The biochemical mechanisms of action of ~~the~~ TIGLIANE AND phorbol esters.

Submitted by
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for the degree of
Doctor of Philosophy,
University of London, 1991.

Abstract.

Initial studies adding TPA and Sap A to Human Mononuclear cell(HMNC) cultures found that the mitogenic action of Sap A was abolished if cells were incubated for 3 days prior to phorbol ester addition.Previously the phorbol ester receptor has been found to be a family of protein kinases (PKC's).The relationship between biochemical action and biological effect was investigated using rat brain as the enzyme source.Phorbol ester-stimulated kinase activities in rat brain hydroxylapatite FPLC fractions were investigated using a modified PS/Triton micellar assay.Proteins in the elution profile were separated by SDS-PAGE and immuno-blotted with antisera specific for PKC $\alpha,\beta 1,\gamma,\delta$ and ε to identify the PKC isotypes present.These investigations were coordinated with phorbol ester activation studies on PKC isotypes ($\alpha,\beta 1,\gamma,\delta$ and ε) purified from bovine brain.This methodology was then applied to screen eluted proteins from subpopulations of HMNC as well as Daudi cells and mouse macrophages.

These approaches revealed several phorbol ester-sensitive protein kinase activities,which could not be identified as PKC $\alpha,\beta 1$, γ,δ or ε isotypes by elution or immunological definition,in crudely fractionated brain and cell extracts.Some of these activities demon-strated tissue specificity when elution positions characteristic of brain were compared to those seen for cultured cells.Prepar-ations of HMNC's were also found to contain . . . *(Continued overleaf)*

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(cond) . . . .an entirely novel kinase activity which was stimulatable by Resiniferatoxin (Rx, a daphnane ester) only in the absence of added calcium (termed Rx-Kinase).A similar activity was also isolated,in greater quantity,from starch-elicited mouse peritoneal macrophages under identical conditions.

Fractions of mouse Rx-Kinase activity were found to potently activate the reconstituted mouse NADPH oxidase system to generate superoxide *in vitro* in the presence of Rx and the absence of calcium (*i.e.* conditions corresponding to it's histone kinase requirements *in vitro*).This suggests major differences between this putative phorbol ester receptor and the PKC family of isotypes.

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Glossary.

A23187 = A calcium ionophore.
AA = Arachidonic acid.
ADP = Adenosine diphosphate.
ATP = Adenosine triphosphate.
cAMP = Cyclic Adenosine monophosphate.
β-cell = β-lymphocytes.
βγ complex = The βγ-dimer of a G-protein.
βMe = β-Mercaptoethanol.
BSA = Bovine serum albumin.
C-1,C-2,etc.. = Carbon 1,Carbon 2,etc..
Ca^{2+} = Calcium ion.
(Ca)i = Intercellular calcium.
CON A = Concanavalin A.
CNS = Central nervous system.
DAG = Diacylglycerol.
Daudi (WT) = Wild type Daudi (transformed β-cell line)
Daudi (TPA-WT) = TPA-treated Daudi cells.
Daudi (αIR-WT) = α-Interferon-treated Daudi cells.
Daudi (αIR Resist) =α-Interferon resistant Daudi cells.
DEAE = Diethylaminoethyl-cellulose.
DiC_8 = 1,2-sn-Dioctanoylglycerol.
Diol = A racemic mixture of 1,2-and 1,3-Dioleoylglycerol.
DNA = Deoxyribonucleicacid.
DTT = Dithiothreitol.
EDTA = Ethylene diamine tetraacetic acid.
EGF = Epidermal growth factor.
EGTA = Ethylene glycol bis (β-aminoethyl ether)-N,N,N',N',-tetraacetic acid.
FCS = Foetal calf serum.
FDGF = Fibroblast derived growth factor.
fMLP = N-formyl-methionyl-leucyl-phenylalanine.
FPLC = Fast protein liquid chromatography.
GDP = Guanosine diphosphate.
Gi = Inhibitory G-protein.
cGMP = Cyclic Guanosine monophosphate.
G-Protein = GTP-binding regulatory protein (α,β and γ subunits).
Gs = Stimulatory G-protein.
GTP = Guanosine triphosphate.
H^+ = Hydrogen ion.
HCl = Hydrochloric acid.
H7 =1-(5-isoquinoline sulphonyl)-2-methyl piperazine (dihydro chloride).
HEPES = 4(2-hydroxyethyl)-1-piperazine ethane sulphonic acid.
HL-60 = Human leukemic cell line.
HMBA = Hexamethyl bisacetamide.
HMNC = Human mononuclear cells from blood.
HPLC = High pressure liquid chromatography.
Ig = Immunoglobulin.
IL-2 = Interleukin 2.
IL-2R = Interleukin 2 receptor.
IP_3 = Inositol-1,4,5-triphosphate.
IP_4 = Inositol-1,3,4,5-tetrakisphosphate.
IP_5 = Inositol-1,3,4,5,6-pentaphosphate.
IP_6 = Inositol hexaphosphate.
K^+ = Potassium ion.
Kd = Kilodaltons.
LTP = Long-term potentiation.
Lyso-PC =Lysophosphatidylcholine.
M = Molar.

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Glossary(cond).

MAP-2 = Microtubule associated protein 2.
Mr = Relative molecular mass.
mRNA = Messenger ribonucleicacid.
N-protein = The α-subunit of a G-protein.
Na^+ = Sodium ion.
NaCl = Sodium chloride (salt).
O_2^- = Superoxide.
OAG = 1-sn-Oleoyl-2-acetylglycerol.
P_{32} = Radiolabled phosphate group.
PA = Phosphatidic acid.
PBS = Phosphate buffered saline (NaCl/Pi).
PC = Phosphatidylcholine.
PDGF = Platelet derived growth factor.
PGE_2 = Prostaglandin E_2.
PHA = Phytoheamagglutanin.
Pi = Inorganic phosphate.
PI = Phosphatidylinositol.
PIP = Phosphatidylinositol-4-phosphate.
PIP_2 = Phosphatidylinositol-4,5-bisphosphate.
PKA = cAMP-dependent protein kinase.
PKC = Protein kinase C.
PKCal = Calmodulin dependent protein kinase.
PKG = cGMP-dependent protein kinase.
PKM = Proteolytic fragment of PKC.
PKTyr = Tyrosine specific protein kinase.
PLA_2 = Phospholipase A_2.
PLipC = Phospholipase C.
PMSF = Phenyl methyl sulphonyl flouride.
pp68 = 68 Kd product of the *ros* oncogene in virally transformed cells (v-*ros*),or normal cells (c-*ros*).
pp60 = 60 Kd product of the *src* oncogene in virally transformed cells (v-*src*),or normal cells (c-*src*).
PS = Phosphatidylserine.
PWM = Pokeweed mitogen.
PYS = Parietal yolk sac.
S.E. = Sheep erythrocytes.
S.E.M = Stardard error of the mean.
SDS/PAGE = Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
StMM = Starch-elicited mouse macrophages.
T-cell = T-lymphocytes.
TCA = Trichloracetic acid.
TEMED = N,N,N',N',Tetra methyl ethylene diamine.
TFP = Trifluoroperazine.
Tris = Tris(hydroxymethyl)aminomethane.
TRE = TPA responsive element.
TSH = Thyroid stimulating hormone.
W7 = N-(6-aminohexyl) 5-chloro-1-naphthalene sulphonamide.

Phorbol ester abbreviations used.
4 α PDD = 4 α phorbol-12,13-didecanoate.
DOPP = 12-Deoxyphorbol-13-phenylacetate.
Doppa = 12-Deoxyphorbol-13-phenylacetate-20-acetate.
PdBu = Phorbol-12,13-dibutyrate.
Rx = Resiniferatoxin = 9,13,14-Orthophenylacetyl resiniferonol-20-(3-methoxy,4-hydroxy)-phenylacetate.
Sap A = Sapintoxin A =12-O-(2-methylaminobenzoyl)-4-deoxyphorbol-13-acetate.
Thy Tox A = Thymeleatoxin A = 9,13,14-Orthobenzoyl-6,7-epoxy, 5-hydroxy resiniferonol-12-cinnamate.
TPA = 12-O-Tetradecanoyl phorbol-13-acetate.

Dedication.

To Dad who planted the seed,
Mum who nurtured it,
The family who shaped it,
Kings for the water,
The Fellows for the sun,
Edinburgh for the spring,
To Fred and colleagues for the pruning,
And to Anne who owns its heart.

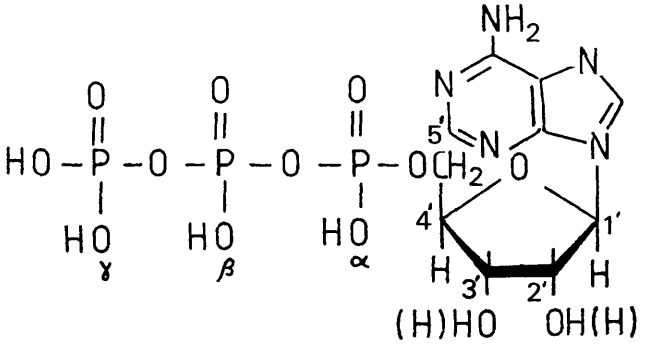
CHAPTER 1

SECTION A: PROTEIN KINASE C

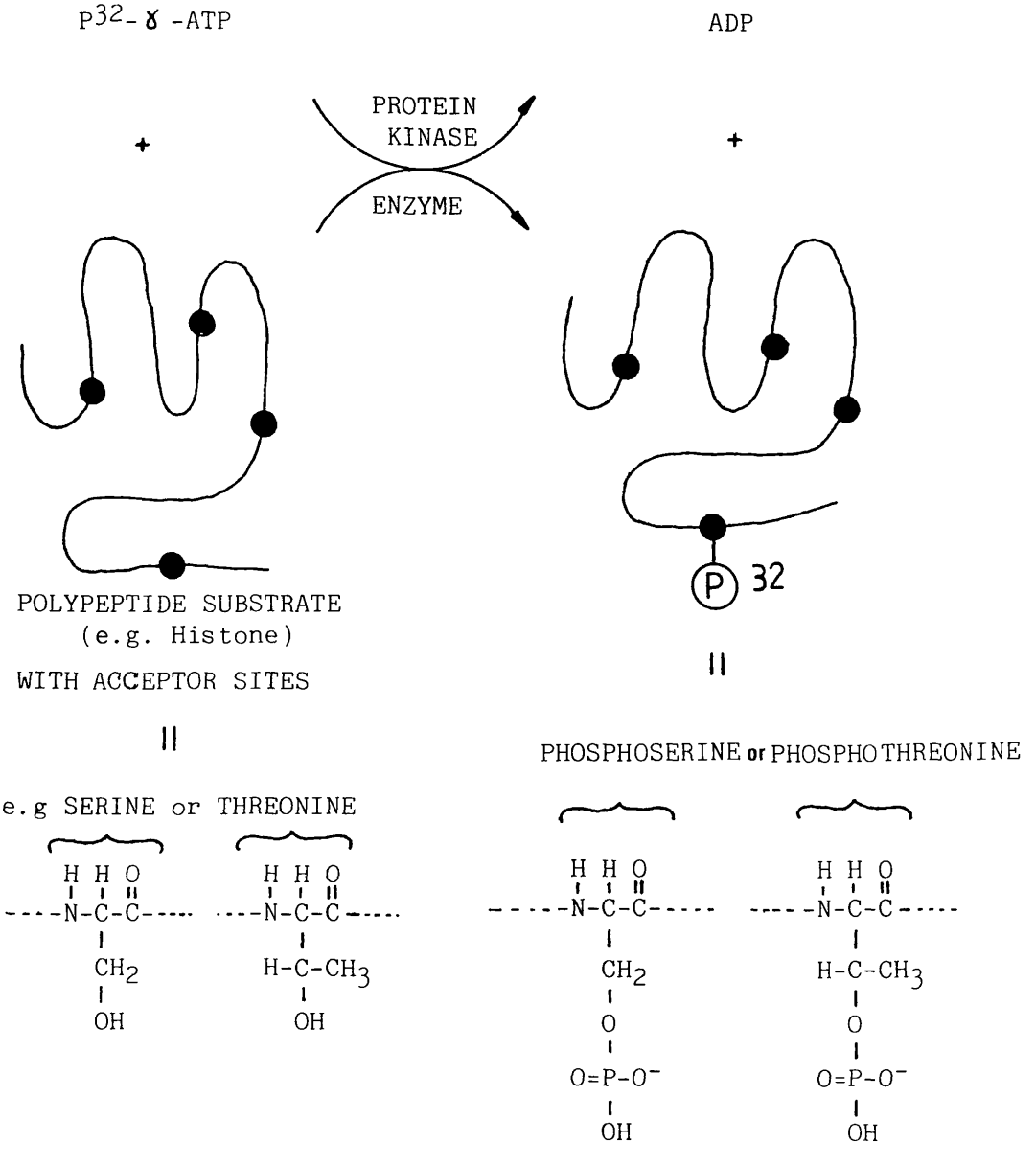
The discovery by Inoue & colleagues in 1977 of a novel protein kinase enzymatic activity in rat brain *(1,2)* opened up a field of biochemistry which has been the subject of intense investigation since that date. The protein kinase activity was produced from a larger pro-enzyme by incubation of samples with the protease Trypsin or an endogenous calcium-dependent neutral thiol protease (termed Calpain). It was assayed by measurement of radiolabled γ-Phosphate^{32} transferred from radiolabled P^{32} - γ - Adenosine Triphosphate (ATP) onto calf thymus whole histone (protein) preparation *(See Fig. 1a)*. In the proenzyme form the kinase could phosphorylate Salmon Protamine (protein) as effectively as the proteolysed fragment, but was inactive on other substrates, and neither cyclic Adenosine Monophosphate (c-AMP) or cyclic Guanosine Monophosphate (c-GMP) affected the reactions, indicating the enzyme was distinct from both cyclic AMP - dependent protein kinase (PKA) and cyclic GMP-dependent kinase (PKG) known at that time. Partial purification, achieved by chromatography of crude extracts through a Diethylaminoethyl (DEAE) cellulose column, revealed two peaks of proenzyme present which density gradient ultracentrifugation showed to have an estimated molecular weight of 77,000 daltons (77 Kd). The active fragment, termed Protein kinase M(PKM) had a molecular weight of between 51Kd and 64 Kd depending on the conditions and type of protease used. Further work *(3)* showed that the proenzyme could be

Fig 1a. Radiolabled-phosphate transfer from ATP (a) onto an acceptor protein by protein kinase C (b).

(a) P^{32}-γ ADENOSINE TRIPHOSPHATE (P^{32}-γ-ATP)



(b) PHOSPHATE TRANSFER BY PROTEIN KINASE ACTION



activated without proteolysis by reversible attachment to membrane phospholipids in the presence of calcium. The addition of Diacylglycerols (e.g. Diolein a racemic mixture of 1,2 and 1,3 Dioleoylglycerols) was found to increase this affinity activation *(4)*. The calcium-activated phospholipid-dependent protein kinase was termed Protein kinase C (PKC).

Many techniques for purification of PKC have been developed. One widely used method, developed by Kikkawa & colleagues *(5,6)* employs several sequential chromatography steps where a crude supernatant is first applied to DEAE-52 cellulose (Anionic exchange) column, activity eluting at a concentration of ~0.2M NaCl buffers. Pooled peaks undergo Sephadex g-150 chromatography then isoelectric - focusing electrophoresis (in an ampholyte pH range of 5 to 7 and a sucrose density gradient of 0-50% w/v) where the PKC activity focuses at pH 5.6. Active fractions are then applied to a Blue-Sepharose column where PKM is separated from PKC (0.75 and 1 M NaCl respectively) and the latter is finally applied to a Phenyl-Sepharose column where a decreasing NaCl gradient elutes PKC as a single peak. On Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) the PKC appears as a single band of apparent molecular weight of 82Kd. This method was later modified to increase the purity and speed of the final preparation by using Threonine-Sepharose, T.S.K. gel Phenyl-5PW, and TSK gel G3000 sW chromatography sequentially after the DEAE-52cellulose step, employing High Performance Liquid Chromatography (HPLC) automated apparatus (7). SDS-PAGE showed that the PKC, although appearing to be a single band with coomasie blue

stain, had some contaminant protein bands when sensitive silver staining techniques were used, and some preparations showed a duplex PKC stain. Other techniques have employed ATP in the buffers which changed the eluting characteristics of PKC sufficiently to enable separation from contaminant proteins *(8)*. Use of the binding ability to phospholipids has also been utilised to separate PKC, where the DEAE cellulose peak is either, applied to a column of phosphatidylserine (PS) immobilized in Polyacrylamide gel in the presence of calcium and eluted with a calcium chelating agent *(9)*, or mixed with PS/Diolein liposomes in a high calcium buffer applied to a gel filtration column and eluted with a lower calcium buffer *(10,11)*. Studies on PKC distribution in the animal kingdom have established its presence in the Annelid, Mollusc, Arthropod and Chordate Phyla *(12)*. PKC has been found in a wide variety of mammalian tissues in varying quantities *(5,13,14,15)*. Platelets, spleen and lymphocytes contain large activity levels as well as brain, with less observed in lung, kidney, liver, heart, muscle, granulocytes and adipose tissue. In certain tissues, such as spleen and brain, PKC activity was found to be present at much higher levels than PKA or PKG and different regions of the brain exhibited marked variation. Additionally PKC has been found in erythrocytes (16) and a similar enzyme has been isolated from plant tissue *(17,18)*.

The sub-cellular distribution of PKC was also found to vary, with activity equally divided in rat brain between soluble (cytosolic), insoluble (membranes) and mitochondrial fractions *(5)*. The mitochondrial fraction was found to be associated with synaptosomal

membranes in brain, but rat liver mitochandria also contain the enzyme *(19)*. The appearance of PKC in Nucleii and subnuclear fractions has also been reported in rat liver *(20,21)*. Other studies showed PKC to be either predominantly cytosolic (e.g. heart), equally distributed in the cytosol and particulate membrane fractions (e.g. spleen) or mostly particulate (e.g. brain) *(22)*. Furthermore a developmental variation was noted in rodent brain *(14)* with little PKC activity in embryonic animals, an increase and a peak in levels at 2-4 weeks after birth, declining to a steady (adult) state. thus these early studies suggested a fundamentally important role for PKC in cells and tissues and prepared a platform from which research mushroomed.

The proteolytic susceptibilty of PKC, has been investigated in an effort to elucidate the physiological significance of PKM generation in a cell. PKM was found to be unaffected by phospholipid-interacting drugs *(See Section D2)* which inhibit PKC *(23)* and to be wholly independent of calcium, phospholipid and diacylglycerol *(24)*. At least two Calpain proteases were found to cleave PKC in this fashion, Protease-I activity was enhanced when PKC was present together with the co-factors phospholipid and diacylglycerol and required micro-molar levels of Calcium for activity, whereas protease -II activity was unaltered by these co-factors and required higher (milli-molar) calcium for activity. Trypsin was found to cleave PKC into a 32Kd fragment which exclusively bound Phospholipid, suggesting it was the co-factor binding 'regulatory domain', and PKM a 50Kd fragment *(25)*. Since PKM was found to be soluble in addition to

being co-factor independent *(26)* the involvement of Calpain has been proposed to function physiologically as either (A) liberating PKC activity into the cell cytosol the amplifying the activity or (B) negatively regulating the PKC activity by repartitioning (possibly channeling the PKM into further proteolytic degradation and ultimate elimination).

Early in vivo investigation of the proteolysis employed antibodies raised to the homogenous enzyme preparation and confirmed the calcium activated proteolysis of ~80Kd PKC in brain into two fragments *(27)* and the existence of both the 82Kd and 67Kd species in mouse β- lymphocytes and skin extracts *(28,29)*. Immunoblotting on crude extracts from human leukemic cell lines (HL-60 and K562) however, found PKM to be the most abundant form *(22)* and have a Mr of 50Kd rather than 67Kd found in brain and spleen. Rat liver nuclear and sub-nuclear fractions had a doublet of 77Kd and 74Kd, where rat brain in these same gels showed 80Kd and 67Kd species *(21)*. Thus PKC and its proteolytic fragment are found in vivo exhibiting differing proportions and mocecular weights between different cell types.

SECTION B1: THE PHOSPHOINOSITIDE SIGNALLING CYCLE

One of the very early responses of cells to a variety of stimuli has been found to be the hydrolysis ("turnover") of phosphoinositides (PI), a class of lipids, in the target cell membranes *(30)*. In many cases the agonists did not appear to generate their biological effects through cyclic nucleotides but a role for an intracellular calcium rise did feature as important. The activation of PKC by diacylglycerols and calcium suggested an intimate coupling of these membrane events and it was proposed that the diacylglycerol was generated by PI hydrolysis *(31,32 and See Fig. 1b)*.

Work on the metabolism and interconversion of phosphoinositides revealed a battery of phospho-monoesterases, -diesterases and -kinases at work *(33,34)*. Kinetic analysis showed that phosphatidylinositol 4,5 bisphosphate *(PI4,5 P_2)* was effectively hydrolysed by agonist-receptor coupling, since inositol 1,4,5 trisphosphate *(IP_3)* appeared to increase first, closely followed by the appearance of inositol 1,4 bisphosphate (Ins 1,4 P_2) *(See Fig. 1c)*. Possibilities remained however that other inositol phospholipids (e.g. phosphatidylinositol (PI) and phosphatidylinositol - 4 - phosphate (PI_4P)) were also broken down during receptor-mediated hydrolysis followed by dynamic phosphate exchange resulting in interconversion of forms *(Reviewed 35)*. Alternatively, individual inositol phospholipid pools could be the source of diacylglycerols and inositol phosphates in different biological contexts which could be achieved by enzymatic

Figure 1b GENERATION OF DIACYLGLYCEROL FROM PHOSPHATIDYL INOSITOL AND MECHANISMS OF REMOVAL

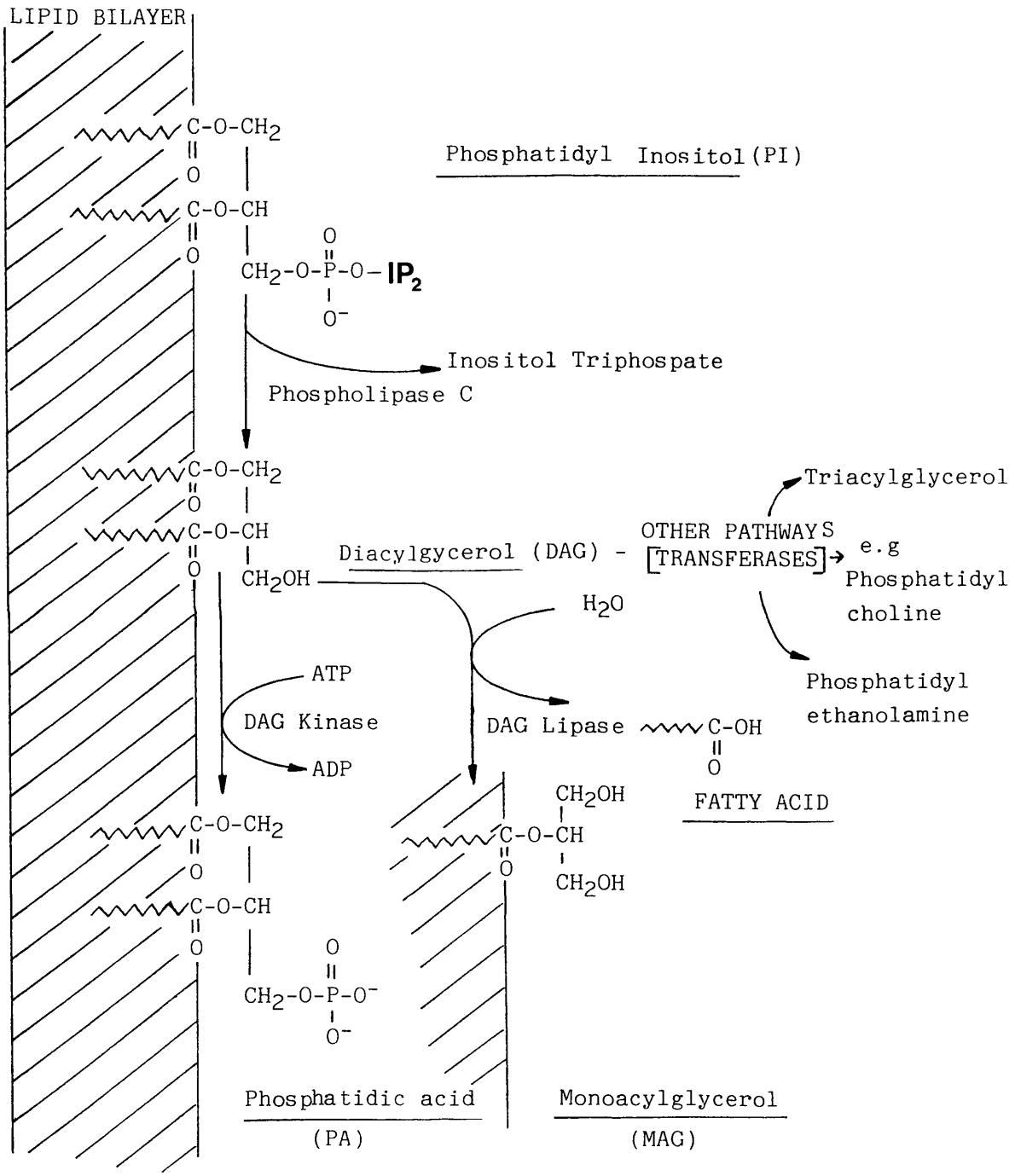
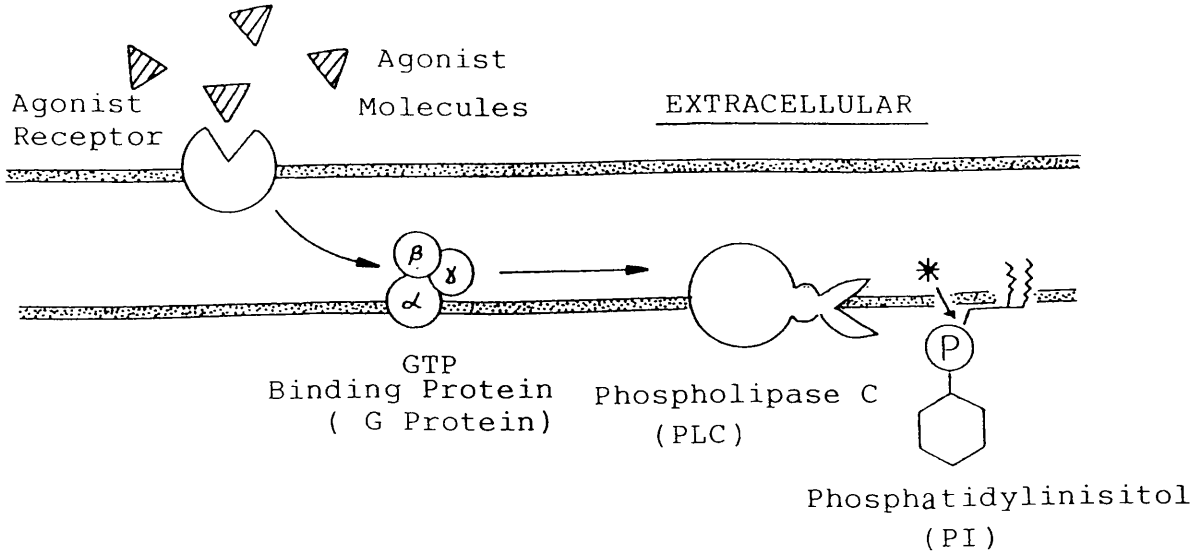


Fig 1c. Turnover of Phospatidylinositol (the P I Cycle).(from ref 34)

(a) The pathway from extracellular hormone agonist (primary messenger) through its membrane-bound receptor.The G-Protein activated by an active agonist/receptor splits, complex binds GTP,and activates a phospholipase C.The action of this type of phospholipase cleaves phosphatidylinositides into diacylglycerols and inositol phosphates (secondary messengers) on the cytosolic surface of the cell membrane.



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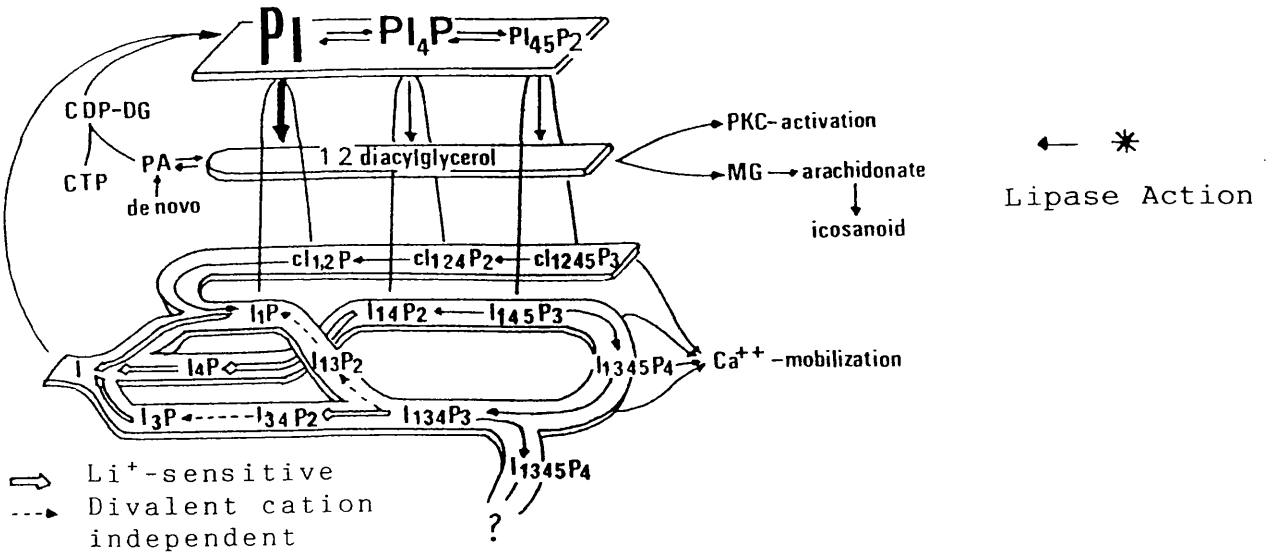
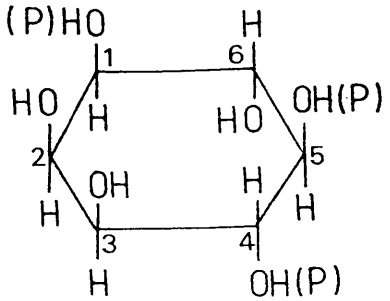


(b)Pathway for inositol phosphate metabolism. TOP = PI indicates phosphatidylinositol;PI_4P, phosphatidylinositol-4-phosphate;$PI_{4,5}P_2$ phos -phatidylinositol-4,5-bisphosphate.BOTTOM = I,inositol;P,phosphate;The numbers before the P refer to the phosphate position on the inositol ring,and after the P to number of these groups, PA,phosphatidic acid;CPP-DG,cytidine diphos-phate diacylglycerol;PKC,protein kinase C.



(c) Myo-Inositol.Numbering for the D isomer is shown above . Positions that contain phosphate esters in the precursor lipid is indicated by (P).The phosphodi-ester linkage to diacylglycerol is at position 1.

specialization. The rapid appearance (5 seconds) and removal of the soluble inositol phosphates lend these molecules the attributes of physiological "messenger" molecules and the connection between agonist-receptor interaction and calcium mobilization was postulated *(36,37)*.

Evidence in support of this came when IP_3 was found to elicit release of calcium from the endoplasmic reticulum compartment from various permeabilized cells. Binding of radiolabled IP_3 to membranes could be inhibited by free calcium, suggesting that IP_3 could have a role in calcium homeostasis *(38)*. However cells do differ in their main source of calcium elicited by stimuli, with many (e.g. Adrenal medullary, nerve, leukemic 2H3 and mast cells), relying on extracellular calcium whereas others (e.g. hepatocyte, pancreatic and parotid gland cells and platelets) use intracellular stores *(Reviewed 39,40)*. Candidate agents for mediating extracellular intake mechanisms range from the agonist-receptor complex itself, phosphatidic acid (acting as a calcium ionophore) or inositol lipid breakdown products, although it is possible that inhibition of proteins pumping calcium out of the cell may be involved in a dynamic system. Recent work has implicated inositol 1,3,4,5 tetrakisphosphate (IP_4) in membrane calcium flux and inositol 1,3,4,5,6 pentaphosphate (IP_5) and inositol hexaphosphate (IP_6) may have a neurotransmitter function *(41,34)*.

The other product of PI hydrolysis, diacylglycerol, has its own messenger function activating PKC and is rapidly and transiently

produced concommitantly *(Reviewed 40,42,43)*. Removal is effected by the action of DAG kinase (producing phosphatidic acid (PA)), Acyltransferases *(44)* and DAG lipase generating monoacylglycerols and fatty acid products from the cleaved tail *(35, and See Fig. 1b)*. The fatty acid most commonly produced is arachidonic acid (AA) which can be generated from PA in platelets *(45)*, or from other phospholipids by Phospholipase A_2 (PLA_2) action. This is of interest since AA itself has been implicated in modulating PKC activity *(See Section D1 and 46)*, and is released into the medium and converted to eicosanoid metabolites (themselves bioactive molecules) in many cells in response to PI turnover *(37,47,48)*. Thus the theory of a bifurcating messenger system has been emerging with PKC activation by diacylglycerol forming one 'arm' and calcium mobilization by inositol phosphate(s) forming the other, both engaged from the same source *(Summarized in Fig. 1c)*. Some agonists stimulating the PI cycle are shown in Table 1A.

Table 1A. Agonists found to stimulate P.I. turnover.

P.I. cycle agonist	Tissue/Cell system	Non-P.I. overlap evident	Refs.
Adenosine diphosphate (ADP)	Platelets.	?	49,but see 50
Adenosine triphosphate (ATP)	Liver.	?	49
Acetylcholine	Smooth muscle,parotid, pancreas,neural and exocrine tissues.	Yes	42,51
Adrenalin	Liver,adipose,neural and exocrine tissues.	Yes	32,51,52, 49
Adrenocorticotrophic hormone (ACTH)	Adrenal cortex.	?	42
Angiotensin 2	Liver,Adrenal cortex.	Yes	49,52
Antibodies to;			
CD2 antigen	T-lymphocytes.	?	53
Ti/CD3 antigen	T-lymphocytes.	Yes	53,54,91
Ig complex	β-lymphocytes.	Yes	55
Antigen	Basiphil leukemia cells.	?	40,49
Bombesin	Fibroblasts,pancreas.	possible	56,57
Bradykinin	Heart endothelia,neuro-blastoma.	?	42,49
Caerulein	Smooth muscle,pancreas.	?	49,51
Cholecystokinin	Neurones.	?	39,58
Collagen	Platelets.	?	42
Embryonal carcinoma-derived growth factor	Fibroblasts.	?	59
Epidermal growth factor (EGF)	Epidermal carcinoma.	Yes	60
Gamma-amino-butyric acid (GABA)	Dorsal ganglia.	?	61
Glucagon	Liver.	Yes	62,63
Glucose	Pancreatic islets.	?	49

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Table 1A. Agonists found to stimulate P.I. turnover(cond).

P.I. cycle agonist	Tissue/Cell system	Non-P.I. overlap	Refs.
Histamine	Smooth muscle.	Yes	*39,51*
5-Hydroxytryptamine	Platelets,insect salivary gland.	?	*33,35,64*
Insulin	Adipose tissue.	Yes	*42,65*
Interferon α,β γ	Lymphoblastoid cells. Endothelial cells.	Yes Yes	*66,67* *68*
Interleukin 2	T-lymphocytes.	suspected	*69,70*
Lectins (*eg* Concanavalin A)	T-lymphocytes.	Yes	*49*
Leukotriene D_4	Lung,macrophages.	Yes	*71,72*
Light	Retina photoreceptors.	Yes	*73*
Nerve growth factor (NGF)	Sympathetic ganglia.	?	*42*
N-formyl-methionyl-leucyl-phenylalanine (fMLP)	Neutrophils.	Yes	*74,75*
Platelet activating factor (PAF)	Liver,platelets.	?	*35,49*
Platelet derived growth factor (PDGF)	Fibroblasts, pre-adipocyte cells.	Yes	*49,76*
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)	Fibroblasts	?	*77*
Serotonin	Insect salivary gland.	?	*49*
Substance P	Pancreas,parotid.	?	*49,57*
Thrombin	Platelets.	?	*43,78,79*
Thyrotropin releasing hormone (TRH)	Pituitary cells.	?	*35*
Thyroid stimulating hormone (TSH)	Thyroid.	?	*42*
Vasopressin	Liver,fibroblasts platelets.	Yes	*49,52,80*

SECTION B2: COMPLEXITY AND REGULATION OF THE PI CYCLE

Closer investigation of the different enzymes involved in the PI cycle has revealed a system of rapidly expanding complexity. The first step is the activation of a family of highly conserved membrane proteins, (comprised of 3 subunits α, β and γ) termed G-proteins, by an activated agonist-receptor complex *(Reviewed 81,82,83,84)*. This activation step catalyses the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) bound to the α-subunit ("N" protein) of a specific G-protein. The α-GTP dissociates from the $\beta\gamma$ regulatory complex and is free to interact with and regulate an effector enzyme in the membrane (in the case of the PI cycle agonists, this is Phospholipase C (PLipC). To date there are at least 12 types of α subunit, 3 types of β subunit and 2 γ subunits identified, with cells seeming to contain a fairly varied complement of subunits - a situation further confused by their apparent promiscuity of interaction <u>in vitro</u> reconstitution experiments *(84)*.

The G-protein α-subunits are classified according to their sensitivity to undergo ADP-ribosylation (covalent modification at the nucleotide binding site) by the bacterial cholera and pertussis toxins. Recognition of the G-protein involvement with the PI cycle came with the observation that PLipC activity relied upon GTP *(85)* and cells could generate diacylglycerol utilizing the nonhydrolysable analogue GTP-γ-S *(86)* or soluble fluoride (NaF) *(87)* which both stabilize the subunit in the active form. The use of bacterial toxins demonstrated pertussis-sensitive PLipC in some cells (e.g.

bone marrow cells responding to Interleukin 3) *(88,89)* whereas others show no pertussis sensitivity but can show cholera toxin sensitivity (e.g. T lymphocytes) *(54,90)*. It should be noted that these toxins have other effects on cells not limited to G-proteins *(91,41)*. Recently a G-protein (termed Gzα) which is not ribosylated by either toxin has been found in platelets *(92)* with the proposal that it could be the pertussis-insensitive PLipC activator, other studies in bovine retina rod cells suggest that the special G-protein, transducin (Gt), stimulates PLipC activity through the α-subunit but the $\beta\gamma$subunits inhibit it in the dark (indirectly?) *(93)*.

Phospholipase C has been found to have many isozymes which occur as soluble form (e.g. 2 in rat brain cytosol) *(94)* and membrane-integrated forms (e.g. bovine brain) *(95)*. Immunological and DNA sequencing/probing studies have revealed at least 3 isozymes in rat brain *(96)* and purification in other tissues and species have revealed a multiplicity of forms and abundances. The isolated purified forms of PLipC are, however, fully active making elucidation of the activating pathway through G-proteins difficult to assess <u>in vitro</u>. These enzymes exhibit specificity for phosphoinositides and can cleave PI,PIP_2 and PIP forming 1,2 diacylglycerol and six different inositol products <u>in vitro</u> *(See Fig. 1c and 34)*. The liberated inositol phosphates (of which there are 66 possible derivatives) contain in their number compounds responsible for calcium mobilization and regulation.

Receptors for IP_3 and IP_4 have been purified and characterized

and appear to occur on the rough endoplasmic reticulum and in nuclear locales *(38,97)*. These appear to operate in a subtle harmony by controlling calcium release from distinct calcium stores in most cell types, and in any particular cell type the complement of calcium stores, receptors and enzymes will determine the responses and interplay with agonist stimulation *(Reviewed 98,99)*. Such an interplay would closely control cell calcium and may be critical factors in agonists which partially mobilise calcium (e.g. Leukotriene D_4/E_4 stimulated U937 monocytic leukemia cells experience an increase in IP_4 without any changes in IP_3 levels *(71)*). Mechanisms concerning calcium entry from the extra-cellular compartments are unresolved but possibly involve inositol phosphates acting on calcium permeable plasma membrane channels *(100)*.

The PI cycle is under a variety of feedback mechanisms serving to efficiently terminate and reverse the response (already greatly amplified from the original ligand-receptor interaction). Calcium is thought to play an important part in this feedback by;

(1) Inhibition of IP_3 binding to its receptor through an intermediary protein termed 'Calcimedin' *(97,38)*.

(2) Stimulation of the IP_3 conversion to IP_4 by an effect of Inositol 1,4,5 Trisphosphate kinase increasing the IP_4/IP_3 ratio where sequestration of calcium is favoured *(98)* and inhibition of Inositol Polyphosphate 1 Phosphomonoesterase *(34)*.

(3) The dependence of Phospholipases on calcium for activity *(95)*.

(4) The activation of Calpains which will act on PKC *(101)*.

(5) The activation of calcium binding proteins which inhibit PKC activity directly *(102)*.

(6) The sensitivity of mitochondrial enzymes, involved in ATP production, to calcium levels may limit the supply of ATP required for kinase activities *(103)*.

In addition the effect of Calmodulin-dependent protein kinase (which bind calcium for activity) and other calcium binding proteins, on the receptor and enzymes in the cascade, may function to buffer free calcium and/or attenuate the signal transducing proteins.

PKC has also been found to exert negative feedback by modulation of components of the PI cycle. Potential targets include;

(1) Activation of Inositol 5' monoesterase resulting in removal of Inositol 1,4,5,P_3, Inositol 1,3,4,5,P_4 and cyclic Inositol 1:2,4,5,P_4 *(34,104,105)*.

(2) Activation of Inositol 1,4,5 Trisphosphate kinase *(105)*.

(3) Modulating elements of the transducing G-protein *(92)*.

(4) Attenuation at the level of the (occupied ?) PI cycle activating receptors *(57)*.

(5) Autophosphorylation of PKC itself has been suggested to activate it initially but cause inhibition later by an unknown mechanism *(106,107)*.

Substrates for PKC are reviewed in Section G.

Other processes are likely to be physiologically important in the progression and termination of these PI cascade events. PKC may be modulated endogenously by agents down stream such as arachidonic acid, and the action of Phosphatases on the substrates of PKC will also be important. Other kinase systems have been found to affect the PI/PKC messenger system, e.g. transforming Oncogene products which have cellular counterparts have been found to modulate diacylglycerol and phosphatidylinositol phosphorylation (e.g. pp68 v-ros *(108)* and pp60 v-src *(109)*), and the IP_3 - receptor is phosphorylated and modulated by PKA *(38)*.

The complexity of the system is further increased by observations conflicting with the basic PI cycle hypothesis. There is growing evidence that the $\beta\gamma$subunit dimer of the G-proteins have their own messenger function. They have been implicated in inhibition of Calmodulin-stimulated cyclic nucleotide

Phosphodiesterase *(110)* and modulation of Phospholipase A_2 action leading to activation of muscarinic potassium ion channels in heart *(111,112, but See 113)*. In bovine retina, $\beta\gamma$subunits have also been found to affect PLA_2 stimulation, and PLipC inhibition in this tissue and in turkey erythrocytes under certain conditions has been reported *(93,114)*.

Research has also indicated that elevation of calcium and generation of diacylglycerol can occur distinct from each other. GTP analoge studies in a permeabilised neuronal cell line have shown G-protein mediated release of calcium from endoplasmic reticulum separate from IP_3 mediated release *(115)* and subsequently calcium ion channels in adrenal, pituitary and dorsal root ganglia cells have been reported to respond directly to G-proteins without apparent second messenger involvement *(Reviewed 113)*. Diacylglycerol generation without concommitant change in calcium levels from non-phospatidylinositol precursors has also been reported. The phospholipid phosphatidylcholine (PC) has been implicated as the source (e.g. in pre adipocyte cells treated with PDGF *(76)*, FDCP-mix 1 stem cell line treated with Interleukin-3 *(116)*, various types of T-lymphocytes treated with interleukin-1 *(117)* and in neutrophils treated with fMLP *(75)* possibly by transphosphatidylation through Phospholipase D or PC-specific phospholipase action.

Other sources of diacylglycerol have been postulated such as the specific hydrolysis of glucosyl-phosphatidylinositol resulting from Insulin treatment *(118)*. In support of this some Central Nervous

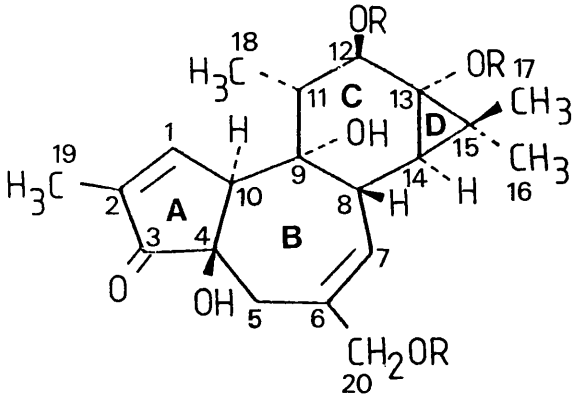
System (CNS) tissues possess abundant PKC levels yet have no IP_3 -receptor, suggesting specialised use of the bifurcating signal apparatus *(38)*.

Alternative sources of diacylglycerol are likely to produce other signalling molecules into the cell and be triggered by specific receptor G-protein relationships unknown at present. The story of diversity does not end here, PKC itslef has been found to comprise of a family of isozymes (See Section E) and the possibility of activation by agents other than diacylglycerol remains. While the protein kinase C story was in its early stages, other research was focussing on a group of natural compounds with a remarkable range of biological activities. The two fields converged providing the natural chemist with a receptor for these compounds and the protein kinase biochemist with invaluable research tools in the shape of the phorbol esters.

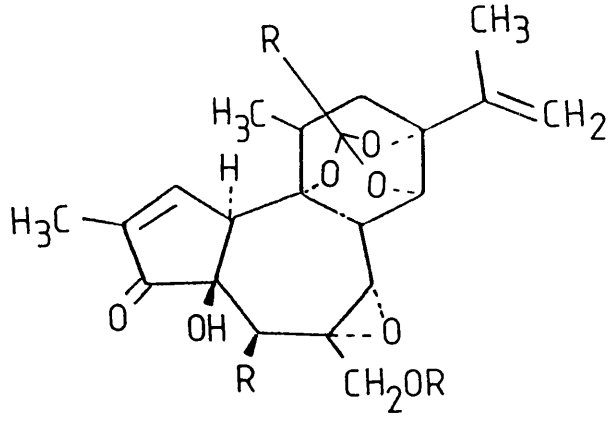
SECTION C1: THE PHORBOL ESTERS

Phorbol ester is a general collective term for the diterpenoid derivatives of the Tigliane, Daphnane and Ingenane hydrocarbon nucleii *(See Fig. 1d)* found naturally occurring in several genera of the Euphorbiacaea and Thymelaeaceae families *(Reviewed 119,120)*. X-ray crystallographic and chemical evidence *(121)* revealed that the central structure of the tigliane derivative,Phorbol,was composed of a semi-rigid carbon backbone arranged into 4 rings *(Tetracyclic See Fig. 1d)*. Ring A, connected in trans-linkage to ring B, has five Carbons and is non-planar. Ring B has 7 carbons and is stabilised into an 'envelope' conformation which is cis-linked to ring C. Ring C has 6 carbons and a 'chair' conformation imposed upon it by cis-linkage to the small 3-carbon ring D. Around the structure are 6 Oxygen atoms, one in an unsaturated Ketol in ring A at C-3 and 5 forming Hydroxyl groups t C-4, C-12 (in the β position) at C-13, C-9 (in the α position) and a free Hydroxyl group at the C-20. The first isolation of Tigliane diterpenes was from the seed oil of the euphorbia Croton tiglium 1 *(122)*. This natural source contained Phorbol derivatives esterified with acyl groups at C-12, C-13, and triesters esterified at C-12, C-13 and C-20. Subsequently Tigliane esters of deoxyphorbol have been isolated including 4-deoxy, 4-deoxy-5-hydroxy, 4,-20-dideoxy, 4,-20-dideoxy-5-hydroxy, 12-deoxy, 12-deoxy-5-hydroxy, 12-deoxy- 16-hydroxy, 12,-20-dideoxy and 16-hydroxy derivatives *(Reviewed 123)*. The ester moiety is highly variable commonly being with a saturated fatty acid, although unsaturated fatty acids are found *(124)* and more recently esters

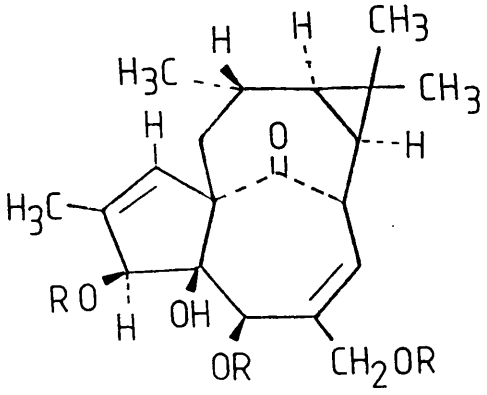
Figure 1d HYDROCARBON NUCLEII STRUCTURES OF TIGLIANE, DAPHNANE AND INGENANE-TYPE PHORBOL ESTERS



TIGLIANE



DAPHNANE



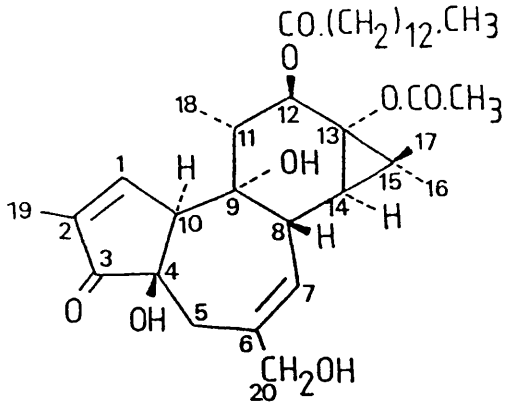
INGENANE

containing aromatic nitrogen at C-12 (the fluorescent sapintoxins from unripe Sapium indicum *(125,126)* have been characterised.

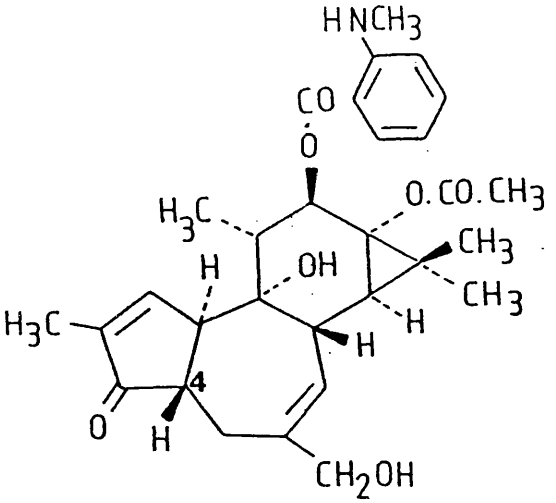
The Daphnane diterpenes have a slightly different Carbon skeleton to the Tigliane diterpenes, where the Cyclopropane ring (ring D) has opened giving the C-13 an isopropylene side chain *(See Fig 1d)*. In these compounds the hydroxy groups of ring C (C-9,C-14,C-13) are linked in an orthoester bond to aromatic or aliphatic groups. Some such as Mezerein and Thymeleatoxin A, have lost the double carbon-carbon bond in ring B having instead an epoxide bridge (C-6,C-7) and an extra C-5 hydroxy group *(127,128)*.

The Ingenance diterpenes are found in genus Euphorbia. They have many basic differences to the Tigliane mucleus with ring B attached to ring C at C-10 (instead of C-9) leaving C-9 to form a $\beta\gamma$ ketone between C-8 and C-10. There is a difference in hydroxyl groups also with C-12 and C-13 both absent and new hydroxyl groups at C-3 and C-5 thus esterification occurs in different parts of the molecule *(See Fig. 1d) (129)*.

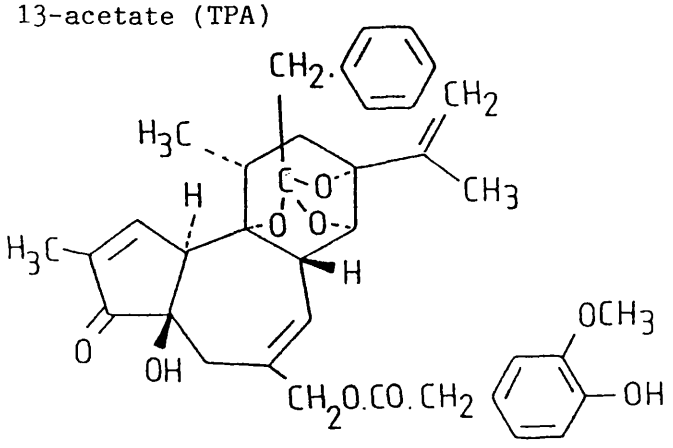
For a list of phorbol esters discussed see Glossary, and Fig. 1e.



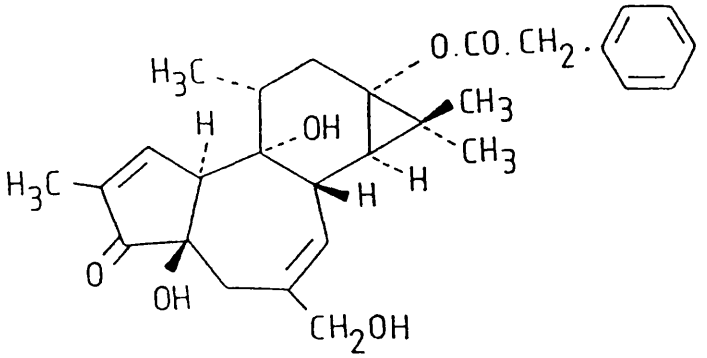
12-O-tetradecanoylphorbol
13-acetate (TPA)



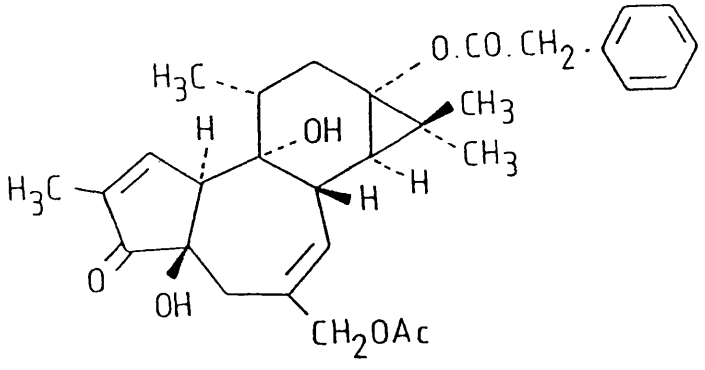
Sapintoxin A (SAP A)



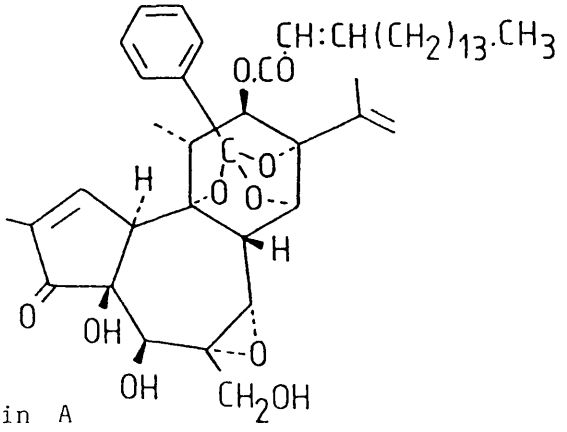
Resiniferatoxin (RX)



12-deoxyphorbol 13-
phenylacetate (DOPP)



12-deoxyphorbol 13-phenylacetate
20-acetate (DOPPA)



Thymeleatoxin A

SECTION C2: PKC - THE PHORBOL ESTER RECEPTOR

The biological actions of the phorbol esters at nano-molar concentrations *(See section F)* suggested the existence of specific binding sites to mediate such actions. Binding assays were employed using Phorbol 12, 13 Dibutyrate (PdBu) rather than 12-O-Tetradecanoyl Phorbol-13-Acetate (TPA the most active Phorbol ester) since PdBu was more soluble and did not bind non-specifically to lipids as TPA does *(130)*. Specific receptors were shown to have a wide distribution in intact cells, mouse epidermis and membrane fractions *(131,132,133)* as well as soluble fractions *(15)*. TPA and other phorbol esters were then shown to directly activate PKC in vitro *(134)* by substituting for diacylglycerol in increasing the calcium affinity of the enzyme. Subsequently the phorbol ester binding activity and PKC activity were found to co-purify together to apparent homogeneity in mouse, rat and bovine brain *(135,136,137, 138,139)*. In vitro work showed that the phorbol ester binding was dependent on phosphatidylserine (PS) and calcium and could be competitively inhibited by diacylglycerol *(140,141,142,143)*.

SECTION C3: TRANSLOCATION OF PKC

Treatment of cells with phorbol esters had other effects on PKC concommitant with activation. In Parietal Yolk Sac (PYS) cells a decrease in cytosolic PKC activity accompanied by an increase in particulate PKC upon phorbol ester treatment was observed within five minutes *(144)*. This suggested that translocation of the enzyme was occuring which could play a significant role in the biological action of phorbol esters. When the translocating action of 1,2 dioctanoyl glycerol (DiC_8 a membrane-permeable diacylglycerol analogue) was compared to phorbol ester action in T-lymphocytes, it was found that both agents elicited similar rapid translocation of cytosolic PKC to the particulate fraction (~50%) but the phorbol ester response was prolonged (4 hours +) compared to DiC_8 (transient rise peaking at 20 minutes) *(145)*. This stabilisation of membrane PKC has been found to reflect differences between the slow metabolism of phorbol esters compared to the rapid breakdown of diacylglycerols *(44,80,120)* although in some systems this is not the case *(146)*. Transient translocation has been demonstrated with physiological agonists of PI turnover, with maximum translocation varying (e.g. 10 minutes with interleukin - 3 treatment of WEH1-3 T-cell line *(147)* and 40 minutes in TSH - treated Thyroid cells *(148)*) before PKC repartitions to resting distributions. When phorbol esters are compared with PI agonists in the same cell type (e.g. Anti-immunoglobulin in β-lymphocytes *(149)* and Interleukin -2 translocation in T-lymphocyte lines *(150,69)*) the translocation induced by phorbol ester significantly exceeded the agonist in quantity as well as duration.

While agonist - induced plasma membrane translocation is transient and total levels of PKC remain constant, in some phorbol ester treated cells (e.g. NIH3T3 fibroblasts) only some translocated activity was found in the plasma membrane the rest being dispersed to other membrane fractions *(42)*. Since PKC has been detected in many membrane compartments (e.g. Nuclear and subnuclear *(21)*) immunofluorescent antibodies to PKC have been employed to address the problem in whole cells. A comparison between sn-1-Oleoyl-2-acetylglycerol (OAG a permeable diacylglycerol analogue) and TPA treatment in T-lymphocytes revealed that diffuse cytoplasmic PKC (in resting cells) became concentrated with both compounds to a focal area adjacent to the nucleus *(151)*. Only the duration of translocation differed (TPA lasting over 24 hours, OAG only 10 minutes). Similar studies in NIH3T3 fibroblasts found nuclear translocation of PKC apparent within 20 minutes of Phorbol ester exposure *(152,153)*. Recent work on HL-60 cells suggests phorbol ester treatment for prolonged periods gives rise to secondary translocation *(154)*. In this study initial translocation of PKC to the plasma membrane was followed by 'inward' translocation to the nuclear region within 24 hours. After 48 hours PKC was detected associated with the nucleus and after 72 hours the progressive redistribution to normal levels occured.

Immunocytochemical studies on resistant cell lines (i.e. cells resistant to the biological effects of phorbol esters) showed impaired membrane translocations (e.g. in HL-60 variants *(155)*) or perinuclear instead of plasma membrane translocation (fibroblastic

cell lines and immature leukemic cells *(156,157)*) of PKC in response to TPA treatment. Evidence underscoring the non-physiological nature of phorbol ester induced PKC translocation comes from GTP analogue treatment of permeabilised cells *(86)*. Endogenous diacylglycerols generated in these cells required calcium levels to be elevated for PKC translocation to occur, although phorbol ester treatment was effective at PKC translocation independent of calcium levels. Furthermore multiple applications of calcium ionophore and DiC_8 to T-cells could only elicit 50% of the biological responses achieved by calcium ionophore and a single phorbol ester application *(145)*.

SECTION C4: DOWN-REGULATION OF PKC

In many cell types incubation with phorbol esters results in the decrease in total PKC levels from the cell. The disappearance in neutrophils and platelets is rapid (6-8 minutes) following translocation of PKC to the membrane fraction but could be blocked by pre-loading the cells with the Calpain inhibitor, leupeptin *(26,158,159)*. Investigation of the rates of PKC synthesis de novo revealed no apparent change upon phorbol ester treatment (in rat glioma cells *(160)*) supporting the case for proteolysis. Immunological evidence suggested the loss after phorbol ester treatment followed maximal translocation (i.e. 15 minutes in GH_3 pituitary cells *(161)*, 30 minutes in mouse skin *(28)*) and was sustained for over three days. Comparison of 'down-regulation' of PKC between phorbol esters and the diacylglycerol analogues (OAG and DiC_8) in swiss 3T3 fibroblasts showed that phorbol esters could elicit almost total loss of PKC (40 hour treatment) but the diacylglycerol analogues could not alter PKC levels *(162)*. The extent of down regulation by the phorbol esters varies considerably with cell type (e.g. in MDB kidney cells 30% of immunoreactive material was lost compared to 100% in swiss 3T3 cells at similar TPA doses *(163)*) and the rates of loss also vary. In some cells down regulation will lead to insensitivity to the bioligical effects of the phorbol esters (e.g. swiss 3T3 cells *(164,56)*, T-lymphocytes *(165)* and macrophages *(166)*) although not in others (e.g. Friend Erythroleukemia cells *(167)*). This may be due to the presence of a PKC 'pool' which is resistant to the down-regulating effect of the

phorbol esters as has been suggested in rabbit aortic smooth muscle *(168)*. The evidence gathered from various systems indicates several ways in which phorbol ester action on PKC translocation and fate differ from physiological responses (diacylglycerols direct or hormone/agonist action).

(1) Endogenous translocation of PKC is regulated by rapid metabolism of diacylglycerol whereas the phorbol esters persist in the system.

(2) The translocated PKC has a greater membrane stability with phorbol ester.

(3) Phorbol esters bypass the calcium-sensitive nature of endogenous PKC translocation.

(4) Phorbol esters can induce a greater translocatory movement than physiological activators.

(5) Phorbol esters cause down-regulation of PKC due to proteolysis to PKM and subsequent loss from the cell whereas diacylglycerol does not.

(6) Phorbol esters may cause different patterns of translocation of PKC within the cell.

Although PKC has been established as the major receptor for

phorbol ester action, binding studies have revealed an heterogeneity of binding curves in many cases (e.g. brain cytosol *(141)* and liver nucleii *(169)*). Both OAG and phorbol esters have been found to exert biological effects on dorsal root ganglion cells when applied to the extracellular but not the intracellular side *(170)*. This effect was additionally elicited equi-effectively by phorbol esters which do not activate PKC in vitro and do not otherwise have biological activity. In platelets Quin -2 fluorescence studies, measuring free calcium, revealed OAG and DiC_8 at high levels (100 μM) induced calcium release from intracellular stores *(171)* and in T-lymphocytes DiC_8 at 12.5 μM (yet not OAG) had a similar effect *(172)*. Phorbol esters could not mimic such an effect in either of these systems. Similarly other studies with these diacylglycerol analogues have suggested that they can elicit various biological effects which are not elicited by phorbol esters and are unrelated to PKC activation *(173,174,175)*.

SECTION D1: ACTIVATION OF PKC

The calcium-dependent activation of PKC initiated by the presence of phospholipid and diacylglycerol cofactors can be influenced by a number of agents *(See Table 1B)*. The hydrophilic regions of phorbol esters which activate PKC show structural similarity with non-phorbol amphiphilic compounds (eg Teleocidin and Aplysiatoxin) allowing them to interact with the enzyme, while lipophilic regions of the molecule have been proposed to interact with the phospholipid microenvironment associated with the protein *(188)*. In vitro activation by the phorbol esters requires very little calcium since saturating amounts of TPA achieve little enhancement, in a calcium-free assay, when calcium is added *(188)*. Measurement of resting levels of free calcium in a number of cells has found that the levels observed (e.g. 200 nM in T-lymphocytes *(197)*, 140 nM in Fibroblasts *(198)* and 100 nM in Neutrophils *(199)*) are adequate for PKC activation by TPA. TPA has been found to be unimpaired in PKC activation when free cell calcium is reduced to as low as 10 nM in Neutrophils *(199)*. Conversely calcium itself is inadequate to induce PKC activation in vitro *(189)* and in vivo phorbol ester responses could not be mimicked by the calcium Ionophore,ionomycin,in T-lymphocytes *(197)*. High concentrations ($5x10^{-4}$ M) of calcium were needed for half-maximal PKC stimulation with phospholipid alone *(32)*. Addition of diacylglycerol or phorbol ester however decreased the concentration required to the micromolar range (10^{-7}M), so by shifting the calcium dependence curve they enable the enzyme to be active at lower (physiological) ranges. The structures are given in *Fig. 1f*.

Table 1B . Activators of protein kinase C.

Unsatturated fatty acids	Effective range	Refs.
Arachidonic acid (C20:4)	50-100 μM(α,β PKC)* 5 μM (γ PKC)*	*46,176,177* *178*
Bryostatin (macrocyclic lactone)	10 nM	*179,180,181*
Cardiolipin	1-20 μM	*182*
Diol-phosphatidylserine	5-10 μM	*183*
Linoleic acid (C18:2)	30-80 μM*	*46,178,183*
Linolenic acid (γ-linoleic acid)	50-100 μM*	*46,178,183*
Lipid A	30-50 μg/ml	*184*
Lipoxin A	1-10 μM	*177,178*
Oleic acid (C18:1cis)	50-120 μM*	*46,183*
Phosphatidylserine	1-20 μM	*185,186*
Retinoic acid (Vitamin A analogues)	10-20 μg/ml	*186*

Phorbol Esters	Effective range	Refs.
TPA	3-10 nM	*187,188*
PdBu	8-20 nM	*188*
Mezerein	30-60 nM	*188,189*
Sapintoxin A	50-80 nM	*125,190*
Doppa	100-1000 nM	*191*

contined overleaf...

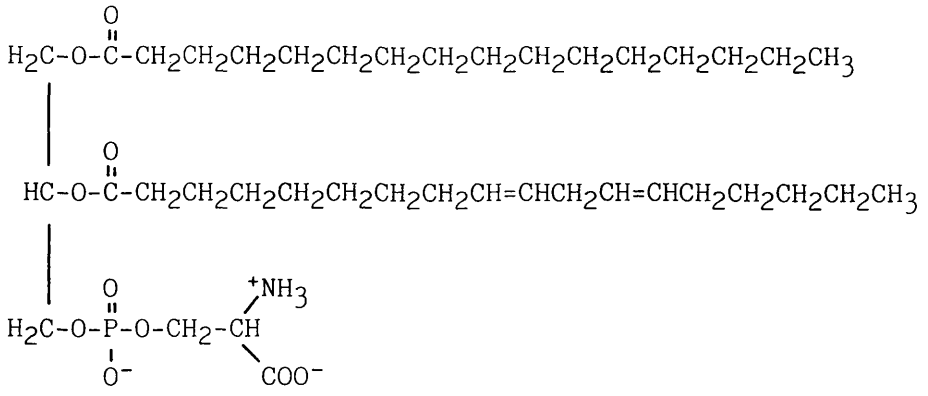
Table 1B . Activators of protein kinase C.(cond)

Diacyl glycerol analogues	Effective range	Refs.
sn-1,2-Dihexanoylglycerol (DiC_6)	11 μM	*192*
sn-1,2-Dioctanoylglycerol (DiC_8)	1-5 μM	*187,192*
sn-1,2-Didecanoylglycerol (DiC_{10})	1-5 μM	*192*
1-Oleoyl-2-acetylglycerol (OAG)	20-100 μg/ml	*193*
Lysophospholipids	10-20 μM*	*194,195*
Teleocidin (indole alkaloid)	20-50 nM	*188,196*
Aplysiatoxin (polyacetate)	10-30 nM	*188*
6-(N-decyl-N-methyl amino)-4-hydroxy-methyl indole (DHMI)	30-100 μM	*180*

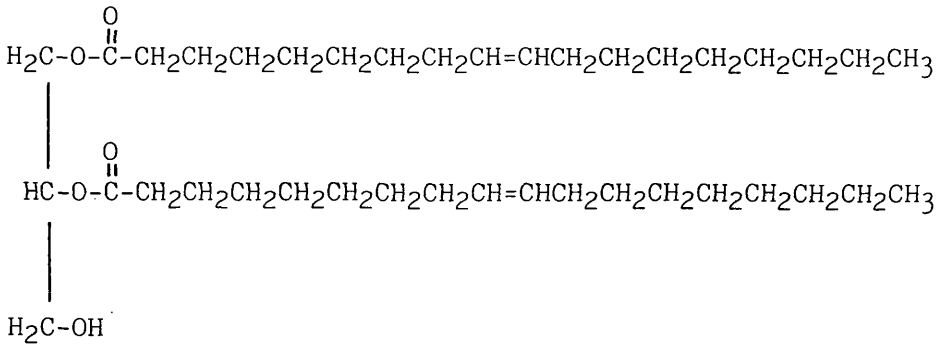
*Inhibitory to PKC activity at higher concentrations.

Figure 1f STRUCTURES OF COFACTORS OF PKC

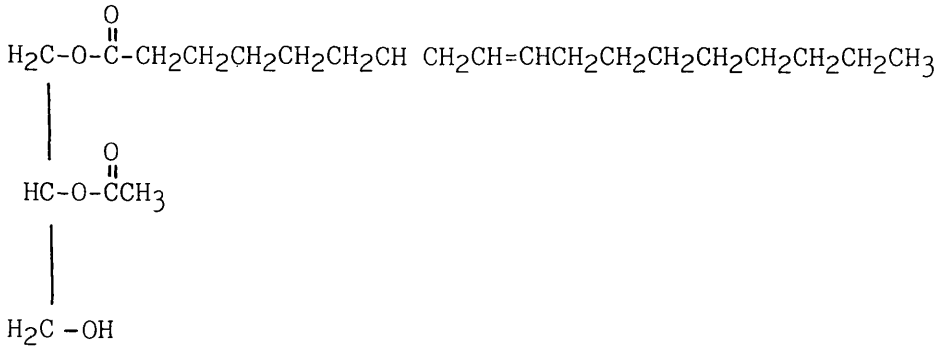
L α -PHOSPHATIDYL SERINE (PS)



SN 1,2 DIOLEOYL GLYCEROL ($DiC_{18:1}$)



1OLEOYL-2-ACETYL GLYCEROL (OAG)



In studies on diacylglycerol analogues it was found that saturated fatty acyl chains at positions 1 and 2 were effective in activating PKC whereas unsaturated acyl chains were less effective and mono- or tri-acylglycerol were ineffective *(31)*. Studies on chain length found that a chain of 6-10 carbons were effective with 8 being optimal *(192)*. Phospholipids capable of supporting enzymatic activity included phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) but not phosphatidylcholine (PC) *(200)*. In the presence of diacylglycerol, PS was more effective than PE or PI in activating the enzyme at a fixed calcium level. Mixtures of phospholipids were found to vary the effectiveness of PS thus cooperation was seen with PS/PE mixture but diminished activation with PS and PC or sphingomyelin. The phospholipids are activators when arranged in a lamellar or micelle structure and PS was most effective present at 8 mole% of the micelle, indicating the cooperation of 4-10 PS molecules for maximal PKC activation *(201,202)*. Two apparent Ka values for PS have been found (0.6-2 and 35-80 μg/ml <u>in vitro</u> *(135)*) - these could be lowered by the presence of the PKC activators. Arachidonate was reported to enhance PKC activity in detergent extracts from neutrophils at 200 μM concentration or at 100μM with diolein or PS *(46)*. With optimum levels of PS and diolein no additional activity was seen suggesting arachidonate was simulating the effects of phospholipid. Other oxidated derivatives of arachidonate including linoleate, oleate and lipoxin A shared this property in parallel with the number of <u>cis</u>-orientated double carbon bonds *(178,203)*. Interestingly <u>trans</u>-forms

of oleic acid were inactive, as were micelles of oleic acid *(183)* only the monomeic form being able to activate PKC. Furthermore such activation did not require calcium for its effect, fuelling speculation that arachidonic acid metabolites (possibly generated through PLA_2 activity) may be able to activate cytosolic forms of PKC without membrane interaction. Other PS-like activators include retinoic acid *(186)* and Lipid A *(184)* from bacteria.

Recently a series of lysophospholipids (especially lysophosphocholine;Lyso-PC) have been found to be able to regulate PKC activity <u>in vitro</u> *(195)*. Up to 20 μM Lyso-PC was found to stimulate PKC in the presence of PS but over 30 μM progressively inhibited the enzyme suggesting interaction with PS (and the substrate?) could alter their affinity for PKC.

Other PKC activators such as the indole alkaloids (e.g. Teleocidin) and polyacetates (e.g. Aplysiatoxin) appear to act in a similar manner as phorbol esters/diacylglycerols and bind to PKC <u>in vitro</u> and mimic some of the biological actions of phorbol esters <u>in vivo</u>. Some activators such as the macrocyclic lactones Bryostatin I and II competitively inhibit phorbol esters binding to PKC, but do not have similar biological effects and even antagonise/reverse them. This appears to be a combination of PS - like action, anchoring the enzyme to the membrane, and a blocking action of the activator site *(180,181)*.

The evidence for these <u>in vitro</u> studies suggests a quaternary

complex is formed by the activated enzyme [4-10 Phospholipid molecules/1 activator molecule of diacylglycerol *(135)* or phorbol ester *(201)*/calcium and PKC]. Interestingly the proteolytic action of Calpain on PKC (generating the calcium, phospholipid - and activator - independent PKM) is greatly enhanced by an active enzyme complex *(204)*. The role for PKM, whether a route for enzyme degradation and/or a further intracellular signal is unknown. The use of antibodies recognising and protecting the proteolytic site may be useful in addressing this *(205)*.

Although the phorbol esters vary in their potency for PKC activation, Resiniferatoxin and DOPPA, at optimum doses, have been found to stimulate PKC activation to only 25% of the maximum elicited by other phorbol esters *(191)*. Biologically inactive phorbol esters can still bind effectively to PKC but activate with 1/100th the potency of active derivatives *(185)*. These results and other binding studies *(189,206)* have indicated a heterogeneity of receptors in PKC preparations.

SECTION D2: INHIBITORS OF PKC

Many inhibitors of PKC have been used in studies aimed at elucidating the effects of PKC activation. Inhibitors can be classed according to their interaction with the enzyme as determined by in vitro experiments *(See Table 1C)*.

The 'analogues' of ATP compete with and block the ATP-binding site on PKC and PKM but will also have this action on other kinases such as PKA, PKG and Calmodulin dependent protein kinases (PKCAL) *(244)* so that modifiction of crosstalk between kinase systems in vivo cannot be ignored. Hence 'H7' has been shown to be unable to inhibit phorbol ester effects completely in some systems *(166)* and to elicit effects in cells depleted of PKC *(70)*. Similarly Staurosporine has biological effects in neutrophils in the absence of PKC activators *(219)*. ATP competitors with greater specificity for PKC, such as sangivamycin *(216)* have been developed and may prove more useful for in vivo work.

Inhibitors which compete with phospholipid binding sites on PKC have also been widely used. Their potency ranges from high specificity (e.g. the polypeptide Cobra cytotoxin-I Ki 1.3 μM) to the relatively unspecific phospholipid interacting drugs (e.g. Dibucaine Ki 0.5 - 1 mM). Many such as polymixin B, adriamycin and DL-palmitoylcarnitine are effective inhibitors of PKCAL and are unsuitable as in vivo PKC inhibitors. Hydrophobic - interacting compounds such as Trifluoroperazine (TFP) and W7 have been used

Table 1C. Inhibitors of protein kinase C

ATP -binding site competitors.

Compound	Effective range	PKC specific inhibition	Limited effect *in vivo*	Refs.
Amiloride	0.5-1 mM	No	Yes	*207*
1-(5-isoquinoline sulphonyl)-2-methyl piperazine (dihydro chloride) (H7)	10-100 µM	No	Yes	*68,70,166, 208,209,210, 211,212,213*
K252a (antibiotic)	5 µM	No	Yes	*75,214*
Phloretin/Quercetin	2-20 µM	No	?	*11,215*
Sangivamycin	10 µM	No	?	*216*
Staurosporine	10-200 nM	No	Yes	*217,218,219*

Inhibitors of PKC/Phospholipid interaction.

Compound	Effective range	PKC specific inhibition	Limited effect *in vivo*	Refs.
Dibucaine	0.3-0.8 mM	?	?	*23,32*
Chlorpromazine	30-100 µM	?	?	*12,23,32*
Cytotoxin AIV(marine worm polypeptide)	2-6 µM	?	?	*220*
Cytotoxin 1 (cobra venom polypeptide)	1-3 µM	?	?	*220*
Lysophospholipids (*eg* lyso Phosphatidyl carnitine)	30-100 µM	?	?	*194,195,221*
DL-Palmitoyl carnitine	1-30 µM	No	?	*221,222,223*
Polymixin B	1-10 µM	No	Yes	*213,220,224, 225*
Retinoids(*eg* retinal)	10-100 µM	?	Yes	*73,159,186, 211,213,226, 227*
Tamoxifen(triphenyl ethylene derivatives)	30-50 µM	No	?	*228,229*

(Continued overleaf)

Table 1C. Inhibitors of protein kinase C (cond)

Membrane disruptive inhibitors.

Compound	Effective range	PKC specific inhibition	Limited effect *in vivo*	Refs.
Adriamycin	50-80 μM	No	?	*222,230*
Polyamines (*eg* spermine)	1-5 μM	No	?	*225*
Trifluoroperazine	10-100 μM	No	Yes	*12,159, 198,211, 213*
N-(6-aminohexyl) 5-chloro-1-naph-thalenesulphonamide (W7)	50-150 μM	No	Yes	*68,198, 213,222, 231*

Active site competitive inhibitors.

Compound	Effective range	PKC specific inhibition	Limited effect *in vivo*	Refs.
1-O-Hexadecyl-2 -O-methylglycerol (HMG-C_{16})	30-100 μM	?	?	*74*
α Sapenine (phorbol ester derivative)	50-100 nM	?	Yes	*185,191, 232*
Sphingosine (lysosphingolipids)	2.5-10 μM	?	Yes	*68,166, 194,233, 234*

Protein inhibitors.

Compound	Effective range	PKC specific inhibition	Limited effect *in vivo*	Refs.
Retinoid holo binding proteins	6 μM	?	?	*235*
Calciproteins				*102*
eg CaBP 12	3-5 μM	?	?	*236,237*
CaBP 17	2-3 μM	?	?	*238*

(Continued overleaf)

Table 1C. Inhibitors of protein kinase C (cond)

Chemical inhibitors.

Compound	Effective range	PKC specific inhibition	Limited effect *in vivo*	Refs.
Oxidising agents (*eg* Chlorosuccinimide)	10-30 μM	No	?	*239*
N-ethylmaleimide (NEM)	0.5-2 mM	No	?	*198,240*

Miscellaneous inhibitors.

Compound	Effective range	PKC specific inhibition	Limited effect *in vivo*	Refs.
Antibodies raised to PKC.	?	Yes	?	*27,241*
Di-(2-ethylhexyl) -phthalate (DEHP)	100-400 nM	?	?	*242*
Neurotoxin 1 (cobra venom polypeptide)	70 μM	?	?	*220*
Pseudosubstrate synthetic peptides	20-80 μM	?	?	*53,243*

widely in vivo. These have been found to exert effects in cell-free systems, independent of their inhibitory properties on PKC and PKCAL, by non-specific action *(213,245)*.

Compounds interacting with the putative activator site include the ether lipid 1-O-Hexadecyl-2-O-methyglycerol (HMG-C_{16} — a diacylglycerol analogue *(74)*) and sphingosine (present in animal cells within complex membrane sphingolipids *(234)*) which compete with phorbol ester binding to PKC in a dose dependent manner. In vivo sphingosine has proved effective in inhibiting the effects of TPA in macrophages and platelets (but only partially effective in inhibiting DiC_8 effects in the former system) *(166)*.

Many of the inhibitors tested (e.g. lipophilic inhibitors TFP and retinal, and ATP inhibitors H7 and Sphingosine) have been found to be unable to prevent PKC binding to membranes in response to PKC activators, thus potentially the enzyme may be exposed to proteolytic effect (and its unknown consequences). Staurosporine has additionally been found to promote PKC translocation. Cell type has also been found to influence inhibitor effects in vivo (e.g. HL-60 and neutrophils differ in their response to H7, polymixin B and retinoids *(213)*). Similarly PS and calcium dependent kinase activity in rat brain extracts but not rat heart is inhibitable by TFP *(12)*.

More recent work has centred on a number of more specific inhibitors which directly interact with PKC in vitro, but are difficult to introduce into intact cells. These include antibodies

raised to PKC, itself, pseudosubstrate peptides (compete for substate binding sites) and a family of calcium-binding proteins isolated from rat brain.

The physiological relevance of endogenous protein inhibitors, sphingosine, retinoids (and their binding proteins) is not understood at present. It is of interest however, that OAG and DiC_8 have been reported to stimulate sphingomyelin hydrolysis which could generate PKC inhibitors in a negative feedback fashion whereas TPA did not elicit this response *(173)*. The in vivo production of lysophospholipids (e.g. by phospholipase A_2 - type activity) could also constitute a regulatory system since these agents show a biphasic stimulation/inhibition of PKC (at 1%/10% mole fraction respectively) in vitro *(195)*.

The variable effects of the inhibitors presently available has led to claims being made, possibly with undue haste, concerning the role of PKC in vivo. The advent of multiple subtypes of PKC has clarified the need for caution in interpreting the results of such studies.

