson *et al,* 1990) and also in GH_3 cells (R Mitchell, unpublished observations) suggest that any such action does not contribute in a significant way to the inhibition of influx caused by AA here. The AA-induced inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx into GH_3 cells was reversed by the PKC inhibitors H7 and staurosporine, but not by their control analogues HA1004 and K252a respectively (see Figures 4.2 and 4.3). The pharmacology of the reversal of AA-induced inhibition (where the AA-induced response can be almost fully reversed by the PKC inhibitors), indicates a critical

involvement of PKC in the full inhibition of K^+-induced $^{45}Ca^{2+}$ entry seen with AA (ie, the inhibition of calcium entry occurs almost exclusively by a PKC-mediated process and not a process induced by AA which is additional to PKC activation). The protocol used here to down-regulate PKC levels has been previously shown by Ballester and Rosen (1985) to reduce PKC levels and PKC-mediated responses. Prolonged treatment of GH_3 cells with PDBu in order to down-regulate PKC levels resulted in a marked reduction in K^+-induced $^{45}Ca^{2+}$ influx. This reduction in 'L'-type Ca^{2+} channel function implicates a role for PKC in maintaining the 'L'-channel in a fully active state and these implications require further investigation. In GH_3 cells which had been so treated to down-regulate PKC, the critical involvement of PKC in the AA-induced $^{45}Ca^{2+}$ influx was again seen (see Figure 4.4), although again AA-mediated inhibition of K^+-induced $^{45}Ca^{2+}$ influx was still possible but with greatly reduced potency. This may be due to appreciable amounts of activatable PKC still being present after the PKC down-regulation process or may be due to the reduction of K^+-induced $^{45}Ca^{2+}$ influx by AA occurring through a process which is in some part independent of PKC activation. The latter possibility may be less convincing, as AA-inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells is fully reversed by PKC inhibitors (but not by their control analogues (see above)); furthermore, our present protocol would not deplete all PKC from GH_3 cells but only reduce PKC levels and its actions (Ballester and Rosen, 1985). Agents with some structural similarity to AA such as linoleic acid and AA-methyl ester, are rather poorer activators of PKC, than AA (Seifert *et al,* 1988). The structurally-related compounds could not appreciably mimic AA at inhibiting K^+-induced $^{45}Ca^{2+}$ influx (see Figure 4.1) further suggesting an effect of AA on PKC in GH_3 cells. The temporal pattern of the AA-induced inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx is not dissimilar to the temporal pattern seen with the PDBu-induced inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx into GH_3 cells. Likewise, the potencies for reversal by H7 and staurosporine of the effect of PDBu and AA match, again suggesting a role for PKC in the AA-response.

Inhibitors of AA metabolism were without effect on the AA-response (Table 4.2) indicating that it may be AA itself and not a metabolite which is responsible for the inhibition of K^+-induced $^{45}Ca^{2+}$ influx seen, although the specific involvement of metabolic products of AA cannot be ruled out on the basis of a few of metabolic inhibitors. Inhibitors of Ca^{2+}-extrusion processes were also without effect on the AA-response (Figure 4.3); with the slight apparent reversal of the AA-inhibition of K^+-induced $^{45}Ca^{2+}$ influx seen with N-methyl-d-glucamine and Na_3VO_4 probably reflecting a slowing in the extrusion of the radioactive calcium, which was also seen with the inhibitors in the absence of AA (Table 4.3). Therefore, the reduction of K^+-induced $^{45}Ca^{2+}$ influx seen with AA in GH_3 cells cannot simply be due to an enhancement of extrusion processes (or at least those investigated here) by AA in addition to the inhibition of 'L'-channel activity.

$^{45}Ca^{2+}$ efflux studies are a method for investigating the rate by which calcium is extruded (against its concentration gradient) from pre-loaded cells and is a useful method which can complement studies in which the entry of radioactive calcium under certain conditions is also assessed. Together, $^{45}Ca^{2+}$ influx and efflux studies provide a good overall picture of the calcium-flux mechanisms which occur in GH_3 cells but both have their disadvantages in that they require incubations with high specific activities of $^{45}Ca^{2+}$. Efflux studies are further hindered by the need for rather lengthy incubation times to allow sufficient labelling of exchanging intracellular calcium stores which can be deleterious to the viability of the tissue investigated. A methodology which has been developed more recently than radioactive ion flux measurements is the assessment of cytosolic calcium concentrations by use of newly-developed fluorescent Ca^{2+}-indication dyes such as Fura-2 and Indo-1. Both dyes are more powerful tools for investigating calcium-handling mechanism than their predecessor dye, Quin-2, and for reasons explained in section 2.2.5, Indo-1 was the preferred dye for use in GH_3 cells.

In the fluorimetric studies, AA raised basal cytosolic calcium concentrations in GH_3 cells. This finding was matched by the $^{45}Ca^{2+}$ efflux studies in GH_3 cells, in which AA induced an increase in the rate of preloaded $^{45}Ca^{2+}$ extruded from the cell. Presumably, the increased rate of calcium efflux reflects the rise in cytosolic calcium, which is seen in the fluorimetric studies, and the calcium extruded is simply the buffering mechanisms of the cells, extruding the raised concentration of calcium (some of which is radioactive). The effect of 50 μM AA on $^{45}Ca^{2+}$ efflux from GH_3 cells was to induce a increased rate of calcium efflux which was considerably less (about 36%) than the calcium efflux induced by 100 μM of the calcium ionophore ionomycin (see Figure 4.5 and Table 4.5), suggesting that AA acts to mobilise calcium from a distinct subset of stores within GH_3 cells. The efflux response to AA was completely prevented by Na_3VO_4 (Table 4.5), which is known to block Ca^{2+}-ATPase enzyme activity (DiPolo *et al,* 1979). The efflux response to AA was unaffected by Na^+-replacement with N-methyl-d-glucamine which blocks Na^+/Ca^{2+} exchanger mechanisms (Gill *et al,* 1981). Thus, the efflux of $^{45}Ca^{2+}$ induced by AA appears to be extruded specifically by Ca^{2+}-ATPase activity and not through a Na^+/Ca^{2+} exchanger mechanism of calcium extrusion. The AA-induced rise in $^{45}Ca^{2+}$ efflux rates was not blocked by inhibitors of its metabolism suggesting that it may be AA itself and not a metabolite which is responsible for the increased calcium efflux. The metabolic inhibitor, NDGA which inhibits both cyclo-oxygenase and lipogenase metabolic pathways, slightly inhibited the response to AA. However, the significance of this effect of NDGA was always in question as the inhibition is seen only at one time point and other cyclo-oxygenase and lipogenase inhibitors, indomethacin and ETYA respectively, were without effect. The effect of AA to raise $^{45}Ca^{2+}$ efflux rates from GH_3 cells appears to be independent of AA's ability to activate PKC, as PDBu was unable to mimic AA at inducing $^{45}Ca^{2+}$ efflux. The response to AA was also unaffected by incubation of the cells with the PKC inhibitor H7, again suggesting the lack of PKC (or other kinases) in the AA-induced $^{45}Ca^{2+}$ efflux in GH_3 cells.

Similarly, in the cytosolic calcium concentration measurements, PDBu could not raise basal cytosolic calcium concentrations in GH_3 cells. Curiously, in the related cell line, GH_4C_1, Albert *et al* (1987) found that phorbol esters could raise cytosolic calcium levels, but the reasons for this discrepancy between GH_3 and GH_4C_1 cells are unknown. Both AA and AA-methyl ester were able to raise cytosolic calcium levels (see Figures 4.6 and 4.7) which is in agreement with other work in which Chan and Turk (1987) demonstrated that these fatty acids could release intracellular calcium stores. Prior addition of AA (but not AA-methyl ester) caused a diminution of the rise in calcium concentrations subsequently induced by 40 mM K^+ (not 60 mM as in influx studies due to a high stock concentration being necessary for fluorimetric studies). This reduction by AA (but not AA-methyl ester) of K^+-induced calcium influx, matches the $^{45}Ca^{2+}$ influx studies (Figure 4.1). It appears that, as discussed earlier, the reduction in calcium channel influx in response to depolarising-concentrations of K^+, is dependent on PKC, as the AA-induced reduction in K^+-evoked raised cytosolic calcium concentrations can be reversed by the PKC inhibitor H7 (see Figure 4.6). Incubation of GH_3 cells with PDBu will also reduce K^+-induced increases in cytosolic calcium, which can again be reversed by H7 (Figure 4.7), but AA-methyl ester (although raising cytosolic calcium to similar levels as AA)does not influence subsequent K^+-induced rises in cytosolic calcium (Figure 4.7). In accordance with this, GH_3 cells which had PKC levels down-regulated by prolonged PDBu treatment were still able to show rises in basal cytosolic calcium concentrations with AA and AA-methyl ester, but no diminution in the subsequent K^+-response. This is supportive of the contention that AA and AA-methyl ester raise cytosolic Ca^{2+} independent of PKC function, but that the AA-induced reduction in K^+-evoked increases in cytosolic calcium concentration may involve activation of PKC. The pharmacological differences seen here with (AA and AA-methyl ester match the differences in ability of these fatty acids to activate PKC with AA-methyl ester being inactive (Seifert *et al,* 1988)).

It therefore appears that AA is having at least two actions in GH_3 cells: (i) an ability to activate the PKC isoforms which are responsible for reducing 'L'-channel activity (at least α- and ß-isoforms, as discussed in section 3.4), and (ii) the ability of AA to raise cytosolic calcium levels. Activation of PKC can be achieved by PDBu but not AA-methyl ester, and raising cytosolic calcium concentrations can be achieved by AA-methyl ester but not PDBu. Arachidonic acid can achieve both PKC activation and raise cytosolic calcium levels. It appears that these two actions of AA are responsible for the full inhibition of K^+-induced $^{45}Ca^{2+}$ influx, as pre-incubation of GH_3 cells with both PDBu and AA-methyl ester (at concentrations which do not induce full inhibition individually) resulted in the full inhibition of K^+-induced $^{45}Ca^{2+}$ influx, thus fully mimicking the response of the cells to AA (see Figure 4.1). In conclusion, it appears that AA fully inhibits K^+-induced $^{45}Ca^{2+}$ influx into GH_3 by its ability both to activate the PKC isoforms mediating the reduction in calcium influx and by raising cytosolic calcium concentrations. As the isoforms responsible for a reduction in 'L'-channel activity are possibly the α- and ß-isoforms, then the ability of AA to increase cytosolic calcium ion concentrations in addition to activating α- and ß-PKC may lead to α- and ß-PKC (which are Ca^{2+}-dependent with respect to their activation) somehow becoming more active with AA than with PDBu. Treatment of GH_3 cells with PDBu does not raise cytosolic calcium concentrations, therefore the α- and ß-isoforms of PKC will be activated by PDBu under conditions of basal Ca^{2+}. However, given sufficient concentrations of the activator, basal cytosolic Ca^{2+} concentrations (in the order of 1 - 200 nM) may still be enough to maximally activate the α- and ß-isoforms of PKC and lead to their full activity (and perhaps a complete reduction in 'L'-channel activity as occurs with AA). For these reasons, a PKC activity assay was developed in which the activity of the enzyme was assessed under conditions of zero Ca^{2+}/EGTA (which is physiologically irrelevant), basal cytosolic Ca^{2+} concentrations (100 nM free Ca^{2+}) or stimulated cytosolic Ca^{2+} concentrations (100 μM free Ca^{2+}) (see section 6.2). The results indicate that PDBu-induced activity of PKC(s) from midbrain for any set

concentration of PDBu (eg, 300 nM) with basal cytosolic Ca^{2+} levels is considerably greater than the appropriate PKC activity in the presence of excess EGTA. Therefore, more physiologically-relevant information may be derived from assessing PKC activity at basal *in vivo* calcium concentrations rather than comparing the activity of the enzyme in excess EGTA conditions. Although basal calcium concentrations can induce enzyme activity with 300 nM PDBu, raised calcium concentrations elicit larger amounts of PKC activity with 300 nM PDBu which is consistent with AA being able to raise cytosolic Ca^{2+} concentrations and fully activate the PKCs which inhibit 'L'-channel function whereas PDBu alone can produce only a 50% maximal inhibition. Another factor which may contribute to the full inhibition of 'L'-channel influx seen with AA and not PDBu, is that AA may be more selectively activating the isoforms of PKC which induce an inhibition of 'L'-type Ca^{2+} channel activity rather than activating the isoform(s) of PKC which results in enhanced 'L'-type Ca^{2+} channel activity, as is usually seen in anterior pituitary prisms (but not markedly with AA, see Figure 3.7).

The activation of PKC by AA resulting in a reduced 'L'-type Ca^{2+} channel activity may be a process of physiological relevance. Although not statistically significant, stimulation of GH_3 cells with N-methyl TRH caused a release of $[^3H]$-AA (previously incorporated into GH_3 cell phospholipids) to be liberated into the bathing medium (see Table 4.7). Metabolites of AA could also be detected in the medium. Thus, N-methyl TRH may induce the generation of AA in GH_3 cells. N-methyl TRH-induced AA production might serve as a negative feedback mechanism by which the amount of Ca^{2+} influx is restricted once TRH receptors are activated. Activation of PKC is already known to reduce the TRH-induced influx of Ca^{2+} (Drummond, 1985) and since it has been recently reported that AA and diacylglycerol can synergise together to activate PKC (Shinomura *et al,* 1991) a functional role for AA to reduce Ca^{2+} influx (and enhance other PKC-directed events) seems more probable. The effect of the AA-activated PKC seems to be restricted mostly to 'L'-type Ca^{2+} channel influx, as AA only fully blocks K^+-stimulated $^{45}Ca^{2+}$ influx, but not ionomycin- or N-methyl

TRH-stimulated influx (see Table 4.6). The ineffectiveness of AA on ionomycin-induced $^{45}Ca^{2+}$ influx into GH_3 cells indicates that the AA effect is not mediated by enhanced Ca^{2+} efflux mechanisms (as discussed above) whereas the slight reduction in total N-methyl TRH-induced $^{45}Ca^{2+}$ influx may simply reflect the minor amount of TRH receptor-induced influx of calcium which may occur through 'L'-type Ca^{2+} channels (Mitchell *et al,* 1989). In this way, mechanisms may act to exert a significant amount of selectivity between different receptor-mediated responses, and result in a 'fine-tuning' of cellular function.

CHAPTER 5

ATTEMPTS TO CHARACTERISE PHORBOL DIBUTYRATE-BINDING TO DISTINCT PROTEIN KINASE C ISOFORMS

5.1. INTRODUCTION

1,2-Diacylglycerols are one of the endogenous activators of protein kinase C (PKC), whereas arachidonic acid (AA) is another more recently discovered endogenous activator of PKC (Nishizuka, 1988). The mechanism by which 1,2-diacylglycerols activate PKC is to bind to a specific binding site which is found on the enzyme. This 1,2-diacylglycerol-binding site is also the site on the enzyme for the interaction of the phorbol ester class of tumour promoters (Blumberg, 1988). Phorbol esters, such as phorbol 12,13-dibutyrate (PDBu) can bind with high affinity and specificity to PKC, and its binding to PKC is competitive with 1,2-diacyl-*sn*-glycerols (Nishizuka, 1984; Blumberg, 1988). More lipophilic phorbol esters such as phorbol 12-myristate 13-acetate (PMA) may be more potent at activating PKC than other less lipophilic analogues such as PDBu, but they also display more non-specific interactions with cell components other than PKC (eg, membranes (Blumberg, 1988)). For this reason, PDBu is the phorbol ester of choice in our studies.

Radioisotope-labelled PDBu is now the most commonly used radioligand for PKC-binding studies. For example, work in Blumberg's laboratories has extensively characterised [^{3}H]-PDBu binding to PKC. Some structural similarity between the 12- and 13-positions of phorbol esters and the 1- and 2-positions of 1,2-diacyl-*sn*-glycerols could account for a similar mechanism of action between both lipids (Nishizuka, 1984), however, the exact sites of structural similarity between 1,2-diacylglycerols and phorbol esters is not certain (Gschwendt *et al,* 1991). The mechanism by which 1,2-diacylglycerols displace [^{3}H]-PDBu binding was assessed to be of a competitive mechanism, and not one simply of 1,2-diacylglycerols simply perturbing the lipid environment that was required by binding activity (Sharkey and Blumberg, 1985). The stoichiometry of the co-factor-requirements of PKC *in vivo* was discerned by Bell and colleagues to be one Ca^{2+} ion to one diacylglycerol or phorbol ester molecule to 4 - 10 phosphatidylserine molecules for every molecule of

monomeric PKC (Hannun *et al,* 1986; Ganong *et al,* 1986; Hannun and Bell, 1990). The mechanism by which AA interacts with PKC is less certain. The interaction of AA with PKC has been suggested to be both competitive with 1,2-diacylglycerols (Leach and Blumberg, 1985), partly competitive with 1,2-diacylglycerols (Sharkey and Blumberg, 1985) or competitive with phosphatidylserine, not 1,2-diacylglycerols (Murakami and Routtenberg, 1985). Preparations of cell membranes were for a time the only available method to study PKC, as specific [^{3}H]-PDBu binding to cytosolic tissue was always low. In 1983, Leach *et al* described a method of presenting phosphatidylserine vesicles to cytosolic PKC, which enabled marked specific [^{3}H]-PDBu binding to occur. It was, therefore, necessary for PKC to be surrounded by a phospholipid environment in order for its ability to specifically bind phorbol esters (and presumably 1,2-diacylglycerols).

In this chapter, we have adopted the methodology of Leach *et al* (1983) to investigate the interaction of PKC with the putative PKC isoform-selective agents which were investigated on K^+-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary and GH_3 cells (Chapter 3).

5.2. SPECIFIC METHODOLOGY

Specific [^{3}H]-PDBu to membranous and cytosolic PKC was performed as described in section 2.2.7, with the exception of studies on the Ca^{2+}-dependency of [^{3}H]-PDBu binding in Table 5.3 and Figure 5.13, which were as follows. Cytosol was prepared by homogenisation of tissue in 2 vol of ice-cold 'Kuo' homogenisation buffer (20 mM Tris HCl (pH 7.5), 50 mM 20-mercaptoethanol, 2 mM EDTA plus 1 mM phenylmethylsulfonyl fluoride). The homogenate was then spun (16,000 g, 20 min, 4°C). The pellet was discarded and the supernatant was spun again (16,000 g, 5 min, 4°C). The supernatant from the second spin was considered as cytosol and stored on ice. Cytosolic [^{3}H]-PDBu binding was assessed in the presence of 300 μg/ml (final) phosphatidylserine micelles reconstituted in 20 mM Tris HCl (pH 7.5)

supplemented with 0.5 mg/ml bovine serum albumin (essential fatty acid-free) and 5 nM [^{3}H]-PDBu (approximately 0.03 μCi/tube). Assay tubes were supplemented with either 1 mM $CaCl_2$ or 5 mM EGTA (final concentrations). In some assay tubes, DTPA (0.5 mM final) may have been additionally present with the Ca^{2+} or EGTA. Total and non-specific binding was determined in the presence of dimethylformamide (1% final) alone or in the presence of 20 μM PDBu dissolved in dimethylformamide respectively. The cytosol (25 μl) was added to the tubes (final volume 250 μl) which were then capped, vortexed and incubated for 30 min at 37°C. Precipitation of cytosolic proteins with bovine -γ-globulin and polyethylene glycol 8,000, centrifugation and aspiration was performed as described in the Methods section (2.2.7), which accounts the usual specific cytosolic [^{3}H]-PDBu methodology. This method of [^{3}H]-PDBu binding, which attempted to determine the Ca^{2+}-dependency of specific [^{3}H]-PDBu binding to PKC, was termed the 'Kuo' method of [^{3}H]-PDBu binding.

Phosphatidylserine was dissolved in chloroform, dried under a stream of N_2 and reconstituted into the appropriate buffer using a Ystral high frequency homogeniser (3 x 30 s, setting 3). Various compounds were added to the assay dissolved in dimethylformamide (< 1% final) unless otherwise indicated (H7 was added to the assay dissolved in distilled water). Compounds such as arachidonic acid and long acyl chain diglycerides were added to the assay with phosphatidylserine in the mixed micellar method described in section 2.2.7.

5.3. RESULTS

Effects of phorbol ester-analogues on [^{3}H]-PDBu binding

Rat lung, frontal cerebral cortex and cerebellum are reported to be tissues relatively enriched in α-,ß- and γ-PKC respectively (73, 63 and 52% of PKC isoform-content (Shearman *et al,* 1988)). In an attempt to characterise any pharmacological differences of various compounds at this binding site, the binding of [^{3}H]-PDBu in the three tissues was used to estimate the properties of α, ß and γ isoforms of PKC, and

the effects of a number of activators of PKC on the specific [^{3}H]-PDBu binding was so investigated. Initial experiments were carried out measuring specific binding of [^{3}H]-PDBu in membranes from male rat frontal cerebral cortex and cerebellum. In these tissues, specific [^{3}H]-PDBu binding was displaced by PDBu (1 to 200 nM) in a concentration-dependent manner, resulting in calculated IC_{50} = 22 ± 2 and 19 ± 4 nM respectively (n = 3; Figure 5.2). In view of the interesting results in the $^{45}Ca^{2+}$ influx studies with anterior pituitary and GH_3 cells and of their possible PKC isoform-selective nature, the effect of mezerein and MPMA were investigated on [^{3}H]-PDBu binding in frontal cerebral cortex and lung membranes. The compounds displaced specific [^{3}H]-PDBu in a concentration-dependent manner giving IC_{50} concentrations of 8.7 ± 1.3 and 207 ± 10 nM respectively in frontal cerebral cortex (n = 3). Therefore, these phorboid analogues displaced PDBu binding to PKC in frontal cerebral cortex membranes, as expected, because of their structural similarities. Specific binding of [^{3}H]-PDBu binding to rat lung membrane was too low to determine accurately a binding displacement value for PDBu (n = 3), therefore, liver and spleen (tissue enriched in α-(69%) and ß-(67%)PKC) membranes were tested, but specific [^{3}H]-PDBu binding to the membrane fractions of these membraneous tissues was also too low (n = 3; Figure 5.1) to be of analytical use. It was at this point that all experimental efforts for investigating PDBu binding were transferred to studies on specific [^{3}H]-PDBu binding from the cytosol of various tissues with known PKC-isoform content (Shearman *et al,* 1988). The move was successful, as not only was there signal available from frontal cerebral cortex and cerebellum cytosols, but sufficient specific [^{3}H]-PDBu was accessible in lung cytosol (Figure 5.3), although higher tissue concentrations of lung cytosol were required. Specific [^{3}H]-PDBu binding in lung, frontal cerebral cortex and cerebellum cytosols was displaced by PDBu (1 - 200 nM) in a concentration-dependent fashion with similar potencies for all three tissues (IC_{50} = 6 ± 1, 5 ± 2 and 7 ± 1 nM respectively, n = 4) indicating that the sensitivity to PDBu in all three preparations was similar, potent and of the same

potency reported previously (Leach *et al,* 1985). Again, the phorbol ester analogues, mezerein and DPB (which showed interesting profiles of modulation in the anterior pituitary and GH_3 cell $^{45}Ca^{2+}$ influx studies) were investigated. In cytosol from male rat frontal cerebral cortex and lung, mezerein displaced specific [^{3}H]-PDBu with very high potency (IC_{50} = 0.6 ± 0.1 and 0.7 ± 0.1 nM respectively (n = 4)). As was seen with PDBu, mezerein was more potent at displacing [^{3}H]-PDBu binding in cytosolic than membrane fractions and therefore the shift in sensitivity to the agents is probably due to the altered assay conditions. No difference in affinity for PDBu binding displacement existed between frontal cerebral cortex and lung cytosol with either PDBu or mezerein. The similar affinities of mezerein in the [^{3}H]-PDBu binding displacement experiments in the two tissues tested could be due to several factors: (i) that the PKCs present in the two cytosols have no difference in affinity towards mezerein (or, indeed, PDBu) and that mezerein may possess altered efficacy towards some PKC-isoforms; (ii) the PKC(s) which are responsible for the mezerein-action in anterior pituitary cells are not present in frontal cerebral cortex and lung cytosols or (iii) the mezerein activity in anterior pituitary cells $^{45}Ca^{2+}$ influx studies, is not caused by different PKC-isoforms. For these reasons, any assessment of the possible PKC isoform-selectivity of mezerein would be better served by investigation in a PKC activity assay. The affinity of DPB was higher in lung cytosol than in frontal cerebral cortex cytosol (IC_{50}= 15 ± 1 and 44 ± 2 nM respectively (n = 6)). Thus a more DPB-sensitive PKC isoform(s) may be present in lung cytosol than in frontal cerebral cortex. However, mezerein showed a similar profile to DPB in $^{45}Ca^{2+}$ influx studies. As mezerein showed no difference in sensitivity in the binding studies and DPB was less promising than mezerein at being PKC isoform-selective in the $^{45}Ca^{2+}$ influx studies, further investigation of DPB on displacement of specific [^{3}H]-PDBu binding was abandoned.

Effect of a variety of protein kinase C activators and inhibitors on [^{3}H]-PDBu binding in rat frontal cerebral cortex, cerebellum and lung cytosol preparations

As certain lipid activators of PKC were shown to be PKC isoform-selective in the $^{45}Ca^{2+}$ influx and [^{3}H]-PDBu binding studies, the effects of various other PKC activators and inhibitors were investigated on cytosol from lung, frontal cerebral cortex and cerebellum (regions enriched in α-, ß- and γ-PKC respectively) to assess whether any were acting in a PKC isoform-selective manner. The PKC inhibitor gossypol (Nakadate *et al,* 1988) (10 - 250 μM) caused a concentration-dependent displacement of specific cytosolic [^{3}H]-PDBu binding in rat frontal cerebral cortex, cerebellum and lung cytosols with IC_{50}s of 120 ± 12, 131 ± 12 and 150 ± 27 μM respectively (n = 3; Figure 5.5). In rat lung, frontal cerebral cortex and cerebellum cytosols, the inhibitor sphingosine (Nakadate *et al,* 1988, 10 - 250 μM) displaced phorbol ester binding in a concentration-dependent manner, with IC_{50} values of 206 ± 21, 180 ± 8 and 192 ± 7 μM respectively (n = 4; Figure 5.5). The active isomer of the PKC activator (-)-indolactam-V (Fujiki *et al,* 1984) at concentrations of 3 - 100 μM in lung, frontal cerebral cortex and cerebellum cytosols, displaced specific cytosolic [^{3}H]-PDBu binding to 20 ± 1, 24 ± 1, 20 ± 1% of control specific binding by 100 μM (-)-indolactam-V, with IC_{50}s = 0.2 ± 0.1, 0.2 ± 0.1 and 0.4 ± 0.2 μM respectively (n = 3; Figure 5.5). The (+) isomer of indolactam-V is the inactive enantiomer of the PKC activator. (+)-Indolactam-V from 3 - 100 μM could not produce 50% displacement of specific binding even at the highest concentration used. In lung, frontal cerebral cortex and cerebellum cytosols, (+)-indolactam-V at 100 μM inhibited specific [^{3}H]-PDBu binding to 91 ± 5, 97 ± 8 and 87 ± 10% of total binding respectively (n = 3; Figure 5.5). Therefore, the above PKC modulators do indeed seem to be directing their action towards the [^{3}H]-PDBu binding site on PKC, but none of them seem to be displaying any PKC isoform-selective characteristics in the tissues

tested here. A purported PKC inhibitor, Cremaphor EL (Zhao *et al,* 1989), at dilutions of 10^{-4} to 10^{-7} in dimethylformamide had little effect on cytosolic binding in frontal cerebral cortex, cerebellum and lung 90 ± 3, 94 ± 2 and 114 ± 5 of control specific [^{3}H]-PDBu binding levels at 10^{-4} dilution respectively (n = 3). 1-0-Hexadecyl-2-0-methyl-*rac*-glycerol (AMG-C16) is a diacylglycerol analogue which is reported to have PKC inhibitory actions (Kramer *et al,* 1989). In view of the PKC isoform-selective nature of the diacylglycerol, DOG, we investigated AMG-C16 to see if it acted as a PKC isoform-selective inhibitor. From 10 - 300 μM, AMG-C16 had a slight potentiatory effect on [^{3}H]-PDBu binding in lung and frontal cerebral cortex cytosols. At 300 μM AMG-C16, specific [^{3}H]-PDBu binding in lung, frontal cerebral cortex and cerebellum cytosol was 111 ± 8, 120 ± 7 and 95 ± 8% of control specific binding levels respectively (n = 3; Figure 5.4). The potentiatory effect of AMG-C16 was more evident in conditions of reduced ($^1/_{10}$th) phosphatidylserine in the micelles (from 1 mg/ml to 100 μg/ml). Under those conditions, AMG-C16 (10 - 100 μM) caused a concentration-dependent increase in specific [^{3}H]-PDBu binding to cytosol from lung, frontal cerebral cortex and cerebellum cytosols, reaching levels of 196 ± 6, 210 ± 25 and 181 ± 14% (n = 3; Figure 5.4(b)) of total specific binding levels with 100 μM AMG-C16 present. In the case of lung cytosol, 300 μM AMG-C16 continued to concentration-dependently enhance binding to levels of 245 ± 25% (n = 3) of control specific binding, whereas, in frontal cerebral cortex and cerebellum cytosol, 300 μM AMG-C16 started to displace the enhanced phorbol binding to levels of 205 ± 11 and 173 ± 21% (n = 3) of control specific ^{3}H-PDBu binding levels. Therefore, AMG-C16 seems to have a complex dual effect on cytosolic [^{3}H]-PDBu binding, with it being able to replace the role of phosphatidylserine in allowing cytosolic [^{3}H]-PDBu binding to occur; and displacing [^{3}H]-PDBu binding at higher concentrations. The latter observation is expected because of the structural analogy to diacylglycerols, however the displacement occurred in frontal cerebral cortex and cerebellum cytosols only and was not apparent in lung. This apparently tissue-specific effect may not be due to any

PKC isoform-selective influence, but simply that PKC from lung cytosol is more susceptible than frontal cerebral cortex and cerebellum cytosols to lipid enhancement of [^{3}H]-PDBu binding in the absence of phosphatidylserine (as seen with AA later in this section). 6-(N-decylamino)-4-hydroxymethylindole (DHI) is another reported PKC activator (Wender *et al,* 1988) which is designed around the presumed active configuration with diacylglycerols and phorbol esters. However, DHI from 20 - 600 μM only slightly displaced specific [^{3}H]-PDBu binding in a concentration-dependent fashion in lung, frontal cerebral cortex and cerebellum cytosols, where 600 μM DHI produced specific binding levels of 101 ± 4, 92 ± 9 and 94 ± 11% of control specific binding levels respectively (n = 3; Figure 5.4). The PKC inhibitors H7 (Hidaka *et al,* 1984) and staurosporine (Kase *et al,* 1987) were unable to displace specific [^{3}H]-PDBu binding (Figure 5.4). Both H7 (3 - 100 μM) and staurosporine (0.1 - 3 μM) could not displace specific cytosolic PDBu binding in lung cytosol (102 ± 9 and 94 ± 6%), frontal cerebral cortex cytosol (92 ± 5 and 102 ± 4%) or cerebellum cytosol (94 ± 7 and 99 ± 4% respectively at 100 μM H7 and 3 μM staurosporine (n = 3)). These results are not surprising, as both H7 and staurosporine are thought to interact with PKC, not at its [^{3}H]-PDBu binding site, but near to its ATP-binding site in the catalytic domain of the enzyme (Huang, 1989).

In summary, although there are a number of PKC activators and inhibitors which are reported to interact with specific [^{3}H]-PDBu binding sites, none of the inhibitors show any obvious isoform-selectivity on the binding studies here. As H7 is known to have actions which may be PKC isoform-selective, and no influence of H7 could be seen in the PDBu binding studies, then a PKC activity assay would perhaps be more fruitful in revealing any PKC isoform-selective agents.

Effect of diacylglycerol analogues on [^{3}H]-PDBu binding in rat frontal cerebral cortex, cerebellum and lung cytosol preparations

In the $^{45}Ca^{2+}$ influx studies into anterior pituitary pieces and GH_3 cells, 1,2-dioctanoyl-*sn*-glycerol mimicked PDBu at enhancing K^+-induced influx into anterior pituitary pieces but was ineffective at mimicking the action of PDBu in GH_3 cells. In order to assess any selective nature of DOG, the affinity of DOG to displace specific [^{3}H]-PDBu binding from cytosols with known PKC isoform-content was investigated. The *sn*-1,2-entantiomers of diacylglycerols are able to activate PKC (see section 1.2.1) with the *sn*-2,3- and 1,3-configurations being inactive. The *sn*-configuration is a system of characterisation of diglyceride analogues which stereospecifically numbers the acyl chains joined to a glycerol backbone (IUPAC-IUB Commission on Biochemical Nomenclature (1978) J Lipid Res **19:** 114-128), enabling a differentiation of stereoisomers (1,2-*sn* being the mirror-image of 2,3-*sn*). The *rac*-designation signifies that both stereoisomers are present in equal amounts (*X*-denoting an undefined stereoisomer). Both 1,2-dioctanoyl-*sn*-glycerol (DOG) and 1,2-dioctanoyl-*rac*-glycerol throughout the concentration range 0.5 - 500 μM displaced specific [^{3}H]-PDBu binding in frontal cerebral cortex, cerebellum and lung cytosols (Figure 5.7). Little difference could be seen when comparing the affinity of PDBu-displacement by 1,2-dioctanoyl-*sn*-glycerol and 1,2-dioctanoyl-*rac*-glycerol, with the *rac*-configuration being only slightly less potent. In frontal cerebral cortex cytosol, *sn*-DOG and *rac*-DOG displaced specific [^{3}H]-PDBu binding in a concentration-dependent manner with similar affinities (IC_{50} = 21 ± 1 and 25 ± 2 μM (n = 6 and 3) respectively). The same was true of the affinities of *sn*-DOG and *rac*-DOG in cerebellum cytosol (IC_{50} = 20 ± 2 and 25 ± 2 μM (n = 6 and 3) respectively). Interestingly, both the *sn*- and *rac*-configurations of DOG displaced [^{3}H]-PDBu binding in lung cytosol in a concentration-dependent fashion, but the affinities for displacement were significantly (($P \leq 0.05$) for *sn*-DOG) lower than those observed in

frontal cerebral cortex or cerebellum (estimated IC_{50}s in lung of 1722 ± 598 and 1894 ± 710 μM (n = 6 and 3) respectively). Therefore, consistent with theory, the *rac*-DOG mixture showed a slightly lower potency of action than the *sn*-DOG isomer alone. The large difference in affinity of DOG in lung and frontal cerebral cortex/cerebellum cytosols could be due to a PKC isoform-selective action of DOG which was reflected in the selective actions of DOG in the $^{45}Ca^{2+}$ influx studies. To test whether the tissue difference was consistent, we tested a range of diacylglycerol analogues with different acyl-chain composition on displacement of specific [^{3}H]-PDBu binding in lung, frontal cerebral cortex and cerebellum cytosols (Figure 5.6). 1,2-Dilauroyl-*rac*-glycerol did not dissolve fully at higher concentrations in dimethylformamide, which led to incomplete and erratic reversal of specific [^{3}H]-PDBu binding in cytosols from all three tissues (n = 2). Therefore, to test less hydrophilic diacylglycerol derivatives than DOG, a mixed micellar method of [^{3}H]-PDBu binding was developed. In this procedure, a range of diacylglycerol analogues were dissolved in chloroform, dried and reconstituted into micelles along with phosphatidylserine which was present throughout the assay. Various concentrations of diglycerides (or AA) were added to the assay in the mixed micellar method where the drug concentration was diluted by mixing with micelles which contained phosphatidylserine only. The binding characteristics of the diacylglycerols was investigated on [^{3}H]-PDBu binding using lung, frontal cerebral cortex and cerebellum cytosols. The 1,2-diacylglycerol analogues tested contained matched saturated acyl chains, with chain lengths from 6 - 18 carbons (C6:0 - C18:0) and a matched unsaturated acyl chain diglyceride, 1,2-dioleoyl-*sn*-glycerol (C18:1, cis9). Also tested was the mixed acyl chain diacylglycerol, 1-stearoyl-2-arachidonoyl-*sn*-glycerol (C18:0/20:4, [cis,cis,cis,cis]-5,8,11,14) which is reported to be a naturally occurring diacylglycerol analogue and is produced upon phosphoinositol-specific phospholipase C activity (Meldrum *et al*, 1991). The displacement of specific [^{3}H]-PDBu binding by the diacylglycerols with unsaturated acyl chains occurred with high potency in lung, frontal cerebral cortex and cerebellum cytosols, the IC_{50}s being similar

at 23 ± 6, 14 ± 2 and 11 ± 2 μM for 1,2-dioleoyl-*sn*-glycerol in the respective tissues (n = 4) and 6 ± 2, 4 ± 1 and 4 ± 1 μM for 1-stearoyl-2-arachidonoyl-*sn*-glycerol (n = 4). This result indicates that unsaturated chain diglycerides do not have selective affinity for the PKC in the tissues tested. However, saturated acyl chain diacylglycerol analogues showed generally lower potency in lung cytosol than in cytosol form rat frontal cerebral cortex and cerebellum. The IC_{50} values for displacement by the diacylglycerol analogues of specific [^{3}H]-PDBu binding in frontal cerebral cortex and cerebellum cytosols ranged from 17 ± 3 μM to 176 ± 19 μM (n = 4), whereas for the same diglyceride analogues, the IC_{50} values in lung cytosol ranged from 46 ± 9 μM to estimated IC_{50} values of 1354 ± 451 μM (n = 4). The potency of all the saturated acyl chain diacylglycerol analogues tested in lung cytosol, was markedly lower than that displayed in frontal cerebral cortex and cerebellum cytosols (the latter two cytosols consistently gave similar values). For the saturated analogues, there was a biphasic relationship between acyl chain length and affinity for the [^{3}H]-PDBu binding site; the optimal chain length being 14 carbons in all three tissues. This biphasic relationship was similar to that found previously (Ebeling *et al,* 1982; Hannun *et al,* 1986; Sekiguchi *et al,* 1988), where the biphasic relationship was attributed to the hydrophilicity of the acyl chain and to the optimal fit of the diacylglycerol-binding site within PKC. The difference in affinity between lung and frontal cerebral cortex/cerebellum cytosols was most marked with diacylglycerols containing acyl chain lengths of 6 - 10 carbons; DOG (8 carbons) showing the greatest separation in affinity. The reduced affinity for DOG in lung cytosol was not due to its degradation occurring in lung only, as DOG (0.5 - 500 μM) preincubated for 30 min (37°C) in lung, frontal cerebral cortex and cerebellum cytosols showed similar high affinities to displace [^{3}H]-PDBu binding when subsequently tested with lung, frontal cerebral cortex and cerebellum cytosols (Table 5.1).

As well as using lung, frontal cerebral cortex and cerebellum cytosols (Figure 5.7), the affinity of DOG (0.5 - 500 μM) (added to the assay dissolved in

dimethylformamide) for specific $[^3H]$-PDBu binding was investigated in cytosols from tissues with known PKC-isoform content (Figure 5.8). As $[^3H]$-PDBu binding was not fully displaced in some tissues, and IC_{50} concentrations for DOG were greater than the maximum concentration of DOG used, the data are expressed as the amount of specific $[^3H]$-PDBu binding remaining with 500 μM DOG, although full concentration response curves were always determined as far as possible. Cytosol from cerebellum, frontal cerebral cortex, spleen, spinal cord, testes, liver, lung, kidney, sciatic nerve and COS 7 cells have been reported to contain 14, 17, 32, 47, 48, 69, 73, 82, 93 and 100% α-isoform of PKC respectively (Shearman *et al,* 1988; Kosaka *et al,* 1988). Using the cytosol from these tissues, the amount of specific $[^3H]$-PDBu binding remaining at 500 μM DOG was determined to be 5 ± 2, 9 ± 2, 15 ± 1, 26 ± 3, 55 ± 4, 70 ± 2, 70 ± 3, 65 ± 2, 89 ± 6 and 70 ± 6% of control specific binding levels respectively (n = 6). An inverse relationship existed between the amount of $[^3H]$-PDBu binding displaced by 500 μM DOG and the reported proportion of α-PKC, suggesting that DOG has reduced affinity towards α-PKC. However, it is important to consider that the reported PKC isoform content of the tissues only takes into consideration the α, ß and γ isoforms of PKC, and not the more recently discovered δ, ε and ζ isoforms.

Effect of arachidonic acid on $[^3H]$-PDBu binding in rat cytosol preparations

Since AA was known to selectively activate certain forms of PKC (Naor *et al,* 1988a) and AA appeared to be acting as a selective activator of certain PKCs in the $^{45}Ca^{2+}$ influx experiments, we assessed its ability to interact with $[^3H]$-PDBu binding in regions enriched in different isoforms. Arachidonic acid (0.2 - 200 μM) added to the binding assay from concentrated stocks in ethanol and in the presence of phosphatidylserine did not displace specific $[^3H]$-PDBu binding from rat lung, frontal cerebral cortex and cerebellum cytosols, regions relatively enriched in α-, ß- and γ-

PKC respectively. The binding levels in the presence of phosphatidylserine for lung, frontal cerebral cortex and cerebellum cytosol with 200 μM AA were 109 ± 5, 100 ± 6 and 100 ± 6% of control specific binding levels (n = 3; Figure 5.9). Experiments were also carried out to assess the ability of AA (0.2 - 200 μM added to the assay dissolved in ethanol) to substitute for the role of phosphatidylserine, which is reportedly necessary to enable cytosolic PKC [^{3}H]-PDBu binding (Leach *et al,* 1983). The AA could not markedly substitute for the permissive role of phosphatidylserine in lung, frontal cerebral cortex or cerebellum cytosols, with [^{3}H]-PDBu binding levels with 200 μM AA of 24 ± 2, 8 ± 2 and 10 ± 3% of control specific binding levels in the presence of phosphatidylserine respectively (n = 4; Figure 5.11). It is interesting to note that the PKC from lung cytosol was somewhat more susceptible to AA here than the PKC from frontal cerebral cortex or cerebellum cytosols. As with experiments examining diacylglycerol effects on [^{3}H]-PDBu binding, solubility limitations of AA in ethanol encouraged us to employ a mixed micellar method of [^{3}H]-PDBu binding in order to investigate the effect of higher concentrations of AA (0.5 - 500 μM) here. The specific [^{3}H]-PDBu binding in lung cytosol was increased to levels of up to 159 ± 9% of control specific binding by AA and had not plateaued by the highest concentration used (n = 4; Figure 5.10). In contrast, the [^{3}H]-PDBu binding in frontal cerebral cortex and cerebellum cytosols was not significantly enhanced by 0.5 - 500 μM AA (109 ± 7 and 110 ± 2% of control specific binding levels with 500 μM AA respectively (n = 4)). Interestingly, lung cytosol has a higher content of α-PKC than does frontal cerebral cortex and cerebellum cytosols (Shearman *et al,* 1988), and thus cytosols from various tissues with known α-PKC content were used to assess whether any relationship existed between AA (0.5 - 500 μM)-induced enhancement of specific [^{3}H]-PDBu binding and the content of α-PKC in the tissue tested (Figure 5.12). A marked AA (500 μM)-induced enhancement of binding occurred not only in lung, but also with cytosols from kidney, sciatic nerve and COS 7 cells (139 ± 3, 238 ± 20 and 248 ± 17% of control specific binding levels respectively (n = 4)). Interestingly, the 4 tissues

which displayed an enhancement of [^{3}H]-PDBu binding by AA were the 4 tissues with the highest α-PKC content of the 10 tissues tested. However, no simple linear relationship existed between AA-induced enhancement of binding and α-PKC content (or ß- or γ-PKC content either); and once more, caution must be used, as only the α-, ß- and γ-PKC content of the tissues is considered, and not that of any other known PKC isoform.

The concentrations of AA at which enhancement of [^{3}H]-PDBu binding occurs are extremely high (typically 50 - 500 μM without plateauing) and are higher than those concentrations of AA needed in cellular responses involving PKC, for example the inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells (IC_{50} = 19 ± 3 μM). The specificity of the AA-induced enhancement of binding was then investigated. Cytosolic PKC from lung, frontal cerebral cortex and cerebellum, which had been partially purified using DE52 diethylaminoethyl cellulose, showed enhancements of specific [^{3}H]-PDBu binding with 500 μM as crude cytosols (mean % of control specific [^{3}H]-PDBu binding of 145, 113 and 108 respectively, n = 2). Fatty acids with similar structures to AA, such as arachidic acid, AA-methyl ester and linoleic acid, were tested on [^{3}H]-PDBu binding in lung, frontal cerebral cortex and cerebellum cytosols, to see whether they could mimic AA. In frontal cerebral cortex and cerebellum cytosols, the slight enhancement of binding with 500 μM AA (109 ± 7 and 110 ± 2% of control specific binding levels respectively) showed a similar trend with arachidic acid, AA-methyl ester and linoleic acid (all at 500 μM). Thus the slight enhancement was mimicked by all the AA analogues, indicating that the enhancement of [^{3}H]-PDBu binding in frontal cerebral cortex and cerebellum cytosols by AA may be due to its lipophilic actions. In contrast, the marked signal enhancement of binding with 500 μM AA in lung cytosol enabled us to assess quantitatively with reasonable accuracy, the ability of the AA-related lipids to mimic AA here. Linoleic acid (500 μM) was almost as good as AA at enhancing [^{3}H]-PDBu binding in lung cytosol (90 ± 7% of the effect of AA at the same concentration, n = 4. However, AA-methyl ester and arachidic acid

(both at 500 μM) either poorly mimicked or could not mimic the action of AA in lung (30 ± 12 and 2 ± 2% of the effect of AA at the same concentration, n = 4). These structural requirements matched those described by Seifert *et al* (1988) for activation of PKC from whole brain cytosol by analogues of AA. However, the concentrations of the fatty acids required to show an effect on lung cytosol binding are higher than those described by Seifert *et al* (1988) on PKC activity. The activation of PKC by AA and linoleic acid, but not arachidic acid and AA-methyl ester, recorded by Seifert *et al* (1988) was in experiments performed in the absence of phosphatidylserine. It is interesting that their PKC activation and the enhancement of lung cytosol [^{3}H]-PDBu binding here should display similar pharmacologies, but at different concentrations of phosphatidylserine, perhaps indicating an interaction of the phosphatidylserine and AA effects on PKC. Leach and Blumberg (1985) showed that AA displaced [^{3}H]-PDBu binding in whole mouse brain cytosol, but only at concentrations of phosphatidylserine that are extremely submaximal for [^{3}H]-PDBu binding (Leach *et al,* 1983), particularly 20 μg/ml. However, at 200 μg/ml phosphatidylserine, the AA-induced displacement of [^{3}H]-PDBu was markedly reduced (Leach and Blumberg, 1985). When we reduced the phosphatidylserine concentration in our [^{3}H]-PDBu binding assay from 1,000 to 750 μg/ml, we too observed a modest displacement of [^{3}H]-PDBu binding to lung, frontal cerebral cortex and cerebellum cytosols by AA (0.5 - 500 μM), but only at very high AA concentrations (82 ± 6, 65 ± 15 and 56 ± 15% of control specific binding levels with 500 μM AA respectively, n = 4 (Figure 5.11 (b))). Thus the AA-displacement of [^{3}H]-PDBu binding to cytosolic PKC is dependent upon phosphatidylserine concentration. Consistent with this idea was previous reports that AA could substitute for the permissive role of phosphatidylserine in PKC activation (Murakami and Routtenberg, 1985; Murakami *et al,* 1986). The interaction of AA, phosphatidylserine and PKC is obviously a complex one which requires more work to fully characterise; however, the fatty acid enhancement of [^{3}H]-PDBu binding seen

here in regions enriched in α-PKC (as well as the correlation of DOG displacement of binding and α-PKC content) may serve as a tool for predicting α-PKC content.

Effect of DOG and AA on specific [^{3}H]-PDBu binding to rat anterior pituitary and GH_3 cell cytosol and membrane preparations

The effects of the two agents (ie, AA and DOG which had shown selectivity between PKCs both in $^{45}Ca^{2+}$ influx experiments and in [^{3}H]-PDBu binding studies) were tested in the binding assay using anterior pituitary and GH_3 cell cytosols, in an attempt to estimate the relative content of α-PKC in these tissues. Arachidonic acid (0.5 - 500 μM) enhanced specific [^{3}H]-PDBu binding in both cytosols in a concentration-dependent manner. The level of enhancement of binding in anterior pituitary and GH_3 cell cytosols with 500 μM AA was 120 ± 7% and 141 ± 10% of control specific [^{3}H]-PDBu binding levels respectively (n = 4). Using the correlation for AA (500 μM)-induced enhancement of [^{3}H]-PDBu binding and cytosolic α-PKC content, this would suggest that anterior pituitary cytosol contains between 48 - 66% α-PKC (Table 5.2). This value is in agreement with that reported previously (Naor, 1990) of 59 ± 8% α-PKC in anterior pituitary cytosol, as assessed by immunoblot analysis with various PKC-isoform antibodies. Similarly, with GH_3 cell cytosol contains between 68 - 80% α-PKC, whereas Naor (1990) reported that GH_3 cell cytosol contained around 83% α-PKC. Displacement of specific [^{3}H]-PDBu binding in both anterior pituitary and GH_3 cells cytosols by DOG (0.5 - 500 μM) was concentration-dependent (IC_{50} = 112 ± 34 and 541 ± 89 μM respectively, n = 6). The amount of specific [^{3}H]-PDBu remaining at 500 μM DOG in anterior pituitary and GH_3 cell cytosols was 35 ± 6 and 60 ± 8% respectively. Using the % binding remaining at 500 μM DOG and α-PKC content correlation would suggest that anterior pituitary cytosol contained between 40 and 53% α-PKC, whereas in GH_3 cytosol, the amount of α-PKC present should be between 65 and 82%. The amount of α-PKC in anterior pituitary and GH_3 cell cytosol estimated by the DOG/α-PKC correlation thus match

those estimated values from the AA/α-PKC correlation, and are close to the relative amount of α-PKC detected by immunoblot analysis in the two cytosols as determined by Naor (1990). Therefore, the two pharmacological correlations presented here may be useful tools for assessing the α-PKC content of a tissue cytosol with unknown isoform content, although again it must be stressed that the reported PKC isoform contents only assess α-, ß- and γ-PKC isoforms. The evidence available from the literature suggests that both anterior pituitary and GH_3 cells (or the related strain, GH_4C_1 cells) contain no γ-PKC, but possess α-, ß- and ε-PKC with other isoforms unknown (Kiley *et al,* 1990). The approach to estimate PKC isoform content shown here may be a useful one with AA, since AA has only been shown to influence the A series of isoforms (γ and α in particular (Nishizuka, 1988)). However, there is no evidence that AA cannot influence any of the B series isoforms too. Attempts to derive proportional α-PKC content for correlating DOG effects are more fraught with difficulty as it seems most unlikely that diglycerides such as DOG would not interact with B series isoforms, and their complement in most tissues remains unknown.

Using membranes from anterior pituitary and GH_3 cells in [^{3}H]-PDBu binding studies revealed nothing informative about the relative isoform amount (partly due to the amount of specific [^{3}H]-PDBu binding being far lower than the levels found in cytosol). Arachidonic acid (0.5 - 500 μM, added to the assay dissolved in ethanol) did not markedly enhance specific [^{3}H]-PDBu binding levels in anterior pituitary or GH_3 cell membranes (115 ± 8 and 103 ± 2% of control specific binding levels respectively, n = 4). Unlike results with cytosols, anterior pituitary and GH_3 cells gave [^{3}H]-PDBu binding displacement by DOG (0.5 - 500 μM added to the assay dissolved in dimethylformamide) only at exceedingly low potency. It appears that the proportion of α-isoform in the membranes of these tissues (or at least the responsiveness of the [^{3}H]-PDBu binding sites to AA and DOG) differs greatly from that in cytosol. It could be that the membranes are extremely high in proportional α-PKC content, as suggested by the DOG response, but total [^{3}H]-PDBu binding levels were relatively low

compared to cytosol. What seems more likely, is that physicochemical or physiological factors may also be capable of influencing these properties, perhaps irrespective of isoform. These findings are consistent with the work of Bazzi and Nelsestuen (1988), who found that PKC markedly changes its regulatory characteristics once it had been inserted into the membrane.

Calcium-dependence of specific [^{3}H]-PDBu binding

The action of arachidonic acid (AA) to activate protein kinase C (PKC) subspecies may depend on the presence of Ca^{2+} ions. For example, γ-PKC is potently activated by AA in the absence or presence of Ca^{2+} (Naor *et al,* 1988a), whereas α (and to a lesser extent ß)-PKC are activated by AA only in the presence of Ca^{2+}. In the hope of resolving the isoforms of PKC into either Ca^{2+}-dependent (A series) or Ca^{2+}-independent (B series), the Ca^{2+}-dependency of specific cytosolic [^{3}H]-PDBu binding was investigated. Using cytosolic preparations as used in the phorbol ester binding studies above (and described in section 2.2.7), the calcium-dependency of [^{3}H]-PDBu binding was assessed by incubation in the presence of Ca^{2+} (1 mM final) or in the absence of Ca^{2+} (+ 5 mM EGTA final). Under these conditions, EGTA did not reduce the specific binding of [^{3}H]-PDBu binding to cytosolic PKCs from frontal cerebral cortex, cerebellum, spleen, testis, spinal cord or sciatic nerve cytosols with values for binding being 119 ± 4, 102 ± 9, 109 ± 4, 112 ± 5, 104 ± 9 and 100 ± 8, n = 3; Table 5.3) of total specific binding in the presence of Ca^{2+} (no EGTA) respectively. In some tissues (lung, kidney and liver), a marked enhancement apparently occurred with EGTA (166 ± 8, 204 ± 16 and 133 ± 10% of total binding in the presence of Ca^{2+} (n = 3; Table 5.3 and Figure 5.13(a))). The latter three tissues, which showed the most enhancement of [^{3}H]-PDBu binding with EGTA, were also the tissues with the highest incorporated erythrocyte content, as assessed by the reddish colour of the cytosolic preparation. With a view to assessing any possible effect of Fe^{3+} here, a heavy metal ion-chelator, DTPA (0.5 mM final) was included in binding assays for lung, frontal

cerebral cortex and kidney cytosols to assess any effect of heavy metal cations on the EGTA-induced enhancement of binding (Figure 5.13(a)). Under these conditions, EGTA enhancement of specific [^{3}H]-PDBu binding in lung, frontal cerebral cortex and kidney cytosolic preparations was similar to the values found in the absence of DTPA (156 ± 6, 112 ± 5 and 205 ± 19% (n = 3) of total specific binding in the presence of Ca^{2+}. In case free Mg^{2+} concentrations were being significantly depleted by DTPA, and since PKC activity requires Mg^{2+} (Nishizuka, 1984a), additional Mg^{2+} (5 mM final) was added to the binding assays of lung, frontal cerebral cortex and kidney cytosolic preparations, but this further addition of Mg as well as DTPA did not alter either the total binding levels or the enhancement by EGTA (n = 3). Slightly different methods have been used previously by other groups to show Ca^{2+}-dependence of [^{3}H]-PDBu to PKC (Ohno *et al,* 1988b; Kiley *et al,* 1990). We therefore used a modified technique on that of these workers so as to more directly highlight the question of Ca^{2+}-dependence of specific [^{3}H]-PDBu binding. Midbrain from male rats was homogenised in 'Kuo' buffer as described in section 5.2, and the supernatant from the procedure was regarded as cytosol. Employing this 'Kuo' method of binding for midbrain cytosolic PKCs, EGTA enhanced specific [^{3}H]-PDBu binding to levels of 129 ± 8% (n = 3; Figure 5.13(b)). In case of degradation of the binding site properties during freeze: thawing procedures, further experiments were carried out with freshly prepared rat material, where midbrain cytosolic PKCs were partially-purified. Cytosolic PKCs freshly obtained from male rat midbrain, were partially purified by an ammonium sulphate-'cut' between 21 - 45% ammonium sulphate. Using this cytosol, EGTA (5 mM final) was still found to enhance binding modestly, with levels of 127 ± 6% of control specific binding in the presence of Ca^{2+} (1 mM final) (n = 3; Figure 5.13). In other experiments, the PKCs from midbrain cytosolic preparation which had been frozen previously, were partially-purified by ion-exchange chromatography on a 1.5 ml DE-52 diethylaminoethyl cellulose column (a volume which in preliminary experiment, gave the highest yield of specific PDBu binding). A batchwise,

discontinuous elution was performed and after column washing, the 0 - 80 mM NaCl (in 'Kuo' buffer) fraction was taken to assay specific [^{3}H]-PDBu binding. Specific binding to aliquots of the DE-52 purified PKCs from midbrain cytosol accounted for around 600 dpm. EGTA (5 mM) reduced this specific [^{3}H]-PDBu binding to 93 ± 6% of control binding levels in the presence of 1 mM Ca^{2+} (n = 3; Figure 5.13(b)). Nor did the presence of the protease inhibitors, 0.01% leupeptin and 20 μM E-64 alter the levels of specific [^{3}H]-PDBu binding to 0 - 150 mM NaCl, DE52-purified midbrain cytosol under conditions of no added Ca^{2+}, low Ca^{2+}, high Ca^{2+} or excess EGTA (n = 3; Figure 5.13(c)), where rat midbrain was partially-purified on DE-52 (0 - 150 mM NaCl), having been prepared and assayed in the absence or presence of the Ca^{2+}-activated protease inhibitors, 0.01% leupeptin + 20 μM E-64. For equal amounts of initial tissue used, the yield of specific [^{3}H]-PDBu binding in both preparations was much higher (14250 and 34500 dpm binding in DE-52-purified preparations, without or with protease inhibitors respectively) when compared to cytosolic preparations that had been previously frozen (specific binding = 600 dpm). Despite the increased signal, the fresh midbrain cytosolic preparations still failed to show any Ca^{2+}-dependence of binding. In experiments using fresh unpurified cytosolic preparations from midbrain, specific binding levels in the presence of low Ca^{2+} (230 μM), high Ca^{2+} (1.4 mM) and EGTA (5 mM) were no different from binding in the absence of either Ca^{2+} or EGTA (101 ± 1, 99 ± 1 and 102 ± 1% of control binding respectively, n = 3). Similarly, when using cytosolic preparation from midbrain which had been partially purified with DE-52, specific binding levels did not change in the presence of low or high Ca^{2+} or EGTA (100 ± 1, 98 ± 1 and 100 ± 1% of control total binding levels in the absence of Ca^{2+} and EGTA respectively (n = 3; Figure 5.13(c))). Therefore, no Ca^{2+}-dependency of [^{3}H]-PDBu binding to the cytosolic preparations of PKCs, unlike the apparent Ca^{2+}-dependency of binding found by some workers (Ohno *et al,* 1988b; Kiley *et al,* 1990). However, the initial indications of an increase in binding with EGTA were eliminated by a convenient procedure which enabled us to partially-purify PKCs in a readily

standardised manner and which was subsequently used as routine in our PKC activity assay (see Chapter 6).

Figure 5.1. **Low levels of specific [^{3}H]-PDBu binding to membranes from non-neuronal tissues.**

Total and non-specific binding (nsb) of [^{3}H]-PDBu to membranes from frontal cerebral cortex (fc cortex), cerebellum, spleen, liver and lung were determined. The method of [^{3}H]-PDBu binding was that described in section 2.2.7. The final concentrations of membranes used in the assay were (mg/ml): frontal cerebral cortex (2); cerebellum (4); spleen (6); liver and lung (20). The data represents the means ± SEM of 3 determinations.

Figure 5.1

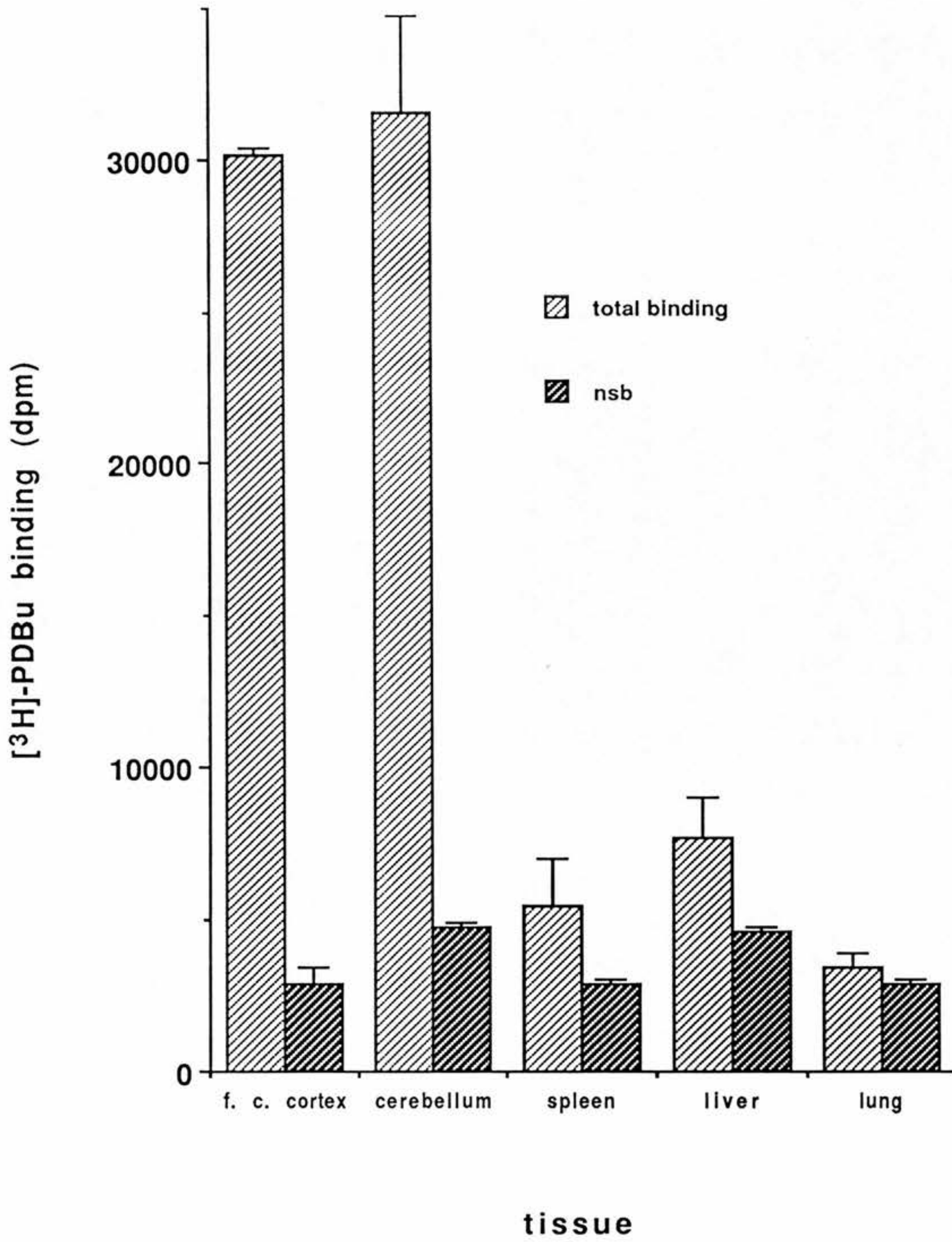


Figure 5.2. Concentration-response relationship of PDBu-displacement of [^{3}H]-PDBu binding to frontal cerebral cortex and cerebellum membranes.

Frontal cerebral cortex (closed circles) and cerebellum (open circles) membraneous [^{3}H]-PDBu binding was performed as described in section 2.2.7. Total and non-specific binding levels are shown in the absence and presence of 20 μM unlabelled PDBu respectively. The final concentrations of membranes used in the assay were 2 and 4 mg/ml for frontal cerebral cortex and cerebellum respectively. The data represent the means ± SEM of 3 determinations.

