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**Partial Purification and Characterisation of a Membrane-  
Associated Phospholipase D from Bovine Spleen.**

**Andrew Paul**

A thesis submitted to the Faculty of Science for the degree  
of Doctor of Philosophy

September 1995  
Department of Biochemistry  
University of Glasgow

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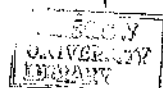
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For my parents

In memory of Colin

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## Abbreviations

ADP	Adenosine 5'-diphosphate.
ARF	ADP-Ribosylation Factor.
ATP	Adenosine 5'-triphosphate.
[Ca] <sub>i</sub>	Intracellular calcium concentration.
cDNA	Complimentary deoxyribonucleic acid.
CDP-choline	Cytidine 5'-diphosphocholine.
cmc	critical micellar concentration.
CMP	Cytidine 5'-monophosphate.
CMP-phosphatidic acid	Cytidine 5'-monophosphate-phosphatidic acid.
CTP	Cytidine 5'-triphosphate.
DAG	<i>sn</i> -1, 2-Diacylglycerol.
DAG kinase	Diacylglycerol kinase.
dpm	disintegrations per minute.
E 64	<i>trans</i> -Epoxysuccinyl-L-leucylamido(4-guanido)butane.
EDTA	Ethylenediaminetetraacetic acid.
EGF	Epidermal growth factor.
EGTA	[Ethylenebis(oxyethylenenitril)]-tetraacetic acid.
fMetLeuPhe	N-Formylmethionylleucylphenylalanine.
GAP	GTPase Activating Protein.
GDI	GDP Dissociation Inhibitor.
GIP	GTPase Inhibitory Protein.
GLC	Gas/liquid chromatography.
GTP	Guanosine 5'-triphosphate.
GTPγS	Guanosine 5'-O -(3-thiotriphosphate).
h	Hour.
HPLC	High pressure liquid chromatography.
Ins(1, 4, 5)P <sub>3</sub>	D- <i>myo</i> -inositol 1, 4, 5-triphosphate.

Ins(1, 3, 4, 5)P <sub>4</sub>	D- <i>myo</i> -inositol 1, 3, 4, 5-tetrakisphosphate.
MARCKS protein	Myristoylated alanine-rich C-kinase substrate protein.
min	Minute.
NEM	<i>N</i> -ethylmaleimide.
NGF	Nerve Growth Factor.
OD	Optical Density.
<sup>32</sup> P <sub>i</sub>	[ <sup>32</sup> P]-orthophosphate.
PAP	Phosphatidic acid phosphohydrolase.
PDGF	Platelet derived growth factor.
PtdInsTP	Phosphatidylinositol Transfer Protein.
PKC	Protein kinase C.
PLA <sub>2</sub>	Phospholipase A <sub>2</sub>
PLC	Phospholipase C.
PLD	Phospholipase D.
PMA	Phorbol 12-myristate 13-acetate.
PMSF	Phenylmethanesulphonyl fluoride.
PtdCho	Phosphatidylcholine.
PtdEtn	Phosphatidylethanolamine.
PtdIns	Phosphatidylinositol.
PtdIns(4)P	Phosphatidylinositol-4-phosphate.
PtdIns(4, 5)P <sub>2</sub>	Phosphatidylinositol-4, 5-bisphosphate.
PtdIns-PLC	Phosphoinositide-specific phospholipase C.
PtdOH	Phosphatidic acid.
PtdSer	Phosphatidylserine.
SD	Standard deviation.
sec	Second.
tlc	Thin layer chromatography.
TNF $\alpha$	Tumour Necrosis Factor $\alpha$ .
TRH	Thyrotropin-releasing hormone.

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## Summary

Phospholipase D (PLD; EC 3.1.4.4) catalyses the hydrolysis of the phosphate ester bond of phospholipid molecules, releasing the hydroxyl containing head group with the concomitant formation of phosphatidic acid (PtdOH). Evidence now exists that PLD catalyses the hydrolysis of membrane phospholipids, particularly PtdCho, in an agonist-dependent manner. This may serve as a novel source of the established second messenger diacylglycerol with its immediate lipid product, phosphatidic acid, functioning as a putative second messenger. The relationship between agonist-stimulated PLD activity *in vivo* and observed *in vitro* activity remains unclear. This study examines the purification and characterisation of membrane-associated PtdCho-PLD activity from bovine spleen.

PLD activity was primarily associated with the membrane compartment of the bovine spleen homogenate. Incubation of these freeze-thawed membranes at high pH resulted in efficient solubilisation of the membrane-associated PLD activity. Purification of the solubilised PLD was subsequently investigated. Use of cation-exchange chromatography resolved two PtdCho-hydrolysing PLD activities, the major one being further purified by successive chromatographic separation on heparin-agarose, hydroxyapatite, cation-exchange and gel-filtration chromatography. SDS-PAGE revealed the final protein preparation to be heterogeneous and it was not possible to identify a particular protein that co-migrated with the PLD activity on gel-filtration which displayed an apparent native molecular weight of 69 kDa. The solubilised PLD remains to be purified to homogeneity.

Using mixed micellar methodology the catalytic activity of the partially purified enzyme towards PtdCho was characterised. At all substrate concentrations the post heparin enzyme displayed pseudo-first order kinetics and values for  $V_{\max}$  and  $K_m$  could not be determined by this assay methodology, however, the reaction velocity displayed a pH optimum of 7.0 and was independent of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Finally, investigation of catalytic activity towards PtdCho, PtdEtn, PtdIns and PtdSer confirmed the partially purified preparation to be a distinct PLD activity that was

selective between the major phospholipids and displayed substrate specificity towards PtdCho.

**Chapter 1**  
**Introduction.**

## **Chapter 1. Introduction.**

Signal transduction mechanisms provide the cell with a method with which to sense and respond to their extracellular environment. Thus, in response to a range of stimuli various physiological processes such as differentiation and proliferation can be both induced and controlled in a co-ordinated manner. The presence of a particular extracellular signal in the extracellular environment is often detected by a specific cell surface membrane receptor. Occupation of this receptor leads to the initiation of a signal transduction cascade and the generation of second messenger molecules. Whilst there are many extracellular stimuli, there are relatively few second messenger systems and this may reflect a degree of evolutionary constriction within signal transduction processes. However, it is becoming increasingly apparent that cells respond to multiple agents by activating multiple second messenger systems that can act in concert to potentiate, synergise or antagonise specific cellular responses (see Malarkey *et al.*, 1995). The second messenger systems involved have been extensively examined and include adenylyate cyclase-mediated elevation of intracellular cAMP, activation of tyrosine kinases and the hydrolysis of membrane phospholipids (see Billah & Anthes, 1990; Berridge, 1993; Dennis, 1994; Malarkey *et al.*, 1995). The introduction to this thesis will focus upon the regulation of agonist-stimulated membrane phospholipid hydrolysis, particularly that of phospholipase D-catalysed hydrolysis of phosphatidylcholine and its functional significance.

### **1.1 Agonist-Stimulated Turnover of Phospholipids.**

#### **1.1.1 Regulation of Agonist-Stimulated Hydrolysis of Phosphatidylinositol-4,5 Bisphosphate.**

The early work of Hokin & Hokin in the 50s and the seminal work of Michell, Berridge, Irvine and co-workers in the late 70s and early 80s identified the hydrolysis of inositol-containing phospholipids as a universal signalling pathway activated in response to calcium mobilising agonists (Hokin & Hokin, 1953; Hokin & Hokin, 1958a; Hokin & Hokin, 1958b; Hokin *et al.*, 1958a; Hokin *et al.*, 1958b; Berridge &

Irvine, 1989). Michell (1974) proposed that the receptor-mediated elevation of intracellular calcium concentrations ( $[Ca^{2+}]_i$ ) was in fact consequent to agonist-stimulated PtdIns hydrolysis. This resultant elevation of  $[Ca^{2+}]_i$  as a consequence of PtdIns(4, 5)P<sub>2</sub> hydrolysis is now recognised as being associated with the initial cellular actions of many growth factors, hormones and neurotransmitters upon a variety of cell and tissue types (reviewed by Berridge & Irvine, 1989; Whitman & Cantley, 1988, Cockcroft & Thomas, 1992; Michell, 1992; Berridge, 1993). Following receptor activation, hydrolysis of PtdIns(4, 5)P<sub>2</sub> is catalysed by a phosphoinositide-specific phospholipase C (PLC) to generate two second messengers, inositol 1, 4, 5-trisphosphate (Ins(1,4, 5)P<sub>3</sub>) and *sn* 1, 2-diacylglycerol (DAG).

Ins(1, 4, 5)P<sub>3</sub> has been identified as the molecule responsible for stimulating the release of intracellular  $Ca^{2+}$  (Streb *et al.*, 1983) from the hormone-sensitive calcium store in the smooth endoplasmic reticulum, by binding to its own intracellular receptor (reviewed by Berridge & Irvine, 1984; Berridge & Irvine, 1989). In parallel, the production of *sn* -1, 2-diacylglycerol, acting in concert with phosphatidylserine (PtdSer) and  $Ca^{2+}$ , activates protein kinase C (PKC) (Nishizuka, 1984, 1986, 1988; Parker *et al.*, 1989). Consequently, elevation of *sn* -1, 2-diacylglycerol is responsible for the increase in PKC activity associated with the action of  $Ca^{2+}$ -mobilising agonists.

As intracellular second messengers both are rapidly removed by metabolism involving a number of specific enzymes. Ins(1, 4, 5)P<sub>3</sub> is sequentially dephosphorylated by a series of phosphatases to free inositol making it available for reincorporation into PtdIns (see Berridge & Irvine, 1989). Ins(1,4,5)P<sub>3</sub> may also be phosphorylated to Ins(1, 3,4, 5)P<sub>4</sub> which itself may play a role in regulating intracellular calcium homeostasis. This tetrakisphosphate can be both broken down to inositol or further phosphorylated to higher inositol phosphates (Berridge, 1993).

Coordinate resynthesis of the phosphoinositides involves a number of integrated phosphorylation reactions catalysed by a multitude of kinases. *sn*-1, 2-diacylglycerol is initially phosphorylated to phosphatidic acid by DAG kinase in



readiness for committed incorporation into the resynthetic pathway for phosphoinositides (see Berridge & Irvine, 1989). Phosphatidic acid (PtdOH) is then condensed with cytidine 5'-triphosphate (CTP) to form cytidine 5'-monophosphate(CMP)-PtdOH, catalysed by CMP-PtdOH synthetase. PtdIns Synthase catalyses the reaction of the generated CMP-PtdOH with free inositol derived from the inositol phosphate pool to produce PtdIns. This then serves as the precursor of the polyphosphoinositides. The steady state levels of PtdIns, PtdIns(4)P and PtdIns(4, 5)P<sub>2</sub> are regulated by the action of specific kinase and phosphatase activities including, PtdIns 4-kinase, PtdIns(4)P 5-kinase, PtdIns(4)P phosphomonoesterase and PtdIns(4, 5)P<sub>2</sub> phosphomonoesterase (see Majerus *et al.*, 1986; Berridge & Irvine, 1989; Carpenter & Cantley, 1990). The relative sizes of these pools are such that approximately 90% of total phosphoinositides is in the form of PtdIns. The equilibrium is transiently disturbed upon agonist stimulation of PLC activities that metabolise PtdIns(4, 5)P<sub>2</sub> and detectable decreases in the PtdIns(4, 5)P<sub>2</sub> pool have been measured. However, the equilibrium is quickly restored. Therefore, it is apparent that hydrolysis and resynthesis of the phosphoinositides occurs by means of an integrated cyclical pathway termed the 'PtdIns-cycle'.

Outwith the 'PtdIns-cycle', PtdIns(4, 5)P<sub>2</sub> may also be further phosphorylated to produce PtdIns(3, 4, 5)P<sub>3</sub> (Whitman *et al.*, 1988; Auger *et al.*, 1989). This reaction is catalysed by PtdIns 3-kinase, primarily a heterodimer consisting of an 85kDa and a 110kDa subunit; however, several isoforms of this enzymic activity are now known to exist (L. Stephens, personal communication). The function of PtdIns(3, 4, 5)P<sub>3</sub> in cells is not clear, although a role for this lipid has been reported in the regulation of platelet aggregation, histamine secretion, translocation of glucose transporters and membrane ruffling (reviewed by Stephens, 1994). These reports perhaps point to a general role for this lipid in cytoskeletal regulation.

Several phosphoinositide-specific phospholipase C isoenzymes are present in mammalian tissues, as deduced from protein isolation and molecular cloning studies (reviewed by Rhee *et al.*, 1989; Meldrum *et al.*, 1991; Cockcroft, 1992). These

activities have been categorised into four groups:  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ . The  $\alpha$  subfamily represents a number of PtdIns-PLC activities classified by an apparent molecular mass of between 60-70 kDa (Rhee *et al.*, 1989). Cloning of a cDNA for one of these 60kDa activities has been reported, though its authenticity has been debatable as its expression did not encode any functional PtdIns-PLC activity comparable to the expression of cDNA encoding  $\beta$ ,  $\gamma$  and  $\delta$  isoforms (Bennet & Crooke, 1987; Meldrum *et al.*, 1991). Further, the PLC $\alpha$  activities possess little sequence or structural similarity to cloned PLC $\beta$ ,  $\gamma$  and  $\delta$  (Meldrum *et al.*, 1991). The classification of Rhee *et al.* (1989) gave rise to the subdivision of the PtdIns-PLC activities previously purified from bovine brain; the  $\beta_1$  to correspond to the 154 kDa activity,  $\gamma_1$  to the 145 kDa activity and  $\delta_1$  to the 85 kDa activity. Further purification and cloning studies resulted in description of more members of these sub-families, designated  $\beta_2$ ,  $\beta_3$  and  $\beta_4$ ,  $\gamma_2$ ,  $\delta_2$  and  $\delta_3$  based upon analysis of apparent sequence and immunoreactivity. In summary, the PtdIns-PLC isoforms exist as a complex family of inter-related activities allowing diverse agonist signalling by means of varied phosphoinositide hydrolysis.

For agonists whose receptors comprise seven transmembrane spanning domains, the signal generated by the agonist receptor complex is well recognised to be modulated and transduced across the plasma membrane by members of the heterotrimeric guanine nucleotide binding proteins (G-proteins) (reviewed by Cockcroft, 1987). This family of proteins, consisting of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, regulate a number of intracellular effector molecules (Simon *et al.*, 1991). Further, these proteins have been arranged into four sub-classes based upon the primary sequence identity of their respective  $\alpha$ -subunits (Simon *et al.*, 1991). Within this subdivision, the G $\alpha_q$  class members were identified as the G-proteins responsible for the regulation of the PtdIns-PLC activity (Gutowski *et al.*, 1991; Smrcka *et al.*, 1991; Taylor *et al.*, 1991; Wu *et al.*, 1992a). Partially purified PtdIns-PLC could be activated by a mixture of purified G $\alpha_q$ /G $\alpha_{11}$  from GTP $\gamma$ S treated bovine liver plasma membranes (Taylor *et al.*, 1990). Moreover, when these activators were reconstituted

with individually purified PtdIns-PLC isoforms, they specifically activated the  $\beta_1$  isoform, with no effect upon the catalytic rates of PtdIns-PLC $\gamma_1$  nor PtdIns-PLC  $\delta_1$  (Taylor *et al.*, 1991). Similarly, the PtdIns-PLC $\beta_1$  homologue purified from turkey erythrocytes could be activated by the  $G\alpha_q$  ( $G\alpha_{11}$ ) homologue from the same source (Waldo *et al.*, 1991).

The regulation of catalysed PtdIns(4, 5) $P_2$  hydrolysis has been refined further by the ability of heterotrimeric G-protein  $\beta\gamma$  subunits to activate PtdIns-PLC isoforms (see Clapham & Neer, 1993). Activation of an unidentified PtdIns-PLC isoform was first observed in a range of cytosolic extracts from bovine liver, human and bovine peripheral neutrophils and HL-60 granulocytes in a similar manner to a PLC purified from turkey erythrocytes (Blank *et al.*, 1992; Boyer *et al.*, 1992; Camps *et al.*, 1992). Moreover, Park *et al.* (1993) described ordered activation of individual purified PtdIns-PLC isoforms upon reconstitution with  $\beta\gamma$  subunits in lipid vesicles containing PtdIns(4, 5) $P_2$ . PLC $\beta_2$  and PLC $\beta_3$  were activated whilst PLC $\beta_1$  was activated to a lesser extent. Efforts to demonstrate the activation of mammalian PtdIns-PLC activity by pertussis toxin-sensitive  $\alpha$  subunits has been unsuccessful (Blank *et al.*, 1992; Camps *et al.*, 1992). Further, agonist-mediated initiation of PLC $\beta_2$  activity has been reported in Cos cells transfected with muscarinic receptors, but only if  $\beta\gamma$  subunits were co-expressed (Katz *et al.*, 1992). The fourth member of the mammalian  $\beta$ -type PtdIns-PLC family has recently been purified, sequenced and cloned (Lee *et al.*, 1993) and in common with the other three sub-family members is activated by all the  $\alpha$  subunits of the  $G_q$  class heterotrimeric G-proteins yet is unresponsive to  $\beta\gamma$  subunits in a similar manner to the  $\beta_1$  isoform (Jiang *et al.*, 1994; Lee *et al.*, 1994). Consequently, this may represent the mechanism by which pertussis toxin-sensitive heterotrimeric G-proteins regulate catalysed PtdIns(4, 5) $P_2$  hydrolysis.

PtdIns-PLC $\gamma$  activities are regulated by receptor or non-receptor tyrosine kinases (reviewed by Rhee & Choi, 1992). Growth factor receptors (R) (eg. EGFR, PDGFR & NGFR) bind cognate ligands and thus activate their intrinsic tyrosine kinase activities leading to PtdIns-PLC $\gamma$  phosphorylation and thus enhanced rates of

PtdIns(4, 5)P<sub>2</sub> hydrolysis. In a number of cell types EGF-mediated activation of PLC $\gamma$  requires the involvement of a pertussis-toxin sensitive G<sub>i</sub> protein in an undefined role (see Rhee, 1991 and references therein).

Receptors that do not possess intrinsic kinase activity can in a number of instances also activate PLC $\gamma$  isoforms. This occurs through the recruitment and activation of non-receptor/soluble tyrosine kinases, typified by the Src family, eg. the T-cell receptor/CD3 complex. This complex does not possess intrinsic tyrosine kinase activity, but upon suitable activation induces tyrosine phosphorylation and activation of PLC $\gamma$  (see Rhee, 1991 and references therein)

A number of agonists that utilise G-protein coupled receptors can also stimulate the tyrosine phosphorylation and activation of PLC $\gamma$ . In CHO cells transfected with the M5 subtype muscarinic receptors, carbachol stimulated the tyrosine phosphorylation of PLC $\gamma$  (Gusovsky *et al.*, 1993). This was inhibited by receptor operated Ca<sup>2+</sup> channels blockers and implicates receptor-mediated Ca<sup>2+</sup> entry and subsequent tyrosine kinase activity in the activation of PLC $\gamma$ . Similar observations have been described in Angiotensin II (AII)-stimulated smooth muscle cells (Marrero *et al.*, 1994; 1995) and consequently may represent an alternative mechanism of activating PLC $\gamma$ -catalysed phosphoinositide hydrolysis.

In contrast to the  $\beta$  and  $\gamma$  isoforms, the cellular mechanisms by which PtdIns-PLC $\delta$  isoforms are regulated is less clear. The  $\delta$  isoforms contain Ca<sup>2+</sup>-binding EF-hand motifs (Bairoch & Cox, 1990) and therefore the regulation by Ca<sup>2+</sup> *in vivo* has been investigated. In CHO cells overexpressing PLC $\delta$ 1, thrombin-stimulated phosphoinositide hydrolysis was completely inhibited by Ca<sup>2+</sup> chelation, while addition of calcium ionophore (ionomycin) stimulated phosphoinositide hydrolysis (Banno *et al.*, 1994). However, the precise mechanisms by which PLC $\delta$ 1 is activated remain unclear and the elucidation of this may require further reconstitution experiments.

## **1.2 Receptor-Generated *sn* -1, 2-Diacylglycerol from Non-PtdIns(4, 5)P<sub>2</sub> Sources: Phosphatidylcholine As A Source of Sustained *sn* -1, 2-Diacylglycerol.**

In a number of cell types, including fibroblasts and vascular smooth muscle cells, it was recognised that agonist-stimulated formation of Ins(1, 4, 5)P<sub>3</sub> did not correlate with the formation of DAG. Ins(1, 4, 5)P<sub>3</sub> formation was transient whilst DAG formation was sustained (Griendling *et al.*, 1986; Cook *et al.*, 1990; Sunako *et al.*, 1990; Cook & Wakelam, 1992; Plevin & Wakelam, 1992). The transient nature of the Ins(1, 4, 5)P<sub>3</sub> signal was not due to enhanced removal of Ins(1, 4, 5)P<sub>3</sub> by phosphatase or kinase activities but reflected a rapid desensitisation of phosphoinositide hydrolysis (eg. Plevin & Wakelam, 1992). This suggested that a lipid source other than PtdIns(4,5)P<sub>2</sub> provided the source of the sustained DAG. Consequently the source of this secondary DAG phase has been extensively examined.

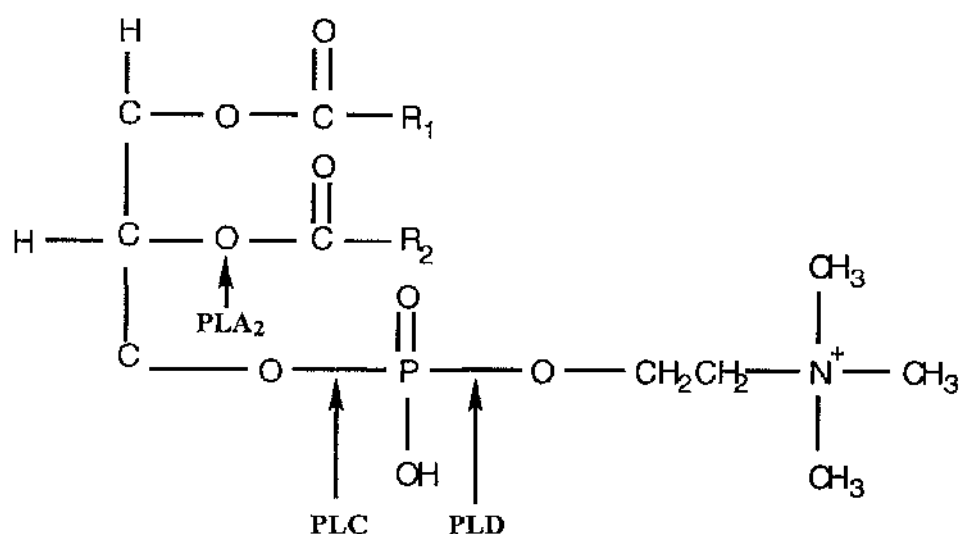
Initial studies in thrombin-stimulated IIC9 fibroblasts (Wright *et al.*, 1988) revealed biphasic formation of DAG, with only the first phase associated with the generation of Ins(1, 4, 5)P<sub>3</sub>. A similar profile of DAG generation was also reported in AII- and endothelin-stimulated smooth muscle cells (Griendling *et al.*, 1986; Sunako *et al.*, 1989), vasopressin-stimulated fibroblasts (Huang & Cabot, 1990a) and bombesin-stimulated Swiss 3T3 fibroblasts (Cook *et al.*, 1990). Thus, dissociation of Ins(1, 4, 5)P<sub>3</sub> formation from DAG generation has been observed in multiple cell types and has questioned the nature and source of the sustained phase of DAG generation.

The measurement of [<sup>3</sup>H]-water soluble metabolites released from prelabelled phospholipids allowed the identification of PtdCho as the putative source of this DAG in a number of cell types upon receptor activation (Besterman *et al.*, 1986a; Cabot *et al.*, 1988; Cook & Wakelam, 1989; Martin & Michaelis, 1989). DAG molecular species analysis has further pointed to PtdCho as the phospholipid source; however, this did not identify unequivocally the mechanism by which the DAG was generated following receptor stimulation. Potentially a PLC- or phospholipase D (PLD) could have catalysed the reaction.

By means of combined DAG derivatisation and gas liquid chromatography techniques Pessin & Raben (1989) described the acyl chain content of different DAGs present at different time points during  $\alpha$ -thrombin stimulation in IIC9 fibroblasts. The DAG generated during the early initial transient phase (at 15 secs) displayed molecular species with acyl content similar to that of the phosphoinositides which reflected the activation of PtdIns-PLC following receptor activation. During the sustained phase (5min-1h) these appeared to be similar to the acyl chain profile of PtdCho in unstimulated cells, consistent with the observed release of [ $^3$ H]-choline from these cells. Based upon this comparison, these workers proposed that PtdCho served as the putative source of this sustained DAG. EGF and PDGF stimulated sustained DAG generation also apparently derived from PtdCho (Pessin & Raben, 1989).

In fMet LeuPhe-stimulated neutrophils biphasic DAG generation also occurred and corresponded to initial phosphoinositide hydrolysis and further sustained phospholipid hydrolysis (Thompson *et al.*, 1990). When measured quantitatively, the amount of DAG formed during the sustained secondary phase was similar to the amount of free choline generated. This suggested that all of this DAG generated was purely PtdCho derived.

Extensive molecular species analysis of DAGs generated upon agonist stimulation in a wide variety of cells have been conducted. In general, these have concluded that the DAG of the initial early phase contains fatty acids predominantly found in the phosphoinositides and the second phase those predominantly found in PtdCho (see Besterman *et al.*, 1986a; Bocchino *et al.*, 1987a; Kennerly, 1987; Pessin *et al.*, 1990; Huang & Cabot, 1990a; Van Blitterswijk *et al.*, 1991; Pettitt & Wakelam, 1993). Although these studies represent the identification of PtdCho as a major alternative source of DAG it is important to emphasise that other phospholipids such as PtdEtn have been reported to be sensitive to phospholipase-catalysed hydrolysis (Kiss & Anderson, 1990) and may therefore also contribute to the observed sustained phase.



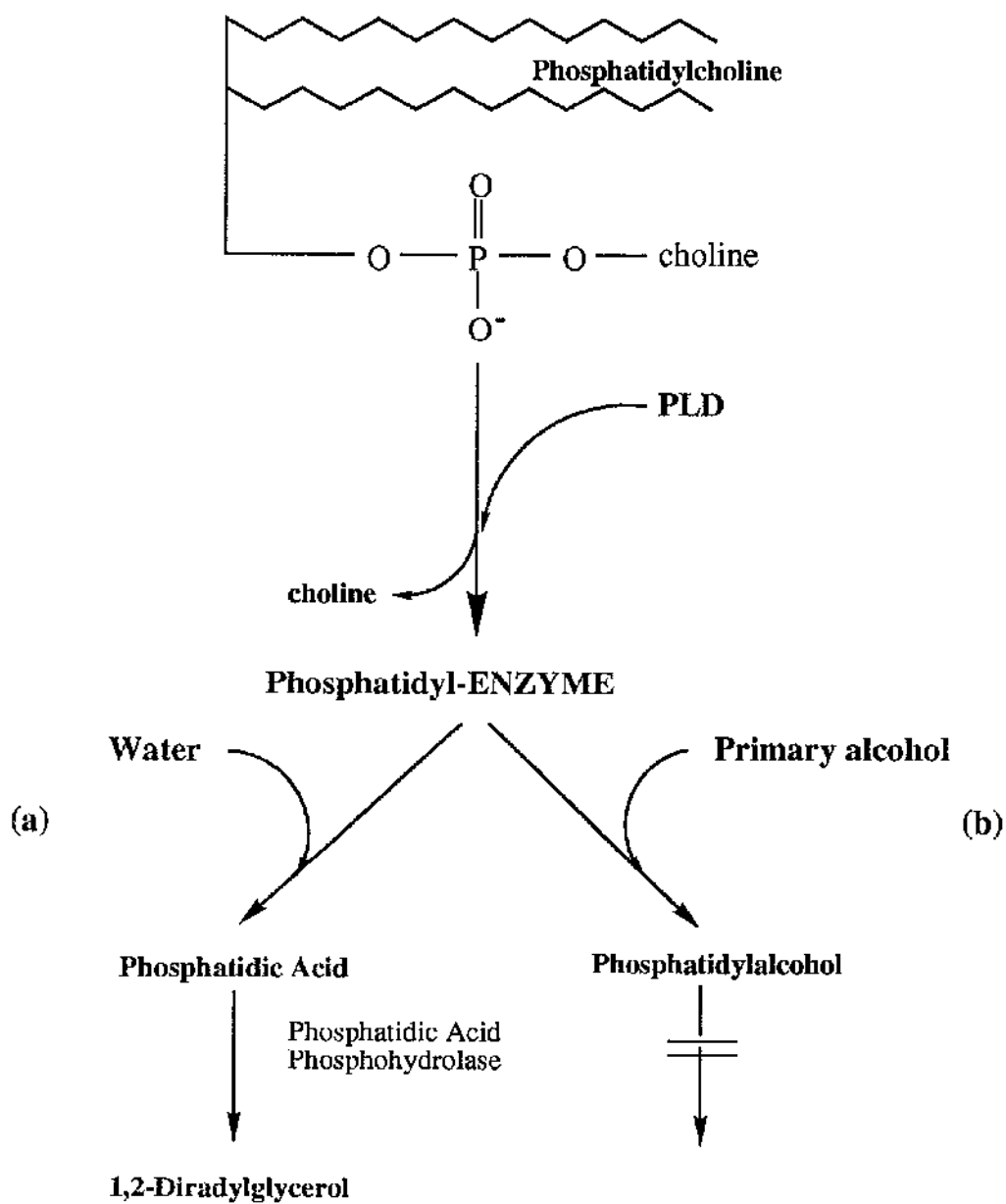
**Fig 1.1 Schematic representation of PtdCho and the potential sites for cleavage catalysed by hydrolytic phospholipases PLA<sub>2</sub>, PLC and PLD. R<sub>1</sub> and R<sub>2</sub> represent the hydrocarbon chains of fatty acids.**

### 1.3 Receptor-Mediated Phosphatidylcholine Hydrolysis Catalysed by Phospholipase D.

In mammals, PtdCho can be metabolised by a number of different phospholipases including PLA<sub>2</sub>, PLC and PLD (see Fig 1.1). PLA<sub>2</sub> enzymes are active against the fatty acid at the *n*-2 position of the phospholipid glycerol backbone. PtdCho-specific PLC catalyses the formation of DAG and phosphocholine directly. A PLC catalysed hydrolytic event may be the predominant pathway by which sustained DAG generation occurs. However, since PtdOH can be rapidly dephosphorylated by phosphatidate phosphohydrolase (PAP), this hydrolytic pathway could serve as an alternative pathway of sustained DAG formation (Fig 1.1). This has been investigated further by examination of the time courses of the formation of both PtdCho-derived lipid molecules and their water soluble co-products. However, as the PtdCho-derived molecules are substrates for PAP, DAG kinase and choline kinase enzymes (see Pelech & Vance, 1989) careful interpretation of their formation and interconversion must be made. PtdCho-hydrolysis would appear to occur predominantly by means of a PLD-catalysed reaction though evidence is apparent for receptor-stimulated PtdCho-PLC activation (see below).

In neutrophils stimulated with fMetLeuPhe, phorbol 12-myristate 13-acetate (PMA) or Ca<sup>2+</sup> ionophore it was reported that not only *sn*-1, 2-diacylglycerol is formed, but also *sn*-1-*O*-alkyl-2-acylglycerol (Agwu *et al.*, 1989; Tyagi *et al.*, 1989). Examination of the 'parent' PtdCho showed that *sn*-1-*O*-alkyl-2-acylglycerophosphocholine constitutes up to 70% of the total PtdCho pool, a characteristic common to other haematopoietic cells (eosinophils and macrophages, Mueller *et al.*, 1982; Ojima-Uchiyama *et al.*, 1988). By labelling of the PtdCho pool specifically with 1-*O*-alkyl-2-lyso-[<sup>32</sup>P]PtdCho without incorporating [<sup>32</sup>P] into the cellular ATP pool the subsequent observation of 1-*O*-alkyl[<sup>32</sup>P]PtdOH formation has identified agonist-stimulated PLD activation in haematopoietic cells (see Billah *et al.*, 1989a & b; Agwu *et al.*, 1989; Billah & Anthes, 1990).





**Fig 1.2 Schematic representation of PLD-catalysed hydrolytic and transphosphatidylation reactions:** (a) the coupled PLD/phosphatidic acid phosphohydrolase pathway and (b) the transferase pathway responsible for formation of phosphatidylalcohols in the presence of primary aliphatic alcohols.

By direct labelling of cells with [ $^{32}\text{P}$ ]-phosphate, receptor-stimulated PtdOH formation has been observed. The specific activity of the [ $^{32}\text{P}$ ]PtdOH formed upon fMetLeuPhe stimulation of neutrophils was significantly less than the cellular ATP pool (Cockcroft, 1984) and therefore argued against the formation of PtdOH as a result of DAG kinase-catalysed reaction. If this was indeed true then the source of PtdOH was from PLD catalysed phospholipid hydrolysis. It must be noted in this example that the observation of [ $^{32}\text{P}$ ]PtdOH formation represents total agonist-stimulated PLD activity and is not necessarily restricted to PtdCho.

Evidence of receptor-regulated PLD activation has been demonstrated by exploitation of a catalytic property unique to this phospholipase. Dawson (1967) described the ability of PLD to catalyse the formation of phosphatidylalcohols in the presence of short chain aliphatic primary alcohols by means of a reaction termed transphosphatidylolation. Formation of these molecules in an agonist-dependent manner is at the 'expense' of PtdOH, whereby the primary alcohols rather than water act as the preferential nucleophilic acceptors (see Fig 1.2). This feature of PLD activation was utilised in the initial identification of agonist-stimulated PLD activity in mammalian cell types (Bocckino *et al.*, 1987a & b; Tetterborn & Mueller, 1988; Liscovitch, 1989; Liscovitch & Amsterdam, 1989). Phosphatidylalcohols are poor substrates for PtdOH phosphohydrolase (PAP) (Metz & Dunlop, 1991) and therefore accumulate within the cell. PLD-catalysed formation of these compounds has been measured by either the incorporation of radiolabelled primary alcohols into unlabelled phosphatidyl moieties or incorporation of unlabelled primary alcohol into previously labelled phosphatidyl moieties. This has involved the use of [ $^3\text{H}$ ]butan-1-ol in neutrophils or cell free fractions (eg. Randall *et al.*, 1990; Horwitz & Davis, 1993) or metabolic labelling of PtdCho with saturated fatty acids such as palmitate (eg. Martin & Michaelis, 1989, Cook *et al.*, 1991). As previously described, cells of haematopoietic origin have also been successfully labelled with 1-*O*-alkyl-2-lyso-[ $^3\text{H}$ ]PtdCho or 1-*O*-alkyl-2-lyso-[ $^{32}\text{P}$ ]PtdCho which is preferentially incorporated into PtdCho. This has allowed observation of both [ $^3\text{H}$ ]- and [ $^{32}\text{P}$ ]-labelled phosphatidylalcohol formation in the

presence of ethanol in a number of studies (Pai *et al.*, 1988a & b; Anthes *et al.*, 1989, 1991; Billah *et al.*, 1989a & b). Hence, formation of these compounds has served as both a selective and definitive assay for agonist-dependent PLD activation *in vivo*.

The use of primary alcohols to 'trap' phosphatidyl moieties, thereby inhibiting PtdOH formation and sequential DAG formation by the PLD/PPH pathway, permits the relative contribution of PLD-derived PtdOH to the sustained phase of DAG production and a number of physiological responses to be determined. For instance, following pretreatment with 30mM butanol, DAG formation in bombesin-stimulated Swiss 3T3 fibroblasts and vasopressin-stimulated A10 vascular smooth muscle cells DAG formation were inhibited 30 and 40-50% respectively (Cook *et al.*, 1991; Plevin & Wakelam, 1992). This has questioned the contribution of PLD-catalysed PtdCho hydrolysis to the sustained phase of DAG generation and focused attention upon other alternative phospholipid sources of DAG.

#### **1.4 Regulation of Receptor-Mediated Phosphatidylcholine-Phospholipase D Activity.**

The regulation of agonist-stimulated PLD activation involves multiple mechanisms. A number of receptor agonists activate a number of phospholipases including PtdIns-PLC, PLA<sub>2</sub> and PLD. In a number of instances observation of PLD activity requires activation of PtdIns-PLC (reviewed by Billah & Anthes, 1990; Thompson *et al.*, 1993; Exton, 1994). This has been interpreted as the requirement for Ins(1, 4, 5)P<sub>3</sub> and DAG generation responsible for concomitant increases in intracellular Ca<sup>2+</sup> release and PKC activation; both responses are relevant to the modulation of PLD activation. Modulation by GTP-binding proteins and a number of protein kinases have also been extensively detailed in the regulation of receptor-stimulated PLD activation (reviewed by Billah & Anthes, 1990; Exton, 1994) and are examined below.

#### 1.4.1 Calcium dependence.

The role of  $\text{Ca}^{2+}$  ions in the regulation of receptor-mediated PLD activation has been examined by the use of  $\text{Ca}^{2+}$  ionophores and the chelation of extracellular  $\text{Ca}^{2+}$ . Addition of the  $\text{Ca}^{2+}$ -ionophore A23187 to a number of cell types results in extracellular  $\text{Ca}^{2+}$ -mediated PLD activation while chelation of extracellular  $\text{Ca}^{2+}$  could attenuate this to varying degrees (Pai *et al.*, 1988a & b; Billah *et al.*, 1989a; Cook *et al.*, 1991; Huang *et al.*, 1991, Llahi & Fain, 1992).

Loading of cells with the  $\text{Ca}^{2+}$  chelator BAPTA has also demonstrated the inhibition of agonist-stimulated PLD activation in a number of cell types; eg  $\text{PGE}_2$ -stimulated HEL cells (Wu *et al.*, 1992b), suggesting that mobilisation of intracellular  $\text{Ca}^{2+}$  is important in agonist-stimulated PLD activation. Use of BAPTA in combination with chelation of extracellular  $\text{Ca}^{2+}$  completely inhibited fMetLeuPhe-stimulated PLD activity which indicated that modulation of both intracellular and extracellular  $\text{Ca}^{2+}$  concentrations directly affects receptor-stimulated PLD activation. The involvement of intracellular  $\text{Ca}^{2+}$  mobilisation in this role has been examined further by the use of the tumour promoter thapsigargin. This acts as a  $\text{Ca}^{2+}$ -ATPase inhibitor (Thastrup *et al.*, 1990) and prevents refilling of the  $\text{Ins}(1, 4, 5)\text{P}_3$ -sensitive  $\text{Ca}^{2+}$ -store. However, thapsigargin added at concentration that resulted in the elevation of  $[\text{Ca}^{2+}]_i$  did not stimulate PLD activation and had no effect upon the dose dependent activation of PLD stimulated by bombesin in Swiss 3T3 fibroblasts (Cook *et al.*, 1991).

Therefore, an obvious question that remains to be addressed is whether  $\text{Ca}^{2+}$  exerts its affect upon PLD itself or plays a contributory role in modulation of the upstream activators of PLD. In cell free experiments with neutrophil membrane and cytosolic fractions, *in vitro* PLD activation required  $\text{Ca}^{2+}$  concentrations of greater than 100nM (Olson *et al.*, 1991). This is consistent with  $[\text{Ca}^{2+}]_i$  in stimulated cells, however, due to the protein heterogeneity of the cellular fractions utilised in this study it remains unclear if the effect of  $\text{Ca}^{2+}$  is upon the enzyme directly.

In examining the site of  $\text{Ca}^{2+}$  ion involvement, A23187-stimulated PLD activation in Swiss 3T3 fibroblasts can be partially inhibited by pretreatment with the selective PKC inhibitor Ro-31-8220 (Cook *et al.*, 1991). Additionally, the inhibition of bombesin-stimulated PLD activation by EGTA and Ro-31-8220 in combination were less than additive suggesting they exerted their effects at a common site. This may signify that the requirement for  $\text{Ca}^{2+}$  in PLD activation is as a cofactor for PKC activation, PKC activation being required for bombesin-stimulated PLD activation in these cells (Cook *et al.*, 1991).

Therefore, the modulation of receptor-stimulated PLD activity by  $\text{Ca}^{2+}$  does not appear to be by a universal mechanism. PLD activation may be influenced by the mobilisation of intracellular  $\text{Ca}^{2+}$  and entry of extracellular  $\text{Ca}^{2+}$  into the cell. However, it remains to be clarified whether  $\text{Ca}^{2+}$  activates components of the pathway regulating PLD or whether it activates PLD directly.

#### 1.4.2 Regulation by PKC.

It has been extensively reported that addition of phorbol esters to a variety of cell types results in PLD activation, implicating PKC in enzyme regulation (Ben-Av & Liscovitch, 1989; Billah *et al.*, 1989b; Cabot *et al.*, 1989; Hii *et al.*, 1989; Huang & Cabot, 1990b; Balsinde & Mollinedo, 1991; Cook *et al.*, 1991; Plevin *et al.*, 1991; Llahi & Fain, 1992; Plevin & Wakelam, 1992). Consequently, activation of PLD by phorbol esters has been demonstrated to be sensitive to a number of inhibitors of PKC (Llahi & Fain, 1992; Liscovitch, 1989; Gustavsson & Hansson, 1990; Metz & Dunlop, 1990; Sandman & Wurtman, 1991; Cook *et al.*, 1991). However, in a number of cases agonist-stimulated PLD activation has appeared insensitive to these compounds. In HL-60 cells, fMetLeuPhe-stimulated PLD activity was only partially inhibited by pretreatment with the PKC inhibitor K252a (Billah *et al.*, 1989b), staurosporine did not inhibit endothelin-stimulated PLD activity in rat aortic preparations and noradrenaline-stimulated PLD activation in rat cerebral cortex (Liu *et al.*, 1992; Llahi & Fain, 1992) and the selective PKC inhibitor, Ro-31-8220,

failed to inhibit PLD activation in fMetLeuPhe-stimulated neutrophils (Uings *et al.*, 1992), EGF-stimulated Swiss 3T3 fibroblasts (Cook & Wakelam, 1992) and  $\alpha_2$ -adrenergic stimulation of fibroblasts (MacNulty *et al.*, 1992). Thus, the requirement for PKC in agonist-stimulated PLD activation is not universal.

The involvement of PKC has been examined further by modulation of cellular PKC expression, relying on chronic exposure of cells to high doses of phorbol ester to downregulate DAG-sensitive PKC isotypes (Ways *et al.*, 1992). This approach has illustrated the inhibition of PLD activation in a range of cell types in response to a variety of agonists or re-addition of phorbol ester; PMA and endothelin-1 in Rat-1 fibroblasts (MacNulty *et al.*, 1990); PMA, bombesin, PDGF and vasopressin in Swiss 3T3 fibroblasts (Cook & Wakelam, 1991; Plevin *et al.*, 1991); bradykinin in bovine pulmonary artery endothelial cells (Martin *et al.*, 1989) and PMA and gonadotrophin-releasing hormone in ovarian granulosa cells (Liscovitch & Amsterdam, 1989). By use of differential down regulation protocols, individual isotypes of PKC have been suggested to regulate PLD activation. Rat renal mesangial cells treated with PMA for 8h showed no loss of PLD activity upon addition of PMA, despite complete downregulation of PKC- $\alpha$ , and  $\delta$ , but not PKC- $\epsilon$  (Huwiler *et al.*, 1991; 1992; 1993). Stimulated PLD activity could only be blocked by 24h pretreatment with PMA, consistent with PKC- $\epsilon$  downregulation in these cells (Huwiler *et al.*, 1993). Under these conditions, PKC- $\zeta$  expression remained unchanged as expected (Ways *et al.*, 1992). In view of this, PKC- $\epsilon$  was proposed to be the main regulator of PLD activation. Further, ATP- and UTP-stimulated PLD activity appeared to be regulated by PKC- $\epsilon$  in a similar manner (Pfielschifter & Merriweather, 1993).