SECTION E: PKC ISOZYMES

The complex cofactor requirements of PKC and its apparent pivotal role in varied signalling pathways prompted closer investigation of the structure of the protein itself. Studies on purified bovine kinase C digested with Trypsin enabled the primary sequence of amino acids to be determined *(246)*. Using predicted-sequences oligonucleotide probes, clones containing a complementary DNA library from calf brain were screened and hybrids isolated. These were found to contain not one but several closely related but distinct cDNA strands which yielded three sequences upon transcription; PKC-α, β and γ *(247,248,249,250)*. PKC-β was found to comprise of 2 isozymes (β_1 and β_2) which are derived from different splicing of a single gene resulting in a different carboxy-terminus *(251)*. All isozymes consisted of a single polypeptide chain with four conserved regions (C_1 -C_4) and five variable regions (V_1 -V_5) *(See Fig 1g)*. The C_1 region contains tandem repeats of cysteine rich sequences (showing homology to metallo-and DNA binding sequences on other proteins) and C_3 was identified as the ATP binding sequence. No characteristic calcium or phospholipid binding site was identifiable from the primary sequence such as have been found in other proteins. Recent low-stringency probing of DNA libraries with oligonucleotides prepared from the β,αandγ-PKC isozymes revealed 3 more distinctly related members of the PKC family termed ϵ, δ and ζ *(253)*(epsilon, delta and zeta PKC). These isozymes appear in brain as well as other tissues such as lung and kidney *(254)*. These isozymes are poorly characterized at present although initial studies suggested PKC-ϵ is

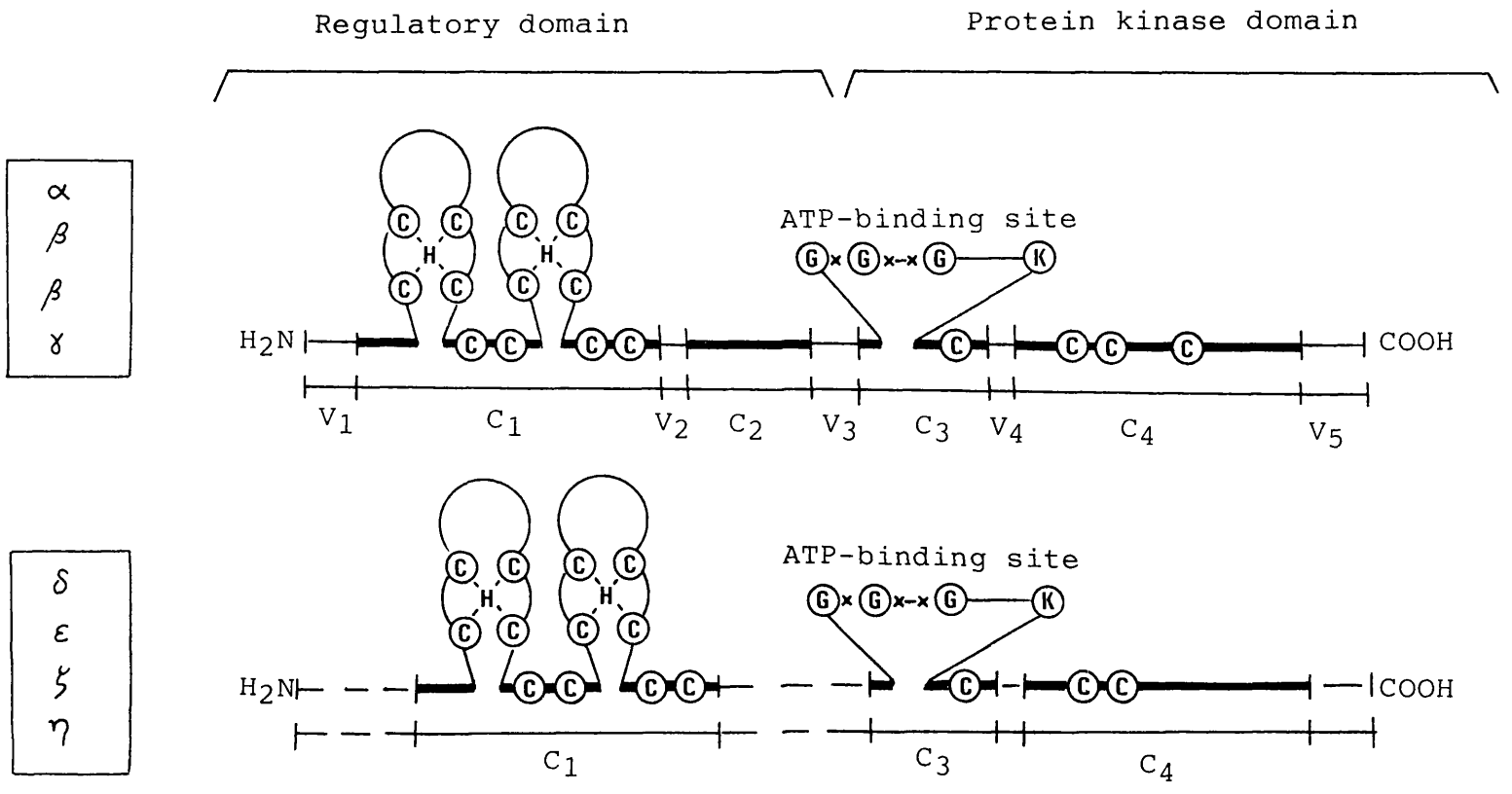


Fig 1g. Structures PKC isotypes.

C,G,K,X and H represent cysteine,lysine,glycine,any amino acid and metal respectively.Four conserved (C_1-C_4) and five variable (V_1-V_5) regions of the larger group are indicated. The main difference between the two groups is that the δ,ε,ξ and η subspecies lack the second conserved region C_2.The β_1 and β_2 subspecies,which seem to be derived from a single messenger RNA by alternative splicing, differ from eachother only in 50 amino acid residues at their carboxy-terminal end regions,V_5,and even in this area they possess a high degree of sequence homology.(from ref *252*)

calcium indpendent and is a poor kinase for histone (used in the majority of PKC assays) *(255)*. The absence of the C_2 region in PKC ϵ, δ and ζ isozymes implicates it with calcium regulation in the α, β and γ isozymes. Additional modified versions of the PKC isozymes seem to exist in a tissue specific manner. PKC isozymes in heart and lung have modified properties to PKC from brain *(256)* and a truncated PKC-ϵ has also been reported *(254)*. Recently yet another isoform of PKC, related to the ϵ, δ and ζ isotypes in the absence of a C_2 region, has been found in lung, skin and heart with very little in brain and termed PKC-η (eta) *(257,258)*.

The existence of many isoforms could be an excellent mechanism to explain the diverse biological activities of phorbol esters, and discrepancies in PKC activation, down regulation and inhibition studies. Thus recent work has been directed to find (i) Functional differences between the occurence and levels of PKC isozymes <u>in vitro</u>. (ii) Differences between the occurence and levels of PKC isozymes <u>in vivo</u>.

<u>In vitro</u> work on α, β and γ isozymes from rat brain has indicated only subtle differences in activity using PS and diolein as activators *(182)*. In one study, rat brain β isozymes were apparently less sensitive to calcium *(See 252)*. Isozymes isolated from rabbit brain however were found to have different calcium requirements in saturating PS conditions without diacylglycerols such that the velocity of kinase activity (Vmax) increased with calcium 5,10 and 20 fold for γ, β and α isozymes respectively *(259)*. Conversely in bovine brain the γ isozyme was the least calcium dependent enzyme *(260)*.

In the absence of PS and diolein, arachidonic acid and derivatives (e.g. Lipoxin A) have been found to partially activate the γ-PKC isozyme from rat brain (~25% without calcium 45-90% with calcium of the PS/Diol elicited activity) *(177)*. This activation only occured in a narrow concentration range (10-20 μM) for PKC and did not activate α or β-PKC until higher concentrations (>100 μM) were used *(176)*. Addition of PKC isozymes to preparations of cell membranes rich in a substrate for PKC (A431 carcinoma cells containing the EGF-receptor substrate *(261)*) revealed that all isozymes could effectively phosphorylate the substrate, although as judged by specific activity of histone phosphorylation, α-PKC was more efficient than β, γ isozymes. Recent approaches have found a distinct specificity for γ-PKC compared to the action of α and β -PKC (which were identical) using artificial peptide substrates *(262)*. Kinase action exclusive to α, β or γ isozymes, known to exhibit substrate and cofactor differences, await further investigation.

Differential tissue distribution of the α, β and γ isozymes has been more promising. γ-PKC was found to be expressed only in rat central nervous tissue (brain and spinal cord) where it is expressed slowly after birth *(240,252)*. The α and β isozymes are more ubiquitous among the tissues *(See Table 1D)*. PKC-δ, ϵ, ζ and η isozyme distributions are less well-defined; ϵ appearing in brain, heart and lung and less so in other tissues and ζ, δ and η isozymes seen with Northern blotting in many tissues *(252,255,257)*. The relative proportions as measured by histone phosphorylation vary amongst the

Table 1D. Examples of PKC isozyme distribution.

Tissue/Cell type	Isoymes found	Refs.
A431 carcinoma cells	α,η	*256,257*
Adrenocortical tissue	α	*263*
Brain	α,β,γ,δ,ε,ξ,(η)	*255,256,257 258,264,265 266*
Cos-7 cells	α	*256*
Erythroleukemia cells (MELC)	α,β	*267*
Epidermis	δ,η	*257,258*
Fibroblasts (NIH 3T3 and Swiss 3T3)	α	*256,268*
Heart	α,β,ε,η	*255,256,258*
Kidney	α,β,(ε?),η	*255,256,258*
Leukemic cells		
HL-60	α,β,+other	*269,270,271*
Rbl-2H3	α,β	*272*
Liver	α,β,(ε?),η	*255,256,258*
Lung	α,β,ε,η	*255,256,257 258*
Neutrophils	α,β,ξ	*176,273,274*
Pancreatic acinar cells	α	*275*
Pituitary cells (GH_4)	α,β,ε	*276*
Platelets	'a' and 'b'	*277*
Pre-β pre-T cell (KM3)	α,β	*278*
Spleen	α,β,η	*256,258,264*
Testes	α,β,η	*256,258*
T-lymphocytes		
Human	α,β	*151,279*
Mouse	β mRNA	*70*

tissues, e.g. α-PKC being dominant over β in lung tissue, whereas in spleen the reverse occurs *(256)* and in monkey brain, regional distribution is exhibited *(266)*.

The distribution of isozymes intracellularly has been found to vary. Phorbol ester binding and messenger RNA hybridization studies in rat brain suggested γ-PKC to be located post-synaptically in cerebellar purkinje cells whereas α and β appeared in cerebellar granule cells and striatonigral fibres pre-synaptically *(264,38)*. Such a distribution suggests that PKC-α and β may be involved in modulating neurotransmitter release while PKC -γ could modulate postsynaptic transduction. Immunocytochemical staining for α and β isozymes inside T-lymphocytes has suggested a diffuse cytoplasmic distribution for both isozymes *(279)*. Phorbol ester treatment caused a redistribution of both isozymes to discrete areas within the cell *(151)*. A similar pattern was observed for PKC-α in resting GH_3 pituitary or rat embryo fibroblasts *(276)* with phorbol ester or hormone treatment causing particulate membrane association. In transformed rat embryo fibroblasts α-PKC could not associate with the cytoskeletal compartment associated with 'focal contact' proteins. This suggests that appropriate cellular growth control may be regulated by appropriate subcellular locations of PKC (mediated by growth hormones), somehow impaired in transformed (growth factor independent) cells. Further work on compartmentalization integrated with the expression of PKC isozymes is needed to build up a picture of functional differences.

SECTIONAL F1: BIOLOGICAL ACTIONS OF THE PHORBOL ESTER: STRUCTURAL REQUIREMENTS

The biological actions of the phorbol esters largely have been investigated with commercially available preparations and has been limited by the availability of source plant material. Consequently investigators have largely concentrated on TPA and PdBu as active derivatives and phorbol and 4- α - PDD as inactive controls. Comparative studies conducted on a range of phorbol derivatives have elucidated many structural requirements for biological activity *(See TAble 1E)*. If the primary hydroxy group at C-20 is substituted (e.g. esterified), the bioactivity is decreased in proportion to the type of substitution *(280,281)*. The spatial position of this C-20 seems important since slight adjustment (e.g. caused by double bond shift from C-6/C-7 to C-7/C-8) results in activity loss. Similarly switching the hydroxy group on the C4 position to the α conformation or derivitization results in lessening of activity. The importance of such hydrophilic groups appears to involve orientating the phorbol ester to facilitate interaction with the phospholipid head groups and/or PKC protein groups in the membrane. The phorbol esters also require lipophilic moieties in the molecule (typically at C-12 and C-13) which allow anchorage and spatial organisation in the lipid bilayer according to acyl chain length and acyl type. These appear to be crucial in the biological activity since short chain acyl groups can bind to PKC but are inactive themselves and ihibitory to biological action (e.g. Tumour promotion by TPA). The trans configuration of the A and B ring linkage is also considered

Table 1E. Structure/activity requirements for tumour-promoting and hyperplastic activity in the phorbol esters.*

(1) An AB ring in the *trans* formation is required for activity.(AB *cis* epimers are inactive)

(2) A tertiary hydroxyl group at the C-4 position is necessary for complete tumour promoting action. O-methylation of the C-4 hydroxyl results in the loss of complete promoting activity although stage 1 promoting activity is retained. 4-deoxyphorbol esters are inactive as complete and stage 2 promoters and as hyperplastic agents.

(3) An ester moiety at C-12 is not required for activity,although a secondary hydroxyl group at this position reduces activity dramatically.

(4) A free primary hydroxyl group at C-20 is required for activity.

(5) Increasing unsaturation in an ester moiety results in decreased complete promoting activity but increased stage 2 promoting activity.

(6) A secondary hydroxyl group at C-16 does not affect activity.

* from G.brooks PhD thesis 1989 Univ.London

necessary for activity although Cis - α - Sapintoxin has been found to be biologically active at high concentrations *(282)*. Similar studies on diacylglycerols have revealed an optimum length for acyl chains (6-10 carbons) and the presence of a free hydroxy group at C-3 of the glycerol backbone (which is exposed by PLip C cleavage) was essential for activity *(192)*. The high activity of OAG and Ingenol-3-monoesters has suggested that a single esterified acyl group is sufficient (and not restricted to the C-12 or C-13 position on the phorbol esters) for activity (283).

Computer modelling techniques have more recently been employed to compare the structural similarities between energetically favourable conformations between phorbol esters and diacylglycerols *(284)*. Interestingly in this study the C-1 ester in diacylglycerol and the C-3 free hydroxy group correspond to the C-13 ester and C-9 hydroxy group (rather than the C-20 *(196)*) of the phorbol derivatives in terms of spatial positioning. Furthermore it was suggested that the C-4 hydroxy group (and possibly other parts of the molecule such as C-20) which had no correlate in the diacylglycerol molecule, but which are involved in the biological potencies of the phorbol esters, may play functional roles on separate receptors or different PKC isoforms. In support of this such groups have been found to have correlates with groups in non-phorbol ester molecules (e.g. Teleocidin) which share some biological activities with phorbol esters in vivo and can activate PKC in vitro *(141,196,285)* without having any resemblance to diacylglycerols.

SECTION F2: TUMOUR PROMOTION

From a medical point of view the most interesting biological action of phorbol esters is tumour promotion. As early as 1941 Berenblum reported that oil from croton tiglium applied with small doses of a carcinogen was observed to potently promote the formation of skin tumours (papillomas) on mouse *(286)* which became progressively dysplastic, aneuploid and finally malignant *(287,288,289)*. This led to establishment of a two-stage model for carcinogenesis *(290)* where the carcinogen is applied in a single subthreshold dose (initiation) followed by application of the croton oil repeated at intervals (promotion) leading to papilloma formation. The effect was found to occur in mouse but not rat, rabbit or guinea pig skin *(291)*. Subsequently the phorbol 12,13 diesters were found to be the agents in croton oil responsible for its tumour promoting activity *(122)*, with TPA being the most potent. Tumour-promoting phorbol esters have been found in derivatives of the Daphnane and Ingenane phorbol esters as well as the Tiglianes *(See Table 1F)*.

The promotion stage has been further subdivided into stage 1 and stage 2 promotion (progression/conversion *(280,293)*, thus if the doses of a complete promoter (e.g. TPA) were limited to give a low tumour induction, doses of a weak promoter (e.g. Mezerein) following would give an equal response as that of high TPA doses *(206)*. At a cellular level the complete tumour promoter TPA induces several distinct changes in both the mouse dermis and epidermis *(294)* including a persistant hyperplasia, a decrease in Thy-1+ epidermal

Table 1F.Examples of biological effects elicited by a selection of phorbol esters.

Phorbol Ester.	Relative potency as a tumour promoter.(a)	Irritancy I.D.50 (n.moles)(b)	Lymphocyte mitogenic action E.C.40 (ng/ml)(c)	Platelet aggregation E.D.50 (μM)(d)	Comitogenic action relative to TPA.(e)
Resiniferatoxin (Rx)	-	0.00021	0	0	N/D
Doppa	-	0.075	560	0	0.97
DOPP	-	0.064	45	0.4	1.0
Thymelia Toxin A (Thy Tox A)	+/- complete ++ 2nd stage	0.070	N/D	0.3	N/D
Sapintoxin A (Sap A)	-	0.042	35	1.8	1.0
TPA	++++ complete	0.016	6	0.3	1.0

(a)Initiation /promotion standard assay on NMR1 mice with DMBA as initiator.
(b)Irritant dose for 50% inflammation (reddening) of mouse ear after 24 hours.
(c)Dose for 40% of Maximal increase of human lymphocyte mitogenesis as optimised by TPA.
(d)Relative mitogenic effect,compared with TPA, on mixed allogeneic human lymphocytes.
(e)Concentration required for 50% of the maximum aggregation response in human platelets.

(Compiled from refs 191,280,281,282,292)

dendritic cells, an increase in dark keratinocytes and infiltration of the area with small mononuclear lymphocytes, neutrophils and macrophages. The stage 2 promoter mezerein, in comparison induced only slow, transient hyperplasia, did not affect Thy-1+ cell levels but increased 1aP+ epidermal dendritic cell levels and induced infiltration of eosinophil lymphocytes. The initial application of a stage 1 promoter has some permanent effects since the commencement of stage 2 promoter application could be delayed for up to 2 months without impairing the response. However if the time between repetition of the stage 2 application was too long (allowing hyperplasia to reverse) the tumour response greatly diminished *(295)*. The possible involvement of various cell types (e.g. phargocytic cells stimulated by TPA to produce reactive oxygen radicals) supplementing the direct epidermal cell effects of the promoting phorbol esters has been suggested *(296)*.

The observation that PKC is a major cellular receptor for the phorbol esters has led to investigation of its involvement in tumour promotion. Using normal and TPA-resistant mice strains no differences were apparent in epidermal PKC levels or activities *(187,297)*. Not all PKC activators are able to promote tumours however, e.g. Sapintoxin A was efficient at activating heterogenous preparations of PKC <u>in vitro</u> but was totally inactive as a stage 1 or a stage 2 promoter unless applied with the calcium ionophore A23187 *(190,232)*. Conversely the sesquiterpene lactone, Thapsigargin, is a tumour promoter which does not activate PKC but has been found to raise cell calcium by inhibition of the endoplasmic reticulum calcium

ATP-ase *(285)*. While PKC activation alone therefore, seems insufficient for tumour promotion, there is however much evidence implicating PKC as playing a supportive role in the transformation of cells. Non-phorbol activators of PKC such as Teleocidin and Aplysiatoxin have been found to be potent tumour promoters *(188,298)* and DiC_8 is a potent stage 2 promoter in mouse skin *(299)*. Cancer chemotherapeutic drugs (e.g. Nonsteroidal antiestrogens such as tamoxifen, and vitamin A analogues such as retinal) have been found to inhibit PKC-activity in vitro *(300,289)* and many anti-inflammatory drugs (e.g. retinoic acid, indomethacin and Triterpenoids) will protect against tumour promotion in vivo *(300,289)*. Similarly a metabolite of AMGP (1-O-alkyl-2-O-methylglycero-3-phosphocholine) an anti-tumour agent has been shown to inhibit PKC activity *(74)*. Point mutations of the α-PKC gene induced by ultraviolet radiation, have been found to induce transformation of mouse Balb/c 3T3 fibroblasts and enable those cells to induce tumours in immunologically 'nude' mice *(301)*. The effect correlated with increased levels and activity of PKC in the transformed cells as has been reported in ultraviolet irradiation of normal cells *(302)*. Increase of diacylglycerol levels have been observed in kidney or fibroblast transformed by Kirsten and Simian sarcoma retroviruses (ras-genes) *(303,304)* and modification of the PI cycle is observed in rous and avian sarcoma virus transformed cells *(108,109)* suggesting that alterations in the PI/PKC pathway may be one mechanism for oncogenesis.

Tumour promotion by the phorbol esters appears to reflect their ability to induce complex interactions at both the cellular level

(between different cell types) and the biochemical level (involving the PKC signalling machinery).

SECTION F3: INFLAMMATION

The irritancy of phorbol esters manifests itself in severe reddening and blistering of the skin when applied externally, and a burning sensation on mucous membranes, intestinal pain and severe purging/vomiting if ingested *(123)*. Observation of mouse ear erythema caused by single applications of irritant phorbol esters detected two types of response. A transient response (e.g. as elicited by resiniferatoxin with 100 times the potency of TPA) peaking within 1-2 hours and then declining to normal and a persistant response (e.g. as elicited by DOPP) evident in one hour and able to remain for 24 hours *(See Table 1F and 305)*. TPA was able to achieve either non-persistent (at low doses) or persistant (at high doses) responses *(306)*. Examination of phorbol ester effects at a cellular level in rabbit skin detected vasoconstriction *(281)* whereas in mouse skin vasodilation, oedema and cellular infiltration were evident *(307)*. . The response as monitored by ear thickness indicated that oedema peaked within 6 hours for TPA and declined over 24 hours whereas that of resiniferatoxin peaked within 1 hour and had disappeared within 4 hours *(308)*. The possibility of a neurogenic effect for the phorbol esters, by causing polymodal nociceptors to release multiple peptides (such as substance P, a PI cycle agonist) which then potently mediate neurogenic inflammation *(309,310)* has been suggested *(311)*. Resinferatoxin (RX) has been suggested to act as an 'ultrapotent' analogue of capsaicin (a pungent compound from hot capsicum peppers) which elicits inflammation by neuropeptide release *(312,313)* in experiments on an isolated population of dorsal

root ganglion neurones which were voltage-clamped both RX at 2 nM and capsaicin at 300 nM could induce the opening of an unidentified mono/divalent ion channel, without PKC involvement, causing cell depolarisation *(314)* and in the case of RX, osmotic death follows. However while pretreatment of mouse ear with RX causes densitization to RX and capsaicin-induced oedema *(312)*, capsaicin pretreatment only inhibited early (transient) erythema induced by RX and had an enhancing effect on persistent erythema *(315)*. Higher doses of RX could reduce the latency time to erythema and caused the erythema to be prolonged (over 12 hours) whereas higher doses of other irritant phorbol esters (e.g. Sapintoxin D) only prolonged the duration and had little effect on latency time. Inflammation appears to be a complex interaction recruiting endogenous mediators from neurogenic and inflammatory cell sources which synergise in the final observable response *(e.g. See Section F8)*.

SECTION F4: MITOGENIC EFFECTS AND LYMPHOCYTE ACTIVATION

Among the biological activities of phorbol esters their role in mitogenesis in cells is under intense investigation. Phorbol esters have been found to promote DNA synthesis and induce proliferation in skin *(316)*, cultured β- and T-lymphocytes *(55,317,318,319)* and fibroblasts *(153)*. Additionally phorbol esters induce macrophages to produce a variety of cytokines (monokines and lymphokines) which themselves act on lymphocyte populations as soluble mediators and mitogens *(320,321)*. Without accessory cells (macrophages and monocytes) TPA-induced mitogenic activity in lymphocyte cultures is poor, but addition of calcium ionophores *(322)*, plant-derived lectins *(323,324)*, or cytokines (e.g Interleukin -1 *(320)*) can serve to augment the response. It has thus been possible to dissect components of lymphocyte activation and mitogenesis at a biochemical level by study and manipulation of physiological activators (such as antigens and antibodies), phorbol esters and other PKC activators, calcium levels and plant lectins.

Lectins such as concanavalin A (CON A), phytohaemagglutanin (PHA) and pokeweed mitogen (PWM) act by coupling surface sugars (attached to protein as glycoproteins) and mimicking the activation process of physiological ligands. PHA and CON A have been found to enhance PI turnover and raise intracellular calcium *(325,326)* - factors implicated in T-lymphocyte activation *(91)*. It has been found that mimicking the intracellular calcium rise ([Ca}i rise) with calcium ionophores (Ionomycin or A23187) in mouse T-cell cultures does not

stimulate mitogenesis in the absence of TPA *(322)*. Combined, these agents induce interleukin-2 (IL-2) to appear in the culture after 24 hours which is responsible for driving mitogenesis. TPA also enhanced IL-2 production induced by CON A. Concomitant with IL-2 production is the appearance of the surface IL-2 receptor (IL-2R) through which the ligand exerts its mitogenic effect. Experiments on an IL-2 dependant clone (CTL-L) demonstrated that CON A or combined TPA + calcium ionophore could not bypass this requirement for IL-2. Similarly in purified guinea-pig T-lymphocytes TPA could only induce proliferation in conjunction with PHA *(323)*.

In human T-lymphocytes calcium rise was not found to be obligatory in the proliferative response *(327)*. Purified T-lymphocytes, in the absence of macrophages, were unable to proliferate with PHA if EGTA was present in the medium or if a mitogen was used which does not raise [Ca}i (e.g. SpA from Staphylcoccus aureus bacillus). TPA however could restore the response without inducing a calcium rise *(328,329)*.

In heterogenous lymphocyte cultures from peripheral blood, the mitogenic effects of phorbol esters have been found to vary between species *(319)*. Thus TPA on its own is mitogenic in Chimpanzee, Marmoset, Baboon, Rhesus monkey and Human cultures but not those from Guinea pig, Rat, Mouse and Dog. Mouse thymocytes, however, are mitogenically responsive to TPA alone *(330)*. Further work showed that TPA stimulated (sub-optimal) proliferation through an IL-2 independent pathway in human T cell-enriched cultures *(317,298)*.

While cells were induced to express IL-2R and Transferrin receptors no IL-2 was detectable in the medium. In heterogenous human lymphocytes application of non-mitogenic doses of CON A, PHA or TPA also did not induce IL-2 production and cells entered and remained in the early G1 phase of the cell cycle *(331)*. Optimum doses led to progression to late G1 phase where induction of IL-2 synthesis and sufficient production to drive cells into the S phase could occur *(332)*. Higher doses still had the effect of accumulating cells in the late G1 phase, the sub-optimal proliferation being associated with the excess IL-2 in the medium. A balance of mechanisms involving IL-2 and IL-2R production and expression appear to be vital to proliferation in T lymphocytes, but phorbol esters can bypass those mechanisms to some extent.

In mouse β-cells synergy between calcium ionophore and TPA mimicks the Anti-Immunoglobulin-antibody (Anti-IgD) stimulation of proliferation *(55)*. Down-regulation of PKC abrogates the Anti-IgD proliferative signal but other early responses are still observed and proliferation can be restored by 8-methylguanosine. The suggestion that multivalency of proliferative pathways are present in lymphocytes is supported by experiments replacing serum in the culture medium with growth factors (e.g. Insulin, EGF, FDGF, PGE_2 and cAMP analogues) and demonstrating stimulation of proliferation with TPA in mouse 3T3 fibroblasts and epidermal cells, and human embryonic fibroblasts *(173,333)*.

Crosslinking experiments to human T-cell surface antigens using 'double antibodies' *(334)* showed that [Ca]i increased in many cases. Only the Ti/CD3 receptor complex was proliferative when crosslinked and showed 2 different [Ca]i rises, while CD2 and Tp44 antigen crosslinking caused IL-2R expression and proliferation with the addition of TPA. Other work demonstrated that CON A binds to the Ti/CD3 receptor and at super-optimal doses, crosslinks this antigen *(335)* and reduces proliferation. Succinyl-CON A was found to bind but not to crosslink to Ti/CD3 complex and exerted no proliferative inhibition even at high doses. TPA furthermore, could reduce proliferation at the optimal dose of CON A but not succinyl-CON A. The Ti/CD3 receptor and the CD2 antigen are linked to PI turnover in T-cells *(53)* and the translocation of PKC has been found to be induced by PHA (transiently) and TPA (sustained) in human T-lymphocytes *(326)*. The TPA-induced translocation is dose dependent and correlated with mitogenic activity *(317)*. Interleukin-2 has also been shown to promote transient translocation of PKC in mouse CT6 cells (IL-2 dependent clone) *(69)*. However in cells lacking PKC, IL-2 is still able to induce proliferation *(70)* suggesting that although PKC is required in early 'priming' of T-cells, it cannot complete the proliferation process without other pathways (e.g. supplied by serum, acessory cells or calcium).

The effectiveness of phorbol esters on mitogenesis does not correlate with the ability and degree of tumour promoting activity as has been suggested *(324,336)* since both promoting and non-promoting phorbol esters are mitogenic in human lymphocyte cultures *(292 see*

Table 1F). The non promoting phorbol esters DOPPA and SAP A could induce 95% and 70% of the mitogenic effect of TPA. Similarly these effects of DOPPA do not fit in with its poor activation of PKC in vitro. In co-mitogenic experiments on allogeneic mixed lymphocyte cultures, DOPPA was more potent then SAP A or TPA in achieving the maximal response. Inhibition of phorbol ester induced mitogenesis by dexamethazone (a glucocorticosteroid selectively inhibiting IL-2 production *(337)*) was similar for these compounds whereas cyclosporin A (a fungal metabolite) was ineffective at inhibiting TPA response (10%) at a concentration that inhibited 50% of the Doppa response *(292)*. Further experiments indicated that cyclosporin A could not negate proliferation induced by both Doppa and TPA in IL-2 dependent human and (CTLL) mouse T-lymphocyte clones *(338,325)*. It is thought that cyclosporin A acts in a different manner on IL-2 systems than dexamethazone by modifying the IL-2R mechanism so that cells fail to acquire responsiveness to IL-2 at concentrations where IL-2 production is unaffected *(339)*. Other effects include inhibition of lymphokine production and diminished responsiveness of T-helper population to Interleukin-1 *(340,341)*. This implies that non-promoting phorbol esters may mediate mitogenic effects only through accessory cell mechanisms. Phorbol esters have also been found to influence growth and activation of lymphocyte specialized subpopulations (e.g. LAK (lymphokine activated killer) T-lymphocytes *(342)*, Lymphnode suppressor T-lymphocytes *(343)* and cytotoxic T - lymphocytes *(344)*).

SECTION F5: DIFFERENTIATION

In certain transformed or leukemic cell lines derived from pro-erythroid cells the effects of the phorbol esters integrate with the stage of differentation or maturation which the cell has reached and exerts effects reflecting the different developmental histories of these cell lines.

Phorbol esters have different and opposite effects on virally transformed cells e.g. inhibiting the differentation of Friend-virus transformed pro-erythroid cells yet inducing the differentation of Friend Rauscher-virus transformed lines *(345)*. TPA effectively blocks the H.M.B.A. induced differentation of Friend erythroleukemia cells as long as it is present *(346)*. The mechanism is uncertain since a TPA-resistant clone exhibited normal levels and activity of PKC.

Growth inhibition is a common response in many cell lines treated with TPA (e.g. Human proleukemic cells (HL-60) *(347,348)*, human colon cancer cell lines *(349)*, U937 monocytic leukemia cell line *(350,196)* and Daudi human β-cell line *(351)*). Not all of these cells undergo differentation as a result (e.g. Daudi cells appear to be arrested in G1/G0 phase of the cell cycle). In HL-60 cells differentiation has been linked with the translocation and down-regulation of PKC induced by TPA, since resistant strains did not exhibit such a decline *(352,155)*. Furthermore Bryostatin which actively translocates PKC but does not induce down-regulation, does not induce HL-60 cells to

differentiate and blocks the effect of TPA *(181)*. Immunocytochemical studies indicated that differentiating action of TPA coincided with the ability to induce outward translocation to the plasma membrane whereas in other fibroblastic and leukemic cell lines resistant to differentiation, TPA induced inward (nuclear) translocation *(157,153)*. More recently temporal studies on HL-60 cells found that after outward translocation, PKC subsequently translocated to the nuclear compartment for the duration of differentiation *(154)*. Cooperation with other signalling systems has been observed during HL-60 differentiation *(353)*. OAG, even when frequently applied to compensate for metabolic loss, was unable to induce differentiation in HL-60 cells *(193,146)*. The effects of phorbol esters on this system are not confined to tumour promoting derivatives *(354)*. Weak promoters (e.g. Thymeleatoxin A) and non-promoters (e.g. Sap A and DOPP) could equally induce differentiation while DOPPA (a non promoter and weak PKC activator) had this effect at high doses. Resiniferatoxin (of limited biological activity) was unable to induce differentiation but shared the effect of immediate inhibition of cell growth/division with the other phorbol esters above.

In normal (i.e. non-leukemic)β-lymphocytes, translocation of PKC is also observed following treatment with cAMP analogues or anti Ia-antigen antibodies which cause differentiation *(29)*. Immunohistochemical analysis demonstrated a transient translocation to the nucleus as well as the plasma membrane, suggesting the inward translocation plays an important role in differentiation whereas outward movement alone was a proliferation response involving the PI

cycle. In mouse erythroleukemia cell lines induced to differentiate by HMBA, the down regulation and disappearance of α and β -PKC isozymes is found to occur *(267)*. The generation of PKM seems vital here since in TPA-resistant lines, PKM persits whereas in sensitive cells TPA removes PKC rapidly from the cell.

SECTION F6: NUCLEAR EVENTS

Phorbol esters have been found to alter gene expression in many systems, e.g. in mouse skin c-fos, c-myc and ornithine decarboxylase genes are induced by TPA as detected by increased mRNA levels *(316)* within 1/2-2 hours. The effect of TPA in several cell types is also to cause gene suppression as well as gene induction *(See Table 1G)*. These effects are not believed to be due to direct interaction of phorbol esters with the DNA molecule *(296,289)*. The events which link activation of PKC and the nuclear and genetic events which follow are uncertain, and the elements (whether PKC, or fragment of PKC or a substrate) which transduce the message are unknown. TPA has been found to increase the activity of viral enhancer elements (e.g. SV-40 and HTLV-1) and cellular gene promoter sequences (e.g. for H-ras and prolactin genes) through cellular factors *(355,356,357,358)*. It has been found that genes inducable by TPA action have one or more TPA responsive elements (TRE's) consisting of short stretches of bases which act as recognition sites on the DNA for at least 3 trans-acting factors. (Activating proteins in the cell). TPA treatment appears to induce or suppress gene transcription by a combination of (1) modification of transcriptional stimulatory activity of DNA-bound activating proteins (2) modification of the DNA affinity of soluble activating proteins. Induction of cellular proto-oncogenes (e.g. c-fos, c-myc, c-sis) could be responsible for potentiation of initiating carcinogens by tumour promoting phorbol esters. The cystine-rich structure of PKC has been suggested to bear homology to the DNA binding regions ('zinc fingers') of other DNA binding

Table 1G. Modification of gene transcription by TPA.

Tissue or Cell.	Genes induced (+) or supressed (-).	Refs.
Mouse skin	+ c-fos,c-myc, ornithine decarboxylase	*187,316*
Virally infected cells lymphoblastoid lines (*eg* Raji)	+ Epstein-Barr and oncogenic herpes viral antigens	*119,280, 359,360*
Rat endothelial cells	+ class 2 MHC antigens	*68*
Chicken embryonic fibroblasts (CEF)	- CEF 10 gene	*361*
Daudi lymphoblastoid cell line	- p34 protein kinase	*351*
Fibroblast (L929) cell line	+ nerve growth factor	*214*
Friend erythro-leukemia cell line	- globin	*167,346*
K562 erythro-leukemia cell line	+ vimentin,actin - 2 glycoproteins	*16,362*
HL-60 leukemic cell line	+ c-fos	*363*
U937 monocytic cell line	+ c-fos,collagenase IL-2 receptor	*145,350, 363*
Medullary thyroid carcinoma	+ calcitonin - c-myc	*364*
Fibroblast (swiss 3T3) cell line	+ c-fos,c-myc	*365*
HTLV-infected T-lymphocytes	+ p40	*357*
Pituitary (GH_4) cell line	+prolactin	*276*
Skin papillomas	+ transin	*366*
T-lymphocytes	+ CD2,CD5 antigens	*367*

proteins *(249,368)*. Simultaneous transfection of a marker gene linked to TRE sequences and plasmids with either PKC or PKM into rat fibroblast cells, showed that the marker gene transcription could be induced by PKM but not by PKC *(369)* suggesting the DNA binding motif was not necessary to promote gene expression and that active PKC activity was required. Several substates of PKC which could transduce the message from PKC to the nuclear machinery have been postulated. These include ribosomal S6 Kinase *(370)* and topoisomerase (since TPA induced differentiation in serum-starved chicken embryonic fibroblast cells is blocked by the topoisomerase inhibitor Novobiocin *(371)*) *See Phosphorylation Section G.*

Gene expression changes depending on the state of the cell cycle (e.g. whether genetically programmed towards differentiation or growth), hence phorbol esters will affect gene transcription and processing according to the cellular factors then present in the cell. Induction of gene expression has been found with non-promoters and weak activators of PKC in addition to TPA in Raji cells (as measured by Epstein - Barr virus early antigen induction *(359)*), indicating that the correlation with tumour promoting ability was poor.

SECTION F7: SECRETION

Phorbol esters have a variety of effects on cellular secretion. Some of these effects are slow, taking many hours to manifest themselves (e.g. enhancement of collagenase secretion in U937 monoblast line *(350)*, Interleukin -1 in macrophages *(320)*) and reflect the induction of genes, or indirectly reflect the influence of cells communicating amongst each other (e.g. the suppression of immunoglobulin secretion in lymphocyte preparations *(372)*). Rapid effects are seen in (A) cells with specific secretory functions whose actions are primed and occur through exocytosis and (B) cells which secrete excess metabolites as they accumulate. Exocytosis has been most extensively studied in platelets and neutrophils and invoke in each, different mechanisms. For some secretion effects of phorbol esters *see Table 1H*.

In neutrophils production of oxygen radicals and secretion of lysosomal enzymes are associated with the activation -response (an antimicrobial phargocytic defence of the host) elicited by TPA *(383)*. Calcium ionophores were found to be poor effectors of oxygen radical generation and, with TPA present at optimum levels, elicited little increase *(385)*. However high levels of calcium or TPA alone could effect granule secretion containing numerous lysosomal enzymes *(382)*. TPA treatment has been linked to the phosphorylation of a protein termed P47 (Mr 47Kd), a component of the complex NADPH oxidase system *(74,386)*. The phosphorylation resulted in the protein translocating to the membrane fraction and the concommitant assembly and activation

Table 1H. Examples of phorbol ester induced secretion

Secreted substance.	Tissue or Cell.	Calcium requirement	Refs.
Acetylcholine	Neuromuscular junction	Required	*373*
Aldosterone	Adrenal glomerulosa	Synergy	*374*
Amylase	Pancreatic acini	Nil	*275*
Arachidonic acid	Macrophages,kidney cells Mast cells	Nil Required	*48,166,* *314*
Catecholamines	Adrenal chromaffin cells	Synergy	*233*
Collagenase	U937 monocytes, Fibroblasts	Nil	*350*
Dopamine	Foetal brain neurones	Required	*375*
Histamine	Mast cells	Required	*376*
Insulin	Pancreatic islets Adrenal cell line	Synergy Nil	*374* *86*
Interleukin-1	Macrophages	Nil	*320*
Leukotrienes	Macrophages	Required	*166*
Prolactin	GH_4 pituitary cells	Nil	*276*
Prostaglandins	Macrophages,rheumatoid synovial cells,mast cells	Nil	*282,341* *166*
Thromboxanes	Platelets	Required	*377*
Transferable aggregating substance (TAS)	Platelets	Synergy	*281,378*
α Granules: (Serotonin,ATP, Platelet factor 4 β-Thromboglobulin 5-Hydroxy -tryptamine	Platelets	Synergy	*43,80,* *210,379,* *380,381*
Superoxide (O_2^-) and H_2O_2	Neutrophils,macrophages	Nil	*227,382,* *383*
Azurophil granules Secretory granules Specific granules	Neutrophils	Synergy	*159,199,* *384*

of the oxygen radical generating apparatus *(387)*. Interestingly fMLP (N-formyl-methionyl-leucyl-phenylanaline, a physiological chemoattractant which elicits exocytosis and to a lesser extent superoxide generation) did not appear to generate superoxide by P47 phosphorylation at low doses *(74)* suggesting the existance of other pathways. The generation of PKM has been suggested as a route for TPA and fMLP to induce exocytosis of neutrophil granules *(159)*, however when calcium has been reduced to 10nM (where fMLP and presumably calpain is ineffective) the effects of TPA and IgG-or C3b/b1- opsonised yeast are barely diminished *(199,384)*. The involvement of non-PI cycle pathways in the process could account for this.

In platelets, natural agonists (e.g. Thrombin, vasopressin) have been found to elicit secretion (from dense α-granules or from the cytosol) and platelet aggregation through the PI cycle turnover *(379)*. PKC activators such as phorbol esters and diacylglycerol analogues as well as inhibitors of diacylglycerol breakdown are effective in amplification of these responses *(80)*. At a biochemical level the responses are believed to be mediated through contractile activity in the platelet which can be modified by several kinase systems by phosphorylation of proteins in the 20 Kd and 40 Kd range *(210,381)*. Although there is synergy in the response with calcium ionophore, the phorbol esters and diacylglycerol analogues can elicit these responses without a rise in cytosolic calcium *(43,380)*. Recently the phorbol esters were found to induce the secretion of a potent agent from human but not rabbit platelets that would itself

cause aggregation (termed Transferable Aggregating Substance, TAS) acting through a novel mechanism *(281,378)*. These responses can be elicited by non-promoting phorbol esters (e.g. SAP A and DOPP) but are not elicited by DOPPA or RX suggesting no obvious correlation with other biological actions of the phorbol esters. DOPPA has been found to synergise with calcium ionophore in phosphorylation of the 40Kd substrate to maximum TPA levels in vivo *(277)*. It was suggested that this reflected the presence of a PKC isozyme which DOPPA could activate only with calcium.

Recently it was suggested that a putative G protein (termed G_E) may be involved in mediating exocytosis reactions induced by both arms of the PI turnover *(41)*. The regulatory portion of PKC itself possesses homology with a protein implicated in membrane-membrane interactions in synaptic secretion *(388)* but a uniform mechanism is not clear at present.

SECTION F8: ARACHIDONIC ACID METABOLISM

TPA has been found to influence arachidonic acid metabolism and prostaglandin production in many cell types. In dog kidney cells (MDCK cells) TPA stimulates deacylation of cellular phospholipids through enhanced Phospholipase A_2 activity resulting in arachidonic acid production and release, and subsequent prostaglandin production and release *(48)*. In platelets Calcium ionophore A23187 - induced production of thromboxane is enhanced by TPA thought to be through suppression of enzymes involved in resynthesizing phospholipids (thus causing the accumulation of arachidonic acid *(377)*). Effects on PLA_2 through a PKC substrate Lipocortin *(389)* or on phospholipase D *(75,390)* have also been suggested. The increased arachidonic acid appears to be derived from the middle acyl group of diacylglycerol during the breakdown and cycling of PI components *(45,391)*. In mouse macrophages TPA alone is sufficient to stimulate prostaglandin synthesis and synergises with calcium ionophore to stimulate thromboxane synthesis from the increased arachidonic acid pool *(166)*. Such evidence points to a central involvement of PKC in eicosanoid synthesis *(See Fig 1h)* implicating these biologically active derivatives of arach idonic acid in inflammation, and allergic reaction (lipoxygenase derived compounds); smooth muscle contraction and platelet aggregation (cycloxygenase derived compounds) *(50,125,391,392,393)* and possibly tumour promotion *(300)*. Recently TPA has been found to greatly increase cell 1,2 diacylglycerol content with phosphatidylcholine (PC) as the source through stimulation of choline lipid metabolism *(146,390,394,395)*. The

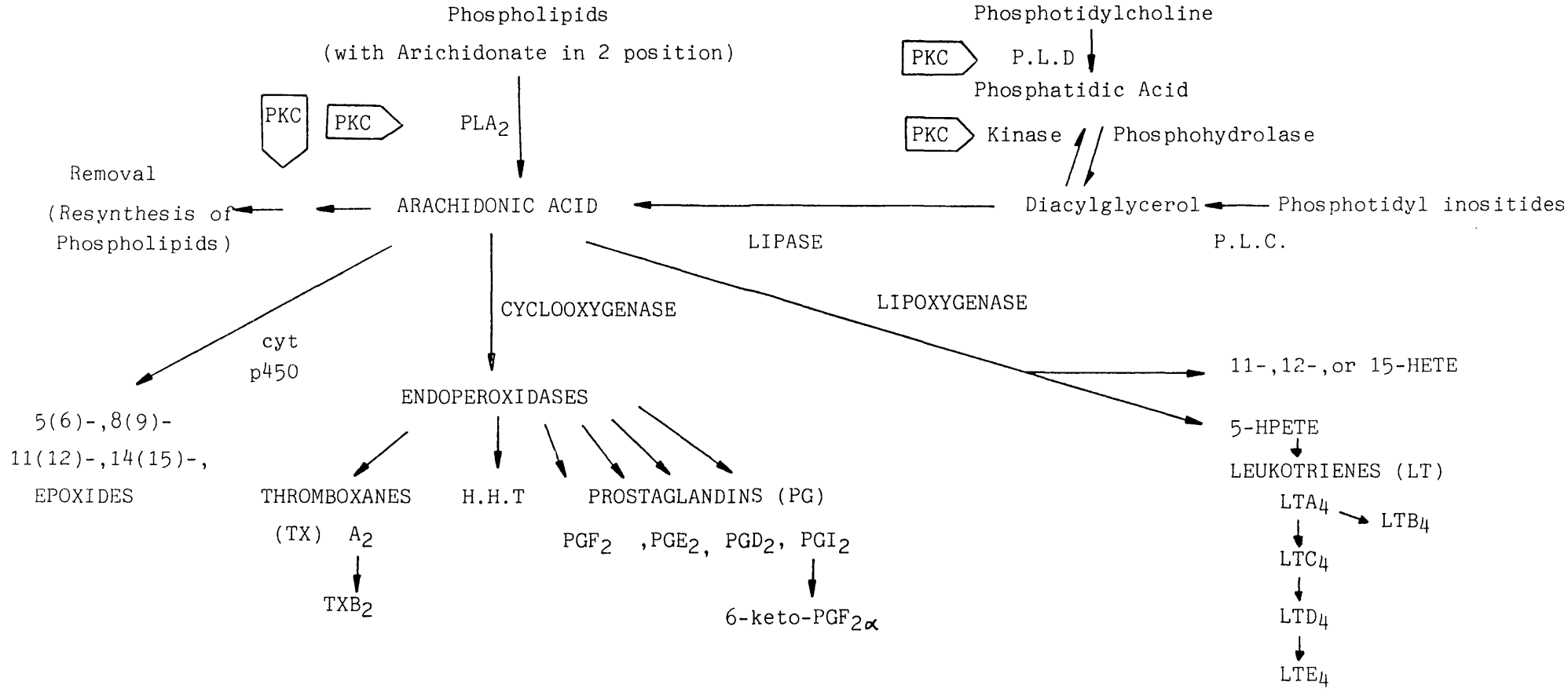


Fig 1h. Arachidonic acid and eicosanoid metabolism.

Arachidonic acid liberated from phospholipids is metabolised to prostaglandins (PG),prostacyclin, thromboxanes (Tx),leukotrienes (LT) and epoxides.Arachidonic acid is metabolised by cyclooxygenase, lipoxygenase and cytochrome p450 to produce biologically active compounds.The possible involvement of protein kinase C (PKC) are shown.(from ref *377,391*)

specific messenger effects of phosphatidylcholine turnover and the increased diacylglycerol, also noted with other hormones, (e.g. Interleukin -1 *(117)* and growth hormone *(396)*) are unknown.

SECTION F9: IONIC AND ELECTROPHYSIOLOGICAL EFFECTS

The phorbol esters have been found to influence ionic parameters in cells specialised for synaptic transmission as well as non exitable cells. In hippocampal neurones the phorbol esters induce longterm potentiation (LTP) (potentiation of neurone firing resulting from exitatory high-frequency stimulation *(397,398)*) which has been linked to the blockade of two persistant Potassium (K+) channels (termed I_{K-Ca} and I_K) as well as a reduction of N(and L) - type calcium currents *(399)*. The translocation of dendritic PKC has been implicated in the mediation of these responses *(400)*, possibly through the persistent phosphorylation of a protein termed neuromodulin *(401)*, and could be mimicked with intracellular injection of active PKC *(402)*. Evidence that PKC mediates a decrease in calcium currents has also been obtained in snail neurones (with cholecystokinin a PI agonist, TPA and injected PKC *(58)*), in dorsal root ganglia (with phorbol ester and OAG *(403)*) and in mouse neurones (with TPA *(52)*). However the opposite effect on calcium current has been found in Aplysia bag cells and Hermissenda photoreceptors treated with phorbol ester *(404,405)* showing that extensive variation exists in neuronal cell response to PKC activation (reviewed *406)*. The enhancement by phorbol esters of electrically stimulated neurotransmitter release in a variety of neuronal tissue and exitable cells has also been reported *(373,375,407,408)*. PKC mediated modulation of ion channels has been suggested as a possible mechanism in cell types where calcium and potassium conductances are central to their neurophysiology and the putative L-type calcium channel has

been found to be a PKC substrate in vitro *(409)*. Recently, however PKC activators OAG and TPA were found to be effective in reducing calcium curents when applied externally to chicken dorsal root ganglia cells but not when applied internally *(170)*. Furthermore, the inactive phorbol derivative 4-α-PDD which does not activate PKC, was as effective as TPA, and PKC inhibitors were ineffective at abolishing the response. Some of the ionic effects of the phorbol esters may therefore be independent of PKC activation.

Phorbol esters have also been found to change calcium flux in some non-neurological exitable cells. While TPA had no effects on resting calcium levels in an Insulin secreting cell line, it inhibited depolarization-induced calcium influx through a voltage-gated calcium channel *(408)*. In smooth muscle cells calcium 'transients' induced by depolarization were increased by TPA, involving a Na^+/Ca^{2+} antiport mechanism *(410)*.

Many non-exitable cells such as thymocytes, fibroblasts, HL-60 cells, neuroblastoma cells, and T-lymphocytes (although not all, *411*) shown no calcium change but do show a slight alkalinization of the cytosol when treated with TPA, OAG or DiC_8, *(198,317,329,412,413)* thought to be generated through a Na^+/H^+ antiport mechanism. The increase in cell Na^+ subsequently leads to stimulation of the Na^+/K^+ antiport is swiss 3T3 fibroblasts indicating that PKC is involved indirectly in the flux of several monovalent ions *(414)*. Similar fluxes without PKC involvement are characteristics of many growth factors *(164,415)*. Interestingly DiC_8 at doses of 12.5 μM was found

to induce calcium release from cytosolic stores and acidification through PKC indpendent mechanisms *(172)*.

Indirectly phorbol esters can negatively regulate calcium rises and other ionic changes induced by stimuli through interaction with the stimulus receptor (transmodulation *see Section F10*).

SECTION F10: RECEPTOR TRANSMODULATION

Experiments using combinations of physiological ligands (especially growth factos) and phorbol esters have revealed an important role for PKC in alteration of the cullular response to many physiological ligands (termed receptor transmodulation *See Table II*).

Many of the effects of the phorbol esters appear to be of a negative feedback moiety on receptors which induce PI turnover but variation in the mechanism between different receptor types and even similar receptor types in different tissues is evident *(rev 57)*. In cases of PKC mediated desensitization the process can be (i) direct desensitization of the receptor (e.g. EGF receptor *(431)* and α-Adrenergic receptor *(417)*) (ii) an indirect uncoupling of the receptor from signalling pathways (e.g. β-Adrenergic receptor is uncoupled from adenylate cyclase in T-cells *(442)*) distal to receptor occupancy, or (iii) a reduction in the number of receptors at the cell surface (e.g. internalization of the occupied or unoccupied transferrin receptor *(443)*). Other effects include increased sensitization - e.g. lymphoma cells experience an enhancement of response to β-Adrenergic agonist when pretreated with TPA *(426)* which appears to be due to a stimulatory effect on G-protein interaction with the adenylate cyclase. In some systems a much greater complexity is evident e.g. Glucagon in hepatocytes operates through two signalling system; primarily through adenylate cyclase and secondly through PI turnover *(63)*. In such a system TPA induces densitization closely followed by resensitization by the putative

Table 1I. Receptors undergoing transmodulation of activity in response to phorbol ester treatment.

Receptor.	Biological system.	Observed response.	Refs.
α-Adrenergic	Hepatocytes Smooth muscle	Inhibition of response, decreased binding.*	*416,417, 418,419, 420*
β-Adrenergic	Avain erythrocytes,Human mononuclear cells	Inhibition of response	*421,422, 423*
β-Adrenergic	Epidermis	Inhibition of response**	*424,425*
β-Adrenergic	Lymphoma (S49)cells	Increased response**	*426*
n-Acetylcholine	Cultured myotubes	Inhibition of response, increased desensitization rate	*427*
m-Acetylcholine	Neuroblastoma	Inhibition of response, increased internalization, degradation,binding (?)	*428,429*
Angiotensin 2	Kidney cells, mesangial cells	Inhibition of response, desensitization of receptor (?)	*57,418*
Epidermal growth factor	Epidermal carcinoma	Inhibition of response, decreased receptor autophosphorylation and binding,decreased receptor/ligand tyrosine kinase activity,conversion of high to low affinity binding sites	*192,430, 431,432*
N-Formyl-methionyl-leucyl-phenylalanine (fMLP)	Neutrophils	Inhibition of response*,**	*433,434*
Glucagon	Hepatocytes	Transient inhibition of response**	*63*
Glucocorticoid	Epidermal cells	Inhibition of response, decreased binding	*418*

(Continued overleaf)

Table 1I. Receptors undergoing transmodulation of activity in response to phorbol ester treatment(cond).

Receptor.	Biological system.	Observed response.	Refs.
Insulin	Hepatoma (HEP G2)	Increase of binding, internalization and processing.	*435*
Insulin	Hepatoma (FAO)	Inhibition of response, decreased receptor tyrosine kinase activity, no change in binding.	*436*
Insulin	Adipocytes	Both increase and inhibition of different ligand responses seen. No change in binding.	*65*
Leukotriene B_4	Neutrophils	Inhibition of response, decrease in binding and high affinity ligand sites.	*434,437*
Somatostatin	Pituitary	Inhibition of response, decreased binding.	*418*
T-cell Ti/CD3	T-lymphocytes	Increased response, increased internalization and cycling of receptor.	*54,151, 212,217, 438,439*
T-cell CD2 and CD5	T-lymphocytes	Increase in numbers, (mRNA synthesis)	*367,439*
T-cell CD4 and CD8	T-lymphocytes	Decrease in numbers.	*212,438*
Thrombin	Platelets	Inhibition of response.	*41,43, 78,209*
Transferrin	Erythroleuk-emic cells (K562,HL-60)	Inhibition of response, decreased numbers of receptor and binding, increased internalization.	*440,441, 443*
Vasopressin 1	Hepatocytes	Inhibition of response.**	*416,418*

*Negative feedback through PKC activation possible in the case of receptors known to stimulate PI turnover.
** No change in ligand/receptor binding characteristics or receptor number observed.

mechanism of PKC deactivating the stimulatory G_s and inhibitory G_i transducer proteins of the adenylate cyclase component. The consequences of transmodulation between phorbol ester-stimulated PKC activity and physiological agonist-receptor systems are largely unknown but the deregulation of cellular systems may contribute to the wider biological effects of the phorbol esters.

SECTION F11: SYSTEM INTERACTION

The major kinase systems discovered (PKCAL, PKA and PKG) are involved in cellular processes in much the same manner as PKC (receptor-ligand recruitment of second messengers). Although the extent and importance of interaction remains largely theoretical, 'crosstalk' is not limited to overlap of kinase activity since many cases of one system influencing agonist-mediated activation of another are apparent. It has been found that in some cells PKC ad PKA signalling systems synergise (e.g. in swiss 3T3 fibroblast mitogenesis *(365)*, in HL-60 cell differentiation *(353)* and in endocrine and platelet secretion *(47)*) whereas in others these systems counteract each other (e.g. mitogenesis in thymocytes *(330)*, IP_3 formation in platelets *41)*). PKG pathway interactions with PKC are more poorly defined. Phorbol ester treatment of T-cells has been found to elevate cGMP levels, activate guanylate cyclase and cGMP Phosphodiesterase *(442,444)*. Use of lipoxygenase inhibitors suggested that these effects were mediated by arachidonic acid metabolites although guanylate cyclase has been found to be activated by PKC in vitro *(445)*. It has been suggested that PKG synergises with PKC in mitogenesis in $\beta$and T-lymphocytes *(55)* although in other cells evidence that cGMP generation plays a negative feedback role on PKC has been found *(45)*.

TPA has a negative effect on cAMP accumulation by β-adrenergic agonists of the PKA system in mouse epidermis *(424,425)* and by PGE_2 in Jurkat leukemia T-cell line *(446)*. Positive effects by TPA are

seen on cAMP accumulation in Jurkat cells responding to Adenosine receptor agonists *(446)* and β-adrenergic agonists in lymphoma cells *(426)* and frog erythrocytes *(447)*. These experiments show that opposite system interactions occur with the same agonist-receptor in different cells and that even in the same cells TPA can show opposite effects on cAMP accumulation by different PKA agonists.

The mechanisms of phorbol ester induced modulation of the cAMP kinase system via PKC has been found to involve not only trans-modulation of receptors *(see Section F10)* but modulation of G protein action, (e.g. phosphorylation and inactivation of the inhibitory pathway Gi, in platelets, *(448,449)* stimulation of G_s in S49 lymphoma cells *(426)* and inhibition of adenylate cyclase action apparently through $\beta\gamma$ subunits in Turkey erythrocytes *(114)*) and adenylate cyclase itself (e.g. activated in pituitary cells *(450)*). Conversely PKA modulates PKC mediated responses in many cells through alteration of IP_3 generation. PKA is found to have both negative and positive effects on phosphorylation patterns induced by TPA in S49 Lymphoma cells, in a study comparing normal and PKA-deficient mutants *(451)* indicating interaction varies at the kinase level. <u>In vitro</u> PKA has been found to decrease the potency of IP_3 to interact with its receptor, and PKA increased the calcium ATPase in the endoplasmic reticulum responsible for sequestering calcium <u>in vivo</u> *(38)*. This could be a mechanism for negatively regulating the PKC system. Effects by cAMP on the translocation of PKC have also been observed in skeletal myoblasts (a decrease in cytosolic PKC *(452)*), HL-60 cells and β-lymphocytes (Inward translocation to the nuclear region

(29,353)) by unknown mechanisms. Further exploration of interactions between the signalling systems in individual cell types will be important to understand the effects of different phorbol esters on tumour promotion, differentiation, mitogenesis and other cellular functions.

SECTION G: PHOSPHORYLATION AND SUBSTRATES OF PKC

The implication of PKC in tumour promotion and mitogenesis, its ubiquitous distribution in tissues across the animal kingdoms and its specific receptor relationship with the phorbol esters, has inspired a great effort to elucidate the physiological substrate proteins over the last decade.

To ascribe a protein as a physiological target for phosphorylation by PKC, it is necessary to critically assess;

A. In vitro stoichiometric phosphorylation of the target protein at a significant rate dependent on the presence of cofactors in conditions necessary for PKC activation.

B. Correlation between the identity of the phosphorylation sites on the protein in vitro with in vivo.

C. Functional differences between the phosphorylated and dephosphorylated form of the protein which can be reversed by protein phosphatase activity.

D. Evidence that activators of PKC induce in vivo phosphorylation through PKC rather than alternative (indirect) pathways.

E. Spatial possibility of the target protein and activated PKC to meet under physiological conditions.

Both in vitro and in vivo experimentation has been used to define substrates for PKC to satisfy these criteria. Radiolabled phosphorous [^{32}P] is utilized to enable detection of phosphorylated

proteins (phosphoproteins).

Intact cells are 'loaded' with radiolabled ortho-phosphate by addition to the culture media until the intracellular phosphate pool has been labled to equilibrium. Cells can then be treated with phorbol esters or diacylglycerol analogues and fractionated to analyse P^{32}-phosphoproteins on SDS-PAGE gels *(453)*. Bands of phosphorylated proteins can be evaluated by autoradiographic scanning and compared with controls (untreated cells or cells treated with inactive phorbol esters eg. 4-α-PDD). This enables the proteins showing enhanced phosphorylation to be focussed on as putative PKC substrates. Examples of such experiments are given on *Table 1J*.

If a putative target protein is characterised well enough enabling antibodies to be raised against it, these can be used to identify phosphoproteins from fractionated cells. In these experiments gels are transferred onto nitrocellulose using Western blotting where they can be detected by double-antibody techniques *(461)*. It is also possible to extract P^{32}-phosphoproteins from crude preparations by double antibody precipitation (e.g. adhering the antibody to protein-A sepharose *(462)*, fixed Staphloccal Aureus bacteria *(463)* or beads coupled with the 2nd antibody *(464)*) prior to running them on SDS-PAGE. Affinity chromatography using target protein characteristics (e.g. a ligand *(465)* or an antibody *(431)*) to retard it in a column. The phosphoprotein, on bands exised from gels, is treated with a protease to sever it into (reproducible) peptides. These are subjected to 2-dimensional electrophoresis to

Table 1J. Some general studies of endogenous phosphorylation enhanced by PKC activators.

Tissue.	System.	Activator used.*	Phosphorlation pattern.	Refs.
Rat liver	Membranes Membranes	TPA/PS Diol/PS	10 Proteins 6 Proteins	*454*
Mouse pre-neoplastic JB6 cells	Cytosol	Diol/PS	13 Proteins	*455*
Human neutrophils	*vivo* Membranes	TPA Ca++/PS	5+ Proteins 5 Proteins	*456* *231*
Human leukemic HL-60 cells	*vivo* *vivo* *vivo*	TPA OAG TPA	2 Proteins 9 Proteins 14 Proteins	*146,* *353*
Rabbit peritoneal neutrophils	Membranes	Diol/PS	6 Proteins	*457*
Canine cardiac ventricle	Sarcolemma membranes	TPA	7 Proteins	*458*
Rat brain	Cytosol	Ca++/PS	6 Proteins	*228*
Rat cerebral cortex	Cytosol	Ca++/PS	6 Proteins	*459*
Mouse β-lymphocytes	*vivo*	TPA	6 Proteins	*460*
Bovine retina rod outer segments	Cytosol	Diol/PS	9 Proteins	*73*
Human T-lymphocytes	*vivo* (membrane)	TPA	7 membrane antigens	*208*

*TPA,tetradecanoylphorbolacetate; Diol,diolein; PS,phosphatidylserine; Ca++,calcium; OAG,oleoylacetylglycerol.

obtain a characteristic 'finger print' of the protein. Fingerprints can then be compared between experiments and individual phosphopeptides can be sequenced to determine which residue has been phosphorylated, or can be hydrolysed to identify which phosphoamino acids are present (e.g. *466*). Such techniques are invaluable when a single protein is subject to multiple phosphorylation by a variety of kinases.

In vitro experiments use purified protein targets accepting ^{32}P from radiolabled ATP catalysed by purified PKC. It is easier to establish kinetic and stoichiometric parameters of the phosphorylation reaction under such conditions, since problems of loaded cell ^{32}P -specific activity or interference from other kinases or phosphatases are largely eliminated. Such experiments can also be used to elucidate the effect of phosphorylation of the target although the extent of endogenous phosphorylation already present must be considered *(467)*. Through such a biochemical approach the physiological effects of PKC activity can be linked with changes in protein target function.

The site of phosphorylation in various substrates has been observed to be a serine (e.g. Neuromodulin *(401)*) or a threonine residue (e.g. EGF-Receptor *(468)*). There does not appear to be a simple consensus sequence which PKC recognises although it is apparent that recognition shows independence from other serine/ threonine kinases *(464)*. In experiments with synthetic substrates, clusters of basic amino acids, (Arginine or lysine) on the carboxyl-

side of the target residue seems a preferred arrangement for cofactor-dependent PKC phosphorylation *(469)*, although a close proximity of amino-side basic residues seems to play an (uncertain) role *(255,470) See Fig 1i* for some sequences.

The numerous substrates claimed for PKC are presented in Tables *1K-O*. The receptor proteins and other stimulus-response coupling proteins *(Table 1K)* have been subject to the greatest scrutiny, involved as they are with the great diversity of phorbol ester action in cell systems. The enzyme *(Table 1L)* and structural *(Table 1M)* substrates are implicated in mediation of cell-specific responses, while the nuclear substrates *(Table 1N)* are (more tentatively) associated with the longer-term effects of PKC activation involving genetic transcription. *Table 10* shows some regularly found substrates which have not been identified.

In vivo investigation of PKC-mediated phosphorylation and effects must proceed with caution. Apart from variation between the tissues, levels of PKC have been found to change between foetal and adult brain *(14,22)* and differences in cofactor requirements between different species (e.g. mammalian and avian kinase *(11)*) have been observed. Localization and quantity of PKC may also vary between phases of the cell division cycle and between transformed and normal cells *(42,463)* and the use of cells down-regulated in PKC as controls, may not abrogate the effects of PKC activators *(167)*. Similarly use of TPA-insensitive cells has not demonstrated uniqueness in endogenous substrates phosphorylated *(455)*, although in certain

Fig 1h. Examples of amino acid sequences phosphorylated by protein kinase C.

C-terminal of IL-2 receptor.(*464*)

Gln-Arg-Arg-Gln-Arg-Lys-**Ser**-Arg-Arg-Thr-Ile

N-terminal of pp60 (v- and c-src).(*468*)

Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Asp-Pro-**Ser**-Gln-Arg-Arg-Arg-Ser-Leu-Glu-Pro-Pro

Calmodulin-binding region of Neuromodulin.(*401*)

Lys-Ala-His-Lys-Ala-Ala-Thr-Lys-Ile-Gln-Ala-**Ser**-Phe-Arg-Gly-His-Thr-Arg-Lys-Lys-Leu-Lys-Gly-Glu-Lys-
-Lys-Gly-Asp-Ala-Pro

Synthetic peptide substrate for PKC-e,also PKC-a (*255*).

Glu-Arg-Met-Arg-Pro-Arg-Lys-Arg-His-Gly-**Ser**-Val-Arg-Arg-Arg-Val

KEY: **SER** = phosphorylated residue.
Lys = basic residue.
Note; N-terminal is on the Left side.

Table 1K. Substrates for protein kinase C:

Receptor and signalling proteins.

Protein	Tissue	Conditions	Effector	Effects of phosphorylation	Refs
n-Acetylcholine receptor.*(nicotinic) (nAChR) α chain 40K δ chain 65K	Torpedo electric organ	*vitro*	TPA	Increased rate of desensitization	*406* *423*
Adenylate cyclase catalytic unit.130K	Bovine brain frog erythrocytes	*vivo/vitro*	TPA	Enhancement of hormone-sensitive activity	*447*
α-Adrenergic receptor* 55K subunit.	Hamster DD1 MF2 cells vas deferens smooth muscle	*vivo/vitro*	TPA	Decreased binding affinity for adrenaline,uncoupling from PI metabolism	*419*
β-Adrenergic receptor* (52K).	Duck/turkey erythrocytes	*vivo*	TPA	Desensitization to agonist,uncoupling from adenylate cyclase activation	*421* *422*
Dihydropyridine receptor.(L-type calcium channel)* 165K,55K	Rabbit skeletal muscle	*vitro*	PS/ Diol	Regulation of calcium channel	*409*
Epidermal growth factor receptor.* (EGFR)170K	Fibroblasts A431 cells (carcinoma)	*vivo/vitro* membranes	TPA PdBu	Decrease in EGFR affinity for EGF, decreased tyrosine kinase activity and autophosphorylation	*192* *261* *430* *431* *471* *472*
G.A.B.A.modulin receptor.*16.5K	Rat brain	synaptosomes. *vitro*	PS/ Ca++	No effect (?)	*473*
Glucose transporter. 50-60K	Human fibroblasts and erythrocytes	*vivo/vitro*	TPA	?	*474* *475*
G-Protein ($G_{i}\alpha$)* free α subunit 41K	Rabbit liver human platelets,S49 lymphoma line	*vitro* membranes	PS/ Ca++	Decreases ability to interact with adenylate cyclase	*51* *56* *92*
G-Protein ($G_{z}\alpha$)* free α subunit 41K	Human platelets	*vivo*	PS/ Ca++	Regulation of signal transduction	*92* *476*
Guanylate cyclase 150K	Rat brain	*vitro*	TPA	Increases activity	*445*
H.L.A.class 1 antigens.* h chains 47K	Human platelets,lymphocytes and HL-60 cells	*vitro*	TPA	?	*477*

(Continued overleaf)

Table 1K. Substrates for protein kinase C:

Receptor and signalling proteins.(cond)

Protein	Tissue	Conditions	Effector	Effects of phosphorylation	Refs.
Insulin receptor*. β subunit 95K	Human placenta β cell line IM-9 Rat liver,FAO hepatoma cells.	*vivo/vitro*	TPA	Decrease in receptor tyrosine kinase activity	*241 436 478*
Interleukin 2 receptor*(T-cell activation antigen ,Tac) 50-55K	Human T-cell line HUT102B2 Transfected mouse C127 line.	*vivo/vitro*	TPA	?	*463 464 479*
Mouse T-cell antigen receptor (TCR) ε chain and δ chain	Mouse T-cell hybridoma 2B4 line.	*vivo*	TPA	Down regulation of the receptor complex	*165*
Ovotransferrin 78K	Chicken oviduct	*vitro*	TPA	?	*11*
Somatomedin C receptor* 92-98K (insulin like growth factor receptor)	Human β cell line IM-9.	*vivo*	TPA	?	*478*
T_3/T-cell antigen receptor (Ti)*. T_3 γ chain 26K CD3 δ chain 21K	Human lympho-blasts and T-cell line HPB-ALL.	*vivo/vitro*	TPA PdBu	Down regulation of the T_3/T-cell receptor complex.	*53, 208 462 but see 197*
Transducin*(Gt,a G-protein). 41K free α subunit in the GDP-bound form	Bovine retina	*vitro*	PS/ Ca++	?	*480*
Transferrin receptor 180K	Human leukemic HL-60 cell line.	*vivo/vitro*	TPA PdBu	Phosphorylation mediates receptor internalization.	*440 441*
Voltage sensitive sodium channel* α subunit 260K	Rat Brain	*vitro,* synapt-osomes	PC/ Ca++	Regulation of the sodium channel.	*481*

*Protein is known target for other protein kinase enzymes.
K=Mr in Kiladaltons (Kd)
TPA,tetradecanoylphorbolacetate; Diol,diolein; PC,phosphatidylcholine; PS,phosphatidylserine; Ca++,calcium; PdBu,phorboldibutyrate.

Table 1L. Substrates for protein kinase C:

Enzyme proteins.

Protein	Tissue	Conditions	Effector	Effects of phosphorylation	Refs.
Acetyl Coenzyme A carboxylase* 230-250K	Rat lactating mammary gland	*vitro*	PS/ Ca++	Inhibition of activity	*482*
ATP-citrate lyase*	Rat lactating mammary gland	*vitro*	PS/ Ca++	No effect (?)	*482*
Cytochrome $p450_{scc}$* 50K	Bovine adrenal cortex	*vitro*	TPA	Increases stereogenesis activity	*483*
Enolase 41K	Rabbit muscle	*vitro*	TPA	Modification of bidirectional enzyme activity	*484*
Glycogen synthase* 85K subunit	Rabbit liver, skeletal muscle.Rat hepatocytes	*vivo/vitro*	PS/ Diol	Inactivation (?)	*485* *486* *but see* *487*
Harvey-ras oncogene product* 21K	Bacterial expression vector	*vitro*	PS/ Ca++	?	*488*
3-Hydroxy-3-methyl -glutaryl Coenzyme A reductase (HMG-CoA reductase)100K (53K)	Rat liver	*vitro*	Diol	Inactivation	*489*
Inositol triphosphate 5'phosphomonoesterase (40K protein,p47) 40K	Rabbit/human platelets	*vivo/vitro*	TPA	Increase of phosphatase activity	*104* *490* *491* *but see* *389*
Neutral protease 74K subunit	Bovine heart	*vitro*	Diol	?	*492*
Phosphofructokinase* 58K (82K)	Bovine heart, Rabbit/rat skeletal muscle Rat liver	*vitro*	PS/ Ca++ Diol	No effect (?)	*493* *494* *but see* *495*
pp60 v-src* 60K	RSV-trans -formed vole, BALB/C and chicken embryo fibroblast cells	*vivo*	TPA	?	*468* *496*

(Continued overleaf)

Table 1L. Substrates for protein kinase C:

Enzyme proteins.(cond)

Protein	Tissue	Conditions	Effector	Effects of phosphorylation	Refs.
pp 60 c-src* 60K	Mink lung, chicken embryo fibroblasts, human platelets.	*vivo*	TPA	?	*465* *468*
Tyrosine hydroxylase* 62K (66K)	Rat brain PC12 cells.	*vitro*	Diol	?	*497*

*Protein is known target for other protein kinase enzymes.
K=Mr in Kiladaltons (Kd)
TPA,tetradecanoylphorbolacetate; Diol,diolein;
PS,phosphatidylserine; Ca++,calcium;

Table 1M. Substrates for protein kinase C: Structural/Contractile proteins.

Protein	Tissue	Conditions	Effector	Effects of phosphorylation	Refs.
Caldesmon*130 K	Chicken sm. muscle.	*vitro*	PS/ Ca++	Decreased activation of myosin light chain kinase	*498*
Calmodulin-binding protein*(CaM-BP) α 130K,β 97K	Human erythrocytes.	*vivo/vitro ghost mem -branes.*	TPA	Modification of membrane skeleton	*499*
C-Protein*145K	Bovine myocardium.	*vitro*	Diol	?	*466*
Cytoskeletal proteins Band 4.1* 78-80K Band 4.9* 45-49K	Human erythrocytes.	*vivo/vitro ghost mem -branes.*	TPA	Modification of membrane skeleton	*461 499 500 501*
Fibrinogen* α chain 60-70K	Human plasma.	*vitro*	Diol	?	*502*
Filamin 68K	Chicken sm. muscle.	*vitro*	TPA	?	*503*
Microtubule associated protein* (MAP-2) 270K	Rat/pig brain.	*vitro*	Diol	Decreases interaction with actin.	*467 504*
Myelin basic protein* 14K,18K	Mammalian brain.	*vivo/vitro*	PS/ Ca++	?	*228 243 505 506*
Myosin light chain* 20K	Human/rabbit platelets,turkey sm.muscle.	*vivo/vitro*	TPA	Modification of activated Mg/ATPase activity	*210 381 507 508*
Myosin light chain kinase* 130K	Turkey sm. muscle.	*vitro*	PS/ Ca++	Decreased kinase activity and Ka for calmodulin	*210 509 510*
Talin 215K	Chicken gizzard.	*vitro*	PS/ Ca++	?	*511*
Troponin I* 27K T* 36K	Bovine heart, rabbit skeletal muscle.	*vitro*	PS/ Ca++	Modulation of contractility	*222 512*
Vinculin* 130K	Chicken embryo fibroblasts,sm. muscle mouse 3T3 cells.	*vivo/vitro*	TPA PdBu	?	*503 513 514*

*Protein is known target for other protein kinase enzymes.
K=Mr in Kiladaltons (Kd)
TPA,tetradecanoylphorbolacetate; Diol,diolein;
PS,phosphatidylserine; Ca++,calcium; PdBu,phorboldibutyrate.

Table 1N. Substrates for protein kinase C:

Nuclear proteins.

Protein	Tissue	Conditions	Effector	Effects of phosphorylation	Refs.
DNA methyl-transferase. 126K	Human placenta.	*vitro*	TPA	Stimulation	*515*
Eukaryotic initiation factors (eIF) 2B* 52K 3 * 120K 3 * 170K 4B* 80K 4F* 220K 4F* 25K	Rabbit reticulocytes.	*vivo/vitro*	TPA, PS/ Ca++	Regulation of mRNA trans-lation (?)	*516* *517* *518* *519* *520*
Histone proteins H-1* 33K H-2B* 33K H-4* 33K	Calf thymus, Rat H-35 hepatoma cells.	*vivo/vitro*	TPA	?	*485* *521* *522* *523*
Ribosomal S6 protein* 40 S subunit	Rat H-35 hepatoma cells mouse C127 cells,chicken embryo fibro-blasts.	*vivo/vitro*	TPA	?	*10* *518* *524* *525* *526*
RNA polymerase 2 subunits 220K 180K 150K	Chicken myo-blastosis leu-kemia cells.	*vitro*	Diol	Modification of RNA synthesis	*527*
Topoisomerase 2* 320K	*Drosophila* embryos.	*vitro*	TPA	Increased activity	*371* but see *528*

*Protein is known target for other protein kinase enzymes.
K=Mr in Kiladaltons (Kd) or Svedbergs (S).
TPA,tetradecanoylphorbolacetate; Diol,diolein;
PS,phosphatidylserine; Ca++,calcium;

Table 10. Substrates for protein kinase C: Miscellaneous and uncharacterized proteins.

Protein	Tissue	Conditions	Effector	Effects of phosphorylation	Refs.
Chromaffin granule binding protein 9 (CB 9) 37K	Rat adrenal medulla.	*vitro,granule membranes*	TPA	Regulation of exocytosis	*529*
Lipocortin * 1 (p35) 35K 2 (p36) 36K (calpactin, lipomodulin)	Bovine lung, kidney(MDBK) line,human AG1523 fibroblast line.	*vivo/vitro*	TPA, Diol	Decreases the inhibition of lipocortin on lipase activity	*490,491, 530,531*
Middle-T antigen* (polyoma viral antigen) 58K	Polyoma infected mouse 3T6 cells.	*vitro*	PS/ Ca++	Stimulates tyrosine kinase activity	*330*
Neuromodulin* (GAP-43,F1,pp46, P57,B50) 24.7K	Bovine/rat brain,hippocampus.	*vivo/vitro synaptosomes*	TPA	Inhibition of calmodulin binding activity, modulation of synaptic plasticity.	*401,532, 533,534*
Phospholamban* 27K	Canine heart sarcolemma.	*vesicles*	TPA	Modification of Ca++ uptake	*458,535*
Retinoid binding proteins 14.6K (retinol;cRBP, retinoic acid;cRABP	Calf thymus, liver.	*vitro*	PS/ Ca++	?	*235*
Stress proteins* (B and C) 28K	Rat embryo fibroblasts.	vivo	TPA	?	*536*
Vitamin D binding protein 56K (G C group specific component)	Rat pancreatic acinar cells.	*vivo/vitro*	PS/ Ca++	?	*537*
32-47K	Neutrophils	*vivo,membranes*	TPA	?	*74,447, 456,538, 539*
80-87K	All tissues	*vivo/vitro*	TPA, PdBu SapA	?	*432,455, 522,540, 541,542, 543,544*
17,37 and 69K	Rat liver mitochondria	vitro	TPA	?	*19*
40K*	Rat adipocytes	*vivo,membranes*	TPA	?	*545*
70K	A341 epidermal carcimoma cells.	*membranes*	Diol	?	*261,430*

*Protein is known target for other protein kinase enzymes.
K=Mr in Kiladaltons (Kd)
TPA,tetradecanoylphorbolacetate; Diol,diolein; SapA,sapintoxin A, PS,phosphatidylserine; Ca++,calcium; PdBu,phorboldibutyrate.

diseased states (e.g. Autosomal chronic granulomatous neutrophils *(546)* and leukemic T-lymphocytes *(221)*) defects in PKC-activator induced phosphorylation has been found. In some cells, activators may not be able to permeate the cell effectively *(491)* and have little effect unless the cell is artificially permeabilised (e.g. Turkey erythrocytes *(421,422)*).

The identification of substrates phosphorylated must be critically assessed, especially under <u>in vivo</u> conditions where molecular weights may be insufficient to identify proteins. Proteins have been found to migrate according to the electrophoretic gel system which they are run in (e.g. Identification of the "87Kd" substrate as "68Kd" *(547)*). The "47Kd" protein in platelets has been identified as 5' monoesterase *(104)*, Lipocortin *(389)* and class I histocompatability antigens *(477)* possibly due to comigration of many proteins at this molecular weight range. Similarly a number of other factors can hamper the identification of phosphoproteins such as; (A) variation between species (e.g. The 87Kd protein in bovine preparations is identical to the 80Kd in rat *(543)*), (B) The protein may experience a change of isoelectric properties upon (multiple) phosphorylation *(548)*, (C) Existance of tissue-specific forms of substrate protein showing differences in phosphorylation *(494,544)*, (D) Differences in post-translational processing of substrate between cell types *(479)*, (E) Degradation of phosphoproteins <u>in situ</u> (e.g. full size band '4.1' protein in erythrocytes disappears in the presence of calcium *(461)*), (F) Availability of substrate protein as a factor of maturation *(505)*, (G) Protein kinase C itself undergoes

autophosphorylation on both catalytic and regulatory domains *(106,107)*. Fragments of PKC phosphoproteins may be generated (H) Action of phosphatases *(197,201)*.

