The source of *sn*-1,2-diradylglycerol in mitogen-stimulated Swiss 3T3 fibroblasts.

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Abbreviations.

[Arg^8]Vp	[Arginine^8] Vasopressin.
ATP	Adenosine 5'-triphosphate.
Bomb	Bombesin.
BSA	Bovine serum albumin (fraction V).
cAMP	Adenosine 3',5'-cyclicmonophosphate.
$[Ca^{2+}]_{i/o}$	Calcium concentration (intra or extracellular).
CDP Cho	Cytidine 5'-diphosphocholine.
Cho	Choline.
ChoP	Phosphocholine.
CMP PtdOH	Cytidine 5'-monophosphate-phosphatidic acid.
CTP	Cytidine 5'-triphosphate
DG	*sn*-1,2-diradylglycerol.
DGK	DG kinase (EC 2.7.1.107).
DGL	DG lipase (EC 3.1.1.34).
DMBH	Serum-free Dulbecco's modified Eagle's medium, 1% (v/v) BSA and 20mM Hepes.
DMEM	Dulbecco's modified Eagle's medium.
EGF	Epidermal growth factor.
EGTA	Ethylene glycol-bis (β-amino-ethyl ether) N,N,N',N'- tetra acetic acid.
FGF	Fibroblast growth factor.
GroPCho	Glycerophosphocholine.
HBG	Hanks' buffered saline solution with 1% (v/v) BSA and 10mM glucose.

Hepes	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])
$InsP_t$	Total D-*myo*-inositol phosphates.
Ins(1)P	D-*myo*-Inositol-1-monophosphate.
Ins(3)P	D-*myo*-Inositol-3-monophosphate.
Ins(4)P	D-*myo*-Inositol-4-monophosphate.
$Ins(1,3)P_2$	D-*myo*-inositol-1,3-bisphosphate.
$Ins(1,4)P_2$	D-*myo*-inositol-1,4-bisphosphate.
$Ins(3,4)P_2$	D-*myo*-inositol-3,4-bisphosphate.
$Ins(1,3,4)P_3$	D-*myo*-inositol(1,3,4)trisphosphate.
$Ins(1,4,5)P_3$	D-*myo*-inositol(1,4,5)trisphosphate.
$Ins(1,3,4,5)P_4$	D-*myo*-inositol(1,3,4,5)tetrakisphosphate.
$InsP_5$	Inositol pentakisphosphate (unspecified isomer).
$InsP_6$	Inositol hexakisphosphate (phytic acid).
PDGF	Platelet-derived growth factor.
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$.
pH_i	Intracellular pH.
PIC	Phosphoinositidase C (PtdIns(4,5)P_2 phosphodiesterase, EC 3.1.4.11).
PKC	Protein kinase C (EC 2.7.1.37).
PLA_2	Phospholipase A_2 (EC 3.1.1.4).
PLC	Phospholipase C (EC 3.1.4.3).
PLD	Phospholipase D (EC 3.1.4.4).
PMA	Phorbol-12-myristate-13-acetate.
PPH	Phosphatidate phosphohydrolase (EC 3.1.3.4).
PtdBut	Phosphatidylbutanol.
PtdCho	Phosphatidylcholine.
PtdEth	Phosphatidylethanol.
PtdEtn	Phosphatidylethanolamine.
PtdIns	Phosphatidylinositol.
PtdIns(4)P	Phosphatidylinositol-4-phosphate.

PtdIns(4,5)P_2	Phosphatidylinositol-4,5-bisphosphate.
PtdOH	Phosphatidic acid.
PtdSer	Phosphatidylserine.
SAG	1-stearoyl-2-arachidonoyl-*sn*-glycerol
SphM	Sphingomyelin.

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Summary.

The aim of the work in this thesis was to characterise the formation of *sn*-1,2-diglyceride (DG) in the Swiss 3T3 fibroblast, a model system for mitogenic signalling, and to assess the source of that DG.

The generation of inositol phosphates was assessed using isotopic and mass assays in Swiss 3T3 cells stimulated with bombesin. The generation of $[^3H]InsP_3$ was rapid and essentially transient, whilst the elevation of $[^3H]InsP_2$ and $[^3H]InsP$ was consistent with the rapid metabolism of $[^3H]InsP_3$. In addition $Ins(1,4,5)P_3$ mass rose to peak after 5 sec stimulation and returned to pre-stimulated levels by 30 seconds. The accumulation of total $[^3H]$inositol phosphates ($InsP_t$) and $Ins(1,4,5)P_3$ mass stimulated by bombesin was dose-dependent with similar, though not identical, EC_{50} values (0.58 ± 0.34 nM and 5.88 ± 3.66 nM respectively).

The phorbol ester phorbol-12-myristate-13-acetate (PMA) did not stimulate $[^3H]InsP_t$ accumulation but inhibited by 50% $[^3H]InsP_t$ accumulation stimulated by bombesin, $[Arg^8]$vasopressin ($[Arg^8]$Vp) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Prolonged treatment of Swiss 3T3 cells with 400nM PMA to down-regulate protein kinase C (PKC) resulted in the potentiation of maximum $[^3H]InsP_t$ accumulation stimulated by bombesin, $[Arg^8]$Vp and $PGF_{2\alpha}$; in addition these responses were no longer inhibited by acute PMA pre-treatment.

The generation of DG mass in response to bombesin was biphasic; the first phase corresponded with the elevation of $Ins(1,4,5)P_3$ mass but the second, sustained elevation from 30 seconds up to 30 min was dissociated from $Ins(1,4,5)P_3$ mass accumulation; the net generation of DG mass in the first phase exceeded that of $Ins(1,4,5)P_3$ by at least four-fold. Stimulation of Swiss 3T3 cells with PMA resulted in the accumulation of DG mass which exhibited similar kinetics to the second phase of bombesin-stimulated DG formation and was dose-dependent; the EC_{50} (2 nM) was similar to the IC_{50} for PMA-mediated inhibition of bombesin-stimulated $[^3H]InsP_t$ accumulation (5.58 ± 3.84 nM). PMA-stimulated $[^3H]$1,2-DG formation was also observed in Swiss 3T3 cells labelled with $[^3H]$palmitic acid in which the fatty acid was preferentially incorporated into

phosphatidylcholine (PtdCho).

Stimulation of [^{3}H] palmitate labelled Swiss 3T3 cells with bombesin or PMA in the presence of 0.3% (v/v) butan-1-ol resulted in the formation of [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut) indicating that both agents were activating phospholipase D. The EC_{50} for [^{3}H]PtdBut formation was essentially identical to that for DG formation for both bombesin and PMA. For PMA and bombesin, the onset of PLD activity preceded that of the sustained phase of DG formation suggesting that phosphatidic acid (PtdOH), the normal lipid product of PLD, might actually serve as a precursor of DG via a coupled PLD/PtdOH phosphohydrolase pathway. Use of butan-1-ol to 'trap' phosphatidate moieties as the metabolically stable PtdBut inhibited 75% of PMA-stimulated DG formation but only 30% of bombesin-stimulated DG formation indicating different roles for a PLD/PPH pathway in contributing to DG formation by bombesin and PMA.

Down-regulation of PKC resulted in the complete loss of bombesin- and PMA-stimulated PLD activity suggesting an absolute requirement for PKC. Studies with the PKC inhibitor Ro-31-8220 confirmed the PKC-dependency of PMA-stimulated PLD activity. However, Ro-31-8220 only inhibited bombesin-stimulated PLD activity by 45% suggesting that there might be PKC-independent regulation of PLD.

Chelation of extracellular [Ca^{2+}] to approximately 150 nM with EGTA resulted in 50% inhibition of bombesin-stimulated PLD activity; the PMA response was unaffected. The combination of Ro-31-8220 and EGTA was less than additive in inhibiting bombesin-stimulated PLD activity; at least 30% of the response to bombesin remained intact in the presence of both agents. The ionophore, A23187, stimulated PLD activity though to a lesser extent than bombesin or PMA. This response was inhibited by chelation of extracellular Ca^{2+}, though not completely, and also by the PKC inhibitor Ro-31-8220; combination of both treatments inhibited the majority of A23187-stimulated PLD activity.

A simple ion-exchange system was developed to resolve choline (Cho), phosphocholine (ChoP) and glycerophosphocholine (GroPCho). This technique was used to assay changes in the level of [^{3}H]choline metabolites in radiolabelled

Swiss 3T3 cells. Bombesin and PMA elicited an increase in [^{3}H]Cho prior to any significant increase in [^{3}H]ChoP; the kinetics and dose dependency of [^{3}H]Cho formation were the same as those for [^{3}H]PtdBut formation indicating that PtdCho was a major substrate for bombesin- and PMA-stimulated PLD activity. [Arg^8]Vp and $PGF_{2\alpha}$ also stimulated the same apparent PtdCho-PLD activity with EC_{50} values corresponding to those for accumulation of [^{3}H]$InsP_t$ in each case. The ability of PMA, bombesin, [Arg^8]Vp and $PGF_{2\alpha}$ to stimulate accumulation of [^{3}H]Cho was completely abolished in cells which had been treated with 400nM PMA for 48 hours to 'down-regulate' PKC.

Epidermal growth factor (EGF) stimulated an increase in DG mass which was sustained above control for up to 60 min but was of smaller magnitude than the response to bombesin or PMA. Half-maximal effects of EGF were obtained at doses at which EGF elicited no significant accumulation of [^{3}H]$InsP_t$. EGF did stimulate PtdCho-PLD activity as measured by the release of [^{3}H]Cho and the formation of [^{3}H]PtdBut. However, butan-1-ol did not inhibit EGF-stimulated DG formation suggesting that it did not arise by a PLD/PPH pathway. EGF-stimulated PLD activity was not inhibited by the PKC inhibitor Ro-31-8220 but was inhibited by the EGF-receptor tyrosine kinase inhibitor, AG 18. Platelet-derived growth factor (PDGF) was also able to stimulate the formation of [^{3}H]PtdBut and the release of [^{3}H]Cho indicating that it also activates a PtdCho-PLD activity. PDGF-stimulated [^{3}H]PtdBut formation was apparent after 30 seconds and therefore occurred at least as quickly as increases in $Ins(1,4,5)P_3$ mass. PDGF-stimulated release of [^{3}H]Cho and [^{3}H]PtdBut formation were abolished in cells which had been depleted of cellular PKC.

The results are considered in terms of the role of inositol lipid and PtdCho hydrolysis in mitogen-stimulated DG formation in Swiss 3T3 cells and the role of sustained DG formation as an early signal in mitogenesis. In the light of potential messenger functions for PtdOH the regulation of PLD activity by growth factors is discussed.

Chapter 1.

Introduction; growth factor signal transduction.

1.1 Regulation of the cell cycle.

Cells grow and divide by progressing through the 'cell cycle', a programmed series of events involving synthesis of proteins and DNA which culminates in mitosis and division into two daughter cells. This process is under rigorous control such that the majority of higher eukaryote cells exist *in vivo* in a fully viable but non-proliferating state known as quiescence. Progress through the cell cycle is controlled both from within and outside the cell. Within the cell, the products of a number of cell division cycle (CDC) genes such as CDC2HS, the human homologue of the yeast cdc2 gene, play vital regulatory roles in controlling the transition between different phases of the cell cycle (reviewed by Nurse, 1990). Outwith the cell, proliferation is controlled by extracellular signals, known collectively as growth factors, which produce chemical messages within the cell resulting ultimately in activation of CDC gene products and initiation of the cell cycle. These growth factors can include neuropeptides, polypeptides, hormones, prostaglandins, and immune complexes depending on the type of recipient cell.

The mechanisms by which these factors stimulate cells are one of the keys to our understanding the whole process of cell division in normal cells and may allow us to identify lesions and thus potential sites of intervention in cancer cells exhibiting unrestrained growth in the absence of a functional requirement for cell division.

The study of many of the biochemical events culminating in cell division has been facilitated by the use of homogenous clonal cell populations grown *in vitro* in biochemically defined growth medium i.e. the process of cell culture. Many cell types can be cultured in a balanced salt solution containing serum which is rich in growth factors. Cultured cells can be made quiescent by allowing them to reach confluency, thereby depleting serum growth factors and contact inhibiting growth, or by replacing the growth medium with serum-free or low-serum containing medium; by adding back whole serum or defined growth factors DNA synthesis can be re-initiated. Such approaches have allowed the identification of distinct phases of the cell cycle and the biochemical signals controlling initiation of these phases. The main disadvantage of using these 'immortal' cell lines *in vitro* is that

cells are maintained and handled in an unnatural state and 'immortal' cell lines are prone to spontaneous 'transformation' to tumour-like cells during extended culture periods. Indeed, 'immortality' is considered one of the stages in the progression from a 'normal' to a 'transformed' phenotype. As such, their use in probing the normal processes of cell division is a necessary compromise.

A typical mammalian cell cycle can be anything from 10-30 hours long. G_1, represents the gap between the previous nuclear division (M for mitosis) and the beginning of DNA synthesis (S-phase) and is the major variable whereas G_2 is the gap between DNA synthesis and nuclear division (M). Quiescent cells are generally considered to be in a distinct physiological state- G_0, though some consider quisecence to be an extension of G_1 in slow growing cells. In this latter model, rapidly growing cells have a very short G_1 whilst terminally differentiated cells such as striated muscle fibres or neurones have an infinite G_1.

Addition of exogenous growth factors to quiescent 3T3 fibroblasts results in a transition from G_0 into a series of events culminating in DNA synthesis and mitosis. A model in which cells, given an initial stimulus, become 'competent' to further stimulation and enter the cell cycle, has been proposed in the Balb/c 3T3 cell line but is not universally applicable (Pledger *et al.*, 1978; O'Keefe & Pledger, 1983). Quiescent Balb/c 3T3 cells, stimulated with a 'competence factor', will remain 12 hours from S phase but will complete G_1 transit and initiate DNA synthesis when stimulated by factors in platelet-poor plasma. In CCL39 cells α-thrombin is required to be present for the entire pre-replicative phase (8-10 hours) for DNA synthesis to subsequently occurr (Van Obberghen-Schilling *et al.*, 1982). What is clear is that, after a given time (between 8-12 hours), cells are said to be 'committed' to progression through the cell cycle and traverse through this variable pre-synthetic phase of G_1 is independent of the presence of serum or growth factors (Zetterberg & Larson, 1985). Whilst cells still remain responsive to various growth factors through the cell cycle, it seems likely that the regulation of entry into and exit from mitosis as well as the committment to DNA synthesis come increasingly under the control of CDC gene products, especially the pp34 product of the CDC2 gene (reviewed by Nurse, 1990).

1.2 Early growth factor-stimulated events in the pre-replicative phase of the cell cycle.

Some of the early biochemical and molecular signals initiated following stimulation with growth factors include ion fluxes, changes in the phosphorylation state of key regulatory proteins and activation of the so-called 'early' CDC genes such as c-*fos*, c-*myc* and c-*jun*

1.2.1 Ion fluxes.

The addition of serum or defined growth factors to quiescent Swiss 3T3 cells and other cells in culture leads to a rapid increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Hesketh *et al.*, 1985; 1988), typically from 100-200nM up to as much as 1μM.

Different growth factors exert different effects upon $[Ca^{2+}]_i$ as defined by the kinetics of the response and its dependency upon extracellular Ca^{2+} ($[Ca^{2+}]_o$). In Swiss 3T3 cells, bombesin or vasopressin elicit a biphasic increase: an initial 'spike' apparent within a few seconds and largely independent of $[Ca^{2+}]_o$ followed by a smaller, sustained rise which is dependent upon $[Ca^{2+}]_o$ (Hesketh *et al.*, 1985 & 1988; Lopez-Rivas *et al.*, 1987; Hasegawa-Sasaki *et al.*, 1988; Bierman *et al.*, 1990). In contrast, EGF induces a slower rise in $[Ca^{2+}]_i$ which is absolutely dependent upon the presence of external Ca^{2+} (Hesketh *et al.*, 1985; 1988).

Mitogen-stimulation of a variety of quiescent cells, including Swiss 3T3 cells and thymocytes (Hesketh *et al.*, 1985) results in a gradual, sustained elevation of intracellular pH (pH_i), the so-called 'alkalinisation' signal. This pH_i increase can also be achieved by addition of the Ca^{2+} ionophore, A23187, as well as the tumour promoting phorbol ester phorbol-12-myristate-13-acetate (PMA), which act synergistically to stimulate early gene transcription prior to DNA synthesis (Kaibuchi *et al.*, 1986). The cytoplasmic alkalinisation is a result of stimulated Na^+/H^+ exchange by an electroneutral, amiloride sensitive antiporter (reviewed by Pouysségur, 1985a). The resulting rise in $[Na^+]_i$ may be buffered by a oubain sensitive Na^+/K^+ pump so that the early changes in monovalent ion fluxes

and pH_i may be integrated into a $[Na^+]_i$ equilibrium cycle (reviewed by Rozengurt, 1985).

The purpose of the $[Ca^{2+}]_i$ and pH_i changes in mitogenesis is still not fully understood. The transient rise in $[Ca^{2+}]_i$ followed by the sustained alkalinisation of the cytosol are obligatory for progression to DNA synthesis in the fertilised sea urchin egg (Whitaker & Steinhardt, 1982). In Swiss 3T3 cells, addition of A23187 to artificially elevate $[Ca^{2+}]_i$ actually stimulates a subsequent alkalinisation (Hesketh *et al.*, 1985) and can mimic EGF and PDGF induced c-*myc* expression (Tsuda *et al.*, 1985). Elevation of $[Ca^{2+}]_i$ will certainly contribute to the assembly of Ca^{2+}-calmodulin (CaM) complexes and the subsequent activation of Ca^{2+}-CaM dependent processes such as activation of cyclic AMP phosphodiesterases (Tanner *et al.*, 1987; Whitfield *et al.*, 1987; reviewed by Wang *et al.*, 1990). This may contribute to the initial drop in [cAMP] seen in a variety of cell lines upon mitogenic stimulation (Pastan *et al.*, 1975). Furthermore, $[Ca^{2+}]_i$ increases are implicated in various non-mitogenic responses to growth factors including cytoskeletal reorganisation, fluid endocytosis and chemotaxis. In sea urchin eggs, $[Ca^{2+}]_i$ is clearly implicated in cortical granule secretion and the raising of a 'fertilization envelope' after sperm entry; indeed every parameter of normal activation by sperm is also observed when eggs are exposed to A23187, including the respiratory burst and pH_i increases (Whitaker & Steinhardt, 1985). In addition to its role as an early signal, Ca^{2+} is very important at later stages in the cell cycle including S and M phases, particularly in construction of the mitotic spindle and the migration of condensed chromosomes (reviewed by Whitfield *et al.*, 1987).

The increase in pH_i associated with growth factor stimulation, typically of 0.2 units, is thought to have a permissive rather than a triggering role. In sea urchin eggs, pH_i must rise by 0.2 units to permit DNA synthesis to begin (reviewed by Whitaker & Steinhardt, 1982). By using mutant fibroblasts which lack a functional Na^+/H^+ antiporter, Pouysségur and colleagues have shown that below a threshold of pH 7.2, pH_i is limiting for cell proliferation and one major pH dependent step is the mitogenic stimulation of protein synthesis (Pouysségur *et al.*,

1985a & b). In this regard, it is interesting to note that the ribosomal protein S6 kinase may be activated by increases in pH_i (Pouysségur *et al.*, 1982) as can another early mitogenic signal, protein phosphorylation (Martin-Pérez *et al.*, 1984).

1.2.2 Protein phosphorylation.

Phosphorylation of proteins upon serine, threonine and tyrosine residues appears to be a common consequence of mitogenic stimulation by growth factors. Regulation of protein function by phosphorylation/de-phosphorylation cycles is recognized as a common feature of biologically responsive sytems, the phosphorylation state of target proteins being determined by the action of specific kinases and phosphatases. The enzymes responsible for regulating protein phosphorylation during mitogenic stimulation vary according to the signal transduction pathway employed by the growth factor and as such will be considered when the relevant pathways are discussed. However, a number of elements involved in controlling cell division are regulated by changes in phosphorylation state.

One example is the Na^+/H^+ antiporter which is responsible for the early increase in pH_i. Recent studies have shown that the antiporter is phosphorylated upon serine residues in a variety of cells upon stimulation with serum, phorbol esters, thrombin and EGF with a stoichiometry of 1 mol of phosphate per mole of anti porter (Sardet *et al.*, 1990).

Another common substrate for growth factor-stimulated phosphorylation is pp42, a mitogen activated protein kinase (MAP-2 kinase, formerly termed microtubule-associated protein), a mitogen-activated, serine- and threonine- directed kinase (Rossomand *et al.*, 1989). It has been shown that activation of MAP-2 kinase requires its phosphorylation upon both threonine and tyrosine residues and, as such, it may function as an important integrator of distinct signal transduction pathways (Anderson *et al.*, 1990b).

A variety of proto-oncogene products are phosphorylated in response to

mitogenic stimulation, though it is not clear in all cases what physiological relevance these events have. The c-*raf* proto-oncogene product, Raf-1, a serine- and threonine-directed kinase, is phosphorylated by activated PDGF β receptors in 3T3 cells and CHO cells whilst PDGF stimulated phosphorylation of Raf-1 on tyrosine residues correlates with a 4-6 fold increase in activity (Morrison *et al.*, 1989). Oncogenic activation of Raf-1 can occur by amino-terminal truncation (Rapp *et al.*, 1987a) and injection of this truncated form of Raf-1 into serum-starved NIH3T3 cells results in the initiation of DNA synthesis (Rapp *et al.*, 1987b). Clearly, phosphorylation and activation of Raf-1 in response to defined growth factors may play a role in mitogenesis. In addition, the product of the c-*src* proto-oncogene, a membrane associated tyrosine kinase is also phosphorylated (Gould & Hunter, 1988) and activated (Kypta *et al.*, 1990) by growth factors such as PDGF.

Activation of protein synthesis is an early, obligatory step in mitogenesis and is regulated in part by multiple phosphorylations of the 40S ribosomal protein, S6 (Duncan & McConkey, 1982; Martin-Pérez *et al.*, 1984). A mitogen-stimulated S6 kinase has been purified from Swiss 3T3 cells which catalyses an identical phosphorylation pattern to that obtained *in vivo* (Jenö *et al.*, 1988) and is highly specific for S6 (Jenö *et al.*, 1989). It is activated *in vivo* by serine and threonine phosphorylation (Ballou *et al.*, 1988) and this may be stimulated by a variety of growth factors including EGF, insulin and $PGF_{2\alpha}$ (Martin-Pérez *et al.*, 1984) as well as a kinase closely related to MAP-2 kinase (Ahn *et al.*, 1990).

Finally the activity of $pp34^{cdc2}$, the product of the cdc2 gene, is regulated by the phosphorylation/de-phosphorylation of Tyr 15 which dictates, in part, the entry into mitosis (Gould & Nurse, 1989; reviewed by Norbury & Nurse, 1989 and Nurse, 1990).

1.2.3 Transcription of 'immediate early' or 'competence' genes.

A common response to many growth factors is the transcription of the so-called 'early-response' or 'competence' genes such as the nuclear proto-oncogenes

c-*fos* and c-*myc*.

Transient expression of c-*fos* and c-*myc* mRNA occurs in Swiss 3T3 cells in response to stimulation with PDGF, FGF, PMA and A23187 (Kaibuchi *et al.*, 1986) and bombesin (Palumbo *et al.*, 1986; Rozengurt & Sinnett-Smith, 1987). Expression of c-*fos* mRNA typically precedes c-*myc*; for example, in bombesin-stimulated Swiss 3T3 cells c-*fos* mRNA is elevated after 15 minutes, maximal at 30 minutes and declines after 60 minutes whereas c-*myc* mRNA is only apparent after 60 minutes and is maintained for upto 6 hours (Palumbo *et al.*, 1986; Rozengurt & Sinnett-Smith, 1987). The product of the c-*fos* gene is thought to act as a *trans*-acting factor, capable of stimulating gene expression by interaction with the transcription factor c-Jun/AP1 (Chiu *et al.*, 1988) though it has no intrinsic, specific DNA-binding properties of its own.

Expression of c-*fos* and c-*myc*, whilst generally associated with the transition from G_0 is not confined to quiescent cells. Thus, PDGF induced c-*fos* expression has been demonstrated in Balb/c 3T3 cells undergoing synchronous transit through S phase and G_2 (Morgan & Pledger, 1989) and in serum-stimulated NIH 3T3 cells synchronized in S phase by hydroxyurea treatment (Bravo *et al.*,1986). The precise role of c-*fos* and c-*myc* in the transition from quiescence is still unclear; in Swiss 3T3 cells in the presence of insulin, bombesin stimulated DNA synthesis at sub-nanomolar concentrations which had little effect upon 'early gene' tr anscription. Furthermore, when PKC was depleted from the cells bombesin was still co-mitogenic with insulin at sub-nanomolar concentrations which had no effect upon 'early gene' induction (Rozengurt & Sinnett-Smith, 1987). Despite this, c-*fos* is thought to act as a'third messenger' in mitogenesis, involved in transmitting signals encoded by second messengers to the transcriptional machinery.

1.3 Signal transduction pathways implicated in the control of cell proliferation.

Like many hormones and neurotransmitters, growth factors bind to specific plasma membrane receptors and activate signal transduction pathways within the cell to exert their effect. Growth factor receptors exhibit many of the properties of classical hormone receptors such as saturable binding with characteristic K_d values for a given growth factor and target cell, amplification of the signal by transducing elements and effector systems and the generation of second messengers which initiate a chain of events leading to the physiological response. The difficulty in studying growth factor action is that since the physiological response, DNA synthesis or cell division, occurs at least 18-20 hours after stimulation with a growth factor the chain of events may be very long and difficult to dissect.

The majority of known growth factors can be broadly divided into three categories based upon cognate receptor structure and post-receptor signalling pathways. These are:

(i) Growth factors which bind to receptors possessing intrinsic, ligand-activated tyrosine kinase activity (receptor tyrosine kinase or RTK-type)

or growth factors which bind to receptors coupling to a separate effector enzyme such as

(ii) Adenylyl cyclase or

(iii) Polyphosphoinositide phospholipase C (or Phosphoinositidase C).

By analogy with many hormones and neurotransmitters, growth factors in the second and third class bind to receptors and activate an effector system through the mediation of a guanine nucleotide regulatory protein (G-protein) (Pouysségur, 1990). Such growth factors (Receptor-G-protein-Effector or RGE-type) are exemplified by bombesin, vasopressin, α-thrombin, $PGF_{2\alpha}$, Substance K and endothelin and the receptors for these mitogens typically possess seven transmembrane domains (Masu *et al.*, 1987; Battey *et al.*, 1991).

These three signal transduction pathways have been implicated in the control of cell proliferation and are now described in further detail with particular emphasis on the role of inositol lipid hydrolysis and the generation of DG in proliferation.

However, these transduction pathways cannot be considered in isolation and so the role of other signalling pathways in mitogenesis is also reviewed.

1.3.1 A class of growth factors bind to receptors which possess an intrinsic, ligand activated, tyrosine kinase activity.

A class of growth factors, typified by EGF, PDGF and FGF, bind to plasma membrane spanning receptors whose intracellular domains exhibit an intrinsic, ligand activated, tyrosine kinase activity. The study of mutant EGF and PDGF receptors, constructed by site-directed deletions and insertions, has provided a great deal of information concerning the mechanism of action of the activated receptor (reviewed by Ullrich & Schlessinger, 1990).

These receptors typically possess a single external domain for ligand binding, a single membrane spanning domain and a cytosolic, catalytic domain. Growth factor receptor binding induces oligomerization either by receptor-receptor interactions (EGF) or by dimerization of neighbouring receptors. Oligomerization leads to activation of the receptors intrinsic tyrosine kinase and an enhanced ligand binding affinity. A common consequence of growth factor binding is receptor autophosphorylation catalysed by its own tyrosine kinase activity; in the case of EGF and insulin receptors, this takes the form of cross-phosphorylation of receptor dimers (Ullrich & Schlessinger, 1990). Autophosphorylation of the insulin receptor β subunit increases the V_{max} of the kinase activity and maintains it in the active state (Rosen *et al.*, 1983). In the case of the PDGF and EGF receptors, autophosphorylation in the kinase insert domain (Tyr 751) and on a C-terminal cluster of tyrosines respectively, may dictate interactions with and phosphorylation of substrates (Kazlauskas & Cooper, 1989).

Activation of the tyrosine kinase activity of EGF and PDGF receptors absolutely requires the Lys in the ATP binding site consensus sequence Gly X Gly X X Gly X(15-20)Lys. Furthermore, the kinase activity itself is a prerequisite for signal transduction and initiation of early and late mitogenic responses including

Ca^{2+} influx, Na^+/H^+ exchange, c-*fos* and c-*myc* expression, S6 kinase activity and DNA synthesis (Honegger *et al.*, 1987a, b; Chen *et al.*, 1987; Moolenaar *et al*, 1988). In the case of the PDGF and FGF receptors, the kinase domain is divided into two halves by a kinase insert sequence containing the autophosphorylation site (Tyr 751). Site directed mutagenesis studies have suggested that this insert region may be the site of interaction between the receptor and cellular substrates and effector proteins (Kazlauskas & Cooper, 1989).

Whilst it has long been assumed that growth factor receptor tyrosine kinases exert their effects by tyrosine phosphorylation of target proteins, the identity of some of these substrates is only now becoming apparent. Interestingly, many of them are proteins implicated in the control of cell proliferation and include proto-oncogene products, their allosteric modulators and enzymes controlling signal transduction pathways known to be involved in mitogenesis. PDGF and EGF can induce the tyrosine phosphorylation (Meisenhelder *et al.*, 1989) and activation (Nishibe *et al.*, 1990) of phospholipase C-γ1, an enzyme controlling polyphosphoinositide hydrolysis which is also involved in mitogenesis (Ullrich & Schlessinger, 1990). In addition, the activated PDGF receptor is capable of phosphorylating and associating with a type I PtdIns kinase the enzyme responsible for the synthesis of inositol lipids phosphorylated upon the 3' position of the inositol ring whose function in mitogenic signalling remains unclear (see section 1.4.2). The PDGF receptor is also able to phosphorylate and activate the Raf-1 serine/threonine kinase, (Morrison *et al.*, 1989) and both the PDGF and EGF receptors are also able to phosphorylate the $p21^{ras}$ GTPase activating protein (GAP) (Kaplan *et al.*, 1989; Molloy *et al.*, 1989; Ellis *et al.*, 1990) though the functional relevance of this event remains unclear. Finally, a recent report has shown that the PDGF receptor is able to associate with and activate the $pp60^{c\text{-}src}$, $p59^{fyn}$ and $pp62^{c\text{-}yes}$ proteins all of which are members of the *src* family of non-receptor tyrosine kinases (Kypta *et al.*, 1990). In the case of $pp60^{c\text{-}src}$, PDGF treatment also results in tyrosine phosphorylation of the protein though this has not been shown to be the case for the others as yet.

1.3.2 Growth factors which regulate adenylyl cyclase activity.

A variety of hormones and neurotransmitters bind to plasma membrane receptors and stimulate an elevation of adenosine 3'5'-cyclicmonophosphate (cAMP) by activating adenylyl cyclase (Ad.Cyc.). Transduction of the extracellular signal to activation of Ad.Cyc. is mediated by a specific stimulatory G-protein, G_s, which is a substrate for cholera toxin-catalysed ADP-ribosylation. Inhibition of Ad.Cyc. by activated receptors occurs via an inhibitory G-protein, G_i, which is a substrate for pertussis toxin-catalysed ADP-ribosylation. Cyclic AMP is a second messenger which binds to the regulatory subunit of protein kinase A (PKA), thereby relieving the inhibitory effect upon the catalytic domain which is then able to phosphorylate distinct substrates upon serine and, occa sionally, threonine residues.

Receptor coupled activation and inhibition of adenylate cyclase is the best understood signal transduction system and has been reviewed extensively (e.g., Levitski, 1990b). Perhaps the best example of the system is the action of adrenaline at the β-adrenergic receptor (e.g., Bahouth & Malbon, 1988). Briefly, the levels of cAMP are regulated by a combination of the following:-

(1) receptor and G_s mediated activation of Ad.Cyc.,

(2) receptor and G_i mediated inhibition of Ad.Cyc.,

(3) activation of cAMP specific phosphodiesterases, to remove cAMP,

(4) competition between G_s & G_i for shared βγ subunits, and

(5) covalent modification of receptors, G-proteins and/or Ad.Cyc. as a means of desensitization/cross talk.

These facets of the adenylate cyclase system have been studied in a variety of model systems (reviewed by Casey & Gilman, 1988). Despite this, the role of cAMP as a second messenger in the regulation of cell proliferation remains unclear. During the 1970s, a body of evidence accumulated to suggest that cAMP was a negative modulator of mammalian cell proliferation: cAMP analogues or cAMP elevating agents inhibited growth (Johnson & Pastan, 1971; Rozengurt & Pardee, 1972); elevation of cAMP did not correlate with proliferative status (Otten *et al.*, 1972; Bannai & Sheppard, 1974); raising cAMP levels in 3T3 cells inhibited serum

stimulated DNA synthesis (Kram *et al.*, 1973) and cAMP levels dropped when growth arrested cells were stimulated with mitogenic agents (Sheppard, 1972; Seifert & Rudland, 1974).

This issue has been been examined extensively by Pouysségur and co-workers (reviewed by Pouysségur, 1990) in α-thrombin-stimulated CCL39 cells. Chambard *et al.* (1987) have shown that α-thrombin-stimulated proliferation was entirely blocked by pretreatment of the cells with pertussis toxin and yet α-thrombin-stimulated inositol lipid hydrolysis is only inhibited by approximately 50% (Paris & Pouysségur, 1986). Furthermore, α-thrombin induced a 30% reduction in PGE_1- and cholera toxin-stimulated cAMP levels, an effect which was 100% pertussis toxin sensitive (Magnaldo *et al.*, 1988). The dose-dependency of the effects of pertussis toxin on cAMP levels, mitogenesis and ADP-ribosylation of G_i-like protein were identical. In the same cell line pertussis toxin completely abolished the mitogenicity of 5-HT. Using selective 5-HT antagonists Seuwen *et al.*(1988a) showed that inositol lipid hydrolysis (via the $5\text{-}HT_2$ receptor) could be abolished without inhibiting mitogenesis, but that $5\text{-}HT_{1B}$ antagonists prevented both inhibition of adenylate cyclase and mitogenesis. Thus a major pathway for α-thrombin and 5-HT mitogenicity may be via G_i mediated inhibition of Ad.Cyc. Similar conclusions have recently been reached for the mitogenic effects of lyso-phosphatidic acid (van Corven *et al.*, 1989).

Despite this, there is some evidence of a role for cAMP in mitogenesis in certain cell types. Cholera toxin treatment of Swiss 3T3 cells, to raise cAMP, promoted serum-stimulated DNA synthesis with similar dose dependencies for both effects; the effects of cholera toxin being potentiated by inhibitors of cAMP-specific phosphodiesterases (Rozengurt *et al.*, 1981). β-adrenergic agonists, acting through cAMP generation, stimulated the proliferation of rat parotid cells *in vivo* and in culture (Tsang *et al.*, 1980) and the regenerating rat liver also employs this pathway (reviewed in Whitfield *et al.*, 1987; Dumont *et al.*, 1989). Stimulation of Swiss 3T3 cells with partially purified porcine PDGF and highly purified human PDGF resulted in an elevation of intracellular cAMP which was maintained for up to four hours (Rozengurt *et al.*, 1983). The use of cAMP phosphodiesterase

inhibitors resulted in a slight leftward shift in the dose response curve for PDGF stimulated [^{3}H]thymidine incorporation suggesting that elevated cAMP facilitates the mitogenic effect of PDGF. However, the elevation of cAMP by PDGF is an indirect effect mediated through autocrine production of E-type prostaglandins.

The conflicting results obtained when considering a role for cAMP in mitogenesis may be due to the variety of tissues studied since cAMP clearly acts as a mitogenic signal in certain cell types (reviewed by Dumont *et al.*, 1989). In this regard, a number of growth hormone-secreting pituitary tumours are associated with GTPase-abolishing mutations in G_s. These cell types exhibit constitutive activation of G_s and Ad.Cyc. as well as elevated cAMP levels (Vallar *et al.*, 1987; Landis *et al.*, 1989). Not only does this implicate cAMP in the regulation of cell growth and proliferation but it also confers the status of oncogene upon the mutated G_s gene, *Gsp*. Furthermore, transfection of the same G_s mutant (Glut 227->Leu α_s) into Swiss 3T3 cells resulted in elevated basal Ad. Cyc. activity, enhanced cAMP accumulation and increased mitogenic responsiveness to forskolin and the phosphodiesterase inhibitor Ro-20-1724 (Zachary *et al.*, 1990).

In addition to cell type variations, there may be a number of explanations for the opposing results obtained when studying the mitogenic effects of cAMP. One of these relates to temporal variations in cAMP levels during the cell cycle. In quiescent fibroblast cell lines, Boynton & Whitfield (1983) identified three distinct phases where cAMP levels decline, rise and again fall during the pre-replicative phase; the characteristic early drop in total cAMP content after mitogenic stimulation may correlate with suggestions that an early inhibition of Ad.Cyc. is required for mitogenesis (Pouysségur, 1990).

A second reason for the confusion may stem from the fact that depending on the dose the same mitogen may inhibit or stimulate Ad.Cyc.; thus, in CCL39 fibroblasts low concentrations of α-thrombin inhibit cholera toxin, forskolin and PGE_1 stimulated Ad.Cyc. whilst higher concentrations fully activate inositol lipid hydrolysis and PKC which can then stimulate Ad.Cyc activity thereby increasing $[cAMP]_i$ (Magnaldo *et al.*, 1988).

A third possible explanation of the cAMP paradox is the existence of two

distinct isozymes of cAMP dependent PKA, sharing identical catalytic subunits but having different regulatory subunits. It has been suggested that selective modulation of the two isozymes may be a function of cAMP in regulating cell proliferation. Use of site specific cAMP analogues has led to suggestions that Type II PKA may be associated with inhibition of the growth of human cancer cell lines, Type II levels being elevated and Type I decreased (Katsaros *et al.*, 1987). Thus, some of the conflicting results obtained for cAMP as a mitogenic second messenger may reflect temporal variations in $[cAMP]_i$ during the cell cycle or variations in the target A-kinase activated by cAMP.

1.3.3 Growth factors which activate phosphoinositidase C.

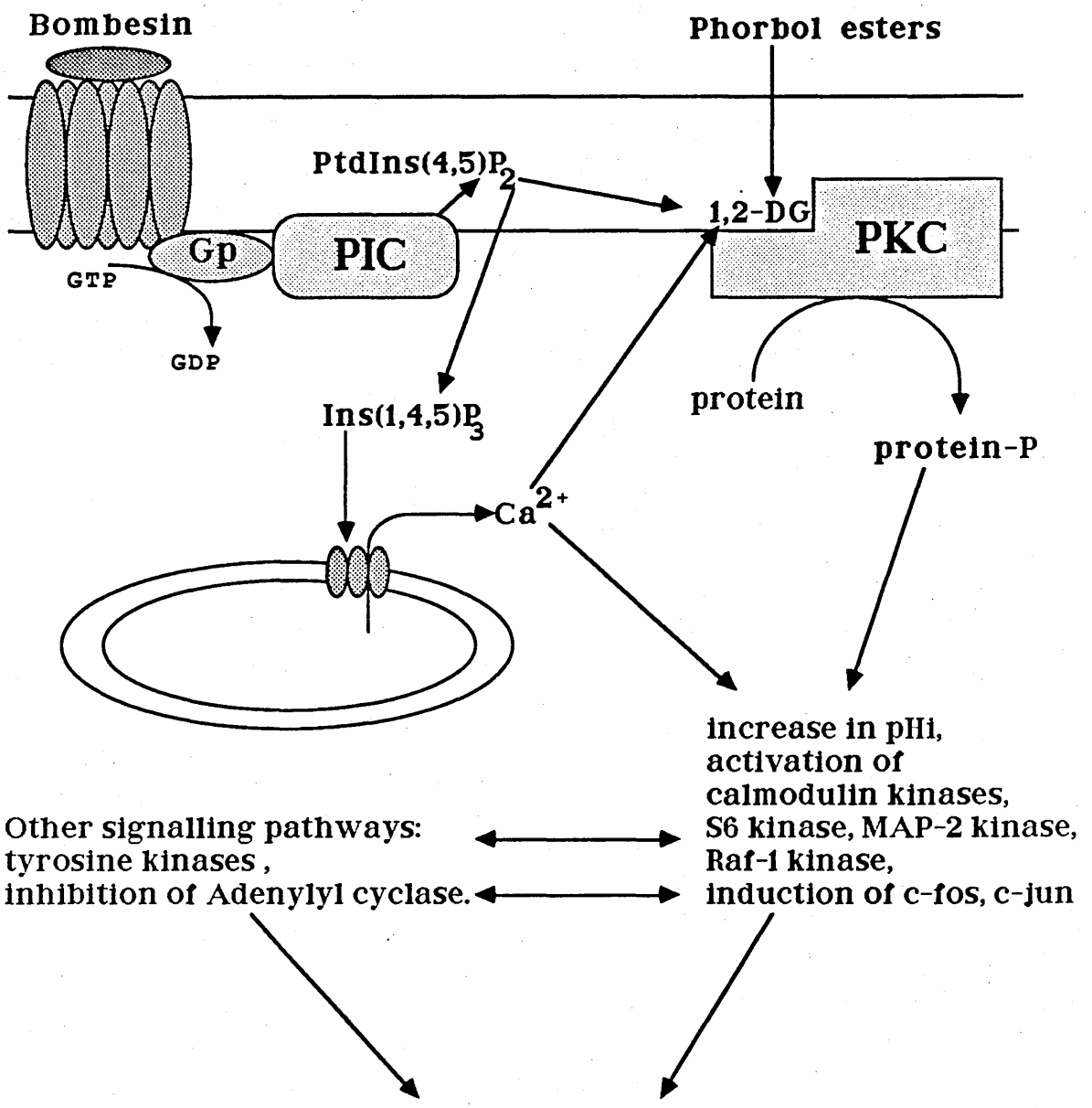
A large number of growth factors, neurotransmitters and hormones bind to plasma membrane receptors and stimulate the hydrolysis of polyphosphoinositides, a process which is coupled to the subsequent increase in $[Ca^{2+}]_i$ (Michell, 1975). Phosphoinositides are minor components of total cellular lipid: phosphatidylinositol (PtdIns) constitutes approximately 10% of total phospholipids and is distributed throughout all cellular membranes whereas phosphatidylinositol-4-phosphate (PtdIns(4)P) and phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) make up only 1-2% of total inositol lipids and are confined to the plasma membrane (reviewed by Michell, 1975; Downes & Michell, 1985). The three lipids are interconverted by specific kinases and phosphatases which are active even in unstimulated cells. It has been estimated that the half life for metabolic renewal of the 4- and 5- phosphate groups in polyphosphoinositides is no more than 5 min in unstimulated, isolated rat hepatocytes (Palmer *et al.*, 1986). Binding of a Ca^{2+}-mobilizing ligand to its receptor (e.g. acetylcholine acting at the M_1 muscarinic receptor) results in the activation of phosphoinositidase C which catalyses the phosphodiesteric hydrolysis of $PtdIns(4,5)P_2$. The products of this reaction are the two second messen ger molecules, $inositol(1,4,5)trisphosphate$ ($Ins(1,4,5)P_3$) and *sn*-1,2-diacylglycerol (DG), which mobilize Ca^{2+} from an intracellular store and activate protein kinase C (PKC) respectively.

1.3.4 Evidence for a role for polyphosphoinositide hydrolysis in the control of cell proliferation.

The observation that a variety of growth factors stimulate the hydrolysis of PtdIns(4,5)P_2 and generation of Ins(1,4,5)P_3 and DG implicates stimulated inositol lipid hydrolysis as an early signalling pathway in mitogenesis (reviewed by Whitman & Cantley, 1988; see Fig. 1.1). The resulting second messengers are able to activate effector systems which are clearly involved in the transition from G_0 through G_1 to S phase. Ins(1,4,5)P_3 increases $[Ca^{2+}]_i$ (see section 1.5.1 & 1.5.2) which is implicated in the control of proliferation (section 1.2) and DG activates PKC (see section 1.5.3-1.5.5) which regulates Na^+/H^+ antiporter activity, S6 kinase activity and c-*fos* and c-*myc* expression (section 1.2). Micro-injection of DG stimulates Balb/c 3T3 cells to enter the cell cycle (Suzuki-Sekimori *et al.*, 1989), whilst micro-injection of antibodies to PtdIns(4,5)P_2 abolishes PDGF- and bombesin-stimulated mitogenesis (Matuoka *et al.*, 1988). Over-expression of PKC in 3T3 cell lines causes disordered growth patterns and increased tumorigenicity of cells when injected into nude mice (Persons *et al.*, 1988; Housey *et al.*, 1988) and PKC itself is now recognized as the receptor for the tumour promoting phorbol esters (Castagna *et al.*, 1982) which are structural analogues of DG (see section 1.4). Phosphorylation of nuclear lamin B is stimulated by bryostatin-1 and dioctanoylglycerol, both activators of PKC, and correlates with translocation of PKC activity to the nuclear envelope in FDC-P1 cells and HL-60 cells (Fields *et al.*, 1989; Hocevar & Fields, 1991). The eukaryote initiation factor complex, eIF-4F, is phosphorylated by addition of PMA to [^{32}Pi]-labelled rabbit reticulocytes and the 2-D phosphopeptide map generated by proteolytic digestion of eIF-4F is the same as that obtained from the purified protein after incubation with PKC *in vitro* (Morley & Traugh, 1989), though to date it is not known what effect this has upon the eIF-4F activity. In addition, expression of a variety of proto-oncogenes and oncogenes, including *ras*, *src*, *ros*, and *abl*, in fibroblasts causes increases in the basal rates of inositol lipid hydrolysis and levels of inositol phosphates and DG (reviewed by Whitman & Cantley, 1988). This may not be a direct effect of the oncogene products but there is certainly a strong correlation

Figure 1.1 Schematic representation of the role inositol lipid hydrolysis in early mitogenic signalling events.

Binding of a growth factor of the RGE class, such as bombesin, to its cognate receptor results activation of phosphoinositidase C (PIC) (Heslop *et al.*, 1986); this appears to be mediated by a guanine nucleotide regulatory protein, G_p (Plevin *et al.*, 1990). PIC-catalysed hydrolysis of PtdIns(4,5)P_2 results in the formation of the second messengers Ins(1,4,5)P_3 and *sn*-1,2-DG. Ins(1,4,5)P_3 binds to a specific receptor thereby releasing stores of intracellular Ca^{2+}, whereas *sn*-1,2-DG binds to protein kinase C (PKC), forms a complex with PtdSer and Ca^{2+} and thereby activates the serine/threonine phosphorylation of key target proteins. These two signals, increased $[Ca^{2+}]_i$ and protein phosphorylation, cooperate in initiating many of the early events associated with the transit from quiescence (G_0) into the cell cycle (G_1 and ultimately DNA synthesis). PKC is the receptor for the tumour promoting phorbol esters (Castagna *et al.*, 1982) and is involved in the regulation of a Na^+/H^+ antiporter and, thereby, pH_i. In addition, PKC is implicated in the regulation of S6 kinase, MAP-2 kinase, Raf-1 kinase activities as well the expression of the 'competence' proto-oncogenes such as c-*fos* and c-*myc*.



Protein synthesis
and
DNA synthesis.

between enhanced growth rates and elevated levels of Ins Ps and DG.

In contrast, there are a number of growth factors which exert their effect without enhancing inositol lipid hydrolysis such as EGF in Swiss 3T3 cells (Hesketh *et al.*, 1985; 1988; Taylor *et al.*, 1988) and PDGF in IIC9 cells (Pessin *et al.*, 1990). Clearly, for many growth factors, hydrolysis of inositol lipids is neither necessary nor sufficient for a full mitogenic signal, but it may play an important role in generating signals for the transition to a state analagous to 'competence' (Whitman & Cantley, 1988). Thus, in cooperation with other pathways the generation of Ins(1,4,5)P_3 and DG will constitute a potent mitogenic signal (Fig 1.1).

1.3.5 Phosphoinositidase C (PIC).

PIC catalyses the hydrolysis of PtdIns(4,5)P_2 to generate the second messengers Ins(1,4,5)P_3 and DG (reviewed by Downes & Michell, 1985). The first inositol lipid specific PLC purified to homogeneity was a 68 kDa form from rat liver (Takenawa & Nagai, 1981), now referred to as PLC-α according to the nomenclature of Rhee *et al.* (1989). Antibodies to this protein were able to recognize a 62 kDa form in guinea pig uterus (Bennett & Crooke, 1987) and a 65-68 kDa form in sheep seminal vesicles (Hofmann & Majerus, 1982). It seems likely that these are isoforms or proteolytic products of PLC-α which are certainly immunologically distinct from the three isozymes which have been purified, cloned and sequenced from bovine brain: PLC-β-1 (mol.wt. 150 kDa), PLC-γ (145 kDa) and PLC-δ (85-88)kDa. They are distinct gene products but exhibit primary sequence homology particularly in two regions, designated X and Y (Suh *et al.*, 1988 a & b), which are thought to be involved in catalytic function although these regions are missing from PLC-α. PLC-α is also unique in possessing a 24 amino acid N-terminal membrane anchoring sequence (Bennett *et al.*, 1988) and exhibiting significant activity in the absence of Ca^{2+}. In addition to the described classification there are reported to be three forms of PLC-β of molecular weights

150 kDa (β-1), 140 kDa (β-2) & 100 kDa (β-3) (Rhee *et al.*, 1989) though the β-3 form may be a proteolytic product. In addition, there are known to be two forms of PLC-γ, (Rhee *et al.*, 1989; Emori *et al.*, 1989).

Unique amongst the PLC isozymes identified to date PLC-γ possesses three regions of homology to the non-receptor tyrosine kinases, A, B & C, which divide the X and Y sequences. These sequences, termed SH (*src* homology) regions, are found in c-*src* and the *crk* oncogene product and suggest the possibility of interactions with various receptor and non-receptor tyrosine kinases (Mayer *et al.*, 1988; Suh *et al.*, 1988 a & b ; Stahl *et al.*, 1988; Anderson *et al.*, 1990a). This is supported by the observation of tyrosine phosphorylation of PLC-γ1 by growth factor receptor tyrosine kinases (Meisenhelder *et al.*, 1989; discussed in 1.4.3). However, this is not the case with other isozymes of PLC and these may be the forms involved in the 'classical' coupling of receptors to PIC via G-proteins or other as yet unidentified regulatory mechanisms.

The PIC isozymes possess in common the ability to hydrolyse PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 but not PtdCho or PtdEtn. Their preferred substrate differs as Ca^{2+} and pH are varied but at low Ca^{2+} concentrations PtdIns(4,5)P_2 is the preferred substrate.

Phosphorylation of PLC is a candidate machanism for PKC-mediated feedback inhibition of PtdIns(4,5)P_2 hydrolysis (Brown *et al.*, 1987). Phosphorylation of PLC by PKC has been demonstrated *in vivo* for PLC-α (Bennett & Crooke, 1987) and *in vitro* for the α, β, γ and δ isozymes (Rhee *et al.*, 1989). However, this has not been shown to affect PLC activity as yet; alternatively it may affect the ability of PLC to interact with positive (G_p, see section 1.4.1) and negative regulatory elements.

1.4 Receptor coupled PtdIns(4,5)P_2 hydrolysis.

Unlike receptor tyrosine kinases where ligand binding, transducing and catalytic domains reside in the same molecule, receptors do not possess an intrinsic

PIC activity. Rather, they interact with PIC directly or via transducing elements such as guanine nucleotide regulatory proteins. These various methods of coupling between receptors and PIC are reviewed.

1.4.1 A role for guanine nucleotide regulatory proteins.

Guanine-nucleotide binding proteins (G-proteins) play a central role in the transduction of a variety of extracellular stimuli to effector systems as diverse as adenylyl cyclase, guanylyl cyclase and ion channels (reviewed by Gilman, 1987; Birnbaumer *et al.*, 1990). The classical, high molecular weight, G-proteins are heterotrimers consisting of an α-subunit of molecular weight 39-52 kDa and a βγ-subunit complex of molecular weights 35-36 kDa and 9-13 kDa respectively. They function in a cyclical manner with binding of an agonist to its receptor stimulating exchange of GTP for bound GDP on the α-subunit. In the presence of Mg^{2+}, the α-GTP complex may dissociate from the βγ-subunit complex and is then able to modulate an effector system. The signal is terminated by the intrinsic GTPase activity of the α-subunit and the cycle is completed by the recomplexing of the subunits to form the heterotrimer. Since it is the α-subunit which is the site of guanine nucleotide binding and interacts with both receptor and effector entities it is likely to confer individuality and specificity upon the G-protein.

The high molecular weight G-proteins have been classified on the basis of their sensitivity to pertussis (G_i) and cholera toxins (G_s). In addition there are a number of G-proteins which are not substrates for toxins e.g., G_z. Finally there are an increasing group of G-proteins, identified by PCR cloning and low stringency hybridization (Strathmann *et al.*, 1989), for which there remains no known function.

The proposal that receptor-activated inositol lipid hydrolysis may be modulated by a G-protein, G_p, was first made by Cockcroft (1987). This was based on the observation that ligand-stimulated Ins(1,4,5)P_3 or InsP_t formation was potentiated by non-hydrolysable guanine-nucleotide analogues such as GTPγS or guanosine 5'-[βγ-imido]triphosphate (GppNHp) and inhibited by GDPβS in

various permeabilized cells (e.g., Geny *et al.*, 1989; Plevin *et al*, 1990). In addition, fluoroaluminate ions (AlF_4^-) which bind to GDP in the nucleotide binding site of G-proteins mimicking the terminal phosphate of GTP but are non-hydrolysable, can elevate inositol phosphate formation in the absence of ligand (Cockcroft & Taylor, 1987).

Despite the number of systems where guanine nucleotide-dependent $InsP_t$ formation has been identified, the molecular identity of G_p remains unresolved. Recent reports have described the purification of a G-protein α-subunit which is not a bacterial toxin substrate and appears to exhibit the properties of G_p in a reconstituted system (Taylor *et al.*, 1990; Pang &Sternweiss, 1990). Other reports have suggested that, in some cell lines where InsP formation is inhibited to varying degrees by pre-treatment with pertussis toxin, G_p may actually be a member of the G_i family (Lad *et al.*, 1985; Pfeilschifter *et al.*, 1986) but again the identity of such a protein remains unknown.

A second and rapidly expanding family of guanine nucleotide regulatory proteins comprises the low or small molecular weight G-proteins (reviewed by Sanders, 1990) which differ markedly from the classical αβγ heterotrimers. This group is exemplified by the members of the *ras* gene super family; protein products of the cellular *ras* proto-oncogene. *Ras* genes encode proteins of molecular weight 21kDa ($p21^{ras}$), possessing a single subunit with an intrinsic GTPase activity. Like the classical G-proteins, *ras* undergoes a GDP/GTP exchange cycle but this is not modulated by interaction with a βγ complex. Indeed, *ras* proteins possess a much lower intrinsic GTPase activity than the classical G-proteins and *in vivo* and this is normally controlled by a cellular protein known as GTPase activating protein (GAP) (Trahey & McCormick, 1987) which stimulates GTP hydrolysis by *ras*. The majority of transforming mutations found in *ras*-induced tumors are due to point mutations in the GTP-binding domain or at the site at which GAP interacts with *ras* preventing GTP hydrolysis, with the result that *ras* remains permanently in the GTP-bound, active state.

It is proposed that in its constitutively active state *ras* generates an uncontrolled proliferative signal resulting in transformation; by analogy with other

G-proteins this would suggest that normal c-*ras* is involved in a signal transduction pathway which regulates mitogenesis. Mammalian *ras* proteins exhibit extensive sequence homology with the yeast RAS genes which function in the regulation of the mating pathway and adenylate cyclase, but in higher eukaryotes *ras* genes do not regulate adenylate cyclase (reviewed by Hall, 1990). A number of observations have suggested a role for $p21^{ras}$ as G_p, the G-protein controlling inositol lipid hydrolysis (Fleischmann *et al.*, 1986; Wakelam *et al.*, 1986; Lloyd *et al.*, 1989). For example, elevated InsP levels correlate with transforming mutations of *ras* which abolish GTPase activity (Hancock *et al.*, 1988). Furthermore, in the $T15^+$ clone of NIH3T3 cells, containing the $p21^{N\text{-}ras}$ gene under the control of a steroid-inducible promoter, expression of the $p21^{N\text{-}ras}$ gene confers responsiveness to bombesin resulting in the stimulated formation of $Ins(1,4,5)P_3$ and elevation of $[Ca^{2+}]_i$ which is negligible in the control cell line $T15^-$ (Lloyd *et al.*, 1989). However, other studies have been unable to show similar effects (e.g., Seuwen *et al.*, 1988b), whilst elevated InsP levels have been reported in cell lines transformed by a variety of oncogenes and may simply reflect elevated growth rates and/or overexpression of growth factor receptors in transformed cells (Downward *et al.*, 1988). Clearly, expression of the $p21^{N\text{-}ras}$ gene in the $T15^+$ cell line does increase bombesin receptor-PIC coupling either directly or indirectly, but it is unlikely that *ras* simply acts as G_p.

1.4.2 Activation of PtdIns-3-kinase.

PtdIns 3-kinase was first demonstrated in immunoprecipitates from polyoma middle T-transformed cells (Whitman *et al.*, 1988) and has recently been purified from rat liver (Carpenter *et al.*, 1990). The enzyme consists of an 85kDa protein, the same as that found to associate with activated PDGF receptors and the middle T antigen (Kaplan *et al.*, 1987), and a 110kDa protein which form a native complex of 190kDa (Carpenter *et al.*, 1990). Since the 85kDa subunit is phosphorylated extensively upon association with PDGF receptors (Kaplan *et al.*, 1987) whilst the 110kDa subunit has not been shown to associate with receptors to

date it is possible that the smaller subunit exerts a regulatory role upon the catalytic activity of the holoenzyme. Non-transforming mutants of Middle T antigen and tyrosine kinase-negative mutants of the PDGF receptor which do not transduce a mitogenic signal do not have an associated PtdIns 3-kinase activity suggesting that this pathway does produce a signal involved in mitogenesis. However, the nature of this signal is unclear (Majerus *et al.*, 1990); it is not yet known if PtdIns(3)P is the precursor for the two novel polyphosphoinositides, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Auger *et al.*, 1989), but none of the 3-phosphate containing inositol lipids are good substrates for phosphoinositidase C from liver or brain (Serunian *et al.*, 1989). Recent reports have implicated this pathway in the response to some extracellular ligands (Auger *et al.*, 1989; Traynor-Kaplan *et al.*, 1989), but its relevance to either second messenger generation or a physiological response remains to be rigorously defined.

1.4.3 Phosphorylation of PLC-γ1.

Immunoprecipitation of activated growth factor receptors followed by blotting with anti-phosphotyrosine or anti-PIC antibodies has shown that a PIC subtype, type II or PLC-γ1, associates with and is phosphorylated upon tyrosine residues in a ligand-dependent manner by PDGF and EGF receptors (Meisenhelder *et al.*, 1989). The receptor tyrosine kinase activity is essential for growth factor-induced PLC activation, and it has recently been shown directly that this tyrosine phosphorylation results in the activation of PLC-γ1 as well as its translocation to the plasma membrane wherein resides its substrate (Nishibe *et al.*, 1990; Todderud *et al.*, 1990). Such coupling of growth factor receptors to PLC-γ1 is fundamentally different to that for mitogenic peptides such as bombesin (Plevin *et al.*, 1990) since it is not modulated by guanine nucleotide analogues. These results are consistent with what is known about the primary structure of PLC-γ1 since it contains two SH2 regions which are thought to play a regulatory role in directing specific interactions with cellular components (Anderson *et al.*, 1990a; reviewed by Rhee *et al.*, 1989).

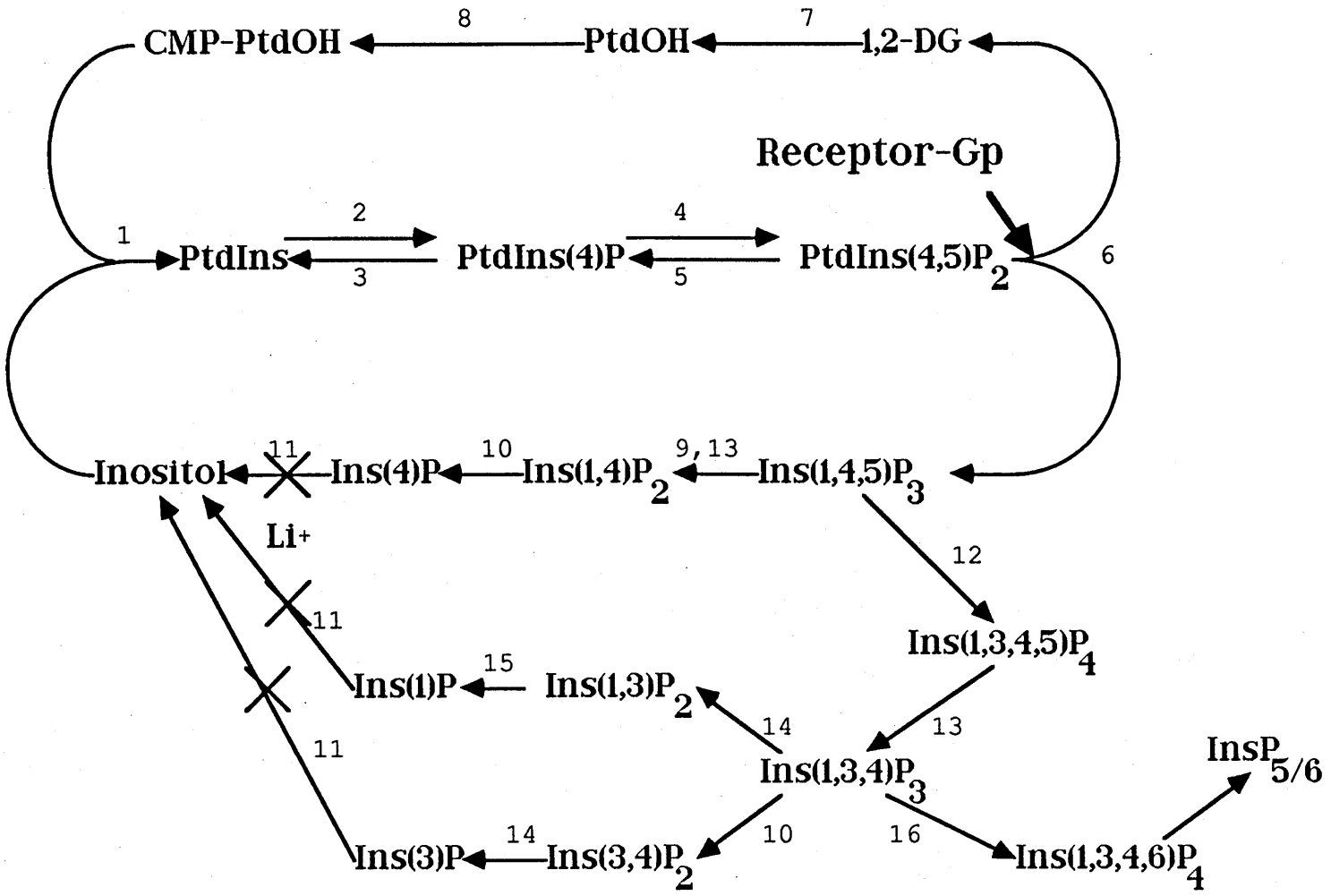
This apparent diversity of coupling mechanisms for activation of PIC presumably reflects some evolutionary advantage in having multiple pathways for regulating inositol lipid hydrolysis. Such speculation will await the identification of G_p, if such a function can be attributed to a single molecular species. The physiological significance of the PLC-γ1 pathway remains to be fully defined since overexpression of PLC-γ1 does not result in increased PDGF-stimulated mitogenesis (Margolis *et al.*, 1990). However different modes of regulation of PIC activity may account for the diversity of responses seen to different agents in the same or different cell lines such as the differing effects of bombesin and PDGF in Swiss 3T3 cells (Nanberg & Rozengurt, 1988; Blakeley *et al.*, 1989).

1.5 Agonist-stimulated polyphosphoinositide hydrolysis generates two second messengers.

A major feature of the signal transduction pathway involving the hydrolysis of the polyphosphoinositides is that both the products of PIC activity are second messengers with distinct cellular functions. Consequently this bifurcating signal constitutes a flexible pathway for initiating diverse effects within the cell (reviewed by Downes & Michell, 1985; Berridge, 1987a) including many of the early events associated with proliferation (Berridge, 1987b; Whitman & Cantley, 1988). The major elements of the hormone sensitive inositol lipid cycle are outlined in Fig. 1.2. This figure does not include details of the metabolism of the 3phosphatecontaining inositol lipids since their physiological relevance remains unclear; however, this issue has recently been reviewed extensively by Majerus *et al.* (1990) and Cantley *et al.* (1991).

Figure 1.2 The major elements of the hormone-sensitive inositol lipid cycle.

A summary of the major elements of the hormone-sensitive inositol lipid cycle is shown; details of the 3' phosphate-containing inositol lipids and the higher phosphorylated forms of inositol ($InsP_{5/6}$) are not shown since their physiological relevance remains unclear. The enzymes are (1) PtdIns synthetase (CMP-PtdOH; inositol phosphatidyltransferase); (2) PtdIns-4-kinase; (3) PtdIns(4)P phosphomonoesterase; (4) PtdIns(4)P-5'-kinase; (5) PtdIns(4,5)P_2 phosphomonoesterase; (6) phosphoinositidase C; (7) DG kinase; (8) CMP-PtdOH synthetase; (9) Ins(1,4,5)P_3-5'-phosphatase; (10) inositolpolyphosphate-1'-phosphatase; (11) Ins(x)P phosphatase; (12) Ins(1,4,5)P_3-3'-kinase; (13) Ins(1,4,5)P_3/Ins(1,3,4,5)P_4-5'-phosphatase; (14) inositolpolyphosphate-4'-phosphatase; (15) Ins(1,3)P_2-3'-phosphatase; (16) Ins(1,3,4)P_3-6'-kinase. All inositol phosphates are numbered as the D-*myo*-inositol configurations as described by Berridge & Irvine, (1989).


CMP-PtdOH
8
PtdOH
7
1,2-DG
Receptor-Gp
1
PtdIns
2
3
PtdIns(4)P
4
5
PtdIns(4,5)P2
6
Inositol
11
Ins(4)P
10
Ins(1,4)P2
9,13
Ins(1,4,5)P3
Li+
12
11
Ins(1,3,4,5)P4
Ins(1)P
15
Ins(1,3)P2
14
13
11
Ins(1,3,4)P3
InsP5/6
Ins(3)P
14
Ins(3,4)P2
10
16
Ins(1,3,4,6)P4

1.5.1 Ins(1,4,5)P_3 and intracellular calcium homeostasis.

Whilst agonist-stimulated inositol lipid turnover was first documented in 1953 (Hokin & Hokin, 1953) it was not until 1975 that Michell (1975) identified stimulated inositol lipid turnover as the common denominator for a variety of agonists which elicit a rapid increase in intracellular free calcium concentration ($[Ca^{2+}]_i$). Indeed, it was 1983 before it was recognised that PtdIns(4,5)P_2 is the major precursor (Creba *et al.*, 1983) and Ins(1,4,5)P_3 is the initial product (Berridge, 1983) of agonist-stimulated inositol lipid hydrolysis, whilst Streb *et al.* (1983) showed that Ins(1,4,5)P_3 was effective in releasing Ca^{2+} from permeabilized pancreatic acinar cells. Such observations have now been repeated in a variety of cell types including Swiss 3T3 cells, GH_3 pituitary cells, hepatocytes and vascular smooth muscle cells (reviewed by Berridge, 1987a). To satisfy the role of 'Ca^{2+} mobiliser' Ins(1,4,5)P_3 must, and indeed is, produced very rapidly upon stimulation of responsive cells. Relatively few studies have looked at timepoints below 10 seconds but in bradykinin-stimulated NG115-401L cells [^{3}H]Ins(1,4,5)P_3 levels are significantly above control at two seconds (Jackson *et al.*, 1987).

In permeabilized cells, the reponse to Ins(1,4,5)P_3 takes the form of saturable release of between 30 and 70% of sequestered Ca^{2+} with an EC_{50} in the range of 0.1-1μM. There is marked selectivity for inositol phosphate induced Ca^{2+} release with orders of potency of Ins(1,4,5)P_3> GroPtdIns(4,5)P_2= Ins(2,4,5)P_3> Ins(4,5)P_2 suggesting an absolute requiremant for vic inal 4' and 5' phosphate groups with a 1' phosphate enhancing the effect. These observations of saturability and selectivity are consistent with Ins(1,4,5)P_3 exerting its effect by binding to a specific receptor. Consequently, it is now accepted that Ins(1,4,5)P_3, released by hydrolysis of PtdIns(4,5)P_2, binds to an intracellular receptor mediating the release of Ca^{2+} from an intracellular store into the cytosol.

The Ins(1,4,5)P_3 receptor has now been purified from brain homogenates (Supattapone *et al.*, 1988) and cloned from cerebellar Purkinje neurons (Furuichi *et al.*, 1989). The receptor is a glycoprotein with predicted molecular weight of 313 kDa which probably exists as a tetramer *in vivo* and when solubilized giving a

molecular weight of around 1000 kDa. In overall topography as well as at the C-terminus, the Ins(1,4,5)P_3 receptor exhibits significant homology with the ryanodine Ca^{2+} channel from the sarcoplasmic reticulum of skeletal muscle (Takeshima *et al.*, 1989).

The subcellular location and identity of the Ins(1,4,5)P_3 sensitive Ca^{2+} store remains to be rigorously defined and indeed may be different depending on the nature of the cell or tissue concerned. However, it is clear that the Ins(1,4,5)P_3 sensitive store is not part of the mitochondria (Burgess *et al.*, 1984; Volpe *et al.*, 1987; Ross *et al.*, 1989) which constitutes a major store of intracellular Ca^{2+}. Recent immunocytochemical data suggest that the rough E.R. and the nuclear envelope are principal sites of the Ins(1,4,5)P_3 receptor as is peripheral smooth E.R., close to the plasma membrane (Ross *et al.*, 1989). The more uniform labelling of the E.R. reported by Mignery *et al.* (1989) would seem to argue against a role for a specialized Ins(1,4,5)P_3-responsive organelle such as the 'calciosome' proposed by Volpe *et al.* (1988).

The transient release of Ca^{2+} from the Ins(1,4,5)P_3-sensitive store is not due to the desensitization of the Ins(1,4,5)P_3 receptor since use of the non-metabolised analogue inositol(1,4,5)trisphosphorothioate results in a sustained Ca^{2+} efflux from hepatocytes with no net re-sequestration suggesting that the Ins(1,4,5)P_3-gated channel remains open (Taylor *et al.*, 1990).

Phosphorylation of Ins(1,4,5)P_3 by a 3' kinase (see Fig. 1.2) may serve to generate another second messenger molecule, Ins(1,3,4,5)P_4. The sustained, though smaller, elevation of $[Ca^{2+}]_i$ represents Ca^{2+} entry into the cell and it has been suggested that this may be due to a second messenger-operated Ca^{2+} channel (reviewed by Berridge & Irvine, 1989; Irvine, 1990). The precise role of Ins(1,3,4,5)P_4 in regulating Ca^{2+} entry is unclear since it exerts no effect unless a Ca^{2+}-mobilizing $InsP_3$ (Ins(1,4,5)P_3 or Ins(2,4,5)P_3) is present (Irvine *et al.*, 1988). Irvine (1990) has speculated that there are three 'players' in the regulation of Ca^{2+}entry: the intraluminal $[Ca^{2+}]$ in the E.R., Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. He argued that Ins(1,4,5)P_3 binding to its receptor discharges

Ca^{2+} from the E.R. lumen and this results in the dissociation of the $Ins(1,4,5)P_3$ receptor from an $Ins(1,3,4,5)_4$ receptor at the plasma membrane, thus removing a tonic inhibition of Ca^{2+} entry. $Ins(1,3,4,5)P_4$ may also contribute to this by binding to its receptor and promoting dissociation of the $Ins(1,4,5)P_3$ receptor. In support of this Irvine cited the example of the proposed communication between the ryanodine receptor on the sarcoplasmic reticulum and the sarcolemma but confirmation of such a model must await the purification and identification of the $Ins(1,3,4,5)P_4$ receptor.

1.5.2 The metabolism of $Ins(1,4,5)P_3$; physiological relevance.

The transient nature of many Ca^{2+} responses suggests that $Ins(1,4,5)P_3$ is rapidly removed and/or responses are rapidly desensitized at the receptor-G_p-PIC level. It is well established that, in eukaryotes, $Ins(1,4,5)P_3$ may be removed rapidly by two pathways (see Fig. 1.2) and there is some evidence to suggest that a rise in $[Ca^{2+}]_i$ may actually facilitate the removal of $Ins(1,4,5)P_3$, thereby ensuring the transient nature of the response.

The first pathway involves the rapid dephosphorylation of $Ins(1,4,5)P_3$ by a 5'-phosphatase to yield $Ins(1,4)P_2$ (Downes *et al.*, 1982; Storey *et al.*, 1984) which, lacking the 4' and 5' vic inal phosphate groups, is unable to release Ca^{2+} from the internal store. The observation that Ca^{2+} activates the 5'-phosphatase in a variety of cells (Kikita *et al.*, 1986; Sasaguri *et al.*, 1985) thereby ensuring the removal of the second messenger is attractive but not universal (Connolly *et al.*, 1987). $Ins(1,4)P_2$ then undergoes a de-phosphorylation reaction to InsP. It would appear that the favoured reaction is a 1'-phosphatase giving Ins(4)P as the product; less than 5% of $Ins(1,4)P_2$ is metabolized by the 4'-phosphatase (Morris *et al.*, 1988). It is known that a single enzyme de-phosphorylates Ins(1)P, Ins(3)P and Ins(4)P to yield *myo*-inositol (Gee *et al.*, 1988). The Ins(x)P-phosphatase is inhibited in an uncompetitive manner by millimolar concentrations of Li^+, providing a useful block in the de-phosphorylation of inositol phosphates. This allows a convenient measure of receptor-mediated inositol lipid hydrolysis to be

made in terms of the accumulation of total inositol phosphates ($InsP_t$) (Berridge *et al.*, 1982).

The second pathway for the removal of $Ins(1,4,5)P_3$ is its rapid phosphorylation to inositol(1,3,4,5)tetrakisphosphate ($Ins(1,3,4,5)P_4$) by a 3'-kinase. This reaction was first observed in parotid gland (Batty *et al.*, 1985) and has subsequently been demonstrated in a variety of tissues. Again, in terms of removal of a Ca^{2+}-mobilising second messenger, the 3' kinase is activated by elevation of $[Ca^{2+}]_i$ and, in brain and neural tissues, by Ca^{2+}-calmodulin complexes (Biden *et al.*, 1987; Morris *et al.*, 1987), allowing fine control of intracellular Ca^{2+} elevation. $Ins(1,3,4,5)P_4$ is rapidly de-phosphorylated by a Mg^{2+}-dependent 5'-phosphatase which is probably the same enzyme which de-phosphorylates $Ins(1,4,5)P_3$ (Connolly *et al.*, 1987). $Ins(1,3,4)P_3$ is ineffective at mobilizing intracellular Ca^{2+} and is probably de-phosphorylated by the same 1'-phosphatase which acts on $Ins(1,4)P_2$ (Inhorn & Majerus, 1987) The product, $Ins(3,4)P_2$ may be de-phosphorylated by a Mg^{2+}-independent 4'-phosphatase (Bansal *et al.*, 1987).

The complex metabolism of inositol phosphates, including the higher phosphorylated forms (e.g., $InsP_5$ and $InsP_6$) has been reviewed by Shears (1989) and is not considered here but recent articles by Stephens & Downes (1990) and Stephens & Irvine (1990) provide new insights.

The rapid decline in $Ins(1,4,5)P_3$ levels may not simply reflect metabolism but also a negative feedback. In Swiss 3T3 cells it has been proposed that DG, the other product of $PtdIns(4,5)P_2$ hydrolysis may, through the activation of protein kinase C lead to the inhibition of further $PtdIns(4,5)P_2$ breakdown (Brown *et al.*, 1987). This inhibition of responses can be mimicked by pre-treating cells with the phobol ester, PMA, for 10 minutes prior to stimulation with bombesin, but is abolished in Swiss 3T3 cells where PKC activity has been down-regulated by prolonged exposure to phorbol ester (Brown *et al.*, 1987).

1.5.3 The diglyceride/protein kinase-C arm of the signalling pathway.

The term *sn*-1,2-diradylglycerol refers to all forms of diglyceride with the *sn*-1,2- configuration and includes ester-linked diacyl species (DAG) and ether-linked 1-*O*-alky-2-acyl and 1-*O*-alk-1'enyl-2-acyl species (AAG). Unless referring to a diglyceride with a particular linkage the abbreviation DG will be used as a general term encompassing *sn*-1,2-diglycerides.

DG is a neutral lipid which remains in the inner leaflet of the plasma membrane when formed as a result of stimulated inositol lipid hydrolysis. It is the endogenous activator of the Ca^{2+}- and phospholipid-dependent serine/threonine directed phosphotransferase, protein kinase C (PKC), isolated in Nishizukas laboratory in 1977 (Inoue *et al.*, 1977; Takai *et al.*, 1979a & b; Kishimoto *et al.*, 1980; reviewed by Nishizuka, 1984). The mechanism by which DG activates PKC is well documented. Briefly, DG acts by lowering the enzymes' K_m for Ca^{2+} by as much as one thousand-fold (Kishimoto *et al.*, 1980; Kaibuchi *et al.*, 1981); PKC may be activated by millimolar concentrations of Ca^{2+} *in vitro*, but inclusion of DG allows activation at $[Ca^{2+}]_i$ in the range 0.1-1μM. Since these Ca^{2+} concentrations are attained in cells as a consequence of Ins(1,4,5)P_3-induced Ca^{2+} mobilisation, this represents cooperation between the two arms of what is essentially a bifurcating signal pathway. It is only the *sn*-1,2- forms of DG (derived from phospholipid hydrolysis) which activate PKC; neither 1,3-DG or *rac*-1,2-DG (*sn*-2,3-DG) are able to support PKC activity (Rando & Young, 1984; Boni & Rando, 1985; Nomura *et al.*, 1986). There is some debate as to whether the AAG forms of DG are able to activate PKC (Ganong *et al.*, 1986; Daniel *et al.*, 1988), but a recent report shows quite clearly that naturally occurring plasmalogenic diglycerides do support rabbit myocardial PKC activity (Ford & Gross, 1990a).

It is proposed that, upon stimulation, soluble PKC translocates to the plasma membrane (Kraft *et al.*, 1982; Kraft & Anderson, 1983) where it combines with its endogenous activator (DG) forming an active quaternary complex of PKC, DG, Ca^{2+} and PtdSer (Ganong *et al.*, 1986). However, quite how PKC rapidly

moves from an aqueous, hydrophilic environment to associate with the plasma membrane is unclear, though increased $[Ca^{2+}]_i$ may play a role. Perhaps a more realistic model envisages PKC normally loosely associated with the plasma membrane, with this association strengthened by complexing with DG and PtdSer in stimulated cells. This model will still allow for the appearance of soluble PKC in control cells since the looser association would result in displacement from the membrane during subcellular fractionation.

1.5.4 Protein kinase C, an intracellular receptor for the tumour-promoting phorbol esters.

In 1982, Castagna *et al.* (1982) demonstrated a crucial link between tumour induction and a signal transduction pathway by showing that the phorbol ester-type tumour promoters could activate PKC and that this activation correlated with their tumour promoting ability (Castagna *et al.*, 1982; Niedel *et al.*, 1983). As a result protein kinase C is now considered to occupy a central role in the pathways proposed to control cell division and proliferation (reviewed by Berridge, 1987b; Whitfield *et al.*, 1987; Housey *et al.*, 1988; Persons *et al.*, 1988; Whitman & Cantley, 1988).

Phorbol esters, typified by phorbol-12-myristate-13-acetate (PMA) act as structural homologues of the diradylglycerols; the "diradyl" portion of PMA is structually analagous to the *sn*-1,2-DGs and studies have shown that, like DG, PMA activates PKC by increasing its Ca^{2+} sensitivity (Castagna *et al.*, 1982). In addition, PMA and DG compete for binding to PKC with a stoichiometry of 1 mol per mol of protein (reviewed by Downes & Michell, 1985). The potent tumour promoting properties of PMA can be explained by the fact that unlike DG it is not readily metabolized within cells and so accumulates, resulting in the prolonged activation of PKC. This is reflected in the potentcy of the phorbol esters in activating PKC; PMA is active at nanomolar concentrations whereas cell permeant diglycerides, which are metabolized, require micromolar concentrations.

Phorbol esters, being membrane permeable, have proved to be useful tools for dissecting the involvement of DG production and PKC activation in cell responses and, being poorly metabolized, accentuate and lengthen normal responses. Their use, coupled with that of membrane permeable DG analogues, such as 1-oleoyl-2-acetyl-glycerol (OAG), has allowed the identification of PKC dependency in a variety of signal-response systems (reviewed by Nishizuka, 1984; Kikkawa *et al.*, 1989; Parker *et al.*, 1989).

In addition, for some cells, most notably the Swiss 3T3 fibroblast, prolonged exposure to super-maximal concentrations of PMA (400nM) for up to 48 hours depletes cellular PKC as measured by binding of [^{3}H]PDBu (Collins & Rozengurt, 1984), histone phosphorylation (Rodriguez-Pena & Rozengurt, 1984) and western blotting for the protein itself (Brown *et al.*, 1990). This loss of PKC activity, known as 'down-regulation', has allowed the identification of PKC-dependent events in a number of hormone and growth factor-stimulated cell lines such as the inhibition of DNA synthesis in down-regulated Swiss 3T3 cells (Rozengurt & Sinnett-Smith, 1987).

1.5.5 Multiple isoforms of protein kinase C with distinct properties.

The relatively simple model of DG and Ca^{2+} cooperating in the activation of PKC is now no longer sufficient to account for the complexity revealed by the purification and cloning of at least seven different isozymes of protein kinase C (reviewed by Kikkawa *et al.*, 1989; Parker *et al.*, 1989). The α, βI, βII and γ forms exhibit the same general structural organization (Kikkawa *et al.*, 1988; Parker *et al.*, 1989) being divided into cysteine-rich regulatory and catalytic domains; the βI and βII subspecies are derived by alternate splicing of a single RNA transcript (Coussens *et al.*, 1987; Kubo *et al.*, 1987). In the last two years cDNAs for at least three further subspecies of PKC (δ-, ε- and ζ-PKC) have been isolated using a mixture of α-, βII and γ- cDNA clones as probes under low stringency conditions (Ono *et al.*, 1987; 1988). These novel forms exhibit extensive clusters of sequence homology to α-, β- & γ- species in their catalytic

domains and in cysteine-rich clusters in the regulatory domains, whilst their overall molecular weights are of a similar order. Calpain-catalysed proteolysis results in the cleavage of PKC into separate regulatory and catalytic domains and, under such conditions, the catalytic domain is fully active in a manner independent of PtdSer or DG, suggesting that the regulatory domain exerts a negative influence over the catalytic site which is overcome by complexing with DG, Ca^{2+} and PtdSer.

In terms of activation, α-type PKC is most sensitive to 1-stearoyl-2-arachidonoylglycerol, derived from PtdIns(4,5)P_2 hydrolysis, whilst βI and βII exhibit substantial activity without raised Ca^{2+} levels. The γ-subspecies is less sensitive to DG but is activated by relatively low concentrations of arachidonic acid; indeed, activation by arachidonic acid does not appear to require Ca^{2+}, DG or phospholipid (reviewed by Kikkawa *et al.*, 1989). Recently nPKC (Ohno *et al.*, 1988), PKC-ε (Schaap & Parker, 1990) and PKC-L (Bacher *et al.*, 1991) have been characterised as Ca^{2+}-independent, DG/PMA activated PKCs.

The various isozymes appear to have distinct substrate specificities aswell as distinct specific activities against well defined PKC substrates such as histone IIIS (Schaap & Parker, 1990; Ohno *et al.*, 1988). Furthermore, what was previously considered to be a homogenous PKC substrate, the 80k phosphoprotein, is now known to consist of at least two isoforms (Morris & Rozengurt, 1988; Hirai & Shimizu, 1990) phosphorylated to different extents by α-, β- & γ-PKCs. Digestion with trypsin generated different phosphopeptide maps (Hirai & Shimizu, 1990) and one isoform (80K-H) was substantially phosphorylated in the absence of Ca^{2+}.

These data suggest that different cells may possess a number of PKC subspecies which may be differentially activated during different phases of a cellular response under conditions in which DG levels or other phospholipid metabolites are elevated in the presence or absence of increased $[Ca^{2+}]_i$, such as stimulated hydrolysis of PtdIns(4,5)P_2 or PtdCho (and other phospholipids) by phospholipases A_2, C or D. This potentially broad repertoire of PKC activities may result in different substrates being phosphorylated at various stages of the response and may be of relevance in long term effects such as smooth muscle contraction, long term potentiation and cell proliferation.

1.5.6 The removal of *sn*-1,2-DG; physiological relevance.

As with $Ins(1,4,5)P_3$, the primary function of the rapid removal of DG is the termination of the physiological response, that is PKC activation. Two major pathways exist for the removal of DG, catalysed by DG kinase and DG lipase. However, it is becoming increasingly apparent that the products of both of these reactions, PtdOH and free fatty acids (particularly arachidonate), may themselves have potent biological effects (Putney *et al.*, 1980; Moolenaar *et al.*, 1986; van Corven *et al.*, 1989; Tsai *et al*, 1989; 1990).

The phosphorylation of DG to yield *sn*-1,2-diradylglycerol-3-phosphate, i.e. phosphatidic acid (PtdOH), is probably the most quantitatively significant pathway for the removal of DG (Bishop *et al.*, 1986). It is catalysed by the enzyme diacylglycerol kinase (ATP:1,2-diacylglycerol 3-phosphotransferase), a Mg^{2+} and ATP dependent kinase first described by Hokin & Hokin (1959). Until recently, there have been few detailed studies of this enzyme in higher eukaryote cells; the realisation of its role as a major 'switch' which turns off protein kinase C activity has focussed attention on what is, by analogy with the cyclic nucleotide phosphodiesterases, likely to prove a major control point in PKC mediated signals (reviewed by Kanoh *et al.*, 1990).

In common with PKC, there is an increase in DG kinase activity recovered from the plasma membrane and a loss from the cytosol when cells are stimulated with Ca^{2+}-mobilizing agonists which cause hydrolysis of inositol lipids or phorbol esters (Ishitoya *et al.*, 1987; Maroney & Macara, 1989). This may represent an important control point since DG is produced in, and confined to, the plasma membrane. Coupled with this, recent reports suggest that porcine DG kinase activity may be enhanced at the physiological $[Ca^{2+}]_i$ attainable in stimulated cells (Sakane *et al.*, 1990). Indeed, the primary sequence for the 80kDa form of DG kinase purified from porcine thymus cytosol and cloned from a porcine thymus cDNA library contains two Ca^{2+}-binding EF hands as well as homologies to PKC, calmodulin and other Ca^{2+} regulated proteins (Sakane *et al.*, 1990). Interestingly, this form of DG kinase also possesses two cysteine-rich motifs which are

conserved in a variety of proteins which bind phorbol esters such as PKC and n-chimaerin (Ahmed *et al.*, 1990); consequently this region may represent the DG/phorbol ester binding region. Cysteine-rich, zinc-finger-like motifs are implicated in DNA binding though there is no evidence of DG kinase activity in the nucleus to date. However, recent reports of insulin-like growth factor 1-stimulated phospholipid metabolism in the nucleus of Swiss 3T3 cells (Cocco *et al.*, 1988) may be of relevance to reports of PKC activity in the nucleus (Fields *et al.*, 1989; 1990; Hocevar & Fields, 1991).

Studies of the substrate specificity of DG kinase from Swiss 3T3 membranes (MacDonald *et al.*, 1988) has demonstrated the presence of an isoform which exhibits greatest activity against the arachidonoyl containing diglycerides. In particular, 1-stearoyl-2-arachidonoyl glycerol (SAG) is an especially good substrate for the DG kinase of Swiss 3T3 membranes and this specificity may provide a means by which inositol lipids become enriched in arachidonoyl containing diglycerides (MacDonald *et al.*, 1988). Furthermore, rabbit brain microsomal DG kinase exhibits 30-fold greater activity against diacylglycerols (DAG) than alkyl-acyl (Alkyl AG) and alkenyl acyl glycerols (AAG) (Ford & Gross, 1990b). This may explain why increases in plasmalogenic diglycerides are more sustained than the increases in DAG in those cells which produce significant quantities of plasmalogenic DG upon stimulation e.g., neutrophils and HL60 cells (Truett *et al.*, 1989a; Bonser *et al.*, 1989; Billah *et al.*, 1989b).

These observations can be brought together in a model to explain the rapid removal of DG. The increase in 1-stearoyl-2-arachidonoyl glycerol (SAG) accompanying stimulated inositol lipid hydrolysis will result in the 'translocation' and activation of an arachidonoyl-diglyceride specific DG kinase. This activation might be enhanced by the concomitant rise in $[Ca^{2+}]_i$ as a result of $Ins(1,4,5)P_3$ elevation. These factors, combined with the potentsubstrate-induced activation reported by MacDonald *et al.* (1988) would result in the rapid removal of SAG. Until recently the accumulation of PtdOH was taken to infer this, but observations of agonist-stimulated phospholipase D activation may also contribute to this elevation of PtdOH and ultimately DG (see section 1.8).

The second major pathway for removing diglyceride is its deacylation at either the *sn*-1, or particularly, the *sn*-2 position. This reaction is catalysed by DG lipase. Despite relatively little evidence, it is thought that DG lipase is of less quantitative importance in removing DG than DG kinase (Bishop *et al.*, 1986). However the enzyme may be of qualitative importance in generating free arachidonic acid which serves as the precursor for the eicosanoids, leukotrienes, and thromboxanes and may well have second messenger properties of its own (reviewed by Kikkawa *et al.*, 1989; Smith, 1989; Millar & Rozengurt, 1990). Consequently the DG lipase reaction may be of importance in generating a variety of other biologically active compounds such as the prostaglandins (reviewed by Smith, 1989).

The use of the putative inhibitors of either DG lipase (RHC 80267) or DG kinase (R 59022) has been reported to raise both control and stimulated levels of diglyceride. However the efficacy of both compounds has been questioned and the former is no longer commercially available. Clearly a thorough study of the contributions of DG lipase and DG kinase in DG and arachidonate homeostasis is required, especially since arachidonate has recently been reported to be an activator of PKC (reviewed by Kikkawa *et al.*, 1989). The purification, cloning and expression of these enzymes in a model system should go some way towards addressing these issues.

Following phosphorylation of DG by DG kinase the PtdOH so produced is combined with CTP to form CMP-PtdOH (CMP-PtdOH synthetase, Fig. 1.2) which serves as the immediate lipid precursor for PtdIns by the action of PtdIns synthetase (Fig. 1.2). In this way the cycle is completed and inositol moieties are recycled for replenishing of inositol lipids.

1.6 Evidence for alternative sources of *sn*-1,2-DG in stimulated cells.

In the last three or four years, a number of observations have suggested that *sn*-1,2-diglyceride can be generated either in the absence of Ins(1,4,5)P_3 accumulation or from non-inositide sources in response to a variety of mitogenic and non-mitogenic stimuli (reviewed by Billah & Anthes, 1990). In particular some agonists which stimulate the rapid generation of Ins(1,4,5)P_3 and DG also elicit a slower sustained elevation of DG which is not accompanied by Ins(1,4,5)P_3 formation (Wright *et al.*, 1988; Truett *et al.*, 1989a; Reibmann *et al.*, 1988). Other agonists can apparently stimulate an increase in DG which is completely divorced from inositol lipid hydrolysis (e.g., Wright *et al.*, 1990). Three major alternative sources of DG which have been considered and are outlined below, but recent attention has focussed particularly on a possible role for hydrolysis of phosphatidylcholine in generating diglyceride and this is therefore reviewed in greatest depth.

1.6.1 Hydrolysis of PtdIns.

Agonist-stimulated formation of Ins(1)P or Ins(1,4)P_2 or Ins(1,4,5)P_3 from PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 respectively must be accompanied by DG formation since PLC-catalysed cleavage of these lipids attacks the ester bond between the 1' phosphate and the diglyceride moiety leaving the phosphate group on the inositol ring. Consequently, PLC-catalysed hydrolysis of any phosphoinositide, or indeed any glycerophospholipid, can conceivably give rise to DG formation. A number of studies have suggested a role for the hydrolysis of PtdIns in signalling events. In vascular smooth muscle cells labelled with [^{3}H]inositol, Griendling *et al.* (1986) have argued that hydrolysis of PtdIns(4,5)P_2 is the primary, but transient, event which is followed by a later calcium-dependent hydrolysis of PtdIns to yield InsP. PIC catalysed hydrolysis of PtdIns would generate diglyceride for activation of protein kinase C, but in the absence of elevated $[Ca^{2+}]_i$ since the water soluble products would not include

$Ins(1,4,5)P_3$. However, the results of radiochemical changes in the specific activity of individual [^{3}H]inositol lipids are difficult to interpret as hydrolytic events. Since $PtdIns(4,5)P_2$ is such a minor component of the inositol lipid pool, changes in its associated radioactivity are readily seen (Creba *et al.*, 1983; Takuwa *et al.*, 1987a). In contrast, PtdIns, the major inositol lipid, needs to undergo much greater changes before differences in its radioactivity can be detected.

Imai & Gershengorn (1986) also suggest a similar temporal relationship between primary $PtdIns(4,5)P_2$ hydrolysis and secondary, sustained PtdIns hydrolysis in TRH-stimulated GH_3 pituitary cells. They observed that in GH_3 cells labelled with ^{32}Pi for only 1 min and then stimulated with TRH the increase in labelling of $PtdIns(4,5)P_2$ was transient, lasting less than 2 min, whereas the increase in labelling of PtdIns and PtdOH was sustained. Furthermore, if GH_3 cells were stimulated with TRH for 4 min and then exposed to ^{32}Pi there was a sustained increase in ^{32}Pi labelling of PtdOH and PtdIns without any labelling of $PtdIns(4,5)P_2$ at all suggesting that hydrolysis of $PtdIns(4,5)P_2$ was finished but that PtdIns hydrolysis was continuing.

