The regulation of mitogen-stimulated phospholipase D activity in Swiss 3T3 fibroblasts

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This thesis is dedicated to the memory of my Grandfather, Ronald Moore.

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Abbreviations

AVP	[Arginine^8] Vasopressin.
ATP	Adenosine 5'-triphosphate.
BSA	Bovine serum albumin (fraction V).
β-AR	β-adrenergic receptor.
β-ARK	β-adrenoreceptor kinase
cyclic AMP	adenosine 3',5' cyclic-mono-phosphate.
$[Ca^{2+}]_{i/o}$	Calcium concentration (intra or extracellular).
ChoP	Phosphocholine.
DAG	*sn* - 1,2-diacylglycerol.
DMBGH	DMEM containing 20mM hepes pH 7.4, 1% (w/v) BSA and 10mM glucose.
DMEM	Dulbeccos' modified Eagles' medium.
DMSO	Dimethylsulphoxide
EGF	Epidermal growth factor.
EGTA	Ethylene glycol-bis (β-amino-ethyl ether) N, N, N', N' - tetra acetic acid.
fMLP	fMetLeuPhe..
GroPCho	Glycerophosphocholine.
HHBG	Hanks' buffered saline containing 20mM hepes pH 7.4, 1% (w/v) BSA and 10mM glucose.
Hepes	(N-[2-Hydroxyethyl]piperazine-N'-[2-etanesulphonic acid]).
$Ins(1,4)P_2$	D-*myo* - inositol-1,4-bisphosphate.
$Ins(1,4,5)P_3$	D-*myo* - inositol(1,4,5)trisphosphate.
$Ins(1,3,4,5)P_4$	D-*myo* - inositol(1,3,4,5)tetrakisphosphate.

LysoPtdOH	Lysophosphatidic acid.
PAP	Phosphatidate phosphohydrolase.
PCA	Perchloric acid.
PDGF	Platelet-derived growth factor.
PtdIns-PLC	Phosphoinositidase C (PtdIns(4,5)P_2 phosphodiesterase
PKA	cyclic AMP-dependent kinase.
PKC	Protein kinase C.
PLA_2	Phospholipase A_2.
PLC	Phospholipase C.
PLD	Phospholipase D.
PMA	Phorbol-12-myristate-13-acetate.
PtdBut	Phosphatidylbutanol.
PtdCho	Phosphatidylcholine.
PtdEtn	Phosphatidylethanolamine.
PtdIns	Phosphatidylinositol.
PtdIns(4)P	Phosphatidylinositol-4-phosphate.
PtdIns(4,5)P_2	Phosphatidylinositol-4,5-bisphosphate.
PtdOH	Phosphatidic acid.
PtdSer	Phosphatidylserine.
SphM	Sphingomyelin.
TBST	Tris buffered saline containing 0.05% Tween 20.
TCA	Trichloroacetic acid.
t.l.c.	Thin layer chromatography.

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Summary

The work in this thesis aimed to characterise the regulation of phospholipase D (PLD) activity by receptor occupancy in Swiss 3T3 fibroblasts.

Bombesin-stimulated PLD activity had previously been found to be partially dependent on protein kinase C (PKC) suggesting that activation of PLD was to some extent down stream of the G-protein coupled receptor activated phosphatidylinositol 4,5, bis phosphate (PtdIns(4,5)P_2) hydrolysis (Cook *et al.*, 1992). Using a permeabilised cell system to introduce non-hydrolysable analogues of guanine-nucleotides, bombesin-stimulated PLD activity was found to be indirectly regulated by a G-protein.

The phosphorylation of proteins on tyrosine was determined using immunoblotting with a monoclonal anti-phosphotyrosine antibody. The elevation of tyrosine phosphorylation using pervanadate, an inhibitor of tyrosine phosphatases, was found to activate PLD activity. PLD activity was found to be reduced by pretreatment with the tyrosine kinase inhibitor Genistein. Pretreatment with both Genistein and the selective PKC inhibitor Ro-31-8220 reduced bombesin-stimulated PLD activity to basal.

Elevation of Ca^{2+} from intracellular pools using thapsigargin, an inhibitor of the Ca^{2+}-ATPase on the intracellular stores was found to be insufficient to activate PLD alone nor did it affect bombesin-stimulated PLD activity. Elevation of cyclic AMP using forskolin and dibutyrylcyclicAMP did not affect bombesin-stimulated PLD activity nor did it alone affect the enzyme.

A common event in cells exposed to agonist is a loss of responsiveness to the stimulant, referred to as desensitisation. Bombesin-stimulated PLD activity was found to desensitise rapidly with a complete loss in the response after a 40 second agonist exposure. Desensitisation was reversible and occurred within 4.5 minutes of agonist removal, however was never complete. In the continuous presence of agonist resensitisation of PLD activity occurred. The resensitised rate was reduced from that generated in response to the initial stimulus but activity continued over a period of an hour. The

recovery of bombesin-stimulated total inositol phosphate generation commenced later than that of bombesin-stimulated PLD activation and reached completion within 11.5 minutes after removal of the desensitising stimulus.

Radio-receptor binding studies showed that there was a transient loss of binding sites from the cell surface which was due to internalisation and recycling of the receptor. Although the rate of receptor loss and recovery paralleled that of the desensitisation and resensitisation of PLD activity, differences were observed in the extent of the two processes. Readdition of bombesin was essential for a stimulation of PLD activity to be observed on resensitisation of the enzyme. Hence degradation of the ligand was occurring before the receptor was recycled.

Desensitisation and resensitisation was independent of PKC activation and a desensitising pretreatment with bombesin did not affect the PMA- stimulated PLD activity. A23187-stimulated PLD activity also underwent homologous desensitisation and could not stimulate enzyme activity after prior desensitisation by bombesin pretreatment. Thus desensitisation was not due to a decrease in intracellular Ca^{2+} levels.

Bombesin also induced heterologous desensitisation. Pretreatment with bombesin or vasopressin reduced a subsequent stimulation with the other agonist by approximately 70%. A desensitising pretreatment with either agonist attenuated the GTPγS-stimulated PLD activity in permeabilised cells by approximately 50%. It was thus proposed that desensitisation not only affected the cell surface receptors but also occurred at or distal to the receptor-coupled G-protein.

These findings support a role for PLD-derived PtdOH in mitogenic signalling, in view of the resensitisation and subsequent maintenance of PLD activity in an early mitogenic situation when the agonist is continually present. The kinetics of the agonist-stimulated PLD activity are discussed with relation to the regulatory pathways of PLD activation and mitogenic signalling.

Chapter 1

Introduction

Introduction

The binding of hormones and growth factors to specific receptors on the plasma membrane of cells initiates a cascade of signalling pathways which are involved in a wide variety of physiological and pathological responses including the propagation of the signal into the nucleus. The tight control of the cell cycle is crucial for the regulation of growth and proliferation. Loss of coordination of the cell cycle gives rise to uncontrolled cell proliferation and cancer. Research has focussed on early events in signalling since initiation of DNA synthesis generally occurs 10-15 hours after exposure to mitogens. The identification of second messengers pathways, their regulation and interaction, is thus paramount for full understanding of cell biology.

Several signalling transduction processes exist in cells involving: receptor-linked cyclic AMP generation; tyrosine phosphorylation; ion-channel modification and phospholipid hydrolysis. These systems may regulate each other through their interaction in a process known as 'cross-talk'.

The work in this thesis has focussed on receptors coupled to the phospholipase-catalysed hydrolysis of phospholipids. Phospholipase C-stimulated breakdown of phosphoinositides is known to be required for a variety of cellular functions including cell proliferation, secretion, smooth muscle contraction and neuronal activity. However, it is now clear that the stimulation of phospholipase D catalysed hydrolysis of phosphatidylcholine (PtdCho) is commonly observed in response to a wide range of growth factors and may thus be of significant relevance to cellular physiology.

1.1 Phosphoinositide specific -phospholipase C.

1.1.1 Agonist-stimulated phosphoinositide metabolism.

Phosphoinositides constitute approximately 10% of the total eukaryotic membrane phospholipids (Michell 1975). The majority of the inositol containing phospholipids are in the form of phosphatidylinositol (PtdIns), phosphatidylinositol 4-monophosphate (PtdIns(4)P) and phosphatidyl 4,5-bisphosphate (PtdIns(4,5)P_2). The first evidence of agonist-stimulated inositol phospholipid metabolism arose from the finding that acetylcholine stimulated the incorporation of ^{32}P into the phospholipids of pancreatic slices (Hokin and Hokin, 1953). The incorporation of radiolabel into phosphoinositides was later found to be a common response to agonist-stimulation and was proposed to be due to a phospholipase C-catalysed hydrolysis of PtdIns, generating diacylglycerol and the inositol phosphate head group (Michell, 1975; Downes, 1982: Hokin, 1985). However, it was subsequently realised that PtdIns(4,5)P_2 was actually the initial substrate for phospholipase C (PLC) (Michell and Kirk, 1981; Kirk *et al.*, 1981; Creba *et al.*, 1983).

Recently a series of 3-phosphorylated phospholipids were found to be generated in response to growth factors such as PDGF in Balb/C3T3 fibroblasts and NGF in PC12 cells (Carter and Downes, 1992) suggesting a role for these molecules in mitogenesis and neuronal development (reviewed Downes and Carter 1991). The reaction was found to be catalysed by a PtdIns-3-kinase which has been isolated from a number of sources including bovine brain (Otsu *et al.*, 1991). The *in vivo* substrate for PtdIns-3-kinase has been shown to be PtdIns(4,5)P_2 which is phosphorylated to PtdIns(3,4,5)P_3 (Traynor-Kaplan *et al.*, 1988; Stephens *et al.*, 1991; Irvine, 1992). PtdIns(3,4,5)P_3 is thought to be subsequently metabolised to produce PtdIns(3,4)P_2 and PtdIns3P.

PtdIns-3-kinase contains the *src* homology domains SH2 and SH3 (Skolnik *et al.*, 1991). *Src* homology regions resemble the conserved, non-catalytic regions in cytoplasmic tyrosine kinases such as the *src* gene product and are found in many

proteins involved in signalling pathways (reviewed Pawson and Gish, 1992). Phosphotyrosine contained within a specific amino acid sequence is recognised by SH2 domains (Cantley *et al.*, 1991), whilst SH3 regions have been proposed to target molecules to cytoskeletal components. Although the function of the PtdIns-3-kinase pathway is unclear the presence of such domains in PtdIns-3-kinase suggests that the enzyme may be involved in second-messenger generation through the activation of tyrosine kinases.

1.1.2 Identification of phosphoinositide specific- phospholipase C.

Allan and Michell (1974) identified a neutral phospholipase C which catalysed the hydrolysis of PtdIns (PtdIns-PLC) producing cyclic and non-cyclic phosphates. The enzyme was reported to be cytosolic, requiring micromolar Ca^{2+} for maximal activity. This was followed by the discovery of a neutral membrane associated PtdIns-PLC activity in rat brain (Oran *et al.*, 1975). However, the first identification of heterogeneity in PtdIns-PLC arose from the purification of two immunologically and functionally distinct enzymes from sheep seminal vesicles (Hofmann and Majerus, 1982). Despite the use of PtdIns to analyse purification products most PtdIns-PLCs were subsequently found to preferentially hydrolyse polyphosphoinositides at physiological Ca^{2+} concentrations, such as the isoform purified from bovine brain (Katan and Parker, 1987) and that from turkey erythrocytes (Morris *et al.*, 1990a,b). This was consistent with the discovery of receptor-mediated PtdIns(4,5)P_2 breakdown

1.1.3 Structure and classification of PtdIns-PLC isoforms.

A total of 16 distinct PtdIns-PLCs have been identified, consisting of 14 mammalian and 2 *Drosophila* enzymes (reviewed Rhee and Choi, 1992). The PtdIns-PLC family have been divided into three sub-families based on amino acid similarity, PLC-β, PLC-γ and PLC-δ, with more variants within each family. Although PLCδ has been purified and cloned, its regulation is unknown.

There are two regions of sequence common to all three enzyme families which are designated the X and Y regions. These domains have been proposed to constitute the catalytic region of the molecule. PLCβ1 and δ1 contain a short region of 50-70 highly charged amino acids between the X and Y domains which may interact with other proteins. In PLCγ1 this intervening region comprises of 400 amino acids which contain SH2 and SH3 domains, implying a role in tyrosine kinase-induced signalling pathways.

The PtdIns-PLC isoforms are regulated to varying extents by Ca^{2+} which is thought to interact with residues in the C-terminal domain of the Y-region. The different isoenzymes all hydrolyse PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, but display distinct substrate specificities *in vitro* , with selectivity for the preferred substrate PtdIns(4,5)P_2 decreasing in the order PLC-β1 > PLC-δ1 > PLC-γ1 (Ryu et al., 1987).

1.1.4 G-protein coupled regulation of receptor activated PLCβ.

Evidence for the regulation of signal transduction pathways by guanine nucleotides was first presented for the glucagon-sensitive adenylyl cyclase system in the plasma membrane of the rat liver (Rodbell *et al.*, 1971a,b). Guanyl nucleotides were found to be obligatory for the activation of adenylyl cyclase, they decreased the affinity of glucagon binding sites and inhibited the fluoride-stimulated enzyme response. GTP binding proteins (G-proteins) are now known to control a variety signal transduction processes including receptor-coupled PLCβ activation and the regulation of ion channels (reviewed Simon *et al.*, 1991; Saverese and Fraser, 1992).

(i) G-protein coupled receptors

The secondary and tertiary structure of G-protein coupled receptors was modelled predominantly on that of the bacteriorhodopsin protein which consists of seven transmembrane helices (Henderson *et al.*, 1990). Studies on the structure of the β_2-adrenergic receptor (β_2-AR) used site-directed anti-peptide antibodies to

define its topography (Wang *et al.*, 1989). Immunofluorescence of intact and permeabilised CHO cells transfected with the cDNA for the β_2-AR supported the existence of a seven transmembrane structure, with an extracellular amino-terminus and cytoplasmic C-terminal tail. The transmembrane domains show significant homology between most receptor types.

(ii) Receptor-coupled G-protein activation.

The activated, ligand bound receptor, binds to the G-protein and elicits a conformational change promoting GDP/GTP exchange (reviewed Conklin and Bourne, 1992). The activated receptor has a high affinity for the heterotrimeric G-protein in a conformation where GDP is absent and the guanine nucleotide binding site on the α-subunit is open (Chabre *et al.*, 1988) Subsequent association of GTP with the α-subunit promotes dissociation of the heterotrimer from the receptor with release of the activated α and the $\beta\gamma$ subunits. Hydrolysis of GTP to GDP by the intrinsic GTPase of the α-subunit (Godchaux and Zimmerman, 1979) increase its affinity for $\beta\gamma$ and allows reassociation of the $\beta\gamma$ subunits and termination of the signal.

Mutagenesis studies have attempted to determine the structure-function relationship between G-protein coupled receptors (reviewed Savarese and Fraser, 1992). The N and C-terminal regions of the third intracellular loop were found to be the major interaction sites with G-proteins, though the C-terminal regions of the tail and second intracellular loop were also possibly involved (Conklin and Bourne, 1993).

(iii) Classification of Gα subunits.

G-protein sub-classes are defined by the amino acid sequence similarity of their α-subunits. Structural division resulted in four classes G_s, G_i/G_o, G_q and G_{12}. Within each class, there are sub-classes which are highly conserved in amino acid

sequence. The use of bacterial toxins has enabled further functional assignment to G-proteins in cells.

Pertussis toxin catalyses the ADP-ribosylation of the G_i/G_o class of α subunits on a C-terminal cysteine, thereby uncoupling the receptor/G-protein interaction and inhibiting signal transduction. The G_i family consists of $G\alpha_o$, $G\alpha_i$, $G\alpha_z$ and $G\alpha_t$ and several variants arising from differential splicing. Whereas G_i lowers cyclic AMP levels, both G_i and G_o have been found to modulate ion channels (Brown and Birnbaumer, 1990; Yatani *et al.*, 1990) and G_t functions to activate cyclic GMP phosphodiesterase in the visual transduction system (reviewed Hamm *et al.*, 1990). The functions of $G\alpha_z$ and that of the G_{12} remain undefined.

Cholera toxin catalyses the ADP ribosylation of an N-terminal arginine in the G_s group of G-proteins leading to constitutive activation of G_s-effector signals. The Gs class of G-proteins activate adenylyl cyclase to increase intracellular cyclic AMP levels and also regulate Ca^{2+} channels (Yatani *et al.*, 1988).

The G_q and G_{12} class of G-proteins lack the C-terminal cysteine and are hence insensitive to pertussis toxin. Partial purification and reconstitution studies (Taylor *et al.*, 1991, Smrcka *et al.*, 1991) showed that the pertussis toxin insensitive G-protein that activated PLCβ1 was similar in amino acid sequence to purified $G\alpha_q$ (Pang and Sternweiss, 1990). Other members of the G_q class, $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$ may also interact with PLC enzymes (reviewed Majerus, 1992). In support of this proposal, membrane extracts from Sf9 insect cells expressing $G_{16}\alpha$ were found to activate PLCβ1, PLCβ2 and PLCβ3 in the presence of GTPγS with similar effectiveness to $G_q\alpha$ (Kozasa *et al.*, 1993). In view of the differences between $G_q\alpha$ and $G_{16}\alpha$ in sequence and distribution it was suggested that the two $G\alpha$ subunits may interact with different receptors or effector systems.

iv) Regulation of PtdIns-PLCβ1 by $G\alpha_q$ subunits.

In 1983, Gomperts proposed that G-proteins were involved in the activation of PtdIns-PLC, from the observation that guanine nucleotides promoted histamine

secretion in mast cells on addition of Ca^{2+}. Many agonists have been shown to activate PtdIns-PLC through a pertussis-toxin insensitive G-protein including vasopressin in vascular smooth muscle cells (Socorro *et al.*, 1990) and bombesin in Swiss 3T3 fibroblasts (Plevin *et al.*, 1990). Bombesin is a tetradecapeptide originally isolated from frog skin (Anastasi *et al.*, 1971). More than twenty peptides structurally related to bombesin have been reported but there are only three related mammalian peptides, gastrin-releasing peptide (GRP) and neuromedins B and C. Bombesin-like peptides have a variety of pharmacological and physiological effects in mammals such as secretion of gut hormones, the regulation of smooth muscle contraction and the regulation of homeostasis. However the finding of a mammalian bombesin in human lung small-cell carcinoma (SCCL) and in SCCL cell lines (Erisman et al., 1982; Moody et al., 1981) suggested that it may act as an autocrine growth factor. Furthermore bombesin has been reported to exert mitogenic effects when added to SCCL cell lines (Cuttita *et al.*, 1985), human breast cancer cells (Nelson, *et al.*, 1991) and Swiss 3T3 fibroblasts (Rozengurt and Sinnett-Smith, 1983). Bombesin-binds to high affinity receptors on Swiss 3T3 cells (Zachary and Rozengurt, 1985) which have been purified and cloned (Feldman *et al.*, 1990; Spindel *et al.*, 1990; Battey.*et al.*, 1991) and found to contain seven transmembrane helices, characteristic of G-protein coupled receptors.

However, it was not until the development of exogenous substrate systems that the relationship between inositol phospholipid breakdown and G-proteins could be fully characterised. In 1991 Taylor *et al.* purified a G-protein belonging to the G_q family of G-proteins from bovine liver membranes. The G-protein was found to activate partially purified PLCβ1 but not the γ1 or δ1 isozymes. A similar conclusion was reached by Smrcka *et al.* (1991) who used an *in vitro* assay to demonstrate that a purified Gq preparation reconstituted a partially purified PLCβ1 activity. In support of these findings, agonist-stimulated PtdIns(4,5)P_2 hydrolysis in several cell lines was attenuated by an antibody raised to the common carboxyl terminal of the α subunits of G_q and G_{11} (Gutowski *et al.*, 1991). Furthermore,

transient transfection of the cDNA to $G\alpha_q$ and $G_{\alpha}11$ together with PLCβ1 was found to increase the generation of inositol phosphates in the presence of GTPγS, whilst other Gα subunits were without effect (Wu *et al.*, 1992).

Activation of PLCβ1 by agonist-stimulated muscarinic receptors, was recently reported to act as a GTPase-activating protein (GAP) specifically for $G\alpha_{q/11}$ (Berstein *et al.*, 1992). This implied that PLCβ1 accelerated the termination of its own activation and thus enhanced the control of G-protein linked signalling.

Both cytosolic and membrane associated forms of PLCβ have been reported to be regulated by guanine nucleotides. Taylor *et al.* (1991) showed that PLCβ1 purified from bovine brain cytosol stimulated PtdIns(4,5)P_2 hydrolysis when reconstituted with a purified activated Gα subunit from bovine liver membranes. However in turkey erythrocytes where the majority of PLC is cytosolic, the remaining PtdIns-PLC present after preparation of ghosts was found to be associated with the cytoskeleton and was sufficient to account for all the receptor/G -protein coupled activity (Vaziri and Downes, 1992). It has been proposed therefore that membrane association of PLCβ may be important in growth-factor stimulated PtdIns(4,5)P_2 hydrolysis.

(v) The regulation of PLCβ isoforms by βγ subunits.

Not all receptor coupled PtdIns-PLC activity is pertussis-toxin insensitive. For example, stimulation of PtdIns(4,5)P_2 hydrolysis in HL 60 cells by the chemotactic peptide formylmethionine-leucine-phenylalanine (fMLP) was inhibited by the toxin (Paris and Pouyssegur, 1987) implying that G_o or G_i may interact with PtdIns-PLC. Though direct evidence for this is lacking, it has been shown that purified Go and G_i could reconsitute the generation of inositol phosphates in membranes from differentiated HL60 cells (Kikuchi *et al.*, 1986).

Camps *et al.* (1992) reported that $\beta\gamma_t$ subunits purified from bovine retinal transducin activated one of the two unidentified PtdIns-PLC enzymes present in soluble fractions from HL-60 granulocytes. $\beta\gamma_t$-stimulation of PtdIns(4,5)P_2

hydrolysis was additive to that activated by GTPγS and was proposed to occur by interaction of the βγ subunits with the enzyme itself or a regulatory component apart from G-protein α-subunits. Further studies on co-transfected COS-7 cells showed that βγ-subunits stimulated PLCβ2 activity but not the β1 isoform (Katz *et al.*, 1992), whereas PLCβ3 and PLCβ2 catalysed PtdIns(4,5)P_2 hydrolysis was activated on the addition of βγ subunits to reconstituted lipid vesicles (Park *et al* ., 1993). Moreover, cotransfection of the M2 muscarinic receptor with PLCβ2 in COS-7 cells elicited the generation of inositol phosphates in response to carbachol which could be inhibited by pertussis toxin (Katz *et al.*, 1992). These reports led to suggestions that βγ subunits released from G_i/G_o may account for pertussis-toxin sensitive PtdIns-PLC activity.

The βγ complex have been found to modulate various other components of the signal transduction system in their own right (reviewed Clapham and Neer, 1993) including adenylyl cyclase (Tang and Gilman, 1992), phospholipase A_2 (Jelsema and Axelrod, 1987), ion channels (Logothetis *et al.*, 1988) and the β-adrenergic receptor kinase, which is involved in phosphorylation and desensitisation of the β-adrenergic receptor (Kim *et al.*, 1993).

(vi) The regulation of PLCβ–catalysed PtdIns(4,5)P_2 hydrolysis by PKC.

Pretreatment of many cells with PMA attenuates agonist-stimulated PtdIns(4,5)P_2 hydrolysis (Brown *et al.*, 1987; Lo and Hughes, 1988; Bishop *et al.*, 1990) though the mechanism of uncoupling is unclear. PMA treatment of PC12 and NIH 3T3 cells was reported to stimulate the phosphorylation on serine residues of PLCβ (Ryu *et al.*, 1990). However, proteolytically activated rat brain PKCβ catalysed the phosphorylation of bovine brain PLCβ *in vitro* but did not alter its activity, suggesting that such a modification may alter the interaction of PLCβ with the G-protein. The PKC-catalysed phosphorylation of PLCβ1 was therefore suggested to explain the PMA-induced attenuation of agonist-stimulated generation of inositol phosphates in some cells. A similar mechanism was proposed to account

for the observation that overexpression of PKCβ1 attenuated the GTPγS-stimulated hydrolysis of PtdIns(4,5)P_2 in permeabilised Rat-1 fibroblasts (Pachter *et al.*, 1992). However, this proposed mechanism for PMA-mediated inhibition of receptor-activated PLCβ does not hold for cells such as Swiss 3T3 fibroblasts in which PKCα is the predominant isoform and PKCβ is absent (Olivier and Parker, 1992). PMA-induced inhibition of agonist-stimulated Ins(1,4,5)P_3 production in Swiss 3T3 cells appears to occur at the level of the receptor/G-protein interaction (Plevin *et al.*, 1990).

1.1.5 Regulation of PLCγ

(i) Regulation by receptors possessing tyrosine kinase activity.

Receptors possessing tyrosine kinase activity stimulate the hydrolysis of PtdIns(4,5)P_2 through activation of PLCγ. PDGF and EGF were found to increase the phosphorylation of PLCγ on tyrosine residues and serine residues whereas the PLCβ1 and PLCδ forms were unaffected (Meisenhelder *et al.*, 1989). Furthermore, the tyrosine phosphorylation of PLC-γ has been shown to coincide temporally with turnover of inositol phosphates (Todderud *et al.*, 1990). Mutant receptors that lacked intrinsic tyrosine kinase activity were defective in stimulating PtdIns(4,5)P_2 hydrolysis suggesting that activation of PLCγ was dependent on the enzyme activity of the receptor (Margolis *et al.*, 1990).

Purified EGF and PDGF receptors phosphorylated PLCγ *in vitro* on tyrosine residues 771, 783, 1254, the same sites that are modified in cells (Meisenhelder *et al.*, 1989; Kim *et al.*, 1990; Wahl *et al.*, 1990). Stimulation of PtdIns(4,5)P_2 hydrolysis by the EGF receptor tyrosine kinase *in vitro* or in A-431 cells suggested that tyrosine phosphorylation increased the catalytic activity of PLCγ (Nishibe *et al.*, 1991). Moreover, treatment of EGF-activated PLCγ with a tyrosine phosphatase decreased the catalytic activity of the enzyme. Tyrosine phosphorylation of PLCγ is thus considered to be the mechanism by which receptors

such as that of PDGF and EGF (such as in A431 cells) (Meisenhelder *et al.*, 1989), stimulate PtdIns(4,5)P_2 hydrolysis. Furthermore, both receptors have been reported to physically associate with PLCγ such that they were co-precipitated with an antibody to PLCγ or receptor (Wahl *et al.*, 1988; Nishibe *et al.*, 1990).

The role of tyrosine phosphorylation in the PDGF stimulation of PLCγ activity was investigated by over expression of NIH 3T3 cells with mutant enzyme, in which phenylalanine was exchanged for tyrosine at each of the phosphorylation sites (Kim *et al.*, 1991). Mutation at tyrosine 783 abolished the PDGF-stimulated PLCγ activity, whereas the enzyme activity was inhibited by 40% when phenylalanine was present at Tyr 1254 and enhanced by 50% when Tyr 771 was mutated. This report indicated that phosphorylation at Tyr 783 was essential to activation of PDGF-stimulated PLCγ activity and that the enzyme was that responsible for PDGF-mediated PtdIns(4,5)P_2 hydrolysis.

In unstimulated cells most of the PLC-γ is present in the cytosol. Upon EGF receptor activation several residues in the C-terminal domain of the receptor become phosphorylated upon tyrosine (Carpenter and Cohen, 1990) and PLCγ translocates to the membrane (Todderud *et al.*, 1990). This presumably occurs through the recognition of the tyrosine phosphorylated proteins on the carboxyl terminus of the receptor by SH2 domains of PLCγ. Such an event would generate the immunoprecipitable complex reported by Nishibe *et al.* (1990) and lead to phosphorylation of PLCγ tyrosine residues. This proposal is supported by the finding that proteins containing SH2 domains, bound in a tyrosine phosphorylation dependent manner to a truncated C-terminal region of the EGF receptor containing all known tyrosine autophosphorylation sites (Margolis *et al.*, 1990). Following tyrosine phosphorylation of PLC-γ the enzyme is thought to be released from the receptor. A model has been suggested whereby an intramolecular reaction allowing the SH2 domains to bind to the tyrosine phosphorylated residues in PLCγ elicits a conformational change permitting a SH3/cytoskeleton interaction (Rhee, 1991). Hence, the catalytic sites of the enzyme would be exposed to its substrate

$PtdIns(4,5)P_2$. Although there is no evidence for such an intramolecular reaction, this proposal is partially supported by the reports that the SH3 domain of proteins are responsible for their targetting to the cytoskeleton (Bar-Sagi *et al.*, 1993).

(ii) Activation of PLC-γ by nonreceptor tyrosine kinases.

Activation and tyrosine phosphorylation of PLC-γ also occurs through receptors which regulate cytosolic tyrosine kinases, for example in CD3 activated T cells (Park *et al.*, 1991) and anti-IgG activated B lymphocytes (reviewed Cushley and Harnett, 1993). The sites of tyrosine phosphorylation in PLCγ in activated T-cells are identical to those phosphorylated in PDGF or EGF-treated cells. Recent reports have shown that stimulation of several G-protein-coupled receptors which activate cytosolic tyrosine kinases can also stimulate PLC-γ activity. Thrombin -stimulated phosphoinositide metabolism in human platelets was inhibited by tyrphostins suggesting regulation of $PtdIns(4,5)P_2$ breakdown by PLCγ activation (Guinebault *et al.*, 1993). Furthermore, immunoprecipitation of carbachol-stimulation of CHO cells transfected with muscarinic receptors showed that PLCγ was phosphorylated upon tyrosine in a Ca^{2+} dependent manner (Gusovsky *et al.*, 1993). Carbachol-stimulated $Ins(1,4,5)P_3$ generation was partially inhibited by a tyrosine kinase inhibitor or removal of extracellular Ca^{2+}. This suggested that muscarinic-stimulated tyrosine phosphorylation of PLCγ was involved in $PtdIns(4,5)P_2$ hydrolysis and was due to activation of Ca^{2+}-sensitive tyrosine kinases.

Although the mechanism of PLC-γ activation by cytosolic tyrosine kinases is unclear, regulation may involve the *src* family of protein tyrosine kinases. This proposal is supported by the finding that immunoprecipitated *src* related kinases were found to phosphorylate both PLC-γ1 and PLC-γ2 (Liao *et al.*, 1993).

1.2 Phosphatidylcholine specific phospholipase D.

Phosphatidylcholine (PtdCho) is the major phospholipid in mammalian tissues, accounting for approximately 50% of the total phospholipid. The hydrolysis of PtdCho occurs predominantly through the action of three phospholipases (Fig.1.1): phospholipase A_2 (PLA_2), generating a fatty acid (generally arachidonic acid) and lysophosphatidyl choline; phospholipase C, to form DAG and phosphatidylcholine; phospholipase D, which generates phosphatidic acid (PtdOH) and choline.

1.2.1 Identification of phospholipase D

Phospholipase D catalyses a reaction where water acts as a nucleophilic acceptor for a putative enzyme/substrate intermediate (Fig 1.2):

phosphatidyl base A + H_20 $\leftrightarrow$ phosphatidic acid+ base A

Using the generation of PtdOH from exogenenous PtdCho to monitor the hydrolytic activity of PLD, the enzyme was originally identified in plants (Hanahan and Chaikoff, 1947) and later in fungi and bacteria (reviewed Heller, 1978). A partially pure PLD from cabbage was characterised by incubation with egg PtdCho dispersions (Dawson and Hemington, 1967). The PLD-catalysed production of PtdOH was optimal at pH 5.4 and was stimulated by Ca^{2+} and ether. However, inhibition by p-chloromercuribenzoate, suggested the presence of an essential sulphydryl group. A later purification of cabbage PLD demonstrated that the enzyme activity was inhibited at high ionic strength and the pH optimum of PtOH generation from exogenous PtdCho depended on the concentration of Ca^{2+} (Allgyer and Wells, 1979). At 0.5mM Ca^{2+} the pH optimum was 7.25 but at 50mM Ca^{2+} it was 6.

Figure 1.1 Sites of action of phospholipases A_2, C and D on phosphatidylcholine.

The figure shows the phosphatidylcholine molecule with the ester-linked fatty acid groups in the *sn*- 1 and *sn*- 2 position represented by R1 and R2 respectively.

Phospholipase A_2 catalyses the hydrolysis of the ester bond at the sn-2 position of the molecule to generate a free fatty acid, typically arachidonic acid and 1-acyl-2-lyso-PtdCho.

Phospholipase C catalyses the cleavage of the phospho-ester linkage between the DAG moiety and phosphocholine (PCho) group thereby producing *sn* -1,2-DAG and PCho.

Phospholipase D catalyses the hydrolysis of the phosphoester bond between the choline head group and the PtdOH moiety to generate PtdOH and choline.

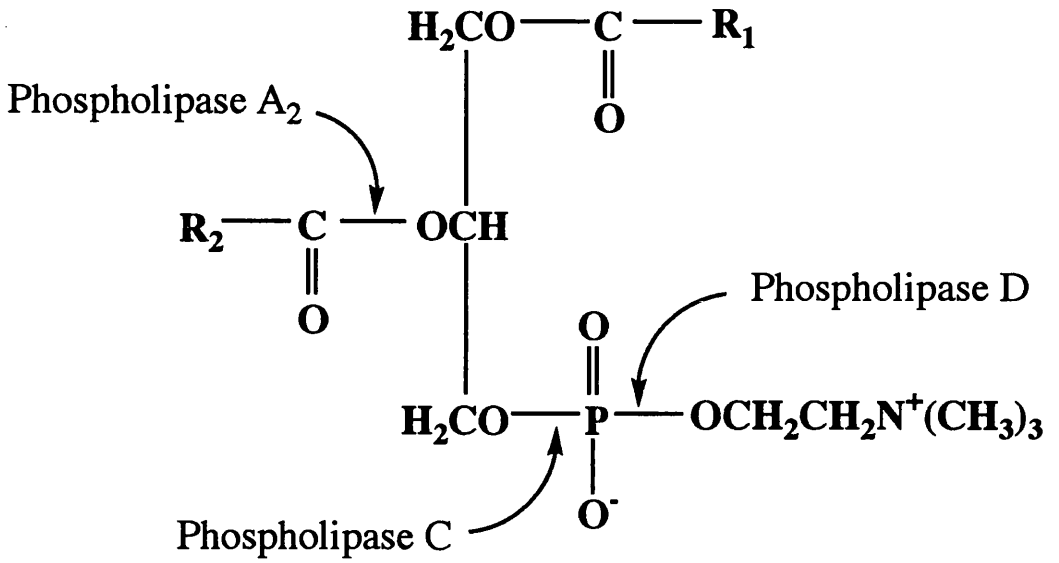
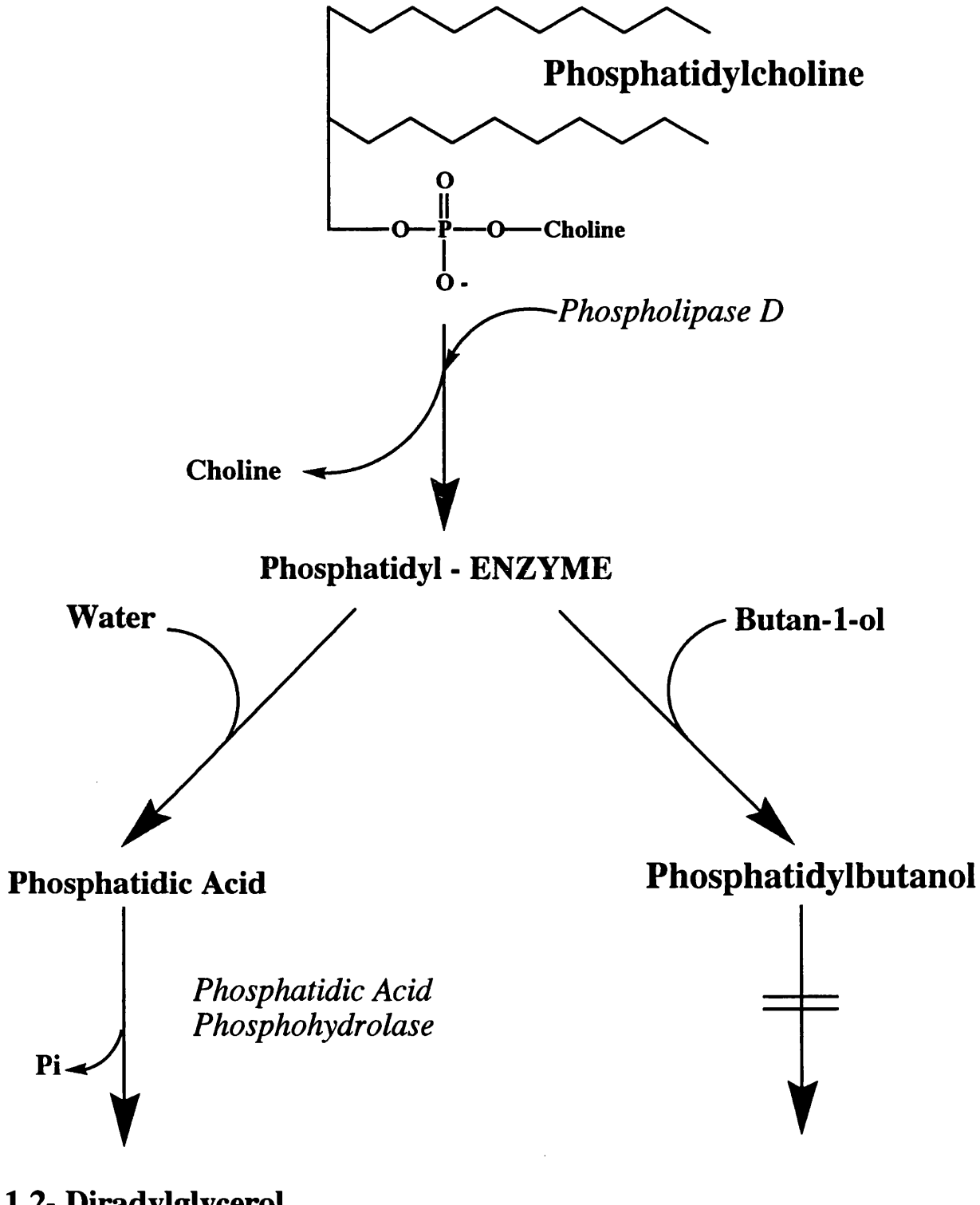

H2CO—C—R1
O
Phospholipase A2
R2—C—OCH
O
Phospholipase D
O
H2CO—P—OCH2CH2N+(CH3)3
O-
Phospholipase C

Figure 1.2 The hydrolytic and transphosphatidylation activities of phospholipase D

The schematic diagram shown opposite represents a proposed mechanism for PLD-catalysed PtdCho hydrolysis. Activation of the enzyme results in release of the head group and the formation of a phosphatidyl-PLD intermediate. In the presence of water as a nucleophilic acceptor for the phosphatidyl moiety (left-hand side of diagram) the hydrolytic activity of PLD generates PtdOH which may then be metabolised to DAG by phosphatidate phosphohydrolase (PAP). However, in the presence of short chain primary alcohols such as butanol which act as a stronger nucleophilic acceptor than water (right-hand side of diagram), the intrinsic transphosphatidylation activity of PLD generates a phosphatidylalcohol at the expense of PtdOH.

The transphosphatidylation activity of PLD forms the basis of an assay for the enzyme activity in whole cells. As phosphatidylalcohol can not be metabolised by the cells it accumulates and can be quantified. Furthermore, as phosphatidylalcohol is a poor substrate for PAP the relative contribution of the stimulated PLD pathway to the generation of DAG can be assessed.


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

PLD also catalyses a transphosphatidylation reaction where in the presence of a preferential nucleophilic acceptor such as a short chain alcohol a phosphatidylation of the alcohol occurs at the expense of PtdOH (Fig. 1.2).

phosphatidylbase A + alcohol ↔ phophatidylalcohol+ base A

The transphosphatidylation activity of PLD was first demonstrated using the cabbage enzyme. In the presence of exogenous PtdCho and glycerol or ethanol, the enzyme generated Ptdglycerol or Ptdethanol (PtdEtOH) and PtdOH (Hemington and Dawson, 1967; Yang *et al.*, 1967). The PLD-catalysed transphosphatidylation occurred simulataneously with the hydrolytic activity and was similarly activated by Ca^{2+}.

It was not until 1975 that Saito and Kanfer reported a mammalian PLD activity in a detergent solubilised preparation from particulate rat brain fraction. Using the generation of radiolabelled PtdOH and choline from a [U-^{14}C]PtdCho microdispersion as an assay for enzyme activity, the K_m was 8.3 x 10^{-4} M. The pH optimum for PtdCho hydrolysis was reported to be 6 and that for transphosphatidylation was 7.2. Ca^{2+} was not essential but maximum stimulation was achieved at a concentration of 5mM and Mg^{2+} could replace Ca^{2+}, whereas blockage of -SH groups was inhibitory. Partially purified rat brain PLD catalysed the hydrolysis of exogenous PtdCho with a K_m of 0.75mM and an optimum pH of 6 (Taki and Kanfer, 1979). The calculated molecular weight was 200 kDa, similar to that estimated for peanut seed PLD (Heller *et al.*, 1974). In an early study by Kater *et al.* (1976) PLD isolated from human eosinophils had an apparent molecular weight of 60,000Da and generated choline from exogenously added PtdCho at a pH optimum of 4.5-6. A later study on rat brain microsomal PLD activity showed that the production of [^{3}H]choline from exogenous [^{3}H]PtdCho and the production of [^{3}H]Ptdglycerol monitored by introduction of [^{3}H]glycerol into the microsomal membrane, was highly dependent on bile acids such as deoxycholate and free

unsaturated fatty acids (Chalifour and Kanfer, 1982). Oleate was reported to be the best *in vitro* activator. The concentration of oleate required for maximal enzyme activity present in a rat brain synaptosomal enriched preparation was found to vary with the concentration of exogenous PtdCho as assessed by the accumulation of [^{14}C]PtdEtOH (Kobayashi and Kanfer, 1987).

Martin (1988), identified a neutral PLD activity in endothelial cells which exhibited strict specificity for PtdCho. The production of [^{3}H]choline and [^{3}H]PtdOH from exogenous 1-oleoyl-2-[^{3}H]oleoyl-PtdCho and Ptd[^{3}H]choline catalysed by the enzyme was dependent on the presence of Triton-X-100 and was inhibited by Mg^{2+} and Ca^{2+}. Chalifa *et al.*, 1990 also partially characterised a neutral PLD activity from rat brain synaptic plasma membranes. Oleate was found to be essential for the production of [^{3}H]PtdOH and [^{3}H]PtdEtOH from [^{3}H]PtdCho in the presence or absence of ethanol and Mg^{2+} also strongly stimulated PLD activity. Triton -X-100 stimulated the PLD activity but with a much lower specific activity than that for oleate. Ca^{2+} up to 0.25mM activated the enzyme but was inhibitory at 2mM. The authors proposed that the failure of earlier studies to observe a neutral PLD activity in rat brain microsomes was due to the use of a buffer system that was inhibitory to the neutral enzyme.

The existence of a cytosolic PLD activity that constituted the majority of the PLD activity in bovine tissues was reported by Wang *et al.* (1991). Using exogenous [^{14}C]PtdCho as a substrate in the presence or absence of ethanol to detect both the hydrolytic and transphosphatidylation activities of PLD, the cytosolic PLD activity was only detected in the presence of 160μM PtdCho. This was a concentration 10-fold greater than that needed to detect membrane bound activity. The K_m for cytosolic PLD activity was 1.45mM whereas the membrane bound activity could only be detected at substrate concentrations of less than 32μM. Furthermore, whereas the membrane-bound enzyme was specific for PtdCho, the cytosolic PLD was more active against PtdEtn than PtdCho. Ca^{2+} was not

essential for either enzyme activity but maximal stimulation was observed at 10mM, whereas Mg^{2+} inhibited the membrane bound but not the cytosolic enzyme activity.

Further characterisation of the rat brain microsomal enzyme was achieved using the generation of [^{3}H]PtdBut from enzyme incubated in the presence of [^{3}H]butanol and exogenously added PtdCho (Horwitz and Davis, 1993). As this method obviated the need for a labelled substrate the phospholipid specificity of the enzyme was investigated. Although a small activity was detected against PtdEtn and PtdSer as substrates, the enzyme preparation hydrolysed PtdCho most efficiently with an EC_{50} of 0.4mM. Furthermore the substrate specificity was independent of fatty acid composition. Full characterisation of mammalian PLD activity awaits complete purification.

1.2.2 PLD and base-exchange activites.

It is possible that the formation of phosphatidylalcohol is derived from a base exchange reaction between the choline head group and alcohol. Base-exchange reactions exchange the bases on phospholipids with free bases such as serine, ethanolamine and choline as follows:

phosphatidyl base A + base B ↔ phosphatidylbase B + base A

The base exchange reactions were found to closely resemble transphosphatidylation activity in plant PLD (Yang *et al.*, 1967). However later investigations compared the phosphohydrolase and base exchange activity of a cabbage PLD preparation (Saito *et al.*, 1974). Base-exchange activities, detected by incorporation of labeled ethanolamine and choline into phospholipids were found to have a pH optimum of 9 whereas that for the formation of PtdOH was 5.6. The two activites could also be distinguished by their Ca^{2+} requirements, the base exchange reaction was optimal at a concentration of 4mM Ca^{2+}, whereas the phosphohydrolase activity required concentrations of Ca^{2+} greater than 28mM for optimal activity. However, though

the activities could be clearly differentiated the study could not resolve whether separate proteins were responsible.

Such reactions have also been shown to occur in several tissues such as brain (Porcellati *et al.*, 1971). The partial purification of the rat brain enzyme suggested that the base exchange enzymes were distinct from PLD (Taki and Kanfer, 1979). In support of this, a partially purified phospholipid serine base-exchange enzyme from rat brain did not exhibit any PLD activity (Taki and Kanfer, 1978). Furthermore the properties of this enzyme were distinct from that of the partially pure rat brain PLD (Taki and Kanfer, 1979) as L-serine incorporation into the most effective acceptor, ethanolamine phospholipid, had a K_m of 0.4mM and an optimum pH of 8. Furthermore, maximal activity was observed at 10mM Ca^{2+}. However, in bovine-retinal microsomes, PLD-like activity was suggested to play a role in Ca^{2+}-dependent base incorporation as the characteristics of both base incorporation and PtdOH generation were similar (Mori *et al.*, 1989). The pH optimum for both the amount of base incorporated and the formation of PtdOH was 8-8.5 whereas the optimum concentration of Ca^{2+} was 3mM for both activities. Hence it appears the relationship between PLD and base exchange may be tissue or species specific and a contribution of base exchange to phosphatidylalcohol formation can not entirely be eliminated.

1.2.3 The identification of cellular PtdCho-specific PLD activity.

Evidence for the cellular breakdown of PtdCho arose from a study in 1981 in which phorbol esters were found to stimulate the release of [^{3}H]choline and [^{3}H]phosphocholine from cells prelabelled with [^{3}H]choline (Mufson *et al.*, 1981). The first report of an agonist-stimulated turnover of PtdCho came from experiments with muscarinic agonists in perfused hearts *in vitro* and *in vivo* in rat cortex (Corradetti *et al.*, 1982; Corradetti *et al.*, 1983). Further evidence for agonist-stimulated PtdCho hydrolysis has arisen from studies in intact cells using fatty acids such as [^{3}H]myristate in rat embryo fibroblast cells (Cabot *et al.*, 1988) or labelled

phospholipids such as [^{3}H]lysophosphatidylcholine in neutrophils (Domino *et al.*, 1989; Billah *et al.*, 1989) to selectively label PtdCho. Stimulation of labelled cells resulted in sustained [^{3}H]DAG or [^{3}H]PtdOH, supporting the theory of growth factor stimulated phospholipase-catalysed PtdCho hydrolysis.

However, the differentiation between PLD and PLC-catalysed hydrolysis of PtdCho has necessitated the use of several techniques. Double-labelling of cells, with [^{32}P] and [^{3}H] in the acyl chain enabled the relative contribution of PLD and PLC to PtdCho hydrolysis to be determined in fMLP-stimulated neutrophils (Billah *et al.*, 1989). [^{3}H]PtdOH would be generated both by the PLD and PLC pathway (from metabolism of [^{3}H]DAG by DAG kinase), whereas [^{32}P]PtdOH would be formed from the PLD pathway only. The relationship between the DAG kinase pathway and PLD-catalysed PtdCho hydrolysis to PtdOH generation could therefore be assessed by comparing the ^{3}H/^{32}P ratios of PtdCho and PtdOH. It was found that in neutrophils at early time points, the ratio was identical to that of PtdCho suggesting that PtdOH was primarily generated through PLD-catalysed PtdCho hydrolysis. At later time points the ^{3}H/^{32}P ratio was higher than that of PtdOH, suggesting a PLC-catalysed hydrolysis of PtdCho and subsequent conversion of DAG to PtdOH.

Cells have also been labelled with lyso[^{32}P]PtdCho such that there was no labelling of the cellular ATP pool. Any [^{32}P]PtdOH generated upon agonist-stimulation thus unequivocally demonstrated PLD-catalysed hydrolysis of PtdCho. Such strategies were used to define fMLP, phorbol ester and Ca^{2+}-ionophore stimulated PLD activity in neutrophils (Billah *et al.*, 1989).

Activation of PLD by agonists has definitively been assessed utilising the transphosphatidylation property of the enzyme to accumulate phosphatidylalcohols following cell stimulation in the presence of short chain alcohols such as ethanol or butanol (Fig. 1.2). As phosphatidylalcohols are poor substrates for phosphatidate phosphohydrolase (PAP) (Metz and Dunlop, 1991), they accumulate in the cell at the expense of PtdOH formation and can thus be quantified. The accumulation of

[^{3}H]Ptdbutanol ([^{3}H]PtdBut) was detected in unlabelled neutrophils stimulated with fMLP in the presence of [^{3}H]butanol (Randall *et al.*, 1990). Alternatively stimulation of cells labelled in PtdCho with fatty acids such as [^{3}H]palmitate, in the presence of unlabelled butanol has also been used to determine activation of PLD by quantification of the [^{3}H]PtdBut generated . The latter technique has been used to definitively assess the stimulation of PLD activity in many cell types including EGF, PDGF, bombesin and vasopressin stimulated Swiss 3T3 cells (Cook, 1991; Cook *et al.*, 1992; Cook and Wakelam, 1992), α-thrombin-, ATP- and bradykinin stimulated-endothelial cells (Martin and Michaelis, 1989) and vasopressin-stimulated A10 vascular smooth muscle cells (Plevin *et al.*, 1992).

1.2.4 The regulation of cellular PLD activity.

Many agonists that activate PLD activity also promote PtdIns(4,5)P_2 hydrolysis such as observed for bombesin-stimulated Swiss 3T3 cells (Cook *et al.*, 1992) and fMLP-stimulated HL60 cells (Geny and Cockcroft, 1992) implying a possible interaction between the two pathways. The modulatory effect of the second-messengers arising from inositol phospholipid hydrolysis appears to vary between cell type, perhaps reflecting different isoforms of PLD.

As agonist-stimulated PtdIns-PLC-catalysed PtdIns(4,5)P_2 hydrolysis is regulated through a G-protein, the possible regulation of PLD activity by such a pathway has also been investigated. The addition of non-hydrolysable GTP to permeabilised cells (Xie and Dubyak, 1991; Geny and Cockcroft, 1992) and cell membranes (Hurst *et al.*, 1990; Van der Meulen and Haslam, 1990) have shown that receptors can regulate PLD activity through the activation of G-proteins. The use of cell-free systems from neutrophils and granulocytes (Olson *et al.*, 1991; Anthes *et al.*, 1991) established that stimulation of PLD activity with GTPγS required protein factors in both the plasma membrane and the cytosol. Although G-protein regulated PLD activity has been reported in a variety of cell types, whether this requirement for protein factors is a general phenomenon has yet to be fully investigated.

It appears that the sub-type of G-protein involved in the regulation of PLD activity can vary which may relate to activation of PLD as an event downstream of receptor-stimulated Ptd(4.5)P_2 hydrolysis. This is exemplified by the reports that fMLP-stimulated PLD activity in granulocytes was found to be pertussis toxin sensitive (Agwu *et al.*, 1989), whereas that in prostaglandin treated erytroleukemia cells (Wu *et al.*, 1992), vasopressin-stimulated rat hepatocytes (Bocckino *et al.*, 1987), and A10 aortic smooth muscle (Grillone *et al.*, 1988) was insensitive.

The nature of G-protein coupled PLD activity is still unclear. In HL6O cells coupling was proposed to be partly direct, rather than completely downstream of G-protein coupled PtdIns-PLC or kinase activation. This was based on the finding that GTPγS-stimulated PLD activity could be partially dissociated from PtdIns(4,5)P_2 hydrolysis when cellular ATP was depleted (Geny and Cockcroft, 1993). Regulation of stimulated PLD activity in neutrophils was therefore suggested to be under the control of two G-proteins. One G-protein is thought to be coupled to PtdIns(4,5)P_2 hydrolysis and hence regulates PLD activity indirectly, in a Ca^{2+}-dependent manner. The other G-protein is proposed to couple directly to PLD and is thought to regulate Ca^{2+}-independent secretion (Cockcroft, 1992).

Ca^{2+} appears to play an important regulatory role in the stimulation of PLD activity in most cells types irrespective on their requirement for PKC. Depletion of extracellular Ca^{2+} in Swiss 3T3 cells reduced bombesin-stimulated PLD activity by approximately 50% (Cook *et al.*, 1992), though in neutrophils fMLP-stimulated PLD activity was completely attenuated (Billah *et al.*, 1989; Pai *et al.*, 1988). Ca^{2+} was also obligatory for fMLP- and GTPγS-stimulated PLD activity in neutrophil homogenates and permeabilised cells (Olson *et al.*, 1991; Geny and Cockcroft, 1992). However, in rabbit platelet membranes GTPγS-stimulated PLD activity was Ca^{2+} independent, although the presence of Ca^{2+} greatly augmented the response (Van der Meulen and Haslam. 1990). Common to many cell types, the Ca^{2+} ionophore A23187 stimulated PLD activity in Swiss 3T3 fibroblasts (Cook *et al.*, 1992). However, the exact mechanism of this is unclear as the response was only

50% inhibited in the presence of EGTA and the protein kinase C inhibitor Ro-31-8220. .

The hydrolysis of PtdIns(4,5)P_2 is however not obligatory for activation of PLD. PMA-stimulated PLD activity is observed in a wide variety of cell types (reviewed Billah and Anthes, 1990), suggesting the potential for regulation of the enzyme activity by PKC. PKC plays a major role in the bombesin-stimulated PLD activity, since the selective PKC inhibitor Ro-31-8220 attenuated the response by 50-70%. Similarly involvement of PKC in the regulation of stimulated PLD activity has been reported for many cell types for example in α-thrombin stimulated endothelial cells (Garcia *et al.*, 1992) and vasopressin-stimulated Rat-1 fibroblasts (Plevin *et al.*, 1992). Overexpression PKCβ1 in rat fibroblasts was found to upregulate stimulated PLD activity, although PtdIns(4,5)P_2 hydrolysis was attenuated (Pachter *et al.*, 1992). However, in Swiss 3T3 cells overexpressing PKCα , PLD activity was also upregulated, though the fold change in activity upon stimulation remained unaltered (Eldar *et al.*, 1993). This was proposed to be due to an increase in PLD expression leading to a higher level of cellular activity. The authors proposed that the PKC isozyme that regulated PLD activation in Swiss 3T3 cells did not appear to be PKCα but may involve another sub-type.