Figure 5.2

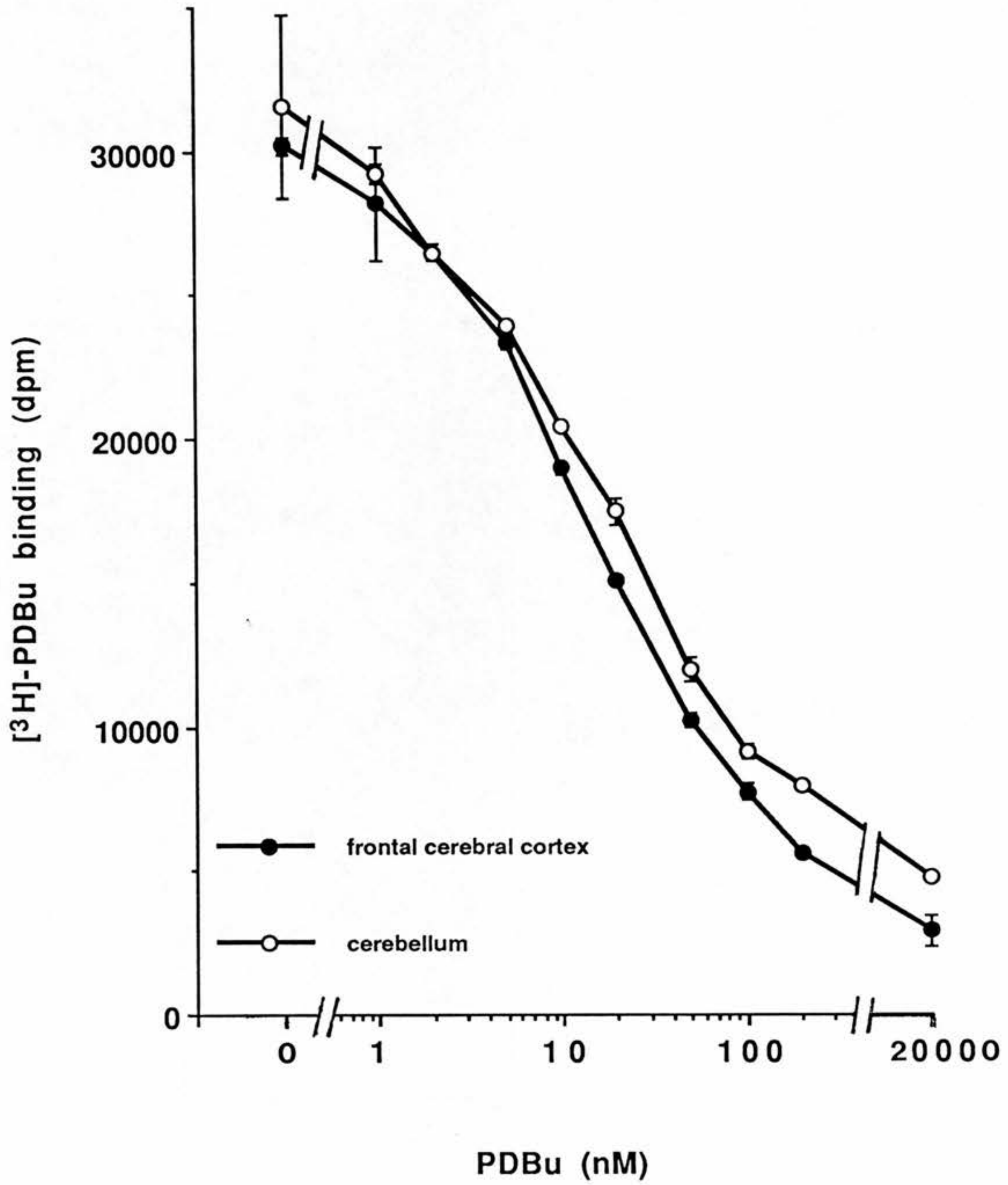


Figure 5.3. Concentration-response relationship of PDBu-displacement of [^{3}H]-PDBu binding to lung, frontal cerebral cortex and cerebellum cytosols.

Lung (squares), frontal cerebral cortex (circles) and cerebellum (triangles) cytosolic [^{3}H]-PDBu binding was performed as described in the Methods section. Total and non-specific binding levels are shown in the absence and presence of 20 μM unlabelled PDBu respectively. The final concentrations of cytosols used in the assay were (mg/ml): lung (200); frontal cerebral cortex and cerebellum (20). The data represent the means ± SEM of 4 determinations. The concentration-response curve for frontal cerebral cortex is the same as that shown in Figure 2.10.

Figure 5.3

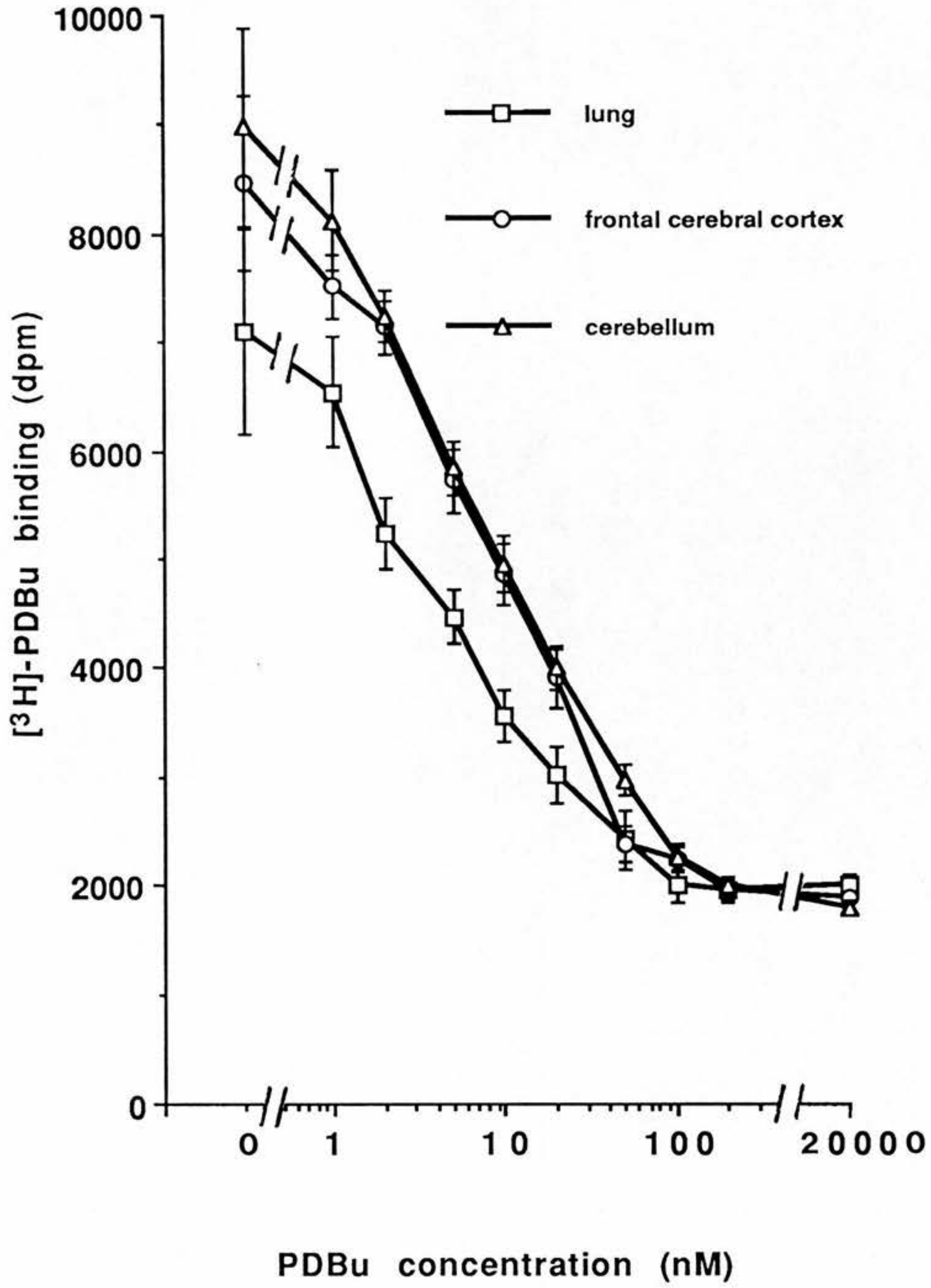


Figure 5.4. Effect of AMG-C16, staurosporine and H7 on specific [^{3}H]-PDBu binding to lung, frontal cerebral cortex and cerebellum cytosols.

Specific [^{3}H]-PDBu binding to lung (squares), frontal cerebral cortex (circles) and cerebellum (triangles) cytosol preparations was performed as described in the Methods section. The effect of AMG-C16 in conditions of normal (1 mg/ml) phosphatidylserine (a) and low (100 μg/ml) phosphatidylserine (b) was determined, as was the effect of staurosporine (c) and H7 (d) under conditions of normal (1 mg/ml) phosphatidylserine concentration. These agents were added to the assay dissolved in dimethylformamide (1% final) except H7 which was dissolved in water. The solvents alone had no effect on total or non-specific binding levels. The data represents the means ± SEM of 3 determinations.

Figure 5.4

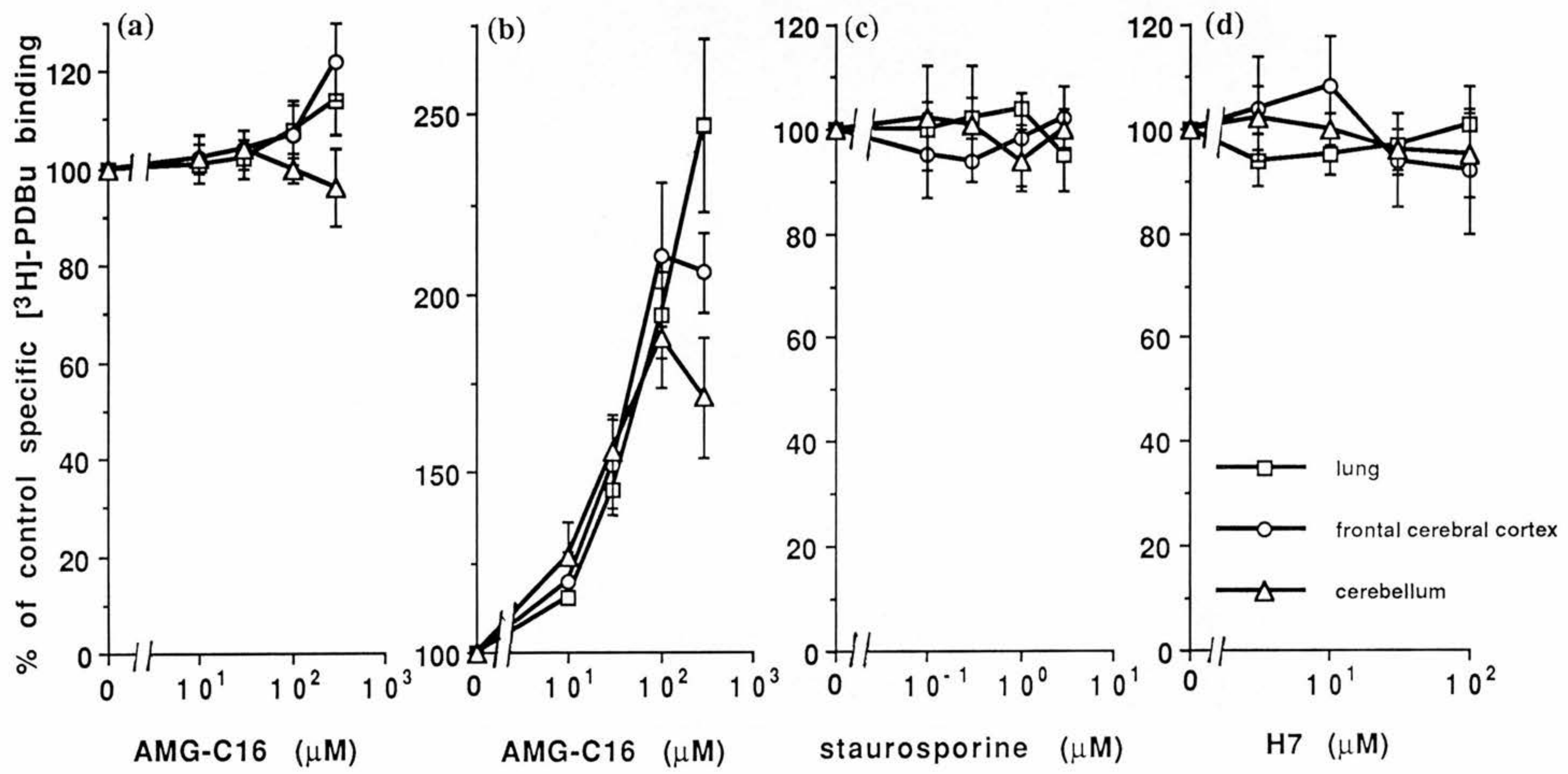


Figure 5.5. The effect of gossypol, sphingosine, indolactam-V and DHI on specific [^{3}H]-PDBu binding to lung, frontal cerebral cortex (circles) and cerebellum cytosols.

The specific [^{3}H]-PDBu binding to lung (squares), frontal cerebral cortex (circles) and cerebellum (triangles) cytosol preparations was assessed as described in the methods section. The effect of gossypol (a), sphingosine (b), the indolactam-V stereoisomers ((+) open symbols, (-) closed symbols (c)) and DHI (d) was determined. All agents were added to the assay dissolved in dimethylformamide. The data represent the means ± SEM of 3 determinations, with the exception of the sphingosine data which is derived from 4 determinations. None of these agents showed significantly ($P < 0.05$) different affinity when comparing the displacement curves for all 3 tissues.

Figure 5.5

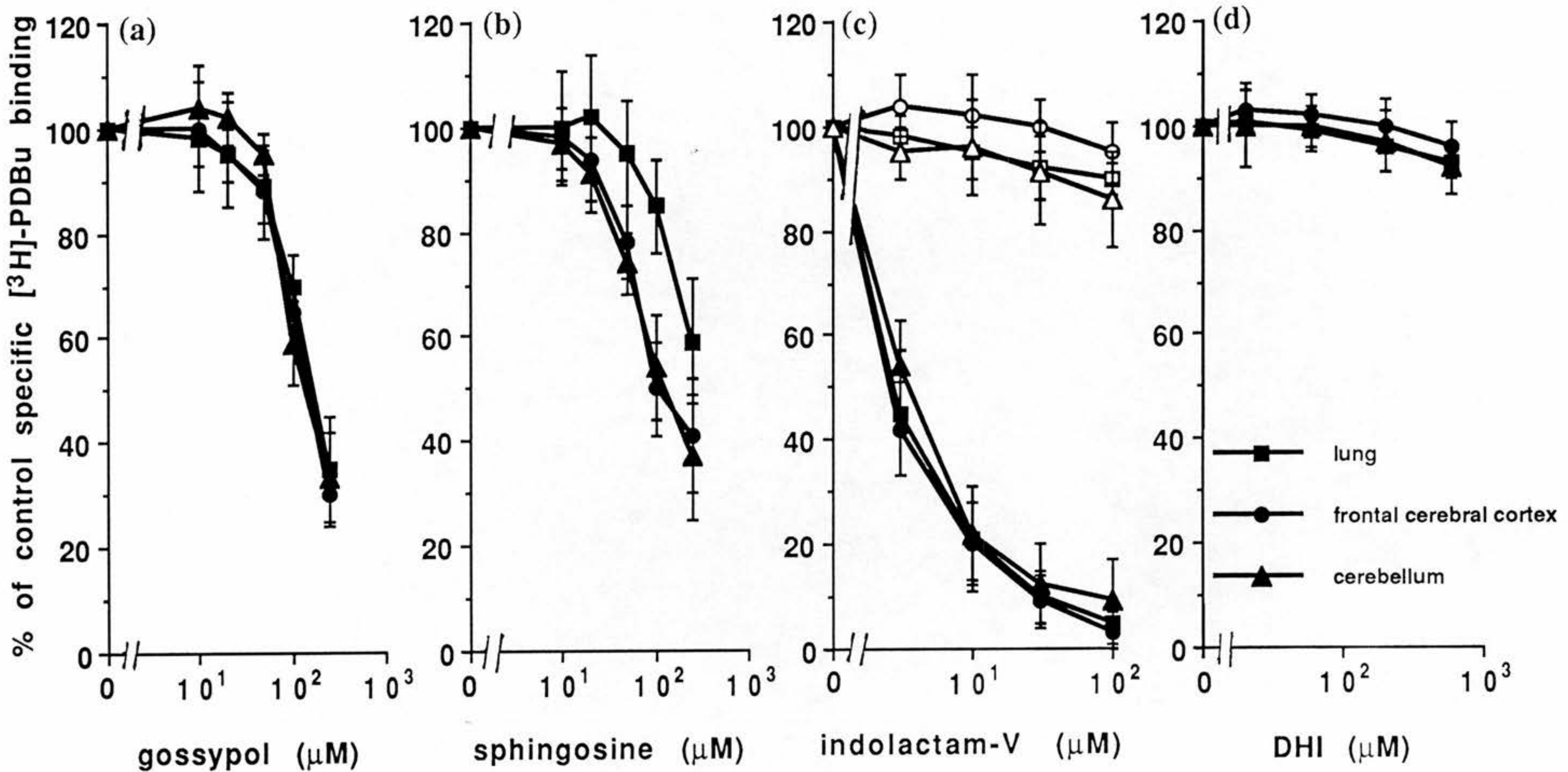


Figure 5.6. The relationship between diacylglycerol acyl chain length and affinity for [^{3}H]-PDBu binding sites in lung, frontal cerebral cortex and cerebellum cytosols.

The IC_{50} values for displacement of specific [^{3}H]-PDBu binding were calculated as described in the Methods section for a range of 1,2-diacylglycerols in lung (squares), frontal cerebral cortex (circles) and cerebellum (triangles) cytosols. The diacylglycerols shown are those with saturated acyl chains: 1,2-dihexanoyl-*sn*-glycerol (6 carbons); 1,2-dioctanoyl-*sn*-glycerol (8); 1,2-didecanoyl-*rac*-glycerol (10); 1,2-dilauroyl-*rac*-glycerol (12); 1,2-dimyristoyl-*rac*-glycerol (14); 1,2-dipalmitoyl-*sn*-glycerol (16); 1,2-distearoyl-*rac*-glycerol (18) and those with unsaturated acyl chain composition: 1,2-dioleoyl-*sn*-glycerol (18:1) and 1-stearoyl-2-arachidonoyl-*sn*-glycerol (18/20:4). The diacylglycerols were added to the assay, suspended by sonication with phosphatidylserine (mixed micelles). The data represent the means ± SEM from 4 determinations, and each of the data points for lung cytosol are significantly ($P < 0.05$) different from the data for frontal cerebral cortex and cerebellum cytosols.

Figure 5.6

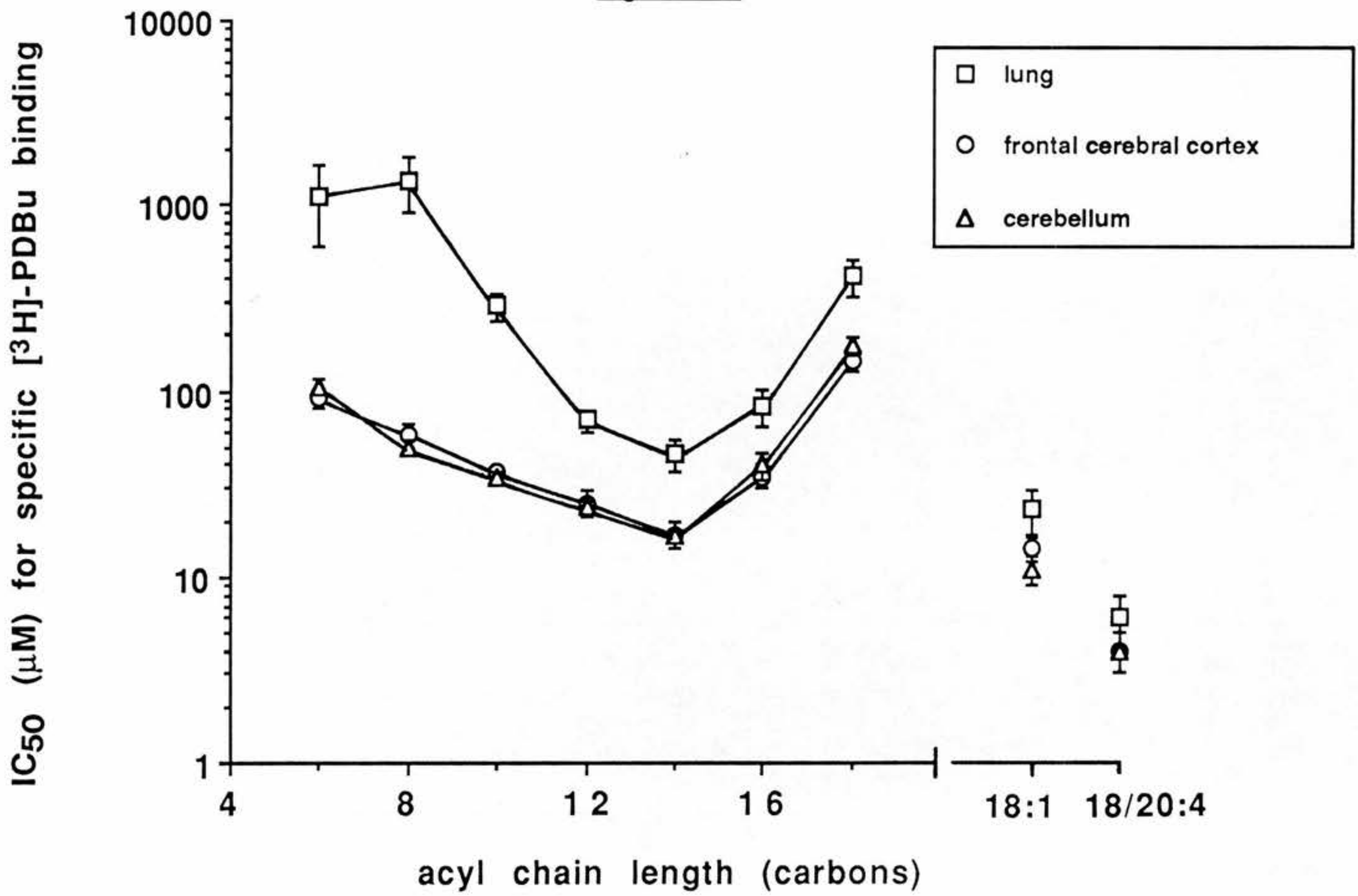


Figure 5.7. Regional differences in displacement by 1,2-dioctanoyl-*sn*-glycerol of specific cytosolic [^{3}H]-PDBu binding.

The effect of 1,2-dioctanoyl-*sn*-glycerol (DOG) on specific binding in lung (squares), frontal cerebral cortex (circles) and cerebellum (triangles) cytosols. DOG was added to the assay dissolved in dimethylformamide. The data represent the means ± SEM from 6 determinations. The concentration-response curve for lung cytosol is significantly ($P < 0.05$) different from those in frontal cerebral cortex and cerebellum at concentration of DOG of 5 μM and greater.

Figure 5.7

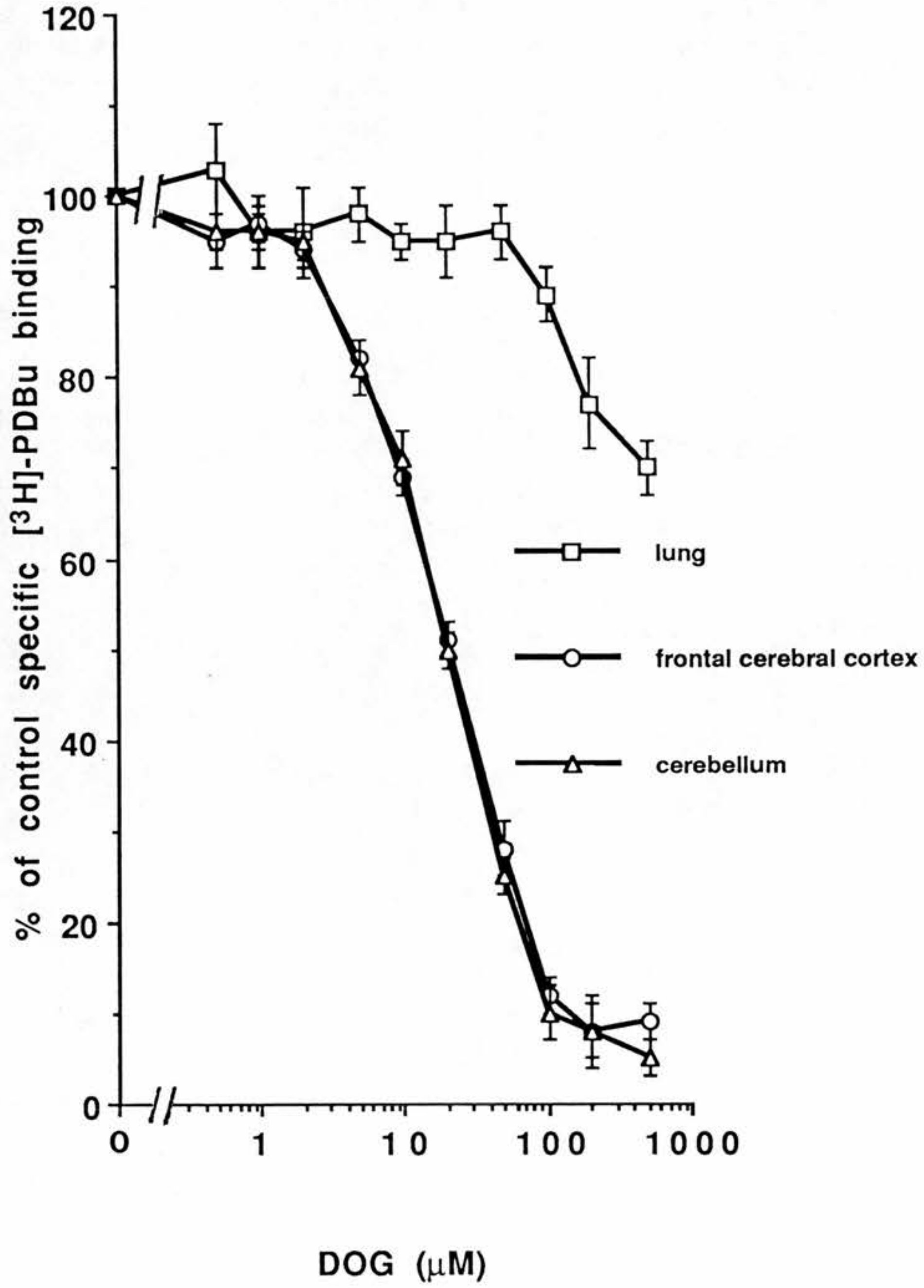


Figure 5.8. Relationship between tissue content of α-PKC and the effects of 1,2-dioctanoyl-*sn*-glycerol on specific cytosolic [^{3}H]-PDBu binding.

The mean values ± SEM for displacement by DOG of specific cytosolic [^{3}H]-PDBu binding is shown for: cerebellum (data point 1); frontal cerebral cortex (2); spleen (3); spinal cord (4); testes (5); liver (6); kidney (7); lung (8); sciatic nerve (9) and COS-7 cell (10) cytosols. Responses are expressed as the % change from control specific binding in the presence of 500 μM *sn*-DOG (added in dimethylformamide). Full concentration-response curves were always determined but the present data at a single concentration is quoted because IC_{50} concentrations were not reached in some tissues. The data represent the mean ± SEM of 6 determinations. The correlation co-efficient of the line through the data was 0.821.

Figure 5.8

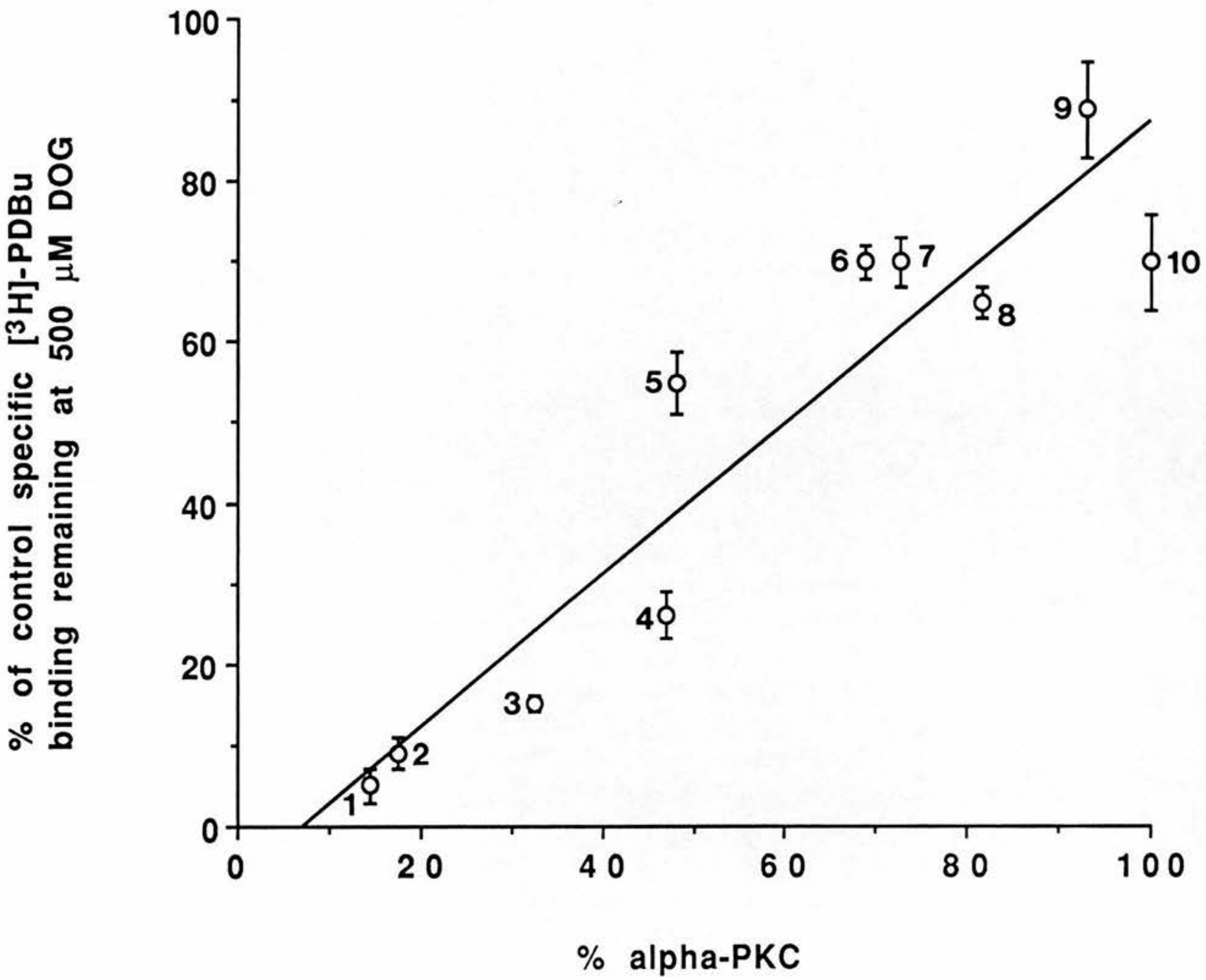


Figure 5.9. **Effect of AA added to the [^{3}H]-PDBu binding assay dissolved in ethanol.**

The effect of AA (dissolved in ethanol, 1% final) on specific [^{3}H]-PDBu binding to lung (squares), frontal cerebral cortex (circles) and cerebellum (triangles) cytosols was determined. The data represent the means ± SEM of 3 determinations.

Figure 5.9

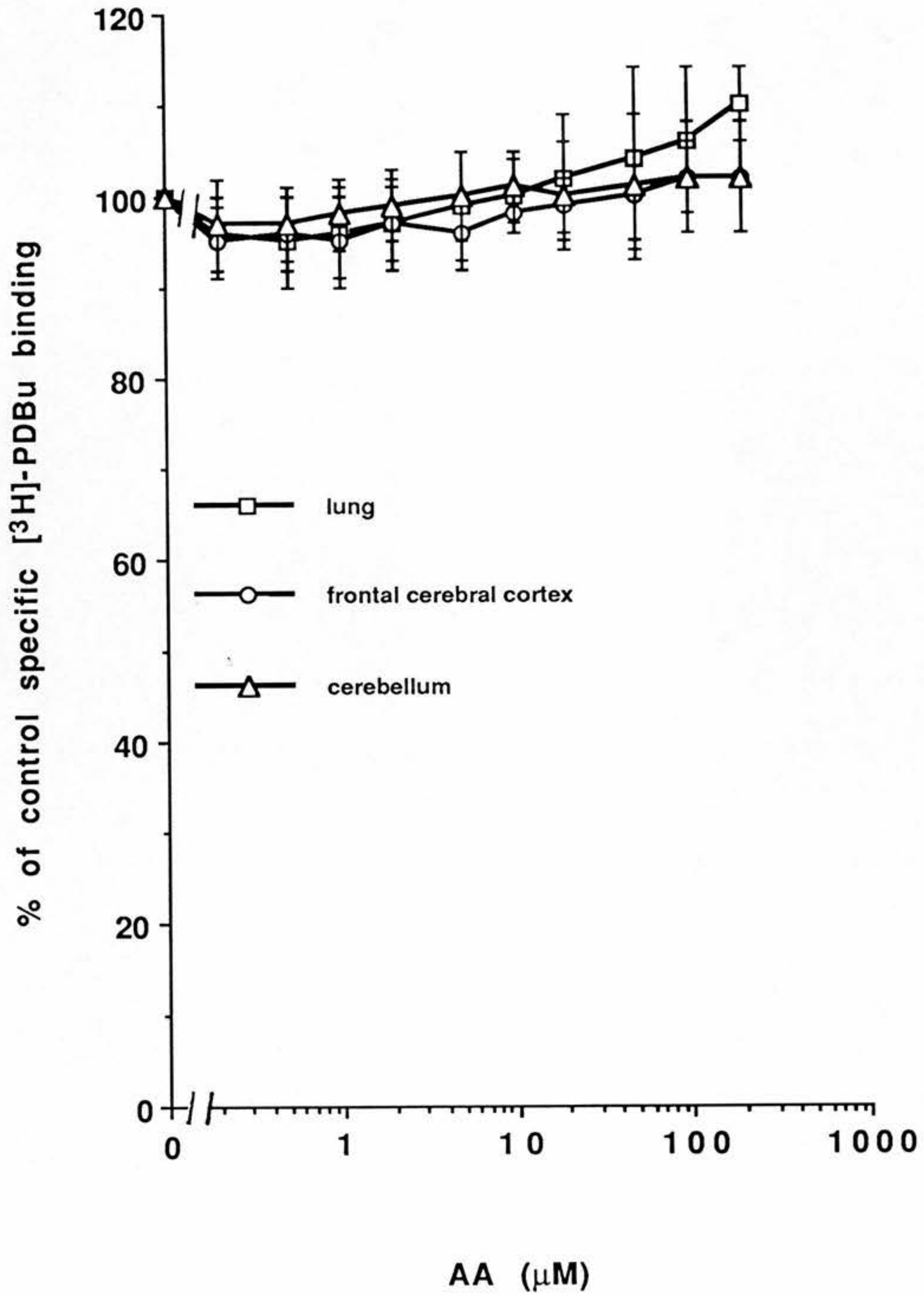


Figure 5.10. Regional differences in enhancement by arachidonic acid (added to the assay in mixed micelles) of specific cytosolic [^{3}H]-PDBu binding.

The effect of AA (added to the assay along with phosphatidylserine (1 mg/ml final throughout) in mixed micelles) on specific [^{3}H]-PDBu binding to lung (squares), frontal cerebral cortex (circles) and cerebellum (triangles) cytosols. The data represent the means ± SEM of 4 determinations. The enhancement by 200 and 500 μM AA in lung cytosol was significantly ($P \leq 0.05$) different from the corresponding binding levels in frontal cerebral cortex or cerebellum cytosol preparations or indeed from lung controls.

Figure 5.10

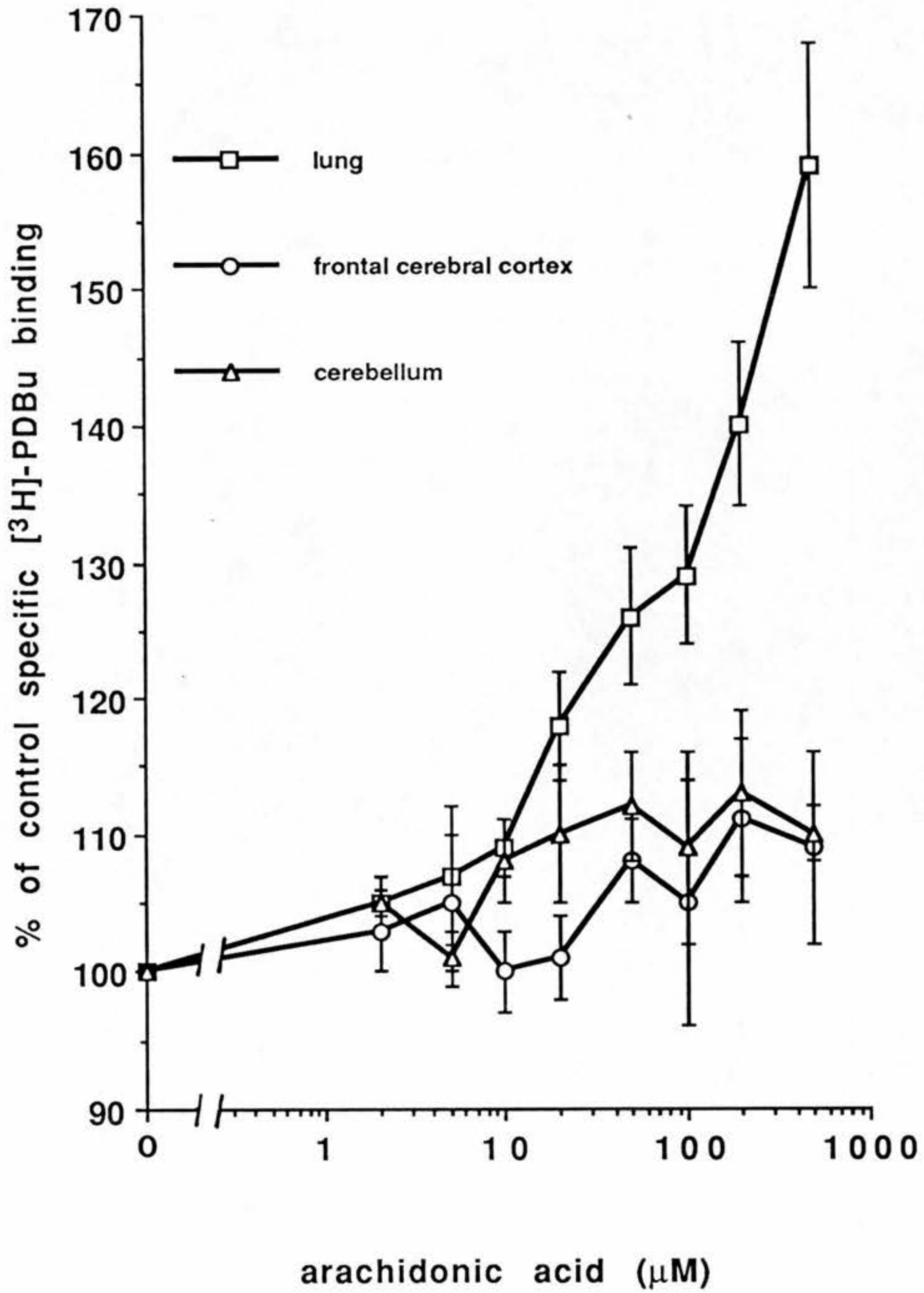


Figure 5.11. Effect of AA on specific cytosolic [^{3}H]-PDBu binding under conditions of zero or reduced phosphatidylserine.

The effect of AA (added to the assay in mixed micelles (or dissolved in ethanol for zero phosphatidylserine conditions)) on specific [^{3}H]-PDBu binding to lung (squares), frontal cerebral cortex (circles) and cerebellum (triangles) cytosols was investigate under conditions of zero (a) and reduced (750 μg/ml (b)) phosphatidylserine. The data represent the means ± SEM of 4 determinations. The enhancement with 100 and 200 μM AA of specific [^{3}H]-PDBu binding to lung cytosol under conditions of zero phosphatidylserine (a), was significantly ($P \leq 0.05$) different from the corresponding binding levels seen with frontal cerebral cortex and cerebellum cytosols.

Figure 5.11

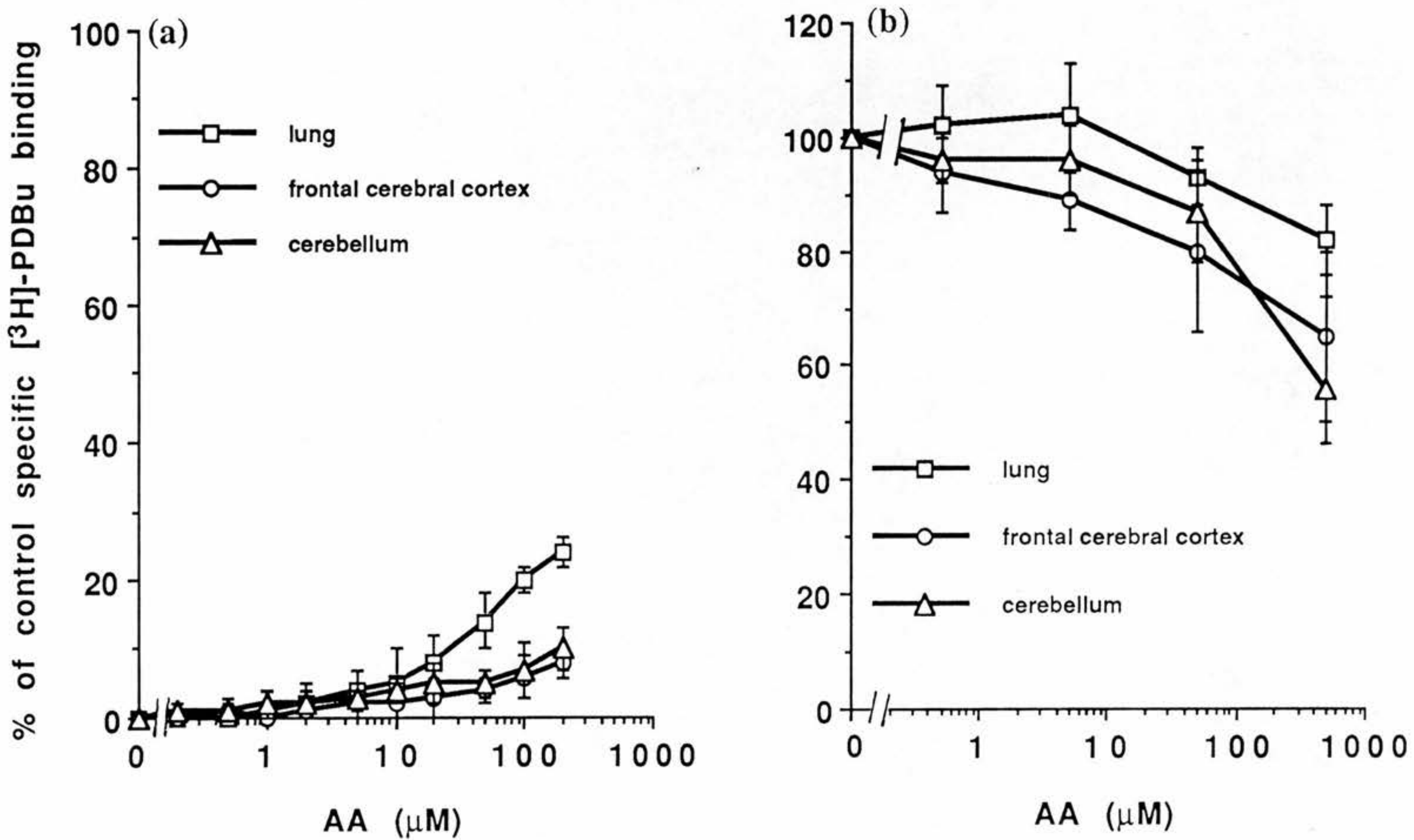


Figure 5.12. Relationship between tissue concentration of α-PKC and the effect of arachidonic acid on specific cytosolic [^{3}H]-PDBu binding. The mean values ± SEM for enhancement by AA of specific cytosolic [^{3}H]-PDBu binding is shown for cerebellum (data point 1); frontal cerebral cortex (2); spleen (3); spinal cord (4); testes (5); liver (6); kidney (7); lung (8); sciatic nerve (9) and COS-7 cells (10) cytosol preparations. Responses are expressed as the % change from control specific binding in the presence of 500 μM AA (added as mixed micelles). Full concentration-response curves were always determined but the data at a single concentration are quoted because EC_{50} values were not reached. The data represent the mean ± SEM of 4 determinations.

Figure 5.12

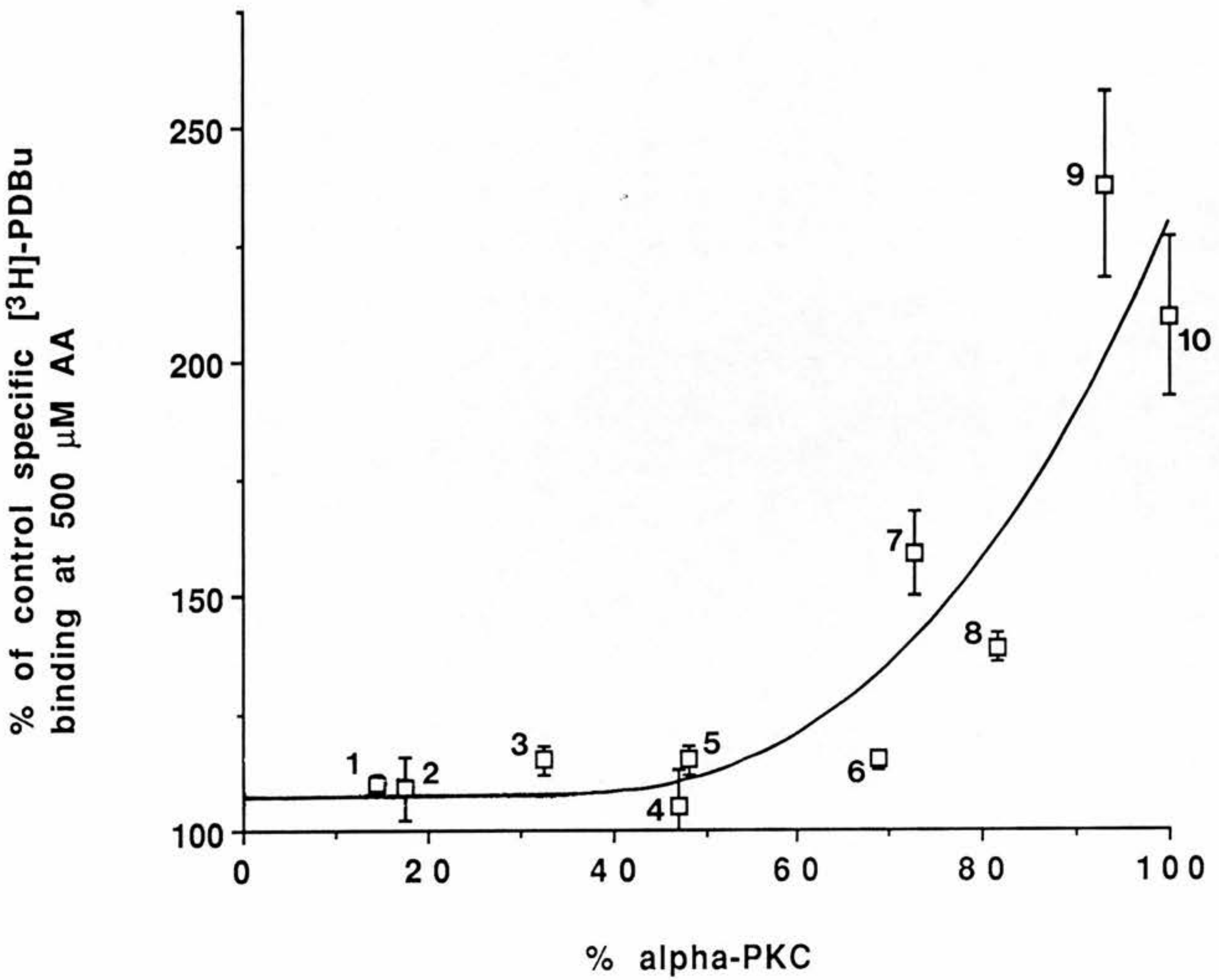


Figure 5.13. Effect of various purification steps on the EGTA-induced enhancement of specific [^{3}H]-PDBu binding to cytosolic PKCs. Specific binding of [^{3}H]-PDBu to cytosolic preparations was assessed as described in section 5.2. In (a), the effect of EGTA (5 mM final) and EGTA (5 mM) plus DTPA (0.5 mM final) on crude cytosol preparations from lung, frontal cerebral cortex (f.c. cortex) and kidney was determined. Values for binding are expressed as % of specific binding levels from the same cytosol, in the presence of 1 mM $CaCl_2$. EGTA or DTPA had no effect on non-specific binding levels. The enhancement of binding by EGTA on cytosol from midbrain (b) was investigated with midbrain which was either unpurified (i) or was partially-purified by a 21 - 45% ammonium sulphate 'cut' (ii) or by DE52 ion exchange chromatography (0 - 80 mM NaCl (iii)). In (c), the effect of protease inhibitors (0.01% final leupeptin and 20 μM final E64) on the specific [^{3}H]-PDBu binding of DE52 partially-purified (0 - 80 mM NaCl) midbrain cytosol under conditions of low Ca^{2+} (230 μM final), high Ca^{2+} (1.4 mM final) or excess EGTA (5 mM final). Values are expressed as a % of specific [^{3}H]-PDBu binding under conditions of no added Ca^{2+} or EGTA. The data represent the means ± SEM of 3 determinations.

Figure 5.13

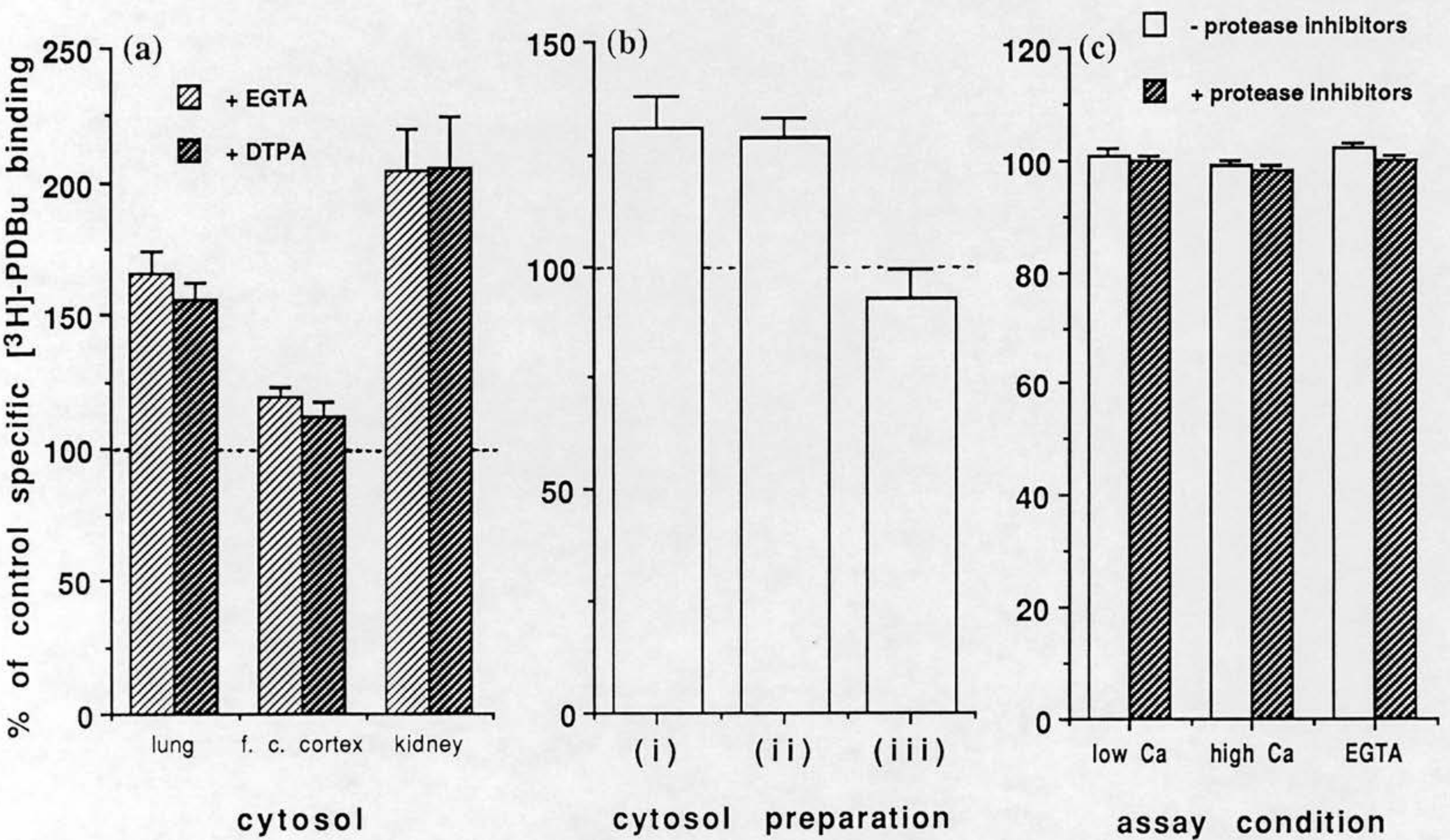


Table 5.1. **The ability of 1,2-dioctanoyl-*sn*-glycerol preincubated in cytosol preparations to displace specific cytosolic [^{3}H]-PDBu binding.** Fresh DOG (500 μM) was preincubated with the appropriate cytosol preparation for 30 min (37°C) as of the [^{3}H]-PDBu binding method described in the Methods section except that [^{3}H]-PDBu in incubation buffer was replaced with incubation buffer alone. Protein was then precipitated with bovine γ-globulin and polyethylene glycol and pelletted by centrifugation as described. The ability of the supernatant from the spin to displace specific [^{3}H]-PDBu binding to cytosolic PKC from lung, frontal cerebral cortex and cerebellum was determined as usual. The final concentration of DOG in the second incubation assay was calculated to be 77 μM. The corresponding values for displacement of [^{3}H]-PDBu by 77 μM DOG which had not been previously preincubated (fresh DOG) in lung, frontal cerebral cortex and cerebellum cytosols, was estimated from Figure 5.7. All other values are the means of 2 determinations (n values indicated in parentheses), with values varying by < 10%. Note that DOG preincubated in lung cytosol was still markedly poorer at displacing specific [^{3}H]-PDBu binding in lung cytosol than in frontal cerebral cortex and cerebellum cytosols.

Table 5.1

	Estimated affinity of cytosolic PKCs for DOG (% of specific [^{3}H]-PDBu binding remaining with 77 μM DOG)		
	Lung	**Frontal cerebral cortex**	**Cerebellum**
Fresh DOG	~ 95% (6)	~ 20% (6)	~ 20% (6)
DOG preincubated in frontal cerebral cortex cytosol	101% (2)	52% (2)	55% (2)
DOG preincubated in cerebellum cytosol	92% (2)	47% (2)	51% (2)
DOG preincubated in lung cytosol	98% (2)	50% (2)	49% (2)

Table 5.2. **Prediction of α-PKC content in rat anterior pituitary and GH_3 cell cytosols.**

The range of % α-PKC was determined by translating the extreme error limits around the mean (ie, mean + SEM and mean - SEM values) of the amounts of DOG displacement and AA-enhancement of specific [^{3}H]-PDBu binding found in anterior pituitary and GH_3 cytosols. The bottom row indicates the proportion of α-PKC found by Naor (1990) using PKC isoform-specific antibodies. As can be seen, the values derived from both correlations match well to the findings of Naor (1990) and confirm that α-PKC content in GH_3 cell cytosol is markedly higher than the content in rat anterior pituitary cytosol.

Table 5.2

	Predicted α-PKC content (%)	
	Rat anterior pituitary cytosol	**GH_3 cell cytosol**
From DOG-displacement correlation (Figure 5.8)	40 - 53%	65 - 82%
From AA-enhancement correlation (Figure 5.12)	48 - 66%	68 - 80%
Naor (1990) immunoblot analysis	59 ± 8%	83%

Table 5.3. **Effect of EGTA on levels of specific [^{3}H]-PDBu binding to various cytosols.**

The method of binding employed was that described in section 5.2. The specific [^{3}H]-PDBu binding with EGTA is expressed as a % of the amount of specific [^{3}H]-PDBu binding in the presence of 1 mM $CaCl_2$ in the same cytosol preparation. EGTA had no effect on non-specific binding levels. The values represent the means ± SEM of 3 determinations.

Table 5.3

Cytosol	[^{3}H]-PDBu binding with EGTA/no Ca^{2+} (% of control specific binding in presence of Ca^{2+})
lung	166 ± 8%
frontal cerebral cortex	119 ± 4%
cerebellum	102 ± 9%
spleen	109 ± 4%
testes	112 ± 5%
spinal cord	104 ± 9%
sciatic nerve	100 ± 8%
kidney	204 ± 16%
liver	133 ± 10%

5.4. DISCUSSION

There is considerable evidence that the major receptor for phorbol ester analogues is PKC (see section 1.2). Phorbol esters can bind to PKC with high affinity and with good specificity, and thereby radioactively-labelled phorbol ester ligands can provide an excellent means of quantifying the amount of PKC which is present in certain tissues. Furthermore, as phorbol esters appear to bind to PKC at the diacylglycerol-binding site of the enzyme (Sharkey and Blumberg, 1985), phorbol ester competition binding studies can provide useful information as to the characteristics of the diacylglycerol/PKC interaction. Phorbol esters are in general quite lipophilic and can thus be difficult to use when they are required to be dissolved in aqueous solutions. A more hydrophilic analogue, PDBu, is considered to be the phorbol ester analogue of experimental choice (Blumberg, 1988) as it can dissolve well in solvents such as ethanol but still retains its potent PKC-activating properties. Radiolabelled PDBu is therefore the most widely used phorbol ester when investigating phorbol ester-binding to PKC. Unlike membrane-bound PKC, cytosolic PKC requires an exogenously-supplied phospholipid environment to enable binding of $[^3H]$-PDBu (Leach *et al,* 1983). Cytosolic $[^3H]$-PDBu binding studies therefore require this additional factor which is not necessary when measuring $[^3H]$-PDBu binding to membranes. At present, the extent to which certain isoforms of PKC require phorbol esters for activation is not wholly clear, for example the ζ-isoform may be less well activated by PMA than the other known isoforms (Nishizuka, 1988). Originally, PKC was characterised as a phospholipid-dependent, Ca^{2+}-activated kinase (see section 1.2) but with the discovery of the Ca^{2+}-independent isoforms of PKC that are activated by phorbol esters, this means of PKC-classification has receded and now PKCs are classified as phospholipid-dependent, phorbol ester-activated kinases. It is possible that other such kinases distinct from the known PKC isoforms may yet be discovered. No such kinase has been characterised, but the possibility for these 'PKC-related'

kinases is real. Specific binding of [^{3}H]-PDBu may therefore contain such 'PKC-related' kinases as well as an amount of any phospholipase D, which is suggested to be another site for phorbol esters (see section 1.2.5). However, the high specificity of phorbol esters for PKC(s) has yet to be conclusively refuted and [^{3}H]-PDBu binding remains an excellent method for investigating the presence of PKC and the characteristics of its diacylglycerol-binding site.

Initial studies here were centred on membraneous [^{3}H]-PDBu binding from different regions in an attempt to characterise any PKC-isoform differences which may exist. Unfortunately, only frontal cerebral cortex and cerebellum membranes showed enough specific binding to enable accurate analysis. This region-specific difference is consistent with brain having the largest amounts of PKC activity (Kuo *et al,* 1980). Furthermore, these regions have a high content of ß-PKC which, unlike α-PKC, tends to translocate readily and be significantly present in membranes under basal conditions (Pelech *et al,* 1991), perhaps reflecting the high amounts of specific [^{3}H]-PDBu binding to membranes in these regions. Displacement of [^{3}H]-PDBu binding in frontal cerebral cortex and cerebellum membranes by unlabelled PDBu yielded lower IC_{50} values than those for PDBu-displacement of specific [^{3}H]-PDBu binding to cytosolic PKC (see Figures 5.2 and 5.3). Both results match previous work investigating PDBu-displacement of specific [^{3}H]-PDBu binding to mouse fibroblast membranes and brain cytosol preparations (Dunn and Blumberg, 1983 and Leach *et al,* 1983 respectively). Investigation of cytosolic [^{3}H]-PDBu binding proved useful, as high amounts of specific binding were detected in almost all tissues tested. In cytosol preparations from frontal cerebral cortex, cerebellum and lung, the displacement of specific [^{3}H]-PDBu binding by unlabelled PDBu showed almost identical IC_{50} values for all three regions (Figure 5.3).

Specific [^{3}H]-PDBu binding in lung, frontal cerebral cortex and cerebellum cytosols (tissue relatively enriched in α-, ß- and γ-PKC respectively) was displaced in a concentration-dependent manner by DPB and mezerein (section 5.3), as would be

expected of these phorbol ester analogues. The displacement of [^{3}H]-PDBu binding with these compounds has been shown previously (Dunn and Blumberg, 1985), however, here these agents which showed interesting selective-profiles on the two models of Ca^{2+} influx (Chapter 3), showed little or no difference in affinity for [^{3}H]-PDBu binding in the three tissue, suggestive of these compounds displaying no selective affinity for various PKC isoforms (at least the isoforms present in the three tissues investigated). Perhaps the responsible isoform(s) is present only in pituitary (and certain other) tissues, a possibility presently being investigated within our laboratory. One other such possibility for the similar affinities for PKC of DPB and mezerein, but still behaving in an isoform-selective nature, is that the compounds could be acting as partial agonists (ie, same affinity but reduced efficacy) towards certain isoforms of PKC. However, PKC activity studies with mezerein and PDBu as activators of PKC (M S Johnson and R Mitchell, unpublished observations) suggests that PDBu and mezerein show similar maximal activity towards rat midbrain cytosolic PKC and are in fact not acting as partial agonists. Curiously, the affinity for [^{3}H]-PDBu displacement by mezerein was markedly higher (IC_{50}s < 1 nM) than the concentration required for half maximal activity in the $^{45}Ca^{2+}$ influx studies (EC_{50}s of > 25 nM). The reasons for this discrepancy are unclear. It is, of course, conceivable that access is a contributory factor in whole cell preparations. It is possible, however, that the affinity of the mezerein-interaction with [^{3}H]-PDBu binding is not representative of the potency of the compound to actually activate PKC in functional assays (compare, for example, with the similarly low reduced affinity of staurosporine in the mixed micellar PKC activity assay (see section 6.3)). Other compounds which were investigated in [^{3}H]-PDBu binding studies in cytosolic preparations from lung, frontal cerebral cortex and cerebellum were the activators DHI, (-)-indolactam-V (and its inactive stereoisomer (+)-indolactam-V) and the inhibitors cremaphor EL, AMG-C16, H7 and staurosporine. Displacement of specific [^{3}H]-PDBu binding occurred with DHI, (-)-indolactam-V and (+)-indolactam-V (albeit with far lower

affinity than its active enantiomer). Each of these compounds displayed no marked differences in affinity for the three tissues tested suggesting they may not be PKC-isoform-selective. Cremaphor EL is a highly viscous substance and the erratic effects it has on [^{3}H]-PDBu binding are probably due to the difficulties experienced in diluting this agent and its probable non-specific effects on perturbing the lipid environment of the PKC. The biphasic effect of the diglyceride-analogue AMG-C16 on specific [^{3}H]-PDBu binding to lung frontal cerebral cortex and cerebellum cytosol preparations, may indicate that it can act in an analogous fashion to phosphatidylserine at lower concentrations to enhance [^{3}H]-PDBu binding, and perhaps also mimic diacylglycerols at higher concentrations to displace [^{3}H]-PDBu binding. The amount of enhancement seen with AMG-C16 is thus dependent on the phosphatidylserine concentration in the assay (see Figure 5.4). Similar observations were also made with the fatty acid AA (discussed later in this section). The greater enhancement of specific [^{3}H]-PDBu binding by AMG-C16 in lung cytosol preparations may reflect a greater susceptibility of α-PKC to the lipid environment surrounding the isoenzyme, although many other interpretations could be possible. Staurosporine and H7 were unable to reverse specific [^{3}H]-PDBu binding in lung, frontal cerebral cortex or cerebellum cytosol preparations (see Figure 5.4) entirely consistent with these inhibitors acting at a site on PKC which is not the diacylglycerol/phorbol ester-binding site (see section 1.2.3). This inability of staurosporine and H7 to affect [^{3}H]-PDBu binding was also reported by Nakadate *et al,* 1988. In order to investigate any PKC-isoform selective nature of H7, it was necessary to assess the effect of this inhibitor on a PKC activity assay; the findings of these studies are reported in the following chapter.