In a variety of systems, $Ins(1,4,5)P_3$ levels return rapidly towards control after stimulation and this has been taken as an indication that the cells are desensitized and $PtdIns(4,5)P_2$ is diminished. Whilst this may well be the case in some cells, an additional explanation is likely to be the high activity of $Ins(1,4,5)P_3$ 5'-phosphatase and 3'-kinase which quickly remove $Ins(1,4,5)P_3$. Thus, the low levels of $Ins(1,4,5)P_3$ may represent a new 'steady state' where stimulated formation is effectively matched by rapid removal. Therefore, in the presence of Li^+, the prolonged accumulation of InsP may reflect a simple shunting of inositol moieties from $Ins(1,4,5)P_3$ to InsP.

1.6.2 *de novo* synthesis of DG.

Relatively few reports have described *de novo* synthesis from glycerol as a pathway of stimulated DG formation. In the main these studies have looked at the stimulated incorporation of [^{3}H]glycerol into DG and attributed this to synthesis.

However, it seems likely that even a short labelling period, under these conditions, will result in significant incorporation into the major phospholipids, so that the appearance of [^{3}H]DG may be potentially explained by phospholipid hydrolysis. Despite this caveat, Farese and co-workers have been able to demonstrate insulin-stimulated increases in [^{3}H]DG in rat diaphragms (Ishizuka *et al.*, 1990) when the [^{3}H]glycerol was added at the same time as the insulin. However, the increase in labelling of DG was preceded by significant increases in the labelling of PtdOH at 5 minutes therefore suggesting that the increase in DG is due to enhanced synthesis of PtdOH *de novo* followed by its conversion to DG. In BC3H-1 myocytes, the case is less clear (Farese *et al.*, 1988): whilst there is a good correlation between the time courses of insulin-stimulated increases in DG mass and [^{3}H]glycerol incorporation into DG, it is not clear if some of the increase in [^{3}H]DG may reflect hydrolysis of pre-existing phospholipids such as PtdCho and PtdEtn which incorporate significant [^{3}H]glycerol over a 2 hour exposure.

It seems likely that in those sytems where *de novo* synthesis of DG may play a role in stimulated DG formation, the onset of the response may be slower than that for the classical pathway for DG formation from phospholipid hydrolysis which is apparent within seconds of addition of the appropriate stimulus. Thus, it may be that *de novo* synthesis may be of relevance to long term increases in DG.

1.6.3 Agonist-stimulated hydrolysis of phosphatidylcholine (PtdCho) and other phospholipids.

A role for phosphatidylcholine metabolism in signal transduction dates back to a number of observations made in the late 1970s and early 1980s. In cultured chick embryo myoblasts Grove and Schimmel showed that addition of the phorbol ester, PMA, stimulated the synthesis PtdIns, PtdCho and an elevation of PtdOH and 1,2-DG levels (Grove & Schimmel, 1982). The fatty acid composition of this DG was similar to that of PtdCho. A number of studies have demonstrated incorporation of [^{32}P]Pi and [^{3}H]choline into PtdCho over a matter of minutes in

response to a variety of stimuli (Grove & Schimmel, 1982; Kolesnick, 1987; Muir & Murray, 1987; Monaco *et al.*, 1988). Furthermore, many hormones, growth factors, cell permeant DGs and phorbol esters can stimulate PtdCho synthesis through the activation of choline kinase (Warden & Friedkin, 1984; 1985) and CTP:phosphocholine cytidylyltransferase (Kolesnick, 1987; Muir & Murray, 1987). However, in considering a role for PtdCho hydrolysis in generating DG, a primary hydrolytic event must be identified; even then assaying the secondary event of PtdCho resynthesis provides little kinetic or mechanistic information.

One of the earliest reports of stimulated PtdCho hydrolysis was by Mufson *et al.* (1981) who demonstrated that addition of PMA to [^{3}H]choline labelled C3H10T1/2 mouse embryo fibroblasts resulted in the release of choline and phosphocholine. Similar effects of PMA have subsequently been identified in a variety of cell types in the last few years including cultured human epidermal keratinocytes (DeLeo *et al.*, 1986), NG108-15 neuroblastoma x glioma hybridoma cells (Liscovitch *et al.*, 1987),Swiss 3T3 fibroblasts (Muir & Murray, 1987; Price *et al.*, 1989) and REF52 fibroblasts (Welsh *et al.*, 1988; Cabot *et al.*, 1989). Furthemore, agonist-stimulated hydrolysis of PtdCho and generation of choline and phosphocholine has been demonstrated in reponse to a variety of agonists which bind to plasma membrane receptors including bradykinin (Martin & Michaelis, 1988), bombesin (Muir & Murray, 1987; Price *et al.*, 1989), PDGF (Besterman *et al.*, 1986b; Price *et al.*, 1989) and carbachol (Martinson *et al.*, 1989). Perhaps the most convincing evidence for PtdCho hydrolysis serving as a source of DG has come from the studies of Raben and co-workers (Pessin & Raben, 1989; Pessin *et al.*, 1990). By comparing the fatty acid composition of DG derived from stimulated cells with that of the individual cellular phospholipids, they showed that only at early time points (15 seconds) were inositol lipids making a significant contribution to DG in α-thrombin-stimulated IIC9 cells. At later times (5 and 60 min), the DG has a fatty acid profile similar to that of PtdCho. These results correlate well with the biphasic increase in DG mass seen in these cells when stimulated with α-thrombin (Wright *et al.*, 1988); the early phase parallelled increases in $InsP_3$ whereas the later phase was divorced from $InsP_3$ formation.

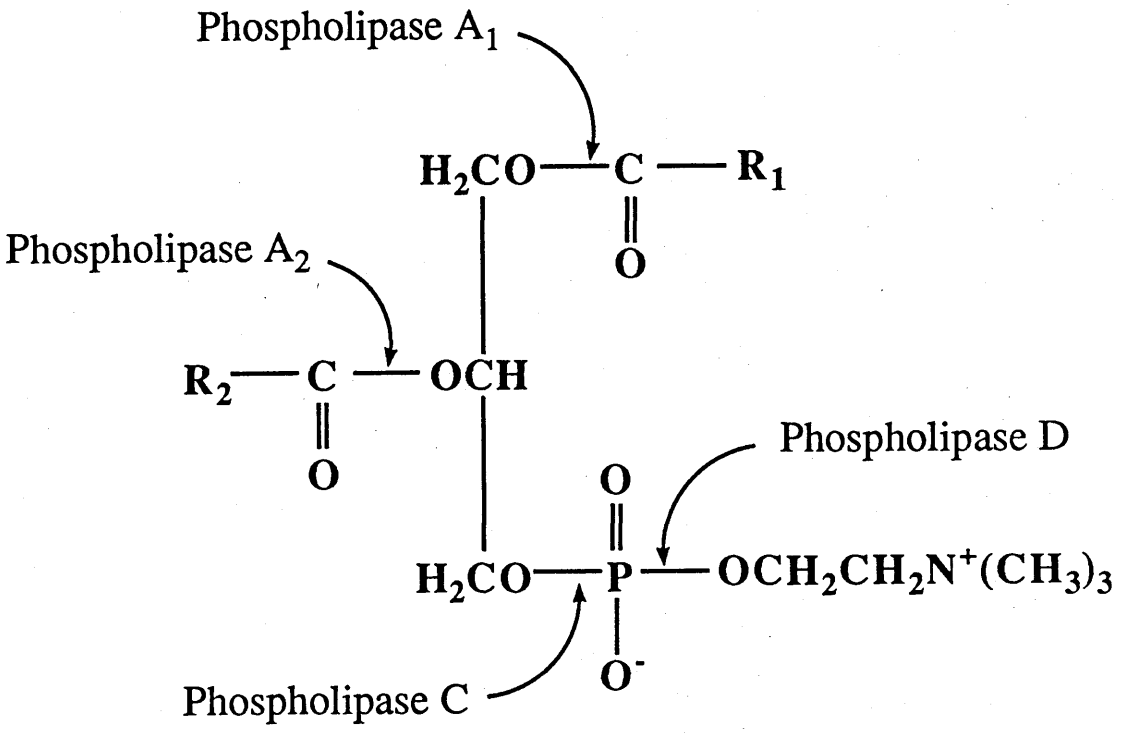
Hydrolysis of PtdCho to generate DG could occur by two distinct mechanisms: activation of a PtdCho specific phospholipase C activity to yield DG and phosphocholine (ChoP) (Fig. 1.3) or activation of a PtdCho specific phospholipase D to generate PtdOH and choline (Cho) (Fig. 1.3) followed by de-phosphorylation of PtdOH to DG by 3-*sn*-phosphatidate phosphohydrolase (PPH) (a PLD/PPH pathway, Fig. 1.4). It is certainly necessary to define which of these two pathways is activated since, whilst both can provide DG, PPH may de-phosphorylate the PtdOH produced by DG kinase: thus, activation of both enzymes could constitute a futile cycle wasting ATP. As such, the PLD/PPH coupled pathway might have major ramifications not only for lipid metabolism but also for cell function and cellular ATP conservation.

With this in mind, it is interesting to note that, to date, the majority of reports of stimulated PtdCho breakdown have identified the PLD-catalysed route (discussed in section 1.7 & 1.8). There are relatively few accounts which provide strong evidence for stimulated PtdCho hydrolysis occurring by a PLC pathway. Thus, Besterman *et al.*(1986b) in 3T3-L1 cells and Larrodera *et al.* (1990) in Swiss 3T3 cells reported that PDGF stimulated an increase in DG which was accompanied by release of phosphocholine but not choline. However, in the latter case, the earliest timepoint at which the increase in ChoP was observed was 4 hours after addition of PDGF. Since, in Swiss 3T3 cells, Price *et al.* (1989) have shown that PDGF can stimulate increases in Cho within 20 minutes, the later elevation of ChoP may be explained by phosphorylation of liberated Cho by Cho kinase (Warden & Friedkin, 1984; 1985). There are a number of instances where DG formation is accompanied by the release of both Cho and ChoP and, in these cases, it is not clear which is the pathway responsible for the DG generation (Martinson *et al.*, 1989; Price *et al.*, 1989; Wright *et al.*, 1990).

In addition to the hydrolysis of PtdCho there is also some evidence that phorbol esters can stimulate the hydrolysis of phosphatidylethanolamine (PtdEtn) in NIH3T3 fibroblasts and HL-60 granulocytes (Kiss & Anderson, 1989). This may represent another potential source of lipid-derived second messengers and future studies are likely to make a more thorough analysis of this phenomenon.

Figure 1.3 Sites of action of phospholipases A, C & D on phosphatidylcholine.

A simplified structure of PtdCho is shown in which the ester linked fatty acid groups are represented by R_1 and R_2. Phospholipase A_1 hydrolyses the ester linkage at the *sn*-1 position to produce free fatty acid and 1-lyso-2-acyl-PtdCho; there is no evidence, to date, of a hormone-sensitive or receptor-activated PLA_1 activity. Phospholipase A_2 hydrolyses the ester linkage at the *sn*-2 position to produce free fatty acid and 1-acyl-2-lyso-PtdCho; there is increasing evidence for a hormone-sensitive PtdCho-PLA_2 activity which may serve as a major source for free arachidonic acid. Phospholipase C, by analogy with PIC, hydrolyses the phospho-ester bond linking the DG moiety to the phosphocholine (ChoP) group; the products are *sn*-1,2-DG and ChoP. Phospholipase D catalyses the release the choline head group by attacking the phospho-ester bond between the head group and the phosphatidate moiety; the products are phosphatidic acid (PtdOH) and choline (Cho). There is now significant evidence, discussed in the text, to suggest that PtdCho-PLC and PLD activity may be regulated by hormones and growth factors as well as phorbol esters (reviewed by Billah & Anthes, 1990).


Phospholipase A_1
$H_2CO-C-R_1$
O
Phospholipase A_2
$R_2-C-OCH$
O
Phospholipase D
O
$H_2CO-P-OCH_2CH_2N^+(CH_3)_3$
O^-
Phospholipase C

1.7 Phospholipase D (PLD).

Phospholipase D (PLD) catalyses the cleavage of the terminal phosphodiester bond of phospholipids, according to the following general reaction scheme :-

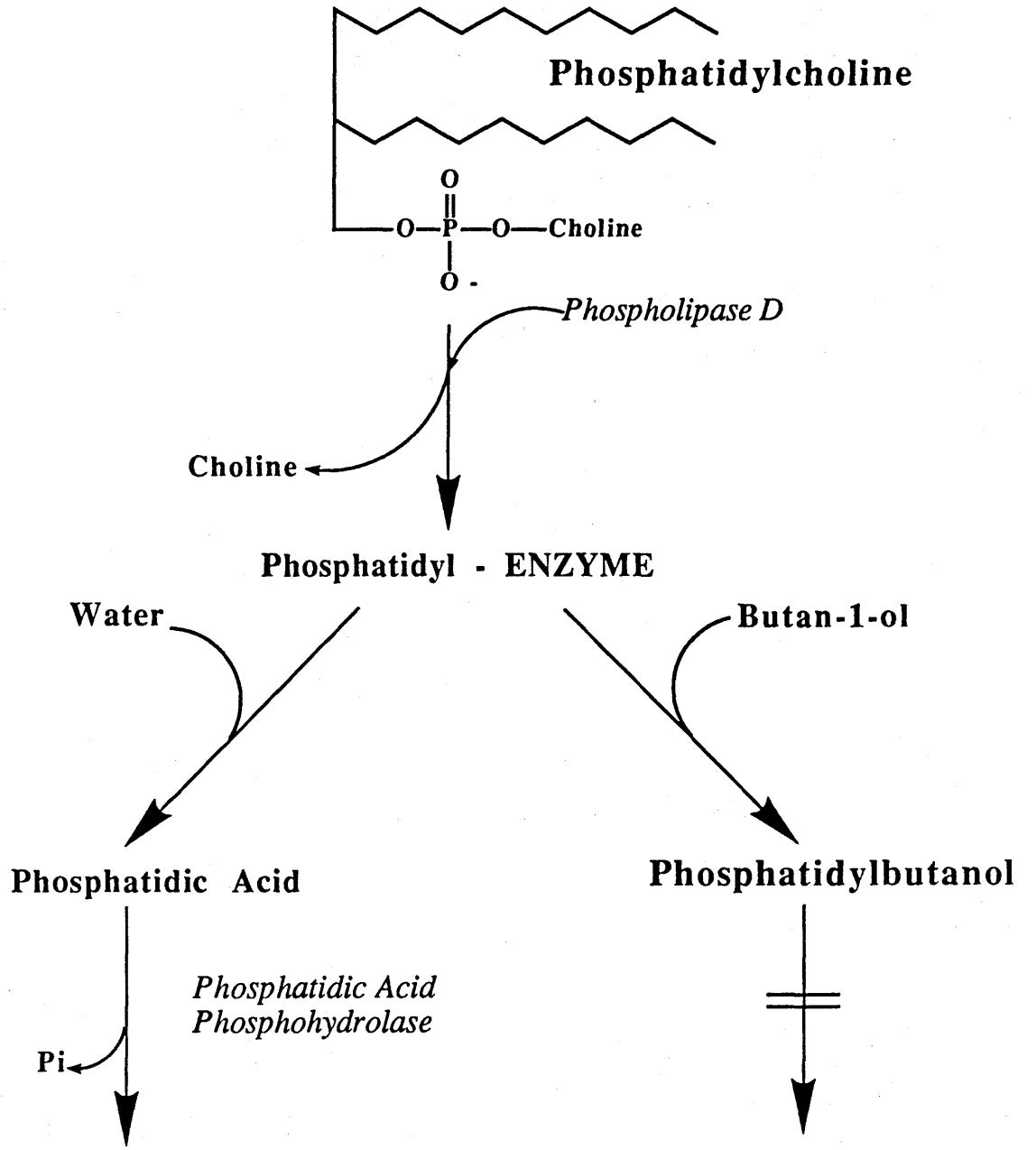
Phosphatidyl-X + X'-OH ----------> Phosphatidyl-X' + X-OH.

The enzyme catalyses a transphosphatidylation reaction (Fig. 1.4) in which the phosphatidyl moiety liberated by choline release is transferred on to an accepting nucleophile, X'-OH. The so-called *hydrolytic* activity is the normal reaction *in vivo* but can be considered a specific example of transphosphatidylation where the acceptor is water (i.e. when X' = H); the products are phosphatidic acid (PtdOH) and the free, polar head group (i.e. choline, ethanolamine). However, in the presence of relatively low concentrations of primary, short chain, aliphatic alcohols (0.1-1%, v/v) the enzyme catalyses a so-called *transferase* reaction in which the phosphatidyl moiety is transferred on to the alcohol to form the corresponding phosphatidylalcohol (e.g., PtdEthanol (PtdEth), PtdButanol (PtdBut), etc.).

Originally studied extensively in plant and bacterial systems (e.g., Dawson, 1967) PLD activity has been reported in a variety of mammalian tissues since its first description in a 'solubilized' rat brain preparation (Saito & Kanfer, 1973). The transferase activity of PLD was discovered fortuitously by Dawson & Hemington (1967) by the inclusion of glycerol in an incubation containing cabbage PLD and PtdCho. In addition, phosphatidylethanol formation has been reported in a variety of rat organs after ethanol intoxication (Alling *et al.*, 1984). More recently, both the hydrolytic and transferase activities have been demonstrated in rat brain microsomes (Witter & Kanfer, 1985) and rat brain synaptosomes (Kobayashi & Kanfer, 1987) and the rat brain enzyme has been partially purified (Taki & Kanfer, 1979). The precise reaction mechanism is unclear but Saito & Kanfer (1975) have shown that PLD activity from a rat brain particulate fraction is inhibited by *p*-chloromercuriophenyl sulphonate and this is in turn relieved by dithiothreitol suggesting a role for a sulphydryl group in formation of an enzyme-substrate

Figure 1.4 The hydrolytic and transferase activities of PLD.

The precise mechanism of action of phospholipase D (PLD) remains unknown but the evidence to date is consistent with the scheme shown opposite. Transphosphatidylation can be considered as the general reaction with hydrolysis being a specific case in which the acceptor for the phosphatidate moiety is water. However, because the phosphatidyl alcohols are unique to PLD activity but distinct from PtdOH in terms of subsequent metabolism, the two are often considered as products of different activities of the same enzyme. It is thought that the first step in the reaction is the PLD-catalysed head goup release and the formation of a phosphatidyl-PLD intermediate. This species is then the target for nucleophilic attack by water (the *hydrolytic* function) giving rise to the normal product phosphatidic acid (PtdOH). PtdOH may then be de-phosphorylated by the enzyme phosphatidate phosphohydrolase (PPH) to yield DG (Billah *et al.*, 1989b; the left hand scheme) and this represents the coupled PLD/PPH pathway of DG formation. In the transphosphatidylation reaction short chain primary alcohols (e.g., ethanol, butan-1-ol) are stronger nucleophiles and compete with water to accept the phosphatidyl moiety (i.e., a *phosphatidyltransferase* activity) giving rise to the phosphatidylalcohol (Pai *et al.*, 1988a & b; the right hand scheme). The formation of phosphatidylalcohols serves as a useful diagnostic tool in whole cells since they are not formed by other metabolic pathways and require only low concentrations of alcohol (0.1-0.3%, v/v) which are not cytotoxic. Furthermore, they allow intervention in a PLD/PPH pathway of DG formation since the phosphatidylalcohols are poor substrates for PPH (Metz & Dunlop, 1991; right hand scheme).


Phosphatidylcholine
O
O—P—O—Choline
O -
Phospholipase D
Choline
Phosphatidyl - ENZYME
Water
Butan-1-ol
Phosphatidic Acid
Phosphatidylbutanol
Phosphatidic Acid
Phosphohydrolase
Pi
1,2- Diradylglycerol
Coupled PLD/PPH pathway
Transferase pathway

complex. This complex is then presumably the target for the attacking nucleophile, water or a primary alcohol (Fig. 1.4).

Phospholipase D activity in rat brain microsomes exhibits a pH optimum of 6.5 (Witter & Kanfer, 1985) whereas that from post-nuclear frations of human neutrophils possesses a major pH optimum of 7.5 and a minor activity at pH 5 (Balsinde *et al.*, 1989). In neutrophils, the 'neutral' PLD requires millimolar concentrations of Ca^{2+} for maximal activity and is cytosolic in location whereas the 'acid' PLD is Ca^{2+}-independent and associated with the lysosomes (Balsinde *et al.*, 1989). Furthermore, addition of A23187 to human neutrophils is reported to activate PLD suggesting a role for Ca^{2+} in its activation (Billah *et al.*, 1989a). However, 10mM Ca^{2+} had no effect upon PLD activity in rat brain microsomes whilst A23187 caused a slight inhibition (Witter & Kanfer, 1985). These contrasting observations suggest that a number of isoforms of PLD may exist with distinct sensitivities to Ca^{2+} and perhaps organelle, tissue and species specific distribution. Monosaturated fatty acids, particularly sodium oleate, acting as detergents, are very potent activators of PLD in rat brain microsomes and synaptosomes (Witter & Kanfer, 1985; Kobayashi & Kanfer, 1987). Finally, recent evidence suggests that PLD may be regulated by activated cell surface receptors, second messengers and protein kinases involved in signal transduction (reviewed by Billah & Anthes, 1990).

Until recently, the importance of PLD was considered to be confined to phospholipid re-modelling events associated with head group exchange in membranes, supplying PtdOH for triglyceride biosynthesis and choline for acetylcholine biosynthesis (reviewed by Löffelholz, 1989). However, there is now an increasing body of evidence, reviewed in section 1.8, to support a role for PLD activation in the signal transduction pathways of a variety of hormones and neurotransmitters.

1.7.1 A distinction between PLD and the base-exchange enzymes ?

Assaying the release of head group bases from phospholipids is not

unequivocal evidence for activation of PLD. The Ca^{2+}-dependent, ATP independent, base exchange reactions replace the head group moieties on phospholipids with free bases (serine, choline & ethanolamine) without a net generation of PtdOH, according to the following scheme :-

Phosphatidyl base A + base B ----------> Phosphatidyl base B + base A.

As such, the release of choline, serine or ethanolamine from phospholipids is only indicative of a 'PLD-like activity' including base exchange reactions. However, it seems likely, from a variety of studies in rat brain, that the two activities can be resolved. Thus, a purified base exchange enzyme from rat brain did not exhibit PLD activity (Taki & Kanfer,1978; Suzuki & Kanfer, 1985) whilst a partially purified PLD from rat brain showed no ability to incorporate bases into phospholipids (Taki & Kanfer, 1979). Furthermore, in rat brain, under conditions supporting Ca^{2+}- dependent base exchange, Gustavsson & Alling (1987) were unable to demonstrate Ca^{2+}-stimulated PtdEt formation which could still be formed by the brain, oleate activated, Ca^{2+}- independent PLD.

Despite this, a recent report by Mori *et al.* (1989) has demonstrated a Ca^{2+}-dependent PLD activity in bovine retinal microsomes which is accompanied by base liberation and generation of PtdOH. When excess exogenous base was added, the PtdOH accumulation was decreased and the base was incorporated into phospholipids. However, the total amount of bases released far exceeded PtdOH formation suggesting that base release activity could not be explained by the PLD activity alone. In addition, the PLD activity in bovine retinal microsomes differed markedly from that in rat brain, having a much higher pH optima (8-8.5) and an absolute requirement for Ca^{2+}.

Clearly, these conflicting observations provide further evidence for isoforms of PLD which may differ markedly in their biochemical properties, both in terms of activation and in terms of their ability to catalyse base exchange reactions. Whilst the final proof of whether PLD possesses base exchange/incorporation

properties awaits purification of what may be a multi-enzyme family, the majority of evidence suggests that the two activities reside in distinct molecules.

1.8 A role for PLD activity in signal transduction ?

1.8.1 Agonist-stimulated PLD activation.

The first unequivocal demonstration of agonist-stimulated PLD activation came from the work of Billah and co-workers (Pai *et al.*, 1988a & b; Billah *et al.*, 1989a & b) in neutrophils and HL-60 granulocytes stimulated with either fMLP or PMA. Using 1-*O*-[^{3}H]alkyl-lyso[^{32}P]PtdCho to specifically label the PtdCho pool with [^{32}P] they were able to demonstrate formation of 1-*O*-[^{3}H]alkyl-lyso[^{32}P]PtdOH. Since, under these conditions, the cellular ATP pool was not labelled with ^{32}P, the [^{32}P]PtdOH could not have been formed by the action of DG kinase and must have been due to PLD catalysed hydrolysis of 1-*O*-[^{3}H]alkyl-lyso[^{32}P]PtdCho (Pai *et al.*, 1988b; Billah *et al.*, 1989b). In hindsight, this phenomenon was probably first documented by Cockcroft (1984) who showed that in ^{32}Pi-labelled neutrophils stimulated with fMet-Leu-Phe the specific activity of the [^{32}P]PtdOH actually decreased markedly within 5-10 seconds; an observation inconsistent with the incorporation of ^{32}Pi from the cellular ATP pool via the DG kinase route which would increase the specific activity of the [^{32}P]PtdOH. Cockcroft (1984) concluded that the mass of PtdOH was increasing by activation of a PLD activity, though at the time PtdIns was proposed as the substrate.

In addition, the ability of primary alcohols to substitute for water in the PLD catalysed-transphosphatidylation reaction (Pai *et al.*, 1988a; Fig. 1.4) has been used as a definitive marker for PLD activity in a number of cells including bovine pulmonary artery endothelial cells (Martin *et al.*, 1989; 1990; Martin & Michaelis, 1989), adrenal glomerulosa cells (Liscovitch & Amsterdam, 1989), 1321N1 astrocytoma cells (Martinson *et al.*, 1989), NIH3T3 fibroblasts (Ben-Av & Liscovitch, 1989), He-La cells (Hii *et al.*, 1989) and spermatozoa stimulated to

undergo the acrosome reaction by a fucose sulphate glycoconjugate (Domino *et al.*, 1989).

There are also a number of reports which provide strong evidence for activation of PLD in response to extracellular stimuli (reviewed by Exton, 1990; Billah & Anthes, 1990). The release of choline prior to, or in the absence of, phosphocholine strongly suggests that activation of PLD occurs in NG108-15 cells (Liscovitch *et al.*, 1987), bovine pulmonary endothelial cells (Martin & Michaelis, 1988) and 1321N1 astrocytoma cells (Martinson *et al.*, 1989). In all these cases the kinetics of this Cho release are similar to either Ptdalcohol and/or PtdOH formation suggesting activation of a PtdCho-PLD activity.

1.8.2 The product of PLD, PtdOH, is a phospholipid with diverse biological functions.

One function of PtdOH produced by PLD activity, to serve as a precursor for DG via the action of PPH, is reviewed below (see section 1.8.3). However, a PLD/PPH pathway of DG presents the cell with significant regulatory problems because of the possibility of a futile cycle of DGK and PPH activities wasting ATP and, for this reason alone, a much simpler pathway would involve activation of a PtdCho-PLC. It therefore seems pertinent to ask what other purpose PLD activation may serve ?

It seems likely that a major function for PLD is to produce PtdOH which may function as a second messenger. PtdOH has had various functions ascribed to it in the past including that of a Ca^{2+} ionophore (Putney *et al.*, 1980), a growth factor acting via activation of inositol lipid hydrolysis and inhibition of adenylyl cyclase (Moolenaar *et al.*, 1986; van Corven *et al.*, 1989) and activator of PKC (Epand & Stafford, 1990). PtdOH and PtdSer have been reported to stimulate PIC activity (Jackowski & Rock, 1989): thus, PtdOH produced in the plasma membrane may have receptor independent effects upon PIC activity. The formation of PtdOH correlates with the release of secretory granules in fMet-Leu-Phe-stimulated neutrophils (Cockcroft, 1984) which have been shown to produce PtdOH by

stimulated PLD activity (Pai *et al.*, 1988 a & b; Billah *et al.*, 1989a & b). It seems likely that the formation of PtdOH will be a common result of PtdIns(4,5)P_2 hydrolysis (via DG kinase) and PtdCho hydrolysis (via PLD) and so future studies may be directed towards potential biological functions for this molecule in stimulus-response systems. This issue is discussed in more detail in Chapter 7.

1.8.3 PtdOH as a precursor of DG.

The human neutrophil, primed with cytochalasin B, has proved a useful model for demonstrating a coupled PLD/PPH pathway of DG formation (Fig 1.4) in response to fMet-Leu-Phe and phorbol esters (Billah *et al.*, 1989b; Bonser *et al.*, 1989). In cells labelled with 1-*O*-[^{3}H]alkyl-lyso-PtdCho, fMet-Leu-Phe-stimulated [^{3}H]alkyl PtdOH formation clearly precedes [^{3}H]DG formation suggesting that PtdOH is the precursor for DG, rather than the product of DG kinase activity. Furthermore, in cells labelled with 1-*O*-[^{3}H]alkyl-lyso-[^{32}P]PtdCho, [^{32}P]ChoP is not formed; rather, the release of [^{32}P]PO_4 accompanies the rise in [^{3}H]DG indicating dephosphorylation of the [^{3}H]alkyl[^{32}P]PtdOH by PPH. The use of the PPH inhibitor propranolol results in the complete inhibition of fMet-Leu-Phe-stimulated [^{3}H]DG formation but actually potentiates [^{3}H]alkyl[^{32}P]PtdOH.

The transferase activity of PLD gives rise to phosphatidylalcohols which are poor substrates for PPH; it has been estimated that the half-life of PtdOH is less than 15 min in intact pancreatic islets whilst that of PtdEth is greater than 2 hours (Metz & Dunlop, 1991). The presence of butan-1-ol, and the consequent formation of PtdBut, inhibit both DG generation and the respiratory burst in fMet-Leu-Phe-stimulated neutrophils by directing phosphatidyl moieties away from PtdOH and towards PtdBut (Bonser *et al.*, 1989; Fig. 1.4). This provides strong evidence for the PLD/PPH pathway of DG formation in neutrophils and suggests that this pathway is necessary for the functional response. Similar inhibition of DG formation using primary alcohols has now been documented in a other cell types

including MDCK epithelial cells and bovine pulmonary endothelial cells (Huang & Cabot, 1990a). As such it seems likely that in a variety of cell types the coupled pathway of PLD and PPH will serve as a route for the stimulated formation of DG.

In addition to these studies, when cells are isotopically labelled with [^{3}H] or [^{14}C] labelled fatty acids the stimulated formation of PtdOH prior to, or commensurate with DG has also been reported in a variety of cell types (Domino *et al.*, 1989; Martinson *et al.*, 1989; Huang & Cabot, 1990a & b; Martin *et al.*, 1990) and is suggestive of a sequential PLD-PPH pathway. Furthermore, when a fatty acid is used to label PtdCho preferentially over PtdIns, for example oleic, myristic or palmitic, the formation of labelled DG has provided further evidence for DG being derived from a non-inositide source (Martinson *et al.*, 1989; Huang & Cabot, 1990a & b).

However, whilst there are now a number of documented examples of agonist-stimulated PLD activation, not all of these studies have been able to demonstrate that the resulting PtdOH is de-phosphorylated to DG to provide sustained increases in cellular DG (Huang & Cabot, 1990a). Thus DG formation may occur by an additional pathway and the prime purpose of PLD may be to form PtdOH, a candidate second messenger.

1.8.4 Is PLD regulated directly by receptors or is it dependent upon prior inositol lipid hydrolysis ?

For those agonists which couple to activation of PIC via G_p the formation of Ins(1,4,5)P_3 is very rapid being maximally elevated within 5 or 10 seconds of addition of the agonist and without a measurable lag time (e.g., Wright *et al.*, 1988; Truett *et al.*, 1989a). The generation of DG is equally rapid, but unlike the transient Ins(1,4,5)P_3 response, rises in a second phase. In all the reports of agonist-stimulated PLD activity to date the kinetics of PtdOH or Ptdalcohol formation are clearly slower than those for Ins(1,4,5)P_3 (e.g., Domino *et al.*, 1989; Martin & Michaelis, 1988, 1989; Agwu *et al.*, 1989) with lag times of 5-15 sec before elevation is apparent, whilst maximal responses are not attained until

after 1-2 min. This raises the question of whether receptors couple directly to PLD or whether this response is downstream of some other early event. Candidates for such early modulatory events are the rapid increase in $[Ca^{2+}]_i$ and PKC activity due to PtdIns(4,5)P_2 hydrolysis.

A number of studies have shown that phorbol esters and cell permeant DG analogues are able to activate PLD and/or PtdCho hydrolysis (Agwu *et al.*, 1989; Price *et al.*, 1989; Martin *et al.*, 1990) suggesting a role for PKC in the regulation of PtdCho-PLD activity. This is supported further by the observation that 'down-regulation' of PKC or the use of PKC inhibitors greatly reduces or abolishes agonist- and phorbol ester-stimulated PtdCho hydrolysis and/or PLD activation (Cabot *et al.*, 1989; Martin *et al.*, 1989; Price *et al.*, 1989; Huang & Cabot, 1990b; Liscovitch & Amsterdam, 1990). However, in neutrophils fMet-Leu-Phe-stimulated PLD activity is only inhibited by approximately 30% by the PKC inhibitor K252a even though PKC-mediated protein phosphorylation is abolished (Billah *et al.*, 1989a). This suggests that there is another major path for regulation of PLD and in neutrophils this appears to be regulation by Ca^{2+}. The formation of PtdOH or PtdEth in neutrophils can be stimulated by the sole addition of A23187 suggesting a major role for Ca^{2+} in regulation of PLD; indeed, the effect of ionophore is more efficacious than that of PMA, giving a greater fold stimulation (Billah *et al.*, 1989a). It is not clear whether this effect is mimicking Ins(1,4,5)P_3-induced Ca^{2+} mobilization or whether it represents a Ca^{2+} entry phenomenon since depletion of extracellular Ca^{2+} with EGTA abolishes both receptor- and A23187-stimulated PLD activity.

There is evidence for regulation of PLD activity by guanine nucleotides in hepatocyte membranes (Bocckino *et al.*, 1987), granulocyte homogenates (Anthes *et al.*, 1989) and permeabilised endothelial cells (Martin & Michaelis, 1989). However, in the light of the possible role of PKC in activating PLD such an interpretation must depend upon the effect of the guanine nucleotide not being due to elevated inositol lipid hydrolysis, by interaction with G_p, and therefore activation of PKC.

Thus, there is certainly evidence of a role for PKC and Ca^{2+} in the

regulation of PLD activity by agonists; in addition, preliminary studies suggest the possiblity of regulation by guanine nucleotide regulatory proteins and therefore perhaps by direct RGE interactions (e.g., Anthes *et al.*, 1989).

1.9 The Swiss 3T3 mouse fibroblast as a model for examining early signals during mitogenic stimulation.

The Swiss 3T3 mouse embryo fibroblast cell is now widely used as a model system for studying the early signals associated with stimulation by growth factors (reviewed by Rozengurt, 1985; 1986). Cultures synchronised in the G_0/G_1 phase of the cell cycle by contact inhibition of growth and serum depletion can be stimulated to re-enter the cell cycle by whole serum or defined mitogenic agents. Such an approach has allowed the identification of a variety of growth factors which, when added alone (complete mitogens), or in combination with other agents (co-mitogens), will initiate DNA synthesis in their target cells. Agonists which are now known to be mitogenic for the Swiss 3T3 cell line include insulin, aFGF, bFGF, PDGF, EGF, vasopressin, prostaglandin $F_{2\alpha}$ bombesin and endothelin-1.

In the last five years much interest has focussed on the mechanism of action of bombesin as a mitogen since the discovery that bombesin-like peptides secreted by Small Cell Lung Carcinoma (SCLC) cells may act as autocrine growth factors in the genesis of this particular type of neoplasia (Cuttitta *et al.*, 1985). Bombesin is a tetradecapeptide isolated from the skin of the frog *Bombina bombina* (Anastasi *et al.*, 1971) which possesses a range of biological activities in mammalian tissues. It stimulates amylase secretion in exocrine glands such as pancreatic acini (Jensen *et al.*, 1978), smooth muscle contraction (Minamino *et al.*, 1984) and may also possess neurotransmitter activity within the brain (Gibbs *et al.*, 1979). Bombesin exhibits a high degree of structural homology with the mammalian gastrin releasing peptide (GRP) and neuromedin B, particularly in the C-terminal eight amino acids.

In Swiss 3T3 cells, and a number of cell lines of murine and rat origin, bombesin is a potent mitogen when added alone aswell as acting synergistically

with insulin at nanomolar concentrations (Rozengurt & Sinnett-Smith, 1983). Bombesin and GRP appear to bind to the same receptor on Swiss 3T3 whole cells (Zachary & Rozengurt, 1985) and membranes (Sinnett-Smith *et al.*, 1990) based on displacement studies using [^{125}I-Tyr^4]bombesin and [^{125}I-Tyr^4]GRP. The K_d for binding is typically in the range of 0.1-5 nM and Scatchard analysis suggests that Swiss 3T3 cells possess a single class of high affinity receptor sites which have an approximate molecular weight of 75-85 kDa as determined by cross linking studies with [^{125}I-Tyr^4]GRP. The receptor has recently been purified to apparent homogeneity from Swiss 3T3 cell membranes (Feldman *et al.*, 1990). Cross-linking studies on SDS-PAGE revealed a diffuse band of 75-100 kDa which after treatment with N-glycanase migrated as a sharper band of 38 kDa suggesting the native receptor is substantially N-glycosylated.

In recent years a number of studies have sought to identify the signal transduction pathways utilized by bombesin in exerting its mitogenic effect. It is known that bombesin stimulates the rapid hydrolysis of PtdIns(4,5)P_2 to generate Ins(1,4,5)P_3 and DG and the resulting increases in $[Ca^{2+}]_i$ and PKC activity have been extensively studied (Lopez-Rivas *et al.*, 1987; Takuwa *et al.*, 1987a; Erusalimsky *et al.*, 1988; Fischer & Schonbrunn, 1988). Furthermore this response is thought to be mediated via a GTP-binding protein (G_p) (Erusalimsky *et al.*, 1988; Plevin *et al.*, 1990) and consistent with this the recent cloning of the bombesin receptor from Swiss 3T3 cells has identified it as belonging to the family of seven-membrane spanning domain receptors which couple to G-proteins (Battey *et al.*, 1991). The activation of PKC by bombesin is implicated in the induction of c-*fos* and c-*myc* mRNAs, alkalinisation of the cytosol and activation of S6 kinase (discussed in section 1.2). Thus, although not sufficient on its own to fully stimulate DNA synthesis, the inositol lipid pathway is of considerable importance in generating many of the early signals associated with mitogenesis (see section 1.2 & 1.3).

Despite this there has been no thorough comparison of Ins(1,4,5)P_3 and DG formation in bombesin-stimulated Swiss 3T3 cells. The studies by Heslop *et*

al. (1986) and Nanberg & Rozengurt (1988) did not examine DG formation, whilst that of Takuwa *et al.* (1987a) looked at the formation of Ins(1,4,5)P_3 and DG at different time points thereby precluding a direct comparison. In the light of recent evidence for alternative sources of DG, the Swiss 3T3 cell was chosen as a model system for defining the kinetics of changes in DG and Ins(1,4,5)P_3 levels to determine what contribution to stimulated DG levels was made by hydrolysis of PtdIns(4,5)P_2 and, if relevant, other phospholipid classes. The Swiss 3T3 cell has the added advantage of being well characterised in terms of inositol phosphate formation (Heslop *et al.*, 1986; Takuwa *et al.*, 1987a; Nanberg & Rozengurt, 1988), and feedback inhibition of inositol phosphate accumulation by PKC activation (Brown *et al.*, 1987), as well as being one of the best model systems in which to 'down-regulate' PKC by chronic phorbol ester treatment (Rodruigez-Pena & Rozengurt, 1984).

The majority of the work presented in this thesis has utilized bombesin as an RGE-type of growth factor to assess the role of inositol and choline phospholipid hydrolysis as sources of DG during mitogenic stimulation. Preliminary investigations (Chapter 3) aimed to assess the kinetics of Ins(1,4,5)P_3 and $InsP_t$ formation in bombesin-stimulated Swiss 3T3 cells making comparisons with mechanistically related mitogens such as vasopressin and prostaglandin $F_{2\alpha}$. In Chapter 4 bombesin-stimulated DG formation was examined and a possible role for activation of phospholipase D in DG formation was investigated. In Chapter 5 evidence is presented to suggest that PtdCho is a major substrate for PLD activity in Swiss 3T3 cells and may play a role in the generation of DG in response to bombesin and mechanistically related mitogens. In addition, these studies have been widened to examine the effects of EGF and PDGF upon phospholipid metabolism and DG formation (Chapter 6). The results are discussed in terms of potential alternative sources of DG and the role of DG and PtdOH in cellular proliferation.

Chapter 2.

Materials & methods.

2.1 Cell lines and materials.

2.1.1 Cell lines.

Swiss mouse 3T3 fibroblasts.

Kindly donated by Dr. Kenneth D. Brown, A.F.R.C. Institute of Animal Physiology, Babraham, Cambridge, CB2 4AT, U.K.

2.1.2 Materials.

The following is a list of the sources for materials used during the course of this research:

Aldrich Chemical Company Ltd, Gillingham, England.

2,2,4-trimethylpentane (iso-octane).

Amersham International plc, Amersham, Buckinghamshire, England.

Adenosine 5'-[γ-^{32}P]triphosphate (specific activity 3Ci mmol^{-1}),

[*methyl*-^{3}H]Choline chloride (spec. ac. 75-85Ci mmol^{-1}),

[2-^{3}H]*myo*-inositol (spec. ac. 10-20Ci mmol^{-1}),

[2-^{3}H]D-*myo*-inositol(1,4,5) trisphosphate (spec. ac. 20-60Ci mmol^{-1}),

[9,10(n)-^{3}H]Palmitic acid (spec. ac.40-60Ci mmol^{-1}),

L-3-Phosphatidyl[*N-methyl*-^{3}H]choline, 1,2-dipalmitoyl (spec. ac. 50-60mCi mmol^{-1}),

Phosphoryl [*methyl*-^{14}C]choline, ammonium salt (spec. ac. 50-60mCi mmol^{-1}),

platelet derived growth factor (c-*sis*).

Glycerophospho[*N-methyl*-^{3}H]choline (GroPCho) had previously been prepared from [^{3}H]PtdCho by transacylation with monoethylamine according to the method of Clarke & Dawson (1981).

Avanti Polar Lipids Inc., 5001-A, Whitling Drive, Pelham, Alabama, U.S.A.

Cardiolipin.

BDH Chemical Company, Poole, England.

Ammonium formate, butan-1-ol, diethyl ether, EDTA, ethanol, glycerol, Hepes, orthophosphoric acid, potassium hydrogen phosphate, potassium di-hydrogen phosphate, perchloric acid, sodium hydroxide, sodium carbonate, trichloroacetic acid and universal indicator.

Biogenesis Ltd. (formerly Biomedical Technologies Inc.), Bournemouth, England.

Epidermal growth factor (receptor grade) purified from mouse submaxillary glands.

Boehringer (UK) Ltd, Lewes, England.

ATP, dithiothreitol and Tris.

Calbiochem (Novabiochem (U.K.) Ltd), University Boulevard, Nottingham, England.

n-octyl-β-D-glucopyranoside.

Cambridge Research Biomedicals, Cambridge, England.

Bombesin, [Arg^8]vasopressin.

Fisons Scientific Apparatus,

Acetic acid (glacial), chloroform, ethyl acetate, hexane, methanol.

Formachem (Research International)plc, Strathaven, Scotland.

D-glucose, sodium hydrogen carbonate

Gibco, Paisley, Scotland.

Dulbeccos modified eagles medium (DMEM, 10x), gentamycin, glutamine (100x), penicillin/streptomycin, sodium bicarbonate (7.5%).

Koch-Light Ltd, Suffolk, England.

Calcium chloride, dimethyl sulphoxide, magnesium sulphate, potassium chloride.

Lipid Products., Nutfield Nurseries, South Nutfield, Surrey.

Phosphatidylserine.

Lipidex Inc., New Jersey, U.S.A.

E. coli DG kinase; crude membranes from an over expressing strain.

May and Baker, Dagenham, England.

Formic acid, hydrochloric acid and sodium tetraborate.

Riedel-DeHaenAg Seelze-Hannover, Germany.

Magnesium chloride and sodium di-hydrogen carbonate.

Roche (UK) Ltd, Welwyn Garden City, England.

The provision of the drug Ro-31-8220, a PKC inhibitor, by Dr. P. D. Davis is gratefully acknowledged.

Wellcome Research Laboratories, Beckenham, UK.

The provision of a [^{14}C]PtdBut standard by Dr. L.G. Garland and the gift of [^{3}H]butan-1-ol is gratefully acknowledged.

Whatman Ltd, Maidstone, England.

LK5DF glass backed thin layer chromatography plates, pre-laned with pre-adsor bent strip.

The gift of the tyrphostin, AG18, by Dr. Alexander Levitski, Department of Biological Chemistry, Hebrew University of Jerusalem, Jerusalem 91904, Israel is gratefully acknowledged.

All other chemicals including ion-exchange resins, phorbol esters and prostaglandins were obtained from the Sigma Chemical Company, Poole, England.

2.2 Buffer composition.

Phosphate-buffered saline (PBS).

146mM sodium chioride, 5.4mM potassium chloride, 9.6mM di-sodium hydrogen orthophosphate, 1.5mM potassium di-hydrogen orthophosphate.

When freshly prepared PBS was adjusted to pH 7.2-7.4.

Sterile trypsin solution for cell passage.

Trypsin was prepared as a solution of 0.1% (w/v) trypsin, 0.025% (w/v) EDTA and 10mM glucose in PBS at pH 7.4 and and filtered before aliquoting through a sterile 0.22μm membrane (Flow pore D).

Hanks buffered saline (Hanks).

1.26mM calcium chloride, 0.5mM magnesium chloride, 0.9mM magnesium sulphate, 5.37 mM potassium chloride, 137mM sodium chloride, 4.2mM sodium hydrogen carbonate, 0.35mM sodium di-hydrogen phosphate.

When freshly prepared the pH was between 7.2 - 7.4.

Hanks buffered saline with glucose and BSA (HBG).

Buffer prepared as above containing 10mM D-glucose and 1% (w/v) BSA (fraction V) and pH adjusted to 7.4.

DMEM buffered with Hepes (DMBH).

Serum-free DMEM was prepared without antibiotics or glutamine supplement but containing 1% (w/v) bovine serum albumin. Buffering was with 20mM Hepes and when preparing for use the buffer was adjusted to pH 7.4 using 1M sodium hydroxide.

2.3 Ion exchange resins.

2.3.1 Preparation of Dowex-formate.

Dowex 1x8-200 chloride form, strongly basic anion exchange resin, 8% cross linked with a dry mesh of 100-200 was treated as described below to obtain the formate form for separation of inositol phosphates. A known packed volume of Dowex was washed with distilled water, left to settle and the 'fines' discarded. This wash was repeated twice. The Dowex was transferred to a scintered glass funnel and washed with 20 volumes of 2M sodium hydroxide. The Dowex was then washed with 10 volumes of water followed by 5 volumes of 1M formic acid. Finally, the Dowex was washed with 50 volumes of water until the pH of the slurry was constant at approximately 5.5. Following each preparation of Dowex formate it was characterised by performing ml by ml elutions of a mixture of [^{3}H]Ins(1)P, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3.

2.3.2 Preparation of Dowex-50W-H^+.

Dowex-50W-H^+ strongly acidic cation exchange resin, 8% cross linked, with a dry mesh of 200-400 was prepared as described below. Dowex was washed three times in three volumes of distilled water, allowing the resin to settle and discarding the 'fines' each time. The dowex was then washed three times with three volumes of 1M HCl accompanied by gentle stirring with a plastic rod. Washings were discarded each time. Finally, the dowex was washed four or five times with three volumes of distilled water until the pH of the wash was constant at about pH 5.5.

Each newly prepared batch of dowex was characterised by performing an elution profile of a mixture of radiolabelled 'standards' of GroPCho, ChoP and Cho added to a Swiss 3T3 cell extract.

2.4 Culture of Swiss 3T3 mouse fibroblast cells.

Swiss 3T3 cells were routinely maintained in Dulbeco's modified Eagle's medium (DMEM) supplemented with glutamine (2mM), penicillin/streptomycin (100 IU/ml.) and newborn calf serum (10%). Medium was changed every two days as it became acidified and depleted of serum. To prevent selection of resistant strains of bacteria, gentamycin was used in rotation with pen/strep. Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. Except for experimental purposes cells were grown in 25 or 75 cm^2 culture flasks and were passaged when sub-confluent. Every two or three weeks a single flask was allowed to grow to confluency to ensure that cells were contact inhibiting and were not exhibiting abberrant morphology. Cells which exhibited transformed morphology, showed reduced responsiveness to given agonists or reached passage numbers higher than 40 were discarded.

2.4.1 Cell passage.

Cells were passaged at a ratio of 1 to 10 or 12 according to the following general protocol.

(1) Medium was aspirated and replaced with 1ml of sterile trypsin solution per 25 cm^2 flask (3ml per 75cm^2 flask).

(2) Flasks were returned to the incubator for 2-3 minutes until cells were detaching from the flask bottom. Trypsin activity was inhibited by addition of 3ml of complete medium. The cells were then suspended by tituration to ensure maximum harvest.

(3) After transfer to a sterile tube, the cell suspension was centrifuged at 1000rpm for 3 minutes and the supernatant discarded.

(4) Cells were resuspended in complete medium and and aliqouted into new sterile flasks containing 3ml of fresh medium. Flasks were rocked gently to ensure even distribution of cells across the flask bottom and returned to the incubator.

2.4.2 Cryogenic preservation of cell lines.

Cell lines were stored in liquid nitrogen according to the following protocol.

(1) Cells were trypsinized as outlined above (cell passage 1-3)

(2) Cells were resuspended in DMEM containing 20% (v/v) donor calf serum and 8% (v/v) DMSO at a density of approximately 10^6 cells per ml of freezing medium and aliquoted into freezing vials.

(3) Cells were slow frozen for 24 hours at -80°C before transfer to a vat of liquid N_2.

To bring cells up from storage, vials were removed from liquid N_2 and thawed rapidly in a water bath at 37°C. Each aliquot of cells was plated out in 3ml of fresh medium and left to attach overnight. The next day the medium and suspended dead cells were removed and the surviving cells were fed normally.

2.5 Measurement of stimulated-inositol phosphate formation.

2.5.1 Incubation of cells with test reagents.

After plating on 24 well plates in DMEM + 10% donor calf serum for 24 hours, Swiss 3T3 cells were grown in 1ml of inositol-free DMEM containing 1% dialysed donor calf serum with either 1μCi ml^{-1} (total InsPs) or 5μCi ml^{-1} (individual InsP fractions) of [2-^{3}H]*myo*-inositol for 48 hours, by which time the cells were confluent, quiescent and labelled to isotopic equilibrium. Labelling medium was aspirated and cells were incubated in Hanks buffered saline (pH 7.4) for 5 minutes at 37°C followed by incubation in HBG and 10mM LiCl (HBG.Li) for 20 minutes. Finally, cells were incubated with 150μl of HBG.Li containing the test reagent at the final concentrations and for the times indicated.

For assays of total inositol phosphates incubations were terminated by direct addition of 500 μl of ice cold methanol. After scraping the cell debris and removing to a 2ml plastic polytube each well was washed again with a further 200 μl of methanol and the two washes pooled. Phospholipids were extracted from the cell

debris by addition of 310 µl of chloroform followed by vortexing and standing at on ice for 30 minutes. After addition of 500µl of ice cold distilled water samples were centrifuged at 14,000g for 3 minutes to split phases and 1 ml of the upper aqueuos/methanolic phase was transferred to a 5 ml insert vial for isolation of total inositol phosphates. In addition, an aliquot of the chloroform phase was removed, dried down in a polytube and, after addition of 1.5 ml of Optiphase Hi-safe scintillation fluid, the radioactivity in the total inositol lipids determined by scintillation counting.

For assays of individual inositol phosphate fractions incubations were terminated by aspiration of the test solution an d addition of 150µl of ice cold 10% (v/v) perchloric acid. Inositol phosphates were extracted on ice for 20 minutes followed by scraping and removal of the cell debris into a plastic eppendorf tube. The 24 well plates were washed with a further 150µl of water, pooled with the acidified extracts and centrifuged at 14,000g for 10 minutes at 4°C. The supernatants were removed to a second tube, neutralized by addition of 1.5M-KOH/ 60mM Hepes in the presence of Universal Indicator and the precipitated potassium perchloate removed by centrifugation at 14,000g for 10 minutes at 4°C. The supernatants were analysed by ion-exchange chromatography on Dowex 1 X8 formate columns. Assays of individual inositol phosphates extracted using the neutral methanol method above gave qualitatively similar results, but quantitatively lower recovery of the higher phosphorylated forms of inositol ($InsP_3$ and $InsP_4$; see Chapter 3). This was probably due to the reported lower solubility of these inositol phosphates in methanol (Wregget *et al.*, 1987; reviewed by Palmer & Wakelam, 1989).

2.5.2 Preparation of samples for measurement of Ins(1,4,5)P_3 mass.

Swiss 3T3 cells were grown in 75cm^2 culture flasks containing DMEM + 10% donor calf serum until confluent and quiescent. Cells were then scraped in the growth conditioned DMEM using a rubber policeman and incubated for 45 minutes

at 37°C with gentle shaking. After washing twice in HBG (pH 7.4) cells were resuspended in a volume of HBG sufficient to give a density of approximately 2 x 10^7 cells/ml (an aliquot was taken for cell counting). After incubation at 37°C for a further 30 minutes cells were used for experiments. This incubation period has been shown to allow full recovery of Ins(1,4,5)P_3 to basal levels in Swiss 3T3 cells after the elevation caused by the physical perturbation of scraping (S. Palmer, personal communication).

For experimental purposes 50µl of cell suspension in HBG was added to a plastic polytube containing 25µl of the test reagent at the given final concentration, and cells were incubated at 37°C with shaking for the required time. Incubations were terminated by the addition of 25µl of ice cold 10% perchloric acid followed by extraction on ice for 20 minutes. Samples were neutralized by addition of 1.5M KOH/ 60mM Hepes containing Universal Indicator and the cell debris and precipitated potassium perchlorate were removed by centrifugation at 14,000g for 10 minutes at 4°C. A 25µl aliquot of the supernatant was then removed for assay of Ins(1,4,5)P_3 mass using an assay based upon a specific binding protein (Palmer *et al.*, 1989) as described in appendix I.

2.5.3 Assay of total inositol phosphates.

Total inositol phosphates were assayed by accumulation in the presence of 10mM LiCl essentially by the method of Berridge *et al.* (1982). Dowex formate (1x8 200-400 mesh) (300µl) was added to samples, followed by 3 ml of distilled water to mix. After the Dowex had settled the supernatant was aspirated. The removal of glycerophosphoinositides was achieved by two washes with 3 ml of 60 mM ammonium formate/5mM sodium tetraborate followed by a further 3 ml wash with water. Finally total inositol phosphates (mainly InsP and $InsP_2$) were eluted by two washes with 0.6ml of 1M ammonium formate/ 0.1M formic acid. After each addition, 0.6ml of the supernatant was removed to a 20ml scintillation vial and the pooled washes for each sample were mixed with 16 ml of Optiphase Hi-safe

scintillation fluid. The radioactivity was estimated by liquid scintillation counting.

2.5.4 Assay of individual inositol phosphate fractions.

Neutralized supernatants were diluted in 3ml of 5mM sodium tetraborate/ 0.5mM-EDTA, pH 6.7 and applied to 1 ml columns of Dowex formate (1x8; 200-400 mesh) prepared in glass wool plugged pasteur pipettes. The columns were then washed with 12 ml of water followed by 12ml of 60 mM ammonium formate/ 5mMsodium tetraborate to remove free inositol and glycerophosphoinositides, respectively. Inositol mono-, bis-, tris-, and tetrakisphosphates were eluted with sequential 18 ml washes of 0.1M-formic acid containing 0.2M-, 0.4M-, 0.8M- and 1.2M- ammonium formate respectively. Aliquots of each fraction (3ml) were mixed with Optiphase Hi-safe scintillant and the radioactivity determined by liquid scintillation counting.

2.6 Assay of *sn*-1,2-diradylglycerol mass.

Mass measurement of DG was essentially by the method of Preiss *et al.* (1986), with modifications developed during the course of studies. The assay employs a DG kinase enzyme to convert the DG from chloroform extracts of cells to phosphatidic acid (PtdOH) in the presence of [γ-^{32}P] ATP. Since the assay is linear, with a stoichiometry of one DG molecule to one ATP molecule, the amount of radioactivity in the PtdOH will be directly proportional to the amount of DG in the sample and the specific activity of the [γ-^{32}P] ATP, allowing estimation of the mass of *sn*-1,2-DG in cell samples.

Two variations of the basic assay method were employed during the course of the studies presented in this thesis. The major differences were the detergent system used to solubilize the cellular DG samples and form mixed micelles and the use of glass reaction tubes rather than plastic with the Triton/PtdSer system which was found to increase conversion, recovery and also yielded much closer agreement

between standard replicates (R. Plevin & A. Paterson, personal communication).

2.6.1 Cardiolipin/*n*-octyl-β-D-glucopyranoside detergent system.

Aliquots from the chloroform phase of chloroform/methanol/water phase splits or standards of 1-stearoyl-2-arachidonoyl-glycerol (typically 0, 125, 250, 500 & 1000 pmoles), were dried down *in vacuo* in plastic polytubes and sonicated in 20µl of a detergent cocktail containing 7.5% *n*-octyl-β-D-glucopyranoside, 5mM cardiolipin and 1mM diethylenetriaminepentacetic acid (DETAPAC). The following reagents were added to each sample;

50µl of incubation buffer containing 100mM Imidazole, 100mM NaCl 25mM $MgCl_2$ and 2mM EGTA at pH 6.6.,

10µl of dithiothreitol to a final concentration of 2mM,

10µl of *E. coli* DG kinase enzyme,

Incubations were started by adding 1.25µCi of [γ-^{32}P]ATP in a solution of 5mM ATP prepared in 100mM Imidazole and 1mM DETAPAC at pH 6.6 (ATP buffer) to each sample followed by vortexing. The actual volume of this ATP cocktail used was determined on each occasion by calculating the precise concentration of ATP from its absorbance at 259nm and the decay factor for the [γ-^{32}P]ATP. Incubations were performed at 30°C for 30 min by which time the reaction had reached completion (data not shown).

Incubations were terminated by the addition of 470µl of chloroform/methanol (1/2; v/v) containing 1% perchloric acid (v/v).and samples were extracted at room temperature for 20 minutes. Phases were resolved by addition of 150µl of chloroform and 1ml of 1% perchloric acid, and following vigorous vortexing samples were centrifuged at 14,000g for 2 minutes. The upper aqueous methanolic phase containing the majority of unincorpoated [γ-^{32}P]ATP was removed and discarded to radioactive waste and a further 1ml pechloric acid wash performed.

2.6.2 Triton / PtdSer detergent system.

Samples or standards of DG were dried down *in vacuo* in glass vials and solubilised in 50µl of a detergent mix containing 6 Mol% PtdSer (0.288mM) in 0.6% (w/v) Triton X-100 by vortexing and then sonicating at 4°C. Incubations were started by adding 50 µl of a mix containing:-

20µl of 5x incubation buffer (250mM Imidazole/HCl, pH 6.6; 250mM NaCl; 62.5mM $MgCl_2$; 5mM EGTA),

10µl DG kinase (from *E.coli.*),

10µl 10x DTT (20mM),

10µl of ATP cocktail containing 1.25µCi [γ-^{32}P]ATP in 5mM ATP (50 nmoles/sample) in 100mM Imidazole and 1mM DETAPAC at pH 6.6 (ATP buffer).

The precise volume added was determined on the day by spectrophotometric assay of the ATP solution and estimation of the decay factor for the [γ-^{32}P]ATP. Incubations were started by vortexing and proceeded at 30°C for 30min. Incubations were terminated by addition of 470 µl of chloroform/methanol (1/2, v/v) and samples were extracted for 20 min at room temperature. Phases were resolved by addition of 150µl of chloroform and 1ml of water and after centrifugation at 3000rpm for 2 min the upper aqueous-methanolic phase was discarded to radioactive waste. A further 1ml water wash was performed and all the traces of the upper aqueous phase were removed before drying down the chloroform layer *in vacuo*.

2.6.3 Resolution of DG kinase assay products.

For both assay procedures the dried chloroform phase was redissolved in 50µl of chloroform/methanol (19/1;v/v) and 25µl spotted on to acetone washed, air dried silica gel 60 t.l.c. plates. The loaded plates were developed in a solvent of chloroform/methanol/acetic acid (65/15/5;v/v), air dried and subjected to autoradiography at -80°C for 12 hours to locate the reaction products. In some experiments the solvent system was modified to chloroform/methanol/acetic acid

(65/15/10) to increase the mobility of the PtdOH (A. Paterson & R. Plevin, personal communication). The uppermost band on the tlc plates was identified as PtdOH on the basis that it had identical chromatographic properties as the PtdOH obtained when pure *sn*-1-stearoyl-2-arachidonoyl glycerol was employed in the assay. The PtdOH was excised from the t.l.c. plate, placed in 6ml vial with 4ml of Optiphase Hi-safe scintillant and the radioactivity determined by scintillation counting.

Standard curves of cpm in PtdOH versus pmoles of DG were plotted as simple straight lines and pmoles of DG in the unknown samples were calculated from the cpm in PtdOH and the equation of the standard curve straight line. After correcting for the total cell lipid extract volume DG was expressed as either pmoles/ 10^6 cells or as Mol% (nmoles of DG/100nmoles of lipid phosphorous).

2.7 Assay of [^{3}H]DG formation in [^{3}H]palmitate labelled Swiss 3T3 cells.

2.7.1 Labelling of Swiss 3T3 cells with [^{3}H] palmitic acid.

To establish conditions for optimum labelling of lipids, Swiss 3T3 cells were sub-cultured onto a 24 well plate in DMEM+10% newborn calf serum until approximately 70-80% confluent. They were then labelled with DMEM+1% newborn calf serum containing 2μCi ml^{-1}[^{3}H] palmitic acid for various times up to 49 hours using a staggered protocol. At the end of the incorporation period medium was aspirated, cells washed in HBG for 2 minutes and killed by addition of 500μl of ice cold methanol. After scraping, washing again with 200μl of methanol and pooling, the cell debris was extracted into chloroform (310μl) for 60 minutes. Phases were split by addition of 650μl of water, and the entire chloroform phase was dried down *in vacuo* and stored under N_2 vapour until analysis.

Isolation of the major individual phospholipids was performed by one

dimensional t.l.c. Silica gel 60 plates (20 x 20 cm) were sprayed with 1mM EDTA and allowed to air dry, before heat activating. The samples, prepared as above, and various phospholipid standards were dissolved in chloroform/methanol (19/1,v/v), applied to the origin and air dried before developing in a solvent system of chloroform:methanol:acetic acid:water (75:45:3:1, v/v) until the solvent front was approximately 17.5cm from the origin. Following visualisation with I_2 vapour, each lane was divided into 0.5cm slices and those sections corresponding to phospholipid standards were scraped into 5ml insert vials. The radioactivity associated with each slice and attributed to each phospholipid by co-migration with standards, was determined by scintillation counting after addition of 4ml of Optiphase Hi-safe scintillant.

2.7.2 Assay of [^{3}H]DG formation in [^{3}H]palmitate labelled Swiss 3T3 cells.

Swiss 3T3 cells were sub-cultured into six-well culture plates and grown in DMEM + 10% newborn calf serum until approximately 80% confluent. The growth media was then replaced with 2ml of DMEM + 2% newborn calf serum containing 2 μCi ml^{-1} of [^{3}H]palmitic acid and cells were returned to the incubator for a further 48 hours. For experimental purposes the labelling medium was removed by aspiration and cells were equilibrated in 1ml of serum-free DMEM containing 1% bovine serum albumin and 20mM Hepes (DMBH) (pH 7.4) at 37^o C for 20 minutes. For kinetic studies cells were incubated in DMBH containing the required concentration of test reagent for the required time at 37^{o}C. Incubations were terminated by aspiration of DMBH followed by the addition of 0.5ml of ice-cold methanol. Cell debris was scraped off the plate surface and placed in screw-top glass tubes together with a further 0.2ml of methanol wash. Phospholipids in these pooled scrapings were extracted by the addition of 0.7ml of chloroform, vigorous vortexing and standing at room temperature for 15 minutes. After addition of 0.585ml of water tubes were centrifuged for 5 minutes at 3000 rpm, the upper aqueous/methanolic phase discarded and the chloroform phase dried *in*

vacuo. Samples were routinely analysed by thin layer chromatography immediately. If chromatography was delayed for 24 hours sample tubes were purged with oxygen-free N_2 and stored at -20° C.

[^{3}H]1,2-DG was resolved from 1,3-DG by thin layer chromatography. Samples were re-dissolved in 100µl of chloroform/methanol (19/1;v/v) and applied to Whatman LK5DF t.l.c. plates. Plates were fully developed once in a lined and fully equilibrated t.l.c. tank in a solvent system of hexane:diethyl ether:acetic acid (60:40:1 v/v). 1,2-DG, identified by its co-migration with an authentic standard after I_2 staining, migrated just below 1,3-DG. The plate lanes were divided into 0.5cm strips and those corresponding to 1,2-DG were scraped off and placed in a scintillation vial with 4ml of Opti-phase Hi-safe scintillant fluid. Radioactivity was determined by scintillation counting.

2.8 Assay of PtdCho hydrolysis.

2.8.1 Incubation of cells with test reagents.

Swiss 3T3 cells were plated at a density of approximately 10^4 cells/ml on 24 well plates and grown in DMEM + 10% donor calf serum for 24 hours. They were then grown in DMEM + 1% donor calf serum + 1.5µCi ml^{-1} [^{3}H] choline chloride for 48 hours by which time they were confluent and quiescent. Following removal of the labelling medium, cells were incubated at 37°C in Hank's buffered saline, pH 7.4, for 5 minutes followed by two washes in HBG for 10 and 30 minutes respectively. Finally, cells were incubated in 150µl of HBG containing the given concentration of test reagent for the required time.

For experiments where intracellular or cell associated choline metabolites were studied, incubations were terminated by aspiration of the test solution and addition of 500µl of ice cold methanol. For experiments where intracellular and extracellular metabolites were studied incubations were terminated by addititon of 500µl of ice cold methanol to the incubation buffer. Cell debris was scraped, transferredto a plastic polytube and pooled with a further 200µl methanol wash.

Phospholipids were extracted by addition of 310µl chloroform followed by vortexing. After standing at -20°C for 24 hours, 500µl of water was added and the phases were split by centrifuging at 14,000g for 3 minutes. A 1ml sample of the aqueous methanolic phase was taken for separation of the major water soluble metabolites of choline by cation exchange chromatography. During the initial characterisation of this method it was found that similar results were obtained if incubations were terminated with perchloric acid and neutralized either by addition of 1.5M KOH/60mM Hepes or Freon/tri-n-octylamine (data not shown).

To allow correction for variation in sample size an aliquot of the chloroform phase was dried down and mixed with 1.5 ml of Optiphase Hi-safe scintillant and subjected to liquid scintillation counting to estimate the activity incorporated into total choline containing lipids.

2.8.2 Separation of the major water soluble metabolites of choline.

The 1ml aqueous methanolic samples from experiments loaded on to 1ml Dowex-50W-H^+ columns prepared in glass wool plugged pasteur pipettes. The flow through and a further 8 ml water wash were pooled and collected as the glycerophosphocholine fraction. A further wash with 20 ml of water was collected as the phosphocholine fraction, whilst a final wash with 20 ml of 1M HCl eluted off the choline fraction. Two ml aliquots of each fraction were mixed with Optiphase Hi-safe scintillant and radioactivity determined by liquid scintillation counting.

2.8.3 Analysis of [^{3}H]choline containing lipids.

[^{3}H]choline labelled Swiss 3T3 cells were stimulated with mitogen or vehicle as described above. Following extraction in chloroform and methanol as described above, the aqueous methanolic phase was removed and the chloroform phase dried *in vacuo*. Choline-containing lipids were re-dissolved in 100µl of

chloroform/methanol (19/1; v/v) and half the sample was applied to silica gel 60 t.l.c. plates. The t.l.c. plates were developed fully in a lined, equilibrated tank in a solvent of chloroform:methanol:acetic acid:water (25:15:4:2, v/v) (Skipski *et al.*, 1964). PtdCho and sphingomyelin were identified by their R_f values and co-migration with authentic standards. After excision the radioactivity in PtdCho and SphM was determined by scintillation counting using 4ml of Optiphase Hi-safe.

2.9 Measurement of Phospholipase D transferase activities in whole cells.

2.9.1 Assay of PLD phosphatidyltransferase activity in intact cells.

PLD catalysed phosphatidyltransferase activity was assayed by measuring the incorporation of butan-1-ol into phosphatidyl moieties by two isotopic labelling methods. In initial studies, unlabelled cells were incubated in the presence of the test reagent and carrier-free [^{3}H] butan-1-ol essentially according to the method of Randall *et al* (1990). However because of the expense and the danger of using such high specific activity [^{3}H]butan-1-ol and of disposing of such high activity waste most studies employed cells pre-labelled with [^{3}H]palmitic and incubated in the presence of 30mM non-radioactive butan-1-ol which equilibrated more rapidly with the cell interior. This second method had the advantage of defining the fatty acid composition of at least some of the PtdBut formed by virtue of the label employed. Thus a combination of both methods allowed identification of PtdBut formation in response to defined agonists.

2.9.2 Assay of PtdBut formation in [^{3}H]palmitate labelled Swiss 3T3 cells.

Swiss 3T3 cells were sub-cultured into six-well culture plates and grown in DMEM + 10% newborn calf serum until approximately 80% confluent. The

growth media was then replaced with 2ml of DMEM + 1% newborn calf serum containing 2 μCi ml^{-1} of [^{3}H]palmitic acid and the cells were returned to the incubator for a further 48 hours. For experimental purposes the labelling medium was removed by aspiration and cells were equilibrated in 1ml of DMBH at 37° C for 20 minutes. For kinetic studies cells were incubated for a further 5 minutes in 1ml of DMBH containing 30 mM butan-1-ol (0.3%, v/v) at 37° C prior to addition of agonists. Finally cells were incubated in DMBH + 30mM butan-1-ol + the required concentration test reagent for the indicated time. For dose dependency studies there was no pre-incubation with butan-1-ol and the appropriate concentration of test reagent in DMBH + 30mM butan-1-ol was added directly to the cells for 15 or 30 minutes. Incubations were terminated by aspiration of DMBH followed by the addition of 0.5ml of ice-cold methanol. Cell debris was scraped off the plate surface and placed in screw-top glass tubes together with a further 0.2ml of methanol wash. Phospholipids in these pooled scrapings were extracted by the addition of 0.7ml of chloroform, vigorous vortexing and standing at room temperature for 15 minutes. After addition of 0.585ml of water tubes were centrifuged for 5 minutes at 3000 rpm, the upper aqueous/methanolic phase discarded and the chloroform phase dried *in vacuo*. Samples were routinely analysed by thin layer chromatography immediately. If chromatography was delayed for 24 hours sample tubes were purged with oxygen-free N_2 and stored for at -20° C. Samples were not stored in chloroform solution as PtdBut was not stable under these conditions.

2.9.3 Assay of PtdBut formation using [^{3}H]butan-1-ol as the phosphatidyl acceptor.

All manipulations with [^{3}H]butan-1-ol were performed in a fume hood to minimize inhalation of [^{3}H]butan-1-ol vapour. Confluent, quiescent cultures of Swiss 3T3 cells grown on 10cm petri dishes were incubated in 5 ml of DMBH (pH 7.4) containing [^{3}H]butan-1-ol (100μCi ml^{-1}) at 37°C for 20 minutes. Test reagents in DMBH were added at the required concentrations and for the given

times with mixing and incubated at 37°C. Incubations were terminated by aspiration of DMBH into a radioactive waste trap and addition of 1.5ml of methanol previously chilled on dry ice. Following scraping of cell debris into polypropylene tubes, lipids were extracted by the addition of chloroform and water as described by Randall *et al.* (1990) and, after removal of the aqueous phase, chloroform layers were evaporated to dryness under N_2. Dry lipid extracts were re-dissolved, with vortexing, in 1ml of chloroform and washed three times with 2ml of theoretical upper phase (upper phase of chloroform: methanol: 1M NaCl: water (2:2:1:1 v/v)). The chloroform layers were than dried down under N_2 and either used directly for t.l.c. analysis or stored dry under N_2 at -20°C for 24 hours.

2.9.4 Isolation and Identification of Phosphatidylbutanol.

Dried chloroform extracts were re-dissolved in 100µl of chloroform/methanol (19/1) with vigorous vortexing and applied to Whatman LK5DF, 20cm x 20cm, pre-laned silica gel thin layer chromatography plates. Once the samples had dried, the plates were fully developed once in the organic phase of 2,2,4-trimethylpentane:ethyl acetate:acetic acid:water (50/110/20/100, v/v) under non-equilibrium conditions in an un-lined chromatography tank.

After drying, plate lanes were divided into 0.5cm strips, the silica gel scraped into scintillation vials and radioactivity counted in 4ml of Optiphase Hi-safe scintillant. The band of radioactivity corresponding to PtdBut was identified by the following criteria:-

(a) its co-migration with an authentic [^{14}C]PtdBut standard developed on the same t.l.c. plate,

(b) its unique appearance in the presence of butan-1-ol in response to a suitable stimulus,

(c) its dose dependent appearance with increasing butan-1-ol concentration up to 100mM in response to a suitable stimulus.

In the t.l.c. solvent system used PtdBut (R_f= 0.42 ± 0.06) was resolved

completely from all other radiolabelled bands including major phospholipids (PtdCho, PtdEtn, PtdSer & PtdIns) which remained at the origin. [^{3}H]PtdOH ran slightly further than the phospholipids (typical R_f value= 0.15) but was not completely resolved from them without a double development in the same dimension. Monoglyceride, di and triglycerides and free fatty acids ran closer to the solvent front (Pai *et al.*, 1988); typical R_f values were 0.78 for MG and 0.9 for DG, TG and free fatty acid which were incompletely resolved.

2.10 Assay of lipid phosphate/phosphorus mass.

Two colorimetric methods were used to assay the mass of total phosphorus containing lipid in chloroform extracts from Swiss 3T3 cells during the course of these studies.

2.10.1 Assay of lipid phosphate.

Reagent A. 1mM K_2HPO_4 prepared in distilled water.

Reagent B. ANS = 2.72g of $Na_2S_2O_5$, 0.2g of Na_2SO_3 and 0.1g of 1-amino-2-napthol-4-sulphonic acid dissolved, in that order, in 100ml of distilled water and filtered before use. Shelf life 1 day.

Reagent C. 2.5% (w/v) ammonium molybdate.

All glassware used was either new or washed thoroughly in distilled water. Samples from the chloroform phase of cellular extracts were evaporated to dryness in a 17 mm test tube. 0.6 ml of concentrated perchloric acid was added to the test tubes containing the dried sample or an appropriate standard (reagent A). Tubes were covered with either aluminium foil or glass marbles to minimise evaporation and heated at 260°C for 2 hours in a fume hood. After cooling 1.6 ml of water and 0.5 ml of reagent B were added and mixed. 1 ml of reagent C followed by 1.3 ml of water was then added and samples were mixed thoroughly by vortexing. Colour development was at 90°C for 30 min and samples were assayed in plastic cuvettes by reading the absorbance at 830nm.

Standard curves of μg of PO_4 versus OD_{830} were plotted and μg of PO_4 in

the unknown samples was estimated from the equation of the standard curve. The assay was linear over the range 0.5 to 20 μg of PO_4.

2.10.2 Assay of lipid phosphorus.

Reagent A. 10% (w/v) $Mg(NO_3)_2.6H_2O$ in methanol.

Reagent B. 4.2% (w/v) ammonium molybdate in 4.5M HCl.

Reagent C. 0.3% (w/v) malachite green (oxalate) Gürr Certistain (BDH product No. 34045, colour 42000) dissolved in distilled water just before use.

Reagent D. Dye-Molybdate reagent. Reagent B (1 vol) mixed with reagent C (3 vol) and left at room temperature for 2-3 hours (or overnight). Filtered through a Whatman No.1 filter paper. Reagent stored at room temperature; stable for 3-5 weeks with limited precipitation. Discarded when further precipitate appears.

Reagent E. 1% (w/v) Triton X-100 in distilled water. Refrigerated and replaced every 3 months.

Assay of total lipid phosphorus was essentially by the method of Duck-Chong (1979). All manipulations were performed in glassware which had been immersed for 24 hours in concentrated sulphuric acid containing 5% concentrated nitric acid followed by vigorous washing with distilled water. A sample of lipid containing approximately no more than 0.5μg of phosphorus in upto 4 ml of chloroform/methanol (1/2, v/v), or a series of 'blanks' for standards, was mixed with 30μl of reagent A in a 17mm test tube and evaporated to dryness at 50°C under a stream of N_2. Lipid was digested by heating the sample for 15 sec in the top of a bunsen flame followed by lowering into the tip of the blue cone of the flame for a further 10 sec; brown fumes were driven off. After cooling to room temperature 1 ml of 1M HCl was added, samples covered with glass marbles to minimise evaporation and heated at 95°C for 15 min before cooling to room temperature. Standards, KH_2PO_4 in 1M HCl, were added to 'blank', digested tubes.

Reagent E (30μl) was added to test solutions or standards followed by addition of 2ml of reagent D with immediate mixing. Colour was allowed to

develop at room temperature for 30 min and the absorbance at 650 nm was determined using plastic disposable cuvettes. Standards were assayed in duplicate. Samples were divided and assayed in duplicate such that they fell at low and high points on the standard curve so as to negate any loss of linearity affecting the validity of the results. The assay was linear from 10 to 500ng of phosphorus.

2.11 Analysis and presentation of results.

Unless otherwise stated all experiments were performed at least three times and each data point represents the mean ± standard deviation (S.D.) of triplicate determinations. Statistical significance was estimated by a paired Students 't' test on an Apple Macintosh Stat-Works program and significance was generally taken as values of $p \leq 0.05$.

EC_{50} and IC_{50} values were calculated from dose-response curves fitted to a logistic equation (non-linear regression analysis) as defined by DeLean *et al.* (1980). However, for presentation purposes dose-response curves were presented as simple line graphs from the Apple Macintosh Cricket Graph program.

Chapter 3.

Mitogen-stimulated inositol lipid hydrolysis and $Ins(1,4,5)P_3$ formation in Swiss 3T3 cells.

3.1 Introduction.

The results presented in this chapter describe some of the kinetic, pharmacological and mechanistic aspects of inositol phosphate generation in response to stimulation of Swiss 3T3 cells with defined mitogenic agents.

The mitogen-stimulated hydrolysis of PtdIns(4,5)P_2 to yield Ins(1,4,5)P_3, and its subsequent metabolism to higher and lower phosphorylated forms of inositol has been studied extensively in Swiss 3T3 fibroblasts (Brown *et al.*, 1984; Heslop *et al.*, 1986; Takuwa *et al.*, 1987a; Brown *et al.*, 1987; Nanberg & Rozengurt, 1988; Blakeley *et al.*, 1989). These studies have demonstrated that a variety of neuropeptides, growth factors and other mitogenic agents stimulate the hydrolysis of inositol lipids at doses similar to those required to stimulate entry into the cell cycle and DNA synthesis (Rozengurt & Sinnett-Smith, 1983; Corps *et al.*, 1985).

The majority of these studies have employed cells labelled to isotopic equilibrium with [^{3}H]inositol and stimulated with an appropriate agonist and the [^{3}H]inositol phosphates produced have been analysed by various ion-exchange techniques. The inclusion of 10mM LiCl inhibits the Ins(x)P phosphatase and thus results in the accumulation of total inositol monophosphates ($InsP_t$) (Berridge *et al.*, 1982). This method provides a very sensitive assay for receptor-mediated inositol lipid hydrolysis during prolonged stimulation, but provides little information on the kinetics of formation of individual inositol phosphates.

Individual inositol phosphate fractions (InsP, $InsP_2$ & $InsP_3$) can be resolved by anion-exchange chromatography (Downes & Michell, 1981). This method was used to show that $InsP_3$, presumed to be Ins(1,4,5)P_3, was the initial product of inositol lipid hydrolysis and was generated fast enough to mediate Ca^{2+} mobilization and therefore fulfil a possible second messenger role (Berridge, 1983). The discovery of Ins(1,3,4)P_3 (Irvine *et al.*, 1984), and subsequently isomers of $InsP_4$ and $InsP_5$, has required the development of more sophisticated ion-exchange techniques for the separation of [^{3}H]inositol phosphates. A number of h.p.l.c. methods are now widely used for this purpose.

For isotopic studies to reflect the changes in mass of inositol phosphates,

the precursor lipids must be 'labelled' to isotopic equilibrium with the intracellular inositol pool. This can be achieved by prolonged preincubation of cells with [^{3}H]inositol in inositol-free culture medium. However, it has been suggested by some workers (Monaco, 1982), though not all (Michell *et al.*, 1988), that distinct hormone sensitive and insensitive pools of inositol lipid might exist. If this is the case, the distribution of radioactivity between the pools must be taken into account. Finally, if continuous labelling is not employed, the specific activity of the precursor lipids may decline throughout the experiment. Clearly, isotopic studies, whilst useful in themselves, have their limitations.

A variety of methods have been developed to measure the mass of inositol phosphates in cells and this topic has been reviewed recently (Palmer & Wakelam, 1990). Perhaps the most useful of these is the Ins(1,4,5)P_3-specific binding assay described by Palmer *et al.* (1989) in which neutralized acid extracts of unlabelled cells compete with a high specific activity [2-^{3}H]*myo*-inositol(1,4,5)P_3 for binding to bovine adrenal cortex microsomes. Since this method determines Ins(1,4,5)P_3 mass directly it negates the problems of the isotopic studies.

The following results are based largely on experiments performed with cells labelled to isotopic equilibrium with [^{3}H]inositol. In the case of bombesin, changes in Ins(1,4,5)P_3 mass have also been performed for a direct comparison with stimulated changes in DG mass (Chapter 4). The experiments describing changes in Ins(1,4,5)P_3 mass were performed as a collaboration with Drs Susan Palmer & Robin Plevin, Institute of Biochemistry, University of Glasgow.

3.2 Results.

The kinetics of inositol phosphate generation in response to stimulation with a maximal dose of bombesin (617nM) were determined.

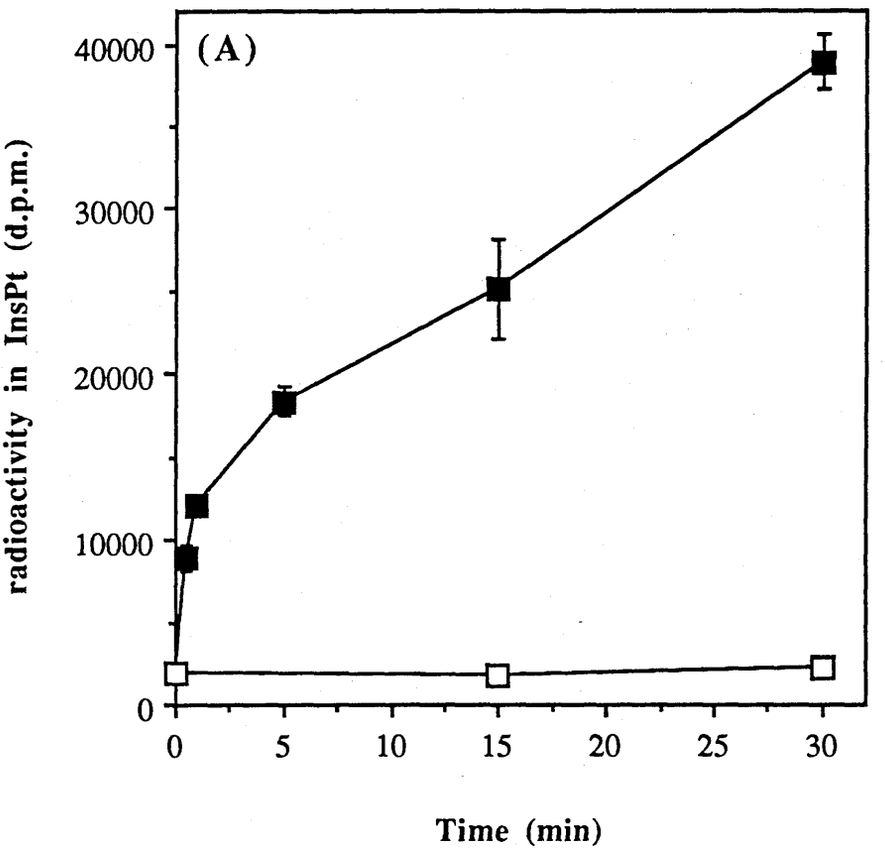
The results in Figure 3.1 show the time-course (a) and dose-response curve (b) for bombesin-stimulated accumulation of $[^3H]InsP_t$ in the presence of 10mM LiCl. Since incubations were performed in the presence of 10mM LiCl the major labelled species would be InsP. Stimulation with bombesin resulted in accumulation of $[^3H]InsP_t$ which was significant above control at 30 seconds (p= 0.001) and rose rapidly over the first minute of stimulation. After one minute $[^3H]InsP_t$ continued to accumulate but at a reduced rate (results from a single representative experiment: 0 to 1 minute, rate = 14,087 dpm min^{-1}; 1 to 15 minutes, rate = 1,787 dpm min^{-1}).The second, slower, rate of $[^3H]InsP_t$ accumulation continued in a near linear fashion for up to 60 minutes (data not shown). The increase in bombesin-stimulated $[^3H]InsP_t$ accumulation, based upon a 20 minute incubation, was dose dependent with a concentration required to give a half maximal increase (EC_{50}) of 0.58 ± 0.34 nM (n=3 determinations in triplicate).

Swiss 3T3 cells, labelled to a higher specific activity with $[^3H]$inositol (5 $\mu Ciml^{-1}$ in inositol-free DMEM) were used to study the kinetics of formation of $[^3H]InsP$, $[^3H]InsP_2$ and $[^3H]InsP_3$ in the presence of 10mM LiCl (Figure 3.2 a-c). Stimulation with bombesin (617nM) resulted in rapid increases in $[^3H]InsP_3$ and $[^3H]InsP_2$ both of which reached a maximum (3-fold and 7-fold) after 10 and 20 seconds of stimulation respectively, before declining again (Figure 3.2 c & b). $[^3H]InsP_3$ levels were significantly above control after 10 sec (p= 0.086) and $[^3H]InsP_2$ levels were clearly significant at 20 sec, the time of maximal elevation (p= <0.001). $[^3H]InsP_3$ levels returned to control values by 5 minutes whereas $[^3H]InsP_2$ levels declined to a half maximal value and were maintained thus for 15 minutes. The accumulation of $[^3H]InsP$ occurred at two distinct rates (Figure 3.2a); rapidly from 0 to 1 minute (3,817 dpm min^{-1}) and slower from 1 to 15 minutes (489 dpm min^{-1}) (values from a single experiment typical of three). Furthermore, $[^3H]InsP$ levels were significantly above control after 5 sec (p=

Figure 3.1 (a & b). Timecourse and dose-response curve for bombesin-stimulated total [^{3}H]inositol phosphate formation.

(a) Swiss 3T3 cells, labelled for 48 hours with 1μCi of [^{3}H]inositol in 1 ml of inositol-free DMEM containing 1% calf serum, were washed in Hanks for 5 minutes followed by HBG.Li for 20 min at 37^{o}C before replacing with HBG.Li (□) or HBG.Li containing 617 nM bombesin (■) for the times indicated. Following extraction in chloroform/methanol total [^{3}H]InsP$_t$ were assayed as described in the Methods section. Results are expressed as radioactivity associated with [^{3}H]InsP$_t$ (d.p.m.; mean ± S.D. (n=3)) from a single experiment, representative of five.

(b) Swiss 3T3 cells labelled for 48 hours with 1μCi of [^{3}H]inositol in 1 ml of inositol-free DMEM were washed in Hanks for 5 min followed by HBG.Li for 20 min at 37^{o}C before replacing with HBG.Li containing the indicated final concentration of bombesin. After 20 min, incubations were terminated and [^{3}H]InsP$_t$ were assayed as described in Chapter 2. Results are expressed as radioactivity in [^{3}H]InsP$_t$ (d.p.m.; mean ± S.D. (n=3)) from a single experiment, representative of three.


(A)
radioactivity in InsPt (d.p.m.)
40000
30000
20000
10000
0
0
5
10
15
20
25
30
Time (min)

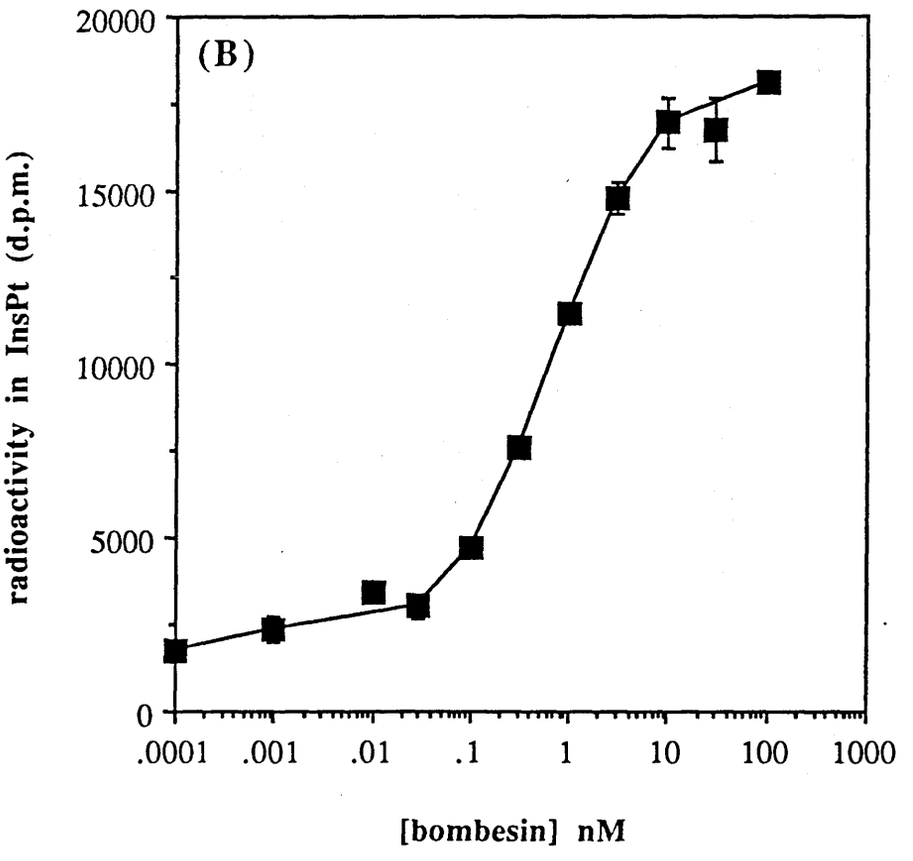
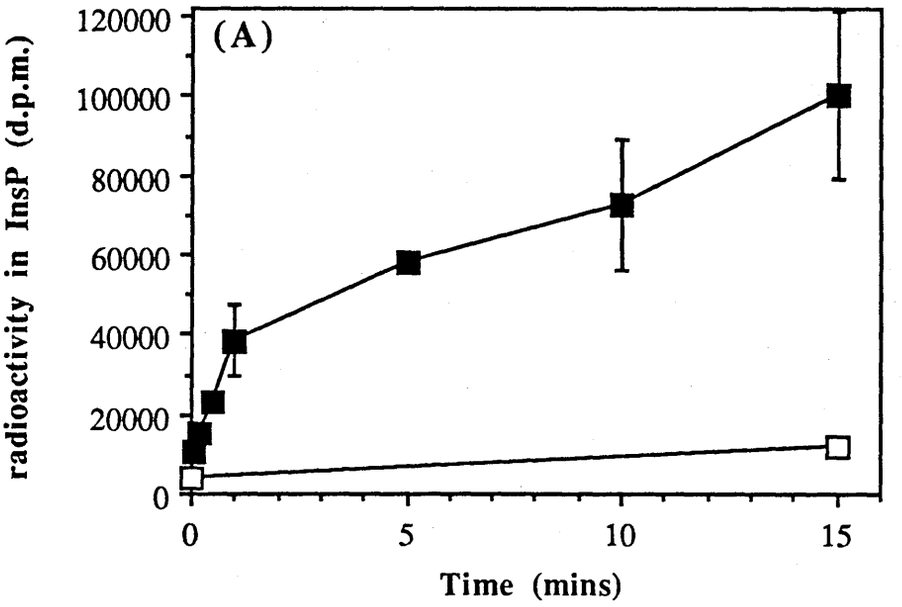
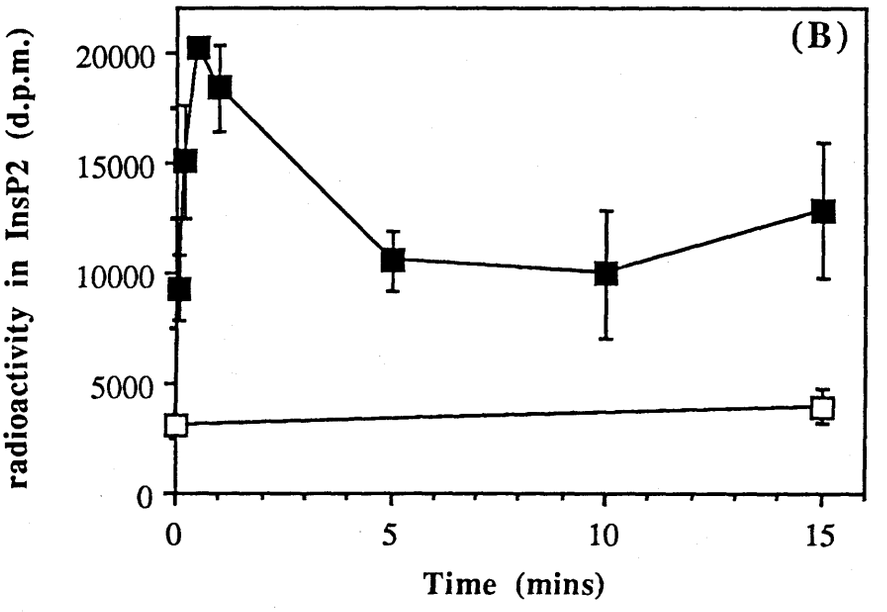
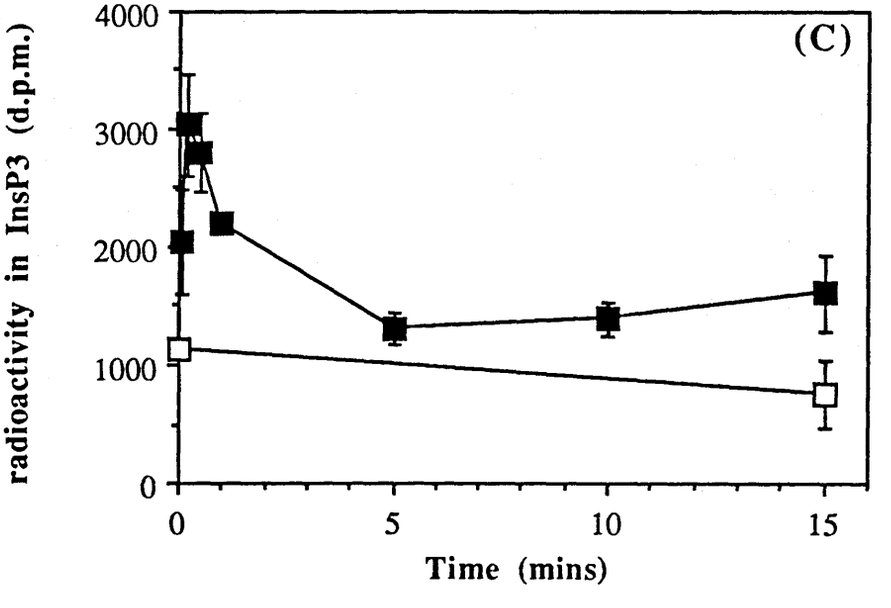

(B)
radioactivity in InsPt (d.p.m.)
20000
15000
10000
5000
0
.0001
.001
.01
.1
1
10
100
1000
[bombesin] nM

Figure 3.2 (a-c). Timecourse of bombesin-stimulated increases in total (a) [^{3}H] InsP, (b) [^{3}H]InsP$_2$ and (c) [^{3}H]InsP$_3$ using a neutral extraction procedure.

