Regulation of Phospholipase D in Submandibular Glands

by

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A thesis
submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Dedicated to my parents

Acknowledgements

I would like to thank:

Dr. N. Fleming, my Ph.D. supervisor, for his guidance and all the time he spent with me in discussion during the past five years.
Dr. R. P. Bhullar, for providing valuable antibodies, and for showing me the techniques of protein purification.
Dr. A. McNicol and Dr. G. Arthur for helpful advice.
Lynne Mellow, for memorable companionship, and fun English lessons.

I deeply thank all the people in Oral Biology and Faculty of Dentistry who have helped me during my difficult time.

This thesis is especially for:

My grandmother, who raised me from a baby to a pre-school boy during the years of the cultural revolution in China.
My father, who has always had high hopes for me, and who has taught me, by his example, to be an honest, loyal and conscientious person.
My mother, who has always helped me to make the right choice in my life.
My wife, Danzhu, who has taught me in our marriage, always to face life with a big smile.
My parents-in-law and sister-in-law, who have given a lot of unconditional love to my son Victor.
And finally, this big book is for you, Victor, I hope you will be able to read it soon.

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List of abbreviations

AA	arachidonic acid
Ach	acetylcholine
AlF_4^-	aluminum fluoride
Arf	ADP-ribosylation factor
ATP	adenosine 5'-triphosphate
BFA	brefeldin A
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5'-monophosphate
CLS	cardiolipin synthase
COPI	coatomer coated protein I
DAG	diacylglycerol
DEF	defroxamine
DTT	dithiothreitol
ECL	enhanced chemiluminescene
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)tetraacetic acid
ER	endoplasmic reticulum
fMetLeuPhe	N-formylmethionyl-leucyl-phenylalanine
GAP	GTPase activating protein

GCT	granular convoluted tubule
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GDS	GDP dissociation stimulator
GEP	guanine nucleotide exchange factor
GTP	guanosine-5'-triphosphate
GTPγS	guanosine 5'-0-[γ-thio]triphosphate
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IP_3	inositol 1,4,5-trisphosphate
IPTG	isopropyl-thio-β-D-galactoside
KDa	kilodalton
K_m	Michaelis constant
LPA	lysophosphatidic acid
MAP kinase	mitogen activated protein kinase
MES	2-[N-Morpholino] ethanesulfonic acid
NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaF	sodium fluoride
n_{app}	apparent Hill coefficient
PA	phosphatidic acid

PAGE	polyacrylamide gel electrophoresis
PAP	phosphatidate phosphohydrolase
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEth	phosphatidylethanol
PH domain	pleckstrin homology domain
PI	phosphatidylinositol
PIP	phosphatidylinositol monophosphate
PI(4)P 5-kinase	phosphatidylinositol 4-phosphate 5-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	12-0-tetradecanoylphorbol-13-acetate
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
PSS	phosphatidylserine synthases
PTPase	protein tyrosine phosphatase
RIA	radioimmunoassay
RSM	rat submandibular mucin
SDS	sodium dodecyl sulfate

SMG	submandibular gland
TCA	trichloroacetic acid
TGN	trans-Golgi network
TLC	thin layer chromatography
Ymt	yersinia pestis murine toxin

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Abstract

Phospholipase D (PLD) is a centrally important enzyme in signal transduction, since its hydrolysis of membrane phospholipids initiates a series of reactions that release several intracellular regulators, including phosphatidic acid, diacylglycerol and arachidonic acid. Studies were carried out on the regulation of PLD and its physiological significance, in enzymatically-dispersed acinar cells of rat submandibular glands.

In permeabilized submandibular cells, the guanine nucleotide, GTPγS, activated PLD by a mechanism that involved both a heterotrimeric GTP-binding protein and the small GTPase, Arf. Purified recombinant Arf activated PLD, and endogenous Arf translocated from the cytosolic to the particulate fraction after GTPγS stimulation. Activation of PLD by the muscarinic agonist, carbachol, was significantly inhibited by the Arf-specific blocker brefeldin A. A receptor-coupled Arf-PLD pathway is proposed, which is largely independent of the PIP_2/PLC signal transduction system.

Aluminum fluoride (AlF_4^-), an established G-protein activator, was found to inhibit GTPγS stimulation of PLD in permeabilized cells. This phenomenon was confirmed and characterized in detail in a carefully controlled cell-free PLD assay system. AlF_4^- inhibited both guanine nucleotide- and oleate-sensitive PLD in dose- and time-dependent ways. Several lines of evidence indicated that AlF_4^- inhibited PLD activation by a mechanism that was independent of G-proteins, the cAMP

signal transduction pathway or regulation of cellular phosphatases. Aluminum fluoride also potently inhibited PLD activity in submandibular Golgi membrane-enriched fractions, suggesting an additional possible regulatory role in intracellular membrane fusion/vesicle transport.

In subsequent studies, purified plant PLD from cabbage was used to confirm the AlF_4^- inhibitory effect in another PLD superfamily member, and to investigate the possible mechanism of this inhibition. AlF_4^-, but not F^- or Al^{3+} alone, directly inhibited purified PLD activity. Inhibition occurred in both PIP_2-dependent and -independent PLD assays. Since AlF_4^- is a structural analog of phosphate, the hypothesis of a phosphate-mimicking mechanism was tested. Two additional phosphate analogs, berrylium fluoride and orthovanadate, as well as phosphate itself, inhibited PLD in a dose-dependent way.

Kinetic studies suggested that cabbage PLD follows the Hill kinetic model, with a possible functional oligomerization and a high degree of cooperativity among multiple substrate-binding sites. Kinetic studies also indicated that AlF_4^- inhibits cabbage PLD activity by direct interaction with a phosphate-binding site, through a competitive mode of inhibition in which the formation of a PLD-substrate phosphatidyl-linked intermediate is blocked.

Chapter 1. Review of the literature

1.1 Salivary glands and cell signalling

1.1.1 Introduction

Salivary glands are those glands that discharge their secretory products into the oral cavity. Saliva, the final product of salivary glands, is a dilute aqueous solution containing both inorganic and organic constituents. Human saliva is produced by three pairs of major salivary glands (the parotid, submandibular and sublingual glands), and several minor salivary glands (Scheneyer and Schneyer, 1967). The latter are distributed as small groups of cells throughout the oral mucosa, and are relatively unimportant in the overall secretory response.

Saliva is a critical component of the oral environment. It plays an essential role during mastication in both bolus formation and in acting as a lubricant in swallowing and speech production. Ingested substances have to be solubilized in saliva in order to be tasted. Salivary glands, particularly the parotid, initiate the digestive process by secreting salivary amylase – an enzyme responsible for the initial stage in the breakdown of starch and glycogen. Saliva also has important buffering and antibacterial action, which inhibits or prevents the onset of dental caries (Scheneyer and Schneyer, 1967). The oral mucosa and teeth become diseased if insufficient saliva is produced. The importance of saliva and salivary glands is sometimes not apparent until the saliva supply becomes insufficient e.g. as in the case of xerostomia (dry mouth), which may be caused by degenerative diseases of the salivary glands, including Sjogren's syndrome (Siefert et al, 1986).

An estimated 500 to 750 ml of fluid is produced by the salivary glands every day (Lagerlof and Dawes 1984). This is about one fifth of the total plasma volume. The three large paired salivary glands are responsible for the bulk of saliva production. In human, the parotid and submandibular glands contribute about 90% of the total saliva volume, and of this, 60-70% is secreted by the submandibular glands.

1.1.2 Structure of major salivary glands

Salivary glands are composed of a large number of secretory units, and take the form of branching structures with terminal secretory end pieces, the acini. Each secretory unit has an acinus, an intercalated duct and a striated duct (See Diagram 1). Among different species, there may be variations on this basic plan. For example, some rodents have an additional duct, the granular convoluted tubule (GCT), lined by cells that release kallikrein-like enzymes (Ellis et al, 1993). Numerous secretory units converge on a major excretory duct which opens into the oral cavity.

Saliva is initially formed at the acini and acinar cells are the predominant cell type in a salivary gland tissue. Two types of acinar cells are found in salivary glands, mucous cells and serous cells (Scheneyer and Schneyer, 1967). These cell types have distinct histologic differences. Serous cells contain small, discrete, electron dense granules, while those of mucous cells are more diffuse and amorphous in appearance. Their secretory products can vary considerably, from

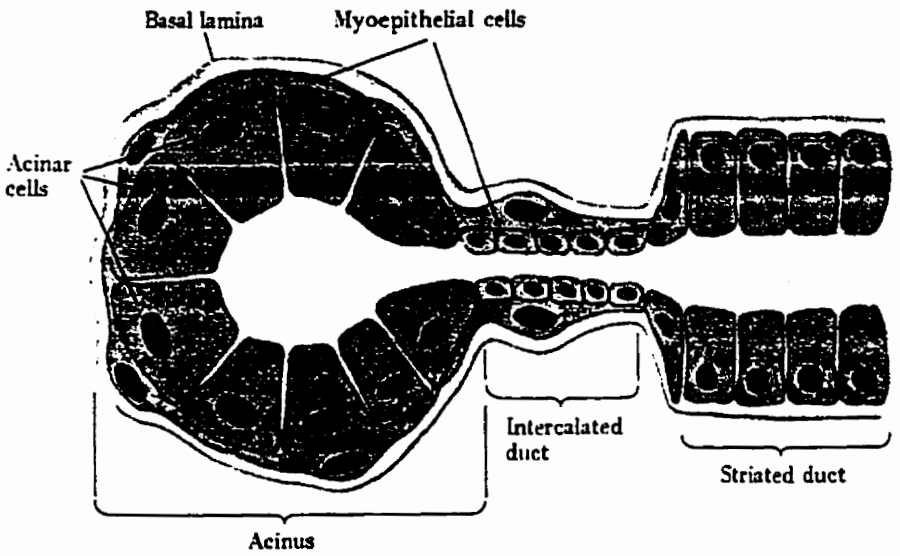


Diagram 1. Diagramatic representation of the structure of a secretory unit of a salivary gland. Experiments described in this thesis were carried out on enzymatically dispersed rat submandibular acinar cells. [Based on Johnson LR, Gastrointestinal Physiology, 2nd ed. St. Louis, CV Mosby, 1981, page 47].

viscous and rich in glycoprotein (e.g. the mucin of mucous cells) to watery and rich in proteins (e.g. the amylase of serous cells).

The water and electrolyte balance of the primary saliva is adjusted in the duct system. The intercalated ducts are short and lined by a single layer of cuboidal epithelium. They are believed to play little part in electrolyte and water transport into or out of the lumen and their main function may be as simple connectors between the acini and the ducts of the salivary gland. The striated ducts are lined by columnar epithelium, rich in mitochondria and with membrane invaginations on the basal and luminal sides to increase surface area for transport reactions. The major function of these ducts is the resorption and secretion of electrolytes from and into the primary fluid so that saliva passing into the collecting duct and on into the oral cavity is increasingly hypotonic to plasma (Scheneyer and Schneyer, 1967).

In addition to the secretory units, myoepithelial cells envelop acini and intercalated ducts. The contractile property of myoepithelial cells helps to expel the primary saliva into the duct system.

1.1.3 Physiology of salivary secretion

Salivary secretion is controlled by the autonomic nervous system. A continuous resting secretion of saliva is due to spontaneous activity of the salivatory nuclei in the brain stem. Increased amounts of saliva are secreted in response to the stimulation of chewing, taste and smell. The impulses for salivary

secretion arise in the cerebral centers and reach the acini via both sympathetic and parasympathetic fibers. Hormone-dependent stimulation via the blood stream is considered of little significance in salivary secretion. Both adrenergic and cholinergic agonists – noradrenaline (NA) and acetylcholine (Ach) are released at the synapses by the terminal nerve fibers, which target receptors on the basolateral membrane of the acinar cells (Edgar and O'Mullane, 1996). Salivary acinar cells are highly polarized. Receptors for neurotransmitters are located on the basolateral plasma membrane. On the apical (luminal) side of the cytoplasm, there are secretory granules ready to be exocytosed through the apical membrane. From excitation to secretion, the whole process of exocytosis is controlled by receptor-coupled signal transduction pathways and the generation of second messengers within the cell.

Salivary secretion can be considered as a "two-stage" process. The secretory end piece – the acinus, first produces a primary saliva from the plasma, which is isotonic with serum (Edgar and O'Mullane, 1996). Primary saliva is then modified during its passage through the duct system. Most of the modification occurs in the striated ducts, where striated duct cells actively reabsorb sodium ions to render the saliva progressively more hypotonic as it flows towards the mouth (Edgar and O'Mullane, 1996).

Salivary secretion can be broadly divided into two types: fluid secretion and macromolecular secretion. The first includes water and electrolytes, the second includes glycoproteins, enzymes, immunoglobulins and other proteins (Edgar and

O'Mullane, 1996). Fluid and macromolecular secretion occur by separate processes. However, both are under the control of the autonomic nervous system. Parasympathetic nerves release acetylcholine (Ach) and stimulate mainly fluid secretion. Sympathetic nerves release noradrenaline (NA) and stimulate mainly protein secretion. However, in some glands, parasympathetic stimulation also causes significant release of proteins or glycoproteins, such as salivary mucin (see below). There are several signal transduction mechanisms that regulate secretion of salivary mucin.

1.1.4 Signal transduction and the control of salivary mucin secretion in rat submandibular acini

Mucins are the principal organic constituents of the mucus that coats all mucosal surfaces of the body, including the oral cavity. Mucus is the first line of defense against mechanical, chemical and microbial insults to the underlying hard and soft oral tissues (Tabak, 1990). It is also of central importance in bolus formation in swallowing. Mucins are high molecular weight glycoproteins comprising a peptide core (apomucin) enriched in serine, threonine and proline residues, plus carbohydrate side chains (oligosaccharides) that are linked O-glycosidically to threonine or serine residues of the protein backbone. The unique physical and chemical properties of the mucus coat are largely attributed to the heavily glycosylated mucins. Sumandibular acinar cells are mucin-secreting cells,

in contrast to amylase-secreting parotid acini. Most of the mucus in rat saliva is contributed by the acinar cells of the submandibular glands (Leon et al, 1995). The secretory mechanism of salivary mucin has been studied in different models, including slices of parotid or submandibular glands (Bogart, 1995), and the enzymatically-dispersed cells of rat submandibular acini (Quissell and Redman, 1979). These cells are obtained by the use of collagenase- and hyaluronidase digestion, chelation of divalent cations, and mild disruptive shearing forces, followed by filtration through a nylon mesh (Kanamura and Barka, 1975). This treatment effectively removes connective tissue and duct cells, so that the resulting preparation consists of mainly acinar cells which retain their three-dimensional relationship, their typical morphology and their sensitivity to different secretagogue classes and other regulators of signalling mechanisms.

It is well established that sympathetic stimulation through β-adrenergic receptors stimulates mucin secretion in rat submandibular cells (Bogart and Picarelli, 1978; Quissell and Redman, 1979; Quissell and Barzen, 1980; Fleming et al, 1980). Early studies were done by measuring the secretion of D-[1-^{14}C] glucosamine hydrochloride-labeled mucin following sympathomimetic stimulation. Quissell et al (1981), found that cellular cAMP levels increased dramatically following β-adrenergic stimulation. Furthermore, cAMP analogues were able to elicit a secretory response in the absence of β-adrenergic receptor activation. Isoproterenol (IPR), a β-adrenergic agonist, or dibutyryl cAMP

stimulation was associated with an enhanced phosphorylation of three proteins with apparent molecular weights of 34, 26, and 21 Kda respectively (Quissell et al, 1983a, b). However, the nature or function of these proteins was not established and still remains unclear. cAMP-dependent protein kinase activity in rat submandibular gland cells was markedly and rapidly increased following β-adrenergic receptor activation. The dose-response relationship of the kinase activation correlated well with isoproterenol-induced mucin release. It was found that type I protein kinase A activation occurred rapidly throughout the entire secretory period, followed by inactivation after β-adrenergic receptor blockade (Quissell et al, 1988). These studies, mainly by Quissell and colleagues, established that a major control system of mucin secretion in rat submandibular glands is the β-adrenergic/cAMP signal transduction pathway.

Bogart and Picarelli (1978), observed that α-adrenergic and muscarinic cholinergic, as well as β-adrenergic stimulation of rat submandibular gland slices evoked mucin secretion, as quantitated by assay of sialic acid, a common terminal sugar on mucin oligosaccharide side chains. Taylor et al (1992a), confirmed that muscarinic (carbachol) stimulation of rat submandibular acinar cells caused a significant protein secretion, and also strongly potentiated IPR-induced mucin secretion. In dog submandibular gland cells, the mucosecretory response to acetylcholine was also strongly enhanced (Dohi et al, 1991). These studies indicated that signalling mechanisms coupled to both sympathetic and

parasympathetic stimulation are involved in the regulation of mucin secretion in salivary glands.

Significant advances were made in the 1980s by the Fleming group, with the purification of rat submandibular mucin, the generation of an anti-mucin antibody and the development of a radioimmunoassay (RIA) for mucin quantitation (Fleming et al, 1982, 1983). Rat submandibular mucin (RSM) was, for the first time, isolated as a large molecular species (Mr > 200 Kda), and shown to contain 63% carbohydrate, with high sialic acid and N-acetylgalactosamine content, and a protein backbone rich in serine, threonine and proline residues (Fleming et al, 1982). Antibody to RSM was prepared in rabbit and produced a single precipitin line on immunoelectro-osmophoresis with the mucin. Immunofluorescence studies showed that the antibody localized only to submandibular acinar cells and confirmed that these cells were the source of RSM (Fleming et al, 1982). A solid phase radioimmunoassay (RIA) for rat sumandibular mucin was subsequently developed (Fleming et al, 1983) and applied to studies of mucin secretion. This technique was capable of detecting as little as 3 ng mucin protein, and showed clear advantages over earlier methods of mucin quantitation - sialic acid assay (Bogart, 1975), ^{3}H-glucosamine labelling (Quissell and Redman, 1979) - in both specificity and sensitivity. By using this RIA, a series of studies was carried out in this laboratory to investigate the signal transduction mechanism of mucin secretion in rat submandibular model (Fleming et al, 1986; 1987a,b; 1992). It was first confirmed that the β-adrenergic/cAMP pathway was the major stimulator of mucin

secretion in rat submandibular glands. However, significant mucin release (> 40%) could also be elicited by stimulating cells with a muscarinic cholinergic agonist (carbachol), α_1-adrenergic agonist (methoxamine), or tachykinin agonist (substance P). All three of these agonists are coupled to the phosphatidylinositol (PI)-phospholipase C (PLC) signalling pathway. This led to speculation that this pathway may play a central role in the preliminary phase of stimulus-secretion coupling in submandibular glands, in which receptor signals are converted into intracellular messengers by signal transduction mechanisms. Therefore an interest in the phosphoinositide signalling pathway, and in the role of phospholipids in general, in salivary gland secretion was developed.

1.1.5. The PIP_2 hydrolysis pathway and signal transduction in rat submandibular glands acinar cells.

The hydrolysis of membrane phosphoinositides by PLC was described by Hokin in the mid 1970s (Hokin, 1974), but the significance of this as the basis for signal transduction reactions of many classes of agonist was not realized until the early 1980s (Berridge, 1983, 1984). Briefly, receptor stimulation activates PLC via a Gq heterotrimeric regulatory G protein, to cause the hydrolysis of membrane phosphoinositides, predominantly phosphatidylinositol 4,5 bisphosphate (PIP_2). Cleavage of PIP_2 generates two signalling products, diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP_3). DAG activates protein kinase C (PKC), a ubiquitous multi-isoform enzyme that regulates many cellular functions (Nishizuka,

1988, 1992). IP_3 acts on endoplasmic reticulum receptors to cause the release of stored calcium, also a multifunctional regulator, into the cytosol (Irvine et al, 1984, 1986). Some of the PKC isoforms (the so-called classical forms) are calcium requiring. The PLC-PIP_2 signal transduction pathway is therefore unique, in that a single signal switches on two separate second messenger pathways that are often cooperative.

Although there was a flurry of research in PLC-PIP_2 signalling mechanisms in the 1980s, this field was largely ignored in salivary glands. This, and the observation that PIP_2-coupled agonists caused mucin secretion (see above), prompted Fleming and coworkers to undertake a series of studies on PIP_2-related signalling in the submandibular model. It was established that protein kinase C (stimulated by phorbol esters or diacylglycerols), caused the time- and dose-dependent secretion of mucin in dispersed rat submandibular cells (Fleming et al, 1986). It was subsequently shown that muscarinic-, α1-adrenergic, and tachykinin agonists stimulated PIP_2-hydrolysis, liberated IP_3 and elevated mucin release in the model (Fleming et al, 1987a). Furthermore, exogenous IP_3 , as well as carbachol and guanine nucleotides were found to increase cytosolic Ca^{2+} levels in acinar cells. The work of Laniyonu et al (1990), developed these findings further by showing that the predominant PIP_2-coupled muscarinic receptor was the M3 subtype, and that NK1 type tachykinin receptors also coupled to the same system. The observation that stimulation of these receptors could also modify the cAMP signalling pathway (Laniyonu et al, 1988,1990) suggested possible cross-talk

regulation between the two transduction pathways. This was subsequently indicated by the inhibition of cAMP levels and of the cAMP-induced mucosecretory response by PKC in the submandibular model (Fleming et al, 1992). These studies clearly indicated a central role for the phosphoinositide signal transduction pathway in the regulation of salivary gland physiology, and stimulated a wider interest in the possible functions of phospholipids in general, and their breakdown products, in salivary gland signalling.

1.1.6 Arachidonic acid and signal transduction in submandibular glands

Arachidonic acid (AA, *sn* 20:4,ω6), is an essential fatty acid found in membrane phospholipids, where it is esterified at the *sn*-2 position on the glycerol backbone. AA is well known as the precursor of a series of extremely bioactive metabolites (prostaglandins, thromboxanes, leukotrienes) known collectively as eicosanoids. These have widespread regulatory roles in many physiological processes, including GI and respiratory function, parturition, mast cell degranulation etc. (Shimizu and Wolfe, 1990). There is increasing evidence that AA itself may be a significant regulatory molecule with several functions that do not require its conversion into eicosanoids. In submandibular cell studies, Chung and Fleming (1992), found that AA inhibited the carbachol-induced generation of IP_3 by 75%. This effect was not duplicated by a range of prostaglandins or leukotrienes, and was not inhibited by blockers of eicosanoid synthesis. Studies on phosphoinositide turnover suggested that AA acted in the synthetic phase of the PI cycle, at a locus

downstream to PI generation (Chung and Fleming, 1992). Subsequent kinetic studies showed for the first time that AA inhibited the synthesis of PIP and PIP_2 by acting as a classical noncompetitive inhibitor of both synthetic enzymes, PI-4 kinase and PI(4)P-5 kinase (Chung and Fleming, 1995a). These findings suggested the possibility of regulatory feedback control of phospholipid-related signalling systems by AA.

Additional potential regulatory functions of AA were investigated in the submandibular model. It was found that AA potently inhibited protein synthesis and lowered ATP levels to 25% of control values (Fleming and Mellow, 1995a). AA also stimulated the release of up to 73% of Ca^{2+} stored in the endoplasmic reticulum and provoked mucin secretion in dispersed submandibular cells (Fleming and Mellow, 1995a). It was therefore concluded that arachidonate was an important, central and multipotent regulator of key physiological functions in salivary glands. This led to studies on the phospholipid source of released AA and the characteristics of its enzymatic cleavage pathways, particularly those involving an initial cleavage of membrane phospholipids by phospholipase D.

1.1.7 Role of phospholipase D in the release of ararchidonic acid from phospholipids in submandibular cells.

Muscarinic cholinergic stimulation is one of the major stimuli for AA release in submandibular cells (Chung and Fleming, 1995b). Since AA is esterified at the *sn*-2 position in phospholipids, the acid may be releasd by direct action of

phospholipase A_2 (PLA_2), as is the case in many other models (Dennis et al, 1991). However, measurements of the appropriate lysophospholipid metabolites showed that PLA_2 did not act significantly on either phosphoinositides or the major membrane phospholipid, phosphatidylcholine (PC), to release AA (Chung and Fleming, 1995b). Accumulating evidence from other cell models suggested that an alternative pathway for AA release might involve a series of reactions beginning with the cleavage of PC by phospholipase D (PLD) (Axelrod et al, 1998). This possibility was investigated in the submandibular model by Chung and Fleming (1995b). Carbachol treatment of submandibular cells produced a dose-dependent activation of PLD to around 5-fold control values. This response was inhibited by 60% by U73122, a blocker of PIP_2-specific phospholipase C (PLC), suggesting that cleavage of PC by PLD was, in part, secondary to PIP_2 hydrolysis. This was confirmed by the observation that both the PKC- and Ca^{2+}-mobilizing arms of the PIP_2 response activated PLD. It was shown in another study that the predominant form of PKC isoform in rat submandibular acinar cells is PKCε (Fleming and Mellow, 1995b).

In the same study, sequential reactions that might release AA from PC were investigated. These involved the stepwise conversion of PC to phosphatidic acid (PA), the conversion of PA to DAG (by the enzyme phosphatidate phosphohydrolase), and the cleavage of AA from DAG (by the enzyme diacylglycerol lipase). This pathway has been proposed in mast cells (Ishimoto et al, 1994), endothelial cells (Matilla et al, 1993) and other models. It was

determined by measuring the intermediate metabolites and by using the phosphatidate phosphohydrolase blocker, propranolol, that PC is a major source of regulatory AA, released by the PC - PA - DAG - AA pathway (Chung and Fleming, 1995b). Therefore, since AA is a multipotent regulator in salivary glands, and since its precursors PA and DAG themselves may also have important regulatory functions, the importance of agonist-coupled PLD cleavage of PC as a key preliminary signal transduction reaction was established. Moreover, it is likely that PLD may also play a central role in intracellular vesicle transport and exocrine secretion, a prime function of salivary glands (see below). Thus, knowledge of the regulation of PLD would be of obvious use in developing our understanding of salivary gland function and regulation.

1.2 Phospholipase D

1.2.1 Introduction

Phospholipase D is a phosphodiesterase that catalyses the hydrolysis of phospholipids into phosphatidic acid (PA) and the corresponding head groups. The predominant substrate for mammalian PLD is phosphatidylcholine (PC) – the most abundant phospholipid in cell membranes. The cleavage reaction generates phosphatidic acid and free choline. In the presence of a primary alcohol, e.g. ethanol, PLD catalyzes a unique transphosphatidylation reaction, which is a special characteristic of PLD (Morris et al, 1997b; see Diagram 2).

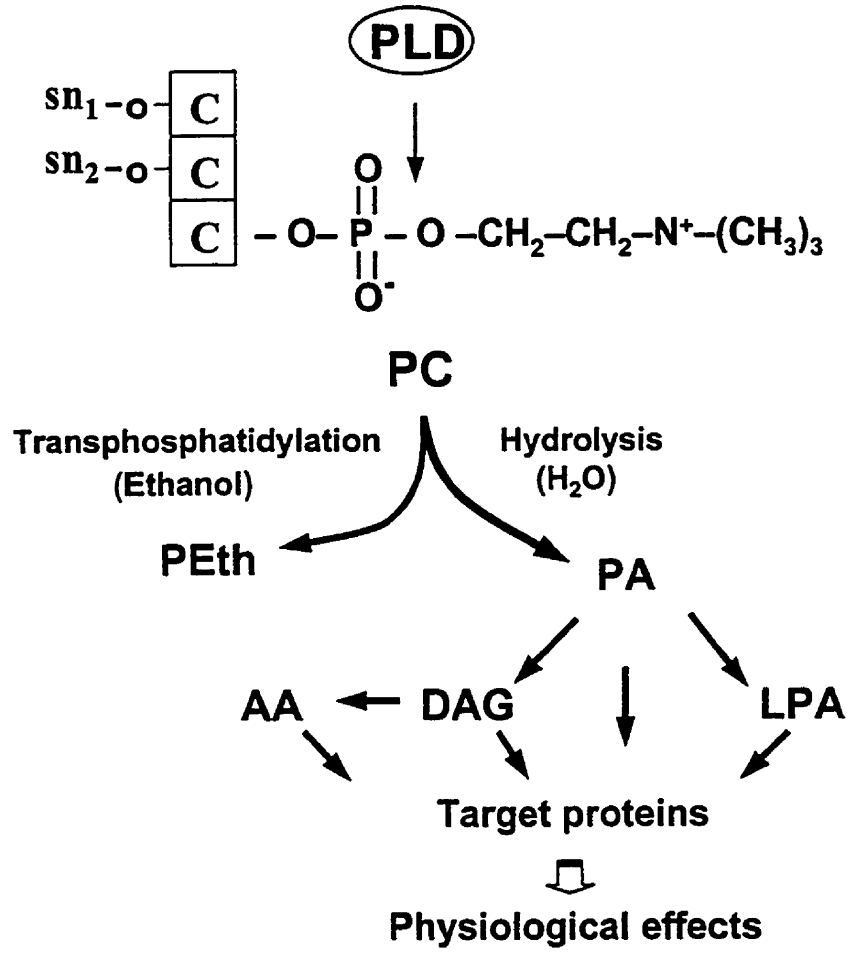


Diagram 2. PLD-catalyzed reaction and signaling pathway

Phospholipase D (PLD) hydrolyzes phosphatidylcholine (PC) to release phosphatidic acid (PA) and free choline. PA may be metabolized to diacylglycerol (DAG), then arachidonic acid (AA) or to lysophosphatidic acid (LPA). These products and PA itself may target several proteins to regulate physiological responses (see text). In the presence of a primary alcohol (e.g. ethanol), PLD catalyzes the transphosphatidylation reaction to produce phosphatidyl-alcohol, at the expense of PA.

