The role of priming in the regulation of cytosolic Phospholipase A_2.

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This thesis is dedicated to the memory of my Gran, Elizabeth Stewart, who was always there to talk to and who never beat about the bush with her opinions, and to the memory of my Di, Hugh Steel, who told me when very young, "Use your computer Hen, always use your computer!".

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ABBREVIATIONS

APS	10% (w/v) Ammonium persulphate
ATP	Adenosine 5'-triphosphate
BAPTA-AM	1,2-bis-(O-Aminophenoxy)-ethane-N,N,N',N'-tetraaceric acid tetra-(acetoxymethyl)-ester
BCA	Bicinchoninic acid
$CaCl_2$	Calcium chloride
CaLB	Calcium-phospholipid binding site
$cPLA_2$	Cytosolic phospholipase A2
CDP	Cysteine diphosphate
CTP	Cysteine triphosphate
DABCO	1,4 Diazabicyclo[2.2.2.] octane
DAG	Diacylglycerol
DBG	DMEM with Hepes, glucose and BSA
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DMV	Demethoxyviridine
DTT	DL-Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid
ERK	Extracellular regulated kinase
fMLP	formylMethylLeucinePhenylalanine
GDPβS	Guanosine 5'-O-(2-thiodiphosphate)
GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
HCl	Hydrochloric acid
HIFCS	Heat-inactivated foetal calf serum
HRP	Horseradish peroxidase

IP_3	Inositol-1,4,5-trisphosphate
IP_{20}	cyclic AMP-dependent protein kinase inhibitor
KCl	Potassium chloride
LiCl	Lithium chloride
LPA	Lysophosphatidic acid
MAPK	Microtubule-associated protein kinase
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NaF	Sodium fluoride
NaOH	Sodium hydroxide
NaPPi	Sodium tetrapyrophosphate
NaTB	Sodium tetraborate
NBCS	Newborn calf serum
NFM	Non-fat milk
PAGE	Polyacrylamide gel electrophoresis
PAP	Phosphatidic acid phosphohydrolase
PBS	Phosphate buffered saline
PC-PLC	Phosphatidylcholine-specific phospholipase C
PIC	Phosphatidylinositol-specific phospholipase C
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLD	Phospholipase D
PMSF	Phenylmethylsulfonyl fluoride
RBG	RPMI 1640 with Hepes, glucose and BSA
s.d.	standard deviation
SDS	Sodium Lauryl sulphate
$sPLA_2$	secretory phospholipase A_2
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-Tetramethylethylenediamine
TPA	Phorbol ester

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SUMMARY

SUMMARY

Work carried out in this Thesis tried to elucidate the factors involved in the regulation and priming of cytosolic phospholipase A_2 ($cPLA_2$).

The 85 kDa $cPLA_2$ originally identified and purified from U937 cells (Clark *et al.*, 1991) was shown to be present in Rat-1 Raf ER4 fibroblasts, Swiss 3T3 fibroblasts and HL60 cells. This enzyme was shown to be acutely primed by cytochalasin B in 5 day DMSO-differentiated HL60 cells and chronically primed by serum in Swiss 3T3 fibroblasts. These priming agents increased tyrosine phosphorylation, which did not result in arachidonate release, but enhanced subsequent agonist-stimulated arachidonate release. This increased tyrosine phosphorylation did not enhance the known regulatory moieties of cytosolic phospholipase A_2, such as MAP kinase (Lin *et al.*, 1993), calcium (Channon & Leslie, 1990) and GTP-binding proteins (Cockcroft, 1992), therefore suggesting that an additional factor was involved in both the acute and chronic priming of $cPLA_2$.

However, all of the above had a role to play in the regulation of $cPLA_2$. GTPγS did not enhance agonist-stimulated arachidonate release; however, a GTP-binding protein was involved in both LPA- and bombesin-stimulated arachidonate release as GDPβS inhibited both agonist-medited responses. *Raf-1*-stimulated MAP kinase activity enhanced LPA-stimulated arachidonate release, but did not stimulate arachidonate release alone, demonstrating that an additional agonist-stimulated signal was required. LPA, bombesin and fMLP all stimulated an immediate increase in intracellular calcium concentrations in their appropriate cells. The calcium chelator BAPTA inhibited agonist-stimulated arachidonate release to varying degrees in the cells studied, suggesting that calcium is essential for maximal agonist-stimulated arachidonate release. Channon & Leslie (1990) demonstrated that increased calcium concentrations resulted in a translocation of $cPLA_2$ from the cytosol to a membrane fraction. Under all priming conditions, western blotting demonstrated that there was a translocation of phosphorylated $cPLA_2$ from the cytosol to a membrane fraction, although there was no increased calcium concentrations. Therefore, calcium was not responsible for the translocation of the enzyme, but was essential for maximum arachidonate release. The

membrane fraction to which $cPLA_2$ translocated under both priming and agonist stimulation was shown to be in the nuclear area and not the plasma membrane as had been suggested. In the fibroblastic cell lines and HL60 cells, $cPLA_2$ was expressed under all conditions studied. However, there was a differential expression of $cPLA_2$ in B-lymphoid cell lines representative of distinct stages in B-lymphocyte development.

Therefore, work presented within this Thesis will try to examine the regulation and priming of cytosolic phospholipase A_2 in both the adherent and suspension cells studied. The involvement of the factors responsible will be discussed in relation to the nuclear location of the enzyme, compared to the predicted plasma membrane location.

CHAPTER 1

INTRODUCTION

1.1 PHOSPHOLIPID CYCLES

Agonist stimulation of cells has been shown to activate a number of phospholipases generating a range of messenger molecules, the properties of which are reviewed below.

1.1.1 Inositol phospholipids as signalling molecules

Phosphatidylinositol (PtdIns) is synthesized *de novo* by a PtdIns synthase activity that catalyzes phosphatidyl transfer from CMP-phosphatidate to inositol. The site of this PtdIns synthase activity has been shown to be predominately the endoplasmic reticulum (Imai & Gershengorn, 1987), but activity has also been demonstrated in the plasma membrane (Cubitt & Gershengorn, 1989), which is thought to correspond to the distribution of PtdIns between different cell membranes. After various deacylation/ reacylation steps, a PtdIns complement is produced which is dominated by the 1-stearoyl, 2-arachidonyl species (Darnell *et al.*, 1991; Darnell & Saltiel, 1991).

A potential signalling role for inositol-derived lipids was first recognized in the 1950's, when Hokin & Hokin (1953) demonstrated that the muscarinic cholinergic agonists, acetylcholine and carbamylcholine, could stimulate the incorporation of [^{32}P]Pi into a total phospholipid fraction of pigeon and duck pancreatic slices. Analysis of deacylated lipids showed the agonist-sensitive molecule to be "diphosphoinositide" (Dawson, 1954), which was subsequently identified as phosphatidylinositol (Hokin & Hokin, 1958). Michell (1975) proposed the 'Phosphatidylinositol response', where the turnover of PtdIns was inextricably linked to intracellular calcium mobilization. Kirk *et al.*(1981) and Creba *et al.*(1983) demonstrated that it was in fact phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) that was specifically degraded upon agonist stimulation. PtdIns-4,5-P_2 is synthesized by the sequential action of two MgATP-dependent inositol lipid kinases: PtdIns 4-kinase, which acts upon PtdIns to generate PtdIns-4-P and PtdIns4P 5-kinase, which phosphorylates PtdIns-4-P to PtdIns-4,5-P_2 (for review see Carpenter & Cantley,

1990)[Fig.1.1.1]. These lipids and the enzymes responsible for their synthesis have been found to be associated with the endoplasmic reticulum (Helms *et al.*, 1991), nuclear fractions (Payrastre *et al.*, 1992) and the plasma membrane to generate the agonist-sensitive pools of PtdIns-4,5-P_2. These different locations may suggest an involvement of PtdIns-4,5-P_2 in multiple cellular functions.

Two agonist-stimulated enzymes which utilize PtdIns-4,5-P_2 as a substrate are phosphoinositidases C (PICs), which generate inositol-1,4,5-trisphosphate (Ins-1,4,5-P_3) and diacylglycerol (DAG)(Berridge, 1987; Whitman & Cantley, 1988), and phosphoinositide 3-kinases (PI3Ks), generating PtdIns-3,4,5-P_3 (Hawkins *et al.*, 1992; Stephens *et al.*, 1993)(see Section 1.1.1.4). Streb *et al.*(1983) demonstrated that Ins-1,4,5-P_3 triggers the release of calcium from intracellular stores, via specific receptors (Ross *et al.*, 1989) and DAG release activates various protein kinase C isozymes, as described in Section 1.3.2.1 (Nishizuka, 1986).

Three families of PIC have been identified, β, γ and δ, within which there are several subtypes. Surprisingly, there are only two regions of homology shared by these three families, termed the X and Y regions, which comprise of 150 and 240 amino acids respectively (Rhee *et al.*, 1991).

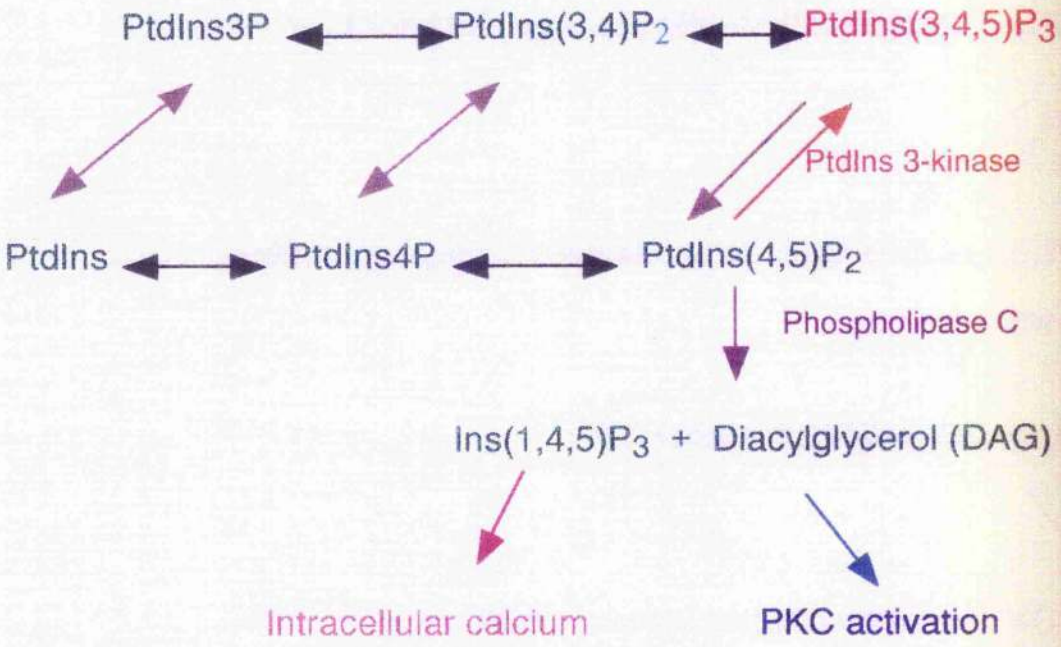
1.2.1.1 The PIC-β family

This family contains four members, PIC-β1 (150-154 kDa), -β2 (134kDa), -β3 and -β4, which are shown to be regulated by heterotrimeric G-proteins. Taylor & Exton (1987) initially purified the G-protein responsible from liver, which was found to belong to the Gq class of G-proteins (Taylor *et al.*, 1990). The G_q family comprises at least five G-proteins, G_q, G_{11}, G_{14}, G_{15} and G_{16} (Simon *et al.*, 1991). $G\alpha_q$ purified from a βγ-affinity gel was found to stimulate PIC-β1 but not PIC-β2 (Park *et al.*, 1992) and $G\alpha_{16}$, when expressed in Cos-7 cells was most effective in reconstituting with PIC-β2 (Lee *et al.*, 1992), suggesting there is a specificity to the coupling, perhaps producing the first level of distinction between which signalling pathways are activated. The newest member of this family, PIC-β4,

Figure 1.1.1 The phosphatidylinositol cycle

This figure is pictorial representation of the phosphatidylinositol cycle. The double-headed arrows (⟷)represent reversable kinase/ phosphatase reactions to produce the phosphatidylinositol moieties demonstrated, for example PtdIns(3,4)P_2 represents phosphatidylinositol phosphorylated upon positions 3 and 4 of the inositol ring. Highlighted in red is phosphatidylinositol 3-kinase, which has been shown to be important in various pathways as described in section 1.1.1.4. The substrate of this enzyme is phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2), which is hydrolysed by PtdIns-specific Phospholipase C/Phosphatidase C (PIC) to produce the intracellular calcium mobiliser inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and the protein kinase C (PKC) activator diacylglycerol (DAG).

Figure 1.1.1 Phosphatidylinositol cycle



was purified, cloned and sequenced from bovine retina (Lee *et al.*, 1993; Lee *et al.*, 1994b) and bovine cerebellum (Min *et al.*, 1993). The mRNA encoding this enzyme is also found in high concentrations within mature rat brain (Tanaka and Kondo, 1994). Like the other members of the family, PIC-β4 is activated by G_q (Jiang *et al.*, 1994), but differs in that it shows no preference for any of G_q α subunits and is not activated by βγ subunits (Lee *et al.*, 1994a). However, this PIC-β isozyme is inhibited by ribonucleotides, which are thought to harness the activity of this enzyme, which is 4-5 fold higher than that of the other 3 β isozymes *in vitro* (Lee *et al.*, 1994a).

1.1.1.2 The PIC-γ family

This family consists of PIC-γ1 (145 kDa) and -γ2 (146 kDa), which are differentially expressed in cells, with the initial purification being from bovine brain (Suh *et al.*, 1988a), HL60 cells, spleen and lung (Ohta *et al.*, 1988; Emori *et al.*, 1989; Banno *et al.*, 1990; Homma *et al.*, 1990). PICγs contain SH2 and SH3 domains, which are homologus to the products of various tyrosine kinase-related oncogenes, such as *src*, *abl*, *fes*, *tck* and *crk* (Mayer *et al.*, 1988). PIC-γ1 is a substrate for receptor protein-tyrosine kinases, such as EGF and PDGF (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Nishibe *et al.*, 1989; Wahl *et al.*, 1989). However, not all receptor tyrosine kinases, e.g. insulin, phosphorylate PIC-γ1 (Downing *et al.*, 1989; Nishibe *et al.*, 1990a). PIC-γ1 is found in the cytosol in unstimulated cells, however upon EGF and PDGF stimulation there is translocation of the enzyme to a membrane fraction, which is dependent upon the affinity of the phosphorylated tyrosine residues upon the receptor for the SH2 domains of PIC-γ1 (Kim *et al.*, 1990b; Todderud *et al.*, 1990). The PICγ is then phosphorylated (Nishibe *et al.*, 1990b). PIC-γ1 is phosphorylated on Tyr 771, 783, 1254 and to a lesser extent, Tyr-472, upon EGF and PDGF stimulation (Kim *et al.*, 1990a; Wahl *et al.*, 1990); of these Tyr-783 and Tyr-1254 are essential for activation (Kim *et al.*, 1991b). PIC-γ1 can also be phosphorylated by cyclic AMP-dependent protein kinase

(PKA) on serine-1248 (Olashaw *et al.*, 1990), which does not affect the activity of the enzyme *in vitro* (Granja *et al.*, 1991), but does inhibit subsequent agonist stimulation *in vivo* (Kim *et al.*, 1989). This suggests that the interaction of PIC-γ1 with the activating receptor is affected by PKA-dependent phosphorylation. Inhibition of PIC-γ1 activity by phosphorylation on serine-1248 by protein kinase C (PKC) has also been demonstrated (Park *et al.*, 1992), but this is dependent on the system studied (Ward & Cantrell, 1990). PIC-γ2 has been shown to be regulated in a similar way (Sultzman *et al.*, 1991; Totzke *et al.*, 1992), but does not contain a tyrosine residue equivalent to Tyr-1254 of PIC-γ1.

1.1.1.3 The PIC-δ family

This family contains PIC-δ1 (85 kDa), -δ2 (85 kDa) and -δ3 (84 kDa) and were initially purified from rat brain (Homma *et al.*, 1988; Suh *et al.*, 1988b), bovine brain (Meldrum *et al.*, 1989; Meldrum *et al.*, 1991) and human fibroblasts (Kritz *et al.*, 1990) respectively. The regulation of this family of PIC's remains to be clarified.

1.1.1.4 Phosphatidylinositol-specific 3-kinase

Two families of agonist-stimulated PtdIns 3-kinases have been identified. One is regulated by tyrosine kinases and consists of a p85 regulatory subunit and a p110 catalytic subunit (Carpenter *et al.*, 1990). This PtdIns 3-kinase binds to activated tyrosine kinase receptors, such as PDGF, via a SH2 domain present within the regulatory p85 domain (Escobedo *et al.*, 1991). This association with the activated PDGF receptor does not lead to phosphorylation of PtdIns 3-kinase (Backer *et al.*, 1992). This suggests that the association with the membrane, via a CaLB domain within the p110 subunit (End *et al.*, 1993), is essential for activity (Varticovski *et al.*, 1991). This form of PtdIns 3-kinase is a dual specificity enzyme, with the p110 subunit phosphorylating the p85 subunit on serine-608 (Carpenter *et al.*, 1993; Dhand *et al.*, 1994), leading to a decrease in the PtdIns 3-kinase activity. However, Lam *et*

al. (1994) have shown that this intrinsic serine/threonine kinase activity can also phophorylate insulin receptor substrate 1.

A G-protein regulated PtdIns 3-kinase (215kDa) has also been identified (Stephens *et al.*, 1994). FMLP- and ATP-stimulated PtdIns 3-kinase in U937 myeloid cells involves a pertussis toxin sensitive G-protein, perhaps a member of the G_i family (Stephens *et al.*, 1993), and βγ subunit-mediated activation (Morris *et al.*, 1995). However, GTP-binding proteins have also been shown to be involved in p85/p110 PtdIns 3-kinase activation, as the majority of the GTP-sensitive PtdIns 3-kinase activity, which had been attributed to the 215kDa PtdIns 3-kinase, could be immunoprecipitated by anti-p85 monoclonal antibodies, even though no similarities between the two forms have been found (Thomason *et al.*, 1994). This could be explained by the small molecular weight GTP-binding proteins *ras* and *rho*. *rho* has been shown to be essential for GTPγS- (Zhang *et al.*, 1993) and LPA-stimulated (Kumagai *et al.*, 1993) p85/p110 PtdIns 3-kinase activity. Rodriguez-Viciana *et al.* (1994) and Kodaki *et al.* (1994) identified the direct interaction of GTP-bound *ras* with p85/p110 PtdIns 3-kinase. This was substantiated by expression of N17 dominant negative *ras* leading to a 5-fold decrease in the levels of PtdIns-3,4,5-P_3 induced by EGF (Rodriguez-Viciana *et al.*, 1994). Therefore, both heterotrimeric and small molecular weight G-proteins maybe involved in the activation of PtdIns 3-kinase activity.

The activation of this enzyme has been shown to be involved in cell growth, chemotaxis, intracellular trafficking of the platelet-derived growth factor receptor, respiratory burst generation in neutrophils, insulin-stimulated glucose transport in adipocytes and the membrane ruffling response of fibroblasts to mitogens (Joly *et al.*, 1994; Kundra *et al.*, 1994; Okada *et al.*, 1994; Roche *et al.*, 1994; Wennstrom *et al.*, 1994). PtdIns-3,4,5-P_3 is the product of PtdIns 3-kinase activity (Stephens *et al.*, 1993) and has been implicated in the regulation of the GTP-state of the small molecular weight G-protein *rac* (Wennstrom *et al.*, 1994). *rac* is the common factor in membrane ruffling (Ridley *et al.*, 1992) and superoxide generation in neutrophils

(Segal & Abo, 1993), which are stimulated by PtdIns 3-kinase. Evidence has also been presented that suggests that PtdIns-3,4,5-P_3 can activate Ca^{2+}-insensitive PKC isozymes (Nakanishi *et al.*, 1993; Toker *et al.*, 1994).

1.1.2 Phosphatidylcholine cycle

As described above, cell activation triggers a phosphatidylinositol cycle. In 1989, Pelech & Vance proposed a cycle involving the most abundant phospholipid in eukaryotic cells, phosphatidylcholine (PtdCho). The PtdCho cycle exhibits several features as compared with the PtdIns cycle:

1. The PtdCho pool is 5 times greater than that of phosphatidylinositols.
2. The resynthesis of phosphatidylcholines from diacylglycerols only needs one step instead of 5 for phosphatidylinositol-4,5-bisphosphate synthesis.
3. This resynthesis needs less energy, with only one CTP molecule being required to resynthesize one PtdCho from one phosphocholine, whereas one CTP and 3 ATP are needed to produce 1 molecule of phosphatidylinositol-4,5-bisphosphate.

These differences strongly suggest a specific role for a phosphatidylcholine cycle in the signal transduction cascade [Fig.1.1.2].

1.1.2.1 Synthesis of phosphatidylcholine (PtdCho)

Three enzymatic steps are required for the *de novo* synthesis of PtdCho in what is termed the Kennedy pathway. The enzymes involved are successively choline kinase, CTP:phosphocholine cytidylyltransferase and choline phosphotransferase. Choline kinase is located within the cytosol and is responsible for phosphorylating choline, to produce phosphocholine. Choline phosphotransferase is located within the particulate fraction and is responsible for transferring CDP-choline onto diacylglycerol to produce phosphatidylcholine (for reviews of these two enzymes see Cornell, 1989 and Ishidate, 1989). The rate-limiting enzyme within this pathway is CTP:phosphocholine cytidylyltransferase (CT), which has been detected in both the

Figure 1.1.2 The phosphatidylcholine cycle

This is a schematic diagram of the phospyhatidylcholine cycle. The enzymes involved include:

PAP=phosphatidic acid phosphohydrolase

DAGK=diacylglycerol kinase

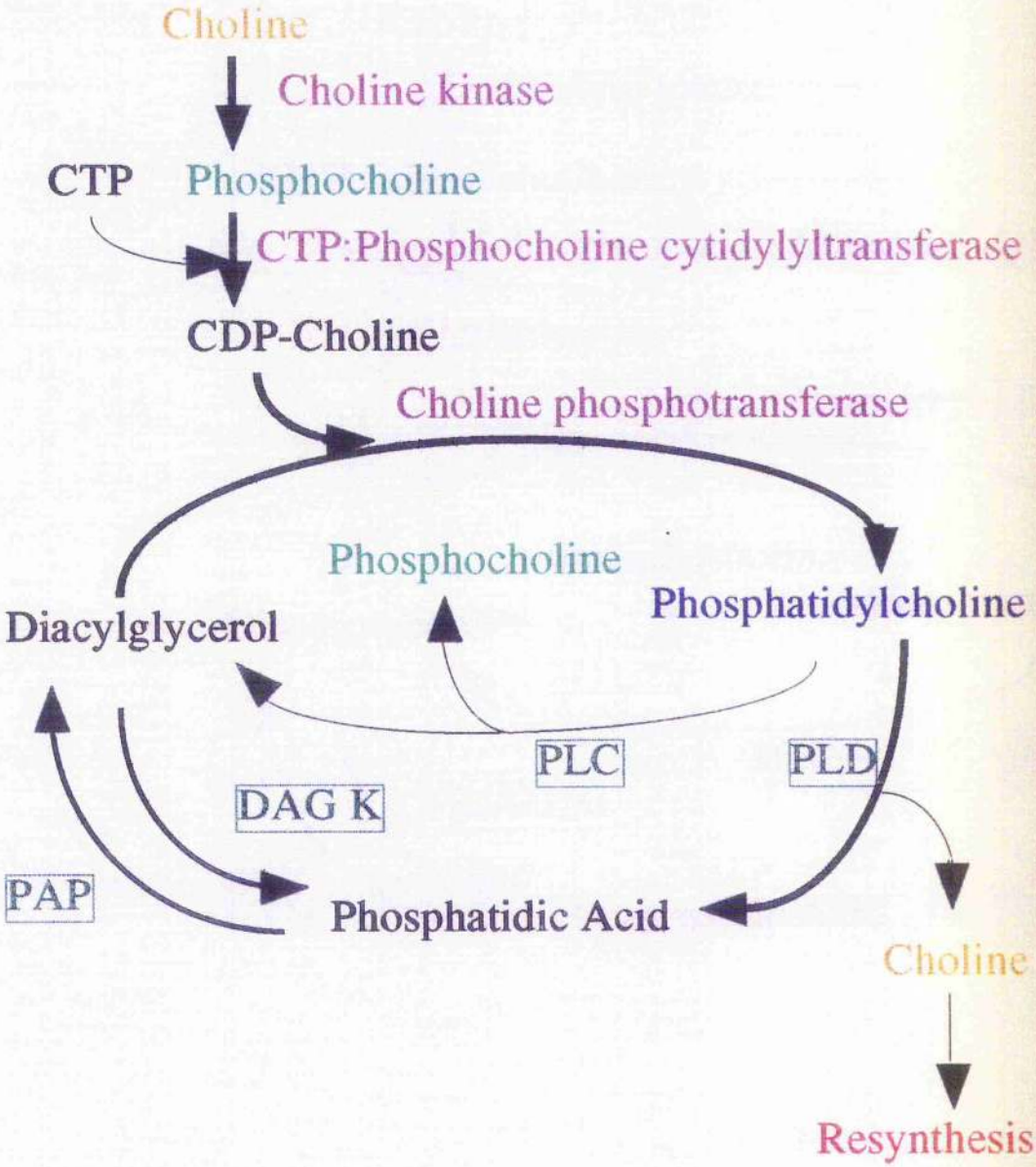
PLC=phospholipase C

PLD=phospholipase D.

This reaction involves the utilisation of one CTP (cysteine triphosphate) molecule, which is converted to CDP (cysteine diphosphate).

[illegible] and Phosphocholine are recycled.

Figure 1.1.2 **Phosphatidylcholine cycle**



cytosol and in the membranes. This enzyme catalyzes, in the presence of CTP, the transformation of phosphocholine to CDP-choline which is not reversible *in vivo* (Choy *et al.*, 1977), as the cell concentrations of CDP-choline and pyrophosphate are too low (Vance, 1989). The native enzyme exists as a non-covalently linked dimer, comprising of two 42kDa subunits (Cornell, 1989), and requires a mixture of fatty acids/phospholipids for activity *in vivo*, with negatively charged phospholipids, such as phosphatidylserine or phosphatidylinositols, producing the highest CT activity (Feldman *et al.*, 1978; Feldman *et al.*, 1985; Cornell & Vance, 1987). This requirement for activity would suggest that the enzyme is only active while associated with a membrane, therefore, the enzyme which has been detected within the cytosol is probably in the inactive form (Vance & Pelech, 1984). The active form of this enzyme is located in the Golgi, endoplasmic reticulum and the nuclear envelope (Vance & Vance, 1988; Morand & Kent, 1989; Jamil *et al.*, 1990; Yao *et al.*, 1990; Wang *et al.*, 1993).

The major event in CT regulation is its membrane translocation (Sleight & Kent, 1980). This is mediated by CT phosphorylation and the enzyme only associates with membranes when phosphorylated (Watkins & Kent, 1991; Hatch *et al.*, 1992). The identity of the protein kinase involved has still to be determined. Diacylglycerol also increases the association of CT to the membrane targets (Kolesnick & Hemer, 1990), however, the mechanism is still unknown, but it has been suggested that DAG may act directly upon CT, or modify the membrane hydrophobicity to allow CT interaction (Utal *et al.*, 1991). This also raises the possibility that DAG activates an isoform of PKC, which may be the unidentified protein kinase involved.

1.1.2.2 Hydrolysis of phosphatidylcholine

PtdCho hydrolysis by PLA_2, PLC or phospholipase D (PLD) is a widespresd response elicited by most growth factors, cytokines, neurotransmitters and hormones (MacNulty *et al.*, 1990; Lin *et al.*, 1991; Cockcroft, 1992)[Fig.1.1.2.2]. In general, the agonists that hydrolyze PtdCho also promote PtdIns-4,5-P_2 hydrolysis generating

Figure 1.1.2.2 Hydrolysis of phosphatidylcholine by the phospholipases.

The abbreviations used in this diagram include:

R_1=fatty acid chain attached to the *sn*-1 position of the diacylglycerol backbone

R_2=fatty acid chain attached to the *sn*-2 position of the diacylglycerol backbone

PLA_1=phospholipase A_1

PLA_2=phospholipase A_2

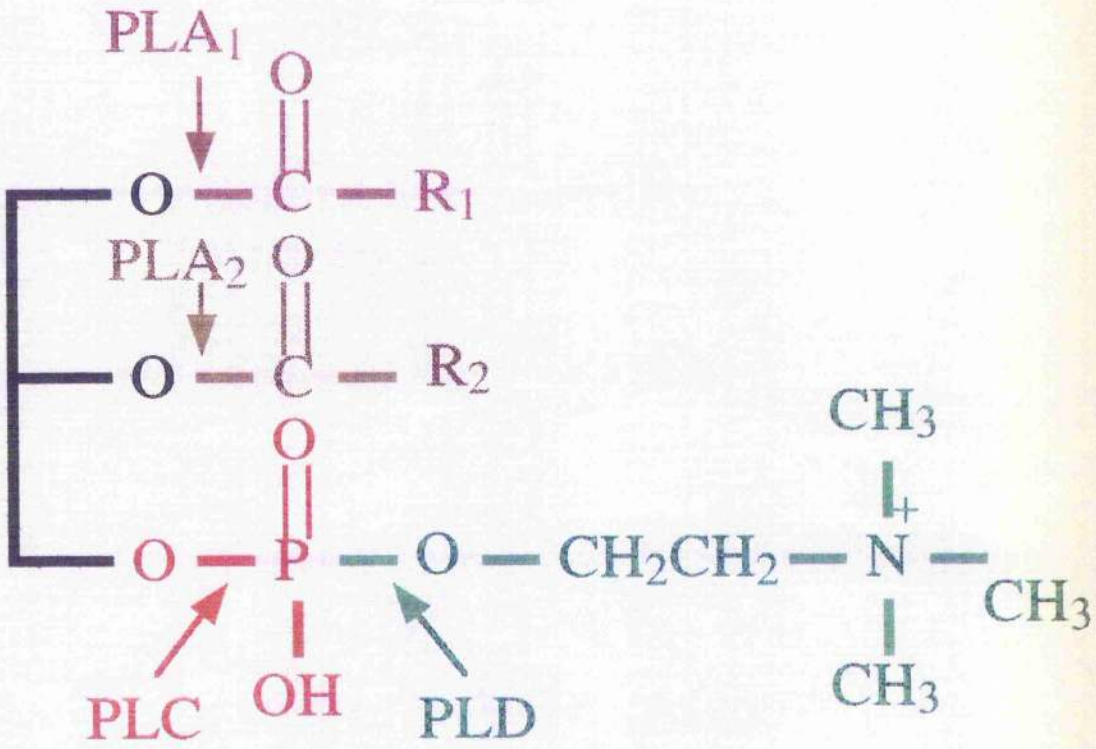
PLC=phospholipase C

PLD=phospholipase D

The arrows indicate the site of action of each of the phospholipases, with the diacylglycerol backbone being outlined in black.

Figure 1.1.2.2

Hydrolysis of phosphatidylcholine by the phospholipases



a biphasic increase in DAG, with an initial rapid, transient peak followed by a more sustained accumulation (Exton, 1990). The initial DAG peak is due to PtdIns hydrolysis (Pettitt & Wakelam, 1993) and the second to PtdCho hydrolysis (Cook *et al.*, 1991). It has also been demonstrated that although PtdCho is the major source of the second phase DAG (Zeisel, 1993), some PtdIns hydrolysis continues and in some cells the major source of this phase of DAG is phosphatidylethanolamine (PtdEth)(Kester *et al.*, 1989; Kiss & Anderson, 1990; Kiss, 1992).

1.1.2.2.1 Agonist-stimulated PtdCho hydrolysis by phospholipase C

It was initially thought that PtdCho hydrolysis in response to agonists mainly resulted from activation of phospholipase A_2 and C, since the products observed were arachidonic acid (AA) and diacylglycerol (DAG). The activation and regulation of PLA_2 will be discussed in more detail in sections 1.2 and 1.3.

A PtdCho-PLC has been partially purified from heart cytosol (Wolff & Gross, 1985), seminal plasma (Sheikhnejad & Srivastava, 1986), and U937 promyelocytes (Clark *et al.*, 1986). These appear to be different isoforms as the enzyme in heart was stimulated by calcium, but the one from the seminal fluid was unresponsive to this divalent cation (Hinkovska-Galchev & Srivastava, 1992). Both forms are responsive to magnesium (Xie & Dubyak, 1991). This enzyme has been suggested to be regulated by an unidentified GTP-binding protein (Billah & Anthes, 1990; Exton, 1990; Cockcroft, 1992). Choudhury *et al.*(1991) demonstrated that colony stimulating factor (CSF-1)-stimulated PtdCho-PLC activity in NIH 3T3 fibroblasts was pertussis toxin sensitive, suggesting the involvement of a member of the G_o/G_i family. However, this group also demonstrated that a large proportion of the activity measured could be immunoprecipitated with anti-phosphotyrosine antibodies. This activity was abolished upon phosphatase treatment, strongly implying a role for tyrosine phosphorylation in the activation of PtdCho-PLC, however, whether this is due to direct or indirect regulation by a tyrosine kinase has still to be elucidated. One possibility, is the activation of the MAP kinase cascade by *ras*, which has been shown

to stimulate PtdCho-PLC activity in NIH 3T3 fibroblats (Lopez-Barahona *et al.*, 1990; Cai *et al.*, 1992; Fu *et al.*, 1992). However, all these studies used phosphocholine production as a measure of PtdCho-PLC activity, which could in fact have been a measure of choline kinase activity upon the product of PtdCho-phospholipase D-mediated hydrolysis of PtdCho, choline. Choline kinase is also stimulated by *ras* (Macara, 1989),

1.1.2.2.2 Agonist-stimulated PtdCho hydrolysis by phospholipase D

The recognition that PLD is a major enzyme involved in PtdCho hydrolysis, came from the observation that accumulation of phosphatidic acid (PA) sometimes preceeds that of DAG and that much of the DAG arises due to the action of phosphatidate phosphohydrolase (PAP) on PA (for a review of PAP see Martin *et al.*, 1994). A major break through in the ability to study PLD specifically, was the observation that PA produced by PLD hydrolysis of PtdCho is "trapped" by increasing addition of a primary alcohol, such as butanol or ethanol. These primary alcohols are stronger nucleophiles than the physiological water molecule, so producing phosphatidylalcohol (Bonser *et al.*, 1989). Unlike PA, this molecule is not a substrate for PAP, so accumulates in the cell. This unique ability displayed by PLD is termed transphosphatidylation, which has been shown to occur in a wide variety of cells (for review see Exton, 1994).

The regulation of agonist-stimulated PLD by PKC and calcium has been widely studied. PKC (Pai *et al.*, 1991; Pachter *et al.*, 1992; Eldar *et al.*, 1993) activation by phorbol esters stimulated PLD activity (Shukla & Halden, 1991), however, PKC inhibitors had varying effects on agonist stimulated PLD activity (Liscovitch & Amsterdam, 1989; Llahi & Fain, 1992). Down regulation of PKC by chronic phorbol ester treatment, has been shown to impair the effect of agonist-stimulated PLD (Cook *et al.*, 1991; Plevin *et al.*, 1991; Stewart *et al.*, 1993). Thus agonist-stimulated PtdCho-PLD activity could be secondary to PtdIns-4,5-P_2 hydrolysis by PIC. This cannot be the complete picture as many agents including

interleukins and EGF, can promote PLD activity in the absence of an input from PtdIns-4,5-P_2 breakdown (Slivka *et al.*, 1988; Cook & Wakelam, 1991; Ha & Exton, 1993).

Recent evidence suggests that PLD can be activated by an increase in tyrosine phosphorylation, but whether it is a direct substrate for a tyrosine kinase or another kinase involved in a phosphorylation cascade remains to be elucidated. Briscoe *et al.*(1995) demonstrated that perhydrovanadate, a tyrosine phosphophosphatase inhibitor, stimulated PLD activity and genistein, a tyrosine kinase inhibitor, partially inhibited the activity as measured by the transphosphatidylation reaction. Similar results have been reported by various groups (Bourgoin & Grinstein, 1992; Uings *et al.*, 1992; Kumada *et al.*, 1993), lending support to the involvement of tyrosine kinases in the regulation of PLD, however, the kinases have still to be identified. One possibility is a non-receptor-associated tyrosine kinase, since v-*src* increased transphosphatidylation activity in Rat-1 fibroblasts (Song *et al.*, 1991; Wyke *et al.*, 1992).

The regulation of PLD has also been shown to be via GTP-binding proteins, however, the evidence to date is confusing. It has been shown that in various cell types, GTPγS stimulates PLD and that this effect is mimicked by aluminum fluoride, suggesting that a heterotrimeric G-protein is involved (Diaz-Meco *et al.*, 1989; Qian & Drewes, 1989; Hurst *et al.*, 1990; Van der Meulen & Haslam, 1990). GDPβS also inhibits the agonist-stimulated response (Kanaho *et al.*, 1991; Liscovitch & Eli, 1991) and in neutrophils (Agwu *et al.*, 1989; Kanaho *et al.*, 1992) and HL60 cells (Xie *et al.*, 1991) the agonist-stimulated response is sensitive to pertussis toxin, suggesting that it is a member of the G_o/G_i family involved. However, in PC12 cells (Kanoh *et al.*, 1992) and liver plasma membranes (Irving & Exton, 1987), the GTP-binding protein involved in PLD activation is pertussis toxin insensitive. The PLD in these liver membranes and membranes prepared from other tissues and cells, can be activated by GTP analogues in the absence of cytosol (Bocckino *et al.*, 1987). However, membranes from neutrophils and HL60 cells could only be activated by the

GTP analogues in the presence of cytosol (Anthes *et al.*, 1989; Olson *et al.*, 1991), suggesting that a factor within the cytosol was essential for GTP-stimulated PLD activity. Brown *et al.*(1993) and Cockcroft *et al.*(1994) identified this essential factor as the small molecular weight GTP-binding protein ARF (ADP ribosylation factor), which ADP-ribosylates $G_{s\alpha}$ in response to cholera toxin (Moss & Vaughan, 1993). This 21 kDa protein has been shown to be involved in the regulation of vesicle protein trafficking (Rothman & Orei, 1992) and perhaps PLD mediates the action of ARF on membrane traffic by its ability to cause changes in the lipid composition of membranes (Kahn *et al.*, 1993). Another low molecular weight GTP-binding protein, *rho*, has also been implicated in GTPγS-stimulated PLD activity in neutrophil membranes (Bowman *et al.*, 1993). This G-protein is membrane bound, unlike ARF, but its role in the activation of PLD has still to be elucidated.

1.1.2.2.3 Physiological effects of phosphatidylcholine hydrolysis

The increase in DAG produced either by PtdCho-PLC or by the combined action of PtdCho-PLD and PAP, is prolonged, providing chronic PKC activation (Fallman *et al.*, 1992), which does not cause downregulation. However, initial data indicated that PtdCho-derived DAG was unable to activate PKC (Martin *et al.*, 1990; Leach *et al.*, 1991), whereas PtdIns-derived DAG in conjunction with elevated intracellular calcium was shown to translocate PKC-α, -β, -δ and -ε (Kiley *et al.*, 1991). When studied in more detail it was shown that the biphasic release of DAG is mediated by the hydrolysis of different substrates (Pettit *et al.*, 1994). Fatty acid composition analysis of the DAG species demonstrated that the initial transient phase was from PtdIns hydrolysis and the second sustained phase from PtdCho hydrolysis (Pettitt & Wakelam, 1993). Both phases of DAG are able to stimulate Ca^{2+}-independent PKC isoforms, such as PKC-ε (Ha & Exton, 1993), but only the initial phase of DAG stimulates Ca^{2+}-dependent PKC isoforms, such as PKC-α. The activity of PKC can also be regulated by the enzyme DAG kinase, which competes with PKC for DAG, which it rapidly phosphorylates to produce phosphatidic acid

(PA) (Besterman *et al.*, 1986). The role of PKC is discussed in section 1.3.2.1.

The PLD product phosphatidic acid (PA) may act as a signalling molecule itself or be hydrolised by a phospholipase A_2 to lysophosphatidic acid (LPA), as well as be converted to DAG by PAP. Exogenously applied PA has been reported to stimulate calcium influx and calcium-mediated processes in various cell types. The effects of PA on cells were previously explained by its ability to mobilize extracellular calcium by an ionophore effect (Tyson *et al.*, 1976; Harris *et al.*, 1981). However, PA has been shown to stimulate phosphoinositide turnover in various cells including fibroblasts (Murayama & Ui, 1987), A431 cells (Moolenaar *et al.*, 1986) and human keratinocytes (Ryder *et al.*, 1993), thereby leading to release of intracellular calcium reserves. PA has also been shown to inhibit Luteinizing hormone-stimulated testosterone production in rat Leydig cells (Lauritzen *et al.*, 1994), lysophospholipase transacylase from rat liver (Sugimoto & Yamashita, 1994) and association of *rac* with the *rho*GDI, under non-activating conditions (Chung *et al.*, 1993). It does however, stimulate ARFGAP activity (Randazzo & Kahn, 1994), therefore possibly inactivating PLD. PA may also have an important role to play in the regulation of the respiratory burst, as it has been shown to stimulate arachidonate release in peritoneal macrophages (Fernandez *et al.*, 1994) and phosphorylates p74 of the NADPH oxidase complex (McPhail *et al.*, 1995), perhaps via a PA-activated kinase, which has been partially purified from human platelets (Khan *et al.*, 1994).

LPA can act as an extracellular signal and addition of this lipid to many cell types often produces rapid and dramatic results. This agonist will be discussed in more detail in section 1.6.3. As yet no signalling role has been established for the water soluble products of PtdCho hydrolysis, choline and phosphocholine, but they are essential for the synthesis of phosphatidylcholine *de novo*.

1.2 PHOSPHOLIPASE A_2

Phospholipase A_2 catalyzes the hydrolysis of the *sn*-2 fatty acyl chain of phospholipid substrates to yield fatty acids and lysophospholipids. These products may themselves act as intracellular second messengers or can be further metabolised as the precursors in the production of specific proinflammatory lipid mediators. The diversity of mammalian *sn*-2 acylhydrolases has increased with 2 main enzyme groups arising. These are the 14kDa low molecular weight phospholipase A_2's, which have been further catogerised into type I, II and III and the 85kDa cytosolic phospholipase A_2, which has been termed type IV. However, even these classifications are incomplete with the new phospholipase A_2 enzymes being discovered, such as the 42kDa enzyme isolated from human placenta (Buhl *et al.*, 1995) and a 14kDa, Ca^{2+}-dependent phospholipase A_2 which contains 16 cysteines, compared to the normal 14 identified in all low molecular weight phospholipase A_2 isoenzymes so far (Chen *et al.*, 1994).

1.3.1 Secretory phospholipase A_2 ($sPLA_2$)

Phospholipase A_2 activity was first studied in pancreatic juice and cobra venom in the early 1950's. The PLA_2's in venom have some digestive functions, but are also directly involved in toxicity (Kini and Evans, 1990). Subsequently, PLA_2's obtained from various snake and bee venoms and from mammalian pancreas have been well characterized, mechanistically elucidated, and structurally defined. These extracellular enzymes have been divided into three main groups and several subgroups based on their amino acid sequences, as follows: Group I (Cobras and kraits; porcine/human pancreas), Group II (rattlesnakes and vipers; human synovial fluid/platelets), Group III (bee/lizard). Group I, II and III have molecular weights of 13-18 kDa and are perhaps the most elementary of the PLA_2's (Davidson and Dennis, 1990). The amino acid sequences and seven disulphide bonds are conserved among the Group I and II species, whilst the Group III bee venom enzyme has distinct and rearranged sequence homologies, however x-ray crystallography has revealed identical

catalytic residues (Thunnissen *et al.*, 1990). These secretory enzymes do not form a classical acyl enzyme intermediate characteristic of serine proteases, but utilize the catalytic site His 48, assisted by an Asp, to polarize a bound H_2O which then attacks the carbonyl group. This transition state is stabilized by the binding of a Ca^{2+} ion in the conserved Ca^{2+}-binding loop, which explains the requirement for millimolar calcium for activity (Verheij *et al.*, 1980). While the majority of the Group I, II and III enzymes that have been studied are extracellular non-human enzymes, a growing number of Group I and II enzymes have been found in human tissues. The best characterized is the Group II PLA_2 originally isolated from human synovial fluid (Wong and Dennis, 1990). However, Group II enzymes have also been found in non-secreted locations, for example in rat liver mitochondria (Van den Bosch *et al.*, 1989).

The human Group I and II phospholipase A_2 enzymes have a number of diverse roles. Group I is mainly secreted from the pancreas as an inactive zymogen, which is converted into the active form by proteases, therefore it has a suggested role as a digestive enzyme (de Haas *et al.*, 1968). This enzyme has also been detected in rat stomach (Yasuda *et al.*, 1990) and human serum (Nishijima *et al.*, 1983). These enzymes have also been suggested to have a role in vascular function, as Group I binding sites have been found on vasculature (Hanasaki and Arita, 1992), with the same binding sites being found on Swiss 3T3 fibroblasts. Binding of Group I PLA_2 to these 200kDa binding sites induced DNA synthesis, increased proliferation and glucose transport (Arita *et al.*, 1991). Group II phospholipase A_2 enzymes may serve as a link joining the proximal and distal limbs of the inflammatory response (Larsen and Henson, 1983; Murakami *et al.*, 1990). This has been suggested to be due to a high level of Group II PLA_2 in peritoneal exudate fluid (Franson *et al.*, 1988) and ascitic fluid after intraperitoneal injection of casein or zymosan (Gans *et al.*, 1989). In addition Group II PLA_2 degrades the membrane phospholipids of gram-negative bacteria (Kaplan-Harris *et al.*, 1980), thus a host defence function has been assigned to this enzyme. However, the observation of enhanced Group II PLA_2 secretion

during activation of certain cell systems and the demonstration of its contribution to prostaglandin production using antisense RNA (Barbour and Dennis, 1993) argue that this enzyme also plays a role in signal transduction and eicosanoid production.

1.2.2 Cytosolic phospholipase A_2 ($cPLA_2$)

Several hormones, neurotransmitters, growth factors and cytokines have been found to stimulate phospholipase A_2 activity via occupation of extracellular receptors, resulting in the hydrolysis of phospholipids and the consequent release of free fatty acids. These include basic fibroblast growth factor (Sa & Fox, 1994); thrombin (Kramer *et al.*, 1993a); macrophage colony stimulating factor (Nakamura *et al.*, 1992); platelet-derived growth factor (Domin &Rozengurt, 1993); bradykinin (Kaya *et al.*, 1989); carabacol (Konrad *et al.*, 1992); transforming growth factor-α (Liu *et al.*, 1993); interleukin-1α (Lin *et al.*, 1992); bombesin (Takuwa *et al.*, 1991; Currie *et al.*, 1992); f-methyl-leucine-phenylalanine (Suga *et al.*, 1990) and lysophosphatidic acid (van Corven *et al.*, 1989), some of which may mediate their effects by increasing intracellular calcium concentrations.

A phospholipase A_2, which has different structural and biochemical properties to secretory phospholipase A_2, but performs the same catalytic function, was purified from the human monocytic leukemic U937 cell (Clark, *et al.*, 1990; Sharp *et al.*, 1991); rat kidney (Gronich *et al.*, 1990) and platelets (Kim *et al.*, 1991a; Takayama, *et al.*, 1991). As this phospholipase A_2 activity was recovered predominately in the cytosolic fraction of cells and tissues it was termed cytosolic phospholipase A_2 ($cPLA_2$). The primary structure of $cPLA_2$ has been elucidated by cDNA cloning and the deduced $cPLA_2$ protein sequence has several interesting structural features. These include a 68-amino acid stretch at the N-terminus termed CaLB, which is a sequence motif associated with calcium and phospholipid binding. This motif has been identified in various proteins and has been shown to confer a terniary configuration between a single calcium atom, phospholipid and the enzyme of interest. This is followed by a 109-amino acid segment that exhibits 33% homology with

phospholipase B from *P. notatum* (Masuda *et al.*, 1991), which contains the active site. In the middle of the sequence there is a 34-amino acid segment that is deficient in hydrophobic amino acids and may represent a flexible 'hinge' region. Towards the C-terminus, there is a proline rich domain, which is preceeded by serine 505, the MAP kinase phosphorylation site. Throughout the sequence there are numerous diverse consensus phosphorylation sites for both serine/threonine and tyrosine protein kinases (Kemp & Pearson, 1990). The inferred sequence of murine $cPLA_2$ is greater than 95% homologous to the human $cPLA_2$ (Clark *et al.*, 1991), indicating great structural similarity between $cPLA_2$ from different species. From the purifications highlighted, $cPLA_2$ was shown to be a polypeptide of approximately 100kDa, however from subsequent cDNA cloning the predicted molecular mass was found to be 85.2kDa (Sharp *et al.*, 1991). This inconsistency between predicted and electrophoretic molecular weight could be due to post-translational modifications or to the proline-rich (>12% of total residues) domain towards the C-terminus. The structure and function of such proline-rich regions in proteins is reviewed by Williamson (1994).

The 85 kDa $cPLA_2$ has been shown to have a 5- to 10-fold preference for *sn*-2-arachidonyl-containing phospholipid substrates, but there is debate to whether or not there is a preference for a choline head group (Diez, *et al.*, 1992; Currie *et al.*, 1992). The enzyme does not discriminate between 1-acyl or 1-alkyl linked phospholipids (Kramer *et al.*, 1988), which it does not hydrolyse in intact phospholipids (Kramer *et al.*, 1986), but readily acts upon lyso-phosphatidylcholine micelles, with no preference for the available fatty acid at the *sn*-1 position (Leslie, 1991). This lysophospholipase activity will be discussed below, but it has recently been shown that both the phospholipase A_2 and the lysophospholipase activities of $cPLA_2$ are catalyzed by the same active site residue, serine 228 (Sharp *et al.*, 1994).

$cPLA_2$ is a calcium-responsive enzyme, which can be activated by submicromolar concentrations of calcium (300nM-2μM) as would be achieved during cell stimulation (Leslie *et al.*, 1988), however, *in vitro* other divalent metal ions can substitute for calcium in the catalytic mechanism of $cPLA_2$ (Kramer *et al.*, 1986;

Gronich *et al.*, 1988). Divalent cations are essential as $cPLA_2$ is inactive in the presence of excess chelating agents. The primary structure of $cPLA_2$ highlighted a 68-amino acid stretch in the N-terminal portion termed the 'CaLB domain' that shows 38% sequence homology with the C2 region of protein kinase C. This sequence has been implicated in the Ca^{2+}-dependent binding of PKC to lipids and is absent in Ca^{2+}-independent forms of PKC (Masuda *et al.*, 1991). This CaLB domain has also been identified in the GTPase activating protein (GAP), phospholipase $C_{\gamma 1}$ and the synaptic vesicle protein p65 (Clark *et al.*, 1991), but it has been demonstrated that the C2 domain in PKC merely confers the specificity for Ca^{2+} and that Ca^{2+} is bound mainly to the C1 domain (Luo & Weinstein, 1993). The possible role of calcium in the regulation of $cPLA_2$ will be discussed below.

Another probable regulator of $cPLA_2$ activity is phosphorylation as the protein sequence contains consensus phosphorylation sites for protein kinase A (PKA), PKC, casein kinase II (Kemp and Pearson, 1990) and MAP kinase (Nemenoff *et al.*, 1993).

1.3 REGULATION OF CYTOSOLIC PHOSPHOLIPASE A_2

The regulation of cytosolic phospholipase A_2 is complicated by the presence of multiple phospholipase A_2 forms within cells. For instance, three distinct *sn*-2 acylhydrolase activities have been characterised in cultured mast cells (Murakami *et al.*, 1992b) and gerbil brain (Rodorf *et al.*, 1991), whilst an additional form of phospholipase A_2 has been purified and characterized from heart (Gross, 1992) and been shown to exist as 2 isoforms (Hazen *et al.*, 1993). Both isoforms have a molecular weight of 40kDa and preferentially hydrolyze arachidonate at the *sn*-2 position, however, these myocardial cytosolic phospholipase A_2 isoforms are calcium-independent.

1.3.1 Calcium

Mobilization or availability of intracellular calcium appears to be an essential step for activation of both the type II 14-kDa PLA_2 and the 85kDa PLA_2. Many studies have demonstrated that treatment of cells with a calcium ionophore induces maximal arachidonate release from intracellular phospholipids. Thus, the activation of PLA_2 may be secondary to receptor-mediated activation of PLC leading to the formation of Ins-1,4,5-P_3 and thus an increase in intracellular free calcium concentrations (Meade *et al.*, 1986). However, Ins-1,4,5-P_3 production and arachidonate release can be dissociated (Burch & Axelrod, 1987; Slivka & Insel, 1988) since arachidonate can be released in the absence of PtdIns-4,5-P_2 hydrolysis (Kanterman *et al*, 1990; Gil *et al.*, 1991; Virdee *et al.*, 1994). $cPLA_2$ activity has also been shown to be dependent upon extracellular calcium in C62B glioma (Brooks *et al.*, 1989), endothelial (Whately *et al.*, 1989) and MDCK-D1 cells (Insel *et al.*, 1991).

As the regulation of PLA_2 activity by increased calcium shows some cell type specificity, separate roles for the ion are suggested. $cPLA_2$ binds to membranes in a calcium dependent manner through the N-terminal domain. Therefore, increased intracellular calcium concentrations from either Ins-1,4,5-P_3-sensitive pools or from increased flux from the extracellular medium could induce the association of $cPLA_2$ with cellular membranes. Channon & Leslie (1990) demonstrated that $cPLA_2$ activity was translocated from the cytosol to a membrane fraction in the presence of micromolar concentrations of calcium and many groups observed that during purification of $cPLA_2$ from cells broken in the presence of the calcium chelator EGTA, the majority of PLA_2 activity was in the cytosol, whereas when broken in the presence of calcium concentrations approaching those measured in activated cells, activity was mainly found in the membrane fraction (Krause *et al.*, 1991; Rehfeldt *et al.*, 1991). This translocation of $cPLA_2$ was also observed in thrombin-stimulated human platelets (Kramer *et al.*, 1993b), suggesting that the Ca^{2+}-mediated translocation occurs *in vivo*. Wijkander & Sundler (1992a) demonstrated using $^{45}Ca^{2+}$, that there was a single binding site for calcium in a ternary complex with the 85 kDa phospholipase A_2

and the phospholipid. However, hydrolytic activity could be obtained in the absence of calcium using high conentrations of NaCl or other salt, implying that calcium is required for interfacial association with lipid but not for catalysis.

1.3.2 Phosphorylation

As described earlier, the sequence of cloned $cPLA_2$ demonstrated various possible phosphorylation sites. As calcium has been shown not to be involved in catalysis, it is perhaps phosphorylation which activates phospholipase A_2.

1.3.2.1 Protein kinase C (PKC)

In many cell types the activation of PKC is not sufficient to induce liberation of *cis*-unsaturated fatty acids, however, several observations support the notion that PKC may be important in the regulation of phospholipase A_2. For example, the cytosolic phospholipase A_2 activity recovered from phorbol ester-stimulated mesangial cells was increased several fold, suggesting a stable modification of the enzyme (Gronich *et al.*, 1988) and in CHO cells overexpressing $cPLA_2$ phorbol ester stimulated the phosphorylation of $cPLA_2$ on serine residues (Lin *et al.*, 1992). This phorbol ester-stimulated phosphorylation of $cPLA_2$ has been shown by various groups (Wijkander & Sundler, 1992b; Kramer *et al.*, 1993a; Qui *et al.*, 1993) and correlates to an increase in the *in vitro* V_{max} of $cPLA_2$ but with no effect on calcium requirements or substrate specificity. This was supported by the observed decrease in PLA_2-mediated arachidonate release in response to phorbol ester when expression of PKCα was inhibited by an antisense cDNA (Godson *et al.*, 1993). However, down-regulation of PKC by chronic phorbol ester treatment, did not effect the EGF-stimulated PLA_2 in mesangial cells (Bonventre *et al.*, 1990) or ATP-stimulated arachidonate release in FRTL-5 rat thyroid cells (Smallridge & Gist, 1994).

1.3.2.2 Tyrosine phosphorylation

It has been suggested that intrinsic tyrosine kinase activity is required for apparent PKC-independent activation of phospholipase A_2. CHO cells transfected with a truncated, tyrosine kinase defective, EGF receptor produced no EGF-stimulated phospholipase A_2 activity, compared to control cells transfected with the complete EGF receptor (Clark & Dunlop, 1991). However, the phospholipase A_2 present in the cells containing the truncated receptor was activatable, as calcium ionophore-stimulated arachidonate release was detected (Goldberg *et al.*, 1990). Kast *et al.*(1993) demonstrated that $cPLA_2$ was specifically immunoprecipitated with antiphosphotyrosine antibodies after TGFα stimulation, which exerts its biological activity via the EGF receptor (Hunter, 1989), but not phorbol ester-treated or control cells. These results suggest that $cPLA_2$ or a tightly associated protein, which is coupled to the tyrosine kinase receptors described, maybe phosphorylated upon tyrosine residues . $cPLA_2$ has been shown to be phosphorylated upon tyrosine residues, but there was no increase in phosphorylation upon agonist stimulation (Currie *et al.*, 1994). It appears that the involvement of tyrosine kinases in the regulation of $cPLA_2$ is not by direct agonist-stimulated tyrosine phosphorylation of $cPLA_2$, but the tyrosine phosphorylation of some regulatory protein. The tyrosine phosphorylation of a regulatory protein may then lead to the phosphorylation of $cPLA_2$ upon serine/threonine residues.

1.3.2.3 MAP Kinase

Growth factor signalling is mediated by growth factor receptors containing an integral tyrosine kinase. In response to growth factors the receptor tyrosine kinases are activated to initiate intracellular signalling events. This tyrosine phosphorylation can be rapidly translated to changes in the phosphorylation of cytoplasmic and nuclear proteins on serine and threonine residues, leading to alteration in the properties of the target proteins. In general, two broad approaches have been adopted in the study of these phosphorylation events in growth factor signal transduction. One was to work

'down' from the receptor in a stepwise manner to identify relevant components in the signalling pathway. The second approach was to start with a cellular event known to be affected by the growth factors and to work 'up' toward the receptor. These approaches have led to the identification of a signal transduction pathway initiated by growth factor receptors [Fig.1.3.2.3].

The MAP kinase cascade was elucidated primarily by the upstream approach. One of the first identified target proteins for enhanced serine/threonine phosphorylation upon growth factor stimulation, was ribosomal protein S6, which produced a convenient marker for the elucidation of upstream events. Two enzymes that can phosphorylate ribosomal protein S6 on growth factor stimulation were identified, $p70^{s6k}$ (Cobb & Rosen, 1983; Novak-Hofer & Thomas, 1984; Kozma *et al.*, 1990) and $p90^{rsk}$ (Erikson & Maller, 1985; Alcorta *et al.*, 1989). These S6 kinases are themselves activated by serine/threonine phosphorylation and it was later shown that partially purified microtubule-associated protein-2 (MAP-2) protein kinase obtained from insulin-stimulated 3T3-L1 cells (Ray & Sturgill, 1987) could phosphorylate and reactivate phosphatase-treated S6 kinase (RSK) obtained from *Xenopus laevis* oocytes (Sturgill *et al.*, 1988). This observation was subsequently confirmed in experiments using insulin-activated MAP-2 kinase and non-stimulated RSK from rat liver (Gregory *et al.*, 1989) or epidermal growth factor (EGF)-stimulated MAP-2 kinase and non-stimulated RSK from Swiss 3T3 fibroblasts (Ahn & Krebs, 1990), implicating MAP-2 kinase as an S6 kinase kinase. The original name, MAP-2 kinase, was later changed to MAP kinase, and when it was cloned, the gene was designated as extracellular regulated kinase (ERK)(Boulton *et al.*, 1990). MAPK and ERK are often used interchangeably, but it has been suggested that ERK-1 ($p44^{MAPK}$) and ERK-2 ($p42^{MAPK}$), are the name for one particular group of isoforms within the MAPK family.

MAP kinase can be inactivated by either the protein tyrosine phosphatase, CD45, or by the protein serine/threonine phosphatase, PP2A, indicating that both tyrosine and serine/threonine phosphorylations are required for its activity (Anderson,

Figure 1.3.2.3 The MAP kinase signalling cascade

This diagram demonstrates the possible components of this signalling cascade such as:

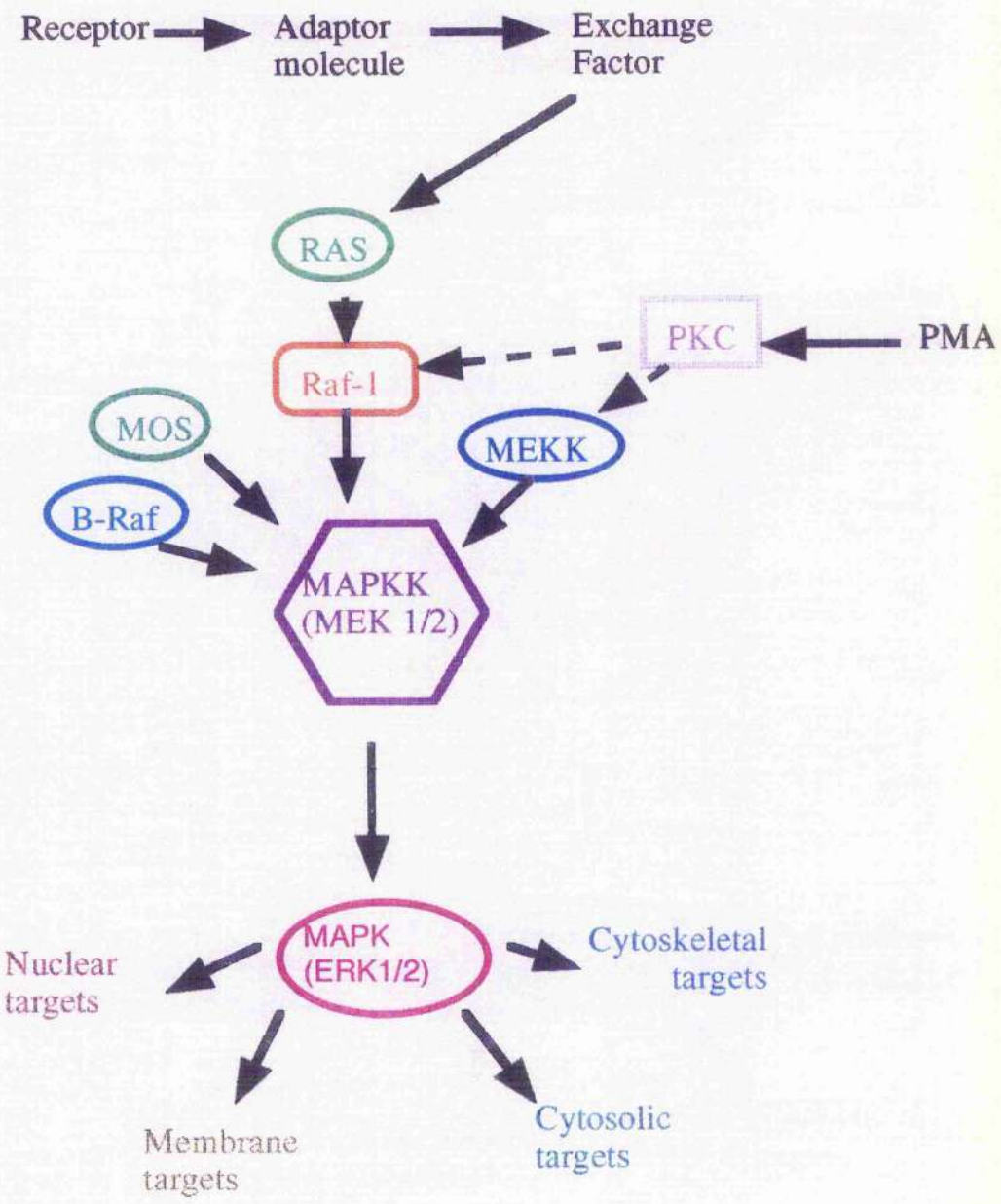
RAS	21kDa small molecular weight G-protein
Raf-1	74kDa MAP kinase kinase kinase which phosphorylates serine/threonine residues
PKC	Protein kinase C
MOS	Germ cell-specific serine/threonine protein kinase
B-Raf	Serine/threonine kinase expressed primarily in brain and in the nervous system which is 54% homologous to c-Raf-1
MEKK	MEK kinase
MEK	Mitogen-activated, ERK-activating kinase
MAPKK	MAPK kinase
MAPK	Mitogen-activated protein kinase / microtubule-associated protein kinase
ERK	Extracellular regulated kinase
PMA	Phorbol myristyl acetate

It must be noted that the following terms can be interchanged:

1. MAPKK and MEK
2. MAPK and ERK

The arrows highlight the targets of the component of interest.

Figure 1.3.2.3 **MAP kinase signalling cascade**



et al., 1990). In quiescent or in unstimulated cells, the MAPK isoforms exist in a dephosphorylated form, and are only activated when phosphorylated on both Thr 183 and Tyr 185, for mammalian 41kDa MAPK(ERK2)(Payne *et al.*, 1991), and Thr 188 and Tyr 190, for *Xenopus* 42kDa MAPK (Posada & Cooper, 1992). The phosphorylation of the two residues seems to be a sequential reaction in which tyrosine phosphorylation preceeds threonine phosphorylation (Haystead *et al.*, 1992). These TEY sites are located in subdomain VIII just upstream of the conserved Ala-Pro-Glu (APE) motif found in all serine/threonine kinases (Hanks *et al.*, 1988). Phosphorylation of both regulatory residues or each one individually causes shifts in the mobility of the ERK on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which is often used as a marker for their activities. However, this is not always accurate as singly phosphorylated ERK lacks kinase activity. As well as the requirement for catalytic activity, phosphorylation of both regulatory residues also appears to be the main requirement for translocation of ERK to the nucleus (Chen *et al.*, 1992; Lenormand *et al.*, 1993). These proline-directed protein kinases can phosphorylate a large number of proteins, which have serine or threonine residues next to proline residues (Gonzalez *et al.*, 1991). These ERK substrates are located in both the cytoplasm and the nucleus, including cytosolic phospholipase A_2 (Lin *et al.*, 1993), nuclear transcription factors, such as *Elk*-1 (Rao & Reddy, 1993), upstream proteins of the MAPK cascade, such as *raf*-1, and cytoskeletal elements such as *Tau*. The phosphorylation of these cytoskeletal elements appears to regulate cytoskeletal rearrangements and cellular morphology (Minshull *et al.*, 1994). Therefore, it is possible that ERK activation controls events leading to the G_0/G_1 transition of the cell cycle (Boulton *et al.*, 1990). Tamemoto *et al.*(1992) reported a biphasic activation of two MAPKs during the cell cycle in CHO cells. In these cells ERK-1 and -2 showed enhanced activities in the G_1 through S and G_2/M phases and were activated biphasically in the G_1 phase and around the M phase.

MAPK is capable of autophosphorylation (Seger *et al.*, 1991), but is activated by MAPK kinases (MAPKK) (Posada & Cooper, 1992; Seger *et al.*, 1992).

Purification of these kinases confirmed that MAPKK (also referred to as MEK) is a single kinase that catalyzes serine/threonine as well as tyrosine phosphorylation, making it a member of a small family of dual specificity kinases (Matsuda *et al.*, 1992; Seger *et al.*, 1992). Cloning of MAPKK revealed five isoforms of the enzyme (Yashar *et al.*, 1993). These are regulated by phosphorylation on serine/threonine residues, with phosphorylation of serine 218 and 222 being essential for activity (Alessi *et al.*, 1994; Seger *et al.*, 1994) and phosphorylation of serine 212, threonine 286, 292 and 386 have a role to play in the inactivation of MAPKK (Rossomando *et al.*, 1994; Zheng & Guan, 1994). Sontag *et al.* (1993) also demonstrated that activation of MEK occurs through the inhibition of serine/threonine phosphatases, such as phosphatase PP2A.

