REGULATION OF CELL SIGNALLING IN SUBMANDIBULAR GLANDS BY ARACHIDONIC ACID

BY

HYUN CHO CHUNG

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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DEDICATION

To Kyung Hee, my wife

and

to Seok Soon Kim, my mother in law,

who was called by God on February 29, 1992.

... Of making many books there is no end, and much study wearies the body.

Now all has been heard; here is the conclusion of the matter: Fear God and keep his commandments, for this is the whole duty of man.

Ecclesiastes 12:12-13.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ADP	adenosine 5′-diphosphate
aPKC	atypical protein kinase C
ARF	ADP-ribosylating factor
ATP	adenosine 5′-triphosphate
BAPTA/AM	1,2-bis-(0-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid tetra-(acetoxymethyl)-ester
BSA	bovine serum albumin
BW755C	3-amino-1-(-3 trifluoromethyl penyl)-2-pyrazoline
cAMP	cyclic adenosine 3′5′-monophosphate
CDP-DAG	cytidine diphosphate diacylglycerol
CGP	choline glycerophospholipid
cPKC	classical protein kinase C
$cPLA_2$	cytosolic phospholipase A_2
CTX	cholera toxin
DAG	diacylglycerol
EGF	epidermal growth factor
EGP	ethanolamine glycerophospholipid
EGTA	ethylenebis(oxonitrilo)tetraacetate
ER	endoplasmic reticulum
GP	guanine nucleotide regulatory protein
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$I(1,4,5)P_3$	inositol 1,4,5-trisphosphate
INF_γ	interferon-γ
kDa	kilo Dalton
K_m	Michaelis constant
LPC	lysophosphatidylcholine
LPI	lysophosphatidylinositol
LPL	lysophospholipid
2-LPL	2-acyl-lysophospholipid
$LPLA_2$	lysophospholipase A_2
LT	leukotriene
MAP	mitogen-activated protein
MEM	minimum essential medium
NDGA	nordihydroguaiaretic acid
nPKC	novel protein kinase C
OAG	1-oleoyl-2-acetyl-glycerol
PA	phosphatidic acid
PAP	phosphatidic acid phosphohydrolase
PC	phosphatidylcholine
PC-PLC	phosphatidylcholine specific phospholipase C
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PEt	phosphatidylethanol
PG	prostaglandin
PI	phosphatidylinositol
PI 3-kinase	phosphatidylinositol 3-kinase
PI 4-kinase	phosphatidylinositol 4-kinase

PI-PLC	phosphatidylinositol specific phospholipase C
PI(3)P	phosphatidylinositol 3-monophosphate
PI(4)P	phosphatidylinositol 4-monophosphate
PI(4)P 5-kinase	phosphatidylinositol 4-phosphate 5-kinase
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLA_1	phospholipase A_1
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PPI	polyphosphoinositides
PS	phosphatidylserine
PTK	protein-tyrosine kinase
PTX	pertussis toxin
RHC80267	1,6-bis-(cyclohexyloximinocarbonylamino)-hexane
Ro-31-8220	3-{1-[3-(amidinothio)propyl]-3-indolyl}-4-(1-methyl-3-indolyl)-1H-pyrrole 2,5-dione
SH	*src* homology
SGP	serine glycerophospholipid
$sPLA_2$	secretory phospholipase A_2
TCA	trichloroacetic acid
TLC	thin layer chromatography
TPA	12-0-tetradecanoylphorbol-13-acetate
U73122	1-{6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl-1H-pyrrole-2,5-dione
U73343	1-{6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-2,5-pyrrolidine-dione
V_{max}	maximum rate

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ABSTRACT

Arachidonic acid (AA) is an essential unsaturated fatty acid, esterified at the *sn*-2 position of several membrane phospholipids. The liberated acid is the precursor of a range of bioactive compounds, collectively known as eicosanoids (*e.g.*, prostaglandins, thromboxanes, leukotrienes) which have widespread regulatory roles in many physiological processes. There is growing evidence that AA itself may act as a second messenger in many cell types, independent of its conversion to eicosanoid products. The present study was undertaken to examine the potential regulatory role for AA in the muscarinic agonist-coupled phosphoinositide pathway in mucus-secreting rat submandibular gland acinar cells. The specific aims were to (1) determine whether AA regulates phosphoinositide cycle activity and establish the mechanism of the regulatory effect; (2) investigate the phospholipid sources of endogenous AA and (3) to examine the agonist-stimulated routes of enzymatic cleavage of phospholipids resulting in the release of AA.

The muscarinic agonist, carbachol, stimulated phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] hydrolysis and the generation of inositol 1,4,5-trisphosphate [I(1,4,5)P_3] in rat submandibular acinar cells. The carbachol-induced I(1,4,5)P_3 response was inhibited by pretreatment of cells with AA. The AA inhibitory effect was not mediated by prostaglandins or leukotrienes, or the enzyme protein kinase

C (PKC). Inositol phospholipid turnover studies indicated that AA may regulate the phosphoinositide response by inhibiting one or both of PI 4-kinase and PI(4)P 5-kinase in the phosphoinositide cycle. Thus, both PI kinases were characterized in submandibular gland membranes. Enzyme kinetic studies showed that AA significantly reduced V_{max} values but had no effect on the apparent K_m values of PI 4-kinase and PI(4)P 5-kinase, for both phosphoinositide- and ATP substrates. Therefore, it is proposed that AA regulates phosphoinositide cycle activity in submandibular cells by acting as a non-competitive inhibitor of both PI kinases.

Carbachol also stimulated phospholipase D (PLD) activity in submandibular acinar cells. This was associated with the elevation of free choline, phosphatidic acid (PA) and AA, indicating that the PLD substrate was phosphatidylcholine (PC). The carbachol response was inhibited by U73122, a blocker of PI(4,5)P_2-specific phospholipase C (PLC), suggesting that PC-PLD cleavage was, at least in part, secondary to agonist-coupled PI(4,5)P_2-PLC hydrolysis. Consistent with this, PLD was also activated by the phorbol ester, TPA and the Ca^{2+}-mobilizer, thapsigargin, two agents that respectively mimic diacylglycerol (DAG) and I(1,4,5)P_3 in the phosphoinositide effect. The Ca^{2+} chelator, BAPTA/AM abolished the thapsigargin-induced activation of PLD and partially inhibited the carbachol- and TPA-PLD responses. The PKC inhibitor, Ro-31-8220, also inhibited the carbachol- and

TPA-PLD responses, but had no effect on the thapsigargin-induced enhancement of PLD. These results support the idea that both the PKC-associated and Ca^{2+}-mobilizing arms of the PI(4,5)P_2-PLC pathway are associated with PLD regulation. Pretreatment of cells with the PA-phosphohydrolase blocker, propranolol, significantly enhanced the carbachol-induced elevation of PA, but decreased agonist-stimulated DAG and AA, indicating that PC was the likely source of AA.