For the past 10 years research has identified PLD as a pivotally important signalling enzyme (for reviews see: Cockcroft, 1992; Exton, 1994; 1997a,b,c; 1998; Singer et al, 1997; Frohman and Morris, 1999; Liscovitch et al, 2000) . Receptor-mediated PLD activation has been reported in a wide variety of cell types. The best-studied cell models included HL60 cells and neutrophils (Olson et al, 1991; Siddiqi et al, 1995). Effective extracellular stimuli consist of hormones, neurotransmitters, growth factors, and cytokines. These agonists, such as fMLP, carbochol, and insulin, stimulate PLD through both G-protein coupled receptors and receptor tyrosine kinases (Billah et al, 1989; Fensome et al, 1998; Rumenapp et al, 1995; Shome et al, 1997).

The immediate signalling molecule produced by PLD is generally considered to be PA, rather than the released headgroup choline. The reason is that, in most cells, the resting levels of choline are high (0.1-0.3 mM). Therefore, the generation of choline molecules after PLD activation cannot normally cause a significant rise in intracellular choline levels to fullfill a signalling purpose (Klein et al, 1995). However, the resting level of choline in neurons is very low (30 nmol/g). Thus, PLD-generated choline is believed to be involved in the synthesis of the neurotransmitter–acetylcholine (Ach) (Klein et al, 1995). Under the action of a phosphatidate phosphohydrolase (PAP), PA is converted into 1,2-diacylglycerol (DAG) (Brindley and Waggoner, 1996). DAG is the lipid second messenger that stimulates protein kinase C (PKC), an enzyme that regulates many key cellular functions such as cell growth, cell differentiation and cell death (Nishizuka, 1988;

1992). Receptor-mediated activation of PKC is mediated by two phases of intracellular DAG elevation. The first one is the rapid phase of DAG generation which comes from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by receptor-coupled phospholipase C (PLC) (Berridge, 1984; Nishizuka, 1992). The second persistent phase of DAG generation is now believed to come from the hydrolysis of PC by PLD and the subsequent conversion of PA by PAP (Billah et al, 1989; Hodgkin et al, 1998; Pettitt et al, 1997). However, DAG derived from PC may be less effective in activating PKC than the DAG that results from the PLC hydrolysis of phosphoinositides, possibly because of the relative fatty acid profiles and/or location on the membrane.

PA itself, without subsequent metabolism, may also have signalling functions. *In vitro,* PA has been found to modulate the activities of several important regulatory proteins, including a GTPase-activating protein, Ras-GAP, phosphatidylinositol 4-kinase, and certain PKC isoforms (Singer et al, 1997). *In vivo*, PA plays a role in the activation of NADPH oxidase and the resulting respiratory burst in neutrophils (Olson and Lambeth, 1996). PA also directly mediates the stimulation of actin stress-fiber assembly by PLD (Exton, 1997a). Therefore, PA itself may function as an independent lipid messenger released after PLD activation.

PA can be further converted into lyso-PA by the action of a PA-specific phospholipase A_2 (PLA_2). Lyso-PA is widely recognized as an important intercellular messenger, which acts through specific cell-surface receptors coupled

to G-proteins of the Gq and Gi family (Moolenaar, 1995). The best-known effect of LPA is its mitogenic action, which confers much of the growth-stimulating effect of serum. This effect of LPA is mediated through a pertusis toxin-sensitive G protein, and involves the Ras/MAP kinase signalling pathway (Moolenaar, 1995). In addition to LPA, the hydrolysis of arachidonyl-PA by PLA_2 also releases AA. AA can either participate in the synthesis of prostaglandins and leukotrienes, or function as an independent intracellular messenger (Axelrod et al, 1998; Ishimoto, et al, 1994).

To summarize, phosphatidylcholine-sepecific phospholipase D is an important signalling enzyme. Its cleavage of PC generates crucial lipid messengers, such as phosphatidic acid, diacylglycerol, lyso-phosphatidic acid, and arachidonic acid.

1.2.2 Enzymology of PLD

1.2.2.1 History

PLD activity was first described in plants (cabbage leaves) by Hanahan and Chaikoff (1947). It was considered to be a typical plant enzyme until in 1975, Saito and Kanfer demonstrated PLD activity in rat brain tissues (Saito and Kanfer, 1975). PLD has been found in bacteria, fungi, plants and animals and it is widely distributed in mammalian cells. Intense research on this enzyme did not start until about a decade ago, when studies on neutrophils and hepatocytes found that PLD may link extracellular agonist stimulation to intracellular signal generation (Cockroft, 1992; Exton, 1994). So, compared with other phospholipases (e.g. PLC

and PLA2), PLD may be regarded as a fairly "new" enzyme in the cell signalling field.

1.2.2.2 Distribution of different types of PLD activities

There are multiple PLD activities in mammalian tissues. PLD activity in animal tissues was first identified in a detergent extract of a rat brain particulate fraction. Sodium oleate was found to stimulate this activity, which has been predominantly detected in neuronal tissues (Saito and Kanfer, 1975). In HL60 cell membranes and porcine brain, a PLD activity was found to require PIP_2 as a cofactor and could be stimulated by the small GTP-binding protein Arf (Brown et al, 1993; Brown and Sternweis, 1995). This is the predominant guanine-nucleotide and phorbol ester–sensitive PLD activity which has been widely detected in the animal cells and tissues. Arf-sensitive PLD activity and oleate-sensitive PLD have been separated by HPLC of a Triton X-100 extract of rat brain membranes (Massenburg et al, 1994). Both these forms of PLDs use PC exclusively as their substrate. There are also reports of PLD activities toward phosphatidylinositol (PI) and phosphatidylethanolamine (PE). PI-PLD seems to be located in the cytosol (Ching et al, 1999) and PE-PLD seems to be located predominantly in mitochondria (Madesh and Balasubramanian, 1997). The biological significance of these activities is not clear at the present time.

PLD is considered to be predominantly membrane-bound. However, with the development of a cell-free PLD assay, cytosolic PLD activity has also been

detected in several cell models (Weng et al, 1991). Siddiqi et al, (1995), reported a cytosolic PLD activity in HL60 cells, which had a very low basal activity and was stimulated by Arf and mM levels of Ca^{2+}. Overall, cytosolic PLD is much less well characterized than the membrane-associated forms. It is still not known whether these two activities represent different isoforms of PLDs or different physical states of the same enzyme.

1.2.2.3 Purification of PLD

Plant PLDs were purified more than two decades ago (Heller, 1978; Yang et al, 1967). Mammalian PLDs have proven difficult to purify, because of their interaction with lipid components and relatively low level of expression (Brown et al, 1995). The earliest limited success was from the laboratory of Kanfer, who purified an oleate-sensitive form of the enzyme 240-fold from solubilized rat brain membranes (Kobayashi and Kanfer, 1991). A molecular weight of 200 Kda for this species was suggested by size exclusion chromatography. Brown et al, (1995) extracted an Arf-stimulated PLD activity from porcine brain membranes, and partially purified the enzyme by chromatography on DEAE-sepharose, sephadex G-100 and heparin-sepharose. The resulting enriched enzyme was found to have a molecular weight of about 95 Kda (Brown et al, 1995). To date, the PLD activity which has been reported to be purified to the highest degree is that purified 2,200 fold from pig lung microsomes (Okamura and Yamashita, 1994). This enzyme utilizes exclusively PC as the substrate. It had an optimum pH of 6.6 and a

molecular weight of 190 Kda. Unsaturated fatty acids, such as arachidonic acid and oleate acid, are markedly stimulatory, which indicates it is likely a fatty acid-sensitive form of PLD (Okamura and Yamashita, 1994). The numerous reported PLD activities differ in their phospholipid dependence, detergent requirement, pH optimum, metal ion dependence and regulatory mechanisms. These indicate the existence of multiple PLD isozymes. Difficulties in producing purified forms of PLD by traditional means has hampered the progress of research in this area. This problem has been partially overcome by the recent advances in cloning mammalian PLDs.

1.2.2.4 Molecular structure of PLD

Due to difficulties in PLD purification, the use of conventional cloning methods (e.g. enzyme purification followed by the generation of antibodies and amino acid sequences for library screening) has been hindered, and progress has been hampered by lack of appropriate molecular probes. The first PLD gene was cloned from castor bean (Wang et al, 1994). This gene encodes a 92 Kda protein, and its activity requires millimolar Ca^{2+}. This isoform was later named PLDα. Subsequently two other plant PLDs - PLDβ and PLDγ were cloned (Pappan et al, 1997; Qin et al, 1997), both of which require micromolar Ca^{2+} and PIP_2 for their activation. Meanwhile, a yeast SPO14 gene was cloned, and was found to encode a PLD similar to mammalian PIP_2-dependent PLD (Rose et al, 1995). The SPO14 protein contained several short regions of discrete homology to the 92 Kda plant

PLD mentioned above. The identification of sequence homologies between the plant and yeast PLDs led to the cloning of a cDNA encoding a human PLD.

By using the yeast PLD gene (SPO14) to identify a human expressed sequence tag for screening a HeLa cDNA library, Morris and Frohman's lab cloned the first mammalian PLD, designated as human PLD1 (hPLD1) (Hammond et al, 1995). This enzyme had 1072 amino acids, and a theoretical molecular mass of 124 Kda. hPLD1 was expressed in Sf9 insect cells and COS-7 cells. A recombinant protein of approximately 120 Kda was generated. The recombinant enzyme used only PC as the substrate. It catalyzed both the hydrolysis and transphosphatidylation reactions. In a later report, a short splice variant of hPLD1 (now designated as hPLD1a) which has 1034 amino acids was identified and named as hPLD1b (Hammond et al, 1997). Both splice variants had similar regulatory properties and were catalytically indistinguishable. hPLD1 was dependent on PIP_2 and inhibited by oleate. It had a low basal rate of catalysis and in the *in vitro* reconstituted conditions, was activated by recombinant Arf , RhoA and PKCα (Hammond et al, 1997). When these activators were added together, a synergistic effect was observed, suggesting that these factors bind to different regulatory sites on the enzyme. Studies on the regulation of human recombinant PLD1 therefore match well with those of biochemically purified PLD.

A second PLD family member was cloned from mouse embryo by the same group of researchers (Colley et al, 1997a). This new PLD gene was designated mammalian PLD2 (mPLD2). Overall, human PLD1 and mouse PLD2 proteins are

51% identical. Recombinant PLD2 was also expressed and purified using the same approach as with PLD1. Unexpectedly, PLD2 exhibited a high basal activity, about 1,500 fold greater than that of PLD1, in an *in vitro* assay. Both forms of PLD needed PIP_2 for their activities, but only PLD1 responds to Arf, RhoA and PKCα. High basal activity was also identified in intact COS-7 cells transfected with PLD2 expression vectors but not with PLD1 vectors (Colley et al, 1997a). This identification of a constitutively active PLD was unexpected. PLD activity has been extensively studied, but no such activity has previously been identified and purified. Instead, only PLD1-like activities (i.e. low basal activity) have been reported. This raises the possibility that endogenous PLD2 might be regulated and masked by inhibitory factors. If so, PLD2 activity could be transiently suppressed upon agonist stimulation. PLD2 is expressed at relatively high levels in the brain. A heat-stable factor from bovine brain cytosol, which preferentially inhibited PLD2 activity has been identified and enriched by chromatography. The fraction contained a major detectable protein of about 18 Kda (Colley et al, 1997a). The major factor responsible for this inhibitory activity was purified and identified as a mixture of α- and β- synnceins (Jenco et al, 1998). PLD2 is 5-100 fold more sensitive to this inhibition than PLD1.

Studies from this group also found that PLD1 and PLD2 are differently located within the cell. PLD1 was found exclusively in the peri-nuclear region of the cell (Golgi, ER, and late endosomes). In contrast, PLD2 was predominantly associated with the plasma membrane. The apparent different location of these two PLDs

indicates different cellular functions and possibly different regulatory mechanisms. PLD1 has been suggested to play a role in intracellular vesicle transport. PLD2 is believed to have a function in signal-induced cytoskeleton rearrangement and endocytosis (Colley et al, 1997a).

Following the initial reports of PLD cloning, PLD1 and PLD2 from different species were also cloned and characterized from other labs (Colley et al, 1997b, Katayama et al, 1998; Kodaki and Yamashita, 1997; Lopez et al, 1998; Min et al, 1998; Park et al, 1997; Steed et al, 1998; Sung et al, 1999). They corresponded to the human PLD1 and mouse PLD2 enzymes discussed above. Following the cloning of PLD genes, the availability of structural information and molecular tools have allowed rapid progress in studying the molecular mechanisms of PLD action and regulation.

1.2.3 Regulation of PLD

1.2.3.1 Regulation of PLD by PIP_2

The complex regulatory mechanisms of PLD began to be elucidated in the 1990s. During the development of an exogenous substrate assay for PLD, Brown and colleagues first used lipid vesicles of different lipid compositions to test their effects on PLD activity (Brown et al, 1993). They found that phosphatidylinositol 4,5-bisphosphate (PIP_2) was required for the optimal stimulation of PLD by guanine nucleotide in the HL60 cell preparations. It was further found that the requirement for this lipid was observed in the absence of detectable accumulation

of inositol phosphates (IPs). Therefore, it was the PIP_2 itself, but not its hydrolysis product, IP_3, that was involved in PLD activation. If phosphatidylinositol 4-bisphosphate (PIP) or phosphatidylserine (PS) were substituted for PIP_2 (they are all negatively charged phospholipids), the assay failed to work for HL60-PLD, but still worked for plant PLD (purified from peanut), although at a lower activity. PIP_2 was thus identified as a specific cofactor for PLD activation. The cell-free, exogenous substrate PLD assay containing PE/PIP_2/PC was established in this work by Brown and has since been used widely.

The absolute requirement for PIP_2 in PLD activation was also shown by Liscovitch et al, (1994) in rat brain membranes. They originally found that neomycin inhibited brain membrane PLD activity, as well as GTPγS-induced activation of PLD in permeabilized NG108-15 cells, derived from neural tissue. They further tested the hypothesis that PIP_2 may act as an essential cofactor for PLD. They assayed a PLD activity partially purified from rat brain membrane by using C6-NBD-PC as a fluorescent substrate. The incoporation of PIP_2 into the micellar system was found to dose-dependently stimulate PLD activity *in vitro*. The maximal effect was about 10-fold stimulation at a PIP_2 concentration of 6 mol % compared with PIP_2-absent conditions. The stimulation was specific, since other acidic phospholipids, including PIP, PI, PS and PA were ineffective. Neomycin, a high affinity ligand of PIP_2, inhibited membrane-bound PLD but had no effect on the activity of a detergent-solubilized or a partially purified enzyme. The addition

of PIP_2 restored the sensitivity of partially purified PLD to neomycin, confirming that neomycin blocks membrane PLD activity by binding to endogenous PIP_2.

In addition to the above *in vitro* (cell-free) studies, the requirement of PIP_2 for PLD activity *in vivo* was also suggested in experiments with permeabilized U937 cells (Pertile et al, 1995). Treatment of permeabilized U937 cells with an antibody against PI 4-kinase, the enzyme responsible for the first step in PIP_2 synthesis, resulted in a marked reduction of PIP_2 levels, and that correlated with the inhibition of PLD activity stimulated by GTPγS and PMA.

Several groups have reported the inhibition of crude PLD activity (from membranes or permeabilized cells) by protein factors that include fodrin and synaptojanin, both of which have been found to exert their inhibitory action on PLD through decreasing PIP_2 levels (Lukowski et al, 1998, Chung et al, 1997).

The novel role of PIP_2 as a cofactor for PLD was further confirmed recently, in *in vitro* assays on purified recombinant hPLD1 and mPLD2. Both of these two known isoforms of mammalian PLD absolutely require PIP_2 for their activities (Hammond et al, 1997; Colley et al, 1997a).

Despite the sound evidence cited above, the mechanism of how PIP_2 actually functions as a cofactor for PLD activation is largely unknown. Since a number of proteins have been shown to bind to PIP_2, it was originally postulated that PIP_2 may serve as a binding site for Arf (Randazzo, 1997), PLD or other potential PLD cofactors (Brown et al, 1993). Some published data suggest that the PLD enzyme itself may bind to PIP_2. A partially purified RhoA-dependent PLD activity bound

to PE/PIP_2/PC vesicles in a PIP_2-dependent manner (Yokozeki et al, 1996). Binding studies with sucrose-loaded phospholipid vesicles and photoaffinity labeling experiments suggested that PLD enzymes interact directly with PIP_2 (Morris, personal communication; see also Morris 1997a). The elucidation of this mechanism should prove of central importance in understanding PLD mechanisms.

1.2.3.2 Regulation of PLD by Arf

1.2.3.2.1 Arf – ADP ribosylation factor

Arf (ADP-ribosylation factor) was originally described as a cofactor of cholera toxin-catalyzed ADP-ribosylation of Gsα of the cAMP signal transduction pathway (Kahn and Gilman, 1984). Cholera toxin-catalyses ADP-ribosylation of Gsα and irreversibly activates adenylate cyclase. Arf was first purified as a 21 Kda protein from rabbit liver (Kahn and Gilman, 1986). Subsequently, it was found that Arf has a high-affinity binding site for guanine nucleotides. Binding of GTP or GTPγS to Arf is necessary for the activity of the cofactor. Thus, Arf, is itself a GTP-binding protein (Kahn and Gilman, 1986). It was later found that Arf is predominantly located in the cytosol. Its activity and/or immunoreactivity have been detected in every mammalian cell tested, but have not been dectected in bacteria (Kahn et al, 1988). The expression level is very high in many tissues, e.g. in neural tissues, it may comprise as much as 1% of the total cellular protein.

Shortly after its purification, Arf was cloned from cow, human, and yeast (Kahn et al, 1991a). The mammalian Arf family has six members, from Arf1 to Arf6. Consistent with their designation as "Arfs", all six mammalian Arf proteins stimulate cholera toxin-catalysed ADP-ribosylation (Massenburg et al, 1994).

1.2.3.2.2 The Arf family of small molecular weight GTP-binding proteins

At the turn of the 80s and 90s, following the cloning and sequencing of the α subunits of the trimeric G-proteins, a large number of structurally related monomeric GTP-binding proteins with molecular masses between 20 and 30 Kda were identified (Takai, 1994; Bhullar, 1997). The identification of many of these proteins came in large part from low stringency hybridization of cDNA libararies with probes derived from previously cloned proteins, primarily ras p21 (Olson and Marais, 2000). Most of these proteins, do not have clearly identified activities and functions in the cell, except that of guanine nucleotide binding and hydrolysis (Bhullar and Haslam, 1987). Arf was identified as one of the 5 subfamilies in this superfamily (the others are Ras, Rho, Rab, and Ran). Arf was the first of the superfamily of low molecular weight monomeric GTP-binding proteins purified from tissues, and one of a few such proteins with a well-defined *in vivo* activity (ADP-ribosylation cofactor) (Weiss et al, 1989; Kahn et al, 1992). The small GTP-binding proteins differ from heterotrimeric GTP-binding proteins in many aspects. For example, Kahn (1991b), demonstrated that AlF_4^-, the well-known activator of

heterotrimeric GTP-binding proteins did not stimulate Arf, and they are not associated with βγ subunits.

Like other GTP-binding proteins, Arf becomes activated upon the binding of GTP. Subsequently, GTP hydrolysis acts as a turn-off signal. Arf proteins alone have negligible GTPase activity. GTP hydrolysis by Arf is dependent on GTPase-activating proteins (GAP) (Cukierman et al, 1995; Makler et al, 1995). The GAP facilitates GTP hydrolysis and results in protein inactivation. The activation step, the exchange of GDP for GTP involves a guanine nucleotide exchange protein (GEP), which can be inhibited by brefeldin A (BFA) (Donaldson et al, 1992b; Helms and Rothman, 1992; Tsai et al, 1996).

1.2.3.2.3 Identification of a cytosolic factor in PLD activation

Early studies with granulocytes (neutrophils and HL60 cells) provided some intriguing findings on PLD (Cockcroft, 1992). One striking observation was that cytosol was required for GTPγS-stimulated PLD activation in membranes. Lambeth's group examined PLD activity in the plasma membrane and cytosol fractions of human neurophils prelabelled with [^{3}H] alkyllysophosphatidylcholine (Olson et al, 1991). A cell-free PLD assay was established using these radiolabelled membrane fractions and activity was measured by the transphosphatidylation reaction. They found that GTPγS failed to stimulat PLD in isolated plasma membranes, although it stimulated PLD in whole cell

homogenates. When the cytosol fraction was added to the plasma membrane, a potent activation was observed with GTPγS. Cytosol itself had little PLD activity. This experiment indicated that a cytosolic factor was required for the activation of a membrane PLD by GTPγS. It was further shown that the cytosolic fraction dose-dependently facilitated this activity and that cytosol from a variety of tissues could substitute for the neutrophil cytosol (Olson et al, 1991). Thus, the requirement for a cytosolic factor in GTPγS-stimulated PLD activation appears to be a widely distributed phenomenon.

This observation was later confirmed by studies in permeabilized cells (Geny et al, 1993). Addition of GTPγS to HL60 cells, together with the permeabilization agent streptolysin O, led to a rapid and prolonged activation of PLD. The authors took advantage of the fact that there is a time-dependent leakage of cytosolic proteins from cells after permeabilization, and showed that permeabilization led to a time-dependent decrease in the ability of GTPγS to stimulate PLD activity. Furthermore, they found that addition of GTPγS to the system before permeabilization prevented the time-dependent decline of PLD activity. This indicated that GTPγS is capable of protecting the loss of a cytosolic component by recruiting it to the membrane. The role of this unknown cytosolic factor was suspected to be essential in PLD regulation. This factor was initially suspected to be a heterotrimeric GTP-binding protein (Brown et al, 1993), but was subsequently identified as the small GTP-binding protein, Arf.

1.2.3.2.4 Identificaion of Arf as a PLD activator

After the development of a cell-free assay which used exogenous lipid vesicles as the PLD substrate (Brown et al, 1993). Brown and colleagues found that PLD activity in HL60 cell membranes stimulated with GTPγS could be enhanced by the addition of cytosol. In order to purify the active cytosolic factor, they first extracted the PLD activity from the crude HL60 cell membranes by salt extraction or with detergent. The extracted activity was enriched by stepwise chromatography. As the PLD activity was enriched, the sensitivity of GTPγS became more dependent on the addition of cytosol. This partially purified PLD from the HL60 cell membranes was used to detect the activating effects of the cytosolic factor. A factor that stimulated PLD activity could be detected in the HL60 cytosol and bovine brain cytosol. They used bovine brain cytosol as an abundant supply to proceed with isolation of the stimulatory cytosolic factor. This factor was purified by standard chromatography techniques (including DEAE-Sepharose, AcA44, hydroxylapatite, Sephadex G-75, and Mono Q columns). Elutions from each step were tested in the cell-free assay system, the fractions which enhanced the activity of partially purified PLD from the HL60 membranes were further enriched until the activating component was purified. In the final steps of purification, this cytosolic, PLD-stimulating activity corresponded to a polypeptide that migrated through SDS polyacrylamide gel with a molecular weight of 21 Kda. The yield of this factor was 100-300 μg from 7 g of cytosolic

protein. Fractions that contained the purified 21 Kda polypeptide were subjected to partial amino acid sequence analysis. The derived sequence matched exactly the amino acid sequence predicted by the cDNAs from bovine Arf1, and human Arf3 (the Arf1 and Arf3 proteins are 96% identical). The activation of resolved PLD activity from HL60 membranes by Arf proteins was further characterized by using purified Arfs. Purified bovine brain Arf, non-myristoylated recombinant Arf1, and myristoylated Arf1 were all found able to stimulate PLD. The most potent one was the native Arf, then the myristoylated rArf. Non-myristoylated Arf had to be added at a higher dose to show the stimulatory effect (Brown et al, 1993). A simultaneous study by Cockcroft's group found the same result (Cockcroft et al, 1994). This group used cytosol-depleted HL60 cells (through streptolysin-O permeabilization) to set up a reconstitution assay. The stimulatory factor in the cytosol was purified and identified as a mixture of Arf1 and Arf3.

Recent studies with purified recombinant PLD1 confirmed the stimulatory effect of Arf on PLD, and indicated a direct interaction between these two proteins (Hammond et al, 1997).

Although reconstitution studies using purified recombinant enzymes indicated that Arf may interact directly with PLD, the mechanism of this interaction remains largely unknown. Initial information came from a couple of studies based on chimeric Arf proteins. Zhang et al (1995), found that different Arf domains were required for the activation of both cholera toxin and PLD. The amino-terminal region (the first 73 amino acids) of Arf 1 appeared to be critical for PLD

activation. More recently, a study based on chimeras between mammalian ARF1 and *S. Cerevisiae* Arf2 found that region II (residues 35-94) of mammalian Arf is necessary for PLD1 activation (Liang et al, 1997). Further elucidation of this interaction at the molecular level will enhance understanding of the regulation of PLD.

1.2.3.3 Regulation of PLD by Rho

The Rho family of small GTP-binding proteins comprises another class of cytosolic activators of PLD. The mammalian Rho family of small GTPases contains the members of Rho A, Rho B, Rho C , Rho G, Rac 1, Rac 2, CDC 42 and TC10 (Frohman and Morris,1996). Many different cellular functions have been associated with this family of proteins (Takai et al, 1994), including cytoskeletal rearrangements (e.g. assembly of actin stress fibres and focal adhesions), membrane movement (e.g. actin polymerization and protrusion of the cell surface to produce membrane ruffles) and cell growth (e.g. mediation of some growth factor pathways). Like other GTP-binding proteins, the exchange of GDP and GTP on Rho controls its activity. The resting Rho is bound to GDP. The exchange of GDP for GTP can be stimulated by smg GDS (small molecular weight GTPase GDP dissociation stimulator). The inhibition of this exchange is controlled by Rho GDI (GDP dissociation inhibitor) (Takai et al, 1994). In fact, Rho GDI binds to the Rho family of proteins, inhibiting the release of GDP and the subsequent exchange with GTP or GTPγS, thus maintaining the inactive GDP

bound form of Rho. Post-translational modification of these proteins by isoprenylation at their C-terminal is important for their biological function (Malcolm et al, 1994). Rho action can also be blocked through ADP-ribosylation by *Clostridium botulinum* C3 exoenzyme (Rho A, B, C) and through glucosylation by *Clostridium botulinum* toxin B (Rho A, Rac 1, and CDC42) (Kuribara et al, 1995; Schmidt et al, 1996). These probes have proven useful for the investigation of Rho family proteins in cell physiology.

In an original study from Lambeth's lab, Olson et al (1991) found in neutrophils that GTPγS-stimulated PLD activity required both membrane and cytosol fractions. At the same time that Arf was identified as the cytosolic activator for PLD in HL60 cells, further studies were undertaken to localize and identify this GTPγS-sensitive component in neutrophils (Bowman et al, 1993). Bowman and colleagues used plasma membrane and cytosol fractions isolated from [^{3}H] alkyl-lyso-PC labelled neutrophils to set up a reconstituted PLD assay. Although GTPγS stimulation of PLD required the presence of cytosol, the GTPγS-binding protein was found to be associated with the membrane. The requirement of low magnesium concentration indicated that it was likely to be a small GTP-binding protein. This was further supported by the finding that a GDP dissociation stimulatory protein (GDS) for small G proteins increased the ability of GTP to stimulate PLD under normal conditions (GTP is a weak activator of PLD due to the GTPase activity of the activating GTP-binding protein). Rho GDI specifically

inhibits the Rho family of GTP-binding proteins. When Rho GDI was incubated with membrane and cytosol, it dose-dependently and fully inhibited GTPγS-stimulated PLD activity, suggesting the involvement of Rho in PLD activation (Bowman et al, 1993). This finding was quickly confirmed by a study on PLD in rat liver (Malcolm et al, 1994). Rho GDI dose-dependently lowered GTPγS-stimulated PLD activity in rat liver plasma membrane. Further experimentation was carried out to determine which member of the Rho family was involved. RhoA, Rac1, and CDC42 were identified in rat liver plasma membrane. Rho GDI preincubation removed RhoA and CDC42 from the membrane. When added to GDI-treated membranes, recombinant RhoA restored PEth formation in the presence of GTPγS, confirming the role of RhoA in PLD activation in this system. In contrast, the addition of recombinant Arf had no effect (Malcolm et al, 1994).