Whether PKC activates PLD by a direct phosphorylation is unknown, however Conricorde *et al.* (1992) have suggested that PMA-stimulated activation of PLD occurs via a phosphorylation independent mechanism in fibroblast membranes. PMA-stimulated choline release and accumulation of [^{14}C]phosphatidylpropanol from membranes labelled with [^{14}C]myristate was observed in the in absence of exogenous ATP. Furthermore, addition of an ATPase did not affect PMA-stimulated PLD activity although autophosphorylation of PKC was abolished. It was therefore proposed that PKC may bind directly to PLD or alternatively may bind to another membrane protein responsible for activating PLD.

However, agonist-stimulation of PLD activity is not dependent on PKC in all cells. Although PMA can stimulated PLD activity in neutrophils, kinase

inhibitors do not attenuate fMLP-stimulated PLD activity (Geny and Cockcroft, 1992). Furthermore, agonists which do not stimulate the hydrolysis of PtdIns(4,5)P_2 such as EGF in Swiss 3T3 cells (Cook *et al.*, 1992), have been shown to stimulate PLD activity, suggesting that mechanisms other than PKC activation and inositol phospholipid turnover are required.

Recent evidence has emerged showing that receptor tyrosine kinases and receptor-stimulated cytosolic tyrosine kinases may also regulate PLD activity. EGF-stimulated PLD acivity was found to be completely inhibited by incubation with a tyrphostin, whereas inhibition of PKC activity had no effect (Cook and Wakelam, 1992). The use of tyrosine kinase inhibitors such as Genistein, Erbstatin, Lavendstin and ST271 showed that fMLP-stimulated PLD activity was regulated through a tyrosine kinase in neutrophils (Uings *et al.*, 1992) and also in endothelin-stimulated A10 vascular smooth muscle cells (Wilkes *et al.*, 1993). Furthermore, activation of PLD was found to correlate with tyrosine phosphorylation in cells stimulated with pervanadate (Bourgoin and Grinstein, 1992; Dubyak *et al.*, 1993). This treatment artificially elevates the levels of tyrosine phosphorylation by inhibition of tyrosine phosphatases (Fantus *et al.*, 1989).

Bombesin is among several peptide agonists known to induce the rapid tyrosine phosphorylation of a number of proteins in Swiss 3T3 cells (Zachary *et al.*, 1991). The identity of the kinases, or mechanisms involved remains to be clarified. However, several of the proteins phosphorylated by bombesin in Swiss 3T3 fibroblasts may be substrates for *src* or releated kinases such as the 125kDa focal adhesion protein tyrosine kinase p125^{fak} (Schaller et al., 1992) which was found to be activated and localised to focal adhesions in *v-src* transfomed fibroblasts (Zachary and Rozengurt, 1992).

Regulation by the adenylyl-cyclase signalling pathway has been suggested in certain cells. The elevation of cyclic AMP has been reported to inhibit fMLP-stimulated PLD activation in neutrophils, possibly mediating its effect at the G-protein or enzyme itself (Tyagi *et al.*, 1991). However in thrombin-stimulated

endothelial cells PLD activity was augmented by elevation of cyclic AMP (Garcia *et al.*, 1992).

Interplay between the various modulatory pathways has also been shown to occur. In permeabilised HL60 cells GTPγS-stimulated PLD activity was found to be synergistic with that stimulated by PMA or through the tyrosine kinase based pathway (Geny and Cockcroft, 1992; Dubyak *et al.*, 1993). Thus the regulation of PLD activity involves the coordination of separate stimulatory pathways.

1.2.5 Functions of cellular PLD activation.

Inferences on the function of stimulated PLD activity are based on correlation between PtdOH production and other cellular events. The characterisation of fMLP-stimulated PLD activity in the neutrophils has suggested a role for the enzyme in the inflammatory response. Several reports have suggested that PtdOH-derived from PLD-catalysed PtdCho hydrolysis is the important mediator in the respiratory burst, critical for the killing of bacteria in neutrophils. The respiratory burst is characterised by the production of reduced oxygen species such as superoxide and hydrogen peroxide, catalysed by a multi-component electron-transfer complex termed NADPH oxidase. Several studies have shown that stimulated superoxide production is inhibited in the presence of a primary alcohol (Bauldry *et al.*, 1991). Using a permeabilised cell system, Bauldry *et al.* (1992) showed that under conditions where DAG formation was not observed, PtdOH production occurred before or concurrently with NADPH oxidase activity. However at high concentrations of Ca^{2+}, PtdOH formation occurred in the absence of superoxide production. Furthermore, stimulation of cells with GTPγS and Ca^{2+} in the presence of ethanol inhibited PtdOH production but has no effect on oxidase activity. This suggested that superoxide production did not correlate with DAG production and that PtdOH was not the only second-messenger involved in the response.

PLD activation has also been proposed to play a regulatory role in secretion. Agonist-stimulated secretion has been shown to correlate temporally with PLD activation and to be inhibited in the presence of primary alcohols in various cells including: platelets (Benistant and Rubin, 1990), mast cells (Lin *et al.*, 1991) and differentiated HL 60 cells (Stutchfield and Cockcroft, 1993). As previously described (section 1.2.4) a G-protein other than that coupled to receptor activated PtdIns-PLC is thought to control Ca^{2+}-independent secretion by the activation of PLD (Stutchfield and Cockcroft, 1993; Cockcroft, 1992). Evidence for such a mechanism has emerged from studies in various cells including mast cells (Cockcroft *et al.*, 1987) and platelets (Wheeler-Jones, 1992; Coorssen and Haslam, 1993). However PtdOH formation was not an absolute requirement for secretion in HL60 cells, though could provide an initiating signal (Stutchfield and Cockcroft, 1993). As the NADPH oxidase system is partly under the control of small molecular weight G-proteins *rac* and *rho* (Abo *et al.*, 1991), the regulation of secretion by PLD activity was suggested to also involve non- heterotrimeric G-proteins (see section 5.1.2).

The activation of PLD activity by mitogenic agonists has also implied a role for PLD in the control of growth and proliferation. Huang and Cabot (1992) reported that PLD activity in non-transformed fibroblasts was activatable to a lesser extent than in transformed fibroblasts. However, when the cell-free PLD activity of the normal and transformed cells was investigated using an exogenous PtdCho substrate in the presence of ethanol the accumulation of PtdEtOH generated was very similar in both cell types. Furthermore, when the assay was repeated using endogenous membrane-bound substrates the formation of [^{3}H]PtdEtOH in transformed cell homogenates was 4-fold lower than that accumulated from normal cells. It was thus conluded that PLD activity was dependent on the source of the PtdCho substrate rather than the enzyme. As many factors may be involved in comparisons between transformed and non-transformed systems including isoenzyme expression and receptor number a further insight into PLD and

mitogenesis has arisen through the relationship between its product, PtdOH and cell signalling (section 1.4.3 (ii)).

1.3 Phospholipase-catalysed phosphatidylcholine hydrolysis and the source of sustained DAG.

1.3.1 The source of sustained DAG generation.

Agonists known to stimulate the hydrolysis PtdIns(4,5)P_2 were found to generate a biphasic DAG profile with the first transient phase corresponding to the production of Ins(1,4,5)P_3, and a second more sustained phase (Cook et al., 1990). The source of the sustained DAG was initially thought to originate from PtdIns (Griendling *et al.*, 1986). However Bocckino *et al.* (1985), suggested that stimulated DAG generation may arise from other phospholipid sources. This was supported by the reports that growth factors and agonists that did not hydrolyse PtdIns(4,5)P_2, generated DAG in a single sustained phase (Wright *et al.*, 1990).

The suggestion that PtdCho could potentially be the source of DAG arose from early studies with PMA-stimulated myoblasts (Grove and Schimmel, 1982) and in neutrophils stimulated with the chemotactic peptide fMLP (Cockcroft, 1984), in which the fatty acid composition of DAG was found to resemble that of PtdCho. Definitive evidence for PtdCho as the source of the late-phase DAG was achieved through the separation of derivatised DAG from α-thrombin stimulated IIC9 fibroblasts (Pessin and Raben, 1989) and mitogen-stimulated fibroblasts (Pessin *et al.*, 1990) by gas-liquid chromatography on the basis of their fatty acyl composition. Subsequent analysis of the molecular species by mass spectrometry showed that the DAG profile of the initial phase was comparable to that attained from PLC-catalysed inositol phospholipid hydrolysis. At later time points the DAG was found to be derived from PtdCho. The profile of EGF- and PDGF-stimulated generation of DAG was similar though not identical to that of PtdCho (Pessin *et al.*, 1990). This led to the proposal that there could be distinct pools of PtdCho sensitive to hormones

which may differ in their fatty acid composition or DAG metabolism. This work was further supported by the observation that nine species of polyunsaturated DAG were found to be elevated in a time-dependent manner in bombesin-stimulated Swiss 3T3 cells (Pettit and Wakelam, 1993). Comparison of the species with the acyl chain structure of phospholipids showed that the elevation of DAG at early stimulation points could have arisen from the inositol lipids whereas PtdCho could have been the source of DAG at all time points.

1.3.2 The generation of DAG by phospholipase D-catalysed PtdCho hydrolysis.

Growth-factor stimulated DAG generation may be produced from phosphatidate phosphohydrolase (PAP) catalysed dephosphorylation of PtdOH produced from PLD-catalysed PtdCho hydrolysis. Two distinct forms of PAP have been identified in rat liver (Jamal *et al.*, 1991). PAP-1 is a Mg^{2+}-dependent cytosolic enzyme that can reversibly associate with the endoplasmic reticulum and is inhibited by sulphydryl reagents such as NEM (Brindley, 1984). PAP-1 is thought to be involved in glycerolipid synthesis and is activated by translocation to the endoplasmic reticulum (Brindley, 1984; Martin *et al.*, 1986). PAP-2 is an integral plasma membrane protein and therefore proposed to be involved in signal transduction. It can be distinguished from PAP-1 by virtue of its Mg^{2+} independency and lack of inhibition by sulphydryl reagents.

However, evidence is accumulating to show that PLD-catalysed hydrolysis of PtdCho is not primarily responsible for DAG generation in response to agonist in several cell types. For example, in Swiss 3T3 cells the inclusion of butanol inhibited bombesin-stimulated DAG generation by only 30% whereas that formed in response to PMA was diminished by up to 70% (Cook *et al.*, 1992).

1.3.3 The generation of DAG by PtdCho-specific phospholipase C.

Several reports have suggested that PLC-catalysed PtdCho hydrolysis may be responsible for the sustained phase of DAG. Huang and Cabot (1991)

investigated the pathways of PtdCho derived DAG generation in response to PMA in three cell lines of diverse origin. PMA-stimulated PLD activity in all cell types examined. The relationship between a PLC and PLD-mediated production of DAG was determined by comparing the production of [^{3}H]PtdOH and [^{3}H]DAG in the presence or absence of ethanol, in cells labelled with [^{3}H]myristate. In a rat smooth muscle cell line ethanol had no effect on PMA-stimulated [^{3}H]DAG generation although in cells prelabelled with [^{3}H]choline PMA generated both [^{3}H]choline and [^{3}H]PCho. This suggested that both PLC and PLD-catalysed PtdCho hydrolysis was responsible for DAG formation. A similar pathway for DAG generation was reported in PMA-stimulated bovine artery endothelial cells. However in Madine Derby Canine Kidney (MDCK) cells ethanol inhibited PMA-stimulated [^{3}H]DAG suggesting that activation of PLD-stimulated PtdCho hydrolysis indirectly generated DAG. This suggested that PMA-stimulated DAG formation may be produced either by PLC- or PLD-catalysed PtdCho hydrolysis, the relative contribution of each pathway appearing to be cell specific.

However, the existence of PtdCho-specific PLC activity remains a matter for debate. Partial purification of PtdCho hydrolysing PLC activity from dog heart cytosol (Wolf and Gross, 1985), bull seminal plasma (Sheikhnejad, 1986) and promonocytic U937 cells (Clark *et al.*, 1986) has been reported. PLC activity was optimal at neutral pH and was inhibited by Ca^{2+} chelation.

Detection of PLC-catalysed PtdCho hydrolysis has relied upon the production of PCho which is subject to metabolism as the first comitted step in the biosynthesis of PtdCho (section 1.4.4) and could also be produced from choline-kinase catalysed phosphorylation of choline produced by PLD-catalysed PtdCho hydrolysis. PCho production was observed in bombesin and PMA-stimulated Swiss 3T3 cells but always subsequent to activation of PLD (Cook *et al.*, 1989). *Ha-ras* transformed cells displayed an increased level of PCho and DAG suggesting activation of PtdCho-PLC (Lacal *et al.*, 1987;Teegarden *et al.*, 1990). However, this could be explained by increases in activity of choline kinase and CTP

phosphocholine cytidyltransferase, both enzymes involved in resynthesis of PtdCho (section 1.4.4) or by elevation of *de novo* DAG synthesis (Chiarugi *et al.*, 1989). Exogenous addition of PtdCho-PLC purified from *B. cereus* was reported to be mitogenic for Swiss 3T3 cells (Larrodera, 1990). Although the downregulation of PKC by prolonged phorbol ester treatment had no effect on the PtdCho-PLC induced mitogenesis, the finding that PKCζ was not degraded by the phorbol ester treatment led to difficulties in interpreting the PKC dependency of the effect. The physiological relevence of exogenously added PtdCho-PLC is however debatable.

1.3.4 Alternative sources of DAG.

An alternative possibility for the generation of DAG in response to growth factors is synthesis *de novo,* thought to occur at the endoplasmic reticulum (revie ed Bishop and Bell, 1988). PtdOH and DAG generation *de novo* from glycerol in insulin-stimulated BC3H-1 myocytes (Farese *et al.*, 1987) has been reported and from glucose in human neutrophils subsequent to phagocytosis of β-glucan particles (Rossi *et al.*, 1991). However in 3T3-L1 cells insulin was without effect upon DAG mass levels (Merrall *et al.*, 1993).

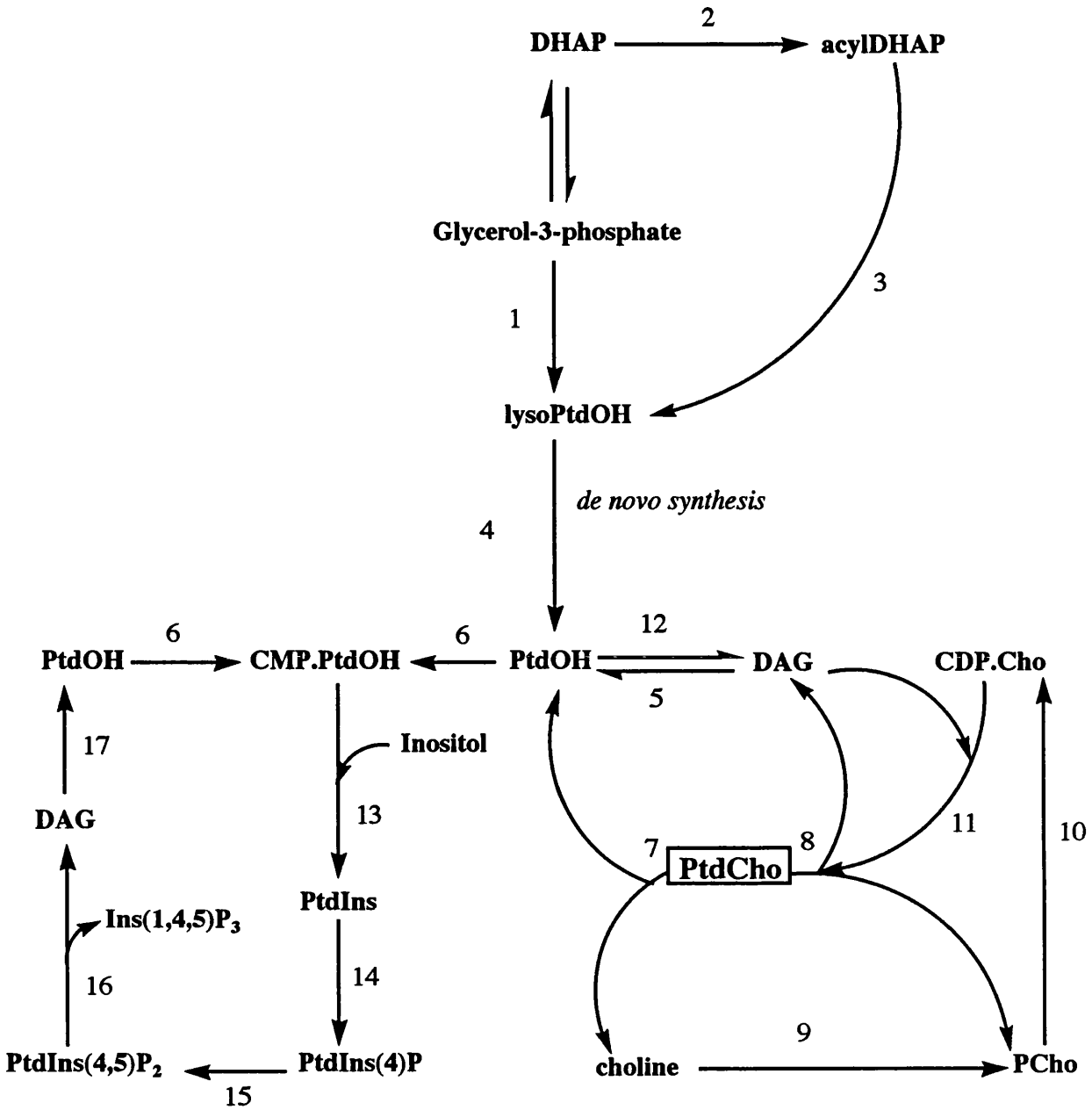
De novo synthesis occurs predominantly through phosphorylation of glycerol to produce glycerol-3-phosphate which can then be acylated to PtdOH (Fig. 1.3). However in some cell types such as adipose with low levels of glycerokinase, lysoPtdOH is formed by acylation of dihydroxyacetone phosphate (DHAP), an intermediate in the glycolytic pathway. LysoPtdOH is then further acylated to produce PtdOH. PtdOH formed *de novo* is dephosphorylated to DAG by PAP-1.

It is also conceivable that the continued hydrolysis of inositol phospholipids observed over at least 30 minutes both in bombesin-stimulated Swiss 3T3 cells (Cook *et al.*, 1991) and vasopressin-stimulated A10 vascular smooth muscle cells (Plevin and Wakelam, 1992) could contribute to the sustained generation of DAG observed in these cells.

Figure 1.3 Summary of phosphatidylcholine metabolising pathways involved in the production of PtdOH and DAG.

The numbered enzymes are:

1. *sn* - glycerol-3-phosphate acyltransferase.
2. dihydroxyacetonephosphate (DHAP) acyltransferase.
3. acyl (alkyl) DHAP oxidoreductase.
4. lysophosphatidic acid (lysoPtdOH) acyltransferase.
5. phosphatidate (PtdOH) phosphohydrolase-2 (PAP-2).
6. PtdOH.cytidyl transferase.
7. phospholipase D (PLD).
8. phosphoinositide specific phospholipase C (PtdIns-PLC).
9. choline kinase.
10. choline phosphate (PCho) cytidyl transferase.
11. diacylglycerol (DAG) choline phosphotransferase.
12 DAG kinase (cytosolic).
13. phosphatidylinositol (PtdIns) synthase.
14. phosphatidylinositol-4-kinase.
15. phosphatidylinositol-4-phosphate (PtdIns(4)P) kinase.
16. phosphatidylinositol specific phospholipase C (PtdIns-PLC).
17. DAG kinase (membrane).


DHAP
2
acylDHAP
Glycerol-3-phosphate
1
3
lysoPtdOH
de novo synthesis
4
PtdOH
6
CMP.PtdOH
6
PtdOH
12
DAG
5
CDP.Cho
17
Inositol
DAG
13
11
10
7
PtdCho
8
PtdIns
Ins(1,4,5)P3
16
14
9
PtdIns(4,5)P2
PtdIns(4)P
15
choline
PCho

1.4 The function and metabolism of products derived from phospholipase-catalysed phospholipid breakdown.

1.4. 1 Ins(1,4,5)P_3 and elevation of intracellular Ca^{2+}.

(i) The Ins(1,4,5)P_3 receptor and mobilisation of Ca^{2+} from intracellular stores.

A relationship between agonist-stimulated inositol phospholipid hydrolysis and the elicited rise in intracellular Ca^{2+} was first proposed by Michell (1975). In 1983 Streb *et al.* found that in permeabilised rat pancreatic acinar cell, Ins(1,4,5)P_3 could mobilise Ca^{2+} from non-mitochondrial stores. Further evidence that Ins(1,4,5)P_3 was a second messenger emerged from studies by Berridge and Irvine (1984), who demonstrated that Ins(1,4,5)P_3 production coincided temporally with Ca^{2+} dependent processes in liver and GH_3 cells. This led to the suggestion that Ins(1,4,5)P_3 may bind to an intracellular membrane site to release Ca^{2+}.The purified Ins(1,4,5)P_3 receptor from rat cerebellar membranes (Supattapone *et al.*, 1988) has been shown to be a protein of molecular weight of 260kDa on SDS-PAGE. However the native molecular weight of the receptor was 1000kDa implying that the protein is a homotetramer. Incorporation of the purified protein into phospholipid vesicles (Ferris *et al.*, 1989) and bilayers (Maeda *et al.*, 1991), showed that the same protein contained the Ins(1,4,5)P_3 binding site and the Ca^{2+} release mechanism and thus was both a receptor and Ca^{2+} channel. The receptor has been shown to be a substrate for PKA, PKC and Ca^{2+}-calmodulin-dependent kinase (Suppatapone *et al.*, 1989; Ferris *et al.*, 1991). Hence phosphorylation of the Ins(1,4,5)P_3 receptor is thought to regulate the release of Ca^{2+} (reviewed Ferris and Snyder, 1992).

There are now known to be at least four members of the Ins(1,4,5)P_3 receptor family, arising from products of different genes with splice site variants (Sudhof *et al.*, 1991; Ross *et al.*, 1992). Analysis of the cloned sequence of the Ins(1,4,5)P_3 receptor (Mignary *et al.*, 1990) has predicted membrane spanning regions localised to the C-terminal end of the molecule, with the N-terminus free in

the cytoplasm. Comparison with the ryanodine receptor, responsible for mobilising stored Ca^{2+} primarily from skeletal-muscle (Takeshima *et al.*, 1989; reviewed Sorrentino and Volpe, 1993), has indicated that the four membrane spanning domains constitute the Ca^{2+} channel. Studies of Ins(1,4,5)P_3 binding to truncated receptors showed that the ligand binding site was located at the N-terminus (Mignery and Sudhof, 1990). Binding of Ins(1,4,5)P_3 to the receptor produced a decrease in apparent molecular weight determined by gel filtration experiments (Mignery and Sudhof, 1990). This suggested that a large conformational change occurred on the interaction of Ins(1,4,5)P_3 with the receptor, consistent with the idea that the binding to the receptor must transmit a signal to the relatively distant Ca^{2+} channel.

Evidence that the Ins(1,4,5)P_3 receptor was localised to the endoplasmic reticulum has arisen from several studies. Subcellular fractionation of pancreatic acinar cells showed that the characteristics of ATP-dependent Ca^{2+} uptake into permeabilised cells and isolated endoplasmic reticulum were similar (Beyerdorffer *et al.*, 1984). Later studies using electron microscopic immunocytochemistry showed that Ins(1,4,5)P_3 receptors in cerebellar Purkinje cells were localised to the endoplasmic reticulum (Ross *et al.*, 1989).

(ii) Metabolism of Ins(1,4,5)P_3 and calcium influx.

In mammalian cells Ins(1,4,5)P_3 is metabolised via a 5-phosphatase to Ins(1,4)P_2 and a 3-kinase to Ins(1,3,4,5)P_4. Ins(1,3,4,5)P_4 is also a substrate for the 5-phosphatase that catalyses the dephosphorylation of Ins(1,4,5)P_3, producing Ins(1,3,4)P_3 (reviewed Erneux and Takazawa, 1991). Since Ins(1,3,4,5)P_4 is made from Ins(1,4,5)P_3 at the expense of ATP it has been proposed that Ins(1,3,4,5)P_4 may itself be a second messenger involved in the control of intracellular Ca^{2+}. However the respective roles of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 and the mechanism behind Ca^{2+} entry remains a matter of controversy (reviewed Irvine, 1991; Berridge, 1993).

1.4.2 DAG and activation of PKC

(i) The protein kinase C family; classification and structure.

In 1977 Inoue *et al.* isolated a histone protein kinase from brain which was activated by limited proteolysis. Further investigations found that it was also activated by Ca^{2+} or phorbol esters in the presence of phospholipids (Takai, *et al.*, 1979; Castagna, 1982). Subsequently these enzymes have been termed "PKC". Molecular cloning has revealed ten members of the PKC family all of which are dependent on phosphatidylserine and diacylglycerol for activation apart from PKCζ (reviewed; Bell and Burns, 1991; Hug and Sarre, 1993). The Ca^{2+}-dependent PKC forms include the α, β1, β2 and γ enzymes. The presence of DAG lowers the Ca^{2+} requirement of these enzymes to the μM range which is consistent with the role of DAG as a second messenger (Lee and Bell, 1991). The Ca^{2+} independent or nPKC isoforms include the δ, ε, ζ, θ, λ and η enzymes.

The cDNA sequence for each PKC enzyme is composed of four conserved domains interspersed with five variable domains (Coussens *et al.*, 1986). The C-terminal region contains the catalytic domain along with the protein substrate and ATP binding sites. The regulatory domain, in the N-terminal region of the molecule (V_1-C_2), contains a cysteine-rich sequence which is thought to form two Zn-finger motifs, commonly found in DNA-binding proteins (Freedman *et al.*, 1988). The generation of deletion mutants enabled the function of the cysteine rich domains to be assigned to the binding of phorbol esters and DAG (Burns and Bell, 1991; Muramatsu *et al.*, 1989). PKCζ only contains one Zn finger and does not bind DAG or phorbol ester (Ono *et al.*, 1989). The flexible hinge region consists of the domain between the regulatory and catalytic domains and is sensitive to proteolysis by the Ca^{2+}-dependent calpain proteases even in the Ca^{2+}-independent PKC isoform, PKCε (Schaap *et al.*, 1990).

The regulatory domain also contains a pseudosubstrate domain, a region homologous to that of the consensus phosphorylation site observed in the substrates of PKC (xRxxS/Tx), except that the serine and threonine residues are changed to non-phosphorylatable alanine residues. This region was proposed to serve as an autoregulatory domain by binding to the catalytic domain of the molecule (House and Kemp, 1987). The lipid activators of PKC, phosphatidylserine (PtdSer) and DAG were found to induce a reversible conformational change in this region of PKCβII thus exposing the pseudosubstrate domain which was subsequently cleaved (Orr *et al.*, 1992). Whether this mechanism of activation holds for the Ca^{2+}-independent isoforms or for activation of the enzyme by other molecules such as fatty acids has yet to be determined.

Binding of Ca^{2+} to the C2 region present in the α,β and γ enzymes induces translocation of PKC to the membrane and hence activation through interaction with PtdSer present in the membrane and DAG. However the mechanism of activation for the nPKC enzymes is unclear.

The role of PKC in cell signalling may thus depend on the cell-specific distribution of the isozymes. Swiss 3T3 fibroblasts contain predominantly PKCα though PKC δ, PKCε and PKCζ are also present (Olivier and Parker, 1992).

(ii) PKC substrates.

Physiological substrates for PKC have mainly been assessed using *in vitro* assays or stimulation *in vivo* with phorbol esters, and appear to fall into three categories. Those substrates involved in signal transduction include the 80 kDa myristoylated alanine-rich C kinase substrate (MARCKS) protein which was reported to be phosphorylated in response to phorbol esters both in brain synaptosomes (Wu *et al.*, 1982) and Swiss 3T3 fibroblasts (Rozengurt *et al.*, 1983). The ubiquitous distribution of the MARCKS protein has led to its frequent use as in assays for PKC activity. PKC activation also mediates negative feedback regulation in second-messenger pathways. Phosphorylation of the EGF receptor in response to

PMA was found to decrease its ligand-induced tyrosine kinase activity and reduce its binding capacity, eventually leading to receptor downregulation (Schlessinger, 1986; Countaway *et al.*, 1990). Furthermore, PKC-catalysed phosphorylation regulates the production of metabolites leading to its activation. This is observed in the PMA-induced inhibition of receptor-mediated hydrolysis of inositol phospholipids, for example in Swiss 3T3 cells (Plevin et al., 1990) (see section 1.1.4(vi)) and the PMA-stimulation of DAG kinase activity (Kanoh *et al.*, 1990) (section 1.4.3(ii)).

PKC substrates also include membrane transport proteins and ion channels. PKC may regulate the $[Ca^{2+}]_i$ of cells by activating the Ca^{2+}-ATPase (Chen *et al.*, 1986) and control intracellular pH by catalysing the phosphorylation of the Na^+/H^+ exchange protein (Moolenaar *et al.*, 1984). PKC also plays an important role in the modulation of neuronal ion-channels, regulating the release of nerotransmitters, thus indicating the importance of the enzyme in behavioural control. In this respect activation of PKC has been reported to have both positive and negative effects on voltage-gated Ca^{2+} channels. For example, in the bag cell neurons of Aplysia, PKC-catalysed phosphorylation was found to enhance the Ca^{2+} influx through voltage-gated Ca^{2+} channels during action potentials (Kaczmarek, 1986), whereas attenuation was observed in *Helix* neurons (Hammond *et al.*, 1987).

The control of nuclear events such as the regulation of transcription factors is also under the influence of PKC-catalysed phosphorylation (reviewed by Meek and Street, 1992). Phorbol esters stimulate the induction of a number of transcription factors, including *c-jun* and *c-fos* (reviewed Borner and Fabbro, 1992), predominantly through interaction with a DNA-motif termed the TPA-responsive element (TRE), present in many TPA-inducible genes.(Boyle *et al.*, 1991). Evidence for a nuclear PKC is increasing and experiments have indicated that PKC-catalyses the phosphorylation and activation of DNA topoisomerase 1, an enyme crucial for efficient gene transcription (Pommier *et al.*, 1990) and also lamin B, involved in disruption of the nuclear envelope during mitosis (Peter *et al.*, 1990).

It is thus clear from the diversity of PKC substrates that enzyme activation plays a pivotol role not only in controlling cellular homeostasis, growth and proliferation but also in neuronal activity.

(iii) Activation of PKC during the sustained phase of DAG generation.

In vitro experiments have shown that most forms of unsaturated DAG species can activate PKC (Go *et al.*, 1987). However other reports *in vivo* have suggested that the second phase of growth factor-stimulated DAG generation in several cell lines does not activate α, β or γ isoforms PKC even in the presence of Ca^{2+} (Martin *et al.*, 1990; Leach *et al.*, 1991). Martin *et al.* (1990) suggested that the loss of activity may have been due to compartmentalisation of the DAG species. However, Ha and Exton (1993) showed that the transient translocation of PKCα and β enzymes in thrombin-stimulated IIC9 fibroblasts was due to the failure of PC hydrolysis to elevate Ca^{2+}. Bombesin-stimulated Swiss 3T3 cells generated time-dependent increase in nine different polyunsaturated DAG species (Pettit and Wakelam, 1993). This suggested that activation of different isoforms of PKC may change over the period of agonist stimulation.

The loss in membrane association of PKC could be attributable to a number of effects. Autophosphorylation is a general phenomenon for all PKC enzymes, usually occurring concomitantly with activation. Autophosphorylation in other protein kinases often modulates activity (Galabru and Hovanessian, 1987), but whether it is important in PKC activation or degradation remains to be clarified (Hug and Sarre, 1993).

Proteolytic cleavage by the calpain enzymes follows enzyme activation (Kishimoto *et al.*, 1983; Saido *et al.*, 1992) and results in a protein containing the regulatory domain and a constitutively active fragment. Whether proteolytic degradation terminates the kinase activity or serves to release the active fragment into the cytosol and other intracellular compartments remains unclear. In support of the latter argument, bombesin stimulation of Swiss 3T3 cells was reported to

translocate native and truncated protein containing the catalytic region of PKC to the nucleus, following turnover of nuclear phosphoinositides (Martelli *et al.*, 1991). However the down regulation of PKC by phorbol ester treatment, reported to be due to an increased rate of degradation (Young *et al.*, 1987), suggests that proteolysis inactivates the kinase activity.

(iv) Activation by fatty acids.

PKC has been reported to be activated *in vitro* by *cis* -unsaturated fatty acids such as arachidonic and oleic acids, both potential products of phospholipase A_2-catalysed phospholipid hydrolysis. Fatty acids have been reported to preferentially activate soluble forms of PKC α and β in the absence of phospholipids (Murakami and Routtenberg, 1986; Khan *et al.*, 1992), although reports vary as to the Ca^{2+} dependency of the fatty acid activation possibly due to the PKC species used and the assay conditions. For example, arachidonic acid-stimulated PKCγ has been reported to be predominantly independent of Ca^{2+} (Sekiguchi *et al.*, 1987) whilst El Touny *et al.* (1990) showed that the presence of Ca^{2+} was essential for the activation of PKC α and β. Furthermore, fatty acids were unable to inhibit phorbol ester binding suggesting that the mechanism by which oleate and arachidonate stimulated PKC activity was distinct from that of PtdSer/DAG (Sharkey and Blumberg, 1985; El Touny *et al.*, 1990). This proposal was supported by reports of synergy between *cis* -unsaturated fatty acids and DAG in the stimulation of PKCα activity (Chen and Murakami, 1992).

Investigations *in vivo* have led to suggestions as to the physiological role of the fatty acids with respect to PKC activation. Whilst oleate induced a translocation of PKC α and β from the cytosol to the membrane in isolated hepatocytes, it was proposed that activation of PKC also involved the increase in $[Ca^{2+}]_i$ elicited by oleate and the DAG synthesised from the fatty acid (Diaz-Guerra *et al.*, 1991) Furthermore Yoshida *et al.*, 1992 reported that in platelets fatty acids acted as enhancers of PKC activity, as phosphorylation of an endogenous PKC substrate was

absolutely dependent on the additional presence of DAG. Although the fatty acids themselves did not increase $[Ca^{2+}]_i$ they were reported to increase the sensitivity of PKC to Ca^{2+} (Yoshida *et al.*, 1992). These findings therefore implicated that the initial activation of PKC arose from PtdIns(4,5)P_2 hydrolysis but activity could remain sustained, despite the decrease in intracellular Ca^{2+}, provided that the levels of DAG and *cis* -unsaturated fatty acids remained elevated.

(v) Activation by other phospholipids.

Other lipid regulators of PKC have been reported including PtdIns(4,5)P_2 (Huang and Huang, 1991; Kochs, 1993), sphingosine / lysosphingolipids (Merrill and Stevens, 1989), phosphatidylcholine (Kaibuchi, *et al.*, 1981; Chen et al., 1992) and lysophosphatidylcholine (Oishi *et al.*, 1988).

(vi) Phorbol-esters and PKC.

Phorbol esters have been shown to provoke a variety of biological effects in cells including tumour promotion (Blumberg, 1980), platelet activation (Zucker *et al.*, 1974), cellular proliferation (Dicker and Rozengurt, 1980) and prostaglandin production (Edwards *et al.*, 1985). These effects are presumed to be mediated by the activation of PKC. PMA activates PKC by interacting with the DAG binding site and dramatically lowering the Ca^{2+} requirement of the enzyme (Castagna *et al.*, 1982).

As previously described prolonged exposure to PMA down-regulates PKC isoforms. However, PMA has a differential effect on PKC isozymes *in vitro* (Huang *et al.*, 1989) and *in vivo* (Olivier and Parker, 1992). In Swiss 3T3 cells PMA causes translocation of PKCα, PKCδ, PKCε, but not PKC ζ (Olivier and Parker, 1992).

1.4 Function and metabolism of phospholipase-derived PtdOH.

The growth factor-stimulated elevation of PtdOH may be derived not only from PLD-catalysed PtdCho hydrolysis, but also from *de novo* synthesis (section 1.3.4) and from metabolism of DAG by DAG kinase.

(i) The production of PtdOH from DAG.

DAG kinase is thought to phosphorylate DAG produced from growth factor activation of PtdIns-PLC hence initiating the resynthesis of phosphatidylinositols (reviewed Bishop and Bell, 1988) and regulating the activation of PKC (reviewed Kanoh *et al.*, 1990). The reaction is thought to be catalysed by a membrane form of DAG kinase specific for arachidonyl containing DAG species which has been identified in several tissues including porcine testis (Hodgkin *et al.*, 1993) and in Swiss 3T3 cells (Macdonald *et al.*, 1988b). However, a cytosolic form of the enzyme was identified in Swiss 3T3 cells (Macdonald *et al.*, 1988a) and subsequently an 80kDa soluble DAG kinase was purified and cloned from porcine thymus (Sakane *et al.*, 1990) which did not display substrate specificity. The enzyme activity was found to be enhanced by μM Ca^{2+} and the protein contained two Zn fingers as found in PKC. Translocation of the 80kDa enzyme from the cytosol to the paticulate fraction of porcine thymus cytosol was detected using antibodies to the enzyme (Sakane *et al.*, 1990). However the effect of translocation on the catalytic activity of DAG kinase is unclear.

DAG kinase was also found to be phosphorylated by PKC and cyclic AMP dependent protein kinase *in vitro*. Although the phosphorylation did not alter the catalytic enzyme activity it did increase the binding to PtdSer vesicles. A recent report showed that DAG kinase was a substrate for PKC α and ε when both were transfected into COS 7 cells (Schaap *et al.*, 1993). Furthermore, stimulation of a transfected EGF receptor resulted in tyrosine phosphorylation of DAG kinase. The 80kDa form of DAG kinase has therefore been proposed to translocate to the

membrane in response to elevation of Ca^{2+} and phosphorylation by PKC to metabolise DAG formed from PtdCho hydrolysis (Sakane *et al.*, 1990).

(ii) PtdOH and lysoPtdOH.

PtdOH is at the crux of *de novo* glycerolipid synthesis as it can be converted to CMP-PtdOH for biosynthesis of phosphatidylinositol, phosphatidylglycerol and cardiolipin or dephosphorylated by PAP-1 to DAG (section 1.3.4). DAG can then be used to synthesise PtdCho, PtdEtn, PtdSer and triacylglycerol.

Alternatively PtdOH could be degraded by a PLA_2 to generate lysoPtdOH and a free fatty acid. Evidence for the interconversion of PtdOH and lysoPtdOH is scarce but both PtdOH and lysoPtdOH were reported to accumulate in PDGF-stimulated Balb/c3T3 cells (Fukami and Takenawa, 1992).

Both lysoPtdOH and PtdOH added exogenously to cells have been shown to have second messenger functions. Although a previous report of the Ca^{2+} mobilising effect of PtdOH (Moolenaar *et al.*, 1986) was found to be due to contaminating lysoPtdOH (Jalink *et al.*, 1990) both phospholipids were found to be equally mitogenic for fibroblasts (Van Corven *et al.*, 1992).

LysoPtdOH added exogenously to Rat-1 fibroblasts was found to stimulate PtdIns-PLC activity (Van Corven *et al.*, 1991; Plevin *et al.*, 1992). Addition of GTPγS enhanced the lysoPtdOH response by decreasing the EC_{50} for the agonist suggesting that lysoPtdOH acted through a G-protein coupled receptor (Plevin *et al.*, 1992). The identification of a putative receptor for lysoPtdOH of molecular weight 38-40kDa suggested that the phospholipid mediated its effects extracellularly (Van der bend *et al.*, 1992a). This may be physiologically significant as lysoPtdOH was recently reported to be released from thrombin stimulated platelets (Eichholtz *et al.*, 1993). Although lysoPtdOH-stimulated PtdIns(4,5)P_2 hydrolysis (Murayama and Ui, 1987) and PLD activity (Van der bend *et al.*, 1992b) were reported to be insensitive to pertussis-toxin the putative receptor may also be coupled to a

pertussis-toxin sensitive G-protein. This is supported by the finding that in fibroblasts the lysoPtdOH-stimulated increase in the GTP content of *ras* , mitogenesis and inhibition of adenylyl cyclase were attenuated by the toxin (Van corven *et al.*, 1993; Murayama and Ui, 1987). Furthermore, Van Corven *et al.* (1993) showed that the incease in the GTP content of *ras* by lysoPtdOH was inhibited by Genistein and that activation of PtdIns-PLC was neither required nor sufficient for DNA synthesis. It was therefore proposed that a member of the G_i subfamily of G-proteins may couple the lysoPtdOH receptor to the regulation of *ras* via a tyrosine kinase.

Although PtdOH was shown not to activate PtdIns-PLC (Jalink *et al.*, 1990 nor increase the GTP content of *ras* (Van Corven *et al.*, 1993) *in vivo* , it has been found to inhibit adenylyl cyclase (Murayami and Ui, 1987) and stimulate arachidonic acid release (Van Corven *et al.*, 1992) as reported for lysoPtdOH. However PtdOH has also been reported *in vitro* to inhibit *ras* GTPase activating protein (GAP) (Tsai *et al.*, 1989a) and activate *ras* GTPase-inhibitory protein (GIP) (Tsai *et al.*, 1989b). Both PtdOH and lysoPtdOH have been shown to activate various kinases including PKC (Epand and Stafford, 1990), phosphatidyl-4-phosphate kinase purified from bovine brain (Moritz *et al.*, 1992) and a phosphatidate dependent kinase in soluble extracts from rat liver, brain, lung and testis (Bocckino *et al.*, 1991).

1.4.4 The role of choline.

There is no evidence for a role of choline in signalling. However a relationship between choline produced from the stimulation of PLD activity and acetylcholine synthesis has been suggested. Incubation of the PLD from a synaptosomal rat brain preparation in the presence of oleate and acetyl-coenzyme A was reported to form acetylcholine (Hattori and Kanfer, 1985). Furthermore the generation of choline in muscarinic-stimulated rat cortex was reported to be used in the resynthesis of the acetylcholine pool (Corradetti, 1983).

However, choline is generally assumed to be used in the resynthesis of PtdCho (reviewed Bishop and Bell, 1988; Pelech and Vance, 1984) with phosphorylation to PCho by choline kinase being the first committed step (Fig. 1.3). Evidence for the regulatory role of choline kinase in PtdCho biosynthesis arises from reports of increased activity in *ras* -transformed cells (Macara *et al.*, 1989; Wakelam *et al.*, 1989) and serum-stimulated Swiss 3T3 fibroblasts (Warden and Friedkin, 1985). The rate limiting step in PtdCho synthesis is catalysed by CTP:phosphocholine cytidyl transferase and generates CDP choline (Pelech and Vance, 1984). Stimulation of PtdCho biosynthesis such as in PMA and thyrotropin-releasing hormone treated GH_3 cells (Kolesnick, 1987) or by treatment of cells with PtdIns- PLC (Terce *et al.*, 1988; Wright *et al.*, 1985) elicits translocation of the inactive cytosolic enzyme to the membrane where it is active . Regulation of the enzyme may involve PKC as it is activated by PMA and DAG. The final step in the synthesis of PtdCho involves the transfer of choline onto DAG with the release of CMP and is catalysed by an intrinsic microsomal protein choline phosphotransferase.

Aims of Thesis

The project aims were to study the stimulation of PLD activity in Swiss 3T3 fibroblasts in response to bombesin. Primarily investigations were directed to the determination of the PKC-independent arm of the regulatory pathway and the relationship between PLD activation, receptor occupancy and PtdIns(4,5)P_2 hydrolysis. With regard to the regulation of stimulated PLD activity in other cell types involvement of several components of the signal transduction machinery were investigated as follows;.

1. The regulation by G-proteins
2. Regulation through increases in cytosolic Ca^{2+}

3. Regulation through interaction with the adenylate cyclase signal transduction system i.e. through 'cross-talk'

4. Regulation through protein tyrosine phosphorylation.

Chapter 2

Materials and Methods

2.1 Cell lines and materials

2.1.1 Cell lines

Swiss mouse 3T3 fibroblasts were obtained from the European collection of animal cell cultures.

2.1.2 Materials

Affiniti Research Products Ltd. Nottingham, England.

Mouse monoclonal antibody to phosphotyrosine PY54.

Aldrich Chemical Company Ltd. Gillingham, England.

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

Amersham International plc, Amersham, Buckinghamshire, England.

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

(4-[^{125}I]*iodotyrosyl*) Gastrin Releasing Peptide

(specific activity 2000 Ci.mmol^{-1}).

[2-^{3}H]*myo*-Inositol (specific activity 10-20 Ci.mmol^{-1}).

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

Phosphoryl[*methyl*-^{14}C]choline, ammonium salt

(specific activity 50-60 mCi.mmol^{-1}).

[5;8-^{3}H]Adenosine 3'5' cyclic phosphate.

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

Enhanced chemiluminescence (ECL) reagent kit.

BDH Chemical Company, Poole, England.

Ammonium formate, butan-1-ol, ethanol, glycerol, potassium di-hydrogen phosphate, perchloric acid, sodium hydroxide, sodium carbonate, sodium dodecylsulphate, trichloroacetic acid, universal indicator and urea.

Boehringer (UK) Ltd, Lewes, England.

ATP and EGTA.

Cambridge Research Biomedicals, Cambridge, England.

Bombesin and [Arg^8]vasopressin.

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

Dithiothreitol.

Canberra Packard, Pangbourne, England.

Ultima-Flo A.F. scintillation fluid.

Costar, Cambridge, M.A., U.S.A.

Nitrocellulose

Dupont (U.K.) Ltd., New England Nuclear research products, Stevenage, England.

[phenylalanyl] 3,4,5-^{3}H(N)]vasopressin (specific activity 40-87Ci.mmol^{-1})

[β-Mercapto-β-β-cyclopenta-methylenepropionyl^1, 0-Et-Tyr^2, Val^4, Arg^8]-vasopressin (specific activity 40-87Ci.mmol^{-1}), (V_{1a} antagonist).

Fisons Scientific Apparatus.

Acetic acid (glacial), chloroform, ethyl acetate, methanol, magnesium chloride, potassium chloride, glycine, hepes and calcium chloride.

Formachem (Research International)plc, Strathaven, Scotland.

D-glucose and sodium hydrogen carbonate.

Genetic Research Instrumentation Ltd., Felsted, Essex, England.

Fuji film.

Gibco, Paisley, Scotland.

Dulbeccos' modified Eagles' medium (DMEM, 10x), glutamine (100x), penicillin/streptomycin (100x), sodium bicarbonate (7.5%) and Tris.

Hopkin and Williams,Essex, England

Potassium iodide.

Koch-Light Ltd. Suffolk, England.

Dimethyl sulphoxide and magnesium sulphate.

May and Baker, Dagenham, England.

Formic acid, hydrochloric acid and sodium tetraborate.

Murex, Dartford, England

Streptolysin-O.

Pierce and Warriner, Chester, England.

Micro BCA protein assay reagent kit.

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

The provision of the drug Ro-31-8220, a PKC inhibitor, by Drs. G. Lawton and T. Hallam is gratefully acknowledged.

Sapu, Lanarkshire, Scotland.

Sheep serum.

Upstate Biotechnology, Inc. (U.B.I.), Buckingham, England.

Anti-phosphotyrosine monoclonal antibody IgG2bK.

Whatman Ltd, Maidstone, England.

LK5DF thin layer chromatography plates.

The gift of [8-Arginine][*Phe*--3,4,5-^{3}H(n)]vasopressin ([^{3}H]AVP) and [^{3}H]-labelled vasopressin (V_{1a}) antagonist, [β-Mercapto-β-β-cyclopenta-methylenepropionyl^{1}, 0-Et-Tyr^{2}, Val^{4}, Arg^{8}]-vasopressin ([^{3}H]AVP antagonist), by Dr. M. Wheatley Department of Biochemistry, University of Birmingham, Birmingham B15 2TT U.K., is gratefully acknowledged.

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

2.2 Culture and preservation of Swiss mouse 3T3 fibroblast cells.

Swiss 3T3 cells were maintained in DMEM containing 10% (v/v) newborn calf serum, 27mg glutamine/ml and penicillin/streptomycin (250 units/ml and 250mg/ml respectively) at 37^{0}C, in a humidified atmosphere of air/CO_2 (19:1). Medium was changed every 2-3 days. Cells were routinely grown in 75cm^{2} tissue culture flasks and passaged when approximately 70% confluent.

After each passage a single flask of cells was allowed to reach confluency, to ensure that the cells were contact inhibiting. Cells were discarded at passage number 15 or when cells exhibited aberrant or transformed morphology.

2.2.1 Cell passage

Sterile trypsin for cell pasage

0.1% (w/v) Trypsin, 0.025% (w/v) EDTA, 10mM glucose was prepared in PBS pH 7.4. Aliquots were filtered and stored at -20^{0}C.

Cells were routinely passaged when approximately 70% confluent, at a ratio of 1 to 5. Counting of cells was performed using a haemocytometer.

2.2.2 Cryogenic preservation of cell lines

Cells were removed from the flask surface by trypsinisation. The cells were then resuspended in medium containing 20% (v/v) calf serum and 8% (v/v) DMSO in DMEM and 1ml aliquots containing approximately 10^6 cells were placed into freezing vials. The vials were frozen at -80^{0}C overnight before being placed into liquid nitrogen.

Cells brought up from storage in liquid nitrogen were thawed rapidly at 37^{0}C and the contents of one vial added to 4ml of complete medium in a 25cm^2 flask. The cells were then cultured overnight, the medium containing dead cells was then replaced with fresh medium. When the cells were sub-confluent they were passaged into a 75cm^2 flask, then grown and passaged as described.

2.3 Measurement of second-messengers in Swiss 3T3 cells

Unless otherwise indicated, cells were washed and stimulated in hepes buffered Hanks buffered saline with glucose and BSA (HHBG) pH 7.4, prepared as below and supplemented with 10mM glucose and 1% (w/v)BSA (fraction V).

Hanks buffered saline (Hanks).

1.26mM calcium chloride, 0.5mM magnesium chloride, 0.4mM magnesium sulphate, 5.37mM potassium chloride, 137mM sodium chloride, 4.2mM sodium hydrogen carbonate, 0.35mM sodium di-hydrogen phosphate, pH 7.4.

2.3.1 Assays for phospholipase D activity

(a) Measurement of phospholipase D transferase activities in [^{3}H]palmitate labelled Swiss 3T3 cells

(i) Incorporation of [^{3}H]palmitic acid into the major phospholipids in Swiss 3T3 cells

Equilibrium labelling of lipids with 2μCiml^{-1} [^{3}H]palmitate (Cook, 1991) in DMEM + 1% calf serum, has been shown to be complete after 24 hours, though [^{3}H]palmitate labelling of cells for 48 hours was routinely used. After 48 hours the majority of [^{3}H]palmitate was found to be incorporated into PtdCho with only minor incorporation into PtdIns. However, after 48 hours in DMEM containing 2% serum, Swiss 3T3 cells often detached from the plate. The experiment previously performed by Cook (1991), to determine equilibrium labelling conditions was repeated, using DMEM + 2% calf serum + 4μCi.ml^{-1} [^{3}H]palmitate to investigate the use of a shorter labelling time.

Swiss 3T3 cells were grown in a 24-well plate in DMEM + 10% newborn calf serum until approximately 70% confluent. The medium was then replaced with DMEM + 2% newborn calf serum containing 4μCi/ml [^{3}H]palmitate for times up to 48 hours, using a time staggered protocol. At the end of the labelling period, medium was removed by aspiration and cells were washed in 1ml of HHBG for 2 minutes, the medium was then replaced with 0.5ml ice-cold methanol. After scraping the well and washing with 0.2ml of methanol, the pooled cell debris was extracted into 0.7ml chloroform, vortexed and incubated at room temperature for 15 minutes. Phases were split by addition of 0.585ml of water and centrifuged at 1000*g* for 5 minutes. The chloroform phase was dried down *in vacuo* prior to analysis by t.l.c.

Isolation of the major individual phospholipids was performed by double development in a single direction, on Whatman LK5DF, 20 x 20cm, pre-laned silica

gel thin layer chromatography plates which had been sprayed with 1mM EDTA and allowed to air dry, before heat activating. The samples and various phospholipid standards were dissolved in chloroform/methanol (19/1, v/v) and applied to the plates. The plates were developed until the solvent front was approximately two-thirds of the way up the plate, in a lined and fully equilibrated t.l.c. tank, using a solvent system of chloroform:methanol:acetic acid:water (75:45:3:1, (v/v)). The plate was removed from the tank, allowed to air dry for 5 minutes, then replaced in the tank until the solvent front had run three-quarters of the way up the plate. Following visualisation with I_2 vapour, each lane was divided into 0.5cm sections and those sections corresponding to phospholipid standards were scraped and the radioactivity quantified by scintillation counting.

Figure 2.1 shows that equilibrium labelling of all the major phospholipids was attained by 24 hours of labelling as previously reported (Cook, 1991). However, incorporation of [^{3}H]palmitate label into PtdCho and sphingomyelin (SphM) decreased after 48 hours of labelling;.this decrease may have reflected a decrease in cell number.

The solvent system was unable to exclusively resolve PtdSer from PtdIns or PtdOH from PtdEtn, but, as previously shown, the majority of [^{3}H]palmitate was incorporated into PtdCho, with SphM, PtdSer, PtdOH/PtdEtn labelled to a lesser extent and only minor incorporation into PtdIns. After 24 hours of labelling, PtdCho contained 65% of the total radioactivity incorporated into total phospholipids (Table 2.1), whereas PtdIns contained 3%.

(ii) Preparation of samples.

Measurement of PtdBut formation was by the method of Cook *et al* (1990). Swiss 3T3 cells were grown in 24-well plates in DMEM + 10% newborn calf serum for 2 days, until approximately 80% confluent. The growth medium was then replaced with 0.5ml of DMEM + 2% newborn calf serum containing 4μCiml^{-1} of

[^{3}H]palmitate and the cells further cultured for 24-36 hours. Prior to the experiment the labelling medium was removed by aspiration and the cells were washed for 20 minutes at 37^{0}C with 0.5ml of HHBG. For kinetic studies cells were incubated for a further 5 minutes in 0.5ml of HHBG containing 30mM butan-1-ol at 37^{0}C, prior to the addition of HHBG + butan-1-ol + the test reagent at the indicated concentration and time. For dose-dependency studies there was no pre-incubation with butan-1-ol. Incubations were terminated by aspiration of the medium followed by the addition of 0.5ml of ice-cold methanol. Cell debris was scraped into screw-top glass tubes, together with a further 0.2ml methanol wash. Lipids were extracted by the addition of 0.7ml of chloroform and incubation at room temperature for 15 minutes. After addition of 0.585ml of water, tubes were centrifuged for 5 minutes at 1000*g*, the upper aqueous/methanolic phase discarded and the chloroform phase dried *in vacuo*. Samples were analysed immediately by thin layer chromatography.

(iii) Isolation and identification of phosphatidylbutanol

Dried chloroform extracts were resuspended in 50µl of chloroform/methanol (19/1) 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 unlined 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 scintillant. The band of radioactivity corresponding to PtdBut was identified by either of 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.

Figure 2.1 Time course of incorporation of [^{3}H]palmitic acid into the majo phospholipid classes in Swiss 3T3 cells.

Swiss 3T3 cells were grown in a 24-well plate until approximately 70% confluent. The medium was replaced with DMEM containing 2% calf serum whic at staggered intervals was removed and substituted with 0.5ml DMEM containin 2% calf serum and 4μCiml^{-1} of [^{3}H]palmitic acid. After 48 hours, the medium wa removed and the incubations terminated by the addition of ice-cold methano Chloroform extracts were separated by thin layer chromatography and the lipic PtdCho (▲), SphM (Δ), PtdEtn/PtdOH, (o), PtdSer/PtdIns (●) identified as describe The corresponding regions of the silica gel were scraped and the associate radioactivity quantified by scintillation counting. Results are expressed a radioactivity in phospholipids (d.p.m., mean ± S.D., n=3) from a single experiment.