Unsaturated diglycerides showed a similar high affinity for displacement of [^{3}H]-PDBu binding in lung, frontal cerebral cortex and cerebellum cytosols (Figure 5.6). The naturally-occurring diacylglycerols, 1-stearoyl-2-arachidonyl-*sn*-glycerol and 1,2-dioleoyl-*sn*-glycerol displaced specific [^{3}H]-PDBu binding in the three tissues with very similar potencies (analagous to PDBu displacement of binding in the three

tissues) suggesting that neither of these diglycerides nor PDBu have differential affinity towards the PKC-isoforms present in the three tissues. Diglycerides with saturated acyl chains generally showed lower potency than their unsaturated acyl chain counterparts. For the saturated analogues, there was a biphasic relationship between acyl chain length and affinity for the [^{3}H]-PDBu binding site (Figure 5.6). These findings were consistent with previous work where the biphasic nature of diglyceride affinity was attributed to the hydrophilicity of the acyl chain and to the optimal fit of the diglyceride at the diacylglycerol-binding site within PKC (Ebding *et al*, 1982; Hannun *et al*, 1986; Sekiguchi *et al*, 1988). The IC_{50} values for any particular diacylglycerol were always similar in frontal cerebral cortex and cerebellum cytosols (enriched in ß- and α-PKC respectively) but the binding site in lung cytosol (relatively enriched in γ-PKC) displayed markedly lower affinity for all the saturated diglycerides (Figure 5.6). The reduced affinity in lung was most marked for saturated chain lengths of 6 - 10 carbons, with DOG having the greatest separation in affinity between frontal cerebral cortex/cerebellum and lung cytosol preparations. This reduced affinity of the saturated diglycerides in lung cytosol was not due to their degradation in lung only, as DOG pre-incubated in lung cytosol showed the same affinity for frontal cerebral cortex or cerebellum binding as DOG preincubated for the same period with either frontal cerebral cortex or cerebellum cytosols (Table 5.1). Thus, the possibility that lung-specific degradation of saturated diglycerides (particular short-acyl chain analogues) was responsible for their reduced affinity for the [^{3}H]-PDBu binding site in lung does not seem to be a significant factor here. What may account for these differences, is that α-PKC may have reduced affinity towards saturated diacylglycerols (but not unsaturated diacylglycerols) and that this reduced affinity of α-PKC compared to ß- and γ-PKC, is most apparent with short-acyl chain diglycerides (particularly DOG). This contention of a reduced affinity of DOG for α-PKC is supported by the investigation of DOG affinity for specific [^{3}H]-PDBu binding to a range of cytosols with known PKC-isoform-content (Figures 5.7 and 5.8). Reduced affinity of DOG

correlates well with the α-PKC isoform content. Such a correlation was not possible with ß- or γ-PKC contents but it must be stressed that only the % content of A series PKC-isoforms is known, and the relative contribution from B series isoforms or any undiscovered specific PDBu binding entities is unknown. However, such a selective nature of DOG could explain the ineffectiveness of DOG on the inhibition of 'L'-type Ca^{2+} channels in GH_3 cells (but not on the enhancement of 'L'-type Ca^{2+} channels in anterior pituitary pieces (Figure 3.5)) as the inhibition of 'L'-channel activity may be mediated (at least in part) by α-PKC, as judged by its AA-sensitivity (Figure 3.7).

Dissolving AA in ethanol to investigate its effects on [^{3}H]-PDBu binding to lung, frontal cerebral cortex and cerebellum cytosol preparations, originally suggested that AA was without much effect (Figure 5.9). However, employing the mixed micellar method of adding lipids for [^{3}H]-PDBu binding analysis, revealed that AA can markedly enhance [^{3}H]-PDBu binding in lung cytosol but not frontal cerebral cortex cytosols (Figure 5.10). Arachidonic acid may be partially substituting for the role of phosphatidylserine which enhances cytosolic [^{3}H]-PDBu binding, as binding levels in lung, frontal cerebral cortex and cerebellum cytosols were modestly raised by AA in a concentration-dependent fashion in the absence of phosphatidylserine, but AA could never fully substitute for the permissive role of phosphatidylserine (Figure 5.11). The enhancing effect of AA on [^{3}H]-PDBu binding interacted with the effect of phosphatidylserine of PKC, as AA-enhancement was reduced in assay conditions in which the phosphatidylserine concentration was lowered (Figure 5.11(b)). Under these conditions, a slight displacement of [^{3}H]-PDBu binding could be seen with higher concentrations of AA. A displacement of specific cytosolic [^{3}H]-PDBu binding by AA has been described previously (Leach and Blumberg, 1985; Sharkey and Blumberg, 1985) which has been attributed to non-competitive mechanisms. However, the displacement of [^{3}H]-PDBu binding by AA occurs only with phosphatidylserine concentrations which are extremely submaximal for [^{3}H]-PDBu binding to cytosolic PKC (Leach *et al,* 1983). Therefore, a mixed mechanism of AA

interaction with PKC may exist: firstly, an incomplete displacement of [^{3}H]-PDBu binding by AA, which is overcome by higher concentrations of phosphatidylserine, and secondly, arachidonic acid may also partially substitute for phosphatidylserine, although its effectiveness in the permissive role of phosphatidylserine on binding is greatly reduced. The enhancement of [^{3}H]-PDBu binding by AA was tested in a range of cytosols from various sources with known PKC isoform content (again only fully quantitated for A series isoforms) and it was found that only tissues with high content of α-PKC showed a marked enhancement (Figure 5.12). This may suggest that the AA-induced activation of α-PKC (see section 1.2.3) is occurring through such a mechanism viewed here. However, enhancement of binding occurs at concentrations of AA which are far higher than those concentrations observed to activate PKC in other assays (eg, the AA-induced activation of PKC in GH_3 cells described in Chapter 4). Furthermore, γ-PKC is reported to be more potently activated by AA than α-PKC, but those regions which possess γ-PKC (eg, cerebellum) show no enhancement of [^{3}H]-PDBu binding by AA. What seems a more likely possibility, is that the AA-induced enhancement of [^{3}H]-PDBu binding is an allosteric or even physico-chemical effect of AA which may be particular to α-PKC. On a similar note, Huang and Huang (1990) have also described an inactivation of PKCs by lipids (such as phosphatidylserine) which appears to occur predominantly with γ-PKC.

In an attempt to assess the relative amount of α-PKC in our two $^{45}Ca^{2+}$ influx models, male rat anterior pituitary and GH_3 cells (Chapter 3), the effect of DOG and AA on [^{3}H]-PDBu binding to cytosol preparations from these tissues was determined. The predicted amount of α-PKC in both tissues was determined by reading-off the value of % α-PKC corresponding to the % displacement by 500 μM DOG or the % enhancement with 500 μM AA on specific [^{3}H]-PDBu binding. For male rat anterior pituitary cytosol, the predicted amounts of α-PKC are 40 - 53% and 48 - 66% as assessed using the correlations between % displacement by 500 μM DOG/% α-PKC and % enhancement with 500 μM AA/% α-PKC respectively. As can

be seen, the assessments of % α-PKC match well between the two methods of binding. Similarly well matched is the study on GH_3 cell cytosol, in which the relative % α-PKC predicted from the same correlation plots are 65 - 82% and 68 - 80% α-PKC respectively. Since our assessment of the relative amount of α-PKC in both tissues was carried out, a study by Naor (1990) was published in which the % α-PKC in the two tissue cytosols was determined by PKC isoform-specific immunoblot analysis. The quoted % α-PKC content of rat anterior pituitary and GH_3 cell cytosols was 59 ± 8% and 83% respectively. These results match well with our predicted α-PKC levels in the two tissues (Table 5.2) and therefore, determination of the affinity of DOG and enhancement by AA could provide a useful tool for estimating the relative amount of α-PKC in a tissue cytosol. From whatever method the % α-PKC was derived, it is clear that GH_3 cell cytosol possesses markedly greater amounts of α-PKC than does anterior pituitary cytosol, which may account for the differences in profiles of PKC-mediated modulation of 'L'-type Ca^{2+} channel activity seen in Chapter 3. If indeed α-PKC is responsible for an inhibition of 'L'-channel activity, the higher amounts of α-PKC in GH_3 cells compared to anterior pituitary pieces, may be responsible for the inhibition of K^+-induced $^{45}Ca^{2+}$ influx that is produced by PDBu treatment in GH_3 cells. The net activity of the large amounts of α-PKC in GH_3 cells may overcome the PDBu-activated isoform(s) of PKC which are responsible for enhanced 'L'-channel activity (and which may be selectively activated by mezerein in GH_3 cells). In contrast, PKCs which can facilitate the activity of 'L'-channels may be of a greater proportion in anterior pituitary pieces, with a greater net influence than the 'L'-channel-inhibitory isoforms of PKC which predominate in GH_3 cells.

The dependence of specific [^3H]-PDBu binding on Ca^{2+} appears to rely on the method employed for assaying binding, with some workers being able to see partially Ca^{2+}-dependent binding (Ohno *et al,* 1988b; Kiley *et al,* 1990). However, we were surprised to see that total [^3H]-PDBu binding actually greater in the presence of EGTA than total [^3H]-PDBu binding in the presence of Ca^{2+}, with no effect of EGTA

on non-specific binding (Figure 5.13). Furthermore, this EGTA-induced increase in total [^{3}H]-PDBu binding appeared to be restricted mostly to lung, kidney, liver and testes cytosol preparations with frontal cerebral cortex cytosolic binding showing a very slight increase with EGTA, and [^{3}H]-PDBu binding being unaffected by EGTA in the other tissues tested. This EGTA induced increase in binding was not mimicked by DTPA (a heavy metal-selective chelator, Figure 5.13). The increase in [^{3}H]-PDBu binding to cytosol prepared from midbrain total binding with EGTA was reduced when employing homogenisation methods similar to other reports of Ca^{2+}-dependent [^{3}H]-PDBu binding. With such methods, ammonium sulphate-fractionated midbrain cytosol still displayed an increase in binding with EGTA (although the increase was less than that seen in 'crude' preparations). However, this increase in binding with EGTA was abolished when midbrain cytosol was partially-purified by ion-exchange chromatography and was also seen for a range of Ca^{2+} ion concentration, and indeed with no added Ca^{2+} or EGTA (Figure 5.13). In conclusion, employing any of our methods of [^{3}H]-PDBu binding, no Ca^{2+}-dependency of binding could be seen, which may be surprising since the Ca^{2+}/phospholipid interaction of PKC is considered essential for an optimal conformation for diacylglycerol (but perhaps not phorbol ester)-binding to PKC (see section 1.2.1). No reasons could be discerned for the qualitative differences between the present results and those of Ohno *et al* (1988b) and Kiley *et al* (1990). Nevertheless, in the process of trying to derive conditions that would display Ca^{2+}-dependency of binding, we developed a convenient method for the purification and enrichment of PKC which proved to be important in the procedure for measuring PKC activity from tissues of various sources (see following chapter).

CHAPTER 6

ATTEMPTS TO CHARACTERISE ACTIVATION OF DISTINCT PROTEIN KINASE C ISOFORMS

6.1. INTRODUCTION

The original classification of protein kinase C (PKC) was as a calcium-activated, phospholipid-dependent kinase (see section 1.2.1) and since that time, kinase activity assays have been a major means of assessing the properties of the enzyme. This approach complements other means of studying PKC, such as [^{3}H]-PDBu binding, immunohistochemical analysis, mRNA *in situ* hybridisation, cDNA transfection studies, mRNA microinjection and incorporation of PKC through cell-permeabilisation. Pharmacological assessment of compounds which interact with PKC, almost inevitably has to involve the determination of their affinity and efficacy using a PKC activity assay. Activity studies on highly purified isoforms of PKC have revealed that α, ß and γ subspecies are (as predicted from their sequences) indeed Ca^{2+}-activated, although slight differences do exist in the activation potency of Ca^{2+} at each isoform (Sekiguchi *et al,* 1987; Sekiguchi *et al,* 1988; Nishizuka, 1988; Naor *et al,* 1988a; Huang *et al,* 1988; Marais and Parker, 1989; Naor, 1990). Each of these studies had used histone as a substrate for the isoenzymes, however, the ability of histone to act as a substrate for B series isoforms is more controversial. Schaap *et al* (1989) described how histone was a poor substrate for PKC-ε expressed from its cDNA in COS 1 cells, and also for PKC-ε expressed from its cDNA by a baculovirus vector (Schaap and Parker, 1990). It was suggested that a short chain peptide which corresponded to the pseudosubstrate site (serine substituting for alanine) was a preferred substrate when measuring PKC-ε activity (Schaap *et al,* 1989). Short chain peptides are known to be excellent substrates for PKCs, however in contrast to protein substrate such as histone and myosin light chain, show little cofactor dependence but kinase activity, which is both phospholipid- and Ca^{2+}-independent (Bazzi and Nelsestuen, 1987). Furthermore, studies on PKC-δ (Mischak *et al,* 1991) and an unidentified Ca^{2+}-independent form of protein kinase C from human platelets (Nishizuka, 1988) showed that these isoforms used histone as a substrate. It may be

only PKC-ε that poorly phosphorylates histone but it is clear that histone is an adequate substrate for ε as this was its means of detection on expression of its cDNA (Ono *et al,* 1987b; Ono *et al,* 1988a; Ono *et al,* 1989b).

In mixed isoform preparations (as used mostly here), the choice of substrate is therefore critical to observe both phospholipid- and Ca^{2+}-stimulated PKC activity. Substrates that will reveal such co-factor-dependence are histone, troponin I, troponin complex and mysoin light chain (Bazzi and Nelsestuen, 1987), and one of these substrates (histone) is the substrate of choice in our studies due to imparting Ca^{2+}-dependence on our kinase investigations (see section 2.2.8). The diacylglycerol, 1,2-dioctanoyl-*sn*-glycerol (DOG) displayed a selective profile on our models of K^+-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary cells (see Chapter 3) and was shown to have reduced affinity towards cytosol preparations enriched in PKC-α (see Chapter 5). The effectiveness of DOG was investigated here in our PKC activity assay. The PKC inhibitor, H7 also showed a preferential inhibition of the PDBu-effect on GH_3 cell K^+-induced $^{45}Ca^{2+}$ influx, rather than the corresponding modulation in anterior pituitary prisms (see Chapter 3). However, the effectiveness of H7 as a PKC isoform-selective pharmacological agent could not be assessed on [^{3}H]-PDBu binding studies (Chapter 5), as unlike DOG, the site of action of H7 is not at the [^{3}H]-PDBu binding site of PKC (Nakadate *et al,* 1988). Instead, the site of action of H7 is thought to be at or around the ATP-binding site of PKC (Ohta *et al,* 1988) and therefore a PKC activity assay is an ideal method of investigating the pharmacological properties of H7 at different forms of PKC.

6.2. SPECIFIC METHODOLOGY

PKC activity assays were as described in section 2.2.7, measuring the incorporation of [^{35}S] thiophosphate into the substrate, histone-IIIS. Kinase activity assessments in the absence of inhibitors had inhibitors replaced by buffer to maintain a constant assay volume of 100 μl.

6.3. RESULTS

Effect of PDBu and DOG on cytosolic protein kinase C activity

The effect of phorbol 12,13-dibutyrate (PDBu) in our models of pituitary cell K^+-induced $^{45}Ca^{2+}$ influx showed no selectivity between the two forms of modulation (see Chapter 3). Due to the selectivity of 1,2-dioctanoyl-*sn*-glycerol (DOG) seen on the models of rat anterior pituitary cell K^+-induced $^{45}Ca^{2+}$ influx, the ability of DOG to activate cytosolic PKCs was investigated and compared to the ability of PDBu to activate the same PKCs. The preparation used for these studies was initially rat midbrain cytosol from which PKC-like activity had been partially-purified by DE52, as described in section 2.2.8. Rat midbrain is a region which had been previously shown to contain mRNA for all seven known PKC isoforms (α, ßI, ßII, γ, δ, ε and ζ; Young, 1989). Using kinases from partially-purified midbrain cytosol, PDBu (0.01 - 10 μM) in the presence of excess EGTA, was found to enhance [^{35}S] thiophosphate incorporation into histone-IIIS in a concentration-dependent manner (Figure 6.1). The calculated EC_{50} for this enhancement was 906 ± 73 nM PDBu (n = 4), with the enhancement at concentrations of 1 μM PDBu and greater being significantly ($P \leq 0.05$) different from basal incorporation (in presence of phosphatidylserine alone). The presence of 100 nM free Ca^{2+} in the assay enhanced total PKC activity (measured at 10 μM PDBu) from values of kinase activity in the absence of Ca^{2+} which were 61 ± 4% of the maximal PKC activity in the presence of 100 nM free Ca^{2+} (n = 4; Figure 6.1). In the presence of 100 nM Ca^{2+}, PDBu (0.01 - 10 μM) also enhanced PKC activity in a concentration-dependent manner, resulting in an EC_{50} = 357 ± 89 nM. The enhancement of PKC activity in the presence of 100 nM free Ca^{2+} was significantly ($P \leq 0.05$) greater than basal activity and significantly different from activity in the absence of Ca^{2+} at concentrations of PDBu of 100 nM and greater. In the presence of 100 μM free Ca^{2+}, total PKC activity (measured at 10 μM PDBu) was not enhanced above the total PKC activity measured in the presence of 100 nM free Ca^{2+} (97 ± 6%) of

maximum inducible PKC activity, n = 4; Figure 6.1). PDBu (0.01 - 10 μM) also enhanced PKC activity in a concentration-dependent manner, resulting in an EC_{50} = 94 $\pm$ 62 nM. The enhancement of PKC activity in the presence of 100 μM PDBu was significantly ($P \leq 0.05$) different from basal activity at concentrations of PDBu of 30 nM and greater. Activity with 100 μM Ca^{2+} was also significantly greater ($P \leq 0.05$) than the activity with 100 nM Ca^{2+} at 30, 100, 300 and 1,000 nM PDBu.

The diacylglycerol, DOG was also able to enhance cytosolic PKC activity from midbrain, with the enhancement occurring in the absence and presence of 100 μM free Ca^{2+} (Figure 6.2(a)). In the absence of Ca^{2+} (excess EGTA), DOG (0.1 - 1,000 μM) enhanced PKC activity in a concentration-dependent manner with an EC_{50} = 75 $\pm$ 21 μM, with the maximal PKC activity in the absence of Ca^{2+} (measured at 1,000 μM DOG) being 29 $\pm$ 4% of maximal inducible PKC activity (n = 4). In the absence of Ca^{2+}, PKC activity was significantly ($P \leq 0.05$) enhanced above basal activity levels at concentrations of DOG of 100 μM and greater. However, in the presence of 100 μM Ca^{2+}, DOG (0.1 - 1,000 μM) enhanced midbrain PKC activity with an EC_{50} = 1.4 $\pm$ 0.6 μM (n = 4). The enhancement of PKC activity in the presence of 100 μM Ca^{2+} was significantly ($P \leq 0.05$) greater than basal activity and significantly ($P \leq 0.05$) greater than activity in the absence of Ca^{2+}, at concentrations of DOG of 1 μM and greater. The maximal inducible midbrain cytosolic PKC activity (with 1,000 μM DOG and 10 μM PDBu) was of a similar magnitude for both activators. The relatively high potency of DOG on Ca^{2+}-stimulated activity seen in midbrain is in contrast to the effect of DOG on COS 7 cell PKC. COS cells are reported to contain only α-PKC (Kosaka *et al,* 1988; Schaap *et al,* 1989) and the partially-purified cytosolic PKC activity from these cells is stimulated by DOG (0.1 - 1,000 μM) in a concentration-dependent manner (EC_{50} = 28 $\pm$ 12 μM in the presence of 100 μM Ca^{2+}, n = 4; (Figure 6.2(b))). This potency of DOG at enriched α-PKC was markedly lower than the potency of DOG in midbrain cytosol preparations which contain a mixture of all known isoforms. Furthermore, DOG cannot stimulate kinase activity at all in the absence of Ca^{2+} in

COS 7 cell cytosol preparations, which is consistent with α-PKC being a Ca^{2+}-dependent enzyme (Nishizuka, 1988). The PKC activity in COS 7 cells in the presence of Ca^{2+} was significantly ($P \leq 0.05$) greater than basal activity (or activity in absence of Ca^{2+}) with concentrations of DOG of 30 μM and greater.

Effect of H7, staurosporine and Ro318220 on cytosolic protein kinase C activity from various tissues

Partially-purified male rat midbrain cytosol was again used as a source of the known PKCs (Young, 1989) to examine the effects of the PKC inhibitors staurosporine and Ro318220, which showed no selectivity in the dual regulation of Ca^{2+} channels by PKC and H7, which did show selective inhibition of the response to PKC activation in GH_3 cells (see Chapter 3). Kinase activity was stimulated with 1 μM PDBu and the enhancement in activity in the absence (excess EGTA) and presence of 100 μM Ca^{2+}, was termed Ca^{2+}-independent and Ca^{2+}-dependent PKC activity respectively.

Using PKCs from midbrain cytosol partially-purified in DE52 by elution with 0 - 150 mM NaCl, H7 (10 - 300 μM) inhibited (in a concentration-dependent manner) both Ca^{2+}-dependent and Ca^{2+}-independent PKC activity with similar potency (34 ± 5 and 27 ± 9 μM respectively, n = 4; Figure 6.3 and Table 6.1). Equivalent results were obtained with staurosporine (30 - 1,000 nM) and Ro318220 (10 - 3,000 nM) (Figure 6.3). Staurosporine inhibited Ca^{2+}-dependent and Ca^{2+}-independent PDBu-stimulated kinase activity with potencies of 450 ± 53 and 382 ± 34 nM respectively (n = 4), whereas the potencies of Ro318220 were 252 ± 40 and 213 ± 47 nM respectively (n = 4). Therefore, no difference in potency was seen with the inhibitors H7, staurosporine and Ro318220 between Ca^{2+}-dependent and Ca^{2+}-independent PKC activity extracted from male rat midbrain.

In the light of the differential sensitivity of phorbol ester effects to H7 (but not staurosporine or Ro318220) in male rat anterior pituitaries (Chapter 3), the

sensitivity of PKCs from a number of tissue cytosol preparations (including male rat anterior pituitary) to inhibition by H7 and staurosporine was investigated (Figure 6.4 and Table 6.1). PKCs partially-purified (0 - 150 mM NaCl, DE52) from cytosol preparations of male anterior pituitary were used to test the sensitivity of Ca^{2+}-dependent and Ca^{2+}-independent PDBu (1 μM)-stimulated PKC activity to the inhibitors H7 and staurosporine. Staurosporine (10 - 1,000 nM) inhibited both Ca^{2+}-dependent and Ca^{2+}-independent PKC activity in a concentration-dependent manner (Figure 6.4(d)), with IC_{50} values of 107 ± 39 and 117 ± 46 nM respectively (n = 4). At none of the tested concentrations of staurosporine were the relative amounts of Ca^{2+}-dependent or Ca^{2+}-independent PKC activities significantly different. In contrast, the concentration-dependent inhibition by H7 (10 - 300 μM) of Ca^{2+}-dependent PKC activity (IC_{50}= 17 ± 4 μM, n = 4) was of much higher potency than the concentration-dependent inhibition by H7 of Ca^{2+}-independent PKC activity (IC_{50} = 121 ± 18 μM; Figure 6.4(c)). The Ca^{2+}-dependent and Ca^{2+}-independent PKC activities from male anterior pituitary cytosol were inhibited to significantly different extents with concentrations of H7 of 30 and 100 μM. Similarly, a reduced potency of H7 on Ca^{2+}-independent PKC activity was also seen in partially-purified (0 - 150 mM NaCl, DE52) cytosol preparations from male rat lung (Figure 6.4(b)), although with not as marked a reduction in potency as seen using anterior pituitary cytosol preparations. The inhibitor H7 (10 - 300 μM) reduced PDBu-stimulated Ca^{2+}-dependent and Ca^{2+}-independent PKC activity in a concentration-dependent fashion (IC_{50}s = 39 ± 5 and 81 ± 10 μM) respectively (n = 4). The difference in Ca^{2+}-dependent and Ca^{2+}-independent lung PKC activity was significantly ($P \leq 0.05$) different with a concentration of 30 and 100 μM H7. However, the reduced potency of H7 was not seen in cytosol preparations from all tissues. As described above, male rat midbrain cytosolic PKC activity showed similar inhibitory potency of H7. This was also the case for partially-purified PKC activity from rat spleen (Figure 6.4(a)), frontal cerebral cortex, cerebellum and COS 7 cell cytosols (Table 6.1). Staurosporine also

showed no difference in potency between Ca^{2+}-dependent and Ca^{2+}-independent PKC activity (Table 6.1) in male midbrain and anterior pituitary cytosols, however, the potency of staurosporine appeared somewhat greater in anterior pituitary cytosol than the potency of inhibitors in midbrain cytosol.

The relative amounts of Ca^{2+}-dependent PKC activity (with 100 μM free Ca^{2+} and 1 μM PDBu) as a proportion of total PKC activity in lung, frontal cerebral cortex, cerebellum, spleen COS 7 cell, male midbrain and male pituitary cytosols was 23 ± 9, 17 ± 4, 33 ± 3, 34 ± 6, 97 ± 12, 45 ± 4 and 54 ± 11% respectively (n = 4), with the remainder of the total activity being Ca^{2+}-independent.

Figure 6.1

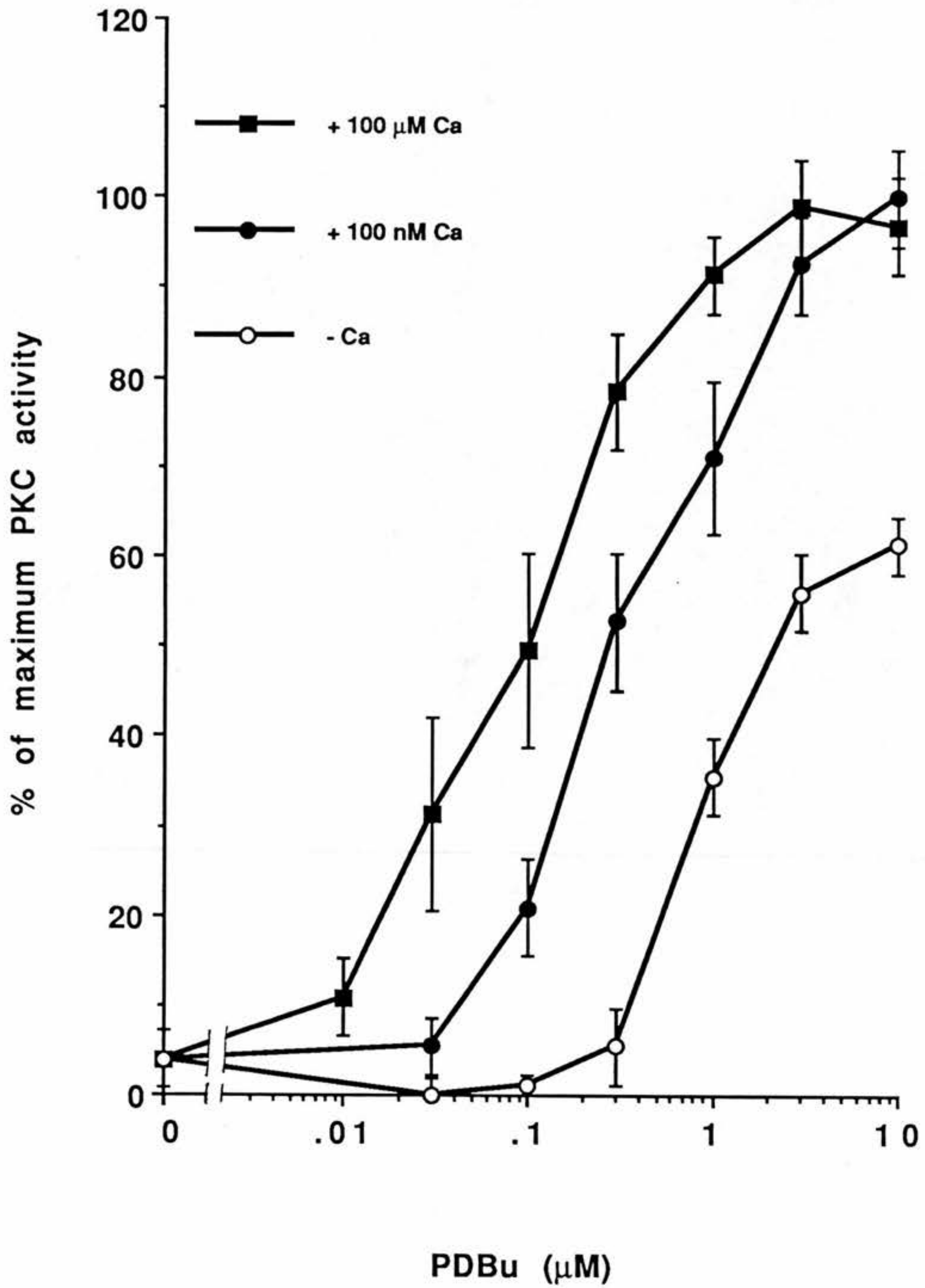
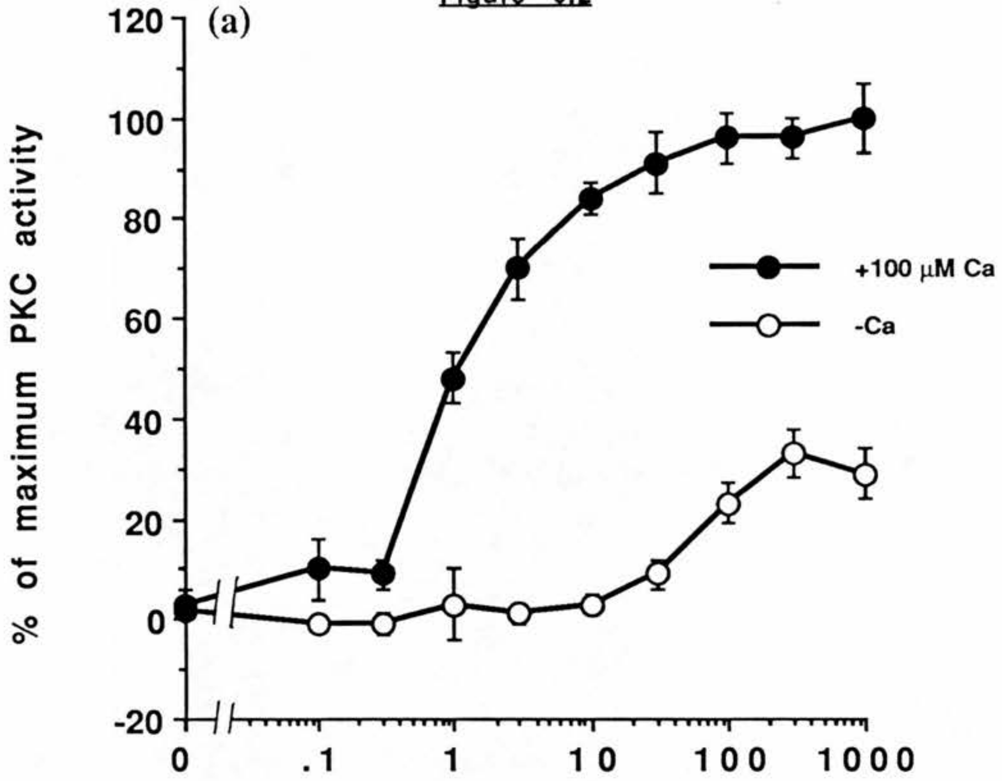
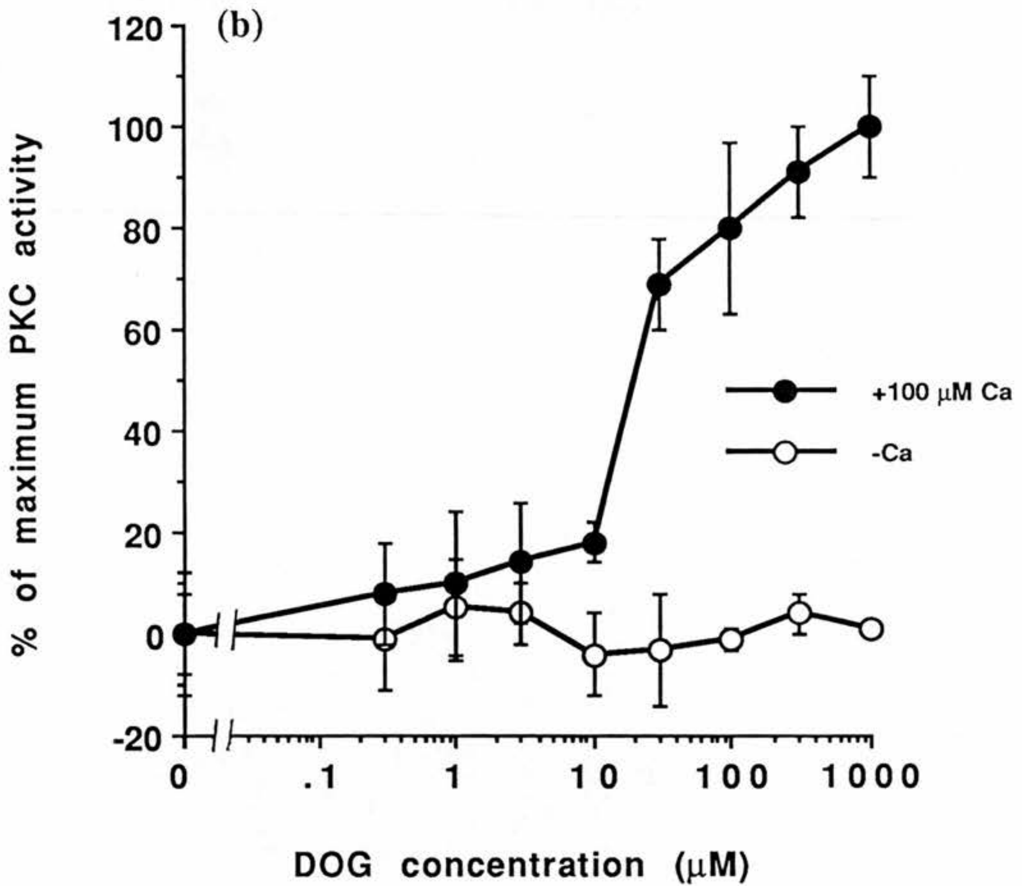


Figure 6.1. Effect of PDBu on partially-purified male rat midbrain cytosolic PKC activity.

Concentration-response relationship of PDBu stimulation of PKC activity from male rat midbrain cytosol whose PKCs were partially-purified on DE52 with 0 -150 mM NaCl. Stimulated PKC activity is shown in the absence of Ca^{2+} (open circles), the presence of 100 nM free Ca^{2+} (closed circles) or presence of 100 μM free Ca^{2+} (closed squares). The methodology employed was the same as that described in section 2.2.8. The data represent the mean ± SEM of 4 determinations, with values expressed as a % of the maximal activity seen. The incorporation of ^{35}S into the substrate (histone) in the presence of phosphatidylserine (100 μg/ml final with 0.04% Nonidet-P40) alone was subtracted from the data.

Figure 6.2





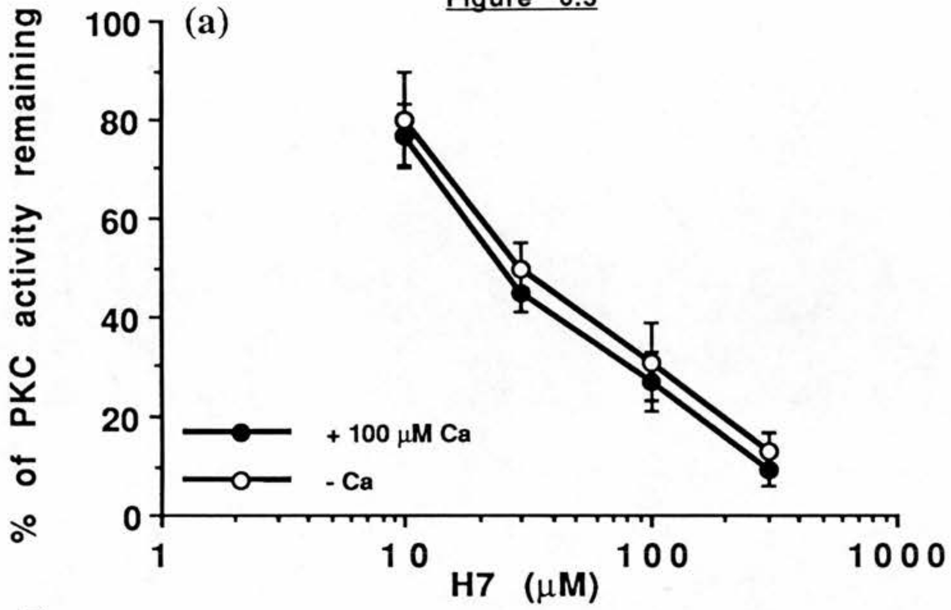
<u>Figure 6.2</u>. Ability of DOG to stimulate partially-purified PKC activity from male rat midbrain and COS-7 cell cytosol.

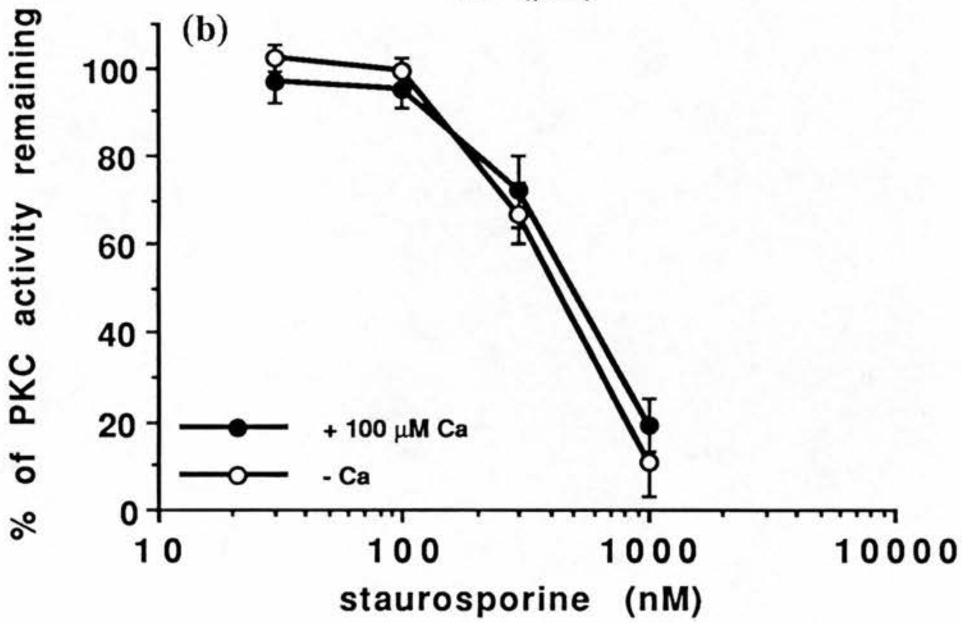
Concentration-response relationships for DOG to stimulate Ca^{2+}-independent (open symbols) and 100 μM Ca^{2+}-dependent (closed symbols) PKC activity from male rat midbrain (a) and COS-7 cell (b) cytosolic preparations that were partially-purified for PKCs on DE52 with 0 - 150 mM DE52. The methodology employed was the same as that described in section 6.2. The incorporation of ^{35}S into the substrate (histone) in the presence of phosphatidylserine (100 μg/ml with 0.04% Nonidet-P40) alone was subtracted from the data, which are expressed as a % of the maximal PKC activity of the tissue seen with 1 μM PDBu and 100 μM free Ca^{2+}. The data represent the mean ± SEM of 4 determinations.

Figure 6.3. **Effect of H7, staurosporine and Ro318220 on PKC activity from partially-purified male rat midbrain cytosol.**

Concentration-response relationships for H7 (a), staurosporine (b) or Ro318220 (c) to inhibit Ca^{2+}-independent (open symbols) and 100 μM free Ca^{2+}-dependent (closed symbols) PKC activity from male rat midbrain cytosol that had its PKCs partially-purified on DE52 with 0 - 150 mM NaCl. The methodology employed was that described in section 2.2.8. Incorporation of ^{35}S into the substrate (histone) under basal conditions (100 μg/ml phosphatidylserine with 0.04% Nonidet-P40 alone) was subtracted from the values of PKC activity. No significant differences between levels of Ca^{2+}-dependent and Ca^{2+}-independent PKC activity was seen for either H7, staurosporine or Ro318220. The data represent the mean ± SEM of 4 determinations.

Figure 6.3





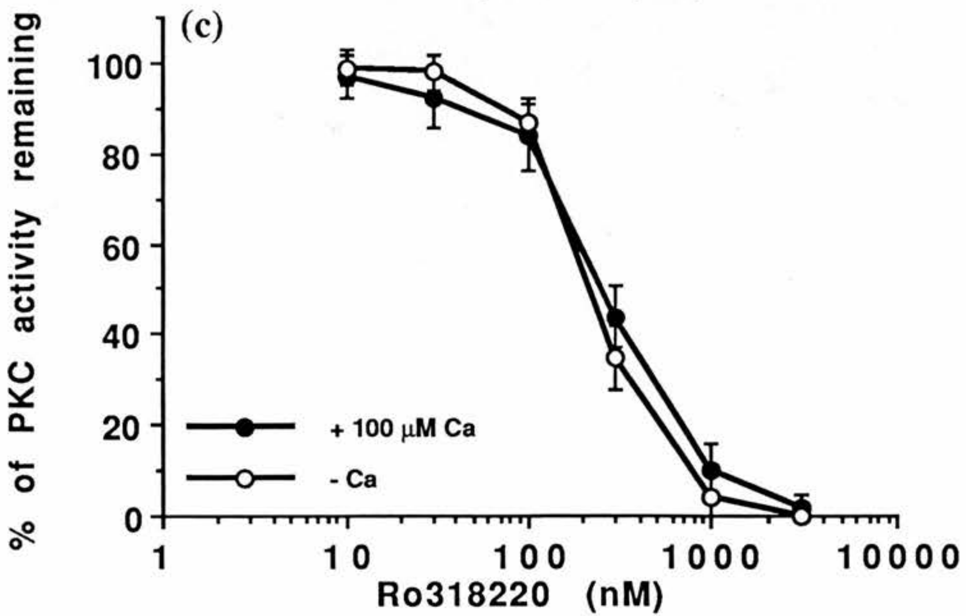


Figure 6.4. Effect of H7 on partially-purified PKC activity from male rat spleen, lung and anterior pituitary cytosol.

Concentration-response relationships for H7 (a, b, c) and staurosporine (d) to inhibit Ca^{2+}-independent (open symbols) and 100 μM Ca^{2+}-dependent (closed symbols) PKC activity from male rat spleen (a), lung (b) and anterior pituitary (c, d) cytosol that had its PKCs partially-purified on DE52 with 0 - 150 mM NaCl. The methodology employed was as described in section 2.2.8. Incorporation of ^{35}S into the substrate (histone) under basal conditions (100 μg.ml phosphatidylserine with 0.04% Nonidet-P40 alone) was subtracted from the values of PKC activity. The levels of inhibition seen between Ca^{2+}-dependent and Ca^{2+}-independent PKC activity with 30 and 100 μM H7 in lung and anterior pituitary were significantly ($P \leq 0.05$) different. The data represent the mean ± SEM of 4 determinations.

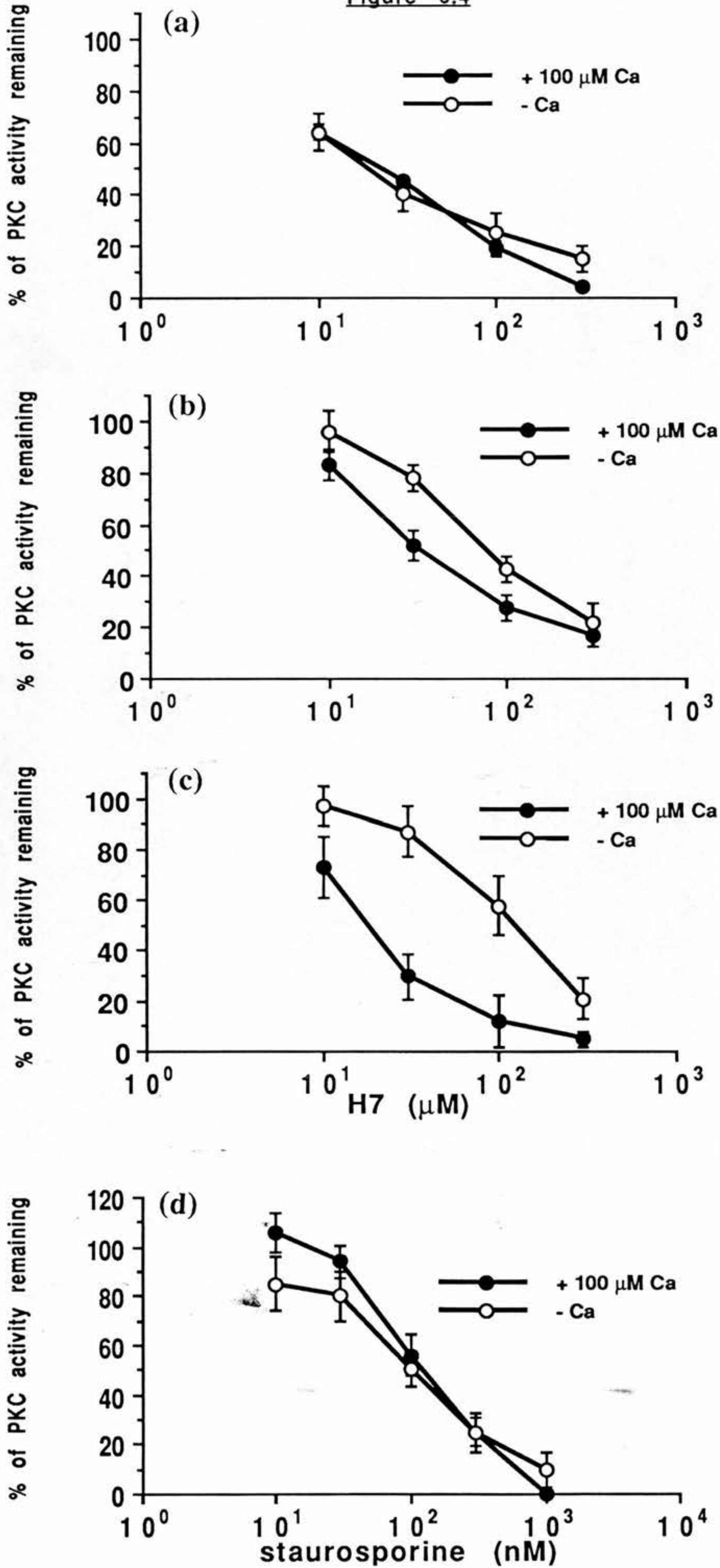


Figure 6.4
(a)
% of PKC activity remaining
+ 100 μM Ca
- Ca
(b)
% of PKC activity remaining
+ 100 μM Ca
- Ca
(c)
% of PKC activity remaining
+ 100 μM Ca
- Ca
H7 (μM)
(d)
% of PKC activity remaining
+ 100 μM Ca
- Ca
staurosporine (nM)

Table 6.1. **Potencies of H7 and staurosporine to inhibit PKC activity from various male rat tissue and COS-7 cells cytosolic preparations.**

The IC_{50} values for H7 and staurosporine to inhibit 100 μM Ca^{2+}-dependent and Ca^{2+}-independent PKC activity form the cytosols of various tissues was calculated by the program, 'P-fit' (section 2.2.10). The cytosol from each tissue had their PKCs partially-purified on DE52 with 0 - 150 mM NaCl, as described in section 2.2.8. The substrate for PKC used in each assay was histone, and ^{35}S incorporation into substrate under basal conditions (100 μg/ml phosphatidylserine with 0.04% Nonidet) was subtracted from 1 μM PDBu-stimulated and 1 μM PDBu plus 100 μM free Ca^{2+}-stimulated PKC activity (Ca^{2+}-independent and Ca^{2+}-independent plus Ca^{2+}-dependent PKC activity respectively). The data represent the mean ± SEM of 4 determinations.

Table 6.1

	IC$_{50}$s for H7 (μM)	
cytosol	**Ca^{2+}-dependent**	**Ca^{2+}-independent**
lung	39 ± 5	81 ± 10
frontal cerebral cortex	31 ± 5	35 ± 5
cerebellum	34 ± 9	38 ± 15
spleen	19 ± 3	25 ± 3
COS 7 cells	36 ± 11	No signal
male midbrain	34 ± 5	27 ± 9
male pituitary	17 ± 4	121 ± 18

	IC$_{50}$s for staurosporine (nM)	
cytosol	**Ca^{2+}-dependent**	**Ca^{2+}-independent**
male midbrain	450 ± 53	382 ± 34
male pituitary	101 ± 39	117 ± 46

6.4. DISCUSSION

Phorbol esters have been reported to have some actions which are independent of PKC (Doerner *et al,* 1990; Kolesnick and Paley, 1987; Kolesnick and Hemer, 1990; Billah and Anthes, 1990). However, these non-PKC-mediated effects of phorbol esters either occur at very high concentrations of phorbol esters (Doerner *et al,* 1990), in which the action of the compounds may be due to disruption of the lipid environment of cells; or are poorly understood. The main action of phorbol esters, such as PDBu, is still thought to be almost exclusively to activate PKC. In the kinase activity assay used here, we have termed the associated kinase activity to be that of the enzyme(s), PKC. However, it is possible that PDBu could be activating a kinase activity which is not PKC, but some other kinase which is also activated by PDBu. Such an enzyme has not been discovered, although Ryves *et al* (1989) have described a kinase which is not PKC, is selectively activated by a structurally related compound, resiniferatoxin and yet is also activated (although poorly (to approximately 15% of maximum)) by phorbol esters. This resiniferatoxin-sensitive kinase does not seem to have a role in our two models of PKC action in rat anterior pituitary cells (Chapter 3) as resiniferatoxin is without effect on either model (R Mitchell, unpublished observations). Thus, until disproved, the main action of PDBu here, is likely to involve a PKC, and the kinase activity assay used in this chapter is thought to represent PDBu-stimulated PKC activity. Also uncertain is the extent to which Ca^{2+}-dependent and Ca^{2+}-independent PKC activity represents the A and B series PKC isoforms respectively. Pelech *et al* (1991) have described forms of PKC-ß which lose their Ca^{2+}-dependence and are produced upon the phosphorylation of the enzyme after PMA stimulation of platelets. The elution from Mono Q ion-exchange chromatography columns of the various forms of phosphorylated PKC-ß was retarded, and alkaline phosphatase treatment of these forms resulted in the production of the original PKC-ß species. These Ca^{2+}-independent forms of PKC-ß also displayed a high histone

phosphorylating capacity. It is possible that the Ca^{2+}-independent PKC activity displayed here, could represent (at least in part) such Ca^{2+}-independent forms of A series PKC isoforms. However, several lines of evidence suggest this not to be the case. Firstly, PDBu-stimulation of Ca^{2+}-independent PKC activity in midbrain (which is known to contain substantial amounts of B series isoforms) resulted in a 50% maximal activity of the total PKC activity inducible in the added presence of Ca^{2+} (Figure 6.1), suggesting that a specific subset of PKCs is being activated, and the activation of Ca^{2+}-independent PKC activity is not simply a 'breakthrough' in activity of Ca^{2+}-dependent PKCs in the absence of Ca^{2+} at higher concentrations of PDBu (similar results were also obtained with DOG activation of midbrain cytosolic PKCs (Figure 6.2(a))). Furthermore, DOG-activation of α-PKC from COS 7 cells, resulted in no increase in activity in the absence of Ca^{2+}, with only Ca^{2+}-dependent PKC activity being seen, as would be expected for an A series PKC isoform. Lastly, although histone was suggested to be a poor substrate for PKC-ε (Schaap *et al,* 1989), it is known that other B series isoforms can use histone as a convenient substrate (Ono *et al,* 1987b; Ono *et al,* 1988a; Ono *et al,* 1989b; Nishizuka, 1988; Mischak *et al,* 1991), and for midbrain at least, B series isoforms of PKC are known to be present.

Activation kinetics of PKC by activators is a complex issue (Bazzi and Nelsestuen, 1989). In Figure 6.1, it is seen that free Ca^{2+} at a concentration of 100 nM (which approximates to the basal cytosolic calcium concentration within cells) is sufficient to activate Ca^{2+}-dependent PKCs in addition to Ca^{2+}-independent PKCs. This suggests that basal cytosolic Ca^{2+} within a cell is sufficient to activate significantly Ca^{2+}-dependent PKCs but only under cytosolic conditions of higher free Ca^{2+} concentration will the activity of PKC be enhanced sufficiently to become fully active at lower concentrations of activator (in this case, PDBu). Thus any activator of the Ca^{2+}-dependent PKCs will be more efficacious under stimulated cellular conditions of high Ca^{2+}. Activity assays of PKC have always been carried out under conditions of high Ca^{2+} or zero Ca^{2+} (in excess EGTA). However, physiologically, the free cytosolic

Ca^{2+} concentration never would be reduced to the levels of free Ca^{2+} seen in the presence of excess EGTA, and perhaps a more physiological assessment of PKC activity should be carried out under conditions of high and low Ca^{2+}, and not high and zero (< 3 nM) Ca^{2+}. The presence of 100 μM Ca^{2+} induced a leftward shift in the potency of PDBu towards cytosolic midbrain PKC activity (Figure 6.1). This is consistent with the work of others (Sekiguchi *et al,* 1988; Huang *et al,* 1988; Naor, 1990) who have also shown that high concentrations of Ca^{2+} enhance the activity of A series isoforms of PKC to any set concentration of activator, but always induce the maximal level of PKC activity seen in low Ca^{2+} concentrations, and never any additional activity in the presence of high concentrations of Ca^{2+}. The results in Figure 6.1 would suggest that PDBu has reduced potency towards Ca^{2+}-independent forms of PKC compared to the PDBu potency towards Ca^{2+}-activated forms of PKC. Furthermore, the DOG potency towards Ca^{2+}-independent PKCs is also reduced compared to the potency of DOG towards Ca^{2+}-dependent forms of PKC from midbrain cytosol (Figure 6.2(a)). It also appears that DOG is slightly less efficacious towards Ca^{2+}-independent forms of PKC from midbrain, as the maximal PKC activity in the presence of Ca^{2+} was similar for both DOG- and PDBu-activation. However, the maximal activity of the Ca^{2+}-independent forms of PKC was less for DOG than it was using PDBu as an activator. The functional significance of this is uncertain but may represent a partially-selective profile of activation being exhibited by DOG rather than by PDBu. In this vein, the potency of DOG to activate PKC-α from COS 7 cells was markedly reduced compared to the potency of DOG to activate Ca^{2+}-dependent forms of PKC from midbrain (which contains a mixture of all known PKC isoforms (Young, 1989)) (EC_{50}= 28 ± 12 and 1.4 ± 0.6 μM respectively). The exact content of PKC-α in male rat midbrain is unknown as the identification of the isoforms was carried out by mRNA *in situ* hybridisation, which is not quantitative, only qualitative. However, the presumed pure PKC-α from COS 7 cells was still activated by DOG (but only in the presence of Ca^{2+}). This suggests that DOG has reduced potency towards

PKC-α and is consistent with the findings in Chapter 5, in which the affinity of DOG for displacement of specific [^{3}H]-PDBu binding was reduced in cytosol preparations with a high PKC-α content (Figure 5.8). These results are supportive of the contention that the α-isoform of PKC is involved in the reduction of 'L'-channel activity in anterior pituitary cells (Chapters 3 and 4) and that DOG up to 100 μM only poorly activates this isoform *in vitro* and in cell responses (Figure 3.5).

One other putative PKC isoform-selective compound which emerged from our studies on anterior pituitary cell K^+-induced $^{45}Ca^{2+}$ influx (Chapter 3) was the PKC inhibitor, H7 (Figure 3.10), with the effect of PDBu on anterior pituitary prisms being more resistant to inhibition by H7 than that in GH_3 cells. In male rat midbrain cytosol preparations (Figure 6.3), neither H7, staurosporine nor Ro318220 showed any disparity in their potencies of inhibition of Ca^{2+}-independent PKC activity. However, an 'H7-resistant' Ca^{2+}-independent PKC activity was found in male anterior pituitary cytosol, whereas both Ca^{2+}-dependent and Ca^{2+}-independent PKC activities were inhibited by staurosporine with a similar potency (Figure 6.4). This finding was similar to those on modulation of 'L'-channel activity by PDBu in rat anterior pituitary prisms and GH_3 cells with the enhancement of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells being less sensitive to inhibition by H7 than the H7-inhibition of the corresponding PDBu-response in GH_3 cells (staurosporine being equally sensitive on both responses (see Chapter 3)), and it may be that it is this Ca^{2+}-independent form which is involved in the enhancement by PDBu of 'L'-channel activity in anterior pituitary pieces (and possibly by other agents in GH_3 cells too). Nakadate *et al* (1989) have described a PKC inhibitor profile of H7 that is similar to that found here. In mouse epidermal cells, quercetin inhibited with matched potencies, phorbol ester-induced ornithine decarboxylase induction and phorbol ester-induced suppression of epidermal growth factor binding. However, the phorbol ester-caused inhibition of epidermal growth factor binding was markedly less sensitive to inhibition by H7 than phorbol ester-induction of ornithine decarboxylase (Nakadate *et al,* 1989). The authors

suggested that the disparity with H7 could be due to the differences in substrate used by PKC in whole mouse epidermal cells, whereas here a similar pharmacological profile was seen with H7, but on the same substrate (histone-IIIS; Figure 6.4), and it may be that the reduced potency to H7 in mouse epidermal cells could reflect such an H7-insensitive form of PKC (a possibility not raised by Nakadate *et al,* 1989). A range of tissue cytosol preparations was also investigated to see whether they possessed an 'H7-resistant' kinase activity (Table 6.1). The majority of tissues tested did not possess an 'H7-resistant' PKC activity, but displayed more normal sensitivity to H7 ($< 40\ \mu M$) as was seen for baculovirus expressed ε-PKC and purified $\alpha/\beta/\gamma$ mixtures from bovine brain (Schaap and Parker, 1990). However, male rat anterior pituitary, lung (Figure 6.4) both contained an 'H7-resistant', Ca^{2+}-independent PKC activity (with lung being slightly more sensitive than pituitary). The distribution of this activity does not coincide with the content of any particular known isoform (Ono *et al,* 1987a; Ono *et al,* 1988a; Ono *et al,* 1989b; Shearman *et al,* 1988; Young, 1989). Further extensive study has so far not revealed any other tissues that possess a kinase activity which has reduced affinity towards H7 inhibition (M S Johnson and R Mitchell, unpublished observations) and it may be that this pharmacologically-distinct form of PKC is one which is previously unclassified, is present selectively in rat anterior pituitary (and to a lesser extent in lung) and is a novel form of PKC (possibly a novel sequence isoform).

Curiously, the sensitivity of the PDBu-stimulated kinase activities is lower to staurosporine-inhibition (ie, $IC_{50}s > 100$ nM) than the potency of the inhibition by staurosporine seen in other models ($IC_{50}s = 19 \pm 8$ and 15 ± 10 nM on PDBu-induced modulation of K^+-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms and GH_3 cells respectively (see section 3.3)) and reported by others (Kase *et al,* 1987). The reasons for this reduced potency to staurosporine are unclear, but were also reported in a mixed-micellar PKC activity assay similar to ours (Schaap and Parker, 1990) and may represent a physicochemical interaction of staurosporine with the detergent, Nonidet P-40. Also of interest was the fact that the potency of staurosporine in male

and female anterior pituitary cytosol was somewhat higher than that found using male and female midbrain cytosols (Table 6.1). The reasons for this discrepancy are presently unclear (not being obviously reflected in the experiments on pharmacology of Ca^{2+} channel regulation). Full characterisation of the different forms of PKCs underlying these phenomena and their properties clearly warrants further extensive study. Moreover, characterisation of a kinase activity which is particularly sensitive to mezerein, is currently being investigated within our laboratory.

CHAPTER 7

OVERVIEW

OVERVIEW

A large number of cellular communication molecules signal within their target cells by transduction processes which, in part, involve the activation of protein kinase C (PKC). Not only the various neurotransmitters, hormones and growth factors that primarily signal through hydrolysis of phosphoinositides (Michell, 1975; Berridge and Irvine, 1984; Downes and Michell, 1985), but also those which can induce a hydrolysis of phosphatidylcholine (Billah and Anthes, 1990; Exton, 1990) are able to generate diacylglycerol, which is an endogenous activator of PKC (Nishizuka, 1984a). Both transmitters which can cause activation of the enzymes, phospholipase A_2 and phospholipase D, have the potential to generate lipid products which can activate PKC (Axelrod *et al,* 1988; Pelech and Vance, 1988; Löffelhotz, 1989). It is clear that PKC has a crucial role in the signalling of a vast array of extracellular stimuli. However, although PKC has a large number of target proteins (see Table 1.1), extracellular signals may act to produce 'signal-specific' responses in their target cells. This may be achieved in part through auxiliary signalling factors (eg, concomitant production of various inositol phosphates, steroidal environment, intracellular ionic environment and regulation of ion channel function, metabolic sufficiency, etc), but could also be influenced by any activator and/or substrate specificity of the isoforms of PKC (Huang, 1989; Parker *et al,* 1989; Pfeffer *et al,* 1990). The mechanisms by which extracellular stimuli can control the activity of specific PKC isoforms is of immense interest as clarification of these mechanisms may lead to the generation of novel classes of therapeutically-useful compounds. Such agents may also be used to advance our basic knowledge of cellular function/dysfunction, and thus in turn, generate novel therapeutic opportunities. The present investigations here were an attempt to advance our knowledge of the pharmacology of various forms of PKC, and to relate this knowledge to physiological events in functioning cells.

Depolarisation-induced 'L'-type Ca^{2+} channel activity in rat anterior pituitary cells was used as a model of the actions of distinct forms of PKC. It was clear (Chapter 3) that overtly similar 'L'-channels existed in rat anterior pituitary tissue and in the GH_3 anterior pituitary cell line, however activation of PKC by PDBu leads to either an enhancement or inhibition of the 'L'-type Ca^{2+} channel respectively. This tissue-specific dual modulation may be due to the differing PKC isoform content of each tissue (Chapter 5) (although, of course, other factors such as endogenous modulators, auto-phosphorylations or other unknown influences may contribute). The α-PKC content of GH_3 cells is markedly higher than the content of α-PKC in whole rat anterior pituitary. The α-PKC content in each tissue could be successfully predicted by the novel means of associations between the effects of AA and DOG on [^{3}H]-PDBu binding aids, discerning the content of cytosolic α-PKC in a tissue. These predictions were closely in agreement with the work of Naor (1990) (who assessed the α-PKC content through Western immunoblot). It is possible that distinct PKC isoforms may enhance or inhibit 'L'-type Ca^{2+} channel function by influencing the phosphorylation of separate PKC phosphorylation sites that are known to exist on the α_1 and ß subunits of the 'L'-type Ca^{2+} channel and such a mechanism may account for the differences seen between rat anterior pituitary pieces and GH_3 cells. A dual control by PKC was also seen for the epithelial Cl^--channel (Li *et al,* 1989) where the differential action of PKC isoforms was also suggested. Our evidence for a dual PKC-mediated modulation of 'L'-type Ca^{2+} channels which is tissue-determined (see section 1.3) and is a result of the prevailing PKC isoform-content of the cell (MacEwan and Mitchell, 1991) may be of considerable importance in consideration of the control of voltage-sensitive Ca^{2+} (and other ion) channels in a variety of tissues.

Further support for our hypothesis that distinct forms of PKC mediate an enhancement or inhibition of 'L'-channel activity in anterior pituitary cells comes from work on a series of agents with apparent selectivity for different forms of PKC (Chapter 3). Indeed, not only can an enhancement of 'L'-channel activity be seen in

both anterior pituitary prisms and GH_3 cells with compounds such as mezerein, but an inhibition of 'L'-channel activity can be shown in both anterior pituitary prisms and GH_3 cells with the ß-PKC isoform-selective phorbol ester, DOPPA (section 3.4). Therefore, it is not assumed to be not an invariant, tonic influence on 'L'-type Ca^{2+} channel in anterior pituitary prisms or GH_3 cells which governs an enhancement or inhibition of the channel activity, but the outcome of at least 2 inverse influences which are already seen to be amenable to pharmacological manipulation with putative PKC isoform-selective compounds. The ability of DOPPA to reduce K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells and anterior pituitary prisms (section 3.4) implicates a role for ß-PKC in the reduction of 'L'-channel activity, and suggests that it is not ß-PKC that is involved in enhancing 'L'-channel activity. In addition to this, arachidonic acid (AA) could reduce 'L'-channel activity in GH_3 cells. Arachidonic acid can selectively activate the γ-isoform of PKC, and to a lesser extent, the α- (and then ß-) isoforms of PKC (Naor *et al,* 1988a). However, γ-PKC is absent from GH_3 cells, but the cells do contain at least α-, ß- and ε-PKC (Naor (1990) and R Mitchell, unpublished observations), a profile which was also found in the related GH_4C_1 cell line (Kiley *et al,* 1991). The inhibition by AA (without its metabolic conversion) of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells is mediated by PKC (Chapter 4). However, the known content of PKC isoforms in the cells indicates that AA-activation of γ-PKC is not responsible for the inhibition of 'L'-channel activity in GH_3 cells (as γ-PKC is absent from the cells), thus suggesting that it may well be α-PKC (and perhaps also ß-PKC) which is mediating the AA-induced inhibition of 'L'-channel activity in GH_3 cells. The responsiveness of ε-PKC (or other unknown isoforms of PKC in GH_3 cells) to AA-activation is unknown. It is thought that a Ca^{2+}-dependent form of PKC (such as α or ß) is responsible for the AA-induced inhibition of 'L'-channel activity in GH_3 cells, as agents which raise intracellular calcium concentrations without activating PKC (such as AA-methyl ester (Chapter 4 and Chan and Turk, 1987; Seifert *et al,* 1988)) enhanced a PDBu-induced inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells to levels of

inhibition similar to those seen maximally with AA (Chapter 4). Unlike AA, PDBu did not raise intracellular Ca^{2+} concentrations (Chapter 4). Therefore, it seems that AA can fully reduce K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells by PKC activation (as occurs with PDBu) with the additional raising of intracellular Ca^{2+} concentrations to thereby further enhance the activity of a Ca^{2+}-dependent form of PKC. Such a presumably Ca^{2+}-dependent form of PKC could well be predominantly α-PKC as the AA-activation of α-PKC in the presence of Ca^{2+} is markedly greater than the activation of ß-PKC (Naor *et al,* 1988a).