Further work by Lambeth's group found that RhoA, and a 50 Kda cytosolic factor were both required to reconstitute GTPγS-stimulated PLD activity in neutrophil plasma membranes (Kwak et al, 1995). However, this 50 Kda cytosolic protein has not been characterized so far. In HL60 cells, Rho GDI inhibited GTPγS stimulation of membrane PLD activity in the presence and absence of cytosol. The addition of RhoA, Rac1, and CDC42 could restore PLD activity in both GDI pretreated or untreated membrane. However, only RhoA but not the other two were shown to be present on the HL60 membranes, indicating that RhoA was the endogenous small G protein involved (Siddiqi et al, 1995). Arf

(located in the cytosol) also stimulated the HL60 cell membrane PLD, and the combination of RhoA and Arf showed a synergistic effect.

In an independent study on rat brain PLD, Kuribara et al (1995), found that bovine brain cytosol contained a PLD-activating factor that was distinct from Arf. This factor was partially purified and found to react strongly with anti-RhoA antibody, but not other antibodies. In addition, this factor could be co-purified by the C3-exoenzyme catalyzed ADP-ribosylation assay, an acitivity specific for the Rho family proteins. It was further shown that recombinant RhoA activated rat brain PLD in a concentration-and GTPγS-dependent manner, and RhoA plus Arf had a synergistic effect.

The activation of PLD by the Rho family of small GTP-binding proteins was further confirmed after the recent cloning of PLD. Recombinant PLD1 (both PLD1a and PLD1b) was shown to be stimulated by RhoA, Rac1 and CDC42, and activation was synergistic with Arf (Hammond et al, 1997).

Recent studies also indicate the role of RhoA in agonist-stimulated PLD activation (such as carbachol and fMLP) in HL 60 cells, human neutrophils and rat fibroblasts (Fensome et al, 1998; Guillemain and Exton, 1998; Keller et al, 1997; Malcolm et al, 1996). There is some recent evidence that at least one heterotrimeric G protein, G_{13}, may be located upstream in a receptor-coupled RhoA-PLD signalling pathway (Plonk et al, 1998).

1.2.3.4 Regulation of PLD by Protein Kinase C

The PKC family has numerous members that have been studied extensively (for reviews see Nishizuka, 1988; 1992). This family of enzymes is currently divided into three groups. The classical PKCs (cPKC) consist of α, β and γ isoforms, and are stimulated by Ca^{2+}, diacylglycerol and phosphatidylserine. The new isoforms (nPKC) consist of σ, ε, η, θ and υ isoforms, and are not regulated by Ca^{2+}. The atypical members (aPKC) consist of ζ and λ, and appear to be regulated by second messengers other than diacylglycerol and Ca^{2+}. It is known that activators of PKC bind to the enzyme and cause conformational changes that expose membrane attachment sites in the amino terminal regulatory domain and remove inhibition of the kinase active site in the carboxy terminal.

Regulation of PLD by protein kinase C (PKC) has been known for some time. Phorbol esters, such as PMA, mimic DAG (derived from PLC cleavage of phosphoinositides – see 1.1.5. above), and activate PKC. PMA has been shown to activate PLD in intact cells from almost every cell type tested, although in some situations PMA failed to work in cell-free conditions or permeabilized cells due to unknown reasons (Cockcroft, 1992; Geny and Cockcroft, 1992; Exton, 1994;). Various PKC inhibitors have been extensively used in many systems to assess the role of the kinase in signalling pathways. In most studies, these inhibitors blocked or attenuated agonist-stimulated PLD activities (Exton, 1997a). Extended incubation of cells with PMA down regulates PKC activity, and this is generally

accompanied by a decreased PLD activity (Exton, 1997a). Although the use of phorbol esters, PKC inhibitors, and down regulation approaches have sometimes given inconsistent and even conflicting results, by far the majority of investigations have suggested PKC as a universial regulator for PLD.

More direct evidence for the role of PKC in PLD activation comes from cell-free studies. The first study of this kind showed that in membrane from Chinese hamster lung fibroblasts (CCL39), the addition of PMA stimulated PLD activity when cytosol was also present (Conricode et al, 1994). Pure rat brain PKC dose- and time-dependently stimulated PLD in the presence of PMA in CCL39 membranes. Surprisingly, PKC stimulation of PLD occurred in the absence of added ATP. Moreover, similar stimulation of PLD was observed in the presence of a high concentration of apyrase, a ATPase which completely abolished autophosphorylation of PKC and phosphorylation of other proteins. The stimulation was also shown to be insensitive to treatment with H-7, a catalytic site inhibitor of PKC. These data confirmed the involvement of PKC in the activation of PLD, but suggested that ATP-dependent phosphorylation was not required for this activation (Conricode et al, 1994). Further studies in this system found that conventional PKCs including α and β were involved. The third member of the cPKC group, PKCγ, was ineffective.

The phosphorylation-independent mechanism found in the above study was totally unexpected, but was confirmed 2 years later in additional studies by the Sternweis group (Singer et al, 1996). A previous study from this lab found a

cytosolic factor that could stimulate the activity of a partially purified PLD from porcine brain in the absence of any nucleotide. This cytosolic factor was further purified to an 80 Kda band by stepwise chromatography. It was identified as PKCα according to the exact match with sequences from rat brain PKCα, and by specific immunoblot analysis (Singer et al, 1996). Purified or recombinant PKCα dose-dependently stimulated PLD in the absence of ATP, Ca^{2+} and PMA. This action was synergistic with either Arf or RhoA. It is known that treatment of PKC by phosphatase removes a permissive phosphorylation site of PKC and therefore eliminates its kinase activity. Dephosphorylation of rPKCα with protein phosphatase 1 or 2A resulted in a loss of its kinase activity, but had little effect on its ability to stimulate PLD. Staurosporine also inhibited the kinase activity of PKCα, but did not affect the activation of PLD. Under controlled conditions, trypsin cleaves PKC to produce a free lipid-binding regulatory domain and a permanently activated kinase domain. Gel filtration was used to separate the 50-52 Kda kinase domain and the 34 Kda regulatory domain from trypsinized enzyme. It was found that the stimulatory activity for PLD coeluted with the regulatory domain of the enzyme (Singer et al, 1996). This work suggested PKC could activate PLD through direct molecular interaction that is independent of its kinase activity.

However, in a study on neutrophils, the activation of PLD by PKC was shown to be achieved through a classical kinase-dependent mechanism (Lopez et al,

1995). Work from Lambeth's lab found that PMA (as well as GTPγS) stimulated plasma membrane PLD in permeabilized neutrophils, when cytosol was present (Olson et al, 1991). Further work by Lopez and colleagues showed that this activation was dependent upon the presence of ATP. A significantly greater response (2-3 fold more) was seen when ATPγS was used in place of ATP, presumably because of increased stability of the thio-phosphoryl group after phosphorylation. In contrast, AppNHp, the adenosine triphosphate analogue which can not be utilized by protein kinase, was ineffective. Supporting the role for phosphorylation, staurosporine, a protein kinase inhibitor, almost completely inhibited PMA-stimulated PLD activity in neutrophils. When cytosol was depleted of PKC by chromatography, PMA-dependent activation was lost, but could be restored by the addition of purified rat brain PKC. The target protein for PKC phosphorylation was found to be in the plasma membrane. Finally, recombinant isoforms of conventional PKC family members α, β and γ but not other subfamily isoforms were found to reconstitute PLD activation in a kinase activity-dependent manner (Lopez et al, 1995).

In HL60 cells, activation of PLD by PKC α, β and γ also required ATP, but was not affected by the catalytic site inhibitor of PKC activity (Ohguchi et al, 1995). A subsequent study suggested that the actual role of ATP in this system is to maintain the PIP_2 level in the membranes, presumably to support PIP_2 as a PLD-activating cofactor (Ohguchi et al, 1996).

A recent study on recombinant hPLD1 clearly showed that it can be stimulated by rPKCα in an ATP-independent manner (Hammond et al, 1997). In fact, the inclusion of ATP in the assay dose-dependently inhibited PLD1 activity. If ATP was included, PKCα and βII could phosphorylate PLD1, and the phosphorylation of rPLD1 by PKC was shown to inhibit its lipase activity (Min et al, 1998). The physiological significance of these findings is not clear, since at present, there are no reports of Ser/Thr phosphorylation of PLD *in vivo*. The mechanism of how PKC interacts with PLD is presently unknown. A recent study indicated that the site of interaction of PKC with rPLD1 is located at the N-terminal region of PLD1 (Park et al, 1998).

In *in vitro* conditions, conventional PKC family members α and β seem to stimulate PLD through kinase-independent interaction. However, whether this mechanism occurs also *in vivo* and especially in agonist-stimulated PLD pathways is unknown. This *in vitro* evidence of PLD regulation by PKC through direct protein-protein interaction does not preclude an additional phosphorylation-dependent mechanism *in vivo*. It should be kept in mind that, *in vivo*, many identified and unidentified factors can regulate PLD, either positively or negatively, and one or several of these proteins, including PLD itself could be the target of PKC phosphorylation. The phosphorylation of PLD itself, if it occurs *in vivo*, may further render the enzyme more susceptible to its activators or inhibitors. Thus, although the regulation of PLD by PKC *in vitro* seems fairly

clear and simple, the mechanism by which PLD is regulated by PKC *in vivo* may be far more complex and is far from clear.

1.2.3.5 Synergistic effects on PLD activition

Studies on crude preparations from HL60 cells and neutrophils found that GTPγS and PMA stimulated PLD activity in a synergistic manner (Geny and Cockcroft, 1992; Olson et al, 1991). Joint activation of PLD by a PKC and G-protein related pathway was suggested by these studies. The Sternweis group used a partially purified PLD from porcine brain membranes in a reconstituted assay system. When they added Arf, RhoA and PKCα to the assay, they found that a combination of any two of these activators gave synergistic stimulation and all three of them added together gave the highest effect (Singer et al, 1996). All three activators rely on the presence of PIP_2. More direct evidence followed with a study that demonstrated synergistic activation of purified recombinant hPLD1 by combination of these four activators (Hammond et al, 1997). This synergism phenomenon indicates that PLD1 contains different sites for interaction with PIP_2, Arf, Rho and PKCα. Moreover, the occupancy of these sites by their respective ligands causes a cooperative increase in the enzyme activity. However, how the cooperative mechanism increases the catalytic activity of the enzyme is presently unknown. The synergism observed *in vitro* may operate also *in vivo*. The substrate PC is located on cell membranes. For hydrolysis to occur, PLD has to bind with

the substrate. The essential cofactor of PLD1 and PLD2, PIP_2, is also located on the membranes. Therefore, PLD must be or at least must become membrane-bound for activation. In fact, most PLD activities identified so far are membrane-associated. It has been shown that upon agonist stimulation, Arf, RhoA and PKC are all translocated from cytosol to the membranes (Houle et al, 1995; Keller et al, 1997; Lopez et al, 1995). This enables all three regulators to interact with PLD via their respective interaction domains. Presumably, PIP_2 also binds to PLD and provides a permissive environment for the other three regulators. As the result of this cooperative interaction, PLD is fully activated and its catalytic site exposed to the substrate. This current model of PLD activation, although it has not been shown *in vivo*, is generally predicted and widely accepted according to the *in vitro* data.

1.2.3.6 Inhibitory factors of PLD

The activation of PLD has been extensively studied, as noted in the above five sections. In contrast, little is known about how the enzyme may be inhibited. Several phenomena indicate the importance of inhibitory regulation on PLD *in vivo*. First, cytosolic fractions from several tissues have been shown to inhibit membrane PLD activity (Han et al, 1996; Morris et al, 1997a). Arf was originally purified from bovine brain cytosol as an activator for the partially purified HL60 membrane PLD (Brown et al, 1993). However, it was also shown that, in fact, bovine brain cytosol itself as a whole, was inhibitory to this PLD activity,

although this inhibitory component could be separated from the activating factor (Arf). The identity of this cytosolic inhibitor is unknown. Second, PLD2 is expressed in a variety of tissues (Steed et al, 1998). Purified PLD2 has a very high intrinsic activity (1,500 fold more than that of PLD1) (Colley et al, 1997a). However, such a high level of activity has never been found in those tissues, where PLD2 has been shown to be present. This suggests that PLD2 activity may normally be masked by inhibitory factor(s) *in vivo*. Third, PLD1 is under the regulation of PIP_2, Arf, Rho and PKCα (Hammond et al, 1997). In the absence of these activators, this PLD showed almost no activity. It is well known that these factors can be deactivated or degraded *in vivo*. For example, GTPase activating proteins (GAPs) for Arf and Rho could deactivate them, and therefore could inhibit PLD activation *in vivo* (Takai et al, 1994). Inhibition of PLD is poorly understood at present and is an area that clearly needs more investigation.

1.2.4 Cellular function of mammalian PLD

1.2.4.1 Phosphatidic Acid – a potential second messenger

A variety of extracellular agonists are capable of causing rapid and transient increase in intracellular PA level (Exton, 1994). For example, in neutrophils, N-formylmethionyl-leucyl-phenylalanine (fMetLeuPhe) stimulates optimal PA formation within 20 seconds (Stutchfield and Cockcroft, 1993). These studies suggested that PA may serve as an intracellular second messenger. The studies on the cellular function of PLD have largely focused on the identification of cellular

targets affected by its product, PA. Under *in vitro* conditions, a variety of regulatory proteins can be activated directly by PA. These include protein kinases C η and ζ (Ballas et al, 1993; Limatola et al, 1994), PI-4-kinase (PI kinase) (Moritz et al, 1992), PI 4-phosphate 5-kinase (PIP kinase) (Jenkins et al, 1994), protein tyrosine phosphatase 1C (Zhao et al, 1993), PLCγ (Jones and Carpenter,1993) and Raf-1 kinase (Ghosh et al, 1996). In addition, PA was found to inhibit Ras-GAP (GTPase-activating protein) (Tsai et al, 1989). Whether any of the above proteins can be regulated by PA *in vivo* has not been determined, and the mechanism of PA action remain to be elucidated.

Recent studies in neutrophils indicated that PA may act through a PA specific protein kinase. In neutrophils, fMetLeuPhe receptor activation stimulates NADPH oxidase and leads to the respiratory burst (resulting in $O_2^{\cdot}$ formation) (Olson and Lambeth, 1996). Qualliothine-Mann et al (1993), established a cell-free system from neutrophils to measure NADPH oxidase and found that PA played a major role in chemotactic peptide stimulated NADPH oxidase activation. A subsequent study found that PA may act through a PA-regulated protein kinase, by phosphorylating p47-Phox, a component of NADPH oxidase (McPhail et al, 1995). This PA-dependent protein kinase was localized in the cytosol. The partially purified protein kinase had an apparent molecular size of 125 Kda. It was selectively activated by PA, and phosphorylated the p47-Phox component of NADPH oxidase on both serine and tyrosine residues (Waite et al, 1997). The kinase activity is present in a variety of cell lines and in rat brain, suggesting a

widespread distribution. Therefore, this protein kinase may be a novel target for the proposed second messenger function of PA (McPhail et al, 1999).

1.2.4.2 The role of PLD in intracellular vesicle transport

In the initial experiments using intact or permeabilized HL60 cells, a correlation was observed between the requirement for activation of PLD and that for secretion of β-glucuronidase (Stutchfield and Cockcroft, 1993). In the presence of 2% ethanol, the production of PA by fMetLeuPhe and other agonists is largely reduced through the transphosphatidylation reaction (with the concomitant phosphatidylethanol formation). The same concentration of ethanol has been found to block secretion (Cockcroft, 1992), suggesting that normal PLD reaction products (PA and its metabolites, generated in the absence of ethanol), may play a role in secretion.

A close relationship is generally found between mechanisms of secretion and those of intracellular membrane trafficking. The intense interest in PLD as a potential mediator of membrane trafficking was started by the identification of Arf as a potent activator of PLD (Brown et al, 1993; Kahn et al, 1993; Cockcroft et al, 1994;). It has been well established that Arf promotes the formation of coated vesicles in conjunction with a protein complex called coatomer (Rothman, 1994). This process is regulated by the activation and inactivation of Arf by GTP and GDP (Donaldson et al, 1992a; Palmer et al, 1993). Intracellular membrane transport within the Golgi complex as well as between the endoplasmic reticulum

and the Golgi is regulated by Arf-related mechanisms (Balch et al, 1984, 1992; Taylor et al, 1992b; Tsai et al, 1993; Chen and Shields, 1996). Ktistakis et al (1995), for the first time found abundant PLD activity in Golgi-enriched membranes from several cell lines. This Golgi PLD activity was greatly stimulated by exogenous Arf and GTPγS and the stimulation could be inhibited by brefeldin A (BFA), a drug that specifically blocks Arf binding to the Golgi membranes (see above) (Donaldson et al, 1992b; Helms and Rothman, 1992). In Golgi membranes from BFA-resistant Ptk1 cells, basal PLD activity was high and not stimulated by Arf and GTPγS (Ktistakis et al, 1995). Thus, the authors suggested that Arf activates PLD on the Golgi membranes and this may relate to the underlying membrane trafficking. Studies on human neutrophils found that Arf-regulated PLD activity was also located on secretory vesicles. In fact, the activity mobilizes to the plasma membrane following fMetLeuPhe stimulation (Whatmore et al, 1996; Morgan et al, 1997).

Subsequent studies by Ktistakis et al (1996), found that formation of coatomer-coated vesicles from Golgi-enriched membranes required not only Arf activation, but more importantly, PLD activation. In fact, cytosolic Arf was not necessary for initiating coat assembly on Golgi membranes from cell lines with high constitutive PLD activity. The formation of coated vesicles was sensitive to ethanol at concentrations that inhibit the production of PA by PLD. Coatomer bound to Glogi membranes when PA was produced by an exogenous bacterial PLD. In addition, purified coatomer also bound selectively to artificial lipid vesicles

containing PA and PIP_2 (Ktistakis et al, 1996). Therefore, the production of PA by PLD was proposed to facilitate coatomer binding to Golgi membranes. The same group of researchers also found that PA formation by PLD was required for transport from ER to the Golgi complex (Bi et al, 1997).

Using a permeabilized cell system derived from growth hormone and prolactin-secreting pituitary GH3 cells, another research group demonstrated that immunoaffinity-purified human PLD1 stimulated nascent secretory vesicle budding from the trans-Golgi network (TGN) (Chen et al, 1997). An enzymatically inactive form of PLD1, with one amino acid mutation, was found to have no effect. The release of nascent secretory vesicles form the TGN was sensitive to 1% 1-butanol, a concentration that inhibited PLD-catalyzed PA formation. Recombinant Arf1 stimulated vesicle budding, which correlated well with its activation of endogenous Golgi PLD activity (Chen et al, 1997). It was further shown that secretory vesicle budding from the TGN is mediated by PA itself, rather than its breakdown product, DAG, (Siddhanta and Shield, 1998).

The above studies provided some fundamental evidence for the involvement of PLD in membrane trafficking and secretion. However, controversy on the role of PLD in these cellular activities still exists (Ktistakis, 1998). One study found that Golgi membrane PA levels do not rise, but in fact, decline, during cell-free budding reactions (Stamnes et al, 1998). The direct evidence for a role of PLD in vesicle transport awaits the development of selective inhibitors of its enzymatic activity. One major question remains to be answered - if PLD is important for

membrane transport, what is the mechanism of action of PLD or its products during vesicle formation and transport? Current information tends to suggest the alteration of the local lipid environment and the biophysical properties of the membrane, which may facilitate the interaction of lipid components with certain regulatory proteins (Moss and Vaughan, 1995; Roth and Sternweis, 1997; Roth et al, 1999). However, the exact mechanism of this proposed action is still far from being fully understood.

1.3 Major aims of the present study in the submandibular model

The present work was undertaken to investigate the regulation of phospholipase D in the rat submandibular acinar cell model. The specific goals were (1) to examine the role of GTP-binding proteins, in particular Arf, on the regulation of PLD in this model; (2) to characterize and attempt to identify the mechanism of the inhibition of PLD by aluminum fluoride in permeabilized or cell-free systems which was observed in preliminary studies; (3) to use purified PLD under controlled reaction conditions to confirm the inhibitory effect and gain more direct information on the enzyme's mode of action and inhibition. Chapter 2, 3 and 4 have been written in a manuscript format to correspond closely with three publications that have resulted from these studies.

Chapter 2. Activation of phospholipase D by ADP-ribosylation factor in rat submandibular acinar cells

2.1 Abstract

The hydrolysis of phosphatidylcholine (PC) by phospholipase (PLD) generates signalling molecules such as phosphatidic acid, diacylglycerol and arachidonic acid. Guanine nucleotide activation of PLD was investigated in enzymatically-dispersed rat submandibular gland acinar cells (SMG cells). GTPγS caused the time- and dose- dependent stimulation of PLD in permeabilized cells. This effect was lost in prepermeabilized cells, from which cytosolic components had been allowed to leak, but was restored when endogenous cytosol, or cytosol from platelets was added back to such cells. PLD was also activated in cytosol-depleted cells by GTPγS in combination with purified recombinant Arf (ADP-ribosylation factor). Brefeldin A, a specific blocker of Arf action, inhibited carbachol- and GTPγS- induced stimulation of PLD. Arf translocated from cytosol to membrane upon GTPγS treatment in permeabilized SMG cells. The heterotrimetic G protein stimualtor, AlF_4^-, also activated PLD in intact SMG cells, this response too, was inhibited by brefeldin A, suggesting the downstream involvement of Arf in coupling AlF_4^- action to lipase activation. PLD activation caused by both GTPγS and AlF_4^- was only partially reduced after treatment of cells with U73122, a demonstrated inhibitor of phospholipase C. It is therefore proposed that in submandibular mucous acinar cells, guanine nucleotide-regulated PLD activation

pathway may exist that involves the sequential actions of a heterotrimeric GTP-binding protein, and Arf. It is further suggested that this pathway is independent of the Gq/PLC/PIP_2 signal transduction system.

2.2 Introduction

Previous work in this laboratory has shown that arachidonic acid (*sn* 20:4 ω6) is a multifunctional regulatory molecule in submandibular glands, controlling several physiological functions, such as protein synthesis, mucin secretion, calcium mobilization and ATP metabolism (Fleming and Mellow, 1995a). A prime source of the fatty acid is the membrane phospholipid, phosphatidylcholine (PC), in which AA is esterified at the *sn*-2 position. Muscarinic cholinergic stimulation of submandibular cells causes the release of arachidonate via a series of reactions, in which the initial stage is the receptor-coupled cleavage of PC by the enzyme, phospholipase D (Chung and Fleming, 1995b). This produces phosphatidic acid, which in turn generates diacylglycerol and AA. All three of these lipid-derived metabolites have probable intracellular signalling or regulatory roles (Exton, 1994). Thus, the activation of PLD is a key central factor in the control of several physiological processes in the submandibular gland.

It was further demonstrated that muscarinic stimulation of PLD was partially mediated by the agonist-coupled phospholipase C/PIP_2 pathway (Chung and Fleming, 1995b). In this system, PLC cleaves the membrane inositol phospholipid, PIP_2, to produce IP_3 and diacylglycerol, which respectively release Ca^{2+} from

endoplasmic reticulum stores, and activate the enzyme protein kinase C. Both Ca^{2+} and PKC play a role in PLD activation in the submandibular model (Chung and Fleming, 1995b). However, approximately 40% of the ligand-induced PLD effect remains after complete blockade of the PLC/PIP_2 pathway, leading to the speculation that a second, PLC-independent pathway is involved in PLD activation in the submandibular model. This is consistent with the proposal that such a pathway, which is guanine nucleotide-dependent, but PLC/Gq- independent may operate in neutrophils (Kanaho et al, 1991; Cockcroft, 1992; Geny and Cockcroft, 1992).

A component which has been implicated in GTP-associated pathways of PLD activation is the ADP-ribosylation factor, Arf (Brown et al, 1993; Cockcroft et al, 1994;). Arf was first identified as the protein cofactor for the cholera toxin-induced ADP-ribosylation of the α subunit of Gs of the adenylyl cyclase/cAMP signalling pathway (Kahn and Gilman, 1984). This protein is classified as a member of the *ras* superfamily of the low molecular weight GTP-binding proteins (Kahn and Gilman, 1986). Arf has an additional established role in intracellular vesicle transport (Rothman, 1994; Moss and Vaughan, 1995), and so may have a central regulatory function in the secretory process of exocrine glands, including salivary acinar cells. A further role for Arf has been proposed in the activation of PLD in HL60 cells and kidney cells (Cockcroft, 1994, Rumenapp et al, 1995), although the protein's mechanism of action is unclear. No such function has so far been examined in salivary glands.

The present study was therefore undertaken on the submandibular mucous acinar cell model to investigate whether PLD may be activated in a guanine nucleotide-associated signalling pathway that is mediated by Arf, and to determine if this pathway is discrete from the phosphoionositide signal transduction system.

2.3 Materials and methods

2.3.1 Materials

Purified collagenase, CLSPA grade, was obtained from Worthington (Freehold, NJ, USA). [5.6.8.9.11.12.14.15-^{3}H] arachidonic acid, specific activity 7.73 TBq/mmol was a product of Amersham (Arlington Heights, IL, USA). Silica gel-coated thin layer chromatography (TLC) plates were from Whatman International (Maidstone, Kent, UK). The phosphatidylethanol standard was supplied by Avanti Polar Lipids (Alabaster, ALA, USA). Compound U73122 and brefeldin A were obtained from Biomol Research Laboratory (Plymouth Meeting, PA, USA). Guanosine 5'-0-[γ-thio] triphosphate (GTPγS) was from Boehringer Mannheim (Laval, Quebec). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO, USA).

2.3.2 Preparation of rat submandibular cells

Male, Sprague-Dawley rats of 250-300 g weight were anaesthetized by an i.p. injection of sodium pentobarbital (Nembutal, 50 mg per kg body weight) then exsanguinated via the vena cava. The submandibular glands were removed,

chopped into small pieces and pooled. The fragments were incubated at 37°C for 60 min in 50 ml of modified Hank's Balanced Salt Solution (HBSS) containing 2,000 U purified collagenase and 25 mg hyaluronidase. Mechanical shearing forces were applied to the tissue by repeated pipetting at 15 min intervals and the preparation was filtered through a 320 μm nylon mesh. This technique effectively removes cells of the gland's duct system and produces an enriched population of over 90% mucous acinar cells. The preparations were maintained at 37°C under atmospheric conditions in supplemented HBSS. Dispersed cells were washed twice in fresh culture medium and resuspended for experimental treatments.

2.3.3 Radiolabelling of cell lipids

Cell lipids were radiolabelled by incubation with 60 KBq [^{3}H] arachidonic acid per ml in the incubation medium without BSA for 90 min. Preparations were then washed in three changes of fresh culture medium and resuspended in 200 μl aliquots for experimental treatment.

2.3.4 Phospholipase D assay in intact SMG cells

Activtion of PLD by carbachol or AlF_4^- was measured in intact SMG cells. PLD was assayed by measuring the formation of [^{3}H] phosphatidylethanol, which is produced by the PLD-specific transphosphatidylation reaction. In this reaction, PEth is produced at the expense of phosphatidic acid in the presence of 1% ethanol. Total reaction volume per sample was 200 µl. Reaction time was 20 min. Reactions were terminated by the addition of 600 µl of a mixture of chloroform, methanol and HCl (100:200:1, by vol). The lipids were isolated according to the method of Bligh and Dyer (1959). The organic and inorganic phases were separated by centrifugation at 2,000 g for 10 min. The extracted organic phase of cells was dried under nitrogen, resolubilized in 100 µl of a mixture of chloroform : methanol : H_2O (75:25:2, by vol), and spotted on to silica gel TLC plates which had been activated at 110 °C for 1 h. The chromatograms were developed at room temperature in the organic phase of ethyl acetate : 2,2,4-trimethyl pentane : acetic acid : H_2O (13:2:3:10, by vol). Lipids were visualized by iodine vapour. The phosphatidylethanol bands (R_f = 0.34) were scraped from the TLC plates and radioactive PEth was quantitated by scintillation counting.