Therefore, it is concluded that, in rat submandibular acinar cells, AA acts as a regulator of the phosphoinositide cycle by non-competitive inhibition of both PI 4-kinase and PI(4)P 5-kinase. Free AA is liberated from membrane phospholipids by muscarinic agonist stimulation. One route of cleavage involves the activation of PC-specific PLD which is secondary to the agonist-coupled PI(4,5)P_2 effect. Phosphatidic acid, produced by PC-PLD action, is further metabolized to DAG from which free AA is released by DAG-lipase.

CHAPTER I

GENERAL INTRODUCTION

Background

Introduction

Saliva plays essential roles in maintaining the organs and tissues of the oral cavity, such as protection, buffering, antimicrobial action and tooth integrity. It is also important in the initial stage of digestion including bolus formation and starch digestion. Saliva consists of two principal components, fluid and macromolecules, respectively derived from blood plasma and secretory granules of the salivary glands. Three major paired salivary glands, the parotid, the submandibular (also called the submaxillary), and the sublingual, are responsible for the bulk of saliva production. In addition, various minor salivary glands are scattered in the oral mucous membranes. It has been estimated that in humans the parotid and submandibular glands contribute about 90% of the total saliva volume (Dawes, 1978).

Structure of the Rat Submandibular Glands

Salivary glands are composed of a large number of secretory units which consist of an acinus, an intercalated duct, and a striated duct (Fig. 1). Many secretory units converge on an excretory duct which in turn opens into the oral cavity. Saliva forms at the proximal end of the duct in pyramid-shaped

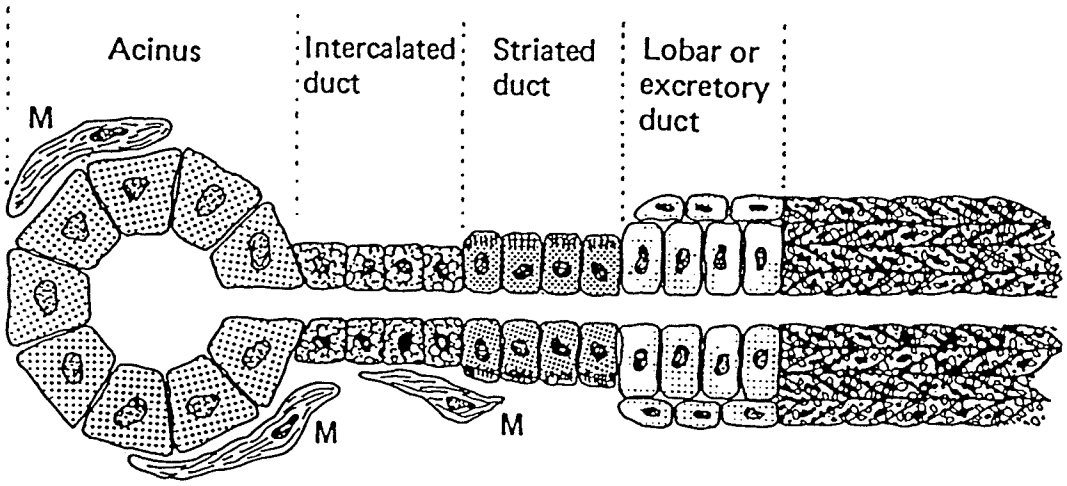

Fig. 1. Schematic illustration of a secretory subunit of a major salivary gland.

M, myoepithelial cell. Adapted, with permission, from Lavelle (1988).

acinar cells arranged in spherical collections called acini. Salivary acini have been classified into three major types, mucous-, serous- and seromucous cells (Munger, 1964). Mucous secretion (mucin) is viscous and rich in glycoproteins, whereas serous secretion is watery and rich in protein, but contains no demonstrable polysaccharides. Seromucous glands contain both serous and mucous cells. The rat submandibular gland is seromucous, consisting of mucous acinar cells with some serous cells in the ducts (Scott and Pease, 1959). Another cell type associated with the acini is the myoepithelial cell. These cells extend over the acini and intercalated ducts in long filaments that have contractile properties. Their contraction probably contributes to the exocrine secretory response.

The acini converge on intercalated ducts, which consist of short cuboidal cells and are associated with myoepithelial cells on their basal surface. The intercalated ducts assemble to form striated ducts, which are lined by tall columnar cells with marked membrane interdigitations projecting towards neighbouring cells. The cytoplasm in this region is packed with mitochondria. The striated ducts are involved in resorption of electrolytes, mainly sodium, ultimately making the saliva hypotonic (Young and Schögel, 1966). The striated ducts empty into two-epithelial cell-layered excretory ducts that extend from the gland mass to drain into the oral cavity.

Control of Secretion in Rat Submandibular Glands

The rat submandibular glands are innervated by the sympathetic and parasympathetic nervous systems and secretion is controlled by both adrenergic and cholinergic neurotransmitters (Schneyer *et al.*, 1972). The rat submandibular acini are mucous, and these cells synthesize and secrete high-molecular-weight glycoproteins (mucins), that are rich in carbohydrate, which comprises 70-85% of dry weight (Herp *et al.*, 1979).

It has been well established that the major control system of mucin secretion in the rat submandibular glands is the β-adrenergic/adenylate cyclase/cyclic adenosine 3′5′-monophosphate (cAMP) signal transduction pathway (Bogart and Picarelli, 1978; Quissell and Barzen, 1980; Fleming *et al.*, 1984). In this classical signal transduction system, the interaction of a β-adrenergic agonist with its specific surface receptor promotes the activation of a GTP binding regulatory protein (G_s), which in turn activates adenylate cyclase and increases the level of cAMP in the cytosol. The cAMP activates a cAMP-dependent protein kinase (PKA), which in turn phosphorylates other enzymes to activate exocytosis in the mucous submandibular cells. In addition to the β-adrenergic control, mucin secretion in response to other classes of agonists has also been described. Bogart and Picarelli (1978) observed that α_1-adrenergic- and muscarinic

cholinergic-, as well as β-adrenergic stimulation of submandibular gland slices evoked mucin release, as quantitated by sialic acid assay. However, using a radioactive glucosamine labelling technique in dispersed cells, Quissell and Barzen (1980) reported that neither α_1-adrenergic nor cholinergic stimulation was able to cause mucin secretion, and suggested that the activation of the β-adrenergic receptor/cAMP pathway may be a prerequisite for the exocrine response in submandibular glands. The disparate results from these groups may be due to the methods they used, including mucin assay techniques.