Swiss 3T3 cells, labelled for 48 hours 5μCi ml^{-1} of [^{3}H]inositol in inositol-free DMEM containing 1% dialysed calf serum, were washed for 5 minutes in Hanks followed by 20 min in HBG.Li at 37^{o}C. Incubations were started by replacing with HBG.Li (□) or HBG.Li containing 617 nM bombesin (■) and terminated at the times indicated by aspiration and addition of ice-cold methanol. Individual fractions of total [^{3}H] InsP (a), [^{3}H]InsP$_2$ (b) and [^{3}H]InsP$_3$ (c) were isolated by ion-exchange chromatography as described in the Methods section and radioactivity was determined by liquid scintillation counting. The results are expressed as radioactivity in[^{3}H]inositol phosphate fraction (d.p.m.; mean ± S.D. (n=3)) from a single experiment, representative of three.


(A)
radioactivity in InsP (d.p.m.)
120000
100000
80000
60000
40000
20000
0
0
5
10
15
Time (mins)


(B)
radioactivity in InsP2 (d.p.m.)
20000
15000
10000
5000
0
0
5
10
15
Time (mins)


(C)
radioactivity in InsP3 (d.p.m.)
4000
3000
2000
1000
0
0
5
10
15
Time (mins)

0.008).

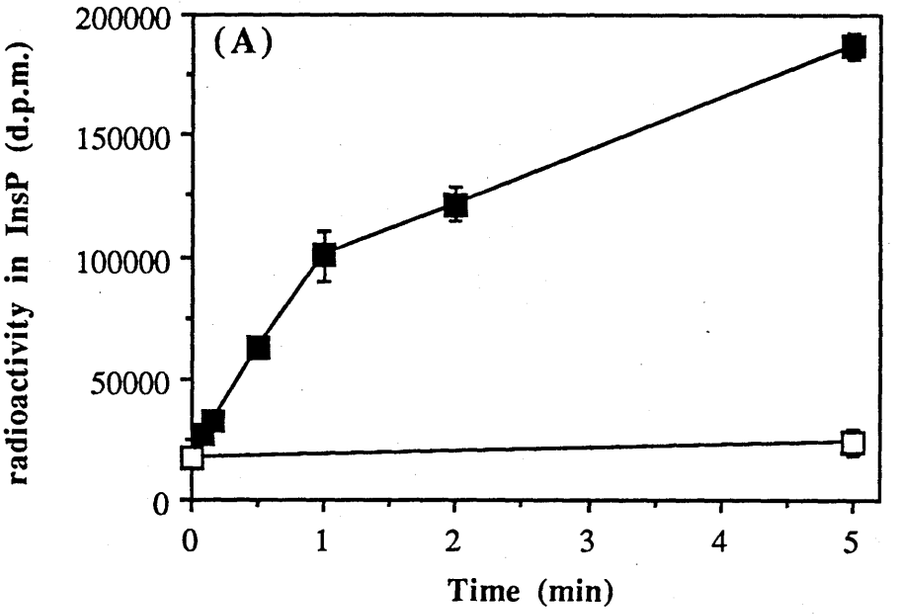
Similar experiments were performed but an acid extraction procedure was employed to recover a greater proportion of $InsP_3$. Incubations were terminated by addition of ice-cold 10% (v/v) $HClO_4$, the samples neutralized with KOH/Hepes and applied to Dowex formate ion-exchange columns for isolation of the [^{3}H]InsP, [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ fractions (Fig 3.3 a-c)

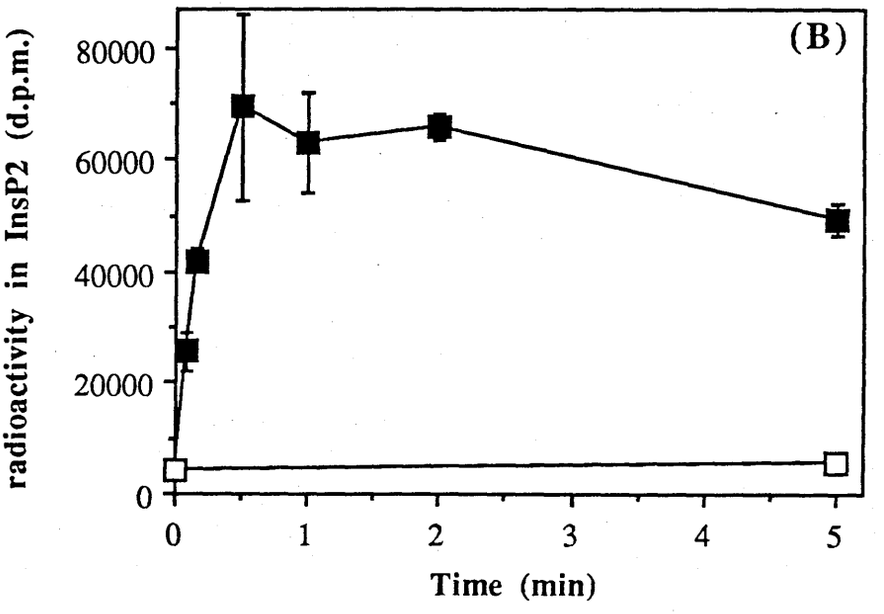
In this instance, the increase in [^{3}H]$InsP_3$ was much greater (typically 9-fold over control at maximum) and significant (p= 0.011) and maximal 5 sec after stimulation with bombesin before declining towards control at 30 sec. From 30 seconds onwards [^{3}H]$InsP_3$ rose slightly for the remaining 5 min (Fig3.3c). The radioactivity associated with the [^{3}H]$InsP_2$ fraction was also significantly elevated above control at 5 seconds (p= 0.004), but was not maximal until after 30 sec or 1 min of stimulation (Fig 3.3b) following which the [^{3}H]$InsP_2$ levels declined. The degree of this decline was variable; in one experiment [^{3}H]$InsP_2$ levels returned almost to basal values by 5 min whilst, generally, they declined to about two thirds of the maximal response and were maintained at this level for up to 5 min. The level of [^{3}H]InsP (Fig 3.3a) rose rapidly from 0 to 1 minute (typically at a rate of 82,618 dpm min^{-1}) being significantly elevated above control after 5 sec (p= 0.014), but again the rise occurred more slowly between 1 and 5 min (typically 21,804 dpm min^{-1}). These two distinct rates of InsP accumulation were similar to those previously described (Fig 3.1a & Fig 3.2a).

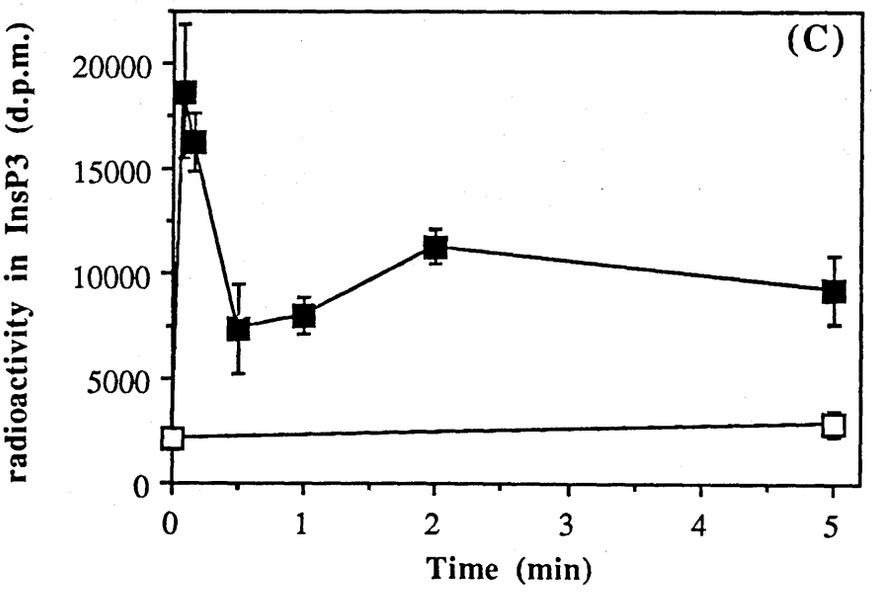
These results suggest that $InsP_3$ was the primary product of bombesin-stimulated inositol lipid hydrolysis. Furthermore, the production of $InsP_3$ was essentially transient; it was rapidly dephosphorylated to $InsP_2$ and thence to InsP. However, measuring changes in total $InsP_3$ provides limited information about what is happening to the levels of Ins(1,4,5)P_3. The initial transient elevation of $InsP_3$ was presumably Ins(1,4,5)P_3 (Heslop *et al.*,1986 and Takuwa *et al.*, 1987a), but the later, sustained elevation of $InsP_3$ could represent an alternative isomer of $InsP_3$, Ins(1,3,4)P_3, which is produced upon bombesin stimulation of Swiss 3T3 cells (Heslop *et al.*, 1986; Takuwa *et al.*, 1987a).

Figure 3.3 (a-c). Timecourse of bombesin-stimulated increases in total (a) [^{3}H]InsP, (b) [^{3}H]InsP$_2$ and (c) [^{3}H]InsP$_3$ (c) using the perchloric acid extraction procedure.

Swiss 3T3 cells were grown on 24 well plates and labelled for 48 hours with 5 μCi of [^{3}H]inositol in 1 ml of inositol-free DMEM containing 1% dialysed calf serum. Cells were washed in HBS and then incubated in HBG.Li for 20 minutes at 37^{o}C. Incubations were initiated by replacing the medium with either HBG.Li (□) or HBG.Li containing 617 nM bombesin (■) and terminated at the times indicated by aspiration and addition of ice-cold 10% (v/v) perchloric acid. Following extraction on ice and neutralization, (a) total [^{3}H] InsP, (b) [^{3}H]InsP$_2$ and (c) [^{3}H]InsP$_3$ were isolated by ion-exchange chromatography and the radioactivity associated with each fraction determined by liquid scintillation counting. Results are expressed as radioactivity in each [^{3}H]inositol phosphate fraction (d.p.m.; mean ± S.D. (n=3)) from a single experiment, representative of three.


(A)
radioactivity in InsP (d.p.m.)
200000
150000
100000
50000
0
0
1
2
3
4
5
Time (min)


(B)
radioactivity in InsP2 (d.p.m.)
80000
60000
40000
20000
0
0
1
2
3
4
5
Time (min)


(C)
radioactivity in InsP3 (d.p.m.)
20000
15000
10000
5000
0
0
1
2
3
4
5
Time (min)

Since the primary purpose of studying inositol phosphate generation was to assess the overall contribution made by inositol lipids to the generation of DG mass, it was necessary to analyse the kinetics of Ins(1,4,5)P_3 generation. This was achieved by analysis of changes in Ins(1,4,5)P_3 mass using the Ins(1,4,5)P_3 binding assay. Samples, prepared from Swiss 3T3 cells stimulated with bombesin were assayed for Ins(1,4,5)P_3 mass by Dr Susan Palmer according to the detailed protocol of Palmer *et al.*(1989) and as outlined in Appendix I.

The results (Fig 3.4) show the kinetics of changes in Ins(1,4,5)P_3 mass in bombesin-stimulated Swiss 3T3 cells over 5 and 15 min timecourses. The level of Ins(1,4,5)P_3 rose rapidly to peak at 5 seconds, the first stimulated time point studied, at which they were 12.52 ± 4.02- fold above controls and significantly elevated (p=0.003). Thereafter, Ins(1,4,5)P_3 mass quickly declined to pre-stimulated levels by 30 seconds and remained thus for up to 15 minutes with no further net increase. These results confirmed that, in mass terms, the bombesin-stimulated increase in Ins(1,4,5)P_3 was rapid in onset and transient. The elevation of Ins(1,4,5)P_3 mass by bombesin was dose-dependent (Fig. 3.5) with half-maximal increases occurring at doses of 5.88 ± 3.66 nM (n=3 determinations in triplicate). (Normalisation of Ins(1,4,5)P_3 mass to mol/10^6 cells revealed that bombesin stimulated a peak elevation of 27.52 ± 3.8 pmoles/10^6 cells at 5 seconds from a basal of.2.33 ± 0.7 pmoles/10^6 cells; mean of 3 experiments).

It has been proposed that bombesin-stimulated generation of DG in Swiss 3T3 cells leads to a protein kinase C-mediated inhibition of PtdIns(4,5)P_2 hydrolysis and that this may serve to limit the response *in vivo* (Brown *et al.*, 1987). This can be mimicked in cultured cells by pre-treating with C-kinase activating phorbol esters, such as PMA, for 10 or 15 minutes resulting in the inhibition of subsequent bombesin-stimulated inositol phosphate generation. The effect of PMA is absent in cells which have been pre-treated with supra-maximal doses of PMA for 48 hours to 'down-regulate' protein kinase C (Brown *et al.*, 1987).

The effects of acute PMA pre-treatment upon agonist-stimulated [^{3}H]InsP_t

Figure 3.4. Timecourse of bombesin-stimulated increases in Ins(1,4,5)P_3 mass.

Swiss 3T3 cells were grown to confluence and quiescence in 75cm^2 flasks and harvested mechanically in their own conditioned medium at 37°C. Harvested cells were maintained in suspension at 37°C for 45 minutes to recover from scraping before being washed twice in HBG; cells were resuspended to a final density of approximately 2 x 10^7 cells ml^{-1}. Incubations were started by addition of 50µl of cell suspension to 25µl aliquots of HBG (□) or HBG containing bombesin (■) at a final concentration of 617 nM. Incubations, at 37°C, were terminated at the indicated times by addition of 25µl of ice-cold 10% perchloric acid and vortexing. After extraction on ice for 20 min samples were neutralised and Ins(1,4,5)P_3 mass was assayed using the competition binding assay of Palmer *et al.* (1989) (Appendix I). In addition an aliquot of cell suspension was taken for cell number determination. Results are expressed as pmoles of Ins(1,4,5)P_3/10^6 cells, mean ± S.D. (n=3) from a single experiment, representative of three.

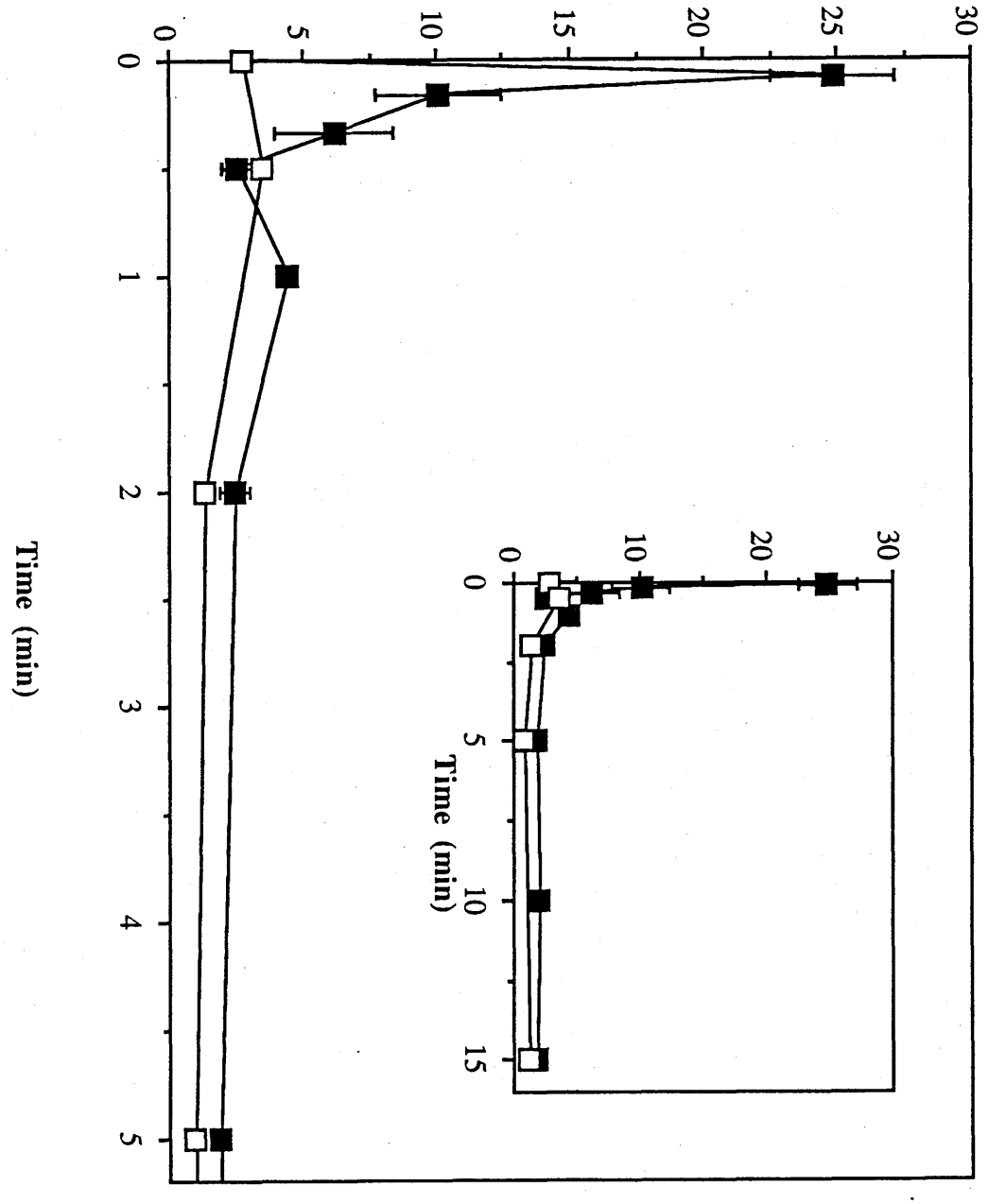
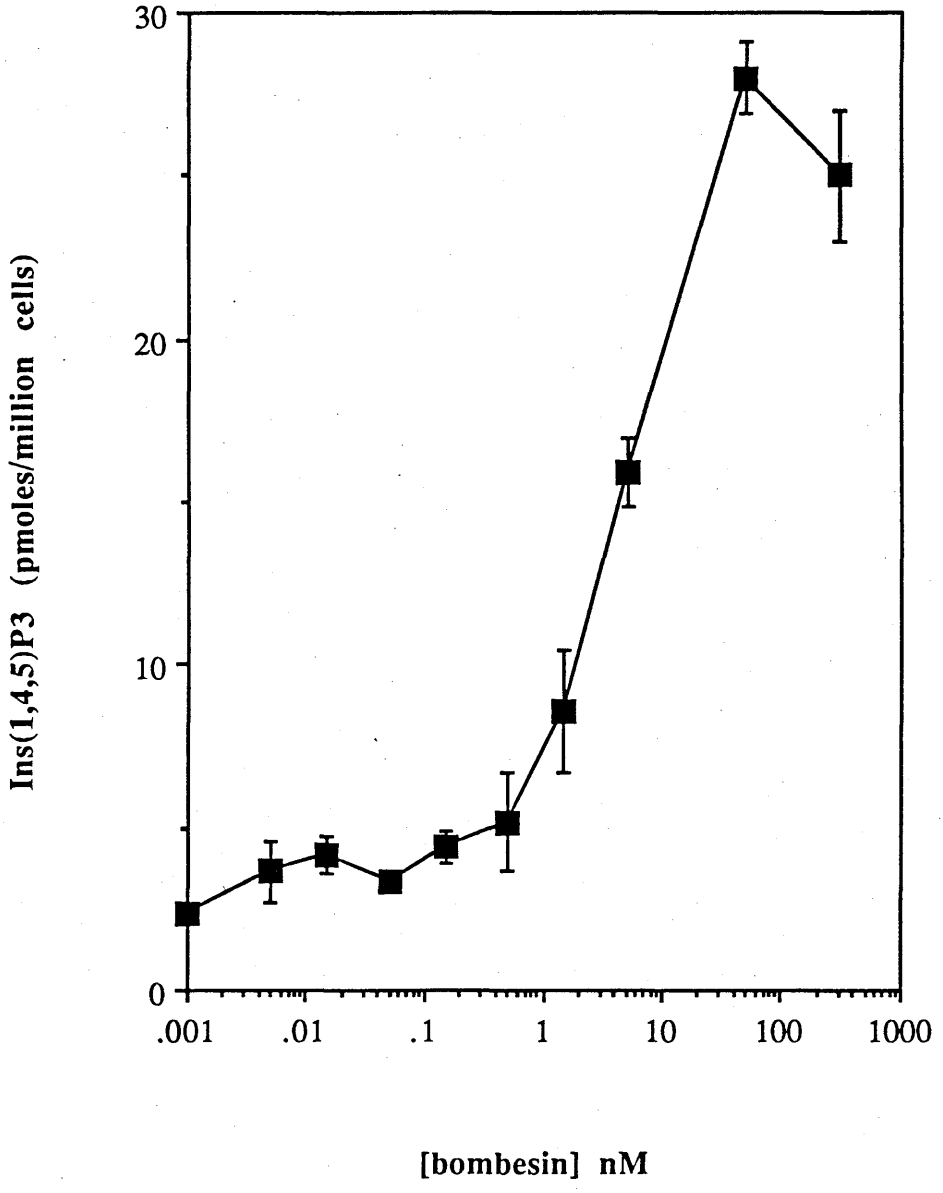

Ins(1,4,5)P3 (pmoles/million cells)
0
5
10
15
20
25
30
0
1
2
3
4
5
Time (min)
0
10
20
30
0
5
10
15
Time (min)

Figure 3.5. Dose-response curve for bombesin-stimulated increases in $Ins(1,4,5)P_3$ mass.

Swiss 3T3 cells, grown to confluence and quiescence, were harvested mechanically and incubated in suspension in their own depleted growth medium for 45 min at 37°C. Following two washes in HBG at 37°C cells were resuspended in HBG at approximately 2×10^7 cells ml^{-1}. Incubations were started by adding 50µl aliquots of cell suspension to 25µl of bombesin, giving the final concentrations indicated, and terminated after 5 sec by addition of 25µl of ice-cold 10% perchloric acid with vortexing. Following neutralisation samples were assayed for $Ins(1,4,5)P_3$ mass using the competition binding assay of Palmer *et al.* (1989) (Appendix I). In addition an aliquot of cell suspension was taken for a cell number determination. Results are expressed as pmoles of $Ins(1,4,5)P_3/10^6$ cells, mean ± S.D. (n=3) from a single experiment, representative of three.


30
20
10
0
.001
.01
.1
1
10
100
1000
Ins(1,4,5)P3 (pmoles/million cells)
[bombesin] nM

generation were examined in an effort to define some mechanistic aspects of the coupling of receptors to the activation of PIC. In addition to bombesin, [Arg^8]vasopressin ([Arg^8]Vp) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were employed. These latter two agonists are also co-mitogenic for the Swiss 3T3 cell line (Corps *et al.*, 1985; MacPhee *et al.*, 1985) and are reported to stimulate inositol lipid hydrolysis in a manner similar to bombesin (Nanberg & Rozengurt, 1988; Black & Wakelam, 1990). Since all three agents stimulate inositol phosphate generation by a similar mechanism the following studies employed accumulation of total inositol phosphates ([^{3}H]$InsP_t$) in the presence of 10mM LiCl as a measure of receptor-mediated inositol lipid hydrolysis.

Figure 3.6 (a-c) shows the dose-response curves for [^{3}H]$InsP_t$ accumulation in Swiss 3T3 cells stimulated for 20 min with bombesin, [Arg^8]Vp and $PGF_{2\alpha}$, respectively. In the case of bombesin and [Arg^8]Vp, this was performed with or without a 15 min pre-treatment with 400nM PMA as indicated in the legend. Each mitogen stimulated a dose-dependent accumulation of [^{3}H]$InsP_t$ with EC_{50} values of 0.58 ± 0.34 nM for bombesin (n=3), 0.48 ± 0.15 nM for [Arg^8]Vp (n=3) and 0.16 ± 0.14 μM for $PGF_{2\alpha}$ (n=4). Pre-treatment of Swiss 3T3 cells with 400 nM PMA inhibited each agonist-stimulated response by some 50% (Fig 3.6 a & b; Fig 3.8 c). In addition, pre-treatment with PMA resulted in a rightward shift of the dose-response curves to bombesin and [Arg^8]Vp giving new EC_{50} values of 4.4 ± 4.5nM (n=2 determinations) and 1.12 ± 0.52nM (n=2 determinations) respectively.

The PMA mediated inhibition of agonist-stimulated [^{3}H]$InsP_t$ accumulation was time- and dose-dependent. Figure 3.7 shows the time course of the effect of PMA pre-treatment on subsequent bombesin-stimulated [^{3}H]$InsP_t$ accumulation. There was a lag of approximately 1 min before the onset of inhibition, whilst maximal inhibition was attained from 5 min onwards. The vehicle (0.1% DMSO) containing β-phorbol was without any apparent effect upon bombesin stimulated [^{3}H]$InsP_t$ accumulation.

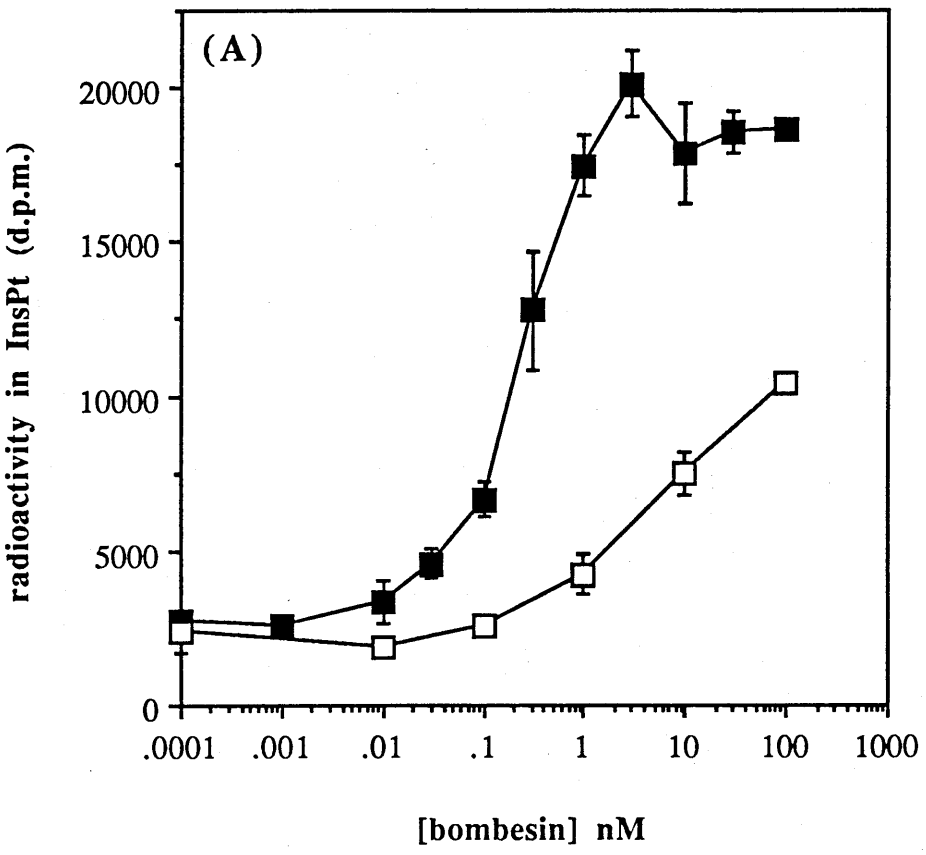
Figure 3.8 (a-c) shows the dose-response curves for PMA-mediated inhibition of subsequent [^{3}H]$InsP_t$ accumulation stimulated by bombesin,

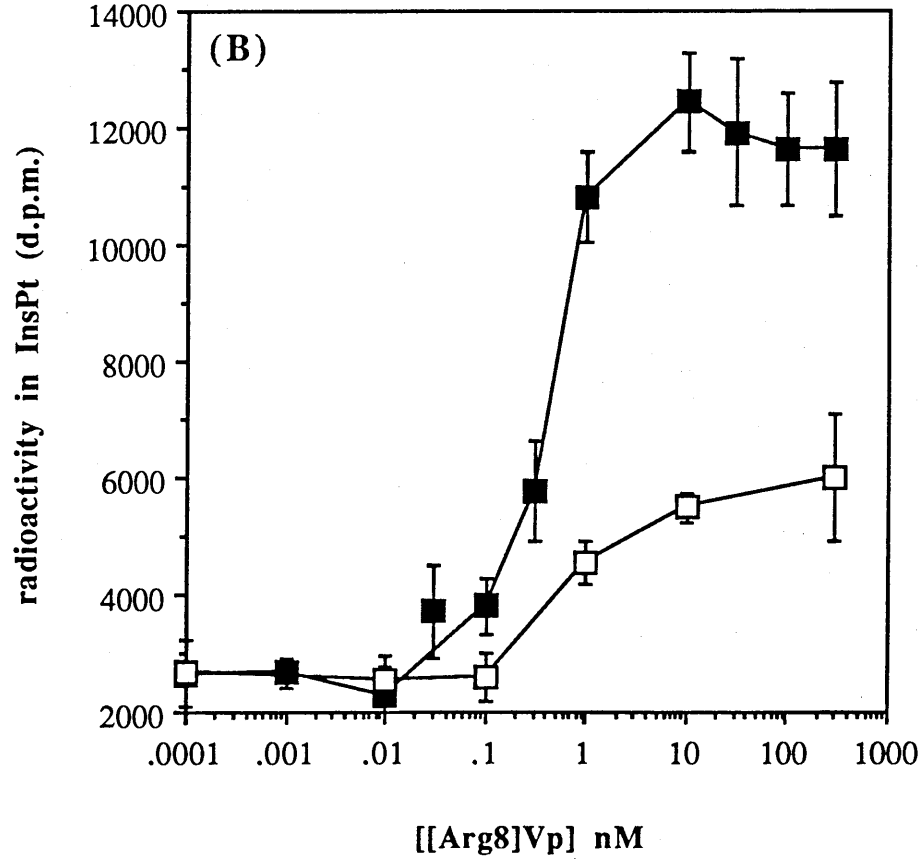
Figure 3.6 (a & b). Dose-response curves for (a) bombesin- and (b) [Arg^8]vasopressin-stimulated total [^{3}H]inositol phosphate formation in control and PMA-pretreated(15 min) Swiss 3T3 cells.

Swiss 3T3 cells, labelled for 48 hours with 1μCi of [^{3}H]inositol in 1ml of inositol-free DMEM containing 1% calf serum, were washed in Hanks for 5 min and then HBG.Li containing 0.1% DMSO (□) or 400 nM PMA in DMSO (■) for 15 minutes at 37°C. Incubations were started by replacing with HBG.Li containing the indicated concentrations of bombesin (a) or [Arg^8]vasopressin (b) and continued at 37°C for 20 minutes. Incubations were terminated by aspiration and addition of ice-cold methanol and formation of [^{3}H]InsP$_t$ was assayed as described in Chapter 2. Results are expressed as radioactivity in [^{3}H]InsP$_t$ (d.p.m., mean ± S.D. (n=3)) from a single experiment, representative of three.

Figure 3.6 (c). Dose-response curve for prostaglandin F$_{2\alpha}$-stimulated total [^{3}H]inositol phosphate formation in Swiss 3T3 cells.

Swiss 3T3 cells, labelled with 1μCi of [^{3}H]inositol in 1 ml of inositol-free DMEM containing 1% serum were washed in Hanks for 5 min followed by HBG.Li for 15 min at 37°C. Incubations were started by replacing with HBG.Li containing the indicated final concentrations of PGF$_{2\alpha}$ and continued for 20 minutes before aspiration and addition of ice-cold methanol. [^{3}H]InsP$_t$ formation was assayed as described in the Methods section. Results are expressed as radioactivity in [^{3}H]InsP$_t$ (d.p.m., mean ± S.D. (n=3)) from a single experiment, representative of four.


(A)
20000
15000
10000
5000
0
radioactivity in InsPt (d.p.m.)
.0001
.001
.01
.1
1
10
100
1000
[bombesin] nM


(B)
14000
12000
10000
8000
6000
4000
2000
radioactivity in InsPt (d.p.m.)
.0001
.001
.01
.1
1
10
100
1000
[[Arg8]Vp] nM

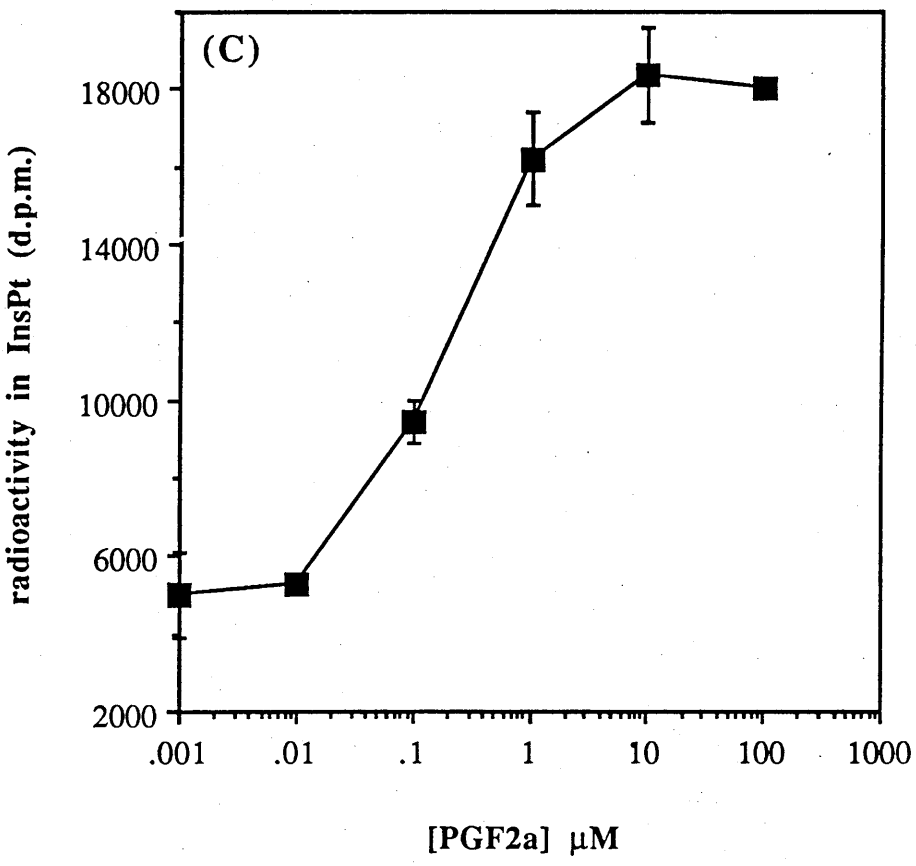

(C)
18000
14000
10000
6000
2000
radioactivity in InsPt (d.p.m.)
.001
.01
.1
1
10
100
1000
[PGF2a] μM

Figure 3.7. Timecourse for inhibition of bombesin-stimulated [^{3}H]InsP$_t$ formation by 400nM PMA.

Swiss 3T3 cells were labelled with 1μCi of [^{3}H]inositol in 1ml of inositol-free DMEM containing 1% calf serum for 48 hours. Following a 5 min wash in Hanks cells were washed in HBG.Li for the times indicated either in the presence of 0.1% DMSO (□) or 400nM PMA in DMSO (■). After this pre-incubation period bombesin was added to give a final concentration of 617nM and incubations were continued for a further 20 min. Incubations were terminated by aspiration and addition of ice-cold methanol and [^{3}H]InsP$_t$ formation was assayed as described in the Methods section. Results are expressed as radioactivity in [^{3}H]InsP$_t$ (d.p.m., mean ± S.D. (n=3)) from a single experiment, representative of two.

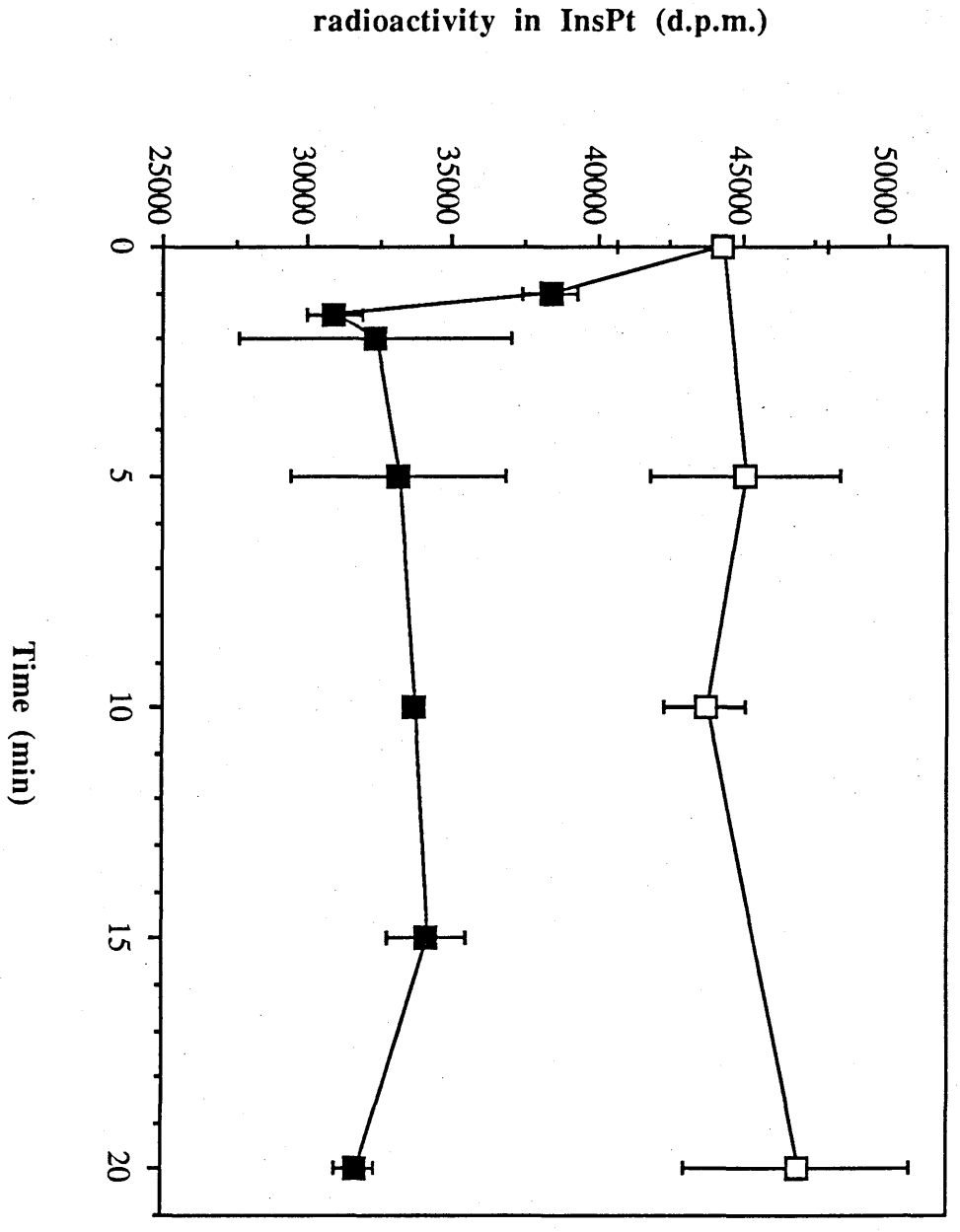
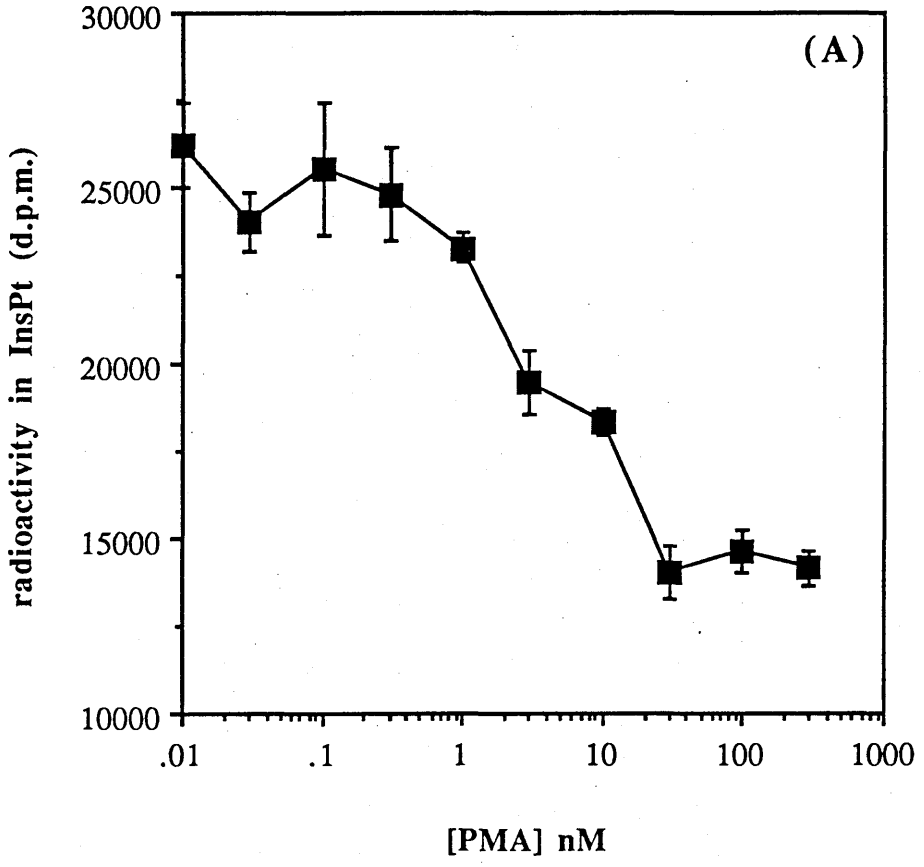

radioactivity in InsPt (d.p.m.)
25000
30000
35000
40000
45000
50000
0
5
10
15
20
Time (min)

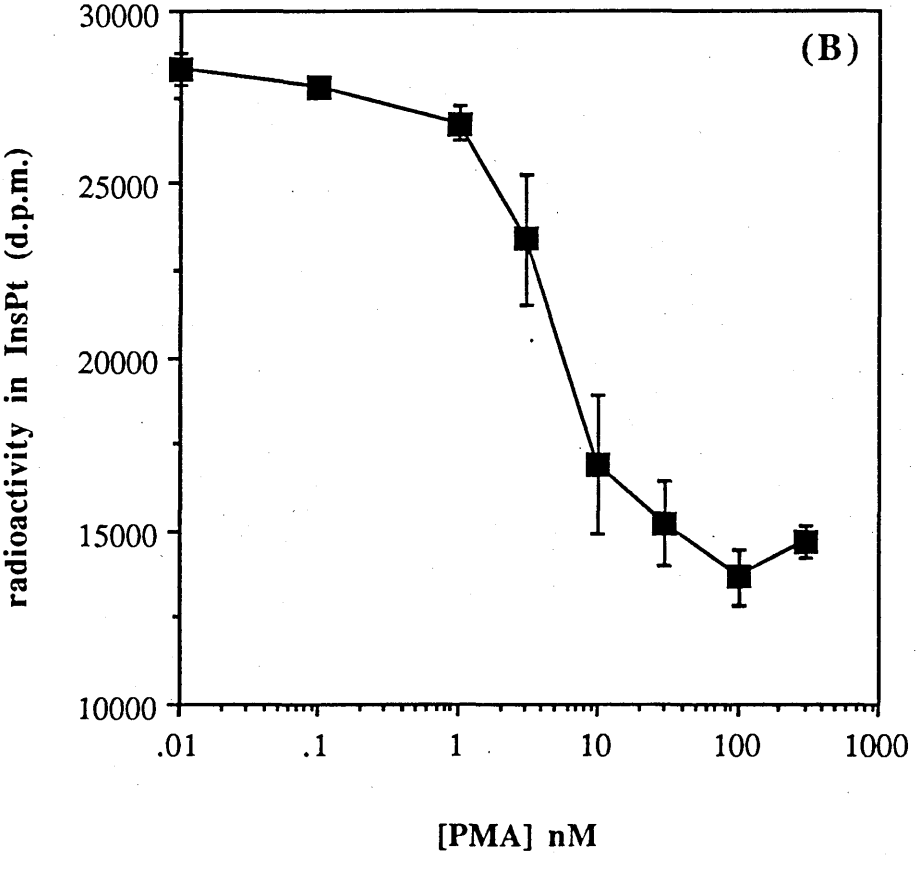
Figure 3.8 (a-c). Dose-response curves for PMA-mediated inhibition of (a) bombesin, (b) [Arg^8]Vp and (c) $PGF_{2\alpha}$-stimulated [^{3}H]$InsP_t$ formation in Swiss 3T3 cells.

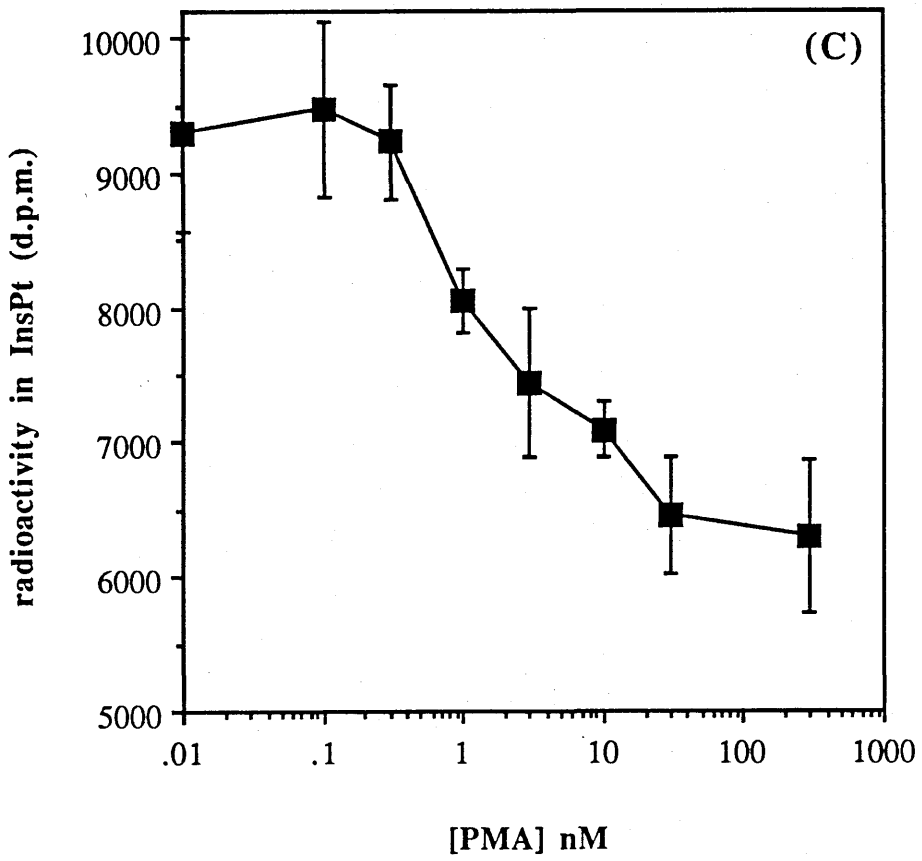
Swiss 3T3 cells were labelled with 1μCi of [^{3}H]inositol in 1ml of inositol-free DMEM containing 1% calf serum for 48 hours. After washing with Hanks for 5 min, cells were incubated in HBG.Li containing the indicated concentration of PMA for 15 min at 37^{o}C. Incubations were initiated by adding mitogens to a final concentration of 617 nM bombesin (a), 100nM [Arg^8]Vp (b) or 2μM $PGF_{2\alpha}$ (c) and incubating at 37^{o}C for a further 20 min. After aspiration and addition of ice-cold methanol [^{3}H]$InsP_t$ was assayed as described in the methods section. The results are expressed as radioactivity in [^{3}H]$InsP_t$ (d.p.m., mean ± S.D. (n=3)) from a single experiment, representative of three in each case. The basal, unstimulated level of radioactivity associated with [^{3}H]$InsP_t$ was 4997 ± 1072 d.p.m. for bombesin, 2566 ± 829 d.p.m. for [Arg^8]Vp and 2722 ± 151 d.p.m. for $PGF_{2\alpha}$.

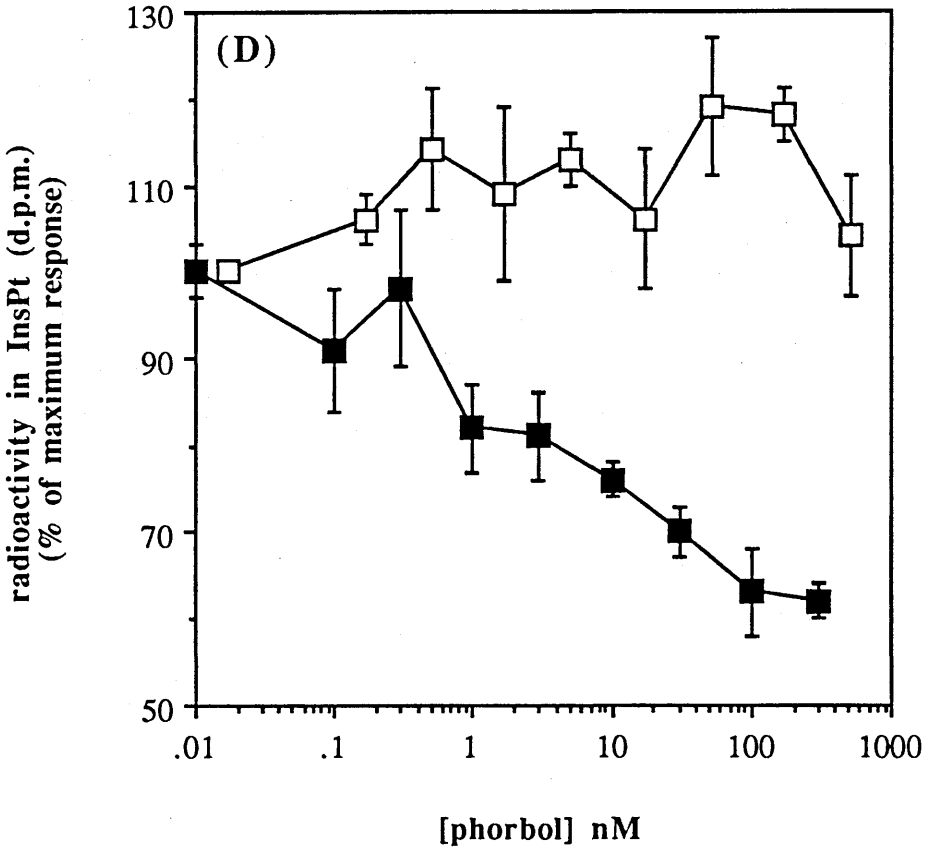
Figure 3.8 (d). Comparison of the effect of PMA and β-phorbol upon bombesin-stimulated [^{3}H]$InsP_t$ accumulation in Swiss 3T3 cells.

Swiss 3T3 cells were labelled with 1μCi of [^{3}H]inositol in 1ml of inositol-free DMEM containing 1% calf serum for 48 hours. After washing with Hanks for 5 min, cells were washed in HBG.Li containing the indicated concentration of PMA (■) or β-phorbol (❑) for 15 min at 37^{o}C. Incubations were initiated by adding bombesin to a final concentration of 617 nM. After 20 min incubations were terminated by aspiration and addition of ice-cold methanol and [^{3}H]$InsP_t$ was assayed as described in the Methods section. The results, radioactivity in [^{3}H]$InsP_t$ (d.p.m., mean ± S.D. (n=3)), are expressed as percent of maximum response and are from a single experiment, representative of three in each case.


(A)
radioactivity in InsPt (d.p.m.)
30000
25000
20000
15000
10000
.01
.1
1
10
100
1000
[PMA] nM


(B)
radioactivity in InsPt (d.p.m.)
30000
25000
20000
15000
10000
.01
.1
1
10
100
1000
[PMA] nM


(C)
radioactivity in InsPt (d.p.m.)
10000
9000
8000
7000
6000
5000
.01
.1
1
10
100
1000
[PMA] nM


(D)
radioactivity in InsPt (d.p.m.)
(% of maximum response)
130
110
90
70
50
.01
.1
1
10
100
1000
[phorbol] nM

[Arg^8]Vp and $PGF_{2\alpha}$, respectively. Typically a 50% inhibition of each response was observed with the IC_{50} value for PMA being similar for each agonist (bombesin, 5.58 ± 3.84 nM (n=3); [Arg^8]Vp, 2.36 ± 1.68 nM (n=3); $PGF_{2\alpha}$, 2.16 ± 0.52 nM (n=3)). These results suggest a common element in the mechanism of coupling of the receptors for bombesin, [Arg^8]Vp and $PGF_{2\alpha}$ to PIC. Pre-treatment of Swiss 3T3 cells with increasing doses of β-phorbol (Fig 3.8d) did not result in a dose-dependent inhibition of bombesin-stimulated [^{3}H]$InsP_t$ accumulation.

To further investigate the role of protein kinase C in phorbol ester-mediated inhibition of agonist-stimulated [^{3}H]$InsP_t$ accumulation, the effects of acute pre-treatment with PMA were compared in normal and PKC down-regulated Swiss 3T3 cells (pre-treated with 400nM PMA for 48 hours to deplete cellular protein kinase C activity (Rodriguez-Pena & Rozengurt, 1984)). Figure 3.9 illustrates that PMA alone did not significantly elevate [^{3}H]$InsP_t$ accumulation in the presence of 10mM LiCl over a 20 min incubation in either control or PKC down-regulated cells. Furthermore, bombesin, [Arg^8]Vp and $PGF_{2\alpha}$-stimulated [^{3}H]$InsP_t$ accumulation in the control cells was not inhibited by down-regulation of PKC. To the contrary, the responses to all three mitogens were reproducibly enhanced in the down-regulated cells (the significance of enhancement of each response in the down-regulated compared to the 'control'cells was: bombesin, $p=0.021$; [Arg^8]Vp, $p<0.001$; $PGF_{2\alpha}$, $p<0.001$). The degree of enhancement of responses varied for each mitogen in the down-regulated cells. Thus, $PGF_{2\alpha}$ showed the greatest enhancement (typically 2.5- 3.2-fold) followed by [Arg^8]Vp (2.2- 2.5-fold enhancement) and, finally, bombesin (1.2- 1.5-fold enhancement). Interestingly, the degree of enhancement in the down-regulated cells was inversely proportional to the magnitude of the response in the control cells. Thus, $PGF_{2\alpha}$, which exhibited the greatest enhancement in down-regulated cells gave the smallest response in control cells, whilst bombesin exhibited much smaller enhancement in the down-regulated cells but showed the biggest response in the control cells. The magnitude of responses in the control cells was also reflected in the degree of depletion of

Figure 3.9. Effect of chronic pre-treatment with 400nM PMA upon mitogen-stimulated [^{3}H]InsP$_t$ formation in Swiss 3T3 cells.

Swiss 3T3 cells were labelled with 1μCi of [^{3}H]inositol in 1ml of inositol-free DMEM containing 1% calf serum for 48 hours in the presence of 400nM PMA (hatched bars) or β-phorbol (open bars). After washing in Hanks for 5 min cells were incubated in HBG.Li for 15 min at 37^{o}C before replacing with HBG.Li alone or containing bombesin (100nM), [Arg^8]Vp (100nM), PGF$_{2\alpha}$ (2μM) or PMA (100nM). After 20 min medium was aspirated and ice-cold methanol added to quench the incubations. [^{3}H]InsP$_t$ was assayed as described in the methods section. Results are expressed as radioactivity in [^{3}H]InsP$_t$ (d.p.m., mean ± S.D. (n=3)) from a single experiment, representative of three.

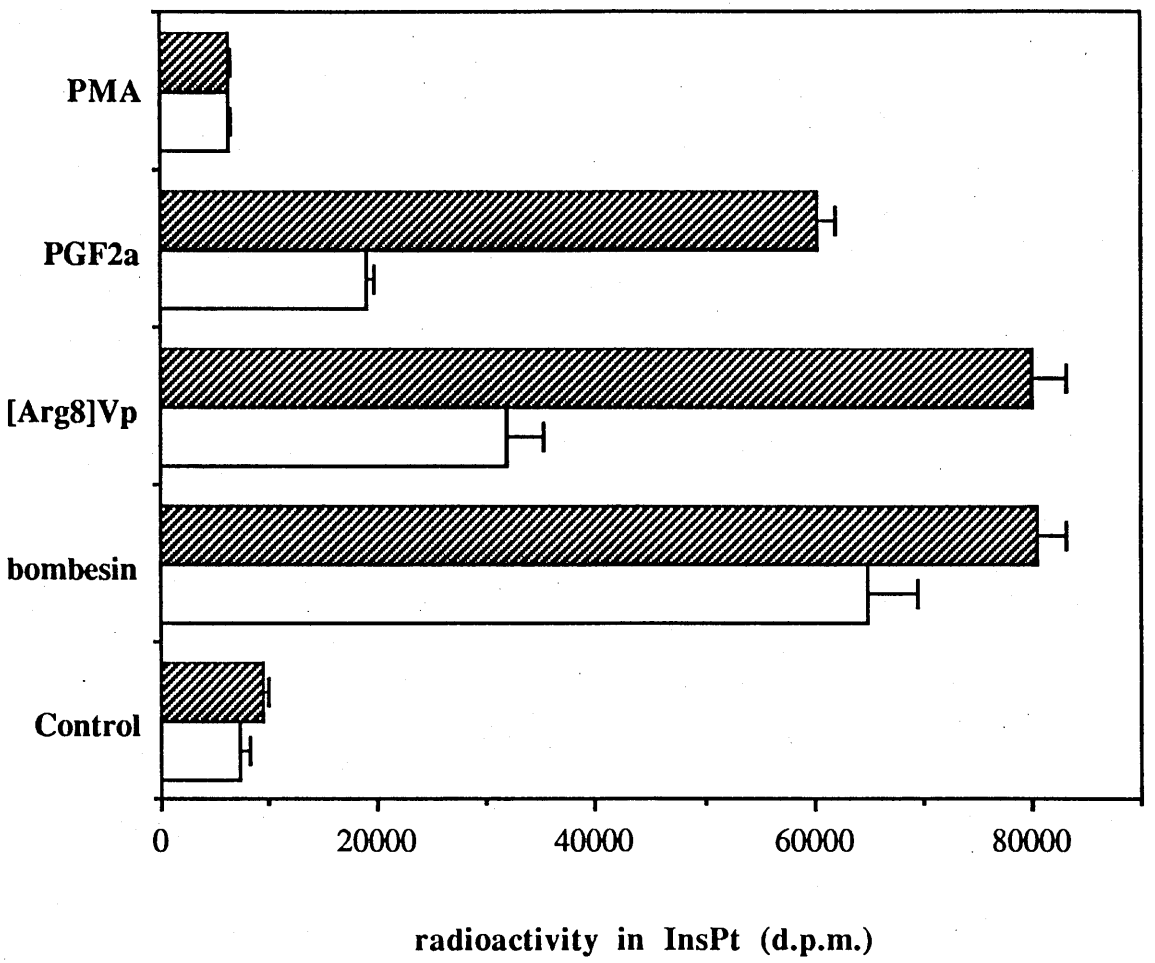

PMA
PGF2a
[Arg8]Vp
bombesin
Control
0
20000
40000
60000
80000
radioactivity in InsPt (d.p.m.)

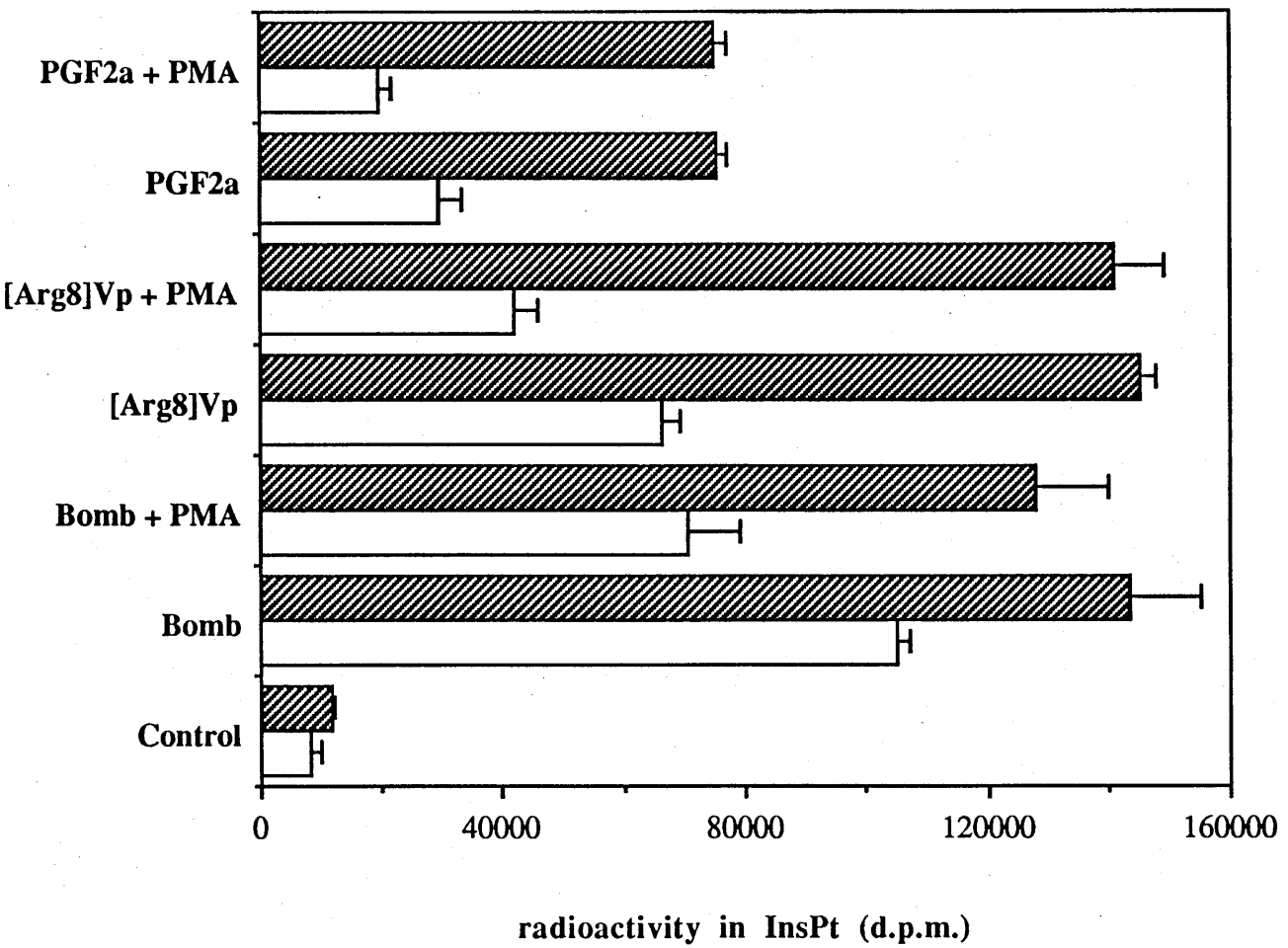
Table 3.1. The effect of agonist stimulation on the radioactivity associated with total $InsP_t$ and inositol lipids in the presence of 10mM LiCl.

Swiss 3T3 cells, labelled for 48 hours with [^{3}H]inositol, were stimulated for 20 minutes with the indicated agonist, or control, in HBG containing 10mM LiCl. Following extraction and removal of total inositol phosphates in the aqueous/methanolic phase, the chloroform phase was dried down *in vacuo*. Radioactivity associated with total inositol phosphates was assayed as previously described in the Methods section. The radioactivity associated with total inositol lipids was determined by liquid scintillation counting after addition of 4mls of Opti phase Hi-safe scintillant cocktail. Results are means of radioactivity in inositol phosphates or inositol lipids (d.p.m.) ± S.D. (n=3) from a single experiment, representative of three.

Treatment.	**radioactivity in $InsP_t$ (d.p.m.).**	**radioactivity in Ins lipids (d.p.m.).**
Control	5,211 ± 2,259	130,714 ± 8,774
bombesin (100nM)	90,142 ± 4,736	70,120 ± 3,239
[Arg^8]Vp (100nM)	60,246 ± 812	107,748 ± 10,859
$PGF_{2\alpha}$ (1μM)	20,961 ± 1,924	136,826 ± 8,123

Figure 3.10. Effect of chronic pre-treatment with 400nM PMA on the ability of acute PMA treatment to inhibit mitogen-stimulated [^{3}H]InsP$_t$ accumulation in Swiss 3T3 cells.

Swiss 3T3 cells were labelled with 1μCi of [^{3}H]inositol in 1ml of inositol-free DMEM containing 1% calf serum for 48 hours in the presence of 400nM PMA (hatched bars) or β-phorbol (open bars). Cells were then washed in Hanks for 5 min before incubating in HBG.Li for 15min at 37°C in the presence or absence of 400nM PMA as indicated. Incubations were started by the addition of bombesin (100nM), [Arg^8]Vp (100nM) or PGF$_{2\alpha}$ (2μM) and were terminated after 20 min by addition of ice-cold methanol. [^{3}H]InsP$_t$ was assayed as described in the methods section. Results are expressed as radioactivity in [^{3}H]InsP$_t$ (d.p.m., mean ± S.D. (n=3)) from a single experiment, representative of three.


PGF2a + PMA
PGF2a
[Arg8]Vp + PMA
[Arg8]Vp
Bomb + PMA
Bomb
Control
0
40000
80000
120000
160000
radioactivity in InsPt (d.p.m.)

radioactivity from total inositol lipids after a 20 minute stimulation in the presence of 10mM LiCl (Table 3.1).

Finally, acute PMA pre-treatment was without effect upon mitogen-stimulated [^{3}H]InsP_t accumulation in PKC down-regulated cells (Figure 3.10). For example, in down-regulated cells pre-treated for 15 min with vehicle, InsP_t accumulation after a 20 minute stimulation in the presence of 10mM LiCl was 80,451 ± 2716 dpm for bombesin, 80,112 ± 3134 dpm for [Arg^8]Vp and 60,255 ± 1703 dpm for $PGF_{2\alpha}$ whilst in down-regulated cells, pre-treated with 400nM PMA for 15 minutes, the values were 82,619 ± 3334 dpm, 83,002 ± 2620 dpm and 61,603 ± 4482 dpm respectively; the basal value was 9,436 ± 488 dpm (values from a single experiment representative of three). The loss of the acute effects of PMA in the down-regulated cells was consistent with the removal of an inhibitory or limiting component, i.e., C-kinase.

3.3 Discussion.

The results presented in this chapter allow three major conclusions to be reached:

(1) The accumulation of the Ca^{2+}-mobilizing second messenger $Ins(1,4,5)P_3$ in bombesin-stimulated Swiss 3T3 cells is essentially transient and the response may be rapidly, though incompletely, desensitized.

(2) Acute pre-treatment of Swiss 3T3 cells with PMA, a potent activator of protein kinase C, to Swiss 3T3 cells does not stimulate the hydrolysis of inositol lipids or the accumulation of $InsP_t$ but, rather, inhibits these responses suggesting a negative, regulatory role for PKC in activation of PIC by bombesin. This effect is absent in PKC down-regulated cells.

(3) Bombesin, $[Arg^8]$Vp and $PGF_{2\alpha}$ are functionally related in their ability to stimulate inositol lipid hydrolysis and $InsP_t$ accumulation as defined by their sensitivity to acute and chronic phorbol ester treatment.

3.3.1 Transient accumulation of $Ins(1,4,5)P_3$ in bombesin-stimulated Swiss 3T3 fibroblasts.

It has previously been shown that bombesin stimulates inositol lipid turnover in Swiss 3T3 cells by stimulating the PIC-catalysed hydrolysis of $PtdIns(4,5)P_2$ to generate $Ins(1,4,5)P_3$ (Heslop *et al.*, 1986, Takuwa *et al.*, 1987a). The coupling of ligand-activated receptors to the activation of PIC is thought to occur through the activation of a guanine nucleotide regulatory protein (G_p; Cockcroft, 1987). Both bombesin-stimulated $Ins(1,4,5)P_3$ and $InsP_t$ accumulation are greatly potentiated by GTPγS in electro-permeabilized Swiss 3T3 cells and the onset of $InsP_3$ formation is much more rapid in the presence of GTPγS suggesting that receptor-stimulated guanine nucleotide exchange is a rate limiting component in the response (Plevin *et al.*, 1990). Furthermore, events downstream of inositol lipid hydrolysis such as PKC-mediated phosphorylation of an 80 kDa protein are also modulated by guanine nucleotides (Erusalimsky *et al.*, 1988).

The initial product of bombesin-stimulated inositol lipid hydrolysis was $InsP_3$ when measured by radiochemical assay (Figs. 3.2c and 3.3c). Mass assay (Fig. 3.4) demonstrated that the initial product was indeed Ins(1,4,5)P_3 confirming the h.p.l.c. analysis performed by Heslop *et al.* (1986) and Takuwa *et al.* (1987a) which identified [^{3}H]Ins(1,4,5)P_3 as the earliest product of bombesin-stimulated inositol lipid hydrolysis in [^{3}H]inositol labelled Swiss 3T3 cells. The onset of the response was rapid being maximal at 5 sec (Figs. 3.3c & 3.4), the earliest time point tested.

Both radiochemical assays (Figs. 3.2c & 3.3c) yielded essentially the same kinetics of [^{3}H]$InsP_3$ formation; namely that [^{3}H]$InsP_3$ formation was maximal within 5 or 10 sec. However, in quantitative terms the magnitude of the response was quite different and this may be due to the different extraction techniques employed. When samples were extracted in methanol the magnitude of the response (typically 3-fold) was much smaller than that using the perchloric acid extraction technique (9-fold over control) and Ins(1,4,5)P_3 mass (12-fold over control). This discrepancy may reflect the poorer extraction of higher phosphorylated forms of inositol in chloroform-methanol (Wregget *et al.*, 1987; reviewed by Palmer & Wakelam, 1989) and may also explain the slightly slower onset of $InsP_3$ formation using the methanol extraction (Fig. 3.2c) as opposed to the perchloric acid extraction (Fig. 3.3c & 3.4). The original reason for using a neutral methanol extraction was to allow [^{3}H]$InsP_3$ and DG mass formation to be assayed from the same samples; the preparation of samples for DG mass analysis must be performed under neutral conditions (Preiss *et al.*, 1986). However, the differences in magnitude of [^{3}H]$InsP_3$ formation indicates that increases in $InsP_3$ would be greatly underestimated under these conditions and the two second messengers were therefore assayed from separate experiments under different extraction conditions.

Despite these differences in magnitude, all experiments revealed a similar qualitative time course of $InsP_3$ formation. Thus, the onset of $InsP_3$ formation was extremely rapid but this increase was essentially transient. The rapid decline in

[^{3}H]$InsP_3$ levels after 5 seconds stimulation with bombesin probably represents Ins(1,4,5)P_3-5'phosphatase activity. This is reflected in the rapid elevation of [^{3}H]$InsP_2$ levels which were significant above control at 5 seconds and continued to rise, peaking at between 30 sec and 1 min (Fig 3.2b) or 2 min (Fig 3.3b) stimulation. [^{3}H]$InsP_2$ levels declined towards 5 minutes (Fig 3.3b) to between 50 and 75% of the peak response and were maintained at this level up to 15 minutes (Fig 3.2b). The rapid elevation in [^{3}H]$InsP_3$ and [^{3}H]$InsP_2$ levels was also reflected in [^{3}H]InsP levels which were significantly elevated above control after 5 seconds stimulation with bombesin and these results are consistent with InsP being derived from the rapid sequential dephosphorylation of $InsP_3$ via $InsP_2$. In such a scenario the apparent new 'steady state' of $InsP_2$ levels between 5 and 15 minutes presumably reflects the input of $InsP_3$ de-phosphorylation and the output to InsP being nearly equal.

The decline in Ins(1,4,5)P_3 levels from 5 sec may also be due, in part, to phosphorylation of Ins(1,4,5)P_3 by a specific 3' kinase to yield Ins(1,3,4,5)P_4 (Batty *et al.*, 1985). Consistent with this is the report of increases in [^{3}H]Ins(1,3,4,5)P_4 in bombesin-stimulated, [^{3}H]inositol labelled Swiss 3T3 cells (Heslop *et al.*, 1986). In some experiments, under perchloric acid extraction conditions, small increases in total [^{3}H]$InsP_4$ were seen in response to bombesin though these were not reproducible (data not shown). This probably reflects the poor sensitivity of radiochemical assays since mass measurement of Ins(1,3,4,5)P_4 reveals much greater increases than [^{3}H]Ins(1,3,4,5)P_4 in carbachol-stimulated bovine tracheal smooth muscle (Chilvers *et al.*, 1990a; 1990b) and in carbachol-stimulated SH-SY5Y neuroblastoma cells (Lambert *et al.*, 1991).

There was one clear discrepancy between the time course of [^{3}H]$InsP_3$ and Ins(1,4,5)P_3 mass, under identical extraction conditions. Bombesin-stimulated increases in [^{3}H]$InsP_3$ declined from 5 sec but were clearly sustained 4-fold above control from 30 sec up to 5 min (fig 3.3c), whereas Ins(1,4,5)P_3 mass declined from 5 sec to pre-stimulated levels at 30 sec after which there was no further net increase up to 15 min (Fig 3.4). Whilst the mass assay is selective for Ins(1,4,5)P_3 (Palmer *et al.*, 1989) the [^{3}H]$InsP_3$ fraction from a Dowex formate

column will contain other isomers of $InsP_3$ in addition to Ins(1,4,5)P_3. The phosphorylation of Ins(1,4,5)P_3 by a 3'-kinase to yield Ins(1,3,4,5)P_4 followed by its subsequent de-phosphorylation by a 5'-phosphatase will yield Ins(1,3,4)P_3 (Batty *et al.*, 1985; Irvine *et al.*, 1986). Such a sequence of events is likely to account for the slower and sustained rise in total [^{3}H]$InsP_3$, which is not due to Ins(1,4,5)P_3 (Fig. 3.4), and is confirmed by the observation that [^{3}H]Ins(1,3,4)P_3 levels do indeed rise in bombesin-stimulated Swiss 3T3 cells over a time course consistent with their contributing to the sustained elevation of total [^{3}H]$InsP_3$ (Heslop *et al.*, 1986; Takuwa *et al.*, 1987a). In particular Takuwa *et al.* (1987a) in super-imposing the time course of bombesin-stimulated increases in [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4)P_3 derive a time course which is very similar to that for total [^{3}H]$InsP_3$ in Fig. 3.3c, suggesting that the sustained elevation is due to the coupled activity of 3'-kinase and 5'-phosphatase.

The rapid increase in [^{3}H]$InsP_3$ and particularly Ins(1,4,5)P_3 mass compares favourably with the time course of increases in $[Ca^{2+}]_i$ stimulated by bombesin. Lopez-Rivas *et al.* (1987) have shown that upon addition of bombesin to quiescent Swiss 3T3 cells, elevations in $[Ca^{2+}]_i$ were apparent without any measureable delay whilst peak values were attained 17 ± 1.1 seconds after addition. Similar results were obtained in bombesin-stimulated WFB rat fibroblasts (Hasegawa-Sasaki *et al*, 1988). Since elevation of Ins(1,4,5)P_3 would be required to precede increases in $[Ca^{2+}]_i$ for it to be responsible for those increases the time course for increases in Ins(1,4,5)P_3 is consistent with its role as a Ca^{2+}-mobilizing second messenger; indeed Ins(1,4,5)P_3 has been shown to stimulate Ca^{2+} release from permeabilized Swiss 3T3 cells (Berridge *et al.*, 1984). These observations are entirely consistent with the hypothesis that Ins(1,4,5)P_3 is an intracellular messenger which stimulates the release of intracellular stores of Ca^{2+} into the cytoplasm as originally proposed by Streb *et al.* (1983). However, the elevation of $[Ca^{2+}]_i$ by bombesin is transient and quickly declines to leave a smaller, more sustained elevation which is dependent on extracellular $[Ca^{2+}]$ (Lopez-Rivas *et al.*, 1987; Hesketh *et al.*, 1988; Hasegawa-Sasaki *et al.*, 1988).

The rapid decline in Ins(1,4,5)P_3 levels is consistent with both the transient bombesin-stimulated Ca^{2+} 'spike' seen in Swiss 3T3 cells (Lopez-Rivas *et al.*, 1987) and the rapid elevation of [^{3}H]InsP_2 and [^{3}H]InsP levels. Thus removal of Ins(1,4,5)P_3 by the 5'-phosphatase or 3'-kinase, will serve to terminate the physiological response (Ca^{2+}-mobilization from intracellular stores) and will account for the elevation of [^{3}H]InsP_2 levels.

The 3'-kinase pathway will also contribute to the rapid decline in Ins(1,4,5)P_3 levels and termination of Ca^{2+} release from intracellular stores and this may be enhanced by the activation of Ins(1,4,5)P_3 3'-kinase by Ca^{2+} and, in some tissues calmodulin, as reported for the brain enzyme (Biden *et al.*, 1987; Morris *et al.*, 1987). In addition the rapid phosphorylation of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 may result in the generation of a further biologically active second messenger derived from inositol lipid hydrolysis. It has been proposed that Ins(1,3,4,5)P_4 may play a role in the entry of Ca^{2+} across the plasma membrane accounting for the smaller, sustained elevation of $[Ca^{2+}]_i$ seen in response to a variety of agonists (reviewed by Irvine, 1990).

The transient nature of bombesin-stimulated increases in $[Ca^{2+}]_i$ would require that Ins(1,4,5)P_3 be removed rapidly, which seems to be the case (Fig 3.4), or that bombesin-stimulated PtdIns(4,5)P_2 hydrolysis be desensitized. The results in Figure 3.1 suggest that bombesin-stimulated [^{3}H]InsP_t accumulation occurred at two distinct rates. The initial rate of [^{3}H]InsP_t accumulation from 0 to 1 min was nearly 8-fold greater than that over the period from 1 to 15 minutes, suggesting that the response was quickly desensitized. The results in Figure 3.2a and 3.3a show that, in the presence of 10mM LiCl, accumulation of [^{3}H]InsP occurred at two distinct rates in all experiments performed. The initial rate of increase in [^{3}H]InsP from 0-1minute was between 4- and 9-fold greater than the rate of accumulation from 1-5 (Fig3.3a) or 1-15minutes (Fig 3.2a) respectively. Since Ins(1,4,5)P_3 has been identified as the initial product of bombesin-stimulated inositol lipid hydrolysis and LiCl allows the accumulation of InsP derived from the dephosphorylation of that Ins(1,4,5)P_3, the rate of [^{3}H]InsP accumulation will reflect the rate of

PtdIns(4,5)P_2 hydrolysis assuming that the rates of Ins(1,4,5)P_3 and Ins(1,4)P_2 de-phosphorylation are not limiting in stimulated cells. Indeed, the rapid decline in peak [^{3}H]InsP_3 levels and the elevation of [^{3}H]InsP at 5 seconds suggests that these steps occur at a very high rate whilst the high activity of Ins(1,4,5)P_3-5'-phosphatase is well documented in a variety of cells (reviewed by Shears, 1989). If Ins(1,4,5)P_3 de-phosphorylation is not limiting then the change in rate of accumulation of [^{3}H]InsP (and [^{3}H]InsP_t in Fig. 3.1a) may reflect some sort of feedback inhibition and/or desensitization of PtdIns(4,5)P_2 hydrolysis. That PtdIns(4,5)P_2 hydrolysis is indeed desensitized is supported by work from this laboratory showing that following removal of an initial dose of bombesin to Swiss 3T3 cells and washing, cells are desensitized to a second dose of bombesin within 30 seconds of the original dose (S. Palmer, R. Plevin & M.J.O. Wakelam, personal communication). Quite what the mechanism of this desensitization event is remains unclear but it continues to operate in cells in which PKC activity has been down regulated and is unaffected by PKC inhibitors or acute pre-treatment with phorbol esters suggesting that it is not mediated by PKC.

It is proposed that the initial, rapid rise in [^{3}H]InsP_t, [^{3}H]InsP and [^{3}H]InsP_2 reflects the rapid formation and subsequent metabolism of Ins(1,4,5)P_3 upon addition of bombesin, whilst the slower rate may represent a partially desensitized, but continued, hydrolysis of PtdIns(4,5)P_2. No evidence is presented to support or discount a role for PtdIns hydrolysis in the rapid accumulation of [^{3}H]InsP in the presence of LiCl. The significant increase in both [^{3}H]InsP and [^{3}H]InsP_2 at 5 seconds, also seen by Takuwa *et al.* (1987a), is very fast compared with the responses in some cells such as bradykinin-stimulated NG105-40IL cells (Jackson *et al.*, 1987) and might argue in favour of hydrolysis of either PtdIns or PtdInsP. However, it seems unlikely that this rapid elevation of [^{3}H]InsP and [^{3}H]InsP_2 is due to [^{3}H]PtdIns or [^{3}H]PtdInsP hydrolysis in Swiss 3T3 cells since Takuwa *et al.* (1987a) have shown quite clearly that there was no significant loss of radioactivity from PtdIns until the loss from PtdInsP_2 had reached maximum and PtdInsP_2 levels were beginning to recover. Since levels of PtdInsP remained constant throughout the time course these results suggest that

bombesin-stimulated PIC activation leads to the hydrolysis of PtdIns(4,5)P_2 which is replenished by conversion of PtdIns to PtdInsP and then to PtdIns(4,5)P_2. Consequently, there seems no need to propose any other explanation for the early increases in [^{3}H]InsP and [^{3}H]InsP_2 other than the rapid, sequential, dephosphorylation of Ins(1,4,5)P_3.

In contrast the later and sustained accumulation of [^{3}H]InsP_t and [^{3}H]InsP, albeit at a reduced rate, may be due to continued PtdIns(4,5)P_2 hydrolysis or the later initiation of PtdIns hydrolysis (Imai & Gershengorn, 1986). The observation that [^{3}H]InsP_2 levels appeared to reach a new 'steady state' between 5 and 15 minutes supports the notion of a continued net flux from InsP_3 to InsP_2 and then to InsP in cells continually stimulated with bombesin. From 30 seconds onwards to 5 (Fig 3.4 main) and 15 minutes (Fig 3.4 inset) there was no further net increase in Ins(1,4,5)P_3 mass above the pre-stimulated level. It is likely that this represents both the desensitization described above and also the possibility that 5'-phosphatase and 3'-kinase activity may be so high that the effective half life of newly produced Ins(1,4,5)P_3 will be negligible; if so it may not accumulate to levels sufficient to monitor. This continued formation of Ins(1,4,5)P_3 and its rapid de-phosphorylation might account for the continued, though slower, accumulation of [^{3}H]InsP and [^{3}H]InsP_t from 1 minute onwards (Fig. 3.1a, 3.2a & 3.3a). Alternatively, Imai & Gershengorn (1986) have provided evidence for TRH-stimulated PtdIns hydrolysis in GH_3 pituitary cells which occurs after the transient hydrolysis of PtdIns(4,5)P_2 is complete, and is sustained for at least 30 min. Thus it is possible that the sustained accumulation of [^{3}H]InsP may represent either a desensitized rate of PtdIns(4,5)P_2 or PtdIns hydrolysis and future studies should aim to assess which is the major source.

The dose-response curve for bombesin-stimulated increases in Ins(1,4,5)P_3 mass (Fig 3.5) generated an EC_{50} of 5.88 ± 3.66 nM (n=3); this value is slightly greater than that for accumulation of [^{3}H]InsP_t (EC_{50}= 0.58 ± 0.34 nM). The anomaly between these two values may simply reflect the errors associated with performing a dose-response curve for a 5 sec stimulus (Ins(1,4,5)P_3 mass) as compared with a 20 min stimulus for [^{3}H]InsP_t. Furthermore, Ins(1,4,5)P_3 levels

at 5 seconds will be a composite of formation and breakdown rates whilst [^{3}H]InsP$_t$ levels will be a much more sensitive assay by virtue of the inhibition of InsP phosphatases by LiCl allowing accumulation of all inositol moieties produced upon stimulation of PtdIns(4,5)P$_2$ hydrolysis. In the former case even a small error will be greatly amplified by minute variations in start-stop times. Despite this the EC$_{50}$ value quoted for Ins(1,4,5)P$_3$ mass elevation agrees well with various parameters previously described for Swiss 3T3 cells including the IC$_{50}$ for bombesin-stimulated inhibition of specific ^{125}I-GRP binding to cell membranes (1.5 nM, Sinnett-Smith *et al.*, 1990), bombesin-stimulated Ca^{2+} mobilization (1-2 nM, Lopez-Rivas *et al.*, 1987) and bombesin-stimulated phosphorylation of an 80kDa protein kinase C substrate (4 nM, Erusalimsky *et al.*, 1988).

3.3.2 Activation of protein kinase C, by administration of PMA, inhibits bombesin-stimulated inositol lipid hydrolysis.

A number of studies have reported sustained elevations of DG in response to a variety of agonists (rerviewed by Billah & Anthes, 1990). Typical of these, Griendling *et al.* (1986) reported sustained elevation of [^{3}H]DG in response to angiotensin II in VSMC labelled with [^{3}H]arachidonic acid. Since the sustained phase of DG elevation was kinetically downstream of InsP$_3$ formation and was accompanied by a sustained depletion of [^{3}H]PtdIns they suggested that PtdIns was the source of sustained DG elevation. They proposed that DG produced from the primary PtdIns(4,5)P$_2$ hydrolysis would act, via PKC, to switch from PtdIns(4,5)P$_2$ to PtdIns hydrolysis and showed that addition of PMA to mimic DG resulted in the inhibition of InsP$_3$ formation without inhibiting the sustained phase of DG elevation. The logical extension of such a model is that addition of PMA to cells would result in the elevation of DG and InsP by stimulating PtdIns hydrolysis. However there is no evidence to date, in any eukaryotic system, that activation of PKC by addition of PMA or DG analogues actually stimulates inositol lipid hydrolysis. In contrast there is evidence from a number of sources to suggest that

addition of PMA or DG analogues to cells does result in an increase in cellular DG content (Takuwa *et al.*, 1987b; Agwu *et al.*, 1989; Huang & Cabot, 1990a). To address this anomaly the effect of PMA upon inositol lipid hydrolysis required thorough characterization.

The effect of PMA upon bombesin-stimulated $InsP_t$ accumulation in Swiss 3T3 cells was assessed using accumulation of $[^3H]InsP_t$ in the presence of 10mM LiCl as an assay of receptor-mediated inositol lipid hydrolysis. Pre-treating Swiss 3T3 cells with 400nM PMA for 15 minutes inhibited bombesin-stimulated $[^3H]InsP_t$ accumulation by approximately 50% (Figure 3.6a) and shifted both the dose-curve and the EC_{50} to the right.

These results confirm those of Brown *et al.* (1987) who showed that acute pre-treatment of Swiss 3T3 cells with the C-kinase activating phorbol ester PMA results in marked inhibition of subsequent bombesin-stimulated inositol phosphate accumulation. The shift in EC_{50} for bombesin in the PMA-treated cells is consistent with the coupling between the activated receptor and PIC being impaired in some way as described by Brown *et al.* (1987) and Plevin *et al.* (1990). Indeed, Plevin *et al.* (1990) in studying the role of guanine nucleotides in the regulation of bombesin-stimulated inositol phosphate formation in electro-permeabilized Swiss 3T3 cells have shown that PMA will inhibit bombesin-stimulated $[^3H]InsP_t$ accumulation and the synergy between bombesin and GTPγS but not the response to GTPγS alone. This suggests that PMA in some way impairs the linkage between the bombesin receptor and the G-protein, G_p. There seems to be no consensus as to where PMA exerts its effect. In DDT_1MF_2 smooth-muscle cells PMA-stimulated phosphorylation of α_1 adrenoceptors decreases agonist-receptor binding (Leeb-Lundberg *et al.*, 1985), though this is clearly not the case for the bombesin receptor in Swiss 3T3 cells (Brown*et al.*, 1987). In HL60 cells PMA inhibits both GTPγS- and Ca^{2+}-stimulated inositol phosphate accumulation suggesting that both coupling between the G-protein and PIC aswell as the PIC itself may be impaired in a PMA-dependent manner (Geny *et al.*, 1989).

The effect of PMA upon bombesin-stimulated $[^3H]InsP_t$ accumulation was

time-dependent. Figure 3.7 shows that there was a 30 sec to 1 min lag before the onset of PMA-mediated inhibition of bombesin-stimulated [^{3}H]InsP$_t$ accumulation. This lag may represent the time taken for the phorbol ester to cross the plasma membrane and enter its receptor site.

It is well known that addition of PMA to Swiss 3T3 cells results in activation of PKC as assessed by phosphorylation of a candidate marker substrate, an 80 kDa acidic phoshoprotein (Eruslalimsky *et al.*, 1988). That this is due to activation of PKC is supported by the ability of cell permeant DG analogues and growth factors which stimulate inositol lipid hydrolysis to mimic the effect of PMA (Erusalimsky *et al.*, 1988; Kazlauskas & Cooper, 1988). Phosphorylation of the 80 kDa protein is lost in cells which have been pre-treated with high doses of phorbol ester to down regulate PKC.

That the effect of PMA upon bombesin-stimulated [^{3}H]InsP$_t$ formation described in Fig 3.6a was mediated by protein kinase C was supported by a number of lines of evidence. First, the ability of PMA to inhibit bombesin-stimulated [^{3}H]InsP$_t$ accumulation was not shared by β-phorbol (Fig 3.8d), a phorbol ester which is not able to activate PKC (Castagna *et al.*, 1982). Second, the IC$_{50}$ for PMA-mediated inhibition of bombesin-stimulated [^{3}H]InsP$_t$ accumulation (approximately 5 nM) was essentially the same as that value previously reported by Brown *et al.* (1987) and is the same as the EC$_{50}$ for PMA-stimulated PKC activity (Castagna *et al.*, 1982). The anomaly between the IC$_{50}$ reported here and that described by Plevin *et al.* (1990) of 50-100nM PMA is probably due to the latter study being in cells which were electro-permeabilized after treatment with PMA. Such a treatment would result in loss of PMA and perhaps other cellular components required for a phosphorylation event from the cytosol, or might activate phosphatases, resulting in a shift in effective dose of the phorbol ester.

Treatment of Swiss 3T3 cells for 48 hours with 400nM PMA down-regulates protein kinase C (Collins & Rozengurt, 1984; Rodriguez-Pena & Rozengurt, 1984; Brown *et al.*, 1990). In this laboratory it has been shown that such a treatment results in the loss of [^{3}H]PDBu binding to Swiss 3T3 cells (S. Currie & M.J.O. Wakelam, personal communication). In Swiss 3T3 cells pre-

treated with 400nM PMA for 48 hours the ability of bombesin to stimulate the accumulation of [^{3}H]InsP$_t$ was reproducibly enhanced as compared to the control cells treated with β-phorbol (Fig 3.9), consistent with the removal of a limiting component, presumably PKC. Furthermore, in the PKC down-regulated cells acute pre-treatment with PMA was no longer effective at inhibiting bombesin stimulated [^{3}H]InsP$_t$ accumulation (Fig 3.10) supporting the proposal that the inhibitory effects of PMA were exerted through protein kinase C.

Finally, addition of PMA to quiescent, [^{3}H]inositol labelled Swiss 3T3 cells did not result in any statistically significant accumulation of [^{3}H]InsP$_t$ over 20 minutes in the presence of 10mM LiCl (Fig 3.9) under the same conditions in which bombesin elicited a near 10-fold response. These results clearly suggest that activation of protein kinase C, by addition of PMA or by the generation of DG from PtdIns(4,5)P$_2$ hydrolysis, does not stimulate inositol lipid hydrolysis but may actually serve to limit bombesin-stimulated PtdIns(4,5)P$_2$ hydrolysis as suggested by Brown *et al.* (1987).

Such evidence clearly argues against the original prediction of Griendling *et al.* (1986), that PKC acti vates PtdIns hydrolysis which is responsible for sustained elevation of DG, being pertinent to Swiss 3T3 cells. Nevertheless, in Swiss 3T3 cells (Takuwa *et al.*, 1987b), as well as a number of other cell types, administration of PMA is clearly able to increase the cellular DG content. Since this is accompanied by the inhibition of inositol lipid hydrolysis it strongly suggests that PMA-stimulated DG formation must be derived from a source other than inositol lipids.

3.3.3 Bombesin, [Arg^8]Vp and PGF$_{2\alpha}$ stimulate inositol lipid hydrolysis by a similar mechanism.

The neuropeptide [Arg^8]vasopressin ([Arg^8]Vp) and the eicosanoid prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) are also potent co-mitogens for the Swiss 3T3 cell line (Corps *et al.*, 1985; MacPhee *et al.*, 1984). Both mitogens stimulate the

hydrolysis of PtdIns(4,5)P_2 and the rapid generation of Ins(1,4,5)P_3 (Nanberg & Rozengurt, 1988; Black & Wakelam, 1990) and these responses are modulated by guanine nucleotides and their analogues (Erusalimsky & Rozengurt, 1989; Plevin *et al.*, 1990) in much the same way as the bombesin response. Because of these similarities experiments were performed to compare the effects of PMA treatment upon [Arg^8]Vp- and $PGF_{2\alpha}$-stimulated [^{3}H]InsP_t accumulation.