2.3.5 Phospholipase D assay in permeabilized SMG cells

In experiments where permeabilized cells were used (e.g. GTPγS treatment), cells were maintained in a cytosolic buffer containing 100 mM KCl, 20 mM NaCl,

25 mM $NaHCO_3$, 0.96 mM NaH_2PO_4, 5 mM $MgSO_4$, 0.01% soybean trypsin inhibitor, 1.5 mM MgATP, 2 μM $CaCl_2$, 15 mM Hepes, pH 7.2. Most assay procedures were the same as that in intact cells, except that the permeabilizing reagent - digitonin (50 μg/ml) was added to the cells together with GTPγS and 1% ethanol right at the start of the reaction. In prepermeabilization experiments, cells in cytosolic medium were treated for 20 min with 50 μg/ml digitonin in the absence of GTPγS and ethanol, followed by several washings with the same medium, to deplete cytosolic components before the addition of nucleotide and 1% ethanol.

2.3.6 Purification of recombinant Arf

Recombinant Arf1 was purified from *E.Coli* strain BL21(DE3), transformed with Arf expression plasmid pOW12, provided by Dr. R. Kahn (Emory University, Atlanta, GA, USA). The expression of recombinant Arf1 was induced with isopropylthio-β-galactoside (IPTG) at a final concentration of 1 mM. BL21(DE) cells transformed with pOW12 were grown in LB medium containing 50 μg/ml ampicillin at 37°C. The bacteria were lysed by incubation for 10 min in 0.2% Triton X-100, 50 mM Tris-HCl, 100 mM $MgCl_2$, pH 8.0 with a brief sonication at the end of the lysis period. The preparation was centrifuged at 100,000 g for 1 h at 4°C and the supernatant used as a source of rArf1. Purification of rArf1 was carried out by successive column chromatogaphy techniques on

DEAE-Sephacel, Ultrogel AcA-54, essentially as described by Randazzo et al, (1992). In the present study, a subsequent additional purification step consisted of the elution of rArf–containing fractions from a hydroxyapatite column. The column was equilibrated in 20 mM Hepes, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, pH 7.4. Bound proteins were eluted with a gradient of NaH_2PO_4/Na_2HPO_4 (0-250 mM). The fractions from each step of the purification procedure were subjected to SDS-PAGE and gels stained with Coomassie blue. Fractions containing species around the 21 Kda range (the Mr of Arf) were combined in each case for the subsequent phase of purification. These fractions were also checked for rArf content by reactivity with a polyclonal anti-Arf antibody (provided by Dr. R. Bhullar, University of Manitoba). Fractions from the hydroxyapatite column that showed a single, Arf-positive band were pooled, dialysed in 10 mM Hepes, 10% glycerol, pH 7.2, and concentrated (Centricon 10, Amicon) to a protein content of 0.8 mg/ml.

2.3.7 Determination of Arf translocation by Western Blotting

Final concentrations of 100 μM GTPγS plus 50 μg/ml digitonin were added to a 1 ml acinar cell suspension. After 20 min, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, and 0.3 mM PMSF were added to the samples. Cells were disrupted by three 10-second pulses of sonication. The unbroken cells and nuclei (pellet) were discarded after 10 min centrifugation at 500 g. The suspension was subjected to ultracentrifugation at 100,000 g for 1h at 4 °C. The retained pellet and supernatant

were regarded as membrane and cytosol fractions respectively. Samples containing the total amount of cytosolic and membrane extract representing 200 μg original total cell protein were subjected to SDS-PAGE with 13% acrylamide. Separated proteins were transferred to a nitrocellulose membrane by electroblotting in buffer containing 0.5% SDS (Fleming and Mellow, 1995b). The blots were blocked with 5% milk powder for 1 h at room temperature in a buffer containing 100 mM Tris, pH 7.5, 100 mM NaCl and 0.1% Tween 20. A polyclonal anti-Arf antibody (1:1,000 dilution) was then added to the incubation mixture. After 1h incubation at room temperature, the blots were washed (5x5 min) in a buffer lacking milk powder. The blots were reacted with goat anti-(rabbit-IgG)-horseradish peroxidase conjugate (1:3,000 dilution, 1 h, 20°C) followed by 5x5 min washing. The antigen-aniboy complex was visualized on Hyperfilm-ECL by the enhanced chemiluminescence procedure (Amersham).

2.3.8 Preparation of membrane and cytosol fractions

Rat submandibular gland acini were suspended in cytosolic buffer plus 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, and 0.3 mM PMSF. The cells were disrupted by sonication for 2x15 sec then centrifugated at 500 g for 10 min. Unbroken cells and nuclei (pellet) were discarded, and the supernatant fluid was further centrifuged at 100,000 g for 1h at 4°C . The supernatant was retained as the cytosolic fraction, and pellet was regarded as the membrane. Human platelets

were lysed by three freeze-thaw cycles in liquid nitrogen, and the cytosolic fraction was prepared by centrifugation at 100,000 g for 1 h at 4°C (Bhullar and Haslam, 1987). The membrane and cytosol fractions from both sources were stored at -70°C for further use in the add- back PLD assays.

2.3.9 Protein determination

The protein concentration in various samples was determined by using the Bio-Rad dye reagent assay, based on the method of Bradford (1976) with bovine serum albumin as standard.

2.4 Results

2.4.1 GTPγS stimulation of PLD in permeabilized SMG cells

Initial experiments were carried out to establish the optimal permeabilization conditions. It was found that the permeabilization agent digitonin, at the concentration of 50 μg/ml, gave the best PLD activation by GTPγS, and this concentration was therefore used in the later experiments (Fig. 1). In a dose-response study, GTPγS, at an optimal concentration of 100 μM, activated PLD to approximately five fold control values (Fig. 2A). In time course experiments, PLD

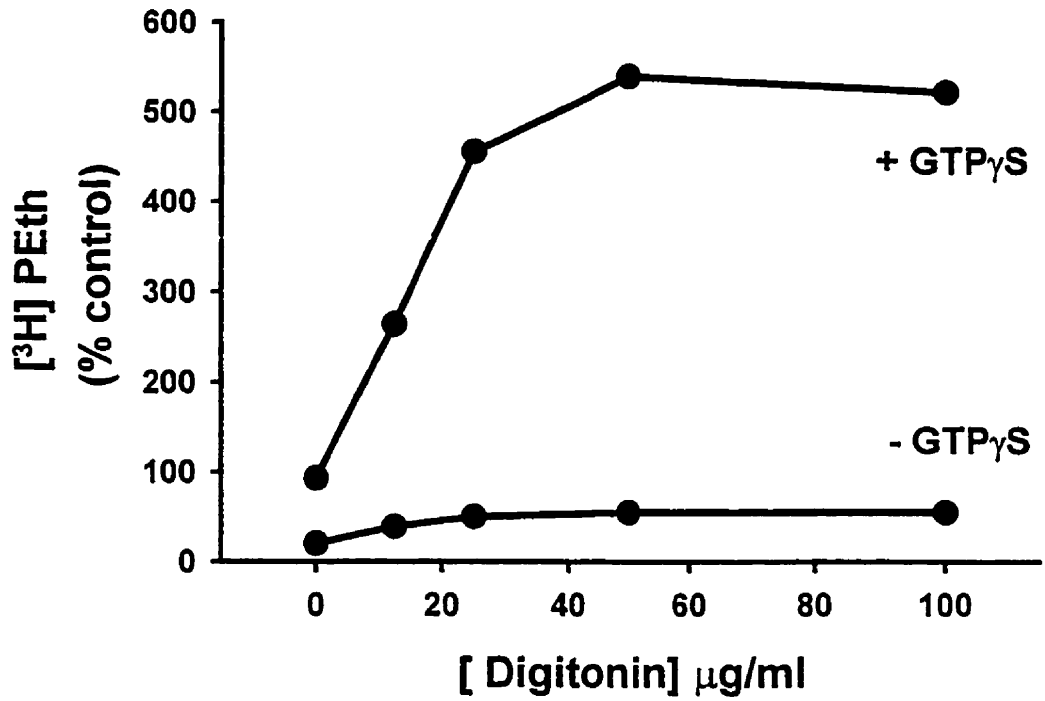


Fig 1. Dose response of digitonin on GTPγS stimulation of PLD in permeabilized rat submandibular gland cells. Different concentrations of digitonin (0-100 μg/ml) were used to permeabilize SMG cells, and GTPγS (100 μM) stimulation of PLD was measured under these condition by the transphosphatidylation reaction in the presence of 1% ethanol. The total reaction time was 20 min. In this experiment, digitonin was dissolved in ethanol (1% in the final reaction mixture), and added after GTPγS to start the reaction.

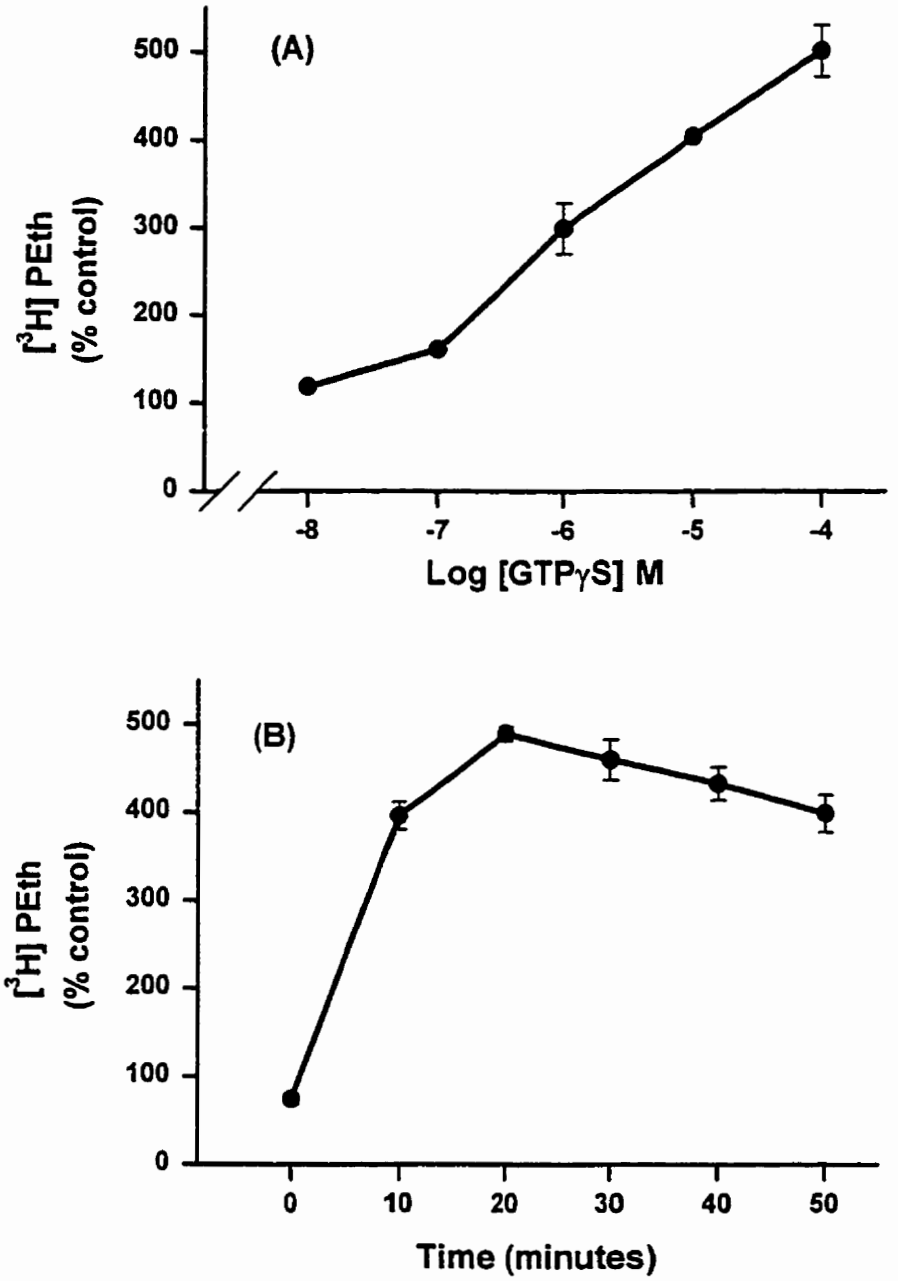


Fig.2 (A) Concentration response of GTPγS activation of PLD. PLD activity was measured by the transphosphatidylation reaction ([^{3}H] PtdEtOH formation), in permeabilized rat submandibular acinar cells. Controls were not exposed to GTPγS. Reaction time was 20 min. Values are means ± SEM, n = 6. **(B) Time course of the GTPγS (10^{-4} μM) activation of PLD in permeabilized acinar cells.** Values are means ± SEM, n= 4.

activity was maximal at 20 min incubation with GTPγS, and dropped only slightly over the following 30 min (Fig. 2B). The standard conditions of 100 μM GTPγS and a 20 min incubation period were therefore used in subsequent experiments, unless otherwise indicated.

2.4.2 A cytosolic factor is involved in PLD activation in SMG cells

Permeabilization conditions proved to be crucial in demonstrating the stimulation of PLD by GTPγS. When cells were permeabilized by treatment with digitonin for 15 min in the absence of nucleotide to allow depletion of cytoplasmic components (prepermeabilization), the subsequent addition of GTPγS had no effect on PLD activity (Fig.3). When GTPγS was included with digitonin at the beginning of the permeabilization period, the nucleotide did elevate PLD activity up to 5 times control (see above). This observation suggested a requirement for a cytosolic component in the stimulation of PLD by GTPγS, which was investigated further in add-back experiments with cytosolic extracts of submandibular acinar cells and human platelets. Exogenous cytosol from both these sources (200 μg protein) restored the guanine nuleotide activating effect on PLD in prepermeabilized, cytosol-depleted cells (Fig. 4), though not to the level found in non-prepermeabilized, GTPγS-treated preparations.

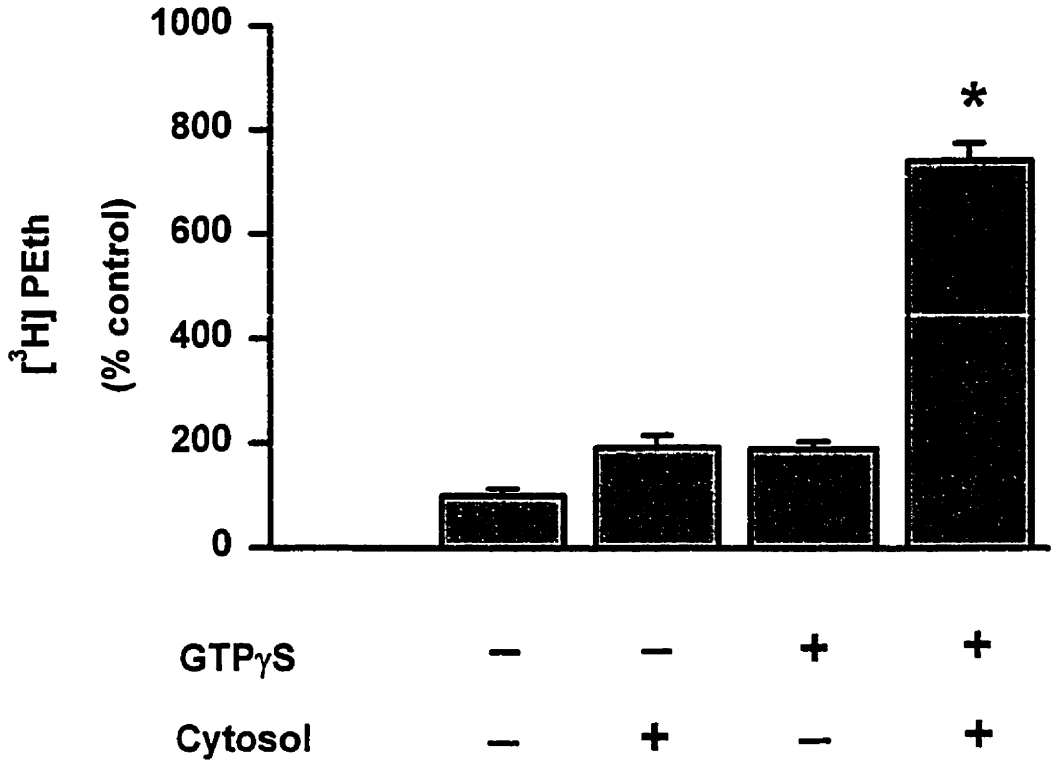


Fig 3. Effect of cytosol depletion on PLD activation by GTPγS in SMG cells. Cells prelabelled with [^{3}H] arachidonic acid were permeabilized with 50 μg/ml digitonin in the presence of 100μM GTPγS (+ cytosol), or permeabilized, then washed three times with fresh buffer to remove cytosolic components before exposure to GTPγS (-cytosol). PLD activity was measured over a 20 min period as described in the text. Values are means ± SEM, n= 4. *$p<0.01$ relative to the other three treatments. Results are expressed as a percentage of basal activity, measured in the absence of cytosol (control). All preparations contained 1% ethanol.

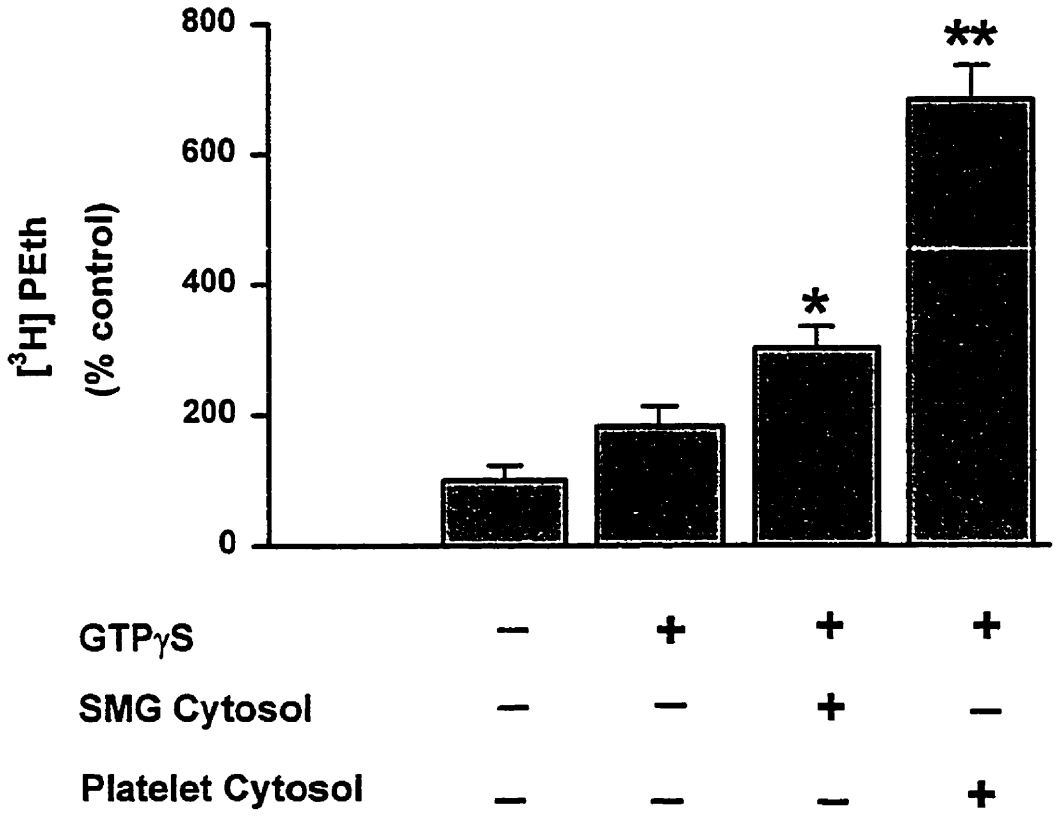


Fig 4. Effect of cytosolic extracts on PLD activation in prepermeabilized SMG cells. Acinar cells were permeabilized with digitonin then washed three times with fresh buffer over a 15 min period to deplete cytosolic components. Extracts of endogenous cytosol (SMG), or platelet cytosol (both approximately 200 μg protein) were added back to 200 μl cell suspensions in the presence of 100 μM GTPγS and PLD was assayed as before. Values are means ± SEM, n= 4. *and ** $p<0.01$ relative to the other three treatments. Results are expressed as a percentage of PLD activity found in the absence of GTPγS and cytosol (control).

2.4.3 Arf is a crucial cytosolic factor for PLD activation in SMG cells

In an additional series of add-back experiments, the effect of exogenous rArf on the nucleotide stimulation of PLD was investigated. Recombinant Arf was purified to a single 21 Kda species that reacted with an anti-Arf antibody (Fig. 5). The purified protein, at a concentration of 220 μg/ml, significantly enhanced the GTPγS-elevation of PLD activity by approximately 2.3 fold (Fig. 6). Recombinant Arf had no effect on enzyme activity in the absence of nucleotide.

The movement of endogenous Arf between soluble and particulate components of submandibular acinar cells after GTPγS treatment was examined in Western blotting experiments with anti-Arf antibody. The nucleotide clearly stimulated the translocation of Arf from the cytosol to the membrane fraction in permeabilized cells (Fig. 7).

Additional evidence of a role for Arf in PLD activation was generated in studies with brefeldin A, an inhibitor of Arf action. PLD activity was elevated in permeabilized cells by GTPγS, the muscarinic agonist, carbachol, and the heterotrimeric G protein activator, AlF_4^- (Fig. 8). In all cases, this effect was significantly inhibited by preincubation of cells with 400 μM brefeldin A (Fig. 8).

2.4.4 PLD activation by GTPγS or AlF_4^- is largely independent of the PLC/PIP_2 signalling pathway

In a final series of experiments, 10^{-5} M U73122 reduced the PLD activities induced by GTPγS and AlF_4^- by only 21% and 19% respectively, indicating that the action of these agents is not predominantly mediated by the PLC/PIP_2 pathway (Fig. 9). Previous study in our lab has shown that 10^{-5} M U73122 completely inhibited carbachol-stimulated inositol phosphate production (Chung and Fleming, 1995b).

2.5 Discussion

The involvement of Arf in the activation of PC-specific PLD has been proposed in HL60 cells (Brown et al, 1993; Cockcroft et al, 1994). However, no data yet exist to indicate a role for Arf in PLD activation in exocrine glands in general and salivary glands in particular. The present study provides several lines of evidence suggesting that Arf does have such a function in submandibular gland cells, and may thus occupy a central position as a signalling molecule in the generation of several PC-derived regulatory metabolites, including phosphatidic acid, diacylglycerol and arachidonic acid.

GTPγS produced both a concentration- and time- dependent stimulation of PLD, consistent with the involvement of a guanine nucleotide-binding protein in the

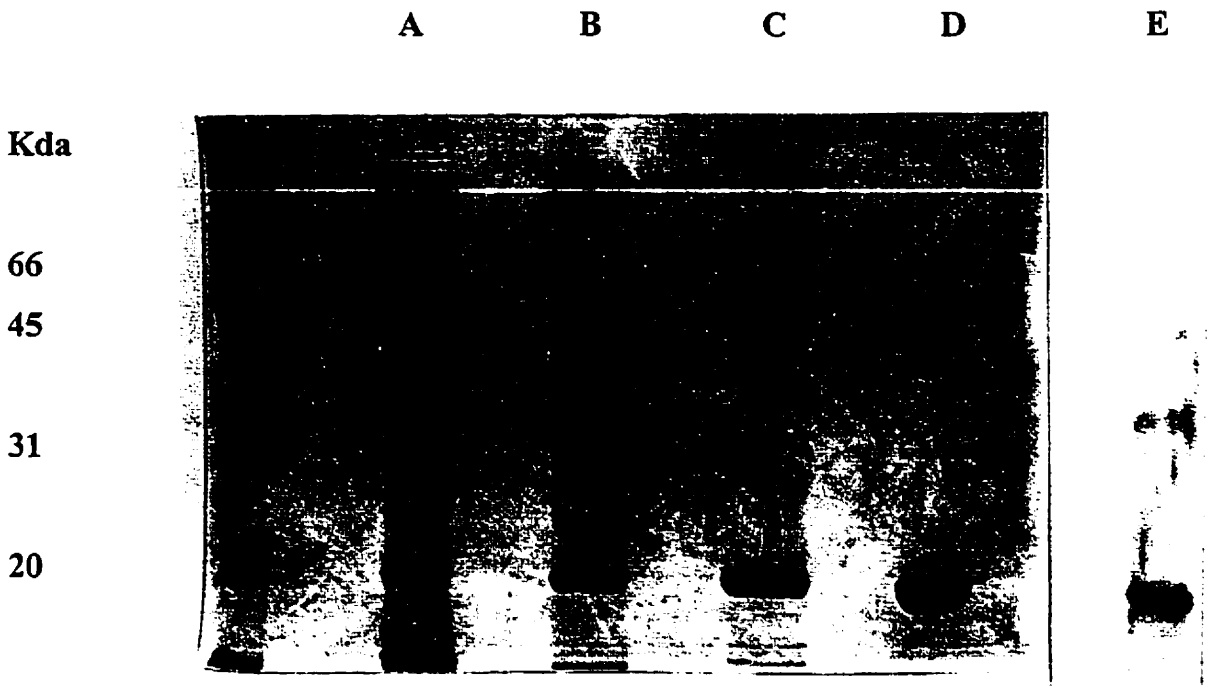


Fig 5. Stepwise purification of rArf from *E.Coli* strain BL21(DE3) transformed with Arf expression plasmid pOW12. Expression of Arf was induced with isopropylthio-β-galactoside (IPTG). Proteins in lanes A-D are stained with Coomassie blue. Lane A: total bacterial protein after IPTG induction. Lane B: after DEAE-Sephacel chromatography. Lane C: after Ultrogel AcA chromatography. Lane D: after hydroxyapatite fractionation. The purified, single 21 Kda species in lane D was identified as Arf by a positive immunostaining reaction with anti-Arf antibody (Lane E).

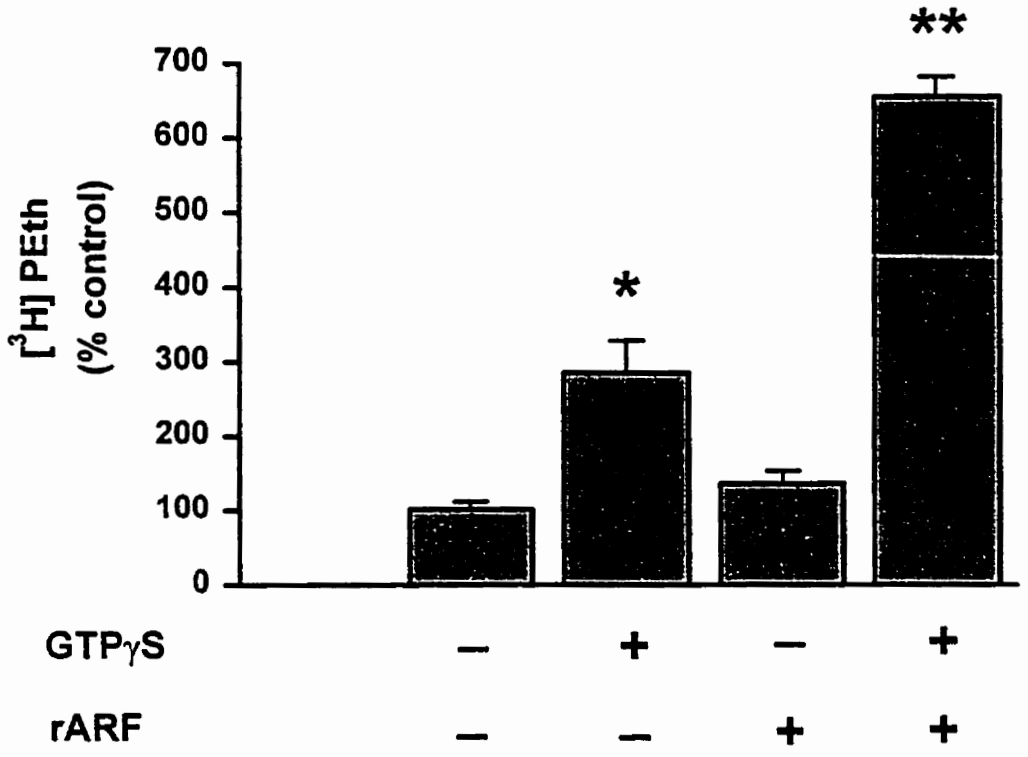


Fig 6. Effect of Arf on PLD activation in cytosol-depleted SMG cells. Recombinant Arf was purified as a single 21 kDa species (see Fig. 5) and added at a concentration of 220 μg protein per ml to prepermeabilized cells in the presence or absence of GTPγS (100 μM). PLD activation was assayed over 20 min. Values are means ± SEM, n=3. *and ** $p<0.01$ relative to the other three treatments. Results are expressed as a percentage of PLD activity measured in the absence of GTPγS and rArf (control).