Kyriakis *et al.*(1992) demonstrated that *raf*-1, a known serine/threonine kinase, is capable of phosphorylating and activating MAPKK after growth factor stimulation. *raf*-1 is the product of c-*raf*-1, the cellular homologue of v-*raf*, the transforming gene of the murine sarcoma virus 3611 (Rapp *et al.*, 1988) and is a 70-75 kDa serine/threonine kinase, which contains a kinase domain in the COOH-terminal half of the molecule and a regulatory domain occupying the remainder. The mechanism of *raf*-1 has been studied extensively and has been shown to be downstream of *ras* (Moodie *et al.*, 1993), which does not activate *raf*-1, but recruits the enzyme to the plasma membrane, where it is activated (Stoke *et al.*, 1994; Leevers *et al.*, 1994). The activation of *raf*-1 coincides with hyperphosphorylation of the enzyme possibly by protein kinase C (PKC), which has been shown to phosphorylate *raf*-1 directly (Morrison *et al.*, 1993) or ceramide-activated protein kinase (Yao *et al.*, 1995). Tyrosine kinases may also be involved, as it has been shown that $p56^{lck}$ can phosphorylate and activate *raf*-1 and tyrosine phosphorylation is essential for activation (Fabian *et al.*, 1993).

$I_\kappa B$ is an additional substrate for *raf*-1 which may transmit signals by releasing cytoplasmic, active $NF_\kappa B$, which can than be translocated to the nucleus (Li & Sedivy, 1993). In addition to *raf*-1, other MAPKKKs (MAP3K) have been

identified, including MEK kinase (Lange-Carter *et al.*, 1993), c-*mos* (Posada *et al.*, 1993), *ras*-dependent ERK kinase stimulator from *Xenopus* oocytes (Itoh *et al.*, 1993), a high molecular weight complex from unfertilised *Xenopus* eggs (Matsuda *et al.*, 1993) and an insulin-stimulated MAP3K from adipocytes (Haystead *et al.*, 1994). However, it has been suggested that each of these MAP3Ks activate a different group in the MAPKK family (Yau *et al.*, 1994).

Physiologically the MAP kinase cascade has been implicated in proliferation and differentiation. Miltenberger *et al.* (1993) used antisense c-*raf*-1 to show interference with the NIH-3T3 cell proliferation, whereas constitutively-activated *raf*-1 had an accelerated effect. Pages *et al.* (1993) demonstrated that a dominant negative form of ERK-1 caused a reduction in the number of NIH 3T3 fibroblasts and their ability to proliferate. However, there is also evidence that cell proliferation is independent of the MAP kinase cascade (Wang *et al.*, 1992; Casillas *et al.*, 1993). The MAP kinase cascade have also been implicated in processes such as monocytic differentiation (Han *et al.*, 1993), neurite outgrowth of PC12 cells (Qui & Green, 1992), T-cell maturation (Alberola-Ila *et al.*, 1995) and mast cell development (Tsai *et al.*, 1993).

1.3.3 GTP-binding proteins

Studies with different cells provided evidence to support a role for GTP-binding proteins in the regulation of cytosoic phospholipase A_2. Both the classical heterotrimeric and the small molecular weight, *ras*-like, G-proteins have been implicated (for review see Burch, 1989; Axelrod, 1990; Cockcroft, 1992).

1.3.3.1 Heterotrimeric G-proteins

Heterotrimeric G-proteins comprise 3 polypeptides : an α-subunit that binds and hydrolyzes GTP, a β- and a γ-subunit. The βγ subunits only dissociate under denaturing conditions and when the α subunit is in the GDP-bound state it associates with βγ. When GTP is bound to the active site, the α subunit dissociates from both

the βγ subunit and the activated receptor and activates its effector. The intrinsic GTPase activity of the α subunit inactivates it by hydrolyzing the bound GTP to GDP, stimulating the reassociation with βγ subunits and the inactive receptor, ready for subsequent agonist stimulation. Therefore, the rate of GTP-hydrolysis controls the duration of both α and βγ subunit activation, as re-association inactivates both subunits (for review see Neer, 1995).

Over 20 different G-protein α subunits have been identified and divided into 4 major classes, according to the similarity of their amino acid sequences, with individual cells usually containing at least 4 types of α subunits (for review see Neer, 1994). There are five known mammalian β subunits, which are 53-90% identical (for review see Simon *et al.*, 1991; Watson *et al.*, 1994), whereas the six known γ subunits vary (for review see Cali *et al.*, 1992) and can interact with most but not all of the β subunits. For example, the β_1 subunit can form a dimer with only γ_2 and γ_3 subunits and β_2 can only interact with γ_2 (Schmidt *et al.*, 1992).

The heterotrimeric complex has been shown in some cases to be located to membranes via lipid attachments (for review see Yamane & Funf, 1993). G_o and G_i α subunits are irreversibly myristolated at the N-terminal glycine, which facilitates the association with βγ. G_s α subunit is palmitoylated at cys-3, which can be removed upon β-adrenergic receptor occupation, leading to adenylyl cyclase inhibition (for review see Casey, 1994). All the γ subunits are prenylated, which is essential for membrane attachment of the βγ subunit and may influence the specificity of the βγ/receptor interaction (Kisselev *et al.*, 1994). This coupling of heterotrimeric G-proteins to an activated receptor is also regulated by a group of serine-threonine protein kinases, termed G-protein coupled receptor kinases (GRKs)(for review see Premont *et al.*, 1995), which may act conversely to βγ subunits by desensitizing the receptor.

The pathways activated by these heterotrimeric G-proteins have been extensively studied, with the various α and βγ subunits having a myriad of effects. In the regulation of phospholipase A_2, both α and βγ subunits have been implicated.

Using antiserum to the pertussis toxin-sensitive G_i and G_o α subunits, histamine-, thrombin- and noradrenalin-stimulated arachidonate release was inhibited in rabbit platelets (Kajiyama *et al.*, 1990; Murayama *et al.*, 1990). A dominant-negative G_{i2} α subunit inhibited thrombin and purinergic receptor activation of $cPLA_2$, suggesting that it is this specific member of the G_i family which is involved (Gupta *et al.*, 1990). However, the very closely related member of this family, G_{i3} α subunit, has been shown to stimulate phospholipase A_2, leading to the activation of K^+ channels in epithelia (Cantiello *et al.*, 1990) and mediate the m2 muscarinic acetylcholine receptor-mediated arachidonate release (Hunt *et al.*, 1994). Agonist-stimulated arachidonate release has also been shown to be pertussis toxin-insensitive. This pertussis toxin-insensitive PLA_2 activity could be stimulated by calcium mobilization via receptor-stimulated, G_q-mediated PIC activation (Conklin *et al.*, 1992). Recently the first strong evidence for a direct involvement of G_{i2} in receptor stimulated arachidonic acid release was shown by Winitz *et al.*(1994). This group demonstrated that a genetic mutant of the G_{i2} α subunit (α_{i2}G203T) expressed in CHO cells, inhibited thrombin- and ATP-stimulated arachidonate release, but had no effect upon agonist-stimulated calcium mobilization. This mutant was engineered by mutating the conserved residue Gly203 to Thr203 of the G_{i2} α subunit. Gly203 corresponds to Gly 225 in the G_s α subunit which inhibited receptor-stimulated adenylyl cyclase when mutated to a Thr (Osawa & Johnson, 1991). It must be noted that this has yet to be repeated using other cell types and/or agonists.

$\beta\gamma$ subunits have been shown to be positive regulators of adenylyl cyclase, PIC-β1–3, phospholipase A_2, PtdIns-3 kinase and the β adrenergic receptor kinase (reviewed in Clapham & Neer, 1993). The interaction of $\beta\gamma$ subunits with their effectors has been suggested to be via plextrin homology (PH) domains (Inglese *et al.*, 1995) and as 71 proteins including phospholipases, kinases and cytoskeletal proteins, have been identified to contain PH domains (Auffray *et al.*, 1980), $\beta\gamma$ subunits could play a vast role in the regulation of intracellular activities . One pathway which has been demonstrated to be activated by $\beta\gamma$ subunits is MAP kinase, which is dependent

upon *ras* activation (Crespo *et al.*, 1994). The other major signalling kinase which may be activated by βγ subunits, is protein kinase C (PKC). Ron *et al.*(1994) isolated an intracellular receptor/docking protein for PKC, which enhanced PKC-stimulated Histone 1 phosphorylation when bound. As this receptor is a homolog of the β subunit of heterotrimeric G-proteins, it raises the possibility that the βγ subunits may act upon PKC directly.

1.3.3.2 Small molecular weight G-proteins

Mammalian cells contain at least 50 related small molecular weight GTP-binding proteins, including *ras*, *rab*, ARF, *rho* and *rac*, which regulate processes as diverse as cell replication and differentiation, cytoskeletal organization, secretion and endocytosis (for review see Glomset & Farnsworth, 1994; Hall, 1994). Their role in GTP-dependent signalling pathways can be distinguished from heterotrimeric G-proteins, as aluminium fluoride does not stimulate activity (Kahn, 1991). The best characterized of these are the *ras* proteins, which are involved in receptor-mediated signal transduction, such as EGF-stimulated arachidonate release in Rat-1 fibroblasts (Warner *et al.*, 1993) and direct activation of phosphatidylinositol-3 kinase (Stokoe *et al.*, 1994). The regulation of *ras*-mediated signalling is reviewed in Burgering & Bos (1995), but involves GTPase-activating proteins (GAP's) and guanine nucleotide-exchange factors (GEF's) (for review see Boguski & McCormick, 1993). Due to their low level of intrinsic GTPase activity, *ras* proteins are dependent on GAP's for deactivation. *ras*GAP proteins undergo a calcium-dependent translocation to cellular membranes mediated via a CaLB domain and are deactivated by phosphorylation upon tyrosine residues (Serth *et al.*, 1992). *ras*GEF exchanges bound GDP for free GTP, thus activating *ras*. In EGF-stimulated *ras* activity, this GEF has been identified as *sos* 1, which is found in the inactive form within the cytosol. *sos*-1 is translocated to the membrane by binding to *grb* 2, via SH3 domains, which then binds to activated EGF-receptors via SH2 domains (Li *et al.*, 1993). However, the subsequent activation of *ras* depends upon the prenylation state of the protein (Porfini *et al.*,

1994). The insulin receptor and non-receptor tyrosine kinases may also directly or indirectly phosphorylate *shc*, which has been shown to bind to the *grb* 2-*sos* 1 complex via SH2 domains, and so recruit it to the membrane and thus activate *ras* (Lai *et al.*, 1993; Skolnik *et al.*, 1993). The activity of this complex is inactivated by MAP kinase phosphorylation of *sos* 1 (Cherniack *et al.*, 1994) producing a negative feedback loop, as MAP kinase is activated via *ras*-dependent *raf-1* activation. This *ras*-dependent activation of MAP kinase has been demonstrated for both LPA (Howe & Marshall, 1993) and fMLP (Worthen *et al.*, 1994) and was initially thought to be mediated via a direct interaction of *ras* with *raf*. However, recently it has been demonstrated that *ras* activation is not required for *raf* activity, only for plasma membrane targeting of *raf*-1. This membrane associated *raf* is constitutively active (Stokoe *et al.*, 1994; Leevers *et al.*, 1994) when phosphorylated upon tyrosine residues (Marais *et al.*, 1995) and interacts with the cytoskeleton in a complex with various proteins including two chaperones hsp90 and p50 (Wartmann & Davis, 1994). The *ras*-mediated membrane localization of *raf* has been shown to be inhibited by increased levels of cAMP. This led to the suggestion that *raf* is phosphorylated by cAMP-dependent protein kinase (PKA), disrupting the docking complex, as *ras* activity is unaffected (Cook & McCormick, 1993; Hordijk *et al.*, 1994). One of the major substrates of MAP kinase is $cPLA_2$, which may have a role in prolonging *ras*-stimulated MAP kinase activity, as fatty acids have been shown to sustain a high level of GTP-bound *ras* (Alblas *et al.*, 1993; Bandyopadhyay *et al.*, 1995) perhaps by inhibiting the GTPase activating protein (Tsai *et al.*, 1989).

The vectorial movement of proteins between compartments of the exocytotic and endocytotic pathways of eukaryotic cells is mediated by carrier vesicles, that bud from a donor organelle and are targetted to and fuse with, the appropriate acceptor organelle. ARF, *rab*, and the *rho* family have been implicated in vesicular transport and are likely to control the assembly and disassembly of protein complexes, maintaining the structural and functional integrity of subcellular compartments (for review see Nuoffer & Balch, 1994).

ADP-ribosylation factor (ARF) was initially of interest due to the fact it stimulated ADP-ribosylation of the α subunit of G_s by cholera toxin (Kahn & Gilman, 1984). This small molecular weight GTP-binding protein was also found to regulate the reversible binding of cytosolic coat proteins to Golgi membranes (for review see Donaldson & Klauser, 1994) and nuclear/endosome fusion (Boman *et al.*, 1992; Taylor *et al.*, 1992). However, recently work has focused on its role in regulating PLD either directly or indirectly (Brown *et al.*, 1993; Zhang *et al.*, 1995).

rab was shown to be mainly involved in the process by which transport vesicles dock and/or fuse with their target membranes (for review see Pfeffer, 1994; Schell *et al.*, 1995). These are also regulated by both GAP and GEF proteins, but have an additional regulatory protein termed guanine nucleotide-dissociation inhibitor (GDI), which inhibits GDP/GTP exchange, preventing the GDP bound form from binding to membranes, where *rab* is normally found in complex with the GDI (Bollag & McCormick, 1991).

Another GDI has been identified which is specific for the *rho* family (Ohga *et al.*, 1989; Segal & Abo, 1993). This family of GTP-binding proteins have been shown to be involved in the organisation of the cytoskeleton and consists of nine proteins, including *rho* A, B and C as well as *rac* 1 and 2, which have 50-55% homology with each other and around 30% homology with *ras* (Ridley & Hall, 1993). In serum-starved Swiss 3T3 fibroblasts, there is almost a complete dissolution of the actin cytoskeleton and disassembly of focal adhesions, which leaves only a faint cortical ring of polymerized actin (Ridley & Hall, 1992). Upon addition of serum or growth factors such as LPA, the formation of stress fibres, focal adhesions and membrane ruffling are observed. By microinjection studies, it was shown that both stress fibre and focal adhesion formation was *rho*-dependent and membrane ruffling was *rac*-dependent (Ridley & Hall, 1992; Ridley *et al.*, 1992). The signal transduction pathways leading to stress fibre formation and membrane ruffling are of great interest (for review see Ridley, 1994). For stress fibre and focal adhesion formation, tyrosine kinases have been implicated (Ridley & Hall, 1994) as well as

kinases involved in the synthesis of phosphatidylinositol species, for example PtdIns-4 kinase (Chong *et al.*, 1994) and PtdIns-3 kinase (Zhang *et al.*, 1993). These were all thought to be mediated via *rho*, as ADP-ribosylation of *rho* by the botulinum C3 exoenzyme, inhibited LPA-induced protein tyrosine phosphorylation and PtdIns-3 kinase activation in cultured Swiss 3T3 fibroblasts (Kumagai *et al.*, 1993), correlating with the inhibition of LPA-stimulated stress fibre formation reported by Ridley & Hall (1992). The products and substrates of both PtdIns-3 and PtdIns-4 kinase have also been implicated in the uncapping of F-actin in permeabilized platelets, leading to actin assembly. However, this effect is mediated by *rac* and not *rho* (Hartwig *et al.*, 1995). This could also be mediated via the activation of a tyrosine kinase, as a serine/threonine protein kinase has been demonstrated to be activated by *rac*, termed p65, which has sequence homology to the putative protein product of *S. cerevisiae* STE20 gene. The product of this gene is required to transmit the pheremone signal from G-protein βγ-subunits to downstream components, including the tyrosine kinase $p120^{ACK}$ (Manser *et al.*, 1994). *rac* has mainly been studied in relation to its role in the NADPH oxidase complex in phagocytic cells (for review see Segal & Abo, 1993). The activation of this complex is dependent upon the presence of *rac*, but is not required to be translocated with the complex to the membrane, for activity. It is thus thought to play a role in the assembly/configuration of the active complex within the cytosol (Le Cabec *et al.*, 1994; Phillips *et al.*, 1995). Both *rac* and *rho* have been shown to activate MAP kinase cascades (Vojtek & Cooper, 1995), one substrate of which is $cPLA_2$. The metabolites of $cPLA_2$ activity have been implicated in EGF-stimulated actin remodelling in A431 cells, with leukotrienes being involved in cortical actin polymerization and prostaglandins being involved in the degradation of cytoplasmic actin filaments (Peppelenbosch *et al.*, 1993). This has been suggested to be due to the regulation of $cPLA_2$ by *rac*, as a constitutively active *rac* mutant, *rac*V12, generated leukotrienes in a growth-factor-independent manner (Peppelenbosch *et al.*, 1995). Therefore, this could be the pertussis-toxin insensitive route of agonist-stimulated arachidonic acid release.

1.3.4 Priming

Polymorphonuclear neutrophilic leucocytes or neutrophils play a critical role in the defence against infecting micro-organisms. Endocytosed bacteria are killed by the triggering of a non-mitochondrial oxidase and the subsequent production of a number of reactive oxygen species (Babior, 1984). In human neutrophils, the activated NADPH-oxidase is a multi-component electron transport chain composed of a membrane flavocytochrome b_{558}, containing haem, flavin and NADPH-binding sites (Rotrosen *et al.*, 1992) and 3 cytosolic components p47, p67 and *rac* (Abo *et al.*, 1991). This $p21^{rac}$ (Didsbury *et al.*, 1989) was purified in a complex with rhoGDI (GDP-dissociation inhibition factor)(Fukumoto *et al.*, 1990). The activation of the oxidase has been shown to be mediated by phosphorylation and arachidonic acid (Dana *et al.*, 1994). Translocation of *rac* to the membrane maybe involved, however, GTPγS-stimulated NADPH-oxidase activity has been observed without this movement of *rac* (Phillips *et al.*, 1995). The membrane composition may play a role in the regulation of NADPH-oxidase, since both phosphatidic acid (Qualliotine-Mann *et al.*, 1993) and DAG (Park & Babior, 1993) activate NADPH-oxidase, suggesting roles for PLD, PKC or membrane fluidity changes.

The Respiratory burst is a two-step process involving an initial priming phase followed by a subsequent triggering event. There are a number of physiologically important primers, including IL-1, IL-6, interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α)(Berkow *et al.*, 1987; Worthen *et al.*, 1988; Borish *et al.*, 1989; Humphreys *et al.*, 1989; Yuo *et al.*, 1990; Sample & Czuprynski, 1991; Yuo *et al.*, 1991) and the neurotransmitter undecapeptide substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly=Leu-Met-NH_2)(Perianin *et al.*, 1989; Wozniak *et al.*, 1989; Lloyds & Hallett, 1993). 'Priming' of neutrophils leads to an exaggerated oxidase response to agonists such as the peptide fMLP, the complement fragment C5a, interleukin-8 (IL-8) and platelet activating factor (PAF) (Phillips & Hamilton, 1989; Phillips & Hamilton, 1990). The mechanisms by which priming occurs are as yet unclear.

A number of studies have implicated a rise in intracellular calcium in the mechanism of priming (Kuhns *et al.*, 1988; Forehand *et al.*, 1989; Koenderman *et al.*, 1989), which could induce translocation of PKC to the cell membrane (Phillips *et al.*, 1989). Later, this same group reported an increase in the expression of an uncharacterized isozyme of PKC, in response to various priming agents (Phillips *et al.*, 1992). Priming has also been shown to occur in a calcium-independent manner (Berkow *et al.*,1987; Naccache *et al.*, 1988; Karnad *et al.*, 1989; Lloyds & Hallett, 1993). The most recent studies into the mechanism of priming have suggested a role for tyrosine phosphorylation. Substance P, TNFα and GM-CSF stimulate tyrosine phosphorylation in neutrophils (McColl *et al.*, 1991; Grinstein & Furuya, 1992; Lloyds *et al.*, 1993), which can be mimicked by the tyrosine phosphophosphatase inhibitor, orthovanadate (Swarup *et al.*, 1982). This artificial elevation of tyrosine phosphorylation has been shown to prime both neutrophils and differentiated HL60 cells (Lloyds *et al.*, 1993; Lloyds & Hallett, 1994; Lloyds and Hallett, 1995). This group suggest that a 74kDa tyrosine phosphorylated protein is involved in this tyrosine phosphorylation-mediated priming response. However, this has still to be identified. The main enzyme known to be activated by a tyrosine kinase cascade is MAP kinase (Thompson *et al.*, 1993). TNFα may prime by activating the sphingomyelin pathway to stimulate ceramide-activated protein kinase, which activates *raf*-1 by phosphorylating Thr 269 (Yao *et al.*, 1995). One of the major substrates of MAP kinase is $cPLA_2$ (Clark *et al.*, 1991), which has been shown to be translocated to a membrane compartment by the priming agent GM-CSF in human neutrophils (Durstin *et al.*, 1994). This priming agent increases MAP kinase activity and produces a small $cPLA_2$ response on its own, however, it potentiates the arachidonate release to a subsequent fMLP stimulation. This could be due to the priming agent causing translocation of $cPLA_2$ to a membrane compartment, with the subsequent fMLP-stimulated calcium release activating the enzyme. This maximum activation of the enzyme is not seen in response to GM-CSF, perhaps since this agonist does not stimulate a rise in intracellular calcium concentrations. Therefore, tyrosine

phosphorylation caused by the priming agents may be involved in a relocation of the enzyme of interest into a more enzymatically favourable enviroment and/or confirmation.

Another factor which has been shown to enhance many physiological responses in neutrophils is cytochalasin B (CytB)(Honeycutt & Niedel, 1986). The action of CytB is poorly understood, but is most likely to manipulate the cytoskeletal network (for review see Cooper, 1987). This compound is a member of a family of cell permeable, fungal metabolites, which inhibit cell ruffling and stimulate adherent cells to round up (Schliwa, 1982; Yahara *et al.*, 1982). These effects are thought to be due to cytochalasins binding to the 'barbed' end of actin filaments and "capping" them. Actin-binding proteins (for review see Weeds, 1982), such as profilins (Reichstein & Korn, 1979), cap the 'barbed' end of the actin filaments causing monomer dissociation from the 'pointed' end, to establish a new monomer:polymer equilibrium (Kirschner, 1980). At physiological KCl and with $MgCl_2$ in excess of 1mM, actin exists predominately in the polymerised form, with a very small (<1μM) concentration of free monomer. The 'capping' effect results in inhibition of actin filament growth, producing shortened filaments and a weakened cytoskeleton (Tilney & Inoue, 1982).

1.3.5 Substrate regulation of $cPLA_2$ activity

The detailed characterization of the kinetic properties of $cPLA_2$ is complicated by the fact that binding of $cPLA_2$ to the lipid-water interface must occur prior to catalysis. To study the affinity of the enzyme for its substrate and the factors in the membrane which may affect it, various *in vitro* assays have been utilised. $cPLA_2$ is expressed as an activated phosphoprotein in SF9 cells (de Carvalho *et al.*, 1993). Using this it was found that mixed liposomes consisting of the synthetic negatively charged phospholipid dimyristoyl-glycero-phosphomethanol (PM) with 3-5 mol% of natural phospholipids, bound $cPLA_2$ irreversibly, but did not produce linear, time-dependent phospholipid hydrolysis (Diez *et al.*, 1992). These non-linear kinetics can

be explained by the reaction products of $cPLA_2$ preventing the dissociation of the enzyme from the water/substrate interphase, therefore restricting its access to new substrate. In an *in vivo* system, these products would be metabolized, possibly via the enzyme's intrinsic lysophospholipase activity, as discussed in the following section.

However, lipid moieties do play a role in $cPLA_2$ regulation as it was shown that incorporation of long-chain unsaturated diacylglycerols into substrate phospholipid liposomes, markedly enhance $cPLA_2$ activity (Dawson *et al.*, 1983; Dawson *et al.*, 1984). These DAGs promote alterations in the structure of phospholipid membranes (Das & Rand, 1986), rendering them more susceptible to phospholipase degradation. Anionic phospholipids were also shown to enhance $cPLA_2$-hydrolysis of PtdCho liposomes, with phosphatidylinositol-4,5-P_2 producing the most pronounced effect (Leslie & Channon, 1990). This may act in a similar manner to the synthetic substrate PM, i.e. mediating the tight association of $cPLA_2$ with the substrate and thus lowering the concentration of calcium required for maximum activation.

All of this evidence demonstrates that the composition of the membrane and the lipid products generated upon cell activation via PIC and/or PLD hydrolysis may regulate $cPLA_2$ activity.

1.4 ROLE OF PHOSPHOLIPASE A_2 PRODUCTS

Phospholipids are hydrolyzed to produce free fatty acids and lysophospholipids, both of which have been suggested to have a role in cellular responses.

1.4.1 Lysophospholipids

Lysophospholipids have been shown to play an important role in the pathogenesis of a broad spectrum of diseases and have been associated with myocardial infarctions, infections, neoplasia and asthma (Weltzien, 1979; Bar-sagi &

Feramisco, 1986; Mock & Man, 1990; Hall & Laubach, 1991; Langton & Cesaveo, 1992). The least active of the lysophospholipids is lysophosphatidylethanolamine (LysoPE), which appears to be utilized in the ethanolamine cycle to resynthesize phosphatidylethanolamine (PtdEth), which is found in high concentrations in platelets, but in very low amounts elsewhere. Lysophosphatidylserine (LysoPS) is involved in the synthesis of PtdCho by enhancing the CDP-choline pathway (Bruni *et al.*, 1992). One of the most studied lysophospholipids is lysophosphatidic acid (LPA), which has a myriad of effects as described in section 1.6.3. Finally, lysophosphatidylcholine (LysoPC) has been shown to be a stereospecific chemoattractant for mouse lymphoblastic cells (Hoffman *et al.*, 1982), human monocytes (Quinn *et al.*, 1988) and has been postulated to be generated at sites of inflammation, tissue injury and repair. Golan *et al.*(1986) demonstrated that the parasite *Schistosoma mansoni* utilises LysoPC to lyse adherent human red blood cells and to fuse to human neutrophils. The mechanism by which this extracellular LysoPC mediates many of its effects is yet unclear, but Besterman & Domanico (1992) have postulated the possible mechanisms and kinetics.

Given the varied functions of lysophospholipids, the concentrations of these lipids must be regulated. It has been shown that there is a vast number of lysophospholipases, whose activity is increased in the protection of cells from the cytotoxic effects of lysophospholipids (Weller *et al.*, 1984), in attenuation of signal transduction processes (Asaoka *et al.*, 1992) and in host-mediated destruction of parasites (Golan *et al.*, 1986). These lysophospholipases have been isolated from a number of different tissues and cell types, including brain (Gross & Sobel, 1991), porcine gastric mucosa (Sunaga *et al.*, 1995) and neutrophils (Laubach & Hall, 1991). Of major interest are the proteins which have been identified to contain both lysophospholipase and phospholipase A_2 activities, such as cytosolic phospholipase A_2. In view of the proposed role of this enzyme in the regulation of non-esterified arachidonate by its phospholipase A_2 activity (discussed in section 1.4.2), the lysophospholipase activity, which was 10-fold higher than that of PLA_2 *in vitro*, may

serve to remove the other product of this activity, the lysophospholipid (Leslie, 1991; Pind & Kuksis, 1991). She *et al.*(1994) demonstrated that $cPLA_2$ was able to hydrolyse all lysoPC species, i.e. with oleoyl, palmitoyl, myristyl or stearoyl in the *sn*-1 position, 1-oleoyl-lysoPS and 1-oleoyl-lysoPA. However, any lysophospholipase activity was abolished when the *sn*-2 positon was acetylated.

1.4.2 Free fatty acids produced by $cPLA_2$

1.4.2.1 From lysophospholipase activity

This activity hydrolyses the fatty acid at the *sn*-1 position, which is normally saturated, of the lysophospholipid produced after the phospholipase A_2 hydrolysis. As described above this activity hydrolyses all the lysoPC substrates, releasing myristate, stearate, oleate and palmitate. More oleate is produced from the hydrolysis of lysoPS and lysoPA. These saturated fatty acids play a role in the homeostasis of the cell, but are probably utilized to a greater extent in the reacylation of cellular phospholipids via an acyl-CoA synthetase activity with a broad acyl specificity (Chern & Kinsella, 1983), which has been identified in liver and adipose tissue (Normann *et al.*, 1981).

1.4.2.2 From phospholipase A_2 activity

Cytosolic phospholipase A_2 activity hydrolyzes the *cis*-unsaturated fatty acid at the *sn*-2 position. This particular enzyme has a preference for arachidonic acid (C20:4(n-6)), which can account for as much as 25% of the total phospholipid fatty acid content (Buttke *et al.*, 1985) and is principally synthesized in the liver from dietary linoleic acid (Habenicht *et al.*, 1990). Arachidonate is transported to various cell types by serum albumin or lipoproteins and the serum levels are low in comparison to other fatty acids. The transported arachidonic acid is incorporated into membrane phospholipids, which requires conversion of the fatty acid into arachidonyl-coenzyme A (AA-CoA) by arachidonyl-CoA synthetase, and its

subsequent esterification by arachidonyl-lysophospholipid transferase. Electron microscopy autoradiography has been used to study the distribution of arachidonate in $HSDM_1C_1$ murine fibrosarcoma cells (Neufeld *et al.*, 1985). At steady states of labelling, i.e. after 24 hours, the plasma membrane had obtained a specific activity similar to that of the endoplasmic reticulum, which contained more than half of the cell-associated arachidonate at all times. Thus, the ER is a major site for both incorporation and storage of arachidonate. Mitochondria were labelled less intensely than other membranes, while both the nuclear membrane and other cytoplasmic structures were highly labelled.

Arachidonyl-CoA synthetase has been demonstrated in mouse peritoneal macrophages (Fernandez *et al.*, 1992), brain (Jakschitz *et al.*, 1983) and platelets (Wilson *et al.*, 1982), which specifically activates arachidonate (20:4(n-6)), 8,11,14-eicosatrienoate (20:3(n-9)) and 5,8,11-eicosatrienoate (20:3(n-9)) (Banerjee & Rosenthal, 1985). These fatty acids can inhibit this enzyme when in excess, which would suggest that this enzyme is the rate limiting step of arachidonate incorporation. However, at physiological arachidonate concentrations, this enzyme is able to keep the level of free arachidonate low, thus minimizing basal synthesis of eicosanoids (Neufeld *et al.*, 1983). These eicosanoids are metabolites of arachidonic acid which is released from intracellular phospholipids upon agonist stimulation. It has been suggested that arachidonate acts as an intracellular signal, independent of eicosanoid production, as it has been shown to activate calcium channels in ventricular myocytes (Huang *et al.*, 1992), release calcium from intracellular stores (Currie *et al.*, 1992) and inhibit the expression of stearoyl-CoA desaturase 2 (SCD2) gene (Tebbey & Buttke, 1993).

The eicosanoids are produced from the metabolism of arachidonic acid via the cyclooxygenase, lipoxygenase and epoxygenase enzymes. Cyclooxygenase or prostaglandin G/H synthase, which catalyzes the conversion of arachidonate into two reactive intermediates, prostaglandin G and H, which are precursors of the prostaglandins (PG's), prostacyclin (PGI_2) and thromboxane (TXA_2). Two cDNA's

encoding cyclooxygenase have been isolated from cDNA libraries of sheep seminal fluid. One has a 2.8kDa mRNA, which may have splice variants (Diaz *et al.*, 1991) and appears not to have a role in the inflammatory process. The second cDNA is a 4.1kDa mRNA, the level of which rapidly increases in human monocytes stimulated with pro-inflammatory agents, such as interleukin 1β and decreases in response to anti-inflammatory agents such as dexamethasone (O'Banion *et al.*, 1991; O'Banion *et al.*, 1992). The protein expressed by this 4.1kDa mRNA is identical to TIS10, the product of a primary response gene, which supports the role of arachidonate metabolism in the regulation of cell division (Kujubu *et al.*, 1991; Kujubu & Herschman, 1992). The role of cyclooxygenase products as mediators of cell division was supported by the demonstration that EGF-stimulated prostaglandin E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) generation in BALB/c3T3 fibroblasts, EGF-dependent DNA synthesis and c-*myc* expression, could be inhibited by specific cyclooxygenase inhibitors (Handler *et al.*, 1990). However, the products of the cyclooxygenase pathway may also act transcellularly via specific membrane receptors. A high-affinity receptor for TXA_2 has been identified (Hirata *et al.*, 1991) along with a PGE_2 receptor, which inhibits adenylyl cyclase (Sugimoto *et al.*, 1992).

The second pathway of arachidonic acid metabolism is the lipoxygenase pathway, which produces hydroperoxyeicosatetraenoic acids (HPETE), which undergo a complex metabolism to the corresponding hydroxyacids (HETE) or leukotrienes. Leukotriene B_4 (LTB_4) has been shown to be an important modulator of immune cell function and is one of the most powerful chemokinetic and chemotactic agents known (Rola-Pleszczynski, 1989). High- and low-affinity receptors have been identified, which appear to mediate signal transduction pathways via a GTP-binding protein (Mong *et al.*, 1986). The leukotrienes and other lipoxygenase products have been shown to activate K^+ channels in a variety of cell types (Margalit & Livne, 1991) and modulate hormone and neurotransmitter secretion (Freeman *et al.*, 1991; Bordeau *et al.*, 1992; Landt *et al.*, 1992). It has also been suggested that other polyunsaturated fatty acids, such as linoleic, may be metabolized via this enzyme pathway and play a

role in mitogenesis (Glasgow & Eling, 1990). Of interest, is that members of both the cyclooxygenase (Rollins & Smith, 1980) and the lipoxygenase (Woods *et al.*, 1995) pathways have been detected at the nuclear membrane and the endoplasmic reticulum.

A further pathway is the epoxygenase enzymes, which include the cytochrome p450 enzyme. This pathway catalyzes the conversion of arachidonate into epoxyeicosatrienoic acids (ETT), which are hydrolyzed to corresponding diols by epoxide hydrolase (Needleman *et al.*, 1986). The products of this pathway are potent inhibitors of the Na^+/K^+ ATPase activity (McGiff, 1991), which supplies the driving force for transcellular transport of electrolytes and organic solutes, playing a central role in the reabsoptive capacity of the kidney.

1.4.3 Regulation of PKC by phospholipase A_2 activation

McPhail *et al.* (1984) initially demonstarted that PKC could be activated by *cis*-unsaturated fatty acids. Further investigations showed that *cis*-unsaturated fatty acids, which are normally located in the *sn*-2 position of the diacylglycerol backbone, such as oleic, lioleic, linolenic, arachidonic and docosahexanoic acids, greatly enhanced the diacylglycerol (DAG)- and phorbol ester-dependent activation of PKC (Seifert *et al.*, 1988; Shinomura *et al.*, 1991; Chen & Murakami, 1992). This enhancement of these responses by *cis*-unsaturated fatty acids is similar to that seen in the differentiation of HL60 cells to macrophages (Asaoka *et al.*, 1993) and the glutamate release from isolated nerve terminals (Herrero *et al.*, 1992). However, *cis*-unsaturated fatty acids cannot activate PKC alone, but only in the presence of DAG or phorbol ester, suggesting that these fatty acids prime PKC, by either changing the conformation of the enzyme or manipulating the lipid enviroment to one that produces a higher activity. The other product of phospholipase A_2 activity, lysophospholipid, has a very similar role in the regulation of PKC. Lysophosphatidylcholine (LysoPC) the product of PC hydrolysis by PLA_2 has some biological activities such as enhancement of sustained Na^+ current (Undrovinas *et al.*, 1992), chemotaxis (Quinn *et al.*, 1988), smooth muscle relaxation (Saito *et al.*, 1988) and induction of EGF-like growth factor in

monocytes (Nakano *et al.*, 1994), but cannot activate PKC alone. Only when added to intact cells together with a membrane-permeant DAG or phorbol ester, LysoPC significantly potentiates subsequent cellular responses, such as T-lymphocyte activation (Berry & Nishizuka, 1990) and HL60 cell differentiation (Asaoka *et al.*, 1992).

1.5 INHIBITION OF PHOSPHOLIPASE A_2 ACTIVITY AND CONSEQUENCES TO THE CELL

Type II or non-pancreatic secreted phospholipase A_2 is secreted from platelets, mast cells and other cell types, exerting its actions in the degradation of foreign bacteria (Elsbach & Weiss, 1993), the release of histamine from mast cells (Murakami *et al.*, 1992a) and arachidonate release from macrophages and endothelial cell lines (Murakami *et al.*, 1993; Barbour & Dennis, 1993). Pancreatic secretory phospholipase A_2 or Type I appears to have a proinflammatory role mediated via a specific receptor, leading to a rise in prostaglandins (Arita & Hanasaki, 1993). The calcium-independent phospholipase A_2 may be involved in the degradation of membranes that accompanies myocardial infarction (Gross, 1992) and in the liberation of free arachidonate in hormone-stimulated aortic smooth muscle cells (Lehman *et al.*, 1993). Finally, cytosolic phospholipase A_2 functions in a receptor-mediated intracellular signal transduction pathway, leading to the rapid release of arachidonate from membrane phospholipids and the biosynthesis of eicosanoids (Kramer *et al.*, 1993a). Therefore, possible phospholipase A_2 inhibitors would have a great therapeutic value.

The analysis of PLA_2 inhibition is more difficult than for most enzymes, as the enzymatic hydrolysis of naturally occurring, long-chain phospholipids necessarily occurs at the lipid/water interface (for review see Gelb *et al.*, 1994). Inhibition of the secretory PLA_2's by various compounds has been extensively studied. Various compounds have been isolated from marine organisms, which inhibit both Type I and II phospholipase A_2 activities (Potts *et al.*, 1992). These naturally occuring products

do not resemble the substrate of the enzyme, unlike phospholipid analogues which have a phosphonate or phosphate in the place of the ester at the *sn*-2 position of the phospholipid. The analogues are tight-binding inhibitors of $sPLA_2$, but have no effect on cytosolic phospholipase A_2 (Jain *et al.*, 1991; Baybunt *et al.*, 1993). However, an analogue of arachidonic acid in which the COOH group is replaced with a trifluoromethyl ketone ($COCF_3$), is a tight binding, reversible inhibitor of $cPLA_2$. This compound ($AACOCF_3$) is more than 1000-fold less potent as an inhibitor of $sPLA_2$'s, probably due to the non-specificity exerted by these enzymes for the fatty acid at the *sn*-2 position (Street *et al.*, 1993). Utilization of this inhibitor demonstrated that $cPLA_2$ plays an important role in the generation of thrombin-stimulated arachidonate release (Bartoli *et al.*, 1994), which results in the inhibition of 12-HETE biosynthesis in human platelets (Riendeau *et al.*, 1994). NMR studies suggested that $AACOCF_3$ forms a hemiketal adduct with an active site nucleophile [enzyme-X-$C(OH)(CF_3)$-AA](Trimble *et al.*, 1993).

In considering the factors that determine the magnitude of enzyme-inhibitor dissociation constants, one must examine not only the interactions of the inhibitor and the enzyme, but also the interactions that the inhibitor forms with its enviroment, when not bound to the enzyme. Some drugs, such as Chlorpromazine, interact with phospholipids and exert their actions by blocking the interaction of proteins with their lipid substrates or activators. Chlorpromazine has been demonstrated to inhibit transcription initiation in Ehrlich ascites tumour cells, by blocking the interaction of PLA_2 with anionic phospholipids (Hirai *et al.*, 1991). The PLA_2 affected here has still to be determined as crude lysates were used, which has also been the problem with a further group of PLA_2 inhibitors. These inhibitors include α-Tocopherol, which has antioxidant properties in biological membranes, where it prevents the peroxidation of membrane lipids (Burton *et al.*, 1986). Potentially, the effects of tocopherols on eicosanoid synthesis may be limited by their poor solubility in aqueous solutions. When the phytol chain was shortened, the hydrophobicity of these molecules was increased, corresponding to an increase in the inhibition of

prostaglandin production. This effect is not due to an inhibition of the cyclooxygenase pathway, but by a direct or indirect inhibiton of phospholipase A_2 (Pentland *et al.*, 1992). Indirect inhibition of PLA_2 has also been shown to occur in human neutrophils, pretreated with BIRM270 (Farina *et al.*, 1994) and in bombesin-stimulated Swiss 3T3 fibroblasts treated with Demethoxyviridine (DMV) and wortmannin (Cross *et al.*, 1995).

DMV and wortmannin belong to the viridine family of steroidal antibiotics (for review see Hanson, 1995) and were shown to inhibit bombesin-stimulated arachidonate release in Swiss 3T3 fibroblasts *in vivo*, but had only a small (30%) effect upon baculovirus expressed or immunoprecipitated $cPLA_2$ activity. Wortmannin has been shown to inhibit various agonist-stimulated responses, including insulin- and serum-stimulated glycogen synthase kinase-3 (GSK-3) and c-*raf* activity, resulting in MAP kinase inhibition. However, this data was interpreted to be due to the specific inhibition of phosphatidylinositol-3 kinase (PI-3 kinase), by this compound and not to a direct inhibition of the enzyme being studied. PtdIns 3-kinase is not activated by bombesin in Swiss 3T3 fibroblasts and it has been shown that c-*raf*, an upstream activator of MAP kinase, cannot be activated by phosphatidylinositol-3,4,5-P_3, the product of PI-3 kinase activity (Cross *et al.*, 1994). Therefore, much of the data published based on the specific inhibition of PI-3 kinase by wortmannin, must be re-examined.

1.6 AGONISTS STUDIED

1.6.1 Bombesin

Bombesin is a tetrapeptide isolated from frog skin and has many biological roles. The mammalian equivalents of this peptide are gastrin release peptide (GRP) and neuromedin B (NMB), which have been shown to play a role in neurotransmission (Minamino *et al.*, 1985), exocrine secretion (Knigge *et al.*, 1984), to act as growth factors for human foetal (Spindel *et al.*, 1987) and small cell lung carcinoma (Cuttitta *et al.*, 1985), as well as be a potent mitogen for Swiss 3T3 fibroblasts *in vitro* (Takuwa *et al.*, 1991).

Bombesin binds to a single class of high affinity receptors (Brown *et al.*, 1988; Sinnett-Smith *et al.*, 1988; Battey *et al.*, 1991) and is a member of the G-protein linked, seven transmembrane spanning receptor superfamily. Three subtypes of this receptor have been identified, however, there appears to be a preference for one specific subtype in any specific cell type (Schrenck *et al.*, 1989; Shapira *et al.*, 1991), with Swiss 3T3 fibroblasts expressing a GRP receptor subtype as determined by using a specific GRP receptor antagonist (Shapira *et al.*, 1991).

The bombesin receptor in Swiss 3T3 fibroblasts is linked to a pertussis toxin insensitive G-protein, which may be a member of the G_q/G_{11} family (Fischer & Schonbrunn, 1988). This group also demonstrated that occupation of this receptor activated PIC, which could lead to a rise in intracellular calcium concentrations via inositol-1,4,5-P_3 sensitive pools. The transient rise in inositol-1,4,5-P_3 in response to bombesin has been well characterised (Takuwa *et al.*, 1987; Cook *et al.*, 1990; Plevin *et al.*, 1990). Cook *et al.*(1990) demonstrated that bombesin stimulated a biphasic diacylglycerol (DAG) response, with the initial phase corresponding to inositol-1,4,5-P_3 generation produced from PIC-stimulated PtdIns-4,5-P_2 hydrolysis and the second phase has been proposed to be from either PLC- (Muir & Murray, 1987) or PLD-mediated (Cook & Wakelam, 1989) hydrolysis of phosphatidylcholine. This DAG activates protein kinase C (PKC) as described in section 1.3.2.1, with

evidence that this PKC activity maybe involved in the expression of the cellular oncogenes c-*fos* and c-*myc* (Bravo *et al.*, 1987).

Bombesin may have a role in cAMP accumulation in Swiss 3T3 fibroblasts, but this has still to be clarified (Fischer & Schonbrunn, 1988; Millar & Rozengurt, 1988; Takuwa *et al.*, 1991). Another regulatory pathway stimulated by bombesin is tyrosine phosphorylation (Cirrillo *et al.*, 1986), which has been shown to have a major role in bombesin-stimulated PLD activation (Briscoe *et al.*, 1995) and in the regulation of cytosolic phospholipase A_2 as discussed in section 1.3.2.2.

1.6.2 Formyl-methyl-leucine-phenylalanine (fMLP)

The neutrophil plays a critical role in nonspecific host defence by responding to chemoattractants, such as formyl-Methyl-Leucine-Phenylalanine (fMLP) (a peptide derived from a bacterial chemoattractant (Collins, 1987)) and C5a (a peptide derived from the C5 complement factor)), leading to accumulation of neutrophils at an inflammatory site (Sandborg & Smolen, 1988).

The mechanism by which neutrophils respond to fMLP, is mediated via a specific seven transmembrane-spanning, G-protein-linked receptor (Thomas *et al.*, 1990). This receptor has been shown to exist in both a low- and high-affinity state (Fay *et al.*, 1991; Posner *et al.*, 1994; Prossnitz *et al.*, 1995a), with only the high affinity, liganded state interacting with a pertussis toxin-sensitive G-protein (Schreiber *et al.*, 1993a), via the second intracellular loop and the carboxyl-terminal domain (Prossnitz *et al.*, 1993; Schreiber *et al.*, 1993b). This carboxyl-terminal domain is a substrate for G-protein coupled receptor kinase 2, which may play a role in desensitization of the fMLP-receptor (Prossnitz *et al.*, 1995b).

The G-protein involved in the fMLP response was found to be G_{i2} (Bokoch & Gilman, 1984) and has been shown to mediate adenylyl cyclase inhibition (Gilman, 1987). The α-subunit of G_{i2} can activate potassium ion channels (Yatani *et al.*, 1988) and $\beta\gamma$-subunits have been shown to regulate specific isoforms of PICβ (Camps *et al.*, 1992). The other phospholipases, i.e. PLA_2 and PLD, have also been shown to be

regulated by GTP-binding proteins and both are activated by fMLP (Cockcroft, 1992), however, whether it is G_{i2} involved has still to be elucidated (Cockcroft & Stutchfield, 1989).

Recently it has been demonstrated that fMLP stimulated tyrosine phosphorylation (Grinstein & Furuya, 1992; Thompson *et al.*, 1993; Torres *et al.*, 1993), which led to increased MAP kinase activity. This produced a flurry of interest in the pathway leading from the fMLP receptor occupation and MAP kinase activation. Grinstein *et al.*(1994) highlighted the involvement of MEK-1 and Worthen *et al.*(1994) demonstrated the activation of *ras* and *raf*-1 by fMLP. This *raf*-1 activation is not via PKC, as a potent PKC inhibitor had no effect on fMLP-stimulated *raf*-1 activity (Toullec *et al.*, 1991). Therefore, the activation of MEK-1 in response to tyrosine kinase-linked receptors (Lange-Carter *et al.*, 1993), appears to be involved in fMLP-stimulated MAP kinase activation. G_{i2}-mediated activation of *ras* and *raf*-1 has been demonstrated in Rat-1a cells transfected with the m2 muscarinic receptor (Winitz *et al.*, 1993), Rat-1 cells transfected with the α2-adrenergic receptor (Alblas *et al.*, 1993) and fibroblasts stimulated with lysophosphatidic acid (Howe & Marshall, 1993). However, evidence for a direct involvement of a tyrosine kinase in *ras* or *raf* activation by seven transmembrane-spanning receptors in neutrophils is currently lacking.

1.6.3 Lysophosphatidic acid

The best known examples of phospholipid-derived signalling molecules include diacylglycerol (DAG), inositol-1,4,5-trisphosphate (Ins-1,4,5-P_3) and prostaglandins, which are all rapidly generated when cells are stimulated by certain growth factors and hormones. The simplest naturally occuring phospholipids, phosphatidic acid (PA) and lysophosphatidic acid (LPA), are of particular interest in that they are not only critical intermediates in *de novo* lipid biosynthesis (for review see Bishop & Bell, 1988) and are rapidly produced in activated cells but can also stimulate cell proliferation when added to appropriate target cells in culture.

In agonist-stimulated cells, it has been shown that the level of PA rapidly rises within the plasma membrane. PA can be generated via phosphorylation of newly formed diacylglycerol (DAG) by DAG kinase (Kanoh *et al.*, 1990) or more directly, through the action of cellular phospholipase D (PLD) acting on phosphatidylcholine (PC) and perhaps also phosphatidylethanolamine (PE) (Bocckino *et al.*, 1987; Exton, 1990). The generation of LPA during cell activation has been less thoroughly examined, however, it has been shown that LPA accumulates rapidly in thrombin-stimulated platelets (Lapetina *et al.*, 1981; Watson *et al.*, 1985; Gerrard & Robinson, 1989). The production of LPA may be secondary to PA formation through the activation of a PA-specific phospholipase A_2, which has been identified in platelets (Billah *et al.*, 1981).

In addition to being rapidly produced in stimulated cells, PA and LPA can exert their own biological effects upon target cells. Tokumura *et al.*(1978) demonstrated effects on blood pressure of intravenously administered soybean lecithin, of which LPA was shown to be the active compound. Subsequent studies demonstrated that LPA was in fact a contractile agonist on smooth muscle (Tokumara *et al.*, 1980). Both lipid moieties can stimulate thymidine incorporation in Rat-1 fibroblasts (Van Corven *et al.*, 1992) and LPA is as potent as EGF or 10% foetal calf serum. This effect on cell proliferation appears to be highly specific and dependent upon the acyl chain length of the LPA species. It has been shown that the long-chain LPA species, such as $C_{18:1}$, are more active than the short chain analogues (Van Corven *et al.*, 1992). This would be consistent with a model in which the degree of partitioning into the lipid bilayer is a major determinant of mitogenic potency of these phospholipids. However, the partitioning of LPA into the lipid bilayer cannot fully explain the specific intracellular signalling pathways stimulated by this phospholipid.

Nanomolar concentrations of LPA have been shown to induce transient increases in intracellular calcium concentrations in many cell types, including Rat-1 fibroblasts (van Corven *et al.*, 1989), A431 carcinoma cells (Moolenaar *et al.*, 1986), platelets (Watson *et al.*, 1985), PC12 cells (Dyer *et al.*, 1992) and *Xenopus laevis*

oocytes (Durieux *et al.*, 1992; Fernhout *et al.*, 1992; Ferguson & Hanley, 1992; Tigyi and Miledi, 1992). LPA has also been shown to induce an increase in overall tyrosine phosphorylation (Hordijk *et al.*, 1994; Seufferlein & Rozengurt, 1994) and more specifically, increases the phosphorylation of focal adhesion kinase ($p125^{FAK}$) (Saville *et al.*, 1994). This latter finding may be involved in the observation, that LPA induced rapid formation of actin stress fibres and assembly of focal adhesions in Swiss 3T3 fibroblasts, which was shown to be inhibited by the microinjection of the low molecular weight G-protein, rho (Ridley & Hall, 1992; Barry & Critchley, 1994). Further evidence indicating a positive role for G-proteins in LPA signalling, is that LPA inhibited forskolin-stimulated cAMP accumulation, DNA synthesis (van Corven *et al.*, 1989) and $p21^{ras}$ activation was sensitive to pertussis toxin (van Corven *et al.*, 1993). Plevin *et al.*(1991) demonstrated that GTPγS potentiated LPA-stimulated phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) hydrolysis and GDPβS abolished the response. This evidence led to the possibility that LPA-stimulated intracellular signalling pathways were mediated via a G-protein-linked, specific cell surface receptor. However, the existence of a membrane receptor for the compound has been difficult to prove, as the lipophilic nature of LPA causes unacceptably high background in binding assays. Therefore, a radiolabelled, photoactivateable crosslinking LPA analogue, [^{32}P] diazirine-LPA, which is less lipopholic, was utilized and found to label a plasma membrane protein with an apparent molecular mass of 38kDa (van der Bend *et al.*, 1992). However, the identification of this protein has yet to be published. Even though this putative receptor has yet to be identified, the evidence supporting the existence of a receptor-mediated LPA response outweighs the support for the detergent-like properties of LPA stimulating the activities. For example, LPA does not permeabilize cells as measured by Ca^{2+} leakage, even at millimolar concentrations (Jalink *et al.*, 1990) and several cell types, such as fresh human neutrophils (Jalink *et al.*, 1990) and K562 cells (a human erythroleukaemia line) (Durieux *et al.*, 1993), are unresponsive to LPA, which would not be expected of a nonspecific detergent.

1.9 AIMS

The aim of this study was to determine the physiological regulation of stimulated cytosolic phospholipase A_2 and to examine the role of priming in cell responsiveness.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 CELL LINES AND MATERIAL

2.1.1 Cell lines

Swiss mouse 3T3 fibroblasts

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

Human promyelocytic leukemic HL60 cells

Obtained from Dr George Birnie, Beatson Institute for Cancer Research, Bearsden, Glasgow, G61 1BD.

Rat-1 and Raf ER4 Rat-1 fibroblasts

Kindly donated by Dr.S.Cook, Onyx Pharmaceuticals, Richmond, CA., USA.

2.1.2.Materials

The following is a list of sources of the materials used during the course of this project.

Affiniti Research Products Ltd., Nottingham, England.

Monoclonal anti-MAP kinase antibody.

Amersham International plc, Buckinghamshire, England

[5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (214.3Ci/mmol)

[1-^{14}C]arachidonic acid (55mCi/mmol)

L-3-Phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl (55.6mCi/mmol)

[9,10-^{3}H] oleic acid (2-10Ci/mmol)

[2-^{3}H] *myo*-inositol (10-20Ci/mmol)

Rainbow Markers

Enhanced chemiluminescence (ECL) reagent kit

Sheep anti-mouse HRP-linked antibody

BDH Chemical company, Poole, England

HEPES

Biorad Laboratories Ltd., Biorad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD

Analytical Grade Anion Exchange Resin, AG 1-X8, 200-400 mesh

Boehringer Mannheim (UK) Ltd., Lewes, England

Bovine serum albumin (fraction V)

ATP

GDPβS

GTPγS

Wortmannin

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

BAPTA/AM

Microcystin LR

Pertussis Toxin

Canberra Packard, Pangbourne, England

Ultima Flo A.F. scintillation fluid

Costar, Cambridge, MA., USA

Nitrocellulose

Difco Laboratories, Detroit, Michigan, USA

Streptolysin O

Fisons scientific apparatus, Loughborough, England

Magnesium chloride, sodium chloride, glycine, Tris, sodium dodecyl sulphate, D-glucose, potassium chloride

Gibco Life Technologies, Paisley, Scotland

Glutamax, heat-inactivated foetal calf serum, newborn calf serum, sodium bicarbonate (7.5%), Dulbecco's Modified Eagle's Medium with Glutamax (DMEM), RPMI 1640 with Glutamax, RPMI 1640 (phenol red free) with Glutamax.

J.& W. Scientific, 91 Blueravine Road, Folsom, CA95630, USA.

DB-23 Gas chromatography column

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 greatfully acknowledged.

SAPU, Law Hospital, Law, Scotland

Goat Serum

Sheep Serum

Whatman Ltd., Maidstone, England

PE SIL G polyester backed Silica G TLC plates

P81 paper

Filter paper

All other chemicals were obtained from the Sigma Chemical Co., Poole, England.

Rabbit anti-cPLA$_2$ antibody and partially purified cPLA$_2$ was kindly donated by Dr.C.Jackson, Fisons Pharmaceuticals, Loughborough, England.
Mouse anti-MAP kinase antibody (ERK 16) was kindly donated by Dr.J.Tavare, Department of Biochemistry, University of Bristol, Bristol.

2.2 BUFFER COMPOSITION

Phosphate buffered saline (PBS)

146mM sodium chloride, 5.4mM potassium chloride, 9.6mM di-sodium hydrogen orthophosphate, 1.5mM potassium di-hydrogen orthophosphate.
PBS was adjusted to between pH7.2-7.4 with 1M sodium hydroxide and 1M hydrochloric acid.

Hank's buffered saline

1.26mM calcium chloride, 5mM potassium chloride, 0.5mM magnesium chloride, 0.85mM magnesium sulphate, 137mM sodium chloride, 4mM sodium hydrogen carbonate, 0.27mM sodium di-hydrogen phosphate.

When freshly prepared, the pH was adjusted to between 7.2-7.4.

Low calcium (150nM) Hank's buffered saline (Hanks)

1.26mM calcium chloride, 0.5mM magnesium chloride, 0.9mM magnesium sulphate, 5.37mM potassium chloride, 137mM sodium chloride, 4.2mM sodium hydrogen carbonate, 0.35mM sodium di-hydrogen phosphate, 2.5mM EGTA

When freshly prepared, the pH was between 7.2-7.4

Low calcium Hank's buffered saline with glucose and BSA (lcHBG)

Hanks was prepared as above and 10mM D-glucose, 20mM Hepes and 0.1% (w/v) BSA (fraction V) added and the pH was adjusted to 7.4.

DMEM with glucose and BSA (DBG)

Powdered Dulbecco's modified eagle's medium was reconstituted in distilled water as per manufacturers instructions and 10mM D-glucose (final concentration), 0.1% (w/v) BSA (fraction V), 20mM Hepes, 0.375% (w/v) sodium bicarbonate added. The pH was adjusted to 7.4 at 37°C.

RPMI 1640 with glucose and BSA (RBG)

RPMI 1640 with or without phenol red with 10mM D-glucose (final concentration), 0.1% (w/v) BSA (fraction V), 20mM Hepes, 0.375% (w/v) sodium bicarbonate added. The pH was adjusted to 7.4 at 37°C.

Permeabilisation buffer

20mM Hepes, 120mM potassium chloride, 6mM magnesium chloride, 2mM potassium di-hydrogen orthophosphate, 0.1mM EGTA, 61μM calcium chloride (0.5mM calcium), 2.5mM ATP and 1mg/ml BSA.

The pH was adjusted to 7.4 with 1M potassium hydroxide.

2.3 CULTURE AND PRESERVATION OF CELL LINES.

2.3.1 Routine culture

Cells were incubated at 37°C, in a humified atmosphere of air/CO_2 (19:1). Cells were discarded at passage 14 or if they exhibited aberrant or transformed morphology.

Adherent cell lines

Swiss 3T3 mouse and Rat-1 fibroblasts were maintained in Dulbecco's modified eagle's medium with Glutamax containing 10% (v/v) newborn calf serum. Rat-1 Raf ER4 fibroblasts were maintained in this medium supplemented with 400µg/ml Geneticin.

Suspension cell lines

Human promyelocytic leukemic HL60 cells were maintained in RPMI 1640 medium with Glutamax, containing 15% (v/v) heat-inactivated foetal calf serum, at a density of $2x10^5$ cells per ml medium.

2.3.2 Cell passage

Adherent cell lines

Sterile trypsin for cell passage

0.1% (w/v) Trypsin, 0.85mM EDTA, 10mM glucose was prepared in PBS (pH7.4). Aliquots were filtered and stored at -20°C.

When no greater than 70% confluent, the cells were passaged at a ratio of 1 to 5.

Suspension cells

HL60 cells were passaged every 3 days when the density was normally $1x10^6$ cells per ml (determined using a haemocytometer). A specific volume of cell suspension was removed and added to fresh medium to give a final cell density of $2x10^5$ cells per ml.

2.3.3 Cryogenic preservation of cell lines

Adherent cells

Cells were removed from the flask surface by trypsinisation. Swiss 3T3 and Rat-1 fibroblasts were then resuspended in medium containing 20% (v/v) newborn calf serum and 8% (v/v) DMSO in DMEM with Glutamax. 1ml aliquots containing approximately 10^7 cells were frozen in cryogenic freezing vials at -20°C for 2 hours and then left overnight at -80°C, before being submerged in liquid nitrogen. Rat-1 Raf ER4 cells were treated exactly as Swiss 3T3 fibroblasts, but they were resuspended in foetal calf serum containing 10% DMSO (v/v), after trypsinisation. They were aliquoted and frozen as described.

Suspension cells

The cell density of the HL60 cells was determined and then the cell suspension was centrifuged at 100xg for 3mins. The cell pellet was then resuspended in medium containing 20% (v/v) foetal calf serum and 8% (v/v) DMSO in RPMI 1640 with Glutamax, to give a density of 10^7 cells per ml. The cells were then aliquoted into cryogenic freezing vials and frozen down exactly as described for the adherent cells.

Cells brought up from storage in liquid nitrogen were thawed rapidly at 37°C and the contents of one vial was added to 10ml of the appropriate complete medium in a 25ml centrifuge tube. The cells were then pelleted by centrifugation at 100 xg for 3 minutes. The supernatant was discarded and the cells resuspended in fresh complete medium. This washing process was repeated 3 times and the adherent cells were

resuspended in 5ml of the appropriate complete medium and transferred to a 25cm^2 tissue culture flask and incubated overnight.

In the case of the suspension cells, the final pellet was resuspended in 10ml of fresh medium and transferred to a 80cm^2 tissue culture flask and incubated. After 24 hours, the adherent cells were either passaged into 80cm^2 flasks, or fed with fresh medium.

2.4 MEASUREMENT OF ARACHIDONIC ACID GENERATION

2.4.1 Preparation of samples for radioactive measurements

Adherent Cells

Swiss 3T3 fibroblasts were seeded at a density of 2x10^4 cells per ml into 24 well plates in DMEM containing 10% (v/v) NBCS. When the cells were between 80-90% confluent, the media was removed and replaced with DMEM containing either 2% (v/v) or 10% (v/v) NBCS and 0.5μCi [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid per well. Rat-1 and Raf ER4 Rat-1 fibroblasts were seeded at 1x10^4 cells per ml in DMEM containing 10% (v/v) NBCS and 400μg/ml Geneticin in the case of the Rat-1 Raf ER4 fibroblasts. When between 80-90% confluent, the media was aspirated and replaced with DMEM containing 0.5μCi [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid per well.

After the labelling period of 24-36 hours, cells were washed 3 times with 0.5ml DBG containing 0.1% BSA (w/v) (FractionV), 20mM Hepes (pH7.4) and 10mM glucose at 37°C for a total of 45mins. 200μl of agonist, which was diluted in DBG containing 0.1% (w/v) BSA, was used for the stimulations as described. For control time points, buffer alone was added.

To terminate the reaction, when measuring intracellular arachidonate release, the media was aspirated and 500μl ice-cold methanol:acetic acid (100:1.5) was added. There was no initial aspiration when the total arachidonate level was being studied. The wells were then scraped using a wax-filled pipette tip and the suspension added to

a 2ml glass vial on ice. The wells were washed with a further 200µl methanol:acetic acid (100:1.5), which was transferred to the glass vial. Lipids were extracted by the addition of 350µl chloroform and 4µg arachidonic acid. Vials were vortexed and either left at room temperature for 30min or overnight at -20°C.

Following extraction, the phases were split by adding 350µl of chloroform and 630µl, in the case of intracellular arachidonate samples, or 430µl, in the case of total arachidonate measurements, of water to each vial and vortexed. The samples were then centrifuged at 1000 xg for 5min. The upper aqueous phase and the interface of cell debris was removed and the lower, organic phase was dried down under vacuum in a vacuum centrifuge. The samples were then returned to ice, ready for thin layer chromatography.

Suspension Cells

HL60 cells were seeded at $5x10^5$ cells per ml in RPMI 1640 with glutamax medium containing 15%(v/v) heat-inactivated foetal calf serum (HIFCS) and 1.3% (v/v) DMSO. They were allowed to differentiate for 3 days before 0.25µCi per ml [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid was added for 17 hours.

To remove labelling medium, cells were pelleted by centrifugation at 100 xg for 3 minutes. The cell pellet was resuspended in RPMI 1640 containing 0.1% BSA (w/v) (fraction V) and incubated for 2 hours. The cells were pelleted again and resuspended to a density of $1x10^7$ cells per ml in RPMI 1640 containing 0.1% BSA (w/v), 20mM Hepes (pH7.4) and 10mM Glucose. 100µl of the cell suspension was added to 2ml glass vials at 37°C. Agonists were made up in RPMI 1640 containing 0.1% BSA (w/v), 20mM Hepes (pH7.4) and 10mM Glucose. The same buffer was added for the control time points. 50µl of agonist or buffer was added and the suspension agitated and incubated for the desired time. Reactions were terminated by adding 700µl methanol:acetic acid (100:1.5) and incubation on ice. Lipids were extracted by the addition of 350µl chloroform and 4µg arachidonic acid. Vials were vortexed and left either at room temperature for 30min. or overnight at -20°C.

After extraction, phases were split by the addition of 350µl chloroform and a volume of water, which when added to the volume of the reaction volume, gave 630µl total. The vials were then centrifuged at 1000 xg for 5 mins and the upper and inter-phase removed. The lower phase was dried down in a vacuum centrifuge in preparation for thin-layer chromatography.

2.4.2 Thin Layer Chromatography

Samples were resuspended in 30µl chloroform:methanol (2:1) and spotted onto Silica Gel, PE SIL G polyester backed plates which had been heat activated at 120°C for 30 min. A further 15µl was then used to wash out the vials and this was spotted onto the silica plate. The plates were developed in a pre-equilibrated thin-layer chromatography tank containing hexane:diethylether:acetic acid(glacial) (70:30:2 v/v). When the solvent front was at the top of the plates, they were removed and allowed to air dry, before being placed in an iodine tank to visualise the arachidonic acid.

The iodine-stained arachidonate spot was identified and each spot was marked in such a way that an equal area was outlined. The plates were then placed face down over a steam bath, in order to get rid of any final traces of iodine and to soften the silica. The silica spots were excised from the plate and placed in a scintillation vial. 1ml of methanol was added to the silica and left for 15minutes, before 10ml scintillation fluid was added. The scintillation vials were then capped, vortexed and placed in the dark overnight, before the radioactivity content was determined using liquid scintillation spectrophotometry.

2.5 WESTERN BLOT ANALYSIS

2.5.1 $cPLA_2$ LOCATION AND PHOSPHORYLATION STATE

2.5.1.1 Preparation of samples

Adherent cells

Swiss 3T3 fibroblasts were grown in 80cm^2 flasks in DMEM containing 10% (v/v) newborn calf serum until 80-90% confluent. After this time the medium was changed to contain either 10% (v/v) or 2% (v/v) NBCS and left for 24 hours, to synchronise both populations into the G_o/G_1 phase of the cell cycle. This occured by either contact inhibition for the cells in high serum or by serum starvation as in the case of those in DMEM containing 2% (v/v) NBCS.

Rat-1 fibroblasts were grown in 80cm^2 flasks in DMEM containing 10% (v/v) NBCS until 80-90% confluent. The medium was then changed to serum-free DMEM for 24 hours.

Raf ER4 Rat-1 fibroblasts were grown in 80cm^2 flasks in DMEM containing 10% (v/v) NBCS and 400µg/ml geneticin until 80-90% confluent. The medium was then changed to serum-free DMEM for 24 hours.

For experiments, the cells were washed 3 times with DBG containing 0.1% (w/v) BSA at 37°C in a total of 45minutes, before treatment with the test reagent. Reactions were terminated by media aspiration and 2 rapid washes with ice-cold PBS, before adding 5ml PBS and placing on ice. The cells were scraped into the PBS and the cell suspension transferred to a 25ml centrifuge tube. The flask was then washed with a further 5ml PBS which was added to the cell suspension.

Suspension cells

HL60 cells were seeded at 5x10^5 cells per ml in RPMI 1640 medium containing Glutamax, 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO and differentiated for 4 days. The cells were pelleted by centrifugation at 100

xg for 3 minutes and resuspended in 20ml RPMI 1640 containing 0.1% (w/v) BSA, 10mM glucose and 10mM Hepes (pH7.4). This washing procedure was repeated, before the cell pellet was resuspended to a density of $1x10^7$ cells per ml. 1ml cell suspension was incubated at 37°C in a 15ml centrifuge tube. Test reagents were added as indicated, with gentle agitation after each addition. Reactions were terminated by addition of 10ml ice-cold PBS and transfer to ice.

2.5.1.2 Lysis procedure

The cells were pelleted by centrifuging at 1000xg for 10minutes. The supernatant was discarded and the pellet resuspended in 0.5ml of ice-cold Lysis buffer 1 (20mM Hepes(pH7.4), 2.5mM EGTA, 20mM PMSF, 2mM orthovanadate, 40μg/ml leupeptin and 40μg/ml aprotinin) and placed on ice for 1 hour. The cell suspension was homogenised in a 1ml glass-on-glass homogeniser. The homogenate was then transferred to an eppendorf tube and spun at 50xg for 5 minutes to pellet the nuclei and any unbroken cells. The supernatant was then transferred to 1ml ultracentrifuge tubes and centrifuged at 150000xg for 30minutes.

The supernatant was carefully removed and transferred to an eppendorf, to which 0.5ml ice-cold lysis buffer 2 (lysis buffer 1 plus 100mM NaF, 10mM $MgCl_2$, 2mM EDTA, 2mM NaCl, 80mM NaPPi and 2% (w/v) Triton X-100) was added and the samples vortexed, before being stored on ice. The membrane pellet was resuspended in 0.5ml lysis buffer 3 (lysis buffer 1 with 50mM NaF, 5mM $MgCl_2$, 1mM EDTA, 1mM NaCl, 40mM NaPPi and 1% (w/v) TritonX-100) transferred to an eppendorf tube and vortexed. Both the cytosolic and membrane fractions were then sonicated in a sonicating water bath for 5 minutes to ensure solubilisation. Insoluble material was pelleted by centrifugation at 4°C at 14000 xg for 15mins and the supernatants were transferred to fresh eppendorf tubes. Lysates were stored at -80°C. The protein concentrations of the lysates were determined using the micro BCA protein assay kit.