Fleming *et al*. (1983) developed a radioimmunoassay (RIA) for rat submandibular mucin, which is highly specific and sensitive, and can measure picogram quantities of secreted mucin. Work in this laboratory (Fleming *et al*., 1984; 1986; 1987; 1992), using RIA and other methods, confirmed that the β-adrenergic/cAMP pathway was the major stimulation of the submandibular mucin secretion (70% of the total mucin secretion). However, a significant mucin release (more than 40%) could also be elicited when the cells were stimulated with other class of agonists, such as carbachol (muscarinic), methoxamine (α_1-adrenergic), and substance P (tachykinin). It was further demonstrated that all three agonists induced the activation of phosphoinositide-specific phospholipase C (PLC) to stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] (Fleming *et al*., 1987). The receptor-

mediated activation of phosphoinositide signal transduction pathway leads to the generation of two important second messengers, inositol 1,4,5-trisphosphate [I(1,4,5)P_3], which mobilizes Ca^{2+} from the endoplasmic reticulum (ER), and diacylglycerol (DAG), which activates protein kinase C (PKC), a ubiquitous enzyme that regulates many physiological processes (Berridge, 1987; Nishizuka, 1992) in most cell types so far examined. Stimulation of submandibular glands with the PKC activators, tetradecanoylphorbol acetate (TPA) and exogenous DAG also elevated mucin secretion up to 30% total mucin (Fleming *et al.*, 1986). Therefore, it is suggested that both the cAMP and phosphoinositide transduction pathways are involved in regulating mucin secretion in the rat submandibular gland cells.

Inositol Phospholipids and Cell Signalling

Inositol-containing phospholipids, collectively called phosphoinositides or polyphosphoinositides (PPI) are unique membrane phospholipids in that *myo*-inositol headgroup can be phosphorylated. There are three major PPI in eukaryotic cells: phosphatidylinositol (PI), phosphatidylinositol 4-phosphate [PI(4)P], and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2]. Although these lipids comprise less than 10% of the total membrane phospholipids (Hokin, 1985), they are important sources of second messengers.

Interests in phosphoinositides and their roles in cell functions were initiated from the landmark observations of Mabel and Lowell Hokin, made in 1950's that treatment of pancreas with cholinergic agonists stimulated an increase in the incorporation of ^{32}P into PPI and phosphatidic acid (PA) (Hokin and Hokin, 1953; 1955; 1958). Progress was slow until Michell (1975) published a provocative review suggesting that a breakdown product of PPI acted as a second messenger for calcium entry into cells. Subsequently, interest in this field developed very rapidly to the point where PPI metabolism is one of the most investigated areas in cell biology.

The Phosphoinositide Cycle

As shown in Fig. 2, phosphoinositides are synthesized through

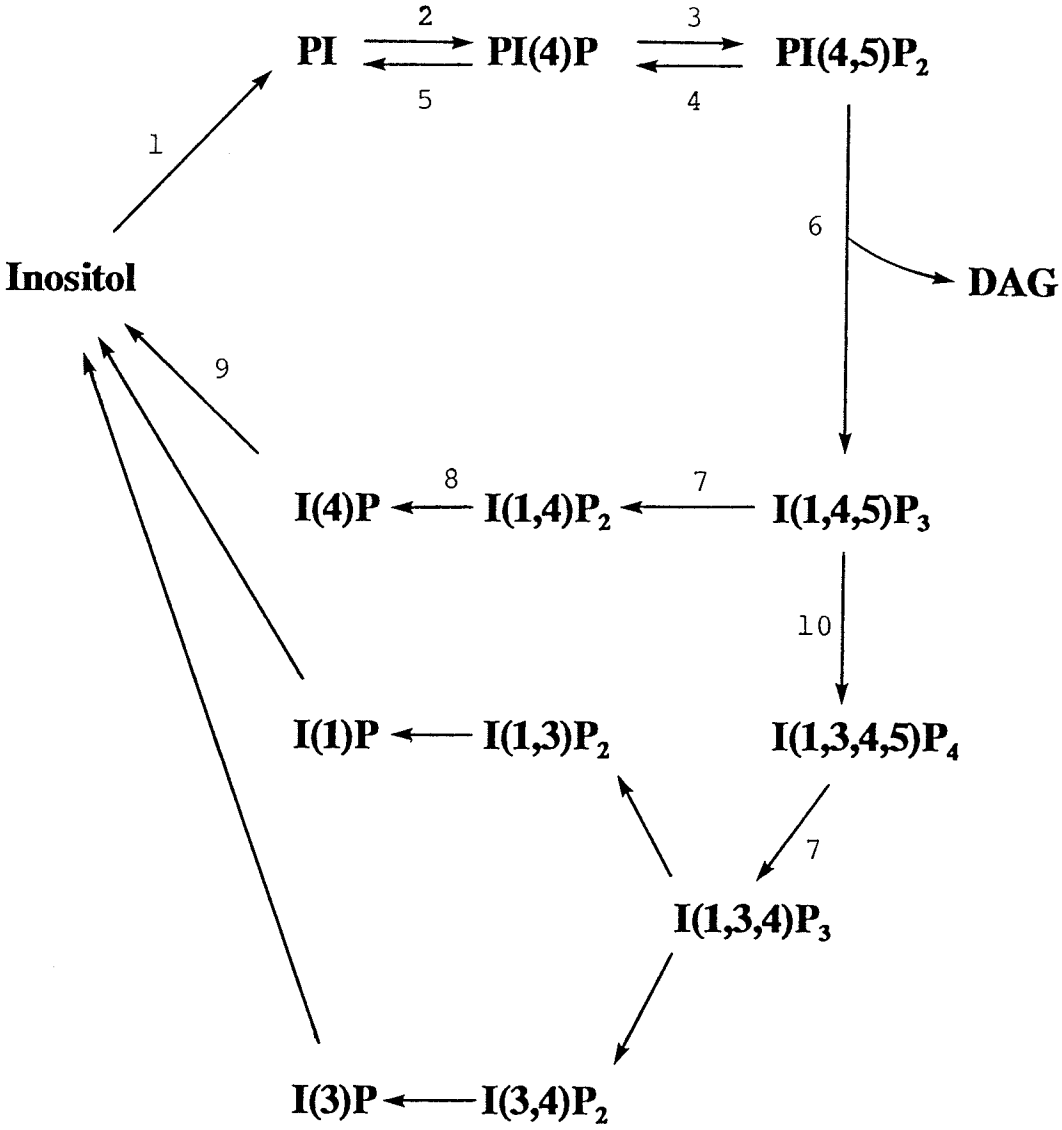

Fig. 2. The phosphoinositide cycle.