Figure 3.6 (b & c) showed that both agents did indeed stimulate the accumulation of [^{3}H]InsP_t with EC_{50} values which agreed well with their potency in stimulating [^{3}H]thymidine incorporation as previously cited (Corps *et al.*, 1985). Furthermore, in the case of [Arg^8]Vp, pre-treatment of cells with 400nM PMA inhibited [^{3}H]InsP_t accumulation and shifted the EC_{50} to the right, as was the case for bombesin, suggesting that PMA impaired the coupling between the V_1 vasopressin receptor and PIC. Pre-treatment of Swiss 3T3 cells with increasing doses of PMA revealed that the inhibitory effect of PMA was dose-dependent (Figure 3.8b & c) with maximal inhibition occurring at 30-100 nM PMA and IC_{50} values of the same order as that for bombesin (2-6 nM).

When Swiss 3T3 cells were treated for 48 hours with 400nM PMA to down-regulate PKC, the ability of both [Arg^8]Vp and $PGF_{2\alpha}$ to stimulate [^{3}H]InsP_t accumulation was enhanced (Fig 3.9). Furthermore, under these conditions acute pre-treatment with PMA no longer inhibited the responses to either mitogen (Fig 3.10). These results are similar to those for bombesin and the similarity in IC_{50} values for PMA-mediated inhibition of mitogen-stimulated [^{3}H]InsP_t accumulation with that for activation of PKC (Castagna *et al.*, 1982) suggests a common mechanism of action which probably involves PKC.

The degree of enhancement of InsP_t accumulation in down-regulated cells was different for each of the three agonists. The greatest enhancement was always observed with $PGF_{2\alpha}$ (2.5- 3.2-fold) > [Arg^8]Vp (2.2- 2.5-fold) > bombesin (1.2- 1.4-fold). A similar trend has recently been reported by Brown *et al.* (1990) and may represent subtle differences in the role of PKC in regulating coupling of the receptors to PIC or different sites for intervention between receptor, G_p and effector by PKC. However, a simpler interpretation may lie in the fact that, in

control cells, $PGF_{2\alpha}$ gives the smallest accumulation of [^{3}H]$InsP_t$ and, consequently, the smallest depletion of [^{3}H]inositol lipids over a 20 minute stimulation in the presence of 10mM LiCl (Table 3.1). Removal of a C-kinase-mediated inhibition by down-regulation of PKC would then allow large potentiation of the response. Such a mechanism would apply for [Arg^8]Vp and bombesin, but since they give successively greater responses in control cells and much greater depletion of [^{3}H]inositol lipids (Table 3.1), potentiation of [^{3}H]$InsP_t$ accumulation in down-regulated cells may be limited by the amount of [^{3}H]inositol lipids available as substrate for PIC.

There was a clear rank order in the magnitude of $InsP_t$ accumulation in response to the three mitogens; bombesin > [Arg^8]Vp > $PGF_{2\alpha}$. This agrees broadly with the rank order of bombesin > [Arg^8]Vp ≥ $PGF_{2\alpha}$ for the magnitude of mitogen-stimulated [^{3}H]thymidine incorporation seen in Swiss 3T3 cells (Corps *et al*, 1985). Whilst this proportionality between mitogenic potency and inositol lipid hydrolysis suggests an important role for the latter in mitogenic signal transduction, this is clearly not the case in all systems. For example, Black & Wakelam (1990) reported that $PGF_{2\alpha}$ stimulates similar inositol phosphate generation in two distinct clones of NIH3T3 cells but is only mitogenic in one of the cell lines. Furthermore a number of growth factors, for example EGF in Swiss 3T3 cells (Hesketh *et al.*, 1988) and CSF-1 in human monocytes (Imamura *et al.*, 1990), are mitogens despite being unable to stimulate measureable inositol lipid hydrolysis. Clearly, whilst inositol lipid hydrolysis may be an important early event in response to some mitogens, it is not in itself sufficient, or necessary for, mitogenesis in response to all growth factors (Whitman & Cantley, 1988).

Thus, the three mitogens, bombesin, [Arg^8]Vp and $PGF_{2\alpha}$, exhibit marked similarities in the mechanism by which they stimulate hydrolysis of inositol lipids. The generation of $Ins(1,4,5)P_3$ was rapid in onset and essentially transient (Fig 3.4; Nanberg & Rozengurt, 1988; Black & Wakelam, 1990), the responses are modulated by guanine nucleotides in a manner consistent with a general model for receptor - G-protein interactions (Plevin *et al*, 1990; Erusalimsky & Rozengurt,

1989) and they display similar sensitivity to acute pre-treament with phorbol esters (Fig 3.8, 3.9 & 3.10), a response which is most likely to be mediated by protein kinase C. These charcteristics are typical of agonists which couple to inositol lipid hydrolysis in a classical RGE type manner.

Chapter 4.

Multiple sources of *sn*-1,2-diradylglycerol in bombesin-stimulated Swiss 3T3 cells; evidence for activation of phospholipase D.

4.1 Introduction.

In Chapter 3, the ability of bombesin and other mitogenic agents to stimulate inositol lipid hydrolysis in Swiss 3T3 cells was characterised in terms of kinetics, dose-dependency and sensitivity to acute- and long term- phorbol ester pre-treatment. In this chapter experiments are described which investigate the ability of bombesin to stimulate increases in the cellular content of *sn*-1,2-diglyceride and these changes are compared with the generation of Ins(1,4,5)P_3 mass. Furthermore, evidence is presented to suggest that bombesin and phorbol esters may contribute to increases in cellular diglyceride content by hydrolysing a non-inositide phospholipid by a phospholipase D catalysed reaction.

Mitogen-stimulated increases in *sn*-1,2-DG have been less extensively studied in Swiss 3T3 cells than increases in inositol phosphates, with many reports making no attempt to assess stimulated increases in DG (Brown *et al.*, 1984; Heslop *et al.*, 1986). Historically, this probably represents the inadequacies of the assay methods available. Until recently the commonest method employed was labelling of cellular glycerophospholipids with either [^{3}H]glycerol or a [^{3}H]fatty acid to isotopic equilibrium followed by monitoring changes in the radioactivity associated with 1,2-DG upon stimulation; radiolabelled diglyceride was usually resolved by thin layer chromatography. The major criticism of these methods is that they cannot distinguish between *sn*-1,2-DG and *sn*-2,3-DG, an important consideration since it is only the *sn*-1,2-forms of DG that are able to activate protein kinase C (Rando & Young, 1984; Nomura *et al.*, 1986). The use of a radioactive fatty acid to label cellular phospholipids provides both advantages and disadvantages. The major advantage of such an approach is that, to some extent, it allows one to investigate which phospholipid species may be contributing to increases in DG since some phospholipids are enriched in particular fatty acids. Thus the inositol lipids, particularly PtdIns(4)P and PtdIns(4,5)P_2, are characterised by generally possessing stearic acid at position 1 and arachidonic acid at position 2 (reviewed by Downes & Michell, 1985; Pessin & Raben, 1989). In contrast, PtdCho, whilst containing a considerable portion of arachidonate also contains large

amounts of oleic acid, linoleic acid, palmitic, stearic or myristic acid. However, this potential specificity of labelling can also be a disadvantage since it excludes those species of diglyceride which do not contain the particular fatty acid used in the labelling, thereby leading to an under-estimation of both basal and stimulated amounts of DG.

The development of a radioenzymatic assay for total *sn*-1,2-DG mass by Preiss *et al.* (1986) has allowed for much more accurate determinations of cellular diglyceride content. The assay employs DG kinase (from an over producing strain of *E.coli*) to phosphorylate the DG in cellular lipid samples in the presence of [γ-^{32}P]ATP. The conversion of DG to [^{32}P]PtdOH can be monitored by constructing a standard curve, allowing quantitation of cellular DG mass. This method has been applied successfully by Wright *et al.* (1988) to show that α-thrombin stimulates a biphasic increase in *sn*-1,2-DG in IIC9 fibroblasts. A criticism of this method is that it gives no indication of the fatty acyl structure of the DG; however it is absolutely specific for *sn*-1,2-DGs since they are the only diglyceride substrates for DG kinase (Bishop *et al.*, 1986).

The use of chemical derivitizations for *sn*-1,2-diglycerides has meant that the mass amounts of individual species of DG can now be analysed with great accuracy. DG derivatives can be separated by h.p.l.c. or g.l.c. whilst 'on-line' mass spectrometry allows mass analysis of individual diglyceride species (e.g., Pessin & Raben, 1989). These techniques have been used to great effect in showing that agonist-stimulated increases in DG are derived largely from non-inositol lipid sources from 30 seconds onwards in α-thrombin-stimulated IIC9 fibroblasts (Pessin & Raben, 1989); in fact, the profile of different DG species at later timepoints was consistent with them being derived from PtdCho. A role for the hydrolysis of non-inositide phospholipids in generating DG in stimulated cells is now increasingly accepted (reviewed by Billah & Anthes, 1990).

The major question which must be addressed when considering the role of other phospholipids in generating DG is the route of hydrolysis since both PLC- and PLD-catalysed pathways have been variously proposed (reviewed by Billah & Anthes, 1990). PLC-catalysed hydrolysis of PtdCho yields DG and

phosphocholine (ChoP) whereas the activation of PLD yields phosphatidic acid (PtdOH) and free choline (Cho); PtdOH may then be dephosphorylated by phosphatidate phosphohydrolase (PPH) to yield DG. This latter route of DG formation, referred to as a coupled PLD/PPH pathway, is certainly more complex since it involves what is effectively the reversal of the DG kinase reaction by PtdOH phosphohydrolase. Using two definitive assays in cells labelled with 1-*O*-[^{3}H]alkyl-2-lyso-glycero-3-[^{32}P]phosphocholine Pai *et al.* (1988a & b) and Billah *et al.* (1989a &b) have shown quite clearly that in HL-60 granulocytes and human neutrophils, both phorbol esters and the chemotactic peptide fMet-Leu-Phe rapidly activate a PLD/PPH pathway which serves to elevate DG levels. The formation of ^{32}P-labelled PtdOH under these conditions rather than ^{32}P-labelled ChoP allows definitive identification of PLD rather than PLC activity; ^{32}P labelled PtdOH cannot have been formed from DG since the cellular ATP pool is not labelled in this system. The formation of phosphatidylalcohols by the transferase reaction also serves as a useful marker for PLD activity in whole cells.

To date, the majority of reports describing generation of DG from a non-inositol lipid source have reported activation of a PLD/PPH pathway though a limited number of reports have suggested the involvement of a PLC (reviewed by Billah & Anthes, 1990). This is an important distinction since the former pathway produces another phospholipid with a potential messenger function, PtdOH (Putney *et al.*, 1980; Moolenaar *et al.*, 1986).

In this chapter, evidence is presented to show that bombesin stimulates a biphasic increase in DG mass. The first phase correlates with Ins(1,4,5)P_3 generation in terms of kinetics whilst the second phase is sustained and divorced from a further increase in Ins(1,4,5)P_3 mass and can be mimicked by addition of PKC-activating phorbol esters. The sustained formation of DG is associated with the activation of PLD which may, in part, be mediated by the activation of PKC. The results are discussed in terms of a possible functional link between PtdIns(4,5)P_2 hydrolysis and PLD.

4.2 Results.

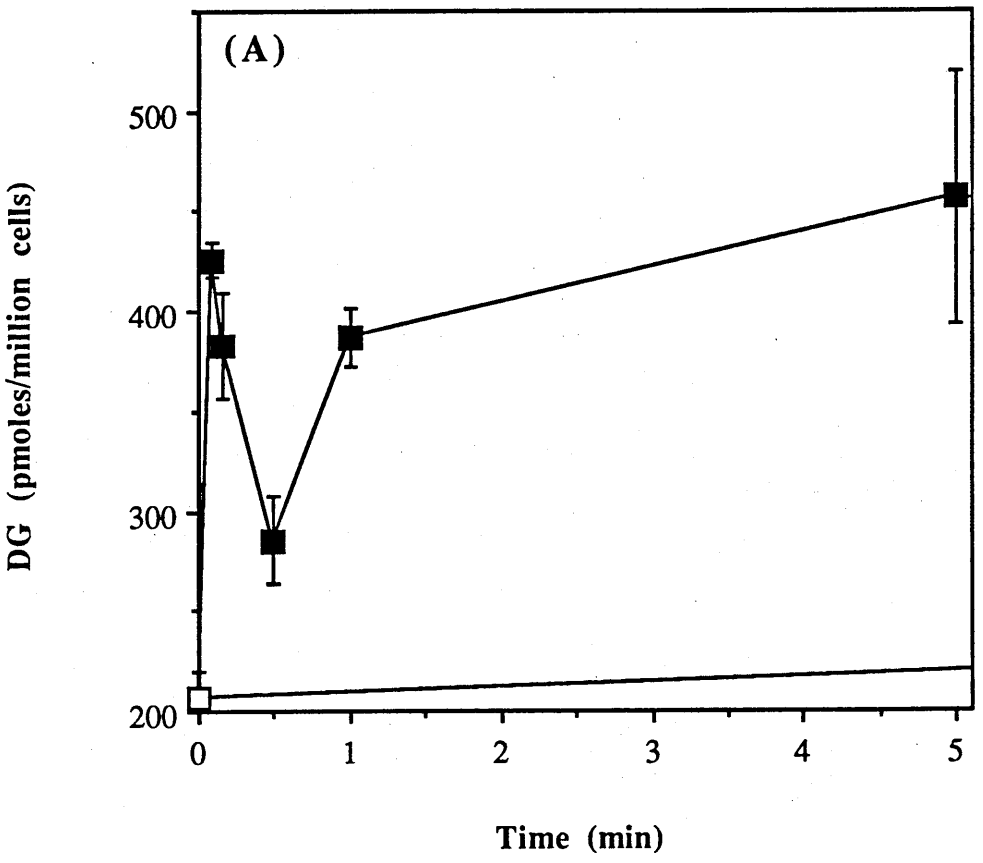
4.2.1 Bombesin- and phorbol ester-stimulated DG formation in Swiss 3T3 cells.

Preliminary experiments examined the changes in DG mass upon stimulation of quiescent Swiss 3T3 cells with a maximal dose (617nM) of bombesin. Fig. 4.1a shows the timecourse for changes in DG mass in response to bombesin. DG levels rose rapidly upon stimulation to give a pronounced peak after 5 sec before declining towards control at 30 sec. However, in contrast to $Ins(1,4,5)P_3$ (Fig. 3.4), DG levels did not return to basal but rose again in a second phase from between 30 sec or 1 min onwards. This second phase was maintained for up to 30 min (Fig. 4.1b) and, in some experiments, DG mass remained significantly above control at 60 min (data not shown). This sustained increase in diglyceride is in agreement with the work of Takuwa *et al.* (1989) who reported that bombesin-stimulated DG elevation persisted for up to 4 hours in Swiss 3T3 cells. In some experiments the initial peak of DG formation was seen as a less pronounced shoulder on a greater sustained second phase of the response (for example see Fig. 4.2). The time course shown in Fig. 4.2 is actually from the same experimental samples as Fig. 3.2, allowing direct comparison of $[^3H]InsP_3$ and DG mass from the same experiment.

The basal level of DG in unstimulated Swiss 3T3 cells was 210 ± 61 pmoles/10^6 cells (mean ± S.D. of 21 determinations conducted throughout the course of these studies). This is similar to that recently reported for Swiss 3T3 cells by Staddon *et al.* (1990). A comparison of the net increase in $Ins(1,4,5)P_3$ and DG mass indicated that after 5 sec stimulation with bombesin the net increase in DG mass (179 ± 64 pmoles/10^6 cells, n=6 determinations) exceeded that of $Ins(1,4,5)P_3$ (25.2 ± 3.8 pmoles/10^6 cells) by 7.2 ± 2.6 fold. The net increase in DG at 5 or 15 min (the second phase) was 606 ± 387 pmoles/10^6 cells (mean ± S.D., n=6 experiments in triplicate). Because of the variation in basal DG level during the course of these studies, fold increases were calculated against the corresponding experimental basal and then averaged rather than calculating the

Figure 4.1 (a & b) Time course of increases in *sn*-1,2-DG mass in bombesin-stimulated Swiss 3T3 cells.

Confluent monolayers of quiescent Swiss 3T3 cells were incubated at 37°C for 60 min in HBG before replacing with HBG containing 617nM bombesin (■) or HBG alone (❑) for the times indicated. Incubations were terminated by aspiration and the addition of ice-cold methanol and, after harvesting, the cell debris was extracted in chloroform by the method described in Chapter 2. An aliquot of the chloroform phase was dried down, solubilised in a cardiolipin/*n*-octyl-β-D-glucopyranoside mixed micelle detergent preparation and assayed for *sn*-1,2-DG mass as described in the Methods section. Samples were taken for analysis of total lipid phosphorous and for cell number determination. The results are expressed as mass of DG (pmoles of DG/10^6 cells, mean ± S.D., n= 3) from a single, representative experiment. Fig. 4.1 (a & b) show the time course up to 5 and 30 min respectively.


(A)
500
400
300
200
0
1
2
3
4
5
DG (pmoles/million cells)
Time (min)

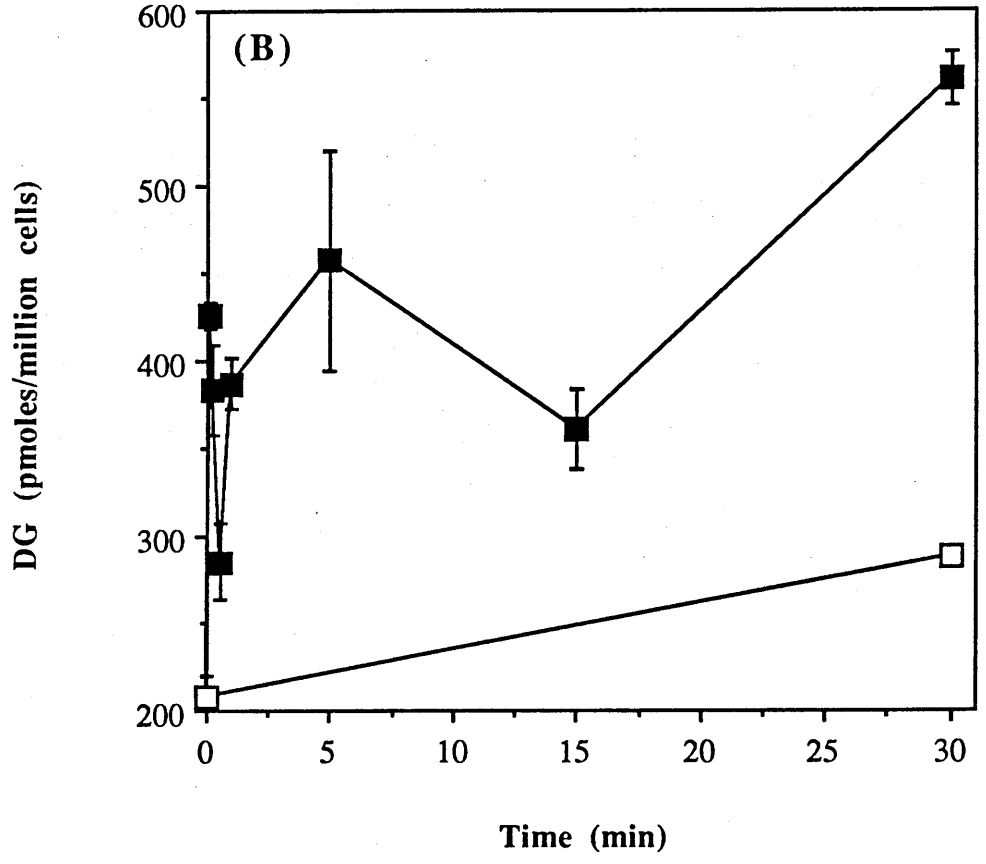
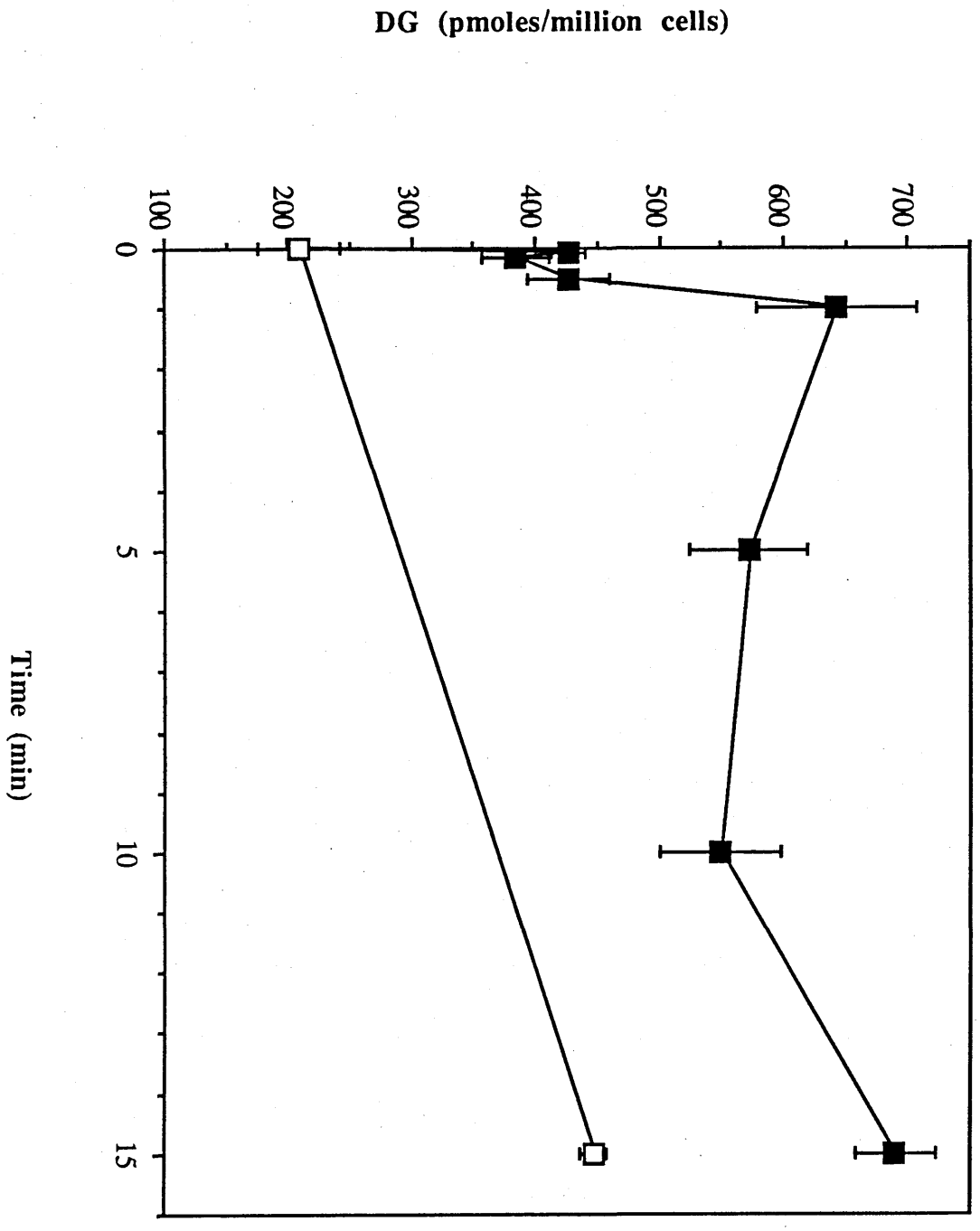

(B)
600
500
400
300
200
0
5
10
15
20
25
30
DG (pmoles/million cells)
Time (min)

Figure 4.2 Time course of increases in *sn*-1,2-DG mass in bombesin-stimulated Swiss 3T3 cells.

Confluent monolayers of quiescent Swiss 3T3 cells were washed in HBG at 37°C for 60 min before replacing with HBG containing 617nM bombesin (■) or HBG alone (❑) for the indicated times. Incubations were terminated by aspiration and addition of ice-cold methanol and, after harvesting, the cell debris was extracted in chloroform as described in the methods section. An aliquot of the chloroform phase was dried down, solubilised in a cardiolipin/*n*-octyl-β-D-glucopyranoside mixed micelle detergent preparation and assayed for *sn*-1,2-DG mass as described in the Methods section. Samples were taken for analysis of total lipid phosphorous and for cell number determination. The results are expressed as mass of DG (pmoles of DG/10^6 cells, mean ± S.D., n= 3) from a single, representative experiment.


DG (pmoles/million cells)
100
200
300
400
500
600
700
0
5
10
15
Time (min)

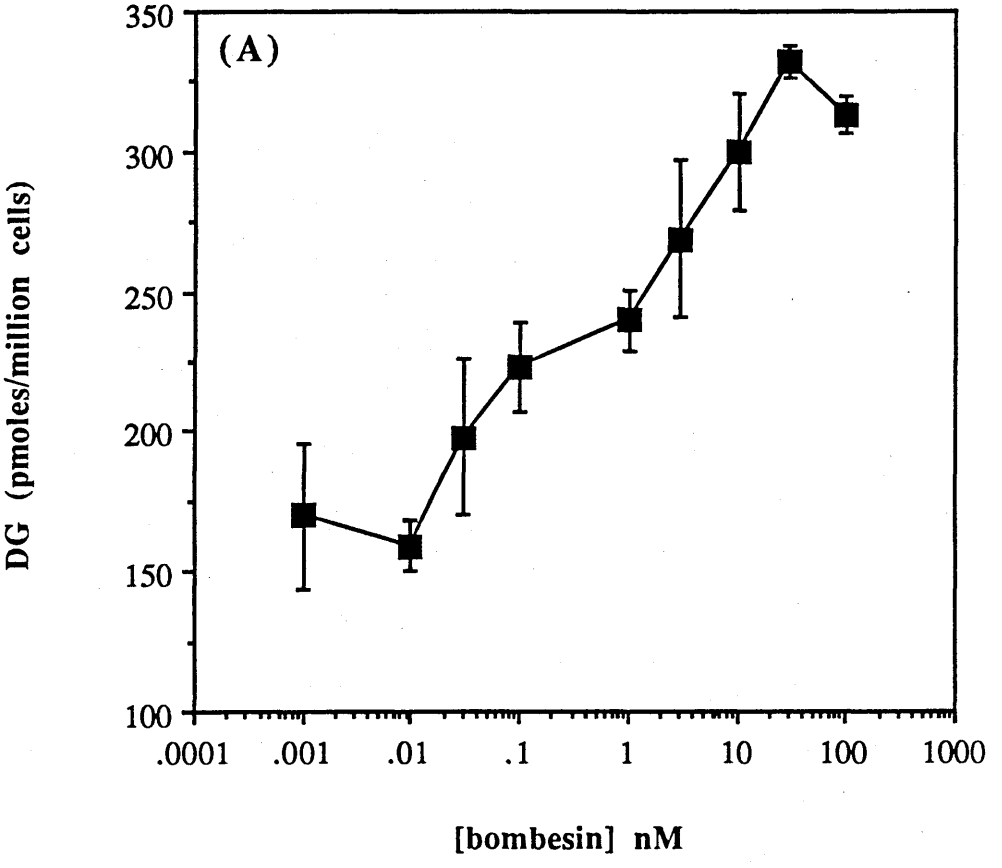
increase over the mean basal. The fold increases in DG over the corresponding experimental control in response to bombesin were 1.98 ± 0.36 at 5 sec (the first phase) and 3.07 ± 0.92 at 5 or 15 min (the second phase). A number of recent studies (Takuwa *et al.*, 1987 a & b; Wright *et al.*, 1988; 1990) have expressed DG mass as nmoles/100nmoles of lipid phosphorous (mol %). To allow comparisons between these studies samples were assayed for total lipid phosphorous as described in the Methods section. Using two different assay methods during the course of these studies there were found to be 70 ± 19 nmoles of lipid phosphorous/10^6 Swiss 3T3 cells (mean ± S.D., of three determinations where n=3-9); this value is of a similar order to that described by Wright *et al.* (1988) in IIC9 fibroblasts. In unstimulated cells DG mass levels were 0.3 ± 0.09 mol %. In cells stimulated with bombesin for 5 sec the net increase in DG was equivalent to an increase of 0.25 mol %, whilst the second phase of DG formation was equivalent to a net increase of 0.87 mol %. The precise details of the timecourse varied between experiments; for example, in some cases, the second phase of the response was of equivalent or slightly smaller magnitude than the first (Fig. 4.1b) whereas, in other experiments, the second phase was the greater (e.g., Fig. 4.2). It is thus evident that the accumulation of DG in response to bombesin was sustained at times when the levels of Ins(1,4,5)P_3 had declined to pre-stimulated levels (30 sec, Fig. 3.4).

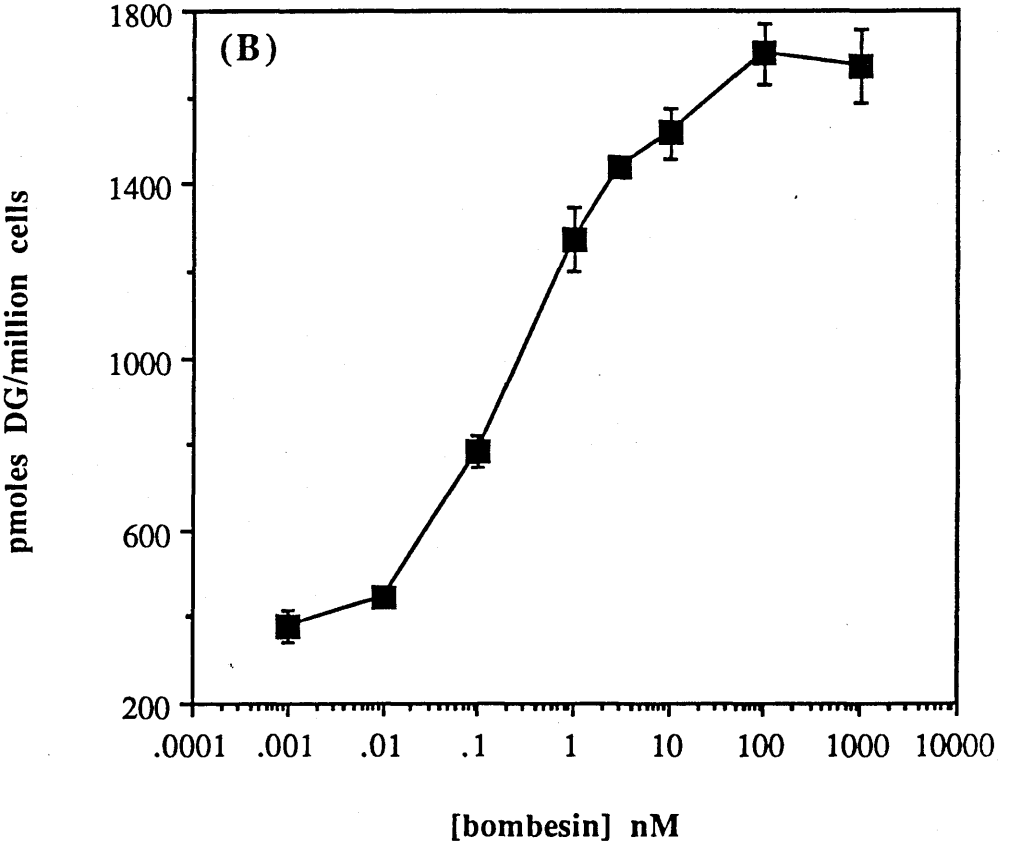
To assess whether both phases of the response were occurring over the same dose range of bombesin and presumably, therefore, being mediated by the activation of a single population of receptors, dose-response experiments were performed at both 5 sec and 5 or 15 min stimulation times. The results in Fig. 4.3 (a & b) show that, at both phases of the response, bombesin stimulated a dose-dependent increase in DG mass with EC_{50} values of 2.04 ± 1.68 nM at 5 sec and 0.42 ± 0.15 nM at 5 or 15 min (mean ± S.D., n=3 experiments in each case). These results are similar to the EC_{50} values for bombesin stimulated Ins(1,4,5)P_3 generation and accumulation of [^{3}H]InsP_t reported in Chapter 3.

Further experiments measuring DG mass examined the effects of adding

Figure 4.3 (a & b) Dose response curves for increases in DG in Swiss 3T3 cells stimulated with bombesin for (a) 5 sec or (b) 15 min.

Confluent monolayers of quiescent Swiss 3T3 cells were washed in DMBH for 60 min at 37^{o}C before replacing with buffer containing the indicated concentration of bombesin for (a) 5 sec or (b) 15 min. Incubations were terminated by aspiration and addition of ice-cold methanol and, after harvesting, the cell debris was extracted in chloroform as described in the methods section. An aliquot of the chloroform phase was dried down, solubilised in either (a) a cardiolipin/*n*-octyl-β-D-glucopyranoside detergent preparation or (b) a Triton X-100/PtdSer detergent mix and assayed for *sn*-1,2-DG mass as described in the Methods section. Samples were taken for analysis of total lipid-containing phosphorous and for cell number determination. The results are expressed as mass of DG (pmoles of DG/10^{6} cells, mean ± S.D., n= 3) from a single, representative experiment.


(A)
DG (pmoles/million cells)
350
300
250
200
150
100
.0001
.001
.01
.1
1
10
100
1000
[bombesin] nM

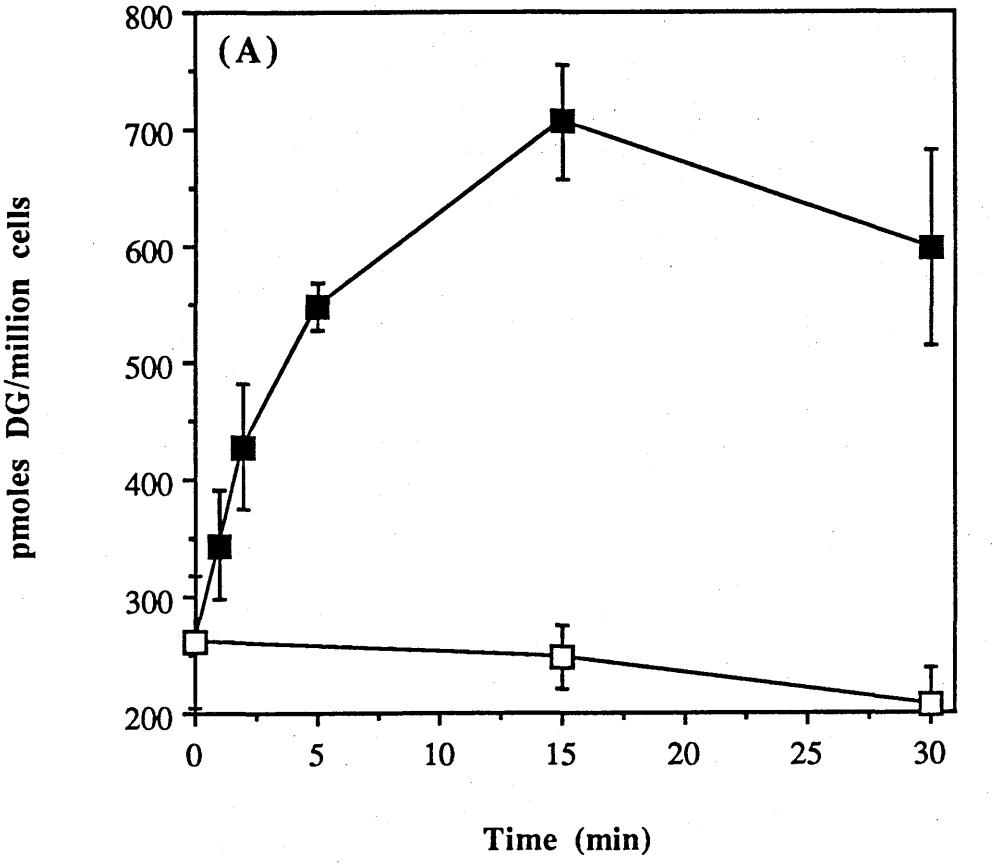

(B)
pmoles DG/million cells
1800
1400
1000
600
200
.0001
.001
.01
.1
1
10
100
1000
10000
[bombesin] nM

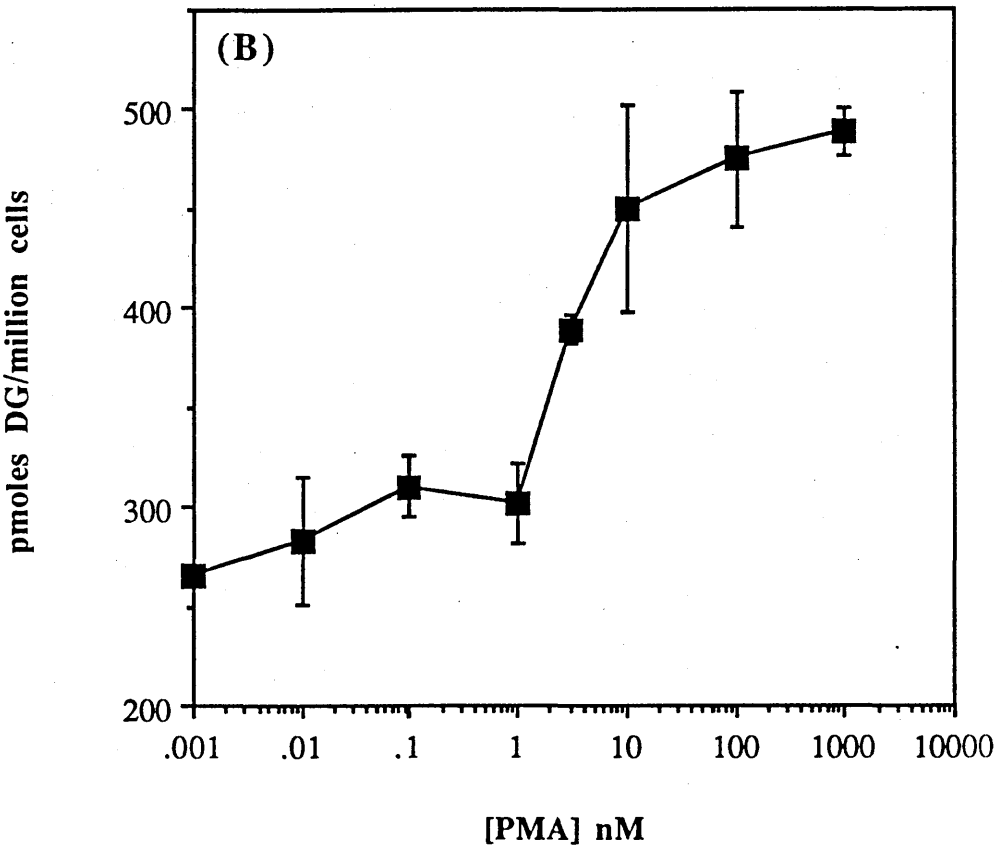
phorbol esters to quiescent Swiss 3T3 cells since their reported effects upon inositol lipid hydrolysis and DG generation seemed, at first sight, anomalous. It is well characterised in a variety of systems, including Swiss 3T3 cells, that phorbol esters do not stimulate inositol lipid hydrolysis but rather inhibit inositol phosphate accumulation stimulated by subsequent agonist challenge. (see Brown *et al.*, 1987 and Chapter 3 of this thesis). Despite this, a number of groups have reported that addition of phorbol esters to cells results in an increase in DG content. In the Swiss 3T3 cell, Takuwa *et al.* (1987b) showed that PMA stimulated an increase in DG mass after a lag of about 2 min. This response was sustained for up to 30 min, and occured at doses at which PMA is mitogenic, but was not accompanied by the accumulation of inositol phosphates. To confirm and advance these observations, the effect of PMA upon cellular DG content was investigated using the DG mass assay and in cells labelled with [^{3}H]palmitic acid.

First, addition of PMA to quiescent Swiss 3T3 cells resulted in a time dependent increase in DG mass (Fig 4.4a) which was apparent, though not significant, after 1 min. DG mass was significantly elevated after 2 min ($p= 0.003$); the rate of accumulation from 5 min onwards was somewhat reduced but the elevation of DG was sustained above control for up to 30 min (Fig. 4.4a) and 60 min (data not shown). PMA stimulated increases in DG mass were dose-dependent (Fig. 4.4b); the concentration of PMA giving half-maximal accumulation of DG mass was 2.6 ± 0.59 nM (mean ± S.D. of 2 experiments). The net increase in DG stimulated by PMA was 435 ± 113 pmoles/10^6 cells (mean ± S.D., n=6 experiments in triplicate) equivalent to an increase of 0.62 mol % and representing a fold increase over the corresponding basal of 2.78 ± 0.65. The lag time of approximately 1 min before the onset of the response was probably due to the time taken for the phorbol ester to cross the plasma membrane and enter its receptor site. Thus, the time course of PMA-stimulated increase in DG was similar to the second phase of bombesin-stimulated DG accumulation. Furthermore, both the PMA response and the second phase of the bombesin response were divorced from stimulated inositol lipid hydrolysis and

Figure 4.4 (a & b) Increases in *sn*-1,2-DG mass in PMA-stimulated Swiss 3T3 cells.

Confluent monolayers of quiescent Swiss 3T3 cells were incubated at 37°C for 60 min in DMBH before replacing with DMBH containing 100nM PMA (■) or β-phorbol (❑) for (a) the times indicated; dose response curves (b) were performed at the concentration indicated for 10 min. Incubations were terminated by aspiration of the medium and addition of ice-cold methanol. After harvesting, the cell debris was extracted in chloroform by the method described in Chapter 2. An aliquot of the chloroform phase was dried down, solubilised in a Triton X-100/PtdSer mixed micelle detergent preparation and assayed for *sn*-1,2-DG mass as described in the Methods section. Samples were taken for analysis of total lipid-containing phosphorous and for cell number determination. The results are expressed as mass of DG (pmoles of DG/10^6 cells, mean ± S.D., n= 3) from a single, representative experiment.


(A)
800
700
600
500
400
300
200
pmoles DG/million cells
0
5
10
15
20
25
30
Time (min)


(B)
500
400
300
200
pmoles DG/million cells
.001
.01
.1
1
10
100
1000
10000
[PMA] nM

further net Ins(1,4,5)P_3 generation respectively. This suggested that PMA might mimic the second phase of bombesin-stimulated DG accumulation.

Since PMA can stimulate accumulation of DG without increasing inositol lipid hydrolysis, the DG may be derived by hydrolysis of an alternative phospholipid or increased *de novo* synthesis. However, since the effects of PMA were apparent within 1 or 2 min, it seemed unlikely to be due to the latter possibility. In those systems where enhanced *de novo* synthesis is thought to play a role in agonist-induced elevation of DG, the effects are thought to be more important over longer stimulation times e.g., tens of minutes (discussed in section 1.6). For this reason the source of the second phase of DG elevation stimulated in bombesin- and PMA-stimulated Swiss 3T3 cells was investigated.

In recent years it has become apparent that, in a variety of stimulus-response sytems, both PtdCho (reviewed by Exton, 1990; Billah & Anthes, 1990) and, to a lesser extent, PtdEtn (Kiss & Anderson, 1989) are significantly hydrolysed within minutes of addition of phorbol esters and receptor agonists. To investigate the possibility of DG being derived from one of these sources, an isotopic labelling protocol was chosen which would preferentially label all the major phospholipid classes other than the inositol lipids. Since the inositol lipids are particularly characterised by a 1-stearoyl-2-archidonoyl acyl composition (reviewed by Downes & Michell, 1985; Pessin & Raben, 1989), [^{3}H]palmitic acid was chosen as a means of selectively labelling the diglyceride moiety of non-inositol-containing phospholipids.

Fig. 4.5 shows the timecourse for incorporation of [^{3}H]palmitic acid into the major phospholipid species in Swiss 3T3 cells. The results indicate that equilibrium labelling of all the major phospholipids was achieved over a 49 hour period. The rapid incorporation of [^{3}H]palmitate into PtdCho, PtdOH and PtdSer suggested that these lipids were rapidly turning over in control cells. Of the other major phospholipids, PtdEtn incorporated label more slowly whilst PtdIns incorporated very little at 4 hours and showed no significant increase over the remaining labelling period. The amount of [^{3}H]palmitate incorporated into the different phospholipid classes after 49 hours varied quite markedly (Table

Figure 4.5 Time course of incorporation of [^{3}H]palmitic acid into the major phospholipid classes in Swiss 3T3 cells.

Swiss 3T3 cells were plated on to 24 well plates and grown in normal medium for 24 hours until judged to be approximately 80% confluent. The medium was then replaced with DMEM containing 1% calf serum and cells were returned to the incubator. At staggered intervals, medium was replaced with 1ml of DMEM containing 1% calf serum and 2μCi of [^{3}H]palmitic acid so that cells were incubated in the presence of isotope for the times indicated. After 49 hours, medium was aspirated and incubations were terminated by the addition of ice-cold methanol. Chloroform extracts, prepared as described in the Methods section, were loaded on to silica gel 60 t.l.c. plates and developed in the solvent system described in Chapter 2. PtdCho (■), PtdSer (▲), PtdEtn (●), PtdIns (○) and PtdOH (□), identified by their co-migration with authentic standards after visualising with I_2 vapour, were excised from the silica gel and the associated radioactivity determined by scintillation counting. Results are expressed as radioactivity in phospholipids (d.p.m., mean ± S.D., n= 3) from a single experiment.

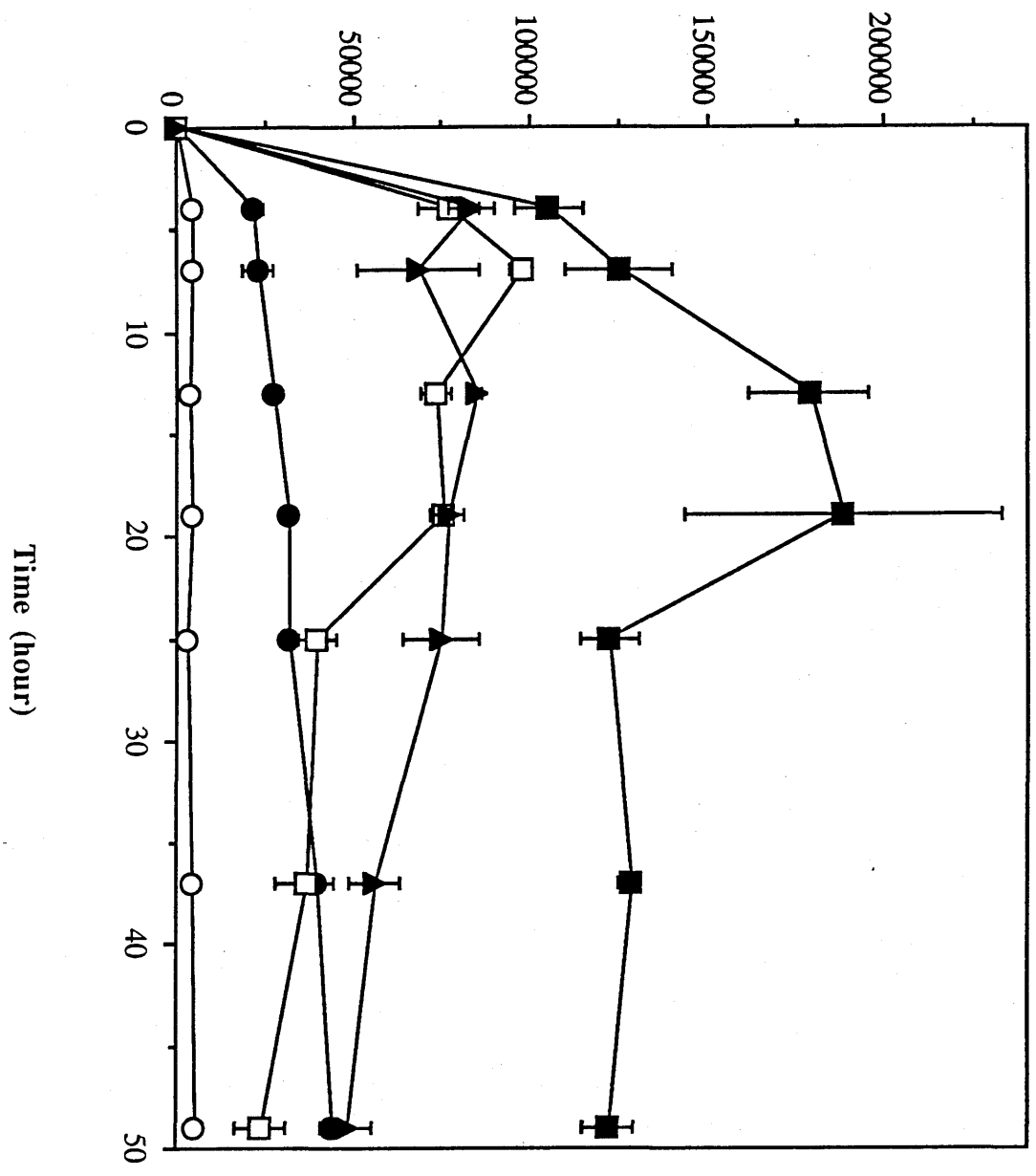

radioactivity in phospholipid (d.p.m.)
0
50000
100000
150000
200000
0
10
20
30
40
50
Time (hour)

Table 4.1 Distribution of [^{3}H]palmitic acid in the major phospholipid classes in Swiss 3T3 cells.

Swiss 3T3 cells were seeded on to 24 well plates and grown in normal medium for 24 hours until judged to be approximately 80% confluent. The medium was then replaced with DMEM containing 1% calf serum and cells were returned to the incubator. At staggered intervals, medium was replaced with 1ml of DMEM containing 1% calf serum and 2μCi of [^{3}H]palmitic acid so that cells were incubated in the presence of isotope for the times indicated. After 49 hours, medium was aspirated and incubations were terminated by the addition of ice-cold methanol. Chloroform extracts, prepared as described in the Methods section, were loaded on to silica gel 60 t.l.c. plates and developed in the solvent system described in Chapter 2. PtdCho, PtdSer, PtdEtn, PtdOh, PtdIns and SphM were identified by their co-migration with authentic standards after visualising with I_2 vapour. The corresponding regions of the silica gel were excised the associated radioactivity determined by scintillation counting. Results are expressed as radioactivity in phospholipids (d.p.m., mean ± S.D., n=3) from a single experiment and as percentage of total radioactivity incorporated into total phospholipids after 49 hours.

Distribution of [^{3}H]palmitic acid in major phospholipid classes.

Lipid.	Radioactivity (d.p.m.)	% of radioactivity in total phospholipids.
PtdCho	122,416 ± 7,256	50.3
PtdSer	48,049 ± 7,144	19.8
PtdSer	43,976 ± 1,807	18.1
PtdOH	23,651 ± 7,132	9.7
PtdIns	5,032 ± 629	2.1
SphM	109,330 ± 13,189	Not applicable.

4.1). Clearly the majority of [^{3}H]palmitate was incorporated into PtdCho (50% of total radioactivity incorporated into total phospholipids) with sphingomyelin, PtdSer and PtdEtn labelled to a lesser extent. Only 2% of the total label incorporated into the major phospholipids was found into PtdIns. Therefore, under these conditions PtdCho and other major phospholipids were labelled preferentially to inositol lipids.

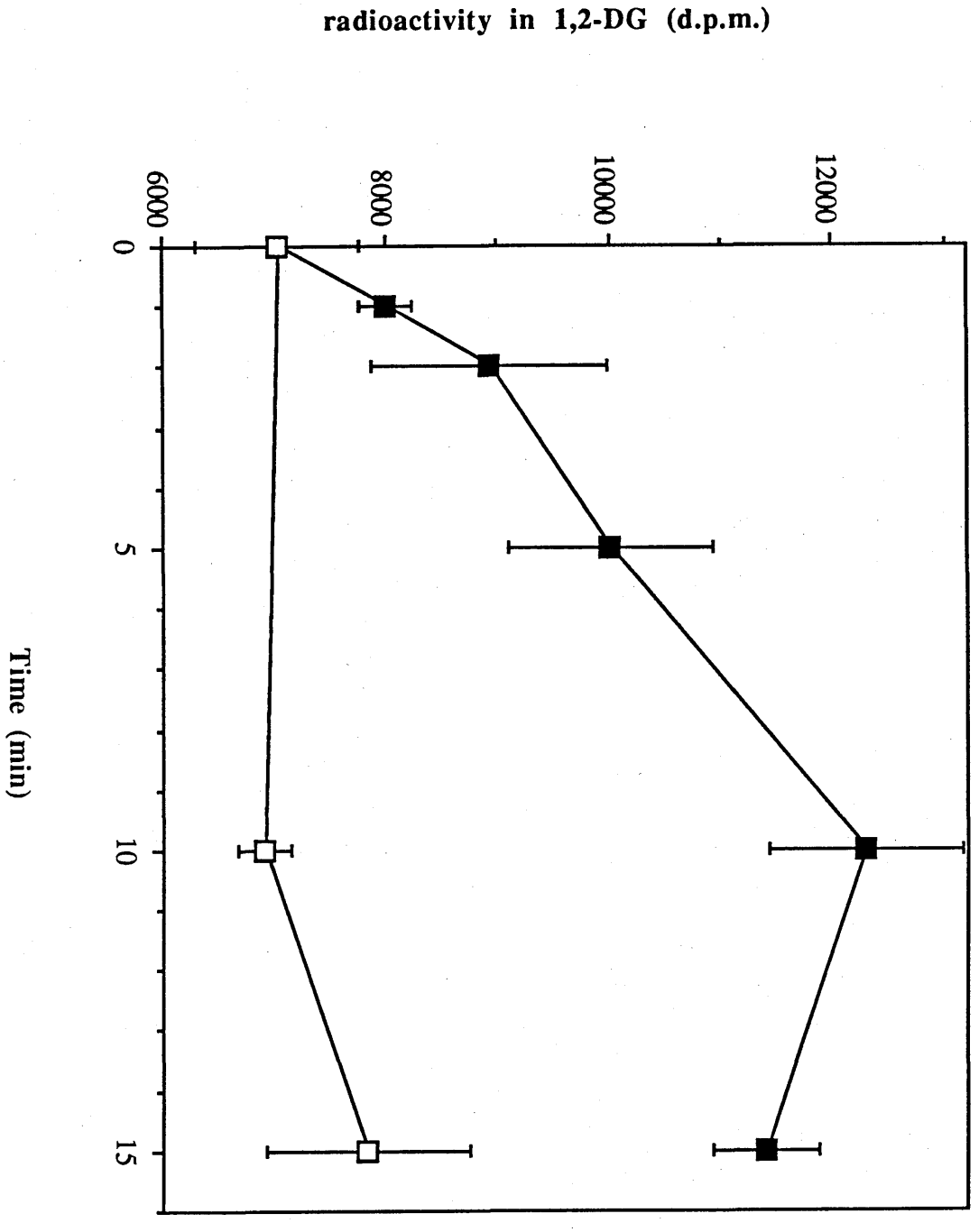
Stimulation of [^{3}H]palmitate labelled Swiss 3T3 cells with 100nM PMA resulted in a time-dependent increase in radioactivity associated with total [^{3}H]1,2-DG. PMA-stimulated increases in [^{3}H]1,2-DG were apparent after 1 min but were never significantly elevated until 2 min ($p=0.009$) (Fig. 4.6). Increases in [^{3}H]1,2-DG exhibited similar kinetics to those for PMA-stimulated increases in DG mass but were of a smaller magnitude. PMA-stimulated increases in [^{3}H]PtdOH were difficult to observe since there was an unexpectedly high amount of radioactivity in PtdOH even in the absence of a stimulus. Since it is known that PtdOH is a minor phospholipid component of Swiss 3T3 cells by mass (Takuwa *et al.*, 1987b), this may suggest that palmitate containing PtdOH makes a significant contribution to the total mass of PtdOH. PMA-stimulated increases in [^{3}H]PtdOH were sometimes seen after 1 min rising to a maximal elevation at 2-5 min, after which the increased level was maintained for up to 15 min (data not shown). However, it was not clear if the basal levels were accurate or elevated due to contamination with other lipid species. Nevertheless , [^{3}H]PtdOH was resolved by three different t.l.c. systems in these experiments.

4.2.2 Bombesin and phorbol ester stimulate phospholipase D transferase activity in [^{3}H]palmitate-labelled Swiss 3T3 cells.

Bombesin- and PMA-stimulated activation of PLD was investigated by assessing the formation of phosphatidylalcohols. This method has the added advantage of acting as a definitive cellular marker for PLD since the

Figure 4.6 Time course of increases in [^{3}H]1,2-DG in PMA-stimulated, [^{3}H]palmitate-labelled Swiss 3T3 cells.

Swiss 3T3 cells, grown on 6 well plates until judged to be 80% confluent, were grown for a further 48 hours in 2 ml of DMEM containing 1% calf serum and a total of 4μCi of [^{3}H]palmitic acid. Monolayer cultures were then washed in DMBH for 60 min at 37^{o}C before incubating in DMBH containing 100nM PMA (■) or β-phorbol (❑) for the times indicated. Incubations were terminated by aspiration of the medium and addition of ice-cold methanol: chloroform extracts were prepared as described in Chapter 2. Samples were resolved on Whatman LK5DF t.l.c. plates using the solvent system described the Methods section. 1,2-DG, resolved from 1,3-DG and identified by its co-migration with an authentic *sn*-1,2-DG standard visualised by I_2 vapour, was excised from the t.l.c. plate in 0.5 cm slices and associated radioactivity determined by scintillation counting. Results are expressed as radioactivity (d.p.m., mean ± S.D., n=3) from a single experiment typical of three.


radioactivity in 1,2-DG (d.p.m.)
6000
8000
10000
12000
0
5
10
15
Time (min)

phosphatidylalcohols are not formed by other pathways in whole cells (Pai *et al.*, 1988a). In contrast, the normal product of PLD, PtdOH, can be formed *de novo*, or by the action of DG kinase and is, therefore, not a definitive marker for PLD activity in whole cells.

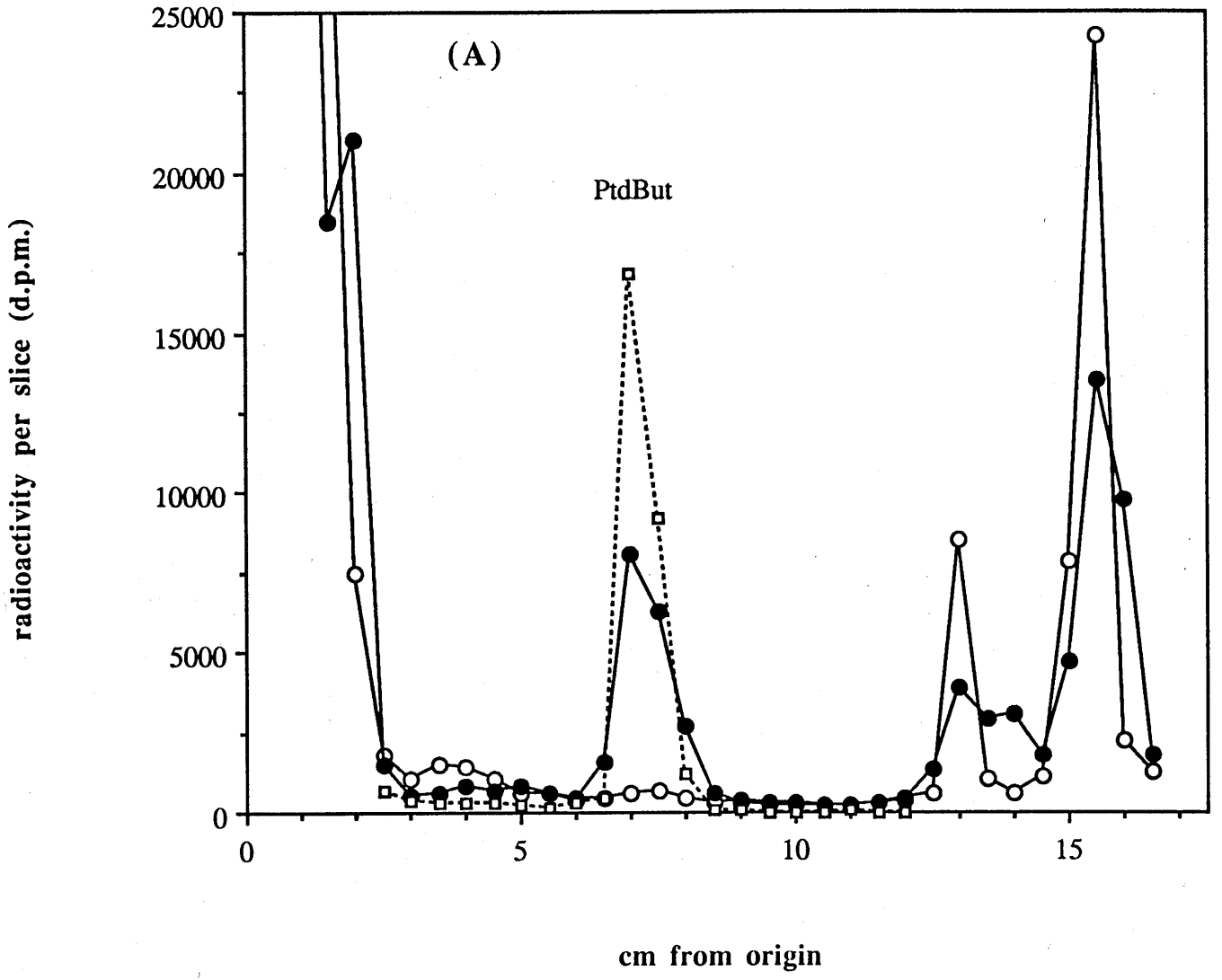
Phosphatidylalcohols are formed by the phosphatidyltransferase activity of PLD in the presence of a suitable primary short chain alcohol. For the experiments described herein butan-1-ol was chosen since Bonser *et al.*(1989) had previously shown that in neutrophils PtdBut was formed at lower concentrations of the alcohol than were required for ethanol in forming PtdEth. Furthermore, on the t.l.c. system chosen PtdBut migrated further than PtdEth by virtue of the longer chain alcohol group, making for complete resolution of PtdBut from other species. Finally, the provision of a [^{14}C]PtdBut 'standard' by Dr. R Randall, The Wellcome Laboratories, Beckenham, Kent, allowed identification of PtdBut.

Preliminary experiments aimed to assess whether [^{3}H]palmitate-labelled Swiss 3T3 cells could form PtdBut under appropriate conditions and to rigorously identify this product as PtdBut. Fig. 4.7a shows the distribution of radioactivity along two lanes of a t.l.c. plate after development of samples from control and PMA-stimulated, [^{3}H]palmitate-labelled Swiss 3T3 cells as described in the methods section. The appearance of a peak of radioactivity with an R_f of 0.40 ± 0.06 can be seen in response to PMA-stimulation. The identity of this peak was confirmed as PtdBut by a number of criteria. Firstly, it co-migrated upon t.l.c. with an authentic [^{14}C]PtdBut standard which had been prepared by incubating [^{14}C]arachidonoyl-PtdCho with butan-1-ol and cabbage PLD. In addition, it co-migrated with a 'dual-labelled' [^{14}C/^{3}H]PtdBut standard prepared as above using [^{3}H]butan-1-ol. Secondly, when quiescent, unlabelled Swiss 3T3 cells were incubated with carrier-free [^{3}H]butan-1-ol and either bombesin, PMA or DMSO, the formation of a [^{3}H] labelled product with identical R_f as the PtdBut standards and the [^{3}H]PtdBut formed in [^{3}H]palmitate-labelled cells was observed only in response to PMA (Fig. 4.7b) and bombesin (data not shown).

Figure 4.7 (a & b) Resolution and identification of [^{3}H]PtdBut in PMA-stimulated Swiss 3T3 cells.

(a) Swiss 3T3 cells, grown on 6 well plates until judged to be 80% confluent, were cultured for a further 48 hours in 2 ml of DMEM containing 1% calf serum and a total of 4μCi of [^{3}H]palmitic acid. Monolayer cultures were then washed in DMBH for 60 min at 37^{o}C before incubating in DMBH containing 100nM PMA (●) or β-phorbol (○) for 30 min. Incubations were terminated by aspiration of the medium and addition of ice-cold methanol: chloroform extracts were resolved on Whatman LK5DF t.l.c. plates in parallel with a [^{14}C]PtdBut standard (❑) using the solvent system described in Chapter 2. Each lane of the t.l.c. plate was divided into 0.5 cm slices which were excised and the associated radioactivity determined by scintillation counting. The results are expressed as radioactivity per 0.5 cm slice (d.p.m.) and are from a single experiment, representative of nine.

(b) Confluent monolayer cultures of Swiss 3T3 cells were incubated in DMBH for 30 min at 37^{o}C before replacing with DMBH containing 500μCi of carrier free [^{3}H]butan-1-ol and either 500nM PMA (●) or 0.1% (v/v) DMSO as a vehicle control (○) for a further 30 min at 37^{o}C. Incubations were terminated by aspiration of the medium and addition of ice-cold methanol. Chloroform extracts, prepared by the method described by Randall *et al.* (1990), were washed three times in theoretical upper phase before applying to Whatman LK5DF t.l.c. plates together with a dual labelled [^{14}C/^{3}H]PtdBut standard (❑). Samples were developed in the solvent system described in Chapter 2. Each lane was divided into 0.5 cm strips, which were excised from the plate and the associated radioactivity determined by scintillation counting. Results are expressed as radioactivity in PtdBut (d.p.m.) from a single experiment representative of three.


(A)
PtdBut
25000
20000
15000
10000
5000
0
0
5
10
15
radioactivity per slice (d.p.m.)
cm from origin

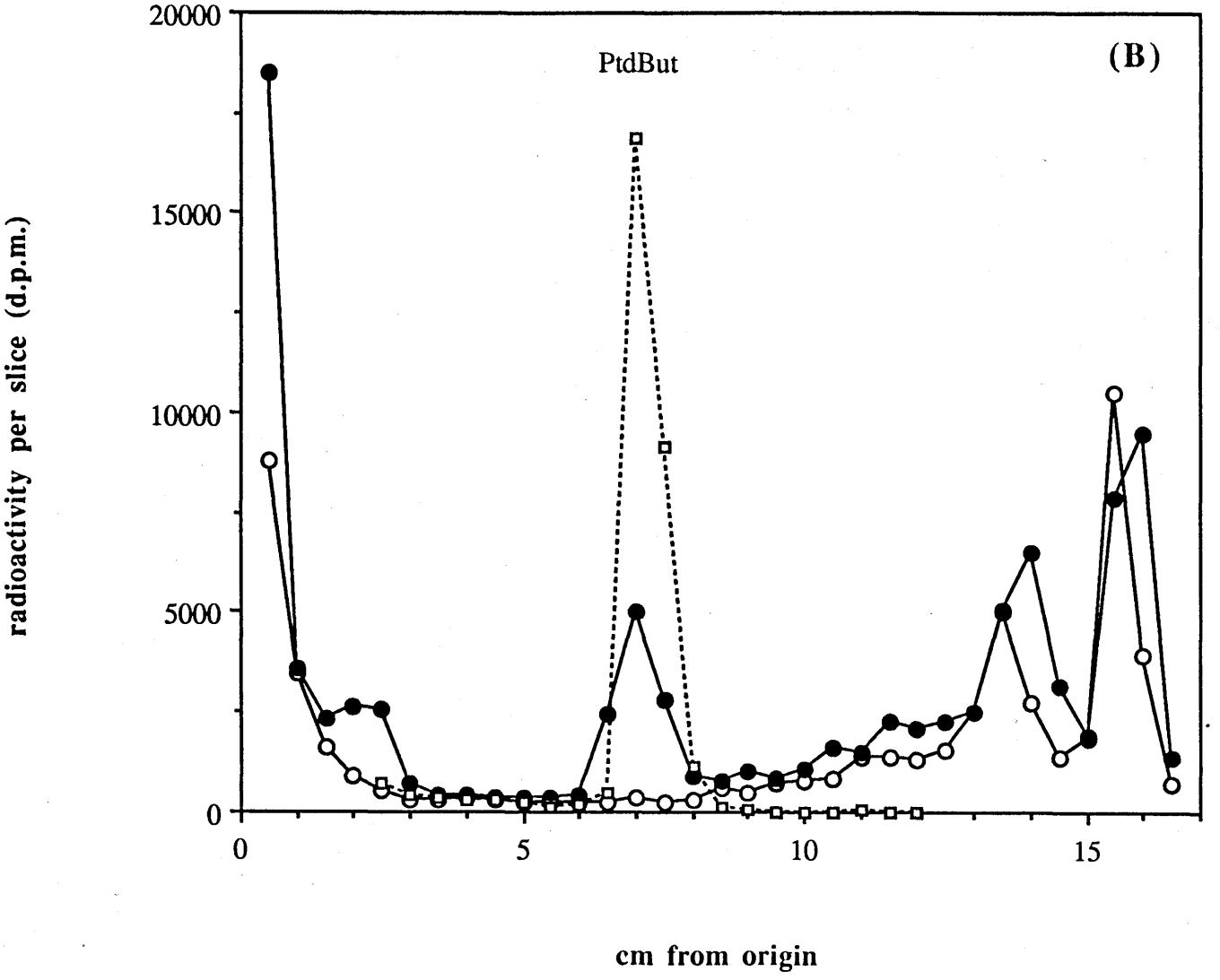
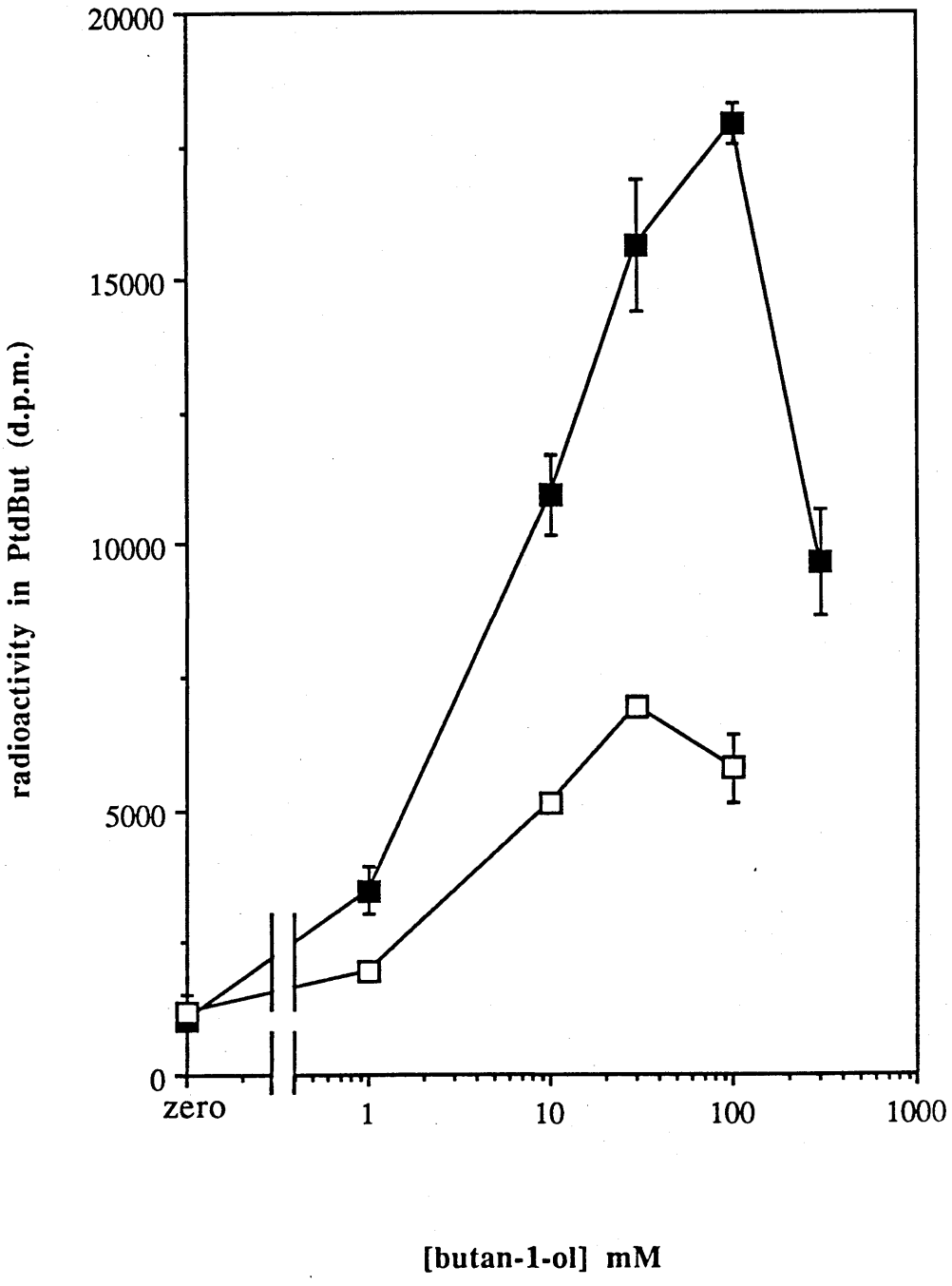

PtdBut
(B)
radioactivity per slice (d.p.m.)
20000
15000
10000
5000
0
0
5
10
15
cm from origin

Figure 4.8 The effect of butan-1-ol concentration on agonist-stimulated [^{3}H]PtdBut formation in [^{3}H]palmitate-labelled Swiss 3T3 cells.

Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent when the medium was replaced with 2ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid and the cells cultured for a further 48 hours. The medium was then replaced with DMBH for 30 min at 37^{o}C and finally DMBH containing the indicated concentration of butan-1-ol and either 617 nM bombesin (□) or 100nM PMA (■); incubations were continued for 20 min before aspiration and addition of ice-cold mathanol. Chloroform extracts, prepared as described in the Methods section, were loaded on to Whatman LK5DF t.l.c. plates and resolved using the solvent system described in Chapter 2. [^{3}H]PtdBut, identified by its co-migration with a [^{14}C]PtdBut standard, was excised in 0.5 cm slices from each lane and its radioactivity determined. The results, expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=2) are from a single experiment representative of two.


20000
15000
10000
5000
0
radioactivity in PtdBut (d.p.m.)
zero
1
10
100
1000
[butan-1-ol] mM

Finally, the ability of bombesin and PMA to stimulate an increase in radioactivity co-migrating with the PtdBut standard was absolutely dependent on the presence and concentration of butan-1-ol (Fig. 4.8). Bombesin- and PMA-stimulated [^{3}H]PtdBut formation were essentially maximal at 30mM butan-1-ol (0.3%, v/v); concentrations of butan-1-ol higher than 100mM gave a reduction in PMA-and bombesin-stimulated [^{3}H]PtdBut formation resulting in a bell-shaped dose response curve. The EC_{50} values for butan-1-ol-dependent, agonist-stimulated [^{3}H]PtdBut formation were 8.95 ± 1.11 mM for PMA and 3.75 ± 0.33 mM for bombesin. Microscopic examination of cells exposed to buffer containing 0.9% (v/v, 100 mM) butan-1-ol for 20 min revealed that many cells became detached from the culture plate, whilst those remaining exhibited morphological changes and some lysis. For further studies, 30mM butan-1-ol was chosen as a concentration which supported near-maximal [^{3}H]PtdBut formation without compromising cellular responsiveness or viability over short term exposure. Having optimised the conditions for formation and identification of [^{3}H]PtdBut, further studies concentrated upon characterizing the responses of Swiss 3T3 cells to stimulation with bombesin and PMA.

Addition of 100nM PMA to quiescent, [^{3}H]palmitate-labelled Swiss 3T3 cells resulted in a time- and dose-dependent increase in the levels of [^{3}H]PtdBut (Fig. 4.9 a & b). Statistically significant formation of [^{3}H]PtdBut in response to PMA occurred after 1 min ($p=0.004$) and continued in a near linear fashion, without any apparent desensitization, for up to 30 min. There was no significant elevation over the zero time control seen in response to vehicle (0.01% DMSO containing β-phorbol). The concentration of PMA required to elicit half-maximal accumulation of [^{3}H]PtdBut was 2.80 ± 1.02 nM (mean ± S.D., n=3 determinations in triplicate) (Fig. 4.9b).

Addition of a maximal dose (617nM) of bombesin to quiescent, [^{3}H]palmitate-labelled Swiss 3T3 cells in the presence of 30mM butan-1-ol resulted in the time- and dose-dependent formation of [^{3}H]PtdBut (Fig. 4.10 a & b). Formation of [^{3}H]PtdBut was apparent and significantly above control

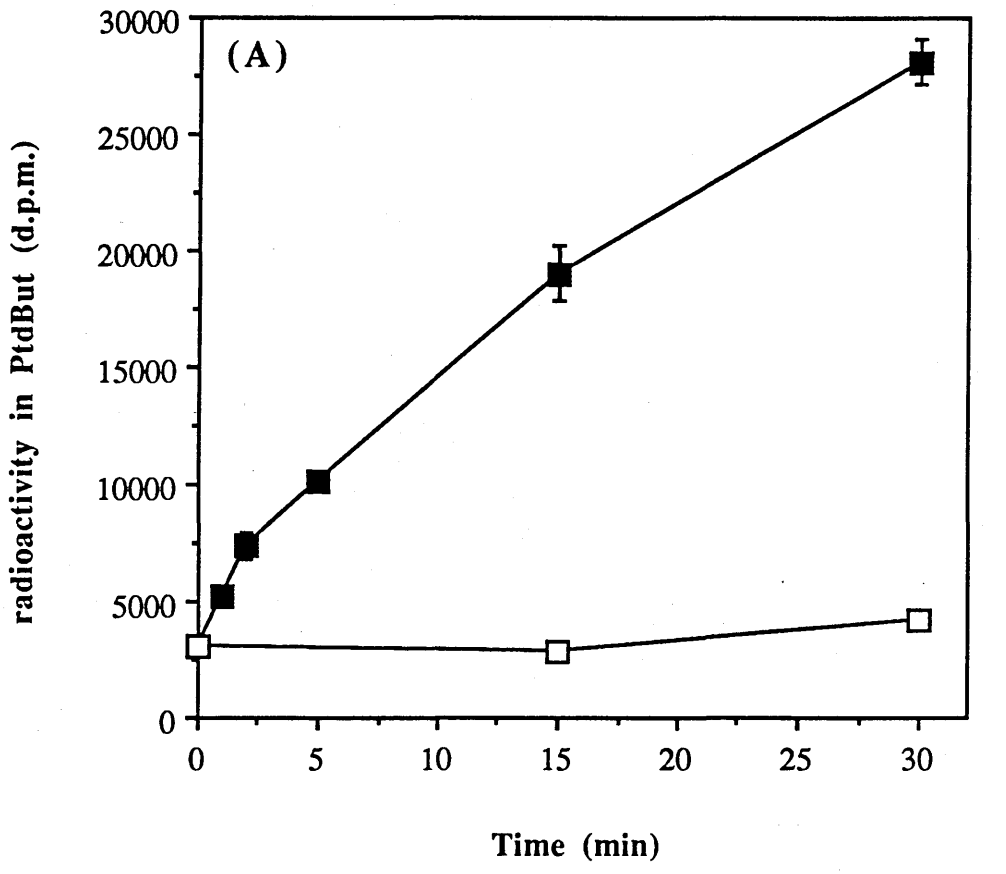
Figure 4.9 (a & b) Time course (a) and dose-response curve (b) for PMA-stimulated [^{3}H]PtdBut formation in Swiss 3T3 cells.

Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent when the medium was replaced with 2ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid and the cells maintained for a further 48 hours. The medium was then replaced with DMBH for 30 min at 37°C.

(a) For time course studies medium was replaced with DMBH containing 0.3% (v/v) butan-1-ol for 5 min and incubations started by replacing with DMBH containing 0.3% (v/v) butan-1-ol and either 100nM PMA (■) or β-phorbol (□). At the indicated times, medium was aspirated and the incubations were terminated by addition of ice-cold methanol.

(b) For dose response studies medium was replaced with DMBH containing 0.3% (v/v) butan-1-ol and the indicated final concentration of PMA (■). Incubations were for 20 min before terminating by aspiration of the medium and addition of ice-cold methanol.

In both cases, chloroform extracts were prepared and resolved on Whatman LK5DF t.l.c. plates as described in the Methods section. [^{3}H]PtdBut was identified by its co-migration with a [^{14}C]PtdBut standard and excised from the silica gel in 0.5 cm slices. The associated radioactivity was determined: results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) from a single experiment, representataive of three.


(A)
radioactivity in PtdBut (d.p.m.)
30000
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
30
Time (min)

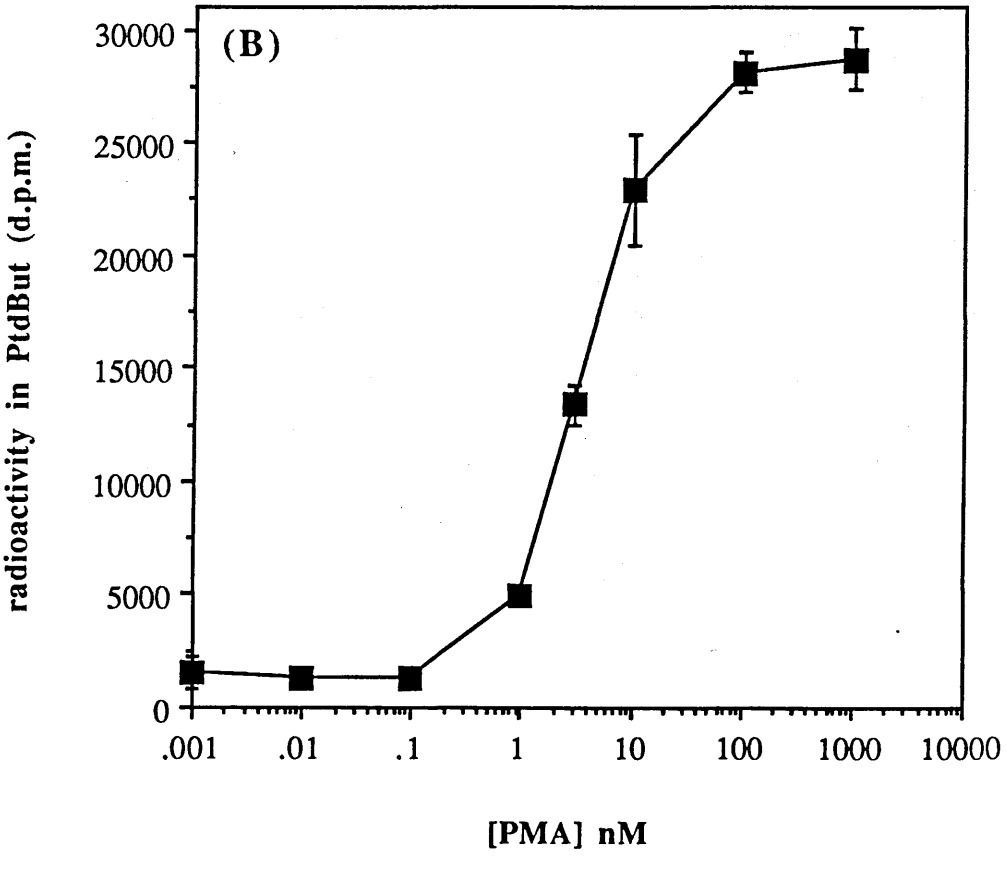

(B)
radioactivity in PtdBut (d.p.m.)
30000
25000
20000
15000
10000
5000
0
.001
.01
.1
1
10
100
1000
10000
[PMA] nM

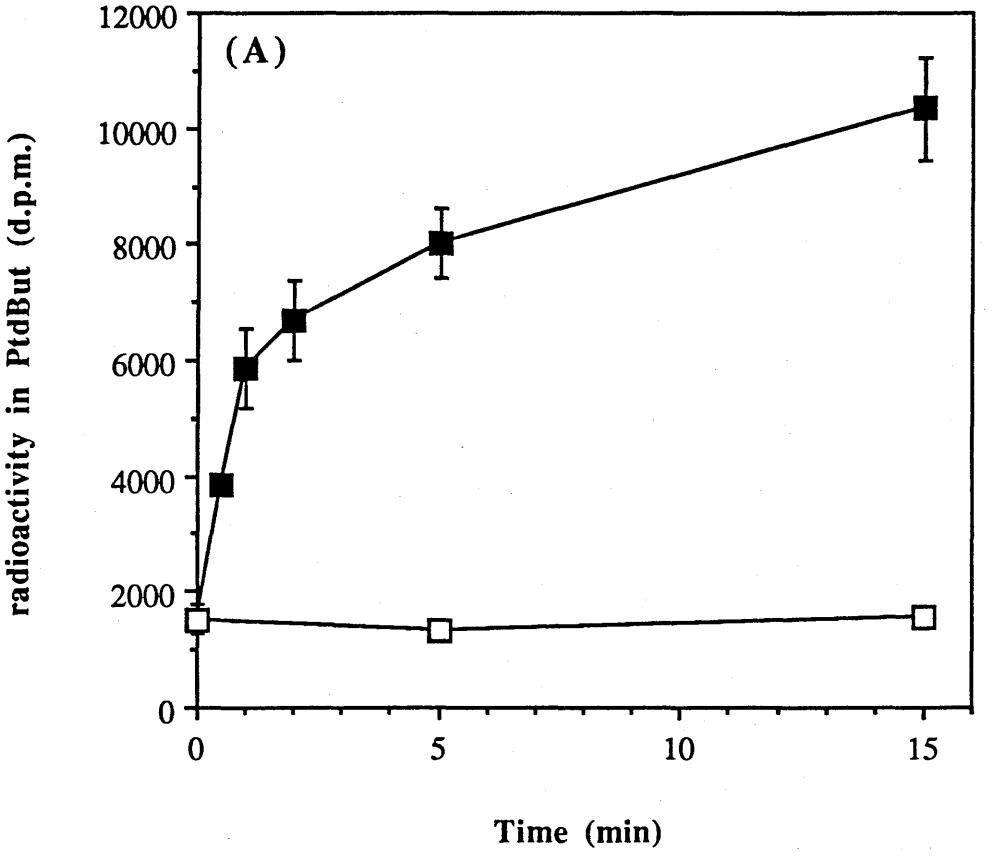
Figure 4.10 (a & b) Time course (a) and dose-dependence (b) for bombesin-stimulated [^{3}H]PtdBut formation in Swiss 3T3 cells.

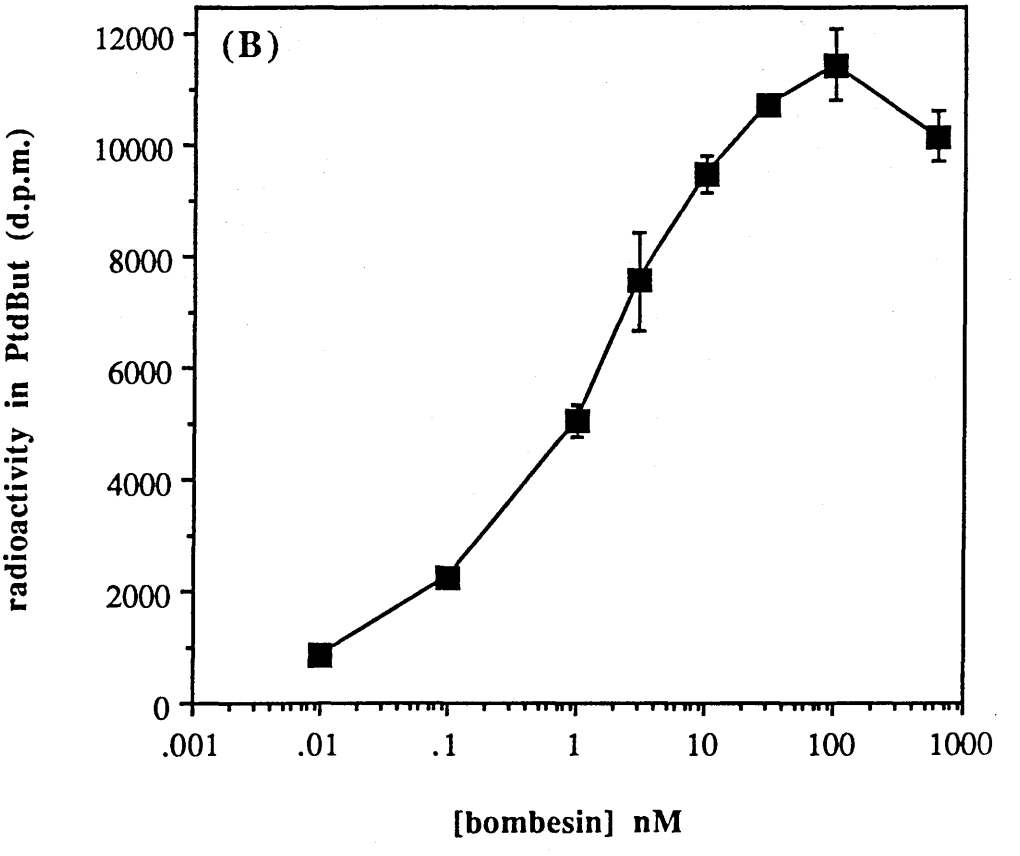
Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent when the medium was replaced with 2ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid and the cells maintained for a further 48 hours. The medium was then replaced with DMBH for 30 min at 37^{o}C.

(a) For time course studies medium was replaced with DMBH containing 0.3% (v/v) butan-1-ol for 5 min and incubations started by replacing with DMBH containing 0.3% (v/v) butan-1-ol and either 617nM bombesin (■) or DMBH alone (❑). At the indicated times, the medium was aspirated and incubations terminated by addition of ice-cold methanol.

(b) For dose-response studies medium was replaced with DMBH containing 0.3% (v/v) butan-1-ol and the indicated final concentration of bombesin (■). Incubations were for 20 min before terminating by aspiration of the medium and addition of ice-cold methanol.

In both cases, chloroform extracts were prepared and resolved on Whatman LK5DF t.l.c. plates as described in the Methods section. [^{3}H]PtdBut was identified by its co-migration with a [^{14}C]PtdBut standard and excised from the t.l.c. plates in 0.5 cm slices. The associated radioactivity was determined: results are radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) from a single experiment, representataive of three.


(A)
radioactivity in PtdBut (d.p.m.)
12000
10000
8000
6000
4000
2000
0
0
5
10
15
Time (min)


(B)
radioactivity in PtdBut (d.p.m.)
12000
10000
8000
6000
4000
2000
0
.001
.01
.1
1
10
100
1000
[bombesin] nM

(p=0.009) after a 30 sec stimulation with bombesin though, in some cases, significant elevation was observed after 15 sec ($p<0.001$, Fig 4.15). [^{3}H]PtdBut levels rose up to 2 min after which time, the rate of accumulation was reduced up to 5 and 15 min. This apparent desensitization of the response was not always complete since the increase in [^{3}H]PtdBut from 5 min to 15 min was in some instances statistically significant ($p=0.026$) suggesting that accumulation of [^{3}H]PtdBut continued, albeit at a reduced rate. The basal, unstimulated level of [^{3}H]PtdBut did not vary significantly from the zero time control over 15 min. [^{3}H]PtdBut formation appeared to be slower than bombesin stimulated increases in [^{3}H]InsP_3 and Ins(1,4,5)P_3 mass and the initial phase of DG elevation all of which were maximal after 5 sec, suggesting that it occurred later than PtdIns(4,5)P_2 hydrolysis. Interestingly, the increase in PtdBut actually seemed to precede the second phase of the DG response which was not apparent until after 30 sec or 1 minute. The ability of bombesin to stimulate accumulation of PtdBut was dose-dependent (Fig. 4.10b), the EC_{50} being 1.3 ± 0.31nM (mean $\pm$ S.D., n=3 determinations in triplicate). These results indicated that both bombesin and PMA were able to activate phospholipase D; furthermore, under these labelling conditions, it was unlikely that inositol lipids were substrates for this PLD activity.

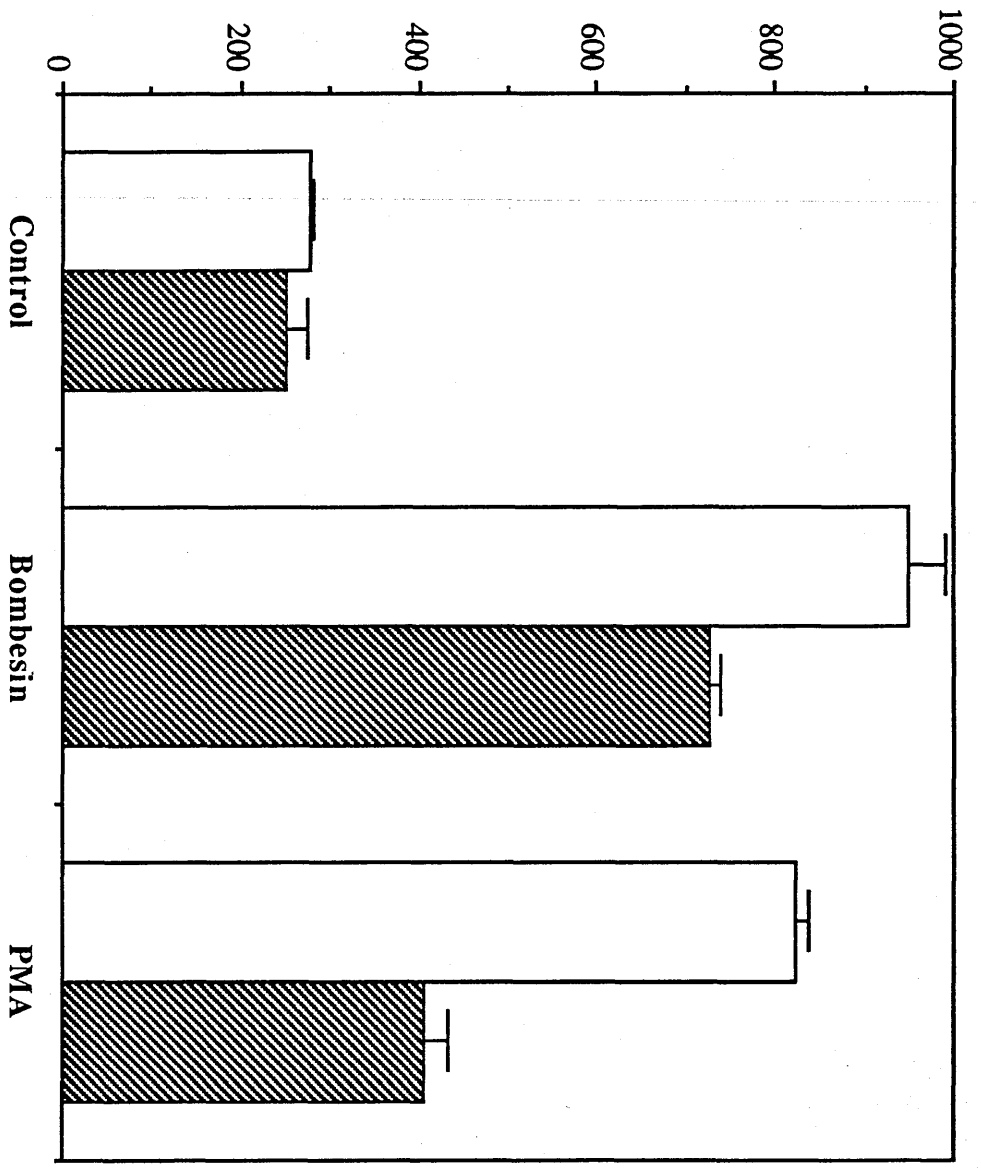
4.2.3 What role does the PLD/PPH pathway play in bombesin- and PMA-stimulated DG formation ?