There have been many reports of inhibition of PKC-mediated phosphorylation implicating Calmodulin (a calcium binding protein) due to the coincidence of the Ca^{2+}:Calmodulin complex binding site and the phosphorylation site on a substrate. This effect has been found for myosin light chain kinase at two sites *(509,510)*, caldesmon *(498)*, the '87kd' protein *(544)* and Neuromodulin *(401)*. In vitro other calmodulin-binding proteins have been found to similarly inhibit phosphorylation (e.g. Troponin C and S-100a *(512)*, Calcineurin and Intestinal Calcium binding protein *(238)*) by "protecting" certain substrates. Direct inhibition of PKC by endogenous proteins has also been detected in rat and bovine brain *(236,238)*. These potent inhibitors comprise of a growing family of small (12-17Kd) Calcium-binding proteins *(102,237)*. Other (non-calcium binding) proteins have also been observed to inhibit phosphorylation (e.g. retinoid binding proteins *(235)*). Little is known about endogenous phosphatase action in negatively regulating PKC effects in cells although a phosphatase specific for PKC substrates has recently been purified *(549)*. The time-course of (de)phosphorylation has been observed to vary enormously between substrates in vivo. With phorbol ester treatment synaptosomal 87Kd is observed as a phosphoprotein within 15 seconds and its level plateaued at 2 minutes *(542)*, GABA-modulin reaches maximum levels in 3 hours and remained phosphorylated for over 20 hours *(473)* and

Neuromodulin phosphorylation persisted for as long as 3 days *(532)*. Other activators which do not maintain PKC activation have different time courses (e.g. whereas TPA maintained pp 60 C-SRC and V-SRC phosphorylation for over 6 hours, OAG-induced phosphorylation dropped to background levels in 30 minutes*(468)*). The phorbol esters often stimulate different phosphorylation patterns to those observed with hormone agonists using the PI turnover (e.g. TRH in GH_3 cells *(550)*, IgM in β-lymphocytes *(460)* and fMLP in neutrophils *(456)*) and with diacylglycerol analogues (e.g. OAG *(146,454)*), suggesting caution is required on the choice of PKC activator. The proteolysis and activation of PKC also presents problems <u>in vivo</u> since the role of PKM is not understood. There are indications that activators of PKC (phospholipids, diacylglycerol and phorbol esters) act in concert to enhance such proteolysis *(204,272)*.

TPA induced activation of phosphorylation has been found to occur using partially purified PKC without added phospholipid *(551)*, without calcium *(208,552)* and without either *(458)*. Basal phosphorylation (without any cofactors) has been found associated with nuclear membranes *(154)* and can occur <u>in vitro</u> with full length PKC on synthetic substrates (peptides *553*) or protamines *(508)* suggesting that some substrates may possess structures at the site of phosphorylation which greatly influence PKC action. Other factors also affect the ability of a protein to act as a phosphate acceptor from PKC e.g. GDP-bound G_i α-subunits of transducin, but not GTP-bound, are substrates <u>in vitro</u> *(480)*. There is also good evidence of myristoylation (post-translational addition of myristic acid) of

proteins playing an important role in this context, by stimulating cytosolic proteins to associate with membrances (e.g. the '87Kd' protein *(547)*). In macrophages myristoylation of at least 5 proteins occurs in a stimulus dependent fashion and leads to their enhanced phosphorylation *(554)* and in chicken embryo cells $pp60^{V\text{-}SRC}$ and $^{C\text{-}SRC}$ require myristoylation to be substrates for PKC *(468,555)*. Furthermore, artificial myristoylation of the soluble protein lysozyme caused it to associate with membranes and be phosphorylated by PKC <u>in vitro</u> *(556)*. Such data suggests that substrate compartmentalization plays a critical role in the action of PKC. What of the compartmentalization of PKC itself? The presence of PKC in the nucleus has been found by immunological techniques *(21)* whilst other approaches suggest exogenous PKC can bind to isolated nucleii in the presence of calcium, augmenting the endogenous activity in a cofactor-independent manner *(20)*. Differential translocation of PKC has been suggested to play a role in the transformed phenotype of some cells *(276)*. The 'your-place-or-mine' relationship between PKC and substrates merits further investigation.

To complete the view of substrate-kinase relationship it is necessary to conduct <u>in vitro</u> investigation with purified reagents. However some substrates, e.g. the sodium channel *(481)*, cannot be purified and reconstituted without detergents which interfere with the phospholipid requirements of PKC. Immunoprecipitation has been suggested as being responsible for activation of $pp60^{CSRC}$ protein since no effect of phosphorylation by PKC can be found *(468)*. Other proteins eg. Vinculin *(513)* and AcetylCoA carboxylase *(482)* show a

number of phosphory lation sites in vitro which cannot be detected in vivo.

Crossphosphorylation by different kinases is found to occur at (A) the same serine/threonine residues (e.g. C-Protein *(466)*), (B) at different residues in the same protein (e.g. GABA-modulin *(473)*, Ribosomal S6 kinase *(10)*), (C) at a mixture of these (e.g. M.A.P.2 *(504)*). Interaction with other kinase systems is discussed in *Section F11*.

Even where phosphorylation can be demonstrated the physiological relevance may be far from certain. Phosphorylation of the Glucose transporter by TPA treatment has been found not to correlate with its activity *(474)*, Calcium-activated neutral protease was only a substrate for PKC when proteolytic inhibitors were present *(492)* and the phosphorylation of P21 ras gene product was found to proceed very slowly *(488)*. Discrepancies have also arisen as to the effects of phosphorylating Glycogen synthase *(485,487)* and phosphofructokinase *(493,495)*.

The actions of TPA have also been reported to stimulate Tyrosine-specific phosphorylation of two Mr40Kd proteins in chicken embryo fibroblasts *(557,558,559)*, the EGF-R in A431 membranes *(560)*, and the Insulin -R in U937 monocyte line *(561)*. Later work suggested that the 40Kd proteins were part of the Lipomodulin complex and were phosphorylated on tyrosine by $pp60^{C\text{-}SRC}$ (a response stimulated by PKC activation) *(330)*. Recently lymphocytes have been found to contain

a related PKTyr (termed $pp56^{LCK}$) which is modified in response to TPA *(211)*. The EGF and Insulin receptors both have tyrosine kinase activities and are thought to autophosphorylate. Increases in PKTyr and phosphotyrosine phosphatases have been found in differentiating HL-60 cells following TPA treatment *(243)* resulting in net decrease of phosphotyrosine residues, suggesting that PKC activation effects may be mediated by other Tyrosine kinases.

A further factor which has not been widely considered is the heterogeneity of the PKC enzymes themselves. Many experimenters have used "partially purified" or "purified to homogeneity" prepartions of PKC which will contain a mixture of isozymes. Similarly brain preparations are rich in γ-PKC, an isozyme which is missing in non-neuronal tissue *(45)*, which could lead to non-physiological phosphorylation results. Distinct roles for PKC isozymes have not been domonstrated, although <u>in vitro</u> tests on EGF-R phosphorylation detected slight variation ($\alpha > \beta > \gamma$, *(261)*). The recently cloned PKC-ϵ isozyme has been found to exhibit totally different substrate specificity (e.g. histone is a poor substrate) and δ ζ and η await characterization *(255)*.

The effect of the phorbol esters have not been tested in an isozyme-specific manner. However in swiss 3T3 cells, expressing only α-PKC, no difference in 80Kd substrate phosphorylation was seen with tumour promoting and non promoting phorbol esters which activated PKC <u>in vitro</u> *(432)*. In GH_3 pituitary cells however, differences in the phosphorylation patterns were observed with a range of phorbol

esters, with even the biologically "inactive" α-sapenine acetate eliciting some increased phosphorylation *(550)*. Whether the variation of the biological activities of the phorbol esters lies with the PKC isotypes, other kinases or some other receptor interaction, is unknown at present.

CHAPTER 2

MATERIALS AND APPARATUS

Section A

During this project a range of chemicals, biochemicals and reagents were employed. Below are listed simpler reagents and the supplier used.

From Sigma:

Hydrochloric acid (HCL)
Potassium hydroxide (KOH)
Sodium hydroxide (NaOH)
Trichloracetic acid (TCA)
Dithiothreitol (DTT)
β-Mercaptoethanol (β-Me)
Ammonium persulphate
Sodium dodecyl sulphate (SDS)
Hydrogen peroxide
Phenylmethylsulphonylfluoride (PMSF)
Histone IIIS =("Type1") (histone)
Leupeptin hemisulphate (Leupeptin)
Trypan blue stain
Brilliant blue G (coomasie blue)
Zymosan
Starch
Trisma (TRIS)
Glycerol
Phosphate buffered saline (PBS)
Histopaque 1077^{o}
Adenosine triphosphate (ATP)
Bovine serum albumin (BSA)
Concanavalin A (CON A)
Bromophenol blue
Potassium dihydrogen orthophosphate (KH_2PO_4)
Sodium dihydrogen orthophosphate (NaH_2PO_4)
Dipotassium hydrogen orthophosphate (K_2HPO_4)
Disodium hydrogen orthophosphate (Na_2HPO_4)
sn-1,2-Dioleoylglycerol ($DiC_{18:1}$)
Ethylene diamine tetracetic acid (EDTA)
Ethyleneglycol bis (β-aminoethyl ether) -N,N,N^1,N^1 tetra acetic acid (EGTA)
Calcium Ionophore A23187
Silver protein staining kit
Staurosporine

From BDH:

Sucrose
Scintran cocktail T
Acetic acid
Ethanol
Water "Hypersolv" and "Analar"
Methanol
Isopropanol
Ficoll hypaque (FICOLL)
Magnesium chloride ($MgCl_2$)
Calcium chloride ($CaCl_2$)
Triton X-100 (Triton)
Tween-20 (Tween)
Butanol
Glycine
Chloronaphthol
Sodium chloride (NaCl)
N-2-hydroxyethylpiperazine-N-ethane sulphonic acid (HEPES)

From Whatman:

Glass microfibre filters (2.5cm GF/c)

Ion exchange chromatography paper (p81)
0.2 Micro-Metre fiters

From Bio-rad:

N,N,N^1,N^1,- tetramethylethylene diamine (TEMED)
DNA Grade Hydroxyapatite (0520 HTP)
Hydroxyapatite biogel (0420HTP)

From LKB: Bromophenol blue
From Gibco: Gentamycin
L-Glutamine
Foetal calf serum (FCS)
RPMI 1640 culture medium
superose 12 prepacked column.

From Pharmacia: low-molecular-mass protein standard mixture (Mr 94, 66, 43, 30, 20.1, 14.4 Kdal)

From May and Baker: Tripotassium citrate
Orthophosphoric acid

From Boehringer-Mannheim:

Acrylamide Solution
(contains 30% Acrylamide 0.8% N,N^1,bis methylene acrylamide)
Protein standards for gel exclusion chromatography

From Jansen:

Autodye Forte colloidal gold reagent

From Schleicher and Schüll:

0.45 Micro-Meter diameter nitrocellulose BA85 filter

From Amersham:

Hybond-C extra nitrocellulose 0.45 Micro-Meter diameter
Rainbow molecular weight markers (Mr 14.3-200Kd) (radiolabled or not)
(I^{125}-labled) Anti-rabbit IgG antibodies (15 Ci/ g)
Anti-mouse IgG peroxidase conjugated antibodies
Mouse anti-protein kinase C IgG monoclonal antibodies
(MC-5 *See Reference 205*)
Adenosine-5-[γ P^{32}]- Triphosphate Triethylammonium salt (^{32}P- γ - ATP at 5000 Ci/mmol)

From ICN Biomedicals:

Ad enosine-5-[γ P^{32}]-Triphosphate Triethylammonium salt [^{32}P- γ - ATP at 4500 Ci/m mol]

From New England Nuclear: [^{3}H]-Thymidine (1mCi/ml)

From Lipid Products: L-α- Phosphatidyl Serine (PS)

All reagents were of "Analar" grade or purer and were handled according to the suppliers recommendations.

Other Reagents

AntiSera

Anti-isozyme PKC antibodies were generously donated by Dr. Peter Parker, Imperial Cancer Research Fund, and were obtained according to references *(255,260)*.

Antisera to PKC	dilution used
α	1 in 1000
β_1	1 in 500
β_2	1 in 1000
γ	1 in 1000
δ	1 in 2000
ϵ	1 in 3000

Epitope Peptides for these sera were also supplied (in 10mM solutions) and were mixed 1:1 with antisera in competition experiments.

Phorbol Esters

The phorbol esters used were isolated and purified from natural sources in the London School of Pharmacy.

Thymeleatoxin A (THY TOX A) = (9,13,14-ORTHOBENZOYL-6,7-EPOXY, 5 HYDROXY-RESINIFERONOL-12-CINNAMATE) Dr. G. Brooks

Sapintoxin A (SAP A) = (12-O-[2-METHYLAMINOBENZOYL]-4, DEOXYPHORBOL-13-ACETATE) Dr. G. Brooks

DOPP = (12-DEOXYPHORBOL-13-PHENYLACETATE) Miss P. Darcy

DOPPA = (12-DEOXYPHORBOL-13-PHENYLACETATE-20-ACETATE) Miss P. Darcy

RESINIFERATOXIN (RX) = (9,13,14-ORTHOPHENYLACETYL-RESINIFERONOL-20 - [3METHOXY, 4HYDROXY]-PHENYLACETATE) Miss P. Darcy

TPA (12-O-TETRADECANOYL PHORBOL-13-ACETATE) was purchased from Scientific Marketing Associates.

These were stored at -20^{o}C in the dark and stock solutions made up in acetone, similarly stored, were employed throughout.

Plastic-ware

For measurement and cell culture work plasticware was supplied by Sterilin, Eppendorf, Gilson, BCL, Flow, Packard, Nalgene, Nunclon,

Surgicos and Monoinject.

Section B: BUFFERS

The reagents were used to make the following buffers in deionised water (Milli-Q water system, Millipore) or "analar"/"hypersolv" water (BDH).

Cell Culture Medium

1. RPMI 1640 medium supplemented with 10% v/v FCS, L-Glutamine 2mM, Gentamycin 50μg ml^{-1}
   Sterile conditions. L-Glutamine was added before use. Foetal Calf Serum was heat inactivated (56°C for 30 minutes) before addition.

2. Blood collection Citrate supplement 10% v/v of 100 mM Trisodium citrate

Protein Separation Buffers

3. Homogenization buffer
   Sucrose 0.25M
   EGTA 10mM
   EDTA 2mM
   20mM TRIS/HCl pH 7.5 at 4°C
   DTT 1mM (added fresh)
   Leupeptin 100μg ml^{-1} (added fresh)

4. Chromatography buffer A

   Potassium Phosphate
   - A mix of mono-and di-potassium salt (190/810 ratio respectively) 20 mM
   - 10% v/v glycerol
   - EGTA 1mM
   - DTT 1mM (added fresh)

   pH to 7.5, filtered through 0.2 μm filter, degrassed by vacuum or by aeration with helium gas.

5. Chromatography buffer B - As above but 500 mM potassium phosphate.

6. Chromatography Sodium buffer A - As potassium buffer A but replacing mono- and divalent potassium salts with sodium salts, and β-Mercaptoethanol used instead of DTT.

7. Chromatography Sodium buffer B - as Sodium buffer A but 500 mM.

8. Storage buffer 1 Glycerol and 2% TRITON x 100 solution mixed in the ration 16.36/1 and added to the fraction to be stored 1 to 4 ratio. After mixing each was frozen in liquid Nitrogen.

9. Storage buffer 2 90% Glycerol v/v, Triton x-100 0.02% v/v, DTT 4mM, EDTA 4mM added 1 to 1 with fraction. After mixing each was frozen in liquid Nitrogen.

Electrophoresis [Method 1]

10. La emmli denaturing buffer [see Ref*453*]
    62.5mM Tris/HCL — Glycerol 10% v/v
    2% SDS — Bromophenol blue 0.001% w/v
    5% βMe or DTT 1%

    pH 6.8, boiled for 5 minutes with sample.

11. Gel Buffer 11 — 1.5M Tris/HCL
    0.4% SDS
    pH to 8.8

12. Gel Buffer 12 — 0.5M Tris/HCL
    0.4% SDS
    pH to 6.8

13.

36mls Separation Gel:(10%)		10mls Stacking Gel:(4.5%)	
Buffer 11	: 9 mls	Buffer 12	: 2.5 mls
Acrylamide soln	: 12 mls	Acrylamide soln	: 1.5 mls
H_2O	: 15 mls	H_2O	: 6 mls

    both gels were polymerised by addition of:

TEMED	: 20 μl	10μl
0.1g ml^{-1} Ammonium persulphate:	120 μl	30μl

14. Gel Running buffer: — 25mM Tris/HCl
    192 mM Glycine
    0.1% SDS
    pH to 8.3

    Western blotting and MC5 antibody buffers:

15. Western transfer buffer — 25 mM Tris
    192 mM Glycine
    20% v/v Methanol

16. Tris buffered saline buffer (TBS) — 10 mM Tris/HCl
    140 mM NaCl
    pH to 7.4

17. As 16 (TBS) + 3% w/v BSA

18. As 16 (TBS) + 0.1% BSA w/v

19. AS 16 (TBS) + 0.1% Tween-20 v/v

20. As 16 (TBS) + 0.1% BSA w/v, 0.1% TWEEN 20 v/v

21. As 16 (TBS) + 6% Methanol + 0.018% Chloronaphthol

22. 30% Hydrogen Peroxide w/v

    Electrophoresis [Method 2]

23. Double-strength Laemmli denaturing buffer *(see ref 453)*

125 mM Tris/HCl	20% Glycerol
4% SDS	0.0025% Bromophenol blue
2% DTT	

pH 6.8 boiled with sample in 1:1 ratio for 5 minutes.

24.

(A) 40 mls Separation Gel: (7.5%)		(B) 20 mls Stacking Gel:(4%)	
1.5M Tris/HCl (pH8:8):10 ml		0.5M Tris/Hcl (pH 6.8): 5ml	
H_2O	: 19.4 ml	H_2O	: 12.2 ml
10% SDS stock	: 400 μl	10% SDS stock	: 200 μl
Acrylamide soln	: 10 ml	Acrylamide soln	: 2.6 ml
Added to polymerise the gels			
TEMED	: 20μl		20μl
0.1 g ml^{-1} Ammonium Persulphate	: 200 μl		100 μl

25. Gel Running buffer (as 14)

Western blotting and PKC-isozyme antisera antibody buffers

26. Western transfer buffer (as 15)

27. Washing buffer

PBS plus;
0.05% Tween-20
0.05% Azide
0.05% Triton X100

28. As (27) + 10% marvel dried milk.

29. As (27) + 3% BSA.

Gel Staining

30. Coomasssie blue stain
0.3% Brilliant blue G stain
50% Methanol
1% Acetic Acid

31. Coomassie blue destain
10% Acetic Acid
10% Isopropanol

32. Silver stain
3.06 mg ml^{-1} Silver nitrate ($AgNO_3$)

33. Oxidiser
1 mg ml^{-1} Potassium dichromate ($K_2Cr_2O_7$)
0.0142% v/v Nitric acid

34. Developer
29.68 mg ml^{-1} Sodium Carbonate (Na_2CO_3)
0.019% w/v Formaldehyde

35. Fixative 1
10% Acetic acid
50% methanol

36. Fixative 2 — 5% Acetic acid
10% ethanol

Section C: Protein Kinase C Assays

Method 1: Crude Detection

37. Assay Buffer — 6.63 mM EGTA
28.57 mM Tris/HCl
pH to 7.5 (STOCK)

38. Micelle Buffer — 28.57 mM Tris/HCl
2.4% w/v Triton X100
pH to 7.5 (STOCK)

39. Histone mix — Assay buffer (37) plus 4 mg ml^{-1} Histone III-S FRESH

40. Micelle mix

PS 6mg ml^{-1} } Phorbol esters (varies)} — Dried down under nitrogen and mixed} with Micelle buffer (38) by bath sonication and vortexing. } FRESH

41. ATP mix — Assay buffer (37) plus 200 μM ATP
300,000 cpm/assay ^{32}P-γ- ATP
20 mM $MgCl_2$
+/- 10.2 mM $CaCl_2$ } FRESH

The ^{32}P-γ-ATP quantities vary with the source material and is calculated from the specific activity and the time elapsed from 100% activity.

42. The enzyme fraction is typically
0.02% Triton
16 % glycerol
0.125mM EGTA, and stored at -70°C

- The assay is prepared by mixing 25μl Micelle mix (40)
25μl Enzyme (42)
50μl Histone mix (39)

- The assay is started by mixing in 100 μl of ATP mix (41). For final concentrations of reagents see *Table 2A*.

- The assay is terminated by addition of 1 ml 25% w/v TCA followed by 100 μl of 2 mg ml^{-1} BSA as a protein carrier.

- The precipitate is filtered and counted (QV).

Method 2: Purified Isozyme PKC Assay

43. Substrate mix 100 mM HEPES
1 mM EGTA
25 mM $MgCl_2$
2.5 mg ml^{-1} Histone or Epsilon/delta pseudosubstrates
pH to 7.5 FRESH +/- 1.5mM $CaCl_2$

44. Micelle buffer Tris/HCl 2 mM
1% v/v Triton X 100
pH to 7.5 (STOCK)

45. Micelle mix PS 5mg/ml } Dried down under nitrogen and
Phorbol ester (varies) } mixed with Micelle buffer (44) by bath sonification and vortexing. FRESH.

46. ATP mix 1mM ATP (ACIDIC pH)
500,000 cpm/assay ^{32}P-γ- ATP
FRESH
The ^{32}P quantities required vary according to source of radiolabled ATP and the time elapsed from 100% activity.

47. Enzyme is typically stored at -20°C in 2mM EDTA
0.02% TRITON X 100
20 mM Tris/HCl
50% Glycerol
1mM DTT pH 7.5

- They are diluted with a similar buffer (without Glycerol according to their concentration) to give an activity in the range of 1-4 units (n moles/min/ml) of kinase activity and glycerol volume of 10%, prior to use.

- The assay is prepared by mixing 20 µl of substrate mix (43)
10 µl of micelle mix (45)
5 µl of Enzyme (47)

- The assay is started by mixing in 5 µl of ATP mix (46). For final concentrations see *Table 2A*.

- The assay is terminated by spotting a 25 µl aliquot onto Ion exchange paper and washing three times in 30% acetic acid for 10 minutes each time.

- The washed paper is counted (QV).

Table 2A. Final concentrations of reagents used in the Protein Kinase assays.

Reagent.	Method 1 Concentration.	Method 2 Concentration.
Buffer (pH 7.5)	25mM (Tris)	50mM (HEPES)
ATP (cold)	100μM	100μM
ATP (radioactive)	300,000 cpm/assay (15 cpm/p.mol)	500,000 cpm/assay (100 cpm/p.mol)
MgCl2	10mM	10mM
CaCl2* (free Calcium ions)	5.1mM (100μM)	0.75mM (100μM)
EGTA	5.1mM	0.5mM
EDTA	-	0.25mM
Histone**	1 mg/ml	1.25 mg/ml**
DTT	0.25mM	0.125mM
Triton X-100	3 mg/ml (81.6 mol %)	2.5 mg/ml (69 mol %)
PS	0.75 mg/ml (18.4 mol %)	1.25 mg/ml (31 mol %)
Phorbol Esters	100 ng/ml (0.003 mol %)	10-10000 ng/ml (0.0003-0.3 mol %)
Final Volume	200μl	40μl

* Where present in the assay, free calcium concentration was calculated according to the reference (*562*).

** For PKC δ and ε, synthetic pseudosubstrate peptides were used at final concentrations of 0.1 mM and 0.35 mM respectively.

Section D: Apparatus Used

Mundane

pH meter - PYE UNICAM PW9418
Ice Maker - SCOTSMAN AF10
Vortex - ROTAMIXER
Bath sonicator - type 6442AE ULTRASONICS Ltd
Centrifuge small MSE Microcentaur
chilled spin MSE CHILSPIN 2
high speed MSE High Speed 18, Minstral, Sorval
Ultracentrifugation with AMICON CENTRIFLO CONES CF 50A at 1000 x G
Bag Sealer - CALOR
Bench shaker - ROTATEST R100 SHAKER

Cell Work

Sterile cabinet - SLEE, London
CO_2 cabinet - LEEC MKII
Cell counting - A haemocytometer (Weber Scientific International Ltd.) was used to monitor Trypan blue exclusion (according to Sigma catalogue) using microscope inspection.
Cell harvester (1) A Millipore 12-well-manifold taking glass fibre filters was used washed with PBS.
(2) A Titertek semi-automatic cell harvester (Flow) washed with PBS.

Protein Separation

Homogenization of tissue/cells was done in three ways according to the sample:

1. Probe sonication/disruption on ice (MSE probe sonicator) 3 x 10 seconds
2. Teflon/glass homogenizer (Jencans Scientific Ltd.) on ice with 10 strokes
3. Ultra-turrax tissue homogenizer on ice for 30 seconds.

Hydroxyapatite FPLC

Protein chromatography was conducted on a pharmacia Fast Liquid Protein chromatography (FPLC) system (LCC 500 plus, Frac-100 collector) using 10 ml- volume glass columns (omnifit) packed with hydroxyapatite (made up fresh in the first chromatography buffer). The sample was injected direct or through a super loop (Pharmacia). The super loop, column, buffers and fraction collector were on ice and samples were immediately stored. The FPLC was kept in 30% Ethanol solution between runs.

PKC Assay Harvesting with Method 1 Assay

2 methods were employed.
(A) The millipore manifold with glass fibre filters was used with 2 x 5 ml 10% TCA washes.

(B) The Titertek apparatus was used by conducting the assay in microlitre wells, terminating with 50 μl 112.5% TCA and 25 μl of 8 mg/ml BSA and filtering onto titertek mats with a 10% TCA wash.

Note: The titertek cell harvester was cleaned with Orthophosphoric acid 3.0% v/v and washed with 25% ethanol between runs. It was eventually unable to function in this manner effectively.

Electrophoresis Equipment

Method 1

For SDS-PAGE LKB (Bromma) electrophoresis apparatus was used running overnight at 30 Milliamps.

For Western transfer "scotch-brite" and 3mm filter paper sandwich apparatus was used running overnight at 200 milliamps.

Method 2

For SDS-PAGE a Protein II (or mini-protean II) system was used (Biorad) running at 23 milliamps per gel for 2-3 hours (or 200 Volts for ~1/2 hour).

For Western transfer an Atto electrophoresis western transfer apparatus was used (Genetic research Instrumentation Ltd) running for 30 minutes at 0.5 Amps.

Radioactive Counting

The various radiolabled chemicals were counted with one of three methods on a Packard Tri-carb L.S spectrometer model 3255.

1. For ^{3}H-Thymidine counting filters were dried overnight and 4 mls of Scintron-T cocktail were added. Vials were then counted for liquid scintillation for 4 minutes.

2. For [^{32}P] counting wet filters or ion exchange strips were placed in plastic vials and counted with the open window method for CerenkoV radiation for 4 minutes.

3. For electrophoretic method 2 ^{125}I-labled second antibodies were visualised on the nitrocellulose western blot by autoradiography. A "flashed" X-ray film (Kodak or Amersham) was enclosed over the blot in an X-ray film case (Kodak), placed in a -70^{o}C freezer and developed at University College Hospital X-ray department.

   Throughout radiochemical work a laboratory monitor (Mini-instruments), badge and finger detectors and a perspex screen (Nalgene) were used.

Further techniques are given in each chapter.

CHAPTER 3

PROLIFERATION STUDIES ON HUMAN MONONUCLEAR CELLS

The finding that the phorbol derivative Sapintoxin A (SAP A) is mitogenic in lymphocyte cultures yet is unable to promote first or second stage tumourgenesis (without calcium Ionophore, *232*), indicates differences in the mechanisms of the SAP A action compared to the tumour promoting derivatives (e.g. Tetradecanoyl phorbol acetate. TPA). The mitogenic response of peripheral mononuclear bloods cells from human donors to SAP A and TPA was therefore used as a model system to investigate their action at a biological level.

Culturing of Cells

Freshly drawn venous blood (50-100 mls) was collected from healthy human donors and mixed 9:1 with citrate buffer (methods section, buffer 2) to prevent coagulation. Under sterile conditions this was centrifuged at 1300 x g for 10 minutes and the mononuclear rich boundary, between the red blood cell fraction and the clear plasma, was carefully aspirated onto a denser medium (e.g. Histopaque 1077, or Ficol Hypaque in ratio 2 blood: 1 dense medium). Tubes prepared in this way were centrifuged at 400 x g for 45 minutes resulting in the retardation of the mononuclear cells in the dense medium, separating them from platelets and red blood cells (and some white blood cells such as neutrophils). The mononuclear cells were aspirated off, pooled and pelleted by centrifugation at 200 x g for 10 minutes. The pellet of mononuclear cells was resuspended in

Culture medium (RPMI 1640 medium supplemented as indicated in methods, buffer 1) and a sample was taken to determine cell count and viability (See Methods). The cells were 'washed' by pelleting and resuspension in culture medium as above. Cells were then plated out in 96-well microtitre plates (100μl each well) and all experiments were conducted in triplicate (typically starting with 5-10 x 10^5 cells/ml). Cultures were all 95% viable at this stage.

Addition of Phorbols

Phorbol esters Sapintoxin A and TPA stored in acetone (see Methods) were added to culture medium under sterile conditions just prior to use. These solutions were serially diluted with culture medium to give the desired range of concentrations. The microtitre wells received 100μl of the phorbol ester solutions (or 100μl of culture medium for control experiments) and were incubated in a 5% CO_2/95% air mixture at 37^{o}C for varying amounts of time (CO_2 incubator).

Assessment of Proliferative Response

Radiolabled Thymidine ([^{3}H] Thymidine) was used as measure of the growth rate of cell cultures. 50μl of[^{3}H]Thymidine (300,000 cpm, 0.4 μCi-per well in culture medium) was added before the cells were harvested and the plates were returned to the CO_2 incubator. Thymidine (5-methyluracil linked to deoxyribose,- a Nucleoside) is incorporated into DNA being synthesized de Novo during this period and the uptake of radioactivity reflects the rate at which DNA synthesis, and thus proliferation, is occuring. Cultures incubated

with ^{3}H Thymidine for extended periods (e.g. 24 hours) were found to show depressed stimulation (i.e. control values were higher relative to stimulated cultures) while short incubation periods (e.g. 2 hours) produced too much variation between triplicates (data not shown). It was therefore decided to maintain the time of incubation with [^{3}H] Thymidine at 6 hours.

Harvesting and Radiation Counting

After incubation with [^{3}H] Thymidine, cells were harvested by filtration using a millipore filter manifold and washed with Phosphate buffered saline (2 x 10 ml washes - see Methods). Filters were then dried overnight and placed in scintillation vials with 4 mls of scintillation cocktail for aqueous scintillation counting (See Methods).

RESULTS

Stimulation of Lymphocyte Proliferation by TPA and SAP A

Studies conducted on freshly cultured human mononuclear cells (HMNC's) showed that both TPA and SAP A were able to act as mitogens in this system *(Fig 3a)*. The mitogenic response to both phorbol esters was dose-dependent, appearing from the second day and increasing to a peak around day 4. The concentrations of the phorbol esters required to produce 40% of the maximal response *(Table 3A)* showed that both were equal in effectiveness but the potency of TPA was 30-50 times greater than SAP A.

In an experiment with SAP A and TPA combined, the addition did not provoke increased values of mitogenesis when compared with TPA or SAP A alone (data not shown). SAP A stored in sterile culture medium for 30 days only slowly lost its mitogenic capacity (23% lost) (data not shown). The mitogenic potency of SAP A at 100 ng ml^{-1} in fresh cultures harvested at 3 days was not enhanced by addition of Calcium Ionophore A23187 (at 12.5 to 50 μM) but rather a slight reduction was observed (data not shown).

Between different batches of HMNC's the values of thymidine uptake (counts per minute and stimulation percentages) were found to vary. Even so the patterns of dose dependent stimulation observed for SAP A and TPA were found to be consistent (e.g. compare *Fig 3a* with *Fig 3b*).

TPA and Sap A stimulation of fresh HMNC cultures.

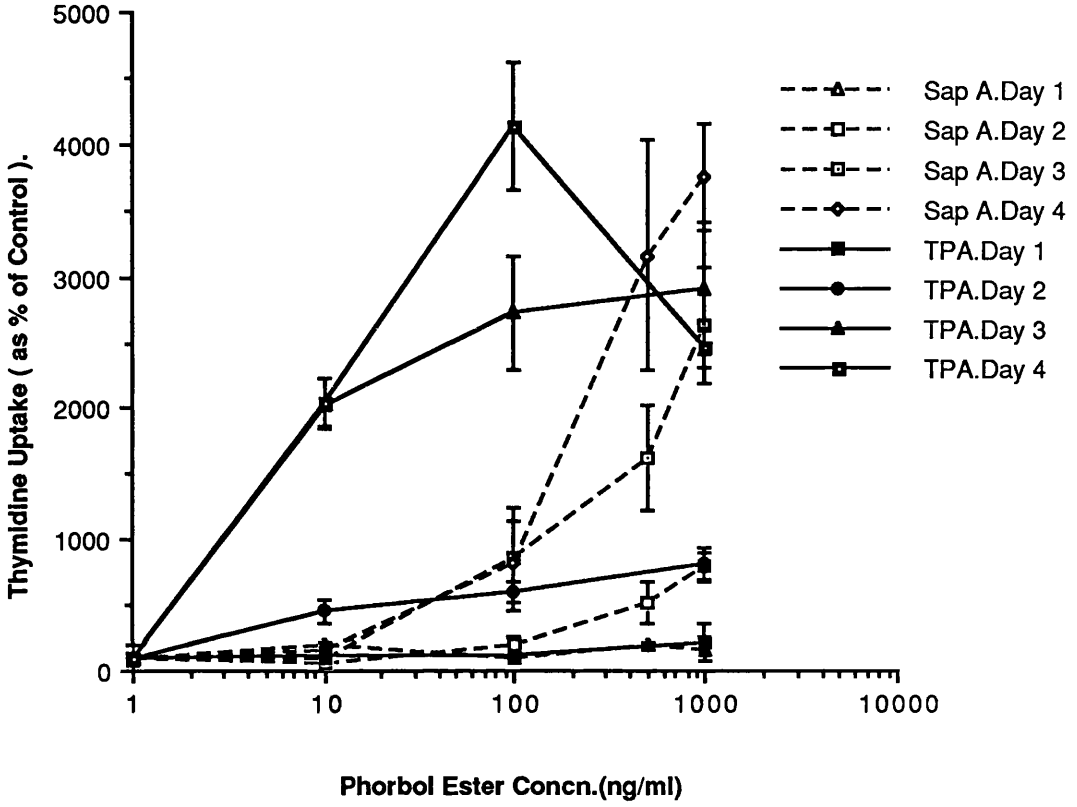


Fig.3a Phorbol esters were added to freshly prepared HMNC cultures at the concentrations given.Cultures were maintained for 1-4 days prior to incubation with radiolabelled thymidine and harvesting.The results are expressed as % of untreated (control) cultures with S.E.M. error bars from triplicate cultures.

Table 3A. Comparison of TPA and Sap A stimulation of fresh HMNC cultures.

Phorbol Ester	Day of harvest	Proliferation (%of TPA max.)	EC 40* (ng/ml)
TPA	1	nil	-
Sap A		nil	-
TPA	2	100	5.5
Sap A		96	275
TPA	3	100	5.0
Sap A		91	250
TPA	4	100	7.3
Sap A		91	200

Table 3B. Comparison of TPA and Sap A stimulation of delayed HMNC cultures.

Phorbol Ester	Day of** harvest	Proliferation (%of TPA max.)	EC 40* (ng/ml)
TPA	3	100	6.7
Sap A		108	24
TPA	4	100	2.6
Sap A		101	30
TPA	5	100	2.4
Sap A		126	12
TPA	7	100	2.1
Sap A		158	2.4

*Concentration required to give 40 % of the maximal proliferation elicited by TPA.

**Cells were in culture 3 days before phorbol ester addition.Day of harvest is in days after addition.

Fig 3b. TPA and Sap A stimulation of fresh and delayed HMNC cultures.

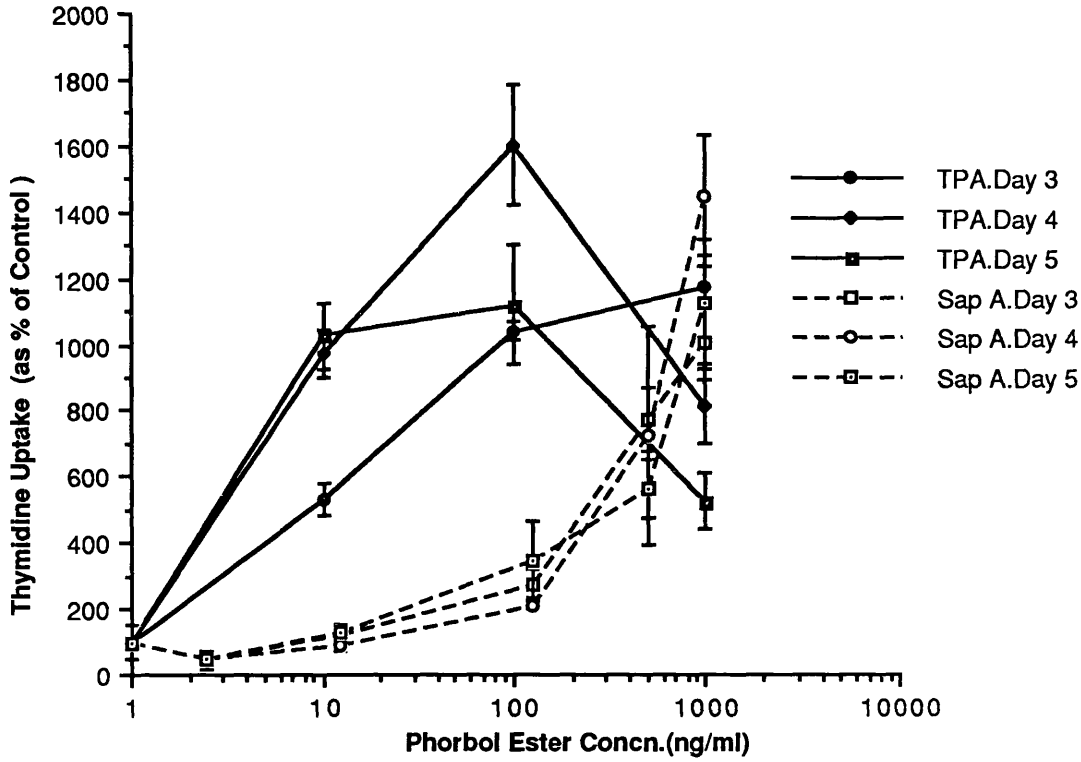
A single preparation of Human Mononuclear cells was divided into two parts.

(a)Phorbol Esters were added to the first HMNC culture immediatly after preparation (fresh).

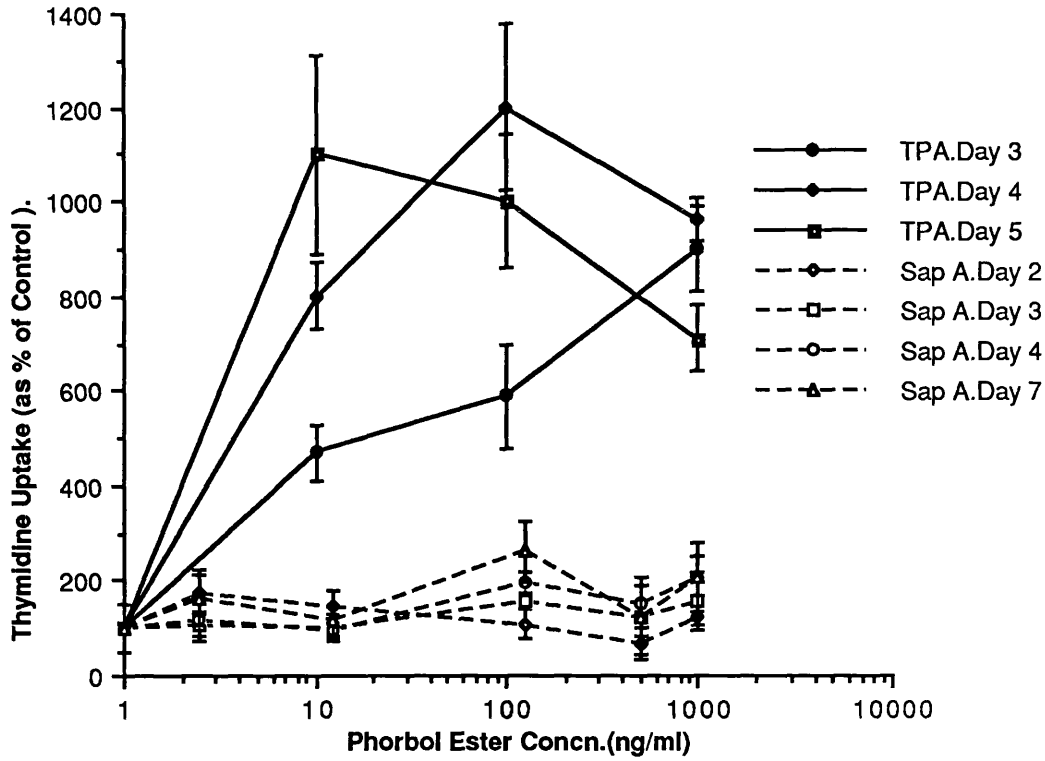
(b)Phorbol Esters were added to the second HMNC culture after the culture had been maintained for 3 days .

After addition of the Phorbol Esters ,cultures were maintained for 2-7 days (as indicated) prior to incubation with radiolabelled thymidine and harvesting.The results are expressed as % of un-treated (Control) cultures with S.E.M.error bars from triplicate cultures.

(a) TPA and Sap A stimulation of fresh HMNC cultures.



(b) TPA and Sap A stimulation of delayed HMNC cultures.



Stimulation of Proliferation in Delayed and Fresh Cultures

The effect of delaying the addition of phorbol esters to HMNC cultures was investigated. A fresh culture was split into 2 parts each plated into microtitre wells. One culture was immediately stimulated with phorbol esters (="Fresh" culture) while the other was left in the CO_2 incubator for 3 days prior to addition of phorbol esters (="Delayed" cultures). The results of such an experiment are shown in *Figure 3b*. Delaying the addition of SAP A had the effect of abrogating the mitogenic response seen in fresh cultures. The mitogenic response of TPA was still evident in delayed cultures.

Effect of Cell Density on Proliferative Responses

When low density cultures (4×10^4 cells/ml) were stimulated after a delay of 3 days, the mitogenic response to SAP A was found to be restored (See *Figure 3c*). The dose-dependency was markedly altered to that observed with fresh cultures however, with the potency of SAP A gradually shifting towards that of TPA over successive days from day 4. Maximal response similarly increased with day of culture relative to TPA and exceeded the latter at day 5 and 7 (*(see Table 3B)*. For TPA, maximal activity experienced a shift towards lower concentrations with successive days of culture similar to that seen in fresh cultures.

An experiment to test this observation was conducted by splitting a culture into two parts. The first culture was plated at a starting concentration of 10^6 cells/ ml (100μl) while the second was plated at 10^5 cells/ml (100μl) by dilution with culture medium. Both cultures

Fig 3c. TPA and Sap A stimulation of delayed HMNC cultures plated at low density.

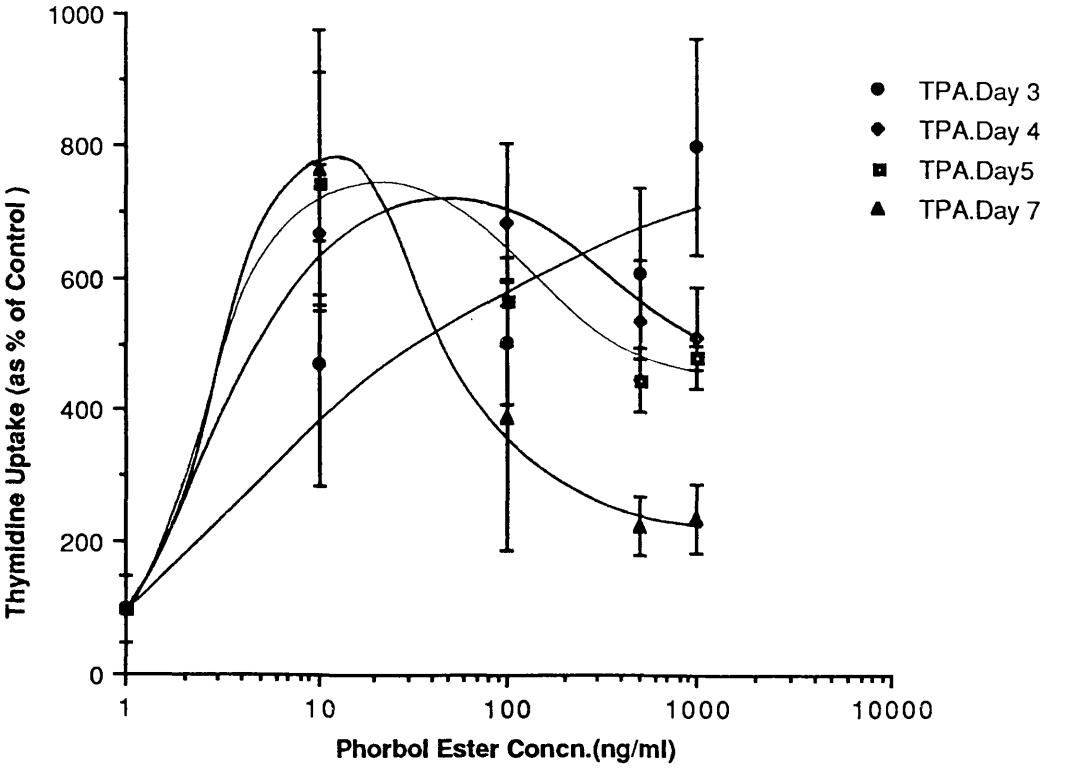
A single preparation of Human Mononuclear cells was diluted to lower the density of the culture,and maintained for 3 days prior to Phorbol Ester addition.

(a)TPA

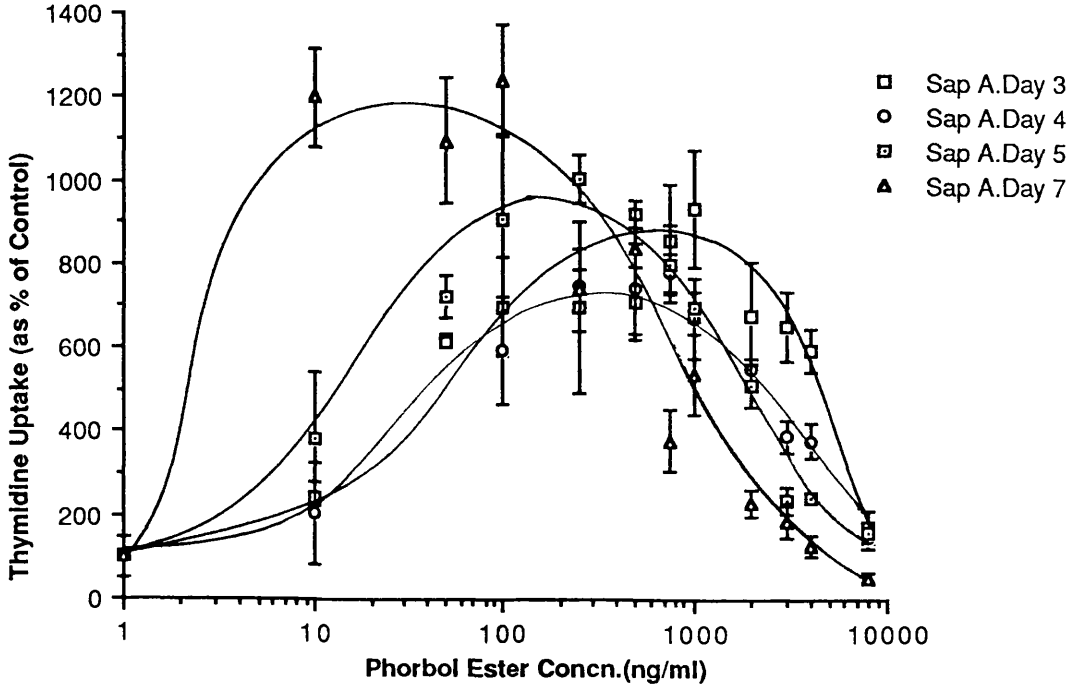
(b)Sap A

After addition of the Phorbol Esters , cultures were maintained for 3-7 days (as indicated) prior to incubation with radiolabelled thymidine and harvesting.The results are expressed as % of untreated (Control) cultures with S.E.M. error bars from triplicate cultures.

(a) TPA stimulation of delayed HMNC cultures plated at low density.



(b) Sap A stimulation of delayed HMNC cultures plated at low density.



were stimulated with SAP A (100μl) after a delay of 3 days. The cultures of high density did not respond to SAP A whereas the low density cultures showed mitogenic responses even with low doses of SAP A *(Figure 3d)*. The quantity of cells was monitored for untreated cells (100μl) in this experiment using the haemocytometer/trypan blue stain counting technique *(Figure 3e)*. Cultures of high density appeared to experience a loss in cell numbers over 6 days whereas in low density cultures numbers had remained stable over 3 days before declining. After 6 days a growth in both cultures was observed. Cells plated in higher density were additionally observed to have formed a whitish 'skein' on the surface of each microtitre well which light microscopy revealed as a gel-matrix containing a carpet of cells. With the cell counting technique used here the carpeted cells were clumped and it was difficult to accurately assess numbers.

DISCUSSION

The results show that both tumour promoting and non-promoting phorbol esters share the capability of acting as potent lymphocyte mitogens in cultured human MNC's. In fresh cultures the response for both mitogens was dose dependent, with maximal rate of growth seen after 3-4 days of administration. Although SAP A gave similar levels of maximal proliferation to TPA over 5 days, a comparison of EC_{40} values showed that SAP A did so with a potency 30-50 times lower than TPA. The potency differences seen here differ from earlier lymphocyte mitogenesis experiments *(292)* where EC_{40} values were given as 17 and 35 ng/ml for TPA and SAP A respectively. Closer scrutiny

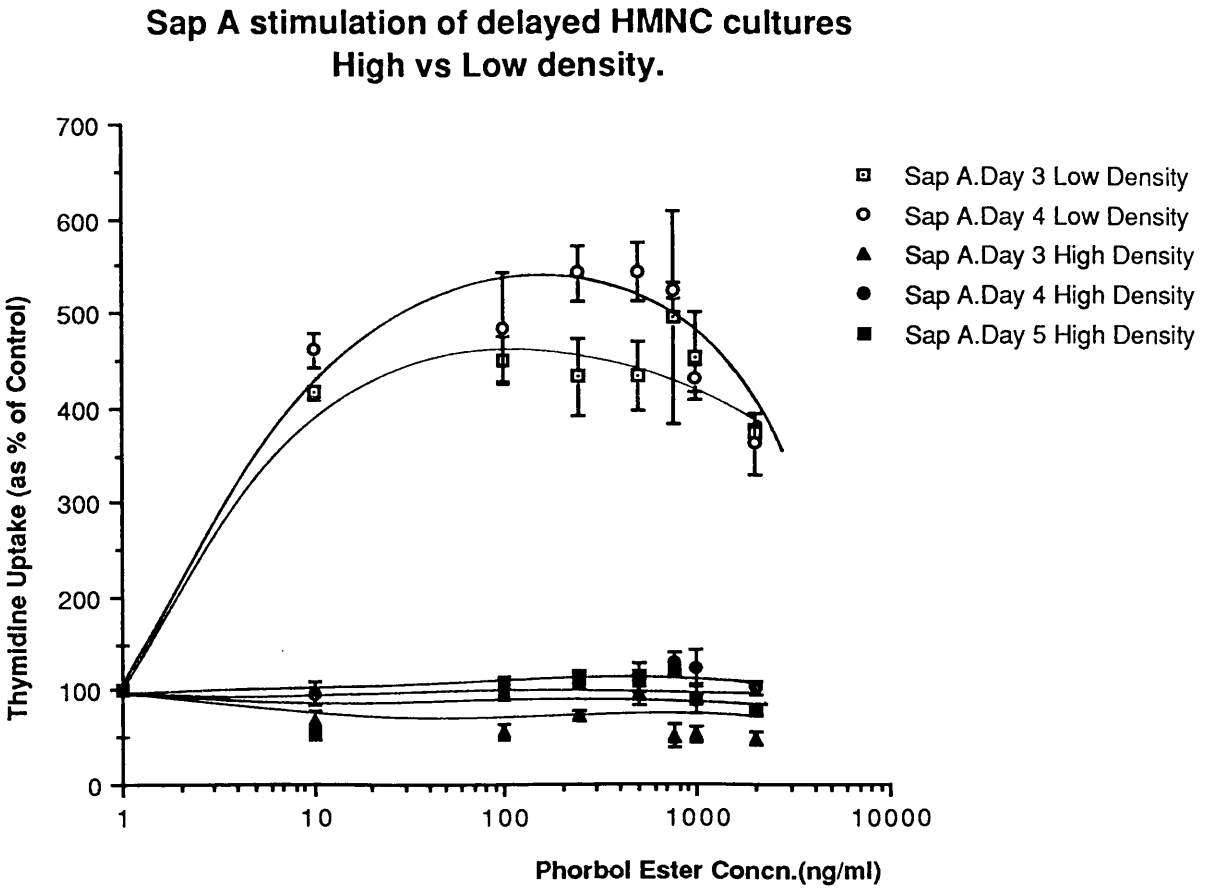


Fig 3d. Sap A stimulation of 3-Day-Delayed HMNC cultures at both high density,and low density prepared from the same source.After the delay ,cultures were treated with Sap A and tested for radio-labelled thymidine uptake at the indicated time from phorbol ester addition (3-5 Days),as detailed in the Methods,Chap.3.

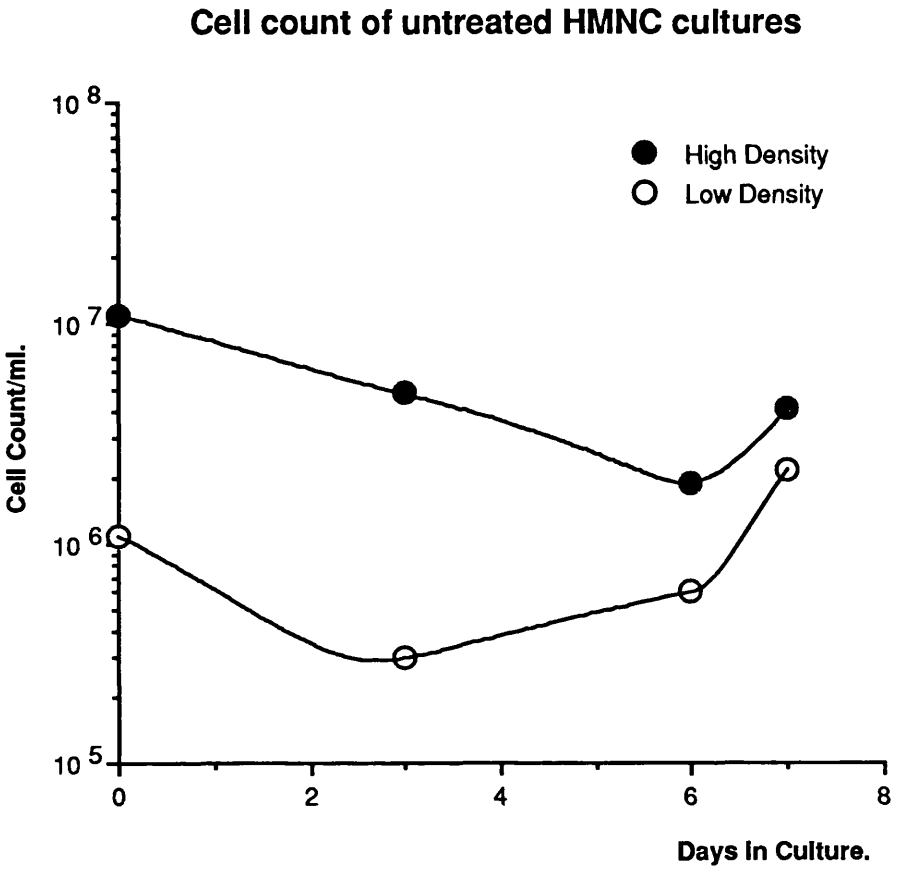


Fig 3e.Samples of untreated cells (Cells/ml)from the experiment featured in figure 3d,were counted with an heamocytometer at the indicated times.

of these results suggests EC_{40} for TPA to be ~6 ng/ml however, bringing closer agreement with the present findings. The results here agree with the potencies of these compounds in epidermal hyperproliferation experiments *(232)*.

Maximal stimulation of mitogenesis occured with 1000ng/ml with TPA at 3 days and shifted to 100ng/ml at 4 days as has been reported *(318)*. The mitogenic actin of SAP A was not markedly affected by prolonged suspension in sterile culture medium suggesting that the conversion of SAP A to its inactive isomer (4-α-deoxyderivatives *119*) in aqueous solution was not responsible for the difference in potency with TPA. Metabolism by the cells themselves, however, cannot be ruled out. The mitogenic response of submaximal SAP A doses harvested at 3 days could not be augmented by addition of a calcium ionophore which can switch SAP A into a tumour promoter when simultaneously applied to mouse skin *(232)*.

Interestingly TPA and SAP A did markedly differ in their mitogenic activity in lymphocyte cultures which had been maintained for 3 days prior to pherbol ester addition. Whereas the mitogenic activity of TPA remained intact, SAP A could not invoke mitogenesis at doses of 10-4000 ng/ml.

Since the mononuclear cell cultures contain a variety of cell types which interact in a complex manner to effect proliferation, the results suggested SAP A (but not TPA) - stimulated proliferation was abolished by changes in cell-to-cell communication occurring when

cultures were allowed to settle. The difference was investigated by attempting to disrupt communication by means of cell dilution. This was aimed at achieving dilution of monokine-type soluble messenger concentration in the culture as well as reducing cell contact. With such an approach SAP A was able to recover its stimulatory effect on proliferation in delayed cultures. The stimulation of such cultures occured over 3-7 days after phorbol ester addition, with maximal stimulation on successive days experiencing a shift of optimal concentration from 1000 to 10 ng/ml. The potency of SAP A in low-density delayed cultures was greatly increased to its potency in fresh cultures (EC_{40} values 2.4-30 ng/ml compared with 200-275 ng/ml in fresh cultures) and equalled TPA by day 7 (although lower doses of TPA were not tested.

Observation of cell number in untreated experiments indicated a process of culture reorganisation (cell death followed by growth) in both densities examined. The significance of this is uncertain, however the observation of the formation of a thick matrix of cells adhered to the substratum of high (but not low) density cultures indicates that the concen tration at which cells are plated may have an effect on cell to cell interaction. The approach was limited to observing cell number and the various types of cell and their properties in cultures was not determined. The results are consistent with the possibility that a cell population(s) which is regulated (or becomes depleted) in high density cultures and which proliferate (or mediate proliferation) in response to SAP A are unregulated in fresh cultures (before communicatin has been

established) or become so in low density delayed cultures. Furthermore the results suggest TPA would be able to influence alternative cell populations in addition to SAP A-influenced population(s), to bypass these effects of changes in cell-to-cell communiction.

Other studies have indicated a variety of effects can be elicited from mononuclear cells by phorbol esters. These include Eicosanoid and Monokine production and secretion *(166,320)*, influencing of several subsets of lymphocytes involved in cytotoxicity *(342,343,344)*, changes in expression of surface antigens *(298)*, and growth restriction *(354)*. A study on HMNCs depleted of specific subpopulations (using complement-linked antibodies to lyse cells) has suggested the proliferative response to TPA differs from that of DOPPA (another non-promoting phorbol ester, *338*) supporting the case for cell-based differences of SAP A and TPA action.

It would be necessary to conduct further studies on subset-depleted or purified subpopulations of lymphocytes, in parallel with investigation of phorbol ester-induced changes in cell types and numbers, to determine the interaction of different phorbol esters with heterogenous lymphocyte cultures.

CHAPTER 4

INVESTIGATION OF PHORBOL ESTER-STIMULATABLE KINASE ACTIVITY USING RAT BRAIN ENZYME

In view of the differences in biological effects of TPA and SAP A on lymphocyte proliferation it was decided to extend the investigation of phorbol ester action to the biochemical level. It has been reported that both non-promoting and promoting-phorbol esters share the ability to activate protein kinase C in vitro *(134,191)*. The enzyme used in these preparations was purified through several steps of chromatography to "homogeneity", yet further work has revealed a family of closely related PKC isozymes *(252)*. The biochemical investigation therefore required a procedure to fractionate source material and investigate (possibly multiple) kinase activity which could be stimulated by phorbol esters.

Rat brain was initially used as an enzyme source since it was readily available in large quantities and has been demonstrated to contain large amounts of the known PKC isozymes *(247,253)*. Consequently a system of crude separation combined with a kinase assay detecting phorbol ester-stimulation was developed to probe rat brain. These pilot experiments could then be applied to address the lymphocyte system.

Tissue Preparation and Chromatography

Fresh rat brain (1-2 brains, ~8g each) were minced in 10 mls ice-

cold homogenization buffer (buffer 3) using a tissue homogenizer (30 seconds max speed). The homogenate was then centrifuged for 30 minutes at 15,000 x g and the supernatant loaded into a 10 ml superloop (pharmacia) in ice connected to a Fast Protein Chromatography system (FPLC, pharmacia). The supernatant was pumped onto a hydroxyapatite column (0420 HTP Biorad made up in buffer 4 or 6) and washed with ice-cold low phosphate buffer (20mM Potassium phosphate - see buffer 4 or 6) until the unbound proteins had flowed through (see example-trace *Fig 4a*). A linear gradient from 20 to 500 mM phosphate (buffer 5 or 7) was then initiated running through at a flow rate of 1 ml per minute. 36 fractions of 1ml were collected on ice and immediately mixed with 200 μl of storage buffer (buffer 8), vortexed and frozen in liquid nitrogen for storage at -70^{o}C. A delay of ~5 minutes for any protein to be eluted from the column was observed upon initiation of the gradient.

Assay for Protein Kinase Activity

The fractions from rat brain were assayed for protein kinase activity using a modified version of the micellar assay of Hannun et al *(201)*. The assay measured the transfer of ^{32}P from radiolabled ^{32}P -γ- ATP to histone IIIs substrate by incubation under various conditions for 10 minutes at room temperature. *(See Kinase assay method 1 for solutions used and TAble 2A for final concentrations of all reagents.)* The reaction was terminated by precipitating all protein with ice-cold Trichloracetic acid, filtering and washing of all unassociated radioactivity. Filters were then counted for ^{32}P radioactivity (See Methods).

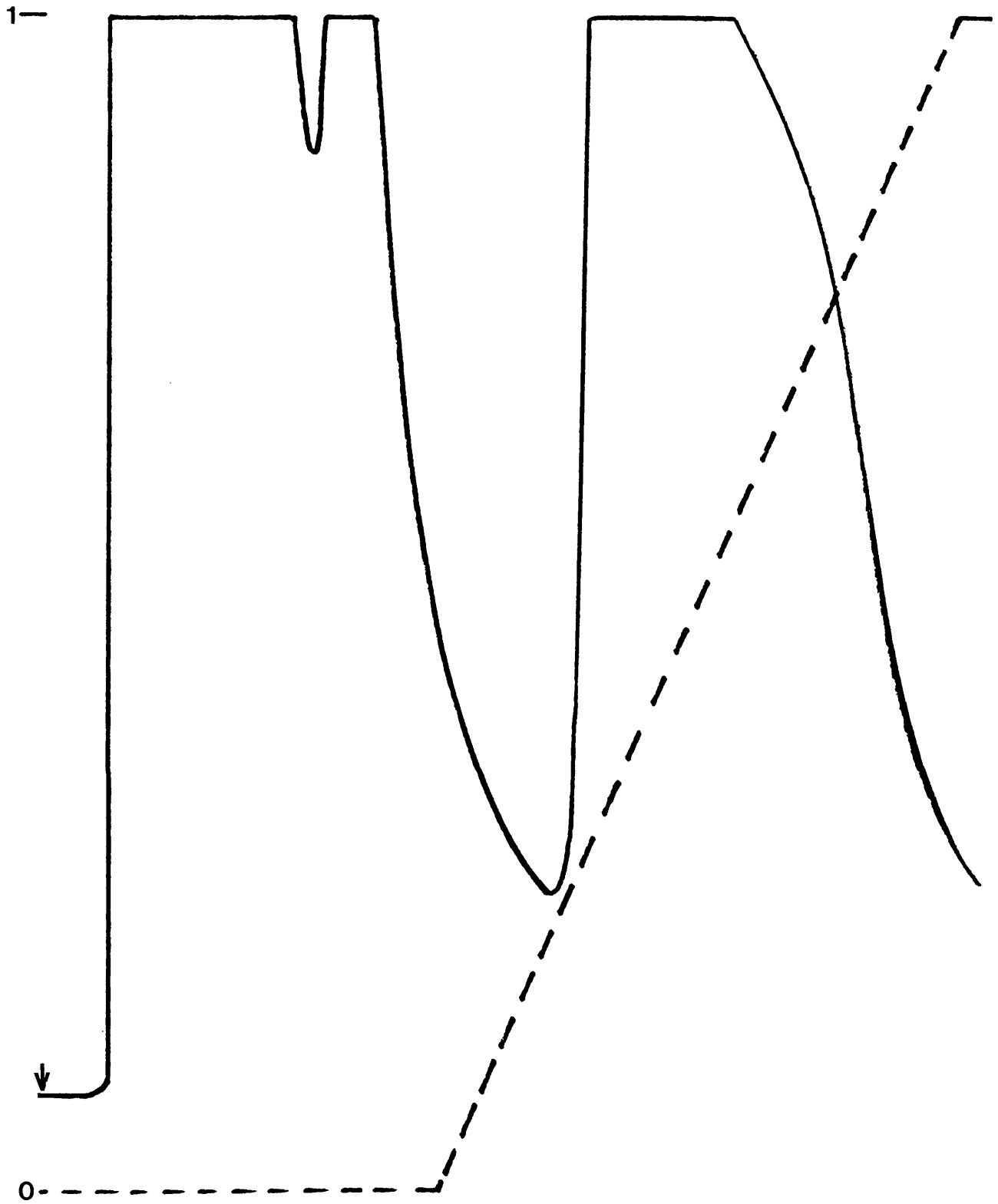


Fig 4a. Example of protein absorbance chart recording for hydroxyapatite FPLC chromatography of rat brain.Centrifuged supernatents from 2 rat brains were loaded onto the column in 20 mM phosphate buffer.After washing through with this buffer,a linear gradient up to 500 mM was initiated (- - - -). Protein absorbance during the run is shown (A_{280} nm,0-1 unit range (———) with chart speed 0.25 cm/min.(▼) marks the application of the sample.

Assay Handling for method 1

The micelle mix (buffer 40) was vortexed and sonicated until the phosphatidyl serine and phorbol ester (where present) were completely dissolved, as indicatd by the solution becoming clear. This micelle solution and the histone solution (buffer 39) were added to the assay tubes first and kept on ice while both ATP mixes were prepared (one without and one with added calcium (see buffer 41)). Tubes were arranged in 2 parallel trays each destined to receive one ATP mix and each test point was conducted in duplicate. Enzyme fractions to be tested were thawed, vortexed and immediately added to the assay tubes. The ATP mix (+ or - Calcium) were then added by autopipette to initiate the assay. The addition of 100μl of ATP mix to 100μl of micelle/histone/enzyme was judged to be sufficient for mixing of the components although the trays were also shaken between additions (12 in 30 seconds).

Assay Components

The micelle assay was conducted with L-α-Phosphatidylserine (PS) from Nutfield laboratories UK. TLC analysis showed this preparation to be of significantly greater purity than PS from Sigma (data not shown). Histone preparations also varied significantly in their ability to accept phosphate from batch to batch, so where possible assays were conducted with tried and trusted histone from identical batch numbers. A titertek cell harvester was employed in large-scale assays with mixed success. For these experiments the final assay volume was reduced to 175μl (adjusting the concentration of all

components accordingly) and terminated with 50 μl of 112.5% w/v TCA solution (final concentration 25% w/v). This was then harvested with a Titertek cell harvester using Titertek filtermats and 2 x 10% TCA reservoir washes. While this gave excellent service initially, the apparatus became increasingly unable to handle the TCA wash (in spite of manufacturers claims) and was discontinued in favour of filtration by a manual Millipore apparatus. At its best the Titertek system proved to be a fast and efficient tool for biochemical filtration assays and would be invaluable if developed upon such lines.

Assay Parameters

Various parameters were tested in order to investigate the assay and optimise the detection of kinase activity in crude fractions. Using the fractions from hydroxyapatite chromatography of 1 rat brain a 'profile' of kinase activity was constructed. The presence of TPA (Present at 100 ng/ml representing 0.003 mole % of micelle) and calcium, either excluded or present at a final concentration of 100 μM calculated free concentration (using a calculator program for multiple metal/ligand solutions *(562)*) was tested in duplicate. A typical profile is shown in *Fig 4b*, and appeared to be readily reproducible using the FPLC system. Fractions from the flow-through on the hydroxyapatile column with low phosphate (buffer 4 or 6) or from the pellet left after the 15,000 x g spin did not appear to contain any TPA or PS stimulatable kinase activity (data not shown).

Rat brain kinase activity.

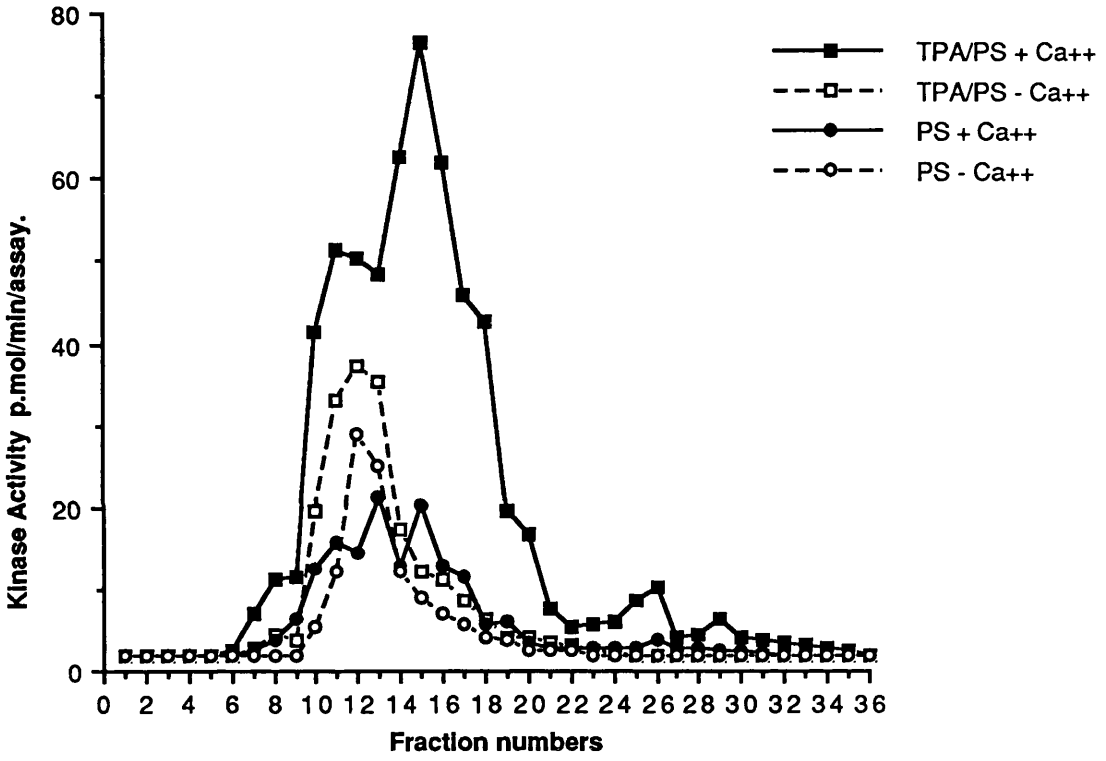


Fig 4b. Typical profile of kinase activity (p.mol/min/assay) of hydroxyapatite chromatography fractions (20-500mM phosphate gradient) from 1 rat brain.Calcium,where present ,had a calculated free concentration of 100µM.TPA,where present ,had a final concentration of 100ng/ml,representing 0.003% of the micelle.Histone was present at 1 mg/ml (final concentration).

Micelle Composition

The crude supernatant and fractions selected from hydroxyapatite profiles were tested for the effect of TPA, PS and calcium on the kinase activity observed *(See Fig 4c)*. TPA stimulated Kinase activity to the greatest extent when both Calcium and Phospholipid were present. Some calcium- and Phospholipid- independent Kinase activity was observed with TPA stimulation in hydroxyapatite fractions but not in crude supernatant.

Phorbol Ester Concentration

Increasing the concentration of TPA from 100 ng/ml to 1μg/ml raised the kinase activity in the absence of added calcium but had little effect in its presence *(Table 4A)*.

Substitute Concentration

Histone when tested between 1 and 3 mg/ml final concentration was found to increase the amounts of phosphate incorporation with TPA/PS + Calcium significantly *(Fig 4d)*. This increase was also observed to a lesser extent for TPA/PS - calcium but had no effect on the Kinase activity supported by PS alone. A profile with both high histone (3mg/ml) and TPA (1μg/ml) final concentrations was constructed. A 'Difference profile' was calculated by subtracting the Kinase activity supported by PS alone from TPA/PS values in the presence or absence of calcium *(Fig 4e)*. This profile represents the stimulation of kinase activity elicited by phorbol ester and when compared with a difference profile of histone at 1 mg/ml and TPA at 100 ng/ml *(Fig 4h)*, shows a general increase of kinase stimulation. However, the

Kinase activity in Rat Brain.

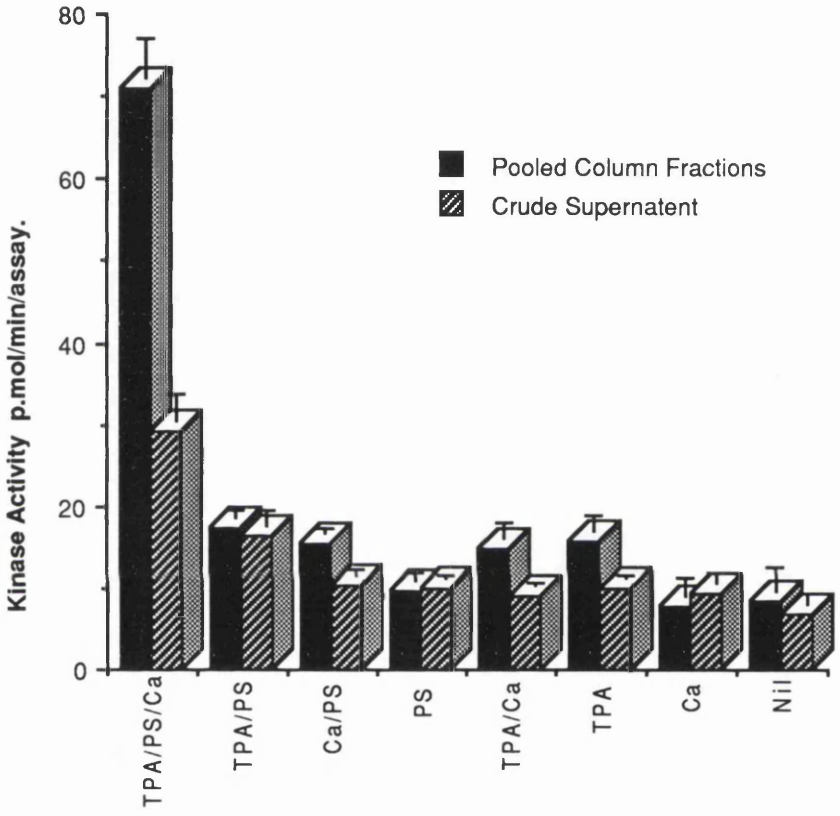


Fig. 4c Protein kinase activity observed in either ;pooled fractions nos.12,14 and 18 from hydroxyapatite column chromatography,or crude supernatant prior to chromatography, using 1 rat brain.TPA was used at 100 ng/ml final concn., histone at 1mg/ml in the triton micellar assay terminated after 10 minutes (For composition see Assay 1 methods in chapter 2).In the absence of added enzyme,the non-specific amount of radioactivity bound was 2 p.mol/min/assay.

TPA effects on kinase activity in rat brain

Fraction.	Ca++	TPA (ng/ml) 100	1000	Difference (%)
11	+	73.6	75.8	+2.3
	-	34.8	45.0	+29.3
16	+	141.8	141.0	-1.0
	-	77.8	95.9	+23.3

Table 4A. Mean kinase activities (p.mol/min/assay) ,with final concentrations of TPA (present at 100 or 1000 ng/ml) and histone (1 mg/ml) in a Triton/PS micellar assay,using fractions from a 2 rat brain preparation.

Dilution effects on kinase activity in rat brain.

	Triton micelle	Ca++	Enzyme (µl) 12.5	25.0	Difference (%)	Stimulation difference
(1)	TPA/PS	+	61.3	108.5	+77	x 2.1
		-	43.3	63.7	+47	x 1.8
	PS	+	24.5	31.0	+27	-
		-	29.5	38.9	+32	-
(2)	TPA/PS	+	53.0	90.5	+71	x 2.1
		-	32.7	49.8	+52	x 1.8
	PS	+	22.0	25.0	+14	-
		-	18.5	24.0	+30	-
(3)	TPA/PS	+	49.8	74.8	+50	x 1.7
		-	28.0	37.2	+33	x 1.7
	PS	+	21.0	26.0	+24	-
		-	21.0	25.0	+19	-

Table 4B. Mean kinase activities (p.mol/min/assay less non-specific level), with TPA at 1000 ng/ml where present,in rat brain; (1) Fraction 16 from a 2 brain preparation with histone at 3 mg/ml;(2)Pooled fractions 10-14 from a 2 brain preparation with histone at either 3 mg/ml or (3) 2 mg/ml .The stimulation difference is the ratio of (TPA/PS - PS) value for 25 µl compared to 12.5 µl enzyme.

Substrate concentration effects on kinase activity in rat brain fractions.

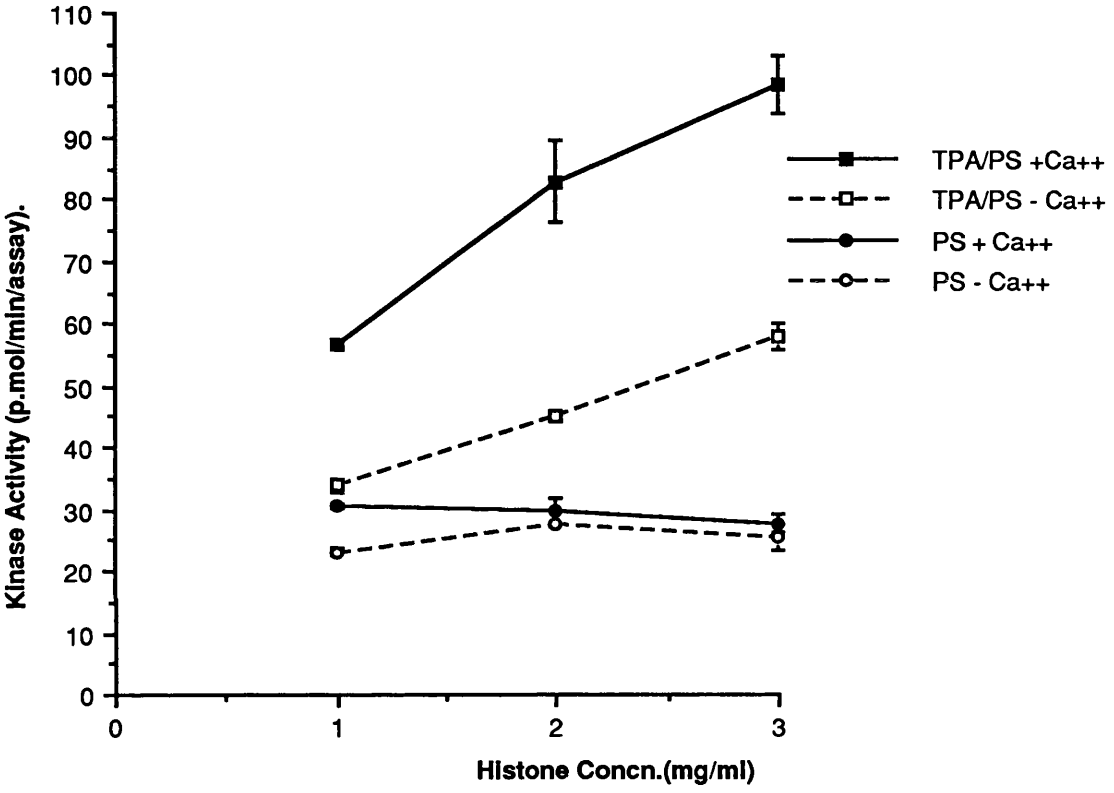


Fig 4d. Effect of histone concentration on kinase activity of rat brain fractions (Nos.10-14 pooled from a 2 brain preparation).TPA,where present,had a final concentration of 1000 ng/ml,representing 0.03% of the Triton/PS micelle.Results are shown as mean values with the range from duplicate assays.

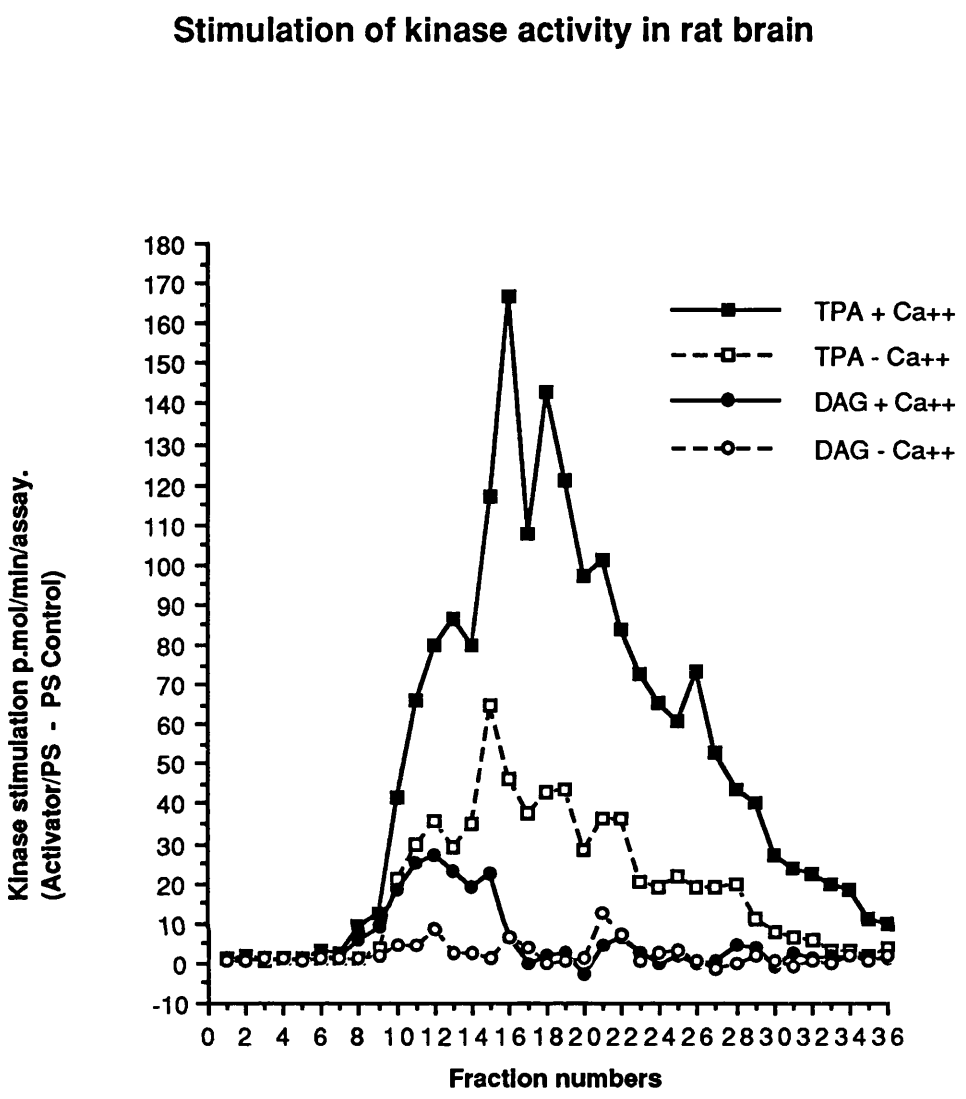


Fig 4e. Stimulation by TPA or a diacylglycerol (present at final concentrations of 1000 ng/ml or 40 μg/ml respectively ,representing 0.03 or 1.2 mol % of the Triton/PS micelles) of rat brain kinase activity with basal levels (PS alone) subtracted. Brains from 2 rats were taken and subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile. Histone was present at a final concentration of 3 mg/ml.

increase caused by higher histone and TPA levels seems varied in different regions of the profile with the effect that the definition of smaller peaks becomes impaired.