Some enzymes and metabolites are omitted for clarity. Abbreviations: PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-monophosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; I(4)P, inositol 4-monophosphate; I(1,4)P_2, inositol 1,4-bisphosphate; I(1,4,5)P_3, inositol 1,4,5-trisphosphate; I(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate. The enzymes are 1, inositol phosphatidyltransferase; 2, phosphatidylinositol 4-kinase; 3, phosphatidylinositol(4)P 5-kinase; 4, PI(4,5)P_2 phosphomonoesterase; 5, PI(4)P phosphomonoesterase; 6, phospholipase C; 7, I(1,4,5)P_3/I(1,3,4,5)P_4 5-phosphatase; 8, inositolpolyphosphate 1-phosphatase; 9, IP phosphatase; 10, I(1,4,5)P_3 3-kinase.

several enzymatic reactions (for reviews, Berridge and Irvine, 1989; Rana and Hokin, 1990). The cellular *myo*-inositol is transferred to a cytidine diphosphate-DAG (CDP-DAG) by a *myo*-inositol 3-phosphatidyltransferase to synthesize PI. The newly synthesized PI is sequentially phosphorylated to PI(4)P and then to $PI(4,5)P_2$ by two membrane bound enzymes, phosphatidylinositol 4-kinase (PI 4-kinase) and phosphatidylinositol 4-phosphate 5-kinase [PI(4)P 5-kinase], respectively. As described, $PI(4,5)P_2$ plays a central role in signal transduction since the hormone-stimulated hydrolysis of this compound by PLC generates the two second messengers, $I(1,4,5)P_3$ and DAG. The released $I(1,4,5)P_3$ and DAG can act as the cellular regulators (see below), or can be metabolized to recycle into the so-called the phosphoinositide cycle (Fig. 2).

Inositol 1,4,5-trisphosphate and diacylglycerol

The $I(1,4,5)P_3$ may either be rapidly dephosphorylated by a specific phosphatase (may also be phosphorylated to inositol 1,3,4,5-tetrakisphosphate [$I(1,3,4,5)P_4$] by a specific kinase) (Fig. 2), or diffuse through the cytoplasm to the specific receptor proteins located on portions of the endoplasmic reticulum (ER) (Ross *et al.*, 1989; Mignery *et al.*, 1989; Ferris and Snyder, 1992). By binding to these specific receptor proteins, $I(1,4,5)P_3$ is able potently and selectively to mobilize sequestered Ca^{2+} from ER stores, which plays

diverse roles in many physiological processes in various cell types (Berridge, 1987). Some of the $I(1,4,5)P_3$ can be further phosphorylated (Sears, 1992). Functions of the phosphorylated metabolites of $I(1,4,5)P_3$ are not clear. Berridge and Irvine (1989) suggested that $I(1,3,4,5)P_4$ might mediate a slower and more prolonged Ca^{2+} response, and together with $I(1,4,5)P_3$ stimulate Ca^{2+} entry in the cells.

Diacylglycerol, the other cleavage product of the PLC-$PI(4,5)P_2$ hydrolysis, has at least three potential signalling roles. First, by the action of DAG-kinase, it can be phosphorylated to phosphatidic acid (PA), which appears to have regulatory roles in such functions as DNA synthesis, increase in cytosolic Ca^{2+} and smooth muscle contraction (reviewed by Thompson *et al.*, 1993). Second, by a specific DAG-lipase it can be cleaved to release free arachidonic acid (AA), a precursor of eicosanoids (*e.g.*, prostaglandins, thromboxanes and leukotrienes). Some of the eicosanoids and AA itself may act as second messengers (see below). Third, it can activate PKC, a central enzyme in signal transduction which phosphorylates regulatory proteins and regulates physiological functions in various cell types. The DAG produced by receptor activation, together with a phosphatidylserine (PS) cofactor in the cytoplasmic half of the plasma membrane, binds to the PKC, thereby increasing the affinity of the enzyme for Ca^{2+} so that the PKC becomes active at the usual concentrations of Ca^{2+} in the cytosol (Nishizuka, 1988). In many cells, PKC may be

activated by the cooperative or synergistic effect of DAG and increased cytosolic Ca^{2+}, brought about by $I(1,4,5)P_3$. To date, the mammalian PKC family consists of at least 12 isoforms which are classified into three groups: the classical PKCs (cPKC), which are Ca^{2+}-dependent and include α, βI, βII and γ; the novel PKCs (nPKC), which are Ca^{2+}-independent and include δ, ε, ε', η, θ and μ; and the atypical PKCs (aPKC) such as ζ and λ (Nishizuka, 1992: Hug and Sarre, 1993). These species show variations in tissue expression, substrate specificity and intracellular localization, which may explain functional diversities (Dekker and Parker, 1994).

Regulation of the Phosphoinositide Cycle

The phosphoinositide cycle can be regulated at several sites of enzymatic action in the cycle (Fig. 2). Three important regulatory enzymes in the cycle are PI 4-kinase, PI(4)P 5-kinase and PLC as discussed below.

Phosphatidylinositol 4-kinase

PI 4-kinase phosphorylates PI at D-4 position on the inositol ring, producing PI(4)P. Endenmann *et al.* (1987) first separated two types of PI 4-kinase from the bovine brain. These two enzymes have apparent sizes of 55 kDa and 230 kDa. The 55-kDa PI 4-kinase has been characterized in the membranes of several cell types, including human red cells (Endenmann *et*

al., 1987), fibroblasts (Whitman *et al.*, 1987), rat liver (Hou *et al.*, 1988), and A431 cells (Walker *et al.*, 1988). This enzyme is an integral membrane protein and requires detergent for extraction from the membranes (Carpenter and Cantley, 1990). This form of PI 4-kinase has a K_m of approximately 20-100 μM for ATP and a K_m of 20 μM for PI, is activated by detergent, but inhibited by adenosine (Whitman *et al.*, 1987; Hou *et al.*, 1988; Carpenter and Cantley, 1990). Divalent cations such as Mg^{2+} and Mn^{2+} support its activity but the kinase is inhibited by high concentration of Ca^{2+} (Walker *et al.*, 1988; Mesaeli *et al.*, 1991; Pike, 1992).

The other PI 4-kinase is also a membrane-associated protein, and appears to have different sizes (80 to 230 kDa) in the various tissues studied, *e.g.*, 80 kDa in rat brain (Yamakawa and Takenawa, 1988), 200 kDa in bovine uterus (Li *et al.*, 1988), and 230 kDa in bovine brain (Endenmann *et al.*, 1987). Compared with the 55-kDa PI 4-kinase, this enzyme exhibits higher K_m values for its substrates, 250-742 μM for ATP and about 115 μM for PI (Yamakawa and Takenawa, 1988; Pike, 1992), and is less sensitive to activation by detergent or inhibition by adenosine (Endenmann *et al.*, 1987: Carpenter and Cantley, 1990).