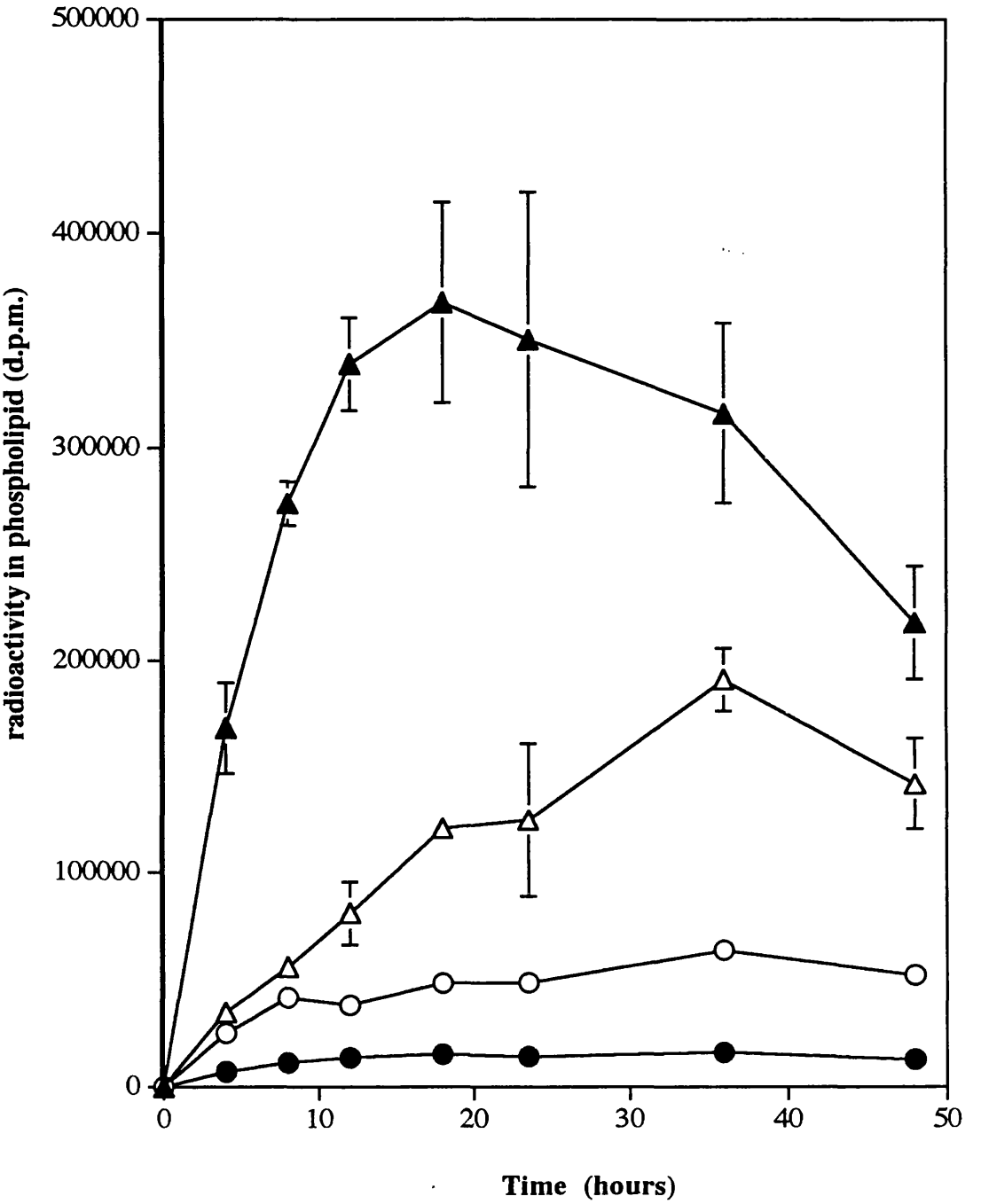

500000
400000
300000
200000
100000
0
radioactivity in phospholipid (d.p.m.)
0
10
20
30
40
50
Time (hours)

Table 2.1 Distribution of [^{3}H]palmitic acid in the major phospholipid classes in Swiss 3T3 cells after 24 hours of labelling.

Swiss 3T3 cells were grown in a 24-well plate until approximately 70% confluent. The medium was replaced with DMEM containing 2% calf serum which was removed after 24 hours and substituted with 0.5ml DMEM containing 2% calf serum and 4μCi.ml^{-1} of [^{3}H]palmitic acid. After 48 hours, the medium was removed, and the incubations terminated by the addition of ice-cold methanol. Chloroform extracts were separated by thin layer chromatography and the lipids identified as described. The corresponding regions of the silica gel were scraped and the associated radioactivity quantified 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 [^{3}H]palmitate incorporated into the phospholipids after 24 hours.

Lipid	Radioactivity in [^{3}H]palmitate containing lipid (d.p.m.)	% of total [^{3}H]palmitate incorporated
PtdCho	350560 ± 36218	65%
SphM	124959 ± 36218	23%
PtdEtn/PtdOH	48463 ± 2552	9%
PtdSer/PtdIns	13916 ± 2030	3%

The t.l.c. system used, separated PtdBut with R_f of approximately, 0.42 ± 0.06 (Cook, 1991) though the R_f varied slightly with each batch of running solvent. PtdCho, PtdEtn, PtdSer and PtdIns remained at the origin and monoglyceride, di and triglycerides and free fatty acids ran close to the solvent front (Pai *et al.*, 1988). [^{3}H]PtdOH was reported to run slightly further than the phospholipids but could not be completely resolved from them without a double development in the same dimension. The small peak running before that of [^{3}H]PtdBut (Fig. 2.2) may be due to [^{3}H]PtdOH or the production of [^{3}H]bisphosphatidic acid ([^{3}H]bisPtdOH) (van Blitterswijk and Hilkmann, 1993), which was found to be produced by stimulants capable of elevating DAG levels and activating PLD simultaneously. Using a slightly different solvent system of ethylacetate:2,2,4,trimethylpentane:acetic acid:water (13/2/3/10, v/v) and double development in the same direction, van Blitterswijk and Hilkmann, (1993) separated PtdBut from PtdOH and bisPtdOH.

(b) Assay of PtdCho hydrolysis

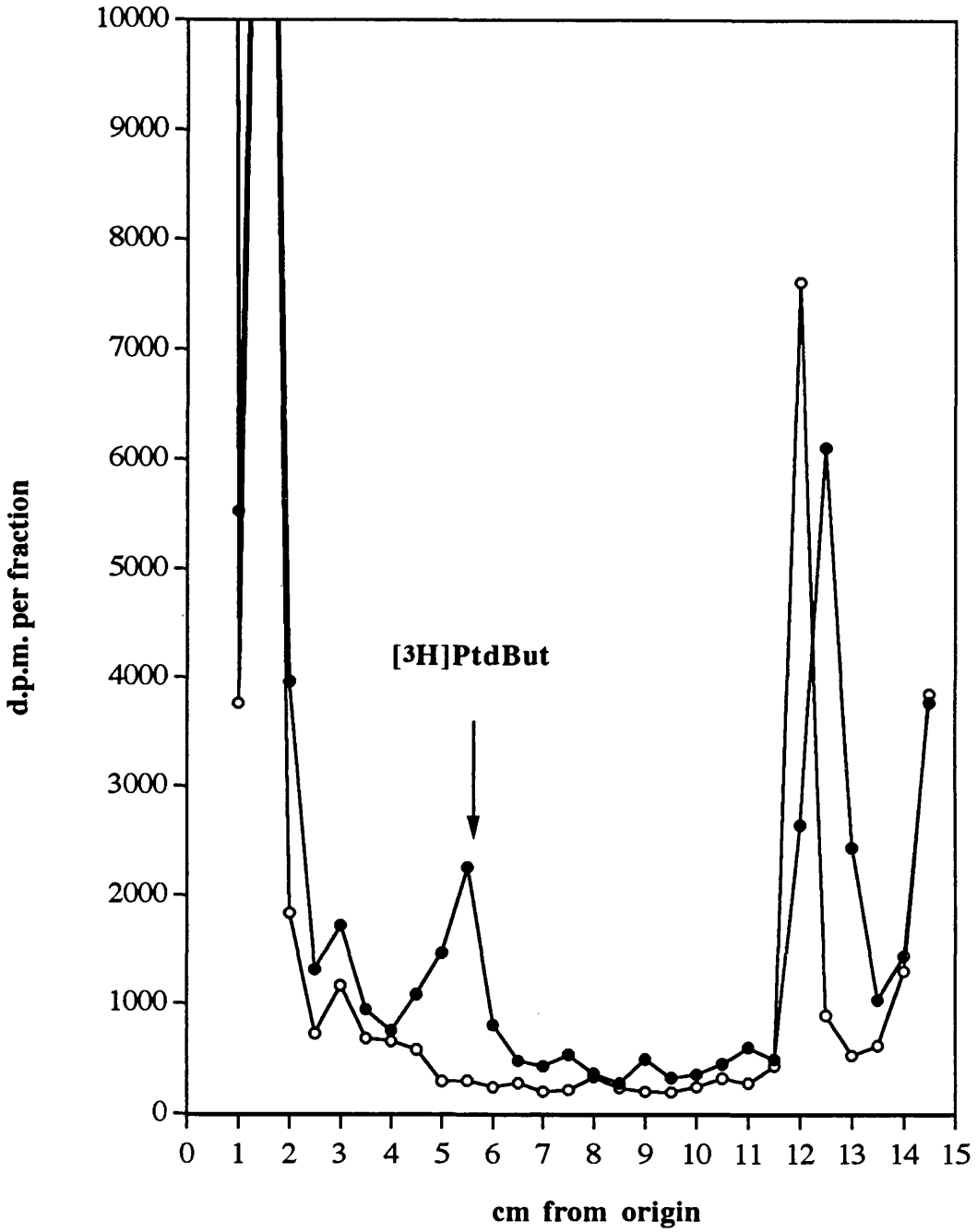
(i) Preparation of Dowex-50W-H$^+$

Dowex-50W-H$^+$ cation exchange resin (200-400 mesh) was washed three times in three volumes of distilled water and the 'fines' were removed each time. The Dowex was then washed three times with three volumes of 1M HCl. Finally, the Dowex was washed four or five times with three volumes of distilled water until the pH of the wash was approximately 5.5.

Each newly prepared batch of Dowex was characterised by performing an elution profile of a mixture of radiolabelled standards of glycerophosphocholine (GroPCho), phosphocholine (PCho) and choline, added to a Swiss 3T3 cell extract. For experimental use 1ml Dowex-50W-H$^+$ columns were prepared in glass wool plugged pasteur pipettes.

Figure 2.2 Resolution and identification of [^{3}H]PtdBut in bombesin-stimulated Swiss 3T3 cells

Swiss 3T3 cells were grown in 24-well plates until approximately 80% confluent before replacing the medium for 24 hours with 0.5ml of DMEMcontaining 2% calf serum and 4μCi.ml^{-1} of [^{3}H]palmitic acid. Cells were washed for 20 minutes in HHBG at 37^{0}C before incubating in HHBG containing 30mM butan-1-ol for 5 minutes. Cells were then stimulated with HHBG containing 30mM butan-1-ol and 100nM bombesin (•), or a vehicle control (o), for a further 1 minute at 37^{0}C. Incubations were terminated by removal of the medium and addition of 0.5ml of ice-cold methanol. Chloroform extracts were resolved on Whatman LK5DF t.l.c. plates, using the solvent system described. Each lane of the t.l.c. plate was divided into 0.5cm sections which were removed by scraping and the associated radioactivity determined by scintillation counting. The results are from a single experiment representative of nine.


10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
d.p.m. per fraction
[3H]PtdBut
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
cm from origin

(ii) Preparation of samples

Swiss 3T3 cells were plated onto 24-well plates and grown until approximately 70% confluent in DMEM + 10% calf serum. The cells were then labelled to equilibrium for 36-48 hours in DMEM + 2% calf serum + 2μCiml^{-1} [*methyl* -^{3}H]choline chloride. Prior to the experiment the medium was replaced with 0.5ml of unlabelled DMEM for 2 hours. Cells were then washed at 37^{0}C in medium containing DMEM, 1% (w/v) BSA, 10mM glucose and 20mM hepes (DMBGH) for 3 washes of 5, 10 and 30 minutes. Incubations were performed with 150μl of test reagent in DMBGH at the indicated concentration and time. Incubations were terminated by the addition of 0.5ml methanol. Cell debris was scraped and transferred to a plastic tube and pooled with a further 0.2ml methanol wash. Phospholipids were extracted by the addition of 310μl of chloroform, vortexed and incubated at room temperature for 15-20 minutes or overnight at 4^{0}C. The phases were split by the addition of 390μl chloroform and 480μl of water and centrifugation at 1000*g* for 5 minutes. A 0.8ml sample of the aqueous methanolic phase was analysed for [^{3}H]choline content by cation-exchange chromatography.

(iii) Measurement of total choline generation

Analysis of [^{3}H]choline content was performed essentially as described by Cook and Wakelam (1989) except that 1M KCl rather than 1M HCl was used to elute the choline fraction as described by Kanoh *et al.*, (1991). Figure 2.3 shows that 12ml of 1M HCl was required to recover 85% of the [^{3}H]choline in a relatively broad peak, whereas only 7ml of 1M KCl was needed to give a similar recovery of [^{3}H]choline in a sharper peak. Hence the use of KCl considerably reduced [^{3}H]choline elution volume and enabled the radioactivity in the entire eluant to be determined, whereas the larger volume of 1M HCl required had necessitated the counting of a 2ml aliquot.

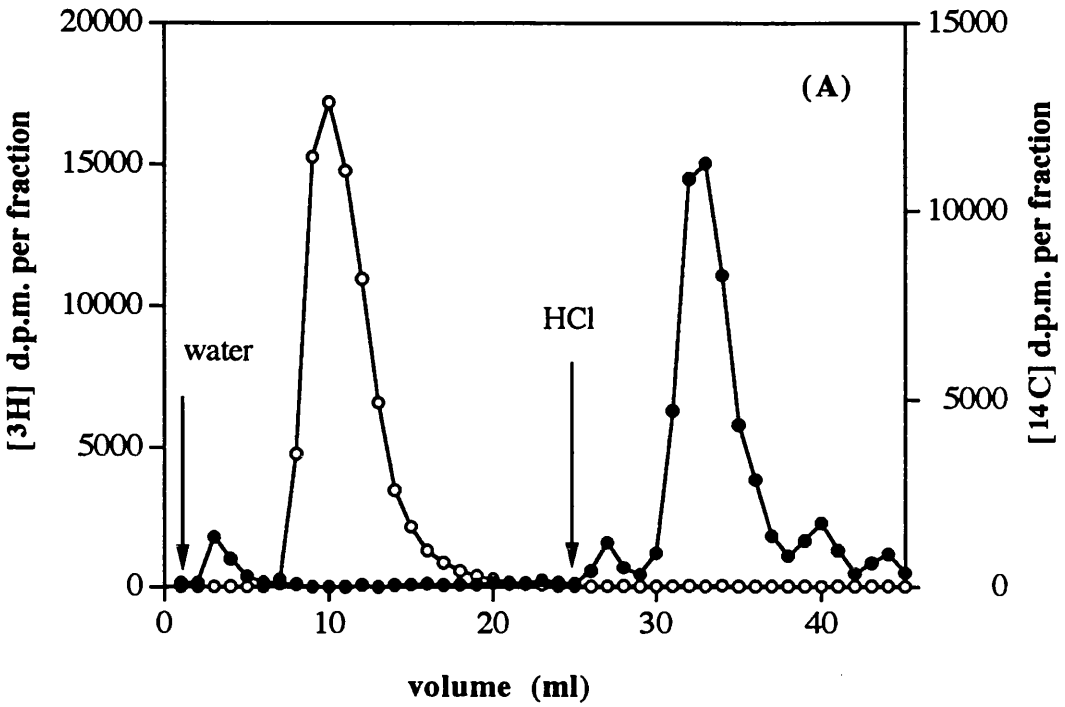
The sample of cell extract was added to 4.2ml of water and loaded onto 1ml Dowex-50W-H^{+} columns (section 2.3.1(bi)). The flow through and a further 20ml

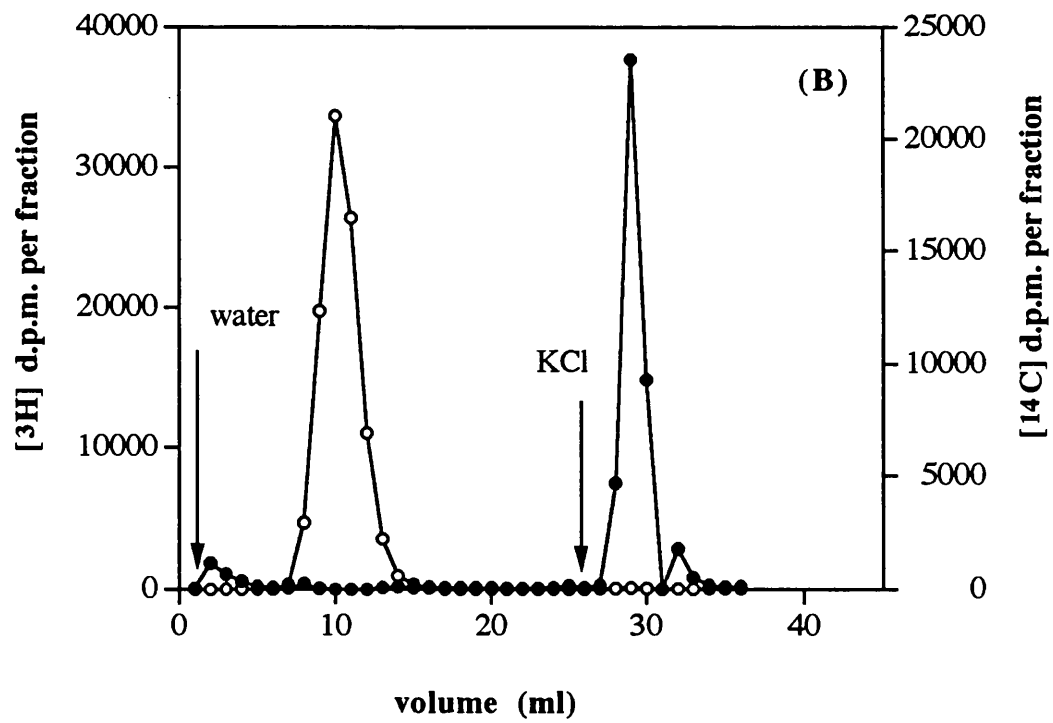
Figure 2.3 (A & B). Elution profile of [^{3}H]GroPCho, [^{14}C]ChoP an [^{3}H]choline:

(A) [^{3}H]choline eluted with1M HCl

(B) [^{3}H]choline eluted with1M KCl.

A mixture of standards were diluted to 1ml with an aqueous cell extra prepared from quiescent, unlabelled Swiss 3T3 cells by chloroform/methanol extraction. Each mixture was applied to separate 1ml Dowex-50-WH$^+$ columns i plugged pasteur pipettes and ml by ml elutions were performed with water followe by; **(A)** 1M HCl or **(B)** 1M KCl, as indicated. Each fraction was collected an counted on a [^{14}C] (o) and [^{3}H] (•), dual label programme. The profile show represents radioactivity in each fraction (d.p.m.), and is typical of at least three oth experiments


(A)
water
HCl
[3H] d.p.m. per fraction
[14C] d.p.m. per fraction
volume (ml)
20000
15000
10000
5000
0
0
10
20
30
40


(B)
water
KCl
[3H] d.p.m. per fraction
[14C] d.p.m. per fraction
volume (ml)
40000
30000
20000
10000
0
25000
20000
15000
10000
5000
0
0
10
20
30
40

water wash containing glycerophosphocholine (GroPCho) and phosphocholine (PCho) was discarded, whilst a final wash with 7ml of 1M KCl was collected as the choline fraction. The radioactivity in the eluant was determined by scintillation counting.

2.3.2 Measurement of stimulated formation of total inositol phosphates.

(i) Preparation of dowex-formate.

100g of Dowex 1x8-200 anion exchange resin in the chloride form, was washed 3 times in distilled water, allowed to settle and the 'fines' discarded after each wash. The resin was then washed with 2l of 2M NaOH followed by 6l of water and then 0.5l of formic acid. Finally the Dowex was washed with 20l water until the pH of the slurry was approximately 5.5. Each preparation of formate form Dowex was characterised for measurement of total inositol phosphates by performing ml by ml elutions of [^{3}H]Ins(1,4,5)P_3.

(ii) Preparation of samples

Total inositol phosphates were assayed by accumulation in the presence of 10mM LiCl as described by Berridge *et al.*, (1982). Swiss 3T3 cells were plated onto 24-well plates at a density of approximately 10^4 cells.ml^{-1} in DMEM + 10% newborn calf-serum, until approximately 80% confluent. They were then grown in 0.5ml inositol-free DMEM containing 2% dialysed newborn calf-serum and 1μCi.ml^{-1} of [2-^{3}H]*myo*-inositol for 24 hours, after which time the cells were confluent, quiescent and labelled to radioactive equilibrium. Prior to the experiment the labelling medium was removed and the cells washed twice in 0.5ml HHBG containing 0.1% (v/v) BSA (fraction V) for 10 minutes at 37^{0}C, prior to incubation for a further 5 minutes in 0.5ml HHBG containing 0.1% (v/v) BSA (fraction V) + 10mM LiCl (HHBG.Li). Incubations were commenced by replacing the medium with 200μl (or 150μl if reaction was to be terminated with methanol) of the test

reagent in HHBG.Li, at the times and concentrations indicated. Incubations were terminated either by addition of 100µl of 10% (v/v) perchloric acid (PCA) or 500µl methanol and the samples were extracted on ice.

In assays where PCA was used, the wells were scraped and the material transferred to an eppendorf tube. Each well was then washed with 50µl water which was pooled with the scraped cell debris. The cell extracts were neutralised by addition of KOH/hepes (1.5M/60mM) containing universal indicator, after which the samples could be stored at -20^{0}C. After thawing, the neutralised extracts were centrifuged at 14,000*g* for 3 minutes at 4^{0}C to remove the precipitated potassium perchlorate and the supernatant assayed for total inositol phosphates (section 2.3.2(iii)).

In assays where methanol was used, the cell debris was scraped and removed to a 2ml plastic tube. Each well was then washed with 200µl of methanol and pooled with the scraped cell debris. Phospholipids were extracted by addition of 310µl of chloroform, vortexed and incubated at room temperature for 15 minutes. After addition of 390µl of chloroform and distilled water, samples were centrifuged at 1,000g for 5 minutes to split phases. 800µl of the upper aqueous/methanolic phases was assayed for total inositol phosphates. In addition, an aliquot of the chloroform phase was removed and dried down and the radioactivity in total inositol containing lipids determined by scintillation counting.

(iii) Assay of total inositol phosphates

Samples prepared as described above, were mixed with 3ml of 0.5mM EDTA/5mM sodiumtetraborate, pH6.7. and 0.5ml Dowex formate (1x8;200-400 mesh, (section 2.3.2.(i)). Most of the sample was aspirated off and the Dowex washed twice with 4ml water. Glycerophosphoinositides were eluted with two 4ml washes of 5mM sodiumtetraborate/60mM ammonium formate. The dowex was washed twice with 4ml water and was centrifuged for 1 minute at 1000*g* before the final wash was aspirated. Finally, 1ml of 1M ammonium formate/0.1M formic acid

was added to elute total inositol phosphates. The radioactivity associated with the total inositol phosphates in 800µl of the supernatant was determined by scintillation counting.

2.3.3 Determination of the generation of total inositol phosphates and phospholipase D transferase activities in the same cell monolayer.

Swiss 3T3 cells were plated onto 24-well plates at a density of approximately 10^4 cells.ml^{-1} in DMEM + 10% newborn calf-serum until approximately 80% confluent. They were then grown in 0.5ml inositol-free DMEM containing 2% dialysed newborn calf-serum and 1µCi.ml^{-1} of [2-^{3}H]*myo*-inositol + 4µCi.ml^{-1} [^{3}H]palmitic acid for 24 hours. Samples were prepared as described for measurement of total inositol phosphates, using methanol to terminate the reaction, except that 30mM butan-1-ol was included in addition to 10mM LiCl. The samples were processed as described for the assay of total inositol phosphates (section 2.3.2(iii)) except that cell-debris was transferred to screw-top glass vials instead of plastic tubes. On splitting the phases the whole of the aqueous phase was taken for total inositol phosphate measurement and the whole of the organic phase dried down and analysed for [^{3}H]PtdBut generation (section 2.3.1(ii - iii)). Table 2.2 shows that in cells labelled with [^{3}H]palmitic acid the presence of 10mM LiCl did not affect stimulated [^{3}H]PtdBut generation, nor did the presence of butan-1-ol in [^{3}H]inositol labelled cells affect stimulated inositol phosphate generation.

2.3.4 Measurement of cyclic AMP generation

Binding protein prepared from bovine heart was kindly donated by Professor. M. Houslay.

(i) Preparation of samples

Swiss 3T3 cells were grown in 6-well plates in DMEM + 10% calf serum until approximately 80% confluent. The medium was removed and the cells

Table 2.2 Effect of (A) 10mM LiCl on bombesin-stimulated [^{3}H]PtdBut generation in [^{3}H]palmitate labelled Swiss 3T3 cells and (B) 30mM butan-1-ol on bombesin-stimulated generation of [^{3}H]total inositol phosphates in [^{3}H]inositol labelled cells.

Swiss 3T3 cells were grown in 24-well plates until approximately 70% confluent then labelled for 24 hours with either; (A) 4μCiml^{-1} of [^{3}H]palmitate in DMEM + 2% calf serum, or (B) 1μCiml^{-1} of [^{3}H]inositol in 0.5ml of inositol-free DMEM containing 2% dialysed calfserum.

(A) After washing with HHBG for 20 minutes, cells were preincubated for 10 minutes in HHBG containing 30mM butan-1-ol in the presence or absence of 10mM LiCl. Incubations were commenced by replacing the medium with HHBG + 30mM butanol + 100nM bombesin in the presence or absence of LiCl for 1 minute. Control cells were incubated with HHBG + 30mM butan-1-ol, with or without 10mM LiCl. Incubations were terminated by replacement of the medium with 0.5ml ice-cold methanol Chloroform extracts were prepared and [^{3}H]PtdBut generation quantified by t.l.c. as described. Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) from a single experiment representative of two.

(B) After washing twice with HHBG over 20 minutes, cells were preincubated for 10 minutes in HHBG containing 10mM LiCl in the presnce or absence of 30mM butan-1-ol. Incubations were commenced by replacing the medium with HHBG + 10mM LiCl + 100nM bombesin in the presence or absence of 30mM butan-1-ol for 1 minute. Control cells were incubated with HHBG + LiCl with or without 30mM butan-1-ol. Incubations were terminated by addition of PCA and the generation of [^{3}H]total inositol phosphates determined as described. Results are expressed as radioactivity in [^{3}H]InsP_t (d.p.m., mean ± S.D., n=3) and are from a single experiment representative of three.

(A) Treatment	d.p.m. in PtdBut
ButOH preincubation ButOH stimulation	655 ± 101
ButOH + LiCl preincubation ButOH + LiCl stimulation	633 ± 37
ButOH preincubation ButOH + bombesin stimulation	2088 ± 134
ButOH + LiCl preincubation ButOH + LiCl + bombesin stimulation	2219 ± 258

(B) Treatment	d.p.m. in total inositol phosphates
LiCl preincubation LiCl stimulation	927 + 236
LiCl + ButOH preincubation LiCl + ButOH stimulation	981 ± 141
LiCl preincubation LiCl + bombesin stimulation	2671 ± 356
LiCl + ButOH preincubation LiCl + ButOH + bombesin stimulation	2690 ± 202

quiesced in DMEM + 2% calf serum for 24 hours prior to the experiment. Cells were washed in HHBG for 20 minutes at 37^0C , then the medium replaced with the test reagent in HHBG at the required concentrations and for the times indicated. Incubations were terminated by aspiration of the medium and addition of 0.25ml of 2% (v/v) PCA. The cell debris was removed by scraping into eppendorf tubes and the samples neutralised by the addition of 2M potassium hydroxide / 0.5M triethanolamine hydrochloride containing universal indicator. The precipitated potassium perchlorate and cell debris were pelleted by centrifugation at 14,000*g* for 10 minutes at 4^0C. A 50µl aliquot was then removed for assay of cyclic AMP.

(ii) Measurement of cyclic AMP generation

Determination of cyclic AMP content was based on the saturation assay of Brown *et al,* (1972), as described by Whetton *et al,* (1983). A standard curve was constructed by preparing a series of unlabelled-cyclic AMP solutions ranging from 0-320pmol.ml^{-1}, in a buffer containing 50mM Tris pH 7.4 and 5mM EDTA. (5;8 -[^{3}H]-adenosine 3'5' cyclic phosphate) in 50% ethanol was diluted in this buffer to give approximately 500,000 c.p.m.ml^{-1}. 100µl of this solution was added to tubes containing 50µl of assay buffer. 50µl of standard cyclic AMP solution or a 50µl aliquot of Swiss 3T3 cell extract was then added. After mixing, 100µl of binding protein diluted in assay buffer, was added to the samples, which were mixed once again. Tubes were incubated at 4^0C for at least 2 hours. Total cyclic AMP binding to the protein was determined by incubating the tritiated cyclic AMP in the absence of the unlabelled species, whilst non-specific-binding was estimated by the incubation of tritiated cyclic AMP in the absence of binding protein as well as unlabelled cyclic AMP. Non-specific binding was typically between 10 and 20% of total binding. Incubations were terminated by addition of 250µl of ice-cold assay buffer containing 2% activated charcoal and 1% bovine serum albumin in assay buffer, followed by centrifugation at 14,000g at 4^0C. A 300µl aliquot of the supernatant was removed to a 5ml insert vial and the radioactivity associated was

determined. The program used to count the samples had an RIA curve fitting program and hence cyclic AMP content was automatically calculated from the standard curve. The binding assay gave optimum sensitivity between 0.25 and 8pmoles/sample.

2.3.5 Western-blot analysis of tyrosine-phosphorylated proteins.

(i) Preparation of cell lysates.

Swiss 3T3 cells were grown in 6-well plates or 25cm^2 flasks in DMEM + 10% (v/v) newborn calf serum for 48 hours, then allowed to quiesce in DMEM + 2%(v/v) calf serum for a further 24 hours. Cells were washed for 20 minutes in HHBG containing 0.1% BSA (w/v) at 37^{0}C, before treatment with the test reagent as indicated. Incubations were terminated by rapidly washing the cells three times with lysis wash buffer [50mM hepes pH 7.4, 5mM EGTA, 150mM sodium chloride, 100μM sodium vanadate, 200μM phenylmethanesulphonyl fluoride], lysis buffer was then added [50mM hepes pH 7.4, 1% (v/v) Triton x-100, 1mM EDTA, 1mM EGTA, 50μg/ml aprotinin, 25mM benzamidine, 5μg/ml leupeptin, 100μM sodium vanadate] and the cells placed on ice. The flasks or plates were shaken at 4^{0}C for 10-20 minutes subsequent to lysis, then the lysed cells removed by scraping into eppendorf tubes. The lysates were centrifuged at 4^{0}C at 14,000*g* for 10 minutes to remove the insoluble material and the nuclei and the supernatant transferred to fresh eppendorf tubes. Lysates were stored at -20^{0}C until required. The protein concentration of the lysates was determined using the micro BCA protein assay kit.

(ii) TCA precipitation of samples for Western-blot analysis.

The protein content of each lysate was corrected to that required in 0.7ml with distilled water. 6.2µl of 2% (w/v) sodium deoxycholate was added and the solution vortex mixed. The protein was precipitated by the addition of 0.25ml of 24% (w/v) trichloroacetic acid and the suspension vortexed and incubated at 4°C for 15 minutes. The precipitated protein was pelleted by centrifugation at 4°C for 5 minutes at 14,000*g* and the supernatant removed by aspiration. 20µl Laemmli buffer [30% (w/v) urea, 5% (w/v) SDS, 6% (w/v) DTT, 20mM Tris/HCl pH 8.0, 0.2% (w/v) bromophenol blue].and 20µl of 2M Tris base pH 8 was added to the pellet. For mini-gels the TCA precipitate was acetone washed. Following removal of the supernatant, 200µl of ice-cold acetone was added and the pellet again centrifuged at 4°C for 5 minutes at 14,000*g*. The supernatant was removed and the process repeated. After the second acetone wash, 10µl of Laemmli buffer and 10µl of 2M Tris base pH 8 was added to the pellet. The pellets were then vortex mixed and boiled for 5 minutes to dissolve the precipitated protein.

(iii) SDS-PAGE gel preparation

SDS-PAGE analysis of cell lysates was performed according to the method of Laemmli *et al,* (1970) in 1.5mm or 0.75mm (mini-gels) thick, polyacrylamide gels, employing a vertical slab gel or mini-gel apparatus (BioRad) as described by the manufacturers.

Proteins were separated using 10% acrylamide resolving gels and 3% acrylamide stacking gels. Immediately after preparation, the resolving gel was poured into the gel apparatus, overlaid with water-saturated butanol and left to polymerise at room temperature. After preparation of the stacking gel, the overlay was removed from the polymerised resolving gel and the stacking gel solution poured into the electrophoresis apparatus. The well comb was positioned and the solution left to polymerise at room temperature

(iv) SDS-PAGE gel electrophoresis

The polymerised gels were assembled into the buffer reservoir as described by the manufacturer's instructions, the well comb removed and the reservoirs filled with running buffer [25mM Tris, 190mM glycine, 0.1% (w/v) SDS]. The prepared samples (section 2.3.5(ii)) were placed directly into the wells of the stacking gel with a Hamilton syringe. Mini-gels were run for 1 hour at 20mA, 150V per gel. Large gels were run over-night at 15mA, 100V for 2 gels until the dye-front reached the end of the resolving gel

(v) Immuno-blotting of SDS-PAGE gels

After electrophoresis the proteins were transferred to nitrocellulose according to the manufacturers instructions (wet-blotting - Hoeffer; semi-dry blotting - Sartoblot). Wet-blotting was performed for large gels in buffer containing, 25mM Tris, 0.19M glycine and 20% (v/v) methanol. For mini-gels blotting was performed in buffer containing, 48mM Tris pH 9.2, 39mM glycine, 1.3mM SDS, 20% (v/v) and methanol, using the semi-dry apparatus. Gels were routinely stained with Coomassie-blue [stain; 500ml distilled water, 400ml methanol, 100ml acetic acid, 0.1% w/v Coomassie blue: destain; 500ml distilled water, 400ml methanol, 100ml, acetic acid] subsequent to blotting, to check the efficiency of the transfer.

The nitrocellulose was rinsed briefly in Tris buffered saline (TBS) [150mM sodium chloride, 20mM Tris pH 7.4], then blocked for 3 hours at 4^0C in TBS containing 0.05% Tween 20 (TBST), with 3% (w/v) BSA (fraction V, electrophoresis grade) and 10% (v/v) sheep-serum added. The blot was then incubated overnight at 4^0C in TBST containing 3% (w/v) BSA and a 1:5000 dilution of anti-phosphotyrosine PY54 antibody (Ruff-Jamison *et al.*, 1991). In earlier experiments a mouse monoclonal antibody from U.B.I was used, however due to expense and problems in stability the antibody was changed. Blots developed using either antibody showed essentially the same results.

After incubation with first antibody, the blot was washed in TBST for 2 hours, changing the wash regularly. The nitrocellulose was then incubated at 4^0C for 1hour in TBST supplemented with 3% (w/v) BSA and a 1:2000 dilution of sheep anti-mouse IgG linked to horse radish peroxidase and preadsorbed with human serum proteins. The blot was then washed repeatedly over 2 hours in TBST. The horse-radish peroxidase-labelled proteins were detected by using ECL. Luminescence was detected by exposing autoradiograph film to the blot for times ranging from 10 seconds to 5 minutes.

2.4 Radio-ligand receptor binding assays

2.4.1 Binding of [^{3}H]AVP and [^{3}H]AVP antagonist to Swiss 3T3 cells

Binding to intact cells was performed as described by Gardner *et al.*, 1989. Cells were grown in 6- or 12-well plates until approximately 80% confluent. The medium was then replaced with DMEM + 2% calf serum and the cells quiesced for 24 hours. The medium was removed and the cells washed three times over 20 minutes at 37^0C in first wash buffer [serum-free DMEM supplemented with glutamine, 10mM hepes pH 7.4 and 0.2% (w/v) bovine serum albumin]. Pretreatments were performed at 37^0C for the times indicated with the test reagent at the required concentration in incubation buffer [serum free DMEM supplemented with glutamine, 10mM hepes pH 7.4, 0.05% (w/v) bovine serum albumin, 10mM glucose and 2mM bacitracin]. After replacing the medium with ice-cold incubation buffer the cells were placed on ice and washed 4 times over 15 minutes with 0.5ml ice-cold incubation buffer. Incubations were commenced by replacing the medium for the times indicated with incubation buffer containing [^{3}H]vasopressin antagonist [β–Mercapto-β-β-cyclopenta-methylenepropionyl^1, 0-Et-Tyr^2, Val^4, Arg^8]-vasopressin ([^{3}H]AVP antagonist or [8-Arginine][Phe-3,4,5-^{3}H(n)]vasopressin ([^{3}H]AVP) at the required concentration. The medium was then removed and the wells washed 4 times with ice-cold second wash buffer [serum-free DMEM

supplemented with glutamine, 10mM hepes pH 7.4 and 0.1% (w/v) bovine serum albumin] before the cells were solubilised.

2.4.2 Solubilization of cells and determination of cell-associated radioactivity.

Following the final wash, 0.5ml of solubilization buffer containing 0.5M NaOH, 1% (w/v) SDS and 10mM hepes, was added to the wells and the cells left at room temperature for 30 minutes. A 0.45ml portion of this solution was transferred to a scintillation vial containing 0.45ml 0.5M HCl and the radioactivity associated with the cells determined by liquid-scintillation counting. Non-specific binding, was determined as the radioligand not displaced by a 200-fold excess of unlabelled antagonist or agonist as appropriate. Cell number was determined using one well per plate which was treated in the same way, except that no agonist pretreatment or radiolabel was added and after the final wash, cells were removed from the well with trypsin and counted using a heamocytometer.

2.4.3 Quantification of Internalized Ligand

Measurement of internalised ligand was established using an acidic wash to remove surface bound agonist as described by Kuppuswamy and Pike (1989). Cells were treated initially as in section 2.4.1. Following the initial wash period, the cells were incubated for indicated times with [^{3}H]AVP in incubation buffer (section 2.4.1) at 37^{0}C. Medium was aspirated and replaced with ice-cold incubation buffer before the cells were placed on ice to prevent further internalisation. Surface-bound [^{3}H]AVP was determined by washing twice for 2 minutes in buffer containing 50mM glycine pH 3 and 100mM sodium chloride, before further washing for four times in ice-cold second wash buffer (section 2.4.1). Determination of cell-associated radioactivity and cell number was as in section 2.4.2 Internalised [^{3}H]AVP was taken as that portion of the total cell-bound [^{3}H]AVP not removed by the acid wash treatment.

2.4.4 Measurement of internalization of ([^{125}I]-Tyr^4) gastrin releasing peptide in intact Swiss 3T3 cells

Cells were grown in 24-well plates and quiesced as described in section 2.4.1. Binding experiments were essentially as described by Brown *et al.* (1987). The wells were washed in HHBG containing 1mM potassium iodide (HHBG.KI) for 20 minutes at 37^{0}C, prior to incubation for 1 minute with 100nM bombesin in HHBG.KI containing 0.1μCi/150μl of ([^{125}I]-Tyr^4) gastrin releasing peptide (([^{125}I]-Tyr^4)GRP). The medium was then replaced with ice-cold HHBG.KI and the cells placed on ice to prevent any further internalisation. Cells were washed twice for 2 minutes with buffer pH 3 (section 2.4.4), or with HHBG.KI., before further washing for four times in ice-cold HHBG.KI. Cells were solubilised in 0.5M sodium hydroxide for 10-20 minutes at 37^{0}C. 400μl of the solubilised sample from each well was counted in a gamma counter. Non-specific binding was determined as the radioligand not displaced by a 200-fold excess of bombesin. Cell number was determined by using one well per plate which was treated in the same way, except that no radiolabel was added and after the final wash, cells were removed from the well with trypsin and counted using a haemocytometer.

2.5 Permeabilisation of cells using Streptolysin-O

Permeabilisation of cells and all subsequent washes and treatments were performed in permeabilisation buffer made up as follows;

Permeabilisation buffer (final free calcium concentration 150nM)

120mM potassium chloride, 20mM hepes pH7.4, 8.49mM magnesium chloride, 61μM calcium chloride, 0.1mM EGTA .

2.5mM ATP and 0.1 % (w/v) BSA (fraction V) were added on day of use and pH was adjusted to 7.4.

2.5.1 Determination of cell permeabilisation using entry of fluorescent dye

Swiss 3T3 cells were scraped from a 75cm^2 flask and transferred to a centrifuge tube at 37^{0}C for 45 minutes to allow recovery. Cells were then washed three times in HHBG and resuspended in permeabilisation buffer containing streptolysin-O (0.6U.ml^{-1}) and the fluorescent stain bisbenzamide (10μg.ml^{-1}). After 5 minutes an aliquot of cells was transferred to a slide and examined under a fluorescent microscope. At least 90% of cells were found to contain the fluorescent dye (Fig. 2.4). Previous findings had demonstrated that 0.6Uml^{-1} of streptolysin-O resulted in maximal release of lactate dehydrogenase from Swiss 3T3 cells (Currie, 1992)

2.5.2 Protocol for Permeabilisation

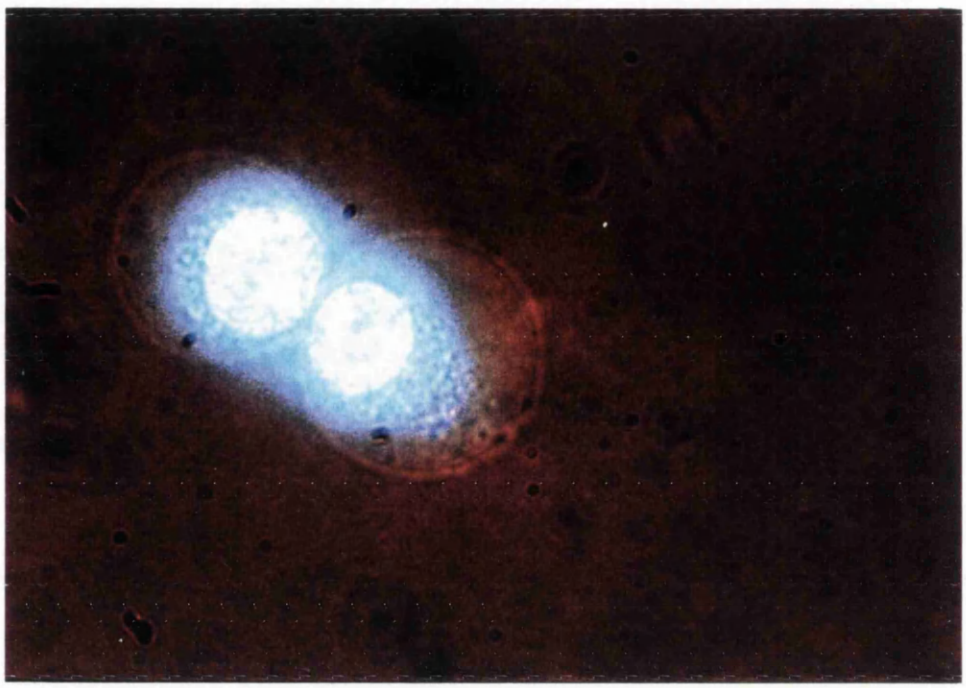
Swiss 3T3 cells were grown on 24-well plates and labelled with the appropriate radioisotopes as described for the relevant assay in intact cells (sections 2.3.1(i),.2.3.2(ii), 2.3.3). After labelling, the monolayers were washed in 0.5ml HHBG for 20 minutes. Cells were permeabilised by incubation with 150μl streptolysin-O (0.6U.ml$^{-1)}$ in permeabilisation buffer for 5 minutes as previously described by Currie and Wakelam (1992). This was followed by washing twice with 150μl of permeabilisation buffer over 10-15 seconds and stimulation with 150μl of the test reagent in permeabilisation buffer at the concentrations and times indicated. Following permeabilisation cells were treated exactly as described in the sections referring to the appropriate assay in intact cells

Figure 2.4 Entry of fluorescent dye into permeabilised Swiss 3T3 cells

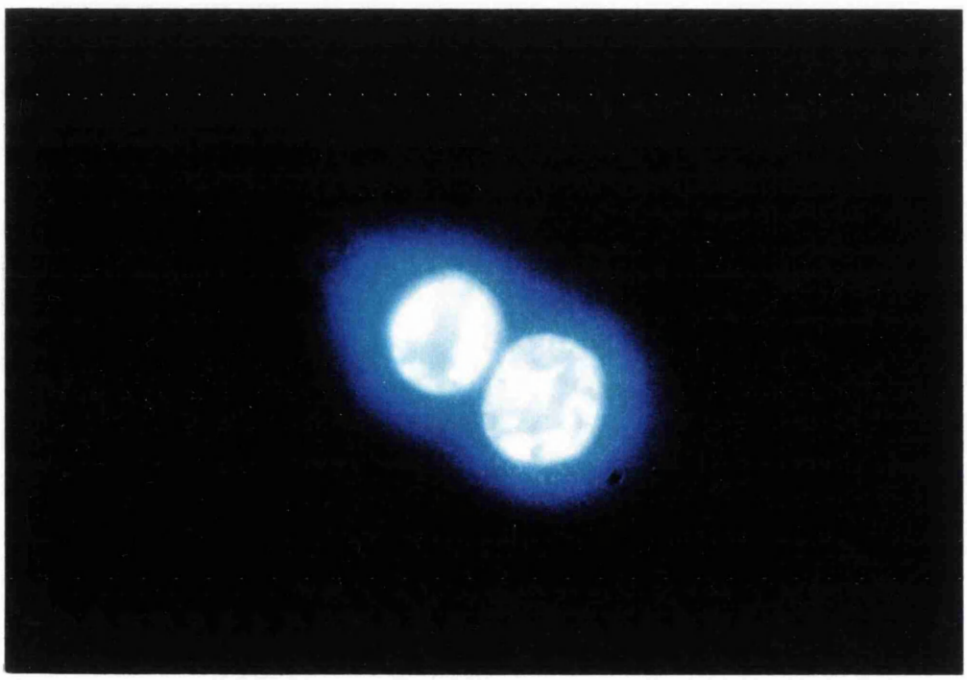
Swiss 3T3 cells were scraped from a $75cm^2$ flask, transferred to a centrifuge tube kept at 37^0C for 45 minutes to allow recovery. Cells were then permeabilised with streptolysin-O as described, together with the fluorescent stain bisbenzamide. The photographs show the cells as seen under a fluorescent microscope and are from.a single experiment

Both photographs (A) and (B) are of cells from the same experiment but viewed using different light intensities.

(A)



(B)



2.6 Miscellaneous procedures

2.6.1 Activation of sodium orthovanadate

The pH of a 10mM solution of sodium orthovanadate was adjusted to pH 10 using 1MNaOH and 1MHCl. The yellow solution was boiled until clear, then cooled to room temperature and the pH readjusted to pH 10. This procedure was repeated until the solution remained clear and the pH was stable. Aliquots of sodium orthovanadate were stored frozen at -20^{0}C.

2.6.2 Preparation of Peroxovanadate

Pervanadate was prepared as described in (Bianchini *et al* ., 1993). 10mM sodium orthovanadate and 10mM hydrogen peroxide were mixed and incubated at 25^{0}C for 15 minutes. Residual hydrogen peroxide was removed by the addition catalase (200μg.ml^{-1} final concentration). Control cells were treated with a solution where Hanks was substituted for sodium orthovanadate.

2.6.3 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 an unpaired 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

The Regulation of Phospholipase D Activity by Second- Messengers

3.1 Introduction

Phospholipase D can be activated both by G-protein coupled receptors which stimulate PLC-catalysed PtdIns(4,5)P_2 hydrolysis, such as those for bombesin (Cook and Wakelam, 1989, Cook *et al.*, 1991) and acetylcholine muscarinic cholinergic receptors (Qian and Drewes, 1989) and by receptor tyrosine kinases, such as that of the EGF receptor (Wright *et al.*, 1992). A plethora of factors are potentially involved in receptor-stimulated PLD activity. The relative contribution of the regulatory components to stimulated PLD activity is cell and agonist specific (reviewed Billah *et al.*, 1993). The experiments in this chapter aimed to characterise the regulatory mechanisms involved in bombesin-stimulated PLD activity in Swiss 3T3 fibroblasts.

The regulation of PtdIns-PLC by guanine-nucleotides is now well established (reviewed Rhee and Choi, 1992). Evidence is accumulating for the involvement of G-proteins in the regulation of PLD activity in a number of cells (reviewed Cockcroft, 1992). The differential sensitivity of stimulated PLD activity to pertussis-toxin suggested that different G-proteins could regulate PLD activity, in an analogous manner to the G-protein regulated PtdIns-PLC activity (Rhee and Choi, 1992). The bombesin receptor couples to PtdIns-PLC through the pertussis-toxin insensitive G_q (Plevin *et al.*, 1990; Smrcka *et al.*, 1991). Therefore the possibility that bombesin may regulate PLD activity through a G-protein, possibly through the same G-protein and/or a different one to that coupled to the hydrolysis of PtdIns(4,5)P_2 was investigated.

The extent to which receptor-stimulated PLD activity is dependent on PKC activation appears to vary with the system studied. Activation of PLD-catalysed PtdCho hydrolysis in prostaglandin E-stimulated human erythroleukaemia cells (Wu *et al.*, 1992) and fMetLeuPhe (fMLP)-stimulated HL60 cells was PKC independent (Rheinhold *et al.*, 1990). However, bombesin-stimulated PLD activity was inhibited by approximately 50% by 10μM of the selective PKC inhibitor Ro-31-8220, a concentration at which PMA-stimulated PLD activity was completely abolished

(Cook *et al.*, 1991). The pathway of PKC-independent activation of bombesin-stimulated PLD activity awaits clarification and thus is addressed in this chapter.

A possible PKC-independent regulatory pathway of PLD activity is through tyrosine phosphorylation, particularly as bombesin-stimulates the PKC-independent tyrosine phosphorylation of a number of proteins in Swiss 3T3 cells (Zachary *et al.*, 1991). The potential involvement of tyrosine kinases in the activation of PLD activity was realised by EGF-stimulation of the enzyme activity (Cook and Wakelam, 1992). This was found to occur independently of inositol lipid hydrolysis and PKC activation, but activation of the receptor kinase was essential. Furthermore, the stimulation of PLD activity through cytosolic tyrosine kinases has been reported in cells such as fMLP-stimulated neutrophils (Uings *et al.*, 1992).

The regulation of PLD by Ca^{2+} appeared to be mediated predominantly by PKC (reviewed Cook and Wakelam 1992). Ro-31-8220 inhibited A23187-stimulated PLD activity by approximately 50%, whereas the combined presence of Ro-31-8220 and EGTA was less than additive in inhibiting bombesin-stimulated PLD activity. A possibility to be investigated was therefore that a Ca^{2+}-stimulated pathway of PLD activation , independent of PKC activation may exist and that this could involve a rise in Ca^{2+} from intracellular stores alone.

Experiments to elucidate the pathways involved in agonist-stimulated PLD activity were therefore undertaken by determining the contributions of components of the signal transduction pathway including; G-proteins, tyrosine kinases, Ca^{2+} and cyclic AMP.

3.2 Results

3.2.1 The regulation of bombesin-stimulated PLD activity by mobilisation of Ca^{2+} from intracellular stores

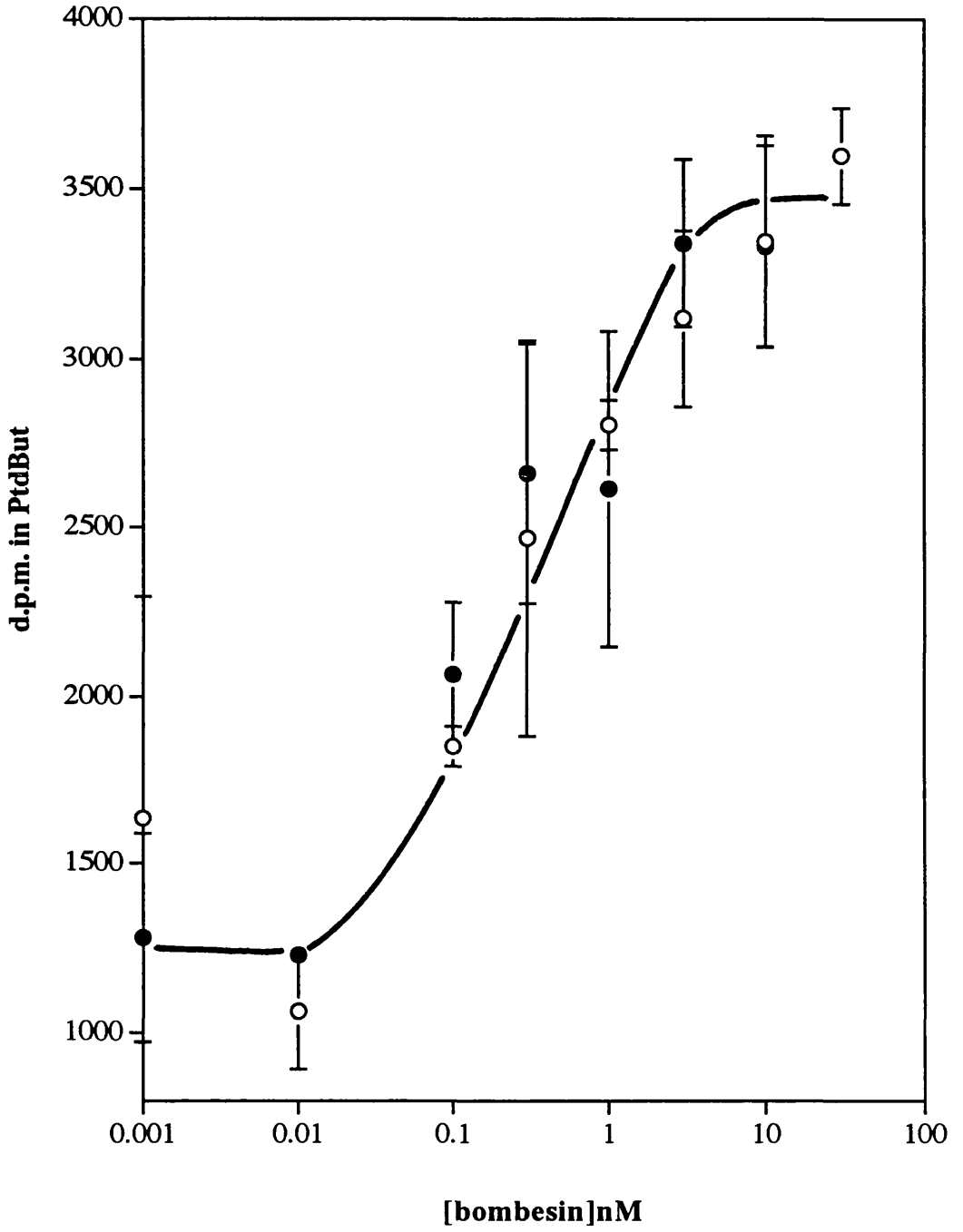
Experiments were performed to determine if PLD could be activated solely through the release of Ca^{2+} from intracellular stores. This store can be emptied by addition of the tumour promoter thapsigargin, an inhibitor of the Ca^{2+}-ATPase in non-mitochondrial stores (Thastrup *et al.*, 1990). In Swiss 3T3 cells 1μM thapsigargin was shown to stimulate an increase in $[Ca^{2+}]_i$ comparable with that stimulated by 100nM bombesin. However, kinetics of the thapsigargin-induced response were slower, with the maximal $[Ca^{2+}]_i$ not attained until approximately 22 seconds, whereas the bombesin-stimulated $[Ca^{2+}]_i$ was maximal by 6 seconds (Currie *et al.*, 1992). Figure 3.1 shows that 1μM thapsigargin was without effect on the dose/response curve of bombesin-stimulated PtdBut formation. At doses ranging from 10nM to 10μM, thapsigargin alone was unable to stimulate PLD activity during a 90s stimulus (results not shown). Longer incubation times were not employed because of the possibility of thapsigargin stimulating Ca^{2+} entry (Takemura *et al.*, 1989).