Supportive of the contention that it is the α-isoform of PKC which mainly mediates the inhibition of K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells is the inability of DOG to cause an inhibition of 'L'-channel activity in GH_3 cells (Chapter 3). In cytosolic [^{3}H]-PDBu binding studies (Chapter 5), DOG was found to have a reduced affinity towards α-PKC. Furthermore, DOG had reduced potency in activation of α-PKC from COS 7 cells compared to its activation of a mixture of α, ßI, ßII, γ, δ, ε and ζ isoforms of PKC from rat midbrain (Chapter 6). Therefore, these reduced activities of DOG towards α-PKC may well be sufficient to account for the inability of the synthetic short chain diacylglycerol (up to 100 μM) to reduce 'L'-channel activity in GH_3 cells. Due to its cell permeability, DOG is often used to activate PKC in cell preparations, but its reduced ability to activate α-PKC as exemplified here should be taken into account in any such cases comparing DOG with phorbol esters.

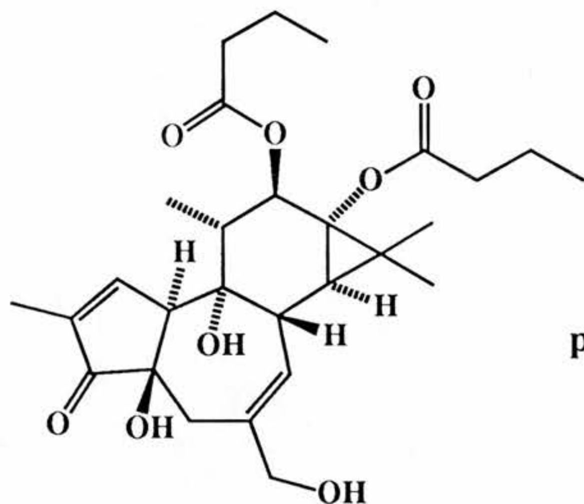
It seems that it is, therefore, mainly the α-isoform of PKC (and to some extent the ß-isoform) which is responsible for the inhibition of 'L'-type Ca^{2+} channels in rat anterior pituitary cells. However, the isoform(s) which is responsible for enhancement of 'L'-type Ca^{2+} channel activity in rat anterior pituitary cells is less certain. Mezerein appears to activate selectively the form(s) of PKC which causes a facilitation of 'L'-channel activity in both anterior pituitary prisms and GH_3 cells (Chapter 3). The PDBu-activated form of PKC which enhances 'L'-channel activity in anterior pituitary prisms is relatively insensitive to inhibition by H7, compared to the

PDBu-activated form(s) of PKC which inhibits 'L'-channel activity in GH_3 cells (Chapter 3). It is not known whether the mezerein-activated form of PKC is the same 'H7-insensitive' form of PKC which is activated by PDBu in anterior pituitary prisms, or if indeed these are two separate forms of PKC which are present in pituitary cells. Interestingly, an H7-resistant PDBu-activated PKC activity was found in cytosol from male rat anterior pituitary (Chapter 6), but not from male rat midbrain, which was reported to contain mRNA for all the known seven isoforms of PKC (Young, 1989). This suggests that anterior pituitary contains a novel form of PKC or related kinase which is neither α-, ßI-, ßII-, γ-, δ-, ε- nor ζ-PKC (or at least their unmodified holoenzymes). This novel form of PKC was implicated to be involved in the mechanism by which the LHRH self-priming phenomenon (Chapter 1.4) in female rat anterior pituitary occurred (M S Johnson, R Mitchell and F J Thomson, unpublished observations), but its exact targets *in vivo* have still to be assessed. This 'H7-insensitive' form of PKC is thought to be a Ca^{2+}-independent form of PKC, as it is found in the proportion of total PKC activity that is active in the absence of Ca^{2+} ions. The PKC activity which is 'H7-insensitive' has a distinct tissue distribution, with its presence not only in the cytosolic fractions from male anterior pituitary, but also from lung, female anterior pituitary and female midbrain. Whether the existence of the 'H7-insensitive' PKC in female midbrain (but not male midbrain) is an issue of gender-specific transcriptional regulation or steroidal-control is unknown. The further characterisation of this 'H7-insensitive' PKC in terms of its biochemistry, pharmacology and physiological roles, is a topic of current active investigation within our laboratory.

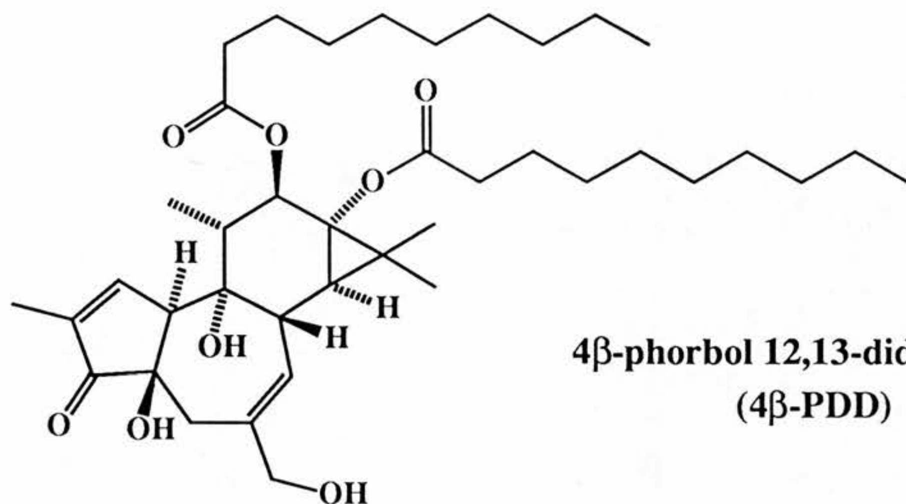
In Chapter 4, experiments were reported that had been designed to assess whether TRH receptor activation could lead to PLA_2 activation. Although the results did not reach statistical significance, the physiological implications of such findings would be of great interest with respect to signalling mechanisms in GH_3 cells, especially since AA has been reported to interact with diacylglycerol to synergistically

activate PKC isoforms (Shinomura *et al,* 1991) as well as directly activating some of them alone. It is known that activation of TRH receptors on GH_3 cells leads to the production of both Ins(1,4,5)P_3 and 1,2-diacylglycerol (Drummond, 1986). The exact mechanism by which TRH receptors might induce increased AA liberation in the present experiments is unknown. Activation of TRH receptors could perhaps lead to the direct stimulation of phospholipase A_2 (PLA_2) by a putative G-protein link (α or $\beta\gamma$ subunits), or the AA production could be in indirect consequence of either an Ins(1,4,5)P_3-induced rise in intracellular Ca^{2+} concentration or a diacylglycerol-induced activation of PKC (or both). Each of these mechanisms of PLA_2 activation has been shown in other systems (see section 1.2.4). It is also possible that AA production is occurring as a consequence of diacylglycerol deacylation (see section 1.2.5). In addition to the fact that AA is metabolised to eicosanoids in GH_3 cells, it has been clearly demonstrated here that AA can enhance activation of PKC within GH_3 cells. The physiological implications of this action of AA are widespread, and include a possible role for AA in the regulation of Ca^{2+} channels by means of acting either as a direct activator, or synergistic factor for certain forms of PKC. TRH receptor-operated, influx of $^{45}Ca^{2+}$ into anterior pituitary tissue occurs through a channel of which only a small proportion are 'L'-type Ca^{2+} channels (Mitchell *et al,* 1989). In GH_3 cells, it has been reported that PKC can reduce TRH-induced increases in cytosolic Ca^{2+} concentrations (Drummond, 1985). It could therefore be, that TRH-induced activation of PKC will result in closing of 'L'-type Ca^{2+} channels (possibly in a selective fashion via the α-isoform of PKC). Moreover, the TRH-induced production of AA may further contribute to the closure of 'L'-type Ca^{2+} channels, as was seen here for AA-induced reductions in K^+-induced $^{45}Ca^{2+}$ influx in GH_3 cells (Chapter 3). The extent to which AA and diacylglycerol synergise to activate (possibly selected) isoforms of PKC is unclear, but each of these mechanisms will lead to a reduction in calcium influx through 'L'-type Ca^{2+} channels.

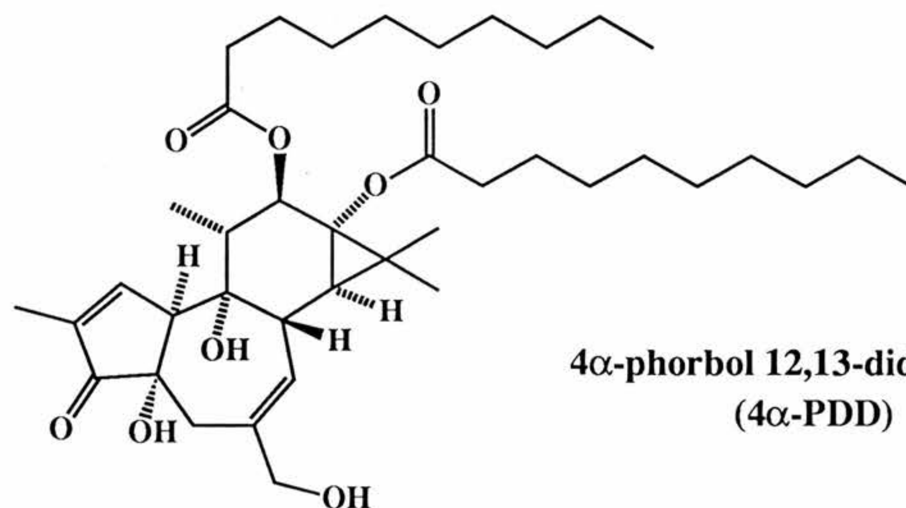
The present results support the idea that cellular actions of hormones and neurotransmitters, which are specific for the target cell encountered, may be determined by the regulation of the cell-specific expression of key components of the signal transduction machinery. The expression of such components may be governed by factors such as steroids, and indeed other secondary-messenger systems to produce a complex and interactive network of control. Such factors are likely also to control the expression of distinct forms of PKC in a manner which is appropriate to both the function of the expressing cell, and the environment and history of the cell type. Once generated, the various forms of PKC are susceptible to regulation by processes such as phosphorylation, which will govern the activity of the enzymes and their specificity towards various endogenous activators, inhibitors and substrates. Any differences in the rates of degradation of different forms of PKC may also contribute to the balance of functional outcome. The selective nature of PKC isoforms towards their substrate targets may have an important role *in vivo,* resulting in such controlled processes as secretion and electrical excitability. The modest differences seen here in our biochemical assays *in vivo,* may well translate into more profound differences on whole cell/tissue systems and *in vivo* due to the presence of selective endogenous activators and distinct protein targets highly preferred by some forms of the enzyme. The preliminary evidence obtained here for heterogeneity in the pharmacology of distinct forms of PKC may provide leads for the development of novel therapeutic agents.



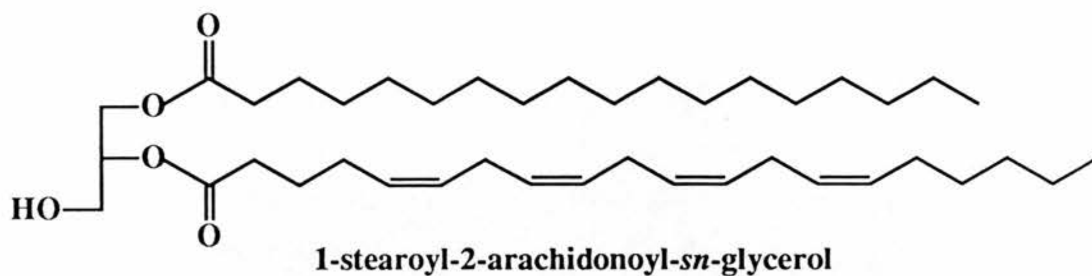
phorbol 12,13-dibutyrate
(PDBu)



4β-phorbol 12,13-didecanoate
(4β-PDD)



4α-phorbol 12,13-didecanoate
(4α-PDD)



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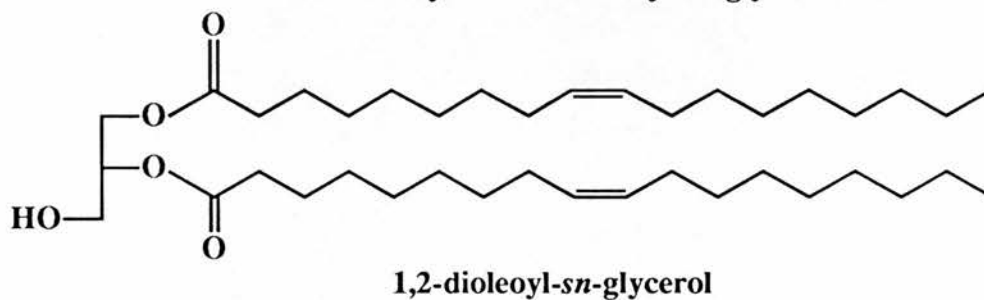

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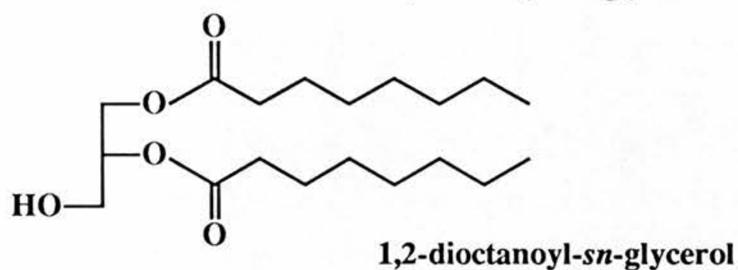

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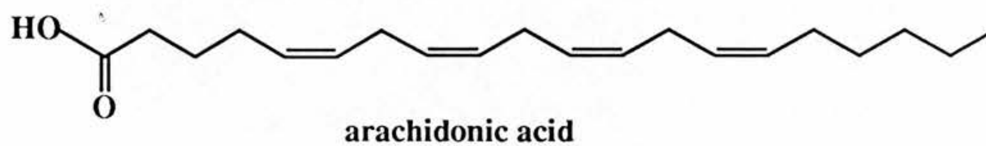
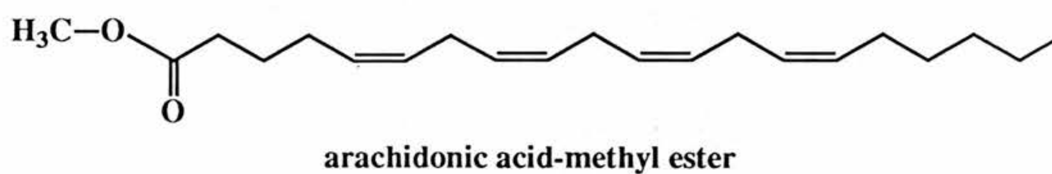


arachidonic acid



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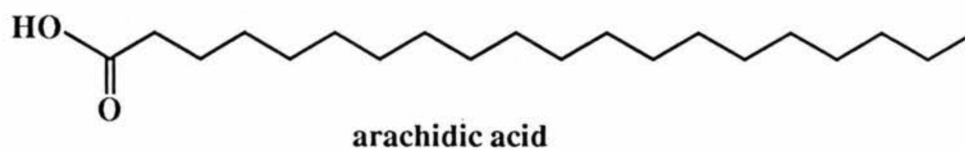
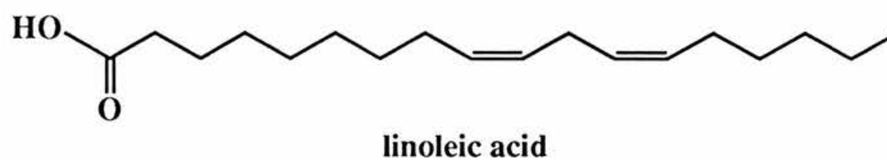
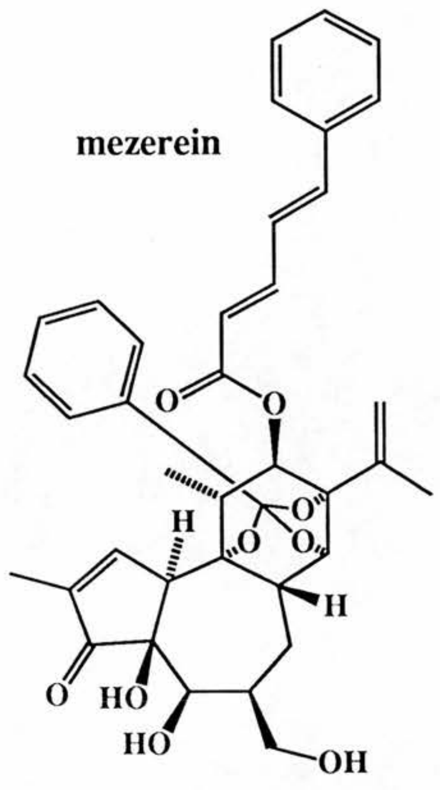
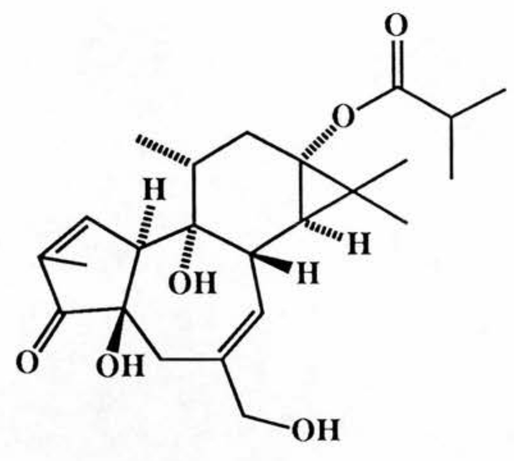


arachidic acid

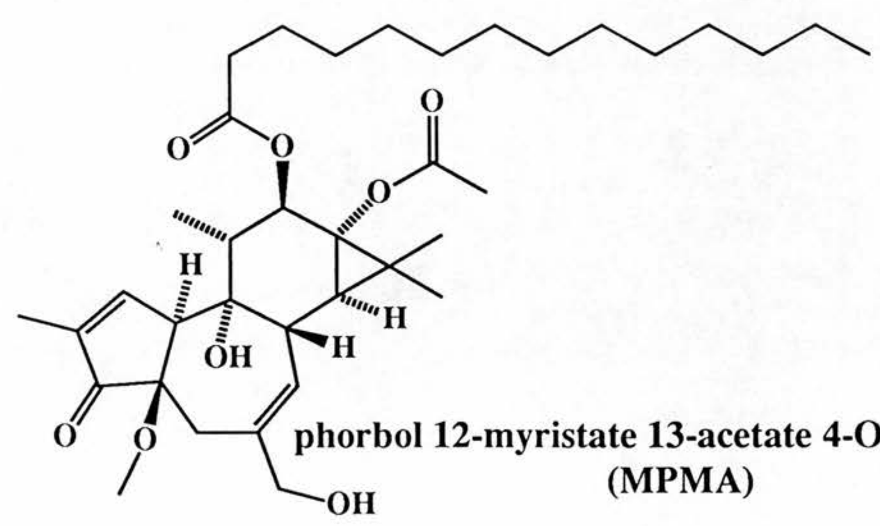


linoleic acid

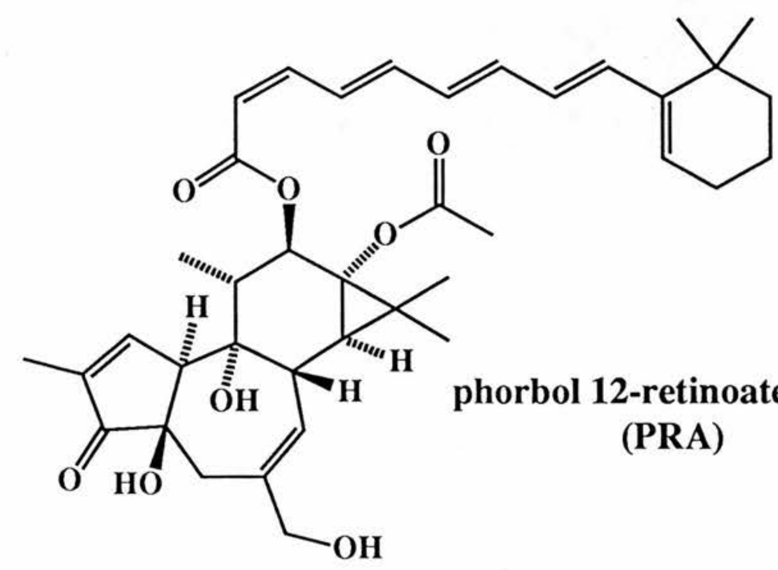




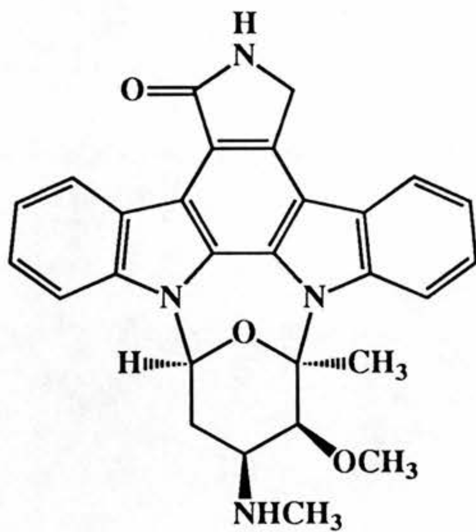
12-deoxyphorbol 13-isobutyrate
(DPB)



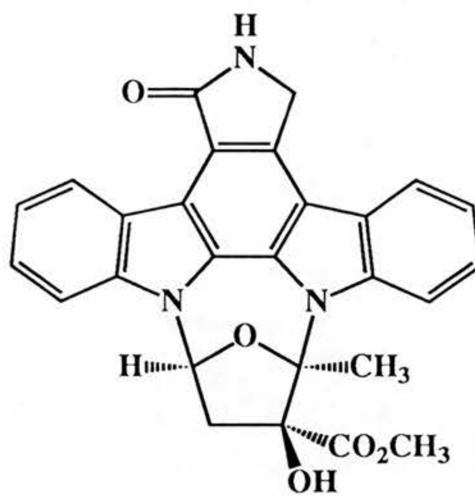
phorbol 12-myristate 13-acetate 4-O-methyl ether
(MPMA)



phorbol 12-retinoate 13-acetate
(PRA)



Staurosporine



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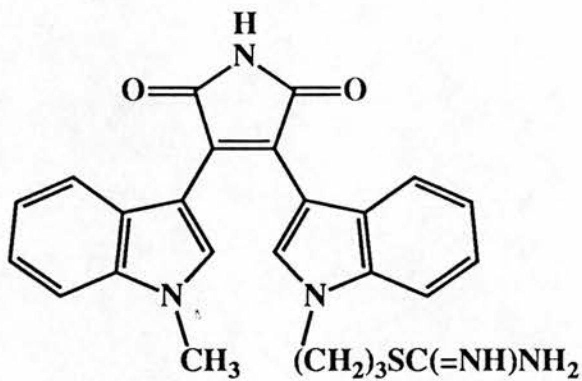

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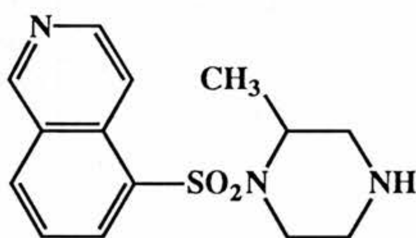
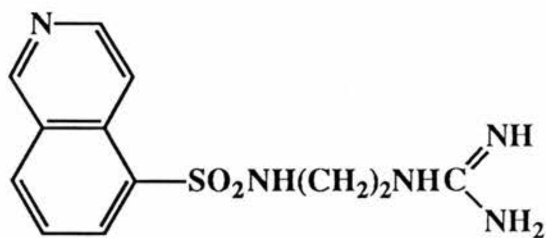
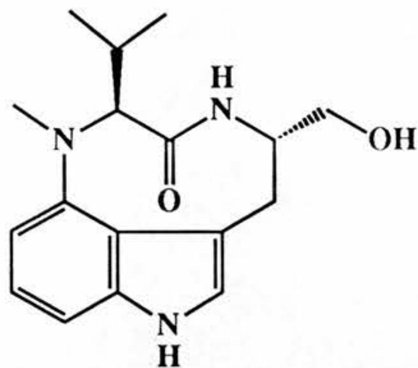


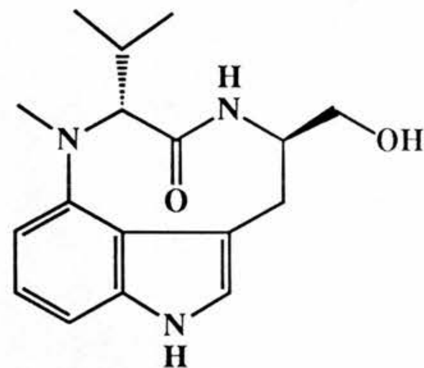


H7

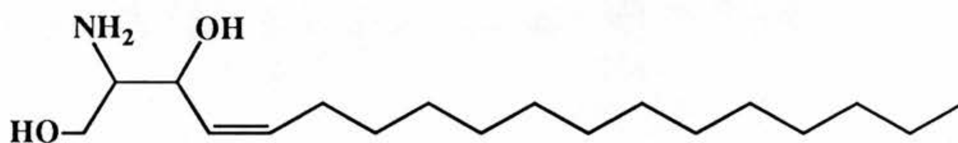
HA1004



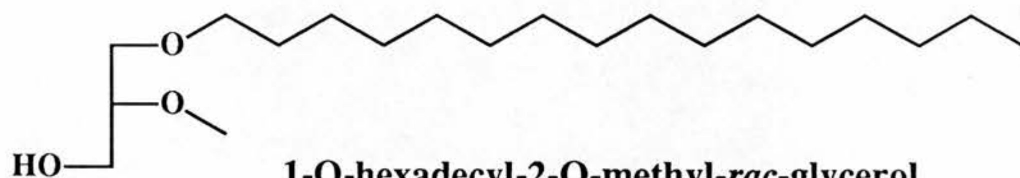
(-)-indolactam V



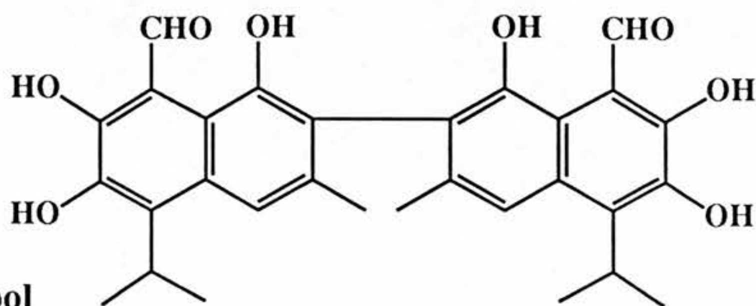
(+)-indolactam V



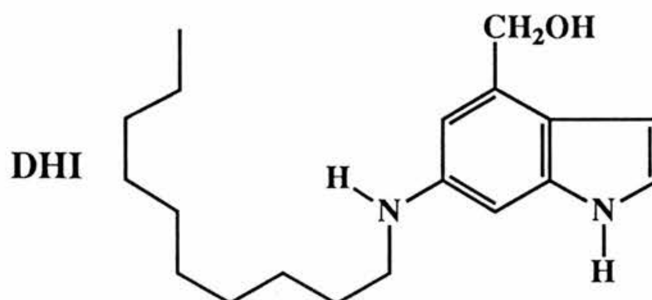
sphingosine



1-O-hexadecyl-2-O-methyl-*rac*-glycerol
(AMG-C_{16})



gossypol



DHI

BIBLIOGRAPHY

Aiyer, M.S., Chiappa, S.A. & Fink, G. (1974). A priming effect of luteinizing hormone-releasing factor on the anterior pituitary gland in the female rat. *Journal of Endocrinology* **62,** 573-588.

Abou-Samra, A.-B., Catt, K. J. & Aguilera, G. (1986). Role of arachidonic acid in the regulation of adrenocorticotropin release from rat anterior pituitary culture cells. *Endocrinology* **119**, 1427-1431.

Aiyer, M. S., Chiappa, S. A. & G, F. (1974). A priming effect of luteinizing hormone-releasing factor on the anterior pituitary gland in the female rat. *Journal of Endocrinology* **62**, 573-588.

Akiba, S., Sato, T. & Fujii, T. (1990). Involvement of a guanine-nucleotide-binding protein-mediated mechanism in the enhancement of arachidonic acid liberation by phorbol 12-myristate 13-acetate and Ca^{2+} in saponin-permeabilized platelets. *Biochimica et Biophysica Acta* **1044**, 291-296.

Albert, P. R. & Tashjian, A. H. (1984a). Thyrotropin-releasing hormone-induced spike and plateau in cytosolic free calcium concentrations in pituitary cells: in relation to prolactin release. *Journal of Biological Chemistry* **259**, 5827-5832.

Albert, P. R. & Tashjian, A. H. (1984b). Relationship of thyrotropin-releasing hormone-induced spike and plateau phases in cytosolic free calcium concentrations to hormone secretion: selective blockade using ionomycin and nifedipine. *Journal of Biological Chemistry* **259**, 15350-15363.

Albert, P. R., Wolfson, G. & Tashijian, A. H. (1987). Diacylglycerolincreases cytosolic free Ca^{2+} concentration in rat pituitary cells. Relationship to thyrotropin-releasing hormone action. *Journal of Biological Chemistry* **262**, 6577-6581.

Alexander, D. R., Graves, J. D., Lucas, S. C., Cantrell, D. A. & Crumpton, M. J. (1990). A method for measuring protein kinase C activity in permeabilized T lymphocytes by using peptide substrates. Evidence for multiple pathways of kinase activation. *Biochemical Journal* **268**, 303-308.

Anderson, M. P. & Welsh, M. J. (1990). Fatty acids inhibit apical membrane chloride channels in airway epithelia. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 7334-7338.

Andrews, W. V. & Conn, P. M. (1986). Gonadotropin-releasing hormone stimulates mass changes in phosphoinositides and diacylglycerol accumulation in purified gonadotrope cell cultures. *Endocrinology* **118**, 1148-1158.

Armstrong, D. & Eckert, R. (1987). Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarisation. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 2518-2522.

Armstrong, D. L. (1989). Calcium channel regulation by calcineurin, a Ca^{2+}-activated phosphatase in mammalian brain. *Trends in Neurosciences* **12**, 117-122.

Ashendel, C. L., Staller, J. M. & Boutwell, R. K. (1983). Protein kinase activity associated with a phorbol ester receptor purified from mouse brain. *Cancer Research* **43**, 4333-4337.

Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P. & Cantley, L. (1989). PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* **57**, 167-175.

Axelrod, J., Burch, R. M. & Jelsema, C. L. (1988). Receptor-mediated activation of phospholipase A_2 via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *Trends in Neurosciences* **11**, 117-123.

Ballester, R. & Rosen, O. M. (1985). Fate of immunoprecipitable protein kinase C in GH_3 cells treated with phorbol 12-myristate 13-acetate. *Journal of Biochemistry* **260**, 15194-15199.

Barbour, B., Szatkowski, M., Ingledew, N. & Attwell, D. (1989). Arachidonic acid induces a prolonged inhibition of glutamate uptake into glial cells. *Nature* **342**, 918-920.

Batty, I. R. & Nahorski, S. R. (1987). Lithium inhibits muscarinic-receptor-stimulated inositol tetrakisphosphate accumulation in rat cerebral cortex. *Biochemical Journal* **247**, 797-800.

Bazzi, M. D. & Nelsestuen, G. L. (1987). Role of substrate in imparting calcium and phospholipid requirements to protein kinase C activation. *Biochemistry* **26**, 1974-1982.

Bazzi, M. D. & Nelsestuen, G. L. (1988). Constitutive activity of membrane-inserted protein kinase C. *Biochemical and Biophysical Research Communications* **152**, 336-343.

Bazzi, M. D. & Nelsestuen, G. L. (1989). Properties of the protein kinase C-phorbol ester interaction. *Biochemistry* **28**, 3577-3585.

Bean, B. P. (1985). Two kinds of calcium channels in canine atrial cells. Differences in kinetics, selectivity, and pharmacology. *Journal of General Physiology* **86**, 1-30.

Bean, B. P., Sturek, M., Puga, A. & Hermsmeyer, K. (1986). Calcium channels in muscle cells isolated from rat mesenteric arteries: modulation by dihydropyridine drugs. *Circulation Research* **59**, 229-235.

Beaumier, L., Faucher, N. & Naccache, P. H. (1987). Arachidonic acid-induced release of calcium in permeabilized human neutrophils. *FEBS Letters* **221**, 289-292.

Behrman, H. R. (1979). Prostaglandins in hypothalamo-pituitary and ovarian function. *Annual Review of Physiology* **41**, 685-700.

Bell, R. L., Kennerly, D. A., Stanford, N. & Majeras, P. W. (1979). Diglyceride lipase: a pathway for arachidonate release from human platelets. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 3238-3241.

Benham, C. D. & Tsien, R. W. (1987). A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature* **328**, 275-278.

Berg, J. (1986). Potential metal-binding domains in nucleic acid binding proteins. *Science* **232**, 485-487.

Berridge, M. J. (1987). Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annual Review of Biochemistry* **56**, 159-193.

Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982). Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochemistry Journal* **206**, 587-595.

Berridge, M. J. & Irvine, R. F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315-321.

Berridge, M. J. & Irvine, R. F. (1989). Inositol phosphates and cell signalling. *Nature* **341**, 197-205.

Billah, M. M. & Anthes, J. C. (1990). The regulation and cellular functions of phosphatidyl choline hydrolysis. *Biochemical Journal* **269**, 281-291.

Billah, M. M., Anthes, J. C. & Mullmann, T. J. (1991). Receptor-coupled phospholipase D: regulation and functional significance. *Biochemical Society Transactions* **19**, 324-328.

Billah, M. M., Pai, J.-K., Mullmann, T. J., Egan, R. W. & Siegel, M. I. (1989). Regulation of phospholipase D in HL-60 granulocytes. Activation by phorbol esters, diglyceride, and calcium ionopore via protein kinase C-independent mechanisms. *Journal of Biological Chemistry* **264**, 9069-9076.

Bird, G. S. J., Rossier, M. F., Hughes, A. R., Shears, S. B., Armstrong, D. L. & Putney, J. W., Jr. (1991). Activation of Ca^{2+} entry into acinar cells by a non-phosphorylatable inositol trisphosphate. *Nature* **352**, 162-165.

Bjorge, J. D., Chan, T.-O., Antczak, M., Kung, H.-J. & Fujita, D. (1990). Activated type I phosphatidylinositol kinase is associated with the epidermal growth factor (EGF) receptor following EGF stimulation. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 3816-3820.

Blaustein, M. P. (1988). Calcium transport and buffering in neurons. *Trends in Neurosciences* **11**, 438-443.

Blumberg, P. M. (1988). Protein kinase C as the receptor for the phorbol ester tumor promoters: Sixth Rhoads Memorial Award Lecture. *Cancer Research* **48**, 1-8.

Bocckino, S. B., Blackmore, P. F. & Exton, J. H. (1985). Stimulation of 1,2-diacylglycerol accumulation in hepatocytes by vasopressin, epinephrine and Angiotensin II. *Journal of Biological Chemistry* **260**, 14201-14207.

Bocckino, S. B., Blackmore, P. F., Wilson, P. B. & Exton, J. H. (1987). Phosphatidate accumulation in hormone-treated hepatocytes via a phospholipase D mechanism. *Journal of Biological Chemistry* **262**, 15309-15315.

Boni, T. & Rando, R. R. (1985). The nature of protein kinase C activation by physically defined phospholipid vesicles and diacylglycerols. *Journal of Biological Chemistry* **260**, 10819-10825.

Boscá, L., Junco, M. & Díaz-Guerra, M. J. M. (1990). Substrate specificity of protein kinase C inhibitors. *Trends in Pharmacological Sciences* **11**, 477.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of micrograms of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Brooks, S. F., Evans, F. J. & Aitken, A. (1987). The stimulation of phosphorylation of intracellular proteins in GH_3 rat pituitary tumour cells by phorbol esters of distinct biological activity. *FEBS Letters* **224**, 109-116.

Brooks, S. F., Gordge, P. C., Toker, A., Evans, A. T., Evans, F. J. & Aitken, A. (1990). Platelet protein phosphorylation and protein kinase C activation by phorbol esters with different biological activity and a novel synergistic response with Ca^{2+} ionophore. *European Journal of Biochemistry* **188**, 431-437.

Buonassisi, V., Sato, G. & Cohen, A. I. (1962). Hormone producing cultures of adrenal and pituitary tumour origin. *Proceedings of the National Academy of Sciences of the United States of America* **48**, 1184-1190.

Burch, R. M., Luini, A. & Axelrod, J. (1986). Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in repsonse to α_1-adrenergic stimulation in FRTL5 thyroid cells. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 7201-7205.

Burns, D. J., Bloomenthal, J., Lee, M.-H. & Bell, R. M. (1990). Expression of the α, ßII, and γ protein kinase C isozymes in the baculovirus-insect cell expression system. Purification and characterisation of the individual isoforms. *Journal of Biological Chemistry* **265**, 12044-12051.

Bushfield, M., Murphy, G. J., Lavan, B. E., Parker, P. J., Hruby, V. J., Milligan, G. & Houslay, M. D. (1990). Hormonal regulation of G_i2 α-subunit phosphorylation in intact hepatocytes. *Biochemical Journal* **268**, 449-457.

Cabot, M. C., Welsh, C. J., Cao, H. & Chabbott, H. (1988). The phosphatidylcholine pathway of diacylglycerol formation stimulated by phorbol diesters occurs via phospholipase D activation. *FEBS Letters* **233**, 153-157.

Calignano, A., Piomelli, D., Sacktor, T. C. & Schwartz, J. H. (1991). A phospholipase A_2-stimulating protein regulated by protein kinase C in aplysia neurons. *Molecular Brain Research* **9**, 347-351.

Campbell, K. P., Leung, A. T. & Sharp, A. H. (1988). The biochemistry and molecular biology of the dihydropyridine-sensitive calcium channel. *Trends in Neurosciences* **11**, 425-430.

Catterall, W. A. (1988). Structure and function of voltage-sensitive ion channels. *Science* **242**, 50-61.

Chan, K.-W. & Turk, J. (1987). Mechanism of arachidonic acid-induced Ca^{2+} mobilation from rat liver microsomes. *Biochimica et Biophysica Acta* **928**, 186-193.

Chang, J., Musser, J. H. & McGregor, H. (1987). Phospholipase A_2: function and pharmacological regulation. *Biochemical Pharmacology* **36**, 2429-2436.

Chang, J. P., Morgan, R. O. & Catt, K. J. (1988). Dependence of secretory responses to gonadotropin-releasing hormone on diacylglycerol metabolism. Studies with a diacylglycerol lipase inhibitor, RHC 80267. *Journal of Biological Chemistry* **263**, 18614-18620.

Charest, R., Prpic, V., Exton, J. H. & Blackmore, P. F. (1985). Stimulation of inositol trisphosphate formation in hepatocytes by vasopressin, adrenaline and angiotensin II and its relationship to changes in cytosolic free Ca^{2+}. *Biochemical Journal* **227**, 79-90.

Chau, L. Y. & Tai, H. H. (1981). Release of arachidonate from diglyceride in human platelets requires the sequential action of a diglyceride lipase and a monoglyceride lipase. *Biochemical and Biophysical Research Communications* **100**, 1688-1695.

Chaudhry, A., Conway, B. R., Laychock, S. G. & Rubin, R. P. (1989). Analysis of the regulation of phosphatidylinositol-4,5-bisphosphate synthesis by arachidonic acid in exocrine pancreas. *Archives of Biochemistry and Biophysics* **272**, 488-495.

Chow, S. C. & Jondal, M. (1990). Polyunsaturated free fatty acids stimulate an increase in cytosolic Ca^{2+} by mobilizing the inositol 1,4,5-triphosphate-sensitive Ca^{2+} pool in T cells through a mechanism independent of phosphoinositide turnover. *Journal of Biological Chemistry* **265**, 902-907.

Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzmann, L. A., Lin, A. Y., Milona, N. & Knopf, J. L. (1991a). A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell* **65**, 1043-1051.

Clark, M. A., Chen, M. J., Crooke, S. T. & Bomalaski, J. S. (1988). Tumour necrosis factor (cachectin) induced phospholipase A_2-activating protein in endothelial cells. *Biochemical Journal* **250**, 125-132.

Clark, M. A., Conway, T. M., Shorr, R. G. L. & Crooke, S. T. (1987). Identification and isolation of a mammalian protein which is antigenically and functionally related to a phospholipase A_2 stimulatory peptide melittin. *Journal of Biological Chemistry* **262**, 4402-4406.

Clark, M. A., Littlejohn, D., Conway, T. M., Mong, S., Steiner, S. & Crooke, S. T. (1986). Leukotriene D_4 treatment of bovine aortic endothelial cells and murine smooth muscle cells in culture results in an increase in phospholipase A_2 activity. *Journal of Biological Chemistry* **261**, 10713-10718.

Clark, M. A., Özgür, L. E., Conway, T. M., Dispoto, J., Crooke, S. T. & Bomalaski, J. S. (1991b). Cloning of a phospholipase A_2 activating protein. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 5418-5422.

Clayton, R. R. & Catt, K. J. (1981). Gonadotropin-releasing hormone receptors: characterization, physiological regulation, and relationship to reproductive function. *Endocrine Reviews* **2**, 186-209.

Cohen, C. J. & McCarthy, R. T. (1985). Differential effects of dihydropyridines on two populations of Ca channels in anterior pituitary cells. *Biophysical Journal* **47**, 513a.

Conklin, B. R., Brann, M. R., Buckley, N. J., Ma, A. L., Bonner, T. I. & Axelrod, J. (1988). Stimulation of arachidonic acid release and inhibition of mitogenesis by cloned genes for muscarinic receptor subtypes stably expressed in A9L cells. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 8698-8702.

Conn, P. M. (1989). Does protein kinase C mediate pituitary actions of gonadotropin-releasing hormone? *Molecular Endocrinology* **3**, 755-757.

Conn, P. M., Marian, J., McMillan, M. & Rogers, D. (1980). Evidence for calcium mediation of gonadotropin releasing hormone action in the pituitary. *Cell Calcium* **1**, 7-20.

Conolly, T. M., Lawing, W. J. & Majerus, P. W. (1986). Protein kinase C phosphorylates human platelet inositol trisphosphate 5'-phosphomonoesterase, increasing the phosphatase activity. *Cell* **46**, 951-958.

Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U. & Ullrich, A. (1986). Multiple, distinct forms of bovine and human protein kinase C suggests diversity in cellular signaling pathways. *Science* **233**, 859-866.

Coyne, M. D., Lusczczynska, H. M. & Kunzi, M. (1987). Evidence for protein kinase C in bovine adrenocortical membrane preparations using $[^{35}S]\gamma$-thio-ATP as a phosphate donor. *Journal of Cyclic Nucleic Protein Phosphorylation Research* **11**, 433-444.

Crooke, S. T. & Bennett, C. F. (1989). Mammalian phosphoinositide-specific phospholipase C isoenzymes. *Cell Calcium* **10**, 309-323.

Crooke, S. T., Mttern, M., Sarau, H. M., Winkler, J. D., Balcarek, J., Wong, A. & Bennet, C. F. (1989). The signal transduction system of the leukotriene D_4 receptor. *Trends in Pharmacological Sciences* **10**, 103-107.

Currie, A. J., Fraser, H. M. & Sharp, R. M. (1981). Human placental receptors for luteinizing hormone releasing hormone. *Biochemical and Biophysical Research Communications* **99**, 332-337.

Curtis, A., Lyons, V. & Fink, G. (1985). The priming effect of LH-releasing hormone: effects of cold and involvement of new protein synthesis. *Journal of Endocrinology* **105**, 163-168.

Daniel, L. W., Waite, M. & Wykle, R. L. (1986). A novel mechanism of diglyceride formation. 12-0-tetradecanoylphorbol-13-acetate stimulates the cyclic breakdown and resynthesis of phosphatidyl choline. *Journal of Biological Chemistry* **261**, 9128-9132.

Davis, P. D., Hill, C. H., Keech, E., Lawton, G., Nixon, J. S., Sedgwick, A. D., Wadsworth, J., Westmacott, D. & Wilkinson, S. E. (1989). Potent selective inhibitors of protein kinase C. *FEBS Letters* **259**, 61-63.

Davis, P. D., Nixon, J. S., Wilkinson, S. E. & Russell, M. G. N. (1988). Inhibition of phospholipase A_2 by some long chain alkylamines. *Biochemical Society Transactions* **16**, 816-817.

de Chaffoy de Courcelles, D., Roeverns, P. & Van Belle, H. (1985). R59022, a diacylglycerol kinase inhibitor. Its effect on diacylglycerol and thrombin-induced C kinase activation in the intact platelet. *Journal of Biological Chemistry* **260**, 15762-15770.

De Vries, D. J. & Blumberg, P. M. (1989). Thermoregulatory effects of resiniferatoxin in the mouse: comparison with capsaicin. *Life Sciences* **44**, 711-715.

Delclos, K. B., Nagle, D. S. & Blumberg, P. M. (1980). Specific binding of phorbol ester tumor promoters to mouse skin. *Cell* **19**, 1025-1032.

Denef, C., Swennen, L. & Andries, M. (1982). Separated anterior pituitary cells and their response to hypophysiotrophic hormones. *International Review of Cytology* **76**, 225-244.

DeRiemer, S. A., Strong, J. A., Albert, K. A., Greengard, P. & Kaczmarek, L. K. (1985). Enhancement of calcium current in *Aplysia* neurones by phorbol ester and protein kinase C. *Nature* **313**, 313-316.

Di Polo, R., Rojas, H. R. & Beauge, L. (1979). Vanadate inhibits uncoupled Ca efflux but not Na-Ca exchange in squid axons. *Nature* **281**, 228-229.

DiVirgilio, F., Pozzan, T., Wollheim, C. B., Vicentini, L. M. & Meldolesi, J. (1986). Tumour promoter phorbol myristate acetate inhibits Ca^{2+} influx through

voltage-gated Ca^{2+} channels in two secretory cell lines, PC12 and RINm5F. *Journal of Biological Chemistry* **261**, 32-35.

Doerner, D., Abdel-Latif, M., Rogers, T. B. & Algers, B. E. (1990). Protein kinase C-dependent and -independent effects of phorbol esters on hippocampal calcium channel current. *Journal of Neuroscience* **10**, 1699-1706.

Dolphin, A. C. & Scott, R. H. (1988). Ca^{2+} agonists/antagonists and GTP. *Trends in Pharmacological Sciences* **9**, 394-395.

Downes, C. P. & Michell, R. H. (1985). Molecular Mechanisms of Transmembrane Signalling. In: *Molecular aspects of cellular regulation*, Vol 4, pp 3-56. Eds. Cohen, P. and Houslay, M.D. Elsevier, Amsterdam.

Driedger, P. E. & Blumberg, P. M. (1980). Specific binding of phorbol ester tumor promoters. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 567-571.

Drouva, S. V., Gorenne, I., Laplante, E., Rérat, E., Enjalbert, A. & Kordon, C. (1990). Estradiol modulates protein kinase C activity in the rat pituitary *in vivo* and *in vitro*. *Endocrinology* **126**, 536-544.

Drummond, A. H. (1985). Bidirectional control of cytosolic free calcium by thyrotropin-releasing hormone in pituitary cells. *Nature* **315**, 752-755.

Drummond, A. H. (1986). Inositol lipid metabolism and signal transduction in clonal pituitary cells. *Journal of Experimental Biology* **124**, 337-358.

Drummond, A. H., Bushfield, M. & Macphee, C. H. (1984). Thyrotropin-releasing hormone stimulated [^{3}H]-inositol metabolism in GH_3 pituitary cells: studies with lithium. *Molecular Pharmacology* **25**, 201-208.

Drummond, A. H. & Macphee, C. H. (1981). Phosphatidylinositol metabolism in GH_3 pituitary tumour cells stimulated by TRH. *British Journal of Pharmacology* **74**, 967-968P.

Dulbecco, R. & Freeman, G. (1959). Plaque production by the polyoma virus. *Virology* **8**, 396-401.

Dumuis, A., Sebben, M., Haynes, L., Pin, J.-P. & Bockaert, J. (1988). NMDA receptors activate the arachidonic acid cascade system in striatal neurons. *Nature* **336**, 68-70.

Dunn, J. A. & Blumberg, P. M. (1983). Specific binding of [20-^{3}H] 12-deoxyphorbol 13-isobutyrate to phorbol ester receptor subclasses in mouse skin particulate preparations. *Cancer Research* **43**, 4632-4637.

Durrell, J., Garland, J. T. & Friedel, R. O. (1969). Acetylcholine action: biochemical aspects. Two major approaches to understanding the mechanism of action of acetylcholine are examined. *Science* **165**, 862-866.

Ebeling, J. G., Vanderbark, G. R., Kuhn, L., Ganong, B., Bell, R. & Niedel, J. (1982). Diacylglycerols mimic phorbol diester induction of leukemic cell differentiation. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 815-819.

Eckstein, F. (1985). Nucleoside phosphorothioates. *Annual Review of Biochemistry* **54**, 367-402.

Edgar, A. D. & Freysz, L. (1982). Phospholipase activities of rat brain cytosol. Occurrence of phospholipase C activity with phosphatidylcholine. *Biochimica et Biophysica Acta* **711**, 224-228.

Eicholtz, T., Alblas, J., van Oververd, M., Moolenaar, W. & Ploegh, H. (1990). A pseudosubstrate peptide inhibits protein kinase C-mediated phosphorylation in permeabilized Rat-1 cells. *FEBS Letters* **261**, 147-150.

Ellis, C. A., Brooks, S. F., Brooks, G., Evans, A. T., Morrice, N., Evans, F. J. & Aitken, A. (1987). The effects of phorbol esters with different biological activities on protein kinase C. *Pytother Research* **1**, 187-190.

Epand, R. M. & Lester, D. S. (1990). The role of membrane biophysical properties in the regulation of protein kinase C activity. *Trends in Pharmacological Sciences* **11**, 317-320.

Evans, F. J., Parker, P. J., Olivier, A. R., Thomsas, S., Ryves, W. J., Evans, A. T., Gordge, P. & Sharma, P. (1991). Phorbol ester activation of the isotypes of protein kinase C from bovine and rat brain. *Biochemical Society Transactions* **19**, 397-402.

Ewald, D. A., Pang, I.-H., Sternweis, P. C. & Miller, R. J. (1989). Differential G protein-mediated coupling of neurotransmitter receptors to Ca^{2+} channels in rat dorsal root ganglion neurons *in vitro*. *Neuron* **2**, 1185-1193.

Ewald, D. A., Sternweis, P. C. & Miller, R. J. (1988). Guanine nucleotide-binding protein G_o-induced coupling of neuropeptide Y receptors to Ca^{2+} channels in sensory neurons. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 3633-3637.

Exton, J. H. (1990). Signaling through phosphatidylcholine breakdown. *Journal of Biological Chemistry* **265**, 1-4.

Faber, L. H., Wilson, F. J. & Wolff, D. J. (1987). Calmodulin-dependent phosphatases of PC12, GH_3, and C_6 cells: physical, kinetic, and immunochemical properties. *Journal of Neurochemistry* **49**, 404-414.

Fain, J. N. (1990). Regulation of phosphoinositide-specific phospholipase C. *Biochimica et Biophysica Acta* **1053**, 81-88.

Farquhar, M. G., Skutelsky, E. H. & Hopkins, C. R. (1975). In: *The anteior pituitary* pp 83-135. Eds. Tixier-Vidal, A. and Farquhar, M. G. Academic Press, New York.

Fearon, C. W. & Tashjian, A. H., Jr. (1985). Thyrotropin-releasing hormone induces redistribution of protein kinase C in GH_4C_1 rat pituitary cells. *Journal of Biological Chemistry* **260**, 8366-8371.

Ferriola, P. C., Cody, V. & Middleton, E., Jr. (1989). Protein kinase C inhibition by plant flavanoids. *Biochemical Pharmacology* **38**, 1617-1624.

Fink, G. (1979). Neuroendocrine control of gonadotrophin secretion. *British Medical Bulletin* **35**, 155-160.

Fink, G. (1986). The endocrine control of ovulation. *Science Progress* **70**, 403-423.

Fink, G. (1988). The G.W. Harris lecture. Steroid control of brain and pituitary function. *Quarterly Journal of Experimental Physiology* **73**, 257-293.

Fink, G., Aiyer, M., Chiappa, S., Henderson, S., Jamieson, M., Levy-Perez, V., Pickering, A., Sarkar, D., Sherwood, N., Speight, A. & Watts, A. (1982).

Fink, G., Johnson, M. S. & Mitchell, R. (1990). Effects of protein kinase C inhibitors on the priming effect of LHRH in rat anterior pituitary glands *in vitro*. *Journal of Physiology* **420**, 76P.

Fish, R. D., Sperti, G., Colucci, W. S. & Clapham, D. E. (1988). Phorbol ester increases the dihydropyridine-sensitive calcium conductance in a vascular smooth muscle cell line. *Circulation Research* **62**, 1049-1054.

Fisher, S. K., Van Rooijen, L. A. A. & Agranoff, B. W. (1984). Renewed interest in the polyphosphoinositides. *Trends in Biochemical Sciences* **9**, 53-56.

Flint, A. J., Paladini, R. D. & Koshland, D. E., Jr. (1990). Autophosphorylation of protein kinase C at three separated regions of its primary sequence. *Science* **249**, 408-411.

Flockerzi, V., Oeken, H.-J., Hoffman, F., Pelzer, D., Cavalie, A. & Trautwein, W. (1986). Purified dihydropyridine-binding site from skeletal muscle t-tubules is a functional calcium channel. *Nature* **323**, 66-68.

Flower, R. J. (1988). Lipocortin and the mechanism of action of the glucocorticoids. *British Journal of Pharmacology* **94**, 987-1015.

Froissart, P., Unligil, P., Aubrey, H. & Proulx, P. (1989). Modulation by phorbol 12-myristate 13-acetate of arachidonic acid release from rat basophilic leukemia cells stimulated with A23187. *Biochimica et Biophysica Acta* **1002**, 376-381.

Fukiji, H., Tanaka, Y., Miyake, R., Kikkawa, U., Nishizuka, Y. & Sugimura, T. (1984). Activation of calcium-activated, phospholipid-dependent protein kinase (protein kinase C) by new classes of tumor promoters: teleocidin and debromoaplysiatoxin. *Biochemical and Biophysical Research Communications* **120**, 339-343.

Fukui, Y., Kornbluth, S., Jong, S.-M., Wang, L.-H. & Hanafusa, H. (1989). Phosphatidylinositol kinase type I activity associates with various oncogene products. *Oncogene Research* **4**, 283-292.

Furth, J. (1955). Experimental pituitary tumours. *Recent Progress in Hormone Research* **11**, 221-255.

Galizzi, J., Qar, J., Fosset, M., Van Rentergham, C. & Lazdunski, M. (1987). Regulation of calcium channels in aortic muscle cells by protein kinase C activators (diacylglycerol and phorbol esters) and by peptides (vasopressin and bombesin) that stimulate phosphoinositide breakdown. *Journal of Biological Chemistry* **262**, 6947-6950.

Ganong, B. R., Loomis, C. R., Hannun, Y. A. & Bell, R. M. (1986). Specificity and mechanism of protein kinase C activation by *sn*-1,2-diacylglycerols. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 1184-1188.

Gershengorn, M. C. (1982). Thyrotropin releasing hormone. *Molecular and Cellular Biochemistry* **45**, 163-179.

Gershengorn, M. C., Hoffstein, S. T., Rebecchi, M. J., Geras, E. & Rubin, B. G. (1981). Thyrotropin-releasing hormone stimulation of prolactin release from clonal rat pituitary cells. Evidence for action independent of extracellular calcium. *Journal of Clinical Investigation* **67**, 1769-1776.

Gershengorn, M. C. & Thaw, C. (1985). Thyrotropin-releasing hormone (TRH) stimulates biphasic elevation of cytoplasmic free calcium in GH_3 cells. Further evidence that TRH mobilises cellular and extracellular calcium. *Endocrinology* **116**, 591-596.

Gill, D. L., Grollman, E. F. & Kohn, L. D. (1981). Calcium transport mechanisms in membrane vesicles from guinea pig brain synaptosomes. *Journal of Biological Chemistry* **256**, 184-192.

Gill, D. L., Ueada, T., Cheuh, S.-H. & Noel, M. W. (1986). Ca^{2+} release from endoplasmic reticulum is mediated by a guanine nucleotide regulatory mechanism. *Nature* **320**, 461-464.

Ginsborg, B. L., Martin, R. J. & Patmore, L. (1991). On the sodium and potassium currents of a human neuroblastoma cell line. *Journal of Physiology* **434**, 121-149.

Girard, P. R., Mazzei, G. J. & Kuo, J. F. (1986). Immunological quantitation of phospholipid/Ca^{2+}-dependent protein kinase and its fragments (tissue levels, subcellular distribution, and ontogenetic changes in brain and heart). *Journal of Biological Chemistry* **261**, 370-375.

Glass, D. G., Frey, W., Carr, D. W. & Goldberg, N. D. (1977). Stimulation of human platelet guanylate cyclase by fatty acids. *Journal of Biological Chemistry* **252**, 1279-1285.

Grandison, L. (1984). Stimulation of anterior pituitary prolactin release by melittin, an activator of phospholipase A_2. *Endocrinology* **114**, 1-7.

Grausz, J. D., Fradelizi, D., Dautry, F., Monier, R. & Lehn, P. (1986). Modulation of c-fos and c-myc mRNA levels in normal human lymphocytes by calcium ionophore A21387 and phorbol ester. *European Journal of Immunology* **16**, 1217-1221.

Graziano, M. P. & Gilman, A. G. (1987). Guanine nucleotide-binding regulatory proteins: mediators of transmembrane signaling. *Trends in Pharmacological Sciences* **8**, 478-481.

Griffiths, E. C. & Bennett, G. W. (1983). *Thyrotropin-Releasing Hormone*. Raven Press, New York.

Grove, R. I. & Schimmel, S. D. (1982). Effects of 12-*0*-tetradecanoylphorbol 13-acetate on glycerolipid metabolism in cultured myoblasts. *Biochimica et Biophysica Acta* **711**, 272-280.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440-3450.

Gschwendt, M., Kittstein, W., Horn, F., Leibersperger, H. & Marks, F. (1989a). A phorbol ester and pospholipid-activate, calcium unresponsive protein kinase in mouse epidermis: characterization and separation from protein kinase C. *Journal of Cellular Biochemistry* **40**, 295-307.

Gschwendt, M., Kittstein, W. & Marks, F. (1991). Protein kinase C activation by phorbol esters: do cystein-rich regions and pseudosubstrate motifs play a role? *Trends in Biochemical Sciences* **16**, 167-169.

Gschwendt, M., Leibersperger, H. & Marks, F. (1989b). Differentiative actions of K252a on protein kinase C and calcium-unresponsive, phorbol ester/phospholipid-activated protein kinase. *Biochemical and Biophysical Research Communications* **164**, 974-982.

Haberman, E. (1972). Bee and wasp venom. *Science* **177**, 314-322.

Halenda, S. P. & Rehm, A. G. (1987). Thrombin and C-kinase activators potentiate calcium-stimulated arachidonic acid release in human platelets. *Biochemical Journal* **248**, 471-475.

Hallam, T. J., Jacob, R. & Merritt, J. E. (1988). Evidence that agonist stimulate bivalent-cation influx into human endothelial cells. *Biochemical Journal* **255**, 179-184.

Hallam, T. J. & Rink, T. J. (1989). Receptor-mediated Ca^{2+} entry: diversity of function and mechanism. *Trends in Pharmacological Sciences* **10**, 8-10.

Ham, R. G. (1963). An improved nutrient solution for diploid chinese hamster and human cell lines. *Experimental Cellular Research* **29**, 515-519.

Han, C., Abel, P. W. & Minreman, K. P. (1987). α_1-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. *Nature* **329**, 333-335.

Hanks, J. H. & Wallace, R. E. (1949). Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proceedings of the Society for Experimental Biology and Medicine* **71**, 196-200.

Hannun, Y., Loomis, C. R. & Bell, R. M. (1985). Activation of protein kinase C by TritonX-100 mixed micelles containing diacylglycerol and phosphatidylserine. *Journal of Biological Chemistry* **260**, 10039-10043.

Hannun, Y. A. & Bell, R. M. (1988). Aminoacridines, potent inhibitors of protein kinase C. *Journal of Biological Chemistry* **263**, 5124-5131.

Hannun, Y. A. & Bell, R. M. (1990). Rat brain protein kinase C. Kinetic analysis of substrate dependence, allosteric regulation, and autophosphorylation. *Journal of Biochemistry* **265**, 2962-2972.

Hannun, Y. A., Loomis, C. R. & Bell, R. M. (1986a). Protein kinase C activation in mixed micelles (mechanistic implications of phospholiped, diacylglycerols, and calcium interdependencies). *Journal of Biological Chemistry* **261**, 7184-7190.

Hannun, Y. A., Loomis, C. R., Merrill, A. J., Jr. & Bell, R. M. (1986b). Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding *in vitro* and in human platelets. *Journal of Biological Chemistry* **261**, 12604-12609.

Hanson, A., Serhan, C. N., Haeggström, J., Inglelman-Sundberg, M. & Samuelsson, B. (1986). Activation of protein kinase C by lipoxin A and other eicosanoids. Intracellular action of oxygenation products of arachidonic acid. *Biochemical and Biophysical Research Communications* **134**, 1215-1222.

Harris, G. W. (1955). *Neural control of the pituitary gland.* Edward Arnold, London.

Hattori, H. & Kanfer, J. N. (1985). Synaptosomal phospholipase D potential role in providing choline for acetylcholine synthesis. *Journal of Neurochemistry* **45**, 1578-1584.

Hedqvist, P. (1977). Basic mechanisms of prostaglandin action on autonomic neurotransmission. *Annual Review of Pharmacology and Toxicology* **17**, 259-279.

Hensey, C. E., Boscoboinik, D. & Azzi, A. (1989). Suramin, an anti-cancer drug, inhibits protein kinase C and induces differentiation in neuroblastoma cell clone NBZA. *FEBS Letters* **258**, 156-158.

Herbert, J. M., Augereau, J. M., Gleye, J. & Maffrand, J. P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochemical and Biophysical Research Communications* **172**, 993-999.

Herlant, M. (1964). Cells of the adenohypophysis and their functional significance. *International Review of Cytology* **17**, 299-382.

Hescheler, J., Rosenthal, W., Trantweih, W. & Schultz, G. (1987). The GTP-binding protein, Go, regulates neuronal calcium channels. *Nature* **325**, 445-447.

Hidaka, H. & Hagiwara, M. (1987). Pharmacology of the isoquinoline sulfonamide protein kinase C inhibitors. *Trends in Pharmacological Sciences* **8**, 162-164.

Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**, 5036-5041.

Hinkle, P. M. & Tashjian, A. H. (1973). Receptors for thyrotropin-releasing hormone in prolactin-producing rat pituitary cells in culture. *Journal of Biological Chemistry* **248**, 6180-6186.

Hirota, K., Hirota, T., Aguilera, G. & Catt, K. J. (1985). Hormone-induced redistribution of calcium-activated phospholipid-dependent protein kinase in pituitary gonadotrophs. *Journal of Biological Chemistry* **260**, 3243-3246.

Ho, A. K. & Klem, D. C. (1987). Activation of α_1-adrenoceptors, protein kinase C, or treatment with intracellular free Ca^{2+} elevating agents increases pineal phospholipase A_2 activity. Evidence that protein kinase C may participate in Ca^{2+}-dependent α_1-adrenergic stimulation of pineal phospholipase A_2 activity. *Journal of Biological Chemistry* **262**, 11764-11770.

Hockberger, P., Toselli, M., Swandulla, D. & Lux, H. D. (1989). A diacylglycerol analogue reduces neuronal calcium currents independently of protein kinase C activation. *Nature* **338**, 340-342.

Hoffman, F., Nastainczyk, W., Röhrkasten, A., Schneider, T. & Sieber, M. (1987). Regulation of the L-type calcium channel. *Trends in Pharmacological Sciences* **8**, 393-398.

Hokin, L. E. & Hokin, M. R. (1963). Diaglyceride kinase and other pathways for phosphatidic acid synthesis in the erythrocyte membrane. *Biochimica et Biophysica Acta* **67**, 470-484.

Hokin, M. R. & Hokin, L. E. (1953). Enzyme secretion and the incorporation of P^{32} into phospholipids of pancreas slices. *Journal of Biological Chemistry* **203**, 967-977.