Regulation of PI 4-kinase activity is poorly understood. Analysis of the available data is difficult and complex since several enzymes and factors are involved in the phosphoinositide cycle, *e.g.*, phosphatases, phospholipases,

PI(4)P 5-kinase and availability of ATP and PI substrates (Carpenter and Cantley, 1990). Despite these complexities, there are some data on enzyme regulation. High concentrations of Ca^{2+} (mM) and the kinase product PI(4)P inhibit PI 4-kinase activity (Rana and MacDonald, 1986; Stubbs *et al.*, 1988). Adenosine inactivates the enzyme activity since it depletes ATP in the cell (Whitman *et al.*, 1987). Taylor *et al.* (1984) demonstrated that treatment of mouse thymocytes with the phorbol ester, TPA, the calcium ionophore A23187, or concanavalin A led to an increase in the level of PI(4)P. Walker and Pike (1987) found an increase in the PI 4-kinase activity of membranes isolated from A431 cells after treatment of the cells with epidermal growth factor (EGF), suggesting that the enzyme may be regulated by protein kinases or G proteins. However, no evidence of the direct regulation of PI 4-kinase by a protein kinase or a G protein has been reported. It has been suggested that PI 4-kinase may be activated by cAMP-dependent phosphorylation in several cell types such as platelets (De Chaffoy de Courcelles *et al.*, 1986) and erythrocytes (Giraud *et al.*, 1988).

Phosphatidylinositol 4-phosphate 5-kinase

PI(4)P 5-kinase phosphorylates PI(4)P at the D-5 position to form PI(4,5)P_2, the substrate of PLC. The kinase is found in both soluble and membrane-bound forms (Cochet and Chambaz, 1986), and can be extracted from the membranes by high salt

concentrations (Ling *et al.*, 1989). The reported molecular masses of the PI(4)P 5-kinases range from 45 kDa to 190 kDa depending on cell type and assay technique (see review, Carpenter and Cantley, 1990). This kinase has an apparent K_m for ATP of 2 μM (Downes and MacPhee, 1990), and its activity is Mg^{2+}-dependent and requires nM levels of Ca^{2+} (Lundberg *et al.*, 1986).

The regulation of PI(4)P 5-kinase is also poorly established. The enzyme is activated by $GTP_\gamma S$ in placental membranes (Urumow and Wieland, 1986) and rat brain membranes (Smith and Chang, 1989), suggesting regulatory role for a heterotrimeric G protein. As with PI 4-kinase, polyamines activate a PI(4) 5 P-kinase, partially purified from rat brain cells (Lundberg *et al.*, 1986). Pike *et al.* (1990) found that chemoattractants and leukotriene B_4 activated the enzyme associated with neutrophil membranes. PI(4)P 5-kinase is also activated by its substrate, PI(4)P (Stubbs *et al.*, 1988) and inhibited by its product, PI(4,5)P_2 (Lundberg *et al.*, 1986).

Phosphatidylinositol 3-kinase

Phosphatidylinositol 3-kinase (PI 3-kinase) is distinctive in that it phosphorylates PI at the D-3 position of the inositol ring to form phosphatidylinositol 3-phosphate [PI(3)P] (Whitman *et al.*, 1988; Auger *et al.*, 1989). Unlike other types of PI kinase, PI 3-kinase is inhibited by non-ionic detergents

but not by adenosine (Whitman *et al.*, 1987). This kinase has been purified and shown to contain a 110 kDa (p110) catalytic subunit and an 85 kDa (p85) regulatory subunit (Skolnik *et al.*, 1991; Hu *et al.*, 1993). PI 3-kinase accounts for only a small percent of the total PI kinase activity found in cells (Majerus *et al.*, 1990), and utilizes PI, PI(4)P and PI(4,5)P_2 as substrates *in vitro* (Carpenter *et al.*, 1990).

PI 3-kinase is associated with various growth factor receptors, including platelet-derived growth factor (PDGF) receptor and EGF receptor, which have intrinsic or associated protein-tyrosine kinase activity (Cantley *et al.*, 1991; Stephens *et al.*, 1993). Association of PI 3-kinase with growth factor receptors appears to be mediated by a p85 regulatory subunit which has two SH-2 domains (Gout *et al.*, 1992). SH-2 domains are known to bind to sequences containing phosphorylated tyrosine residues (McGlade *et al.*, 1992). Thus, the regulation of PI 3-kinase activity appears to be mediated by the association of phosphotyrosine-containing proteins with the p85 of PI 3-kinase, which in turn integrates the signals, then activates the associated 110 kDa catalytic subunit (p110) (McGlade *et al.*, 1992). One current model for activation of PI 3-kinase activity is that the SH-2 domains of p85 recognize and bind to specific phosphotyrosine-containing sequences in activated receptors, promoting the relocation of the kinase to the plasma membrane in close association with its substrates (Kapeller and Cantley, 1994). Stephens *et al.* (1991) found

that, in neutrophils, the stimulated PI 3-kinases rapidly generated $PI(3,4)P_2$ and $PI(3,4,5)P_3$. These 3-phosphorylated lipids are not hydrolyzed by any known PLC and are not components of the conventional phosphoinositide turnover pathway (Majerus *et al.*, 1990; Cantley *et al.*, 1991; Stephens *et al.*, 1993). The rapid appearance of PI 3-kinase lipid products and their resistance to PLC-mediated hydrolysis suggested that the 3-phosphoinositides themselves may act as intracellular mediators (Kapeller and Cantley, 1994). However, more work is required to elucidate the downstream targets of the 3-phosphorylated inositol lipids and establish the clear mechanisms of their functions in the cells.

Phospholipase C

Another point of regulation of the phosphoinositide cycle is the action of phospholipase C (PLC) which plays a crucial role in generating $I(1,4,5)P_3$ and DAG via receptor-mediated $PI(4,5)P_2$ hydrolysis as described.

There are at least 16 isozymes of PLC that can be classified into three types, PLC-β, PLC-γ and PLC-δ (Meldrum *et al.*, 1991; Rhee and Choi, 1992). Each type is a single polypeptide protein from a single specific gene, and has a different molecular weight; PLC-β (150-154 kDa), PLC-γ (145-148 kDa) and PLC-δ (85-88 kDa). These isozymes have two regions of high (40-60%) homology that may be important in the catalytic function of the PLC (Rhee, 1991). PLC-γ isoform

contains the additional domains, SH-2 and SH-3, which are homologous to conserved regions in the regulatory regions of the *src*-encoded family of tyrosine kinases (Rhee and Choi, 1992).