3.2.2 The regulation of PLD activity by guanine nucleotides

The effects of GTP analogues on PLD activity was investigated in streptolysin-O permeabilised cells. Streptolysin-O, a bacterial cytolysin, has been used to permeabilise several cell types including HL60 cells (Stutchfield and Cockcroft, 1988) and T lymphocytes (Alexander *et al.*, 1989). This agent generates pores in cholesterol-containing membranes of up to 12nm, which permits access of nucleotides but not the toxin, hence permeabilisation of intracellular membranes is prevented. Streptolysin-O permeabilisation of Swiss 3T3 cells was maximal at a concentration of $0.6U.ml^{-1}$.

Figure 3.1 Effect of thapsigargin upon bombesin-stimulated PLD activity.

[^{3}H]Palmitate-labelled cells were stimulated for 1 minute with increasing concentrations of bombesin as indicated, in the presence of 1μM-thapsigargin (•), or vehicle (0.1% DMSO), (o). Incubations were terminated by replacement of the medium with methanol. Chloroform extracts were prepared and [^{3}H]PtdBut quantified as descibed. Results are expressed as radioactivity in [^{3}H]PtdBut (mean ± S.D., d.p.m., n=3) and are from one experiment typical of three.


4000
3500
3000
2500
2000
1500
1000
d.p.m. in PtdBut
0.001
0.01
0.1
1
10
100
[bombesin]nM

measured by the release of LDH activity (Wakelam and Currie, 1992). This concentration caused approximately 90% permeabilisation of Swiss 3T3 cells as determined by entry of the fluorescent dye bisbenzimide (Fig. 2.4).

All permeabilisation experiments were performed at a resting Ca^{2+} concentration of 150nM in order to maintain physiologically relevant conditions as closely as possible. Permeabilisation of cells was found to activate PLD activity as determined by [^{3}H]PtdBut accumulation when streptolysin-O was added to cells in the presence or absence of 30mM butan-1-ol (radioactivity in [^{3}H]PtdBut, mean ± S.D., d.p.m., n=3: permeabilisation -ButOH, 1480 ± 832; permeabilisation + ButOH, 4547 ± 380, from a single experiment typical of three). All permeabilisations were therefore performed in the absence of alcohol, followed by two rapid washes in buffer before stimulation in the presence of butanol.

Figure 3.2 shows the time course of GTPγS-stimulated PLD activity in Swiss 3T3 cells. In some experiments GTPγS-stimulated [^{3}H]PtdBut accumulation was elevated above basal after 1 minute, but was always significant after 5 minutes (p=0.015 ±0.015, n=3). The extent of [^{3}H]PtdBut accumulation in response to 5 minute GTPγS treatment was not affected by washing cells for times up to 15 minutes subsequent to permeabilisation (results not shown). Fig 3.3(A) shows that 30μM GTPγS did not affect bombesin-stimulated PLD activity at any concentration of bombesin tested. Fig. 3.3(B) shows that at low [GTPγS] the presence of bombesin potentiated stimulated-[^{3}H]PtdBut generation. However in all experiments, there was no potentiating effect of bombesin on concentrations of [GTPγS] of 30μM and above. Elevation of GTPγS-stimulated PLD activity above basal was always significant at a 1μM guanine nucleotide concentration (p= 0.004±0.004, n=3). 30μM GTPγS and 100nM PMA were synergistic in the generation of [^{3}H]PtdBut over a 10 minute stimulation period (p=0.01 ± 0.01, n=3) (Fig. 3.4).

Figure 3.2 Time course of GTPγS-stimulated PLD activity.

[^{3}H]Palmitate-labelled cells were permeabilised with streptolysin-O as described (section 2.5), then stimulated for times indicated with 30μM GTPγS in the presence of 30mM butanol. Incubations were terminated by addition of methanol. Chloroform extracts were prepared and [^{3}H]PtdBut quantified as described. Results are expressed as radioactivity in [^{3}H]PtdBut ± S.D. (mean ± S.D., d.p.m., n=3) and are from a single experiment representative of three.

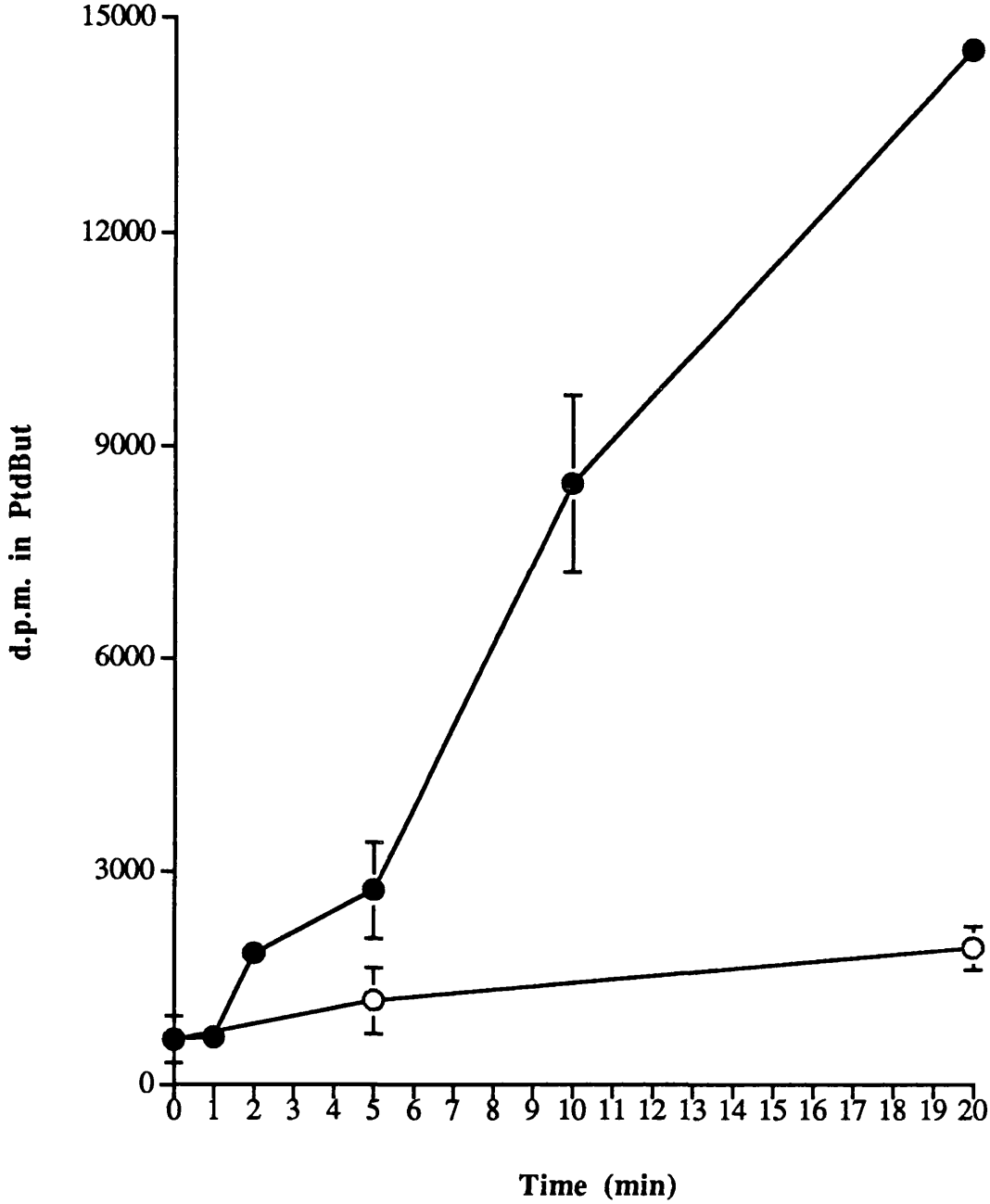

15000
12000
9000
6000
3000
0
d.p.m. in PtdBut
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time (min)

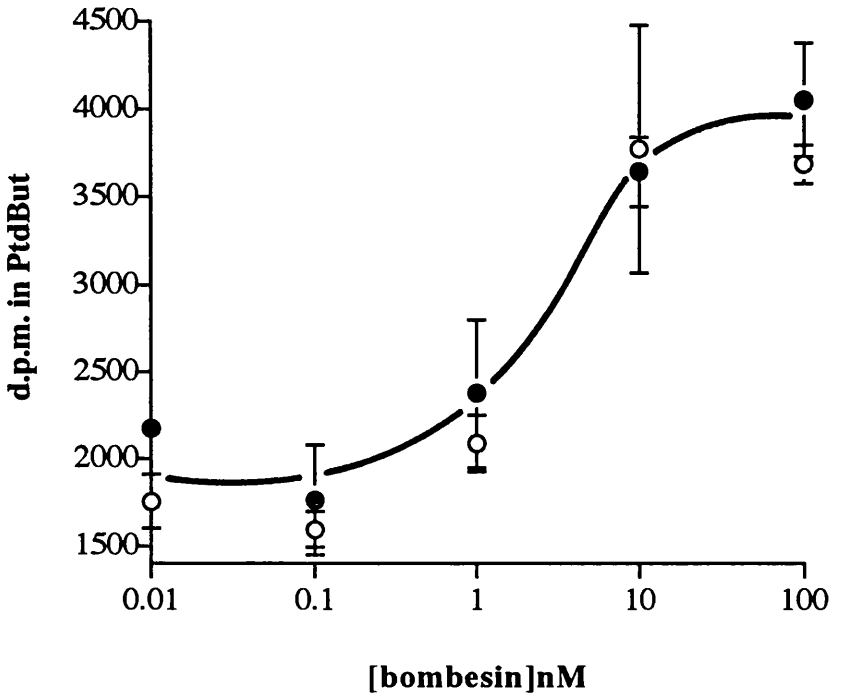
Figure 3.3 (A) Effect of bombesin on 30μM GTPγS-stimulated PLD activity; (B) Effect of GTPγS on 3nM bombesin-stimulated PLD activity.

(A) Effect of 30μM GTPγS on bombesin-stimulated PLD activity

[^{3}H]Palmitate labelled cells were permeabilised using streptolysin-O (0.6U.ml^{-1}) and stimulated for 1 minute with increasing concentrations of bombesin and 30mM butanol in the presence (•), or absence (o), of 30μM GTPγS . Incubations were terminated by the addition of chloroform. Chloroform extracts were prepared and [^{3}H]PtdBut quantified as described. Results are expressed as radioactivity in [^{3}H]PtdBut generated (mean ± S.D., d.p.m., n=3) and are from a single experiment typical of three.

(B) Effect of 3nM bombesin on GTPγS-stimulated PLD activity.

[^{3}H]Palmitate labelled cells were permeabilised using streptolysin-O (0.6U.ml^{-1}) and stimulated for 5 minutes with increasing concentrations of GTPγS and 30mM butanol in the presence (•), or absence (o), of 3nM bombesin. Incubations were terminated by addition of methanol. Chloroform extracts were prepared and [^{3}H]PtdBut generated was determined as described. Results are expressed as radioactivity in [^{3}H]PtdBut, (mean ± S.D., d.p.m., n=3) and are from a single experiment typical of three.


(A)
4500
4000
3500
3000
2500
2000
1500
d.p.m. in PtdBut
0.01
0.1
1
10
100
[bombesin]nM

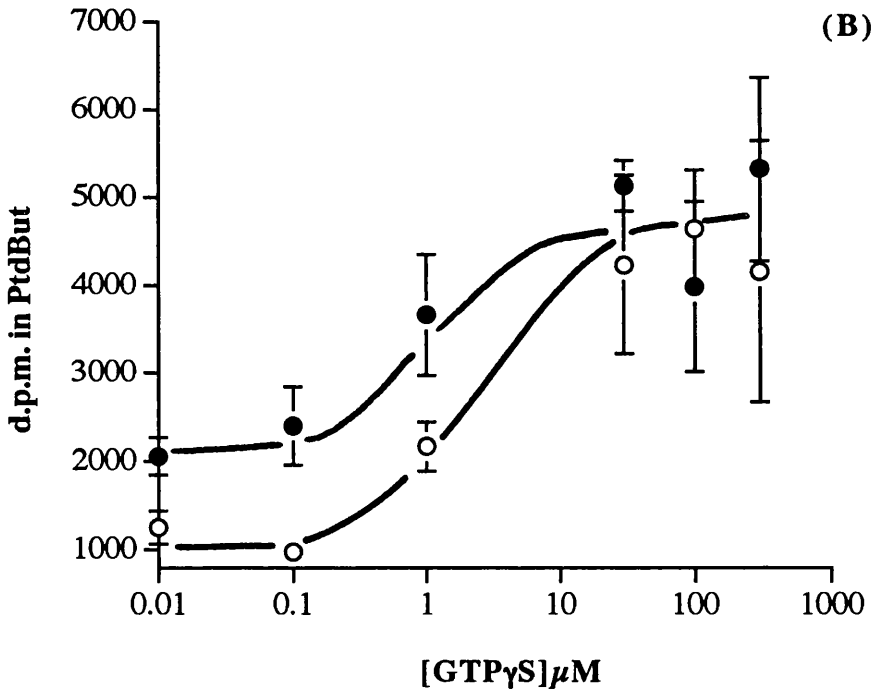
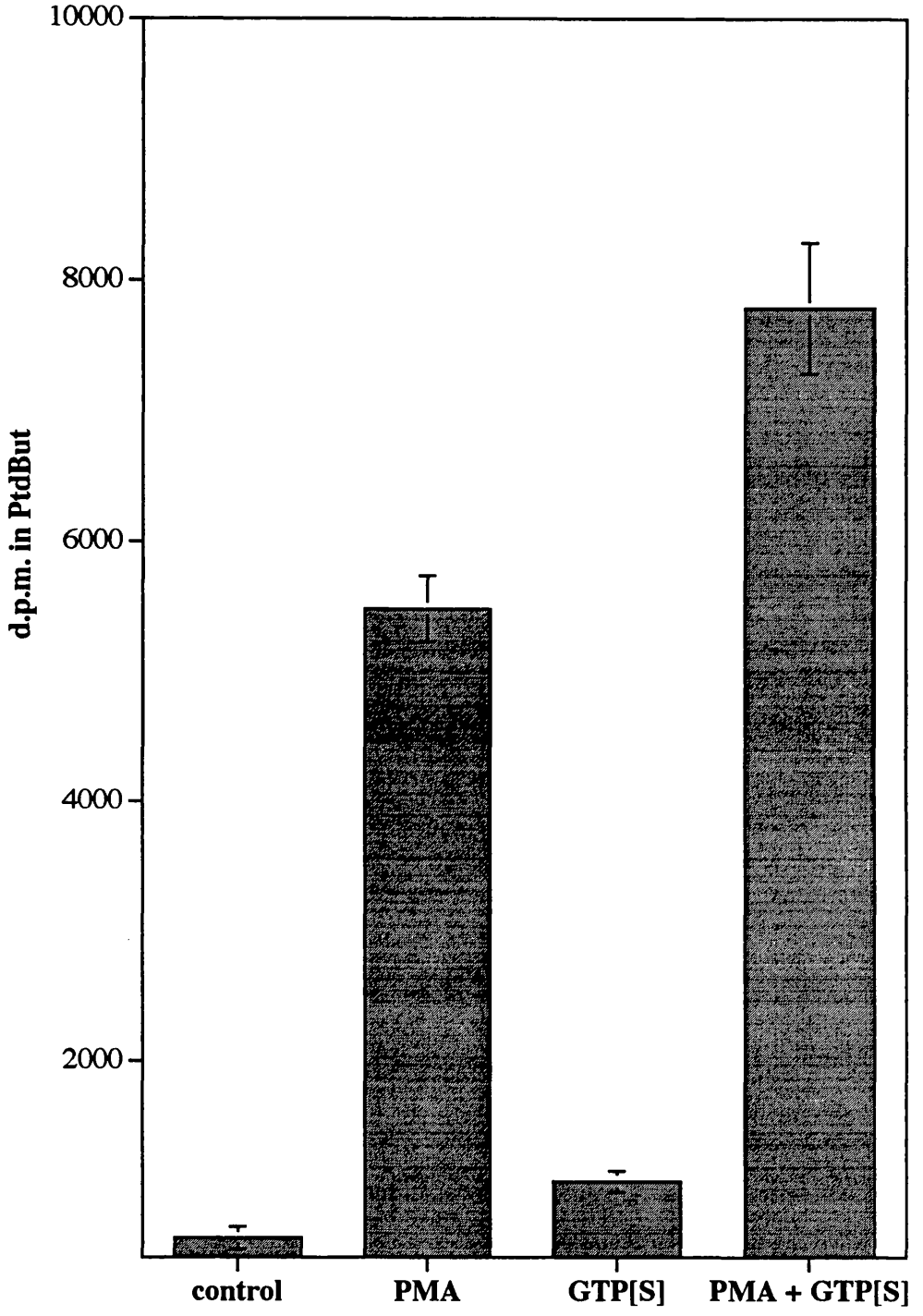

(B)
7000
6000
5000
4000
3000
2000
1000
d.p.m. in PtdBut
0.01
0.1
1
10
100
1000
[GTPγS]μM

Figure 3.4 Effect of GTPγS on PMA-stimulated PLD activity.

[^{3}H]Palmitate labelled cells were permeabilised with streptolysin-O (0.6U.ml^{-1}) and stimulated for 10 minutes with vehicle, 30μM GTPγS, 100nM PMA or 100nM PMA + 30μM GTPγS. All additions contained a final concentration of 0.01% DMSO and 30mM butanol. Incubations were terminated by the addition of ice-cold methanol. Chloroform extracts were prepared and the generation of [^{3}H]PtdBut was quantified as described. Results are expressed as radioactivity in [^{3}H]PtdBut, (mean ± S.D., d.p.m., n=3) and are from a single experiment typical of three.


10000
8000
6000
4000
2000
d.p.m. in PtdBut
control
PMA
GTP[S]
PMA + GTP[S]
Treatment

The response to bombesin was significantly reduced by pre-incubation of permeabilised cells with 2mM GDPβS the non-hydrolysable GDP analogue ($p=0.02\pm 0.01$), n=3), whereas activation of PKC using PMA, stimulated PLD activity in a manner that was unaffected by GDPβS (Table 3.1). The percentage inhibition of bombesin-stimulated [^{3}H]PtdBut generation, in the presence of GDPβS, over three separate experiments was, $58 \pm 19\%$

Cells were depleted of cellular ATP by treatment with the metabolic inhibitors antimycin A and deoxyglucose, thereby preventing the activation of PLC and kinases (Nielson *et al.*, 1991; Geny and Cockcroft, 1992). Double-labelling of cells enabled the generation of [^{3}H]PtdBut and total [^{3}H]inositol phosphates to be measured in the same well. Figure 3.5(B) shows that pretreatment with the metabolic inhibitors reduced the stimulation of total [^{3}H] inositol phosphates by 30μM GTPγS, 100nM bombesin or 30μM GTPγS + 100nM bombesin, to near basal levels. Stimulation of PLD activity was reduced to similar levels, or in some experiments, was completely abolished. (Fig 3.5(A)).

3.2.3 Regulation of bombesin-stimulated PLD activity by tyrosine phosphorylation

The analysis of protein tyrosine phosphorylation after exposure of cells to stimulant, was determined by immunoblotting of cell lysates with an anti-phosphotyrosine monoclonal antibody.

Experiments were performed to control for the antibody specificity. Figure 3.6 shows that in cells stimulated with pervanadate detetection of tyrosine phosphorylated proteins was only possible when both primary and the secondary antibody were present. Pervanadate elevates tyrosine phosphorylation of cellular proteins through inhibition of phosphotyrosine phosphatase (see later for fuller description). In the presence of secondary antibody only, several non-specific bands consistently appeared at molecular weights of approximately 200, 97 and 60 kDa,

Table 3.1 Effect of GDPβS on bombesin- and PMA-stimulated PLD activity.

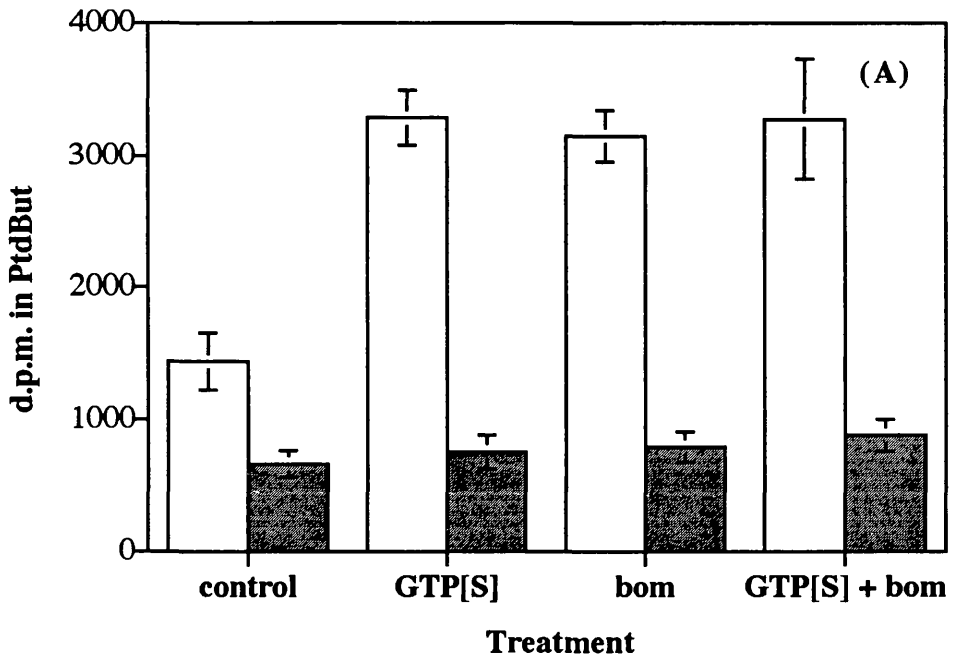
[^{3}H]Palmitate labelled cells were permeabilised with streptolysin-O ($0.6Uml^{-1}$), then preincubated with 2mM GDPβS for 1 minute. Cells were then stimulated in the presence of 30mM butanol with: **(A)** 100nM bombesin for 5 minutes; **(B)** 100nM PMA for 10 minutes, where **(A)** and **(B)** are from the same experiment. Incubations were terminated by addition of methanol. Chloroform extracts were prepared and the generation of [^{3}H]Ptdbut was determined as described. Results are expressed as radioactivity in [^{3}H]PtdBut (mean ± S.D., d.p.m., n=3) and are from a single experiment typical of three.

Treatment	(A) bombesin	(B) PMA
	d.p.m. in PtdBut	d.p.m. in PtdBut
control	455 ± 40	562 ± 32
control + GDPβ S	711 ± 203	550 ± 42
stimulated	2559 ± 427	5127 ± 839
stimulated + GDPβ S	1664 ± 137	6725 ± 550

Figure 3.5 Effect of cellular ATP depletion on; (A) stimulated PLD activity and (B) stimulated generation of total inositol phosphates.

Cells labelled with [^{3}H] inositol and [^{3}H] palmitate were washed with buffer in the presence or absence of 10mM glucose for 20 minutes. 4 minutes prior to permeabilisation with streptolysin-O, cells to be metabolically inhibited were preincubated in buffer minus glucose containing 6mM deoxyglucose and 5μM antimycin A. Cells that were not to be metabolically inhibited were preincubated in buffer containg 0.01% DMSO and 10mM glucose. Permeabilisations, subsequent washes, preincubations and stimulations were performed in the presence or absence of metabolic inhibitors. Cells were preincubated for 10 minutes subsequent to permeabilisation, with 10mM LiCl and 30mM butanol then stimulated for 5 minutes with 30μM GTPγ S, 3nM bombesin or 30μMGTPγS + 3nM bombesin. Incubations were terminated by addition of methanol. Chloroform extracts were prepared as described and [^{3}H]PtdBut and total [^{3}H]inositol phosphates quantified as previously described. Results are expressed as radioactivity in; **(A)** [^{3}H]PtdBut and **(B)** total [^{3}H]inositol phosphates, (mean ± S.D., d.p.m., n=3) and are from a single experiment representative of three. Unshaded columns, control cells; shaded columns, cells depleted of cellular ATP.

For cells that were to be depleted of cellular ATP, ATP was omitted from the permeabilisation buffer. Cells stimulated with: vehicle, control; 3nM bombesin, bom; 3nM bombesin + GTPγS, GTP[S] + bom; GTPγS, GTP[S].


4000
3000
2000
1000
0
(A)
d.p.m. in PtdBut
control
GTP[S]
bom
GTP[S] + bom
Treatment

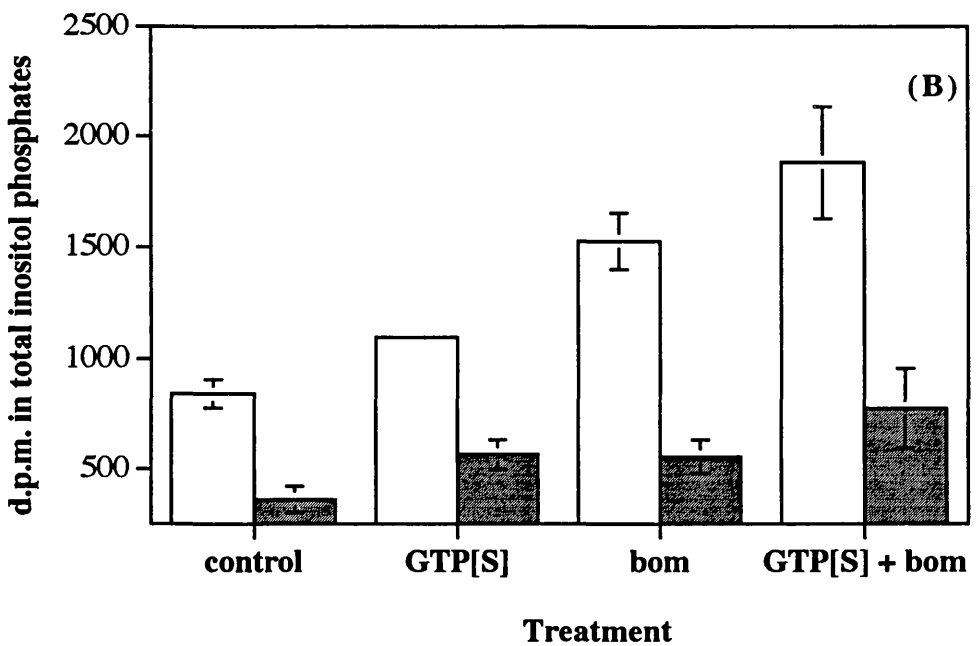

2500
2000
1500
1000
500
(B)
d.p.m. in total inositol phosphates
control
GTP[S]
bom
GTP[S] + bom
Treatment

Figure 3.6 Effect of omitting primary or secondary antibody on detection of pervanadate-stimulated protein tyrosine phosphorylation.

Cells were stimulated with 0.5mM pervanadate for 10 minutes before being lysed. An aliquot of cell lysate containing approximately 30μg of protein was analysed for protein phosphotyrosine phosphorylation as previously described. The blots were incubated in the presence or absence of the primary anti-phosphotyrosine antibody and secondary anti-mouse IgG peroxidase conjugated antibody. Where the primary antibody was omitted, blots were probed in TBST containing 3% BSA (fraction V, electrophoresis grade). Where the secondary antibody was omitted, blots were probed in TBST containing 5% BSA. The numbered photograph corresponds to the lanes of the blot incubated as follows;

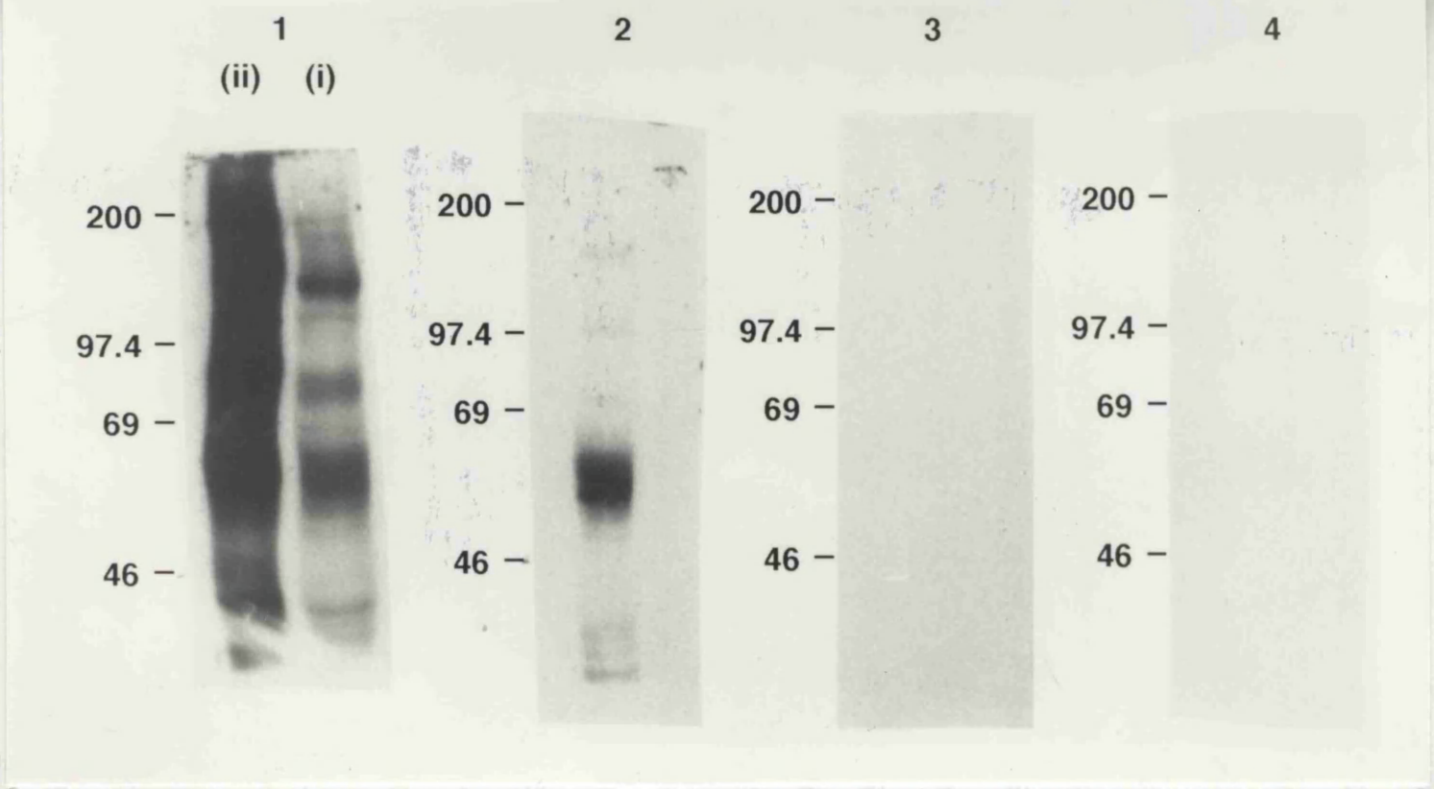
1(i) control cells probed with primary and secondary antibody

1(ii) pervanadate-treated cells probed with primary and secondary antibody

(2) pervanadate-treated cells probed with secondary antibody only

(3) pervanadate-treated cells probed with primary antibody only.

(4) pervanadate-treated cells incubated with no primary or secondary antibody.


1
(ii)
(i)
2
3
4
200
97.4
69
46
200
97.4
69
46
200
97.4
69
46
200
97.4
69
46

with the most prominent being that at 60kDa. In some cases these were only apparent after longer exposure to the film than would normally be necessary for observation of the bands specific to tyrosine phosphorylated proteins. As the secondary antibody was anti-mouse IgG, the bands present after incubation with secondary antibody alone may have been due to non-specific interactions with the mouse Swiss 3T3 cell-lysates. After incubation with primary antibody alone or with neither antibody there was no detectable signal after 'ECl' development.

Competition experiments were performed using 1mM phosphotyrosine to inhibit the binding of the antibody and with 1mM phosphoserine and 1mM phosphothreonine to control for cross-reactivity with other phosphoamino acids. Figure 3.7 shows that whereas the presence of 1mM phosphotyrosine prevented the appearance of most bands observable in stimulated cells, 1mM phosphothreonine and phosphoserine had no detectable effect, either in lysates from pervanadate (Fig. 3.7(A)) or bombesin-stimulated cells (Fig. 3.7(B)). The faint bands observed after incubation with 1mM phosphotyrosine are probably the non-specific bands due to the presence of the secondary antibody alone, as shown in Figure 3.6.

The time course and dose-response of bombesin-stimulated tyrosine phosphorylated proteins was comparable with that first reported by Zachary *et al.*, (1991) using immunoprecipitation and incubation with [γ-^{32}P]ATP. The time course showed a rapid increase in bands of molecular weight of approximately, 110-130kDa, 80-85kDa, 65-70kDa. and 40kDa (results not shown).

Pervanadate (peroxide of vanadate) was used to investigate whether increasing tyrosine phosphorylation in the absence of a receptor stimulation event could activate PLD. Pervanadate, prepared by combining vanadate and H_2O_2 (section 2.6.2), is a known insulin mimetic being 10^2-10^3 more potent than vanadate alone (Kadota *et al.*, 1987). It has been reported to be a potent inhibitor of a phosphotyrosine phosphatase (Fantus *et al.*, 1989) and also to stimulate tyrosine

Figure 3.7 Effect of probing with anti-phosphotyrosine antibody in the presence of phosphotyrosine, phosphothreonine and phosphoserine on; (A(a)) and (A(b)) Cells stimulated with 100nM bombesin for 2 minutes. (B) Cells stimulated with 0.5mM pervanadate for 10 minutes.

Cells were stimulated: **(A(a))** and **(A(b))** for 2 minutes with 100nM bombesin or HHBG; **(B)** for 10 minutes with vehicle or 0.5mM pervanadate. Cells were lysed and an aliquot of the lysates, corresponding to approximately 30μg of protein was analysed for protein phosphotyrosine phosphorylation as previously described. Incubations with anti-phosphotyrosine anti-body were performed in the presence of 1mM phosphotyrosine, 1mM phosphothreonine, 1mM phosphoserine or with the anti-phosphotyrosine antibody alone. The numbered lanes in the photograph correspond to proteins from cell lysates analysed for phosphotyrosine content under the different incubation conditions as follows;

1(i) Control cell lysates incubated with primary antibody alone.

1(ii) Stimulated cell lysates incubated with primary antibody alone.

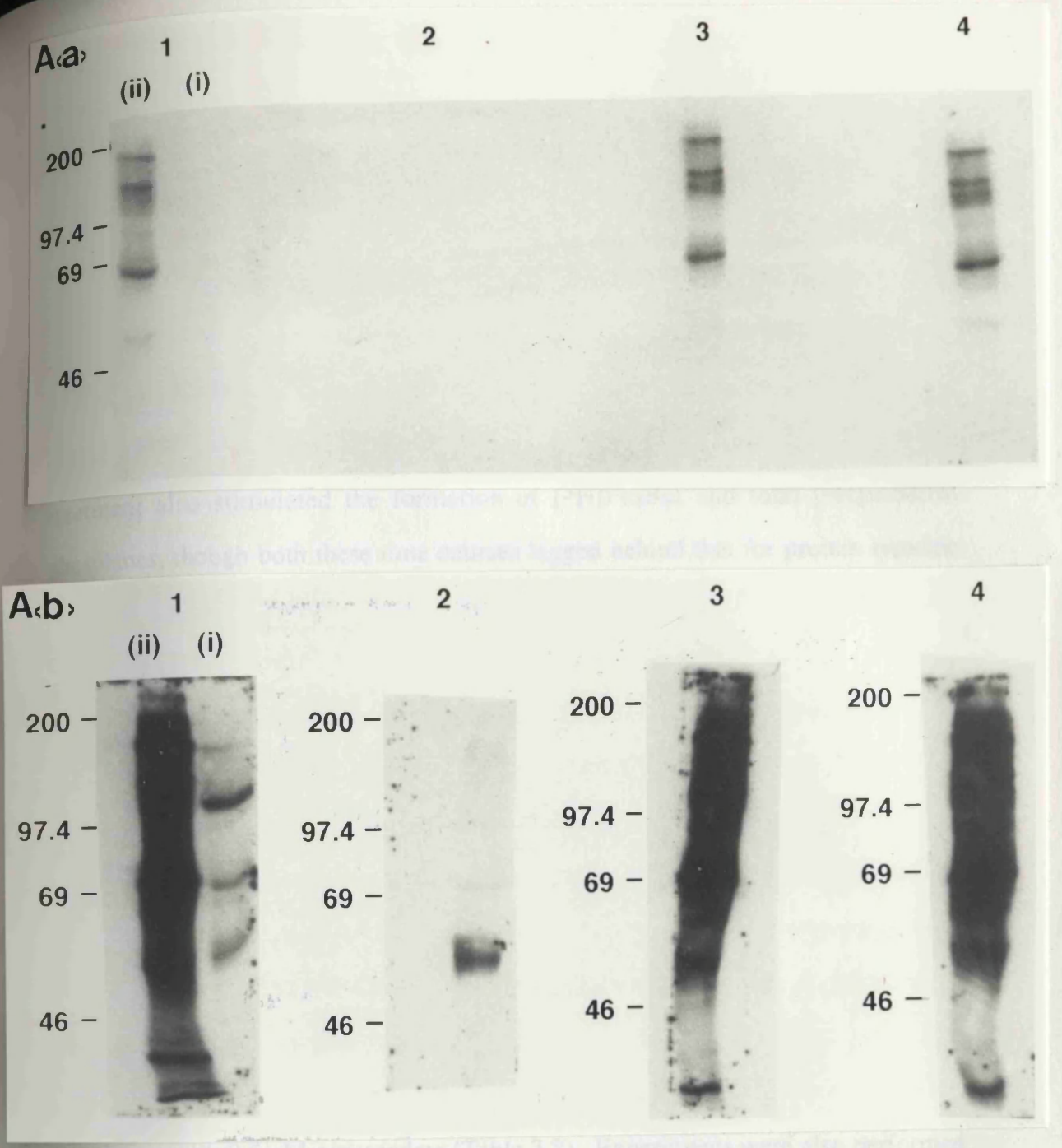
(2) Stimulated cell lysates incubated with primary antibody + 1mM phosphotyrosine.

(3) Lysates from stimulated cells, incubated with primary antibody + 1mM phosphothreonine.

(4) Lysates from stimulated cells, incubated with primary antibody + 1mM phosphoserine.

The two photographs **(A(a))** and **(A(b))** are from the same experiment but after different lengths of exposure of the film to the blot.

Results are from a single experiment representative of three.


A‹a›
1
2
3
4
(ii)
(i)
200
97.4
69
46
A‹b›
1
2
3
4
(ii)
(i)
200
97.4
69
46
B
1
2
3
4
(ii)
(i)
200
97.4
69
46

phosphorylation in a number of cells including human platelets and T-cells (Blake *et al.*, 1993; Secrist *et al.*, 1993).

Figure 3.8 shows that the time course of 0.5mM pervanadate-stimulated tyrosine phosphorylation was rapid, commencing by 30 seconds and increasing steadily over the time measured, up to 30 minutes. Tyrosine phosphorylation of protein bands with a molecular weight of approximately; 200kDa, 110-130kDa, 97kDa, 80kDa, 65kd and 45kDa were observed. Figure 3.9 shows that pervanadate treatment also stimulated the formation of [^{3}H]PtdBut and total [^{3}H]inositol phosphates, though both these time courses lagged behind that for protein tyrosine phosphorylation. Elevation of [^{3}H]PtdBut and total [^{3}H]inositol phosphates were always significant above basal after 10 minutes of stimulation. Whilst the extent of stimulation varied considerably between experiments, the generation of total [^{3}H]inositol phosphates was generally less than that of [^{3}H]PtdBut.

Figure 3.10 (A) shows that 10μM Ro-31-8220 had a negligible effect on a 10 minute stimulation of tyrosine phosphorylation with 0.5mM pervanadate, but slightly reduced the phosphotyrosine content of proteins in cells stimulated with 100nM bombesin and pervanadate. Similar results were achieved using a 5 minute stimulation with 0.5mM pervanadate and 3nM bombesin (Fig. 3.10(B)). 10μM Ro-31-8220 did not attenuate the [^{3}H]PtdBut accumulation attained after a 10 minute stimulation with 0.5mM pervanadate (Table 3.2). Experiments were also performed where 0.1nM bombesin was used to activate PLD for 5 minutes in the presence of 0.5mM pervanadate. After this time tyrosine phosphorylation was elevated but no pervanadate-stimulated PLD activity was observed. These experiments showed that bombesin-stimulated PLD activity was not potentiated in the presence of pervanadate (results not shown).

Having shown that PLD could potentially be activated by elevation of protein tyrosine phosphorylation, the effect of tyrosine kinase inhibitors on bombesin-stimulated PLD activity was investigated. Lavendustin, the erbstatin analogue methyl 2,5-dihydroxycinnamate and Genistein (reviewed Workman *et al.*,

Figure 3.8 Time course of protein tyrosine-phosphorylation in pervanadate-treated cells.

Cells were stimulated with vehicle or 0.5mM pervanadate for times as indicated. Cells were lysed and an aliquot, containing 30μg of protein, was analysed for protein tyrosine phosphorylation as described The Western blot was probed with the U.B.I. anti-phosphotyrosine antibody. Results are from a single experiment representative of three.

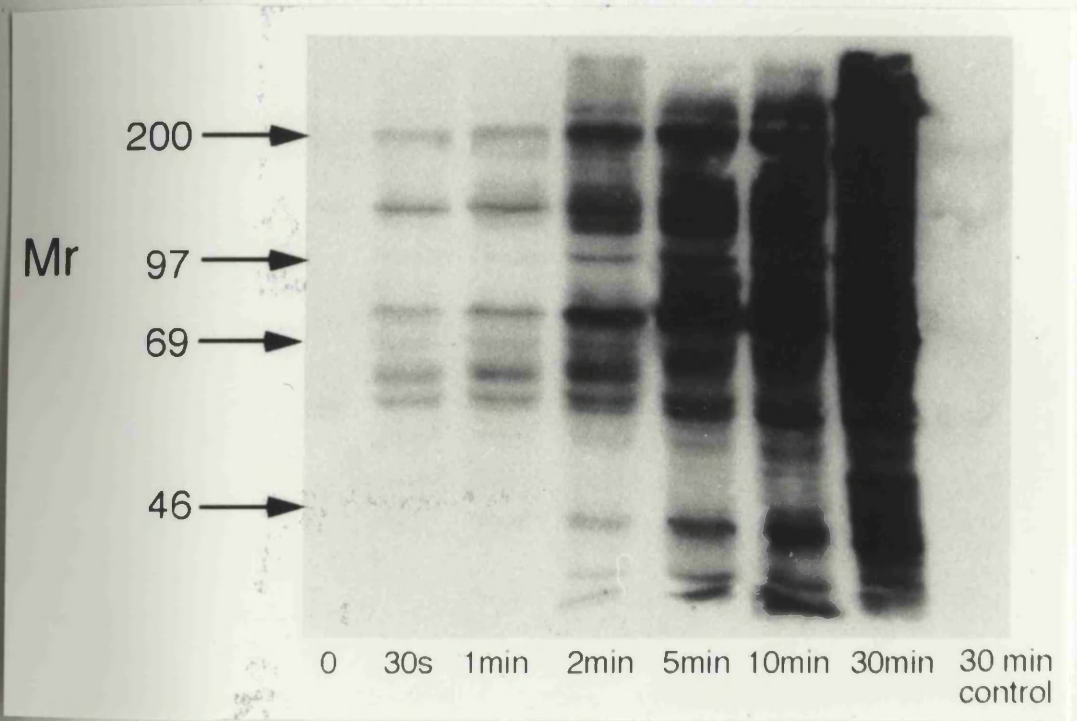
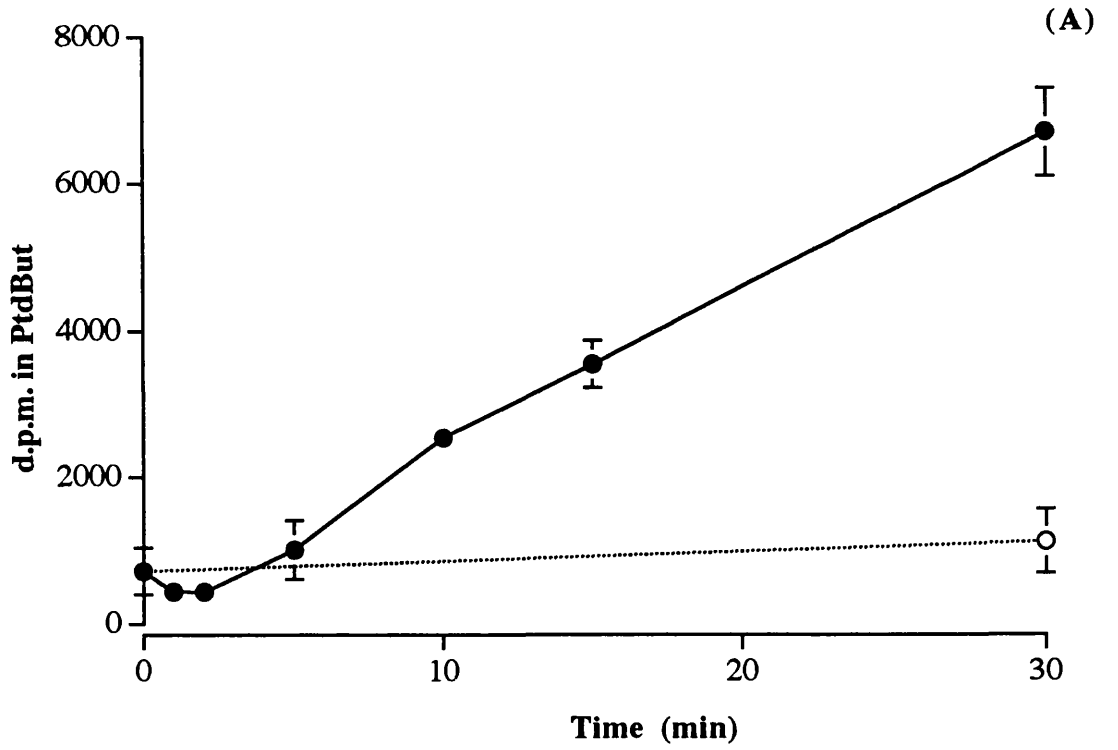

Mr
200
97
69
46
0
30s
1min
2min
5min
10min
30min
30 min
control

Figure 3.9 Time course of PLD activity and generation of total inositol phosphates in pervanadate-treated, double-labelled cells

Cells labelled with [^{3}H]palmitate and [^{3}H]inositol were preincubated for 10 minutes with 10mM LiCl and 30mM butanol, then stimulated for times indicated with 0.5mM pervanadate (•), or vehicle (o). Chloroform extracts were prepared and the generation of **(A)** [^{3}H]PtdBut and **(B)** [^{3}H]total inositol phosphates, quantified as described. Results are expressed as radioactivity in **(A)** [^{3}H]PtdBut or **(B)** total [^{3}H]inositol phosphates, (mean ± S.D., d.p.m., n=3) and are from a single experiment representative of three.


(A)
8000
6000
4000
2000
0
d.p.m. in PtdBut
0
10
20
30
Time (min)

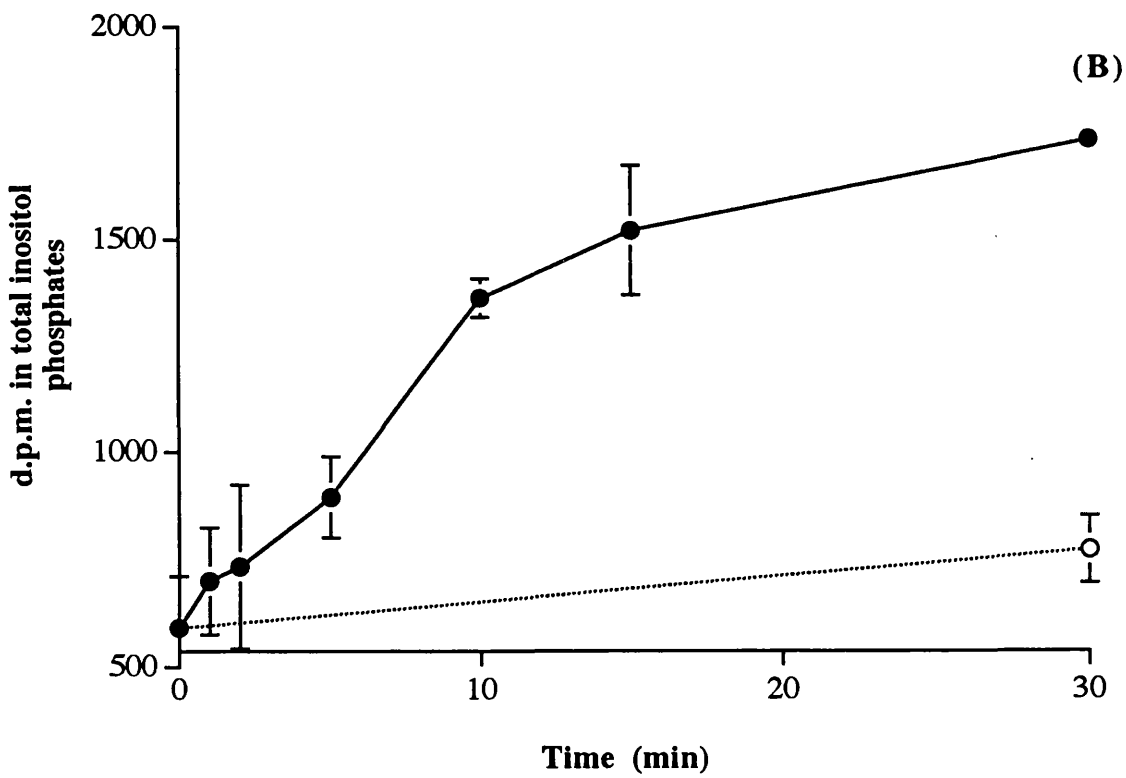

(B)
2000
1500
1000
500
d.p.m. in total inositol phosphates
0
10
20
30
Time (min)

Figure 3.10 Effect of bombesin and Ro-31-8220 on protein tyrosine phosphorylation in pervanadate-treated cells.

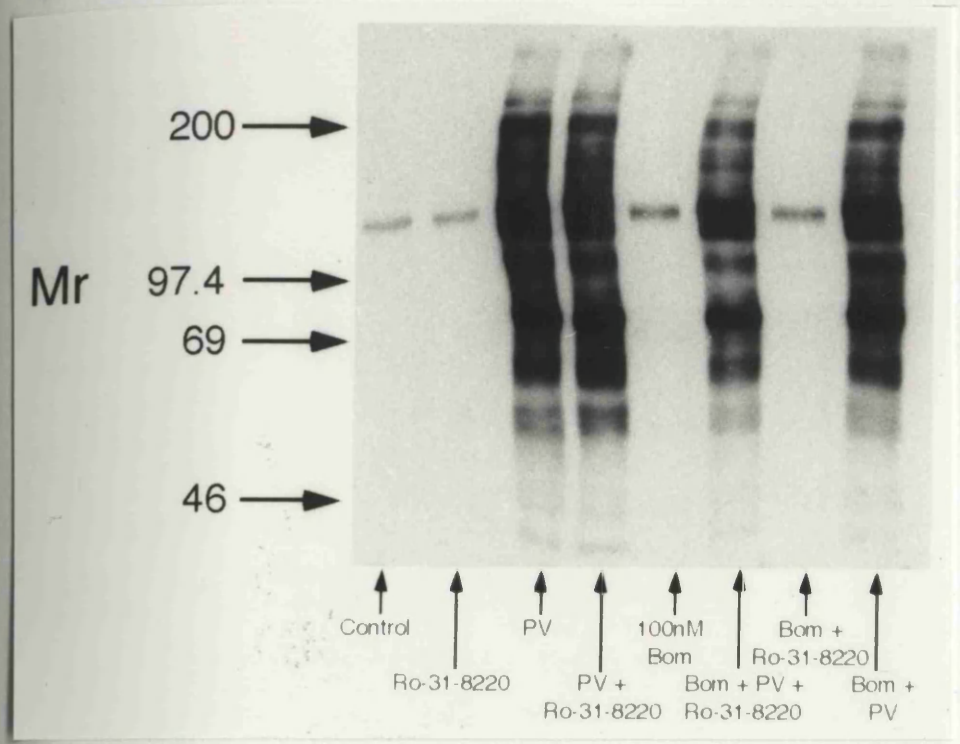
Cells were preincubated with vehicle (0.1% DMSO) or 10μM Ro-31-8220 for 10 minutes, then stimulated in the presence of Ro-31-8220 or vehicle (0.1% DMSO) as follows;

(A) For 10 minutes with 0.5mM pervanadate, vehicle or 100nM bombesin. Cells were lysed and an aliquot of cell lysate corresponding to approximately 15μg of protein was analysed for protein phosphotyrosine phosphorylation as previously described.

(B) For 5 minutes with 0.5mM pervanadate, vehicle or 3nM bombesin. Cells were lysed and a portion of cell lysate containing approximately 30μg protein was analysed for protein phosphotyrosine phosphorylation as previously described. Phosphotyrosine containing proteins were detected using the U.B.I. anti-phosphotyrosine antibody.

Results are from single experiments representative of three.
Pervanadate abbreviated to PV; bombesin to Bom.

A


200
Mr
97.4
69
46
Control
Ro-31-8220
PV
PV +
Ro-31-8220
100nM
Bom
Bom + PV +
Ro-31-8220
Bom +
Ro-31-8220
Bom +
PV

B

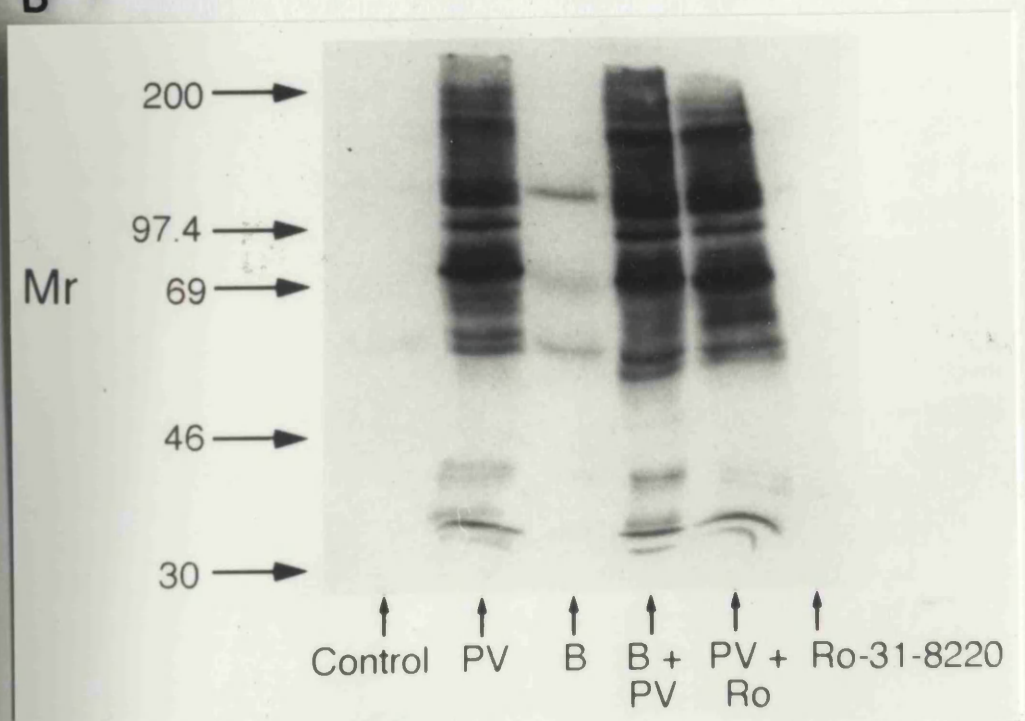

200
97.4
Mr
69
46
30
Control
PV
B
B +
PV
PV +
Ro
Ro-31-8220

Table 3.2 Effect of bombesin and Ro-31-8220 on pervanadate-stimulated PLD activity

[^{3}H]Palmitate labelled cells were preincubated with vehicle (0.1% DMSO) or 10μM Ro-31-8220 for 10 minutes in the presence of 30mM butanol, then stimulated for 10 minutes in the presence or absence of 0.5mM pervanadate, with vehicle or 100nM bombesin. Incubations were terminated by the addition of ice-cold methanol. Chloroform extracts were prepared and the generation of [^{3}H]PtdBut quantified as described. Results are expressed as radioactivity in [^{3}H]PtdBut (mean ± S.D., d.p.m., n=3) and are from a single experiment representative of three.