The involvement of a PKC typical of the  $\text{Ca}^{2+}$ -independent isoforms may explain the observed insensitivity to a number of the commonly used PKC inhibitors described earlier. Compounds such as staurosporine and K252a have been reported to show selectivity towards  $\text{Ca}^{2+}$ -dependent isoforms (McGlynn *et al.*, 1992; Gschwendt *et al.*, 1989), however, it is still apparent that PLD activation may occur by PKC-independent mechanisms (discussed later). In contrast to rat mesangial cells, PKC- $\epsilon$

has been suggested to play only a minor role in the regulation of PLD activity in C3H/10T1/2 fibroblasts (Kiss & Anderson, 1994). Selective downregulation of PKC- $\epsilon$  with carcinogenic compounds over 24 h had no effect upon the expression of the other PKC isoforms present in these cells ( $\alpha$ ,  $\delta$  and  $\zeta$ ). Under these conditions both PMA- and PDGF-stimulated PLD activity remained unaltered.

Similar intervention at the level of PKC isoforms, demonstrated that incubation of mouse epidermal (HEL-37) and human skin (SF 3155) fibroblasts with sphingomyelin-derived ceramide specifically inhibited membrane-association of the PKC- $\alpha$  isoform and PLD activation in response to bradykinin (Jones & Murray, 1995). Further, supplementation of a related fibroblastic cell type (SF 3271) with 1-*O*-hexadecylglycerol, increasing membrane ether lipid content and decreasing PKC- $\alpha$  translocation, prevented bradykinin-stimulated PLD activation (Clark & Murray, 1995). Thus, a common role for PKC- $\alpha$  in the regulation of agonist-stimulated PLD activation may exist in these fibroblasts.

Regulation of PLD activity has also been examined following the overexpression of individual PKC isoforms in a variety of cell types. PMA and endothelin-1 stimulated PLD activity in Rat-6 fibroblasts over-expressing PKC- $\beta_1$  was observed to be markedly elevated (Pai *et al.*, 1991a & b). The effect of PKC- $\alpha$  overexpression upon PLD activation in Swiss 3T3 fibroblasts has been examined and the authors concluded that while acute PLD activation was not affected, the actual expression of PLD itself may be upregulated by PKC- $\alpha$  overexpression (Eldar *et al.*, 1993). Further, they proposed PKC- $\epsilon$  may mediate PLD activation in these cells as PKC- $\beta_1$  is not normally expressed in mouse fibroblasts. This suggestion contrasts with the minor 1.5-fold increase in PMA-induced PLD activation in NIH3T3 fibroblasts overexpressing PKC- $\epsilon$  some 15-fold (Kiss & Garamszegi, 1993).

Regulation of PLD activation by PKC has also been investigated by addition of cytosolic extracts and individually purified isoforms of PKC to isolated membrane preparations. Conricode *et al.* (1992) originally described PMA-stimulated PLD activity in CCL39 fibroblast membranes, but only in the presence of cytosol, partially

purified PKC or purified PKC from this fraction. In contrast to a number of other cell types (see Section 1.4.2), this activation did not require ATP-dependent phosphorylation, as apyrase-catalysed ATP depletion was without effect. This suggested that PKC may interact with PLD directly and potentially activate it by an allosteric mechanism. Consequently, these workers described the ability of the conventional PKC's,  $\alpha$  and  $\beta$ , purified from rat brain to activate PLD in these membranes, in either a PMA- or  $\text{Ca}^{2+}$ -dependent manner (Conricode *et al.*, 1994). Moreover, rat brain PKC- $\gamma$  and recombinant PKC-  $\delta$ ,  $\epsilon$  and  $\zeta$  were all ineffective at substituting in this role. Immunoblot analysis of the soluble fraction confirmed the presence of only  $\alpha$  and  $\zeta$  isoforms, however, recombinant PKC- $\beta_1$  was also able to activate PLD in these membranes. Therefore, PKC's  $\alpha$  and  $\beta$  were identified as activators of PLD.

It would appear that both  $\text{Ca}^{2+}$ -dependent and independent isoforms of PKC are involved in the regulation of PLD activation. Agonists that stimulate  $\text{PtdIns}(4, 5)\text{P}_2$  hydrolysis prior to PLD activation may utilise  $\text{Ca}^{2+}$ -dependent PKC isoforms activated by  $\text{PtdIns}(4, 5)\text{P}_2$ -derived DAG and concomitant intracellular  $\text{Ca}^{2+}$  release. In addition, multiple PLD isoforms may exist and their activities may be modulated by different PKC isoforms in an agonist and cell specific manner. It remains to be determined if activation occurs by phosphorylation-dependent and/or independent mechanisms, as inhibitors of PKC that are active competitors at the enzyme ATP binding site, are known to inhibit PLD activation (see earlier text).

#### 1.4.3 Involvement of GTP-Binding Proteins.

G-protein involvement in the regulation of stimulated PLD activity were illustrated by the differing sensitivity to pertussis-toxin pretreatment and activation of PLD with non-hydrolysable GTP analogues.

Pertussis-toxin pretreatment has been shown to completely block PLD activity in stimulated granulocytes (Pai *et al.*, 1988b; Kanaho *et al.*, 1991; Agwu *et al.*, 1989) and pretreatment also inhibited  $\alpha_2$ -stimulated PLD activation in  $\alpha_2$ -adrenoceptor



transfected fibroblasts (MacNulty *et al.*, 1992). In the latter study ADP-ribosylation of both G<sub>12</sub> and G<sub>13</sub> by toxin pre-treatment corresponded with impaired receptor stimulated GTPase activity and PLD activity. In this instance, it was also reported that no significant  $\alpha_2$ -stimulated Ins(1, 4, 5)P<sub>3</sub> generation occurred and suggested that PLD activation was regulated by direct G-protein/PLD effector coupling and not as a downstream consequence of PLC activation. This mechanism of regulation is discounted in granulocytes as PLC, like PLD, is sensitive to toxin pretreatment (Verghese *et al.*, 1985). In hepatocytes, Bocckino *et al.* (1987a & b) reported that activation of PLD was not impaired by pertussis toxin pre-treatment and could be observed upon addition of GTP $\gamma$ S to membrane fractions prepared from these cells, suggesting the possible involvement of a G-protein not of the pertussis-sensitive G<sub>i</sub> or G<sub>o</sub> class and distinct from that in fibroblasts.

Extensive evidence for G-protein involvement in PLD activation has been based upon permeabilised cell systems or broken cell fractions and the introduction of non-hydrolysable GTP and GDP analogues. In electroporated HL-60 cells, sustained PLD activation and phosphatidylethanol accumulation was observed upon addition of GTP $\gamma$ S. Further, this accumulation displayed Mg<sup>2+</sup>-dependence and also required ATP, though ATP could not substitute for GTP $\gamma$ S (Xie & Dubyak, 1991). Similar potentiation of GTP $\gamma$ S-stimulated PLD activity has been observed in permeabilised NG108-15 hybridoma cells (Liscovitch & Eli, 1991). Moreover, adenine nucleotide-stimulated hydrolysis of PtdEtn in isolated membranes of NIH 3T3 fibroblasts was potentiated by the addition of GTP $\gamma$ S, while nonhydrolysable analogues of ATP failed to activate PLD even in the presence of GTP (Kiss & Anderson, 1990). In broken cell fractions, particularly membrane preparations, GTP $\gamma$ S also stimulates PLD activity; this has been documented in synaptosomes and microsomal membranes from canine brain (Qian & Drewes, 1989; Qian *et al.*, 1990), hepatocyte plasma membranes as described above, rabbit platelet plasma membranes (Van Der Meulen & Haslam, 1990) and neutrophil preparations (Anthes *et al.*, 1989, Olson *et al.*, 1990).

In addition the activation of the PLC $\beta_2$  isoform by  $\beta\gamma$  subunits of pertussis-toxin sensitive heterotrimeric G-proteins (Camps *et al.*, 1992; Katz *et al.*, 1992; see Section 1.1) may indicate a possible means of PLD regulation and activation. This has been examined, whereby both  $\alpha$  and  $\beta\gamma$  subunits were added to neutrophil subcellular fractions (Bowman *et al.*, 1993). No modulation of PLD activity was noted; however, it still remains a possible means of activation in other cell types and requires further investigation.

#### 1.4.4 PKC-Independent Mechanisms.

An observed potentiation of GTP $\gamma$ S-stimulated PLD activity in the presence of ATP has been described in a number of permeabilised cell systems (see earlier text). Although ATP may interact and mediate a number of signalling events via interaction with its own receptor it has been suggested that provision of ATP allows the completion of a kinase event required for PLD activation. The involvement of both  $\text{Ca}^{2+}$  and particularly PKC (see above) in the regulation of receptor-stimulated PLD activation has suggested that the upstream activation of PLC is obligatory. However, in a number of studies PLD activation occurs in the absence of PLC-catalysed PtdIns(4, 5)P $_2$  hydrolysis (Cook & Wakelam, 1992; MacNulty *et al.*, 1992; Ahmed *et al.*, 1994). The pathways by which PLD activation occurs in the absence of PtdIns-PLC activity have been partly elucidated. Involvement of receptor tyrosine kinases has been suggested in EGF-stimulated Swiss 3T3 fibroblasts and basic FGF-stimulated vascular endothelial cells (Cook & Wakelam, 1992; Ahmed *et al.*, 1994), where agonist-stimulated PLD activation was sensitive to tyrosine kinase inhibitor compounds. In fMetLeuPhe-stimulated human neutrophils, PLD activation was also sensitive to tyrosine kinase inhibitors though the structural nature of the fMetLeuPhe receptor would suggest the involvement of non-receptor tyrosine kinases (Uings *et al.*, 1992). Additionally, tyrosine kinase inhibitors were ineffective against phorbol ester-stimulated PLD activation suggesting they act proximal to PLD itself. Moreover, fMetLeuPhe-stimulated tyrosine phosphorylation of target proteins was

attenuated while fMetLeuPhe-stimulated PLC activation was unaffected and was clearly dissociated from PLD activation (Uings *et al.*, 1992). Consequently, an alternative pathway to PLD activation must exist and is not simply as a result of PtdIns(4, 5)P<sub>2</sub> hydrolysis. Similar observations have been made in endothelin-stimulated A10 vascular smooth muscle cells (Wilkes *et al.*, 1993) and the putative involvement of tyrosine kinase activity has been strengthened by the observed parallel increases in tyrosine phosphorylation and PLD activation upon treatment of cells with tyrosine phosphatase inhibitor pervanadate (Bourgoin & Grinstein, 1992; Uings *et al.*, 1992; Dubyak *et al.*, 1993). In contrast, the role of receptor stimulated tyrosine kinase activity in the regulation of PLD activity in Swiss 3T3 cells is only a minor one (Briscoe *et al.*, 1995). Therefore, the involvement and relative contributions of tyrosine kinases in the regulation of PLD activation may be cell type specific.

The regulation of PLD activation by the cAMP-dependent protein kinase (PKA) pathway has been reported; however, this varied in a cell type specific manner. Attenuation of fMetLeuPhe-stimulated PLD activity occurs in human neutrophils pretreated with cAMP-elevating agents (Agwu *et al.*, 1991a; Tyagi *et al.*, 1991). In contrast, human endothelial cells treated with similar agents display enhanced thrombin-stimulated PLD activity consistent with cholera toxin pretreatment of the same cells (Garcia *et al.*, 1992). However, modulation of the PKA pathway has no effect upon bombesin-stimulated PLD activity in Swiss 3T3 fibroblasts (Cook *et al.*, 1991). Therefore, there is no clear universal mechanism for regulation of the PLD pathway by PKA, though, in neutrophils intervention with the agents described above did not affect phorbol ester-stimulated PLD activity. Hence, PKA regulation in this situation may function as part of another converging pathway or be distal to the activation of PKC.

Thus, the identification of the multiple kinases involved and their spatial placement in the signalling cascades responsible for PLD activation are unknown and requires further investigation. This may rely upon reconstitution studies with purified proteins.

### **1.5 A Summary of The Regulation of Receptor-Mediated Phospholipase D Activation.**

Evidence now exists for the involvement of multiple effector molecules in the regulation of receptor-stimulated PLD activity. Further clarification of G-protein involvement in PLD activation is required and whether it is purely restricted to PtdIns(4, 5)P<sub>2</sub> hydrolysis as a prerequisite to PLD activation by defined agonists in defined cell types. PKC and Ca<sup>2+</sup> ions also differentially contribute to the regulation of stimulated PLD activation, however, no universal mechanism is apparent. PLD activities, in common with PLC and PKC isoforms, displays varied cytosolic and membrane localisation (see Section 1.11.1). It is unclear whether receptor-mediated activation involves the translocation of cytosolic PLD to a membrane location for full activation or if it is already membrane-associated for activation and requires the translocation of other regulatory components such as PKC and/or protein kinases. PLD activities may display characteristics similar to other phospholipases such as PtdIns-PLC or PtdCho-PLA<sub>2</sub> which are known to associate with the plasma membrane in a Ca<sup>2+</sup>-dependent fashion (Channon & Leslie, 1990; Clark *et al.*, 1991). Elucidation of the contribution of these various effector molecules may rely upon reconstitution studies with purified proteins. In addition the generation of specific anti-PLD antisera may allow immunological examination of agonist specific changes in PLD subcellular localisation in both non-activated and activated cells.

### **1.6 Phospholipase C Catalysed Hydrolysis of Phosphatidylcholine.**

Where PtdCho-PLD activation is rapidly desensitized it has been documented that prolonged phosphocholine production is observed (Cook *et al.*, 1991; McKenzie *et al.*, 1992; Plevin & Wakelam, 1992; Briscoe *et al.*, 1994). This has implicated a PtdCho-PLC pathway as contributing to sustained generation of DAG. Indeed, in a number of cell types the contribution of this pathway may be the major source of DAG (Besterman *et al.*, 1986a; Cook *et al.*, 1991; Purkiss *et al.*, 1991; Schutze *et al.*, 1992). More recent studies of the PtdCho-derived DAG species from EGF-stimulated Swiss

3T3 fibroblasts has suggested the presence of a potential PtdCho-PLC catalysed pathway (Pettitt & Wakelam, 1994).

For many growth factors and G-protein-coupled agonists, generated water soluble metabolites of PtdCho have been examined. PLD activation and subsequent choline generation were observed to precede phosphocholine production (eg Cook & Wakelam, 1991; Plevin & Wakelam, 1992; see Billah & Anthes, 1990; Thompson *et al.*, 1993). This may reflect metabolism of choline by choline kinase as part of PtdCho resynthesis. The proposal of agonist-stimulated PtdCho-PLC activity has relied upon the elucidation of the temporal relationship between the production of these two water-soluble PtdCho metabolites.

The observed stimulation of prolonged phosphocholine production in PDGF-stimulated Swiss 3T3 fibroblasts (Larrodera *et al.*, 1990) instigated a series of studies by Moscat and co-workers investigating the contribution of a putative PtdCho-PLC catalysed hydrolytic pathway to mitogenic signalling (Cai *et al.*, 1993 and references therein). This utilised either the addition of an exogenous bacterially-derived PtdCho-PLC from *Bacillus cereus* directly to cells or transfection of these cells with an expression plasmid containing the phospholipase. Although sustained DAG and phosphocholine generation and a number of cellular effects were observed it remains debatable what relevance these results have to the elucidation of mammalian signal transduction cascades. It remains unclear what similarities, if any, the bacterial protein shares with the putative mammalian form(s).

Despite this, PtdCho-PLC activities have been identified and isolated from a number of mammalian sources. Neutral activities displaying PtdCho substrate specificity have been partially purified from heart cytosol (Wolf & Gross, 1985), bull seminal plasma (Sheikhnejad & Srivastava, 1986) and U937 cells (Clark *et al.*, 1986). The heart cytosol and U937-derived enzymes appeared to be sensitive to millimolar concentrations of divalent cations whereas the seminal plasma enzyme appeared to be unaffected by divalent cations. This activity was further characterised and displayed an

apparent molecular weight of 125kDa, consisting of 69 and 55 kDa subunits (Sheikhnejad & Srivastava, 1986).

The relationship between these cell free activities and putative agonist-stimulated PtdCho-PLC remains unknown. Therefore, the existence of mammalian PtdCho-PLC activities intrinsic to signal transduction remains speculative and awaits the isolation of an enzyme activity sensitive to agonist stimulation.

### **1.7 Phospholipase A<sub>2</sub> Catalysed Hydrolysis of Phosphatidylcholine.**

The hydrolysis of PtdCho can also occur by PLA<sub>2</sub> catalysed hydrolysis, generating free fatty acid and lysophospholipid (see Fig 1.1). There are presently four cellular PLA<sub>2</sub> activities which have either been purified or whose corresponding cDNA have been cloned and sequenced (Glaser *et al.*, 1993; Dennis, 1994). One activity is a 30kDa activity and has been characterised as a member of the 14.3.3 protein family (Zupan *et al.*, 1992). A 40 kDa Ca<sup>2+</sup>-independent activity has also been purified from canine heart (Hazen & Gross, 1993). However, the two forms of PLA<sub>2</sub> activity that have been best characterised are the low molecular weight (12-20kDa) secreted isoforms of PLA<sub>2</sub>, designated Groups I-III and the high molecular weight (approximately 85kDa) cytosolic activities termed cPLA<sub>2</sub> (Group IV) (Dennis, 1994).

The extracellular Group I-III forms are present in secretory granules, snake and bee venoms, human synovial fluid and pancreatic secretions (see Dennis, 1994 for summary). Cytosolic PLA<sub>2</sub> has been purified from a number of sources including and human promyolecytic U937 cells (Clark *et al.*, 1990; Kramer *et al.*, 1991; Wijkander & Sundler, 1989). The cPLA<sub>2</sub> from U937 cells was sequenced and the cDNA subsequently cloned (Sharp *et al.*, 1991; Clark *et al.*, 1991). Expression of the cDNA for this activity in fibroblastic cells demonstrated that this form that was responsive to agonist stimulation of cells (Lin *et al.*, 1992; Lin *et al.*, 1993). The PLA<sub>2</sub> been further characterised to translocate to membranes in a calcium dependent manner. This is by virtue of its N-terminal Ca<sup>2+</sup>-dependent phospholipid binding domain (Clark *et al.*,

1991) common to a number of signal transduction proteins, including isoforms of PKC (Hug & Sarre, 1993) and PLC $\gamma$  (see Rhce, 1991).

Growth factor receptors and G-protein coupled receptors have been shown to stimulate and regulate cPLA<sub>2</sub> (see Dennis, 1994; Exton, 1994). Recently, studies have revealed that the regulation of cPLA<sub>2</sub> involves changes in [Ca<sup>2+</sup>]<sub>i</sub> mediated by PLC activation and phosphorylation of the cPLA<sub>2</sub> polypeptide. Phospholipase A<sub>2</sub> has been suggested to be directly coupled and regulated by heterotrimeric G-proteins (Glaser *et al.*, 1993). Consistent with this are extensive reports of the ability of GTP analogues to stimulate arachidonic acid release or enhance agonist-stimulated release in a wide variety of cells (see Exton, 1994). These events can also be inhibited by GDP analogues and, in some cases, blocked by pertussis toxin (see Exton, 1994). The  $\beta\gamma$  subunit of heterotrimeric G-proteins has also been proposed to regulate cPLA<sub>2</sub>. When the  $\beta\gamma$  subunits of transducin were added to membranes cPLA<sub>2</sub> activity was markedly stimulated (Jelsema & Axelrod, 1987). Similarly, the use of antiserum against  $\beta\gamma$  subunits have been shown to inhibit cPLA<sub>2</sub> activation by histamine and thrombin in permeabilised platelets (Murayama *et al.*, 1990). However, antisera raised against G<sub>i</sub> and G<sub>o</sub> inhibited both agonist-stimulated events. Differential involvement of heterotrimeric G-proteins in agonist-stimulated cPLA<sub>2</sub> activation has been suggested by studies utilising antisera raised against different heterotrimeric G-proteins and their constituent subunits. Antiserum against  $\alpha$  and  $\beta$  subunits of G<sub>o</sub> inhibited agonist stimulated cPLA<sub>2</sub> activity in platelet membranes (Kajiyama *et al.*, 1990). However, antiserum to G<sub>i</sub> inhibited noradrenaline-stimulated activity but not that of thrombin stimulation (Kajiyama *et al.*, 1990). Further re-addition of G<sub>o</sub> and G<sub>i</sub> proteins to isolated pertussis toxin pretreated platelet membranes restored thrombin and noradrenaline responses respectively (Kajiyama *et al.*, 1990). The precise nature of G-protein regulation of agonist-stimulated cPLA<sub>2</sub> activation has remained controversial as the specificity and purity of the tools described above remain unclear.

Expression of a dominant negative heterotrimeric G-protein chimera, consisting of the  $\alpha$  subunit of G<sub>s</sub> with its C-terminal 38 residues replaced with those of G<sub>i</sub> $\alpha$ <sub>2</sub>, in

CHO cells has demonstrated inhibition of both thrombin and ATP-stimulated cPLA<sub>2</sub> activation (Gupta *et al.*, 1990). This suggests the involvement of G<sub>i2</sub> in the regulation of the enzyme by these agonists. A similar involvement of G<sub>i2</sub> may be apparent from studies of NIH3T3 fibroblasts transfected with wild type G<sub>iα2</sub> and a mutant G<sub>iα2</sub> whereby the mutated G<sub>α</sub> possessed a lower intrinsic GTPase activity, resulting in enhanced arachidonic acid generation in the presence of serum (Xu *et al.*, 1993). Although these results predict G-protein regulation of agonist-stimulated cPLA<sub>2</sub> activation, this does not appear to be restricted to the involvement of heterotrimeric G-proteins. Recent studies have also implicated a regulatory role for low molecular weight monomeric G-proteins in growth factor-stimulated activation of cPLA<sub>2</sub>. The Rac and Rho proteins and the generation of leukotrienes are well characterised to be involved in the regulation of growth factor induced actin stress-fibre formation (Ridley & Hall, 1992; Peppelenbosch *et al.*, 1993). In investigating the relationship between these effector molecules and their relative contribution to stress fibre formation, it was recently reported that Rac was essential for EGF-stimulated arachidonic acid production and subsequent generation of leukotrienes (Peppelenbosch *et al.*, 1995). Cellular expression of a constitutively active Rac mutant (RacV12) resulted in arachidonic acid generation and leukotriene production independent of growth factor stimulation. The mechanism by which Rac mediates cPLA<sub>2</sub> activation remains unclear, though this does not appear to be by means of direct interaction of the Rac and cPLA<sub>2</sub> polypeptides (Peppelenbosch *et al.*, 1995).

Intermediate roles for various protein kinases, including protein kinase A (PKA), PKC and mitogen-activated protein (MAP) kinase, have been proposed to mediate in part PLA<sub>2</sub> activation involving G-proteins. Direct phosphorylation of cPLA<sub>2</sub> at serine residue 505 (Ser-505) by MAP kinase has been demonstrated *in vitro* (Lin *et al.*, 1993). Mutation of this residue to alanine abolished phosphorylation and dramatically decreased agonist-stimulated cPLA<sub>2</sub> activation and arachidonic acid generation in cells transfected with the mutated cPLA<sub>2</sub> protein (Lin *et al.*, 1993). PKC is well known to participate in the regulation of MAP kinase (Malarkey *et al.*, 1995)



and PMA treatment of intact cells leads to phosphorylation of PLA<sub>2</sub> at Ser-505 (Lin *et al.*, 1993). However, the role of MAP kinase in the serine phosphorylation and activation of cPLA<sub>2</sub> does not appear to be an exclusive, as phosphorylation of cPLA<sub>2</sub> by MAP kinase remains to be demonstrated *in vivo*. MAP kinase independent activation of cPLA<sub>2</sub> has also been reported. The expression of a mutant G<sub>i</sub>α<sub>2</sub> subunit in CHO cells, where glycine-203 was mutated to threonine, inhibited thrombin and ATP-stimulated arachidonic acid release without effects upon adenylyl cyclase inhibition, mobilisation of intracellular Ca<sup>2+</sup> or MAP kinase activation (Winitz *et al.*, 1994). Overexpression of wild type G<sub>i</sub>α<sub>2</sub> subunit or an inactive mutant polypeptide had no effect upon agonist-stimulated arachidonic acid release (Winitz *et al.*, 1994). Therefore, these studies demonstrated a role for G<sub>i</sub>α<sub>2</sub> in the regulation of cPLA<sub>2</sub> activation dissociated from the roles of Ca<sup>2+</sup> and MAP kinase. Very recently, the stimulation of platelets with thrombin receptor agonist peptide has demonstrated differential activation of cPLA<sub>2</sub> relative to thrombin itself (Kramer *et al.*, 1995). While thrombin stimulated Ca<sup>2+</sup> mobilisation and activation of MAP kinase, the receptor agonist peptide evoked a weaker and less sustained Ca<sup>2+</sup> response and failed to activate the MAP kinases. However, the 'activating' phosphorylation of cPLA<sub>2</sub>, though weaker than that stimulated by thrombin, was observed and suggested the involvement of a proline-directed kinase distinct from the MAP kinases.

The functional significance of cPLA<sub>2</sub> activation is generally accepted to be its participation in the generation of arachidonic acid for production of leukotrienes, prostaglandins and thromboxanes. The physiological function of these compounds has been extensively detailed elsewhere (Needleman *et al.*, 1986). Generation of arachidonic acid is rate limiting for these synthetic pathways and therefore dependent upon activation of cPLA<sub>2</sub>.

Arachidonic acid itself may have a number of putative second messenger functions and directly interact with a number of cellular proteins affecting their physical or activation status. This has developed primarily from *in vitro* studies. Activation of α, β, γ, ε and ζ isoforms of PKC in the absence of Ca<sup>2+</sup>, DAG and phospholipid have been

described (Murakami & Routtenberg, 1985; Sekiguchi *et al.*, 1987) and this may be further enhanced in the presence of  $\text{Ca}^{2+}$ , DAG and PtdSer, depending on PKC isoform (Shinomura *et al.*, 1991; Chen & Murakami, 1992). In common with other fatty acids, arachidonic acid can activate cell free preparations of PtdCho-PLD (eg Chalifour & Kanfer, 1982; Chalifa *et al.*, 1990) and PAP (Gomez-Munoz *et al.*, 1992a). Consequently, arachidonic acid may have distinct cellular protein targets, however, these effects remain to be demonstrated in intact cells and in an agonist dependent manner.

Cytosolic PLA<sub>2</sub>-catalysed hydrolysis of PtdCho also results in the production of lysoPtdCho and this too has been suggested to have signalling functionality. LysoPtdCho, like arachidonic acid, potentiated DAG-induced activation of the  $\alpha$ ,  $\beta$  and  $\gamma$  isoforms of PKC *in vitro* (Sasaki *et al.*, 1993). Exogenously added lysoPtdCho in combination with cell permeable DAG and  $\text{Ca}^{2+}$ -ionophore activated T-lymphocytes as assessed by the upregulation of cell surface expression of Interleukin-2 receptors and the initiation of DNA synthesis (Asaoka *et al.*, 1992). Similarly, exogenous lysoPtdCho also potentiated phorbol ester or DAG-induced differentiation of HL-60 cells to the macrophage-like lineage (Asaoka *et al.*, 1993).

### **1.8 Other Glycerophospholipids Are Substrates For Phospholipase D.**

Although PtdCho appears to be the major phospholipid substrate for receptor-coupled PLD, other glycerophospholipids may serve as substrates for PLD enzymes. By specific labelling of individual membrane phospholipids, hydrolysis of other glycerophospholipids has been demonstrated. PtdEtn hydrolysis in response to phorbol esters has been documented in NIH 3T3 fibroblasts, HL-60 and HeLa cells (Kiss & Anderson, 1990; Kiss & Anderson, 1991). Similarly, labelling of the endogenous PtdIns pool has demonstrated bradykinin-stimulated PtdIns-PLD activities in Madin-Darby canine kidney cells (Huang & Cabot, 1992).

The consequences of these hydrolytic events remain unclear. Generation of PtdOH from these distinct phospholipid pools may have separate putative second

messenger functions to those PtdOH species derived from PtdCho. Further, hydrolysis of PtdEtn and PtdIns may contribute to sustained DAG generation observed in response to a number of agonists.

This may be consistent with the predicted hydrolysis of an alternative inositol lipid in thrombin-stimulated platelets (Wilson *et al.*, 1985), AII-stimulated vascular smooth muscle cells (Griendling *et al.*, 1986, 1987), TRH-stimulated pituitary cells (Imai & Gershengorn, 1986), bombesin-stimulated Swiss 3T3 fibroblasts (Cook *et al.*, 1991) and vasopressin-stimulated A10 vascular smooth muscle cells (Plevin & Wakelam, 1992). However, the contribution of a PLD-catalysed hydrolytic pathway in this context remains debatable. Hydrolysis could simply occur as a result of sustained PtdIns-PLC hydrolysis. This could be assessed by specific labelling of cellular PtdEtn and PtdIns and incubation of cells in the presence of primary alcohols during stimulation. This approach, as previously described, has been utilised in the assessment of the contribution PtdCho-PLD activation to sustained DAG production in bombesin-stimulated Swiss 3T3 fibroblasts and vasopressin-stimulated A10 vascular smooth muscle cells (Cook *et al.*, 1991; Plevin & Wakelam, 1992).

## **1.9 Functional Significance of Phospholipase D Activation.**

### **1.9.1 Does Diacylglycerol Derived From Phosphatidylcholine-PLD Activate Protein Kinases C ?**

The fate of PtdOH, by its conversion to DAG, has questioned whether PtdCho hydrolysis serves as an alternative means of PKC activation distinct from that subsequent to PtdIns(4, 5)P<sub>2</sub> hydrolysis. The lipid product of phosphoinositide hydrolysis, *sn*-1, 2-DAG, is recognised to be the main physiological activator of a family of phosphatidyl serine-dependent serine/threonine-directed kinases collectively called protein kinase C. Since their discovery by Nishizuka and co-workers, at least eleven isoforms have been identified by purification and molecular cloning and these can be divided into two main groupings based upon structural and regulatory properties (Nishizuka, 1988; Parker *et al.*, 1989; Hug & Sarre, 1993). Group 1 consisting of  $\alpha$ ,  $\beta$ <sub>1</sub>,

$\beta_{II}$  and  $\gamma$  isoforms are activated by both DAG and  $Ca^{2+}$ . Group 2 isoforms consisting of  $\delta$ ,  $\epsilon$  and  $\theta$  lack the  $Ca^{2+}$ -binding domain and though activated by DAG are  $Ca^{2+}$ -independent. Isoforms from both groups are known to translocate to the plasma membrane following agonist stimulation (Hug & Sarre, 1993). A third group, that possesses only one rather than two cysteine-rich regions and lacks a  $Ca^{2+}$ -binding domain has recently been identified (Hug & Sarre 1993). This consists of the  $\lambda$ ,  $\mu$  and  $\zeta$  isoforms. They are not activated by DAG or  $Ca^{2+}$  and they have been shown not to undergo translocation to the plasma membrane following agonist stimulation (Ways *et al.*, 1992; Olivier *et al.*, 1994). These PKC isoforms are phorbol ester insensitive and do not appear to undergo down regulation following chronic PMA pretreatment (Ways *et al.*, 1992).

Upon receptor stimulation, DAGs with different fatty acid compositions are generated. PtdIns(4, 5)P<sub>2</sub>-derived DAG contains predominantly unsaturated carbon chains while PtdCho-derived species are predominantly saturated. The production of divergent DAG, including 1-*O*-alkyl-2-acyl species in some cells (see Billah *et al.*, 1989a & b), has suggested that selective PKC isoform activation may be initiated. Evidence in support of this has been based upon the observed effects of 1, 2-diacyl, 1-*O*-alkyl-2-acyl and 1-*O*-alkenyl-2-acyl glycerol species upon PKC activity *in vitro* (see Stabel & Parker, 1991). The potential correlation between sustained generation of PtdCho-derived DAG and the activation of PKC has involved assessment of PKC activation by isoform translocation to the membrane compartment in conjunction with the phosphorylation of the 80kDa MARCKS protein, an endogenous PKC substrate (Aderem, 1992).

In  $\alpha$ -thrombin (500ng/ml)-stimulated IIC9 fibroblasts, translocation of the  $\alpha$  form of PKC occurred in parallel with the generation of early PtdIns(4, 5)P<sub>2</sub>-derived DAG and correlated with phosphorylation of the 80kDa MARCKS protein (Leach *et al.*, 1991). Translocation of PKC $\alpha$  nor phosphorylation of the 80kDa MARCKS protein did not correlate with the generation of PtdCho derived DAG (Leach *et al.*, 1991). At lower concentrations of  $\alpha$ -thrombin (100pg/ml) PtdCho

hydrolysis was observed in the absence of PtdIns(4, 5)P<sub>2</sub> hydrolysis.

Correspondingly, no PKC $\alpha$  translocation was observed (Leach *et al.*, 1991). Despite the lack of PtdCho-derived DAG activation of PKC in whole cell stimulations, it was reported that a PKC activity from these cells could be activated *in vitro* by DAG derived from exogenous PLC catalysed hydrolysis of PtdCho extracted from these cells (Leach *et al.*, 1991). Therefore, as PtdCho-derived DAG can activate PKC *in vitro*, PKC may not be exposed to these species *in vivo*. This theory has been confirmed in TRH-stimulated GH<sub>3</sub> pituitary cells, whereby the observed biphasic generation of DAG consisted of an early transient PtdIns(4, 5)P<sub>2</sub>-derived phase localised to the plasma membrane and a sustained PtdCho-derived phase at the intracellular membranes (Martin *et al.*, 1990). Physical separation of generated DAG and the various PKC isoforms, each having varied subcellular localisation, may account for this. Moreover, the initial phase of generation corresponded to the observed translocation of the Ca<sup>2+</sup>-dependent  $\alpha$ ,  $\beta$  and  $\gamma$  PKC isoforms, whilst the latter phase showed no correlation with PKC translocation.

Although PtdCho-derived DAG did not appear to activate PKC in the above studies, three points regarding the assessment of PKC activity must be taken into account. Firstly, assessment of translocation of Ca<sup>2+</sup>-dependent isoforms, at time points when intracellular Ca<sup>2+</sup> levels will be near basal, may not reflect PKC activity induced by the formation of PtdCho-derived DAG. Therefore, translocation of these isoforms is only pertinent to the generation of PtdIns(4, 5)P<sub>2</sub>-derived DAGs.

Secondly, if PtdCho-derived DAG activates a Ca<sup>2+</sup>-independent isoform then 80kDa protein phosphorylation may not serve as a means of assessment of PKC activation as it has been reported that this protein is not an endogenous substrate for these isoforms (see Olivier & Parker, 1991). Thirdly, it is apparent that the redistribution of Ca<sup>2+</sup>-dependent PKC isoforms can be dissociated from the phosphorylation of their substrates and that phosphorylation can be maintained after translocation events have ceased (Trilivas *et al.*, 1991).

With the isolation of further PKC isoforms, activation of  $\text{Ca}^{2+}$ -independent sub-family members by PtdCho-derived DAG has been investigated. Subsequent work upon IIC9 fibroblasts described above illustrated that  $\alpha$ -thrombin (500ng/ml)-stimulated PKC $\alpha$  translocation lasted for approximately 1 minute, however, the addition of a  $\text{Ca}^{2+}$  ionophore at 5 min, could re-induce PKC $\alpha$  translocation after it had returned to the cytosol (Ha & Exton, 1993). Translocation of PKC $\alpha$  could also be induced by addition of both dioctanoylglycerol and ionomycin, but not each alone. Therefore, the availability of  $\text{Ca}^{2+}$  and not the species of DAG present in the membrane appeared to confer translocation. The reported lack of translocation of this isoform upon stimulation with 100pg/ml  $\alpha$ -thrombin in the presence of ionomycin remains to be resolved. However, in parallel to sustained DAG generation, PKC $\epsilon$  was observed to translocate and remain membrane associated for up to 1 h. This was consistent with the proposed activation of  $\text{Ca}^{2+}$ -independent PKC isoforms by sustained PtdCho-derived DAG. Similarly, in GH4C1 cells, TRH-stimulated biphasic DAG generation corresponded to early and transient PKC  $\alpha$  and  $\beta$  translocation whilst that of PKC $\epsilon$  occurred in parallel with the sustained phase of DAG generation (Akita *et al.*, 1990).

In the absence of inositol lipid hydrolysis, interferon- $\alpha$  stimulated HeLa cells display PKC $\beta$  translocation in association with PtdCho hydrolysis (Pfeffer *et al.*, 1990). It remains unclear whether PtdCho hydrolysis was catalysed by a PLC or PLD mechanism, however, these investigations illustrated that activation of PKC isoforms by PtdCho-derived DAG may not be restricted to  $\text{Ca}^{2+}$ -independent sub-family members as described earlier. This may reflect agonist specificity of activation. Interferon- $\gamma$  was also reported to activated an unidentified PKC family member in endothelial cells in a triphasic manner (Mattila & Renkonen, 1992). PKC activation was induced by PLD-derived DAG produced after concomitant PtdOH and choline generation.

Thus, no clear consensus is evident for the relationship between the generation of PtdCho-derived DAG and the activation of distinct PKC isoforms. The

contribution of the PLD pathway to the generation of sustained DAG still remains unclear and therefore the activation of PKC may be an agonist specific phenomena. Further, as a number of DAG molecules with diverse fatty acid content do not appear to discriminate in their activation of individual PKC isoforms *in vitro*, physiological activation may be agonist specific requiring appropriate membrane localisation of both DAG generation and PKC isoforms. These points remain to be clarified by further study.

#### 1.9.2 Phosphatidic Acid As A Distinct Second Messenger In Whole Cells.

The transient generation of PtdOH and the associated rapid desensitisation of agonist-stimulated PLD activity in a number of cells (McKenzie *et al.*, 1992; Plevin & Wakelam, 1992; Briscoe *et al.*, 1994) have suggested that PtdOH itself may mediate acute responses rather than sustained ones. Therefore the potential role of PtdOH as a putative second messenger has been studied.

Investigations of the cellular responses initiated by PtdOH have involved its addition directly to whole cells and observed effects have included hydrolysis of PtdIns(4, 5)P<sub>2</sub>, inhibition of adenylate cyclase (Murayama & Ui, 1987), elevation of intracellular Ca<sup>2+</sup> and DNA synthesis (Moolenaar *et al.*, 1986), enhancement of cPLA<sub>2</sub> activation (Sato *et al.*, 1993) and contraction of smooth muscle (Salmon & Honeyman, 1980). However, the reported contamination of commercial preparations of PtdOH with lysoPtdOH (Jalink *et al.*, 1990) and the recent identification of a putative lysoPtdOH receptor (Van der Bend, 1992a) questions to what extent these observed cellular responses are in fact attributable to lysoPtdOH. This is primarily due to lyso PtdOH, but not PtdOH, being a potent Ca<sup>2+</sup>-mobilising stimulus for fibroblasts (Jalink *et al.*, 1990).

PtdOH has been shown to have a number of actions *in vitro* that may suggest it could function as a distinct second messenger. Studies of PtdOH action suggested it could act as a potent Ca<sup>2+</sup>-ionophore (Putney *et al.*, 1980) and to transfer Ca<sup>2+</sup> across artificial phospholipid membranes (Serhan *et al.*, 1981) though this was not

confirmed by a further study (Holmes & Yoss, 1983). More direct effects of PtdOH upon PtdIns(4, 5)P<sub>2</sub> hydrolysis have been described. Jackowski & Rock (1989) observed PtdOH activation of PtdIns-PLC in both membrane and soluble platelet extracts which was attributed to either an allosteric effect upon the enzyme or due to structural modification of the enzyme's lipid environment. PtdOH was also reported to mediate up to 20-fold enhanced *in vitro* catalytic activity of PtdIns(4)P 5-kinase (Moritz *et al.*, 1992). This occurred in a concentration dependent manner and suggested PtdIns(4)P 5-kinase was sensitive to PtdOH intercalated in cellular membranes. This has been suggested to play a role in the maintenance of PLD activity *in vivo* (Liscovitch *et al.*, 1994) by stimulating the resynthesis of PtdIns(4, 5)P<sub>2</sub> as a cofactor for PLD activation (discussed further in section 1.1.1.3). Similarly, the activity status of a number of small molecular weight G-proteins and their associated protein regulators has been proposed to be modulated by PtdOH directly (Chuang *et al.*, 1993). In addition to these, the proto-oncogene product, p21<sup>ras</sup>, may be influenced by PtdOH-mediated inhibition of its GTPase-activating protein (Tsai *et al.*, 1988) and activation of its GTPase inhibitory protein (Tsai *et al.*, 1990). Functionally, this would predict that proportionally more Ras would exist in the GTP-bound and active state, leading to activation of the ras-signal transduction pathways and cellular proliferation (see Mulcahy *et al.*, 1985). This is consistent with the observed inhibition of PtdOH-mediated proliferation in fibroblasts by anti-Ras antibodies (Yu *et al.*, 1988). However, this may not reflect the effects of PtdOH directly, but the contaminating effects of lysoPtdOH. LysoPtdOH is now known to utilise and activate Ras-dependent pathways (Hordijk *et al.*, 1994). Additionally, the abrogation of agonist-stimulated PLD activation and its effect upon Ras status has not been investigated in whole cells and therefore the direct modulation of Ras by PtdOH remains speculative.

PtdOH-mediated initiation of protein phosphorylation in cytosolic extracts from a number of rat tissues (Bocckino *et al.*, 1991) has suggested the existence of a distinct PtdOH-sensitive kinase. The observed pattern of target protein



phosphorylation was distinct from those initiated by PtdSer in combination with 1, 2-dioleoylglycerol, excluding the involvement of classical PKC isoforms. Existence of a such a protein, biochemically and immunologically distinct from PKC, has been confirmed by the partial purification of a phospholipid-dependent and fatty acid-activated kinase from platelets (Khan *et al.*, 1994). This kinase was selectively dependent upon PtdOH and may represent the identification of a novel kinase signalling cascade.

It would appear that the generation of PtdOH as a consequence of receptor-stimulated PLD activation may initiate a number of diverse signalling events and be regarded as a distinct second messenger in its own right. However, there is no identified universal mechanism to explain all its effects. This may involve both the structural effects of PtdOH generation in a membrane environment or its interaction with distinct protein targets. These aspects and their physiological relevance *in vivo* remain to be investigated further.

### 1.9.3 Regulation of Agonist-stimulated Superoxide Generation in Hematopoietic Cells.

There are a number of reports that have correlated PtdOH production with agonist-stimulated respiratory burst in neutrophils (Bonser *et al.*, 1989; Koenderman *et al.*, 1989; Rossi *et al.*, 1990; Agwu *et al.*, 1991b; English & Taylor, 1991; Gay & Murray, 1991; Bauldry *et al.*, 1992). Activation of a membrane-bound multiprotein NADPH oxidase complex catalyses the conversion of  $O_2$  to a reactive superoxide ( $O_2^-$ ) species (Heyworth *et al.*, 1992). These products are active in the killing of bacteria and are mediators of inflammation.

The evidence for the involvement of PtdOH in the regulation of NADPH oxidase activation has relied upon two approaches. Firstly, stimulation of intact cells in the presence of primary alcohols, which results in the accumulation of phosphatidylalcohols at the expense of PtdOH, inhibited oxidase activation (Bonser *et al.*, 1989; Rossi *et al.*, 1990; Bauldry *et al.*, 1992). Secondly, a number of workers

have utilised propranolol as an inhibitor of PAP to suppress PAP-catalysed dephosphorylation of PtdOH. This approach led to sustained PtdOH levels and enhanced superoxide production (Rossi *et al.*, 1990; Bauldry *et al.*, 1991; English & Taylor, 1991). The close relationship between PtdOH production and NADPH oxidase activation is further illustrated in neutrophils primed with tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and stimulated with fMLP (Bauldry *et al.*, 1991). This protocol resulted in enhanced PtdOH generation and superoxide production, in the absence of DAG formation (Bauldry *et al.*, 1991).

The precise role PtdOH plays in the regulation of NADPH oxidase activity remains to be elucidated. This may involve modulation of the assembly of the NADPH oxidase complex itself or changes in the membrane environment required for activation of this complex. The NADPH oxidase complex contains, and is regulated by, low molecular weight G-proteins of the Rho subfamily (Rac1/2; Heyworth *et al.*, 1992). Rho is regulated by Rho-associated GTP Dissociation Inhibitor (GDI) that binds to Rho family members to prevent GDP to GTP exchange and consequently their activation (Downward, 1992; Takai *et al.*, 1995). The dissociation of Rho family members from GDI is regulated by phospholipids, in particular PtdOH (Chuang *et al.*, 1993). Therefore, agonist-stimulated generation of PtdOH may regulate Rho/Rac status and hence the activation of the respiratory burst complex.