Since both bombesin and PMA appeared to stimulate PLD activity in a manner consistent with it contributing to sustained increases in DG formation, experiments were performed to confirm that this pathway did indeed operate.

To assess the role of the PLD/PPH pathway in bombesin- and PMA-stimulated DG formation, the ability of butan-1-ol to inhibit DG formation was examined since it has been shown in neutrophils that butan-1-ol effectively 'traps' phosphatidyl moieties as PtdBut thereby interupting the PLD/PPH pathway of DG formation (Bonser *et al.*, 1989). Metz & Dunlop (1991) have

Figure 4.11 The effect of 0.3% (v/v) butan-1-ol upon bombesin- and PMA-stimulated *sn*-1,2-DG mass formation in Swiss 3T3 cells.

Confluent monolayers of quiescent Swiss 3T3 cells were incubated at 37°C for 60 min in DMBH before replacing with DMBH with (hatched bars) or without (open bars) 0.3% (v/v) butan-1-ol for 5 min. Incubations were started by replacing with DMBH alone, or with 100nM PMA or 100nM bombesin in the presence (hatched bars) or absence (open bars) of 0.3% (v/v) butan-1-ol. Incubations were terminated by aspiration of the medium and the addition of ice-cold methanol; after harvesting, the cell debris was extracted in chloroform as described in Chapter 2. An aliquot of the chloroform phase was dried down, solubilised in a Triton X-100/PtdSer mixed micelle detergent preparation and assayed for *sn*-1,2-DG mass as described in the Methods section. Samples were taken for analysis of total lipid phosphorous and for cell number determination. The results are expressed as mass of DG (pmoles of DG/10^6 cells, mean ± S.D., n=3) from a single experiment, representative of three.


DG mass (pmoles/million cells)
0
200
400
600
800
1000
Control
Bombesin
PMA

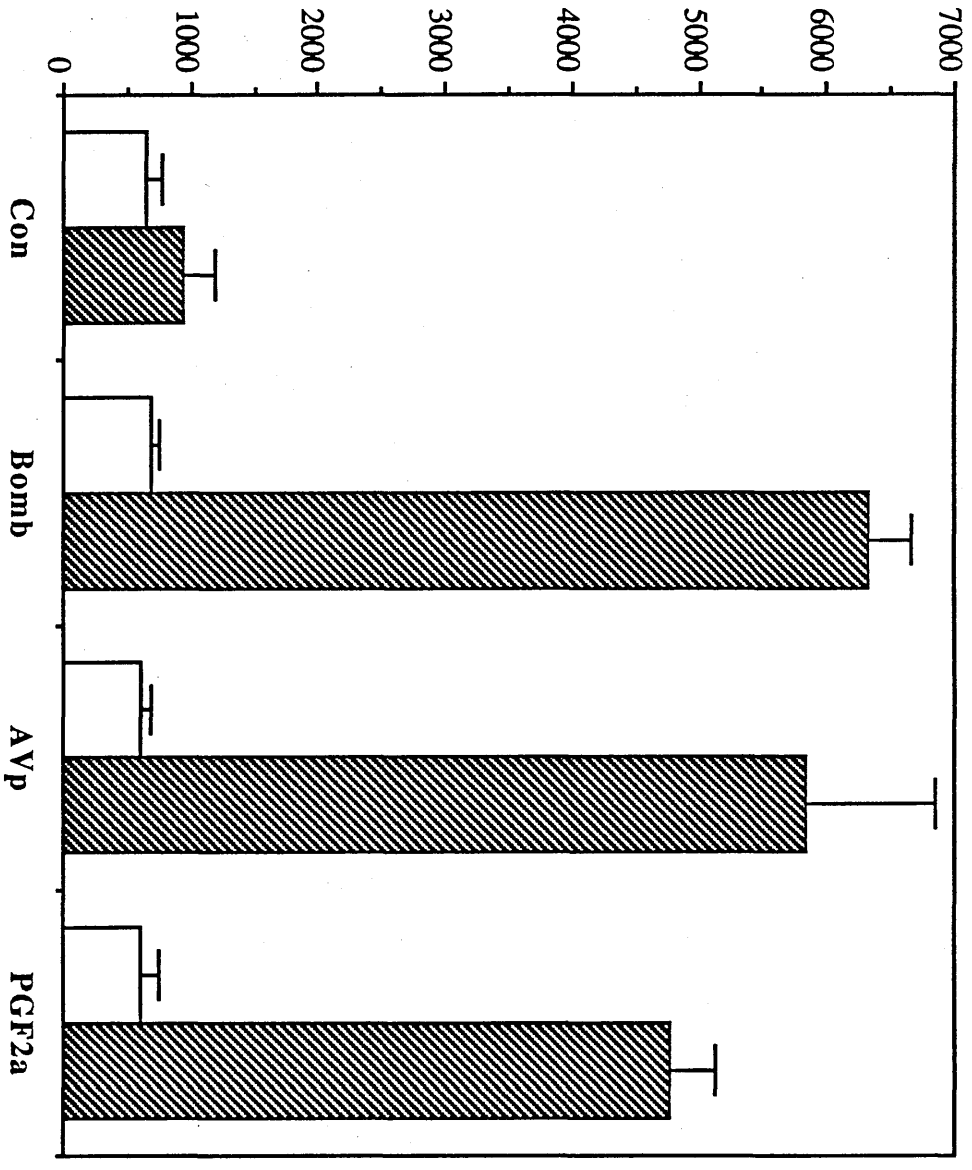
recently shown that the metabolic half-life of phosphatidylethanol in pancreatic islets is greater than two hours whereas that for PtdOH is only 15 min. The results in figure 4.11 show that whilst PMA-stimulated increases in DG mass were inhibited by 74 ± 14 % (n=3 determinations in triplicate) by the inclusion of butan-1-ol, bombesin-stimulated increases were only inhibited by some 30 ± 5 % (n=3) at the same concentration of the alcohol (30mM). These results suggest marked differences in the role of the PLD/PPH pathway in DG formation for a receptor-linked mitogen (bombesin) and the tumour-promoting phorbol esters.

4.2.4 The mechanism of activation of phospholipase D by bombesin and phorbol esters.

Because the formation of PtdBut appeared to occur after Ins(1,4,5)P_3 elevation and before the second, sustained phase of DG accumulation, it seemed possible that PLD activation might be involved in the sustained elevation of DG as has been shown in other systems (Bonser *et al.*, 1989; Huang & Cabot, 1990a). The results in figure 4.11 suggested that this was the case for PMA and to a lesser extent for bombesin. Such a model would require a functional link between the bombesin receptor and PLD. Whilst this could be achieved by PLD being directly coupled to the receptor in a manner analagous to PIC, two observations suggested that this might not be the case. Firstly, the appearance of PtdBut seemed to occur more slowly than Ins(1,4,5)P_3 suggesting that PLD activation was kinetically downstream of PIC activation. Secondly, PMA, a potent activator of protein kinase C, could mimic the sustained accumulation of DG, though not to the same magnitude as bombesin (see Fig 4.4 and particularly Fig. 4.11), and activate PLD (Figs. 4.7, 4.8 & 4.9). Since activation of PKC is an early consequence of bombesin-stimulated PtdIns(4,5)P_2 hydrolysis, this suggested that PKC might represent a functional link between PtdIns(4,5)P_2 hydrolysis and activation of PLD. Furthermore, [Arg^8]Vp and $PGF_{2\alpha}$, agonists which were able to stimulate inositol lipid hydrolysis in a similar manner to bombesin, were also able to stimulate PLD-transferase activity in the presence of

Figure 4.12 [Arg^8]Vp and $PGF_{2\alpha}$ activate PLD transferase activity in Swiss 3T3 cells.

Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent when the medium was replaced with 2ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid and the cells cultured for a further 48 hours. The medium was then replaced with DMBH for 30 min at 37^{o}C. Incubations were started by replacing with DMBH alone or DMBH containing 100nM bombesin , 100nM [Arg^8]Vp or 2μM $PGF_{2\alpha}$ in the presence (hatched bars) or absence (open bars) of 0.3% (v/v) butan-1-ol as indicated. Incubations were continued for 15 min before terminating by apiration of the medium and addition of ice-cold methanol. Chloroform extracts were prepared and resolved on Whatman LK5DF t.l.c. plates as described in the Methods section. [^{3}H]PtdBut was identified by its co-migration with a [^{14}C]PtdBut standard and excised from the t.l.c. plate in 0.5 cm slices. The associated radioactivity was determined: results are radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) from a single experiment, representative of two.


radioactivity in PtdBut (d.p.m.)
0
1000
2000
3000
4000
5000
6000
7000
Con
Bomb
AVp
PGF2a

butan-1-ol (Fig. 4.12). This suggested that activation of PLD might be a common consequence of inositol lipid hydrolysis and, therefore, activation of PKC or increases in $[Ca^{2+}]_i$. Thus, experiments were performed to investigate the requirement for PKC activity in the activation of PLD.

Treatment of Swiss 3T3 cells with 400nM PMA for 48 hours results in the depletion of cellular PKC as measured by loss of [^{3}H]PDBu binding (Collins & Rozengurt, 1984), loss of susbstrate phosphorylation (Rodriguez-Pena & Rozengurt, 1984) and loss of the protein itself as determined by western blotting (Brown *et al.*, 1990). Under these conditions, the ability of both bombesin and PMA to stimulate the accumulation of [^{3}H]PtdBut was completely abolished compared to cells pre-treated with a DMSO vehicle containing β-phorbol (Fig. 4.13). These results suggested that both bombesin and PMA absolutely required the presence of functional PKC to be able to activate PLD.

To further confirm this, a recently described analogue of staurosporine, Ro-31-8220, was used as a PKC inhibitor (Davis *et al.*, 1989). Whilst acting at the catalytic domain of PKC , Ro-31-8220, is much more selective for PKC than PKA or Ca^{2+}-calmodulin dependent protein kinase and is half-maximally effective at sub-micromolar concentrations. Pre-incubation of Swiss 3T3 cells with Ro-31-8220 for 5 min resulted in a dose-dependent inhibition of bombesin- and PMA-stimulated [^{3}H]PtdBut formation (Fig. 4.14 a & b). In the case of PMA, Ro-31-8220 resulted in 92 ± 7% (n=4 experiments) inhibition of [^{3}H]PtdBut formation at 10μM whilst half-maximal inhibition (IC_{50}) occurred at a concentration of 0.87 ± 0.43μM. The almost complete inhibition of PMA-stimulated [^{3}H]PtdBut formation by Ro-31-8220 was consistent with PMA exerting its effects via activation of protein kinase C, agreeing with the result obtained in PKC-down-regulated Swiss 3T3 cells (Fig. 4.13). In contrast bombesin-stimulated [^{3}H]PtdBut formation was only inhibited by 45 ± 5% (n=3) at 10μM Ro-31-8220 with an apparent IC_{50} value of 1.12 ± 0.47 μM.

To assess whether Ro-31-8220 might be exerting different effects at various stages during the response the effects of the drug were compared at

Figure 4.13 The effect of a 48 hour pre-treatment with 400nM PMA upon bombesin- and PMA-stimulated [^{3}H]PtdBut formation in Swiss 3T3 cells.

Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent. They were then cultured for a further 48 hours in DMEM containing 1% calf serum, 4μCi of [^{3}H]palmitic acid and either 400nM PMA (hatched bars) or β-phorbol (open bars). After 48 hours the medium was replaced with DMBH for 30 min before starting incubations by addition of DMBH containing 0.3% (v/v) butan-1-ol alone or with 100nM bombesin or 100nM PMA as indicated. Incubations were terminated after 20 min by aspiration of the medium and addition of ice-cold methanol; [^{3}H]PtdBut formation was assayed as previously described after resolution by t.l.c. Radioactivity in [^{3}H]PtdBut was normalised to the degree of labelling in total [^{3}H]phospholipids which was enhanced some four-fold by the PMA pre-treatment. The results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) from a single experiment, representative of two which gave qualitatively identical results.

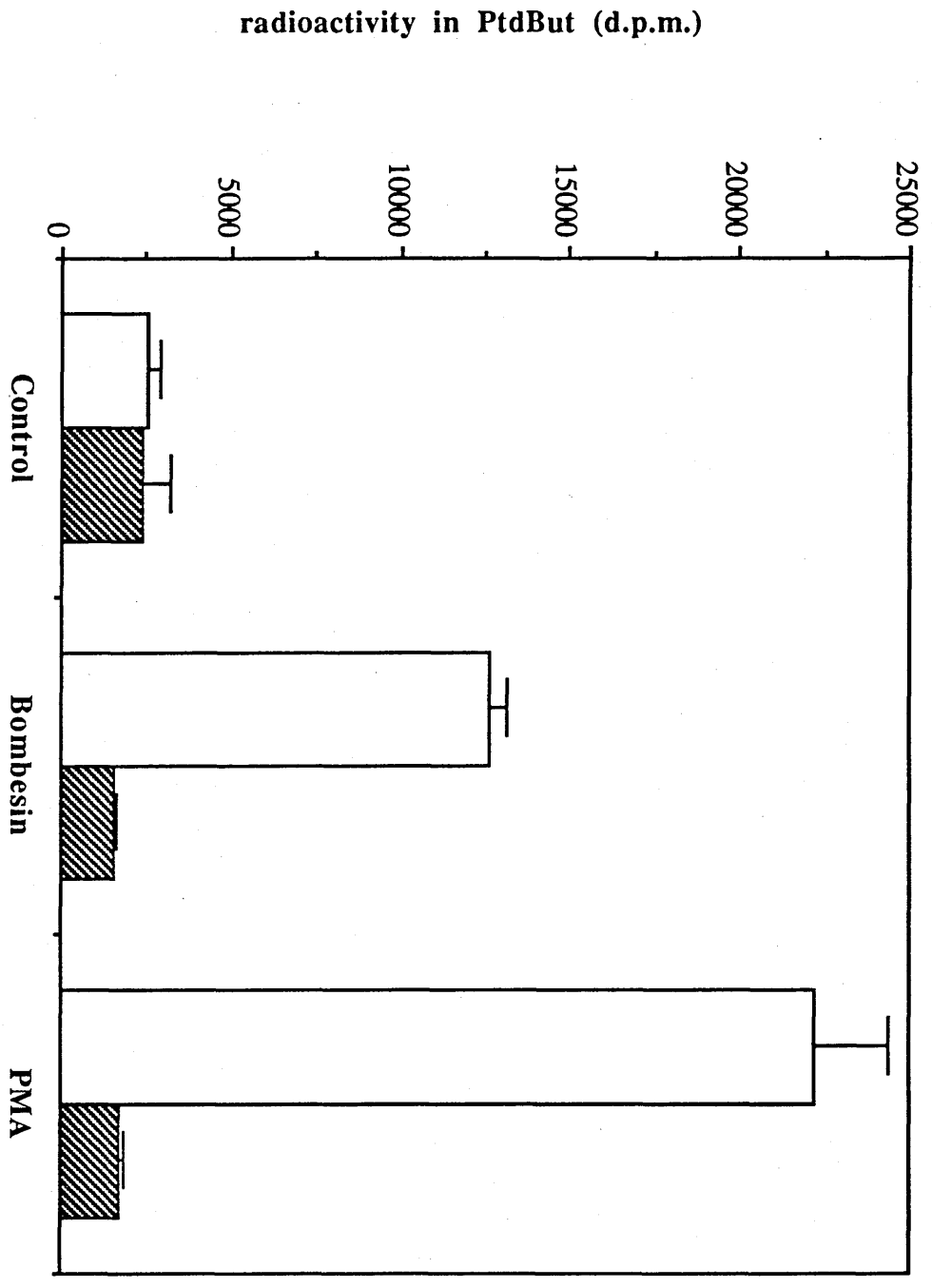
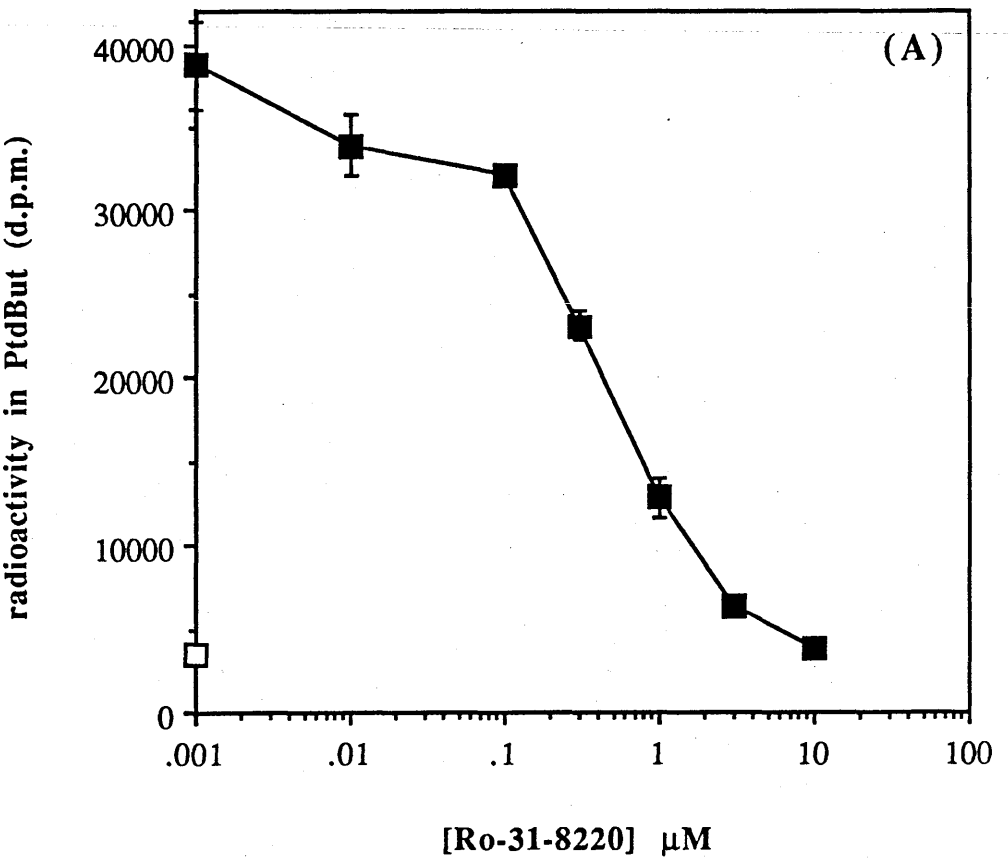
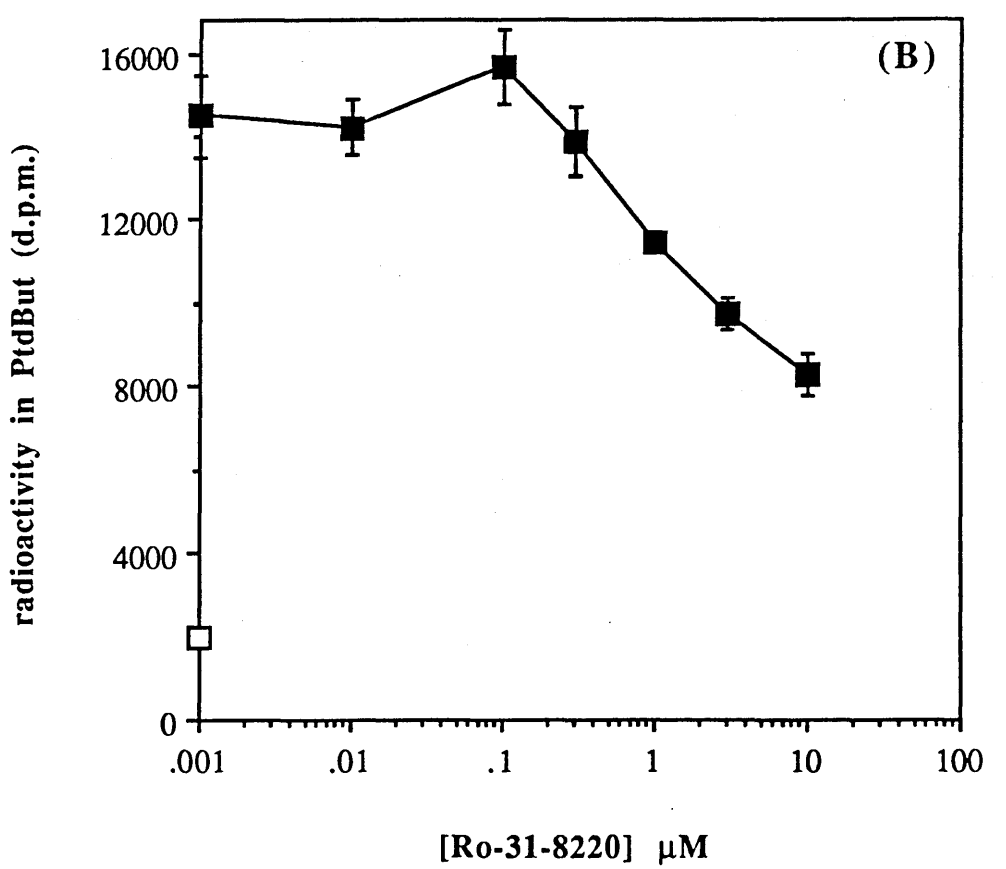

radioactivity in PtdBut (d.p.m.)
0
5000
10000
15000
20000
25000
Control
Bombesin
PMA

Figure 4.14 The effect of Ro-31-8220 concentration upon (a) PMA- and (b) bombesin-stimulated [^{3}H]PtdBut formation in Swiss 3T3 cells.

Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent when the medium was replaced with 2ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid and the cells maintained for a further 48 hours. The medium was then replaced with DMBH for 30 min at 37^{o}C and then DMBH containing 0.3% (v/v) butan-1-ol and the indicated concentration of Ro-31-8220 for 5 min. Finally, incubations were started by addition of agonist to give final concentrations of 100nM PMA (a) or 100nM bombesin (b). After 15 min, incubations were terminated by aspiration of the medium and addition of ice-cold methanol. [^{3}H]PtdBut formation was assayed as described in the Methods section after resolution by t.l.c. The results are mean radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) from a single experiment, representative of (a) four and (b) three. In both cases ■ = the effect of Ro-31-8220 upon (a) PMA- and (b) bombesin-stimulated [^{3}H]PtdBut formation and ❑ = the effect of a 0.3% DMSO vehicle control in the absence of agonist.


(A)
radioactivity in PtdBut (d.p.m.)
40000
30000
20000
10000
0
.001
.01
.1
1
10
100
[Ro-31-8220] μM


(B)
radioactivity in PtdBut (d.p.m.)
16000
12000
8000
4000
0
.001
.01
.1
1
10
100
[Ro-31-8220] μM

different time points in bombesin-stimulated cells. However the results in Fig. 4.15 indicate that Ro-31-8220 inhibited [^{3}H]PtdBut formation by approximately 50% at all time points tested from 15 sec to 15 min.

The inability of Ro-31-8220 to completely inhibit bombesin stimulated [^{3}H]PtdBut formation suggested that this response, unlike that of PMA, might not be entirely mediated by PKC. Another early signal elicited by bombesin is the increase in $[Ca^{2+}]_i$, initially from an intracellular store and later by entry of Ca^{2+} into the cell (Lopez-Rivas *et al.*, 1987; Hesketh *et al.*, 1988). To assess if entry of Ca^{2+} was playing any part in the activation of PLD by bombesin, the extracellular medium concentration of Ca^{2+} was lowered by chelation with EGTA. Using a computer programme (IONS, Microsoft Basic on Apple Macintosh), based upon the concentration of Mg^{2+}, Ca^{2+}, ATP and glutamate in DMEM, it was calculated that inclusion of 2.5mM EGTA would result in a free Ca^{2+} concentration of 150nM. Pre-treatment of Swiss 3T3 cells with DMBH/EGTA for 15 min had no effect upon control [^{3}H]PtdBut levels (Fig. 4.16). The ability of PMA to stimulate [^{3}H]PtdBut formation was unaffected by the inclusion of EGTA in the incubation medium suggesting that entry of Ca^{2+} was not required for phorbol ester-stimulated PLD activity (Fig. 4.16). However, the ability of bombesin to stimulate [^{3}H]PtdBut formation was inhibited by 57 ± 5 % (n=3) by the inclusion of EGTA in the incubation medium (Fig. 4.16).

Since increases in Ca^{2+} synergise with DG in activating PKC, the effect of Ca^{2+} chelation might not have been directly upon PLD but rather upon PKC activity. To address this, the effect of both EGTA and Ro-31-8220 upon bombesin-stimulated PLD activity was examined by pre-treating cells with EGTA in the presence or absence of the PKC inhibitor. As previously shown, pre-treatment with Ro-31-8220 for 5 min resulted in a 45 ± 12 % inhibition of bombesin stimulated [^{3}H]PtdBut formation. However, when a combination of Ro-31-8220 and EGTA was used, the inhibition of bombesin-stimulated [^{3}H]PtdBut formation (70 ± 9 %, n=3) was not fully additive (Fig. 4.16).

Finally, to further investigate the role of Ca^{2+} in activation of PLD, the

Figure 4.15 The effect of Ro-31-8220 upon the time course of bombesin-stimulated [^{3}H]PtdBut formation in Swiss 3T3 cells.

Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent when the medium was replaced with 2ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid and the cells maintained for a further 48 hours. The medium was replaced with DMBH for 30 min at 37°C and then DMBH containing 0.3% (v/v) butan-1-ol alone (■) or with 10μM Ro-31-8220 (❑) for 5 min. Incubations were started by replacing the medium with DMBH containing 0.3% (v/v) butan-1-ol and 100nM bombesin alone (■) or with 10μM Ro-31-8220 (❑) and were terminated at the indicated times by aspiration of the medium and addition of ice-cold methanol. [^{3}H]PtdBut formation was assayed as described in the Methods section. The results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) from a single experiment, representative of two.

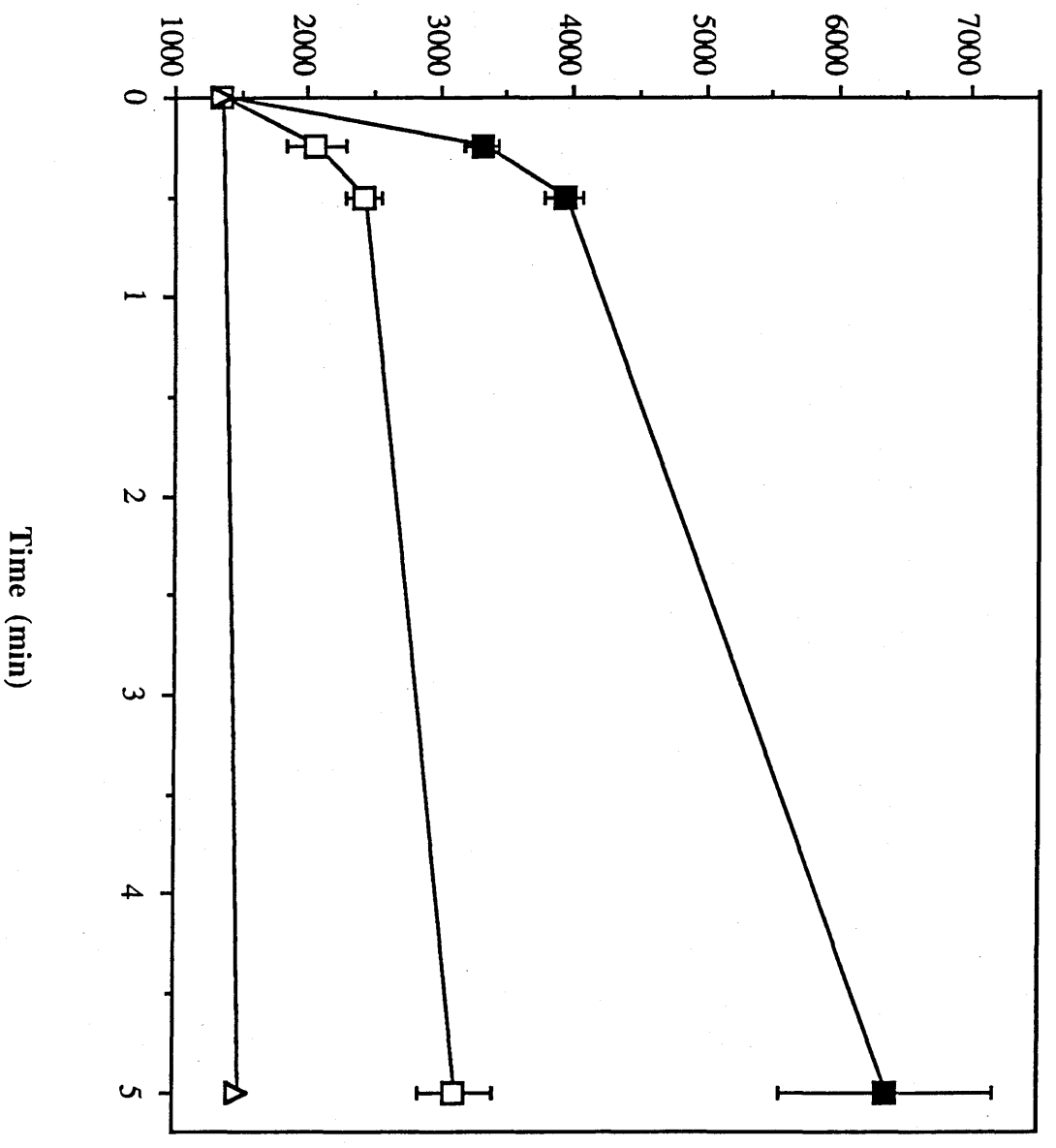

radioactivity in PtdBut (d.p.m.)
1000
2000
3000
4000
5000
6000
7000
0
1
2
3
4
5
Time (min)

Figure 4.16 The effect of EGTA and Ro-31-8220, alone and in combination, upon PMA- and bombesin-stimulated [^{3}H]PtdBut formation in Swiss 3T3 cells.

Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent when the medium was replaced with 2ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid and the cells maintained for a further 48 hours. The medium was then replaced with DMBH for 15 min at 37^{o}C and then DMBH containing 0.3% (v/v) butan-1-ol alone (open bars) or with 2.5mM EGTA (hatched bars) for 15 min. In some experiments, as indicated, 10μM Ro-31-8220 was included in the last 5 min of pre-incubation. Incubations were started by replacing with DMBH alone (open bars) or with EGTA (hatched bars) in the presence or absence of Ro-31-8220 and containing 100nM PMA or 100nM bombesin as indicated. Incubations were continued for 20 min and were terminated by aspiration of the medium and addition of ice-cold methanol. [^{3}H]PtdBut formation was assayed as described previously. The results are expressed radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) from a single experiment, representative of three.

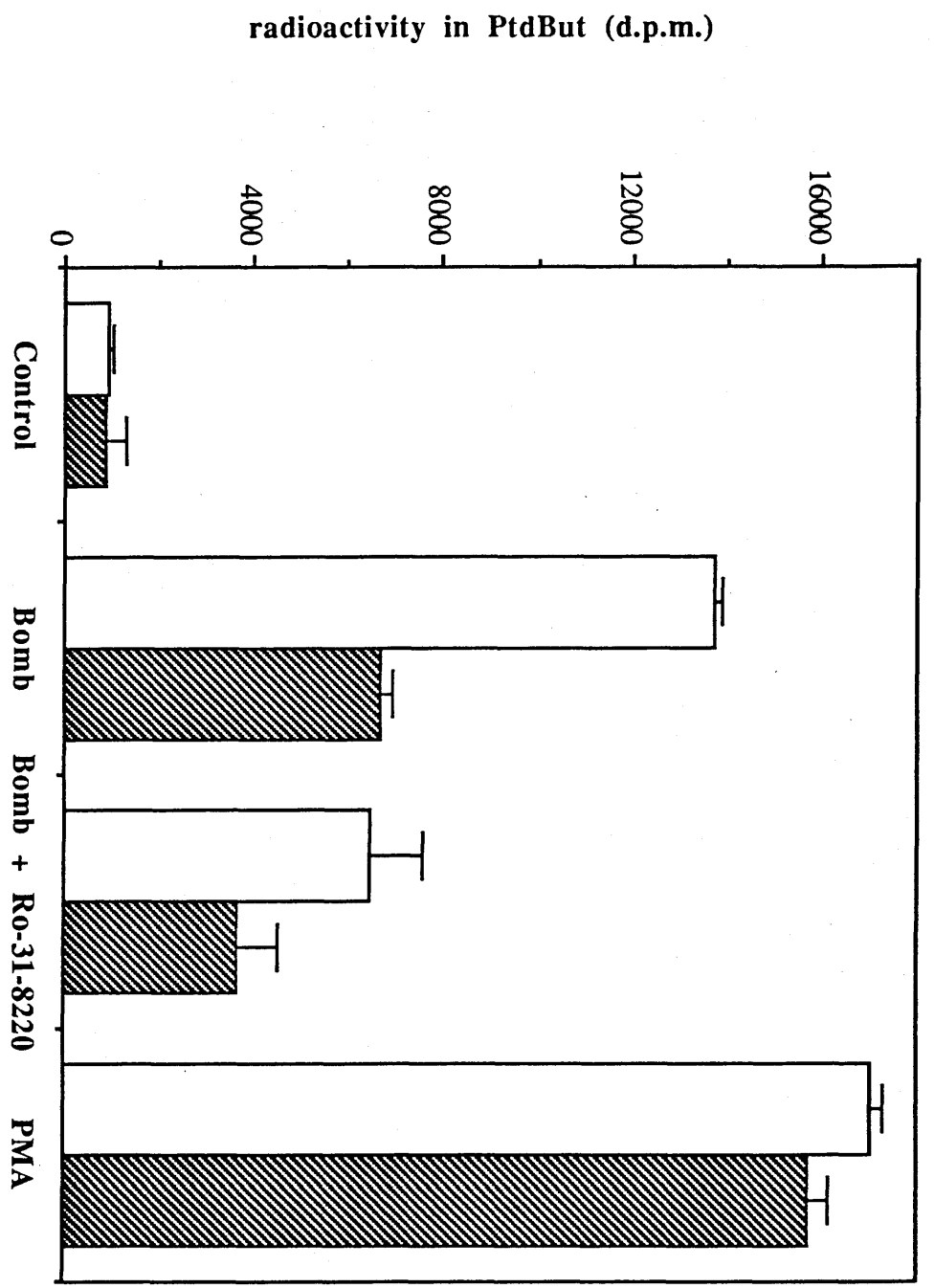
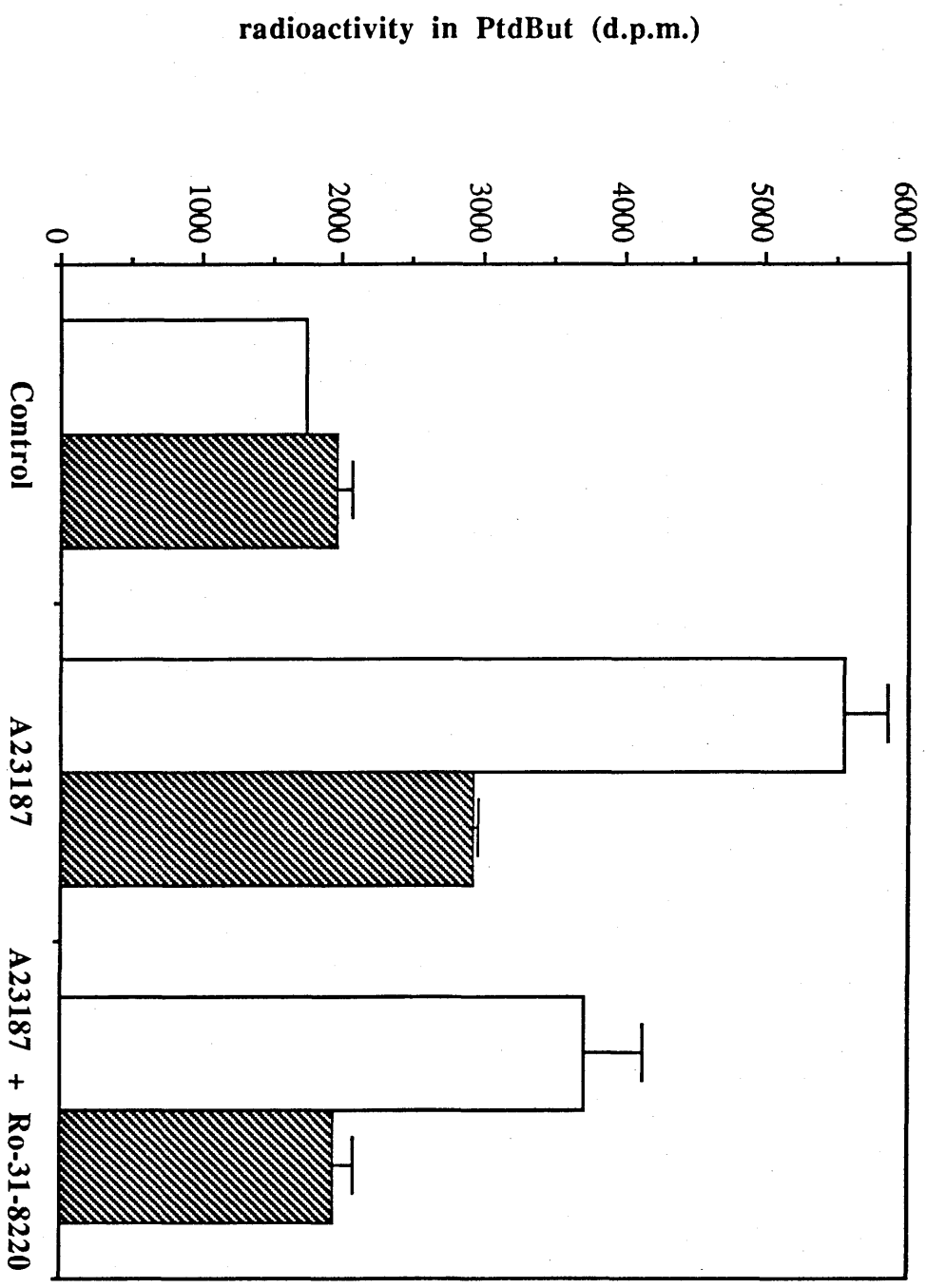

radioactivity in PtdBut (d.p.m.)
0
4000
8000
12000
16000
Control
Bomb
Bomb + Ro-31-8220
PMA

Figure 4.17 The effect of EGTA and Ro-31-8220, alone and in combination, on A23187-stimulated [^{3}H]PtdBut formation in Swiss 3T3 cells.

Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent when the medium was replaced with 2ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid and the cells maintained for a further 48 hours. The medium was replaced with DMBH for 15 min at 37^{o}C and then DMBH containing 0.3% (v/v) butan-1-ol alone (open bars) or with 2.5mM EGTA (hatched bars) for 15 min. Where indicated, 10μM Ro-31-8220 was included in the last 5 min of pre-incubation. Incubations were started by replacing the medium with DMBH alone (open bars) or with EGTA (hatched bars) in the presence or absence of Ro-31-8220 and 5μM A23187 as indicated. Incubations were continued for 20 min and terminated by aspiration of the mediumand addition of ice-cold methanol. [^{3}H]PtdBut formation was assayed as described previously. The results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) from a single experiment representative of two.


radioactivity in PtdBut (d.p.m.)
0
1000
2000
3000
4000
5000
6000
Control
A23187
A23187 + Ro-31-8220

ability of the calcium ionophore A23187 to stimulate [^{3}H]PtdBut formation was examined. Addition of A23187 to [^{3}H]palmitate-labelled Swiss 3T3 cells in the presence of butan-1-ol resulted in the formation of [^{3}H]PtdBut over a 15 minute incubation (Fig. 4.17) indicating that an increase in intracellular [Ca^{2+}] was able to activate PLD. However, the previous results in Swiss 3T3 cells pre-treated with high doses of PMA (Fig 4.13) or Ro-31-8220 (Fig. 4.14) had indicated a role for PKC in the activation of PLD. Since Ca^{2+} is involved in the activation of PKC, it was necessary to investigate if the increase in PLD activity stimulated by A23187 might simply represent Ca^{2+}-mediated activation of PKC rather than a direct effect upon the PLD. To address this issue [^{3}H]palmitate-labelled Swiss 3T3 cells were pre-treated with 10μM Ro-31-8220 for 5 min in the presence of 0.3% (v/v) butan-1-ol before addition of A23187. Such a treatment resulted in a 58 ± 14 % (n=2) inhibition of A23187 stimulated [^{3}H]PtdBut formation (Fig. 4.17) suggesting that at least half of the A23187 response was due to Ca^{2+} induced PKC activation of PLD. Suprisingly, the chelation of extracellular Ca^{2+} did not entirely inhibit the response to A23187; 55 ± 20 % (n=2) of the response was inhibited by the inclusion of EGTA. However, combination of both EGTA and Ro-31-8220 resulted in the majority of the response to A23187 (82 ± 18 %, n=2) being inhibited (Fig. 4.17).

4.3 Discussion.

The results presented in this chapter allow three major conclusions to be reached:

(1) Bombesin stimulates a biphasic increase in DG; the sustained phase is partially mimicked by phorbol esters.

(2) Both bombesin and PMA stimulate phospholipase D activity. The kinetics of PLD activation are consistent with a role in the sustained phase of DG elevation, but there are marked differences between bombesin and PMA in the contribution made by a PLD/PPH pathway to DG formation.

(3) Activation of phospholipase D appears to be largely dependent upon protein kinase C activity which may therefore represent a major link between PtdIns(4,5)P_2 hydrolysis and PLD activation.

4.3.1 Bombesin stimulates a biphasic increase in DG mass in Swiss 3T3 cells.

In Chapter 3, results were presented to indicate that bombesin-stimulated increases in Ins(1,4,5)P_3 mass were transient and returned to pre-stimulated levels within 30 sec (Fig. 3.4). However, a number of the early mitogenic signals associated with the inositol lipid pathway are sustained for some time after Ins(1,4,5)P_3 levels and the initial rapid increase in $[Ca^{2+}]_i$ have declined, including phosphorylation of an 80 kDa PKC substrate (Isacke *et al.*, 1986; Erusalimsky *et al.*, 1988) and increases in pH_i (Bierman *et al.*, 1990), whereas others occur tens of minutes later e.g., increases in c-*fos* and c-*myc* mRNA (Sinnett-Smith & Rozengurt, 1987). Since many of these events are particularly associated with the DG/PKC arm of the signal pathway, the kinetics of increases in DG mass were compared with those of Ins(1,4,5)P_3 mass.

Fig.4.1 shows that there were two kinetically distinct phases of DG mass accumulation in response to bombesin stimulation. The initial increase, peaking at 5 to 10 sec before declining at 30 sec, mirrored the transient increase in Ins(1,4,5)P_3 mass, presumably because they are both derived from PIC-

catalysed hydrolysis of PtdIns(4,5)P_2. However, the mass of DG generated in this early phase exceeded, by at least four-fold, the mass of Ins(1,4,5)P_3 formed. This might represent evidence for receptor-stimulated PtdIns hydrolysis which would generate DG without a concomitant increase in Ins(1,4,5)P_3. Alternatively, it may simply be that the removal of Ins(1,4,5)P_3 by the 5'-phosphatase and 3'-kinase is so rapid that the effective half life of Ins(1,4,5)P_3 is much shorter than that for DG resulting in an under-estimation of mass increases. The equally rapid drop in DG mass from 5 sec to 30 sec or 1 minute is consistent with its rapid removal by DG kinase and/or lipase. In particular, since the DG from PtdIns(4,5)P_2 hydrolysis is likely to be 1-stearoyl-2-arachidonoyl (Pessin & Raben, 1989), this rapid removal of DG may reflect the high activity of a membrane-associated DG kinase selective for arachidonoyl-containing DG as reported in Swiss 3T3 cells by MacDonald *et al.* (1988). Equally the rapid increase in free arachidonic acid in Swiss 3T3 cells in response to bombesin may represent rapid, coupled activation of DG lipase and MG lipase or direct activation of PLA_2 (S. Currie & M.J.O. Wakelam, unpublished results).

The transient nature of the first phase of DG formation was consistent with the transient increase in Ins(1,4,5)P_3 and PtdIns(4,5)P_2 hydrolysis and yet from between 30sec and 1 min onwards DG levels rose again in response to continued bombesin stimulation. This second phase was sustained for at least 30 min (Fig 4.1b) and in some cases 60 min (data not shown) whilst a recent report from Takuwa *et al.* (1989) has shown that bombesin-stimulated increases in DG mass in Swiss 3T3 cells are sustained above control for at least 4 hours. Whilst the pronounced initial 'spike' of DG formation was not always as clearly resolved from the second phase (Fig 4.2), DG levels were reproducibly maintained above control long after Ins(1,4,5)P_3 levels had declined. The magnitude of the second phase was variable but was generally greater than that of the first and this is perhaps consistent with the second phase being derived from a potentially larger lipid pool than PtdIns(4,5)P_2; candidates for this pool might be PtdIns or other phospholipids such as PtdCho.

There are two potential reasons for the sustained increase in DG mass:

either that the DG derived from PtdIns(4,5)P_2 hydrolysis was not removed as rapidly as Ins(1,4,5)P_3 or that DG was being generated from another source. Since in most experiments there was a distinct initial peak of DG formation which declined before rising again in a second phase of DG accumulation argues against the former possibility as does the observation of a high DG kinase activity in Swiss 3T3 membranes (MacDonald *et al.*, 1988). Thus it would seem that the second phase of DG formation, which is sustained, divorced from net formation of Ins(1,4,5)P_3, and occurs at a time at which PtdIns(4,5)P_2 hydrolysis is apparently desensitized, is derived from an alternative source. These results are not without precedent, either within the Swiss 3T3 cell line or in other cell lines. Takuwa *et al.* (1987a) have previously shown that bombesin stimulated a sustained increase in DG levels in Swiss 3T3 cells; however, the earliest timepoint which they examined was 15 sec by which time DG levels are declining (Fig 4.1a), explaining why they were unable to detect biphasic kinetics. In addition, Wright *et al.* (1988) have demonstrated pronounced biphasic kinetics of DG formation in response to α-thrombin stimulation of IIC9 fibroblasts. In this case the two phases of DG formation exhibited quite different dose-dependency; thus, with 500ng/ml α-thrombin Wright *et al.* (1988) observed a clear biphasic response whilst using 100pg/ml they were only able to detect the second sustained phase of DG formation. Furthermore, at the lower concentrations α-thrombin did not stimulate increases in [^{3}H]InsP_3 or arachidonic acid. This raises the intruiging possibility that the two phases of DG formation may be associated with activation of different receptor subtypes with different affinities for the same ligand. Whether this is the case in Swiss 3T3 cells stimulated with bombesin is not clear since the dose-dependency for the two-phases of bombesin-stimulated DG formation whilst not identical were not as strikingly different as that for α-thrombin (Fig. 4.3). The EC_{50} for bombesin-stimulated DG formation at 5 sec (2.04 ± 1.68 nM) was of a similar order to that for Ins(1,4,5)P_3 formation (5.88 ± 3.66 nM), again suggesting that the two were derived from the same source. The EC_{50} for DG formation after a 5 or 15 min exposure to bombesin (0.48 ± 0.15 nM) was not significantly different from that

at 5 sec but was clearly sub-nanomolar. This may warrant further examination by performing timecourses at different concentrations of bombesin as described for α-thrombin (Wright *et al.*, 1988). Alternatively, if PKC is involved in the initiation of the second phase of DG elevation the lower EC_{50} may be due to the amplification of the response by PKC resulting in a lower effective concentration required to give the same response.

The observation that addition of PMA to Swiss 3T3 cells stimulated an increase in DG mass, with kinetics very similar to the sustained phase of the bombesin response (Fig. 4.4), confirms the initial observation of Takuwa *et al.*(1987b) and is of great interest in the context of its mitogenic effects. PMA circumvents receptor activation and inositol lipid hydrolysis by entering the cell and, acting as a DG analogue, binding to and activating PKC (Castagna *et al.*, 1982). The observed increase in DG in response to PMA was not accompanied by accumulation of inositol phosphates (Chapter 3, Fig. 3.9) suggesting that the DG was derived from a non-inositide source. Indeed, the timecourse of increases in DG formation in response to PMA was similar to the timecourse for inhibition of bombesin-stimulated $InsP_t$ accumulation (Chapter 3, Fig. 3.7) suggesting that the two events were related, presumably through the action of PKC. This is supported further by the fact that the EC_{50} for PMA-stimulated increases in DG mass (Fig 4.4b 2.6 ± 0.59 nM) is essentially the same as the IC_{50} for PMA mediated inhibition of bombesin-stimulated inositol phosphate accumulation (Brown *et al.*, 1987; Chapter 3 of this thesis).

Since the sustained elevation of DG in response to bombesin and PMA was clearly not associated with the accumulation of $Ins(1,4,5)P_3$ or $[^3H]Ins_t$ respectively, further experiments sought to define the kinetics of DG formation in Swiss 3T3 cells isotopically labelled so as to largely exclude the inositol lipids as a possible source. Since the inositol lipids, and particularly the polyphosphoinositides, are defined by a 1-stearoyl-2-arachidonoyl composition, [^{3}H]palmitic acid was chosen to label PtdCho, PtdEtn & PtdSer preferentially over PtdIns. The results in Fig. 4.5 show that after a 49 hour incubation with

[^{3}H]palmitic acid all the major phospholipids had been labelled to a constant specific activity. Furthermore, they show that the label was initially incorporated into PtdCho suggesting that PtdCho was a metabolically active pool of palmitate-containing phospholipid. The rapid increase in labelling of PtdCho was followed by a slower incorporation into the other phospholipid classes which may serve to distribute fatty acids to other lipids. Table 4.1 shows that after 49 hours the majority of [^{3}H]palmitic acid incorporated into total phospholipids was in PtdCho (50%) with only 3% of the label in PtdIns. Whilst incorporation into the polyphosphoinositides was not analysed it is likely to be negligible since they are minor components of total cellular inositol lipids which themseleves make up less than 10% of the total cell phospholipid and are characterised by a 1-stearoyl-2-arachidonoyl fatty acid composition. Clearly, this protocol allowed preferential labelling of PtdCho, PtdEtn and PtdSer and was therefore used to determine if phorbol esters could stimulate an increase in [^{3}H]DG under these conditions.

The formation of [^{3}H]DG in response to PMA exhibited very similar kinetics to the increases in DG mass (Fig. 4.6) but were of smaller magnitude. Taken together with the lack of inositol phosphate accumulation this result confirms that phorbol esters are able to increase DG levels in cells from a non-inositide source since under these conditions PtdIns was poorly labelled.

Since bombesin-stimulated inositol lipid hydrolysis and DG formation will necessarily result in activation of PKC, which will also result from PMA stimulation, it seems likely that a consequence of PKC activation will be to stimulate the hydrolysis of a non-inositide lipid which may serve as the source for the second phase of DG formation. In addition to the observation that PMA does not stimulate inositol lipid hydrolysis, evidence in support of the sustained phase of DG formation being derived from an alternative phospholipid source has been provided most conclusively from the work of Pessin & Raben (1989). By analysing the molecular species of DG in α-thrombin-stimulated IIC9 fibroblasts by gas chromatography they have shown that the fatty acid composition of DG was similar to that of PtdIns in the first 15 sec of stimulation whereas DG produced at 5 and 60 min stimulation was derived from PtdCho. In addition,

Muir & Murray, (1987) and Takuwa *et al.*(1987) have shown that stimulation of Swiss 3T3 cells with bombesin or PMA results in the formation of DG which is associated with the increased metabolic turnover of PtdCho.

4.3.2 Bombesin and PMA stimulate phospholipase D activity in Swiss 3T3 cells.

Since the elevation in DG levels could conceivably be due to the action of phospholipases C or D the ability of PMA or bombesin to stimulate increases in [^{3}H]PtdBut was examined as an assay of PLD activity. The formation of [^{3}H]PtdOH was not used since it was not a definitive assay for PLD activity and the high basal counts associated with [^{3}H]PtdOH meant that only small increases were apparent making this is a relatively insensitive assay. Whether the high degree of labelling of [^{3}H]PtdOH indicates that palmitate is a major constituent of PtdOH or that there are hormone-sensitive pools of phospholipid which are poorly enriched with palmitate is not clear. Other groups have used the same t.l.c. methodology described here to resolve PtdOH from cells labelled with [^{3}H]oleic or [^{3}H]myristic acid with much lower associated radioactivity (Huang & Cabot, 1990 a & b; Liscovitch & Amsterdam, 1989). This again suggests that there may be a distinct pool of phospholipid, made up of certain fatty acids, which is sensitive to hormone stimulation as has been suggested by Pessin *et al.* (1990). To more clearly define the kinetics of PtdOH formation it seems likely that either a convenient and reliable mass assay must be developed or some form of isotopic labelling and t.l.c. separation protocol analagous to that described by Billah *et al.* (1989b) but suitable for all cell types. Since some small increases in [^{3}H]PtdOHwere seen in response to PMA which exhibited similar kinetics to DG formation the possibility of activation of PLD was examined.

To investigate if stimulation by PMA or bombesin resulted in activation of PLD the ability of Swiss 3T3 cells to form phosphatidylalcohols was assessed. As previously described PLD can catalyse the formation of phosphatidylalcohols by its phosphatidyltransferase activity in the presence of low, non-cytotoxic,

concentrations of primary alcohols such as ethanol and butan-1-ol. Whilst this is probably of little physiological relevance it serves as a useful marker for PLD activity in whole cells since it is not formed by any other pathway in cells including base-exchange (Kanfer, 1980), a putative alcohol kinase/CDP-alcohol transferase pathway (Pai *et al.*, 1988a), or by transfer of alcohols on to CMP-PtdOH (Pai *et al.*, 1988a).

Preliminary experiments confirmed that stimulation of [^{3}H]palmitate-labelled Swiss 3T3 cells with bombesin or PMA in the presence of non-cytotoxic concentrations of butan-1-ol resulted in the formation of a [^{3}H]PtdBut as confirmed by its co-migration with various authentic PtdBut 'standards' (Fig 4.7, a & b) and by the absolute dependency upon butan-1-ol concentration (Fig 4.8). These experiments allowed the unequivocal identification of [^{3}H]PtdBut formation, and therefore PLD activity, in response to bombesin and PMA in Swiss 3T3 cells and optimised the conditions for maximal cellular responsiveness without adverse effects by butan-1-ol.

Stimulation of [^{3}H]palmitate-labelled Swiss 3T3 cells with PMA resulted in increases in [^{3}H]PtdBut (Fig. 4.9) which were significantly above control after 1 min and continued in a near linear fashion for up to 30 min suggesting that the response was not desensitized since phosphatidylalcohols are poor substrates for PPH (Billah *et al.*, 1989b; Bonser *et al.*, 1989; Metz & Dunlop, 1991). Over the same time course the small increase in [^{3}H]PtdOH levels increased at 1 and 2 min but then reached a plateau, suggesting that PMA-stimulated [^{3}H]PtdOH produced by PLD activity is then removed, presumably by PPH. Additional evidence for [^{3}H]PtdOH produced by PLD activity being the precursor of DG comes from the observation that formation of [^{3}H]PtdBut clearly precedes [^{3}H]DG formation; the former being significantly elevated at 1min whilst the latter was increased between 1 and 2 min. Since the kinetics of PLD activation preceded the onset of PMA-stimulated [^{3}H]DG formation the normal product of PLD, PtdOH, could be a precursor for [^{3}H]DG (Martin, 1988; Billah *et al.*, 1989b); this may also explain why increases in PtdOH were so

hard to detect since in this case the molecule would be rapidly de-phosphorylated by PPH. Furthermore, the EC_{50} for PMA-stimulated [^{3}H]PtdBut formation was virtually identical to that for PMA-stimulated DG formation suggesting that both arose from the phorbol ester interacting at a common site.

Stimulation [^{3}H]palmitate-labelled Swiss 3T3 cells with bombesin resulted in the time- and dose-dependent formation of [^{3}H]PtdBut (Fig. 4.10, a & b). Bombesin-stimulated increases in [^{3}H]PtdBut were apparent as early as 15 sec (Fig 4.15) after addition of bombesin and rose rapidly from 30 sec to 2 min after which the rate of increase was greatly reduced but sustained in some cases up to 15 min (Fig. 4.10a). The drop in the rate of [^{3}H]tdBut formation after 2 min may reflect desensitization. However, the observation that the levels of [^{3}H]PtdBut continued to rise from 2 to 15 min in some experiments, albeit at a reduced rate, questions whether this desensitization was complete. The levels of [^{3}H]PtdBut remained unchanged in the absence of bombesin indicating that PLD activity was largely ligand-dependent, occurring at a very slow rate in unstimulated cells.

The EC_{50} for bombesin stimulated [^{3}H]PtdBut formation (Fig 4.10b) was of a very similar order to that for previously described parameters such as Ins(1,4,5)P_3 and DG formation, as well as [^{3}H]InsP_t accumulation, suggesting that all these events were perhaps mediated through the same population of receptors.

In the case of bombesin, using [^{3}H]PtdBut formation as an indicator of PLD activation, there was a clear temporal dissociation of PLD activation from Ins(1,4,5)P_3 formation and the second phase of bombesin-stimulated DG formation. Thus, bombesin-stimulated [^{3}H]PtdBut formation was apparent after 15 sec at which time Ins(1,4,5)P_3 mass formation and the first phase of DG formation were declining from their peak values. Despite this, [^{3}H]PtdBut formation preceded the onset of the second phase of bombesin-stimulated DG formation, again arguing that PLD activation and therefore PtdOH formation, occurred prior to, rather than as a consequence of, the sustained elevation of DG. This together with the fact that the EC_{50} values for bombesin-stimulated

Ins(1,4,5)P_3, DG mass and [^{3}H]PtdBut formation were essentially the same suggested that the activation of PLD might occur via the same receptor as that stimulating inositol lipid hydrolysis, but downstream of this event. As such the PtdOH normally produced by PLD activity might serve as the precursor of the second phase of DG formation. In such a scenario a link between the two phospholipases would be required and since PMA can stimulate both PLD and a sustained increase in cellular DG content in the absence of inositol lipid hydrolysis this would suggest that protein kinase C may represent such a link between transient, PtdIns(4,5)P_2-derived and sustained, PLD/PPH-derived DG formation.

4.3.3 The PLD/PPH pathway makes different contributions to bombesin- and PMA-stimulated DG formation.

The use of ethanol or butan-1-ol to inhibit DG formation has been demonstrated by a number of groups in various cells types (e.g., Bonser *et al.*, 1989; Huang & Cabot, 1990a). Since the phosphatidylalcohol formed by the transferase reaction of PLD is a poor substrate for PPH (Billah *et al.*, 1989b; Bonser *et al.*, 1989; Metz & Dunlop, 1991) the inclusion of butan-1-ol effectively interrupts the PLD/PPH pathway of DG formation. Perhaps the most significant observations have been by Bonser *et al.* (1989) who showed that butan-1-ol caused a dose-dependent inhibition of f-Met-Leu-Phe-stimulated DG formation in the human neutrophil which correlated with a loss of PtdOH formation and the appearance PtdBut. Furthermore, over the same dose range, butan-1-ol inhibited f-Met-Leu-Phe-stimulated O_2^- generation indicating that the PLD/PPH pathway of DG formation was required for the physiological response, the oxidative burst.

Since the kinetics of PLD activation in response to both PMA and bombesin suggested that the PLD/PPH pathway could make a contribution to sustained DG elevation, the ability of butan-1-ol to inhibit DG formation by both

agonists was examined. The results in Fig. 4.11 indicate striking differences between PMA and bombesin in the role of the PLD/PPH pathway of DG formation. The presence of butan-1-ol at 0.3% (v/v), a concentration giving maximal [^{3}H]PtdBut formation, resulted in inhibition of 76 ± 14% of PMA-stimulated DG formation suggesting that the major pathway was that of PLD/PPH. In contrast, at the same concentration of butan-1-ol, only 30 ± 5% of bombesin-stimulated DG formation was inhibited suggesting that the PLD/PPH pathway was of less quantitative importance for bombesin-stimulated diglyceride generation.

These results are of interest for a number of reasons. Firstly, they suggest that the PMA-stimulated increase in [^{3}H]PtdBut does indeed reflect PLD activity and that the normal product, PtdOH, would usually be dephosphorylated to DG. As such this would confirm the reports in a wide variety of cell types that phorbol esters do activate PLD (Huang & Cabot, 1990a; Martin *et al.*, 1990; reviewed by Billah & Anthes, 1990). However, it seems likely that not in all cases will the PtdOH produced by PLD activity serve as the precursor of DG by the PPH reaction. Huang & Cabot (1990a) showed that PMA is able to activate PLD in a variety of cell lines including MDCK epithelial cells, bovine pulmonary endothelial cells and two smooth muscle cell lines. However, in the smooth muscle cell lines they were unable to demonstrate inhibition of PMA-stimulated DG formation by ethanol suggesting that in these cell lines the purpose of PLD activation is not to form DG by the PLD/PPH pathway. The demonstration that 30% of the bombesin-stimulated DG formation is inhibited by butan-1-ol indicates that some of the sustained DG formation is occurring via the PLD/PPH pathway; however, in comparison with PMA this is clearly a minor pathway. This may reflect the observation that bombesin-stimulated PLD activity is essentially transient (Fig. 4.10); [^{3}H]PtdBut formation was rapid in onset up to 2 min but was then greatly reduced from 2 min onwards. The effect of butan-1-ol upon bombesin-stimulated DG formation was assessed at 15 min when PLD activity was desensitised; it will be of interest to examine the effect of butan-1-ol upon DG formation at earlier points in the bombesin time course when PLD

activity is still maximal (e.g., 1-1.5 min).

Since the assay for [^{3}H]PtdOH formation was not of adequate sensitivity it was not possible to determine if the differences between bombesin and PMA were due to butan-1-ol not completely inhibiting bombesin-stimulated PtdOH formation. Such an explanation seems unlikely since 0.3% butan-1-ol resulted in maximal [^{3}H]PtdBut formation for both agents suggesting that butan-1-ol was competing with water to the same extent in both bombesin- and PMA-stimulated PLD reactions. For this reason these results must be considered preliminary and should be confirmed by studying the inhibition of PtdOH formation by butan-1-ol as well as the effect of proposed inhibitors of PPH. However, with these caveats in mind, the results suggest that in comparison with PMA the PLD/PPH route is a relatively minor pathway of bombesin-stimulated DG formation indicating that the action of PMA is not simply to mimic the second phase of bombesin-stimulated DG formation, some of which may be formed by an additional pathway. The studies of Huang & Cabot (1990) suggest that an alternative pathway may be a PtdCho-PLC acting in parallel with the PLD activity described above. In addition there remains the observation that accumulation of [^{3}H]InsP_t in response to bombesin continued for up to 30 min (Fig. 3.1a) and 1 hour (data not shown) and this may also contribute to sustained bombesin-stimulated DG accumulation after PLD activity is largely desensitised.

4.3.4 A role for PKC in activation of phospholipase D ?

Evidence for a role for PKC in the activation of phospholipase D is widespread having been reported in neutrophils (Billah *et al.*, 1989a), HeLa cells (Hii *et al.*, 1989), pulmonary endothelial cells (Martin *et al.*, 1990), NIH3T3 cells (Ben-Avi & Liscovitch, 1989) and NG108-15 cells (Liscovitch, 1988). Studies have typically involved the use of phorbol esters and cell permeant DG analogues, PKC inhibitors such as staurosporine and H7 (Huang & Cabot, 1990b; Liscovitch & Amsterdam, 1989) and also the use of chronic treatment with high doses of phorbol ester to down-regulate PKC (e.g.,

Liscovitch & Amsterdam, 1989). Addition of both bombesin and PMA to Swiss 3T3 cells results in the rapid activation of PKC as assayed by phosphorylation of an 80kDa phosphoprotein (Kazlauskas & Cooper, 1988; Erusalimsky *et al.*, 1988). Since both [Arg^8]Vp and $PGF_{2\alpha}$ were able to activate PLD (Fig 4.12) and appeared to stimulate inositol lipid hydrolysis by similar mechanisms it seemed possible that activation of PLD might occur through a common pathway via activation of PKC.

To address the question of whether PKC was involved in the activation of PLD by bombesin and PMA, Swiss 3T3 cells were pre-treated for 48 hours with 400nM PMA to down-regulate PKC. The fact that under these conditions the ability of both mitogens to stimulate [^{3}H]PtdBut formation was completely abolished (Fig. 4.13), suggests an absolute requirement for PKC in the activation of PLD. A simple working hypothesis to fit this observation might be that activation of PKC resulting from the first phase of bombesin-stimulated DG formation might lead to the phosphorylation of PLD resulting in its activation. To confirm such an idea use was made of a recently described PKC inhibitor, Ro-31-8220 (Davis *et al.*, 1989). The observation that pre-treatment of Swiss 3T3 cells with Ro-31-8220 for 5 min resulted in the dose-dependent, near total, inhibition of PMA-stimulated [^{3}H]PtdBut formation (Fig 4.14a) is consistent with the result in down-regulated cells suggesting that PMA exerts its effect upon PLD by activation of PKC. This is supported by the IC_{50} value for inhibition of the PMA response (0.87 ± 0.43 μM) which was similar to the value for inhibition of PKC by Ro-31-8220 in an *in vivo* assay (Davis *et al.*, 1989). However, in the same series of experiments, using the same stock preparation of the inhibitor, bombesin-stimulated [^{3}H]PtdBut formation was only inhibited by approximately 50% (Fig 4.14b), with the same IC_{50} value (1.12 ± 0.47 μM), and yet, like PMA, the response to bombesin was completely abolished in down-regulated cells. This 50% inhibition of bombesin-stimulated [^{3}H]PtdBut formation was observed at all stimulated time points tested, from 15 sec (Fig 4.15) to 15 min (Fig 4.14), ruling out the possibility of a PKC-independent PLD activity which was only observed at early times in the time course. These results

cannot easily be reconciled with a simple model in which activation of PKC as a consequence of PtdIns(4,5)P_2 hydrolysis is solely responsible for the activation of PLD and, therefore, the sustained generation of DG.

There may be a number of possible explanations for the apparently anomalous results obtained using both PKC 'down-regulation' and PKC inhibitors as approaches in defining PKC-dependent PLD activity. Perhaps one potential model lies in the idea that PMA may not exert all its effects via activation of PKC; this has also been suggested by Billah and co-workers (Billah & Anthes, 1990) and is supported by the discovery of novel non-PKC type phorbol ester receptors (Ahmed *et al.*, 1990). If PMA is able to bind to PLD and activate it in a manner analagous to its activation of PKC then PLD might also be 'down-regulated' and this could explain the loss of PLD activity stimulated by both bombesin and PMA. Again, however, this model does not account for the total inhibition of PMA-stimulated PLD activity by the PKC inhibitor Ro-31-8220 which suggests that the effects of PMA can be accounted for solely by activation of PKC.

The fact that Ro-31-8220 inhibition of bombesin-stimulated PLD activity was only ever approximately 50% of the total maximum response implies that at least some of the response might be activated by a PKC-independent pathway. Alternatively the observation that PKC is now known to constitute a large family of isozymes with different properties may point to bombesin-stimulated PLD activity being mediated by an isozyme which is sensitive to down-regulation but relatively less sensitive to Ro-31-8220. To examine whether some of the bombesin-stimulated PLD activity could be accounted for by PKC-independent mecahnisms its dependency upon extracellular Ca^{2+} concentration, and therefore Ca^{2+} entry, was examined.

The 57 ± 5 % inhibition of bombesin-stimulated PLD activity by the buffering of extracellular Ca^{2+} to approximately 140 nM (Fig 4.16) suggests that increases in intracellular Ca^{2+} play some role in regulation of PLD activity *per se*. The major problem in interpreting such results is that it is not clear whether

the inhibition of PLD activity is due to loss of Ca^{2+} entry , loss of Ca^{2+} mobilization from intracellular stores due to their depletion during the EGTA treatment period, or depletion of intracellular Ca^{2+} which is required as a co-factor for PLD but does not, alone, activate or stimulate the enzyme.

Although A23187-stimulated increases in $[Ca^{2+}]_i$ stimulated [^{3}H]PtdBut formation this does not mean that PLD is simply activated by Ca^{2+} which could exert its effects at some other site. For example, it is known that increases in $[Ca^{2+}]_i$ are involved in activation of PKC which has already been shown to play a role in activation of PLD (Figs. 4.13 and 4.14). The ability of A23187 to activate PLD alone (Fig. 4.17) indicates that increasing $[Ca^{2+}]_i$ will activate PLD; however the 58 ± 14 % inhibition of A23187-stimulated PLD activity by pre-treating cells with Ro-31-8220 suggests that at least half of this response may be due to Ca^{2+}-induced activation of PKC which is then responsible for activating PLD. This is supported further by the fact that the inhibition of bombesin-stimulated PLD activity by EGTA or Ro-31-8220 is not greatly enhanced by combination of the two treatments suggesting that they are acting at the same site; in the presence of EGTA and Ro-31-8220 30 ± 9 % of the bombesin response remained intact (Fig. 4.16). A simple model to explain this might suggest that bombesin-stimulated increases in DG and Ca^{2+} entry due to bombesin-stimulated PtdIns(4,5)P_2 hydrolysis cooperate to stimulate PKC which is then responsible for activation of PLD. However, the results in Fig. 4.16 suggest that this cannot be a full explanation since there is still residual bombesin-stimulated PLD activity in the presence of Ro-31-8220 and EGTA whilst some A23187-stimulated PLD activity remains in the presence of Ro-31-8220 (Fig. 4.17). Thus it seems possible that some of the effects of increased $[Ca^{2+}]_i$ upon PLD activity may be independent of PKC and represent effects upon PLD itself.

In some experiments the ability of A23187 to stimulate [^{3}H]PtdBut formation was not completely inhibited by the chelation of extracellular Ca^{2+} with EGTA. At first site this is an unusual result since the role of A23187 is to transport Ca^{2+} into the cell and so chelation of Ca^{2+} would be expected to inhibit its effects completely. There may be two possible explanations for this. Firstly,

there was no access to a Ca^{2+} electrode to confirm the final concentration of Ca^{2+} in DMBH/EGTA and thus there may have been a higher than calculated $[Ca^{2+}]_o$ allowing sufficient to be taken into the cell for activation of PLD, by whatever means. Secondly, A23187 acts by carrying Ca^{2+} ions across membranes; it is possible that some of the ionophore entered the cell and actually carried Ca^{2+} out of intracellular stores into the cytosol thereby overcoming the depletion of extracellular Ca^{2+}.

It seems likely that a definition of the roles played by Ca^{2+} entry and Ca^{2+} mobilization will require the use of specific drugs which inhibit plasma membrane Ca^{2+} channels as well as the use of thapsigargin, a potent inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, which will allow depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} store. Treatment of cells with thapsigargin results in a small increase in $[Ca^{2+}]_i$ but results in the inhibition of subsequent Ins(1,4,5)P_3-stimulated Ca^{2+} mobilisation (Thastrup *et al.*, 1990). In addition agents such as BAPTA may be used which will allow buffering of changes in $[Ca^{2+}]_i$ and so prevent any input from release of intracellular stored Ca^{2+}. Such approaches should allow future definition of the relative importance of Ca^{2+} mobilization from intracellular stores and Ca^{2+} entry on activation of PLD. What is clear is that there may be marked differences in the role of Ca^{2+} in activation of PLD in different cell types. For example in neutrophils (Agwu *et al.*, 1989) and in human erythroleukaemia cells (Halenda *et al.*, 1990) A23187 seems to be even more effective at activating PLD than PMA. In Swiss 3T3 fibroblasts the role of Ca^{2+} seems to be subordinate to that of the DG/PMA-PKC signal. Quite how these differences in activation of PLD correlate with the physiological responses of these cell to the various agonists is not clear.

In addition to the possible role of Ca^{2+} entry in PLD regulation, there remains the observation that bombesin-stimulated PLD activity is still apparent in the presence of both EGTA and Ro-31-8220. Whether this points to a possible direct coupling of receptor to PLD independently of inositol lipid hydrolysis, activation of PLD by Ca^{2+} mobilised from Ins(1,4,5)P_3-sensitive intracellular

stores or a role for DG or arachidonic acid in activating PLD is not clear and future studies should aim to address these points.

Chapter 5.

Hydrolysis of phosphatidylcholine by phospholipase D is a common response to mitogens which stimulate inositol lipid hydrolysis in Swiss 3T3 cells.

5.1 Introduction.

In the previous chapters results have been presented to show that bombesin stimulates a biphasic increase in cellular DG content; the first corresponds to the transient elevation of Ins(1,4,5)P_3 mass whilst the second phase is sustained and divorced from any elevation of Ins(1,4,5)P_3. The sustained phase is mimicked by addition of phorbol esters but this occurs in the absence of inositol lipid hydrolysis; indeed PMA inhibits bombesin-stimulated inositol lipid hydrolysis. Kinetic and functional studies suggest that some part of this sustained phase of DG formation may occur via a PLD/PPH pathway which is mediated in part by prior activation of PKC. Furthermore, it seems likely that the substrate for this mitogen stimulated PLD is a non-inositide phospholipid.

Since the sustained elevation of DG stimulated by bombesin and phorbol ester is essentially divorced from inositol lipid hydrolysis it is important to identify the phospholipid source. In theory any glycerophospholipid may serve as a source of DG either directly by PLC-catalysed hydrolysis or via the PLD/PPH pathway. A number of recent studies have reported that phosphatidylcholine (PtdCho) hydrolysis and synthesis are increased in response to a variety of stimuli (Billah & Anthes, 1990). Consequently studies were undertaken to see if PtdCho hydrolysis was enhanced by mitogenic stimuli in Swiss 3T3 cells.

Evidence for stimulated PtdCho metabolism playing a role in signal transduction events dates back to the late 1970s and early 1980s. Some of these observations have remained unsubstantiated, for example the suggestion that activation of PtdEtn methyltransferase played a role signal transduction in mast cells (reviewed by Axelrod & Hirata, 1982), whilst the significance of others is only now being realised. One of the earliest studies of relevance to increased cellular DG content was the work of Grove & Schimmel (1982) who showed that addition of PMA to cultured chick embryo myoblasts resulted in an elevation of 1,2-DG levels and stimulated the synthesis of PtdOH and PtdCho. A number of studies have demonstrated that addition of phorbol esters, serum, and a variety of receptor agonists to various cell types results in the synthesis of PtdCho as measured by the incorporation of [^{3}H]choline or [^{32}P]Pi into PtdCho.

In Swiss 3T3 cells there is a rapid 2-3 fold increase in choline kinase activity following stimulation with either whole serum or phorbol esters (Warden & Friedkin, 1984 &1985) and this is reflected in an increase in levels of phosphocholine. There is also evidence that the half-life of phosphocholine is altered in response to serum and phorbol esters and that this is likely to be due to the translocation and activation of CTP:phosphocholine cytidylyltransferase (Lim *et al.*, 1983; reviewed by Pelech & Vance, 1984), a rate limiting step in the synthesis of PtdCho.

Despite extensive studies on agonist-stimulated PtdCho synthesis a primary hydrolytic event must be identified if considering a role for PtdCho as a source of DG. Mufson *et al.* (1981) were one of the first groups to report stimulated PtdCho hydrolysis when they showed that addition of PMA to C3H10T1/2 mouse embryo fibroblasts resulted in the release of [^{3}H]choline (Cho) and [^{3}H]phosphocholine (ChoP) and similar effects of PMA have now been reported in a variety of cell types including Swiss 3T3 fibroblasts (Muir & Murray, 1987; Price *et al*, 1989), NG108-15 cells (Liscovitch *et al*, 1987) and endothelial cells (Martin *et al.*, 1990).

Evidence for receptor-coupled PtdCho hydrolysis was a little slower in appearing and was probably first observed in fMet-Leu-Phe-stimulated neutrophils by Cockcroft (1984) though at the time it was attributed to PtdIns hydrolysis by phospholipase D. The list of agonists now known to stimulate the hydrolysis of PtdCho is quite wide ranging and this topic has recently been reviewed extensively (Pelech & Vance, 1988; Loffelholz,1989; Exton, 1990; Billah and Anthes, 1990). The best characterised systems remain the fMet-Leu-Phe-stimulated human neutrophil and HL-60 cell (Pai *et al.*, 1988a & b; Billah *et al.*, 1989a & b) but other agonists known to stimulate PtdCho hydrolysis include bombesin, vasopressin & PDGF in Swiss 3T3 cells (Muir & Murray, 1987; Price *et al.*, 1989), bradykinin and purinergic agonists in endothelial cells (Martin & Michaelis, 1988 & 1989b), carbachol in 1321N1 astrocytoma cells (Martinson *et al.*, 1989), gonadotrophin-releasing hormone in ovarian glomerulosa cells (Liscovitch & Amsterdam, 1989) and also in spermatozoa stimulated to undergo the acrosome

reaction by a fucose sulphate glycoconjugate (Domino *et al*, 1989).

Hydrolysis of PtdCho to generate DG may proceed by either activation of a PtdCho-PLC to generate DG and phosphocholine (ChoP) or by activation of a PtdCho-PLD to generate PtdOH and choline (Cho) followed by de-phosphorylation of PtdOH to DG by PtdOH phosphohydrolase (PPH). There are a number of difficulties in unequivocally identifying which is the primary hydrolytic event. For example, the elevation of ChoP (a PLC product) can be achieved by the rapid phosphorylation of Cho (a PLD product) by choline kinase (Warden & Friedkin, 1985) though the reverse reaction, ChoP phosphatase, seems unlikely to function (Cabot *et al.*, 1988). Equally, the generation of [^{32}P]PtdOH in [^{32}P]Pi-labelled cells can also be achieved by phosphorylation of DG (a PLC product) by DG kinase, a reaction occurring rapidly following stimulation of inositol lipid hydrolysis.

Definitive assays for PLD activity are now available and the use of 1-*O*-alkyl-2-acyl-*sn*-glycerol[^{32}P]phosphate ([^{32}P]PtdOH) in neutrophils and the formation of phosphatidylalcohols in the presence of short chain primary alcohols have already been described (Chapters 1 & 4 of this thesis).

Having identified PLD activity in mitogen-stimulated Swiss 3T3 cells it was necessary to confirm that this activity was utilizing PtdCho as a substrate. One obvious way to approach this is to label cells with [^{3}H]choline and examine the release of the water soluble head groups Cho or ChoP. Not only will this confirm or refute PtdCho as a substrate, but by examining the kinetics of formation of either Cho or ChoP one should be able to confirm a PLD or PLC activity previously characterized. Many early studies in this field made no attempt to separate individual metabolites and simply attributed any increase in 'total water soluble metabolites' as evidence for PtdCho-PLC activity (e.g., Muir & Murray, 1987; Monaco *et al.*, 1988; Welsh *et al.*, 1988).

The following results describe the development of a simple ion-exchange chromatography procedure to separate the major water soluble metabolites of endogenous [^{3}H]PtdCho hydrolysis in Swiss 3T3 cells. This method has been used to study the effects of defined mitogenic agents upon PtdCho hydrolysis. The

results confirm that activation of PtdCho hydrolysis by PLD is a common response to mitogens which stimulate hydrolysis of inositol lipids in Swiss 3T3 cells.

5.2 Results.

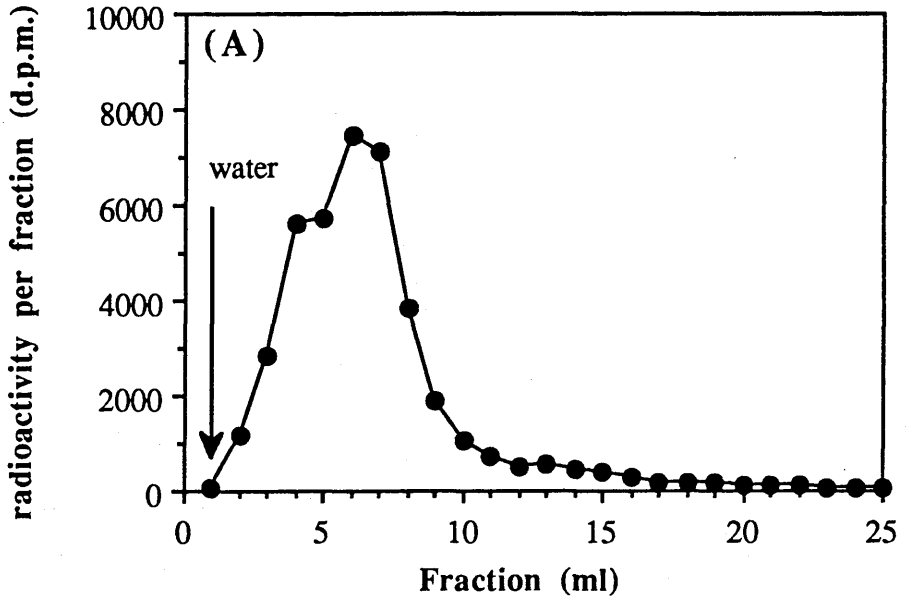
5.2.1 Analysis of the water soluble products of PtdCho hydrolysis by ion-exchange chromatography.

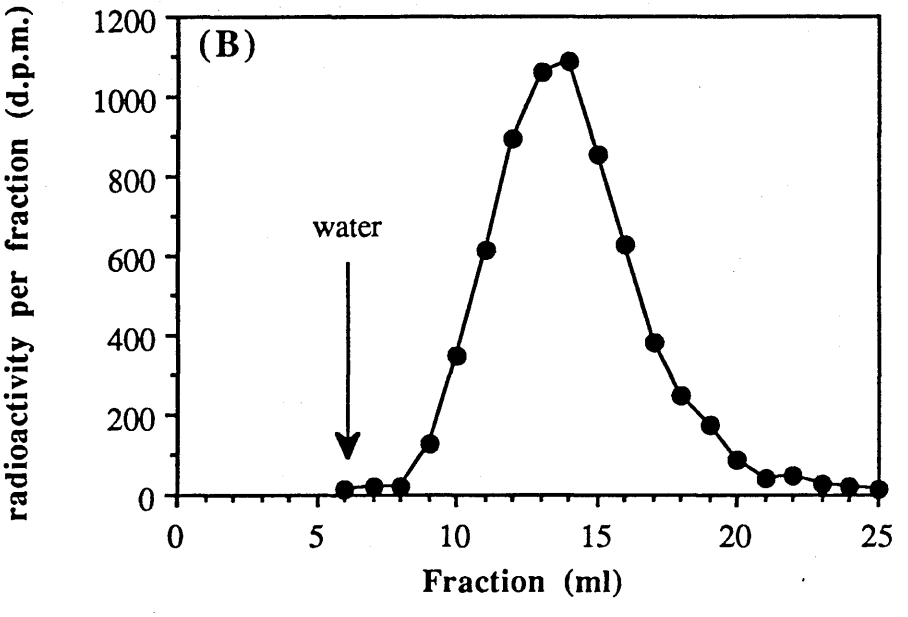
The separation of choline metabolites has been most commonly reported by the method of Yavin (1976) which involves thin layer chromatography. However, preliminary work suggested that this method, as well as being time-consuming and laborious, did not adequately resolve the major water soluble metabolites of PtdCho hydrolysis; a fundamental drawback since in many cells phosphocholine levels exceed those of choline by up to six-fold making even minor cross-contamination a serious problem. The modifications of Kolesnick & Paley (1987) go some way to overcoming these problems but it was decided that better resolution might be obtained by an ion-exchange system which would also be quicker and easier to use. The use of Dowex-50W-H^+ cation exchange resin was originally suggested by the work of Dowdall *et al.* (1972) who used it to isolate phosphocholine from neutral extracts of guinea pig cerebral cortex after the intraventricular injection of [N-*methyl*-^{3}H] choline.

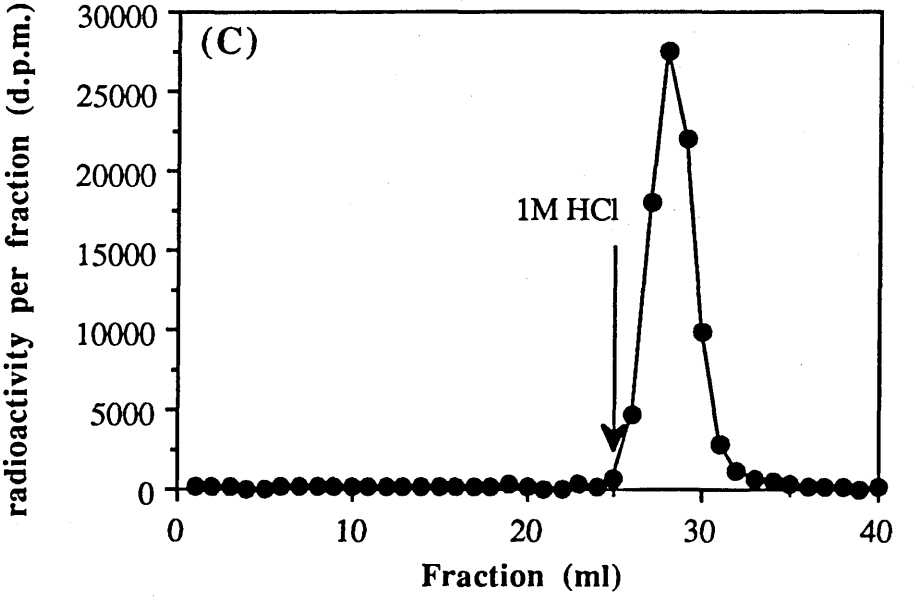
From the structures of choline, phosphocholine and glycerophosphocholine it was decided that the strongly cationic quarternary amino group on the choline moiety provided the best oppurtunity for binding to an acidic cation exchange resin. A sample of [^{3}H] choline chloride diluted to 1ml with water was loaded onto a 1ml column prepared in a glass wool plugged pasteur pipette. Washing with 1ml fractions of water did not elute any radioactivity from the column. Using 1M HCl as a competing cation, 20 x 1ml washes eluted a single peak of radioactivity which corresponded to 100 % recovery of the load and was found to be completely reproducible (Figure 5.1c).

Figure 5.1 (a-c). Elution of (a) [^{3}H]GroPCho, (b) [^{14}C]ChoP and (c) [^{3}H]Cho from 1 ml Dowex-50-WH^{+} columns.

Samples of each 'standard' were diluted with water to 1 ml and loaded on to separate 1 ml columns of Dowex-50-WH^{+} in plugged pasteur pipettes. Elutions were performed with either water or 1M HCl as indicated in a ml by ml manner and each sample was collected and the associated radioactivity (d.p.m.) determined by liquid scintillation counting. The results shown are from a single experiment performed in singlicate but representative of 8 other similar elutions.


(A)
water
radioactivity per fraction (d.p.m.)
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
Fraction (ml)


(B)
water
radioactivity per fraction (d.p.m.)
1200
1000
800
600
400
200
0
0
5
10
15
20
25
Fraction (ml)


(C)
1M HCl
radioactivity per fraction (d.p.m.)
30000
25000
20000
15000
10000
5000
0
0
10
20
30
40
Fraction (ml)

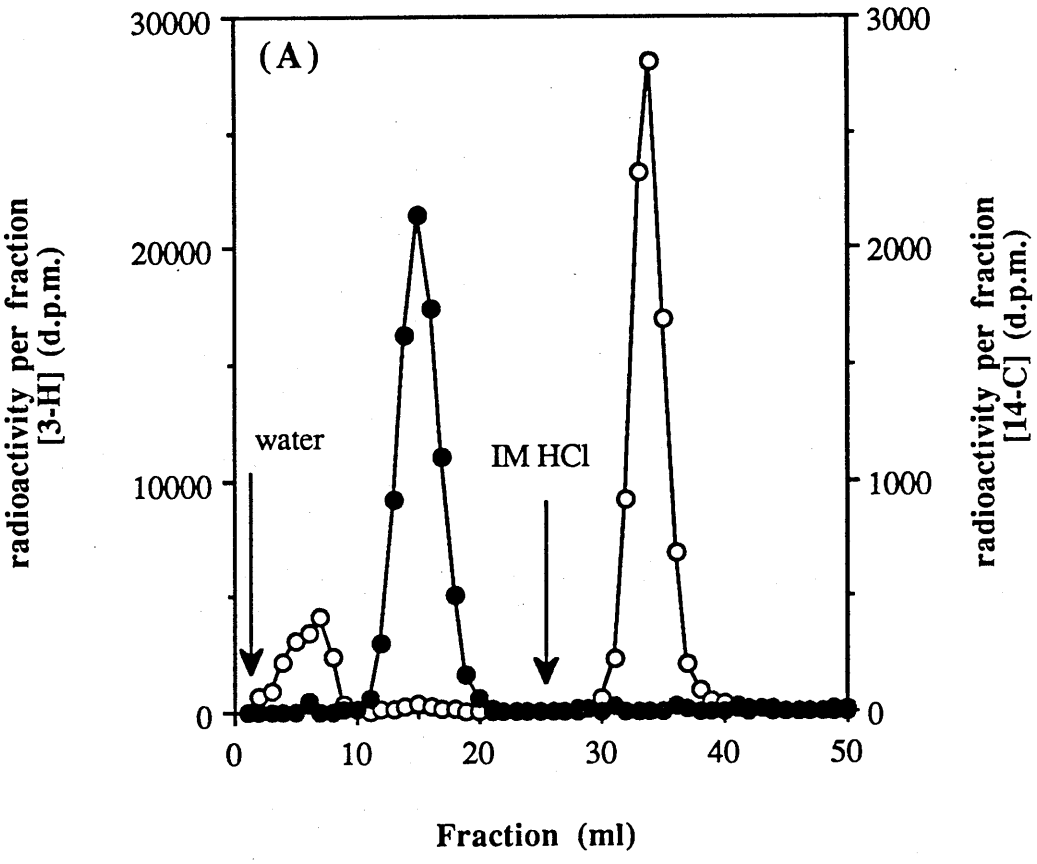
On the basis that the phosphate group on phosphocholine would to some extent counteract the strong positive charge of choline resulting in weaker binding, a sample of [^{14}C] phosphocholine was loaded on to a 1ml Dowex-50W-H^+ column in 1ml of water. Washing with 15 x 1ml fractions of water eluted off a single peak of activity after an initial lag of 9ml (Figure 5.1b). Recovery of the loaded [^{14}C] phosphocholine was 96%. When a sample of [methyl-^{3}H] glycerophosphocholine was loaded in 1ml of water and washed with 1ml fractions of water it was found to elute as a single peak of activity in the first 10 ml of water with a recovery of 95% of the loaded activity (Figure 5.1a). Further characterisations using Hanks buffered saline as a 'synthetic cell extract' to which was added a mixture of the three standards yielded similar results (Figure 5.2a).

All other chracterisations were performed by adding a mixture of the standards to a Swiss 3T3 cell extract prepared either by chloroform/methanol extraction or 10% perchloric acid extraction so as to mimic experimental conditions. Fractions were counted on a [^{3}H]/[^{14}C] dual label programme allowing estimation of recoveries and crossovers. Batch elutions were found to yield the same results as ml by ml elution profiles. In addition this method was routinely employed to characterise each newly prepared sample of Dowex-50W-H^+ throughout the series of experiments described herein (Fig 5.2b). The average percent recoveries of 'standards' as well as the average percentage cross-overs between different fractions are shown in Table 5.1.

When this procedure was applied to an aqueous methanolic extract from cells which had been labelled with 1μCi/ml of [methyl-^{3}H]choline chloride for 48 hours three peaks of radioactivity were eluted with identical mobility to the standards previously characterised. Furthermore, after elution of [^{3}H]GroPCho and [^{3}H]ChoP the competing cation concentration was increased in four 10 x 1ml steps from 0.25M-, 0.5M-, 0.75M- to 1M HCl to see if any other peaks of activity were eluted off before [^{3}H]Cho. Such stepwise elutions did not reveal any peaks of activity until [^{3}H]Cho which came off as a broad band between 0.5- and 0.75M HCl (Fig 5.3).

Figure 5.2 (a & b). Elution profile of a mixture of [^{3}H]GroPCho, [^{14}C]ChoP and [^{3}H]Cho loaded in either (a) a synthetic cell extract (HBG) or (b) in a Swiss 3T3 cell extract.

A mixture of standards was diluted to 1 ml with either Hanks buffered saline solution as a synthetic cell extract (a) or with a cell extract prepared from unlabelled Swiss 3T3 cells by chloroform/methanol extraction (b). Each mix was then applied to separate 1ml Dowex-50-WH^{+} columns in plugged pasteur pipettes and ml by ml elutions were performed with water followed by 1M HCl as indicated. Each 1 ml fraction was collected and counted on a [^{14}C] (●) and [^{3}H] (○) dual label programme. The profiles shown represent radioactivity in each fraction (d.p.m.), and are typical of seven other similar experiments.


(A)
30000
20000
10000
0
3000
2000
1000
0
water
IM HCl
0
10
20
30
40
50
radioactivity per fraction
[3-H] (d.p.m.)
radioactivity per fraction
[14-C] (d.p.m.)
Fraction (ml)

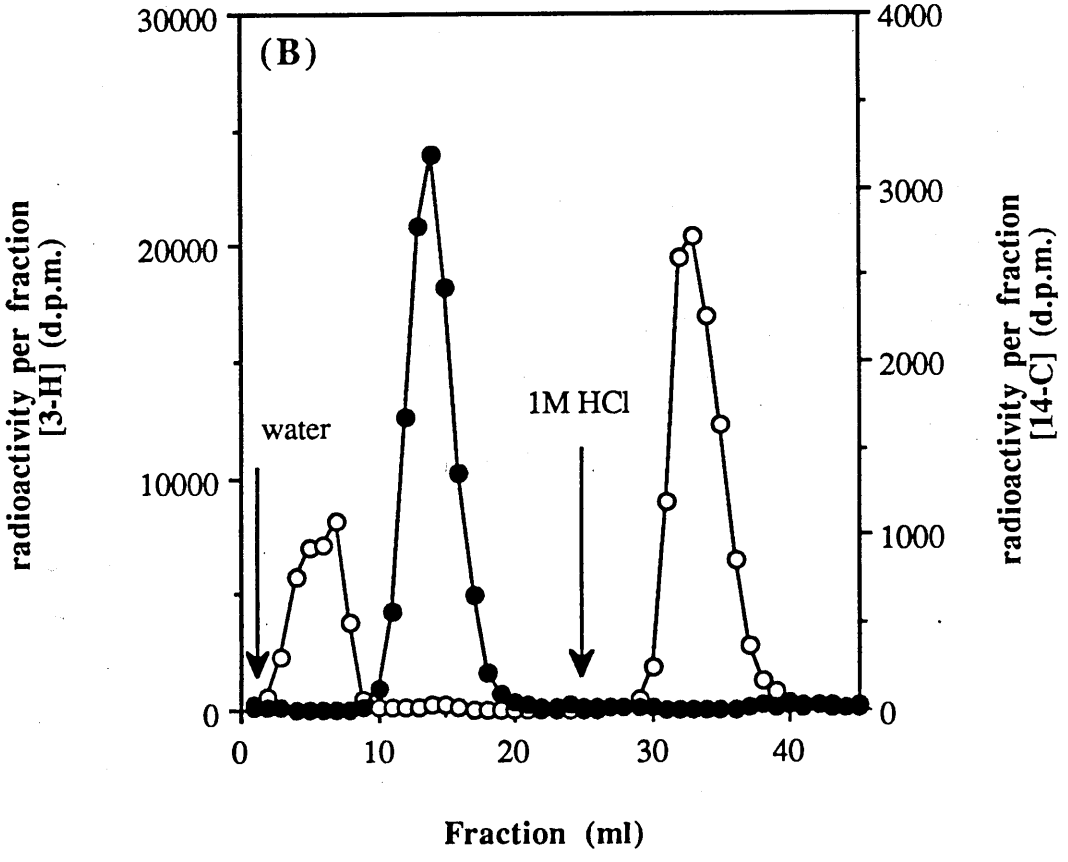

(B)
30000
20000
10000
0
4000
3000
2000
1000
0
water
1M HCl
0
10
20
30
40
radioactivity per fraction
[3-H] (d.p.m.)
radioactivity per fraction
[14-C] (d.p.m.)
Fraction (ml)

Figure 5.3. The elution of [^{3}H]Cho by stepwise increases of HCl reveals no further [^{3}H]choline labelled bands in Swiss 3T3 cells.