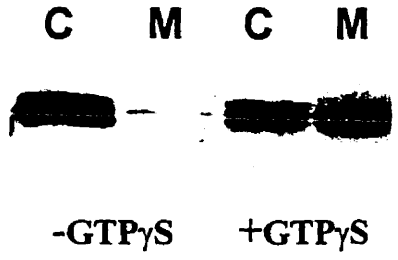


Fig. 7. Translocation of Arf from cytosol to membrane in submandibular acinar cells after GTPγS treatment. Cells were permeabilized in digitonin in the presence (+) or absence (-) of GTPγS for 20 min. The cytosol and membrane fractions were obtained by centrifugation at 100,000 g. Proteins were separated by SDS-PAGE, electroblotted to nitrocellulose membranes and immunostained with anti-Arf antibody as described in the text. C = cytosolic preparation, M = membrane preparation.

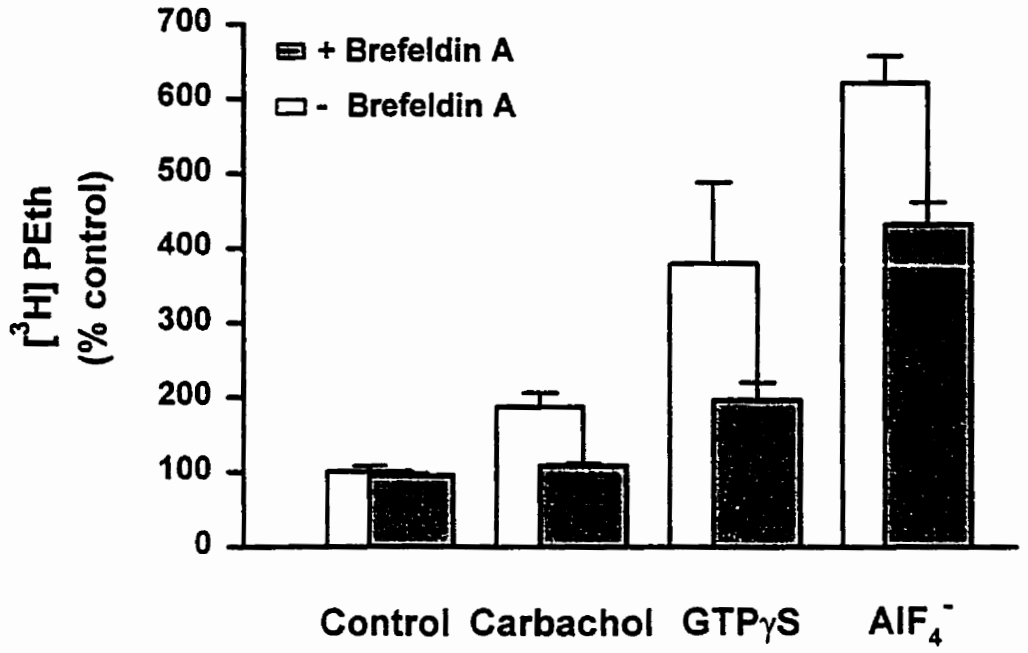


Fig 8. Effect of brefeldin A on PLD activation in submandibular acinar cells. Cells were prelabelled with [^{3}H] arachidonic acid, then treated with 400 μM brefeldin A for 10 min or left as controls. The preparations were then incubated with carbachol (100 μM) or AlF_4^- (10 mM NaF plus 50 μM $AlCl_3$) for 10 or 40 min respectively (these optimal times were predetermined empirically). In GTPγS (100 μM; 20 min) -treated samples, cells were permeabilized by digitonin treatment (50 μg/ml). PLD was assayed as described in the text. Values are means ± SEM, n = 4. All brefeldin A-treated samples showed significantly lower PLD activities than their brefeldin A-free counterparts ($p<0.01$).

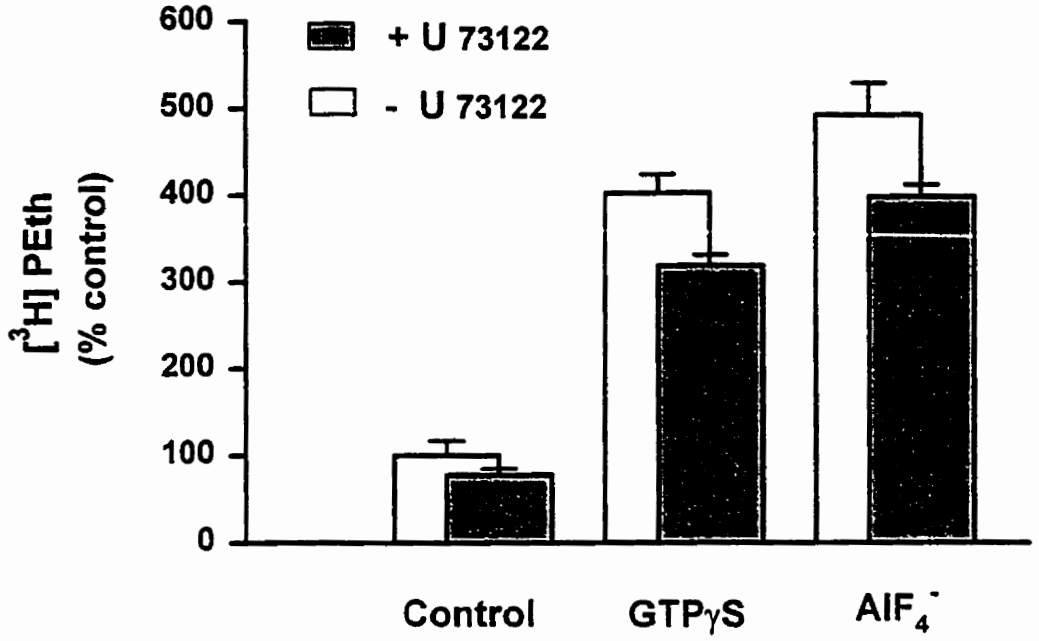


Fig 9. Effect of U73122 on PLD activity in SMG cells. Permeabilized cells (GTPγS treated), or intact cells (control and AlF_4^- treated) were incubated with or without 10 μM U73122, a phospholipase C blocker, for 10 min then treated with 100 μM GTPγS for 20 min or AlF_4^- for 40 min. Cells were assayed for PLD as described in the text. Values are means ± SEM, n = 4. Cells incubated with U73122 in the presence of GTPγS or AlF_4^- had PLD values tha were lower than their U73122-free counterparts (p,0.05), but still significantly greater than the control values of non-stimulated cells ($p<0.01$).

activation process. Such a protein could represent a heterotrimeric G protein, or a member of the extensive family of small GTP-binding proteins. Signal transduction pathways involving Gq (phospholiapse C coupled) and Gs/Gi (adenylyl cyclase coupled) have been confirmed in submandibular acinar cells (Fleming et al, 1987, 1992). However, these G proteins are membrane-associated, and so, the lack of effect of GTPγS on PLD in cytosol-depleted cells suggests that the stimulation of heterotrimeric GTP-binding proteins alone was insufficient to activate the enzyme in the permeabilized cell model. This idea is reinforced by the findings from add-back experiments, in which cytosol replacement by endogenous submandibular gland cell cytosol, or platelet cytosol, in prepermeailized cells restored guanine nucleotide-induced PLD activation. These results are consistent with those of Geny et al (1993), who demonstrated that a cytosolic factor from rat brain regulated PLD in prepermeabilized HL60 cells. This factor was later shown to be Arf (Brown et al, 1993; Cockcroft et al, 1994) and it was subsequently confirmed that the same protein enhanced PLD activity in human embryonic kidney cells (Rumenapp et al, 1995).

In the present study, purified rArf in combination with GTPγS, activated PLD in cytosol-depleted submandibular cells. PLD is known to be predominantly membrane bound (Cockcroft, 1996). The fact that Arf plus GTPγS restored PLD activity to prepermeabilized cells confirms that the targeted form of PLD in this case was the membrane-associated enzyme. The movement of Arf from cytosol to

membrane has been proposed as a key step in the protein's activation of PLD (Cockcroft, 1996). In the submandibular model we have now clearly shown by Western Blotting that translocation of Arf from the cytosolic to the particulate fraction occurs after GTPγS treatment. A third line of evidence which indicates Arf involvement in PLD activation, was our observation that brefeldin A, a known Arf inhibitor (Ktistakis et al, 1995; Tsai et al, 1996), significantly reduced PLD activation by carbachol, GTPγS and AlF_4^-. Taken together, these findings strongly indicate a central involvement of Arf in the activation of PLD, leading to PC hydrolysis in mucous acinar cells of the rat submandibular gland.

Chung and Fleming (1995b) found that in SMG cells, PLD was stimulated by the muscarinic cholinergic agonist, carbachol. It was further demonstrated that this effect was only partially secondary to the carbachol-induced hydrolysis of PIP_2 by PLC. This reaction liberates diacylglycerol, which activates PKC, an enzyme known to elevate PLD in submandibular cells (Chung and Fleming, 1995b) and other cell types (Cockcroft, 1992; Exton, 1994). When the carbachol/PIP_2 effect was totally blocked at the level of PLC by U73122, approximately 40% of the agonist's PLD stimulating capacity was retained (Chung and Fleming, 1995b). Downstream blockade of PKC activity by Ro-31-8220 also had an inhibitory effect on PLD. This led us to speculate on the existence of a muscarinic agonist-coupled PLD activation pathway that is PLC/PIP_2–independent. Muscarinic agonists are known to be of the seven membrane domain spanning, heterotrimeric

G protein- linked type, and their coupling pathways should therefore be responsive to guanine nucleotide treatment. In the present study, GTPγS significantly stimulated PLD in permeabilized cells, and this effect was inhibited by only 21% after PLC inhibition by U73122. This finding indicates that a PLC/PIP_2– independent pathway does exist in PLD activation in the salivary gland cell model.

Since guanine nucleotides can activate both heterotrimeric G proteins and small GTP-binding proteins, we attempted to differentiate between the involvement of these classes of proteins by using two probes – brefeldin A, a specific inhibitor of Arf (Ktistakis et al, 1995), and AlF_4^-, which activates heterotrimeric G proteins but not small GTP-binding proteins, such as Arf (Kahn et al, 1991). In intact SMG cells, AlF_4^- enhanced PLD activity to approximately five fold control values, but this response was reduced by only 19% under conditions of complete or almost complete PLC blockade by U73122 (Fig. 9). This finding indicates the involvement of a heterotrimeric G protein, other than the Gq of the PLC/PIP_2 transduction pathway, in PLD activation. In Arf inhibition experiments, the PLD-stimulating effects of carbachol, GTPγS and AlF_4^- were all significantly reduced by brefeldin A (30-50%). Since AlF_4^- stimulates only the heterotrimeric G proteins, the inhibition of its PLD-activating effect by brefeldin A suggests the possible downstream involvement of Arf in coupling AlF_4^- action to lipase activation. Donaldson et al (1991), presented evidence that Arf binding to Golgi membranes in normal rat kidney (NRK) cells was regulated by heterotrimeric G

proteins. In a study on embryonic kidney cells, Rumenapp et al (1995), proposed that muscarinic stimulation activated PLD by a pathway involving sequential roles for G proteins and Arf. When the previous (Chung and Fleming, 1995b), and present studies in our lab are considered together in light of this work, it may be proposed that in submandibular acinar cells, muscarinic stimulation of PLD proceeds by at least two different signalling pathways. One of these is the PLC/PIP_2/PKC pathway already described (Chung and Fleming, 1995b). The second, investigated here, involves the sequential coupling of receptor, G protein (other than Gq) and Arf in PLD activation. Using a kidney cell line expressing human muscarinic M3 receptors, Rumenapp et al (1995), presented evidence that this receptor subtype mediated a G protein-Arf-PLD response. Previous studies in this lab have established that M3 is the predominant, G protein-linked muscarinic receptor in rat submandibular cells (Laniyonu et al, 1990), so it is most likely that the M3 subtype preferentially activates Arf-associated pathways in our experimental model. However, the involvement of additional coupling proteins within the proposed G-protein-Arf-PLD sequence cannot be ruled out.

In an earlier study on the submandibular model, a role was established for arachidonic acid as a multipotent regulator of several physiological functions, including mucin secretion, protein synthesis and calcium mobilization (Fleming and Mellow, 1995a). It was later demonstrated that muscarinic-induced cleavage of PC by PLD was a key initial stage in a series of coupled reactions leading to the

release of AA, as well as phosphatidic acid and diacylglycerol (Chung and Fleming, 1995b). All three of these PC-derived metabolites are believed to have signalling roles (Exton, 1994; Cockcoft, 1996). The present study shows that Arf activtes PLD in submandibular cells and thus establishes a central position for the small GTP-binding protein in controlling the generation of membrane phospholipid-associated regulatory molecules in salivary gland.

Arf is known to play a prime role in membrane trafficking and intracellular vesicle transport (Kahn et al, 1993; Exton, 1994; Rothman, 1994; Moss and Vaughan, 1995). Arf, in combination with GTP, is believed to be involved in the assembly of a set of cytosolic coat proteins, termed coatomer (coat protomer complex), on the membrane of Golgi transport vesicles (Waters et al, 1991; Rothman, 1994). More specifically, cytosolic Arf (which is bound to GDP) is activated by a GTP-GDP exchange factor at the Golgi membrane surface. After binding GTP, Arf/GTP is bound to Golgi membrane, which would then recruit coatomer from the cytosol to assemble the bud. Subsequent hydrolysis of the bound GTP (through an Arf GTPase activating protein) would induce uncoating by releasing Arf and coatomer. The uncoated vesicles could therefore fuse with the acceptor membranes. There is now evidence that PLD may mediate the Arf-dependent assembly of Golgi coated vesicles (Ktistakis et al, 1996). The most clearly described model at the present hypothesizes that the GTP-bound form of Arf induces the assembly of coated vesicles on membranes and their budding off.

Meanwhile, PLD is activated by a GTP bound form of Arf. The subsequent generated PA from PC hydrolysis will stimulate phosphatidylinositol 4-phosphate-5-kinase, and produce PIP_2. This newly formed PIP_2 will cause further stimulation of PLD. Such a positive feedback will cause a rapid and profound change in the lipid composition of the vesicular membrane, and promote vesicle fusion with the target membrane. The local accumulation of PA and PIP_2 also stimulates Arf/GAP, and converts active Arf/GTP into inactive Arf/GDP. This shuts off the PLD activation and therefore halt the positive loop. At the same time, Arf and coatomer are disassembled from the vesicles to allow their fusion with the acceptor membrane.

The primary function of the submandubular gland is the synthesis and exocrine secretion of salivary mucin, and the acinar cells are particularly rich in Golgi- and secretory vesicles. Thus, it maybe speculated that Arf translocation/activation of PLD in the sumandibular model observed here reflects a role for the phospholipase in vesicle transport in the exocytotic response. The precise mechanism of Arf activation of PLD, and the possible function of PLD in vesicle transport or fusion in salivary gland exocytosis remain to be clarified.

Chapter 3. Aluminum fluoride inhibits phospholipase D activation by a GTP-binding protein-independent mechanism

3.1 Abstract

Aluminum fluoride (AlF_4^-) inhibited guanine nucleotide-activated phospholipase D (PLD) in rat submandibular gland cell-free lysates in a concentration-dependent response. This effect was consistent in permeabilized cells with endogenous phospholipid PLD substrates. Inhibition was not caused by either fluoride or aluminum alone and was prevented by aluminum chelation. Inhibition of PLD by aluminum fluoride was not mediated by cAMP, phosphatases 1, 2A or 2B, or phosphatidate phosphohydrolase. AlF_4^- had a similar inhibitory effect on rArf-stimulated PLD, but did not block the translocation of Arf from cytosol to membranes, indicating a post GTP-binding-protein site of action. Oleate-sensitive PLD, which is not guanine nucleotide-dependent, was also inhibited by AlF_4^-, supporting a G protein-independent mechanism of action. A submandibular Golgi-enriched membrane preparation had high PLD activity which was also potently inhibited by AlF_4^-, leading to speculation that the known fluoride inhibition of Golgi vesicle transport may be PLD-mediated. It is proposed that aluminum fluoride inhibits different forms of PLD by a mechanism that is independent of GTP-binding proteins and that acts via a membrane-associated target which may be the enzyme itself.

3.2 Introduction

The results reported in Chapter 2 above (see Li et al, 1998) established a role for Arf in the muscarinic receptor-coupled activation of PLD in the rat salivary gland model. The involvement in the Arf-PLD pathway of a heterotrimeric G protein, discrete from Gq of the PLC/PIP_2 system, was also suggested by the stimulatory effect of aluminum fluoride, a G protein activator, on PLD. AlF_4^- stimulates only heterotrimeric G proteins and not small GTPases such as Rho and Arf (Kahn, 1991), and so, could theoretically be used to distinguish the effects of these two groups of GTP-binding proteins. The AlF_4^- stimulatory mechanism is based on its capacity to mimic the γ-phosphate group of GTP in stablizing the Gα subunit of the regulatory protein in the transitional state (Bigay et al, 1987; Coleman et al, 1994).

Although AlF_4^- activates PLD in intact cells, it has so far failed to activate the enzyme in cell-free preparations, including extracts of HL60 cells and neutrophils (Brown et al, 1993; Bowman et al, 1993). This inconsistency with its action on whole cells, led us to examine the possible effect of aluminum fluoride on PLD in lysates of submandibular cells, in an attempt to gain a better understanding of PLD regulation in this model. It was found that aluminum fluoride did not activate PLD in broken cell preparations of rat submandibular gland, but rather, had an inhibitory effect on the enzyme by an undefined mechanism that appeared to be independent of its stimulation of heterotrimeric GTP-binding proteins.

3.3 Materials and methods

3.3.1 Materials

Dipalmitoyl-phosphatidylcholine (PC), dioleoyl-phosphatidylethanolamine (PE), and phosphatidylethanol (PEth) were purchased from Avanti Polar Lipids, Alabaster, AL. Phosphatidylinositol 4,5-bisphosphate (PIP_2) purified from bovine brain was obtained from Sigma-Aldrich, Oakville, ONT, Canada. Dipalmitoyl-[2-palmitoyl-9,10 ^3H] phosphatidylcholine and dipalmitoylphosphatidyl-[methyl-^3H] choline were from Dupont NEN (Mandel Scientific, Guelph, ONT, Canada). [5,6,8,9,11,12,14,15-^3H] arachidonic acid was a product of Amersham Pharmacia, Baie d'Urfe, QUE, Canada. Complete Mini^{TM} Protease inhibitor tablets were from Boehringer Mannheim, Laval, QUE, Canada. Whatman Silica Gel 60 TLC plates were purchased from Fisher Scientific, Winnipeg, MB, Canada. Ultrapure sodium fluoride and all the other reagents were from Sigma-Aldrich. Materials that are the same as that in Chapter 2 are not repeated here.

3.3.2 Preparation of rat submandibular acinar cells

see Chapter 2 – 2.3.2

3.3.3 Preparation of submandibular membrane and cytosol fractions

Fresh isolated rat submandibular acinar cells were resuspended in buffer A (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 1

mM DTT, pH 7.0). One tablet of Complete^{TM}-Mini protease inhibitor cocktail (Boehringer Mannheim) was added per 10 ml buffer A. The cells were disrupted by homogenization in a ground glass homogenizer plus brief sonication. Unbroken cells and nuclei were removed by centrifugation at 500 g for 10 min. The resulting suspension is referred to as postnuclear supernatant. Total membrane and cytosol were obtained by centrifugation at 100,000 g for 90 min. The supernatant (cytosol) was removed. The pellet (membrane) was resuspended in buffer A with the same concentration of the protease inhibitors as above. Membrane and cytosol fractions were aliquoted and stored at -80°C.

3.3.4 PLD assays

3.3.4.1 Cell-free PLD assay

3.3.4.1.1 Preparation of lipid vesicles

Cell-free PLD assays were carried out by using phospholipid vesicles (PE/PIP_2/PC, in a molar ratio of 16:1.4:1) prepared according to Brown et al (1995). Lipid substrate vesicles were prepared fresh each day at 6 x final concentration. Phospholipids were added to a thick glass tube in the following concentrations: 100 μM PE, 15μM PIP_2, 10 μM PC plus 0.075 μCi dipalmitoyl[2-palmitoyl-9-10-^{3}H] PC. Chloroform was removed with a stream of nitrogen and a desired volume of 2x reaction solution (without $MgCl_2$ or $CaCl_2$) was added into the glass tube (reaction solution: 50 mM Hepes, 80 mM KCl, 3.0 mM $MgCl_2$, 1

mM DTT, 1 mM EGTA, 1 mM $CaCl_2$, pH 7.0). The lipids were suspended by sonication for 10 min at 25°C in a bath sonicator. The vesicles were stored at 4°C until use.

3.3.4.1.2 Assay procedures

PLD activity was assayed by quantifying radiolabelled PEth, produced in the transphosphatidylation reaction and isolated by thin layer chromatography (Chung and Fleming, 1995b). To start the reaction, 10 µl lipid vesicles and 1 % ethanol were added to the assay system which contained 50-100 µg membrane and/or cytosol fraction. In most experiments, 10 µM GTPγS was used to stimulate PLD activity. The assays were conducted at 37^0C for 20 min in a total assay volume of 60 µl. The reaction was stopped, PEth was extracted and quantified by TLC as described previously (Li et al, 1998).

3.3.4.2 PLD assay in intact or permeabilized SMG cells

See Chapter 2 – 2.3.4 and 2.3.5

3.3.4.3 Oleate – dependent PLD assay

Oleate-dependent PLD activity was assayed as described by Massenburg et al (1994). 10 μl phosphatidylcholine vesicles containing 10 nmol of dipalmitoyl-PC and 0.3 μCi of dipalmitoyl-[2-palmitoyl-9,10-^{3}H] PC were incubated for 30 min in a reaction system that included Na-Hepes (50 mM, pH 7.2), 4 mM sodium oleate, 1 mM EGTA, 1 mM $MgCl_2$, 50-100 μg submandibular cell membrane and 1% ethanol. Generated radiolabelled PEth, reflecting PLD activity, was measured as above.

3.3.5 Purification of recombinant myristoylated Arf from *E.Coli*

Recombinant myristoylated Arf1 protein was purified from bacteria according to the same protocol described in Chapter 2-2.3.6. The *E.Coli* strain which co-expressed the Arf1 gene and the yeast N-myristoyltransferase gene (NMT1) was kindly provided by Dr. R. Bhullar, University of Manitoba.

3.3.6 Determination of Arf translocation by Western blotting

Arf translocation was determined in a permeabilized cell system by an immunoblotting technique as described in Chapter 2-2.3.7.

3.3.7 Preparation of Golgi-enriched membrane fractions

A crude homogenate from broken rat submandibular cells was prepared as described in Chapter 2 – 2.3.8. It was homogenously mixed and adjusted to 1.3 M sucrose in 10 mM Tris-HCl, pH 7.4. A discontinuous sucrose gradient was formed by overlaying 1.2 M sucrose and 0.8 M sucrose on top of cell homogenate in 1.3 M sucrose. A hanging bucket ultracentrifugation was performed at 70,000 rpm for 4 hr with an SW28 rotor (Beckman). Material that migrated to the 0.8/1.2 M sucrose interface (Golgi enriched membrane) was carefully collected by a long tip pipette, and stored at -70°C. Protein concentration was determined by the Bradford method with BSA standards (Bradford, 1976).

3.3.8 Determination of the Golgi marker enzyme activity - galactosyltransferase assay

The enrichment of the membrane preparations was assessed by assay of the Golgi marker enzyme, galactosyltransferase as described by Dunphy et al (1981).

3.4 Results

3.4.1 Activation of PLD by AlF_4^- in intact SMG cells

Consistent with our previous observation (Li et al, 1998), AlF_4^-, a widely-used activator of heterotrimeric GTP-binding proteins, stimulated PLD to around four fold control levels in intact rat submandibular acinar cells. This effect was

dependent on aluminum ion concentration, with a maximal response at 3 mM NaF/2 μM $AlCl_3$ (Fig.10A). Since fluoride can etch aluminum ions from laboratory glassware to form AlF_4^- (Sternweis and Gilman, 1982), all experiments were carried out in plastic tubes and with ultrapure NaF to rule out uncontrolled aluminum contamination. NaF alone did not stimulate PLD. The stimulatory effect of AlF_4^- on PLD could be blocked by the aluminum ion chelator-deferoxamine. From our previous studies on this model, it is assumed that activation of PLD by aluminum fluoride in intact cells results from a stimulatory effect on a heterotrimeric GTP-binding protein (Chung and Fleming, 1995b; Li et al, 1998).

3.4.2 Inhibition of GTPγS -stimulated PLD by AlF_4^- in a cell-free assay system

In total submandibular lysates (membrane plus cytosol), 10 μM GTPγS stimulated PLD activity to about 5 fold basal level (Fig.10B). As in other experimental models (Bowman et al, 1993; Brown et al, 1993), AlF_4^- did not activate PLD in this cell-free system. Unexpectedly, we found that AlF_4^- potently inhibited GTPγS-stimulated PLD activity. Inhibition was prevented by the aluminum chelator, deferoxamine (Fig.10B). In concentration-effect studies, either Al^{3+}or F^- was used at a fixed dose, while the other was varied. At 5 μM $AlCl_3$, NaF inhibited GTPγS-activated PLD in a concentration-dependent response

(Fig.11A). Similarly, with NaF held at 3 mM, inhibition was dependent on Al^{3+} concentration over the range of 0-12 μM $AlCl_3$ (Fig. 11B). NaF alone had no effect on PLD activity. AlF_4^- inhibited PLD not only when it was preincubated with cell lysates before GTPγS stimulation, but also lowered enzyme activity in a time-dependent way when it was added to the assay system after GTPγS stimulation (Fig.11C). The GTP analog, GTPγS, is unhydrolysable, and not readily displaced from a G protein. Thus, the capacity of AlF_4^- to inhibit PLD after GTPγS stimulation, suggests that this effect may be unrelated to its property of activating heterotrimeric G proteins.

3.4.3 Inhibition of GTPγS-dependent PLD by AlF_4^- in permeabilized cells

The inhibition of PLD by aluminum fluoride was confirmed in permeabilized cells in a concentration-response effect (Fig. 11D). GTPγS enhanced PLD to approximately 5.5 fold basal levels. An optimal combination of 20 μM $AlCl_3$/10 mM NaF inhibited GTPγS-induced PLD activity by 80%. In this assay system, the PLD substrates are prelabelled endogenous membrane phospholipids, suggesting that the AlF_4^- inhibition of PLD observed in the cell-free assay was not an artifact related to the use of exogenous phospholipid vesicles as substrate.

3.4.4 Investigation of potential mechanism of AlF_4^- inhibition of PLD

The effects of known aluminum fluoride-sensitive pathways on PLD activation were investigated. Stimulation of the heterotrimeric Gs protein, leading to cAMP elevation is one of the classic effects of AlF_4^- (Sternweis and Gilman, 1982), and the elevation of intracellular cAMP has been reported to have an inhibitory effect on fMetLeuPhe-mediated PLD activation in neutrophils (Tyagi et al, 1991). We previously showed cross-talk regulation between the cAMP and phospholipid signaling pathways in submandibular cells (Fleming et al, 1992). Therefore, the possibility of cAMP regulation of PLD in the submandibular model exists. However, in the present study, three activators of the cAMP pathway, cholera toxin, forskolin, and *db* cAMP failed to show any significant effects on either basal or GTPγS-stimulated PLD activity (Table 1).

Fluoride has been reported to inhibit different classes of protein phosphatases (Beg et al, 1978). The alteration of phosphorylation status of regulatory proteins or of PLD itself may potentially affect PLD activation. Okadaic acid is a known inhibitor of protein phosphatases 1, 2A and 2B (Hardie et al, 1991). In our experiments, at the concentration range of 0.1 nM - 10 μM, okadaic had no effect on either basal or GTPγS-stimulated PLD activity (results not shown).

Fluoride is also known as an inhibitor of phosphatidate phosphohydrolase (PAP) (Hosaka et al, 1975), an enzyme that converts the PC hydrolysis product of PLD action, phosphatidic acid (PA), to diacylglycerol.