Time Course

The time dependence of histone phosphorylation by active rat brain fractions was assessed over 12 minutes *(See Fig 4f)*. The pattern of phosphate incorporation with time appeared to follow the same course, irrespective of calcium addition or TPA, with the initial rate trailing off until a plateau approached after 10-12 minutes. Kinase stimulation elicited by TPA was observable at each time point tested.

Dilution Tests

Assays conducted with 25μl of enzyme fraction per assay was compared with assays with 12.5μl *(Table 4B)*. The assay was found to be poorly linear, with PS alone kinase activity dropping by 20-25% and TPA/PS kinase activity by 30-40% rather than a linear 50%. The stimulation of kinase activity by TPA expressed as [TPA/PS - PS control], however, was approximately linear (45-55%) in the presence or absence of calcium.

Conclusions on the Assay

Using the FPLC system, the elution profile of TPA-stimulated kinase activity in rat brain was consistently reproducible. Detection of kinase activity in these crudely fractionated rat brain has been investigated and found to depend on PS and be stimulatable

Time course of kinase assay.

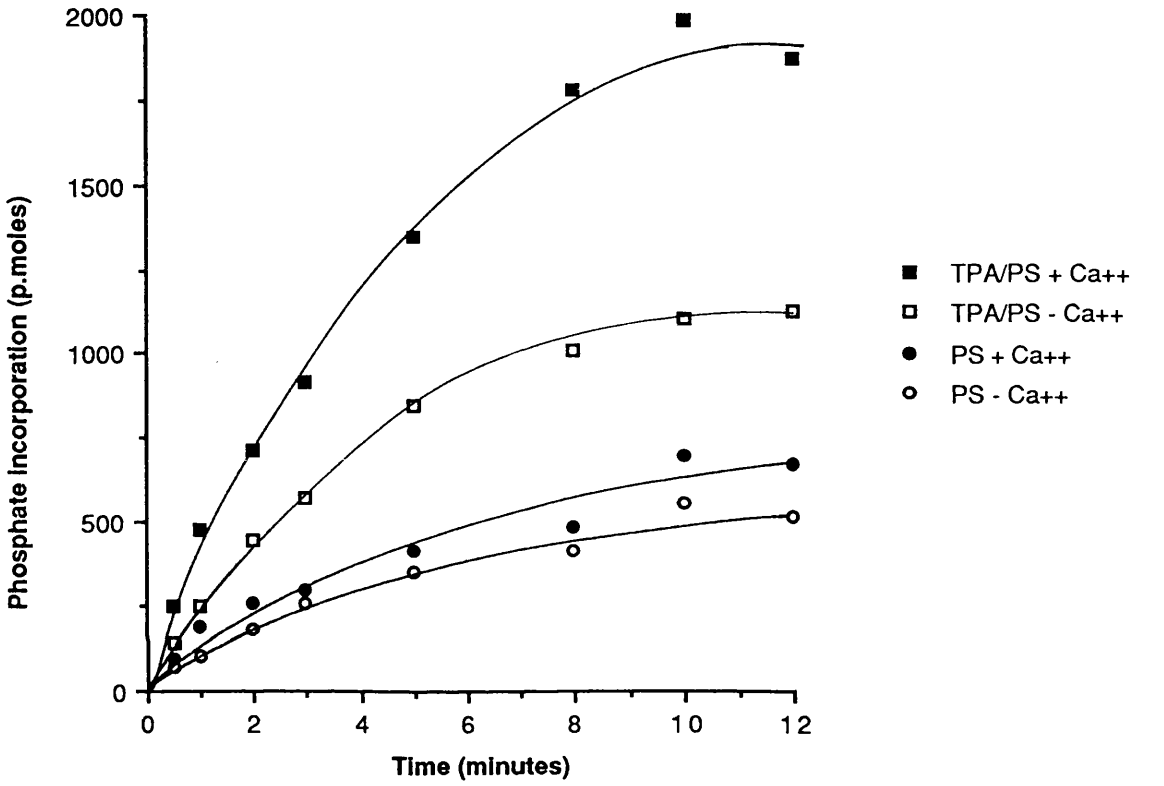


Fig 4f. Pooled fractions 12 and 13 from a preparation of 2 rat brains were assayed for phosphate incorporation (p.moles incorporated minus 80 p.moles non-specific level).At the time points indicated ,an aliquot of 25 µl of each reaction mix was pipetted into 25% TCA to terminate the reaction.TPA,where present,had a final concentration of 1000 ng/ml (0.03 mol % of micelles),and histone had a final concentration of 2 mg/ml.

by phorbol ester in the range of 100-1000 ng/ml. The large amounts of kinase activity present in rat brain fractions appear to shift the assay out of the linear range of histone phosphorylation. Increased substrate concentration appears to increase phorbol-ester stimulatable activity but not the activity with PS alone. This histone effect is reduced however if the enzyme fraction is diluted suggesting that high enzyme concentration is responsible in crude fractions. In terms of quantity of extra phosphorylation elicited by phorbol esters, the difference between kinase activity stimulated by PS and TPA/PS was linear with the enzyme concentrations tested.

Differential substrate effects on the level of PKC phosphorylation have been reported previously comparing Histone IIIs with HMG-8 as phosphate acceptors *(563)*. In these studies, an increase in histone concentration did not affect the Km for diacylglycerol-stimulated activity, whereas the Km and Vmax values increased dramatically with increases in HMG-8 concentration.

In view of these parameters an assay of 10 minutes was used to give easily measurable quantities of transferred radioactivity and allow the simultaneous assay of large numbers of tubes. To emphasise the definition of peaks of kinase activity across the fractions, conditions of low histone (1mg/ml) and Phorbol ester (100 ng/ml) were chosen. Results are given in terms of the difference profile (i.e. Phorbol ester/PS - PS alone) in the presence and absence of calcium.

Stimulation of Kinase Activity from Rat Brain by a Variety of Phorbol Esters

2 rat brains were homogenised and supernatents run on a potassium phosphate gradient using an FPLC apparatus. Each fraction was subsequently assessed for stimulation of kinase activity above the PS alone activity *(See Fig 4g)* by a range of phorbol esters present at 100 ng/ml in the micellar assay. Difference profiles are shown for TPA *(Fig 4h)*, Thymeleatoxin (THY TOX; *Fig 4i)*, Sapintoxin A (SAP A; *Fig 4k*), Resiniferatoxin (RX; *Fig 4j*) and the 12-Deoxyphorbols; DOPP (-13-phenylacetate derivative; *Fig 4l*) and Doppa (-13 phenylacetate-20-acetate derivative, *Fig 4m*). These phorbol esters uncovered several peaks of stimulatable kinase activity across the elution gradient. 8 potential peaks are discussed below in the order of their elution. A column void-volume of 5 mls was assumed and the phosphate molarities calculated from this lag period;

Peak I: Fraction 7-8/45-60 mM phosphate.

A small peak seen as a low shoulder or a discrete peak running into the central body of kinase activity. Activated in the presence of calcium by TPA, THY TOX, DOPP and to a lesser extent by SAP A. In the absence of calcium THYTOX and to a lesser extent TPA stimulated kinase activity.

Peak II: Fraction 10-12/85-115 mM phosphate.

This was a large rounded shoulder on peak III. Phorbol ester stimulation was able to distinguish it from the other peaks. Activated in the presence of calcium by THY TOX whilst DOPP and

Basal kinase activity in rat brain fractions

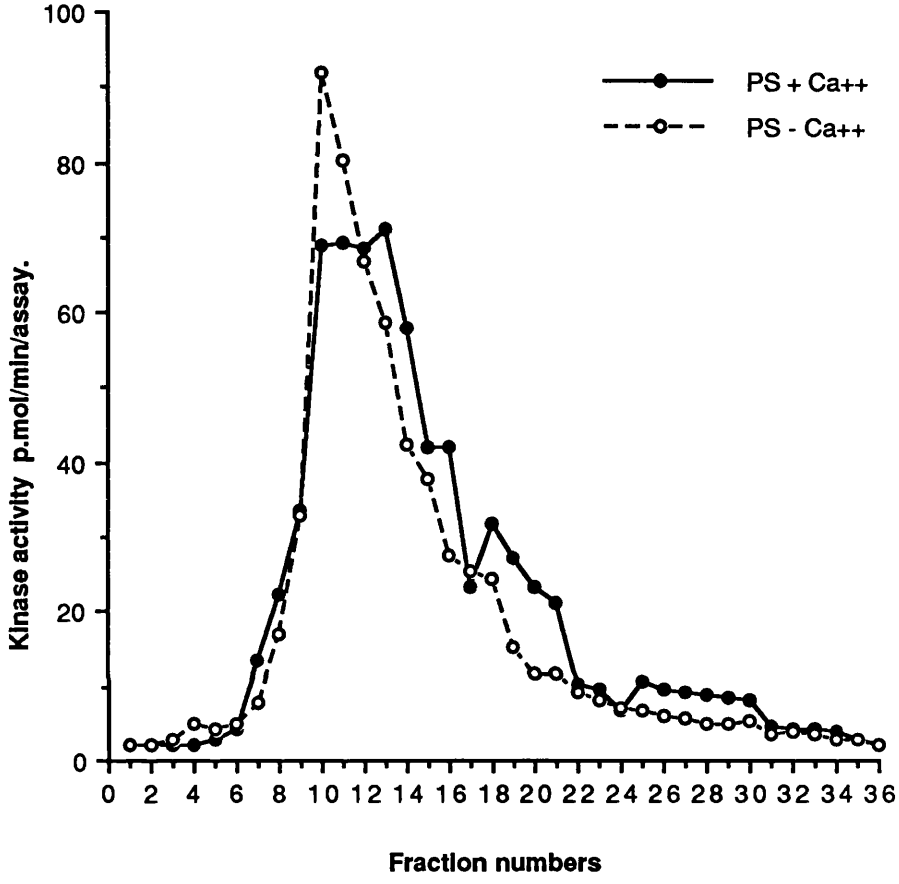


Fig 4g. Brains from 2 rats were taken and subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.The basal level of kinase activity (PS alone without activators) is shown above in the presence or absence of 100μM of free calcium added.Histone was present in a final concentration of 1 mg/ml.

Stimulation of kinase activity in rat brain by TPA.

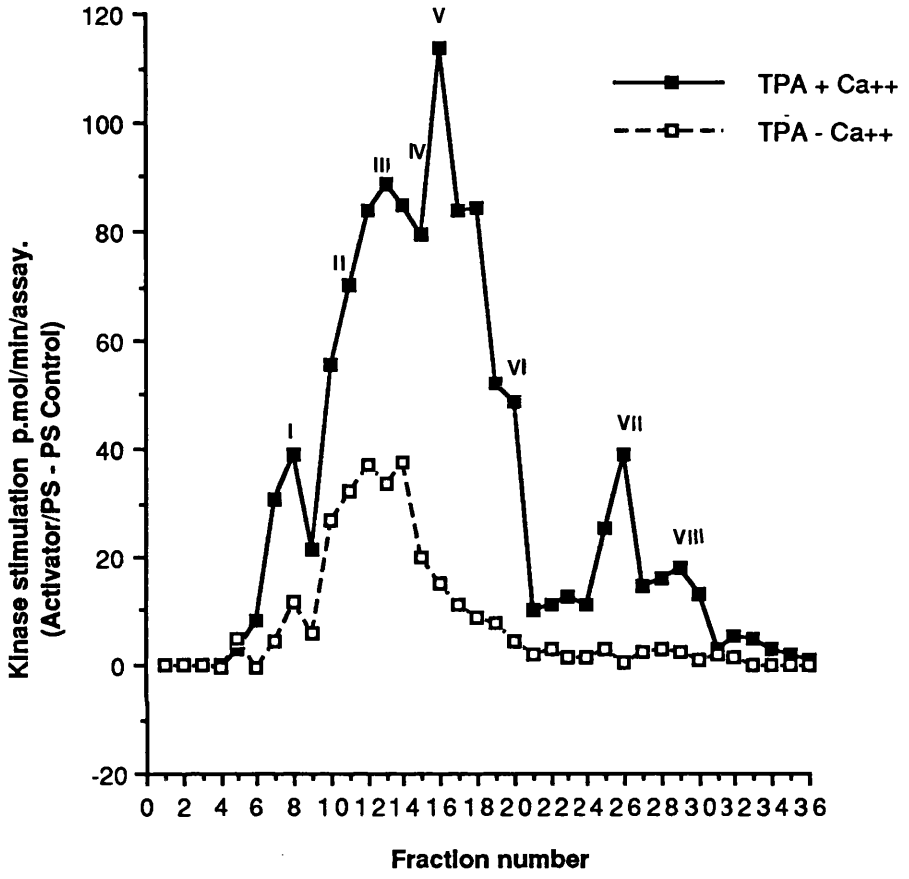


Fig 4h. Stimulation by TPA (present at a final concentration of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of rat brain kinase activity with basal levels (PS alone,from the same preparation shown in Fig 4g)subtracted. Calcium,where present, had a final free concentration of 100μM and histone had a final concentration of 1 mg/ml.

Stimulation of kinase activity in rat brain by Thymeleatoxin A.

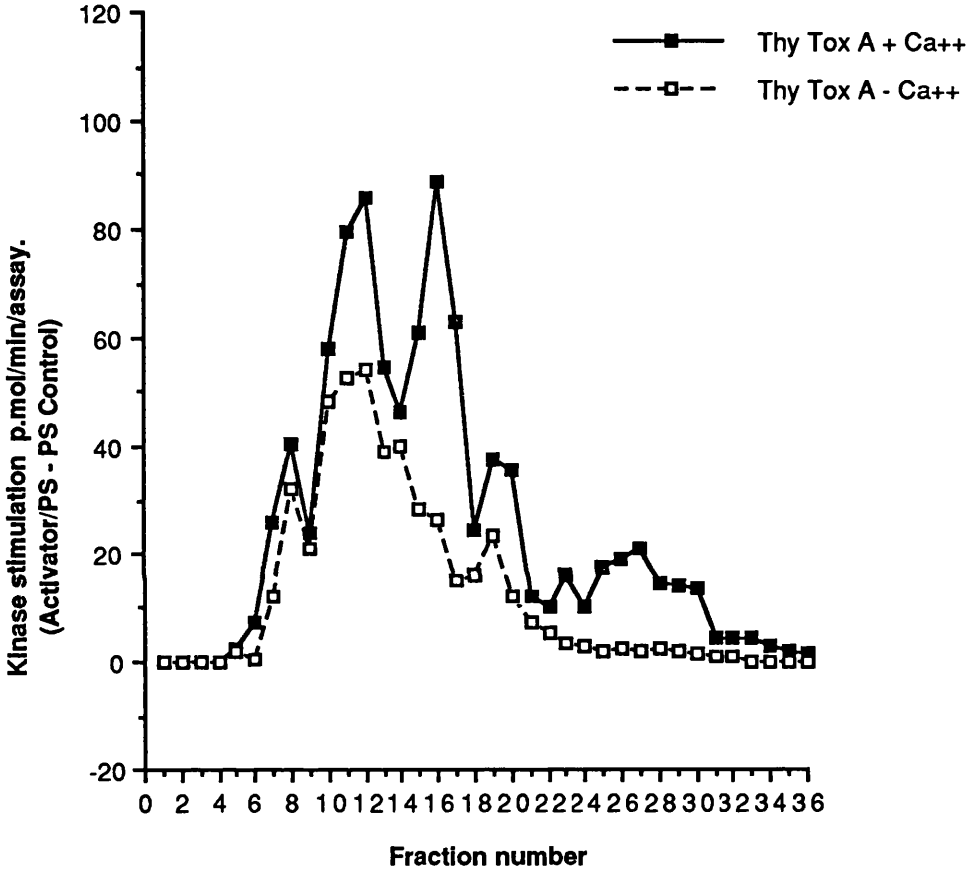


Fig 4i. Stimulation by Thymeleatoxin A (present at a final concentration of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of rat brain kinase activity with basal levels (PS alone,from the same preparation shown in Fig 4g) subtracted.Calcium where present,had a final free concentration of100μM and histone had a final concentration of 1 mg/ml.

Stimulation of kinase activity in rat brain by Resiniferatoxin.

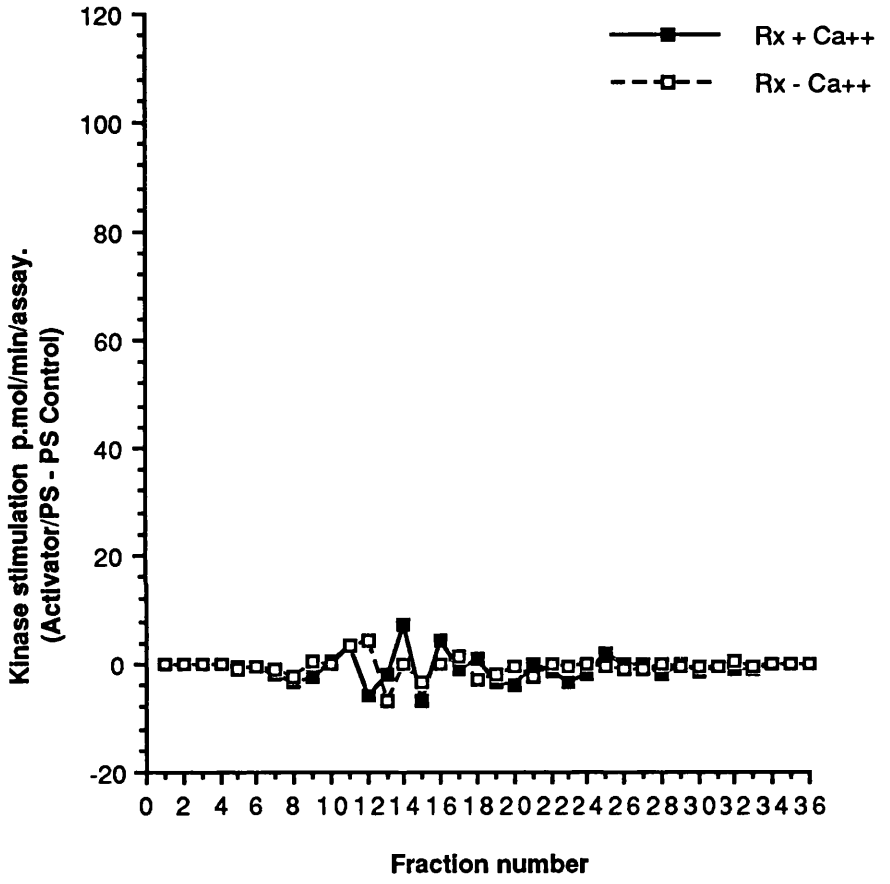


Fig 4j. Stimulation by Resiniferatoxin (present at a final concentration of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of rat brain kinase activity with basal levels (PS alone,from the same preparation shown in Fig 4g) subtracted. Calcium,where present, had a final free concentration of100μM and histone had a final concentration of 1 mg/ml.

Stimulation of kinase activity in rat brain by Sap A.

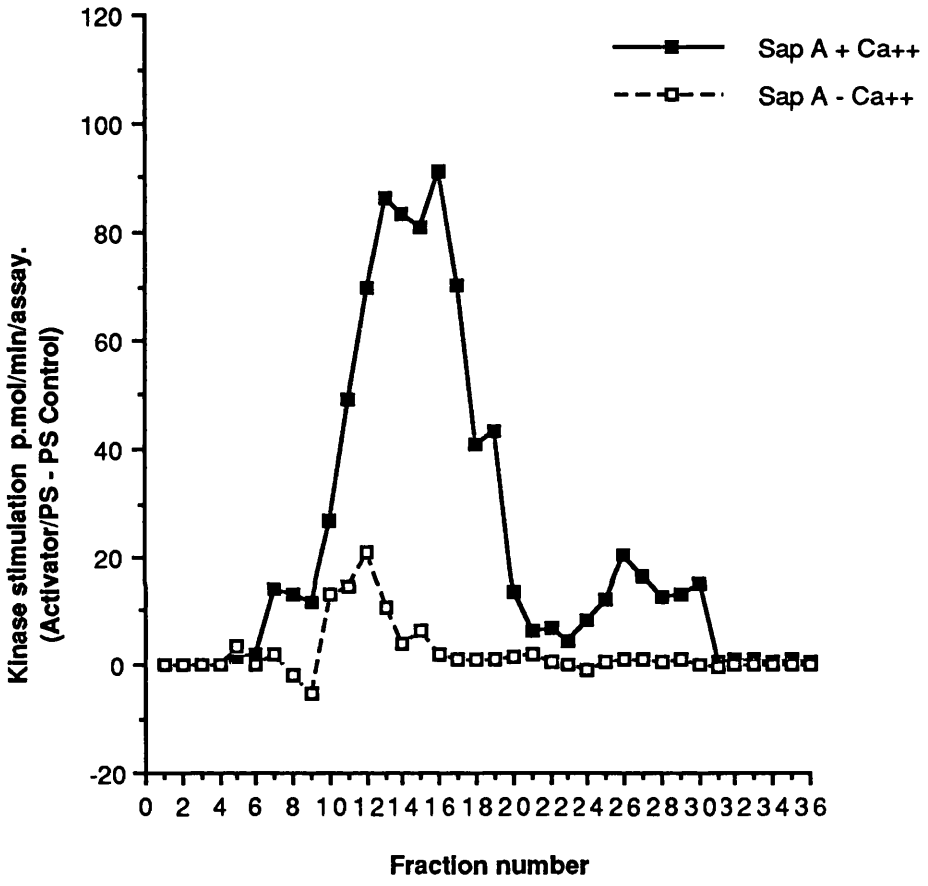


Fig 4k. Stimulation by Sapintoxin A (present at a final concentration of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of rat brain kinase activity with basal levels (PS alone,from the same preparation shown in Fig 4g) subtracted. Calcium,where present, had a final free concentration of100μM and histone had a final concentration of 1 mg/ml.

Stimulation of kinase activity in rat brain by DOPP.

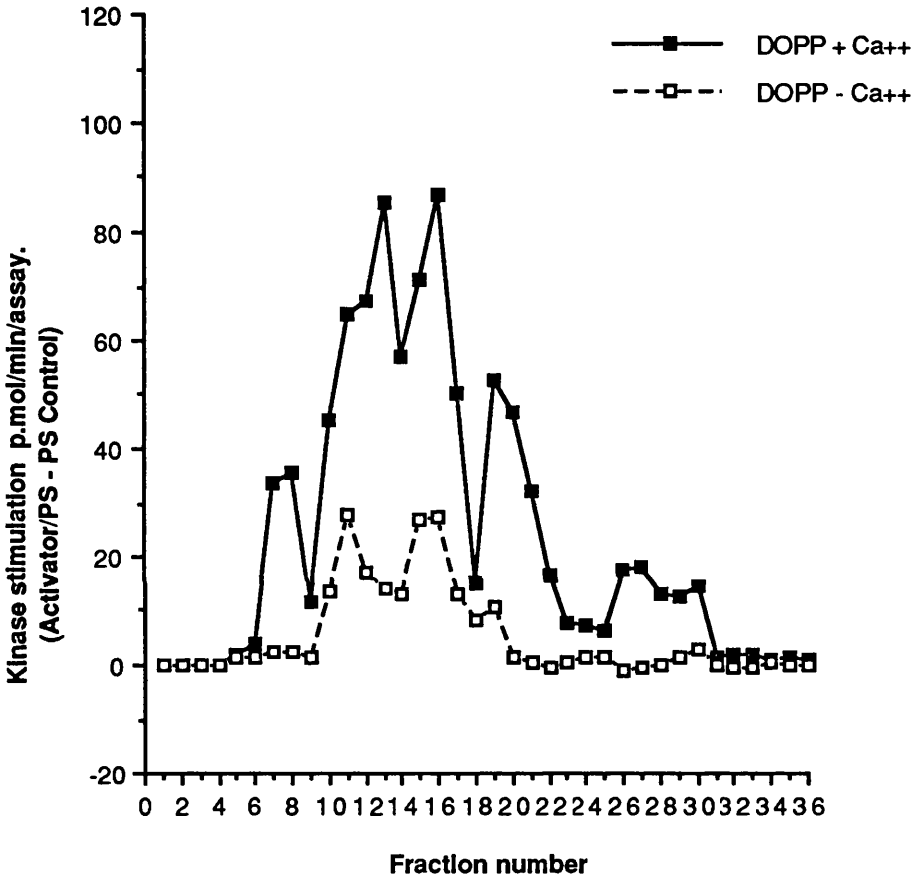


Fig 4l.Stimulation by DOPP (present at a final concentration of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of rat brain kinase activity with basal levels (PS alone,from the same preparation shown in Fig 4g)subtracted. Calcium,where present, had a final free concentration of 100μM and histone had a final concentration of 1 mg/ml.

Stimulation of kinase activity in rat brain by Doppa.

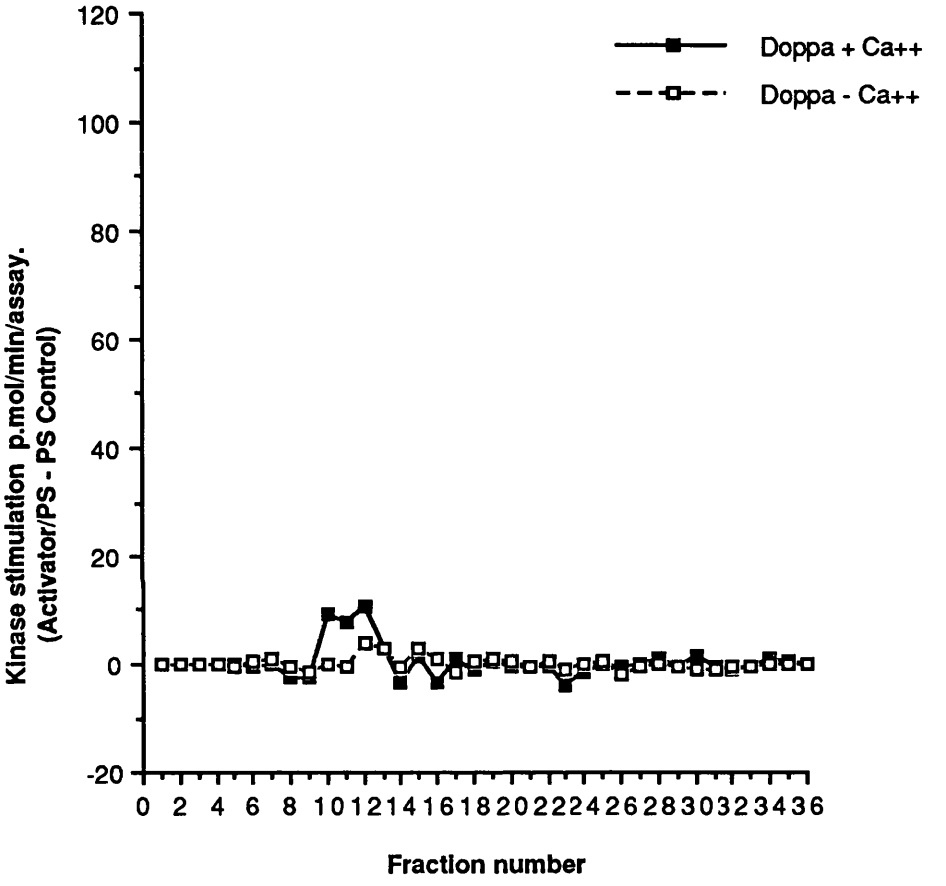


Fig 4m.Stimulation by Doppa(present at a final concentration of100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of rat brain kinase activity with basal levels (PS alone,from the same preparation shown in Fig 4g)subtracted. Calcium,where present, had a final free concentration of 100μM and histone had a final concentration of 1 mg/ml.

TPA appear to incorporate it into a shoulder of activity. In the absence of calcium, TPA, DOPP and SAP A appear to activate it. The effect of THY TOX is uncertain due to masking from peak I run-over in the absence of calcium.

Peak III and IV: Fractions 11-15/100-150 mM phosphate.
Two peaks are suspected here from the stimulation patterns observed with different phorbol esters. With calcium both appear to be stimulated by TPA and SAP A whilst DOPP and THY TOX appear more selective for the first peak (Peak III). In the absence of calcium, TPA and THY TOX stimulate both peaks, SAP A appears to preferentially stimulate the first peak whilst DOPP stimulated activity is reduced in this region.

Peak V: Fractions 14-17/140-180 mM phosphate.
A large peak sharply stimulated by TPA, THY TOX, SAP A and DOPP in the presence of calcium. In the absence of calcium DOPP and to a lesser extent TPA and THY TOX cause stimulation (possibly run over from previous peaks).

Peak VI: Fractions 18-20/190-220 mM phosphate.
This peak appeared as a shoulder off peak V for TPA in the presence of calcium. THY TOX and to a lesser extent DOPP caused stimulation in the absence of calcium. Interestingly a further peak (VIa) could be seen in high histone/TPA assays *(Fig 4e fractions 21 and 22)*.

Peak VII and VIII: Fractions 25-31/285-365 mM phosphate. This doublet of peaks was separated from other peaks of kinase stimulation in assays with low histone and phorbol ester concentration. Both peaks were stimulated by TPA, THY TOX, DOPP and SAP A in the presence but not the absence of calcium. TPA caused same stimulation in the absence of calcium with high TPA/Histone concentrations.

Doppa was seen to stimulate poorly around fractions 10-12 in the presence of calcium whilst RX gave no stimulation of kinase activity over PS alone. In another experiment stimulation of the profile was conducted with the diacylglycerol analogue, sn1,2 Dioleoylglycerol ($DiC_{18:1}$) present at the minimum optimum concentration *(201)* of 1.2 mol% = 40μg/ml final concentration) see *Fig 4e*. This caused stimulation across the fractions 8-16 (peaks II-V and possibly peak I) in the presence (and less so in the absence) of calcium. Traces of slight activation of a post peak VI activity (fraction 21-22, peak VIa) independent of calcium were also observed. All stimulation was significantly less than the corresponding stimulation seen with TPA (present at 1000ng/ml here).

These results demonstrate a variety of kinase activities can be separately identified, from a hydroxyapatite chromatography profile of crude rat brain supernatent, using phorbol esters as stimulating agents. The identification of these peaks, with respect to PKC isozymes, cannot be made with any certainty. However when the pattern of kinase stimulation elicited by TPA was compared with a

diacylglycerol ($DiC_{18:1}$ here), the putative endogenous activator of PKC, only the peaks II to V (eluting at 60-160 mM phosphate) were stimulated in a classical PKC, calcium-dependent manner, suggesting the PKC isozymes are eluted within this region. Other investigators have effected PKC isozyme elution between 40 and 150 mM phosphate using hydroxyapatite chromatography *(247,260,263,265)* in the order γ, β(1 and 2) and α, supporting this suggestion. The elution of other isozymes in this system has yet to be reported. It is known that full-length PKC can be cleaved into an active kinase fragment (PKM) which could account for the observation of multiple peaks *(ref 26)*. PKM has been found to be independent of calcium, phospholipid phorbol ester/DAG for activation (The alternative cleavage product - the 'regulatory domain' - accomodates such binding sites), thus in these "difference profile" graphs, PKM activity would be effectively excluded (being similar in phorbol ester/PS and PS alone assays). The possibility of any phorbol-ester stimulated kinase peaks being due to PKM stimulation can be discounted on these grounds. However, the possibility of PKM being generated in the assay (due to the presence of phorbol esters) has not been investigated here.

To address the question of identification of these kinase peaks it was necessary to employ antibody techniques detecting the location of PKC in the elution fractions from hydroxyapatite chromatography. This is the subject of the following chapter.

CHAPTER 5

IDENTIFICATION OF PKC ISOZYMES IN RAT BRAIN

The discovery that multiple peaks of kinase activity in rat brain were stimulatable by phorbol esters in a micellar assay was unsurprising in view of the widely accepted hypothesis that the PKC family of isozymes serve as the cellular receptor for phorbol ester action. The question of identity - whether peaks of activity could be assigned to a particular isozyme of PKC - remained unanswered. Two avenues of approaching the question suggested themselves; the first and most direct approach, involved the use of antibodies, raised to specific PKC isozymes to probe the profile fractions, and is reported in this chapter. The second approach investigated the kinase activity of purified PKC isozymes challenged with the phorbol esters. The latter approach is reported in the following chapter which is concluded by an evaluation of the rat brain data.

ANALYTIC PROCEDURES

MATERIALS

Antisera and Competition Peptides

Antisera to the PKC isozymes $\alpha, \beta_1, \beta_2, \gamma, \delta$ and ϵ were used, generously supplied by Dr. Peter Parker (I.C.R.F. laboratories, England). The anti-sera was isolated from rabbit blood previously immunised with synthetic peptides corresponding to the carboxy-terminal (V_5 region) of each isozyme (as reported; *255,260*). These same peptides (except for δ-PKC) were used in competition

experiments to define specific interactions (i.e. between specific epitopes and the antigenic (Fab) subunits of the antibody molecules) against the non-specific interactions.

Peptides used;

α	PQFVHPILQSAV
β_1	SEFLKPEVKS
β_2	NPEFVINV
γ	PDARSPISPTPVPVM
ϵ	NQEEFKGFSYFGEDLMP
δ	MNRRGSIKQAKI

Second Antibody

The second antibody, ^{125}I-radiolabled (15 μCi mg^{-1}) for autoradiography detection, was donkey anti-rabbit IgG (Amersham). It was used at a 1/1000 dilution.

METHODS

Samples

Part of each fraction from rat brain hydroxyapatite chromatography (taken from the Sodium phosphate gradient figured in 4e to avoid the precipitation experienced with Potassium phosphate in Laemmli buffer) was mixed 1:1 with double-strength Laemmli (buffer 23) and boiled for 5 minutes. Fractions treated in this way were stored at -20°C and re-boiled just prior to use.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sample fractions were run (5-10μl following the order of elution in profile) on 7.5% SDS-polyacrylamide gels with 4% stacking gel

(buffer 24(a) and (b)) according to the method of Laemmli *(453)* with protean II or mini-protean II systems (see electrophoresis method 2 using running buffer 25). Electrophoresis was graduated using coloured/ radioactive markers of known molecular weight (Amersham Low molecular weight 'rainbow' markers) and a PKC standard (containing the isozyme under investigation) was also included as a positive control (a gift from Dr. Peter Parker, ICRF London).

Western Transfer

Protein samples separated on SDS-PAGE gels were then transferred onto nitrocellulose (Hybond C-extra, Amersham) by the western technique *(564)*, using appropriate apparatus (method 2) and buffer 26.

Immunoblotting

Primary Antibody

Nitrocellulose transfers were rocked for 25 minutes in high-protein buffer no. 28 to block background adherence of antibodies. After a quick rinse in washing buffer (no. 27) the nitrocellulose was incubated with anti-PKC antibody (diluted appropiately in buffer no. 29) overnight at 4^{o}C. In competition experiments antibody was first mixed 1:1 with the epitope peptide (30 minutes room temperature) before dilution and incubation. Such experiments were conducted by running a duplicate sample on the same gel, cutting the nitrocellulose western transfer down the centre, and incubating one half with antibody, and the other with the antibody/epitope peptide mix.

Secondary Antibody

Immunoblots were washed (3x15 minutes rocking in buffer no 27) and incubated with radiolabled 2nd antibody (diluted 1 in 1000 in buffer 29) for 1 hour at room temperature. They were then washed (3 x 15 minutes rocking in buffer 27) and dried on filter paper.

Autoradiography

Bands of radioactivity from the transfer were visualised by autoradiography (see Methods).

Reprobing Epsilon Immunoblots

When transfers were challenged with PKC-ϵ antisera it was possible to reprobe the nitrocellulose transfer with 1st and 2nd antibody if the first antibody was anti-PKC α , β or γ , since PKC-ϵ has a higher molecular weight (~92Kd) than these other isozymes (Mr ~77Kd).

Results

Samples of fractions from rat brain in elution sequence were probed with the antisera specific for α , β_1 , β_2 , γ , δ and ϵ isozymes. The antisera for PKC-β_2 was found to be unable to detect standard and gave high erratic background on the blots (data not shown) when tested at Dilutions from 1/1000 to 1/10,000. Titrations of β_1 antisera were conducted and the antisera was used at 1/500. Dilutions for the other antisera were effective in the concentration given in Methods (Chapter 2). To accommodate the elution profile, 2

gels (poured and run "back to back") were used enabling direct comparison of immunoreactivity between the two gels. Where it was not possible to run all 36 fractions, early and late fractions (with low or negligible kinase activity) were omitted.

PKC Epsilon

Immunoreactivity detectable with PKC-ϵ antisera are shown in *Fig 5a*. The bands first appeared at fraction 9 increasing in density to a peak (fractions 13-16) and trailing away over the profile. The apparent Mr was 90Kd and the trace appeared as a doublet possibly due to the comigration of another unreactive band (e.g. see fractions 17-20).

PKC Delta

Immunoreactive bands with PKC-δ antisera are shown in *Fig 5b*. The bands, of an apparent Mr of 77Kd, appeared across fractions 10-22. A lower molecular weight band (~45Kd) was also seen throughout fractions 10-30.

PKC Alpha

With PKC-α antisera reblotting over PKC-ϵ blots immunoreactive bands appeared from fractions 12, peaking across fractions 14-16 before trailing away over the profile *(Fig 5c)*. The single PKC-α immunoreactive band (Mr ~ 77Kdal) was clearly separated from the PKC-ϵ trace. A smaller fragment (Mr ~ 45Kdal) was also detectable in fractions 9-12, which was not present in the PKC-ϵ immunoblot.

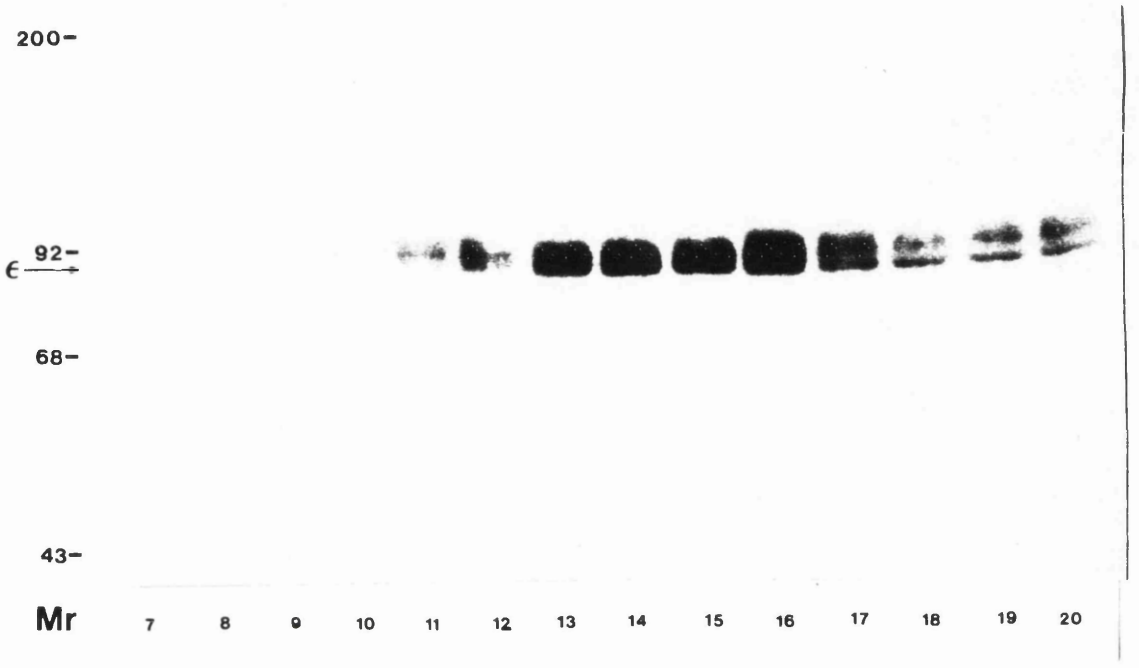


Fig 5a. Autoradiography of rat brain samples probed for PKC ε. Samples from rat brain hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC ε.Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (overnight exposure). ε = PKC ε standard , Mr = Molecular weight markers ,Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 4e.

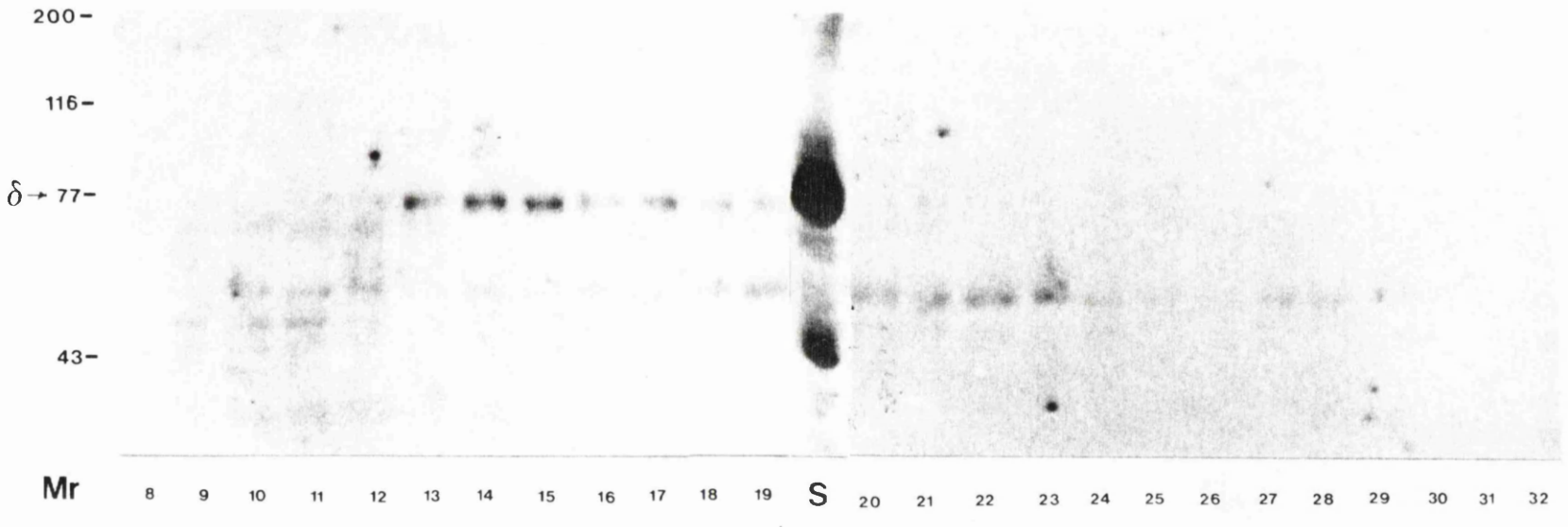


Fig 5b. Autoradiography of rat brain samples probed for PKC δ.Samples from rat brain hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC δ. Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (5 days exposure). Lane S = PKC standard , Mr = Molecular weight markers ,Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 4e.

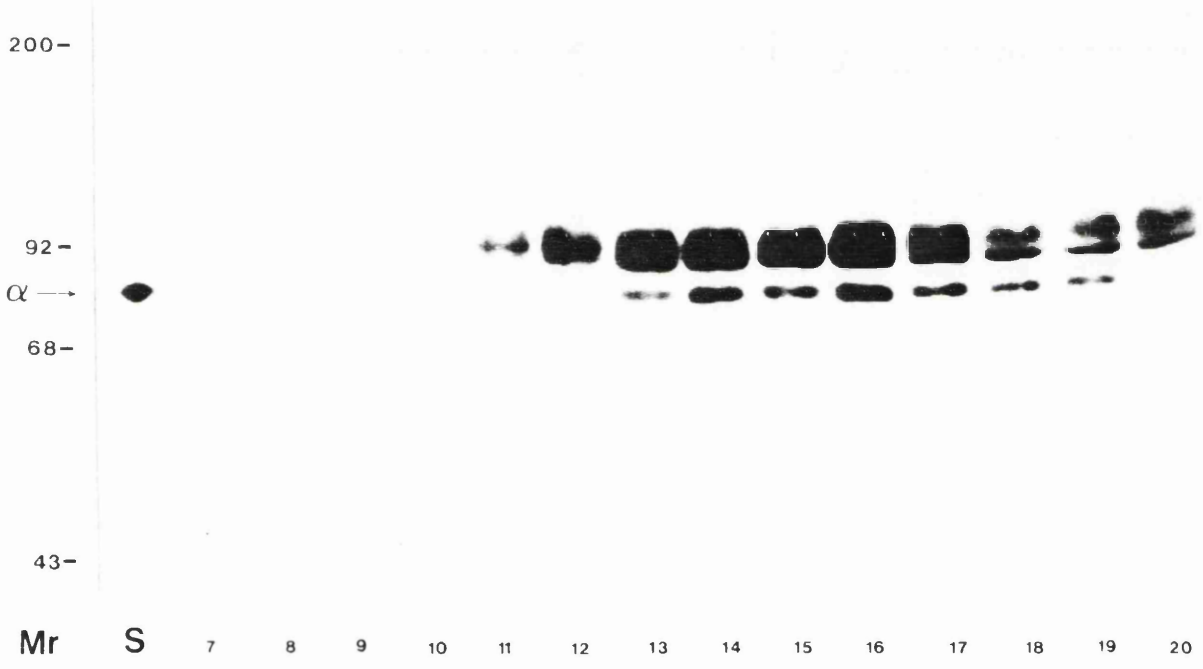


Fig 5c. Autoradiography of rat brain samples probed for PKC ε and reprobed for PKC α. Samples from rat brain hydroxyapatite chromatography, subjected to SDS-PAGE/Western blotting and probed with antisera for PKC ε, (see Fig 5a), were reprobed for PKC α. Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (3 day exposure). Lane S = PKC α standard, Mr = Molecular weight markers, Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 4e.

PKC Beta-1

The β_1-PKC antisera detected an immunoreactive band appearing in fraction 10 and peaking over fractions 11-14 before tailing off *(Fig 5d)*. The apparent molecular weight was in the 77Kdal range clearly dissociated from the PKC-ϵ trace.

PKC Gamma

PKC-γ gave a single band starting from fraction 9 and peaking over fractions 10-12 before trailing off *(Fig 5e)*. The apparent Mr was 77 Kdal.

Competition Experiments

Selected fractions in which immunoreactivity had been detected were tested with antisera incubated with competing epitope peptides to determine whether the immunoreactivity was specific. The disappearance of bands in such competition experiments indicated that the interaction was specific for the epitope *(Fig 5f for α, β_1 and ϵ PKC Fig 8r for γ-PKC)*. The results demonstrated that all the bands (77Kd for α, β_1 and γ, 90Kd for ϵ) could be competed with successfully by the specific epitope peptide.

Conclusions and Discussion

The rat brain elution profile for hydroxyapatite chromatography was probed for the presence of PKC α, β_1, γ, δ and ϵ isozymes. The presence and apparent molecular weights of these isozymes in rat brain has been reported *(249,255,263,265)* and are confirmed by the present results. Immunoreaction with specific antisera for all

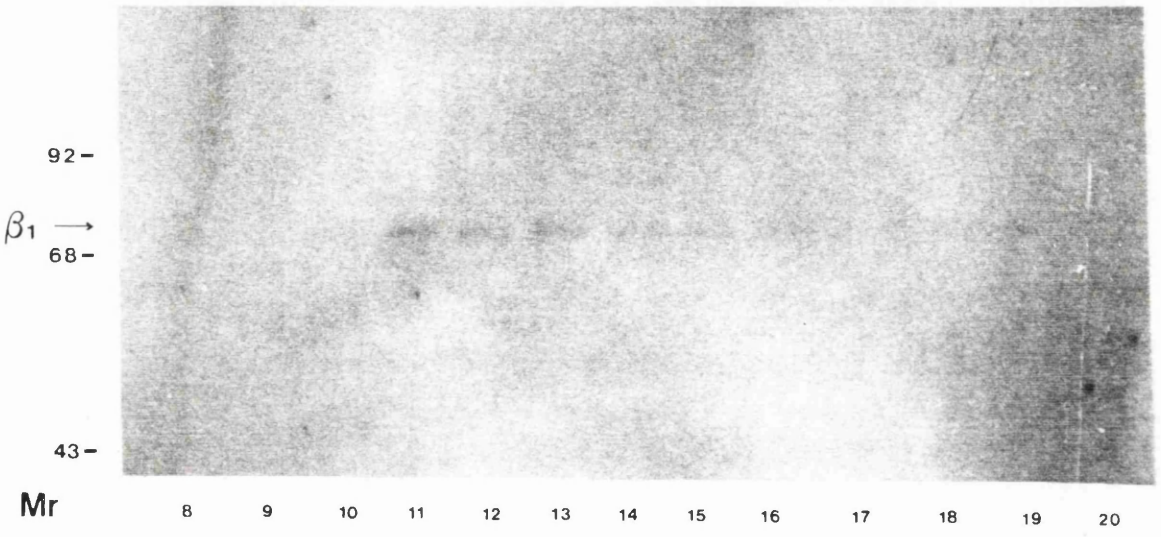


Fig 5d. Autoradiography of rat brain samples probed for PKC β1. Samples from rat brain hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC β1. Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (3 days exposure). Mr = Molecular weight markers ,Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profileshown in Fig 4e.

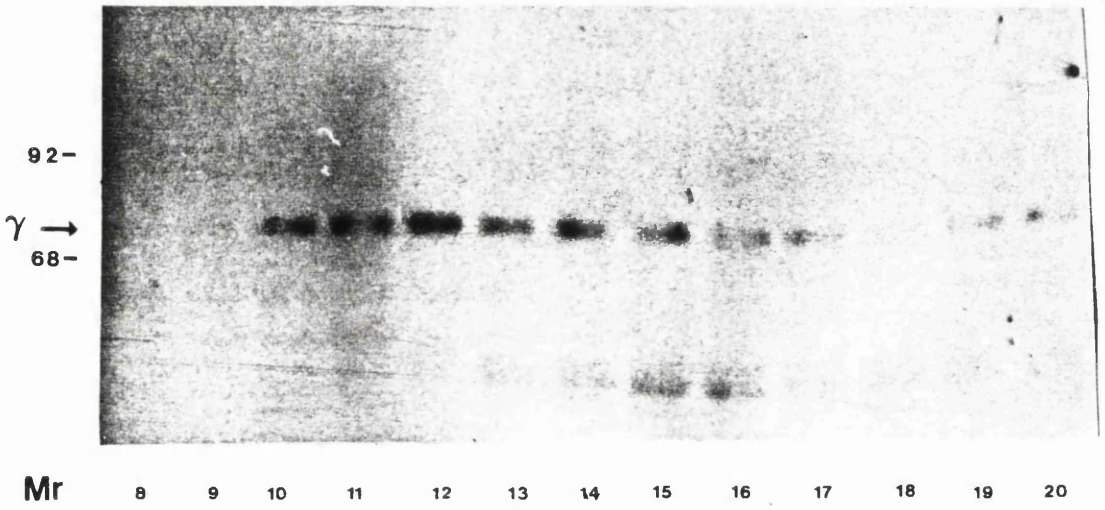


Fig 5e. Autoradiography of rat brain samples probed for PKC γ. Samples from rat brain hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC γ.Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (14 days exposure). Mr = Molecular weight markers ,Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 4e.

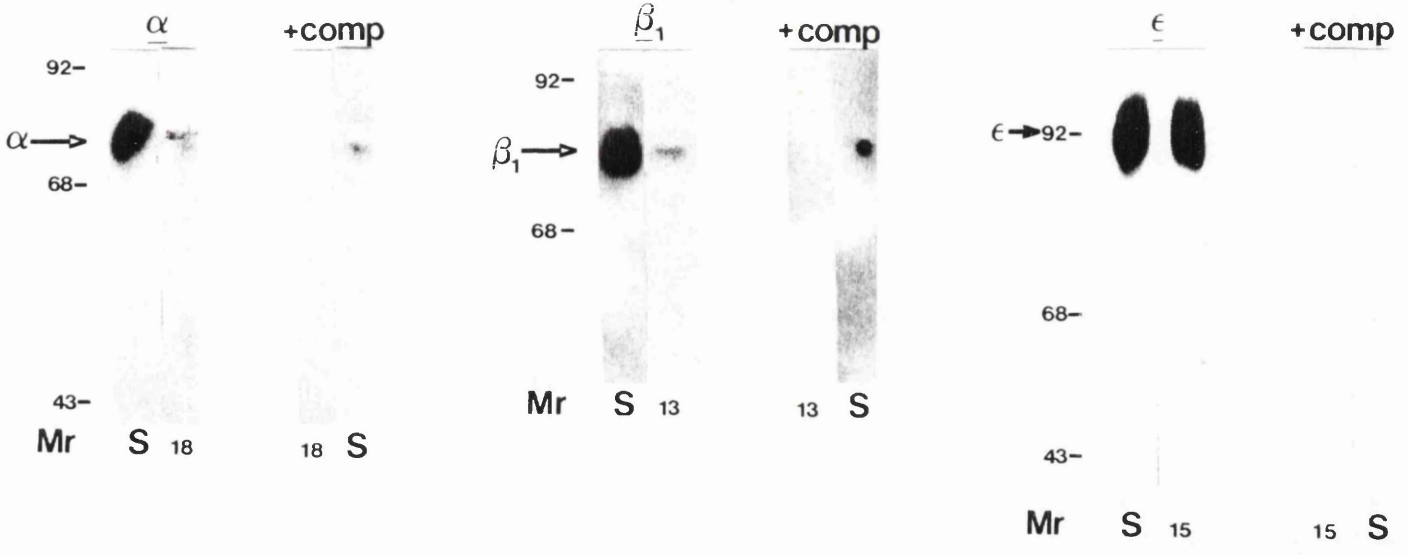


Fig 5f. Competion autoradiography of rat brain α,β1 and ε PKC.Samples of PKC standards (= S) and selected fractions (13,15 or 18) from rat brain hydroxyapatite chromatography (shown in Fig 4e) were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC α,β1 and ε,or with antisera previously incubated with its corresponding competing peptide (+ Comp).Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (3 day,7 day and overnight exposure for α, β1 and ε respectively).Mr = Molecular weight markers.

isozymes tested, could be competed with by prior incubation with their corresponding epitopes, indicating specific interaction was responsible for the immunoreactive bands seen (as opposed to non-specific interaction visualised in bands that remained unaffected). A duplex band was evident in samples immunoreacting with PKC-ϵ antisera. The sharpness of the division indicates a co-migrating protein is screening the centre of a large, single immunoreactive band. A weak immunoreactive band (45Kd) was detected with PKC-α antisera in addition to full length PKC-α . This could be a proteolytic fragment of PKC or a 47Kd protein containing a similar epitope as has been observed in U937 monocytes using PKC-α antisera from the same source (unpublished results Prof. A. Segall, U.C.L.)

The immunoreactive profile in terms of phosphate concentration is within the range of PKC elution from other investigations of rat brain (See Chapter 4). Use of hydroxyapatite biogel (0420HTP) rather than DNA grade hydroxyapatite (0520HTP) has been inefficient at separating the PKC isozymes from each other, resulting in the immunoreactive peaks spread over only ~7 fractions. The order of elution can be approximately estimated by the peaks of immunoreactivity as being PKC-γ (peak fractions 10-12), -β_1 (peak fractions 11-14) and -α(peak fractions 14-16), with PKC-δ and ϵ peaking over a wider range (fractions 13-17) and tailing off slowly (summarised in *Fig 5g* compared with TPA kinase stimulation). This pattern of elution for α, β_1 and γ isozymes is consistent with other investigations using hydroxyapatite choromatography *(247,263)*. PKC-δ and PKC-ϵ elution on hydroxyapatite has not been reported however.

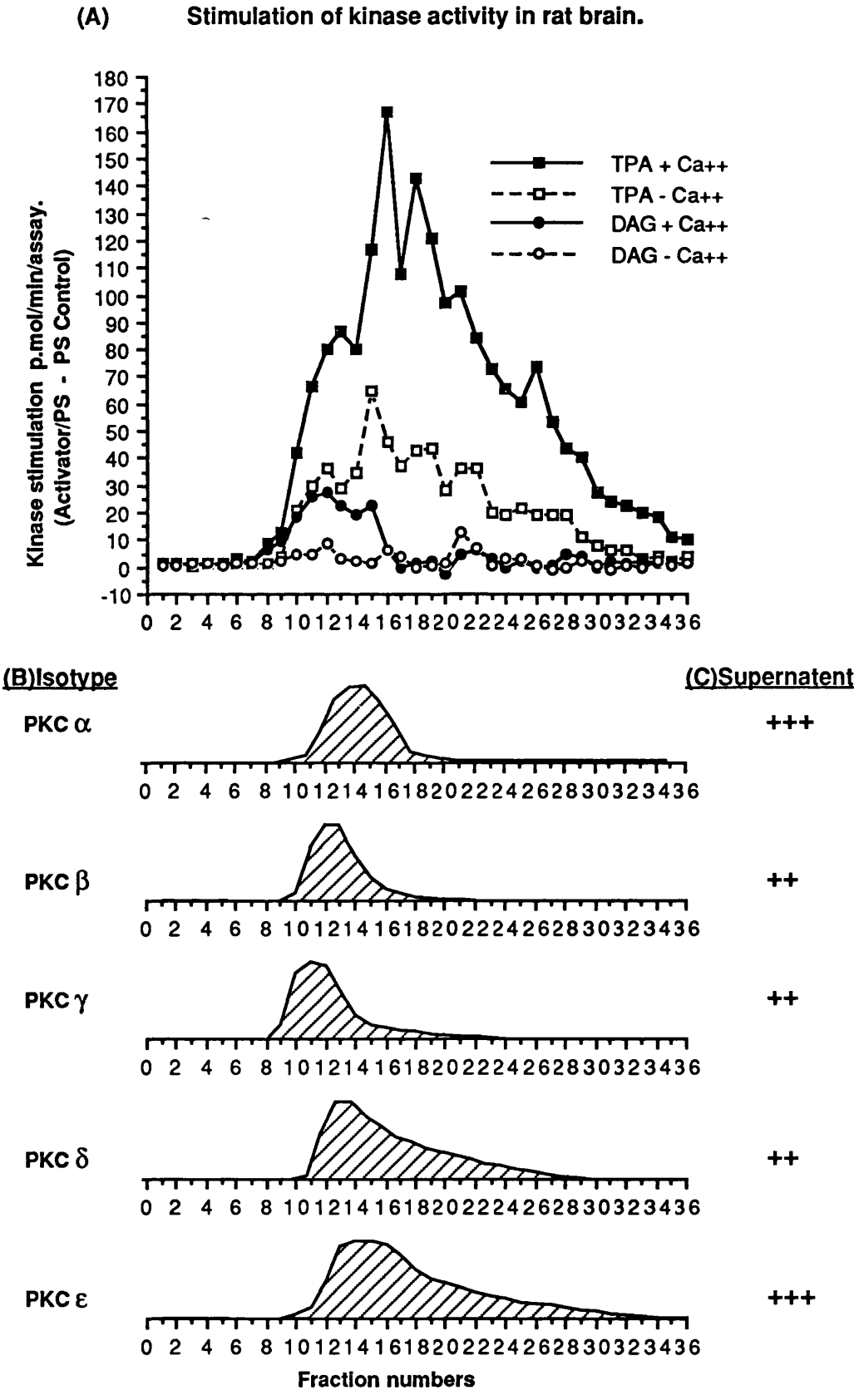


Fig 5g. Comparison of TPA-stimulated kinase activity in rat brain fractions (A),with immunological data (B) using fractions from the same FPLC run. Immunological bands for each isotype were given values from 0 (no trace seen with autoradiograph) to1 (peak fraction).The relative abundance for each isotype apparent in pre-FPLC supernatent is shown in (C).

In terms of isozyme quantity, using concentrations of antisera to give similar specific titres against peptides, if we assume ease of detection to be proportional to the presence of isozyme, the apparent order of abundance of PKC isozymes in rat brain is $\epsilon > \alpha > \beta_1, \gamma, \delta$. Other investigators have found PKC-β_2 isozyme to be dominant in rat brain followed by γ and α and finally β_1 *(247)* using immunoblotting techniques.

When the phorbol ester stimulation of kinase activity in rat brain fractions is compared with the immunological analysis of the PKC isozymes, the central block of stimulated activity can be seen to coincide with the peaks of α , β_1 , γ , δ and ϵ PKC isozymes *(See Fig 5g)*. The TPA and Ca^{2+} dependence of the α , β and γ-PKC isozymes for activation has been variously reported (e.g. *134,260*). Purified PKC-ϵ however has been found to be almost inactive with histone as a substrate, although TPA appeared to stimulate some activity in the presence of calcium *(255)*. This suggests that some contribution to the phorbol ester stimulated kinase activity by PKC-ϵ may occur at a subdued level with calcium, but not without. Other studies conversely suggest PKC-ϵ can phosphorylate histone although the enzyme used was only partially pure and was poorly characterised *(254)*. In this present study the PKC-ϵ immunoreactive fractions showed an increased phorbol ester stimulation when assayed with high levels of histone and TPA (compare *Fig 4e* with *Fig 4h*) possibly reflecting increased PKC-δ or PKC-ϵ involvement (e.g. via PKM generation in the assay).

The other phorbol ester stimulatable peaks (Peaks I, VI, VII and VIII) cannot however be assigned to any of the isozymes tested for here, in so far as their peaks of immunoreactivity occur elsewhere in the elution profile. These peaks do have low level contamination of the PKC isozymes, but these cannot account for their presence. The antisera to the PKC isozymes will also detect PKM (containing the V_5 epitope) but no significant amounts of cleavage products are evident with immunoblotting supporting the notion that these other kinase peaks also stimulated by phorbol esters are not due to PKM. (The subsequent degradation of PKC to active PKM in the assay itself cannot be excluded, since immunoreactive bands would be expected to appear as full-length PKC in immunoblotting).

The location of β_2, ζ and η-PKC isozymes in the elution profile remain unknown in this study. PKC-β_2 has been reported to co-elute with PKC-β_1 on hydroxyapatite and to possess similar kinetic characteristics *(247,251)*, suggesting it occurs in the central block of phorbol ester-stimulatable activity. The situation regarding PKC-ζ is uncertain since a study revealing its presence with β-PKC in bovine neutrophils *(274)* could only find one peak of PS/Ca^{2+} dependent kinase activity suggesting that PKC-ζ also coelutes with the other PKC isozymes during hydroxyapatite chromatography.

In so far as the peaks II-V of phorbol ester stimulatable activity correspond to the well-characterised PKC isozymes (γ, β_1 and α - with β_2 also suspected to lie therein), and only a

background level effect can be tentatively ascribed to the presence of PKC-δ and ϵ, the other peaks (I, VI,VII,VIII) cannot be identified at present. In view of the putative calcium-independent kinase activity of the unplaced PKC isozymes, the calcium dependence of these peaks may represent activity of one or more previously uncharacterised kinases.

To further clarify the pattern of phorbol ester stimulated kinase activity in rat brain, the investigation was directed towards PKC isozymes purified from bovine brain (See following chapter).

CHAPTER 6

Phorbol Ester Stimulation of Purified PKC Isozymes

The diffeent patterns of phorbol ester stimulation of kinase activity in rat brain chromatography fractions, indicted selectivity of phorbol ester action was responsible for the complexity observed. The close proximity of the PKC isozymes, found with subsequent immunological analysis, however precluded a more detailed investigation of individual peaks. Such an approach would be undermined by the heterogeneity of peak fractions (as witnessed by the immunological investigation) and the unknown location of remaining PKC isozymes. Instead individually purified isozymes were investigated for their ability to be stimulated by the range of phorbol esters under a variety of conditions. By comparing the effect of the phorbol esters on pure isozymes and collating this information with both profile stimulation and immunological work, it was hoped to gain an overall view of kinase stimulation in rat brain which could then be applied to other tissue/systems.

MATERIALS AND METHODS

PURIFIED ISOZYMES

The PKC-α, β_1 and γ isozymes were separated from each other using bovine brain as a source according to *(260)*. PKC-δ and ϵ were separated from COS-1 cells transfected with bovine plasmid DNA vectors for PKC-δor ϵ according to *(255)*. All purified enzymes were gifts from Dr. Peter Parker (I.C.R.F. London, England), were immunologically pure and were stored at -20^{o}C (in buffer 47) in

concentrated form until used.

PURIFIED ISOZYME SUBSTRATES

PKC-α, β_1 and γ were assayed for their ability to transfer P^{32} to Histone III-s as before. Synthetic peptide substrates were employed for PKC-δ and ϵ supplied by Dr. Peter Parker (ICRF, London, England);

δ synthetic substrate: MNRRGSIKQAKI

ϵ synthetic substrate: ERMRPRKRQGSVRRRV

(The target residues for phosphorylation are underlined)

ASSAY FOR PURIFIED PKC ISOZYMES

A modified assay to that used in crude rat brain fractions was used throughout (See Kinase assay method 2) . While final concentrations of components was essentially similar (See *Table 2A*), the assay used smaller volumes of purified enzyme (5μl), had a final volume of 40μl and used radiolabeled ATP at a higher specific activity (~ 500,000 cpm/assay). The assay was calibrated to be linear when kinase activity of up to 4 units (n mol/min/ml) was used, and PKC isozymes of known activity (Units/ml) were diluted accordingly. Phosphate incorporation was measured over a 5 minute period which was approximately within the linear region of the time-course (data not shown). For termination of the assay 25μl aliquots were spotted onto ion exchange paper (P 81) and washed with 30% acetic acid (3 x 10 minute washes). Wet filters were then counted for radioactivity (See methods).

Handling

All Phorbol esters were tested at 10, 100, 1000 and 10,000 ng/ml final concentrations range (0.0003 to 0.3 Mol % micelle), in the absence or presence of PS (31 Mol % micelle). Duplicates of each test point were assayed in the presence or absence of added calcium (100 μM free) for each isozyme. Controls (without phorbol esters) were conducted in parallel. Assays were conducted with eppendorf tubes in groups of 20. A single test point was initiated every 15 seconds by addition of ATP mix (buffer 46) and terminated in sequence. Enzyme, once diluted, was kept on ice and added just prior to initiation, being utilised invariably within 1 hour.

Results

The stimulation of purified PKC isozyme kinase activity for all phorbol esters was found to be wholly dependent on the presence of PS, no stimulation being observed at any concentration (10-10,000 ng/ml) without this phospholipid (data not shown). With PS, TPA stimulation of PKC-α, β_1, γ, δ and ϵ in the presence and absence of calcium are shown in *Figs 6a-e* respectively. At each concentration of TPA, the stimulation (expressed in p. moles/min/assay) above the PS alone control, was compared to the maximally obtained TPA/PS activity less the PS alone control, and expressed as a %age for each isozyme.

i.e. For isozyme δ

$$\frac{(\text{TPA/PS} - \text{PS})\ \text{Isozyme}\,\delta\ (10\text{-}10{,}000)}{(\text{TPA/PS} - \text{PS})\ \text{Isozyme}\,\delta\ \text{Maximum}} \times 100\ \%$$

For the maximal value of TPA/PS stimulation, the largest value in the

TPA activation of PKC Alpha.

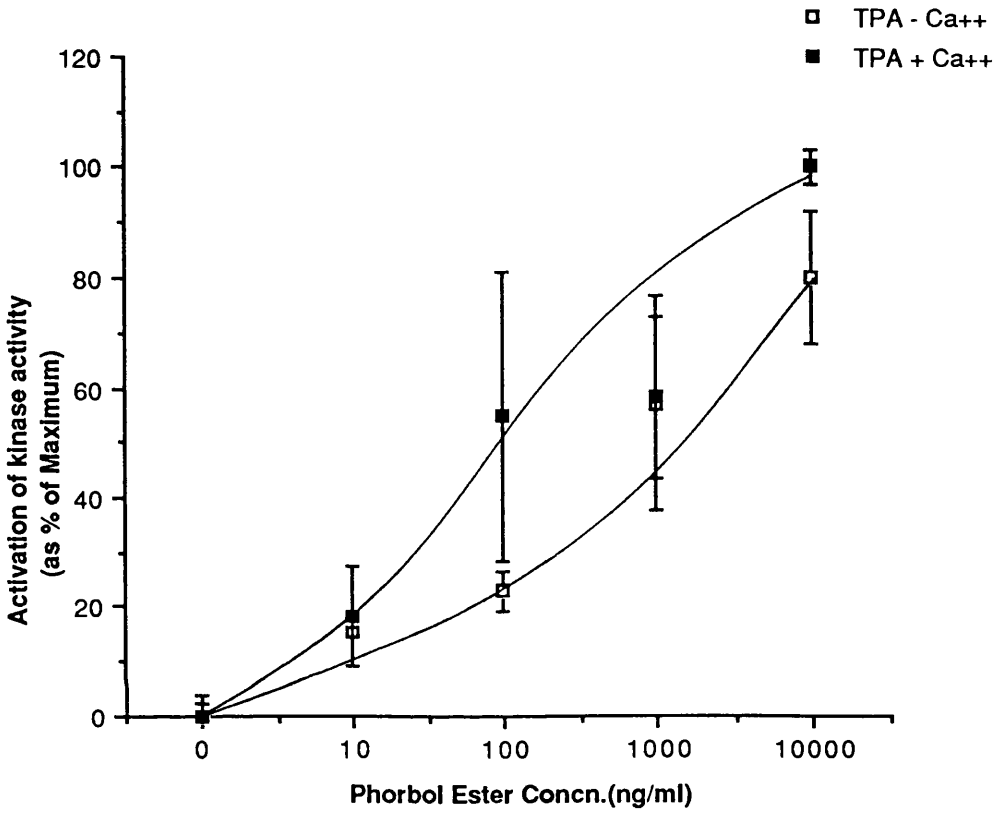


Fig 6a. TPA,at the final concentrations indicated,was tested for the ability to activate purified PKC-α in the presence or absence of 100μM free calcium.At each point the phosphate incorporation into histone was determined and compared with the maximum incorporation with TPA as activator.Non-specific incorporation was subtracted from all values and the results are expressed as a percentage of the maximum TPA activity with the range from duplicate assays.

TPA activation of PKC Beta$_1$.

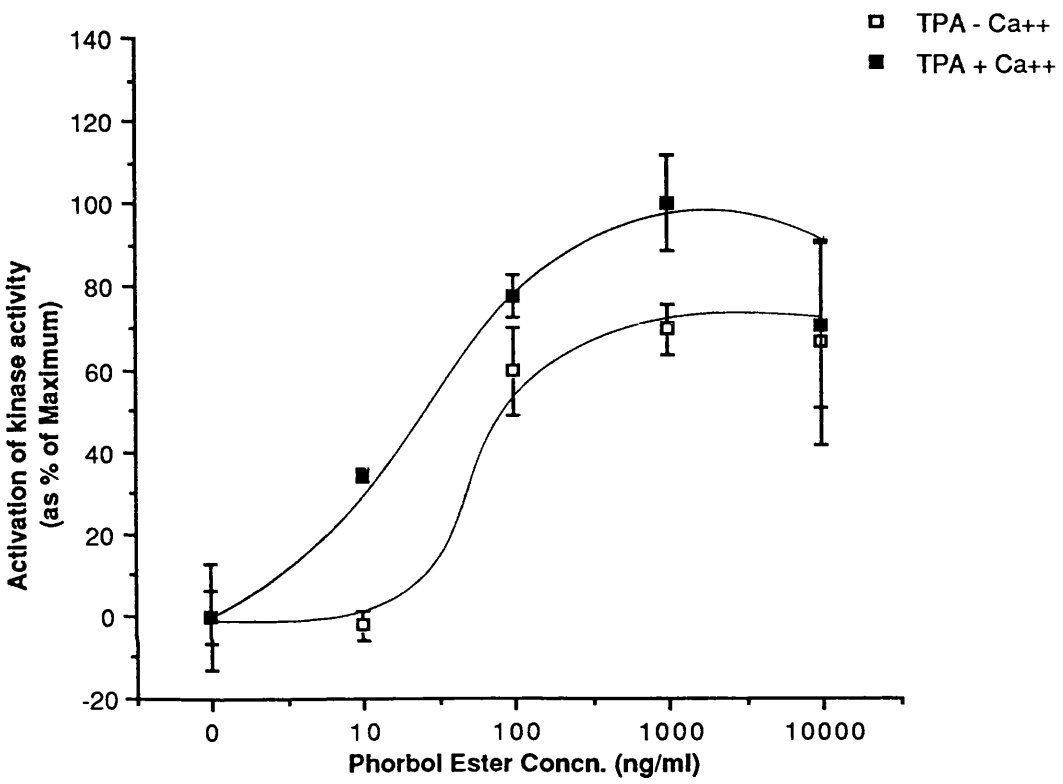


Fig 6b. TPA,at the final concentrations indicated,was tested for the ability to activate purified PKC-β_1 in the presence or absence of 100μM free calcium.At each point the phosphate incorporation into histone was determined and compared with the maximum incorporation with TPA as activator.Non-specific incorporation was subtracted from all values and the results are expressed as a percentage of the maximum TPA activity with the range from duplicate assays.

TPA activation of PKC Gamma.

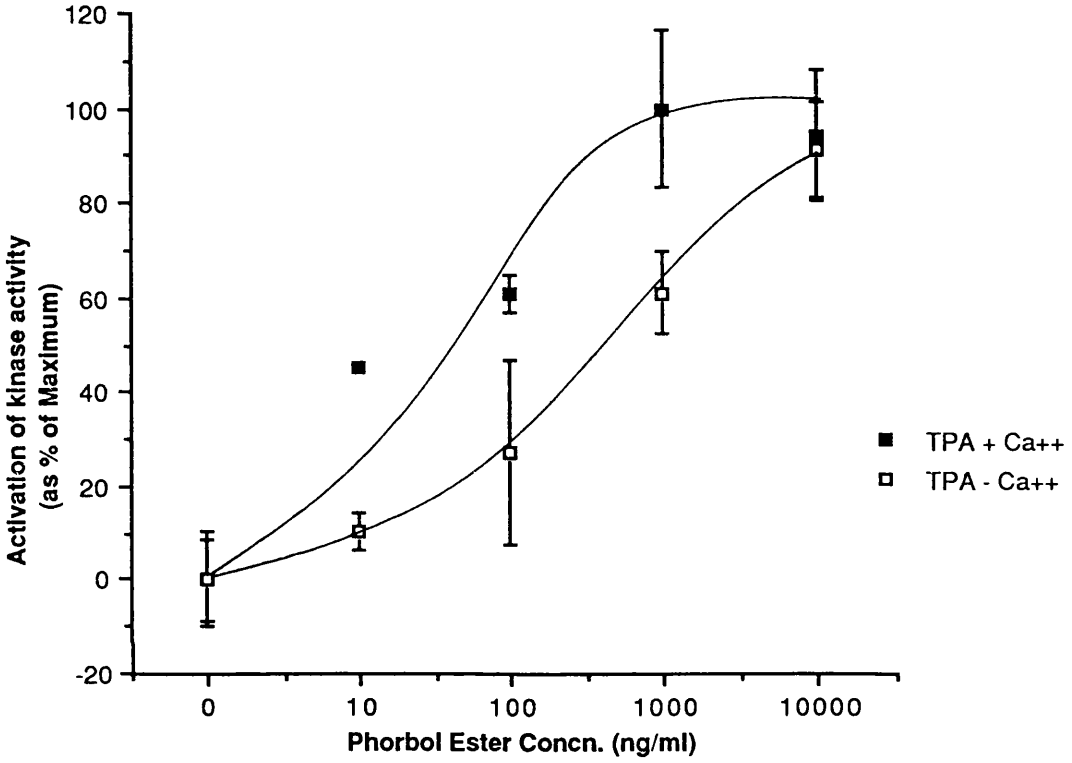


Fig 6c. TPA,at the final concentrations indicated,was tested for the ability to activate purified PKC-γ in the presence or absence of 100μM free calcium.At each point the phosphate incorporation into histone was determined and compared with the maximum incorporation with TPA as activator.Non-specific incorporation was subtracted from all values and the results are expressed as a percentage of the maximum TPA activity with the range from duplicate assays.

TPA activation of PKC Delta.

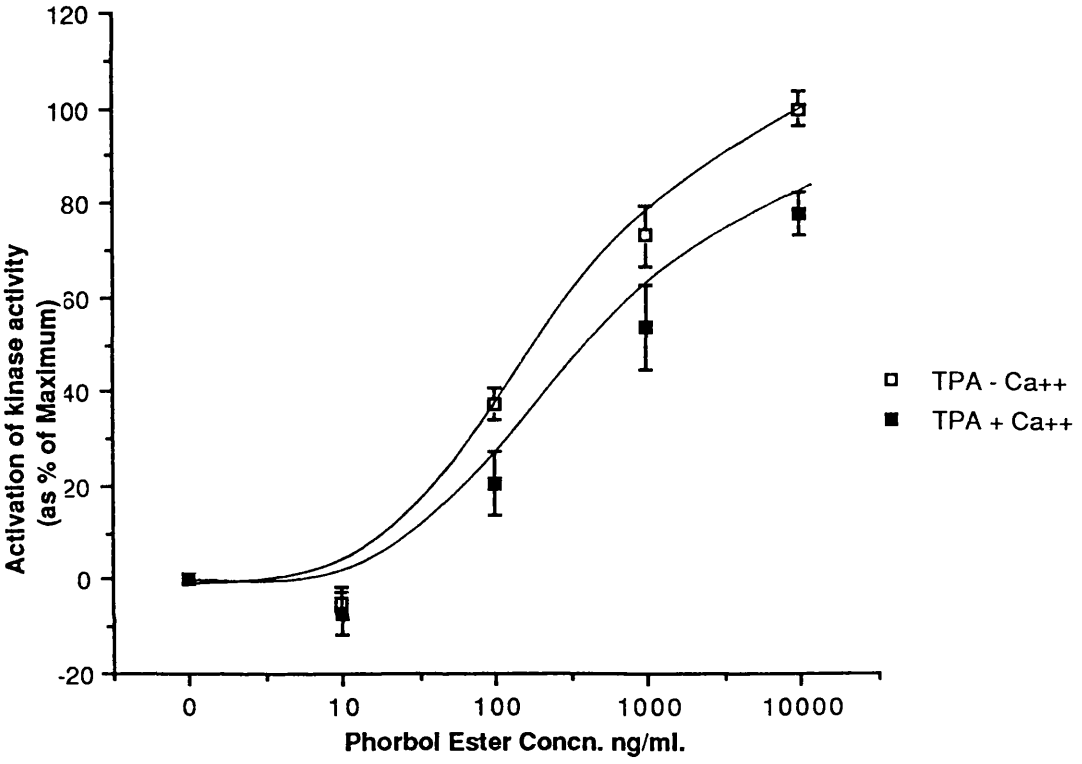


Fig 6d. TPA,at the final concentrations indicated,was tested for the ability to activate purified PKC-δ in the presence or absence of 100μM free calcium.At each point the phosphate incorporation into an artificial substrate was determined and compared with the maximum incorporation with TPA as activator.Non-specific incorporation was subtracted from all values and the results are expressed as a percentage of the maximum TPA activity with the range from duplicate assays.

TPA activation of PKC Epsilon.

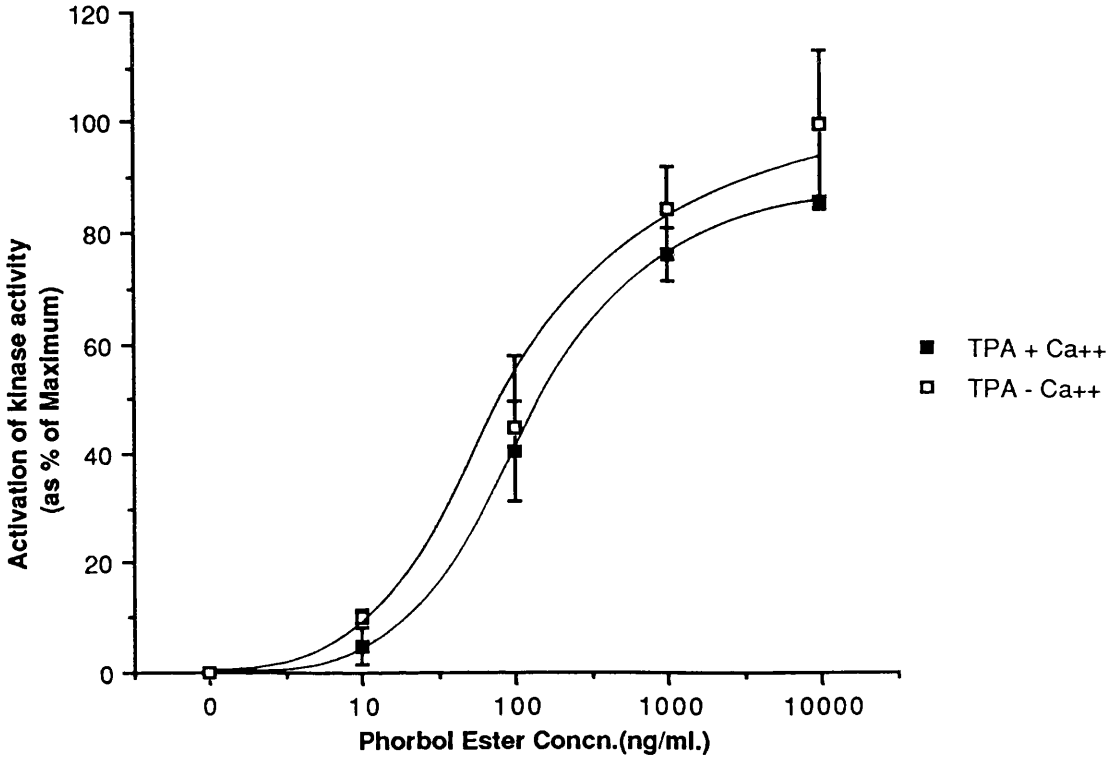


Fig 6e. TPA,at the final concentrations indicated,was tested for the ability to activate purified PKC-ε in the presence or absence of 100μM free calcium.At each point the phosphate incorporation into an artificial substrate was determined and compared with the maximum incorporation with TPA as activator.Non-specific incorporation was subtracted from all values and the results are expressed as a percentage of the maximum TPA activity with the range from duplicate assays.

presence or absence of calcium was taken, and used throughout. Stimulation by all other phorbal esters was similarly compared to this maximal (TPA/PS - PS) value, at each concentration for each isozyme. An example of how Sapintoxin A was able to stimulate the PKC isozymes-α, β_1, γ, δ and ϵ are shown in *Figs 6f-j* respectively. From these stimulation graphs for SAP A, TPA and the other phorbol esters (THY TOX, DOPP, RX, Doppa - data not shown in full), a table was constructed showing the maximal stimulation compared to TPA (=100%) and the concentration of the phorbol ester required to give 50% of the maximal TPA stimulation, for each isozyme *(Table 6A)*.