A temporal relationship between the formation of PtdOH, rather than DAG, has also been observed in other physiological settings such as insulin secretion in pancreatic islets (Dunlop & Metz, 1989; Konrad *et al.*, 1991), proliferation in fibroblasts (Zhang *et al.*, 1990) and smooth muscle contraction (Salmon & Honeyman, 1980).

#### 1.9.4 Regulation of Cellular Cytoskeleton Assembly.

Another function of PtdOH generation may be in mediating agonist-stimulated cytoskeletal rearrangements. Stimulation of IIC9 fibroblasts with  $\alpha$ -thrombin and lysoPtdOH induced changes in cell morphology, from rounded to elongated cells,

consistent with actin-stress fibre formation (Ha & Exton, 1994; Ha *et al.*, 1994). This correlated with increased PtdOH formation and F-actin content. These agonist specific events could be mimicked by addition of exogenous PtdCho-PLD from bacteria or PtdOH micelles (Ha & Exton, 1994). However, addition of PtdCho-PLC or cell permeable diglycerides or inhibition of PKC had no effect upon stress fibre formation (Ha & Exton, 1994). Similar studies in Swiss 3T3 fibroblasts have also implicated lysoPtdOH in regulation of actin-stress fibre formation (Ridley & Hall, 1992). In addition, PtdOII, thrombin and bombesin also stimulated small changes in actin polymerisation, whereas growth factors appeared ineffective or stimulated alternative changes in cell morphology, particularly membrane ruffling (Ridley & Hall, 1992; Ridley *et al.*, 1992). The effects of lysoPtdOH were suggested to be regulated by a mechanism dependent on the activation of the low molecular weight G-protein Rho (Ridley & Hall, 1992). This was based upon the inhibition of rho following microinjection of C3 ADP-ribosyl transferase which inactivated Rho by ribosylation (Ridley & Hall, 1992) and lowered stress fibre formation.

The mechanism(s) that regulate lysoPtdOII-stimulated actin polymerisation and the role of Rho within this have been developed further during recent studies upon porcine aortic endothelial (PAE) cells. In this cell type lysoPtdOH stimulated actin polymerisation and stress fibre formation (Cross *et al.*, 1995) in the absence of PLC, PLA<sub>2</sub> and PtdIns 3-kinase activation and suggested a critical functional role for PtdOH formation in this cell type. Consistent with PLD activation playing a central role in the regulation of stress fibre formation, was the observed inhibition of lyso PtdOII-stimulated stress fibre formation in the presence of butan-1-ol, which resulted in the accumulation of phosphatidylbutanol at the expense of PtdOH. Further, direct addition of PtdOH stimulated stress fibre formation which was not susceptible to butanol preincubation (Cross *et al.*, 1995). A functional role for Rho was suggested by the inhibition of both lysoPtdOH and PtdOH-stimulated stress fibre formation by C3 ADP-ribosyl transferase-catalysed ribosylation of Rho and microinjection of ADP-ribosylated Rho protein (Cross *et al.*, 1995). Therefore, Rho may function as a

component of this signalling cascade downstream of PLD activation, however it remains to be elucidated if PtdOH directly activates Rho itself or an intermediate factor required for Rho activation. Potential candidates include the Rho-associated GDI proteins as previously described (see previous section). Again, the dissociation of these proteins from Rho family members may be regulated by PtdOH (Chuang *et al.*, 1993) and therefore agonist-stimulated generation of this bioactive lipid may regulate Rho status and hence cytoskeletal assembly in a manner similar to that proposed for the NADPH oxidase complex.

#### 1.9.5 A Potential Role for Phospholipase D in Mitogenesis.

In addition to a number of putative second messenger functions for PtdOH (see previous sections) its proposed involvement as a causal factor in the stimulation of mitogenesis has been closely considered (reviewed by Boarder, 1994). It has been reported that PDGF-stimulated mitogenesis in Balb/c 3T3 fibroblasts correlated with PtdOH generation (Fukami & Takenawa, 1992) in the absence of DAG formation. PtdOH also stimulated DNA synthesis. This could be mimicked by exogenous addition of PLD derived from the fungi *Streptomyces chromofuscus*, yet addition of exogenous bacterial PtdCho-PLC from *Bacillus cereus* had no effect. The observed correlation between PtdOH generation and mitogenesis in the absence of other growth factors would suggest PLD activation and PtdOH formation to be a causal factor involved in mitogenesis in these cells.

Investigations in CCL39 fibroblasts transfected with the M1 subtype of muscarinic receptor demonstrated that carbachol could elicit strong phosphoinositide hydrolysis also stimulated by  $\alpha$ -thrombin, however, only  $\alpha$ -thrombin stimulation was mitogenic (Seuwen *et al.*, 1990). Therefore, these workers concluded that strong and persistent inositol lipid hydrolysis may initiate a number of early mitogenic signals but was not sufficient to maintain cellular proliferation. It was proposed however, that these events induced a state of 'competence' in readiness for other 'progression' factors required for successful mitogenesis. In subsequent studies on the same transfectants,

carbachol stimulated substantial activation of PLD with a negligible mitogenic response while the reverse was apparent for  $\alpha$ -thrombin (McKenzie *et al.*, 1992) again suggesting that receptor-mediated phospholipid hydrolysis was not the causal factor in the mitogenic response. ET-1 activation of PtdCho hydrolysis in Rat-1 fibroblasts was large though did not elicit a mitogenic response (Van der Bend *et al.*, 1992b). Therefore, these data suggested that although a number of G-protein coupled receptor agonists could induce robust PtdCho hydrolysis, they did not appear to function as full mitogens in a variety of cell types.

It is also well established that growth factors also activate PLD (see Cook & Wakelam, 1992; Plevin *et al.*, 1992), however, relative to the G-protein coupled receptor agonists described above they are less efficacious (McKenzie *et al.*, 1992). In a number of cases, particularly PDGF, PLD activation is regulated by PLC $\gamma$  activity (Plevin *et al.*, 1992; Yeo *et al.*, 1994) and additionally dependent upon the expressed levels of PLC $\gamma$ 1 (Lee *et al.*, 1994). Molecular studies of growth factor receptors have demonstrated that mutation of the intracellular binding site for PLC $\gamma$  abrogates PLC $\gamma$ -catalysed inositol lipid hydrolysis yet shows no impairment of mitogenesis in response to growth factor (Peters *et al.*, 1992; Mohammadi *et al.*, 1992). Additionally, overexpression of the  $\gamma$  isoform of PtdIns-PLC in NIH 3T3 fibroblasts does not alter the effect of PDGF-induced mitogenesis and once again indicates that inositol lipid hydrolysis and mitogenesis can be dissociated (Margolis *et al.*, 1990).

The involvement of PLD-catalysed PtdOH generation has been addressed more directly in smooth muscle cells (Kondo *et al.*, 1992). Exogenous addition of PLD from *S.chromofuscus* and subsequent generation of PtdOH appeared to elicit a mitogenic response and function in a similar manner as the addition of PDGF. However, these experiments were conducted in the presence of insulin, a known mitogen and non-activator of PLD in smooth muscle cells. The abrogation of ET-1-stimulated PLD activity in A10 vascular smooth muscle cells by addition of primary alcohols and the preferential formation of phosphatidylalcohols at the expense of PtdOH has also suggested a potential role for this putative lipid messenger in the

mitogenic response. However, in these experiments as above, the presence of insulin was required for the observation of a mitogenic response (Wilkes *et al.*, 1993). Both studies may suggest PLD activation functions as a competence factor yet cell cycle progression and mitogenesis only occurs in the presence of growth factors.

Down regulation of PKC isoforms by chronic PMA pretreatment reduced PDGF-stimulated mitogenesis by 50% but PLD activation was completely abolished (Leach *et al.*, 1991), suggesting pathways other than PLD activation are required for mitogenesis. Similarly, Sharma & Bhalla (1993) reported that subsequent to chronic phorbol ester pretreatment and PKC isoform down regulation, PKC-dependent pathways were not involved in PDGF-stimulated mitogenesis in rat aortic vascular smooth muscle cells. As PDGF-stimulated activation of PLD is regulated by PKC in a number of cell types (see above) this again questions the proposed role for PLD in the mitogenic response. Therefore, conflicting evidence as to the role of PLD activation in mitogenic responses is apparent. The observed effects of PtdOH generation in Balb/c 3T3 fibroblasts (Fukami & Takenawa, 1992) remains unresolved relative to other cell types. In some cases PLD activation may function in a capacity similar to competence factors but true mitogenesis requires the presence of synergising peptide growth factors. However, as described above this may be cell type specific. Thus, the proposed causal relationship between PLD activation and mitogenesis requires further investigation.

#### 1.9.6 Choline As A Second Messenger.

To date no evidence exists to indicate a distinct second messenger role for choline. However, the release of choline as a consequence of PLD activity has been suggested to be functionally coupled to the resynthesis of acetylcholine in the brain (Corradetti *et al.*, 1983; Hattori & Kanfer, 1985). As PLD activation has been identified in cultured neuronal cells stimulated with muscarinic agonists (Liscovitch, 1989; Martinson *et al.*, 1989) it may provide a source of PtdCho-derived lipid

messengers involved in the release of acetylcholine but also mediate the replenishment of neurotransmitter stores.

### **1.10 Metabolism of Phosphatidic Acid and Choline.**

#### **1.10.1 Metabolism of PtdOH by Phosphatidic Acid Phosphohydrolase.**

Phosphatidic Acid Phosphohydrolase (PAP) catalyses the hydrolysis of PtdOH to DAG and  $P_i$ . This may be relevant to the transient production of PtdOH that is observed in cells stimulated by a number of receptor agonists. Furthermore the rapid removal of this bioactive lipid is consistent with its putative role as a second messenger. In addition, PAP plays a fundamental role in the biosynthesis of glycerophospholipids (Brindley, 1988). To date, two distinct PAP activities have been described, termed PAP-1 and PAP-2 (Brindley, 1988, Jamal *et al.*, 1991). These activities can be distinguished by their sensitivity to divalent cations and the sulphhydryl-modifying agent, *N*-ethylmaleimide (NEM). PAP-1 was dependent on the presence of  $Mg^{2+}$  for activity and inhibited by NEM (Brindley, 1988), whereas PAP-2 was independent of  $Mg^{2+}$  and insensitive to NEM (Jamal *et al.*, 1991).

PAP-1 exists as an inactive pool in the cytosol and upon translocation to the endoplasmic reticulum becomes active (Brindley, 1988). This can be stimulated by free fatty acid, particularly oleic acid and specificity for long chain unsaturated fatty acid has been described (Aridor-Piterman *et al.*, 1992; Cascales *et al.*, 1984; Gomez-Munoz *et al.*, 1992a). PAP-2 is predominantly plasma membrane bound (Jamal *et al.*, 1991) which is consistent with its proposed involvement in signal transduction events.

In common with a number of other signal transduction proteins, particularly PKC, PAP activity can be regulated by a number of lipids. These include sphingosine, derived from sphingomyelin, which can block oleate-stimulated PAP-1 translocation to the endoplasmic reticulum and inhibit activity (Aridor-Piterman *et al.*, 1992; Gomez-Munoz *et al.*, 1992a). Sphingosine was proposed to interact with PtdOH intercalated in the endoplasmic reticulum membranes rather than having a direct effect upon PAP-1 (Gomez-Munoz *et al.*, 1992a). The translocation of PAP-1 to the

endoplasmic reticulum has been described to be subject to agonist regulation. Translocation was inhibited by the presence of cAMP or agonists that elevate cAMP, such as glucagon (Butterwith *et al.*, 1984; Pittner *et al.*, 1985). This may suggest that regulation is mediated by phosphorylation events; in this instance PKA may be responsible. Consistent with this mechanism of regulation, it has been reported that okadaic acid, a protein phosphatase 2/2A inhibitor, inhibited oleate-stimulated translocation of the enzyme and displaced PAP-1 activity from microsomal membranes (Gomez-Munoz *et al.*, 1992b).

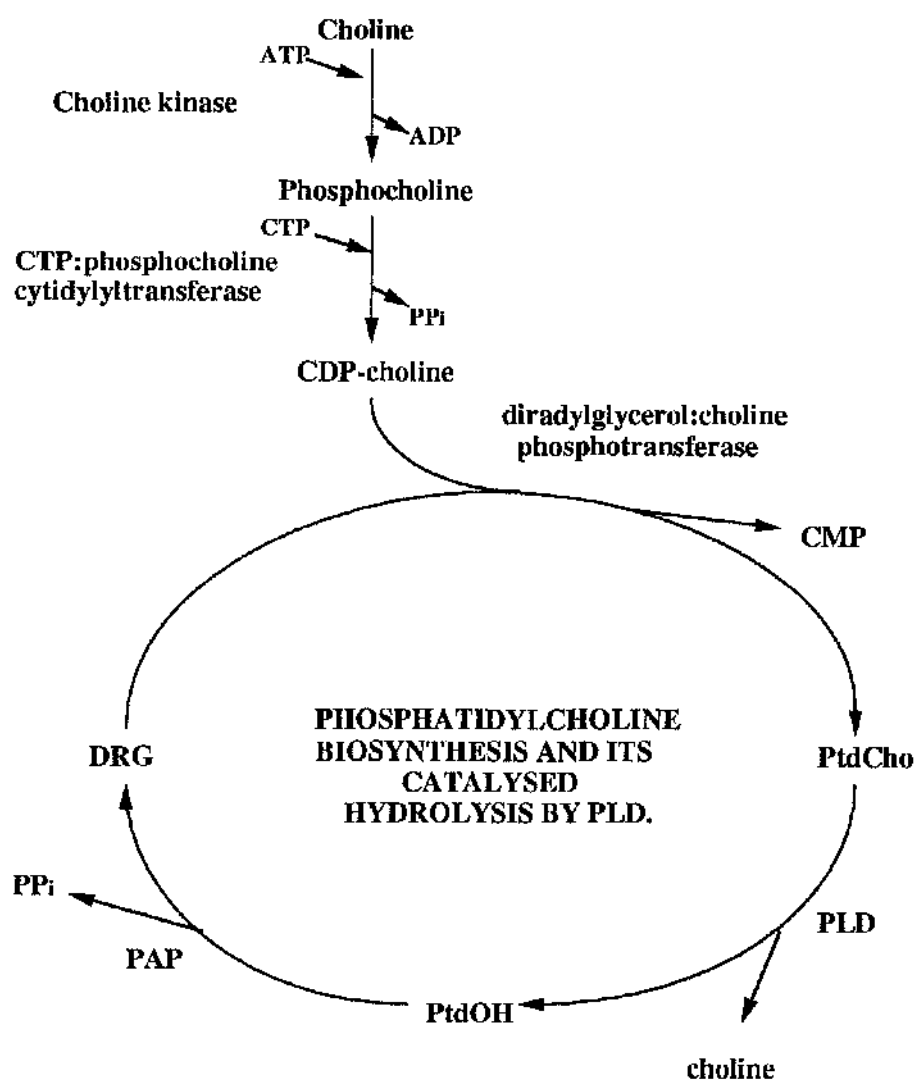
Similar investigations relevant to the regulation of PAP-2 have also been conducted. Inclusion of protein tyrosine-phosphate phosphatase inhibitors such as vanadate and  $Zn^{2+}$  during membrane preparation increased the PAP-2 activity recovered from rat fibroblasts (Martín *et al.*, 1993). This may infer that *in vivo* PAP-2 is subject to regulation by tyrosine phosphorylation. In contrast to PAP-1 activity, inclusion of okadaic acid had no effect upon recoverable PAP-2 activity (Gomez-Munoz *et al.*, 1992b) and suggested serine/threonine phosphorylation and dephosphorylation did not contribute to enzyme regulation.

Further insight into PAP enzymology and regulation may come from the purification of these enzymes. A membrane-associated PAP-2 activity from porcine thymus has been partially purified (Kano *et al.*, 1992) and a similar activity more recently has been purified to homogeneity from rat liver (Waggoner *et al.*, 1995).

#### 1.10.2 Removal of Choline and the Phosphatidylcholine Biosynthetic Pathway.

As previously described agonist stimulation of a number of cell types results in the generation of water soluble PtdCho metabolites. Free choline generated by receptor stimulated PLD activation may have second messenger functions, though these may be limited (see Section 1.9.6). Alternatively, free choline can be utilised in the resynthesis of cellular PtdCho in whole cells. Such a relationship between PtdCho hydrolysis and resynthesis was proposed to form a 'phosphatidylcholine cycle' by Pelech & Vance, (1989). Within this cycle, the predominant route of PtdCho biosynthesis in eukaryotic





**Fig 1.3** Schematic representation of the biosynthesis of phosphatidylcholine via the CDP-choline (Kennedy) pathway and its relationship to agonist-stimulated PLD-activation.

cells occurs via the CDP-choline or 'Kennedy' pathway (Bishop & Bell, 1988; Pelech & Vance, 1989; Tronchere *et al.*, 1994). This relies upon incorporation of choline into PtdCho mediated by the sequential action of choline kinase, CTP:phosphocholine cytidylyltransferase and choline phosphotransferase (see Fig1.3).

Cytosolic choline kinase activity represents the first step in PtdCho resynthesis and enzyme activation by mitogenic growth factors in cultured fibroblasts has been described (Warden & Freidkin, 1985). Consistent with this, the isolation of a fibroblastic mutant cell type with an approximate 3-fold decrease in choline kinase activity displayed a similar 3-fold decrease in PtdCho synthesis (Nishijima *et al.*, 1984) and highlights its requirement as the initial step to PtdCho synthesis.

The second step is catalysed by key regulatory enzyme CTP:phosphocholine cytidylyltransferase. This catalyses the rate limiting step in the pathway (Wright *et al.*, 1985). The enzyme has been purified from rat liver cytosol and exists as a heterodimer of 38 and 45kDa polypeptides, with the 45kDa polypeptide representing the catalytic subunit (Feldman & Weinhold, 1987). Similar to PAP (see previous section) cytidylyltransferase appears to be localised to both cytosolic and microsomal compartments. The cytosolic form has been described as an inactive pool of the enzyme and upon translocation to and association with microsomal membranes becomes active. This occurs under conditions that stimulate PtdCho synthesis including regulation by addition of free fatty acids (Pelech & Vance, 1984; Weinhold *et al.*, 1991), stimulation with phorbol esters that stimulate DAG generation (Pelech & Vance, 1984), treatment of cells with exogenous PLC (Wright *et al.*, 1985) and by phosphorylation and dephosphorylation dependent mechanisms (Hatch *et al.*, 1990; Utal *et al.*, 1991; Jamil *et al.*, 1992).

The final step is catalysed by choline phosphotransferase localised to the microsomal compartment (Bishop & Bell, 1988). Multiple forms of this enzyme have been isolated which catalyse the synthesis of distinct species of PtdCho, one is responsible for the synthesis of diacyl and alkylalkyl forms and another appears to be specific for *de novo* synthesis of platelet activating factor (PAF) (Woodard *et al.*, 1987).

Homologous enzymes have also been studied in yeast (*Saccharomyces cerevisiae*) and mutant strains have been isolated with defects in choline phosphotransferase and consequently PtdCho synthesis (Hjelmstad & Bell, 1987). This enzyme is specific for choline and distinct from the analogous protein required for PtdEtn synthesis. However, yeast exhibiting mutations in both transferase enzymes were viable and highlighted the ability of other pathways to support PtdCho and PtdEtn synthesis (Bishop & Bell, 1988). Alternative pathways may include the successive transfer of methyl groups to PtdEtn from S-adenosyl methionine to generate PtdCho (Ridgeway & Vance, 1987).

More recently attention has focused directly upon the relationship between receptor-stimulated hydrolysis and synthesis of PtdCho. In human neutrophils both fMetLeuPhe and PMA stimulate PtdCho hydrolysis catalysed by PLD (eg Thompson *et al.*, 1990). Tronchere *et al.*, (1995) reported that fMLP alone stimulated PtdOH production but not PtdCho synthesis. However, addition of fMLP in combination with cytochalasin B led to stimulated PtdOH formation, its conversion to DAG and resulted in choline incorporation into PtdCho. This was mediated by the CDP-choline pathway and relied upon membrane-association and activation of CTP:phosphocholine cytidyltransferase (Tronchere *et al.*, 1995). Therefore, PtdCho-derived DAG and not formed PtdOH appeared to regulate the activity status of the transferase and the synthesis of PtdCho. PMA also stimulated the generation of DAG from PtdCho and caused similar effects upon cytidyltransferase membrane association. This too resulted in PtdCho synthesis (Tronchere *et al.*, 1995). Therefore, this study demonstrated that only DAG derived from PtdCho, catalysed by the PLD/PAP pathway, was involved in stimulated choline incorporation into PtdCho. This may serve as a model demonstrating the close association of PtdCho hydrolysis and its resynthesis in a similar manner to that described for the catalysed hydrolysis and resynthesis of the phosphoinositides in the extensively described 'PtdIns cycle' (see Section 1.1). Moreover, the relationship between hydrolytic and synthetic pathways in this cell type may serve as an example to be extrapolated to others. Further study is obviously

required to elucidate whether this represents a universal mechanism of 'phospholipid cycling' common to all mammalian cell types.

## **1.11 Cell Free Phospholipase D.**

### **1.11.1 Assessment of Cell Free PLD Activity.**

The assessment of cell free PLD activity has relied upon the development of suitable *in vitro* assay conditions, such that both hydrolytic and transphosphatidylation activities of PLD enzymes can be monitored by presentation of substrate to the enzyme in an appropriate manner. This has included investigations utilising cell free preparations from cells and tissue whereby endogenous PtdCho substrate has been radiochemically labelled prior to cellular fractionation or secondly, by the presentation of exogenous substrate in a lipid/detergent micelle or as a dispersion.

Metabolic labelling of endogenous membrane glycerophospholipids (PtdCho & PtdEtn) by preferential incorporation of labelled fatty acids, choline or ethanolamine prior to cell fractionation has facilitated the identification of PLD activities in membrane fractions of rat brain (Chalifour & Kanfer, 1979; Mohn *et al.*, 1992; Horvitz & Davis, 1993) CCL39 fibroblasts (Conricode *et al.*, 1992; Conricode *et al.*, 1994) Swiss 3T3 fibroblasts (Eldar *et al.*, 1993) and leukemic HL-60 granulocytes (Kiss & Anderson, 1989). PLD enzymes active against exogenously presented substrate have also been widely documented, however, the technical approach to these assay protocols was diverse. Presentation of exogenous substrate has been performed in three main ways.

- 1: PtdCho substrate in a mixed micellar assay system with the non-ionic detergent Triton X-100 (TX-100) (see Martin, 1988; Kanoh *et al.*, 1991)
- 2: A dispersion of PtdCho with the free fatty acid oleate (see Chalifour & Kanfer, 1982, Chalifa *et al.*, 1990).

- 3a: A dispersion of PtdCho and the non-ionic detergent *n*-octyl- $\beta$ , D-glucopyranoside (see Wang *et al.*, 1991).
- 3b. Radiolabelled endogenous PtdCho in native cellular membranes.  
(Kiss and Anderson, 1990).

Studies utilising assay methodology based upon the above have demonstrated that PLD activity in cell free preparations was highly latent and required the presence of a suitable activator. In these situations it is likely that TX-100, oleate and octylglucoside have fulfilled this role. These assays were used in the preliminary characterisation and purification of various PLD activities. Prior to purification of an enzyme it is necessary to identify a suitable tissue source and if possible a subcellular fraction enriched in the protein of interest. The subcellular distribution of PLD activities has been examined in detail. Crude subcellular fractionation by differential centrifugation has demonstrated detectable PLD activities in various organelle fractions.

Cytosolic PLD activities have been observed upon presentation of lipid substrate in the form of a PtdCho/octylglucoside dispersion (Wang *et al.*, 1991). Although this approach also demonstrated the existence of membrane-associated PLD activities in the same bovine tissues, the cytosolic PLD activities were described to be the predominant form of the enzyme under these assay conditions (Wang *et al.*, 1991). A cytosolic PtdIns-hydrolysing PLD activity in addition to the previously described PtdCho-hydrolysing activity present in Madin-Darby kidney cells has also been identified (Huang *et al.*, 1992). Detection of this enzyme activity required altered detergent conditions to those in the assay of PtdCho-hydrolysing PLD (Huang *et al.*, 1992). Again, this suggests not only distinct isoforms of PtdCho hydrolysing PLD, but also PLD isoforms active against other glycerophospholipids may be activated in an agonist specific manner.

However, in the majority of studies PLD activity has been described as almost exclusively particulate. For example in bovine pulmonary artery endothelial cells,

PLD activity was present in all fractions recovered (Martin *et al.*, 1988). An intermediate pellet fraction (15,000g) contained the most PLD activity arguing for localisation to be in a cellular organelle or plasma membrane fraction. A similar PtdCho-selective PLD activity has also been described in Madin-Darby cells (Huang *et al.*, 1992). PLD activity in rat ventricular myocardium was localised primarily to particulate fractions, though these were the 40,000 & 100,000g pellets respectively suggesting PLD localisation to be in a less dense organelle than plasma membranes (Panagia *et al.*, 1991). Studies of rat brain (Kano *et al.*, 1991) have demonstrated that PLD activity in TX-100-solubilised crude plasma membranes displayed a greater specific activity than that of microsomal membranes in a PtdCho/TX-100 micellar assay while the reverse relationship is apparent upon assaying these fractions by means of a PtdCho/oleate dispersion. This was clarified following the identification of distinct PLD isoforms in rat brain, where an oleate-dependent and an ARF-activated enzyme were separated from solubilised membranes by column chromatography on heparin (Massenburg *et al.*, 1994).

The membrane bound, or associated, forms of PLD have been most successfully characterised in neutrophils and HL-60 granulocytes, where PLD specific activity is considerably higher than tissues such as brain and spleen (M.Hodgkin, personal communication). Prelabelling of endogenous phospholipids in neutrophils and HL-60 cells led to the observation of TPA- and GTP $\gamma$ S-stimulated PLD activity. However, this was only detectable if both membrane and cytosolic fractions were combined in cell free assays (Olson *et al.*, 1991; Anthes *et al.*, 1991; Bowman *et al.*, 1993). Similar observations were made in HL-60 cells depleted of their cytosol following streptolysin-O permeabilisation (Geny *et al.*, 1992). Upon re-addition of cytosol, GTP $\gamma$ S-stimulated PLD activity was restored (Geny *et al.*, 1992). This model system allowed the purification and identification of ARF as a component required for GTP $\gamma$ S-dependent PLD activation (Cockcroft *et al.*, 1994). A defined *in vitro* assay for PLD activity was developed by Brown *et al.*, (1993) which used exogenous lipids. The PtdCho was presented in the form of liposomes composed of

PtdCho, PtdIns(4, 5)P<sub>2</sub> and PtdEtn in specific molar ratios (1:1.4:16). PLD activity in the membrane fraction of HL-60 cells could be observed in a GTPγS-dependent manner that also required PtdIns(4, 5)P<sub>2</sub>. Other acidic phospholipids, such as PtdSer, could not replace PtdIns(4, 5)P<sub>2</sub> in the assay. This too served as a pseudo-reconstitution assay for the purification and identification of ARF as a PLD activator (Brown *et al.*, 1993). Similarly, PtdIns(4, 5)P<sub>2</sub> activates rat brain membrane PLD activity up to 10-fold *in vitro* in a specific manner (Liscovitch *et al.*, 1994) which could not be fulfilled by PtdIns(4)P, PtdIns nor other acidic phospholipids. The physiological relevance of exogenous lipid addition remains unclear and does not prove the requirement for PtdIns(4, 5)P<sub>2</sub> as an endogenous cofactor. Estimations of PtdIns(4, 5)P<sub>2</sub> concentration in the plasma membranes of cells approaches 2 mol% (White, 1973), a concentration at which Liscovitch and coworkers (1994) reported near maximal activation of membrane-bound PLD. In the presence of neomycin, which binds anionic phospholipids with high affinity (Gabev *et al.*, 1989), inhibition of membrane-bound PLD but not solubilised or partially purified PLD was apparent (Liscovitch *et al.*, 1994). This was not due to varying detergent/phospholipid concentrations as neomycin sensitivity could be restored upon addition of PtdIns(4, 5)P<sub>2</sub> to the partially purified enzyme (Liscovitch *et al.*, 1994). Further evidence in support of PtdIns(4, 5)P<sub>2</sub> functioning as an endogenous cofactor is the observed neomycin-mediated inhibition of GTPγS-stimulated PLD activation in permeabilised NG108-15 cells (Liscovitch *et al.*, 1991) and may confirm its involvement in the activation of membrane-associated PLD activity in whole cells.

#### 1.11.2 Regulation of *in vitro* PLD Activity By Small Monomeric GTPases.

Recently, attention has focused upon the nature of the regulation of PLD activity in fractions of neutrophils and granulocytes. In neutrophil subcellular fractions, Olson *et al.*, (1991) reported that for optimal GTPγS-stimulated PLD activity both plasma membranes and cytosol were required in the presence of Ca<sup>2+</sup>. This was consistent with the previous observation of a requirement for GTP and Ca<sup>2+</sup>

for PLD activation in neutrophil homogenates (Anthes *et al.*, 1989). The use of HL-60 cells to study PLD and its regulation quickly led to the identification of ARF as a GTP-dependent step in PLD activation (see section 1.11.1). ADP-Ribosylation Factor (ARF) is a member of the Ras superfamily of small molecular weight GTP-binding proteins (Brown *et al.*, 1993; Cockcroft *et al.*, 1994).

ARF was originally identified as a protein cofactor for cholera toxin-catalysed ADP-ribosylation of  $\alpha$  subunit of the heterotrimeric G-protein  $G_s$ , responsible for the activation of adenylate cyclase (Kahn & Gilman, 1984; Kahn & Gilman, 1986). The ARF proteins (ARF 1, 3, 4, 5, and 6) represent a family of highly conserved and ubiquitously expressed GTP-binding proteins related to both the Ras superfamily and heterotrimeric G-protein  $\alpha$  subunits (see Moss & Vaughan, 1993). However, the ARF proteins are distinct from heterotrimeric GTP-binding proteins in that binding of GTP initiates their translocation from a cytosolic to membrane localisation following N-terminal myristoylation (Kahn *et al.*, 1988; Huan *et al.*, 1993). Nucleotide exchange can be modulated by lipids and detergents (Bobak *et al.*, 1990). It is also apparent that ARF binding to Golgi membranes is mediated by both receptor agonists and PKC activation (De Matteis *et al.*, 1993). In mammalian cells ARF is localised to the Golgi network and plasma membranes upon GTP $\gamma$ S activation (Stearns *et al.*, 1990; Regazzi *et al.*, 1991; Walker *et al.*, 1992). Golgi transport assays identified ARF as being required for a number of GTP $\gamma$ S sensitive-vesicular fusion processes, including endoplasmic reticulum-golgi fusion (Balch *et al.*, 1992), endosome-endosome fusion and nuclear vesicle fusion (Lenhard *et al.*, 1992; Boman *et al.*, 1992). Thus, the identification of ARF as a putative activator of PLD in HL-60 granulocytes suggests a potential role for PLD activation and PtdOH generation in the regulation of intracellular membrane trafficking (see Khan *et al.*, 1993).

Other small molecular weight GTP-binding proteins have also been implicated in the regulation of PLD activity in neutrophils (Bowman *et al.*, 1993) and hepatocyte membranes (Malcolm *et al.*, 1994). This is likely to be a member of the Rho family of proteins which, like ARF, are also members of the Ras-superfamily. The involvement



of Rho, or Rho-like proteins, was suggested based upon the ability of *smgGDP* dissociation stimulator, which stimulates GDP for GTP exchange on Rac and Rho (Downward, 1992), to stimulate GTP-dependent PLD activity (Bowman *et al.*, 1993). The RhoGDP Dissociation Inhibitor (RhoGDI) protein, which binds specifically to Rho proteins and maintains their inactive GDP-bound status, was observed to inhibit GTP $\gamma$ S-dependent PLD activity (Bowman *et al.*, 1993). In hepatocyte membranes treated with RhoGDI, GTP $\gamma$ S-stimulated PLD activity was diminished which correlated with the removal of membrane-associated RhoA (Malcolm *et al.*, 1994). Readdition of recombinant RhoA fully reconstituted GTP $\gamma$ S-stimulated PLD activity. Only partial recovery of PLD activity was observed upon re-addition of Rac1, another Rho family member and no enhancement was observed upon addition of ARF. These data may clarify the previously observed pertussis-toxin insensitivity of GTP $\gamma$ S-stimulated PLD activation in hepatocytes membrane fractions (Bocckino *et al.*, 1987a & b) as these small molecular weight G-proteins are insensitive to modification by pertussis-toxin-catalysed ADP-ribosylation. Therefore, the regulation of PLD activation would appear to occur by a G-protein dependent mechanism but the individual small molecular weight G-proteins involved in the activation of membrane-associated PLD may be diverse and cell type specific.

It is also now apparent that PtdOH itself, in common with a number of acidic phospholipids may function to modulate the physical association of the Rho proteins, particularly Rac, with their GDI proteins (Chuang *et al.*, 1993). This complex only exists in resting cells and thus may be regulated by the generation of lipid molecules in an agonist-dependent manner. Therefore, the production of PtdOH may regulate further Rho-mediated activation of PLD or allow activated Rho to initiate other divergent signalling events. The activity of ARF also appears to be modulated by acidic phospholipids (Randazzo & Kahn, 1994). PtdIns(4, 5)P<sub>2</sub> stimulates the ARF GTP-ase activating protein and GTP-hydrolysis up to 30-fold. However, the requirement for PtdIns(4, 5)P<sub>2</sub> is reduced or increased in the presence of PtdOH and PtdCho respectively. Recently Terui *et al.* (1994) have extended this by showing that

PtdIns(4, 5)P<sub>2</sub> increases GTP binding to ARF and mediates this effect by binding to ARF. As ARF functions as an activator of PLD, the finding that substrate for PLD (PtdCho) inhibits ARFGAP activity while the product (PtdOH) potentiates it suggests a negative feedback loop for the coordinate regulation of PLD and ARF activities may exist.

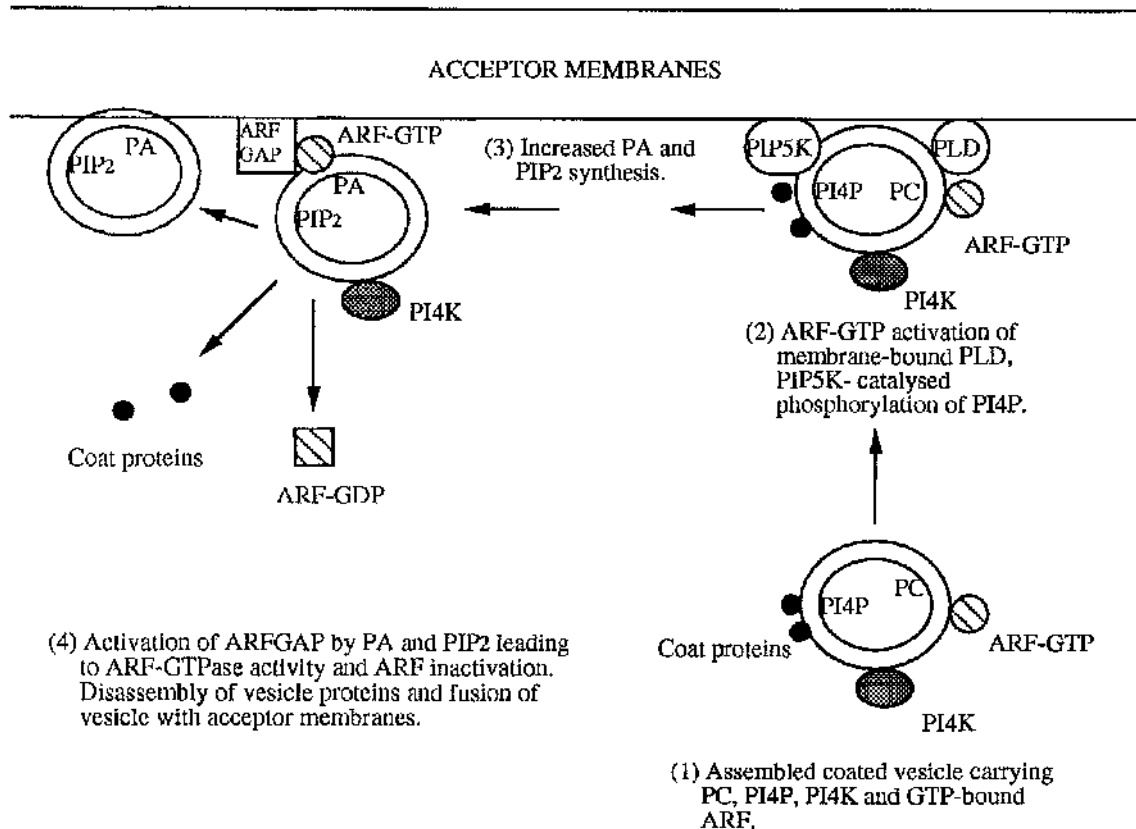
### 1.11.3 Is PLD Regulated Via PtdIns(4, 5)P<sub>2</sub> Synthesis ?

The formation of PtdIns(4, 5)P<sub>2</sub>, catalysed by PtdIns(4)P 5-kinase activity, may influence PLD activation. In this context, PtdIns(4)P 5-kinase activity has been suggested to be regulated by GTP-binding proteins (Smith & Chang, 1989) and more recently the small monomeric GTPase, Rho, has been identified as fulfilling this role (Chong *et al.*, 1994). This was based upon the use of lavostatin, an inhibitor of small molecular weight G-protein isoprenylation, in reducing PtdIns(4, 5)P<sub>2</sub> levels in stimulated cells. Further, in cell lysates, *botulinum* C3 exoenzyme pretreatment, which catalyses ADP-ribosylation of Rho and impairs its interaction with effector molecules, blocked GTPγS-stimulated PtdIns(4)P 5-kinase activity (Chong *et al.*, 1994). Conversely, microinjection of GTP-bound Rho into cells, stimulated PtdIns(4)P 5-kinase activity, whereas GDP-bound Rho and GTP-bound Rac were ineffective. Therefore a Rho family member, regulates PtdIns(4)P 5-kinase activity and therefore cellular PtdIns(4, 5)P<sub>2</sub> content. This may potentially influence the nature and magnitude of PLD activation and is consistent with the proposed regulation of PLD activation by Rho or an alternative member of the Rho subfamily, that has been observed in both rat liver plasma membranes (Malcolm *et al.*, 1994) and human neutrophils (Bowman *et al.*, 1993). However, C3 exoenzyme-catalysed ADP-ribosylation of RhoA had no effect upon GTPγS-stimulated PLD activation in either study and which suggests that PtdIns(4)P 5-kinase and PLD activity are in fact dissociable. It remains to be examined whether C3 exoenzyme-catalysed ADP-ribosylation of Rho proteins inhibits agonist-stimulated PLD activation in whole cells.

#### 1.11.4 A Model for Co-ordinate Regulation of ARF Activity, Phosphoinositide Metabolism and PLD Activity; A Potential Role in the Regulation of Receptor-mediated Vesicular Membrane Trafficking.

The identification of ARF proteins as activators of PLD has implicated PLD itself in vesicular trafficking (see Kahn *et al.*, 1993). In addition, polyphosphoinositide metabolism has emerged as a candidate mediating membrane transport in both yeast and a number of cell types. This is due to the apparent association of the enzymatic machinery of phosphoinositide metabolism with that of membrane trafficking. For example, the phosphatidylinositol transfer protein (PtdInsTP), the product of the SEC14 gene in yeast, is required for constitutive secretion in yeast (Cleves *et al.*, 1991; Skinner *et al.*, 1993). Its mammalian homologue plays a similar role in neurotransmitter release in PC 12 cells (Hay & Martin, 1993). The VSP34 mutant of yeast, deficient in membrane trafficking, has been established as a lesion at the level of a PtdIns 3-kinase enzymatic activity (Schu *et al.*, 1993) and PtdIns 4-kinase activity is localised in secretory granules from adrenal chromaffin cells (Husebye & Flatmark, 1988), coated vesicles (Campbell *et al.*, 1985) and glucose transporter carrying vesicles (Del Vicchio & Pilch, 1991). This therefore suggests that the activation of PLD by ARF and PtdIns(4, 5)P<sub>2</sub> in concert coordinately regulates membrane trafficking. This is supported by the stimulation of PtdIns(4)P 5-kinase activity by PtdOH, dramatically influencing the synthesis of PtdIns(4, 5)P<sub>2</sub> (Moritz *et al.*, 1992). Consequently the ability of PtdIns(4, 5)P<sub>2</sub> to enhance activation of PLD and the ability of PtdOH to influence the activity of PtdIns(4)P 5-kinase has formed the basis for the model, proposed by Liscovitch *et al.*, (1994), in which the formation of PtdOH, by PLD, and PtdIns(4, 5)P<sub>2</sub> participate in a positive feedback loop regulating cellular vesicular and membrane trafficking (see Fig 1.4).

Information generated from a number of studies upon the phosphoinositide kinases, ARF proteins, PLD and membrane trafficking may be relevant in defining this proposed model system. It has been proposed that the GTP-bound form of ARF



**Fig 1.4 A hypothetical model for the co-ordinate regulation of vesicular membrane trafficking involving the activation of ARF, PLD and the phosphoinositide kinases.** Abbreviations used: PC, phosphatidylcholine; PI4P, phosphatidylinositol 4-phosphate; PI4K, phosphatidylinositol 4-kinase; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; PIP2, phosphatidylinositol (4, 5)-bisphosphate.

initiates the assembly of coated vesicles on donor membranes and their subsequent budding off (Orci *et al.*, 1993). These vesicles are likely to contain PtdIns(4)P due to the presence of PtdIns 4-kinase (Campbell *et al.*, 1985; Del Vicchio & Pilch, 1991; Husebye & Flatmark, 1988) and their subsequent interaction with acceptor membranes would result in the ARF-GTP activating membrane bound PLD (Brown *et al.*, 1993; Cockcroft *et al.*, 1994). Activation of PLD would result in an increase in local PtdOH concentration, stimulate PtdIns(4)P 5-kinase activity, putatively co-localised at acceptor membranes and dramatically increase local PtdIns(4, 5)P<sub>2</sub> concentrations further augmenting PLD activation. Responsibility for termination of such activity may rest with the associated GTPase-activating protein for ARF, ARFGAP. This regulatory enzyme is synergistically activated by PtdIns(4, 5)P<sub>2</sub> and PtdOH (Randazzo & Kahn, 1994). Therefore, the interaction of ARFGAP with these vesicles enriched in PtdIns(4, 5)P<sub>2</sub> and PtdOH may lead to its activation, stimulating GTP hydrolysis by ARF converting it to the GDP bound and inactive form. Consequently, PLD activity would cease, the positive feedback loop would terminate and the coat proteins disassemble and the lipid vesicle fuse with the acceptor membranes.

Further evidence in support of such a model has come from investigations conducted in permeabilised U937 myeloma cells where MgATP potentiates GTPγS-stimulated PLD activation (Pertile *et al.*, 1995). This was proposed to represent ongoing PtdIns(4, 5)P<sub>2</sub> synthesis, MgATP being required for activity of the phosphoinositide kinases and maintenance of both PtdIns(4)P and PtdIns(4, 5)P<sub>2</sub> levels. Use of an inhibitory antibody directed against PtdIns 4-kinase completely inhibited PtdIns 4-kinase activity and dramatically reduced cellular PtdIns(4)P and PtdIns(4, 5)P<sub>2</sub> levels in the permeabilised cells. This coincided with complete abrogation of GTPγS-stimulated PLD activity and approximately 60% inhibition of PMA-stimulated activation (Pertile *et al.*, 1995) and again suggests a critical functional role for PtdIns(4, 5)P<sub>2</sub> and its synthesis in the initiation and maintenance of PLD activation.