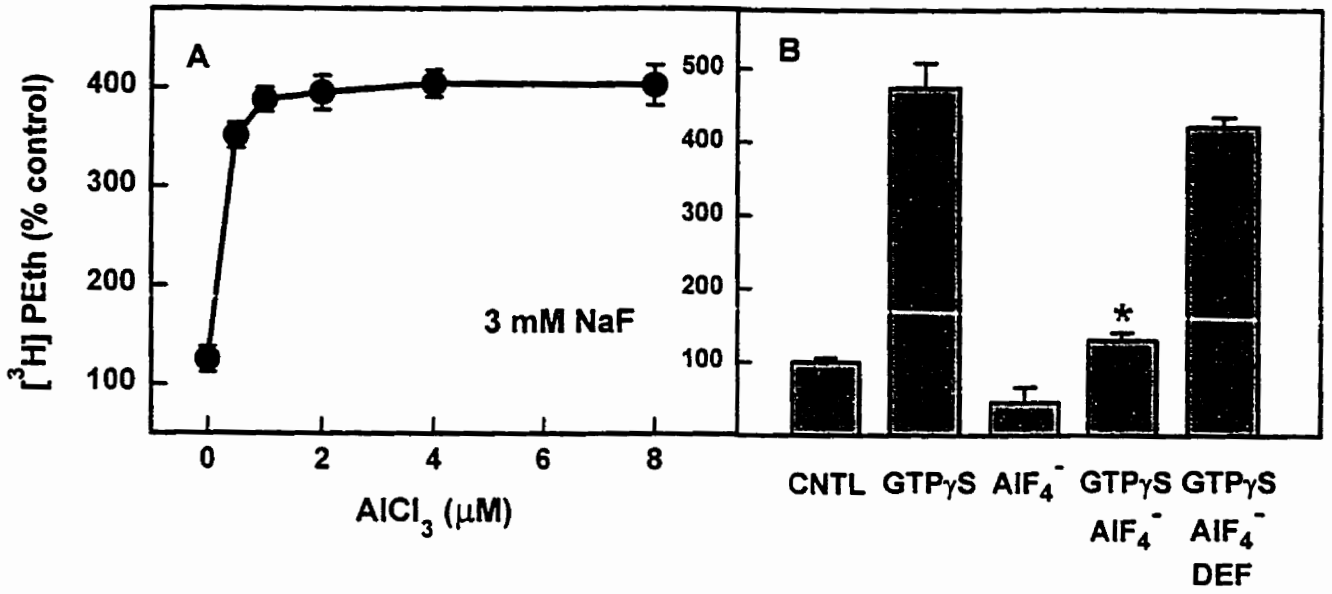


Fig 10. Aluminum fluoride activates PLD in intact cells but inhibits PLD in cell extracts. (A) Aluminum-dependence of AlF_4^- stimulation of PLD in intact submandibular acinar cells. Cells were radiolabeled with [^{3}H] arachidonic acid then treated with 3 mM NaF plus the indicated concentrations of $AlCl_3$ in the presence of 1% ethanol as substrate for the transphosphatidylation reaction. PLD activity was assayed as described in Methods. Values are means ± S.D., n = 3. **(B) Inhibition of GTPγS-stimulated PLD activity by AlF_4^- in submandibular cell extracts.** Cell extracts (54 μg membrane protein plus 30 μg cytosol protein) were preincubated with or without AlF_4^- (3 mM NaF, 20 μM $AlCl_3$) for 5 min before the addition of 10 μM GTPγS. In one group, 100 μM deferoxamine (DEF) was preincubated with cell extracts for 5 min before the addition of AlF_4^-. Values are means ± S.D., n = 3. The average GTPγS response represents the formation of 17.4 pmol PEth in 30 min. * = $p<0.01$ compared with the response to GTPγS. Control (100%) received no treatment.

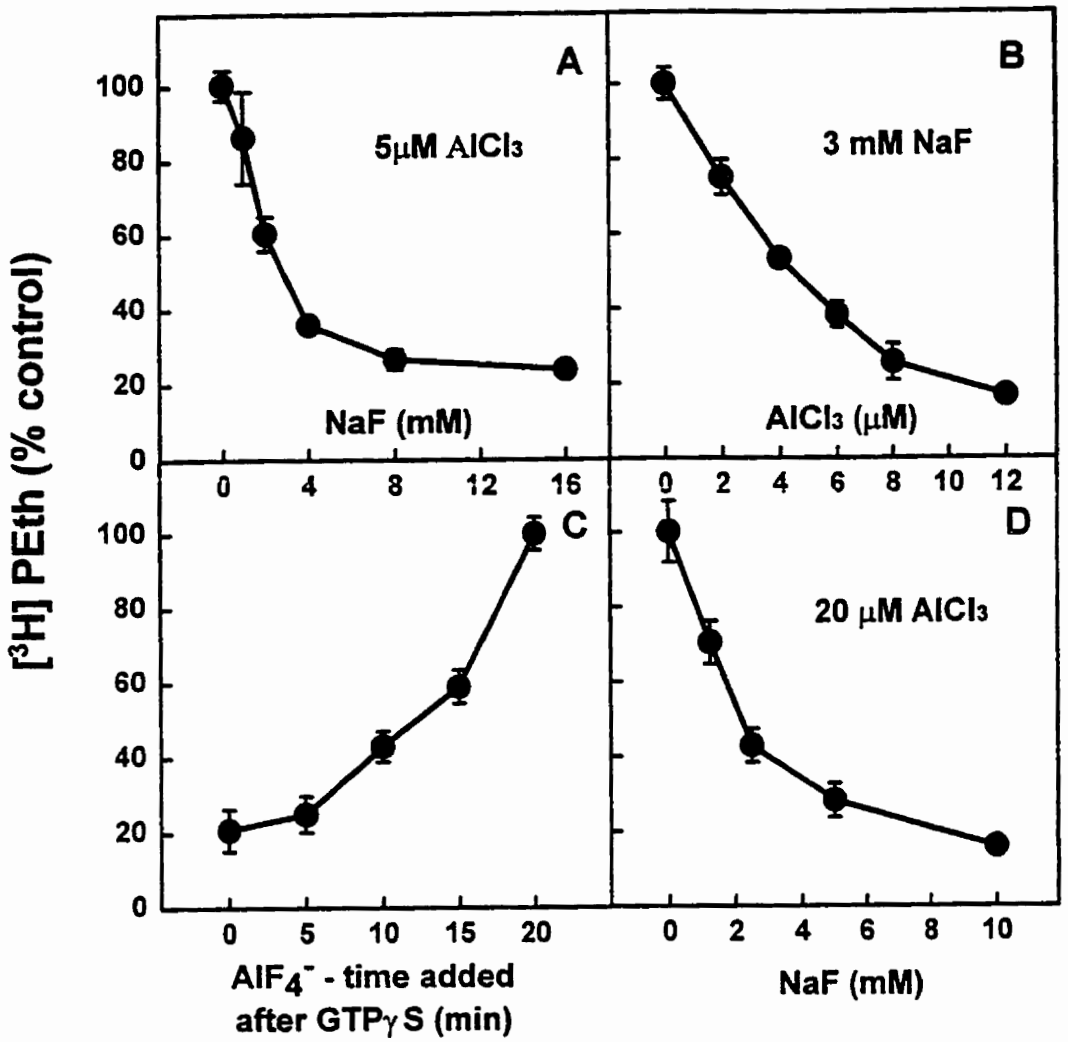


Fig 11. Concentration/time dependence of AlF_4^- inhibition of GTPγS - stimulated PLD activity in cell extracts or permeabilized cells. **(A)** Submandibular cell extracts were preincubated with the indicated concentrations of NaF together with a fixed concentration of 5 μM $AlCl_3$. **(B)** Cell extracts were preincubated with indicated concentrations of $AlCl_3$ with a fixed concentration of 3 mM NaF. **(C).** Time-response of AlF_4^- inhibition of GTPγS-stimulated PLD activity. AlF_4^- (3 mM NaF + 20 μM $AlCl_3$) was added to the cell-free assay system at different time points during the 20 min reaction time course after the reaction had been started by 10 μM GTPγS. **(D).** AlF_4^- effect on GTPγS (100 μM)-stimulated PLD activity in permeabilized submandibular cells. Cells prelabelled with [^{3}H] arachidonic acid were permeabilized by 50 μg/ml digitonin with different concentrations of NaF and 20 μM $AlCl_3$. Values are means ± S.D., n = 3.

	Cholera toxin(2μg/ml)	Forskolin (10^{-4}M)	*db* cAMP (5×10^{-4}M)
Control	665 ± 12	773 ± 55	724 ± 21
Agent alone	602 ± 7	892 ± 61	985 ± 59
GTPγS (10^{-4}M)	2327 ± 163	4659 ± 83	3246 ± 97
GTPγS+Agent	2580 ± 111	4862 ± 67	3817 ± 62

Table 1. Effects of cholera toxin, foskolin and *db* cAMP on basal and GTPγS stimulated PLD activity in permeabilized SMG cells. Rat submandibular cells radiolabelled with [^{3}H] arachidonic acid were pretreated with activators of the cAMP pathway - cholera toxin, forskolin or *db*cAMP for 10 min. Then these cells were stimulated with or without 100 μM GTPγS together with 50 μg/ml digitonin to permeabilize cells. PLD activity was measured as d.p.m. of radioactive phosphatidylethanol formed in the presence of 1% ethanol by transphosphatidylation reaction. Values are means ± SD, n=3.

We previously demonstrated that the PAP inhibitor, propranolol (up to 100 μM), lowered DAG and elevated PA in submandibular cells after carbachol stimulation of PLD, raising the possibility of product inhibitory feedback of the enzyme by elevated PA (Chung and Fleming, 1995b). In the present study, however, it was found that inhibition of PAP by propranolol had no effect on PLD activity. More directly, we found that phosphatidic acid itself at the concentration range of 1-100μM had no effect on PLD activation by GTPγS (results not shown). The above results suggest that the AlF_4^- inhibitory effect was independent of its possible action on PAP.

In a previous study, we showed that Arf/GTPγS stimulation of PLD involved the translocation of Arf from cytosol to membranes (Li et al, 1998). In the present study, rArf-stimulation of PLD in submandibular membranes was also inhibited by AlF_4^- (Fig.12A) However, Western blotting experiments with an anti-Arf antibody showed that AlF_4^- treatment did not prevent GTPγS-induced Arf translocation from cytosol to membranes (Fig.12B), suggesting a post-translocation site of inhibitory action.

3.4.5 Effect of AlF_4^- on oleate-sensitive PLD

Oleate-sensitive PLD, which is not guanine nucleotide-dependent, was also detected in submandibular membranes. Stimulation with oleate in membrane preparations elevated PLD to approximately six fold control values (Fig.13).

Again, AlF_4^- inhibited PLD in a concentration-dependent way (Fig.13). This effect in a GTP-binding protein-independent pathway supports the proposal that GTP-binding proteins are not involved in the aluminum fluoride inhibition of PLD.

3.4.6. Effect of AlF_4^- on PLD in Golgi-enriched membranes

Golgi-enriched membranes, which are essentially devoid of contaminating endoplasmic reticulum, endosomes and multivesicular vesicles (Ktistakis et al, 1996), have been used in several cell types to study PLD activation and function (Kitistakis et al, 1995, 1996). In the present study, enrichment of the preparations from submandibular cells was confirmed by assay for galactosyltransferase, a Golgi enzyme marker. The specific enzyme activity in extracts of the 0.8/1.2 M sucrose interface was 19 fold that of total cell membranes.

Golgi enriched membranes from submandibular cells had a high basal PLD activity, which was approximately ten fold the value of that for total cell membranes (Fig.14). Treatment with GTPγS increased basal levels by a factor of 2.4. AlF_4^- potently inhibited Golgi PLD in both basal- and guanine nucleotide-stimulated preparations to almost negligible activities (Fig.14).

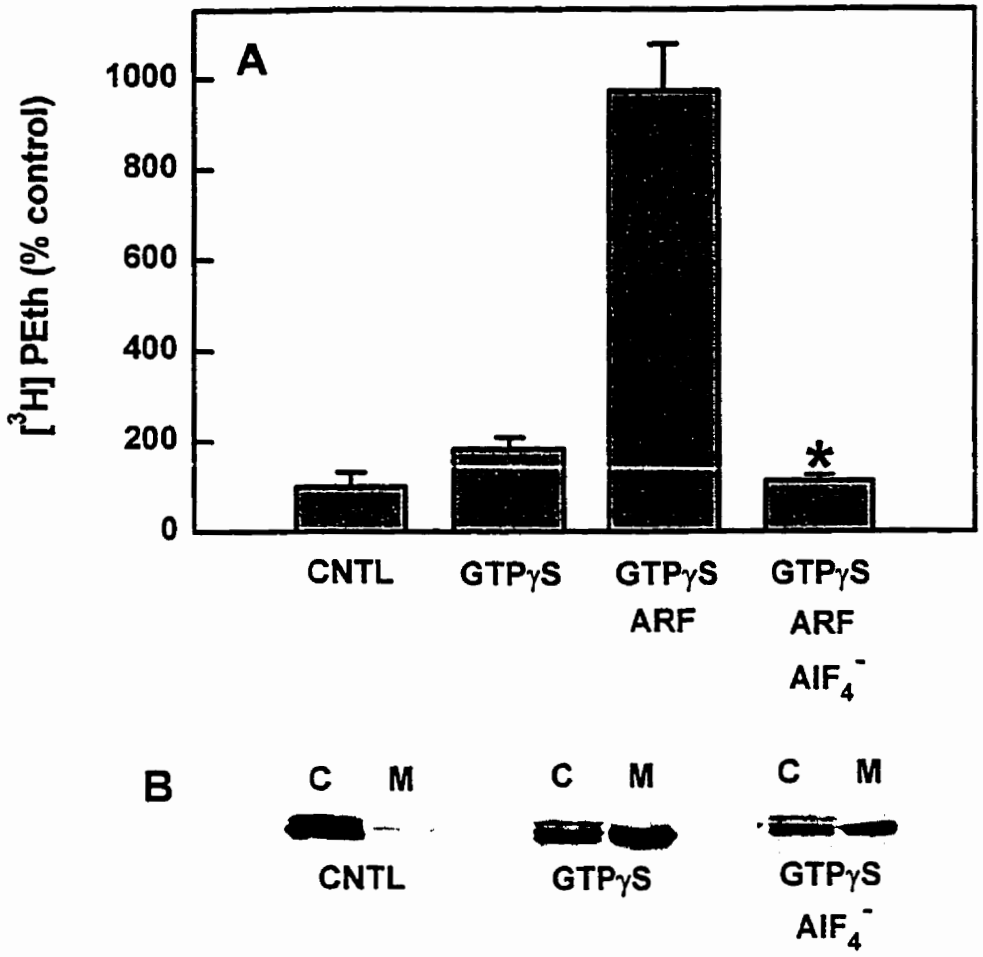


Fig 12. AlF_4^- effect on rArf-stimulated PLD activity and Arf translocation.

(A) [^{3}H] arachidonic acid-labeled cells were permeabilized with 50 μg/ml digitonin, followed by several washings to deplete the cytosolic components. Cytosol-depleted cells were preincubated with or without AlF_4^- (10 mM NaF + 50 μM) for 5 min, then 10 μM GTPγS plus or minus 1.8 μM recombinant myristoylated Arf were added for a 20 min assay period. Values are means of duplicate assays in a representative experiment, bars show the variations from the mean. The Arf response represents the formation of 35 pmol PEth. *= $p<0.01$ compared with the response to Arf. **(B)** Lack of effect of aluminum fluoride on the translocation of Arf from cytosol to membranes. Cells were permeabilized by 50 μg/ml digitonin in the presence or absence of AlF_4^- (10 mM NaF + 20 μM $AlCl_3$) and 10 μM GTPγS for 20 min.. Proteins in the membrane and cytosol fractions were separated by SDS-PAGE and Western blotting was performed with anti-Arf polyclonal antibody as described in Methods.

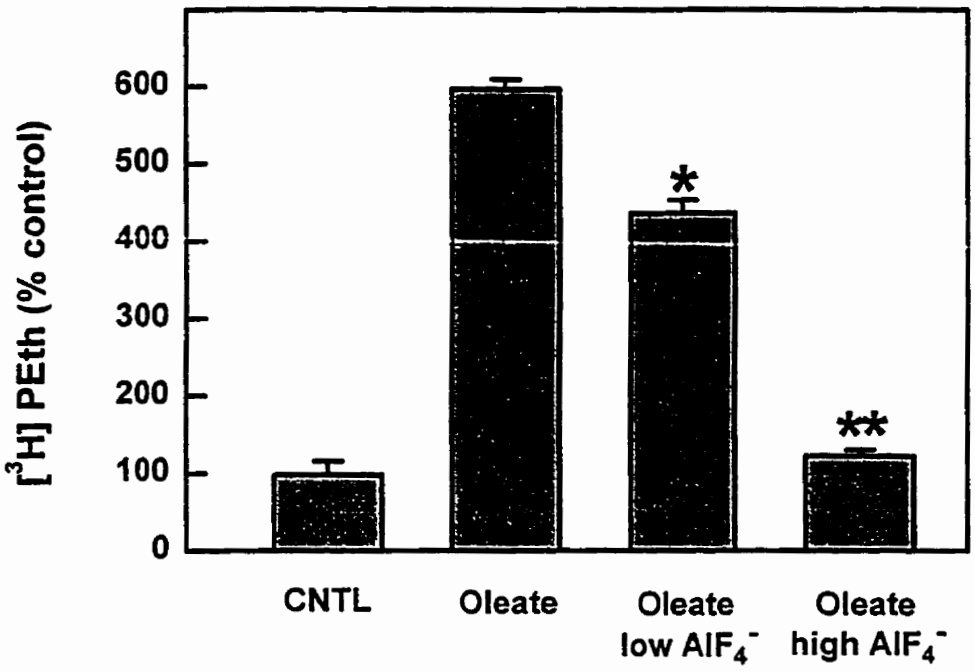


Fig 13. AlF_4^- effect on oleate-sensitive PLD in SMG membrane. Cell membranes (60 μg protein) were preincubated with or without AlF_4^- (10 mM NaF plus 2* or 20** μM $AlCl_3$) for 5 min. PLD was activated by 4 mM sodium oleate as described in the Methods. PEth was measured as before. Values are means ± S.D., n = 3. The average activity without inhibitor represents 16.1 pmol PEth formation. *= $p<0.05$, ** =$p<0.01$ compared with oleate-sensitive PLD activity. All preparations contained 1% ethanol. Control (CNTL) received no treatment.

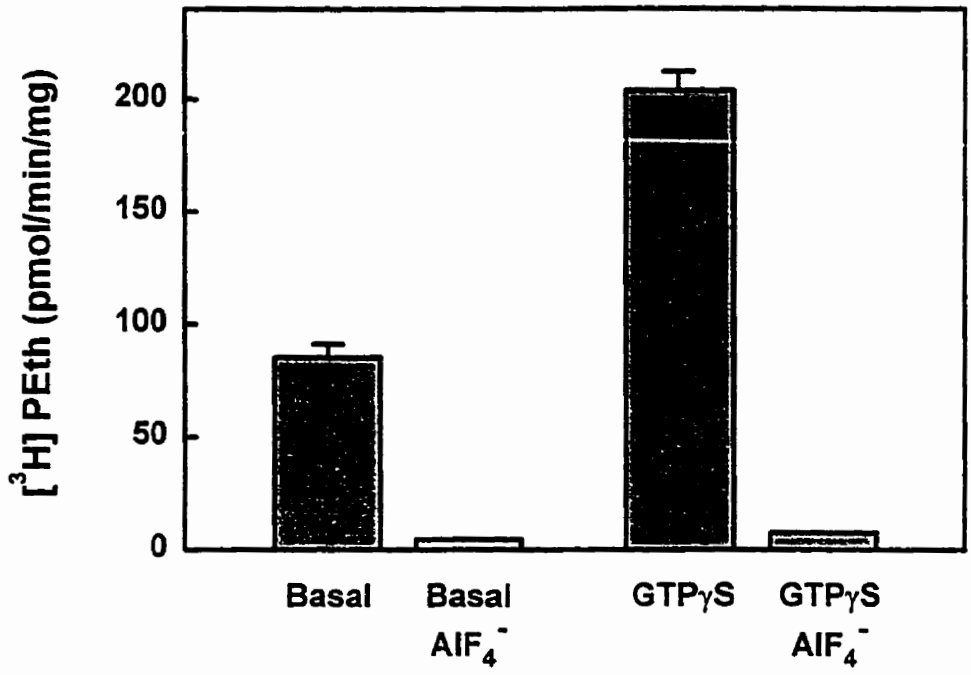


Fig 14. Effect of AlF_4^- on basal and GTPγS-stimulated PLD in a Golgi membrane-enriched preparation from rat submandibular cells. Membranes (5 μg/assay) were incubated with or without AlF_4^- (10 mM NaF + 20 μM $AlCl_3$) for 5 min then in the presence or absence of 10 μM GTPγS for 20 min. PLD was assayed as before. Values are means ± S.D., n = 3.

3.5 Discussion

Aluminum fluoride is a well-known activator of heterotrimeric GTP-binding proteins in receptor-coupled signalling pathways. Fluoride alone is often found to have the same effect, attributed to its capacity to etch aluminum from laboratory glassware to form the active AlF_4^- species (Sternweis and Gilman, 1982). In intact cells, including neutrophils (English et al, 1991; Kanaho et al, 1991), Cos-7 cells (Plonk et al, 1998) and submandibular acinar cells (Li et al, 1998; present study), fluoride or AlF_4^- activates PLD, presumably via a heterotrimeric GTP-binding protein. Recent evidence suggests that the heterotrimeric GTP-binding protein, G_{13}, couples to PLD (Plonk et al, 1998). We previously showed that in submandibular acinar cells, a heterotrimeric G protein and the small GTPase, Arf, may be sequentially involved in muscarinic receptor-coupled PLD activation in the model (see chapter 3 above; Li et al, 1998).

In spite of its widely-found stimulatory effect in intact cells, aluminum fluoride is unable to activate PLD in several types of cell extracts or in assays with purified components (Brown et al, 1993; Bowman et al, 1993; Exton J., personal communication). However, AlF_4^- does activate another phospholipase, PLC, in intact cells, permeabilized cells and cell extracts (Fain, 1990; Smrcka et al, 1991), by its action on the PLC-coupled Gq regulatory protein. Thus, the inhibitory effect on PLD found in the present study is not universal for phospholipases and is not the result of some difference between AlF_4^- effects in cell-free versus intact cell

conditions. Our findings also confirmed that AlF_4^- does not inhibit the enzyme via a Gs-coupled cAMP elevation in a crosstalk regulatory effect.

Several lines of evidence also suggest that the AlF_4^- inhibitory effect is not dependent on its well known activation of heterotrimeric GTP-binding proteins. Guanine nucleotide-sensitive PLD could be inhibited by AlF_4^- added after GTPγS, a non-hydrolysable GTP analog which binds GTP-binding proteins with a negligible off-rate (Coleman et al, 1994), and is not, therefore easily replaced by GDP-AlF_4^-. In addition, oleate-sensitive PLD, which is not regulated by guanine nucleotide binding proteins (Massenburg et al, 1994; Lee et al, 1998) was also inhibited by AlF_4^-. Thus there may be a common regulatory mechanism for different forms of PLD. Furthermore, AlF_4^- inhibited rArf-stimulated PLD activity but did not affect the translocation of Arf from the cytosol to membranes. This indicates that the site of action of AlF_4^- in PLD inhibition is located at a post Arf translocation locus. Since AlF_4^- inhibited PLD in the membrane fraction alone, a non GTP-binding membrane-associated target is proposed. This could be a PLD regulatory protein, or the enzyme itself.

PLD has been proposed to regulate several stages of intracellular vesicle transport (Kahn et al, 1993; Ktistakis, 1998). The enzyme mediates the formation of COPI-coated vesicles from Golgi-enriched membranes (Ktistakis et al, 1996) and stimulates the release of nascent secretory vesicles from the trans-Golgi network (Chen et al, 1997). Abundant PLD activity has recently been identified in Golgi-enriched membranes from several cell lines (Ktistakis et al, 1995). It is well

known that intra-Golgi transport in a cell-free systems is inhibited by AlF_4^- (Rothman, 1994; Melancon et al, 1987; Taylor et al, 1992b). A recent study found that AlF_4^- inhibits COPI-mediated transport through an AlF_4^--sensitive factor located on the Golgi membrane (Helms et al, 1998). In the present study, we found that PLD activity was high in Golgi-enriched membranes from submandibular cells and that this activity was potently inhibited by AlF_4^- in both the basal- and GTPγS-stimulated condition. It is therefore possible that AlF_4^- blocks vesicle transport in the Golgi by inhibition of PLD. In other words, the AlF_4^--sensitive factor on Golgi-membrane which confers its inhibition on vesicle transport may be the same as the AlF_4^--sensitive target which confers its inhibition on PLD. If so, aluminum fluoride should prove a useful probe in future studies on the role and mechanism of action of PLD in intracellular vesicle transport.

In preliminary experiments with rat brain extracts and purified plant PLD, AlF_4^- also inhibited PLD activation, so that this effect may represent a universal inhibitory mechanism on different forms of the enzyme. This is supported by our findings on GTPγS/Arf-sensitive and oleate-sensitive PLD, discussed above. The mode of action of aluminum fluoride in this effect is unknown. Since plant PLD requires only a combination of enzyme and simple PC substrate to cause activation (Wang et al, 1993), the enzyme itself may be the target for AlF_4^- action. Kinetic studies on purified phospholipase D may clarify this.

Though it is well-documented that AlF_4^- activates PLD in intact cells, we believe that this is the first report of its inhibition of the enzyme in cell extracts. This

difference most likely reflects the fact that in extracts, aluminum fluoride acts on an inhibition-causing target which is shielded from it in intact cells, and which may be the enzyme itself. The use of AlF_4^- in PLD studies has potential value in expanding our knowledge of the enzyme's regulation and function.

Chapter 4. Aluminum fluoride inhibition of cabbage phospholipase D by a phosphate-mimicking mechanism

4.1 Abstract

Aluminum fluoride (AlF_4^-) inhibited phospholipase D (PLD) purified from cabbage in both PIP_2-dependent and PIP_2-independent assays, consistent with its previously observed effect on mammalian PLD. The possibility that AlF_4^- may exert this effect through its known phosphate-mimicking property was examined. Inorganic phosphate, as well as two phosphate analogs, berryllium fluoride and orthovanadate, also inhibited cabbage PLD. Enzyme kinetic studies confirmed that PLD followed Hill kinetics, characteristic for allosteric enzymes, with an apparent Hill coefficient (n_{app}) of 3.8, indicating positive cooperativity among multiple substrate binding sites, and suggesting the possible functional oligomerization of the enzyme. Aluminum fluoride modification of PLD kinetics was consistent with a competitive mode of enzyme inhibition. It is therefore proposed that aluminum fluoride, and other phosphate analogs, inhibit plant PLD by competing with a substrate phosphate group for a substrate binding site,

thereby preventing the formation of an ezyme-phosphatidyl intermediate. This may be a conserved feature of PLD superfamily enzymes.

4.2 Introduction

The studies described in chapter 3 above (Li and Fleming, 1999a) showed that aluminum fluoride inhibited both PIP_2- and oleate- sensitive forms of PLD in submandibular gland extracts. It was further confirmed that this action of AlF_4^- was not mediated by cAMP, three different classes of phosphatases or phosphatidate phosphohydrolyase. Inhibition was G protein-independent and involved AlF_4^- interaction with a membrane –associated target, which may be PLD itself.

This possibility was followed up in the present study. The rationale was to use purified PLD to examine more directly the nature of possible inhibitory interaction between AlF_4^- and the enzyme in a controlled cell-free assay system. Ideally, the purified PLD of choice would be that from rat submandibular gland, or failing that, from some other mammalian source. This would involve the application of molecular biological- or biochemical PLD purification procedures, which are not yet developed in this laboratory. It was therefore decided to use commercially available plant (cabbage) PLD in the preliminary studies here, given that plant and mammalian PLDs have similar catalytic mechanisms (Ponting and Kerr, 1996). The added benefit of such an approach would be to establish whether AlF_4^- can inhibit PLDs of different origins and to generate new information on a potentially

universal mechanism of inhibition that may apply to the PLD superfamily of enzymes.

Plant phospholipase D was first described in 1947 (Hanahan and Chaikoff, 1947). It is widespread in the plant kingdom, and partially purified enzyme, particularly from cabbage and peanut (Heller, 1978; Ryu et al, 1997; Becker et al, 1997), has been widely investigated. Plant PLD and mammalian PLD are highly homologous and contain the same putative catalytic domains. Unlike mammalian enzymes, plant PLDs are insensitive to GTP-binding proteins such as Arf and Rho, and to GTPγS stimulation (Pappan et al, 1997). Three plant PLD isoforms have recently been cloned from *Arabidopsis*. PLDα, the prevalent form of the enzyme, which has been extensively studied in plant species (Wang et al, 1993), is PIP_2-independent and shows high activity at millimolar Ca^{2+} concentrations with PC as the only substrate (Wang et al, 1994). Two additional forms, PLDβ and PLDγ, exhibit no activity under the above assay conditions. Instead they reqire PIP_2 and are optimally active at micromolar Ca^{2+} levels (Pappan et al, 1997; Qin et al, 1997). Conversely, PLDα exhibits very low activity under the PIP_2-dependent assay conditions.