The structural differences of PLC iosforms and the existence of numerous receptors to activate them suggest the possibility of multiple regulatory systems of these enzymes (Exton, 1994a). The receptor-mediated regulation of PLC occurs by two distinct mechanisms. PLC-β is activated by G proteins, whereas PLC-γ is activated by receptor-tyrosine kinases. The PLC activated by muscarinic, α_1-adrenergic and tachykinin agonists in submandibular gland cells is PLC-β (Fleming *et al.*, 1987). All references to PLC in the body of this thesis will thus refer to this form of the enzyme.

The idea that a G protein couples cell surface receptors to the PLC hydrolysis of PI has existed for more than a decade (Cockcroft and Gomperts, 1985; Litosch *et al.*, 1985). Evidence for the involvement of pertussis toxin (PTX)- and cholera toxin (CTX)-insensitive G proteins in receptor-mediated activation of PLC has been reported (Fain *et al.*, 1988; Martin, 1989). G proteins that can stimulate PLC have been purified from the bovine brain (Pang and Sternweis, 1990) and rat liver (Taylor *et al.*, 1990). These proteins had molecular weights of 42-kDa and 43-kDa, and were not recognized by antiserum to any of the known G protein α-subunits (Taylor *et al.*, 1990), indicating that they were novel G proteins, and

designated α_q and α_{11} (Strathmann and Simon, 1990). These α-subunits are now known to be members of a new family of G proteins, the G_q family. To date at least eight α-subunits of G_q proteins (α_q, α_{11-16}) which can activate PLC have been identified (Sternweis and Smrcka, 1992; Hepler and Gilman, 1992). Reconstitution experiments showed that α_q and α_{11} specifically activate PLC-β_1 but not PLC-δ or PLC-γ (Smrcka *et al.*, 1991). Further experiments demonstrated that the members of the G_q family can interact with appropriate receptors and function as the coupler in hormonal stimulation of PLC. Reconstitution of purified G_q with isolated muscarinic receptors showed that M_1 receptors were much more efficient stimulators of the protein than M_2 receptors (Berstein *et al.*, 1992). This finding is compatible to the linkage of M_1 and M_2 receptors to PTX-insensitive and -sensitive pathways, respectively (Sternweis and Smrcka, 1992). Other members of G_q family, α_{14},α_{15}, and α_{16} can also stimulate PLC-β activities (Lee *et al.*, 1992).

Although the stimulation of effectors by G proteins is generally mediated by the activated α-subunits, Camps *et al.* (1992) reported that βγ-subunits purified from transducin (G_t) or brain activated PLC from HL60 human promyeolocytes and neutrophils. Similar stimulatory effects of βγ-subunits on PLC have been reported from several preparations such as turkey erythrocytes (Boyer *et al.*, 1992), bovine liver and brain cells (Blank *et al.*, 1992), and lipid vesicles (Park *et al.*,

1993). These findings suggest that activation of PLC by PTX-sensitive G proteins involves their $\beta\gamma$-complexes of G_i and G_o (Exton, 1994a).

PLC-γ appears to be regulated by growth factor tyrosine kinases. Polypeptide growth factors such as EGF and PDGF mediate their actions by binding to and activating receptors that possess intrinsic protein-tyrosine kinase (PTK) activity (Ullrich and Schlessinger, 1990). The early cellular events induced by the binding of growth factors to these respective receptors include receptor autophosphorylation at several tyrosine residues and the stimulation of PLC activity (Ullrich and Schlessinger, 1990). This activation of PLC appears to be independent of G proteins and requires the intrinsic tyrosine kinase activity (Hasegawa-Sasaki *et al.*, 1988). Studies in intact cells have shown that EGF and PDGF elicit increased tyrosine phosphorylation of PLC-γ_1, but not of PLC-β_1 or PLC-δ (Wahl *et al.*, 1989; Meisenhelder *et al.*, 1989; Rhee and Choi, 1992). *In vitro* phosphorylation experiments indicated that three tyrosine residues (771, 783 and 1254) on bovine PLC-γ_1 were highly phosphorylated by the purified EGF receptor (Kim *et al.*, 1990). Like the p85 of PI 3-kinase, PLC-γ_1 has been identified as a tyrosine phosphorylation substrate, and contains two SH-2 and one SH-3 domains (Wahl *et al.*, 1989; Koch *et al.*, 1991).

Molecular events that may happen during the activation of PLC-γ_1 by a growth factor receptor PTK have been proposed

(Rhee, 1991; Rhee and Choi, 1992). The proposed model for PLC-γ_1 activation is that the SH-2 domains of the cytosolic PLC-γ_1 bind to phosphotyrosine residue in activated receptor, followed by the phosphorylation of PLC-γ_1 tyrosine residues 771, 783, and 1254 by the receptor kinase. Tyrosine phosphorylation may induce conformational change in PLC-γ_1, then promote the translocation of the enzyme from the cytosol to the membrane where the PLC substrate, $PI(4,5)P_2$, is located.

To date the mechanism for regulation of PLC-δ remains unknown.

Arachidonic Acid

It is well established that the fatty acids of cellular phospholipids are distributed asymmetrically on the glycerol backbone. In general, saturated fatty acids are esterified at the *sn*-1 position, while unsaturated fatty acids are frequently found at the *sn*-2 position. Under normal conditions, most fatty acids are stored as esterified forms in glycerolipids. When cells are stimulated either chemically or electrically, they are released from cellular phospholipids by the action of phospholipases (see below).

5,8,11,14-*cis*-eicosatetraenoic acid (20:4ω6), conventionally known as arachidonic acid (AA), is one of the most important unsaturated fatty acids because it is the precursor of a range of extremely bioactive metabolites. For example, AA can be converted to prostaglandins and thromboxanes by cyclooxygenase; to leukotrienes, lipoxins, and various hydroperoxy acids by lipoxygenase; to epoxyeicosatrienoic acid by P-450. These AA metabolites, known collectively as eicosanoids have widespread regulatory roles in many physiological processes of the cardiovascular, immune, and reproductive systems and others (see reviews, Samuelsson, 1983; Gerrard, 1984; Smith 1989; Shimizu and Wolfe, 1990). Recently, there has been mounting evidence that AA itself plays a second messenger role in the signal transduction mechanism that modulates key cell regulators in various cell types.

Arachidonic Acid as a Signalling Molecule

Several main criteria that are necessary to define a molecule as a second messenger have been suggested (Robinson *et al.*, 1971; Jones and Persaud, 1993): (1) its intracellular concentration must be altered in response to the primary stimulus; (2) specific mechanisms must exist for its removal; (3) inhibition of its degradation should mimic or potentiate the physiological response to the primary stimulus; (4) its exogenous addition can mimic the physiological effects of the primary stimulus. In addition to the well known second messengers such as cAMP, $I(1,4,5)P_3$ and DAG, evidence has been increasing that AA may act as a second messenger by satisfying the above mentioned criteria. Arachidonate may regulate PKC, ion channels, phosphoinositide turn over and other biochemical effects associated with stimulus-response coupling mechanisms by systems that are independent of its conversion into eicosanoid products (Naor, 1991; Jones and Persaud, 1993; Graber *et al.*, 1994).