Further support for this proposed model is the inhibition of secretory events in varying cell types upon pretreatment with a competing peptide corresponding to the N-terminus of ARF. This includes both inhibition of catecholamine release from adrenal chromaffin cells (Morgan & Burgoyne, 1993) and exocytotic activity in HL-60 cells (Fensome *et al.*, 1994). Again, ARF activity is both required and fundamental to trafficking events. In addition these peptides also inhibit PLD activation (Fensome *et al.*, 1994; R. Randall, personal communication) and indicate the potential involvement of PLD, in association with ARF, in these vesicular trafficking events.

Therefore, a distinct physiological role for ARF-sensitive PLD activities involving the coordinate regulation of ARF, phosphoinositide kinases and PLD may be evident. It must be stressed, however, that relationship of such a model to receptor-stimulated PLD activation in intact cells remains to be extensively examined. Indications of receptor mediated regulation of ARF-activity and status have been described. ARF binding to Golgi membranes is mediated by both receptor agonists and PKC activation (De Matteis *et al.*, 1993) and may indicate the potential regulation of such a system in an agonist-dependent manner. In addition, this does not account for the functionality of the sodium oleate-dependent PLD isolated from porcine lung (Okamura & Yamashita, 1994) and the related oleate-activated PLD activity from rat brain (Massenburg *et al.*, 1994). Their contribution to agonist-stimulated PLD activation, the physiological significance of the PtdOH generated by their activation and their relationship to ARF-sensitive activities remains to be elucidated.

#### 1.11.5 PtdCho-PLD Enzymes Are Distinct From Phospholipid Base-Exchange Enzymes and the GPI-PLD Enzymes.

The group of enzymes that catalyse phospholipid remodelling are the base-exchange enzymes and have previously been confused with PLD activities. They are both phosphatidyl transferases. However, despite these similarities base-exchange enzymes are not hydrolases and do not produce PtdOH. PLD activities can also utilise primary alcohol at millimolar concentrations in the generation of phosphatidylalcohols

by means of a transphosphatidyl transfer reactions. Base-exchange enzymes are unable to catalyse such a reaction and fail to generate phosphatidylalcohols (Gustavsson & Alling, 1987; Siddiqui & Exton, 1992). In addition, noticeable differences in the enzymological characteristics of base-exchange and PLD activities have been demonstrated by studies *in vitro* (reviewed by Kanfer, 1980). In one outstanding example, the base-exchange and PLD activities associated with subcellular fractions of rat liver were described to be clearly dissociated and displayed different subcellular localisation and sensitivity to divalent cations (Siddiqui & Exton, 1992). However, it was suggested that base-exchange activity, like that of PLD, could be regulated in both an agonist and G-protein dependent manner. The relationship of these events to the mechanisms that regulate agonist-stimulated PLD activation remains to be clarified, though molecular cloning of the mammalian base-exchange and PLD enzymes will allow this to be further elucidated.

It is now well established that mammalian plasma contains a PLD activity which is able to remove PtdOH from glycosylphosphatidylinositols (GPI), termed GPI-PLD (reviewed by Low, 1989). This PLD activity is produced as a secretory protein by the Islets of Langerhans (Metz *et al.*, 1991) and a range of myeloid cell types (Xie & Low, 1994) and releases GPI-anchored proteins from membranes either at the cell surface or in an intracellular compartment (see Low, 1989). This enzyme has been purified, cloned and sequenced and demonstrated the existence of at least two distinct human cDNAs (see Huang *et al.*, 1990; Low & Huang, 1993). However, this GPI-PLD activity can be distinguished from agonist-stimulated PtdCho-PLD and PtdIns-PLD activities as glycerophospholipids do not serve as substrates for the enzyme (Hoener *et al.*, 1990). Again, the relationship between the GPI-PLD activities and the signalling PtdCho-PLD activities awaits further clarification by molecular studies.

#### 1.11.6 Purification of PLD Isoenzymes.

Information regarding the purification of PLD enzymes initially came from its isolation from non-mammalian sources, including plant tissues and bacteria. This is

best illustrated by the series of investigations in which PLD was purified 680-fold from Savoy cabbage (Davidson & Long, 1958; Dawson & Hemington, 1967, Yang *et al.*, 1967; Allgyer & Wells, 1979). The enzyme was inactive at high ionic strength, displayed pH optima of 7.25 and 6.0 at low and high  $\text{Ca}^{2+}$  concentrations respectively and displayed both parabolic and hyperbolic kinetics towards substrate at set pH and ionic strength conditions (Allgyer & Wells, 1979).

A 31kDa PLD has been directly cloned and sequenced from the bacteria *Corynebacterium pseudotuberculosis* (*C.pseudotuberculosis*) and expressed in *E. coli* (Hodgson *et al.*, 1990). Comparison of the of protein sequence with the PLA<sub>2</sub> protein of *Laticauda laticaudata* showed a region of homology conferring a putative metal ion binding site. As the PLD of *C.pseudotuberculosis* was  $\text{Mg}^{2+}$  dependent, this structural motif was proposed to fulfil this role and be required for activity (Hodgson *et al.*, 1990). Similar approaches have allowed identification of PLD enzymes in *Ricinus communis* (Wang *et al.*, 1994) and the fungi *Streptomyces antibioticus* (Iwasaki *et al.*, 1994). Despite the isolation of these PLD activities it is unknown how these relate to mammalian enzymes and their stimulus-dependent activation. As yet, none of the molecular information of these non-mammalian enzymes has been utilised in novel approaches to purify or clone their mammalian counterparts.

The first partial purification of a mammalian PLD was that of a cytosolic activity from human eosinophils (Kater *et al.*, 1976). Following ion-exchange and gel filtration the enzyme was characterised and displaying a pH optimum of 4.5-6.0 and a molecular weight of 60kDa. These characteristics were directly compared to PLD of Savoy cabbage and found to be similar except that upon gel-filtration the cabbage enzyme had a molecular weight between 100-200kDa (Kater *et al.*, 1976).

A more extensive purification of a mammalian PLD distinct from base-exchange activities was that of a membrane localised activity from rat brain (Taki & Kanfer, 1979). This involved the partial purification (240-fold) of the microsomal enzyme from Miranol H2M-solubilised rat brain that utilised both PtdCho and PtdEtn as substrates and displayed an acidic pH optima (6.5). The protein had an molecular



weight of 200kDa upon gel filtration. Subsequent studies, identified the unsaturated fatty acid oleate as a potent activator of this PLD activity *in vitro* and allowed identification of PLD activity in a range of rat tissues (Chalifour & Kanfer, 1982). A similar activity has been identified in rat brain microsomes (Chalifa *et al.*, 1990) and partially purified (M. Liscovitch, personal communication), however, this enzyme displays a neutral pH optima (7.2). It remains unclear as to whether this represents another PLD isoform or reflects variation in tissue preparation and assay techniques.

A PLD activity with characteristics common to the ones identified in the above studies has been purified to homogeneity from heptylthioglucoside-solubilised pig lung microsomes (Okamura & Yamashita, 1994). The enzyme displayed specificity for PtdCho, a pH optimum of 6.6 and molecular weight of 190kDa.

A neutral membrane-associated PLD activity has been partially purified from TX-100-solubilised crude plasma membranes of rat lung (Martin, 1988). This enzyme was dependent upon TX-100 for hydrolytic activity and its relationship to the activities described above remains unresolved since no investigations into the effect of oleate upon activity were made. Despite this, further studies illustrated that purification of oleate-activated rat brain PLD could also be initiated by solubilisation of microsomes with TX-100 (Kobayashi & Kanfer, 1991; Liscovitch *et al.*, 1994). Additionally, suitable dilution of TX-100 during *in vitro* assay allowed oleate activation of the enzyme to be observed (Kobayashi & Kanfer, 1991). The relative effects of TX-100 and oleate upon each of these enzymes and whether they represent a means of distinguishing between individual isoforms remains to be reconciled. However, the chromatographic separation of two membrane-associated PLD activities from solubilised rat brain particulate fraction has demonstrated the presence of an oleate-activated PLD activity distinct from an additional ARF-regulated PLD activity (Massenberg *et al.*, 1994).

The partial purification of the previously described ARF-stimulated, membrane-associated PLD in HL-60 granulocytes has been described (Brown *et al.*, 1993). Washing of crude membranes from these cells with hypertonic salt solutions

generated a solubilised activity and was subsequently partially purified by a number of chromatographic steps. This has been further extended to the purification of a similar ARF-sensitive membrane-associated PLD from porcine brain (Brown *et al.*, 1995)

During these studies, the presence of *n*-octyl- $\beta$ , D-glucoside was required for maintenance of activity and this may infer similarities to the previously described membrane-associated and cytosolic activities partially purified 20-fold from bovine lung also solubilised and chromatographed in the presence of *n*-octyl- $\beta$ , D-glucoside (Wang *et al.*, 1991). These enzymes were dependent upon the presence of *n*-octyl- $\beta$ , D-glucoside for activity, characterised as a membrane-associated PtdCho-specific PLD and a cytosolic activity that hydrolysed PtdEtn>PtdCho>PtdIns. Therefore, with differences in biochemical characteristics under the same *in vitro* assay conditions these observed cytosolic and membrane bound activities may indeed be distinct isoforms as previously described. The relationship of these *n*-octyl- $\beta$ , D-glucoside-dependent activities to both the ARF-activated and oleate-dependent activities remains to be clarified.

Thus, partial purification studies have highlighted the potential existence of multiple PLD isoforms, two distinct isoforms being identified in rat brain (Massenberg *et al.*, 1994) with a comparable oleate-dependent isoform being purified to homogeneity from pig lung (Okamura & Yamashita, 1994). The ARF-activated PLD activity remains to be purified to homogeneity yet its identification in parallel to the oleate-dependent activity may represent the emergence of a multiple PLD enzyme family.

### **1.12 Aim of Project.**

Receptor-stimulated phospholipid hydrolysis is a common response to a number of hormones and growth factors. Within this, PLD catalysed hydrolysis results in the generation of free base and the putative second messenger PtdOH. The PLD activity involved appears to preferentially catalyse the hydrolysis of PtdCho,

however, this substrate specificity is not shared by all the PLD activities monitored in crude fractions from a range of cells and tissues. Additionally, the relationship between the activities monitored *in vitro* and *in vivo* remains unclear.

At the initiation of this work no mammalian PLD enzymes had been purified to homogeneity. Therefore, the aim of this project was the purification of a PtdCho-specific PLD activity responsible for agonist-stimulated hydrolysis. Tissue distribution studies in a number of mammalian tissues and tissue availability suggested bovine spleen to serve as an appropriate source of enzyme.

To purify a PtdCho-specific PLD, PtdCho was to be utilised as substrate for monitoring reaction velocities at all stages during the development of the purification protocol and allow resolution of this activity from other non-selective PLD isoforms. This selectivity would be confirmed by comparison of the purified preparations' reaction velocities towards other glycerophospholipids under identical assay conditions.

## **Chapter 2**

### **Materials and Methods**

## **2.1 Materials**

All reagents were of analytical or similar grade; purchased from the following suppliers.

### **Amersham International p.l.c. , Aylesbury, U.K.**

n-[3, 4(n)-<sup>3</sup>H] butanol.

L-3-Phosphatidyl [N-methyl-<sup>3</sup>H] choline, 1, 2-dipalmitoyl.

L-3-Phosphatidyl [2-<sup>14</sup>C] ethanolamine, 1, 2-dioleoyl.

L-3-Phosphatidyl [2-<sup>3</sup>H] inositol, mixed acyls.

L-3-Phosphatidyl [3-<sup>14</sup>C] serine, 1, 2-dioleoyl.

Donkey anti-rabbit Ig horseradish-peroxidase conjugated antibodies.

Enhanced chemiluminescence (ECL) reagents.

### **Amicon Ltd., Stonehouse, U.K.**

Centriprep 10 microconcentrators.

### **B.D.H. Ltd. Poole, U.K.**

EDTA, formaldehyde and gluteraldehyde.

### **Bio-Rad Laboratories Ltd., Hemel Hempstead, U.K.**

BioGel HTP (hydroxyapatite), Econo-Pac hydroxyapatite cartridges (pre-packed, 5ml) and Detergent Compatible Protein Assay Kit.

### **Boehringer Mannheim., Lewes, U.K.**

DTT and Tris base.

**Fisons Scientific Equipment, Loughborough, U.K.**

Absolute alcohol, acetic acid (glacial), acrylamide, chloroform, citric acid, glycine, hydrochloric acid, methanol, *N, N'*-methylenebisacrylamide,  $\text{MgCl}_2 \cdot 6(\text{H}_2\text{O})$ , Mops,  $\text{Na}_2\text{CO}_3$ , NaCl, NaOH,  $\text{Na}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$ , sodium acetate and urea.

**Janssen Chimica, Newton, Hyde, U.K.**

Heptan-4-one and Mes.

**Lipid Products, Redhill, U.K.**

Lecithin (bovine spinal cord)

**May and Baker Ltd., Dagenham, U.K.**

Ammonium persulphate.

**Pharmacia, Milton Keynes, U.K.**

Mono-S HR 5/5 FPLC column, Phenyl-Sepharose, Q-Sepharose FF, SR-12 Gel Filtration FPLC column, and S-Sepharose FF.

**Scientific Instrument Centre Ltd. Eastleigh, U.K.**

Dialysis tubing (Visking).

**Scientific Marketing Associates, Barnet, U.K.**

E-64 [*L-trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane], leupeptin and pepstatin A.

**Sigma, Poole, U.K.**

Aprotinin (bovine lung), bovine serum albumin (fraction V), bromophenol blue, choline chloride, ethanolamine hydrochloride, *myo*-inositol, heparin-agarose (Type I), phosphatidylinositol (soybean), phosphatidylethanolamine (bovine brain),

phosphatidylserine (bovine brain), PMSF, L-serine, sodium cholate, sodium deoxycholate, sodium fluoride, TEMED, sodium tetraphenylboron, Triton X-100 and Tween 20.

**Whatman LabSales Ltd., Maidstone, U.K.**

LK5DF pre-laned TLC plates.

## **2.2 Methods: Determination of protein concentration.**

### **2.2.1 Detergent Compatible Method.**

Protein concentration was determined using the Bio-Rad DC Protein Assay Kit based upon the method of Lowry *et al.* (1951). Samples (200µl) were prepared in duplicate and 100µl of Solution A (Copper Sulphate solution) and 800µl of Solution B (Folin's Reagent) added as described in the manufacturers Micro assay protocol. Standards (0-50µg) constructed from 1.0mg/ml bovine serum albumin were prepared in the same manner. All samples were incubated for 15 min at room temperature and absorbance recorded at 750nm.

### **2.2.2 U.V. Absorbance Method.**

The protein concentration of chromatography column eluants was determined by the 'on line' monitors connected in series with the apparatus utilised for routine preparative chromatography; UVS II with the Pharmacia Hi-Load system and LCC500/UVM with the Pharmacia FPLC system. The absorbance of the eluant was monitored at 280nm in a 3mm quartz flow cell and recorded by the connected pen recorder.

## **2.3 SDS-Polyacrylamide Gel Electrophoresis.**

Electrophoresis was performed using the method of Laemmli (1970). Slab gels were routinely performed employing either 100mm x 140mm x 1.5mm gel plates

as part of a Bio-Rad Protean system, or as mini-slab gels using a Hoeffer 'Mighty Small' system.

### 2.3.1 Lower Resolving Gels

Separating gels contained 10% (w/v) acrylamide, and 0.27% (w/v) bisacrylamide with 0.375M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.033% (w/v) glycerol, 0.0003% (v/v) TEMED and 0.0004% (w/v) ammonium persulphate. For 1.5mm thick slab gel a final volume of 16ml was used and for mini-gels 4.5ml was used.

### 2.3.2 Upper Stacking Gels.

The upper stacking gels contained 3% (w/v) acrylamide and 0.08% (w/v) bisacrylamide with 0.125M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.0005% (v/v) TEMED and 0.001% (w/v) ammonium persulphate. An appropriate volume of the stacking gel solution was poured into the electrophoresis apparatus and the well comb introduced before polymerisation.

### 2.3.3 Sample Preparation.

The protein samples for electrophoresis were corrected to 740µl with H<sub>2</sub>O and 10µl 2% (w/v) sodium deoxycholate added. Subsequent to mixing, proteins were precipitated by addition of 250µl of 24% (w/v) trichloroacetic acid and the samples incubated at room temperature for 5 min after further vigorous mixing. The precipitate was pelleted by centrifugation (18,000 x g, 15 min, 4 °C) and the supernatant removed by careful aspiration. Appropriate equal volumes of 1.0M Tris base and Laemmli buffer were added to the pellet and the samples solubilised by incubation in a boiling water bath for 3 min [Laemmli buffer: 30% (w/v) urea, 5% (w/v) SDS, 6% (w/v) DTT, 20mM Tris-HCl pH 8.0, 0.2% (w/v) bromophenol blue].



#### 2.3.4 Electrophoresis Running Buffers.

The running buffer contained 25mM Tris-HCl pH 8.5, 0.192M glycine and 0.1 % (w/v) SDS. Samples were introduced to the wells of the stacking gel using Gilson 'Ducktail' pipette tips and electrophoresis performed towards the anode at 150V, 25mA until the dye front was 0.5cm from the end of the resolving gel.

#### 2.3.5 Silver Staining of SDS-Polyacrylamide Gels .

Upon completion of electrophoresis, gels were silver stained according to the method of Morrissey (1981). Gels were prefixed for 30 min in 50% (v/v) methanol, 10% (v/v) acetic acid followed by 5% (v/v) methanol, 7% (v/v) acetic acid again for 30 min. The fixed gels were placed in 10% (v/v) glutaraldehyde and incubated for 30 min. The glutaraldehyde was removed by washing the gels with multiple changes of distilled water over 3 hours. After washing, the gels were placed in an aqueous solution of 5µg/ml DTT for 30 min followed by immersion in 0.1% (w/v) silver nitrate for 30 min. Excess silver nitrate solution was removed by aspiration and the gels washed briefly in a small volume of developer solution (3% (w/v) Na<sub>2</sub>CO<sub>3</sub>, 0.019% (v/v) formaldehyde). This was rapidly removed by aspiration and development continued by immersion of the gel in 100ml of developer solution. When the desired level of staining was achieved the reaction was terminated by the addition of 12ml of 2.3M citric acid to the developer solution and agitated for 10 min. The developed gels were then transferred to 10% (v/v) glacial acetic acid and left overnight. In preparation for photography, the gels were rinsed and stored in distilled water at 4°C in the dark.

#### 2.4 Assay of Phospholipase D Activity.

The reaction velocity of bovine spleen PLD was measured by means of a phosphatidylcholine (PtdCho)/Triton X-100 (TX-100) mixed micellar assay. This methodology was also employed for the kinetic characterisation of the purified PLD.

The bovine spleen PLD was incubated in a final reaction volume of 120 $\mu$ l containing 0.1% (w/v) TX-100, 2.8mol % (39 $\mu$ M) phosphatidylcholine [0.26 $\mu$ Ci [ $^3$ H]PtdCho/nmol PtdCho], 50mM Hepes pH 7.5, 2mM EDTA. An appropriate amount of PtdCho and labelled PtdCho were dried under a stream of nitrogen and resolubilised in 2mM EDTA pH 7.5, containing an appropriate concentration of TX-100, by vigorous vortexing and sonication in a bath type sonicator at 4 $^{\circ}$ C for 15 min. The final concentration of the PtdCho in the sonicate was 78 $\mu$ M and served as a 2x strength substrate for aliquoting to the final assay tubes. Concentrated buffer (600mM Hepes pH 7.5, 2mM EDTA) was added in a volume of 10 $\mu$ l. The tubes were vortexed, sonicated for 5 min as above and incubated at 37 $^{\circ}$ C for 3 min.

Reactions were then initiated by addition of protein samples and appropriate controls in a volume of 50 $\mu$ l. Assay tubes were briefly vortexed and maintained at 37 $^{\circ}$ C for 45 min. The reactions were terminated by the addition of 750 $\mu$ l of CHCl<sub>3</sub>:MeOH (1:2, v/v) and incubated at room temperature for 15 min. Organic and aqueous phases were resolved by the addition of 250 $\mu$ l CHCl<sub>3</sub> and 330 $\mu$ l H<sub>2</sub>O followed by brief centrifugation (2500 x g, 5 min). The upper aqueous phase was removed to a clear 5ml insert vial and 650 $\mu$ l H<sub>2</sub>O added. Tritiated choline produced, was recovered from the collected aqueous products by means of an ion-pair extraction as described by Murray *et al.*, (1990). One ml of heptan-4-one, containing tetraphenylboron (5mg/ml), was added to each insert vial, vigorously vortexed and centrifuged (2500 x g, 5 min). The upper heptanone phase, containing tetraphenylboron-extracted  $^3$ H-choline, was removed and radioactivity determined by liquid scintillation counting in 10ml of Optiphase scintillant.

## **2.5 Preparation of Solubilised PLD.**

### **2.5.1 Preparation of Bovine Spleen Crude Plasma Membrane Fraction.**

A fresh bovine spleen obtained from a local abattoir was rapidly decapsulated and finely chopped into ice-cold homogenisation buffer (25mM Hepes pH 7.0, 154mM NaCl, 2mM EDTA, 5 $\mu$ g/ml leupeptin, 5 $\mu$ g/ml aprotinin, 1 $\mu$ M E-64, 200 $\mu$ M

PMSF, 1 $\mu$ M pepstatin A). The buffer was decanted and the chopped tissue homogenised in 2-3 volumes of the same buffer; initially by 4 x 30 sec periods in a tissue blender, followed by 5 strokes of a teflon pestle in a glass homogenisation vessel at approximately 800rpm. The homogenate was centrifuged (1000 x g, 15 min) and the pellet discarded. The supernatant was filtered through cheesecloth and then centrifuged (35,000 x g, 45 min) to yield a pellet representing the crude plasma membrane fraction and a supernatant representing a crude cytosolic fraction. The cytosolic fraction was discarded and the pellet resuspended in an equal volume of the homogenisation buffer and washed by centrifugation (35,000 x g, 45 min). The final pellet was resuspended in homogenisation buffer at an approximate protein concentration of 15mg/ml and stored overnight at -80°C.

#### 2.5.2 Solubilisation of Membrane-Associated PLD Activity from the Crude Plasma Membrane Fraction of Bovine Spleen.

The stored crude plasma membranes were thawed and pelleted by centrifugation (40,000 x g, 45 min), the supernatant collected and termed the 'pre-spin' activity. The particulate material was resuspended in an equal volume of 50mM Na<sub>2</sub>HPO<sub>4</sub> pH 12 supplemented with leupeptin, aprotinin, E-64, PMSF and pepstatin A (at the concentrations described in Section 2.5.1) and incubated at 4°C for 30 min with agitation. Generation of soluble PLD activity was achieved by centrifugation (40,000 x g, 45 min) of this suspension and collection of the resultant supernatant. This 'phosphate-extracted' supernatant was pooled with the 'pre-spin' supernatant and brought to pH 7.5 by gradual addition of 500mM NaH<sub>2</sub>PO<sub>4</sub> and termed the solubilised PLD activity for ammonium sulphate precipitation and further purification of the membrane-associated PLD.

### 2.5.3 Ammonium Sulphate Precipitation of the Solubilised PLD from the Crude Plasma Membrane Fraction of Bovine Spleen.

The solubilised PLD activity was maintained at 4°C with constant mixing and solid ammonium sulphate added gradually (calculated from Dixon, 1953) over 45 min until saturation of 50% achieved, pH being maintained by the addition of NH<sub>4</sub>OH. The suspension was mixed for 30 min, and the precipitate collected by centrifugation (10,000 x g, 15 min). The supernatant was decanted and the pellet discarded. Further solid ammonium sulphate was added to the recovered supernatant over 30 min until saturation of 70% achieved. Again, after 30 min mixing the precipitate was recovered by centrifugation as described above and the supernatant discarded. The pellet containing the PLD activity was resuspended in 20-40ml of homogenisation buffer and dialysed against 2 x 5 litres of chromatography equilibration buffer (25mM Mes pH 6.0, 2mM EDTA, 0.1% (w/v) TX-100, supplemented with leupeptin, aprotinin, E-64, PMSF, pepstatin A as described in Section 2.5.1) to remove ammonium sulphate. The dialysate was collected and clarified by centrifugation (10,000 x g, 15 min) and the supernatant used for further purification of the membrane-associated PLD.

### 2.6 Preparation of Chromatography Resins: S-Sepharose FF and Heparin-Agarose.

The following procedures were performed at 4°C. An appropriate volume of each resin was transferred to a clean glass bottle and allowed to settle. The supernatant was decanted and the settled gel resuspended in 10 volumes of 250mM Mes pH 6.0, 1M NaCl. The resin was again allowed to settle and the supernatant removed by aspiration. The above procedure was repeated twice and a further three times with chromatography equilibration buffer as described in Section 2.5.3. Finally, the supernatant was aspirated to generate a resin slurry of 75% which was used for column packing. The column was then equilibrated with 10 volumes of chromatography buffer.

## **2.7 Scanning Spectrophotometry.**

Continuous scanning spectrophotometry of partially purified enzyme preparations was performed between 200 and 600nm in a Shimadzu UV-2101PC UV-Visible scanning spectrophotometer. Absorbance profiles were recorded by a Roland Sketchmate printer.

## **2.8 Anti-p47 and p67<sup>phox</sup> Immunoblotting.**

Aliquots of whole cell lysates and partially purified PLD from chromatography steps prepared in SDS-sample buffer, as described in Section 2.3.3, were subjected to SDS-PAGE on 10% polyacrylamide slab gels and then transferred onto nitrocellulose. The nitrocellulose membranes were incubated for three hours in 10mM Hepes, 0.5M NaCl pH 7.4, containing 3% BSA and 10% donkey serum then incubated overnight in 10mM Hepes, 0.5M NaCl pH 7.4, containing 1% BSA, 0.2% (w/v) Tween 20, 3% donkey serum and  $1\mu\text{gml}^{-1}$  of rabbit polyclonal anti-p47 and p67<sup>phox</sup> antibody respectively. Following six washes over 30 min in 10mM Hepes, 0.5M NaCl pH 7.4 containing 0.2% Tween 20 (w/v) the membranes were incubated with anti-rabbit Ig-horseradish-peroxidase conjugate (HRP) for 1 hour. The membranes were then washed a further 4 times over 20 min and then finally for 5 min in 10 mM Hepes, 0.5M NaCl pH 7.4 in the absence of Tween 20 before development using the ECL chemiluminescent detection system (Amersham).

## **2.9 Miscellaneous Procedures.**

### **2.9.1 Concentration of Protein Solutions.**

Pooled chromatography eluants requiring concentration, usually 1-5ml, were placed in Centrprep 10 microconcentrators and centrifuged (2500 x g, 4°C) until the desired volume was obtained.

### 2.9.2 Preparation of Dialysis Tubing.

Dialysis tubing was hydrated in distilled H<sub>2</sub>O and then boiled twice for 30 min in 10mM EDTA. Before use, the tubing was allowed to cool and extensively rinsed with distilled H<sub>2</sub>O.

### 2.9.3 Critical Micelle Concentrations.

Values for detergent critical micelle concentration (cmc) employed in this work were as described by Neugebauer (1990).

### 2.9.4 Chromatography Buffers.

Unless otherwise stated the chromatography buffer used was 25mM Mes pH 6.0, containing 2mM EDTA and 0.1% (w/v) TX-100. All buffers throughout the purification protocol contained the following protease inhibitors; 5µg/ml leupeptin, 5µg/ml aprotinin, 1µM E-64, 200µM PMSF and 1µM pepstatin A.

### 2.9.5 Statistical Analysis.

Unless otherwise stated, the results presented within this thesis are representative of at least three experiments.

The PLD activities measured in fractions recovered from chromatography columns are single point determinations. Otherwise, values stated are expressed as the mean of a triplicate determination and the error expressed as  $\pm$  Standard Deviation.

**Chapter 3**  
**Partial Purification of the Membrane-Associated PLD.**

### 3.1 Introduction.

#### 3.1.1. Preparation of an Appropriate Source of Enzyme Activity for Purification.

The formation of phosphatidylalcohols in a stimulus-dependent manner has allowed the description of agonist-dependent PLD activation in a wide variety of cells and tissues (reviewed by Billah & Anthes, 1990; Thompson *et al.*, 1993; Exton, 1994). Although the physiological importance and function of PLD is still unclear, insight can be gained in understanding enzyme function by the preparation of highly purified protein. In order to purify any protein, both assay conditions and the potential source of protein must be carefully considered.

Using a variety of *in vitro* assays PLD activity has been described in subcellular fractions from a number of cell types and most commonly in bovine and rat tissues (for summary see Thompson *et al.*, 1993). Cytosolic PLD activity in bovine tissues varies greatly, with the following order:

lung>brain=spleen>heart=kidney>thymus>liver (Wang *et al.*, 1991). Membrane-bound and microsomal PLD activity in various rat tissues exhibited a similar distribution to the bovine tissues where

lung>brain=spleen>heart=kidney>thymus>liver>testis (Martin, 1988; Chalifour & Kanfer, 1982). From these reports, it was apparent that PLD activity was consistently highest in lung, brain and spleen tissues of both rat and bovine tissues even when lipid substrates were presented in differing assay environments. Abundant PLD activity has also been reported in subcellular fractions of neutrophils and related myeloid cell types such as HL-60 granulocytes (see Thompson *et al.*, 1993). PLD activity in membranes from these cell types appeared to be regulated in a GTP $\gamma$ S-dependent manner by a required cytosolic factor (Anthes *et al.*, 1991; Geny *et al.*, 1992), proposed to be a G-protein (Xie & Dubyak, 1991; Geny & Cockcroft, 1992).

At the initiation of this study, no mammalian PLD enzymes had been purified to homogeneity. However, a 30 and 80kDa PLD enzyme had been partially purified from bovine lung cytosol using anion-exchange and gel filtration chromatography (Wang *et al.*, 1991). Subsequently, a PtdCho-specific PLD was identified in the



particulate fraction of bovine lung and partially purified by anion-exchange chromatography in the presence of *n*-octyl- $\beta$ , D-glucopyranoside (Wang *et al.*, 1991). This was different from the observed 50 kDa activity from human neutrophil cytosol (Bowman *et al.*, 1993) and the partially purified rat brain 200 kDa activity (Taki & Kanfer, 1978)

In accordance with tissue availability and the apparent abundance of PLD in haematopoietic cells, bovine spleen was selected as the source of PLD activity for purification of a PtdCho specific enzyme.

### 3.1.2 Particulate and Cytosolic PLD Activities of Bovine Spleen.

Bovine spleen was homogenised and centrifuged at 35,000 x g to prepare crude cytosolic and plasma membrane fractions. Using a PtdCho/TX-100 mixed micellar assay (Martin, 1988) PLD activity was found to be approximately equally distributed between the two fractions (Table 3.1). The highest specific PLD activity was expressed in the membrane fraction.

**Table 3.1 PLD Activity in Crude Fractions Prepared from Bovine Spleen.**

	<u>Specific PLD</u> <u>Activity</u> <u>(pmol/min/mg)</u>	<u>Fold</u>	<u>Total PLD</u> <u>Activity</u> <u>(pmol/min)</u>	<u>Recovery</u> <u>(%)</u>
Homogenate	0.12	1	6083	100
Cytosol	0.21	2	3042	50
Membranes	1.21	10	4594	76

It was also apparent that upon fractionation of the homogenate, the total activity recovered in cytosolic and membrane fractions represented a greater amount than in the homogenate alone. This may represent the cytosolic localisation of an inhibitory factor consistent with the observed fold increase in PLD activity recovered in the membrane compartment.

Very little PLD activity was recovered from these fractions when assessed by the PtdCho/*n*-octyl- $\beta$ , D-glucopyranoside mixed micellar method described by Wang *et al.* (1991) whereas the use of a PtdCho/oleate dispersion assay (Chalifour & Kanfer, 1982) resulted in specific activities of up to 10-fold higher than those shown here. However, the dispersion assay was not detergent compatible (data not shown) and it was therefore considered to be of limited use in this study. In accordance with the aim of purifying a PtdCho-hydrolysing PLD activity involved in signal transduction, the membrane fraction was used as a source of PLD activity.

### **3.2 Solubilisation of the Membrane-Associated PLD.**

Several groups have reported the solubilisation of membrane-associated PLD activities with a number of non-ionic detergents, including TX-100 and *n*-octyl- $\beta$ , D-glucopyranoside (Martin, 1988; Kanfer, 1991; Kanoh *et al.*, 1991; Wang *et al.*, 1991), however, from these studies to date it has not been possible to assign PLD to an integral or peripheral membrane location. Further, it remains possible that the PLD exists as a cytoskeletally-associated protein with localisation dependent upon protein to protein interactions via cytoskeletal elements anchored to the plasma membrane. Therefore, generation of a soluble PLD activity from bovine spleen membranes was approached utilising a range of solubilising agents. Extraction with detergents to solubilise membrane-bound and associated proteins and either hypertonic concentrations of salt and solutions of high pH to solubilise peripheral membrane-associated proteins (Shariff & Luna, 1990; Shariff & Luna, 1992) were examined.

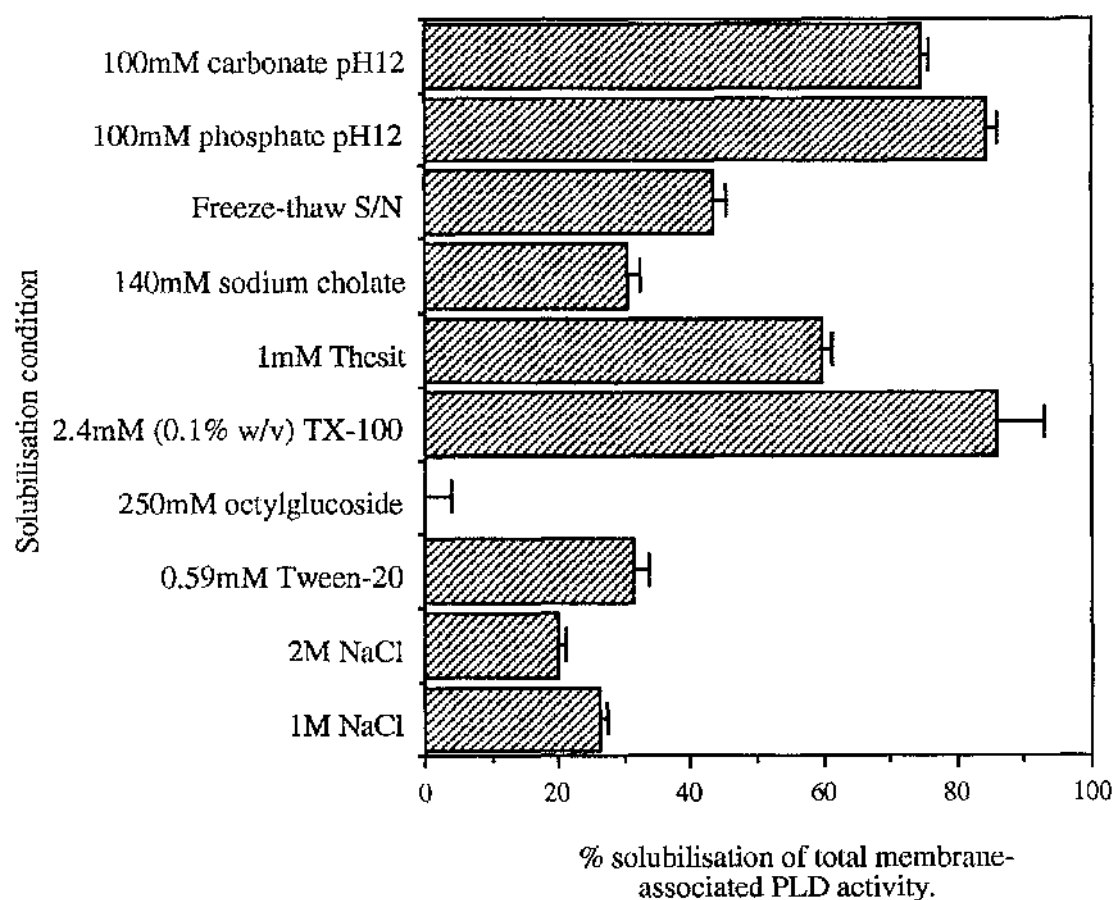
Frozen crude plasma membranes stored at -80°C were thawed and an equal volume of homogenisation buffer, containing the appropriate concentration of detergent or salt, added. The non-ionic detergents TX-100, Tween-20, *n*-octyl- $\beta$ , D-glucopyranoside and Thesit and the anionic detergent cholate were examined at a concentration equivalent to 10 x their critical micelle concentration. In parallel, salt extraction was performed with 1M and 2M respectively. Frozen crude plasma membranes were also thawed, pelleted by centrifugation (100,000 x g, 1 h),

resuspended to 2 x their original volume and exposed to high pH; in either phosphate or carbonate solution at 100 mM, pH 12. All samples were agitated on a rotary wheel for 1 hr at 4°C and solubilisation by these agents assessed as their ability to release PLD activity to the supernatant recovered following centrifugation (100,000 x g, 1 hr) of the extracted membranes (Fig 3.1).

All the agents examined released PLD activity to the supernatant, except *n*-octyl- $\beta$ , D-glucopyranoside (Fig 3.1). Extraction with 1M and 2M NaCl respectively, each resulted in the recovery of a small percentage of the total membrane-associated PLD activity. Detergent extraction was also successful using Tween-20, Thesit and cholate, however, maximal detergent solubilisation was achieved using TX-100 and allowed the recovery of approximately 85 % of the total membrane-associated PLD activity (Fig 3.1).

Membranes were pelleted before incubation with phosphate and carbonate solutions of high pH. It was apparent that up to 45% of the total membrane-associated PLD was present in the 100,000 x g supernatant (Fig 3.1), indicating release of PLD activity upon freeze-thawing and centrifugation. This was not attributed to contaminating cytosolic activity as successive washing of the membranes with homogenisation buffer removed all soluble activity without significantly altering the apparent specific and total PLD activity recovered in freshly prepared crude membranes before freezing (data not shown). Consequently, pH extraction with phosphate and carbonate solutions at alkaline pH resulted in a further  $40.8 \pm 1.6\%$  and  $31.2 \pm 1.0\%$  solubilisation of the total membrane-associated PLD activity respectively (Fig 3.1).

The recovery of soluble activity without treatment of the membranes questioned the ability of the previously described salt and detergent extractions to genuinely solubilise PLD activity. Therefore, it was concluded that TX-100 and pH extraction, in combination with prior freeze-thawing and high speed centrifugation



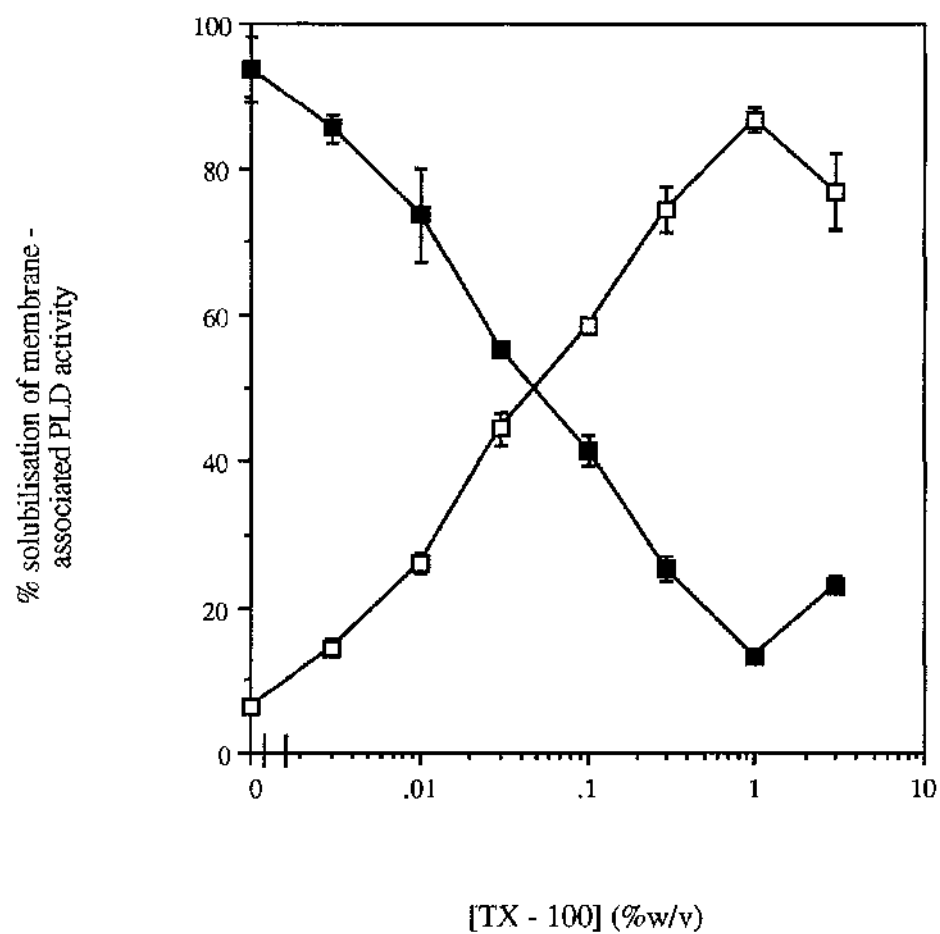
**Fig 3.1 Solubilisation of Membrane-Associated PLD Activity from the Crude Plasma Membrane Fraction of Bovine Spleen.**

Crude plasma membranes from bovine spleen were prepared by homogenisation and differential centrifugation as described in Section 2.5.1 and stored at  $-80^{\circ}\text{C}$ . Frozen membranes were thawed and 0.4 ml aliquots (4mg protein, 7.26 pmol/min PLD activity) were diluted with an equal volume of homogenisation buffer containing detergent or salt as appropriate. For alkaline extraction at pH 12, 0.4 ml aliquots were centrifuged ( $100,000 \times g$ , 1 h) and resuspended in 0.8 ml of 100mM phosphate or carbonate solution pH 12. Samples were then agitated on a rotary wheel for 1 hr at  $4^{\circ}\text{C}$ , centrifuged ( $100,000 \times g$ , 1 h) and the solubilised PLD recovered in the supernatants. 5  $\mu\text{l}$  of the post-solubilisation supernatants were assayed for PLD activity as described in Section 2.4 and protein determined as described in Section 2.2.1. Total PLD activity extracted, expressed as a percentage of the total membrane-associated PLD activity, for each condition is shown opposite. This experiment is representative of another identical experiment.

of the frozen membranes, resulted in maximal release of solubilised PLD activity. For alkaline pH extraction,  $\text{Na}_2\text{HPO}_4$  was chosen in preference to carbonate as a neutral pH could be re-established by direct addition of 500mM  $\text{NaH}_2\text{PO}_4$ . Further, as these agents would effectively solubilise differing amounts of membrane lipid, minimum volumes of recovered solubilised and unsolubilised material (5 $\mu\text{l}$  from each fraction) were assayed for PLD activity. This would minimise the contribution of endogenous lipid to the overall concentration of exogenous lipid present in the *in vitro* PLD assay and therefore have limited influence upon the measured reaction rates of each sample.

The concentration dependence of TX-100 solubilisation of membrane-associated PLD activity was investigated (Fig 3.2). PLD activity was released in a concentration dependent manner with maximal solubilisation achieved at a TX-100 concentration of 16mM (1% w/v). Consistent with the recovery of PLD in the soluble supernatant fraction, a concentration-dependent reduction of PLD activity associated with the pelleted insoluble membrane fraction was also observed (Fig 3.2). Membranes that were not exposed to detergent and further centrifuged showed little release of PLD activity (approximately 6%; data not shown).