2.5.1.3 TCA precipitation of samples for western blot analysis.

The protein content of each lysate was adjusted to that required in 0.95ml distilled water. 10µl of 2% (w/v) deoxycholate was added and the samples vortexed. The proteins were then precipitated by the addition of 0.25ml of 24% (w/v) trichloroacetic acid (TCA) and incubation at 4°C for 15 minutes. The proteins were then pelleted by centrifuging at 14000 xg for 20 minutes at 4°C. The supernatant was removed and 20 µl of 2M Tris/HCl (pH 8.3) and Lamelli (30% (w/v) urea, 5% (w/v) SDS, 6% (w/v) DTT, 20mM Tris/HCl (pH8.3) and 0.2% (w/v) bromophenol blue) was added to each pellet. The samples were vortexed and allowed to solubilise for at least one hour at room temperature. The samples were pulsed in a microcentrifuge in preparation for SDS-PAGE.

2.5.1.4 SDS-PAGE for $cPLA_2$

Phosphorylated and non-phosphorylated $cPLA_2$ were separated on 22x16cm, 1.5mm thick, vertical polyacrylamide gels using 12.5% "low bis" polyacrylamide resolving gels as kindly explained by Dr.Lin-Ling Lin (Genetics Institute, Boston, MA, USA). These gels have the following constituents :

30% (w/v) Acrylamide	18.66ml
1% (w/v) Bisacrylamide	4.50ml (0.1% final)
1.5M Tris/HCl (pH 8.3)	11.00ml
10% (w/v) SDS	0.45ml
Water	9.23ml
10% (w/v) ammonium persulphate (APS)	220µl
TEMED	25µl

Prior to the addition of the 10% (w/v) APS and the TEMED the pH was always checked to be pH 8.3, as this is critical for the separation of phosphorylated and non-phosphorylated forms of $cPLA_2$. Immediately after preparation, the resolving gel was poured into the gel apparatus and overlaid with 0.1% (w/v) SDS.

When polymerised, the overlay was removed and 3% polyacrylamide stacking gels (pH 6.8) were poured and the comb positioned.

2.5.1.5 SDS-PAGE gel electrophoresis

Polymerised gels were assembled into the gel apparatus as described by the manufacturers and the wells filled with running buffer (25mM Tris, 190mM Glycine, 0.1% (w/v) SDS). Samples were loaded into the wells using a Hamilton syringe and run at 35 mAmps, 60 volts per gel for 36 hours until the 30kDa marker had run off the resolving gel. 20µg of $cPLA_2$ partially purified protein was also loaded.

2.5.1.6 Immuno-blotting of $cPLA_2$

After electrophoresis the higher molecular weight proteins (200-68kDa) were transferred to nitrocellulose by wet blotting in a buffer containing 25mM Tris, 190mM Glycine, 0.1% (w/v) SDS and 20% (v/v) methanol, according to manufacturers instructions. Gels were routinely stained with Coomassie-blue stain (500ml distilled water, 400ml methanol, 100ml acetic acid and 0.1% (w/v) coomassie blue) and destained (500ml distilled water, 400ml methanol and 1100ml acetic acid) subsequent to blotting, to check for efficiency of transfer.

The nitrocellulose was rinsed briefly in NaTT (150mM NaCl, 20mM Tris/HCl pH 7.2. 0.1% (v/v) Tween 20), then blocked for 4 hours at 4°C in NaTT containing 5% (w/v) non-fat milk (NFM) and 5% (v/v) goat serum. The blot was then incubated overnight at 4°C or for 2 hours at room temperature in NaTT containing 1% (w/v) NFM, 1% (v/v) goat serum and a 1:10,000 dilution of rabbit anti-$cPLA_2$ antibody. After incubation with first antibody, the blot was washed in NaTT containing 1% (w/v) NFM for 2 hours, changing the wash buffer frequently. The nitrocellulose was then incubated at 4°C for 1.5 hours in NaTT supplemented with 1% (w/v) NFM and a 1:2000 dilution of goat anti-rabbit IgG linked to horseradish peroxidase and pre-absorbed with human serum proteins (IgG-HRP). The blot was then repeatedly washed over 2 hours with NaTT. The horseradish peroxidase labelled proteins were

detected using enhanced chemical luminescence (ECL). Luminescence was detected by autoradiography film for times ranging from 10 seconds to 10 minutes.

2.5.2 MAP KINASE DETECTION AND PHOSPHORYLATION STATE

2.5.2.1 Preparation of cell lysates

Adherent cells

Swiss 3T3 and Rat-1 Raf ER4 fibroblasts were grown in 6-well tissue culture plates and quiesced as described in section 2.4.1. After agonist stimulation, the reactions were terminated by washing twice with ice-cold PBS. To 5 wells of each plate, 0.5ml Laemilli buffer was added. The remaining well of each tissue culture plate was used for the determination of protein content, by incubating with 1ml PBS containing 0.1% (v/v) NP 40 at 37°C for 1 hour. The samples were then transferred to eppendorf tubes and sonicated in a water bath for 5 minutes. Any insoluble material was removed by centrifuging at 14000xg for 15 minutes. Protein content was determined using the micro BCA protein assay kit.

Suspension cells

HL60 cells were grown at a density of $5x10^5$ cells per ml in RPMI medium containing 15% (v/v) HIFCS and 1.3% (v/v) DMSO for 5 days. Cells were pelleted and resuspended in RPMI containing 0.1% (w/v) BSA and 10mM glucose, to a density of $1x10^7$ cells per ml. 1ml of cell suspension was added to 15ml centrifuge tubes and stimulated. The reaction was terminated by the addition of 10ml ice-cold PBS and transfer to ice. Cells were pelleted by centrifugation and the supernatant discarded. To each pellet, 0.5ml Lamelli buffer was added and the samples solubilised at 37°C for 1 hour. One pellet was used for protein determination as described above. Insoluble material was pelleted by centrifugation.

2.5.2.2 SDS-PAGE for MAP Kinase

SDS-PAGE analysis of cell lysates was performed in vertical 1.5mm thick, polyacrylamide gels, with the size and constituents of the gels being identical to those for $cPLA_2$ described in Section 2.5.1.5. A volume of sample corresponding to 50µg total protein was added to each well and the gels run for 36 hours at 35mA, 60 volts per gel or until the 30kDa marker had run off the resolving gel.

2.5.2.3 Immuno-blotting of SDS-PAGE gels for MAP kinase

After electrophoresis the lower molecular weight (68-30kDa) proteins were transferred to nitrocellulose by wet blotting in buffer containing 25mM Tris, 190mM Glycine, 0.1% (w/v) SDS and 20% (v/v) methanol, according to manufacturers instructions. Gels were routinely stained with Coomassie-blue stain subsequent to blotting, to check the efficiency of transfer.

The nitrocellulose was rinsed briefly in NaTT (150mM NaCl, 20mM Tris/HCl pH 7.2. 0.1% (v/v) Tween 20), before blocking at 4°C for 4 hours in NaTT containing 3% (w/v) BSA. Nitrocellulose was then incubated overnight at 4°C or for 2 hours at room temperature, with a 1:500,000 dilution of a monoclonal anti-ERK 1/2 antibody in NaTT containing 1% BSA (w/v). Membranes were then washed extensively for a total of 2 hours in NaTT, prior to a 2 hour incubation at 4°C with a 1:10,000 dilution of a sheep anti-mouse IgG linked to horseradish peroxidase in NaTT. The membranes were then washed for 2 hours in NaTT, with the wash buffer being changed frequently. The horseradish peroxidase linked proteins were then detected using ECL. Luminescence was detected by autoradiography for times ranging from 10 seconds to 10 minutes.

2.5.3 DETECTION OF TYROSINE PHOSPHORYLATED PROTEINS

2.5.3.1 SDS-PAGE gel preparation for Tyrosine phosphorylation

The preparation of samples for tyrosine phosphorylation detection was identical to that described in Section 2.5.2.1. SDS-PAGE analysis of samples carried out on vertical, 14x16cm, 1.5mm thick, 10% (w/v) polyacrylamide gels, which comprised of:

30% (w/v) acrylamide : 0.8% (w/v) bisacrylamide	8.0ml
1.5M Tris/HCl : 0.4% (w/v) SDS (pH 8.8)	6.0ml
50% (v/v) Glycerol	1.6ml
Water	8.2ml
10% (w/v) APS	90µl
TEMED	8µl

Prior to the addition of the 10% (w/v) APS and the TEMED the pH was readjusted to 8.8. Immediately after preparation, the resolving gel was poured into the assembled gel apparatus and overlaid with 0.1% (w/v) SDS. Following polymerisation, the overlay was removed and a 3% (w/v) polyacrylamide stacking gel (pH 6.8) was poured and the comb positioned.

Polymerised gels were assembled and the wells filled with running buffer (25mM Tris, 190mM glycine, 0.1% (w/v) SDS). A volume of sample corresponding to 50µg total protein was loaded and the gels run overnight at 12mA, 30volts per gel until the dye front reached the end of the resolving gel.

2.5.3.2 Immunoblotting of SDS-PAGE gels for Phosphotyrosine

After electrophoresis, the proteins were transferred to nitrocellulose by wet-blotting with efficiency of transfer being checked, as previously described.

The nitrocellulose was rinsed briefly in NaTT, then blocked for 4 hours in NaTT containing 3% (w/v) BSA. The blot was then incubated overnight at 4°C in NaTT containing 3% (w/v) BSA and a 1:5000 dilution of PY54 antibody. After incubation with the first antibody, the blot was washed in NaTT for 2 hours, changing the wash regularly. The nitrocellulose was incubated at 4°C for 1 hour in NaTT containing 3% (w/v) BSA and a 1: 10,000 dilution of sheep anti-mouse IgG linked to horseradish peroxidase and pre-absorbed with human serum proteins. The blot was then washed repeatedly over the next 2 hours with NaTT.

ECL was used to detect horse radish peroxidase linked proteins, with luminescence being detected by autoradiography for times ranging from 10seconds to 10 minutes.

2.6 MAP KINASE *IN VITRO* ACTIVITY MEASUREMENTS

2.6.1 Preparation of samples

Adherent cells

Swiss 3T3 and Rat 1 Raf ER4 fibroblasts were seeded into 60x15mm^3 petri dishes at 2x10^4 cells per ml, cultured and quiesced as described in Section 2.5.1.1.

On the day of the experiment, the petri dishes were washed 3 times for a total of 30 minutes at 37°C with DBG. Stimulations were carried out as described and reactions terminated by washing twice with ice-cold PBS and transfer to ice. The PBS was replaced with 0.5ml Lysis buffer (1mM potassium dihydrogen phosphate, 1mM EDTA, 10mM $MgCl_2$, 50mM β-glycerophosphate, 5mM EGTA, 1mM sodium orthovanadate, 40μg/ml aprotinin, 40μg/ml leupeptin, 0.1mM PMSF and 0.5% (w/v) Triton X-100) and incubated on ice for 1 hour. The dishes were scraped and the suspension transferred to eppendorf tubes, which were vortexed and centrifuged at

14000 xg for 15 minutes to pellet insoluble material. The supernatant was transferred to a fresh eppendorf and the protein content of each sample determined using the BCA protein assay kit. A volume corresponding to 50µg of total protein was then removed to assay MAP kinase activity.

Suspension cells

HL60 cells were seeded at $5x10^5$ cells per ml, maintained and differentiated as described in Section 2.5.1.2. The cell density was determined using a haemocytometer and the cells washed twice in RBG. The final washed pellet was resuspended in the RBG at a density of $2x10^7$ cells per ml and 100µl of this suspension was incubated at 37°C in eppendorfs. Agonists were diluted in RBG and stimulations carried out as described. Reactions were terminated by washing twice in 1ml ice-cold PBS, with the final washed pellet resuspended in 0.5ml lysis buffer and incubated on ice for 1 hour. The samples were vortexed and centrifuged at 14000xg for 15minutes to pellet insoluble material. The supernatant was transferred to fresh eppendorfs and the protein content determined using the BCA protein assay kit. A volume corresponding to 50µg total protein was transferred to a clean eppendorf, ready for MAP kinase activity measurements.

2.6.2 MAP kinase activity measurements

The samples containing 50µg protein were made up to 500µl with lysis buffer, to which 5µl of rabbit anti-ERK 1/2 was added and allowed to mix for 2 hours at 4°C. The immunocomplexes were captured by addition of protein-G to the samples and mixing for 1 hour at 4°C. Protein G complexes were pelleted by centrifuging at 14000 xg for 1minute. The pellet was then washed twice in buffer A (1mM Tris/HCl [pH7.2], 0.5% (w/v) sodium deoxycholate, 100mM NaCl, 1mM EDTA, 1mM sodium orthovanadate and 1% (w/v) Triton X-100), twice in buffer B (1mM Tris/HCl [pH7.2], 1M NaCl, 1mM sodium orthovanadate and 0.1% (w/v) Triton X-100) and once in buffer C (5mM Tris/HCl [pH7.2] and 150mM NaCl).

To the final pellet, 20µl assay buffer (50mM MOPS (pH7.4), 3.75mM EGTA, 25mM $MgCl_2$, 0.25mM sodium orthovanadate, 2µM IP_{20} and 5µM microcystin LR) was added along with 0.5mg myelin basic protein in a final total volume of 45µl. The total reaction mixture was then mixed gently and pelleted by pulsing in a bench top microfuge. All additions were carried out at 4°C.

Reactions were initiated by the addition of 5µl 0.5mM ATP containing 5µCi [γ-^{32}P] ATP, mixing and incubating at 30°C for 10minutes. Reactions were terminated by the addition of 50µl ice-cold 2M HCl and tubes were transfered to ice. The reaction mixture was pelleted by centrifugation at 14000xg for 1 minute at 4°C. The samples were then carefully resuspended and 50µl of each suspension spotted onto P81 paper, which was carefully washed with a total of 1 litre of 150mM orthophosphoric acid, with the wash being changed frequently. Finally, the papers were washed briefly in 100% ethanol and allowed to air dry. The samples had been spotted onto defined areas, which were removed and added to scintillation vials along with 1ml of methanol. After 30minutes at room temperature, 10ml of scintillation fluid was added, the vials vortexed and placed in the dark overnight, before the radioactivity was determined by liquid scintillation spectrophotometry.

2.7 CYTOSOLIC PHOSPHOLIPASE A_2 *IN VITRO* ACTIVITY MEASUREMENTS.

2.7.1 Preparation of substrate

A stock of liposome was prepared as follows:

167.5µg stearoyl-arachidonyl phosphatidylcholine, 2µCi stearoyl-[^{14}C] arachidonyl phosphatidylcholine, 8µg phosphatidylinositol-4,5-bisphosphate (PIP_2) and 29µg 1-stearoyl-2-arachidonyl glycerol (DAG) were mixed and dried under a stream of nitrogen. 15ml 50mM Tris/HCl (pH 7.4) was then added and the solution vortexed for 30 seconds, prior to freezing in dry ice for 15 minutes.

Prior to use, the substrate was thawed whilst sonicating in a sonicating water bath at room temperature and quickly vortexed.

2.7.2 Assay procedure

The liposomes were diluted to a final volume of 0.5ml in 100μM KCl, 10μM $CaCl_2$ and 50mM Tris/HCl (pH7.4) to give a liposome containing 7μM 1-stearoyl-2-arachidonyl phosphatidyl choline (containing [^{14}C] in the two position), 2.3μM 1-stearoyl-2-arachidonyl glycerol and 0.3μM phosphatidylinositol-4,5-bisphosphate. The liposome mixture was added to round bottom glass tubes and placed on ice. The liposomes were then pre-incubated for 5 minutes at 37°C in the presence of inhibitor or vehicle.

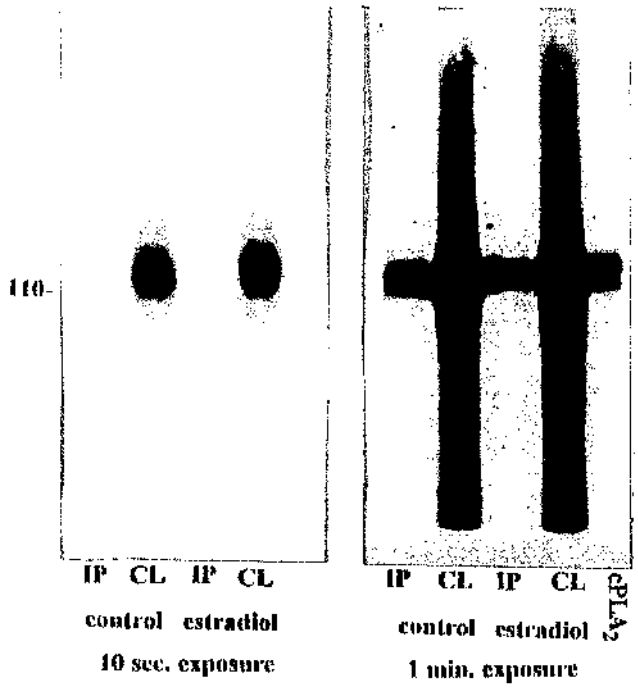
The reaction was initiated by the addition of either partially purified cytosolic phospholipase A_2 from platelet cytosol, total cell lysate from Swiss 3T3 fibroblasts or immunoprecipitated $cPLA_2$ from Swiss 3T3 fibroblasts. Immunoprecipitation was carried out as described in section 2.6.2, using a 1:10 dilution of rabbit anti-$cPLA_2$ antibody. After washing, the final pellet was resuspended in 50μl 50mM Tris/HCl (pH7.2) and added to the prepared liposomes. This immunoprecipitation isolated both phosphorylated and non-phosphorylated $cPLA_2$ as shown in figure 2.7.2. Upon addition of enzyme, the samples were vortexed and returned to 37°C for a further 5 minutes. The reaction was terminated by the addition of 700μl ice-cold methanol:acetic acid (100:1.5) and transfer to ice.

350μl of chloroform was added and the samples vortexed. They were then left at room temperature for 30 minutes to allow extraction of the lipids into the organic solvent. The phases were split by the addition of 350μl of chloroform, 130μl of water, vortexing and centrifuging at 1000xg for 5 minutes. 600μl of the lower organic phase was then transferred to a 2ml glass vial and dried in a vacuum centrifuge. Free [^{14}C]arachidonate was isolated by thin-layer chromatography and quantified by liquid scintillation spectrometry as previously described in Section 2.4.2.

Figure 2.7.2 Immunoprecipitation of cytosolic phospholipase A_2

Rat-1 Raf ER4 fibroblasts were grown in 80cm^2 flasks in DMEM containing 10% (v/v) NBCS and 400mg/ml geneticin until 80-90% confluent. The medium was then changed to serum-free DMEM for 24 hours. Quiesced cells were washed twice with DBG and incubated with either DBG or 1mM β-estradiol for 1 hour at 37°C. Termination of reaction and preparation of lysates as described in sections 2.5.1.1 and 2.5.1.2. 200μg total protein was used for immunoprecipitation as described in section 2.6.2. Immunoprecipitated (IP) $cPLA_2$ was utilised as described in section 2.7.2 with 50μg of total protein being used for cell lysate (CL) experiments. Result is representative of 2 individual experiments.

Immunoprecipitation of cPLA$_2$ from control and estradiol-treated Rat-1 Raf ER4 fibroblasts



2.8 MEASUREMENT OF CALCIUM RELEASE

2.8.1 Adherent cells

Swiss 3T3 and Rat-1 Raf ER4 fibroblasts were seeded onto a coverslip in a $35x10mm^2$ petri dish, at a density of $1x10^5$ cells per ml, cultured and quiesced as described in Section 2.5.1.1. Prior to stimulation, the Rat-1 Raf ER4 cells were treated with or without 1μM β-estradiol in HBG at 37°C for 1 hour.

All cells were transferred to HBG prior to loading with Indo-1-AM, which was added at 37°C for 20 minutes, at a final concentration of 1μM. After this time, the coverslips were transferred to a fluorescent microscope stage, linked to a laser which monitored the fluorescence at 351nm and 363nm, corresponding to Ca^{2+}-bound and -unbound Indo-1. The cells were submerged in HBG until the cells to be monitored were selected. The HBG was then removed and the agonist perfused on for a total of 5 minutes. The change in the ratio of Ca^{2+}-bound and -unbound Indo-1 in the cells selected was monitored and recorded.

2.8.2 Suspension Cells

HL60 cells were seeded at $5x10^5$ cells per ml, cultured and differentiated as described in Section 2.5.1.2. The cells were washed twice in RBG and the cell density determined using a haemocytometer. The washed pellet was resuspended to a density of 10^7 cells per ml in RBG and incubated with 2μM Fura-2-AM at 37°C for 45 minutes. The cells were washed twice in RBG and resuspended at a density of $5x10^6$ cells per ml. Agonists to be tested were reconstituted to the required concentration in RBG.

1ml of cell suspension was preincubated with RBG or RBG containing 5μM cytochalasin B or 0.5mM pervanadate at 37°C in a fluorimeter which monitored the excitation wavelengths 340nm and 380nm, corresponding to Ca^{2+}-bound and -unbound Fura-2 respectively. The emission wavelength was 510nm. Agonists were

added through a porthole, therefore reducing any effects of stray light upon the fluorescence.

Incubations proceded for between 5-20minutes, with the ratio of the Ca^{2+}-bound and -unbound fluorescence being recorded. To quantitate the amount of Ca^{2+} released, the total calcium concentration in the cells was measured by lysing with Triton X-100. A background level was determined by chelating the total calcium with excess EGTA. Using these values, the actual amount of Ca^{2+} released could be determined using the software supplied by the manufacturer.

2.9 CONFOCAL MICROSCOPY

2.9.1 Preparation of samples

Adherent cells

Swiss 3T3 and Rat-1 Raf ER4 fibroblasts were seeded onto 'microspot' adherent microscope slides at $2x10^4$ cells per ml in $100x15mm^3$ petri dishes, cultured and quiesced as described in Section 2.5.1.1. The quiesced cells were washed twice with DBG at 37°C prior to the incubations described. Reactions were terminated by washing twice with ice-cold PBS and transfer to ice. The cells were then immunostained.

Suspension cells

HL60 cells were seeded at $5x10^5$ cells per ml in RPMI 1640 containing 15% (v/v) HIFCS and 1.3% (v/v) DMSO in the case of the differentiated cells. The cells were then grown for 5 days and the cell density determined by using a haemocytometer. Cells were washed twice in RBG, with the washed pellets being resuspended in RBG at a density of 10^7 cells per ml. 100µl aliquots of cell suspension were transferred to eppendorf tubes and incubated at 37°C. Agonists were dissolved in RBG and the incubations carried out as described. Reactions were terminated by washing twice in ice-cold PBS. The final cell pellet was resuspended in

1ml ice-cold PBS and 20µl of cell suspension spotted onto clean 'microspot' microscope slides. The cells were then immunostained as described below.

2.9.2 Immunostaining of cells

Cells adhered to 'microspot' slides were fixed in 4% (v/v) paraformaldehyde for 8 minutes, washed in PBS and then furthered fixed in ice-cold acetone for 10 minutes. The acetone was allowed to evaporate and the slides placed into a prepared damp box, where all antibody additions would be carried out. The slides were blocked with 10% (v/v) foetal calf serum containing 0.1% (w/v) sodium azide in PBS, at room temperature for 30 minutes. The slides were washed briefly after blocking and a 1:50 dilution of either rabbit anti-cPLA$_2$ antisera or pre-immune sera in blocking solution, was added to individual microscope wells and incubated for 90 minutes at 37°C in a pre-prepared damp box. The slides were washed 3x10minutes in PBS at room temperature. After washing, the slides were returned to the damp-box and a 1:50 dilution of anti-rabbit FITC-linked antibody diluted in blocking solution, was added to the wells treated with anti-cPLA$_2$, preimmune serum and a control well, in order to detect any non-specific staining by the second antibody. The damp box was then incubated at 37°C for 60 minutes. After this time, the slides were briefly washed in ice-cold PBS and transferred to fresh PBS for 30 minutes. The PBS in the bath was continually agitated. The cell nuclei were then stained by placing the cells in a bath of PBS containing 10µg/ml propidium iodide for 2 minutes at room temperature. Following propidium iodide staining the slides were washed briefly in PBS and any non-immuno stained areas gently dried off. To each treated well, a drop of 2.5% (w/v) Diazabicyclo[2.2.2]octane (DABCO) in glycerol was added and a glass coverslip overlaid, with great care being taken to remove any excess from the slide. The effectiveness of staining was checked under a fluorescent microscope, prior to confocal microscopy.

2.9.3 Measurement of fluorescence intensity.

Fluorescence was measured using an argon/ion confocal laser scanning microscope. The wavelengths utilised for the dual fluorescence were 488nm for Fluorescein and 568nm for propidium iodide, with additional enhancement filters attached. Images were colour-banded to give an instant visual analysis for the intensity of staining and quantitative data was obtained for intra- and extra-nuclear staining by extracting the specific staining observed in the nucleus, which was well defined when propidium iodine was used. Using the computer-operated control of the focus level, a series of optical '*xy* sections' in the z (vertical) axis were obtained as well as mean pixel intensity measurements. All images were stored on flexible disk catridges and hard-copy records were obtained using an on-line Matrix colour graphic camera.

2.10 CHEMICAL MEASUREMENT OF BASAL AND STIMULATED ARACHIDONIC ACID LEVELS

2.10.1 Preparation of samples

Five 80cm^2 flasks of Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS, with or without 0.1μg/ml arachidonate or 0.1μg/ml deuterated arachidonate. When 80-90% confluent, fresh DMEM containing 10% (v/v) NBCS was added and the cells allowed to quiesce by contact inhibition.

The flasks were washed 2x15 minutes in DBG at 37°C. Agonists were made up in DBG and the incubations described carried out at 37°C. Reactions were terminated by aspiration and the addition of ice-cold methanol:acetic acid (100:1.5) before being transferred to ice. Flasks were scraped and the suspension transferred to a glass screw-top test-tube with a further 3ml methanol: acetic acid (100:1.5), which had been used to wash the scraped flask. The lipids were then extracted and the free fatty acids isolated as descibed in Sections 2.4.1 and 2.4.2.

The fatty acids were eluted from the silica by washing with 10ml redistilled diethyl ether, which was collected into clean glass screw-topped test-tubes. The diethyl ether was then evaporated under a stream of nitrogen, leaving the concentrated free fatty acids ready for derivatisation.

2.10.2 Derivitisation of free fatty acids.

To the extracted free fatty acids, 2ml of dry methanol containing 2.5% (v/v) sulphuric acid was added and the capped test-tubes incubated at 70°C for 2 hours. The solution was allowed to cool to room temperature and the reaction terminated by the addition of 5ml 5% (w/v) NaCl. The methylated free fatty acids were extracted by washing the NaCl solution with 3x3ml redistilled hexane, with the top phase of hexane being transferred to clean test-tubes each time. The hexane was evaporated under nitrogen. A series of hexane washes were performed with the volume being reduced on each occasion, until the derivatised free fatty acids were resuspended in 25µl redistilled hexane and transferred to a insert vial. The insert vial was placed in a 2ml glass vial which was sealed with a metal cap containing a rubber speculum.

2.10.3 Gas chromatography

The prepared samples were placed on the autosampler of a Hewlett Packard 5890 Series II gas chromatographer which was linked to a Hewlett Packard 5972 Mass selective detector. 1µl of the sample was auto injected onto a DB-23 column (30m x 0.25mm, with a filter thickness of 0.25 microns), where the carrier gas was high purity Helium. The GC Mass Spectometer was set at the following parameters:

Injection head pressure	12 psi
Flash vapouriser	220°C
Purge time	1 minute
Initial flow rate	1.5ml/min
Detection inlet	270°C
Temperature gradients	55°C (2min) > 140°C (70°C/min) > 210°C (1°C/min)

All detectable products were recorded and manipulated using MS Chem Station software (G1034C), supplied by the manufacturer.

2.11 PERMEABILISATION OF CELLS USING STREPTOLYSIN-O

Permeabilisation of cells and all subsequent washes and treatments were performed in permeabilisation buffer as described for whole cell experiments described in Section 2.2.

2.11.1 Determination of cell permeabilisation using entry of Ethidium Bromide

Swiss 3T3 fibroblasts were seeded at $1x10^4$ cells per ml into 6 well plates in DMEM containing 10% (v/v) NBCS. When 80-90% confluent, fresh culture medium was added and the cells allowed to quiesce by contact inhibition. On the day of the experiment, the cells were washed 3 times for a total of 30 minutes with HBG at 37°C. Permeabilisation buffer containing 0.0-1.0 unit/ml Streptolysin-O and 1µg/ml ethidium bromide was added to individual wells and incubated at 37°C for 5 minutes. The cells were then washed twice with ice-cold PBS and viewed under the fluorescent microscope. Figure 2.11 shows that 0.6 unit per ml Streptolysin-O permeabilised 90% of the cells, without disrupting their morphology.

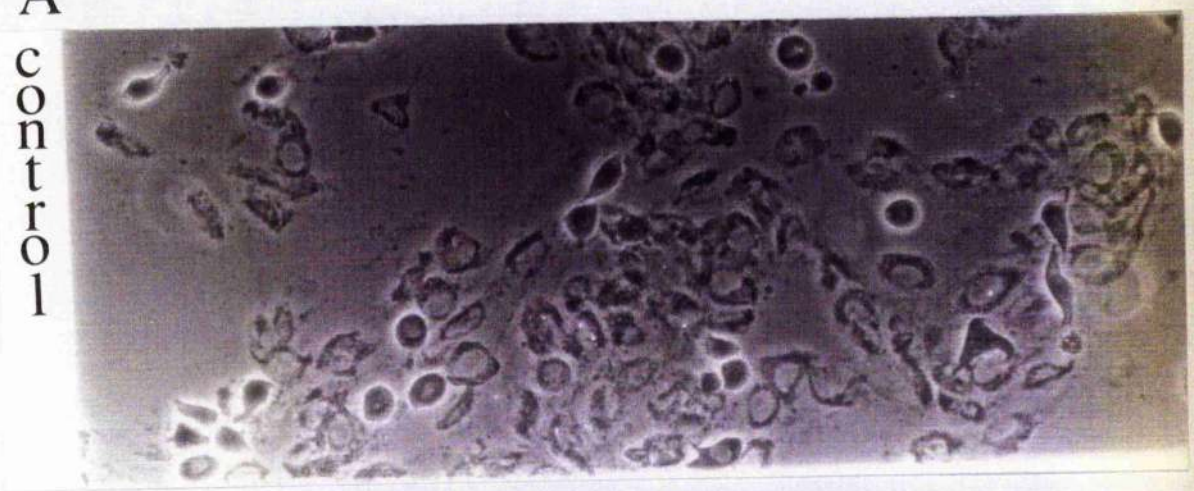
2.11.2 Protocol for permeabilisation

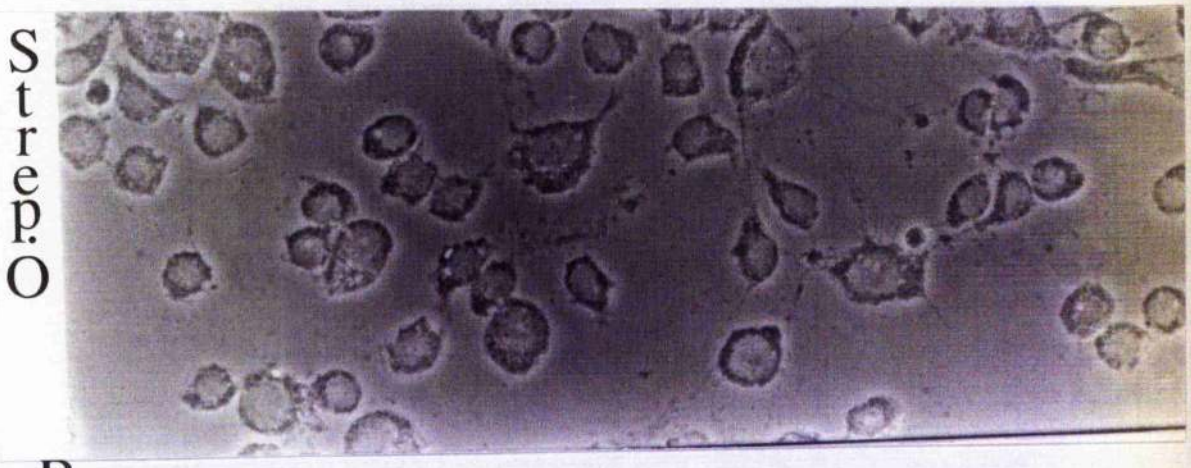
Swiss 3T3 fibroblasts were grown on 24 well plates, quiesced and labelled as described for intact cells in Section 2.4.1. After labelling, the cells were washed 2x10 minutes with DBG and then permeabilised by incubation with 150µl 0.6 units/ml Streptolysin-O in permeabilisation buffer for 5 minutes at 37°C. This was followed by washing the cells twice with 200µl permeabilisation buffer over 10-15 seconds, prior to stimulation with 150µl agonist at the concentrations and times described.

Figure 2.11 Permeabilisation of Swiss 3T3 fibroblasts by 0.6 units/ml streptolysin O

Cells were treated ass described in section 2.11.1. Results presented are representative of 2 individual experiments and demonstrate (a) Morphology of permeabilised cells and (b) Nuclear staining by ethidium bromide.

A





B

Reactions were terminated by the addition of 0.5ml ice-cold methanol:acetic acid (100:1.5) and the fatty acids isolated and quantified as described in Sections 2.4.1 and 2.4.2.

2.12 MEASUREMENT OF STIMULATED INTRACELLULAR INOSITOL PHOSPHATE GENERATION

2.12.1 Preparation of samples

Rat-1 Raf ER4 fibroblasts were seeded at $1x10^4$ cells per ml in 24-well plates in DMEM containing 10% (v/v) NBCS and 400µg/ml Geneticin. When 80-90% confluent, the medium was changed to serum-free, inositol-free DMEM containing 3µCi/ml [2-^{3}H] *myo*-inositol for 48 hours. The cells were then washed 2x15minutes with DBG before incubation with DBG containing 10mM LiCl for 10 minutes at 37°C. Incubations were commenced by replacing the medium with 200µl of the test reagent in DBG containing 10mM LiCl for the times indicated. Reactions were terminated by aspiration and the addition of 400µl ice-cold 5% (v/v) perchloric acid.

The wells were scraped and the debris transferred to an eppendorf tube and each well washed with 200µl water which was added to the corresponding eppendorf tube. The cell extracts were neutralised by the addition of KOH/Hepes (1.5M/60mM) containing universal indicator. All manipulations were carried out at 4°C. The neutralised extracts were centrifuged at 14,000 xg for 10 minutes at 4°C to remove the precipitated potassium perchlorate and the supernatent transferred to a fresh eppendorf tube before being stored at 4°C overnight.

2.12.2 Determination of individual inositol phosphate species

Pre-prepared Dowex AG 1-X8 anion exchange resin columns were washed through with 5ml distilled water. 5ml sodium tetraborate (NaTB)/EDTA (5mM/0.5mM)(pH6.7) was added to the column reservoir, to which the neutralised sample was added prior to addition to the column. The column was washed through

with a further 5ml NaTB/EDTA followed by 10ml distilled water. Finally, 10ml NaTB/ammonium formate (AF) (5mM/60mM), followed by 10ml distilled water were washed through the columns. The washes that have been described, have cleared any free inositol and glycerophosphoinositol from the columns, leaving only the inositol phosphates bound. 10ml of the following reagents were added to the column in the order listed, eluting the inositol phosphate species specified and collected for scintillation spectrophotometry :

0.2M ammonium formate/0.1M formic acid	Inositol Monophosphates
0.4M ammonium formate/0.1M formic acid	Inositol Bisphosphates
0.8M ammonium formate/0.1M formic acid	Inositol Trisphosphates
1.2M ammonium formate/0.1M formic acid	Inositol Tetrakisphosphates
2.0M ammonium formate/0.1M formic acid	Inositol Penta/Hexakisphosphate

For 0.2-0.8M ammonium formate, 5ml of elutant was transferred to a fresh scintillation vial and 15ml scintillation fluid added. For 1.2M and 2.0M ammonium formate elutants, 10ml scintillation fluid was added directly. All samples were vortexed and radioactivity quantitated using liquid scintillation spectrophotometry.

2.13 MISCELLANEOUS PROCEDURES

2.13.1 Activation of sodium orthovanadate

The pH of a 10mM solution of sodium orthovanadate was adjusted to pH10 using 1M NaOH and 1M HCl. The yellow solution was boiled until clear, then cooled to room temperature and the pH readjusted to pH10. This procedure was repeated until the solution remained clear and the pH stable. Aliquots were then stored at -20°C until required.

2.13.2 Preparation of Perhydrovanadate

10mM activated sodium orthovanadate and 10mM hydrogen peroxide were mixed and incubated at 25°C for 15 minutes. Residual hydrogen peroxide was removed by the addition of Catalase (200μg final concentration), giving a final concentration of 5mM perhydrovanadate. Control cells were treated with a solution where DBG or RBG was substituted for sodium orthovanadate.

2.13.3 Analysis and presentation of results

Unless otherwise stated all experiments were performed at least 3 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 the significance was generally taken as values of $p<0.05$.

EC_{50} and IC_{50} values were calculated from dose-response curves fitted to logistic equation (non-linear regression analysis). However, for presentation puposes dose-response curves were presented as simple line graphs from the Apple Macintosh Cricket Graph programme.

CHAPTER 3

PRIMING OF CYTOSOLIC PHOSPHOLIPASE A_2

3.1 INTRODUCTION

Various components of serum have been documented to have effects upon the growth and differentiation of cells (Perkins *et al.*, 1994). Such effects have been mainly determined at the DNA level (Hill & Triesman, 1995) with a number of serum response elements having been identified (Triesman, 1992). However, serum is a complex mixture containing, for example, growth factors, immunoglobulins, lipid moieties such as lysophosphatidic acid (LPA) and albumin, all of which have been shown to have a marked effect upon the cell.

The sensitivity of the cell to these factors determines the conditions used to both maintain them in culture and to quiesce them for experimental procedures. The procedure of quiescing is where the cells are synchronised into the G_O/G_1 phase of the cell cycle and therefore, not dividing or differentiating. The cells are thus prepared for a stimulus such as a growth factor. Quiesced cells can be achieved by manipulating the serum content of the growth medium in two ways. Firstly, there is serum deprivation, where the serum concentration is lowered to such a concentration that there are not enough growth factors available to stimulate division, but enough to maintain the cells' viability. Secondly, there is contact inhibition, which is only applicable to cells of an adherent nature which stop growing when they physically contact neighbouring cells. This procedure is normally carried out in complete cell culture medium, which is replenished at regular intervals until a dense monolayer of cells is visualised. Both methods are widely used in signal transduction studies, but the possible differences in the response of the cells to subsequent agonist stimulation has not been investigated.

The data in this chapter suggest that $cPLA_2$ in Swiss 3T3 fibroblasts quiesced by contact inhibition in normal serum concentrations is more responsive to stimulation by the peptide agonist bombesin than cells quiesced by serum withdrawal. This could be described as a 'priming' event. Priming by such agents as granulocytic macrophage colony stimulating factor (GMCSF) (Yuo *et al.*, 1991) and the cytochalasins (Honeycutt & Niedel, 1980), such as cytochalasin B, has been widely

studied in haemopoietic cells. These latter priming agents do not in themselves stimulate a signalling pathway, but do enhance the response of the signalling enzyme in question upon agonist stimulation. The mechanism of action of these priming agents has not been fully elucidated, but several possibilities have been suggested, such as increased tyrosine kinase activity (Lloyds *et al.*, 1993), increased MAP kinase activity (Thompson *et al.*, 1993) and intracellular calcium release (Forehand *et al* ., 1989). Specifically, in the case of the cytochalasins, it has been shown that there is a capping of G-actin, which results in the truncation or disassembly of the F-actin cytoskeletal structure (for review see Weeds, 1982).

The premyelocytic human leukemic HL60 cell is a well defined model in which to examine the possible effects of priming upon cytosolic phospholipase A_2 activity using cytochalasin B as the priming agent. These cells have been shown to have a requirement for priming for various signalling components, such as the stimulation of phospholipase D by the the non-hydrolysable forms of guanosine triphosphate (GTPγS) (Cockcroft, 1992). The HL60 cells have been treated with dimethyl sulphoxide (DMSO), to differentiate these cells to a neutrophil-like cell, in order to study the effect of priming upon an agonist-stimulated response. When differentiated in this way, there is an induction in the expression of the chemotactic peptide formylMethylLeucinePhenylalanine (fMLP) receptor (Collins, 1987), over a period of 3-5 days DMSO treatment. fMLP has been shown to stimulate PLD and $cPLA_2$ activity in both GMCSF- and cytochalasin B-primed DMSO-differentiated HL60 cells (for review see Cockcroft, 1992).

3.2 RESULTS

3.2.1 Product of $cPLA_2$ activity.

Arachidonic acid (20:4) is considered to be the preferred acyl group on phospholipids hydrolysed by $cPLA_2$. However, other *cis*-unsaturated fatty acids such as oleic acid (18:1) have been found to be released upon agonist stimulation. Therefore, using both [4,5,8,9,11,12,14,15-^{3}H] arachidonate and [9,10-^{3}H] oleate labelled Rat-1 fibroblasts, the kinetics of the partial mitogen endothelin 1-stimulated release of these fatty acids was studied. Figure 3.1(a) shows that endothelin 1-stimulated oleate release was biphasic, with an initial 3-fold over basal transient phase which was maximal at 5 seconds and returned to basal by 2 minutes. The second sustained phase was maximal, also 3-fold, at 10 minutes, remaining elevated at 20 minutes. The EC_{50} for this response at 5 seconds was 3nM (± 2.5nM) and maximal at 10nM as shown in Figure 3.1(b). The arachidonate release in response to endothelin 1 was also biphasic, but shows differences in kinetics as shown in Figure 3.2(a). The initial transient phase is maximal at 15 seconds, 4-5 fold over basal, but does not return to basal. The second sustained phase was still elevated at 20 minutes, 3-fold above basal. The EC_{50} value at 15 seconds was also 3nM (±1.5nM) and maximal at 30nM as shown in Figure 3.2(b).

The bombesin-stimulated Swiss 3T3 fibroblast has been an extensively studied model for phospholipase A_2 activation (Currie *et al.*, 1992) and being a fibroblast cell it is a good comparison to the Rat-1 fibroblast. Using the chemical technique of Gas-chromatography, Figure 3.3(a) demonstrates that various fatty acids are released in bombesin-stimulted Swiss 3T3 fibroblasts. However, due to modifications to the small amount of fatty acids that occur during the various steps in the preparation of the samples, it is difficult to determine the exact nature of the majority of these fatty acid species. Therefore, since arachidonate has historically been the major fatty acid of interest, but is of such low abundance, the cells were cultured in the presence of 1μg/ml arachidonic acid. The inclusion of arachidonate in the culture medium of

Swiss 3T3 fibroblasts was to produce maximum incorporation of arachidonate into the *sn*-2 position of the phospholipid glycerol backbone, therefore providing a larger pool of substrate for bombesin-stimulated phospholipase A_2 activity. Detailed quantitative analysis was carried out on the fatty acids released upon bombesin-stimulation and Figure 3.3(b) demonstrates that arachidonate was indeed released. The basal level of free intracellular arachidonate from five $75cm^2$ flasks, approximately $4x10^6$ cells, of contact inhibited confluent Swiss 3T3 fibroblasts was approximately 30pg, which rose to 90pg, as determined by standard curve analysis, upon bombesin-stimulation.

3.2.2 Kinetics of arachidonate release in bombesin-stimulated Swiss 3T3 fibroblasts quiesced by both contact inhibition and serum deprivation.

Using [4,5,8,9,11,12,14,15-^{3}H] arachidonate labelled contact inhibited Swiss 3T3 fibroblasts, the effect of bombesin stimulation was measured. As shown in Figure 3.4(b), the arachidonate release was biphasic, with an initial 2-fold, transient phase which was maximal at 30 seconds and returned to basal around 10 minutes. The second sustained phase was 60% above basal, but remaining elevated at 30 minutes. When the cells were quiesced by serum deprivation, where the cells were cultured in 2% (v/v) newborn calf serum for 24 hours, the response to bombesin was also biphasic as shown in Figure 3.4(a). However, there were clear differences in the magnitude and kinetics of both the initial transient and second sustained phases of the response. The initial transient phase was maximal at 30 seconds, 2.3-fold above basal, returning to basal by 10 minutes. The second sustained phase was still increasing at 30 minutes, 3-fold above basal and returned to basal by 60 minutes (result not shown).

The initial phase of arachidonate release differed in magnitude between the quiescing method used (Figure 3.4(a) and (b)). Figure 3.5(a) shows that in 2% (v/v) NBCS the response is indeed maximal at 30 seconds, however, in cells cultured by contact inhibition in 10% (v/v)NBCS, as shown in Figure 3.5(b), the response is

maximal at 10 seconds, returning towards basal by 30 seconds. This appears to show that some component of serum is at a high enough concentration to prime $cPLA_2$ activity in 10% (v/v) NBCS, but does not stimulate arachidonate release in isolation, since the basal levels are identical under both serum conditions.

3.2.2.1 Possible serum components which may chronically prime $cPLA_2$ in Swiss 3T3 fibroblasts.

Serum fractionation was not attempted, however two possible components insulin and lysophosphatidic acid (LPA) were examined.

Inclusion of insulin (10ng/ml) for 24 hours with 2% (v/v) NBCS resulted in a rapid bombesin-stimulated arachidonate release, identical to that observed in cells cultured in 10% (v/v) NBCS (Figure 3.6(a)). The inclusion of insulin prolonged the response, which remained elevated at 60 seconds, at which time the response in non-treated cells had returned to basal. Insulin did not affect the kinetics of bombesin-stimulated arachidonate release in 10% quiesced cells (results not shown).

When Swiss 3T3 fibroblasts were quiesced in 2% (v/v) NBCS containing 10μM LPA for 24 hours, the basal level of arachidonate increased, as shown in Figure 3.6(b). Non-LPA treated cells gave a 2-fold response to a 1 minute stimulation with 100nM bombesin, however, in LPA-treated cells this response was 4-5 fold above basal. For cells quiesced in 10% (v/v) NBCS, inclusion of 10μM LPA in the quiescing medium did not increase the basal level of free intracellular arachidonate. In the absence of 10μM LPA, there was no significant bombesin-stimulated arachidonate release at the time point chosen, however, bombesin stimulation produced a 4-fold response in cells quiesced in the presence of LPA. When studied in more detail, the enhanced bombesin-stimulated arachidonate release observed in cells quiesced in the presence of LPA is dependent upon the quiescing method adopted. Figure 3.7 demonstrates that cells quiesced in 2% NBCS produce an enhanced bombesin response at very low concentrations of LPA (where the basal level is taken as the bombesin-stimulated arachidonate release in the absence of LPA), where as cells

quiesced in 10% NBCS do not. When the concentration of LPA in the quiescing medium increased, the magnitude of respose in both 2% (v/v) and 10% (v/v) converged at a threshold concentration of 1μM LPA.

3.2.2.2 Effect of priming at the receptor-G-protein level

Swiss 3T3 fibroblasts quiesced under both conditions were stimulated with various concentrations of bombesin for times which corresponded to the maximum release of arachidonate in the initial transient phase. Figure 3.8(a) and (b) demonstrate that the apparent affinity of the receptor for bombesin was unaffected by priming, with both conditions giving an EC_{50} value of 3nM ± 2.5nM.

To examine the role of GTP-binding proteins, streptolysin O-permeabilised cells and the non-hydrolysable analogues of GTP and GDP, GTPγS and GDPβS respectively, were utilised. Arachidonate release was observed in response to bombesin which was potentiated by GTPγS in cells cultured in 2% (v/v) NBCS, but was not in the cells cultured at the high serum concentration (Figure 3.9(a) and (b)). This bombesin concentration was just maximal (10nM), however, when a supramaximal concentration of 100nM bombesin was used, there appeared to be a small inhibition of bombesin-stimulated arachidonate release by GTPγS, which was more pronounced in 10% (v/v) NBCS cultured Swiss 3T3 fibroblasts (Figure 3.10(a)). This inhibition can be mimicked to a further extent by using GDPβS, which fully inhibits bombesin-stimulated arachidonate release under both conditions, an example of which is shown in Figure 3.10(b) for 10% (v/v) NBCS cultured cells.

In an attempt to examine the type of GTP-binding protein involved in this response, cells were incubated with pertussis toxin for 18 hours. Figure 3.11(a) demonstrates that bombesin-stimulated arachidonate is pertussis toxin insensitive. However, Figure 3.11(b) demonstrates that the heterotrimeric GTP-binding protein activator, Aluminium Fluoride, produced no arachidonate release until after 5 minutes, in both intact and Streptolysin O permeabilised cells.

3.2.3 HL60 cells as a model to study acute priming of $cPLA_2$

Premyelocytic human leukemic HL60 cells were differentiated to a neutrophil-like cell using 1.3% (v/v) dimethyl sulphoxide (DMSO) over a period of 5 days. Over this time, the arachidonate release in response to the chemotactic peptide fMLP, the phorbol ester PMA and the tyrosine phosphophosphatase inhibitor perhydrovanadate was studied, with the cells being primed by cytochalasin B prior to agonist stimulation. Figure 3.12(a) demonstrates that a tyrosine kinase regulated phospholipase A_2 was highly active after only 2 days of differentiation. The phorbol ester did not produce a response until after 5 days differentiation and the fMLP receptor was apparently only at high enough levels to produce a significant response after 3 day DMSO treatment.

When 5 day DMSO-differentiated HL60 cells were screened with various agonists in the absence of cytochalasin B, no arachidonate release was detectable at the time point chosen (Figure 3.12(b)). Preincubation for 5 minutes with 5μM cytochalasin B, produced a significant increase in fMLP-stimulated arachidonate release. This response was both time- and dose-dependent as shown in Figure 3.13(a) and (b). fMLP-stimulated arachidonate release in cytochalasin B primed 5 day DMSO-differentiated HL60 cells is a rapid monophasic response, maximum at 1 minute and remaining elevated at 10 minutes. At 5 minutes, the EC_{50} value was approximately 30nM (± 5nM) and maximal at 100nM, the concentration used for all kinetic studies.

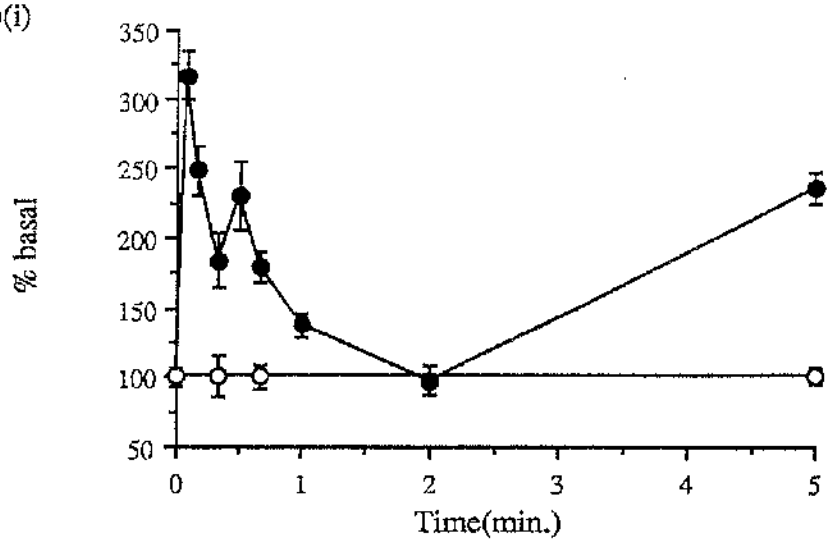
Figure 3.1(a) **Endothelin 1-stimulated intracellular [^{3}H] oleate release in Rat-1 fibroblasts**

Cells labelled for 24 hours in DMEM containing 10% (v/v) newborn calf serum and 5μCi/ml [9,10-^{3}H] oleic acid. Cells stimulated with 100nM endothelin 1 (●) or HBG (○) for the times indicated upto (i) 5 minutes and (ii) 20 minutes. Result representative of 3 individual experiments. Presented as % basal ± s.d., with basal levels of 2000 ± 65 dpm.

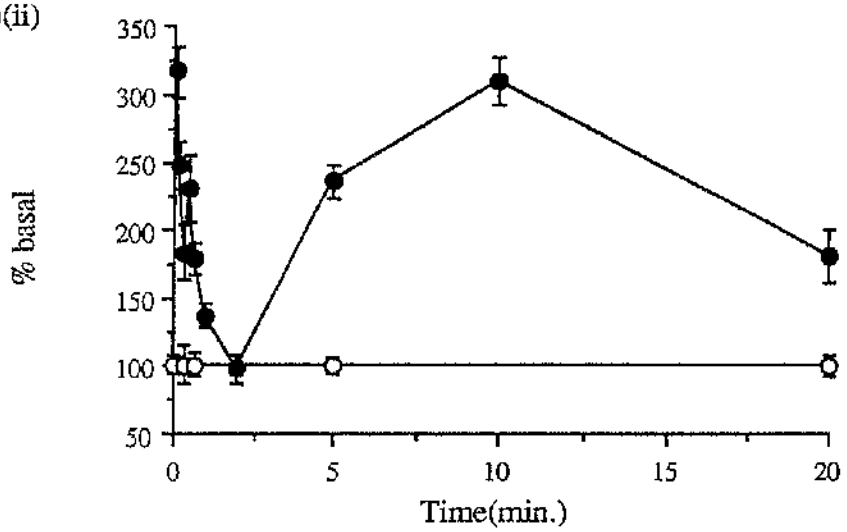
Figure 3.1(b) **Dose-dependency of ET-1-stimulated intracellular [^{3}H] oleate release at 5 seconds.**

Cells labelled as above and stimulated for 5 seconds with increasing concentrations of ET-1 shown (●). Results presented as % basal ± s.d., obtained by stimualting with HBG for 5 seconds, which gave a value of 2000 ± 230 dpm. Experiment representative of 3 similar experiments.

(a)(i)


350
300
250
200
150
100
50
% basal
0
1
2
3
4
5
Time(min.)

(a)(ii)


350
300
250
200
150
100
50
% basal
0
5
10
15
20
Time(min.)

(b)

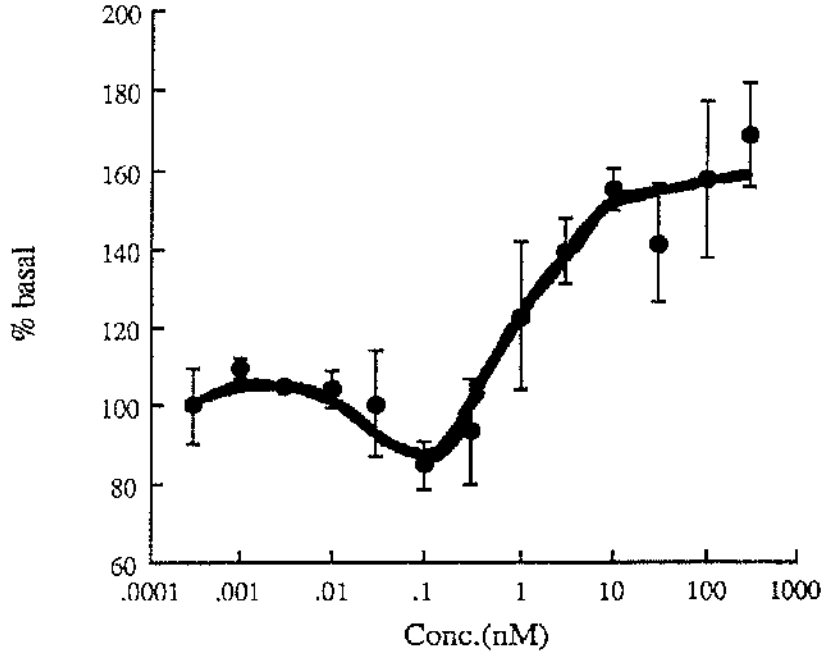

200
180
160
140
120
100
80
60
% basal
.0001
.001
.01
.1
1
10
100
1000
Conc.(nM)

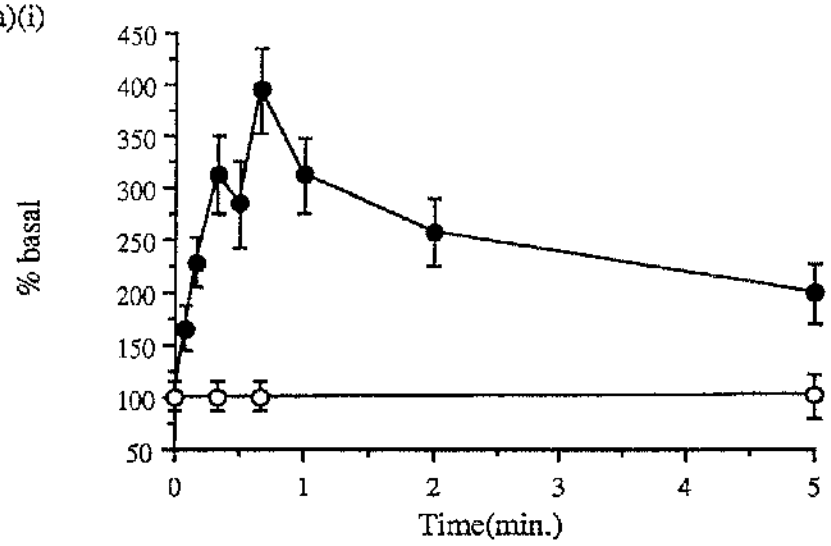
Figure 3.2(a) **Endothelin 1-stimulated intracellular [^{3}H] arachidonate release in Rat-1 fibroblasts.**

Rat-1 fibroblasts labelled with 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid for 24 hours in DMEM containing 10% (v/v) newborn calf serum. Cells stimulated with 100nM ET-1 (●) or HBG (○) for the times indicated upto (i) 5 minutes and (ii) 20 minutes. Result shown is representative of 3 separate experiments and values given as % basal ± s.d., where the basal level is 1000 ± 150 dpm.

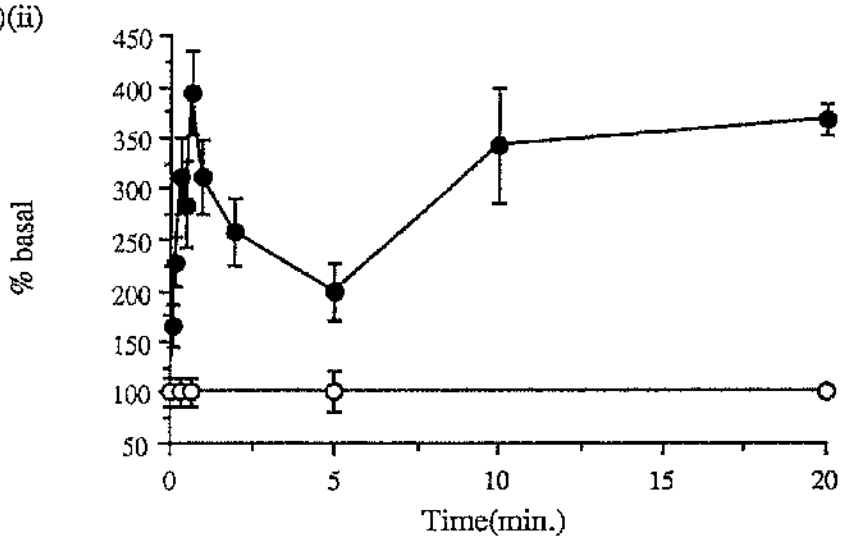
Figure 3.2(b) **Dose-dependency of ET-1-stimulated intracellular [^{3}H] arachidonate release at 15 seconds in Rat-1 fibroblasts.**

Cells labelled as above and stimulated with the concentrations of ET-1 shown for 15 seconds (●). Values depicted as % basal ± s.d., with the basal level being 400 ± 35 dpm when the cells stimulated with HBG for 15 seconds.

(a)(i)


% basal
450
400
350
300
250
200
150
100
50
0
1
2
3
4
5
Time(min.)

(a)(ii)


% basal
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400
350
300
250
200
150
100
50
0
5
10
15
20
Time(min.)

(b)

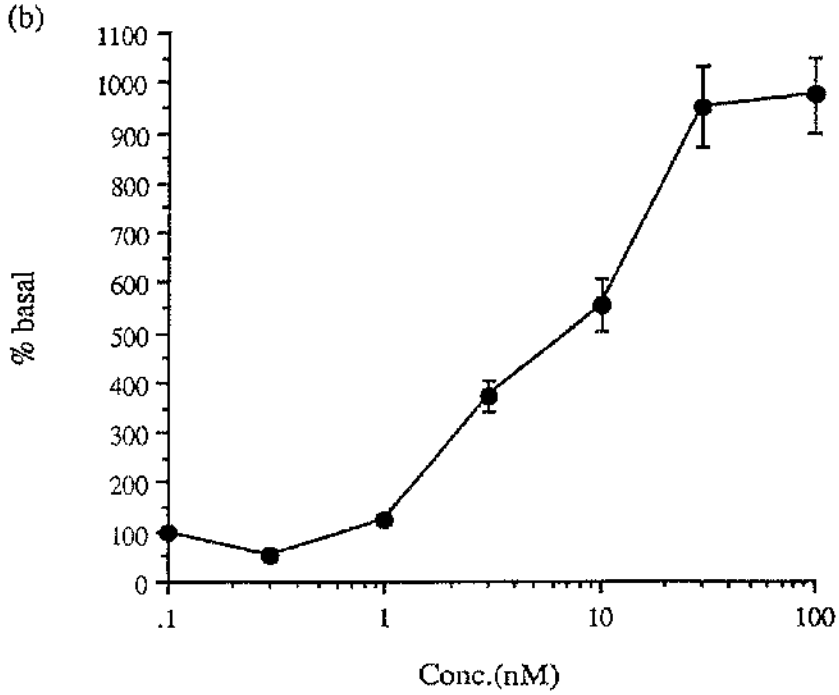

% basal
1100
1000
900
800
700
600
500
400
300
200
100
0
.1
1
10
100
Conc.(nM)

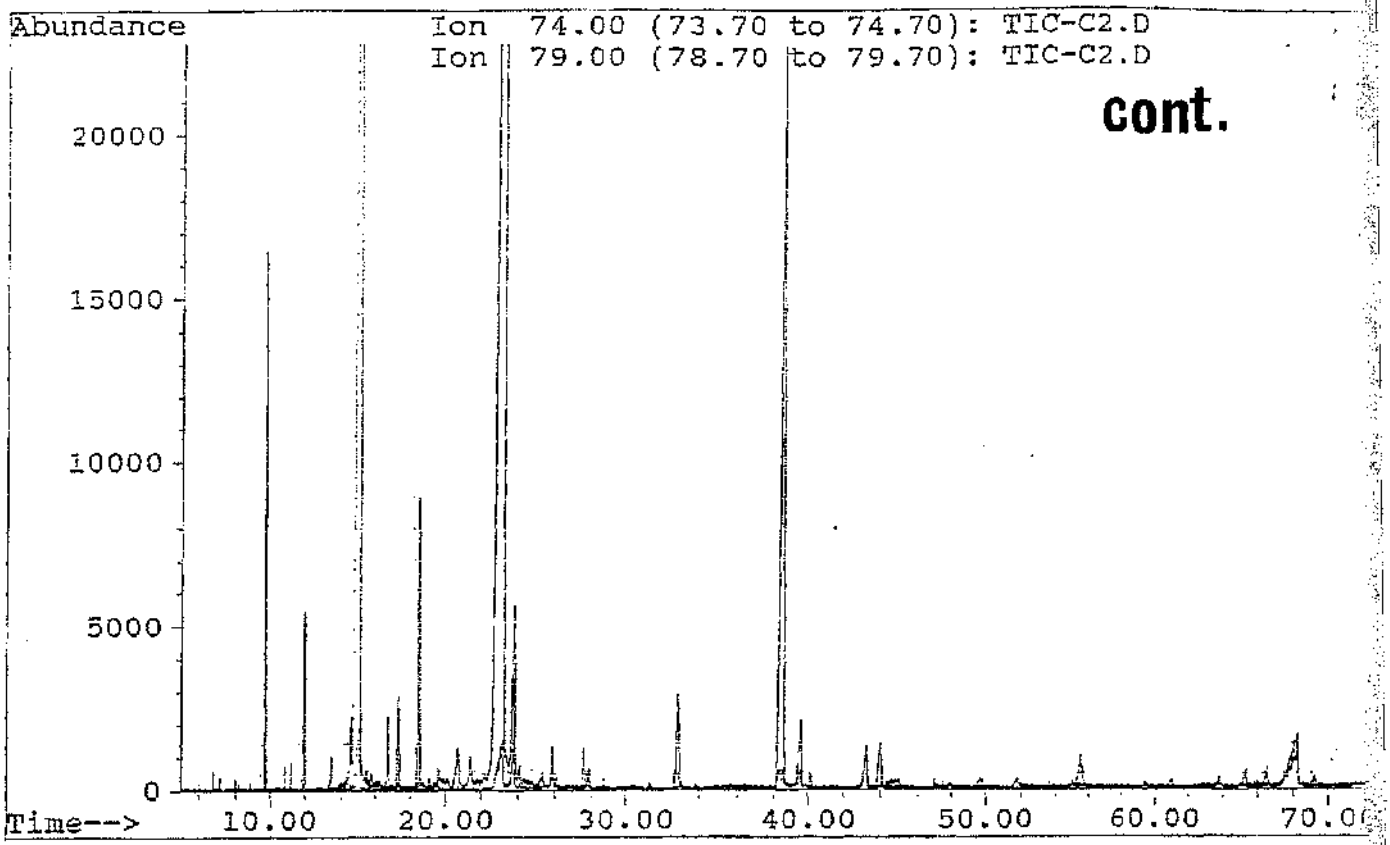
Figure 3.3(a) **Bombesin-stimulated free fatty acid release in Swiss 3T3 fibroblasts as determined by gas chromatography.**

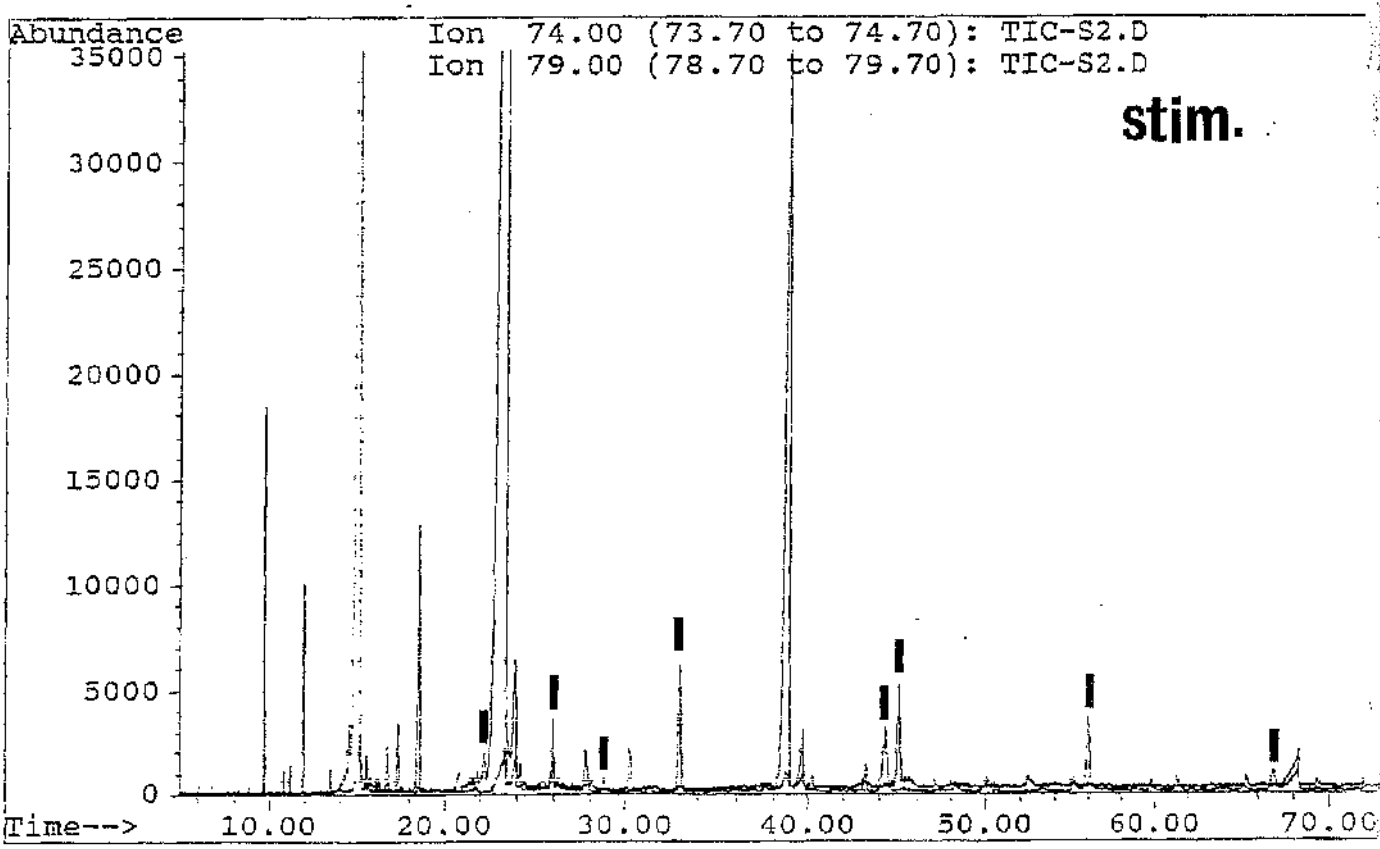
Cells were cultured in DMEM containing 10% (v/v) newborn calf serum until a confluent monolayer was obtained. Cells were stimulated with either 100nM bombesin or HBG for 1 minute at 37°C and the methylated free fatty acids prepared and separated by Gas chromatography using an attached mass spectophotometer facility. Possible increases as marked upon the figure.