One 25 cm^2 flask of Swiss 3T3 cells was labelled with 1μCi ml^{-1} for 48 hours before harvesting and preparation of an aqueous methanolic extract as described in the Methods section. The sample, final volume 1 ml, was applied to a 1ml Dowex-50-WH^+ column and [^{3}H]GroPCho and [^{3}H]ChoP eluted with 9 and 15 ml of water respectively. The concentration of the competing cation, HCl (---), was increased by four stepwise 10 x 1 ml elutions with 0.25, 0.5, 0.75 and 1M HCl. All fractions were collected and the radioactivity determined by liquid scintillation counting. Results are radioactivity in fraction (d.p.m.), (●); similar results were obtained in two experiments

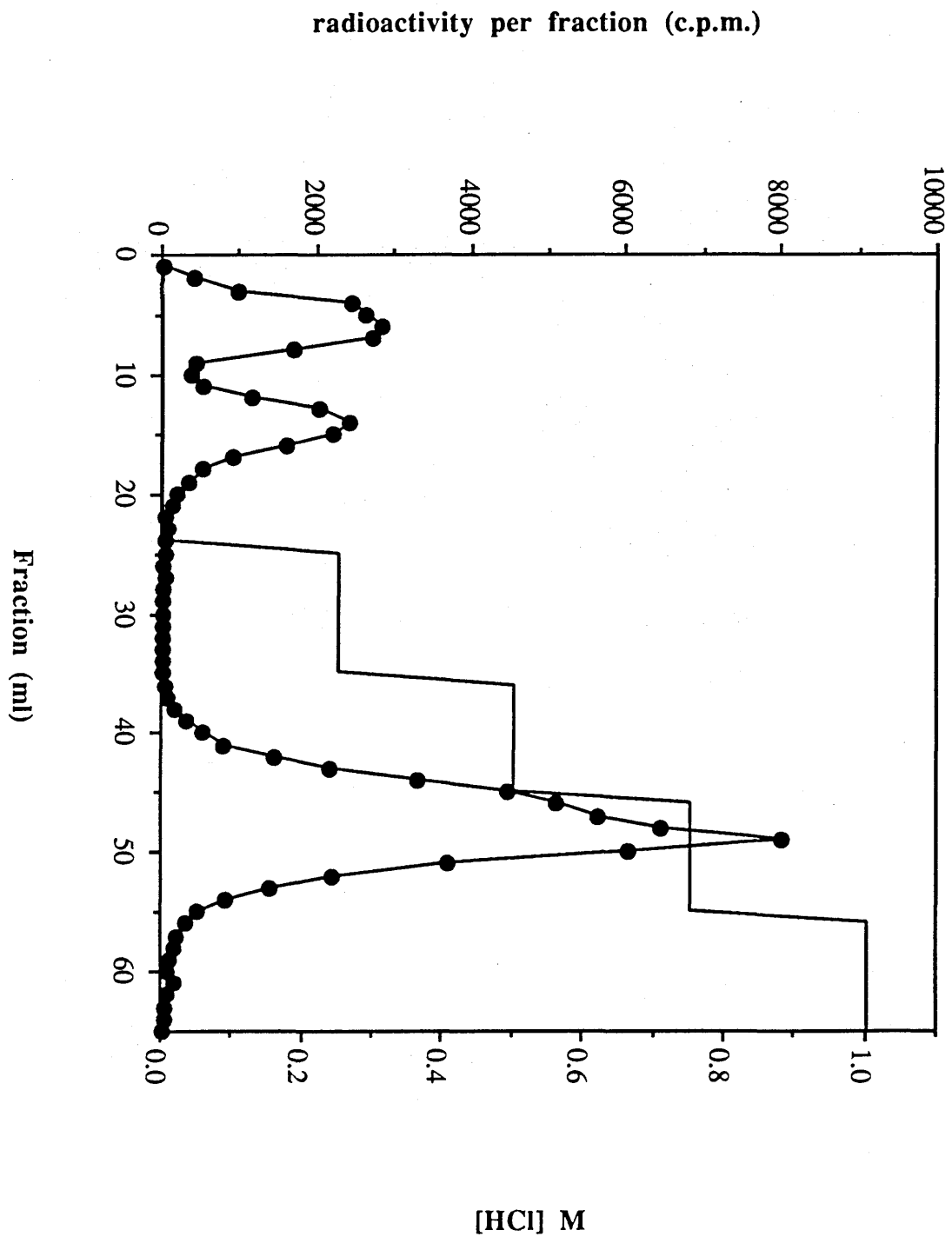

radioactivity per fraction (c.p.m.)
0
2000
4000
6000
8000
10000
Fraction (ml)
0
10
20
30
40
50
60
[HCl] M
0.0
0.2
0.4
0.6
0.8
1.0

Table 5.1. Analysis of recoveries of, and cross-contamination between, [^{3}H] or [^{14}C]choline-labelled metabolites separated on Dowex-50-WH^{+}.

Standard samples of [^{3}H]GroPCho, [^{14}H]ChoP and [^{3}H]Cho were separated on Dowex-50-WH^{+} columns in the presence of an aqueous methanolic extract prepared from Swiss 3T3 cells. Recoveries and cross-overs were determined by dual-label liquid scintillation counting. The results are means ± S.D. calculated from eight separate experiments. Other details are as in the Methods section.

N.A. = not applicable; N.D. = not detectable.

	Recovery (%)	% contamination with:		
Fraction		**Cho**	**ChoP**	**GroPCho**
Cho	94.8 ± 8.4	N.A.	2.51 ± 4	N.D.
ChoP	92.6 ± 9.1	2.5 ± 4	N.A.	2.9 ± 2.8
GroPCho	91.5 ± 7.5	N.D.	6.1 ±2.8	N.A.

Finally, to assay the integrity of the separation of GroPCho and ChoP batchwise elutions of a [methyl-^{3}H]choline chloride labelled cell extract were freeze dried, redissolved in ethanol/water (50/50; v/v), spotted on to heat activated silica gel 60 thin layer chromatography plates and developed by the method of Kolesnick and Paley (1987). By comparing mobility with known standards it was found that GroPCho and ChoP were resolved well with a cross contamination of only about 8% of each fraction, agreeing well with the values from dual labelling studies.

Analysis of a labelled cell extract by this t.l.c. method revealed that GroPCho, ChoP and Cho were the only major labelled products; betaine, an oxidative metabolite of choline, was found to be a very minor component of labelled cells representing only 1% of the total activity of an aqueous cell extract, and seemed to remain unchanged when cells were stimulated with either bombesin or PMA. Similar low levels of betaine have been reported previously in Swiss 3T3 cells (Warden & Friedkin, 1984), NG108-15 cells (Liscovitch *et al*, 1987) and brain cells (Yavin, 1976). Isolation of CDP.choline by t.l.c. also revealed it to be a minor component of Swiss 3T3 cells under the labelling conditions employed.

On the basis of these experiments it was concluded that the method of separation devised gave both good resolution and recovery of GroPCho, ChoP and Cho and was suitable for analysing their relative levels in control and stimulated cells. This method has subsequently and successfully been applied to the analysis of choline metabolites in Rat-1 fibroblasts stimulated with Endothelin-1 (MacNulty *et al*, 1990).

5.2.2 The effect of defined growth factors upon PtdCho hydrolysis in Swiss 3T3 cells.

Swiss 3T3 cells were grown to approximately 80-90% confluence in 24-well culture plates and incubated for a further 48 hours, or part thereof, with DMEM containing 1% newborn calf serum and 1μCi ml^{-1}[^{3}H]choline. At given times the medium was aspirated, cells harvested and lipid extracts prepared as

described in the Methods section. Following phase resolution the entire chloroform phase was dried down *in vacuo* and the radioactivity in total choline containing lipids was determined by liquid scintillation counting. The results indicated that equilibrium labelling of the choline containing lipids was essentially reached after 30 hours (data not shown). However, all experiments were performed on cells which had been labelled for at least 48 hours in low serum containing medium to ensure quiescence.

Of the three major choline-containing lipids in Swiss 3T3 cells [^{3}H]choline was preferentially incorporated into PtdCho (85%) with the remainder in sphingomyelin (13-14%). Negligible label was found to be associated with lyso-PtdCho. Upon stimulation of [^{3}H]choline-labelled Swiss 3T3 cells with bombesin or PMA there was a loss of radioactivity from the PtdCho pool with little change in the radioactivity associated with sphingomyelin (Table 5.2). The amount of label lost from PtdCho was small representing only about 5% of the total associated radioactivity. This is in agreement with the finding of Liscovitch *et al.*(1987) in PMA-stimulated NG108-15 cells and probably represents the relatively small generation of Cho seen (Fig 5.5a) as compared with the large size of the cellular PtdCho pool.

Since the loss of label presumably represented a hydrolytic event, the effect of both bombesin and PMA upon Cho, ChoP and GroPCho levels was examined as an indication of which pathway of hydrolysis was being activated. Figure 5.4 shows the elution profile of a cell samples from [^{3}H]choline labelled Swiss 3T3 cells incubated for 5 minutes with either a control buffer or 617nM bombesin. The results indicated that both Cho and ChoP levels increased upon stimulation with bombesin whilst GroPCho appeared to remain unchanged. Further studies sought to define the kinetics of these changes in choline metabolites.

Figure 5.5 (a-c) shows the time course of effects of stimulating [^{3}H]choline labelled Swiss 3T3 cells with 617nM bombesin upon [^{3}H]Cho, [^{3}H]ChoP and [^{3}H]GroPCho levels. Bombesin stimulated a rapid increase in [^{3}H]Cho levels which was apparent at 10 sec, significant between 30 sec and 1 min ($p=0.002$) and was maximally above control (2-2.5 -fold) after 2 min (Fig 5.5a). The rate of

Table 5.2. Effect of bombesin- and PMA-stimulation on the level of PtdCho and sphingomyelin in [^{3}H]choline labelled Swiss 3T3 cells.

Following stimulation of [^{3}H]choline labelled Swiss 3T3 cells with bombesin (617nM), PMA (100nM) or appropriate vehicle (0.1% DMSO) organic extracts were spotted on to heat-activated silica gel 60 t.l.c. plates and developed according to the method of Skipski *et al.* (1964) as described in Chapter 2. PtdCho and sphingomyelin (SphM) were visualised by I_2 staining alongside authentic standards. Radioactivity associated with phosphatidylcholine (PtdCho) and SphM was determined by scintillation counting following excision of the appropriate spots from the silica gel. The results are mean radioactivity in PtdCho or SphM (d.p.m.) ± S.D. of triplicate determinations from a single experiment typical of three.

Incubation.	radioactivity in PtdCho (d.p.m.)	radioactivity in SphM (d.p.m.)
Control, 2 min.	76,961 ± 5,195	7,247 ± 158
Bombesin, 2 min	66,205 ± 1,176	8,644 ± 549
Vehicle, 5 min	80,802 ± 363	8,003 ± 138
PMA, 5 min	75,735 ± 1,926	8,983 ± 194

Figure 5.4 Elution profile from control and bombesin-stimulated, [^{3}H]choline labelled Swiss 3T3 cells.

Swiss 3T3 cells were labelled with [^{3}H]choline, 1.5μCi ml^{-1} in 1 ml of DMEM containing 2% calf serum, for 48 hours by which time they were confluent and quiescent. After washing and incubating at 37°C for 5 min in HBG (○) or HBG containing 617 nM bombesin (●) aqueous methanolic extracts were prepared as described in the Methods section. Samples were applied to 1 ml Dowex-50-WH$^+$ columns in plugged pasteur pipettes and GroPCho, ChoP and Cho eluted in a ml by ml manner. The radioactivity associated with each fraction (d.p.m.) was determined by liquid scintillation counting.

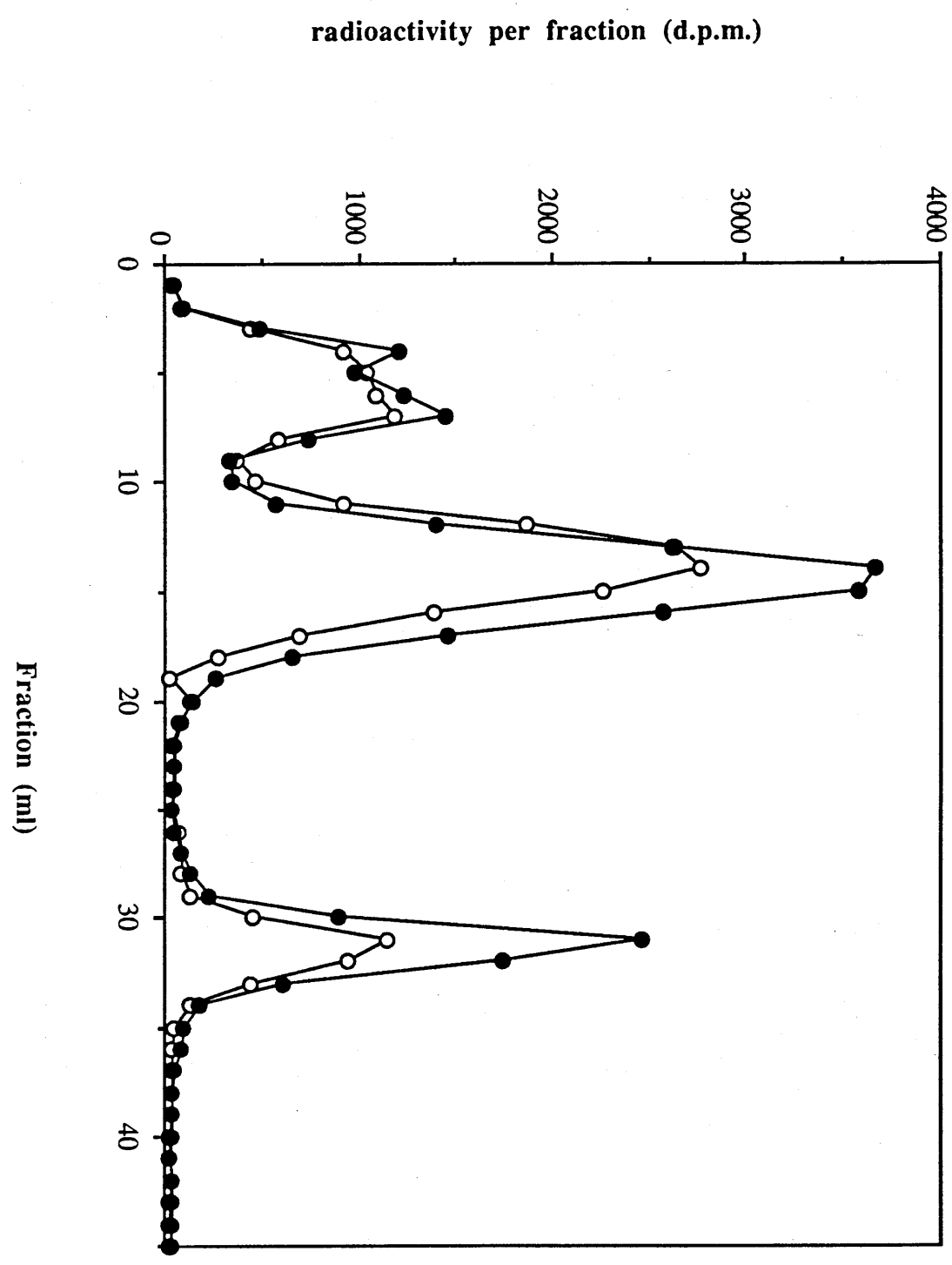
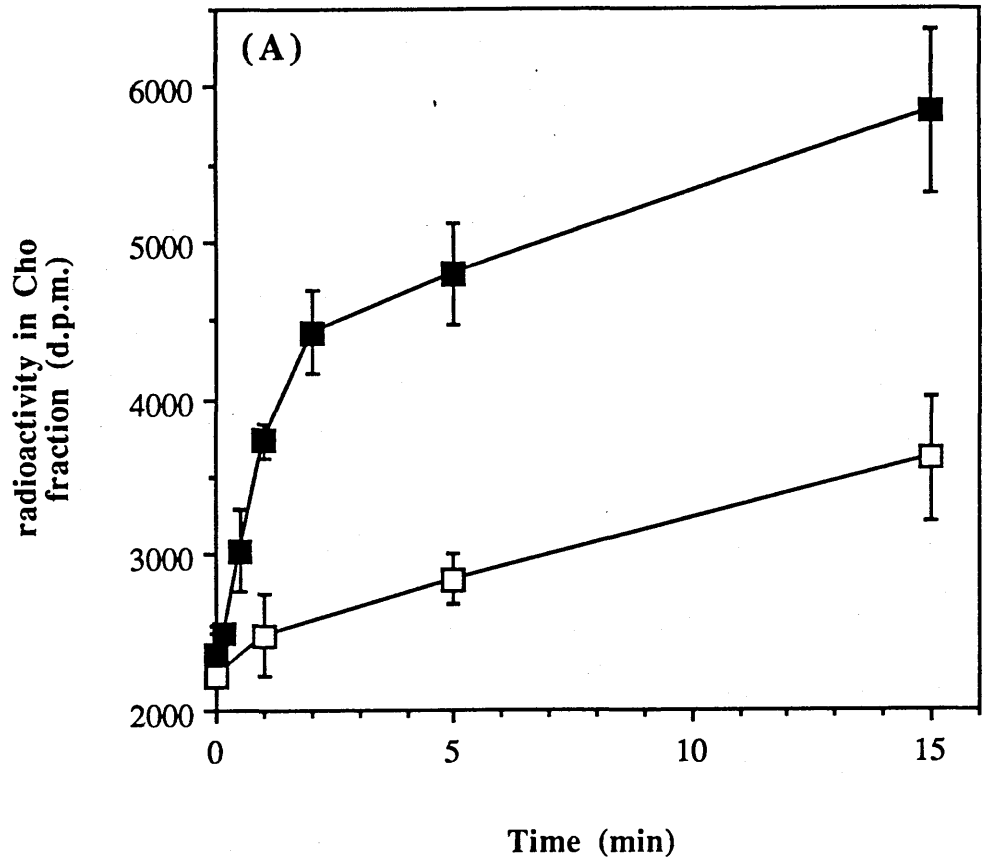
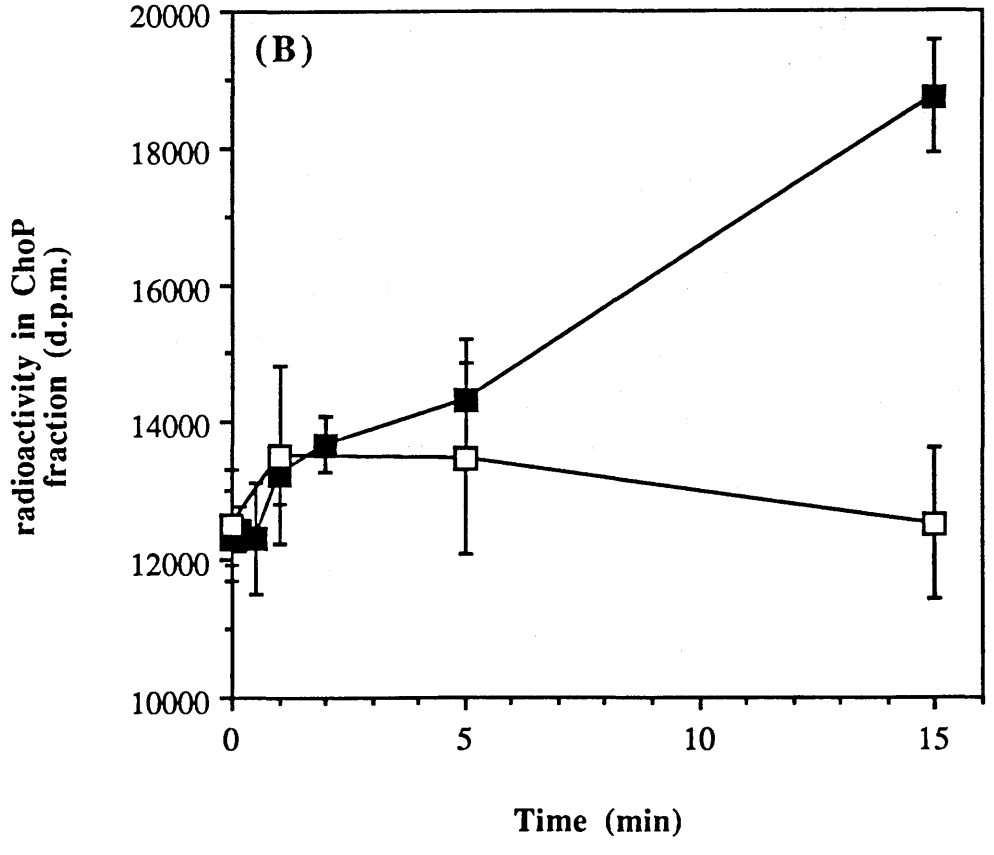
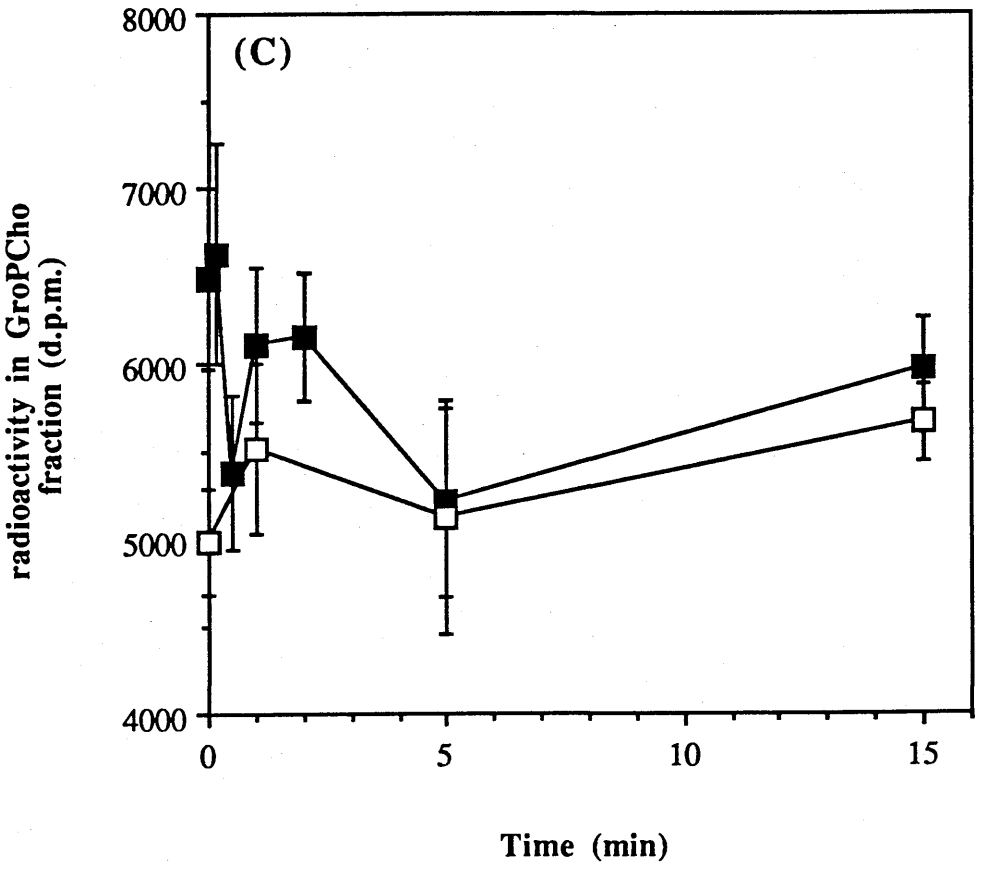

radioactivity per fraction (d.p.m.)
0
1000
2000
3000
4000
0
10
20
30
40
Fraction (ml)

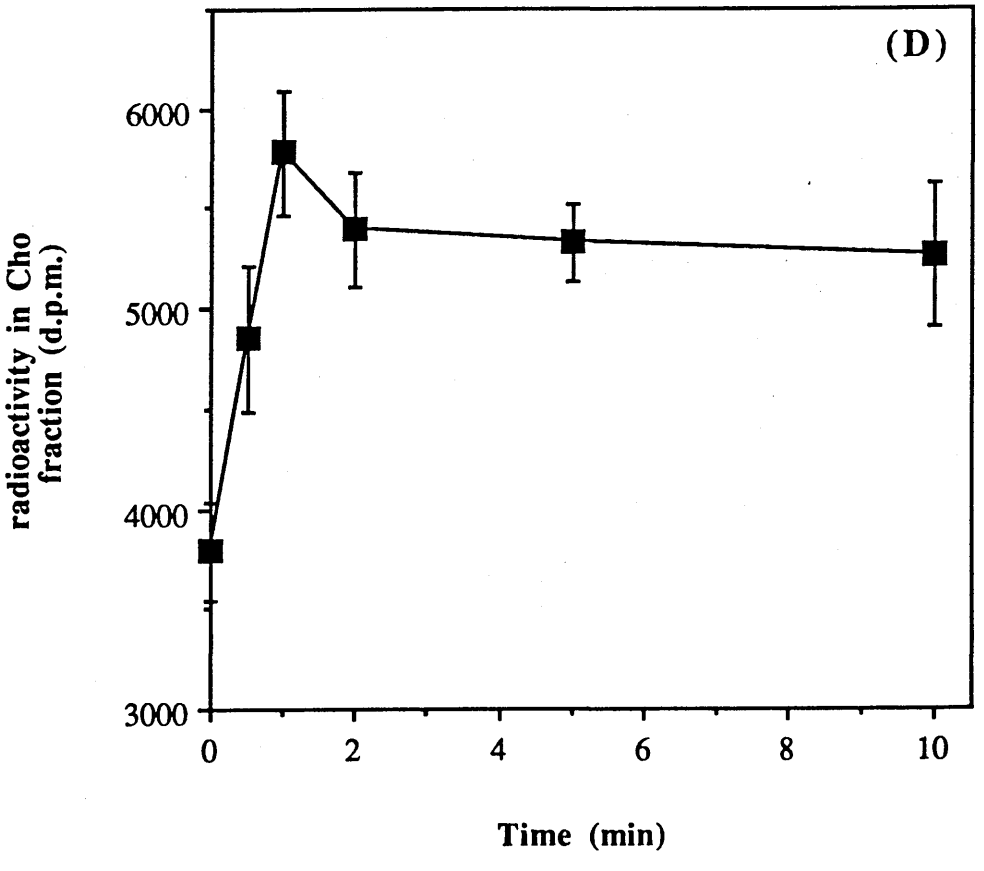
Figure 5.5. Bombesin-stimulated changes in (a) total [^{3}H]Cho, (b) total [^{3}H]ChoP, (c) total [^{3}H]GroPCho and (d) cell associated [^{3}H]Cho in [^{3}H]choline labelled Swiss 3T3 cells.

Swiss 3T3 cells grown on 24-well plates were labelled for 48 hours with 1.5μCi ml^{-1} [^{3}H]choline in 1 ml of DMEM containing 2% calf serum by which time they were confluent and quiescent. Cells were washed with HBG at 37^{o}C as described in the Methods section before incubating with either HBG (❑) or HBG containing 617 nM bombesin (■) for the times indicated. Incubations were terminated by direct addition of ice-cold methanol (a-c) or by aspiration prior to addition of methanol so that total or intracellular choline metabolites were included in the assay. After preparation of aqueous methanolic extracts (a & d) [^{3}H]Cho, (b) [^{3}H]ChoP and (c) [^{3}H]GroPCho were resolved by batch elution from Dowex-50-WH$^+$ columns and the associated radioactivity determined by liquid scintillation counting. Results are mean radioactivity in Cho, ChoP or GroPCho (d.p.m.) ± S.D. (n=3) from a single experiment typical of four.


(A)
radioactivity in Cho fraction (d.p.m.)
6000
5000
4000
3000
2000
0
5
10
15
Time (min)


(B)
radioactivity in ChoP fraction (d.p.m.)
20000
18000
16000
14000
12000
10000
0
5
10
15
Time (min)


(C)
radioactivity in GroPCho fraction (d.p.m.)
8000
7000
6000
5000
4000
0
5
10
15
Time (min)


(D)
radioactivity in Cho fraction (d.p.m.)
6000
5000
4000
3000
0
2
4
6
8
10
Time (min)

[^{3}H]Cho generation was reduced between 2 and 5 minutes after which it paralleled the increase in basal up to 15 and 30 minutes (data not shown). The precise details of the time course varied between experiments and in one case [^{3}H]Cho levels were significantly elevated after 10 seconds. Increases in [^{3}H]ChoP levels in response to bombesin were generally smaller in fold terms, varying in magnitude from 20 to 58% above basal and were generally not significantly elevated above control until between 5 (Fig 5.4) and 15 minutes of stimulation ($p<0.001$) (Fig 5.5b) though in some experiments non-significant increases were apparent at 2 min. No significant increase in the radioactivity in the [^{3}H]GroPCho fraction over basal was observed in response to bombesin stimulation for up to 15 and 30 minutes in any experiment (Fig 5.5c).

These experiments represented total intracellular and extracellular choline since incubations were terminated by the direct addition of ice-cold methanol to the incubation medium. By aspirating the incubation medium prior to addition of ice-cold methanol, experiments were performed to see if increases in [^{3}H]Cho occurred within the cell or represented leakage from the cell. Figure 5.5d shows the time course for increases in cell-associated [^{3}H]Cho upon stimulation with bombesin. Intracellular [^{3}H]Cho levels mirrored the rise in total [^{3}H]Cho described in Fig 5.5a up to 2 min after which the levels declined slightly, reaching a new apparent 'steady state' which was maintained for at least 10 min; basal intracellular [^{3}H]Cho levels remained unchanged over this time course (data not shown).

The increase in total [^{3}H]Cho levels in response to a 5 min stimulation with bombesin was clearly dose-dependent (Fig 5.6); the EC_{50} for bombesin was 1.44 ± 1.02 nM (mean ± S.D. of four separate experiments performed in triplicate).

In contrast to bombesin there was a lag of 1 min before any increase in [^{3}H]Cho was observed (Fig 5.7a) when [^{3}H]choline labelled Swiss 3T3 cells were stimulated with 100nM PMA. Details varied between experiments but generally [^{3}H]Cho levels rose above control in a sustained manner from 1 min up to 30 min (Figure 5.7a) and continued to rise in a near linear fashion above control for at least 60 min (data not shown). [^{3}H]Cho elevation was sometimes apparent, though not

Figure 5.6. Bombesin stimulates a dose-dependent increase in [^{3}H]Cho.

[^{3}H]Choline labelled Swiss 3T3 cells were washed in HBG and stimulated with the indicated final concentration of bombesin in HBG (■) for 5 min. Incubations were terminated by the direct addition of ice-cold methanol and aqueous methanolic extracts were prepared as described previously. [^{3}H]Cho was resolved by batch elution from Dowex-50-WH$^+$ columns after the prior elution of [^{3}H]GroPCho and [^{3}H]ChoP. Radioactivity was determined by liquid scintillation counting. The results are mean radioactivity in Cho (d.p.m.) ± S.D. (n=3) from a single experiment representative of three.

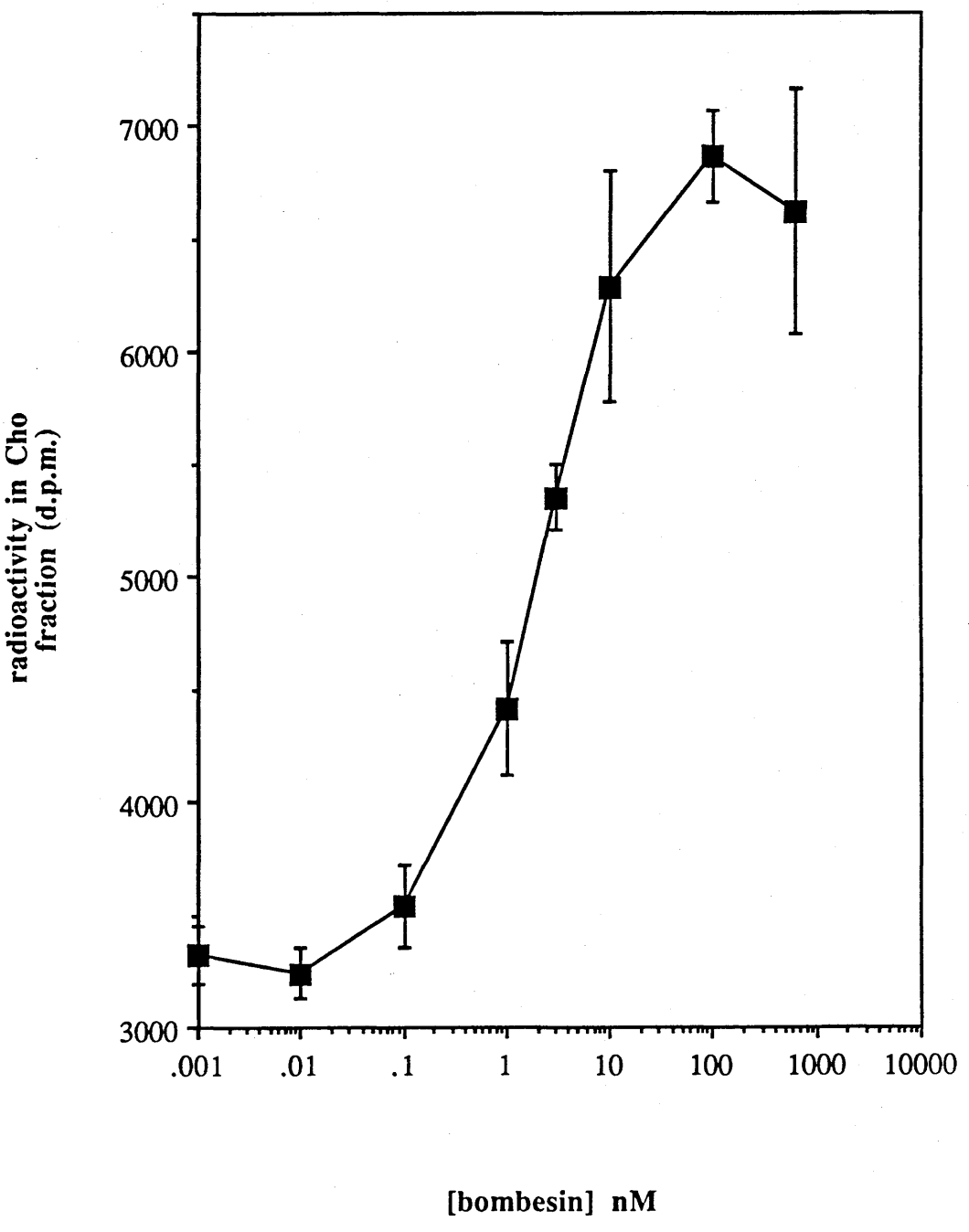
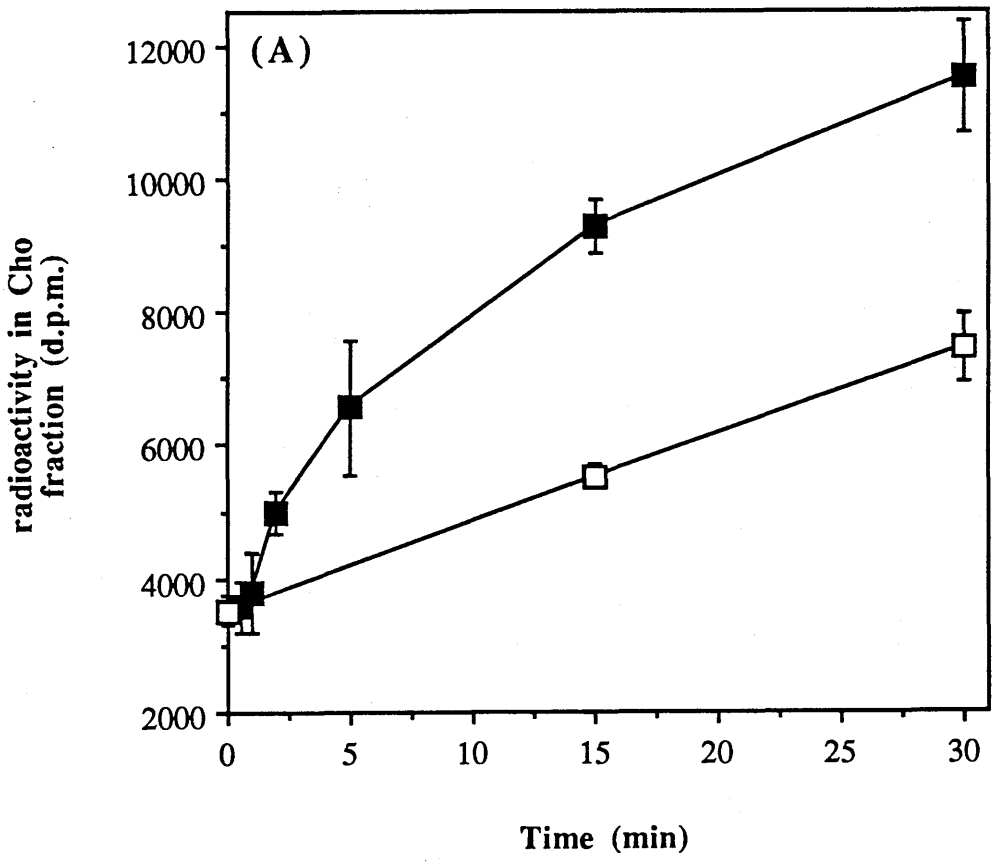
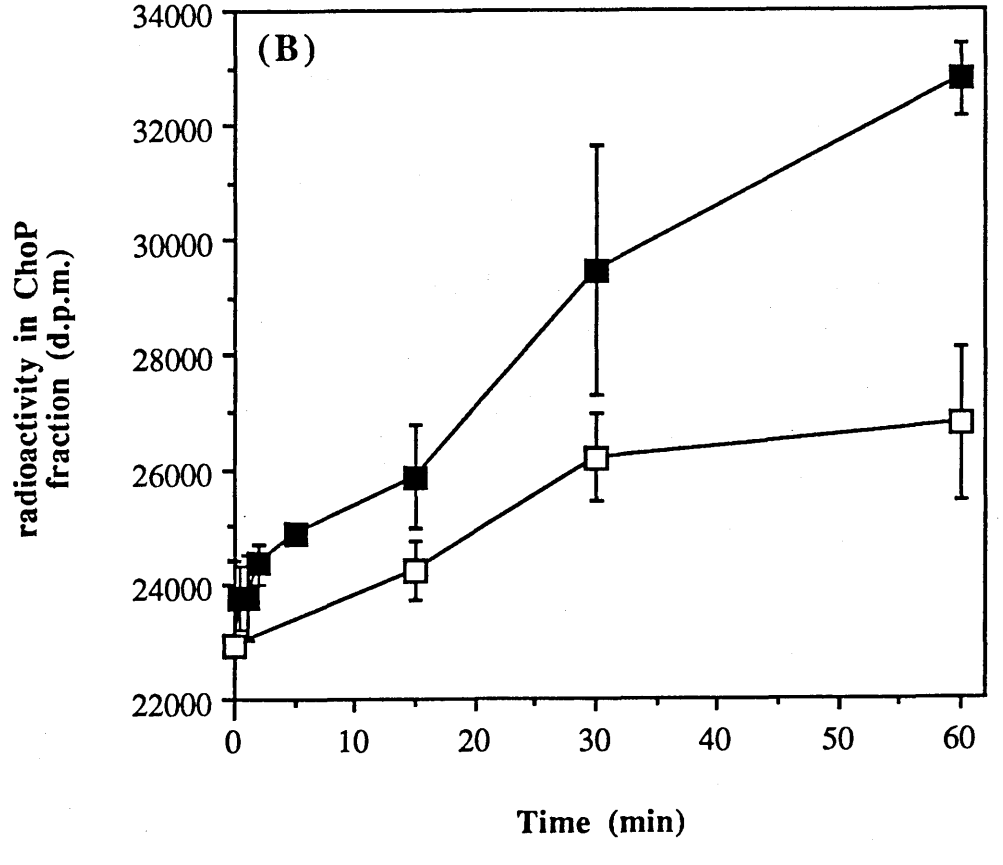

radioactivity in Cho
fraction (d.p.m.)
7000
6000
5000
4000
3000
.001
.01
.1
1
10
100
1000
10000
[bombesin] nM

Figure 5.7. PMA-stimulated changes in (a) total [^{3}H]Cho, (b) total [^{3}H]ChoP, (c) total [^{3}H]GroPCho and (d) cell associated [^{3}H]Cho in [^{3}H]choline-labelled Swiss 3T3 cells.

Swiss 3T3 cells were labelled with 1.5μCi ml^{-1} of [^{3}H]choline in 1 ml of DMEM containing 2% (v/v) calf serum for 48 hours by which time they were confluent and quiescent. Cell were washed with HBG at 37°C before incubating with HBG containing either 0.1% DMSO (□) or 100nM PMA in DMSO (■) at 37°C for the indicated times. Incubations were terminated by the direct addition of ice-cold methanol (a-c) or by aspiration prior to addition of methanol (d) and aqueous methanolic extracts were prepared and resolved on Dowex-50-WH$^+$ columns as described in the Methods section. Radioactivity associated with each fraction was determined by liquid scintillation counting. The results are mean radioactivity in Cho, ChoP or GroPCho (d.p.m.) ± S.D. (n=3) from a single experiment representative of four.


(A)
radioactivity in Cho fraction (d.p.m.)
12000
10000
8000
6000
4000
2000
0
5
10
15
20
25
30
Time (min)


(B)
radioactivity in ChoP fraction (d.p.m.)
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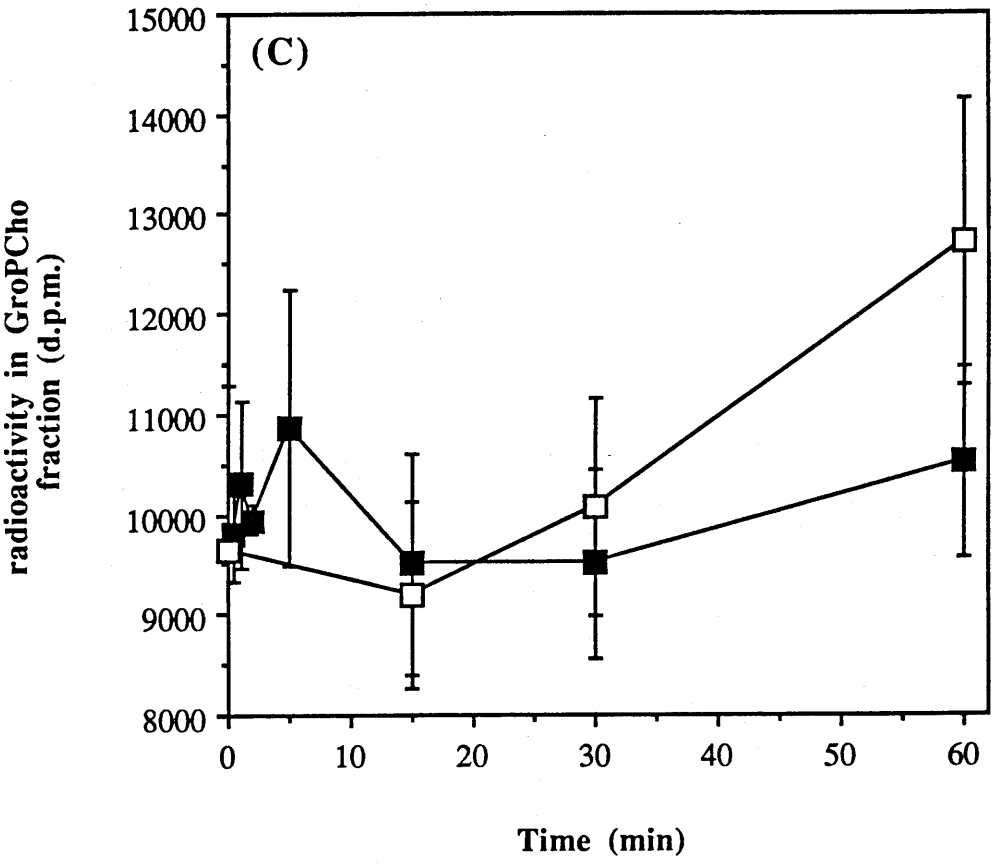
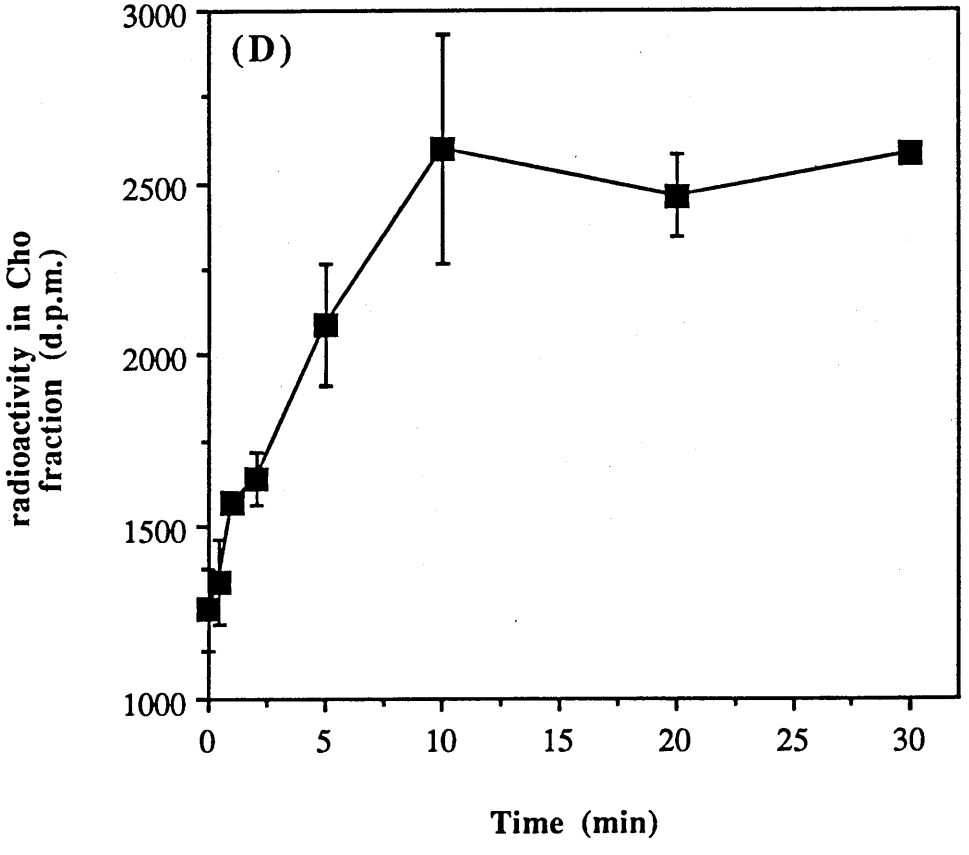
32000
30000
28000
26000
24000
22000
0
10
20
30
40
50
60
Time (min)


(C)
radioactivity in GroPCho fraction (d.p.m.)
15000
14000
13000
12000
11000
10000
9000
8000
0
10
20
30
40
50
60
Time (min)


(D)
radioactivity in Cho fraction (d.p.m.)
3000
2500
2000
1500
1000
0
5
10
15
20
25
30
Time (min)

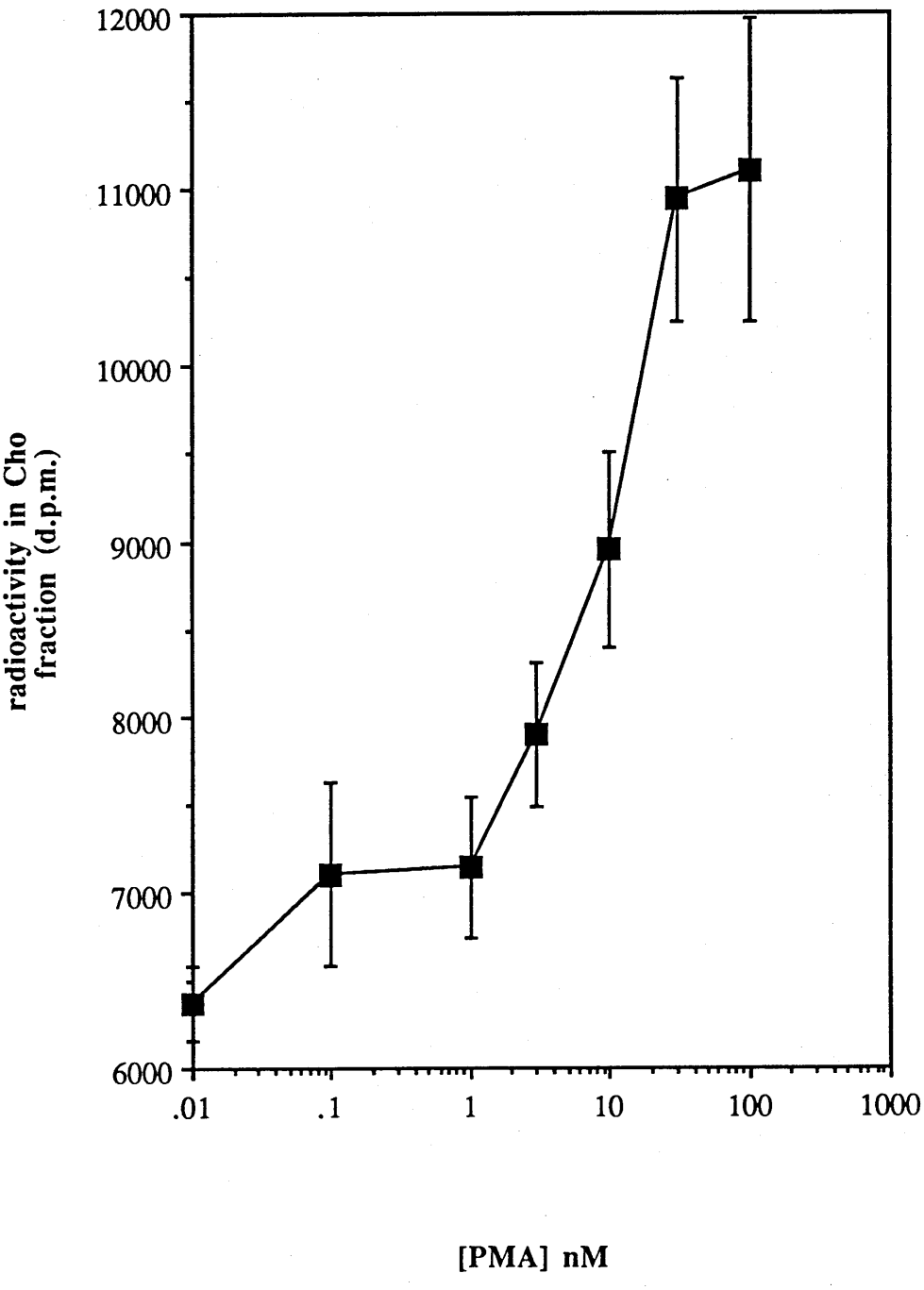
significant, at 1 min but was significantly above control from 2 min onwards (p=0.004). PMA-stimulated elevation of [^{3}H]ChoP, as observed for bombesin, was generally much slower than [^{3}H]Cho and was not significantly above control until 15 or 30 minutes (p= 0.093) after addition of the phorbol ester (Figure 5.7b). The levels of [^{3}H]GroPCho remained unchanged by addition of PMA over the time course examined, up to 60 minutes (Figure 5.7c).

When the effect of PMA upon intracellular [^{3}H]Cho levels was examined it was found that, like bombesin, a significant increase in [^{3}H]Cho was occurring within or associated with the cells. Figure 5.7d shows that stimulation with PMA resulted in an increase in cell associated [^{3}H]Cho, the onset of which exhibited virtually identical kinetics to that previously described (Fig 5.7c). However, from 10 min onwards PMA-stimulated increases in [^{3}H]Cho reached a plateau and were maintained at this level for at least 30 min. Cell associated [^{3}H]ChoP and [^{3}H]GroPCho levels did not change significantly. The effect of PMA upon [^{3}H]Cho levels was dose-dependent (shown in Figure 5.8) with half-maximal elevation occurring at doses of 4.95 ± 3.63 nM (mean ± S.D. of n=4 experiments).

Since both [Arg^8]Vp and $PGF_{2\alpha}$ appeared to stimulate inositol lipid hydrolysis by a similar mechanism to bombesin (Chapter 3) experiments were performed to determine if these mitogens were also able to stimulate the hydrolysis of PtdCho in [^{3}H]choline labelled Swiss 3T3 cells. Figure 5.9a shows the time course for increases in [^{3}H]Cho levels in Swiss 3T3 cells stimulated with 100nM [Arg^8]Vp. The kinetics of the response were essentially similar to that for bombesin; elevation of [^{3}H]Cho levels was clearly significant at the first time-point tested (1 minute, p= 0.015) and maximally above control (2-fold) at 5 minutes after which these levels were maintained above basal for up to 15 minutes. No significant increases in the radioactivity associated with [^{3}H]ChoP or [^{3}H]GroPCho was observed in response to [Arg^8]Vp. Furthermore, whilst no thorough kinetic analysis was performed, it was also observed that stimulation with 1μM $PGF_{2\alpha}$ also resulted in a significant elevation of [^{3}H]Cho levels though this was not of the magnitude of the responses to bombesin or [Arg^8]Vp, being

Figure 5.8. PMA-stimulates a dose-dependent increase in [^{3}H]Cho.

[^{3}H]Choline labelled Swiss 3T3 cells were washed with HBG before incubating at 37^{o}C with HBG containing the given concentration of PMA (■) for 10 min. Incubations were terminated by the direct addition of ice-cold methanol and aqueous methanolic extracts were prepared as described in the Methods section. Aqueous methanolic extracts were loaded on to Dowex-50-WH$^+$ columns and [^{3}H]Cho eluted with 1M HCl after prior elution of [^{3}H]GroPCho and [^{3}H]ChoP. Radioactivity in [^{3}H]Cho fraction was determined by liquid scintillation counting. Results are mean radioactivity in Cho (d.p.m.) ± S.D. (n=3) of triplicate determinations from a single experiment representative of four.


radioactivity in Cho fraction (d.p.m.)
12000
11000
10000
9000
8000
7000
6000
.01
.1
1
10
100
1000
[PMA] nM

typically 1.5-fold above basal after 5 minutes. No significant elevation of [^{3}H]ChoP or [^{3}H]GroPCho levels was observed upon stimulation with $PGF_{2\alpha}$. The dose response curves for elevation of [^{3}H]Cho levels by [Arg^8]Vp and $PGF_{2\alpha}$ are shown in Figure 5.9b. The concentrations of each mitogen required to give half-maximal elevation of [^{3}H]Cho levels were 0.92 ± 0.57 nM for [Arg^8]Vp and 0.51 ± 0.32 μM for $PGF_{2\alpha}$.

Since activation of phospholipase D appeared to be dependent upon functional PKC activity and the stimulated release of [^{3}H]Cho exhibited identical kinetics to [^{3}H]PtdBut formation for both bombesin and PMA, experiments were performed to see if the release of [^{3}H]Cho from PtdCho had the same dependency upon PKC activity.

Firstly, in the case of PMA, the ability of β-phorbol to stimulate increases in cellular [^{3}H]Cho content was compared with that of PMA. β-phorbol, a phorbol ester analogue which does not activate PKC (Castagna *et al.*, 1982), was ineffective in elevating [^{3}H]Cho levels above control, based on a 15 minute stimulation in which the identical final concentration of PMA was quite clearly effective. The radioactivity associated with [^{3}H]Cho was for control (15 min, 0.1% DMSO) 2,851±196 dpm, β-phorbol (15 min, 100nM) 3,220±164 dpm and PMA (15 min, 100nM) 6,151±476 dpm; results from a single typical experiment, n=3).

Secondly, when [^{3}H]choline labelled Swiss 3T3 cells were pre-incubated for 15 minutes with 1μM staurosporine (a proposed PKC inhibitor) prior to stimulation with bombesin or PMA, the elevation of [^{3}H]Cho levels by each mitogen was inhibited by 30-40% (Table 5.3).

Furthermore, chronic treatment of Swiss 3T3 cells with PMA results in the loss of cellular PKC (Collins & Rozengurt, 1984; Rodriguez-Pena & Rozengurt, 1984; Brown *et al.*, 1990). When Swiss 3T3 cells were labelled with [^{3}H]choline for 48 hours in the presence of 400nM PMA there was a 1.7- 2-fold increase in [^{3}H]choline incorporation into both lipid and aqueous pools, probably reflecting the reported stimulation of PtdCho synthesis by PMA (e.g., Muir & Murray, 1987). To allow a real comparison between down-regulated and control cells, [^{3}H]Cho

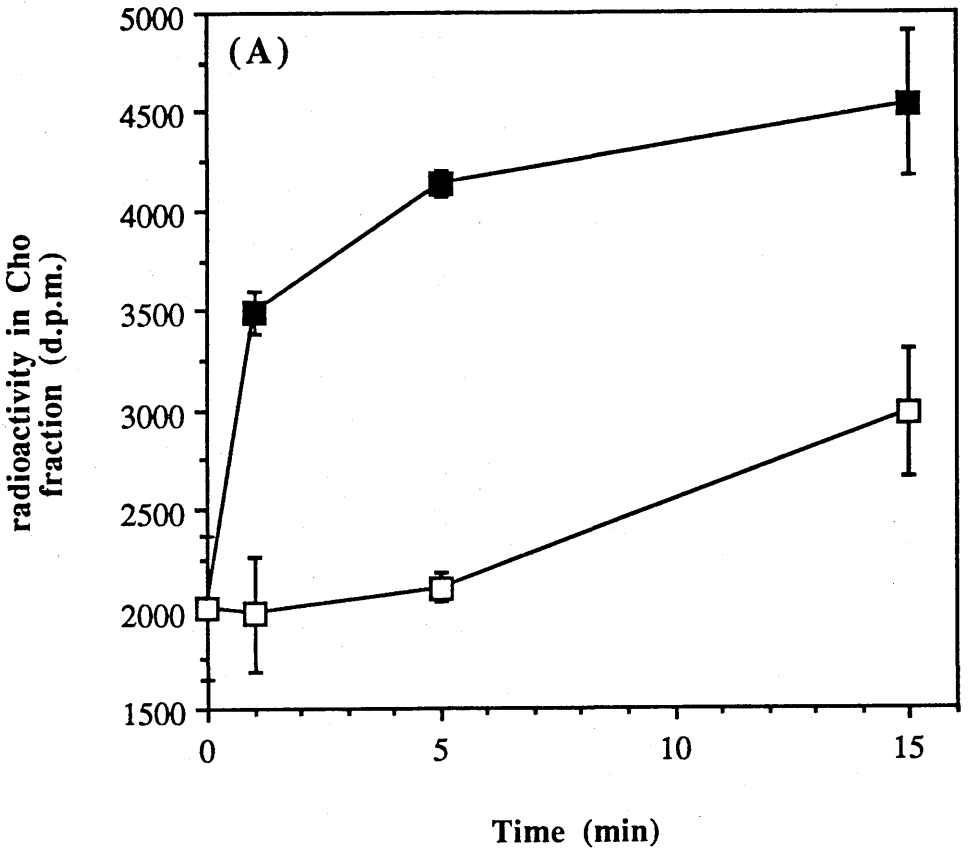
Figure 5.9

(a) [Arg^8]Vasopressin stimulates the release of [^{3}H]Cho from [^{3}H]choline labelled Swiss 3T3 cells.

[^{3}H]Choline labelled Swiss 3T3 cells were washed with HBG before incubating with HBG (□) or HBG containing100nM [Arg^8]Vp (■) at 37°C for the indicated times. Incubations were terminated by the direct addition of ice-cold methanol and aqueous methanolic extracts were assayed for [^{3}H]Cho after resolution on Dowex-50-WH$^+$ columns. Results are mean radioactivity in Cho (d.p.m.) ± S.D. (n=3) from a single experiment representative of four.

(b) [Arg^8]vasopressin and prostaglandin $F_{2\alpha}$ both stimulate the dose-dependent accumulation of [^{3}H]Cho.

[^{3}H]Choline labelled Swiss 3T3 cells were washed with HBG and incubated at 37°C with HBG containing the given concentration of either [Arg^8]Vp (■) or $PGF_{2\alpha}$ (□) for 5 min. Incubations were terminated by direct addition of ice-cold methanol and aqueous methanolic extracts were assayed for [^{3}H]Cho after resolution on Dowex-50-WH$^+$ columns. Results are normalised to % of control to include both sets of data on a single graph; the basal dpm values were 3290 ± 151 for [Arg^8]Vp and 5992 ± 368 for $PGF_{2\alpha}$. Results are mean radioactivity in Cho (d.p.m.) ± S.D. (n=3) from a single experiment representative of three in each case.


(A)
radioactivity in Cho fraction (d.p.m.)
5000
4500
4000
3500
3000
2500
2000
1500
0
5
10
15
Time (min)

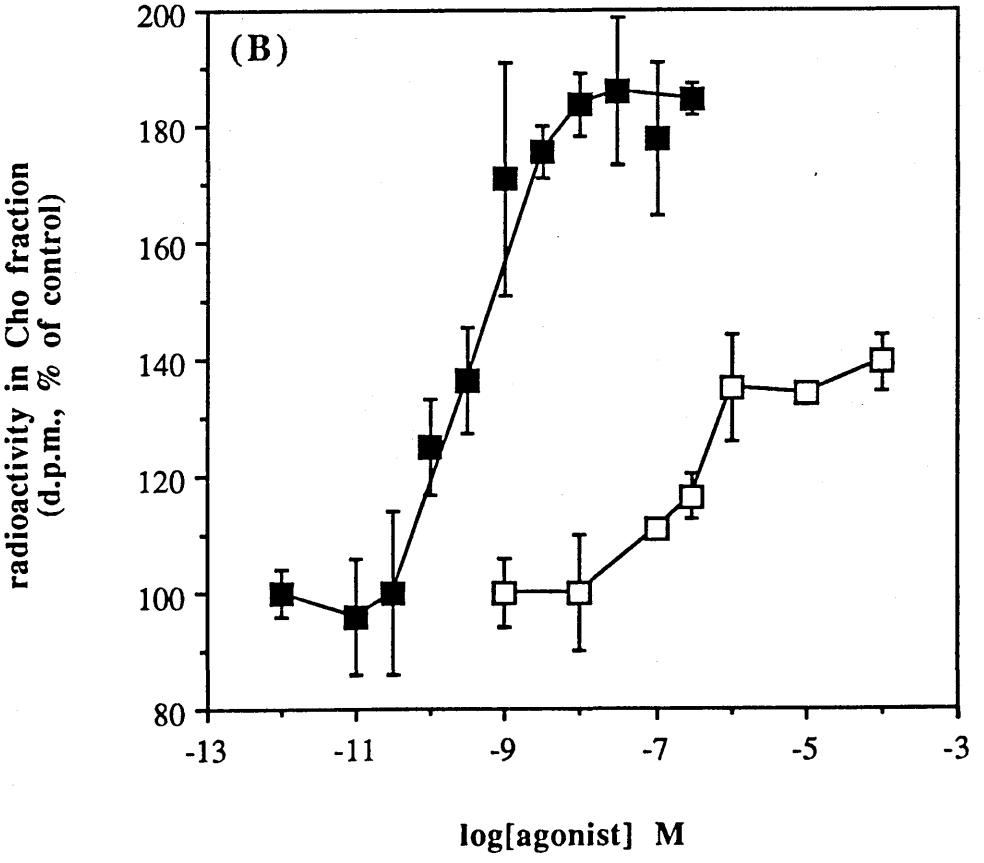

(B)
radioactivity in Cho fraction (d.p.m., % of control)
200
180
160
140
120
100
80
-13
-11
-9
-7
-5
-3
log[agonist] M

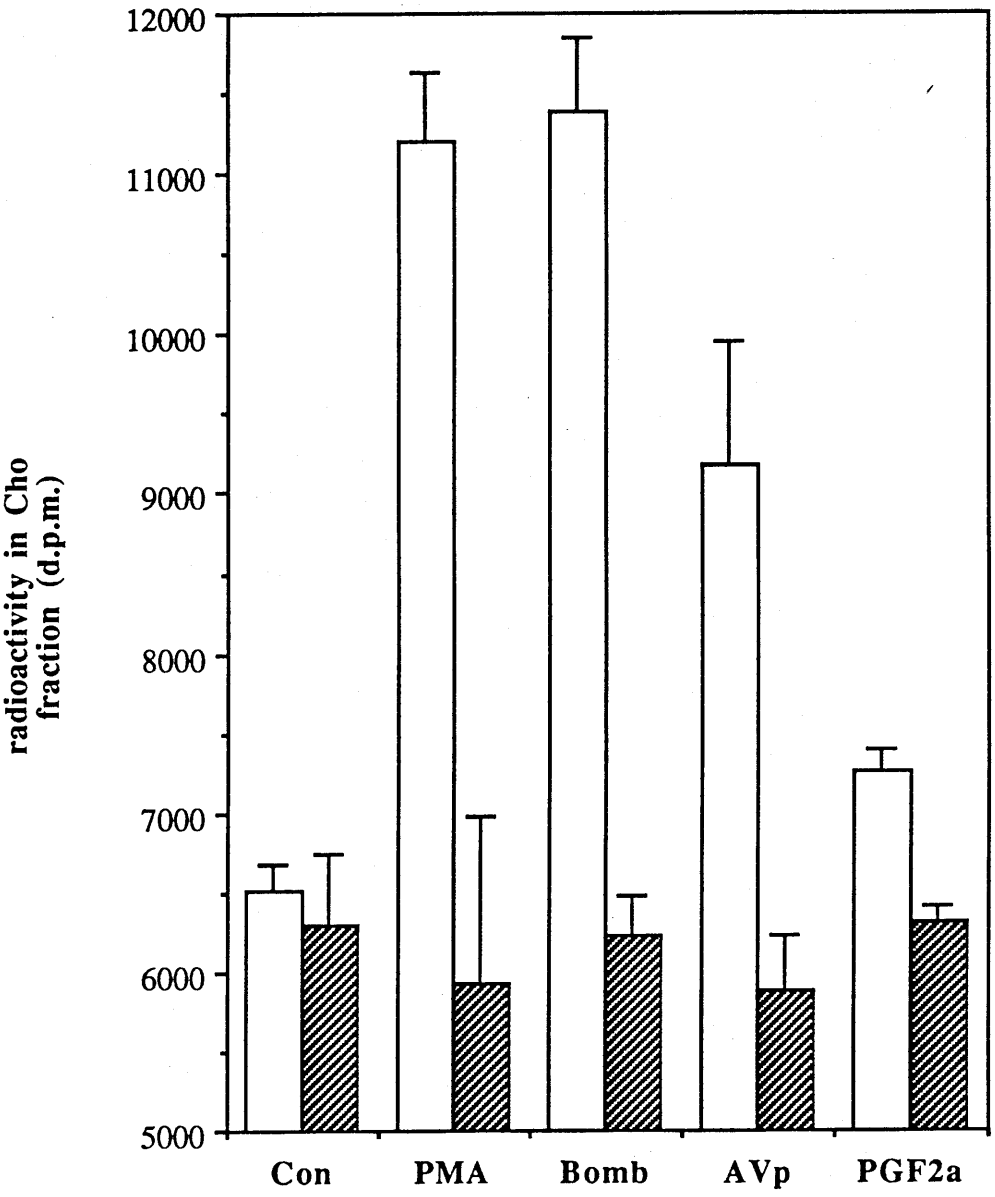
Table 5.3. The effect of acute pre-treatment with staurosporine upon bombesin- and PMA-stimulated [^{3}H]Cho elevation.

[^{3}H]choline labelled Swiss 3T3 cells were incubated for 15 min with 1μM staurosporine or vehicle (0.1% DMSO) as indicated prior to addition of agonist. Cells were then incubated with bombesin (617nM, 2 min) or PMA (100 nM, 15 min) and radioactivity associated with [^{3}H]Cho determined as before. Results are mean radioactivity in Cho fraction (d.p.m.) ± S.D. of triplicate determinations from a single experiment typical of three.

	Radioactivity in Cho (d.p.m.).	
Incubation.	**Control**	**+ Staurosporine**
Control, 2 min.	3,439 ± 540	3,692 ± 706.
Bombesin, 2 min.	5,029 ± 394	4,358 ± 209.
DMSO (0.1%), 15 min.	4,217 ± 624	4,481 ± 937.
PMA, 15 min.	6,739 ± 751	5,416 ± 247.

Figure 5.10 Effect of 48 hour pre-treatment with 400nM PMA upon agonist stimulated [^{3}H]Cho accumulation.

Swiss 3T3 cells were labelled for 48 hours with 1.5μCi ml^{-1} of [^{3}H]choline in 1 ml of DMEM containing 2% (v/v) calf serum and either 400nM β-phorbol (open bars) or 400nM PMA (hatched bars). Cells were then washed in HBG and incubated for 10 min with PMA (100nM), bombesin (100nM), [Arg^8]Vp (100nM) or $PGF_{2\alpha}$ (2μM), at 37°C. Incubations were terminated by the direct addition of ice-cold methanol and aqueous-methanolic extracts were assayed for [^{3}H]Cho following resolution on Dowex-50-WH$^+$ columns. Results were normalised to the amount of label incorporated into total choline containing lipids. Each data set is the radioactivity associated with [^{3}H]Cho (d.p.m.) ± S.D. (n=3) from a single experiment representative of six (PMA and bombesin) or three ([Arg^8]Vp and $PGF_{2\alpha}$).


radioactivity in Cho fraction (d.p.m.)
12000
11000
10000
9000
8000
7000
6000
5000
Con
PMA
Bomb
AVp
PGF2a

levels were normalised by equating the radioactivity in total choline containing lipids in down-regulated cells with a mean value for radioactivity associated with choline containing lipids in control cells. The results, shown in Figure 5.10, show that the ability of PMA, bombesin, [Arg^8]Vp and $PGF_{2\alpha}$ to stimulate increases in cellular [^{3}H]Cho content was completely abolished in cells which had been treated with PMA for 48 hours whilst in cells treated with the vehicle control containing β-phorbol the responses remained intact.

The ability of bombesin, [Arg^8]Vp and $PGF_{2\alpha}$ to increase levels of [^{3}H]Cho exhibited the same rank order as that for [^{3}H]$InsP_t$ accumulation, i.e. bombesin≥ [Arg^8]Vp> $PGF_{2\alpha}$ (Figure 5.10 and compare Figs 5.6b and 5.9b). Thus, fold increases in [^{3}H]Cho were typically 2- 2.5-fold for bombesin, 1.8- 2-fold for [Arg^8]Vp and 1.2- 1.5-fold for $PGF_{2\alpha}$.

Finally, if bombesin, [Arg^8]Vp and $PGF_{2\alpha}$ were activating PtdCho hydrolysis by a common mechanism involving PKC then one would expect that combination of two of these agonists at sub-maximal doses would not synergise to stimulate [^{3}H]Cho accumulation. Consistent with this, combination of sub-maximal doses of [Arg^8]Vp and bombesin resulted in accumulation of [^{3}H]Cho which was only additive compared to the two responses alone. For example, the net increase in radioactivity in [^{3}H]Cho in response to a 5 min stimulation with 0.1nM bombesin was 1,530 ± 179 d.p.m.; 5 min, with 0.1 nM[Arg^8]Vp= 882 ± 42; 5 min with 0.1 nM each of bombesin and [Arg^8]Vp= 2,334 ± 147 (the radioactivity in [^{3}H]Cho in control cells was 7,214 ± 424 d.p.m.; results from a single experiment representative of two).

5.3 Discussion.

5.3.1. Analysis of the water soluble products of PtdCho hydrolysis by cation exchange chromatography.

The analysis of the water soluble metabolites of PtdCho hydrolysis has been hampered by the lack of a suitable assay method. Much of the work in the literature has used variations of the t.l.c. separation method of Yavin (1976) which is time-consuming and in these studies often proved unreliable. Many studies (e.g. Muir & Murray, 1987; Welsh *et al.*, 1988) simply measured the radioactivity associated with the total water-soluble metabolites without identifying the primary product and therefore the route of lipid hydrolysis; an important point if PtdCho is being viewed as a potential source of DG. Recently an ion-exchange separation of Cho metabolites was described by Liscovitch *et al.* (1987) but this procedure involved the use of h.p.l.c. The method described in this chapter is quick, reproducible and yields quantitative separation and recovery of Cho, ChoP and GroPCho. It is not clear if the method will also separate betaine, but in the cell system examined here this metabolite was such a minor, and seemingly inert, component that it did not interfere with analysis. Similar extremely low levels of betaine have been reported before in Swiss 3T3 cells (Warden & Friedkin, 1984) aswell as NG108-15 cells (Liscovitch *et al.*, 1987) and brain cells (Yavin, 1976). Furthermore, the total radioactivity associated with CDP-choline when isolated from a [^{3}H]choline labelled Swiss 3T3 cell extract was also so low as to not interfere with analysis of levels of [^{3}HCho, [^{3}H]ChoP and [^{3}H]GroPCho. It therefore seems likely that this method should prove applicable to a wide range of cell types following suitable, thorough characterization.

Recently methods have also been described for assay of mass amounts of both Cho and ChoP respectively (Murray *et al.*, 1990; Truett *et al.*, 1989b). Whilst these will be very useful along side mass assays for Ins(1,4,5)P_3 and DG in determining the contributions made by PtdIns(4,5)P_2 and PtdCho to DG they are relatively time-consuming.

5.3.2 Bombesin and PMA stimulate the hydrolysis of PtdCho by a phospholipase D catalysed pathway.

Having defined a mitogen-stimulated PLD activity in Swiss 3T3 cells (Chapter 4) it remained to identify the major substrate(s) for that PLD. Stimulation of [^{3}H]choline-labelled Swiss 3T3 cells with bombesin or PMA resulted in the loss of radioactivity from [^{3}H]PtdCho with only small and variable changes in sphingomyelin, the other major Cho-containing lipid in Swiss 3T3 cells (Table 5.2). The amount of label lost was very small representing only about 5% of total radioactivity associated with PtdCho. This is in agreement with the result of Liscovitch *et al.* (1987) in PMA-treated NG108-15 cells and is probably due to the relatively small generation of [^{3}H]Cho and [^{3}H]ChoP (Figure 5.4) as compared to the large size of the cellular PtdCho pool. Since the loss of label fom [^{3}H]PtdCho was accompanied by increases in [^{3}H]Cho and [^{3}H]ChoP (Figure 5.4) it clearly suggests that PtdCho is a substrate for a mitogen-stimulated PtdCho hydrolytic activity.

The initial product of PtdCho hydrolysis generated in response to bombesin was [^{3}H]Cho with an increase in [^{3}H]ChoP only being observed at later time points (Figure 5.5a & b). Thus, in agreement with the results in Chapter 4, it appears that the primary hydrolytic activity is that of a phospholipase D. In essence the time course for [^{3}H]Cho generation was identical to that previously described for bombesin-stimulated [^{3}H]PtdBut formation in the presence of 0.3% butan-1-ol (Chapter 4) and the two results confirm that a PLD, acting upon PtdCho, is activated in bombesin-stimulated Swiss 3T3 cells.

The slower increase in [^{3}H]ChoP in response to bombesin stimulation may represent activation of a PtdCho-specific phospholipase C enzyme. However, the increase in [^{3}H]ChoP could also be due to phosphorylation of liberated [^{3}H]Cho by choline kinase. The ratio of [^{3}H]Cho to [^{3}H]ChoP in equilibrium-labelled Swiss 3T3 cells is of the order of 1:5 or 1:6 (see Figure 5.5a & b and Price *et al*, 1989) and phosphorylation by choline kinase following a rapid increase in [^{3}H]Cho levels as seen in Figure 5.5a might simply reflect the activation of a PtdCho synthetic pathway to replenish the parent lipid. In support of this Muir & Murray

(1987) noted that bombesin stimulated PtdCho synthesis in Swiss 3T3 cells, as measured by the incorporation of ^{32}Pi into PtdCho, after a lag of approximately 10 minutes; this is similar to the lag time before increases in [^{3}H]ChoP were observed in response to bombesin. Furthermore, Warden & Friedkin (1984; 1985) have demonstrated a 2- 3-fold increase in choline kinase activity and PtdCho biosynthesis in Swiss 3T3 cells following stimulation with either PMA or whole serum; these agents have in common with bombesin the ability to stimulate PtdCho hydrolysis and activate PKC. It is not possible to predict which of these two sources of [^{3}H]ChoP (PtdCho-PLC or choline kinase) is the more likely due to the lack of selective inhibitors of choline kinase and the difficulties in comparing the inter-relationships between the Cho and ChoP pools in a stimulated cell system. In this respect the magnitude of increases in radioactivity associated with [^{3}H]ChoP was variable and it was therefore difficult to determine if this could be explained in terms of the increase in radioactivity in [^{3}H]Cho followed by its phosphorylation. Such questions will require mass analysis of changes in Cho and ChoP to be adequately addressed.

The metabolic fate of liberated [^{3}H]Cho is further complicated by the exit from the cell. Figure 5.5d shows that the bombesin-stimulated increases in [^{3}H]Cho actually occurred within the cells rather than simply reflecting a bombesin-stimulated leakage of Cho. Cell associated [^{3}H]Cho reached a plateau after 2 minutes beyond which its levels were maintained for up to 30 minutes. Over the same time course total [^{3}H]Cho continued to rise in parallel with the control. Since elevated intracellular Cho levels were maintained from 2 minutes when [^{3}H]Cho was being lost from the cells and choline kinase activity was likely to be high (Warden & Friedkin, 1984), this implies that bombesin-stimulated PtdCho hydrolysis and generation of [^{3}H]Cho was sustained. This is supported by the observation that significant increases in PtdBut continue, albeit at a reduced rate, for at least 15 minutes (Chapter 4 of this thesis). The EC_{50} for bombesin-stimulated increases in [^{3}H]Cho (1-2 nM) was essentially the same as that for its effects upon [^{3}H]PtdBut accumulation suggesting that the two responses were related or linked, presumably because they reflect the same PtdCho-PLD activity. Furthermore, the

EC_{50} value was similar to that for [^{3}H]InsP_t accumulation, Ins(1,4,5)P_3 formation and DG formation suggesting that all these events might be mediated through the same receptor.

The second, sustained phase of bombesin-stimulated DG formation can be mimicked by addition of PMA which can also stimulate increases in [^{3}H]palmitate-labelled DG from a non-inositide source (Chapter 4 of this thesis). Further, the kinetics of this PMA-stimulated DG formation are similar to the onset of PMA stimulated PLD activity as measured by the formation of [^{3}H]PtdBut. The ability of PMA to stimulate increases in [^{3}H]Cho levels following a lag of approximately 1 minute (shown in Figure 5.7a) agrees very well with the time course of increases in [^{3}H]PtdBut confirming that PMA-stimulated PLD activity utilises PtdCho as a major substrate. Like bombesin, the primary product of PtdCho hydrolysis was [^{3}H]Cho with increases in [^{3}H]ChoP occurring over much longer time points confirming activation of a PtdCho-PLD. The increase in [^{3}H]ChoP may reflect either activation of a phospholipase C or choline kinase activity as previously discussed. The continued rise of both total and cell associated [^{3}H]Cho for up to 60 minutes following stimulation clearly indicated that PMA addition resulted in the sustained activation of phospholipase D confirming the result obtained for [^{3}H]PtdBut formation which continued, unabated, for at least 30 minutes (Chapter 4 of this thesis). The EC_{50} for PMA-stimulated increases in [^{3}H]Cho (4-5 nM) was in close agreement with the value for [^{3}H]PtdBut formation and that for activation of protein kinase C previously described (Castagna *et al.*, 1982).

5.3.3 A role for PKC in activating PtdCho hydrolysis

Both PMA and bombesin can activate PKC and since both mitogens were able to activate PLD with the same dose dependency as their effects upon PKC, it seemed possible that both exerted their effects through the activation of PKC. In the case of bombesin this was supported by the fact that the onset of both [^{3}H]Cho generation and [^{3}H]PtdBut formation were kinetically downstream of Ins(1,4,5)P_3 formation and the first phase of DG formation; Ins(1,4,5)P_3 levels peaked at 5

seconds and were declining at 15 sec when [^{3}H]Cho was elevated above control and had fully declined by 30 seconds when [^{3}H]PtdBut formation was significantly above control. Therefore, it seemed possible that activation of PKC in response to the first phase of bombesin-stimulated DG formation might in some way serve to 'switch on' PtdCho hydrolysis as seemed to be the case for PLD activity assayed by [^{3}H]PtdBut formation (Chapter 4 of this thesis).

The results in this chapter (Table 5.3 and Fig 5.10) support such a role for PKC in the activation of PtdCho hydrolysis in response to both PMA and bombesin. Firstly, PMA was able to activate [^{3}H]Cho generation whereas β-phorbol, a phorbol ester which cannot activate PKC (Castagna *et al.*, 1982), was unable to elevate [^{3}H]Cho levels above the vehicle control. Second, pre-treatment of Swiss 3T3 cells with staurosporine, a proposed PKC inhibitor, resulted in the partial inhibition of both bombesin- and PMA-stimulated [^{3}H]Cho generation (Table 5.3). It should be stressed that staurosporine is not a selective inhibitor of PKC since it competes with ATP at the catalytic domain and therefore will have effects upon a variety of protein kinases. Indeed, this non-selectivity may explain the poorer inhibition of mitogen-stimulated PtdCho hydrolysis compared with the effects of Ro-31-8220 which abolished 90% of PMA-stimulated and 50% of bombesin-stimulated [^{3}H]PtdBut formation (Chapter 4). Ro-31-8220, whilst based upon the staurosporine structure, has been shown to be at least two orders of magnitude more selective for PKC than for PKA or Ca^{2+}-CaM kinase (Davis *et al.*, 1989). Nevertheless the fact that a kinase inhibitor did inhibit both bombesin- and, particularly, PMA-stimulated [^{3}H]Cho generation argues in favour of a role for PKC in the activation of PtdCho hydrolysis when considered together with the results in Fig 5.10 which show that 'down-regulation' of PKC, resulted in the complete abolition of both bombesin- and PMA-stimulated [^{3}H]Cho elevation.

Taken together the results in Chapters 4 and 5 suggest that upon stimulation of Swiss 3T3 cells with bombesin, phospholipase D catalysed PtdCho hydrolysis is stimulated within at least 15 seconds and that this activation is at least partially dependent upon activation of protein kinase C. As such PKC may represent a major functional link between bombesin-stimulated inositol lipid hydrolysis and

activation of PtdCho-PLD. In such a model PMA would exert its effects by mimicking the action of DG derived from PtdIns(4,5)P_2 hydrolysis in activating PKC and 'switching on' PLD; however the possibility that PMA may exert effects upon PLD independently of PKC, as mentioned in Chapter 4, must not be ignored. There remains the intruiging anomaly that PMA- and bombesin-stimulated increases in [^{3}H]Cho and [^{3}H]PtdBut levels are completely abolished by down-regulation of PKC and yet Ro-31-8220 will abolish essentially all PMA-stimulated [^{3}H]PtdBut formation but only 50% of the bombesin-stimulated response.

One model which might go some way to explain this assumes that as well as the PKC-mediated effects, PMA may be able to bind to, and activate, PLD in a manner analagous to its activation of PKC at the DG binding site. In such a scenario PLD would be predicted to have a DG/PMA binding site and therefore might also be 'down-regulated' in response to chronic PMA treatment. However this model would not explain the total loss of PMA-stimulated PLD activity by Ro-31-8220 since PMA would be predicted to exert some effects independently of PKC. It is possible that PLD possesses both PKC-dependent (presumably phosphorylation) and -independent (DG-binding) regulatory sites which may exhibit cooperative and non-cooperative interactions to achieve activation in response to diverse stimuli. PMA and DG (derived from PtdIns(4,5)P_2 hydrolysis) may also exert different effects on PLD activity. In this respect there are a number of reports suggesting that PMA and DG do exert different effects upon phospholipid metabolism. In particular Kolesnick & Paley (1987) demonstrated that the effects of diC_8 and PMA upon PtdCho hydrolysis were additive at maximal concentrations whilst diC_8, but not PMA, was able to stimulate the release of water soluble [^{3}H]choline metabolites from PKC down regulated GH_3 pituitary cells. These results are consistent with PMA and diC_8 being able to exert separate and distinct effects. In addition, Muir & Murray (1988) have shown that diC_8 but not PMA will stimulate PtdCho synthesis in platelets.

5.3.4 Bombesin, [Arg^8]Vp and $PGF_{2\alpha}$ activate PtdCho hydrolysis by a common mechanism involving protein kinase C.

Bombesin, [Arg^8]Vp and $PGF_{2\alpha}$ possess the common property of stimulating the rapid hydrolysis of PtdIns(4,5)P_2 to generate Ins(1,4,5)P_3 and DG. Furthermore there are a number of similarities between the three mitogens in the mechanism by which their receptors are coupled to the activation of PIC. The generation of inositol phosphates stimulated by each mitogen is modulated by a G-protein (Erusalimsky & Rozengurt, 1989; Plevin *et al.*, 1990) and is also subject to PKC-mediated negative feedback inhibition (Brown *et al.*, 1987; Chapter 3 of this thesis). Since these three mitogens exhibited marked similarities in their ability to stimulate [^{3}H]InsP_t accumulation (Chapter 3) experiments sought to determine if [Arg^8]Vp and $PGF_{2\alpha}$ would also be able to stimulate PtdCho hydrolysis.

The observation that both [Arg^8]Vp and $PGF_{2\alpha}$ were able to stimulate the generation of [^{3}H]Cho from Swiss 3T3 cells at concentrations at which both are mitogenic (MacPhee *et al.*, 1984; Corps *et al.*, 1985) and at which both stimulate InsP_t accumulation suggests that, by analogy with bombesin, PtdCho hydrolysis by a PLD might be a common response to mitogens which stimulate the rapid hydrolysis of inositol lipids. In the case of [Arg^8]Vp and $PGF_{2\alpha}$ formation of [^{3}H]Cho does represent PLD activity since both these agonists stimulated the formation of [^{3}H]PtdBut in [^{3}H]palmitate labelled Swiss 3T3 cells in the presence of butan-1-ol. The time course for [Arg^8]Vp-stimulated [^{3}H]Cho generation was similar to that for bombesin, not reaching maximal elevation above basal until between 1 and 5 minutes. Since this was kinetically downstream of [Arg^8]Vp-stimulated increases in Ins(1,4,5)P_3 (Nanberg & Rozengurt, 1988) it seemed possible that, like bombesin, [Arg^8]Vp-stimulated PtdCho hydrolysis might be dependent upon prior activation of PKC via inositol lipid hdrolysis. Since both [Arg^8]Vp- and $PGF_{2\alpha}$-stimulated [^{3}H]Cho generation was completely abolished in cells which had been treated with 400nM PMA for 48 hours to down-regulate protein kinase C this would suggest that, like bombesin, PKC activity is required for activation of PtdCho-PLD.

The fact that bombesin, [Arg^8]Vp and $PGF_{2\alpha}$ exhibited broadly the same

rank order of increases in [^{3}H]InsP_t (Chapter 3) and [^{3}H]Cho accumulation was consistent with the degree of PtdCho hydrolysis being dependent upon the magnitude of prior inositol lipid hydrolysis and, therefore, activation of PKC. Finally, the observation that sub-maximal concentations of bombesin and [Arg^8]Vp were additive rather than synergistic in stimulating [^{3}H]Cho accumulation in combination is also consistent with the the two agonists stimulating PtdCho hydrolysis through a common pathway.

Thus, it seems that for RGE-type mitogens which couple to inositol lipid hydrolysis via a G-protein, a common consequence of mitogen-stimulated inositol lipid hydrolysis will be the activation of PtdCho-PLD. The kinetics of bombesin-stimulated PtdCho hydrolysis and formation of [^{3}H]Cho and [^{3}H]PtdBut are fully consistent with PtdCho-PLD contributing to the second phase of bombesin-stimulated DG elevation and this has been shown to be the case by using butan-1-ol to block DG formation (Chapter 4 of this thesis). By analogy with bombesin, it would seem likely that PLD activation will contribute to the formation of DG by vasopressin and $PGF_{2\alpha}$; consistent with this, Huang & Cabot (1990b) have recently shown that vasopressin does indeed stimulate a PLD activity which may perform just this function in REF52 fibroblasts.

Chapter 6.

Growth factor receptors with tyrosine kinase activity can couple to DG formation and activation of PtdCho-phospholipase D.

6.1 Introduction.

In bombesin-stimulated Swiss 3T3 cells neither the kinetics or mass increase in Ins(1,4,5)P_3 can completely account for the observed increases in DG (Chapters 3 and 4). Furthermore, the sustained increase in DG levels can be dissociated from inositol lipid hydrolysis by the phorbol ester PMA. It seems that phospholipase D-catalysed hydrolysis of PtdCho contributes to the sustained phase of DG production and this may require prior activation of protein kinase C resulting from initial PtdIns(4,5)P_2 hydrolysis. Furthermore, it would seem that activation of a PtdCho-PLD may be a common early response to those mitogens which activate PtdIns(4,5)P_2 hydrolysis via a 'classical' receptor- G_p- PIC (RGE) mechanism. These results suggest that PtdCho hydrolysis by PLD might be a widespread signal transduction pathway involved in mitogenic signalling events. Using the Swiss 3T3 cell as a model system experiments were performed to determine if this pathway of stimulated PtdCho hydrolysis was activated by growth factors of the 'receptor tyrosine kinase' (RTK) family. These studies concentrated upon epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) whose receptors possess ligand activated tyrosine kinase activity. EGF and PDGF serve as particularly useful comparisons to bombesin since PDGF is able to stimulate Ins(1,4,5)P_3 formation, albeit by a fundamentally different mechanism (Blakeley *et al.*, 1989), whilst EGF is reported not to stimulate inositol lipid hydrolysis in Swiss 3T3 cells (Hesketh *et al.*, 1988).

In this chapter the ability of EGF and PDGF to stimulate inositol lipid hydrolysis, PLD activity and, in the case of EGF, DG formation is compared with bombesin and phorbol ester. The results suggest that in addition to regulation by PKC, PLD activity may be regulated by other pathways including growth factor receptor tyrosine kinases. The results are discussed in the context of possible multiple pathways of PLD regulation and DG formation in response to mitogenic stimuli.

6.2 Results.

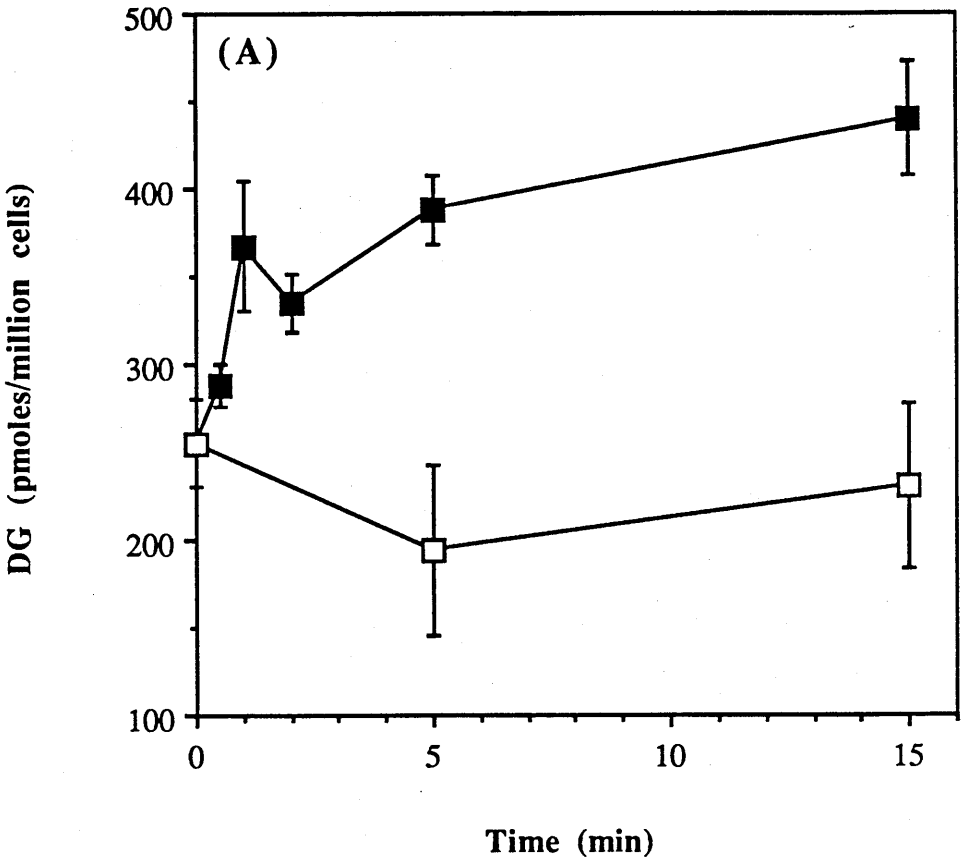
6.2.1 EGF stimulates an increase in DG mass in the absence of significant inositol lipid hydrolysis.