DISCUSSION

The effect of phorbol esters on the kinase activity of PKC isozymes, purified from bovine brain, was investigated. All isozymes tested were found to be dependent on Phosphatidylserine (PS) to enable phorbol ester stimulation and were effectively inactive in its absence. for each PKC isozyme used, kinase activity stimulated with each phorbol ester was compared to the maximal TPA-elicited kinase activity (after PS control values had been subtracted from them). The results demonstrated that full tumour promoters (e.g. TPA), weak tumour promoters (e.g. DOPP and THY TOX) and non-promoting phorbol esters (e.g. SAP A) share the ability to potently activate PKC-α, β_1 and γ isozymes in a calcium-dependent manner. In the micellar assay used here, the co-factor action of calcium was found to exert the greatest effect on the PKC γ-isozyme when TPA was the activator, as

Sapintoxin A activation of PKC Alpha.

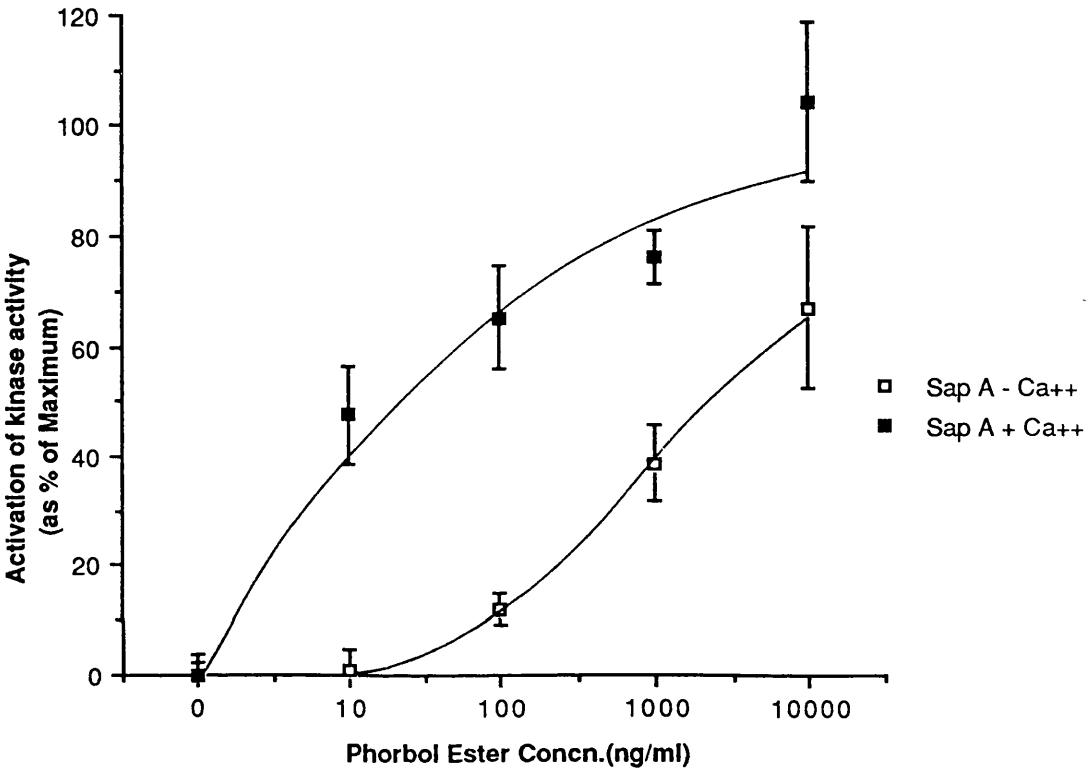


Fig 6f. Sap A,at the final concentrations indicated,was tested for the ability to activate purified PKC-α in the presence or absence of 100μM free calcium.At each point the phosphate incorporation into histone was determined and compared with the maximum incorporation with TPA as activator.Non-specific incorporation was subtracted from all values and the results are expressed as a percentage of the maximum TPA activity with the range from duplicate assays.

Sapintoxin A activation of PKC Beta$_1$.

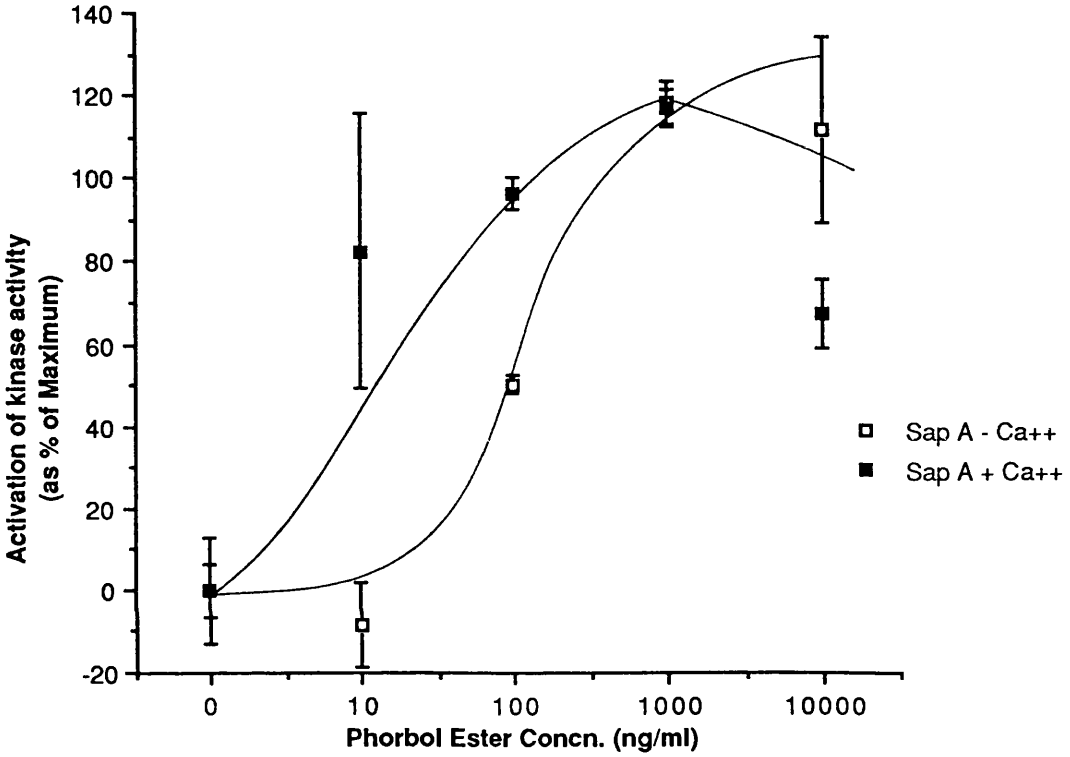


Fig 6g. Sap A,at the final concentrations indicated,was tested for the ability to activate purified PKC-$\beta_1$in the presence or absence of 100μM free calcium.At each point the phosphate incorporation into histone was determined and compared with the maximum incorporation with TPA as activator.Non-specific incorporation was subtracted from all values and the results are expressed as a percentage of the maximum TPA activity with the range from duplicate assays.

Sapintoxin A activation of PKC Gamma.

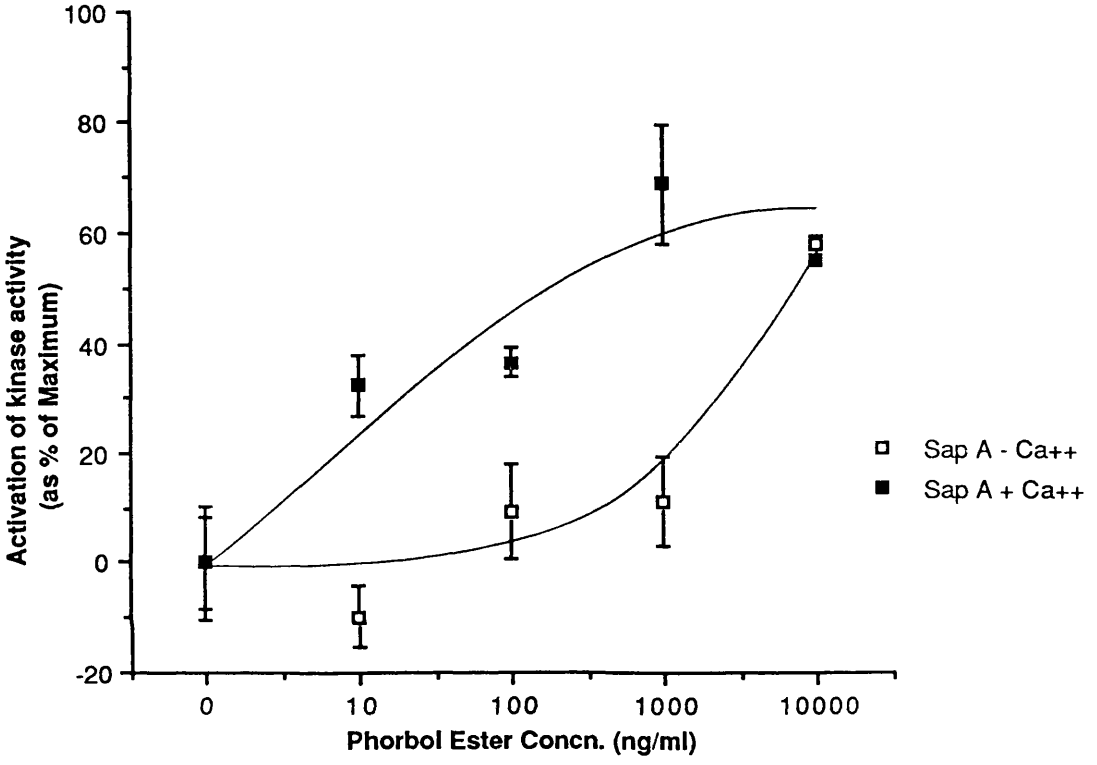


Fig 6h. Sap A,at the final concentrations indicated,was tested for the ability to activate purified PKC-γ in the presence or absence of 100μM free calcium.At each point the phosphate incorporation into histone was determined and compared with the maximum incorporation with TPA as activator.Non-specific incorporation was subtracted from all values and the results are expressed as a percentage of the maximum TPA activity with the range from duplicate assays.

Sapintoxin A activation of PKC Delta.

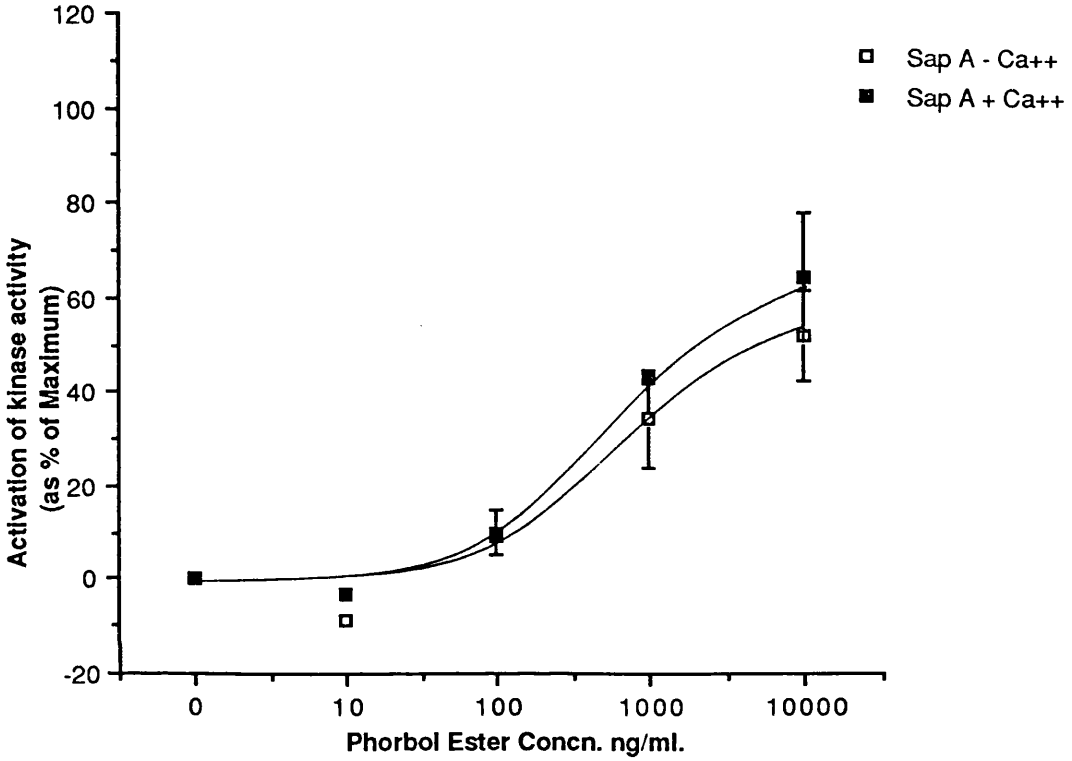


Fig 6i. Sap A,at the final concentrations indicated,was tested for the ability to activate purified PKC-δ in the presence or absence of 100μM free calcium.At each point the phosphate incorporation into an artificial substrate was determined and compared with the maximum incorporation with TPA as activator.Non-specific incorporation was subtracted from all values and the results are expressed as a percentage of the maximum TPA activity with the range from duplicate assays.

Sapintoxin A activation of PKC Epsilon.

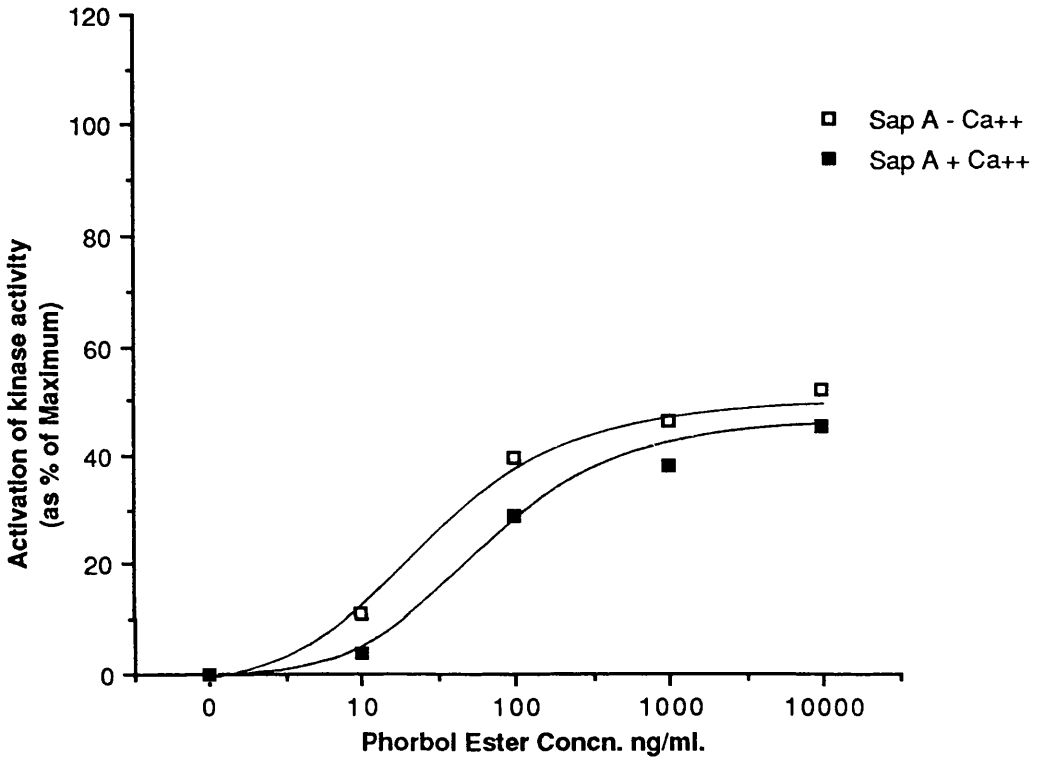


Fig 6j. Sap A,at the final concentrations indicated,was tested for the ability to activate purified PKC-ε in the presence or absence of 100μM free calcium.At each point the phosphate incorporation into an artificial substrate was determined and compared with the maximum incorporation with TPA as activator.Non-specific incorporation was subtracted from all values and the results are expressed as a percentage of the maximum TPA activity with the range from duplicate assays.

Table 6A. Activation of purified PKC-isotypes by phorbol esters *in vitro.*

PKC isotype	Phorbol Ester	Activation (% of TPA max.) +Ca	-Ca	Max 50* (ng/ml) +Ca	-Ca
α	TPA	100	80*	80	1000
	Thy Tox A	105	50*	100	10000
	DOPP	80	85*	5	630
	Sap A	85	70	125	1200
	Doppa	-	-	-	-
	Rx	20	-	-	-
β	TPA	100	70	25	100
	Thy Tox A	95	88*	60	1500
	DOPP	170	125*	3	140
	Sap A	115	115	6	105
	Doppa	45	-	-	-
	Rx	90	-	500	-
γ	TPA	100	90	16	560
	Thy Tox A	93	66*	160	5000
	DOPP	75	70	50	1600
	Sap A	60	60*	160	5000
	Doppa	50	-	10000	-
	Rx	46	20*	-	-
δ	TPA	80	100	600	200
	Thy Tox A	60*	60*	8400	7500
	DOPP	65*	70*	1900	1500
	Sap A	55*	65*	2500	5000
	Doppa	-	-	-	-
	Rx	15*	-	-	-
ε	TPA	90	100	150	120
	Thy Tox A	35*	50*	-	10000
	DOPP	80	90	280	200
	Sap A	45	50	-	7200
	Doppa	-	-	-	-
	Rx	-	-	-	-

* Activity still increasing at 10,000 ng/ml concentration.

** Concentration required to stimulate 50 % of the maximal activity observed with TPA under optimal conditions.

indicated by the potency shift between the presence and absence of 100 μM free calcium. In agreement with other studies this calcium effect followed the order: PKC-γ > PKC-α > PKC-β_1 *(260)*. Phorbol esters of limited biological activity (e.g. Doppa and RX) were much less potent and effective at the stimulation of these isozymes, when compared with the other phorbol esters used.

The stimulation of PKC-δ and ϵ isozymes by a range of phorbol esters is reported here for the first time. Both isozymes were stimulatable in an essentially calcium-independent manner in the presence (but not the absence) of PS, by TPA. PKC-δ was stimulated by DOPP and SAP A with 5 and 10 times less potency respectively than TPA, while THY TOX was 20-30 times less potent. PKC-ϵ was stimulated by DOPP with a similar (~1/2) potency to TPA whereas SAP A and THY TOX were much less potent (60 and 80 times less potent respectively *See Table 6A*). No stimulation of either isozymes was seen with RX or Doppa, although an upward drift in PKC-δ activity seemed to be occuring in the presence of Calcium. This was thought to be a consequence of the enzyme and PS micelle awaiting initiation of the assay. When PS alone controls were monitored for this effect, the drift from first to last initiation sequence (~15% of the TPA maximal stimulation) was averaged to obtain the baseline (reflected in a slightly higher value of the '0' compared to the '10' ng/ml phorbol ester points - *See Fig 6d and 6i*). In experiments replacing the pseudosubstrate peptide with Histone III-S as phosphate acceptor no drift was observed with PS controls and all phorbol esters (tested at 100 or 1000 ng ml^{-1}) failed to stimulate any kinase

activity (data not shown) suggesting the effect is not due to the generation of a constituitively active kinase fragment (PKM). In further support of this conjecture, leupeptin (widely used to inhibit PKM generation e.g. *159*) did not affect the activity of either isozymes (PS micelles $\pm$ Ca^{2+} and $\pm$ 1000 ng ml^{-1}TPA) when present in the assay at 1mM (data not shown).

The selective action of Doppa on purified PKC isozymes merits further notice. Earlier reports have found certain biological activities <u>in vivo</u> and the activtion of pooled PKC <u>in vitro</u> by Doppa to be incomplete ($\sim$ 40% of maximal effect seen by TPA and other phorbol esters) *(191,513)*. The selective activation of the β_1-PKC isozyme by Doppa may thus represent the biochemical receptor responsible for these observations. These findings argue in favour of a difference in phorbol ester binding site characteristics between different isozymes and suggests Doppa may be a useful predictive probe in β_1-PKC functional studies. In support of this, a recent study of platelet PKC found a peak with similar Doppa-activated characteristics *(277)*.

These studies on purified PKC isozymes (widely accepted as the cellular phorbol-ester-receptor) can be used in the analysis of kinase stimulation in rat brain hydroxyapatite fractions by phorbol esters (Chapter 4). The expected stimulation of kinase activity in crude fractions by the various phorbol esters can be estimated by comparing their relative action on the pure isozymes at the same concentration (100 ng/ml *See Table 6B*). This approach engages a

Table 6B. Activation of PKC isotypes *in vitro* by a range of phorbol esters present at 100 ng/ml.

In the presence of Calcium (100 mM free concentration)

PKC isotype	TPA	Thy Tox A	Sap A	DOPP	Doppa	Rx
α	70	60	65	80	0	0
β	80	80	90	130	40	0
γ	70	45	40	50	0	10
δ	25	0	10	10	0	0
ε	40	0	30	30	0	0

In the absence of added Calcium.

PKC isotype	TPA	Thy Tox A	Sap A	DOPP	Doppa	Rx
α	25	5	10	25	0	0
β	60	0	50	50	0	0
γ	30	0	10	0	0	0
δ	35	0	10	10	0	0
ε	45	0	40	35	0	0

Note: All values are given as a % of the maximal activation elicited by TPA,under optimal concentration and conditions, for each isotype.

number of assumptions in yielding information from the data; (I) Phorbol ester-stimulated phosphate transfer onto histone substrates is a direct consequence of calcium and phospholipid-dependent protein kinase action, (II) Contaminant proteins in the elution profile do not interfere with the phorbol-ester stimulation of such kinase activities (causing them to behave in a different manner than the purified enzymes), (III) PKC isozymes do not affect each others' activities when a heterogenous mixture of isozymes is assayed. (IV) The rat brain enzymes share the characteristics of bovine brain enzymes when challenged with the phorbol esters. The effect of the PKC-δ and ϵ isozymes on the elution profile is uncertain. In purified form these isozymes demonstrated negligible kinase activity, in the presence or absence of phorbol esters, with histone III-s as a substrate. However, under conditions favouring the generation of their respective PKM's,these isozymes may influence the stimulation profiles in rat brain. The possiblity that endogenous phosphate-accepting substrates co-elute with these isozymes in rat brain fractions, must also be considered.

With these assumptions and limitations in mind, the immunological data can be brought in to provide a framework for the identification of major isozymes present in the peaks of kinase stimulation (see Chapter 5).

That peak II-stimulation is mainly due to the presence of γ-PKC (elevated by the β isozyme(s)) is supported by its stimulation by TPA, THY TOX, DOPP and SAP A in the presence of calcium. In its absence

TPA and SAP A stimulation also agree with this data, although the large peak observed with THY TOX and DOPP is not supported. To some extent the stimulation of Peak I by THY TOX, in the absence of calcium, may be expected to enhance that seen in Peak II as overlapping elution occurs.

The calcium dependent stimulation by TPA, DOPP, SAP A and partial stimulation by THY TOX and Doppa suggest peaks III and IV are mainly due to the stimulation of the β-PKC isozymes. These peak fractions also contain traces of γ-and the emerging α, δ and ϵ-isozymes. In the absence of calcium, TPA and SAP A substantiate this claim but again the THY TOX (too high) and DOPP (too low) stimulation profiles appear contrary. The β peaks cannot be clearly defined from each other without the investigation of the β_2 presence (reported to be as high as 55% of rat brain PKC *(247)*), and the effect of phorbol esters on its kinase activity in purified enzymes, being assessed.

Peak V appears to be mainly due to the presence of α-PKC with PKC-$\delta$and ϵ as major-, and PKC-$\beta_1$and γ as minor-contaminants. This is supported by the effects of TPA, THY TOX, SAP A and DOPP in the presence of calcium and SAP A and DOPP in its absence. THY TOX and TPA stimulate in this area but are receeding rather than peaking in the absence of calcium.

RX, which causes no PKC-δ or PKC-ϵ phosphorylation of pseudosubstrate peptides or any isozyme phosphorylation of histone III-s at 100 ng/ml, fails to cause any kinase stimulation in the

elution profile as expected.

The results from these three approaches to the effects of phorbol esters on rat brain kinase activity are in broad agreement with each other. Stimulation of elution fractions with THY TOX and DOPP appear to produce some anomalies when calcium is not present in the assay however, which cannot be interpreted by reference to the purified isozyme data. The remaining peaks of kinase activity stimulatable by phorbol esters (peaks I, VI, VII and VIII) failed to correspond to any of the purified PKC isozymes, were not stimulated by diacylglycerol ($DiC_{18:1}$ present at 1.2 mole % of micelles) and were not within the peak fractions of known PKC immunological activity. The identify of these novel kinase activities remains to be elucidated.

More information is required about the relationship between the PKC isozymes and the corresponding active fragments (PKM) which they are degraded to. The coelution of proteases was implicit in the discovery of PKC in the first instance, and subsequent work has suggested that the same co-factors which stimulate PKC activity (calcium, phospholipid and activators such as diacylglycerol and phorbol esters) combine to exaccerbate such proteolytic action *(204)*. Hence in crudely separated elution profiles (differential) phorbol ester mediated PKM generation may give rise to the appearance of difference peaks, relative to PS controls, where proteases elute. Diacylglycerol would also be expected to produce this effect yet only the central block of activity responded to $DiC_{18:1}$ in this study

(i.e. not peaks I, VI, VII and VIII). The immunological analysis presented in Chapter 5 cannot be of assistance in this question since elution fractions for analysis were immediately boiled with denaturing buffer so preventing PKM generation. It would be necessary to compare fractions before and after kinase assays (or to include protease inhibitors) immunologically, to determine if PKM fragments are being generated in the assay itself. Conversely phorbol esters could be tested for their ability to enhance proteolytic degradation (e.g. by Calpain) of purified isozymes within the micellar assay. It would be highly desirable to effect complete isolation of the novel kinases from the PKC isozymes in order to investigate their biochemical/biophysical characteristics. With the FPLC apparatus this might be achieved by stepwise elution of the central block followed by a slower graded elution (for peaks VII and VIII) and/or variation of the column packing material.

CHAPTER 7

PROTEIN KINASE STIMULATION IN HUMAN MONONUCLEAR CELL EXTRACTS

The penchant phorbol esters possess for illuminating the presence of stimulatable phospholipid-dependent kinases in crudely fractionated rat brain make these invaluable biochemical tools. It was decided to investigate kinase activity in fractionated human mononuclear cells using the same procedures resolved for rat brain. For those studies large quantities of cells were used.

Methods

Blood Separation

2 methods were used to obtain human mononuclear cells (HMNC's). (I) Freshly drawn venous blood was collected in a similar manner to Chapter 3 but using large quantities (250-2000 ml) from up to 4 donors. Cell cultures, prepared as before, were used for fractionation immediately or after 1-2 days in a CO_2 incubator. Cells from different donors were not mixed except after fractionation. (II) HMNC-enriched blood was also obtained using a cell separator unit (with permission from the University College Hospital, London). HMNC's were extracted from this preparation as before.

Cell Fractionation

HMNC's (fresh or cultured) were pelleted (10 mins at 400 x g) and resuspended in ice-cold homogenization buffer (buffer 3). The cells

were then fractionated by sonication and/or a teflon/glass homogenizer (method 1 or 2). Hydroxyapatite chromatography (0420 HTP Biorad) with an FPLC system was then conductd on the homogenate following the same procedures used with rat brain extracts. A typical protein trace measuring absorbance at 280 nm, is shown in *Fig 7a*.

Kinase Assays

The hydroxyapatite fractions were assayed for protein kinase activity following the crude assay method 1 used on rat brain *(see Chapter 4)*. Pelleted material and pooled breakthrough proteins from hydroxyapatite columns (prior to running a phosphate gradient) did not exhibit TPA stimulatable kinase activity (data not shown).

Immunological Analysis

Part of each fraction eluted from the columns were taken for immunological analysis. These samples were probed for the presence of Protein kinase C using one of two methods.

Method 1 Immunoblotting with the anti-PKC MC-5 antibody

The MC-5 antibody (Amersham data sheet 11800) is a monoclonal antibody from mouse, detecting an epitope (residues 312-23) located in the "hinge" region (where proteolysis occurs) in full length PKC -α and β_1 / β_2 isozymes but not PKC-γ. Samples [500μl] from potassium phosphate gradients were first placed in Amiga centriflo-cones and ultracentrifuged [1000 x g] until no liquid remained. The protein residue was then taken up in laemmli denaturing buffer (100μl buffer 10). Proteins in the sample (25μl) were then separated by SDS-PAGE,

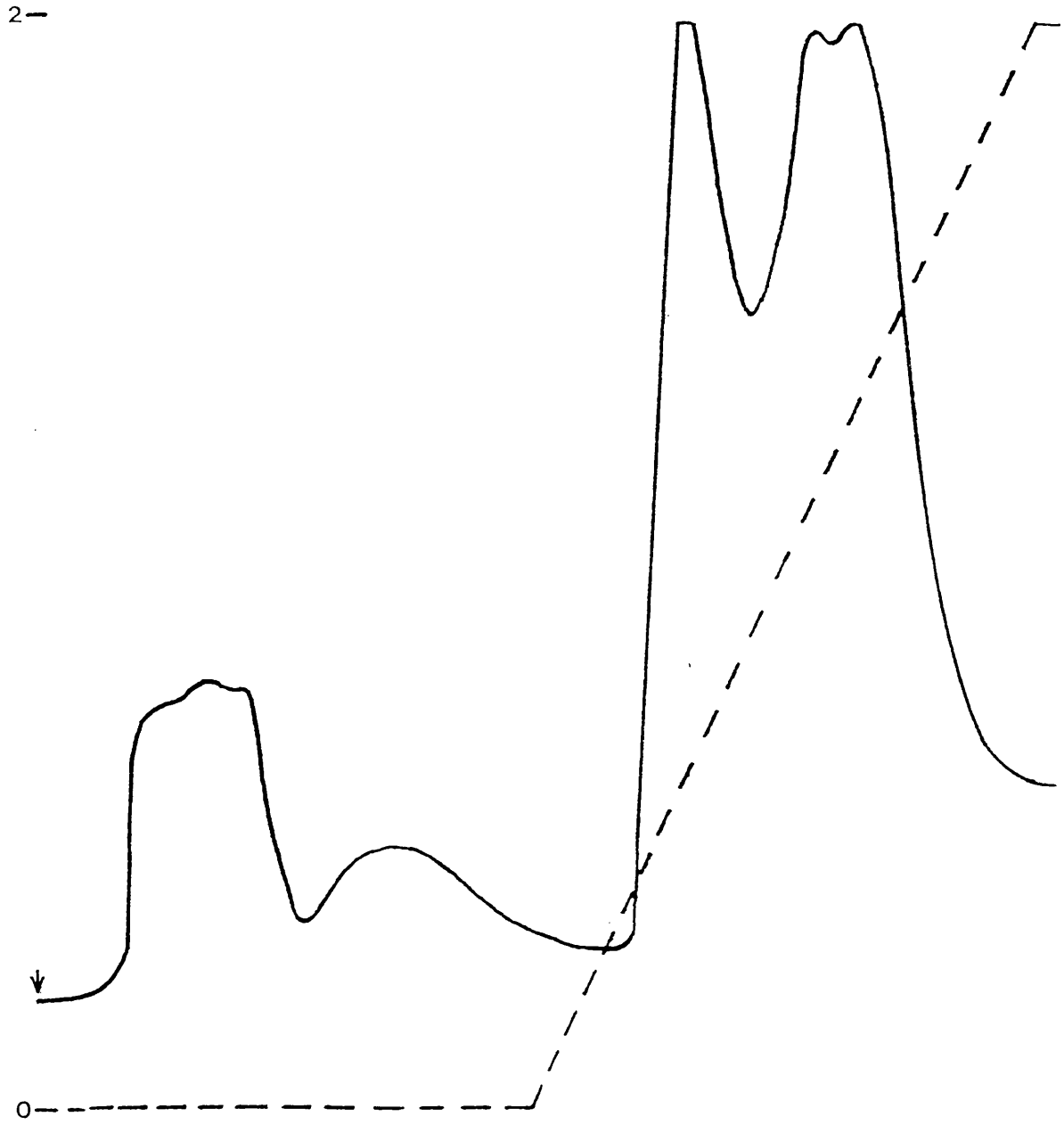


Fig 7a. Example of protein absorbance chart recording for hydroxyapatite FPLC chromatography of HMNC's.Centrifuged supernatents from 1.9 x 10_9 cells were loaded onto the column in 20 mM phosphate buffer.After washing through with this buffer,a linear gradient up to 500 mM was initiated (- - - -).Protein absorbance during the run is shown (A_{280} nm, 0-2 unit range (———) with chart speed 0.21cm/min .(▼) marks the application of the sample.

using 10% Polyacrylamide separating gels and 4.5% stacking gels (buffers 11-13), with LKB electrophoresis apparatus (method 1) according to Laemmli *(453)*. Electrophoresis was calibrated with molecular mass protein standards (Pharmacia). Proteins separated on the gels were transferred onto nitrocellulose (Hybond C extra, Amersham) using the western technique (using buffer 15 and see *564*). The nonspecific sites on the nitrocellulose membranes were blocked with high protein buffer (no 17 for 1 hour 37°C). The primary antibody (MC-5) was then incubated with the 'western' blot (diluted 1:100 with buffer 18) for 4 hours at room temperature. Immunoblots were then washed (4x5 minutes in buffer 19) and incubated for 30 minutes at room temperature with secondary antibody. This was anti-mouse IgG antibody conjugated to peroxidase (Amersham) diluted 1:100 with buffer. After washing (4x5 minutes buffer 19), immunoblots were incubated with 4-chloro-1-naphthol solution (buffer 21). Violet immunological bands were visualized within 5 minutes of addition of hydrogen peroxide (to final concentration of 0.03% v/v buffer 22), and when their intensity was sufficient the reaction was terminated with a water wash. Protein standards run adjacent to the samples were cut away, after western transfer, and visualized by incubation with colloidal gold protein stain (Jensen Autodye forte).

Method 2 Immunoblotting with PKC-isozyme specific antisera

FPLC samples eluting from a sodium phosphate gradient were immediately boiled with denaturing buffer (see buffer 23) for immunological analysis. 15-20μl of each was immunoblotted for the presence of PKC-α, β_1, γ, δ and ϵ isozymes following the

method used on rat brain fractions (Chapter 5).

Results

Kinase activity with TPA or sn1,2 Dioleoylglycerol ($DiC_{18:1}$ a diacylglycerol analogue) was assayed across the hydroxyapatite profile using the phospholipid micellar assay (Chapter 4). The results expressed as a "difference profile" are shown in *Fig 7b*. Pooled fractions from this profile (fractions 11-13) were used in testing various parametes of the micellar assay.

Time Course

The extent of histone phosphorylation in pooled active fractions was assessed over 12 minutes (see *Fig 7c*). TPA-elicited stimulation was observable throughout this period reaching a maximum level after 10 minutes similar to that seen with rat brain crude fractions. In other tests assays were conducted over a 10 minute period.

Substrate Concentration Effects

When stimulation of kinase activity using 1 mg ml^{-1} histone was assessed for $DiC_{18:1}$ and TPA over the hydroxyapatite profile of another HMNC preparation (obtained from a cell separator), the effectiveness of TPA compared to $DiC_{18:1}$ was much reduced across the profile (see *Fig 7d* compared with *Fig 7b*) suggesting that increased histone contributed to the TPA-, but not the $DiC_{18:1}$ stimulated kinase activity. To test this, concentrations of histone between 1-3 mg/ml were assessed for effect on kinase stimulation by TPA (1000 ng/ml) and $DiC_{18:1}$ (1.2 mol%) in pooled active fractions from the

Stimulation of kinase activity in HMNC cells.

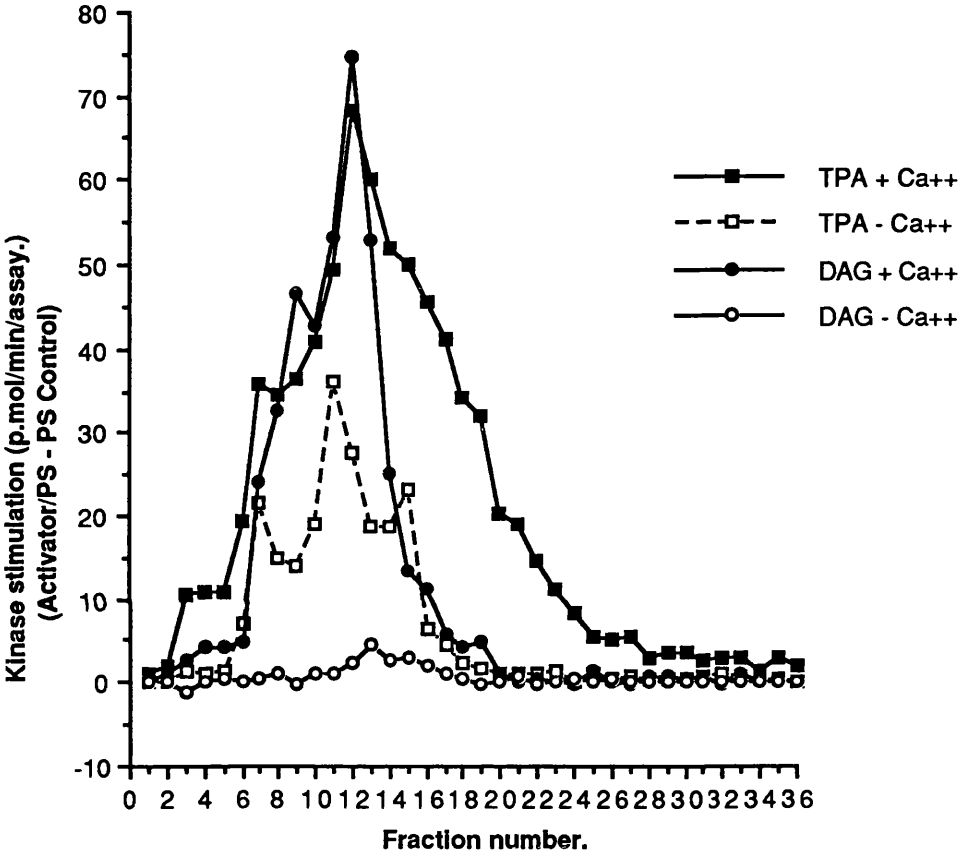


Fig 7b. Stimulation by TPA or a diacylglycerol (present at final concentrations of 1000 ng/ml or 40 μg/ml respectively, representing 0.03 or 1.2 mol % of the Triton/PS micelles) of fresh human mononuclear cells (2.3 x10$_6$cells) with basal levels (PS alone) subtracted. Cell supernatents were taken and subjected to hydroxyapatite chromatography (using a 20-600mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of 2 mg/ml.

Time course of kinase assay.

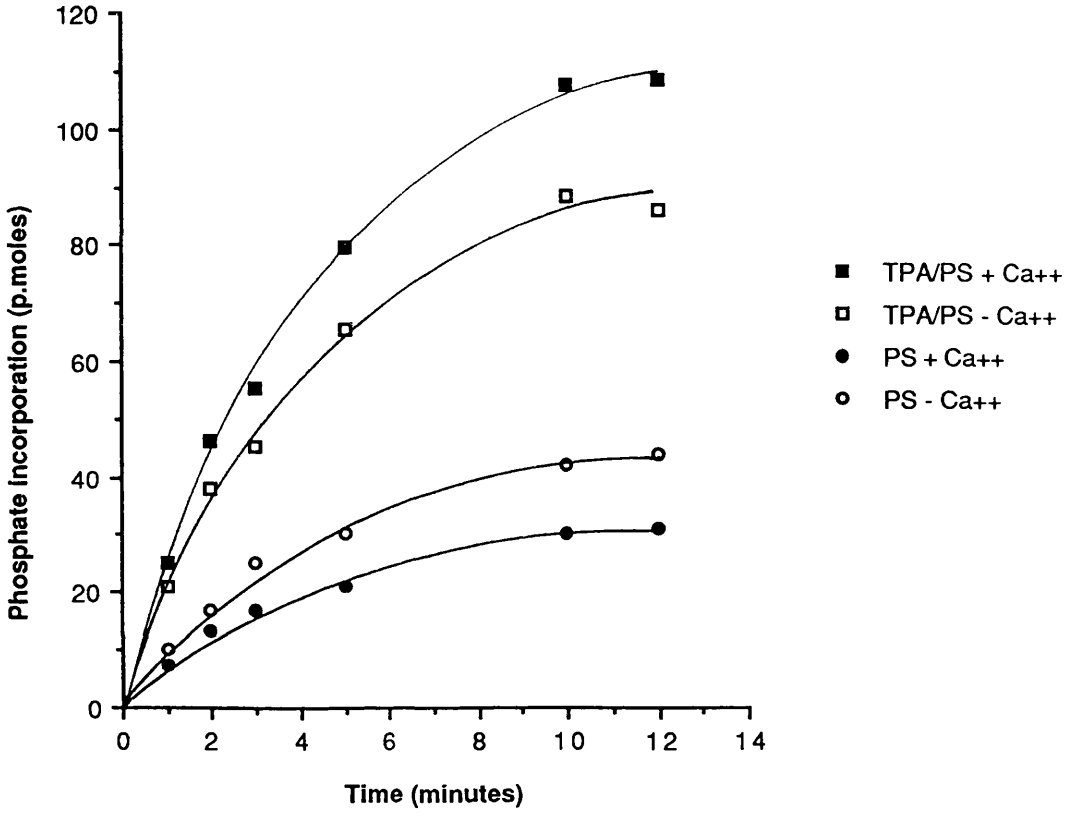


Fig 7c. Pooled fractions 11-13 (from an HMNC preparation obtained from 1.8 x 10^9 cells) were assayed for phosphate incorporation (p.moles incorporated minus 2 p.moles non-specific level).At the time points indicated,an aliquot of 20µl of each reaction mix was pipetted into 25% TCA to terminate the reaction.TPa,where present,had a final concentration of 1000 ng/ml (0.03 mol % of micelles),and histone had a final concentration of 3 mg/ml.

Stimulation of kinase activity in HMNC chromatography fractions.

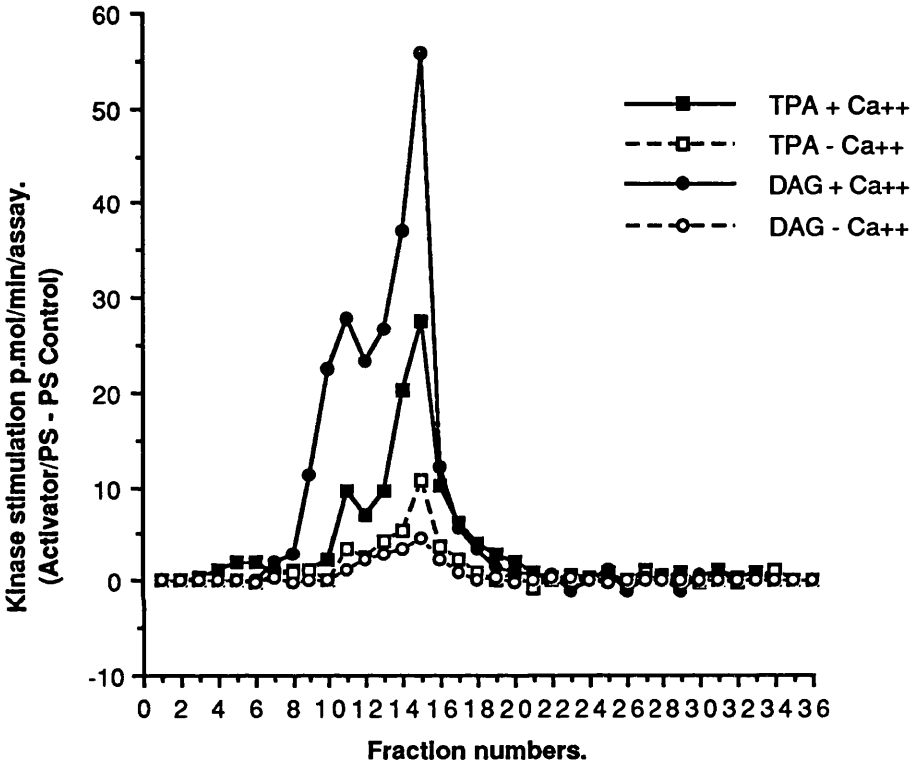


Fig 7d. Stimulation by TPA or a diacylglycerol (Present at final concentrations of 100 ng/ml or 40 µg/ml respectively,representing 0.003 or 1.2 mol % of the Triton /PS micelles) of HMNC kinase activity with basal levels (PS alone) subtracted. 1.8 x10^9 Cells were taken fresh and subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of 1 mg/ml.

initial profile (see *Fig 7e*). The presence of increased histone appeared to have no effect on kinase activity with PS alone or with $DiC_{18:1}$ either in the presence or absence of calcium. However when histone was increased with TPA as activator, kinase activity increased in a linear manner supporting the observation of enhanced histone effects with phorbol ester. It is not known whether this effect would extend to other substrates.

Dilution Tests

The effects of enzyme concentration and kinase activity was monitored for 5-25μl of pooled active fractions *(Fig 7f)*. Within this range the kinase activity increased with enzyme concentration but was not directly linear and levelled off. Interestingly at low concentration of enzyme (5μl) no additional effect was seen when histone levels were increased with TPA as the activator (data not shown). This observation indicated that when the kinase activity was out of the linear detection range TPA, but not $DiC_{18:1}$, could influence the system to take advantage of higher substrate concentrations.

Conclusions on Assay Parameters

The investigations on the assay parameters demonstrate that in crudely fractionated HMNC extracts, increased histone concentrations in the assay can affect the TPA stimulation profile in highly active fractions. To detect kinase activities stimulated by TPA it appears adequate to use histone in the range of 1-3 mg/ml final concentration although with the higher levels, smaller peaks may merge (and become

Substrate concentration effects on kinase activity in HMNC fractions.

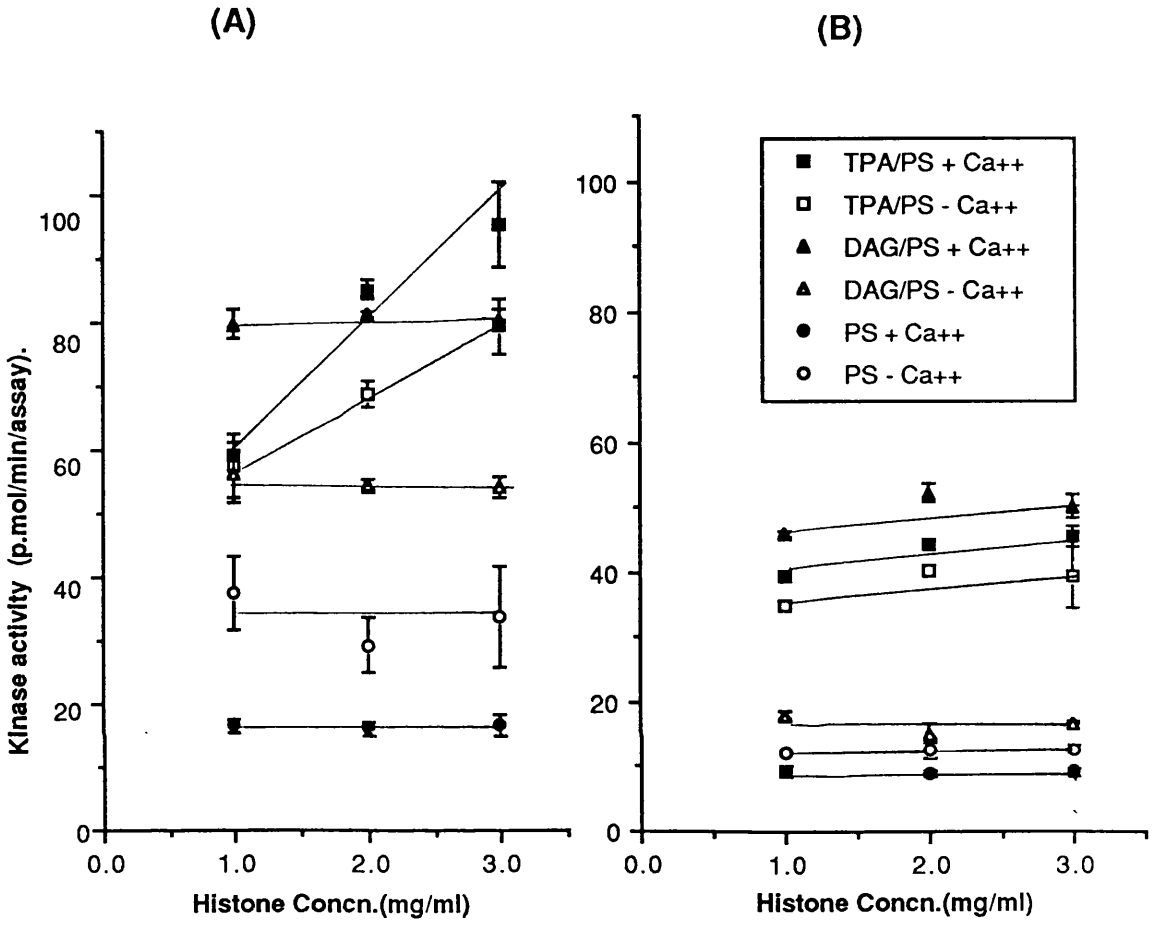


Fig 7e. Effect of histone concentration on kinase activity of pooled HMNC fractions (Nos. 11-13 from the HMNC preparation featured in Fig 7b) using (A) 12.5 µl or (B) 5 µl of enzyme.TPA and DAG had final concentrations of 1000 ng/ml and 40 µg/ml (0.03 or 1.2 mol % of the Triton/PS micelles respectively). Results are shown as mean values with the range from duplicate assays.

Effect of enzyme dilution on kinase activity in HMNC fractions.

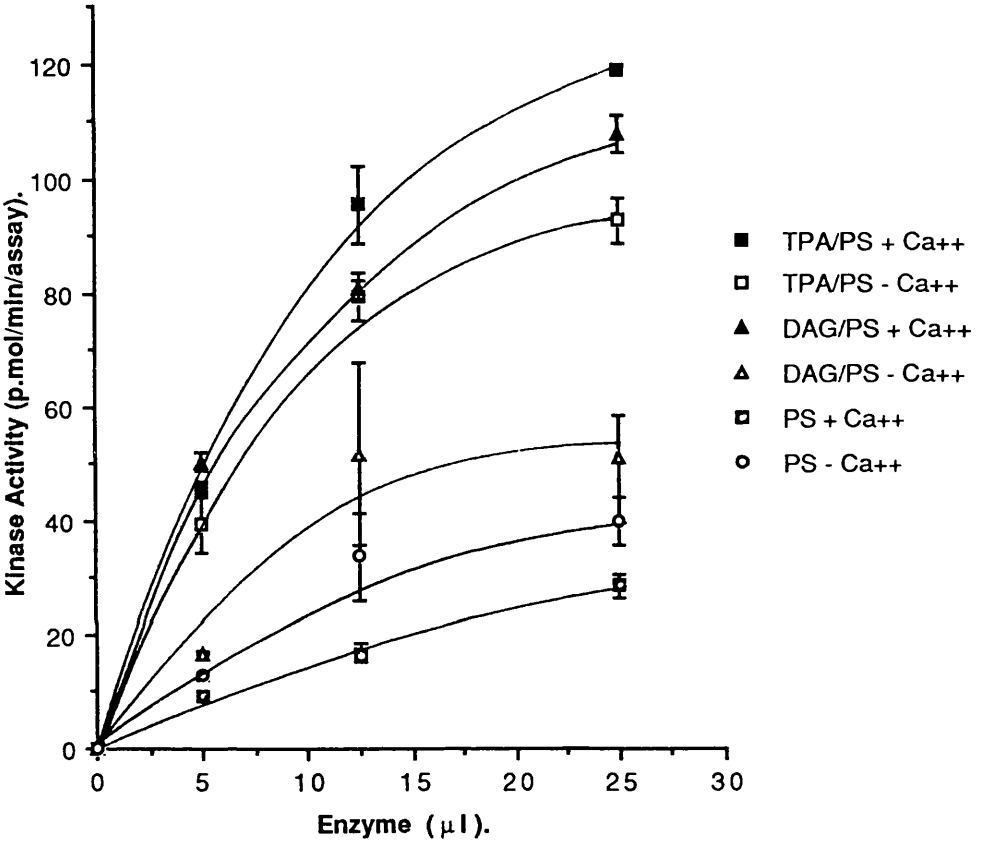


Fig 7f. Dilution effects on kinase activity ofHMNC fractions (Nos.11-13 pooled from the preparation of HMNC featured in Fig 7b).TPA and DAG had final concentrations of 1000 ng/ml and 40 µg/ml (0.03 or1.2 mol % of the Triton/PS micelles respectively) Results are shown as mean values with the range from duplicate assays.

obscured) with major peaks. For ease of assessing kinase activity simultaneously in large numbers of fractions, 10 minute assay periods were used as standard throughout. These protocols preclude kinetic investigation since the enzyme concentration and time course are not linear in this system.

Results: Kinase Stimulation Profiles

Several FPLC runs on HMNC extracts obtained from fresh whole blood were conducted using a starting volume of between 250 and 2000ml of blood. The kinase stimulation profile from 2 litres of blood is shown with TPA (100 ng/ml) and Resiniferatoxin (RX, 100 ng/ml) in *Fig 7g*. Different preparations using HMNC extracts prepared from fresh blood or cell separator HMNC-encriched blood were used in several experiments. The protein traces, levels of kinase activity and the location of major kinase peaks showed some variation between different preparations although the relative patterns of TPA stimulation remained fairly constant. From these different experiments five peaks of stimulatable kinase activities were observed when TPA, $DiC_{18:1}$ or RX were included in the PS micelles.

Peak I. Fractions 4-6, ~45-70 mM Phosphate

A very small peak stimulated by TPA in the presence of calcium and appearing as a shoulder to peak II in some preparations. $DiC_{18:1}$ appeared to stimulate some activity in this area.

Peak II. Fractions 8-12, ~100-150 mM Phosphate

A large peak showing calcium-dependent stimulation by TPA and

Stimulation of kinase activity in HMNC fractions.

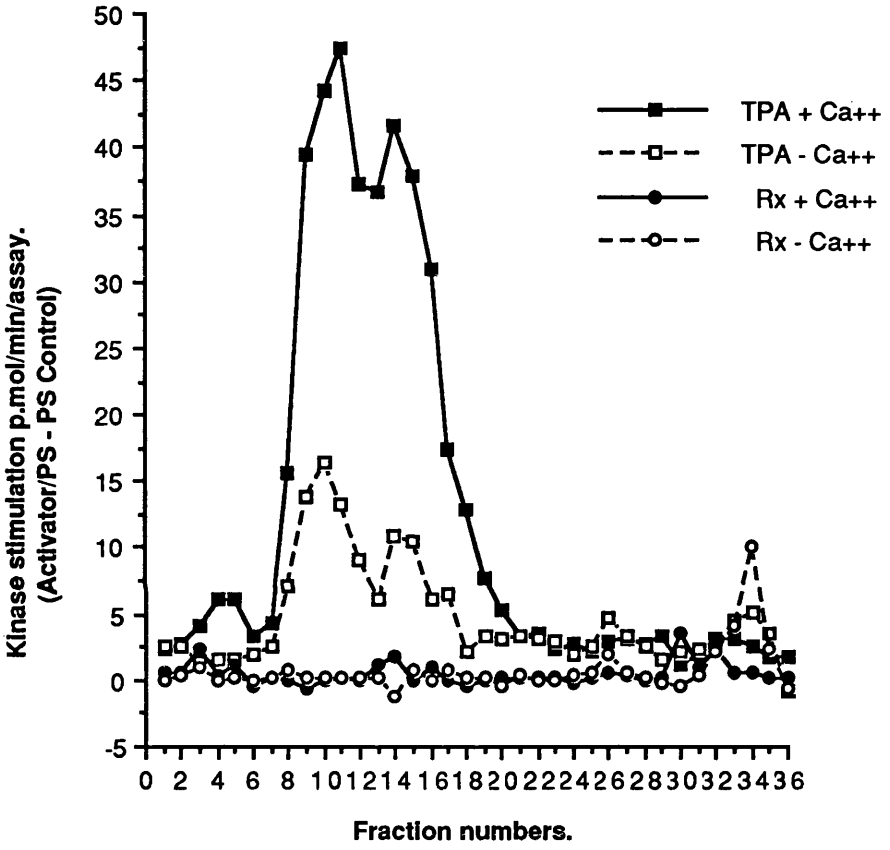


Fig 7g. Stimulation by TPA or Resiniferatoxin (present at final concentrations of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of HMNC kinase activity with basal levels (PS alone) subtracted. Cells from 2 litres of fresh blood were taken and subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile. Histone was present at a final concentration of 1 mg/ml.

$DiC_{18:1}$ running into peak III. TPA, but not $DiC_{18:1}$ was able to stimulate this peak without added calcium.

Peak III. Fractions 13-15, ~160-190 mM Phosphate

A large peak stimulated by TPA and $DiC_{18:1}$, in the presence of calcium, and by TPA and to a lesser extent $DiC_{18:1}$, in the absence of calcium.

Peak IV. Fractions 17-19, ~220-240 mM Phosphate

A shoulder running off Peak III and poorly resolved from the latter. This showed calcium dependent stimulation by TPA while $DiC_{18:1}$ did not appear to stimulate this peak. In the absence of calcium a small stimulation by TPA was observed.

Peak V. Fractions 31-34, ~400-450 mM Phosphate

A small isolated peak well resolved from the main block of kinase activity was stimulated by RX in the absence of calcium (and possibly by TPA also). No stimulation was seen by any activator in the presence of calcium.

Peaks [II] and [III] were also tested for kinase activity stimulated by Sapintoxin A (SAP A) and DOPP, present in PS micelles at 100 ng/ml. Both phorbol esters were able to stimulate these peaks well in the presence of calcium, but only DOPP was able to effect some stimulation of both peaks when calcium was excluded (data not shown).

The activity of peak V was observed, in cells extracts separated from fresh blood, in 4 separate experiments *(See Table 7A)* and was found to be highly sensitive to freeze/thawing, was unstable at room temperatures and was inhibitable by the kinase inhibitor staurosporine, when present at 1μM final concentration (data not shown).

Immunological analysis

Selected fractions from the run depicted in *Fig 7g* were immunoblotted with the MC-5 antibody for PKC (detecting full-length PKC-α and β isozymes). From this analysis only fractions in peaks III and IV showed presence of full length PKC in the molecular weight range 77-79Kd seen as a doublet with this method *(see Fig 7h)*. It was necessary to repeat the immunological analysis using the PKC isozyme- specific antibodies.

HMNC's, obtained using a cell-separator were, subjected to hydroxyapatite FPLC with a sodium phosphate gradient. Eluted samples were split into two halves - one was tested for TPA stimulatable kinase activity, the other for immunogical reactivity. The kinase stimulation profile *(see Fig 7i)* had a similar pattern of peaks previously observed.

Immunoblotting with antisera specific for PKC-α, β_1, γ, δ and ϵ isozymes was conducted on the elution samples. Bands immunoreactive to PKC-α antisera *(Fig 7j)*, PKC-β_1 antisera *(Fig 7k)* and PKC- ϵ

Rx-kinase activity in HMNC preparations.

Quantity of blood (ml)	Days in culture.	Number of Fraction with Rx-Kinase.	Peak Rx-Kinase activity (p.mol/min/assay)
200	8	31	9.3
450	1	31	13.0
450	3	34	8.9
2000	0	33-35	10.1

Table 7A. Stimulation by Resiniferatoxin (present at final concentrations of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of calcium-inhibited kinase activity (Rx-Kinase) with basal levels (PS alone) subtracted.Human mononuclear cells from 200-2000 ml of blood were taken in several experiments and subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of 1 mg/ml.

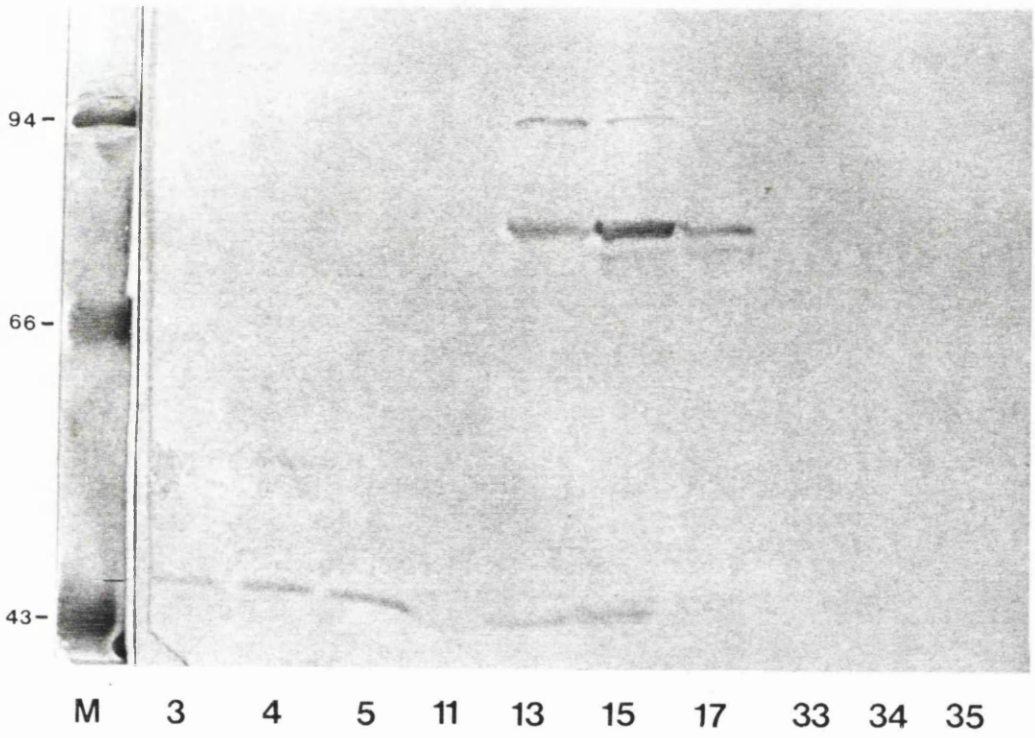


Fig 7h. Peroxidase stain of HMNC samples probed with the MC-5 antibody. HMNC samples from hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with the MC-5 anti PKC monoclonal antibody.Peroxidase conjugated 2nd antibody was used to visualise immunoreactive bands by staining.Lane M = Molecular weight markers (stained with colloidal gold),Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 7g.

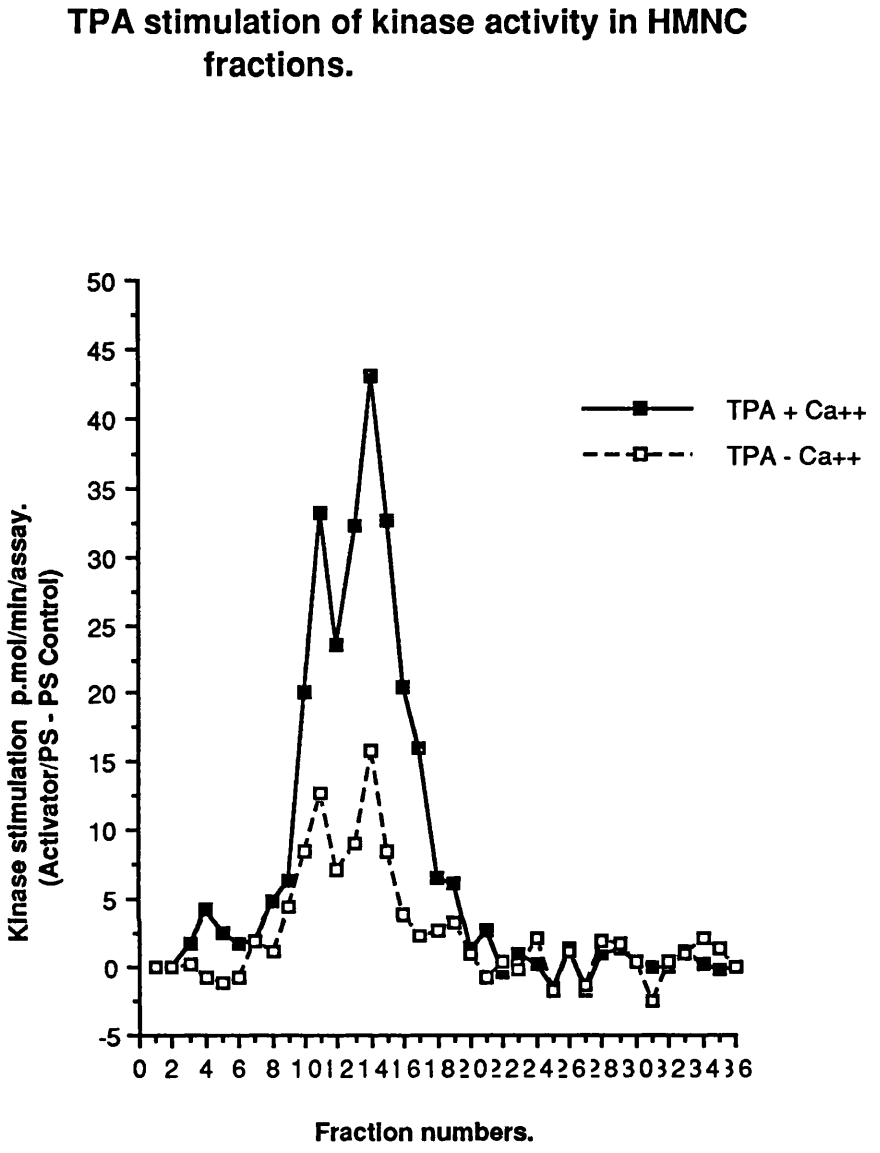


Fig 7i. Stimulation by TPA (present at a final concentrations of 1000 ng/ml representing 0.03 mol % of the Triton/PS micelles) of HMNC kinase activity with basal levels (PS alone) subtracted. 1.9 x 10^9 cells,cultured for two days,were taken and subjected to hydroxyapatite chromatography (20-500 mM Na phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of 3 mg/ml.

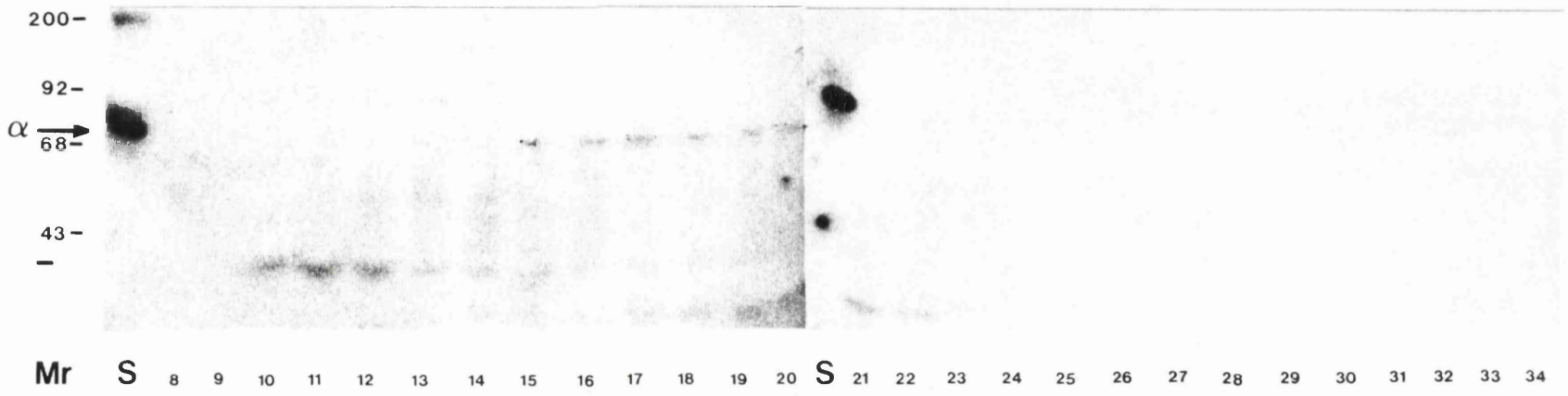


Fig 7j. Autoradiography of HMNC samples probed for PKC α.Samples from HMNC hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC α. Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (7 day exposure). Lane S = PKC standard ,Mr = Molecular weight markers ,Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 7i.

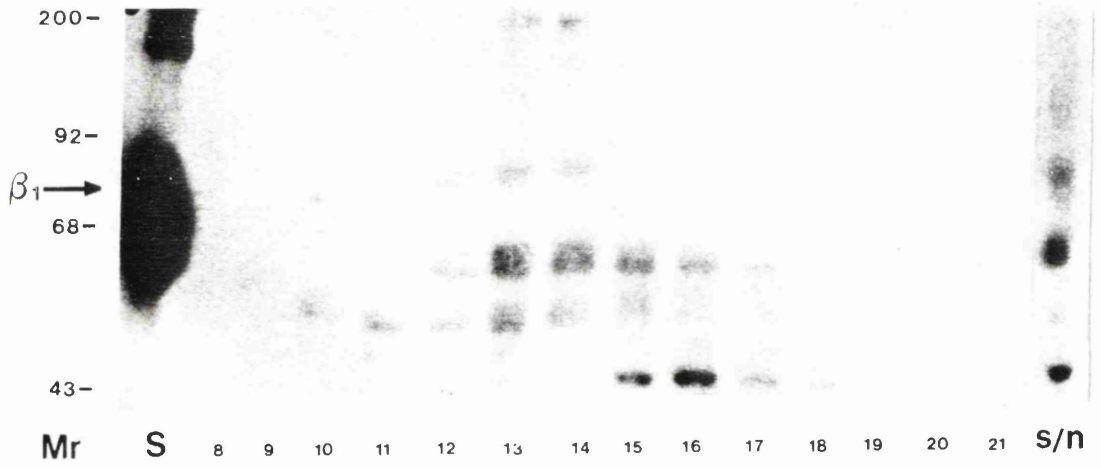


Fig 7k. Autoradiography of HMNC samples probed for PKC β1. Samples from HMNC hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC β1. Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (Overnight exposure) Lane S = PKC standard ,Mr = Molecular weight markers , Lane s/n = Pre-column supernatent. Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 7i.

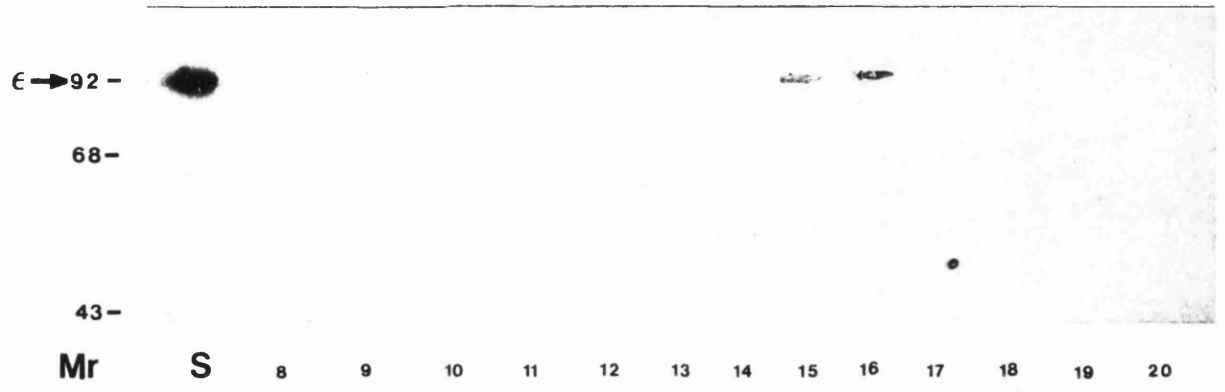


Fig 71. Autoradiography of HMNC samples probed for PKC ε.Samples from HMNC hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC ε.Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (7 day exposure).Lane S = PKC standard ,Mr = Molecular weight markers ,Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 7i.(Full spread of enzyme obscured by film processing)

antisera *(Fig 7I)* were observed, but not for PKC-γ or δ antisera (data not shown). The immunoreactive bands could be competed out with prior incubation of the antisera with the epitope peptide before blotting *(See Fig. 8p* for PKC-α and β_1 and *Fig 8q* for PKC-ϵ).The fractions eluting at the positions of peak II and peak III were found to contain immunoreactive peaks of PKC-β_1 and PKC-α respectively although poorly resolved from each other. The presence of PKC-ϵ was detected in a wide peak diffused over all the fractions. No immunoreactivity was apparent for these antisera in the fractions around peak I and V.

Discussion

Hydroxyapatite chromatography using FPLC apparatus was performed on HMNC extracts from fresh and cultured cells. The elution samples were tested for kinase activity stimulated by the phorbol esters, TPA and Resiniferatoxin (RX) compared to Phosphatidylserine (PS) alone, using the PS-micellar assay protocol developed from the rat brain experiments. Using these techniques we were able to detect 5 peaks of phorbol ester-stimulated kinase activity, in HMNC fractions, eluted at phosphate concentrations around 60, 120, 170, 230 and 440 mM (either sodium or potassium phosphate). The amount of kinase activity, in terms of amounts of basal and "differences" in activity stimulated by TPA (measured in p.moles/min/25μl fraction), was higher in rat brain profiles than in HMNC profiles. Conversely, when a diacylglycerol ($DiC_{18:1}$) was present, the stimulation of kinase activity observed was very high in HMNC's and indeed greater than stimulation values obtained with diacylglycerol in rat brain

samples. The diacylglycerol was used from the same batch in both studies and was used at the same concentrations (1.2 mol%), suggesting that variation was not 'experimental' but occurs in the diacylglycerol requirements of HMNC and Rat brain protein kinase C pools. Apart from the diacylglycerol differences, both profiles featured a large "central block" of stimulatable kinase activity which demonstrated calcium and phospholipid dependence. Assay parameters of mixed fractions from the central block were found to exhibit similar traits to those mixed fractions tested from the central block in Rat brain (e.g. Histone effects on TPA stimulatable but not $DiC_{18:1}$ stimulatable activity, Time course etc.).

When this elution profile was probed for the presence of PKC-α, β_1, γ, δ and ϵ isozymes, only PKC-α, β_1 and ϵ were detected. The immunoreactive bands were found to correspond to the control block of kinase activity (peaks II and III) such that peak II encompassed the peak of β_1-PKC immunoreactivity and peak III that of α-PKC immunoreactivity. ϵ-PKC immunoreactive bands were also detected in this central region but was spread diffusely over the region rather than being concentrated in any one peak. In terms of quantity (in so far as ease of detection by the antisera is equally proportional to presence of antigen), PKC-α and PKC-β_1 were present at similar levels, both being greater than PKC-ϵ in the HMNC elution profile. Comparing these results with the data from Rat brain reveals that the positions of the immunoreactive α, β_1 and ϵ bands in both cases are very similar although the relative quantities differ. Compared to rat brain, the quantities of immunological material in

FPLC samples of HMNC fractions were much smaller (as would have been expected from the lower mass of tissue involved) further supporting the case for the variation in the effect of diacylglycerol lying in the character of the enzyme rather than the quantity.

Previously, human T-lymphocytes have been studied to investigate the presence of PKC isozyme content *(279)*. This study, using polyclonal antibodies raised to PKC-α, β_1, β_2 and γ isozymes identified $\beta_{1 \text{ and } 2}$ and α-PKC subspecies which corresponded to 2 peaks of kinase activity (using 1,2 Diolein as an activator in a non micellar assay). Although our experiments were conducted on HMNC's (which include T-lymphocytes), they agree with the presence of α and β-isozymes and with the finding of PKC-ϵ, expand the number of PKC enzymes found in human mononuclear cells. The absence of PKC-γ from non-neural tissue has been reported *(252)* and is confirmed in the immunological analysis with the PKC isozyme-specific antisera, although absence of MC-5 immunoreactive bands in peak II was observed *(Fig 7h)*. However the prolonged ultracentrifugation of fractions which had a high protein content, in preparation for method 1, may have allowed extensive proteolysis to have occured before addition of Laemmli denaturing buffer, leading to destruction of the 'hinge' region and abrogating the MC-5 immunological reaction. These identities of peaks II and III are supported by the calcium and phospholipid dependent nature of stimulatable kinase activity in this central region by TPA and $DiC_{18:1}$.

Three peaks (termed I, IV and V) however, lie outside the

immunological peaks detected by antisera use. Peaks I and IV appear similar to two peaks observed in the rat brain profiles (see Chapter 4) with TPA. The stimulation of peak I activity was seen with TPA and slightly with $DiC_{18:1}$ in the presence but not the absence of calcium (possibly corresponding to peak I in rat brain). Peak IV was stimulated by TPA strongly in the presence, but poorly in the absence, of calcium while $DiC_{18:1}$ had no apparent effect in this region (corresponding to peak VI in rat brain). Furthermore the approximate phosphate concentrations at which these peaks eluted in HMNC runs were essentially the same as peaks I and VI in rat brain, suggesting that these uncharacterised kinases are fairly widespread in mammalian tissue. No peaks, corresponding to those designated VII and VIII in rat brain, were detected in HMNC kinase profiles.

Peak V represented an entirely novel kinase activity in human mononuclear cell extracts, eluting only under very high phosphate concentrations (~ 430 mM). This activity was observed in several separate experiments with HMNC's, but was not detectable in rat brain preparations. Unexpectedly this enzyme activity was very sensitive to calcium levels such that there was no kinase activity detectable if calcium was added to a free concentration of 100μM in the assay. Resiniferatoxin (RX) and to a lesser extent TPA, was found to stimulate activity of peak V when calcium was excluded from the assay. This stimulation of kinase activity of peak V was the only effect observed with Resiniferatoxin in HMNC elution samples, and was termed RX-Kinase activity. Investigation of the nature of this activity suggested it was a protein as judged by its susceptibility

to freeze/thawing and the inhibition of its activity by staurosporine (an antibiotic-type protein kinase inhibitor ref *218*).

The inhibitory effect apparent on RX-kinase activity with calcium is contrary to the known cofactor-action documented for all the PKC isozymes tested so far. The wider implications of kinases which can be inhibited by calcium extend into the spheres of the cellular function of calcium in regulatory and messenger roles as well as complicating the manner in which phorbol esters are perceived to influence biological systems through interaction with specific receptors.

CHAPTER 8

PHORBOL ESTER STIMULATION OF KINASE ACTIVITY IN DIFFERENT MONONUCLEAR CELL TYPES

The observation of multiple peaks of phorbol ester-stimulatable kinase activities in extracts from human mononuclear blood cells, and the discovery of a calcium-inhibited Resiniferatoxin stimulated kinase activity (RX-Kinase), prompted an investigation directed at specific cell types present in these preparations. This approach was combined with a screening of other sources of mononuclear cells, available in large numbers, for the presence of RX-Kinase activity.

Materials and Methods

Separation of Specific Mononuclear Cell Types from Human Blood

Using a cell separator (courtesy of the department Haematology, University College Hospital London) 200 mls of HMNC-enriched blood was taken from a healthy donor. The cells were separated into Monocyte/adherent cells, β-lymphocyte and T-lymphocyte-enriched fractions in the U.C.H. clinical haematology laboratories after the HMNC fraction was isolated (See Chapter 3).

(I) Monocytes were isolated from HMNC's by resuspending the cells in culture medium (buffer 1) and incubating the culture overnight at 37^{o}C in flat dishes. The culture containing non-adherent cells, was then poured off and the petri dishes were rinsed in culture medium (T- and β-lymphocytes were isolated from this

medium subsequently). The cells adhering to the petri dishes were carefully scraped off with a rubber glove into fresh medium and were pelleted (400 x g for 10 minutes) and frozen until used.

(II) β-and T-lymphocytes remaining in the original culture medium were separated from each other by sheep erythrocyte rosetting. Sheep erythrocytes (S.E. 10^7/ml in 25 mM HEPES treated with 4% 2-Aminoethylisothouronium, AET) kindly supplied by Dept of Haematology, UCH, were mixed 1:1 with the β/T cell culture, spun down (200 x g 5 mins), and left on ice for 1 hour. The T-lymphocytes which possess surface receptors for S.E. antigens (E positive) formed rosette complexes. These dense complexes were then separated from the β-lymphocytes (E negative) by centrifugation (800 x g 45 minutes) through a dense medium (e.g. Histopaque 1077 sigma) as in Chapter 3. β-lymphocytes were aspirated off, washed, pelleted and frozen until used.

(III) T-lymphocytes/SE rosettes were separated by osmotic shock (15 seconds in dilute (hypotonic) saline added to the pellet followed by hypertonic saline to restore the osmotic balance) resulting in the lysis of the SE.T-lymphocytes were then pelleted, washed, repelleted and frozen until use.