Figure 3.3(b) **Bombesin-stimulated arachidonate release in Swiss 3T3 fibroblasts as determined by gas chromatography.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) newborn calf serum and 0.1μg/ml arachidonic acid until a confluent monolayer was observed. Cells were then stimulated with either 100nM bombesin or HBG for 1 minute at 37°C and the methylated free fatty acids prepared and separated using gas chromatography utilising an attached mass spectophotometer. Arachidonate is marked on the figure.

A


Abundance
Ion 74.00 (73.70 to 74.70): TIC-C2.D
Ion 79.00 (78.70 to 79.70): TIC-C2.D
cont.
20000
15000
10000
5000
0
Time-->
10.00
20.00
30.00
40.00
50.00
60.00
70.00


Abundance
Ion 74.00 (73.70 to 74.70): TIC-S2.D
Ion 79.00 (78.70 to 79.70): TIC-S2.D
stim.
35000
30000
25000
20000
15000
10000
5000
0
Time-->
10.00
20.00
30.00
40.00
50.00
60.00
70.00

B

Generation of arachidonic acid following bombesin stimulation of swiss 3T3 mouse fibroblasts as determined by GC-MS using select ion monitoring at m/z 79 and 150

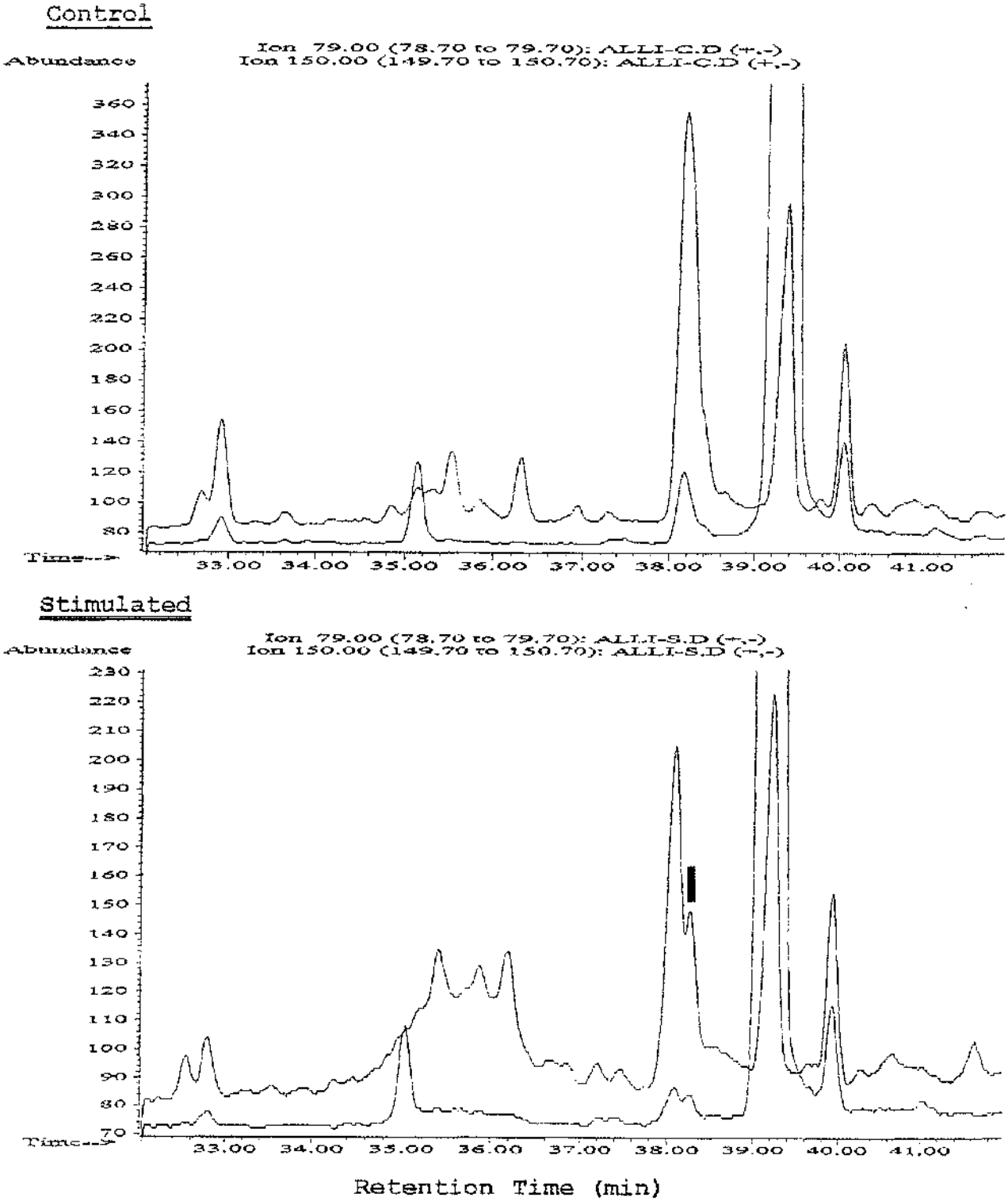


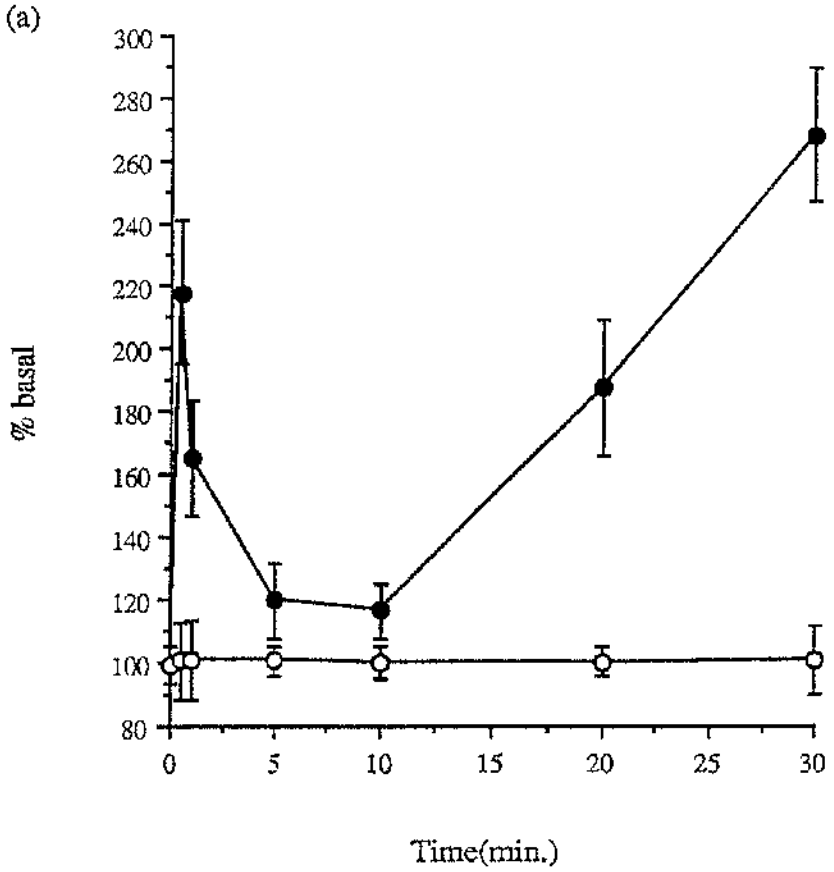
Figure 3.4(a) **Bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced using DMEM containing 2% (v/v) NBCS.**

Cells cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent. Medium then changed to DMEM containing 2% (v/v) NBCS and 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid for 24 hours. Stimulations were carried out for the times indicated with either 100nM bombesin (●) or DBG (○). Result is representative of 3 separate experiments with values being presented as % basal ± s.d., where the basal level is 550 ± 60 dpm.

Figure 3.4(b) **Bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced in DMEM containing 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was changed to DMEM containing 10% (v/v) NBCS and 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid for 24 hours. Cells stimulated for the times indicated with either 100nM bombesin (●) or DBG (○). Result is representative of 3 individual experiments and presented as % basal ± s.d., with a basal level of 500 ± 40 dpm.

(a)


300
280
260
240
220
200
180
160
140
120
100
80
% basal
0
5
10
15
20
25
30
Time(min.)

(b)

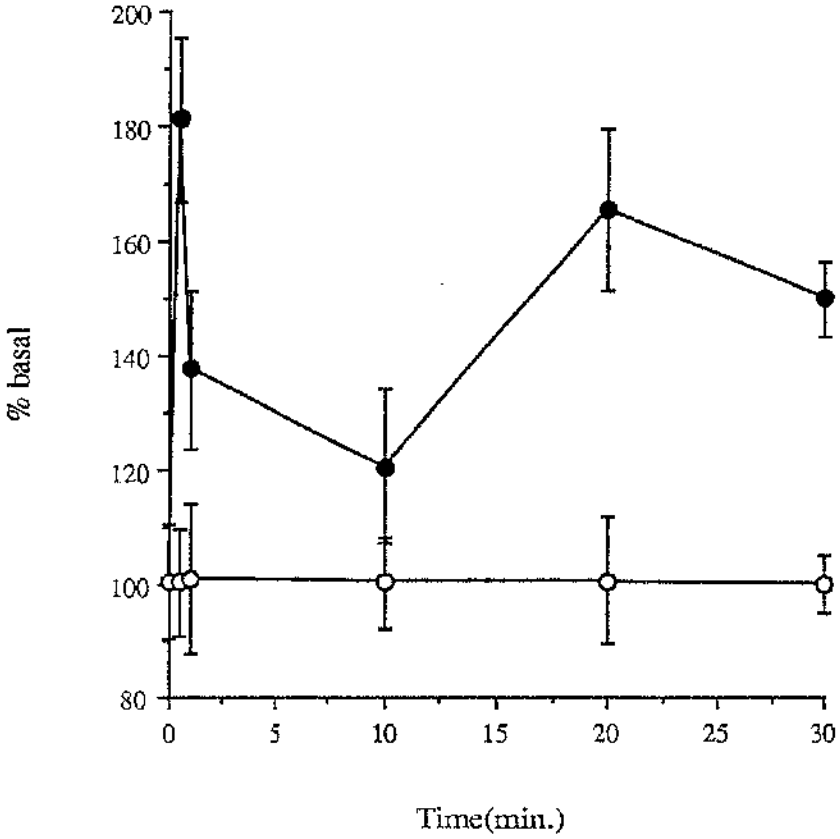

200
180
160
140
120
100
80
% basal
0
5
10
15
20
25
30
Time(min.)

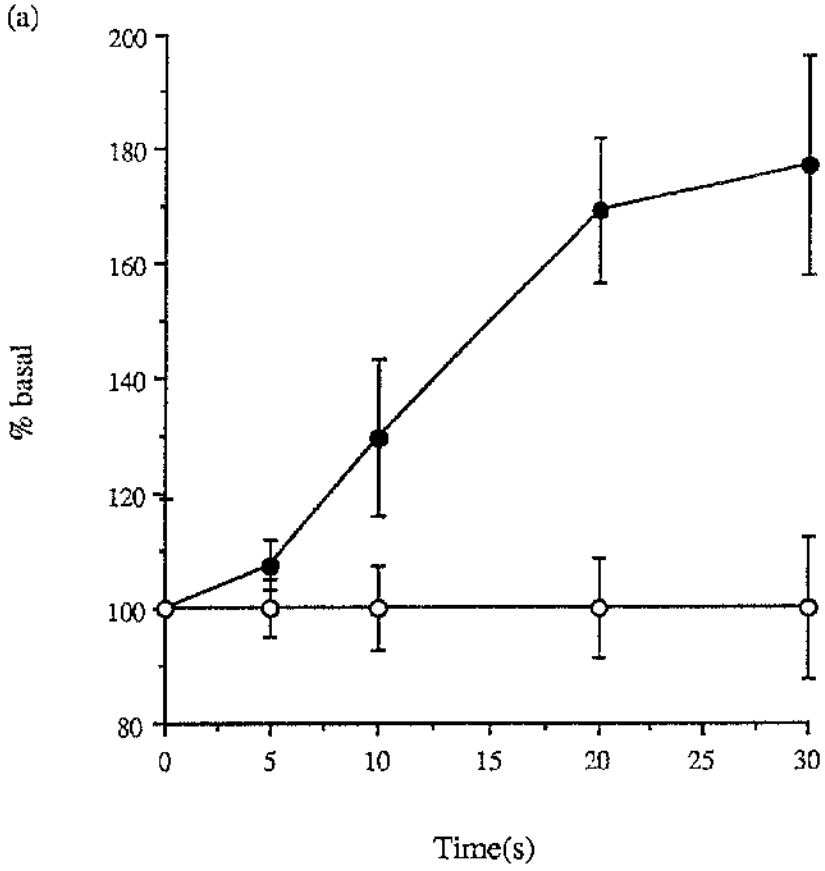
Figure 3.5(a) **Short time course of bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced in DMEM containing 2% (v/v) NBCS.**

Swiss 3T3 fibroblasts cultured as described in Figure 3.4(b) and stimulated for the times shown with either 100nM bombesin (●) or DBG (○). Result shown is representative of 5 individual experiments and the values presented are % basal ± s.d., where the basal level is 475 ± 35 dpm.

Figure 3.5(b) **Short time course of bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced in DMEM containing 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts cultured as described in Figure 3.4(a) and stimulated for the times indicated with either 100nM bombesin (●) or DBG (○). Result is representative of 5 individual experiments and the values presented as % basal ± s.d., where the basal level is equivalent to DBG stimulated arachidonate which was measured at a level of 435 ± 40 dpm for the times shown.

(a)


200
180
160
140
120
100
80
% basal
0
5
10
15
20
25
30
Time(s)

(b)

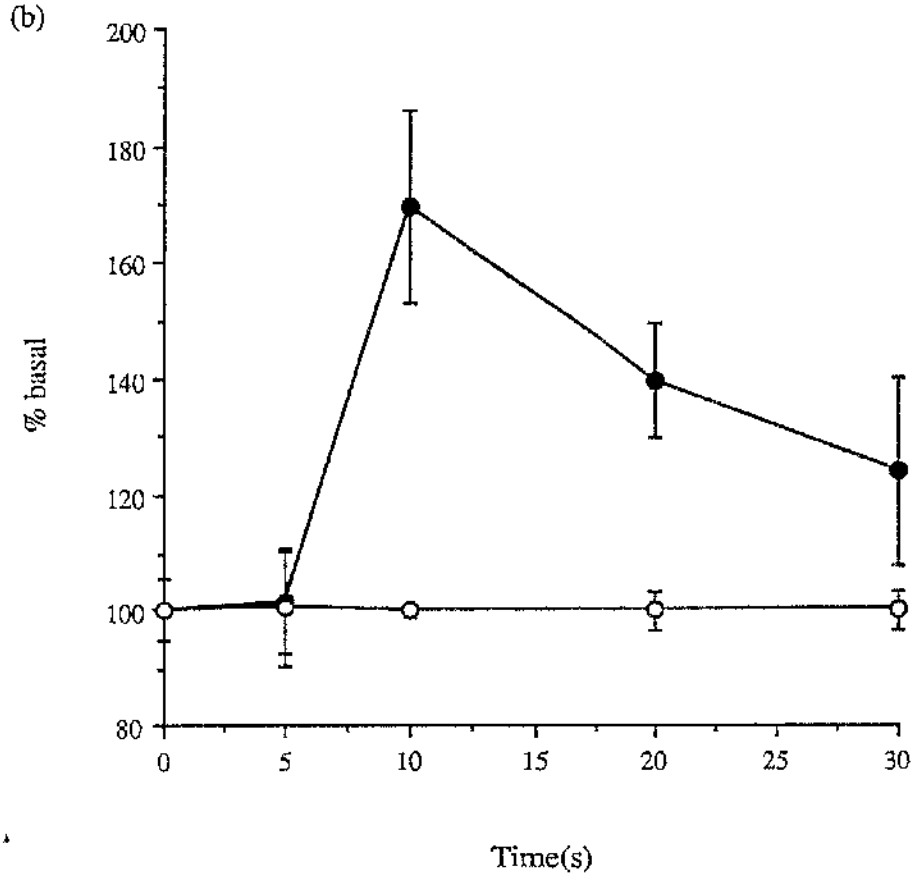
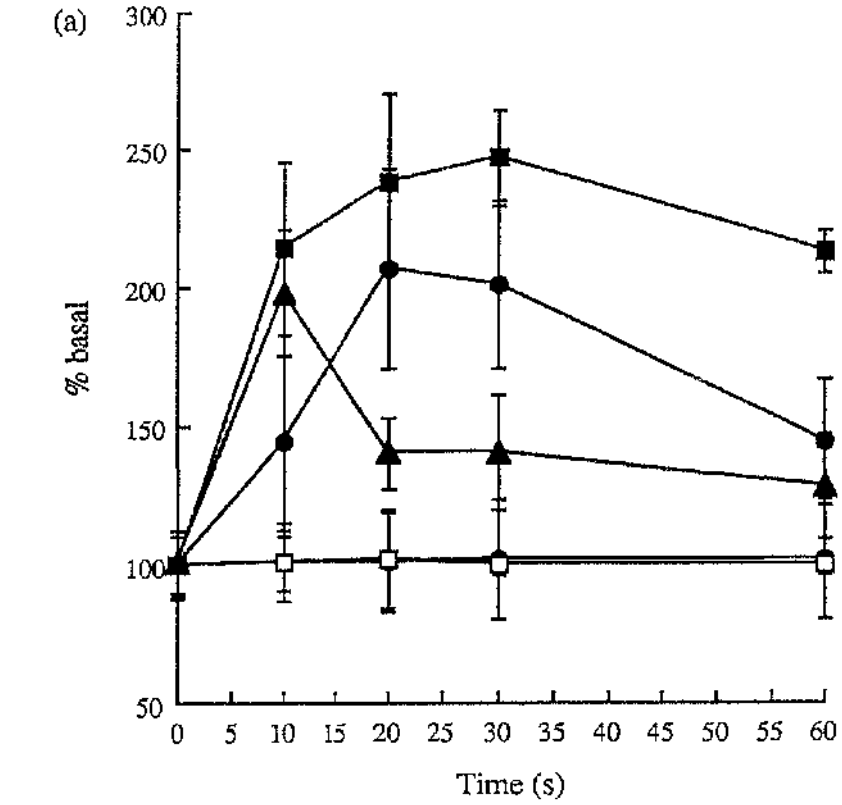

200
180
160
140
120
100
80
% basal
0
5
10
15
20
25
30
Time(s)

Figure 3.6(a) **Effect of Insulin upon bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced in DMEM containing 2% or 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when the medium was changed to DMEM containing 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid and either 2% (v/v) or 10% (v/v) newborn calf serum for 24 hours 10ng/ml human insulin was added to a proportion of the cells treated with both serum concentrations for the 24 hours. Cells were stimulated for the times indicated with either 100nM bombesin or DBG. Result is representative of 3 individual experiments with values presented as % basal ± s.d., which is equivalent to 500 ± 55 dpm in all conditions. The conditions depicted are 2% stimulation (●), 2% control (○), 2% stimulation with insulin (■), 10% control (❑) and 10% stimulated (▲).

Figure 3.6(b) **Effect of lysophosphatidic acid (LPA) upon bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced in DMEM containing either 2% or 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when the medium was changed to DMEM containing 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid and either 2% (❑) or 10% (■) (v/v) NBCS for 24 hours. To a proprtion of the cells under both quiescing conditions, 10μM LPA was added for the 24 hour quiescing period. Cells were then stimulated for 1 minute with 100nM bombesin. Result shown is representative of 2 individual experiments and values presented as % basal ± s.d., which was 450 ± 35 dpm.


(a)
300
250
200
150
100
50
% basal
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (s)

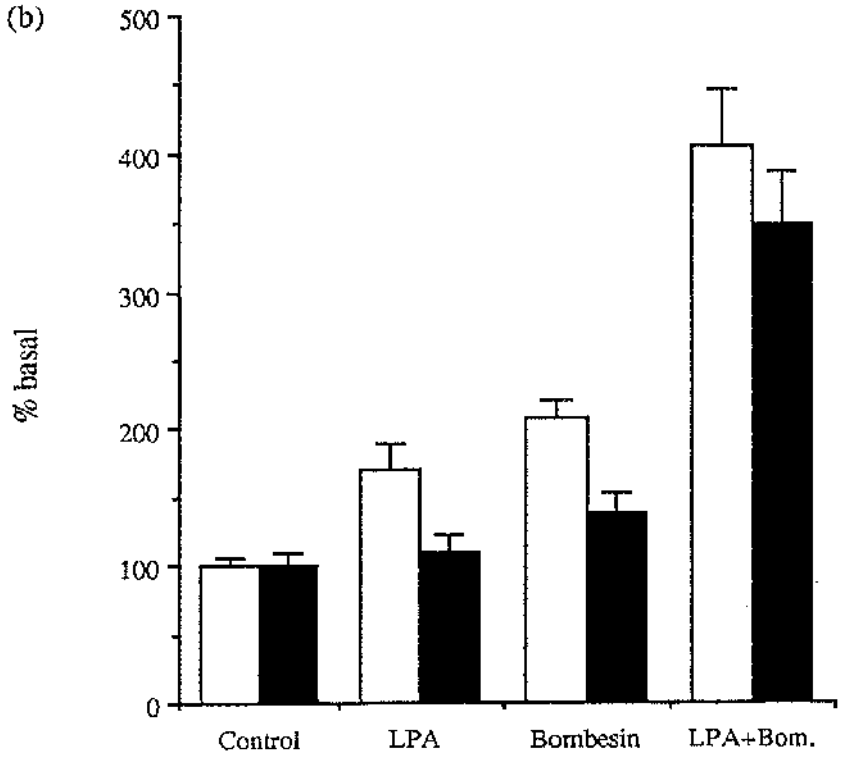

(b)
500
400
300
200
100
0
% basal
Control
LPA
Bombesin
LPA+Bom.

Figure 3.7 **Dose-dependency of LPA attenuation of bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced in DMEM containing either 2% or 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was changed to DMEM containing 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid and either 2% (●) or 10% (○) (v/v) NBCS for 24 hours. To a proportion of cells quiesced under both serum condition various concentrations of LPA were added for the quiescing period. Cells were then stimulated with 100nM bombesin for 1 minute. A basal value of 400 ± 35 dpm was obtained by stimulating with DBG for the times utilised. Result shown is representative of 2 individual experiments and values presented as % basal ± s.d., whereby the basal value is taken as 1200 ± 130 dpm, corresponding to bombesin-stimulated arachidonate release at the times chosen. Therefore, all values presented are compared to the bombesin-stimulated response in the absence of preincubation by LPA.

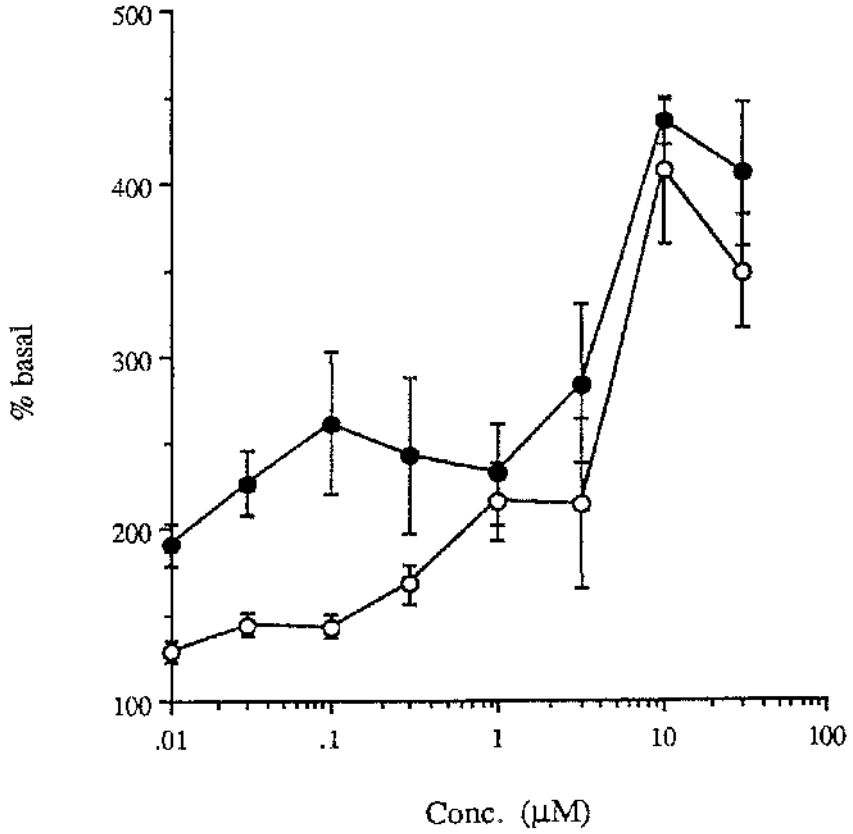

500
400
300
200
100
% basal
.01
.1
1
10
100
Conc. (μM)

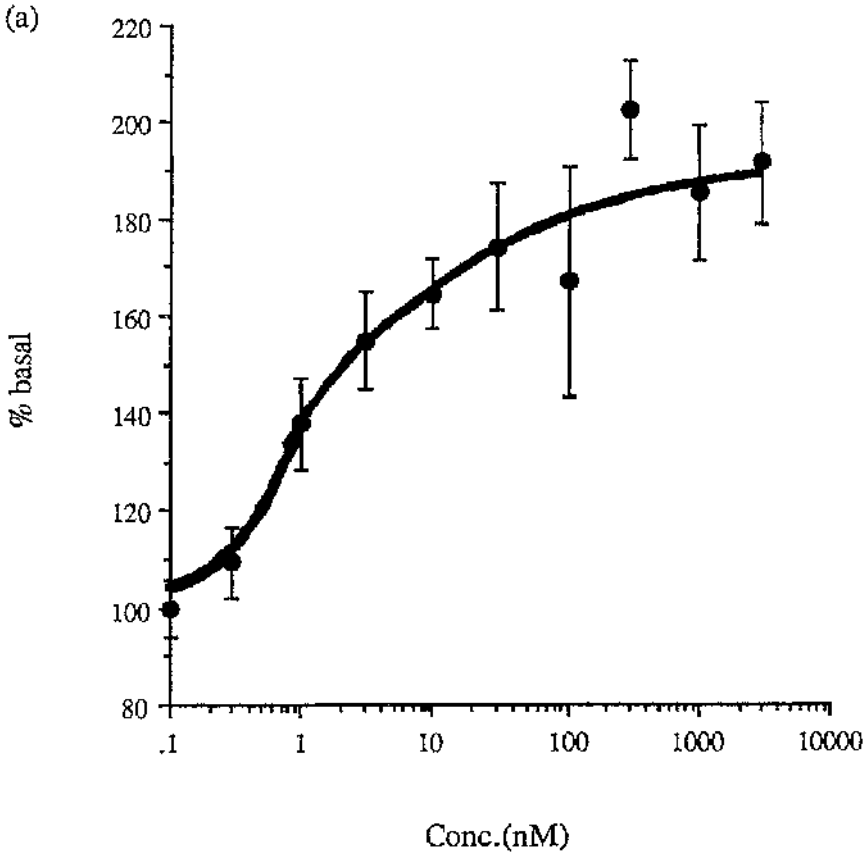
Figure 3.8(a) **Dose-dependency of bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced in 2% (v/v) NBCS.**

Swiss 3T3 fibroblasts were quiesced in DMEM containing 2% (v/v) NBCS and 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid for 24 hours. Cells were then stimulated for 30 seconds with the concentrations of bombesin shown (●). Result shown is representative of 6 individual experiments with the values being presented as % basal ± s.d. corresponding to a basal value of 550 ± 40 dpm.

Figure 3.8(b) **Dose-dependency of bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced in 10% (v/v)NBCS.**

Swiss 3T3 fibrobalsts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was replaced by DMEM containing 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid and 10% (v/v) NBCS for 24 hours. Cells were stimulated for 20 seconds with the concentrations of bombesin shown (●). Result is representative of 5 individual experiments with values being presented as % basal ± s.d. where the basal level is equivalent to 400 ± 30 dpm.

(a)


220
200
180
160
140
120
100
80
% basal
.1
1
10
100
1000
10000
Conc.(nM)

(b)

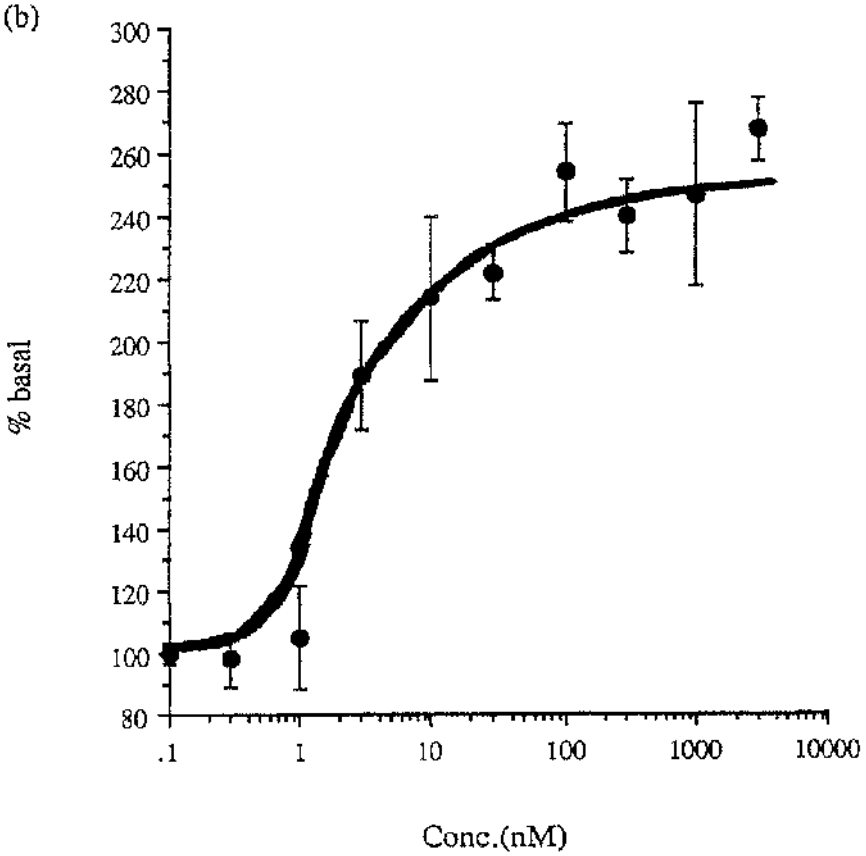

300
280
260
240
220
200
180
160
140
120
100
80
% basal
.1
1
10
100
1000
10000
Conc.(nM)

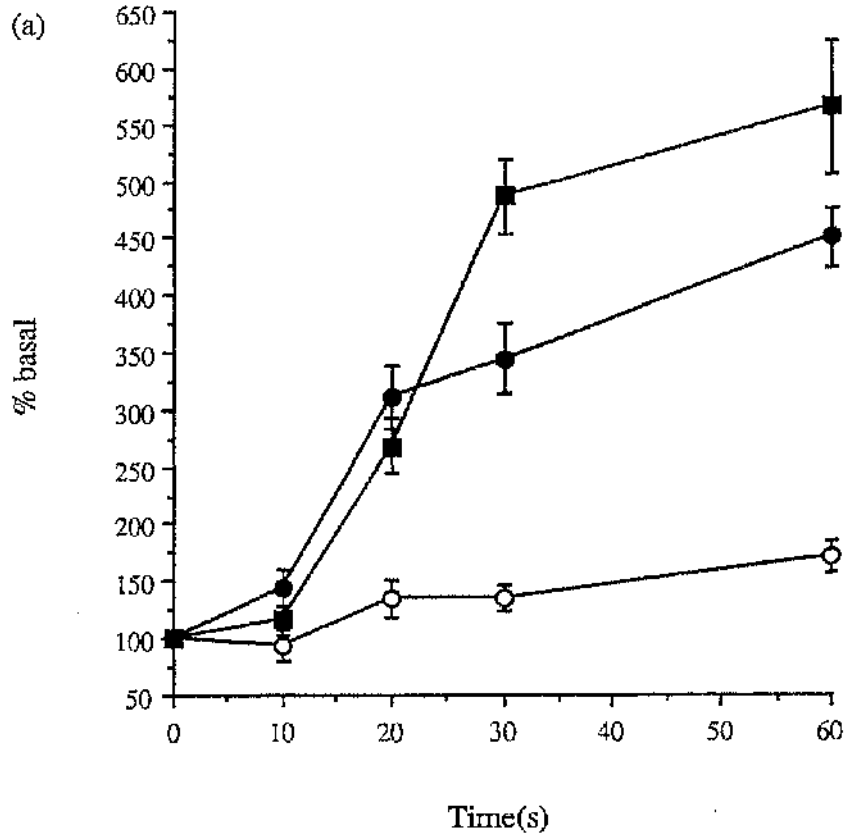
Figure 3.9(a) **Effect of GTPγS upon submaximal bombesin-stimulated total [^{3}H] arachidonate release in Streptolysin O-permeabilised Swiss 3T3 fibroblasts quiesced in 2% (v/v) NBCS.**

Swiss 3T3 fibroblasts cultured in 10% (v/v) NBCS until 80-90% confluent when medium was changed to DMEM containing 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid and 2% (v/v) NBCS for 24 hours. Cells were washed and permeabilised for 5 minutes using 0.6units/ml Streptolysin O in permeabilisation buffer. 10nM bombesin (●), 30μM GTPγS (○) and both together (■) were diluted in permeabilisation buffer and used to stimulate the cells for the times shown. Result shown represents 2 individual experiments with values being represented as % basal ± s.d., where basal value is equivalent to 800 ± 60 dpm.

Figure 3.9(b) **Effect of GTPγS upon submaximal bombesin-stimulated total [^{3}H] arachidonate release in Streptolysin O-permeabilised Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was replaced with DMEM containing 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid and 10% (v/v) NBCS for 24 hours. Cells were then washed and permeabilised for 5 minutes with 0.6 units/ml Streptolysin O in permeabilisation buffer. 10nM bombesin (●), 30μM GTPγS (○) and both together (■) were diluted in permeabilisation buffer and used to stimulate the cells for the times shown. Result shown is representative of 2 individual experiments with the values being presented as % basal ± s.d., where the basal value is 850 ± 75 dpm.

(a)


650
600
550
500
450
400
350
300
250
200
150
100
50
% basal
0
10
20
30
40
50
60
Time(s)

(b)

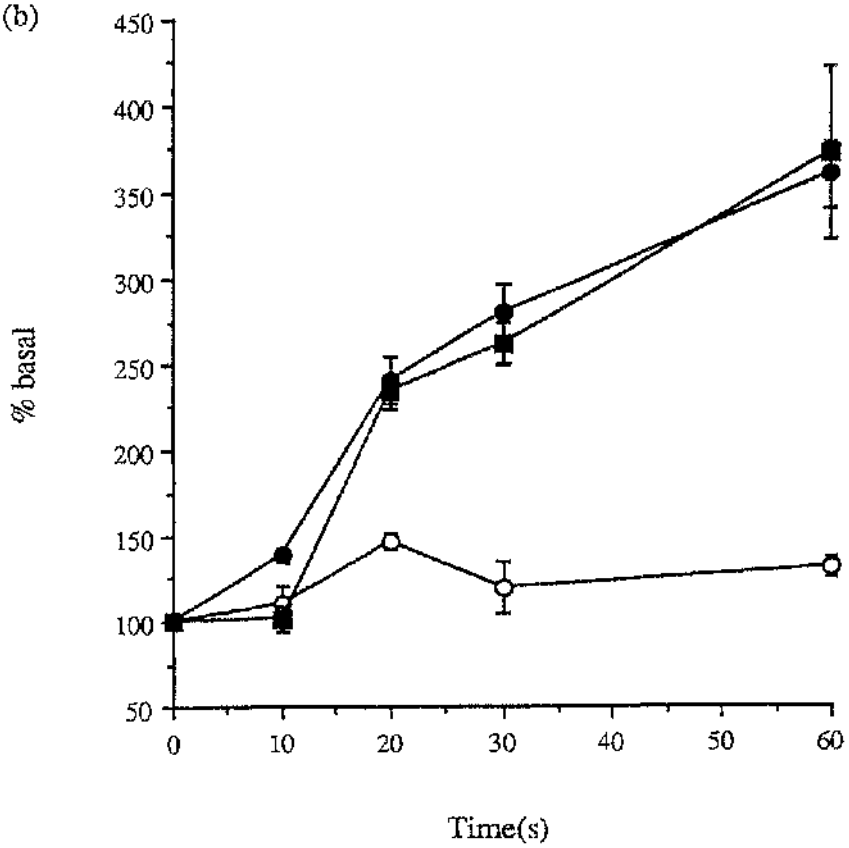

450
400
350
300
250
200
150
100
50
% basal
0
10
20
30
40
50
60
Time(s)

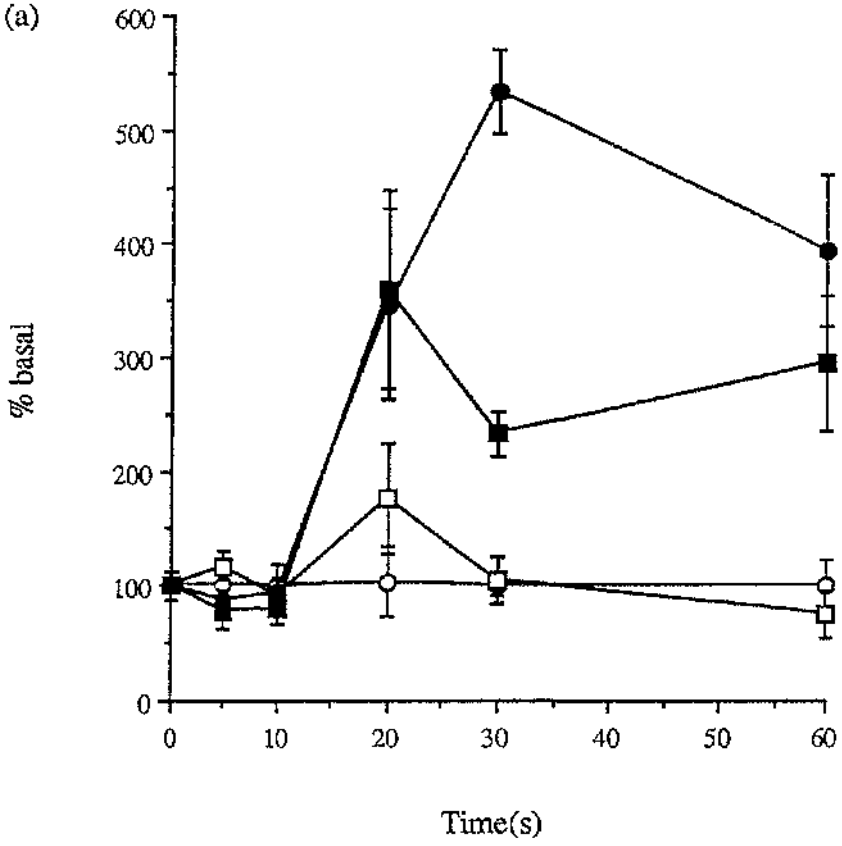
Figure 3.10(a) **Effect of GTPγS upon maximal bombesin-stimulated total [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced using 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when the medium was replaced with DMEM containing 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid and 10% (v/v) NBCS for 24 hours. Cells were washed and permeabilised with 0.6units/ml Streptolysin O in permeabilisation buffer for 5 minutes at 37°C. Vehicle alone (○), 100nM bombesin (●), 30μM GTPγS (□) and both together (■) were diluted in permeabilisation buffer and used to stimulate the cells for the times indicated. Result shown is representative of 2 individual experiments with values presented as % basal ± s.d., where the basal level is equivalent to 900 ± 90 dpm.

Figure 3.10(b) **Effect of GDPβS upon maximal bombesin-stimulated total [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced using 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when the medium was changed to DMEM containing 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid and 10% (v/v) NBCS for 24 hours. Cells were washed and permeabilised usin 0.6units/ml Streptolysin O in permeabilisation buffer for 5 minutes at 37°C. Vehicle alone (○), 100nM bombesin (●), 2mM GDPβS (□) and both together (■) were diluted in permeabilisation buffer and used to stimulate the cells for the times indicated. Result shown is representative of 3 individual experiments with values presented as % basal ± s.d., where the basal level is equivalent to 1250 ± 135 dpm for each of the experiments performed.

(a)


600
500
400
300
200
100
0
% basal
0
10
20
30
40
50
60
Time(s)

(b)

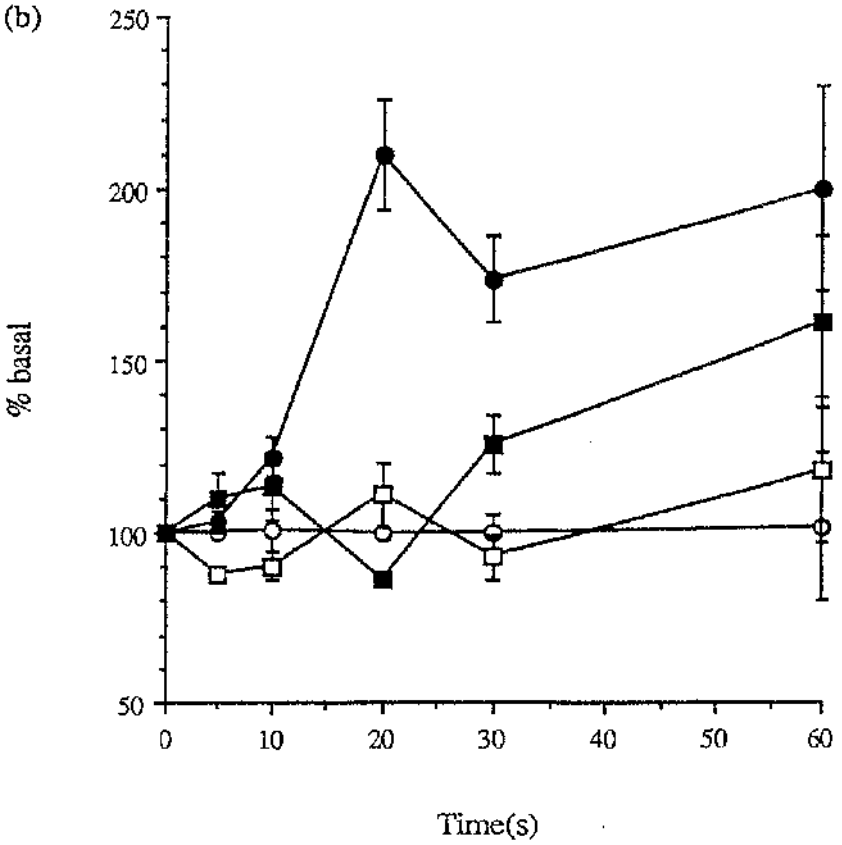
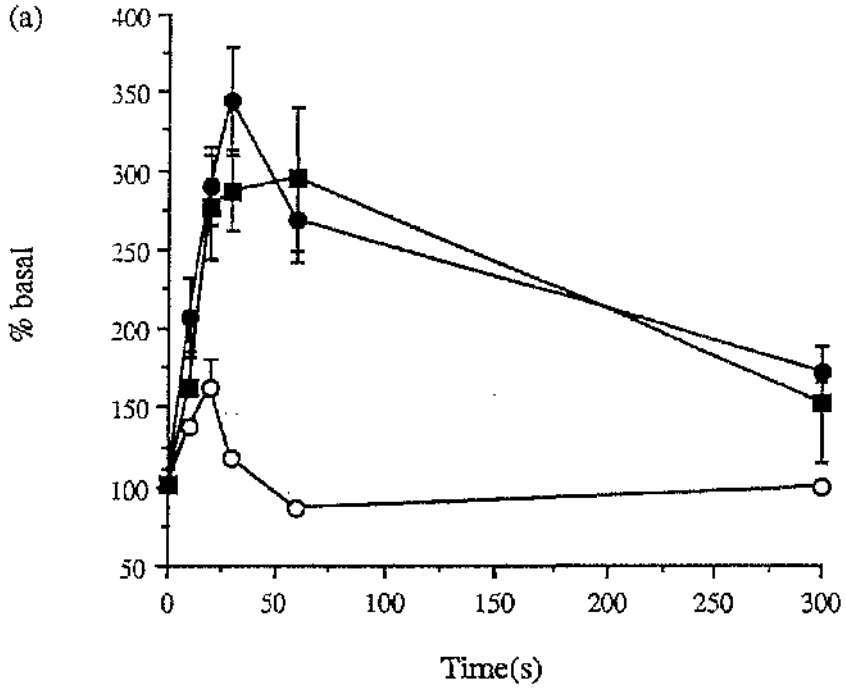

250
200
150
100
50
% basal
0
10
20
30
40
50
60
Time(s)

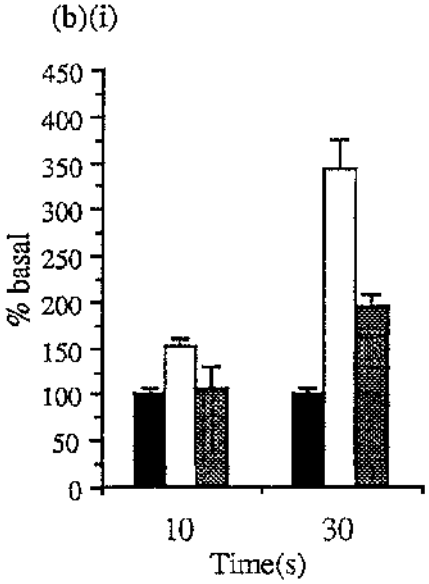
Figure 3.11(a) **Effect of Pertussis toxin upon bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced using 10% (v/v) NBCS.**

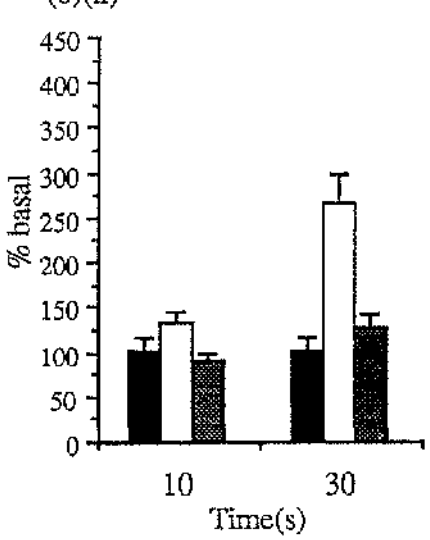
Cells were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when the medium was replaced with DMEM containing 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid and 10% (v/v) NBCS in the presence or absence of 25ng/ml pertussis toxin for 24 hours. Cells were then stimulated for the times indicated with 100nM bombesin. Result shown is a representation of 3 individual experiments and the values are presented as % basal ± s.d., where the basal value is equivalent to 800 ± 55 dpm. Vehicle alone (○), 100nM bombesin (●) and 100nM bombesin after pertussis toxin treatment (■).

Figure 3.11(b) **Aluminium Fluoride-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced using 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was replaced with DMEM containing 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid and either (i) 2% (v/v) or (ii) 10% (v/v) NBCS for 24 hours. Permeabilisation was achieved by preincubation with 0.6units/ml Streptolysin O in permeabilisation buffer for 5 minutes at 37°C. Cells were stimulated for the times shown with vehicle (■), 10nM bombesin (❑) or 20μM aluminium chloride/30mM sodium fluoride (❑) diluted in permeabilisation buffer and total arachidonate measured (iii) Whole cells quiesced in 10% (v/v) NBCS were stimulated with the same concentrations of agonists but diluted in DBG and intracellular arachidonate measured. Result is representative to 3 individual experiments with the values presented as % basal ± s.d., where the basal level is equivalent to vehicle-stimulated arachidonate release and is 900 ± 85 dpm.


(a)
% basal
400
350
300
250
200
150
100
50
0
50
100
150
200
250
300
Time(s)


(b)(i)
% basal
450
400
350
300
250
200
150
100
50
0
10
30
Time(s)


(b)(ii)
% basal
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400
350
300
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200
150
100
50
0
10
30
Time(s)

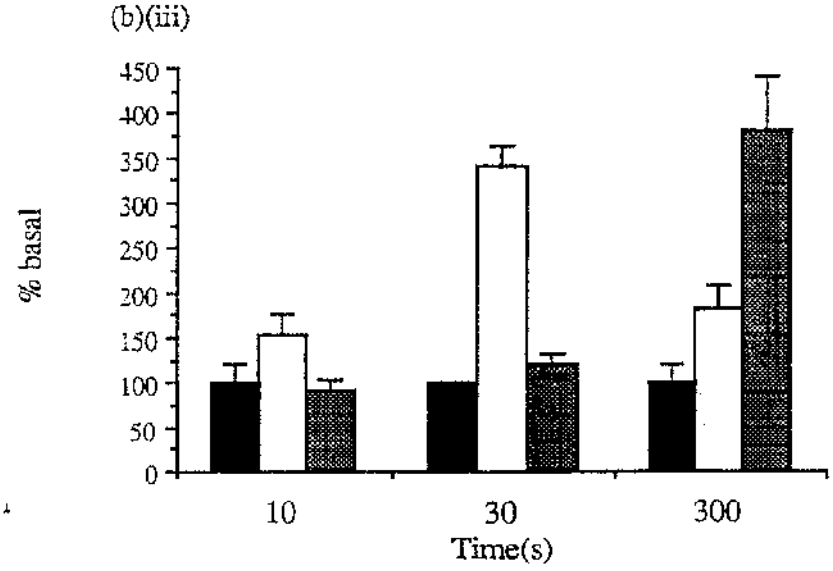
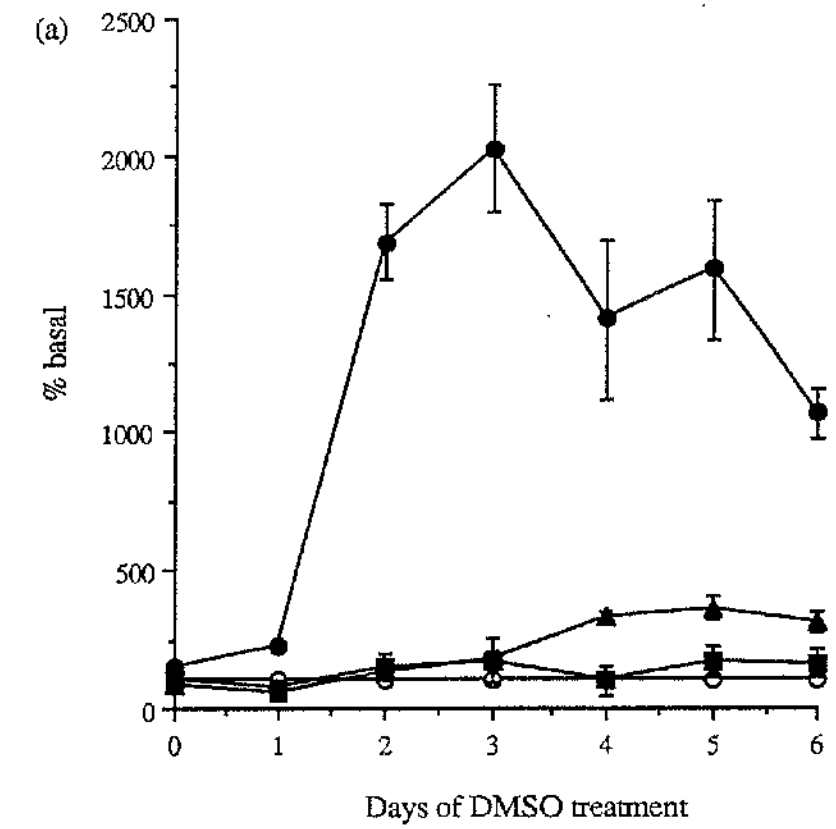

(b)(iii)
% basal
450
400
350
300
250
200
150
100
50
0
10
30
300
Time(s)

Figure 3.12(a) Effect of differentiation upon stimulated total [^{3}H] arachidonate release in HL60 cells.

HL60 cells were seeded at $5x10^5$ cells per ml in RPMI 1640 containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) dimethyl sulphoxide for the times indicated. 17hours prior to harvesting 0.25μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid was added to the cell culture. On harvesting, cells were washed and resuspended at 10^7 cells/ml in RBG. 10^6 cells were pretreated with 5μM cytochalasin B for 5 minutes prior to stimulation with either RBG (○), 100nM TPA (■), 0.5mM perhydrovanadate (●) for 10 minutes or 100nM fMLP (▲) for 5 minutes. Total arachidonate was measured. Result is representative of 3 individual experiments with values presented as % basal ± s.d., where the basal value is equivalent to 1200 ± 115 dpm.

Figure 3.12(b) Comparison of agonist-stimulated total [^{3}H] arachidonate release in cytochalasin B-primed and -unprimed 5 day DMSO-differentiated HL60 cells.

HL60 cells were seeded at $5x10^5$ cell/ml in RPMI 1640 containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO for 5 days. 17 hours prior to harvesting 0.25μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid was added. Cells were washed and resuspended in RBG at 10^7 cells/ml. 10^6 cells were preincubated for 5 minutes with either RBG (❑) or 5μM cytochalasin B (■) and stimulated with either RBG, 100nM TPA, 10μM platelet activating factor, 100μM lysophosphatidic acid for 10 minutes or 100nM fMLP for 5 minutes. Result is a representation of 3 individual experiments with values being presented as % basal ± s.d., where the basal value is equivalent to a value of 400 ± 150 dpm for each experiment.


(a)
2500
2000
1500
1000
500
0
% basal
0
1
2
3
4
5
6
Days of DMSO treatment

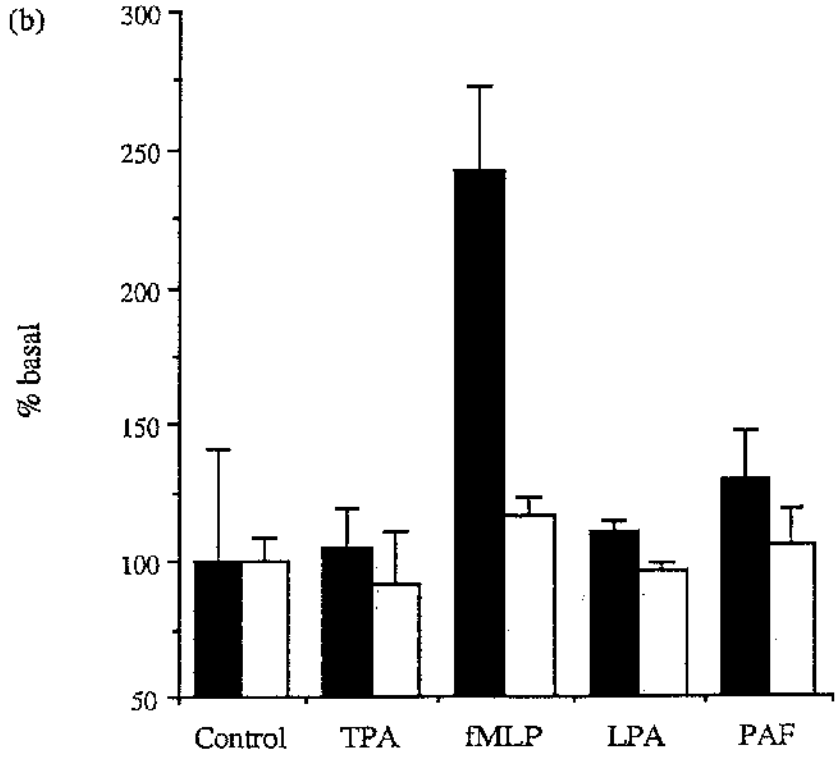

(b)
300
250
200
150
100
50
% basal
Control
TPA
fMLP
LPA
PAF

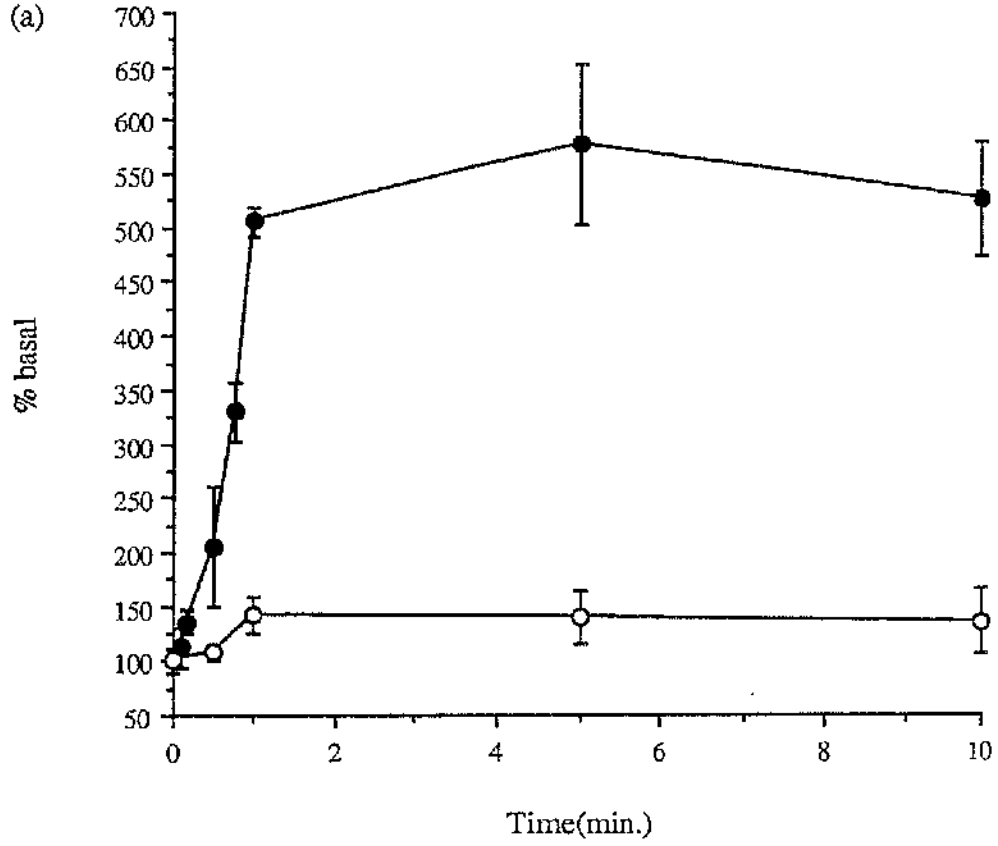
Figure 3.13(a) fMLP-stimulated total [^{3}H] arachidonate release in DMSO-differentiated, cytochalasin B-primed HL60 cells.

HL60 cells were seeded at $5x10^5$ cells/ml in RPMI 1640 containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO. Cells were allowed to differentiate for 5 days with 0.25μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid being added 17 hours prior to harvesting. 10^6 cells were used per sample which were incubated for 5 minutes with 5μM cytrochalasin B at 37°C. The cells were stimulated with either RBG (○) or 100nM fMLP (●) for the times indicated. Result shown is representative of 3 individual experiments with the values being presented as % basal ± s.d., where the basal value is equivalent to 500 ± 45 dpm.

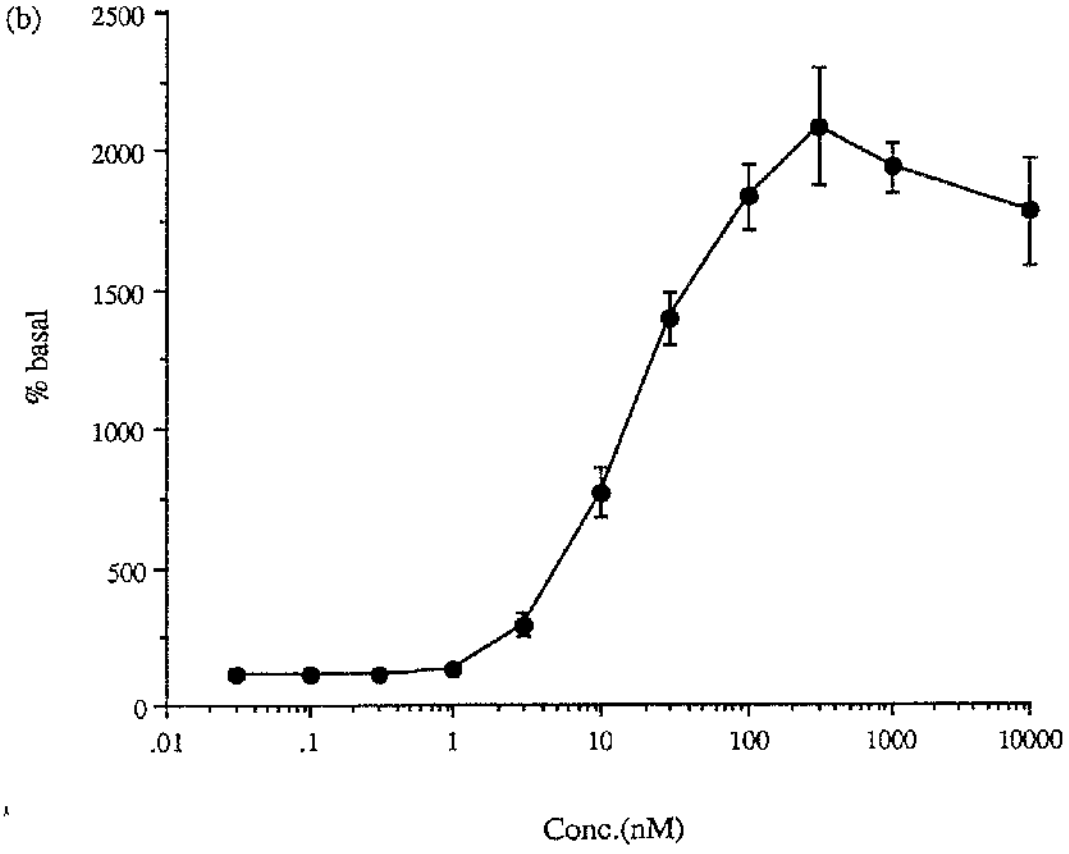
Figure 3.13(b) Dose-dependency of fMLP-stimulated total [^{3}H] arachidonate release in DMSO-differentiated, cytochalasin B-primed HL60 cells.

HL60 cells were seeded at $5x10^5$ cells/ml in RPMI 1640 containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO. Cells were allowed to differentiate for 5 days with 0.25μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid being added 17 hours prior to harvesting. 10^6 cells were used per sample which were incubated with 5μM cytochalasin B for 5 minutes at 37°C prior to stimulation for 5 minutes with the various fMLP concentrations shown (●). Result is representative of 3 individual experiments with the values presented as % basal ± s.d., where the basal level, as determined by incubating with RBG in place of fMLP for 5 minutes, is equivalent to an average value of 560 ± 45 dpm over the times taken.

(a)


700
650
600
550
500
450
400
350
300
250
200
150
100
50
% basal
0
2
4
6
8
10
Time(min.)

(b)


2500
2000
1500
1000
500
0
% basal
.01
.1
1
10
100
1000
10000
Conc.(nM)

3.3 DISCUSSION

3.3.1 Fatty acid product of $cPLA_2$ activation.

The fatty acid product of $cPLA_2$ activity has been widely thought to be arachidonate (20:4), due to the importance of this *cis*-unsaturated fatty acid as the precursor of eicosanoid production. Many groups have reported agonist-stimulated [4,5,8,9,11,12,14,15-^{3}H] arachidonate release, but as shown in Figure 3.3(b), it can also be measured chemically. The amount of material required to measure the physiological levels of arachidonate is much greater than that required for radiochemical analysis. With this in mind, Figure 3.3(a) demonstrates that a large amount of other fatty acids are released upon agonist stimulation. However, these are very difficult to identify and may not be due to cytosolic phospholipase A_2 activity, but maybe due to other lipase actions, including the lysophospholipase activity that $cPLA_2$ also exhibits (Leslie *et al.*, 1991). In particular, most of the saturated species identified are generally found in the *sn*-1 position of the diacylglycerol backbone of phospholipids, which would be the site of action of a lysophospholipase after the hydrolysis of the *sn*-2 position by phospholipase A_2 activity (She *et al.*, 1994). Another factor to be taken into consideration is the abundance of these fatty acids within the phospholipid pools. It has been shown that arachidonate is one of the least abundant of the fatty acids found in eukaryotic cells, therefore, even though such a small amount is released, this maybe a very high proportion of the available arachidonate within the cell, whereas the proportion of other fatty acids released maybe minute in relation to their abundance within the cell (Buttke *et al.*, 1984). One fatty acid that was seen to be released was oleic acid (18:1) which has been reported to be required for maximal activity of certain isoforms of protein kinase C (McPhail *et al.*, 1984) and phospholipase D (PLD) (Chalifa *et al.*, 1990; Massenburg *et al.*, 1994). Using radiochemical analysis, Figure 3.1(a) demonstrates that endothelin 1-stimulated, biphasic release of oleate in Rat-1 fibroblasts is very similar in kinetic terms to that observed for arachidonate release in Figure 3.2(a). This maybe via the

same receptor since Figure 3.2(b) and 3.3(b) show that the EC_{50} for both initial phases was 3nM(± 2nM); however, the concentration of endothelin-1 required to produce maximum arachidonate release differs, which would suggest the occupation of diffferent receptor subtypes. This question could only be answered by receptor binding assays which have shown the existence of three such subtypes of endothelin receptor (for review see Miller *et al.*, 1993). Whether the endothelin-1-stimulated oleate release observed is due to $cPLA_2$ activity remains to be determined. If it is the same enzyme responsible for both, there does seem to be a preference towards arachidonate-containing phospholipids, since the amount of arachidonate released from that available is high compared to the relative amount of oleate released. However, this could also be explained by either different rates of hydrolysis at the *sn*-1 and *sn*-2 position by the cytosolic phospholipase A_2 activity and the same enzyme's lysophospholipase activity or by different rates of reincorporation of the free fatty acids into the phospholipid pools.