Regulatory effects of arachidonic acid on protein kinase C and other kinases

McPhail *et al.* (1984) first reported that in human neutrophils PKC could be activated by AA. This effect was not reversed by inhibitors of the cyclooxygenase (indomethacin) or lipoxygenase (nordihydroguaiaretic acid; NDGA) synthetic

pathways, indicating that AA and not a metabolite was responsible for the activation of PKC. Subsequently, AA involvement in the modulation of the PKC activity has been shown in a number of cell types, including rat brain (Sekiguchi *et al.*, 1987; Shinomura *et al.*, 1991), hypothalamus (Naor *et al.*, 1988), bovine aorta (Dell and Severson, 1989), human platelets (Fan *et al.*, 1990) and pancreatic β-cells (Landt *et al.*, 1992).

The mechanism of PKC activation by AA is not clear to date. A synergistic action of AA and DAG to activate PKC has been suggested (Shinomura *et al.*, 1991; Lester *et al.*, 1991). As described in the previous section, for PKC activation, DAG increases the apparent affinity of PKC for Ca^{2+}, thereby activating the enzyme at micromolar Ca^{2+} concentrations (Nishizuka, 1988). Shinomura *et al.* (1991) and Lester *et al.* (1991) suggested that AA promotes binding of DAG to PKC and thereby acts as a enhancer molecule, synergising with DAG to activate PKC at submicromolar Ca^{2+} concentrations. One characteristic of PKC is that with activation, the enzyme is translocated from the cytosol to the membrane (Nishizuka, 1986; Fleming *et al.*, 1992). In rat hepatocytes, Diaz-Guerra *et al.* (1991) observed that AA induced a translocation of PKC to the plasma membranes and increased PKC activity. However, Sekiguchi *et al.* (1987) and Naor *et al.* (1988) reported that PKC purified from rat brain could be stimulated by AA in the absence of DAG and phosphatidylserine. Recently Khan *et al.*

(1992) found that AA activated preferentially soluble α, β and γ isoforms of PKC, and they postulated that free AA could activate soluble PKC, while DAG activated the membrane-bound fraction of PKC after translocation. Since PKC-α, β and γ isoenzymes are Ca^{2+}-dependent (Nishizuka, 1992), and since AA can enhance the intracellular Ca^{2+} concentrations (see below), it is also possible that AA activates PKC via cytosolic Ca^{2+} enhancement.

In addition to PKC, AA influences the activities of other protein kinases. Speizer *et al.* (1991) found that AA inhibited cAMP-dependent protein kinase (PKA) activity in S49 lymphoma cells. AA is also reported to inhibit Ca^{2+}/calmodulin-dependent protein kinase II activity extracted from rat brain cortex (Piomelli *et al.*, 1989) and from pancreatic islets (Landt *et al.*, 1992), but the mechanism of its inhibitory effect on these enzymes is unknown.

Arachidonic acid effects on ion channels

It has been shown that AA elevated cytosolic Ca^{2+} concentrations in numerous cell types, including pancreatic islets (reviewed by Jones and Persaud, 1993; Turk *et al.*, 1993), human neutrophils (Nacchache *et al.*, 1989), T and B human lymphocytes (Corado *et al.*, 1990), avian granulosa cells (Hertelendy *et al.*, 1992), ciliary ganglion cells (Khurana and Bennett, 1993) and rat submandibular glands (Fleming and Mellow, 1995). In the majority of studies, AA was found to

play the role of second messenger via the elevation of intracellular Ca^{2+} levels by mobilizing Ca^{2+} from intracellular stores and/or by augmenting Ca^{2+} influx from the extracellular space. For example, in pancreatic β-cells, AA increased insulin secretion, which was mediated by the acid's enhancement of cytosolic Ca^{2+} from both Ca^{2+} influx and release from the ER Ca^{2+} stores (Jones and Persaud, 1993; Turk *et al.*, 1993). Recent work in our laboratory (Fleming and Mellow, 1995) showed that AA induced Ca^{2+} mobilization from ER, to a greater degree than that caused by optimal levels of $I(1,4,5)P_3$ or thapsigargin in mucous submandibular cells, suggesting the existence of an AA-sensitive Ca^{2+} pool in the ER that is not responsive to the other two Ca^{2+}-mobilizing agents. AA also provoked Ca^{2+} influx from the extracellular space (Fleming and Mellow, 1995). The elevated intracellular Ca^{2+} concentrations by AA could be associated with the modulation of several physiological functions of the submandibular glands, including mucin secretion and protein synthesis (Fleming and Mellow, 1995).

Arachidonic acid also influences the activities of other ion channels such as K^+-, Na^+-, Cl^--, glutamate-gated-, gap junction, and GABA-gated channels (reviewed by Ordway et al., 1991; Meves, 1994). However, the AA effect on ion channels is variable and may cause activation or inhibition, depending on the types of ion channels and cell preparations (Meves, 1994). Even in the same preparation AA can have opposite effects on

different K^+ channels, for example, in cardiac ventricular cells AA activated ATP-insensitive and inhibited ATP-sensitive K^+ channels (Kim and Duff, 1990).

The mechanism by which AA affects ion channels is not clearly established. It has been suggested that some of the effects of AA on ion channels are mediated by its ability to perturb the order of the membrane lipids, a property known as an increase in 'membrane fluidity' (Klausner *et al.*, 1980; Anel *et al.*, 1993). Béhé *et al.* (1992) observed that in NG108-15 neuroblastoma x glioma hybrid cells, AA and other unsaturated fatty acids increased membrane fluidity, and inhibited the kinetics of the M-current, a non-inactivating K^+ current, whereas stearic acid (saturated) had no effect. The result indicates that the AA effect on ion channels could be mediated at least in part by the increase in the membrane fluidity by the fatty acid (Béhé *et al.*, 1992). However, correlations between fatty acid effects on membrane fluidity and ion channels may be questionable since most fluidity measuring techniques reflect the bulk physical properties rather than the lipid confined to the channel protein (Meves, 1994).

Another possible mechanism by which AA affects ion channels is the interaction of the acid with ion channel proteins themselves. Ordway *et al.* (1991) suggested that fatty acid-binding sites may exist on ion channel protein like those of albumin. Furthermore, Rich (1993) indicated that the AA

molecule with its hairpin structure is very flexible and therefore more suited to interact directly with concave protein surfaces, compared with other fatty acid molecules.