Treatment	d.p.m. in PtdBut
control	1191 ± 418
control + Ro-31-8220	1499 ± 67
pervanadate	6356 ± 1104
bombesin	6770 ± 612
bombesin + Ro-31-8220	3428 ± 433
pervanadate + Ro-31-8220	5162 ± 741

1992), were screened for their ability to affect the protein tyrosine phosphorylation following a 2 minute stimulation with 3nM bombesin. Neither Erbstatin or Lavendustin significantly inhibited bombesin-stimulated protein phosphotyrosine content (Fig. 3.11), nor did they affect bombesin-stimulated [^{3}H]PtdBut accumulation (results not shown). Figure 3.12 shows that 100μM Genistein consistently inhibited protein tyrosine phosphorylation in lysates from cells stimulated with 3nM bombesin. Genistein also decreased the basal protein tyrosine phosphorylation in cell lysates. 100μM Genistein significantly inhibited bombesin-stimulated PLD activity by approximately 50% ($p=0.011 \pm 0.006$). No significant inhibition was observed at lower concentrations of the inhibitor tested (Table 3.3).

The effect of the PKC inhibitor Ro-31-8220 and 100μM Genistein on 100nM bombesin-stimulated [^{3}H]PtdBut was investigated. Fig 3.13 shows that although 100μM Genistein did not significantly attenuate bombesin-stimulated [^{3}H]PtdBut generation, Ro-31-8220 partially inhibited the stimulation of PLD activity, whilst the presence of Genistein and Ro-31-8220 completely abolished the bombesin response.

3.2.4 Regulation of PLD activity by other signalling pathways

The effect of cross-talk between the adenylyl-cyclase signalling pathway and that activating PLD was investigated using agents known to elevate cyclic AMP. Pretreatment with 10μM-forskolin and 100μM isobutylmethylxanthine (IBMX) for 5 or 10 minutes, or 1mM dibutyryl cyclic AMP for 15 minutes were shown to elevate cyclic AMP in cells by 20-30-fold above basal (Table 3.4). Bombesin alone had no effect, neither did it augment cyclic AMP generation in response to forskolin or dibutyryl cyclic AMP. Table 3.5 shows that similar treatments with forskolin and dibutyryl cyclic AMP had no effect upon subsequent bombesin-stimulated PLD activity.

Figure 3.11 Effect of Erbstatin and Lavendustin on bombesin-stimulated protein tyrosine phosphorylation

Cells were preincubated for 1hour with 10μM of the erbstatin analogue methyl [2,5,-dihydroxy] cinnamate, 5μM lavendustin, or vehicle, then stimulated for 2 minutes with a final concentration of 3nM bombesin and lysed. An aliquot of each lysate corresponding to approximately 25μg of protein was analysed for protein phosphotyrosine content as described. The numbered lanes in the phostograph correspond to protein phosphotyrosine in lysates from cells treated as follows;

(1) Preincubated with 0.2% DMSO , stimulated with HHBG.

(2) Preincubated with 0.2% DMSO, stimulated with bombesin.

(3) Preincubated with HHBG, stimulated with HHBG.

(4) Preincubated with HHBG, stimulated with bombesin.

(5) Preincubated with erbstatin, stimulated with HHBG.

(6) Preincubated with erbstatin, stimulated with bombesin.

(7) Preincubated with 0.5% DMSO, stimulated with HHBG.

(8) Preincubated with 0.5% DMSO, stimulated with bombesin.

(9) Preincubated with lavendustin, stimulated with HHBG.

(10) Preincubated with lavendustin, stimulated with bombesin.

Results are from a single experiment typical of three.

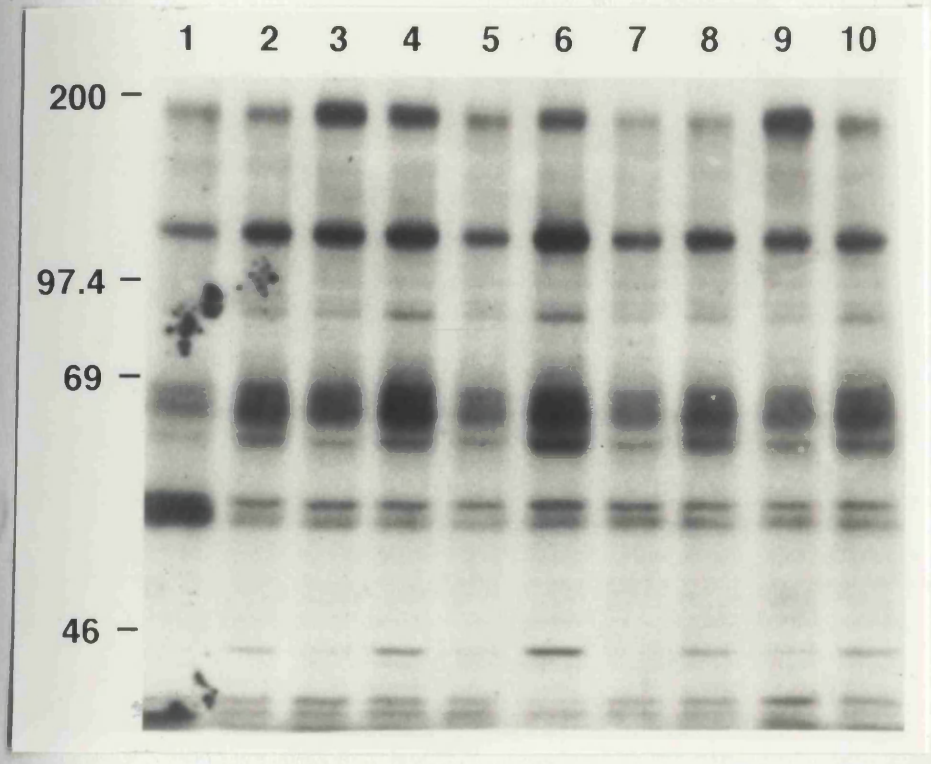

1
2
3
4
5
6
7
8
9
10
200
97.4
69
46

Figure 3.12 Effect of genistein on bombesin-stimulated protein tyrosine phosphorylation

Cells were preincubated for 1hour with vehicle or genistein, stimulated for 2 minutes with vehicle or a final concentration of 3nM bombesin and then lysed. An aliquot of each lysate corresponding to approximately 25µg of protein was analysed for protein tyrosine phosphorylation as described. The numbered lanes in the phostograph correspond to protein phosphotyrosine in lysates from cells treated as follows;

(1) Preincubated with 0.2% DMSO, stimulated with HHBG

(2) Preincubated with 0.2% DMSO, stimulated with 3nM bombesin.

(3) Preincubated with 100µM genistein, stimulated with HHBG.

(4) Preincubated with100µM genistein, stimulated with 3nM bombesin.

(5) Preincubated with 30µM genistein, stimulated with 3nM bombesin.

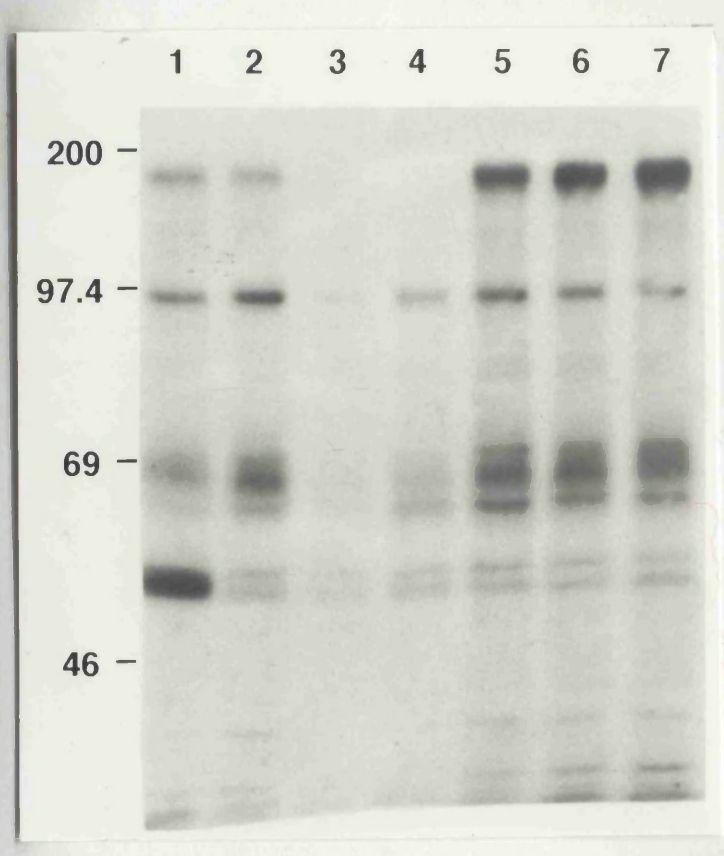
(6) Preincubated with 10µM genistein, stimulated with 3nM bombesin.

(7) Preincubated with 1µM genistein, stimulated with 3nM bombesin.

Results are from a single experiment representative of three.

Table 3.3 Inhibition of bombesin-stimulated PLD activity by genistein.

[^{3}H]Palmitate labelled cells were preincubated for 1 hour with increasing concentrations of genistein or vehicle (0.1%) DMSO, prior to a 10 minute preincubation with 30mM butan-1-ol . Cells were then stimulated for 2 minutes with 3nM bombesin. Incubations were terminated by the addition of ice-cold methanol. Chloroform extracts were prepared and the generation of [^{3}H]PtdBut quantified as described. Results are expressed as mean ± S.D.%, where n=3, of the bombesin-stimulated [^{3}H]PtdBut accumulation in the absence of genistein and are from a single experiment typical of three. Mean basal values where vehicle or genistein alone was used in the preincubation period was subtracted before % values were determined. Mean basal d.p.m.: 0.1% DMSO preincubation, 1145 ± 216; genistein only (100µM) preincubation, 773 ± 233; 3nM bombesin, no genistein, 2185 ± 177.



[genistein]μM	% of bombesin-stimulated [^{3}H]PtdBut in absence of genistein
0	100 ± 17
0.1	124 ± 29
1	104 ± 15
10	99 ± 45
30	81 ± 15
100	40 ± 10

Figure 3.13 Effect of Ro-31-8220 and genistein on bombesin-stimulated PLD activity.

[^{3}H]Palmitate labelled cells were preincubated for 1 hour with vehicle (0.1% DMSO) or 100μM genistein, prior to a 10 minute treatment with 10μM Ro-31-8220 or vehicle (0.1% DMSO v/v) and 30mM butanol. Cells were then stimulated by the addition of vehicle or 100nM bombesin. Incubations were terminated by the addition of ice-cold methanol. Chloroform extracts were prepared and the generation of [^{3}H]PtdBut quantified as described. Results are expressed as mean % ± S.D., where n = 3, of the bombesin-stimulated [^{3}H]PtdBut accumulation in the absence of genistein and are from a single experiment representative of three. Mean basal values where vehicle or genistein alone was used in the preincubation period was subtracted before % values were determined. Mean basal d.p.m.: 0.1% DMSO preincubation, vehicle stimulation, 525 ± 124; 0.1% DMSO preincubation, 100nM bombesin stimulation, 2603 ± 278; 100μM genistein preincubation + Ro-31-8220 preincubation, vehicle stimulation, 988 ± 251.

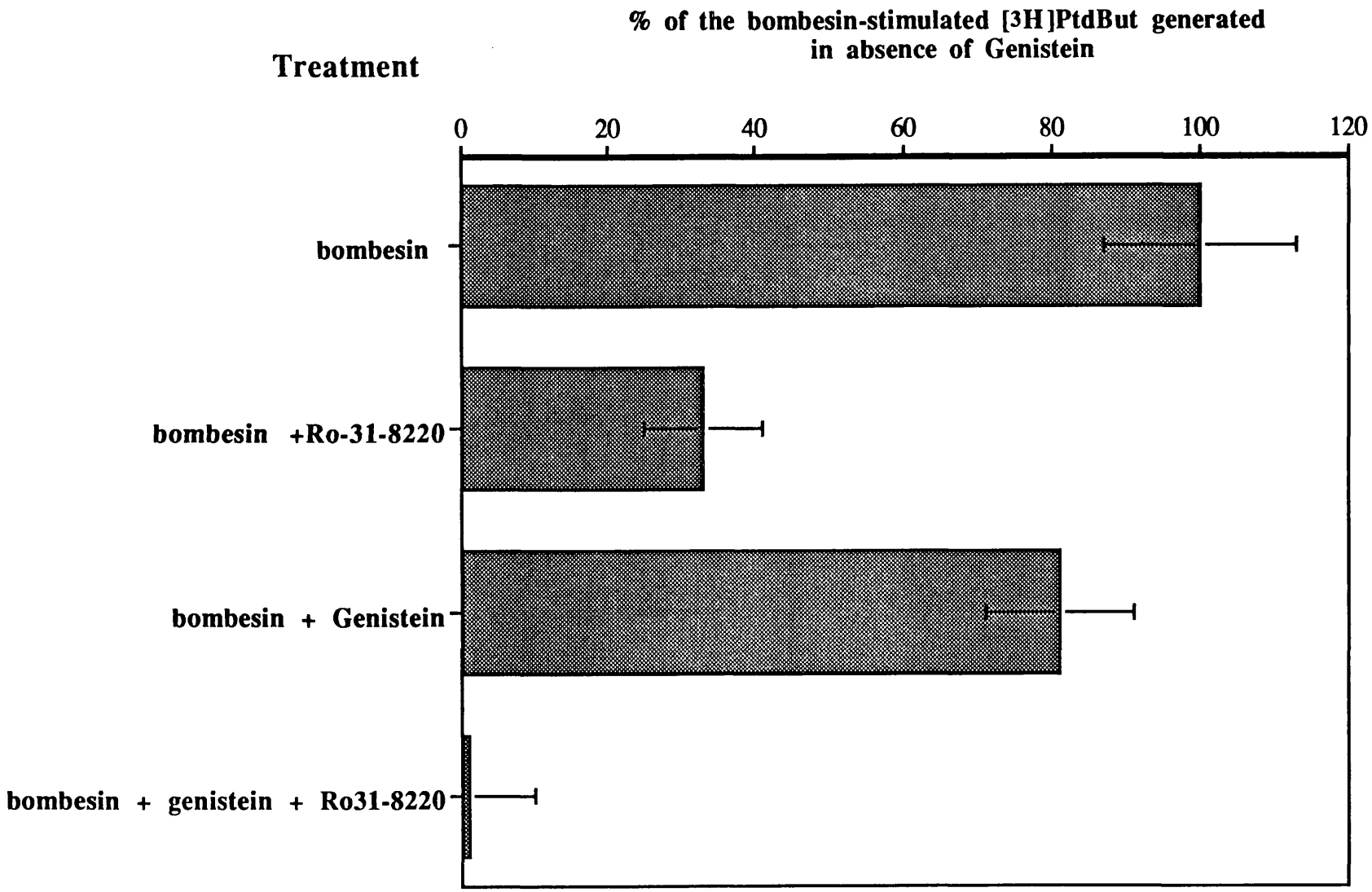

Treatment
% of the bombesin-stimulated [3H]PtdBut generated in absence of Genistein
0
20
40
60
80
100
120
bombesin
bombesin +Ro-31-8220
bombesin + Genistein
bombesin + genistein + Ro31-8220

Table 3.4 Effect of cyclic AMP elevating agents on cyclic AMP levels in Swiss 3T3 cells.

Quiescent cells were pretreated with: (**A**) vehicle, containing 0.1% ethanol and 0.1% DMSO; (**B**) 10μM forskolin and 0.1mM IBMX; (**C**) vehicle, containing 0.1% DMSO; (**D**) 1mM dibutyryl cyclic AMP and IBMX ; for times indicated. Cells were then stimulated with HHBG or 10nM bombesin for 5 minutes or 30 seconds as shown. Incubations were terminated by replacement of the medium with 2% PCA. Samples were neutralised and assayed for their cyclic AMP content as described (section 2.3.4). Results are shown as [cAMP] in $pmol.ml^{-1}$ where n=3 and are from a single experiment representative of three.

Treatment	Control cells [cyclic AMP] pmol.ml^{-1}	bombesin-stimulated cells [cyclic AMP] pmol.ml^{-1}
5 min. vehicle pretreatment,	5 min.stim. 5.3±0.5 30s stim. 4.4 ± 1.2	5 min stim. 3.5 ± 2.42 30s stim. 6.7 ± 4.1
5 min. forskolin + IBMX pretreatment,	N.D.	5 min. stim. 125 ± 28 30s stim. 115 ± 35
10 min. vehicle pretreatment	5 min. stim. 2.2 ± 1.9	N.D.
10 min forskolin + IBMX pretreatment,	5 min. stim. 48.1 ± 33	N.D.
15 min. vehicle pretreatment, 5 min. stimulation	1.48 ± 0.3	N.D.
15 min. dibutyryl cyclic AMP pretreatment, 5 min. stimulation.	34 ± 16	N.D.

Table 3.5 Effect of cyclic AMP elevating agents upon bombesin-stimulated PLD activity.

[^{3}H]Palmitate labelled cells were preincubated with 10μM forskolin, 0.1mM IBMX, or 1mM dibutyryl cyclic AMP as stated, for the indicated times. The cells were then stimulated with bombesin for 5 minutes in the presence of 30mM butanol. Incubations were terminated by the addition of ice-cold methanol. Chloroform extracts were prepared and the generation of [^{3}H]PtdBut quantified as described. Results are expressed as radioactivity in [^{3}H]PtdBut (mean ± S.D., d.p.m., n=3) and are from a single experiment representative of three.

Pre-treatment	[bombesin]nM	Control cells d.p.m. in PtdBut	Pre-treated cells d.p.m. in PtdBut
5 minutes with Forskolin + IBMX	1	1558 ± 300	1629 ± 99
	10	2231 ± 264	2394 ± 289
10 minutes with forskolin and IBMX	0	772 ± 226	1142 ± 104
	1	1882 ± 265	1610 ± 305
	10	2022 ± 124	2338 ± 39
15 minutes with dibutyryl cyclic AMP and IBMX	0	589 ± 115	620 ± 107
	10	2296 ± 749	1687 ± 366

3.3 Discussion

3.3.1 Mobilisation of Ca^{2+} from intracellular stores alone is insufficient to activate PLD activity

Thapsigargin-stimulated Ca^{2+} release from intracellular stores has been reported to occur through inhibition of the Ca^{2+}-ATPase pump on non-mitochondrial stores (Thastrup *et al.*, 1990) and in the absence of inositol phosphate release (Jackson *et al.*, 1988). Treatment with thapsigargin was found to be insufficient to activate PLD directly, nor did it affect the EC_{50} for bombesin-stimulated [^{3}H]PtdBut generation (Fig. 3.1). This suggested that Ca^{2+} did not play an essential stimulatory role in itself, but in view of previous results, appeared to be a co-factor for activation of PKC (Cook *et al.*, 1991). The 30% of bombesin-stimulated PLD activity that remained after treatment with EGTA and Ro-31-8220 and 50% of A23187-stimulated PLD activity that remained after Ro-31-8220 treatment, may therefore be attributable to another pathway. This could involve the interaction of Ca^{2+} with other second-messengers.

3.3.2 Bombesin-stimulated PLD activity is regulated through a G-protein.

Experiments in permeabilised cells with GTPγS and bombesin suggested that bombesin regulated PLD activity through a G-protein. The lag phase of up to 5 minutes before GTPγS-stimulated [^{3}H]PtdBut was elevated above control levels (Fig 3.2) probably reflected the slow exchange rate of GDP for GTP in the absence of receptor activation. Similar findings were reported for GTPγS-stimulated Ins(1,4,5)P_3 generation both in electropermeabilised (Plevin *et al.*, 1990) and streptolysin-O permeabilised Swiss 3T3 cells (Currie, 1992). The failure of a maximal concentration of GTPγS to affect bombesin-stimulated [^{3}H]PtdBut accumulation (Fig. 3.3(A)), however, differed from that of bombesin-stimulated total [^{3}H]inositol phosphates production (Plevin *et al.*, 1990; Currie 1992). Significant potentiation of the accumulation of total [^{3}H] inositol phosphates was observed with

all concentrations of bombesin tested, but as for bombesin-stimulated [^{3}H]PtdBut generation, the EC_{50} for bombesin-stimulated inositol-phosphate generation was not significantly affected. It has been suggested that this may be due to different modes of peptide-receptor coupling to PLC or that there is no receptor reserve for bombesin on Swiss 3T3 cells (Plevin *et al.*, 1990). This contrasted with findings in ATP-stimulated turkey erythrocyte membranes where GTPγS shifted the dose/response curve for agonist-stimulated generation of inositol phosphates to the left (Boyer *et al*, 1989). One difference between the experiments on the bombesin-stimulated generation of total [^{3}H]inositol phosphates and [^{3}H]PtdBut is that experiments on PLC activity were performed for 30 minutes whereas times up to 5 minutes were used for determination of PLD activity.

Fig. 3.3(B) showed that potentiation of 3nM bombesin-stimulated [^{3}H]PtdBut accumulation was only observed at concentrations of GTPγS which were less than maximal and the EC_{50} was not significantly changed in the presence of bombesin. This is probably again a reflection of the accelerated GDP/GTP exchange rate in the presence of agonist, compared to the rate achieved during partial activation of the G-protein mediated pathway. This may occur through either incomplete receptor occupancy or stimulation with sub-maximal concentrations of GTPγS. The inhibition of bombesin-stimulated [^{3}H]PtdBut by GDPβS provided further evidence for a G-protein regulated PLD activity (Table 3.1).

Ca^{2+} was buffered to 150nM in all permeabilised experiments and was hence sufficient to permit agonist and GTPγS-stimulated [^{3}H]PtdBut accumulation, however the effect of increasing [Ca^{2+}] was not examined. In HL60 cells (Billah *et al.*, 1989) and neutrophils (Kessels *et al.*, 1991) fMLP-stimulated PLD activity was dependent on changes in [Ca^{2+}]. GTPγS-stimulated PLD activity in HL60 cells was found to be observable at low [Ca^{2+}] but was greatly potentiated by increases in [Ca^{2+}] to levels greater than 10μM. PMA-stimulated PLD activity was less sensitive to [Ca^{2+}] (Geny and Cockcroft, 1992). The experiments with HL 60 cells however bore little physiological relevance due to the high [Ca^{2+}] used, moreover

the addition of an agonist did not elicit a response (S. Cockcroft, personal communication). Therefore comparisons between findings in this system and those in Swiss 3T3 cells, both in this chapter and in chapter 5 are open to interpretation in various ways. The necessity of increased [Ca^{2+}] for full activation of G-protein regulated PLD activity was also reported in GTPγS-stimulated platelet membranes (Van der Meulen and Haslam 1990) and for PMA and GTPγS-stimulated PLD activity in sub-cellular fractions from neutrophils (Olson *et al.*, 1991). However, these experiments in Swiss 3T3 cells are the first demonstration of agonist-stimulated PLD activity in a permeabilised cell or membrane system.

In HL-60 cells GTPγS-stimulated PLD activity was significantly reduced if cells were left for 15 minutes after permeabilisation prior to stimulation, although the presence of GTPγS exerted some protection against the attenuation (Geny and Cockcroft, 1992). The authors suggested that G-protein dependent cytosolic factors were required for full activation of PLD, supported by experiments in sub-cellular fractions from neutrophils and granulocyte homogenates (Olson *et al.*, 1991; Anthes *et al.*, 1991). The observation in Swiss 3T3 cells that the extent of GTPγS-stimulated [^{3}H]PtdBut accumulation was not significantly reduced even after washing permeabilised cells for up to 15 minutes, implied that a cytosolic factor was unnecessary for stimulation of PLD activity. This was further suggested by the ability of bombesin to stimulate [^{3}H]PtdBut generation in the absence of added GTPγS. Although this could be explained by the presence of residual cellular GTP, this seems unlikely when the dilution factor caused by the presence of the medium in the well is considered.

The synergy observed between PMA and GTPγS production upon PLD activation (Fig. 3.4) and the lack of effect of GDPβS suggested that PMA-stimulated PLD activity was not mediated through a G-protein. Both pathways converge presumably at the point of PKC activation to stimulate [^{3}H]PtdBut formation. Similar synergistic activation of PLD between PMA and non-hydrolysable GTP analogues have been reported in rabbit platelet membranes (Van der Meulen and

Haslam 1990) and streptolysin-O permeabilised HL60 cells (Geny and Cockcroft 1992).

An indirect G-protein/PLD coupling was suggested by the failure of bombesin or GTPγS to activate PLD when total cellular ATP and PtdIns-PLC activity was depleted by the presence of inhibitors of the mitochondrial respiratory chain (Fig. 3.5). This implied that a kinase intermediate was essential to the stimulation of PLD activity in Swiss 3T3 cells. These findings contrasted to similar experiments in HL60 cells. Although PLC activation was minimal, a significant GTPγS stimulation of PLD activity was observed suggestive of a direct G-protein coupling (Geny and Cockcroft, 1992). However as previously described the GTPγS-stimulation was only observable at concentrations of Ca^{2+} above 10μM. Furthermore, in rat pheochromocytoma PC12 cells ATP and Ca^{2+} was found to be essential for GTPγS-stimulated PLD activity (Kanoh *et al.*, 1993) whereas that in U937 promonocytic leukocytes was found to proceed by both ATP dependent and independent pathways (Kusner *et al.*, 1993). Thus the mode of G-protein regulation of PLD activity appears to vary between cell types.

Experiments were unable to conclusively determine whether bombesin-stimulated PLD activity and PtdIns-PLC activity was regulated through the same G-protein. The lack of effect of pertussis toxin on bombesin-stimulated PLD activity was consistent with bombesin-receptor coupling through G_q, as for receptor-mediated activation of PtdIns-PLC (Plevin *et al.*, 1990; Smrcka *et al.*, 1991). However the bombesin-receptor may couple to more than one G-protein as has been suggested for bombesin-stimulated PLA_2 activation in Swiss 3T3 cells (Currie, 1992). In neutrophils it has been proposed that fMLP may not only stimulate PLD through the pertussis-toxin sensitive G-protein activity coupled to PtdIns-PLC but also directly, through a putative G-protein termed G_e, designated as the G-protein regulating exocytosis (Cockcroft, 1992). For further discussion on the theoretical G-protein regulation of stimulated PLD activity see chapter 5.

3.3.3 The PKC-independent pathway of bombesin-stimulated PLD activity is due to activation of tyrosine kinases.

Bombesin-stimulated protein tyrosine phosphorylation, determined by immunoblotting cell lysates with an anti-phosphotyrosine antibody, was similar to that reported by Zachary *et al.* (1991). The increase in tyrosine phosphorylation of these bands was reported not to relate to the bombesin receptor, GTPase activating protein (GAP), PLCγ, or PtdIns-3-kinase. Bombesin was reported to stimulate the tyrosine phosphorylation of the p125 focal adhesion kinase ($p125^{fak}$), which was constitutively associated with GAP (Leeb-Lundberg and Xin-Hua, 1993) and also p130 a major phosphotyrosyl protein in *src* -transformed cells (Zachary *et al.*, 1992). The 70-75kDa bands have been suggested to be paxillin (Zachary and Rozengurt, 1992) which is another cytoskeletal component.

Pervanadate treatment of cells was shown to elevate the tyrosine phosphorylation of proteins with a molecular weight similar to those phosphorylated in response to bombesin (Fig 3.8). The stimulation of [^{3}H]PtdBut and total[^{3}H]inositol phosphates by pervanadate was not significant above basal levels until 10 minutes (Fig. 3.9) and thus lagged considerably behind that of protein tyrosine phosphorylation which was observable within 30 seconds (Fig. 3.8). This suggested that protein tyrosine phosphorylation, through inhibition of tyrosine phosphatases, could lead to activation of PtdIns-PLC and PLD activity. In HL-60 cells pervanadate-stimulated protein tyrosine phosphorylation was found to precede that of PLD activity by about 1 minute, with a high degree of correlation between the two events (Bourgoin and Grinstein, 1992). In most experiments the pervanadate-stimulation of PLD activity occurred concomitantly with that of PtdIns-PLC activity with the accumulation of total [^{3}H] inositol phosphates being generally being lower than that of [^{3}H]PtdBut. The elevation of total [^{3}H]inositol phosphates implied that pervanadate activated a PLCγ. Pervanadate was reported to stimulate protein tyrosine phosphorylation and activation of PLCγ1 in human platelets (Blake *et al.*, 1993) and PLCγ2 in HL60 cells (Bianchini *et al.*, 1993). However, it is difficult to

directly compare pervanadate-stimulated and bombesin-stimulated second-messenger events in view of the different modes of elevation of tyrosine phosphorylation. PDGF and EGF-stimulated production of total [^{3}H]inositol phosphates has been proposed to arise through tyrosine phosphorylation and hence activation of PLCγ (Meisenhelder *et al.*, 1989; Nishibe *et al.*, 1990). However there is no evidence for bombesin-stimulated tyrosine phosphorylation of PLCγ in Swiss 3T3 cells.

Pervanadate-stimulated PLD activity has been reported in human neutrophils (Uings *et al.*, 1992), HL60 cells (Bourgoin and Grinstein, 1992) and phagocytic leucocytes (Dubyak et al., 1993). The observation that a 5 minute stimulation of PLD activity with 0.1nM bombesin was slightly attenuated by the presence of pervanadate, may be explained by the finding that pervanadate was found to inhibit Ca^{2+} uptake in HL60 cells possibly by closure of the influx pathway or by uncoupling of Ca^{2+} entry from the intracellular stores (Bianchini et al., 1993). At longer times of stimulation and at higher concentrations of bombesin the possible inhibitory effect of pervanadate on Ca^{2+} entry may be masked by the greater contribution of other signalling pathways to the activation of PLD.

The failure of the PKC inhibitor Ro-31-8220 to attenuate pervanadate-stimulated protein tyrosine phosphorylation (Fig. 3.10) or PLD activity (Table 3.2), suggested that the contribution of pervanadate stimulated production of total [^{3}H] inositol phosphates must play a minimal role in pervanadate-stimulated PLD activity. A PKC-independent mechanism of pervanadate-stimulated PLD activity and tyrosine phosphorylation was also reported in HL 60 cells (Bourgoin and Grinstein 1992) and for pervanadate-stimulated generation of total [^{3}H] inositol phosphates and protein tyrosine phosphorylation in human platelets (Blake *et al.*, 1993).

Vanadate has been reported to activate G-proteins by forming a stable complex with GDP at the nucleotide binding site, thus acting as an analogue of the γ-phosphate of GTP (Bigay *et al.*, 1987). Furthermore, the effects of vanadate in some

cells were inhibited by pertussis toxin treatment, such as vanadate-stimulated generation of total [^{3}H] inositol phosphates in hamster fibroblasts (Paris and Pouyssegur, 1987). Though the possibility that pervanadate may also affect G-proteins in Swiss 3T3 cells could not be eliminated, the finding that pervanadate-stimulated [^{3}H]PtdBut accumulation was PKC independent argued against the involvement of G_q in pervanadate-stimulated PLD activity. Furthermore mM concentrations of vanadate was required to activate transducin (Bigay et al., 1987) suggesting that the pervanadate concentration of 0.5mM used would be too low to activate G-proteins. Similarly, G-protein stimulation was not responsible for pervanadate-stimulated PLD activity in HL60 cells, as GTPγS and pervanadate-effects were additive in these cells (Bourgoin and Grinstein, 1992).

Genistein, an isoflavone and a specific competitive inhibitor with respect to ATP (Akiyama *et al.*, 1987), was the only tyrosine kinase inhibitor of the range tested, that consistently attenuated tyrosine phosphorylation in cells stimulated with a sub-maximal dose of bombesin (Table 3.3). Concomitant with the decrease in tyrosine phosphorylation, 100μM Genistein partially reduced bombesin-stimulated PLD activity. This concentration of Genistein is below that thought to inhibit PKC activity. The failure of the erbstatin analogue and lavendustin to affect bombesin-stimulated tyrosine phosphorylation may have reflected cell specific tyrosine kinases or poor entry into the cell. In support of this, neither lavendustin nor the erbstatin analogue inhibited stimulated PLD activity in phagocytic leucocytes, whereas genistein was inhibitory (Dubyak *et al.*, 1993). Similarly pervanadate-stimulated PLD activity in HL-60 cells was inhibited by the tyrosine kinase inhibitor ST638, but not by erbstatin or tyrphostins (Bourgoin and Grinstein, 1992). However, erbstatin has been used to inhibit tyrosine phosphorylation and PLD activity in the human neutrophil (Uings *et al.*, 1992). Bradykinin-stimulated generation of total [^{3}H]inositol phosphates in Swiss 3T3 cells was reported to be inhibited 40-50% by Genistein whereas 70-80% of the tyrosine phosphorylation was inhibited. This

suggested that tyrosine phosphorylation may be involved in bradykinin-stimulated formation of total [^{3}H]inositol phosphates (Leeb-Lundberg and Song, 1991).

Genistein had no significant effect on 100nM bombesin-stimulated PLD activity. Though 10μM Ro-31-8220 alone only partially attenuated 100nM bombesin-stimulated PLD activity, the abolishment of [^{3}H]PtdBut accumulation in the additional presence of 100μM Genistein suggested that PLD activity was regulated through both PKC and tyrosine kinases (Fig. 3.13). The failure of Genistein alone to significantly affect 100nM bombesin-stimulated [^{3}H]PtdBut accumulation maybe because the small contribution of tyrosine kinases to PLD activity could not be observed within the limits of experimental error in cases where maximal doses of agonist are used. In support of the PKC-independent nature of tyrosine phosphorylation, PMA and A23187 have been reported to have a weaker effect on tyrosine protein kinase activity compared to that stimulated by bombesin in Swiss 3T3 cells (Zachary *et al.*, 1991). The authors also reported that down-regulation of PKC had no effect on bombesin-stimulated tyrosine kinase activity.

The extent to which tyrosine kinases control the stimulation of PLD activity in different cell types may depend on the regulatory contribution of other second messenger pathways. FMLP- and ET-1- stimulated PLD activity in neutrophils and A10 cells respectively, was completely attenuated by tyrosine kinase inhibitors. fMLP-stimulated PLD activity is PKC independent thus may be entirely regulated by tyrosine kinases. Though ET-1 stimulated PLD activity has a PKC dependent and independent component, reports suggested that mitogenesis was regulated by tyrosine kinases which could be PKC dependent or independent (Simonson and Herman, 1993). ET-1, AVP and Angiotensin II-stimulated tyrosine phosphorylation in glomerular mesangial cells was suggested to occur through PKC-dependent and independent pathways, though PKC activation was not necessary (Force *et al.*, 1991).

The mechanism of PLD activation through elevation of protein tyrosine phosphorylation is unclear especially without knowledge of the kinases involved and

their possible substrates (see chapter 5 for further discussion). Proposed pathways of regulation have included direct phosphorylation of PLD upon tyrosine residues, or removal of a consitutive inhibitor upon protein tyosine phosphorylation (Bourgoin and Grinstein 1992).

3.3.4 Bombesin-stimulated PLD activity is not regulated by cyclic AMP

The failure of cyclic AMP elevation (Table 3.4) to effect bombesin-stimulated [^{3}H]PtdBut accumulation argued against the involvement of the adenylyl cyclase signalling pathway in the regulation of PLD activity in Swiss 3T3 cells (Fig. 3.6). It was notable that in contrast to reports by Millar and Rozengurt (1988), the presence of bombesin did not enhance cyclic AMP generation in the presence of forskolin. A positive effect of 'cross-talk' was reported in human endothelial cells where elevation of cyclic AMP augmented thrombin-stimulated PLD activity (Garcia *et al.*, 1992). This was suggested to be due to the effect of PKA on PKC, PLD or receptor G-protein coupling. However, fMLP-stimulated PLD activity in neutrophils was attenuated by the elevation of cyclic AMP, though there was no effect on phosphoinositide hydrolysis suggesting that cyclic AMP was affecting the receptor-linked activation of PLD (Agwu *et al.*, 1990; Tyagi *et al*, 1990). However, PMA-stimulated PLD activity was unaffected by the cyclic AMP-elevating agents implying that the enzyme itself was not the target. Furthermore Kessels *et al.*. (1993) reported that an inhibitor of PKA enhanced fMLP-stimulated PLD activity. This led to the proposal that activation of PKA in response to fMLP inhibited the activation of PLD at the level of the receptor or receptor/coupled G-protein.

Receptor phosphorylation is commonly associated with uncoupling from the G-protein linked effector mechanisms such as for the β-adrenergic receptor. Moreover phosphorylation of the Gα subunits appears to be sub-type specific. Phosphorylation of $G\alpha_{i2}$, $G\alpha_z$ and $G\alpha_2$ have been reported, however it is unclear how the modification affects function (Gunderson and Devreotes, 1990; Carlson *et al.*, 1989). The ability of cyclic AMP to affect fMLP-stimulated PLD activity may

be therefore be related to the regulatory pertussis-toxin sensitive G-protein involved (Agwu *et al.*, 1989). Bombesin was found not to stimulate the phosphorylation of G_q in Swiss 3T3 cells (P. Kaur, personal communication) nor is there any evidence for phosphorylation of the bombesin receptor. The bombesin receptor or G-protein(s) coupled to PtdIns-PLC and PLD activation may therefore not be substrates for the cyclic AMP mediated modifications.

Bombesin-stimulated PLD activity was found to be regulated through a series of interacting pathways. Regulation of stimulatable PLD activity occurred indirectly, through a pertussis-toxin insensitive G-protein and was mediated by PKC-dependent and -independent pathways. The PKC-independent pathway of bombesin-stimulated PLD activity was found to be mediated through tyrosine kinases. Neither the rise in $[Ca^{2+}]_i$ alone nor the elevation of cyclic AMP was sufficient to activate PLD or affect the extent of bombesin-stimulated [^{3}H]PtdBut generation.

Chapter 4

Desensitisation and Resensitisation of Agonist-stimulated Phospholipase D Activity

4.1 Introduction

Desensitisation is a key characteristic of second-messenger pathways and is represented by diminished reponsiveness to persistent agonist stimulation. A well characterised example of desensitisation is the β-adrenergic receptor coupled to adenylyl cyclase (Hausdorff *et al.*, 1990;Collins *et al.*, 1992). The initial rapid desensitisation of the β-adrenergic receptor (β–AR) is due to phosphorylation of the receptor by protein kinase A (PKA) and β-adrenoreceptor kinase (βARK) leading to uncoupling of the receptor from G_s, followed by sequestration defined as a loss in the ability of receptors to bind hydrophilic but not hydrophobic ligands (Yu *et al.*, 1993). Prolonged agonist exposure results in receptor down regulation, a distinct event from sequestration, in that a decrease in the total number of receptors is observed (reviewed Hausdorff *et al.*, 1990). Receptor internalisation is reversible after short agonist treatments, even in the continual presence of agonist (Kurz and Perkins, 1992).

Recent research has focussed on the desensitisation of phosphoinositidase C-linked receptors. A notable feature of PtdIns-PLC-linked receptors is the very rapid desensitisation to agonist, characterised by a decrease in the rate of phosphoinositide hydrolysis, the extent of which is receptor specific (Sugiya *et al.*, 1987; Martin and Harden 1989; Palmer *et al.*, 1991). The mechanism of this rapid loss of responsiveness has yet to be clarified but may, as for early desensitisation events associated with β-AR, involve receptor phosphorylation and internalisation (reviewed Wojcikiewicz *et al.*, 1993). More prolonged exposure to agonist generally results in receptor downregulation, (Dorn II 1992; Millar and Rozengurt 1990) which is apparently distinct from events involved in acute desensitisation and comparable to desensitisation of the β-AR after longer agonist stimulation.

Structural analysis has suggested that the first phase of bombesin-stimulated DAG generation originates from PtdIns(4,5)P_2 generation, whilst the second is derived from PtdCho (Pettit and Wakelam, 1993). The second sustained phase however, cannot be fully accounted for by dephosphorylation of the PtdOH

generated by PLD-catalysed PtdCho hydrolysis. When butanol was included to 'trap' any PtdOH being formed by PLD activation, the sustained phase of bombesin-stimulated DAG was only attenuated by 30%. PMA-stimulated DAG was diminished by 70% during similar treatment (Cook *et al,* 1991). The time-course of bombesin-stimulated [^{3}H]PtdBut accumulation showed that PLD was activated within 15 seconds, though by 1 minute the generation of [^{3}H]PtdBut appeared to have ceased with no further increase until about 10 minutes. However, PMA-stimulated [^{3}H]PtdBut formation continued to increase after an initial lag phase, for at least 15 minutes. Taken together the data indicated that in fibroblasts, activation of PLD is not exclusively responsible for the sustained phase of DAG generation, indicating a possible desensitisation of bombesin-stimulated PLD activity.

The hydrolysis of both PtdIns(4,5)P_2 and PtdCho were found to both be rapidly desensitised in vasopressin-stimulated A10 vascular-smooth-muscle cells (VSMC) (Plevin and Wakelam, 1992) and in thrombin- and carbachol-stimulated fibroblasts transfected with M1 muscarinic receptors (McKenzie *et al.*, 1992). To date there have been no studies concerning the desensitisation of stimulated PtdCho breakdown in Swiss 3T3 cells.

The research in this chapter characterises the rapid desensitisation and resensitisation of agonist-stimulated PLD activity in Swiss 3T3 cells in an attempt to determine more fully the function and regulation of PLD in mitogenic signalling.

4.2 Results

4.2.1 Rapid desensitisation and resensitisation of agonist-stimulated PLD activity

To investigate the possibility that bombesin-stimulated [^{3}H]PtdBut rapidly desensitised during continual receptor activation, the transphosphatidylation assay was modified as described by Plevin and Wakelam (1992).

The cells were first exposed to 100nM bombesin in the absence of butanol, the alcohol was then added in order to determine any subsequent PLD activity. Figure 4.1 shows that the subsequent loss in stimulated-[^{3}H]PtdBut accumulation with 100nM bombesin was found to be rapid and significant by 15 seconds of pretreatment with 100nM bombesin ($p=0.007$) and had decreased to basal levels by 40 seconds of pretreatment. In some experiments the onset of desensitisation was slightly slower and was only significant after 20 seconds of pretreatment. A similar time course of desensitisation was observed with 3nM bombesin , a concentration close to the EC_{50} for bombesin-stimulated [^{3}H]PtdBut accumulation, and also for 30nM vasopressin (data not shown). Figure 4.2 shows the dose-response curve of the bombesin-stimulated loss in [^{3}H]PtdBut accumulation, determined by pretreating cells for 1 minute with increasing concentrations of bombesin, then adding butanol for 2 minutes in the presence of 100nM bombesin. The IC_{50} for the [bombesin] used in a 1 minute pretreatment, which desensitised the generation of [^{3}H]PtdBut generation to a subsequent stimulation with 100nM bombesin, was 0.2 ± 0.1nM, calculated from three separate experiments.

Desensitisation of bombesin-stimulated PLD activity was reversible. The rate of resensitisation was examined in cells that had been pretreated for 1 minute with 100nM bombesin. The cells were then washed for increasing periods of time and the formation of [^{3}H]PtdBut was determined in response to a 1 minute

Figure 4.1. Time course of bombesin-mediated homologous desensitisation of bombesin-stimulated PLD activity.

[^{3}H]Palmitate-labelled cells were pretreated with buffer or 100nM bombesin for increasing times, in the absence of butanol, then stimulated with a final concentration of buffer or 100nM bombesin for 30 seconds in the presence of 30mM butanol. Incubations were terminated by replacement of the medium with ice-cold methanol. Chloroform extracts were prepared and [^{3}H]PtdBut determined by t.l.c. as described (section 2.3.1(iii)). Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D. n=3) and are from a single typical experiment representative of three. (o), cells pretreated with buffer, stimulated with 100nM bombesin + butanol; (•), cells pretreated with 100nM bombesin, stimulated with 100nM bombesin + butanol; (Δ), cells pretreated with buffer, stimulated with buffer + butanol.

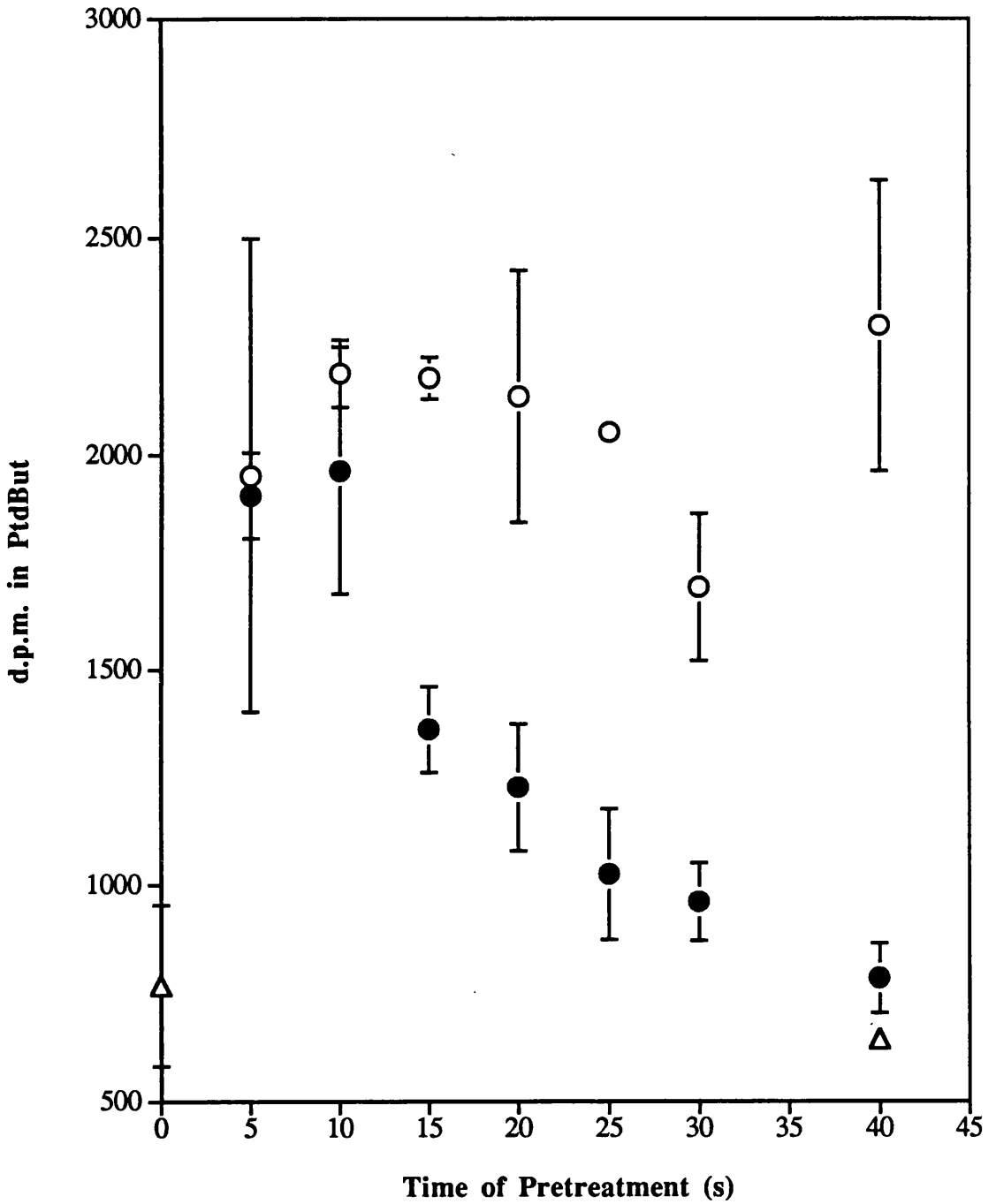
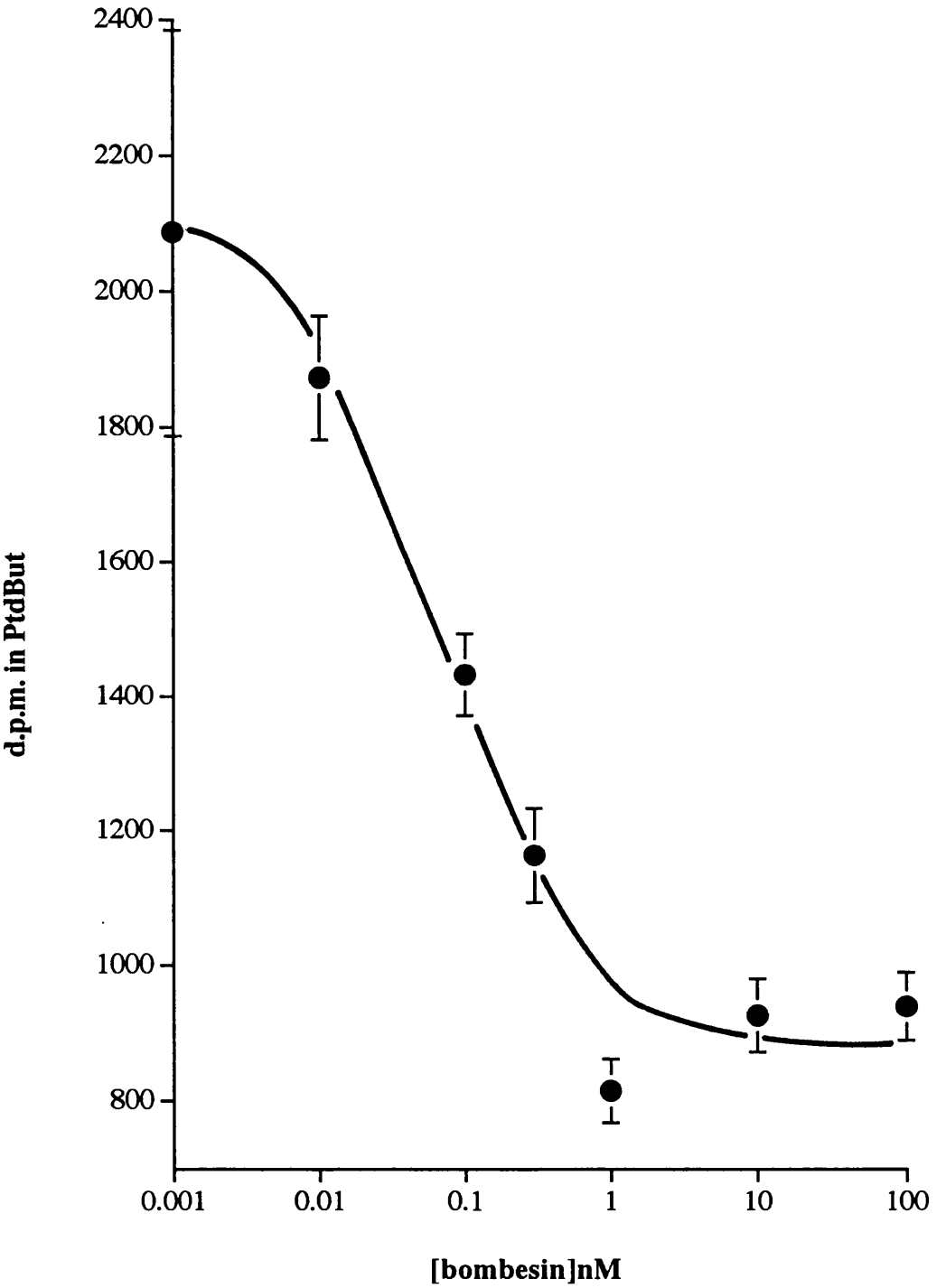

3000
2500
2000
1500
1000
500
d.p.m. in PtdBut
0
5
10
15
20
25
30
35
40
45
Time of Pretreatment (s)

Figure 4.2 Dose-response of bombesin-mediated homologous desensitisation of bombesin-stimulated PLD activity.

[^{3}H]Palmitate-labelled cells were pretreated for 1 minute with increasing concentrations of bombesin, in the absence of butanol, then stimulated for 2 minutes with 100nM bombesin in the presence of 30mM butanol. Incubations were terminated by addition of 0.5ml of ice-cold methanol. Chloroform extracts were prepared and [^{3}H]PtdBut quantified by t.l.c. as described. Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3), from a single experiment representative of three. Mean basal d.p.m., 699±60, where vehicle was used for both the pretreatment and stimulations.


2400
2200
2000
1800
1600
1400
1200
1000
800
d.p.m. in PtdBut
0.001
0.01
0.1
1
10
100
[bombesin]nM

stimulation with 100nM bombesin in the presence of butanol. Figure 4.3 shows that the recovery of bombesin-stimulated PLD activity was rapid, with [^{3}H]PtdBut generation recommencing after a total wash time of 4.5 minutes ($p=0.007$). At best, up to 80% of the control enzyme activity was ever recovered, even after a total wash time of 41.5 minutes. Incomplete resensitisation was not due to depletion of labelled PtdCho, since similar results were obtained when [^{3}H]palmitate was included in the experimental incubation medium (Table 4.1).

To confirm that the desensitisation of bombesin-stimulated [^{3}H]PtdBut accumulation was a direct reflection of PLD-mediated PtdCho hydrolysis, the effect of a 1 minute bombesin pretreatment upon total [^{3}H]choline generation in response to a subsequent bombesin stimulation, was determined in cells labelled with [^{3}H]choline. Cells were washed for the times indicated after the bombesin pretreatment, before addition of buffer or bombesin for a 1 minute period. Figure 4.4 shows that bombesin-stimulated choline production was clearly attenuated to levels comparable with that of cells, which were pretreated with bombesin but restimulated with buffer. Significant choline production, above that of control, was always observed 11.5 minutes after agonist removal ($p=0.01$) and in some experiments after 6.5 minutes of washing.

Table 4.2 shows the effect of rechallenging the cells with bombesin after allowing the cells to partially resensitise. There was no significant difference in the extent of resensitization observed 11.5 minutes after removal of the final pretreatment, between cells that were challenged only once and those that were rechallenged up to three times. The stimulation of [^{3}H]PtdBut in cells could also be completely desensitised to bombesin if, following the initial challenge, 11.5min recovery period and rechallenge, cells were immediately stimulated for 1 minute with bombesin in the presence of butanol (data not shown). The agonist requirement in resensitisation was determined by pretreating cells for 1 minute with bombesin then washing briefly, before exposing cells, in the presence of butanol, to buffer or bombesin. Figure 4.5 shows that unless bombesin was present in the medium, no

Figure 4.3 Resensitisation time course of bombesin-stimulated PLD activity after a 1 minute bombesin pretreatment

Cells labelled with [^{3}H]palmitate, were pretreated for 1minute with 100nM bombesin or HHBG in the absence of butanol, then washed with HHBG for 3 x 30 seconds, followed by a fourth wash for the times indicated. Cells were then stimulated for 1 minute with 100nM bombesin or HHBG, in the presence of 30mM butanol. Incubations were terminated by removal of the medium and addition of ice-cold methanol. Chloroform extracts were prepared and assayed for [^{3}H]PtdBut by t.l.c. as described. Results are expressed as radioactivity in [^{3}H]Ptdbut (d.p.m., mean ± S.D., n=3) and are from a single typical experiment representative of four. (o), cells pretreated with buffer, stimulated with 100nM bombesin + butanol; (●), cells pretreated with 100nM bombesin + butanol, cells stimulated with 100nM bombesin + butanol; (Δ), cells pretreated with buffer, stimulated with buffer + butanol.