(IV) Neutrophil-enriched fractions were obtained from another cell separator run (the cell separator system can modify the neutophil content of the HMNC enriched blood which is collected). The blood was centrifuged on dense medium as before (See Chapter 3),

but the erythrocyte pellet (containing most of the neutrophils) was taken. The neutrophils were separated from the erythrocytes using osmotic shock-lysis as above. They were then pelleted, washed, repelleted and frozen until use.

Separation of human platelets

50mls of fresh human blood was mixed 9:1 with citrate buffer (buffer 2) and centrifuged at 250 x g for 10 minutes. The clear platelet-enriched supernatant was aspirated off and recentrifuged (280 x g 10 minutes) to remove residual erythrocytes and mononuclear cells and the supernatant was centrifuged (1400 x g 10 minutes) to pellet the platelets. These platelets were resuspended directly into ice-cold homogenization buffer (5 mls buffer 3) for fractionation.

Raji Cells

Raji cells (a human lymphoblastoid cell line) were grown using continuous culture techniques by Cancer Research Campaign, Birmingham by Prof. Rickmansworth and transported as a frozen pellet.

Daudi Cells

Daudi cell cultures (a transformed β-lymphoblastoid cell line) were kindly donated by Dr. N. S. B. Thomas, Dept Haematology, UCH, London. Cells were pelleted and resuspended in ice cold homogenization buffer (10mls buffer 3) for fractionation. Daudi cells treated with TPA, α-Interferon and an α-Interferon resistant strain treated with α-Interferon were used in addition to untreated 'wild type' cells.

Mouse Macrophage Cell Line

Cultured macrophage cells (P388D 5th Passage) were obtained from the London School of Hygiene and Tropical Medicine, Keppel Street, London. Cells were pelleted and resuspended in ice - cold homogenization buffer (buffer 3) for fractionation.

Starch -elicited Peritoneal Mouse Macrophages

Cells were obtained from the peritoneal cavities of 30 mice. To obtain large quantities of cells, the resident population was boosted by an interperitoneal injection of starch (2mls of 2% w/v). The starch granules elicit resident cell increase by stimulating invasion and multiplication of macrophage-type cells in the cavity. At the peak of this increase (~3 days) the mice were sacrificed and cells were washed out of the cavity by injection and aspiration of 5mls culture medium (buffer 1). The cells (~5 x 10^6/mouse) were pelleted by centrifugation (400 x g 10 minutes), washed repelleted and suspended in ice cold homogenization buffer (10 mls buffer 3) for fractionation.

Methods

Fractionation of Cell Extracts by Hydroxyapatite FPLC

Cells, resuspended in 2-10 mls of homogenization buffer (buffer 3) were disrupted by homogenization/sonication (see Methods Chapter 2) and centrifuged at 15,000 x g for 30 mins. The resulting supernatant was loaded onto an hydroxyapatite (0420 HTP Biorad) connected to an FPLC system. Fractions of 1ml were eluted from the

column with a 20-500mM phosphate gradient (1ml/min) following the procedures employed in rat brain studies (Chapter 4).

Kinase Assays

Kinase assays were conducted on eluted fractions using the PS micellar assay (see Chapter 4). TPA and RX (100 or 1000ng/ml) were used to stimulate radiolabled phosphate transfer from ^{32}P-ATP to histone III-S (1-3mg/ml) in the presence and absence of added calcium. The results were compared to assays with PS-alone controls and the results presented as the 'difference' (p.moles P^{32}/min/assay) between phorbol ester and control assay across the eluted fractions. Assay parameters were not assessed in these studies.

Immunological Analysis

Immunological investigation with PKC isozyme-specific antisera was limited to starch-elicited mouse macrophages and Daudi FPLC fractions eluted with sodium phosphate gradients. Samples of fractions were boiled immediately after elution after mixing 1:1 with denaturing buffer (buffer 23). 15-20μl of boiled denatured fractions were analysed using Method 2 electrophoresis (See Chapter 2 and Chapter 5). Competition experiments were conducted on selected fractions.

Results

Human Monocytes

The kinase stimulation of ($1x10^8$) human monocyte hydroxyapatite fractions by TPA was monitored in the presence and absence of calcium

(Fig 8a). Activity was low but three peaks were apparent with TPA and Calcium corresponding to ~140, 170 and 200mM phosphate. In the absence of Calcium stimulation of the 140 mM phosphate peak could be observed. RX did not appear to stimulate any kinase activity in any fraction (data not shown).

Human β-lymphocytes (E NEGATIVE CELLS)

The kinase stimulation for TPA of hydroxyapatite fractions from 6 x 10^8 β-lymphocytes is shown in *Fig 8b*. Three main peaks were seen with TPA in fractions corresponding to ~140, 170 and 220 mM phosphate in the presence of calcium. Low stimulation of these peaks was seen with TPA in the absence of calcium. RX did not appear to stimulate any kinase activity above PS controls over the profile (data not shown).

Human T-Lymphocytes (E POSITIVE CELLS)

The kinase stimulation of hydroxyapatite fractions from 1 x 10^8 T-lymphocytes by TPA is shown in *Fig 8c*. 2 low peaks of activity were stimulated by TPA in fractions corresponding to 140 and 170 mM phosphate. In the absence of calcium very poor stimulation was seen with TPA in the 140 mM range. RX did not elicit any stimulation of kinase activity in the profile (data not shown).

Human Neutrophils

The kinase stimulation for the phorbol esters TPA and RX of hydroxyapatite fractions from 5 x 10^8 Neutrophils are shown in *Fig 8d and 8e* respectively. In the presence of calcium low activity

Stimulation of kinase activity in Human Monocyte (Adherent cell) fractions.

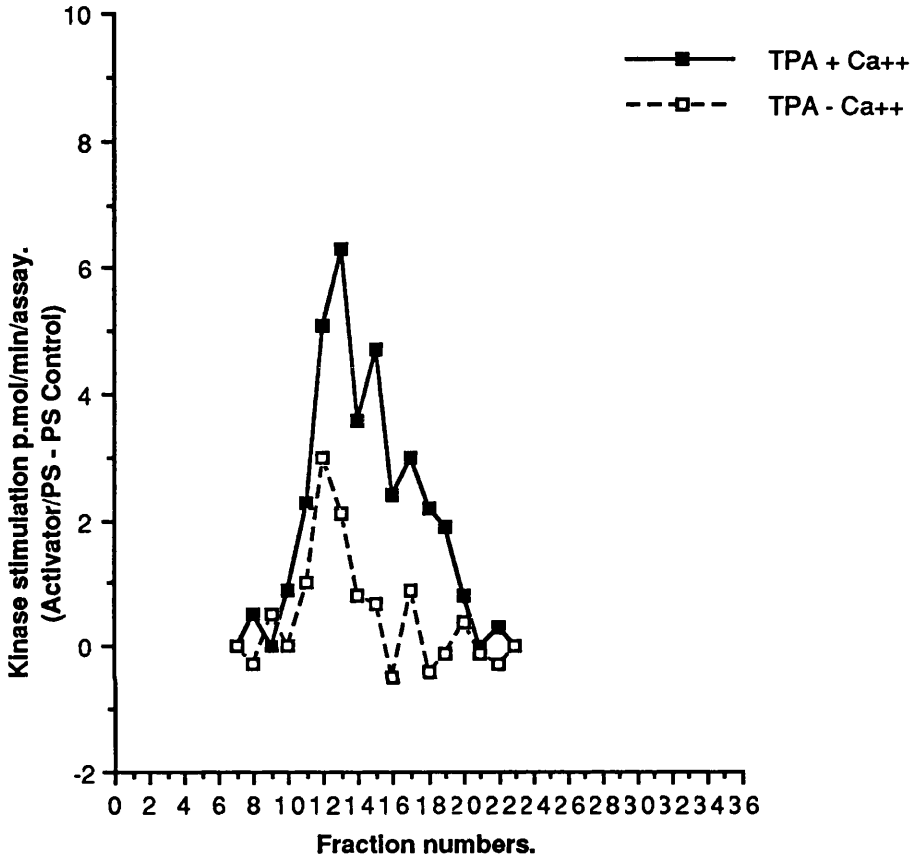


Fig 8a. Stimulation by TPA (present at final concentrations of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of human monocyte (adherent cell) kinase activity,with basal levels (PS alone) subtracted. 1 x 10^8 cells,isolated from whole blood and frozen,were subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of 1 mg/ml.

Stimulation of kinase activity in Human β-lymphocyte (E-Negative) fractions.

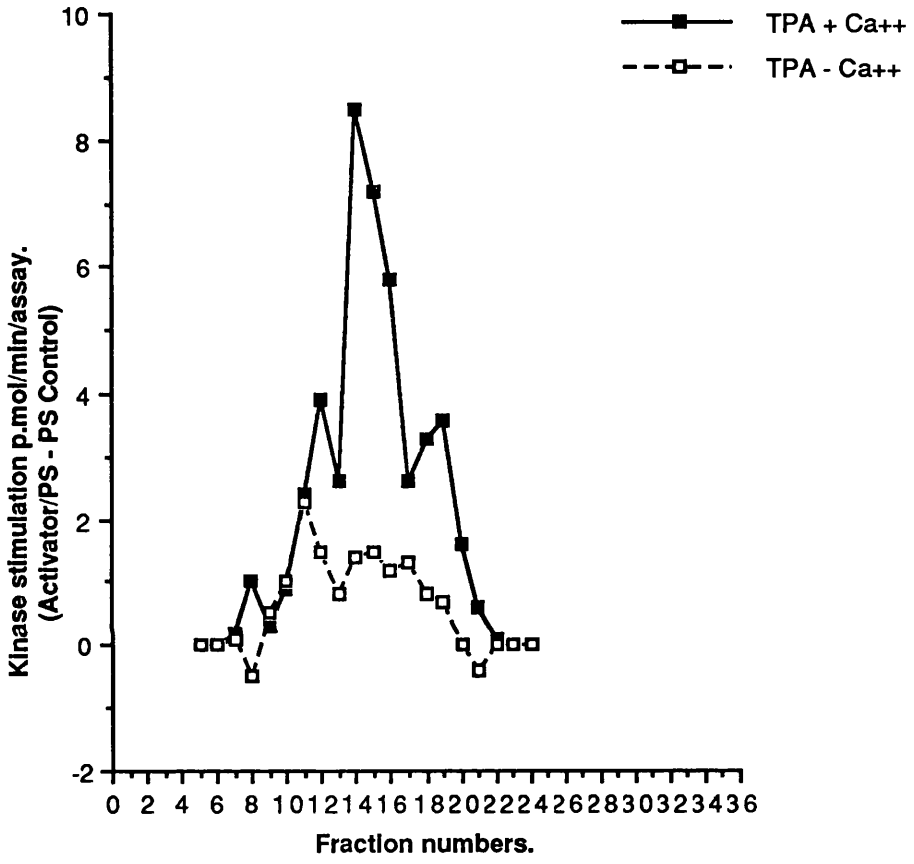


Fig 8b. Stimulation by TPA (present at final concentrations of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of human β–lymphocyte kinase activity,with basal levels (PS alone) subtracted. 6.6 x 10^8 cells,isolated from whole blood and frozen,were subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of1 mg/ml.

Stimulation of kinase activity in Human T-lymphocyte (E-Positive) fractions.

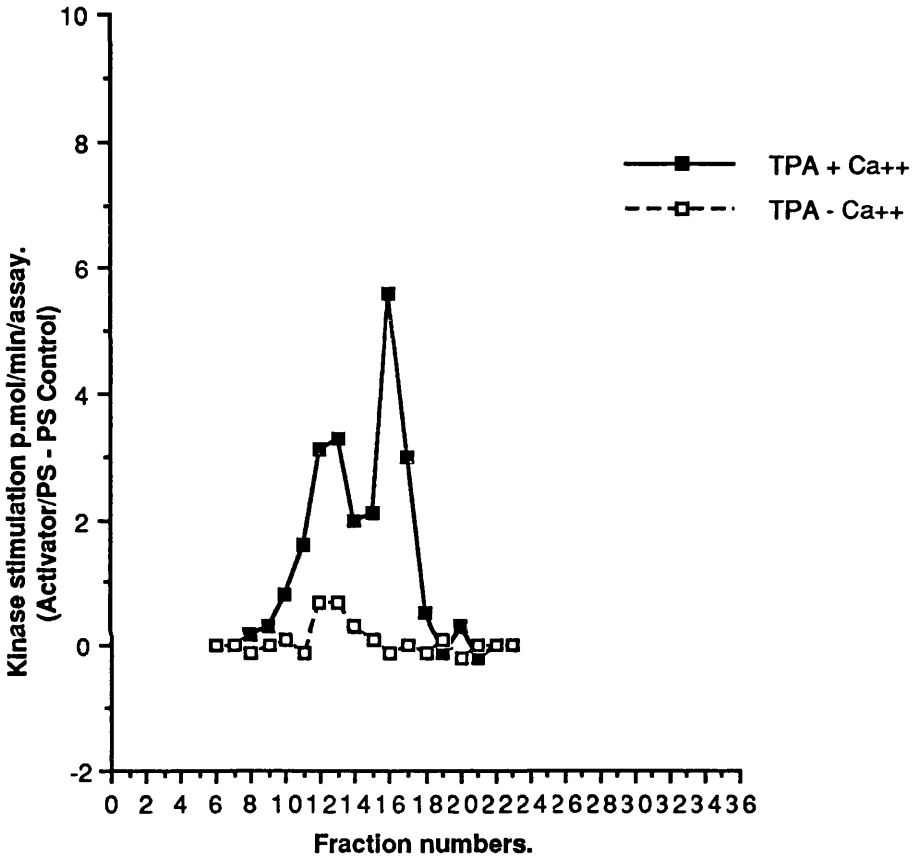


Fig 8c. Stimulation by TPA (present at final concentrations of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of human T–lymphocyte kinase activity,with basal levels (PS alone) subtracted. 1.1 x 10^8 cells,isolated from whole blood and frozen,were subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of1 mg/ml.

Stimulation of kinase activity in Human Neutrophil fractions.

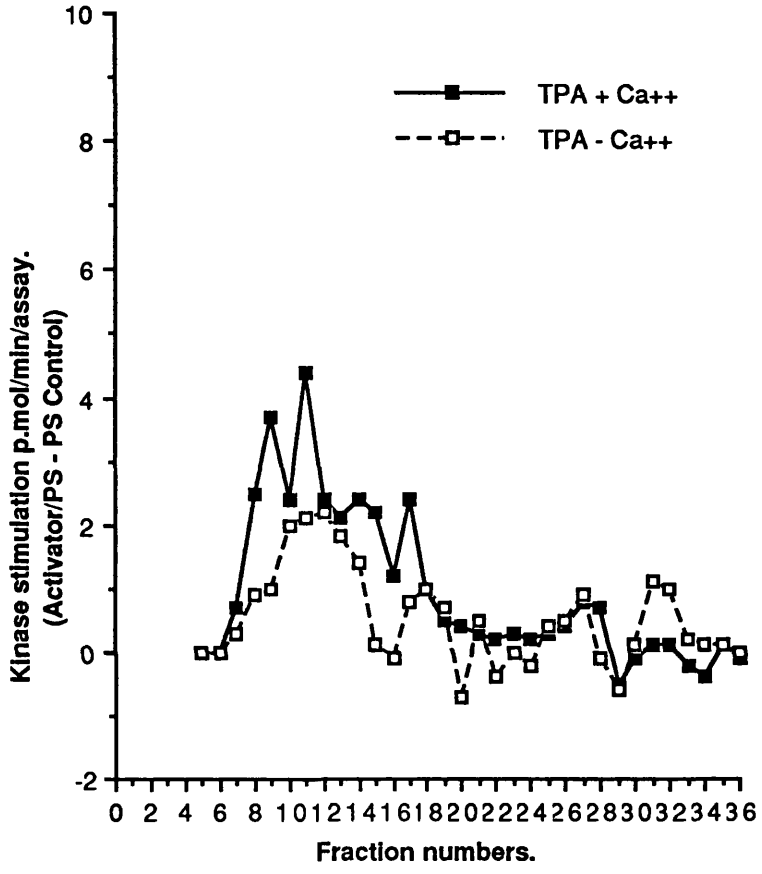


Fig 8d. Stimulation by TPA (present at final concentrations of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of human neutrophil kinase activity,with basal levels (PS alone) subtracted. 5 x 10^8 cells,isolated from whole blood and frozen, were subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of1 mg/ml.

Stimulation of kinase activity in Human Neutrophil fractions.

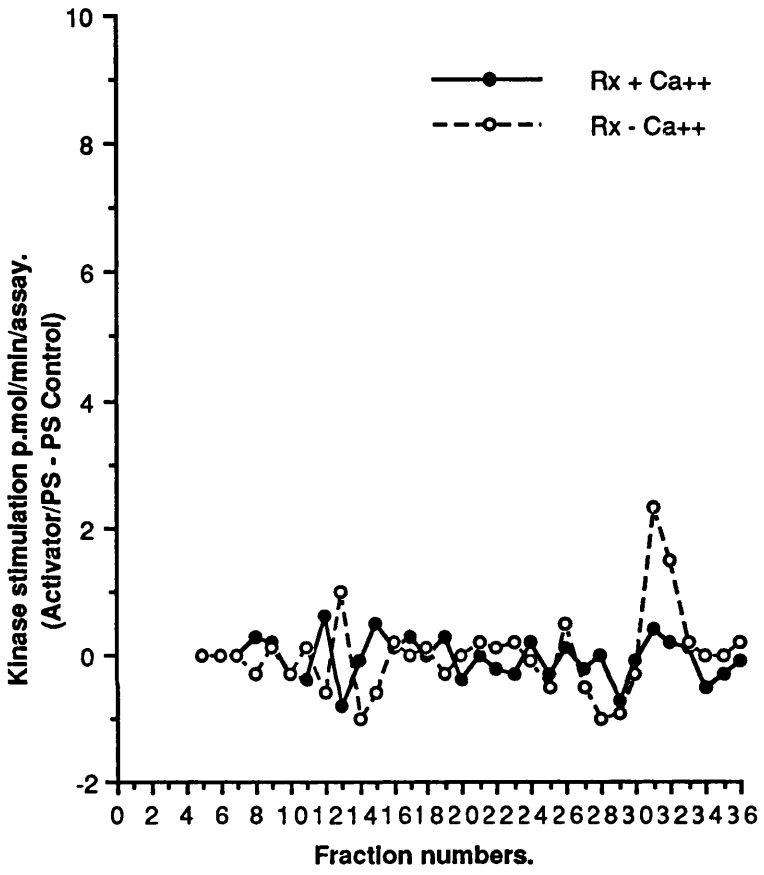


Fig 8e. Stimulation by Resiniferatoxin (present at final concentrations of100 ng/ml representing 0.003 mol % of the Triton/PS micelles) of human neutrophil kinase activity,with basal levels (PS alone) subtracted. 5 x 10^8 cells,isolated from whole blood and frozen,were subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile (see Fig 8d).Histone was present at a final concentration of1 mg/ml.

stimulated by TPA was observed in three or four peaks in fractions corresponding to 100, 140, 170 and 220 mM phosphate. In the absence of calcium there appeared to be some stimulation across fractions corresponding to 100 to 170 mM Phosphate. RX appeared to stimulate a slight peak, in the absence of calcium, eluting at around 400 mM phosphate.

Human Platelets

The kinase stimulation of hydroxyapatite fractions fom 3×10^9 platelets by TPA is shown in *Fig 8f*. 2 peaks of activity were stimulated by TPA in fractions corresponding to 140 and 165 mM Phosphate. Stimulation was also observed in both peaks with TPA in the absence of calcium. No kinase stimulation was seen with RX in the elution samples.

Raji Cell Line

8×10^8 Raji cells were subjected to hydroxyapatite FPLC and assayed for TPA and RX stimulatable kinase activity. No stimulatable kinase activity was observed along the profile in the presence or absence of calcium. Rat brain fractions, employed as internal standards, indicated the assay was working correctly and the protein content present in the profile, as detected during FPLC at 280nm, was similar to other runs (data not shown).

Daudi Cell Lines

The kinase stimulation by TPA and RX of various Daudi cell hydroxyapatite fractions was investigated in the presence and absence

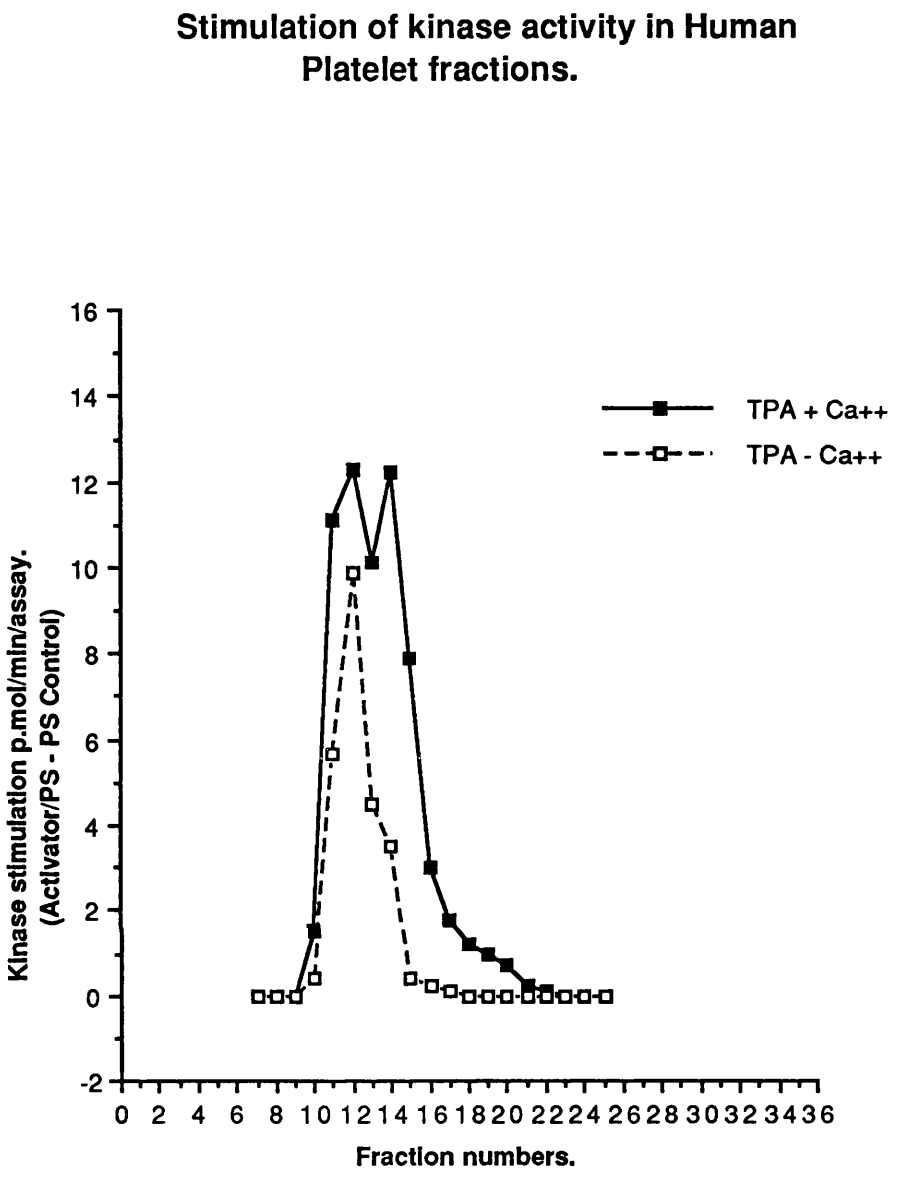


Fig 8f. Stimulation by TPA (present at final concentrations of 1000 ng/ml representing 0.03 mol % of the Triton/PS micelles) of human platelet kinase activity,with basal levels (PS alone) subtracted. 3 x 10^9 platelets,isolated fresh from whole blood (50 ml), were subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of 3 mg/ml.

of calcium. Kinase difference profiles with TPA are shown for untreated 'wild type' Daudi cells (WT: *Fig 8g)*, Daudi cells treated with α-Interferon (300U/ml) for 20 hours (αIF-WT:*Fig 8h*) and α-Interferon-resistant Daudi cells cultured for 4 weeks with α-Interferon (300U/ml) (αIF-RESIST: *Fig 8i*). No activity was observed with Daudi cells treated with TPA (100 nM) for 40 hours (TPA-WT: data not shown). No stimulation of kinase activity was observed with RX in any of these profiles (data not shown). Similar profiles of TPA stimulated activity were observed in (WT) and (αIF-WT) Daudi samples in the presence of calcium, corresponding to ~70, 145, 180 and 220 mM phosphate. αIF-RESIST Daudi profiles showed a depression of stimulatable activity with small peaks at ~ 150 and 220 mM phosphate.

Limited immunological analysis was conducted on Daudi elution samples. PKC-α antisera detected immunological bands of ~ 77Kd across the region corresponding to the 180mM phosphate peak of TPA-stimulated kinase activity in (WT) and (αIF-WT) Daudi cell elution samples only. All profiles however, featured a strongly immunoreactive band of ~45Kd across a wide region (corresponding to 80-190mM Phosphate) *See Figs 8g and 8k.* PKC-ϵ antisera detected a 90/92 Kd doublet of immunoreactive bands in fractions eluting between 150 and 190 mM phosphate in WT, αIF-WT and αIF-RESIST, but not TPA-WT Daudi profiles (e.g. *See Fig 8j and 8k).* When (WT) (αIF-WT) and (TPA-WT) Daudi elution samples were blotted with PKC-β_1 antisera, only (WT) and (αIF-WT) Daudi samples corresponding to fractions in the 140mM phosphate peak exhibited immunoreactive bands (data not shown).

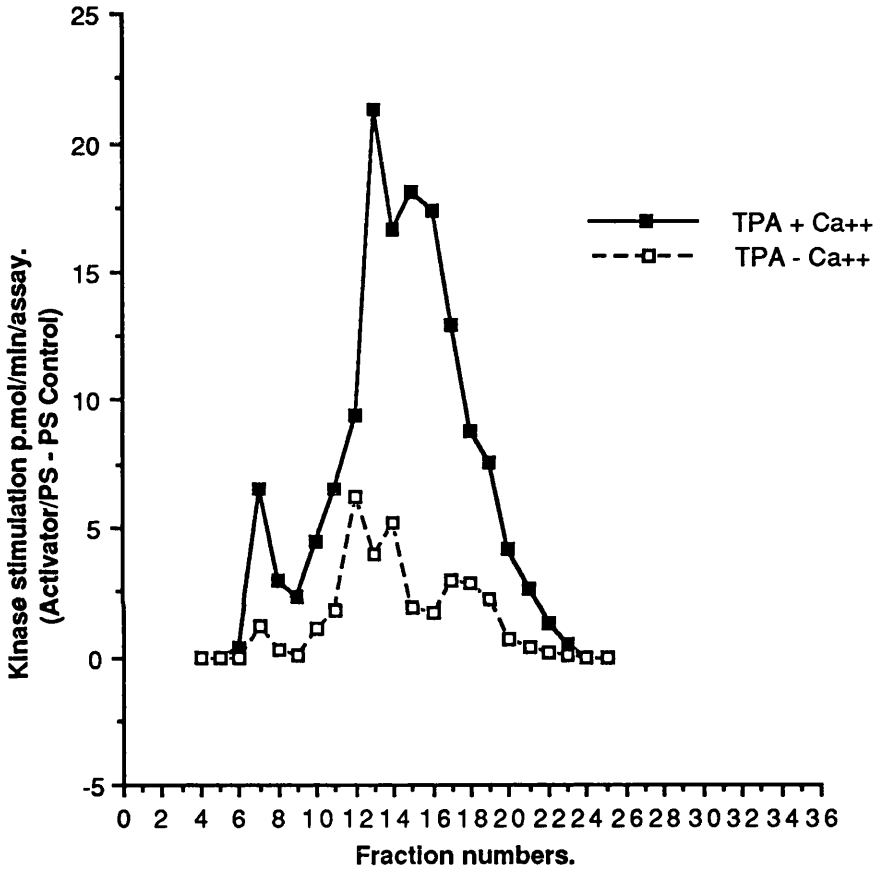


Fig 8g. Stimulation by TPA (present at final concentrations of 1000 ng/ml representing 0.03 mol % of the Triton/PS micelles) of wild type Daudi cell kinase activity,with basal levels (PS alone) subtracted. 3.4 x 10^8 cultured cells,were subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of 2mg/ml.

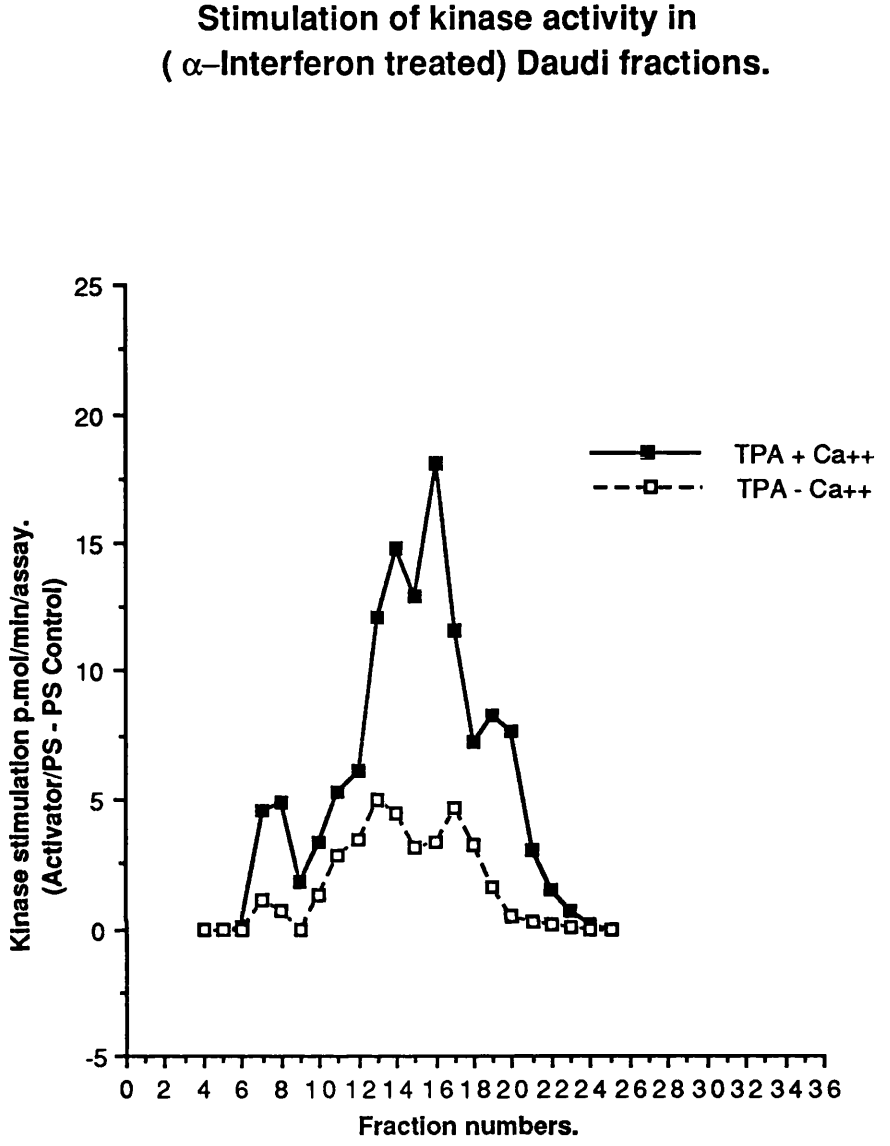


Fig 8h. Stimulation by TPA (present at final concentrations of 1000 ng/ml representing 0.03 mol % of the Triton/PS micelles) of α interferon treated Daudi cell kinase activity,with basal levels (PS alone) subtracted. 3.4 x 10^8 cultured cells,were subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of 2 mg/ml.

Stimulation of kinase activity in fractions from an α-Interferon resistant Daudi strain.

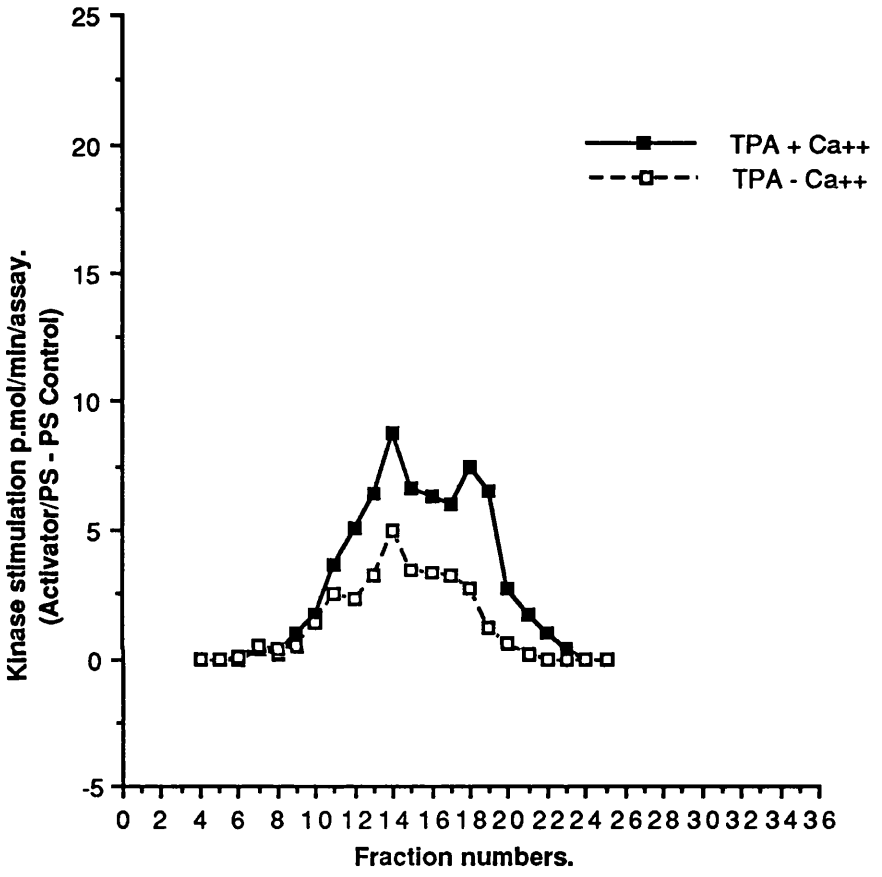


Fig 8i. Stimulation by TPA (present at final concentrations of 1000 ng/ml representing 0.03 mol % of the Triton/PS micelles) of an α interferon resistant Daudi strain kinase activity,with basal levels (PS alone) subtracted. 3.4 x 10^8 cultured cells were subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of 3 mg/ml.

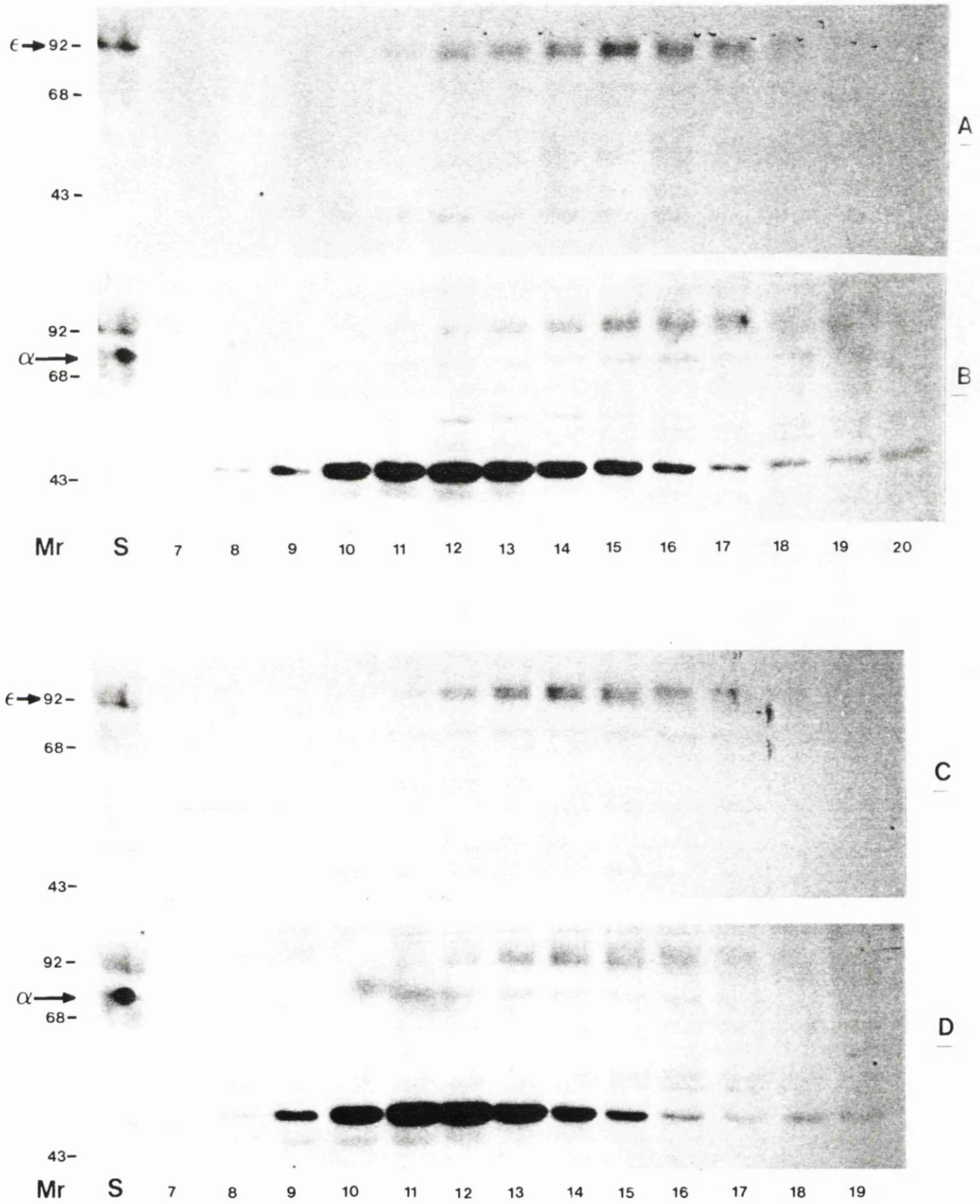


Fig 8j. Autoradiography of Daudi cell samples probed for PKC ε and α. Samples from wild-type Daudi (= A,B) and α-interferon treated Daudi (= C,D) hydroxyapatite chromatography (see Figs. 8g and 8h respectively) were subjected to SDS-PAGE/Western blotting. Blots were initially probed with antisera for PKC ε (A and C) and then reprobed for PKC α (B and D). Radiolabled 2nd antibody was used to visualise the bands on Kodak X-omat film (5 day exposure for PKC ε, 3 day exposure for the reblot with PKC α). Lane S = PKC standard, Mr = Molecular weight markers. Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 8g and 8h.

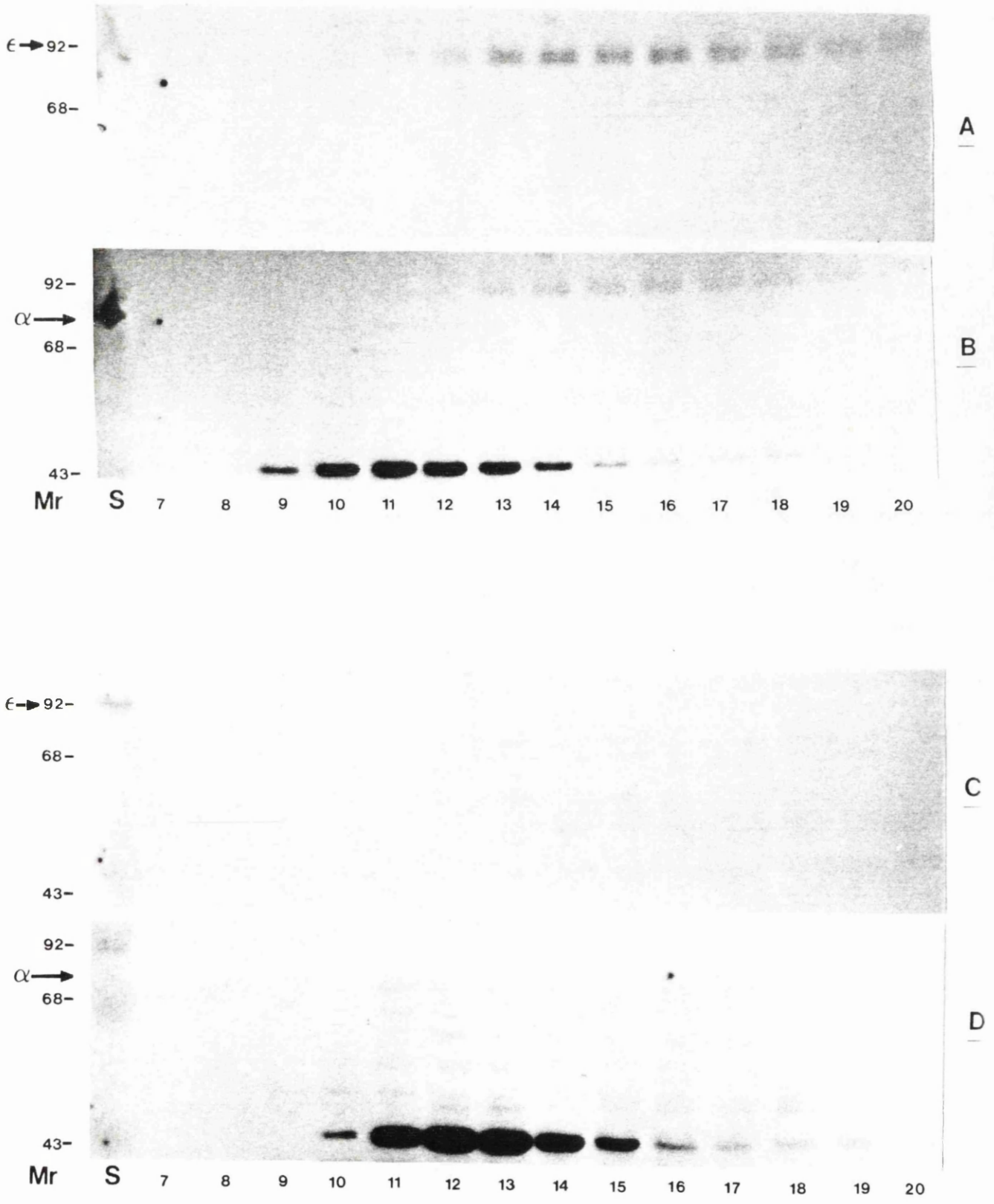


Fig 8k. Autoradiography of Daudi cell samples probed for PKC ε and α Samples from an α-interferon resistant Daudi strain (= A,B) and TPA treated wild-type Daudi (= C,D) hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting.Blots were initially probed with antisera for PKC ε (A and C) and then reprobed for PKC α (B and D) Radiolabled 2nd antibody was used to visualise the bands on Kodak X-omat film (5 day exposure for PKC ε,3 day exposure for the reblot with PKC α) Lane S = PKC standard ,Mr = Molecular weight markers.Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile (shown in Fig 8i for α-interferon resistant Daudi strain)

PKC-γ antisera did not react with any Daudi elution samples (data not shown), and PKC-δ was not tested. Competition experiments conducted on (WT) samples and (WT) supernatant demonstrated that the bands immunoreactive with PKC-α, β_1 and ϵ antisera were competable with corresponding epitope peptides *(See Fig 8p and 8q)*.

Mouse Macrophage Cell Line

3 x 10^8P388D murine macrophage cells were subjected to hydroxyapatite FPLC and assayed for TPA and RX stimulatable kinase activity. No stimulatable kinase activity was observable in the elution profile. Rat brain fractions, employed as internal standards, indicated the assay was working correctly and the protein content present in the profile as detected during FPLC at 280nm, was similar to other runs (data not shown).

Starch-elicited Mouse Peritoneal Macrophages

Mouse cells taken from peritoneal cavities of 30 mice, 3 days after an injection of starch solution, were subjected to hydroxyapatite FPLC. The stimulation of kinase activity by TPA and RX in the presence or absence of calcium was investigated *(see Fig 8l)*. With TPA in the presence of calcium, three peaks of kinase activity were observed corresponding to ~140, 165 and 200 mM phosphate. In the absence of calcium only peaks corresponding to 140mM, and to a lesser extent 200mM were observed with TPA. RX stimulated a peak of kinase activity, which eluted late (~380-400mM phosphate) and was only stimulated when calcium was excluded from the assay.

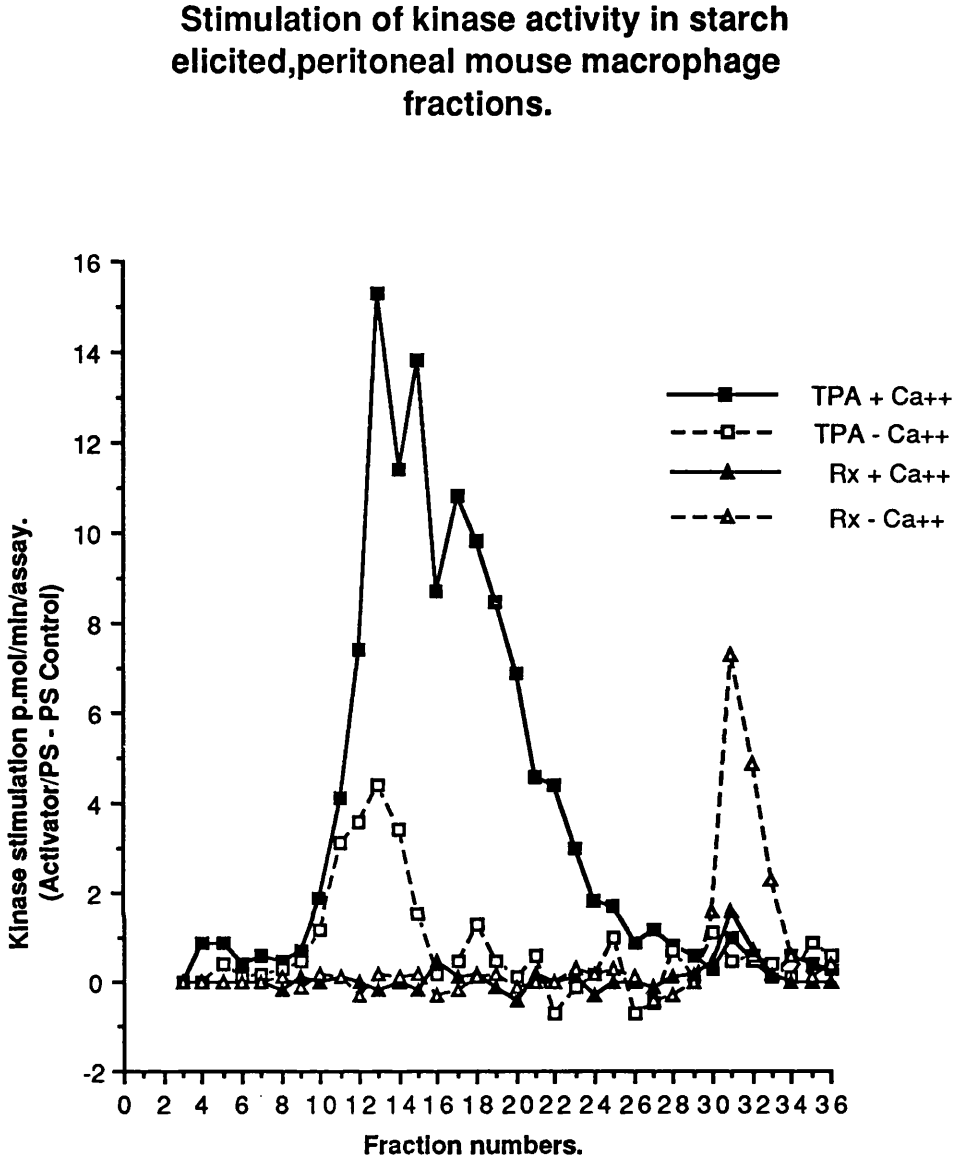


Fig 8l. Stimulation by TPA or Resiniferatoxin (present at final concentrations of 1000 ng/ml representing 0.03 mol % of the Triton/PS micelles) of mouse macrophage kinase activity,with basal levels (PS alone) subtracted. Cells,fresh from the peritoneal cavities of 30 starch-treated mice were taken and subjected to hydroxyapatite chromatography (20-500 mM phosphate gradient) generating 36 fractions in profile.Histone was present at a final concentration of 3 mg/ml.

Immunological analysis of PKC isozymes in elution samples of this profile was conducted using antisera to PKC-$\alpha, \beta_1, \gamma, \delta$ and ϵ. PKC-α antisera detected immunological bands of $\sim$ 77Kd around fractions corresponding to the 160mM region, and also broadly detected a 45Kd immunological band in the 75-170 mM phosphate region *(See Fig 8m)*. PKC-β_1 was detected in the 140mM phosphate region (seen in *Fig 9c)* and a PKC-ϵ doublet, as before, was found diffused over the 150-190mM phosphate region *(See Fig 8n)*. A small immunoreactive band was also detected with PKC-γ antisera in the 200mM region *(See Fig 80)*. PKC-δ was tested for in the supernatant fraction but was absent (data not shown). Competition experiments found that the PKC -α, β_1 and ϵ immunoreactive bands were competable with corresponding epitope peptides *(See Figs 8q and 9c)*, however the PKC-γ immunoreactive band was not competable and must be assumed to be due to a non specific effect *(See Fig 8r)*.

Discussion

Human white blood cells and platelets were separated into subpopulation-enriched preparations and these were individually fractionated by FPLC on (0420 HTP) Hydroxyapatite. Eluted fractions were assayed for the presence of phorbol ester-stimulatable kinase activity using a PS/micellar assay. Five such peaks, which were observed in earlier investigations of human mononuclear cell preparations *(See Chapter 7)*, were found to be represented amongst these subpopulation-enriched preparations. Working under the assumption that similar kinase activities in whole HMNC preparations and subdivided HMNC preparations will elute in a similar manner on

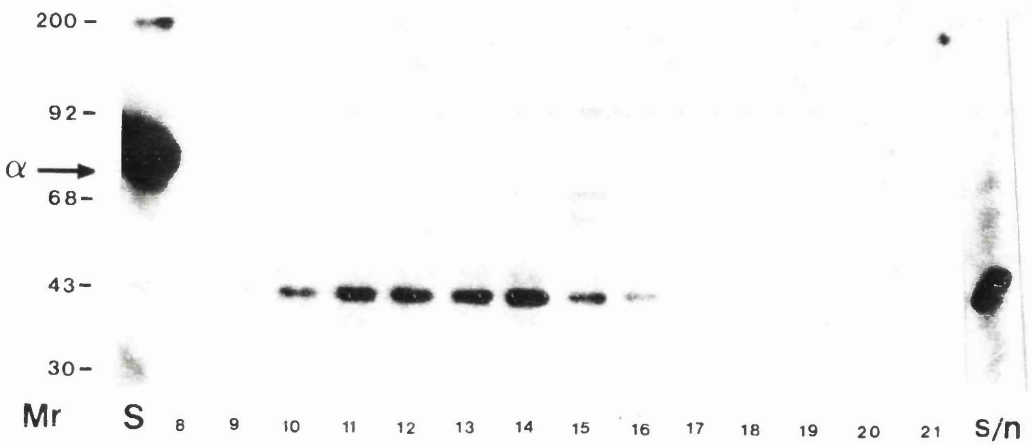


Fig 8m. Autoradiography of mouse macrophage samples probed for PKC α. Samples from mouse macrophage hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC α. Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (3 day exposure). Lane S = PKC standard ,Mr = Molecular weight markers, Lane s/n = Pre-column supernatent. Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 8l.

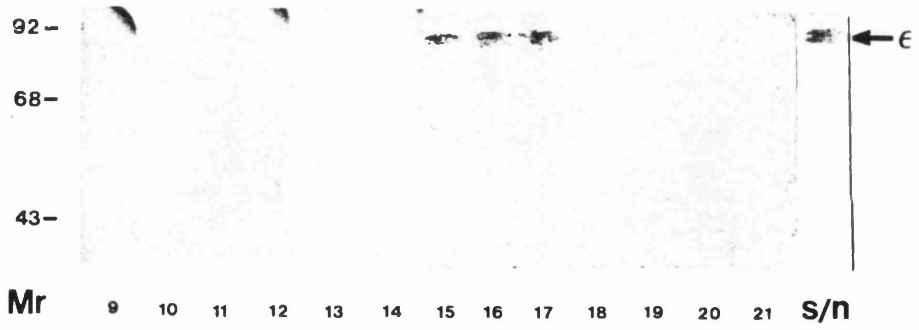


Fig 8n. Autoradiography of mouse macrophage samples probed for PKC ε.Samples from mouse macrophage hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC ε.Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (5 day exposure).Mr = Molecular weight markers,Lane s/n = Pre-column supernatent.Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 8l.

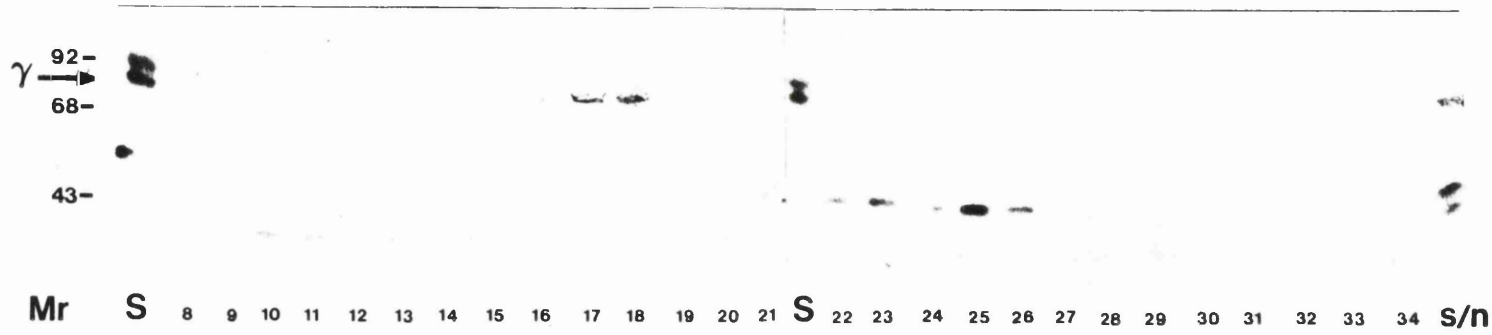


Fig 8o. Autoradiography of mouse macrophage samples probed for PKC γ.Samples from mouse macrophage hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for PKC γ.Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (7 day exposure).Lane S = PKC standard ,Mr = Molecular weight markers, Lane s/n = Pre-column supernatent.Other lanes are numbered in an identical manner to the fractions in the hydroxyapatite chromatography elution profile shown in Fig 8l.

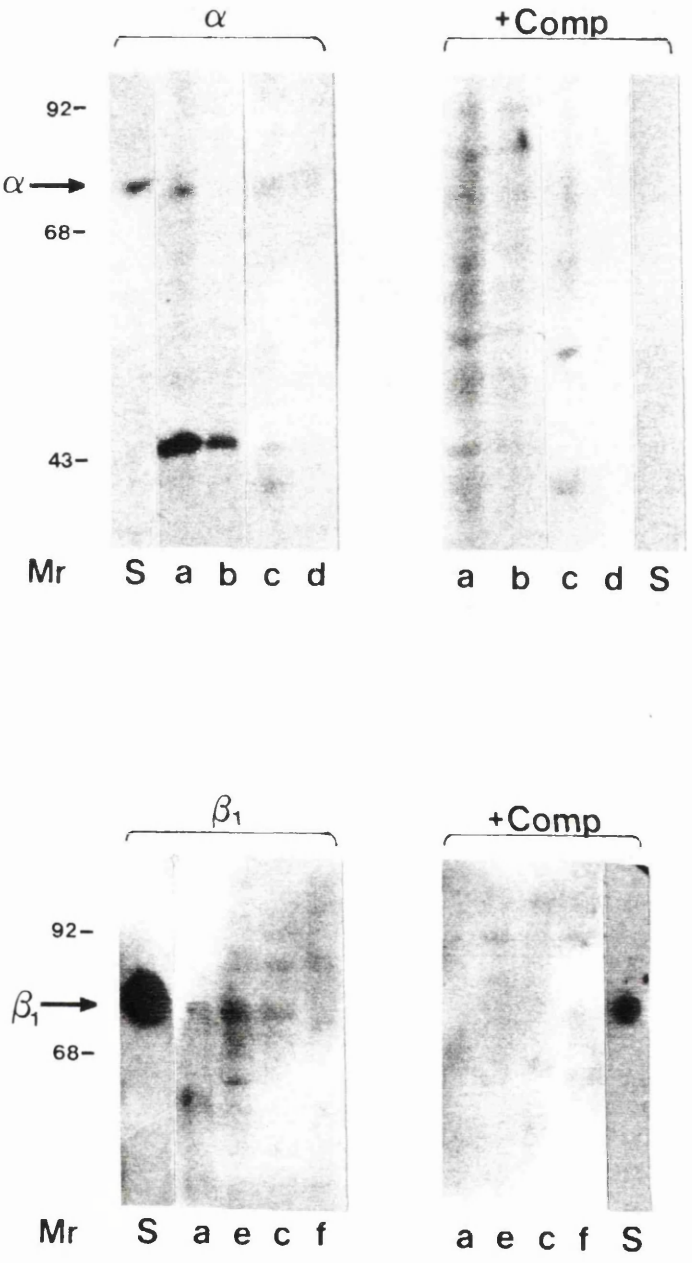


Fig 8p. Competion autoradiography of Daudi and HMNC α and β1 PKC. Samples of PKC standards (= S) and selected fractions from wild-type Daudi and HMNC hydroxyapatite chromatography (shown in Figs 7i and 8g respectively) were subjected to SDS-PAGE/Western blotting. Blots were probed with antisera for PKC α or β1 and with antisera previously incubated with its corresponding competing peptide (+ Comp). Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (3 day and 7 day exposure for α and β1 respectively). Mr = Molecular weight markers, a = Daudi pre-column supernatent, b = HMNC pre-column supernatent, c = Daudi fraction 15, d = HMNC fraction 17, e = Daudi fraction 13, f = HMNC fraction 13.

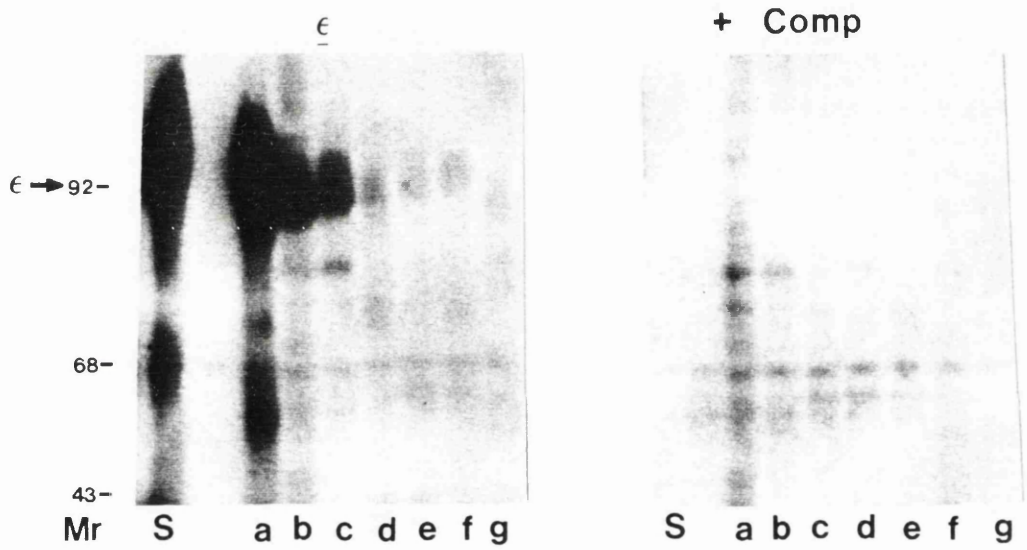


Fig 8q. Competion autoradiography of PKC ε.Samples of PKC standards (= S) and selected fractions from Rat brain,wild-type Daudi,HMNC and mouse macrophage hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting. Blots were probed with antisera for PKC ε and with antisera previously incubated with its corresponding competing peptide (+ Comp).Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (14 day exposure).Mr = Molecular weight markers, a = rat brain fraction 15 (see Fig 4e), b,c = Daudi pre-column supernatent and fraction 15 (see Fig 8g), d,e f= HMNC pre-column supernatent,fraction 15 and fraction 17 (see Fig 7i), g = Mouse macrophage pre-column supernatent (see Fig 8l).

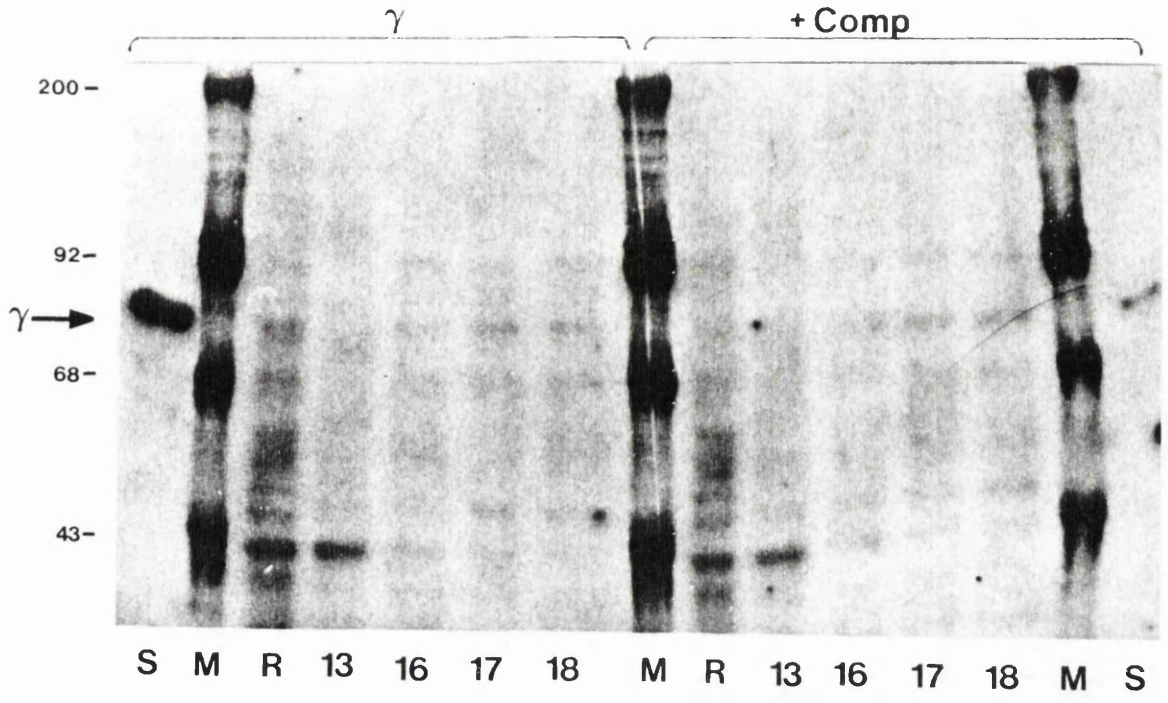


Fig 8r. Competion autoradiography of PKC γ.Samples of PKC standards (= S),fraction 13 from Rat brain (= R) and fractions 13,16,17 and 18 from starch-elicited mouse macrophage hydroxyapatite chromatography (see Fig 4e and 8l respectively) were subjected to SDS-PAGE/Western blotting. Blots were probed with antisera for PKC γ and with antisera previously incubated with its corresponding competing peptide (+ Comp).Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (23 day exposure).M = Molecular weight markers.

hydroxyapatite columns, a table showing the presence of these peaks according to cell type was compiled *(See Table 8A)*.

The first peak (a small peak eluting "pre-β") appears most strongly in neutrophil preparations with an indication of a slight peak evident in β-lymphocytes. The second and third peaks, found to correspond to the peaks of PKC-β_1, (and presumably β_2) and PKC-α respectively in immunological analysis of HMNC preparations *(See Chapter 7)*, were found to be major peaks in all subpopulations tested, eluting at between 100-150mM and 150-190mM phosphate. The forth ("post α") peak was apparent in Neutrophils, β-lymphocytes and monocytes eluting at 200-240mM phosphate. The late eluting peak of activity characteristic of RX-kinase from HMNC preparations was only seen in the Neutrophil preparation at low levels. It should be noted, however that the values of stimulated kinase activity (p moles/min/assay) found in difference profiles of many of those cell-enriched preparations was very low and the presence of other low-levels of stimulatable activity cannot be ruled out. Low activities may be a factor of insufficient numbers of cell types acquired during separation or cell damage sustained by 'snap freezing' pelleted cells in liquid nitrogen for transport. Additionally large quantities of degradative enzymes in certain cell types (e.g. Neutrophils see *273*) may reduce the amounts of stimulatable kinase activity during fractionalization where the only inhibitor of protease action present was leupeptin.

Analysis of the PKC isozymes expressed in a variety of blood

Table 8A.
Peaks of phorbol ester-stimulated kinase activity in human mononuclear blood cells and platelets.

Peak number and name.*	Phosphate buffer range.(mM)	Cell Type.					
		Neutro-phils	β-cells	T-cells	mono-cytes	** All	plate-lets
1 pre β	45-100	+	+/-	-	-	+	-
2 β	100-150	+	++	++	++	++ ++	+++
3 α	150-190	+	+++	++	+	++ ++	+++
4 post α	200-240	+	+	-	+	++	-
5 Rx-kinase	380-450	+	-	-	-	+	-

Table 8B.
Peaks of phorbol ester-stimulated kinase activity in a variety of cell types.

Peak number and name.*	Phosphate buffer range.(mM)	Daudi Cells				Rj	p 388D (mm)	St (mm)
		WT	α-if	TPA	α-ifR			
1 pre β	45-100	++	++	-	-	-	-	-
2 β	100-150	++	++	-	++	-	-	++
3 α	150-190	+++	+++	-	+	-	-	+
4 post α	200-240	+	+	-	++	-	-	+
5 Rx-kinase	380-450	-	-	-	-	-	-	++

Rj = Raji cell line. p388D (mm) = Mouse macrophage line.
St mm = Starch-elicited peritoneal mouse macrophages.
Daudi Cells; WT = wild type ,α-if = α-interferon treated,
TPA = TPA treated,α-ifR = α-interferon resistent strain.

*Nomenclature used here is based upon the hydroxyapatite elution order and the immunological data (see chap 7 & 8).

**All = Whole HMNC preparations.

(+)indicates presence of peak to varying degrees.
(-)= no peak observed.

cells has previously been conducted. mRNA transcripts of both β-PKC (type II) and α-PKC (type III) isozymes have been found in the marginal zone of rat spleen (containing mostly β-lymphocytes) *(264)*, and immunologial analysis of pre-β, pre-T lymphocyte leukemic cell line (KM 3 cells) and fresh human T-lymphocytes have similarly shown the presence of both these isozymes *(151,278,279)* supporting the data presented here. Neutrophils have variously been reported to contain only PKC-α(Type III), and both PKC-α and β in human cells *(176,273)* or PKC-β and ζ in bovine cells *(274)*. An uncharacterised PKC-type activity was also found eluting after α-PKC in human neutrophils which may correspond the 4th (post α) peak reported here *(273)*. Platelets have been found to possess 2 PKC-type enzymes eluting at similar positions to PKC-α and β isozymes (termed a and b) in agreement with present results *(277)*.

Wild type Daudi cells were found to elute 4 peaks of TPA-stimulatable kinase activity at similar locations to the first four peaks described above. Daudi, a human β-lymphoblastoid cell line was found to contain β and α-PKC immunological bands at the region of peaks 2 and 3 respectively, lending support to the identification of these peaks in human β-lymphocytes. Only slight differences were observed in the TPA stimulation profile when (WT) Daudi were compared with similar amounts of α-Interferon treated Daudi cells (αIF-WT). TPA and α-Interferon treatment both cause cell growth inhibition in these cells, associated with a decrease in the phosphorylation and mRNA levels of a protein termed $p34^{CDC2H5}$ *(351)*. The regulation of this protein and its involvement in growth has been suggested to be

mediated in part by protein kinase C, since α-Interferon stimulates phosphatidylinositol turnover *(67)*. The absence of the first peak from α-Interferon resistant Daudi cell stimulation profiles and the reduced kinase activity stimulated by TPA over the remaining peaks suggests that resistance to α-Interferon may involve elements of the PKC-type kinases (e.g. changes in kinase activity or subcellular location). Treatment of (WT) Daudi cells with TPA (40 hours, 100nM) was found to abrogate all TPA-stimulated kinase activity in elution profiles and cause loss of immunoreactive material (PKC-α, β_1 and ϵ). The continued presence of a 45Kd protein detected by PKC-α antisera appears to be a consequence of a shared epitope with a 47Kd Protein rather than a PKM fragment (Prof A. Segall, UCL, unpublished results). This band seen in all Daudi samples demonstrates that the disappearance of full-length PKC bands in TPA-WT fractions was not caused by procedural error. The down regulation of PKC isozymes by phorbol ester treatment has been observed in many other cell types *(163,564)*.

The absence of Phorbol-ester stimulatable kinase activity in Raji and mouse macrophage P388D cell lines is unclear. However an earlier study on TPA- and diacylglycerol-stimulatable activity on proliferating Raji, reported all such activity to be represented in the particulate fraction not the supernatant *(565)*. Similarly mutant cells which have no PKC-α, β or γ immunoreactivity, and are entirely deficient in TPA-stimulated phosphotransferase activity, have been identified *(70)*.

The kinase stimulation of Starch-elicited peritoneal mouse macrophages (StMM) elution fractions with TPA was similar but of greater magnitude than that observed with Human monocyte cells. The StMM, fractionated immediately after pelleting, may have a greater concentration of activity or the conditions (e.g. the freezing of the human cells or the use of starch solution) may be responsible for the variation. RX-kinase activity was found in large amounts in StMM (peak 5) but was not observed in the preparation of human cells used. Rapid changes in quantities of PKC isozymes have been observed with developmental changes in some cell types (e.g. during the process of differentiation of HL-60 (human promyelocytic leukemic) cells into neutrophil-like phenotype *(270)*).

Immunological analysis of the three central peaks in StMM revealed that the first contained the peak of β_1-PKC and the second contained the peak of α-PKC. These peaks eluted at the phosphate concentrations corresponding to peaks 2 and 3 in the other human cells, while the third peak was unrecognised by the PKC antisera used, and eluted in the same region as the post-α peak 4. The peaks of StMM, Daudi and other cells are tabulated in *Table 8B*.

The identity of peaks 1 and 4 (pre-β and post-α) PKC-type peaks has not been established in these studies. The presence of PKC-γ has been found to be limited to brain and spinal cord tissue *(252)* and indeed no immuno-reactivity which was competable with the PKC-epitope was detected in these studies. In HL-60 cells an unidentified TPA-stimulated protein kinase activity has been reported

(eluting between β and α peaks) *(271)* and a possible TPA-responsive peak can also be observed eluting 'post-α' in the same cells *(269)*. However many studies on cells and tissue, by using series of columns consecutively and using only diacylglycerol as the stimulating cofactor in PKC assays (which has a poor effect on peaks 1 and 4, See Chapter 7) may fail to detect kinase activities which can be observed with TPA.

These studies in various cell types while limited, suggest that peak 5 (RX-Kinase) activity is concentrated in neutrophil- and macrophage-type cells. The initial discovery of RX-Kinase activity in HMNC preparations, in the light of present results, represents contamination of such preparations during isolation from blood by such cells. This could be used to explain why the quantity of RX-Kinase activity in HMNC elution profiles does not appear to be linear with the number of human mononuclear cells used in fractionation. The quantity of 'contaminating' RX-containing cell types, and possibly the concentration of RX kinase activity within these cells, may be the subject of variation. The pursuit of RX-Kinase activity was continued using starch-elicted mouse macrophages as the source material (See following chapter).

CHAPTER 9

STUDIES ON RX-KINASE ACTIVITY FROM MOUSE

The strong presence of RX-Kinase activity discovered in starch-elicited peritoneal mouse macrophages was persued further to isolate and characterise this activity. Other mouse tissues were also screened for RX-Kinase activity, as defined by a calcium-inhibited Resiniferatoxin-stimulated kinase activity, by assaying crudely separated supernatants using Histone III-S as phosphate acceptor in the PS/micellar assay. The implications of these studies in biological and biochemical terms are discussed in the concluding chapter (Chapter 10).

Materials and Methods

Starch-elicited mouse macrophages (StMM) were isolated from the peritoneal cavities of 15-90 mice using the techniques detailed in Chapter 8. Mouse lungs, dissected into thin strips, were placed in sealed vessels with 10mls of Oxygen-saturated culture medium (buffer 1) and rocked vigorously for 2 hours at 37^{o}C prior to fractionation. Other mouse tissues were dissected and used immediately.

Fractionation of Mouse Tissues

All cells/tissues were pelleted and resuspended in 2-10 mls of ice-cold homogenization buffer (buffer 3). Suspensions were homogenized (See Methods Chapter 2) and centrifuged at 15000 x g for 30 minutes to obtain supernatants. StMM supernatants were subjected

to hydroxyapatite FPLC modified for RX-Kinase elution. After washing with 20mM phosphate buffer (buffer 4) to elute breakthrough proteins, a rapid gradient up to 350mM phosphate was initiated (20 mins at 1ml/min flow rate) and maintained until eluted protein absorbance at 280nm had dropped to base levels (~ 20 minutes). The kinase activities of the peaks I-IV (See Chapter 8) were eluted by this method. RX-kinase activity was eluted by a subsequent increase of phosphate concentration up to 500mM in a single step, and 10 fractions of 1ml were collected and stored in buffer 8 for assay. Supernatants (2mls) from other mouse tissues were fractionated using a simpler procedure. These were mixed with 1ml of hydroxyapatite slurry in 20mM phosphate (buffer 4) and left to settle on ice for 10 minutes. The top layer was then aspirated off and replaced with 2 x 10ml of 20mM phosphate buffer, stirred and allowed to settle as before, and the top layer discarded. This was followed by similar treatment with 2 x 10 ml of 250mM phosphate buffer (a mixture of buffers 4 and 5) and the top layer was discarded as before. Finally 0.5 ml of 500 mM phosphate (buffer 5) was stirred in, allowed to settle, and aspirated off as "fraction 1" for assay. Another 0.5ml was added and similarly collected and designated as "fraction 2". These two fractions were stored in buffer 9 until assayed for RX-kinase activity.

Kinase Assays

Kinase assays were conducted as before using the PS/micellar assay (See Chapter 4).

Immunological Analysis

15μl of selected fractions were tested for the presence of PKC -α,β_1, γ and ϵ isozymes using the method described in Chapter 5. Samples for analysis had been boiled in denaturing buffer (buffer 23) immediately following elution from hydroxyapatite.

Protein Staining

15μl of selected fractions were run on SDS-polyacrylamide gels as described (See Chapter 5) and silver stained at 37^{o}C, using the procedure below. The gel was fixed in buffer 35 for 30 minutes followed by buffer 36 for 2 x 15 minutes. The gel was then rocked for 10 minutes in oxidiser (buffer 33), followed by silver stain (buffer 32) for 30 minutes. The stain was developed by vigorous rocking in buffer 34 until bands were visualised and the gel was then rinsed in 5% Acetic acid.

Gel Exclusion Chromatography

A Superose 12 prepacked column (pharmacia) was calibrated with protein standards of known molecular mass (cytochrome C:Mr 12.4Kd, Chymotrypsinogen A:Mr 25Kd, hen-egg Albumin: Mr 45Kd and Aldolase: Mr 158Kd, Boehringer-Mannheim) detected by absorbance of 280 nm. 100μl of RX-kinase peak fraction was then run through at a flow rate of 0.4ml minute and eluting fractions (0.4ml) were assayed for RX kinase activity.

Results

Mouse cells taken from the peritoneal cavities of 15, 30, 60 and 90 mice, 3 days after an interperitoneal injection of starch solution, were subjected to modified hydroxyapatite FPLC to elute RX-kinase activity. The RX-kinase activity eluted between 4 and 8 minutes after the gradient was stepped up to 500mM phosphate. This activity was found to possess a linear relationship, in terms of both the total activity spread over several fractions and the activity in the peak fraction, with the numbers of mouse employed in the investigation *(See Fig 9a)*. The peak fraction eluted from a preparation of 90 mice was partitioned and frozen in several tubes to enable several experiments to be conducted.

Effect of Other Phorbol Esters

An RX-kinase peak fraction was tested for activity in the presence of a range of phorbol esters. *Fig 9b* shows the activity of 7.5μl of RX-kinase enzyme with PS alone, the phorbol esters RX, TPA, THY TOX, SAP A, DOPP and Doppa (present at 100 ng/ml in PS micelles) and Capsaicin (present at 10μg /ml in PS micelles), in the presence and absence of calcium. Only Resiniferatoxin in the absence of added calcium was found to stimulate the kinase activity.

Immunological Analysis

Active RX-Kinase samples boiled immediately in denaturing buffer following elution from hydroxyapatite, were immunoblotted using antisera specific for PKC-α, β_1 , γ and ϵ . No immunoreactivity was observed in these samples *(See Fig 9c)*. In another experiment a

Rx-Kinase activity in mouse macrophage preparations.

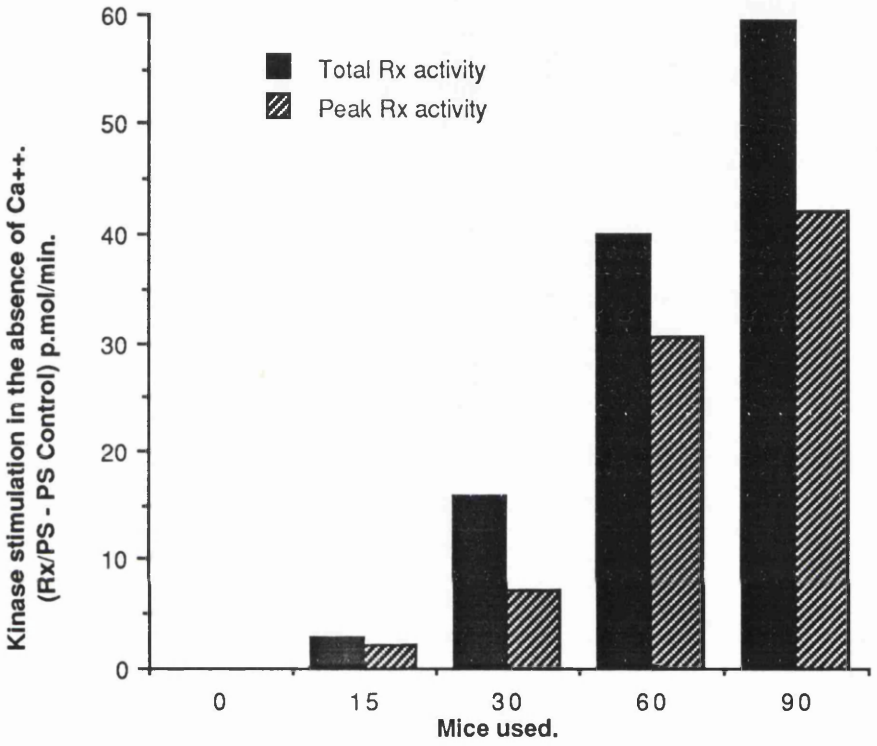


Fig 9a. Resiniferatoxin (present at 100 ng/ml,corresponding to 0.003 mol % of Triton/PS micelles) was assessed for stimulation of histone kinase activity in late-eluting fractions from several preparations of starch-elicited,peritoneal mouse macrophages. Rx stimulated activity (activity of Rx/PS minus that of PS alone,in the absence of added calcium) is shown as a total(in assays of the range of fractions around the peak) and for the peak fraction itself.Histone was present at a final concentration of 1 mg/ml.

Activation of Rx-Kinase activity from mouse macrophages.

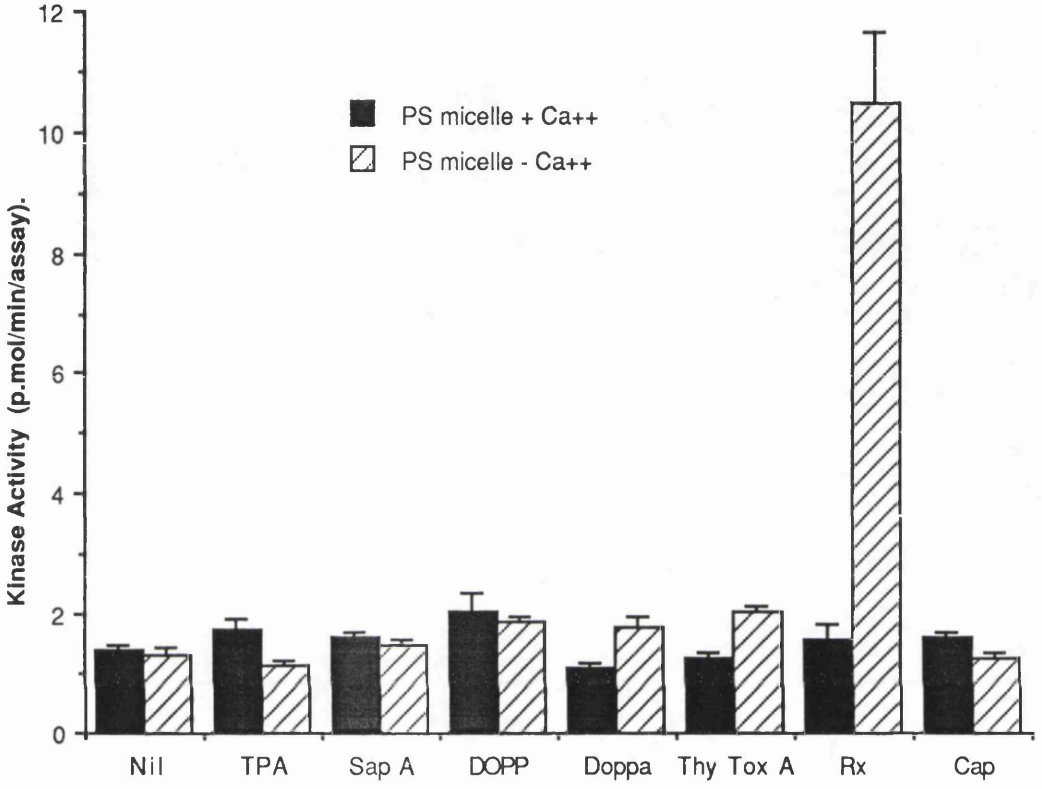


Fig 9b. A variety of phorbol Esters (present at 100 ng/ml, corresponding to 0.003 mol % of Triton/PS micelles),and capsaicin (present at 10 μg/ml final concentration) were assessed for their effect on the histone kinase activity of Rx-Kinase.The enzyme was prepared from the peritoneal starch-elicited macrophages of 90 mice,and the assays were conducted on the peak fraction (10 μl/assay) in the presence and absence of added calcium .Histone was present at a final concentration of 1 mg/ml.Results are shown as the mean values with the range from duplicate assays.