Hollingstead, M., Thompson, M. & Schofield, J. G. (1985). Phosphoinositide metabolism in bovine anterior pituitary. *Biochemical Society Transacations* **13**, 189-190.

Horn, F., Bilezikjian, L. M., Perrin, M. H., Bosma, M. M., Windle, J. J., Huber, K. S., Blount, A. L., Hille, B., Vale, W. & Mellon, P. L. (1991). Intracellular

responses to gonadotropin-releasing hormone in a clonal cell line of the gonadotrope lineage. *Molecular Endocrinology* **5**, 347-355.

Horn, F., Gschwendt, M. & Marks, F. (1985). Partial purification and characterisation of the calcium-dependent and phospholipid-dependent protein kinase C from chick oviduct. *European Journal of Biochemistry* **148**, 533-538.

House, C. & Kemp, B. E. (1987). Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. *Science* **238**, 1726-1728.

House, C., Robinson, P. J. & Kemp, B. E. (1989). A synthetic peptide analogue of the putative substrate-binding motif activates protein kinase C. *FEBS Letters* **249**, 243-247.

Huang, F. L., Yoshida, Y., Melo-Cunha, J. R., Beaven, M. A. & Huang, K.-P. (1989). Differential down-regulation of protein kinase C isozymes. *Journal of Biological Chemistry* **264**, 4238-4243.

Huang, K.-P. (1989). The mechanism of protein kinase C activation. *Trends in Neurosciences* **12**, 425-432.

Huang, K.-P. & Huang, F. L. (1990). Differential sensitivity of protein kinase C isozymes to phospholipid-induced inactivation. *Journal of Biological Chemistry* **265**, 738-744.

Huang, K.-P., Huang, F. L., Nakabayashi, H. & Yoshida, Y. (1988). Biochemical characterisation of rat brain protein kinase C isozymes. *Journal of Biological Chemistry* **263**, 14839-14845.

Huang, K. P., Chan, K.-F. J., Singh, T. J., Nakabayashi, F. & Huang, F. L. (1986). Autophosphorylation of rat brain Ca^{2+}-activated and phospholipid dependent protein kinase. *Journal of Biological Chemistry* **261**, 12134-12140.

Hughes, A. R., Takemura, H. & Putney, J. W. (1988). Kinetics of inositol 1,4,5-trisphosphate and inositol cyclic:1:2,4,5-trisphosphate metabolism in intact rat parotid acinar cells. Relationship to calcium signalling. *Journal of Biological Chemistry* **260**, 10314-10319.

Hulting, A.-L., Lindgren, J.-Å., Hökfelt, T., Eneroth, P., Werner, S., Patrono, C. & Samuelsson, B. (1985). Leukotriene C_4 as a mediator of luteinizing hormone release from rat anteror pituitary cells. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 3834-3838.

Hunter, T., Ling, N. & Cooper, J. A. (1984). Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. *Nature* **311**, 480-483.

Hwang, T.-C., Guggino, S. E. & Guggino, W. B. (1990). Direct modulation of secretory chloride channels by arachidonic acid and other unsaturated fatty acids. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 5706-5709.

Inoue, M., Kishimoto, A., Takai, Y. & Nishizuka, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues (II - proenzyme and its activation by calcium-dependent protease from rat brain. *Journal of Biological Chemistry* **252**, 7610-7616.

Irvine, R. F. (1982). How is the level of free arachidonic acid controlled in mammalian cells? *Biochemical Journal* **204**, 3-16.

Irvine, R. F. (1990). 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates - a possible mechanism. *FEBS Letters* **263**, 5-9.

Irving, H. R. & Exton, J. H. (1987). Phosphatidylcholine breakdown in rat liver plasma membranes. Roles of guanine nucleotides and P_2-purinergic agonists. *Journal of Biological Chemistry* **262**, 3440-3443.

Jaken, S., Feldman, H., Blumberg, P. M. & Tashjian, A. H., Jr. (1983b). Association of phorbol ester receptor down modulation with a cryptic receptor state. *Cancer Research* **43**, 5795-5800.

Jaken, S., Shupnik, M. A., Blumberg, P. M. & Tashjian, A. H., Jr. (1983a). Relationship - between mezerein-mediated biological responses and phorbol ester receptor occupancy. *Cancer Research* **43**, 11-14.

Jefferson, A. B. & Schulman, H. (1988). Sphingosine inhibits calmodulin-dependent enzymes. *Journal of Biological Chemistry* **263**, 15241-15244.

Jeffrey, A. M. & Liskamp, R. M. (1986). Computer-assisted molecular modeling of tumour promoters: rationale for the activity of phorbol esters, teleocidin B , and aplysiatoxin. *Proceedings of the Academy of Sciences of the United States of America* **83**, 241-245.

Jelsema, C. L. (1987). Light activation of phospholipase A_2 in rod outer segments of bovine retina and its modulation by GTP-binding proteins. *Journal of Biological Chemistry* **262**, 163-168.

Jelsema, C. L. & Axelrod, J. (1987). Stimulation of phospholipase A_2 activity in bovine rod outer segments by the $\beta\gamma$-subunits of transducin and its inhibition by the α-subunit. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 3623-3627.

Johnson, M. S., MacEwan, D. J., Mitchell, R. & Thomson, F. J. (1990). Effects of calmodulin antagonists on neurohormone-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary tissue *in vitro*. *Journal of Physiology* **424**, 43P.

Johnson, M. S., Mitchell, R. & Fink, G. (1988). The role of protein kinase C in LHRH-induced LH and FSH release and LHRH self priming in rat anterior pituitary glands *in vitro*. *Journal of Endocrinology* **116**, 231-239.

Johnson, M. S., Thomson, F. J., Avery, J. L., MacEwan, D. J. & Mitchell, R. (1991). Differential actions of protein kinase C modulators on the release of

hormones from rat anterior pituitary tissue *in vitro*. *Journal of Physiology* (in press).

Joseph, S. & Krishnamurthi, S. (1989). Pharmacological manipulation of diacylglycerol-dependent protein kinase C. *Trends in Pharmacological Sciences* **10**, 396.

Junco, M., Díaz-Guerra, M. J. M. & Boscá, L. (1990). Substrate-dependent inhibition of protein kinase C by specific inhibitors. *FEBS Letters* **263**, 169-171.

Kai, M., Salway, J. G., Michell, R. H. & Hawthorne, J. N. (1966). The biosynthesis of triphosphoinositide by rat brain *in vitro*. *Biochemical and Biophysical Research Communications* **22**, 370-375.

Kaibuchi, K., Takai, Y. & Nishizuka, Y. (1981). Cooperative roles of various membrane phospholipids in the activation of calcium-activated, phospholipid-dependent protein kinase. *Journal of Biological Chemistry* **256**, 7146-7149.

Kaplan, D. R., Whitman, M., Schaffhausen, B., Raptis, L., Garcea, R. L., Pallas, D., Roberts, T. M. & Cantley, L. (1986). Phosphatidylinositol metabolism and polyoma-mediated transformation. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 3624-3628.

Kase, H., Iwahashi, K., Nakanashi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A. & Kanedo, M. (1987). K-252 compounds, novel and potent inhibitions of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochemical and Biophysical Research Communications* **142**, 436-440.

Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jakobs, K. H. (1985). Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *European Journal of Biochemistry* **151**, 431-437.

Kemp, B. E. (1979). Phosphorylation of synthetic peptide analogs of rabbit cardiac troponin inhibitory subunit by the cyclic AMP-dependent protein kinase. *Journal of Biological Chemistry* **254**, 2638-2642.

Kihoshita, F., Nakai, Y., Katakami, H., Imura, H., Shimizu, T. & Hayaishi, O. (1982). Suppressive effect of prostaglandin (PG) D_2 on pulsatile luteinizing hormone release in conscious castrated rats. *Endocrinology* **110**, 2207-2209.

Kikkawa, U., Ogita, K., Ono, Y., Asaoka, Y., Shearman, M. S., Fujii, T., Ase, K., Sekiguchi, K., Igarishi, K. & Nishizuka, Y. (1987). The common structure and activities of four subspecies of rat brain protein kinase C family. *FEBS Letters* **223**, 212-216.

Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. & Nishizuka, Y. (1982). Calcium-activated, phospholipid-dependent protein kinase from rat brain

(subcellular distribution, purification and properties). *Journal of Biological Chemistry* **257**, 13341-13348.

Kiley, S., Schaap, D., Parker, P., Hsieh, L.-L. & Jaken, S. (1990). Protein kinase C heterogeneity in GH_4C_1 rat pituitary cells. Characterisation of a Ca^{2+}-independent phorbol ester receptor. *Journal of Biological Chemistry* **265**, 15704-15712.

Kim, D., Lewis, D. L., Graziadei, L., Neer, E. J., Bar-Sagi, D. & Clapham, D. E. (1989). G-protein ßγ-subunits activate the cardiac muscarinic K^+-channel via phospholipase A_2. *Nature* **337**, 557-560.

Kishimoto, A., Kajikawa, N., Shiota, M. & Nishizuka, Y. (1983). Proteolytic activation of calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease. *Journal of Biological Chemistry* **258**, 1156-1164.

Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S.-I., Tominaga, M., Kuroda, T. & Nishizuka, Y. (1989). Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). *Journal of Biological Chemistry* **264**, 4088-4092.

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980). Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *Journal of Biological Chemistry* **255**, 2273-2276.

Kiss, Z. & Anderson, W. B. (1989). Phorbol ester stimulated the hydrolysis of phosphatidyl ethanolamine in leukimic HL-60, NIH3T3, and baby hamster kidney cells. *Journal of Biological Chemistry* **264**, 1483-1487.

Kiss, Z., Deli, E., Girard, P. R., Pettit, G. R. & Kuo, J. F. (1987). Comparative effects of polymixin B, phorbol ester and bryostatin on protein phosphorylation, protein kinase C translocation, phospholipid metabolism and differentiation of HL60 cells. *Biochemical and Biophysical Research Communications* **146**, 208-215.

Knopf, J. L., Lee, M.-H., Sultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. M. & Bell, R. M. (1986). Cloning and expression of multiple protein kinase C cDNAs. *Cell* **46**, 491-502.

Kobayashi, E., Nakano, H., Morimoto, M. & Tamaoki, T. (1989). Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochemical and Biophysical Research Communications* **37**, 2704-2706.

Kolesnick, R. N. (1987). Thyrotropin-releasing hormone and phorbol esters induce phosphatidylcholine synthesis in GH_3 pituitary cells. Evidence for stimulation via protein kinase C. *Journal of Biological Chemistry* **262**, 14525-14530.

Kolesnick, R. N. & Hemer, M. R. (1990). Physiologic 1,2-diacylglycerol levels induce protein kinase C-independent translocation of a regulatory enzyme. *Journal of Biological Chemistry* **265**, 10900-10904.

Kolesnick, R. N. & Paley, A. E. (1987). 1,2-Diacylglycerols and phorbol esters stimulate phosphatidylcholine metabolism in GH_3 pituitary cells. Evidence for separate mechanisms of action. *Journal of Biological Chemistry* **262**, 9204-9210.

Kosaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U. & Nishizuka, Y. (1988). The heterogeneity of protein kinase C in various rat tissues. *Biochemical and Biophysical Research Communications* **151**, 973-981.

Kraft, A. S. & Anderson, W. B. (1983). Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* **301**, 621-623.

Kramer, I. M., van der Bend, R.L., Tool, A.T., van Blitterswijk, W.J., Roos, D. and Verhoeven, A.J. (1989). 1-*0*-Hexadecyl-2-*0*-methylglycerol, a novel inhibitor of protein kinase C, inhibits the respiratory burst in human neutrophils. *Journal of Biological Chemistry* **264**, 5876-5884.

Krauth, J. (1983). The interpretation of significance tests for independent and dependent samples. *Journal of Neuroscience Methods* **9**, 269-281.

Kubo, K., Ohno, S. & Suzuki, K. (1987). Primary structures of human protein kinase C ßI and ßII differ only in their C-terminal sequences. *FEBS Letters* **223**, 138-142.

Kucera, G. L. & Rittenhouse, S. E. (1990). Human platelets form 3-phosphorylated phosphoinositides in response to α-thrombin, U46619, or GTPγS. *Journal of Biological Chemistry* **265**, 5345-5348.

Kuno, M. & Gardner, P. (1987). Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. *Nature* **326**, 301-304.

Kuo, J. F., Anderson, R. G. G., Wise, B. C., Mackerlova, L., Salomonsson, I., Brackett, N. L., Katoh, N., Shoji, M. & Wrenn, R. W. (1980). Calcium-dependent protein kinase: widespread occurrence in various tissues and phyla of the animal kingdom and comparison of effects of phospholipid, calmodulin and trifluoperazine. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 7039-7043.

Kurachi, Y., Ito, H., Ssugimoto, T., Shimizu, T., Miki, I. & Ui, M. (1989). Arachidonic acid metabolites as intracellular modulators of the G protein-gated cardiaac K^+-channel. *Nature* **337**, 555-557.

Lacerda, A. E., Rampe, D. & Brown, A. M. (1988). Effects of protein kinase C activators on cardiac Ca^{2+} channels. *Nature* **335**, 249-251.

Lapetina, E. G. & Hawthorne, J. N. (1971). The diglyceride kinase of rat cerebral cortex. *Biochemical Journal* **122**, 171-179.

Leach, K. L. & Blumberg, P. M. (1985). Modulation of protein kinase C activity and [^{3}H]-phorbol 12,13-dibutyrate binding by various tumour promoters in mouse brain cytosol. *Cancer Research* **45**, 1958-1963.

Leach, K. L., James, M. L. & Blumberg, P. M. (1983). Characterisation of a specific phorbol ester aporeceptor in mouse brain cytosol. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 4208-4212.

Leach, K. L., Ruff, V. A., Wright, T. M., Pessin, M. S. & Raben, D. M. (1991). Dissociation of protein kinase C activation and *sn*-1,2-diacylglycerol formation. comparison of phosphatidylinositol- and phosphatidylcholine-derived diglycerides in α-thrombin-stimulated fibroblasts. *Journal of Biological Chemistry* **266**, 3215-3221.

Leong, D. A., Frawley, S. L. & Neill, J. D. (1983). Neuroendocrine control of prolactin secretion. *Annual Review of Physiology* **45**, 109-127.

Lewis, D. L. & Weight, F. F. (1988). The protein kinase C activator 1-oleoyl-2-acetylglycerol inhibits voltage-dependent Ca^{2+} current in the pituitary cell line AtT-20. *Neuroendocrinology* **47**, 169-175.

Li, M., McCann, J. D., Anderson, M. P., Clancy, J. P., Liedtke, C. M., Nairn, A. C., Greengard, P. & Welsh, M. J. (1989). Regulation of chloride channels by protein kinase C in normal and cystic fibrosis airway epithelia. *Science* **244**, 1353-1356.

Liscovitch, M. & Amsterdam, A. (1989). Identification and purification of sheep platelet phospholipase A_2 isoforms. Activation by physiologic concentrations of calcium ions. *Journal of Biological Chemistry* **264**, 11762-11767.

Loeb, L. A. & Gross, R. W. (1986). Identification and purification of sheep platelet phospholipase A_2 isoforms. Activation by physiologic concentrations of calcium ion. *Journal of Biological Chemistry* **261**, 10467-10470.

Löffelholz, K. (1989). Receptor regulation of choline phospholipid hydrolysis. A novel source of diacylglycerol and phosphatidic acid. *Biochemical Pharmacology* **38**, 1543-1549.

Lotan, I., Dascal, N., Naor, Z. & Boton, R. (1990). Modulation of vertebrate brain Na^+ and K^+ channels by subtypes of protein kinase C. *FEBS Letters* **267**, 25-28.

Luckhoff, A. (1986). Measuring cytosolic free calcium concentration in endothelial cells with indo-1: the pitfall of using the ratio of two fluorescence intensities recorded at different wavelengths. *Cell Calcium* **7**, 233-248.

Luini, A. G. & Axelrod, J. (1985). Inhibitors of the cytochrome. P-450 enzymes block the secretagogue-induced release of corticototropin in mouse pituitary

tumour cells. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 1012-1014.

Macara, I. G. (1989). Oncogenes and cellular signal transduction. *Pharmacological Review* **69**, 797-820.

Macara, I. G., Marinetti, G. V. & Balduzzi, P. C. (1984). Transforming protein of arian sarcoma virus URZ is associated with phosphatidylinositol kinase activity: possible role in tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 2728-2732.

MacEwan, D. J., Johnson, M. S., R, M. & Thomson, F. J. (1991). Protein kinase C may be required to maintain activity of nimodipine-sensitive Ca^{2+} channels in rat anterior pituitary cells *in vitro*. *Journal of Physiology* (in press).

MacEwan, D. J. & Mitchell, R. (1991). Calcium influx through 'L'-type channels into rat anterior pituitary cells can be modulated in two ways by protein kinase C (PKC-isoform selectivity of 1,2-dioctanoyl *sn*-glycerol?). *FEBS Letters* **291**, 79-83.

MacPhee, C. H. & Drummond, A. H. (1984). Thyrotropin-releasing hormone stimulates rapid breakdown of phosphatidylinositol 4,5-biphosphate and phosphatidylinositol 4-phosphate in GH_3 pituitary tumour cells. *Molecular Pharmacology* **25**, 193-200.

Maelicke, A. (1988). Structural similarities between ion channel proteins. *Trends in Biochemical Sciences* **13**, 199-202.

Mahadevappa, V. G. & Sicilia, F. J. (1988). The inhibition of arachidonic acid mobilization in human platelets by R59 022, a diacylglycerol kinase inhibitor. *Biochimica et Biophysica Acta* **961**, 309-315.

Majerus, P. W., Ross, T. S., Cunningham, T. W., Caldwell, K. K., Jefferson, A. B. & Bansal, V. S. (1990). Recent insights in phosphatidylinositol signaling. *Cell* **63**, 459-465.

Makowske, M., Ballester, R., Cayre, Y. & Rosen, O. M. (1988). Immunochemical evidence that three protein kinase C isozymes increase in abundance during H2-60 differentiation induced by dimethylformamide and retinoic acid. *Journal of Biological Chemistry* **263**, 3402-3410.

Marais, R. M. & Parker, P. J. (1989). Purification and characterisation of bovine brain protein kinase C isotypes α, ß and γ. *European Journal of Biochemistry* **182**, 129-137.

Marchetti, C. & Brown, A. M. (1988). Protein kinase C activator 1-oleoyl-2-acetyl-*sn*-glycerol inhibits two types of calcium currents in GH_3 cells. *American Journal of Physiology* **23**, C206-210.

Marcus, A. J., Ullner, H. L. & Safier, L. B. (1969). Lipid composition of subcellular particles of human blood platelets. *Journal of Lipid Research* **10**, 108-114.

Martin, T. F. J. (1983). Thyrotropin-releasing hormone rapidly activates the phosphodiester hydrolysis of polyphosphoinositides in GH_3 pituitary cells. Evidence for the role of polyphosphoinositide-specific phospholipase C in hormone action. *Journal of Biological Chemistry* **258**, 14816-14822.

Martin, T. W., Feldman, D. R. & Michaelis, K. C. (1990). Phosphatidylcholine hydrolysis stimulated by phorbol myristate acetate is mediated by phospholipase D in endothelial cells. *Biochimica et Biophysica Acta* **1053**, 162-172.

Martin, T. W., Wysolmerski, R. B. & Lagunoff, D. (1987). Phosphatidylcholine metabolism in endothelial cells: evidence for phospholipase A and a novel Ca^{2+}-independent phospholipase C. *Biochimica et Biophysica Acta* **917**, 296-307.

Mason, W. T., Rawlings, S. R., Cobbett, P., S K, Zorec, R., Akerman, S. N., Benham, C. D., Berridge, M. J., Cheek, T. & Moreton, R. B. (1988). Control of secretion in anterior pituitary cells-linking ion channels, messengers and exocytosis. *Journal of Experimental Biology* **139**, 287-316.

Matsuzawa, Y. & Hostetler, K. Y. (1980). Inhibition of lysomal phospholipase A and phospholipase C by chloroquine and 4,4,-bis(diethylaminoethoxy)-α,ß-diethyldiphenylethane. *Journal of Biological Chemistry* **255**, 646-652.

Mayer, B. J., Hamaguchi, M. & Hanafusa, H. (1988). A novel viral oncogene with structural similarity to phospholipase C. *Nature* **332**, 272-275.

McArdle, C. A. & Conn, P. M. (1986). Hormone-stimulated redistribution of gonadotrope protein kinase C *in vivo:* dependence in Ca^{2+} influx. *Molecular Pharmacology* **29**, 570-576.

McArdle, G. A. & Conn, P. M. (1989). Use of protein kinase C-depleted cells for investigation of the role of protein kinase C in stimulus-response coupling in the pituitary. *Methods in Enzymology* **168**, 287-301.

McPhail, L. C., Clayton, C. C. & Snyderman, R. (1984). A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase. *Science* **224**, 622-625.

Meldolesi, J. & Pozzan, T. (1987). Pathways of Ca^{2+} influx at the plasma membrane: voltage-, receptor, and second-messenger-operated channels. *Experimental Cell Research* **171**, 271-283.

Meldrum, E., Parker, P. J. & Carozzi, A. (1991). The PtdIns-PLC superfamiily and signal transduction. *Biochimica et Biophysica Acta* **1092**, 49-71.

Michell, R. H. (1975). Inositol phospholipids and cell surface receptor function. *Biochimica et Biophysica Acta* **415**, 81-147.

Michell, R. H. & Hawthorne, J. N. (1965). The site of diphosphoinositide synthesis in rat liver. *Biochemical and Biophysical Research Communications* **21**, 333-338.

Miller, R. J. (1987). Multiple calcium channels and neuronal function. *Science* **235**, 46-52.

Miller, W. R., Scott, W. N., Morris, R., Fraser, H. M. & Sharpe, R. M. (1986). Direct effects of LHRH and agonists on human breast cancer cells, pp 475-488. In: *Neuroendocrine Molecular Biology*. Eds. Fink, G., Harmar, A.J. & McKerns, K.W. Plenum Press, New York.

Mischak, H., Bodenteich, A., Kolch, W., Goodnight, J., Hofer, F. & Mushinski, J. F. (1991). Mouse protein kinase C-δ, the major isoform expressed in mouse Hemopoietic cells: sequence of the cDNA, expression patterns, and characterisation of the protein. *Biochemistry* **30**, 7925-7931.

Missiaen, L., Taylor, C. W. & Berridge, M. J. (1991). Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores. *Nature* **352**, 241-244.

Mitchell, R., Johnson, M., Ogier, S.-A. & Dougan, L. (1989). Regulation of receptor-operated and depolarisation-operated Ca^{2+} influx in anterior pituitary tissue. *Annals of the New York Academy of Sciences* **560**, 451-453.

Mitchell, R., Johnson, M., Ogier, S.-A. & Fink, G. (1988). Facilitated calcium mobilisation and inositol phosphate production in the priming effect of LH-releasing hormone in the rat. *Journal of Endocrinology* **119**, 293-301.

Mitchell, R., Johnson, M. S., Avery, J. L., MacEwan, D. J. & Thomson, F. J. (1992). The effects of different classes of activators on protein kinase c from rat midbrain. *British Journal of Pharmacology* (in press).

Mitchell, R., MacEwan, D. J., Dougan, L., Johnson, M. S. & Thomson, F. J. (1991). Does inositol hexakisphosphate induce Ca^{2+} entry into GH_3 cells? *Biochemical Society Transactions* **19**, 116S.

Mobbs, C. V., Fink, G. & Pfaff, D. W. (1990a). HIP-70: a protein induced by estrogen in the brain and LH-RH in the pituitary. *Science* **247**, 1477-1479.

Mobbs, C. V., Fink, G. & Pfaff, D. W. (1990b). HIP-70: an isoform of phosphoinositol-specific phospholipase C-α. *Science* **249**, 566.

Mochly-Rosen, D. & Koshland, D. E., Jr. (1987). Domain structure and phosphorylation of protein kinase C. *Journal of Biological Chemistry* **262**, 2291-2297.

Moelling, K., Heimann, B., Beimling, P., Rapp, U. R. & Sander, T. (1984). Serine- and threonine-specific protein kinase activities of purified gag-mil and gag-raf proteins. *Nature* **312**, 558-561.

Mollay, C. & Kreil, G. (1974). Enhancement of bee venom phospholipase A_2 activity by melittin, direct lytic factor from cobra venom and polymexin B. *FEBS Letters* **46**, 141-144.

Mori, T., Takai, Y., Yu, B., Takahashi, J., Nishizuka, Y. & Fujikura, T. (1982). Specificity of the fatty acyl moeities of diacylglycerol for the activation of

calcium-activated, phospholipid-dependent protein kinase. *Journal of Biochemistry* **91**, 427-431.

Mori, Y., Friedrich, T., Kim, M.-S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hofmann, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T. & Numa, S. (1991). Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature* **350**, 398-402.

Moriarty, C. M. & Leuschen, M. P. (1981). Role of calcium in acute stimulated release of prolactin in neoplastic GH_3 cells. *Americal Journal of Physiology* **240**, E705-E711.

Morris, A. P., Gallacher, D. V., Irvine, R. F. & Peterson, O. H. (1987). Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channels. *Nature* **330**, 653-655.

Mufson, R. A., Okin, E. & Weinstein, I. B. (1981). Phorbol esters stimulate the rapid release of choline from prelabelled cells. *Carcinogenesis* **2**, 1095-1102.

Muid, R. E., Penfield, A. & Dale, M. M. (1987). The diacylglycerol kinase inhibitor, R59022, enhances the superoxide generation from human neutrophils induced by stimulation of fMet-Leu-Phe, IgG and C3b receptors. *Biochemical and Biophysical Research Communications* **143**, 630-637.

Murad, F. & Haynes, R. C., Jr. (1980). Adenohypophyseal hormones and related substances. In: *The Pharmacological Basis of Therapeutics (6th Edition)* pp 1369-1396. Eds. Gilman, S.G., Goodman, L.S. & Gilman, A. Macmillan Publishing, New York.

Murakami, K., Chan, S. Y. & Routtenberg, A. (1986). Protein kinase C activation by *cis*-fatty acid in the absence of Ca^{2+} and phospholipids. *Journal of Biological Chemistry* **261**, 15424-15429.

Murakami, K. & Routtenberg, A. (1985). Direct activation of purified protein kinase C by unsatured fatty acids (oleate and arachidonate) in the absence of phospholipids and Ca^{2+}. *FEBS Letters* **192**, 189-193.

Murphy, S. & Welk, G. (1989). Arachidonic acid evokes inositol phospholipid hydrolysis in astrocytes. *FEBS Letters* **257**, 68-70.

Nakabayashi, H., Brown, J. H., Morrell, J. L., Chen, H.-C. & Huang, K.-P. (1990). Phosphorylation of magainin-2 by protein kinase C and inhibition of protein kinase C isozymes by a synthetic analogue of magainin-2-amide. *FEBS Letters* **267**, 135-138.

Nakadate, T., Jeng, A. Y. & Blumberg, P. M. (1988). Comparison of protein kinase C functional assays to clarify mechanisms of inhibitor action. *Biochemical Pharmacology* **37**, 1541-1545.

Nakadate, T., Yamamoto, S., Aizu, E., Nishikawa, K. & Kato, R. (1989). H7, a protein kinase C inhibitor, inhibits phorbol ester-caused ornithine decarboxylase induction but fails to inhibit phorbol ester-caused suppression of epidermal

growth factor binding in primary mouse epidermal cultures. *Molecular Pharmacology* **36**, 917-924.

Nakashima, S., Tohmatsu, T., Hattori, H., Suganuma, A. & Nozawa, Y. (1987). Guanine nucleotides stimulate arachidonic acid release by phospholipase A_2 in saponin-permeabilized human platelets. *Journal of Biochemistry* **101**, 1055-1058.

Naor, Z. (1990). Further characterisation kinase-C subspecies in the hypothalamo-pituitary axis: differential activation by phorbol esters. *Endocrinology* **126**, 1521-1526.

Naor, Z., Capponi, A., Rossier, M., Ayalan, D. & Limor, R. (1988b). GnRH-induced rise in cytosolic free Ca^{2+} levels: mobilization of cellular and extracellular Ca^{2+} pools and relationship to gonadotorpin secretion. *Molecular Endocrinology* **2**, 512-518.

Naor, Z. & Catt, K. J. (1981). Mechanism of action of gonadotropin-releasing hormone. Involvement of phospholipid turnover in luteinizing hormone release. *Journal of Biological Chemistry* **256**, 2226-2229.

Naor, Z., Dan-Cohen, H., Hermon, J. & Limor, R. (1989). Induction of exocytosis in permeabilized pituitary cells by α- and ß-type protein kinase C. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 4501-4504.

Naor, Z., Shearman, M. S., Kishimoto, A. & Nishizuka, Y. (1988a). Calcium-independent activation of hypothalamic type I protein kinase C by unsaturated fatty acids. *Molecular Endocrinology* **2**, 1043-1048.

Naor, Z., Vanderhoek, J. Y., Lindner, H. R. & Catt, K. J. (1983). Arachidonic acid products as possible mediators of the action of gonadotropin-releasing hormone. In: *Advances in Prostaglandin, Thromboxane, and Leukotriene Research.* Eds. Samuelsson, B., Paoletti, R. and Ramwell, P. Raven Press, New York.

Nasmith, P. & Grinstein, S. (1989). Diacylglycerol kinase inhibitors R59022 and dioctanoylethylene glycol potentiate the respiratory burst of neutrophils by raising cytosolic Ca^{2+}. *Biochemical and Biophysical Research Communications* **161**, 95-100.

Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. R. & Lefkowith, J. B. (1986). Arachidonic acid metabolism. *Annual Review of Biochemistry* **55**, 69-102.

Neer, E. J. & Clapham, D. E. (1988). Role of G protein subunits in transmembrane signalling. *Nature* **333**, 129-134.

Negishi, M., Ito, S. & Hayaishi, O. (1990). Arachidonic acid stimulates phosphoinositide metabolism and catecholamine release form bovine adrenal chromaffin cells. *Biochemical and Biophysical Research Communications* **169**, 773-779.

Newton, A. C. & Koshland, D. E., Jr. (1990). Phosphatidylserine affects specificity of protein kinase C substrate phosphorylation and autophosphorylation. *Biochemistry* **29**, 6656-6661.

Niedel, J. E., Kuhn, L. J. & Vandenbark, G. R. (1983). Phorbol diester receptor copurifies with protein kinase C. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 36-40.

Nishizuka, Y. (1983). Phospholipid degradation and signal translation for protein phosphorylation. *Trends in Biochemical Sciences* **8**, 13-16.

Nishizuka, Y. (1984a). The role of protein kinase C in cell surface transduction and tumour promotion. *Nature* **308**, 693-698.

Nishizuka, Y. (1984b). Turnover of inositol phospholipids and signal transduction. *Science* **225**, 1365-1370.

Nishizuka, Y. (1984c). Protein kinases in signal transduction. *Trends in Biochemical Sciences* **9**, 163-166.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. *Science* **233**, 305-312.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**, 661-665.

Nishizuka, Y., Takai, Y., Kishimoto, A., Kikkawa, U. & Kaibuchi, K. (1984). Phospholipid turnover in hormone action. *Recent Progress in Hormone Research* **40**, 301-345.

Nowycky, M. C., Fox, A. P. & Tsien, R. W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* **316**, 440-443.

Nunn, D. E. & Watson, S. P. (1987). A diacylglycerol kinase inhibitor, R59022, potentiates secretion by and aggregation of thrombin-stimulated human platelets. *Biochemical Journal* **243**, 809-813.

Offermans, S., Schultz, G. & Rosenthal, W. (1989). Secretion-stimulating and secretion-inhibiting hormones stimulate high-affinity pertussis-toxin-sensitive GTPases in membranes of a pituitary cell line. *European Journal of Biochemistry* **180**, 283-287.

Ohno, S., Akita, Y., Konno, Y., Imajoh, S. & Suzuki, K. (1988b). A novel phorbol ester receptor/protein kinase, nPKC, distantly related to the protein kinase C family. *Cell* **53**, 731-741.

Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T. & Hidaka, H. (1987). Tissue-specific expression of three distinct types of rabbit protein kinase C. *Nature* **325**, 161-166.

Ohno, S., Kawasaki, H., Konno, Y., Inagaki, M., Hidaka, H. & Suzuki, K. (1988a). A fourth type of rabbit protein kinase C. *Biochemistry* **27**, 2083-2087.

Ohta, H., Tanaka, T. & Hidaka, H. (1988). Putative binding site(s) of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) on protein kinase C. *Biochemical Pharmacology* **37**, 2704-2706.

Ojeda, S. R. & Campbell, W. B. (1982). An increase in hypothalamic capacity to synthesize PGE_2 precedes the first preovulatory surge of gonadotropins. *Endocrinology* **111**, 1031-1037.

Ojeda, S. R., Negro-Vilar, A. & McCann, S. M. (1982). Evidence for involvement of α-adrenergic receptors in norepinephrine-induced prostaglandin E_2 and luteinizing hormone-releasing hormone release from the median eminence. *Endocrinology* **110**, 409-412.

Ojeda, S. R., Urbanski, H. F., Junier, M.-P. & Capderila, J. (1989). The role of arachidonic acid and its metabolites in the release of neuropeptides. *Annals of the New York Academy of Sciences* **559**, 192-207.

Ojeda, S. R., Urbanski, H. F., Katz, K. H. & Costa, M. E. (1986). The activation of oestradiol positive feedback at puberty: Estradiol sensitizes the LHRH releasing system at two different biochemical steps. *Neuroendocrinology* **43**, 259-266.

Ono, Y., Fujii, T., Igarashi, K., Kikkawa, U. & Nishizuka, Y. (1988b). Nucleotide sequences of cDNAs for α and γ subspecies of rat brain protein kinase C. *Nucleic Acid Research* **16**, 5199-5200.

Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U. & Nishiuka, Y. (1989a). Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 4867-4871.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarachi, K. & Nishizuka, Y. (1989). Protein kinase C ζ subspecies from rat brain: its structure, expression, and properties. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 3099-3103.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. & Nishizuka, Y. (1987b). Identification of three additional members of rat protein kinase C family: δ-, ε- and ζ-subspecies. *FEBS Letters* **226**, 125-128.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. & Nishizuka, Y. (1988a). The structure, expression, and properties of additional members of the protein kinase C family. *Journal of Biological Chemistry* **263**, 6927-6932.

Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K. & Nishizuka, Y. (1987a). Expression and properties of two types of protein kinase C: alternative splicing from a single gene. *Science* **236**, 1116-1120.

Ono, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarashi, K., Kikkawa, U., Ogita, K. & Nishizuka, Y. (1986). Two types of complementary DNAs of rat brain protein kinase C. *FEBS Letters* **206**, 347-352.

Ordway, R. W., Singer, J. J. & Walsh, J. V., Jr. (1991). Direct regulation of ion channels by fatty acids. *Trends in Neuroesciences* **14**, 96-100.

Ordway, R. W., Walsh, J. V., Jr. & Singer, J. J. (1989). Arachidonic acid and other fatty acids directly activate potassium channels in smooth muscle cells. *Science* **244**, 1176-1179.

Pai, J., Pachter, J. A., Weinstein, I. B. & Bishop, W. R. (1991). Overexpression of protein kinase C ß1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 598-602.

Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Watefield, M. D. & Ullrich, A. (1986). The complete primary structure of protein kinase C - the major phorbol ester receptor. *Science* **238**, 853-859.

Parker, P. J., Kour, G., Marais, R. M., Mitchell, F., Pears, C., Schaap, D., Stabel, S. & Webster, C. (1989). Protein kinase C – a family affair. *Molecular and Cellular Endocrinology* **65**, 1-11.

Pelech, S. L., Charest, D. L., Howard, S. L., Paddon, H. B. & Salari, H. (1990). Protein kinase C activation by platelet activating factor is independent of enzyme translocation. *Biochimica et Biophysica Acta* **1051**, 100-107.

Pelech, S. L., Samiei, M., Charest, D. L., Howard, S. L. & Salari, H. (1991). Characterization of calcium-independent forms of protein kinase C-ß in phorbol ester-treated rabbit platelets. *Journal of Biological Chemistry* **266**, 8696-8705.

Pelech, S. L. & Vance, D. E. (1984). Regulation of phsophatidylcholine biosynthesis. *Biochimica et Biophysica Acta* **919**, 26-36.

Pelech, S. L. & Vance, D. E. (1989). Signal transduction via phosphatidylcholine cycles. *Trends in Biochemical Sciences* **14**, 28-30.

Penner, R., Matthews, G. & Neher, E. (1988). Regulation of calcium influx by second messengers in rat mast cells. *Nature* **334**, 499-504.

Pfaff, D. W. (1980). *Estrogens and brain function.* Springer, New York.

Pfeffer, L. M., Strulovici, B. & Saltiel, A. R. (1990). Interferon-α selectively activates the ß isoform of protein kinase C through phosphatidylcholine hydrolysis. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 6537-6541.

Pietrobon, D. & Hess, P. (1990). Novel mechanism of voltage-dependent gating in L-type calcium channels. *Nature* **346**, 651-655.

Piomelli, D., Volterra, A., Dale, N., Siegelbaum, S. R., Kandel, E. R., Schwartz, J. H. & Belardetti, F. (1987). Lipoxygenase-metabolites of arachidonic acid as second messengers for presynaptic inhibition of *Aplysia* sensory cell. *Nature* **328**, 38-43.

Piomelli, D., Wang, J. K. T., Sihra, T. S., Nairn, A. C., Czernik, A. J. & Greengard, P. (1989). Inhibition of Ca^{2+}/calmodulin-dependent protein kinase II by arachidonic acid and its metabolites. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 8550-8554.

Popkin, R., Bramley, T. A., Currie, A., Shaw, R. W., Baird, D. T. & Fraser, H. M. (1983). Specific binding of luteinizing hormone-releasing hormone to human tuteal tissue. *Biochemical and Biophysical Research Communications* **114**, 750-754.

Powell, W. S. (1982). Rapid extraction of arachidonic acid metabolites from biological samples using octadecylsilyl silica. *Methods in Enzymology* **86**, 467-477.

Poyser, N. L. (1977). Physiological Roles of Prostaglandins in Reproduction. In: *Prostaglandins and Thromboxanes*. pp 205-219. Eds. Berti, F., Samuelson, B. and Velo, G.P. Plenum Publishing, New York.

Poyser, N. L. (1978). Involvement of prostaglandins in the female reproductive cycle. *Biochemical Society Transactions* **6**, 718-722.

Poyser, N. L. (1983). Uterine prostaglandin production: arachidonic acid cascade, fact or fiction? *Advances in Prostaglandin, Thromboxane, and Leukotriene Research* **12**, 443-447.

Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E. & Bell, R. M. (1986). Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and vas- and sis-transformed normal rat kidney cells. *Journal of Biological Chemistry* **261**, 8597-8600.

Putney, J. W., Jr. (1986). A model for receptor-regulated calcium entry. *Cell Calcium* **7**, 1-12.

Putney, J. W., Jr., Weiss, S. J., Van De Walle, C. M. & Haddas, R. A. (1980). Is phosphatidic acid a calcium ionophore under neurohumoral control? *Nature* **284**, 345-347.

Ramsdell, J. S., Pettit, G. R. & Tashijian, A. H., Jr. (1986). Three activators of protein kinase C, bryostatins, dioleins, and phorbol esters, show differing specificities of action on GH_4 pituitary cells. *Journal of Biological Chemistry* **261**, 17073-17080.

Ran, W., Dean, M., Levine, R. A., Henkle, C. & Campisi, J. (1986). Induction of c-fos and c-myc mRNA by epidermal growth factor or calcium ionophore is cyclic AMP dependent. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 8216-8220.

Rando, R. R. & Young, N. (1984). The stereospecific activation of protein kinase C. *Biochemistry and Biophysical Research Communications* **122**, 818-823.

Rane, S. G., Walsh, M. P., McDonald, J. R. & Dunlop, K. (1989). Specific inhibitors of protein kinase C block transmitter-induced modulation of sensory neuron calcium current. *Neuron* **3**, 239-245.

Raymond, V., Leung, P. C. K., Veilleux, R., Le Fevre, G. & Labrie, F. (1984). LHRH rapidly stimulates phosphatidylinositol metabolism in enriched gonadotrophs. *Molecular and Cellular Endocrinology* **36**, 157-164.

Rebecchi, M. J. & Gershengorn, M. C. (1983). Thyroliberin stimulates rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate by phosphodiesterase in rat mammotrophic pituitary cells: evidence for an early calcium-independent action. *Biochemical Journal* **216**, 287-294.

Rebecchi, M. J., Monaco, M. E. & Gershengorn, M. C. (1981). Thyrotropin-releasing hormone rapidly enhances ^{32}P-orthophosphate incorporation into phosphatidic acid in cloned GH_3 cells. *Biochemical and Biophysical Research Communications* **101**, 124-130.

Rebecci, M. J., Kolesnick, R. N. & Gershengorn, M. C. (1983). Thyrotropin-releasing hormone stimulates rapid loss of phosphatidyl inositol and its conversion to 1,2-diacylglycerol and phosphatidic acid in rat mammotrophic pituitary cells. *Journal of Biological Chemistry* **258**, 227-234.

Rhee, S. G., Suh, P.-G., Ryu, S.-H. & Lee, S. Y. (1989). Studies of phospholipid-specific phospholipase C. *Science* **244**, 546-550.

Rink, T. & Pozzan, T. (1985). Using Quin 2 in cell suspensions. *Cell Calcium* **6**, 133-144.

Rink, T. J. (1990). Receptor-mediated calcium entry. *FEBS Letters* **268**, 381-385.

Rodriguez-Pena, A. & Rozengurt, E. (1984). Disappearance of Ca^{2+}-sensitive, phospholipid-dependent protein kinase activity in phorbol ester-treated 3T3 cells. *Biochemical and Biophysical Research Communications* **120**, 1053-1059.

Rosenthal, W., Hescheler, J., Hinsch, K.-D., Spicher, K., Trautwein, W. & Schultz, G. (1988). Cyclic AMP-independent dual regulation of voltage-dependent Ca^{2+} currents by LHRH and somatostatin in a pituitary cell line. *EMBO Journal* **7**, 1627-1633.

Rosenthal, W. & Schultz, G. (1987). Modulation of voltage-dependent ion channels by extracellular signals. *Trends in Pharmacological Sciences* **8**, 351-354.

Rosoff, P. M., Savage, N. & Dinarello, C. A. (1988). Interleukin 1 stimulates diacylglycerol production in T lymphocytes by a novel mechanism. *Cell* **54**, 73-81.

Rüegg, U. T. & Burgess, G. M. (1989). Staurosporine, K252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends in Pharmacological Sciences* **10**, 218-220.

Ryves, W. J., Garland, L. G., Evans, A. T. & Evans, F. J. (1989). A phorbol ester and a daphnane ester stimulate a calcium-independent kinase activity from human mononuclear cells. *FEBS Letters* **245**, 159-163.

Sakuma, Y. & Pfaff, D. W. (1980). LH-RH in the mesencephalic central grey can potentiate lordosis reflex in female rats. *Nature* **283**, 566.

Sandeman, J., Peralta, E. G. & Wurtmann, R. J. (1991). Coupling of transfected muscarinic acetylcholine receptor subtypes to phospholipase D. *Journal of Biological Chemistry* **266**, 6031-6034.

Sando, J. J. & Young, M. C. (1983). Identification of high affinity phorbol ester receptor in cytosol of EL4 thymoma cells: requirement for calcium, magnesium and phospholipids. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 2642-2646.

Schaap, D. & Parker, P. J. (1990). Expression, purification and characterisation of protein kinase C-ε. *Journal of Biological Chemistry* **265**, 7301-7307.

Schaap, D., Parker, P. J., Bristol, A., Kriz, R. & Knopf, J. L. (1989). Unique substrate specificity and regulatory properties of PKC-ε: a rationale for diversity. *FEBS Letters* **243**, 351-357.

Schally, A. V. (1973). Aspects of hypothalamic regulation of the pituitary gland: its implications for the control of reproductive functions. *Science* **202**, 18-28.

Schimmel, R. J. (1988). The α_1-adrenergic transduction system in hamster brown adipocytes. Release of arachidonic acid accompanies activation of phospholipase C. *Biochemical Journal* **253**, 93-102.

Schlegel, W., Rosuit, C. & Zahnd, G. R. (1984). Polyphosphoinositide hydrolysis by phospholipase C is accelerated in GH_3 cells: a possible mechanism in stimulus-response coupling. *FEBS Letters* **134**, 47-49.

Schlegel, W. & Wollheim, C. B. (1984). Thyrotropin-releasing hormone increases free cytosolic calcium in clonal pituitary cells (GH_3 cells). Direct evidence for the mobilisation of cellular calcium. *Journal of Cellular Biology* **99**, 83-87.

Schrey, M. P. (1985). Gonadotrophin-releasing hormone stimulates the formation of inositol phosphates in rat anterior pituitary tissue. *Biochemistry Journal* **226**, 563-569.

Seifert, R., Schächtele, C., Rosenthal, W. & Schultz, G. (1988). Activation of proteinkinase C by *cis*-and *trans*-fatty acids and its potentiation by diacylglycerol. *Biochemical and Biophysical Research Communications* **103**, 759-765.

Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U. & Nishizuka, Y. (1988). Mode of activation and kinetic properties of three distinct forms of protein kinase C from rat brain. *Journal of Biochemistry* **103**, 759-765.

Sekiguchi, K., Tsukuda, M., Ogita, K., Kikkawa, U. & Nishizuka, Y. (1987). Three distinct forms of rat brain protein kinase C: differential response to unsaturated fatty acids. *Biochemical and Biophysical Research Communications* **145**, 797-802.

Serhan, C., Anderson, P., Goodman, E., Dunham, P. & Weissmann, G. (1981). Phosphatidate and oxidised fatty acids are calcium ionophores. Studies employing arsenazo III in liposomes. *Journal of Biological Chemistry* **256**, 2736-2741.

Sharkey, N. A. & Blumberg, P. M. (1985). Kinetic evidence that 1,2-diolein inhibits phorbol ester binding to protein kinase C via a competitive mechanism. *Biochemical and Biophysical Research Communications* **133**, 1051-1056.

Sharkey, N. A., Hennings, H., Yuspa, S. H. & Blumberg, P. M. (1989). Comparison of octahydromezerein and mezerein as protein kinase C activators and as mouse skin tumor promoters. *Carcinogenesis* **10**, 1937-1941.

Shearman, M. S., Kosaka, Y., Ase, K., Kikkawa, U. & Nishizuka, Y. (1988). Type 1 (γ) protein kinase C subspecies appears to be located exclusively in central nervous tissues. *Biochemical Society Transactions* **16**, 307-308.

Shearman, M. S., Naor, Z., Sekiguchi, K., Kishimoto, A. & Nishizuka, Y. (1989a). Selective activation of the γ-subspecies of protein kinase C from bovine cerebellum by arachidonic acid and its lipoxygenase metabolites. *FEBS Letters* **243**, 177-182.

Shearman, M. S., Seikiguchi, K. & Nishizuka, Y. (1989b). Modulation of ion channel activity: a key function of the protein kinase C enzyme family. *Pharmacological Reviews* **41**, 211-237.

Shimizu, T. & Wolfe, L. S. (1990). Arachidonic acid cascade and signal transduction. *Journal of Neurochemistry* **55**, 1-15.

Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. & Nishizuka, Y. (1991). Synergistic action of diacylglycerol and unsaturated fatty acid for protein kinase C activation: its possible implications. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 5149-5153.

Simmonds, A. C. & Halsey, S. A. (1985). General and local anaesthetics perturb the fusion of phospholipid vesicles. *Biochimica et Biophysica Acta* **813**, 331-337.

Simon, M. I., Strathmann, M. P. & Gautam, N. (1991). Diversity of G proteins in signal transduction. *Science* **252**, 802-808.

Slivka, S. R. & Insel, P. A. (1987). α_1-adrenergic receptor-mediated phosphoinositide hydrolysis and prostaglandin E_2 formation in Madin-Darby

canine kidney cells. Possible parallel activation of phospholipase C and phospholipase A_2. *Journal of Biological Chemistry* **262**, 4200-4207.

Smart, R. C., Mills, K. J., Hansen, L. A. & Conney, A. H. (1989). Synthetic lipid second messenger *sn*-1,2-didecanoylglycerol: a complete tumor promoter in mouse skin. *Cancer Research* **49**, 4455-4458.

Smrcka, A. V., Hepler, J. R., Brown, K. O. & Sternweis, P. C. (1991). Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. *Science* **251**, 804-807.

Snyder, G. D. & Bleasdale, J. E. (1982). Effect of LHRH on incorporation of [^{32}P]-orthophosphate into phosphatidylinositol by dispersed anterior pituitary cells. *Molecular and Cellular Endocrinology* **28**, 55-63.

Snyder, S. H. & Supattapore, S. (1989). Isolation and functional characterisation of an inositol trisphosphate receptor from brain. *Cell Calcium* **10**, 337-342.

Stevens, L. R., Hughes, K. T. & Irvine, R. F. (1991). Pathway of phosphatidylinositol (3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature* **351**, 33-39.

Stojilkovic, S. S., Merilli, F., Iida, T., Krsmanovic, L. Z. & Catt, K. J. (1990). Endothelin stimulation of cytosolic calcium and gonadotropin secretion in anterior pituitary cells. *Science* **248**, 1663-1666.

Strathmann, M. & Simon, M. I. (1990). G protein diversity: A distinct class of α subunits is present in vertebrates and invertebrates. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 9113-9117.

Streb, H., Irvine, R. F., Berridge, M. J. & Schultz, I. (1983). Release of Ca^{2+} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* **306**, 67-69.

Strong, J. A., Fox, A. P., Tsien, R. W. & Kaczmarek, L. K. (1987). Stimulation of protein kinase C recruits covert calcium channels in Aplysia bag cell neurons. *Nature* **325**, 714-717.

Strulovici, B., Daniel-Issakani, S., Bazter, G., Knopf, J., Sultzman, L., Cherwinski, H., Nestor, J., Jr, Webb, D. R. & Ransom, J. (1991). Distinct mechanisms of regulation of protein kinase C ε by hormones and phorbol diesters. *Journal of Biological Chemistry* **266**, 168-173.

Strulovici, B., Daniel-Issakani, S., Oto, E., Nestor, J., Jr., Chan, H. & Tsou, A.-P. (1989). Activation of distinct protein kinase C isozymes by phorbol esters: correlation with induction of interleukin 1ß gene expression. *Biochemistry* **28**, 3569-3576.

Sutrave, P., Bonner, T. I., Rapp, U. R., Jansen, H. W., Patschinsky, T. & Bister, K. (1984). Nucleotide sequence of avian retroviral oncogene v-mil: homologue of murine retroviral oncogene v-raf. *Nature* **309**, 85-88.

Suzuki, N. & Yoshioka, T. (1987). Differential blocking action of synthetic ω-conotoxin on components of Ca^{2+} channel current in clonal GH_3 cells. *Neuroscience Letters* **75**, 235-239.

Szallasi, A. & Blumberg, P. M. (1989a). Resiniferatoxin, a phorbol-related diterpine, acts as an ultrapotent analogue of capsaicin, the irritant constituent in red pepper. *Neuroscience* **30**, 515-520.

Szallasi, A. & Blumberg, P. M. (1989b). Neurogenic component of phorbol ester-induced mouse skin inflammation. *Cancer Research* **49**, 6052-6057.

Szallasi, A. & Blumberg, P. M. (1990). Effect of resiniferatoxin pretreatment on the inflammatory response to phorbol-12-myristate-13-acetate in mouse strains with different susceptibilities to phorbol ester tumour promotion. *Carcinogenesis* **11**, 583-587.

Szallasi, A., Joo, F. & Blumberg, P. M. (1989). Duration of desensitization and ultrastructural changes in dorsal root ganglia in rats treated with resiniferatoxin, an ultrapotent capsaicin anolog. *Brain Research* **503**, 68-72.

Takai, Y., Kishimoto, A., Inoue, M. & Nishizuka, Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues (I - purification and characterisation of an active enzyme from bovine cerebellum). *Journal of Biological Chemistry* **252**, 7603-7609.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. & Nishizuka, Y. (1979a). Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *Journal of Biological Chemistry* **254**, 3692-3695.

Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. & Nishizuka, Y. (1979b). Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. *Biochemistry and Biophysical Research Communications* **91**, 1218-1224.

Takemoto, H., Yokoro, K., Furth, J. & Cohen, A. I. (1962). Adrenotropic activity of mammo-somatotropic tumors in rats and mice. *Cancer Research* **22**, 917.

Takemura, H. & Putney, J. W. (1989). Capacitative calcium entry in parotid acinar cells. *Biochemical Journal* **258**, 409-412.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochemical and Biophysical Research Communications* **135**, 397-402.

Tanabe, T., Beam, K. G., Powell, J. A. & Numa, S. (1988). Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature* **336**, 134-139.

Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kengawa, K., Kojima, M., Matsuo, H., Hirose, T. & Numa, S. (1987). Primary structure

of the receptor for calcium channel blockers from skeletal muscle. *Nature* **328**, 313-318.

Tashjian, A. H., Jr. (1979). Clonal strains of hormone-producing pituitary cells. *Methods in Enzymology* **58**, 527-535.

Tashjian, A. H., Borowsky, N. J. & Jensen, D. K. (1971). Thyrotropin-releasing hormone: direct evidence for stimulation of prolactin production by pituitary cells in culture. *Biochemical and Biophysical Research Communications* **43**, 516-523.

Tashjian, A. H., Lomedico, M. E. & Maina, D. (1978). Role of calcium in the thyrotropin-releasing hormone-stimulated release of prolactin from pituitary cells in culture. *Biochemical and Biophysical Research Communications* **81**, 798-806.

Tashjian, A. H., Jr, Yasumura, Y., Levine, L., Sato, G. H. & Parker, M. L. (1968). Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. *Endocrinology* **82**, 342-352.

Taylor, C. W. (1990). Receptor-regulated Ca^{2+} entry: secret pathway or secret messenger? *Trends in Pharmacological Sciences* **11**, 269-271.

Taylor, G. W. & Clarke, S. R. (1986). The leukotriene biosynthetic pathway: a target for pharmacological attack. *Trends in Pharmacological Sciences* **7**, 100-103.

Taylor, S. J., Chae, H. Z., Rhee, S. G. & Exton, J. H. (1991). Activation of the ß1 isozyme of phospholipase C by α subunits of the Gq class of G proteins. *Nature* **350**, 516-518.

Testori, A., Hii, C. S. T., Fournier, A., Burgoyne, L. A. & Murray, A. W. (1988). DNA-binding proteins in protein kinase C preparations. *Biochemistry and Biophysical Research Communications* **156**, 222-227.

Thomson, W. (1969). Positional distribution of fatty acids in brain polyphosphoinositides. *Biochimica et Biophysica Acta* **187**, 150-153.

Thorner, M. O., Holl, R. W. & Leong, D. A. (1988). The somatotrope: an endocrine cell with functional calcium transients. *Journal of Experimental Biology* **139**, 169-179.

Traynor-Kaplan, A. E., Harris, A. L., Thomson, B. L., Taylor, P. & Sklar, L. A. (1988). An inositol tetrakisphosphate-containing phosphilipid in activated neurtophils. *Nature* **334**, 353-356.

Traynor-Kaplan, A. E., Thomson, B. L., Harris, A. L., Taylor, P., Oman, G. M. & Sklar, L. A. (1989). Transient increases in phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol trisphosphate during activation of human neutrophils. *Journal of Biological Chemistry* **264**, 15668-15673.

Tsien, R. W., Ellinor, P. T. & Horne, W. A. (1991). Molecular diversity of voltage-dependent Ca^{2+} channels. *Trends in Pharmacological Sciences* **12**, 349-354.

Tsien, R. W., Lipscombe, D., Madison, D. V., Bley, K. R. & Fox, A. P. (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends in Neurosciences* **11**, 431-438.

Vale, W., Rivier, C. & Brown, M. (1977). Regulatory peptides of the hypothalamus. *Annual Review of Physiology* **39**, 473-527.

Vallejo, M., Jackson, T., Lightman, S., N & Hanley, M. R. (1987). Occurrence and extracellular actions of inositol pentakis- and hexakisphosphate in mammalian brain. *Nature* **330**, 656-658.

van Blitterswijk, W. J., Hilkmann, H., de Widt, J. & van der Bend, R. L. (1991a). Phospholipid metabolsim in bradykinin-stimulated human fibroblasts. I. Biphasic formation of diacylglycerol from phosphatidylinositol and phosphatidylcholine, controlled by protein kinase C. *Journal of Biological Chemistry* **266**, 10337-10343.

van Blitterswijk, W. J., Hilkmann, H., de Widt, J. & van der Bend, R. L. (1991b). Phospholipid metabolism in bradykinin-stimulated human fibroblasts. II. Phosphatidylcholine breakdown by phospholipases C and D; involvement of protein kinase C. *Journal of Biological Chemistry* **266**, 10344-10350.

Van Breemen, C., Farinas, B. R., Casteels, R., Gerba, P., Wuytack, F. & Deth, R. (1973). Factors controlling cytoplasmic Ca^{2+} concentration. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **265**, 57-71.

Van Renterghem, C., Romey, G. & Lazdunski, M. (1988). Vasopressin modulates the spontaneous electrical activity in aortic cells (line A7r5) by acting on three different types of ionic channels. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 9365-9369.

Velasco, J. M. & Petersen, O. H. (1989). The effect of a cell-permeable diacylglycerol analogue on single Ca^{2+} (Ba^{2+}) channel currents in the insulin-secreting cell line RINm5F. *Quarterly Journal of Experimental Physiology* **74**, 367-370.

Volterra, A. & Siegelbaum, S. A. (1988). Role of two different guanine nucleotide-binding proteins in the antagonistic modulation of the S-type K^+ channel by cAMP and arachidonic acid metabolites in *Aplysia* sensory neurons. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 7810-7814.

Von Tscharner, V., Prod'hom, B., Baggliolini, M. & Reuter, H. (1986). Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration. *Nature* **324**, 369-372.

Wagner, P. D. & Vu, N.-D. (1989). Thiophosphorylation causes Ca^{2+}-independent norepinephrine secretion from permeabilized PC12 cells. *Journal of Biological Chemistry* **264**, 19614-19620.

Wakelam, M. J. O., Davies, S. A., Houslay, M. D., McKay, I., Marshall, C. J. & Hall, A. (1986). Normal p21$^{N\text{-}ras}$ couples bombesin and other growth factor receptors to inositol phosphate production. *Nature* **323**, 173-176.

Warden, C. H. & Friedkin, M. (1985). Regulation of choline kinase activity and phosphatidylcholine biosynthesis by mitogenic growth factors in 3T3 fibroblasts. *Journal of Biological Chemistry* **260**, 6006-6011.

Wender, P. A., Cribbs, C. M., Koehler, K. F., Sharkey, N. A., Herald, C. L., Kamano, Y., Petit, G. R. & Blumberg, P. M. (1988). Modeling of the bryostatins to the phorbol ester pharmacophore on protein kinase C. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 7197-7201.

Whetton, A. D., Monk, P. N., Consalavey, S. D., Huang, S. J., Dexter, T. M. & Downes, C. P. (1988). Interleukin 3 stimulates proliferation via protein kinase C activation without increasing inositol lipid turnover. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 3284-3288.

Whitehouse, B. J. (1989). Lipocortins, mediators of the anti-inflammatory actions of corticosteroids? *Journal of Endocrinology* **123**, 363-366.

Whitman, M. & Cantley, L. (1988). Phosphoinositide metabolism and the control of cell proliferation. *Biochimica et Biophysica Acta* **948**, 327-344.

Whitman, M., Downes, C. P., Keeler, M., Keller, T. & Cantley, L. (1988). Type I phosphatidylinolitol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature* **332**, 644-646.

Windle, J. J., Weiner, R. I. & Mellon, P. L. (1990). Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. *Molecular Endocrinology* **4**, 597-603.

Wise, B. C., Glass, D. B., Chou, C.-H. J., Raynor, R. L., Katoh, N., Schatzman, R. C., Turner, R. C., Kibler, R. F. & Kuo, J. F. (1982b). Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. II. substrate specificity and inhibition by various agents. *Journal of Biological Chemistry* **257**, 8489-8495.

Wise, B. C., Raynor, R. L. & Kuo, J. F. (1982a). Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. I. purification and general properties. *Journal of Biological Chemistry* **257**, 8481-8488.

Wolf, M., Guatrecasas, P. & Sahyoun, N. (1985). Interaction of protein kinase C with membranes is regulated by Ca^{2+}, phorbol esters and ATP. *Journal of Biological Chemistry* **260**, 15718-15722.

Worley, P. F., Baraban, J. M., Colvin, J. S. & Snyder, S. H. (1987). Inositol trisphosphate receptor localization in brain: variable stoichiometry with protein kinase C. *Nature* **325**, 159-161.

Yagisawa, H., Simmonds, S. H. & Hawthorne, J. H. (1989). Response to muscarinic stimulation of rat pituitary GH_3 cells in relation to thyrotropin releasing hormone-induced phosphoinositide turnover and activation of membrane GTPase. *Biochemical Society Transactions* **17**, 100-101.

Yamamoto, S., Kiyoto, I., Aizu, E., Nakadate, T., Hosoda, Y. & Kato, R. (1989). Differential inhibition by staurosporine, a potent protein kinase C inhibitor, of 12-0-tetradecanoyl-phorbol-13-acetate-caused skin tumour promotion, epidermal ornithine decarboxylase induction, hyperplasia and inflammation. *Carcinogen* **10**, 1315-1322.

Young, W. S., III. (1989). Levels of transcripts encoding a member of the protein kinase C family in the paraventricular and supraoptic nuclei are increased by hyperosmolality. *Journal of Neuroendocrinology* **1**, 79-82.

Zavioco, G. B., Halenda, S. P., Sha'afi, R. I. & Fenstein, M. B. (1985). Phorbol myristatelacetate inhibits thrombin-stimulated Ca^{2+} mobilisation and phosphatidylinositol 4,5-bisphosphate hydrolysis in human platelets. *Proceedings of the National Academy of Sciences of the Untied States of America* **82**, 3859-3862.

Zernig, G. (1990). Widening potential for Ca^{2+} antagonists: non-L-type Ca^{2+} channel interaction. *Trends in Pharmacological Sciences* **11**, 38-44.

Zhao, F.-K., Chuang, L. F., Israel, M. & Chuang, R. Y. (1989). Cremaphor EL, a widely used parenteral vehicle, is a potent inhibitor of protein kinase C. *Biochemical and Biophysical Research Communications* **159**, 1359-1367.

Zschauer, A., Van Breeman, C., Bühler, F. R. & Nelson, M. T. (1988). Calcium channels in thrombin-activated human platelet membrane. *Nature* **334**, 703-705.

Zwelling, L. A., Chan, D., Altschuler, E., Mayes, J., Hinds, M. & Pettit, G. R. (1991). Effect of bryostatin 1 on drug-induced, topoisomerase II-mediated DNA cleavage and topoisomerase II gene expression in human leukemia cells. *Biochemical Pharmacology* **41**, 829-832.

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Lithium enhances the inhibitory effect of protein kinase C on LHRH-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary tissue *in vitro*

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The metabolism of inositol phosphates is modified by Li^+ (Allison *et al.* 1976), and it has been suggested that this may underlie the therapeutic effects of Li^+ in manic-depressive disorders (Berridge *et al.* 1982). However, agonist-induced diacylglycerol production is enhanced by Li^+ (Drummond & Raeburn, 1984), suggesting that Li^+ may modify several aspects of responses to Ca^{2+}-mobilizing receptors, including the activation of protein kinase C (PKC).

The influx of $^{45}Ca^{2+}$ into prisms of male rat anterior pituitary tissue was measured by an assay involving quenching of uptake after 30 s and extensive washing in ice-cold EGTA-containing medium (Fink *et al.* 1986). The $^{45}Ca^{2+}$ influx induced by 100 nM luteinizing hormone-releasing hormone (LHRH) but not by 300 nM thyrotrophin-releasing hormone (TRH) was inhibited by low concentrations (3–300 nM) of the PKC activator phorbol 12-myristate, 13-acetate (Mitchell *et al.* 1989). The concentration–response curve to LHRH but not TRH was biphasic, and the downturn at high LHRH concentrations was reversed by inhibitors of PKC (Fink *et al.* 1986). Therefore the mechanism of $^{45}Ca^{2+}$ influx induced by LHRH but not TRH can apparently be inhibited by hormone-induced activation of PKC. In the presence of LiCl (45 min pre-incubation), the $^{45}Ca^{2+}$ influx due to 100 nM-LHRH, but not 300 nM TRH or basal accumulation, was reduced, with 50% inhibition at $1{\cdot}0 \pm 0{\cdot}2$ mM (mean ± S.E.M., $n = 4$). This effect was reversed by the PKC inhibitor H7 (30 μM) but not by HA 1004 (Hidaka & Hagiwara, 1987). The biphasic concentration–response curve to LHRH was suppressed by 3 mM-Li^+ such that the maximal response was inhibited by approximately 50% and the downturn phase (from 100 to 1000 nM-LHRH) was amplified. It is possible that enhanced activation of PKC, as well as modified inositol phosphate metabolism, may contribute to the therapeutic actions of Li^+ *in vivo*.