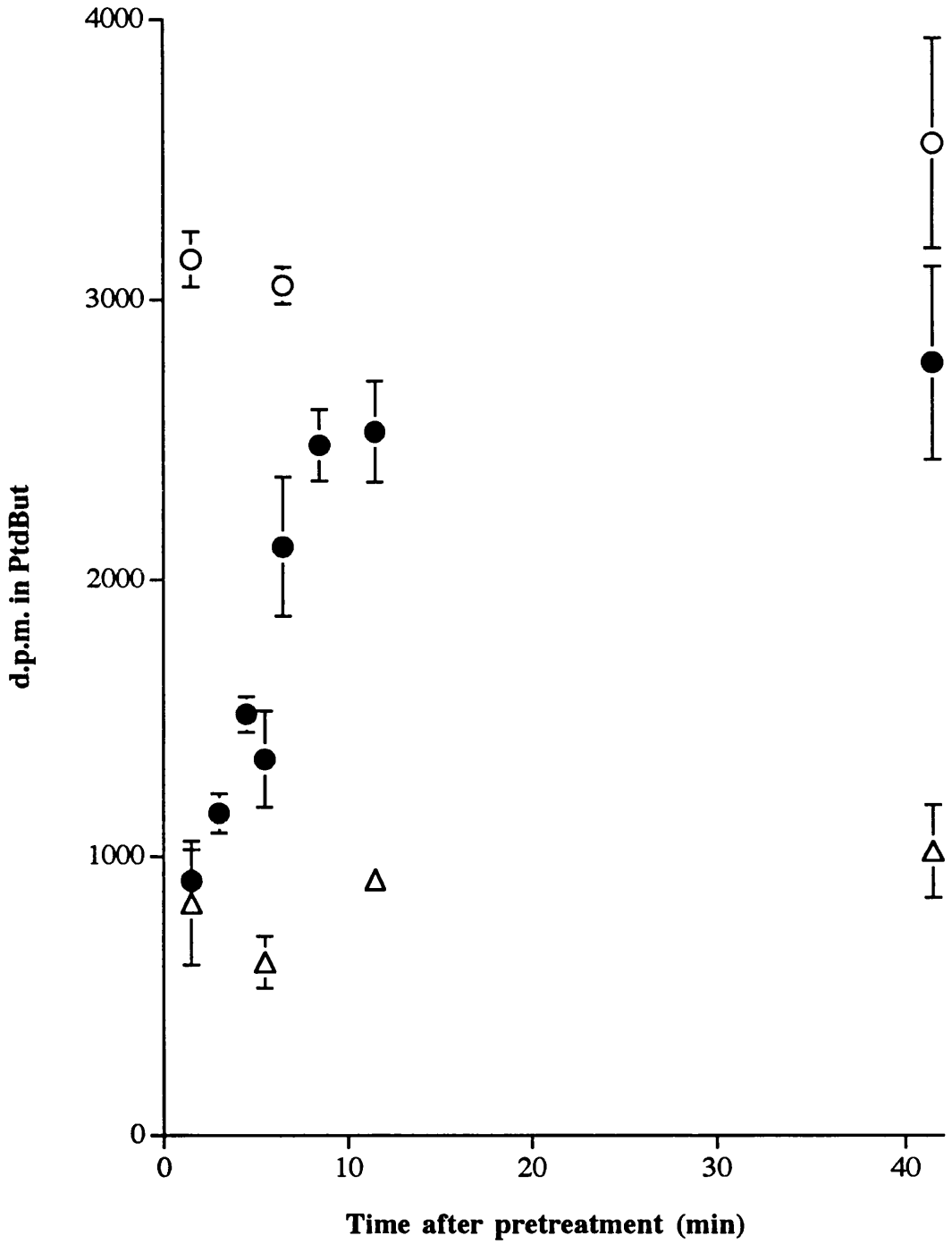

4000
3000
2000
1000
0
0
10
20
30
40
d.p.m. in PtdBut
Time after pretreatment (min)

Table 4.1 The effect of the continual presence of [^{3}H]palmitate on desensitisation and resensitisation of bombesin-stimulated PLD activity.

[^{3}H]Palmitate-labelled cells were pretreated for 1 minute with 100nM bombesin or HHBG, then washed for 3 x 30 seconds with HHBG. The cells were then stimulated for 1 minute with bombesin or HHBG, containing 30mM butanol, or were washed for a further 10 minutes with HHBG. Cells which had been washed for a total of 11.5 minutes, were then stimulated for 1 minute with 100nM bombesin or HHBG, containing 30mM butanol. For cells that were to be treated in the continual presence of [^{3}H]palmitate, all pretreatments, washes and stimulations were performed in medium containing 4μCi.ml^{-1} of [^{3}H]palmitate. Incubations were terminated by replacement of the medium with ice-cold methanol. Chloroform extracts were prepared and [^{3}H]PtdBut quantified by t.l.c. as described. Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) and are from a single experiment representative of three.

Treatment	no [^{3}H]palmitate d.p.m. in PtdBut	+ [^{3}H]palmitate d.p.m. in PtdBut
HHBG pretreatment 1.5min wash HHBG + ButOH stimulation	578 ± 98	744 ± 112
HHBG pretreatment 1.5min wash bombesin + ButOH stimulation	3967 ± 496	3481 ± 725
bombesin pretreatment 1.5min wash bombesin + ButOH stimulation	718 ± 93	877 ± 113
HHBG pretreatment 11.5min wash HHBG + ButOH stimulation	690 ± 126	839 ± 94
HHBG pretreatment 11.5min wash bombesin + ButOH stimulation	4136 ± 366	3785 ± 175
bombesin pretreatment 11.5min wash bombesin + ButOH stimulation	2493 ± 287	2229 ± 61

Figure 4.4 Desensitisation and resensitisation of bombesin-stimulated total choline production after a 1 minute bombesin pretreatment.

[^{3}H]Choline labelled cells were pretreated for 1 minute with 100nM bombesin, then washed for 3 x 30 seconds with DMBGH, followed by a fourth wash for various times. After times as indicated, the cells were treated for 1 minute with 100nM bombesin or DMBGH. Incubations were terminated by addition of ice-cold methanol. Chloroform extracts were prepared and total choline quantified using Dowex-H^+ chromatography as described in the materials and method section. Results are expressed as radioactivity in [^{3}H]choline (d.p.m., mean ± S.D., n=3) and are from a single experiment representative of three. (o), cells pretreated with 100nM bombesin, stimulated with buffer, (●); cells pretreated with 100nM bombesin and stimulated with 100nM bombesin; (Δ), cells pretreated with buffer, stimulated with buffer. Control stimulation in absence of bombesin pretreatment: basal d.p.m., 3535 ±461; 1 minute with 100nM bombesin d.p.m., 5126±385.

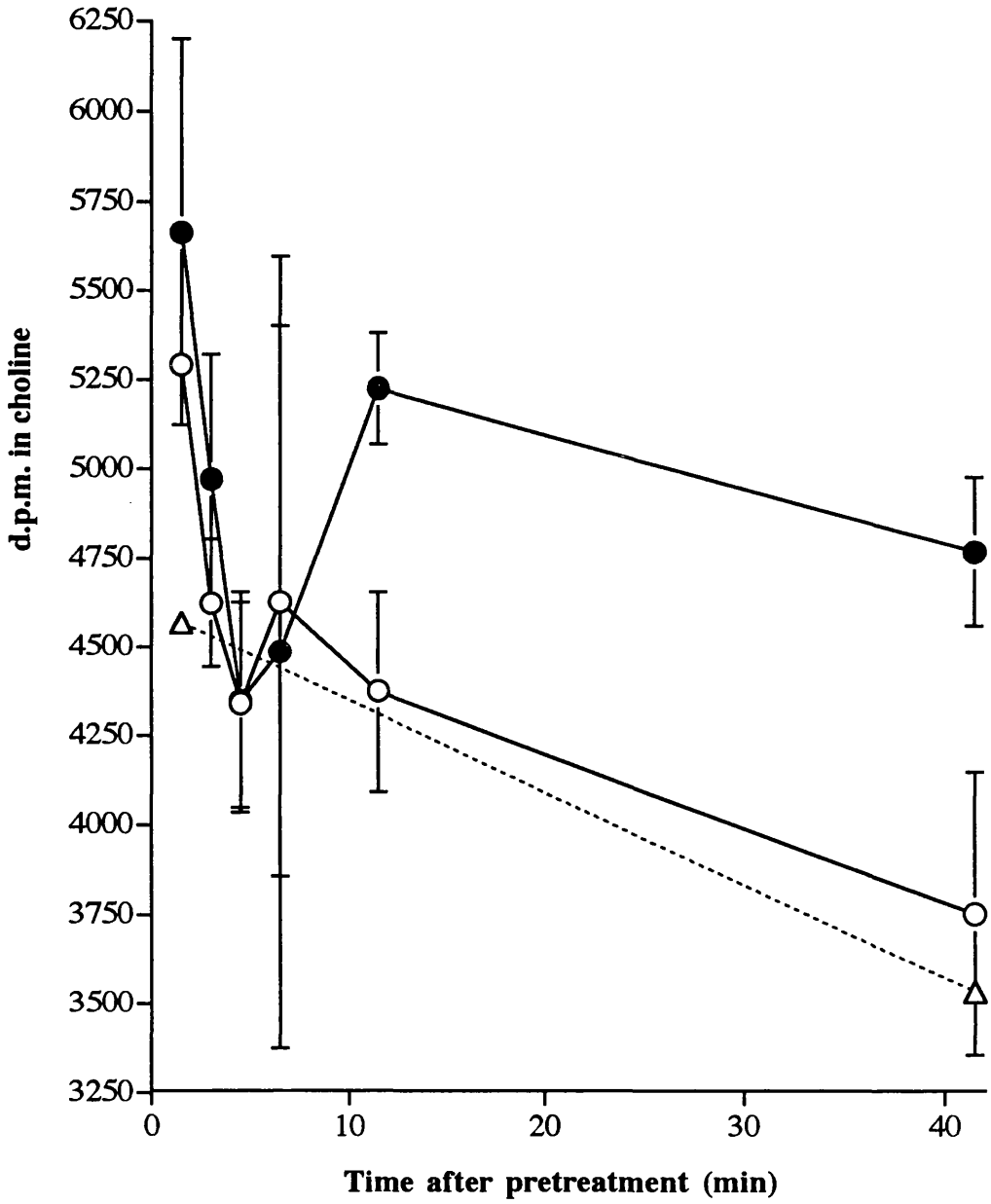

6250
6000
5750
5500
5250
5000
4750
4500
4250
4000
3750
3500
3250
d.p.m. in choline
0
10
20
30
40
Time after pretreatment (min)

Table 4.2 Effect of rechallenging with bombesin on the extent of resensitisation of bombesin-stimulated PLD activity.

[^{3}H]Palmitate-labelled cells were pretreated for 1 minute with HHBG or 100nM bombesin before washing the cells for 3x 30 seconds, followed by a fourth wash for 10 minutes. Cells were then either subjected to a further challenge for 1 minute with HHBG or 100nM bombesin and the washing process repeated as described above, or stimulated for 1 minute with HHBG or 100nM bombesin in the presence of 30mM butanol. The pretreatment and washing procedure was repeated for a maximum of three challenges as indicated. Incubations were terminated by the addition of ice-cold methanol. Chloroform extracts were prepared and [^{3}H]PtdBut quantified as described. Results are expressed as mean ± S.D.% of the [^{3}H]PtdBut generated after the cells were challenged for the appropriate number of times with HHBG before stimulation with bombesin. Mean basal values, where vehicle was used in the pretreatments and stimulations, were subtracted before % values were determined. Results are combined from three separate experiments where n=3 for each experiment.

Number of challenges with 100nM bombesin	% of unpretreated PtdBut generation, 11.5 minutes after removal of final challenge
1	40 ± 14
2	38 ± 12
3	30 ± 8

[^{3}H]PtdBut formation was observed.

Since, readdition of agonist was required for resensitisation of stimulated-[^{3}H]PtdBut accumulation, the effect of sustained stimulation with bombesin was examined. The cells were desensitised by a 1 minute bombesin pretreatment in the absence of butanol, the alcohol was then added and the formation of [^{3}H]PtdBut examined over a 1 hour time course. Figure 4.6 illustrates that there was a pattern of desensitisation/resensitisation which appeared to repeat four times over the 1 hour time period.

4.2.2 Desensitisation and resensitisation of bombesin-stimulated generation of total inositol phosphates.

Bombesin-stimulated PtdIns(4,5)P_2 hydrolysis in Swiss 3T3 cells is also rapidly desensitised. Bombesin-stimulated total [^{3}H]inositol phosphate generation is biphasic, with a rapid increase up to 1 minute, followed by a second phase at a reduced rate (Cook *et al.*, 1991). Resensitisation of bombesin-stimulated generation of total [^{3}H]inositol phosphates was examined in a similar manner to that of [^{3}H]PtdBut, except that following the various wash times LiCl was substituted for butanol during the 1 minute stimulation. A 1 minute pretreatment with 100nM bombesin was found to reduce subsequent bombesin-stimulated production of total [^{3}H]inositol phosphates to between 20-50% of that observed with a vehicle pretreatment, as described by Palmer *et al.*, (1991). Figure 4.7 shows that an increase in total [^{3}H]inositol phosphates generation in cells stimulated for 1 minute with 100nM bombesin, over that remaining after the bombesin pretreatment, varied over several experiments, but was never observed until at least 6.5 minutes after the agonist pretreatment (Fig. 4.7, $p=0.005$ at 8.5 minutes after agonist pretreatment). Subsequently, recovery in the generation of total [^{3}H]inositol phosphates occurred rapidly, being complete after 11.5 minutes.

Figure 4.5 Effect of the presence of bombesin on resensitisation of bombesin-stimulated PLD activity

[^{3}H]Palmitate-labelled cells were pretreated for 1 minute with HHBG or 100nM bombesin then washed for 3x30 seconds, before being stimulated for 1 or 10 minutes with, HHBG or 100nM bombesin containing 30mM butanol. Incubations were terminated by the addition of ice-cold methanol. Chloroform extracts were prepared and [^{3}H]PtdBut generation quantified as described. Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) and are from a single experiment representative of three. - (pt), cells pretreated with buffer; - (st), cells stimulated with buffer + butanol; BOM (pt), cells pretreated with 100nM bombesin; BOM (st), cells stimulated with 100nM bombesin + butanol.

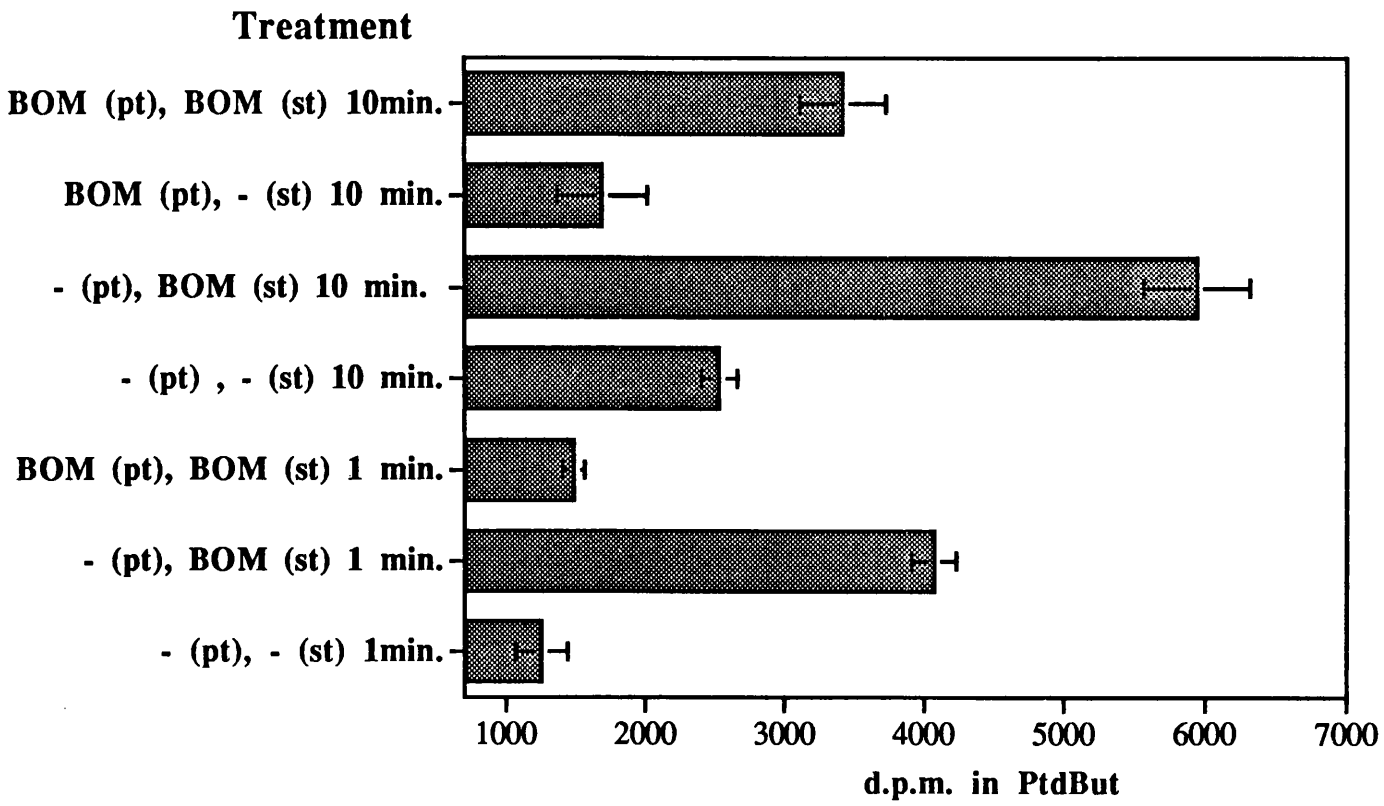

Treatment
BOM (pt), BOM (st) 10min.
BOM (pt), - (st) 10 min.
- (pt), BOM (st) 10 min.
- (pt) , - (st) 10 min.
BOM (pt), BOM (st) 1 min.
- (pt), BOM (st) 1 min.
- (pt), - (st) 1min.
1000
2000
3000
4000
5000
6000
7000
d.p.m. in PtdBut

Figure 4.6 Time course of bombesin-stimulated PLD activity in the continual presence of 100nM bombesin, after a 1 minute pretreatment with 100nM bombesin.

[^{3}H]Palmitate-labelled cells were pretreated for 1 minute with 100nM bombesin, in the absence of butanol, prior to the addition of 30mM butanol for the times indicated. Incubations were terminated by removal of the medium and addition of ice-cold methanol. Chloroform extracts were prepared and [^{3}H]PtdBut generation quantified by t.l.c. as described. Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) and are taken from a single experiment typical of three. (o), cells pretreated with buffer, stimulated with buffer + butanol; (•), cells pretreated with 100nM bombesin, stimulated with 100nM bombesin + butanol. The numbers refer to the significance values obtained from an unpaired t-test comparing the following time points: 1, 4 minutes vs 1minute p=0; 2, 7 minutes vs 4 minutes p=0.003; 3, 16 minutes vs 7 minutes p=0.009; 4, 22 minutes vs 16 minutes p=0.0012; 5, 28 minutes vs 22 minutes p=0.085; 6, 34 minutes vs 28 minutes p=0.041; 7, 49 minutes vs 34 minutes p=0.047.

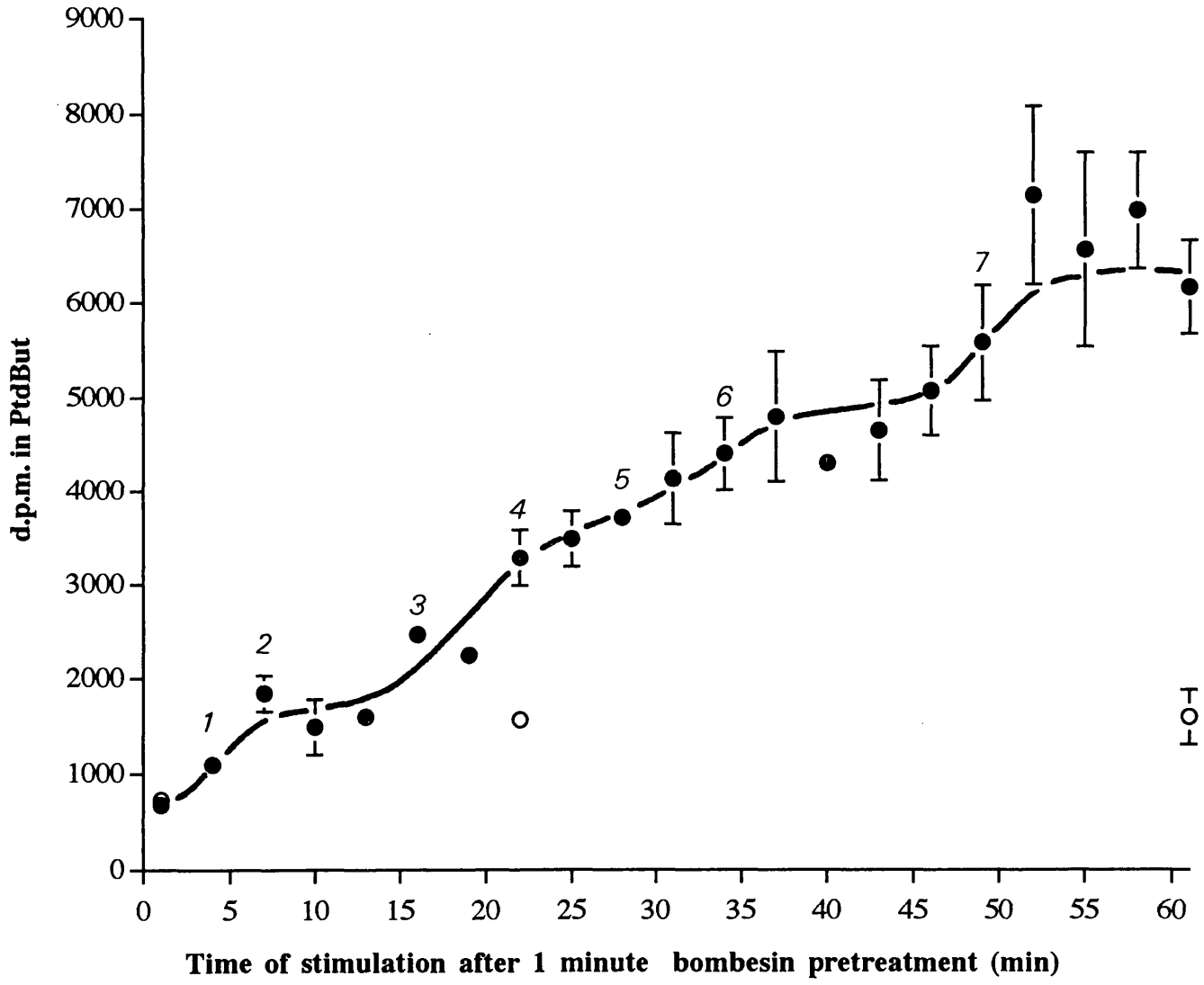
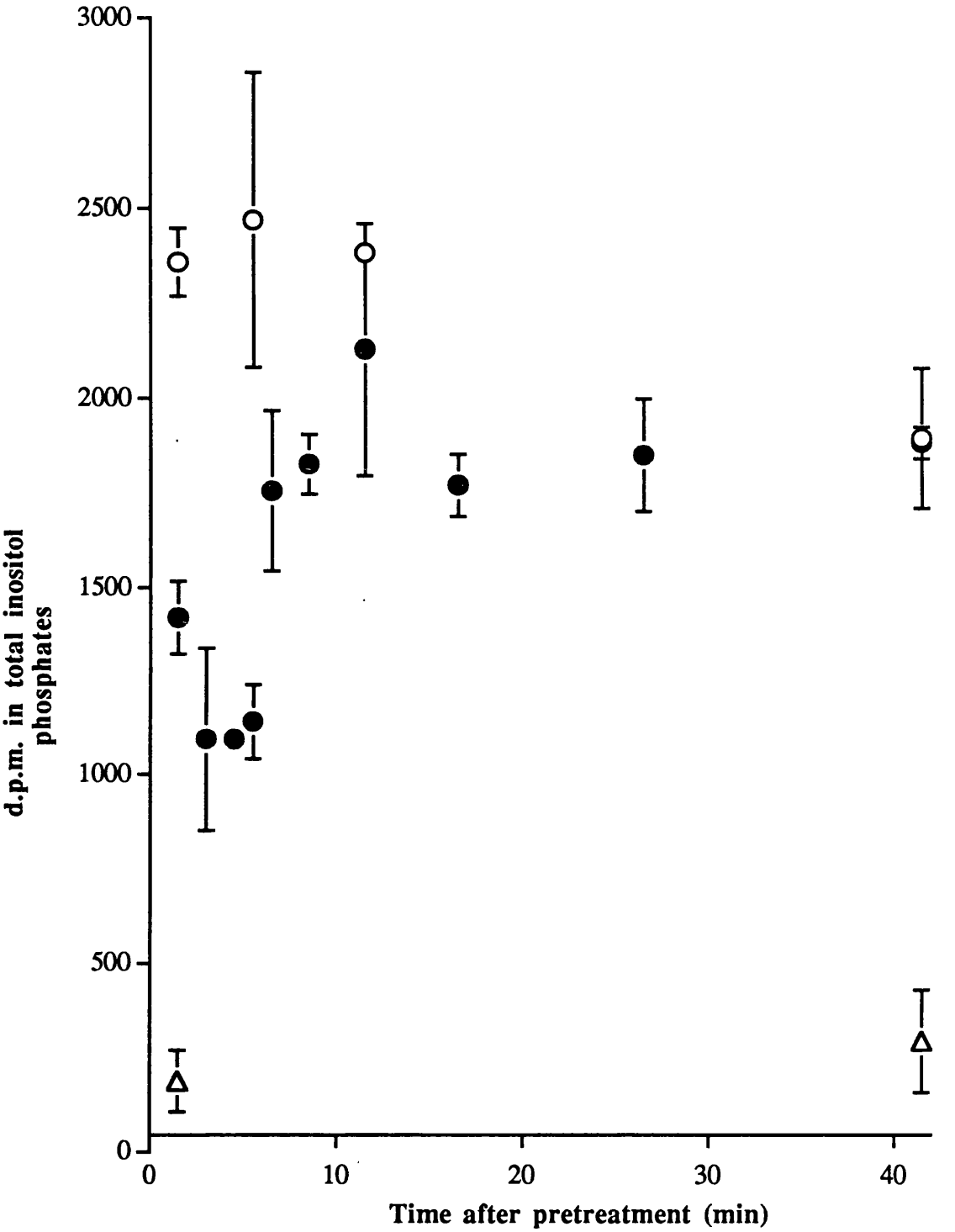

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
d.p.m. in PtdBut
1
2
3
4
5
6
7
0
5
10
15
20
25
30
35
40
45
50
55
60
Time of stimulation after 1 minute bombesin pretreatment (min)

Figure 4.7 Resensitisation time course of bombesin-stimulated generation of total inositol phosphates after a 1 minute bombesin pretreatment.

Cells labelled with [^{3}H] inositol, were pretreated for 1 minute with 100nM bombesin or HHBG, in the absence of LiCl, then washed with buffer for 3 x 30 seconds, followed by a fourth wash for the times indicated. Cells were then stimulated for 1 minute with 100nM bombesin or HHBG, in the presence of 10mM LiCl. Incubations were terminated by replacing the medium with ice-cold methanol. Chloroform extracts were prepared and generation of total [^{3}H]inositol phosphates was determined by batch chromatography, as described in the materials and methods. Results are expressed as radioactivity in total [^{3}H]inositol phosphates (d.p.m., mean ± S.D., n=3) and are from a single experiment representative of three. (o), cells pretreated with buffer, stimulated with bombesin + LiCl; (●), cells pretreated with 100nM bombesin and stimulated with 100nM bombesin + LiCl; (Δ), cells pretreated with buffer and stimulated with buffer + LiCl.


3000
2500
2000
1500
1000
500
0
0
10
20
30
40
d.p.m. in total inositol phosphates
Time after pretreatment (min)

4.2.3 The role of protein kinase C in bombesin-stimulated homologous desensitisation of PLD activity

In contrast to the rapid but transient bombesin-stimulation of [^{3}H]PtdBut accumulation, PMA-induced [^{3}H]PtdBut accumulation did not commence until 2 minutes after addition but continued for at least 15 minutes, at a rate reduced from its initial rapid production (Cook *et al.*, 1991). Table 4.3 shows that there was only a partial loss of [^{3}H]PtdBut generation stimulated with 100nM PMA, after pretreatment with 100nM PMA, compared to the [^{3}H]PtdBut formed in unpretreated cells. These observations suggested that desensitisation of stimulated PLD activity was independent of PKC.

The loss and recovery of 100nM bombesin-stimulated [^{3}H]PtdBut accumulation after a 1 minute pretreatment with 100nM bombesin was investigated, subsequent to preincubation with the selective protein kinase C inhibitor Ro-31-8220 (Davis *et al.*, 1989). Table 4.4 shows that Ro-31-8220 did not prevent bombesin-mediated desensitisation of bombesin-stimulated [^{3}H]PtdBut accumulation, nor did it affect the extent of resensitisation observed after cells had been washed for 11.5 minutes and restimulated.

4.2.4 The internalisation and recycling of cell-surface receptors in intact Swiss 3T3 cells.

Binding studies were performed to compare agonist-mediated desensitisation and resensitisation of stimulated [^{3}H]PtdBut accumulation with the extent of agonist binding to cell-surface receptors. Gastrin releasing peptide (GRP) has the same affinity for the bombesin receptor as bombesin (Nagalla *et al.*, 1992), thus [^{125}I-GRP] was used to investigate the effect of washing the cells on the binding of bombesin to its receptor. Equilibrium binding of [^{125}I-GRP] is achieved at 37^{0}C after 30 minutes (Zachary and Rozengurt, 1985). However a binding period of 1 minute was used to closely mimic the experimental conditions used in the determination of agonist-mediated desensitisation of stimulated PLD activity.

Table 4.3 Effect of a 5 minute PMA pretreatment on PMA-stimulated [^{3}H]PtdBut accumulation

[^{3}H]Palmitate-labelled cells were pretreated for 5 minutes with 100nM PMA or vehicle (0.01% (v/v) DMSO). Cells were then stimulated for times indicated with 100nM PMA or vehicle, containing 30mM butanol. Incubations were terminated by removal of the medium and addition of ice-cold methanol. Chloroform extracts were prepared and [^{3}H]PtdBut generation quantified by t.l.c. as described. Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3), where results are from a single experiment representative of three.

Treatment	d.p.m. in PtdBut
5 min vehicle pretreatment 10 min vehicle stimulation	3394 ± 325
5min vehicle pretreatment 5min PMA stimulation	10390 ± 1527
5min vehicle pretreatment 10min PMA stimulation	14000 ± 467
5 min PMA pretreatment 5 min PMA stimulation	5721 ± 336

Table 4.4 Effect of Ro 31-8220 on bombesin-mediated (A) desensitisation and (B) resensitisation, of bombesin-stimulated PLD activity.

(A) [^{3}H]Palmitate-labelled cells were preincubated with vehicle (containing 0.04% (v/v) DMSO) or 10μM Ro-31-8220 for 5 minutes before pretreating the cells for 1 minute, in the absence of butanol, with vehicle, 10μM Ro-31-8220, 100nM bombesin or 100nM bombesin plus 10μM Ro 31-8220. The cells were then stimulated for 1 minute with vehicle, 10μM Ro-31-8220, 100nM bombesin or 100nM bombesin plus 10μM Ro 31-8220, each in the presence of 30mM butanol. All additions contained 0.04% (v/v) DMSO. Incubations were terminated by replacement of the medium with ice-cold methanol. Chloroform extracts were prepared and [^{3}H]PtdBut quantified as described. Results are expressed as % means ± S.D., where n=3, of the [^{3}H]PtdBut obtained in the absence of Ro 31-8220, after a vehicle pretreatment and stimulation for 1 minute with 100nM bombesin and are from a single experiment typical of three. Mean basal values, where vehicle or 10μM Ro 31-8220 only was used in pretreatments and stimulations, were subtracted before % values were determined. Basal d.p.m.: vehicle, 1100 ± 331; Ro 31-8220, 1023± 238.

(B) The cells were pretreated as above. They were then washed with vehicle ± 10μM Ro 31-8220 for 3 x 30 seconds followed by a fourth wash for 10 minutes. Cells were then stimulated for 1 minute with vehicle, 100nM bombesin or 100nM bombesin plus 10μM Ro 31-8220. Incubations were terminated and [^{3}H]PtdBut generation determined as in **(A)**. Results are % means ± S.D., where n=3, of the [^{3}H]PtdBut obtained in the absence of Ro 31-8220 after a vehicle pretreatment, washes for 3 x 30 seconds and 10 minutes, followed by a 100nM bombesin stimulation for 1 minute. The data is from a single experiment typical of three Mean basal values, where vehicle or 10μM Ro 31-8220 only was used in pretreatments and stimulations were subtracted before % values were determined. Basal d.p.m.: vehicle, 644 ± 51; Ro 31- 8220, 694±106.

(A)

Treatment	% of the PtdBut generated in the absence of pretreatment
vehicle pretreatment + Ro-31-8220 Ro-31-8220 + bombesin stimulation	29 ± 17
bombesin pretreatment bombesin stimulation	9 ± 14
Ro-31-8220 + bombesin pretreatment Ro-31-8220 + bombesin stimulation	-11 ± 5

(B)

Treatment	% of the PtdBut generated in the absence of pretreatment
vehicle pretreatment +Ro-31-8220 Ro-31-8220 + bombesin stimulation after 10min wash	33 ± 8
bombesin pretreatment bombesin stimulation after 10min wash	33 ± 7
Ro-31-8220 + bombesin pretreatment Ro-31-8220 + bombesin stimulation after 10min wash	14 ± 6

Initial experiments suggested that the washing procedure had no effect on the amount of cell-associated [^{125}I-GRP]. To investigate whether the failure of the washing procedure to remove bound ligand was due to its internalisation, the cells were washed with a glycine buffer pH3. Washing cells with a low pH buffer is used to remove all surface-bound ligands and permits an estimation of the extent of receptor internalisation (Kuppuswamy and Pike 1989). All the cell associated radioactivity present after washing at pH 7.4 (section 2.4.4) remained after the acid wash, implying a rapid internalisation of the bombesin receptor (mean specific c.p.m. bound/10^6 cells: no acid wash, 245 +/- 27; with acid wash 285 +/- 65, n=3, results from a single experiment typical of three).

To investigate the possibility that the internalised receptor subsequently returned to the surface at 37^0C, cells were pretreated for 1 minute with 100nM of unlabelled bombesin, then washed for 3 x 30 seconds and a fourth wash of increasing lengths of time. Cells were then incubated for 1 minute at 37^0C with [^{125}I-GRP] then placed on ice. The results suggested that there was indeed an increase in the binding of [^{125}I-GRP] to the cells over time, after removal of the unlabelled bombesin. However it proved impossible to generate statistically valid data due to the very low levels of specific binding under these conditions.

Rather than pursuing the experiments with [^{125}I-GRP], which not only produced difficulties with high levels of non-specific binding, but were also hazardous and expensive, studies were continued on the vasopressin receptor using [^{3}H] [Arg^8] Vasopressin ([^{3}H]AVP) and the radiolabelled antagonist [^{3}H][β-Mercapto-β–β–cyclopenta-methylenepropionyl^1, O-Et-Tyr^2, Val^4, Arg^8] Vasopressin ([^{3}H]AVP antagonist). The presence of a class of high affinity vasopressin (AVP) receptors on Swiss 3T3 cells with a K_d of approximately 10nM, with 10^5 binding sites per cell has previously been reported (Collins and Rozengurt, 1983).

The time course of AVP-stimulated PLD activity (results not shown) and rate of decrease in AVP-stimulated [^{3}H]PtdBut generation following a short AVP

pretreatment was similar to that observed with bombesin (section 4.2.1). Experiments showed that AVP-stimulated [^{3}H]PtdBut accumulation resensitised to an extent comparable with that generated in response to bombesin, after the desensitising pretreatment was removed and the cells washed for 11.5 minutes, prior to a 1 minute stimulation (mean ± S.D.% of unpretreated response: AVP, 57 ± 16; bombesin, 54 ± 10, n=3, results from a single typical experiment of three). [^{3}H]AVP antagonist was used for binding studies, since the coupling of receptors to G-proteins results in a shift in receptor affinity for agonist from a high to lower state whereas the affinity for antagonists remains unchanged. However, in this study, determination of the number of cell-surface receptors, after exposure to unlabelled agonist at 37^{0}C, was performed at 4^{0}C to prevent further receptor internalisation (Briner et al., 1992). Therefore any change in receptor affinity state was limited.

Results presented are those performed with [^{3}H]AVP antagonist, though most experiments were repeated with [^{3}H]AVP. Figure 4.8(A) shows the specific binding of [^{3}H]vasopressin antagonist at 4^{0}C as a function of ligand concentration. Non-specific binding, defined as that observed in the presence of 200-fold excess of unlabelled antagonist, varied linearly with antagonist concentration and was between 15-50% of total binding. Scatchard analysis of [^{3}H]vasopressin antagonist binding gave a K_d of 2.5nM and a Bmax. of approximately 50,000 receptors per cell (Fig. 4.8(b)). These results were comparable to those of Collins and Rozengurt (1983). Figure 4.9 shows that equilibrium binding of [^{3}H]vasopressin antagonist at 4^{0}C was reached after 1 hour of binding, with only a small increase in bound antagonist observed at longer incubation times. A 1 minute pretreatment with unlabelled AVP at 37^{0}C was found to reduce subsequent [^{3}H]vasopressin antagonist binding at 4^{0}C by 70% (Table 4.5). Washing the cells at 37^{0}C for 11.5 minutes allowed subsequent binding at 4^{0}C to recover to 70-90% of that observed in unpretreated cells. Pretreatment with bombesin did not affect the binding of [^{3}H]AVP antagonist to the receptor, implying that the loss of receptor binding sites was agonist specific. Similar results were observed using binding of [^{3}H]AVP at 4^{0}C (n=2). Preliminary

experiments suggested that pretreatment at 37^{0}C with a sub-maximal concentration of AVP only reduced [^{3}H]AVP binding by 30-40% and 11.5 minutes after agonist removal, binding was comparable to that observed in the unpretreated cells (results not shown, n=2).

To determine whether the loss in binding could be due to internalisation of the receptor/ligand complex, cells were incubated with [^{3}H]AVP for increasing times at 37^{0}C, then placed on ice to prevent further receptor internalisation as described previously. Surface bound ligand was then removed by an acidic wash. As for [^{125}I GRP], [^{3}H]vasopressin binding does not reach equilibrium until 30 minutes at 37^{0}C (Collins and Rozengurt, 1983). The experiment was again performed using shorter times of binding, to mimic experimental conditions used for determinations of the desensitisation and resensitisation of agonist-stimulated PLD activity. Figure 4.10 shows that although all the bound ligand was on the surface after a 1 minute exposure [^{3}H]AVP to after 10 minutes all the agonist had apparently been internalised and could not be removed with an acidic wash.

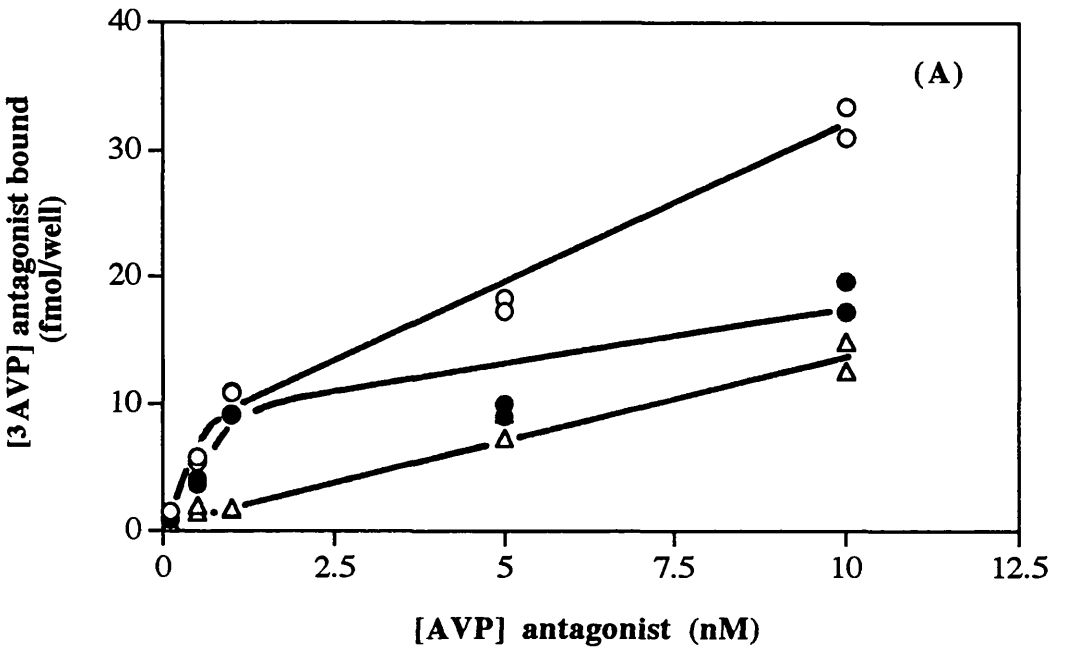
Experiments were performed using pretreatments at 37^{0}C with maximal concentrations of unlabelled AVP antagonist. This reduced the number of binding sites accessible to agonist or antagonist, to a similar extent to that observed with unlabelled agonist pretreatment. The effect of a 1 minute pretreatment with 30nM antagonist did not significantly reduce vasopressin-stimulated PLD activity in most experiments, though in some, vasopressin-stimulated PLD activity was decreased by approximately 25%. In all experiments a 1 minute AVP pretreatment completely desensitised subsequent AVP-stimulated PLD activity (results not shown).

4.2.5 Heterologous desensitisation of agonist-stimulated PLD activity

The nature of agonist-desensitised PLD activity was investigated, by pretreating cells with maximal concentrations of bombesin or vasopressin for 1 minute in the absence of butanol, washing cells briefly, then stimulating with a different agonist in the presence of butanol. Figure 4.11 shows that pretreatment for

Figure 4.8 Scatchard analysis of [^{3}H]AVP antagonist binding to intact Swiss 3T3 cells at 4^{0}C

Swiss 3T3 cells were grown in 6-well plates, in DMEM + 10% calf serum, until 80% confluent. The medium was then removed and the cells allowed to quiesce for a further 24 hours in DMEM + 2% calf serum. Cells were washed at 37^{0}C, then at 4^{0}C before being incubated for 1 hour at 4^{0}C with [^{3}H]AVP antagonist at concentrations indicated. Cell associated radioactivity was determined as described (section 2.4.2). Non-specific binding at each concentration was determined in the presence of a 200-fold excess of AVP antagonist and was subtracted from total binding to give the specific binding shown. Results are expressed as: **(A)** a saturation isotherm of total (o), specific (•) and non-specific (Δ) binding, of [^{3}H]AVP antagonist in fmol/well, **(B)** the derived Scatchard plot where, B_{max} = 24 fmol , K_d = 2.5nM. Each point was performed in duplicate and results were taken from a single experiment representative of three. The number of cells per well determined as described (section 2.4.2) was approximately 2.8 x 10^5.


(A)
[3AVP] antagonist bound (fmol/well)
40
30
20
10
0
0
2.5
5
7.5
10
12.5
[AVP] antagonist (nM)

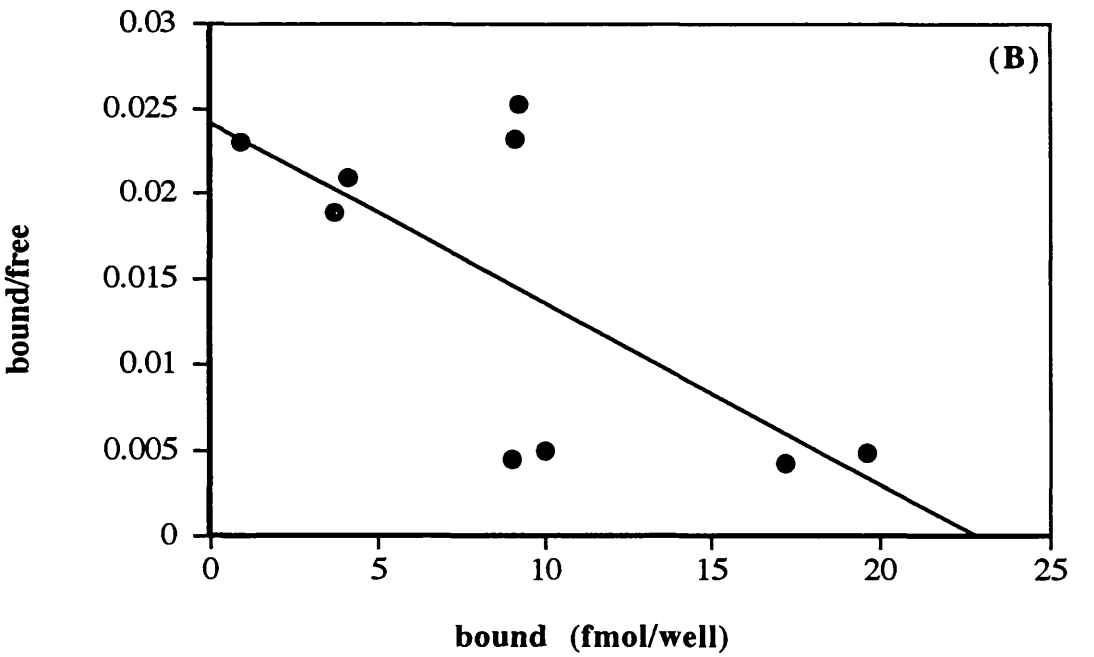

(B)
bound/free
0.03
0.025
0.02
0.015
0.01
0.005
0
0
5
10
15
20
25
bound (fmol/well)

Figure 4.9 Time course of [^{3}H]AVP antagonist binding to intact Swiss 3T3 cells at 4^{0}C.

Quiescent Swiss 3T3 fibroblasts were washed at 37^{0}C, then at 4^{0}C, before being incubated for times indicated with 1nM [^{3}H]AVP antagonist. Cell-associated radioactivity was determined as described in materials and methods. Non-specific binding was determined in the presence of 0.2μM of unlabelled AVP antagonist and was subtracted in each case to give the specific binding shown. Results are expressed as specific bound [^{3}H]AVP antagonist in fmol/well (n=2) and results are taken from a single experiment representative of two. The number of cells per well was approximately 3.1 x 10^5.

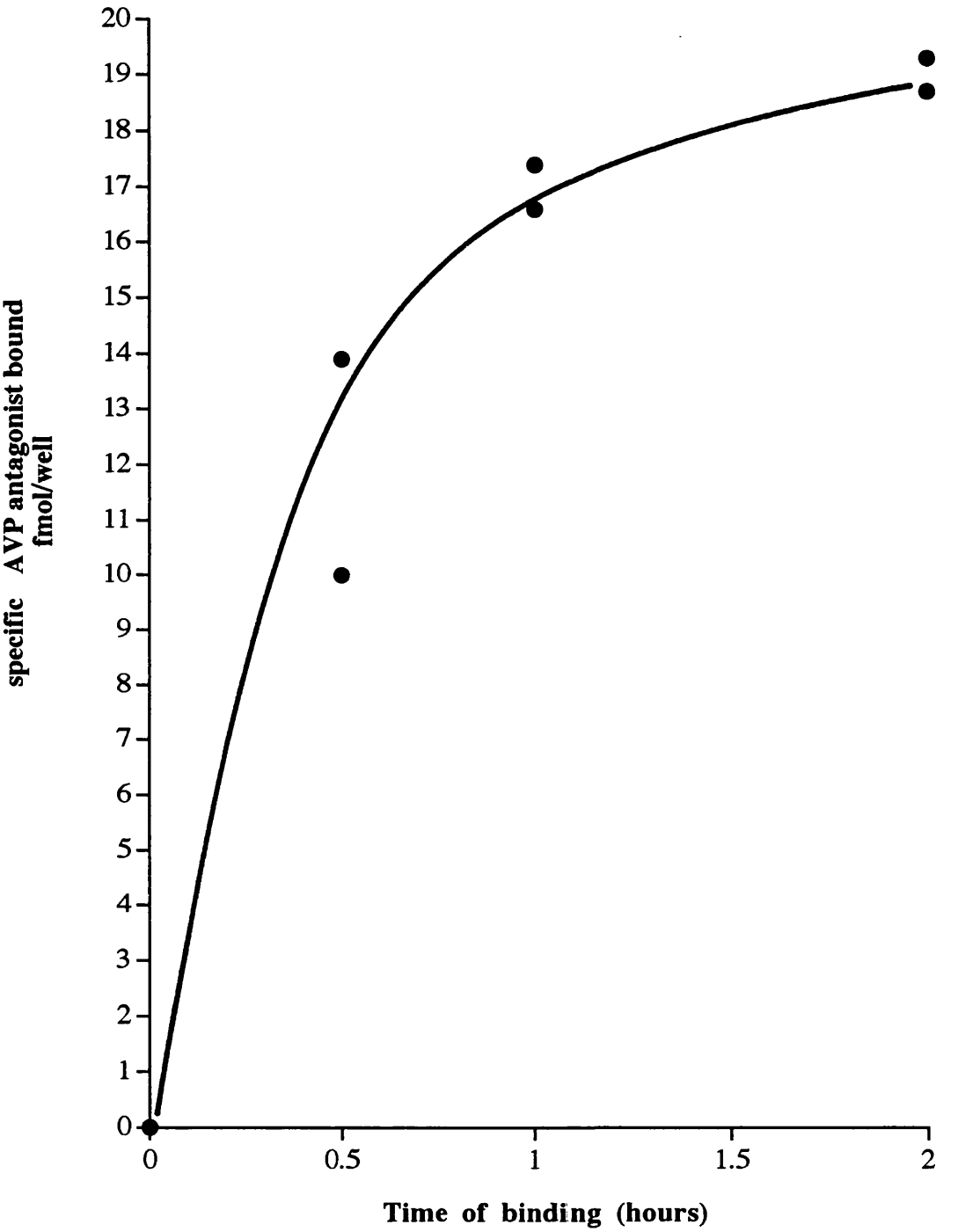

specific AVP antagonist bound
fmol/well
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
0.5
1
1.5
2
Time of binding (hours)

Table 4.5 Effect of a 1 minute pretreatment at 37^0C with 30nM unlabelled AVP or 100nM bombesin on [^{3}H]AVP antagonist binding at 4^0C.

Swiss 3T3 fibroblasts were grown in 6-well plates, in DMEM + 10% calf serum, until 80% confluent. The medium was replaced with DMEM + 2% calf serum and the cells allowed to quiesce for a further 24 hours. After washing, cells were pretreated for 1 minute with 30nM AVP, 100nM bombesin or incubation buffer alone at 37^0C. For cells that were not to be washed further, the medium was then replaced with ice-cold incubation buffer and the cells placed on ice. Cells that were to be washed received 3 x 30 second washes with incubation buffer at 37^0C, followed by a fourth wash for the time indicated. After the final wash the medium was replaced with ice-cold incubation medium and the cells placed on ice. The binding of [^{3}H]AVP antagonist was performed at 4^0C for 2 hours, before the cells were washed and solubilised as described in the materials and method section. Non-specific binding was determined at each time point, by including a 200-fold excess of unlabelled AVP antagonist (1μM) in the binding medium. Results are expressed as : specific bound [^{3}H]AVP antagonist in fmol/well, after subtracting non-specific binding (mean ± S.D., n=2); also as % of [^{3}H]AVP antagonist bound, where incubation buffer was used during the pretreatment period (mean ± S.D.%, n=2). Results are taken from a single experiment representative of two. The number of cells per well was approximately 1.8 x 10^5.

Treatment	Specific [^{3}H]AVP antagonist bound (fmol/well)	% of [^{3}H]AVP antagonist bound in the absence of pretreatment
no pretreatment, 0min wash	15.9 ± 0.6	100±4
1 min AVP pretreatment, 0min wash	5.2 ± 0.1	33 ± 1
1 min bom pretreatment 0 min wash	14.9 ± 1.7	94 ± 9
1 min AVP pretreatment 6.5 min wash	10.5 ± 1.9	66 ± 10
1min AVP pretreatment 11.5 min wash	12.5 ± 0.8	78 ± 5

1 minute with 100nM bombesin or vasopressin completely prevented a subsequent generation of [^{3}H]PtdBut, in response to a 1 minute stimulation in the presence of butanol, with the same agonist. However, if after pretreatment, a different agonist was used for the stimulation, between 20-30% of the agonist-stimulated [^{3}H]PtdBut generation attained in the absence of any pretreatment, remained.

To investigate whether agonist-induced desensitisation was also heterologous with respect to GTPγS-stimulation of PLD activity, permeabilised cells were pretreated for 1 minute with bombesin (Fig 4.12(A)), or vasopressin (Fig 4.12 (B)), washed briefly and stimulated for 5 minutes with 30μM GTPγS. Both 3nM bombesin and 30nM vasopressin partially reduced the subsequent generation of [^{3}H]PtdBut in response to GTPγS in the presence of butanol, to approximately 50% of that attained in the absence of pretreatment. Fig 4.12 (A) also shows that a 1 minute pretreatment with 30μM GTPγS did not reduce the subsequent GTPγS stimulated [^{3}H]PtdBut accumulation

4.2.6 The effect of a short agonist pretreatment on subsequent stimulation of PLD activity by PMA.

Experiments were performed to determine if agonist-induced desensitisation of stimulated [^{3}H]PtdBut generation was also heterologous with respect to the stimulation of PLD by PKC-activating phorbol esters. Figure 4.13 shows that a 1 minute pretreatment with bombesin, which attenuated the activation of PLD by a subsequent 1 minute stimulation with bombesin, had no effect on a 10 minute PMA-stimulation of PLD activity. In the same experiment, the short bombesin pretreatment partially attenuated the generation of [^{3}H]PtdBut in response to a 10 minute bombesin-stimulation, whilst in the presence of 30mM butanol alone no [^{3}H]PtdBut generation above that of basal was detected, as previously described (section 4.2.1).

Figure 4.10 Effect of an acidic wash on cell-associated radioactivity afte preincubation at 37^{0}C with [^{3}H]AVP.

Quiescent Swiss 3T3 fibroblasts were washed thoroughly at 37^{0}C, the incubated for times indicated, with a final concentration of 30nM AVP, of whic 5nM was [^{3}H]AVP. Cells were placed on ice immediately following the incubatio with [^{3}H]vasopressin, to prevent further receptor internalisation. Wells were washe and those indicated subjected to a low pH wash before determination of cel associated radioactivity, as described in materials and methods (section 2.4.3). Nor specific binding was determined by including 6μM unlabelled vasopressin in th binding medium and was subtracted from cell-associated radioactivity to give th specific binding shown. Results are expressed as radioactivity in specific bour [^{3}H]vasopressin per well (d.p.m., mean ± S.D., n=2), where results are from a sing experiment representative of three. The number of cells in each well w approximately 1.8 x 10^5. Unshaded columns represent specific AVP bound in cel that were not washed with the low pH buffer; shaded columns represent specif AVP bound in cells subject to an acidic wash.