PLD has recently been identified as one of a group of enzymes with diverse functions, termed the PLD superfamily (Ponting and Kerr, 1996). Other members include cardiolipin synthases (CLS), phosphatidylserine synthases (PSS), pox viral envelope proteins, a bacterial endonuclease (Nuc) and the Yersina Pestis murine toxin (Ymt) (Ponting and Kerr, 1996; Koonin et al, 1996; Zhao et al, 1997). The

hallmark of the PLD superfamily is the presence of conserved HxKxxxxD motifs in the enzyme amino acid sequence (Hammond et al, 1995; Koonin, 1996). It is believed that duplicated HKD motifs are key structural components of the active site (except in Nuc, which has a single HKD motif), and that members of the superfamily therefore share similar catalytic mechanisms (Koonin, 1996; Ponting and Kerr, 1996). Recent studies on two PLD-related enzymes, Nuc and Ymt, suggested a common catalytic action that proceeds via a phosphohistidine-linked phosphatidyl-enzyme intermediate (Gottlin et al, 1998; Rudolph et al, 1999). Studies on the enzymology of plant PLD, and its inhibition by AlF_4^- and analogs, may therefore expand our understanding of the conserved mechanisms of action and of the regulation of other superfamily members.

From the findings in this study, we propose that aluminum fluoride is a competitive inhibitor of purified cabbage PLD, with a mechanism of action based on its phosphate-mimicking property.

4.3 Materials and methods

4.3.1 Materials

Cabbage PLD purified from cabbage or peanut, deferoxiamine, neomycin, beryllium chloride and sodium orthovanadate were from Sigma-Aldrich, Oakville, ONT, Canada. Materials that are the same as those in the previous chapters are not repeated here.

4.3.2 PIP_2 - dependent PLD assay

4.3.2.1 PIP_2-dependent PLD assayed by the transphosphatidylation reaction

The same protocol was used as described in Chapter 3-3.3.4.1. The free calcium concentration was EGTA-buffered to 7.8 μM Ca^{2+}. 2.5 μg cabbage PLD was used per assay.

4.3.2.2 PIP_2 - dependent PLD activity assayed by choline release

Dipalmitoylphosphatidyl-[methyl-^{3}H] choline was used to prepare lipid vesicles as described above. The assay was carried out by the same procedure as in 3.3.4.1 in a total volume of 60 μl. The reaction was stopped by adding 360 μl chloroform/methanol (2:1, v/v). After vigorous vortexing and centrifugation at 6,000 g for 5 min, radiolabelled choline was measured in 50 μl aliquots of the aqueous phase by scintillation counting.

4.3.3 PIP_2 - independent PLD assay

PIP_2-independent PLD activity was assayed by a modified procedure of Wang et al (1993). The reaction mixture contained 100 mM MES (pH 7.0), 1 mM SDS, 1% ethanol, 2.5 μg cabbage PLD and 0.2 mM dipalmitoyl-[2-palmitoyl-9,10-^{3}H] PC with varying concentrations of $CaCl_2$ at the millimolar level. The reaction was carried out at 37°C in the total volume of 60 μl. The transphosphatidylation reaction product, phosphatidylethanol, was measured as in other PLD assays. In the experiments where phosphatidic acid was assayed to reflect PLD activity,

ethanol was omitted from the reaction system, radiolabelled PA was isolated by TLC with the same solvent system as used for PEth (R_f = 0.25) and measured by scintillation counting. To assay PLD activity with PE as substrate, PC was replaced by unlabelled PE, plus 1-palmitoyl-2-arachidonyl [arachidonyl-1-^{14}C] phosphatidylethanolamine in the assay system. The final concentration of PE was 0.4 mM. The assays were conducted at 37°C for 20 minutes in a total assay volume of 60 µl.

4.3.4 Kinetic study on cabbage PLD

The kinetic experiments were performed with the PIP_2 - independent PLD assay procedure. Two stocks of PC substrate were prepared by bath sonication. Stock one contained only [^{3}H] dipalmitoyl-PC (100,000 dpm/10 µl). Stock two contained the same concentration of [^{3}H] dipalmitoyl-PC and 1 mM cold dipalmitoyl-PC. Different concentrations of PC substrates were prepared by mixing these two stocks with brief sonication. Cabbage PLD (2.5 µg/assay) was preincubated with two concentrations of NaF plus $AlCl_3$ for 5 minutes before addition to the assay system. The reaction was carried out at 37 °C for 5 minutes. The product, phosphatidic acid (PA), was separated by TLC under the same developing solvents as those for PEth and quantitated by scintilation counting.

4.4 Results

4.4.1 Inhibition of plant PLD by AlF_4^- in a PIP_2-dependent PLD assay

Under PIP_2-dependent assay conditions, cabbage PLD exhibited a specific activity of 2.1 nmol PEth formation/min/mg. The inclusion of aluminum fluoride (a combination of 3 mM NaF and 20 μM $AlCl_3$) potently inhibited cabbage enzyme activity to 24% of the control value (Fig. 15A). Neither sodium fluoride nor aluminum chloride alone had this effect (Fig. 15A), indicating a requirement for the formation of AlF_4^- in the inhibitory response. Additional evidence for this requirement was the action of the aluminum ion chelator, deferoxamine (DEF) in reversing the aluminum fluoride inhibition of PLD (Fig.15A). DEF itself had no effect on PLD activity. The inhibitory effect of AlF_4^- was further confirmed by a choline-release PLD assay on cabbage PLD (PLD activity inhibited to 25% of control value) (Fig.15B). The effect was consistent with PLD purified from peanuts (inhibited to 16% control) (Fig.15B), indicating that inhibition by aluminum fluoride may be universal for plant PLDs of different origin.

Under the assay conditions used, cabbage PLD required micromolar levels of free calcium. Enzyme activity was abolished in the absence of added Ca^{2+} plus the presence of 1 mM EGTA. At a concentration of 300 nM free calcium in the assay buffer, PLD activity was only about 1/20 of that at 7.8 μM calcium. Cabbage PLD activity was also concentration-dependent on PIP_2, with a maximal response at about 12 μM phosphoinositide (Fig. 16A). The role of PIP_2 in PLD stimulation was examined by using neomycin, a high affinity ligand that selectively binds to

polyphosphoinositides and blocks their binding to other targets (Gabev et al, 1989). The inclusion of 12 μM PIP_2 in mixed lipid vesicles enhanced PLD activity to approximately 7 fold the level found in samples lacking PIP_2 (Fig. 16B). 1 mM neomycin inhibited PIP_2 -activated PLD to 33% of the control value, and AlF_4^- inhibited it to 9% (Fig. 16B). A combination of neomycin and AlF_4^- produced a maximal inhibitory effect (Fig. 16B)

4.4.2 Effect of different phosphate analogs on PIP_2-dependent cabbage PLD

Aluminum fluoride is a known phosphate analog. It stimulates heterotrimeric G proteins by mimicking the γ- phosphate of GTP and stabilizing the transitional state of the Gα protein (Chabre, 1990; Coleman et al, 1994). A phospho-enzyme intermediate has been proposed in the PLD substrate hydrolysis mechanism (Bruzik and Tsai, 1984). We therefore investigated the possibility that AlF_4^- inhibition of PLD may be caused by its phosphate-mimicking property. When the fluoride concentration was fixed at 3 mM, the inhibitory effect depended on the concentration of aluminum ions, with half maximal Al^{3+} about 15 μM (Fig. 17A). Similar dose-dependent curves were observed with two other known phosphate analogs, beryllium fluoride (BeF_3^-) and sodium orthovanadate (Na_3VO_4), with half maximal inhibition doses of 3 mM NaF + 50 μM $BeCl_3$ and 35 μM Na_3VO_4 respectively (Fig. 17B and 17C). Furthermore, inorganic phosphate itself, at millimolar concentration range, also inhibited cabbage PLD activity in a dose-dependent way (IC_{50} approximately 10 mM, Fig. 17D). These results suggest that

AlF_4^- may inhibit PLD by a phosphate-mimicking effect, and emphasize the central importance of a probable phosphate-related mechanism in the enzyme's action.

4.4.3 Effect of AlF_4^- and related phosphate analogs on cabbage PLD activity in a PIP_2-independent PLD assay

Under the PIP_2-independent assay conditions conventionally used for the plant enzyme, cabbage PLD was dose-dependently activated by Ca^{2+} over a 1-8 mM calcium concentration range (Fig 18A). At 8 mM Ca^{2+}, the specific activity of PLD (dipalmitoyl-PC substrate) was 23 nmol PEth formation/min/mg protein. Enzyme activity was almost completely inhibited by aluminum fluoride at all calcium concentrations (Fig. 18A). Consistent with the PIP_2-dependent assay results (above), both BeF_3^- and $Na_3VO_4^-$ also inhibited PLD activity in the PIP_2-independent assay (Fig. 18B). The effect of inorganic phosphate could not be tested under these assay conditions, since phosphate precipitates millimolar level of calcium (Clapham, 1995). In addition to its utilization of PC substrate, plant PLD is also able to hydrolyze phosphatidylethanolamine (PE) (Pappan et al, 1998). Cabbage PLD hydrolysed PE at about 40% of the rate of PC cleavage (Fig. 18B). PE hydrolysis was also inhibited by three phosphate analogs with potencies comparable to those shown with PC substrate (Fig. 18B). This suggests a consistent mode of inhibitory action on the PLD hydrolysis of both phospholipid substrates.

4.4.3 Kinetics of cabbage PLD

The kinetic behavior of cabbage PLD was studied by the PIP_2-independent assay which uses only PC in the substrate vesicles. The rate of PA formation from PC substrate by cabbage PLD was linear for approximately 20 min. Phosphatidic acid production was measured over a reaction time of 5 min to represent the initial reaction rate. The velocity *versus* substrate concentration experiments generated data that did not fit conventional Michaelis-Menten kinetics, but, instead, produced a sigmoidal curve characteristic of Hill kinetics (Fig.19). This is consistent with a recent study on cabbage PLD which first proposed Hill kinetic behaviour for this enzyme (Dittrich et al, 1998). The Hill model represents the kinetics of allosteric enzymes, in which the sequential binding of substrate molecules to multiple substrate-binding sites increases the binding affinities of the remaining vacant sites (cooperative binding) (Segel, 1975) . The Hill plot (log v/(Vmax-v) *versus* log(S)) of cabbage PLD kinetic data showed an approximately straight line (Fig. 19 inset). The calculated dissociation constant, K', was 0.65 mM, and the apparent Hill coefficient (n_{app}) was 3.8. The effects of two different concentrations of AlF_4^- on the kinetics of PLD were examined. Both concentrations of aluminum fluoride displaced the velocity/substrate concentration curves to the right in a dose-dependent way. Inhibitory potency of AlF_4^- decreased with increasing PC substrate concentration, suggesting a competitive mode of inhibition under Hill kinetic behaviour.

4.5 Discussion

The work described in Chapter 3 (above) demonstrated that both guanine nucleotide- and oleate-sensitive PLD activities in salivary gland cells were inhibited by aluminium fluoride in a GTP-binding protein independent manner. The present study sought to determine whether the inhibition of mammalian PLD by aluminum fluoride (Li and Fleming, 1999a) was consistent for plant PLD, and, if so, to examine possible mechanisms of inhibition.

Our study found that cabbage PLD exhibited high activities in both the PIP_2-dependent and- independent assay systems, indicating the existence of multiple isoforms. The inhibitory effect of AlF_4^- on cabbage PLD was first identified in the PIP_2-dependent assay system, which reflects PLDβ and/or PLDγ activities. A study found that both isoforms were bound to PIP_2, and their activities rely on PIP_2 (Qin et al, 1997). The stimulation by polyphosphoinositide is a property conserved by plant, yeast and mammalian PLDs (Frohman and Morris, 1999). A putative PIP_2-binding sequence rich in basic amino acids has been found in PLDβ and PLDγ (Qin et al, 1997). Recent studies on mammlian PLDs have revealed the existence of putative PIP_2-binding PH domains (Holbrook et al, 1999). Although the exact mechanism is not clear, PIP_2-binding to the enzyme most likely

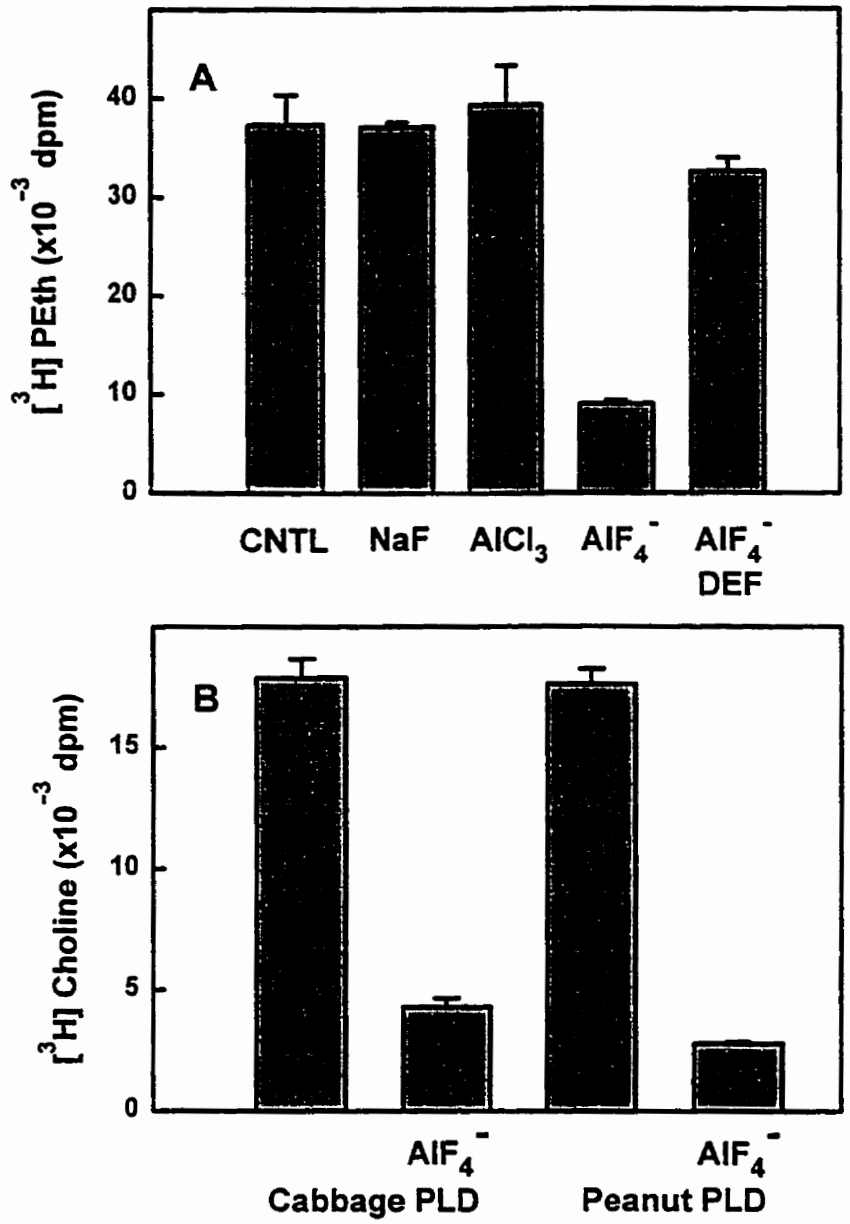


Fig 15. AlF_4^- inhibition of cabbage PLD activity in a PIP_2-dependent assay.

(A) Cabbage PLD (2.5 μg/sample) was assayed by the transphosphatidylation reaction in 1% ethanol over 20 min. The enzyme was preincubated with 3 mM sodium fluoride (NaF), 20 μM aluminum chloride ($AlCl_3$), or 3 mM NaF + 20 μM $AlCl_3$ (AlF_4^-), in the presence or absence of 100 μM deferoxamine (DEF) for 5 min. Controls were untreated.

(B) Cabbage or peanut PLD (2.5 μg/sample) was assayed by the radiolabelled choline-release with or without AlF_4^- (3 mM NaF + 20 μM $AlCl_3$) preincubation for 5 min. The assays were performed as described in Materials and methods. Values are means ± S.D., n = 3.

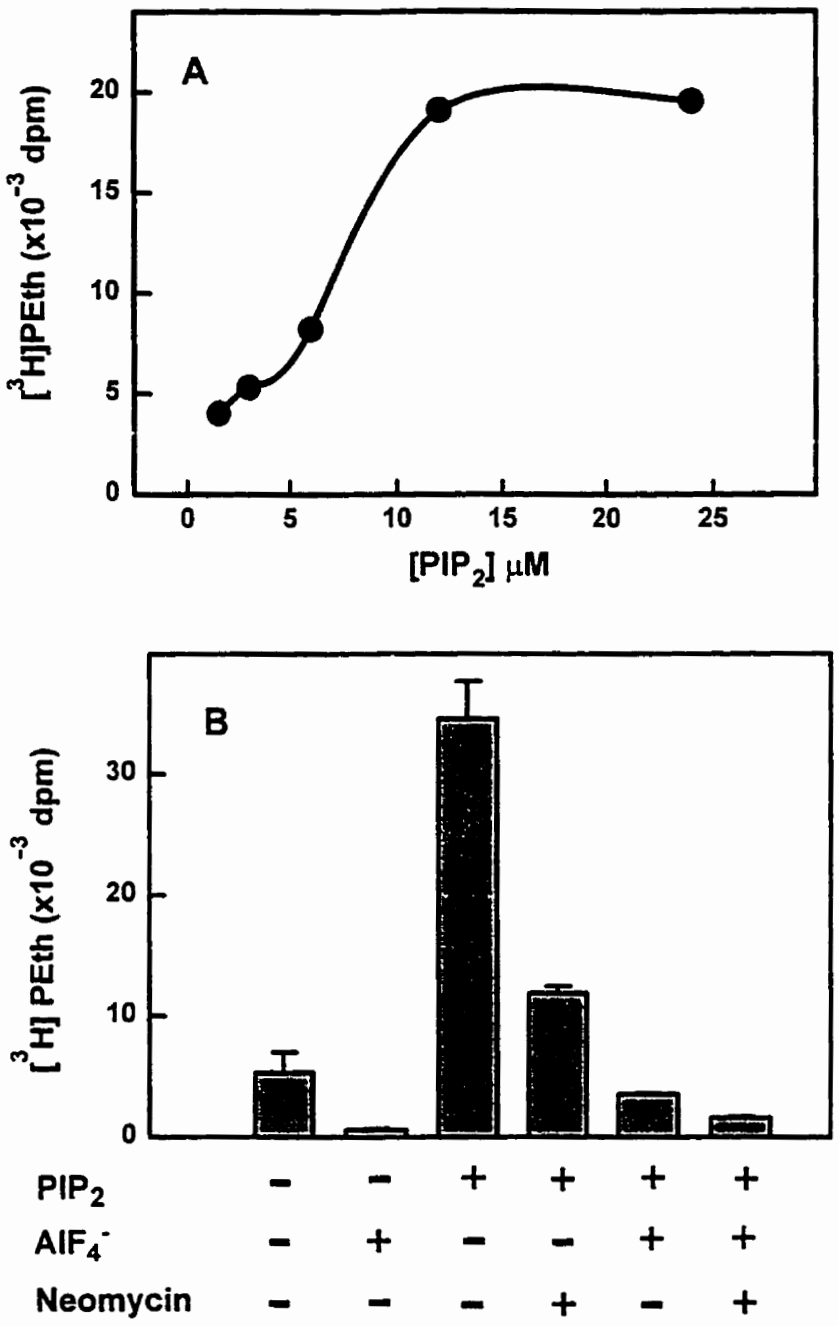


Fig 16. PIP_2-dependence of cabbage PLD. (A) Cabbage PLD activity was assayed in mixed lipid vesicles (PE/PIP_2/PC) with PIP_2 concentration from 1.5 μM to 24 μM. **(B)** Cabbage PLD activity was assayed in the presence or absence of 12 μM PIP_2. The enzyme was pretreated for 5 min with or without neomycin (1 mM) or AlF_4^- (3 mM NaF + 20 μM $AlCl_3$), or neomycin + AlF_4^-. Enzyme activity was measured by the transphosphatidylation reaction in 1% ethanol for 20 min. Values are means ± SD, n = 3.

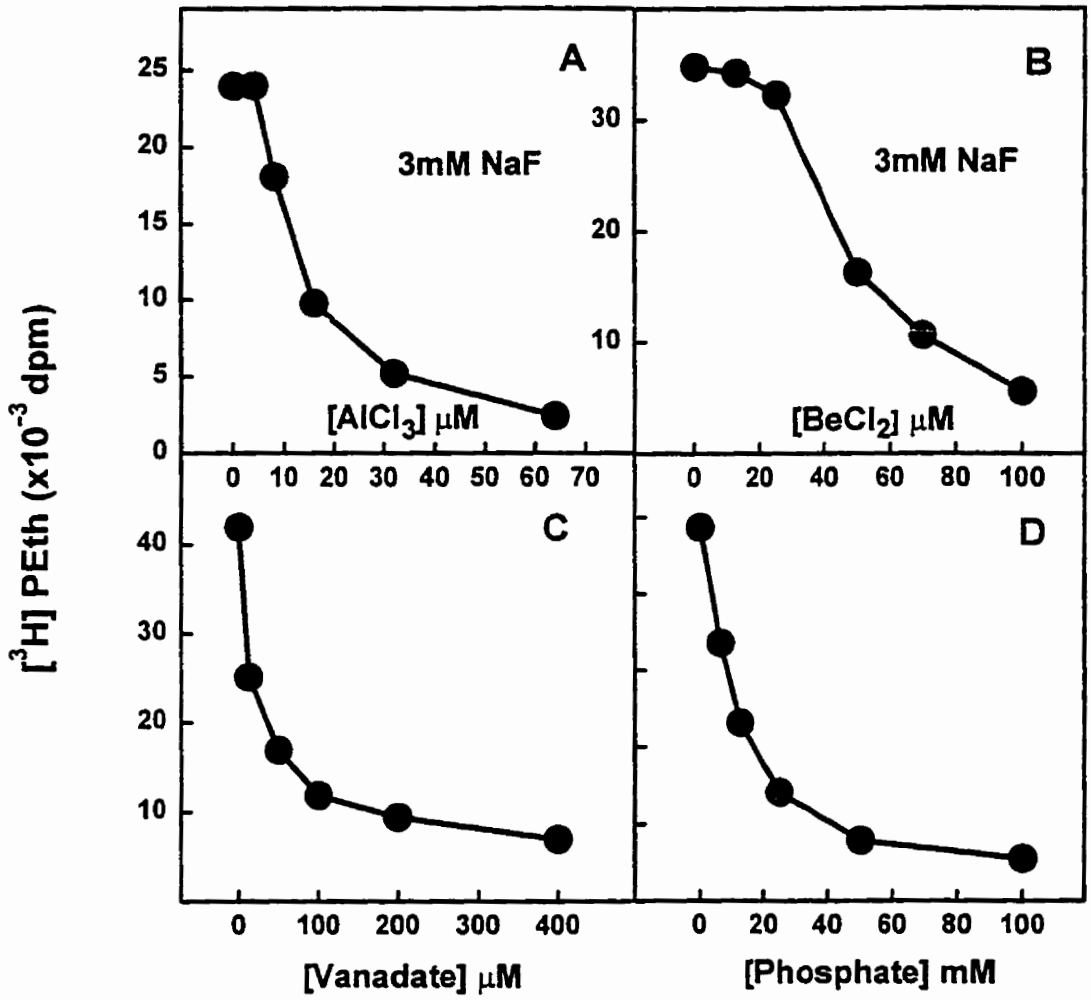


Fig 17. Concentration-dependent inhibition of PIP_2-dependent cabbage PLD activity by phosphate and phosphate analogs. Cabbage PLD (2.5 μg/sample) was pretreated for 5 min with: **(A)** aluminum fluoride (3 mM NaF + 0-64 μM $AlCl_3$). **(B)** beryllium fluoride (3 mM NaF + 100 μM $BeCl_2$). **(C)** sodium orthovanadate (0-400 μM). **(D)** inorganic phosphate (0-100 mM). Values are means from a representative experiment with variations of less than 12%, n = 3.

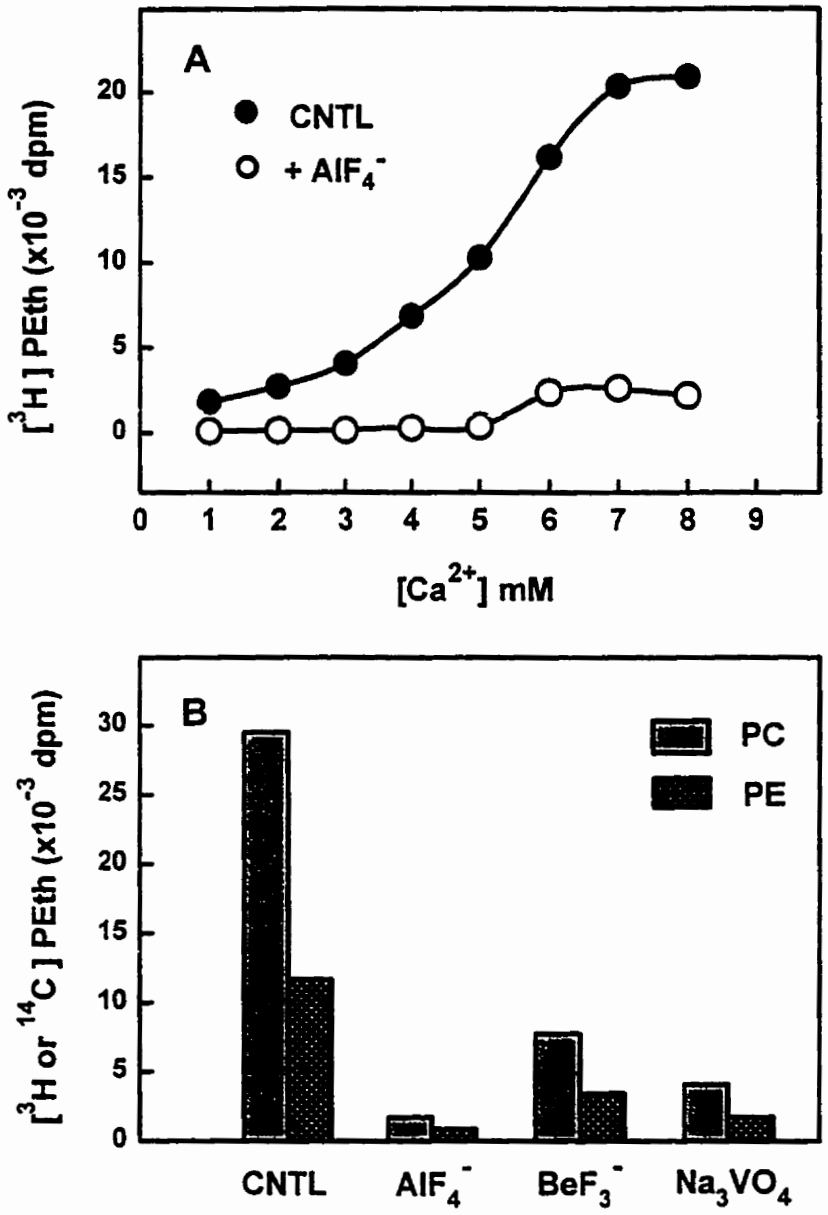


Fig 18. Inhibition of PIP_2-independent cabbage PLD activity by AlF_4^- and other phosphate analogs. **(A)** PLD activity (20 min) over a range of 1-8 mM Ca^{2+} concentrations. Cabbage PLD (2.5 μg/sample) was preincubated with or without AlF_4^- (3 mM NaF + 20 μM $AlCl_3$) for 5 min. **(B)** PLD activity was assayed with phsophatidylcholine (PC) or phosphatidylethanolamine (PE) substrates. The enzyme was preincubated for 5 min without (control) or with AlF_4^- (3 mM NaF + 20 μM $AlCl_3$), BeF_3^- (3 mM NaF + 20 μM $BeCl_2$) or Na_3VO_4 (200 μM). Values are means from a representative experiment with variations less than 10%, n = 3.