3.3.2 Kinetics of arachidonate release

Due to these observations and their correlation with published work, arachidonate was chosen as the fatty acid most appropriate to study the regulation of agonist-stimulated cytosolic phospholipase A_2 activity. Bombesin-stimulated arachidonate release in murine Swiss 3T3 fibroblasts has been previously reported (Currie *et al.*, 1992). In this published work, the cells were quiesced by contact inhibition in DMEM containing 10% (v/v) newborn calf serum. This was of relevance, since other phospholipases studied in bombesin-stimulated Swiss 3T3 fibroblasts, have been quiesced by serum deprivation (DMEM containing 2% (v/v) newborn calf serum 24 hours prior to experimentation) (Cook & Wakelam, 1989). The bombesin-stimulated arachidonate release in cells cultured in each condition is biphasic as shown in Figure 3.4(a) and (b), however, there were subtle differences in the kinetics. In 10% serum, the initial transient phase appeared to be of a smaller magnitude to that produced in 2% serum quiesced cells. Both initial phases return to

basal by 10 minutes, with the second sustained phase being evident at 20 minutes. The response in 10% cells is of a smaller magnitude of those in 2%, however, it appears to be kinetically faster, being maximal at 20 minutes and returning towards basal by 30 minutes. The second phase in cells quiesced in 2% is increasing at 30 minutes, returning to basal by 60 minutes (result not shown). Therefore, it appears that the second phase is kinetically quicker but of a lower magnitude in contact inhibited cells compared to serum starved cells. This observation could be due to an effect of the quiescing method on a multitude of signalling enzymes. In the acute, initial transient phase of the response, however, only a phospholipase of the A_2 type could be responsible and again, it appears that in 10% quiesced cells, the magnitude of the response is less than in 2% quiesced cells at the 30 second time point taken. This is not the true picture as Figure 3.5(a) and (b) demonstrates. Here it is apparent that in contact inhibited cells the onset of the bombesin-stimulated arachidonate release is quicker than in 2% quiesced cells, the magnitude and the duration of the response being identical in both cases. It appears that the cytosolic phospholipase A_2 responsible for the response is more reactive to subsequent bombesin stimulation in 10% quiesced cells. This could be described as a priming event, since the higher concentration of serum used to quiesce the cells produces a more reactive enzyme, but does not activate it itself, as the basal levels of arachidonate are identical to, if not even slightly lower, than those in 2% quiesced cells.

3.3.3 Possible serum components responsible for priming effect

This data suggested that there is a component within serum which is at a high enough concentration to prime $cPLA_2$ in 10% (v/v) NBCS. One such component maybe insulin or insulin-like growth factor I as Figure 3.6(a) demonstrates that quiescing in 2% (v/v) NBCS containing 10μg/ml insulin, result in cells which respond to bombesin in a manner similar to cells quiesced by contact inhibition using DMEM containing 10% (v/v) NBCS. However, the response is more sustained, suggesting that other activators of $cPLA_2$ are stimulated, in addition to those primed by the serum

component responsible in 10% (v/v) NBCS. Inclusion of insulin in cells quiesced in 10% (v/v) NBCS-containing medium had no effect upon the kinetics of subsequent bombesin-stimulated arachidonate release (Result not shown).

Another possible component of serum that may be responsible for priming is lysophosphatidic acid (LPA). Figure 3.6(b) shows that LPA itself produced a small arachidonate release in 2% quiesced cells, but also produced a potentiation of the bombesin-stimulated response in both quiesced conditions. This is probably due to the activation of MAP kinase by LPA as reported by Howe and Marshall (1993). The initial LPA-stimulated MAP kinase activation produced would phosphorylate $cPLA_2$ leading to the small amount of arachidonate released, but also the priming of the enzyme so as to be more sensitive to bombesin stimulation. The small LPA-stimulated arachidonate release was more apparent in 2% quiesced cells compared to 10% cells, probably due to the LPA present in the serum being at a high enough concentration to produce the priming response and thus desensitize the cell to further LPA stimulation.

The sensitivity of the cells quiesced under the two conditions to LPA was a dose dependent phenomenon (Figure 3.7). When cells quiesced in 2% were preincubated with various concentrations of LPA and then stimulated with 100nM bombesin there was a small potentiation of the bombesin-stimulated arachidonate release even at very low concentrations of LPA. Those quiesced in 10% serum showed no additional response at these lower LPA concentrations, suggesting the desensitization of the LPA receptor, due to the higher concentration of LPA in the serum. However, between 1μM and 30μM LPA there was an identical potentiation of bombesin-stimulated arachidonate release in both serum conditions. This may be due to the occupation of a higher number of receptors or of other lower affinity LPA receptors stimulating the same pathways, which may explain the overriding of the desensitization seen at lower LPA concentrations in cells quiesced in 10% NBCS. The activation of additional $cPLA_2$ regulators by these higher concentrations of LPA could explain the very similar responses seen in both conditions. Therefore, it can be

suggested that both LPA and insulin can prime cytosolic phospholipase A_2, probably through MAP kinase activation. This has still to be confirmed.

3.3.4 Role of GTP-binding proteins in both chronically primed and unprimed Swiss 3T3 fibroblasts.

In order to identify the site of action of priming observed in Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS, the possible regulatory proteins of $cPLA_2$ were studied in isolation.

Receptors for a range of agonists can exist in both high and low affinity states (Prossnitz *et al.*, 1995(a)). It was thus possible that the affinity of the bombesin receptor was being affected by priming. There is apparently only one receptor subtype for bombesin expressed upon Swiss 3T3 fibroblasts (Shapira *et al.*, 1991) and as shown in Figure 3.8, the affinity of this receptor was unaffected by the quiescing method employed, with an EC_{50} value of 3nM (± 2nM) in both cases. Since the bombesin receptor is a classical 7 membrane spanning-G-protein linked receptor, the effect of priming upon the GTP-binding proteins involved in the regulation of $cPLA_2$ was studied.

Using maximal concentrations of bombesin (10nM) as determined by the dose-dependency in the preceding figure and 30μM GTPγS, the non-hydrolysable analogue of GTP, in streptolysin O permeabilised Swiss 3T3 fibroblasts, Figure 3.9(a) demonstrates that there was a potentiation of bombesin-stimulated arachidonate release in serum-starved cells. This potentiation was observed at 30 seconds and release remained elevated after 1 minute. There was no such potentiation observed in 10% quiesced cells as shown in Figure 3.9(b). A potentiation of agonist-stimulated arachidonate release has been observed in Swiss 3T3 fibroblasts, quiesced in 10% (v/v) NBCS (Currie *et al.*, 1992), but the method for isolating free arachidonate differed, however it is well known that there is a variation between clones of the same cell type. With this in mind, it appears that the GTP-binding protein involved in bombesin-stimulated arachidonate release is only able to bind GTPγS in 2% quiesced

cells, suggesting that in 10% quiesced cells, the GTP-binding protein is already fully loaded with GTP. In both cases, there must be agonist-stimulation to produce a rapid response, with GTPγS producing a small, significant release of arachidonate after 5 minutes (results not shown), corresponding to the basal GTP-GDP exchange rate within the cell.

GTP-binding proteins have a role in the activation of bombesin-stimulated arachidonate release in 10% quiesced cells, since the non-hydrolysable analogue of GDP, GDPβS, completely abolished the response as shown in Figure 3.10(b). This further substantiates the possibility that the GTP-binding protein involved in bombesin-stimulated arachidonate release in 10% quiesced cells is already loaded with GTP, but requires subsequent agonist stimulation to produce arachidonate release.

Due to the fact that a potentiation by GTPγS could not be produced by maximal bombesin concentrations in 10% quiesced Swiss 3T3 fibroblasts, the effect of supramaximal bombesin concentrations (100nM) used throughout the kinetic studies in this work was examined. Figure 3.10(a) shows an effect, but apparently an inhibition of bombesin-stimulated arachidonate release, rather than a potentiation. This could be explained by the activation of a GTP-dependent feedback inhibition upon $cPLA_2$. The most probable pathway to be activated by GTPγS, that could inhibit bombesin-stimulated arachidonate release, perhaps via an effect upon the MAP kinase pathway, is the production of cyclic AMP by activation of adenylyl cyclases (Millar & Rozengurt, 1988).

The GTP-binding protein involved in bombesin-stimulated arachidonate release in Swiss 3T3 fibroblasts quiesced under both serum conditions was pertussis toxin insensitive as shown in Figure 3.11(a) . To determine the type of GTP-binding protein involved, the heterotrimeric G-protein activator aluminium fluoride (AlF^{4-}) was used on both streptolysin O permeabilised and intact cells. This ion mimics the terminal phosphate of GTP, binding to the GTP-binding site of the α-subunit of the heterotrimeric G-proteins, but cannot be hydrolysed by the intrinsic GTPase activtity. This effect is not seen in small molecular weight GTP-binding proteins, suggesting a

difference in the stoicheimetric binding of GTP and thus providing a tool to discriminate between the two types of agonist-stimulated GTP-binding proteins (Kahn, 1991). In permeabilised 2% quiesced cells (Figure 3.11(b)(i)), there was a small arachidonate release at 30 seconds AlF^{4-} stimulation, whereas there was nothing in permeabilised 10% quiesced cells (Figure 3.11(b)(ii)). In whole cells (Figure 3.11(b)(iii)), arachidonate release was seen at 5 minutes, in response to AlF^{4-}. Taking all of these results together, it appears that there is an involvement of a heterotrimeric G-protein at later time points in both permeabilised and whole cells. The lack of GTP-input in the initial onset of bombesin-stimulated arachidonate release could be explained by the effect of endogenous GTP already bound to the GTP-binding protein involved, or the role of a small molecular weight GTP-binding protein, such as *rac*, in the response. These small molecular weight G-proteins have been suggested to be involved in various signalling pathways, including the regulation of $cPLA_2$ (for review see Hall, 1994). However, more work would have to be carried out to try and elucidate which form of GTP-binding protein is involved.

3.3.5 HL60 cells as a model of acute priming.

To aid the study of the chronic priming observed in Swiss 3T3 fibroblasts, a well established model, HL60 cells, of acute priming was utilised. The premyeolocitic human leukemic cell line HL60 can be differentiated by such agents as the phorbol esters, dibutyryl cyclic AMP and retinoic acid, in order to observe various agonist-stimulated signalling pathways. One such agonist is the chemotactic peptide formlyMethylLeucinePhenylalanine (fMLP). The expression of the receptor for fMLP is induced when HL60 cells are differentiated to a neutrophil-like cell using 1.3% (v/v) dimethyl sulphoxide (DMSO) in the culture medium for 3-5 days (Collins, 1987). As well as differentiation, a requirement for priming with such agents as cytochalasin B has been reported in order to observe fMLP-stimulated signalling responses (for review see Cockcroft, 1992). Figure 3.12(a) demonstrates that cytochalasin B-primed, fMLP-stimulated arachidonate release was observed after 4 days of DMSO-

differentiation. However, a tyrosine phosphorylated activator of $cPLA_2$ was active after only 2 days of DMSO-differentiation as shown by the results using the tyrosine phosphophosphatase inhibitor perhydrovanadate (0.5mM) (Figure 3.12(a)). These results suggest a role for tyrosine kinases in the regulation of arachidonate release in DMSO-differentiated HL60 cells. At the time point chosen, there does not appear to be a role for protein kinase C in the stimulation of arachidonate release, as the phorbol ester TPA did not produce any arachidonate release even after 6 days of DMSO differentiation.

The observed fMLP-stimulated arachidonate release in 5 day DMSO-differentiated HL60 cells was dependent on preincubation with cytochalasin B as shown in Figure 3.12(b) an effect that was both time- and dose-dependent (Figure 3.13(a) and (b)). The response was rapid, plateauing after 1 minute but remaining elevated for the 20 minutes fMLP treatment. This plateauing could be due to an equilibrium between arachidonate release and metabolism by the lipoxygenase and cyclooxygenase pathways, as these cells have been reported to produce high levels of the inflammatory mediators produced by these metabolic pathways. The response to fMLP was variable as can be seen for the fold stimulation differences in Figure 3.13(a) and (b), which can be explained by the length of time the cells are in culture as these cells have been shown to lose agonist responsiveness over a certain period of time in cell culture. However, it was only the magnitude of the response that differed, not the affinity of the receptor to fMLP.

In conclusion, this chapter demonstrates that arachidonate-containing phospholipids are the preferred substrate for $cPLA_2$ in Swiss 3T3 fibroblasts, however other fatty acids can be detected both chemically and by radiolabelling upon bombesin-stimulation, but may not be due to cytosolic phospholipase A_2 activation, but to the lysophospholipase activity that $cPLA_2$ exibits. Bombesin-stimulated arachidonate release as measured by radiolabelling, differed depending on the method adopted to quiesce Swiss 3T3 fibrobalsts, prior to agonist treatment, where cells quiesced in 10% (v/v) NBCS exibited a higher sensitivity to bombesin stimulation.

This priming of $cPLA_2$ by a serum factor, such as insulin or lysophosphatidic acid, did not affect the affinity of the receptor, but the pertussis toxin-insensitive GTP-binding protein associated, appeared to be in a GTP-bound state. The activation of $cPLA_2$ by GTP-binding proteins in the premyelocytic HL60 cell has been well documented and has been shown to require both differentiation and priming (for review see Cockcroft, 1992). The proposed G-protein-mediated fMLP-stimulated arachidonate release also required DMSO-differentiation and cytochalasin B priming, to produce a time- and dose-dependent response. However, at shorter periods of DMSO-differentiation, a tyrosine kinase-stimulated arachidonate release was evident, suggesting the involvement of a tyrosine phosphorylated protein in $cPLA_2$ activation in these cells. Therefore, in the following chapter, the role of tyrosine phosphorylation in the regulation and priming of $cPLA_2$ will be discussed.

CHAPTER 4

THE ROLE OF TYROSINE PHOSPHORYLATION IN PRIMING OF CYTOSOLIC PHOSPHOLIPASE A_2

4.1 INTRODUCTION

The role of tyrosine phosphorylation as a key component of signalling pathways has been extensively studied in T- and B-cells (for review see Abraham *et al.*, 1992; Cushley & Harnett, 1993), human neutrophils (Torres *et al.*, 1993) and Rat-1 fibroblasts (Hordijk *et al.*, 1994). The proteins that have been shown to be phosphorylated upon tyrosine residues, following agonist stimulation, have a wide variety of effects within the cell including cytoskeletal rearrangement (Ridley & Hall, 1994), in the case of focal adhesion kinase (FAK) (Saville *et al.*, 1994), receptor autophosphorylation, in the case of the PDGF receptor (for review see Williams, 1989) and nuclear translocation as in the case of MAP kinase (Lenormand *et al.*, 1993).

The diversity of agonist stimulated tyrosine phosphorylation and the regulation of the tyrosine kinases involved has been studied to great lengths. This opened a whole new field of regulation in signalling pathways, since the serine/threonine directed kinases were thought to be the main kinase regulators of both phospholipase D and A_2 (Nishizuka, 1995). In the case of phospholipase D, a requirement for both protein kinase C phosphorylation and tyrosine kinase phosphorylation in activation has been demonstrated (Briscoe *et al.*, 1995). Cytosolic phospholipase A_2 activation has been shown to be regulated by protein kinase C (Currie *et al.*, 1992), whilst recently an indirect role for tyrosine phosphorylation has become apparent. Recent evidence has demonstrated a MAP kinase phosphorylation site in the sequence of cloned $cPLA_2$ and it has been shown that Ser 505 could be phosphorylated directly by MAP kinase *in vitro*. (Lin *et al.*, 1993)

Bombesin has been shown to stimulate an increase in tyrosine phosphorylation in Swiss 3T3 fibroblasts (Briscoe *et al.*, 1995). The results in this chapter, address the possible involvement of tyrosine phosphorylation in the chronic and acute priming of cytosolic phospholipase A_2 outlined in Chapter 3.

4.2 RESULTS

The priming of cytosolic phospholipase A_2 by cytochalasin B in DMSO-differentiated HL60 cells was shown in Chapter 3 to be essential for fMLP-stimulated arachidonate release. The mechanism by which cytochalasin B produces this priming effect is as yet unclear, however, as Figure 4.1(a) demonstrates, the agent increased overall tyrosine phosphorylation in 5 day DMSO-differentiated HL60 cells, suggesting that a tyrosine kinase maybe involved in the acute priming effects. In the chronic priming of Swiss 3T3 fibroblasts by serum, there was also an increase in basal levels of tyrosine phosphorylation as shown in Figure 4.1(b), where cells quiesced in 10% (v/v) newborn calf serum (NBCS) showed a significant increase in overall tyrosine phosphorylation compared to cells quiesced by serum starvation in DMEM containing 2% (v/v) NBCS.

The level of protein tyrosine phosphorylation can be elevated using the tyrosine phosphophosphatase inhibitor perhydrovanadate. Figure 4.2 (a) demonstrates that 0.5mM perhydrovanadate can stimulate arachidonate release in Swiss 3T3 fibroblasts quiesced in 10% serum. This was also the case in 2% quiesced cells (result not shown). This was also observed in DMSO-differentiated HL60 cells (Figure 4.2(b)), suggesting a role for tyrosine kinases in the regulation of $cPLA_2$. The kinetics of perhydrovanadate-stimulated arachidonate release corresponded to the second sustained phase of bombesin-stimulated arachidonate release in Swiss 3T3 fibroblasts. However, this association cannot be taken as absolute, since the inhibitor has to overcome both the physical barrier of entry into the cell to inhibit the phosphatases and rely upon basal tyrosine kinase activity in order for any significant effect to be observed. Taking this into consideration, DMSO-differentiated HL60 cells preincubated with 0.5mM perhydrovanadate for 2 minutes which does not produce arachidonate release, permitted the measurement of fMLP-stimulated arachidonate release (Figure 4.3(a)). Using this 2 minute preincubation of perhydrovanadate, subsequent fMLP-stimulation produced identical time-dependent kinetics of

arachidonate release (Figure 4.3(b)) to those seen when cytochalasin B was used as the priming agent.

As Figure 4.1(b) demonstrated, there was an increased basal level of tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced in DMEM containing the higher NBCS concentration. The serum factors insulin and lysophosphatidic acid (LPA) were shown to mimic the priming effect observed when they were added to 2% quiesced Swiss 3T3 fibroblasts (Figure 3.6). Both of these serum factors increased basal tyrosine phosphorylation levels (Figure 4.4) with differing banding patterns which might explain the different effects they produce. Figure 4.5(a) clearly shows that bombesin stimulated a transient increase in tyrosine phosphorylation in cells quiesced in 2% NBCS which was maximal at 30 seconds, reaching levels equivalent to the basal levels in cells quiesced in 10% NBCS. In these primed cells there did not appear to be an increase in overall tyrosine phosphorylation until after 1 minute, however, there did appear to be a dephosphorylation of a 69kDa protein at earlier time points (Figure 4.5(b)). The chronic primimg of Swiss 3T3 fibroblasts by serum did not inhibit the effect upon tyrosine phosphorylation by other agonists as shown in Figure 4.6.

To further elucidate the role of tyrosine phosphorylation upon cytosolic phospholipase A_2, an inhibitor demethoxyviridine (DMV) was employed. This analogue of the putative PI-3 kinase inhibitor wortmannin was shown to inhibit bombesin-stimulated tyrosine phosphorylation levels in Swiss 3T3 fibroblasts, markedly on a 69kDa protein, in a dose-dependent manner with an IC_{50} = 10nM (± 2nM) (Figure 4.7). In an *in vitro* bilayer assay utilising baculovirus expressed $cPLA_2$, there was a small inhibition (30%) of arachidonate release at 0.1nM wortmannin (Figure 4.8(a)), when the $cPLA_2$ activity enhancer phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) (Figure 4.8(b)) was included in the micelle. This inhibition was not as complete as speculated at the lower concentrations of wortmannin, suggesting multiple modes of action upon the enzyme by wortmannin, since at the higher concentrations, there was also an inhibition of arachidonate release

in the absence of PtdIns-4,5-P_2. Inhibition could not be detected in immunoprecipitated $cPLA_2$ from Swiss 3T3 fibroblasts (Figure 4.9), suggesting that the site of action of wortmannin was perhaps blocked in the immunoprecipitated enzyme from the whole cell. In whole cell experiments, the wortmannin analogue, DMV, produced a dose-dependent inhibition of both bombesin- and fMLP-stimulated arachidonate release (Figure 4.10(a) and (b) respectively); the IC_{50} in Swiss 3T3 fibroblasts was 2nM (± 2nM) and 200nM (± 20nM) in HL60 cells. These results suggested both direct and indirect effects of DMV and wortmannin upon $cPLA_2$.

Figure 4.1(a) **Western blot analysis of 5μM cytochalasin B-stimulated tyrosine phosphorylation in 5 day DMSO-differentiated HL60 cells.**

HL60 cells were seeded at $5x10^5$ cells per ml in RPMI 1640 medium containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) dimethylsulphoxide (DMSO) and cultured for 5 days. The cells were washed and 10^7 cells were used per sample. Samples were stimulated for 5 minutes at 37°C with either 5μM cytochalsin B or RBG and reactions were terminated by washing with ice-cold PBS. Lysis was carried out as described in Materials and Methods and 10μg total protein was loaded onto 10% (w/v) polyacrylamide gels and SDS-PAGE carried out as described in Chapter 2. Tyrosine phosphorylated proteins were visualised using ECL. Result is representative of 3 individual experiments.

Figure 4.1(b) **Western blot analysis of basal levels of tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced either by serum starvation (2% (v/v) NBCS) or contact inhibition (10% (v/v) NBCS).**

Cells cultured in DMEM containing 10% (v/v) newborn calf serum until 80-90% confluent when medium was replaced with DMEM containing either 10% (v/v) or 2% (v/v) NBCS for 24 hours. Cells were then washed 3 times with ice-cold PBS and lysates prepared as described in Materials and Methods. 10μg total protein was loaded onto 10% (w/v) polyacrylamide gels and SDS-PAGE carried out as described in Chapter 2. Tyrosine phosphorylated proteins were visualised using ECL. Result is representative of 3 individual experiments, which used freshly prepared lysates.

a

Cytochalasin B-stimulated tyrosine phosphorylation in 5 day DMSO-differentiated HL60 cells

b

Basal levels of tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced in 2% and 10% (v/v) NBCS

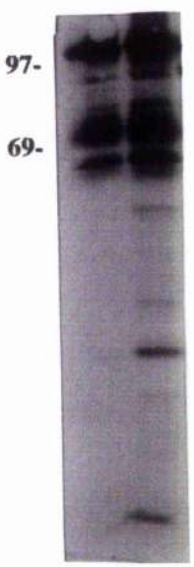
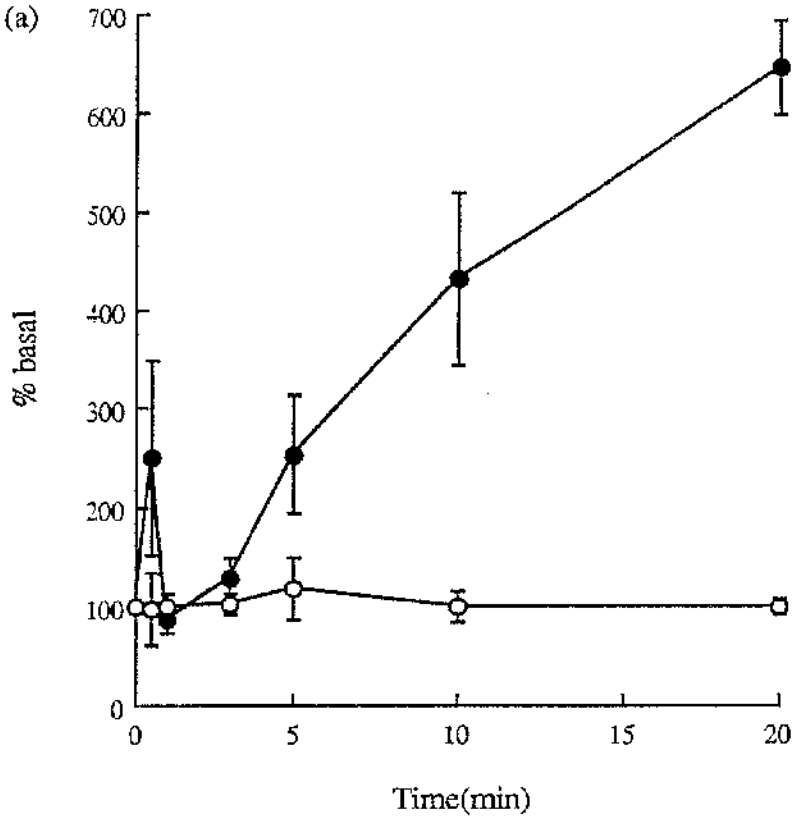


Figure 4.2(a) **Perhydrovanadate-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced in DMEM containing 10% (v/v) newborn calf serum.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was replaced with DMEM containing 10% (v/v) NBCS and 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid for 24 hours. Cells were stimulted with 0.5mM perhydrovanadate (●) in DBG or DBG alone (○) for the times indicated. Result is representative of 3 individual experiments with values being presented as % basal ± s.d., where the basal level was 1000dpm ± 200 dpm.

Figure 4.2(b) **Perhydrovanadate-stimulated total [^{3}H] arachidonate release in cytochalasin B-primed, 5 day DMSO-differentiated HL60 cells.**

HL60 cells were seeded at 5x10^5 cells per ml in RPMI 1640 medium containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO and cultured for 5 days. 17 hours prior to the end of the culturing period, 0.25μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid was added. 10^6 cells which had been preincubated for 5 minutes at 37°C with 5μM cytochalasin B, were stimulated for the times shown with either 0.5mM perhydrovanadate (●) in RBG or RBG alone (○). Result represents 3 individual experiments with values presented as % basal ± s.d., where the basal level was 500dpm ± 100 dpm for all experiments.


(a)
700
600
500
400
300
200
100
0
% basal
0
5
10
15
20
Time(min)

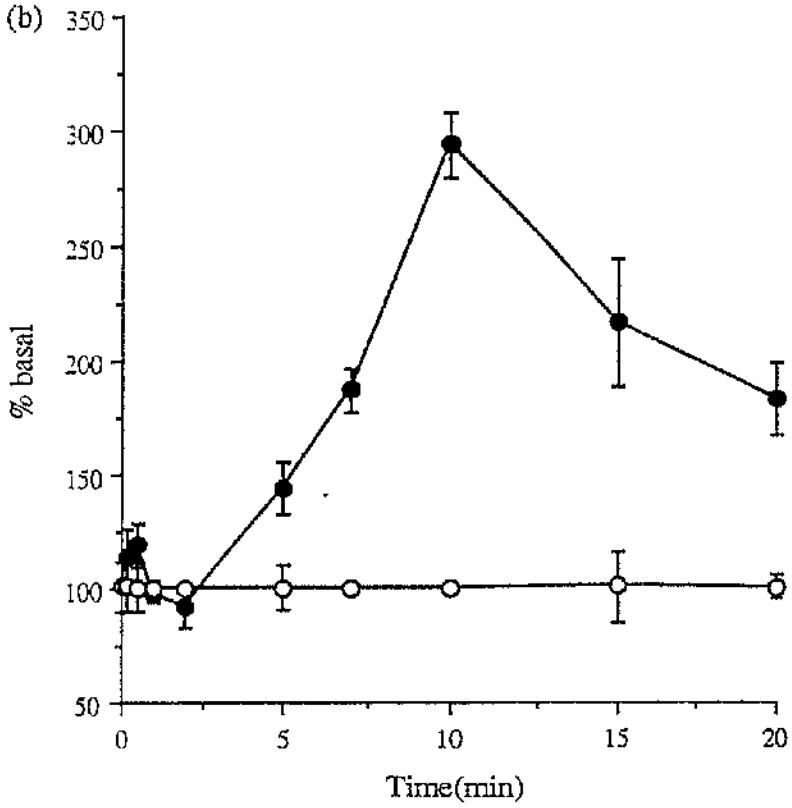

(b)
350
300
250
200
150
100
50
% basal
0
5
10
15
20
Time(min)

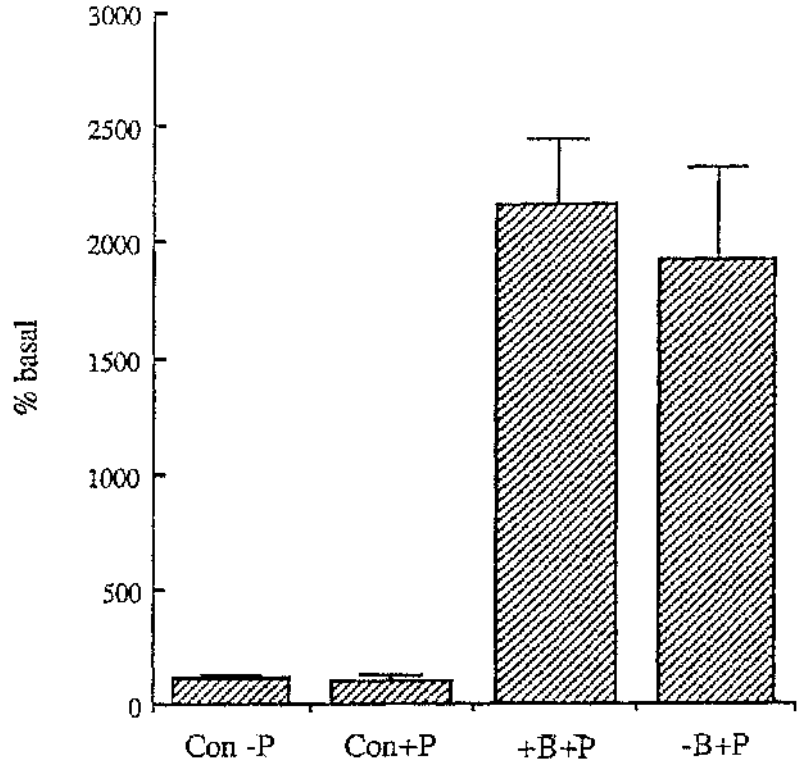
Figure 4.3(a) **Perhydrovanadate-stimulated total [^{3}H] arachidonate release in cytochalasin B-primed and -unprimed 5 day DMSO-differentiated HL60 cells.**

HL60 cells were seeded at $5x10^5$ cells per ml in RPMI 1640 medium containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO and cultured for 5 days. 17 hours prior to the end of the culturing period, 0.25μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid was added. 10^6 cells per sample were preincubated with either 5μM cytochalasin B in RBG or RBG alone for 5 minutes at 37°C. Preincubated samples were then stimulated with either 0.5mM perhydrovanadate in RBG or RBG alone for a further 5 minutes at 37°C. Result is representative of 3 individual experiments with values presented as % basal ± s.d., where the basal level was 800 ± 150 dpm.

Figure 4.3(b) **fMLP-stimulated total [^{3}H] arachidonate release in perhydrovanadate-primed, 5 day DMSO-differentiated HL60 cells.**

HL60 cells were seeded at $5x10^5$ cells per ml in RPMI 1640 medium containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO and cultured for 5 days. 17 hours prior to the end of the culturing period, 0.25μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid was added. 10^6 cells were used for each sample which were incubated with 0.5mM perhydrovanadate in RBG for 2 minutes at 37°C, prior to stimulation with either 100nM fMLP (●) in RBG or RBG alone (○) for the times indicated. Result is representative of 3 individual experiments with values being presented as % basal ± s.d., where the basal level was 700 ± 160 dpm.

(a)


3000
2500
2000
1500
1000
500
0
% basal
Con -P
Con+P
+B+P
-B+P

(b)

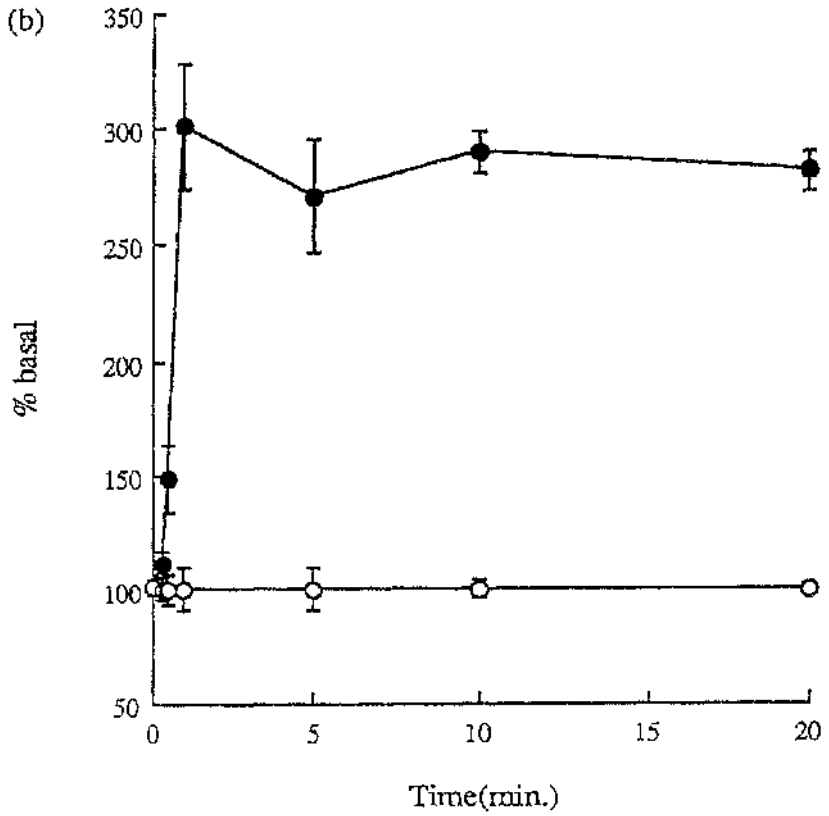

350
300
250
200
150
100
50
% basal
0
5
10
15
20
Time(min.)

Figure 4.4 **Western blot analysis of insulin- and lysophosphatidic acid (LPA)-stimulated tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced in 2% (v/v) NBCS.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent, when the medium was replaced by DMEM containing either 2% (v/v) or 10% (v/v) NBCS for 24 hours. A proportion of the cells quiesced in 2% (v/v) NBCS had either 1μM LPA or 10μg/ml insulin added to the quiescing medium. Quiesced cells were then stimulated with either 100nM bombesin in DBG or DBG alone for 1 minute at 37°C. Lysates were prepared as described in Materials and Methods and 10μg total protein was loaded onto 10% (w/v) polyacrylamide gels and SDS-PAGE carried out as described in Chapter 2. Tyrosine phosphorylated proteins were visualised using ECL and result is representative of 3 individual experiments.

Lysophosphatidic acid (LPA) - and insulin-stimulated tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced in 2% (v/v) NBCS

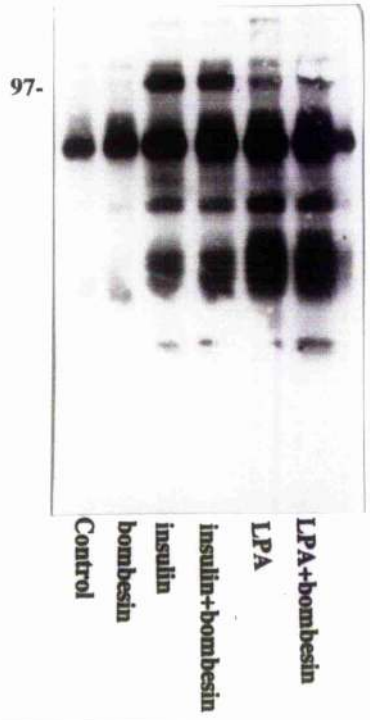


Figure 4.5(a) **Bombesin-stimulated tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced in 2% (v/v) and 10% (v/v) newborn calf serum.**

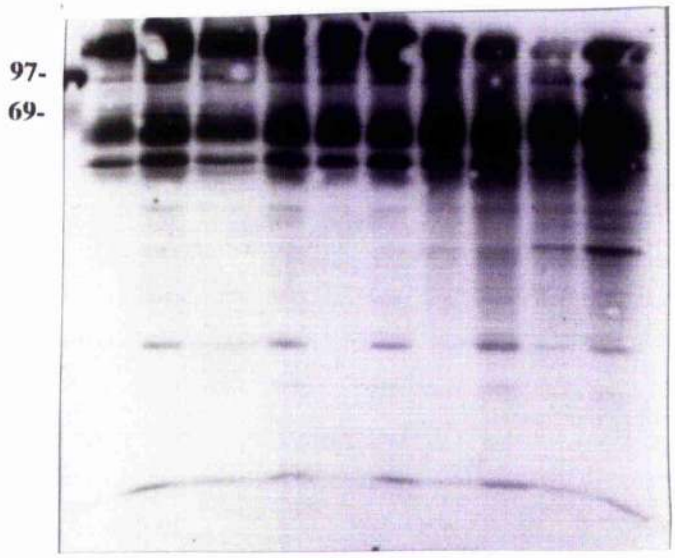
Swiss 3T3 fibroblasts were cultured in DMEM containing 10% NBCS until 80-90% confluent when medium was replaced by DMEM containing either 2% (v/v) or 10% (v/v) NBCS for 24 hours. Quiesced cells were then stimulated with 100nM bombesin or DBG for the times indicated and lysates prepared as described in Materials and Methods. 10µg total protein was then loaded onto 10% (w/v) polyacrylamide gels and SDS-PAGE carried out as described in Chapter 2. Tyrosine phosphorylated proteins were visualised using ECL and result is representative of 3 individual experiments.

Figure 4.5(b) **Western blot analysis of bombesin-stimulated tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was replaced with fresh culture medium for 24 hours. Quiesced cells were stimulated with either 100nM bombesin in DBG or DBG alone for the times indicated. Lysates were prepared as described in Materials and Methods and 10µg total protein was loaded onto 10% (w/v) polyacrylamide gels and SDS-PAGE carried out as described in Chapter 2. Tyrosine phosphorylated proteins were detected using an anti-phosphotyrosine, HRP-linked conjugate antibody, which was modelled on the monoclonal used for all other Phosphotyrosine western blots. Recognised proteins were visualised using ECL and result is representative of 2 individual experiments.

a

Bombesin-stimulated tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced in 2% and 10% (v/v) NBCS



b

Bombesin-stimulated tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS

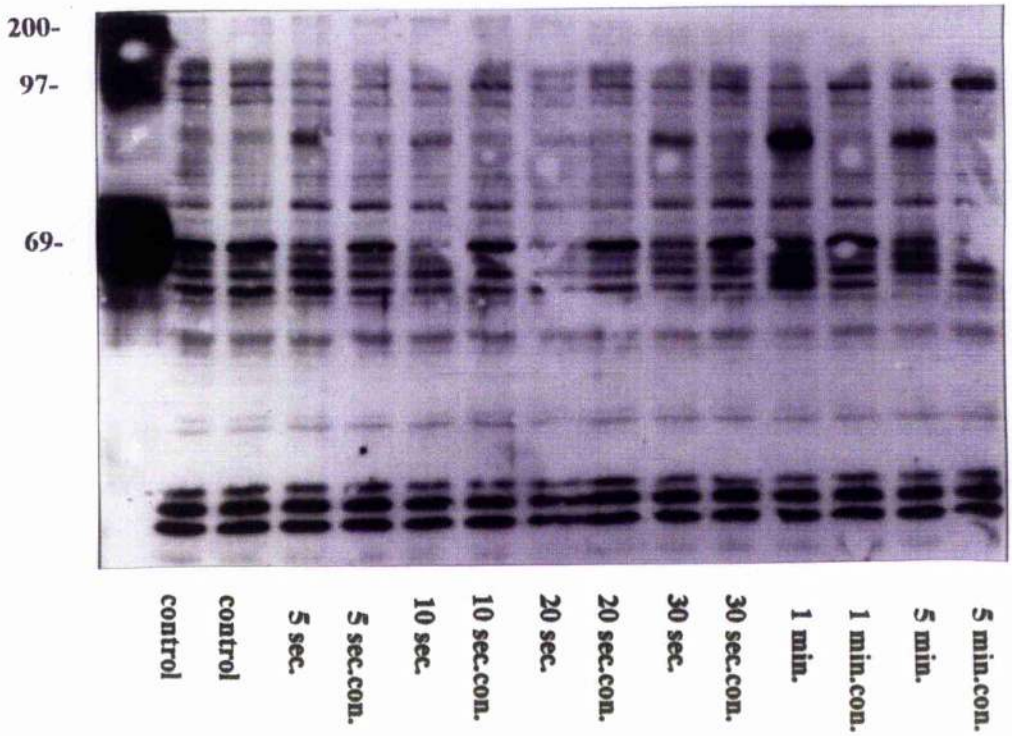


Figure 4.6 **Agonist-stimulated tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced in 2% (v/v) and 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was replaced with DMEM containing either 2% (v/v) or 10% (v/v) NBCS for 24 hours. Cells quiesced under both conditions were then stimulated for 20 minutes at 37°C with either 100nM phorbol ester (TPA), 30μM lysophosphatidic acid (LPA), 10ng/ml insulin, 30ng/ml platelet derived growth factor (PDGF) in DBG or DBG alone. Lysates were prepared as described in Materials and methods with 10μg total protein being loaded onto 10% (w/v) polyacrylamide gels and SDS-PAGE carried out as described in Chapter 2. Tyrosine phosphorylated proteins were visualised using ECL and result is representative of 2 individual experiments.

Agonist-stimulated tyrosine phosphorylation in Swiss 3T3 fibroblasts quiesced in 2% and 10% (v/v) NBCS

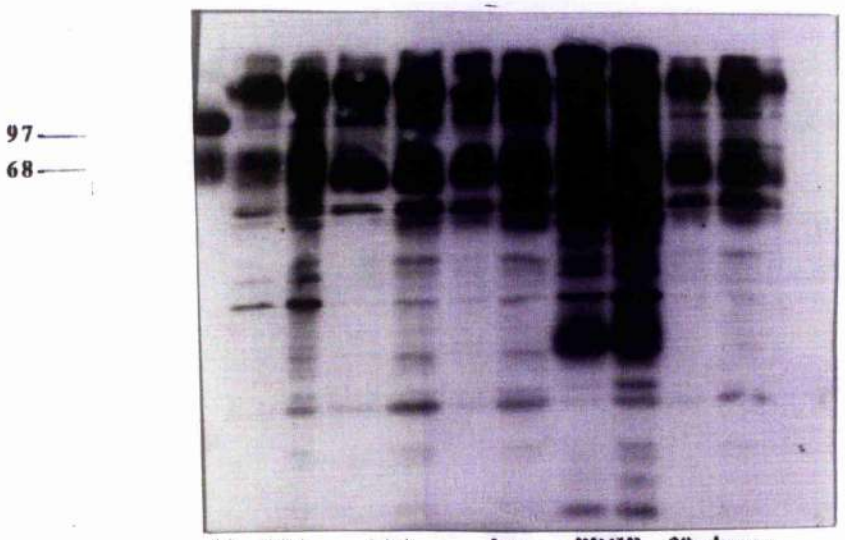


Figure 4.7 **Effect of demethoxyviridine (DMV) upon bombesin-stimulated tyrosine phosphorylation in Swiss 3T3 fibroblasts.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was replaced by DMEM containing 2% (v/v) NBCS for 24 hours. Cells were preincubated for 5 minutes at 37°C with the concentrations of DMV shown. Control cells were preincubated with a vehicle control containing the same amount of DMSO present in the inhibitor samples. Preincubated cells were then stimulated with either 100nM bombesin in DBG or DBG alone for 2 minutes at 37°C. Lysates were prepared as described in Materials and Methods and 10μg total protein was loaded onto 10% (w/v) polyacrylamide gels and SDS-PAGE carried out as described in Chapter 2. Tyrosine phosphorylated proteins were visualised using ECL and result is representative of 4 individual experiments.

M.J. Cross is greatfully acknowledged for this initial inhibitor work.

Effect of DMV upon bombesin-stimulated tyrosine phosphorylation in Swiss 3T3 fibroblasts

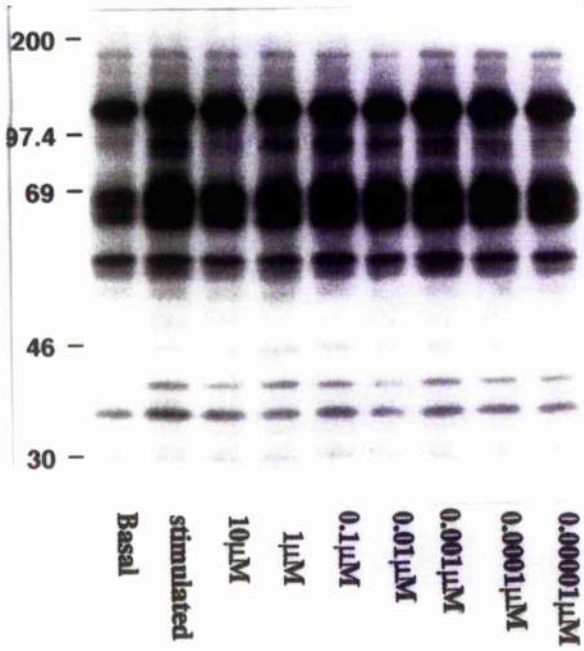


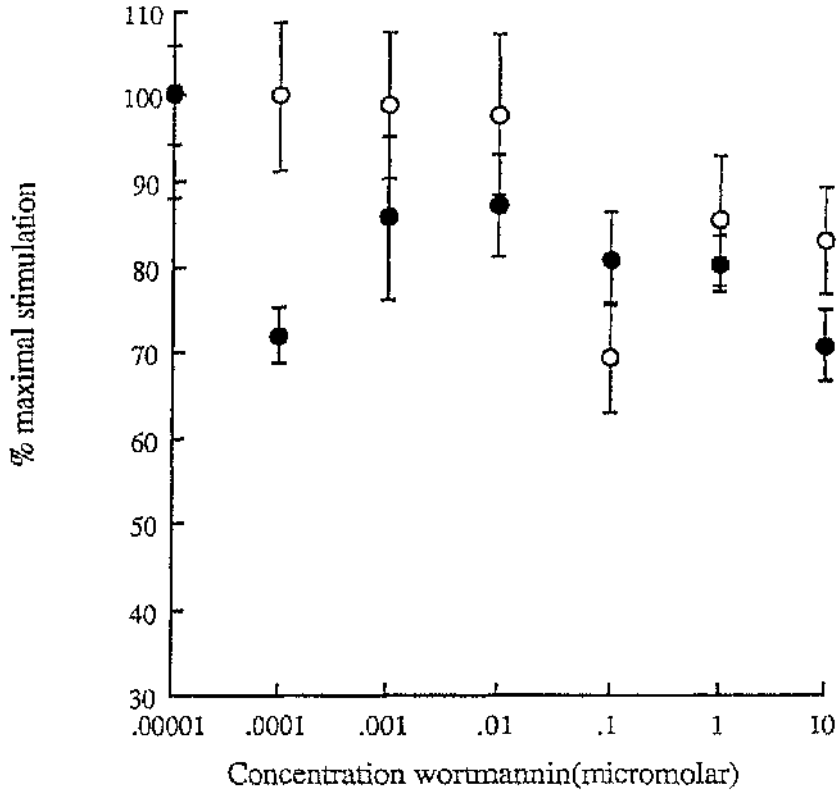
Figure 4.8(a) **Effect of wortmannin upon baculovirus expressed $cPLA_2$ activity in an *in vitro* bilayer assay.**

Bilayer micelles were prepared including and excluding phosphatidylinositol-4,5-bisphosphate (PIP_2) and utilised as described in Materials and Methods. Prepared micelles were incubated for 5 minutes at 37°C with the concentrations of DMV shown and either 13 μg/ml in the absence of PIP_2 (○) or 3.25 μg/ml in the presence of PIP_2 (●), of baculovirus-expressed $cPLA_2$. Reactions were terminated by the addition of stopping buffer containing 0.01μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid. Free arachidonate was extracted and the [^{3}H] and [^{14}C] levels measured by liquid scintillation spectrophotometry. Results is representative of 2 individual experiments.

Figure 4.8(b) **Effect of phosphatidylinositol-4,5-bisphosphate (PIP_2) upon baculovirus-expressed $cPLA_2$ activity in an *in vitro* bilayer assay.**

Micelles containing 1-stearyl-2-[^{14}C] arachidonyl phosphatidyl choline were prepared as described in Materials and Methods with PIP_2 present (■) or excluded (❑) from the final micelle. Different concentrations of baculovirus-expressed $cPLA_2$ was then incubated for 5 minutes at 37°C with both forms of the micelle prepared. Reactions were terminated by the addition of stopping buffer containing 0.01μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid. [^{3}H] and [^{14}C] labelled arachidonate was then extracted and quantified using liquid scintillation spectrophotometry. Result is representative of 2 individual experiments carried out on freshly prepared micelles.

(a)


110
100
90
80
70
60
50
40
30
% maximal stimulation
.00001
.0001
.001
.01
.1
1
10
Concentration wortmannin(micromolar)

(b)

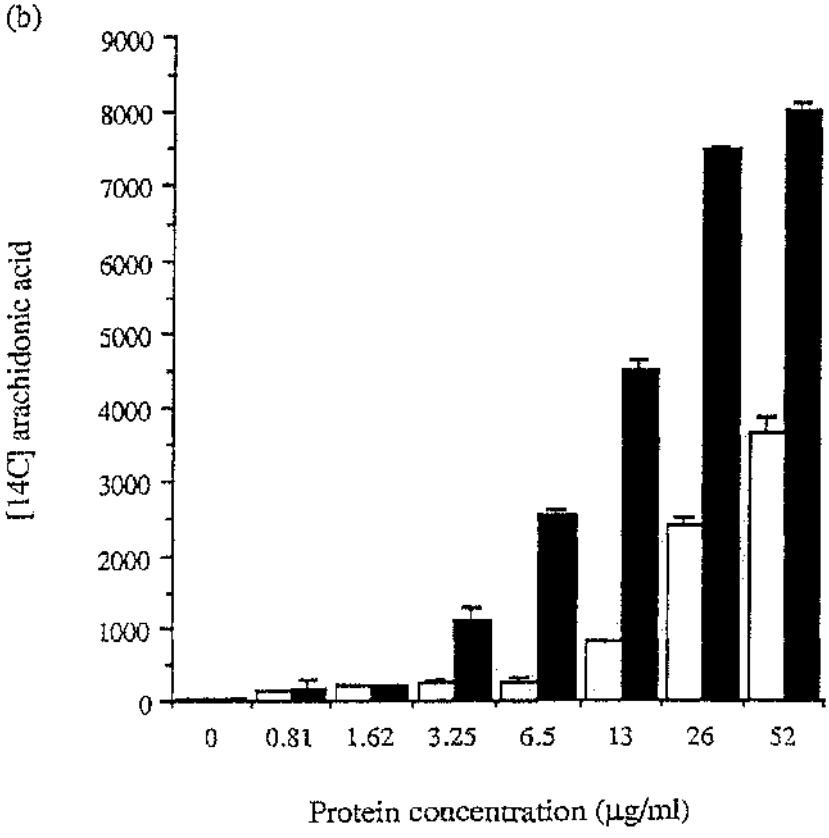

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
[14C] arachidonic acid
0
0.81
1.62
3.25
6.5
13
26
52
Protein concentration (µg/ml)

Figure 4.9 Effect of wortmannin upon *in vitro* arachidonate release produced by immunoprecipitated $cPLA_2$ activity from Swiss 3T3 fibroblasts quiesced in 2% (v/v) NBCS.

Swiss 3T3 fibroblasts were seeded in $75cm^2$ culture flasks in DMEM containing 10% (v/v) NBCS until 80-90% confluent, when medium was replaced by DMEM containing 2% (v/v) NBCS for 24 hours. Quiesced cells were washed 3 times with DBG at 37°C and reactions terminated by washing with ice-cold PBS and placing on ice. Lysates were prepared and immunoprecipitation carried out as described for MAP kinase *in vitro* studies, using a 1:50 dilution of a rabbit anti-$cPLA_2$ antibody. Washed pellets were resuspended in 35µl assay buffer (50mM Tris/HCl (pH7.4)) and incubated for 10 minutes at room temperature with the concentrations of wortmannin shown. The micelle containing PIP_2 suspended in 10µM $CaCl_2$ and 100mM KCl was then added and the total reaction mixture incubated for a further 10 minutes at 37°C. Reaction was stopped and arachidonate release quantified as described in Materials and methods. Result is representative of a single experiment, with duplicate points. Lane 1=control; 2=baculovirus expressed $cPLA_2$; 3=baculovirus expressed $cPLA_2$ with 10 minute wortmannin pretreatment; 4=immunoprecipitate; 5=immunoprecipitate with wortmannin pretreatment; 6=total cell lysate; 7=total cell lysate with wortmannin pretreatment.

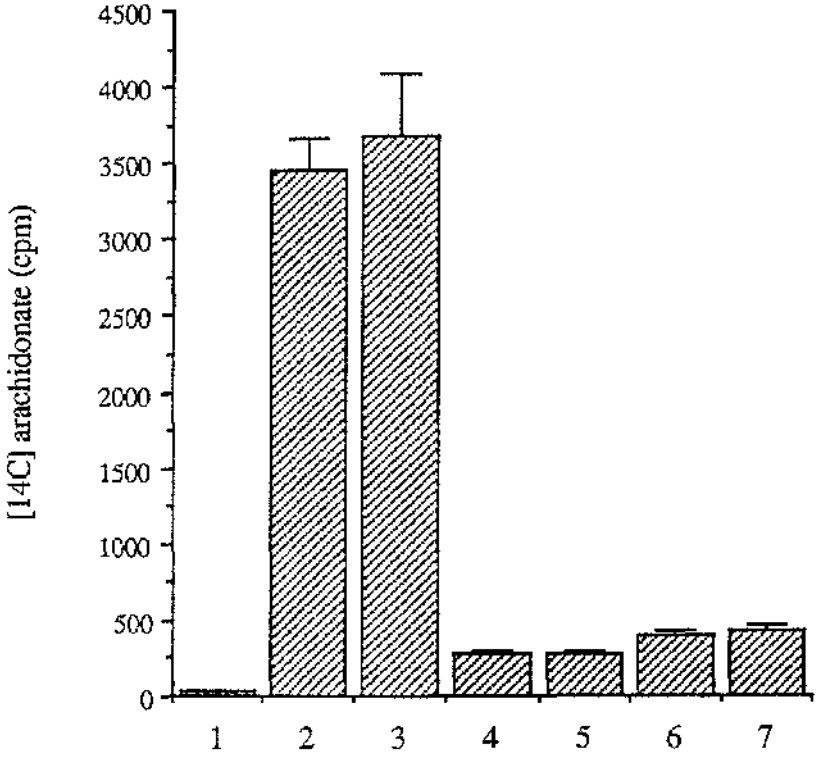

[14C] arachidonate (cpm)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1
2
3
4
5
6
7

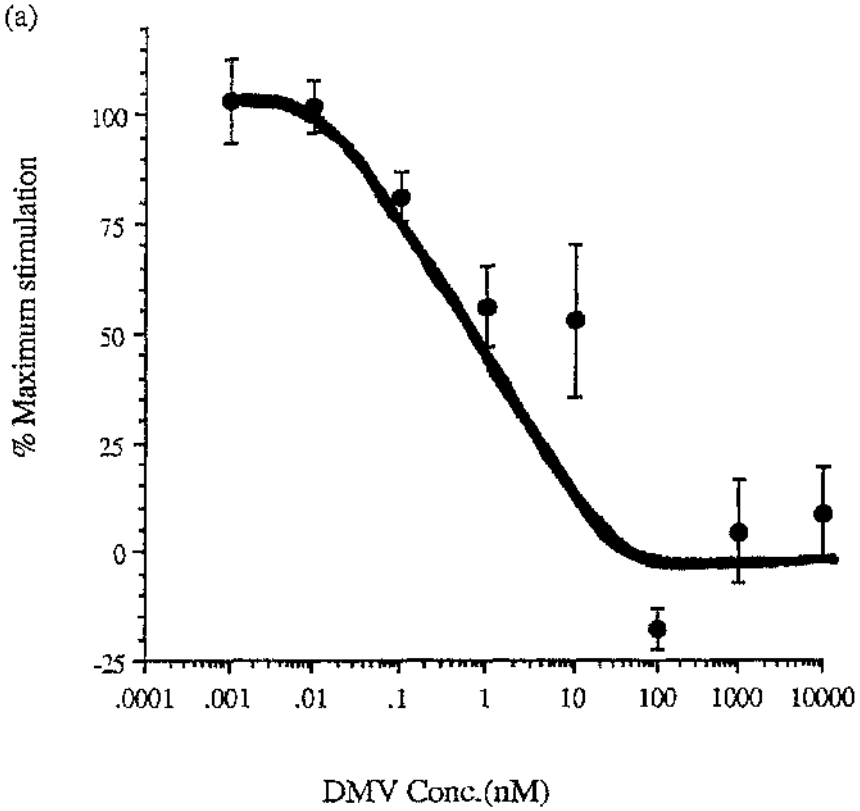
Figure 4.10(a) **Effect of DMV upon bombesin-stimulated intracellular [^{3}H] arachidonate release in Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were cultured in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was replaced with DMEM containing 10% (v/v) NBCS and 1μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid. Cells were preincubated wth the concentrations of DMV shown for 5 minutes at 37°C and then stimulated with 100nM bombesin for 1 minute (●). Intracellular arachidonate was quantified by liquid scintillation spectrophotometry and result is representative of 6 individual experiments. Results are presented as % maximum stimulation ± s.d., which is equivalent to 3500 ± 200 dpm and represents bombesin-stimulated arachidonate release in the absence of any inhibitor. The basal level is equivalent to 800 ± 150 dpm.

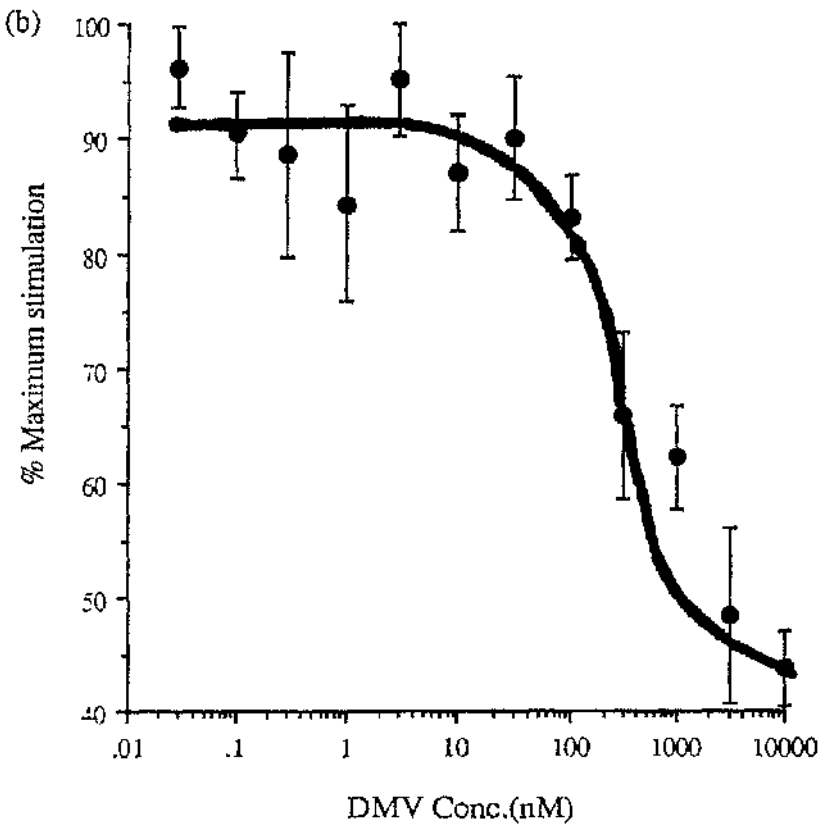
Figure 4.10(b) **Effect of DMV upon fMLP-stimulated total [^{3}H] arachidonate release in 5 day DMSO-differentiated HL60 cells.**

HL60 cells were seeded at 5x10^5 cells per ml in RPMI 1640 medium containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO and cultured for 5 days. 17 hours prior to the end of the culturing period 0.25μCi/ml [4,5,8,9,11,12,14,15-^{3}H] arachidonic acid was added. 10^6 cells per sample were preincubated for 5 minutes at 37°C with the concentrations of DMV shown, prior to a 5 minute stimulation with 100nM fMLP (●) at 37°C. Arachidonate was quantified using liquid scintillation spectrophotometry and results are presented as % maximum stimulation ± s.d., which is equivalent to 4500 ± 230 dpm. The basal level is 800 ± 150 dpm and the result is representative of 2 individual experiments.

(a)


% Maximum stimulation
100
75
50
25
0
-25
.0001
.001
.01
.1
1
10
100
1000
10000
DMV Conc.(nM)

(b)


% Maximum stimulation
100
90
80
70
60
50
40
.01
.1
1
10
100
1000
10000
DMV Conc.(nM)

4.3 DISCUSSION

The work presented in this chapter was performed to elucidate the role of protein tyrosine phosphorylation in the priming of $cPLA_2$ activity in the models presented in Chapter 3.

Published data has suggested that, in differentiated HL60 cells and human neutrophils, the priming agent cytochalasin B increases the activity of a tyrosine kinase leading to increased responsiveness to subsequent agonist stimulation. Figure 4.1(a) supports this finding since a 5 minute preincubation of 5 day DMSO-differentiated HL60 cells with 5μM cytochalasin B increased overall tyrosine phosphorylation as measured by western blotting. This preincubation did not increase basal levels of free arachidonate as described in Chapter 3. This increase in tyrosine phosphorylation is also visible in Swiss 3T3 fibroblasts chronically primed, i.e. when grown in DMEM containing 10% as opposed to 2% (v/v) NBCS (Figure 4.1(b)). Therefore, it appears that an increase in tyrosine phosphorylation maybe responsible for both acute and chronic priming, as in both cases there is no increase in basal levels of arachidonate, but the responsiveness to subsequent agonist stimulation is enhanced. The role of tyrosine phosphorylation in the activation of $cPLA_2$ was further investigated using the general tyrosine phosphophosphatase inhibitor perhydovanadate. By inhibiting the dephosphorylation of tyrosine phosphorylated proteins, a chronic stimulation of arachidonate release was observed in Swiss 3T3 fibroblasts (Figure 4.2(a)) quiesced under both conditions (the data for 10% NBCS-quiesced cells is shown). The kinetics of this response correlated to the second sustained phase of bombesin-stimulated arachidonate release in Swiss 3T3 fibroblasts, but care must be taken in drawing any conclusions from this because of the time taken for the inhibitor to penetrate the cell and to inhibit the phosphatases. With this in mind, Figure 4.2(b) demonstrates that 0.5mM perhydrovanadate can stimulate arachidonate release in 5 day DMSO-differentiated HL60 cells lending further evidence for a tyrosine phosphorylated protein being involved in the activation of $cPLA_2$, however the kinetics of the response was much slower than those seen in response to fMLP-stimulation. Taking

a 2 minute time point from this data, where there is no detectable perhydrovanadate-stimulated arachidonate release, Figure 4.3(a) shows that the cells are primed for subsequent fMLP stimulation. The kinetics of this response (Figure 4.3(b)) are very similar to those seen in the previous chapter, where cytochalasin B was used as the priming agent. Therefore, perhydrovanadate can be substituted for cytochalasin B suggesting that it is indeed a tyrosine phosphorylated protein that is responsible for the acute priming effect observed in HL60 cells. This is not the only enzyme which has been shown to be primed by tyrosine phosphorylation in neutrophils, for instance an involvement in the priming of NADPH oxidase in human neutrophils has been reported (Lloyds & Hallett, 1995; Phillips & Hamilton, 1990). The kinases affected by perhydrovanadate remain to be identified, however, an increase in the phosphorylation of both ERK1, ERK2 and a 74kDa protein has been demonstrated (Lloyds *et al.*, 1995).

In chapter 3, the role of the serum factors insulin and lysophosphatidic acid in the chronic priming of Swiss 3T3 fibroblasts was discussed. These data suggested that both could prime 2% (v/v) NBCS quiesced cells for subsequent agonist stimulation, however the effects varied. Insulin-pretreatment of Swiss 3T3 fibroblasts quiesced in 2% (v/v) NBCS did not stimulate $cPLA_2$, but produced the same kinetics of bombesin-stimulated arachidonate release as seen in Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS, whilst LPA produced a dose-dependent potentiation of bombesin-stimulated arachidonate release. This kinetic effect can be explained by an increase in tyrosine phosphorylation as shown in Figure 4.4. Both insulin and LPA induced an increase in tyrosine phosphorylation similar to that observed in 10% quiesced cells. However, the proteins that are tyrosine phosphorylated differed between the 2 agonists, which might partly explain the differences in the kinetics observed. Bombesin stimulation does not visibly increase the overall level of tyrosine phosphorylation of pretreated cells. This phenomenon was also evident in cells quiesced in 10% (v/v) NBCS as shown in Figure 4.5(a). In this Figure, bombesin-stimulated tyrosine phosphorylation in 10% quiesced cells was only increased after 1

minute. However, bombesin had an effect at the earlier time points upon the tyrosine phosphorylation in 10% quiesced cells as shown in Figure 4.5(b), where there appeared to be a dephosphorylation of an approximately 69kDa protein following bombesin-stimulation. This dephosphorylation upon bombesin stimulation could only be visualised using a 12.5% (w/v) polyacrylamide gel which produced a better resolution of the protein bands around this molecular weight and an HRP-conjugate of the monoclonal anti-phosphotyrosine used in all the other phosphotyrosine western blots presented. In 2% quiesced cells a transient increase in bombesin-stimulated tyrosine phosphorylation was visible, which was maximum at 30 seconds and correlated with the kinetics of bombesin-stimulated arachidonate release under these quiescing conditions. The level of maximum tyrosine phosphorylation in 2% quiesced cells was comparable to the basal level in 10% quiesced cells, suggesting that the tyrosine phosphorylated protein involved in the activation of $cPLA_2$ was already phosphorylated in 10% quiesced cells, i.e. primed, with agonist stimulation producing the additional signal required to fully activate $cPLA_2$. In 2% quiesced cells, agonist stimulation was required for the tyrosine phosphorylation of this putative activating protein in addition to the additional signal required for maximal arachidonate release. This could, therefore, explain the slower kinetics of bombesin-stimulated arachidonate release.

Other agonists also show a differential pattern of protein tyrosine phosphorylation in 2% and 10% quiesced cells (Figure 4.6). The receptor tyrosine kinase agonists insulin (Goldstein, 1992) and platelet derived growth factor (Williams, 1989), significantly increased tyrosine phosphorylation. The lipid agonist lysophosphatidic acid (LPA), which is reported to mediate its effects via a G-protein coupled receptor (van der Bend *et al.*, 1992) also increased the overall level. However, the phorbol ester TPA appeared to stimulate a decrease in basal tyrosine phosphorylation in 2% quiesced cells, but an increase in 10% quiesced cells, suggesting that activation of protein kinase C has different effects depending upon the state of the cells. This could not be studied in greater detail in the time available, but it

can be concluded that the level of tyrosine phosphorylation is higher in 10% quiesced Swiss 3T3 fibroblasts, irrespective of the agonist used. As shown for bombesin-stimulated arachidonate release, an additional signal maybe required for maximal arachidonate release and since the bombesin receptor has been shown to couple to PLCγ (Plevin *et al.*, 1990) and that $cPLA_2$ requires calcium for activation (Kramer *et al.*, 1986), the additional signal required here may be inositol-1,4,5-trisphosphate (IP_3)-stimulated intracellular calcium release. This will be discussed in Chapter 5.

Therefore, from work presented in this chapter so far, it appears that a tyrosine phosphorylated protein is responsible for both the acute and chronic priming of $cPLA_2$ observed. However, there is also a tyrosine phosphorylated protein involved in the agonist-stimulated arachidonate release. To further elucidate this, the inhibitor demethoxyviridin (DMV) was utilised. This compound inhibited bombesin-stimulated tyrosine phosphorylation in a dose-dependent manner with an IC_{50}=10nM (± 2nM) (Figure 4.7, experiment performed by M.J.Cross). This inhibitor is an analogue of the putative potent phosphatidylinositol-3 kinase (PI-3K) inhibitor wortmannin, however, the effect upon bombesin-stimulated tyrosine phosphorylation cannot be due to inhibition of PI-3 kinase, since there is no bombesin-stimulated PI-3 kinase activity in Swiss 3T3 fibroblasts (Cross *et al.*, 1995). There was a small direct inhibition by 0.1nM wortmannin of baculovirus expressed $cPLA_2$ activity as shown in Figure 4.8(a), but only when phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) was included in the micelle adopted in the *in vitro* assay used. PtdIns-4,5-P_2 was a very potent activator of $cPLA_2$, producing a response 5 fold over that observed in the absence of PtdIns-4,5-P_2 with identical levels of enzyme present (Figure 4.8(b)). This may suggest that wortmannin has more than one mode of action upon $cPLA_2$ and that perhaps, the conformation of the enzyme determines whether or not there is an inhibition, as the presence of PtdIns-4,5-P_2 in the micelle appeared to allow inhibition at the lower wortmannin concentrations. This cannot be the only answer, as $cPLA_2$ immunoprecipitated from whole cells was not inhibited by wortmannin (Figure 4.9),

but in the whole cell, bombesin-stimulated arachidonate release was inhibited in a dose-dependent manner to a far greater extent, to that seen for baculovirus expressed $cPLA_2$ (M.J.Cross, personal communication). This suggests that there was both direct and indirect inhibitory effects exerted upon $cPLA_2$ by wortmannin. In Swiss 3T3 fibroblasts (Figure 4.10(a)), bombesin-stimulated arachidonate release was totally inhibited by the wortmannin analogue, demethoxyviridine (DMV), with an IC_{50} value of 2nM (± 2nM), whereas fMLP-stimulated arachidonate release in 5 day DMSO-differentiated HL60 cells (Figure 4.10(b)) was inhibited by 60% with an IC_{50} value of 200nM (± 20nM). This suggests that a different regulatory element, maybe a tyrosine phosphorylation-regulated protein, was affected to a differing degree in the 2 cell types, or that it was affecting a multitude of possible $cPLA_2$-regulatory proteins. However, western blotting was not sensitive enough to isolate any inhibition at these very low concentrations of DMV. One of the main proteins regulated by tyrosine phosphorylation, which has been shown to phosphorylate and activate $cPLA_2$ *in vitro*, is MAP kinase (Lin *et al.*, 1993), the role of which will be discussed in detail in Chapter 5.