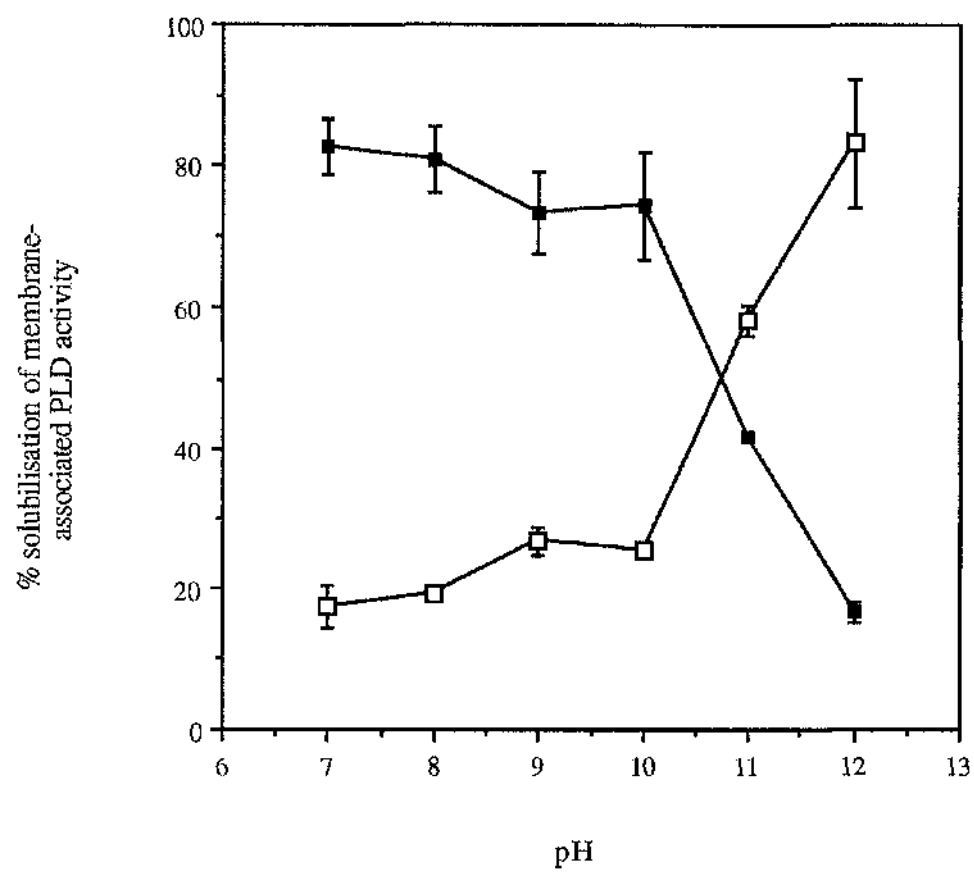
Similar results were obtained when the pH dependence of phosphate extraction was investigated (Fig 3.3). Membranes were thawed and pelleted by centrifugation before exposure to phosphate solutions of varying pH. Up to 45% of the total membrane-associated PLD activity was recovered in freeze-thaw supernatant generated by centrifugation as previously described. After incubation in the presence of phosphate and further centrifugation, additional PLD activity was recovered in the soluble supernatant fraction, but in a pH dependent manner, maximal at pH 12. Parallel reduction of PLD activity in the insoluble membrane fraction was also apparent (Fig 3.3). Incubation of the remaining unsolubilised crude plasma membranes in the presence of 100mM phosphate pH 12 showed little further release (approximately 4%) of PLD activity upon centrifugation (data not shown).



**Fig 3.2 Triton X-100-Dependent Solubilisation of Membrane-Associated PLD Activity from the Crude Plasma Membrane Fraction of Bovine Spleen.**

Crude plasma membranes stored at  $-80^{\circ}\text{C}$  were thawed and 0.4 ml aliquots (4mg protein, 7.26 pmol/min PLD activity) were diluted with an equal volume of homogenisation buffer containing TX-100 as appropriate. Samples were then agitated on a rotary wheel for 1 h at  $4^{\circ}\text{C}$ . The samples were centrifuged ( $100,000 \times g$ , 1 h) and the solubilised PLD recovered in the supernatants. Pellets were resuspended in 0.8 ml of homogenisation buffer. An appropriate volume of the post-solubilisation supernatants and 5 $\mu\text{l}$  of each pellet were assayed for PLD activity as described in Section 2.4; final concentration of TX-100 in the assay was 0.1% (w/v). Protein concentration of each sample was determined as described in Section 2.2.1. Total PLD activity recovered in each of the supernatants and pellets, expressed as a percentage of the total membrane-associated PLD activity, is shown opposite[open squares-solubilised activity; closed squares-unsolubilised activity]. This experiment is representative of another identical experiment.





**Fig 3.3 pH-Dependent Solubilisation of Membrane-Associated PLD Activity from the Crude Plasma Membrane Fraction of Bovine Spleen.**

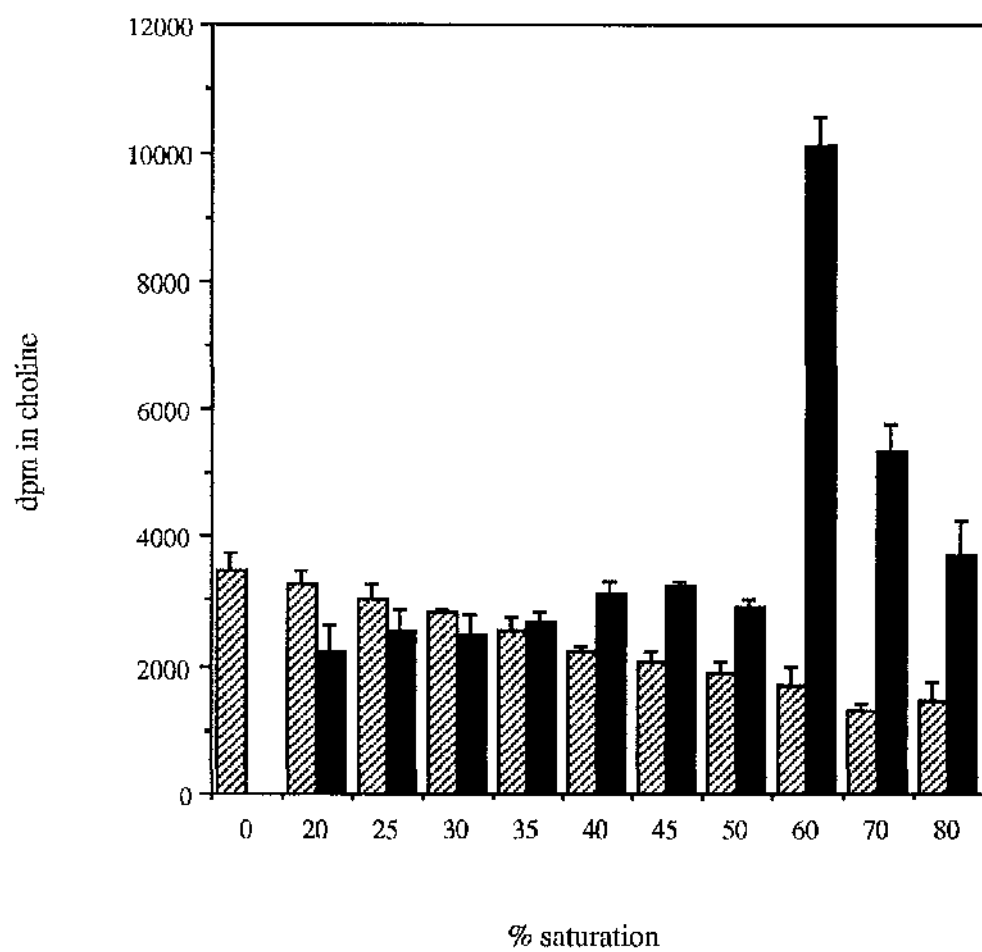
Crude plasma membranes stored at  $-80^{\circ}\text{C}$  were thawed and 0.4 ml aliquots (4mg protein, 7.26 pmol/min PLD activity) were centrifuged ( $100,000 \times g$ , 1 h) and resuspended in 0.8 ml of 100mM phosphate solution of appropriate pH. Samples were then agitated at for 1 h on a rotary wheel at  $4^{\circ}\text{C}$ . The samples were centrifuged ( $100,000 \times g$ , 1 h) and the solubilised PLD recovered in the supernatants. Pellets were resuspended in 0.8 ml of phosphate solution and 5 $\mu\text{l}$  of the post-solubilisation supernatants and pellets were assayed for PLD activity as described in Section 2.4. Protein concentration of each sample was determined as described in Section 2.2.1. Total PLD activity recovered in each of the supernatants and pellets, expressed as a percentage of the total membrane-associated PLD activity, is shown opposite [open squares-solubilised activity; closed squares-unsolubilised activity]. This experiment is representative of another identical experiment.

Therefore, maximal solubilisation could be achieved by either TX-100 extraction or high pH extraction with 100mM phosphate pH 12. Both approaches displayed comparable solubilisation of PLD activity, however, it was recognised that pH extraction after centrifugation allowed recovery of a protein solution depleted of membrane lipid and unlikely to have membrane bound proteins contributing to its heterogeneity. Subsequently, freeze-thawing, centrifugation and alkaline pH extraction of the bovine spleen crude plasma membranes (pH 12) was adopted for solubilisation of the membrane-associated PLD activity. At full preparative scale this solubilisation protocol routinely resulted in  $70 \pm 14$  % (n=5) recovery of the total membrane-associated PLD activity. Further, the absence of detergent allowed the investigation of ammonium sulphate fractionation as a potential means of enzyme concentration and purification (see Section 3.3).

### **3.3 Ammonium Sulphate Precipitation of the Solubilised PLD.**

A number of approaches have been taken towards precipitation of proteins for preparative purposes (reviewed by England & Seifter, 1990). These include perturbation or modification of solvent by changes in pH and temperature or introduction of miscible organic solvents or certain salts. Generation of a soluble PLD activity from the crude plasma membrane fraction (as described in Section 2.5.2) in the absence of detergent allowed the use of ammonium sulphate fractionation as a potential means of concentration and purification of enzyme activity.

Small scale experiments were performed using solubilised PLD preparation and the dissolved bulk protein was precipitated, in increasing steps of 5 or 10% (w/v) saturation, by the successive addition of solid ammonium sulphate at pH 7.5 and 4°C. The salt was added with constant stirring and full equilibration of the solvent achieved by further mixing for 30 min. The precipitate was then collected by centrifugation (10,000 x g, 15 min). The supernatant was decanted for further fractionation and the precipitate resuspended in 25 mM Hepes pH 7.5, 2 mM EDTA. This procedure was repeated until a final saturation of 80 % (w/v) was achieved. A 1



**Fig 3.4 Fractionation of the Solubilised PLD Using Solid Ammonium Sulphate.**

100 ml (207 mg protein) of solubilised PLD was prepared by freeze-thawing, centrifugation and alkaline extraction of crude plasma membranes and adjusted to pH 7.5. Successive fractionations were made by addition, at 4°C with constant stirring, of solid ammonium sulphate. The pH was maintained at 7.5 by addition of concentrated  $\text{NH}_4\text{OH}$ . When all the salt was added the mixture was stirred for a further 15 min. The resultant precipitate was then recovered by centrifugation (10,000 x g, 15 min). The supernatant was decanted and used as the starting material for the next fractionation. Fractions were successively precipitated at saturations of 20, 25, 30, 35, 40, 45, 50, 60, 70 and 80% (w/v) respectively. A 1 ml aliquot of each supernatant was retained and each precipitate was resuspended in 10 ml of 25 mM Hepes pH 7.5, 2 mM EDTA. All fractions were then dialysed overnight against two changes of 1 l of 25 mM Hepes pH 7.5 containing 2 mM EDTA. 20  $\mu\text{l}$  from each fraction was then assayed for PLD activity as described in Section 2.4 [hatched bars-activity remaining in recovered supernatants; solid bars-activity present in recovered precipitates].

ml aliquot of supernatant and precipitate from each saturation point were dialysed overnight against two changes of 1 l of 25 mM Hepes pH 7.0, 2 mM EDTA and assayed for PLD activity (Fig 3.4).

PLD activity was detected in the first precipitate recovered at a saturation of 20% (w/v). As saturation increased to 50% (w/v) the distribution of activity between supernatant and precipitate did not significantly change, however, at 60% (w/v) the majority of the total PLD activity was recovered in the precipitate, with further activity recovered in the precipitates of 70 and 80% (w/v) saturation. However, under these conditions the changes in protein concentration throughout the fractionation protocol were not determined. Therefore, as a step towards full scale preparative fractionation, a similar experiment was performed to investigate the distribution of total PLD activity, specific PLD activity and protein recovered as supernatants or precipitates over the following range of percentage saturation; 0-30%, 30-50%, 50-60%, 60-70% and 70-80% (w/v). This was performed in a similar manner as above, except equilibration of the protein solution achieved by 30 min of constant mixing. Again, samples from each of the recovered precipitates were dialysed overnight and subsequently assayed for protein content, total PLD activity and specific PLD activity (Table 3.2).

**Table 3.2 Changes In Total Protein, Total PLD Activity and Specific PLD Activity During Ammonium Sulphate Fractionation of the Solubilised PLD.**

<u>% saturation</u>	<u>Total protein</u>	<u>Total PLD activity</u>	<u>Specific activity</u>
<u>(w/v)</u>	<u>(mg)</u>	<u>(pmol/min)</u>	<u>(pmol/min/mg)</u>
0-30%	39.7	13.5 ± 1.2	0.34 ± 0.03
30-50%	19.0	19.8 ± 0.9	1.04 ± 0.05
50-60%	8.0	8.7 ± 0.5	1.08 ± 0.06
60-70%	13.8	54.1 ± 1.7	3.93 ± 0.12
70-80%	8.2	16.6 ± 1.0	2.04 ± 0.12

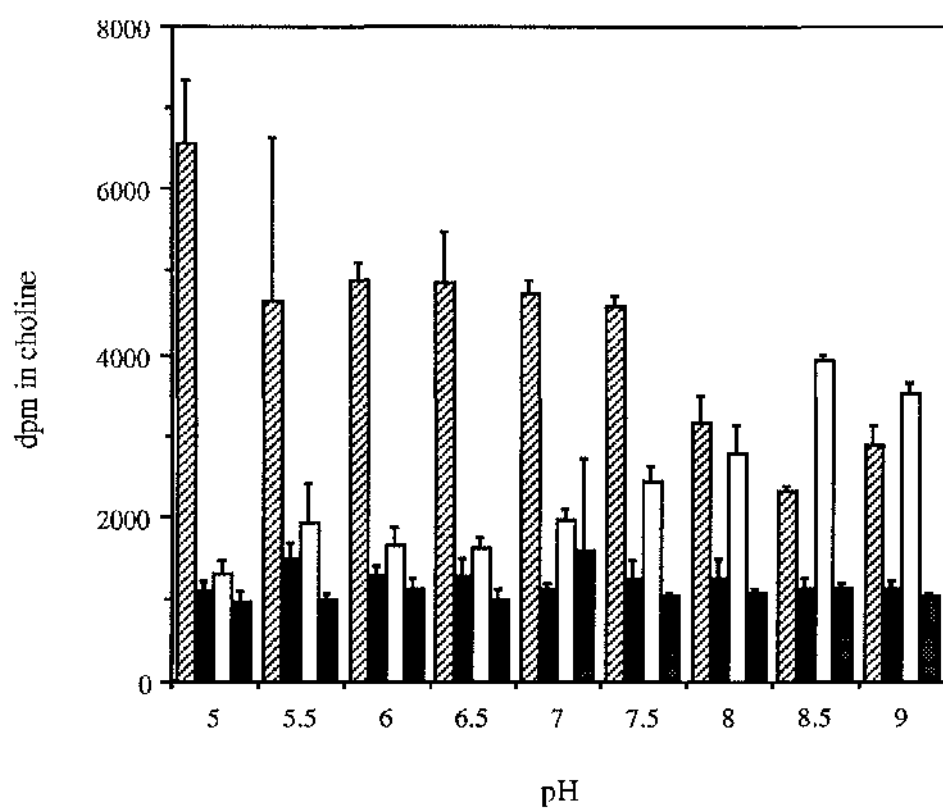
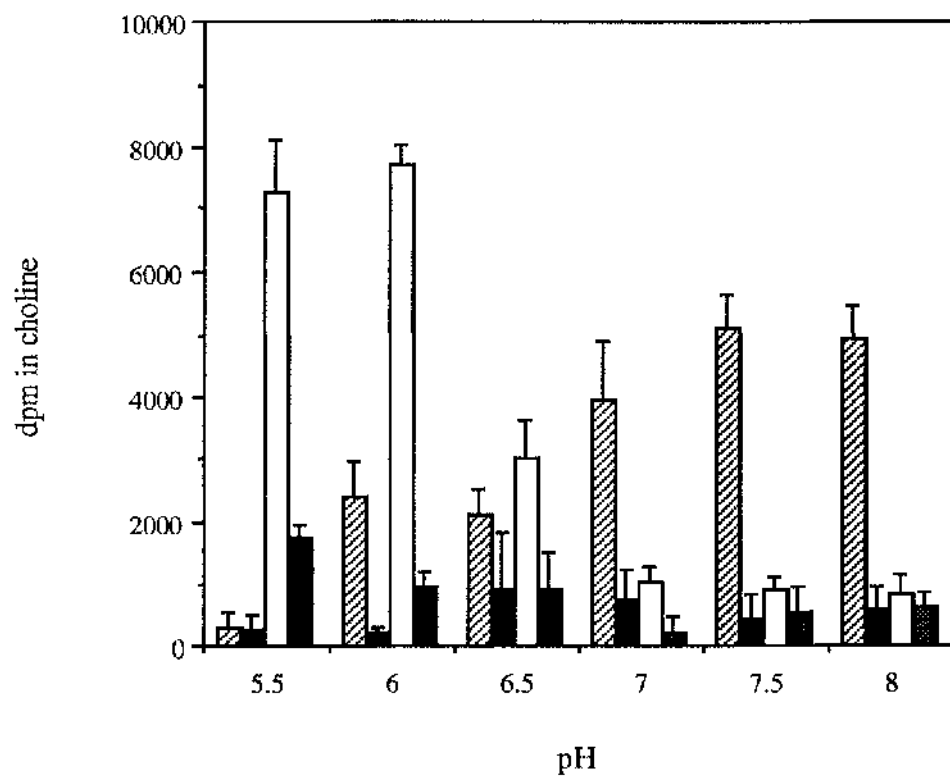
The profile of total protein, total PLD activity and specific PLD activity changed dramatically throughout the fractionation procedure. It was evident that maximal PLD activity was recovered at a saturation interval of 60-70% (w/v) (Table 3.2) with approximately 12% of the total protein.

The specific PLD activity, in common with total PLD activity, was also maximal at 60-70% (w/v) saturation where substrate was hydrolysed at a rate of  $3.93 \pm 0.12$  pmol/min/mg of protein. Therefore, PLD activity was significantly enriched in this fraction with respect to the original starting material which hydrolysed substrate at a rate of  $1.04 \pm 0.08$  pmol/min/mg of protein. Based upon the above data, approximately 20 % of the total protein representing 60-65 % of the total activity was recovered over the saturation interval of 50-80% (w/v). Therefore, fractionation allowing the recovery of such a precipitate was established for full scale preparative purification of the solubilised PLD. Use of this protocol routinely resulted in an approximate 3-fold enrichment with a 30-45 % yield of PLD activity.

### **3.4 Ion-Exchange Chromatography.**

As an initial purification step, ion-exchange chromatography of the solubilised PLD was considered suitable. Initial studies utilised Q-Sepharose FF anion-exchange media due to its high capacity for protein, high chromatographic flow rates, ease of use and economy. A preliminary investigation was initiated to define conditions required for successful anion-exchange chromatography. Q-Sepharose FF 1 ml columns were equilibrated over the pH range of 5.0-9.0 (in 0.5 pH unit intervals) and 1 ml of solubilised PLD activity, equilibrated to the same pH, was applied to each column. The columns were washed in the absence of NaCl and then eluted with buffer containing 1M NaCl and the collected fractions assayed for PLD activity (Fig 3.5A).

The amount of solubilised PLD activity bound to the anion-exchange resin increased as pH increased from the 5.0 to 9.0. Optimum binding occurred at pH 8.5. However, this represented only 33 % of total PLD activity whereas 66 % of the total

**A****B**



### **Fig 3.5 Determination of pH Conditions Required for Ion-Exchange.**

#### **A pH Conditions Required for Anion-Exchange.**

#### **B pH Conditions Required for Cation-Exchange.**

2 ml of Q-Sepharose FF anion-exchange resin or S-Sepharose FF cation-exchange resin were equilibrated to the desired pH (5-9 or 5-8, in 0.5 pH units) by three washes of 10 volumes of 250 mM Buffer of appropriate pH, containing 1 M NaCl and three further washes of 10 volumes of 25 mM Buffer of appropriate pH, containing 2 mM EDTA, 0.1% (w/v) TX-100. After each wash the suspended resin was allowed to settle and the supernatant removed by aspiration. The supernatant of the final wash was aspirated to generate a resin slurry of 50%. Columns of 1 ml bed volume were then packed in glass wool plugged 2 ml syringes with each of the equilibrated resins. To each column, 1 ml of solubilised PLD (prepared as described in Section 2.5.2) equilibrated to the appropriate pH (by overnight dialysis against 25 mM buffer, 2 mM EDTA, 0.1% (w/v) TX-100) was loaded and the following washes performed:

- (i) 3 x 1 ml of final equilibration buffer; pooled as 4 ml 'load' fraction inclusive of loaded volume.
- (ii) 4 x 1 ml of equilibration buffer; pooled as 4 ml 'non-specific wash' fraction.
- (iii) 3 x 1 ml of equilibration buffer containing 1 M NaCl; pooled as 3 ml 'first elution' fraction.
- (iv) 3 x 1 ml of equilibration buffer containing 1 M NaCl; pooled as 3 ml 'second elution' fraction.

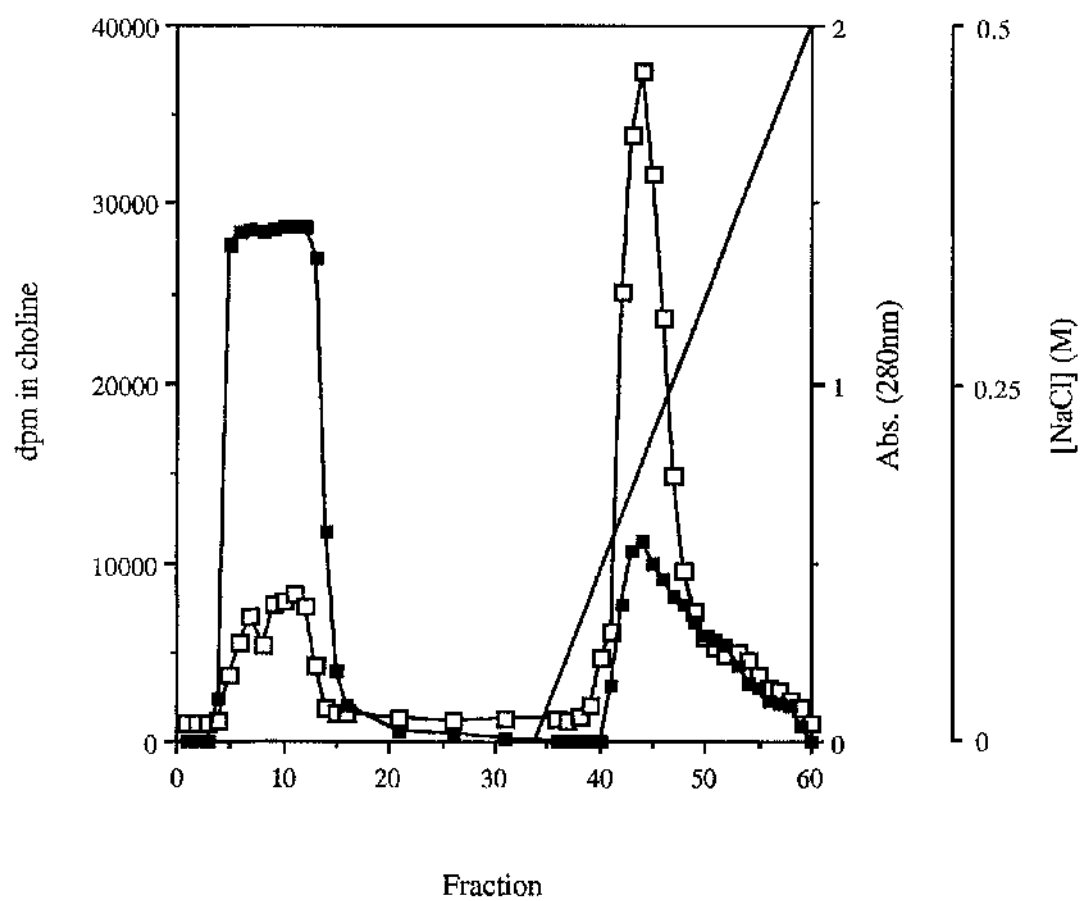
For screening experiments the following buffering systems over the pH range of 5 to 9 were used; pH 5.0-Acetate, pH 5.5/6.0/6.5-Mes, pH 7.0/7.5-Mops and pH 8.0/8.5/9.0-Tricine.

50 µl from each pooled fraction was then assayed for PLD activity as described in Section 2.4 and the activity recovered in each of the collected fractions from each column shown opposite [hatched bars-'load' fraction; solid bars-'non-specific wash' fraction; open bars-'first elution' fraction; stippled bars-'second elution' fraction].

PLD activity remained unbound (Fig 3.5A). Therefore, cation-exchange chromatography using S-Sepharose FF resin was investigated as a potential step in purification of the enzyme. Conditions for successful cation-exchange were determined by preparation of similar 1ml columns, except the pH range of 5.5 to 8.0 was investigated. Solubilised PLD was prepared as appropriate and applied to the equilibrated columns. Columns were then washed and eluted and the collected fractions assayed for PLD activity (Fig 3.5B).

The solubilised PLD preparation bound to the cation-exchange columns in a pH dependent manner and under these conditions, strength of binding increased as pH decreased. At pH 5.5 and 6.0 approximately 80% of the activity was eluted with a buffer containing 1M NaCl. However, it was noted that the protein applied to the column equilibrated to pH 5.5 contained a heavy precipitate. This loaded material was immobilised at the top of the column bed and was resoluted upon development with equilibration buffer containing 1 M NaCl. Therefore, this condition was discarded and a pH of 6.0 was established for full scale preparative cation-exchange chromatography using S-Sepharose FF resin. In addition, TX-100 (0.1% w/v) was included in all chromatography buffers as its exclusion resulted in lower recovery of applied solubilised PLD activity (data not shown).

At full scale, the solubilised PLD activity was prepared as described in Sections 2.5.2 and 2.5.3 and subjected to cation-exchange chromatography on S-Sepharose FF column with a bed volume of 10ml. (Fig 3.6). The majority of applied protein did not bind to the column. Co-migrating with this protein was a minor PLD activity and represented approximately 5 % of the total PLD activity applied to the column. The major PLD activity was recovered upon development of the column with a linear gradient of 0-0.5M NaCl, eluting as a single peak between 120-360 mM NaCl. Development of the column from 0.5-1.0 M NaCl resulted in no further elution of PLD activity (data not shown). Active fractions were pooled and NaCl removed by overnight dialysis against 5 l of chromatography buffer in preparation for further chromatographic purification.



**Fig 3.6 Cation-Exchange Chromatography of the Solubilised PLD on S-Sepharose FF.**

A 10 ml (1.6 x 4.96 cm) column of S-Sepharose FF was prepared as described in Section 2.6.2 and subsequently packed with 10 column volumes of 25mM Mes pH 6.0 containing 2mM EDTA and 0.1% (w/v) TX-100. Solubilised PLD was prepared and dialysed overnight against the same chromatography equilibration buffer as described in Section 2.5.3. The dialysate was clarified by centrifugation (10,000 x g, 15min) and the supernatant (190.01 mg protein) applied to the column (fraction 1-14). The resin was then washed with approximately 100 ml of chromatography buffer (fractions 15-34) and bound protein eluted by development of a 100 ml linear gradient of 0-0.5 M NaCl (*solid line*) in chromatography buffer. Fractions of 4.0 ml were collected and 50 µl from each fraction assayed for PLD activity (*open squares*) as described in Section 2.4. Protein concentration (*closed squares*) of the eluate was measured by absorption at 280 nm.

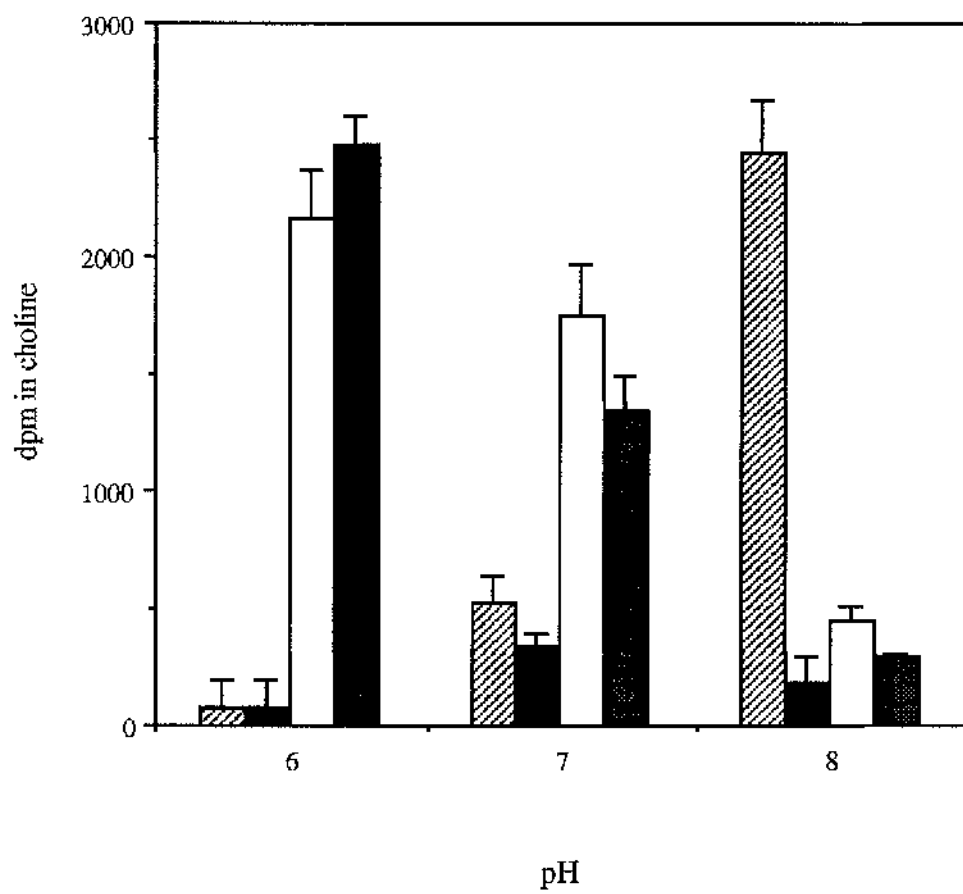
Employment of cation-exchange chromatography routinely resulted in an approximate 2-fold purification and a recovery of 30-50 % of the total PLD activity applied to the column.

### **3.5 Heparin-Agarose Chromatography.**

Chromatography using heparin-substituted agaroses consists of both an ion-exchange process between the applied protein and the immobilised polyanion and also a pseudo-affinity-like binding process (Margalit *et al.*, 1993). Based upon these interactions, immobilised heparin has been successfully utilised in the purification of a number of proteins, particularly kinases, lipases and phospholipases. These include a receptor and G-protein regulated PtdIns-specific phospholipase C from turkey erythrocytes (Morris *et al.*, 1990) and most recently the membrane-associated PLD activities of HL-60 cells (Brown *et al.*, 1993) and rat brain (Massenberg *et al.*, 1994).

The solubilised PLD routinely bound to a 1 ml column of heparin-agarose in the absence of NaCl at pH 6.0 and activity was recovered upon elution of the column with chromatography buffer (25mM Mes pH6.0, 2mM EDTA, 0.1% (w/v)) containing 1 M NaCl (Fig 3.7). Therefore, the post cation-exchange PLD activity was subjected to full scale preparative heparin-agarose chromatography (Fig 3.8).

All the applied protein containing PLD activity bound to the column and subsequent to washing with 10 volumes of chromatography buffer the PLD activity was recovered upon development of the column with a linear gradient of 0-0.85M NaCl. The PLD activity eluting as a single peak between 360-500 mM NaCl, distinct from the major peak of eluted protein. Development of the column from 0.85-2.0 M NaCl resulted in elution of no further protein or PLD activity (data not shown). Active fractions were pooled and dialysed overnight against 5 l of 25 mM Mes pH 6.0, 1 M NaCl, 5 mM MgCl<sub>2</sub> in preparation for adsorption chromatography using hydroxyapatite.



**Fig 3.7 Determination of Conditions Required for Chromatography of the Solubilised PLD on Heparin-agarose.**

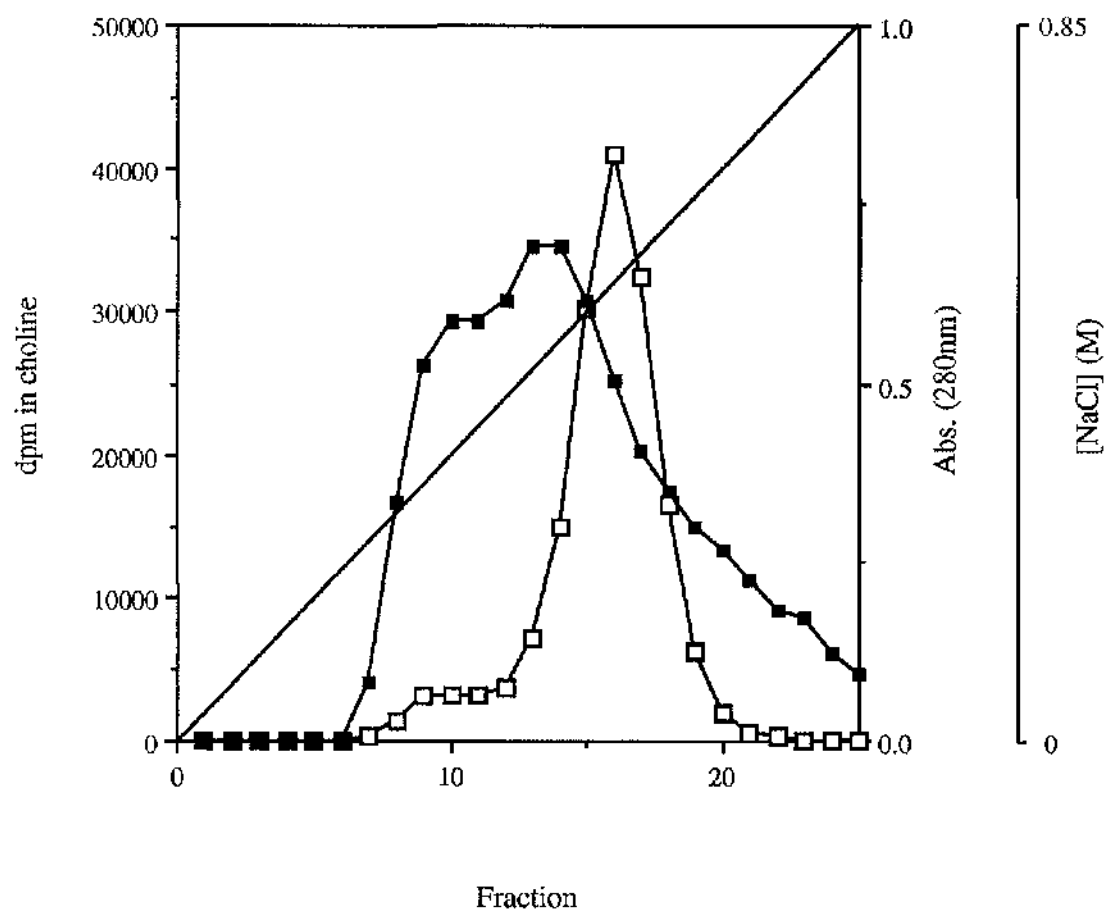
2 ml of heparin-agarose resin were each equilibrated to the desired pH (6, 7 and 8) by three washes of 10 volumes of 250 mM Buffer of appropriate pH, containing 1 M NaCl and three further washes of 10 volumes of 25 mM Buffer of appropriate pH, containing 2 mM EDTA, 0.1% (w/v) TX-100. After each wash the suspended resin was allowed to settle and the supernatant removed by aspiration. The supernatant of the final wash was aspirated to generate a resin slurry of 50%. Columns of 1 ml bed volume were then packed in glass wool plugged 2 ml syringes with each of the equilibrated resins.

To each column, 1 ml of solubilised PLD (prepared as described in Section 2.5.2) equilibrated to the appropriate pH (by overnight dialysis against 25 mM buffer, 2 mM EDTA, 0.1% (w/v) TX-100) was loaded and the following washes performed:

- (i) 3 x 1 ml of final equilibration buffer; pooled as 4 ml 'load' fraction inclusive of loaded volume.
- (ii) 4 x 1 ml of equilibration buffer; pooled as 4 ml 'non-specific wash' fraction.
- (iii) 3 x 1 ml of equilibration buffer containing 1 M NaCl; pooled as 3 ml 'first elution' fraction.
- (iv) 3 x 1 ml of equilibration buffer containing 1 M NaCl; pooled as 3 ml 'second elution' fraction.

For screening experiments the following buffering systems over the pH range of 6 to 8 were used; pH 6.0 & 7.0-Mes and pH 8.0-Tricine.

50 µl from each pooled fraction was then assayed for PLD activity as described in Section 2.4 and the activity recovered in each of the collected fractions from each column shown opposite [hatched bars-'load' fraction; solid bars-'non-specific wash' fraction; open bars-'first elution' fraction; stippled bars-'second elution' fraction].





### **Fig 3.8 Heparin-Agarose Chromatography of the Solubilised PLD.**

A 10 ml (1.6 x 4.96 cm) column of heparin-agarose prepared as described in Section 2.6.2 was packed and equilibrated with 10 column volumes of 25mM Mes pH 6.0 containing 2mM EDTA and 0.1% (w/v) TX-100. The PLD preparation (43.4 mg protein) pooled from cation-exchange chromatography (Fig 3.6) was desalted by overnight dialysis against 5 l of chromatography buffer and applied to the column. The resin was washed with 100 ml of chromatography buffer and bound protein eluted (fractions 1-25) by development of a 100 ml linear gradient of 0-0.85M NaCl in chromatography buffer(*solid line*). Fractions of 4.0 ml were collected and 50 µl from each fraction assayed for PLD activity (*open squares*) as described in Section 2.4. Protein concentration (*closed squares*) of the eluate was measured by absorption at 280 nm.

Employment of heparin-agarose chromatography routinely resulted in an approximate 4-fold purification and a recovery of 125-170 % of the total PLD activity applied to the column. The recovery of a greater amount of PLD activity than loaded onto the column may reflect the removal of an endogenous PLD inhibitor protein or an endogenous factor that interferes with the assessment of PLD activity *in vitro*.

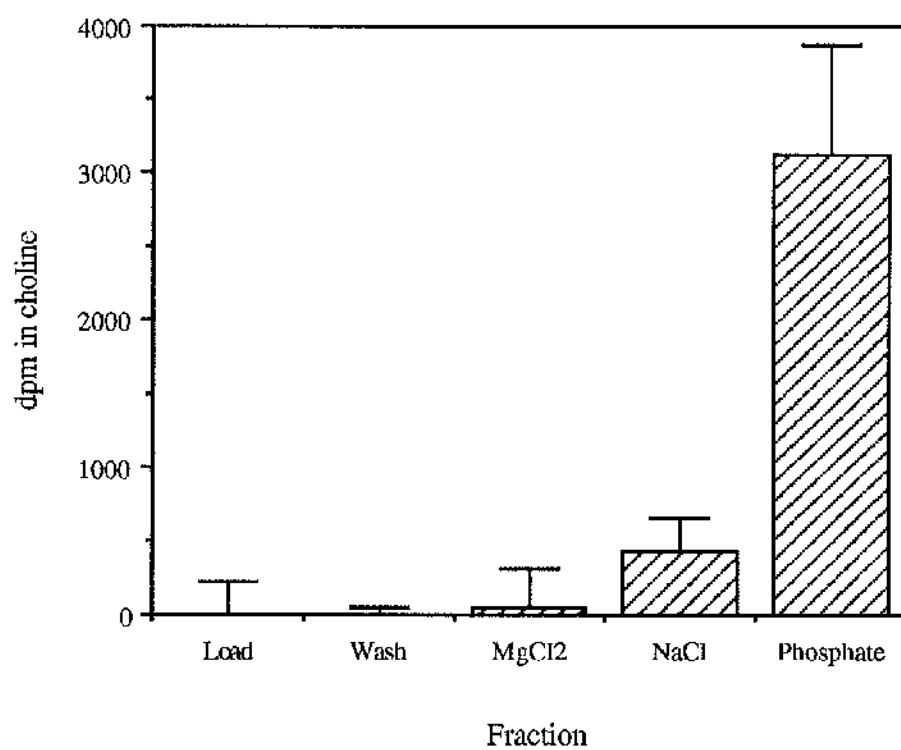
### 3.6 Hydroxyapatite Chromatography.

Proteins adsorbed to an hydroxyapatite chromatography matrix can be specifically eluted based upon the systematic disruption of the complex electrostatic interaction between the amino and carboxyl groups of the proteins and the charged calcium and phosphate of the hydroxyapatite crystal surface. Consequently, procedures that allow specific elution of basic, acidic and neutral proteins were investigated (Gorbunoff, 1990).

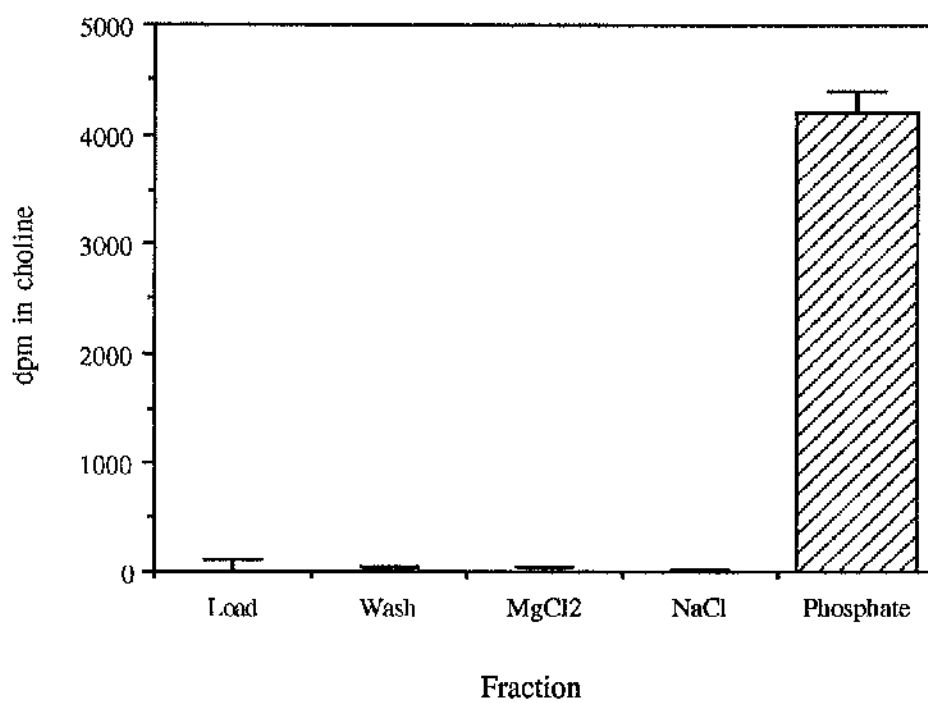
Solubilised PLD bound hydroxyapatite (BioGel HTP and Bio-Rad EconoPac cartridges) and was retained on a 1 ml column equilibrated with 25 mM Mes pH 6.0, 2 mM EDTA 0.1% (w/v) TX-100 (Fig 3.9A). Activity remained bound to the column during washing with 5 mM MgCl<sub>2</sub> and a minor activity was recovered upon washing with 1M NaCl. The majority of activity was recovered upon elution with 0.3M sodium phosphate. Post heparin-agarose PLD activity was fully retained on the column during washing with either 5 mM MgCl<sub>2</sub> or 1 M NaCl in 25 mM Mes pH 6.0 and successful elution was achieved using 0.3 M sodium phosphate (Fig 3.9B). From this information, an elution protocol was constructed. This employed equilibration of the column in 25 mM Mcs pH 6.0 containing 5 mM MgCl<sub>2</sub> and 1 M NaCl, resulting in basic and neutral proteins selectively running through the column. A linear gradient of 0-0.5 M sodium phosphate eluted the bound acidic proteins and the PLD activity.