The ability of EGF to activate PKC in Swiss 3T3 cells is subject to some debate (Isacke *et al.*, 1986; Rodruigez-Pena & Rozengurt, 1986; Kazlauskas & Cooper, 1988). Those reports describing the inablility of EGF to activate PKC in Swiss 3T3 cells are supported by the observation that EGF does not stimulate inositol lipid hydrolysis in this cell line (Hesketh *et al.*, 1988). Because of the anomalous reports concerning the ability of EGF to activate PKC in Swiss 3T3 cells and the recent reports of agonist-stimulated DG formation from PtdCho (Billah & Anthes, 1990), the effect of EGF upon DG levels was examined by mass assay. Figure 6.1a shows the timecourse of EGF-stimulated increases in DG mass. Unlike the response to bombesin there was no rapid increase at 5 sec. Increases above control were only apparent from 30 sec onwards, but were statistically significant from 1 ($p= 0.021$) or 2 min and were maintained at this maximum level for 30 min before declining. At 60 min DG levels had declined, but were still above the corresponding control time point (Fig. 6.1b). The ability of EGF to increase DG levels in Swiss 3T3 cells was dose-dependent (Fig.6.2) with maximal elevation occurring at approximately 1nM and an EC_{50} value of 0.07 ± 0.02 nM. In general the magnitude of EGF-stimulated increases in DG mass was much smaller than that observed for bombesin or PMA; this is particularly apparent in Fig. 6.6 where the three agonists were used in parallel in the same experiment allowing a direct comparison. EGF stimulated a net increase of 150 ± 41 pmoles of DG/10^6 cells (mean $\pm$ S.D. of n= 5 determinations) representing a 1.81 ± 0.48 fold increase over unstimulated levels (mean $\pm$ S.D. from n= 9 determinations). This smaller response, compared with the sustained phase of bombesin-stimulated DG formation, is similar to that seen by Wright *et al.* (1988; 1990a) in EGF-stimulated IIC9 cells in comparison to that stimulated by α-thrombin.

Despite this increase in cellular diglyceride content, EGF was unable to stimulate a reproducible increase in [^{3}H]inositol phosphate accumulation over

Figure 6.1 (a & b). Time course of EGF-stimulated increases in DG mass in Swiss 3T3 cells.

Confluent, quiescent Swiss 3T3 cells were washed in serum-free DMBH for 60 min at 37°C before replacing with DMBH alone (❑) or containing 100nM EGF (■) for the indicated times up to 15 (a) or 60 (b) min. Incubations were terminated by aspiration and addition of ice-cold methanol and chloroform extracts were assayed for DG mass by incubating with DG kinase and [γ-^{32}P]ATP as outlined in Chapter 2. The results are expressed as mass of DG (pmoles/10^6 cells) and each point represents the mean ± S.D. of triplicate determinations from a single experiment representative of three.


(A)
500
400
300
200
100
0
5
10
15
DG (pmoles/million cells)
Time (min)

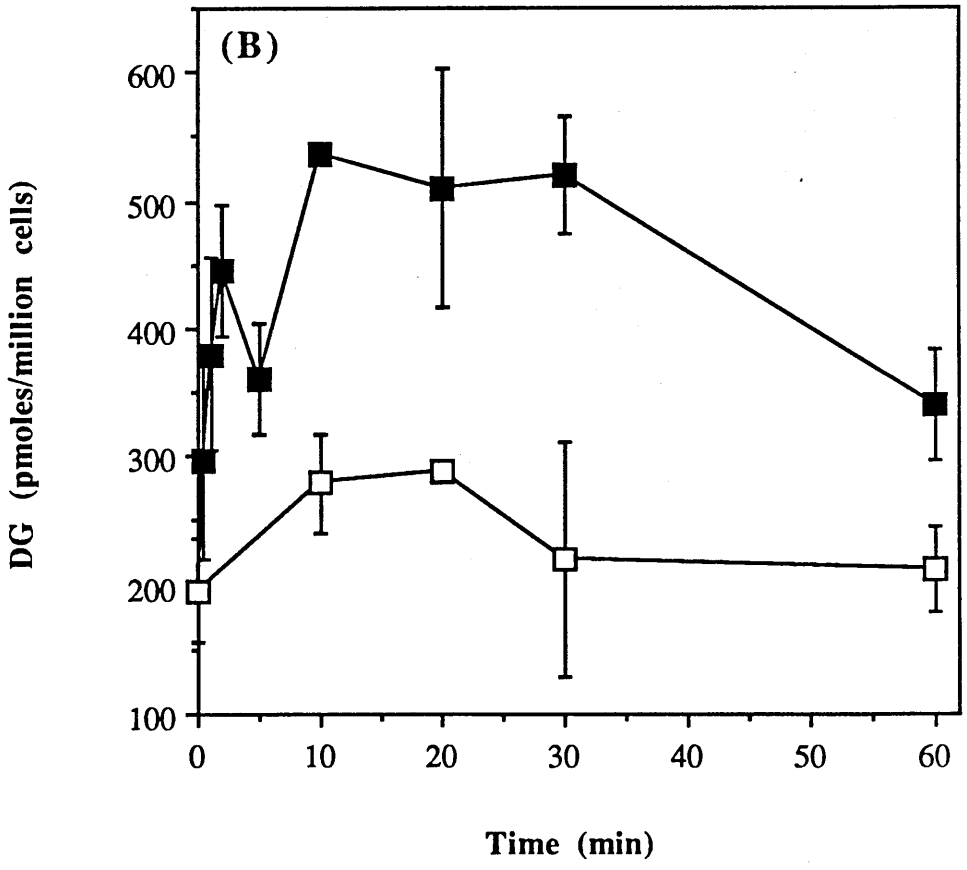

(B)
600
500
400
300
200
100
0
10
20
30
40
50
60
DG (pmoles/million cells)
Time (min)

Figure 6.2. Dose-response curve for EGF-stimulated DG accumulation in Swiss 3T3 cells.

Confluent, quiescent Swiss 3T3 cells were washed in serum-free DMBH for 60 min at 37°C before replacing with DMBH containing the indicated final concentration of EGF (■) for a further 10 min. Following aspiration and addition of ice-cold methanol, chloroform extracts were prepared and an aliquot assayed for DG mass by the method outlined in Chapter 2. The results are expressed as mass of DG (pmoles/10^6 cells) and each point represents the mean ± S.D. of triplicate determinations from a single experiment representative of three.

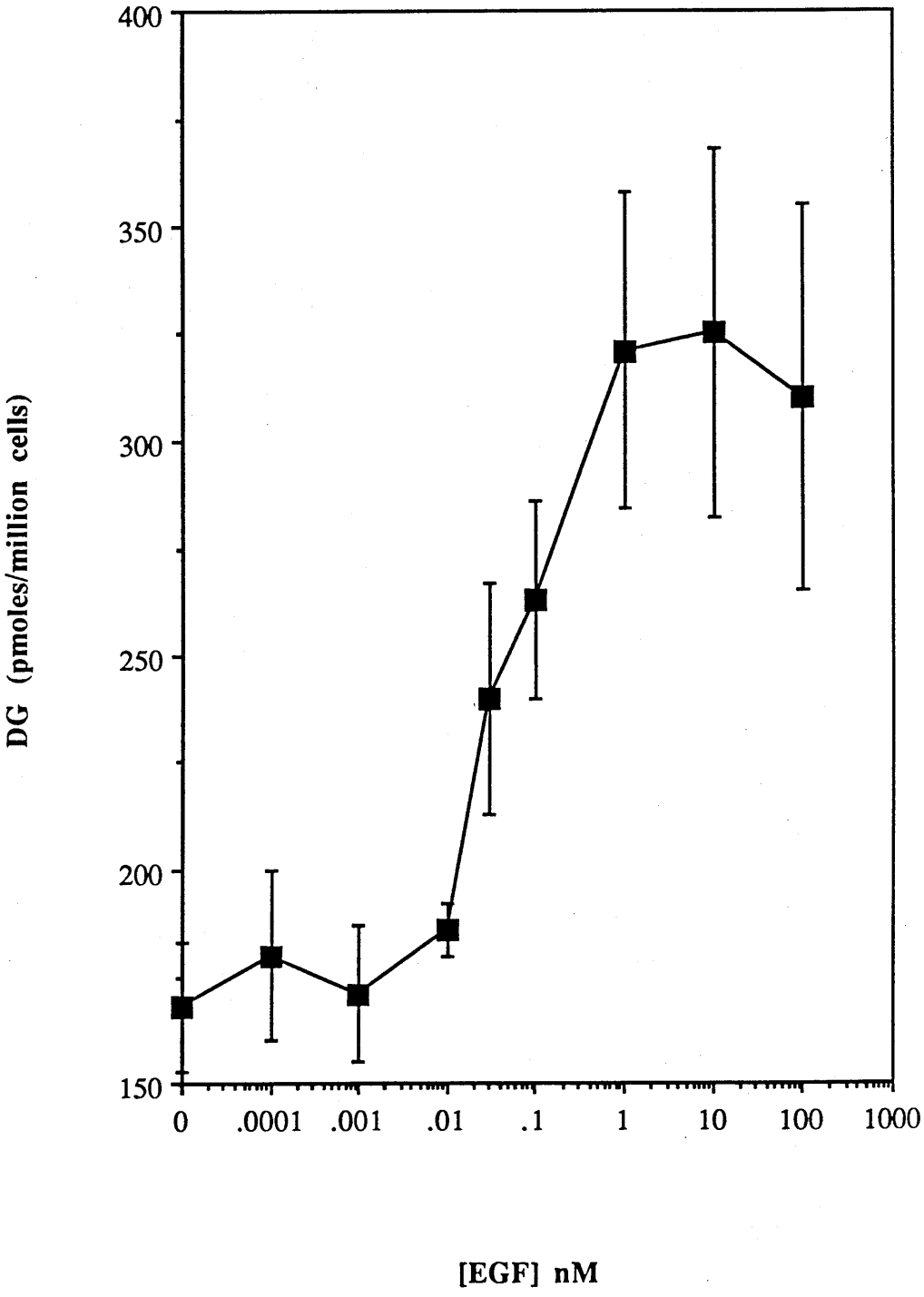
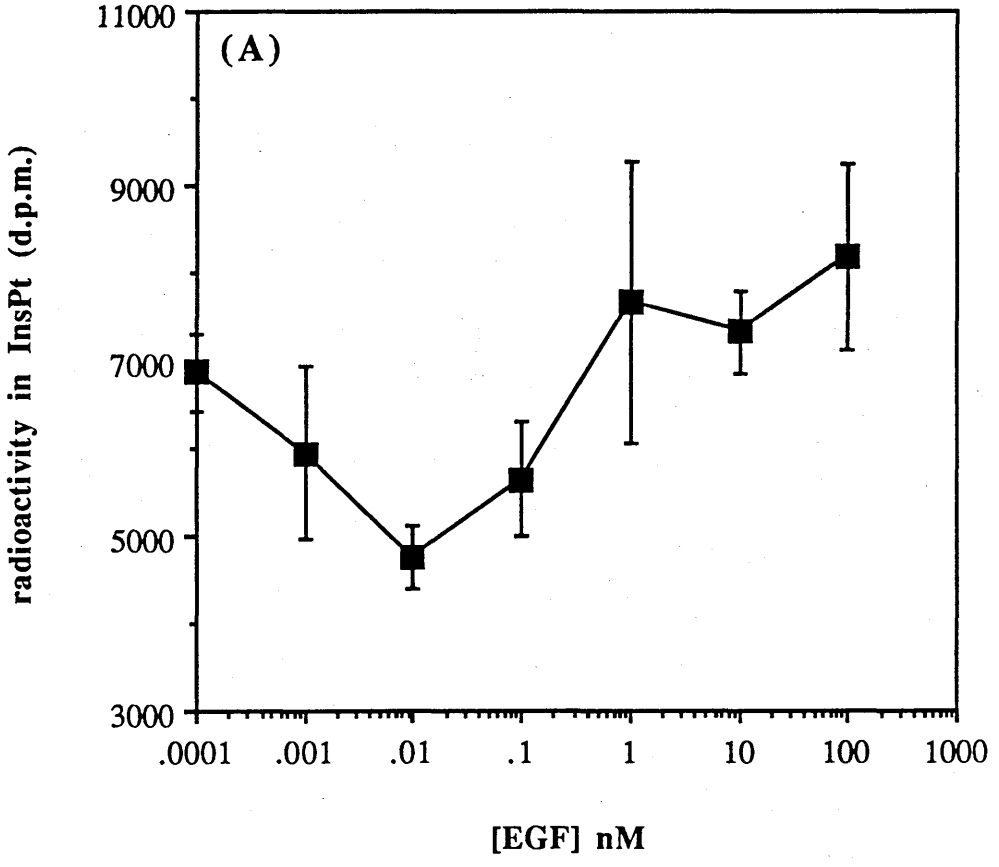
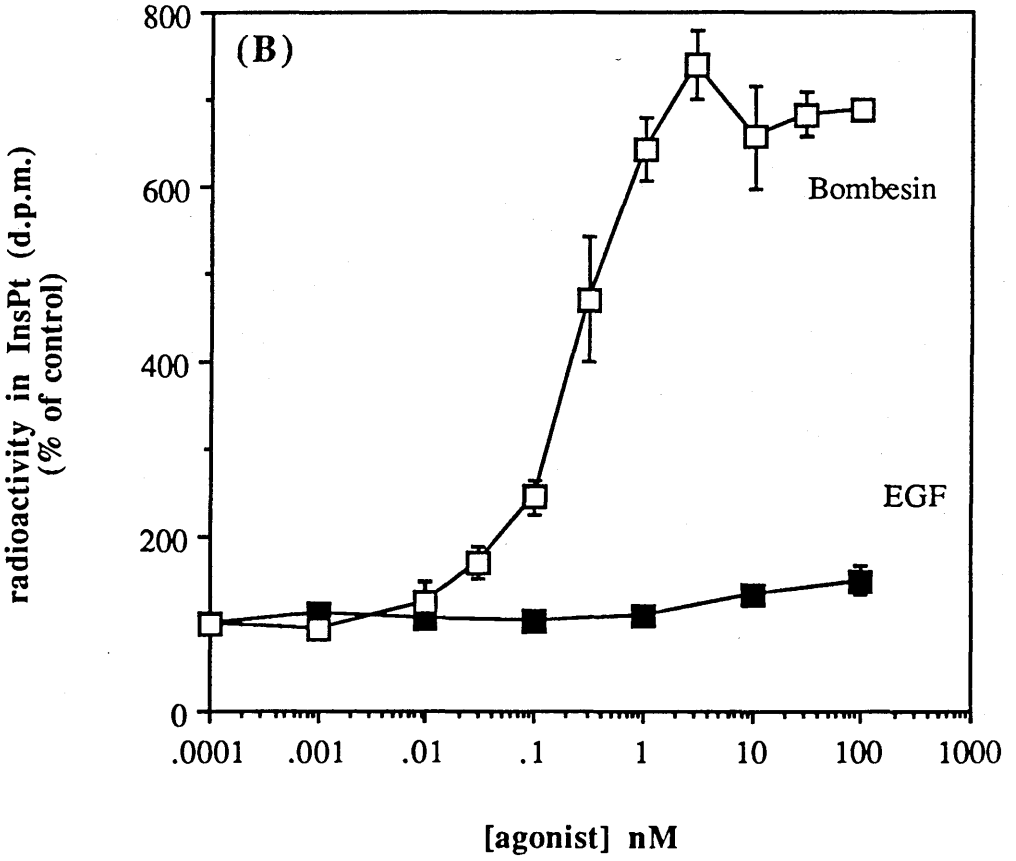

400
350
300
250
200
150
DG (pmoles/million cells)
0
.0001
.001
.01
.1
1
10
100
1000
[EGF] nM

Figure 6.3 (a & b). The effect of EGF upon [^{3}H]InsP$_t$ accumulation in Swiss 3T3 cells; a comparison with bombesin.

Swiss 3T3 cells were grown in 24 well plates until judged to be 80 % confluent and were then grown for a further 48 hours in inositol-free DMEM containing 1% dialysed calf serum and either 0.75μCi ml^{-1} (bombesin in (b)) or 1μCi ml^{-1} ((a) and EGF in (b)) [^{3}H]inositol. After washing in HBG for 5 min cells were incubated in HBG containing 10mM LiCl (HBG.Li) for a further 15 min before replacing with HBG.Li containing the indicated final concentrations of (a) EGF (■) and (b) EGF (■) or bombesin (❑). Incubations were terminated after 20 min by aspiration and addition of ice-cold methanol and aqueous methanolic extracts were prepared as described in the Methods section. [^{3}H]InsP$_t$ were resolved as described in Chapter 2 and the associated radioactivity was determined by liquid scintillation counting. The results are expressed as (a) radioactivity in [^{3}H]InsP$_t$ (d.p.m.) or (b) percentage of control, to allow comparison of the two agonists. Each point represents the mean ± S.D. of triplicate determinations from single experiments representative of three. In the case of (b) the basal radioactivity associated with [^{3}H]InsP$_t$ was 2716 ± 93 dpm for bombesin and 5665 ± 948 dpm for EGF.


(A)
11000
9000
7000
5000
3000
radioactivity in InsPt (d.p.m.)
.0001
.001
.01
.1
1
10
100
1000
[EGF] nM


(B)
800
600
400
200
0
radioactivity in InsPt (d.p.m.)
(% of control)
Bombesin
EGF
.0001
.001
.01
.1
1
10
100
1000
[agonist] nM

controls even in the presence of 10mM LiCl. Figure 6.3a shows the dose response curve for EGF-stimulated changes [^{3}H]InsP_t accumulation; a small elevation was sometimes observed at the highest dose (100nM, Fig. 6.3b), but was not reproducible and was clearly at least two orders of magnitude to the right of the dose response curve for DG accumulation (Fig.6.2.). Figure 6.3b compares the dose-response curves for EGF- and bombesin-stimulated increases in [^{3}H]InsP_t accumulation. The accumulation of [^{3}H]InsP_t in response to EGF was negligible in comparison to that stimulated by bombesin, suggesting that at mitogenically relevant doses the activated EGF receptor did not couple to inositol lipid hydrolysis. Furthermore EGF did not stimulate any apparent increase in Ins(1,4,5)P_3 mass up to 5 min when assayed by the competitive binding assay (S. Palmer, personal communication).

6.2.2 EGF stimulates PtdCho-PLD activity but this does not serve as a pathway for DG formation.

Since a number of reports (Wright *et al.*, 1990a; Billah & Anthes, 1990) and results from Chapter 4 have suggested a role for phospholipase C- & D-catalysed PtdCho hydrolysis as an alternative source of DG, experiments were performed to investigate if EGF did stimulate PtdCho hydrolysis and whether this played a role in DG formation.

Figure 6.4a shows the kinetics of EGF-stimulated increases in [^{3}H]PtdBut in [^{3}H]palmitate-labelled Swiss 3T3 cells stimulated in the presence of butan-1-ol. Following addition of EGF increases in [^{3}H]PtdBut were apparent and significant after 30 sec ($p= 0.006$) and increased rapidly until 2 min. In most experiments [^{3}H]PtdBut levels continued to rise from 2 min up to 15 min. However, the rate of [^{3}H]PtdBut formation from 2 min onwards was much lower than that in the first 2 min (0-2 min + EGF 854 dpm min^{-1}, 2-15 min + EGF 65 dpm min^{-1}, control no added EGF, 12 dpm min^{-1}; results from a single representative experiment). This slower increase in [^{3}H]PtdBut was variable; in some cases the 15 min stimulated point was significantly higher than the 5 min stimulated point ($p= 0.032$, Fig.6.4a)

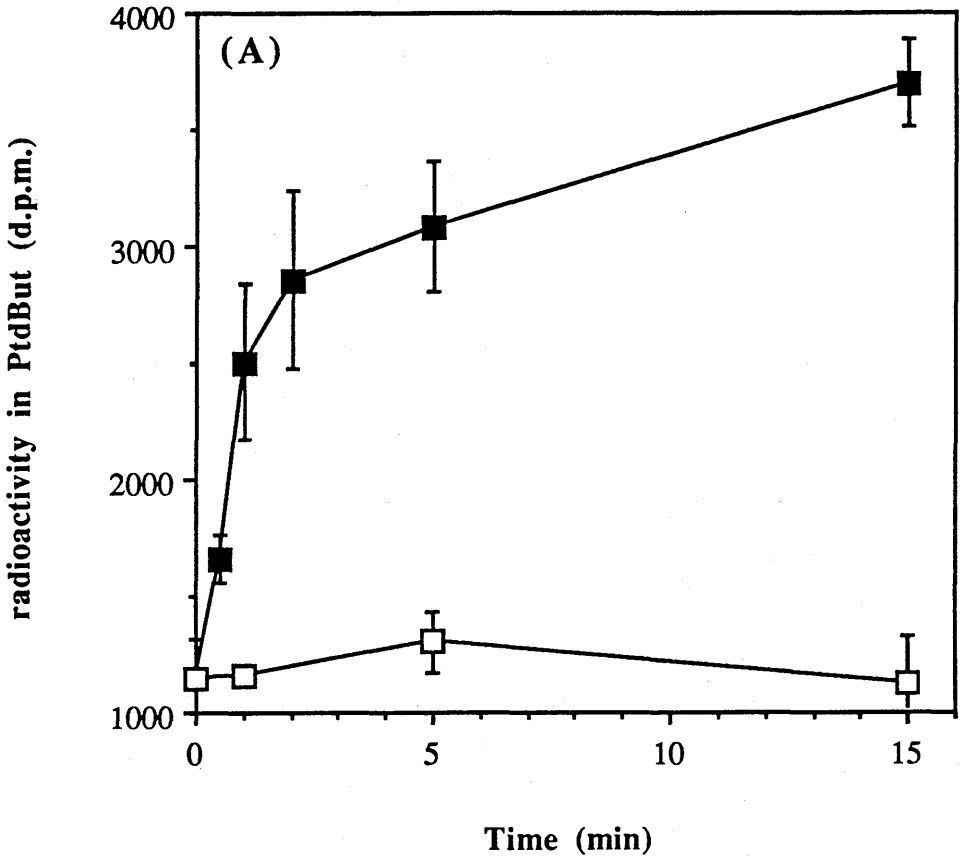
Figure 6.4 (a & b). EGF-stimulated [^{3}H]PtdBut formation in Swiss 3T3 cells.

Swiss 3T3 cells were grown in 6 well plates until judged to be 80% confluent. They were then grown for a further 48 hours in 2ml DMEM containing 1% calf serum and a total of 4μCi of [^{3}H]palmitic acid.

(a) After washing in DMBH for 30 min at 37^{o}C cells were incubated for a further 5 min in DMBH containing 0.3% (v/v) butan-1-ol before replacing with DMBH containing 0.3% (v/v) butan-1-ol alone (□) or with 100nM EGF (■) for the indicated times.

(b) After washing in DMBH for 30 min at 37^{o}C cells were incubated in DMBH containing 0.3% (v/v) butan-1-ol and the indicated concentration of EGF (■) for 15 min.

In both cases incubations were terminated by aspiration and addition of ice-cold methanol and chloroform extracts were prepared and [^{3}H]PtdBut resolved on LK5DF t.l.c. plates as described in the Methods section. [^{3}H]PtdBut was excised from the silica gel and the radioactivity determined by scintillation counting. The results are mean radioactivity in PtdBut (d.p.m.) ± S.D. of triplicate determinations from a single experiment representative of three.


(A)
4000
3000
2000
1000
0
5
10
15
radioactivity in PtdBut (d.p.m.)
Time (min)

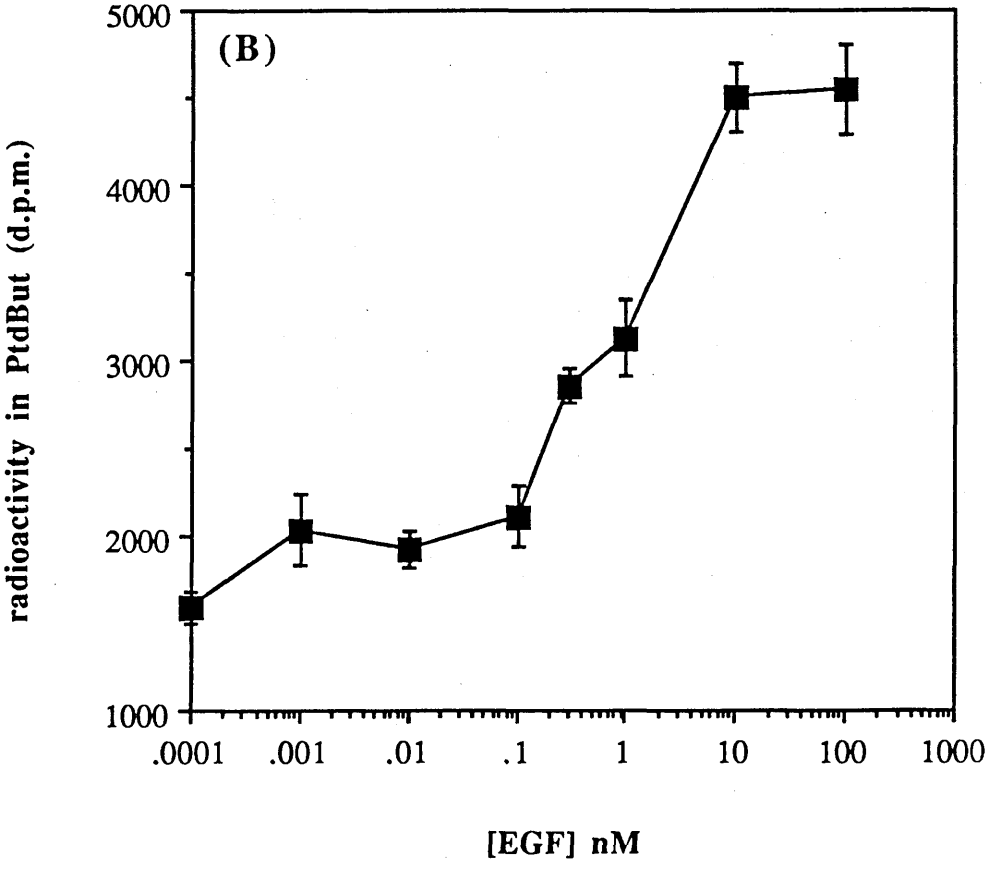
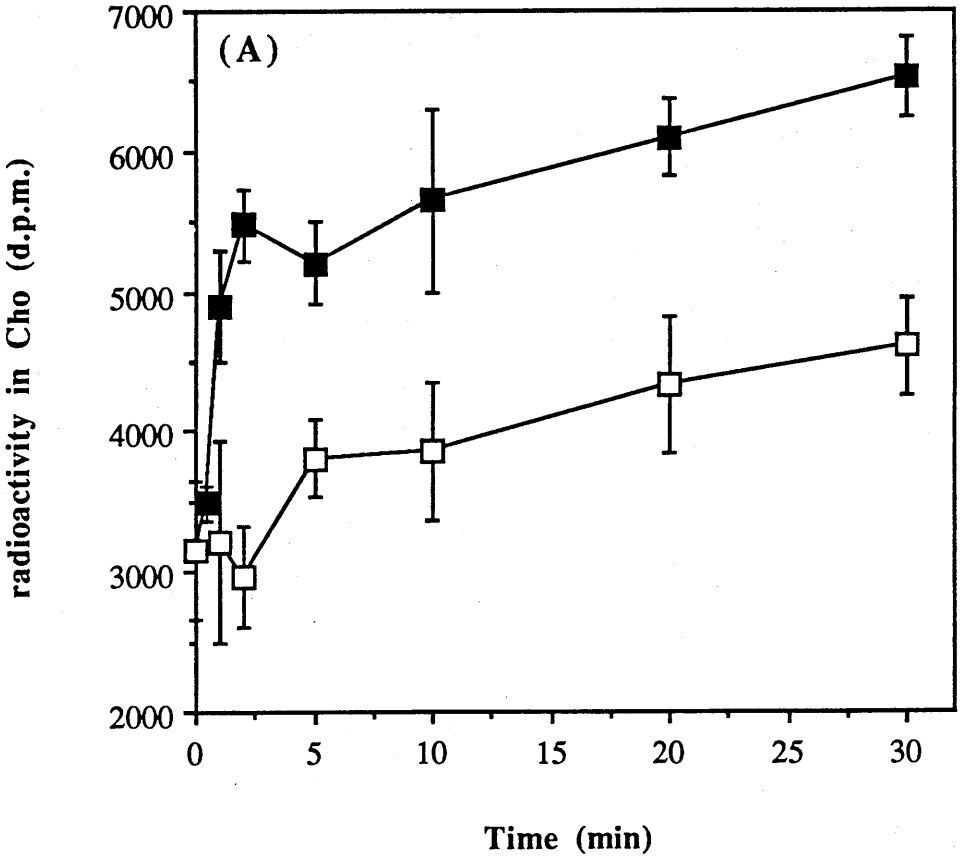
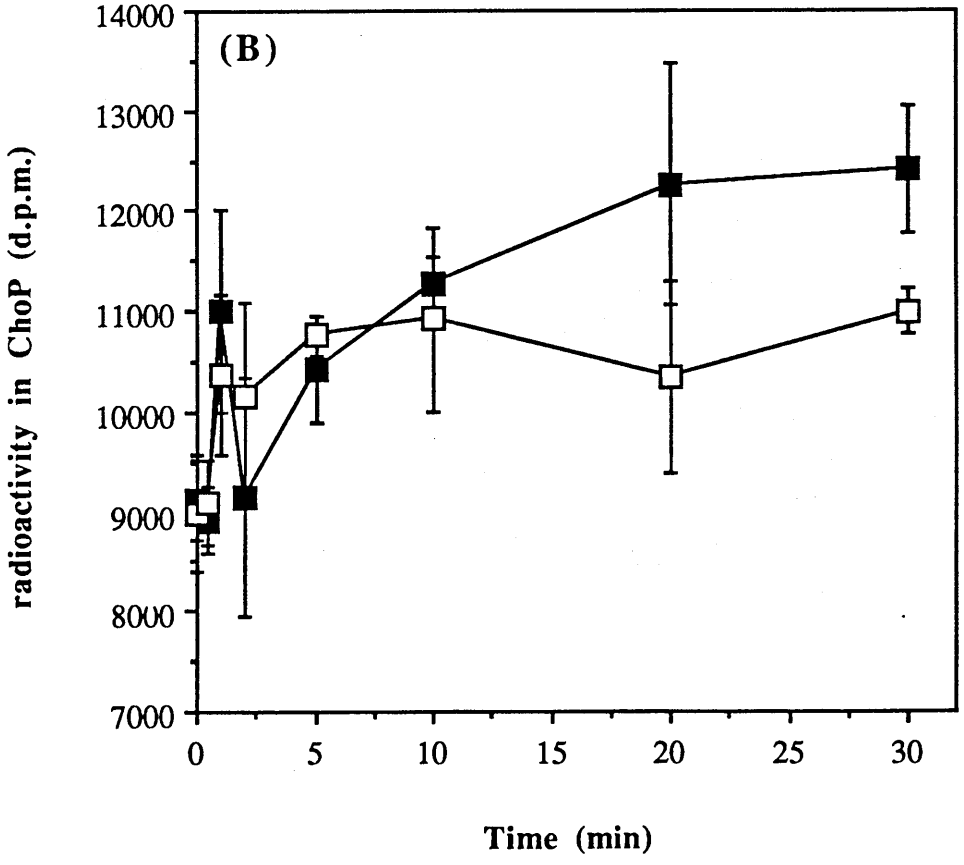

(B)
5000
4000
3000
2000
1000
.0001
.001
.01
.1
1
10
100
1000
radioactivity in PtdBut (d.p.m.)
[EGF] nM

Figure 6.5 (a & b). EGF-stimulated increases in [^{3}H]Cho and [^{3}H]ChoP in Swiss 3T3 cells.

Swiss 3T3 cells were grown in 24 well plates until judged to be 80% confluent after which they were grown for a further 48 hours in 1ml of DMEM containing1% calf serum and 1.5μCi of [^{3}H]choline chloride. Cells were then washed in Hanks and HBG for a total of 45 min as described in the Methods section before replacing with HBG alone (□) or containing 100nM EGF (■) for the indicated times. Incubations were terminated by addition of ice-cold methanol and aqueous methanolic extracts were loaded onto Dowex-50W-H^+ columns for resolution of (a) [^{3}H]Cho and (b) [^{3}H]ChoP. The radioactivity associated with an aliquot of each fraction was determined by scintillation counting. The results are mean radioactivity in each fraction (d.p.m.) ± S.D. of triplicate determinations from a single experiment representative of three.


(A)
radioactivity in Cho (d.p.m.)
7000
6000
5000
4000
3000
2000
0
5
10
15
20
25
30
Time (min)


(B)
radioactivity in ChoP (d.p.m.)
14000
13000
12000
11000
10000
9000
8000
7000
0
5
10
15
20
25
30
Time (min)

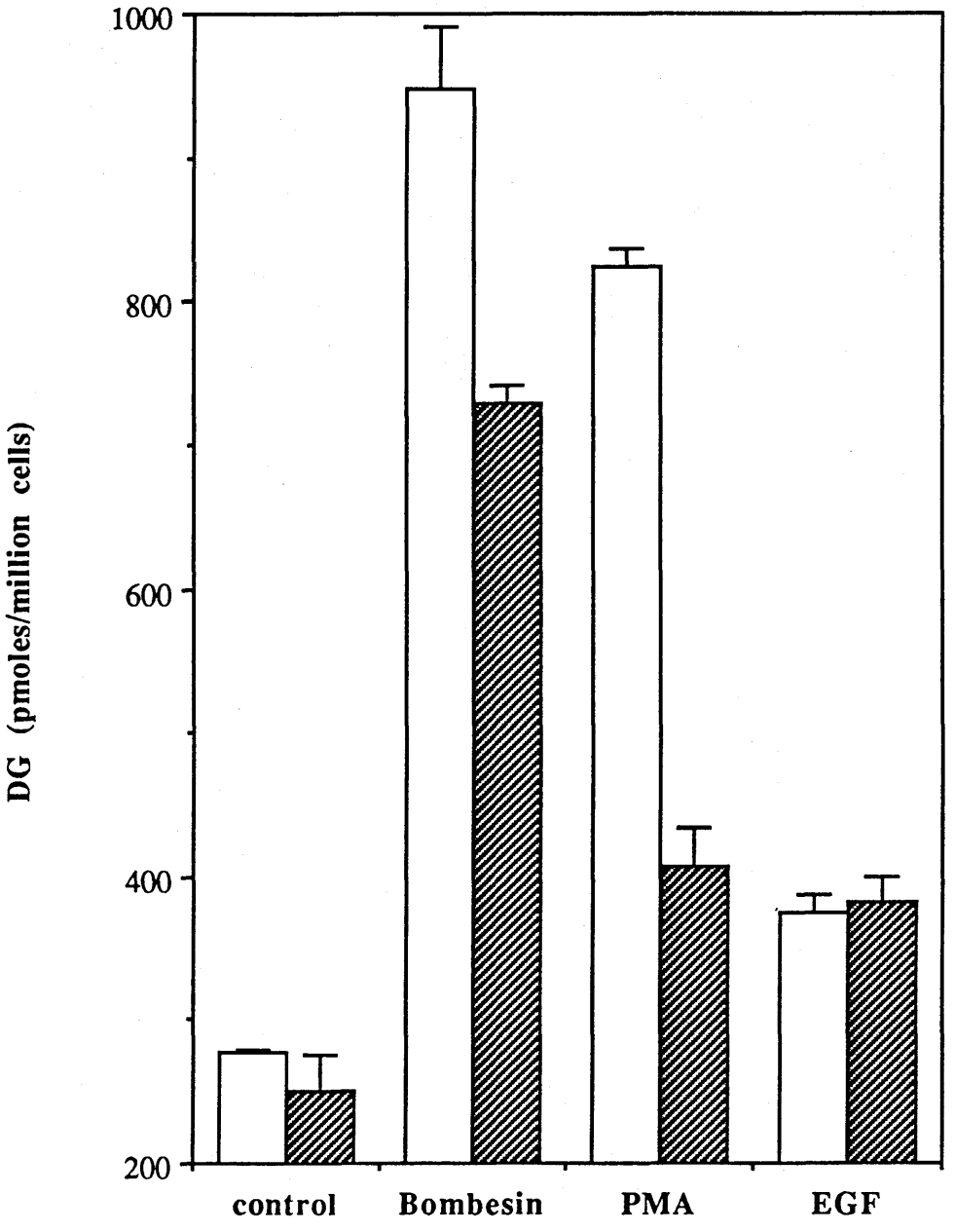
whilst in other experiments the continued rise in [^{3}H]PtdBut was not statistically significant. Fig. 6.4 (b) shows the dose-response curve for EGF-stimulated increases in [^{3}H]PtdBut levels in Swiss 3T3 cells; the EC_{50} value was 1.14 ± 0.54 nM (mean ± S.D., n=3 experiments). These results clearly demonstrated that EGF stimulates PLD activity.

To confirm that this PLD activity was utilizing PtdCho as a major substrate the effect of EGF upon [^{3}H]Cho, [^{3}H]ChoP and [^{3}H]GroPCho levels was examined in Swiss 3T3 cells labelled to isotopic equilibrium with [^{3}H]choline chloride. Figure 6.5 (a & b) shows the time course of changes in the radioactivity associated with [^{3}H]Cho and [^{3}H]ChoP in EGF-stimulated Swiss 3T3 cells; there was no significant increase in [^{3}H]GroPCho in any experiment performed (data not shown). Following addition of EGF, [^{3}H]Cho levels were elevated within 30 sec and were significantly above control at 1 minute (Fig. 6.5a) ($p= 0.025$). Maximal elevation was observed at 2 min after which the rate of increase slowed but [^{3}H]Cho levels were maintained above control for at least 30 and, in some cases, 60 min (data not shown). No significant increases in the radioactivity associated with [^{3}H]ChoP were observed in response to EGF until much later timepoints (typically 30 min, Fig.6.5b).

Incubation in the presence of primary alcohols allows a preliminary assessment of what role a coupled PLD/PPH pathway plays in DG formation since it traps phosphatidyl moieties as the relativeley non-reactive phosphatidylalcohol (Bonser *et al.*, 1989; Metz &Dunlop, 1991; Chapter 4). To determine what contribution, if any, the PLD/PPH pathway made to EGF-stimulated DG formation the effect of butan-1-ol upon EGF-stimulated DG levels was examined. Pre-incubation of Swiss 3T3 cells with 0.3% (v/v) butan-1-ol for 5 min resulted in inhibiton of PMA- and, to a lesser extent, bombesin-stimulated DG formation (Fig. 6.6). Under the same conditions EGF-stimulated increases in DG formation were unaffected by the presence of butan-1-ol prior to and during the stimulation (Fig. 6.6), suggesting that DG formation did not occur via a PLD/PPH coupled pathway in response to EGF.

Figure 6.6. The effect of butan-1-ol upon EGF-stimulated DG formation.

Swiss 3T3 cells were grown to confluence and quiescence and were washed for 60 min in serum-free DMBH at 37°C. Cells were then washed for a further 5 min in DMBH containing 0.3% (v/v) butan-1-ol before replacing with DMBH containing butan-1-ol and either EGF (100nM), bombesin (100nM) or PMA (100nM) for 15 min. Incubations were terminated by aspiration and addition of ice-cold methanol and chloroform extracts were prepared as described in chapter 2. DG mass in these extracts was assayed as described in the Methods section. Results are expressed as mass of DG (pmoles/10^6 cells) and each data set is the mean ± S.D. of triplicate determinations from a single experiment representative of three.


1000
800
600
400
200
DG (pmoles/million cells)
control
Bombesin
PMA
EGF

6.2.3 Regulation of PLD by ligand-activated EGF receptors.

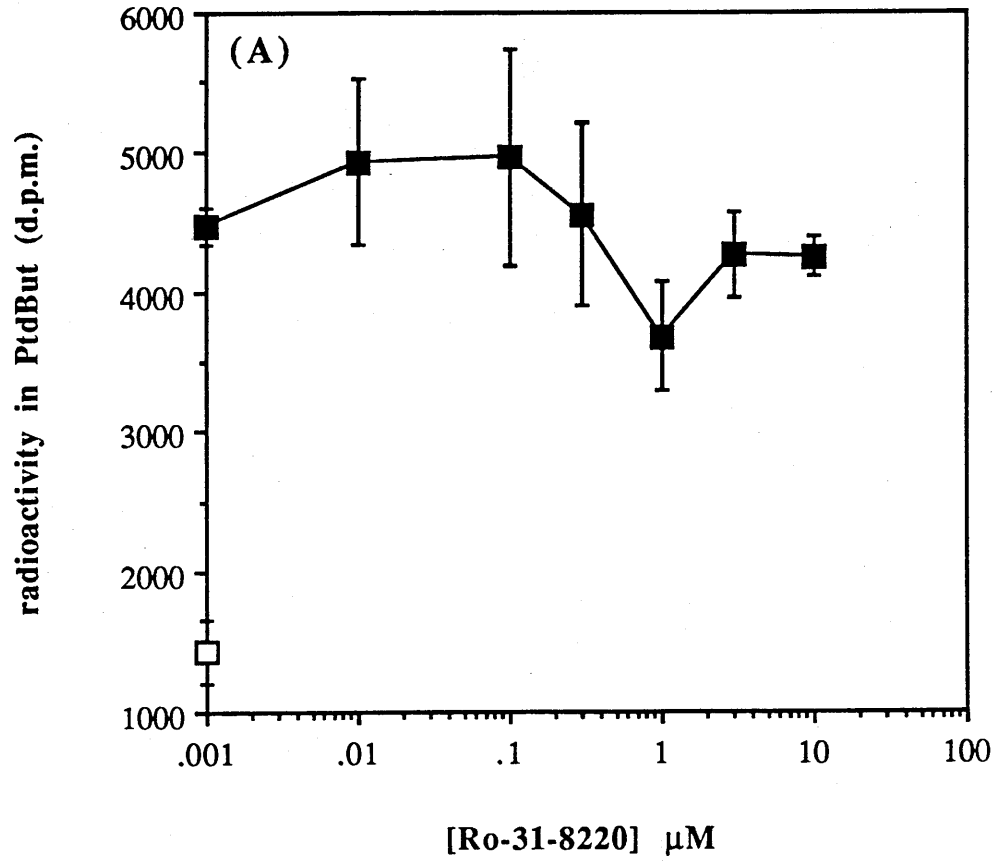
These results suggested that the EGF receptor was able to activate a PtdCho-PLD activity, though this apparently did not serve as a pathway for DG formation. In Chapter 4 evidence was presented to suggest that bombesin-stimulated PtdIns(4,5)P_2 hydroysis might couple to activation of PLD via increases in PKC activity. Since EGF was able to increase DG levels and activate PLD without PtdIns(4,5)P_2 hydrolysis this suggested that the EGF receptor might couple directly to PLD independently of PKC activity. To investigate this, the effect of the PKC inhibitor Ro-31-8220 on EGF-stimulated [^{3}H]PtdBut formation was determined and compared with that of PMA and bombesin. The results are shown in Figure 6.7 (a & b). In Fig.6.7a, [^{3}H]palmitate labelled Swiss 3T3 cells were pre-treated for 5 min with increasing concentrations of Ro-31-8220 followed by addition of a maximal dose of EGF (20 nM). The figure shows that even at the highest concentration of the PKC inhibitor EGF-stimulated [^{3}H]PtdBut formation was not significantly inhibited. When the results were normalised to 'percent of maximum' and compared with that for PMA and bombesin (Fig. 6.7b) the contrasting effect upon the EGF response was clearly seen. PMA-stimulated [^{3}H]PtdBut formation was inhibited by approximately 90% whilst bombesin-stimulated PLD activity was inhibited by about 50% as described in Chapter 5. In contrast EGF-stimulated [^{3}H]PtdBut activity was not inhibited and in some cases there was a clear enhancement at 3μM Ro-31-8220 which was statistically significant ($p= 0.007$) at 10μM Ro-31-8220 (Fig. 6.7b).

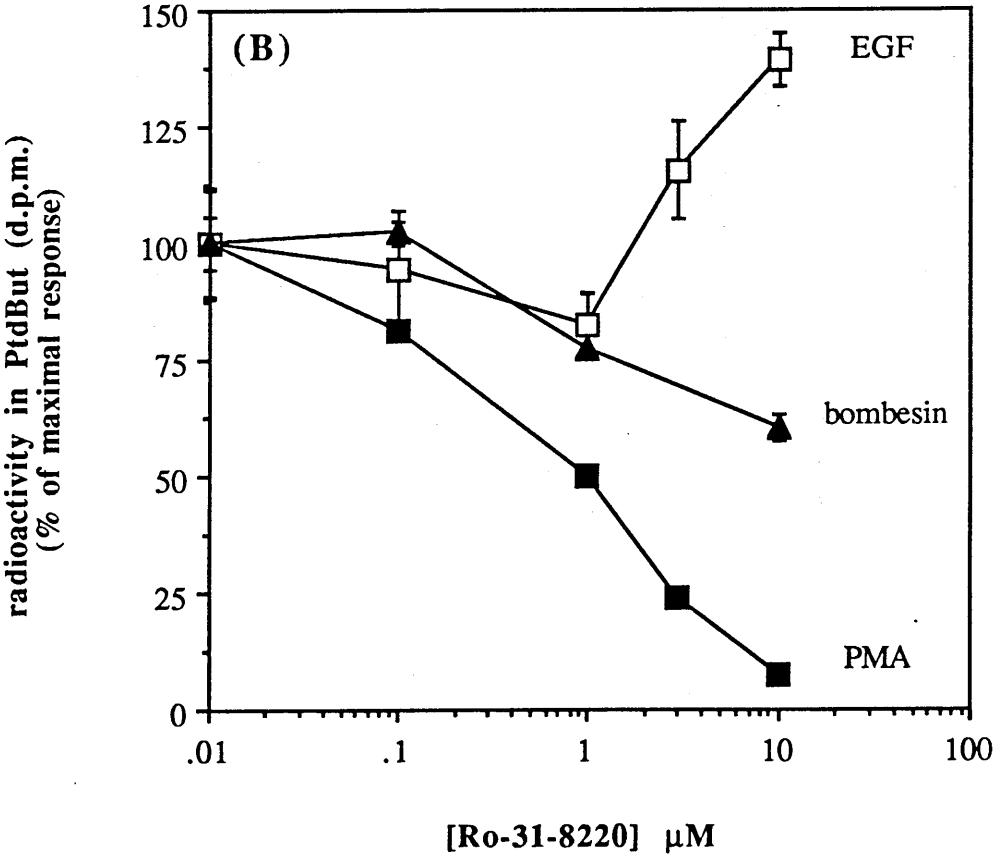
The cytosolic domain of the EGF receptor includes a tyrosine kinase catalytic region which is activated after ligand induced receptor autophosphorylation and dimerisation. It has been proposed that, once activated, this catalytic domain is responsible for interacting with and phosphorylating various key substrates such as effector systems (reviewed by Ullrich & Schlessinger, 1990). In A431 cells and NIH3T3 cells over-expressing the human EGF receptor one of these substrates is PLC-γ1 (Meisenhelder *et al.*, 1989; Margolis *et al.*, 1989) However, in Swiss 3T3 cells possessing their 'native' receptor complement EGF does not stimulate inositol lipid hydrolysis (Hesketh *et al.*, 1985; Fig 6.3) or phosphorylation of

Figure 6.7 (a & b). Comparison of the effect of Ro-31-8220 upon EGF-, PMA- and bombesin-stimulated [^{3}H]PtdBut formation in Swiss 3T3 cells.

Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent whereupon they were grown for a further 48 hours in 2 ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid. After washing in DMBH for 30 min at 37^{o}C cells were incubated for 5 min in 0.9 ml of DMBH containing 0.3% (v/v) butan-1-ol and the indicated concentration of Ro-31-8220. Incubations were started by addition of 0.1 ml of agonist solutions to give final concentrations of 30nM for EGF and 100nM each for PMA and bombesin. After 15 min incubations were terminated by aspiration and addition of ice-cold methanol and chloroform extracts were prepared as described in the Methods section. [^{3}H]PtdBut was resolved by t.l.c. and the associated radioactivity determined by scintillation counting after excision from the plate. The results are expressed as (a) mean radioactivity in [^{3}H]PtdBut (d.p.m.) ± S.D.(n=3) from a single experiment typical of four or (b) are normalised to percentage of maximal response to allow comparison with bombesin and PMA. In the case of bombesin the unstimulated d.p.m. in [^{3}H]PtdBut was 1894 ± 57 and the maximaum response was 12033 ± 1185 d.p.m., whilst for PMA the unstimulated radioactivity in [^{3}HPtdBut was 1306 ± 218 d.p.m. and the maximal response was 32289 ± 1704 d.p.m.

In (a), ■ = EGF and □ = the vehicle control, DMSO, with no EGF stimulus. In (b), □ = EGF, ▲ = bombesin and ■ = PMA.


(A)
radioactivity in PtdBut (d.p.m.)
6000
5000
4000
3000
2000
1000
.001
.01
.1
1
10
100
[Ro-31-8220] μM


(B)
radioactivity in PtdBut (d.p.m.)
(% of maximal response)
150
125
100
75
50
25
0
.01
.1
1
10
100
EGF
bombesin
PMA
[Ro-31-8220] μM

PLC-γ1 (Meisenhelder *et al.*, 1989). To investigate whether the EGF receptor tyrosine kinase activity was required for activation of PLD by EGF the effect of pre-treating Swiss 3T3 cells with the tyrosine kinase inhibitor AG 18 (Levitski, 1990a) was examined. AG 18 exemplifies a group of compounds called Tyrphostins, hydroxylated benzylidene malonitrile compounds, which inhibit receptor tyrosine kinase activities by competing with the substrate rather than inhibiting at the ATP binding site. However, the K_i against the EGF receptor is at least two orders of magnitude lower than that against the insulin receptor and as such it can be used as a reasonably selective inhibitor of the EGF receptor tyrosine kinase (Lyall *et al.*, 1989; Levitski, 1990a).

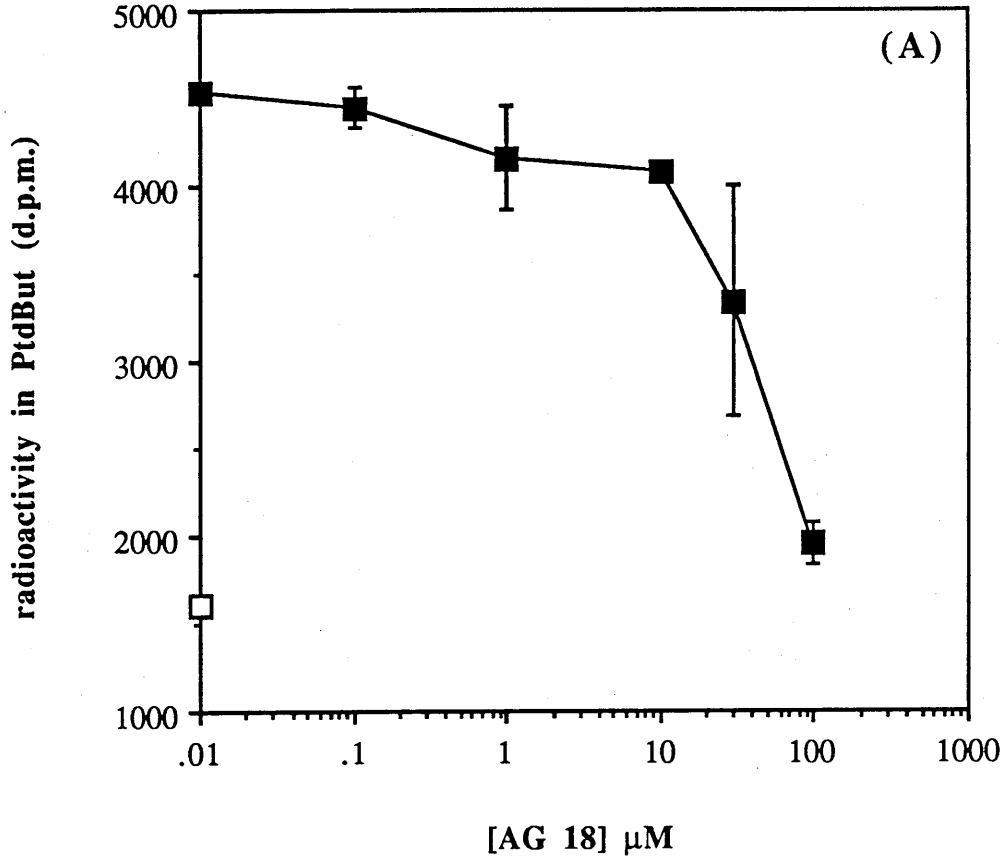
When [^{3}H]palmitate labelled Swiss 3T3 cells were pre-treated with increasing concentrations of AG18 for 18 hours there was a dose-dependent inhibition of EGF-stimulated [^{3}H]PtdBut formation as shown in Fig. 6.8a. In addition, the results were normalised to 'percent of maximum' to allow comparison with bombesin which gave a larger response. Maximal inhibition of EGF-stimulated [^{3}H]PtdBut formation was observed at 100μM AG18 with an apparent IC_{50} of about 30μM; an accurate IC_{50} could not be estimated since the inhibition was not complete. At 100μM AG18 the response to EGF was inhibited by approximately 85-90%. In the same experiments in which 100μM AG18 inhibited 90% of EGF-stimulated [^{3}H]PtdBut formation the response to bombesin was completely unaffected (Fig. 6.8b).

Finally, the role of Ca^{2+} in regulating EGF-stimulated PLD activity was assessed by buffering extracellular $[Ca^{2+}]_i$ to approximately 150nM with EGTA. In Swiss 3T3 cells the ability of EGF to stimulate an increase in $[Ca^{2+}]_i$ is a subject of some debate (Hesketh *et al.*, 1985; 1988; Olsen *et al.*, 1988). However in those cells where EGF does stimulate an increase in $[Ca^{2+}]_i$ it is wholly dependent upon the presence of extracellular Ca^{2+} (Hesketh *et al.*, 1985; 1988), so by buffering extracellular Ca^{2+} with EGTA experiments were performed to see if Ca^{2+} entry played any role in EGF-stimulated PLD activity. The results shown in Fig. 6.9 are preliminary and await the confirmation that EGF does indeed stimulate an

Figure 6.8 (a & b) A comparison of the effect of AG 18 upon EGF- and bombesin-stimulated [^{3}H]PtdBut formation.

Swiss 3T3 cells were grown on 6 well plates until judged to be 80% confluent whereupon they were grown for a further 48 hours in 2 ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid. For the last 18 hours AG 18 was added to the cells at the indicated final concentration such that the concentration of the vehicle, DMSO, was never greater than 0.1% (v/v). After 48 hours cells were washed at 37^{o}C in DMBH for 15 min followed by a further wash in 0.9 ml of DMBH containing the same concentrations of AG 18 and 0.3% (v/v) butan-1-ol for 15 min; incubations were started by the addition of 0.1 ml of agonist to give final concentrations of 30nM for EGF and 100nM for bombesin. After 15 min incubations were terminated by aspiration and addition of ice-cold methanol and chloroform extracts were prepared as described in the Methods section. [^{3}H]PtdBut was resolved by t.l.c., excised from the plate and the associated radioactivity determined by scintillation counting. The results are expressed as (a) radioactivity in [^{3}H]PtdBut (d.p.m.) ± S.D. (n=3) from a single experiment typical of three or (b) as percentage of response in the absence of inhibitor to allow a comparison with bombesin. In the case of (b) the unstimulated radioactivity in [^{3}H]PtdBut was 1461 ± 230 d.p.m. and the maximum response in the absence of AG 18 was 3905 ± 332 d.p.m. for EGF and 11557 ± 115 d.p.m. for bombesin.

In (a) ■ = EGF and ❐ = the vehicle control, DMSO, in the absence of stimulus. In (b) ■ = EGF and ❐ = bombesin.


(A)
radioactivity in PtdBut (d.p.m.)
5000
4000
3000
2000
1000
.01
.1
1
10
100
1000
[AG 18] μM

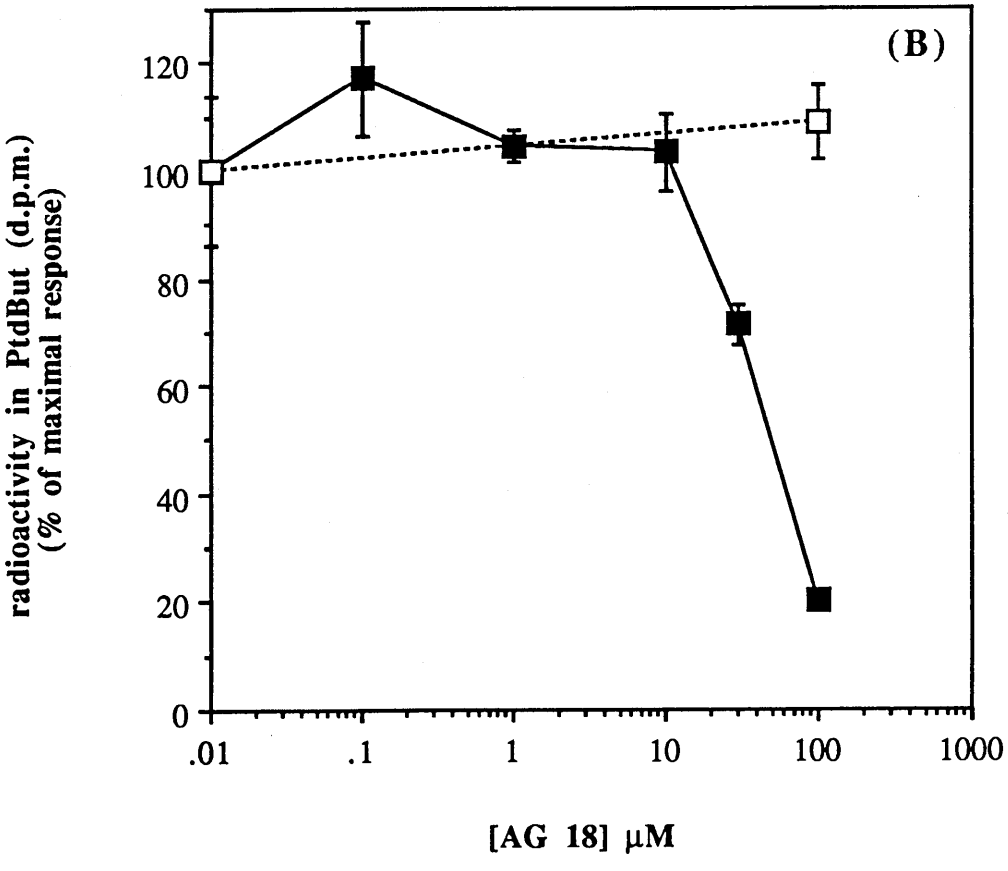
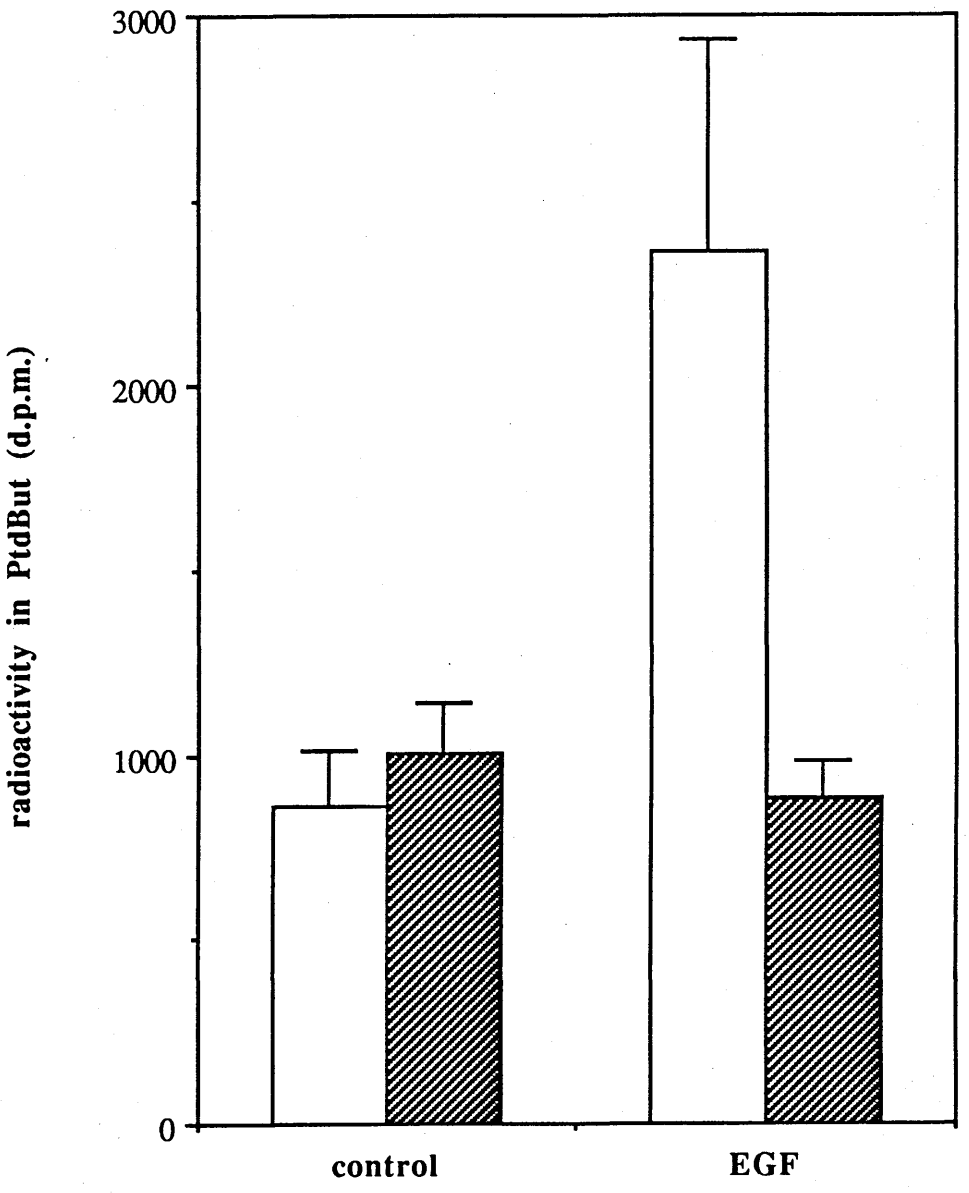

(B)
radioactivity in PtdBut (d.p.m.)
(% of maximal response)
120
100
80
60
40
20
0
.01
.1
1
10
100
1000
[AG 18] μM

Figure 6.9. The effect of EGTA upon EGF-stimulated [^{3}H]PtdBut formation in Swiss 3T3 cells.

Swiss 3T3 cells, grown until judged to be 80% confluent, were grown for a further 48 hours in 2 ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid. Cells were washed at 37°C with DMBH for 15 min followed by DMBH containing 0.3% (v/v) butan-1-ol in the presence (hatched bars) or absence (open bars) of 2.5mM EGTA and incubations were started by replacing with DMBH containing 0.3% (v/v) butan-1-ol ± 2.5mM EGTA and 50nM EGF. After 15 min incubations were terminated by aspiration and addition of ice-cold methanol and chloroform extracts were prepared as described in the Methods section. [^{3}H]PtdBut was resolved by t.l.c., excised from the plate and the associated radioactivity was determined by scintillation counting. The results are expressed as mean radioactivity in [^{3}H]PtdBut (d.p.m.) ± S.D. (n=3) from a single experiment; essentially identical results were obtained in a second experiment.


radioactivity in PtdBut (d.p.m.)
3000
2000
1000
0
control
EGF

increase in $[Ca^{2+}]_i$ in this clone of Swiss 3T3 cells but buffering of extracellular Ca^{2+} resulted in an inhibition of EGF-stimulated PLD activity suggesting that EGF-stimulated PLD activity required the entry of extracellular Ca^{2+} or was Ca^{2+}-dependent in some way.

6.2.4 PDGF stimulates a PtdCho-PLD activity in Swiss 3T3 cells.

Since stimulated PLD activity appears to be a common response to diverse mitogenic growth factors, studies were undertaken to determine if activation of PLD occurred in response to PDGF whose receptor also possesses a ligand activated tyrosine kinase activity. These experiments employed the c-*sis* protein as a BB homodimer of PDGF. Since it is able to interact with both the PDGF α and β type receptor it allowed investigation of the effects of PDGF even though it was not known which receptor sub-type was present in the Swiss 3T3 cell line. Furthermore c-*sis* has been used successfully by other workers studying the effects of PDGF in Swiss 3T3 cells (Blakeley *et al.*, 1989).

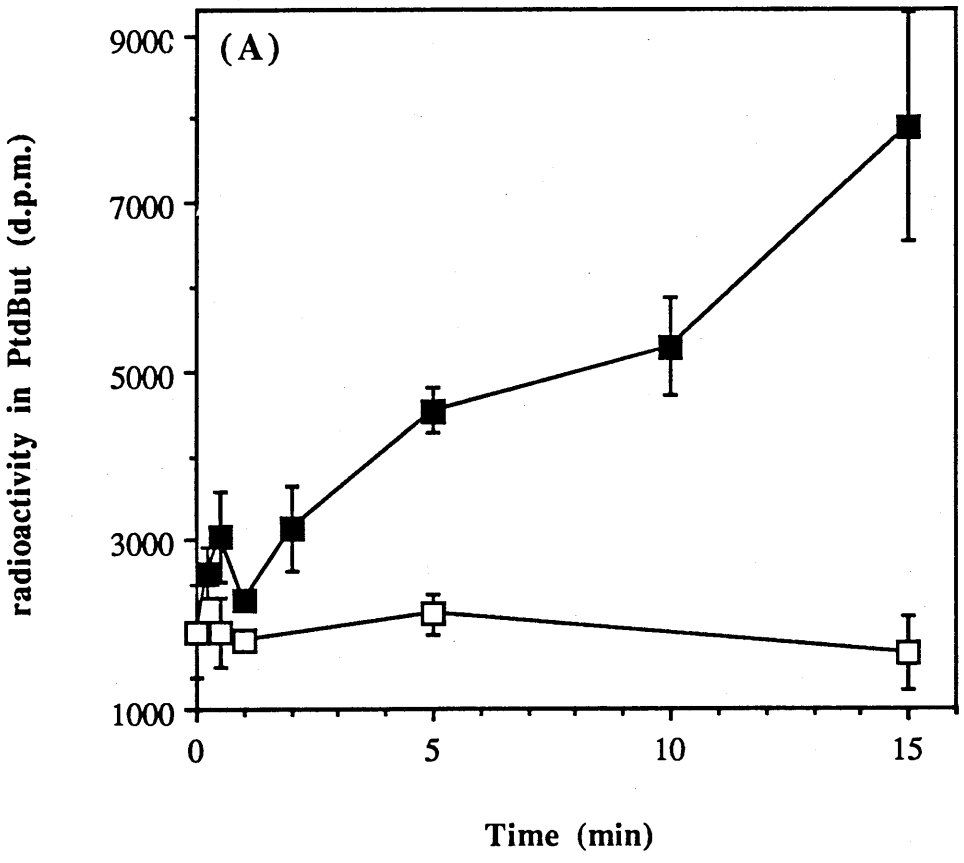
Swiss 3T3 cells labelled for 48 hours with [^{3}H]palmitate were incubated with buffer containing butan-1-ol and PDGF (c-*sis*, 30ng ml^{-1}) for the times indicated. The results in Fig. 6.10a show that PDGF was able to activate two distinct phases of [^{3}H]PtdBut formation. Increases in [^{3}H]PtdBut were apparent and statistically significant after a 30 sec stimulation (Fig.6.10 a & b; p= 0.082 & 0.077 respectively) at which point they were 1.5- 2-fold over control values. Elevated [^{3}H]PtdBut was generally maintained thus for up to 2 min. However, from between 2 and 5 min [^{3}H]PtdBut levels increased again in a second distinct phase which was maintained, and continued to rise for up to 15 min (Fig.6.10, a & b). The initial phase of PDGF-stimulated [^{3}H]PtdBut formation was similar to the response to EGF in both magnitude and kinetics of onset as shown in Fig.6.10b where the two responses are compared; thus it appears that the initial phase of PLD activation was common to both growth factors whilst the later sustained activation was unique to PDGF.

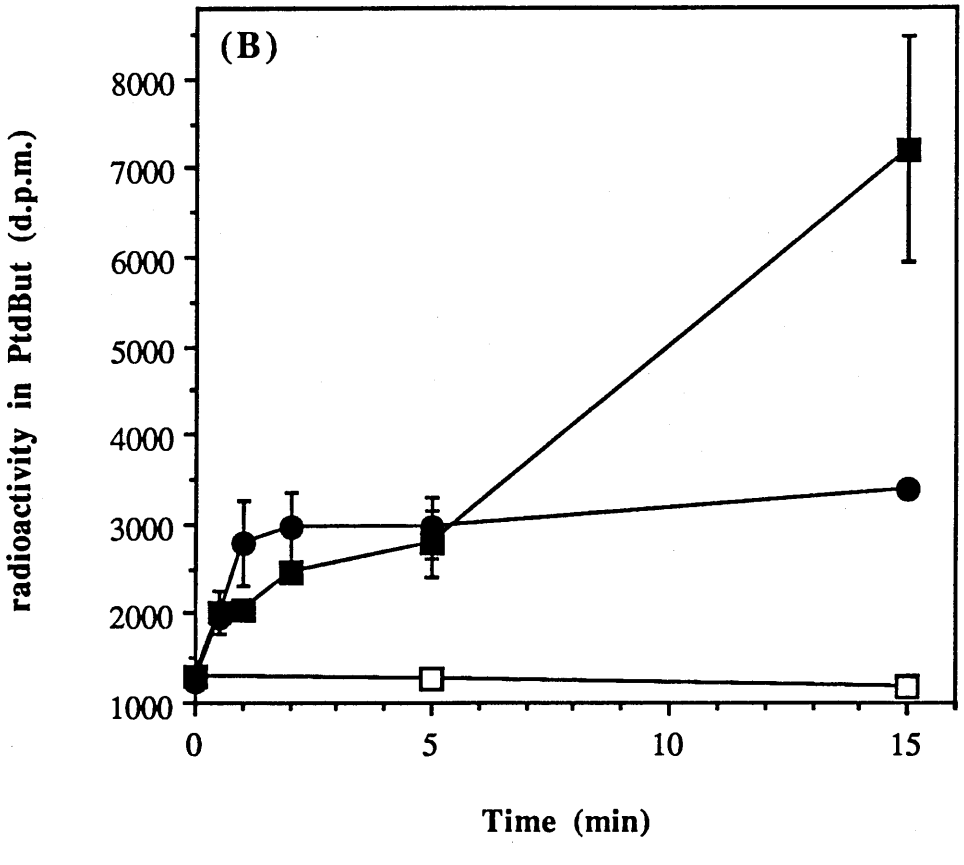
PDGF was also able to stimulate the hydrolysis of [^{3}H]PtdCho and release

Figure 6.10 (a & b). A comparison of the time course of PDGF- and EGF-stimulated [^{3}H]PtdBut formation in [^{3}H]palmitate labelled Swiss 3T3 cells.

Swiss 3T3 cells, judged to be 80% confluent, were grown for a further 48 hours in 2 ml of DMEM containing 1% calf serum and 4μCi of [^{3}H]palmitic acid. Cells were washed at 37^{o}C for 30 min in DMBH followed by a 5 min wash in DMBH containing 0.3% butan-1-ol (v/v). Incubations were started by replacing with DMBH containing 0.3% butan-1-ol ± 30ng ml^{-1} PDGF (c-*sis*) and were terminated after the indicated time by aspiration and addition of ice-cold methanol. Chloroform extracts prepared as described in the methods section were applied to LK5DF plates and [^{3}H]PtdBut was resolved by t.l.c. and the associated radioactivity was determined by scintillation counting. The results are mean radioactivity in [^{3}H]PtdBut (d.p.m.) ± S.D. (n=3) from a single representative experiment. In (b) a time course for EGF-stimulated [^{3}H]PtdBut formation is included for comparison.

In (a) ❐ = control and ■ = PDGF. In (b) ❐ = control, ■ = PDGF and ● = EGF.


(A)
9000
7000
5000
3000
1000
0
5
10
15
radioactivity in PtdBut (d.p.m.)
Time (min)


(B)
8000
7000
6000
5000
4000
3000
2000
1000
0
5
10
15
radioactivity in PtdBut (d.p.m.)
Time (min)

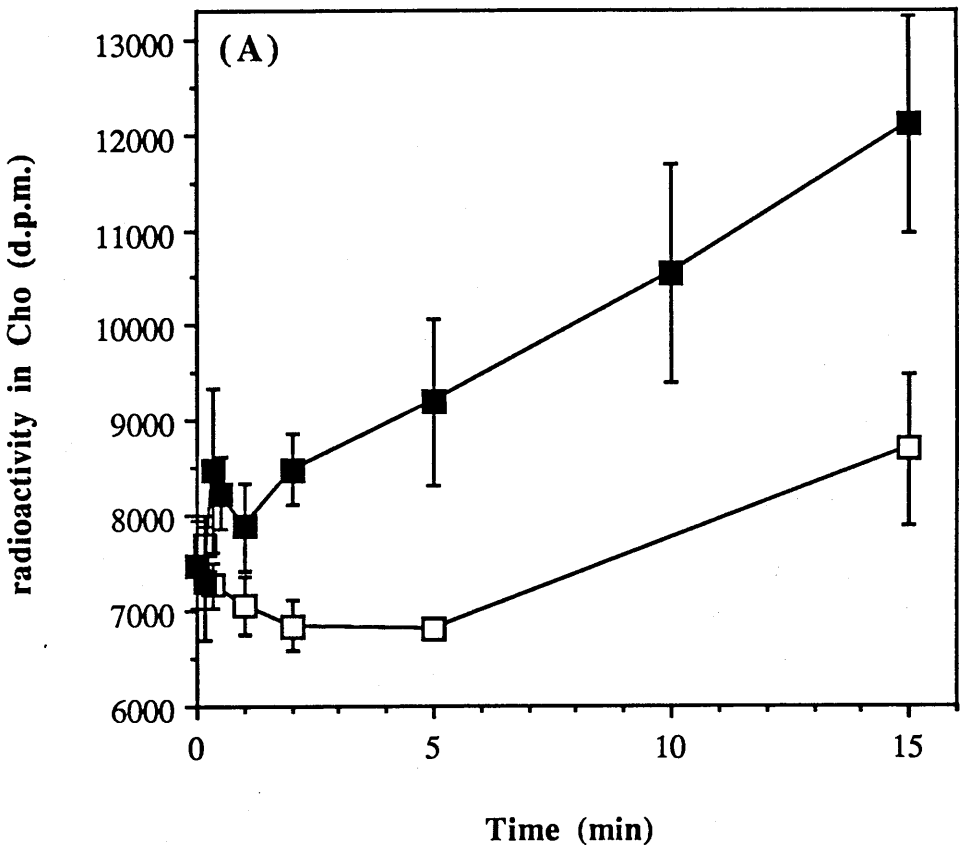
of [^{3}H]choline metabolites (Fig. 6.11, a & b). Addition of PDGF (c-*sis*, 10 ng ml^{-1}) resulted in a small increase in [^{3}H]Cho at 20 and 30 sec (Fig.6.11a) which whilst not always statistically significant was similar to the early increase in [^{3}H]PtdBut (Fig.6.10). From 1 min stimulation with PDGF resulted in a sustained increase in [^{3}H]Cho which was maintained above control for at least 15 min. Whilst the small initial phase of [^{3}H]Cho elevation may be due to the limits of detection of a simple isotopic labelling assay the sustained phase was clearly reproducible. PDGF-stimulated increases in cell associated [^{3}H]Cho were also observed (Fig.6.11b) indicating that Ptdho hydrolysis was ocurring within the cells and the increase in [^{3}H]Cho was not due to leakage from the cells into the medium. In this case the response was again apparent as early as 30 sec after addition of PDGF. Since the early response could be detected as PLD transferase activity which is a more sensitive, cumulative assay, it seems that PDGF is able to activate a PtdCho-PLD activity within 30 sec of addition in Swiss 3T3 cells.

PDGF-stimulated increases in ChoP were smaller in fold magnitude, occurred at later timepoints and were less reproducible (data not shown). However, as discussed in the previous chapter this may be due to an underestimate of increases in ChoP due to a relatively high basal associated radioactivity.

Unlike EGF, PDGF-stimulated PtdCho PLD activity was not dissociated from inositol lipid hydrolysis since PDGF was able to stimulate a dose-dependent increase in [^{3}H]InsP$_t$ (Fig. 6.12). The EC_{50} value for this response was 8.64 ± 0.77 ng ml^{-1}; similar to that previously described by Blakely *et al.* (1989). However, the mechanism of PDGF-stimulated [^{3}H]InsP$_t$ accumulation was quite different to that of bombesin, [Arg^8]Vp or $PGF_{2\alpha}$. Firstly, acute pre-treatment for 15 min with 100nm PMA did not inhibit the response to PDGF (Fig. 6.12, open symbols). Furthermore, chronic pre-treatment with 400nM PMA for 48 hours to down-regulate PKC did not result in potentiation of the response as described for bombesin, [Arg^8]Vp and $PGF_{2\alpha}$ (data not shown; Blakeley *et al.*, 1989); rather there was a small inhibition (approximately 10%) of the response as described by Blakeley *et al.* (1989).

Figure 6.11 (a & b). Time course of PDGF-stimulated increases in [^{3}H]Cho in Swiss 3T3 cells.

Swiss 3T3 cells, grown on 24 well plates until judged to be 80% confluent, were grown for a further 48 hours in 1 ml of DMEM containing 1% calf serum and 1.5μCi of [^{3}H]choline. Cells were washed for 45 min in Hanks and HBG as described in the Methods section before incubating for the indicated times with HBG alone (□) or containing PDGF (30ng ml^{-1}) (■). In (a) total Cho was assayed whereas in (b) intracellular Cho was assayed. Incubations were terminated by addition of ice-cold methanol either (a) directly or (b) after aspirating the medium and aqueous methanolic extracts were loaded on to Dowex-50WH$^+$ columns. [^{3}H]Cho was eluted as described in the Methods section. The associated radioactivity was determined by liquid scintillation counting. The results are expressed as mean radioactivity in [^{3}H]Cho fraction (d.p.m.) ± S.D. (n=3) from a single experiment representative of three.


(A)
13000
12000
11000
10000
9000
8000
7000
6000
0
5
10
15
radioactivity in Cho (d.p.m.)
Time (min)

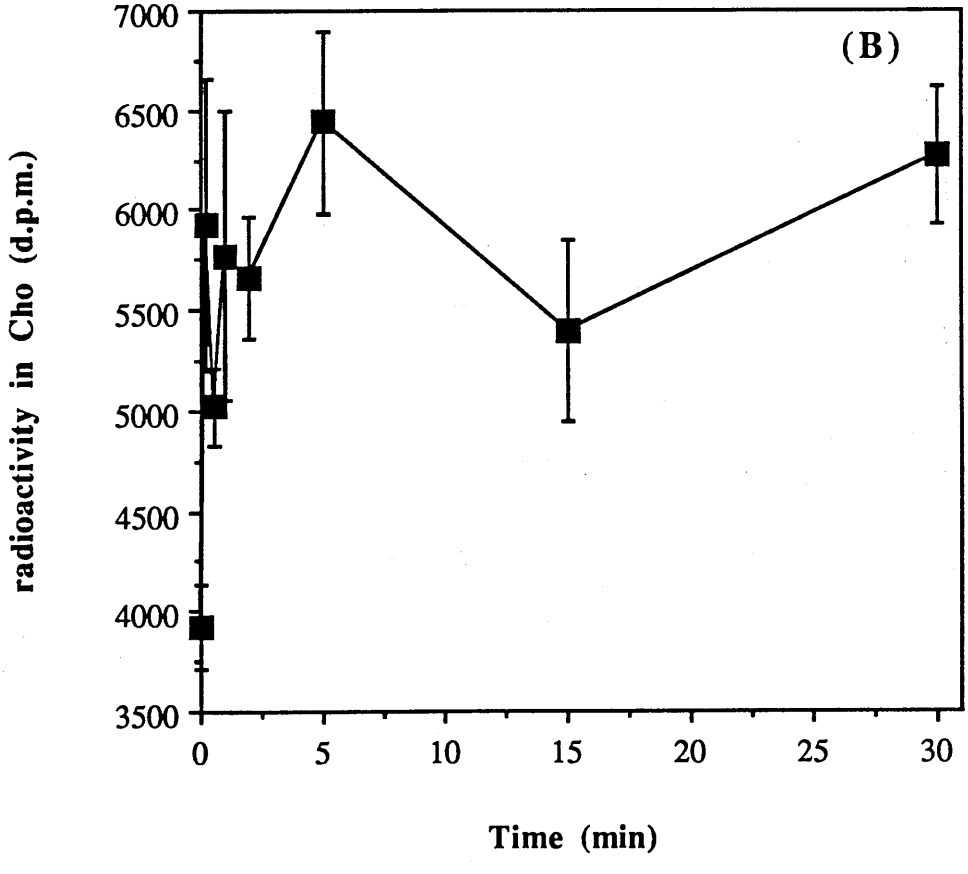
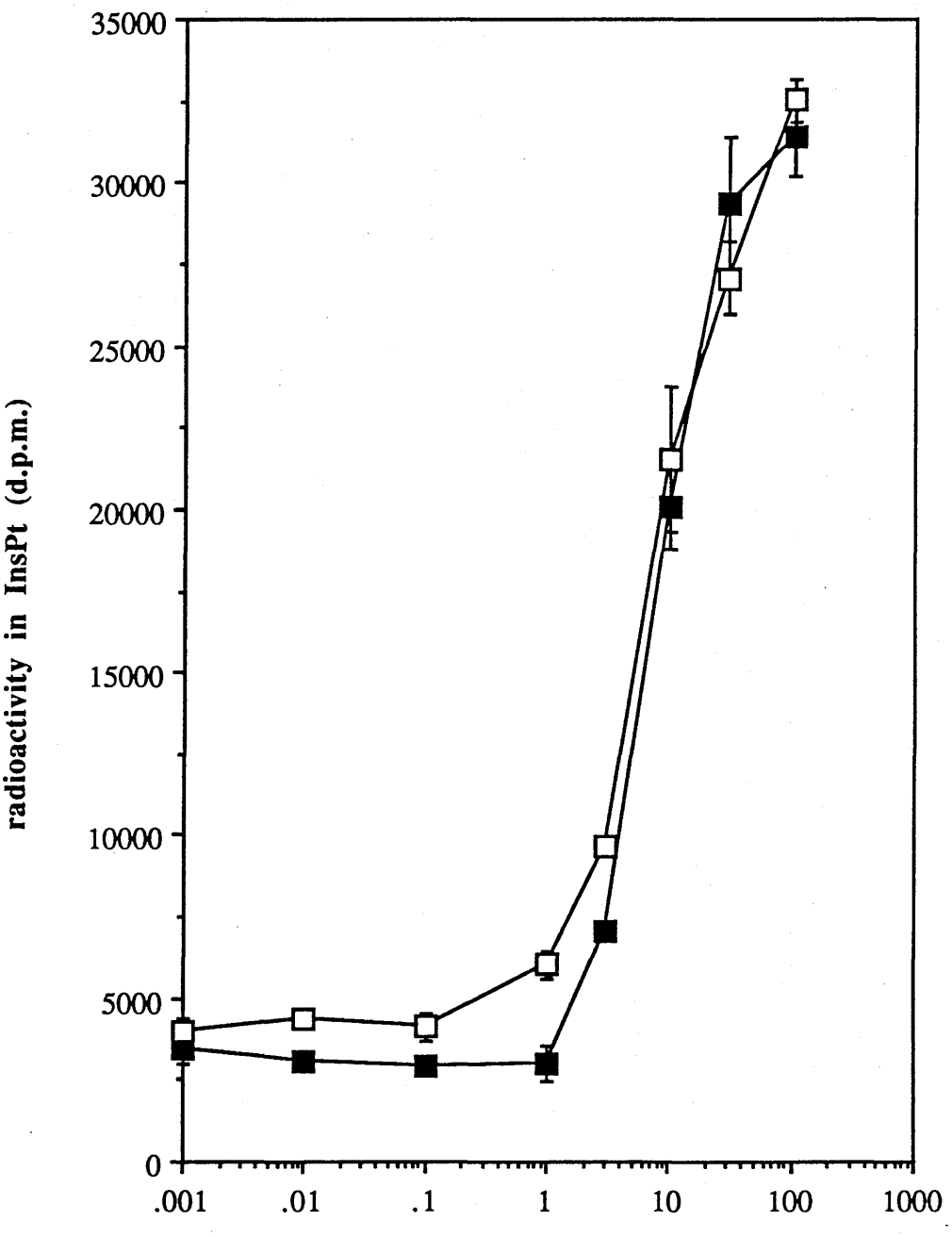

(B)
7000
6500
6000
5500
5000
4500
4000
3500
0
5
10
15
20
25
30
radioactivity in Cho (d.p.m.)
Time (min)

Figure 6.12. Dose-response curve for PDGF-stimulated [^{3}H]InsP$_t$ accumulation in Swiss 3T3 cells.

Swiss 3T3 cells, grown on 24 well plates until judged to be 80% confluent, were grown for a further 48 hours in 1 ml of DMEM containing 1% dialysed calf serum and 1μCi of [^{3}H]inositol. Cells were washed at 37°C in HBG for 5 min followed by HBG containing 10mM LiCl (HBG.Li) with 300nM PMA (❑) or 0.1% DMSO containing β-phorbol (■) for 15 min before incubating in HBG.Li containing the indicated final concentration of PDGF for 20 min. Incubations were terminated by aspiration and addition of ice-cold methanol and aqueous methanolic extracts were assayed for [^{3}H]InsP$_t$ as described in the methods section. Results are expressed as mean radioactivity in [^{3}H]InsP$_t$ (d.p.m.) ± S.D.(n=3) from a single experiment respresentative of two.


35000
30000
25000
20000
15000
10000
5000
0
radioactivity in InsPt (d.p.m.)
.001
.01
.1
1
10
100
1000
[PDGF] ng/ml

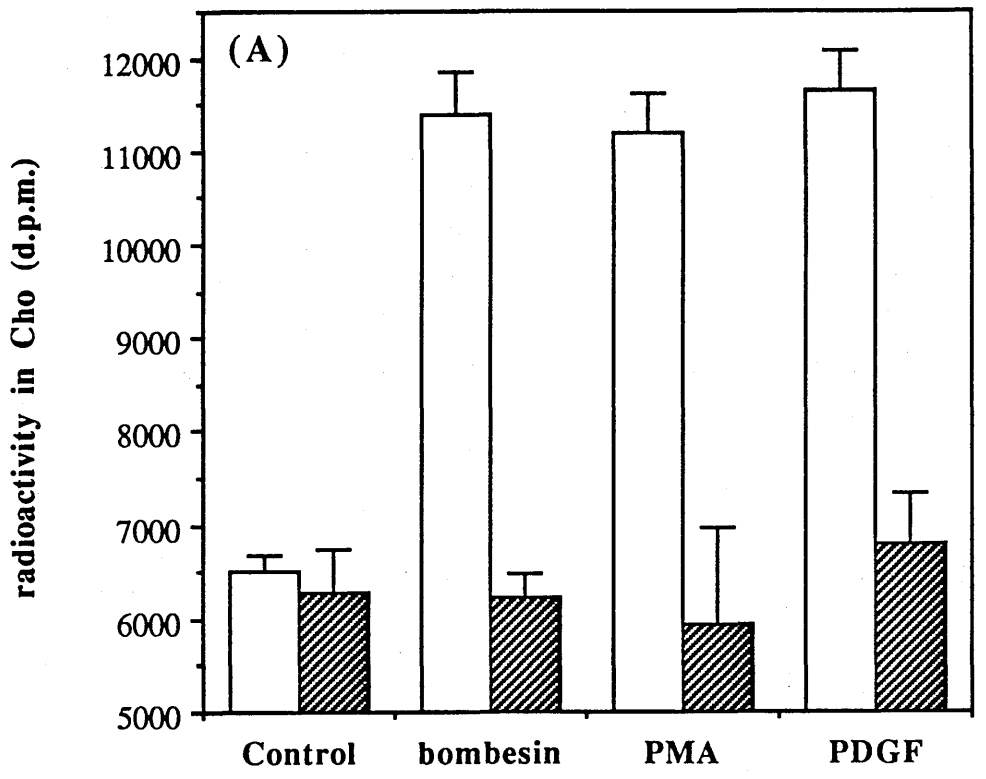
Finally the ability of PDGF to stimulate PLD activity, as measured by [^{3}H]Cho release (Fig.6.13a) or [^{3}H]PtdBut formation (Fig.6.13b), was attenuated in cells which had been pre-treated with 400nM PMA for 48 hours to 'down-regulate' PKC (Fig. 6.13). In addition, 'down-regulation' of PKC exerted a somewhat variable effect upon the ability of EGF to stimulate PtdCho hydrolysis. In some experiments EGF-stimulated [^{3}H]Cho release was apparently unaffected by the 48 hour pre-treatment with PMA (e.g., dpm in [^{3}H]Cho fraction; control cells, unstimulated = 6740 ± 424 dpm, 100nM EGF = 9713 ± 1106 dpm; PMA-treated cells, unstimulated = 6850 ± 466 dpm, 100nM EGF, 10080 ± 933 dpm). These results were consistent with the lack of inhibitory effect of Ro-31-8220 upon EGF-stimulated [^{3}H]PtdBut formation. In contrast, other experiments yielded results suggesting that EGF-stimulated PtdCho hydrolysis was abolished in cells treated with 400nM PMA for 48 hours (e.g., dpm in [^{3}H]Cho fraction; control cells, unstimulated = 6518 ± 155 dpm, 100nM EGF = 12454 ± 1329 dpm; PMA-treated cells, unstimulated = 6292 ± 450 dpm, 100nM EGF = 6767 ± 841 dpm).

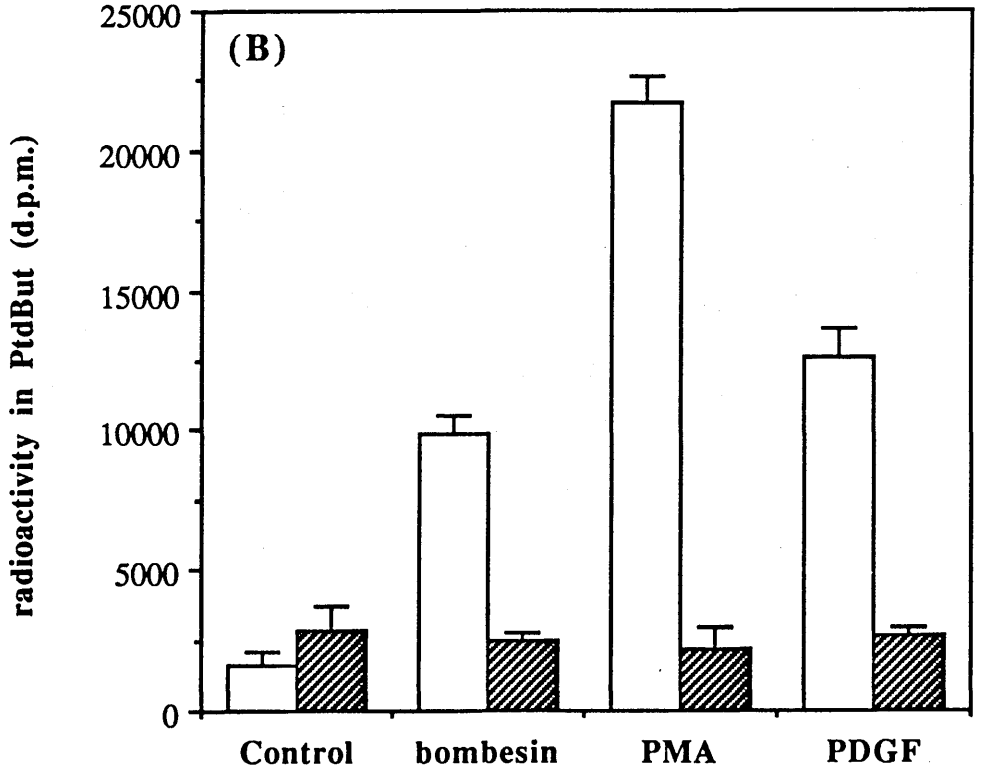
Figure 6.13 (a & b). The effect of 48 hour pre-treatment with 400nM PMA upon PDGF-stimulated PtdCho-PLD activity in Swiss 3T3 cells.

(a) Swiss 3T3 cells, grown on 24 well plates until judged to be 80% confluent, were grown for a further 48 hours in 1 ml of DMEM containing 1% calf serum, 1.5μCi of [^{3}H]choline and either 400nM PMA (hatched bars) or β-phorbol (open bars). Cells were washed for 45 min in Hanks and HBG as described in the Methods section before incubating for the indicated times with HBG alone or containing bombesin (100nM), PMA (100nM) or PDGF (30ng ml^{-1}) as indicated. Total [^{3}H]Cho was isolated as described in the methods section and the associated radioactivity was determined by liquid scintillation counting. The results are expressed as mean radioactivity in [^{3}H]Cho fraction (d.p.m.) ± S.D. (n=3) from a single experiment representative of three.

(b) Swiss 3T3 cells, grown on 6 well plates until 80% confluent, were grown for a further 48 hours in 2 ml of DMEM containing 1% calf serum, 4μCi of [^{3}H]palmitic acid and either 400nM PMA (hatched bars) or β-phorbol (open bars). Cells were washed at 37°C for 30 min in DMBH followed by a 5 min wash in DMBH containing 0.3% butan-1-ol (v/v). Incubations were started by replacing with DMBH containing 0.3% butan-1-ol alone or with bombesin (100nM), PMA (100nM) or PDGF (30ng ml^{-1}) as indicated, and were terminated after 20 min by aspiration and addition of ice-cold methanol. Chloroform extracts prepared as described in the Methods section were applied to LK5DF plates and [^{3}H]PtdBut was resolved by t.l.c. and the associated radioactivity was determined by scintillation counting. The results are mean radioactivity in [^{3}H]PtdBut (d.p.m.) ± S.D. (n=3) from a single representative experiment.

A 48 hour pre-treatment with PMA resulted in a 2-fold increase in radioactivity in total [^{3}H]choline labelled lipids and a 3.5- 4-fold increase in the total radioactivity in [^{3}H]palmitate labelled lipids. Consequently the radioactivity in either [^{3}H]Cho or [^{3}H]PtdBut was normalised to the labelling in the lipids.