CHAPTER 5

REGULATION OF $cPLA_2$ BY MAP KINASE AND INCREASED INTRACELLULAR CALCIUM CONCENTRATION

5.1 Introduction

In chapter 4, it was shown that both acute priming by cytochalasin B in HL60 cells and chronic priming of Swiss 3T3 fibroblasts by serum, resulted in an increase in tyrosine phosphorylation. One of the main signalling enzymes regulated by a tyrosine kinase cascade is MAP kinase (Anderson *et al.*, 1990). This enzyme is activated by phosphorylation upon tyrosine and threonine residues (Haystead *et al.*, 1992), but exibits activity by phosphorylating its target substrates on serine/threonine residues, located within a specified recognition sequence (Rao & Reddy, 1993). *In vitro* studies have shown that $cPLA_2$ can be directly phosphorylated on serine 505 by activated MAP kinase (Lin *et al* ., 1993). The regulation of MAP kinase has been shown to involve the *ras* target *raf-1* which is a MAP kinase kinase kinase (Kyriakis *et al.*, 1992). MAP kinase is a convergence point for both tyrosine kinase and protein kinase C-mediated pathways (Morrison *et al.*, 1993).

$cPLA_2$ has been shown to contain a CaLB domain (Sharp *et al.*, 1991), which has been proposed to bind calcium and potentiate the binding of $cPLA_2$ to the membrane (Masuda *et al.*, 1991). It has been shown that calcium is essential for $cPLA_2$ activity (Leslie *et al.*, 1988) and it has been proposed that it is required for the translocation of the enzyme to its membrane substrate (Channon & Leslie, 1990). However, the mechanism involved has yet to be clarified.

In this chapter the role of MAP kinase activation and increased intracellular calcium concentrations upon $cPLA_2$ activation and priming are described.

5.2 RESULTS

5.2.1 Manipulation of MAP kinase activity using Rat-1 fibroblasts transfected with an estradiol-regulated plasmid expressing phosphorylated MAP kinase kinase kinase (*raf*).

In Rat-1 Raf ER4 fibroblasts, the cDNA for the kinase domain of p74$^{raf\text{-}1}$ was fused to the hormone binding domain cDNA of the human estrogen receptor to generate the fusion gene product Raf ER (Samuels *et al.*, 1993). Upon addition of 1μM β-estradiol for 1 hour, the activated *raf-1* causes complete phosphorylation of ERK 1 and ERK 2 as demonstrated by western blotting (Figure 5.1). This figure also demonstrates that a 1 minute stimulation with the complete mitogen for Rat-1 fibroblasts, lysophosphatidic acid (LPA), did not affect the phosphorylation of either MAP kinase isoform, in both estradiol-treated and -untreated Rat-1 Raf ER4 fibroblasts. Figure 5.2 shows that MAP kinase was not the only tyrosine phosphorylated potein affected by *raf-1* over expression, since anti-phosphotyrosine western blotting showed increased phosphorylation of various proteins. Since *in vitro* analysis has demonstrated the direct phosphorylation of $cPLA_2$ by MAP kinase (Lin *et al.*, 1993), the effect of increased MAP kinase phosphorylation upon LPA-stimulated arachidonate release was studied. Figure 5.3(a) demonstrates that estradiol-induced Raf-1 expression and thus, MAP kinase phosphorylation, produced an enhanced and rapid onset of LPA-stimulated arachidonate release. In estradiol-pretreated Raf ER4 fibroblasts, LPA-stimulated arachidonate release was detectable at 10 seconds and maximal at 30 seconds, 5-6 fold above basal. The cells responded to LPA in the absence of estradiol pretreatment, with the same kinetics, but to only 2-3 fold above basal. In both cases, the response was still elevated at 5 minutes. Estradiol treatment increased the efficacy of LPA-stimulated arachidonate release, but did not alter the affinity of the LPA-receptor, as the EC_{50} in both estradiol-treated and -untreated Raf ER4 fibroblasts was 200nM ±25nM (Figure 5.3(b)).

5.2.2 Effect of acute and chronic priming upon MAP kinase phosphorylation state and activity.

Figure 5.4 shows that chronic priming of Swiss 3T3 fibroblasts by serum apparently increased the level of both MAP kinase isoforms (ERK 1 and ERK 2) within the cells, compared to that in cells quiesced in 2% (v/v) NBCS. Whilst there was no difference in the ratio of phosphorylated to non-phosphorylated enzyme, bombesin stimulated a marked increase in the ratio over time, in cells quiesced under both conditions.

When 5 day DMSO-differentiated HL60 cells were examined, only ERK1 (44kDa) was recognised (Figure 5.5). When lysates from estradiol-treated and -untreated Raf ER4 fibroblasts were examined as a comparison, it was clear that ERK1 in DMSO-differentiated HL60 cells was fully phosphorylated, with 5μM cytochalasin B, 100nM fMLP and both in conjunction, having no effect upon the phosphorylation state. The tyrosine phosphophosphatase inhibitor perhydrovanadate (0.5mM) produced hyper-phosphorylation of ERK1, which was unaffected by cytochalasin B priming.

In order to correlate the phosphorylation of MAP kinase to activity, an *in vitro* assay was utilised. Using myelin basic protein as the substrate, immunoprecipitated MAP kinase activity was assayed (Table 5.1). In HL60 cells, which can be primed by either cytochalasin B or perhydrovanadate, only the latter produced a significant increase in MAP kinase activity. As expected, estradiol-treated Raf ER4 cells showed an increased basal level of MAP kinase activity, which was not further increased in response to LPA. In the case of Swiss 3T3 fibroblasts, even though there was apparently a higher level of both ERK1 and ERK2 in 10% (v/v) quiesced cells, there was no significant increase in the level of basal MAP kinase activity, compared to cells quiesced in 2% (v/v) NBCS. However, in cells quiesced in 10% (v/v) NBCS, there was a biphasic MAP kinase activity, which was not detectable in 2% (v/v) quiesced cells (Figure 5.6).

5.2.3 Role of agonist-stimulated increased intracellular calcium concentrations in the activation and priming of $cPLA_2$.

Cytochalasin B has been reported to induce its priming effects by stimulating the release of intracellular calcium (Forehand *et al.*, 1989). However, this was not the case in 5 day DMSO-differentiated HL60 cells (Figure 5.7(a)). The cells did produce receptor-stimulated intracellular calcium release, with a calcium trace being obtained in response to 100nM fMLP. The intracellular calcium concentration increased from 150nM to approximately 450nM. This showed that the lack of cytochalasin B-stimulated intracellular calcium release was not due to the non-viability of the cells. The other priming agent of 5 day DMSO-differentiated HL60 cells, perhydrovanadate, was also without effect upon the level of intracellular calcium concentration, as shown in Figure 5.7(b). Therefore, an increase in the intracellular calcium concentration was not responsible for the acute priming effects observed in 5 day DMSO-differentiated HL60 cells.

The effect of an increased intracellular calcium concentration upon bombesin-stimulated arachidonate release in Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS was studied using the calcium chelator BAPTA-AM(10μM). This compound enters the cell, where it is cleaved by non-specific lipases, to produce the active chelator BAPTA. Figure 5.8(a) demonstrates that preincubation with BAPTA-AM inhibited bombesin-stimulated arachidonate release by 40-50%. LPA-stimulated arachidonate release in both estradiol-treated and -untreated Raf ER4 fibroblasts was totally inhibited after preincubation with this intracellular calcium chelator (Figure 5.8(b)). These observations demonstrate that calcium plays a major role in the activation of $cPLA_2$ in chronically primed cells, but do not point to a role in the priming *per se*.

Agonist-stimulated increased intracellular calcium concentrations were measured in Swiss 3T3 and Raf ER4 fibroblasts, which had been loaded with the fluorescent dye Indo-1. In Swiss 3T3 fibroblasts quiesced in both 2% (v/v) and 10% (v/v) NBCS, bombesin stimulated a rapid rise in intracellular calcium concentrations, which may be attributed to PIC-stimulated IP_3 generation, which has been previously

demonstrated in bombesin-stimulated Swiss 3T3 fibroblasts (Cook *et al.*, 1990)(Figure 5.9). The basal levels did not vary between the quiescing methods, suggesting that changes in calcium levels are not involved in the serum-induced priming event observed. However, an increase in intracellular calcium concentration does have a role in bombesin-stimulated arachidonate release in primed cells, as disscused earlier, where BAPTA-AM was shown to inhibit this response by 40-50%. To rule out the possibility that the partial inhibition by BAPTA of bombesin-stimulated arachidonate release was due to incomplete chelation of calcium, the effect of BAPTA upon the intracellular calcium concentration was measured. Figure 5.10 demonstrates that BAPTA-AM was able to compete with Indo-1, resulting in a total loss of bombesin-stimulated increased intracellular calcium fluxes. This data suggests that an additional mechanism to calcium changes must be involved in the activation of $cPLA_2$.

BAPTA-AM also effectively chelated LPA-stimulated intracellular calcium release in Raf ER4 fibroblasts (results not shown), explaining the total inhibition of LPA-stimulated arachidonate release. The LPA-stimulated increased intracellular calcium concentration in estradiol treated- and -untreated Raf ER4 fibroblasts was presumably due to an IP_3-mediated response (Figure 5.11(a) and (b)). However, the increase in [calcium] in estradiol-treated Raf ER4 cells was greater than in the control (Figure 5.11(b)), which could not be explained by an estradiol-stimulated release of intracellular calcium (Figure 5.12). The LPA-receptor is proposed to be a 7-membrane spanning, G-protein linked receptor which is coupled to IP_3-mediated intracellular calcium release (van der Bend *et al.*, 1992; van Corven *et al.*, 1993), thus the effect of estradiol-pretreatment upon LPA-stimulated inositol phosphate generation was studied. Figure 5.13 shows that estradiol-pretreatment had no effect upon LPA-stimulated inositol-phosphate (IP_1) and inositol-1,4-bisphosphate (IP_2) generation. However, the level of LPA-stimulated inositol-1,4,5-trisphosphate (IP_3) was greater in estradiol-treated Raf ER4 fibroblasts, which could explain the enhanced intracellular calcium concentration measured. The total IP_4, IP_5 and IP_6 isoforms appeared to be at a lower level in estradiol-treated, compared to estradiol-untreated, Raf ER4

fibroblasts, suggesting that estradiol may have had an effect on the metabolism of inositol-1,4,5-trisphosphate (IP_3).

Figure 5.1 **Western blot analysis of estradiol- and LPA-stimulated phosphorylation of ERK1 and 2 in Rat-1 Raf ER4 fibroblasts.**

Raf ER4 fibroblasts were seeded at $2x10^4$ cells per ml and cultured in DMEM containing 10% (v/v) NBCS and 400μg/ml geneticin until 80-90% confluent, when medium was replaced with DMEM for 48 hours. Cells were washed with DBG and then incubated for 1 hour at 37°C with either DBG alone or DBG containing 1μM β-estradiol. Preincubated cells were then stimulated with either DBG or 30μM LPA in DBG for 30 seconds at 37°C. Cells were then washed with ice-cold PBS and lysed as described in Materials and methods. 40μg total protein was loaded onto 12.5% (w/v) polyacrylamide gels and SDS-PAGE carried out. Detection of ERK 1 and ERK 2 was carried out as described in Chapter 2, with recognised proteins being visualised by ECL. Result is demonstrative of 3 individual experiments.

MAP kinase phosphorylation in Rat-1 ER4 fibroblasts.

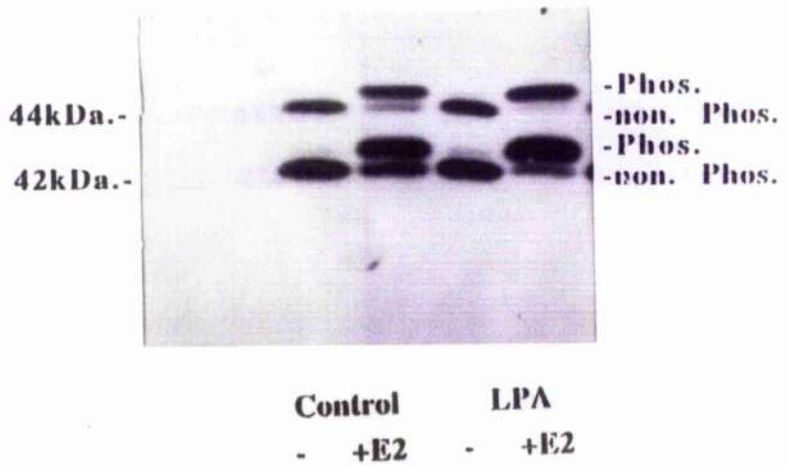


Figure 5.2 Western blot analysis of estradiol- and LPA-stimulated tyrosine phosphorylation in Rat-1 Raf ER4 fibroblasts. Cells were cultured and treated exactly as described in Figure 5.1, with 10μg total protein being loaded onto 10% (w/v) polyacrylamide gels and SDS-PAGE being carried out. Tyrosine phosphorylated proteins were detected as described in Materials and methods and visualised using ECL. Result is representative of 3 individual experiments.

Estradiol- and LPA-stimulated tyrosine phosphorylation in Rat-1 Raf ER4 fibroblasts

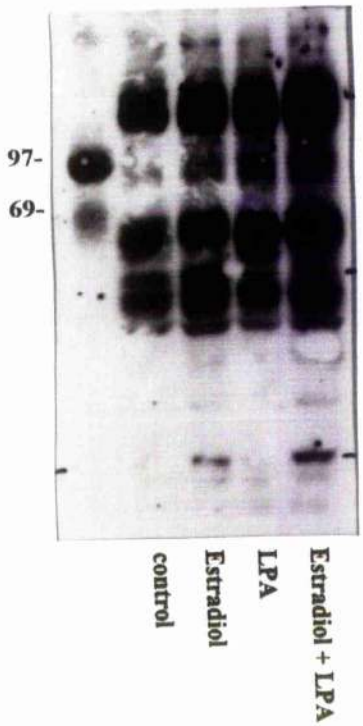


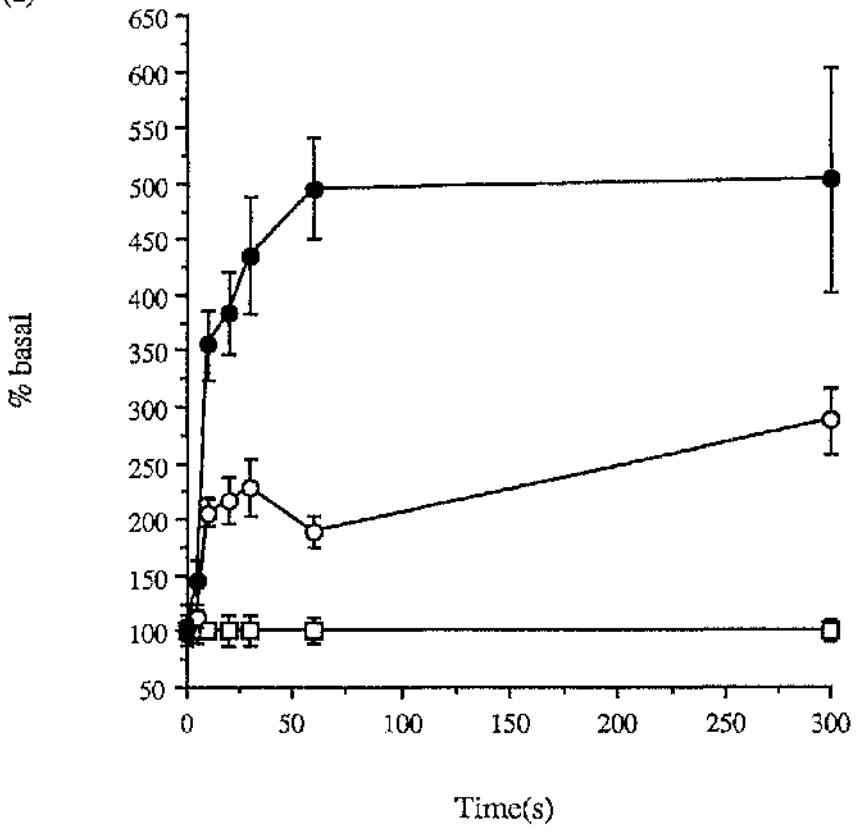
Figure 5.3(a) **Effect of estradiol-pretreatment upon LPA-stimulated intracellular [^{3}H] arachidonate release in Rat-1 Raf ER4 fibroblasts.**

Cells were seeded at $2x10^4$ cells per/ml in DMEM containing 10% (v/v) NBCS and 400μg/ml geneticin until 80-90% confluent. The medium was then replaced with DMEM containing 1μCi/ml [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid for 48 hours. After this time, cells were washed with DBG and then incubated for 1 hour with either DBG alone or DBG containing 1μM β-estradiol. Pretreated cells were then stimulated with either 30μM LPA or DBG alone for the times shown. Control values (❑) represent those obtained for both DBG and estradiol-pretreated cells. The figure represents LPA-stimulated intracellular arachidonate release in estradiol-treated (●) and -untreated cells (○) and is representative of 3 individual experiments, with the results being presented as % basal ± s.d. which is equivalent to 800 ± 35 dpm.

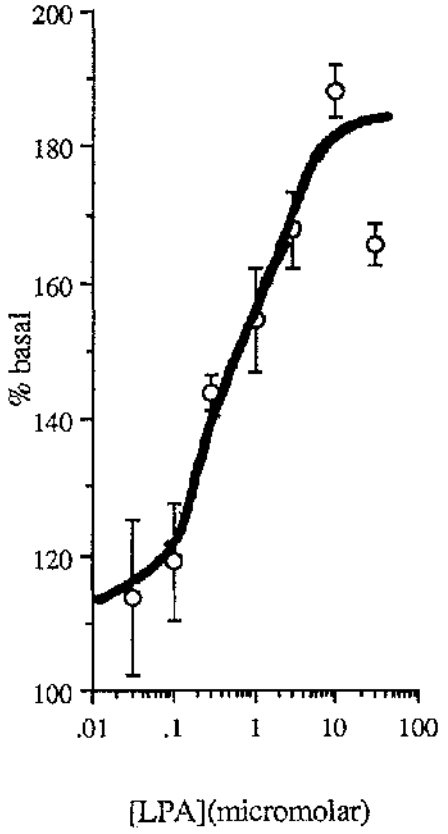
Figure 5.3(b) **Dose-dependency of LPA-stimulated intracellular [^{3}H] arachidonate release in estradiol-treated and -untreated Rat-1 Raf ER4 fibroblasts.**

Rat-1 Raf ER4 fibroblasts were seeded at $2x10^4$ cells/ml in DMEM containing 10% (v/v) NBCS and 400μg/ml geneticin until 80-90% confluent. Medium was replaced with DMEM containing 1μCi/ml [5,6,8,9,11,12,14,15-^{3}H] arachidonaic acid for 48 hours. Labelled cells were then washed with DBG and preincubated with either DBG alone or DBG containing 1μM β-estradiol for 1 hour at 37°C. Pretreated cells were stimulated with the concentrations of LPA shown for 30 seconds. Result represents LPA-stimulated intracellular arachidonate release in (i)control (○) and (ii)estradiol-treated(●)cells. The results are presented as % basal ± s.d, which is equivalent to 500±30 dpm and is representative of 3 individual experiments.

(a)


650
600
550
500
450
400
350
300
250
200
150
100
50
% basal
0
50
100
150
200
250
300
Time(s)

(b)(i)


200
180
160
140
120
100
% basal
.01
.1
1
10
100
[LPA](micromolar)

(b)(ii)

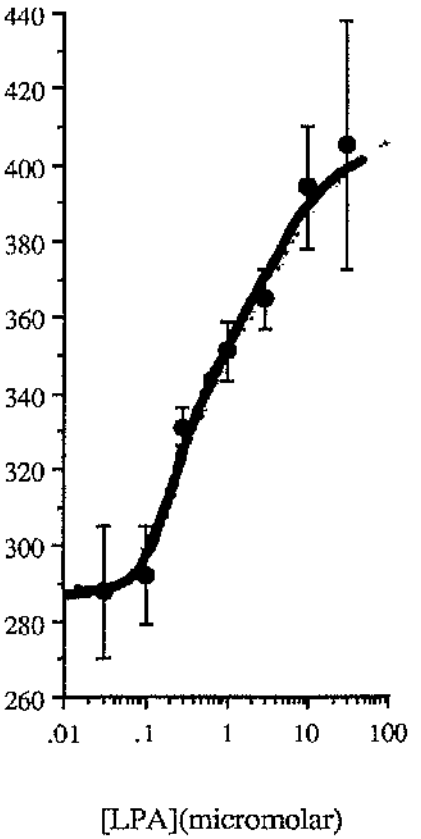

440
420
400
380
360
340
320
300
280
260
.01
.1
1
10
100
[LPA](micromolar)

Figure 5.4 Western blot analysis of bombesin-stimulated ERK1 and ERK2 phosphorylation in Swiss 3T3 fibroblasts.

Swiss 3T3 fibroblasts were seeded at $2x10^4$ cells/ml in DMEM containing 10% (v/v) NBCS until 80-90% confluent, when medium was replaced with DMEM containing either 10% (v/v) or 2% (v/v) NBCS for 24 hours. Cells were then washed with DBG and stimulated with either DBG or 100nM bombesin for the times shown. Stimulated cells were then washed with ice-cold PBS and lysed as described in Materials and methods. 40µg total protein was loaded onto 12.5% (w/v) polyacrylamide gels and SDS-PAGE electrophoresis carried out. ERK1 and ERK2 were identified as described in Chapter 2 and visualised using ECL. Result is representative of 3 individual experiments where freshly stimulated cells were utilised.

Bombesin-stimulated MAP kinase phosphorylation in Swiss 3T3 fibroblasts.

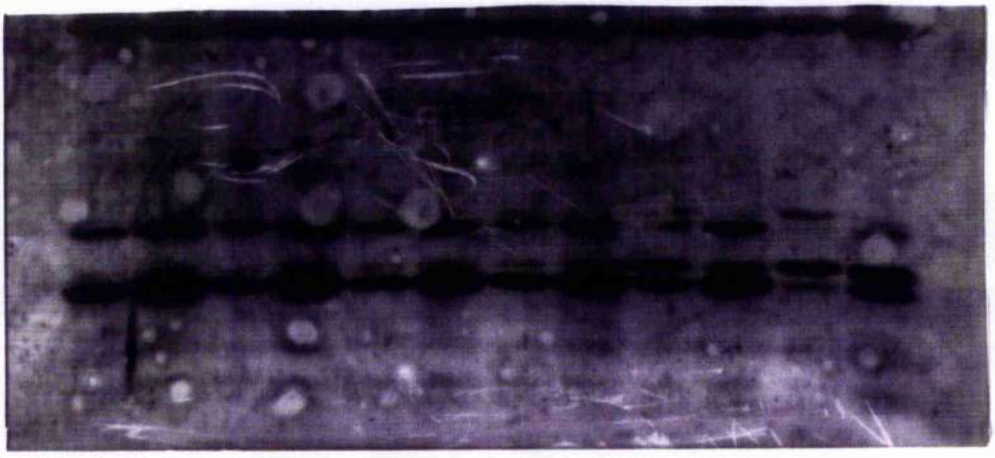


Figure 5.5 Western blot analysis of MAP kinase isoforms present in 5 day DMSO-differentiated HL60 cells.

HL60 cells were seeded at $5x10^5$ cells/ml in RPMI 1640 medium containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO and cultured for 5 days. Differentiated cells were then washed in RBG prior to the stimulation of 10^7 cells with either RBG, 5μM cytochalasin B, 100nM fMLP or both cytochalasin B and fMLP. Stimulated cells were washed with ice-cold PBS and lysed as described in Materials and methods. 40μg total protein was loaded onto 12.5% (w/v) polyacrylamide gels and SDS-PAGE carried out. MAP kinase isoforms were detected as described in Chapter 2 and visualised using ECL. Result is representative of 3 individual experiments.

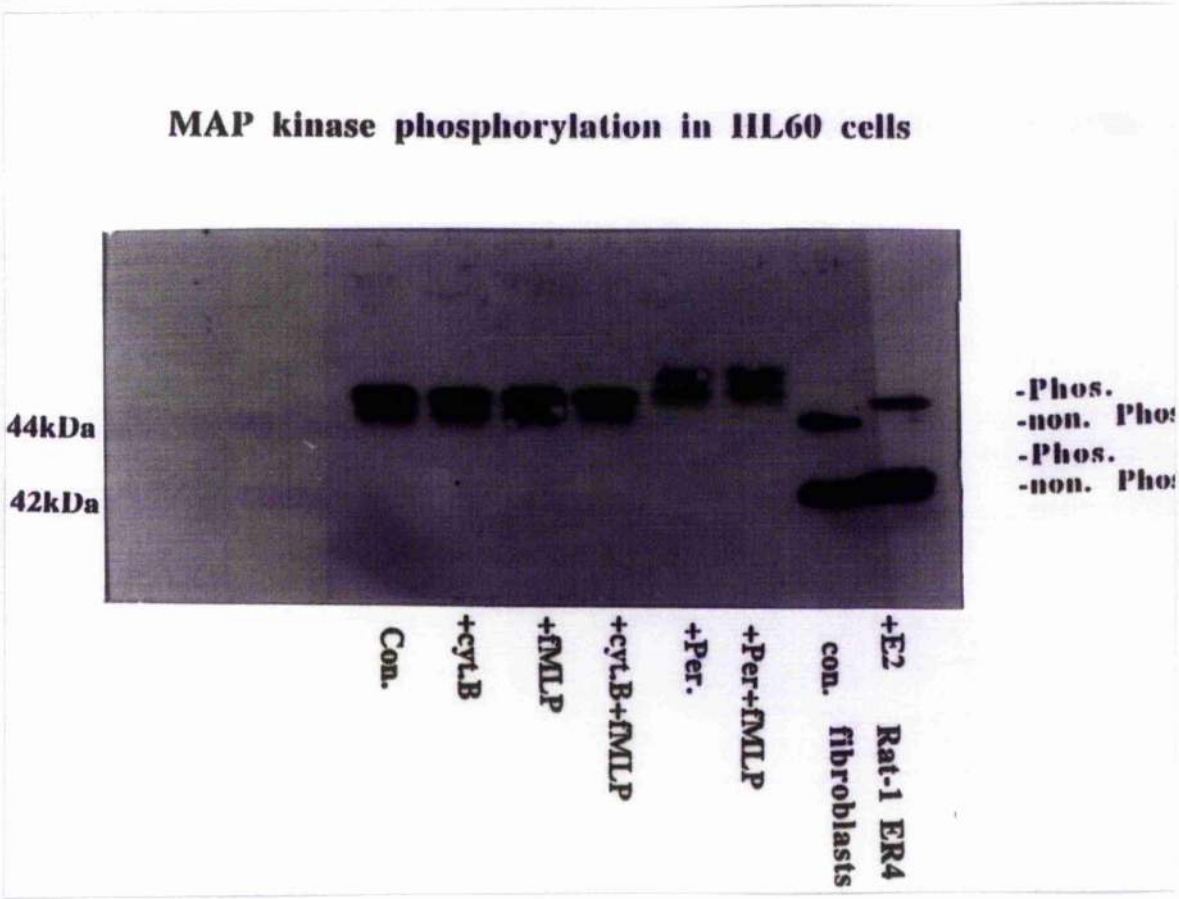

MAP kinase phosphorylation in HL60 cells
44kDa
42kDa
Con.
+cyt.B
+fMLP
+cyt.B+fMLP
+Per.
+Per+fMLP
con. fibroblasts
+E2 Rat-1 ER4
-Phos.
-non. Phos
-Phos.
-non. Phos

Table 5.1 ***In vitro*** **MAP kinase activity in the cell types studied.**

Swiss 3T3 fibroblasts were seeded at $2x10^4$ cells/ml in DMEM containing 10% (v/v) NBCS and cultured until 80-90% confluent. Medium was then replaced with DMEM containing either 2% (v/v) or 10% (v/v) NBCS for 24 hours. Rat-1 Raf ER4 fibroblasts were seeded and cultured in the same way as Swiss 3T3 fibroblasts, but in the presence of 400μg/ml geneticin. When 80-90% confluent the medium was replaced with DMEM for 24 hours. Quiesced cells were washed with DBG and then stimulated with either DBG alone or DBG containing 1μM β-estradiol for 1 hour at 37°C. Pretreated cells were stimulated with either DBG alone or DBG containing 30μM LPA for 30 seconds at 37°C. HL60 cells were seeded at $5x10^5$ cells/ml in RPMI1640 medium containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO and cultured for 5 days. Differentiated cells were washed in RBG and resuspended at 10^7 cells per ml in RBG. 10^6 cells were used per sample which were pretreated with either RBG alone, RBG containing 5μM cytochalasin B for 5 minutes at 37°C or RBG containing 0.5mM perhydrovanadate for 2 minutes at 37°C. Preincubated cells were then stimulated with either RBG alone or RBG containing 100nM fMLP for 5 minutes at 37°C. Reactions were terminated by washing with ice-cold PBS and lysates prepared as described in Materials and methods. Result is representative of 3 individual experiments and is presented as cpm of ^{32}P incorporated into myelin basic protein, ± standard deviation, as described in section 2.6.2.

Table 5.1

Swiss 3T3 cells

2% serum	9407.1	± 60.6
10% serum	9042.3	± 424.6

5 day DMSO-differentiated HL60 cells

Control	7413.9	± 208.0
Cytochalasin B	7022.8	± 200.0
Perhydrovanadate	8992.4	± 94.4

Rat-1 Raf ER4 fibroblasts

Control	2303.6	± 87.4
Estradiol	4240.6	± 320.0
LPA	2388.2	± 295.5
Estradiol+LPA	4336.0	± 100.0

Figure 5.6 ***In vitro* MAP kinase activity in bombesin-stimulated Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were seeded at $2x10^4$ cells/ml in DMEM containing 10% (v/v) NBCS and cultured until 80-90% confluent, when medium was replaced with DMEM containing either 2% (v/v) or 10% (v/v) NBCS for 24 hours. After quiescing cells were washed with DBG and stimulated with either DBG (○) or DBG containing 100nM bombesin (●) for the times indicated. Reactions were terminated by washing with ice-cold PBS and lysates prepared as described in Materials and methods. The measure of *in vitro* MAP kinase activity was determined as described in chapter 2, using myelin basic protein as the substrate. Result is presented as % basal of cpm of ^{32}P incorporated into myelin basic protein ± s.d. with a basal representing 9000±250 cpm and is representative of 3 individual experiments.

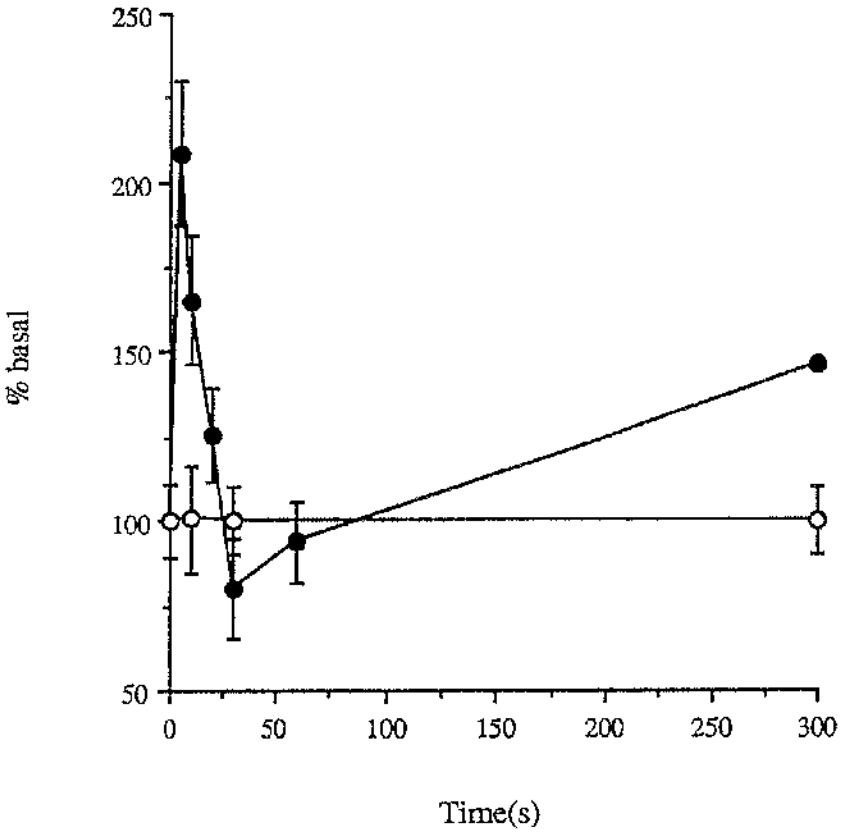

250
200
150
100
50
% basal
0
50
100
150
200
250
300
Time(s)

Figure 5.7(a) **Cytochalasin B- and fMLP-stimulated intracellular calcium concentrations in 5 day DMSO-differentiated HL60 cells.**

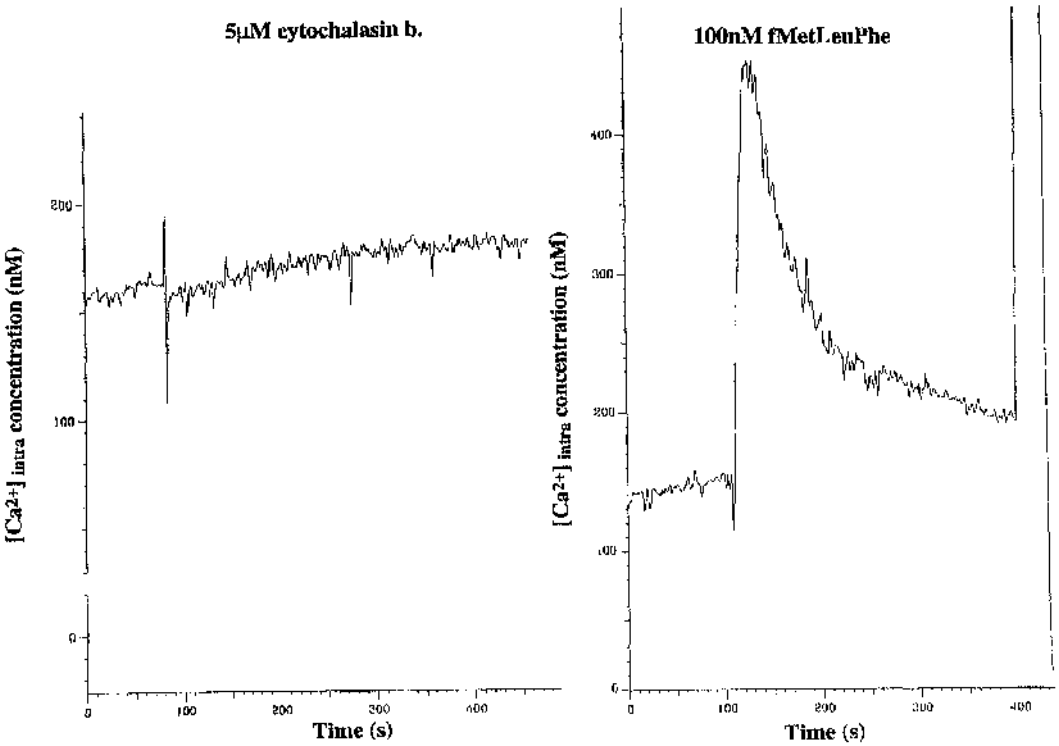
HL60 cells were seeded at $5x10^5$ cells/ml in RPMI 1640 medium containing 15% (v/v) heat inactivated foetal calf serum and 1.3% (v/v) DMSO and cultured for 5 days. DMSO-differentiated HL60 cells were washed in RBG (phenol red free) and then incubated at 37°C for 45 minutes in RBG (phenol red free) containing 1μM Fura-2-am. 10^6 Fura-2 loaded HL60 cells were treated with 5μM cytochalasin B or 100nM fMLP for the time indicated and the ratio of calcium-bound and -unbound Fura-2 measured as described in Materials and methods. Result is representative of 3 individual experiments and the time scale represents the duration of the experiment.

Figure 5.7(b) **Perhydrovanadate-stimulated intracellular calcium concentrations in 5 day DMSO-differentiated HL60 cells.**

Cells were cultured and loaded with Fura-2 as described in Figure 5.7(a). 10^6 cells were stimulated with 0.5mM perhydrovanadate for the time shown and the ratio of calcium-bound and -unbound Fura-2 measured as described in Materials and methods. Result is representative of 3 individual experiments. Time recorded was the duration of the experiment.

a

Cytochalasin B and fMetLeuPhe-stimulated intracellular calcium release



B

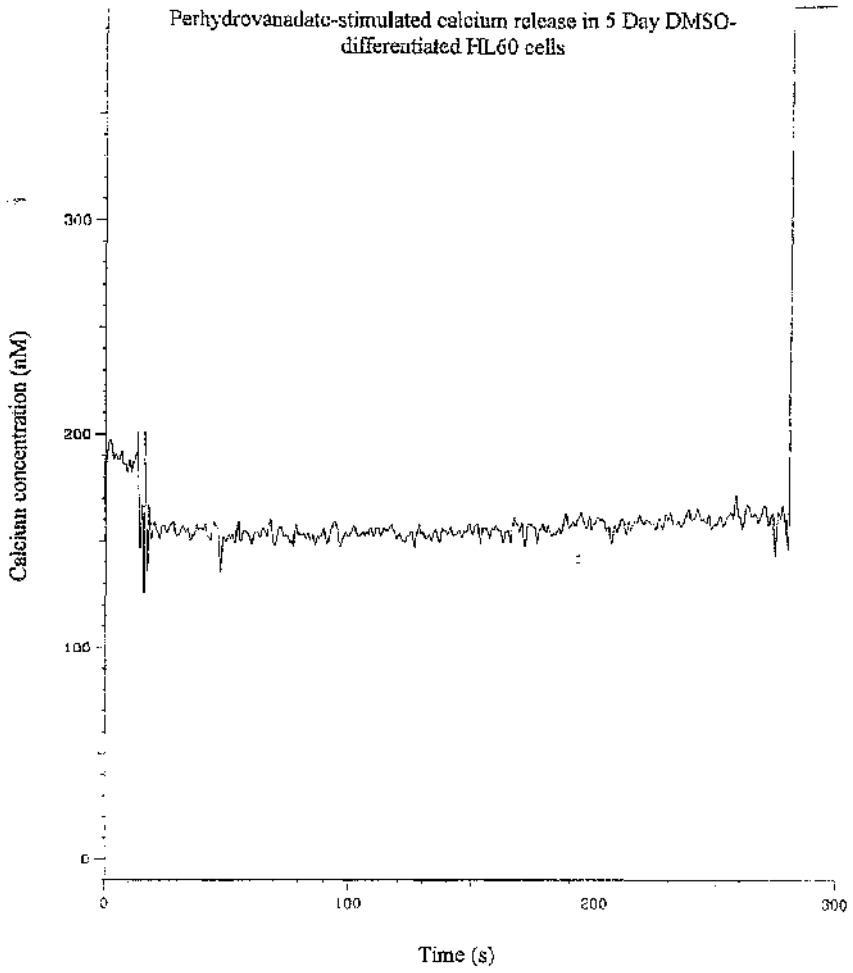


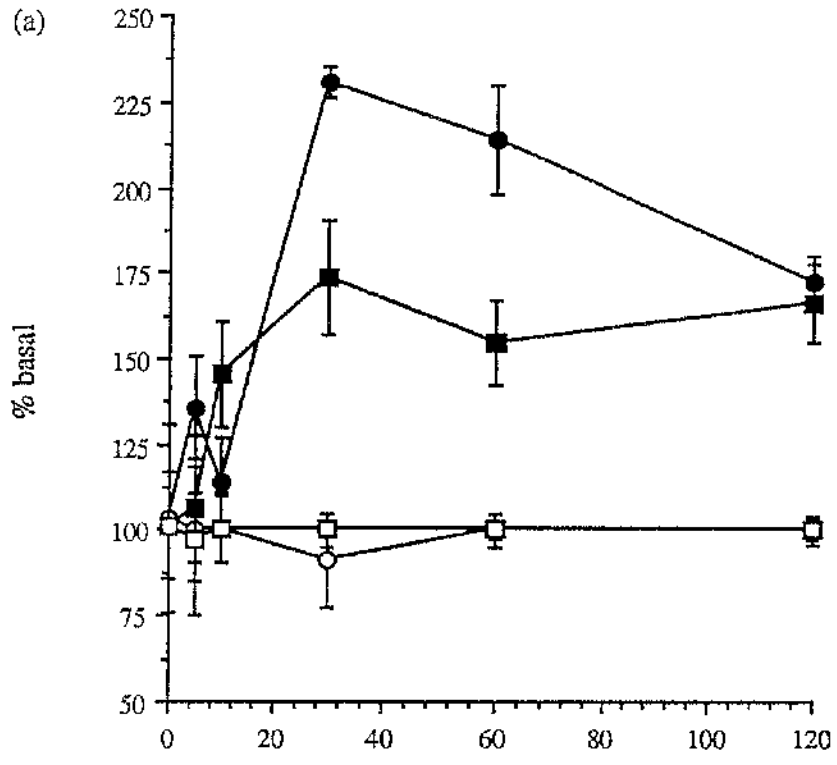
Figure 5.8(a) **Effect of preincubation with BAPTA-AM, upon bombesin-stimulated intracellular [^{3}H]arachidonate release in Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were seeded and labelled as previously described in Chapter 3. Radiolabelled cells were then washed with DBG and preincubated with either DBG alone or DBG containing 10μM BAPTA-AM for 1 hour at 37°C. Preincubated cells were stimulated with either DBG or DBG containing 100nM bombesin for the times shown, resulting in the study of arachidonate levels in contol, non-BAPTA pretreated(○); control, BAPTA pretreated(❑); bombesin-stimulated, non-BAPTA pretreated(●) and bombesin-stimulated, BAPTA pretreated(■) Swiss 3T3 fibroblasts. Result is representative of 2 individual experiments and presented as % basal ± s.d, which is a value equivalent in all cases to 800 ± 45 dpm.

Figure 5.8(b) **Effect of preincubation with BAPTA-AM upon LPA-stimulated intracellular [^{3}H]arachidonate release in estradiol-treated and -untreated Rat-1 Raf ER4 fibroblasts.**

Raf ER4 fibroblasts were seeded and labelled as previously described in Materials and methods. Radiolabelled cells were washed with DBG and preincubated for 1 hour at 37°C with either DBG alone, DBG containing 10μM BAPTA-AM or DBG containing 1μM β-estradiol and 10μM BAPTA-AM. Pretreated cells were then stimulated with either DBG alone or DBG containing 30μM lysophosphatidic acid (LPA) for the times shown at 37°C. This allowed the study of arachidonic acid levels in control, estradiol-treated(▲); LPA-stimulated(○); LPA-stimulated, BAPTA-treated(●); LPA-stimulated, estradiol-treated(❑) and LPA-stimulated, estradiol- and BAPTA-treated(■) Rat-1 Raf ER4 fibroblasts. Result is representative of 2 individual experiments and is presented as % basal ± s.d. which has a value equivalent to 1000 ± 200 dpm.

(a)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225
200
175
150
125
100
75
50
% basal
0
20
40
60
80
100
120
Time(s)

(b)

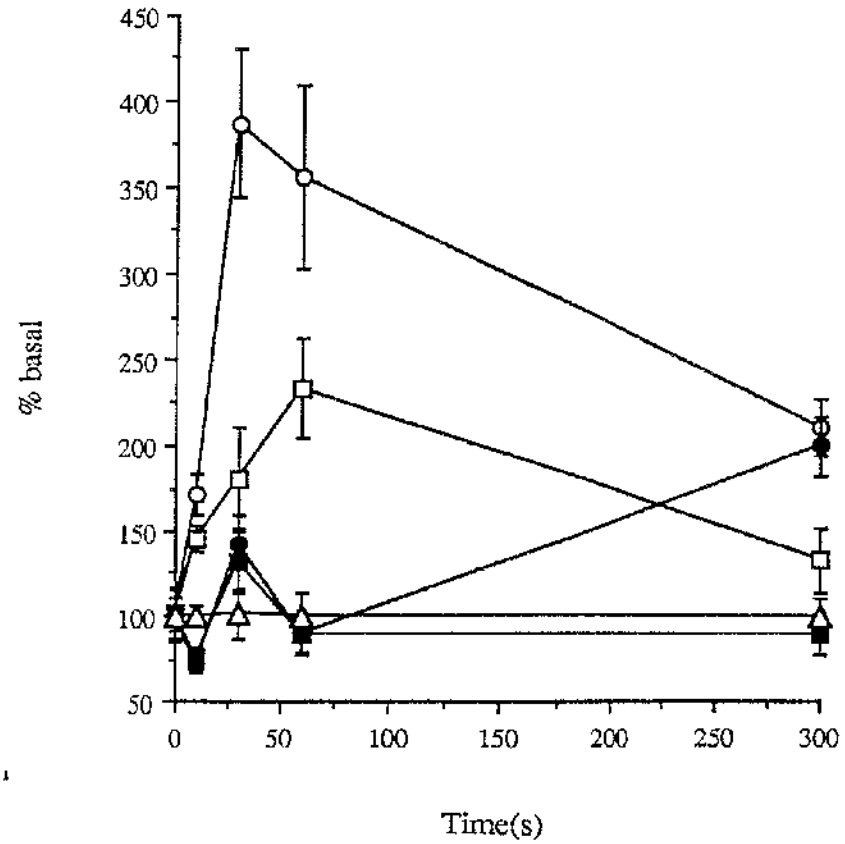
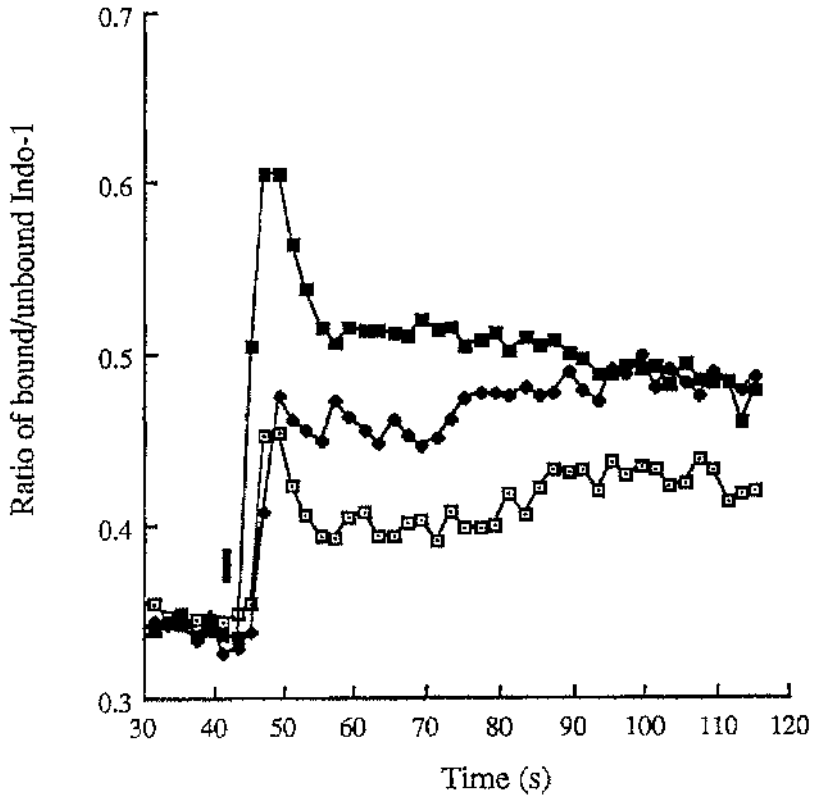

450
400
350
300
250
200
150
100
50
% basal
0
50
100
150
200
250
300
Time(s)

Figure 5.9 **Bombesin-stimulated intracellular calcium concentrations in Swiss 3T3 fibroblasts quiesced in either 2% (v/v) or 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were seeded at $2x10^4$ cells/ml in DMEM containing 10% (v/v) NBCS and cultured on glass coverslips until 80-90% confluent, when medium was replaced with DMEM containing either 2% (v/v) or 10% (v/v) NBCS for 24 hours. Quiesced cells were washed in DBG (phenol red free) and then incubated for 30 minutes at 37°C with DBG (phenol red free) containing 1μM Indo-1. Indo-1 loaded Swiss 3T3 fibroblasts were then stimulated with 100nM bombesin and the consequent increase in intracellular calcium concentrations were measured as described in Materials and methods. Result is representative of 3 individual experiments and is presented as the ratio of the fluorescence obtained for calcium-bound and -unbound Indo-1 at the time shown. Each trace shown is from a single cell measurement and the time scale represents the length of the experiment.

2%


Ratio of bound/unbound Indo-1
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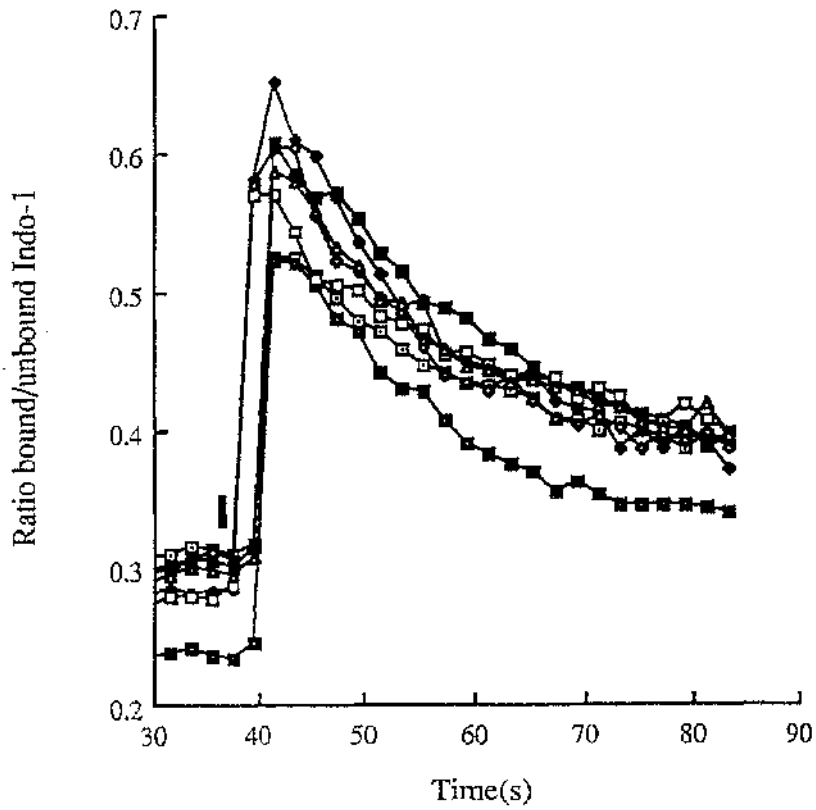
0.6
0.5
0.4
0.3
30
40
50
60
70
80
90
100
110
120
Time (s)

10%


Ratio bound/unbound Indo-1
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0.6
0.5
0.4
0.3
0.2
30
40
50
60
70
80
90
Time(s)

Figure 5.10 **Effect of preincubation with BAPTA-AM upon bombesin-stimulated intracellular calcium concentrations in Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS.**

Swiss 3T3 fibroblasts were seeded at $2x10^4$ cells/ml in DMEM containing 10% (v/v) NBCS and cultured on glass coverslips until 80-90% confluent, at which time the medium was replaced with fresh culture medium. Cells were then washed with DBG (phenol red free), prior to incubation for 1 hour with DBG (phenol red free) containing 10μM BAPTA-AM at 37°C. 30 minutes prior to the end of the incubation period, 1μM Indo-1 was added. Preincubated cells were stimulated with 100nM bombesin for the time shown and the intracellular calcium concentration measured as described in Materials and methods. Result is representative of 3 individual experiments, which utilised freshly cultured Swiss 3T3 fibroblasts, and is presented as the ratio of the fluorescence measured for calcium-bound and -unbound Indo-1. Each trace shown is from an individual cell measurement and the time scale represents the length of the experiment.

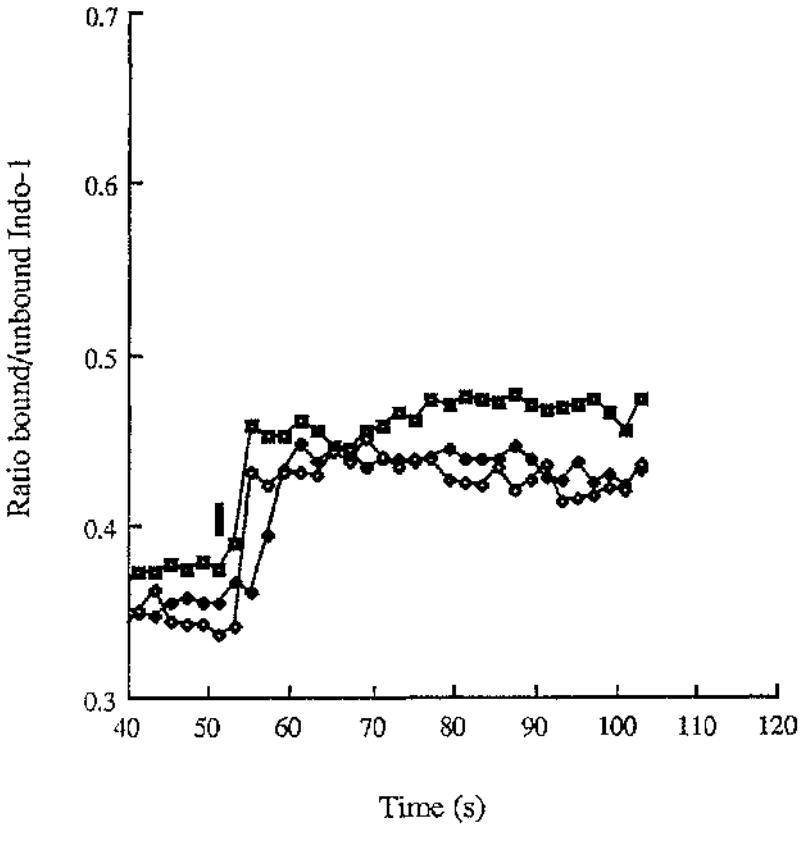

0.7
0.6
0.5
0.4
0.3
Ratio bound/unbound Indo-1
40
50
60
70
80
90
100
110
120
Time (s)

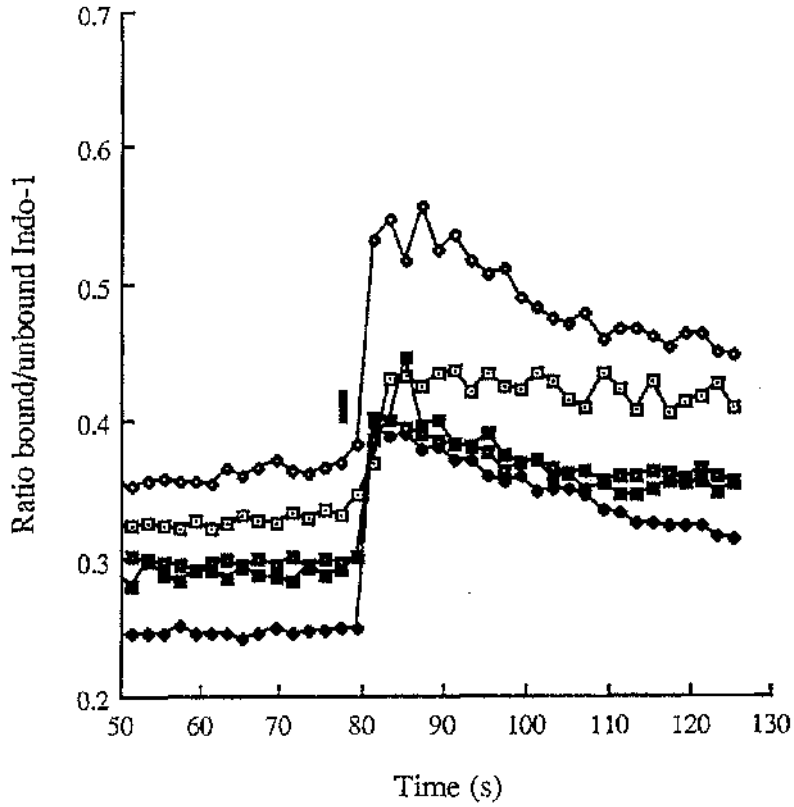
Figure 5.11(a) LPA-stimulated intracellular calcium concentrations in Rat-1 Raf ER4 fibroblasts.

Rat-1 Raf ER4 fibroblasts were seeded onto glass coverslips at $2x10^4$ cells/ml in DMEM containing 10% (v/v) NBCS and 400μg/ml geneticin. When 80-90% confluent, medium was replaced with DMEM for 24 hours. Quiesced cells were washed with DBG (phenol red free) and preincubated for 30 minutes at 37°C with 1μM Indo-1, prior to stimulation with DBG (phenol red free) containing 30mM LPA for the time shown. Intracellular calcium concentrations were measured as described in Materials and methods. Result is representative of 3 individual experiments and presented as the ratio of fluorescence measured for calcium-bound and -unbound Indo-1 over the time shown. Each trace is from an individual cell measurement and the time scale represents the length of the procedure.

Figure 5.11(b) LPA-stimulated intracellular calcium concentrations in estradiol-treated Rat-1 Raf ER4 fibroblasts.

Rat-1 Raf ER4 fibroblasts were seeded at $2x10^4$ cells/ml onto glass coverslips and cultured until 80-90% confluent. Medium was then replaced with DMEM for 24 hours. Quiesced cells were washed with DBG (phenol red free) and preincubated with DBG (phenol red free) containing 1μM β-estradiol for 1 hour at 37°C. 30 minutes prior to the end of the incubation period, 1μM Indo-1 was added. Cells were stimulated with DBG (phenol red free) containing 30μM LPA for the time shown and the intracellular calcium concentration measured as described in Materials and methods. Result is representative of 3 individual experiments and is presented as the ratio of the fluorescence measured for calcium-bound and -unbound Indo-1. Each trace is from an individual cell measurement and the time scale represents the length of the experiment.

(a)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0.2
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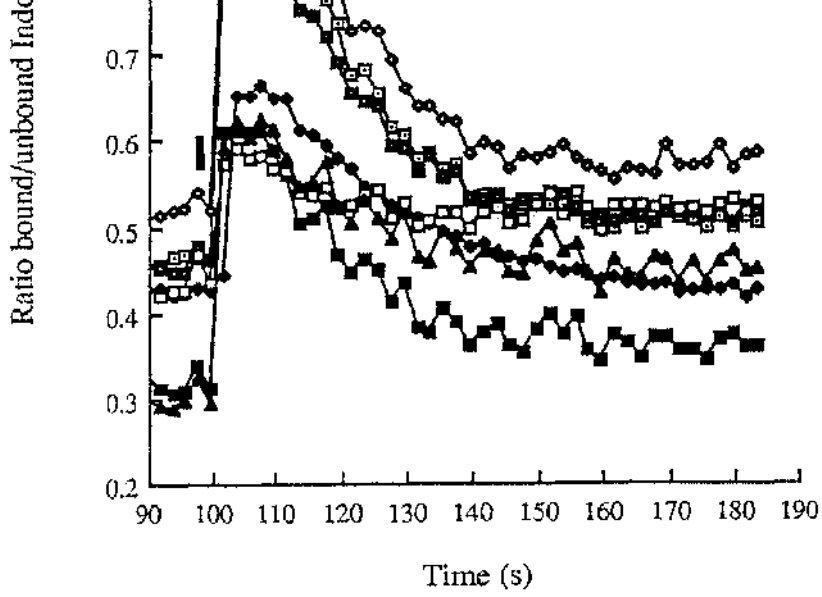
60
70
80
90
100
110
120
130
Time (s)

(b)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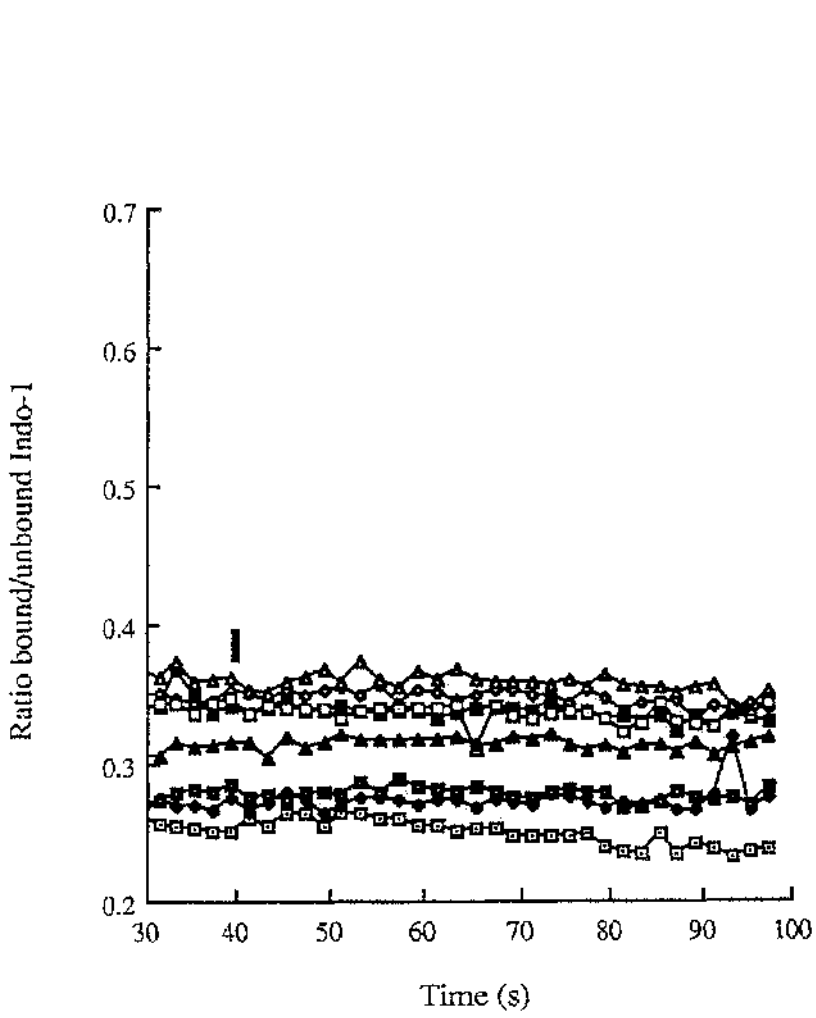
0.6
0.5
0.4
0.3
0.2
90
100
110
120
130
140
150
160
170
180
190
Time (s)

Figure 5.12 **β-estradiol stimulated intracellular calcium concentrations in Rat-1 Raf ER4 fibroblasts.**

Rat-1 Raf ER4 fibroblsts were seeded at $2x10^4$ cells per ml onto glass coverslips in DMEM containing 10% (v/v) NBCS and 400μg/ml geneticin and cultured until 80-90% confluent. Medium was then replaced with DMEM for 24 hours. Quiesced cells were washed with DBG (phenol red free), then incubated with DBG (phenol red free) containing 1μM Indo-1 for 30 minutes at 37°C, prior to stimulation with 1μM β-estradiol for the time shown. Intracellular calcium concentrations were measured as described in Materials and methods. Result is representative of 3 individual experiments and is presented as the ratio of the fluorescence for calcium-bound and -unbound Indo-1. Each trace is from an individual cell measurement and the time scale represents the length of the experiment.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0.6
0.5
0.4
0.3
0.2
Ratio bound/unbound Indo-1
30
40
50
60
70
80
90
100
Time (s)

Figure 5.13 LPA-stimulated intracellular [^{3}H]inositol phosphate release in estradiol-treated and -untreated Rat-1 Raf ER4 fibroblasts.

Rat-1 Raf ER4 fibroblasts were seeded at $2x10^4$ cells per ml in DMEM containing 10% (v/v) NBCS and 400μg/ml geneticin and cultured until 80-90% confluent. Medium was then replaced with DMEM containing 4μCi/ml *myo*-[^{3}H] inositol for 48 hours. Radiolabelled cells were washed with DBG and preincubated for 1hour at 37°C with either DBG alone or DBG containing 1μM β-estradiol. Pretreated cells were then stimulated with either DBG alone or DBG containing 30μM LPA for the times shown. The inositol phosphate groups were separated as described in Materials and methods and quantified using liquid scintilation spectrophotometry. Results for control(○); control, estradiol-treated(❑); LPA-stimulated(●) and LPA-stimulated, estradiol-treated(■) Rat-1 Raf ER4 fibroblasts are representative of 2 individual experiments and presented as dpm ± s.d.