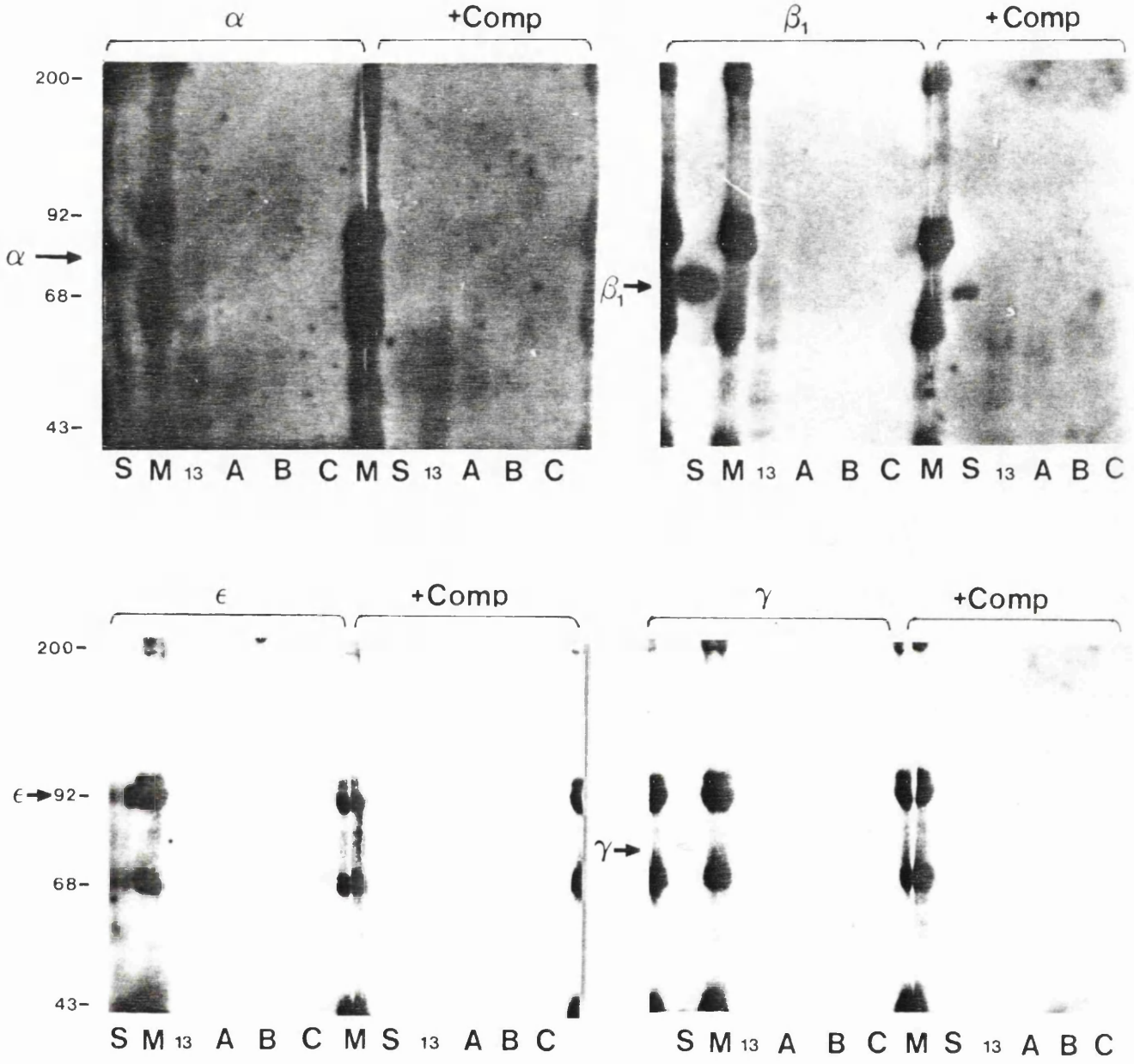


Fig 9c. Competition autoradiography of mouse Rx-kinase samples probed for PKC α,β1,γ and ε. Samples of Rx-kinase from mouse macrophage hydroxyapatite chromatography were subjected to SDS-PAGE/Western blotting and probed with antisera for each PKC isotype and antisera previosly incubated with its competing peptide (+ Comp). Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (3 day exposure). Lane S = PKC standards, Lane M = Molecular weight markers, 13 corresponds to fraction 13 and A, B and C correspond to Rx-kinase active fractions from 90 mice (see Fig 9a), with the peak activity in B.

sample of mixed PKC isozymes, of known kinase activity equivalent to that of the RX-kinase peak for 90 mice (calculated from the relative abilities to phosphorylate Histone III-S), was immunoblotted at various dilutions (x 1, x 1/10, x 1/100 and x 1/1000) with the PKC-specific antisera. Immunoreactive bands were strongly detectable in equivalently active samples and could still be clearly seen at 1/10 dilution *(See Fig 9d)* and 1/100 dilution upon longer exposure (data not shown.

Protein Staining

RX-kinase samples were run on SDS polyacrylamide gels and silver stained *(See Fig 9e)*. Several bands were visible corresponding to molecular weights of 40, 70, 72, 83 and 90 kilodaltons. No bands were visible staining around the 77 Kd region where PKC standards were observed.

Superose Gel Exclusion Chromatography

RX-kinase activity passed through a superose gel exclusion column was found to elute between the Aldolase (Mr 158Kd) and hen-egg Albumin (Mr 45Kd) standards *(See Fig 9f)*. The activity was observed closer to the Aldolase standards suggesting the activity was connected to the 2 protein bands observed in the 80-90Kd region.

RX-kinase Stability

The enzyme activity, even stored at -70^{o}C in protective buffers was found to be unstable, with a loss of 30% experienced in 5 days in the peak fraction from a preparation of 60 mice. Fractions with

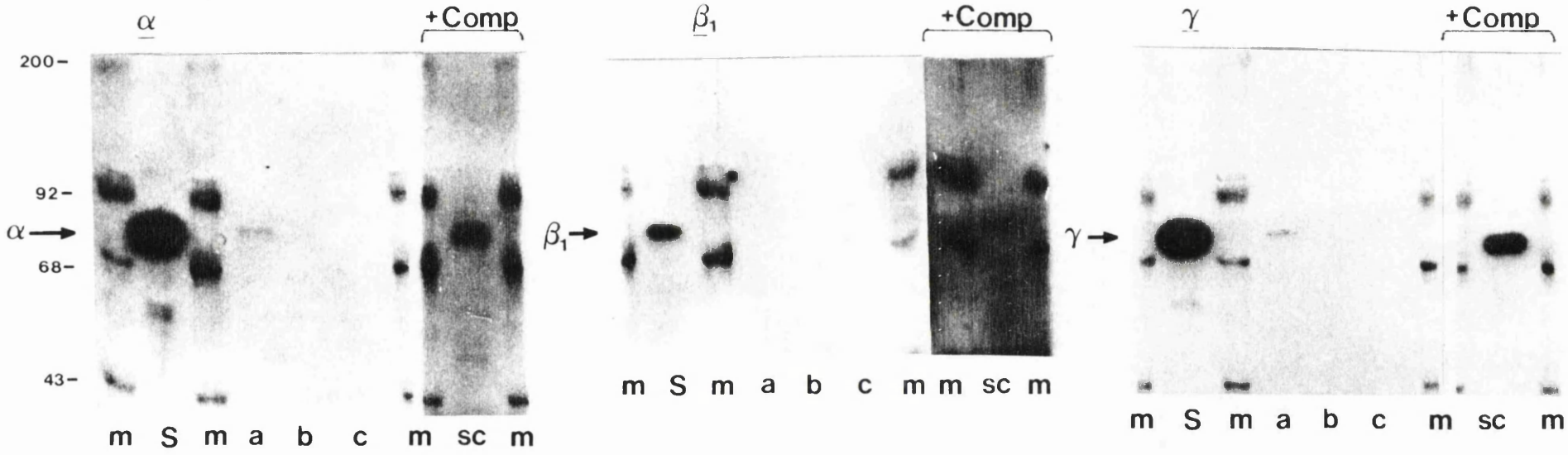


Fig 9d. Autoradiography of dilutions of purified PKC isotypes probed for PKC α,β1 and γ. Samples of each isozyme, with histone-kinase specific activity equivalent to the peak Rx-Kinase fraction from 90 mice, were subjected to SDS-PAGE/Western blotting. Blots were probed with antisera for each PKC isotype (= S) and with antisera previously incubated with its corresponding competing peptide (+ Comp = sc). Radiolabled 2nd antibody was then used to visualise the bands on Kodak X-omat film (Overnight exposure). Lane a,b and c represent 10, 100 and 1000-fold dilutions of (S) respectively, Lane m = Molecular weight markers.

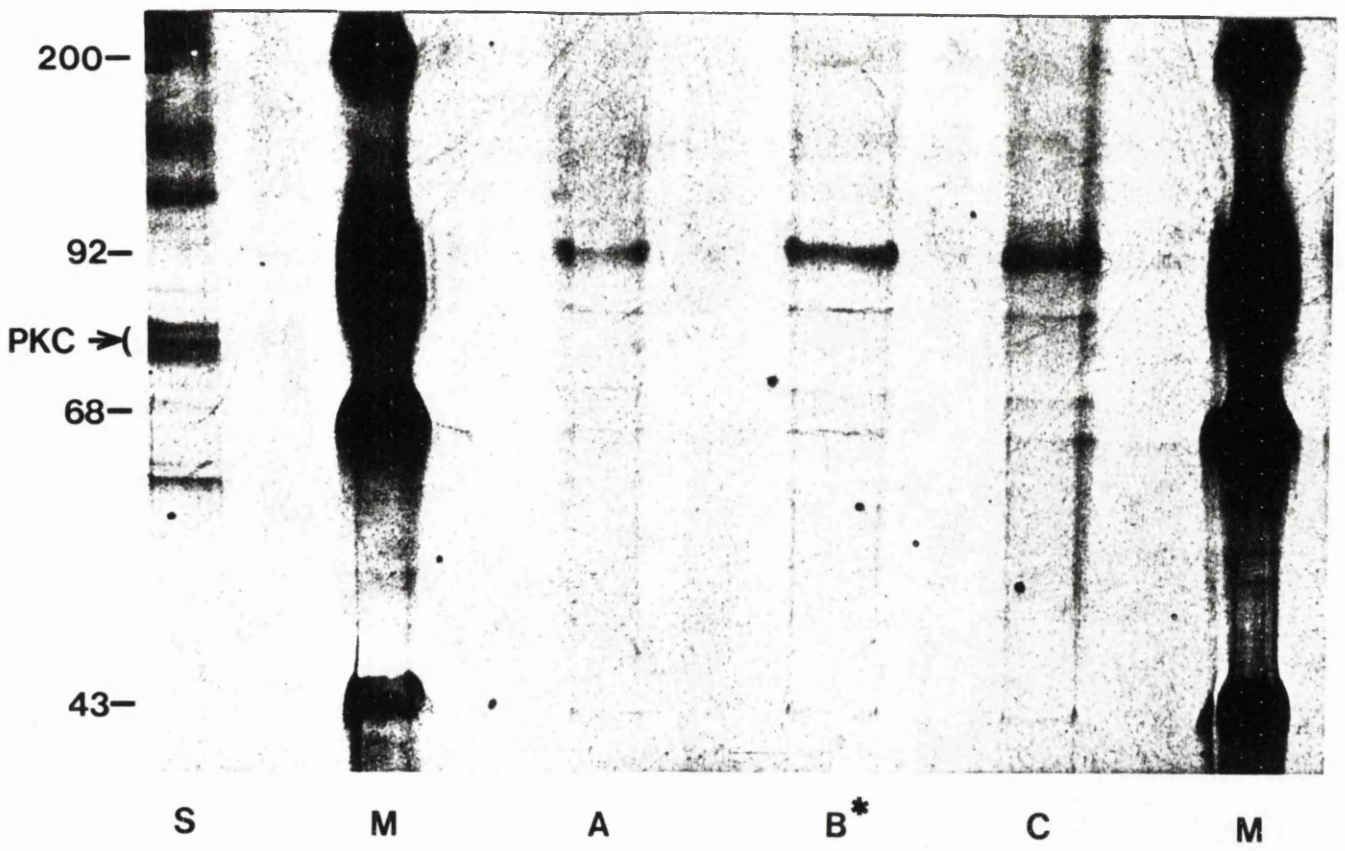


Fig 9e.Silver stain of Rx-kinase fractions.Samples of Rx-kinase from mouse macrophage hydroxyapatite chromatography were subjected to SDS-PAGE and silver stained.Lane S = PKC standards ,Lane M = Molecular weight markers ,A ,B and C correspond to the Rx-kinase active fractions from 90 mice (see Fig 9a),with * representing the peak activity fraction.

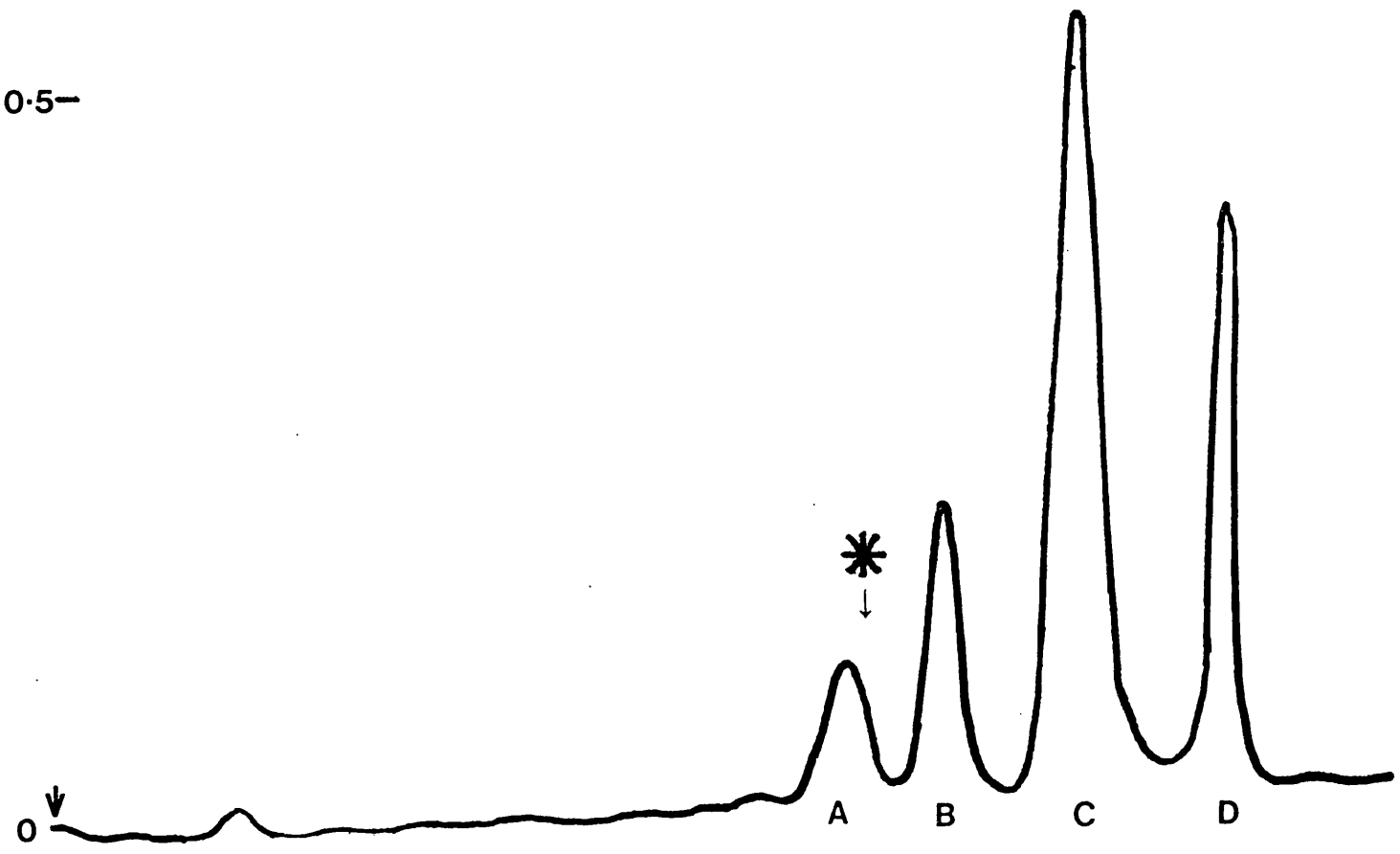


Fig 9f. Superose 12 Gel Exclusion Chromatography .The column was standardized using ; Aldolase (158 Kd = A), Hen-egg albumin (45 Kd = B), Chymotrypsinogen (25 Kd = C) and Cytochrome c (12.4 Kd = D). These markers were observed by detecting protein absorbance at 280 nm (0-1 unit range (—) with chart speed 0.4 cm/min).When 100 µl of mouse Rx-kinase activity was run through the column,kinase activity was found to elute between A and B (marked * above).(↓) marks the application of the sample.

larger amounts of activity appeared less prone to this loss, suggesting surface absorption is a factor in activity loss. Refreezing and thawing of active fractions was also found to cause loss of enzyme activity.

Mouse Tissues

Preliminary investigations were conducted in brain, spleen, liver, testes, thymus, lung, diaphram and intestine mouse supernatants. RX-kinase activity as defined by Resiniferatoxin stimulation of activity in the absence of calcium, was assayed in fractions obtained from a simple stepwise elution method with hydroxyapatite. Mouse lung cultured for 2 hours in oxygen-saturated medium, was found to contain a large amount of RX-kinase activity in such fractions *(Fig 9g)*. Uncultured mouse lung samples were observed to contain smaller amounts of RX-stimulatable activity (data not shown). No appreciable activity stimulatable by RX was found in the other mouse tissues tested.

Discussion

The presence of RX-kinase activity was consistently detected in fractionated preparations of peritoneal starch-elicited mouse macrophages, using histone III-S as a substrate in the PS/micellar assay. The amount of RX-kinase activity was found to have a linear correlation with quantity of mouse cells employed, with the majority of activity eluting at high phosphate levels in a single sharp peak under the conditions used. This linearity, not observed in HMNC preparations, reinforces the notion that a minor mononuclear cell

Rx-Kinase activity in mouse lung.

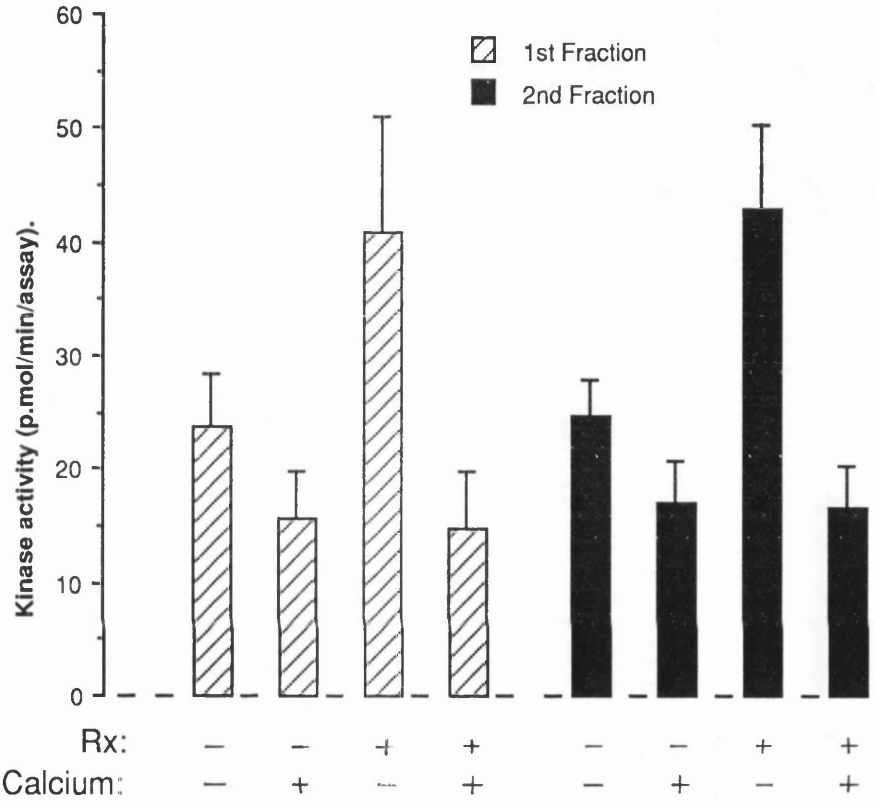


Fig 9g. Rx-Kinase activity observed in the 1st and 2nd fractions,eluted with 0.5ml of 500 mM phosphate from hydroxyapatite,in cultured mouse lung supernatents (4 lungs).Resiniferatoxin (present at final concentrations of 100 ng/ml representing 0.003 mol % of the Triton/PS micelles) and calcium (100 μM free final concentration) were present as indicated in the Triton/PS micellar assay.Histone was present at a final concentration of 1 mg/ml.Results are shown as the mean values with the standard deviation from six experiments with duplicate assays.

population contains RX-kinase activity and suggests macrophage-type cells are the likely source. Highly active fractions were assayed in the presence and absence of calcium with the phorbol esters; TPA, SAP A, DOPP, Doppa and THY TOX, but no significant activation was observed with these compounds at 100ng/ml (at which RX significantly stimulated activity in the absence of calcium). The biological actions of RX have been claimed to be that of an 'ultrapotent capsaicin analogue' (the inflammatory agent of Chili peppers *(312)*), whereupon a proportionally high dose (10μg/ml) of capsaicin was tested for effect on RX-kinase activity. Capsaicin failed to stimulate any RX-kinase activity suggesting that while these compounds may share some pathways in exertion of biological activity, they cannot be classed together in all respects.

Immunological analysis failed to identify the RX-kinase activity as containing PKC-α,β_1,γ or ϵ isozymes, known receptor proteins for other phorbol esters (See Chapter 7). By using dilutions of PKC preparations of known kinase activity in subsequent immunological tests it was established that PKC activity equivalent to 1/100th the RX-kinase activity was still detectable. Thus, immunological analysis combined with the unique calcium-dependent-inhibition of the RX-kinase activity and its elution characteristics, defines RX-kinase activity as being separate from any characterized PKC isozyme or PKM fragment.

The elution profile of a superose gel exclusion column calibrated with proteins of known molecular mass, suggested a likely molecular

mass of 80-100 Kd for RX-kinase protein(s), possibly corresponding to 2 bands silver-staining in this region on SDS-PAGE.

The characteristic instability of RX-kinase activity and the large number of cells required for a large scale preparation from mouse macrophages, prevented a wider investigation into other aspects of this activity. The dependence of activity on cofactors such as calcium, phospholipid, RX and Diacylglycerol and on substrate specificity by RX-Kinase requires investigation. Detection of activity also relied upon pure reagents in buffer preparation since the inclusion of even small quantities of contaminating calcium (e.g. as found in some preparations of phospholipids, *35*) could substantially influence the observed RX activity in assay while having little effect on Rat brain PKC controls.

The detection of RX-kinase activity in simply fractionated lung supernatents (a tissue itself rich in macrophage cells) reinforces the possible link between the kinase activity and accessory cells. The greater quantity of RX-kinase activity found in cultured lung relative to normal lung and the high activity in starch-elicited mouse macrophages (un-elicited (resident) mouse macrophages give a much lower yield of cells and were not tested), may be of significance in the biological action of RX-kinase. Higher yields of RX-kinase activity apparent under stimulated conditions may be due to post-translational changes in a pro-RX-kinase (e.g. phosphorylation, proteolysis), changes in genetic or mRNA processing, or compartmentalization of the enzyme activity. The greater

availability of lung as a source of RX-kinase activity may be helpful in providing the quantities of enzyme required for further purification and characterization on a routine basis.

CHAPTER 10

CONCLUDING REMARKS

The numbers of protein kinases discovered in biological systems has dramatically increased throughout the last ten years *(See review 566)*. The functions of only a handful are well characterized, while others have been identified according to sequences deduced from cDNA clones and can be placed into 'families' *(250,567)*. Many differentiated and specialized cells contribute to the list with their own protein kinases thought to be linked to specialised functions (e.g. Tyrosine protein kinase p.56 ^{LCK} in Lymphocytes *(211)*).

From the 'classical' family of PKC isozymes initially isolated (α, β_1, β_2 and γ isozymes), investigators found less closely related PKC 'isotypes' ($\delta, \epsilon, \zeta, \eta$ isotypes and a truncated PKC-ϵ cDNA sequence) with a greater diversity in characteristics *(254,255)*. Many protein kinases have been isolated which demonstrate characteristics of this growing PKC family; e.g. protein kinase "b" from HL-60 cells (a Ca^{2+} and Phospholipid dependent activity, *271*), "nPKC" from rabbit brain (a calcium independent activity which appears to correspond to PKC-ϵ in the light of subsequent investigation *see 255,568,569*), an apparent second 'type III' PKC activity in rat brain *(570)*, and PKC-η predominantly found in lung and skin *(257,258)*. Other protein kinases which show only passing resemblance to PKC enzymes, e.g. Protease-activated kinase II from reticulocytes ("PAKII" which can be also activated by phospholipids

and diolein, *571*) and a phosphatidylglycerol-stimulated kinase in mouse tissue (Protein kinase "P", *572*).

In these present studies several phorbol ester stimulatable kinase activities, which cannot be immunologically identified with antisera available, have been observed using a variety of developmentally discrete tissue sources (e.g. Rat brain was found to contain several phorbol-ester-stimulatable activities not apparent in human mononuclear cell types and vice versa).

While the unidentified peaks observed in these present studies may converge with the activities in other reports, by reason of their calcium and phorbol ester dependent stimulation, the discovery of a phorbol ester-stimulatable yet calcium-inhibited activity is clearly distinct.

Reports of calcium-inhibited kinase activities have been reported on occasion, e.g. An activity in rat brain DEAE-cellulose chromatography contaminating PKC fractions was found to have a greater activity with EGTA than calcium in the absence of phospholipids *(10)*. This activity was greatest however with the inclusion of phosphatidylserine and calcium, suggesting it was a PKM-type fragment. In other studies using hydroxyapatite fractionated Rabbit and Rat brain extracts, an apparent Ca^{2+}-inhibited activity coeluting with the PKC activity profile was sometimes observed *(570,573)*. These experiments however used a synthetic peptide as a substrate similar to peptide substrates for PKC-ϵ , and the results

would be consistent with the synthetic peptide being used as a substrate for the PKC-ϵ (or δ) type isozymes in the assay system employed. We have found PKC-δ and ϵ to elute within the PKC-α,β_1 and γ peaks *(See Chapter 5)*. These same procedures in pig and human neutrophils found Ca^{2+}-stimulatable kinase levels were low, and the apparent Ca^{2+}-inhibition more pronounced *(573)*. However the presence of endogenous serine proteases activated by calcium in neutrophils *(273,574)* could achieve this effect by coelution.

Preliminary work has been conducted in our laboratories addressing the possible functions of RX-kinase activity from starch elicited peritoneal mouse macrophages (StMM). We found that RX could weakly stimulate an oxidative burst in StMM but not in resident (untreated) peritoneal mouse macrophages *(575)*. Furthermore this effect became equivalent to that of TPA if the StMM were subsequently treated with opsonized zymosan granules (which stimulates the phargocytic action in these cells). Peak fractions of RX-kinase activity were added to a cell-free assay of NADPH oxidase activity using the oxidase system extracted from StMM cells in further studies. It was found to stimulate oxygen radical (superoxide) production with a potency equivalent to mixed PKC stimulated with TPA (by comparison of specific activity of histone III-S phosphorylation). The effect of RX-Kinase was dependent on the dose of RX and was abolished by 1mM added calcium exactly in the manner of histone phosphorylation assays. This action of RX-kinase on the NADPH oxidase system has since been found to be independent of phosphatidylserine (P. Sharma unpublished results) suggesting a

greater divergence of RX kinase from the known PKC characteristics. The effect of other phospholipids remains to be addressed.

In this context the involvement of non-PKC pathways in activating the respiratory burst has been reported in addition to the PKC pathway in human neutrophils *(74,384)*. Another study of psoriatic epidermis (an inflammatory condition associated with leukocyte invasion and epidermal hyperplasia) detected a novel histone-stimulated kinase activity present in affected tissue at a significantly higher level than in normal epidermal tissue *(576)*. This activity was associated with proteins eluting at ~100Kd. Whether the RX-activity described here has involvement in the physiological processes remains conjectural.

The poor potency with which RX was found to stimulate protein kinase C purified isozymes <u>in vitro</u> in comparison to other phorbol esters, is puzzling in view of its greater potency in the inflammatory response compared to these latter compounds. Attempts to explain this anomaly in terms of an ultrapotent capsaicin analogue, effecting inflammation through specialised nerve fibres, have been eroded by other biological effects evidenced with RX in cultured cell systems *(315,575)*. The criteria for alternative signalling pathways to PKC in the com plex pharmacology of resiniferatoxin may be satisfied by a protein kinase activity potently stimulatable by RX at concentrations at which other phorbol esters have no significant effect.

The novel RX kinase activity described here establishes the existence of another class of phorbol ester receptor in cellular systems. As a calcium-inhibited activity this has major implications for a comprehensive physiological role of cellular free calcium in homeostatic and signalling processes.

References

(1) Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. *J. Biol. Chem.* **252** 7603-7609.

(2) Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. *J. Biol. Chem.* **252** 7610-7616.

(3) Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.* **254** 3692-3695.

(4) Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979) Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated phospholipid-dependent protein kinase system. *Biochem. Biophys. Res. Commun.* **91** 1218-1224.

(5) Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) Calcium-activated, phospholipid-dependent protein kinase from rat brain. *J. Biol. Chem.* **257** 13341-13347.

(6) Kikkawa, U., Minakuchi, R., Takai, Y. and Nishizuka, Y. (1983) Calcium-activated, phospholipid dependent protein kinase (protein kinase C) from rat brain. *Methods in Enzymology.* **99** 288-298.

(7) Kikkawa, U., Go, M., Koumoto, J., Nishizuka, Y. (1986) Rapid purification of protein kinase C by high performance liquid chromatography. *Biochem. Biophys. Res. Commun.* **135** 636-643.

(8) Jeng, A.Y., Sharkey, N.A. and Blumberg, P.M. (1986) Purification of stable protein kinase C from mouse brain cytosol by specific ligand elution using fast protein liquid chromatography. *Cancer Res.* **46** 1966-1971.

(9) Uchida, T and Filburn, C.R. (1984) Affinity chromatography of protein kinase C - phorbol ester receptor on polyacrylamide-immobilised phosphatidylserine. *J. Biol. Chem.* **259** 12311-12314.

(10) Le Peuch, C.J., Ballester, R. and Rosen, O.M. (1983) Purified rat brain calcium-and phospholipid-dependent protein kinase phosphorylates ribosomal protein S6. *Proc. Natl. Acad. Sci. USA.* **80** 6858-6862.

(11) Horn, F., Gschwendt, M and Marks, F. (1985) Partial purification and characterization of the calcium-dependent and phospholipid-dependent protein kinase C from chick oviduct. *Eur. J. Biochem.* **148** 533-538.

(12) Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M. and 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. *Proc. Natl. Acad. Sci. USA.* **77** 7039-7043.

(13) Minakuchi, R., Takai, Y., Yu, B. and Nishizuka, Y. (1981) Widespread occurrence of calcium-activated, phospholipid-dependent protein kinase in mammalian tissues. *J. Biochem.* **89** 1651-1654.

(14) Shoyab, M., Warren, T.C. and Todaro, G.J. (1981) Tissue and species distribution and developmental variation of specific receptors for biologically active phorbol and ingenol esters. *Carcinogenesis* **2** 1273-1276.

(15) Ashendel, C.L., Staller, J.M. and Boutwell, R.K. (1983) Identification of a calcium- and phospholipid-dependent phorbol ester binding activity in the soluble fraction of mouse tissue. *Biochem. Biophys. Res. Commun.* **111** 340-345.

(16) Siebert, P.D. and Fukuda, M. (1985) Regulation of glycophorin gene expression by a tumour-promoting phorbol ester in human leukemic K562 cells. *J. Biol. Chem.* **260** 640-645.

(17) Schafer,A.,Bygrave,F.,Matzenauer,S.and Marme,D. (1985)Identification of a calcium- and phospholipid-dependent protein kinase in plant tissue.*FEBS Letts.* **187** 25-28.

(18) Blowers, D.P. and Trewavas, A.J. (1988) Phosphatidylinositol kinase activity of a plasma-membrane associated calcium-activated protein kinase from pea. *FEBS Letts* **238** 87-89.

(19) Backer, J.M., Arcoleo, J.P. and Weinstein, I.B. (1986) Protein phosphorylation in isolated mitochondria and the effects of protein kinase C. *FEBS Letts.* **200** 161-164.

(20) Misra, U.K. and Sahyoun, N. (1987) Protein kinase C binding to isolated nuclei and its activation by a Ca^{2+}/phospholipid-independent mechanism. *Biochem. Biophys. Res. Commun.* **145** 760-768.

(21) Capitani, S., Girard, P.R., Mazzei, G.J., Kuo, J.F., Berezney, R. and Manzoli, F.A. (1987) Immunochemical characterisation of protein kinase C in rat liver nuclei and subnuclear fractions. *Biochem. Biophys. Res. Commun.* **142** 367-375.

(22) Girard, P.R., Mazzei, G.J. and Kuo, J.F. (1986) Immunological quantitation of phospholipid/Ca^{2+}-dependent protein kinase and its fragments. *J. Biol. Chem.* **261** 370-375.

(23) Mori, T., Takai, Y., Minakuchi, R., Yu, B. and Nishizuka, Y. (1980) Inhibitory action of chlorpromazine, dibucaine, and other phospholipid-interacting drugs on calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* **255** 8378-8380.

(24) Kishimoto, A., Kajikawa, N., Shiota, M and Nishizuka, Y. (1983) Proteolytic activation of calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease. *J. Biol. Chem.* **258** 1156-1164.

(25) Lee, M-H. and Bell, R.M. (1986) The lipid binding regulatory domain of protein kinase C. *J. Biol. Chem.* **261** 14867-14870.

(26) Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B. and Horecker, B.L. (1986) The involvement of calpain in the activation of protein kinase C in neutrophils stimulated by phorbol myristic acid. *J. Biol. Chem.* **261** 4101-4105.

(27) Huang, K-P. and Huang, F.L. (1986) Immunochemical characterization of rat brain protein kinase C. *J. Biol. Chem.* **261** 14781-14787.

(28) Fournier, A. and Murray, A.W. (1987) Application of phorbol ester to mouse skin causes a rapid and sustained loss of protein kinase C. *Nature* **330** 767-769.

(29) Cambier, J.C., Newell, M.K., Justement, L.B., McGuire, J.C., Leach, K.L. and Chen, Z.Z. (1987) Ia binding ligands and cAMP stimulate nuclear translocation of PKC in b lymphocytes. *Nature* **327** 629-632.

(30) Hokin, M.R. and Hokin, L.E. (1953) Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreas slices. *J. Biol. Chem.* **203** 967-977.

(31) Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* **255** 2273-2276.

(32) Takai, Y., Kishimoto, A., Kawahara, Y., Minakuchi, R., Sano, K., Kikkawa, U., Mori, T., Yu. B., Kaibuchi, K. and Nishizuka, Y. (1981) Calcium and phosphatidylinositol turnover as signalling for transmembrane control of protein phosphorylation. *Adv. Cyc. Nuc.Res.* **14** 301-313.

(33) Berridge, M.J. (1983) Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinostides instead of phosphatidylinositol. *Biochem. J.* **212** 849-858.

(34) Majerus, P.W., Connolly, T.M., Bansal, V.S., Inhorn, R.C., Ross, T.S. and Lips, D.L. (1988) Inositol phosphates: synthesis and degradation. *J. Biol. Chem.* **263** 3051-3054.

(35) Hirasawa, K. and Nishizuka, Y. (1985) Phosphatidylinositol turnover in receptor mechanism and signal transduction. *Ann. Rev. Pharmacol. Toxicol.* **25** 147-170.

(36) Michell, R.H. (1982) Inositol lipid metabolism in dividing and differentiating cells. *Cell Calcium* **3** 429-400.

(37) Michell, R.H. (1975) Inositol phospholipids and cell surface receptor function. *Biochem. Biophys. Res. Commun.* **415** 81-147.

(38) Snyder, S.H., Ferris, C.D. and Theibert, A.B. (1990) Second messenger receptors in the phosphoinositide cycle. In Biology of Cellular Transducing Signals. (ed. J. Y. Vanderhoek). Plenum Press. New York. pp49-59.

(39) Berridge, M.J. (1984) Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* **220** 345-360.

(40) Bleaven, M.A., Moore, J.P., Smith, G.A., Hesketh, T.R. and Metcalfe, J.C. (1984) The calcium signal and phosphatidylinositol breakdown in 2H3 cells. *J. Biol. Chem.* **259** 7137-7142.

(41) Inositol lipids and transmembrane signalling.(1988) In Philos. Trans. R. Soc. London. B. (Eds. M. J. Berridge and R.H. Michell). Royal Society, London, UK. **320** 1199.

(42) Anderson, W.B., Estival, A., Tapiovaara, H. and Gopalakrishna, R. (1985) Altered subcellular distribution of protein kinase C (a phorbol ester receptor). Possible role in tumour promotion and the regulation of cell growth: relationship to changes in adenylate cyclase activity. *Adv. Cyc. Nuc. Res.* **19** 287-306.

(43) Zavoico, G.B., Halenda, S.P., Sha'afi, R.I. and Feinstein, M.B. (1985) Phorbol myristate acetate inhibits thrombin-stimulated Ca^{2+} mobilization and phosphatidylinositol 4,5 biphosphate hydrolysis in human platelets. *Proc. Natl. Acad. Sci. USA.* **82** 3859-3862.

(44) Mayorek, N. and Bar-tan, J. (1985) Inhibition of diacylglycerol acyltransferase by 2-bromooctanoate in cultured rat hepatocytes. *J. Biol. Chem.* **260** 6528-6532.

(45) Nishizuka, Y. (1984) The role of protein kinase C in cell surface signalling transduction and tumour promotion. *Nature* **308** 693-697.

(46) McPhail, L.C., Clayton, C.C. and Snyderman, R. (1984) A potential second messenger role for unsaturated fatty acids: Activation of Ca^{2+}-dependent protein kinase. *Science* **224** 622-625.

(47) Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. *Science* **233** 305-312.

(48) Levine, L. and Hassid, A. (1977) Effects of phorbol-12,13-diesters on prostaglandin production and phospholipase activity in canine kidney (MDCK) cells. *Biochem. Biophys. Res. Commun.* **79** 477-483.

(49) Berridge, M.J. and Irvine, R.F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312** 315-320.

(50) Sweatt, J.D., Blair, I.A., Cragoe, E.J. and Limbird, L.E. (1986) Inhibitors of Na^+/H^+ exchange, block epinephrine- and ADP-induced stimulation of human platelet phospholipase C by blockade of arachidonic acid release at a prior step. *J. Biol. Chem.* **261** 8660-8666.

(51) Jones, L.M., Cockcroft, S. and Mitchell, A.H. (1979) Stimulation of phosphatidylinositol turnover in various tissues by cholinergic and adrenergic agonists, by histamine and caerulein. *Biochem. J.* **182** 699-676.

(52) Bocckino, S.B., Wilson, P.B. and Exton, J.H. (1987) Ca^{2+} mobilizing hormones elicit phosphatidylethanol accumulation via phospholipase D activation. *FEBS Letts* **225** 201-204.

(53) Alexander, D.R., Graves, J.D., Lucas, S.C., Hexham, J.M., Cantrell, D.A. and Crumpton, M.J. (1990) A protein kinase C pseudosubstrate peptide blocks G-protein and mitogenic lectin induced phosphorylation of the CD3 antigen in human T-lymphocytes. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek). Plenum Press. New York. pp253-262.

(54) Abraham, R.T., Augustine, J.A., Schlager, J.W., Barna, T.J. and Leibson, P.J. (1990) Initiation and modulation of signal output from the T cell antigen. In Biology of Cellular Transducing Signals. (ed. J. Y. Vanderhoek). Plenum Press. New York. pp173-183.

(55) Mond, J.J., Feuerstein, N., Finkelman, F.D., Huang, F., Huang, K.P. and Dennis, G. (1987) b-lymphocyte activation mediated by anti-immunoglobulin antibody in the absence of protein kinase C. *Proc. Natl. Acad. Sci. USA.* **84** 8588-8592.

(56) Mendoza, S.A., Schneider, J.A., Lopez-Rivas, A., Sinnett-Smith, J.W. and Rozengurt, E. (1986) Early events elicited by bombesin and structurally related peptides in quiescent Swiss 3T3 cells. II. Changes in Na^+ and Ca^{2+} fluxes, Na^+/K^+ pump activity, and intracellular pH. *J. Cell. Biol.* **102** 2223-2233.

(57) Menniti, F.S., Takemura, H., Sugiya, H. and Putney, J.W. (1990) Mechanisms of receptor regulation for the phosphoinositide signalling system. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek). Plenum Press. New York. pp61-72.

(58) Hammond, C., Paupardin-Tritsch, D., Nairn, A.C., Greengard, P. and Gerschenfeld, H.M. (1987) Cholecystokinin induces a decrease in Ca^{2+} current in snail neurones that appears to be mediated by protein kinase C. *Nature* **325** 809-811.

(59) Mahadevan, L.C., Aitken, A., Heath, J. and Foulkes, J.G. (1987) Embryonal carcinoma-derived growth factor activates protein kinase C *in vivo* and *in vitro*. *EMBO J.* **6** 921-926.

(60) Sawyer, S.T. and Cohen, S. (1981) Enhancement of calcium uptake and phosphatidylinositol turnover by epidermal growth factor in A431 cells. *Biochem.* **20** 6280-6286.

(61) Dunlap, K. and Holz, G.G. (1990) Receptor-mediated alterations of calcium channel function in the regulation of neurosecretion. In Biology of Cellular Transducing Signals. (ed. J. Y. Vanderhoek). Plenum Press. New York. pp107-116.

(62) Wakelam, M.J.O., Murphy, G.J., Hruby, V.J and Houslay, M.D. (1986) Activation of two signal-transduction systems in hepatocytes by glucagon. *Nature* **323** 68-71.

(63) Houslay, M.D. (1990) 'Crosstalk' and the regulation of hepatocyte adenylate cyclase activity: desensitization, G_1 and cyclic AMP phosphodiesterase regulation. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek) Plenum Press. New York. pp141-153.

(64) De Chaffoy de Courcelles, D., Roevens, P. and Vanbelle, H. (1987) Prostaglandin E_1 and forskolin antagonise C-kinase activation in the human platelets. *Biochem. J.* **244** 93-99.

(65) Van de Werve, G., Proietto, J. and Jeanrenaud, B. (1985) Tumour-promoting phorbol esters increase basal and inhibit insulin-stimulated lipogenesis in rat adipocytes without decreasing insulin binding. *Biochem. J.* **225** 523-527.

(66) Hannigan, G.E. and Williams, B.R.G. (1980) Signal transduction by interferon-α through arachidonic acid metabolism. *Science* **251** 204-207.

(67) Yap, W.H., Teo, T.S., McCoy, E. and Tan, Y.H. (1986) Rapid and transient rise in diacylglyerol concentration in Daudi cells exposed to interferon. *Proc. Natl. Acad. Sci. USA.* **83** 7765-7769.

(68) Mattila, P., Häyry, P. and Renkonen, R. (1989) Protein kinase C is crucial in signal transduction during IFN-g induction in endothelial cells. *FEBS Letts.* **250** 362-366.

(69) Farrar, W.L. and Anderson, W.B. (1985) Interleukin-2 stimulates association of protein kinase C with plasma membrane. *Nature* **315** 233-235.

(70) Mills, G.B., Girard, P., Grinstein, S. and Gelfand, E.N. (1988) Interleukin-2 induces proliferation of T lymphocyte mutants lacking protein kinase C. *Cell* **55** 91-100.

(71) Sarau, H.M., Tzimas, M.N., Foley, J.J., Winkler, J.D., Kennedy, M.E., and Crooke, S.T. (1990) Leukotriene D4 increases the metabolism of an inositol tetrakisphosphate-containing phospholipid in U937 cells. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek). Plenum Press. New York. pp235-244.

(72) Koo, C.H., Sherman, J.W., Baud, L., Jin, W., Mai, K. and Goetzl, E.J. (1990) Mechanisms for diversification of responses of human polymorephonuclear leukoctyes to leukotrienes. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek). Plenum Press. New York. pp21-26.

(73) Kapoor, C.L. and Chader, G.J. (1984) Endogenous phosphorylation of retinal photoreceptor outer segment proteins by calcium phospholipid-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **122** 1397-1403.

(74) Kramer, I.M., Van der Bend, R.L., Tool, A.T.J., 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. *J. Biol. Chem.* **264** 5876-5884.

(75) Billah, M.M., Anthes, J.C., Egan, R.W., Mullmann, T.J. and Siegel, M.I. Stimulus-coupled activation of phospholipase D. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek) Plenum Press. New York. pp301-310.

(76) Besterman, J.M., Duronio, Y. and Cuatrecasas, P. (1986) Rapid formation of diacylglycerol from phosphatidylcholine: A pathway for generation of a second messenger. *Proc. Natl. Acad. Sci. USA.* **83** 6785-6789.

(77) MacPhee, C.H., Drummond, A.H., Otto, A.M. and Jimenez de Asua, L. (1984) Prostaglandin F2a stimulates phosphatidylinositol turnover and increases the cellular content of 1,2-diacylglycerol in confluent resting Swiss 3T3 cells. *J. Cell. Phys.* **119** 35-40.

(78) Tohmatsu, T., Hattori, H., Nagao, S., Ohki Koki, and Nozawa, Y. (1986) Reversal by protein kinase C inhibitor of suppressive actions of phorbol-12-myristate-13-acetate on polyphosphoinositide metabolism and cytosolic Ca^{2+} mobilization in thrombin-stimulated human platelets. *Biochem. Biophys. Res. Commun.* **134** 868-875.

(79) Tysnes, O.B., Verhoeven, A.J.M., Aarbakke, G.M. and Holmsen, H. (1987) Phosphoinositide metabolism in resting and thrombin-stimulated platelets. *FEBS Letts.* **218** 68-72.

(80) De Gaffroy de Courcelles, D., Roevens, P., Van Belle, H., Kennis, L., Somers, Y. and de Clerk, F. (1989) The role of endogenously formed diacylglycerol in the propagation and termination of platelet activation. *J. Biol. Chem.* **264** 3274-3285.

(81) Weiss, E.R., Kelleher, D.J., Woon, C.W., Soparkar, S., Osawa, S., Heasley, L.E. and Johnson, G.L. (1988) Receptor activation of G-proteins. *FASEB J.* **2** 2841-2848.

(82) Bourne, H.R. (1986) One molecular machine can transduce diverse signals. *Nature* **321** 814-816.

(83) Rodbell, M. (1985) Programmable messengers: A new theory of hormone action. *TIBS* **11** 461-464.

(84) Neer, E.J., Chow, Y-K., Garen-Pazio, S., Michel, T., Schmidt, C.J. and Silbert, S. (1990) The family of G proteins. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek) Plenum Press. New York. pp83-91.

(85) Michell, R.H. and Kirk, C. (1986) G-protein control of inositol phosphate hydrolysis. *Nature* **323** 112-113.

(86) Regazzi, R., Li, G., Ullrich, S., Jaggi, C. and Wollhelm, C.B. (1989) Different requirements for protein kinase C activation and Ca^{2+}-independent insulin secretion in response to guanine nucleotides. *J. Biol. Chem.* **264** 9939-9944.

(87) Hauschildt, S., Hirt, W. and Bessler, W. (1988) Modulation of protein kinase C activity by NaF in bovine marrow-derived macrophages. *FEBS Letts* **230** 121-124.

(88) He, Y., Hewlett, E., Temeles, D and Quesenberry, P. (1988) Inhibition of interleukin-3 and colony-stimulating factor-1-stimulated marrow cell proliferation by pertussis toxin. *Blood* **71** 1187-1195.

(89) Kelvin, D.J., Shreeve, M., McAuley, C., McLeod, D.L., Simard, G. and Connolly, J.A. (1989) Interleukin 3-stimulated proliferation is sensitive to pertussis toxin: evidence for a guanyl nucleotide regulatory protein-mediated signal transduction mechanism. *J. Cell. Phys.* **138** 273-280.

(90) Cockroft, S. (1987) Phosphoinositide phoshodiesterase: Regulation by a novel guanine nucleotide binding protein. *TIPS* **12** 75-78.

(91) Gardner, P. (1989) Calcium and T lymphocyte activation. *Cell* **59** 15-20.

(92) Carlson, K.E., Brass, L.F. and Manning, D.R. (1990) Selective phosphorylation of a G-protein within permeabilised and intact platelets caused by activators of protein kinase C. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek) Plenum Press. New York. pp185-192.

(93) Jelsema, C.L. and Ma, A.D. (1990) The role of GTP-binding protein in the regulation of phospholipase A_2 and C in bovine retinal rod outer segments. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek) Plenum Press. New York. pp289-300.

(94) Nakanishi, H., Nomura, H., Kikkawa, U., Kishimoto, A., and Nishizuka, Y. (1985) Rat brain and liver soluble phospholipase C: resolution of two forms with different requirements for calcium. *Biochem. Biophys. Res. Commun.* **132** 582-590.

(95) Katan, M. and Parker, P.J. (1987) Purification of phosphoinositide-specific phospholipase C from a particulate fraction of bovine brain. *Eur. J. Biochem.* **168** 413-418.

(96) Suh, P.G., Ryu, S.H., Moon, K.H., Suh, H.W. and Rhee, S.G. (1988) Cloning and sequence of multiple forms of phospholipase C. *Cell* **54** 161-169.

(97) Supattapone, S., Worley, P.F., Baraban, J.M. and Snyder, S.H. (1988) Solubilization, purification and characterisation of an inositol trisphosphate receptor. *J. Biol. Chem.* **263** 1530-1534.

(98) Irvine, R.F. (1990) Inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate: two second messengers in a duet of calcium regulation. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek) Plenum Press. New York. pp213-225.

(99) Joseph, S.K., Hansen, C.A. and Williamson, J.R. (1987) Inositol 1,3,4,5-tetrakisphosphate increases the duration of the inositol 1,4,5 trisphosphate-mediated Ca^{2+} transient. *FEBS Letts.* **219** 125-129.

(100) Gardner, P., Alcover, A., Kuno, M., Moingeon, P., Weyland, C.M., Goronzy, J. and Reinherz, E.L. (1989) Triggering of T-lymphocytes via either T3 -Ti or T-11 surface structures opens a voltage-insensitive plasma membrane calcium-permeable channel: Requirement for interleukin 2 gene function. *J. Biol. Chem.* **264** 1068-76.

(101) Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Castafiore, B. and Salamino, F. (1985) Binding of protein kinase C to neutrophil membranes in the presence of Ca^{2+} and its activation by a Ca^{2+}-requiring proteinase. *Proc. Natl. Acad. Sci. USA.* **82** 6435-6439.

(102) McDonald, J.R. and Walsh, M.P. (19??) Regulation of protein kinase C activity by natural inhibitors. *Biochemical Society Transactions* **14** 585-586.

(103) McCormack, J.G. and Denton, R.M. (1986) Ca^{2+} as a second messenger within mitochondria. *TIBS* **11** 258-262.

(104) Connolly, T.M., Lawing, W.J. and Majerus, P.W. (1986) Protien kinase C phosphorylates human platelet inositol triphosphate 5'-phosphomonoesterase increasing the phosphatase activity. *Cell* **46** 951-958.

(105) Rittenhouse, S.E., King, W.G., Downes, C.P. and Kucera, G.L. (1990) The role of endogenously stimulated protein kinase C in regulating inositol polyphosphates in platelets. Plenum Press. New York. Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek). Plenum Press. New York.. pp227-234.

(106) Mochly-Rosen, D. and Koshland, D.E. (1987) Domain structure and phosphorylation of protein kinase C. *J. Biol. Chem.* **262** 2291-2297.

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

(108) Sugimoto, Y., Whitman, M., Cantley, L.C. and Lerikson, R.L. (1984) Evidence that the rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. *Proc. Natl. Acad. Sci. USA.* **81** 2177-2121.

(109) Macara, I.G., Marinetti, G.V. and Balduzzi, P.C. (1984) Transforming protein of avain sarcoma virus UR2 is associated with phosphatidylinositol kinase activity: possible role in tumourgenesis. *Proc. Natl. Acad. Sci. USA.* **81** 2728-2732.

(110) Asao, T., Ogasawara, N., Kitajima, S. and Sano, M. (1986) Interaction of GTP-binding protiens with calmodulin. *FEBS Letts.* **203** 135-138.

(111) Logothetis, D.E., Kurachi, Y., Galper, J., Neer, E.J. and Clapham, D.E. (1987) The bg subunits of GTP binding proteins activate the muscarinic K+ channel in heart. *Nature* **325** 321-326.

(112) Kim, D., Lewis, D.L., Graziadei, L., Neer, E.J., Bar-Sagi, D. and Clapham, D.E. (1989) G protein bg subunits activate the cardiac muscarinic K+ channel via phospholipase A_2. *Nature* **337** 557

(113) Birnbaumer, L., Vatani, A., Mattera, R., Codina, J. and Brown, A.M. (1990) Roles of G protein subunits in coupling of receptors to ionic channels and other effector systems. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek). Plenum Press. New York.. pp93-106.

(114) Boyer, J.L., Waldo, G.L., Evans, T., Northup, J.K., Downes, C.P. and Harden, T.K. (1989) Modification of ALF_4 - and receptor-stimulated phospholipase C activity by G-protein $\beta\gamma$ subunits. *J. Biol. Chem.* **264** 13917-13922.

(115) Whetton, A.D., Monk, P.N., Consalvey, S.D. and Downes, C.P. (1986) The Haemopoietic growth factors interleukin-3 and colony stimulating factor-1 stimulate proliferation but do not induce inositol lipid breakdown in murine bone-marrow-derived macrophages. *EMBO* **5** 3281-3286.

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

(117) Rosoff, P.M., Savage, N. and Dinarello, C.A. (1988) Interleukin-1 stimulates diacylglycerol production in T-lymphocytes by a novel mechanism. *Cell* **54** 73-81.

(118) Saltiel, A.R. (1990) Second messengers of insulin action. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek). Plenum Press. New York.. pp391-399.

(119) Evans, F.J. and Taylor, S.E. (1983) Pro-imflammatory, tumour-promoting and anti-tumour diterpenes of the plant families euphorbiaceae and thymelaeaceae. *Progress in the Chemistry of Organic Natural Products.* 44 1-99.

(120) Evans, F.J. and Soper, C.J. (1978) The tigliane, daphnane, and igenane diesters, their chemistry, distribution and biological activities. A review. *Lloydia* **41** 193-233.

(121) Hoppe, W., Brandl, F., Strell, I., Rohrl, M., Gassmann, I, Hecker, E., Bartsch, H., Kreibich, G and Szczepanski, CH.V. (1967) Rontgenstruckturanalyse des Neophorbols agnew. *Chem. Internat. Edit.* **6** 809.

(122) Hecker, E. (1968) Co-carcinogenic principles from the seed oil of croton tiglium and from other euphorbiaceae. *Cancer Res.* **28** 2338.

(123) Evans, F.J. (1986) Phorbol esters. In *Naturally Occuring Phorbol Esters* (ed. F.J. Evans) CRC Press, Boca Raton, USA 1-31.

(124) Ohigashi, H. and Mitsui, T. (1972) Studies on the biologically active substances of sapium japonicum. *Bull. Inst. Chem. Res.*, Kyoto University. **50** 239.

(125) Brooks, G., Morrice, N., Ellis, C.A., Aitken, A., Evans, A.T., Evans, F.J. (1987) Toxic phorbol esters from chinese tallow stimulate protein kinase C. *Toxicon* **25** 1229-1233.

(126) Taylor, S.E., Gafur, M.A., Choudhury, A.K. and Evans, F.J. (1981) Sapintoxin A, a new biologically active nitrogen containing phorbol ester. *Experientia* **37** 681-682.

(127) Evans, F.J. and Schmidt, R.J. (1976) Two new toxins from the latex of *euphorbia poisonii. Phytochem.* **15** 333.

(128) Brooks, G., Evans, A.T., Aitken, A., Evans, F.J., Rizk, A.F.M., Hammouda, F.M., El-Missiry, M.M. and Ismail, S.E. (1990) Daphnane diterpenes of *thymelaea hirsuta*. *Phytochem.* **29** 2235-2237.

(129) Evans, F.J. and Kinghorn, A.D. (1974) The isolation of ingenol from euphorbia desmondi. *Phytochem.* **13** 1011.

(130) Jaken, S. (1987) Measurement of phorbol ester receptors in intact cells and subcellular fractions. *Methods in Enzymology* **141** 274-87.

(131) Shoyab, M. and Todaro, G.J. (1980) Specific high affinity cell membrane receptors for biologically active phorbol and ingenol esters. *Nature* **288** 451-455.

(132) Dunphy, W.G., Delclos, K.B. and Blumberg, P.M.(1980) Characterization of specific binding of (H^3) phorbol-12,13-dibutyrate to mouse brain. *Cancer Res.* **40** 3635-41.

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

(134) Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium-activated,phospholipid-dependent protein kinase by tumour promoting phorbol esters. *J. Biol. Chem.* **257** 7847-7851.

(135) Parker, P.J., Stabel, S., and Waterfield, M.D. (1984) Purification to homogeneity of protein kinase C from bovine brain - identity with the phorbol ester receptor. *EMBO J.* **3** 953-959.

(136) König, B., DiNitto, P.A. and Blumberg, P.M. (1985) Phospholipid and Ca^{2+} dependency of phorbol ester receptors. *J. Cell. Biochem.* **27** 255-265.

(137) Leach, K.L., James, M.L. and Blumberg, P.M. (1983) Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. *Proc. Natl. Acad. Sci. USA*. **80** 4208-4212.

(138) Niedel, J.E., Kuhn, L.J. and Vanderbark, G.R. (1983) Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. USA*. **80** 36-40.

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

(140) Tanaka, Y., Miyake, R., Kikkawa, U., Nishizuka, Y. (1986) Rapid assay of binding of tumour promoting phorbol ester to protein kinase C. *J. Biochem.* **99** 257-261.

(141) Blumberg, P.M., Jaken, S., Konig, B., Sharkey, N.A., Leach, K.L., Jeng, A.Y. and Yeh, E. (1984) Mechanism of action of the phorbol ester tumour promoters: specific receptors for lipophylic ligands. *Biochem. Pharm.* **33** 933-940.

(142) König, B., DiNitto, P.A. and Blumberg, P.M. (1985) Stoichiometric binding of diacylglycerol to the phorbol ester receptor. *J. Cell. Biochem.* **29** 37-44.

(143) Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. and Nishizuka, Y. (1983) Protein kinase C as a possible receptor protein of tumour-promoting phorbol esters. *J. Biol. Chem.* **258** 11442-11445.

(144) Kraft, A.S. and Anderson, W.B. (1983) Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with the plasma membrane. *Nature* **301** 621-623.

(145) Berry, N., Ase, K., Kishimoto, A. and Nishizuka, Y. (1990) Activation of resting human T cells requires prolonged stimulation of protein kinase C. *Proc. Natl. Acad. Sci. USA.* **87** 2294-2298.

(146) Kreutter, D., Caldwell, A.B. and Morin, M.J. (1985) Dissociation of protein kinase C activation from phorbol ester-induced maturation of HL-60 leukemia cells. *J. Biol. Chem.* **260** 5979-5984.

(147) Farrar, W.L., Thomas, T.P. and Anderson, W.B. (1985) Altered cytosol/membrane enzyme redistribution on interleukin-3 activation of protein kinase C. *Nature* **315** 235-237.

(148) Ginsberg, J., Murrayl, P.G., Parente, J.E. and Wong, K. (1988) Translocation of protein kinase C in porcine thyroid cells following exposure to thyrotropin. *FEBS Letts.* **226** 223-226.

(149) Nel, A.E., Wooten, M.W., Landreth, G.E., Goldschmidt-Clermont, P.J., Stevenson, H.C., Miller, P.J. and Galbraith, R.M. (1986) Translocation of phospholipid/Ca^{2+}-dependent protein kinase in *B*-lymphocytes activated by phorbol ester or cross-linking of membrane immunoglobulin. *Biochem. J.* **233** 145-149.

(150) Giri, J.G. (1988) IL-2 signal transduction, IL-2 induced translocation of PKC.*Immunology Abstracts* no 1337.

(151) Berry, N., Ase, K., Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989) Human T cell activation by phorbol esters and diacylglycerol analogues. *J. Immunol.* **143** 1407-1413.

(152) Thomas, T.P. ,Talwar, H.S., Anderson, W.B. (1988) Phorbol ester-mediated association of protein kinase C to the nuclear fraction in NIH 3T3 cells. *Cancer Res.* **48** 1910-1919.

(153) Halsey, D.L., Girard, P.R., Kuo, J.F. and Blackshear, P.J. (1987) Protein kinase C in fibroblasts. Characteristics of its membrane association during growth and after exposure to phorbol esters and other mitogens. *J. Biol. Chem.* **262** 2234-2243.

(154) Kiss, Z., Deli, E. and Kuo, J.F. (1988) Temporal changes in intracellular distribution of protein kinase C during differentiation of human leukemia HL-60 cells induced by phorbol ester. *FEBS Letts.* **231** 41-46.

(155) Homma, Y., Chubb, C.B.H. and Huberman, E. (1986) Translocation of phosphokinase C is associated with susceptibility of human leukemia cells to phorbol 12-myristate-13-acetate induced cell differentiation. *Proc. Am. Assoc. Cancer. Res.* **27** 42

(156) Shoji, M., Girard, P.A., Koeffler, H.P., Vogler, W.R. and Kuo, J.F. (1987) Effects of phorbol esters on translocation and down-regulation of PKC and phosphorylation of endogenous proteins in human acute myeloid leukemia cell line KG1 and its phorbol ester resistant subline KG1a. *Cancer Res.* **47** 6363-6370.

(157) Girard, P.R., Stevens, V.L., Blackshear, P.J., Merrill, A.H., Wood, J.G., and Kuo, J.F. (1987) Immunocytochemical evidence for phorbol ester-induced directional translocations of protein kinase C in HL-60, K562, CHO and E75KS cells: possible role in differentiation. *Cancer Res.* **47** 2892-2898.

(158) Tapley, P.M. and Murray, A.W. (1985) Evidence that treatment of platelets with phorbol ester causes proteolytic activation of Ca^{2+}-activated, phospholipid-dependent protein kinase. *Eur. J. Biochem.* **151** 419-423.

(159) Pontremoli, S., Melloni, E., Michetti, M,. Sacco, O., Salamino, F., Sparatore, B. and Horecker, B.L. (1986) Biochemical responses in activated human neutrophils mediated by protein kinase C and a Ca^{2+}-requiring proteinase. *J. Biol. Chem.* **261** 8309-8313.

(160) Young, S., Parker, P.J., Ullrich, A. and Stabel, S. (1987) Down-regulation of protein kinase C is due to an increased rate of degradation. *Biochem. J.* **244** 775-779.

(161) Ballester, R. and Rosen, O.M. (1985) Fate of immunoprecipitable protein kinase C in GH3 cells treated with phorbol 12-myristate 13-acetate. *J. Biol. Chem.* **260** 15194-15199.

(162) Issandou, M. and Rozengurt, E. (1989) Diacylglycerol, unlike phorbol esters, do not induce homologous desensitization or down-regulation of protein kinase C in Swiss 3T3 cells. *Biochem. Biophys. Res. Commun.* **163** 201-208.

(163) Adams, J.C. and Gullick, W.J. (1989) Differences in phorbol-ester-induced down-regulation of protein kinase C between cell lines. *Biochem. J.* **257** 905-911.

(164) Vara, F. and Rozengurt, E. (1985) Stimulation of Na^+/H^+ antiport activity by epidermal growth factor and insulin occurs without activation of protein kinase C. *Biochem. Biophys. Res. Commun.* **130** 646-653.

(165) Minami, Y., Samelson, L.E., Klausner, R.D. (1987) Internalization and recycling of the T-cell antigen receptor. *J. Biol. Chem.* **262** 13342-11347.

(166) Kaever, V., Pfannkuche, H.J., Wessel, K., Sommermeyer, H., and Resch, K. (1990) Eicosanoid synthesis in resident macrophagaes: role of protein kinase C. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek). Plenum Press. New York. pp359-369.

(167) Yamasaki, Y., Martel, N. and Ostertag, W. (1984) Continuous suppression of globin gene expression and differentiation of friend erythroleukemia cells by phorbol-12-myristate-13-acetate (PMA) despite the loss of PMA binding sites by down-regulation. *Proc. Natl. Acad. Sci. USA.* 81 2075-2079.

(168) Kariya, K. and Takai, Y. (1987) Distinct functions of down-regulation-sensitive and -resistant types of protein kinase C in rabbit aortic smooth muscle cells. *FEBS Letts.* **219** 119-124.

(169) Perrella, F.W., Bussel, P.A. and Boutwell, R.K. (1982) Diversity of nuclear phorbol ester tumour receptors in mouse liver: Evidence for two classes of binding site. *Biochem. Biophys. Res. Commun.* **108** 1722-1727.

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

(171) Bruns, C., Schachtele, C. and Marme, D. (1987) Synthetic diacylglycerols induce a rise of quin 2-detectable free intracellular calcium in human platelets. *FEBS Letts.* **221** 23-27.

(172) Banks, R.E., Roifman, C., Mellors, A. and Mills, G.B. (1989) The diacylglycerol analogue, 1,2-sn-dioctanoylglycerol, induces an increase in cytosolic free Ca^{2+} and cytosolic acidification of T lymphocytes through a protein kinase C-independent process. *Biochem.J.* **258** 689-698.

(173) Kolesnick, R.N. (1987) 1,2-diacylglycerols but not phorbol esters stimulate sphingomyelin hydrolysis in GH3 pituitary cells. *J. Biol. Chem.* **262** 16759-16762.

(174) Kramer, R.M., Checani,. G.C., Deykin, D. (1987) Stimulation of Ca^{2+}-activated human platelet phospholipase A_2 by diacylglycerol. *Biochem. J.* **248** 779-783

(175) Goppelt-Strube, M., Pfannkuche, H.J., Gemsa, D. and Resch, K. (1987) The diacylglycerols dioctanoylglycerol and oleoyl-acetyl-glycerol enhance prostaglandin synthesis by inhibition of the lysophosphatide acyltransferase. *Biochem. J.* **247** 773.

(176) Sekigushi, K., Tsukuda, M., Ogita, K., Kikkawa, U. and Nishizuka, Y. (1987) Three distinct forms of rat protein kinase C : Differential response to unsaturated fatty acids. *Biochem. Biophys. Res. Commun.***145** 797-802.

(177) Shearman, M.S., Naor, Z., Sekiguchi, K., Kishimoto, A.,Nishizuka, Y. (1989) Selective activation of the g-species of protein kinase C from bovine cerebellum by arachidonic acid and its lipoxygenase metabolites". *FEBS Letts* **243** 177-182.

(178) Hansson, A., Serhan, C.N., Haeggstrom, J., Ingelman-Sundberg, M. and Samuelsson, B. (1986) Activation of protein kinase C by lipoxin A and other eicosanoids. Intracellular action of oxygenation products of arachidonic acid. *Biochem. Biophys. Res. Commun.* **134** 1215-1222.

(179) Ramsdell, J.S., Pettit, G.R., Tashjian, A.H. (1986) Three activators of protein kinase C (bryostatins, diolein and phorbol esters) show differing specificities of action on GH3 pituitary cells. *J. Biol. Chem.* **261** 17073-17080.

(180) Blumberg, P.M. (1988) Protein kinase C as the receptor for the phorbol ester tumour promoters : Sixth Rhoads memorial award lecture. *Cancer Res.* **48** 1-8.

(181) Kraft, A.S., Smith, J.B. and Berkow, R.L. (1986) Bryostatin, an activator of the calcium phospholipid-dependent protein kinase, blocks phorbol ester induced differentiation of human promyelocytic leukemia cells HL-60. *Proc. Natl. Acad. Sci. USA* **83** 1334-1338.

(182) Huang, K.P., Huang, F.L., Nakabayashi, H. and Yoshida, Y (1988) Biochemical characterisation of rat brain protein kinase C isozymes. *J. Biol. Chem.* **263** 14839-14845.

(183) Murakami, K., Chan, S.Y. and Routtenberg, A. (1986) Protein kinase C activation by cis fatty acid in the absence of Ca^{2+} and phospholipids. *J. Biol. Chem.* **261** 15424-15429.

(184) Ellis, C.A., Aitken, A., Takayama, K., Qureshi, N. (1987) Substitution of phosphatidylserine by lipid A in activation of purified rabbit brain protein kinase C. *FEBS Letts* **218** 238-242.

(185) Aitken, A. (1987) The activation of protein kinase C by daphnane, ingenane and tigliane diterpenoid esters. *Botanical J. Linnean Soc.* **94** 247-263.

(186) Ohkubo, S., Yamada, E., Endo, T., Itoh, H. and Hidaka, H. (1984) Vitamin A acid-induced activation of Ca^{2+}-activated, phospholipid dependent protein kinase from rabbit retina. *Biochem. Biophys. Res. Commun.* **118** 460-466.

(187) Mills, K.J. and Smart, R.C. (1989) Comparison of epidermal protein kinase C activity, ornithine decarboxylase induction and DNA synthesis stimulated by TPA or dioctyanoylglycerol in mouse strains with differing susceptibility to TPA-induced tumour promotion. *Carcinogenesis* **10** 833-838.

(188) Acroleo, J.P. and Weinstein, I.B. (1985) Activation of protein kinase C by tumour promoting phorbol esters teleocidin and aplysiatoxin in the absence of added calcium. *Carcinogenesis* **6** 213-317.

(189) Miyake, R., Tanaka, Y, Tsuda, T., Kimbuchi, K., Kikkawa, U. and Nishizuka, Y. (1984) Activation of protein kinase C by non phorbol tumour promoter mezerein. *Biochem. Biophys. Res. Commun.* **121** 649-656.

(190) Brooks, G., Evans, A.T., Aitken, A. and Evans, F.J. (1988) Sapintoxin A : A flourescent phorbol ester that is a potent activator of protein kinase C but is not a tumour promoter. *Cancer Letts.* **38** 165-169.

(191) Ellis, C.A., Brooks, S.F., Brooks,G., Evans, A.T., Morrice, N., Evans, F.J. and Aitken, A. (1988) Novel biological activities of phorbol esters; The sapintoxin series includes non-tumour promoters that are potent activators of protein kinase C and a specific inhibitor of this enzyme. *Phytotherapy Res.* **1** 187-90.

(192) Davis, R.J., Ganong, B.R., Bell, R.M. and Czech, M.P. (1985) Structural requirements for diacylglycerols to mimic tumor-promoting phorbol diester action on epidermal growth factor receptor. *J. Biol. Chem.* **260** 5315-5322.

(193) Yamamoto, S., Gotoh, H., Aizu, E. and Kato R. (1985) Failure of 1-oleoyl-2-acetylglycerol (OAG) to mimic the cell differentiating action of 12-O-tetradecanoyl phorbol-13-acetate in HL-60 cells. *J. Biol. Chem.* **260** 14230-14234.

(194) Blackshear, P.J., Nairn, A.C. and Kuo J.F. (1988) Protein kinases 1988: A current perspective. *FASEB J.* **2** 2957-2959.

(195) Oishi, K., Raynor, R.L., Charp, P.A. and Kuo, J.F. (1988) Regulation of protein kinase C by lysophospholipids. *J. Biol. Chem.* **263** 6865-6871.

(196) Wender, P.A., Koehler, K.F., Sharkey, N.A, Dell Aquila, M.L. and Blumberg, P.M. (1986) Analysis of the phorbol ester phamacophore on protein kinase C as a guide to the rational design of new classes of analogs. *Proc. Natl. Acad. Sci. USA.* **83** 4214-4218.

(197) Cantrell D.A., Friedrich, B., Davies, A.A., Gullberg, M. and Crumpton, M.J. (1989) Evidence that a kinase distinct from protein kinase C induces CD3 γ-subunit phosphorylation without a concommitant down-regulation in CD3 antigen expression. *J. Immunol.* **142** 1626-1630.

(198) Moolenaar, W.H., Tertoolen, L.G.J. and de Laat, S.W. (1984) Phorbol ester and diacylglycerol mimic growth factors in raising cytoplasmic pH. *Nature*. **312** 371-374.

(199) Di Virgilio, F., Lew, D.P. and Pozzau, T. (1984) Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels. *Nature* **310** 691-693.

(200) Kaibuchi, K., Takai, Y. and Nishizuka, Y. (1981) Cooperative roles of various membrane phospholipids in the activation of calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* **256** 7146-7149.

(201) Hannun, Y.A., Loomis, C.R. and Bell, R.M. (1985) Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. *J. Biol. Chem.* **260** 10039-10043.

(202) Hannun Y.A. and Bell R.M. (1987) Mixed micellar assay for phorbol ester binding. *Methods in Enzymology* **141** 287-293.

(203) Leach, K.L. and 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 Res.* **45** 1958-1963.

(204) Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tominaga, M., Kuroda, T. and Nishizuka, Y. (1989) Limited proteolysis of protein kinase C subspecies by calcium dependent neutral protease (calpain). *J. Biol. Chem.* **264** 4088-4092.

(205) Young, S., Rothbard, J. and Parker, P.J. (1988) A monoclonal antibody recognising the site of limited proteolysis of protein kinase C. *Eur. J. Biochem.* **173** 247-252.

(206) Slaga, T.J., Fischer, S.M., Nelson, K. and Gleason, G.L. (1980) Studies on the mechanism of skin tumour promotion: evidence for several stages in promotion. *Proc. Natl. Acad. Sci. USA.* **77** 3659-3663.

(207) Besterman, J.M., May, W.S., Levine, H., Cragoe, E.J. and Cuatrecases, P. (1985) Amiloride inhibits phorbol ester-stimulated Na^+/H^+ exchange and protein kinase C. *J. Biol.Chem.* **260** 1155-1159.

(208) Chatila C. and Geha R.S. (1988) Phosphorylation of T-cell membrane proteins by activators of protein kinase C. *J. Immunol.* **140** 4308-14.

(209) Tohmatzu, T., Haytori, H., Nagao, S., Ohki, K. and Nozawa, Y. (1986) Reversal by protein kinase C inhibitor of suppressive actions of phorbol-12-myristate-13-acetate on polyphosphoinositide metabolism and cytosolic Ca^{2+} mobilisation in thrombin-stimulated human platelets. *Biochem. Biophys. Res. Commun.* **134** 868-875.

(210) Inagaki, M., Kawamoto, S. and Hidaka, H. (1984) Serotonin secretion from human platelets may be modified by Ca^{2+}-activated, phospholipid-dependent myosin phosphorylation. *J. Biol. Chem.* **259** 14321-14323,

(211) Marth, J.D., Lewis, D.B., Cooke, M.P., Mellins, E.D., Gearn, M.E., Samelson, L.E., Wilson, C.B., Miller, A.D. and Perlmutter, R.M. (1989) Lymphocyte activation provokes modification of a lymphocyte-specific protein tyrosine kinase (p56*lck*). *J. Immunol.* **142** 2430-2437.

(212) Jung, L.K.L., Bjorndahl, J.M and Fu, S.M. (1988) Dissociation of TPA-induced down-regulation of T-cell antigens from protein kinase C activation. *Cell. Immunol.* **117** 352-359.

(213) Siefert, R. and Schachtele, C. (1988) Studies with protein kinase C inhibitors presently available cannot elucidate the role of protein kinase C in the activation of NADPH oxidase. *Biochem. Biophys. Res. Commun.* **152** 585-592.

(214) Wion, D., MacGrogan, D., Houlgatte, R. and Brachet, P. (1990) Phorbol 12-myristate-13-acetate (PMA) increases the expression of hte nerve growth factor (NGF) gene in mouse L-929 fibroblasts. *FEBS Letts.* **262** 42-44.

(215) Gschwendt, M., Horn, F., Kittstein, W., Furstenberger, G., Besemfelder, E. and Marks, F. (1984) Calcium and phospholipid-dependent protein kinase activity in mouse epidermis cytosol. Stimulation by complete and incomplete tumour promoters and inhibition by various compounds. *Biochem. Biophys. Res. Commun.* **124** 63-68.

(216) Loomis, C.R. and Bell, R.M. (1988) Sangivamycin, a nucleotide analogue, is a potent inhibitor of protein kinase C. *J. Biol. Chem.* **263** 1682-1692.

(217) Ward, S.G., Cantrell, D.A. and Wetnick, J. (1988) Inhibition by staurosporine of mitogen-induced calcium mobilization in human T-lymphocytes. *FEBS.Letts* **239** 363-6.

(218) Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M and Tomita, F. (1986) Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135** 397-402.

(219) Dewald, B., Thelen, M., Wymann, M.P. and Bagglioni, M. (1989) Staurosporine inhibits the respiratory burst and induces exocytosis in human neutrophils. *Biochem. J.* **264** 879-884.

(220) Kuo, J.F., Raynor, R.L., Mazzei, G.J., Schatzman, R.C., Turner, R.S. and Kem, W.R. (1983) Cobra polypeptide cytotoxin I and marine worm polypeptide cytoxin A-IV are potent and selective inhibitors of phospholpid-sensitive Ca^{2+}-dependent protein kinase. *FEBS Letts.* **153** 183-186.

(221) Helfman, D.M., Barnes, K.C., Kinkade, J.M., Vogler, W.R., Shoji, M. and Kuo, J.F. (1983) Phospholipid-sensitive Ca^{2+}-dependent protein phosphorylation system in various types of leukemic cells from human patients and in human leukemic cell lines HL-60 and K562 and its inhibition by alkyl-lysophospholipid. *Cancer Res.* **43** 2955-2961.

(222) Katoh, N., Wise, B.C. and Kuo, J.F. (1983) Phosphorylation of cardiac troponin inhibitory subunit (troponin I) and tropomyosin-binding subunit (trotonin T) by cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase. *Biochem. J.* **209** 189-195.

(223) Nakaki, T., Mita, S., Yamamoto, S., Nakadate, T. and Kato, R. (1984) Inhibition by palmitoylcarnitine of adhesion and morphological changes in HL-60 cells induced by 12-0-tetradecanoylphorbol-12-acetate. *Cancer Res.* **44** 1908-1912.

(224) Nazzei, G.J., Katoh, N. and Kuo, J.F. (1982) Polymixin B is a more selective inhibitor for phospholiad-sensitive Ca^{2+}-dependent protein kinase than for calmodulin-sensitive Ca^{2+}-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **109** 1129-1133.

(225) Qi, D-F., Schatzman, R.C., Mazzei, G.J., Turner, R.S., Raynor, R.L., Liao, S. and Kuo, J.F. (1983) Polyamines inhibit phospholpid-sensitive and calmodulin-sensitive Ca^{2+}-dependent protein kinases. *Biochem. J.* **213** 281-288.

(226) Taffet, S.M., Rissa, A., Greenfield, L and Maddox, M.K. (1983) Retinal inhibits TPA activated, calcium dependent phospholipid dependent protein kinase ("C" kinase). *Biochem. Biophys. Res. Commun.* **114** 1194-1199.

(227) Fujita, I., Irita, K., Takeshige, K and Minakami, S. (1984) Diacylglycerol, 1-oleoyl-2-acetyl-glycerol, stimulates superoxide-generation from human neutrophils. *Biochem. Biophys. Res. Commun.* **120** 318-324.

(228) Su, H-D., Mazzei, G.J., Vogler, W.R. and Kuo, J.F. (1985) Effect of tamoxifen, a nonsteroidal antiestrogen, on phospholipid/calcium-dependent protein kinase and phosphorylation of its endogenous substrate proteins from the rat brain and ovary. *Biochem. Pharmacol.* **34** 3649-3653.

(229) Bignon, E., Pons, M., Gilbert, J. and Nishizuka, Y. (1990) Multiple mechanisms of protein kinase C inhibition by triphenylacrylaonitrile antiestrogens. *FEBS Letts.* **271** 54-58.

(230) Katoh, N., Wise, B.C., Wrenn, R.W. and Kuo, J.F. (1981) Inhibition of adriamycin of calmodulin-sensitive and phospholipid-sensitive calcium-dependent phosphorylation of endogenous proteins from heart. *Biochem. J.* **198** 199-205.

(231) Helfman, D.M., Appelbaum, B.D., Vogler, W.R. and Kuo, J.F. (1983) Phospholipid-sensitive Ca^{2+} dependent protein kinase and its substrates in human neutrophils. *Biochem. Biophys. Res. Commun.* **111** 847-853.

(232) Brooks, G., Evans, A.T., Aitken, A. and Evans, F.J. (1989) Tumour-promoting and hyperplastic effects of phorbol and daphnane esters in CD-1 mouse skin and a synergistic effect of calcium ionophore with the non-promoting activator of protein kinase C, sapintoxin A. *Carcinogenesis* **10** 283-288.

(233) Burgoyne, R.D., Morgan, A. and O'Sullivan, A.J. (1988) A major role for protein kinase C in calcium-activated exocytosis in permeabilised adrenal chromaffin cells. *FEBS.Letts* **238** 151-155.

(234) Hannun, Y.A., Loomis, C.R., Merrill, A.H. and Bell, R.M. (1986) Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding *in vitro* and in human platelets. *J. Biol. Chem.* **261** 12604-12609

(235) Cope, F.O., Staller, J.M., Mahsem, R.A. and Boutwell, R.K. (1984) Retinoid-binding proteins are phosphorylated *in vitro* by soluble Ca^{2+}- and phosphatidylserine-dependent protein kinase from mouse brain. *Biochem. Biophys. Res. Commun.* **10** 593-601.