(A)
radioactivity in Cho (d.p.m.)
12000
11000
10000
9000
8000
7000
6000
5000
Control
bombesin
PMA
PDGF


(B)
radioactivity in PtdBut (d.p.m.)
25000
20000
15000
10000
5000
0
Control
bombesin
PMA
PDGF

6.3 Discussion.

6.3.1 EGF-stimulated DG formation in the absence of inositol lipid hydrolysis.

The ability of EGF to stimulate inositol lipid hydrolysis, and indeed cell growth, appears to vary with cell lineage. In a number of quiescent fibroblast cell lines including Swiss 3T3 cells (Otto *et al.*, 1981; Hesketh *et al.*, 1985; 1988), NIH3T3 fibroblasts (Meisenhelder *et al.*, 1989), Balb/c 3T3 cells (Besterman *et al.*, 1986) and IIC9 cells (Wright *et al.*, 1990a) EGF is mitogenic with EC_{50} values in the 0.1-1 nM range, but does not significantly stimulate the accumulation of inositol phosphates, $Ins(1,4,5)P_3$ or phosphorylation of PLC-γ1. In Swiss 3T3 cells EGF is reported to be a complete mitogen as it can stimulate DNA synthesis in the absence of other growth factors though it is not as efficacious as serum (Otto *et al.*, 1981). EGF is also able to synergise with insulin and $PGF_{2\alpha}$ in stimulating DNA synthesis. In Swiss 3T3 cells EGF elevates $[Ca^{2+}]_i$ and pH_i and stimulates DNA synthesis and cell proliferation in the absence of inositol lipid hydrolysis (Otto *et al.*, 1981; Hesketh *et al.*, 1985; 1988), though the precise mechanism is in some doubt since in another clone of Swiss 3T3 cells EGF was only able to stimulate increases in $[Ca^{2+}]_i$ after priming with $[Arg^8]$Vp or $PGF_{2\alpha}$ (Olsen *et al.*, 1988).

In contrast a number of cells do respond to EGF by tyrosine phosphorylation of PLC-γ1 and stimulation of inositol lipid hydrolysis, but it is not clear that these are physiologically relevant models of mitogenesis. For example, the A431 human epidermoid carcinoma cell line over-expresses EGF receptors to the order of 1.5-2 x 10^6 per cell and addition of EGF to these cells has variously been reported to result in the primary generation of $Ins(1,4,5)P_3$ or $Ins(1,3,4)P_3$ (Hepler *et al.*, 1987; Tilly *et al.*, 1988) but actually inhibits growth (Kamata *et al.*, 1986; Iwashita *et al.*, 1990) whilst the EC_{50} for inositol phosphate generation is much higher than its reported mitogenic effects in other cells being in the range of 10-100nM (Hepler *et al.*, 1987). The results of Tilly *et al.* (1988) are of interest since these authors were able to detect an increase in $Ins(1,3,4)P_3$ in response to

EGF but not Ins(1,4,5)P_3; these results are in contrast to those of Hepler *et al.* (1987) and to date it is not clear what the source of this Ins(1,3,4)P_3 is. EGF can also stimulate Ins(1,4,5)P_3 formation in a number of 'engineered' cell lines such as the murine NIH3T3 cell over-expressing the human EGF receptor (the HER14 cell, Margolis *et al.*, 1989). It is interesting to note that Levitski (1990a) speculates that the EGF receptor/PLC-γ1 interaction may be confined to those cells over-expressing the EGF receptor. Thus, phosphorylation, and presumably activation, of PLC-γ1 may not be an event associated with EGF stimulation of normal cells but it may be of great importance in the unrestrained growth of cells which either over-express, or possess mutant EGF receptors. Such speculation raises the important question of whether receptor/effector coupling specificity is compromised when receptors are over-expressed or mutated. For example, the CSF-1 receptor is unable to couple to inositol lipid hydrolysis (Imamura *et al.*, 1990) whereas its constitutively active counterpart, the *fms* oncogene product, is able to couple to inositol lipid hydrolysis (Jackowski *et al.*, 1986). Furthermore, over-expression of the α-2 C-10 adrenergic receptor in Rat-1 fibroblasts results in the expression of an agonist-dependent PLD activity which is only present in the cells expressing 2-3 fmoles mg^{-1} of membrane protein; not in clones with lower receptor density (Milligan *et al.*, 1991). Whilst such cell lines provide novel approaches to adressing certain questions of structure-function relationship their use in extrapolation to the 'normal' situation is difficult and perhaps questionable.

Since in most cell lines where EGF is mitogenic there is no evidence for EGF-stimulated PKC activity the inability of EGF to stimulate inositol lipid hydrolysis would seem to fit the available facts and few studies have determined if DG levels rise upon EGF stimulation. However, even in different clones of a single cell line, Swiss 3T3, there appears to be no consensus and the ability of EGF to activate PKC in Swiss 3T3 cells is the subject of conflicting reports. Isacke *et al.* (1986) and Rodruigez-Pena & Rozengurt (1986) reported that EGF was unable to stimulate the phosphorylation of an 80 kDa phosphoprotein, which is reported to serve as a marker for PKC activation in Swiss 3T3 cells. This result was deemed

to be consistent with the inability of EGF to activate inositol lipid hydrolysis or inositol phosphate accumulation in Swiss 3T3 cells (Hesketh *et al.*, 1985; 1988). However in the same cell line Kaslauskas & Cooper (1988) were able to demonstrate EGF-stimulated phosphorylation of an 80 kDa protein which was of the same magnitude as that for PDGF and PMA. Since, the response was abolished following the down-regulation of PKC it suggested that EGF was stimulating PKC-mediated 80 kDa protein phosphorylation. Furthermore, in Swiss 3T3 cells Susa *et al.* (1989) have demonstrated that the kinetics of EGF-stimulated S6 kinase activity are biphasic and that the second phase is inhibited by chronic pre-treament with phorbol ester suggesting a role for activation of PKC. A possible explanation for this anomaly has come from the recent report that EGF can elevate DG levels in IIC9 fibroblasts in the absence of inositol lipid hydrolysis; the hydrolysis of PtdCho is implicated as the source (Pessin *et al.*, 1990; Wright *et al.*, 1990a) and the DG produced may then activate PKC in the absence of $Ins(1,4,5)P_3$ formation.

In the light of the results obtained in the previous chapters the ability of EGF to stimulate the formation of DG, the endogenous activator of PKC, was tested directly using the DG mass assay. The results in Fig. 6.1 confirm that DG formation occurs within 30 sec of addition of 100nM EGF to Swiss 3T3 cells. Furthermore, the response is sustained above control levels for at least 1 hour indicating that sustained elevation of DG appears to be an early signal associated with EGF stimulation. The response was clearly of smaller magnitude than that for bombesin or PMA; whether this reflects the lack of input from inositol lipid hydrolysis is not clear but, in agreement with other reports, EGF did not stimulate a reproducible elevation of $[^3H]InsP_t$ in Swiss 3T3 cells (Fig. 6.3). In some experiments there was a very small increase in $[^3H]InsP_t$ accumulation in the presence of 10mM LiCl, but this was observed rarely and then only at the highest concentrations of EGF (100nM). This indicated that EGF-stimulated DG formation was not due to even this small stimulation of inositol lipid hydrolysis since the concentration required was at least two orders of magnitude to the right of the EC_{50} for EGF-stimulated DG formation. Furthermore, EGF was unable to stimulate an

increase in Ins(1,4,5)P_3 mass using the competitive binding assay (S. Palmer, personal communication). Thus, EGF-stimulated DG formation occurred over mitogenically relevant doses at which inositol phosphate accumulation was unaffected.

The ability of EGF to stimulate DG formation, albeit modestly, is in contrast to the recent report of Bierman *et al.* (1990) who were able to demonstrate biphasic bombesin-stimulated DG formation but no EGF-stimulated DG formation in Swiss 3T3 cells. Apart from the possibility that this represents clonal differences between different Swiss 3T3 cell lines, differences in sample preparation for the DG mass assay may explain these conflicting results. After stimulating cells with agonist, incubations were terminated by aspiration and addition of 10% trichloroacetic acid (Bierman *et al.*, 1990); however, Preiss *et al.* (1986) report that exposure of *sn*-1,2-DGs to even mild acid or alkali results in acyl group migration and loss of the *sn*-1,2- specificity of the assay leading to an underestimation of increases in DG mass. The increase in DG mass seen with EGF is clearly much smaller than that seen with bombesin or PMA and might not be observed if samples had been prepared under acid conditions.

The ability of EGF to stimulate increases in DG in the absence of inositol lipid hydrolysis is not unprecedented. Wright *et al.* (1988; 1990a & b) have demonstrated an increase in DG of similar magnitude and duration in EGF-stimulated IIC9 fibroblasts whilst Baldassare *et al.* (1990) have made similar observations in human dermal fibroblasts; in both cases there was no evidence for EGF-stimulated inositol lipid hydrolysis.

6.3.2 EGF stimulates the PLD-catalysed hydrolysis of PtdCho but this does not serve as a pathway for DG formation.

Despite its inability to stimulate the hydrolysis of inositol lipids, EGF clearly stimulated phospholipase D activity as measured by the formation of [^{3}H]PtdBut by the transferase pathway. The kinetics of EGF-stimulated [^{3}H]PtdBut formation (Fig. 6.4a) were very similar to those for increases in DG

(Fig. 6.1). The slowing in rate of PtdBut formation suggested that activation of PLD was rapidly desensitised though this desensitization did not always appear to be complete since [^{3}H]PtdBut formation was in some cases maintained though the rate from 2 min onwards was greatly reduced. The kinetics of [^{3}H]PtdBut formation suggested that a PLD/PPH pathway might be a more likely candidate pathway for DG formation than inositol lipid hydrolysis and this was supported by the dose-dependency studies (Fig. 6.4 b). The EC_{50} for EGF-stimulated [^{3}H]PtdBut formation (1.14 ± 0.54 nM) was closer to that for DG formation than the small increase in [^{3}H]InsP_t accumulation.

The rapid increase in [^{3}H]Cho in response to EGF indicated that a major substrate for the activated PLD was PtdCho. Indeed, the kinetics of [^{3}H]Cho elevation were similar to both EGF-stimulated DG and PtdBut formation. Statistically significant increases in [^{3}H]ChoP were not observed until after 20 or 30 min stimulation though in some instances non-significant increases were apparent at 5 min. As discussed previously (Chapter 5) this may represent phosphorylation of the [^{3}H]Cho liberated in the PLD reaction by choline kinase as part of a PtdCho cycle to replenish the parent lipid. Alternatively, the increases in [^{3}H]ChoP may represent PLC activity. Both Wright *et al.* (1990b) and Baldassare *et al.* (1990) have described EGF-stimulated DG formation which was associated with the release of both [^{3}H]Cho and [^{3}H]ChoP suggesting activation of both PtdCho-PLD and PLC.

Despite the close agreement in the kinetics and dose-dependency of EGF-stimulated DG formation and PLD activation the results in Fig. 6.6 suggest that EGF-stimulated DG formation did not proceed by a PLD/PPH pathway since the inclusion of 0.3% (v/v) butan-1-ol to 'trap' phosphatidyl moieties as PtdBut did not affect EGF-stimulated DG formation. These experiments should be repeated with inhibitors of PPH such as sphingosine or propranolol to confirm that this pathway does not play a role in DG formation. In addition a thorough time course of the effects of butan-1-ol upon EGF-stimulated DG formation should be performed. The onset of PLD activity was 30 sec after addition of EGF but the effect of butan-

1-ol upon DG formation was assessed at a single 15 min time point at which the rate of [^{3}H]PtdBut formation was greatly desensitised and increases in [^{3}H]ChoP were apparent. If PLD activity is transient, as is perhaps suggested by the drop in rate of [^{3}H]PtdBut formation (Fig. 6.4a), then it may make a contribution to DG formation at early time points which would be missed by assessing the effects of butan-1-ol at 15 min when a potential PtdCho-PLC pathway might be more important. Similar results have recently been described by both Wright *et al.* (1990b) and Baldassare *et al.* (1990) suggesting that whilst EGF does indeed stimulate a PLD activity the PtdOH so produced does not serve as a precursor of DG. In both studies the authors propose that both PtdCho-PLD and PLC are activated by EGF but it is the latter reaction which is responsible for DG formation. If this is the case it is reasonable to ask what purpose activation of PLD serves if not as a pathway for DG formation; this issue is considered in Chapter 7. A possible role for PtdCho-PLC in DG formation should be addressed more fully and assessing ChoP mass formation may allow clearer definition of this issue. It is unlikely that the small and non-reproducible increase in inositol lipid hydrolysis contributes to DG formation since this only occurred at 100nM EGF whereas the EC_{50} for DG formation was approximately 0.1 nM.

The ability of EGF to stimulate PtdCho hydrolysis is not entirely without precedent in the Swiss 3T3 cell line. Until recently many studies of stimulated PtdCho hydrolysis only measured the re-synthesis of PtdCho by incorporation of ^{32}Pi into PtdCho (for example Muir & Murray, 1987). In this respect EGF-stimulated PtdCho, but not PtdIns, synthesis was observed in Swiss 3T3 cells by MacPhee *et al.* (1984). In hindsight these results are consistent with the re-synthesis of PtdCho following its stimulated hydrolysis as shown in this thesis.

6.3.3 Regulation of phospholipase D by EGF; evidence of a requirement for the receptor tyrosine kinase activity.

Despite the finding that PLD did not seem to be the pathway for EGF-stimulated DG formation the observation that PLD is activated by diverse mitogenic stimuli suggested that it might play some additional role in mitogenic signal transduction. Therefore studies were undertaken to define the regulation of EGF-stimulated PLD activity in whole cells using bombesin and PMA as comparisons.

By analogy with bombesin, the observation that EGF stimulated an increase in DG without stimulating inositol lipid hydrolysis suggested that EGF-stimulated PLD activity might be independent of protein kinase C. To test this hypothesis the effect of the PKC inhibitor Ro-31-8220 upon PLD activity was assessed. In the same experiments in which PMA-stimulated PLD activity was inhibited by 90% and the response to bombesin by 40-50%, EGF-stimulated PLD activity was never significantly inhibited by Ro-31-8220. This was consistent with the observation that EGF did not stimulate inositol lipid hydrolysis and suggests that activation of PLD is not downstream of prior PKC activation. However, in two out of four experiments the response was significantly enhanced at 10 μM Ro-31-8220 (Fig. 6.7 b). It is well known that the EGF receptor is subject to a PKC-mediated phosphorylation of Thr 654 (Davis & Czech, 1985; Hunter *et al.*, 1984) which results in a shift to a lower affinity binding form of the receptor (Livneh *et al.*, 1987) and an inhibition of signalling capacity (Cochet *et al.*, 1984). This is known as receptor 'transmodulation' and not 'down-regulation' since the number of receptors stays the same (Brown *et al.*, 1979), and can be mimicked in whole cells by addition of PMA, bombesin, PDGF and other agents which activate PKC (Brown *et al.*, 1979; 1984; reviewed by Rozengurt, 1985). Thus inhibition of PKC by Ro-31-8220 might explain the significant potentiation of the EGF response in some instances since this would prevent receptor 'transmodulation' allowing the response to continue for longer at the initial maximal rate. This would require that the DG derived from EGF-stimulated PtdCho hydrolysis would normally activate PKC resulting in receptor transmodulation and so such speculation must await confirmation of whether EGF-stimulated PKC activity does indeed occur in this

clone of Swiss 3T3 cells.

The use of tyrphostins to inhibit EGF, PDGF and insulin-stimulated events is now well documented (reviewed by Levitski, 1990a). These drugs have been used to inhibit EGF-stimulated cell proliferation in fibroblasts over-expressing the human EGF receptor (Lyall *et al.*, 1989) and EGF-stimulated phosphorylation of PLC-γ1 and elevation of $[Ca^{2+}]_i$ (Margolis *et al.*, 1989) and inositol lipid hydrolysis (Posner *et al.*, 1989).

The ability of 100μM AG 18 to inhibit up to 90% of EGF-stimulated PLD activity (Fig. 6.8a) clearly indicates that this response requires a tyrosine phosphorylation event. No higher concentrations were examined since they were deemed to be potentially harmful to the cell; even at 100μM there was some concern that any inhibition observed might be due to non-specific cytotoxic effects of AG18. To address this question the effect of 100μM AG18 upon both EGF- and bombesin-stimulated PtdBut formation was compared since the bombesin receptor couples to PIC via a G-protein rather than via tyrosine kinase activity (Plevin *et al.*, 1990). The observation that even at 100μM AG 18 there was no effect upon bombesin-stimulated PLD activity (Fig.6.8b) indicates that the drug was exerting a selective effect upon tyrosine kinase activity rather than a non-selective or cytotoxic effect. Microscopic examination of the cells after 18 hours treatment with the tyrphostin did not reveal any marked morphological changes (data not shown).

It has recently been shown that a number of proteins implicated as effectors in mitogenic signal transduction pathways are phosphorylated upon tyrosine residues after stimulation with EGF, PDGF and CSF-1. These proteins, which include $p21^{ras}$ GAP (Molloy *et al.*, 1989; Ellis *et al.*, 1990), Raf-1 (Morrison *et al.*, 1989), PLC-γ1 (Meisenhelder *et al.*, 1989) and PtdIns 3'-kinase (Kaplan *et al.*, 1987; Varticovski *et al.*, 1989), appear to physically associate with the growth factor receptors perhaps through interactions with SH2 (*src* homology) domains within these molecules. In the light of these results it is tempting to speculate that the inhibition of EGF-stimulated PLD activity by AG 18 may be due to inhibition of EGF receptor-mediated phosphorylation of PLD as has been shown for PLC-γ1 (Margolis *et al.*, 1989). Such speculation must await the demonstration of PLD

activity in EGF-receptor immune precipitates or the co-immuno precipitation of EGF receptors and PLD once suitable anti-sera are available.

The ability of A23187 to activate PLD, even in the presence of Ro-31-8220 (Chapter 4, Fig 4.17) indicated that an alternative mechanism for regulation of PLD activity might be EGF-stimulated Ca^{2+} entry. The ability of EGF to stimulate increases in $[Ca^{2+}]_i$ in Swiss 3T3 cells is also the subject of conflicting reports which may represent variations between different clones of Swiss 3T3 cells. Thus, Hesketh *et al.* (1985; 1988) were able to demonstrate EGF-stimulated increases in $[Ca^{2+}]_i$ which were completely dependent upon $[Ca^{2+}]_o$ indicating that EGF was able to stimulate Ca^{2+} entry. In contrast Olsen *et al.* (1988) were only able to demonstrate EGF-stimulated increases in $[Ca^{2+}]_i$ when EGF was added after $PGF_{2\alpha}$ or $[Arg^8]$Vp. The results in Fig 6.9 indicate that buffering extracellular Ca^{2+} to 150 nM, the resting $[Ca^{2+}]_i$ in Swiss 3T3 cells, resulted in an inhibition of EGF-stimulated PLD activity. The simplest conclusion from this is that EGF-stimulated increases in $[Ca^{2+}]_i$, as a result of Ca^{2+} entry, are responsible for activating PLD but to date it is not known if this clone of Swiss 3T3 cells is one which exhibits an EGF-stimulated Ca^{2+} entry response. An alternative explanation for these results is that Ca^{2+} represents a vital co-factor for PLD activity, rather than actually stimulating its activity directly, and that during the buffering of $[Ca^{2+}]_o$ there is depletion of intracellular Ca^{2+} so that PLD activity is compromised. Since the regulation of PLD activity by EGF is apparently different from that described for bombesin and PMA (Figs 6.7 & 6.8 and Chapter 4) it may be possible that EGF stimulates a distinct isoform of PLD which has a different Ca^{2+} sensitivity to that regulated by bombesin or PMA. Another possibility is that EGTA inhibits EGF binding to its receptor but this is unlikely since binding of the related growth factor PDGF to its cognate receptor was not affected by omission of extracellular Ca^{2+} (Bowen-Pope & Ross, 1982). Thus it remains to be clearly defined at what site the buffering of extracellular Ca^{2+} exerts its effect. In this context quite how EGF does stimulate Ca^{2+} entry (Hesketh *et al.*, 1985; 1988) is unclear. In those instances where EGF stimulates $Ins(1,4,5)P_3$ formation the entry

of Ca^{2+} may be by a similar mechanism to that described for agonists such as bombesin though this mechanism is also far from resolved (Irvine, 1990). Speculation as to whether the activated EGF receptor is able to phosphorylate and thereby activate a Ca^{2+} channel awaits a thorough pharmacological assessment of Ca^{2+} entry to determine the nature of the channel using defined channel blockers.

6.3.4 PDGF can stimulate PtdCho-PLD activity in Swiss 3T3 cells.

Studying the signal transduction pathways of PDGF is particularly important since PDGF is capable of stimulating DNA synthesis and cell division in a variety of cell types including, Swiss 3T3 cells and IIC9 fibroblasts, without the requirement for a co-mitogen such as insulin.

Recent studies have demonstrated the existence of two distinct receptor sub-types which display different ligand binding affinities for the various divalent forms of PDGF; -AA, -AB & BB (Heldin *et al.*, 1988). Thus, the α-type receptor binds all three isoforms with high affinity whereas the β-type receptor binds PDGF-BB with high affinity, PDGF-AB with lower affinity and does not bind PDGF-AA. cDNAs for both receptor types have been cloned (Yarden *et al.*, 1986; Matsui *et al.*, 1989) and the predicted proteins exhibit similar structural organization including Ig-like ligand-binding domains, a hydrophobic transmembrane segment and an intracellular protein tyrosine kinase domain. This catalytic domain is divided in two by the so-called 'kinase insert sequence' which includes Tyr 751, a major site of *in vivo* receptor autophosphorylation; mutation of this Tyr to Phe or Gly, or mutation of the catalytic domain to abolish kinase activity, blocks association of the PDGF receptor with PtdIns 3'-kinase as well as other cellular proteins. Furthermore, deletion of 82 of the 104 amino acids in this insert region abolishes PDGF-induced mitogenesis without affecting ligand induced receptor auto-phosphorylation or inositol phosphate accumulation (Escobedo & Williams, 1988). Kazlauskas & Cooper (1989) propose that autophosphorylation in this insert sequence regulates interactions with cell proteins. The nature of some of these proteins has been described previously in the Introduction (Chapter 1) and only the

modulation of lipid signalling pathways will be considered.

Within 30 sec of stimulation of Swiss 3T3 (Meisenhelder *et al*, 1989), Balb/c3T3 (Wahl *et al*, 1989) or NIH3T3 cells (Meisenhelder *et al*, 1989) with PDGF, PLC-γ1 associates with and is phosphorylated on tyrosine residues by the PDGF receptor in a ligand-dependent manner. This may well be the mechanism by which PLC-γ1 is activated resulting in the formation of Ins(1,4,5)P_3 and DG since these responses are certainly not modulated by guanine nucleotides (Cattaneo & Vicentini, 1989) as is the case for bombesin (Plevin *et al*, 1990). In fact EGF-stimulated phosphorylation of PLC-γ1 upon tyrosine residues has recently been shown to increase the catalytic activity of the enzyme (Nishibe *et al.*, 1990) as well as resulting in its translocation to the plasma membrane (Todderud *et al*, 1990).

Stimulation of many cells with c-*sis* and/or PDGF-BB, acting at either the α- or β-type receptors, results in the formation of both Ins(1,4,5)P_3 and DG, but it has recently been shown that stimulation with the AA form of PDGF, acting at the α-type receptor may result in the formation of DG and activation of PKC in the absence of inositol phosphate formation (Block *et al.*, 1989; Sachinidis *et al.*, 1990). Furthermore, PDGF can stimulate the formation of DG in the absence of InsP accumulation in IIC9 fibroblasts and the molecular species analysis of this DG reveals that it is most likely derived from PtdCho (Pessin *et al.*, 1990). Thus it appears that production of DG and activation of PKC are a common response to PDGF and can, in some circumstances, be dissociated from inositol lipid hydrolysis at the receptor level. This would suggest that in terms of mitogenicity, DG formation is more important than Ins(1,4,5)P_3 and this is supported by the fact that micro-injection of DG into Balb/c 3T3 cells stimulates proliferation whereas Ins(1,4,5)P_3 is ineffective (Suzuki-Sekimori *et al.*, 1989).

The finding that PDGF was able to activate PtdCho-PLD activity as measured by [^{3}H]Cho release (Fig.6.11) and [^{3}H]PtdBut formation (Fig. 6.10) indicates that this may be a common response to both RGE- and RTK-type mitogens, irrespective of their ability to couple to inositol lipid hydrolysis. The identification of this PDGF-stimulated PtdCho-PLD activity is in contrast to a recent report by Larrodera *et al.* (1990) who suggested that PDGF actually stimulated

hydrolysis of PtdCho via PLC. However, the earliest stimulation time in their study was 2 hours at which point increases in [^{3}H]ChoP might be due to activation of choline kinase (Warden & Friedkin, 1984; 1985). In the same study the authors were unable to demonstrate PDGF-stimulated [^{3}H]Cho generation; this is in contrast to the studies reported in this thesis since increases in [^{3}H]Cho was the earliest and most reproducible manifestation of PtdCho hydrolysis (Fig 6.11). In addition the generation of [^{3}H]ChoP is not a definitive assay for PtdCho-PLC activity (see Billah & Anthes, 1990; Chapter 5 of this thesis) whereas the formation of [^{3}H]PtdBut (Fig 6.10) is definitive for PLD. PDGF-stimulated [^{3}H]Cho formation has also been observed by Price *et al.* (1989) again implicating PLD catalysed PtdCho hydrolysis.

The kinetics of PtdCho-PLD activation are of particular interest since, although PDGF does indeed stimulate inositol lipid hydrolysis in Swiss 3T3 cells the mechanism by which this occurs is clearly different from agonists such as bombesin (Nanberg & Rozengurt, 1988; Blakeley *et al.*, 1989). These two studies have suggested that the onset of the response to PDGF, in terms of generation of [^{3}H] $InsP_3$ or [^{3}H]Ins(1,4,5)P_3, is much slower than that for bombesin being not apparent until after 1.5-2 min stimulation. In the light of the results shown in Fig 6.10 (a & b) this would suggest that PtdCho-PLD activation was actually upstream of inositol lipid hydrolysis. However, recent results from this laboratory (R. Plevin, S. Palmer & M.J.O. Wakelam, personal communication) using the sensitive mass assay for Ins(1,4,5)P_3 as well as h.p.l.c resolution of [^{3}H]Ins(1,4,5)P_3, from cultures labelled with [^{3}H]inositol to a high specific activity, show that Ins(1,4,5)P_3 levels increase 30 sec after addition of PDGF though the magnitude of this response is clearly smaller than that for bombesin (Fig 3.4). Thus, it would seem that the two responses, activation of PLC-γ1 and PLD, are initiated commensurate with each other rather than PLD being kinetically downstream of PIC activation as seems to be the case for bombesin (Chapter 4 of this thesis). Furthermore, mass analysis of the increase in DG and Ins(1,4,5)P_3 in response to PDGF stimulation reveals that the mass of DG formed exceeds that of Ins(1,4,5)P_3 even more so than that for bombesin (R. Plevin, S. Palmer &

M.J.O.Wakelam, personal communication; Chapter 4) suggesting that the majority of DG is derived from another source. The demonstration of PDGF-stimulated PtdCho hydrolysis and PLD activity suggest that this is PtdCho and future experiments should aim to assess the role of this PLD activity in PDGF-stimulated DG formation using butan-1-ol and a selection of PPH inhibitors.

The observation that PDGF-stimulated PtdCho hydrolysis and DG formation are dissociated from inositol lipid hydrolysis in IIC9 cells (Pessin *et al.*, 1990) and that DG formation and PKC activation occur in the absence of InsP formation in response to the the PDGF-AA homodimer (Block *et al.*, 1989; Sachidinis *et al.*, 1990) suggests that the α- and β-type receptors may couple selectively to PtdCho-PLD and PLC-γ1 respectively. In such a case PDGF would be able to activate PtdCho-PLD by two distinct mechanisms; directly, via an α-type receptor (a situation perhaps analagous to the activation of PLD by the EGF reeceptor) or indirectly, via β-type receptor activation of PLC-γ1 leading to activation of PKC and thence PLD. The c-*sis* used in these experiments is a BB homodimer and can therefore interact with both α- and β-type receptors (Heldin *et al.*, 1988). The ability of PDGF to stimulate inositol phosphate accumulation (EC_{50} approximately 8 ng ml^{-1}) exhibited similar dose-dependency to PDGF-stimulated [^{3}H]Cho release (EC_{50} 2.02 ± 0.33 ng ml^{-1}; R. Plevin, personal communication) suggesting that c-*sis* is either activating one class of β-type receptors or is interacting with two classes of receptor with similar affinities. The latter scenario may explain the commensurate initiation of PLD and PLC-γ1 activity whilst the larger sustained activation of PLD may represent the downstream, PKC-mediated component of PLD activation by PDGF which is absent in the EGF response since EGF cannot stimulate inositol lipid hydrolysis (Fig. 6.3). Certainly, the ability of PDGF to activate PKC in Swiss 3T3 cells is in no doubt (Kazlauskas & Cooper, 1988) and the stimulated accumulation of inositol phosphates is sustained and non-desensitising in this cell line (Blakeley *et al.*, 1989) and is not subject to a PKC-mediated feedback inhibition (Fig. 6.12). Thus the sustained activation of PKC and increases in $[Ca^{2+}]_i$ resulting from PDGF-stimulated inositol

lipid hydrolysis may account for the slower but sustained activation of PLD.

An observation which is to some extent consistent with this model is that down-regulation of PKC results in the loss of PDGF-stimulated PLD activity. This is not likely to be due to loss of PDGF-stimulated inositol lipid hydrolysis since down-regulation of PKC resulted in only a very small inhibition of PDGF-stimulated [^{3}H]InsP$_t$ (Blakeley *et al.*, 1989). Like the results with bombesin-stimulated [^{3}H]Cho release and [^{3}H]PtdBut formation, the simplest explanation is that both phases of PDGF-stimulated PLD activity require activation of PKC and yet the model described above would predict that the small initial phase of PLD activity might be receptor-mediated and therefore PKC-independent.

One possible explanation for this returns to an issue raised in Chapters 4 and 5; namely the possibility that PLD is down-regulated by prolonged PMA-treatment. The observation that EGF does not significantly stimulate inositol lipid hydrolysis argues against a role for PKC in mediating EGF-stimulated PLD activity. This is confirmed by the lack of inhibition of EGF-stimulated [^{3}H]PtdBut formation by the PKC inhibitor Ro-31-8220. Despite this in some experiments, under PKC down-regulating conditions, there was some inhibition of EGF-stimulated [^{3}H]Cho release though this was not reproducible (section 6.2.4). If PLD is also down-regulated by prolonged PMA treatment this might imply that the loss of PLD activity is not simply due to loss of PKC but is to some extent artefactual; thus, any study of the role of PKC in a given response should not rely solely upon the use of PKC down-regulation but should also use PKC inhibitors to confirm the results. This matter clearly warrants further and fuller consideration and a starting point would be to assess the effect of the PKC inhibitor Ro-31-8220 upon PDGF-stimulated PLD activity. Other approaches are considered in the discussion, Chapter 7.

Thus, the results in this chapter indicate that growth factors of the tyrosine kinase family are also able to increase DG levels in their target cells and activate PtdCho hydrolysis by a PLD pathway. In the case of EGF the activation of PLD and formation of DG are divorced from inositol lipid hydrolysis and activation of PLD requires the receptor tyrosine kinase activity and appears to be dependent upon

Ca^{2+} entry. In the case of PDGF the activation of PtdCho-PLD occurs at least as fast as the formation of Ins(1,4,5)P_3, but is suceptible to chronic pre-treatment with high doses of PMA. EGF-stimulated DG formation was not inhibited by butan-1-ol suggesting that it does not proceed by a PLD/PPH pathway. Thus it would seem that activation of PLD and/or formation of DG are also common events following stimulation of Swiss 3T3 cells with growth factors which couple to receptor tyrosine kinases, raising the possibility of multiple pathways for regulation of PLD activity.

Chapter 7.

Conclusions and perspectives.

7.1 Evidence for multiple sources of DG in mitogen-stimulated Swiss 3T3 fibroblasts.

The results presented in this thesis indicate that the increases in DG mass in bombesin- and EGF-stimulated Swiss 3T3 cells cannot be explained solely in terms of inositol lipid hydrolysis. Moreover, the hydrolysis of PtdCho and generation of [^{3}H]Cho and [^{3}H]ChoP by these mitogens suggests that PtdCho may contribute to DG formation.

The kinetics and mass of bombesin-stimulated Ins(1,4,5)P_3 formation could not account for the observed increase in DG mass which was biphasic (Chapter 4). The first phase of DG formation accompanied the transient generation of Ins(1,4,5)P_3 (Chapter 3) but exceeded it in mass terms whilst the second phase, from 30 sec onwards for up to 30 min and 4 hours (Takuwa *et al.*, 1989), was divorced from any further elevation of Ins(1,4,5)P_3. These studies have confirmed and advanced those of Takuwa *et al.* (1987a) by making a more thorough comparison of the kinetics of Ins(1,4,5)P_3 and DG formation. Furthermore, recent reports of similar biphasic DG formation in α-thrombin-stimulated IIC9 fibroblasts (Wright *et al.*, 1988), CCK-stimulated pancreatic acini (Matozaki *et al.*, 1990), $PGF_{2\alpha}$-stimulated NIH3T3 cells (Fukami & Takenawa, 1989) and vasopressin-stimulated REF52 fibroblasts (Huang & Cabot, 1990b) indicate that this might be a more general response to agonists which can couple to the hydrolysis of PtdIns(4,5)P_2. In the majority of these studies PtdCho has been implicated as the source of the sustained DG formation by observations of stimulated release of Cho or ChoP. Additionally, molecular species analysis of the sustained phase of DG formation in α-thrombin-stimulated IIC9 cells confirms that it is derived from PtdCho (Pessin & Raben, 1989). In the case of bombesin-stimulated Swiss 3T3 cells the onset of [^{3}H]Cho release (Chapter 5) was consistent with PtdCho hydrolysis contributing to sustained DG formation since it occurred after the initial phase of DG but prior to the second phase. A notable exception to these studies is the markedly transient and monophasic formation of DG in thrombin-stimulated platelets (Bishop *et al.*, 90). Interestingly, this in turn correlates with the poor ability of thrombin to stimulate PtdCho-PLD activity in these cells (Randall *et al.*,

1990) again suggesting that sustained DG formation is derived from PtdCho hydrolysis.

PMA-stimulated DG formation (Chapter 4) occurred in the absence of inositol lipid hydrolysis (Chapter 3) confirming the previous report of Takuwa *et al.* (1987b) and was sustained for at least 1 hour. Furthermore, it occurred over the same dose range at which the phorbol ester inhibited bombesin-stimulated $InsP_t$ formation. Since bombesin, like PMA, can activate PKC this suggests that *in vivo* bombesin-stimulated PKC activation may serve to both inhibit PtdIns(4,5)P_2 hydrolysis (Brown *et al.*, 1987) and stimulate a second phase of DG formation. The observation that PMA-stimulated [^{3}H]1,2-DG formation in Swiss 3T3 cells labelled with [^{3}H]palmitic acid and also stimulated the release of [^{3}H]Cho and [^{3}H]ChoP suggests that accelerated PtdCho hydrolysis is a candidate source for phorbol ester-stimulated DG formation. These results, subsequently confirmed by Price *et al.* (1989) who have also demonstrated bombesin and PMA-stimulated [^{3}H]Cho release in Swiss 3T3 cells, advance our understanding of phorbol ester-stimulated PtdCho hydrolysis considerably from the studies of Muir and Murray (1987) and Takuwa *et al.* (1987b) which measured PtdCho resynthesis and made no thorough kinetic analysis of Cho and ChoP release.

EGF-stimulated DG formation (Chapter 6) was of much smaller magnitude than that for bombesin and PMA but was maximal at doses of 0.1nM in the absence of significant inositol phosphate accumulation which was poorly stimulated and only apparent at 100 nM EGF. EGF-stimulated DG formation was accompanied by the release of [^{3}H]Cho implicating PtdCho as a possible source. Thus, these results agree with those of Wright *et al.* (1988; 1990a & b) who showed that EGF stimulates a small, but sustained, increase in DG mass in IIC9 cells which is associated with PtdCho hydrolysis and not the hydrolysis of inositol lipids. Furthermore, the results are consistent with those of Pessin *et al.* (1990) who demonstrated that the molecular species of the DG produced by EGF was similar to PtdCho rather than PtdIns.

Taken together with the results of Raben and co-workers (Wright *et al.*, 1988; 1990 a & b; Pessin & Raben, 1989; Pessin *et al.* 1990), Price *et al.* (1989),

Takuwa *et al.*, (1987 a & b; 1989) and Huang & Cabot (1990b) these results indicate that in cultured fibroblasts PtdCho represents a metabolically active pool of phospholipid which can be hydrolysed within seconds of addition of growth factors to contribute towards sustained DG elevation. It seems likely that PtdCho serves a similar function in a variety of other cell types stimulated by appropriate ligands (reviewed by Billah & Anthes, 1990; Exton, 1990). As such it now seems that the generation of DG within cells can no longer be considered solely in terms of the hydrolysis of the inositol lipids and the stimulated, cyclical hydrolysis of PtdCho (Fig. 7.1) must now be considered as a widespread and important signal transduction pathway.

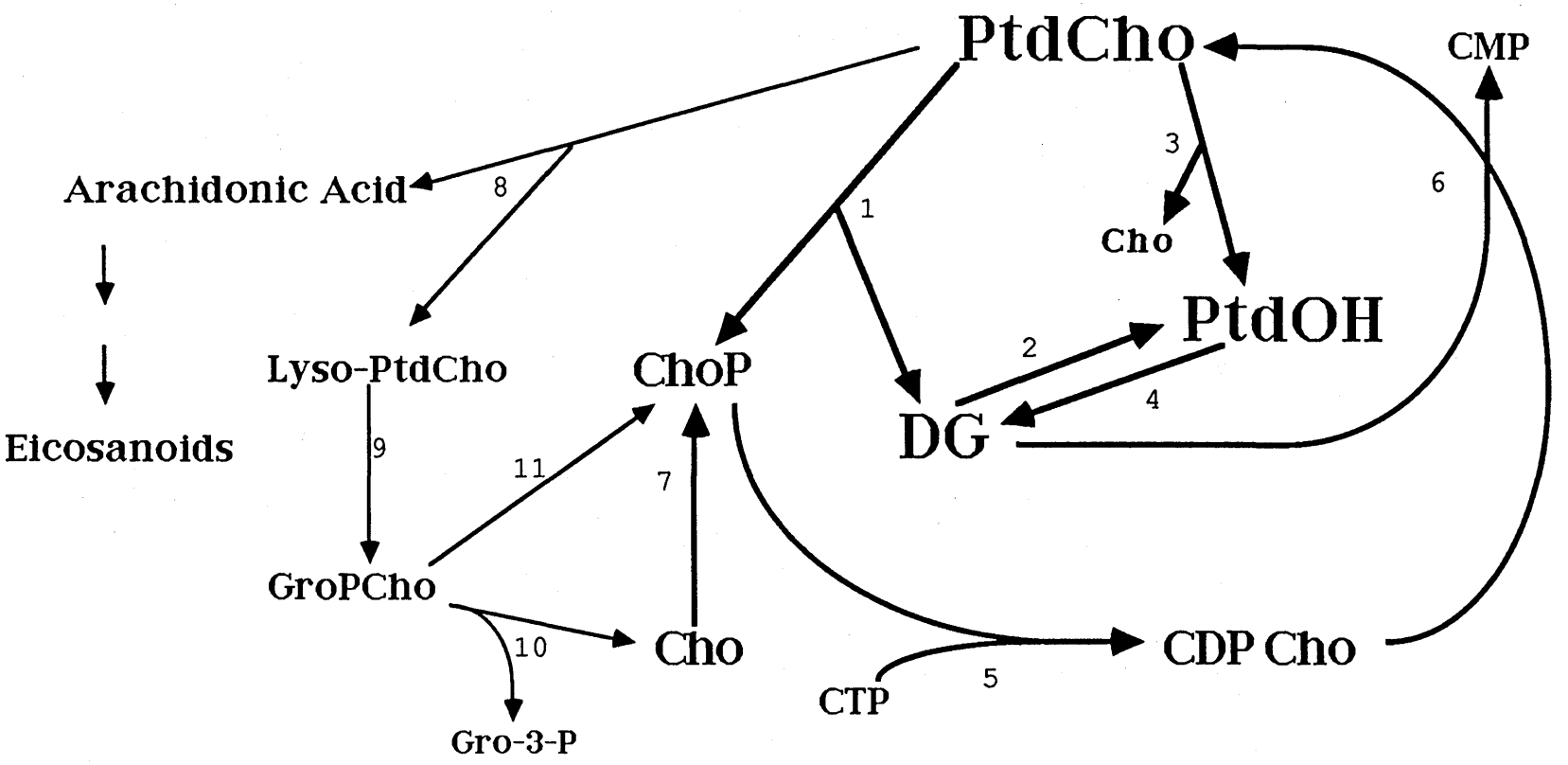
7.1.1 By which pathway is PtdCho hydrolysed, PLD or PLC ?

In Swiss 3T3 cells stimulated with either bombesin, PMA or EGF the earliest apparent manifestation of PtdCho hydrolysis is the release of [^{3}H]Cho. This would suggest that PtdCho is hydrolysed initially by a PLD-like activity (Fig. 7.1) since it seems unlikely that such an increase in Cho would be due to the stimulated dephosphorylation of ChoP (Cabot *et al.*, 1988). The demonstration that all three agonists stimulated the formation of [^{3}H]PtdBut in the presence of butan-1-ol with identical kinetics to the stimulated release of [^{3}H]Cho confirms that the initial pathway of PtdCho hydrolysis was indeed that of PLD.

The observation that bombesin also stimulated the release of [^{3}H]ChoP which was significantly elevated after 15 min suggests that PtdCho may also be hydrolysed to DG by the direct PLC pathway (Fig 7.1), although the possibility of this being due to Cho kinase activity (Warden & Friedkin, 1984 & 1985) cannot be ruled out because of the apparent temporal dissociation of increases in [^{3}H]Cho and [^{3}H]ChoP. PMA-stimulated [^{3}H]Cho formation was slower in onset than the bombesin response and this is reflected in the slower rise in [^{3}H]ChoP. In the case of EGF this temporal dissociation is even more apparent. It seems likely that the question of whether PtdCho-PLC is activated can only be adequately addressed by sensitive assays for Cho and ChoP mass (e.g., Murray *et al.*, 1990) and definitive

Figure 7.1 Major elements of a hormone-sensitive PtdCho cycle.

The major elements of a hormone-sensitive PtdCho cycle are shown. Pathways directly involved in the regulation of DG levels are indicated by thick arrows; other pathways are in thin arrows including PLA_2 which may serve as a source of arachidonic acid from PtdCho. The enzymes are: (1) Phospholipase C; (2) DG kinase; (3) Phospholipase D; (4) Phosphatidate phosphohydrolase; (5) CTP:phosphocholine cytidylyltransferase; (6) CDP choline:1,2-DG phosphocholinetransferase; (7) Choline kinase; (8) Phospholipase A_2; (9) Lysophospholipase; (10) GroPCho phosphodiesterase; (11) GroPCho cholinephosphodiesterase.


PtdCho
CMP
Arachidonic Acid
8
1
3
6
Cho
PtdOH
Lyso-PtdCho
ChoP
2
4
DG
Eicosanoids
9
11
7
GroPCho
Cho
10
CDP Cho
5
CTP
Gro-3-P

identification of PtdCho-PLC activity such as the formation of [^{32}P]ChoP under conditions where the ATP pool is not labelled (e.g., Billah *et al.*, 1989b).

7.1.2 The role of PtdCho-PLD in sustained DG formation.

The use of butan-1-ol to 'trap' phosphatidyl moieties liberated during PLD-catalysed head group release allows intervention in a PLD/PPH pathway of DG formation (Bonser *et al.*, 1989; Metz & Dunlop *et al.*, 1991). There were marked differences between PMA and bombesin in terms of the contribution made by a PLD/PPH pathway to DG formation. The inhibition of approximately 70% of PMA-stimulated DG mass formation by butan-1-ol suggests that the majority of PMA-stimulated DG formation arises by a PLD/PPH coupled pathway. Since DG levels are maintained, despite removal via DGK or DGL, this would require sustained activation of PLD which is demonstrated by the linear, non-desensitising accumulation of [^{3}H]PtdBut in response to PMA. Furthermore, the inability of cells to metabolize PMA would ensure that PLD activity was sustained. However, in the case of bombesin, the inclusion of butan-1-ol inhibited only 30% of bombesin-stimulated DG formation at 15 min suggesting a relatively minor role for a PLD/PPH pathway in bombesin-stimulated DG formation. This seems to correlate with the rapid drop in the rate of [^{3}H]PtdBut accumulation after 2 min stimulation with bombesin suggesting a rapid desensitisation. This raises a number of questions concerning the role of PtdCho hydrolysis in DG generation.

First, if only 30% of the sustained phase of bombesin-stimulated DG formation is due to PLD activity, where is the remaining 70% derived from ? If the slower increase in [^{3}H]ChoP seen in bombesin-stimulated Swiss 3T3 cells does indeed represent PtdCho-PLC activity this may contribute to long term bombesin-stimulated DG generation. If both PLD and PLC are being activated do both pathways make the same contribution to DG formation at the onset of the second phase (e.g., 30 sec-1 min) as they do at later times (e.g, 15 min) ? It may be possible to assess this question by examining the effect of butan-1-ol on DG

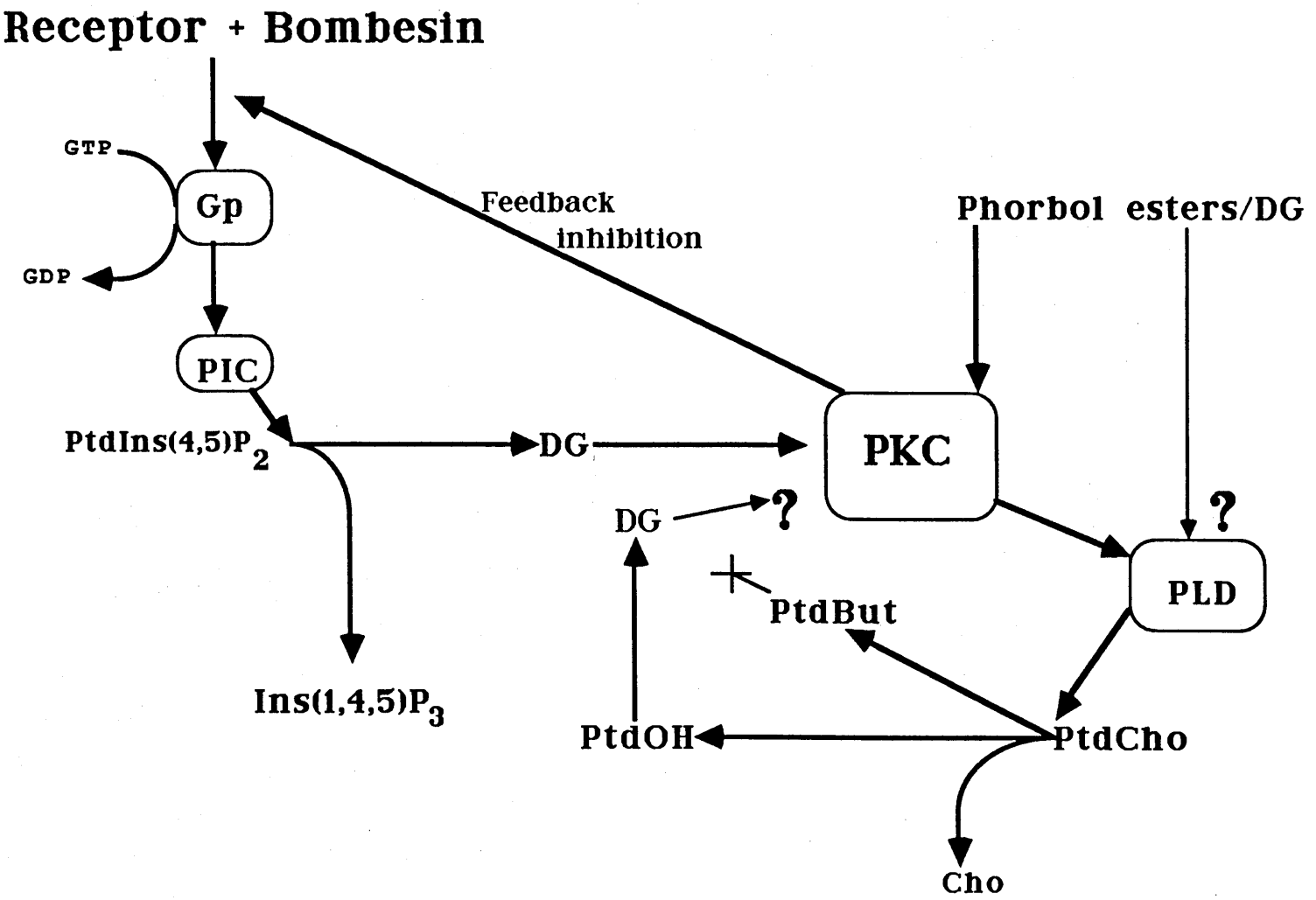
formation at different points in the bombesin timecourse. In addition, the sustained accumulation of [^{3}H]InsP$_t$ and [^{3}H]InsP in bombesin-stimulated Swiss 3T3 cells (Chapter 3, Fig 3.1-3.3 inclusive) indicates sustained hydrolysis of either PtdIns(4,5)P_2 or PtdIns (Imai & Gershengorn, 1986), though at a reduced rate, which will necessarily result in DG formation. This may explain why, in parallel incubations, bombesin-stimulated DG formation was greater than the PMA-stimulated response (Chapter 4, Fig. 4.11); whilst both agents can activate PtdCho-PLD, and perhaps PtdCho-PLC, PMA cannot utilize inositol lipids as a source of DG (Chapter 3, Fig. 3.9).

Second, a major requirement for any hydrolytic event which contributes to the long term generation of DG is that it should be sustained. The generation of [^{3}H]PtdBut in response to bombesin, whilst rapid in onset up to 2 min, was quickly reduced in rate by nearly 10-fold (Chapter 4). This suggests that bombesin-stimulated PLD activity is quickly desensitised and, therefore, might only contribute to the early part of sustained DG formation. In contrast, bombesin-stimulated increases in cell associated or intracellular [^{3}H]Cho were equally rapid in onset up to 1-2 min but then appeared to reach an apparent new 'steady state' where removal of [^{3}H]Cho, by choline kinase or exit from the cell, was apparently in equilibrium with its continued formation (Chapter 5). This would suggest that PtdCho-PLD activity was sustained.

This anomaly could perhaps be explained by the invasive nature of the transphosphatidylation assay for PLD as opposed to assaying Cho or PtdOH formation. If PKC is a major link between transient PtdIns(4,5)P_2 hydrolysis and activation of PLD, as is suggested by the results in Chapter 4, then one can envisage a positive feedback loop (outlined schematically in Fig 7.2), as suggested by Exton (1990). Activation of PKC, consequent with PtdIns(4,5)P_2 hydrolysis and DG formation, would lead to activation of PLD which, coupled to PPH, would provide further DG for activation of PKC, and so on. Such a model would account for sustained increases in both [^{3}H]Cho and DG. By including butan-1-ol one immediately interrupts such a positive feedback loop since PtdBut cannot provide DG for activation of PKC and so activation of PLD cannot be sustained. Thus, the

Figure 7.2 The pathway for activation of PtdCho-PLD in bombesin-stimulated Swiss 3T3 cells.

The major pathway for activation of PLD appears to be via activation of PKC; a possible role for Ca^{2+} is not shown for simplicity and because these studies are only preliminary. Reactions which are known to take place are shown in thick arrows; reactions which are speculative are shown in thin arrows. The activated bombesin receptor couples to PIC via a G-protein (Plevin *et al.*, 1990) resulting in the generation of Ins(1,4,5)P_3 and DG (Chapters 3 & 4). Activation of PKC results in an inhibition of inositol lipid hydrolysis (Brown *et al.*, 1987; Chapter 3). Activation of PLD is kinetically downstream of PIC activation, can be mimicked by phorbol esters and is inhibited by PKC down-regulation or selective kinase inhibitors (Chapters 4 & 5), consistent with a sequential PIC-PKC-PLD pathway. A potential positive feedback loop for DG formation, involving a coupled PLD/PPH pathway, is shown; intervention in this pathway using butan-1-ol is shown by the inability of PtdBut to be de-phosphorylated to DG (see text for discussion).


Receptor + Bombesin
GTP
Gp
GDP
PIC
PtdIns(4,5)P2
DG
Ins(1,4,5)P3
Feedback
inhibition
Phorbol esters/DG
PKC
DG
?
PtdBut
PLD
?
PtdOH
PtdCho
Cho

rapid onset of [^{3}H]PtdBut formation in response to bombesin may represent the initiation of the positive feedback loop by DG derived from PtdIns(4,5)P_2, whereas the drop in the rate of [^{3}H]PtdBut formation may be due to interruption, by butan-1-ol, of the positive feedback loop required to sustain PLD activity when PtdIns(4,5)P_2 hydrolysis has subsided. In such a scenario the slower, sustained accumulation of [^{3}H]PtdBut seen in some experiments from 2 to 15 min may be due to the incomplete competition between butan-1-ol and water for accepting the released phosphatidyl moiety, providing some PtdOH as a source of DG, or the DG derived from sustained inositol lipid hydrolysis serving to maintain a lower level of PKC-mediated PLD activity. An alternative model is that activation of PLD is dependent upon prior bombesin-stimulated inositol lipid hydrolysis but the rapid desensitisation of this response (Chapter 3, Fig. 3.1-3.4 inclusive; S. Palmer, R. Plevin & M.J.O. Wakelam, personal communication) is reflected in a desensitisation of PLD.

If a positive feedback loop does operate a prediction from such a model is that addition of butan-1-ol to bombesin-stimulated Swiss 3T3 cells should result in a gradual drop in stimulated [^{3}H]Cho elevation as the positive feedback loop gradually 'runs down'. An alternative approach would be to stimulate Swiss 3T3 cells with bombesin for 2-5 min and then add a bolus of butan-1-ol to the required final concentration. If [^{3}H]PtdBut is formed as normal over the next 2 min and then slows this would suggest that butan-1-ol is indeed interrupting a feedback loop; alternatively if [^{3}H]PtdBut is not formed this would imply that the response is largely desensitised. Recent work from this laboratory using the latter approach has shown that pre-incubation of Swiss 3T3 cells with bombesin followed by addition of butan-1-ol prevents stimulated formation of [^{3}H]PtdBut suggesting that bombesin-stimulated PLD activity is largely desensitised after 5 min (R. Plevin & M.J.O. Wakelam, personal communication). This suggests that PLD activity remains dependent upon receptor-stimulated inositol lipid hydrolysis, declining as this initial response desensitises, and is not maintained by a self-sustaining PKC loop. Thus, a PLD/PPH pathway may only make a contribution to DG formation over the first 2-5 min of the bombesin response and future experiments should

address where the sustained elevation of DG is derived from.

The apparent anomaly that EGF clearly stimulates a PLD activity, as measured by the release of [^{3}H]Cho and formation of PtdBut, which seems to play no role in EGF-stimulated DG formation, which is not inhibited by butan-1-ol, is not easily explained. The effect of butan-1-ol was only assessed at a single 15 min time point whereas PLD activity appeared to be largely desensitised after 2 min. In this case there was no significant increase in [^{3}H]ChoP until 20 min after addition of EGF. Whether these smaller and later increases in [^{3}H]ChoP are underestimates due to the poor sensitivity of isotopic labelling measurements and a high basal level of [^{3}H]ChoP is not clear but mass analysis of ChoP may help to address this. This should allow an assessment of whether PtdCho-PLC activity may contribute to EGF-stimulated DG formation as has been suggested by Wright *et al.* (1990 a & b) and Baldassare *et al.* (1990).

These results should be considered in the light of the recent work by Huang & Cabot (1990a) who have assessed the role of the PLD/PPH pathway in phorbol ester-stimulated DG formation in MDCK epithelial cells, bovine pulmonary endothelial cells and two smooth muscle cell lines. These authors show that whilst activation of PLD is a common response to stimulation by PMA, only in the MDCK and endothelial cells does the PLD/PPH pathway operate in DG formation. In the two smooth muscle cell lines PtdEth and PtdOH were formed but the latter did not serve as the precursor of DG. This indicates that there may be marked differences between different cell types in their ability or necessity to utilise a PLD/PPH coupled pathway of DG formation even when PLD is clearly activated. Whether these differences are also reflected for different agonists within the same cell remains to be seen.

The results in this thesis concerning the role of a PLD/PPH pathway are preliminary since they rely upon the use of butan-1-ol to trap phosphatidyl moieties. Whilst this appears to be a valid approach in neutrophils (Bonser *et al.*, 1989) it is not clear as yet whether this method will be universally applicable with the same degree of success. Furthermore, the method relies upon butan-1-ol (30 mM)

competing with water as the nucleophile attacking a Ptd-Enz intermediate. Since the effective concentration of water within the cell is very high adequate controls will be required to ensure that PtdOH formation is completely abolished in favour of PtdBut.

It appears that PtdCho hydrolysis does occur by a PLD-catalysed pathway and indeed this may be the initial hydrolytic route. This pathway is involved in DG formation by PMA and, to a lesser extent bombesin, but a clearer assessment of how much it contributes to DG formation requires comparison of the effects of butan-1-ol with proposed PPH inhibitors such as sphingosine and propranolol. In addition to PLD/PPH, other pathways may make a significant contribution to bombesin-stimulated DG formation even when PLD is activated (Huang & Cabot, 1990a). The identity of these other pathways remains unclear but the direct formation of DG via PtdCho-PLC activity or the slower, sustained hydrolysis of inositol lipids are both candidates.

7.1.3 The implications of PLD/PPH and/or PLC as pathways for DG formation.

The hydrolysis of PtdIns(4,5)P_2 to form Ins(1,4,5)P_3 and DG followed by the recycling of the inositol and diglyceride moieties is an energetic process requiring 4 molecules of ATP and 1 of CTP for each turn of the cycle (including Ins(1,4,5)P_3 3'-kinase but not PtdIns 3'-kinase, see Fig 1.2). In the context of mitogenic signalling it appears that DG/PKC mediated signals may be more important in the long term than the transient Ins(1,4,5)P_3-mediated mobilisation of intracellular stores of Ca^{2+} (e.g., Suzuki-Sekimori *et al.*, 1989). Thus, a switch to an energetically cheaper source of DG, such as PtdCho, might be advantageous once the requirement for large increases in $[Ca^{2+}]_i$ and therefore Ins(1,4,5)P_3 has passed. The cyclical turnover of PtdCho, initiated by a PLD pathway, consumes 1 molecule of ATP (the choline kinase reaction) and 1 of CTP (the cytidylyltransferase reaction) per turn of the cycle (Fig. 7.1) whilst the PtdCho-PLC pathway, which may contribute to sustained DG formation in response to

prolonged bombesin stimulation, consumes only 1 molecule of CTP (the cytidylyltransferase reaction). Thus, the sequential activation of PIC, PLD and perhaps PLC could allow sustained DG formation accompanied by the transient formation of Ins(1,4,5)P_3 and PtdOH whilst making progressively smaller demands upon cellular nucleotide triphosphate pools.

The rapid hydrolysis of a significant portion of cellular PtdIns(4,5)P_2 is energetically expensive and may perturb the integrity of the inner plasma membrane if continued for any length of time. Few definitive studies have been made, but in fMet-Leu-Phe-stimulated neutrophils it seems that 3-5% of total PtdCho is hydrolysed (Billah *et al.*, 1989b). Isotopic measurements in other cells indicate that, in general, no more than 10% of labelled PtdCho is hydrolysed in response to a variety of mitogenic and non-mitogenic stimuli (Chapter 5). Since PtdCho accounts for approximately 50% of total cellular phospholipid its hydrolysis may provide large, sustained, quantities of PtdOH and DG without exerting great influences on localised plasma membrane fluidity and permeability.

The generation of second messengers, by definition, must ultimately be terminated to ensure adequate regulation of responses. The sustained elevation of DG levels presents the cell with another regulatory problem if, as seems to be the case in Swiss 3T3 cells, PKC plays a role in activating PtdCho hydrolysis. Whilst there is no clear evidence as to how sustained DG elevation is terminated, the answer may lie in the regulation of CTP:phosphocholine cytidylyltransferase activity which is known to be a rate limiting step in the resynthesis of PtdCho (Lim *et al.*, 1983; Pelech & Vance, 1984). Like PPH and DGK this enzyme is apparently translocated to particulate fractions upon stimulation of cells (Pelech & Vance, 1984; Besterman *et al.*, 1986b; Maroney & Macara, 1989). In addition it is activated by PtdOH and DG (Liscovitch *et al.*, 1986; Kolesnick, 1987; Kolesnick & Paley, 1987). Billah & Anthes (1990) speculate that PtdOH and DG, both products of PtdCho hydrolysis, will activate the cytidylyltransferase so that sustained DG formation will ensure its own conversion to PtdCho thereby leading to an eventual termination of the signal.

The rapid formation of DG by, for example, bombesin-stimulated PIC

activity constitutes a potent mitogenic signal which must be rapidly removed; the 'classical' pathways for this are via DG kinase or DG lipase. However, in the light of the potential roles for PtdOH and arachidonic acid as second messengers can the DGK and DGL pathways simply be considered as routes for signal termination ? Tsai *et al.* (1988; 1990) report that 1-stearoyl-2-arachidonoyl PtdOH is the most potent inhibitor of *ras* GAP and stimulator of *ras* GIP (discussed below) whilst arachidonic acid also exerts considerable effects upon the activities of GAP and GIP as well as activating certain PKC isoforms (reviewed by Kikkawa *et al.*, 1989), stimulating DG formation in Swiss 3T3 cells (Takuwa *et al.*, 1988) and synergising with [Arg^8]vasopressin to stimulate DNA synthesis in Swiss 3T3 cells (Millar & Rozengurt, 1990). Thus, in this context both DGK and DGL can be considered as routes for prolonging and diversifying the signal initiated by DG formation. By the same token, removal of PtdOH by PPH or a putative PLA_2 pathway (Billah *et al.*, 1981) can also be considered as routes for prolonging and diversifying a given signal, namely activation of PLD.

Therefore, which pathways within cellular phospholipid metabolism might fulfil a signal termination role without generating other second messenger-like molecules ? One possible candidate is phosphatidate cytidylyltransferase (or CMP PtdOH synthetase) which catalyses the formation of CMP PtdOH from PtdOH and CTP, though little is known about this enzyme in terms of regulation of its activity. CTP:phosphocholine cytidylyltransferase might be considered to fulfil a similar role since it converts DG to PtdCho thereby terminating a signal and generating a molecule with no second messenger activity. Such speculation must await confirmation of any messenger functions for PtdOH and arachidonic acid. If however PtdOH and arachidonic acid do turn out to be second messengers this will perhaps necessitate a reconsideration of the roles of DGK and DGL as not simply signal terminators but under certain circumstances also signal generating pathways.

Finally, for those agonists which stimulate PtdIns(4,5)P_2 hydrolysis a major pathway of DG removal appears to be DGK (Bishop *et al.*, 1986; reviewed by Kanoh *et al.*, 1990). If such agonists are also capable of activating a PLD/PPH pathway of DG formation the cell is faced with considerable regulatory problems in

balancing the removal of DG by DGK and the formation of DG from PtdOH by PPH. If the two events are clearly temporally divorced, does the cell 'switch' from DGK to PPH activity and if so what is the biochemical/molecular basis of this switch ? Alternatively, the two pathways may operate in distinct compartments of the cell allowing independent regulation of the two enzymes due to spatial separation. Indeed, it is not clear, whether there is any communication between the two pathways for example in terms of DG from PtdIns(4,5)P_2 hydrolysis serving as a substrate for the CDP:phosphocholine cytidylyltransferase or PtdOH from PtdCho-PLD serving as a precursor for CMP.PtdOH. On the latter point MacDonald *et al.* (1989) suggest that the DGK selective for arachidonoyl-containing DG in Swiss 3T3 membranes may play a role in specifically enriching inositol lipids with arachidonic acid at the *sn*-2 position. This is only a preliminary study, but suggests that the DG derived from PtdIns(4,5)P_2 hydrolysis may stay within the inositol lipid cycle; studies of the metabolic fate of 1-stearoyl-2-arachidonoyl glycerol in broken cell preparations may help to address the question of communication between the two phospholipid cycles.

These issues are important since without adequate regulation of DGK and PPH activitites, there exists the possibility of futile cycling of diglyceride moieties and wastage of ATP. PtdCho hydrolysis may serve distinct functions in signal transduction, be it DG formation or the generation of other messenger molecules (discussed below), which it can perform with less energetic expenditure than the hydrolysis of PtdIns(4,5)P_2. However it also places significant regulatory constraints upon the cell in which it operates.

7.2. DG formation and PtdCho hydrolysis; common elements in mitogenic lipid signalling pathways ?

By comparing the results presented in this thesis with those of related model systems of mitogenic signalling a picture begins to emerge of which growth factors employ which pathways of lipid signalling.

One correlation which is immediately apparent is that those mitogens of the RGE type which are able to stimulate the rapid generation of $Ins(1,4,5)P_3$ and DG are also able to activate a PtdCho-PLD and elicit sustained DG elevation. This appears to be the case for a variety of mitogens and co-mitogens of this class including α-thrombin (Wright *et al.*, 1988; Pessin & Raben, 1989), $PGF_{2\alpha}$ (Fukami & Takenawa, 1989) and vasopressin (Huang & Cabot, 1990b) and in many ways the responses resemble those for bombesin described herein. Since all these agonists are able to activate PKC which dissociates $PtdIns(4,5)P_2$ and PtdCho hydrolysis (Brown *et al.*, 1987; Chapters 3 and 5) it would appear that PtdCho hydrolysis and presumably sustained DG formation are common events following inositol lipid hydrolysis and PKC activation. A major exception to this apparent consensus may be the generation of DG by α-thrombin in IIC9 cells. This response is clearly biphasic, but it is possible to distinguish between the two phases on a pharmacological basis. Lowering the α-thrombin concentration from 500ng ml^{-1} to 100pg ml^{-1} results in a loss of $InsP_3$ formation and the first phase of DG formation, but the second phase of DG formation is still observed, if reduced in magnitude (Wright *et al.*, 1988). This suggests that whilst a PKC regulated pathway may contribute to the second phase of DG, some of it may be coupled to activation of a distinct receptor subtype. It is of interest to note that α-thrombin is mitogenic for IIC9 cells at both of these concentrations, suggesting that $Ins(1,4,5)P_3$ formation can be lost without compromising mitogenic potency. As mentioned in Chapter 4 it is not clear whether this is also the case for bombesin for two main reasons; whilst the second phase of bombesin-stimulated DG formation exhibits a slightly lower EC_{50} than the first phase this might be due to amplification of the response by PKC resulting in a lower effective concentration of bombesin required for DG elevation. In addition there is a precedent for two distinct receptor sub-types for α-thrombin since the inhibition of adenylyl cyclase occurs at much lower doses than does the stimulation of $Ins(1,4,5)P_3$ formation (Pouysségur, 1990). Whilst the bombesin receptor has recently been purified (Feldman *et al.*, 1990) and cloned (Battey *et al.*, 1991) there is no evidence to suggest the existence of any receptor sub-types on Swiss 3T3 cells other than the single class of high

affinity sites with a K_d in the range 0.1 - 1 nM (Feldman *et al.*, 1990; Sinnett-Smith *et al.*, 1990).

For the RTK group of mitogens and co-mitogens such as PDGF and EGF it is more difficult to reach a consensus since their ability to stimulate lipid signalling pathways seems to depend upon the cell type studied (discussed in Chapter 6). In those cells where RTK growth factors do stimulate inositol lipid hydrolysis it seems likely that as a consequence of the resulting PKC activation PtdCho hydrolysis will be initiated. EGF does not stimulate inositol lipid hydrolysis in Swiss 3T3 cells (Hesketh *et al.*, 1988), Balb/c 3T3 cells (Besterman *et al.*, 1986a), CCL39 cells (Paris *et al.*, 1988) or IIC9 cells (Pessin *et al.*, 1990) whilst PDGF is unable to couple to inositol lipid hydrolysis in IIC9 cells (Pessin *et al.*, 1990) or CCL39 fibroblasts (Paris *et al.*, 1988). Despite this, evidence to date indicates that PDGF, EGF and FGF are able to stimulate increases in DG levels and/or PtdCho hydrolysis irrespective of their ability to couple to activation of PIC in Swiss 3T3 cells (Price *et al.*, 1989; Larrodera *et al.*, 1990; Nanberg *et al.*, 1990; Chapter 6), IIC9 cells (Pessin *et al.*, 1990) and CCL39 cells (Paris *et al.*, 1990). In those instances where activation of PLD and generation of DG is dissociated from inositol lipid hydrolysis, future studies should aim to assess the mechanism of coupling of the receptor tyrosine kinase catalytic domain to these responses.

The colony stimulating factor 1 (CSF-1) receptor is identical to the *c-fms* proto-oncogene product and undergoes agonist-dependent autophosphorylation. Immamura *et al.* (1990) have shown that in human monocytes CSF-1 activates PKC and increases cellular DG content but does not stimulate the hydrolysis of PtdIns(4,5)P_2. However, CSF-1 does stimulate the hydrolysis of PtdCho and the generation of ChoP (Immamura *et al.*, 1990). Also, Rossof *et al.* (1988) have shown that interleukin-1 is apparently able to stimulate an increase in DG and ChoP in T lymphocytes though they were unable to demonstrate measureable binding of IL-1 to the T-cells used in their study.

Thus, the ability to raise DG levels and activate PtdCho hydrolysis may be common responses to stimulation by diverse mitogens and co-mitogens. In many cases this is accompanied by the hydrolysis of the polyphosphoinositides and

generation of Ins(1,4,5)P_3, though even in these cases the quantitative contribution made by inositol lipid hydrolysis may be minor and PtdCho may be a more significant source. However there are clearly a number of examples, such as EGF, PDGF and CSF-1, where DG elevation proceeds in the absence of inositol lipid hydrolysis and apparently derives from the stimulated hydrolysis of PtdCho. By virtue of this fact there is a case for arguing that PtdCho hydrolysis and DG formation may be common denominators amongst mitogen-stimulated lipid signalling pathways.

A number of reports have shown that growth factors and other hormones and neurotransmitters appear to activate PKC in various target cells but are unable to stimulate inositol lipid hydrolysis These include embryonal carcinoma-derived growth factor (Mahadevan *et al.*, 1987), FGF in a distinct clone of Swiss 3T3 cells (Nanberg *et al.*, 1990) and interleukin-3 in the multipotent stem cell line FDCP-Mix1 (Whetton *et al.*, 1988). In hindsight it will be of interest to examine whether these agonists are able to activate PLD- or PLC-catalysed hydrolysis of PtdCho and generation of DG.

The contention that PtdCho hydrolysis and DG formation are common lipid signalling responses to proliferative stimuli does not, and should not, imply that they represent the vital, overriding mitogenic signals. Indeed such speculation of a single 'master' signal ignores the enormous evidence indicating that growth factors can employ multiple early and later signal pathways to mediate the transition through G_1 to 'commitment' to the cell cycle (reviewed by Rozengurt, 1985; 1986; Pouysségur, 1990; Cantley *et al.*, 1991). Some growth factors are able to elicit different signals in target cells at different stages in the cell cycle. For example, insulin-like growth factor-I stimulates increases in Ca^{2+} entry and DG content in 'primed-competent' Balb/c 3T3 cells but does not in quiesecent cells (Kojima *et al.*, 1990).

If the stimulated transition from quiescence into the cell cycle, culminating in cell division is considered as a 'stimulus-response' system in much the same way as, for example, O_2^- generation in f-Met-Leu-Phe-stimulated neutrophils, the sheer

complexity of events required for completion of the cell cycle is staggering in comparison. To then try and attribute all these events to a single signal transduction pathway or second messenger is to greatly over simplify the whole process.

Micro-injection of DG, but not Ins(1,4,5)P_3, into Balb/c 3T3 cells (Suzuki-Sekimori *et al.*, 1989) and exogenous addition of cell permeant DG analogues to Swiss 3T3 cells (Rozengurt *et al.*, 1984) both synergise with insulin in stimulating DNA synthesis. In addition, overexpression of PKC results in accelerated growth rates and morphological transformation (Housey *et al.*, 1988; Persons *et al.*, 1988). These observations suggest that increases in DG and/or PKC activity lie on a mitogenic signal pathway. However, down-regulation of PKC does not inhibit the mitogenic effects of all growth factors indicating that other parallel, PKC-independent pathways exist such as receptor and non-receptor tyrosine kinases. The discovery of so called 'switch kinases' (e.g., Raf-1 and MAP II), serine- and threonine-directed kinases activated by tyrosine and/or threonine phosphorylation indicates that cross-talk will occur between serine/threonine kinase pathways (PKC) and tyrosine kinase pathways (EGF-R, PDGF-R and *src*). The role of cAMP-dependent PKA in stimulating or inhibiting mitogenesis, depending on the system under investigation, must be considered along side these other pathways.

Consequently we cannot consider a single pathway in isolation and hope to understand mitotic regulation. However, with these caveats in mind one can speculate as to the role of sustained DG formation and PLD activation in the pre-replicative phase of mitogenesis.

7.2.1 The role of sustained DG elevation in mitogenesis.

The simplest and most obvious role for sustained DG elevation is the prolonging of PKC activation. This would seem reasonable since many PKC-mediated events occur either kinetically downstream, or in the absence of, elevated Ins(1,4,5)P_3 levels. Thus phosphorylation of an acidic 80 kDa protein by PKC in Swiss 3T3 cells continues after Ins(1,4,5)P_3 levels and $[Ca^{2+}]_i$ have declined in bombesin-stimulated Swiss 3T3 cells (Isacke *et al.*, 1986). Transcription of the c-

fos and c-*myc* proto-oncogenes, which encode transcription factor elements, is downstream of the initial growth factor stimulus by 30 to 60 min and in the case of bombesin is greatly reduced following 'down-regulation' of PKC (Rozengurt & Sinnett-Smith, 1987). Likewise, in bombesin-stimulated Swiss 3T3 cells increases in pH_i by a PKC-activated Na^+/H^+ antiporter are biphasic, mirroring the biphasic increase in DG mass; the second phase of alkalinisation correlates with the sustained phase of DG mass formation (Bierman *et al.*, 1990).

Despite this recent studies by Leach *et al.* (1991) question the role of sustained DG formation in activating PKC. The authors have isolated total PtdCho from IIC9 cells, treated it with PLC and demonstrated that the DG so derived is able to activate PKC *in vitro* in a mixed micelle assay using IIC9 cytosol as a source of PKC. However, in whole cells PKC activation and translocation in response to α-thrombin is only associated with the first phase of DG activation as assessed by phosphorylation of an 80kDa PKC substrate. At low concentrations of α-thrombin (100pg/ml) there is no phosphorylation of the 80kDa protein associated with the second sustained phase of DG elevation. These results suggest that the DG derived from PtdCho hydrolysis does not activate PKC *in vivo*. In contrast, Block *et al.* (1989) were able to demonstrate activation of PKC by both PDGF AA and BB in vascular smooth muscle cells despite the fact that the same authors showed that PDGF AA is able to elevate DG levels in the absence of $InsP_3$ accumulation (Sachidinis *et al.*, 1990).

PKC is a family of enzyme isoforms with distinct activation requirements (reviewed by Kikkawa *et al.*, 1989; Parker *et al.*, 1990) and possibly distinct substrate specificities (Schaap & Parker, 1990) and tissue/cell distribution (Bacher *et al.*, 1991). DG derived from PtdCho hydrolysis, having a different fatty acid complement to that from $PtdIns(4,5)P_2$ and being produced in the absence of elevated $[Ca^{2+}]_i$, may activate different isoforms of PKC which may not utilise the classical marker substrates such as the 80kDa protein. Alternatively, these isoforms may make an important qualitative rather than quantitative contribution to mitogen-stimulated protein phosphorylation and the cell and tissue distribution of these isoforms may dictate whether PKC activation is associated with PtdCho hydrolysis

(Block *et al.*, 1989) or not (Leach *et al.*, 1991). Certainly there is evidence for distinct tissue-specific distribution even among the Ca^{2+}-independent isoforms such as PKC-ε which is found in neural tissue (Ono *et al.*, 1988) and the highly homologous PKC-L which is absent from brain but is found in lung, skin and heart (Bacher *et al.*, 1991).

Such speculation also calls into question the validity of assessing PKC activation solely in terms of phosphorylation of the 80kDa protein. The 80kDa phosphoprotein is an acidic, cytosolic protein of unknown identity or function; this very fact might make it a poor candidate for use as a marker for PKC activity. At least two 80kDa proteins exist (Morris & Rozengurt, 1988; Patel & Kligman, 1987; Hirai & Shimizu, 1990); in the studies by Hirai & Shimizu (1990) '80K-L' was phosphorylated to a greater extent than '80K-H' by purified PKC but the two exhibited completely different 2-D phosphopeptide maps after digestion with TPCK-treated trypsin. Furthermore, significant phosphorylation of '80K-H' occurred in the presence of EGTA upon four major tryptic peptides, but was reduced by the addition of Ca^{2+}, whilst four novel phosphopeptides were apparent in the presence of Ca^{2+}, PtdSer and PMA. The two forms of 80kDa protein were also phosphorylated to quite different extents by different isoforms of PKC. Thus whilst phosphorylation of 80kDa protein will strongly suggest activation of PKC, lack of phosphorylation need not rule out activation of distinct isoforms of the kinase.

In addition to PKC there exist a number of serine- and threonine-kinases in cells which may be potential candidates for activation by DG. Protease-activated kinase (PAK II) activity is stimulated by phospholipid and DG, inhibited by the inclusion of Ca^{2+} and is resolved from PKC upon ADP-agarose chromatography (Gonzatti-Haces & Traugh, 1986), but gives similar 2-D tryptic phosphopeptide maps of Histone-1 and S6 ribosomal proteins. Krebs and co-workers (Ahn & Krebs, 1990; Ahn *et al.*, 1990) have recently identified six separate EGF-stimulated serine and threonine kinases in Swiss 3T3 cells; at least two of these appear to be novel kinases which may therefore be potential candidiates for regulation by the products of stimulated phospholipid metabolism. The Raf-1

protein also possesses cysteine-rich sequences homologous to the highly conserved motifs in the regulatory domain of PKC (Ohno *et al.*, 1988) though to date no effects of phospholipid or DG upon Raf-1 activity have been shown. Certainly PKC and Raf-1 share similar mechanisms of regulation; in both cases the regulatory domain exerts an inhibitory effect upon the catalytic domain which can be overcome by DG/PMA binding (PKC) or by N-terminal truncation (PKC or Raf-1). Such truncation leads to irreversible activation of the kinase activity which in the case of Raf-1 is oncogenic when injected into cells (Rapp *et al.*, 1987 a & b). There are conserved cysteine rich motifs within the C1 regulatory domain of PKC which are shared by a number of proteins in addition to Raf-1. These include DG kinase (Sakane *et al.*, 1990), the human glucocorticoid receptor (Green *et al.*, 1988) and *n*-chimaerin, a novel high affinity phorbol ester receptor in brain (Ahmed *et al.*, 1990). By producing degenerate oligonucleotides to conserved motifs in the regulatory domain of PKC it may be possible to clone cDNAs for novel DG-activated proteins by low stringency hybridization. Such an approach would be likely to produce a lot of false positives (i.e. clones of PKC itself) and would require a second round of screening to select against such clones, but this could provide a powerful approach to determining novel targets for DG. Similar strategies have recently been successful in isolating PKC-L from a human keratinocyte cDNA library (Bacher *et al.*, 1991) as well as novel receptor-like tyrosine phosphatases from a fibroblast cDNA library using conserved sequences from the catalytic domain of the leukocyte common antigen CD45 as a probe (Kaplan *et al.*, 1990).

In addition to kinases there is some evidence that DG may exert effects upon other proteins involved with mitogenic signal transduction. DG activates a partially purified PLA_2 preparation from Swiss 3T3 cells which exhibits no DG-activated PKC activity (Burch, 1988); DG appears to exert its effect at the level of PLA_2 itself. The highest stimulation of PLA_2 activity was by SAG which is produced rapidly upon mitogenic stimulation of Swiss 3T3 cells. In these experiments PLA_2 was stimulated by as little as 0.3 Mol% DG which is of similar magnitude to the increase in DG observed upon stimulation of Swiss 3T3 cells with bombesin

(Takuwa *et al.*, 1987a; Chapter 4). Furthermore, in bombesin-stimulated Swiss 3T3 cells activation of PLA_2 is independent of PKC activity (S. Currie & M.J.O. Wakelam, personal communication) Thus, production of DG by mitogens may serve to activate PLA_2 as part of a signal transduction pathway. The product of PLA_2, arachidonic acid, may also activate distinct isoforms of PKC aswell as serving as the precursor of prostanoids, leukotrienes and epoxides (reviewed by Smith, 1989)

Tsai *et al.* (1990) have recently partially purified a $p21^{ras}$ GTPase inhibiting protein (*ras* GIP) which is stimulated *in vitro* by DG. The result of activating GIP would be to inhibit the GTPase activity of $p21^{ras}$ thereby increasing the amount of active, GTP-bound, $p21^{ras}$. Since $p21^{ras}$.GTP represents a potent mitogenic signal and constitutively active $p21^{ras}$.GTP a transforming signal, these observations raise the intruiging possibility that DG may act as a second messenger regulating the activation of $p21^{ras}$.

7.2.2 A role for PLD and PtdOH in mitogenic signal transduction.

Activation of PLD appears to be a common response to a variety of mitogenic and non-mitogenic stimuli, but may not always function as a pathway for DG formation (Huang & Cabot, 1990b; Chapters 4 and 6) suggesting that PtdOH may serve other distinct functions. For example the studies presented in this thesis indicate that PLD activity may account for less than half of the sustained phase of bombesin-stimulated DG formation whereas the small increase in DG stimulated by EGF seems not to be produced by a PLD/PPH pathway even though PLD is clearly activated. What then is the purpose of PLD activation in these circumstances and what, if any, might be the role of PtdOH ?

Various possible roles have been proposed for PtdOH. Reports of a Ca^{2+}-ionophore function (Putney *et al.*, 1980) have remained unsubstantiated but in neutrophils the kinetics of PtdOH formation, produced largely by a PLD pathway (Cockcroft, 1984; Billah *et al.*, 1989b), correlate with enzyme secretion (Cockcroft, 1984) suggesting a role for PtdOH in membrane fusion and secretion

events. Certainly the membrane fusogenic properties of PtdOH and DG are well documented (reviewed by Wakelam, 1988).

The exogenous addition of PtdOH and particularly its lyso- derivative, lyso-PtdOH, to fibroblast and non-fibroblast cells results in the hydrolysis of polyphosphoinositides, activation of PKC, inhibition of adenylyl cyclase and stimulation of DNA synthesis (Moolenaar *et al.*, 1986; Van Corven *et al.*, 1989; Murayama & Ui, 1987) suggesting that the two agents may act as growth factors. It seems likely that lyso-PtdOH is more potent than PtdOH and that some of the reported effects of PtdOH may be due to contamination of commercial preparations with lyso-PtdOH (Jalink *et al.*, 1990). Whether PtdOH and lyso-PtdOH produced by intracellular signal pathways actually leave the cell membrane and act as auto- and para-crine mitogens remains unclear; such speculation will await the identification of receptors for these molecules.

In the context of mitogenic messenger properties PtdOH and arachidonic acid are both able to inhibit $p21^{ras}$GTPase activating protein (*ras* GAP) (Tsai *et al.*, 1989) and stimulate *ras* GIP (Tsai *et al.*, 1990) *in vitro*. As described previously, the net effect of both of these events, should they occur *in vivo*, would be to greatly increase the amount of $p21^{ras}$ in its active, GTP-bound state. Both PDGF and EGF have been shown to activate *ras* by increasing the ratio of $p21^{ras}$GTP/GDP (Satoh *et al.*, 1990, a & b). Since EGF and PDGF are also able to activate PLD it is tempting to speculate that the PtdOH/DG produced may be involved in the activation of *ras* by virtue of their effects upon GAP and GIP. However, it seems likely that the ability of EGF and PDGF to phoshorylate GAP upon tyrosine residues (Molloy *et al.*, 1989; Kaplan *et al.*, 1989; Ellis *et al.*, 1990) will also play a major role though what effect this phosphorylation has upon GAP function is not known.

Finally there are some recent reports that PtdOH may actually activate kinases in a manner analagous to DG. Epand & Stanford (1990) have shown that PtdOH will support a PKC activity which is inhibited by Ca^{2+} in a dose-dependent manner. In addition a preliminary report from Bocckino *et al.* (1990) suggests that

PtdOH can stimulate a distinct pattern of protein phosphorylation from that induced by DG in an *in vitro* assay using rat liver cytosol as a source of kinase activity. Thus, it seems likely that PtdOH may serve a role as a second messenger in its own right with perhaps diverse intracellular and extracellular targets, as well as a precursor of DG, and further research into its possible funtions is clearly warranted.

7.3 Suggestions for further work arising from the studies presented in this thesis.

In this chapter a number of ideas have been presented for further work arising out of some of the observations made in this study. These have mostly been speculative, concerning the possible roles of PtdCho-derived DG and PtdOH in mitogenic signal transduction. However, there still remain a number of points raised by this thesis concerning pathways of DG and PtdOH formation which should be addressed and will hopefully yield much information.

Analysis of PtdOH formation in mitogen-stimulated Swiss 3T3 cells.

The formation of [^{3}H]PtdBut serves as a definitive assay for PLD activity since [^{3}H]PtdOH formed in cells labelled with [^{3}H]palmitic acid could also be due to the action of DG kinase. However, in the light of the possible roles for PtdOH as a second messenger a thorough characterisation of the kinetics and mass of PtdOH formation is clearly required. This is particularly important because the transferase assay may give a false indication of the duration of PLD activity since it is not a substrate for PPH. Mass analysis of PtdOH should be possible by assaying for phosphorus, using malachite green, after the PtdOH has been resolved by t.l.c. or h.p.l.c. and digested, or some variation of g.l.c. mass spectrometry should allow assessment of mass changes in the level of PtdOH as well as molecular species analysis. Alternatively, Huang & Cabot (1990b) have recently shown that labelling of REF52 fibroblasts with 1-*O*-[^{3}H]hexadecyl-2-lyso-*sn*-glycero-3-

phosphocholine can be employed to specifically label the cellular PtdCho pool and have demonstrated vasopressin-stimulated PLD activity in cells labelled in this manner. This may prove a better method of detecting increases in isotopically-labelled PtdOH. Finally, the formation of [^{32}P]PtdOH in cells labelled with ^{32}Pi may not just represent DG kinase acting on the DG derived from PtdIns(4,5)P_2 hydrolysis. It now seems likely in a number of sytems that at least some of this may be due to PLD catalysed PtdCho hydrolysis. Formation of [^{32}P]PtdOH may prove a useful assay for PLD since the use of butan-1-ol should allow assessment of how much [^{32}P]PtdOH is derived by the DG kinase pathway and how much by PLD.

Mass analysis of Cho and ChoP formation and assessment of PtdCho-PLC activity.

The observation that in bombesin-stimulated Swiss 3T3 cells a PLD/PPH pathway apparently accounts for only 30% of the sustained phase of DG formation must be addressed. Mass analysis of Cho and ChoP formation should provide two pieces of information. First, it will allow a comparison with the mass of of DG and Ins(1,4,5)P_3 formed, to address which is quantitatively the major pathway for DG formation. Second, it should provide a more sensitive assay for ChoP and allow an assessment of whether increases in ChoP mass can be accounted for solely by choline kinase activity or whether PtdCho-PLC activity is taking place. PtdCho-PLC activity can be identified by developing a labelling protocol similar to that of Billah *et al.* (1989b) to label PtdCho with ^{32}P without labelling the ATP pool. The studies of Huang & Cabot (1990b) suggest that this should be possible in fibroblasts and formation of [^{32}P]ChoP under these conditions will serve as a definitive assay for PtdCho-PLC just as the formation of [^{32}P]PtdOH does for PLD.

The possibility that either PtdIns(4,5)P_2 or PtdIns hydrolysis contributes to the sustained phase of DG formation should be addressed. The continued accumulation of [^{3}H]InsP_t in the presence of LiCl suggests that inositol lipid hydrolysis is continuing by some route, albeit at a reduced rate. Furthermore the observation that in parallel incubations bombesin-stimulated DG formation was

always greater than PMA suggests that bombesin can draw upon a source of DG which PMA is unable to utilise. The inability of PMA to stimulate accumulation of inositol phophates suggests that this source may be an inositol lipid.

Regulation of PLD activity in Swiss 3T3 cells.

The results presented in this thesis on the role of $[Ca^{2+}]_o$ and Ca^{2+} entry in regulating PLD are preliminary since they do not address the role of intracellular stored Ca^{2+}. They do however point the way to further studies since they indicate a role for Ca^{2+} in PLD activity. It must not be assumed that this role will be an active, stimulatory, role but may rather, *in vivo*, be a role as an essential co-factor supporting PLD activity; *in vitro* it may well prove possible to stimulate PLD activity with, for example, millimolar concentrations of Ca^{2+} but this will provide little information concerning the *in vivo* regulation of PLD by Ca^{2+}. A thorough assessment of the role of Ca^{2+} is clearly warranted and will require the use of BAPTA or thapsigargin and the development of a cell-free or membrane system.

The speculation that DG may activate PLD at the level of the enzyme itself is warranted by a number of investigations reviewed by Billah & Anthes (1990). In addition some of the apparently artefactual results obtained with chronic pre-treatment of Swiss 3T3 cells with PMA (Chapter 6) suggest that PLD might also be down-regulated by PMA pre-treatment. A role for DG/phorbol ester in activating PLD directly can only satisfactorily be resolved by studying PLD activity in membranes or partially pure enzyme preparations which exhibit no PKC activity or under conditions which do not support PKC activity (e.g. no ATP). An alternative will be to compare the time course for down-regulation of PKC activity with that of PLD activity in response to treatment with PMA. If the loss of PLD activity simply parallels the loss of PKC activity then this would suggest that PLD activity requires functional PKC activity. Alternatively, if PLD is down-regulated before PKC this might point towards the two enzymes being independently modulated by PMA.

Purification, cloning and expression of mammalian PLD and PPH.

With the increasing evidence of a role for PtdCho-PLD in signal

transduction the purification of the enzyme from a mammalian source is of great importance. Once purified and sequenced the cloning of PLD may well reveal a family of enzymes analagous to the diverse forms of PIC. Once the enzyme is cloned over-expression studies should allow assessment of whether base exchange and PLD activities reside in the same enzyme and indeed whether the hydrolytic and transferase activities of PLD are solely attributable to the same protein.

Furthermore, since there is evidence of a role for a PLD/PPH pathway in DG formation a thorough charcterization of PPH is clearly required; ideally this will involve the purification of the enzyme and ultimately its cloning. This will be of particular interest in terms of the regulation of DG levels since it catalyses the reverse reaction to DG kinase and is therefore a likely point of control for either removal or formation of DG. As such the interplay between these two activities may be crucial in regulating second messenger supply (DG or PtdOH). PPH has largely been studied in liver (Martin *et al.*, 1987) where it is of importance in the synthesis of triglycerides (Brindley, 1984); the results in this thesis, taken together with those of a number of other groups working in similar systems (reviewed by Billah & Anthes, 1990) suggest that both PLD and PPH should now be considered as important enzymes in signal transduction.

Generation of specific neutralizing anti-sera to PLD and PPH may allow micro-injection studies to assess their role in mitogenic signalling in much the same way that the Y13-259 antibody has been used to identify the requirement for $p21^{ras}$ in serum-stimulated DNA synthesis (Mulcahy *et al.*, 1985). Such approaches may lead to a better understanding of the role of stimulated phospholipid metabolism and DG formation in the transition from quiescence into the cell cycle.

Appendix I.

Protocol for assay of $Ins(1,4,5)P_3$ mass.

Samples prepared as described in the Methods section (Chapter 2, 2.5.2) were assayed for $Ins(1,4,5)P_3$ mass by the competitive binding assay of Palmer *et al.* (1989). The assay employs a bovine adrenocortical microsomal preparation which possesses a single population of specific high affinity binding sites for $Ins(1,4,5)P_3$.

Aliquots of adrenal cortex preparation (0.5-1mg of protein) were incubated with an aliquot of Swiss 3T3 cell extract or $Ins(1,4,5)P_3$ standard in 25mM Tris (pH 9), 1mM EDTA, 5mM $NaHCO_3$, 0.25mM dithiothreitol and 1mg ml^{-1} BSA (Fraction V). Incubations were performed on ice in a final volume of 100µl with $[^3H]Ins(1,4,5)P_3$ (approx. 3000 c.p.m. = 52 fmoles = 0.52 nM). Non-specific binding was determined in the presence of 940nM $Ins(1,4,5)P_3$. Incubations were terminated by centrifugation at 12000 r.p.m. in a bench top centrifuge for 3 min at 4^oC and the supernatant was removed by aspiration. The radioactivity bound to the pellet was determined by liquid scintillation counting after solubilisation in Hi-Safe scintillant cocktail. The use of a standard curve of displacement of $[^3H]Ins(1,4,5)P_3$ by unlabelled $Ins(1,4,5)P_3$ with each assay allowed estimation of $Ins(1,4,5)P_3$ mass in cell extracts.

Appendix II.

Publications arising from the work presented in this thesis.

[1] Analysis of the water soluble metabolites of phosphatidylcholine breakdown by ion-exchange chromatography; bombesin and TPA (12-*O*-tetradecanoylphorbol-13-acetate) stimulate choline generation in Swiss 3T3 cells by a common mechanism.
Simon J. Cook & Michael J.O. Wakelam. (1989) *Biochem. J.* **263**, 581-587.

[2] Mass measurement of inositol-1,4,5-trisphosphate and *sn*-1,2-diacylglycerol in bombesin-stimulated Swiss 3T3 mouse fibroblasts.
Simon J. Cook, Susan Palmer, Robin Plevin & Michael J.O. Wakelam. (1990) *Biochem. J.* **265**, 617-620.

[3] Bombesin and platelet-derived growth factor stimulate phosphatidylcholine breakdown by a common mechanism.
Simon J. Cook, Robin Plevin, Susan Palmer & Michael J. O Wakelam. (1990) *Biochem. Soc. Trans.* **18**, 484-485.

[4] Epidermal growth factor stimulates phospholipase D activity in the absence of inositol lipid hydrolysis in Swiss 3T3 cells.
Simon J. Cook & Michael J.O. Wakelam. (1991) *Biochem. Soc. Trans.* **19**, 108S.

[5] Hydrolysis of phosphatidylcholine by phospholipase D is a common response to mitogens which stimulate inositol lipid hydrolysis in Swiss 3T3 cells.
Simon J. Cook & Michael J.O. Wakelam (1991) *Biochim. Biophys. Acta.* in press.

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