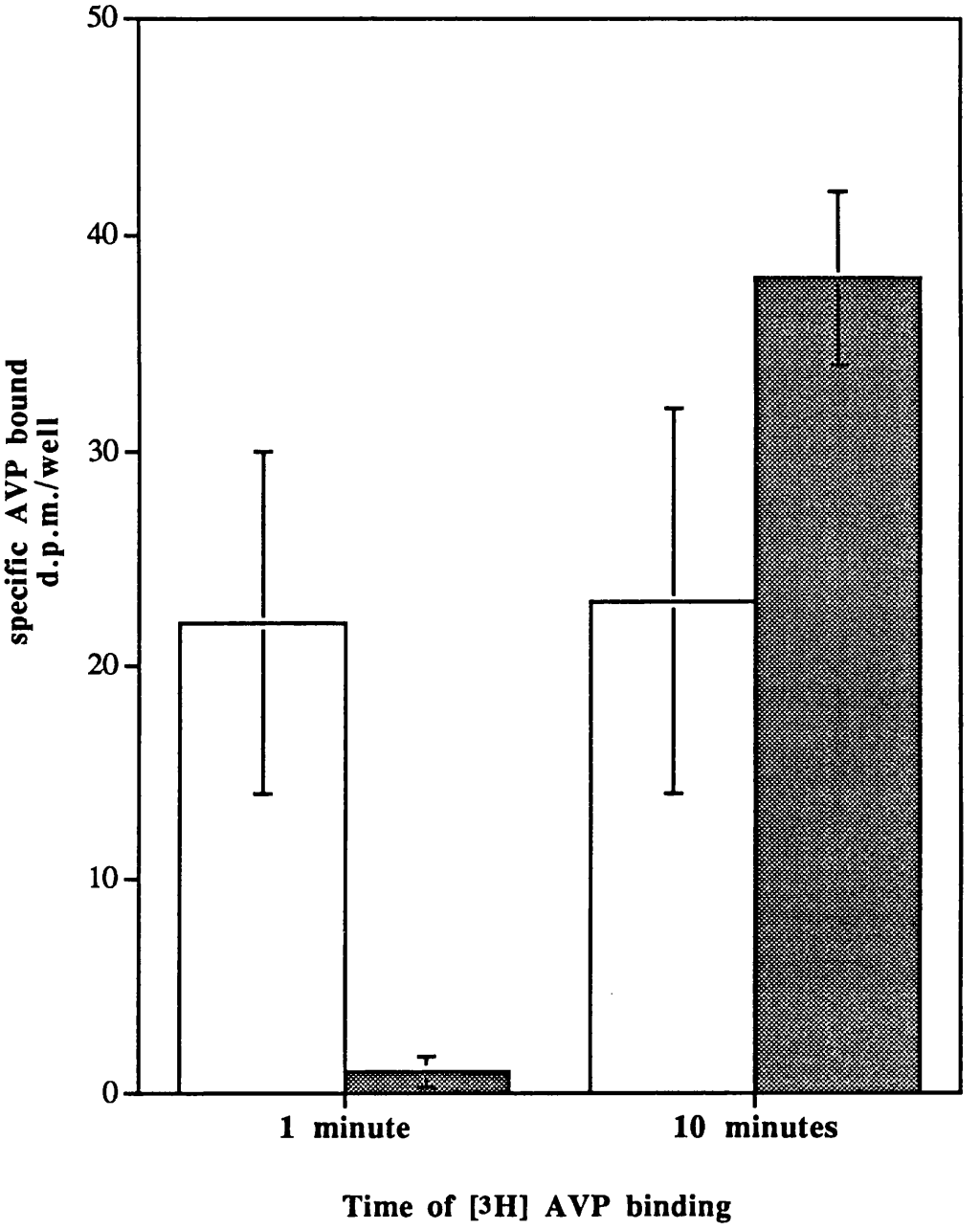

50
40
30
20
10
0
specific AVP bound
d.p.m./well
1 minute
10 minutes
Time of [3H] AVP binding

Figure 4.11 Heterologous desensitisation of agonist-stimulated PLD activity.

[^{3}H]Palmitate-labelled cells were pretreated for 1 minute with HHBG, 100nM vasopressin or 100nM bombesin in the absence of butanol, then washed for 3 x 30 seconds with HHBG. Cells were stimulated for 1 minute in the presence of 30mM butanol with, HHBG, 100nM bombesin or 100nM AVP. Incubations were terminated by replacement of the medium with ice-cold methanol. Chloroform extracts were prepared and the generation of [^{3}H]PtdBut quantified as previously described. Results are expressed as radioactivity in [^{3}H]Ptdbut (mean d.p.m. ± S.D., n=3) and are from a single experiment typical of three. - (pt), cells pretreated with buffer; - (st), cells stimulated with buffer + butanol; BOM (pt), cells pretreated with 100nM bombesin; BOM (st), cells stimulated with 100nM bombesin + butanol; VP (pt), cells pretreated with 100nM AVP; VP (st), cells stimulated with 100nM AVP + butanol.

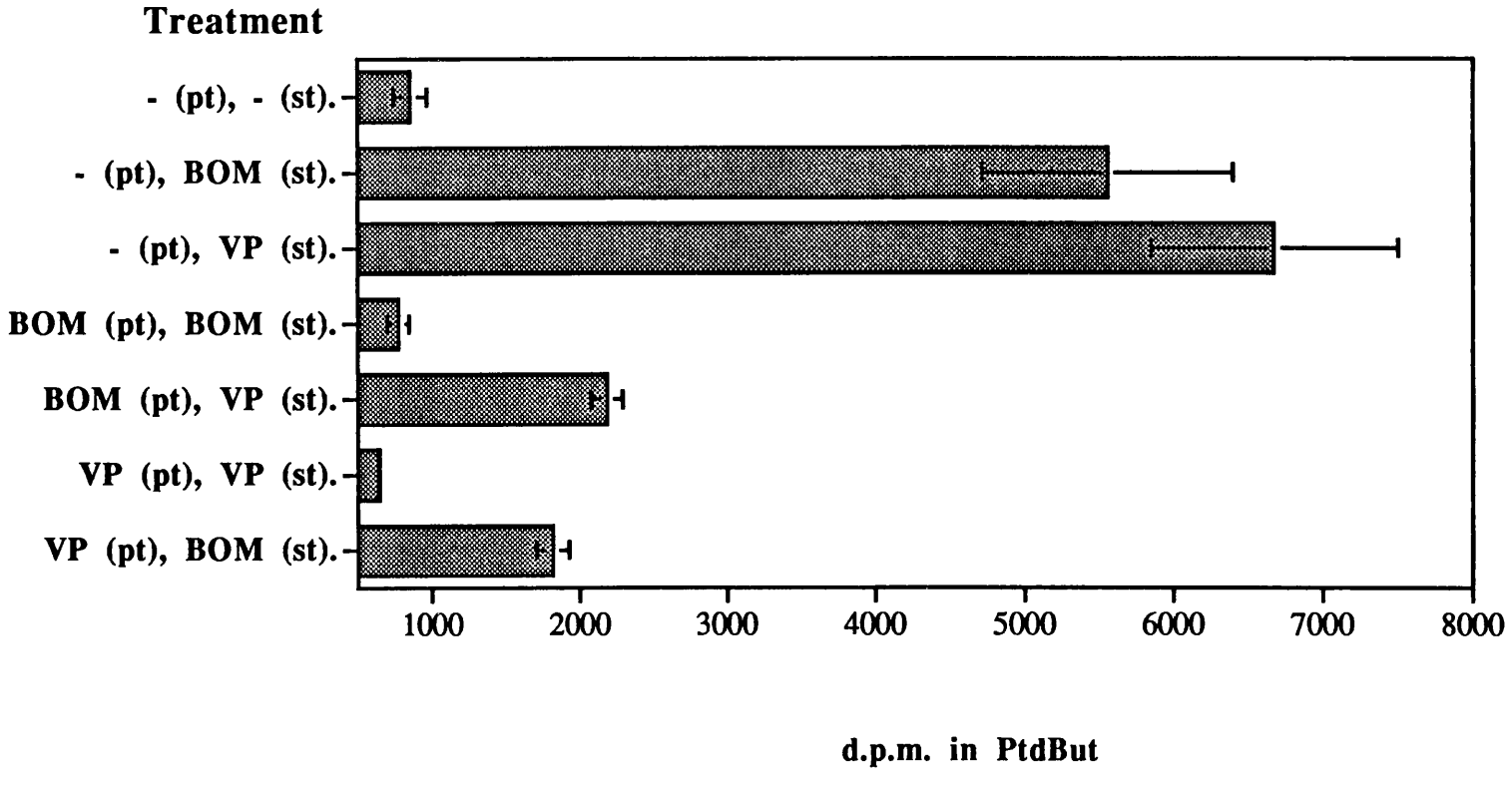
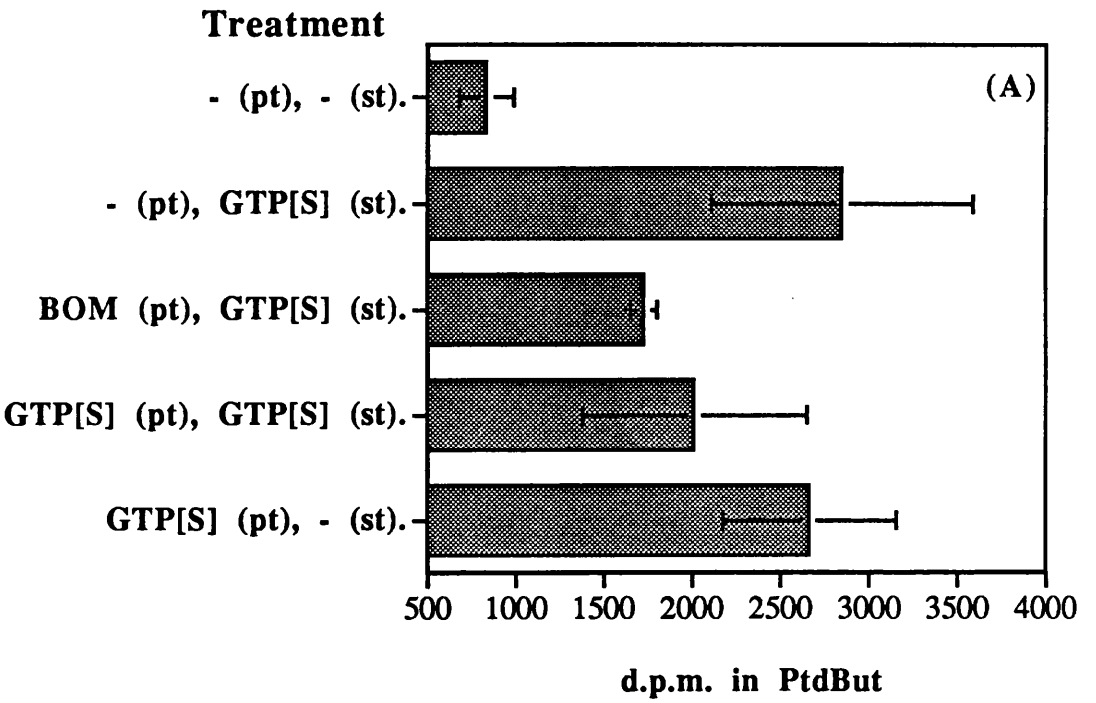

Treatment
- (pt), - (st).
- (pt), BOM (st).
- (pt), VP (st).
BOM (pt), BOM (st).
BOM (pt), VP (st).
VP (pt), VP (st).
VP (pt), BOM (st).
1000
2000
3000
4000
5000
6000
7000
8000
d.p.m. in PtdBut

Figure 4.12 Effect of agonist pretreatment on GTPγS-stimulated PLD activity.

[^{3}H]Palmitate-labelled cells were permeabilised with streptolysin-O ($0.6Uml^{-1}$), then pretreated for 1 minute in the absence of butanol, with HHBG or; **(A)** 3nM bombesin or GTPγS; **(B)** 30nM vasopressin. Cells were washed 3 times over 10s, then stimulated for 5 minutes with vehicle or 30μM GTPγS in the presence of 30mM butanol. Incubations were terminated by direct addition of ice-cold methanol. Chloroform extracts were prepared and the generation of [^{3}H]PtdBut determined by t.l.c. as described. Results are expressed as radioactivity in [^{3}H]PtdBut (mean d.p.m. ± S.D., n=3) and are from a single experiment representative of three. - (pt), cells pretreated with buffer; - (st) cells stimulated with buffer + butanol; BOM (pt), cells pretreated with 100nM bombesin; VP (pt), cells pretreated with 100nM vasopressin; GTP[S] (pt), cells pretreated with GTPγS; GTP[S] (st), cells stimulated with GTPγS + butanol.


Treatment
- (pt), - (st).
- (pt), GTP[S] (st).
BOM (pt), GTP[S] (st).
GTP[S] (pt), GTP[S] (st).
GTP[S] (pt), - (st).
(A)
500 1000 1500 2000 2500 3000 3500 4000
d.p.m. in PtdBut

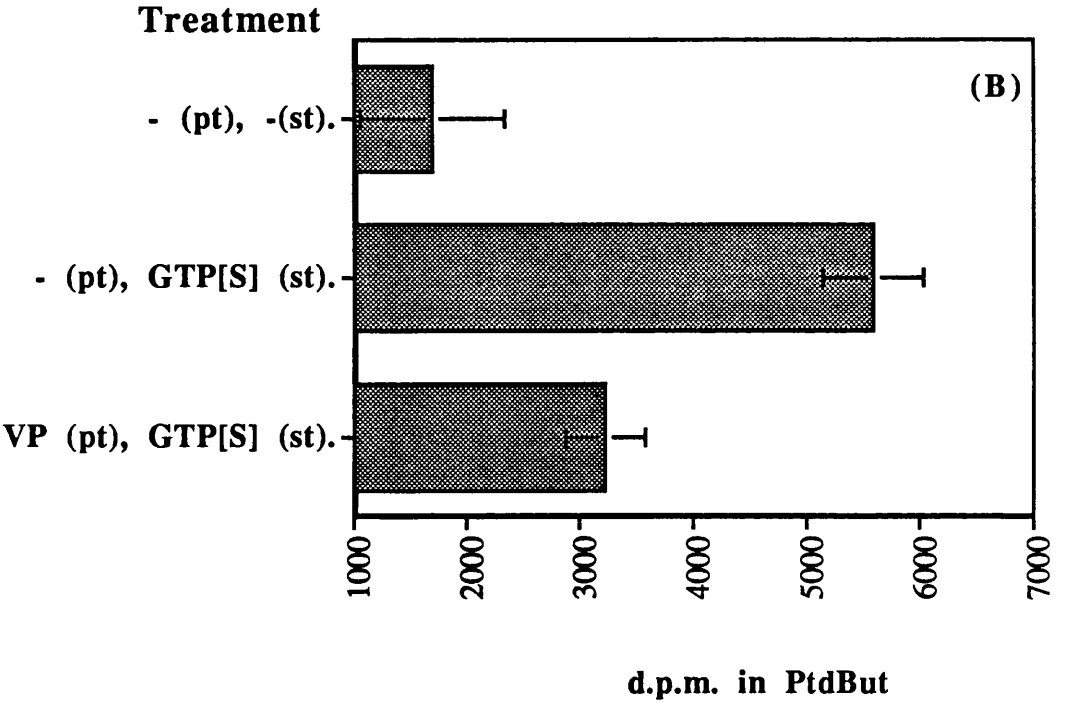
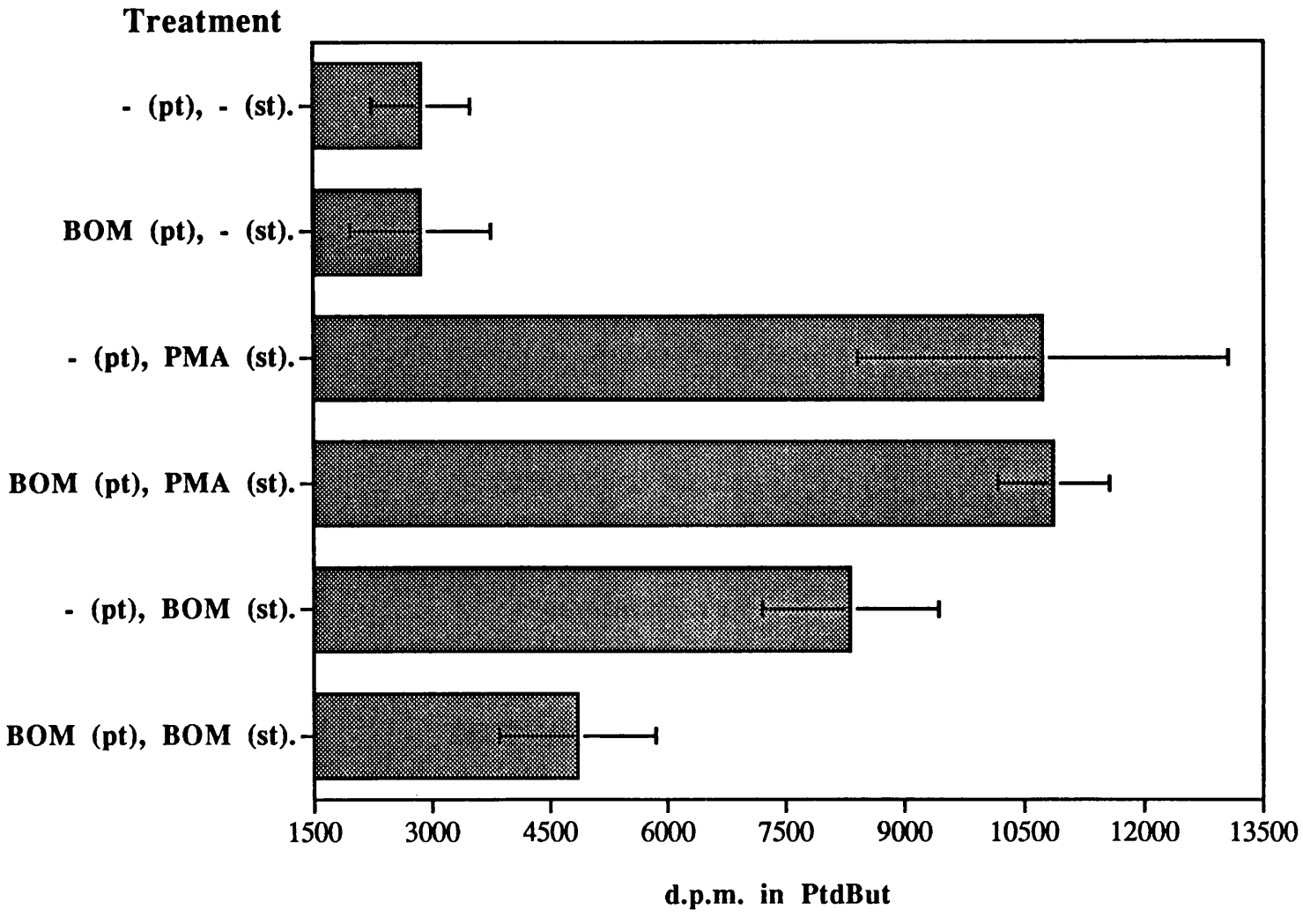

Treatment
- (pt), -(st).
- (pt), GTP[S] (st).
VP (pt), GTP[S] (st).
(B)
1000 2000 3000 4000 5000 6000 7000
d.p.m. in PtdBut

Figure 4.13 Effect of a 1 minute bombesin pretreatment on PMA-stimulated PLD activity

[^{3}H]Palmitate-labelled cells were incubated for 1 minute, with HHBG or 100nM bombesin. Cells were washed for 3x 30 seconds, then stimulated in the presence of 30mM butanol for 10 minute with, HHBG, 100nM bombesin or 100nM PMA. Incubations were terminated by replacement of the medium with ice-cold methanol. Chloroform extracts were prepared and [^{3}H]PtdBut quantified by t.l.c. as described. Results are expressed as radioactivity in [^{3}H]PtdBut (mean ± S.D., d.p.m., n=3) and are from a typical experiment representative of three.


Treatment
- (pt), - (st).
BOM (pt), - (st).
- (pt), PMA (st).
BOM (pt), PMA (st).
- (pt), BOM (st).
BOM (pt), BOM (st).
1500
3000
4500
6000
7500
9000
10500
12000
13500
d.p.m. in PtdBut

4.2.7 The effect of elevating intracellular calcium on bombesin-stimulated PLD activity

The stimulated increase in intracellular calcium by bombesin in Swiss 3T3 cells is transient (Takuwa *et al.*, 1987; Currie *et al.*, 1992), thus the decline in calcium levels could play a role in desensitisation, particularly in light of the suggestion that in some cells, PLD activity may be regulated by changes in intracellular [Ca^{2+}]. It has been suggested that heterologous desensitisation of stimulated-PLD activity is due to short term depletion of an intracellular Ca^{2+} pool(McKenzie *et al.*, 1992). Therefore the effect of maintaining an elevated intracellular calcium level by treating with the calcium ionophore A23187 on stimulated PLD activity was examined. Figure 4.14 shows that A23187 stimulated rapid [^{3}H]PtdBut accumulation, which plateaued between 30 seconds and 1 minute consistent with the theory of homologous desensitisation. Thus maintaining an elevated intracellular [Ca^{2+}] using A23187 could not prevent homologous desensitisation of A23187-stimulated [^{3}H]PtdBut accumulation. However, a pretreatment with ionophore only caused a 40% reduction in bombesin-stimulated PLD activity (Table 4.6). Furthermore pretreating with 100nM bombesin for 1 minute completely abolished any subsequent bombesin- or A23187-stimulated [^{3}H]PtdBut accumulation.

Figure 4.14 Time course of A23187-stimulated PLD activity

[^{3}H]PtdBut accumulation was determined in [^{3}H]palmitate-labelled cells, stimulated with 5μM A23187 for increasing times in the presence of butanol, after a 5 minutes preincubation with 30mM butanol. Incubations were terminated by the addition of ice-cold methanol. Chloroform extracts were prepared and [^{3}H]PtdBut generation quantified by t.l.c. as described. (o), control cells (0.1% (v/v) dimethylsulphoxide); (•), stimulated cells. Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m., mean ± S.D., n=3) and are from a single experiment representative of three.

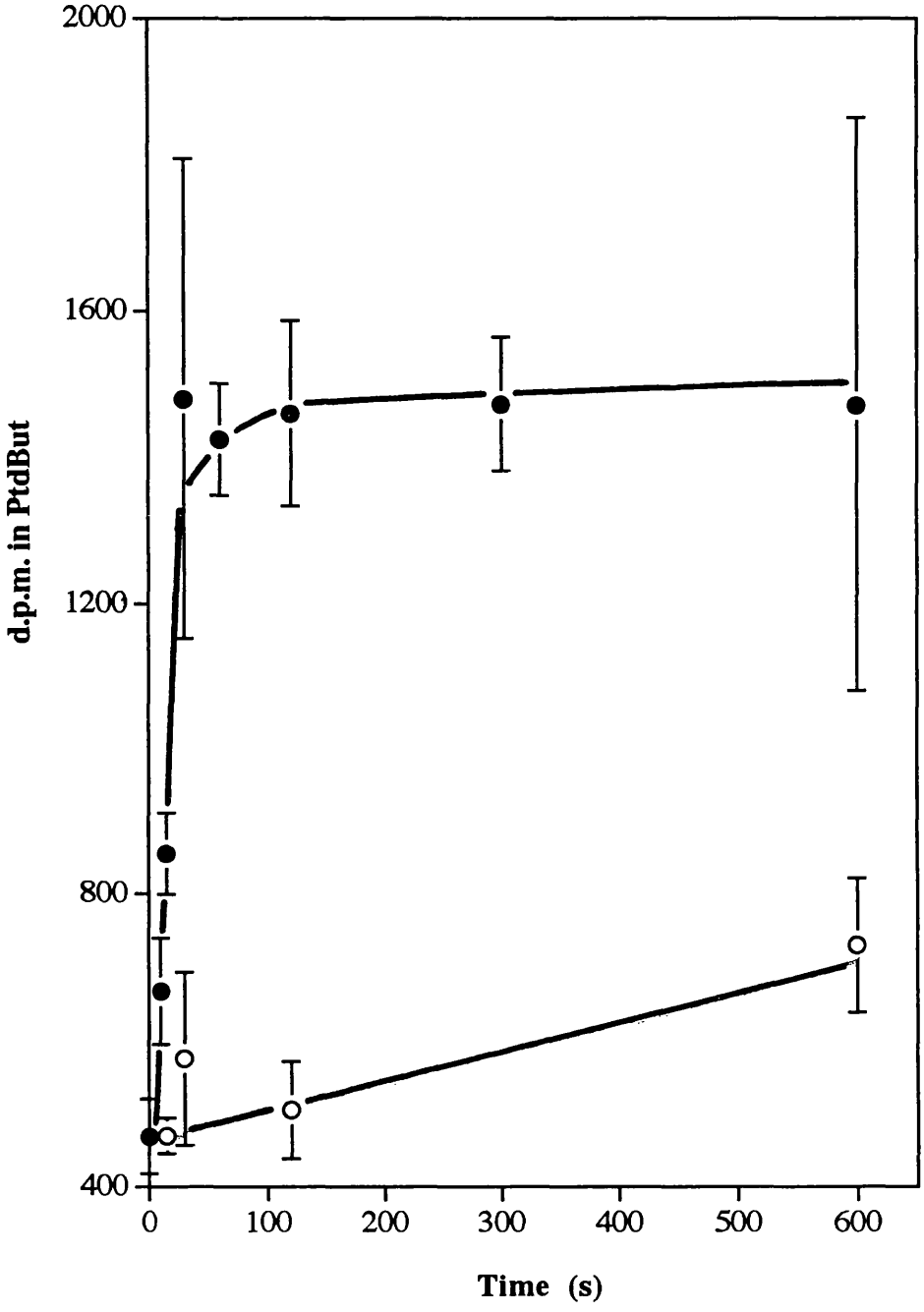

2000
1600
1200
800
400
d.p.m. in PtdBut
0
100
200
300
400
500
600
Time (s)

Table 4.6 Effect of elevating $[Ca^{2+}]_i$ on bombesin-stimulated PLD activity.

[^{3}H]Palmitate-labelled cells were pretreated with vehicle, 5μM A23187 or 100nM bombesin for 1minute, before washing for 3 x 30 seconds with HHBG. The cells were then stimulated for 1 minute with vehicle, 100nM bombesin or 5μM A23187, in the presence of 30mM butan-ol. All pretreatments and stimulations contained 0.1% (v/v) DMSO. Incubations were terminated by the addition of ice-cold methanol. Chloroform extracts were prepared and [^{3}H]Ptdbut was determined by t.l.c. as described. Results are expressed as % mean ± S.D., of basal, where vehicle was used in the pretreatment and stimulation. Results are from a single experiment representative of three, where each point was performed in triplicate. Mean basal d.p.m., 1527 ± 255.

Treatment	d.p.m.in PtdBut as % of basal
no pretreatment 1 min bombesin stimulation	291± 78
no pretreatment 1 min A23187 stimulation	200± 36
bombesin pretreatment 1 min vehicle stimulation	94 ± 18
A23187 pretreatment 1 min vehicle stimulation	86 ± 18
bombesin pretreatment 1 min bombesin stimulation	115 ± 19
bombesin pretreatment 1 min A23187 stimulation	118 ± 17
A23187 pretreatment 1 min A23187 stimulation	100 ± 15
A23187 pretreatment 1 min bombesin stimulation	200 ± 31

4.3 Discussion.

4.3.1 Desensitisation of bombesin-stimulated [^{3}H]PtdBut accumulation is rapid and reversible

Agonist-stimulated PLD activity was found to be tightly regulated and subject to rapid desensitisation (Fig.4.1). The IC_{50} for bombesin-induced desensitisation of a 2 minute 100nM bombesin stimulation of [^{3}H]PtdBut generation was 0.2 ± 0.1nM (Fig. 4.2) and was comparable to the IC_{50} of 0.35 ± 0.47nM obtained for the bombesin-mediated homologous desensitisation of stimulated-Ins(1,4,5)P_3 mass (Palmer *et al.*, 1991). The kinetics of desensitisation of [^{3}H]PtdBut generation to bombesin are very similar those described for the desensitisation of bombesin-stimulated Ins(1,4,5)P_3 generation, suggesting that they may occur through similar processes. Desensitisation of bombesin-stimulated PLD activity may possibly occur downstream from that of stimulated PtdIns(4,5)P_2 hydrolysis. Similar correlations between the time courses of stimulated PtdIns(4,5)P_2 hydroysis and PLD-catalysed PtdCho hydrolysis were reported in A10 vascular smooth muscle cells (Plevin and Wakelam, 1992) and in Chinese hamster lung fibroblasts transfected with the human M1 receptor (McKenzie *et al.*, 1992).

The IC_{50} values for desensitisation of bombesin-stimulated Ins(1,4,5)P_3 mass and [^{3}H]PtdBut accumulation are notably ten-fold lower than the reported EC_{50} values for other bombesin receptor-linked responses in Swiss 3T3 cells such as: DNA synthesis, EC_{50} 1nM (Rozengurt and Sinnett-Smith, 1983); inositol phospholipid hydrolysis, EC_{50} 1.78 ± 0.03 (Plevin *et al.*, 1990); PtdCho generation, EC_{50} 2.30 ± 0.57 (Cook and Wakelam, 1989) and [^{3}H]PtdBut generation, EC_{50} 1.3 ± 0.31nM (Cook *et al.*, 1991). This suggests that desensitisation may be due to post-receptor events, possibly the result of a signalling cascade.

Resensitization of bombesin-stimulated PLD activity, though incomplete after 41.5 minutes of washing, commenced as early as 4.5 minutes after removal of the desensitising pretreatment (Fig 4.3). Similarly in A10 VSMC, removal of AVP

from its receptor for at least 3 minutes, was found to be necessary for response to a fresh stimulus (Caramelo *et al,* 1991).

4.3.2 Bombesin-stimulated desensitisation and resensitisation of PtdCho hydrolysis

The determination of total [^{3}H]choline production at increasing times after the initial pretreatment with bombesin (Fig 4.4), proved that the desensitisation and resensitisation of bombesin-stimulated [^{3}H]Ptdbut generation was reflected in the hydrolysis of PtdCho and was not solely a phenomenon connected with the transphosphatidylation activity of phospholipase D. The difference in resensitisation time between experiments measuring [^{3}H]PtdBut and [^{3}H]choline generation probably reflects the fact that the former is an accumulation assay and the latter is measuring a product that can be metabolised by a number of different pathways. In this respect, choline production from the cells, pretreated with bombesin and stimulated with buffer, decreased with increasing wash time. This presumably reflected metabolism of the choline produced from the initial bombesin stimulation.

4.3.3 Desensitisation is not due to a limitation in labelled substrate or butanol.

PtdCho constitutes approximately 50% of the total membrane lipid and it has previously been reported that stimulation of Swiss 3T3 cells with a maximal concentration of either bombesin or PMA caused the loss of only about 5% of the label from the total PtdCho fraction (Cook and Wakelam 1989). Thus desensitisation is probably not due to limitation of labelled substrate.

This argument was strengthened by the finding that desensitisation of bombesin-stimulated [^{3}H]PtdBut was not affected by experiments performed in the continual presence of [^{3}H]palmitate (Table 4.1). Pretreatments that desensitised the cells to further agonist stimulation of PLD activity, were performed in the absence of butan-1-ol, whereas the stimulations were in the presence of butanol. Hence limitation of butan-1-ol was probably not responsible for desensitisation.

Furthermore, PMA-stimulated [^{3}H]PtdBut continued for at least 15 minutes demonstrating that labelled substrate and butanol was an unlikely limiting factor in desensitisation of agonist-stimulated PLD activity. It was possible however, though unproven, that PMA hydrolyses a different pool of [^{3}H]PtdCho to that affected by bombesin.

4.3.4 The extent and kinetics of resensitisation of bombesin-stimulated generation of total [^{3}H]inositol phosphates differs from that of [^{3}H]PtdBut accumulation.

As desensitisation of bombesin-stimulated [^{3}H]PtdBut generation appeared to be closely correlated with that of PtdIns(4,5)P_2 hydrolysis, the relationship between resensitisation of bombesin-stimulated accumulation of total [^{3}H]inositol phosphates and that of [^{3}H]PtdBut was determined. Li^+ uncompetitively inhibits inositol monophosphatase (reviewed Nahorski *et al.*, 1991), allowing measurement of PtdIns-PLC activity through accumulation of total [^{3}H]inositol phosphates. However, at stimulation times greater than 5 minutes Li^+ can inhibit phosphoinositide synthesis (Berridge, 1993). The resensitisation of bombesin-stimulated production of total [^{3}H]inositol phosphates was determined when LiCl was only present during the 1 minute bombesin stimulation, thus the disruptive effects of LiCl were likely to be minimal. An increase in total [^{3}H]inositol phosphates stimulated by 100nM bombesin, over the attenuated level attained after a 1 minute bombesin pretreatment, was observed within 8.5 minutes after agonist removal with complete recovery after 11.5 minutes (Fig. 4.7). The difference in the kinetics and extent of resensitisation of bombesin-stimulated generation of total [^{3}H]inositol phosphates from that of [^{3}H]PtdBut may suggest mechanistic differences. The resensitisation of bombesin-stimulated accumulation of total inositol phosphates contrasted with the recovery of bombesin-induced loss of stimulated Ins(1,4,5)P_3 mass, which did not commence until at least 10 minutes after removal of the stimulus, not reaching completion even after 60 minutes (Palmer *et*

al., 1991). Measurement of Ins(1,4,5)P_3 mass, however, has the problem that its metabolism is not accounted for. Thus small increases in bombesin-stimulated Ins(1,4,5)P_3, following removal of the desensitising stimulus, may not be detected.

The resensitisation of the stimulated production of total [^{3}H]inositol phosphates determined in the presence of LiCl, apparantly occurred at times when Ins(1,4,5)P_3 mass levels were still at basal. The transient formation of Ins(1,4,5)P_3 could be due to rapid activation of Ins(1,4,5)P_3 5-phosphatase and 3-kinase, a depletion of PtdIns(4,5)P_2 or activation of different isoforms of PtdIns-PLC having different substrate specificities. There is increasing evidence that, although the initial event of receptor-activated PtdIns-PLC is the hydrolysis of PtdIns(4,5)P_2, at later times the hydrolysis of PtdIns may become more important. Plevin and Wakelam (1992) reported that AVP-stimulated Ins(1,4,5)P_3 mass generation and the decrease in PtdIns(4,5)P_2 mass levels was transient and suggested that another inositol-containing phospholipid other than PtdIns(4,5)P_2.may be hydrolysed during longer exposures of A10 VSMC to agonist. Similar findings were reported in hormone-stimulated rat pituitary cells (Imai and Gershengorn 1986), lithium treated rats (Ackerman *et al.*, 1987) and thrombin stimulated platelets (Wilson *et al.*, 1985). However in carbamoyl-stimulated neuroblastoma cells polyphosphoinositides were found to be the major source of inositol phosphates (Fisher *et al.*, 1990).

4.3.5 The internalisation and recycling of agonist occupied receptors is a potential mechanism for desensitisation and resensitisation of bombesin-stimulated PLD activity.

The loss of cell surface receptors after exposure to agonist is a common event in transmembrane signalling systems. Many reports suggest that after short exposure to stimulus, some proportion of the internalised receptors can also recycle to the cell surface (Hoxie, *et al.*, 1993; Briner *et al.*, 1992; Zachary and Rozengurt, 1987). However, experiments with the β_2-AR have shown that such sequestration is not important for the initial desensitisation but is essential for resensitisation after

short agonist exposure (Yu *et al.*, 1993). Receptor binding studies therefore examined the loss of cell surface binding sites with agonist exposure as a possible mechanism of desensitisation and/or a prerequisite for resensitisation.

A receptor-ligand complex can be processed by several different pathways: (a) internalisation of the complex, with subsequent dissociation of the ligand from the receptor, degradation of the ligand and recycling of the receptor to the cell surface, as reported for endothelin in human VSMC (Resink et al., 1990); (b) internalisation of the complex, followed by recycling of intact unit and its externalisation as described for receptor-mediated transport of IgG across the new-born rat intestine (Abrahamson and Rodewald, 1981); (c) internalisation and lysosomal degradation of the whole complex, such that protein synthesis is required for expression of new cell surface receptors, as for EGF in human fibroblasts (Carpenter and Cohen, 1976); (d) transcytosis of the complex without lysosomal processing or recycling, as described for a transmembrane precursor of secretory component in epithelial cells (Mostov and Blobel 1982).

Acid washing of Swiss 3T3 cells, after the addition of [^{125}IGRP] for 1 minute to Swiss 3T3 cells at 37^{0}C, showed that all the cell associated radioactivity was internal after this time. Since non-equilibrium binding conditions were employed to examine GRP-receptor interactions, high non-specific binding varying from 40-85% of total binding was a problem with these experiments. As explained in the results section of this chapter, further binding experiments were performed with [^{3}H]AVP and [^{3}H]AVP antagonist.

A short AVP pretreatment at 37^{0}C which completely abolished subsequent AVP-stimulation of [^{3}H]PtdBut accumulation, only partially reduced [^{3}H]AVP antagonist binding (Table 4.5). Acid-washing removed most of the cell associated radioactivity after 1 minute of [^{3}H]AVP binding at 37^{0}C, but not after 10 minutes of binding (Fig 4.10). This implied that the receptors must be on the surface accessible to acid stripping, but not to ligand binding after 1 minute, perhaps in a stage before entering early endosomes and it is not until later that the receptor/ligand complex is

internalised. These findings are supported by investigations of thrombin receptor internalisation (Hoxie *et al.*, 1993). Using receptor-directed antibodies, thrombin receptors were found to be clustered in and around coated pits in the membrane after 1 minute of stimulation. Prelysosomes were not detected until after 10 minutes of thrombin exposure . The difference between the kinetics of internalisation between [^{125}I]GRP and [^{3}H]AVP, where studies suggested all the [^{125}I]GRP was internalised after 1 minute, may be due to the different receptors or the fact that any [^{125}I]GRP that was removed by the low pH wash was masked by the level of non-specific binding.

It appears that AVP-mediated desensitisation of stimulated [^{3}H]PtdBut generation and loss of cell-surface binding occurs more rapidly than the internalisation of receptors. The reduction in cell-surface binding before the detection of internalisation may be due to a conformational change in the receptor perhaps due to phosphorylation. As previously discuused (section 4.3.1) the IC_{50} for bombesin-stimulated desensitisation of PLD activity suggested a post-receptor mechanism was involved. It is thus possible that a similar mechanism of rapid desensitisation to that occurring for the β-AR also occurs for desensitisation of the bombesin-receptor. Uncoupling of the receptor from G-protein/effector system by receptor phosphorylation is postulated to be the primary mechanism of desensitisation of the β_2-AR. Subsequent receptor sequestration is a necessary event for receptor reactivation (see chapter 5 for fuller discussion). However this comparison is purely speculative as although uncoupling of the receptor/effector mechanism may occur prior to internalisation there is no evidence for bombesin receptor phosphorylation.

Evidence for the involvement of both receptor and post receptor mechanisms in desensitisation exists for other receptors-linked to PtdIns(4,5)P_2 hydrolysis. Although receptor number and binding affinity was not determined after agonist pretreatment in this study, several studies have shown that a decrease in binding of cell surface receptors can occur without a change in receptor affinity. For

example, homologous desensitisation in response to a 2 hour preincubation with AVP in vascular smooth muscle cells was reported to cause a 30-40% decrease in receptor number with no change in receptor affinity and a 60-80% decrease in inositol phosphate production (Grier *et al.*, 1989). This suggested that AVP also has an effect on post-receptor sites in these cells. The decrease in inositol phosphate production was not blocked by the V_{1a} antagonist and was therefore receptor specific. Desensitisation of AVP-induced Ca^{2+} mobilisation in VSMC (Caramelo *et al.*, 1991) and thrombin-stimulated phosphoinositide breakdown in fibroblasts (Paris *et al.*, 1988) was reported to still occur at 4^0C, a temperature at which internalisation is blocked, suggesting that in these cells receptor internalisation was not important for desensitisation .

The increase in [^{3}H]AVP antagonist receptor binding, after removal of the AVP pretreatment, was generally greater than the resensitisation of AVP-stimulated [^{3}H]PtdBut observed (Table 4.5). The recovery of antagonist binding probably reflects a recycling of the internalised receptor. Thus both receptor and post-receptor mechanisms may also be involved in the regulation of resensitised, agonist-stimulated PLD activity. Recycling of the AVP receptor was also reported by Briner *et al.*, (1992), who showed that recovery of AVP binding sites in VSMC occurred in a protein-synthesis independent manner.

Preliminary experiments suggested that pretreatment with [^{3}H]AVP antagonist could also reduce the binding of radiolabelled ligand to cells. However, antagonist pretreatment either had little or no effect on subsequent AVP-stimulated PLD activity. Furthermore, after washing the cells for 11.5 minutes there was no difference between those that were unpretreated and those that had been pretreated with antagonist, though cells pretreated with agonist were still partially desensitised. The antagonist does not have any effect on intracellular signalling events nor should it be internalised. One explanation for the effect of the antagonist on binding is that it binds to the receptor with high affinity hence blocking agonist binding. The failure of the AVP antagonist to have a significant effect on stimulated PLD activity

suggested that binding to the receptor alone is insufficient to induce desensitisation processes. Similarly, the binding of a fluorescent-labelled AVP analog to A-10 VSMC, that bound to the V_{1a} receptor, but did not initiate intracellular events or endocytosis, implied that receptor activation was essential to receptor internalisation (Lutz *et al.*, 1992).

In support of bombesin-receptor internalisation Zachary and Rozengurt (1987) investigated the binding of [^{125}I]GRP to Swiss 3T3 fibroblasts. After 5 minutes at 37^{0}C approximately 50% of specific cell-associated radioactivity was internalised, with 20% remaining resistant to acid after 30 minutes. The internalisation of the receptor-ligand complex was accompanied by lysosomal degradation of [^{125}I]GRP and recycling of the receptor. Similar results were reported using ^{125}I-[Tyr^4]bombesin (Brown *et al.*, 1988; Wang *et al.*, 1993). Bombesin receptor internalisation has also been reported in guinea pig pancreatic acini (Pandol *et al.*, 1982). Although preincubations of 90 minutes were used to study desensitisation, a virtual complete recovery of the response was attained following washing for 2 to 3 minutes, which was proposed to be due to receptor recycling.

Despite the partial resensitisation of bombesin-stimulated [^{3}H]PtdBut accumulation 11.5 minutes after removal of the desensitising pretreatment, a further challenge with bombesin resulted in complete desensitisation to a short stimulation in the presence of butanol. There was no difference in the extent of resensitisation, even if the cells were subjected to several pretreatment/wash cycles before stimulation in the presence of butanol (Table 4.2). This suggests resensitised receptors must be free of any modifications that may have caused the initial desensitisation although post-receptor events may prevent the full resensitisation of receptors. Rechallenge may commence a new cycle of receptor/effector uncoupling and sequestration.

Cells, pretreated with bombesin, in the absence of butanol for 1 minute, then stimulated in the presence of the alcohol, exhibited a continual increase in

[^{3}H]PtdBut accumulation at a rate reduced that produced from the initial stimulus. This suggested that resensitisation occurred even in the continual presence of agonist which is an unusual phenomenon. Transient resensitisations of muscarinic-stimulated muscle contractility was reported to occur even in the continual presence of the agonist, although the phenomenon correlated with changes in the affinity of carbachol for the receptors (Hishinuma *et al.*, 1993). The rate changes of bombesin-stimulated PLD activity may have been due to continuous cycles of receptor internalisation and recycling. Although it was not possible to prove this continuous receptor trafficking from binding studies, it has been reported that prolonged exposure to bombesin or GRP for up to 3 hours did not affect the binding of GRP to its receptor even in the presence of cycloheximide (Zachary and Rozengurt 1987). In contrast to EGF receptors, the bombesin receptors are therefore not down-regulated in Swiss 3T3 cells after short exposure to agonist. The inability of Swiss 3T3 fibroblasts to down-regulate bombesin receptors over relatively short exposure to agonist appears to be cell specific. A decrease in receptor binding during continuous exposure for 90 minutes to bombesin, was reported in both rat pancreatic acini and in HIT-T15 islet cells (Zhu *et al.*, 1991; Swope and Schonbrunn, 1990).

Receptor sequestration and return to the cell surface in the continual presence of agonist has been reported for β-AR (Kurz and Perkins, 1992) and guanylate cyclase/atrial natriuretic factor receptors (Pandey, 1993). The recycling of the β-AR was found to occur through similar pathways irrespective of the continual presence of agonist. The kinetics of bombesin-stimulated PLD activity may have been due to continuous cycles of receptor internalisation and recycling to the plasma membrane. The initial desensitisation may reflect the rapid transfer of receptors to endosomes with the resensitised rate of PLD activity due to the attainment of a steady state of receptor trafficking.

A role for the receptor in resensitisation is supported by the requirement for bombesin to be present in the medium for recovery of [^{3}H]PtdBut accumulation after a bombesin pretreatment (Fig 4.5). This suggested that receptors were recycling to the surface, but reoccupancy of the receptors by agonist was necessary for

restimulation of PLD activity. Bombesin may therefore have dissociated from the receptor before it recycled. Endolysosomal processing of the AVP-receptor complex in vascular smooth muscle cells (Briner *et al.*, 1992) was found to be essential for the recycling process.

It is thus proposed that receptors do recycle in the presence or absence of agonist however the mechanism of PLD activation by the bombesin-activated, recycled receptor is unclear. As previously discussed, at longer times of agonist exposure the production of inositol phosphates may be due to the hydrolysis of PtdIns. The production of DAG at longer stimulation times is apparently not derived solely from PLD-catalysed PtdCho hydrolysis in Swiss 3T3 cells (Cook *et al.*, 1992; Pettit and Wakelam, 1993). In other cell types it has been proposed that DAG may be produced from PtdIns hydrolysis such as in A10 vascular smooth muscle cells (Plevin and Wakelam, 1992) or *de novo* synthesis, for example in neutrophils (Rossi *et al.*, 1991). Receptor recycling may therefore permit the continual production of DAG which in the absence of Ins(1,4,5)P_3-mediated Ca^{2+} rises could activate the Ca^{2+}-independent isoforms of PKC such as PKCδ and hence stimulate PLD activity. In accordance with this proposal, phases of bombesin-stimulated DAG and PKC activity may be expected to precede those of [^{3}H]PtdBut generation. Although there is no evidence for this in Swiss 3T3 fibroblasts, three phases of PKC activity were detected over 60 minutes in IFNγ-stimulated endothelial cells although these were subsequent to increases in choline, PtdOH and DAG (Mattila and Renkonen, 1992). Furthermore, a multiphasic generation of DAG was observed in α-thrombin-stimulated human platelets in response to sub-threshold levels of agonist (Werner *et al.*, 1992). It is also possible that interactions with products from other phospholipase-catalysed phospholipid hydrolysis may regulate PLD activity. Recently it was reported that oleate, presumably generated from PLA_2-catalysed PtdCho hydrolysis, stimulated DAG formation through effects on PLD and PAP (Siddiqui and Exton, 1992).

However, as previously described, resensitisation of stimulated PLD activity is incomplete and may be also under the control of post-receptor events. The mechanism of activation of PLD through PKC and tyrosine kinases is unclear but may directly modify PLD itself or upstream components of the signalling pathway such as the receptor or G-protein. Such phosphorylation may occur transiently and be under the control of tyrosine or serine and threonine kinases and phosphatases. Bombesin has also been reported to activate MAP kinase (Pang *et al.*, 1993) which may feed back to regulate PLD activity . Moreover biphasic activation of MAP kinase was observed in α-thrombin stimulated fibroblasts (Kahan *et al.*, 1992) and a recent report demonstrated that MAP kinase could activate a phosphatase which dephosphorylated the EGF receptor (Griswold-Prenner *et al.*, 1993).

However, whether bombesin stimulates PLD activity through the same pathways irrespective of whether the receptor is recycled in the continual presence or in the absence of the agonist is uncertain.

4.3.6 Bombesin-stimulated desensitisation and resensitisation of PLD activity is PKC independent.

The continuous generation of PMA-stimulated [^{3}H]PtdBut over 15 minutes suggested that desensitisation may be independent of PKC. Table 4.4 shows that complete desensitisation of bombesin-stimulated PLD activity occurred even in the presence of the selective PKC inhibitor Ro-31-8220, at a concentration which reduced PMA-stimulated PLD activity to basal levels and has been reported to be selective for inhibition of PKC (Davis *et al.*, 1989). A recovery in PKC activity is unlikely to be involved in a mechanism by which resensitisation occurs, since preincubation with the kinase inhibitor resulted in a similar percentage inhibition of

the partially resensitised bombesin-stimulated [^{3}H]PtdBut accumulation as that of bombesin-stimulated PLD activity observed in unpretreated cells. Previous reports suggest that bombesin and AVP-stimulated homologous desensitisation of Ins(1,4,5)P_3 (Palmer *et al.*, 1991; P.Kaur personal communication) was also PKC independent. Furthermore, AVP-stimulated desensitisation of Ca^{2+} mobilisation was found to be PKC dependent in VSMC (Caramelo *et al.*, 1991).

4.3.6 Agonist-stimulated desensitisation of PLD activity is both homologous and heterologous.

The loss in stimulated [^{3}H]PtdBut generation induced by a short pretreatment with agonist was found to be partially heterologous. Figure 4.11 shows that after a 1 minute pretreatment in the absence of butanol, the stimulation of PLD activity using an agonist different from that used in the pretreatment, was attenuated by about 70%. As agonist-stimulated PLD activity is reduced by 50-70% with Ro-31-8220 and heterologous desensitisation of stimulated-PLD activity resulted in such a large attenuation of the response, it proved impossible to demonstrate the involvement of PKC in the phenomenon. Figure 4.13 shows that a 1 minute pretreatment with bombesin did not affect a subsequent PMA stimulation of PLD activity. This suggested that agonist pretreatment of cells did not affect the stimulation of PLD through the activation of PKC. AVP and bombesin-stimulated heterologous desensitisation of Ins(1,4,5)P_3 has also been reported in Swiss 3T3 cells (P. Kaur personal communication).

Vasopressin receptor binding studies showed that a 1 minute pretreatment, with a maximal dose of bombesin at 37^{0}C, did not affect the binding of [^{3}H]AVP or [^{3}H]AVP antagonist at 4^{0}C (Table 4.5). This suggested that heterologous desensitisation was not receptor mediated. Prolonged AVP pretreatment was reported not to alter the number, affinity or internalisation capacity of bombesin-receptors in Swiss 3T3 cells (Millar and Rozengurt 1989).

4.3.7 A short agonist pretreatment partially reduces GTPγS-stimulated PLD activity

Bombesin and vasopressin receptors in Swiss 3T3 cells couple through the pertussis-toxin insensitive G-protein, G_q to activate PLCβ1 (Smrcka *et al.*, 1991). Results presented in Chapter Three showed that bombesin-stimulated PLD activity was also G-protein regulated. Experiments were thus performed to investigate whether a short agonist exposure could affect the stimulation of PLD activity through G-protein activation alone. Agonist-pretreated cells were found to reduce GTPγS-stimulated PLD activity in permeabilised cells by approximately 50% (Fig. 4.12). This suggested that agonist-induced desensitisation of PLD activity is not due solely to receptor/G-protein uncoupling which has been suggested to be the site of PMA inhibition of bombesin-stimulated Ins(1,4,5)P_3 generation (Plevin *et al.*, 1990). Therefore a component of the signalling pathway at, or downstream of the G-protein is also affected. The agonist-induced reduction in G-protein regulated PLD activity may account for the attenuation of stimulated PLD activity observed in response to a second agonist (Fig. 4.11). This may occur if the receptors for both agonists use the same pool of G-proteins to activate PLD as proposed for the α_1-adrenoreceptor and the AVP receptor in hepatocytes (Dasso and Taylor, 1992). The finding that GTPγS pretreatment of cells did not decrease a subsequent GTPγS-stimulation of PLD activity suggested that activation of the G-protein itself, is insufficient for desensitisation but that receptor activation and coupling to the G-protein is essential. This contrasts with desensitisation of the β-AR where stimulation of adenylyl cyclase through the activation of G_s was not affected despite loss of responsiveness to the agonist and alterations in the function of G_s did not affect desensitisation of β-AR (Green and Clark, 1981).

It is possible that the receptor and the G-protein regulating PLD activity (possibly G_q) could be internalised together. An internalisation of $G_q\alpha$ with the receptor could perhaps account for the continued generation of inositol phosphates, if PLC and phosphoinositides were accessible in the cytoplasmic structures. In

support of this, internalisation of muscarinic receptors with G-proteins has been reported in human astrocytoma cells and in rat brain homogenates, with the release of $G_{o\alpha}$ and $G_i\alpha$ detected in the latter (Harden *et al.*,1985; Ho *et al.*, 1991). However only muscarinic receptors present at the cell surface were able to activate PtdIns-PLC in neuroblastoma cells (Thompson and Fisher, 1991). Furthermore, G_s was reported to be released from the membrane upon stimulation of the β-adrenergic receptor (Ransnas *et al.*, 1992). Although loss of GTPγS-stimulated PLD activity could be also due to an agonist dependent modification of the G-protein itself, no phosphorylation of $G_q\alpha$ was detected in bombesin-stimulated Swiss 3T3 cells (P. Kaur personal communication).

4.3.9 The calcium dependency of homologous and heterologous desensitisation of stimulated PLD activity.

Heterologous desensitisation of agonist-stimulated Ca^{2+} mobilisation in astrocytoma cells (McDonough*et al.*, 1988), hepatocytes (Joseph *et al.*, 1985) and in bombesin-stimulated human small cell lung cancer cell lines (Heikkila *et al.*, 1987) has been suggested to be due to emptying of the $Ins(1,4,5)P_3$ sensitive Ca^{2+} pools. Carbachol-mediated desensitisation of α-thrombin stimulated PLD activity in Chinesehamster lung fibroblasts was also proposed to be due the depletion of intracellular Ca^{2+} stores (Mckenzie et al., 1992). It was thus possible that $[Ca^{2+}]_i$ may be involved in the decrease in PLD activity in response to bombesin in Swiss 3T3 cells.

However, in addition or alternatively to depletion of Ca^{2+} stores, the $Ins(1,4,5)P_3$ receptor may become insensitive to any further increases in $Ins(1,4,5)P_3$ due to addition of a second agonist. $Ins(1,4,5)P_3$ binding or its effect on the Ca^{2+} release channel of the endoplasmic reticulum is regulated by $[Ca^{2+}]_i$ in a biphasic manner. This mode of regulation is thought to account to a significant extent for the transient rise in $[Ca^{2+}]$ elicited by agonists which stimulate $PtdIns(4,5)P_2$ hydrolysis and for the repetitve Ca^{2+} oscillations observed in some cells in response to

continual elevation of Ins(1,4,5)P_3 (Lino and Endo, 1990). The effect of Ins(1,4,5)P_3 is enhanced at lower $[Ca^{2+}]_i$, whilst the inhibition of Ins(1,4,5)P_3 binding at high $[Ca^{2+}]$ is thought to be mediated partly through the interaction of the Ca^{2+}-binding protein calmedin with the receptor (Danoff *et al.*, 1988). Furthermore, the affinity of the Ins(1,4,5)P_3 receptor was reported to switch from a low to a high affinity, inactive state, in the presence of high $[Ca^{2+}]_i$ (Rouxel *et al.*, 1992; Pietri *et al.*, 1990). Other possible inhibitory mechanisms of Ins(1,4,5)P_3-stimulated Ca^{2+} release may occur through the depletion of ATP levels such that the enhancement of Ca^{2+} release through the high affinity ATP recognition site is reversed (Ferris *et al.*, 1990). Phosphorylation of the receptor by PKC and Ca^{2+} activated calmodulin-dependent protein kinase may also be involved (Ferris et al., 1991; Ferris and Snyder, 1992). Reversal of these inhibitory effects may depend on the refilling of the Ins(1,4,5)P_3-sensitive pool and the return of Ca^{2+} to basal levels. The above effects may therefore explain heterologous desensitisition in cells where agonist-stimulated Ins(1,4,5)P_3 remains elevated such as in astrocytoma cells (McDonough *et al.*, 1988). However in other cell types heterologous desensitisation of Ins(1,4,5)P_3 generation may occur which would also abbrogate the ability of the second agonist to mobilise Ca^{2+} from intracellular stores. In support of this, heterologous desensitisation of agonist-stimulated Ins(1,4,5)P_3 generation has been observed in Swiss 3T3 cells (P.Kaur personal communication).