REFERENCES

Allison, J. H., Blisner, M. E., Holland, W. M., Hipps, P. P. & Sherman, W. R. (1976). *Biochem. Biophys. Res. Comm.* **71**, 664–670.

Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982). *Biochem. J.* **206**, 587–595.

Drummond, A. H. & Raeburn, C. A. (1984). *Biochem. J.* **224**, 129–136.

Fink, G., Johnson, M. S., Minaur, N. J., Mitchell, R. & Ogier, S.-A. (1986). *J. Physiol.* **381**, 44*P*.

Hidaka, H. & Hagiwara, M. (1987). *Trends Pharm. Sci.* **8**, 162–164.

Mitchell, R., Johnson, M. S. & Dougan, L. (1989). *Biochem. Soc. Trans.* **17**, 750–751.

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Depolarization-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary cells *in vitro* can be modulated in two ways by protein kinase C

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The operation of voltage-sensitive Ca^{2+} channels can be either enhanced or inhibited by activators of protein kinase C (PKC) (Lacerda *et al.* 1988). The present experiments explore the effects of a number of PKC activators on depolarization-induced $^{45}Ca^{2+}$ influx into anterior pituitary cells and in the GH_3 cell line.

The influx of $^{45}Ca^{2+}$ was measured by an assay involving quenching of uptake after 30 s and extensive washing in ice-cold EGTA-containing medium (Fink *et al.* 1986). The $^{45}Ca^{2+}$ influx induced in GH_3 cells by 60 mM-K^+ medium was inhibited by 40–50% in a concentration-dependent manner by 4β- but not 4α-phorbol 12,13-didecanoate (PDD) and this effect was reversed by the PKC inhibitor staurosporine (Hidaka & Hagiwara, 1987). Drummond (1985) also found that K^+-induced elevation of cytosolic Ca^{2+} in GH_3 cells was inhibited by phorbol esters. In contrast, in anterior pituitary prisms from male rats, K^+-induced $^{45}Ca^{2+}$ influx was facilitated stereo-selectively by PDD in a staurosporine-sensitive manner. The PKC inhibitor H7 (Hidaka & Hagiwara, 1987) reversed the stimulatory effect of 300 nM-phorbol 12,13-dibutyrate (PDBu) with an IC_{50} of 62 ± 10 μM, mean $\pm$ S.E.M., $n = 5$, but gave 50% reversal of the inhibitory effect in GH_3 cells at only 10 μM. Dioctanoyl-sn-glycerol (DOG), another PKC activator, at 3–100 μM, was active only in pituitary prisms, causing facilitation. The inability of DOG to elicit inhibition was also seen by Lacerda *et al.* (1988), describing dual effects of PKC on cardiac Ca^{2+} channels. Pre-incubation times from 0 to 30 min caused no difference in PDBu effects here. In both preparations nimodipine blocked the K^+-induced influx with 50% inhibition at 3–7 nM.

The two modes of influence of PKC on K^+-induced $^{45}Ca^{2+}$ influx here have different properties which could be consistent with different iso-forms of the enzyme (Nishizuka, 1988) being involved. It is not clear whether the locus of action in each case is the L-type Ca^{2+} channel itself or some other Ca^{2+}-handling component of the cell.

REFERENCES

DRUMMOND, A. H. (1985). *Nature* **351**, 752–755.
FINK, G., JOHNSON, M. S., MINAUR, N. J., MITCHELL, R. & OGIER, S.-A. (1986). *J. Physiol.* **382**, 31*P*.
HIDAKA, H. & HAGIWARA, M. (1987). *Trends Pharmac. Sci.* **8**, 162–164.
LACERDA, A. E., RAMPE, D. & BROWN, A. M. (1988). *Nature* **335**, 249–251.
NISHIZUKA, Y. (1988). *Nature* 661–665.

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Inhibition of depolarization-induced calcium influx into GH_3 cells by arachidonic acid may involve direct activation of protein kinase C

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Recent evidence suggests that arachidonic acid (AA) can be produced by several G protein-linked receptors in response to agents such as bradykinin, noradrenaline and GABA (Axelrod *et al.* 1988). Arachidonic acid can be metabolized to derivatives which modulate cellular function, for example by activation of K^+ channels (Kim *et al.* 1989). However, Nishizuka and his co-workers have reported that AA can activate certain isoforms of protein kinase C (PKC) in cell-free systems (Naor *et al.* 1988). Here we show that exogenous AA, in the presence of inhibitors of its metabolism, can mimic phorbol esters in reducing depolarization-induced $^{45}Ca^{2+}$ influx into GH_3 clonal pituitary cells.

GH_3 cells were grown, harvested and $^{45}Ca^{2+}$-influx measurements were performed as previously described (Johnson *et al.* 1989). The $^{45}Ca^{2+}$-influx elicited by 60 mM-K^+ medium was fully inhibited by nimodipine (IC_{50} 3–7 nM). Arachidonic acid completely suppressed 60 mM-K^+-induced $^{45}Ca^{2+}$ influx in a dose-dependent manner with an IC_{50} of 20 μM ($n = 12$). Phorbol esters also stereoselectively inhibited calcium influx into GH_3 cells induced by K^+-depolarization (Johnson *et al.* 1989). Like the effect of phorbol esters, inhibition by AA was reversed by the PKC inhibitors (Johnson *et al.* 1989) H7 (2–50 μM) and staurosporine (5–300 nM), but not by their less active congeners HA1004 and K252a, respectively. Calcium influx was not reduced by 8-Br cyclic GMP (up to 100 μM), suggesting that guanylate cyclase was not participating in the effect of AA (Glass *et al.* 1977). Inhibitors of AA metabolism such as nordihydroguaiaretic acid, eicosatetraynoic acid, piperonyl butoxide and 2-diethyl-aminoethyl-2,2-diphenylvalerate had no effect on the action of AA.

These results suggest that AA may itself act in GH_3 cells to activate an isoform of PKC which inhibits the influx of calcium through an 'L'-type voltage-sensitive calcium channel. It is possible that not only diacylglycerols, but also AA may be important in the physiological activation of certain PKC isoforms.

REFERENCES

AXELROD, J., BURCH, R. M. & JELSEMA, C. L. (1988). *Trends in Neurosci.* **22**, 117–123.
GLASS, D. G., FREY, W., CARR, D. W. & GOLDBERG, N. D. (1977). *J. biol. Chem.* **252**, 1279–1285.
JOHNSON, M. S., MACEWAN, D. J. & MITCHELL, R. (1989). *J. Physiol.* **418**, 186*P*.
KIM, D., LEWIS, D. L., GRAZIADEI, L., NEER, E. J., BAR-SAGI, D. & CLAPHAM, D. E. (1989). *Nature* **337**, 557–560.
NAOR, Z., SHEARMAN, M. S., KISHIMOTO, A. & NISHIZUKA, Y. (1988). *Mol. Endocrinol.* **2**, 1043–1048.

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Effects of calmodulin antagonists on neurohormone-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary tissue *in vitro*

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Luteinizing hormone-releasing hormone (LHRH) and thyrotrophin-releasing hormone (TRH) both induce $^{45}Ca^{2+}$ influx into rat anterior pituitary tissue, but utilize different routes, which are regulated differentially by protein kinase C (Mitchell *et al.* 1989*b*). The $^{45}Ca^{2+}$ influx induced by LHRH is largely sensitive to nimodipine (suggesting that it may occur indirectly through voltage-sensitive Ca^{2+} channels), whereas the response to TRH is resistant to blockers of the known voltage-sensitive Ca^{2+} channels or of the Na^+/Ca^{2+} exchanger (Mitchell *et al.* 1989*b*). Receptor-activated Ca^{2+} entry (perhaps such as the response to TRH here) may involve a synergistic action of inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) and inositol 1,4,5-trisphosphate ($InsP_3$) (A. P. Morris *et al.* 1987). The production of $InsP_4$ from $InsP_3$ by a 3-kinase may be calmodulin (CM)-dependent (A. J. Morris *et al.* 1987). We have therefore investigated effects of CM antagonists on neurohormone-induced Ca^{2+} entry.

The rapid influx of $^{45}Ca^{2+}$ into prisms of tissue from male rats was measured in 30 s incubations with stimuli, as described previously (Mitchell *et al.* 1989*a*). At this time point, the stimulus-induced influx had reached a maximal increment over the basal $^{45}Ca^{2+}$ accumulation in each case. The influx of $^{45}Ca^{2+}$ induced by LHRH (100 nM) or TRH (100 nM), but not by 60 mM-K^+ medium, was inhibited by a new selective CM antagonist, 5-iodo-1-C_8 (MacNeil *et al.* 1988) at 1–30 μM. The related, but less selective compounds W7 and W5 (MacNeil *et al.* 1988) had similar effects with lower potencies. At concentrations of 100 μM, W7 and W5 also caused significant reduction of K^+-induced $^{45}Ca^{2+}$ influx (as did pimozide at just 10 μM), indicating that the specificity of these compounds is uncertain at such concentrations. The present results suggest that an unknown CM-dependent step is indeed involved in neurohormone-induced $^{45}Ca^{2+}$ influx regardless of whether this occurs via known voltage-sensitive Ca^{2+} channels (in the case of LHRH) or via some other route of receptor-activated Ca^{2+} entry (in the case of TRH).

REFERENCES

MACNEIL, S., GRIFFIN, M., COOKE, A. M., PETTETT, N. J., DAWSON, R. A., OWEN, R. & BLACKBURN, G. M. (1988). *Biochem. Pharmacol.* **37**, 1717–1723.
MITCHELL, R., JOHNSON, M. S., & DOUGAN, L. (1989*a*). *Biochem. Soc. Trans.* **17**, 750–751.
MITCHELL, R., JOHNSON, M. S., OGIER, S.-A. & DOUGAN, L. (1989*b*). *Ann. N.Y. Acad. Sci.* **560**, 451–453.
MORRIS, A. J., DOWNES, C. P., HARDEN, T. K. & MICHELL, R. H. (1987). *Biochem. J.* **248**, 489–493.
MORRIS, A. P., GALLACHER, D. V., IRVINE, R. F. & PETERSEN, O. H. (1987). *Nature* **330**, 653–655.

Comparison of purified D_2-dopamine receptor from bovine brain and pituitary gland

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D_2-dopamine receptors extracted from bovine caudate nucleus, using the detergent cholate, have been purified to apparent homogeneity by affinity chromatography on haloperidol–Sepharose and wheat-germ agglutinin–agarose columns [1]. In this report, we describe a preliminary comparison of purified receptor preparations derived from other D_2-dopamine-receptor-rich regions of bovine brain and the pituitary gland.

A mixed mitochondrial–microsomal preparation of the tissues studied was solubilized using a sodium cholate (0.3% w/v)/sodium chloride (1 M) combination and purified in a stepwise manner on a haloperidol type II- or spiperone type I-affinity column [2, 3], followed by wheat-germ agglutinin–agarose chromatography [1]. The resulting purified D_2-dopamine-receptor preparations were compared by stereospecific [^{3}H]spiperone binding and SDS/PAGE analysis [1]. Bovine cerebellum was adopted as a control tissue, with no detectable D_2-dopamine receptor present in the initial mixed mitochondrial–microsomal preparation (data not shown).

Our purification scheme, adopting both types of affinity columns, isolates an M_r 95000 glycoprotein as defined by SDS/PAGE analysis under reducing conditions. Several lines of evidence cited previously suggest this to be the D_2-dopamine receptor in caudate nucleus [1]. The lack of appearance of an M_r 95000 species when the cerebellum was the chosen tissue is consistent with this suggestion (Table 1).

The M_r 95000 species runs coincidentally on SDS/PAGE with the major glycoprotein(s) isolated from bovine putamen (striatal) and olfactory tubercle (limbic) regions, both shown to be rich in D_2-dopamine receptors in membrane and soluble preparations ([4] and this study). The final purified preparations from these brain regions also contain stereospecific [^{3}H]spiperone binding of a similar magnitude to that observed in caudate nucleus. Thus it seems that in brain the D_2-dopamine receptor purifies as an M_r 95000 species.

The major protein isolated from the pituitary gland using a spiperone type I-affinity column, however, is an M_r 142000–145000 species, although the M_r 95000 species is also purified from this tissue. The identity of the higher molecular mass species awaits pharmacological analysis but, interestingly, corresponds to recent photolabelling data suggesting the existence of a M_r 140000–150000 subtype of the D_2-dopamine receptor in the pituitary gland and brain [5, 6].

Table 1. *Comparison of the major features observed upon SDS/PAGE analysis of various purified D_2-dopamine receptor preparations*

D_2-dopamine receptors were solubilized from the tissues shown and assayed by [^{3}H]spiperone binding as in Hall *et al.* [7]. Receptors were purified from the soluble extract and analysed by SDS/PAGE as in Williamson *et al.* [1]. The relative intensity of the major bands on SDS/PAGE are indicated as follows: ++ very strong, + strong, − absent.

Tissue	[^{3}H]spiperone binding in soluble preparation (fmol/mg)	M_r 95000	M_r 145000
Caudate nucleus	133	++	−
Putamen	104	++	−
Olfactory tubercle	65	++	−
Pituitary gland	115	+	+
Cerebellum	2	−	−

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1. Williamson, R. A., Worrall, S., Chazot, P. L. & Strange, P. G. (1988) *EMBO J.* **7**, 4129–33
2. Juszczak, R. & Strange, P. G. (1985) *Biochem. Pharmacol.* **34**, 3959–63
3. Juszczak, R. & Strange, P. G. (1987) *Neurochem. Intl.* **11**, 389–95
4. Leonard, M. N., Macey, C. A. & Strange, P. G. (1987) *Biochem. J.* **248**, 595–602
5. Jarvie, K. R., Niznik, H. B. & Seeman, P. (1988) *Mol. Pharm.* **34**, 91–97
6. Jarvie, K. R. & Niznik, H. B. (1989) *J. Biochem.* **106**, 17–22
7. Hall, J. M., Frankham, P. A. & Strange, P. G. (1988) *J. Neurochem.* **41**, 1526–32

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Arachidonic acid acts through protein kinase C to inhibit depolarization-induced $^{45}Ca^{2+}$ influx

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Activation of protein kinase C (PKC) in the GH_3 clonal pituitary cell line by phorbol esters [4β-phorbol 12,13-didecanoate and phorbol 12,13-dibutyrate (PDBu) but not 4α-phorbol 12,13-didecanoate] results in a reduction of the $^{45}Ca^{2+}$ influx through 'L'-type Ca^{2+} channels induced by 60 mM-K^+ medium [1]. Briefly, GH_3 cells grown in Ham F10 medium with 15% (v/v) foetal bovine serum, 1 mM-L-glutamine, 100 units of penicillin/ml and 0.1 mg of streptomycin/ml were harvested, washed and resuspended at ~6 × 10^6 cells/ml in 'Ca^{2+}-uptake medium' (concentrations in mM: NaCl, 154; KCl, 5.4; $CaCl_2$, 1.5; D-glucose, 11; Hepes, 6; pH adjusted to 7.4 with Tris base and with 0.1%, w/v, bovine serum albumin). Cells were preincubated (30 min, 37°C, O_2) before a 10 min incubation with drugs (37°C, O_2) then exposure to low-(5.4 mM) or high-(60 mM) K^+ Ca^{2+}-uptake medium containing $^{45}CaCl_2$ (~3 μCi/tube). After 30 s, $^{45}Ca^{2+}$ uptake was halted by quenching with ice-cold 2 mM-EGTA (Ca^{2+}-free) Ca^{2+}-uptake medium and filtration under vacuum. Cellular $^{45}Ca^{2+}$ uptake was measured by liquid scintillation counting. Using the same conditions as those used for experiments with phorbol esters, a similar reduction in K^+-induced Ca^{2+} influx was produced by arachidonic acid [IC_{50} (concentration for 50% inhibition of response) = 20 ± 3 μM, mean ± S.E.M., $n = 12$]. It has been suggested [2] that arachidonic acid (or a metabolite) may reduce cellular Ca^{2+} concentrations by activating guanylate cyclase. Therefore, this possibility was tested in GH_3 cells. 8-Bromo-cyclic GMP (a cell-permeable activator of protein kinase G) had no effect on depolarization-induced $^{45}Ca^{2+}$

Abbreviations used: PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; DMF, dimethylformamide.

influx up to 100 μM at which K^+-induced influx was $105 \pm 9\%$ of control (mean ± S.E.M., $n = 12$). In contrast, the PKC inhibitors H7 (2–50 μM) and staurosporine (5–300 nM) dose-dependently reversed the inhibitory effect of arachidonic acid, but their less active congeners HA1004 and K252a, respectively, were inactive at the same concentrations, thus suggesting that a selective activation of PKC by arachidonic acid in GH_3 cells was the mechanism of inhibition of Ca^{2+} influx through 'L' channels. Arachidonic acid can be metabolized to a range of other bio-active compounds. Inhibitors of any such metabolism [nordihydroguaiaretic acid (30 μM), eicosatetraynoic acid (10 μM), piperonyl butoxide (30 μM) and SKF525A (10 μM)] were unable to modify the arachidonic acid inhibition of K^+-evoked Ca^{2+} influx. Therefore, arachidonic acid itself rather than any of its metabolites appears to be responsible for this inhibition of depolarization-induced Ca^{2+} influx into GH_3 cells.

The relative abundance of different PKC isoforms in GH_3 cells is unknown, but the pituitary gland (from which the GH_3 line is derived) contains both α- and β- but not γ-isoforms [3]. The α- and γ- but not β-isoforms respond to arachidonic acid [3], suggesting that the isoform of PKC mediating the present results with arachidonic acid may be α. To test this hypothesis, we carried out a series of ligand-binding studies at the phorbol ester/diacylglycerol recognition site of cytosolic PKCs derived from regions enriched in different isoforms. Cytosolic [³H]PDBu binding was carried out as described by Leach *et al.* [4]. Briefly, various regions of male COB-Wistar rats were homogenized in 2 vol. of 50 mM-Tris–HCl (pH 9.0) containing 1 mM-phenylmethanesulphonyl fluoride and 1 mM-$MnCl_2$ and ultracentrifuged (100000 ***g*** for 1 h at 4°C then 120000 ***g*** for 1 h, at 4°C). The supernatant from the second spin was regarded as cytosol and stored at −40°C until use. Cytosol at a concentration which gave total binding of ~10% of total radioactivity present, was incubated (30 min, 37°C) with various concentrations (0.5–500 μM) of sonicated arachidonic acid, 1 mg of sonicated phosphatidylserine/ml, [³H]PDBu (−0.03 μCi/tube) and dimethylformamide (DMF) or 2 mM-PDBu in DMF (for non-specific binding). Protein was precipitated with 12 mg of bovine gamma globulin/ml and 24% (w/v) polyethyleneglycol 8000. After 20 min on ice, tubes were spun (12000 ***g*** for 5 min at 4°C), aspirated and the radioactivity in each pellet was determined by liquid scintillation counting. The binding of [³H]PDBu was not displaced by arachidonic acid (1–500 μM) but in certain regions was allosterically enhanced. This effect was marked (+60–135% at 500 μM) using regions such as lung and sciatic nerve which are highly enriched in α-isoform [5] but not (< +18% at 500 μM) in regions such as spleen and cerebral cortex (enriched in β-isoforms) or cerebellum (enriched in γ-isoform). Our data therefore suggest not only that arachidonic acid (as well as diacylglycerols) can act as a physiological activator of PKC, but furthermore that it is the α-isoform of the enzyme that mediates the observed arachidonic acid inhibition of Ca^{2+} influx. It is possible that activation of the α-isoform of PKC by arachidonic acid may be an important physiological mechanism for the regulation of cellular function, at least in part, by inhibition of voltage sensitive Ca^{2+} influx.

1. Johnson, M. S., MacEwan, D. J. & Mitchell, R. (1989) *J. Physiol. (London)* **418**, 186*P*
2. Glass, D. G., Frey, W., Carr, D. W. & Goldberg, N. D. (1977) *J. Biol. Chem.* **252**, 1279–1285
3. Naor, Z., Shearman, M. S., Kishimoto, A. & Nishizuka, Y. (1988) *Mol. Endocrinol.* **2**, 1043–1048
4. Leach, K. L., James, M. L. & Blumberg, P. M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4208–4212
5. Shearman, M. S., Kosata, Y., Ase, K., Kikkawa, U. & Nishizuka, Y. (1988) *Biochem. Soc. Trans.* **16**, 307–308

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Biological activity of some thyrotrophin-releasing hormone analogues substituted at the 2 position

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Receptors for thyrotrophin-releasing hormone (TRH; pGlu-His-Pro-NH_2) are present in a number of neuronal and endocrine cells. Centrally administered TRH analogues produce analeptic, hyperthermic and motor effects and have undergone limited clinical trials for spinal injury, motor neuron disease, circulatory shock and Alzheimer's disease [1]. The affinities of a number of substituted TRH analogues at these sites have been extensively studied by ligand-binding techniques [1]. There is some evidence for heterogeneity of TRH receptors, first suggested by the somewhat differing potency ratios of TRH and [3-MeHis²]TRH on prolactin and thyrotrophin release from pituitary tissue *in vitro* [2]. Regional differences in the affinity of certain benzodiazapines for TRH receptors [3] are also consistent with the contention of receptor heterogeneity. Most recently, studies with a number of analogues substituted at the 2-position have shown that a clear segregation of several *in vivo* actions of TRH can be achieved with analogues such as [Nval²]TRH [4, 5]. We have investigated the activity of these analogues and some novel heteroaromatic substitutions at the 2-position in a functional response assay for TRH receptors *in vitro*.

The rapid influx of $^{45}Ca^{2+}$ into slices of anterior pituitary tissue or cells of the GH_3 pituitary-derived line (as in the present experiments) was assayed as previously described [6]. Briefly, TRH-analogue-induced influx was stopped after 30 s by quenching with ice-cold EGTA-containing medium, filtration and extensive washing. The response to 300 nM-TRH was maximal within 30 s and represented in the order of 180% increase over the basal unstimulated accumulation of $^{45}Ca^{2+}$ by tissue. TRH, [3-MeHis²]TRH and [Nval²]TRH (Peninsula Laboratories Inc.) all produced similar maximum responses of around 170–190% increase in $^{45}Ca^{2+}$ accumulation, with approximate EC_{50} values (concentrations for 50% of maximal response) of 2, 16 and 200 nM, respectively. A number of other 2-substituted TRH analogues were synthesized by solution methods (by C.B.) and their structures verified by fast atom bombardment mass spectra and amino acid analysis. [4-NO_2-His²]TRH produced only $45 \pm 8\%$ increase at a concentration of 10 μM (mean ± S.E.M., $n = 4$). [L-Furyl-Ala²]TRH, and to a lesser extent [L-pyrolyl-Ala²]-TRH and [L-thienyl-Ala²]-TRH were moderately active, producing effects of $152 \pm 10\%$, $89 \pm 11\%$ and $75 \pm 8\%$ (means ± S.E.M., $n = 4$–6), respectively, at a concentration of 10 μM.

The selective activation of the receptor on GH_3 cells by [Nval²]TRH but not [4-NO_2-His²]TRH is consistent with this

Abbreviations used: TRH, thyrotrophin-releasing hormone; pGla, pyroglutamyl; 3-MeHis, 3-methylhistidyl; Nval, norvalyl.

representing one of the two receptor subtypes suggested by data in the literature. We have described the potency of a number of novel analogues at this receptor subtype. The effectiveness of [Nval²]TRH at GH_3 cell TRH receptors is consistent with its effectiveness in promoting prolactin release *in vivo*, since GH_3 cells are considered to be derived from one form of prolactin-secreting pituitary cell, the somatomammotrophe. Recent data describing an inositol phosphate production assay for TRH responses in GH_3 cells have also suggested that [Nval²]TRH is a full agonist of clearly lower potency than TRH [7]. Experiments currently in progress are investigating the potencies of these analogues in causing $^{45}Ca^{2+}$ influx in other pituitary cells and regions of the central nervous system.

1. Sharif, N. A., To, Z., Michel, A. D. & Whiting, R. L. (1989) *Br. J. Pharmacol.* **96**, 45*P*
2. Dannies, P. S. & Markell, M. S. (1980) *Endocrinology* **106**, 107–111
3. Sharif, N. A. & Burt, D. R. (1984) *J. Neurochem.* **43**, 742–746
4. Siren, A.-L., Feurerstein, G., Labroo, V. M., Cohen, L. A. & Lozovsky, D. (1986) *Neuropeptides* **8**, 63–70
5. Labroo, V. M., Cohen, L. A., Lozovsky, D., Siren, L.-A. & Feurerstein, G. (1987) *Neuropeptides* **10**, 29–36
6. Mitchell, R., Johnson, M., Ogier, S.-A. & Dougan, L. (1989) *Ann. N.Y. Acad. Sci.* **560**, 451–453
7. Sharif, N. A., To, Z. & Whiting, R. L. (1989) *Biochem. Biophys. Res. Commun.* **161**, 1306–1311

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Pyroglutamylpeptide amides in rat central nervous system: possible new class of neurotransmitter

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Since the first hypothalmic releasing factor, pyroglutamylhistidylproline amide (pGlu-His-Pro-amide; thyroid hormone-releasing hormone, TRH), was identified [1, 2], there have been a number of reports that this tripeptide occurs in other regions of the central nervous system [3] and in certain peripheral tissues [4, 5]. Recently, a peptide with a related structure, pGlu-Glu-Pro amide, was identified in rabbit prostate [6] and it was shown to occur also in rabbit and human semen [7, 8]. Since rat portal blood has been reported to contain three TRH-immunoreactive components [9], of which only one corresponded chromatographically to TRH, it has become of interest to investigate whether TRH-related peptides such as pGlu-Glu-Pro amide occur in brain. In this communication we describe a chromatographic procedure for distinguishing between TRH and TRH-related peptides and we have used this method to test for the presence of TRH-like peptides in the central nervous system of the rat. In addition, we report chromatographic data showing that the new forms of TRH which were found comprise at least seven components.

Regions of brain tissues were dissected from male Sprague–Dawley rats (200–250 g), weighed and rapidly frozen at −20°C. The tissues were homogenized for 2 min at room temperature in 100 mM-hydrochloric acid or 1 M-acetic acid containing approximately 5000 c.p.m. of ^{125}I-TRH (0.3 μg labelled with 1 mCi of ^{125}I). The resulting suspensions were incubated in the homogenizing medium for 10 min, then were maintained for a further 10 min at 100°C to ensure cyclization of *N*-terminal glutamine. After centrifugation at 20000 rev./min for 30 min, the supernatants were concentrated on a rotary evaporator *in vacuo*, the residues were taken up in 1 ml of 50% (v/v) acetic acid and the resulting solutions were clarified by centrifugation before chromatography on a mini-column of SP-Sephadex C25. The columns (6 cm × 0.5 cm) were prepared in the pyridinium form by washing successively with 5 ml of 1 M-HCl/H_2O/1 M-pyridine, and finally with 50% (v/v) acetic acid. Elution was performed in 50% (v/v) acetic acid providing eight 0.5 ml fractions and in 0.4 M-pyridine in 50% (v/v) acetic acid to provide a further 12 fractions. The ability of the column to resolve neutral and acidic TRH-related peptides from basic peptides was demonstrated at the pmolar level with the aid of synthetic preparations of pGlu-Glu-Pro amide and pGlu-His-Pro amide. After chromatography of the brain extracts on the mini-column, the acetic acid was removed from the eluted fractions by centrifugation *in vacuo* and the concentrations of TRH and TRH-related peptides were determined by radioimmunoassay using a procedure described previously [10].

When 100 m-HCl was used for extraction, it was found that the TRH-immunoreactive peptides obtained were completely retained on the cationic mini-column; their distribution corresponded to the known distribution of TRH. The highest concentrations were in the hypothalamus (13.9 pmol/g) and spinal cord (11.9 pmol/g), whereas lower concentrations were observed in the olfactory bulb, cortex, mid-brain and other regions. In marked contrast, extraction of the tissues in 1 M-acetic acid revealed additionally the presence of neutral or acidic TRH-related peptides which were not retained on the mini-column. These peptides occurred in high concentration in the hippocampus (40.1 pmol/g), brain stem (38.3 pmol/g) and dorsal colliculae (34 pmol/g), and lower concentrations were observed in the cerebellum and mid-brain. The concentrations were assigned by comparison with a TRH standard and, since the TRH-related peptides may be less immunoreactive than TRH, represent minimum values.

It is notable that TRH was extracted by 100 mM-HCl or 1 M-acetic acid in similar quantities, but the acidic or neutral TRH-related peptides were found only in the acetic acid extracts. The same result was observed when extraction was carried out in 50% (v/v) acetic acid, 1 M-acetic acid at 4°C or methanol: TRH was extracted by each of these solvents but no TRH-related peptides were obtained. These findings suggest that the TRH-related peptides extracted in 1 M-acetic acid at room temperature are generated enzymically during the course of the extraction procedure. The precursors of these peptides do not appear to react with the TRH antisera employed.

The TRH-immunoreactive peptides obtained by 1 M-acetic acid extraction of two rat brains were fractionated by gel exclusion chromatography on Sephadex G50 Superfine in 30% (v/v) acetic acid and were separated from TRH by passage through a column (6 cm × 2.5 cm) of SP-Sephadex C25 (H^+ form, eluted in H_2O). The unretained fractions were combined, concentrated *in vacuo* and the peptides present were resolved by h.p.l.c. Seven TRH-immunoreactive components were observed (Fig. 1). Each of the TRH-related peptides was tested for the presence of an acidic amino acid residue by chromatography at neutral pH on a mini-column of DEAE-Sephadex. Fractions I, III and IV were found to contain acidic peptides; fractions II, V, VI and VII contained neutral peptides.

Abbreviations used: TRH, thyroid hormone-releasing hormone; pGlu, pyroglutamyl.

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183 STUDIES ON THE MECHANISM OF LHRH-INDUCED $^{45}Ca^{2+}$ INFLUX IN RAT ANTERIOR PITUITARY CELLS

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The secretion of gonadotrophins in response to LHRH is attenuated by removal of extracellular Ca^{2+}, indicating a partial requirement for Ca^{2+} influx in LHRH action. Both LHRH-induced LH secretion in vitro and the rapid influx of $^{45}Ca^{2+}$ induced by LHRH in anterior pituitary slices[1] are inhibited (the former only partially) by dihydropyridines. In bullfrog sympathetic ganglia and gonadotrophes, LHRH-induced membrane depolarisation, perhaps by inhibition of preactivated outward K^{+} ('M') currents, may be the means of activation of voltage-sensitive Ca^{2+} currents. Indeed the 'M' current blocker uridine 5'-triphosphate (10-300μM) caused a concentration-dependent, nimodipine-sensitive $^{45}Ca^{2+}$ influx here. This response could not be mimicked by phorbol ester (phorbol 12-myristate 13-acetate (PMA) at concentrations below 10μM). Furthermore, the $^{45}Ca^{2+}$ influx induced by LHRH (1-100nM) was unaffected by protein kinase C inhibitors H7 and polymyxin B (10μM), and inhibited by PMA (10-300nM) or by the diacylglycerol kinase inhibitor R 59022 (10μM). Although our results suggest that LHRH-induced Ca^{2+} influx in gonadotrophes may indeed be secondary to 'M' current inhibition, they contrast with those in bullfrog sympathetic ganglia and NG 108-15 cells (but not hippocampal pyramidal cells), where PKC is suggested to mediate M current closure[2].

(1) Mitchell et al. (1989) Ann. N.Y. Acad. Sci 560, 451-453.
(2) Higashida & Brown (1988) Nature 323, 333-335.

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198 SELECTIVITY OF PROTEIN KINASE C ACTIVATORS IN THE REGULATION OF Ca^{2+}-CHANNELS IN RAT ANTERIOR PITUITARY CELLS

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Protein kinase C (PKC) can be activated not only by diacylglycerols and phorbol esters but also by arachidonic acid (AA) which can selectively activate certain PKC-isoforms. Phorbol 12,13-dibutyrate (PDBu) has previously been shown to enhance depolarisation-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary pieces, but inhibits $^{45}Ca^{2+}$ influx into GH_3 clonal cells under similar conditions[1]. This influx occurs through an L-type Ca^{2+}-channel in both pituitary pieces and GH_3 cells. Amongst a range of PKC activators tested, on K^+-induced $^{45}Ca^{2+}$ influx in these models, 1,2-dioctanoyl glycerol (DOG), 12-deoxyphorbol 13-isobutyrate (DPB) and AA could selectively elicit just one of the responses. K^+-induced $^{45}Ca^{2+}$ influx into pituitary pieces was increased by 108 ± 10% in the presence of 100μM DOG and by 30 ± 5% in the presence of 1μM DPB. DOG and DPB were inactive in GH_3 cells. In contrast, AA inhibited K^+-induced $^{45}Ca^{2+}$-influx into GH_3 cells (IC_{50} = 20μM) through activation of PKC[2] but was inactive in pituitary pieces up to 300μM. There is evidence that both α- and β- but not γ- isoforms of PKC are present in the pituitary[3]. It is possible that the selective actions of certain PKC activators here may be due to their preferential activation of particular PKC isoforms.

1. Johnson, M.S. et al. (1989) J. Physiol. (in press).
2. MacEwan, D. and Mitchell, R. Biochem. Soc. Trans. (in press).
3. Naor, Z. et al. (1988) Mol. Endocrinol. 2, 1043-1048.

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PHARMACOLOGY AND CELLULAR ACTIONS OF PROTEIN KINASE C ISOFORMS (*Eur. J. Pharmacol.* **183**, 750-751)

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Protein kinase C (PKC) and phorbol ester binding sites are widely distributed and are likely to play a major role in signal transduction/cellular regulation in a number of systems. It is now clear that at least 7 isoforms of the enzyme are encoded. However, very little is known of the physiological roles of the isoforms and whether they display pharmacological differences that may permit selective intervention. We have developed a number of cellular models of PKC action allowing us to explore in a physiological context the putative selective pharmacology of different isoforms.

Experiments were carried out on anterior pituitary cells from rat or on the GH_3 cell line. It has been reported that α and β, but not γ isoforms of PKC are present in pituitary and that the pituitary-derived GH_3 contains two forms of PKC mRNA. K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells and pituitary pieces (both through L channels) are influenced quite differently by phorbol esters; the former being inhibited and the latter enhanced by 4ß but not 4α-phorbol 12, 13-didecanoate (PDD)(Johnson, MacEwan and Mitchell, 1989). While both effects were readily blocked by staurosporine, only that in GH_3 cells was readily blocked by H7 (IC_{50} = 10 μM). We sought selective agonists for the two effects, finding that arachidonic acid (AA) mimicked the effect in GH_3 cells but not pituitary (being both H7- and staurosporine-sensitive) whereas *sn*-1,2 dioctanoyl glycerol (DOG) and 12-deoxyphorbol 13-isobutyrate (DPB) were effective only in pituitary. Since AA activates α and γ isoforms but β is not considered to be present here, we suggest that the effect shown by AA is via αPKC. Ligand binding studies to cytosolic PKC showed an allosteric enhancement of [^{3}H]-PDBu binding in α-rich but not β– or γ-rich organs and CNS regions, supporting this hypothesis. In contrast, the IC_{50}s for a range of diacyl glycerols, particularly DOG, in displacing [^{3}H]-PDBu were much lower in ß-rich (or γ-rich) regions than in α-rich regions, suggesting that the facilitatory, H7-resistant profile on $^{45}Ca^{2+}$ influx in pituitary pieces was through ß-PKC.

Models at the level of hormone secretion parallel these observations (Johnson and Mitchell, 1989). PDBu-induced secretion of luteinising hormone (LH) but not growth hormone (GH) is mimicked by AA. Again the former effect is H7-sensitive but the latter resistant. Staurosporine is effective on both. The priming effect of LH-releasing hormone (LHRH) is a unique phenomenon of increased secretory responsiveness (Mitchell, Johnson, Ogier & Fink, 1988). This can be mimicked in some aspects by phorbol esters and is sensitive to staurosporine yet not H7. This profile is reminiscent of the extremely high levels of H7 required to block long-term-potentiation and we suggest that this is characteristic of the ß-isoform of PKC. It is clear that less well-characterised isoforms may also contribute to these phenomena to an as yet unknown extent.

References:

Johnson, M.S. and Mitchell, R. (1989), Biochemical Society Transactions 17, 751-752.

Johnson, M.S., MacEwan, D.J. and Mitchell, R. (1989), J. Physiol. (Lond.) 418, 186P

Mitchell, R., Johnson, M.S., Ogier, S.-A. and Fink, G. (1988), J. Endocrinol. 119, 293-301.

PHARMACOLOGY OF PROTEIN KINASE C ISOFORMS AND THEIR CELLULAR ACTIONS IN ANTERIOR PITUITARY CELLS.

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The multiple isoforms of protein kinase C (PKC) may have a range of distinct functions. We are interested in whether they can be differentially manipulated pharmacologically in anterior pituitary cells. A number of distinct pharmacological profiles emerge from experiments on PKC regulation of secretion and ion fluxes in pituitary cells. Arachidonic acid (AA), like phorbol dibutyrate (PDBu),acts through PKC in GH_3 cells to attenuate $^{45}Ca^{2+}$ influx through 'L'-type Ca^{2+} channels. However, in anterior pituitary tissue, AA, cannot mimic the PDBu-induced enhancement of $^{45}Ca^{2+}$ influx through 'L'-channels. Likewise, in pituitary tissue, PDBu-induced secretion of luteinizing hormone (LH), but not growth hormone (GH), is mimicked by AA. These PKC-mediated effects are all sensitive to the PKC inhibitor staurosporine. However, only the influences of PKC on GH_3 (but not pituitary) Ca^{2+} influx and LH (but not GH) secretion were H7-sensitive. Selectivity of dioctanoyl glycerol (DOG) and deoxyphorbol isobutyrate (DPB) will also be discussed. These activators and inhibitors of PKC may be selective for PKC-isoforms in functional pituitary cells.

Protein kinase C-dependent and -independent actions of arachidonic acid in GH_3 cells

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The influx of $^{45}Ca^{2+}$ evoked by exposure of GH_3 cells to high K^+-containing medium is mainly (~85%) through a nimodipine-sensitive route [1]. Pre-exposure of GH_3 cells to arachidonic acid (AA) causes a time- and concentration-dependent inhibition of K^+-induced $^{45}Ca^{2+}$ influx which is reversed by selective inhibitors of protein kinase C (PKC) [2] and attenuated by down-regulation of PKC levels by 24 h pre-treatment with phorbol-12,13-dibutyrate (PDBu). The inhibition of K^+-induced $^{45}Ca^{2+}$-influx by AA and other unsaturated fatty acids occurred in the folowing order of magnitude at 30 µM: AA > linoleic acid > AA methyl ester, which agrees with their order of potency in activation of PKC [3]. A similar inhibition of K^+-induced $^{45}Ca^{2+}$ influx was seen with 10 min PDBu treatment prior to the depolarising stimulus [2]. Either PDBu or 4β (but not α) –phorbol 12,13-didecanoate, however, caused a maximal inhibition of depolarisation-induced $^{45}Ca^{2+}$ influx of ~50%, whereas AA fully inhibited K^+-induced $^{45}Ca^{2+}$ influx at a concentration of 100 µM. A selective inhibitor of PKC, H7 [4], fully reversed the inhibition seen with either PDBu or AA, but HA1004, a less potent and unselective kinase inhibitor, could not reverse the effect of PDBu or AA. The same distinction was apparent with the PKC-selective and -unselective kinase inhibitors staurosporine [5] and K252a [6] respectively. Therefore, the involvement of PKC in AA-induced inhibition of K^+-evoked $^{45}Ca^{2+}$ influx into GH_3 cells seems likely, but an additional (non PDBu-like) action of AA must account for the full inhibition of $^{45}Ca^{2+}$-influx seen with AA and not PDBu.

Fluorimetric studies to determine the cytosolic calcium concentrations within GH_3 cell populations were performed using the fluorescent dye Indo-1 [7]. Briefly, GH_3 cells were loaded with 10 µM Indo-1 acetoxy methyl ester (Indo-1-AM) for 45 min in culture medium (Hams F-10, 15% foetal bovine serum, 1 mM l-glutamine, 100 u/ml penicillin and 0.1 mg/ml streptomycin) at 37°C, 5% CO_2, 95% air. Cells were harvested, washed and resuspended in Hank's Balanced Salt Solution (HBSS (Gibco, Paisley, U.K.)). A 30 min incubation in the dark at room temperature was allowed to provide more complete hydrolysis of intracellular Indo-1-AM. Cells were washed by resuspension-centrifugation (1,000 g, 10 min, 25°C) and again resuspended in HBSS to a density of 5 x 10^6 cells/ml. A sample (2.5 ml) was aliquoted to a quartz cuvette (37°C, stirred magnetically) and the fluorescence was measured in a Shimadzu RF-5000 spectrofluorophotometer interfaced with an IBM PS/2 55 SX micro-computer. Excitation (332 nm, band width 5 nm) and emission (400 nm, band width 5 nm) wavelengths were kept constant so as to reduce the possibility of significant auto fluorescence artefacts in the final signal [8] and a data point was taken every 2 s.

AA (17 µM) induced a rise in GH_3 cytosolic calcium concentrations which was unaffected by the cyclo-oxygenase and lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, 30 µM) and to H7 (30 µM). Higher concentrations of AA could not be tested because of vehicle and solubility artefacts. No rise in cytosolic Ca^{2+} level was apparent with 300 nM PDBu. If a 40 mM K^+ challenge was applied to the cells 5 min after addition of AA, then the K^+-stimulated rise in cytosolic Ca^{2+} concentration was greatly diminished, concurring with the $^{45}Ca^{2+}$ influx data. In the additional presence of H7 (30 µM), the K^+-induced response was fully restored. Therefore, the initial AA-induced rise in cytosolic calcium was insensitive to H7, whereas, the inhibition by AA of K^+-induced elevation of cytosolic Ca^{2+} levels was prevented by H7.

In $^{45}Ca^{2+}$ efflux studies, where the prelabelled cells were exposed to a 50 µM AA challenge, there was a marked increase in the rate of $^{45}Ca^{2+}$ release from the cells. This increase in Ca^{2+} efflux seen with AA was not mimicked by PDBu (300 nM) nor blocked by H7 (30 µM) or cyclo-oxygenase and lipoxygenase inhibitors (indomethacin (10 µM), eicosatetraynoic acid (10 µM) or NDGA (10 µM)). The increased $^{45}Ca^{2+}$ efflux due to AA was blocked by the Ca^{2+} ATPase inhibitor Na_3VO_4 (1 mM) but was unaffected by replacing Na^+ in the medium with N-methyl d-glucamine (in order to inhibit the Na^+/Ca^{2+} exchanger).

The inhibition of depolarisation-induced $^{45}Ca^{2+}$ influx by AA seems to be mediated by PKC activation just like the PDBu-induced inhibition of Ca^{2+}-influx [1]. Unlike PDBu, AA can cause a rise in cytosolic calcium concentrations which is not due to metabolism of AA, but is presumably due to AA itself releasing intracellularly stored Ca^{2+}, as seen in other cellular systems [9-11]. It seems likely that it is this AA-induced rise in cytosolic calcium that underlies the activity of AA in the $^{45}Ca^{2+}$ efflux studies. Thus the AA-stimulated rise in Ca^{2+} efflux may simply reflect the calcium handling processes of GH_3 cells whereby raised cytosolic calcium is extruded from the cells via a Ca^{2+}-ATPase, but not a Na^+/Ca^{2+} exchange mechanism. Both the fluorimetric and $^{45}Ca^{2+}$ efflux studies have shown that this AA-induced rise in cytosolic calcium is unaffected by cyclo-oxygenase or lipoxygenase inhibitors and is not mimicked by PDBu. Our theory, therefore, is that the additional inhibition of K^+-stimulated $^{45}Ca^{2+}$ influx in GH_3 cells when comparing AA to PDBu action, is due to the additional ability of AA to release intracellularly stored Ca^{2+}. This raised cytosolic calcium concentration could act to potentiate the activation of PKC by AA and lead to a full inhibition of nimodipine-sensitive Ca^{2+} entry. Other Ca^{2+}-stimulated mechanisms of diminishing the K^+-stimulated $^{45}Ca^{2+}$ entry apart from PKC cannot be ruled out, although the inhibition of Ca^{2+} entry caused by both AA and PDBu are fully H7- and staurosporine-reversible (but not fully reversed with their inactive congeners, HA1004 and K252a respectively, at the same concentrations).

AA can be metabolised to a wide range of cellular mediators which may be involved in the action of exogenously applied AA in GH_3 anterior pituitary cells, but in the present study have remained uninvestigated. It is likely that AA-induced release of intracellularly stored Ca^{2+} may have a necessary role in the enhanced PKC-mediated inhibition of 'L'-type Ca^{2+} channel activity seen with AA rather than phorbol esters. A dual intracellular calcium release and PKC activating response may be required to see the full inhibition of 'L'-type Ca^{2+} channel activity. Such synergistic dual actions of AA may result in an ability of AA to act as a far more important physiological activator of PKC than has been previously recognised.

References

1. Johnson, M.S., MacEwan, D.J. & Mitchell, R. (1989) J. Physiol. **418,** 186P
2. MacEwan, D. & Mitchell, R. (1990) Biochem. Soc. Trans. **18,** 433-434
3. Seifert, R., Schachtele, C., Rosenthal, W. & Schultz, G. (1988) Biochem. Biophys. Res. Commun. **154,** 20-26
4. Hidaka, H. & Hagiwara, M. (1987) Trends Pharmacol. Sci. **8,** 162-164
5. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986) Biochem. Biophys. Res. Commun. **135,** 397-402
6. Kase, H., Iwahashi, K., Nakanashi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A. & Kaneko, M. (1987) Biochem. Biophys. Res. Comm. **142,** 436-440
7. Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985) J. Biol. Chem. **260,** 3440-3450
8. Lückhoff, A. (1986) Cell Calcium **7,** 233-248
9. Chan, K.-M. & Turk, J. (1987) Biochim. Biophys. Acta **928,** 186-193
10. Beaumier, L., Faucher N. & Naccache, P.H. (1987) FEBS Lett. **221,** 289-292
11. Chow, S.C. & Jondal, M. (1990) J. Biol. Chem. **265,** 902-907

Does inositol hexakisphosphate induce Ca^{2+} entry into GH_3 cells?

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A variety of cells have recently been described to synthesise inositol 1,3,4,5,6-pentakisphosphate ($InsP_5$) and inositol hexakisphosphate ($InsP_6$), (see [1] for review). In contrast to the lower inositol phosphates, the levels of these inositol polyphosphates do not change rapidly in response to Ca^{2+}-mobilising hormones [2]. However, marked excitatory effects were reported when microinjected into the Nucleus Tractus Solitarius of the brain stem [1], consistent with an extracellular site cf action. Inositol polyphosphates are known to be produced in the GH_3 clonal pituitary cell line [2]. We have used the GH_3 cell line to investigate whether inositol polyphosphates might act extracellularly on these cells to influence their mobilisation of Ca^{2+}. The present report describes several series of experiments, begun originally in 1988, and our persistent difficulties in making an unequivocal interpretation of the data.

GH_3 cells were cultured and $^{45}Ca^{2+}$ accumulation determined as described previously [3]. After 30 s at 37°C with 2 μM $^{45}Ca^{2+}$,accumulation was quenched with cold EGTA-medium before rapid filtration and washing.

Concentration-dependent increases in $^{45}Ca^{2+}$ influx were induced by TRH, K^+ and ionomycin with peak increments over basal controls of 148 ± 23% (100 nM TRH) 262 ± 30% (60mM K^+) and 1059 ± 51% (100 μM ionomycin), (mean ± SEM n = 4-8). $InsP_6$ but not its non-physiological analogue, inositol hexasulphate, induced a marked accumulation of $^{45}Ca^{2+}$, much greater than that due to TRH or K^+, but still clearly less than that due to ionomycin. The effect of $InsP_6$ was concentration-dependent showing a statistically significant increment at 3 μM and above, and was saturable with a maximum response of 519 ± 70% increase over basal at 30 μM (n = 6). The $^{45}Ca^{2+}$ accumulation due to 20 μM $InsP_6$ was reduced by 78 ± 4% (n = 4) on inclusion of 100 μM digitonin in the EGTA wash medium. Cell viability (assessed with 0.2% Trypan Blue or 0.5 μg/ml fluorescein diacetate) was 93-100% following incubation with 150 μM $InsP_6$ for 30 min, (n = 4). The effect of $InsP_6$ could not be mimicked by Na_3PO_4, Na glutamate, EGTA, EDTA, 2,3-diphosphoglycerate or Na hydroxide (all at 1mM). At 150 μM, $InsP_6$ caused no change in the pH of the medium, although stock solutions of $InsP_6$ (K^+salt) were extremely alkaline.

The effect of 20 μM $InsP_6$ was unaltered by blockers of L-and N-type voltage-sensitive Ca^{2+} channels (nimodipine, 9 ± 11% inhibition at 1μM and ω-conotoxin, 15 ± 8% inhibition at 1 μM; n = 6 in each case). Polyvalent cations however showed the following IC_{50}'s (μM concentrations producing 50% inhibition): Gd^{3+}, 1.2 ± 0.5; La^{3+}, 5.7 ± 2.3 ; Cd^{2+}, 112 ± 16 ; Co^{2+}, 550 ± 75 ; Ni^{2+}, 956 ± 132 . The low potency of Ni^{2+} is inconsistent with the involvement of a T-type Ca^{2+} channel. Replacement of Na^+ with N-methyl glucamine or the selective inhibitor of the Na^+/Ca^{2+} exchanger (5-(N-4-chlorobenzyl)-2',4'-dimethylbenzamil) at a concentration of 30 μM, had no effect on the response to $InsP_6$. It seemed unlikely that $InsP_6$ was acting by depolarising cells in view of the greater maximal response to $InsP_6$ than to K^+. Indeed when GH_3 cells were loaded with the potential-sensitive dye, 3,3'-dihexyl-oxacarbocyanine iodide, the change in fluorescence (excitation 484 nm, emission 510 nm) induced by 60 mM K^+ could not be mimicked by 20 μM $InsP_6$. Seeking support for the idea that cytosolic Ca^{2+} levels would be raised in response to $InsP_6$, further experiments were carried out with the Ca^{2+} fluorophore INDO-1 AM, with excitation at 332 nm and emission at 400 nm (Ca^{2+}-bound) and 483 nm (free form of INDO-1). Using GH_3 cells or rat hippocampal synaptosomes, 100 μM $InsP_6$ but not Ins $(SO_4)_6$ produced a clear rise in fluorescence at 400 nm or in the 400:483 nm ratio, comparable to the response to 60 mM K^+. Without cells present $InsP_6$ caused an increase in fluorescence of INDO-1 salt but this could be largely suppressed by 100μM Mn^{2+}. Using extracellular Mn^{2+} to suppress dye leakage artefacts, a clear response to $InsP_6$ still occurred with GH_3 cells or synaptosomes. Surprisingly, the response to 100 μM $InsP_6$ was similar even if preceded by 100 μM ionomycin. Unfortunately, $InsP_6$ (3-100μM) caused a clear concentration-dependent increase in emission at 400 nm when only medium (containing 1.5 mM Ca^{2+}), without cells or INDO-1 was present. Spectral analysis revealed a fairly uniform response from 350-600 nm,indicating a non-specific physical phenomenon.We then looked further at the possibility of low-solubility artefacts arising in the $^{45}Ca^{2+}$ experiments.

A hypotonic EGTA wash solution (2 mM EGTA, 6 mM HEPES) failed to release any of the $InsP_6$-induced $^{45}Ca^{2+}$ accumulation. Filters alone showed a small increase in $^{45}Ca^{2+}$ retention due to 100 μM $InsP_6$ but less than 10% of the response in the presence of GH_3 cells. The effect of $InsP_6$ on cellular Ca^{2+} accumulation was not reduced by carrying out the experiment at 0°C rather than 37°C and some 60-105% of the normal response was apparent in zero time blanks. Preincubation of cells at 80°C for 5 min failed to reduce the effect of $InsP_6$, although increasing the temperature to 100°C caused more than 50% reduction, as did pretreatment with 2% paraformaldehyde. Incubation under N_2 with 100 μM carbonyl cyanide m-chlorophenyl hydrazone reduced the effect of 100 μM $InsP_6$ by around 60%. Although obvious turbidity could rarely be detected by eye, 10-300 μM $InsP_6$ in the experimental medium showed clear concentration-dependent increases in OD_{520}. Another anion which forms low-solubility complexes with Ca^{2+}, oxalate, had similar effects from 100-10000 μM. When $^{45}Ca^{2+}$ experiments were carried out with oxalate, large accumulations of $^{45}Ca^{2+}$ were induced at equivalent concentrations. Clear accumulation of $^{45}Ca^{2+}$ in the presence of oxalate was seen with filters alone. In the presence of GH_3 cells (just like with $InsP_6$) there was a marked increase (in the order of 5 fold) in the amount of $^{45}Ca^{2+}$ retained. All of the effects of $InsP_6$ observed in our laboratory can thus be explained as physical phenomena without the need to invoke bio-activity.

The combination of chelation and solubility properties shown by $InsP_6$ and $InsP_5$ but to a much lesser extent by lower inositol phosphates [4] is bound to confound any investigation of their influence on cellular Ca^{2+} movements. In our opinion, both the present results and those provided in recent reports on $^{45}Ca^{2+}$ accumulation [5,6] and Ca^{2+} fluorimetry [7] cannot be considered with any validity to demonstrate biological effects of inositol polyphosphates on cellular Ca^{2+} movements.

1. Vallejo, M., Jackson, T., Lightman, S. & Hanley, M.R. 1987) Nature, **330**, 656-658
2. Dean, N.M. & Moyer, J.D. (1988) Biochem. J. **250**, 493-500
3. MacEwan, D.J. & Mitchell, R. (1990) J. Physiol. **424**, 42P
4. Lönnerdal, B., Sandberg, A.S., Sandström, B. & Kunz, C. (1989) J. Nutr. **119**, 211-214
5. Nicoletti, F., Bruno, V., Fiore, L., Cavallaro, S. & Canonico, P.L. (1989) J. Neurochem. **53**, 1026-1030
6. Sortino, M.A., Nicoletti, F. & Canonico, P.L., (1990) Europ. J. Pharmacol., **189**, 115-118
7. Law, G.J., Pachter, J.A. & Dannies, P.S. (1988) J. Gen. Physiol. **92**, 8a

Differential activation of phospholipase A_2 by protein kinase C in pituitary cells

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Phorbol esters have been shown to induce secretion of luteinizing hormone (LH) and growth hormone (GH) from anterior pituitary tissue *in vitro* [1] implicating a role for protein kinase C (PKC) in triggering and/or modulating release of these hormones. Using pituitary tissue from pro-oestrous female rats, we have previously shown that phorbol 12,13-dibutyrate (PDBu) induced LH release is sensitive to the PKC inhibitors staurosporine and H7 [1]. In contrast, PDBu-induced GH release is sensitive to staurosporine only, thus suggesting that pharmacologically distinct forms of PKC (perhaps different structural isoforms) are involved in the modulation of LH release rather than GH release. Further observations that the temporal pattern of phorbol induced-LH release differs from that of GH release led us to consider that PKCs might exert actions on different targets in gonadotrophes from those in somatotrophes to control hormone secretion. Since arachidonic acid (AA) and its metabolites have been shown to induce release of pituitary hormones [2] we postulated that phospholipase A_2 (PLA_2) may be one such target for PKC in mediating pituitary hormone release. Here, we investigated the possibility that pituitary PLA_2 could be activated by PKC. The differential effects of PKC- and PLA_2-inhibitors on PDBu-induced pituitary hormone release shown here suggest: (1) that only certain forms of PKC can modulate PLA_2 activity and (2) that crosstalk between PKC and PLA_2 may be an important process in secretory responses in some but not all pituitary cell types.

Pituitary hormone release was measured using pituitary tissue obtained from pro-oestrous female cob wistar rats that had been maintained under controlled lighting and temperature. Briefly, pituitary glands were removed by 1300 h on the day of pro-oestrus and the anterior lobes removed and hemisected. Each hemisected lobe was placed into 2 ml of HEPES-buffered Minimal Essential Medium containing Earle's salts (MEM). After 30 minutes preincubation at 37°C, 95%O_2/5%CO_2 in a shaking water bath, the medium was replaced. From then onwards, medium was changed at hourly intervals with or without the appropriate combination of drugs. Medium LH and GH content were measured by radioimmunoassay.

Pituitary PLA_2 activity was determined by measuring [^{3}H]-AA release from prelabelled pituitaries. Pituitary tissue was removed from pro-oestrous rats as described above. Anterior pituitaries were quartered and placed in MEM (4 x 1/4 per flask). After 30 minutes preincubation, the medium was replaced with MEM containing 0.5 μCi [^{3}H]-AA. After a 2 hour incubation period (37°C, 95% O_2 5% CO_2), the label was removed and the tissue washed twice with MEM containing 1% BSA. Tissue was then incubated for a further hour in MEM containing 0.5% BSA with the appropriate combination of drugs. Release of [^{3}H]-AA was measured by extraction of the incubation medium with octadecyl silica. Fractions containing lipids and metabolic derivatives were sequentially eluted with the solvents described by Powell (1982) [3]. The fraction taken to represent [^{3}H]-AA released from the tissue contained over 83% of authentic AA that was added subsequent to tissue incubation and carried through the extraction procedure.

Luteinizing hormone release from pro-oestrous anterior pituitary tissue progressively increased over 3 consecutive hourly incubations with PDBu (300 nM) from a level of 4.3±0.8 ng/ml (mean ±SEM,n=4) in the basal hour to 29.3±4.3ng/ml,n=4 in the third hour of incubation with PDBu. Growth hormone release induced by PDBu was maximal by the first hour of incubation increasing to a level of 3.14 ±0.31, n = 19 fold of basal in the first hour with the phorbol. In the presence of the PLA_2 inhibitor, quinacrine (50 μM), PDBu-induced LH release was significantly reduced by the 3rd hour of phorbol incubation from a level of secretion of 29.3±4.3ng/ml,n=4 in the absence of quinacrine to 13.2±0.9ng/ml,n=4 in the presence of quinacrine. Phorbol 12,13-dibutyrate-stimulated GH release was unaffected by the presence of quinacrine. Similarly, PDBu (300 nM) induced an 87 ± 8% (mean ± SEM, n = 10) increase in pituitary PLA_2 activity, an effect which was completely blocked by quinacrine (50 μM). Phorbol 12,13-dibutyrate-induced PLA_2 activity was also significantly, but only partially reduced to an increment of 48±11% (n=14) over basal activity by 30 μM H7 and was completely inhibited to a basal level of activity by 300 nM staurosporine.

The susceptibility of the PDBu-induced LH secretory response, but not the GH secretory response, to quinacrine indicates that in gonadotrophes, but not somatotrophes, a PKC might act to cause hormone release by a pathway involving increased PLA_2 activity. The evidence that PLA_2 may have a role in mediating phorbol-induced hormone release is strengthened by the observation that PDBu stimulates an increase in pituitary PLA_2 activity. Interestingly, the susceptibility of the PKC form(s) stimulating pituitary PLA_2 activity to PKC inhibitors matches the PKC-inhibitor profile observed in induction of LH but not GH release. That is, the actions of PDBu on LH release and PLA_2 activity are staurosporine and H7 sensitive, whereas PDBu-stimulated GH release is susceptible to staurosporine only. Thus it appears to be unlikely that a PKC-mediated increase in PLA_2 activity occurs in the GH secretory response to PDBu. In contrast, in gonadotrophes, it appears that a form of PKC is present which is capable of acting to enhance PLA_2 activity and consequently evoke hormone release. These results lend support to other evidence [4,5] for pharmacological heterogeneity in the functional cellular actions of PKC(s) and further suggest that distinct forms of PKC may have selective cellular targets.

References

1. Johnson, M.S. & Mitchell, R. (1989) Biochem. Soc. Trans. **17**, 751-752
2. Naor, Z. & Catt, K.J. (1981) J. Biol. Chem. **265**, 2226-2229
3. Powell, W.S. (1982). In Methods of Enzymology, vol. **86**, pp 47-477 Academic Press
4. Johnson, M.S., MacEwan, D.J. & Mitchell, R. (1989) J. Physiol. **418**, 186p
5. Mitchell, R., MacEwan, D., Johnson, M. & Thomson, F. (1990) Eur. J. Pharm. **183**, 750-751

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Modulation by phorbol 12,13-dibutyrate of dihydropyridine-sensitive hormone release from rat anterior pituitary tissue *in vitro*

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Our previous studies revealed quite different influences of activators of protein kinase C (PKC) on hormone secretion from gonadotrophes and somatotrophes (Johnson & Mitchell, 1989; Johnson *et al.* 1989*b*). Phorbol 12,13-dibutyrate (PDBu)-induced release of growth hormone (GH), unlike that of luteinizing hormone (LH), occurs rapidly and is sensitive to the L-type Ca^{2+} channel blocker nimodipine (NMD). It is clear from experiments measuring $^{45}Ca^{2+}$ movements that L-channels in pituitary cells can be directly modulated by PKC (Johnson *et al.* 1989*a*). In order to investigate further the influence of PKC on L-channels in pituitary cells we examined phorbol ester and dihydropyridine effects on K^+-induced release of GH and LH.

Female COB/Wistar rats, that had been maintained under controlled lighting and temperature were killed at 13.30 h of pro-oestrus and their anterior pituitary glands removed and hemisected. The secretion of LH and GH *in vitro* was measured as described previously (Johnson *et al.* 1989*b*). Incubation with 60 mM-K^+ medium for 1 h caused a 6·7-fold increase in GH release over basal and a 9·2-fold increase in LH release. In the presence of 1 μM-NMD these responses were 97% and 87% inhibited respectively. In the presence of 100 nM-PDBu, K^+-induced GH release was unaltered, showing a mean increment of 5733 ± 1236 ng GH/ml compared to 5438 ± 531 ng GH/ml in controls (mean $\pm$ S.E.M., $n = 5$). In contrast K^+-induced LH release was amplified almost 2-fold by PDBu, showing a mean increment of $174{\cdot}6 \pm 10{\cdot}8$ ng LH/ml compared to $90{\cdot}5 \pm 11{\cdot}0$ ng LH/ml in controls. The K^+-induced release of GH in the presence of PDBu was reduced by 5355 ± 718 ng/ml by 1 μM-NMD whereas the corresponding LH response was only reduced by $97{\cdot}3 \pm 8{\cdot}7$ ng/ml (an amount similar to the K^+-induced release without PDBu amplification). However, when PDBu-induced hormone release was studied in the presence of 3 μM-BAY K8644 (which had no effect alone during 1 h of incubation), the LH response was greatly potentiated with an increment of 2·56-fold over that of PDBu-induced LH release alone. The release of GH was completely unaffected. It is possible that in gonadotrophes but not in somatotrophes, L-type Ca^{2+} channels are recruited by PDBu in a form (Hess *et al.* 1984) which requires the additional influence of another factor such as BAY K8644 before optimal activity is expressed.

REFERENCES

Hess, P., Lansman, J. B. & Tsien, R. W. (1984). *Nature, Lond.* **311**, 538–544.
Johnson, M. S. & Mitchell, R. (1989). *J. Endocrinol.*, suppl. **121**, 49.
Johnson, M. S., MacEwan, D. J. & Mitchell, R. (1989*a*). *J. Physiol.* **418**, 186*P*.
Johnson, M. S., Mitchell, R. & Fink, G. (1989*b*). *Biochem. Soc. Trans.* **17**, 751–752.

A possible role for phospholipase A_2 in phorbol ester-induced release of hormones from rat anterior pituitary tissue *in vitro*

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Stimulation of luteinizing hormone (LH) and growth hormone (GH) release by phorbol 12,13-dibutyrate (PDBu) *in vitro* (Johnson & Mitchell, 1989) is consistent with a physiological role for protein kinase C (PKC) in triggering or modulating release of these hormones. Differences in the pharmacology and time course of PDBu-induced LH and GH release suggest that PKC(s) may influence different targets in gonadotrophes and somatotrophes. Since arachidonic acid (AA) has been reported to induce LH release (Naor *et al.* 1981), we investigated the possibility that AA production by phospholipase A_2 (PLA_2) may have a role in PKC-mediated LH and GH release.

Female COB/Wistar rats that had been maintained under controlled lighting and temperature were killed at 13.00 h on the appropriate day of the oestrous cycle. Anterior pituitary glands were removed and hemisected. The release of LH and GH *in vitro* was measured as described previously (Johnson & Mitchell, 1989). Phospholipase A_2 activity was determined in anterior pituitary tissue preincubated for 1 h with [^{3}H]arachidonic acid in minimal essential medium at 37 °C under 95% O_2/5% CO_2. After extensive washing, stimuli were applied for 1 h and the labelled lipids and metabolites separated on octadecylsilyl silica (Powell, 1982).