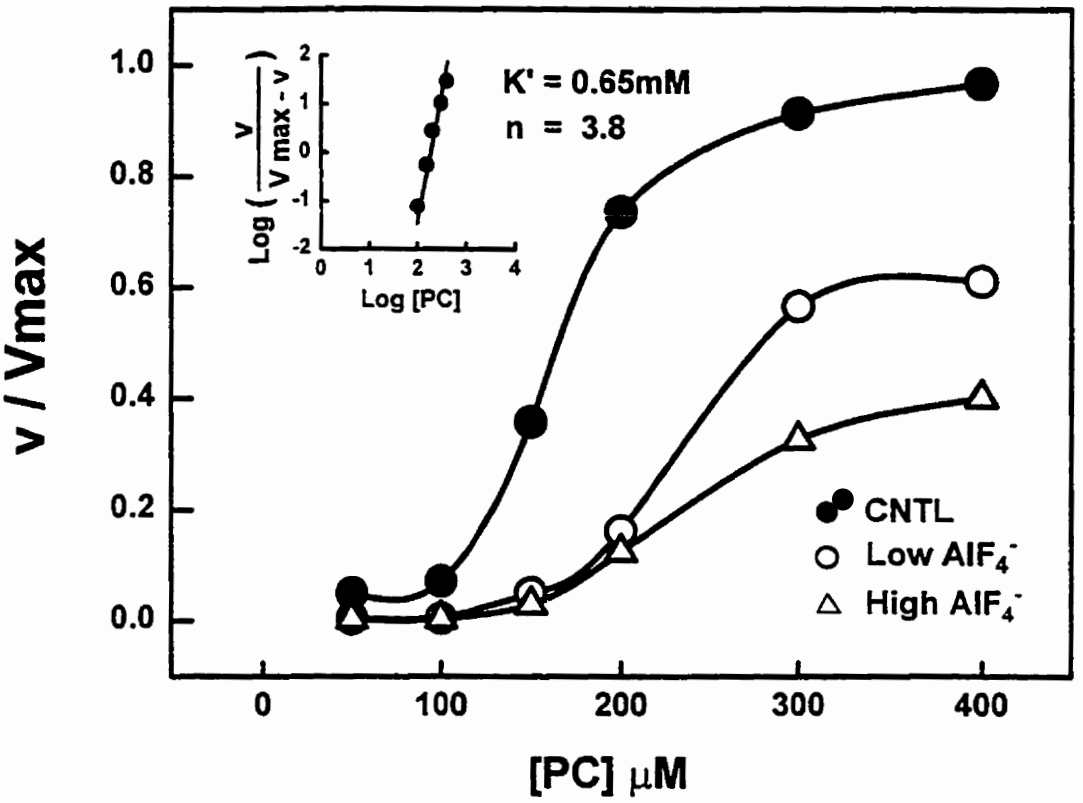


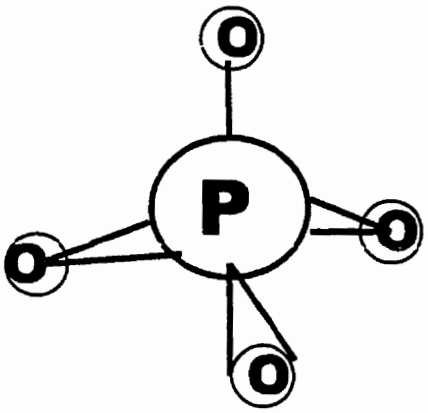
Fig 19. Effect of AlF_4^- on the kinetics of cabbage PLD (PIP_2-independent assay). Two concentrations of AlF_4^- (1 mM NaF + 5 μM $AlCl_3$ or 3 mM NaF + 20 μM $AlCl_3$) were preincubated with the enzyme for 5 min. The assay was conducted for 5 min at different PC concentrations, and phosphatidic acid was measured to reflect PLD activity. **Inset:** The Hill plot from the logarithmic form of the Hill equation.

facilitates the enzyme's association with substrates (Frohman and Morris, 1999) . Several lines of evidence indicate that the observed AlF_4^- inhibition of PLD does not occur through its interaction with PIP_2 or with PIP_2 binding to the enzyme. First, under the present PIP_2 assay conditions, significant levels of PLD activity could be detected in lipid vesicles containing no PIP_2 (about 1/8 of that with optimal PIP_2), and this activity was similarly inhibited by AlF_4^-. Second, the inhibitory effect of AlF_4^- was more potent than that of neomycin, a high affinity ligand of PIP_2 that impairs PIP_2 function. A combination of neomycin and AlF_4^- produced a maximal effect of inhibition. Finally, cabbage PLD assayed in PIP_2-independent conditions with only PC or PE substrates (which reflects PLDα activity) were similarly inhibited by AlF_4^-. Therefore, disruption of polyphosphoinositide binding to PLD, a potential regulatory mechanism (Qin et al, 1997), may be discounted as the common AlF_4^- mode of action.

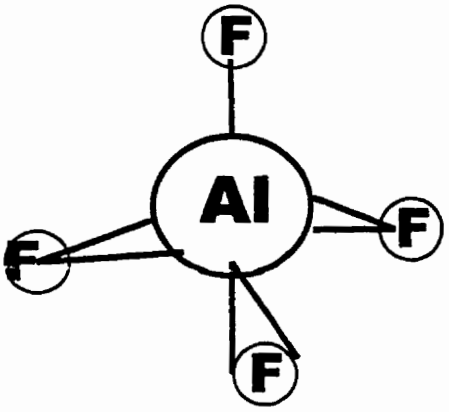
The possibility that AlF_4^- may act through a calcium-related mechanism to inhibit PLD was considered. Plant PLDs contain a Ca^{2+}/phospholipid-binding C_2 domain (Ponting and Parker, 1996) and enzyme activation requires calcium. Cabbage PLD was sensitive to micromolar levels of Ca^{2+} in the PIP_2-dependent assay and to millimolar Ca^{2+} in the PIP_2-independent assay. AlF_4^- inhibited PLD in both assay systems. However, it also inhibits mammalian PLD, which lacks the calcium-binding C_2 domain, as well as mammalian oleate-sensitive PLD, assayed in the absence of free Ca^{2+} (Li and Fleming, 1999a; Chapter 3 above). It is probable, therefore, that AlF_4^- inhibits PLD by a mechanism that does not affect

Ca^{2+} binding to the enzyme. Also compatible with our previous observations on salivary gland PLD (Li and Fleming, 1999a), a mechanism that involves the well-documented property of aluminum fluoride to activate heterotrimeric GTP-binding proteins (Bigay et al, 1987) is ruled out, since plant PLDs are insensitive to guanine nucleotide stimulation (Pappan et al, 1997).

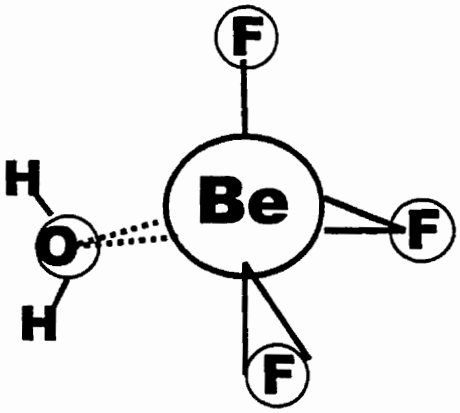
Aluminum fluoride adopts a tetrahedral chemical structure. The Al-F bond length and bond angle closely resemble those of the P-O bond in phosphate. Therefore, AlF_4^- is known as a structural analog of phosphate. So are beryllium fluoride and orthovanadate. The structures of the phosphate analogs used in this study are showed in Diagram 3. Many of the effects of AlF_4^-, including the activation of G proteins, are exerted through its capacity to mimic a phosphate group (Bigay et al, 1987), and aluminum fluoride has been reported to affect enzymes with phosphotransfer activities (GTPase, ATPase, phosphatase) (Chabre, 1990). AlF_4^- may therefore inhibit PLD by acting as a phosphate analog. Supportive evidence for this is provided by our observation that two additional phosphate analogs, beryllium fluoride and orthovanadate, inhibited both PIP_2-sensitive and -insensitive forms of PLD in concentration dependent responses. Inorganic phosphate itself also inhibited PLD in the PIP_2-dependent assay (this could not be tested in the PIP_2-independent assay, where millimolar Ca^{2+} is precipitated by inorganic phosphate). The differences among the concentrations of phosphate (millimolar) and the other three analogs (micromolar) required to inhibit PLD may simply reflect the relative binding affinities of these compounds



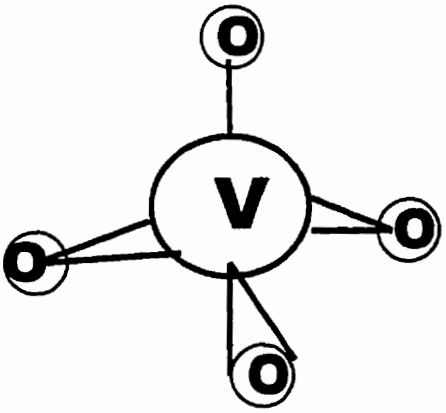
Phosphate



Aluminum fluoride



Beryllium fluoride



Orthovanadate

Diagram 3. The tetrahedral structure of phosphate and its analogs Diagramatic representation of the structure of aluminum fluoride, beryllium fluoride and orthovanadate to show their similarity to the structure of a phosphate group.

to the enzyme (Segel, 1975). The three phosphate analogs also inhibited cabbage PLD conversion of PE substrate, suggesting a possible universal, phosphate-mimicking mechanism of inhibition of different forms of PLD.

PLD has been recognized as a member of the PLD superfamily which is characterized by the conserved HKD domains in the active site (Koonin, 1996). Enzymes in this superfamily may therefore be expected to share a common catalytic mechanism (Rudolph et al, 1999; Gottlin et al, 1998). A sterochemical study on cabbage PLD more than a decade ago indicated that enzyme catalysis may proceed with a mechanism that involves the formation of a phosphatidyl-enzyme intermediate (Bruzik and Tsai, 1984; Sung et al, 1997). A previous study on cabbage PLD also found the inhibition of enzyme acitivity by two histidine-modifying reagents, diethylpyrocarbonate (DEPC) and 4-bromophenacyl bromide (p-BPB) (Lee et al, 1989). Recent studies on two superfamily members, a bacterial endonuclease Nuc and the Yersinia Pestis murine toxin Ymt, have demonstrated that enzyme action of both members proceeds via a two-step mechanism involving the formation and breakdown of a phosphoenzyme intermediate that appears to be phosphohistidine-linked (Rudolph et al, 1999; Gottlin et al, 1998). This information suggests that a similar two-step mechanism with a comparable phosphatidyl-enzyme configuration is most likely employed by cabbage PLD. The only phosphate group in phospholipid PLD substrates, therefore the one that binds to PLD in the reaction, is that linking the phosphatidyl group to the polar head group (e.g. choline, ethanolamine). It is therefore likely that enzyme inhibition by

aluminum fluoride, or the other phosphate analogs examined, is caused by their binding to the phosphate-binding site of the catalytic domain of PLD (possibly to a histidine residue) to prevent the formation of a phosphatidyl-enzyme intermediate.

This model is supported by the competitive mode of inhibition of PLD by AlF_4^- observed in the enzyme kinetics experiments in the present study. It is also consistent with the recent observation that another phosphate analog, tungstate (WO_4^-), which is a competitive inhibitor of the PLD superfamily member Nuc (Gottlin et al, 1998), binds Nuc at one histidine and one lysine residue on each of the two conserved HKD motifs on the catalytic site (Stuckey and Dixon, 1999).

PLD contains two copies of the substrate-binding HKD motif. Both motifs are absolutely required for enzyme activity, and HKD domains from the N- and C-terminals associate to form a single active site (Xie et al, 1998). Functional dimerization of PLD has thus been suggested through a "head to tail" interaction of two molecules to produce an enzyme with two active sites and four HKD motifs (Xie et al, 1998). Supporting this idea, structural studies on the PLD superfamily members Nuc and Ymt, found that both exist as dimers (Rudolph et al, 1999; Stuckey and Dixon, 1999). Kinetic studies on Ymt found that it does not follow the conventional Michaelis-Menten kinetics (Rudolph et al, 1999). Dittrich et al (1998), demonstrated that cabbage PLD follows Hill kinetics, which are characteristic of cooperative allosteric enzymes with multiple substrate binding sites (Segel, 1975). The present study on cabbage PLD confirms the Hill model, which has been proposed to describe the kinetic behaviour of lipases in general

(Marangoni, 1994). The calculated Hill coefficient (n_{app}=3.8) indicates a high degree of positive cooperativity among multiple binding sites, suggesting functional oligomerization of the plant enzyme. This would be consistent with the dimerization of the PLD superfamily members, Nuc and Ymt into a catalytically active form, discussed above. Oligomerization of cabbage PLD, and of PLD superfamily enzymes in general, may therefore be a requirement for activation.

In summary, we propose that aluminum fluoride acts as a competitive inhibitor of cabbage PLD by occupying a phosphate binding locus on the active site(s) to prevent the formation of a phosphatidyl-enzyme intermediate (see Diagram 4). Taken together with our previous observations on the inhibition of different forms of PLD by AlF_4^- in salivary glands (Li and Fleming, 1999a), these findings suggest that this may be a universal mechanism of inhibition of PLDs of animal and plant origin. Aluminum- and beryllium fluoride, as well as metal oxoanion analogs of phosphate (vanadate, molybdatye, tungstate), are known as potent inhibitors of phosphomonoesterases e.g. PTPases (Macara, 1980; Zhang et al, 1997). The present study indicates for the first time that they also inhibit phosphodiesterases, such as PLD, and should thus prove useful probes in studying the enzymology of the PLD superfamily

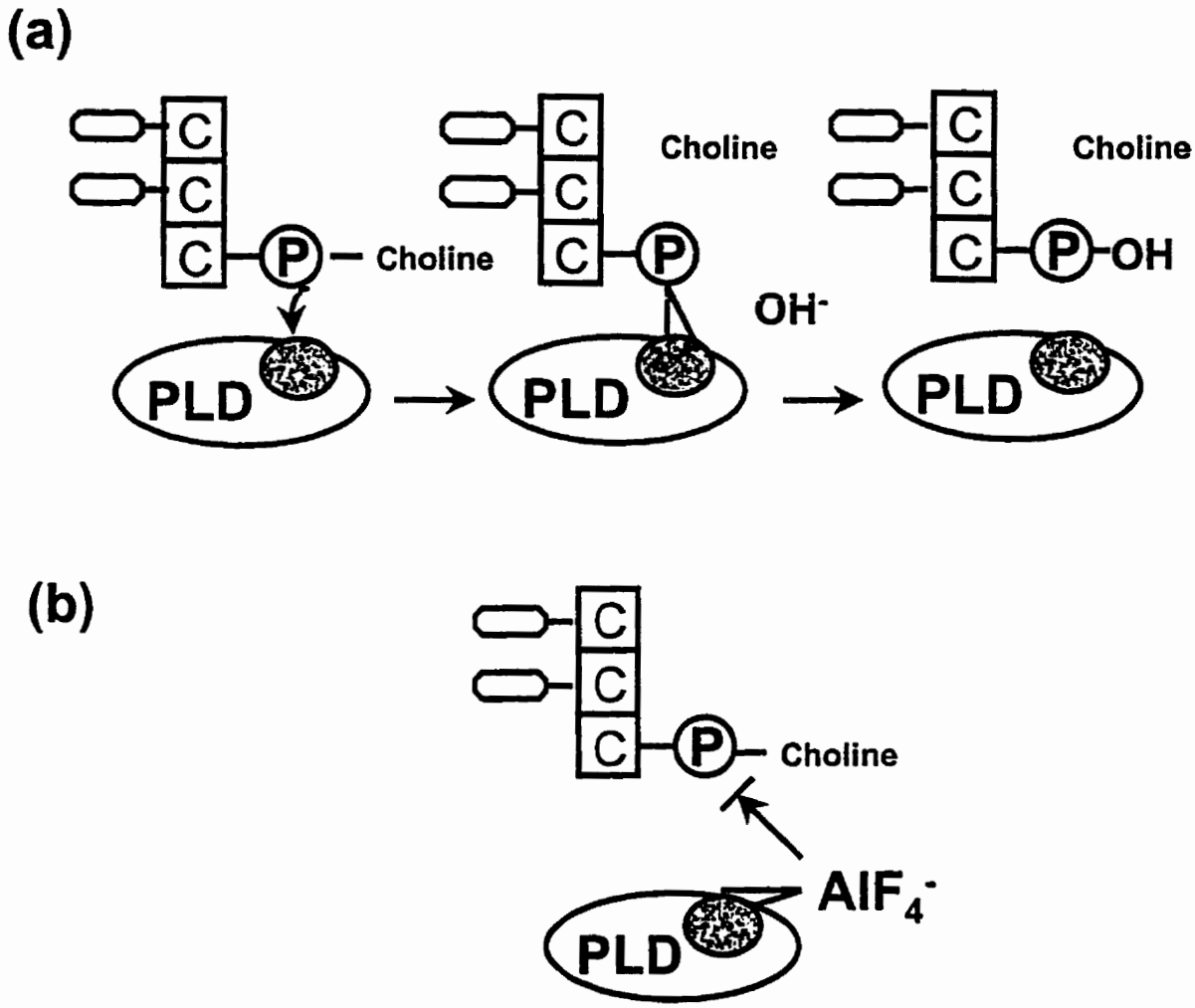


Diagram 4. A proposed mechanism for AlF_4^- inhibition of PLD. **(a)** It is Believed that PLD hydrolysis of PC proceeds by a multistep mechanism that involves the formation of a phosphatidyl-enzyme intermediate, on the binding of the PC-phosphate group with the PLD active site . **(b)** It is proposed that aluminum fluoride competes with the phosphate group for the binding site, and so inhibits enzyme acitivity.

Chapter 5. Conclusions and future directions

Previous work in this laboratory showed that arachidonic acid, released from membrane phospholipids, is a potent regulator of several biological processes in rat submandibular gland acinar cells (Fleming and Mellow, 1995a). It was subsequently established that a major source of AA in the submandibular model was phosphatidylcholine, and that the acid was released through a sequence of reactions, of which the initial one was the muscarinic receptor-coupled activation of phospholipase D, leading to the cleavage of PC into PA and choline (Chung and Fleming, 1995b). These studies confirmed that PLD occupied an important role in a key signal transduction system in the submandibular model, and set the groundwork for the direction of the present thesis. The aim of this project was thus to investigate possible mechanisms of regulation of PLD, with the goal of gaining new insight into the enzyme's regulation and physiological significance in salivary gland cells.

The first part of this study, as outlined in Chapter 2, clearly showed that submandibular PLD is regulated by the small GTP-binding protein, Arf. In addition, a heterotrimeric GTP-binding protein, acting at a locus upstream to Arf, is likely involved in agonist-coupled PLD activation (Li and Fleming, 1998). However, the nature of this G-protein is unknown, although we believe it to be distinct from the Gq protein of the PLC/PIP_2 signal transduction pathway and from the Gs/Gi proteins of the cAMP system. During the course of this part of

investigation, there was an unexpected finding. In permeabilized submandibular cells, AlF_4^-, a well-established G-protein activator, was observed to potently inhibit GTPγS stimulation of PLD. Since this was the first report of aluminum fluoride inhibition of PLD, the second phase of the study was aimed at investigating the possible mechanism of this inhibitory effect. A central tool in this approach was a cell-free PLD assay system in which conditions could be carefully controlled.

AlF_4^- inhibition of guanine nucleotide-activated PLD was confirmed and characterized under various conditions in the cell-free assay. Several lines of evidence indicated that AlF_4^- exerts its inhibitory effect on PLD through a mechanism that is independent of its actions on G-proteins (Li and Fleming 1999a). This finding may shed light on both PLD regulation and the interpretation of the diverse biological effects of AlF_4^-. For example, it now seems reasonable to speculate that the well-known AlF_4^- effect of inhibiting intra-Golgi vesicle transport may be mediated by its inhibition of PLD (see Discussion in Chapter 3 above). Enriched Golgi membrane from submandibular cells has high basal and GTPγS-stimulated PLD activity, and, significantly, both of these activities were potently inhibited by AlF_4^- (Li and Fleming, 1999a). However, whether these PLD activities are involved in Golgi-vesicle transport in salivary glands is yet unknown. To investigate this hypothesis further, it would be crucial to establish a cell-free vesicle formation/transport assay in extracted salivary gland Golgi membranes, so that the role of PLD in vesicle transport could be investigated more directly. In

such a system, enzyme function could be examined by using a range of appropriate probes, such as ethanol (to block PA generation by transphosphatidylation), exogenous PA (to mimic endogenous PA, the direct product of PLD action) , recombinant or biochemically-purified PLDs, and AlF_4^-. In exocrine glands, the formation and transport of secretory vesicles is particularly important and obviously linked to the process of exocrine secretion itself. Therefore an overall investigation of a possible central regulatory role of PLD on exocrine secretion in the salivary gland model should be a future priority. It has already been shown by Fleming and Mellow (1995a) that arachidonic acid, most likely released in a sequence of reactions beginning with PLD hydrolysis of PC, regulates mucin release, as well as calcium and ATP levels, in submandibular cells. It may be that PLD also has a key role in the final phases of exocytosis, where its fusogenic properties could facilitate the joining of secretory granule/plasmalemma membranes to stimulate release of mucin and other exocrine products.

The third part of this study focused on the mechanism of AlF_4^- inhibition of PLD. Because of a lack of availability of purified mammalian PLD, purified plant PLD (from cabbage and also in a few experiments, from peanut) was used in the preliminary series of experiments on regulatory mechanisms reported in Chapter 4 (Li and Fleming, 1999b). The use of a purified enzyme enabled us to examine the potential nature of possible direct interactions between PLD and aluminum fluoride. It was shown that AlF_4^- potently inhibited cabbage PLD in both

phosphoinositide-dependent and - independent assays, and with both PC and PE substrates. This inhibitory effect was duplicated by inorganic phosphate, as well as by two additional phosphate analogs, beryllium fluoride and orthovanadate. We therefore proposed that the AlF_4^- inhibition of PLD may involve a mechanism involving a phosphate-mimicking property (Li and Fleming, 1999b). Subsequent kinetic studies indicated that AlF_4^- acts as a competitive inhibitor of cabbage PLD, which was observed to follow Hill kinetics, rather than the more conventional Michaelis-Menten behavior. A tentative model was therefore proposed, in which AlF_4^-, by its structural similarity with phosphate, competes with the phosphate group of PC for binding to the enzyme active site. This prevents the formation of a phospho-enzyme intermediate, and therefore blocks PLD catalytic activity.

If this model is correct, then AlF_4^- should be able to affect the binding of PC substrate-containing lipid vesicles with the enzyme. This can be measured by an untracentrifugation technique after coincubation of PLD with sucrose-loaded phospholipid vesicles as described by Sciorra et al (1999). Both the supernatant and pellet can be analyzed by Western blotting with anti-PLD antibody, as well as by the cell free PLD assay described in the present study. If AlF_4^- does affect enzyme-lipid binding, we should expect more PLD to remain in the supernatant in the AlF_4^- incubation group. Second, if our proposed model is true, theoretically we should be able to show that PLD binds to a phosphate analog. P^{32}- labeled inorganic phosphate could be used to test this hypothesis. Experimentally, PLD will be coincubated with potassium [P^{32}] phosphate and P^{32} autoradiography

carried out after SDS-PAGE. If this experiment suggests [P^{32}] phosphate binds to the enzyme, we can further investigate the effect of AlF_4^-. Although no radioisotope of AlF_4^- is commercially available, we can overcome this by adding different doses of AlF_4^- together with [P^{32}] phosphate to the enzyme. If AlF_4^- is found to compete with phosphate in a dose-dependent way for binding to PLD, this will strengthen our proposed model when considered together with our previous kinetic data discussed above.

It is realized that our reported work on plant PLD simply indicates the probable nature of aluminum fluoride inhibition of the enzyme in the submandibular model. Clearly, experiments on purified submandibular PLD (recombinant or biochemically purified enzyme) along the lines of those discussed above, will have to be carried out to lend additional weight to the proposal that AlF_4^- inhibits this mammalian enzyme by competitive binding with the phosphate of PC for the PLD active site.

The finding that AlF_4^- and other phosphate analogs bind to phospholipase D could have a significant impact on approaches to studying the structure of PLD and other related superfamily enzymes. Such a binding property has already been used to advantage to characterize some enzymes such as protein tyrosine phosphatases (PTPases), (these are actually phosphomonoesterases, while PLD is a phosphodiesterase). PTPases can bind different phosphate analogs, such as vanadate, and so become stabilized in the transitional state. This has greatly facilitated the determination of their crystal structures (Zhang et al, 1997).

Progress in defining the molecular structure of PLD could certainly provide new insights on the reaction mechanism. This would hopefully lead to the development of specific PLD inhibitors, which are presently lacking in this field, but would be of obvious experimental advantage.

Two forms of mammalian PLD have been cloned, namely PLD1 and PLD2. Most cell types examined so far express both isoforms (Colley et al, 1997b). Some cell lines such as U937 or L1210 cells exclusively express PLD1 or PLD2 (Kim et al, 1999). The regulation and subcellular localization of these two isoforms are different. Therefore, before proceeding with further investigations on PLD in submandibular cells, it is important to know which of these two isoforms are expressed in our model. This can be determined either at the mRNA level (by Northen Blot) or at the protein level (by Western Blot). The use of PLD1 and PLD2 antibodies on detecting the two different forms of PLD in cells or extracts has not been very successful because of the general low expression levels of the enzyme. This problem can be overcome by using immunoprecipitation to concentrate the enzyme before doing Western blot (Kim et al, 1999). PLD1 is located in the intracellular membranes (e.g. Golgi membrane), while PLD2 is predominantly plasma membrane form (Colley et al, 1997b). The availability of a well-purified plasma membrane fraction, together with enriched Golgi membrane, will help linking specific isoforms with their distinct localization and potential function in submandibular cells.

An additional form of PLD activity, which is oleate-sensitive, has also been detected in submandibular cell model. This enzyme has so far not been cloned and its molecular characteristics are not known (Liscovitch et al, 2000). A recent study found that unsaturated fatty acids, such as oleate and arachidonate, selectively stimulate the activity of PLD2, but not of PLD1 (Kim et al, 1999). Therefore, oleate-sensitive PLD and PLD2 may be two closely related enzymes that express different activities under different assay (or physiological) conditions. This is an interesting finding, since in some cell models, including submandibular cells, PLD hydrolysis of PC is the significant initial reaction in the subsequent release of arachidonic acid (Chung and Fleming, 1995b). This suggests the possibility of a self-reinforcing cycle, or positive fedback loop in the regulation of PLD by self-generated AA. It will be interesting to see whether arachidonic acid may stimulate PLD activity in intact submandibular cells, cell lysate or purified particulate fractions.

A recent study found that unsaturated fatty acid-dependent PLD is stimulated during apoptosis, which is preceded by a release of unsaturated fatty acid (Kasai et al, 1998). Apoptosis inducers such as TNFα, hydrogen peroxide or actinomycin D need 5-10 h incubation time to show morphologically detectable evidence of apoptosis (Kasai et al, 1998). Observations in our laboratory suggest that the short-term rat submandibular cell culture system is able to retain structure and function over this time span, and so may have use as a model in apoptosis studies. Apoptosis can be evaluated either by DNA electrophoresis (DNA ladder) or by

morphological methods such as staining cells with Hoechst 33258 (stains the DNA within 10 min), and observing nuclear fragmentation and condensation by confocal fluorescent microscopy. Measurement of associated endogenous arachidonic acid release can be done by a simple TLC technique (Kasai et al, 1998) or by HPLC. The potential connection between apoptosis, fatty acids and and PLD is intriguing, with the possibility of a central role for the enzyme in programmed cell function/death over a defined time span (Nakashima and Nozawa, 1999).

The phosphoinositide, PIP_2, is absolutely required for the activity of both PLD1 and PLD2 (Colley et al, 1997). A recent study suggested that PIP_2 binds directly to a region of basic amino acids on PLD and so influences the enzyme's catalytic action (Sciorra et al, 1999). *In vivo*, PIP_2 is synthesized through phosphorylation of PI(4)P by PI(4)P5-kinase, which has three isoforms α, β and γ. A recent study found that PI(4)P5-kinase α is a downstream effector of the small GTP-binding protein Arf6 (Honda et al, 1999). The activation strictly requires phosphatidic acid, the product of PLD. PI(4)P5-kinase activation leads to PIP_2 synthesis, which further stimulates PLD2. Thus, again there is evidence for a positive feedback cycle in PLD2 stimulation.

Previous kinetic studies in our laboratory showed that AA inhibited PI(4)P5-kinase by non-competitive mechanism (Chung and Fleming, 1995a). If this mechanism operates *in vivo*, then PA and AA, both products of the initial cleavage of PC by PLD, may be capable respectively of stimulating and inhibiting PIP_2

synthesis, with the corresponding downstream regulation of PLD activation. This would represent an extremely sensitive regulatory system with a high degree of fine tuning made possible by small adjustment of the relative amounts of PA and AA present in the system. Future investigations on the nature of interactions between these connected signalling pathways should clarify our understanding of the complex mechanisms of regulation of PLD in salivary glands.

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