The time course of A23187-stimulated [^{3}H]PtdBut accumulation (Fig. 4.14) was similar to that stimulated by bombesin and vasopressin, with stimulation of PLD activity apparently ceasing after about 1 minute. This suggested that continuous elevation of intracellular calcium levels could not prevent homologous desensitisation of A23187-stimulated PLD activity. Pretreatment of cells with bombesin, completely abolished a subsequent A23187 stimulation of [^{3}H]PtdBut accumulation. Pretreatment with A23187 only reduced bombesin-stimulated PLD activity by 40% (Table 4.6). This suggests that bombesin-mediated homologous desensitisation is a Ca^{2+}-independent pathway. The effect of the ionophore

pretreatment on a bombesin stimulation may be a result of the activation of Ca^{2+}-dependent protein kinase C α isozyme as it has previously been shown that Ro-31-8220 can inhibit 50% of the stimulation of PLD activity by A23187 (Cook *et al.*, 1991). However the previous experiments have demonstrated that such a bombesin pretreatment did not affect the PMA-stimulation of PLD activity. These differences may be resolved by the possibility that the Ca^{2+}-independent forms of PKC are unaffected by a short bombesin pretreatment and these can thus stimulate [^{3}H]PtdBut accumulation. The partial attenuation of bombesin-stimulated PLD activity by A23187 pretreatment may therefore reflect the Ca^{2+}-dependent protein kinase C α dependency of heterologous desensitisation.

It is possible that A23187-induced desensitisation of bombesin-stimulated [^{3}H]PtdBut formation is mediated through a different mechanism to agonist-induced heterologous desensitisation, as the ionophore does not stimulate PtdIns(4,5)P_2 hydrolysis. A23187 may also have acted as an ionophore for intracellular Ca^{2+} stores, thus by depleting Ca^{2+} pools it may have mimicked the proposed heterologous effects of agonist pretreatment. It is possible that both depletion of Ca^{2+} pools and PKC are involved. This argument is supported by the finding that a 30 minute carbamoylcholine treatment of permeabilised pancreatic acinar cells, decreased the affinity and maximal response of Ins(1,4,5)P_3 induced Ca^{2+} release (Willems *et al.*, 1989). The decrease in affinity was mimicked by PMA pretreatment, suggesting that the effect was partly PKC mediated. As it was not possible to demonstrate conclusively, that PKC was involved in heterologous desensitization of agonist-stimulated PLD activity, the involvement of other second messengers must also be considered.

The conclusions that can be drawn from this chapter are firstly, that bombesin-stimulates a rapid, dose dependent and PKC independent homologous desensitisation of stimulated [^{3}H]PtdBut accumulation. Homologous desensitisation occurs via receptor and post receptor mediated mechanisms and is reversible, even in the continual presence of agonist. Receptor internalisation and recycling is probably

responsible, not only for the observed partial resensitisation after agonist removal and washing, but also for the rate changes in stimulated-PLD activity observed in the continual presence of bombesin. Agonist-induced heterologous desensitisation is incomplete and apparently mediated through post-receptor mechanisms.

Chapter 5

General Discussion

The experiments in this thesis show that agonist-stimulated PLD activity in Swiss 3T3 fibroblasts can be regulated by a number of interacting pathways involving G-proteins, Ca^{2+}, protein tyrosine phosphorylation and PKC. In addition, the results in Chapter 4 show that the mechanisms by which PLD activity is controlled are finely tuned to generate a rapid deactivation of the signal upon receptor activation followed by a reactivation. The possible mediators of agonist-stimulated desensitisation and resensitisation of PLD activity can be compared and contrasted to those involved in the control of receptor-linked responses in other systems. Perspectives of such tight control through the production of second-messengers are interpretable in a number of different ways.

5.1 Regulation of agonist-stimulated PLD activity through G-proteins and tyrosine kinases.

5.1.1 The involvement of heterotrimeric G-proteins.

Experiments have clearly shown that PLD can be regulated through a receptor coupled G-protein. Interaction between the G-protein and PLD is indirect and occurs probably via a kinase intermediate such as PKC and/or a tyrosine kinase. Although the precise nature of the G-protein that couples to PLD is unclear, at least 50% of bombesin-stimulated PLD activity is regulated through PKC activation, presumably due to receptor coupled $G_q\alpha$ activation.

Interaction between tyrosine kinases and G-proteins have also been reported. Immunopecipitated *c-src* was reported to phosphorylate $G_s\alpha$ and G_t *in vitro*, slightly enhancing their activites (Hausdorff *et al.*, 1992) and epinephrine was found to stimulate direct association of *c-src* with $G_{i\alpha}$ in human platelets (Torti *et al.*, 1992). Although the phosphorylation sites were not determined, the tyrosine residues are conserved, not only in $G\alpha_s$, $G\alpha_i$, $G\alpha_o$ and $G\alpha_t$ but also in $G\alpha_q$. This suggested that a similar interaction may occur for pertussis-toxin insensitive G-proteins coupled to PtdIns(4,5)P_2 hydrolysis. In support of this, *c-src* was found to

be involved in pertussis toxin insensitive endothelin-stimulated transcriptional regulation and mitogenic signalling in glomerular mesangial cells (Simonson and Herman, 1993). Furthermore, it was proposed that ET-1 stimulated cell growth was controlled through $G\alpha_q$. However, phosphorylation of $G\alpha_q$ in response to bombesin in Swiss 3T3 cells was not detected (P. Kaur, personal communication).

It is conceivable that more than one G-protein could be involved in the full activation of agonist-stimulated PLD activity as suggested for fMLP-stimulated PLD activity in neutrophils (Cockcroft, 1992). It is possible that two G-proteins may regulate the PKC-dependent and PKC-independent pathways of PLD activation. This proposal implies that in Swiss 3T3 cells a G-protein other than $G\alpha_q$ (G_D) may be coupled directly to bombesin-stimulated tyrosine kinase activity.

However, bombesin may activate tyrosine kinases independently of G-proteins. Depletion of cellular ATP would have abolished any kinase activity in addition to PLC-catalysed PtdIns(4,5)P_2 hydrolysis. A direct stimulation of PLD through kinases would therefore not have been distinguished from that regulated by G-proteins. The bombesin-stimulated activation of G-protein and tyrosine kinase mediated second-messenger pathways may act in parallel to stimulate the same or different PLD isoforms. Clarification of these possibilities await the purification and cloning of the PLD enzymes. This will allow *in vitro* reconstitution experiments and *in vivo* experiments involving overexpression of PLD enzymes and gene knockout of PLD isoforms using anti-sense mRNA.

5.1.2 The regulation of PLD activity by small molecular weight G-proteins

The monomeric G-proteins are structurally related to the *ras* oncogene and have been divided by sequence homology into four sub-families, *ras*, *rho*, *rab* and *arf* (reviewed Hall, 1990). It has been suggested by both Cockcroft and Sternweiss (personal communications) that the small molecular weight G-protein *arf* is the cytosolic component essential for the full activation of stimulated-PLD activity in neutrophils. Whether *arf* acts downstream, upstream or in parallel with the

heterotrimeric G-protein to regulate PLD activity remains to be determined. Furthermore, it has been proposed that this may constitute the guanine nucleotide regulatory component of PLD-mediated exocytosis.

There are six known members of the *arf* family (reviewed Moss and Vaughan, 1992; Serventi *et al.*, 1992), raising the possibility of differential intracellular localisations and functional properties for each isoform. *Arf* was originally isolated as a cofactor necessary for the ADP-ribosylation of G_s by Cholera toxin (Kahn and Gilman, 1986). Although members of the *arf* family are predominantly cytosolic, N-terminal myristoylation may permit their membrane association (Kahn *et al.*, 1988).

The G-protein regulation of PLD in permeabilised neutrophils is clearly different to that in Swiss 3T3 cells and may be a reflection not only of the function of PLD in the different cell types, but also the fact that neutrophils are suspension cells whereas Swiss 3T3 fibroblasts are adherent. Agonists stimulated PLD activity in permeabilised Swiss 3T3 cells in the absence of added GTPγS and Ca^{2+}, with minimal loss in GTPγS-stimulated PLD activity over 15 minutes. This contrasted with reports in neutrophils where both membrane and cytosol in the presence of Ca^{2+} were required to fully reconstitute GTPγS-stimulated PLD activity (Olson *et al.*, 1991; Anthes *et al.*, 1991). In the absence of GTPγS, stimulatable-PLD activity was rapidly lost from permeabilised HL60 cells (Geny and Cockcroft, 1992). These findings suggest that whereas a soluble form of *arf* has been proposed to be the cytosolic factor essential for the full activation of PLD activity in neutrophils, a membrane associated form, if any, would be involved in Swiss 3T3 cells.

Arf is thought to be involved in intracellular vesicular transport by regulating the assembly of the non-clathrin protein coat and hence the budding, of Golgi-derived vesicles (Orci *et al.*, 1993). The binding of *arf* to Golgi membranes was reported to be essential for the membrane binding of one of the subunits β-COP of the polypeptide complex (coatomer) (Donaldson *et al.*, 1992a) which forms the major constituent of the vesicle coat (Waters *et al.*, 1991). Furthermore, reversible

association of *arf* with Golgi membranes has been found to be under the control of a membrane bound guanine-nucleotide exchange factor (Donaldson *et al.*, 1992b; Helms and Rothman, 1992).

Endocytosis of receptors may occur through clathrin-coated or non-coated vesicles. *Arf* was recently been reported to be important in endocytosis *in vitro* and was found to be present in clathrin-coated vesicles. The association with these vesicles was suggested to be mediated through the adaptins present in the clathrin coat (Lenhard *et al.*, 1992). This proposal is supported by the association between βCOP and *arf* described above and the homology between β-COP and β-adaptin (Waters *et al.*, 1991). Constitutive recycling of receptors, such as the transferrin receptor occurs through clathrin coated vesicles (Brown *et al.*, 1983). Using epitope tagging and immunofluorescence Von Zastrow and Kobilka (1992) reported that ligand-regulated internalisation of β_2-AR occurred through coated pits. Furthermore, the β_2 -AR were found to colocalise with transferrin receptors. However, previous reports have suggested that non-clathrin coated vesicles may also be involved in β_2-AR receptor internalisation, such as in A431 cells (Raposo *et al.*, 1989). Differences in these findings may be due to cell specific regulation or the degree of non-specificity of immunocytochemical staining. Other receptors such as that of vasopressin (Cantau *et al.*, 1988) and the epidermal growth factor (Wiley *et al.*, 1991) were reported to be internalised through coated pits, whereas muscarinic acetylcholine receptors were found to be sequestered in uncoated vesicles (Raposo *et al.*, 1987). The pathway of bombesin and vasopressin receptor sequestration in Swiss 3T3 cells is unknown. This could be resolved by the use of epitope tagging and immunofluorescence to detect the localisation of the receptor. A hypothetical regulation of the internalisation of agonist-occupied receptors in Swiss 3T3 fibroblasts by a membrane associated *arf* could perhaps be envisioned.

Bombesin may however regulate PLD activity through the small GTP-binding protein *rho*. Ridley and Hall, (1992) reported that the *ras* related protein *rho* regulated the formation of focal adhesions and actin stress fibers in response to

bombesin and other growth factors in Swiss 3T3 cells. As previously described (section 1.2.4) bombesin stimulated the tyrosine phosphorylation of the *src* - substrate $p125^{fak}$ in Swiss 3T3 fibroblasts (Zachary and Rozengurt, 1991, 1992). Other cytoskeletal components have also been reported to be tyrosine phosphorylated in *v-src* transformed cells including vinculin and talin (Sefton and Hunter, 1981; Pasquale *et al.*, 1986). Vinculin was also reported to be phosphorylated upon tyrosine in thrombin-stimulated platelets (Vostal and Shulman, 1993). Furthermore, the clustering of integrins (non-covalently linked glycoprotein heterodimers) during the formation of adhesive contacts was found to enhance the tyrosine phosphorylation of $p125^{fak}$ (Kornberg *et al.*, 1991).

It is therefore possible that tyrosine kinases such as *c-src* may be activated through cytoskeletal changes controlled by *rho* and hence constitute the pathway of tyrosine kinase stimulated PLD activity.

5.1.3 Possible sequestration of G-proteins by acylation/ reacylation cycles.

Recent reports have found that thioester-linked palmitoylation of G-protein α-subunits can occur, including that of $G\alpha_q$ (Linder *et al.* 1993, Parenti *et al.*, 1993). As the palmitoylation occurred post-translationally it was proposed that reversible acylation may play a regulatory role in the membrane localisation of G-proteins. The control of membrane association by reversible palmitoylation has also been reported for other proteins such as for the *src* -related tyrosine kinase *lck* (Paige *et al.*, 1993) and for *ras* (Magee *et al.*, 1987). The cellular compartment in which palmitoylation occurs is probably that in which the biosynthesis of the receptor occurs. However the site of reversible acylation may occur in other compartments, as proposed for reacylation/acylation cycles of proteins in erythrocytes (Staufenbiel, 1988).

Experiments showed that a short agonist pretreatment attenuated the GTPγS-stimulated PLD activity. As bombesin- and AVP-stimulated receptors were apparently sequestered following a short agonist exposure it is possible that Gα subunits and small molecular weight G-proteins involved in the stimulation of PLD

activity, may also be rapidly released from the membrane. The G-proteins or Gα subunits may be internalised with the agonist-bound receptor as suggested in section 4.3.7. Release of the GTP binding proteins from the plasma membrane may be due to deacylation, initiated by G-protein coupling to an activated receptor. It has also been suggested that palmitoylation alters the association between receptors and adapters present in the coated pits in which many proteins are thought to be internalised (Chin *et al.*, 1989). Thus deacylation may not only promote release of the proteins from the membrane but also their seqestration in vesicles.

The return to the membrane of Gα could be triggered by its deactivation and/or possible dissociation from the activated receptor. This could be elicited in a number of ways including GTP hydrolysis, dephosphorylation of the receptor or a conformational change in the receptor caused by degradation of the bound agonist. Reacylation or reassociation with βγ subunits (for heterotrimeric G-proteins) could be associated with the return of the G-proteins to the membrane and the recovery of agonist-stimulated responses. In support of the role of acylation in release of G-proteins from the membrane, a palmitoylation mutant of the transferrin receptor was found to be defective in endocytosis. However acylation of the receptor was not required for recycling to the plasma membrane (Alvarez *et al.*, 1990).

Epitope tagging could be utilised to study the relationship between the sequestration of Gα subunits and/or small molecular weight G-proteins and the receptor. Epitope tagging of $G\alpha_s$ was used to study its cellular localisation in response to isoproterenol (Levis and Bourne, 1992). When expressed in α_s-deficient S49 *cyc* $^-$ cells the epitope tagged $G\alpha_s$ was rapidly released into the soluble fraction subsequent to isoproterenol treatment which was followed by a return of the Gα subunit to the particulate fraction.

However, whether the G-protein or Gα subunit is internalised and recycles to the membrane with, or separately from the receptor, or whether it is degraded along with the ligand is a further uncertainty. It could be argued that a degradation of a proportion of internalised G-proteins may play a role in the incomplete

resensitisation of PLD activity. However, if bombesin-stimulated PLD and PtdIns-PLC activity are both regulated through $G\alpha_q$, the incomplete recovery of bombesin-stimulated PLD activity is more likely to be due to down-stream signals as agonist-stimulated generation of total inositol phosphates completely resensitised (section 4.2.2).

5.1.4 The involvement of Ca^{2+} in the tyrosine kinase stimulated PLD activity.

Experiments in Chapter 4 suggested that tyrosine phosphorylation was responsible for the PKC-independent pathway of agonist-stimulated PLD activity. It is probable, though as yet unproven, that the A23187-stimulated PLD activity remaining after treatment with Ro-31-8220 and EGTA could also be due to activation of tyrosine kinases. However, the relationship between $[Ca^{2+}]_i$ and activation of tyrosine kinases remains a matter for investigation. A23187 was however reported to stimulate a small increase in tyrosine phosphorylation in Swiss 3T3 fibroblasts (Zachary and Rozengurt, 1991). It is possible that although the thapsigargin-induced rise in $[Ca^{2+}]_i$ may have stimulated protein tyrosine phosphorylation, the activation of PLD through this pathway alone was too small to be significantly detected above basal.

Ca^{2+}-dependent tyrosine phosphorylation has been reported in angiotensin II-stimulated rat liver epithelial cells (Huckle *et al.*, 1990) and thrombin-stimulated muscle cells (Offermanns *et al.*, 1993). Ca^{2+} was proposed to act directly or indirectly, where indirect activation may occur through a Ca^{2+}-binding protein such as calmodulin. Despite the established role for such proteins in the activation of serine/threonine protein kinases (Edelman *et al.*, 1987), there is no evidence that they may also control tyrosine kinases. It may be however that $[Ca^{2+}]_i$ may contribute to the activation of tyrosine kinases which also require the input of a receptor-coupled G-protein (section 5.1, 5.1.2).

As previously proposed, it is probable that one of the tyrosine kinases involved in bombesin-stimulated PLD activation is *c-src* or a related kinase. In

support of this possibility the activation of *v-src* in Rat-1 cells (Wyke *et al.*, 1992) and Balb/c3T3 cells (Song and Foster, 1993) was found to stimulate PLD-catalysed PtdCho hydrolysis.

Src - related tyrosine kinases (reviewed Cooper and Howell, 1993) are inactive under normal conditions, through an intramolecular reaction involving the binding of an SH2 region to a C-terminal tyrosine phosphorylated residue (Liu *et al.*, 1993). Activation involves both dephosphorylation of this residue and phosphorylation of a residue in the catalytic domain of the protein (Kmiecik *et al.*, 1988). Ca^{2+} may promote the interaction of substrates with tyrosine kinase, for example, Ca^{2+} was found to enhance the association of a *src* substrate with phospholipid and actin (Glenney, 1986). It is also conceivable that Ca^{2+} could inhibit tyrosine phosphatases to enhance tyrosine phosphorylation. Experiments showed that inhibition of tyrosine phosphatases by pervanadate stimulated PLD activity in Swiss 3T3 cells and in liver epithelial cells. Moreover A23187 was found to synergise with pervanadate in stimulating PLD activity (Huckle *et al.*, 1990). Activation of tyrosine kinases rather than inhibition of tyrosine phosphatases however, is likely to be responsible for the elevation of bombesin-stimulated protein tyrosine phosphorylation due to the inhibitory effect of the tyrosine kinase inhibitor Genistein. However, whether artificial elevation of Ca^{2+} through the use of ionophores could increase tyrosine phosphorylation by the same mechanism as agonists is unclear.

5.1.5 Alternative pathways of bombesin-stimulated PLD activity through protein tyrosine phosphorylation.

Elevation of protein tyrosine phosphorylation using pervanadate was found to stimulate the accumulation of inositol phosphates, implying an activation of PLCγ. Whether bombesin could also stimulate PLCγ activity in Swiss 3T3 fibroblasts was not assessed but could be determined by immunoprecipitating agonist-stimulated cell

lysates with an anti-PLCγ antibody, followed by immunublotting with anti-phosphotyrosine antibodies or performing an *in vitro* assay.

The membrane association of PLCγ may be facilitated through the binding of SH2 regions to the phosphotyrosine of cytoskeletal components, aided by the interaction of SH3 regions with the cytoskeleton. The activation of PLCγ could perhaps then arise through tyrosine phosphorylation catalysed by the cytoskeletal associated tyrosine kinases such as $p125^{fak}$, however the substrates of $p125^{fak}$ are not known.

It is possible that bombesin-stimulated tyrosine kinase activity could activate MAP kinase which could then feedback to regulate PLD activity either by a direct effect on the enzyme or on other components of the regulatory pathway. This proposal could be compared to the activation of PLA_2 by MAP kinase which acts in conjunction with mobilisation of intracellular Ca^{2+} to result in full enzyme activation (Lin *et al.*, 1993). In support of this, MAP kinase has been shown to be activated by a number of G-protein coupled receptors such as those in bombesin-stimulated Swiss 3T3 cells (Pang *et al.*, 1993), thrombin-stimulated NIH 3T3 cells and thrombin and LPA-stimulated Rat 1 cells (Cook *et al.*, 1993; Gardner *et al.*, 1993).

Ras -GAP a GTPase-activating protein which not only enhances the intrinsic rate of GTP hydrolysis but is also a putative effector of *ras* (Duchesne *et al.*, 1993) has been reported to associate with *src*- related protein tyrosine kinases both *in vitro* (Park *et al.*, 1992) and in thrombin-stimulated platelets (Cichowski *et al.*, 1992). G-protein linked receptors which activate *src* like tyrosine kinases such as *c-src* may therefore be capable of stimulating *ras* - dependent signalling pathways and hence activate MAP kinase.

Cycling of *ras* between inactive GDP-bound states and active GTP-bound states is like *arf* (section 5.1.2), controlled by nucleotide exchange. The exchange factor for *ras* is thought to be a mammalian homologue of the Drosophila *Son of sevenless gene* (Sos) which resembles a yeast *ras* exchange factor (see Downward and Buday, 1993). Activation of Sos and hence *ras* by growth factor receptors

(Downward and Buday, 1992) occurs through adapter molecules, the SH2 containing protein (Shc) (Pelicci *et al.*, 1992) and growth factor bound protein 2 (GRB2) which contains one SH2 and two SH3 domains (Lowenstein *et al.*, 1992). Although both Shc and GRB2 both contain *src* homology regions they possess no intrinsic catalytic activity. Shc can be phosphorylated by intrinsic receptor tyrosine kinases and was also reported to be phosphorylated in *v-src* transformed cells, thus demonstrating possible regulation by cytosolic tyrosine kinases (Rozakis-Adcock *et al.*, 1992). The GRB2 protein binds to autophosphorylated receptors and also to phosphorylated Shc, hence translocating Sos, to the plasma membrane (Egan *et al.*, 1993).

Activation of *ras* thus leads to the activation of a cascade of kinases, including Raf kinase (Warne *et al.*, 1993; Zhang *et al.*, 1993) and mitogen activated protein kinase kinase (MAPKK) (Adams and Parker, 1992; Gardner *et al.*, 1993). MAP kinase is subsequently activated by MAPKK-mediated phosphorylation on both tyrosine and threonine residues.

Gardner *et al.* (1993), proposed that the thrombin and EGF-stimulated signals converged at MAPKK (reviewed Crews and Erikson, 1993). However, Cook *et al.* (1993), showed that the receptor tyrosine kinases and G-protein coupled receptors used *ras* as a common pathway. Nevertheless, *ras* - independent pathways of MAP kinase activation have been reported (Gallego, *et al.*, 1992), therefore the extent to which mitogens use *ras* in signalling may be both cell and agonist specific.

5.2. The role of stimulated PLD activity in the production of PtdOH and DAG in Swiss 3T3 cells

The rapid desensitisation of agonist-stimulated PLD activity, together with previous reports on the partial attenuation of agonist-stimulated DAG in the presence of butanol (Cook *et al.*, 1992), suggested that the role of PLD activation in Swiss 3T3 fibroblasts was to produce PtdOH as an effector. This contrasts with previous proposals that PtdOH may function solely as a precursor of DAG. As PMA-stimulated PLD activity appeared however to generate DAG, it may be that the PLD

isoform activated through PKC, is responsible for the generation of DAG from PtdOH. PKC-independent pathways of PLD activation may generate DAG due to the production of PtdOH species with a fatty acid content which may be poor substrates for PAP. However there is no evidence that PAP posseses substrate specificity at the level of acyl chain structure.

The possibllity that the activation of PLD by different second-messenger pathways may catalyse the hydrolysis of PtdCho species with different fatty acid composition is supported by the finding that the*v-src* stimulated PLD activity in Balb/c 3T3 cells was found to be distinct from that activated by phorbol esters (Song and Foster, 1993). The two activities were differentiated by labelling with arachidonate or alkyl-lysoPtdCho but not myristate or palmitate, suggesting that they were specific for a sub-population of PtdCho. However, both pathways of stimulation resulted in the same extent of DAG production. The substrate specificity of PLD may therefore relate to the existence of several differentially regulated isoforms.

Although bombesin-stimulated desensitisation of PLD activity was also reflected in choline production, a more definitive determination of the regulation of PLD-catalysed hydrolysis of different pools of PtdCho would require labelling with different fatty acids such as myristate and palmitate. Alternatively, stimulated-PLD activity could be determined in the presence of [^{3}H]butanol thus obviating the need for labelling of cellular PtdCho.

5.3 Regulation of agonist-stimulated PLD activity through receptor inactivation and reactivation

5.3.1 Receptor phosphorylation and its possible contribution to receptor-stimulated PLD activity.

Uncoupling of the β-adrenergic receptor from its G-protein commences initially through phosphorylation of the receptor on serine and threonine residues

catalysed by β-adrenergic receptor kinase (β-ARK) (Benovic *et al.*, 1987) and PKA (Hausdorff *et al.*, 1989), followed by binding of the cytosolic factor β-arrestin (Pitcher *et al.*, 1992). β-ARK phosphorylation sites were found to be predominantly in the C-terminus (Dohlman *et al.*, 1987), whereas PKA preferentially phosphorylated sites in the third cytoplasmic loop (Bouvier *et al.*, 1989). The role of receptor phosphorylation in agonist-stimulated desensitisation was implicated by the findings that β-ARK-catalysed phosphorylation occurred only when agonist was bound to the receptor and that it paralleled the loss of agonist-stimulated adenylyl cyclase activity (Sibley *et al.*, 1987).

Chick heart muscarinic and α_2-adrenergic receptors were also phosphorylated by βARK in an agonist-dependent manner, which coincided with desensitisation of agonist-stimulated responses (Kwatra *et al.*, 1987; Kwatra *et al.*, 1989; Sibley *et al.*, 1987). Other receptors including the muscarinic cholinergic receptors (Richardson and Hosey, 1990; Richardson *et al.*, 1992) and the α1-adrenergic receptor in smooth muscle cells (Leeb-lundberg *et al.*, 1987), were also reported to undergo agonist-dependent phosphorylation.

Hence the phosphorylation of either the bombesin or vasopressin receptor in Swiss 3T3 cells may play a key role in the initiation of desensitisation of receptor-linked responses to agonist. Although there is no direct evidence of bombesin receptor phosphorylation in Swiss 3T3 cells, C-terminal serine and threonine domains of the GRP receptor were found to regulate receptor internalisation when mutant receptors were transfected into Chinese hamster ovary fibroblasts expressing papovavirus large T antigen (CHOP fibroblasts) (Benya *et al.*, 1993). As desensitisation of bombesin-stimulated PLD activity was PKC-independent, a kinase, perhaps that activated by PtdOH (Bocckino *et al.*, 1991) (section 1.4.(ii)) may phosphorylate the agonist-occupied receptor. Alternatively activation of tyrosine kinases may stimulate a serine/threonine kinase such as MAP kinase as previously proposed (section 5.1.5). In this way feed back inhibition of agonist-stimulated PLD activity could be achieved. Phosphorylation of the receptor could be

investigated *in vitro* using the purified receptor in a micellar system, or *in vivo* by incubating cells with bombesin in the presence of ^{32}P, prior to immunoprecipitation with receptor antibody.

5.3.2 Receptor domains involved in sequestration.

Desensitisation of G-protein coupled receptors to agonist-stimulation is also regulated by sequestration (section 4.1). In support of this, the rapid desensitisation of agonist-stimulated [^{3}H]PtdBut generation in Swiss 3T3 cells appeared to reflect to a significant extent the transient loss of receptor binding.

The control of receptor internalisation remains unclear despite numerous studies, but after shorter agonist exposure it appears to be a mechanism by which receptors are reactivated after their uncoupling from effector systems. The generation of mutant receptors has suggested that the C-terminal domain is essential for sequestration, though reports conflict as to the mode of regulation. Mutation of serine and threonine residues in the C-terminal tail of the β–AR prevented agonist-stimulated receptor sequestration (Hausdorff *et al.*, 1991). C-terminal serine and threonine residues were also found to regulate the internalisation of the GRP receptor (Benya *et al.*, 1993). However, removal of the C-terminal tail of β-AR resulted in internalisation that was similar (Strader *et al.*, 1987) to or more rapid than the wild type receptor (Hertel *et al.*, 1990). Similarly, results from other mutant receptors containing deleted C-terminal tails have led to confusion. Mutation of the thyrotropin-releasing hormone receptor (TRHR) resulted in a decreased internalisation rate (Nussenzveig et al., 1993), whereas that in the luteinizing hormone chorionic gonadotropin receptor was increased (Rodririguez *et al.*, 1992). The domains controlling internalisation may be receptor specific as serine and threonine rich domains in the third cytoplasmic loop have been proposed to regulate the sequestration of muscarinic cholinergic receptors (Moro *et al.*, 1993; Lameh *et al.*, 1992).

In addition to the requirement for serine and threonine residues, other amino acids have been found to regulate the transient decrease in cell surface receptors. Nussenveig *et al.* (1993) reported that cysteine residues in the C-terminal domain of the thyrotropin-releasing hormone receptor were important for receptor trafficking. Cysteine residues located in a similar postion near the seventh transmembrane domain are conserved in many G-protein coupled receptors including those of rhodopsin (Ovchinokov *et al.*, 1988) and β-adrenergic agonists (O'dowd *et al.*, 1989). These cysteine residues constitute a possible palmitoylation site, a modification which may be important for internalisation. However, transfection of the GRP receptor containing mutated cysteine residues, into CHOP fibroblasts, resulted in receptor internalisation comparable to that attained with the wild type receptor suggesting that the putative palmitoylation site was not necessary for internalisation (Benya *et al.*, 1993).

The regulation of receptor -mediated desensitisation by receptor C-terminal tyrosine residues has also been suggested. The essential tyrosine residues in lysosomal acid phosphatase and the low density lipoprotein receptor (LDL receptor) were found to adopt a tight turn. This conformation was proposed to be a signal for rapid internalisation (Eberle *et al.*, 1991; Bansal and Gierasch, 1991). However, mutation of tyrosine residues in the β_2-AR was found to affect receptor down-regulation but not sequestration (Valiquette *et al.*, 1990). Whether such a turn is possible in bombesin-receptors, in which the sequence contains a single tyrosine residue one amino acid from the end of the receptor seems unlikely (Battey *et al.*, 1990).

Thus it is possible that certain serine and threonine residues and/or other residues which may be phosphorylated or post-translationally modified, form a specific secondary structure essential for the regulation of receptor internalisation. A critical length of the C-terminal tail may be crucial for formation of the secondary structure, disruption of which may lead to loss of or uncontrolled receptor sequestration. Confirmation of the roles of domains of the bombesin and vasopressin

receptor could be achieved by similar site directed mutagenesis experiments to those described above.

5.3.3 The interaction between receptor/G-protein uncoupling, sequestration and down-regulation.

Receptor sequestration appears to be functionally uncoupled from down-regulation in the β_2-AR. Down-regulation of the receptor is characteristically observed after prolonged exposure to agonist and is modulated by phosphorylation and decreased by mutations impairing receptor/G_s coupling (Bouvier *et al.*, 1989, Campbell, 1991). However mutations that impaired receptor/G_s coupling did not affect sequestration. Conversely, mutations of the β_2-AR C-terminal tail that inhibited receptor phosphorylation and sequestration did not affect down regulation or stimulation of adenylyl cyclase activity (Hausdorff *et al.*, 1991; Strader *et al.*, 1987)). Similarly, truncation of the C-terminal tail in the GRP receptor and TRHR receptors, which attenuated receptor sequestration as previously described , did not affect the hydrolysis of inositol phosphates (Nussenveig *et al.*, 1993; Benya *et al.*, 1993).

5.3.4 Recycling of the receptor.

The proposals that the resensitisation in bombesin-stimulated PLD activity may have been due to recycling of the receptor is supported by studies in CHO cells transfected with the β_2-AR. Blockage of β_2-AR sequestration by mutagenesis or sucrose pretreatment was found to markedly reduce the rate of resensitisation upon removal of the agonist, which would normally have occurred with $t_{1/2}$ of approximately 3 minutes (Yu *et al.*, 1993). The authors proposed that sequestration of β_2-AR was involved in reactivation and recycling of receptors . The recovery of agonist-stimulated adenylyl-cyclase activity therefore occurred with a similar time scale to that observed for the recovery of bombesin-stimulated PLD activation in Swiss 3T3 cells (section 4.2.1).

Sequestered vesicles containing the β_2-AR have been found to be enriched in phosphatases and the internalised receptors were found to be less phosphorylated than the membrane associated ones (Sibley *et al.*, 1986). The function of receptor internalisation was therefore proposed to be to dephosphorylate and hence reactivate the β_2-AR receptor. Similarly, internalisation of a phosphorylated bombesin or vasopressin receptor could result in their dephosphorylation.

Further comparisons with the β_2-AR can be drawn in terms of the finding that the recovery of bombesin-stimulated PLD activity, proposed to be partially due to receptor recycling, occurred even in the continual presence of bombesin. Similarly, recycling of the β_2-adrenergic receptor (Kurz and Perkins, 1992) and guanylate cyclase/atrial natriuretic factor receptors (Pandey, 1993) was reported to occur in the presence of agonist.

5.3.6 Characteristics of agonist-stimulated desensitisation in other systems.

The PKC-independency of the homologous desensitisation of bombesin-stimulated PLD activity is supported by findings in many other systems. For example, the waning of muscarinic-mediated [^{3}H]cyclic GMP responses in neuroblastoma cells (Lai *et al.*, 1990) and the attenuation over time of PtdIns-PLC activity in thrombin-stimulated fibroblasts (Paris *et al.*, 1988) and in histamine and thrombin-stimulated endothelial cells (Halldorssen and Thorgeirsson, 1989) were reported to be independent of PKC activity.

In many systems short-term desensitisation was found to involve both receptor and post receptor events as proposed in Chapter 4 for the desensitisation of vasopressin- and bombesin-stimulated PLD activity. For example, the time course for the desensitisation of intracellular Ca^{2+} mobilisation differed from the loss and recovery of cell surface receptors in neurotensin-stimulated HT29 cells (Turner *et al.*, 1990) and the extent of angiotensin II-stimulated desensitisation of Ins(1,4,5)P_3 production in vascular smooth muscle cells was greater than the loss of angiotensin II receptors (Ullian and Linas, 1990).

A short bombesin pretreatment was found to reduce the GTPγS-stimulated PLD activity, however reports on the G-protein-stimulated effector signalling pathways vary in other systems. AlF^--and thrombin-stimulated phosphoinositide hydrolysis was unaltered by a thrombin pretreatment which attenuated subsequent thrombin-stimulated PtdIns breakdown (Paris *et al.*, 1988). However, in histamine-desensitised Hela cells both the NaF and bradykinin-mediated inositol phosphate production was attenuated suggesting that the site of desensitisation was at, or distal to the G-proteins (Bristow and Zamani, 1993).

5.3.6 Chronic and acute desensitisation are distinct.

In addition to the difference in initiating signals needed for chronic and acute desensitisation, studies on second-messenger generation following prolonged exposure to agonist has also shown differentiation between the two processes. Chronic agonist exposure generally results in receptor down regulation which depending on the system studied may or may not correlate with the extent of desensitisation. The relationship between sequestration and downregulation after longer exposure to agonist has been suggested to be an agonist-controlled change in equilibrium between the recycling of the internalised receptor and its degradation (von Zastrow and Kobilka, 1992).

Down-regulation of the β_2 - adrenergic receptor after chronic agonist exposure is thought to be due to an instability in receptor mRNA (Hadcock *et al.*, 1989). Experiments in Chinese hamster ovary cells investigating the hydrolysis of phosphoinositides stimulated through muscarinic receptors, have suggested that the relative contribution of receptor/effector uncoupling and receptor loss to desensitisation depends on the efficacy of the agonists used in the desensitisation and activation stages (Hu *et al.*, 1991).

Resensitisation of cells exposed for long times to agonist is also reversible. However, in contrast to the recovery after short agonist exposure, the process

requires long incubations in the absence of the desensitising stimulant and is dependent on protein synthesis.

Chronic desensitisation to bombesin in Swiss 3T3 fibroblasts resulted in a loss of binding sites without a change in their affinity, caused by receptor down-regulation. Pretreated cells were also unable to mobilise Ca^{2+} and stimulate DNA synthesis upon readdition of bombesin (Millar and Rozengurt, 1990). In contrast to the heterologous desensitisation observed after a short pretreatment with bombesin, a prolonged exposure did not affect Ca^{2+} mobilisation in response to other agonists. Prolonged pretreatment of Swiss 3T3 cells with vasopressin for at least 12 hours was found to desensitise cells to subsequent vasopressin-stimulated mitogenesis. Desensitisation was proposed to occur partly through post-receptor events as 60% of the vasopressin binding sites were retained (Collins and Rozengurt, 1983). Prolonged vasopressin stimulation was also reported to reduce the mitogenicity of bombesin, although there was no effect on the bombesin receptor (Millar and Rozengurt, 1989). This suggested that heterologous desensitisation after chronic agonist exposure primarily involved post-receptor events as for the heterologous desensitisation observed after short agonist stimulation (section 4.2.4).

A similar dissociation of early and late events during exposure to vasopressin was reported in WRK cells with the time course of VP-sensitive PtdIns-PLC desensitisation occurring more rapidly than the downregulation of receptors (Cantau *et al.*, 1988). However in these cells the reduction in binding sites correlated with the extent of inhibition of vasopressin-stimulated production of inositol phosphates.

Other mechanisms of adaptation can also exist. In cells such as muscarinic-stimulated SH-SY5Y neuroblastoma cells $Ins(1,4,5)P_3$ remains elevated above basal for prolonged periods of time. An adaptive mechanism causes a reduction in the concentration of $Ins(1,4,5)P_3$ receptor such that $Ins(1,4,5)P_3$ -induced Ca^{2+} release is attenuated (Wojcikiewicz and Nahorski, 1991; Wojcikiewicz *et al.*, 1992). This process was found to be PKC- independent and was absolutely dependent on

$Ins(1,4,5)P_3$. Whether the mechanism involved receptor degradation or a reduction in the rate of receptor synthesis was unclear.

5.4. The relationship between continual receptor occupancy and mitogenic signalling pathways.

The continual production of PtdOH that would be produced on resensitisation of PLD activity in the continual presence of agonist, suggest that the activation of downstream effectors of PtdOH (or lysoPtdOH) may also be maintained in a similar manner. Multiple phases of second-messenger production may play a critical role in the control of cellular responses. Three phases of PKC activity were detected in IFNγ-stimulated endothelial cells subsequent to increases of choline, PtdOH and DAG within the first 60 minutes (Mattila and Renkonen, 1992). The authors suggested that in each phase, PtdOH was metabolised to DAG which could then activate PKC. The multiphasic generation of DAG observed in α-thrombin stimulated human platelets in response to sub-threshold levels of agonist, was proposed to be a controlling mechanism for the optimal activation of PKC during secondary aggregation (Werner *et al.*, 1992). Furthermore, the second sustained phase of MAP kinase activation reported in α-thrombin fibroblasts was proposed to account for α-thrombin stimulated cell proliferation (Kahan *et al.*, 1992). The function of the resensitised rate of bombesin-stimulated PLD activity may be to accumulate PtdOH, at least in the first few hours of agonist stimulation. This may act as a control mechanism for mitogenesis, perhaps a critical threshold of PtdOH must be reached for a certain length of time allowing prolonged activation of downstream effectors.

5.5 Proposed models for the regulation of agonist-stimulated PLD activity

A model proposed for bombesin-stimulated regulation of phospholipase D activity is presented in figure 5.1. The bombesin receptor couples to G_q, hence activating PtdIns-PLC. Stimulation of tyrosine kinases, of which an essential one is thought to be *c-src*, may occur directly through a G-protein G_q or G_D, or indirectly through the rise in $[Ca^{2+}]_i$. G_D is thought to be cytoskeletal associated and may belong to the small molecular weight class of G-proteins. Bombesin-stimulated protein tyrosine phosphorylation may be initiated through cytoskeletal changes controlled by *rho* , a possible candidate for G_D. Activation of *c-src* is also proposed to activate PLCγ, contributing to the production of DAG and Ins(1,4,5)P_3. The rise in $[Ca^{2+}]_i$ and DAG activates PKC which stimulates PLD activity. Tyrosine kinases are proposed to activate PLD independently of PKC, possibly acting through an intermediate protein, for example MAP kinase. However, the mechanism of kinase regulation of PLD activity remains to be investigated. Stimulation of PLD activity may also occur by fatty acids such as oleate, produced as a result of bombesin-stimulated PLA_2 activation.

The PtdOH produced from PLD-catalysed PtdCho hydrolysis is proposed to contribute to only a minor extent to DAG generation, which may activate certain PKC isoforms. The main function of PtdOH is to act as a second-messenger, eliciting various effects as previously described either in its own right or by conversion to lysoPtdOH (section 1.4.3(ii)). Some of the effects of PtdOH or lysoPtdOH may necessitate extrusion of the phospholipid from the cell.

A hypothetical scheme for the desensitisation of vasopressin and bombesin receptors is shown in figure 5.2 and is based upon models proposed for the β_2-AR. Stimulation of the receptor leads to its uncoupling from the effector systems regulating PLD activity. This may occur through conformational changes, perhaps initiated by receptor phosphorylation on C-terminal serine and threonine residues and release of the Gα subunit or a small molecular weight G-protein from the membrane. Reactivation of the receptor is proposed to occur by sequestration of the

Figure 5.1 A proposed model for regulation of bombesin-stimulated PLD activity in Swiss 3T3 fibroblasts.

Interaction of bombesin (B ●) with the receptor activates PLCβ via the G-protein G_q. The hydrolysis of PtdIns(4,5)P_2 generates DAG and Ins(1,4,5)P_3. The concentration of $[Ca^{2+}]_i$ is elevated through Ins(1,4,5)P_3-induced Ca^{2+} release from the endoplasmic reticulum and entry of Ca^{2+} from the extracellular medium, through channels in the plasma membrane. PKC isoforms are activated by DAG from PtdIns(4,5)P_2 hydrolysis and for PKC α, β and γ, $[Ca^{2+}]_i$ is also required. PKC activates PLD by an undefined mechanism which may involve direct phosphorylation of the enzyme or an intermediate regulatory protein. Bombesin receptors may regulate PLD activity through a pertussis-toxin insensitive G-protein other than G_q. The hypothetical G-protein, G_D, may belong to the heterotrimeric or low molecular weight classes of G-proteins and could activate PLD activity through a tyrosine kinase proposed to be *c-src*. Such an interaction could occur through bombesin-stimulated cytoskeletal changes mediated by the small molecular weight G-protein *rho*, a possible candidate for G_D. However, G_D may also activate PLD through an unidentified serine/threonine kinase. Stimulation of a tyrosine kinase may activate PLD directly or indirectly, possibly via activation of MAP kinase. Receptor activation of a tyrosine kinase may also lead to PtdIns(4,5)P_2 hydrolysis catalysed by PLCγ. $[Ca^{2+}]_i$ may activate PLD indirectly through PKC, or as a cofactor in the activation of *c-src*. Unsaturated fatty acids from PLA_2-catalysed PtdCho hydrolysis may also contribute to bombesin-stimulated PLD activity.

PLD-catalysed PtdCho hydrolysis generates PtdOH and choline which is not only accumulated inside the cell but is also extruded. For clarity choline is only shown leaving the cell. The PtdOH may act as a second-messenger itself eliciting a range of effects such as activation of kinases or its second-messenger effects may be mediated by conversion to lysoPtdOH. Possible extrusion of PtdOH or lysoPtdOH from the cell is not shown, again for clarity. Alternatively PtdOH may be converted by PAP-2 to DAG which could activate PKC. A line with a question mark above, denotes activation of the enzyme by an undefined mechanism. A dashed line indicates a possible but unproven route of activation.

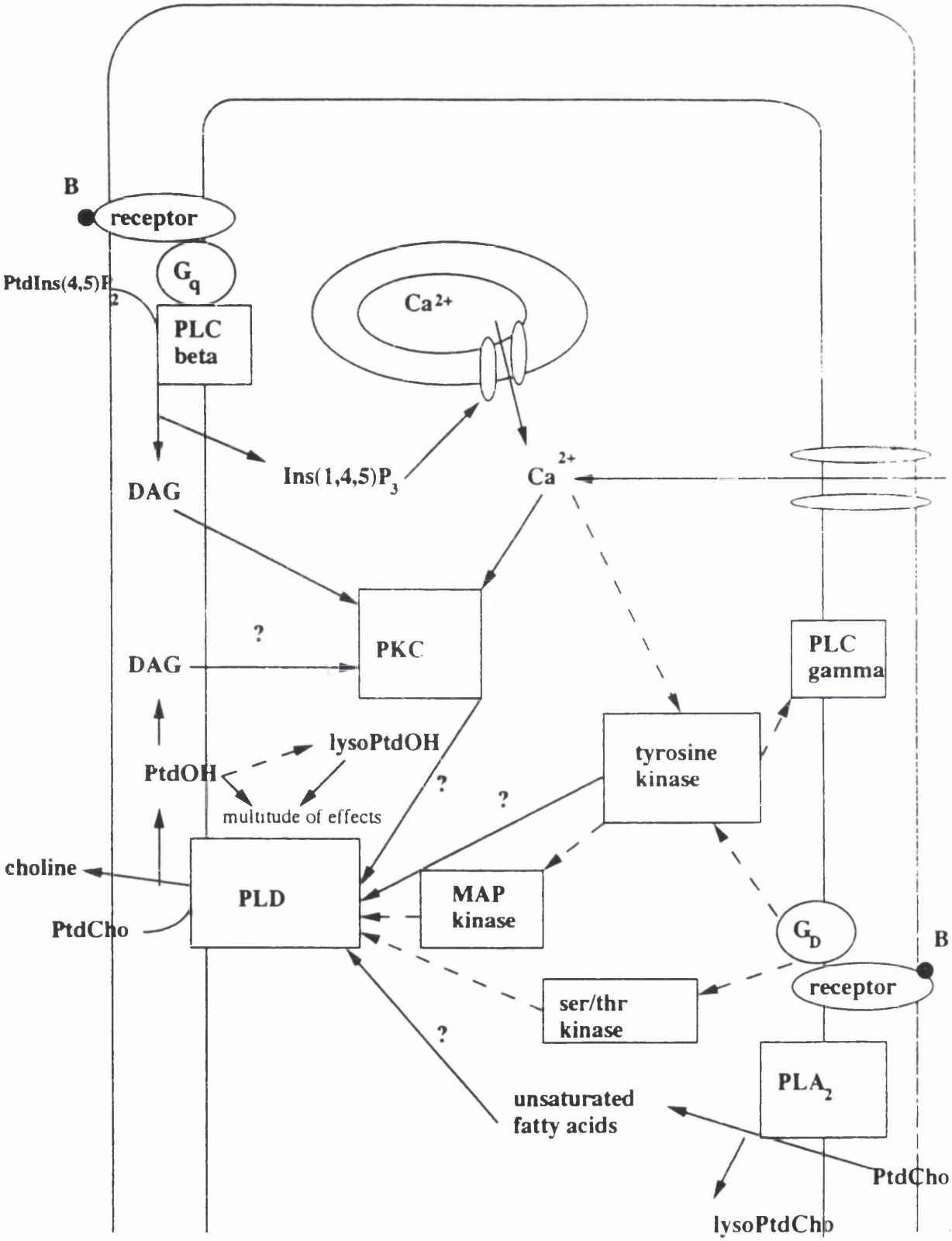
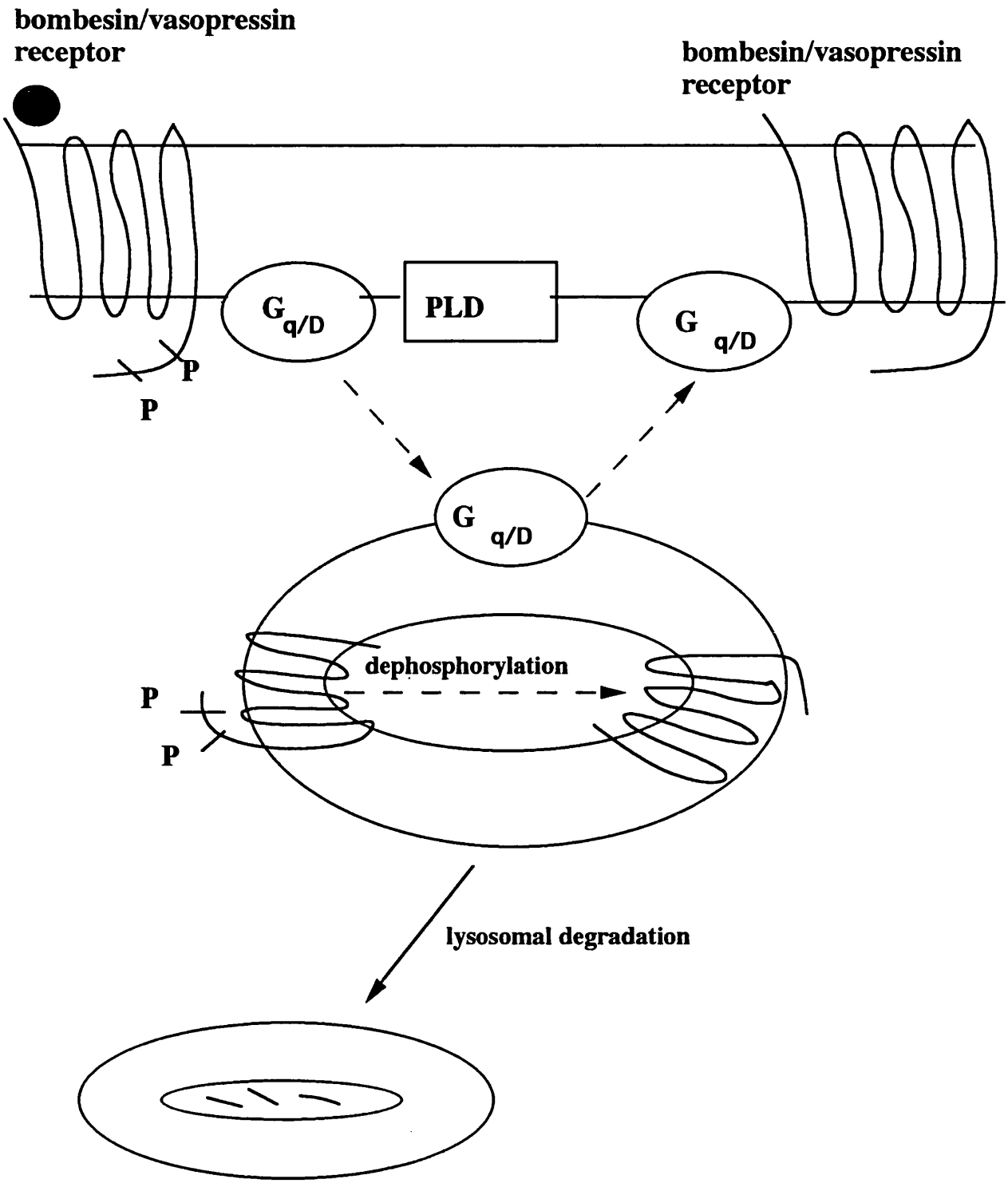

B
receptor
G_q
PtdIns(4,5)P2
PLC beta
Ca^{2+}
$Ins(1,4,5)P_3$
Ca^{2+}
DAG
PKC
?
DAG
PLC gamma
lysoPtdOH
PtdOH
multitude of effects
tyrosine kinase
?
?
choline
PLD
MAP kinase
PtdCho
G_D
B
receptor
ser/thr kinase
?
PLA_2
unsaturated fatty acids
PtdCho
lysoPtdCho

Figure 5.2 Hypothetical scheme of bombesin and vasopressin receptor desensitisation and resensitisation in Swiss 3T3 cells after short exposure to agonist.

Schematic diagram is adapted from the model proposed for desensitisation of β_2 - AR (Yu *et al.*, 1993; von Zastrow and Kobilka, 1992). Dashed lines indicate hypothetical pathways of receptor desensitisation and resensitisation. After a short agonist (•) stimulation the receptor is uncoupled from the effector systems regulating PLD activity (see Fig. 5.1). This may occur via phosphorylation of the receptor as proposed for the β_2 - AR and deacylation of the G-protein regulating PLD activity which may involve G_q and/or another undefined G-protein (G_D). The phosphorylated receptors possibly in conjunction with the $G\alpha_q$ or G_D are sequestered into intracellular vesicles. Reactivation of the receptors may occur after dephosphorylation of the internalised receptors by phosphatases present in the vesicles. A conformational change may also initiate reactivation of the receptor possibly elicited by dissociation and lysosomal degradation of the receptor bound agonist. The GTP-binding protein may be returned to the membrane after hydrolysis of bound GTP, reacylation or reassociation with $\beta\gamma$ subunits. The receptor and G-protein may be recycled to the plasma membrane separately or together.


bombesin/vasopressin
receptor
bombesin/vasopressin
receptor
G q/D
PLD
G q/D
P
P
G q/D
dephosphorylation
P
P
lysosomal degradation

receptor and G-proteins into vesicles, perhaps in a complex. Reactivation of the receptor occurs by degradation of the bound agonist and dephosphorylation which would result in conformational changes, dissociating the receptor from any receptor/G-protein complex. Similarly, the internalised Gα subunit or GTP-binding protein could be reactivated by GTP hydrolysis or dissociation from the receptor and may reassociate with the membrane by interaction with βγ subunits and/or reacylation. More prolonged exposure to the agonist promotes degradation of the sequestered receptor and G-protein sub-units, rather than their recycling to the plasma membrane.

5.6 Concluding remarks

Further characterisation of the modulatory pathways of PLD activity through enzyme purification and molecular biology techniques will extend the current knowledgeof the linkage between initial events induced by receptor occupancy and mitogenesis. Loss of control of the desensitisation process may result in deregulation of agonist-stimulated second-messenger signalling pathways leading to unrestrained proliferation in response to mitogens.

Recent reports have described constitutively activated receptors which are associated with disease. A mutation in the luteinising hormone receptor resulting in agonist-independent elevation of cyclic AMP was associated with familial male precocious puberty (Shenker *et al.*, 1993). Similarly, an alteration in the thyrotropin receptor leading to deregulation of adenyl cyclase was reported to cause hyperfunctioning thyroid adenomas (Parma *et al.*, 1993). Discovery of the mechanisms involved in inactivation of agonist-stimulated receptors will therefore promote the development of novel therapeutic targets.

Appendix

The following is a list of publications arising from the work presented in this thesis.

1. " The regulation of phospholipase D activity and its role in *sn* - 1,2 - diradylglycerol formation in bombesin- and phorbol 12-myristate 13-acetate-stimulated Swiss 3T3 cells."
Cook, S.J., Briscoe, C.P. and Wakelam, M.J.O. (1991) *Biochem. J.* **280,** 431-438.

2. " Phosphatidylcholine hydrolysis - a multiple messenger generating system."
Wakelam, M.J.O., Pettitt, T.R., Kaur, P., Briscoe, C.P., Stewart, A., Paul, A., Paterson, A., Cross, M.J., Gardner, S.D., Currie, S., MacNulty, E.E., Plevin, R. and Cook, S.J. (1992) *Adv. Sec. Mess. Res.* **28,** 73-80.

3. "Rapid desensitisation and resensitisation of bombesin-stimulated phospholipase D activity in Swiss 3T3 cells."
Briscoe, C.P., Plevin, R. and Wakelam, M.J.O. (1993) *Biochem. J. in press* .

4. " The regulation of agonist-stimulated phospholipase D activity in Swiss 3T3 fibroblasts".
Briscoe, C.P. and Wakelam,M.J.O. (1993) *Biochemsoc. Trans.* 492S.

5. "Phosphatidylcholine hydrolysis: A source of multiple lipid messenger molecules"
Wakelam, M.J.O., Briscoe, C.P., Stewart, A., Pettit, T., Cross, M.J., Paul, A., Yule, J.M., Gardner, S.D. and Hodgkin, M. (1993) *Biochem. Soc. Trans.* **21,** *in press.*

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