Luteinizing hormone output increased progressively over three successive hourly incubations with 300 nM-PDBu and was greatest using tissue from pro-oestrous or oestrous rats. The PLA_2 inhibitor quinacrine (50 μM) significantly attenuated PDBu-induced LH release from pro-oestrous and oestrous but not metoestrous or dioestrous tissue. In contrast, the magnitude of PDBu-induced GH release was unaltered throughout the oestrous cycle and was never reduced by quinacrine. Phospholipase A_2 activity in pro-oestrous anterior pituitary tissue was stimulated 1·89 ± 0·09-fold (mean ± S.E.M., $n = 8$) by 300 nM-PDBu. These results indicate that activation of PLA_2 by PKC can occur in some pituitary cells and may have a role in influencing hormone release from particular cell types.

REFERENCES

Johnson, M. S. & Mitchell, R. (1989). *Biochem. Soc. Trans.* **17**, 751–752.
Naor, Z. & Catt, K. J. (1981). *J. biol. Chem.* **265**, 2226–2229.
Powell, W. S. (1982). In *Methods in Enzymology*, vol. 86, pp. 467–477. Academic Press.

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The D_1-like dopamine receptor of the cockroach salivary gland is coupled to phospholipase C in addition to adenylyl cyclase

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A D_1-like dopamine receptor has been characterized on the cockroach salivary gland which appears to be coupled to at least two second messengers (Evans & Green, 1990). One, known to be cyclic-AMP (Grewe & Kebabian, 1982), subserving the secretory response to dopamine (Gray *et al.*, 1984) and the other, as yet unknown, subserving the electrical response. We have carried out biochemical assays to determine (i) whether inositol-1,4,5-trisphosphate (IP_3) and protein kinase C (PKC) were present in the acinar cells and (ii) the effect of dopamine on the levels of these two substances. The protocol for the IP_3 assay was as described by Palmer *et al.* (1989). Salivary glands (3/tube) were incubated at room temperature in physiological solution (see Evans & Green, 1990) in the absence and presence of dopamine (10^{-6}M) ± the D_1 dopamine receptor antagonist SCH23390 (5×10^{-5}M). Incubations were stopped at 5 and 30 s by addition of 50 µl ice cold 10% $HClO_4$ and immediate homogenization. PKC levels were determined by use of a specific [^{3}H]-phorbol dibutyrate (PDBu) binding assay (Leach *et al.*, 1983). Two groups of 20 glands were pre-incubated in physiological solution in the absence and presence of dopamine (10^{-6}M), respectively, for 5 min, homogenized in 25 vol of buffer (Leach *et al.*, 1983), centrifuged (100,000 g, 1 hr, 4°C) then assayed for particulate and cytosolic PKC. Both IP_3 and PKC were present in the acinar cells at rest. In the presence of dopamine (10^{-6}M) the levels of IP_3 were increased 2.3 and 2.1 fold over control levels after 5 ($n = 2$) and 30 s ($n = 2$), respectively. These increases were blocked in the presence of SCH23390 (5×10^{-5}M). The distribution of PKC at rest was (values = mean ± s.e.mean, $n = 6$-12) 56.2 ± 2.1% in the particulate and 43.8 ± 3.1% in the cytosolic fraction. After 5 min stimulation with 10^{-6}M dopamine, total [^{3}H]-PDBu levels were 29% of that in the unstimulated glands. The distribution of PKC in the stimulated cells had also changed to 76.4 ± 4.2% particulate and 23.6 ± 9.0% cytosolic. Thus dopamine can induce translocation and down-regulation of PKC. It therefore seems quite possible that activation of phospholipase C may underlie the electrical response of the gland to dopamine. The inhibition of this response by LiCl (10mM), an inhibitor of the phosphoinositide cycle, and potentiation by the G-protein activator NaF (500 µM) supports this conclusion.

Evans, A.M. & Green, K.L. (1990) *Br. J. Pharmacol.* **101**, 103-108.
Gray, D.C., Ginsborg, B.L. & House, C.R. (1984) *Q. Rev. Exp. Physiol.* **69**, 171-186.
Grewe, C.W. & Kebabian, J.W. (1982) *Cell Molec. Neurobiol.* **2**, 65-69.
Leach, K.L., James, M.L. & Blumberg, P.M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4208-4212.
Palmer, S., Lee, D.Y., Hughes, K.T. & Wakelam, M.J.O. (1989). *Cell. Signal.* **1**, 147-156.

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Heterogeneous profiles of protein kinase C activation

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Depolarisation-induced calcium influx in anterior pituitary prisms and in GH_3 cells are both mediated by a nimodipine-sensitive process and are modulated in two ways by activation of protein kinase C (PKC) (Johnson *et al.,* 1989). Phorbol 12,13-dibutyrate (PDBu) activation of PKC leads to an increase in K^+-induced $^{45}Ca^{2+}$ influx in anterior pituitary pieces, but a decrease in GH_3 cells. These actions are also seen with 4ß-phorbol 12,13-didecanoate (4ß-PDD) but not 4α-PDD. The two opposing profiles may represent the actions of different forms of PKC on 'L'-channels in the two preparations. A number of diterpenes have been described which differentially display certain aspects of phorbol-like actions, for example, inflammation/tumour promotion/ornithine decarboxylase induction (Dunn & Blumberg, 1983). The present experiments investigate the actions of a range of such analogues in the Ca^{2+} influx models.

$^{45}Ca^{2+}$ influx measurements into anterior pituitary prisms and GH_3 cells were as previously described (MacEwan and Mitchell, 1990). In pituitary prisms, the marked facilitation of influx caused by (10-1000nM) PDBu and 4ß-PDD (~+180% at 1000nM) was mimicked, but to a lesser degree, by 1,2-dioctanoyl-sn-glycerol (1-100μM) (DOG), mezerein, phorbol 12-retinoate 13-acetate (PRA), phorbol 12-myristate 13-acetate 4-0-methyl ether (MPMA) and to a small degree by 12-deoxyphorbol 13-isobutyrate (DPB) (all at 10-1000nM). In GH_3 cells, PDBu and 4ß-PDD reduced K^+-induced $^{45}Ca^{2+}$ influx by up to 50% and a similar effect, of greater magnitude, was displayed by arachidonic acid (AA) (MacEwan and Mitchell, 1990). In contrast, DOG, PRA, MPMA and DPB were inactive. Surprisingly, the effect of mezerein in GH_3 cells was a facilitation of K^+-induced $^{45}Ca^{2+}$ influx, apparently identical to that in pituitary prisms. The effects of diterpenes were not altered by differing times of pre-incubation nor by VO_4^{3-} (1mM) or substitution of Na^+ by N-methyl-d-glucamine. The results are consistent with the idea that at least two forms of PKC can exert qualitatively different influences over 'L'-type Ca^{2+} channel function. The two complex profiles could be explained if different relative amounts of two distinct forms of PKC were involved in the two models. Whilst PDBu and 4ß-PDD were non-selective activators, mezerein and AA may be selective activators of the PKC forms that facilitate or attenuate $^{45}Ca^{2+}$ influx respectively. The other analogues may show partial selectively for the facilitatory effect. Although other explanations of these profiles may well be possible, selective antagonism by H7, but not staurosporine, of the effect of PDBu in GH_3 cells but not in pituitary prisms (Johnson *et.al.,* 1989) supports our hypothesis.

Dunn, J.A. & Blumberg, P.M. (1983) *Cancer Res.* **43**, 4632-4637.
Johnson, M.S., MacEwan, D.J. & Mitchell, R. (1989) *J. Physiol.* **418**, 186P.
MacEwan, D. & Mitchell, R. (1990) *Biochem. Soc. Trans.* **18**, 433-434.

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Differences in the effect of putative activators of protein kinase C on secretion of pituitary hormones

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Secretion of pituitary hormones *in vitro* can be induced by activation of protein kinase C (PKC) (Johnson and Mitchell, 1989). Using several putative activators and inhibitors of PKC, we investigated the possibility that PKC-mediated regulation of hormone secretion may be differentially organised in distinct pituitary cell types. Anterior pituitary glands were removed from long term (4 weeks) ovariectomised cob wistar rats and hemisected. Release of luteinizing hormone (LH) and growth hormone (GH), *in vitro*, was measured as previously described (Johnson & Mitchell,1989). Pituitary LH release progressively increased over 3 consecutive hourly incubations with 300nM phorbol 12,13-dibutyrate (PDBu), reaching a level 192±21% (n=16) of basal secretion in the third hour. In contrast, GH was rapidly released by PDBu with a maximal response in the first hour of 558±95% (n=16) of basal secretion. PDBu-induced release of both LH and GH was unaffected by the PKC inhibitor H7(30µM) but was attenuated in the presence of staurosporine (300nM). Selective inhibition by staurosporine but not H7 of certain PKC actions has been described previously in the regulation of L-type calcium channels by PKC (Johnson *et al.*,1989). In the present experiments, 200µM 1,2-dioctanoyl *sn*-glycerol (DOG) induced a small release of LH but not GH. Luteinizing hormone release induced by DOG was inhibited by staurosporine but was unaffected by H7. Release of LH could also be evoked by 300µM arachidonic acid (AA), but this effect was unaffected by PKC inhibitors. Release of GH was reduced by AA, an effect which is also unaltered by PKC inhibitors.

The release of LH and GH from pituitary tissue obtained from ovariectomized rats can be influenced by activation of PKC. However, the lack of effect of H7 on LH release shown here contrasts with those effects observed on PDBu induced LH release from tissue obtained from intact pro-oestrous rats (Johnson & Mitchell,1989.). It appears that AA can exert stimulatory (LH) or inhibitory (GH) effects on hormone secretion by means other than activation of PKC. This is consistent with evidence suggesting a role for AA metabolites in the control of LH and GH release (Naor *et al*, 1983; Schweitzer *et al*, 1990).

Johnson, M.S., MacEwan, D.J. & Mitchell, R. (1989).*J.Physiol.* **418**, 186P.
Johnson, M.S. & Mitchell, R. (1989).*Biochem. Soc. Trans.* **17**,751-752.
Naor, Z.,Vanderhoek, J.Y.,.Lindner, H.R. & Catt, K.J. (1983) in *Advances in Prostaglandin,Thromboxane and Leukotriene Research.*Vol **12**, 259-263 ed.Samuelsson, B., Paoletti, R. & Ramwell, P. New York:Raven Press.
Schweitzer, P., Madamba, S. & Siggins, G.R. (1990) *Nature* **346**,464-467.

ıes [15]. Transport processes such as glutamate up-
e into glial cells may also be influenced by AA [16]
sibly by increasing membrane fluidity.
A further putative target for AA and its metabolites
protein kinase C (PKC); of which at least seven
inct isoforms have been identified so far [17].
hizuka and his co-workers have shown that AA
lf can selectively activate the γ-and α-isoforms of
C in cell-free systems [18]. Little is known however,
whether such events may be of physiological impor-
ce.
n anterior pituitary cells, AA has been reported to
ease the secretion rates of several hormones.
einizing hormone and follicle stimulating hormone
etion were enhanced in response to exposure to
[19]. Prolactin secretion [20] and adrenocorti-
ophic hormone secretion [21] are also increased by
AA stimulus.
n the present experiments we investigated some of
cellular influences of AA on the GH_3 clonal ante-
pituitary cell line; in particular the effects on
olarisation induced Ca^{2+} influx. In view of the
ntitative differences observed in the influences of
and other PKC activators on depolarisation-in-
ed $^{45}Ca^{2+}$ influx, we carried out further studies
$^{+}$ fluorimetry; $^{45}Ca^{2+}$ efflux) to clarify any addi-
al action of AA.

erials and Methods

erials

H_3 cells and foetal bovine serum were obtained
Flow Laboratories, Irvine, Strathclyde, U.K.
's F-10 medium and Hanks' balanced salt solution
supplied by Gibco-BRL, Paisley, Strathclyde, U.K.
cillin, streptomycin, L-glutamine, essential fatty
free bovine serum albumin, arachidonic acid
um salt), arachidonic acid-methyl ester, linoleic
phorbol 12,13-dibutyrate, NDGA, 8-bromo cyclic
(sodium salt), *N*-methyl-D-glucamine, sodium or-
nadate, indomethacin and Indo-1-AM were all
nased from the Sigma, Poole, Dorset, U.K. Piper-
butoxide was supplied by the Aldrich, Gillingham,
et, U.K. SKF-525A ('Proadifen') was obtained
Research Biochemicals, Natick, MA, U.S.A. *N*-
ıyl TRH was purchased from Peninsula Laborato-
St. Helens, Merseyside, U.K. H7 and HA 1004
obtained from Seikagaku America, St. Peters-
FL, U.S.A. Staurosporine and K252a were sup-
by Kyowa Medex Co., Tokyo, Japan. Ionomycin
bought from Novabiochem (U.K.), Nottingham,
ETYA was a gift from Roche Products, Welwyn
en City, U.K. and $^{45}Ca^{2+}$ (specific activity, 17
mg) was purchased from Amersham Interna-
l, Amersham, U.K. All other chemicals were of
ır grade.

Methods

Cell culture. GH_3 cells were grown in Ham's F-10 medium supplemented with 15% foetal bovine serum, 1 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. Cells were harvested by agitation, washed by resuspension centrifugation ($1000 \times g$, 10 min, 25°C) and finally resuspended at the appropriate density in the required medium.

Calcium influx studies. Cells were diluted to a density of $5 \cdot 10^6$ cells/ml in 'calcium uptake medium' (concentrations in mM: NaCl, 154/KCl, 5.4/$CaCl_2$, 1.5/D-glucose, 11/Hepes, 6 (pH adjusted to 7.4 with Tris-base) and with 0.05% fatty acid-free bovine serum albumin). Aliquots of this suspension (0.5 ml/tube) were preincubated (30 min, 37°C, O_2 atmosphere) before a 10 min incubation (37°C, O_2) with drugs or solvent alone. Cells were then exposed to 1 ml of calcium uptake medium containing either low K^+ (5.4 mM final concentration) or high K^+ (60 mM) with 4 μM $^{45}CaCl_2$ (≈ 3 μCi/tube). After 30 s (37 °C), $^{45}Ca^{2+}$ uptake was halted by quenching with 3 ml of ice-cold 2 mM EGTA (Ca^{2+}-free) calcium uptake medium and tissue was separated by vacuum-filtration through Millipore SCWP cellulose acetate/nitrate filters (8 μm pore size) underlain by GF/B filters on Millipore 1225 sampling manifolds (Millipore, Harrow, U.K.). Samples were washed once immediately with 3 ml ice-cold EGTA calcium uptake medium and then washed a further three times for 2 min each in the same medium. The radioactivity associated with the cellulose filters and cells was determined by liquid scintillation counting. Preliminary experiments on GH_3 cells and extensive similar studies on anterior pituitary prisms [22] revealed that these conditions gave the optimal signal-to-noise ratio. The stimulus-induced influx of $^{45}Ca^{2+}$ in excess of basal controls was maximal within 30 s, suggesting that it represented specific response-triggered flux rather than adsorption or steady accumulation by storage pools. Vehicle (ethanol up to 0.4%) was always included in controls as appropriate and was independently determined to have no effect on either basal or K^+-induced $^{45}Ca^{2+}$ accummulation.

Calcium efflux studies. Harvested GH_3 cells were resuspended at a concentration of $1 \cdot 10^7$ cells/ml in calcium uptake medium containing 0.05% essential fatty acid-free bovine serum albumin and incubated for 20 min (37°C, O_2) before addition of 1 ml of medium containing 6 μM $^{45}Ca^{2+}$ (≈ 4.5 μCi/tube). Samples were incubated for 1 h at 37°C under O_2 before loading onto separate GF/B filters pre-washed with calcium uptake medium and under vacuum on a Millipore 1225 sampling manifold. Cells were then washed three times for 2 min with 3 ml of pre-warmed (37°C) calcium uptake medium alone. There then followed a further eight washes with pre-warmed calcium uptake

medium containing the appropriate inhibitor or solvent for controls. Each successive 2 min wash with 3 ml of calcium uptake medium (±inhibitor) was collected. The 4th and subsequent washes contained 50 μM AA. Radioactivity in each fraction was measured by liquid scintillation counting.

Cytosolic calcium measurements. Cytosolic Ca^{2+} concentrations were measured by use of the calcium fluorescent dye Indo-1 [23]. GH_3 cells were loaded with 10 μM Indo-1-AM for 45 min in culture medium at 37°C, 5% CO_2/95% air. Cells were then harvested, washed and resuspended in Hanks' balanced salt solution. A 30 min incubation in the dark at room temperature was then given to allow more complete hydrolysis of intracellular Indo-1-AM. The suspension was then centrifuged ($1000 \times g$, 10 min, 25°C) and the cell pellet was again resuspended in Hanks' balanced salt solution at a concentration of $5 \cdot 10^6$ cells/ml. A sample (2.5 ml) of the cell suspension was loaded into a quartz cuvette which was stirred magnetically and maintained at a constant temperature of 37°C. Fluorescence was measured in a Shimadzu RF-5000 spectrofluorophotometer. Excitation was at 332 nm (band width = 5 nm) and emission was at 400 nm (band width = 5 nm). Emission wavelengths were not ratioed between 400 and 490 nm so that the possibility of introducing significant autofluorescence artefacts in the final signal [24] would be reduced. A fluorescence value was taken every 2 s and at the end of each experiment, 10 μM ionomycin followed by 10 mM $MnCl_2$ (final concentrations) were added to give a measure of the maximum and minimum fluorescent values of the cuvette contents, respectively. An assessment of extracellular fluorescence resulting from dye leakage was made according to the method proposed by Rink and Pozzan [25]. Thus, appropriate corrections were made for the small changes in basal fluorescence induced by the addition of 100 μM $MnCl_2$ which were rapidly and fully reversed by 200 μM DTPA. The experimental values for cytosolic Ca^{2+} concentrations were calculated according to Grynkiewicz et al. [23] and Luckhoff [24]. In view of the limited solubility of AA in aqueous media, we carried out experiments to assess light-scattering using excitation and emission wavelengths of 520 nM and maximum sensitivity of the fluorimeter. Under our conditions (calcium uptake buffer containing 0.4% ethanol vehicle) there was no detectable increase in light-scattering up to and including 32 μM AA. At 38 μM AA, irreversible turbidity was detectable and by 44 μM this was very marked. All cell Ca^{2+} fluorescence experiments were thus carried out with AA at concentrations below the threshold for turbidity (Fig. 6).

Data analysis. Statistical significance of effects was assessed by a non-parametric method (Mann-Whitney *U*-test). Concentration-response curves were analysed by a non-linear iterative curve-fitting program ('P-fit'; Biosoft, Cambridge, U.K.). The calculated concent tion which inhibits 50% of the maximal response (IC is expressed, with the errors representing stand errors of the mean.

Results

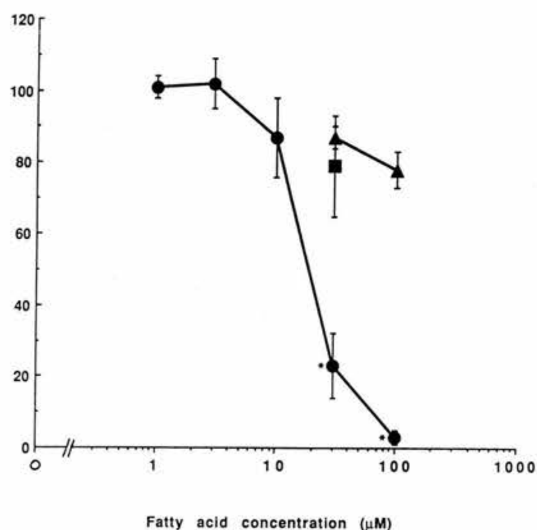
Depolarisation with 60 mM K^+ medium causec marked influx of $^{45}Ca^{2+}$ (see Table I) which was inh ited in a time-dependent manner by 30 μM AA. T inhibitory effect of AA was apparent without preincubation but an additional 1 min preincubat produced a maximal inhibitory effect. At concen tions up to 100 μM, AA had no effect on ba accumulation of $^{45}Ca^{2+}$ (104 ± 8% of control at μM). Inhibition of K^+-induced $^{45}Ca^{2+}$ influx by was concentration-dependent (Fig. 1) with an I (concentration that gives 50% inhibition of maxi response) of 19 ± 3 μM. Agents with some structu similarity to AA; AA-methyl ester (30 and 100 μ and linoleic acid (30 μM) were unable to mimic inhibition seen with AA (30 μM). The inhibitory ef of AA was virtually maximal by the concentration μM) at which AA reached the limit of its solub under these conditions. Higher concentrations of did not significantly affect basal $^{45}Ca^{2+}$ accumula (Table I) or that induced by ionomycin or *N*-me TRH (Table IV) suggesting that even exceeding solubility limit of AA had little influence on the res of $^{45}Ca^{2+}$ influx experiments. Furthermore, it was c that AA-methyl ester (which was less soluble than under our conditions; showing marked light-scatte

TABLE I

Time-course of inhibition of depolarisation-induced calcium infl arachidonic acid (AA) into GH_3 cells

Values represent means ± S.E., $n = 4$–6. The total $^{45}Ca^{2+}$ acc lated in the presence of 60 mM K^+ was typically around 1600 per assay, whereas basal $^{45}Ca^{2+}$ accumulation was around 600 of which non-specific adsorbtion to the filter and cell su (determined in zero-time blanks) was responsible for aroun dpm. $^{45}Ca^{2+}$ accumulation was measured over 30 s and the in tion times with AA are inclusive of the 30 s influx measure period. Incubations were at 37 °C and $^{45}Ca^{2+}$ influx was stopp described under Materials and Methods. Statistically significant bition of K^+-induced $^{45}Ca^{2+}$ influx is indicated by (* $P < 0.05$

Conditions	$^{45}Ca^{2+}$ accumulation (fmol/10^6 cells per min)
Non-specific adsorbtion (zero-time blank)	34 ± 2
Basal	50 ± 4
Basal, 100 μM AA (10 min)	54 ± 6
60 mM K^+	120 ± 8
60 mM K^+, 30 μM AA (30 s)	86 ± 6 *
60 mM K^+, 30 μM AA (45 s)	64 ± 3 *
60 mM K^+, 30 μM AA (90 s)	52 ± 3 *



1. Concentration-response data for the inhibition of depolarisa- -induced calcium influx by fatty acids. Cells were exposed to $^{2+}$ for 30 s at 37 °C and calcium influx was stopped as described er Materials and Methods. Cells were preincubated with arachi- ic acid (AA, circles), arachidonic acid-methyl ester (AA-methyl r, triangles) and linoleic acid (square) for 10 min at 37 °C before sure to 60 mM K^+ and $^{45}Ca^{2+}$. Values represent means ± S.E., 4–12. Statistically significant inhibition of K^+-induced $^{45}Ca^{2+}$ influx is indicated by (* $P < 0.05$).

facts by 24 μM) failed to mimic the inhibition of induced $^{45}Ca^{2+}$ influx caused by AA, even when ed at concentrations of 30 and 100 μM (Fig. 1). vever, 30 μM AA-methyl ester in the presence of nM PDBu reduced K^+-induced $^{45}Ca^{2+}$ influx lev- to 14 ± 2% of control K^+-induced influx ($n = 4$).

he inhibitory effect of 30 μM AA on K^+-induced ium influx (a reduction to 23 ± 9% of control K^+- iced influx) was reversed by the protein kinase C bitor H7 (Fig. 2), with an IC_{50} of 14 ± 4 μM. A gener of H7, HA1004, with much reduced activity PKC inhibitor [26] was inactive at similar concen- ons. Similarly, the potent PKC inhibitor stau- orine also reversed the effect of 30 μM AA on nduced $^{45}Ca^{2+}$ influx (IC_{50} of 52 ± 15 nM, see Fig. ut its less active congener K252a [27,28] failed to e reversal. The reversibility of the effect of AA by e means rules out any simple physicochemical ac- of AA or any chemical oxidation products that arise during the experiment. Down-regulation of by treatment of GH_3 cells with 300 nM phorbol for 24 h results in more than 50% reduction in levels [29]. Using a similar protocol for PKC 1-regulation [30] by treatment of GH_3 cells with nM PDBu for 24 h, reduced the ability of AA to it K^+-induced $^{45}Ca^{2+}$ influx (Fig. 4).

he inhibition of K^+-induced $^{45}Ca^{2+}$ influx by 30 AA was not influenced by inhibitors of its bolism (Table II). The cyclo-oxygenase and lipoxy- se inhibitor ETYA (10 μM), the lipoxygenase in-

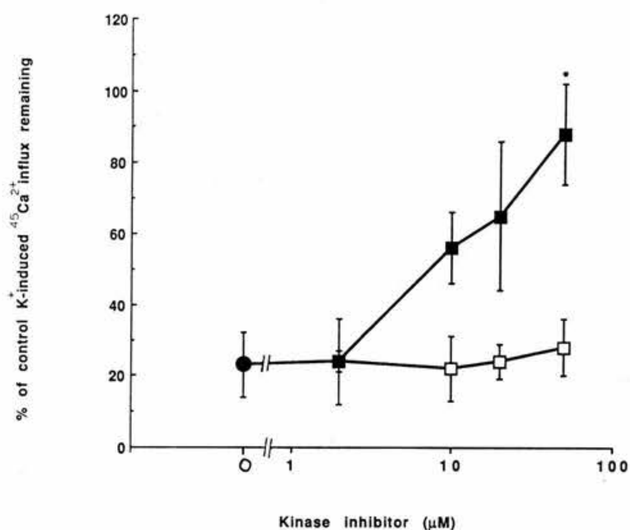


Fig. 2. Reversal by H7 but not HA1004 of the inhibition by AA of depolarisation-induced calcium influx. GH_3 cells were exposed to $^{45}Ca^{2+}$ for 30 s at 37 °C and calcium influx was stopped as described under Materials and Methods. H7 (filled squares) and HA1004 (open squares) were added immediately prior to addition of AA (30 μM), then cells were preincubated for 10 min at 37 °C before exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Control K^+-induced $^{45}Ca^{2+}$ influx data in the presence of AA is shown by the circle. Values represent means ± S.E., $n = 8$–12. Both H7 and HA1004 at 50 μM had no effect alone on basal or K^+-induced $^{45}Ca^{2+}$ influx. The inhibition of K^+-induced $^{45}Ca^{2+}$ influx due to AA was significantly reversed by 50 μM H7 ($P < 0.05$).

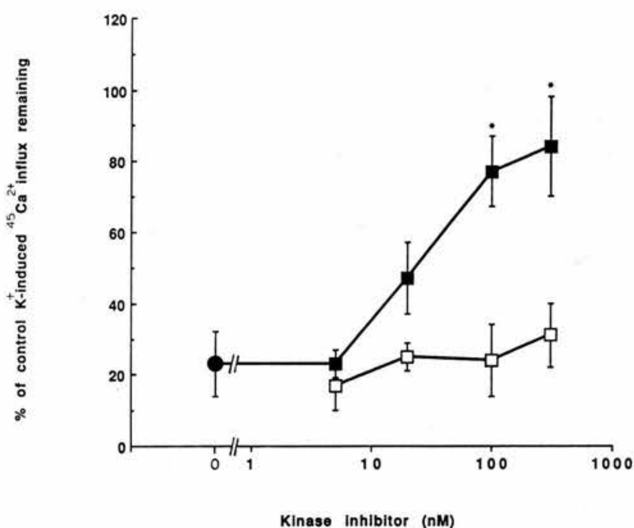


Fig. 3. Reversal by staurosporine but not K252a of the inhibition by AA of depolarisation-induced calcium influx. GH_3 cells were exposed to $^{45}Ca^{2+}$ for 30 s at 37 °C and calcium influx was stopped as described under Materials and Methods. Staurosporine (filled squares) and K252a (open squares) were added immediately prior to addition of AA (30 μM) then cells were preincubated for 10 min at 37°C before exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Control K^+-induced $^{45}Ca^{2+}$ influx data in the presence of AA is shown by the circle. Values represent means ± S.E., $n = 4$–8. Both staurosporine and K252a at 300 nM had no effect alone on basal or K^+-induced $^{45}Ca^{2+}$ influx. The inhibition of K^+-induced $^{45}Ca^{2+}$ influx due to AA was significantly reversed by 100 and 300 nM staurosporine ($P < 0.05$).

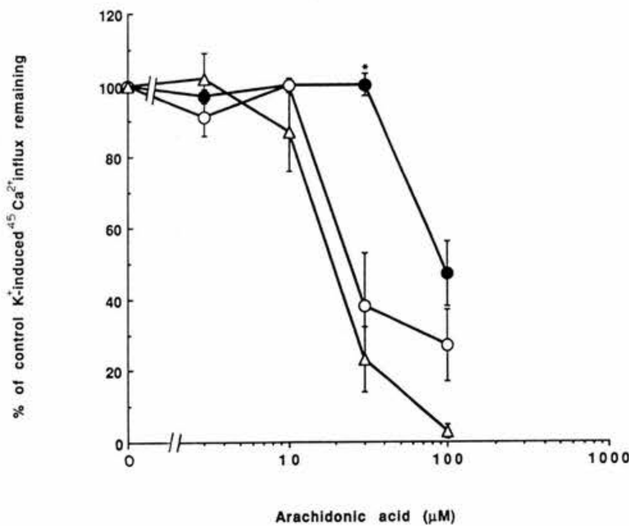


Fig. 4. Effect of PKC-down regulation on arachidonic acid inhibition of depolarisation-induced calcium influx. GH_3 cells were cultured for 24 h with 300 nM PDBu in dimethylformamide (0.01% final volume, filled circles), dimethylformamide alone (open circles) or nothing (triangles) and then extensively washed by centrifugation and resuspension in 'calcium uptake medium' (three times 10 min, 1000 × g, 25°C). Arachidonic acid was added 10 min before a 30 s exposure to 60 mM K^+ and $^{45}Ca^{2+}$. Values represent means ± S.E., $n = 6$. The reduction in $^{45}Ca^{2+}$ influx caused by AA (30 μM) was significantly attenuated (*) by prolonged preincubation with PDBu compared to its solvent alone ($P < 0.05$).

hibitor NDGA (30 μM) and the cytochrome *P*-450 inhibitors piperonyl butoxide (30 μM) and SKF 525A (10 μM) did not significantly modify the effect of AA.

Whereas 50 μM AA inhibited the influx induced by 60 mM K^+ to 3 ± 1% of control, the influx responses to 1 μM *N*-methyl TRH and 30 μM ionomycin were reduced by AA to only 77 ± 16% and 83 ± 10% of the

TABLE II

Effect of inhibitors of arachidonic acid metabolism on its inhibition of depolarisation-induced calcium influx

GH_3 cells were exposed to 30 μM alone, or in combination with 10 μM ETYA, 30 μM NDGA, 10 μM SKF 525A or 30 μM piperonyl butoxide for 10 min before and during addition of 60 mM K^+ plus $^{45}Ca^{2+}$ medium. None of these drugs caused any significant alteration in the inhibition of K^+-induced $^{45}Ca^{2+}$ accumulation due to 30 μM AA or had any apparent effect on basal K^+-induced $^{45}Ca^{2+}$ influx alone. Values represent the means ± S.E., $n = 4$–8.

Drug concentration	% of control K^+-induced $^{45}Ca^{2+}$ influx remaining
60 mM K^+ medium alone	100
+ 30 μM arachidonic acid	23 ± 9
+ 30 μM arachidonic acid + 10 μM ETYA	23 ± 4
+ 30 μM arachidonic acid + 30 μM NDGA	35 ± 8
+ 30 μM arachidonic acid + 10 μM SKF 525A	26 ± 3
+ 30 μM arachidonic acid + 30 μM piperonyl Butoxide	29 ± 5

TABLE III

Effect of inhibitors of calcium extrusion processes on ability of ara donic acid to reduce depolarisation-induced calcium influx

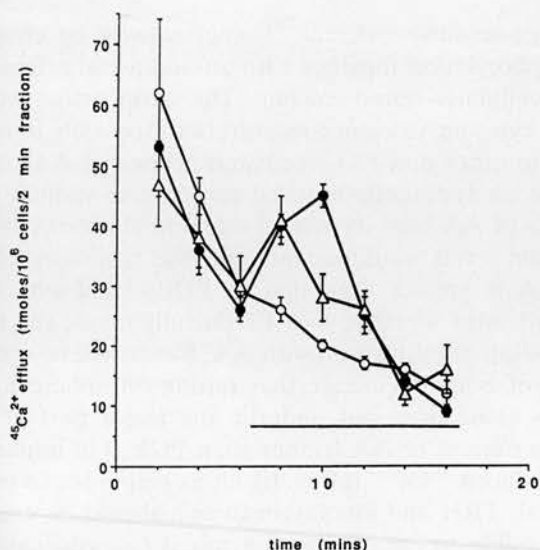
GH_3 cells were harvested and then resuspended in either nor 'calcium uptake medium' with or without 1 mM Na_3VO_4 or sodi free 'calcium uptake medium' with *N*-methyl-D-glucamine repla sodium (154 mM). Cells were incubated with or without 100 arachidonic acid for 10 min before exposure to 60 mM K^+ $^{45}Ca^{2+}$ medium. Values represent the means ± S.E., $n = 4$. Nei K^+-induced $^{45}Ca^{2+}$ nor the inhibition of that response by AA w significantly altered by Na_3VO_4 or *N*-methyl-D-glucamine.

Conditions	% of control K^+-induced $^{45}Ca^{2+}$ influx remaining
60 mM K^+	100
60 mM K^+, *N*-methyl-D-glucamine + Na_3VO_4	130 ± 8
60 mM K^+, 100 μM AA	8 ± 6
60 mM K^+, 100 μM AA, *N*-methyl-D-glucamine	30 ± 6
60 mM K^+, 100 μM AA, Na_3VO_4	16 ± 2

respective control responses (Table IV). The effect AA on K^+-induced $^{45}Ca^{2+}$ influx therefore relates least mostly) to a modulation of the specific entry ro involved in that response, not just a general alterat of Ca^{2+}-handling by the cell.

The cell-permeable activator of cyclic GMP-dep dent protein kinase, 8-Br-cyclic GMP, was unable mimic the effect of AA at any concentration fı 3–100 μM (data not shown). Throughout this conc tration range of 8-Br-cyclic GMP the maximum de tion from control K^+-induced $^{45}Ca^{2+}$ influx was 1 14% (mean ± S.E., $n = 8$). Sodium orthovana (Na_3VO_4) and replacement of sodium with *N*-met D-glucamine will effectively inhibit plasma membr Ca^{2+}-ATPases and the Na + /Ca^{2+} exchanger, res tively [31,32]. Neither 1 mM Na_3VO_4 nor sodium placement could prevent the inhibition of K^+-indu calcium influx caused by 100 μM AA (see Table The minor elevation of Ca^{2+} accumulation due to in the presence of AA that was seen with sod orthovanadate and *N*-methyl-D-glucamine was seen with K^+alone. It seems likely, therefore, these small effects reflect influences of the C ATPase and the Na + /Ca^{2+} exchanger on redistı tion of elevated Ca^{2+} and we could thus find evidence that these Ca^{2+} transporters are involve any specific way in the AA-induced inhibition of stimulated $^{45}Ca^{2+}$ influx. Since the effect on influ AA (Table III) was unaltered in the presence concentration of Na_3VO_4 which fully blocked ^{45}(extrusion (see Fig. 5) it seems unlikely that PKC m lation of Ca^{2+}-ATPase activity [33] plays any major in the inhibition of $^{45}Ca^{2+}$ influx observed here.

In the $^{45}Ca^{2+}$ extrusion experiments, a clea crease in the rate of $^{45}Ca^{2+}$ efflux was seen in resp



. 5. Effect of arachidonic acid on calcium efflux from GH_3 cells. asurement of $^{45}Ca^{2+}$ efflux was performed as described under terials and Methods. Cells were batch-perfused in either the sence (triangles) or absence (filled circles) of 10 μM NDGA. ichidonic acid (50 μM) in ethanol (or ethanol alone for control en circles)) was added to the medium from 6–16 min where icated by the line. Values represent the means ± S.E., $n = 3$.

50 μM AA (Fig. 5). This response was transient, ng over within four efflux fractions, despite the itinued presence of AA, suggesting that it originated m the discharge of a discrete stored pool. The effect 50 μM AA was considerably less than (about 36% the efflux caused by 100 μM ionomycin. In the sence of 10 μM NDGA the response to AA ap- ired to be slightly attenuated and was completely vented in the presence of 1 mM Na_3VO_4 (data itted for clarity). There was no increase in $^{45}Ca^{2+}$ ux rate in response to 300 nM PDBu suggesting the : of involvement of PKC activation in this effect of . The $^{45}Ca^{2+}$ efflux response to AA was unaltered 10 μM ETYA, 10 μM indomethacin, 30 μM H7 or odium-free medium (data not shown).

'he fluorimetric studies revealed that basal cytosolic ium concentrations were clearly elevated by quite a concentration (17 μM) of AA alone (Fig. 6b and c), as also reported by Drummond [33], phorbol esters e ineffective. This response to AA was not inhib- by 30 μM H7 (Fig. 6c) or 30 μM NDGA (data not vn) but quite unlike the inhibition of K^+-induced $^{2+}$ influx caused by AA, was mimicked by AA- hyl ester at an equivalent concentration. The incre- ts in calcium concentration due to AA (17 μM), 3u (300 nM) and AA-methyl ester (17 μM) were : 11, 6 ± 8 and 52 ± 9 nM respectively (means ± , $n = 3$–5). The rise in cytosolic calcium induced by ıM K^+ medium was markedly reduced to 14 ± 9% ontrol (mean ± S.E., $n = 4$) by previous addition of (Fig. 6b) paralleling the $^{45}Ca^{2+}$ influx data. In the presence of 30 μM H7, the rise in basal cytosolic calcium level induced by 17 μM AA was unaltered, but the inhibitory effect of AA on subsequent responses to 40 mM K^+ was markedly attenuated (Fig. 6c); the mean response to K^+ now being 89 ± 10% of control (mean ± S.E., $n = 3$). In accordance with this, presumed down-regulation of PKC in GH_3 cells by pro-

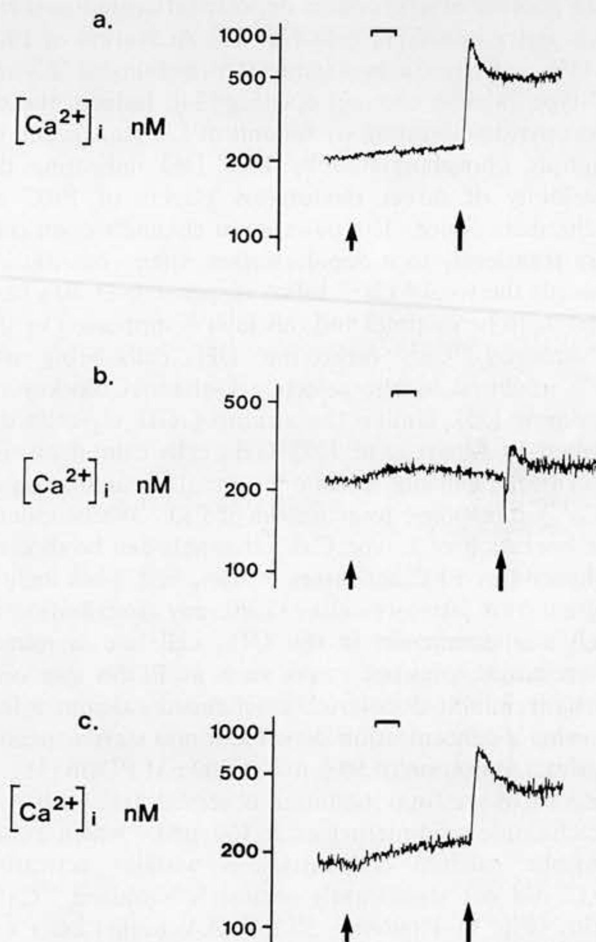


Fig. 6. Effect of arachidonic acid on the cytosolic calcium concentration in GH_3 cells. Cells were prepared and intracellular Ca^{2+} levels estimated by Indo-1 fluorescence as described under Materials and Methods. Arachidonic acid (AA, 17 μM) in ethanol (b and c) or ethanol alone (a) were added where indicated by the first (shorter) arrows. Ethanol at concentrations of up to 0.4% (as used) had no apparent effect on basal or K^+-induced increments in calcium concentrations. At the concentration used, AA produced no turbidity artefact in fluorescence records made in the absence of cells. At the second (longer) arrow, KCl was added to a final concentration of 40 mM. Addition of 40 mM NaCl rather than KCl had no effect. Part (a) illustrates a typical response to K^+ in the presence of ethanol, the vehicle for AA (0.4%). In (b), AA induced a rise in basal Ca^{2+} levels and a diminution of the subsequent response to 40 mM K^+. In (c), when 30 μM H7 had been present from the start of the record, the AA-induced rise in basal Ca^{2+} levels was still present, but the diminution by AA of the response to K^+ was reversed. There was no apparent effect of H7 alone. Staurosporine could not be tested because of the unfavourable fluoresence spectrum of the compound. Each trace is representative of at least three similar results. The abscissa represents time with each scale bar indicating 1 min.

longed preincubation with PDBu (300 nM) greatly diminished the effect of AA on the response to K^+ but not its elevation of basal calcium levels (data not shown).

Discussion

Previous work has shown that activation of PKC with phorbol esters reduces depolarisation-induced calcium entry into GH_3 cells [33–35]. Activation of PKC in GH_3 cells produces a reduced probability of 'L'- and 'T'-type calcium channel opening [34]. Indeed, the dihydropyridine-binding α_1 subunit of L-channels can be multiply phosphorylated by PKC [36] indicating the possibility of direct modulatory effects of PKC on L-channels. Since 'T'-type calcium channels open only very transiently to a depolarisation, their contribution towards the total $^{45}Ca^{2+}$ influx response over 30 s here is likely to be minimal and this idea is supported by the K^+-induced $^{45}Ca^{2+}$ influx into GH_3 cells being over 85% inhibited by the selective L-channel blocker nimodipine [35]. Unlike the strain of GH_4C_1 cells described by Albert et al. [37], GH_3 cells exhibit no rise in cytosolic calcium levels or in basal accumulation of $^{45}Ca^{2+}$ in response to activators of PKC. Whilst indeed the operation of L-type Ca^{2+} channels can be directly enhanced by PKC activators in some cell types including anterior pituitary cells [35,38], any contribution of such a phenomenon in the GH_3 cell line is minor. Interestingly, phorbol esters such as PDBu can only partially inhibit depolarisation-induced calcium influx showing a concentration-dependent and stereo-specific maximal inhibition of $50 \pm 4\%$ at 300 nM PDBu [35], in contrast to the total inhibition observed here with AA. Arachidonic acid-methyl ester (30 μM), which raises cytosolic calcium concentrations without activating PKC, did not significantly reduce K^+-induced $^{45}Ca^{2+}$ influx (Fig. 1). However, 30 μM AA-methyl ester enhanced the reduction of K^+-induced $^{45}Ca^{2+}$ influx caused by 300 nM PDBu to $86 \pm 4\%$.

Furthermore, unlike PDBu, AA also has the ability to raise cytosolic calcium concentrations in GH_3 cells. This effect is apparent in the fluorimetric studies and probably underlies the increase in $^{45}Ca^{2+}$ efflux rate induced by AA. Down-regulation of PKC in GH_3 cells leads to reduced PKC-mediated responses [29]. Although the potency of AA at inhibiting K^+-induced $^{45}Ca^{2+}$ influx was greatly reduced by presumed down-regulation of PKC, the effect was not completely prevented. This probably reflects incomplete down-regulation (as would occur with the present protocol [29]), and the contribution of an additional effect of AA, other than direct activation of PKC. In summary, it seems likely that AA may inhibit $^{45}Ca^{2+}$ influx into GH_3 cells by a mechanism with at least two components: a direct activation of PKC which may reduce voltage-sensitive calcium channel activity by cha phosphorylation together with an additional releas intracellularly-stored calcium. The latter action w raise cytosolic calcium concentrations (possibly in a tion to other non-PKC-mediated actions of AA) l ing to an apparently reduced response to stimuli. ability of AA (and its methyl ester) to elevate cytos calcium levels would explain why the inhibitory e of AA is greater than that of PDBu, and why methyl ester together with PDBu fully mimic the t inhibition of influx seen with AA. Nevertheless, sev lines of evidence dictate that raising cytosolic calc levels alone does not underlie the major part of greater effect of AA (rather than PDBu) in inhibi K^+-induced $^{45}Ca^{2+}$ influx: (i) influx responses to o stimuli, TRH and ionomycin (which should be equ susceptible to the effects of a raised cytosolic calc concentration) were almost unmodified by AA, anc the whole of the effect of AA was reversed by H7 staurosporine (but not their less active congeners) manner suggesting critical involvement of PKC. simplest unifying explanation of the data would s to be that the additional release of Ca^{2+} by AA se to promote the efficiency with which AA can inc activation of PKC. Other explanations may of co be possible.

Arachidonic acid and some of its metabolites h been reported to open potassium channels in h [4,5] resulting in a hyperpolarisation. As here, t effects took seconds to minutes to develop, bu contrast were prevented by lipoxygenase inhibit Such relatively slow development of the effect of here is consistent with the involvement of an enzy mechanism such as kinase action. Similarly, maxi effects of PDBu on GH_3 cells were observed only a a preincubation of at least 1 min. These facts coup to the selective H7 and staurosporine reversal of AA effect (Figs. 2 and 3) support the idea that act tion of PKC by AA mediates the effects observed h

In cell-free systems, AA can selectively activate α- and γ-isoforms of PKC [18]. Our observations i cate that material immunoreactive with polyclonal bodies for α-, β- and ϵ-isoforms of PKC, is preser GH_3 cells (Simpson, J., MacEwan, D.J., Mitchell, Johnson, M.S., Thomson, F.J. and Parker, P.J., unp lished data), similar to the profile reported in GH_4C_1 cell line where the γ-isoform has also t shown to be absent [39]. This suggests that it ma the α-isoform of PKC which mediates AA actio GH_3 cells, although any role of any further PKC forms in these cells cannot yet be excluded.

Linoleic acid and AA-methyl ester are less pc activators of PKC than AA [40], but do cause (release from intracellular stores [10,12]. Linoleic and AA-methyl ester only poorly mimic AA in calcium influx studies (see Fig. 1) but AA-methyl

robustly mimic AA in the fluorimetric studies, her indicating that AA-inhibition of calcium influx not simply be due to release of calcium from intra-ular stores.

t has been suggested [41] that lowered cytosolic ium may result from activation by AA of guanylate ase and thus, activation of cyclic GMP-dependent tein kinase. This appears not to be a major factor in AA inhibition of calcium influx seen here since r-cyclic GMP is totally without effect up to 100 μM. chidonic acid and certain of its metabolites can also bit Ca^{2+}/calmodulin-dependent protein kinase II , but the lack of effect of calmodulin antagonists on induced $^{45}Ca^{2+}$ influx in pituitary pieces [42] and in GH_3 cells (Mitchell, R. and MacEwan, D.J., ublished data) suggests that any action does not ribute significantly to the inhibition of influx caused ιA here.

'he components of the response to AA in GH_3 may well be direct actions of AA, since inhibitors A metabolism are without effect (Table II and Fig. \lthough NDGA caused a slight inhibition of AA-ced $^{45}Ca^{2+}$ efflux, any specific action was in doubt use of the lack of effect with ETYA in the same ies. The increased extrusion of $^{45}Ca^{2+}$ occuring in ɔnse to AA (Fig. 5) may be almost exclusively iated by a Ca^{2+}-ATPase as it was completely ced by sodium orthovanadate [31], whereas Na^+ ɔval was without effect [32]. Table IV shows that does not inhibit all stimulus-induced calcium influx ie same degree suggesting that the AA site(s) of n is at a specific route of Ca^{2+} entry rather than ılcium extrusion.

summary, arachidonic acid inhibits depolarisa-induced calcium influx into GH_3 cells by activa-of PKC (perhaps α-isoform in particular) and by raising cytosolic calcium concentrations. Both of these mechanisms seem to contribute to a reduction in calcium influx. It is likely that it is arachidonic acid itself, and not a metabolite, which mediates these effects. This appears to be the first clear evidence linking activation of PKC by AA with a physiologically-relevent consequence. Further work is required to characterise the exact PKC isoform(s) which mediate the effects of AA here and indeed what specific substrate sites this isoform might have. It seems clear that not only diacylglycerols, but also AA may be important in the physiological activation of PKC.

E IV

of arachidonic acid on calcium influx induced by 60 mM K^+, ıyl TRH and ionomycin

:ell $^{45}Ca^{2+}$ influx was measured as described under Materials ethods. Cells were preincubated with 50 μM arachidonic acid before exposure to either 60 mM K^+-, 1 μM *N*-methyl TRH-ιM ionomycin-containing $^{45}Ca^{2+}$ 'calcium uptake medium' for 37°C. Values represent the means ± S.E., $n = 4$–12. The effect on the response to K^+, but not ionomycin or *N*-methyl TRH ıtistically significant (* $P < 0.05$).

us	$^{45}Ca^{2+}$ accummulation (fmol/10^6 cells per min)		% Inhibition of stimulus-evoked increment in $^{45}Ca^{2+}$ influx by 50 μM AA
	−AA	+50 μM AA	
	50 ± 10	49 ± 15	–
KCl	120 ± 18	52 ± 18	97 ± 16
V-methyl [	72 ± 6	66 ± 4	23 ± 11
ionomycin	230 ± 28	198 ± 19	17 ± 10

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References

1 Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R. and Lefkowith, J.B. (1986) Annu. Rev. Biochem. 55, 65–102.
2 Volterra, A. and Siegelbaum, S.A. (1988) Proc. Natl. Acad. Sci. USA 85, 7810–7814.
3 Piomelli, D., Volterra, A., Dale, N., Siegelbaum, S.A., Kandel, E.R., Schwartz, J.H. and Belardetti, F. (1987) Nature (London) 328, 38–43.
4 Kurachi, Y., Ito, H., Sugimoto, T., Shimizu, T., Miki, I. and Ui, M. (1989) Nature (London) 337, 555–557.
5 Kim, D., Lewis, D.L., Graziadei, L., Neer, E.J., Bar-Sagi, D. and Clapham, D. (1989) Nature (London) 337, 557–560.
6 Conklin, B.R., Brann, M.R., Buckley, N.J., Ma, A.L., Bonner, T.I. and Axelrod, J. (1988) Proc. Natl. Acad. Sci. USA 85, 8698–8702.
7 Dumuis, A., Sebben, M., Haynes, L., Pin, J.-P. and Bockaert, J. (1988) Nature (London) 336, 68–70.
8 Axelrod, J., Burch, R.M. and Jelsema, C.L. (1988) Trends Neurosci. 11, 117–123.
9 Jelsema, C.L. and Axelrod, J. (1987) Proc. Natl. Acad. Sci. USA 84, 3623–3627.
10 Chan, K.-M. and Turk, J. (1987) Biochim. Biophys. Acta 928, 186–193.
11 Beaumier, L., Faucher, N. and Naccache, P.H. (1987) FEBS Lett. 221, 289–292.
12 Chow, S.C. and Jondal, M. (1990) J. Biol. Chem. 265, 902–907.
13 Chaudhry, A., Conway, B.R., Laychock, S.G. and Rubin, P.R. (1989) Arch. Biochem. Biophys. 272, 488–495.
14 Murphy, S. and Welk, G. (1989) FEBS Lett. 257, 68–70.
15 Piomelli, D., Wang, J.K.T., Sihra, T.S., Nairn, A.C., Czernik, A.J. and Greengard, P. (1989) Proc. Natl. Acad. Sci. USA 86, 8550–8554.
16 Barbour, B., Szatkowski, M., Ingledew, N. and Attwell, D. (1989) Nature (London) 342, 918–920.
17 Nishizuka, Y. (1988) Nature (London) 334, 661–665.
18 Naor, Z., Shearman M.S., Kishimoto, A. and Nishizuka, Y. (1988) Mol. Endocrinol. 2, 1043–1048.
19 Naor, Z. and Catt, K.J. (1981) J. Biol. Chem. 256, 2226–2229.
20 Grandison, L. (1984) Endocrinology 114, 1–7.
21 Abou-Samra, A.-B., Catt, K.J. and Aguilera, G. (1986) Endocrinology 119, 1427–1431.
22 Mitchell, R., Johnson, M., Ogier, S.-A. and Fink, G. (1988) J. Endocrinology 119, 293–301.

23 Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
24 Luckhoff, A. (1986) Cell Calcium 7, 233–248.
25 Rink, T.J. and Pozzan, T. (1985) Cell Calcium 6, 133–144.
26 Hidaka, H. and Hagiwara, M. (1987) Trends Pharmacol. Sci. 8, 162–164.
27 Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
28 Kase, H., Iwahashi, K., Nakanashi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A. and Kanedo, M. (1987) Biochem. Biophys. Res. Commun. 142, 436–440.
29 Ballester, R. and Rosen, O.M. (1985) J. Biol. Chem. 260, 15194–15199.
30 McArdle, G.A. and Conn, P.M. (1989) Methods Enzymol. 168, 287–301.
31 DiPolo, R., Rojas, H.R. and Beauge, L. (1979) Nature (London) 281, 228–229.
32 Gill, D.L., Grollman, E.F. and Kohn, L.D. (1981) J. Biol. Chem. 256, 184–192.
33 Drummond, A.H. (1985) Nature (London) 351, 752–755.
34 Marchetti, C. and Brown, A.M. (1988) Am. J. Physiol. C206-
35 Johnson, M.S., MacEwan, D.J. and Mitchell, R. (1989) J. Ph 418, 186P.
36 Campbell, K.P., Leung, A.T. and Sharp, A.H. (1988) Tr Neurosci. 11, 425–430.
37 Albert, P.R., Wolfson, G. and Tashjian, A.H. (1987) J. Chem. 262, 6577–6581.
38 Lacerda, A.E., Rampe, D. and Brown, A.M. (1988) N (London) 335, 249–251.
39 Kiley, S., Schaap, D., Parker, P., Hsieh, L.-L. and Jaken, S. (J. Biol. Chem. 265, 15704–15712.
40 Seifert, R., Schachtele, C., Rosenthal, W. and Schultz, G. (Biochem. Biophys. Res. Commun. 154, 20–26.
41 Glass, D.G., Frey, W., Carr, D.W. and Goldberg, N.D. (19 Biol. Chem. 252, 1279–1285.
42 Johnson, M.S., MacEwan, D.J., Mitchell, R. and Thomson (1990) J. Physiol. 424, 43P.

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:alcium influx through 'L'-type channels into rat anterior pituitary cells can be modulated in two ways by protein kinase C (PKC-isoform selectivity of 1,2-dioctanoyl *sn*-glycerol?)

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e depolarisation-induced influx of $^{45}Ca^{2+}$ into anterior pituitary tissue and GH_3 cells through 'L'-type, nimodipine-sensitive channels was vestigated. In anterior pituitary prisms, phorbol esters, activators of protein kinase C, caused an enhancement of K^+-induced $^{45}Ca^{2+}$ influx. ›wever, in the GH_3 anterior pituitary cell line, phorbol esters inhibited K^+-induced $^{45}Ca^{2+}$ influx. The modulation by phorbol esters in both tissues s stereo-specific and time- and concentration-dependent. The diacylglycerol analogue, 1,2-dioctanoyl *sn*-glycerol was able to mimic the phorbol er-induced enhancement of calcium influx into anterior pituitary pieces, but was ineffective in GH_3 cells. 1,2-Dioctanoyl *sn*-glycerol may selectively activate an isoform of protein kinase C which is responsible for enhanced 'L'-type Ca^{2+}-channel activity.

Phorbol ester; Diacylglycerol; Protein kinase C isoform; Ca^{2+} influx; Rat anterior pituitary cell

INTRODUCTION

·Depolarisation of cells can cause an influx of Ca^{2+} o the cells through 'L'-type, voltage-sensitive calcium annels which are known to be a site of action for a mber of second-messenger-activated enzymes [1,2]. ese nimodipine-sensitive channels can be multiply osphorylated by protein kinase C (PKC) [2]. In some :parations, including GH_3 cells, PKC activity leads to luced 'L'-type calcium channel activity [3–7]. Cardiac -type calcium channels can show both an enhance- nt and an inhibition of their activity upon PKC ivation [8], whereas in other preparations PKC ivation leads to an enhancement of 'L'-channel activ- [9–12]. At the present time, at least seven different forms of PKC have been described with distinct cel- ır expression [13]. Some of the PKC-isoforms show tinctly different activation by agents such as phos- ›lipids, arachidonic acid, other fatty acids and :ium [14–16] and show some substrate selectivity (for iews see [13,16]).

'he present experiments investigate the effects of C activators on the depolarisation-induced influx of $^{45}Ca^{2+}$ in two different models, the GH_3 clonal rat anterior pituitary cell line and rat anterior pituitary tissue prisms.

reviations: PKC, protein kinase C: EGTA, ethyleneglycol-bis-(β-ıoethyl ether)*N,N,N',N'*-tetraacetic acid; PDBu, phorbol 12,13-tyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate; 4β-PDD, 4β-·bol 12,13-didecanoate; DOG, 1,2-dioctanoyl *sn*-glycerol; OAG, :oyl 2-acetyl *sn*-glycerol.

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2. EXPERIMENTAL

2.1. *Materials and chemicals*

All standard laboratory chemicals were of Analar grade and purchased from BDH Ltd. (Glasgow, UK). Staurosporine was bought from Novabiochem (UK) Ltd., (Nottingham, UK). GH_3 cells and foetal bovine serum were obtained from Flow Laboratories (Irvine, UK). Medium F-10 Ham was supplied by Gibco-BRL (Paisley, UK) and radioactive $^{45}CaCl_2$ was supplied by Amersham International PLC (Amersham, UK) (spec. act. = 17 mCi/mg). All other materials were purchased from the Sigma Chemical Co. (Poole, UK).

2.2. *Tissue preparation*

Male Wistar-COB rats (>250 g) supplied by Charles River UK Ltd. (Margate, UK) were maintained under controlled lighting (lights on from 05.00 to 19.00 h) and temperature (22°C) and allowed free access to diet 41B (Oxoid Ltd., Basingstoke, UK) and tap water. Animals were killed by cervical dislocation and anterior pituitaries were rapidly dissected out and hemisected for use in calcium influx studies. GH_3 cells were grown in medium F-10 Ham supplemented with 15% foetal bovine serum, 1 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Cells were harvested by agitation, washed by resuspension/centrifugation (100 × *g*, 10 min, 25°C) and prepared for calcium influx measurement as described below.

2.3. *Calcium influx measurements*

Each fresh, hemisected anterior pituitary was sliced into four equal parts and incubated in separate polypropylene tubes in 0.5 ml of 'calcium uptake medium' (concentrations in mM: NaCl 154, KCl 5.4, $CaCl_2$ 1.5, D-glucose 11, HEPES 6, pH adjusted to 7.4 with Tris base and with 0.05% essential fatty acid-free bovine serum albumin). Washed, harvested GH_3 cells were diluted to a density of 5×10^6

cells/ml in 'calcium uptake medium' and aliquoted at 0.5 ml/tube. Both cellular preparations were preincubated (30 min, 37°C, O_2 atmosphere) before a 10 min incubation (37°C, O_2) with drugs or solvent alone. Cells were then exposed to low K^+-(5.4 mM) or high K^+-(60 mM) calcium uptake medium containing 4 μM $^{45}CaCl_2$ (≈3 μCi/tube, specific). After 30 s (37°C), $^{45}Ca^{2+}$ uptake was halted by quenching with 3 ml of ice-cold 2 mM EGTA (Ca^{2+}-free) calcium uptake medium and tissue was separated by vacuum-filtration through Millipore SCWP cellulose acetate/nitrate filters (8 μm pore size) underlaid by GF/B filters on Millipore 1225 sampling manifolds (Millipore UK Ltd., Harrow, UK). Samples were washed once immediately with 3 ml ice-cold EGTA calcium uptake medium and then a further three times for 2 min each. Cellulose filters were then counted by liquid scintillation counting. Preliminary experiments revealed that these conditions gave the optimal signal-to-noise ratio and that stimulus-induced influx of $^{45}Ca^{2+}$ in excess of basal controls was maximal within 30 s, suggesting that it represented specific response-triggered flux rather than adsorption or steady accumulation by storage pools.

2.4. *Data analysis*

Concentration–response curves were analysed by a non-linear, iterative, individually-weighted curve-fitting program ('P-fit'; Biosoft, Cambridge, UK). The values quoted represent the calculated mean EC_{50} (effective concentration which produces 50% of the maximal response), with errors representing standard errors of the mean.

3. RESULTS

Fig. 1 shows that the high-K^+-stimulated influx of $^{45}Ca^{2+}$ into both pituitary pieces and GH_3 cells was inhibited by the dihydropyridine, nimodipine in a concentration-dependent fashion. The IC_{50} values for nimodipine were 3 ± 2 and 5 ± 2 nM (n = 4) for anterior pituitary prisms and GH_3 cells, respectively. Maximal inhibition of calcium influx was seen with ≥100 nM nimodipine. In both pituitary pieces and GH_3 cells small proportion (≈15%) of the response was resista to block by nimodipine, perhaps occurring throug some route other than dihydropyridine-sensitive 'L channels.

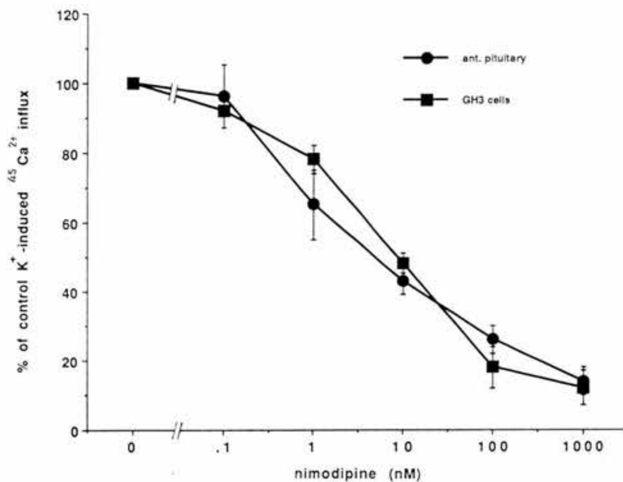


Fig. 1. Concentration-dependent inhibition by nimodipine of K^+-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms and GH_3 cells. Typically, basal accumulation of $^{45}Ca^{2+}$ accounted for around 50 fmol $^{45}Ca^{2+}$/hemi-pituitary or 10^6 GH_3 cells/min of which non-specific adsorption to filter and cell surfaces accounted for 34 fmol $^{45}Ca^{2+}$/min. Stimulation with 60 mM K^+ increased accumulation to around 120 fmol $^{45}Ca^{2+}$/hemi-pituitary or 10^6 GH_3 cells/min (approximately 1600 dpm per assay). $^{45}Ca^{2+}$ represented 1 part in 375 of the total Ca^{2+} concentration. Anterior pituitary prisms (●) or GH_3 cells (■) were preincubated with nimodipine (or solvent alone for control measurements) for 10 min before exposure to 60 mM K^+-containing medium with radioactive Ca^{2+}. After 30 s, $^{45}Ca^{2+}$ influx was halted as described in section 2. The data represent the means ±SEM of 4 determinations.

Preincubation for 10 min with phorbol 12,13-dibut rate (PDBu) and 4β-phorbol 12,13-didecanoate (4β PDD) before 30 s exposure to high-K^+ medium ar measurement of $^{45}Ca^{2+}$ influx caused marked changes the depolarisation-response seen in both tissue prepar tions. In anterior pituitary tissue, PDBu and 4β-PD (3–1000 nM) enhanced calcium influx in a concentr tion-dependent manner (EC_{50} of 55 ± 22 nM for PDB enhancement (Fig. 2)). The enhancement of K^+-induc $^{45}Ca^{2+}$ influx into anterior pituitary prisms seen wi 4β-PDD, was not mimicked by its inactive enantiom 4α-PDD at the same concentrations (3–1000 nM). the presence of maximally-effective concentrations PDBu, or 1000 nM 4β-PDD, K^+-induced $^{45}Ca^{2+}$ infl was ≈280% of control. In contrast, in GH_3 cells, PDB or 4β-PDD (3–1000 nM) pretreatment resulted in a cc centration-dependent inhibition of the K^+-induced i flux of calcium (IC_{50} of 17 ± 12 nM for PDBu inhibiti (Fig. 3)) which also showed stereoselectivity with t 4-position isomers of PDD. The maximal inhibition calcium influx seen with either PDBu or 4β-PDD w ≈50% of total depolarision-evoked Ca^{2+} influx.