Full scale preparative chromatography of the post heparin-agarose activity was performed using a 10 ml hydroxyapatite column and the elution protocol outlined above (Fig 3.10). The solubilised PLD reproducibly eluted as a single peak of activity associated with eluted protein at a position in the gradient equivalent to

**A**



**B**



### **Fig 3.9 Determination of Conditions Required for Chromatography of the Solubilised PLD on Hydroxyapatite.**

**A** Conditions required for chromatography of solubilised PLD.

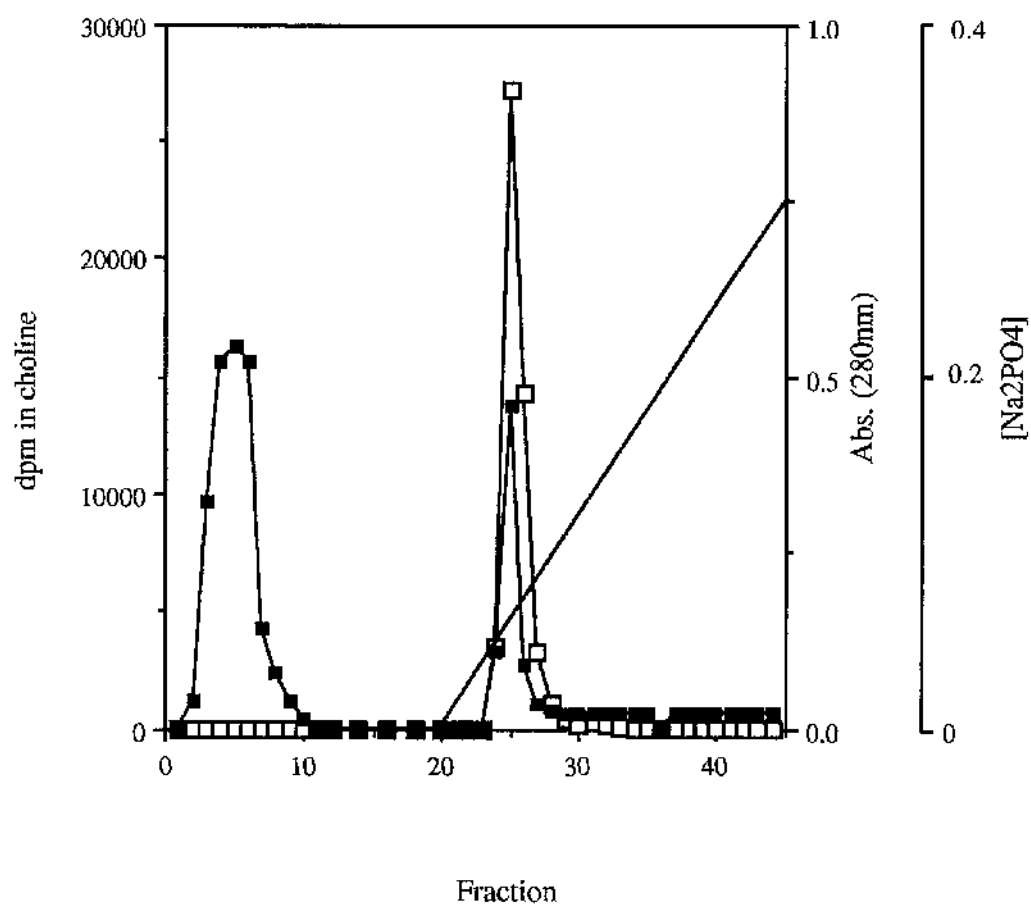
**B** Conditions required for chromatography of post heparin-agarose PLD.

2 ml of hydroxyapatite (BioGel HTP) were initially washed three times with 10 volumes of distilled water to remove fines. The hydroxyapatite was then equilibrated to pH 6.0 by three washes of 10 volumes of 250 mM Mes pH 6.0 containing 1 M NaCl and three further washes of 10 volumes of 25 mM Mes pH 6.0, containing 2 mM EDTA, 0.1% (w/v) TX-100. After each wash the suspended resin was allowed to settle and the supernatant removed by aspiration. The supernatant of the final wash was aspirated to generate a resin slurry of 50%. A column of 1 ml bed volume was then packed in a glass wool plugged 2 ml syringe with the equilibrated resin.

To the column, 1 ml of solubilised PLD (prepared as described in Section 2.5.2) or post heparin-agarose PLD, equilibrated to the same pH (by overnight dialysis against 25 mM Mes pH 6.0, 2 mM EDTA, 0.1% (w/v) TX-100), were loaded and the following washes performed:

- (i) 3 x 1 ml of final equilibration buffer; pooled as 4 ml 'load' fraction inclusive of loaded volume.
- (ii) 4 x 1 ml of equilibration buffer; pooled as 4 ml 'non-specific wash' fraction.
- (iii) 4 x 1 ml of equilibration buffer containing 5 mM  $MgCl_2$ ; pooled as 4 ml  $MgCl_2$  fraction.
- (iv) 4 x 1 ml of equilibration buffer containing 1 M NaCl; pooled as 4 ml 1 M NaCl fraction.
- (v) 4 x 1 ml of equilibration buffer containing 0.5 M  $NaH_2PO_4$  ; pooled as 4 ml  $NaH_2PO_4$  fraction.

50  $\mu$ l from each pooled fraction was then assayed for PLD activity as described in Section 2.4 and the activity recovered in each of the collected fractions from each column shown opposite.



### **Fig 3.10 Hydroxyapatite Chromatography of the Solubilised PLD.**

The PLD preparation (18.97 mg protein) pooled from heparin-agarose chromatography (Fig 3.10) was dialysed overnight against 5 l of 25 mM Mes pH 6.0, 1 M NaCl, 5 mM MgCl<sub>2</sub>, 0.1% (w/v) TX-100 and applied to 10 ml column of hydroxyapatite, consisting of two Bio-Rad 5 ml pre-packed cartridges connected in series, equilibrated with 50 ml of the above buffer. The resin was washed with chromatography buffer until the absorbance trace returned to baseline. Bound protein was eluted (fractions 20-45) by development of a 100 ml linear gradient of 0-0.3 M NaH<sub>2</sub>PO<sub>4</sub> (*solid line*) in chromatography buffer. Fractions of 4.0 ml were collected and 50 µl from each fraction assayed for PLD activity (*open squares*) as described in Section 2.4. Protein concentration (*closed squares*) of the eluate was measured by absorption at 280 nm.

approximately 85 mM sodium phosphate. Active fractions were pooled and dialysed overnight to remove salt and phosphate in preparation for FPLC cation-exchange chromatography. Typically, use of adsorption chromatography on hydroxyapatite resulted in an approximate 1.5-fold purification and 39-42 % recovery of the activity applied to the column.

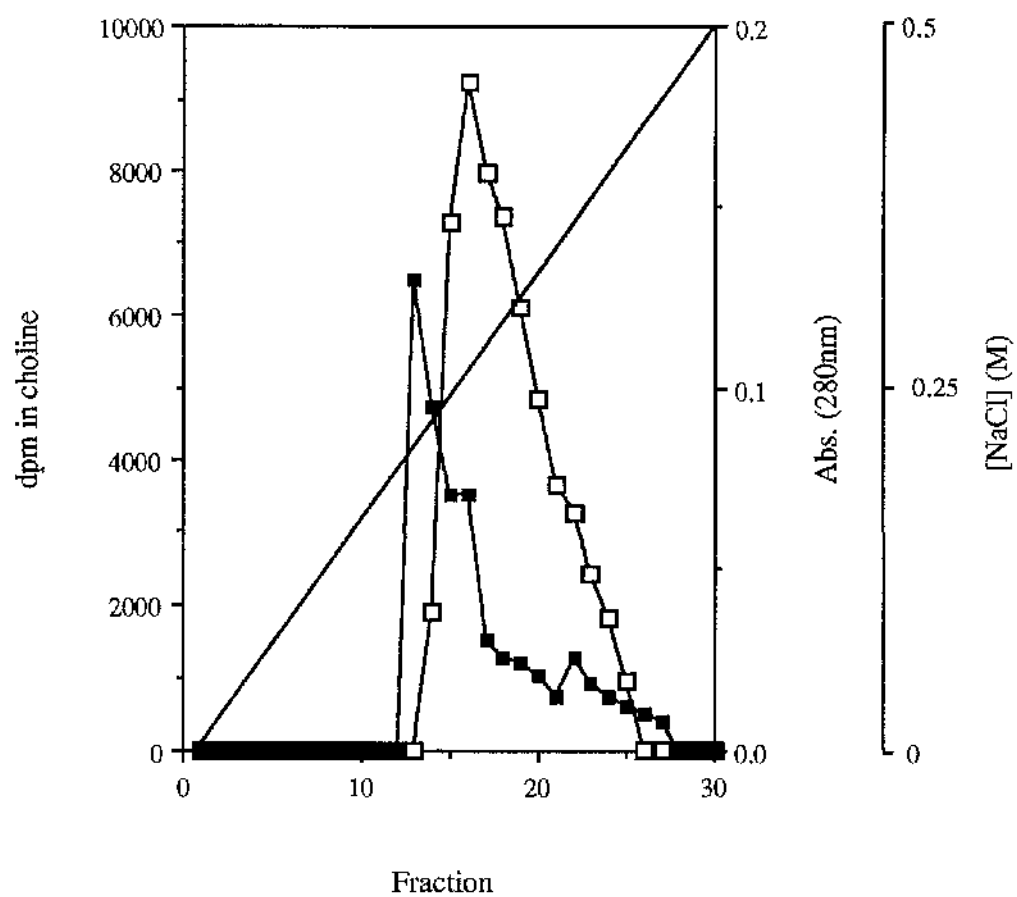
### **3.7 FPLC Cation-Exchange Chromatography.**

Characterisation of conditions required for cation-exchange chromatography using Pharmacia ion-exchange FF resin (see Section 3.4) allowed direct loading of the dialysed post hydroxyapatite preparation to a Pharmacia Mono-S FPLC cation-exchange column. As an intermediate manipulative step, high resolution ion-exchange was investigated as a means of sample concentration and detergent removal that is required for successful gel filtration. Detergent removal was achieved by washing with chromatography buffer excluding TX-100. Initial experiments using gradients of 0-0.5 M NaCl over a small number of column volumes allowed recovery of all of the applied activity as an essentially detergent free sample of 3-4 ml (data not shown). However, development of the column with a gradient of 0-0.5 M NaCl over 20 column volumes, partially resolved the majority of activity from the majority of protein. At preparative scale, 'column dialysis' was performed by washing the immobilised sample with detergent free buffer until the absorbance trace returned to baseline. Fractions were then collected following gradient elution as described above (Fig 3.11). Again, resolution of activity from protein was observed. Activity in fractions 15-20 were pooled and concentrated in preparation for gel filtration chromatography.

Use of this chromatographic step routinely resulted in an approximate 2-fold purification and a 25-30 % recovery of the total activity applied to the column.

### **3.8 FPLC Gel Filtration Chromatography.**

The ability to further purify the solubilised PLD activity by gel filtration of the





### **Fig 3.11 FPLC Cation-Exchange Chromatography of the Solubilised PLD.**

The PLD preparation (5.15 mg protein) pooled from hydroxyapatite chromatography (Fig 3.11) was dialysed overnight against 5 l of 25 mM Mes pH 6.0, 2 mM EDTA, 0.1 % (w/v) TX-100 and applied to a Pharmacia pre-packed 1 ml Mono-S HR 5/5 cation-exchange column equilibrated with 25 mM Mes pH 6.0, 2 mM EDTA. After loading, the resin was washed with detergent-free chromatography buffer until the absorbance trace returned to baseline. Bound protein was eluted by development of a 30 ml linear gradient of 0-0.5M NaCl (*solid line*) in chromatography buffer. Fractions of 1 ml were collected during gradient development and 10  $\mu$ l from each fraction assayed for PLD activity (*open squares*) as described in Section 2.4. Protein concentration (*closed squares*) of the eluate was measured by absorption at 280 nm.

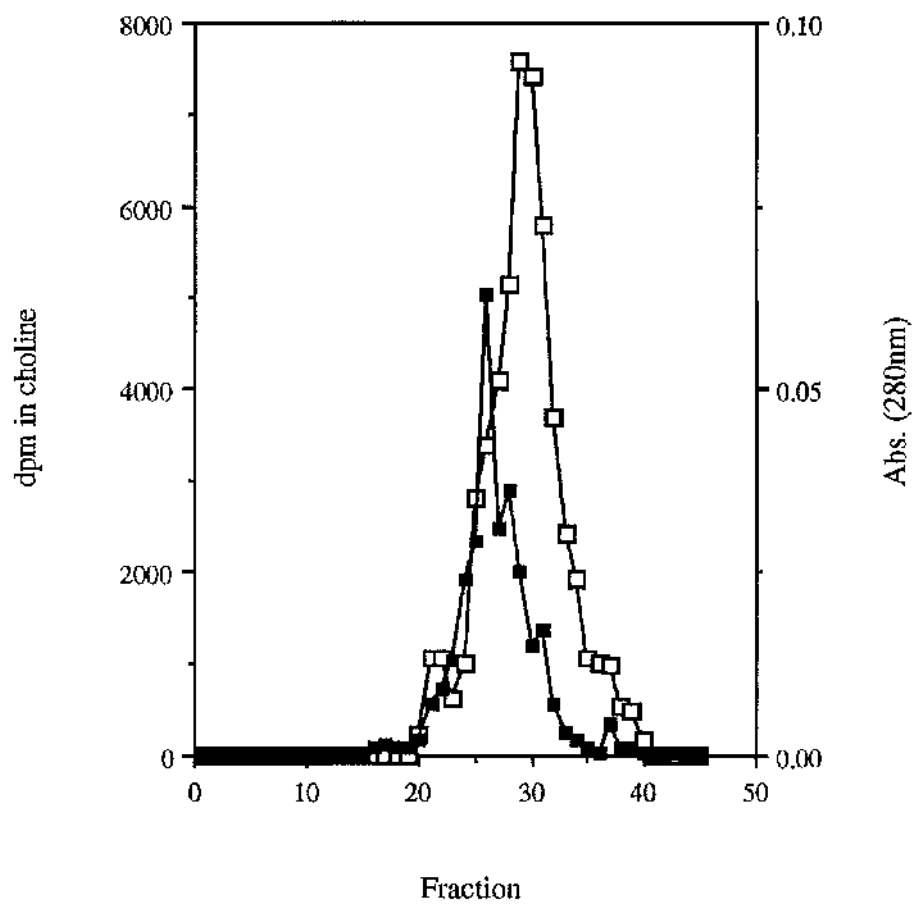
detergent free post Mono-S preparation was investigated. Pooled post Mono-S PLD preparation (5 ml) was concentrated 20-fold and applied to a FPLC SR-12 pre-packed gel filtration column (Fig 3.12). The solubilised PLD activity eluted as a single peak distinct from the major peak of protein. Typically, the activity eluted in a volume of approximately 13 ml of buffer (n=5). Upon the determination of the columns void volume ( $v_0$ , equivalent to 7 ml), the elution volume ( $v_e$ ) of the solubilised PLD was calculated to correspond to  $v_e/v_0$  values ranging between 1.64 and 1.65. Comparison of the elution volume of the solubilised PLD to those of known standard proteins of determined molecular weight allowed calculation of the apparent native molecular weight of the PLD (Fig 3.13). The native molecular weight of the solubilised PLD was calculated to be  $69 \pm 2$  kDa (mean  $\pm$  SD, n=5).

As a preparative chromatography step, FPLC SR-12 gel filtration chromatography routinely resulted in a small enrichment of the post Mono-S preparation and a 82-95 % yield of activity applied to the column.

### **3.9 Table of Purification of the Solubilised PLD and SDS-PAGE Analysis of Chromatography Fractions.**

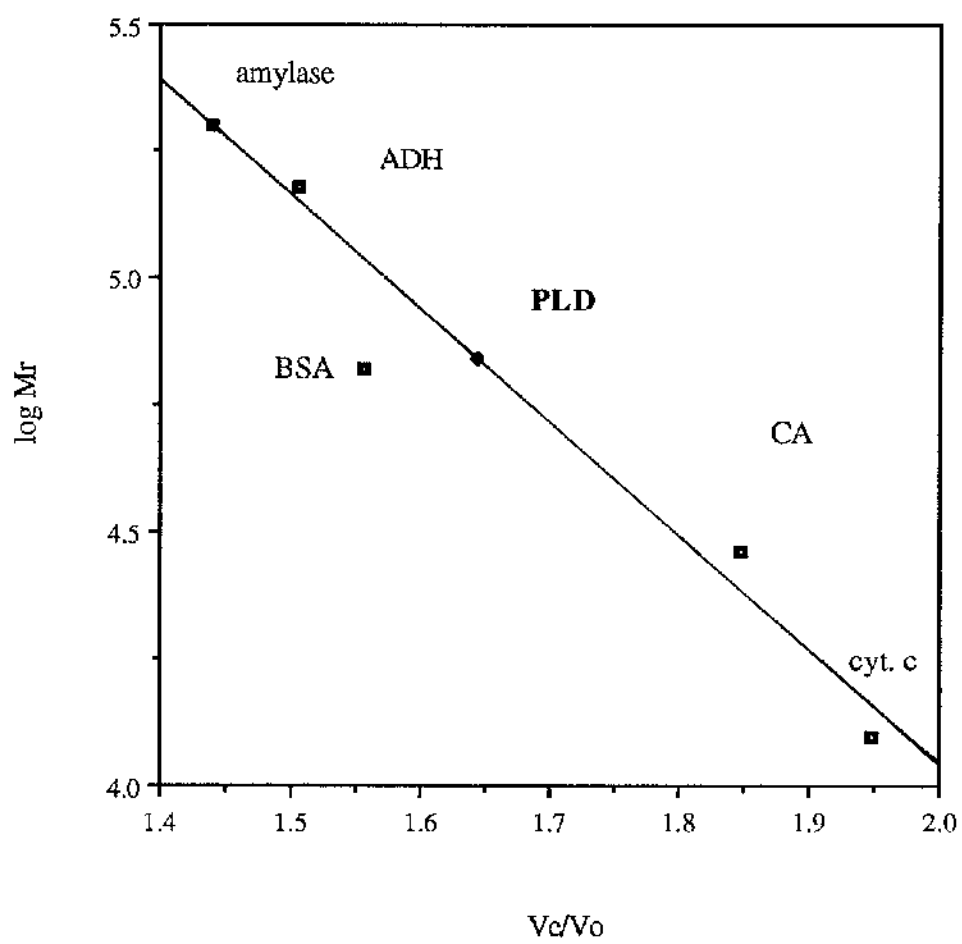
Partial purification of the solubilised PLD by the above protocol has been performed on five separate occasions with similar results. The pooled PLD recovered from each step was analysed by SDS-PAGE (Fig 3.14) and a typical purification summary is shown in Table 3.3. Use of the above protocol resulted in the recovery of a significantly purified PLD preparation; 42.3-64.2 fold purification relative to the total solubilised PLD recovered with a 1.52-1.98% yield. The final post gel filtration preparation exhibited a specific activity towards substrate of 71-111.1 pmol/min/mg of protein.

The individual fractions recovered from gel filtration chromatography that contained PLD activity were also analysed by SDS-PAGE (Fig 3.15). It was not possible to identify any single polypeptide that co-migrating with PLD activity during gel filtration chromatography. However, it was apparent that polypeptides of a similar



### **Fig 3.12 FPLC Gel Filtration Chromatography of the Solubilised PLD.**

The PLD preparation (0.72 mg protein) pooled from FPLC cation-exchange chromatography (Fig 3.10) was concentrated 20-fold to a volume of 250  $\mu$ l in a Centriprep 10 microconcentrator as described in Section 2.7.1. 200  $\mu$ l of the protein concentrate was applied to a Pharmacia pre-packed 30 ml SR-12 gel filtration column equilibrated with 25 mM Mes pH 6.0, 2 mM EDTA, 250 mM NaCl. Fractions of 0.5 ml were collected and 5  $\mu$ l of each fraction assayed for PLD activity (*open squares*) as described in Section 2.4. Protein concentration (*closed squares*) of the eluate was measured by absorption at 280 nm.



### **Fig 3.13 Calibration Curve of the FPLC SR-12 Gel Filtration Column.**

The 30 ml pre-packed FPLC SR-12 column employed in Fig 3.12 was equilibrated with 25 mM Mes pH 6.0, 2 mM EDTA, 250 mM NaCl. Protein standards or post Mono-S PLD preparation were applied to the column in a volume of 200  $\mu$ l and the column developed in the same buffer at a flow rate of 0.5 ml/min. The void volume ( $v_0$ ) of the column was determined from the elution of Blue Dextran monitored at 280 nm. The elution of  $\beta$ -amylase (200 kDa), alcohol dehydrogenase (ADH, 150 kDa), bovine serum albumin (BSA, 66 kDa), carbonic anhydrase (CA, 29 kDa) and cytochrome-c (cyt. c, 12.4 kDa) from the column were also monitored at 280 nm and their elution volume ( $v_e$ ) determined. The  $v_e$  of the solubilised PLD was determined for the elution of PLD activity from the column (see Fig 3.13). The apparent molecular weight of the solubilised PLD was calculated from the calibration curve of  $\log M_r$  against  $v_e/v_0$ .

Lane number

1 2 3 4 5 6 7 8 9 10 11

←200

←97

←68

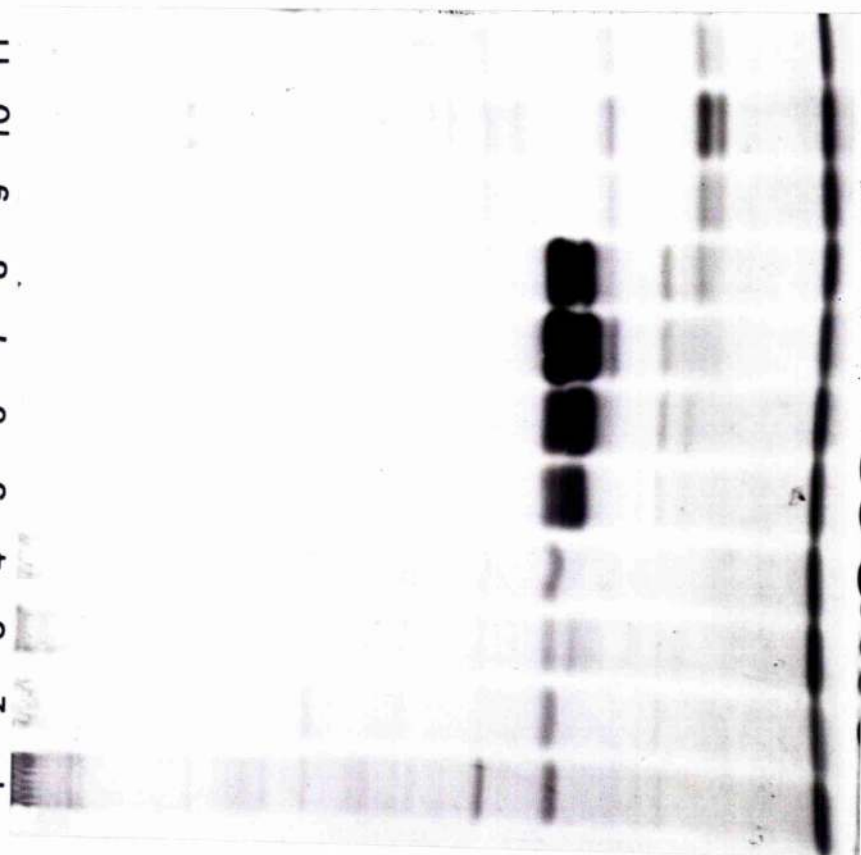
kDa

←43

←29

←18

←14



**Fig 3.14 Purification of the Solubilised PLD: SDS-PAGE Analysis.**

10 µg of the PLD preparation pooled from each stage of the purification protocol was analysed by SDS- PAGE on a 10 % polyacrylamide resolving gel, as described in Section 2.3, and silver stained as described in Section 2.3.5.

Lane 1:	Membranes.
Lane 2:	Freeze-thaw 100,000 x g supernatant.
Lane 3:	Phosphate extracted activity.
Lane 4:	Total Solubilised PLD.
Lane 5:	Ammonium sulphate precipitate (50-80% fraction).
Lane 6:	Clarified load to S-Sepharose FF.
Lane 7:	Post S-Sepharose FF.
Lane 8:	Post heparin-agarose.
Lane 9:	Post hydroxyapatite.
Lane 10:	Post Mono-S HR 5/5.
Lane 11:	Post SR-12 Gel Filtration.



**Table 3.3 Purification of the Membrane-Associated PLD of Bovine Spleen.**

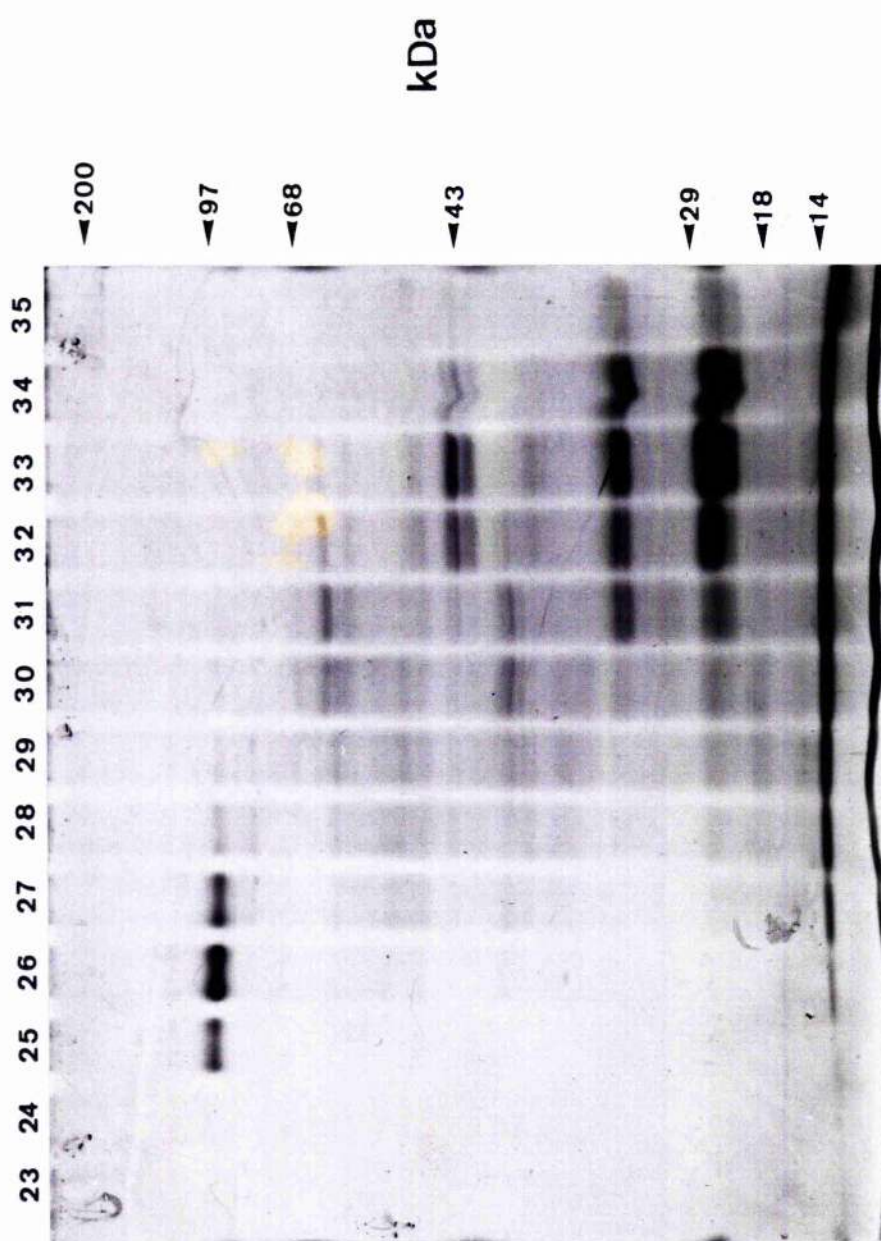
<u>Step</u>	<u>Protein</u> <u>(mg)</u>	<u>Total</u> <u>Activity</u> <u>(pmol/min)</u>	<u>Specific</u> <u>Activity</u> <u>(pmol/min/ mg)</u>	<u>Fold</u> <u>Purification</u>	<u>Yield</u> <u>%</u>
Extracted Membranes	1507.00	2607.11	1.73	1.00	100.00
50-80% Ammonium Sulphate Fraction	190.01	851.24	4.48	2.60	32.65
S-Sepharose FF	43.40	279.50	6.44	3.72	10.72
Heparin- Agarose	18.97	452.81	23.87	13.80	17.37
Hydroxy- apatite	5.15	189.67	36.83	21.29	7.28
Mono-S	0.72	56.56	78.55	45.40	2.17
SR-12 Gel Filtration (Fractions 28-32)	0.68	55.76	82.00	47.40	1.82

2-10  $\mu$ l of enzyme preparation from each stage of the purification protocol was assayed for PLD activity as described in Section 2.4. Protein concentration was determined as described in Section 2.2.1.

599  
1008  
2816  
3380  
4093  
5135  
7590  
7415  
5799  
3703  
2412  
1903  
1068

dpm in choline

Fraction number



**Fig 3.15 SDS-PAGE of the Column Fractions Co-Purifying With PLD Activity During SR-12 Gel Filtration Chromatography.**

50µl of each fraction co-eluting with PLD activity from the SR-12 gel filtration column (see Fig 3.12) was analysed by SDS-PAGE as described in Section 2.3 and silver stained as described in Section 2.3.5. The PLD activity contained in a similar 50 µl aliquot is indicated on each lane as appropriate. PLD activity was measured as described in Section 2.4.

molecular weight to that determined as the native molecular weight of the solubilised PLD (see Section 3.15) were present in the collected fractions. In conclusion, the solubilised PLD was substantially purified by the above protocol, though not to homogeneity and the polypeptide responsible for activity remains to be identified.

### **3.10 Identification of the Solubilised PLD.**

In the absence of a homogeneous preparation of the solubilised PLD, its identification within the mixture of polypeptides present after gel filtration chromatography (illustrated in Fig 3.15) could allow protein sequencing to be initiated. Despite the presence of 69 kDa polypeptides, no individual polypeptide was identified as co-purifying with PLD activity. Therefore, alternative analytical approaches were initiated to identify the polypeptide responsible for PLD activity.

The ability of PLD to catalyse a transphosphatidylation reaction and in the presence of short chain primary aliphatic alcohols form phosphatidylalcohols at the expense of PtdOH formation may predict the existence of a phosphatidyl-enzyme intermediate during hydrolysis of PtdCho by PLD. This may occur prior to the consequent formation of PtdOH and choline products. By presentation of PtdCho, tritium labelled in the acyl chain, during the reaction process, a  $^3\text{H}$ -labelled intermediate should be formed. If such an intermediate was stable then it was proposed that it could be isolated by precipitation, electrophoresed on an SDS-PAGE gel and subsequently visualised by autoradiography. Further, it was concluded that if labelling occurred in a specific manner then it would be possible to specifically 'chase' the labelled phosphatidyl moiety from the PLD by addition of a large excess of unlabelled PtdCho. Additionally, since the formation of the supposed intermediate would occur via a covalent linkage, it would be expected that the unlabelled PtdCho would not 'chase' the labelled PtdCho from those proteins labelled in a non-specific manner. Unfortunately, upon employment of this approach no labelling of the solubilised PLD was apparent (data not shown) and as this was a single investigation

it is difficult to predict whether the solubilised PLD can be identified in this manner under similar or developed conditions. This requires further investigation.

### **3.11 Alternative Chromatographic Approaches Taken Towards the Purification of the Solubilised PLD.**

Despite considerable purification of the solubilised PLD it was apparent that a number of polypeptides co-purified with the PLD activity (Fig 3.15). To allow purification of the solubilised PLD to homogeneity, a number of purification strategies in addition to those utilised could be pursued.

The use of affinity dye matrices were considered during the early stages of development of this purification protocol. It was found that a series of immobilised dyes; Matrex Orange A, AffiGel Blue, Matrex Green A and Matrex Red A were all able to bind the solubilised PLD at pH 6.0 in the absence of salt (data not shown). Washing the gels with 300mM NaCl resulted in complete elution of PLD activity from the Matrex Orange A and partial elution from Affigel Blue. Increasing the NaCl concentration to 1M allowed complete elution from AffiGel Blue and initiated the elution of PLD activity from both Matrex Red A and Matrex Green A. Complete elution from Matrex Green A was achieved by multiple washes of 1M NaCl (data not shown). This ordered elution of PLD activity would indicate an increasing binding affinity of Matrex Orange A<AffiGel Blue<Matrex Green A<Matrex Red (group 1, group 3, group 3 and group 5 respectively, as described by Stellwagen, 1990). Classification of the dye matrices relies upon their individual ability to bind protein; group 1 binding least and group 5 most. Despite characteristic binding to and elution from the affinity dyes, at full preparative chromatography scale, the use of affinity dyes during the early stages of the chromatography protocol did not result in any further enrichment of the solubilised PLD (data not shown). However, it may prove beneficial as a chromatographic step incorporated into the latter stages of the established purification protocol and requires further development.

A series of amino-linked hydrophobic resins of increasing carbon chain length (agarose-NH[CH<sub>2</sub>]<sub>n</sub>NH<sub>2</sub>) were investigated for potential binding of the solubilised PLD. It has previously been reported that  $\omega$ -aminopropyl-agarose chromatography can be utilised for the successful purification of PLD from Savoy Cabbage (Allgyer & Wells, 1979). The immobilised  $\omega$ -aminopropyl group was proposed to confer a mimetic for the choline headgroup of PtdCho and allowed purification by means of a pseudo-affinity interaction. Despite this, in the presence of 1M NaCl, the solubilised PLD did not interact with any of the resins based upon hydrophobicity or otherwise. In a similar manner, the use of hydrophobic interaction chromatography was investigated using a Phenyl-Superose FPLC column. Under varying conditions in the presence of 1M NaCl and TX-100 no consistent binding to and elution from the column was observed.

Therefore, this resin in common with the series of amino-linked hydrophobic resins described above did not offer any further means of purification of the solubilised PLD and were not further investigated.

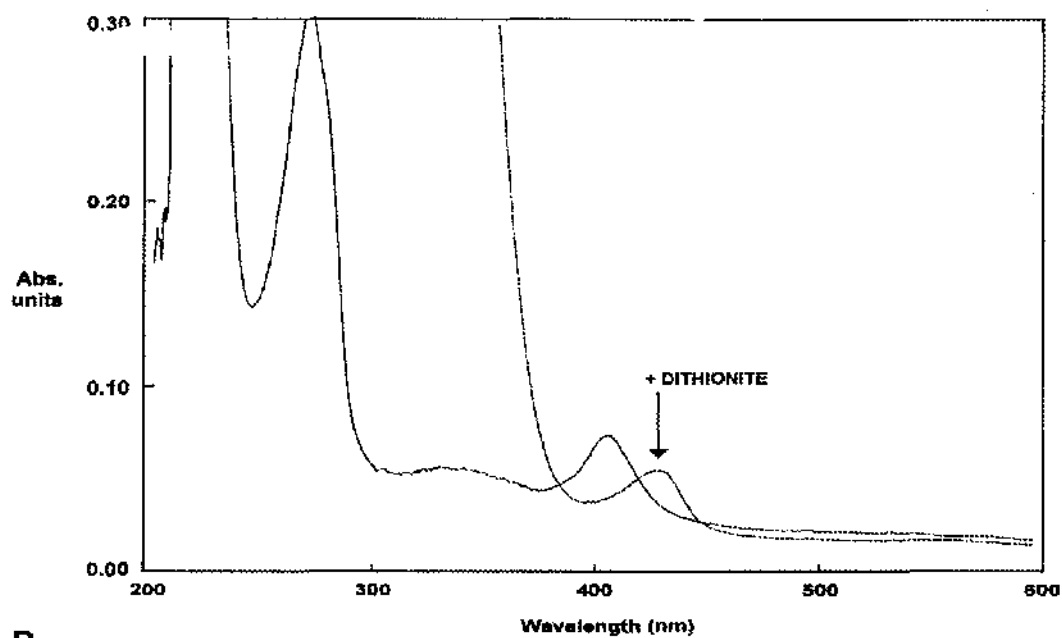
### **3.12 A Number of Proteins Co-Purify with the Solubilised PLD Activity During Chromatography.**

Partial purification of the solubilised PLD by the protocol routinely generated a preparation that displayed pigmentation and suggested the presence of a haem containing protein. This was investigated by the use of scanning spectroscopy of post S-Sepharose and post heparin-agarose PLD activity in the presence and absence of the reducing agent dithionite (Fig 3.16). This generated a maximum peak of absorption at 412nm for the oxidised form which shifted to 428nm upon reduction with dithionite. This was consistent with the observed spectra generated upon scanning of purified cytochrome b558 (cyt b558) from human neutrophils under similar conditions (Harper *et al.*, 1984). Therefore, this suggested a putative cyt b558 co-purified with the PLD activity.

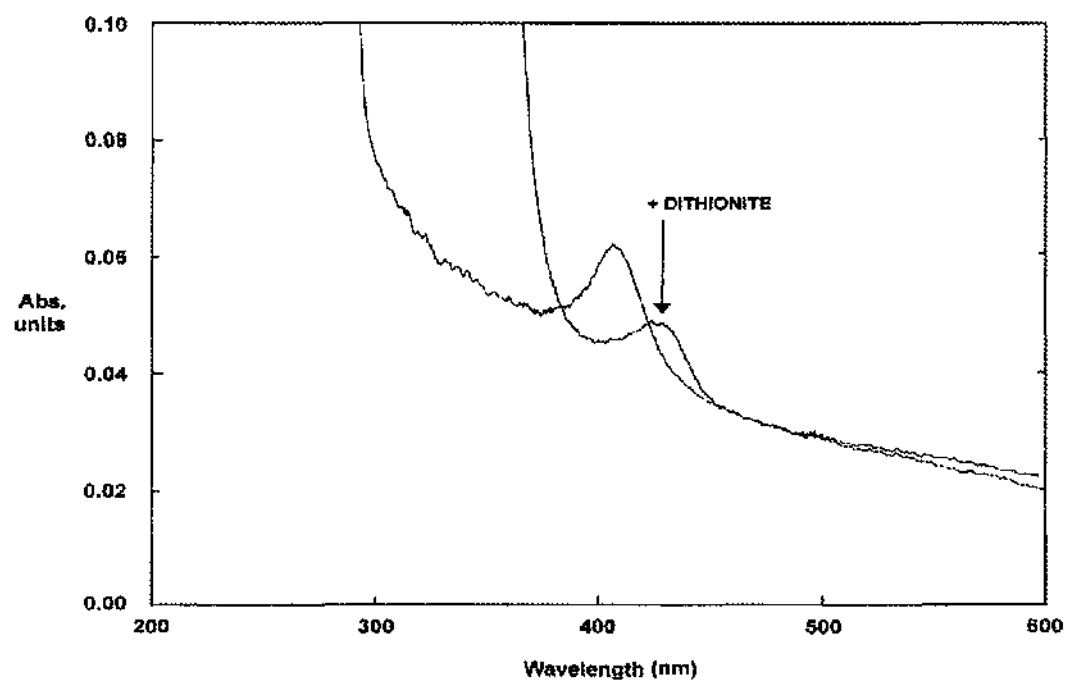
In a number of studies of haematopoietic type cells a temporal relationship between PLD activity and superoxide generation mediated by the formation of the respiratory burst complex has been described (Kocnderman *et al.*, 1989; Rossi *et al.*, 1990; Thompson *et al.*, 1990; Agwu *et al.*, 1991b) placing receptor-mediated activation of PLD kinetically upstream of superoxide production. The active respiratory burst complex is formed upon receptor-mediated assembly of multiple protein components (see Heyworth *et al.*, 1992; Segal & Abo, 1993; Chanock *et al.*, 1994) which consists of the membrane bound cyt b558 (a 22kDa  $\alpha$  subunit, p22<sup>phox</sup> and a heavily glycosylated 91kDa  $\beta$  subunit, gp91<sup>phox</sup>) with two cytosolic proteins of 47<sup>phox</sup> and 67<sup>phox</sup> kDa respectively (p47<sup>phox</sup> and p67<sup>phox</sup>) and one or more small molecular weight G-proteins including Rac1, Rac2 and/or Rap1a (Quinn *et al.*, 1989, Abo *et al.*, 1991; Knaus *et al.*, 1991; Benna *et al.*, 1994; Bromberg *et al.*, 1994). Complex formation involves the movement of these cytosolic proteins to a cytoskeletal location, the successful interaction of p22<sup>phox</sup>, p67<sup>phox</sup> and the small molecular weight G-protein with the cyt b558 is dependent upon p47<sup>phox</sup> and its expressed Src homology 3 domains (Sumimoto *et al.*, 1994). Activation appears to be GTP-dependent, however, the GDP-bound form of Rac1 has recently been described as a potent activator of the complex (Bromberg *et al.*, 1994).

Purification of p47<sup>phox</sup> has been reported to occur in association with cyt b558 (Teahan *et al.*, 1990) and its direct interaction with the C-terminal regions of cyt b558 subunits during activation of the complex has been characterised (Nakanishi *et al.*, 1992). Therefore, based on the putative co-purification of a cyt b558 with PLD activity the possibility of co-purification of the other respiratory burst proteins with the bovine spleen PLD preparation. This was examined by Western blotting (Fig 3.17) utilising polyclonal antibodies raised to the p47<sup>phox</sup> and p67<sup>phox</sup> proteins (kind gift of P. Heyworth). Initial investigations demonstrated that Western blotting of material from each stage of the purification protocol showed p47<sup>phox</sup> to be immunologically detectable to the stage of post heparin agarose-chromatography and corresponded to the expression of p47<sup>phox</sup> in HL-60 cell extracts and the lack of

**A**



**B**





**Fig 3.16 Scanning Spectroscopy of Post S-Separeose and Post Heparin-agarose PLD Preparations.**

A Scanning spectrophotometry of post S-Separeose PLD preparation.

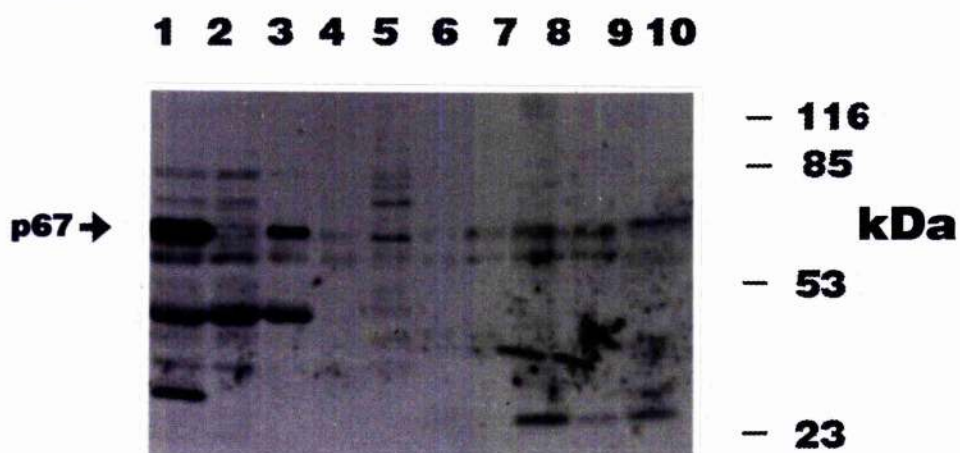
B Scanning spectrophotometry of post heparin-agarose PLD preparation.

Continuous scanning spectrophotometry of the partially purified PLD preparations (200  $\mu$ l) was performed between 200 and 600nm in a Shimadzu UV-2101PC UV-Visible scanning spectrophotometer in the presence and absence of an excess amount of solid dithionite. Absorbance profiles were recorded by a Roland Sketchmate printer.

**A**



**B**



### **Fig 3.17 Anti-p47<sup>phox</sup> and p67<sup>phox</sup> Immunoblotting of Solubilised PLD**

#### **Recovered During Purification.**

30µg of protein from whole cell lysates and the partially purified PLD preparations recovered during chromatography (listed below) were prepared in SDS-sample buffer as described in Section 2.3.3 and subjected to SDS-PAGE on 10% polyacrylamide slab gels. Resolved proteins were transferred onto nitrocellulose and membranes probed with anti-p47<sup>phox</sup> and p67<sup>phox</sup> antibodies, washed and developed using the ECL detection system as described in Section 2.8. This experiment is representative of another identical experiment.