The list of graphs represent: (a) IP_1

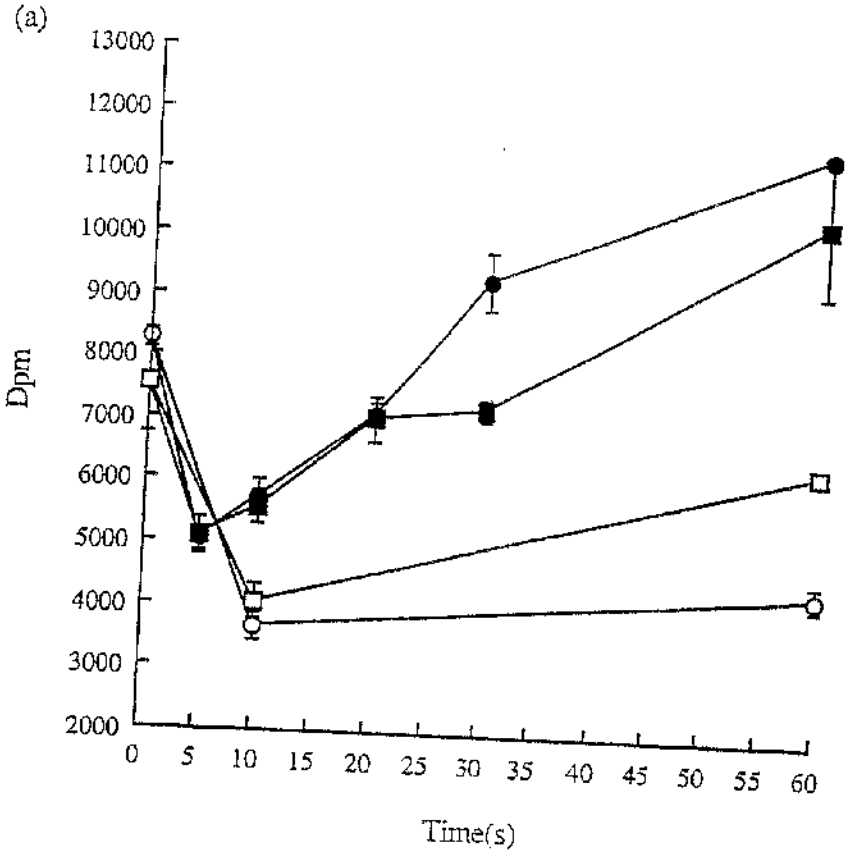
(b) IP_2

(c) IP_3

(d) IP_4

(e) $IP_{5/6}$

(a)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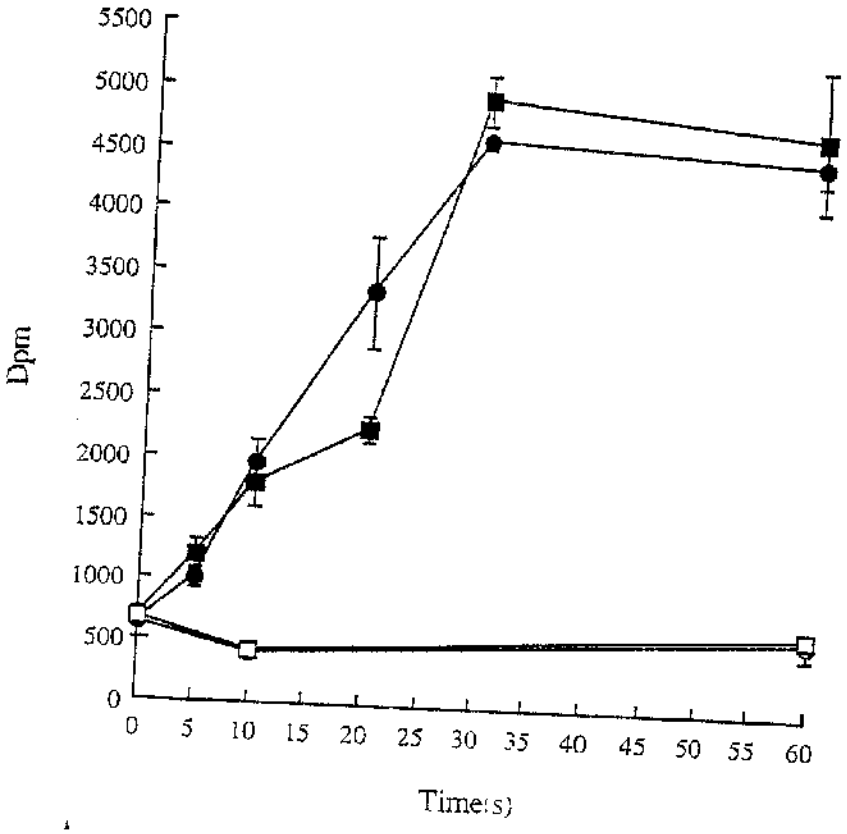
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
Dpm
0 5 10 15 20 25 30 35 40 45 50 55 60
Time(s)

(b)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2500
2000
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1000
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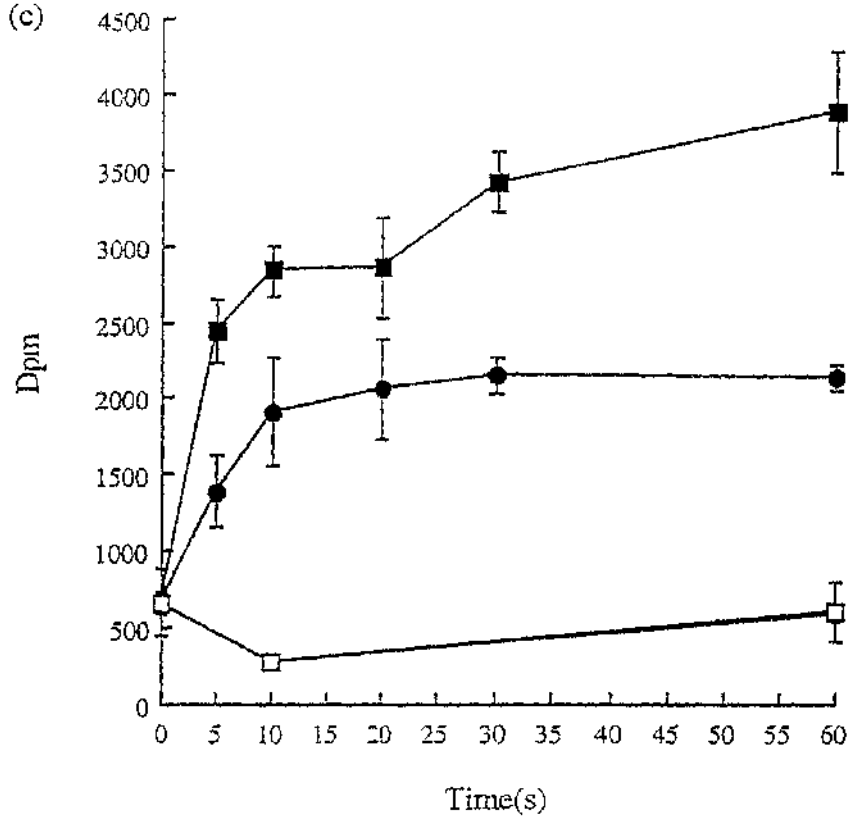
Dpm
0 5 10 15 20 25 30 35 40 45 50 55 60
Time(s)

(c)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2500
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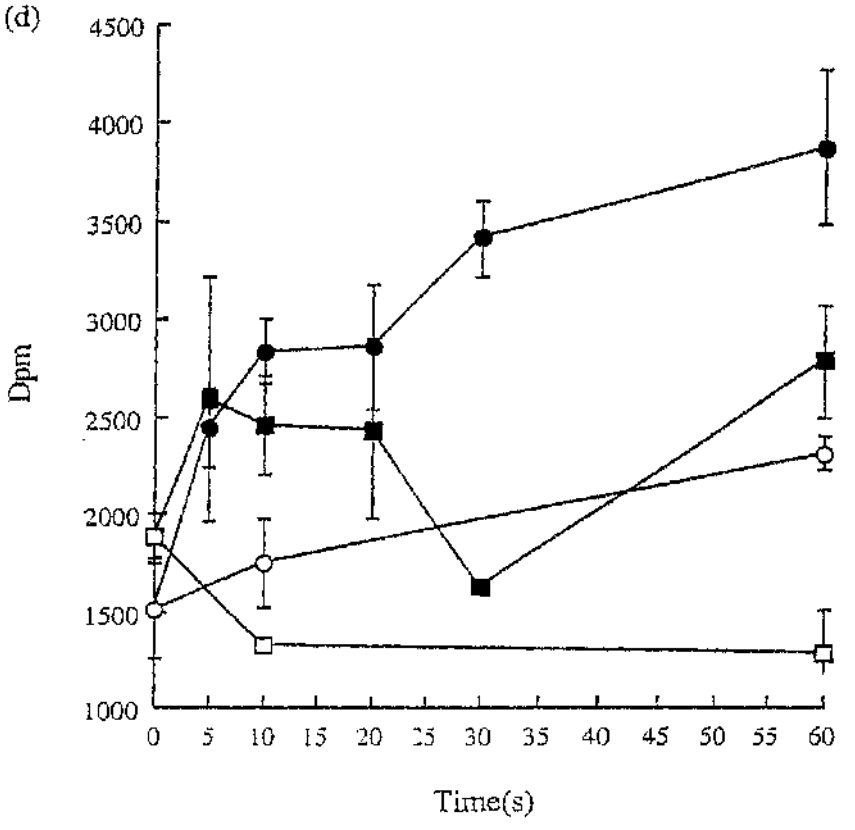
1500
1000
500
0
Dpm
0 5 10 15 20 25 30 35 40 45 50 55 60
Time(s)

(d)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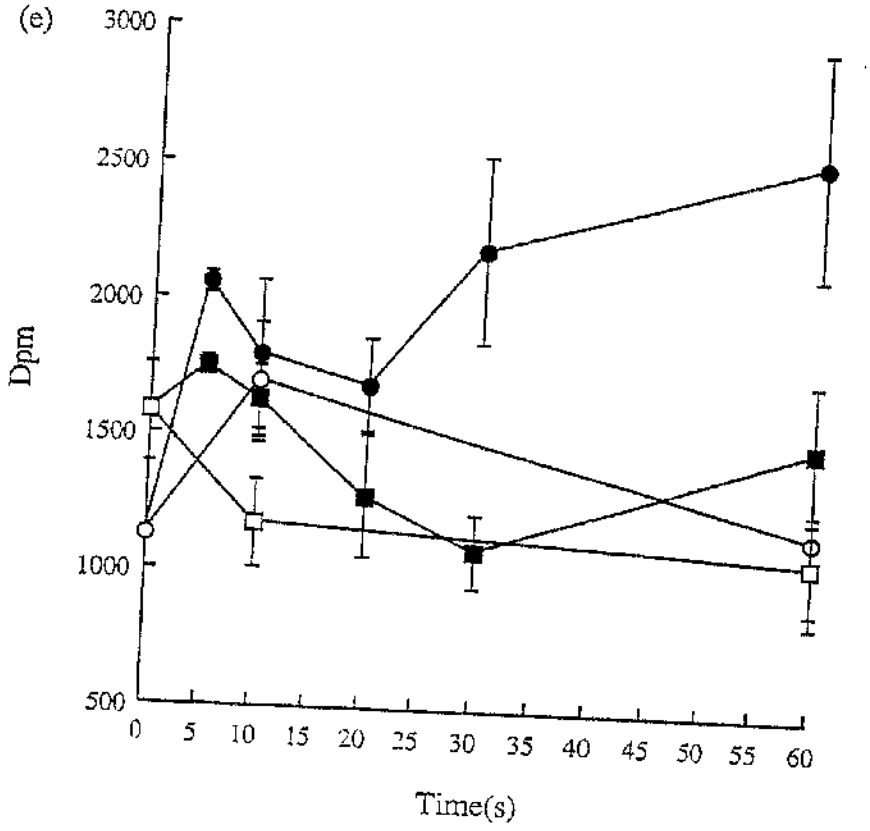
Dpm
0 5 10 15 20 25 30 35 40 45 50 55 60
Time(s)


(e)
3000
2500
2000
1500
1000
500
Dpm
0
5
10
15
20
25
30
35
40
45
50
55
60
Time(s)

5.3 DISCUSSION

5.3.1 Manipulation of MAP kinase using Rat-1 fibroblasts transfected with an estradiol-regulated plasmid expressing activated MAP kinase kinase kinase, p74$^{raf\text{-}1}$.

When discussing the regulation of MAP kinase, all isoforms must be taken into consideration. The isoforms of importance in this work are ERK1 (44kDa) and ERK2 (42kDa) which are both activated by phosphorylation on tyrosine and threonine residues (Hanks *et al.*, 1988). Using western blotting techniques this phosphorylation can be visualised by what is termed the 'band-shift' assay, whereby the phosphorylated MAP kinase isoforms are retarded in the gel, thus running at an apparently higher molecular weight than their non-phosphorylated counterparts (McLees *et al.*, 1995). This bandshift technique has been widely utilised to study many enzymes regulated by phosphorylation. MAP kinase is phosphorylated by MAP kinase kinase (Posada & Cooper, 1992), which is in turn phosphorylated by a MAP kinase kinase kinase, termed *raf-1* (Kyriakis *et al.*, 1992). This 74kDa protein is itself activated by *ras* which has been shown to be activated by many agonists upon receptor activation (Moodie *et al.*, 1993). The dual phosphorylation of MAP kinase on both tyrosine and threonine residues provides a convergence point for both a tyrosine kinase cascade, such as that stimulated by insulin (for review see White & Kahn, 1994) and the classical PKC-mediated response stimulated by agonists which work via 7 membrane spanning receptors and *src* kinases, such as bombesin (Cook *et al.*, 1990). Therefore, the manipulation of *raf-1* is a good tool to study the regulation of MAP kinase, and thus, its effects upon its substrates. One such substrate has been shown to be cytosolic phospholipase A_2 when studied in an *in vitro* system (Lin *et al.*, 1993).

Rat-1 Raf ER4 cells are Rat-1 fibroblasts transfected with a plasmid containing the kinase domain of p74$^{raf\text{-}1}$ fused to the hormone binding domain of the human estrogen receptor, resulting in the fusion gene product Raf ER (Samuels *et al.*, 1993). Upon incubation with β-estradiol, the increased expression of the kinase domain of

p74$^{raf\text{-}1}$, results in fully phosphorylated ERK1 and ERK2 (Figure 5.1). This construct permits the examination of the role of MAP kinase activation in the absense of an input from either p21^{ras} or receptor activation. The full mitogen for Rat-1 fibroblasts, lysophosphatidic acid (LPA), did not alter the phosphorylated state of either ERK isoform after a 1 minute stimulation, irrespective of prior estradiol pretreatment (Figure 5.1). This is probably due to the time point chosen for LPA-stimulation, as a 5 minute stimulation by LPA has been shown to stimulate the phosphorylation of MAP kinase in these cells (Hordijk *et al.*, 1994). The increased MAP kinase phosphorylation correlates with an increase in MAP kinase activity as shown in Table 5.1. However, the pretreatment with 1μM β-estradiol also increases overall tyrosine kinase activity, as various proteins have increased tyrosine phosphorylation, as shown by western blotting in Figure 5.2, which maybe due to the activation of various proteins involved in the tyrosine kinase cascade initiated by *raf-1*, including MAP kinase. This increase in tyrosine phosphorylation may have an effect upon the activation of cPLA$_2$, but MAP kinase is activated to such an extent that it will probably be the major kinase involved in the regulation of cPLA$_2$. Estradiol-treated Raf ER4 fibroblasts produced a substantial increase in LPA-stimulated arachidonate release, compared to untreated cells (Figure 5.3(a)). In untreated cells, there was a monophasic release of arachidonate, which was maximum at 30 seconds, 2-3 fold above basal. In estradiol-treated cells, this response was enhanced in magnitude, with a maximum response at 30 seconds, 5-6 fold above basal. This demonstrates that phosphorylated MAP kinase can increase LPA-stimulated cPLA$_2$ activity but, since the basal level of arachidonate is identical in both the estradiol-treated and -untreated Rat-1 Raf ER4 fibroblasts, does not produce the release of arachidonate alone. This suggests that the reported activation of cPLA$_2$ by MAP kinase *in vitro*, is not the case *in vivo* and that an additional factor in conjunction with MAP kinase phosphorylation is required for arachidonate release. This enhanced magnitude of response is mirrored in the dose-dependency of LPA-stimulated arachidonate release, but does not effect the affinity of the receptor as the EC$_{50}$ is unaffected (Figure 5.3(b)).

5.3.2 Effect of acute and chronic priming upon MAP kinase phosphorylation and activity.

It has been shown that $cPLA_2$ can be primed by the phosphorylation of MAP kinase by a *raf-1*-mediated cascade. This lead to the possibility that the activation of MAP kinase by either or both of the chronic priming agents in serum and cytochalasin B, could be involved in the subsequent priming of $cPLA_2$.

Figure 5.4 demonstrates that Swiss 3T3 fibroblasts quiesced in DMEM containing 10% (v/v) NBCS, have a higher level of both ERK1 and ERK2, compared to cells quiesced in 2% (v/v) NBCS. The ratio of phosphorylated to non-phosphorylated ERK1/2 at basal levels was identical under both quiescing conditions and the increase in MAP kinase phosphorylation in response to bombesin, was unaffected by the quiescing method employed. Therefore, chronic priming by serum does not appear to affect the phosphorylation state of MAP kinase, but does appear to enhance the level expression of the MAP kinase isoforms. The higher level of MAP kinase in 10% quiesced cells, does not result in a higher level of basal MAP kinase enzyme activity as determined by *in vitro* measurements (Table 5.1), providing further evidence that MAP kinase is not involved in the priming event induced by serum in Swiss 3T3 fibroblasts. In 10% (v/v) NBCS, there was a biphasic *in vitro* MAP kinase activity. The initial, transient phase was maximum at 10 seconds, returning to basal by 30 seconds, the second phase was sustained, maximal at 1 minute and remaining elevated at 5 minutes (Figure 5.6). Only the second phase was detectable in cells quiesced in 2% (v/v) NBCS (results not shown), suggesting that MAP kinase is also primed by serum. This may be one of the factors involved in the priming event of $cPLA_2$ observed, but the results highlight the inacuracy of western blot 'band-shifts' as a measure of MAP kinase activity.

In the case of acute priming of $cPLA_2$ by cytochalasin B in 5 day DMSO-differentiated HL60 cells, there was no increased phosphorylation of MAP kinase, in response to the priming agent, the agonist fMLP or both used in conjunction (Figure 5.5). However, when compared to the ERK1 and ERK2 in control and estradiol-

treated Rat-1 Raf ER4 fibroblasts, it was clear that only the phosphorylated ERK1 was present in 5 day DMSO-differentiated HL60 cells. The phosphorylation of ERK1 was enhanced by the other possible priming agent, perhydrovanadate, suggesting that the hyperphosphorylation observed is due to tyrosine phosphorylation. Therefore, in 5 day DMSO-differentiated HL60 cells a number of tyrosine or other kinases can phosphorylate MAP kinase and some of these are active prior to priming. Cytochalasin B priming does not appear to be mediated through MAP kinase, as it does not increase its phosphorylation state, however, the priming event mediated by perhydrovanadate, may be due to increased MAP kinase activity. This activity increase was confirmed by *in vitro* measurements (Table 5.1). Therefore, it appears that in both serum-primed Swiss 3T3 fibroblasts and cytochalasin B-primed DMSO-differentiated HL60 cells, MAP kinase activation is not involved. This may not be the case in perhydrovanadate-primed HL60 cells, as this was able to phosphorylate MAP kinase, providing the possibility that the priming of differentiated HL60 cells maybe via MAP kinase-dependent and -independent pathways.

5.3.3 Role of agonist-stimulated intracellular calcium concentrations in the activation and priming of $cPLA_2$.

$cPLA_2$ can be activated by increased intracellular calcium concentrations (Leslie *et al.*, 1988). From the sequence of $cPLA_2$, a Ca^{2+}-lipid-binding domain (CaLB) was identified (Sharp *et al.*, 1991) and may mediate the translocation of $cPLA_2$ to the membrane upon agonist-stimulated increased intracellular calcium concentrations (Channon & Leslie, 1990). Therefore, any manipulation of intracellular calcium concentration would have an effect upon subsequent cytosolic phospholipase A_2 activity.

It was suggested that cytochalasin B treatment increased intracellular calcium, however, this was not the case in 5 day DMSO-differentiated HL60 cells. The cells used were able to induce a Ca^{2+} signal as the agonist fMLP produced an apparently IP_3-mediated intracellular calcium release (Figure 5.7(a)). Figure 5.7(b) demonstrated

that the alternative priming agent in 5 day DMSO-differentiated HL60 cells, perhydrovanadate, did not effect the concentration of intracellular calcium. Therefore, it can be concluded that the priming of $cPLA_2$ in 5 day DMSO-differentiated HL60 cells, is not mediated via increased intracellular calcium concentrations, but that agonist-stimulated intracellular calcium release is essential for $cPLA_2$ activity.

This calcium requirement for $cPLA_2$ activity was also shown in Swiss3T3 and Rat-1 Raf ER4 fibroblasts. In the presence of the intracellular calcium chelator BAPTA, bombesin-stimulated arachidonate release in Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS was inhibited by 40-50% (Figure 5.8(a)), whereas LPA-stimulated arachidonate release in both estradiol-treated and -untreated Raf ER4 fibroblasts, was totally inhibited (Figure 5.8(b)). The incomplete inhibition of bombesin-stimulated arachidonate release in Swiss 3T3 fibroblasts cannot be attributed to incomplete chelation of intracellular calcium by BAPTA, as there was a complete abolition of bombesin-stimulated increased intracellular concentrations in the presence of BAPTA (Figure 5.10). Therefore, agonist-stimulated intracellular calcium release is essential for LPA-stimulated arachidonate release in Rat-1 Raf ER4 fibroblasts, but there is another factor in conjunction with calcium required for maximal bombesin-stimulated aracidonate release in Swiss 3T3 fibroblasts. This additional regulator may be a GTP-binding protein as in 10% quiesced Swiss 3T3 fibroblasts, bombesin-stimulated arachidonate release was totally inhibited by GDPβS (Figure 3.10(b)). However, the GTP-binding protein involved appeared to be in the GTP-bound state under the exprimental conditions and, since the non-hydrolyzable analogue GTPγS, did not potentiate bombesin-stimulated arachidonate release in streptolysin O-permeabilised Swiss 3T3 fibroblasts (Chapter 3).

Calcium, however, does not appear to play a role in the priming of $cPLA_2$ by serum. In Swiss 3T3 fibroblasts quiesced under both conditions, the basal concentration of intracellular calcium was identical, suggesting that serum does not induce a sustained elevation of the intracellular calcium concentrations. Nevertheless, the bombesin-stimulated intracellular calcium concentration was greater in 10%

quiesced cells (Figure 5.9), suggesting that PLCγ is also affected by serum, since it has been well documented that bombesin-stimulated intracellular calcium release is mediated via an IP_3-stimulated response (Cook *et al.*, 1990). In the case of estradiol-treated and -untreated Rat-1 Raf ER4 fibroblasts, the LPA-stimulated increased intracellular calcium concentration also appeared to be IP_3-mediated (Figure 5.11(a) and (b)), from the trace obtained. Of interest, was the apparent elevation of LPA-stimulated intracellular calcium release in estradiol-treated Raf ER4 fibroblasts. This elevation was not due to estradiol stimulating intracellular calcium release (Figure 5.12), but may be due to a similar priming effect upon PLCγ as observed in Swiss 3T3 fibroblasts in response to serum. The chronic priming of Rat-1 Raf ER4 fibroblasts by estradiol elevated LPA-stimulated IP_3 production, which was detected immediately upon the addition of LPA (Figure 5.13). LPA-stimulated IP_4 levels were also enhanced in estradiol-treated Raf ER4 cells, but were detected at a later time point compared to the LPA-stimulated increase in intracellular calcium, which was instantaneous. This suggests that IP_4-stimulated extracellular calcium entry does not play a role in the onset of LPA-stimulated increased intracellular calcium concentrations, however, this is rather ambiguous as all species of IP_4 are measured, not just that responsible for stimulating extracellular calcium influx.

In conclusion, cytochalasin B-stimulated MAP kinase activity and intracellular calcium release does not play a role in the acute priming of $cPLA_2$ in 5 day DMSO-differentiated HL60 cells. The chronic priming of Swiss 3T3 fibroblasts is not mediated via an increased basal concentration of calcium or MAP kinase activity, but calcium is required for maximal $cPLA_2$ activity. The activation of MAP kinase by estradiol-pretreatment in Rat-1 Raf ER4 fibroblasts, enhances LPA-stimulated arachidonate release, but does not increase the basal levels of arachidonate release, suggesting that another factor is required to stimulate $cPLA_2$. This factor is calcium, as BAPTA-pretreatment totally inhibits LPA-stimulated arachidonate release in estradiol-treated cells. Chronic priming by serum in Swiss 3T3 fibroblasts and by estradiol in Rat-1 Raf ER4 fibroblasts, enhances the concentration of intracellular

calcium released upon agonist stimulation, suggesting that PLCγ is perhaps also primed under these conditions. This priming of PLCγ was demonstrated by an elevation of LPA-stimulated IP_3 levels in estradiol-treated Rat-1 Raf ER4 fibroblasts. Therefore, chronic and acute priming appears to be due to a tyrosine phosphorylated protein (Chapter 3), which is not involved in the MAP kinase cascade. In the case of Swiss 3T3 fibroblasts, this protein may feed into $cPLA_2$ activation via a non-MAP kinase mediated pathway, or affect the cytoskeletal network as shown for cytochalasin B. This possible involvement of the cytoskeleton allows $cPLA_2$ easier access to the substrate, bypassing any agonist-stimulated translocation mechanisms that maybe required for maximal activity. In this new configuration the enzyme would be more susceptible to subsequent agonist-stimulated calcium release, which is essential for activity.

CHAPTER 6

LOCALISATION AND PHOSPHORYLATION STATE OF CYTOSOLIC PHOSPHOLIPASE A_2 UNDER THE CONDITIONS STUDIED.

6.1 INTRODUCTION

In the previous chapters it has been shown that cytosolic phospholipase A_2 activity can be acutely primed by cytochalasin B in 5 day DMSO-differentiated HL60 cells and chronically primed by serum in Swiss 3T3 fibroblasts. Neither cytochalasin B nor serum stimulated phospholipase A_2 activity, as shown by arachidonate release, but their effect produced enhanced agonist-stimulated arachidonate release (Chapter 3). Both cytochalasin B and serum induced an increase in tyrosine phosphorylation, implying that a tyrosine phosphorylated protein was activated, which did not stimulate $cPLA_2$ alone, but enhanced the enzyme's activity to subsequent agonist stimulation (Chapter 4). Tyrosine phosphorylation induced by the priming agents, did not stimulate MAP kinase activity *in vitro*, but *raf-1*-stimulated MAP kinase activity enhanced LPA-stimulated arachidonate release, demonstrating that MAP kinase was a positive regulator of $cPLA_2$ (Chapter 5). However, basal levels of $cPLA_2$ activity were not affected, therefore, an additional agonist-stimulated signal was required for the enhanced activation of cytosolic phospholipase A_2 in cytochalasin B-primed HL60 cells, serum-primed Swiss 3T3 fibroblasts and β-estradiol-treated Rat-1 Raf ER4 fibroblasts.

Cytosolic phospholipase A_2 activity has been shown to be regulated by various factors including protein kinase C (Gronich *et al.*, 1988) and GTP-binding proteins (Cockcroft, 1992). Both G_{i2} (Winitz *et al.*, 1994) and *rac* (Peppelenbosch *et al.*, 1995) have been implicated in the regulation of $cPLA_2$ activity. The enhancement of agonist-stimulated arachidonate release by GTPγS, the non-hydrolysable analogue of GTP, was initially thought to be due to the chronic activation of phospholipase C (PIC), producing increased intracellular levels of the protein kinase C activator diacylglycerol (DAG) and the intracellular calcium mobiliser inositol-1,4,5-trisphosphate (IP_3). The IP_3-mediated response was of more importance when studying the acute agonist-stimulated arachidonate release, as PKC only effects the second sustained phase of arachidonate release (Currie *et al.*, 1992). IP_3 levels increase rapidly and transiently in response to bombesin in Swiss 3T3 fibroblasts

(Cook *et al.*, 1990), producing an immediate increase in intracellular calcium concentrations, which was required for maximal $cPLA_2$ activity (Chapter 5), as bombesin-stimulated arachidonate release was inhibited by 50% by the calcium chelator BAPTA (Figure 5.8(a)). The regulation of $cPLA_2$ by calcium is through the calcium-phospholipid (CaLB) binding site and is thought to confer an active conformational state of the enzyme upon calcium binding (Sharp *et al.*, 1991). This may result in the translocation of the enzyme from the cytosol to the membrane, which has been demonstrated by western blotting upon increased intracellular calcium concentrations (Channon & Leslie, 1990). Using a 'band-shift' SDS-PAGE system, it was shown that it was only phosphorylated $cPLA_2$ that was translocated to the membrane upon calcium binding. This phosphorylated form of $cPLA_2$ has been shown *in vitro* to be the more active state of the enzyme (Kramer *et al.*, 1993(a)), which would be logical as the membrane contains the enzymes natural substrate, as well as many of the suggested regulators of $cPLA_2$.

This chapter addressed the translocation of $cPLA_2$ in response to the priming agents and agonists studied throughout this work. The phosphorylation state of the enzyme was also examined in order to correlate the location of cytosolic phospholipase A_2 with its activity.

6.2 RESULTS

6.2.1 Localisation and phosphorylation state of cytosolic phospholipase A_2 in chronically primed Swiss 3T3 and β-estradiol treated Rat-1 Raf ER4 fibroblasts.

In Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS, the initial, transient, bombesin-stimulated arachidonate release was shown to be kinetically quicker than in cells quiesced in 2% (v/v) NBCS (Figure 3.4). This phenomenon was attributed to priming of cytosolic phospholipase A_2 by some factor which was at a high enough concentration in 10% serum, to enhance the activity of the enzyme to a subsequent agonist stimulation. To examine the localisation and phosphorylation state of $cPLA_2$ under these conditions, samples were blotted with a rabbit anti-peptide, anti-cytosolic phospholipase A_2 antibody. Figure 6.1(a) demonstrates that most of the $cPLA_2$ was present in the cytosolic fraction when Swiss 3T3 fibroblasts were quiesced in either 2% or 10% (v/v) NBCS. However, Figure 6.1(a) also demonstrates that in cells quiesced in 10% (v/v) NBCS more of the enzyme was in the membrane fraction under basal conditions. Only non-phosphorylated $cPLA_2$ was detected in the cytosol and the ratio of phosphorylated to non-phosphorylated enzyme in the membrane fraction under basal conditions, was greater in cells quiesced in 10% serum. Upon stimulation with 100nM bombesin for 20 or 30 seconds, a translocation of $cPLA_2$ from the cytosolic fraction to the membrane fraction was observed in cells quiesced under both conditions (Figure 6.1(b)). To determine which membrane compartment the enzyme was located confocal microscopy using the same antibody utilised for western blotting was performed, with antibody binding being detected using an anti-rabbit fluorescene conjugate. Figure 6.2(a) demonstrates that in cells quiesced in DMEM containing 2% (v/v) NBCS, the majority of the enzyme is located in the cytosol and extranuclear compartment. In cells quiesced in DMEM containing 10% (v/v) NBCS, the enzyme was in both the extra- and intra-nuclear regions (Figure 6.2(b)). Therefore, there is an apparent difference in the localisation of the enzyme within the cell, depending on the quiescing conditions used.

As MAP kinase activation has been shown to phosphorylate $cPLA_2$ directly *in vitro*, the effect upon the phosphorylation state and localisation of $cPLA_2$ by the activation of MAP kinase in Rat-1 Raf ER4 fibroblasts was examined. In Figure 5.1 it was shown that MAP kinase in Rat-1 Raf ER4 fibroblasts was in the fully phosphorylated form after a 1 hour treatment with 1μM β-estradiol. Figure 6.3(a) demonstrates that in control Rat-1 Raf ER4 fibroblasts, $cPLA_2$ was mainly in the non-phosphorylated form. After a 1 hour pretreatment with 1μM β-estradiol a clear retardation in the mobility of the enzyme was visualised, corresponding to the phosphorylation of $cPLA_2$. This phosphorylation was confirmed using phosphatase treatment, where the 'band-shift' was abolished and only dephosphorylated enzyme was detected (results not shown). This figure also demonstrates that a 30 second stimulation with 30μM lysophosphatidic acid, did not alter the phosphorylation state of $cPLA_2$ in either estradiol-treated or -untreated Rat-1 Raf ER4 fibroblasts. This phosphorylation of $cPLA_2$ by estradiol-stimulated MAP kinase phosphorylation, was due to a direct effect on $cPLA_2$ by the kinase cascade initiated by *raf-1*, as both inhibition of protein kinase C by a PKC-specific inhibitor, RO-31-8220 and chelation of intracellular calcium by BAPTA had no effect upon estradiol-stimulated phosphorylation (results not shown). Estradiol pretreatment caused a substantial phosphorylation of $cPLA_2$ in both the membrane and cytosolic fractions. 30μM LPA did not affect the phosphorylation state at 30 seconds stimulation, whereas 100nM thrombin caused a marked increase in phosphorylated enzyme in the membrane fraction (Figure 6.3(b)). Whether this phosphorylated enzyme is due to the phosphorylation of enzyme already located at the membrane, or to a translocation of phosphorylated enzyme from the cytosolic fraction is unclear. The phosphorylation of $cPLA_2$ by estradiol-stimulated MAP kinase activation appeared to determine the localisation of the enzyme visualised by confocal microscopy. In untreated Rat-1 Raf ER4 fibroblasts, $cPLA_2$ was detected mainly in an intranuclear location (Figure 6.4(a)), however, following a 1 hour treatment with 1μM β-estradiol, there was a concentration of the enzyme to a extranuclear compartment (Figure 6.4(b)). There

appeared to be a distinct structural element to which the enzyme localised in estradiol-stimulated cells, rather than being diffusely distributed within the cytosol (Figure 6.5). The identity of the region to which the enzyme became associated remains to be elucidated.

Quantitation of extra- and intra-nuclear $cPLA_2$ localisation was performed by using propidium iodide to stain the nucleus and subtracting the nuclear-associated fluorescence from the total fluorescence attributable to $cPLA_2$ (Table 6.1). This demonstrated that in Swiss 3T3 fibroblasts quiesced in 2% (v/v) NBCS, the majority of $cPLA_2$ was extranuclear and following a 1 minute stimulation of 100nM bombesin, there was translocation of the enzyme into the nucleus. In cells quiesced in 10% (v/v) NBCS, there was a more even distribution between extra- and intra-nuclear compartments, with 100nM bombesin stimulating a translocation of the enzyme into the extranuclear compartment. In Rat-1 Raf ER4 fibroblasts $cPLA_2$ was evenly distributed between the extra- and intra-nuclear compartments, however, estradiol treatment stimulated relocation of the enzyme into an extranuclear location.

As discussed earlier, there was a distinct localisation of the enzyme in the extranuclear compartment in estradiol-treated Rat-1 Raf ER4 fibroblasts (Figure 6.5). To examine this further, a *z*-series was taken of estradiol-treated Rat-1 Raf ER4 fibroblasts (Figure 6.6). In this procedure, latitudinal sections were taken through the cell, to determine the localisation. This demonstrates, that as well as a concentration of the enzyme to a perinuclear fraction, there is also a polarity to the localisation. It was apparent that the enzyme was concentrated on the upper section of the nucleus and is not detected on the adherent surface of the cell.

6.2.2 Localisation and phosphorylation state of cytosolic phospholipase A_2 in acutely primed 5 day DMSO-differentiated HL60 cells and other human cells.

When the phosphorylation state of $cPLA_2$ in 5 day DMSO-differentiated HL60 cells was examined, only the phosphorylated form of the enzyme could be detected (Figure 6.7(a)). Therefore, priming by cytochalasin B and perhydrovanadate had no apparent effect on the phosphorylation of $cPLA_2$, since the enzyme was already in the fully phosphorylated form. Both priming agents stimulated a translocation of $cPLA_2$ from the cytosolic fraction to the membrane fraction (Figure 6.7(b)), which would explain the higher sensitivity of the enzyme to subsequent fMLP stimulation. However, fMLP alone stimulated translocation of the enzyme to the membrane fraction suggesting that an agonist-stimulated factor was involved. This factor could possibly be calcium, which has been shown to stimulate the translocation of $cPLA_2$ (Channon & Leslie, 1990) and intracellular calcium concentrations have been shown to increase upon fMLP stimulation (Figure 5.7(a)). To determine the localisation of the enzyme in more detail, confocal microscopy was utilised, however, specific staining could not be detected using the conditions developed for the fibroblastic cells. This inability to detect specific staining in 5 day DMSO-differentiated cells appeared to be a phenomenon of human cells, including human tonsil germinal centres. These human cells contained cytosolic phospholipase A_2 (Figure 6.8(a)), but the localisation of the enzyme in both the isolated cells and tonsil sections could not be determined directly by confocal microscopy, due to very high non-specific staining.

Examination of the germinal centre B-cells and cell lines derived from different stages of the B- and T-cell maturation pathway demonstrated that cytosolic phospholipase A_2 was not detectable in the more mature B- and T-cell lines, suggesting that there is a difference in the requirements of these cells for cytosolic phospholipase A_2 activity (Figure 6.8(b)).

Figure 6.1(a) **Localisation of cytosolic phospholipase A_2 ($cPLA_2$) by western bloting in Swiss 3T3 fibroblasts quiesced in DMEM containing either 2% (v/v) or 10% (v/v) NBCS.**

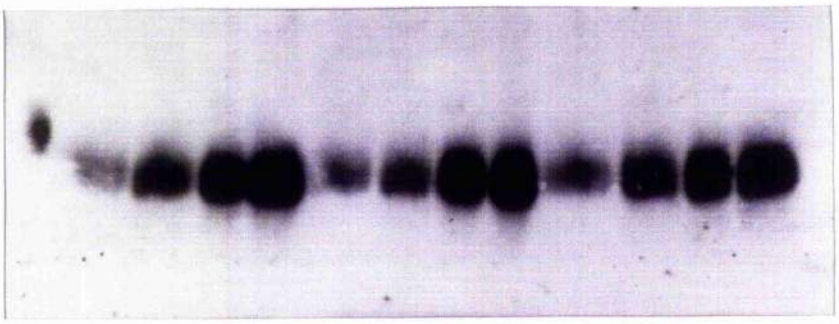
Swiss 3T3 fibroblasts were seeded in 75cm^2 flasks in DMEM containing 10% (v/v) newborn calf serum (NBCS) until 80-90% confluent when medium was replaced with DMEM containing either 2% or 10% (v/v) NBCS for 24 hours. Quiesced cells were washed and stimulated for the times shown with either DBG alone or 100nM bombesin. Reaction was terminated by washing with ice-cold phosphate buffered saline (PBS) and placing on ice. Membrane and cytosolic fractions were prepared as described in Materials and methods. 30µg total protein was loaded onto 12.5% (w/v) polyacrylamide gels and SDS-PAGE carried out. Proteins were transferred to nitrocellulose and western blotting was carried out using an anti-peptide anti-$cPLA_2$ antibody as described in Chapter 2. Detected proteins were visualised using ECL and result is representative of 3 individual experiments.

Figure 6.1(b) **Localisation and phosphorylation state of $cPLA_2$ in Swiss 3T3 fibroblasts quiesced in DMEM containing either 2% or 10% (v/v) NBCS**

Lysates were prepared as described for Figure 6.1(a). 5µg total protein was loaded and SDS-PAGE carried out, allowing the dye front to run off for 3 hours prior to transfer onto nitrocellulose. Transferred proteins were identified as described in Chapter 2 and visualised using ECL. Result is representative of 7 individual experiments.

A

Location of cytosolic phospholipase A_2 ($cPLA_2$) in Swiss 3T3 fibroblasts quiesced in DMEM containing either 2% (v/v) or 10% (v/v) NBCS



B

Location and phosphorylation state of cytosolic phospholipase A_2 ($cPLA_2$) in Swiss 3T3 fibroblasts quiesced in DMEM containing either 2% (v/v) or 10% (v/v) NBCS.

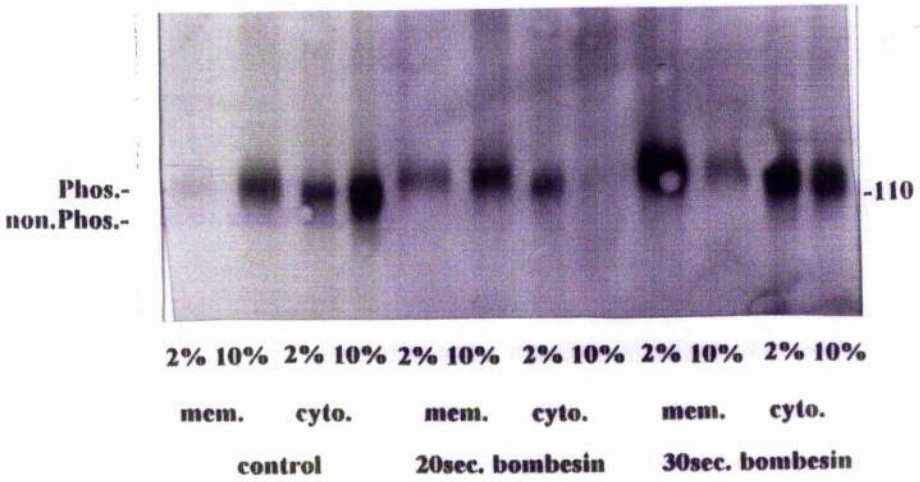


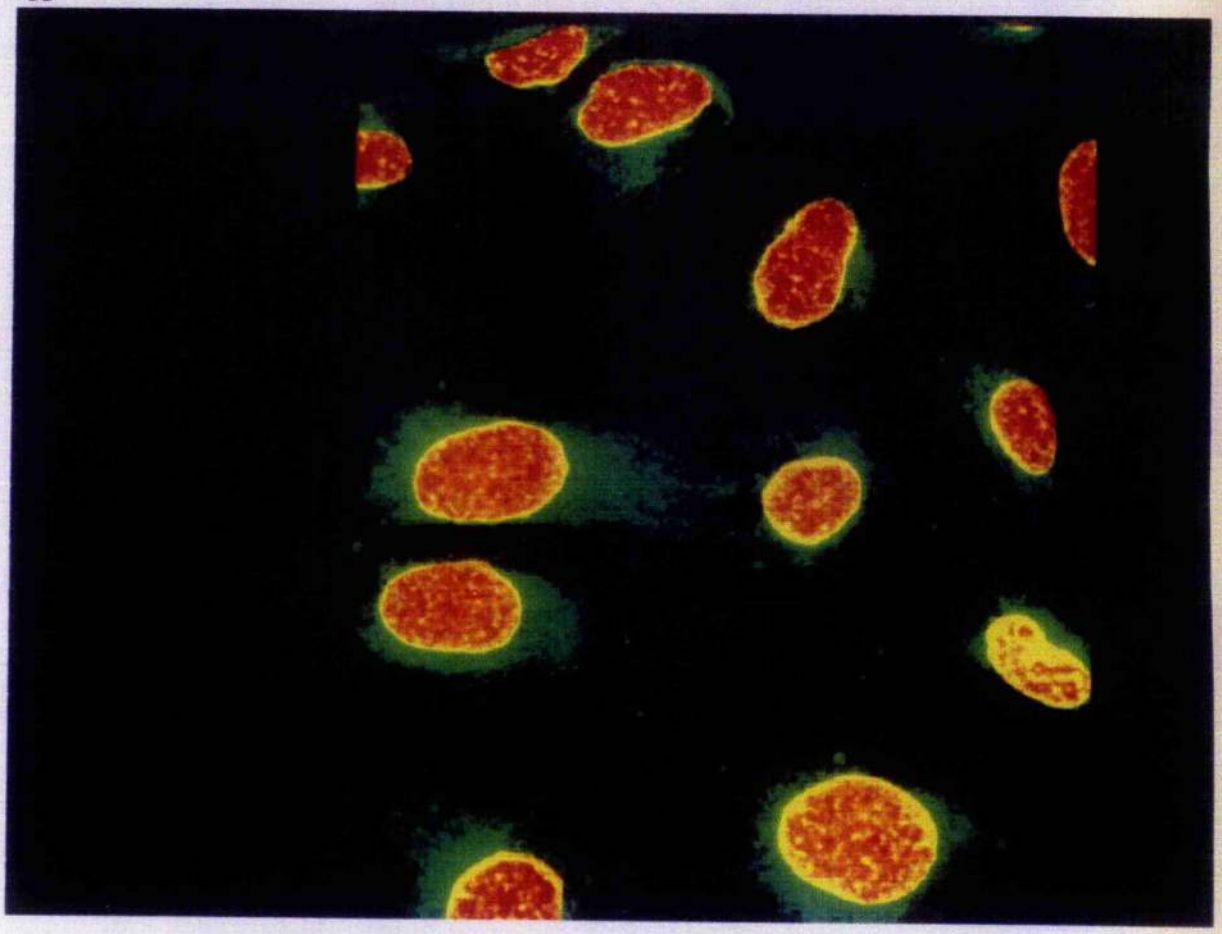
Figure 6.2(a) **Localisation of $cPLA_2$ in Swiss 3T3 fibroblasts quiesced in DMEM containing 2% (v/v) NBCS by confocal microscopy.**

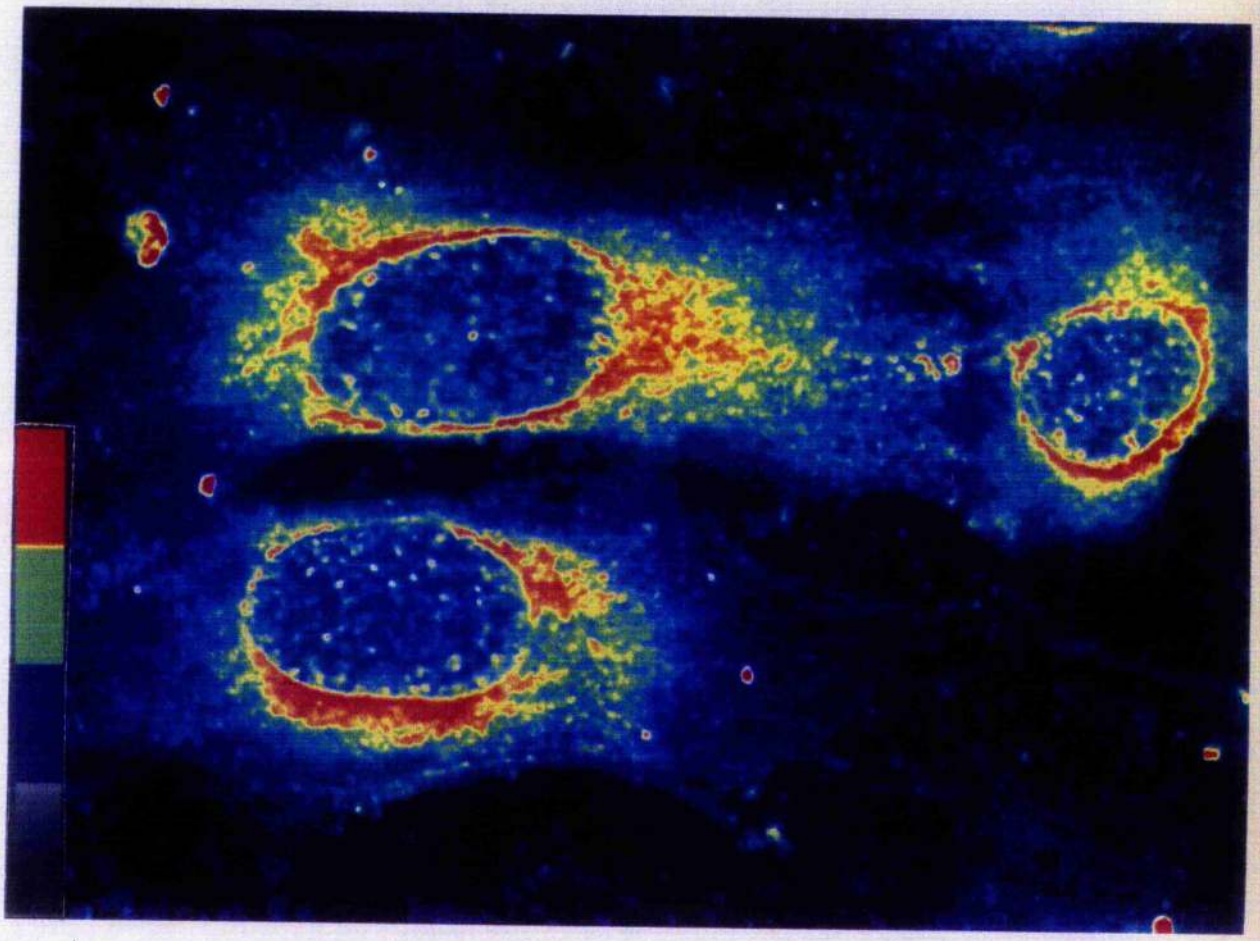
Swiss 3T3 fibroblasts were seeded onto microspot microscope slides at $2x10^4$ cells per ml in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was replaced with DMEM containing 2% (v/v) NBCS for 24 hours. Quiesced cells were washed with DBG and reactions terminated by washing with ice-cold PBS. Cells were then fixed and stained for confocal microscopy as described in Materials and methods. Result is representative of 3 individual experiments. The top panel represents the visible staining observed down the microscope and the lower panel is selected cells from the visible field. These selected cells are magnified and 'banded' by the software available, with red indicating the strongest staining and blue indicating the weakest staining. The bar code shown highlights the colours used for the staining intensities.

Figure 6.2(b) **Localisation of $cPLA_2$ in Swiss 3T3 fibroblasts quiesced in DMEM containing 10% (v/v) NBCS by confocal microscopy.**

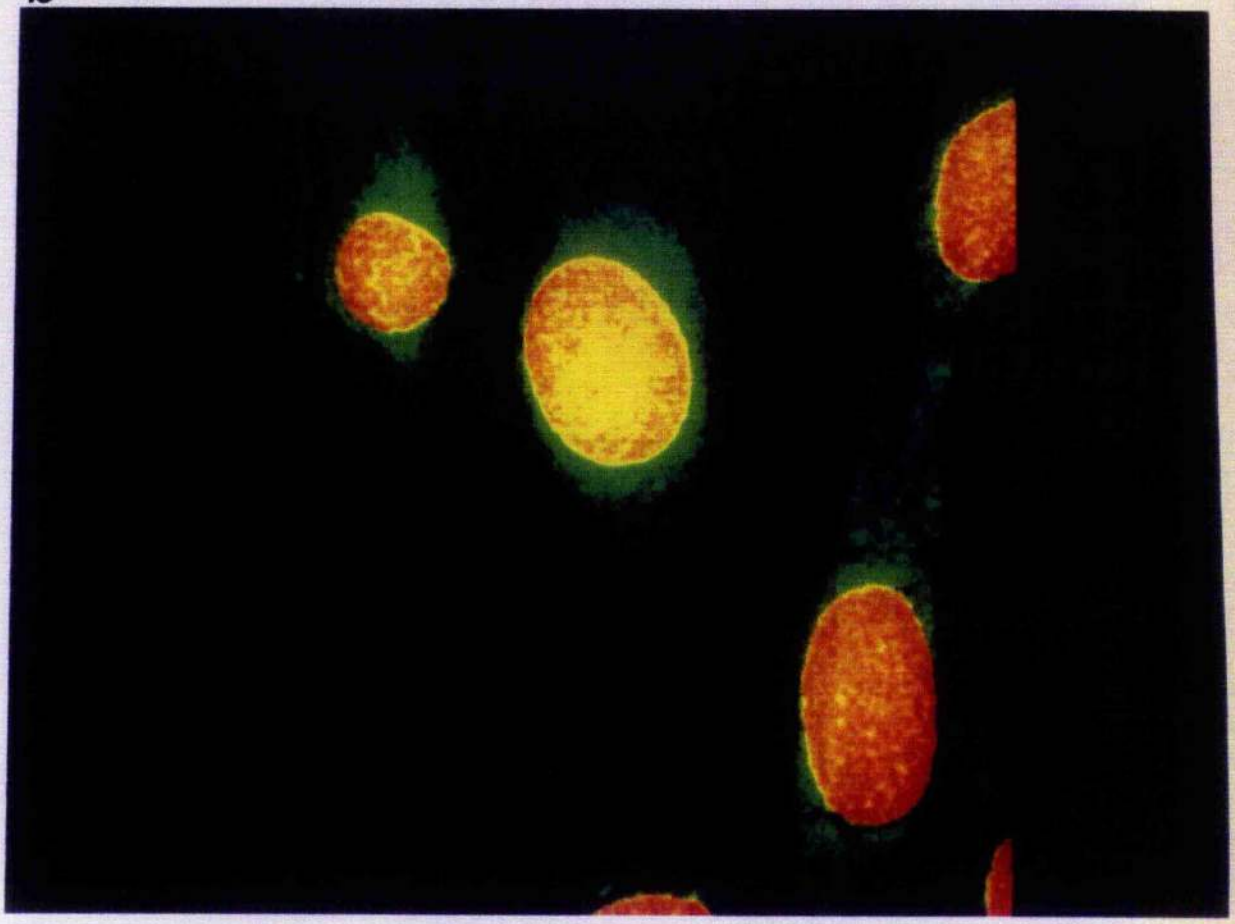
Swiss 3T3 fibroblasts were seeded onto microspot microscope slides at $2x10^4$ cells per ml in DMEM containing 10% (v/v) NBCS until 80-90% confluent when the medium was replaced with fresh culture medium for 24 hours. Quiesced cells were washed with DBG prior to the reaction being terminated by washing thouroughly with ice-cold PBS. Cells were fixed and stained for confocal microscopy as described in Materials and methods. Result is representaive of 3 individual experiments, carried out on freshly prepared cells. The upper panel demonstrates the visible staining as observed down the microscope and the lower panel showns an alternative 'banding' pattern available, with the brightest areas corresponding to the highest amount of staining.

a





b



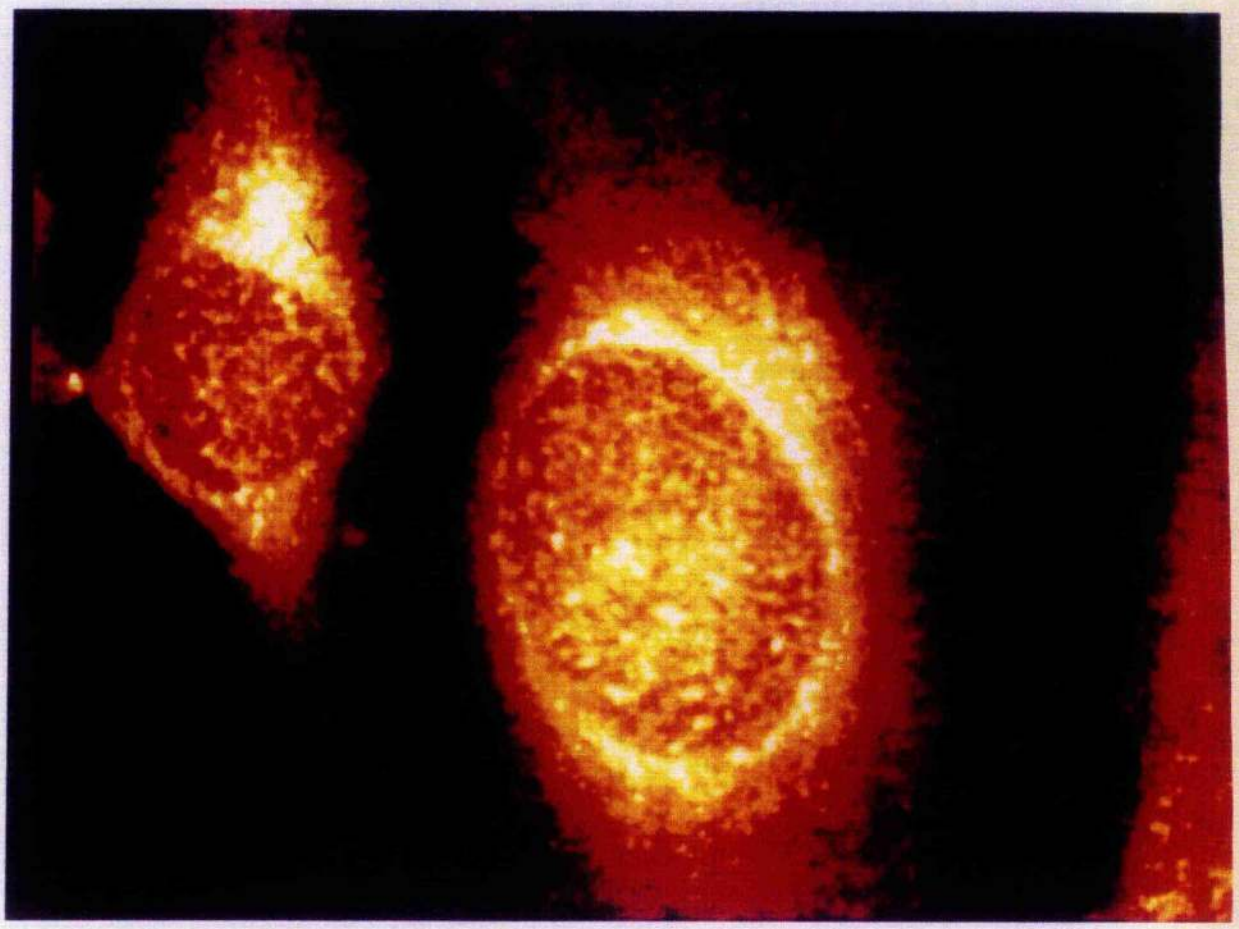


Figure 6.3(a) **Phosphorylation state of $cPLA_2$ in estradiol-treated and -untreated Rat-1 Raf ER4 fibroblasts.**

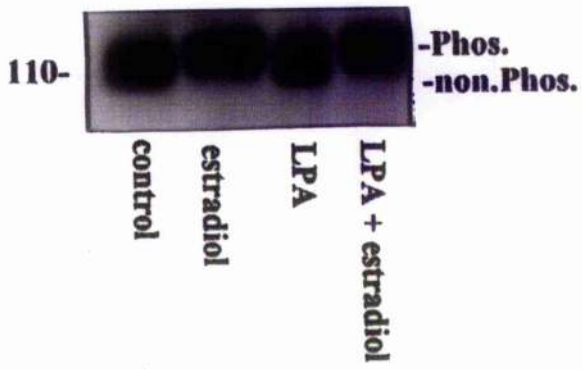
Rat-1 Raf ER4 fibroblasts were seeded at $2x10^4$ cell/ml in DMEM containing 10% (v/v) NBCS and 400μg/ml geneticin until 80-90% confluent when medium was replaced with DMEM alone for 24 hours. Quiesced cells were washed with DBG and then treated with DBG alone or 1μM β-estradiol for 1 hour at 37°C. Treated cells were then stimulated with either DBG alone or 30μM lysophosphatidic acid (LPA) for 30 seconds at 37°C. Reactions were terminated by washing with ice-cold PBS. Lysates were prepared as described for MAP kinase *in vitro* assay in Materials and methods. 10μg total protein was loaded onto 12.5% (w/v) polyacrylamide gels and SDS-PAGE carried out as described in Chapter 2. Result is representative of 2 individual experiments.

Figure 6.3(b) **Localisation and phosphorylation state of $cPLA_2$ in estradiol-treated and -untreated Rat-1 Raf ER4 fibroblasts by western blotting.**

Rat-1 Raf ER4 fibroblasts were seeded in $75cm^2$ flasks in DMEM containing 10% (v/v) NBCS and 400μg/ml geneticin until 80-90% confluent when medium was replaced with DMEM alone for 24 hours. Quiesced cells were then washed with DBG and incubated at 37°C for 1 hour with either DBG alone or 1μM β-estradiol. Pretreated cells were then stimulated for 30 seconds at 37°C with either 30μM LPA, 100nM endothelin-1 or 100nM thrombin. Reactions were terminated by washing with ice-cold PBS and placing on ice. Membrane and cytosolic fractions were prepared as described in Materials and methods. 10μg total protein was loaded onto 12.5% (w/v) polyacrylamide gels and SDS-PAGE carried out as described in Chapter 2. Proteins were transferred onto nitrocellulose and western blotting carried out as described in Materials and methods. Detected proteins were visualised using ECL and result is representative of 3 individual experiments using freshly cultured fibroblasts.

A

Phosphorylation state of cytosolic phospholipase A_2 ($cPLA_2$) in control and estradiol-treated Rat-1 Raf ER4 fibroblasts



B

Location and phosphorylation of cytosolic Phospholipase A_2 in Rat-1 ER4 fibroblasts

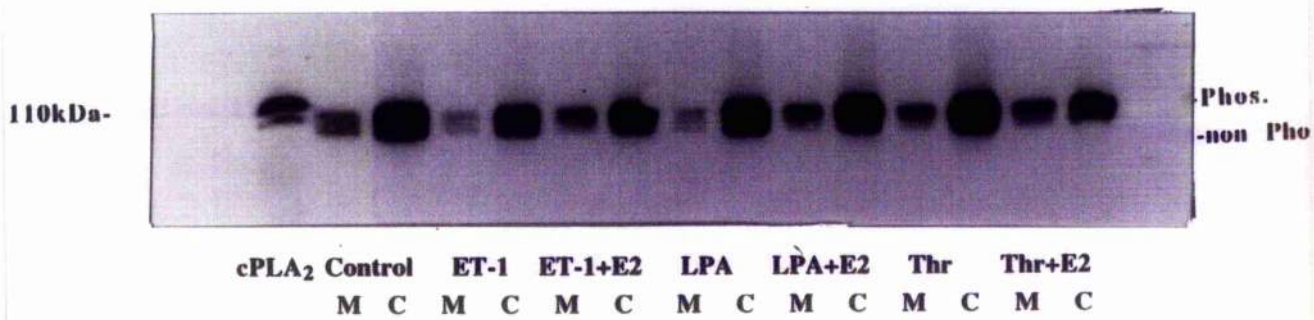


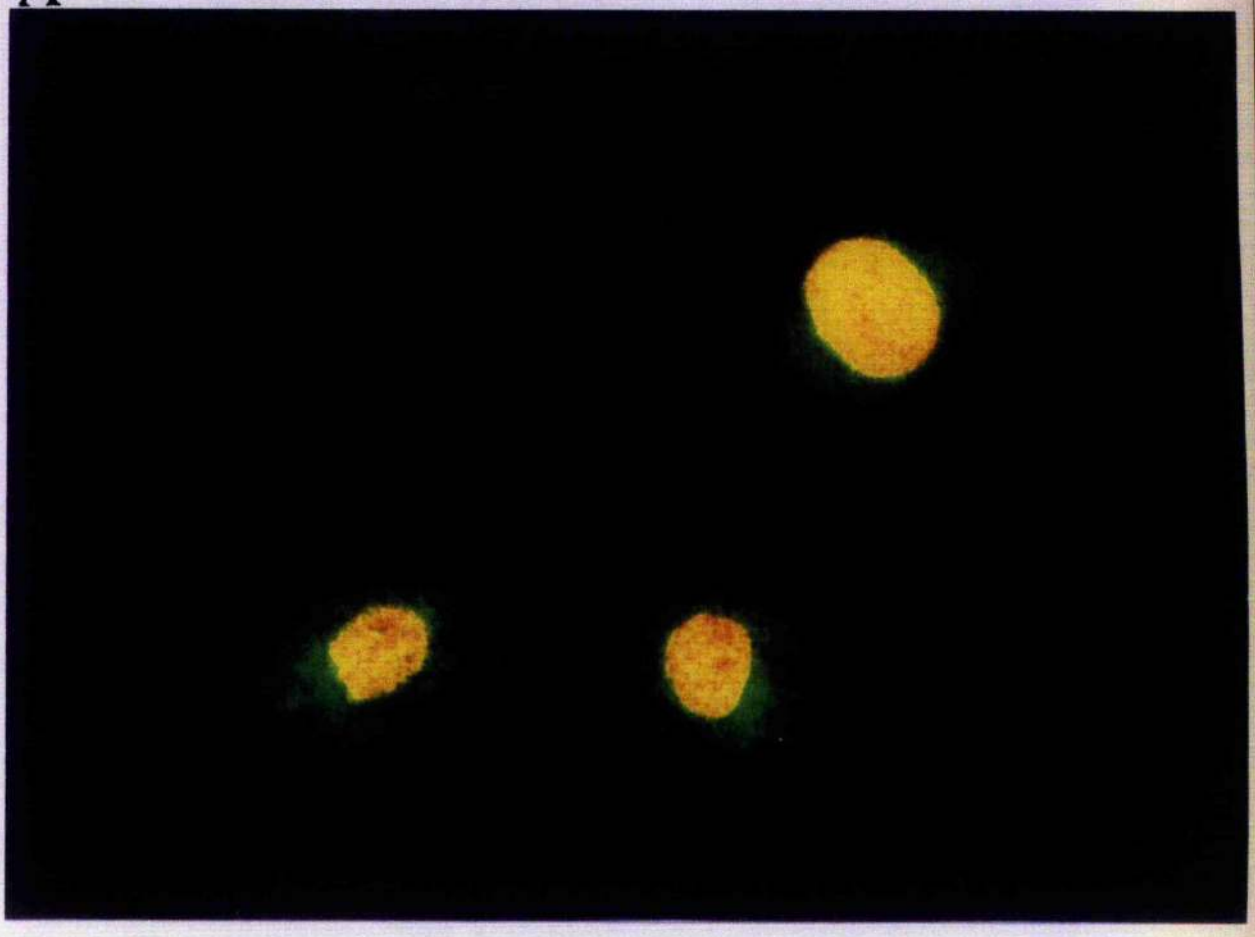
Figure 6.4(a) Localisation of $cPLA_2$ in Rat-1 Raf ER4 fibroblasts by confocal microscopy.

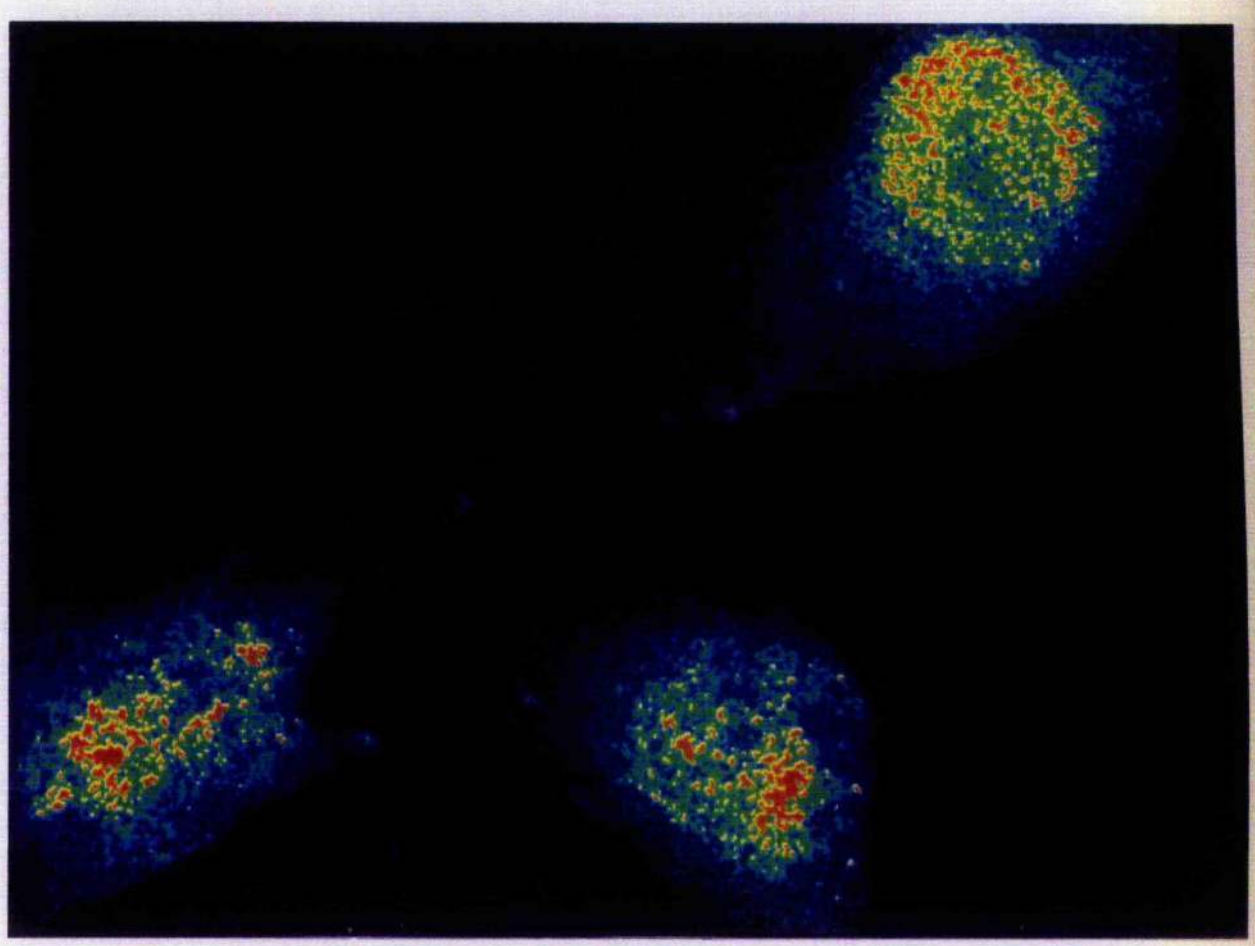
Rat-1 Raf ER4 fibroblasts were seeded at $2x10^4$ cells/ml onto microspot microscope slides in DMEM containing 10% (v/v) NBCS and 400µg/ml geneticin until 80-90% confluent when medium was replaced with DMEM alone for 24 hours. Cells were washed for a total of 30 minutes at 37°C with DBG and then incubated for 1 hour at 37°C with DBG. Reaction was terminated by washing with ice-cold PBS and placing on ice. Slides were fixed and stainined for confocal microscopy as described in Materials and methods. Result is representative of 4 individual experiments. The upper panel demonstrates the visible staining observed under the microscope and the lower panel represents 'banding' of magnified, selected cells from the visible field. The banding adopted is presented and explained in Figure 6.4(b).

Figure 6.4(b) Localisation of $cPLA_2$ in estradiol-treated Rat-1 Raf ER4 fibroblasts by confocal microscopy

Rat-1 Raf ER4 fibroblasts were seeded at $2x10^4$ cells per ml onto microspot microscope slides in DMEM containing 10% (v/v) NBCS and 400µg/ml geneticin until 80-90% confluent when medium was replaced by DMEM alone for 24 hours. Quiesced cells were washed with DBG for 30 minutes at 37°C and then incubated at 37°C for 1 hour with DBG containing 1µM β-estradiol. Reaction was terminated by washing with ice-cold PBS and placing on ice. Slides were fixed and stained for confocal microscopy as described in Materials and methods. Result is representative of 3 individual experiments which utilised freshly set up slides and cell batches. The upper and lower panels correspond to the conditions described in Fig.6.4(a) and the 'banding' utilised is demonstrated with red corresponding to the most highly stained areas and blue for the weakest stained.