(236) Schwantke, N and Le Peuch, C.J. (1984) A protein kinase C inhibitory activity is present in rat brain homogenate. *FEBS Letts.* **177** 36-40.

(237) McDonald, J.R. and Walsh, M.P. (1985) Ca^{2+}-binding proteins from bovine brain including a potent inhibitor of protein kinase C. *Biochem. J.* **232** 559-567.

(238) McDonald, J.R. and Walsh, M.P. (1985) Inhibition of the Ca^{2+}- and phospholipid-dependent protein kinase by a novel Mr 17,000 Ca^{2+}-binding protein. *Biochem. Biophys. Res. Commun.* **129** 603-610.

(239) Gopalakrishna, R. and Anderson, W.B. (1987) Susceptibility of protein kinase C to oxidative inactivation: loss of both phosphotransferase activity and phorbol diester binding. *FEBS Letts* **225** 233-237.

(240) Kikkawa, U., Ogita, K., Ono, Y., Asoako, Y., Shearman, M.S., Fujii, T., Ase, K., Sekigushi, K., Igarashi, K. and Nishizuka, Y. (1987) The common structure and activities of four subspecies of rat brain protein kinase C family. *FEBS Letts.* **223** 212-216.

(241) Bollag, G.E., Roth, R.A., Beaudoin, J., Mochly-Rosen, D. and Koshland, D.E. (1986) Protein kinase C directly phosphorylates the insulin receptor *in vitro* and reduces its protein-tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA.* **83** 5822-5824.

(242) Shukla, R.R., Albro, P.W., Corbett, J.T. and Schroeder, J.L. (1989) *In vitro* studies of the inhibition of protein kinase C from rat brain by di-(2-ethylhexyl)-phthalate. *Chem. Biol. Interactions.* **69** 73-85.

(243) Su, H.D., Kemp, B.E., Turner, R.S. and Kuo, J.F. (1986) Synthetic myelin basic protein peptide analogs are specific inhibitors of phospholipid/calcium-dependent protein kinase (protein kinase C). *Biochem. Biophys. Res. Commun.* **134** 78-84.

(244) Kitajima, Y., Inoue, S., Nagao, S., Nagata, K., Yaoita, H. and Nozawa, Y. (1988) Biphasic effects of 12-O-tetradecanoylphorbol-13-acetate on the cell morphology of low calcium grown human epidermal carcinoma cells : Involvement of translocation and down-regulation of protein kinase C.
Cancer Res. **48** 964-970.

(245) Sakata, A., Ida, E., Tominaga, M. and Onoue, K. (1987) Calmodulin inhibitors, W7 and TFP, block the calmodulin-independent activation of nadph-oxidase by arachidonate in a cell-free system. *Biochem. Biophys. Res. Commun.* **148** 112-119.

(246) Parker, P.J., Coussens, L., Totty, N., Rhee, L., Young, S., Stabel, S., Waterfield, M.D. and Ullrich, A. (1986) The complete primary structure of protein kinase C - the major phorbol ester receptor. *Science* **233** 853-859.

(247) Kikkawa, U. Ono, Y., Ogita, K., Fujii, T., Asoaka, Y., Sekiguishi, K., Kosaka, Y., Igarashi, K. and Nishizuka, Y. (1987) Identification of the structures of multiple subspecies of protein kinase C expressed in rat brain. *FEBS Letts* **217** 227-231.

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

(249) Knopf, J.L., Lee, M-H., Sultzman, L.A., Kriz, R.W., Loomis, C.R., Hewick, R.M., and Bell, R.M. (1986) Cloning and expression of multiple protein kinase C cDNAs. *Cell* **46** 491-502.

(250) Housey, G.M., O'Brian, C.A., Johnson, M.D., Kirschmeier, P. and Weinstein, I.B. (1987) Isolation of cDNA clones encoding protein kinase C evidence for a protein kinase C-related gene family. *Proc. Natl. Acad. Sci. USA.* **84** 1065-1069.

(251) Ono, Y., Kikkawa, U., Fujii, T., Kurokawa, T., Asoaka, Y., Sekigushi, K., Ase, K., Igarashi, K., and Nishizuka, Y. (1987) Expression and properties of two types of protein kinase C : Alternative splicing from a single gene. *Science* **236** 1116-1120.

(252) Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334** 661-665.

(253) Ono, Y., Fujii,T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1987) Identification of three additional members of rat protein kinase C family :δ-,ε-,and ξ-subspecies. *FEBS Letts* **226** 125-128.

(254) Ono, Y., Fuji, T., Ogita, K., Kikkawa, U., Igarisihi, K. and Nishizuka, Y. (1988) The structure, expression and properties of additional members of the protein kinase C family. *J. Biol. Chem.* **263** 6927-6932.

(255) Schaap, D., Parker, P.J., Bristol, A., Kriz, R. and Knopf, J. (1989) Unique substrate specificity and regulatory properties of PKC-e: a rationale for diversity. *FEBS letts.* **243** 351-357.

(256) Kosaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U. and Nishizuka, Y. (1988) The heterogeneity of protein kinase C in various rat tissues. *Biochem. Biophys. Res. Commun.* **151** 973-981.

(257) Bacher, N., Zisman, Y., Berent, E. and Livneh, E. (1991) Isolation and characterisation of PKC-L, a new member of the protein kinase C related gene family specifically expressed in lung skin and heart. *Mol. Cell. Biol.* **11** 126-133.

(258) Osada, S., Mizuno, K., Saido, T.C., Akita, Y., Suzuki, K., Kuroki, T. and Ohno, S. (1990) A phorbol ester receptor/protein kinase, nPKCη, a new member of the protein kinase C family predominalty expressed in lung and skin. *J. Biol. Chem.* **265** 22434-22440.

(259) Jaken, S. and Kiley, S.C. (1987) Purification and characterisation of three types of protein kinase C from rabbit brain cytosol. *Proc. Natl. Acad. Sci. USA.* **84** 4418-4422.

(260) Marais, R.M. and Parker, P.J. (1989) Purification and characterisation of bovine brain protein kinase C isotypes a, b and g. *Eur. J. Biochem.* **182** 129-137.

(261) Ido, M., Sekigushi, K., Kikkawa, U. and Nishizuka, Y. (1987) Phosphorylation of the EGF receptor from A431 epidermoid carcinoma cells by three distinct types of protein kinase C. *FEBS Letts* **219** 215-218.

(262) Marais, R.M., Nguyen, O., Woodgett, J.R. and Parker, P.J. (1990) Studies on the primary sequence requirements for PKC-a , b_1 and g peptide substrates. *FEBS Letts.* **277** 151-155.

(263) Pelosin, J.M., Vilgrain, I. and Chambrez, E.M. (1987) A single form of protein kinase C is expressed in bovine adrenocortical tissue, as compared to four chromatographically resolved enzymes in rat brain. *Biochem. Biophys. Res. Commun.* **147** 382-391.

(264) Brandt, S.J., Niedel,J.E., Bell, R.M. and Young, W.S. (1987) Distinct patterns of expression of different protein kinase C mRNA's in rat tissue. *Cell* **49** 57-63.

(265) Huang, K.P., Nakabayashi, H. and Huang, F.L. (1986) Isozymic forms of rat brain Ca^{2+}-activated and phospholipid dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* **83** 8535-8539.

(266) Huang, F.L., Yoshida, Y., Nakabayashi, H. and Huang, K.P. (1987) Differential distribution of protein kinase C isozymes in the various regions of brain. *J. Biol. Chem.* **262** 15714-15720.

(267) Melloni, E., Pontremoli, S., Viotti, P.L., Patrone, M., Marks, P.A. and Rifkind, R.A. (1989) Differential expression of protein kinase C isozymes and erythroleukemia cell differentiation. *J. Biol. Chem.* **264** 18414-18418.

(268) Rose-john, S., Dietrich, S. and Marks, F. (1988) Molecular cloning of mouse protein kinase C (PKC) cDNA from swiss 3T3 fibroblasts. *Gene* **74** 465-471.

(269) Beh, I., Schmidt, R. and Hecker, E. (1989) Two isozymes of PKC found in HL-60 cells show a difference in activation by the phorbol ester TPA. *FEBS Letts.* **249** 264-266.

(270) Makowske, M., Ballester, R., Cayre, Y. and Rosen, O.M. (1988) Immunochemical evidence that three protein kinase C isozymes increase in abundance during HL-60 differentiation induced by dimethyl sulphoxide and retinoic acid. *J. Biol. Chem.* **263** 3402-3410.

(271) Hashimoto, K., Kishimoto, A., Aihara, H., Yasuda, T., Mikawa, K. and Nishizuka, Y. (1990) Protein kinase C during differentiation of human promyelocytic leukemia cell line, HL-60. *FEBS Letts.* **263** 31-34.

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

(273) Pontremoli, S., Melloni, E., Sparatore, B., Michetti, M., Salamino, F. and Horecker, B.L. (1990) Isozymes of protein kinase C in human neutrophils and their modification by two endogenous proteinases. *J. Biol. Chem.* **265** 706-712.

(274) Stasia, M.J., Strulovici, B., Daniel-Issakani, S., Pelosin, J.M., Dianoux, A.C., Chambaz, E. and Vignais, P.V. (1990) Immunocharacterisation of β- and ξ- subspecies of protein kinase C in bovine neutrophils. *FEBS Letts* **274** 61-64.

(275) Wooten, M.W. and Wrenn, R.W. (1988) Linoleic acid is a potent activator of protein kinase type III-α isoform in pancreatic acinar cells: Its role in amylase secretion. *Biochem. Biophys. Res. Commun.* **153** 67-73.

(276) Jaken, S., Kiley, S.C., Klauck, T., Dong, L. and Hyatt, S. (1990) Immunocytochemical localisation of protein kinase C. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek). Plenum Press. New York. pp245-252.

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

(278) Ase, K., Berry, N., Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1988) Differential down-regulation of protein kinase C subspecies in KM3 cells. *FEBS Letts* **236** 396-400.

(279) Sherman, M.S., Berry, N., Ase, K., Kikkawa, U. and Nishizuka, Y. (1988) Isolation of protein kinase C subspecies from a preparation of human T-lymphocytes. *FEBS Letts.* **234** 387-391.

(280) Hecker, E. (1985) Cell membrane associated protein kinase C as receptor of diterpene ester co-carcinogens of the tumour promoter type, and the phenotypic expression of tumours. Arzneimittel Forshung. **35** 2-15.

(281) Evans F.J. and Edwards M.C. (1987) Activity correlations in the phorbol ester series. *Bot. J. Linn. Soc.* **94** 231-246.

(282) Edwards, M.C., Evans, F.J., Barrett, M.L. and Gordon, D. (1985) Structural correlations of phorbol-ester-induced stimulation of PGE_2 production by human rheumatoid synovial cells. *Inflammation* **9** 33-38.

(283) Blumberg, P.M., Nakadate, T., Warren, B.S., Dell'Aquila, M., Sako, T., Pasti, G. and Sharkey, N.A. (1987) Phorbol esters as probes of the modulatory site on protein kinase C - an overview. *Bot. J. Linn. Soc.* **94** 283-292.

(284) Leli, U., Hauser, G. and Froimowitz, M. (1990) Requirements for the activation of protein kinase C: comparison of the molecular geometrics of phorbol and diacylglycerol. *Molec. Pharmacol.* **37** 286-295.

(285) Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R. and Dawson, A.P. (1990) Thapsigargin, a tumour promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} ATPase. *Proc. Natl. Acad. Sci. USA.* **87** 2466-2470.

(286) Berenblum, I. (1941) The co-carcinogenic action of croton resin. *Cancer Res.* **1** 44.

(287) Mottram, J.C. (1944) A developing factor in experimental blastogenesis. *J. Path. Bact.* **56** 181.

(288) Aldaz, C.M., Conti, C.J., Klein-Szanto, A.J.P. and Slaga, T.J. (1987) Progressive dysplasia and aneuploidy are hallmarks of mouse skin papillomas: relevance to malignancy. *Proc. Natl. Acad. Sci. USA.* **84** 2029-2032.

(289) Cerutti, P.A., Amstad, P. and Emerit, I. (1983) Tumour promoter phorbol-myristate-acetate induces membrane-mediated: chromosomal damage. In Radio Protectors and Anticarcinogens. Academic Press. 527-538.

(290) Berenblum, I. and Shubik, P. (1947) A new quantitative approach to the study of the stages of chemical carcinogenesis in mouse skin. *Br. J. Cancer.* **1** 383.

(291) Shubik, P. (1950) Studies on the promoting phase in the stages of carcinogenesis in mice, rats, rabbits and guinea pigs. *Cancer Res.* **10** 13.

(292) Edwards, M.C., Nouri, A.M.E., Gordon, D. and Evans, F.J. (1982) Tumour promoting and non-promoting pro-inflammatory esters act as human lymphocyte mitogens with different sensitivities to cyclosporin A. *Molec. Pharmacol.* **23** 703-708.

(293) Furstenberger, G., Kinzel, V., Schwarz, M. and Marks, F. (1985) Partial inversion of the initiation-promotion sequence of multistage tumourgenesis in the skin of NMR1 mice. *Science* **230** 76-78.

(294) Baxter, C.S., Andringa, A., Chalfin, K. and Miller, M.L. (1989) Comparative histomophometric changes in sencar mouse epidermis in response to multiple treatments with complete and stage specific tumour promoting agents. *Carcinogenesis* **10** 1855-1861.

(295) Fürstenberger, G., Sorg, B. and Marks, F. (1983) Tumour promotion by phorbol esters in skin evidence for a memory effect. *Science* **220** 89-90.

(296) Birnboim, H.C. (1983) Importance of DNA strand-break damage in tumour promotion. In Radioprotectors and anticarcinogens. Academic Press. 539-556.

(297) Hashimoto, Y., Chida, K., Huang, M., Nishihira, T. and Kuroki, T. (1989) Levels of protein kinase C activity in human gastro intestinal cancers. *Biochem. Biophys. Res. Commun.* **163** 406-411.

(298) Isakov, N., Bleackley, R.C., Shaw, J. and Altman, A. (1985) Teleocidin and phorbol ester tumour promoters exert similar mitogenic effects on human lymphocytes. *Biochem. Biophys. Res. Commun.* **130** 724-731.

(299) Verma, A.K. (1988)The protein kinase C activator L-α-dioctanoylglycerol a potent stage II mouse skin tumour promoter. *Cancer Res.* **48** 1736-1739.

(300) Nishino, H., Nishino, A., Takayasu, J., Hasegawa, T., Iwashima, A., Hirabayashi, K., Iwata, S. and Shibata, S. (1988) Inhibition of the tumour-promoting action of 12-O-tetradecanoylphorbol-13-acetate by some oleane-type triterpenoid compounds. *Cancer Res.* **48** 5210-5215.

(301) Megidish, T. and Mazurek, N. (1989) A mutant protein kinase C that can transform fibroblasts. *Nature* **342** 807-811.

(302) Matsui, M.S. and DeLeo, V.A. (1990) Induction of protein kinase C activity by ultraviolet radiation. *Carcinogenesis* **11** 229-234.

(303) Fleischman, L.F., Chahwala, S.B. and Cantley, L. (1986) Ras-transformed Cells: Altered levels of phosphatidylinositol-4,5-bisphosphate and catabolites. *Science* **231** 407-410.

(304) Preiss, J., Loomis, C.R., Bishop, W.R., Stein, R., Niedel, J.E. and Bell, R.M.(1986) Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and ras- and sis- transformed normal rat kidney cells. *J. Biol. Chem.* **261** 8597-8600.

(305) Schmidt, R.J. and Evans, F.J. (1979) Investigations into the skin-irritant properties of resiniferonol ortho esters. *Inflammation* **3** 273-280.

(306) Evans, F.J. and Schmidt, R.J. (1979) An assay procedure for the comparative irritancy testing of esters in the tigliane and daphnane series. *Inflammation* **3** 215-223.

(307) DeYoung, L.M., Kheifetsm J.B., Ballaron, S.J. and Young, J.M. (1989) Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents. *Agents and Actions* **26** 335-41.

(308) Szallasi, A. and 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.

(309) Barnes, P.J., Belvisi, M.G. and Rogers, D.F. (1990) Modulation of neurogenic inflammation: novel approaches to inflammatory disease. *TIPS* **11** 185-189.

(310) Foreman, J.C. (1987) Peptides and neurogenic inflammation. *Brit Med. Bulletin.* **43** 386-400.

(311) Szallasi, A. and Blumberg, P.M. (1989) A neurogenic component of phorbol ester induced mouse skin inflammation. *Cancer Res.* **49** 6052-6057.

(312) Szallasi, A. and Blumberg, P.M. (1989) Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. *Neuroscience.* **30** 515-520.

(313) Winter, J. (1987) Characterisation of capsaicin-sensitive neurones in adult rat dorsal root ganglion cultures. *Neurosci. Letts* **80** 134-140.

(314) Winter, J., Dray, A., Wood, J.N., Yeats, J.C. and Bevan, S. (1990) Cellular mechanism of action of resiniferatoxin: a potent sensory neuron exitotoxin. *Brain Res.* **520** 131-140.

(315) Gordge, P.C., Evans, A.T. and Evans, F.J. (1990) Evidence of a mixed aetiology in features of the erythema response to the potent irritant, resiniferatoxin. *J. Pharm. Pharmacol.* **43** 32.

(316) Rose-John, S., Furstenberger, G., Krieg, P., Besemfelder, E., Rincke, G. and Marks, F. (1988) Differential effects of phorbol esters on c-fos and c-myc and ornithine decarboxylase gene expression in mouse skin *in vivo*. *Carcinogenesis.* **9** 831-835.

(317) Isakov, N., Scholz, W. and Altman, A. (1986) Signal transduction and intra-cellular events in T - lymphocyte activation. *Immunol Today* **7** 271-277.

(318) Touraine, J.L., Hadden, J.W., Touraine, F., Estensen, R.D. and Good, R.A. (1977) Phorbol myristate acetate: A mitogen selective form a T-lymphocyte subpopulation. *J. Exp. Med.* **145** 460-465.

(319) Abb, J., Bayliss, G.J. and Deinhardt, F. (1979) Lymphocyte activation by the tumour-promoting agent 12-0-tetradecanoyl phorbol-13-acetate (TPA). *J. Immunol.* **122** 1639-1642.

(320) Krakauer, T., Mizel, D and Oppenheim, J.J. (1982) Independent and synergistic thymocyte proliferative activities of PMA and IL 1. *J. Immunol.* **129** 939-941.

(321) Huang, J.J., Newton, R.C., Horuk, R., Matthew, J.B., Covington, M., Pezzela, K. and Lin Y. (1987) Muteins of human interleukin 1 that show enhanced bioactivities. *FEBS Letts* **223** 294-298.

(322) Truneh, A., Albert, F., Golstein, P. and Schmitt-Verhulst, A.-M. (1985) Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature* **313** 318-320.

(323) Rosenstreich, D.L. and Mizel, S.B. (1979) Signal requirements for T lymphocyte activation. I. Replacement of macrophage function with phorbol myristic acetate. *J. Immunol.* **123** 1749-1754.

(324) Mastro, A.M. and Mueller, G.C. (1974) Synergistic action of phorbol esters in mitogen-activated bovine lymphocytes. *Exptl. Cell. Res.* **88** 40-46.

(325) Mizushima, Y., Kosaka, H., Sakuma, S., Kanda, K., Itoh, K., Osugi, T., Mizushima, A., Hamaoka, T., Yoshida, H., Sobue, K. and Fujiwara, H. (1987) Cyclosporin A inhibits late steps of T-lymphocytes activation after transmembrane signalling. *J. Biochem.* **102** 1193-1201.

(326) Mire, A.R., Wickremasinghe, R.G. and Hoffbrand, A.V. (1986) Phytohaemagglutinin treatment of T-lymphocytes stimulates rapid increases in activity of both particulate and cytosolic protein kinase C. *Biochem. Biophys. Res. Commun.* **137** 128-134.

(327) Gelfand, E.W., Cheung, R.K., Mills, G.B. and Grinstein, S. (1985) Mitogens trigger a calcium-independent signal for proliferation in phorbol-ester treated lymphocytes. *Nature* **315** 419-420.

(328) McNeil, P.L., McKenna, M.P. and Taylor, D.L. (1985) A transient rise in cytosolic calcium follows stimulation of quiescent cells with growth factors and is inhibitable with phorbol myristate acetate. *J. Cell. Biol.* **101** 372-379

(329) Hesketh, T.R., Moore, J.P., Morris, J.D.H., Taylor, M.V., Rogers, J., Smith, G.A and Metcalfe, J.C. (1985) A common sequence of calcium and pH signals in the mitogenic stimulation of eukaryotic cells. *Nature* **313** 481-484.

(330) Hirata, F., Matsuda, K., Notsu, Y., Hattori, T. and Delcarmine, R. (1984) Phosphorylation at a tyrosine residue of lipomodulin in mitogen-stimulated murine thymocytes. *Proc. Natl. Acad. Sci. USA.* **81** 4717-4721.

(331) Stadler, B.M., Dougherty, S.F., Farrar, J.J. and Oppenheim, J.J. (1981) Relationship of cell cycle to recovery of IL2 activty from human mononuclear cells, human and mouse T-cell lines. *J. Immunol.* **127** 1936-1940.

(332) Stern, J.B. and Smith, K.A. (1986) Interleukin-2 induction of T-cell G_1 progression and c-myb expression. *Science.* **233** 302-306.

(333) Dicker, P. and Rozengurt, E. (1978) Stimulation of DNA synthesis by tumour promoter and pure mitogenic factors. *Nature* **276** 723-726.

(334) Ledbetter, J.A., June, C.H., Grosmaire, L.S. and Rabinovitch, P.S. (1987) Crosslinking of surface antigens causes mobilisation of intracellular ionized calcium in T-lymphocytes. *Proc. Natl. Acad. Sci. USA.* **84** 1384-1388.

(335) Wang, J.L., McClain, D.A. and Edelman, G.M. (1975) Modulation of lymphocyte mitogenesis. *Proc. Natl. Acad. Sci. USA.* **72** 1917-1921.

(336) Estensen, R.D., Drazick, B.F. and Hadden, J.W. (1978) Phorbol myristate acetate as a lymphocyte mitogen, quantitation of effects in culture and relationship to binding and inhibition of analogs. In Carcinogenesis Mechanisms of Tumour Promotion And Co-Carcinogenesis. Vol.2. Raven Press. New York. pp379-388.

(337) Gillis, S., Crabtree, G.R. and Smith, K.A. (1979) Glucocorticoid induced inhibition of T cell growth factor production. 1. The effect of mitogen-induced lymphocyte proliferation. *J. Immunol.* **123** 1624-1631.

(338) Edwards, M.C., Evans, A.T. and Evans, F.J. (1989) Phorbol esters of different biological activities may preferentially act as mitogens of human suppressor T-cells and are equi-effective mitogens of IL-2 dependent cells. *J. Pharm.Pharmacol.* **41** 164-7.

(339) Larsson, E.L. (1980) Cyclosporin A and dexamethasone suppress T cell responses by selectively acting at distinct sites of the triggering process. *J. Immunol.* **124** 2828-2833.

(340) Uyemura, K., Dixon, J.F.P. and Parker, J.W. (1985) Cyclosporine inhibits macrophage accessory function in mitogen-induced lymphocyte proliferation. *Transplant. Proc.* **17** 1374-1377.

(341) Handschumacher, R.E., Harding, M.W., Rice, J. and Drugge, R.J. (1984) Cyclophilin: a specific cytosolic binding protein for cyclospoin A. *Science* **226** 5440547.

(342) McCrady, C.W., Li, F., Grant, A.J., Merchant, R.E. and Carchman, R.A. (1988) Alteration of human lymphokine-activated killer cell activity by manipulation of protein kinase C and cytosolic Ca^{2+}. *Cancer Res.* **48** 635-640.

(343) Hurley, D.J. and Mastro, A.M. (1987) Induction of suppressor activity by tumour promoting phorbol esters in primary cultures of lymph node cells. *Carcinogenesis* **8** 357-362.

(344) Russell, J.H., McCulley, D.E. and Taylor, A.S. (1986) Antagonistic effects of phorbol esters on lymphocyte activation. *J. Biol. Chem.* **261** 12643-12648.

(345) Miao, R.M., Fieldsteel, A.H. and Fodge, D.W. (1978) Opposing effects of tumour promoters on erythroid differentiation. *Nature* **274** 271-272.

(346) Giroldi, C., Hollstein, M. and Yamasaki, H. (1988) Cellular-oncogene expression in friend erythroleukemia cells : Relationship to differentiation, commitment and TPA effects. *Carcinogenesis* **9** 817-821.

(347) Rovera, G. Santoli, D. and Damsky, C. (1979) Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with phorbol ester. *Proc. Natl. Acad. Sci. USA.* **76** 2779-2783.

(348) Frank, D.A. and Sartorelli, A.C. (1988) Alterations in tyrosine phosphorylation during the granulocytic maturation of HL-60 leukemia cells. *Cancer Res.* **48** 52-58.

(349) McBain, J.M., Pettit, G. and Mueller, G.C. (1988) Bryostatin 1 antagonises the terminal differentiating action of TPA in a human colon cancer cell. *Carcinogenesis* **9** 123-129.

(350) Lipsey Hersh, C., Yeh, R.K., Callaway, J.E., Garcia, J.A. and Gilmore-Herbert, M. (1986) Induction of collagenase production in U937 cells by phorbol ester and partial purification of the induced enzyme. *Biochemistry* **25** 4750-4757.

(351) Thomas, N.S.B. (1989) Regulation of the product of a possible human cell cycle control gene cdc2Hs in B-cells by a-interferon and phorbol ester. *J. Biol. Chem.* **264** 13697-13700.

(352) Solanki, V., Slaga, T.J., Callaham, M. and Huberman, E. (1981) Down-regulation of specific binding of [20-^{3}H] phorbol 12,13-dibutyrate and phorbol ester-induced differentiation of human promyelocytic leukemia cells. *Proc. Natl. Acad. Sci. USA.* **78** 1722-1725.

(353) Deli, E., Kiss, Z. and Kuo J.F. (1988) Cooperative interactions of protein kinase C and cAMP-dependent protein kinase systems in human promyelocytic leukemia HL-60 cells. *FEBS Letts* **231** 407-412.

(354) Evans, A.T., McPhee, C., Beg, F., Evans, F.J. and Aitken, A. (1989) The ability of diterpene esters with selective biological effects to activate protein kinase C and induce HL-60 cell differentiation. *Biochem. Pharmacol.* **38** 2925-2927.

(355) Chiu, R., Imagawa, M., Imbra, R.J., Bockoven, J.R. and Karin, M. (1987) Multiple cis- and trans-acting elements mediate the transcriptional response to phorbol esters. *Nature* **329** 648-651.

(356) Spandidos, D.A., Nichols, R.A.B., Wilkie, N.M. and Pintzas, A. (1988) Phorbol ester-responsive H-ras 1 gene promoter contains multiple TPA-inducible/AP-1 binding consensus sequence elements. *FEBS Letts* **240** 191-5.

(357) Fujii, M., Nakamura, M., Ohtani, K., Sugamura, K. and Hinuma, Y. (1987) 12-O-tetradecanoylphorbol-13-acetate induces the enhancer function of human T-cell leukemia virus type 1. *FEBS Letts* **223** 299-303.

(358) Elsholtz, H.P., Mangalam, H.J., Potter, E., Albert, V.R., Supowit, S., Evans, R.M. and Rosenfeld, M.G. (1986) The different Cis-active elements transfer the transcriptional effects of both EGF and phorbol esters. *Science* **234** 1552-1557.

(359) Evans, A.T., Brooks, G. and Evans, F.J. (1990) Non-promoting diterpene esters can induce epstein-barr virus early antigen expression in the raji cell line. *Cancer Letts.* **49** 25-29.

(360) Zur Hauzen, H., O'Neill, F.J. and Freese, U.K. (1978) Persisting oncogenic herpes virus induced by the tumour promoter TPA. *Nature* **272** 373-375.

(361) Simmons, D.L., Levy, D.B., Yannoni, Y. and Erikson, R.L. (1989) Identification of a phorbol ester-repressible v-src-inducible gene. *Proc. Natl. Acad. Sci. USA.* **86** 1178-1182.

(362) Siebert, P.D. and Fukuda, M. (1985) Induction of cytoskeletal vimentin and actin gene expression of a tumour-promoting phorbol ester in human leukemic cell line K562. *J. Biol. Chem.* **260** 3868-3874.

(363) Mitchell, R.L., Zokas, L., Schreiber, R.D. and Verma, I.M. (1985) Rapid induction of the expression of proto-oncogene fos during human monocytic differentiation. *Cell* **40** 209-217.

(364) De Bustros, A., Baylin, S.B., Berger, C.L., Roos, B.A. and Leong, S.S. (1985) Phorbol esters increased calcitonin gene transcription and decrease c-myc mRNA in cultured human medullary thyroid carcinoma. *J. Biol. Chem.* **260** 98-104.

(365) Rozengurt, E., Erusalimsky, J., Mehmet, H., Morris, C., Nanberg, E. and Sinnet-Smith, J. (1988) Signal transduction in mitogenesis: Further evidence for multiple pathways. Cold Spring Harbor Symposium on Quantitative Biology **53** 945-954.

(366) Matrisian, L.M., Bowden, G.T., Krieg, P., Fursten Berger, G., Briand, J.P., Leroy, P. and Breathnach, R. (1986) The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumours. *Proc. Natl. Acad. Sci. USA.* **83** 9413-9417.

(367) Carrera, A.C., Cardenas, L, Tugores, A., Alonso, M., Sanchez-Madrid, F. and De Landazur, M.O. (1989) Activators of protein kinase C up-regulate the cell surface expression of CD2 and CD5 cell glycoproteins. *J. Biol. Chem.* **264** 15650-15655.

(368) Klug, A. and Rhodes, D. (1987) Zinc fingers: A novel protein motif for nucleic acid recognition. *TIBS* **12** 464-469.

(369) Hata, A., Akita, Y., Konno, Y., Suzuki, Y. and Ohno, S. (1989) Direct evidence that the kinase activity of protein kinase C is involved in transcriptional activation through a TPA-responsive element. *FEBS Letts.* **252** 144-146.

(370) Blenis, J. and Erikson, R.L. (1985) Regulation of a ribosomal protein S6 kinase activity by the Rous sarcoma virus transforming protein, serum, or phorbol ester. *Proc. Natl. Acad. Sci. USA.* **82** 7621-7625.

(371) Sahyoun, N., Wolf, M., Besterman, J., Hsieh, T-S., Sander, M., LeVine, H., Chang, K.-J. and Cuatrecasas, P. (1986) Protein kinase C phosphorylates topoisomerase II: Topoisomerase activation and its possible role in phorbol ester-induced differentiation of HL-60 cells. *Proc. Natl. Acad. Sci. USA.* **83** 1603-1607.

(372) Jagielski, P., Gregg, S.L. and Gajl-peczalka, J. (1983) TPA, tumour promoter-induced suppression of immunoglobulin secretion in human blood lymphocytes. *J. Immunol.* **130** 1159-63.

(373) Haimann, C., Meldolesi, J. and Ceccarelli, B. (1987) The phorbol ester 12-O-tetradecanoylphorbol-13-acetate enhances the evoked quanta release of acetylcholine at the frog neuromuscular junction. *Pfleugers Archives* **408** 27-31.

(374) Zawalich, N., Brown, C. and Rasmussen, H. (1983) Insulin secretion: combined effects of phorbol ester and A23187. *Biochem. Biophys. Res. Commun.* **117** 448-455.

(375) Zurgil, N. and Zisapel, N. (1985) Phorbol ester and calcium act synergistically to enhance neurotransmitter release by brain neurones in culture. *FEBS Letts.* **185** 257-261.

(376) Jacobsen, S., Hansen, H.S. and Jensen, B. (1987) Synergism between thapsigargin and the phorbol ester 12-0-tetradecanoylphorbol-12-acetate on the release of [^{14}C] arachidonic acid and histamine from rat peritoneal mast cells. *Biochem. Pharmacol.* **36** 621-626.

(377) Fuse, I., Iwanaga, T. and Tai, H. (1989) Phorbol ester, 1,2-diacylglycerol and collagen induce inhibition of arachidonic acid incorporation into phospholipids in human platelets. *J. Biol. Chem.* **264** 3890-3895.

(378) Edwards, M.C., Williamson, E.M., Formukong, E., Brooks, G. and Evans, F.J. (1987) Secretion and general properties of a polypeptide factor generated by phorbol ester's stimulation of human blood platelets. *Biochem. Pharmacol.* **36** 2421-4.

(379) Aitken, A. The Biochemical mechanism of action of phorbol esters. In *Naturally Occuring Phorbol Esters.* (ed. F. J. Evans) CRC Press, Boca Raton, USA. pp 271-288.

(380) Rink, T.J., Sanchez, A and Hallam, T.J. (1983) Diacylglyerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature*. **305** 317-319.

(381) Naka, M., Nishizuka, M., Adelstein, R.S. and Hidaka, H. (1983) Phorbol ester-induced activation of human platelets is associated with protein kinase C phosphorylation of myosin light chains. *Nature* **306** 490-492.

(382) Gennaro, R., Pozzan, T. and Romeo, D. (1984) Monitoring of cytosolic free Ca^{2+} in C5a-stimulated neutrophils: Loss of receptor-modulated Ca^{2+} stores and Ca^{2+} uptake in granule-free cytoblasts. Proc. Natl. Acad. Sci. USA. **81** 1416-1420

(383) Pozzan, T., Lew, D.P., Wollheim, C.B. and Tsien, R.Y. (1983) Is cytosolic ionized calcium regulating neutorphil activation? *Science* **221** 1413-1415.

(384) Bianca, V.D., Grzeskowiak, M. and Rossi, F. (1990) Studies on molecular regulation of phargocytosis and activation of the NADPH oxidase in neutrophils. *J. Immunol.* **144** 1411-1417.

(385) Robinson, J.M., Badwey, J.A., Karnovsky, M.L., and Karnovsky, M.J. (1984) Superoxide release by neutrophils: synergistic effects of a phorbol ester and a calcium ionophore. *Biochem. Biophys. Res. Commun.* **122** 734-739.

(386) Brozna, J.P., Hauff, N.F., Phillips, W.A. and Johnston, R.B. (1988) Activation of the respiratory burst in macrophages. *J. Immunol.* **141** 1642-1647.

(387) Ambruso, D.R., Bolscher, B.G.J.M., Stokman, R.M., Verhoeven, A.J. and Roos, D. (1990) Assembly and activation of the NADPH:O^2 oxidoreductase in human neutrophils after stimulation with phorbol myristate acetate. *J. Biol. Chem.* **265** 924-930.

(388) Perin, M.S., Fried, V.A., Mignery, G.A., Jahn., R. and Sudhof, T.C. (1990) Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. *Nature*. **345** 260-263.

(389) Touqui, L, Rothhut, B., Shaw, A.M., Fradin, A., Vargaftig, B.B. and Russo-Marie, F. (1986) Platelet activation - a role for the 40k anti-phospholipase A_2 protein indistinguishable from lipocortin. *Nature* **321** 177-180.

(390) Mullmann, T.J., Siegel, M.L., Egan, R.W., and Motasim, B. (1990) Phorbol-13-myristate-12-acetate activation of phospholipase D in human neutrophils leads to the production of phosphatides and diglycerides. *Biochem. Biophys. Res. Commun.* **170** 1197-1202.

(391) Lapetina, E.G. (1990) Eicosanoid formation and regulation of phospholipase A_2. In Biology of Cellular Transducing Signals. (ed. J.Y. Vanderhoek) Plenum Press. New York. pp275-281.

(392) Inoue, H., Mori, T., Shibata, S. and Koshihara. (1989) Modulation by glycyrrhetinic acid derivatives of TPA-induced mouse ear oedema. *Brit. J. Pharmacol.* **96** 204-10.

(393) Kurachi, Y., Ito, H., Sugimoto, T., Shimizu, T., Miki, I and Ui, M. (1989) Arachidonic acid metabolites as intracellular modulators of the G-protein-gated cardiac K+ channel. *Nature* **337** 555-557.

(394) Glatz, J.A., Muir, J.G. and Murray, A.W. (1987) Direct evidence for phorbol ester-stimulated accumulation of diacylglycerol derived from phosphatidyl-choline. *Carcinogenesis* **8** 1943-5.

(395) Takuwa, N., Takuwa, Y and Rasmussen, H. (1987) A tumour promoter, 12-0-tetradecanoylphorbol-13-acetate, increases cellular 1,2-diacylglycerol content through a mechanism other than phosphoinositide hydrolysis in Swiss mouse 3T3 fibroblasts. *Biochem. J.* **243** 647-653.

(396) Catalioto, R.M., Ailhaud, G. and Negrel, R. (1990) Diacylglycerol production induced by growth hormone in OB1771 preadipocytes arises from phosphatidylcholine breakdown. *Biochem. Biophys. Res. Commun.* **173** 840-848.

(397) Baraban, J.M., Snyder, S.H. and Alger, B.E. (1985) Protein kinase C regulates ionic conductance in hippocampal pyramidal neurones: electrophysiological effects of phorbol esters. *Proc. Natl. Acad. Sci. USA.* **82** 2538-2542.

(398) Malenka, R.C., Madison, D.V. and Nicoll, R.A. (1986) Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature* **321** 175-177.

(399) Doerner, D., Pitler, T.A., and Algar, B.E. (1988) Protein kinase C activators block specific calcium and potassium current components in isolated hippocampal neurones. *J. Neuroscience* **8** 4069-4078.

(400) Akers, R.F., Lovinger, D.M., Colley, P.A., Linden, D.J. and Routtenberg, A. (1986) Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. *Science.* **231** 587-589.

(401) Apel, E.D., Byford, M.F., Au, D., Walsh, K.A. and Storm, D.R. (1990) Identification of the protein kinase C phosphorylation site in neuromodulin. *Biochem.* **29** 2330-2335.

(402) Hu, G.Y., Hvalby, O., Walaas, S.I., Albert, K.A., Skjeflo, P., Anderson, P. and Greengard, P. (1987) Protein kinase C injection into hippocampal pyramidal cells elicits features of long term potentiation. *Nature* **328** 426-429.

(403) Rane, S.G. and Dunlap, K. (1986) Kinase C activator 1,2-oleoylacetylglycerol attenuates voltage-dependent calcium current in sensory neurons. *Proc. Natl. Acad. Sci. USA.* **83** 184-188.

(404) Deriemer, S.A., Strong, J.A., Albert, K.A., Greengard, P. and Kaczmarek, L.K. (1985) Enhancement of current in aplysia neurones by phorbol esters and protein kinase. *Nature* **313** 313-315.

(405) Farley, J. and Auerbach, S. (1986) Protein kinase C activation induces conductane changes in hermissenda photoreceptors like those seen in associative learning. *Nature* **319** 220-223.

(406) Hemmings, H.C., Nairn, A.C., McGuinness, T.L.M., Huganir, R.L. and Greengard, P. (1989) Role of protein phosphorylation in neuronal signal transduction. *FASEB J.* **3** 1583-1592.

(407) Malenka, R.C., Ayoub, G.S. and Nicoll, R.A. (1987) Phorbol esters enhance transmitter release in rat hippocampal slices.*Brain Res.* **403** 198-203.

(408) Di Virgillo, F., Pozzan, T., Wolheim, C.B, Vincentin, L.M. and Meldolesi, J. (1986) Tumor promoter phorbol myristate acetate inhibits Ca^{2+} influx through voltage-gated Ca^{2+} channels in two secretory cell lines, PC12 and RINm5F. *J. Biol. Chem.* **262** 32-35.

(409) Nasainczyk, W., Rohrkasten, A., Sieber, M., Rudolph, C., Schachiele, C., Marme, D. and Hofmann, F. (1987) Phosphorylation of the purified receptor for calcium channel blockers by cAMP kinase and protein kinase C. *Eur. J. Biochem.* **169** 137-142.

(410) Vigne, P., Breittmayer, J.P, Duval, D., Freun, C. and Lazdunski, M. (1988) The Na^{+}/Ca^{2+} antiporter in aortic smooth muscle cells. *J. Biol. Chem.* **263** 8078-8083.

(411) Vincentini, L.M. and Villereal, M.L. (1985) Activation of Na^{+}/H^{+} exchange in cultured fibroblasts: Synergism and antagonism between phorbol esters, Ca^{2+} ionophore, and growth factors. *Proc. Natl. Acad. Sci. USA.* **82** 8053-8056.

(412) Vigne, P., Frelin, C. and Lazdunksi, M. (1985) The Na^{+}/H^{+} antiport is activated by serum and phorbol esters in proliferating myoblasts but not in differentiated myotubes. *J. Biol. Chem.* **260** 8008-8013.

(413) Besterman, J.M. and Cuatrecasas, P. (1984) Phorbol esters rapidly stimulate amiloride-sensitive Na^{+}/H^{+} exchange in a human leukemic cell line. *J. Cell. Biology.* **99** 340-343.

(414) Vara, F., Schneider, J.A. and Rozengurt, E. (1985) Ionic responses rapidly elicited by activation of

protein kinase C in quiescent Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA.* **82** 2384-2388.

(415) Maly, K., Uberall, F., Loferer, H., Doppler, W., Oberhuber, H., Groner, B. and Gruncke, H.H. (1989) Ha-ras activates the Na^+/H^+ antiporter by a protein kinase C-independent mechanism. *J. Biol. Chem.* **264** 11839-11842.

(416) Woods, N.M., Cuthbertson, K.S.R. and Cobbold, P.H. (1987) Phorbol ester induced alterations of free calcium ion transients in single rat hepatocytes. *Biochem. J.* **246** 619-23.

(417) Leeb-Blundberg, L.M.F., Cotecchia, S., DeBlasi and A., Caron, M.G. (1987) Regulation of adrenergic receptor function by phosphorylation of α1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT1 MF-2 smooth muscle cells. *J. Biol. Chem.* **262** 3098-3105.

(418) Lynch, C.J., Charest, R., Bocckino, S.B., Exton, J.H. and Blackmore, P.F. (1985) Inhibition of hepatic α1-adrenergic effects and binding by phorbol myristate acetate. *J. Biol. Chem.* **260** 2844-2851.

(419) Leeb-Lundberg, L.M.F., Cotecchia, S., Lomasney, J.W., DeBernardis, J.F., Lefkowitz, R.J. and Caron, M.G. (1985) Phorbol esters promote α1-adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. *Proc. Natl. Acad. Sci. USA.* **82** 5651-5655.

(420) Corvera, S. and Garcia-Sainz, J.A. (1984) Phorbol esters inhibit α1-adrenergic stimulation of gycogenolysis in isolated rat hepatocytes. *Biochem. Biophys. Res. Commun.* **119** 1128-1133.

(421) Sibley, D.R., Nambi, P., Peters, J.R. and Lefkowitz, R.J. (1984) Phorbol diesters promote β-adrenergic receptor phosphorylation and adenylate cyclase desensitisation in duck erythrocytes. *Biochem. Biophys. Res. Commun.* **121** 973-979.

(422) Kelleher, D.J., Pessin, J.E., Ruoho, A.E. and Johnson, G.L. (1984) Phorbol ester induces desensitisation of adenylate cyclase and phosphorylation of the β-adrenergic receptor in turkey erythrocytes. *Proc. Natl. Acad. Sci. USA.* **81** 4316-4320.

(423) Huganir, R.L., Albert, K.A. and Greengard, P. (1983) Phosphorylation of the nicotinic acetylcholine receptor by Ca^{2+}/phospholipid-dependent protein kinase, and comparison with its phosphorylation by cAMP-dependent protein kinase. *Soc. Neuroscience.* Abstract 168.8.

(424) Garte, S.J. and Belman, S. (1980) Tumour promoter uncouples β-adrenergic receptor from adenylate cyclase in mouse epidermis. *Nature* **284** 171-173.

(425) Garte, S.J., Currie, D. and Belman, S. (1983) Inhibition of β-adrenergic response in cultured epidermal cells by phorbol myristate acetate. *Carcinogenesis* **4** 939-940.

(426) Bell, J.D., Buxton, I.L.O. and Brunton, L.L. (1985) Enhancement of adenylate cyclase activity in S49 lymphoma cells by phorbol esters. *J. Biol. Chem.* **260** 2625-2628.

(427) Eusebi, F., Molinaro, M. and Zani, B.M. (1985) Agents that activate protein kinase C reduce acetylcholine sensitivity in culture myotubes. *J. Cell. Biol.* **100** 1339-1342.

(428) Liles, W.C., Hunter, D.D., Meier, K.E. and Nathanson, N.M. (1986) Activation of protein kinase C induces rapid internalization and subsequent degradation of muscarinic acetylcholine receptors in neuroblastoma cells. *J. Biol. Chem.* **261** 5307-5313.

(429) Orellana, S., Solski, P.A. and Brown, J.H. (1987) Guanosine 5'-O-(thio-phosphate)-dependent inositol triphosphate formation in membranes is inhibited by phorbol ester and protein kinase C. *J. Biol. Chem.* **262** 1638-1643.

(430) Cochet, C., Gill, G.N., Meisenhelder, J., Cooper, J.A and Hunter, T. (1984) C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. *J. Biol. Chem.* **259** 2553-2558.

(431) Downward, J., Waterfield, M.D. and Parker, P.J. (1985) Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* **260** 14538-14546.

(432) Brooks, G. and Brooks, S.F. (1990) Both tumour-promoting and non-promoting phorbol esters inhibit [^{125}I] EGF binding and stimulate the phosphorylation of an 80kD protein kinase C substrate in intact quiescent Swiss 3T3 cells. *Carcinogenesis*. **11** 667-672.

(433) Barrowman, M.M., Cockcroft, S. and Gomperts, B.D. (1986) Potentiation and inhibition of secretion from neutrophils by phorbol ester. *FEBS Letts.* **201** 137 -142.

(434) Naccache, P.H., Molski, T.F.P., Borgeat, P., White, J.R. and Sha'afi, R.I. (1985) Phorbol esters inhibit the fmet-leu-phe- and leukotriene B_4-stimulated calcium mobilisation and enzyme secretion in rabbit neutrophils. *J. Biol. Chem.* **260** 2125-2131.

(435) Blake, A.D. and Strader, C.D. (1986) Potentiation of specific association of insulin with HEP G2 cells by phorbol esters. *Biochem. J.* **236** 227-234.

(436) Takayama, S., White, M.F., Lauris, V. and Kahn, C.R. (1984) Phorbol esters modulate insulin receptor phosphorylation and insulin action in cultured hepatoma cells. *Proc. Natl. Acad. Sci. USA.* **81** 7797-7801.

(437) O'Flaherty, J.T., Redman, J.F and Jacobson, D.P. (1986) Protein kinase C regulates leukotriene B_4 receptors in human neutrophils. *FEBS Letts.* **206** 279-282.

(438) Richie, E.R., McEntire, B., Phillip, J. and Allison J.P. (1988) Altered expression of lymphocyte differentiation antigens on phorbol ester-activated CD4+8+ T-cells. *J. Immunol.* **140** 4115-4122.

(439) Cantrell, D.A., Verbi, W., Davies, A., Parker, P. and Crumpton, M.J. (1988) Evidence that protein kinase C differentially regulates the human T lymphocyte CD2 and CD3 surface antigens. *Eur. J. Immunol.* **18** 1391-1396.

(440) May, W.S., Jacobs, S. and Cuatrecasas, P. (1984) Association of phorbol ester induced hyperphosphorylation and reversible regulation of transferrin membrane receptors in HL-60 cells. *Proc. Natl. Acad. Sci. USA.* **81** 2016-2020.

(441) May, W.S., Sahyoun, N., Jacobs, S., Wolf, M. and Cuatrecasas. P. (1985) Mechanism of phorbol diester-induced regulation of surface transferrin receptor involves the action of activated protein kinase C and an intact cytoskeleton. *J. Biol. Chem.* **260** 9419-9426.

(442) Coffrey, R.G. and Hadden, J.W. (1983) Phorbol myristate acetate stimulation of lymphocyte guanylate cyclase and cyclic guanosine 3'-5'-monophosphate phosphodiesterase and reduction of adenylate cyclase. *Cancer Res.* **43** 150-158.

(443) Klausner, R.D., Hareford, J. and Van Renswoude, J. (1984) Rapid internalisation of the transferrin receptor in K562 cells is triggered by ligand binding or treatment with a phorbol ester. *Proc. Natl. Acad. Sci. USA.* **81** 3005-3009.

(444) Coffrey, R.G. and Hadden, J.W. (1981) Phorbol myristate acetate (PMA) stimulation of lymphocyte guanylate cyclase. *Fed. Proc.* **40** Abstract 1091.

(445) Zwiller, J., Revel, M-O. and Malviya, A.N. (1985) Protein kinase C catalyzes phosphorylation of guanylate cyclase *in vitro*. *J. Biol. Chem.* **260** 1350-1353.

(446) Nordstedt, C., Kuanta, A., Van der Plogg, I. and Fredholm, B.B. (1989) Dual effects of protein kinase C on receptor-stimulated cAMP accumulation in a human T-cell leukemia line. *Eur. J. Biol.* **172** 51-60.

(447) Yoshimasa, T., Sibley, D.R., Bouvier, M., Lefkowitz, R.J. and Caron, M.G. (1987) Cross-talk between signalling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. *Nature* **327** 67-70.

(448) Jakobs, K.H., Bauer, S and Watanabe, Y. (1985) Modulation of adenylate cyclase of human platelets by phorbol ester. Impairment of the hormone-sensitive inhibitory pathway. *Eur. J. Biochem.* **151** 425-530.

(449) Katada, T, Gilman, A.G., Watanabe, Y. Bauer, S and 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. *Eur. J. Biochem.* **151** 431-437.

(450) Summers, S.T. and Cronin, M.J. (1986) Phorbol esters enhance basal and stimulated adenylate cyclase activity in a pituitary cell line. *Biochem. Biophys. Res. Commun.* **135** 276-281.

(451) Kiss, Z. and Steinberg, R.A. (1985) Interactions between cyclic AMP and phorbol ester-dependent phosphorylation systems in S49 mouse lymphoma cells. *J. Cell. Physiol.* **125** 200-206.

(452) Narindrasorasak, S., Brickenden, A., Ball, E. and Sanwal, B.D. (1987) Regulation of protein kinase C by cyclic adenosine 3':5'-monophosphate and a tumour prometer in skeletal myoblasts. *J. Biol. Chem.* **262** 10497-10501.

(453) Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680-5.

(454) Kiss, Z. and Luo, Y. (1986) Phorbol ester and 1,2-diolein are not fully equivalent activators of protein kinase C in respect to phosphorylation of membrane proteins *in vitro*. *FEBS Letts* **198** 203-207.

(455) Smith, B.M. and Colburn, N.H. (1988) Protein kinase C and its substrates in tumour promoter sensitive and resistant cells. *J. Biol. Chem.* **263** 6424-6431.

(456) Reibman, J., Korcha, H.M., Yosshall, L.B., Haines, K.A., Rich, A.M. and Weissmann, G. (1988) Changes in diacylglycerol labelling, cell shape, and protein phosphorylation distinguish "triggering" from "activation" of human neutrophils. *J. Biol. Chem.* **263** 6322-6328.

(457) Huang, C-K., Hill, J.M., Bormann, B-J., Mackin, W.M. and Becker, E.L. (1983) Endogenous substrates for cyclic AMP-dependent and calcium-dependent protein phosphorylation in rabbit peritoneal neutrophils. *Biochim. Biophys. Acta.* **760** 126-135.

(458) Yuan, S. and Sen, A.K. (1986) Characterization of the membrane-bound protein kinase C and its substrate proteins in canine cardiac sarcolemma. *Biochim. Biophys. Acta.* **886** 152-161.

(459) Wrenn, R.W., Katoh, N., Wise, B.C. and Kuo, J.F. (1980) Stimulation by phosphatidylserine and calmodulin of calcium dependent phosphorylation of endogenous proteins from cerebral cortex. *J. Biol. Chem.* **255** 12042-12046.

(460) Hornbeck, P. and Paul, W.E. (1986) Anti-immunoglobulin and phorbol ester induce phosphorylation of proteins associated with the plasma membrane and cytoskeleton in murine β lymphocytes. *J. Biol. Chem.* **261** 14817-14824.

(461) Ling, E. and Sapirstein, V. (1984) Phorbol ester stimulates the phosphorylation of rabbit erythrocyte band 4.1. *Biochem. Biophys. Res. Commun.* **120** 291-298.

(462) Cantrell, D.A., Davies, A.A. and Crumpton, M.J. (1985) Activators of protein kinase C down regulate and phosphorylate the T3/T-cell antigen receptor complex of human T-lymphocytes. *Proc. Natl. Acad. Sci. USA.* **82** 8158-8162.

(463) Shackelford, D.A. and Trowbridge, I.S. (1984) Induction of expression and phosphorylation of the human interleukin 2 receptor by a phorbol diester. *J. Biol. Chem.* **259** 11706-11712.

(464) Gallis, B., Lewis, A., Wignall, U., Alpert, A., Mochizuki Cosman, D., Hopp, T. and Urdal, D. (1986) Phosphorylation of the human interleukin 2 receptor and a synthetic peptide identical to its C-terminal,cytoplasmic domain. *J. Biol. Chem* **261** 5075-5080.

(465) Gentry, L.E., Chaffin, K.E., Shoyab, M. and Purchio, A.F. (1986) Novel serine phosphorylation of pp60-c-src in intact cells after tumour promoter treatment. *Mol. Cell. Biol.* **6** 735-738.

(466) Lim, M.S., Sutherland, C. and Walsh, M.P. (1985) Phosphorylation of bovine cardiac C - protein by protein kinase C. *Biochem. Biophys. Res. Commun.* **132** 1187-1195.

(467) Tsuyama, S., Bramblett, G.T., Huang, K.-P. and Flavin, M. (1986) Calcium/phospholipid-dependent kinase recognizes sites in microtubule-associated protein 2 which are phosphorylated in living brain and are not accessible to other kinases. *J. Biol. Chem.* **261** 4110-4116.

(468) Gould, K.L., Woodgett, J.R., Cooper, J.A., Buss, J.E., Shalloway, D. and Hunter, T. (1985) Protein kinase C phosphorylates pp60src at a novel site. *Cell* **42** 849-857.

(469) O'Brian, C.A., Lawrence, D.S., Kaiser, E.T. and Weinstein, I.B. (1984) Protein kinase C phosphorylates the synthetic peptide arg-arg-lys-ala-gly2-pro-pro-val in the presence of phospholipid plus either Ca^{2+} or a phorbol ester tumor promoter. *Biochem. Biophys. Res. Commun.* **124** 296-302.

(470) Ferrari, S., Marchiori, F., Borin, G. and Pinna, L.A. (1985) Distinct structural requirements of Ca2+/phospholipid-dependent protein kinase (protein kinase C) and cAMP-dependent protein kinase as evidenced by synthetic peptide substrates. *FEBS Letts.* **184** 72-77.

(471) Davis, R.J. and Czech, M.P. (1984) Tumor-promoting phorbol diesters mediate phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* **259** 8545-8549.

(472) Davis, R.J., Ganong, B.R., Bell, R.M. and Czech, M.P. (1985) sn-1,2-diotanoylglycerol. A cell-permeable diacylglycerol that mimics diester action on the epidermal growth factor receptor and mitogenesis. *J. Biol. Chem.* **260** 1562-1566.

(473) Wise, B.C., Guidotti, A. and Costa, E. (1983) Phosphorylation induced a decrease in the biological activity of the protein inhibitor (gaba-modulin) of γ-aminobutyric acid binding sites. *Proc. Natl. Acad. Sci. USA.* **8**0 886-890.

(474) Witters, L.A., Vater, C.A. and Lienhard, G.E. (1985) Phosphorylation of the glucose transporter *in vitro* and *in vivo* by protein kinase C. *Nature* **315** 777-778.

(475) Allard, W.J., Gibbs, E.M., Witters, L.A. and Leinhard, G.E. (1986) Increased glucose transport in response to phorbol ester, growth factors, and insulin: relationship to phosphorylation of the glucose transporter. *Fed. Proc.* **45** Abstract 416.

(476) Carlson, K.E., Brass, L.F. and Manning, D.R. (1989) Thrombin and phorbol esters cause the selective phosphorylation of a G protein other than Gi in human platelets. *J. Biol. Chem.* **264** 13298-13305.

(477) Feuerstein, N., Monos, D.S. and Cooper, H.L. (1985) Phorbol ester effect in platelets, lymphocytes and leukemic cells (HL-60) is associated with enhanced phosphorylation of Class I HLA antigens. *Biochem. Biophys. Res. Commun.* **126** 206-213.

(478) Jacobs, S., Sahyoun, N.E., Saltiel, A.R. and Cuatrecasas, P. (1983) Phorbol esters stimulate the phosphorylation of receptors for insulin and somatomedin C. *Proc. Natl. Acad. Sci. USA.* **80** 6211-6213.

(479) Leonard, W.J., Depper, J.M., Kronke, M., Robb, R.J., Waldmann, T.A. and Greene, W.C. (1985) The human receptor for T-cell growth factor. *J. Biol. Chem.* **260** 1872-1880.

(480) Zick, Y., Sagi-Eisenberg, R., Pines, M., Gierschik, P. and Spiegel, A.M. (1986) Multisite phosphorylation of the α subunit of transducin by the insulin receptor kinase and protein kinase C. *Proc. Natl. Acad. Sci. USA.* **83** 9294-9297.

(481) Costa, M.R.C. and Catterall, W.A. (1985) Phosphorylation of the α subunit of the sodium channel by protein kinase C. *Cell. Mol. Neurobiol.* **4** 291-297.

(482) Hardie, D.G., Carling, D., Ferrari, S., Guy, P.S. and Aitken, A. (1986) Characterisation of the phosphorylation of rat mammary ATP-citrate lyase and acetyl-coA carboxylase by Ca^{2+} and calmodulin-dependent multiprotein kinase and Ca^{2+}- and phospholipid-dependent protein kinase. *Eur. J. Biochem.* **157** 553-561.

(483) Vilgrain, I., Defaye, G. and Chambaz, E.M. (1984) Adrenocortical cytochrome p-450 responsible for cholesterol side chain cleveage (P450scc) is phosphorylated by the calcium-activated phospholipid sensitive protein kinase (protein kinase C). *Biochem. Biophys. Res. Commun.* **125** 554-561.

(484) Nettelblad, F.A. and Engstrom, L. (1987) The kinetic effects of *in vitro* phosphorylation of rabbit muscle enolase by protein kinase C. *FEBS Letts.* **214** 249-252.

(485) Ahmad, Z., Lee, F-T., DePaoli-Roach, A. and Roach, P.J. (1984) Phosphorylation of glycogen synthase by the Ca^{2+}- and phospholipid-activated protein kinase (protein kinase C). *J. Biol. Chem.* **259** 8743-8747.

(486) Arino, J. and Guinovart, J.J. (1986) Phosphorylation and inactivation of rat hepatocyte glycogen synthase by phorbol esters and mezerein. *Biochem. Biophys. Res. Commun.* **134** 113-119.

(487) Imazu, M., Strickland, W.G., Chrisman, T.D. and Exton, J.H. (1984) Phosphorylation and inactivation of liver glycogen synthase by liver protein kinases. *J. Biol. Chem.* **259** 1813-1821.

(488) Jeng, A.Y., Srivastava, S.K., Lacal, J.C. and Blumberg, P.M. (1987) Phosphorylation of ras oncogene product by protein kinase C. *Biochem. Biophys. Res. Commun.* **145** 782-788.

(489) Beg, Z.H., Stonik, J.A. and Brewer, H.B. (1984) Phosphorylation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase and modulation of its enzymic activity by calcium-activated and phospholipid-dependent protein kinase. *J. Biol. Chem.* **260** 1682-1687.

(490) Ieyasu, H., Takai, Y., Kaibuchi, K, Sawamura, M. and Nishizuka, Y. (1982) A role of calcium-activated, phospholipid-dependent protein kinase in platelet-activating factor-induced serotonin release from rabbit platelets. *Biochem. Biophys. Res. Commun.* **108** 1701-1708.

(491) Lapetina, E.G., Reep, B., Ganong, B.R. and Bell, R.M. (1985) Exogenous sn-1,2-diacylglycerols containing saturated fatty acids functions as bioregulators of protein kinase C in human platelets. *J. Biol. Chem.* **260** 1358-1361.

(492) Hincke, M.T. and Toinai, S. (1986) Phosphorylation of bovine cardiac calcium-activated neutral protease by protein kinase-C. *Biochem. Biophys. Res. Commun.* **137** 559-565.

(493) Nettleblad, F.A., Forsberg, P.O., Humble, E. and Engström, L. (1986) Aspects on the phosphorylation of muscle phosphofructokinase by protein kinase C - inhibition by phosphofructokinase stabilisers. *Biochem. Biophys. Res. Commun.* **136** 445-453.

(494) Rider, M.H. and Hue, L. (1986) Phosphorylation of purified bovine heart and rat liver 6-phosphofructo-2-kinase by protein kinase C and comparison of the fructose-2,6-bisphosphate activity of the two enzymes. *Biochem.J.* **240** 57-61.

(495) Hofer, H.W., Schlatter, S and Graefe, M. (1985) Phosphorylation of phosphofructokinase by protein kinase C changes the allosteric properties of the enzyme. *Biochem. Biophys. Res. Commun.* **129** 892-897.

(496) Purchio, A.F., Shoyab, M. and Gentry, L.E. (1985) Site-specific increased phosphorylation of pp60-v-src after treatment of RSV-transformed cells with a tumour promoter. *Science* **229** 1393-1395.

(497) Albert, K.A., Helmer-Matyjek, E., Nairn, A.C., Müller, T.H., Haycock, J.W., Greene, L.A., Goldstein, M. and Greengard, P. (1984) Calcium/phospholipid dependent protein kinase (protein kinase C) phosphorylates and activates tyrosine hydroxylase. *Proc. Natl. Acad. Sci. USA.* **81** 7713-7717.

(498) Umekawa, H. and Hidaka, H. (1985) Phosphorylation of caldesmon by protein kinase C. *Biochem. Biophys. Res. Commun.* **132** 56-62.

(499) Ling, E., Gardner, K. and Bennett, V. (1986) Protein kinase C phosphorylates a recently identified membrane skeleton-associated calmodulin-binding protein in human erythrocytes. *J. Biol. Chem.* **261** 13875-13878.

(500) Horne, W.C., Leto, T.L. and Marchesi, V.T. (1985) Differential phosphorylation of multiple sites in protein 4.1 and protein 4.9 by phorbol ester-activated and cyclic AMP-dependent protein kinases. *J. Biol. Chem.* **260** 9073-9076.

(501) Palfrey, H.C. and Waseem, A. (1985) Protein kinase C in the human erythrocyte. *J. Biol. Chem.* **260** 16021-16029.

(502) Humble, E., Heldin, P., Forsberg, P.O. and Engstrom, L. (1984) Phosphorylation of human fibrinogen *in vitro* by calcium-activated phospholipid-dependent protein kinase from pig spleen. *J. Biochem.* **95** 1435-1443.

(503) Kawamoto, S. and Hidaka, H. (1984) Ca^{2+}-activated, phospholipid-dependent protein kinase catalyses the phosphorylation of actin-binding proteins. *Biochem. Biophys. Res. Commun.* **118** 736-742.

(504) Akiyame, T., Nishida, E., Ishida, J., Saji, N., Ogawara, H., Hoshi, M., Miyata, Y. and Sakai, H. (1986) Purified protein kinase C phosphorylates microtubule-associated protein z. *J. Biol. Chem.* **261** 15648-15648.

(505) Turner, R.S., Raynor, R.L., Mazzei, G.J., Girard, P.R. and Kuo, J.F. (1984) Developmental studies of phospholipid-sensitive Ca^{2+}-dependent protein kinase and its substrates and of phosphoprotein phosphatases in rat brain. *Proc. Natl. Acad. Sci. USA.* **81** 3143-3147.

(506) Kishimoto, A., Nishiyama, K., Nikanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y. and Nishizuka, Y. (1985) Studies on the phosphorylation of myelin basic protein by protein kinase C and adenosine 3':5'-monophosphate-dependent protein kinase. *J. Biol. Chem.* **260** 12492-12499

(507) Nishizuka, M., Hidaka, H. and Adelstein, R.S. (1983) Phosphorylation of smooth muscle heavy meromyosin by calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* **258** 14069-14072.

(508) Endo, T., Naka, M. and Hidaka, H. (1982) Ca^{2+}-phospholipid dependent phosphorylation of smooth muscle myosin. *Biochem. Biophys. Res. Commun.* **105** 942-948.

(509) Ikebe, M., Inagaki, M., Kanamaru, K. and Hidaka, H. (1985) Phosphorylation of smooth muscle myosin light chain kinase by Ca^{2+}-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* **260** 4547-4550.

(510) Nishikawa, M., Shirakana, S. and Adelstein, R.S. (1985) Phosphorylation of smooth muscle myosin light chain kinase by protein kinase C. *J. Biol. Chem.* **260** 8978-8983.

(511) Litchfield, D.W. and Ball, E.H. (1986) Phosphorylation of the cytoskeletal protein talin by protein kinases. *Biochem. Biophys. Res. Commun.* **134** 1276-1283.

(512) Mazzei, G.J. and Kuo, J.F. (1984) Phosphorylation of skeletal-muscle troponin I and troponin T by phospholipid-sensitive Ca^{2+}-dependent protein kinase and its inhibition by troponin C and tropomyosin. *Biochem. J.* **218** 361-369.

(513) Werth, D.K. and Pastan, I. (1984) Vinculin phosphorylation in response to calcium and phorbol esters in intact cells. *J. Biol. Chem.* **259** 5264-5270.

(514) Werth, D.K., Niedel, J.E. and Pastan, I. (1983) Vinculin, a cytoskeletal substrate of protein kinase C. *J. Biol. Chem.* **258** 11423-11426.

(515) DePaoli-Roach, A., Roach, P.J., Zucker, K.E. and Smith, S.S. (1986) Selective phosphorylation of human DNA methyltransferase by protein kinase C. *FEBS Lett.* **197** 149-153.

(516) Tuazon, P.T., Morley, S.J., Dever, T.E., Merrick, W.C., Rhoads, R.E. and Traugh, J.A. (1990) Association of initiation factor eIF-4E in a CAP binding protein complex (eIF-4F) is critical for and enhances phosphorylation by protein kinase C. *J. Biol. Chem.* **265** 10617-10621.

(517) Morley, S.J. and Trough, J.A. (1989) Phorbol esters stimulate phosphorylation of eukaryotic initiation factors 3, 4B and 4F. *J. Biol. Chem.* **264** 2401-2404.

(518) Tuazon, P.T., Merrick, W.C. and Traugh, J.A. (1989) Comparative analysis of phosphorylation of translational initiation and elongation factors by seven protein kinases. *J. Biol. Chem.* **264** 2773-2777.

(519) Morley, S.J. and Traugh, J.A. (1990) Differential stimulation of phosphorylation of initiation facots eIF-4F, eIF-4B, eIF-3 and ribosomal protein S6 by insulin and phorbol esters. *J. Biol. Chem.* **265** 10611-10616.

(520) Shatzman, R.C., Grifo, J.A., Merrick, W.C. and Kuo, J.F. (1983) Phospholipid-sensitive Ca^{2+}-dependent protein kinase phosphorylates the **b** subunit of eukaryotic initiation factor 2 (eIF-2). *FEBS Letts.* **159** 167-170.

(521) Iwasa, Y., Takai, Y., Kikkawa, U. and Nishizuka, Y. (1980) Phosphorylation of calf thymus H1 histone by calcium-activated, phospholipid-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **96** 180-187.

(522) Albert, K.A., Wu, W.C-S., Nairn, A.C. and Greengard, P. (1984) Inhibition by calmodulin of calcium/phospholipid-dependent protein phosphorylation. *Proc. Natl. Acad. Sci. USA.* **81** 3622-3625.

(523) Butler, A.P., Byus, C.V. and Slaga, T.J. (1986) Phosphorylation of histones is stimulated by phorbol esters in quiescent reuber H35 hepatoma cells. *J. Biol. Chem.* **261** 9421-9425.

(524) Burkhard, S.J. and Traugh, J.A. (1983) Changes in ribosome function by cAMP-dependent and cAMP-independent phosphorylation of ribosomal protein S6. *J. Biol. Chem.* **258** 14003-14008.

(525) Blenis, J., Spivack, J.G. and Lerikson, R. (1984) Phorbol ester, serum and rous sarcoma virus transforming gene product induce similar phosphorylations of ribosomal protein S6. *Proc. Natl. Acad. Sci. USA.* **81** 6408-6412.

(526) Trevillyan, J.M., Kulkarni, R.K. and Byus, C.V. (1982) Tumour promoting phorbol ester causes rapid phosphorylation of ribosomal protein S6 in serum starved reuber H35 rat hepatoma cells. *J. Cell. Biol.* **95** Abstract 9052.

(527) Chuang, L.F., Cooper, R.H., Yau, P., Bradbury, E.M. and Chuang, R.Y. (1987) Protein kinase C phosphorylates leukemia RNA polymerase II. *Biochem. Biophys. Res. Commun.* **145** 1376-1383.

(528) Sander, M., Nolan, J.M., Hsieh, T.S. (1984) A protein kinase activity tightly associated with drosophila type II DNA topoisomerase. *Proc. Natl. Acad. Sci. USA.* **81** 6938-6942.

(529) Summers, T.A. and Creutz, C.E. (1985) Phosphorylation of a chromaffin granule-binding protein by protein kinase C. *J. Biol. Chem.* **260** 2437-2443.

(530) Khanna, W.C., Tokuda, M. and Waisman, D.M. (1986) Phosphorylation of lipocortins *in vitro* by protein kinase C. *Biochem. Biophys. Res. Commun.* **141** 547-554.

(531) Gould, K.L., Woodgett, J.R., Isacke, C.M. and Hunter, T. (1986) The protein tyrosine kinas substrate p36 is also a substrate for protein kinase C *in vitro* and *in vivo*. *Mol. Cell. Biol.* **6** 2738-2744.

(532) Akers, R.F. and Routtenberg, A. (1985) Protein kinase C phosphorylates a 47Mr protein (F1) directly related to synaptic plasticity. *Brain Res.* **334** 147-151.

(533) Dekker, L.V., De Graan, P.N.E., Oestreicher, A.B., Versteeg, D.H.G. and Gispen, W.H. (1989) Inhibition of noradrenaline release by antibodies to B50 (GAP-43). *Nature* 3**42** 74-76.

(534) Aloyo, Y.J., Zwiers, H. and Gispen, W.H. (1983) Phosphorylation of B-50 protein by calcium-activated, phospholipid dependent protein kinase and B-50 protein kinase. *J. Neurochem.* **41** 649-653.

(535) Movsesian, M.A., Nishikawa, M and Adelstein, R.S. (1984) Phosphorylation of phospholamban by calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* **259** 8029-8032.

(536) Welsh, W.J. (1985) Phorbol ester, calcium ionophore, or serum added to quiescent rat embryo fibroblast cells all result in the elevated phosphorylation of two 28,000-dalton mammalian stress proteins. *J. Biol. Chem.* **260** 3058-3062.

(537) Wooten, M.W., Nel, A.E., Goldschmidt-Clermont, P.J., Galbraith, R.M. and Wrenn, R.W. (1985) Identification of a major endogenous substrate for phospholipid/Ca^{2+}-dependent kinase in pancreatic acini as G_C (vitamin D-binding protein). *FEBS Letts.* **191** 97-101.

(538) Dewald, B., Payne, T.G. and Baggiolini, M. (1984) Activation of NADPH oxidase of human neutrophils. Potentiation of chemotactic peptide by a diacylglycerol. *Biochem. Biophys. Res. Commun.* **125** 367-373.

(539) Papini, E., Grzeskowiak, M., Bellavite, P. and Rossi, F. (1985) Protein kinase C phosphorylates a component of nadph oxidase of neutrophils. *FEBS Letts*. **190** 204-208.

(540) Witters, L.A. and Blackshear, P.J. (1987) Protein kinase C-mediated phosphorylation in intact cells. *Methods in Enzymology* 1**41** 412-424.

(541) Morris, C. and Rozengurt, E. (1988) Purification of a phosphoprotein from rat brain closely related to the 80KDa substrate of protein kinase C identified in swiss 3T3 fibroblasts. *FEBS Letts* **231** 311-316.

(542) Wang, J.K.T., Walaas, S.I., Sihra, T.S., Aderem, A. and Greengard, P. (1989) Phosphorylation and association translocation of the 87kDa protein, a major protein kinase C substrate, in isolated nerve terminals. *Proc. Natl. Acad. Sci. USA* **86** 2253-2256.

(543) Albert, K.A., Walaas, S.I., Wang, J. K.-T., and Greengard, P. (1986) Widespread occurrence of "87 kDa", a major specific substrate for protein kinase C. *Proc. Natl. Acad. Sci. USA*. **83** 2822-2826.

(544) Wu, W.C.S., Walaas, S.I., Nairn, A.C. and Greengard. P. (1982) Calcium/phospholipid regulates phosphorylation of a Mr "87k" substrate protein in brain synaptosomes. *Proc. Natl. Acad. Sci. USA*. **79** 5249-5253.

(545) Graves, C.B. and McDonald, J.M. (1985) Insulin and phorbol ester stimulate phosphorylation of a 40 kDa protein in adipocyte plasma membranes. *J. Biol. Chem.* **260** 11286-11292.

(546) Kramer, I.M., Verhoeven, A.J., Van der Bend, R.L., Weening, R.S. and Roos, D. (1988) Purified protein kinase C phosphorylates a 47 kDa protein in control neutrophil cytoplasts but not in neutrophil cytoplasts from patients with the autosomal form of chronic granulomatous disease. *J. Biol. Chem.* **263** 2352-2357.

(547) Aderem, A.A., Albert, K.A., Keum, M.M., Wang, J.K.T., Greengard, P. and Cohn, Z.A. (1988) Stimulus-dependent myristoylation of a major substrate for protein kinase C. *Nature* **332** 362-364.

(548) MacFarlane, D.E. (1986) Phorbol diester-induced phosphorylation of nuclear matrix proteins in AL60 promyelocytes. *J. Biol. Chem.* **261** 6947-6953.

(549) Levine, H., Sahyoun, N., McConnell, R., Bronson, D. and Cuatrecasas, P. (1984) Specificity of a phosphatase for phospholipid, Ca^{2+}-dependent protein kinase-phosphorylated histone H1 resides in the catalytic subunit. *Biochem. Biophys. Res. Commun.* **118** 278-283.

(550) Brooks S.F,Evans F.J,Aitken A.(1987)The stimulation of phosphorylation of intracellular proteins in GH3 rat pituitary cells by phorbol esters of distinct biological activity. *FEBS Letts* **224** 109-116.

(551) Mauduit, P., Zoukhri, D and Rossignol, B. (1989) Direct activation of a protein kinase activity from rat lacrimal gland by PMA in a phospholipid-free system. *FEBS Letts* **252** 5-11.

(552) Omri, B., Breton, M.F. and Pavlovic-Hournac, M. (1987) Characteristics of thyroid protein kinase C. *Eur. J. Biochem.* **165** 83-90.

(553) Ferrari, S, Marchiori, F.M., Marin, O. and Pinna, L.A. (1987) Ca2+ phospholipid-dependent and independent phosphorylation of synthetic peptide substrates by protein kinase C. *J. Biol. Chem.* **163** 481-487.

(554) Aderem, A.A., Keum, M.M., Pure, E. and Cohn, Z.A. (1986) Bacterial lipopolysaccharides, phorbol myristate acetate, and zymosan induce the myrisolylation of specific macrophage proteins. P*roc. Natl. Acad. Sci. USA.* **83** 5817-5821.

(555) Kamps, M.P., Buss,J.E. and Sefton, B.M. (1985) Mutation of NH_2-terminal glycine of pp60-src prevents both fatty acylation and morphological transformation. *Proc. Natl. Acad. Sci. USA.* **82** 4625-4629

(556) Utsumi, T., Yoshinaga, K., Koga, D., Ide, A., Nobori, K., Okimasu, E., Terada, S. and Utsumi, K. (1988) Association of a myristoylated protein with a biological membrane and its increased phosphorylation by protein kinase C. *FEBS.Letts* **238** 13-16.

(557) Gilmore, T. and Martin, G.S. (1983) Phorbol ester and diacyglycerol induce protein phosphorylation at tyrosine. *Nature* **306** 487-490.

(558) Bishop, R., Martnez, R., Nakamura, K.D. and Weber, M.J. (1983) A tumour promoter stimulates phosphorylation on tyrosine. *Biochem. Biophys. Res. Commun.* **115** 536-543.

(559) Cooper, J.A., Sefton, B.M. and Hunter, T. (1984) Diverse mitogenic agents induce the phosphorylation of two related 42,000-dalton protein on tyrosine in quiescent chick cells. *Mol. Cell. Biol.* **4** 30-37.

(560) Moon, S.O., Palfrey, H.C. and King, A.C. (1984) Phorbol esters potentiate tyrosine phosphorylation of epidermal growth factor recpetors in A431 membranes by a calcium-independent mechanism. *Proc. Natl. Acad. Sci. USA.* **81** 2298-2302.

(561) Grunberger, G., Zick, Y., Taylor, S.I. and Gorden, P. (1984) Tumour-promoting phorbol ester stimulates tyrosine phosphorylation in U-937 monocytes. *Proc. Natl. Acad. Sci. USA.* **81** 2762-2766.

(562) Fabiato, A. and Fabiato, J. (1979) Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol(Paris).* **75** 463-505.

(563) Wolf, M., Sahyoun, N., LeVine, H. and Cuatrecasas, P. (1984) Protein kinase C: rapid enzyme purification and substrate-dependence of the diacylglycerol effect. Biochem. Biophys. Res. Commun. 122 1268-1275.

(564) Stabel, S., Rodriguez-Pena, A., Young, S., Rozengurt, E. and Parker, P.J. (1987) Quantitiation of protein kinase C by immunoblot-expression in different cell lines and response to phorbol esters. *J. Cell. Physiol* **130** 111-117.

(565) Adamo, S., Caporale, C., Guanno, S.A., Lazdins, J., Faggioni, A., Belli, L., Cortesi, E., Nervi, C., Gastaldi, R., and Molinaro, M. (1986) Proliferating and quiescent cells exhibit different subcellular distribution of protein kinase C activity. *FEBS Letts.* **195** 352-356.

(566) Hunter, T. (1987) A thousand and one protein kinases. *Cell* **50** 823-839.

(567) Hanks, S.K. (1987) Homology probing: Identification of cDNA clones encoding members of the protein-serine kinase family. *Proc.Natl.Acad.Sci.USA.* **84** 388-392.

(568) Ohno, S., Akita, Y., Konno, Y., Imajoh,S and Suzuki, K. (1988) A novel phorbol ester receptor/protein kinase nPKC, distantly related to the protein kinase C family. *Cell* **53** 731-741.

(569) Konno, Y., Ohno, S., Akita, Y., Kawasaki, H. and Suzuki, K. (1989) Enzymatic properties of a novel phorbol ester receptor/protein kinase; nPKC. *J. Biochem.* **106** 673-678.

(570) Buday, L., Meszaros, G. Farkas, G, Seprodi, J., Antoni, F and Farago, A. (1989) Two components of type III protein kinase C with different substrate specificities and a phospholipid-dependent but Ca^{2+} inhibited protein kinase in rat brain. *FEBS Letts.* **249** 324-328.

(571) Gonzatti-Haces, M.I. and Traugh, J.A. (1986) Ca^{2+}-independent activation of protease-activated kinase II by phospholipids/diolein and comparison with the Ca^{2+}/phospholipid-dependent protein kinase. *J. Biol. Chem.* **261** 15266-15272.

(572) Klemm, D.J. and Lias, L.E. (1987) A distinctive phospholipid-stimulated protein kinase of normal and malignant murine hemopoietic cells. *J. Biol. Chem.* **262** 7580-7585.

(573) Farago, A., Farkas, G., Mészáros, G, Buday, L., Antoni, F and Sepródi. (1989) Isoenzyme patterns of protein kinase C and a phospholipid-dependent but Ca^{2+}-inhibited enzyme fraction in the crude extracts of different tissues. *FEBS Letts.* **243** 328-332.

(574) Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F and Horecker, B.L. (1985) Binding of protein kinase C to neutrophil membranes in the presence of Ca^{2+} and its activation by a Ca^{2+}-requiring proteinase. *Proc. Natl. Acad. Sci. USA.* **82** 6435-6439.

(575) Evans, A.T., Sharma, P., Ryves, W.J. and Evans, F.J. (1990) TPA and resiniferatoxin-mediated activation of NADPH-oxidase: A possible role for Rx-kinase augmentation of PKC. *FEBS Letts* **267** 253-256.

(576) Takematzu, H., Fisher, G.J. and Voorhees, J.J. (1989) A novel histone-stimulated protein kinase in normal and psoriatic epidermis. In *The Society for Investigative Dermatology Inc.* **92** 385-390.

Acknowledgements.

During the course of this project I was fortunate enough to recieve help from many different sources.

I should like to give special thanks to Fred Evans for being my knowledgable and determined supervisor at the School of Pharmacy,for giving me the opportunity to expand this project and for the lively discussions which resulted.I am also indebted to Laurie Garland my external supervisor at Wellcome Research, Beckenham,Kent,for his exellent constructive criticism and for his patience with me during my time in his laboratories.

At the School itself I thank my colleagues Tudor Evans, Pawan Sharma and Phil Gordge for making my stay an enjoyable and interesting one.My thanks also go to Margaret Roberts for advice,and Annie Cavanagh and Maureen Picket for photographic and auditing support.

Many institutes and their staff were kind enough to help me with various aspects of the project.I should therefore like to thank;

Shaun Thomas,Mark Jones,Assim Kwaja and Professor Linch (Dept.of Heamatology,University College Hospital,London) for Daudi cells,technical assistance and use of the cell separator unit and the UCH laboratory.

Peter Parker and Andree Olivier (ICRF,Lincoln Inn Fields, London)for supplying PKC isozymes and PKC antibodies, and for the use of the ICRF laboratory facilities.

Neil Thompson,Clive O'Neil,and Martin Merrett (Wellcome Research Laboratories,Beckenham,Kent) for technical advice and laboratory space.

Ian Wood (Sandoz Institute,London) for use of the FPLC.

Susan Brooks and Alastair Aitken (NIMR,Mill Hill,London) for technical advice and laboratory space.

Antony Davies and Prof.Rickmansworth (CRC,Birmingham) for Raji cell line.

I should also like to thank Manda Wilkinson,Mary Keogh Anne Blaikie and Charlie Ogilvy for secretarial assistance.

Finally I should like to thank S.E.R.C who funded this project together with Wellcome Research.