The time-courses of the two opposing influences PDBu in the two preparations are shown in Fig. 4. Bc in anterior pituitary prisms and GH_3 cells, PDBu (3 nM) induced nearly half of its maximal response w total incubation times of 30 s (i.e. present only in t $^{45}Ca^{2+}$ influx measurement period). Inclusion of t

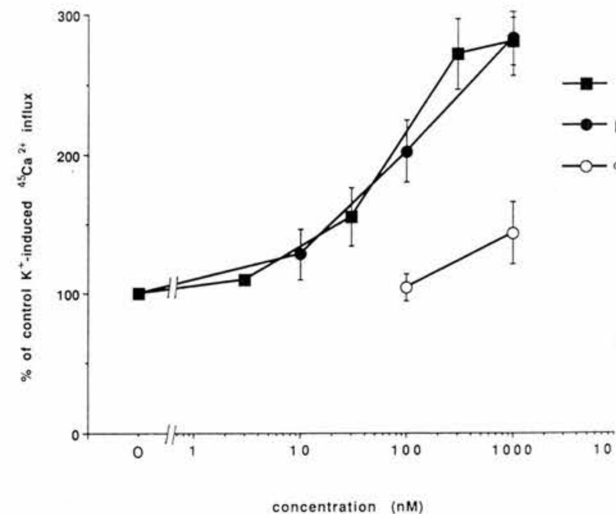
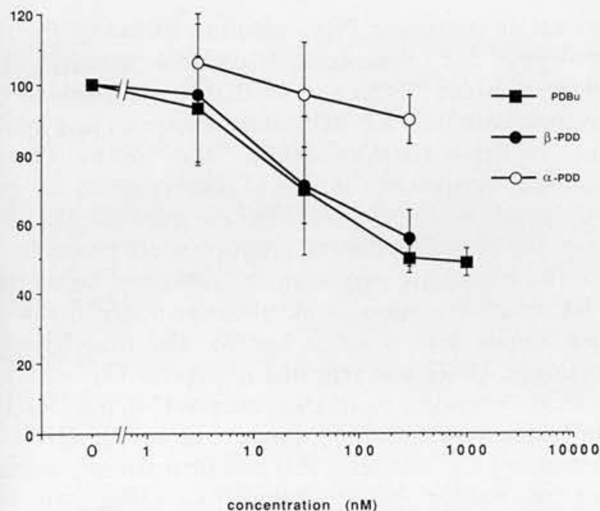


Fig. 2. Concentration-dependent enhancement by phorbol of K duced $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms. Rat ante pituitary prisms were preincubated for 10 min with PDBU (■), PDD (●) or 4α-PDD (○) before exposure to 60 mM K^+-contai medium, $^{45}Ca^{2+}$ influx was measured as described in section 2. N of the compounds had any effects on basal $^{45}Ca^{2+}$ influx at the centrations used. The data represent the means ±SEM from determinations.



. 3. Concentration-dependent inhibition by phorbol esters of K^+-uced $^{45}Ca^{2+}$ influx into GH_3 cells. GH_3 cells were preincubated for min with PDBU (■), 4β-PDD (●) or 4α-PDD (○) before exposure 60 mM K^+-containing medium. $^{45}Ca^{2+}$ influx was measured as cribed in section 2. None of the compounds had any effects on al $^{45}Ca^{2+}$ influx at the concentrations used. The data represent the means ±SEM from 4–8 determinations.

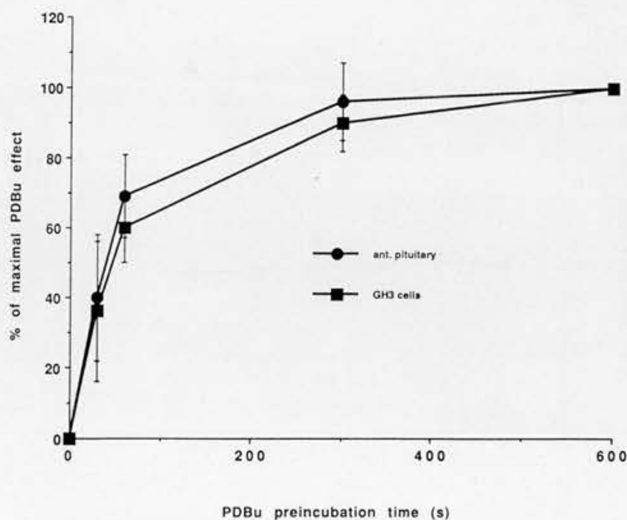


Fig. 4. Time-course of the PDBu modulation of $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms and GH_3 cells. Anterior pituitary prisms (●) and GH_3 cells (■) were preincubated with 300 nM PDBu for the indicated time before exposure to 60 mM K^+. The preincubation time with PDBu is inclusive of the 30 s $^{45}Ca^{2+}$ influx measurement period. The data represent the means ±SEM of 6 determinations.

C inhibitor, staurosporine [17], reversed the re-onse to 300 nM PDBu in both tissues. The PDBu ects were inhibited by staurosporine (1–1000 nM) in oncentration-dependent manner, with 30 nM stauro-orine reversing 59 ± 12 and 60 ± 17% of the 300 nM)Bu response on K^+-induced $^{45}Ca^{2+}$ influx into ante-r pituitary prisms and GH_3 cells, respectively ($n = 8$). ith the highest concentration of staurosporine used, significant amount of the PDBu response remained either preparation ($n = 8$).

When the diacylglycerol analogue 1,2-dioctanoyl *sn*-cerol (DOG) was tested on the two systems (Fig. 5),)G was able to mimic the enhancement by PDBu and -PDD in anterior pituitary prisms, resulting in an hancement to ≈220% of control K^+-induced influx h 100 μM DOG. However, even up to a concentra-n of 100 μM, DOG was unable to inhibit K^+-induced a^{2+} influx into GH_3 cells (maximum of 4% inhibition 100 μM).

DISCUSSION

The data presented here show that activation of C by phorbol esters can modulate (in a stereo-speci-concentration- and time-dependent manner) the de-arisation-induced influx of $^{45}Ca^{2+}$ into both rat ante-r pituitary prisms and GH_3 cells. The K^+-induced ux of $^{45}Ca^{2+}$ into both anterior pituitary prisms and H_3 cells was mediated mainly through a nimodipine-sitive 'L'-channel. The remaining 15% of nimodi-e-resistant influx represents another voltage-sensi- route which may be a 'T' or 'N'-type Ca^{2+}-channel and/or the more recently discovered dihydropyri-e-resistant, slow-inactivating, high voltage-activated Ca^{2+}-channel described by Mori et al. [18]. However, both this channel (designated the 'BI'-type channel by Mori et al.) and the 'T'-type Ca^{2+}-channel are not to-tally insensitive to block by dihydropyridines [18–20] and the presence in pituitary cells of voltage-activated calcium channels other than 'L'- or 'T'-type is uncertain [6]. Both tissues are known to contain 'L'- and 'T'-type Ca^{2+}-channels although the contribution of any tran-siently-opening Ca^{2+}-channels to the total influx seen in this 30 s depolarisation-induced $^{45}Ca^{2+}$ protocol is un-known.

Protein kinase C is known to modulate the activity of other voltage-activated channels, for example α, β- and γ-PKC can decrease transient K^+-currents in *Xenopus* oocytes, whereas only α- and β-PKC reduced transient Na^+-currents [21]. Epithelial Cl^--channels can be either up- or down-regulated by activation of PKC with either phorbol esters or diacylglycerols [22]. Such regulation of the channel may be attributable to different PKC-isoforms phosphorylating different sites on the Cl^--channel, one PKC-isoform increasing, and another iso-form decreasing channel activity [22]. A similar array of PKC influences after activation with phorbol esters was also seen in cardiac 'L'-type Ca^{2+}-channels [8]. In that case, the time-dependent increase followed by a de-crease in cardiac 'L'-channel activity may involve modulation by distinct PKC-isoforms. However, here the PDBu-induced enhancement and inhibition of K^+-induced $^{45}Ca^{2+}$ influx had similar time-courses in the two preparations (Fig. 4). At least two PKC-phospho-rylation sites exist on subunits of 'L'-type Ca^{2+}-channels [2] but the functional effect of such subunit-specific PKC-phosphorylation is unknown. The opposing ac-tions of PKC-activation on 'L'-channel influx seen here

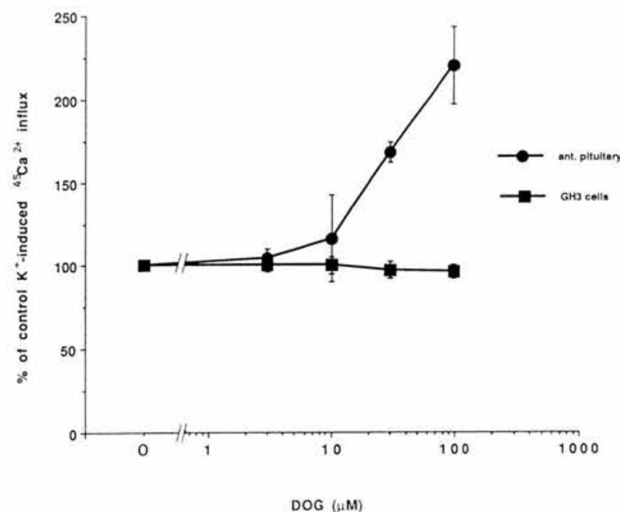


Fig. 5. Concentration-dependent enhancement by DOG of K⁺-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms and inability of DOG to modulate K⁺-induced $^{45}Ca^{2+}$ influx into GH_3 cells. Anterior pituitary prisms (●) and GH_3 cells (■) were preincubated for 10 min with the indicated concentration of DOG before exposure to 60 mM K⁺-containing medium. $^{45}Ca^{2+}$ influx was measured as described in section 2. There was no effect of 100 μM DOG on basal $^{45}Ca^{2+}$ influx, or on non-specific adsorption of $^{45}Ca^{2+}$ to filter blanks. The data represent the means ±SEM of 4 determinations.

may be due to different PKC-isoforms acting in opposing ways on 'L'- channel activity. The identity of these putative, opposing PKC-isoforms is unknown, as is the complete PKC-isoform content of the two tissues (although both tissues contain at least α-, β- and ε-PKC, but not γ-PKC (Simpson, J., MacEwan, D.J., Mitchell, R., Johnson, M.S., Thomson, F.J. and Parker, P.J., unpublished). The exact relative quantity of each of these PKC-isoforms within the two tissues is also uncertain. Naor [23] determined that the α- and β-PKC content of the two tissues varied considerably, with GH_3 cells containing 83% α-PKC and anterior pituitary containing 58% α-PKC (the remainder being β-PKC), but these results do not take account of Ca^{2+}-independent PKC isoforms [13]. The differences in absolute amount of particular PKC isoform(s) in anterior pituitary prisms and GH_3 cells may account for the functional differences seen here between the two preparations with PDBu-induced modulations of Ca^{2+}-channel activity.

Interestingly, Lacerda et al. [8] were unable to elicit the phorbol ester-mediated inhibitory regulation of 'L'-channels using the synthetic diacylglycerol, DOG. Likewise, DOG was only able to activate the PKC-induced enhancement of 'L'-channels in anterior pituitary pieces and was ineffective on the PKC-induced inhibition of 'L'-channel activity in GH_3 cells (this effect is not due to differential degradation of DOG between the two tissues (MacEwan, D.J. and Mitchell, R., unpublished)). If indeed the dual modulation of 'L'-type Ca^{2+}-channels seen in cardiac myocytes and in the present study is due to differences in PKC-isoform action, then DOG, unlike PDBu, may only be capable of activating particular PKC-isoforms, including that re sponsible for enhanced 'L'-channel activity. Th phorbol esters, PDBu and 4β-PDD are capable of ac tivating both the PKC-elicited enhancement and inhib tion of depolarisation-induced $^{45}Ca^{2+}$ influx. Protei kinase C-independent actions of phorbol esters and dia cylglycerol analogues have been reported [24,25], how ever, the concentration of phorbol esters necessary t see the effects are very high (>5 μM) and occur wit PKC-inactive isomers, unlike the stereospecificity of ac tion shown here (Figs. 2 and 3). The diacylglycer analogue, OAG was reported to depress Ca^{2+}-chann activity dependent on its PKC actions [24], but DOG a the same concentrations could not mimic OAG i depressing Ca^{2+}-currents [24] and furthermore, did no act to reduce $^{45}Ca^{2+}$ influx here (Fig. 5) sug gesting that non-PKC activity may only occur wit OAG and not DOG.

Due to its ability to penetrate cells, DOG is often use as an agent to reflect the actions of endogenous diglyc rides. Our evidence suggests that DOG may be pharma cologically selecting for one or more PKC-isoforms an therefore caution must be used in interpretation of re sults arising from use of the agent.

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REFERENCES

[1] Tsien, R.W., Lipscombe, D., Madison, D.V., Bley, K.R. a Fox, A.P. (1988) Trends Neurosci. 11, 431–438.
[2] Campbell, K.P., Leung, A.T. and Sharp, A.H. (1988) Tren Neurosci. 11, 425–430.
[3] Di Virgilio, F., Pozzan, T., Wollheim, C.B., Vicentini, L.M. a Meldolesi, J. (1986) J. Biol. Chem. 261, 32–35.
[4] Lewis, D.L. and Weight, F.F. (1988) Neuroendocrinology 169–175.
[5] Rosenthal, W., Hescheler, J., Hinsch, K.-D., Spicher, K., Tra wein, W. and Schultz, G. (1988) EMBO J. 7, 1627–1633.
[6] Marchetti, C. and Brown, A.M. (1988) Am. J. Physiol. C206–2
[7] Rane, S.G., Walsh, M.P., McDonald, J.R. and Dunlap, K. (19 Neuron 3, 239–245.
[8] Lacerda, A.E., Rampe, D. and Brown, A.M. (1988) Nature 3 249–251.
[9] DeRiemer, S.A., Strong, J.A., Albert, K.A., Greengard, P. a Kaczmarek, L.K. (1985) Nature 313, 313–316.
[10] Strong, J.A., Fox, A.P., Tsien, R.W. and Kaczmarek, L.K. (19 Nature 325, 714–717.
[11] Fish, R.D., Sperti, G., Colucci, W.S. and Clapham, D.E. (19 Circ. Res. 62, 1049–1054.
[12] Velasco, J.M. and Petersen, O.H. (1989) Q.J. Exp. Physiol. 367–370.
[13] Nishizuka, Y. (1988) Nature 334, 661–665.
[14] Shearman, M.S., Naor, Z., Sekiguchi, K., Kishimoto, A. Nishizuka, Y. (1989) FEBS Lett. 243, 177–182.
[15] Huang, K.-P., Huang, F.L., Nakabayashi, H. and Yoshida, (1988) J. Biol. Chem. 263, 14839–14845.
[16] Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, Schaap, D., Stabel, S. and Webster, C. (1989) Mol. Cell. Endo nol. 65, 1–11.

[17] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 142, 436–440.
[18] Mori, Y., Friedrich, T., Kim, M.-S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hofmann, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T. and Numa, S. (1991) Nature 350, 398–402.
[19] Bean, B.P. (1985) J. Gen. Physiol. 86, 1–30.
[20] Bean, B.P., Sturek, M., Puga, A. and Hermsmeyer, K. (1986) Circ. Res. 59, 229–235.
[21] Lotan, I., Dascal, N., Naor, Z. and Boton, R. (1990) FEBS Lett. 267, 25–28.
[22] Li, M., McCann, J.D., Anderson, M.P., Clancy, J.P., Liedtke, C.M., Nairn, A.C., Greengard, P. and Welsh, M.J. (1989) Science 244, 1353–1356.
[23] Naor, Z. (1990) Endocrinology 126, 1521–1526.
[24] Hockberger, P., Toselli, M., Swandulla, D. and Lux, H.D. (1989) Nature 338, 340–342.
[25] Doerner, D., Abdel-Latif, M., Rogers, T.B. and Algers, B.E. (1990) J. Neurosci. 10, 1699–1706.

SELECTIVE PHARMACOLOGY OF PROTEIN KINASE C

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Activation of protein kinase C (PKC) by diglyceride (derived in part from the action of phospho-inositidase C) appears to play a major role in signal transduction/cellular regulation in many systems. More than seven isoforms of PKC are now known, which fall into two series: A; Ca^{2+} dependent and B; Ca^{2+} independent. However whilst many targets of 'PKC' have been described, very little is known of the physiological roles of particular isoforms and indeed whether they display pharmacological differences that may permit selective intervention. We have developed a number of cellular models of PKC action allowing us to explore, in a physiological context, the putative selective pharmacology of different isoforms. With the aim of assigning identities to the kinases active in particular models, we have further assessed the actions of particular drugs in ligand binding and kinase activity assays for PKC using cells and tissues enriched in particular isoforms.

One of our models for PKC(s) action is the regulation of $^{45}Ca^{2+}$ influx through L channels in the GH_3 cell line and in anterior pituitary cells. K^+- induced $^{45}Ca^{2+}$ into these different cells is influenced quite differently by phorbol esters; the former being inhibited and the latter enhanced by 4 β- but not 4α-phorbol 12, 13-didecanoate (PDD) (Johnson, MacEwan and Mitchell, 1989). While both effects were readily blocked by staurosporine, only that in GH_3 cells was readily blocked by H7 ($IC_{50} \cong 10\mu M$). We sought selective agonists for the two effects, finding that arachidonic acid (AA) mimicked the effect in GH_3 cells but not pituitary (being both H7- and staurosporine-sensitive) whereas sn-1,2 dioctanoyl glycerol (DOG) and 12-deoxyphorbol 13-isobutyrate (DPB) were effective only in pituitary. It is known that α, ß, ε and not γ isoforms are present in both tissues, with GH_3 cells being rather more enriched in α. Since AA is reported to activate α and γ isoforms, but γ is not considered to be present here, we suggest that the effect shown by AA is via αPKC. In support of this hypothesis ligand binding studies to cystosolic PKC showed an allosteric enhancement of [^{3}H]-PDBu binding by AA, the magnitude of which correlates with the α content of tissue. In contrast, the affinities for a range of diacylglycerols, particularly DOG, in displacing [^{3}H]-PDBu showed if anything an inverse correlation with α content suggesting that the facilitatory, H7-resistant profile on $^{45}Ca^{2+}$ influx in pituitary pieces was through something other than αPKC.

Models at the level of hormone secretion parallel these observations (Johnson and Mitchell, 1989). PDBu-induced secretion of luteinising hormone (LH) but not growth hormone (GH) is H7-sensitive but the latter resistant, whilst staurosporine is effective on both. The priming effect of LH-releasing hormone (LHRH) is a unique phenomenon of increased secretory responsiveness (Mitchell, Johnson, Ogier & Fink, 1988). This can be mimicked in some aspects by phorbol esters and is sensitive to staurosporine yet not H7. This profile is reminiscent of the very high levels of H7 required to block long-term potential (LTP). It appears that ε as well as α, ß and γ isoforms are H7-sensitive (Schaap and Parker, 1990) so some other form of PKC or related kinase may mediate the H7 resistant events observed.

References:
Johnson, M S and Mitchell, R (1989), Biochemical Society Transactions 17, 751-752.
Johnson, M S, MacEwan, D J and Mitchell, R (1989), J. Physiol. (Lond.) 418, 186P.
Mitchell, R, Johnson, M S, Ogier, S A and Fink, G (1988), J. Endocrinol. 119, 293-301.
Schaap, D and Parker, P J (1990), J. Biol. Chem. 265, 7301-7303.

CELLULAR ACTIONS OF PHARMACOLOGICALLY DISTINCT FORMS OF PROTEIN KINASE C.

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We have developed various cellular models of protein kinase C (PKC) action which have allowed us to explore, in a physiological context, the selective pharmacology of different species of PKC. Depolarisation-induced $^{45}Ca^{2+}$ influx through dihydropyridine-sensitive Ca^{2+} channels into anterior rat pituitary tissue and into GH_3 cells were differently influenced by phorbol esters and putative PKC inhibitors. In GH_3 cells, 4ß-, but not 4α-phorbol 12,13-didecanoate (PDD) inhibited K^+-induced $^{45}Ca^{2+}$ influx in a staurosporine- and H7-sensitive manner. In pituitary tissue, by contrast, PDD enhanced K^+-induced $^{45}Ca^{2+}$ influx in a staurosporine-sensitive, but H7-insensitive manner. We have also found certain PKC actions in other models to show differential sensitivity to H7. For example, H7 blocked phorbol 12,13-dibutyrate (PDBu)-induced release of luteinizing hormone (LH) but not growth hormone (GH), whereas staurosporine inhibited both. These results were supported by the fact that both Ca^{2+}-independent and Ca^{2+}-dependent PDBu-stimulated kinase activities were blocked with similar potency by staurosporine, whereas Ca^{2+}-independent kinase activity was found to be much more resistant to H7 relative to Ca^{2+}-dependent activity. These pharmacologically different PKCs in anterior pituitary also differ in their cellular targets. Phospholipase A_2 (PLA_2) inhibitors (eg quinacrine) blocked PDBu-induced LH but not GH release suggesting that in gonadotrophes, but not somatotrophes, an H7-sensitive PKC(s) can act to modulate PLA_2 activity. These data suggest that PKC species may differ in their sensitivity to PKC inhibitors, and that they may have distinct cellular targets.

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ifferential actions of protein kinase C modulators on the release of hormones from
at anterior pituitary tissue *in vitro*:

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 have previously reported that phorbol 12,13-dibutyrate (PDBu)-induced release of
uteinizing hormone (LH) and growth hormone (GH) from rat anterior pituitary *in
itro* show different time courses and different sensitivity to the protein kinase C
KC) inhibitors H7 and staurosporine (Johnson & Mitchell, 1989). In view of the
ssibility that distinct forms of PKC may be involved, we have examined the effects
 another PKC activator, mezerein (MEZ); known to mimic only some of the effects of
horbol esters (Slaga *et al*, 1980).

e release of LH and GH from pro-oestrous rat hemipituitaries was measured
cording to Johnson & Mitchell (1989). The magnitude of GH release induced by MEZ
s similar to that seen with PDBu, thus in the presence of either 100nM PDBu or
0nM MEZ, GH release was 2.32±0.33 or 2.33±0.33 (mean±s.e.m., n=4-6) fold of basal.
wever, MEZ (100nM) was more effective at releasing LH, where the equivalent
gures were 3.52±0.28 and 13.35±1.92 fold of basal LH release respectively (n=4-6).
e release of GH induced by MEZ or by PDBu (300nM) was unaffected by H7 (30μM). In
ntrast, 10μM H7 inhibited the release of LH induced by MEZ and PDBu by 35±10% and
±15% respectively (n = 4-6).

 compare the actions of these PKC modulators on kinase activity, partially
rified PKCs from male rat midbrain (reported to contain messenger RNA for all of
e known PKC isoforms; Scott Young III, 1989) were investigated using a
osphatidyl serine-dependent, histone III-S kinase assay, similar to that
scribed by Huang *et al* (1988). The Ca^{2+}-independent activity induced by MEZ and
Bu was inhibited similarly by H7 (IC_{50}s of 27±7 and 49±4 μM, n = 4-8). The Ca^{2+}-
pendent activity induced by MEZ was notably more sensitive to H7 than that induced
 PDBu (IC_{50}s of 4±2 and 47±9 μM, n=3-8). Taken together, these data suggest that
Z may show some selectivity for activation of a Ca^{2+}-dependent PKC, which is
ghly sensitive to H7. Both this and other PKCs may participate in the LH release
duced by PKC activators.

ferences

hnson, M.S. & Mitchell, R. (1989) *Biochem. Soc. Trans.* **17**, 751-752.
aga, T.J., Fischer, S.M., Nelsoon, K. & Gleason, G.L. (1980) *Proc. Natl. Acad.
i. USA* **77**, 3659-3663.
ang, K.-P., Huang, F.L., Nakabayashi, H. & Yoshida, Y. (1988) *J. Biol. Chem.* **263**,
839-14845.
ott Young III, W. (1989) *J. Neuroendocrinol.* **1**, 79-82.

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otein kinase C may be required to maintain activity of nimodipine-sensitive Ca^{2+} annels in rat anterior pituitary cells *in vitro*.

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tassium-induced release of growth hormone (GH) from anterior pituitary tissue *in tro* is inhibited by nimodipine (Johnson *et al*, 1991). Correspondingly, K^+-induced Ca^{2+} influx into anterior pituitary prisms and into cells of the GH_3 line ~~are~~ is modipine-sensitive (Johnson *et al*, 1991; Johnson *et al*, 1989). The protein kinase (PKC) activator phorbol 12,13-dibutyrate(PDBu) also releases GH and the response largely inhibited by nimodipine (97 ± 9% inhibition at 1 μM, mean ± s.e.m., = 6), suggesting that a PKC in somatotrophes can enhance the activity of hydropyridine-sensitive Ca^{2+} channels. The K^+-induced influx of $^{45}Ca^{2+}$ in anterior tuitary prisms is facilitated by PDBu and both this response and the effect of Bu on GH release were acutely reversed by the PKC inhibitor staurosporine (3 - 0 nM) but not H7 (1 - 30 μM) (Johnson *et al*, 1989; Johnson & Mitchell, 1989). The esent experiments were carried out to investigate whether there is a tonic fluence of PKC on these Ca^{2+} channels.

asurements of GH secretion and K^+-induced $^{45}Ca^{2+}$ influx were made as previously scribed (Johnson & Mitchell, 1989; MacEwan & Mitchell, 1990). Staurosporine (300) reduced 60 mM K^+-induced GH release during 2 consecutive hours to 44 ± 13 and 10 ± of controls after 1 hour additional preincubation with the drug (mean ± s.e.m., n 8). There was no effect of 10 μM H7, (114 ± 15% of control; mean ± s.e.m., n = , nor did staurosporine reduce ionomycin (50 μM)-induced release (107 ± 14 and 100 12% of controls in 2 consecutive hours, mean ± s.e.m., n = 7). Correspondingly, veral selective PKC inhibitors reduced K^+-induced $^{45}Ca^{2+}$ influx into pituitary isms after preincubation for 60 min but not 10 min: for example, 300 nM aurosporine caused 84 ± 7% and 7 ± 5% inhibition at the two time points spectively (mean ± s.e.m., n = 4). Ionomycin (30 μM)-induced $^{45}Ca^{2+}$ influx was affected by staurosporine (300 nM for 60 min) and K^+-induced $^{45}Ca^{2+}$ influx was ightly enhanced by H7 to 138 ± 10% of control by 20 μM H7 for 60 min (mean ± e.m., n = 4). Furthermore, presumed down-regulation of PKC levels in GH_3 cells by eatment with PDBu (300 nM) for 24 h reduced 60 mM K^+-induced $^{45}Ca^{2+}$ influx to 29 ± % of control levels (mean ± s.e.m., n = 6).

ese results support the hypothesis that PKC activity in somatotrophes may normally ntribute to maintaining nimodipine-sensitive Ca^{2+} channels in a relatively tivated state.

ferences

nson, M.S., MacEwan, D.J. & Mitchell, R. (1989). *J. Physiol.* **418,** 186P.
nson, M.S. & Mitchell, R. (1989). *Biochem. Soc. Trans.* **17,** 751-752.
nson, M.S., Mitchell, R., MacEwan, D.J. & Thomson, F.J. (1991). *J. Physiol.* **434,** ?.
cEwan, D.J. & Mitchell, R. (1990). *Biochem. Soc. Trans.* **18,** 432-433.

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Characterisation of [^{3}H]dimethylstaurosporine binding sites by displacement studies using protein kinase C inhibitors.

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Staurosporine inhibits protein kinase C (PKC) activity by interacting with, or close to, its ATP binding site (Nakadate, *et al,* 1988). Since the ATP binding sites of different serine/threonine- and tyrosine-specific kinases show homology, staurosporine has only modest selectivity for PKC (Rüegg & Burgess, 1989) suggesting that its radiolabelled derivative, [^{3}H]N,N-dimethylstaurosporine ([^{3}H]DMS), is a useful tool for characterisation of different classes of protein kinase within a tissue. We have characterised [^{3}H]DMS binding sites in rat midbrain and lung cytosol by displacement studies using the PKC inhibitors, H7 and staurosporine.

Adult male rat midbrain or lung was homogenised in 20mM Tris-HCl (pH7.5) with 50mM EtSH ,2mM EDTA, and 1mM phenylmethylsulphonyl fluoride, then centrifuged (12,000g, 4°C, 20 mins). The supernatant was recentrifuged (12,000g, 4°C, 5mins) and this supernatant taken to represent cytosol. The binding assay constituents were 50mM Tris-HCl (pH8.0), 1µg/ml bovine gamma globulin (BGG), 1mM dithiothreitol, [^{3}H]DMS (5nM), H7 or staurosporine and cytosol (Gross, *et al,* 1990). Total binding was measured in the absence of PKC inhibitor and non-specific binding (which was approximately 25% of total binding) was defined in the presence of 3µM staurosporine. After a 30 min incubation (4°C), the protein was precipitated on ice with 1mg/ml BGG and 10% polyethyleneglycol 8000 and pelleted by centrifugation (12,000g, 4°C, 5 mins). The pellet was washed once with 1ml of ice cold Tris-HCl then counted. Staurosporine displaced specific [^{3}H]DMS binding to sites in lung and midbrain cytosol with Hill slopes of 0.58±0.07 and 0.22±0.05 respectively, consistent with both tissues having more than one site with different affinities for staurosporine. Binding to midbrain sites was more sensitive to displacement by staurosporine (IC_{50}=0.23±0.06nM) than lung sites (IC_{50}=9.38±1.93nM). Midbrain cytosol contained sites sensitive to very low concentrations of staurosporine (with 27±4% displacement at just 0.02nM), that were apparently absent in lung. The H7 displacement curves were similar in both tissues. However, the potency of H7 was quite low with concentrations up to 500µM displacing only approximately 40% of total binding.These results are consistent with both lung and midbrain cytosol containing more than one kinase type which differ in their affinities for staurosporine. Midbrain, but not lung, appears to express a kinase (or set of kinases) which is highly sensitive to staurosporine,much more so than has previously been reported for staurosporine on purified kinase activity (Gross, *et al,* 1990). However, H7 was unable to distinguish any sites unique to either tissue. The weak displacement of binding by H7 suggests that the PKC recognition sites for staurosporine and H7 are not identical.

Gross, J.L., Herbelin, W.F., Hoi Do, U., Pounds, J.S., Johnson Buenaga, L. & Enders Stephens, L. (1990). Biochem. Pharmacol. **40**, 343 - 350.
Nakadate, T., Jeng, A.Y.& Blumberg, P.M. (1988) Biochem. Pharmacol. **37**, 1541-1545.
Rüegg, U.T. & Burgess, G.M. (1989). Trends Pharmacol. Sci. **10**, 218-220.

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Cholecystokinin-octapeptide stimulates ACTH release from AtT-20 tumour cells through a mechanism independent of polyphosphoinositide hydrolysis

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Cholecystokinin-octapeptide (CCK-8) has been found to be present in the hypothalamus (Beinfeld *et al,* 1981) where it was suggested to co-exist with corticotrophic-releasing factor (CRF) in medial parvocellular neurons in the paraventricular nucleus (Mezey *et al,* 1985). We investigated whether CCK-8 could induce secretion of adrenocorticotrophic hormone (ACTH) from the clonal mouse AtT-20 anterior pituitary tumour cell line, and to see if any release may involve CCK-8-stimulated inositol phosphate production. Culture of AtT-20/D16-16 cells and radioimmunoassay for ACTH were performed as previously described (Hook *et al,* 1982). For measurement of inositol phosphates, AtT-20 cells were preincubated for 48 h in inositol-free culture medium supplemented with 2 µCi/ml [^{3}H]-inositol. Washed, harvested, labelled cells were incubated at a cell density of 10^6 cells/ml in DMEM + 0.25% BSA + 25 mM HEPES + 3 µg/ml bacitracin + 10 mM LiCl. After drug challenge for 0-60 min at 37°C, reactions were stopped by addition of (1:2) chloroform/methanol and vortexed. After 20 min, organic and aqueous phases were separated by addition of (1:1) chloroform/H_2O then centrifugation (1000 g, 5 min). The organic phase was sampled for radioactivity and the inositol phosphate contained in the aqueous phase (4°C) were separated on Dowex-formate anion-exchange columns. The radioactivity incorporated into inositol, IP_1, IP_2, IP_3 and IP_4 was separated by successive elutions with H_2O then with 0.1 M formic acid + 0.2, 0.4, 0.8 and 1.2 M ammonium formate respectively. As expected, CRF (0.1 µM) and forskolin (20 µM) stimulated ACTH release from AtT-20 cells. Likewise, CCK-8 could also stimulate ACTH secretion in a dose-dependent fashion (EC_{50} = 20 nM) and 1 µM CCK-8 induced a release rate of 133 ± 34 pg ACTH/10^6 cells/h, mean ± s.e.m., n = 12 (4.4 fold increase over basal release). Exposure of AtT-20 cells to high (6.8 mM) Ca^{2+} concentrations in the presence of Li^+ caused a time-dependent reduction in radioactivity of the lipid- and inositol-content of the cells at 60 min to 50 ± 10 and 44 ± 6% of unstimulated levels (mean ± s.e.m., n = 3) with a concomitant increase in IP_1 levels to 250 ± 10% of control (n = 3) at 60 min, and a peak rise in IP_2 levels to 350 ± 25% of unstimulated levels (n = 3) at 10 min. Over the concentration range 10^{-11} to 10^{-6} M, CCK-8 (preincubated for up to 60 min) and in the presence of Li^+ produced no marked change in inositol phosphates, inositol or lipid-radioactivities (n = 3)

These results further indicate a role for CCK-8 in ACTH release from corticotrophes. However, although phosphoinositide hydrolysis in the cells could be detected by exposure to high Ca^{2+}, no detectable change in phosphoinositide hydrolysis could be seen with CCK-8. Therefore, in AtT-20 cells, CCK-8 acts as a secretagogue but produces its effects by a cyclic AMP-independent (Reisine *et al,* 1985) and an inositol phosphate-independent mechanism.

Beinfeld, M.C., Meyer, D.K., Eskay, R.L., Jensen, R.T. & Brownstein, M.J. (1981) Brain Res. **212**, 51-57
Hook, V.Y.H., Heisler, S., Sabol, S.L. & Axelrod, J. (1982) Biochem. Biophys. Res. Commun. **106**, 1364-1371
Mezey, E., Reisine, T.D., Skirboll, L., Beinfeld, M. & Kiss, J.Z. (1985) Ann. N.Y. Acad. Sci. **448**, 152-156
Reisine, T., Rougon, G., Barbet, J. & Affolter, H.-U. (1985) Proc. Natl. Acad. Sci. USA **82**, 8261-8265.

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Regional differences in the affinity of diacylglycerol analogues for [^{3}H]-phorbol 12,13-dibutyrate binding sites

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Diacylglycerols are one of the endogenous activators of protein kinase C (PKC) (Nishizuka, 1988). We have previously postulated that the short chain diacylglycerol, 1,2-dioctanoyl *sn*-glycerol (DOG) may selectively activate an isoform(s) of PKC (Johnson *et al,* 1989). Since phorbol esters, such as phorbol 12,13-dibutyrate (PDBu), are known to activate PKC by acting at the diacylglycerol binding site, we investigated the effect of a range of 1,2-diglycerides on cytosolic [^{3}H]-PDBu binding from various regions in the rat with known contents of the different Ca^{2+}-dependent PKC-isoforms.

Cytosolic [^{3}H]-PDBu binding was performed as previously described (MacEwan & Mitchell, 1990). The range of 1,2-diglycerides consisted of unmixed saturated chains of 6 -18 carbon atoms ($C_{6:0}$ - $C_{18:0}$), 1,2-dioleoyl *sn*-glycerol (DO) which has two unmixed chains with one unsaturated double bond in each, and a mixed unsaturated chain ($C_{18:1}$, cis-9) diacylglycerol, 1-stearoyl-2-arachidonoyl *sn*-glycerol (SAG) ($C_{18:0}/C_{20:4}$,[cis,cis,cis,cis]-5,8,11,14). When measuring the IC_{50} for reversal of specific binding by 0 - 500 μM of each compound in rat lung, frontal cerebral cortex and cerebellum, DO and SAG showed the highest affinities, which were similar in each of the three regions tested (11 - 23 μM with DO, and 6, 4 and 4 μM with SAG for lung, cortex and cerebellum respectively (n = 4)). However, the IC_{50} values for $C_{6:0}$ - $C_{18:0}$ saturated diglycerides varied considerably, with their affinities in lung being consistently lower than in the other regions for all the chain lengths tested. Differences in affinity were not due to selective actions of lipases because DOG pre-incubated in lung had the same subsequent affinity for binding in cortex as DOG pre-incubated in cortex or cerebellum. Plots of affinity against chain length were bell-shaped and showed lowest affinity for [^{3}H]-PDBu-binding with $C_{6:0}$ and $C_{18:0}$ (IC_{50}s were 50 - 176 μM for $C_{6:0}$, $C_{8:0}$ and $C_{18:0}$ in cortex and cerebellum and 1120, 1354 and 416 μM in lung respectively). The highest affinity in the saturated diglyceride series was with a C_{14} chain length (IC_{50}s of 17 μM for cortex and cerebellum; 46 μM in lung). Using cytosol from various tissues with known contents of Ca^{2+}-dependent PKC-isoforms (Shearman *et al,* 1988), the affinity of DOG varied not only using lung, cortex and cerebellum, but with all the regions tested and an inverse relationship was apparent between the affinity for [^{3}H]-PDBu binding and the proportion of α-PKC isoform present in the tissue.

There is clearly an optimal chain length and composition for diacylglycerol interaction with [^{3}H]-PDBu binding sites, but it now appears that short chain saturated diglycerides such as DOG may selectively interact with certain isoforms of PKC.

Johnson, M.S., MacEwan, D.J. & Mitchell, R. (1989) J. Physiol. **418**, 186P.
MacEwan, D. & Mitchell, R. (1990) Biochem. Soc. Trans. **18**, 433-434.
Nishizuka, Y. (1988) Nature **334**, 661-665.
Shearman, M.S., Kosaka, Y., Ase, K., Kikkawa, U. & Nishizuka, Y. (1988) Biochem. Soc. Trans. **16**, 307-308.

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The effects of different classes of activators of protein kinase C on the enzyme of rat midbrain

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It is now known that there are at least 7 distinct isoforms of protein kinase C (PKC) (Nishizuka, 1988) and messenger RNAs for all these isoforms have been detected in rat midbrain (Scott Young III, 1989). We have been interested for some time in the possibility that these isoforms may exhibit selective pharmacological properties. This idea is supported by the differential effects of PKC inhibitors on phorbol ester-induced hormone release from pituitary (Johnson & Mitchell, 1989) and the selectivity of some PKC activators on regulation of Ca^{2+} channels in these cells (MacEwan *et al,* 1990).

To directly examine the properties of the kinases, we used a mixed-micelle histone III-S kinase assay similar to that of Huang *et al* (1988). Phosphatidyl serine-dependent kinase activity from male rat brain cytosol (partially-purified on DEAE cellulose) was measured in the presence of 100 μM or zero (< 3 nM) free Ca^{2+}. Phorbol 12,13-dibutyrate (PDBu) activated Ca^{2+}-dependent PKCs with a consensus EC_{50} of 21 ± 5 nM compared to that for Ca-independent activity of 790 ± 120 nM (means ± s.e. mean, n = 8 - 20). Phorbol 12-myristate 13-acetate induced activity with similar maxima in each case and with a potency on Ca^{2+}-dependent activity around 10 fold greater than on Ca^{2+}-independent. Two compounds which showed selectivity in the modulation of Ca^{2+} channels by apparently distinct PKCs (MacEwan *et al,* 1991) were also tested. Deoxyphorbol 13-isobutyrate recruited similar maximal activity of Ca^{2+}-independent kinases (93 ± 3% of that due to PDBu), but rather less (73 ± 5%) of the maximal PDBu-evoked Ca^{2+}-dependent activity (mean ± s.e. mean, n = 8 - 9). Mezerein recruited 82 ± 5% of the maximal Ca^{2+}-independent activity evoked by PDBu but only 37 ± 5% of the Ca^{2+}-dependent increment (mean ± s.e. mean, n = 5 - 9). The diglyceride 1,2-dioctanoyl-sn-glycerol (DOG) activated the overall population of kinases with a consensus EC_{50} of 0.8 ± 0.3 μM in the presence of 100 μM Ca^{2+}. However, even at high concentrations (up to 1 mM), DOG could only elicit about half of the maximal Ca^{2+}-independent activity seen with PDBu (n = 4 - 8).

These data indicate that activity of Ca^{2+}-dependent and independent PKCs can be differentially elicited by some PKC activators. Under certain conditions, the diglyceride, (DOG) appears to be ineffective on a component of the Ca^{2+}-independent activity whereas other compounds such as mezerein may be ineffective on some of the Ca^{2+}-dependent kinases.

Huang, K.-P., Huang, F.L., Nakabayashi, H. & Yoshida, Y. (1988) J. Biol. Chem. **263**, 14839-14845.
Johnson, M.S. & Mitchell, R. (1989) Biochem. Soc. Trans. **17**, 751-752.
MacEwan, D.J., Mitchell, R., Johnson, M.S. & Thomson, F.J. (1991) Brit. J. Pharmacol. **102**, 258P.
Nishizuka, Y. (1988) Nature **334**, 661-665.
Scott Young III, W. (1989) J. Neuroendocrinol. **1**, 79-82.

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PROPERTIES AND [^{32}P] PHOSPHORYLATION TARGETS OF A NOVEL FORM OF PROTEIN KINASE C IN PITUITARY.

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A number of cellular responses in anterior pituitary cells (such as the priming effect of LHRH and a component of phorbol ester-induced phospholipase A_2 activation) are elicited by a form of protein kinase C (PKC) which is distinguished by its resistance to isoquinoline PKC inhibitors such as H7. In a mixed-micelle PKC activity assay, H7-resistant but staurosporine-sensitive activity was observed in cytosol from anterior pituitary and lung but not from a number of other tissues. At least three proteins with weight 16, 16 and 25 kDa were identified as selective targets of this novel PKC (in two-dimensional autoradiographs of anterior pituitary tissue incubated with [^{32}P] orthophosphate).

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Second Messenger Systems as Pharmacological Targets

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REGIONAL SELECTIVITY IN THE INTERACTION OF LIPID ACTIVATORS WITH CYTOSOLIC PROTEIN KINASE C.

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ABSTRACT

The effect of 1,2-diacylglycerols on specific binding of [^{3}H]-phorbol 12,13-dibutyrate to cytosolic protein kinase C was investigated in tissues reported to contain different proportions of protein kinase C isoforms. In lung, frontal cerebral cortex and cerebellum cytosols (enriched in α-, β- and γ-protein kinase C respectively) displacement of specific binding by diacylglycerols containing unsaturated acyl chains was of similar potency for each tissue. 1,2-diacylglycerols containing saturated acyl chains exhibited varying affinities for [^{3}H]-phorbol 12,13-dibutyrate binding sites in each tissue; defining an optimal acyl chain length of around 14 carbons in each case. However, the affinities of saturated diglycerides were consistently lower in lung cytosol than in frontal cerebral cortex and cerebellum cytosols, with the greatest differences occurring at lower acyl chain lengths, especially with 1,2-dioctanoyl-*sn*-glycerol. Binding analysis in several tissues with known protein kinase C isoform content, indicated that 1,2-dioctanoyl-*sn*-glycerol may have reduced affinity towards protein kinase C-α.

Key words: Diacylglycerols; Arachidonic acid; Phorbol ester binding; Protein kinase C isoforms.

Abbreviations: PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; AA, arachidonic acid; DOG, 1,2-dioctanoyl-*sn*-glycerol; EGTA, ethylene glycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid.

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1.INTRODUCTION

Phorbol esters are a class of tumour promoting compounds which bind with high affinity and selectivity to protein kinase C (PKC) [1-5] which exists as a family of at least seven isoenzymes [6]. Phorbol esters such as [^{3}H]-phorbol 12,13-dibutyrate ([^{3}H]-PDBu) bind to PKC at the diacylglycerol binding site of the enzyme [4]. The competitive displacement by diacylglycerols of specific [^{3}H]-PDBu binding can be used as a convenient means of exploring the structural requirements of this site in different PKC isoforms.

Arachidonic acid (AA) increases the activity of the α- and γ-isoforms of PKC in cell-free systems, but is less active at the β-isoform of PKC [7]. The precise site of action of AA on PKC is at present uncertain [8-11], as is the effect of AA on the other three more recently discovered members of the PKC-isozyme family, δ–,ε– and ζ-PKC [6].

The proportions of the different calcium-dependent PKC isoforms (α, β and γ) in the cytosolic component of various tissues from rat have been described [12-14]. In the present study a range of diacylglycerol analogues was examined to assess the effect of acyl chain composition on [^{3}H]-PDBu-binding affinity in several regions with varying PKC isoform content. We have also used these tissues to compare the effects of AA and the diacylglycerol, 1,2-dioctanoyl-*sn*-glycerol (DOG) on the specific binding of [^{3}H]-PDBu, to elucidate any relationship between their binding characteristics and the content of particular PKC isoforms.

2. EXPERIMENTAL

2.1. Materials.

Standard laboratory chemicals of Analar grade were obtained from BDH Chemicals Ltd, Poole, UK. Dulbecco's modification of Eagles medium with 4500 mg/ml glucose, without sodium pyruvate (DMEM), Ham's F-10 medium containing 146 mg/l l-glutamine and foetal bovine serum were supplied by Gibco-BRL, Paisley, UK. All other compounds and drugs were bought from the Sigma Chemical Company Ltd, Poole, UK., except [20-^{3}H(N)]-PDBu (specific

activity = 19.1 mCi/mmol) which was purchased from Du Pont, Dreieich, Germany.

2.2. Animals and cells.

Male COB-Wistar rats (>250 g) were purchased from Charles River UK Ltd, Margate, UK and were maintained under controlled lighting (lights on from 05.00 to 19.00 h) and temperature (22°C) and allowed free access to diet 41B (Oxoid Ltd, Basingstoke, UK.) and tap water. COS 7 cells obtained from the European Collection of Animal Cell Cultures, Porton Down, UK., were cultured in a humidified atmosphere of 95% air/5% CO_2 with DMEM supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, and were harvested by trypsinisation. GH_3 cells purchased from Flow Laboratories Ltd., Irvine, UK., were cultured in Ham's F-10 medium supplemented with 15% foetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, and were harvested by agitation.

2.3. Method for specific binding of [^{3}H]-PDBu to cytosolic PKC.

Cytosolic [^{3}H]-PDBu binding was performed in a method similar to that described by Leach et al [4]. Rats were killed by cervical dislocation and various regions were rapidly dissected and homogenised in 2 volumes of 50 mM Tris HCl (pH 9.0) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM $MnCl_2$ and ultracentrifuged (100,000g, 1 hr, 4°C). The pellet was discarded and the supernatant was again ultracentrifuged (120,000g, 1 hr, 4°C). The supernatant from the second spin was regarded as cytosol and stored at -40°C until use. Cytosol was diluted in 'assay buffer' (50 mM Tris HCl (pH 7.4), 4 mg/ml essential fatty acid-free bovine serum albumin, 1 mM $CaCl_2$ and 75 mM magnesium acetate) to a concentration which gave total binding of approximately 5 - 10% of total radioactivity present, then incubated in a total volume of 250 µl (30 mins, 37°C) with 1 mg/ml phosphatidyl serine (sodium salt), 5 nM [^{3}H]-PDBu (approx. 0.03 µCi per tube), various concentrations (0.5 - 500 µM) of either AA or diacylglycerol, dimethylformamide (<1% final) or 20 µM PDBu in dimethylformamide for total and non-specific binding measurements respectively. Protein was precipitated on ice by the addition of 100 µl 12 mg/ml bovine gamma-globulin and 300 µl 24% polyethyleneglycol 8000 in 50 mM Tris

HCl (pH 7.4). After 20 mins, assay tubes were centrifuged (12,000g, 5 mins, 4°C), spirated and the radioactivity in each pellet determined after solubilisation. Lipids were prepared for addition to the assay as follows. Phosphatidylserine with or without AA or diacylglycerol were dissolved in chloroform and dried under a stream of N_2 before being sonicated in 'assay buffer' with a Ystral high frequency homogeniser (2 x 30 s, setting 4) followed by two full strength 30 s pulses in an MSE micro-tip sonicator. Various concentrations of diacylglycerols or AA were added to the assay in a mixed micellar method where the drug concentration was diluted by mixing with micelles which contained phosphatidylserine only.

2.4. Binding-data analysis

Concentration-response curves were analysed by a non-linear, iterative, individually weighted curve-fitting program ('P-fit'; Biosoft, Cambridge, UK.). The values quoted represent the means with errors representing standard errors of the mean (S.E.M.) of IC_{50} values (concentration required to inhibit 50% of the response) where appropriate.

3. RESULTS and DISCUSSION.

Displacement of specific [^{3}H]-PDBu binding by diacylglycerols with unsaturated acyl chains, such as 1,2-dioleoyl-*sn*-glycerol ($C_{18:1}$) and 1-stearoyl-2-arachidonoyl-*sn*-glycerol ($C_{18:0}/C_{20:4}$) occurred with similar, high potency in lung, frontal cerebral cortex and cerebellum cytosols (Figure 1), Similarly, displacement with unlabelled PDBu showed virtually identical potency in lung, frontal cerebral cortex and cerebellum cytosols (IC_{50}s of 6 ± 1, 5 ± 2 and 7 ± 1 nM respectively, n = 4). However, diglycerides with saturated acyl chains generally showed lower potency than the unsaturated analogues investigated. For the saturated analogues, there was a relationship between chain length and affinity for the binding site that was biphasic. This was consistent with previous findings [15-17] where this biphasic action was attributed to hydrophilicity of the acyl chain and to the optimal fit at the diacylglycerol-binding site within PKC. The IC_{50} values for any particular diacylglycerol were always similar in frontal cerebral cortex and cerebellum cytosols, but the binding sites in lung cytosol

lisplayed markedly lower affinity for all the saturated diglycerides (Figure 1). The reduced affinity in lung was most marked for saturated chain lengths of 6-10 carbons, with 1,2-dioctanoyl-*sn*-glycerol (DOG) having the greatest separation in affinity between frontal cerebral cortex/cerebellum and lung cytosols (Figure 1). The reduced affinity for the diglycerides in lung cytosol was not due to their degradation in lung only, as DOG preincubated for 30 min (37°C) in either lung or frontal cerebral cortex or cerebellum cytosol had the same high affinity when subsequently tested on binding to cytosol from frontal cerebral cortex and cerebellum (data not shown).

Figure 2(a) demonstrates the differences in affinity for DOG towards lung, frontal cerebral cortex and cerebellum cytosolic PKC. The binding method employed in these experiments was slightly different in that DOG was added to the assay dissolved in dimethylformamide. Using this altered method, the IC_{50}s of DOG in frontal cerebral cortex and cerebellum were 21 ± 1 and 20 ± 2 μM respectively (n = 6), whereas the IC_{50} in lung cytosol was over 80 fold greater. No marked difference could be seen when comparing the affinity of *sn*-DOG and a *rac*-DOG mixture on specific [^{3}H]-PDBu binding, nor did 500 μM DOG or AA have any effect on non-specific binding levels (data not shown).

Figure 2(b) shows that increasing concentrations of AA (2 - 500 μM) enhanced the specific binding in cytosol from lung, but not frontal cerebral cortex and cerebellum (159 ± 9, 109 ± 7 and 110 ± 2% of control specific binding with 500 μM AA respectively, n = 4). A marked enhancement of binding was also seen in sciatic nerve, kidney, COS 7 cell and GH_3 cell cytosols with 500 μM AA (238 ± 40, 39 ± 5, 210 ± 20 and 141 ± 10% of control specific binding respectively, n = 4). The enhancement of binding seen in lung cytosol with AA was mimicked to a lesser extent by linoleic acid and AA-methyl ester, but not by arachidic acid (90 ± 7, 0 ± 4 and 2 ± 2% respectively of the effect of AA at the same concentration 500 μM), n = 4). These structural requirements match those described by Seifert et al, [18] for PKC activation by AA analogues in whole brain cytosol. The enhancement of [^{3}H]-PDBu binding seen at 500 μM AA was not inhibited by incubation with 5 mM EGTA (no Ca^{2+}) included in the experiment (data not

hown) indicating the lack of direct Ca^{2+}-dependency of binding under the present conditions.

Blumberg and his co-workers described a non-competitive mechanism for displacement by AA of [^{3}H]-PDBu binding to mouse brain cytosol [8,9] but only under modified assay conditions of low phosphatidylserine concentrations (particularly at 20 µg/ml phosphatidylserine) which are extremely submaximal for allowing [^{3}H]-PDBu binding to cytosolic PKC [4]. At higher phosphatidylserine concentrations (200 µg/ml) Leach and Blumberg [9] found much weaker displacement of [^{3}H]-PDBu binding by AA. When we altered our assay to match their conditions (and reduced the phosphatidylserine concentration from 1 mg/ml to 750 µg/ml), we too observed a modest inhibition by AA, with specific binding to cytosol from lung, frontal cerebral cortex and cerebellum being reduced by 500 µM AA to 82 ± 6, 65 ± 15 and 56 ±16% of control specific binding respectively (n = 3). Whilst there is clearly a close relationship between the site for phosphatidylserine recognition and that mediating AA-inhibition of [^{3}H]-PDBu binding, we found that AA (0.5 - 500 µM) was unable to markedly substitute for phosphatidylserine in enabling [^{3}H]-PDBu binding to cytosolic PKC. Thus it appears that two different influences of AA on PKC may be possible; firstly an inhibitory influence on the phosphatidylserine recognition site and secondly (but only in certain tissues; Figures 2(b) and 3(b)) a facilitatory influence on [^{3}H]-PDBu binding mediated by some distinct site.

Figure 3 illustrates the varying degree to which DOG (a) and AA (b) modify specific [^{3}H]-PDBu binding throughout all the tissues investigated. In each case a relationship can be discerned with the proportion of cytosolic PKC which is of the α isoform [12-14]. The correlation between low potency of DOG and α-content is reasonable (correlation co-efficient = 0.845, Figure 3(a)). Experiments with purified α-PKC [17] and on the partially purified α-PKC from COS 7 cells (our unpublished observations) confirm that under certain conditions, DOG is an extremely poor activator of α-PKC in particular. Although the correlation between the AA effect and α content is less close (Figure 3(b)), it is only those tissues with high α content that show a marked enhancement by AA. In this case, other factors may contribute and it is important to note that the

PKC-isoform content of the tissues reported [12-14] represents only the α-, βI-, βII- and γ-isoforms of PKC and does not take into consideration the more recently-discovered δ-, ε- and ζ-isoforms of PKC [6]. Therefore caution must be exercised when attempting to assign an isoform-identity to any result. Although γ-PKC is more potently activated by AA than either α- or β-PKC [7], rat cerebellum cytosol, a region enriched in the γ-isoform, shows no marked enhancement of binding by AA (Figure 2(a)) and the regions which showed enhancement of binding with AA (sciatic nerve, lung, kidney, COS 7 cell and GH_3 cell cytosols) are devoid of γ-PKC [12-14]. Thus, neither the degree of activity of DOG nor that of AA could be correlated with either the β or γ isoform content.

The present data suggest that DOG will act preferentially on PKC isoforms other than the α-isoform. A calcium-dependent isoform of PKC which is insensitive to DOG, but sensitive to AA has been implicated in the inhibition of K^+-induced calcium influx into GH_3 cells [19]. The PKC isoform responsible is not γ-PKC as it is absent from these cells, and therefore the responsible isoform may well be α-PKC. In contrast, the facilitation by phorbol esters of K^+-induced calcium influx into pituitary prisms was mimicked by DOG (but not by AA) [20] suggesting the involvement of a PKC isoform other than α. Supportive of our findings is previous work in which translocation/activation of α-PKC in U937 cells was induced by treatment with phorbol ester but not by DOG (up to 100 μM) [21]. In myocytes, phorbol esters are able to both increase and decrease 'L'-type Ca^{2+}-channel activity, however DOG only mimics the phorbol ester at enhancing channel activity, being ineffective at the inhibitory modulation of the channel [22]. These selective effects of DOG in myocyte-Ca^{2+}-channel studies may be due to ineffectiveness of DOG at the PKC-isoform which has been implicated in reducing 'L'-type Ca^{2+}-channel activity in other cells *in vitro* [20].

It thus seems likely that not only in cell-free assays but also under more physiological conditions, short chain diacylglycerols such as DOG could display a significant degree of selectivity against activation of α-PKC; a factor which should be taken into consideration in the interpretation of results.

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EFERENCES

.] Ashendel, C.L., Staller, J.M. and Boutwell, R.K. (1983) Cancer Res. 43, 4333-4337.

2] Niedel, J.E., Kuhn, L.J. and Vanderbark, G.R. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 36-40.

3] Sando, J.J. and Young, M.C. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 2642-2646.

4] Leach K.L., James, M.L. and Blumberg, P.M. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 4208-4212.

5] Blumberg, P.M. (1988) Cancer Res. 48, 1-8.

6] Nishizuka, Y. (1988) Nature 334, 661-665

7] Naor, Z., Shearman, M.S., Kishimoto, A. and Nishizuka, Y. (1988) Mol. Endocrinol. 2, 1043-1048

8] Sharkey, N.A. and Blumberg, P.M. (1985) Biochem. Biophys. Res. Commun. 133, 1051-1056.

9] Leach, K.L. and Blumberg, P.M. (1985) Cancer Res. 45, 1958-1963.

10] Murakami, K. and Routtenberg, A. (1985) FEBS lett. 192, 189-193.

11] Murakami, K., Chan, S.Y. and Routtenberg, A. (1986) J. Biol. Chem. 261, 15424-15429.

12] Shearman, M.S., Kosaka, Y., Ase, K., Kikkawa, U. and Nishizuka, Y. (1988) Biochem. Soc. Trans. 16, 307-308.

13] Kosaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U. and Nishizuka, Y. (1988) Biochem. Biophys. Res. Commun. 151, 973-981.

14] Naor, Z. (1990) Endocrinol. 126, 1521-1526.

15] Ebeling, J.G., Vanderbark, G.R., Kuhn, L., Ganong, B., Bell, R. and Niedel, J. (1982) Proc. Natl. Acad. Sci. U.S.A. 82, 815-819.

16] Hannun, Y.A., Loomis, C.R. and Bell, R.M. (1986) J. Biol. Chem. 261, 7184-7190.

17] Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U. and Nishizuka, Y. (1988) J. Biochem. 103, 759-765.

18] Seifert, R., Schachtele, C., Rosenthal, W. and Schultz, G. (1988) Biochem. Biophys. Res. Commun. 154, 20-26.

9] MacEwan, D.J., Mitchell, R., Thomson, F.J. and Johnson, M.S. (1991) Biochim. Biophys. Acta (in press).

.0] MacEwan, D.J. and Mitchell, R. (1991) FEBS Lett. (in press)

.1] Strulovici, B., Daniel-Issakani, S., Oto, E., Nestor, J. Jr., Chan, H. and Tsou, A.-P. (1989) Biochem. 28, 3569-3576.

.2] Lacerda, A.E., Rampe, D. and Brown, A.M. (1988) Nature 335, 249-251.

ure 1. The relationship between acyl chain length of diacylglycerols and inity for [^{3}H]-PDBu binding sites in lung, frontal cerebral cortex and ebellum cytosols.

e IC_{50} values for displacement of specific [^{3}H]-PDBu binding were calculated as scribed in the Experimental section for a range of 1,2-diacylglycerols in lung (■), ntal cerebral cortex (●) and cerebellum (▲) cytosols. The diacylglycerols shown e those with saturated acyl chains: 1,2-dihexanoyl-*sn*-glycerol (6); -dioctanoyl-*sn*-glycerol (8); 1,2-didecanoyl-*rac*-glycerol (10); 1,2-dilauroyl- -glycerol (12); 1,2-dimyristoyl-*rac*-glycerol (14); 1,2-dipalmitoyl-*sn*-glycerol (16); 2-distearoyl-*rac*-glycerol (18) and those with unsaturated acyl chain nposition: 1,2-dioleoyl-*sn*-glycerol (18:1) and 1-stearoyl-2-arachidonoyl- -glycerol (18:0/20:4). The diacylglycerols were added to the assay as mixed celles. The data represent the means ± S.E.M. from 4 determinations.

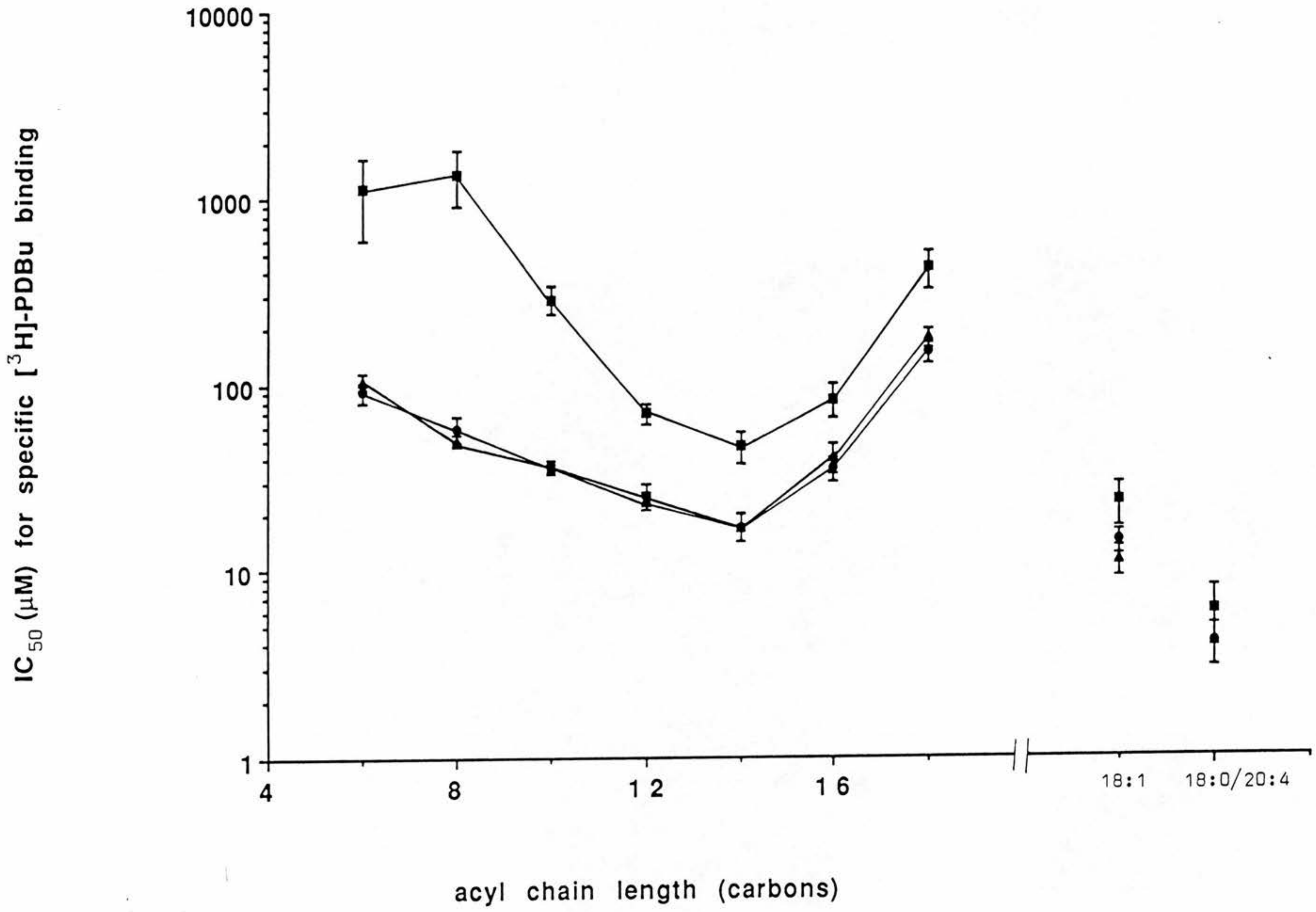
gure 2. Regional differences in displacement by 1,2-dioctanoyl-*sn*-glycerol and hancement by arachidonic acid of cytosolic [^{3}H]-PDBu binding

e effect of 1,2-dioctanoyl-*sn*-glycerol (DOG(a)) and arachidonic acid (AA(b)) on ecific binding in lung (■), frontal cerebral cortex (●) and cerebellum (▲) cytosols.)G was added to the assay dissolved in dimethylformamide whereas AA was ded to the assay in mixed micelles. The data represents the means ± S.E.M. e values expressed in (a) are from 6 determinations, whereas the values in (b) e from 4 determinations.

gure 3. Relationship between tissue content of α-PKC and the effects of -dioctanoyl-*sn*-glycerol and arachidonic acid on cytosolic [^{3}H]-PDBu binding.

e mean values for displacement by DOG in (a) and the enhancement by AA in of specific cytosolic binding is shown for: cerebellum (data point 1); frontal ebral cortex (2); spleen (3); spinal cord (4); testes (5); anterior pituitary (6); liver ; kidney (8); lung (9); GH_3 cell (10); sciatic nerve (11) and COS 7 cell (12) tosols. Responses are expressed as the % change from control specific binding the presence of 500 μM *sn*-DOG (added in dimethylformamide) or 500 μM AA dded as mixed micelles). Full concentration-response curves were always ermined but the data at a single concentration is quoted because IC_{50} and EC_{50}

concentrations were not reached in some tissues. The data represent the mean ± S.E.M. of 4 - 6 determinations.


IC50 (µM) for specific [3H]-PDBu binding
10000
1000
100
10
1
4
8
12
16
18:1
18:0/20:4
acyl chain length (carbons)

(a)

% of control specific [^{3}H]-PDBu binding

DOG (μM)

(b)

% of control specific [^{3}H]-PDBu binding

AA (μM)

Figure 3. MacEwan et al

(a)

binding remaining at 500 μM DOG

100
80
60
40
20
0

(11)
(7) (8)
(12)
(9) (10)
(5)
(6)
(4)
(3)
(1) (2)

0 20 40 60 80 100

% alpha-PKC