Lane 1: Whole-cell lysate from normal human neutrophils.

Lane 2: Whole-cell lysate from CGD-p67<sup>phox</sup> deficient human neutrophils.

Lane 3: Whole-cell lysate from CGD-p47<sup>phox</sup> deficient human neutrophils.

Lane 4: Crude cytosolic fraction of HL-60 cells.

Lane 5: Crude membrane fraction of HL-60 cells.

Lane 6: Solubilised PLD.

Lane 7: Post S-Sepharose.

Lane 8: Post heparin-agarose.

Lane 9: Post hydroxyapatite.

Lane 10: Post Mono-S cation-exchange.

expression of the  $p47^{phox}$  protein in neutrophil extracts obtained from chronic granulomatous disease (CGD) patients deficient in the  $p47^{phox}$  protein (Fig 3.17A). Cross-reactivity with the anti- $p67^{phox}$  antibody was also apparent in these fractions, however, a number of polypeptides of varying molecular weight were detected (Fig 3.17B). The integrity of these polypeptides as being  $p67^{phox}$  or  $p67^{phox}$ -derived remains questionable despite comparison to cellular extracts from HL-60 cells and neutrophils of CGD patients deficient in  $p67^{phox}$ .

### 3.13 Discussion.

#### 3.13.1 Purification of Solubilised PLD from Bovine Spleen.

From initial investigations it was apparent that the membrane fraction of bovine spleen exhibited both a higher specific and higher total PLD activity than the crude cytosolic activity. It was also evident that fractionation of bovine spleen homogenate generated a crude plasma membrane fraction with a 10-fold increase in the specific activity of PLD. Significant PLD activity was also recovered in the cytosol. However, as the cytosolic preparation represented the supernatant from the 35,000 x g pellet, it is possible that this activity is associated with non-pelleted microsomal membranes. The crude membrane fraction contained higher specific and total PLD activity and was adopted as the source of enzyme for purification. This indicated that successful solubilisation or extraction of the membrane-associated activity from the heavy membrane fraction was necessary.

A wide range of agents solubilised PLD activity. It was recognised that these agents may solubilise different amounts and species of lipid to different extents from the particulate samples. These lipids would influence the assay conditions and therefore the specific activity of the recovered solubilised fractions. Since only small amounts of the solubilised and unsolubilised fractions were assayed there was only a small contribution of endogenous factors in the assay. Consequently, the non-ionic detergent TX-100 and high pH extraction appeared to be optimal. Additionally, pH extraction produced a lipid depleted and detergent free protein solution.

The use of TX-100 for successful solubilisation suggested the PLD to be a membrane-bound protein; however, the ability to recover a soluble activity after salt extraction suggested the activity to be membrane-associated. It was also evident that the freeze-thawing of the heavy membrane fraction resulted in release of PLD activity, again suggesting the PLD activity to be membrane-associated rather than membrane bound. Further, high pH extraction of the heavy membrane fraction also released activity in the absence of detergent. Therefore, it was evident that PLD activity existed as a membrane-associated protein that did not require detergent for successful solubilisation.

The existence of multiple membrane-associated isoforms of PLD cannot be discounted. No solubilising agent investigated allowed complete extraction of all the activity and therefore it may be that a number of distinct PLD isoforms are extracted by the different agents: a loosely membrane-associated activity sensitive to salt extraction, a tightly membrane-associated form requiring high pH extraction for successful solubilisation, a membrane-bound form requiring detergent solubilisation and a detergent insoluble form interacting with detergent insoluble elements associated with the plasma membrane, possibly the cytoskeleton.

Generation of a lipid depleted soluble activity, that did not require detergent for solubility allowed the use of ammonium sulphate precipitation in the purification protocol. Recovery of the majority of activity in the precipitate of 50-80 % (w/v) saturation suggested that the activity existed with a highly charged shell of hydration. It was also apparent that activity was recovered at all saturation points in the preliminary experiments. As percentage saturation increased from 0-50 % (w/v), it is probable that any remaining particulate material present in the solubilised preparation would precipitate. Therefore, the activity recovered in these fractions may represent the co-precipitation of any PLD activity in association with this remaining particulate-derived lipid. Further, the solubilising conditions used allowed extraction of peripheral membrane proteins, likely to be predominantly hydrophilic in character. Any hydrophobic regions or domains expressed by these protein may associate

specifically or non-specifically in an aqueous environment, such that complexes of proteins with a highly charged surface were formed. Under these conditions, perturbation of the soluble activity in its aqueous environment would require a high percentage of saturation.

The precipitated PLD activity was dialysed to remove salt and establish a pH of 6.0. Routinely, after dialysis the solubilised PLD preparation required clarification before initiation of cation-exchange chromatography. The recovered precipitate contained little (approximately 1%) or none of the total activity dialysed (data not shown). The precipitate was discarded and the majority of the activity, recovered in the clarified supernatant, utilised for further purification.

It was observed that binding of solubilised activity to anion and cation-exchange resins occurred at pH 8.5 and 6.0 respectively. This suggested that the solubilised activity existed as either a single protein with a neutral pI, with pH manipulation altering its electrostatic charge and hence binding to both ion-exchange resins, or as two separate activities, one anionic and the other cationic. Upon full-scale cation-exchange chromatography, the applied preparation routinely resolved as two separate activities, a minor activity associated with the unbound protein and the major activity recovered upon elution of the column. Separation of such activities may indicate the solubilisation of two membrane-associated PLD isoforms.

In conclusion, two PtdCho-hydrolysing PLD activities were observed to be present in the solubilised bovine spleen preparation, however, it may represent the activity of one single protein that has been partially modified or 'lost' a cationic region required for binding to the initial S-Sepharose column. As gel-filtration of the solubilised PLD preparation was not performed at this stage it remains speculative to suggest that these two activities represent individual PLD isoforms. Thus, this requires confirmation by biochemical characterisation of its molecular mass, true isoelectric point and kinetic parameters.

Initial small-scale chromatography with heparin-agarose suggested that PLD activity interacted in a manner similar to that observed with cation-exchange resin;

the activity interacting in an ionic manner with the immobilised polyanion. However, at full preparative scale, gradient elution of the solubilised PLD required higher concentrations of NaCl than those utilised during cation-exchange chromatography, suggesting a more specific interaction. Such interactions have been attributed to distinct heparin binding sites expressed within protein sequences (Margalit *et al.*, 1993). Such a sequence may be expressed by the solubilised PLD or a protein with which it is in close association. Therefore, it could be suggested that the use of heparin-agarose chromatography results in two chromatographic processes. Firstly, the majority of protein interacts in a manner similar to cation-exchange and secondly a percentage of the post S-Sepharose preparation, including the solubilised PLD interact in a manner based upon affinity.

Use of heparin-agarose chromatography routinely resulted in recovery of a greater amount of PLD activity than the total activity applied. This effect upon activity cannot simply be attributed to the loss of cellular lipid as this would have been removed during prior S-Sepharose cation-exchange chromatography. This suggested the possible existence of a PLD inhibitory protein or factor. Partial purification of cytosolic PLD from bovine lung also enhanced overall PLD activity by up to 20-fold (Wang *et al.*, 1991) and may further suggest the existence of a PLD inhibitory factor. Additionally, regulation of the membrance-associated activity of HL-60 granulocytes may be influenced by the potential existence of a PLD inhibitory protein, illustrated by the fact that ARF derived from bovine brain cytosol, as opposed to cytosol from HL60 cells, was ineffective in stimulating PLD activity in HL-60 membranes until it was partially fractionated by chromatography (Brown *et al.*, 1993). Further, partial purification of an ARF-sensitive PLD activity from porcine brain is only detectable after cation-exchange chromatography, with no measurable activity present in crude fractions nor solubilised extracts prior to this fractionation (Brown *et al.*, 1995). These observations also resemble the existence of an unidentified cytosol-derived inhibitor of soluble PtdCho-PLC from canine myocardial tissue (Wolf & Gross, 1985).

During hydroxyapatite chromatography the post-heparin PLD activity routinely resolved as a single peak of activity recovered by sodium phosphate elution. According to the classification of Gorbunoff (1990), this would suggest the partially purified PLD to be an acidic enzyme of pI less than 7.0. Examination of the small scale hydroxyapatite chromatography (see Section 3.6), utilising the initial solubilised material, suggested that the minor PLD activity recovered upon 1 M NaCl elution may represent another isoform with a neutral pI (7.0-7.6), distinct from the partially purified activity. This may discount the suggestion that the solubilised activity that interacts with both anion and cation-exchange resins, at pH 8.5 and 6.0 respectively, is a single enzyme. Indeed, two distinct isoforms may be present in the initial solubilised preparation, the minor one removed by cation-exchange chromatography (see Section 3.4). However, the assignment of pI values suggested by chromatography on hydroxyapatite remains tentative.

Mono-S cation-exchange chromatography served as a rapid, high resolution means of detergent removal in preparation for gel-filtration chromatography. Development of a shallow linear salt gradient also led to further purification of the eluted solubilised PLD.

Upon gel-filtration chromatography the solubilised PLD migrated with an apparent molecular weight of 69 kDa. This was different from the observed 50 kDa activity from human neutrophil cytosol (Bowman *et al.*, 1993), the 30 and 80 kDa activities partially purified from bovine lung (Wang *et al.*, 1991) and the partially purified rat brain 200 kDa activity (Taki & Kanfer, 1979). The differences in observed molecular weight may be accounted for by the absence or presence of detergents which contribute to the apparent molecular weight.

The purification protocol developed during this work shares a number of similarities to those utilised in the purification of other membrane-bound PLD activities. Ammonium sulphate precipitation has previously been reported in the partial purification of the oleate-activated membrane-associated PLD activity of rat brain (Taki & Kanfer, 1979). In common with the solubilised PLD activity described



here, this activity was precipitated at a saturation of 70 % (w/v). The chromatographic approaches used here are similar to those of published characterisations of PLD isozymes where ion-exchange, heparin, hydroxyapatite and gel-filtration resins have been exploited. These are relevant to the purification of the membrane-associated PLD activities from porcine brain (Brown *et al.*, 1995), porcine lung (Okamura & Yamashita, 1994), rat brain (Taki & Kanfer, 1979; Massenberg *et al.*, 1994) and HL-60 granulocytes (Brown *et al.*, 1993). In the most recently communicated partial purifications of PLD activities (Brown *et al.*, 1993; Massenberg *et al.*, 1994; Okamura & Yamashita, 1994) it has become apparent that successful chromatography has been possible by the use of a number of chromatographic steps that were independently developed during this research. Amongst the above studies, one outstanding example of striking similarity to the work described in this chapter, in both the nature of chromatography and the elution protocols utilised, is the partial purification of the membrane-associated PLD activity of porcine brain (Brown *et al.*, 1995). Within this particular protocol, partial purification utilised S-Sepharose cation-exchange chromatography with a 100-1000mM NaCl gradient, repeated heparin affinity chromatography with a 300mM-1500mM NaCl gradient, hydroxapatite chromatography with a 0-500mM potassium phosphate gradient and gel-filtration resolution. The similarity of these chromatographic steps, though in a different order, suggests that the solubilised PLD activity partially purified in this research may be related to the ARF-sensitive PLD activity characterised from porcine brain. However, this remains to be confirmed under the assay conditions established for identification and characterisation of such an activity (Brown *et al.*, 1993).

### 3.13.2 Does PLD Exist In A Complex With Other Proteins ?

Pertinent to the solubilised PLD described here, it was recognised that removal of detergent during FPLC cation-exchange chromatography and subsequent concentration before gel-filtration may have resulted in specific or non-specific protein:protein interactions, contributing to the determined molecular weight of 69

kDa. Therefore, the profile observed upon gel-filtration may represent that of either a single polypeptide or a number of interacting proteins in a protein complex.

Consistent with multiple proteins interacting in an undefined manner was the observed co-purification of a putative cyt b558 and immunodetectable p47<sup>phox</sup> protein to the stage of heparin-agarose chromatography. These proteins are required for the formation of the agonist-stimulated respiratory burst complex in haematopoietic cell types (see Section 3.12). In a number of cases the involvement of agonist-stimulated PLD and the formation of PtdOH have been reported to be involved in the regulation of the assembly of this complex (see Section 1.9.3). However, the nature of the interaction of PLD, or its lipid product, with these proteins during complex formation remains unknown. This may be by direct or indirect means. Therefore, co-purification of these proteins may merely reflect structural and biochemical similarities to the PLD activity described in this work such that they interact with chromatography supports in a manner independent of PLD. This requires further elucidation.

### 3.13.3 Additional Strategies for the Purification of PLD.

In the absence of purification of the membrane-associated PLD to homogeneity a number of alternative chromatographic approaches to purification could be undertaken. This is highlighted by the successful binding to and elution from the affinity dye resins described in Section 3.11. Although no significant purification was observed upon chromatography of the solubilised PLD during the early stages of development of the purification protocol, it may represent an alternative means of fractionation to be included as a later step. In addition to this, affinity chromatography of the solubilised PLD could be investigated. This would rely upon the successful immobilisation of PtdCho to a suitable chromatography support. Indeed, PtdCho covalently immobilised via its acyl chains to an agarose support has been successfully employed in the purification of *Crotalus adamanteus* venom PLA<sub>2</sub> (Rock & Snyder, 1975). Further, polyacrylamide immobilised *sn*-1, 2-diacylglycerol and *sn*-1, 2-diacylglycerol in combination with PtdSer have been utilised in the purification of

membrane-associated DAG kinase of rat brain and PKC from rabbit renal cortex respectively (Besterman *et al.*, 1986b; Uchida & Filburn, 1984). It is unknown whether the solubilised PLD would interact with immobilised PtdCho, however, both techniques remain attractive alternative chromatographic approaches towards purification of the membrane-associated PLD.

The use of ion-exchange chromatography during the purification protocol may suggest that chromatofocusing could be applied towards purification of the enzyme. During this type of chromatography, proteins are eluted by means of a decreasing pH gradient developed across a weak anion-exchange column, as their net positive charge is lost. This would rely upon the application of the solubilised PLD to a weak anion-exchange medium at a pH above its pI to allow binding. It must be realised that whilst chromatofocusing may allow further purification of the enzyme, conditions for successful chromatography may require some development as it is not known whether the solubilised PLD will bind to an anion-exchange column at a pH above its suggested pI of <7 (see Section 3.9) or whether significant loss of activity would be observed upon manipulation of the pH environment of the enzyme subsequent to partial purification.

Non-chromatographic approaches towards purification of the solubilised PLD could be undertaken, including preparative non-denaturing gel electrophoresis and preparative SDS-PAGE analysis. It is unknown whether electrophoresis of the solubilised PLD can be conducted under non-denaturing conditions. Further, the solubilised PLD remains to be identified on SDS-PAGE analysis of the post gel-filtration preparation (see Section 3.9) and this has prevented the further purification of the enzyme by preparative SDS-PAGE. Therefore, both these techniques remain to be investigated.

The putative formation of a phosphatidyl-enzyme intermediate during PLD catalysed hydrolysis of PtdCho allowed the possible isolation of such a protein/substrate complex to be investigated. This was conducted with radiolabelled lipid substrate such that any formation of a protein/substrate complex could be

detected; however, this was not successful in this study. Despite this, covalent modification of the solubilised PLD's catalytic subunit with a photo-affinity PtdCho analogue containing a photoreactive fatty acid may represent an alternative approach to the enzyme's identification. Use of a lysoPtdOH molecule containing a photo-reactive diazirine group has been utilised in the identification of a putative membrane receptor for this bioactive lipid (van der Bend *et al.*, 1992a). Suitability of such an approach would rely on its characterisation as a PLD substrate and its inhibition of modification by PtdCho substrate. Although this technique has not as yet been employed for the bovine spleen membrane-associated PLD described in this work, such an approach has been taken towards identifying a solubilised membrane-associated PLD from rat brain. Despite labelling of multiple proteins, no single polypeptide was conclusively identified as the solubilised PLD (M. Liscovitch, personal communication).

The solubilised bovine spleen PLD protein has been partially purified in this research; however, the polypeptide responsible for activity has not been unequivocally identified. Alternative approaches to the purification and identification of PLD include the possibility that a phosphatidyl-enzyme intermediate is formed during the catalytic reaction thereby allowing labelling of the protein and subsequent isolation of the protein-label complex without the preservation of enzyme activity.

## **Chapter 4**

### **Characterisation of the Solubilised Phospholipase D.**

## 4.1 Introduction.

The aim of this project was to purify the PLD activity responsible for receptor-stimulated PtdCho hydrolysis. Until recently, no PLD activities had been purified to homogeneity from a mammalian source, however, the existence of distinct isoforms with distinct phospholipid substrate specificities had been suggested (eg. Taki & Kanfer, 1979; Kobayashi & Kanfer, 1987; Wang *et al.*, 1991; Horwitz & Davis, 1993; Okamura & Yamashita, 1994; see Sections 1.8 & 1.11). To facilitate the purification of a PtdCho-specific PLD activity, PtdCho was employed as substrate in the mixed micellar assay. Further, any previous kinetic and biochemical characterisation of PLD activity has been performed upon crude fractions of broken cell preparations and not a highly purified enzyme preparation in a defined mixed micellar assay system as described in this chapter.

The catalytic rate towards PtdCho was investigated by the presentation of lipid substrate in combination with the detergent TX-100, as described by Dennis and coworkers for other lipid hydrolysing enzymes such as PLA<sub>2</sub> (Dennis, 1974; Deems *et al.*, 1975) and the phosphatidic acid phosphohydrolase and PtdIns 4-kinase activities of yeast (Lin & Carman, 1990; Buxeda *et al.*, 1991). This model is known as the 'surface dilution' model for presentation of lipid substrate to lipid hydrolysing enzymes.

Using this methodology, the activity of the partially purified preparation was characterised as a distinct Phospholipase D activity and its substrate specificity within the major glycerophospholipids determined. The divalent cation and pH dependence of the solubilised PLD's activity were also examined.

## 4.2 Phosphatidylcholine Concentration Dependence.

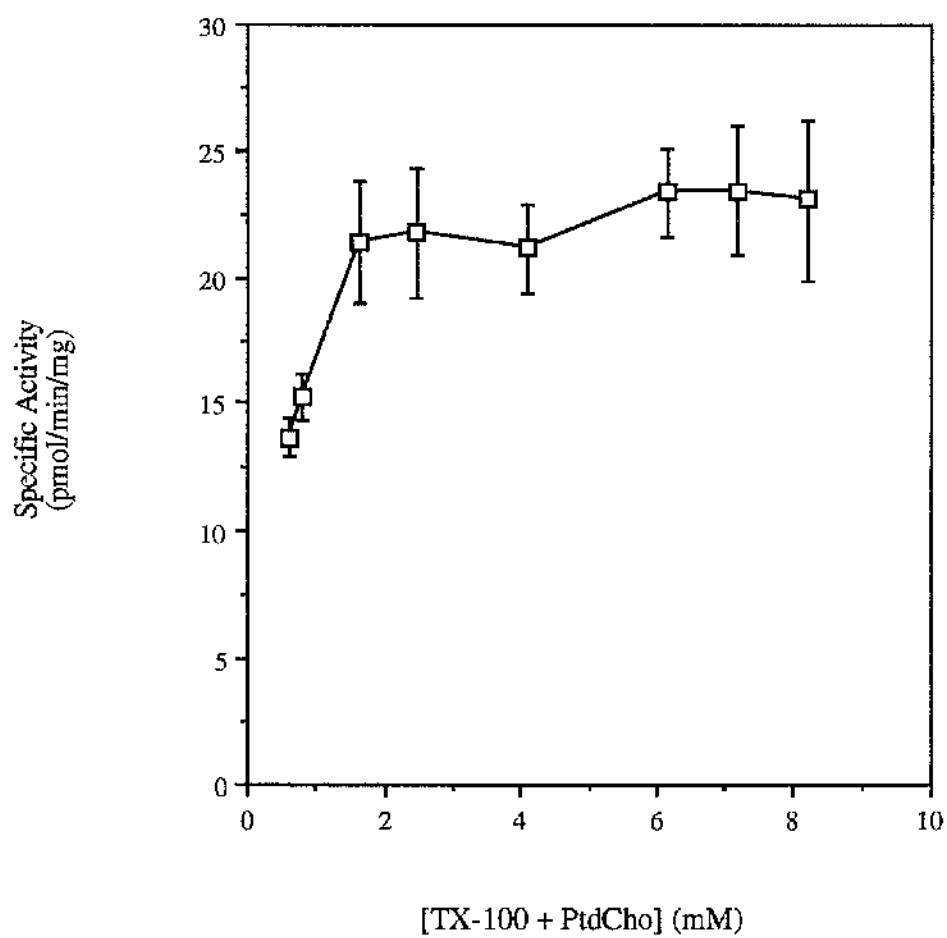
### 4.2.1 Establishment of Mixed Micellar Conditions For Measurement of Catalytic Rates.

The membrane-associated PLD was partially purified by S-Sepharose cation-exchange and heparin-agarose chromatography. This preparation was then utilised in

the kinetic analysis and characterisation of the enzyme. These studies required a defined assay for PLD *in vitro* comparable to those used for studying the detailed 'mode of action' of other lipid hydrolysing and metabolising enzymes. These enzymes normally act upon substrates that are intercalated as part of complex structures such as mixed micelles rather than ones that are dispersed (DeHaas *et al.*, 1971). Dennis and coworkers (Dennis, 1974; Deems *et al.*, 1975) described such methodology for the assessment of the activity of purified PLA<sub>2</sub> (*Naja naja*) towards mixed micelles of TX-100 and PtdCho, establishing the 'surface dilution' kinetic model. This involved the preparation of lipid/detergent micelles at fixed molar ratios whereby fixed concentrations of lipid could be maintained at the surface of the lipid/detergent micelles and 'presented' at the aqueous/lipid interface for enzyme catalysed metabolism. Kinetic analyses of these enzymes were demonstrated to be best performed using mixed micelles (Dennis, 1974; Deems *et al.*, 1975) and this methodology was utilised for the characterisation of the solubilised PLD.

Initial assessment of PLD activity in crude fractions was achieved using an *in vitro* assay based upon that described by Martin (1988); however, detailed characterisation of this assay was not determined. Therefore, in accordance with the 'surface dilution' kinetic model, PLD activity was measured as a function of the sum of the molar concentration of TX-100 plus PtdCho at a set micellar surface concentration of 2.8 mole percentage (mol %), similar to that described in the original assay of Martin (1988) and utilised during the early development of the purification protocol.

At low micellar concentrations of TX-100, where the critical micelle concentration was a significant percentage of the total detergent concentration, activity was dependent on the bulk concentration of PtdCho (Fig 4.1). However, at high detergent concentration in the micelle the contribution of the the critical micelle concentration to the total detergent concentration became less significant and PLD activity was independent of the bulk concentration of PtdCho (Fig 4.1). At these elevated concentrations saturation kinetics were exhibited and the same percentage of





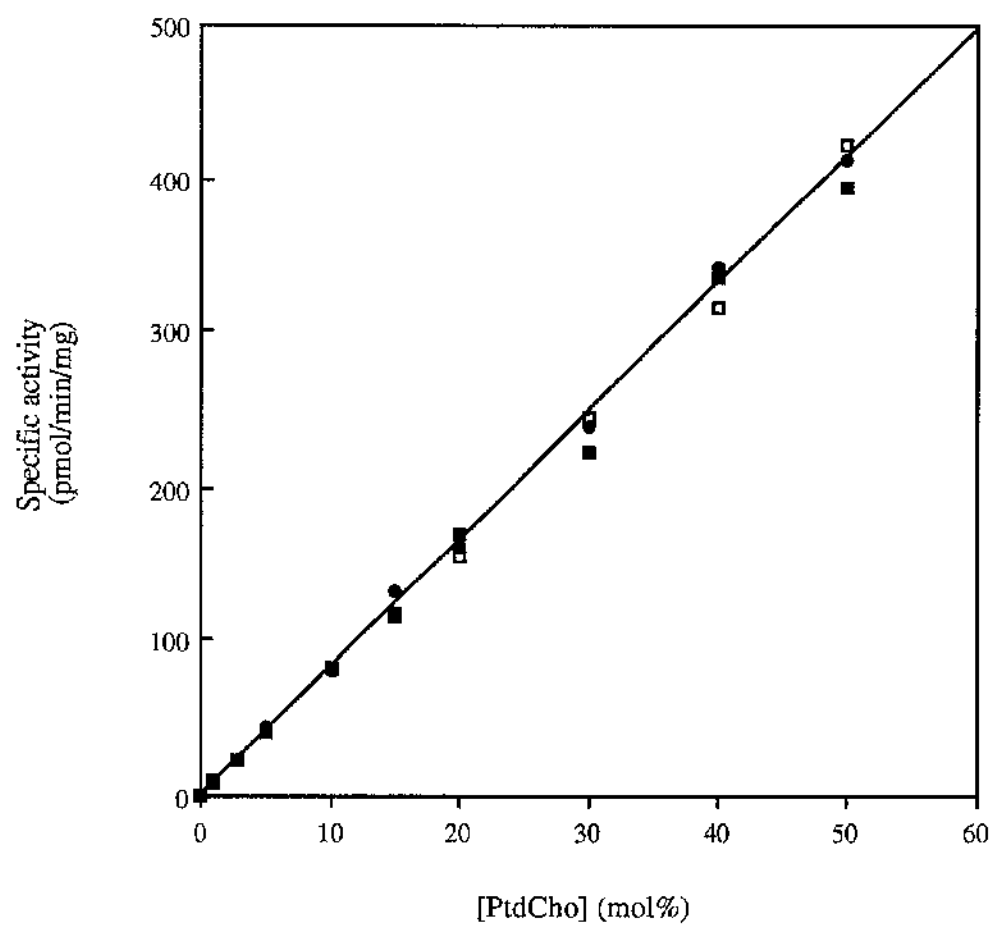
**Fig 4.1 Phospholipase D Activity Measured as a Function of the Sum of the Molar Concentrations Of Triton X-100 Plus Phosphatidylcholine.**

PLD reaction velocity was measured as a function of the sum of the molar concentrations of TX-100 plus PtdCho at a set micellar surface concentration of PtdCho (2.8 mol %). Reaction velocity was determined at the bulk concentrations indicated employing 10  $\mu$ l of the post heparin-agarose preparation. After 60 min, reactions were terminated and choline generation determined as described in Section 4.2.1. Protein concentration of the post heparin-agarose PLD preparation was determined as described in Section 2.2.1.

the total lipid substrate was hydrolysed. It was apparent that the partially purified PLD activity catalysed the hydrolysis of PtdCho substrate in a manner predicted by the model for enzymes hydrolysing substrate intercalated in a lipid/detergent phase. This represented maximal activity at a micellar surface substrate concentration of 2.8 mol% and TX-100 concentrations of 1.6 mM and higher (Fig 4.1). Therefore, the characterisation of PLD activity and its catalytic rates towards PtdCho were examined at TX-100 concentrations of 1.6mM, whereby all of the provided substrate was assumed to be incorporated into the lipid/detergent micelles and the contribution of the critical micelle concentration for TX-100 to be negligible.

#### 4.2.2 Determination of Apparent $K_m$ and $V_{max}$ Values with Phosphatidylcholine as Substrate.

At a fixed TX-100 concentration of 1.6 mM (0.1% w/v), the reaction rate of the partially purified PLD was examined over a range of PtdCho concentrations expressed as a mole percentage of the TX-100/PtdCho mixed micellar phase. The rate of reaction increased as a linear function of micellar surface PtdCho concentration (Fig 4.2). Saturation of activity with respect to substrate did not occur and a maximal rate was not measured. This was in part due to the inability of TX-100 to maintain lipid solubilisation above 40-50 mol % PtdCho. Lipid substrate was visibly insoluble at these high concentrations. Fitting of the data to the Enzfitter Version 1.05 non-linear regression data analysis computer programme revealed that the observed kinetics were typically first order. This was consistent with the mixed micelle 'surface diluton' model in that PLD activity was dependent upon increasing PtdCho concentration, however, the lack of observed saturation of activity with respect to substrate did not allow  $V_{max}$  and  $K_m$  values to be estimated and suggested the enzyme did not conform to Michaelis -Menten kinetics. Therefore, high mol% concentrations of substrate may allow zero order kinetics to be observed, however this appears to be beyond the solubilisation capabilities of TX-100 (see above).



**Fig 4.2 Phospholipase D Reaction Velocity: Concentration Dependence on Phosphatidylcholine.**

The reaction velocity of the solubilised PLD was determined as outlined in Section 4.1 in the presence of 1.6 mM (0.1% w/v) TX-100 except at the indicated increasing mol% micellar surface concentrations of PtdCho using 10  $\mu$ l of the post heparin-agarose enzyme preparation. After 60 min, reactions were terminated and choline generation determined as described in Section 2.4. Protein concentration of the post heparin-agarose PLD preparation was determined as described in Section 2.2.1.

Since saturation of activity with respect to substrate could not be achieved in this assay system, all further assays were performed at 1.6 mM TX-100 and 2.8 mol% PtdCho under conditions of pseudo first-order kinetics. These conditions displayed linear choline generation with respect to protein (50  $\mu$ g partially purified enzyme) and time (60 min) (data not shown).

#### **4.3 Dependence on Divalent Cations for Activity.**

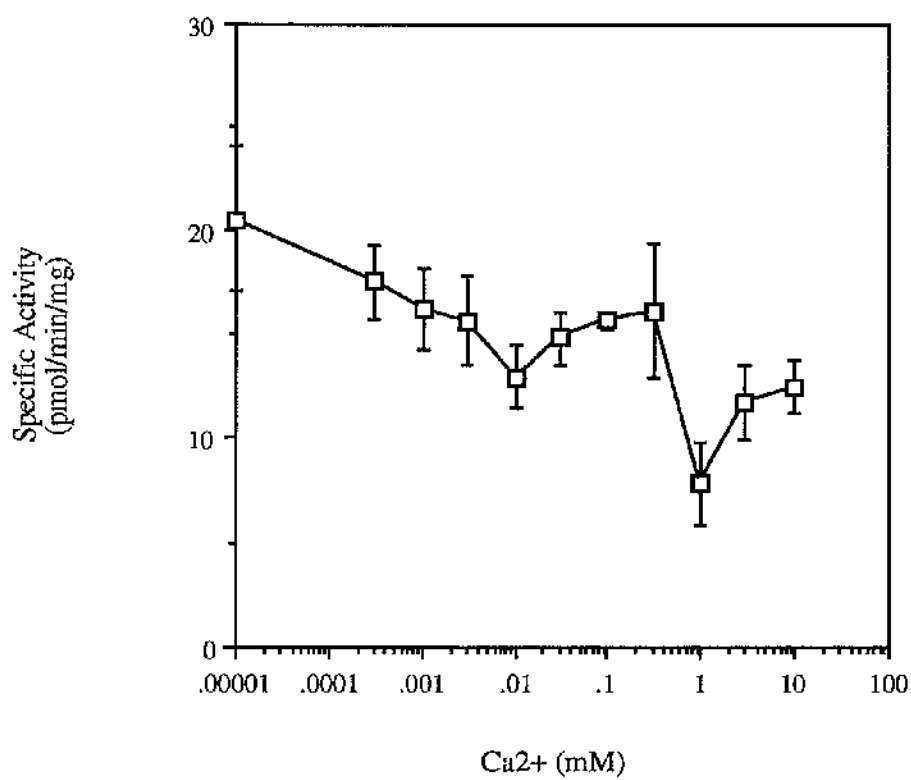
The effect of divalent cations,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , upon the rate of reaction of the solubilised PLD was investigated over the concentration range of  $10^{-10}$ - $10^{-2}$ M and  $10^{-6}$ - $10^{-2}$ M respectively. This was performed using the standard assay conditions described in Section 4.2 with free divalent cation concentrations buffered by means of EGTA/cation buffering calculated by a chelation computer programme.

In each of these investigations, under conditions of no free divalent cation/1mM EGTA, the specific activities of the partially purified PLD preparations were  $20.1 \pm 0.8$  and  $22.3 \pm 1.8$  pmol/min/mg respectively. Subsequently, the activity of the partially purified enzyme was unaltered at physiological  $\text{Ca}^{2+}$  concentrations (0.1 $\mu$ M). However, at higher concentrations of  $\text{Ca}^{2+}$  a small inhibition of activity was observed (Fig 4.3A). In contrast, the partially purified enzyme appeared insensitive to modulation of free  $\text{Mg}^{2+}$  concentration (Fig 4.3B) and no modulation of reaction rate was observed over the  $\text{Mg}^{2+}$  concentration range when assayed in the presence of  $10^{-6}$ M free  $\text{Ca}^{2+}$  (data not shown).

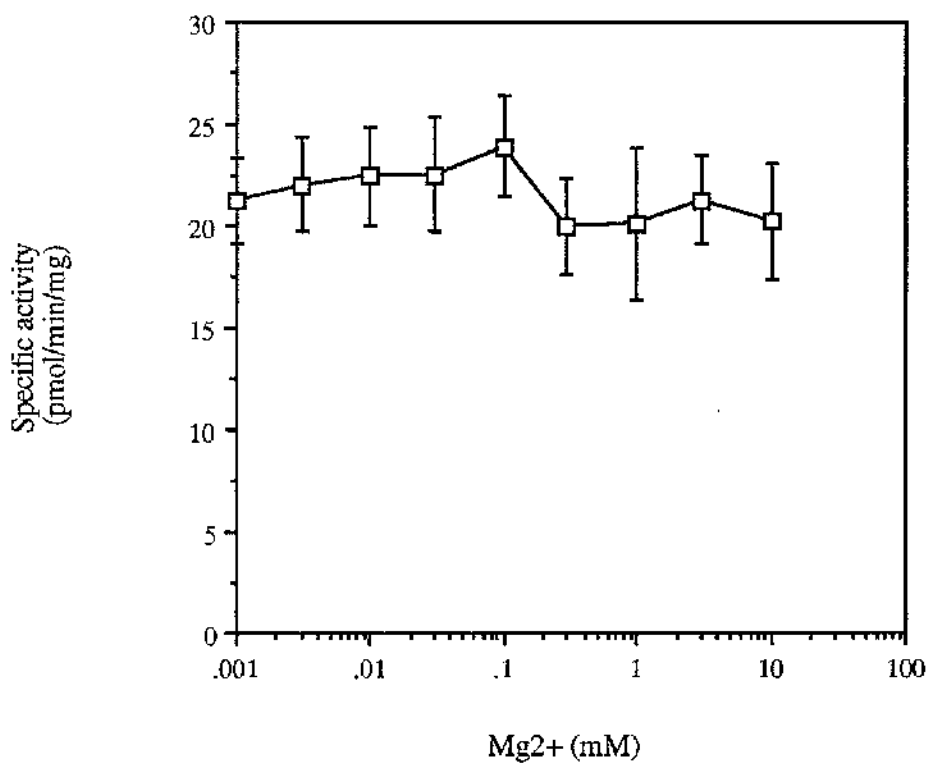
#### **4.4 pH Dependence of Activity.**

The effect of pH upon the rate of reaction of the solubilised PLD was investigated over the pH range of 5.0 to 9.0. This was performed using the standard assay conditions (Section 4.2) and a multicomponent buffering system consisting of 50 mM Acetate/Mes/Mops/Tricine; pH was fixed by addition of HCl or NaOH as appropriate. At pH 7.5, use of this buffering system to replace the customary 50 mM

**A**



**B**

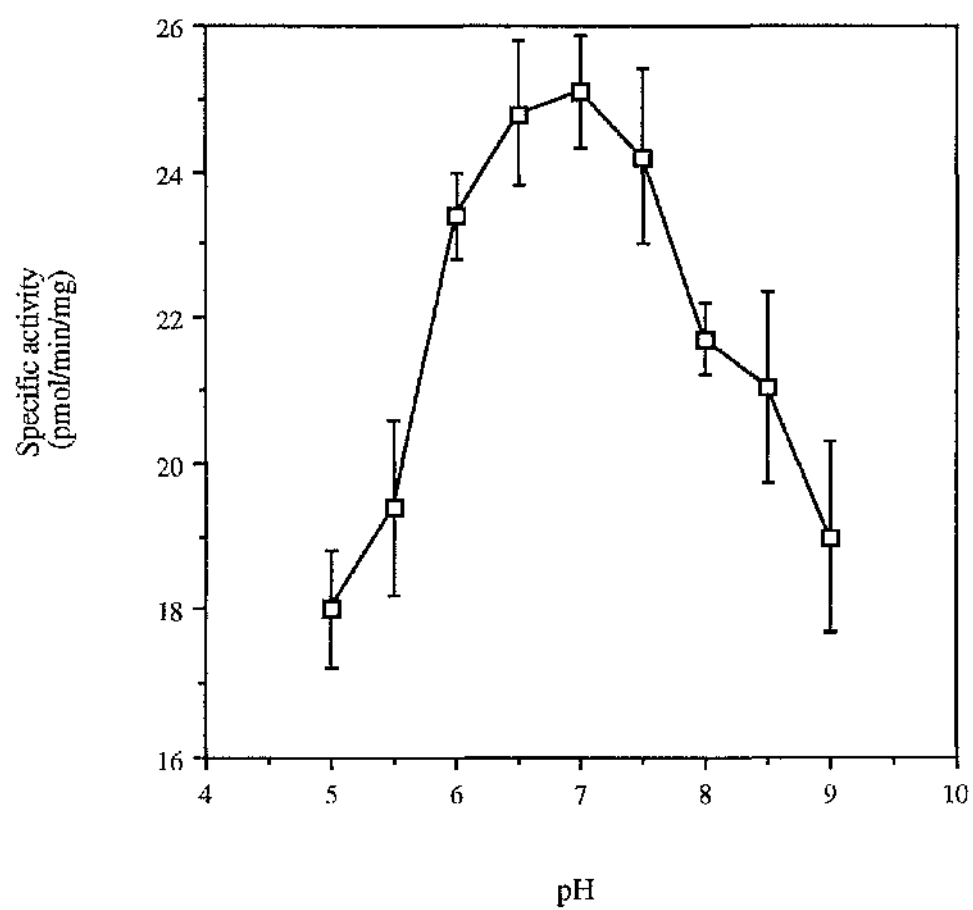


**Fig 4.3 Effect of Calcium and Magnesium Upon Solubilised Phospholipase D  
Reaction Velocity.**

**A** Effect of calcium upon solubilised PLD reaction velocity.

**B** Effect of magnesium upon solubilised PLD reaction velocity.

PLD reaction velocity was determined with 10  $\mu$ l of the post heparin-agarose preparation using the standard assay conditions described in Section 4.2.1 except free divalent cation concentrations, as indicated, were established by means of computer calculated EGTA/cation buffering. Protein concentration of the post heparin-agarose PLD preparation was determined as described in Section 2.2.1.





#### **Fig 4.4 pH Dependence of Solubilised PLD Reaction Velocity.**

PLD reaction velocity was determined with 10  $\mu$ l of the post heparin-agarose preparation using the standard assay conditions described in Section 4.2.1 except pH conditions, as indicated, were established using a multicomponent buffering system of 50 mM Acetate/Mes/Mops/Tricine, adjusted by addition of HCl or NaOH respectively. Protein concentration of the post heparin-agarose PLD preparation was determined as described in Section 2.2.1.

Types in the standard assay protocol (Section 2.4) showed no significant alteration of the observed catalytic rate of the solubilised PLD (data not shown).

Increasing the pH of the reaction mixture from 5.0 to 9.0 resulted in the reaction velocity proceeding as a parabolic function of pH, maximal activity observed at pH 7.0 (Fig 4.4). Therefore, it would appear that the reaction rate of the solubilised PLD does not significantly fluctuate in the physiological pH range and does not alter significantly at the extremes of pH examined here.

#### **4.5 Hydrolytic Activity: Identification of a Distinct Phospholipase D Activity.**

The hydrolysis of membrane phospholipids by PLD activities results in the concomitant generation of PtdOH and free base. Therefore, in pursuit of the purification of a membrane-associated PtdCho-PLD activity, choline generation was utilised as a means of monitoring PLD hydrolytic activity. However, it was recognised that this did not unequivocally identify a PtdCho-PLD activity. A number of enzymes could potentially account for the liberation of free choline from a PtdCho substrate: firstly sequential PtdCho-PLC activity and a non-specific dephosphorylation of the produced phosphocholine, secondly the sequential action of PtdCho-PLC activity and the specific dephosphorylation of phosphocholine to choline by means of a phosphocholine phosphatase and thirdly the direct generation of free choline may occur by means of base-exchange activity upon PtdCho.

These enzymatic events can be partly dispelled due to the lack of evidence in support of the existence of a specific phosphocholine phosphatase (Pelech & Vance, 1989). Further, phospholipid base-exchange enzymes rely upon the provision of appropriate free base and are markedly stimulated by millimolar  $\text{Ca}^{2+}$  concentrations (Saito & Kanfer, 1975; Mori *et al.*, 1989). However, it was apparent from previous studies (see Section 4.3) that millimolar concentrations of  $\text{Ca}^{2+}$  appeared to partially inhibit the hydrolytic activity of the partially purified PLD preparation (Fig 4.3A).

To further address the potential contribution of these enzymatic pathways to the generation of free choline, the reaction velocity of the solubilised PLD was

assessed in the presence of free base and various concentrations of the general phosphatase inhibitor, sodium fluoride. When the reaction velocity of the solubilised PLD was measured utilising the standard assay conditions (Section 2.4) in the presence of 5 mM free choline, ethanolamine, inositol or serine, no significant alteration in specific activity was observed (Table 4.1). Further, in the combined presence of 0.1mM Ca<sup>2+</sup> no further modulation of the reaction velocity was observed (data not shown). This suggested that choline generation was not a consequence of phospholipid base-exchange activity. Inclusion of NaF, at concentrations of 1, 3 and 10 mM was without effect (Table 4.1) and suggested that dephosphorylation of

**Table 4.1 Hydrolytic Activity of Solubilised PLD in the Presence of Free Base and NaF.**

<u>Addition</u>	<u>Specific Activity</u> (pmol/min/mg)
None	22.85 ± 1.9
5 mM choline	23.23 ± 3.1
5 mM ethanolamine	21.70 ± 2.4
5 mM inositol	21.98 ± 3.0
5 mM serine	23.87 ± 1.4
1 mM NaF	22.98 ± 1.7
3 mM NaF	23.68 ± 1.5
10 mM NaF	21.88 ± 3.2

10 µl of post heparin-agarose preparation was assayed for PLD activity as outlined in Section 2.4, except the above additions were made to the incubation mixture. Protein concentration was determined as described in Section 2.2.1.

phosphocholine, if formed, played no role in the generation of free choline from the PtdCho substrate. Further, inclusion of <sup>14</sup>C-labelled phosphocholine in the reaction

did not alter the reaction velocity of the solubilised PLD and did not result in the generation of  $^{14}\text{C}$ -labelled choline (data not shown), discounting the possibility of dephosphorylation of phosphocholine, subsequent to PtdCho-PLC activity.

The unique ability of PLD activities to catalyse a transphosphatidylation reaction and in the presence of short chain aliphatic alcohols form phosphatidylalcohols (Dawson, 1967), has been ubiquitously utilised as a marker for receptor-activated PLD activity in whole cell studies (eg. Bocckino *et al.*, 1987a & b; Liscovitch, 1989; Cook *et al.*, 1991). Therefore, the possible formation of phosphatidylalcohols by the purified preparation was investigated. Presentation of substrate to the enzyme was approached in a number of ways utilising aqueous dispersions of tritium labelled or unlabelled PtdCho substrate in 2 mM EDTA pH 7.5 or as mixed micelles with TX-100 or *n*-octyl- $\beta$ , D-glucoside, incubated in the presence of unlabelled ethanol or unlabelled butanol as appropriate. These conditions are summarised in Table 4.2.