A





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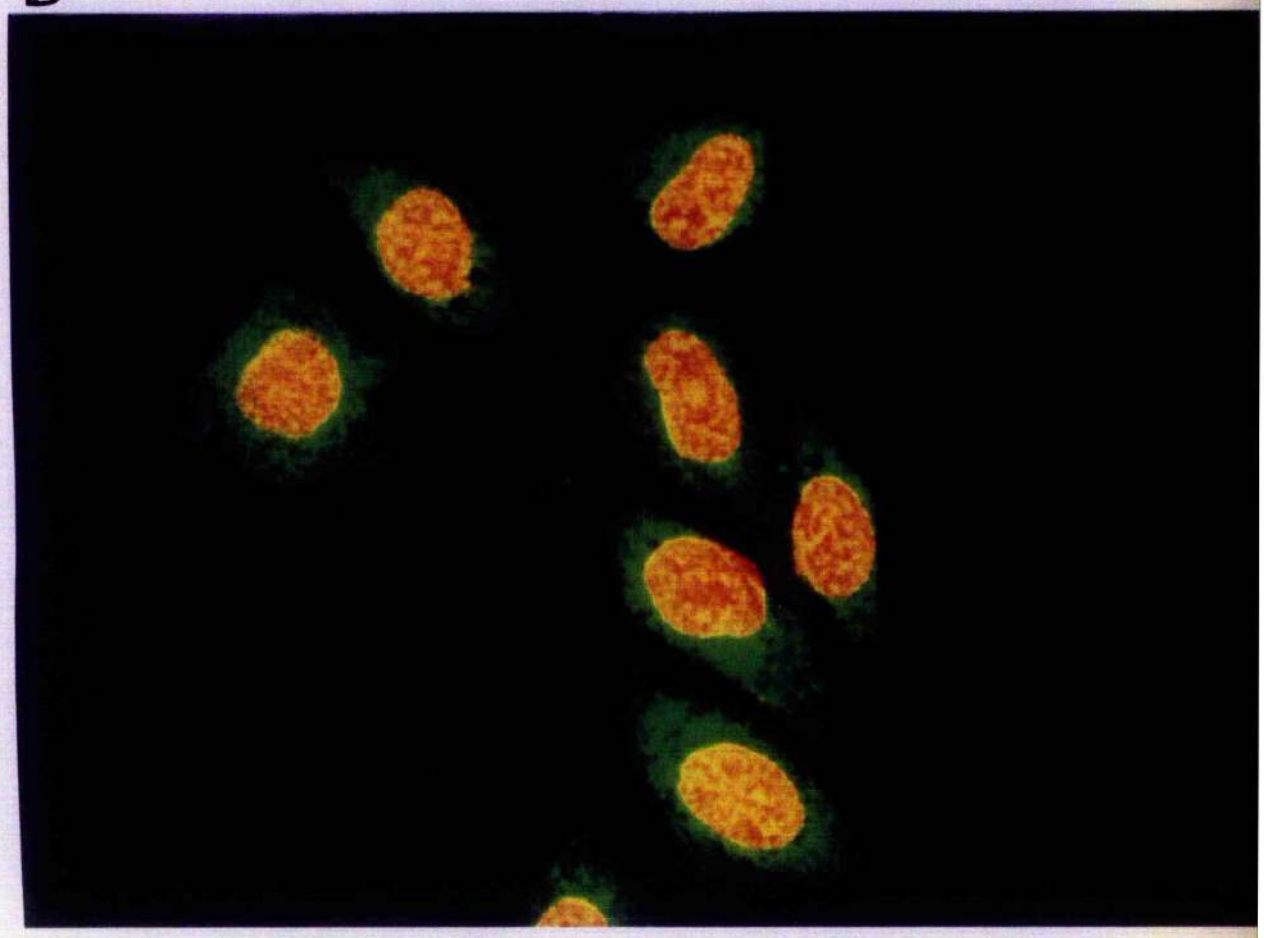


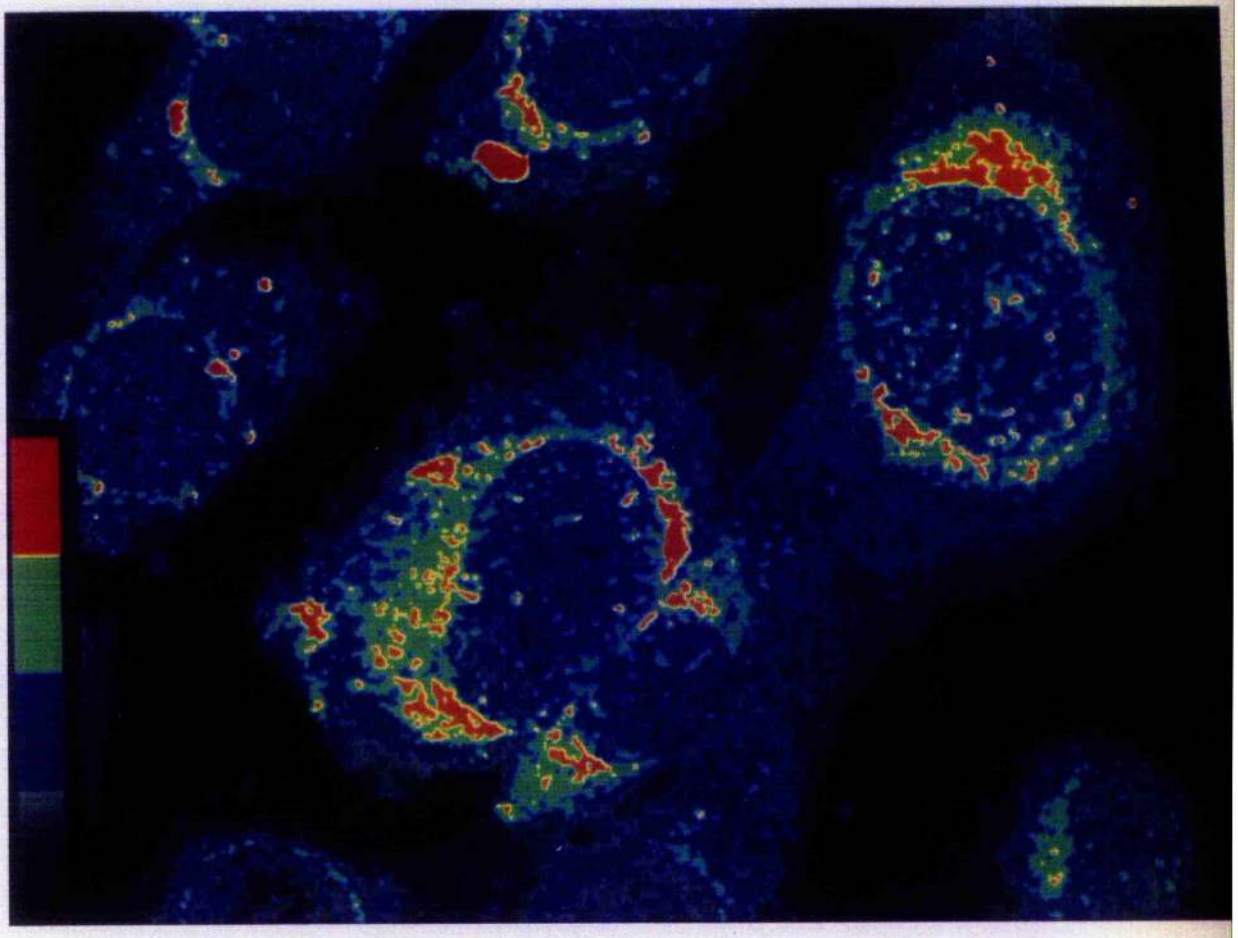


Table 6.1 **Quantitative analysis of fluorescence attributed to $cPLA_2$ localisation extra- and intra-nuclear in Swiss 3T3 and Rat-1 Raf ER4 fibroblasts.**

Swiss 3T3 fibroblasts were seeded at $2x10^4$ cells/ml onto microspot microscope slides in DMEM containing 10% (v/v) NBCS until 80-90% confluent when medium was replaced with DMEM containing either 2% or 10% (v/v) NBCS for 24 hours. Quiesced cells were washed for 30 minutes with DBG at 37°C and then stimulated for 30 seconds at 37°C with either DBG alone or 100nM bombesin. Rat-1 Raf ER4 fibroblasts were seeded at $2x10^4$ cells/ml onto microspot microscope slides in DMEM containing 10% (v/v) NBCS and 400μg/ml geneticin until 80-90% confluent when medium was replaced with DMEM alone for 24 hours. Quiesced cells were washed with DBG for 30 minutes at 37°C and then incubated for 1 hour at 37°C with either DBG alone or 1μM β-estradiol. Reaction was terminated by washing with ice-cold PBS and placing on ice.

All slides were fixed and stained as described in Materials and methods with the nucleus being stainined using propidium iodide (10μg/ml). The nucleus could then be isolated and thus, the fluorescence attributed to intranuclear $cPLA_2$ could be subtracted from the total cell fluorescence, by the software used. Result is representative of 2 individual experiments and presented as % total fluorescence attributed to $cPLA_2$ detection.

Table 6.1

	% Total Fluorescence	
	Intranuclear	Extranuclear
Swiss 3T3 fibroblasts		
2% control	28	72
2% stimulation	42	58
10% control	40	60
10% stimulation	30	70
Rat-1 Raf ER4 fibroblasts		
Control	45	55
β-estradiol treated	29	71

Figure 6.5 **Qualitative measurement of $cPLA_2$ fluorescence in relation to location within estradiol-treated Rat-1 Raf ER4 fibroblasts.**

Rat-1 Raf ER4 fibroblasts were seeded at $2x10^4$ cells per ml onto microspot microscope slides in DMEM containing 10% (v/v) NBCS and 400μg/ml geneticin until 80-90% confluent when medium was replaced with DMEM alone for 24 hours. Cells were washed with DBG for 30 minutes at 37°C and then incubated for 1 hour at 37°C with DBG containing 1μM β-estradiol. Reaction was terminated by washing with ice-cold PBS and placing on ice. Slides were fixed and stained as described in Materials and methods, with the nucleus being stained using propidium iodide. Confocal microscopy was carried out and pixel measurements taken longitudinally through the cell. Result is representative of 2 individual experiments.

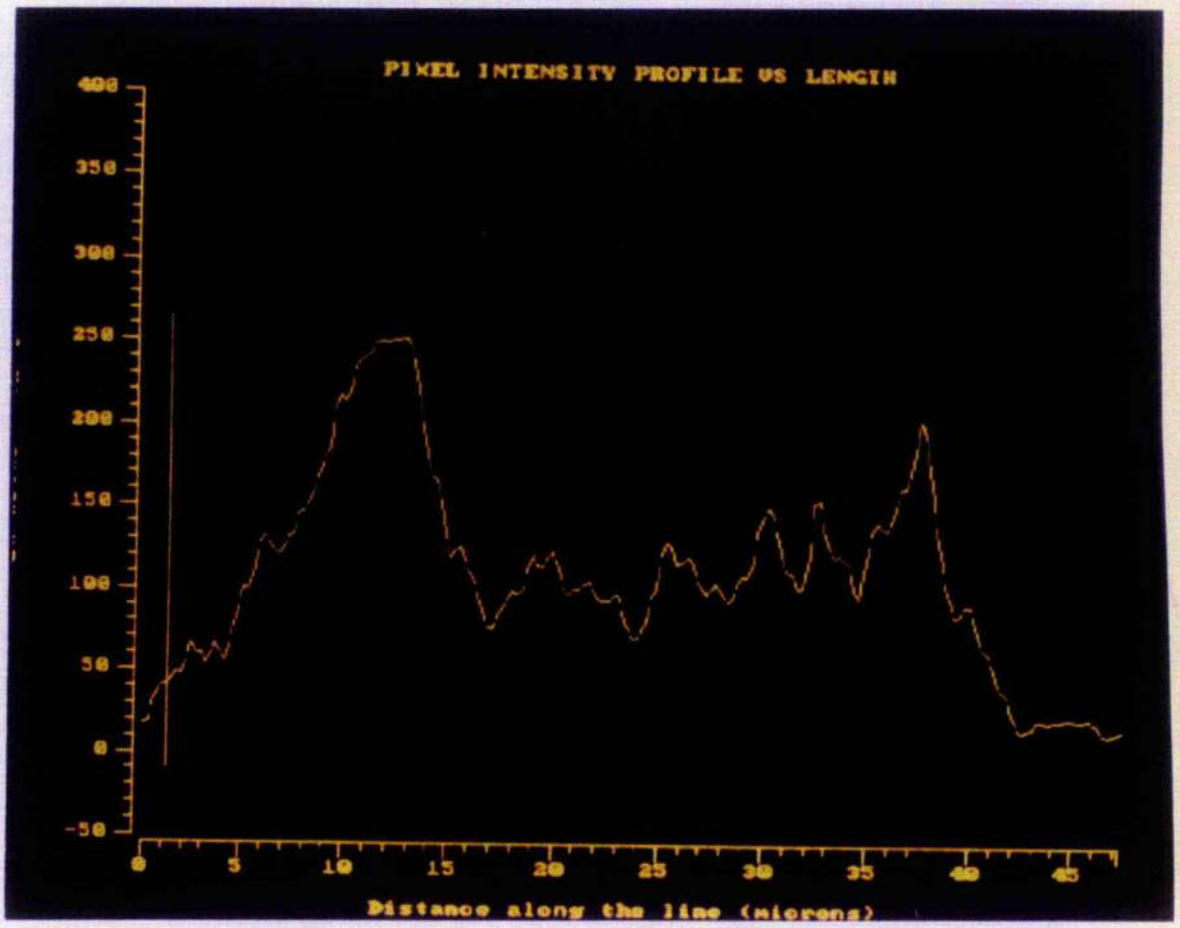

PIXEL INTENSITY PROFILE VS LENGTH
400
350
300
250
200
150
100
50
0
-50
0
5
10
15
20
25
30
35
40
45
Distance along the line (microns)

Figure 6.6 Polarity of $cPLA_2$ localisation in estradiol-treated Rat-1 Raf ER4 fibrobalsts.

Slides of estradiol-treated Rat-1 Raf ER4 fibroblasts were prepared exactly as described in Figure 6.5. Latitudinal, or *z*-sections were taken through the cell, starting at the exposed cell area working down to the basal lateral membrane. There are 4 sections presented here which run from left to right, top to bottom, going further towards the basal lateral membrane. This result is representative of 2 individual experiments.

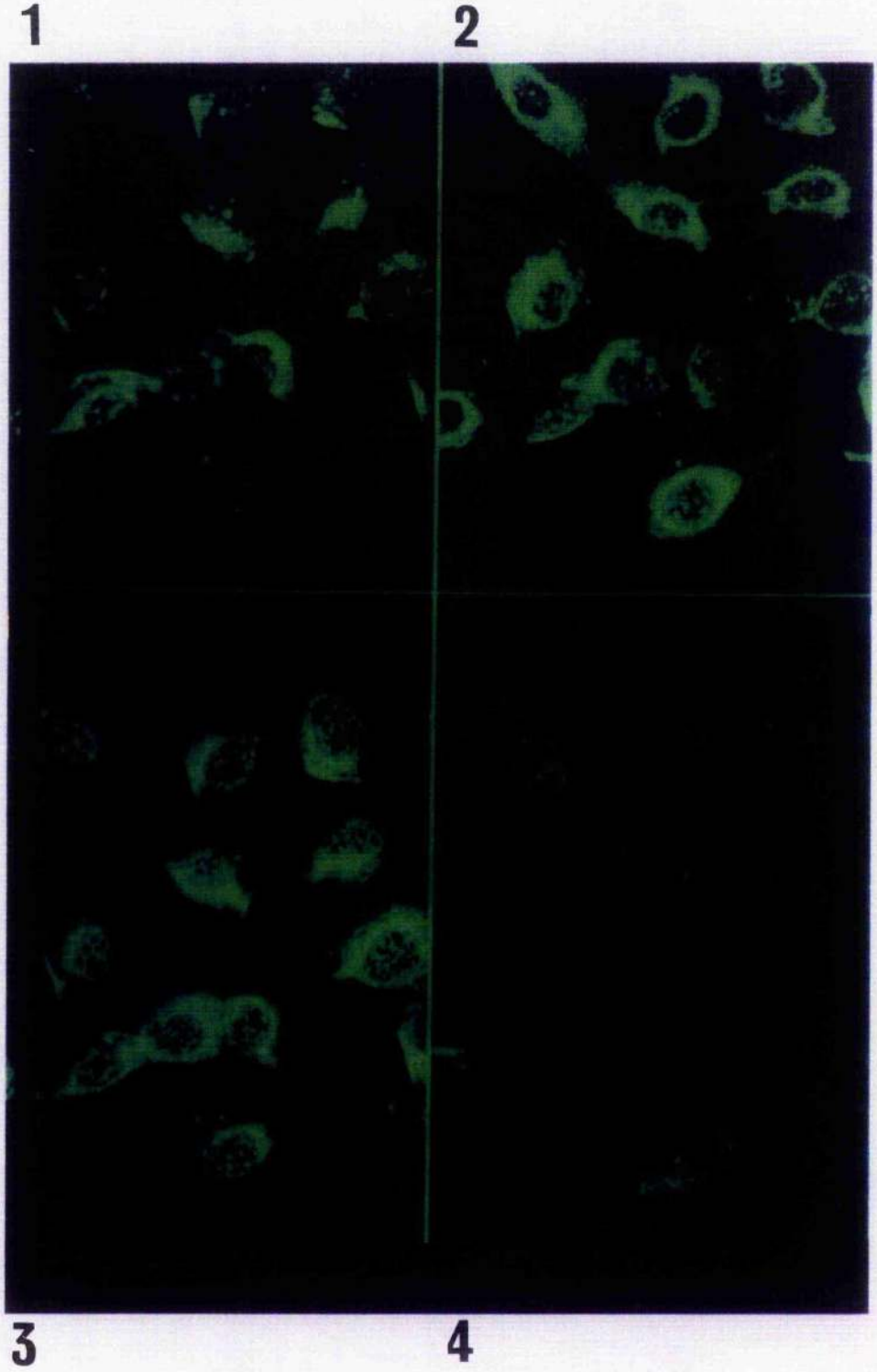

1
2
3
4

Figure 6.7(a) Phosphorylation state of $cPLA_2$ in 5 day DMSO-differentiated HL60 cells.

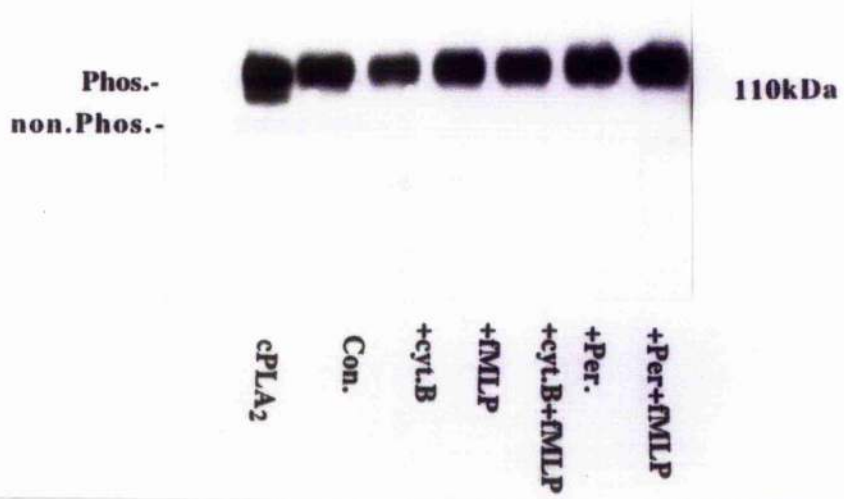
HL60 cells were seeded at $5x10^5$ cells per ml in RPMI 1640 medium containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO for 5 days. Differentiated cells were washed in RBG and resuspended at $2x10^7$ cells/ml in RBG. 10^7 cells were used per sample and preincubated with either RBG alone, 5μM cytochalasin B for 5 minutes or 0.5mM perhydrovanadate for 2 minutes at 37°C. Pretreated cells were then stimulated with either RBG alone, 100nM fMLP or 0.5mM perhydrovanadate for 5 minutes at 37°C. Reaction was terminated by washing twice with ice-cold PBS and storing on ice. Lysates were prepared as described for MAP kinase *in vitro* assay and 10μg total protein was loaded onto 12.5% (w/v) polyacrylamide gels and SDS-PAGE carried out as described in Materials and methods. Proteins were transferred to nitrocellulose and western blotting carried out as described in Chapter 2 using baculovirus expressed $cPLA_2$ as the standard. Result is representative of 3 individual experiments.

Figure 6.7(b) Localisation of $cPLA_2$ as determined by western blotting in 5 day DMSO-differentiated HL60 cells.

HL60 cells were seeded at $5x10^5$ cells per ml in RPMI 1640 medium containing 15% (v/v) heat-inactivated foetal calf serum and 1.3% (v/v) DMSO for 5 days. Differentiated cells were washed twice with RBG and resuspended at $2x10^7$ cells per ml in RBG. 10^7 cells were used per sample and preincubated at 37°C with either RBG or 5μM cytochalasin B for 5 minutes or 0.5mM perhydrovanadate for 2 minutes. Preincubated cells were then stimulated with either RBG or 100nM fMLP for 5 minutes at 37°C. Reaction was terminated by washing twice with ice-cold PBS and cytosolic and membrane fractions prepared as described in Materials and methods. 10μg total protein was loaded onto 12.5% (w/v) polyacrylamide gels and SDS-PAGE carried out. Proteins were transferred to nitrocellulose and western blotting carried out as described in Chapter 2. Detected proteins were visualised using ECL and result is representative of 3 individual experiments, which utilised freshly prepared lysates.

A

Phosphorylation of cytosolic phospholipase A_2 in HL60 cells



B

Location of cytosolic phospholipase A_2 ($cPLA_2$) in 5 day DMSO-differentiated HL60 cells

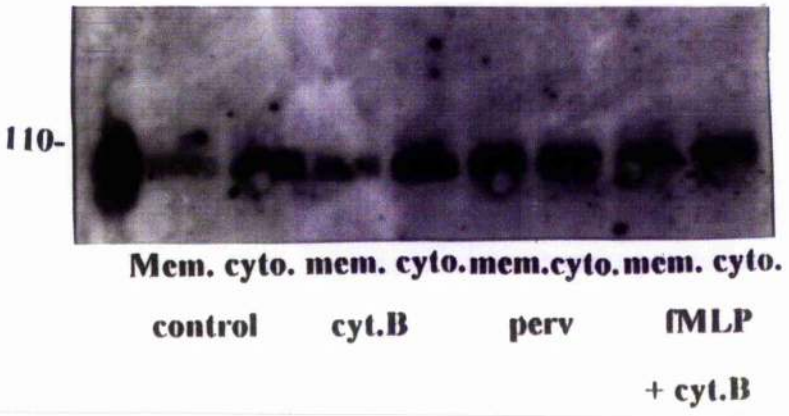


Figure 6.8(a) Detection of $cPLA_2$ in human germinal centres by western blotting.

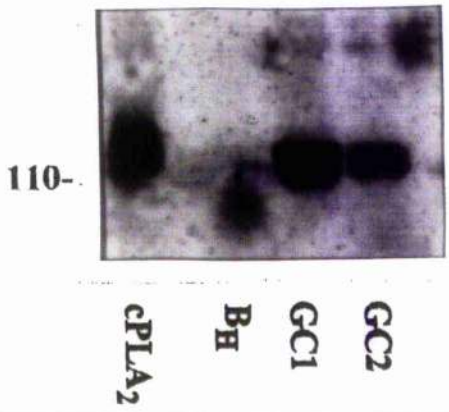
Germinal centres were isolated from human tonsils by I.MacDonald, Department of Immunology, University of Birmingham. Lysates were prepared as described in MAP kinase *in vitro* assay and 20μg total protein was loaded onto 12.5% (w/v) polyacrylamide gels and SDS-PAGE carried out. Proteins were transferred to nitrocellulose and western blotting carried out as described in Chapter 2. Detected proteins were visualised using ECL and result is representative of 3 individual experiments. B_H = B-heavy cells from germinal centre mantle; $Gc_{1/2}$ = Germinal $centre_{1/2}$.

Figure 6.8(b) Detection of $cPLA_2$ in human B- and T-lymphocyte cell lines by western blottting.

Jurkat, Daudi, HL60 and Ramos cells were kindly cultured by Dr.M.M.Harnett, Department of Biochemistry, University of Glasgow and lysates were prepared as described for MAP kinase *in vitro* assay in the Materials and methods chapter. Primary T-lymphocytes and resting B-lymphocytes were isolated by Dr.B.Cushley, Department of Biochemistry, University of Glasgow. 20μg total protein was loaded onto 12.5% (w/v) polyacrylamide gels and SDS-PAGE carried out. Proteins were transferred onto nitrocellulose and western blotting carried out as described in Chapter 2. Detected proteins were visualised using ECL and result is representative of 3 individual experiments.

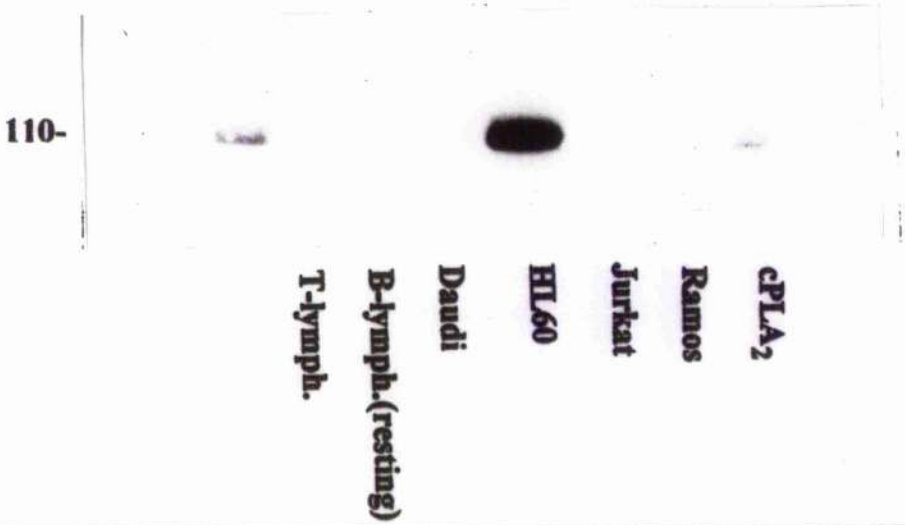
A

Detection of cytosolic phospholipase A_2 ($cPLA_2$) in human germinal centres



B

Detection of cytosolic phospholipase A_2 ($cPLA_2$) in human T- and B-lymphocytes



6.3 DISCUSSION

6.3.1 Localisation and phosphorylation state of cytosolic phospholipase A_2 ($cPLA_2$) in chronically primed Swiss 3T3 and Rat-1 Raf ER4 fibroblasts.

Cytosolic phospholipase A_2 was so named, because it was first purified from the cytosol of U937 cells (Clark *et al.*, 1990). In work carried out on the regulation and activation of cytosolic phospholipase A_2 ($cPLA_2$), evidence has been presented which suggests that for maximum responsiveness and activity, $cPLA_2$ has to be associated with the plasma membrane (Diez *et al.*, 1992). This would be the obvious location for the enzyme, since it is the source of its substrate and regulatory moieties, such as GTP-binding proteins. It has also been shown that it is the phosphorylated form of the enzyme which is the more active form (Kramer *et al.*, 1993(a)). Therefore, to determine the phosphorylation state and the localisation of the enzyme is of prime importance in elucidating its regulation.

In previous Chapters, it has been shown that Swiss 3T3 fibroblasts quiesced in DMEM containing 10% (v/v) NBCS, $cPLA_2$ was more sensitive to bombesin stimulation, resulting in a quicker onset of arachidonate release (Figure 3.5(a)). This priming of $cPLA_2$, may have involved tyrosine phosphorylation and GTP-binding proteins, but also manifested itself in a higher proportion of phosphorylated $cPLA_2$ being located in a membrane fraction, prior to agonist stimulation, compared to cells quiesced in 2% (v/v) NBCS (Figure 6.1(b)). This work could not determine whether $cPLA_2$ was phosphorylated in the cytosol or when attached to the membrane. However, it is more probable that the enzyme is phosphorylated when attached to the membrane, or in transit, as no phosphorylated $cPLA_2$ has ever been detected in the cytosolic fraction, whereas both phosphorylated and non-phosphorylated enzyme has been visible in the membrane fraction. The occurence of phosphorylated enzyme in the membrane fraction in response to priming, did not increase the basal levels of arachidonate release (Chapter 3). However, it could explain the increased responsiveness to bombesin stimulation, since the enzyme is already in its active form

and in close proximity to both its substrate and potential regulatory moieties. This association with a membrane fraction, did indeed appear to have a role in the activation of $cPLA_2$, as the appearance of phosphorylated enzyme in the membrane fraction in response to bombesin stimulation, correlated with the kinetics observed for bombesin-stimulated arachidonate release in cells quiesced in both 2% and 10% (v/v) NBCS (Figure 6.1(b)). However, it has always been suggested that the membrane fraction with which the activated form of $cPLA_2$ associates, is the plasma membrane. This would be the immediate choice of location of a receptor-regulated signalling enzyme, since it is here that the stimulatory elements such as the receptor and GTP-binding proteins are situated. This is not the case in Swiss 3T3 fibroblasts quiesced in either 2% or 10% (v/v) NBCS. Confocal microscopy demonstrated that under both quiescing conditions, there was no detectable levels of $cPLA_2$ at the plasma membrane (Figure 6.2(a) and (b)). This was not due to inaccessibility of the epitope for the antibody, as a second polyclonal antibody raised to an internal sequence of $cPLA_2$ also failed to recognise any enzyme at the plasma membrane (result not shown). In cells quiesced in 2% NBCS, the majority of the enzyme was in the extranuclear area of the cell, whereas in cells quiesced in 10% serum, there was an even distribution between the extranuclear and intranuclear compartments. Upon bombesin stimulation, there was a translocation of the enzyme into the intranuclear compartment in cells quiesced in 2% serum, whereas in cells quiesced in 10%, this translocation was into the extranuclear compartment (Table 6.1). As the cells quiesced in 10% NBCS are primed to bombesin stimulation, this localisation would suggest that $cPLA_2$ is maximally active when situated in the nucleus. This is substantiated by the intranuclear location of $cPLA_2$ quiesced in 2% NBCS, after 30 seconds bombesin stimulation as quantified in Table 6.1, which was the time required for maximum arachidonate release in cells quiesced under these conditions (Figure 3.5(a)).

The distribution of $cPLA_2$ between the intra- and extra-nuclear compartments in Raf ER4 cells at basal levels was very similar to that quantified for Swiss 3T3 fibroblasts quiesced in 10% (v/v) NBCS (Table 6.1 and Figure 6.4(a)). However, the

enhanced responsiveness seen after estradiol treatment, corresponded to a translocation of the enzyme to a membrane fraction (Figure 6.3) in the extranuclear compartment. This membrane fraction appeared to be a defined structure, which remains to be identified (Figure 6.4(b)). This demonstrated that priming of $cPLA_2$ in Swiss 3T3 fibroblasts by some serum factor and the priming of $cPLA_2$ by estradiol-stimulated MAP kinase activity in Rat-1 Raf ER4 fibroblasts resulted in a different location of the enzyme within the cell. This could be explained by the location of the $cPLA_2$ regulatory elements under both conditions. It has been shown that MAP kinase translocates to the nuclear membrane upon activation (Lenormand *et al.*, 1993), therefore in estradiol-treated Rat-1 Raf ER4 fibroblasts, the $cPLA_2$ could be recruited to the nuclear membrane by the activated MAP kinase. In the case of Swiss 3T3 fibroblasts, upon bombesin stimulation a similar localisation was seen, around the perinuclear area, which could suggest a recruitment by activated MAP kinase. However, the intranuclear location observed at basal levels in Swiss 3T3 fibroblasts quiesced in DMEM containing 10% (v/v) NBCS cannot be explained as easily. Work carried out has shown that there is a high arachidonate content in the nuclear lipids and that the small molecular weight, secretory phospholipase A_2 ($sPLA_2$) isozymes can associate with membrane structures within the nucleus, as demonstrated by ultrastructural cytochemistry (Maraldi *et al.*, 1992), making it highly probable that cytosolic phospholipase A_2 could do the same. One possibility that must be addressed, is that the location of $cPLA_2$ in both basal and primed Swiss 3T3 and Rat-1 Raf ER4 fibroblasts, could be the same membraneous structure, which interlaces within the nucleus and the extranuclear compartment. From electron microscopy studies, it has been shown that the golgi and the endoplasmic reticulum, do in fact intersperse between the cytosol and the nucleus through pores in the nuclear membrane (Kessel, 1983). Therefore, $cPLA_2$ may move to areas within this membrane structure, which contain the desired elements required for maximal activity, such as MAP kinase. It would, therefore, be the affinity of association of $cPLA_2$ with the membrane, both intra- and -extranuclear, that would determine whether or not the

enzyme could be detected in a membrane or cytosolic fraction, due to the procedure used to differentiate between cytosol and membrane fractions. The affinity of association with the membrane could be determined by the phosphorylation state of the enzyme, as it was mainly phosphorylated $cPLA_2$ detected in the membrane fraction.

The polarity of the localisation visualised (Figure 6.6) in estradiol-treated Rat-1 Raf ER4 fibroblasts, could be explained by the position of the factors which stimulate $cPLA_2$ activity. Lysophosphatidic acid has been suggested to bind to a 7 membrane-spanning, G-protein linked, plasma membrane receptor, therefore, LPA-stimulated arachidonate release is initiated at the plasma membrane of the cell, but the enzyme was shown to be located in and around the nucleus. As estradiol-mediated priming of $cPLA_2$ did not increase the basal levels of arachidonate and LPA-stimulated arachidonate release had an absolute requirement for increased intracellular calcium concentrations, it could be advocated that it was the increase in intracellular calcium concentrations that mediated the activation of nuclear-associated $cPLA_2$ activity. The response to increased calcium concentrations could be immediate as LPA-stimulated intracellular calcium release was detectable instantaneously and it has been documented that IP_3-sensitive calcium pools are present within the endoplasmic reticulum (Gill *et al.*, 1989), the possible location for $cPLA_2$.

6.3.2 Localisation and phosphorylation state of $cPLA_2$ in acutely primed 5 day DMSO-differentiated HL60 cells and other human cells.

It was shown that in 5 day-DMSO differentiated HL60 cells the cytosolic phospholipase A_2 was already in a fully phosphorylated form (Figure 6.7(a)). This was not a consequence of differentiation, as it was the same in undifferentiated HL60 cells (results not shown), but could be due to the high concentration of serum used to culture these cells. As shown in Swiss 3T3 fibroblasts (Chapter 3 & 4), serum factors can prime $cPLA_2$, resulting in a higher proportion of phosphorylated enzyme being detected, therefore, this could be responsible for the complete phosphorylation of

$cPLA_2$ in HL60 cells. The hyperphosphorylation of ERK1 observed for perhydrovanadate-primed DMSO-differentiated HL60 cells, did not appear to affect the phosphorylation state of $cPLA_2$ under the same conditions. As with ERK1 in these cells (Chapter 5), cytochalasin B and fMLP did not increase the phosphorylation state of cytosolic phospholipase A_2, suggesting that the highly neutrophil-like state of these cells or the culture serum concentration, fully phosphorylated both ERK1 and $cPLA_2$, therefore, fully priming these enzymes for subsequent agonist stimulation, such as fMLP, which produces an additional intracellular signal, leading to full activation of $cPLA_2$. However, priming by cytochalasin B and perhydrovanadate, did induce a translocation of the phosphorylated $cPLA_2$ from the cytosol to a membrane fraction (Figure 6.7(b)), suggesting that the role of the priming agent was to induce a translocation event, placing the enzyme in a cell location advantageous to the production of maximal activity upon agonist stimulation. Therefore, priming appears to be due to an effect upon the structural location of $cPLA_2$, as it requires the translocation of the enzyme from the cytosolic fraction to a membrane structure. It has been suggested that both calcium and MAP kinase-stimulated phosphorylation of $cPLA_2$ is responsible for this translocation, however, in the work carried out here, it has been shown that these factors are only involved in the regulation of agonist-stimulated $cPLA_2$ activity. All priming agents studied stimulate tyrosine phosphorylation, therefore, a possible cytoskeletally-associated protein could be tyrosine phosphorylated, leading to a manipulation of the cytoskeleton, which allows $cPLA_2$ to associate with its substrate and regulatory moities more advantageously.

The location of $cPLA_2$ may have a role to play in other human haemopoietic cell, such as germinal centres (for review see MacLennan *et al.*, 1992). These primary B-lymphocytes isolated from human tonsils had cytosolic phospholipase A_2 present (Figure 6.8(a)), however, a CD40- or IgM-stimulated arachidonate release, could not be detected within these cells (results not shown). This lack of agonist-stimulated arachidonate release was also observed in various B- and T-lymphocyte cell lines (results not shown), however, in this case, there was no detectable cytosolic

phospholipase A_2 present (Figure 6.8(b)). This was very interesting as these cell lines, including HL60 cells, were derived from cells further down the maturation pathway of haemopoietic cells, suggesting that somewhere along the maturation process, there is a loss of $cPLA_2$ in some cell types but not others. Why this is the case would be a very interesting future project.

In conclusion, it was shown that in the fibroblastic cell types studied, cytosolic phospholipase A_2 was not located at the plasma membrane as had been suggested. The location was in and around the nuclear area of the cell, depending on the activation state of the enzyme. Priming by serum in Swiss 3T3 fibroblasts produces a different location of the enzyme, compared to *raf-1*-stimulated MAP kinase activation in Rat-1 Raf ER4 fibroblasts. However, the location under both priming conditions, maybe due to the position of substrate and regulatory elements within the same membrane structure. Acute priming of 5 day DMSO-differentiated HL60 cells, also resulted in the translocation of the enzyme to a membrane fraction, the nature of which could not be elucidated. In all the priming conditions, it appeared that it was the appearance of phosphorylated $cPLA_2$ at the membrane which directed activation.

CHAPTER 7

DISCUSSION

The work presented in this thesis supported proposals presented in the literature regarding the regulation of cytosolic phospholipase A_2, however it also demonstrated that the regulation cannot be related to an assumed plasma membrane location of the enzyme.

7.1 The implications of the nuclear location of cytosolic phospholipase A_2

In Chapter 6, confocal microscopy clearly demonstrated that the enzyme was not detectable at the plasma membrane under any condition studied in the fibroblastic cell lines utilised. Western blot analysis did show that $cPLA_2$ could be detected as a 110kDa protein in both the cytosol and membrane fractions of Swiss 3T3 and Rat-1 Raf ER4 fibroblasts. However, the membrane fraction contained all cellular membranes, including the plasma membrane. In published work the membrane location of $cPLA_2$ had been assumed to be the plasma membrane, however from Chapter 6, it was demonstrated that $cPLA_2$ was associated with a membrane structure which appeared to be located in both the extra- and intra-nuclear compartments. If the 'dogma' of signalling enzymes can be put to one side, this 'nuclear' location of $cPLA_2$ can be shown to be highly beneficial to the cell.

When the first studies demonstarted that $cPLA_2$ was located in both the cytosol and membrane fractions prepared from cell fractionation, (Rehfeldt *et al.*, 1991; Krause *et al.*, 1991) it was assumed that the membrane in question was the plasma membrane. This assumption was due to various pieces of evidence that suggested that $cPLA_2$ was regulated by receptor-coupled heterotrimeric G-proteins (Cockcroft, 1992). The involvement of G-proteins in the regulation of $cPLA_2$ has been widely studied and depending upon the cell and agonist used, a pertussis toxin-sensitive and -insensitive G-protein has been implicated (Cockcroft & Stutchfield, 1989; Fischer & Schonbrunn, 1988). Pertussis toxin ADP-ribosylates G_i and G_o α subunits, preventing association with the effector (for review see McDonald & Moss, 1994), which could include PIC. This is of importance due to the activation of $cPLA_2$ by an

increase in intracellular calcium concentrations (Bonventre *et al.*, 1988), which can be stimulated by calcium release from Ins-1,4,5-P_3-sensitive pools located within the endoplasmic reticulum (for review see Gill *et al.*, 1989). Therefore, the sensitivity of $cPLA_2$ to pertussis toxin, could be due to the sensitivity of the PIC-associated G-protein, therefore inhibiting arachidonate release indirectly. The involvement of a pertussis toxin-insensitive G-protein in agonist-stimulated $cPLA_2$ activity can also be explained by an indirect effect upon Ins-1,4,5-P_3-stimulated intracellular calcium release, as the pertussis toxin insensitive G-protein, G_q, has also been shown to be associated with PIC (Conklin *et al.*, 1992). However, this cannot be the complete story as G_{i2}-stimulated arachidonate release has been shown to be independent of PIC-stimulated calcium release (Winitz *et al.*, 1994). In Swiss 3T3 fibroblasts, the attenuation of bombesin-stimulated arachidonate release by GTPγS, was only observed in cells quiesced by serum deprivation, i.e. in DMEM containing 2% (v/v) NBCS (Figure 3.9(a)). Under both quiesing conditions, however, bombesin-stimulated arachidonate release was completely inhibited by GTPβS (Figure 3.10(b)), suggesting that a GTP-binding protein was indeed involved in the response. However, it appeared that some other factor restricts the activation of $cPLA_2$ by GTPγS, which maybe due to the activation of a GTP binding protein-mediated inhibitory pathway, such as adenylyl cyclase or that $cPLA_2$ has a finite capacity for that particular regulatory molecule enhanced by GTPγS stimulation, for example calcium. Therefore, G_q could be activated by GTPγS, resulting in elevated intracellular calcium concentrations, but $cPLA_2$ would be saturated after a very short time and conversely, $cPLA_2$ would be totally inhibited by GTPβS-mediated inhibition of both G_q and G_i G-proteins. Finally, GTPγS-stimulated arachidonate release may involve the small molecular weight GTP-binding proteins, as in various studies, including the work presented here, a GTP-dependence has been shown, which cannot be mimicked by the heterotrimeric G-protein activator aluminum fluoride, which is unable to activate the small molecular weight G-proteins (Kahn, 1991). These small molecular weight GTP-binding proteins were first shown to be involved in vesicular transport

(for review see Glomset & Farnsworth, 1994), raising the possibility that the GTP-dependence of agonist-stimulated arachidonate release could be due to a cellular location factor. Perhaps the activation of these *ras*-like proteins, stimulates the translocation of $cPLA_2$ to a more favourable enviroment or membrane compartment. This could explain the attenuation of bombesin-stimulated arachidonate release in 2% quiesced cells, as it has been shown by confocal microscopy, that the location of $cPLA_2$ in these cells differs to that observed in the more agonist-responsive 10% quiesced cells (Figure 6.2). As these small molecular weight GTP-binding proteins are located within the cytosol, their mediation in the translocation of $cPLA_2$ is possible, as upon activation they have been shown to translocate to various membrane compartments. The best characterised member of this protein family is $p21^{ras}$, which has been shown to mediate various kinase pathways, by direct receptor coupling (for review see Grand & Owen, 1991). One such pathway is the MAP kinase cascade, which coincides with increased tyrosine phosphorylation of various proteins (Worthen *et al.*, 1994). Therefore, GTPγS-stimulated *ras* activity would explain the pertussis toxin insensitive activation of $cPLA_2$ and the total inhibition with GTPβS. Another member of this family, *rac*, has been shown to associate with $cPLA_2$ in the NADPH-oxidase complex and is essential for maximal superoxide generation (Abo *et al.*, 1991). Therefore, it could be responsible for positioning $cPLA_2$ within the active complex. Therefore, both heterotrimeric, via either direct coupling to $cPLA_2$ or indirect manipulation of PIC-mediated increased intracellular calcium concentrations and small molecular weight GTP-binding proteins play a role in the regulation of $cPLA_2$. However, in all the cases presented, activation of a nuclear-associated $cPLA_2$ would be possible.

It was shown that the $cPLA_2$ isoform present in Swiss 3T3 fibroblasts had a preference for phosphatidylcholine containing arachidonic acid (20:4) at the *sn*-2 position of the diacylglycerol backbone (Currie *et al.*, 1992). This arachidonyl-specific cytosolic phospholipase A_2 has been detected in many cell types, mainly by biasing *in vitro* systems utilising a PC micelle (Diez *et al.*, 1992; Kramer *et al.*, 1988).

However, it has been clearly shown that arachidonate was abundant within the nuclear phospholipids, especially within phosphatidylcholine and phosphatidylethanolamine (Tasca & Galis, 1988). Therefore, the substrate for the enzyme was in plentiful supply within the peri- and nuclear areas. Arachidonate released upon $cPLA_2$ activation may be a second messenger alone, as it has been shown to release calcium from Ins-1,4,5-P_3-insensitive pools (Currie *et al.*, 1992). The possible location of these pools could indeed be the nuclear area, as both Ins-1,4,5-P_3-sensitive and -insensitive pools have been identified there (Miyazaki, 1995). However, the main role of arachidonate is in the production of eicosanoids via the lipoxygenase and cyclooxygenase pathways, with enzymes involved in both arachidonate-metabolising pathways being located at the nuclear membrane (Rollins & Smith, 1980; Woods *et al.*, 1995).

Known regulatory enzymes of $cPLA_2$, including MAP kinase and some isoforms of protein kinase C (PKC), have also been shown to translocate from the cytosol to the nuclear membrane upon agonist stimulation. Both kinase pathways are regulated by receptor associated GTP-binding proteins with many of the components of the kinase cascade being distributed within the cytosol, therefore allowing the transduction of the signal from the plasma membrane to the nucleus (for review see Nishizuka, 1995 and Lenormand *et al.*, 1993)

. Therefore, a nuclear location for $cPLA_2$ is possible and advantageous in both regulation and cellular responses involving arachidonate [for a possible scheme see Figure 7.1].

7.2 The possible protein kinase C 'loop'

In the case of protein kinase C, $cPLA_2$ activation may play a role in the regulation of various PKC isoforms. $cPLA_2$ does not only hydrolyse arachidonate at the *sn*-2 position of the diacylglycerol backbone, but contains a lysophospholipase activity, resulting in the hydrolysis of the mainly saturated fatty acids at the *sn*-1 position (Leslie, 1991). In Chapter 3, it was shown that endothelin stimulated a dose-

Figure 7.1 **Possible regulation of nuclear-associated cytosolic phospholipase A_2.**

This is a schematic diagram of the possible regulation of nuclear-associated cytosolic phospholipase A_2 by extracellular stimuli. The components outlined include:

G-protein-linked receptors

Hetrotrimeric GTP-binding proteins

Tyrosine kinase receptors

MAPK=Microtubule-associated protein kinase

PKC=Protein kinase C

ras = 21kDa small molecular weight G-protein

PIC = Phosphatinositidase C

PtdIns-4,5-P_2= phosphatidylinositol-4,5-bisphosphate

Ins-1,4,5-P_3 = inositol-1,4,5-trisphosphate

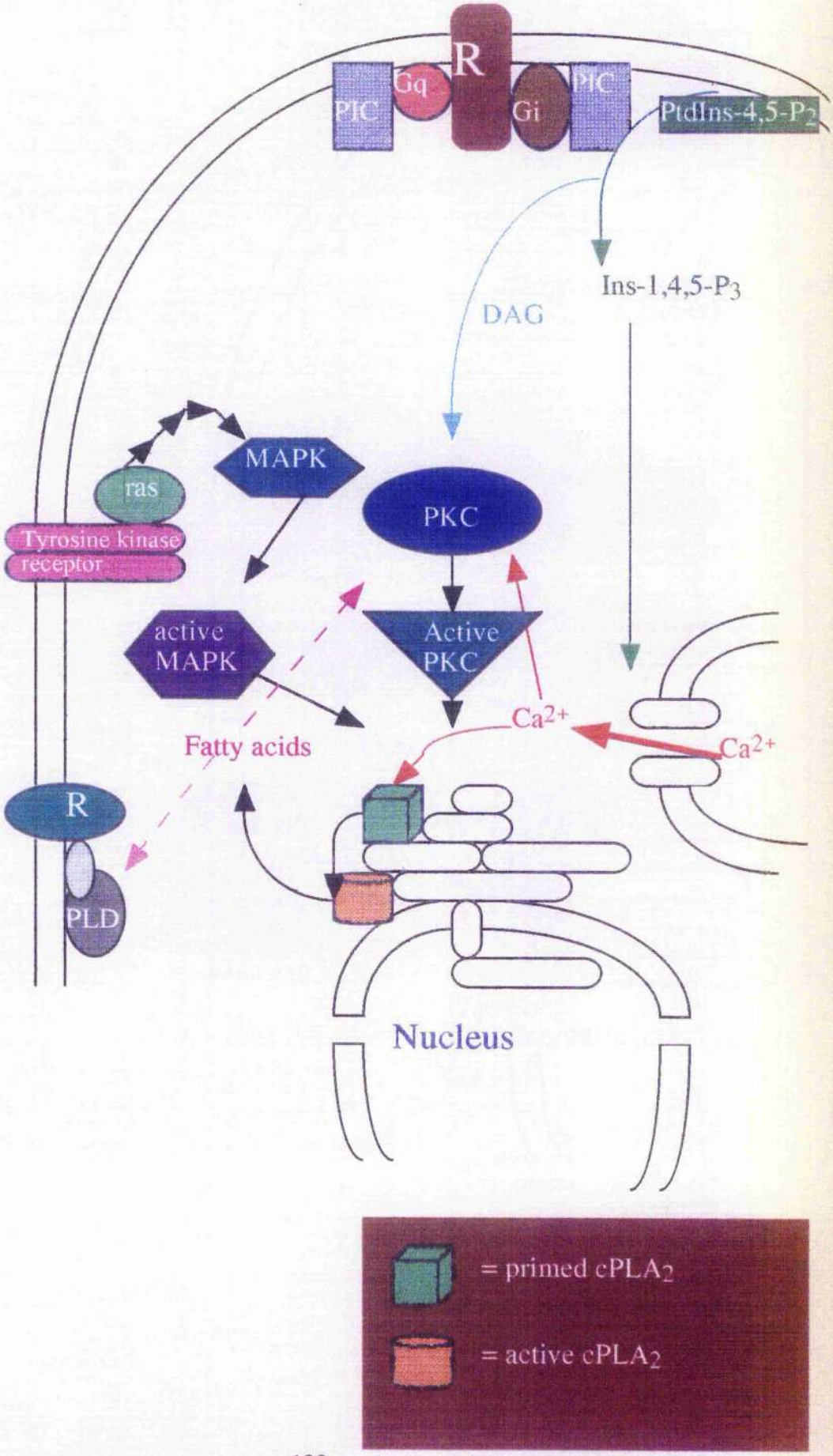
DAG = Diacylglycerol

Ca^{2+} = calcium

Fatty acid possible targets 

All arrows represent the possible effectors of the component in question.

Figure 7.1


R
Gq
Gi
PIC
PIC
PtdIns-4,5-P2
Ins-1,4,5-P3
DAG
MAPK
ras
Tyrosine kinase receptor
PKC
active MAPK
Active PKC
Ca2+
Ca2+
Fatty acids
R
PLD
Nucleus
= primed cPLA2
= active cPLA2

and time-dependent release of oleic acid (18:1) in Rat-1 fibroblasts and GC mass spectrometry demonstrated that many saturated and monounsaturated fatty acids were released upon bombesin-stimulation in Swiss 3T3 fibroblasts, which could be due to the lysophospholipase activity of $cPLA_2$. Evidence has shown that this lysophospholipase activity can hydrolyse any fatty acid at the *sn*-1 position of lysophosphatidylcholine (lysoPtdCho) and oleate at the same position in lysoPtdEth and LysoPtdSer (She *et al.*, 1994). This is of interest as it has been shown that there is a requirement for free fatty acids in the maximum activation of certain PKC isoforms (McPhail *et al.*, 1984) and oleate has been implicated in the activation of one form of phospholipase D (PLD) (Chalifa *et al.*, 1990). As many agonists have been shown to produce biphasic $cPLA_2$ activity (Currie *et al.*, 1992), it can be proposed that the initial transient phase of fatty acid release could be responsible for producing maximal activity of such enzymes as PLD and PKC. The cellular location of PLD has still to be elucidated, however, it has been shown that certain PKC isoforms translocate to the nucleus when free fatty acids, diacylglycerol (DAG) and calcium are available (for review see Nishizuka, 1995). Since many agonists stimulate both $cPLA_2$ and PIC (Takuwa *et al.*, 1987), the factors required for this translocation would be available. Therefore, PKC would come into contact with the nuclear associated $cPLA_2$ and then regulate the activity of this enzyme by phosphorylation. This proposition would explain the phorbol-ester-stimulated arachidonate release, which corresponds kinetically with the second sustained phase of $cPLA_2$ activity produced by many agonists. The only drawback of this arguement is the supply of DAG required. One possibility is the involvement of the fatty acid- and DAG-insensitive PKC isoforms, the regulation of which has still to be elucidated. An other exciting possibility, is the involvement of PLD, which has been shown to produce a sustained phase of PtdCho-derived DAG in response to phorbol esters and bombesin (Cook & Wakelam, 1989), with very similar kinetics to those seen for arachidonate release. Therefore, this raises the possibility that PLD-mediated DAG production is used to produce the chronic stimulation of PKC required for the chronic activation of

$cPLA_2$ and therefore, eicosanoid production, one of which prostaglandin E_2 (PGE_2) was detectable in Swiss 3T3 fibroblasts after a 10 minute bombesin stimulation (Currie, 1991). It also raises the possibility of a regulatory loop for both PKC and PLD by $cPLA_2$-stimulated fatty acid release, resulting in the enhancement of activity of all 3 enzymes, as outlined in Figure 7.2. However, this analogy depends upon the initial, rapid release of fatty acids upon agonist stimulation.

7.3 Role of phosphorylation in the priming and translocation of $cPLA_2$

In both the chronic priming of $cPLA_2$ by serum in Swiss 3T3 fibroblasts and the acute priming of the enzyme by cytochalasin B in differentiated HL60 cells, there was an increase in the level of tyrosine phosphorylation (Figure 4.1), but this did not result in an increased MAP kinase activity (Table 5.1) or arachidonate release (Chapter 5), compared to the unprimed cells. This increased tyrosine phosphorylation may have increased the sensitivity of $cPLA_2$ to subsequent agonist stimulation, by perhaps modulating the enzyme into a more active configuration or location. In 5 day DMSO-differentiated HL60 cells, MAP kinase is in a highly phosphorylated state, perhaps due to priming by the high concentration of serum in which the cells are cultured (Figure 5.5). However, this phosphorylated MAP kinase did not stimulate the translocation of $cPLA_2$ from the cytosol to the membrane, as shown by western blotting (Figure 6.7). Only the addition of the priming agents, perhydrovanadate or cytochalasin B, stimulated translocation (Figure 6.7). Both these priming agents increased tyrosine phosphorylation (Figure 4.1) with only perhyrdrovanadate stimulating any additional *in vitro* MAP kinase activity (Table 5.1). Therefore, the translocation is perhaps via the enhanced tyrosine phosphorylation of a protein involved directly or indirectly in the structural alignment of $cPLA_2$, much in the same way *rac* has been suggested to do in the NADPH-oxidase complex (Abo *et al.*, 1991). A readjustment of the cytoskeleton has been shown for cytochalasin B priming (Cooper, 1987) and has been suggested to be mediated via an increase in tyrosine

Figure 7.2 The possible protein kinase C (PKC) 'loop'

This a schematic diagram of a possible cytosolic phospholipase A_2 ($cPLA_2$) and protein kinase C (PKC) 'loop'. The components described include:

PIP_2 = phosphatidylinositol-4,5-bisphosphate

PtdCho = Phosphatidylcholine

DAG = Diacylglycerol

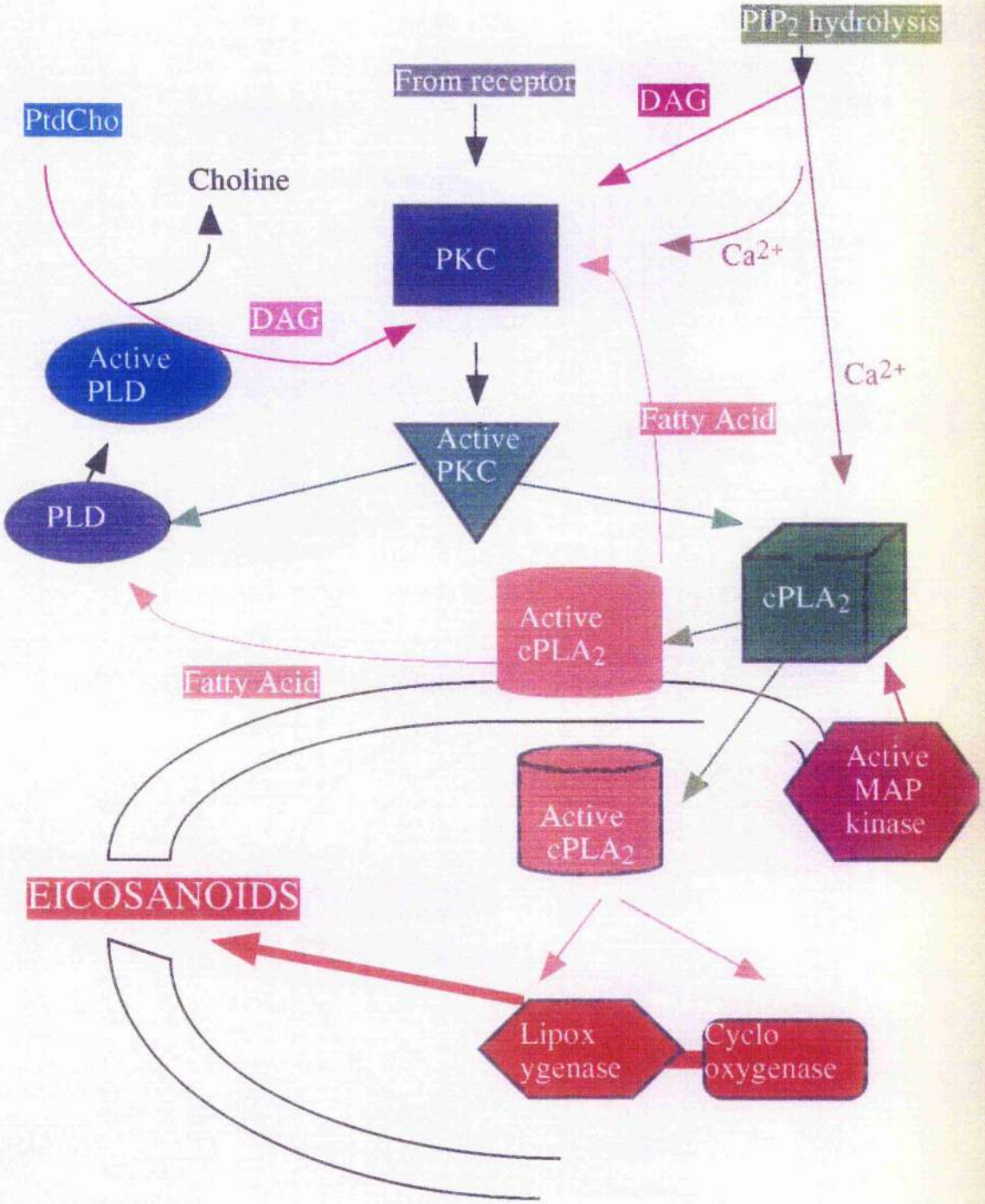
Ca^{2+} = calcium

PLD = Phospholipase D

MAP kinase = microtubule-associated protein kinase

All arrows show the possible targets of the component of interest.

Figure 7.2


PIP2 hydrolysis
From receptor
DAG
PtdCho
Choline
PKC
Ca2+
DAG
Active PLD
Ca2+
Fatty Acid
Active PKC
PLD
cPLA2
Active cPLA2
Fatty Acid
Active MAP kinase
Active cPLA2
EICOSANOIDS
Lipox ygenase
Cyclo oxygenase

phosphorylation (Ridley & Hall, 1994). The association of the cytoskeleton with the nucleus has been shown, therefore, the structural moiety with which $cPLA_2$ is associated with could be the cytoskeleton (Earnshaw & MacKay, 1994). In these cells, MAP kinase is so highly phosphorylated that it may have consigned a definite localisation of $cPLA_2$ prior to priming, however, confocal studies are not available at this moment in time to confirm this.

This definite MAP kinase-stimulated localisation of $cPLA_2$ was observed in Rat-1 Raf ER4 fibrobasts. Confocal microscopy demonstrated that in Rat-1 Raf ER4 fibroblasts $cPLA_2$ is evenly distributed between the extra- and intra-nuclear compartments, with a relocation to a structure in the extranuclear compartment upon estradiol-mediated *raf-1* activity (Figure 6.4). However, this MAP kinase activity only phosphorylated and did not activate $cPLA_2$ (Figure 5.3(a)). Of interest was the fact that in both 5 day DMSO-differentiated HL60 cells and estradiol-treated Rat-1 Raf ER4 fibroblasts, phosphorylated $cPLA_2$ was detected within the cytosol. In the cell fractionation carried out, any weakly membrane associated $cPLA_2$ would appear in the cytosolic fraction, along with any true cytosolic enzyme, whereas $cPLA_2$ which has a high membrane affinity, will be detected only in the membrane fraction. Work carried out in serum-primed Swiss 3T3 fibroblasts showed that only non-phosphorylated $cPLA_2$ was ever found in the cytosol, suggesting that weakly associated membrane-bound and free cytosolic $cPLA_2$, were in the non-phosphoylated state (Figure 6.1). This led to the suggestion that it was phosphorylation of $cPLA_2$ which enhanced the affinity of the enzyme for the membrane. However, in many experiments, both phosphorylated and non-phosphorylated $cPLA_2$ was found in the membrane fraction and as discussed above, phosphorylated enzyme was found in the cytosol of both DMSO-differentiated HL60 cells and estradiol-treated Rat-1 Raf ER4 fibroblasts. Therefore, phosphorylation of $cPLA_2$ does not confer high affinity with membranes, but may only regulate activity as it has been demonstrated *in vitro* that phosphorylated $cPLA_2$ is the more active form (Kramer *et al.*, 1993(a)).

This increased affinity for membrane upon priming appears therefore, to be due to an additional factor, which may or may not be mediated via the common factor in all the priming models studied, namely increased tyrosine phosphorylation. With $cPLA_2$ containing so many as yet unassigned phosphorylation sites, it is possible that the enzyme maybe phosphorylated by some factor, to such an extent that the mobility of the enzyme would be unaffected upon SDS-PAGE, but would confer some possible conformational change upon the enzyme which results in enhanced membrane affinity.

Figure 7.3 demonstrates the possible mechanisms involved in priming in the fibroblast models studied, combining both western blotting and confocal microscopy. In this model it is suggested that for the initial phase of agonist-stimulated $cPLA_2$ activity, the enzyme has to be located within the nucleus and tightly associated with a membrane fraction. Activity is only seen when there is an increase in intracellular calcium concentrations. In serum-primed Swiss 3T3 fibroblasts, both MAP kinase and PIC appear to be primed, producing a small biphasic MAP kinase activity and enhanced calcium release in comparison to serum-deprived cells, which would produce the signals required for maximum $cPLA_2$ activity. At later time points of agonist stimulation, there is a chronic stimulation of both MAP kinase and arachidonate release. The former could mediate the translocation of $cPLA_2$ to the perinuclear region as demonstrated in estradiol-treated Raf ER4 fibroblasts, where it can be regulated by the activated protein kinase C isoforms, as discussed in section 7.2.

In conclusion, the work presented in this thesis highlights the need to be flexible in the visualisation of the physiological localisation of signalling enzymes and to question the 'dogma' which can arise from isolated pieces of investigation. These thorough investigations are correct in their own right, however, the conclusions drawn are perhaps ambigious. As shown, the absence of $cPLA_2$ from the plasma membrane, which was assumed to be the cellular location of activated cytosolic phospholipase A_2, does not affect its regulation by PKC and MAP kinase as these enzymes are

Figure 7.3 Possible mechanism for priming

This is a diagram of the possible mechanism of priming, derived from the data presented in this Thesis.

The abbreviations used are: P = phosphorylated $cPLA_2$

P-ser505 = phosphorylated on serine 505

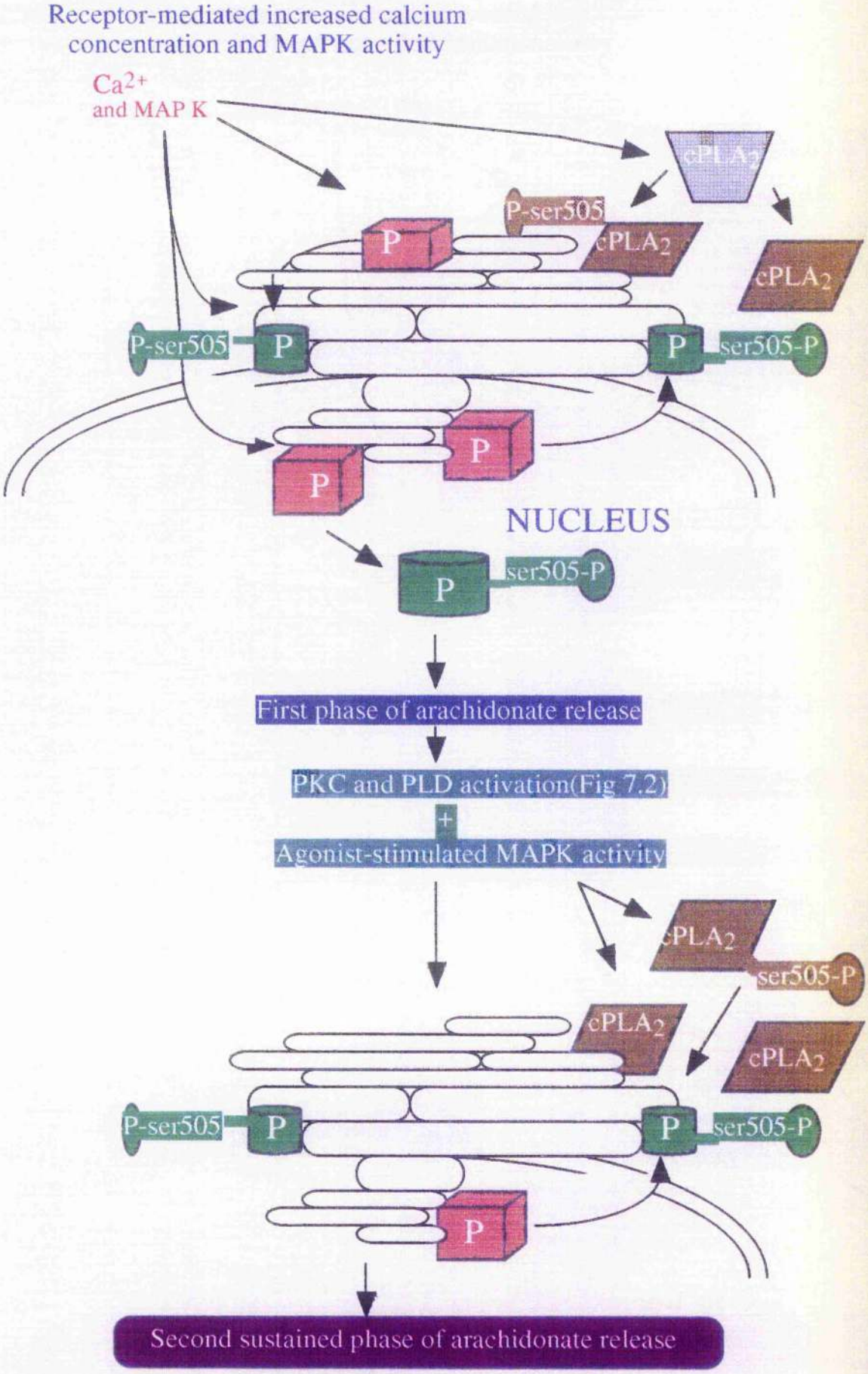
$cPLA_2$ = cytosolic phospholipase A_2

Ca^{2+} = calcium

MAPK = microtubule-associated protein kinase

The arrows represent the possible targets of the component in question. The differences in the shape of $cPLA_2$ depends upon the regulatory factors which have acted upon it.

Figure 7.3


Receptor-mediated increased calcium concentration and MAPK activity
Ca2+ and MAP K
cPLA2
P-ser505
cPLA2
cPLA2
P-ser505
P
P
ser505-P
P
P
P
NUCLEUS
P
ser505-P
First phase of arachidonate release
PKC and PLD activation(Fig 7.2)
+
Agonist-stimulated MAPK activity
cPLA2
ser505-P
cPLA2
cPLA2
P-ser505
P
P
ser505-P
P
Second sustained phase of arachidonate release

translocated to the nuclear membrane upon activation. It does not affect the metabolism of the $cPLA_2$ product, free arachidonic acid, as the lipoxygenase and cyclo-oxygenase enzymes involved have also been shown to be located at the nuclear membrane. Finally, the reported GTP-binding protein involvement can be explained by the effect on both inhibitory and stimulatory regulators of $cPLA_2$, such as cAMP and PIC-mediated increased intracellular calcium concentrations, respectively. Both of these pathways also supply the link from the plasma membrane-associated agonist receptor and the intracellular localisation of $cPLA_2$.

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