**Table 4.2 Summary of *in vitro* Assay Conditions Utilised for Observation of Cell Free PLD-catalysed Transphosphatidylation.**

<u>Assay</u>	<u>Substrate form</u>	<u>Lipid substrate</u>	<u>Alcohol</u>
1	Aqueous dispersion	labelled PtdCho	unlabelled ethanol/butanol
2	Aqueous dispersion	unlabelled PtdCho	labelled ethanol/butanol
3	Mixed micelles (TX-100)	labelled PtdCho	unlabelled ethanol/butanol
4	Mixed micelles (TX-100)	unlabelled PtdCho	labelled ethanol/butanol
5	Mixed micelles (OG)	labelled PtdCho	unlabelled ethanol/butanol
6	Mixed micelles (OG)	unlabelled PtdCho	labelled ethanol/butanol

(OG-*n*-octyl- $\beta$ , D-glucopyranoside)

Under all of these conditions, the partially purified preparation failed to catalyse a transphosphatidylation reaction and no phosphatidylalcohol formation was observed. However, in the presence of TX-100 the membrane-associated activity of bovine spleen catalysed PtdOH formation in the absence of catalysed transphosphatidylation, indicating a PLD reaction (M. Wakelam, personal communication). Similar results have also been observed with membrane-associated PLD from both rat brain and spleen (M. Wakelam, personal communication).

#### **4.6 Phospholipid Substrate Specificity : Identification of a Distinct Phosphatidylcholine Specific Phospholipase D.**

Whole cell studies have suggested the main substrate for PLD catalysed phospholipid hydrolysis is PtdCho. However, the hydrolysis of both PtdEtn and PtdIns have been documented (see Section 1.8). In the context of purifying a PtdCho-specific PLD activity, the major phospholipid substrate specificity was determined. All the potential substrates, PtdCho, PtdEtn, PtdIns and PtdSer, were prepared as mixed micelles of labelled and unlabelled phospholipid with TX-100 (0.1% w/v) at a fixed concentration (2.8 mol%) in a similar manner to that described in Section 2.4. Recovery of total tritium labelled water soluble metabolites was utilised as an indication of phospholipase activity. The partially purified preparation from each stage of the purification protocol outlined in Chapter 3 was examined for hydrolytic activity (Table 4.3). Material from the final gel filtration chromatographic step exhibited activity towards PtdCho, PtdEtn and PtdSer and none towards PtdIns. This final preparation appeared to be specific for PtdCho as substrate, exhibiting an approximate 20-fold higher rate of hydrolysis than that for PtdEtn and a 4-fold higher rate than that for PtdSer (Table 4.3).

**Table 4.3 Phospholipid Hydrolysis by the Solubilised PLD.**

<u>Preparation</u>	<u>Specific Activity (pmol/min/mg)</u>			
	<u>PtdCho</u>	<u>PtdEtn</u>	<u>PtdIns</u>	<u>PtdSer</u>
S - Sepharose	9.82±0.26	0.35±0.01	2.16±0.20	4.24±0.54
Heparin	25.32±2.76	0.55±0.02	4.17±0.40	9.22±0.23
HA	28.45±1.21	1.36±0.15	4.79±0.96	5.70±0.23
Mono - S	71.55±1.87	1.56±0.32	<i>n.d.</i>	30.90±11.36
Gel - filtration	74.40±5.19	4.00±0.96	<i>n.d.</i>	17.76±0.98

2-10 µl of enzyme preparation from each stage of the purification protocol was assayed for phospholipid hydrolysis in a similar manner to that described in Section 2.4 except water soluble metabolites in the total aqueous phase were recovered as an indication of phospholipid hydrolysis. Protein concentration was determined as described in Section 2.2.1. (*n.d.*-none detected, HA-hydroxyapatite)

Further, the specific hydrolytic activity of total water soluble metabolite generation from PtdCho was comparable to the determined specific PLD activity of the post gel-filtration preparation as assessed by <sup>3</sup>H-choline generation (see Table 3.3). Further characterisation of the water soluble metabolites formed upon catalysed PtdCho hydrolysis were assessed by ion-pair extraction as routinely utilised for determination of PLD catalysed <sup>3</sup>H-choline formation during purification (Table 4.4).

Ion-pair extraction of the total aqueous products generated following hydrolysis of PtdCho by the post gel-filtration preparation confirmed choline to be the only aqueous soluble metabolite formed, neither phosphorylcholine nor glycerophosphocholine were formed (Table 4.4). However, the identification of the individual water soluble metabolites produced upon catalysed hydrolysis of the other major phospholipids was not determined and may represent not only a PLD catalysed mechanism, but other lipid hydrolysing activities. This may be consistent with the

changing ratios of PtdCho: PtdEtn, PtdCho: PtdIns and PtdCho: PtdSer hydrolysis observed throughout the purification protocols (see Table 4.5). Indeed, the maintained ratio of PtdCho: PtdEtn (approximately 1:0.04) may suggest that hydrolysis of both PtdCho and PtdEtn was catalysed by the same activity whilst that of PtdSer was by a different activity, distinct from the solubilised PLD as the ratio of PtdCho:PtdSer was observed to vary throughout the purification protocol.

**Table 4.4 Recovery of Water Soluble Metabolites Generated Following Catalysed Hydrolysis of PtdCho.**

<u>Enzyme preparation</u>	<u>dpm recovered in water</u>	
	<u>soluble PtdCho metabolites.</u>	
	<u>dpm in</u>	
	<u>glycerophosphocholine</u>	<u>dpm in choline fraction</u>
	<u>/phosphocholine fraction</u>	
Control	4012±123	778±28
post gel-filtration	4342±345	5543±156

20 µl of post-gel filtration enzyme or buffer were assayed for PtdCho hydrolysis in a similar manner to that described in Section 2.4 except following heptanone/tetraphenylboron ion-pair extraction of the total aqueous soluble products radioactivity recovered in the glycerphosphocholine/phosphocholine and choline fraction was determined by liquid scintillation counting.

**Table 4.5 Ratio of Major Phospholipid Hydrolysis Relative to that of PtdCho.**

<u>Preparation</u>	<u>PtdCho</u>	<u>PtdEtn</u>	<u>PtdIns</u>	<u>PtdSer</u>
S-Sepharose	1	0.03	0.22	0.43
Heparin	1	0.02	0.16	0.36
HA	1	0.05	0.17	0.20
Mono-S	1	0.02	N.A.	0.43
Gel-filtration	1	0.05	N.A.	0.23

In conclusion, the partially purified preparation from each stage of the purification protocol displayed hydrolytic activity towards all of the major glycerophospholipids with differing and changing specificities. However, the final post gel filtration enzyme exhibited hydrolytic activity and distinct specificity towards PtdCho, observed as an apparent PLD activity.

#### **4.7 Is The Membrane-Associated PLD of Bovine Spleen Regulated By a Small Molecular Weight G-protein ?**

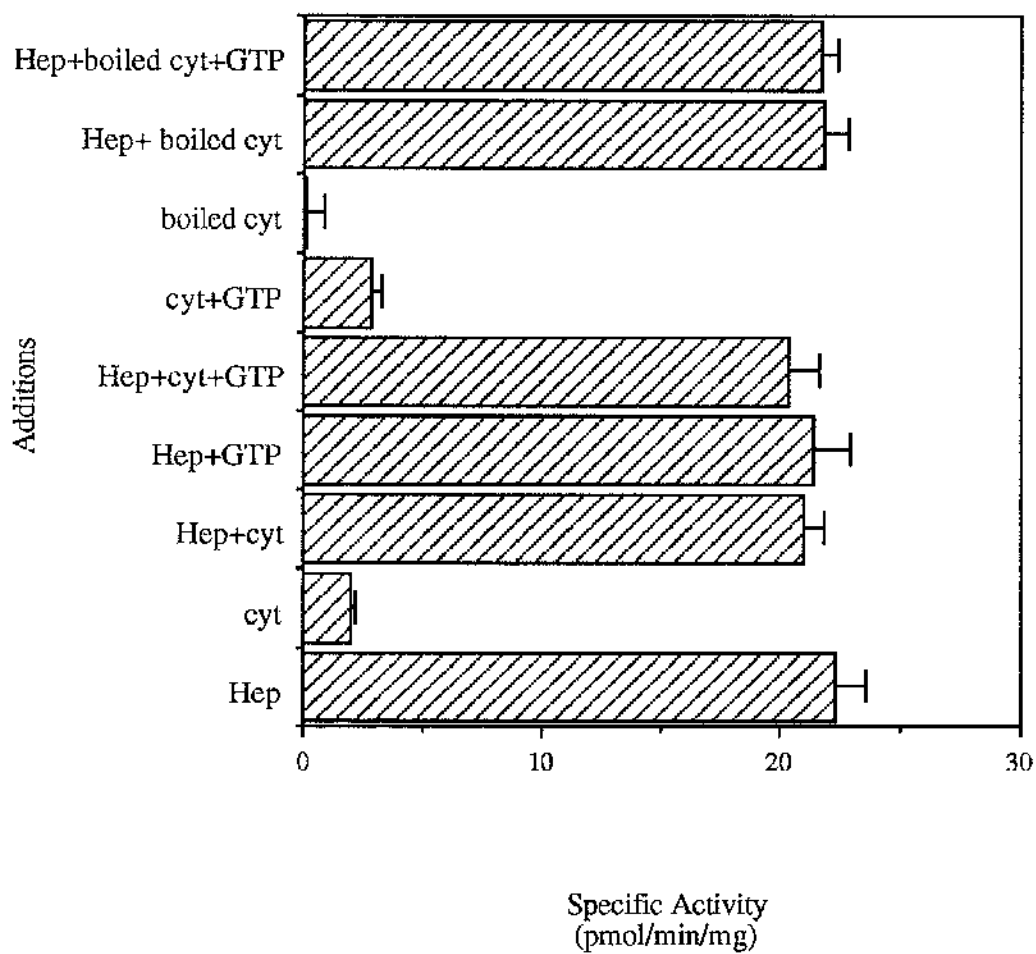
During the completion of the work towards this thesis it was reported that the small molecular weight G-protein, ARF was identified as a putative PLD activator (Brown *et al.*, 1993; Cockcroft *et al.*, 1994). Further studies also revealed the apparent involvement of other small molecular weight G-proteins of the Rho family in the regulation of PLD activity (Bowman *et al.*, 1993; Malcolm *et al.*, 1994). This questioned whether the bovine spleen enzyme described in this work could be activated in a similar manner. Experiments addressing this were initiated after SDS-PAGE analysis of fractions recovered during column chromatography showed they contained polypeptides of approximately 15-25 kDa (see Figs 3.14 & 3.15), the relevant molecular weight for members of the Ras superfamily of small molecular weight G-proteins (Downward, 1990; Hall, 1992).



Initial experiments conducted with post heparin-agarose material showed no sensitivity to 1mM  $Mg^{2+}$ -GTP $\gamma$ S in incubations under standard assay conditions with or without bovine spleen cytosolic fraction as a potential source of small molecular weight G-proteins protein (Fig 4.5). This was attributed to the detergent conditions (0.1% (w/v) TX-100), known to be inhibitory to ARF activity and GTP binding by ARF (Bobak *et al.*, 1990). Incubation of detergent free post Mono-S PLD activity in the presence of detergent/lipid conditions known to be optimal for the ADP-ribosylation activity of ARF (0.2% (w/v) cholate, 2mM PtdCho; Bobak *et al.*, 1990) resulted in enhanced specific activity of the post Mono-S preparation relative to the same material assayed under standard conditions in the presence of TX-100 (Fig 4.6). Further, a modest 2-3 fold  $Mg^{2+}$ -GTP $\gamma$ S dependent activation of PLD activity in the absence of cytosolic protein or in the presence of boiled cytosolic extract was observed (Fig 4.6). The inhibition of  $Mg$ -GTP $\gamma$ S-dependent activation of the post Mono-S preparation in the presence of crude cytosol may suggest the presence of an inhibitory factor, active against the mediator of GTP $\gamma$ S-dependent activation. This suggested that the PLD preparation may potentially be regulated in a manner dependent upon small monomeric G-proteins.

#### **4.8 Characterisation of the Solubilised Phospholipase D: Discussion and Summary.**

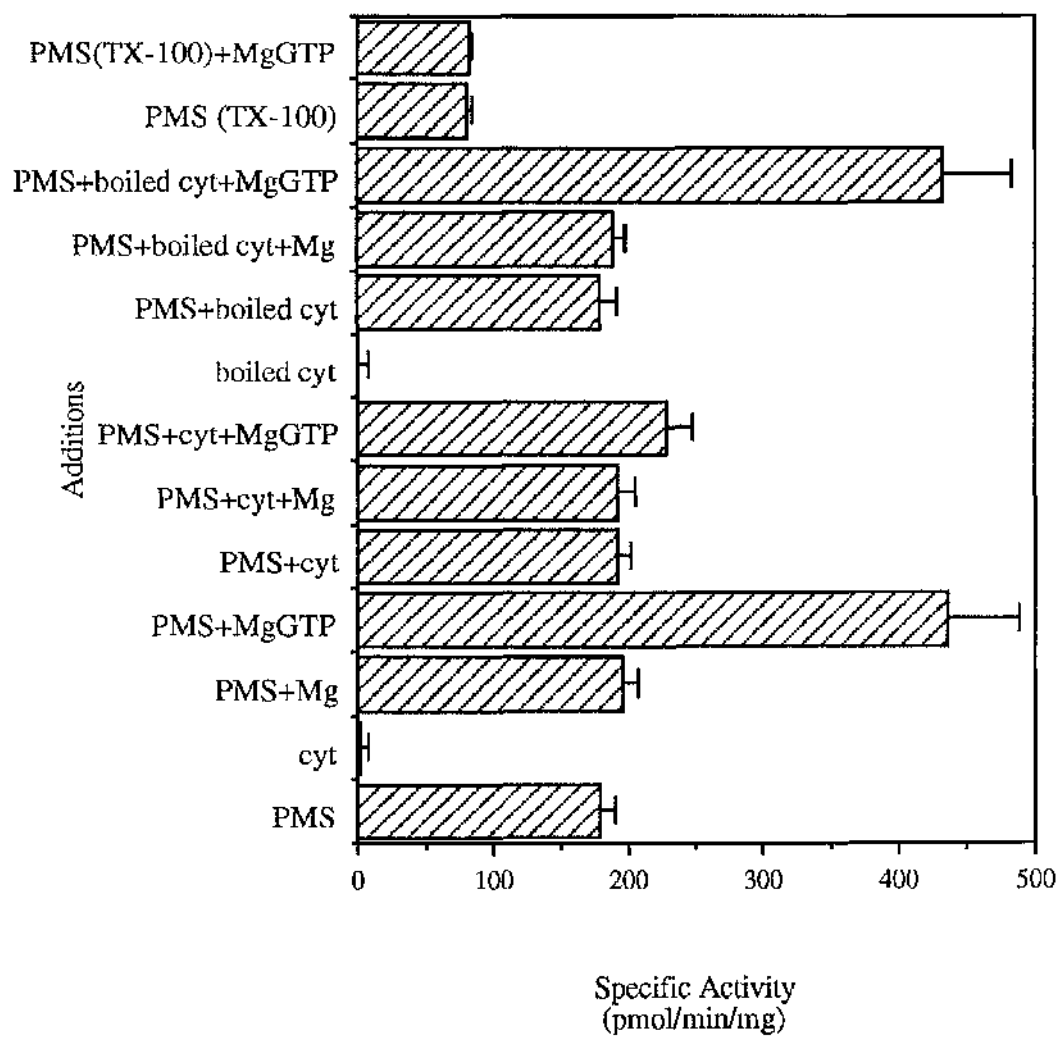
The detergent/phosphatidylcholine mixed micelle may provide an artificial lipid/water interface resembling the surface of mammalian plasma membranes and therefore be applicable to the characterisation of cell free PLD activity. Based upon the kinetic and physical characteristics described by Dennis (1974), the 'surface dilution' kinetic scheme for lipid hydrolysing enzymes appeared appropriate for the solubilised PLD. Consequently, PLD activity was observed to be dependent on both the bulk and the surface concentration of PtdCho. Studies of both rat (Martin, 1988) and rabbit tissues (Kano *et al.*, 1991) utilising PtdCho/TX-100 micelles also



**Fig 4.5 Effect of  $Mg^{2+}$ -GTP $\gamma$ S Upon Catalysed PtdCho Hydrolysis by the Post Heparin-agarose Preparation.**

PLD reaction velocity was determined with 10  $\mu$ l of the post heparin-agarose preparation under standard assay conditions as described in Section 4.2 in the presence and absence of 10  $\mu$ g of crude cytosolic protein, 1 mM  $Mg^{2+}$ , 1 mM  $Mg^{2+}$ -GTP $\gamma$ S and 10  $\mu$ g of denatured crude cytosolic protein (boiled at 100°C for 5 min) as indicated. Protein concentration of the post heparin-agarose PLD preparation was determined as described in Section 2.2.1.

Abbreviations used: Hep-post heparin-agarose PLD preparation; cyt-crude cytosolic fraction; GTP-GTP $\gamma$ S.



**Fig 4.6 Effect of  $Mg^{2+}$ -GTP $\gamma$ S Upon Catalysed PtdCho Hydrolysis by the Post Mono-S Preparation.**

Substrate was prepared as mixed micelles of 0.1% (w/v) TX-100/2.8 mol% PtdCho under standard assay conditions as described in Section 2.4 or as a detergent/lipid dispersion of 0.2% (w/v) cholate/2 mM PtdCho. PLD reaction velocity was then determined with 10  $\mu$ l of the post Mono-S preparation in the presence and absence of 10  $\mu$ g of crude cytosolic protein, 1 mM  $Mg^{2+}$ , 1 mM  $Mg^{2+}$ -GTP $\gamma$ S and 10  $\mu$ g of denatured crude cytosolic protein (boiled at 100°C for 5 min) as indicated. Protein concentration of the post Mono-S PLD preparation was determined as described in Section 2.2.1.

Abbreviations used: PMS-post Mono-S PLD preparation; cyt-crude cytosolic fraction; GTP-GTP $\gamma$ S.

described maximal PLD activity at a TX-100 concentration of 0.05-0.1% (w/v); however, at higher concentrations of detergent activity was observed to decline. This may reflect dilution of substrate, which was not maintained at a fixed surface concentration as detergent concentration was increased. Therefore, the membrane-associated PLD activities of both rat and rabbit tissues appear to hydrolyse PtdCho in a similar manner to the partially purified enzyme described here, though a potentially greater maximal hydrolytic activity may have been observed if the surface concentration was maintained in a similar manner to that proposed by Dennis (1974).

Application of Michaelis-Menten kinetics within this 'surface dilution' model did not allow estimation of both  $V_{\max}$  and  $K_m$  values for the partially purified enzyme. Saturation 'zero order' kinetics were not observed as TX-100 could not maintain PtdCho substrate solubility above 50 mol% concentrations. In conclusion, the kinetics of the solubilised PLD were not extensively characterised within the 'surface dilution' model. However, it may serve as a useful means of more thoroughly characterising the enzyme with regard to defined kinetic parameters (eg interfacial Michaelis constant, dissociation constant for the mixed micelle binding site etc.), substrate specificity towards PtdCho of varying acyl chain structure and modulation of reaction velocity by lipid molecules (discussed further in Chapter 5) or putative inhibitor compounds. The lack of saturation kinetics may reinforce the proposed existence of an inhibitory modulator of the PLD activity present during cation-exchange and heparin-agarose chromatography as suggested in Section 3.5. Further, the recent identification of the small molecular weight G-protein, ARF, as a putative PLD activator (Brown *et al.*, 1994, Cockcroft *et al.*, 1994) may indicate a requirement for a GTP-dependent *in vitro* activation of the enzyme, potentially resulting in elevated *in vitro* specific activity.

The partially purified PLD was insensitive to the modulation of both free  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions at physiologically relevant concentrations (0.1  $\mu\text{M}$  & 1 mM respectively). This is consistent with the similar reported insensitivity of the membrane-associated PLD activities of rat lung (Martin, 1988), rat brain (Kano *et*

*al.*, 1991) and canine brain (Qian *et al.*, 1990). At high concentrations of  $\text{Ca}^{2+}$ , inhibition of PLD reaction velocity was observed. In a similar manner, both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  inhibited the membrane-associated activity of rat brain, but in a concentration dependent manner (Kano *et al.*, 1991) and high millimolar concentrations of both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  slightly inhibited the membrane-associated activities of canine brain and endothelial cells (Qian *et al.*, 1990, Martin, 1988). Therefore, it would appear that a number of membrane-associated activities from various mammalian tissues display similar insensitivity to the modulation of divalent cations when reaction velocity is monitored by means of a mixed micellar assay utilising PtdCho and the non-ionic detergent TX-100 and can be regarded as divalent cation-independent activities.

In contrast to the partially purified preparation, a number of other partially purified PLD activities display different sensitivity to the modulation of free divalent cation concentration. The partially purified cytosolic and membrane-associated activities of bovine lung were reported to be activated by  $\text{Ca}^{2+}$  in a concentration dependent manner, being maximal at 10 mM, however  $\text{Mg}^{2+}$  did not effect the activity of the cytosolic enzyme but inhibited the membrane-associated form in a concentration dependent manner (Wang *et al.*, 1991). This difference may be reconciled by the fact that the investigation of Wang and coworkers (Wang *et al.*, 1991) relied upon the assessment of PLD activity by means of an octylglucoside/PtdCho dispersion assay. Independent studies examining membrane-associated PLD activities of rat brain by means of similar PtdCho/oleate dispersion assays reported differing sensitivities to divalent cations. Kobayashi and Kanfer (1987) reported that under their assay conditions  $\text{Ca}^{2+}$  (up to 5 mM) and  $\text{Mg}^{2+}$  (up to 10 mM) had no effect upon PLD reaction velocity whilst Chalifa *et al.* (1990) described a  $\text{Mg}^{2+}$  induced stimulation of PLD activity *in vitro*. Under the conditions of this assay, inclusion of  $\text{Mg}^{2+}$  prolonged linear PLD activation which may suggest that divalent cation stabilizes enzyme activity or affects enzyme-substrate interaction.

Similarly, Okamura & Yamashita (1994) described in the recent purification of a 190kDa membrane-associated PLD of pig lung that PLD activity showed no

absolute requirement for divalent cations yet their stimulatory effects were dependent upon the presence of fatty acid and may further suggest that interaction of lipid/fatty acid and divalent cation stabilizes enzyme activity as described by Chalifa *et al.* (1990). It may be concluded that a number of different PLD activities exist in a number of tissues yet this may be influenced by the differing assay methodologies utilised for monitoring PLD activity in each of these studies. Indeed, the environment within which the PtdCho substrate is presented to the enzyme may reflect the observed sensitivities to divalent cation modulation. Consequently, the mechanisms of activation or inhibition and whether these effects are upon the assay system or the enzyme itself remain to be clarified.

The partially purified bovine spleen enzyme displayed a pH optimum of 7.0. This was similar to the membrane-associated activities from various tissues described above, where PLD activity was monitored using a PtdCho/TX-100 mixed micellar assay in rat lung (Martin, 1988), rat brain (Kano *et al.*, 1991) and canine brain (Qian *et al.*, 1990) respectively. Chalifa *et al.* (1990) also described the membrane-associated PLD of rat synaptic membranes to display a neutral pH optimum (7.2). Despite the use of a similar PtdCho/oleate dispersion assay, this contrasted with the reports of Kobayashi & Kanfer (1987) and Okamura & Yamashita (1994) whereby similar investigation utilising rat brain and pig lung extracts displayed an acidic pH optimum (6.5). However, in the investigations of Chalifa and coworkers it was apparent that different buffering agents were utilised. Consequently, description of both acidic (Kobayashi & Kanfer, 1987) and neutral PLD activities (Chalifa *et al.*, 1990) of rat brain may reflect this and further evidence is required to address whether this represents the same or distinguishable enzymic entities.

Hydrolysis of L-3-phosphatidyl [N-methyl-<sup>3</sup>H] choline by the partially purified preparation showed <sup>3</sup>H-choline to be the only water soluble metabolite generated. Further, this was not secondary to glycerophosphocholine or phosphocholine formation and was not as a result of phospholipid base-exchange activity. This suggested the partially purified preparation to be a distinct PLD activity.



A unique feature of PLD enzymatic activity is the ability to catalyse a transphosphatidylation reaction and in the presence of primary aliphatic alcohols the formation of phosphatidylalcohols. The ability of the partially purified preparation to catalyse such a reaction was examined. However, under all of the assay conditions utilised no formation of phosphatidylalcohols was observed. This questioned the integrity of the partially purified preparation as a PLD enzyme, however, the ability of lipid free partially purified preparations to catalyse transphosphatidylation requires specific assay conditions. The conditions of substrate presentation, ionic strength, buffering systems, pH and presence of a membrane-like environment subtly influence the observation of *in vitro* transphosphatidylation (J. N. Kanfer, personal communication). Additionally, the competition of short chain primary alcohols with water in the transphosphatidylation reaction in an *in vivo* situation relative to that in an *in vitro* assay system may be more favourable to phosphatidylalcohol formation and consequently indicate the potential requirement for an intact membrane lipid environment to allow intercalation of alcohols and access to the enzyme active site. Hence these factors may reflect the inability of the partially purified preparation to catalyse transphosphatidylation in this study.

In examining the activity of the partially purified PLD towards different phospholipids, specificity towards PtdCho was observed. Hydrolysis of both PtdEtn and PtdSer were apparent though to a much lesser extent. PtdIns was not a substrate for the partially purified PLD. This is consistent with the observed profile of phospholipid hydrolysis in rat microsomes, where PtdCho appeared to be the preferred substrate for PLD and both PtdEtn and PtdSer gave relatively small activities (Horwitz & Davis, 1993). Despite partially purified PLD from rat brain microsomes (Taki & Kanfer, 1979; Kobayashi & Kanfer, 1987) and purified pig lung PLD (Okamura & Yamashita, 1994) displaying similar characteristics in divalent cation sensitivity, pH optima and detergent effects in a PtdCho/oleate dispersion assay the profile of phospholipid hydrolysis was inconsistent. Absolute PtdCho specificity was observed for the microsomal PLD purified from pig lung (Okamura &

Yamashita, 1994) whereas the enzyme preparation described by Taki & Kanfer utilised both PtdCho and PtdEtn, with PtdEtn giving a higher enzymatic  $V_{max}$  than PtdCho. This discrepancy remains to be clarified though confirmation of absolute PtdCho specificity has been reported for octylglucoside solubilised membrane PLD of bovine lung (Wang *et al.*, 1991). A cytosolic PLD activity from bovine lung was also described by Wang *et al.*, (1991) and displayed broader phospholipid substrate specificity, hydrolysing PtdEtn > PtdCho > PtdIns. This may represent the existence of multiple PLD isoforms though how this relates to other activities in other tissues assayed under varying conditions remains unclear. Whole cell studies have described PLD catalysed hydrolysis of phospholipids in response to agonists. This has included the stimulated hydrolysis of PtdCho, PtdEtn and PtdIns (see Section 1.8). Direct comparison of these activities and the ones described above awaits purification to homogeneity of each of these individual activities, assessment of their substrate specificity and elucidation of their regulation upon reconstitution. Purification of microsomal PLD from pig lung (Okamura & Yamashita, 1994) has allowed confirmation of a PtdCho-specific PLD activity while purification of further PLD activities will allow assignation of PtdCho-specific, PtdIns-specific, PtdEtn-specific or general phospholipid-PLD nomenclature.

In addition to the characterisation of the kinetics of PLD activity under defined detergent/substrate conditions it was also evident that the partially purified preparation recovered during Mono-S cation-exchange chromatography appeared sensitive to incubation in the presence of  $Mg^{2+}$ -GTP $\gamma$ S. Under modified detergent/substrate conditions PLD hydrolytic activity was enhanced approximately 2-fold. This suggested that a small molecular weight G-protein was present in the post Mono-S preparation and the modified assay conditions may reflect the desired protein/lipid/detergent conformational requirements for effective GTP binding of a small molecular weight G-protein, its activation of PLD or its interaction with an unidentified intermediate protein required for activation of PLD. This may be consistent with the reported activation of PLD activities by ARF and members of the

Rho subclass of the Ras superfamily of small molecular weight G-proteins (Brown *et al.*, 1993; Bowman *et al.*, 1993; Cockcroft *et al.*, 1994; Malcolm *et al.*, 1994).

Inhibition of enhanced *in vitro* activity was also evident in the presence of crude cytosol and may suggest that the crude cytosolic fraction of bovine spleen contains an inhibitory factor relevant to the regulation of *in vitro* PLD hydrolytic activity. Similar reports of cytosol-mediated inhibition of *in vitro* activity of membrane-associated PLD partially purified from HL-60 cells and porcine brain have been described (Brown *et al.*, 1993; Brown *et al.*, 1995). However, the data described above represent the results from successive purification protocols and requires further investigation and characterisation. Additionally, the presence of a small molecular weight G-protein in the post Mono-S preparation requires confirmation by Western blotting techniques or GTP-binding experiments and therefore the activation of the bovine spleen PLD in a GTP-dependent manner remains speculative.

In summary, the solubilised PLD appeared to conform to the 'surface dilution' kinetic model proposed by Dennis and coworkers, however, the  $V_{\max}$  and hence  $K_m$  for the enzyme could not be determined from the observed pseudo-first order kinetics despite the enzyme exhibiting concentration dependence upon PtdCho. This characteristic has yet to be reconciled with other PLD and lipid metabolising enzymes but may represent the kinetics of a partially active or inhibited enzyme (discussed further in Chapter 5). The reaction rate showed no dependence upon the presence of divalent cations and remained insensitive to any alteration over physiological concentration ranges and a pH optima of 7.0. In addition, the partially purified preparation displayed distinct PLD activity with specificity to PtdCho hydrolysis in accordance with the original aims of the work conducted towards this thesis. Finally, the partially purified preparation may be subject to regulation by small molecular weight G-proteins, however, this remains tentative as further development of initial studies are required.

**Chapter 5**  
**Final Discussion.**

### 5.1 Existence of Multiple PLD Isoenzymes.

Cation-exchange chromatography performed during the purification protocol described in this thesis routinely resulted in the separation of two PtdCho-hydrolysing enzymes (see Section 3.4) and may suggest the presence of two related PLD isoforms in bovine spleen. However, the additional activity was recovered as unbound material during cation-exchange chromatography and was not biochemically characterised further. It remains unclear as to whether this activity represents an additional PtdCho-specific PLD, a general glycerophospholipid-hydrolysing PLD activity or a physically modified but related form of the PLD activity. The existence of two independent PLD isoenzymes in rat brain was suggested by Kanoh *et al.* (1991) and multiple PLD activities, active against various phospholipids are apparent, though their inter-relationship and similarities remain unclear. Plasma membrane PLD preparations have been shown to preferentially metabolise PtdCho presented in detergent micelles, while microsomal preparations have been shown to preferentially hydrolyse PtdCho presented as a dispersion with fatty acid (eg. Kanoh *et al.*, 1991). This suggested existence of distinct PLD isoforms has been clarified by the separation and identification of two distinguishable PLD activities in TX-100-solubilised rat brain membranes chromatographed on heparin-5PW (Massenburg *et al.*, 1994). One PLD form was completely dependent upon sodium oleate for activity and similar to the 190kDa PLD subsequently purified to homogeneity from pig lung (Okamura & Yamashita, 1994) and the other was markedly activated by the addition of purified ARF protein and GTP $\gamma$ S in the presence of PtdIns(4, 5)P $_2$  (Massenburg *et al.*, 1994). Identification of distinguishable PLD activities from the same tissue may allow subsequent purification and characterisation of members of a putative PLD isoenzyme family. Clearly the relationship between the isoforms of PLD would be clarified by understanding the role of these enzymes within cells.

## 5.2 Functional Significance of PLD Activity.

The identification and characterisation of at least two PLD isoforms may reflect the cell's use of PtdCho in multiple metabolic processes. PtdCho represents approximately 50% of the total cellular phospholipid and within it there may be metabolic compartmentation. PLD activities may therefore not only be involved in the generation of PtdOH for second messenger functions such as the control of the cytoskeleton and the respiratory burst (see Section 1.9) but also in the maintenance and metabolism of cellular PtdCho as part of the Kennedy pathway. However, which of the PLD isoforms is involved in signalling and which is involved in general biosynthesis of PtdCho remains unclear. Whether, for example, it is the ARF activated, PtdIns(4, 5)P<sub>2</sub> sensitive PLD that is responsible for PtdCho turnover in response to agonists is not known since further molecular details are required to relate structure to function amongst PLD isoforms.

Recently a model for the co-ordinate regulation of PLD activity with that of ARF and the phosphoinositide kinases has been proposed to regulate cellular membrane trafficking events (see Section 1.11.4). This may not only reflect movement of cellular lipids by means of lipid vesicle trafficking but also cellular proteins. However, it remains unclear as to whether such an integrated transport model is relevant *in vivo* and sensitivity to agonist stimulation remains to be demonstrated.

PtdIns(4, 5)P<sub>2</sub> would appear to function as a PLD cofactor and now questions the role of PtdIns(4, 5)P<sub>2</sub>, its synthesis and its degradation in regulating PLD activation. The requirement for successful interaction of PLD with PtdIns(4, 5)P<sub>2</sub>, whether direct or indirect, may further suggest the potential involvement of cytoskeletal proteins such as gelsolin and profilin, known to bind PtdIns(4, 5)P<sub>2</sub> (Goldschmidt-Clermont *et al.*, 1990), in the regulation of PLD activity. Additionally, a 100 amino acid module within the cytoskeletal protein pleckstrin, defined as a pleckstrin homology (PH) domain, that is conserved in a number of proteins involved in signal transduction has been implicated in their membrane localisation. Within this

domain is a lipid binding site conferring interaction with membrane PtdIns(4, 5)P<sub>2</sub> (Harlan *et al.*, 1994). Such a domain structure may be present in PLD and consequently influence its cellular localisation. However, it is also apparent that ARF, in common with other known signal transduction and cytoskeletal proteins such as cPLA<sub>2</sub>, PLCs, the catalytic subunit of PtdIns 3-kinase, gelsolin and villin contains an amino acid sequence (-R/KXKK-) that apparently confers PtdIns(4, 5)P<sub>2</sub> binding (M. Hodgkin & P. Hughes, personal communication). Therefore, the cofactor requirement of PtdIns(4, 5)P<sub>2</sub> in the activation of ARF-sensitive PLD may indeed reflect direct lipid regulation of ARF activity and consequently activation of PLD.

The sensitivity of the bovine spleen PLD described in this work to other phospholipids including PtdIns(4, 5)P<sub>2</sub> has not been investigated, however, the micellar conditions described in the characterisation of this hydrolytic activity (see Section 4.2) would easily allow its assessment as a potential activator.

### **5.3 A Reappraisal of the Regulation of Receptor-mediated PLD Activation.**

Although a number of the signalling mechanisms involved in the regulation of receptor-stimulated PLD activation, including GTP-binding proteins, phosphoinositide hydrolysis, and PKC activation, have been identified and extensively characterised, it is apparent that other potential mechanisms of regulation of PLD activities may exist. This has been illustrated by recent cell free studies. In particular, a number of anomalies have arisen between the considered established mechanisms of PLD regulation in whole cells studies and observations in cell free assays. This has questioned the nature of the regulation of agonist-stimulated PLD activation and highlighted an alternative interpretation for the observations made in whole cells.

In a number of cell types it has been extensively reported that PKC activation plays a major role in the regulation of agonist-stimulated PLD activation. The ability of tumour promoting phorbol esters to activate PLD-catalysed phospholipid hydrolysis, the use of PKC inhibitors and phorbol ester-mediated down regulation of

PKC expression has confirmed PKC involvement in PLD regulation in a number of cell types. This may be mediated by PKC catalysed phosphorylation of either PLD itself or an intermediate protein required for PLD activation. Despite this, phorbol ester-stimulated activation of PLD in membranes of hamster lung fibroblasts has been suggested to occur in an ATP-independent manner (Conricode *et al.*, 1992). This contrasts with studies utilising PKC inhibitors, which are generally active against the ATP binding site of PKC isoforms, that block PLD activation. This may suggest that phorbol ester-stimulated activation of PLD may be mediated by alternative protein targets for phorbol esters that are not protein kinases. A number of proteins have high amino acid sequence identity with the cysteine-rich lipid/phorbol ester binding domain of PKC isoforms and therefore represent novel functional receptors for phorbol esters (Ahmed *et al.*, 1991). This includes *n*-chimaerin (Ahmed *et al.*, 1993) which functions as a Rac-GTPase activating protein and Vav, a potential GDP/GTP exchange factor (GEF) for the Ras-related Rho subfamily of small molecular weight G-proteins (Feig, 1994). The relative contribution or involvement of these proteins to the activation of PLD in stimulated cells remains to be assessed, but may represent an alternative mechanism of regulation. However, it is interesting to note that Rho has been suggested to be upstream of PLD (Bowman *et al.*, 1993; Malcolm *et al.*, 1994), thereby providing a possible link between phorbol ester regulated PLD *in vivo* and small molecular weight G-proteins.

The *in vitro* activation of PLD by ARF is now well established. The involvement of this monomeric G-protein would suggest that, in common with other small molecular weight G-proteins of the Ras superfamily such as Rho (Feig, 1994), the exchange of GDP for GTP upon ARF, catalysed by a guanine-nucleotide exchange factor (GEF), is required for ARF activation. A golgi membrane-bound GEF for ARF derived from CHO cells (Donaldson *et al.*, 1992) and rat liver (Helms & Rothman, 1992) has been described and this activity is sensitive to the fungal metabolite Brefeldin A (Donaldson *et al.*, 1992; Helms & Rothman, 1992). Brefeldin A has been a powerful tool for investigating membrane trafficking, however, its effect



upon the regulation of PLD activity in whole cells has not been determined. The contribution of ARF activation to agonist-stimulated PLD activation in a wide range of cell types would need to be ascertained to assess whether this represents a universal regulatory mechanism common to a number of receptor agonists.

#### **5.4 Is The Membrane-Associated PLD of Bovine Spleen Regulated By a Small Molecular Weight G-protein ?**

During the completion of the work towards this thesis it was reported that the small molecular weight G-proteins, ARF and Rho were identified as putative PLD activators (Brown *et al.*, 1993; Bowman *et al.*, 1993; Cockcroft *et al.*, 1994; Malcolm *et al.*, 1994). However, whether the bovine spleen enzyme described in this work could be activated in a similar manner remains an open question. SDS-PAGE analysis of fractions recovered during column chromatography showed they contained polypeptides of approximately 15-25 kDa (see Fig 3.14 & 3.15), the relevant molecular weight for members of the Ras superfamily of small molecular weight G-proteins (Downward, 1990; Hall, 1992).

#### **5.5 A Number of Proteins Co-purify with PLD Activity ?**

Partial purification of the solubilised PLD routinely generated a preparation that also contained a haem binding protein. The absorption spectra of this preparation was similar to the spectra generated upon scanning of purified cytochrome b558 (cyt b558) from human neutrophils under similar conditions (Harper *et al.*, 1984). Therefore, a putative cyt b558 appeared to co-purify with PLD activity. In a number of studies of haematopoietic type cells a temporal relationship between PLD activity and superoxide generation mediated by the formation of the respiratory burst complex has been described (Koenderman *et al.*, 1989; Rossi *et al.*, 1990; Thompson *et al.*, 1990; Agwu *et al.*, 1991b) placing receptor-mediated activation of PLD kinetically upstream of superoxide production.

Although further studies are required in support of these initial observations it remains intriguing to speculate about the relationship of the respiratory burst proteins and their apparent co-purification with the partially purified PLD. Further, with the recent description of PLD activation by small molecular weight G-proteins of the Rho sub-class in human neutrophils and hepatocytes (Bowman *et al.*, 1993; Malcolm *et al.*, 1994), the nature of small molecular weight G-protein-mediated signalling events may require reappraisal. This may represent a divergence in their activation of multiple effector molecules or their required involvement in PLD activation with a potentially causal role in the assembly and activation of the respiratory burst complex. Additionally, this may be indirectly related to the potential activation of the partially purified PLD in a GTP-dependent manner.

#### **5.6 An Alternative Strategy to the Purification of the Phosphatidylcholine-Specific Phospholipase D.**

Purification of mammalian PLDs has relied primarily upon effective solubilisation of membrane-associated activities, by means of detergent treatment or hypotonic salt washing and subsequent chromatography. Whilst this approach has allowed the partial purification of membrane-associated activities from a number of rat and bovine tissues and the purification to homogeneity of a microsomal PLD of pig lung (Okamura & Yamashita, 1994) a number of alternative approaches to identification and purification of PLD isoforms could be investigated. As described in Chapter 3, a more direct means of PLD isolation could be initiated by substrate photoaffinity labelling and substrate affinity chromatography. However, the general hydrophobicity of an immobilised lipid substrate, such as PtdCho, possesses particular problems for aqueous chromatography (M.Hodgkin, personal communication). Further, crude or partially purified extracts may be detrimental to the formation of specific substrate-enzyme interactions and therefore requires careful appraisal.

The identification of the small molecular weight G-protein, ARF as a putative PLD activator in HL-60 granulocytes (Brown *et al.*, 1994; Cockcroft *et al.*, 1994) and the potential involvement of other small molecular weight G-proteins of the Rho subclass in PLD activation (Bowman *et al.*, 1993; Malcolm *et al.*, 1994) have allowed partial elucidation of the mechanisms regulating *in vitro* PLD activity. However, it remains to be established whether the activation of PLD by small monomeric GTPases is mediated by direct interaction with PLD or via interaction with another intermediate protein. If the interaction is indeed a direct one then a number of chromatographic approaches could be taken towards the purification of the PLD. The immobilisation of purified small molecular weight G-proteins on a suitable chromatographic support and exposure to solubilised partially purified PLD has been attempted. Extracts from HL-60 granulocytes, were passed through a column of recombinant ARF in the presence of GTP $\gamma$ S. However, there was no detectable loss of PLD activity from the loaded extracts or recovery of PLD activity following a number of elution protocols (A. Martin; G. Thomas, personal communications). This data highlights the possibility that the ARF activation of PLD is indirect and successful affinity chromatography may require the presence of other factors eg. PtdIns(4, 5)P<sub>2</sub> or an appropriate guanine-nucleotide exchange protein to catalyse GDP/GTP exchange upon ARF. Recently it has been observed that a PLD activity prepared from HL-60 membranes is insensitive to activation by ARF (and GTP) unless a cytosolic fraction prepared by anion-exchange is present. This data implies that ARF activation of PLD requires at least one more component found in cytosol and possibly membranes (M. Hodgkin, personal communication). This is an important consideration since the ARF activated PLD has only been partially purified (Brown *et al.*, 1993; 1995). Several recent reports have identified a 50kDa protein that also activates PLD and synergy between ARF and other cytosolic PLD activators has been suggested (Lambeth *et al.*, 1995; Bourgoin *et al.*, 1995). Clearly the exact nature of the GTP dependent step in the regulation of PLD remains contentious.

If ARF-mediated activation of PLD is indirect, then the use of chromatography of this nature in combination with the pseudo-reconstitution assay utilised for the initial identification of ARF as a PLD activator in HL-60 granulocytes (Cockcroft *et al.*, 1994) may serve as a means of purifying activators or enhancers of PLD activity. It is not known whether this approach could be taken towards purification of the PtdCho-specific PLD described in this work, though it would appear from initial investigations that the partially purified enzyme from bovine spleen may be activated in a GTP-dependent fashion, potentially mediated by a small molecular weight G-protein.

### **5.7 Purification of the Phosphatidylcholine-Specific Phospholipase D: Long-Term Aims of the Project.**

The aim of purifying the PtdCho-specific PLD to homogeneity was to provide protein sequence data that would ultimately allow the construction of oligonucleotide probes to screen a bovine spleen cDNA library. Subsequent screening would allow the isolation and sequencing of a full length cDNA for PLD. Currently, sequences from PLD activities from species such as yeast are being utilised to screen human cDNA libraries for mammalian PLD enzymes (M.Wakelam, personal communication).

Comparison of the sequence generated for the PtdCho-specific PLD to those generated for other phospholipases and lipid hydrolysing enzymes may also allow the initiation of structure/function studies and the possible elucidation of protein motifs or domains expressed by the PLD in common with these enzymes. The possibility of a PLD family could also be investigated using PLD specific DNA sequences.

In addition to molecular biological studies, the purification of the PtdCho-specific PLD would allow the production of antisera. The development of activating, neutralising or identifying antisera would allow the initiation of cellular experiments to examine the role, function and localisation of the PtdCho-specific PLD in quiescent and agonist-stimulated cells.

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