Arachidonic acid effects on phosphoinositide turnover

It is suggested that the phosphoinositide effect can be regulated by AA. For example, exogenous AA has been reported to stimulate phosphoinositide hydrolysis and increases in inositol phosphates in several cell types, including rat liver microsomes (Irvine *et al.*, 1979), human placental cells (Zeitler and Handswerger, 1985) and astrocytes (Murphy and Welk, 1989). The AA stimulatory effect on the phosphoinositide effect was not secondary to eicosanoid synthesis (Zeitler and Handswerger, 1985; Murphy and Welk, 1989). The positive effect of AA on phosphoinositide hydrolysis may be mediated via the acid's activation of PLC (Irvine *et al.*, 1979; Zeitler and Handswerger, 1985).

On the other hand, in rat pancreatic cells, AA reduced carbachol-stimulated $I(1,4,5)P_3$ levels (Chaudhry *et al.*, 1987; 1989; Maruyama, 1990). Chaudhry *et al.*, (1989) observed that AA lowered $PI(4,5)P_2$ levels and, in addition, depleted cellular ATP levels in pancreatic cells. Thus, they suggested that reduced $PI(4,5)P_2$ could result from a decreased availability of ATP as the donor of γ phosphate in the phosphorylation of PI to PI(4)P to $PI(4,5)P_2$ by the respective enzymes PI 4-kinase and PI(4)P 5-kinase.

In addition to the PLC-PI(4,5)P_2 effect, AA inhibited phospholipase A_2 (PLA_2) purified from a macrophage line (Lister *et al.*, 1988) and snake venom (Raghupathi and Franson, 1992). Higher concentrations of AA (4 mM), however, stimulated phospholipase D (PLD) in rat brain microsomes (Chalifour and Kanfer, 1982). There is growing evidence that agonists stimulate the release of AA secondary to the PLC-PI(4,5)P_2 effect, or via action of PLA_2 and/or phospholipase D (PLD) on non-inositol phospholipids, and that liberated AA may act as a feedback regulator of the phosphoinositide cycle and phospholipases (Chaudhry *et al.*, 1989; Raghupathi and Franson, 1992).

Sources and Regulation of Arachidonic Acid Release from Membrane Phospholipids

The phospholipases are a group of enzymes that hydrolyse phospholipids. They are classified into four types depending on their sites of attack on the phospholipid molecules (Fig. 3). An enzyme that hydrolyses the acyl ester at the *sn*-1 position of phospholipids is a phospholipase A_1 (PLA_1), and at the *sn*-2 position a phospholipase A_2 (PLA_2). A phospholipase that cleaves the phosphodiester bond on the glycerol side is a phospholipase C (PLC) and on the base side a phospholipase D (PLD) (Fig. 3).

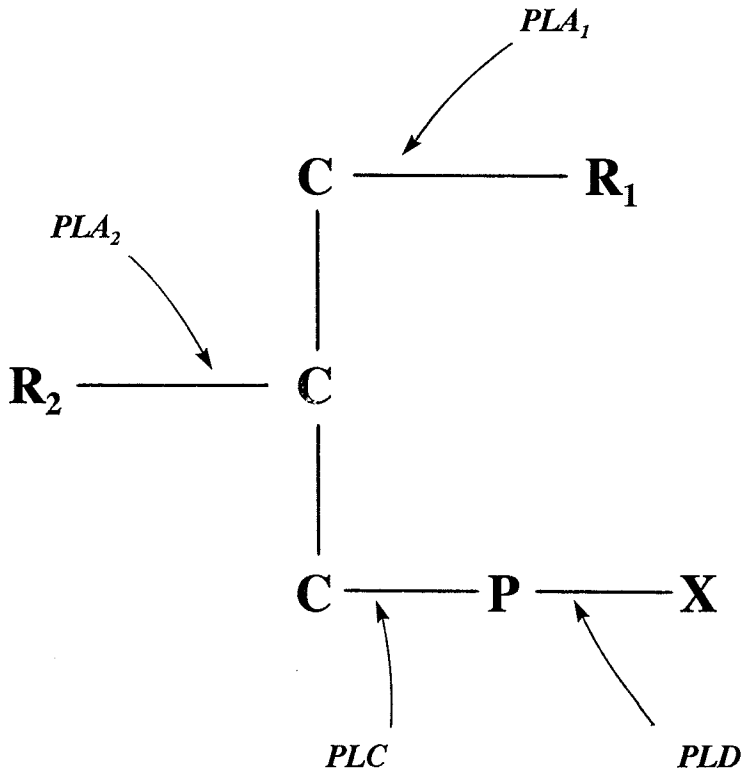

Fig. 3. Hydrolysis of phospholipids by phospholipases.

R_1 and R_2, fatty acids; X, polar head group.

AA, esterified at the *sn*-2 position on the glycerol backbone of the membrane phospholipids can be liberated directly by PLA_2 or indirectly by other phospholipases (Axelrod *et al.*, 1988; Dennis *et al.*, 1991). Mechanisms and regulations of the phospholipases and other related enzymes which liberate AA from membrane phospholipids follow.

Phospholipase A_1 pathway

Phospholipase A_1 (PLA_1) cleaves the *sn*-1 ester bond of phospholipids producing free fatty acids and 2-acyl-lysophospholipids (2-LPL) (Fig. 3). Subsequently, AA-containing 2-LPL can be hydrolysed by lysophospholipase A_2 ($LPLA_2$) resulting in the generation of free AA. Mammalian PLA_1 has been purified and described in the membranes and cytosolic subcellular fractions in a number of cell types (Van den Bosch, 1980; Waite, 1987). Characteristics of the activity of PLA_1 vary depending on source, and sometimes are not specific. However, generally they are independent of Ca^{2+} and preferentially hydrolyse phosphatidylethanolamine (PE) as a substrate. To date, the protein structure of PLA_1 has not been elucidated, and the function of most PLA_1 is not clear. A possible role in remodeling the acyl group at the *sn*-1 position of membrane phospholipids has been suggested (Waite, 1987).

Little information is available on $LPLA_2$, which hydrolyses 2-LPL. Recently, Arthur and coworkers (Arthur,

1989; Badiani *et al.*, 1990; Badiani and Arthur, 1991) described $LPLA_2$ in the mitochondria and microsomes of guinea pig heart. The enzyme does not require Ca^{2+} for activity, and exhibits high specificity for 2-arachidonoyl- and 2-linoleoyl-containing ethanolamine glycerophospholipid (EGP) and choline glycerophospholipid (CGP) substrates over the 2-oleoyl- and 2-palmitoyl-containing counterparts. These findings suggest that the $PLA_1/LPLA_2$ pathway may be involved in the generation of free AA from cellular phospholipids. The regulation of the $PLA_1/LPLA_2$ pathway to generate free AA is yet to be characterized.

Phospholipase A_2 pathway

Phospholipases A_2 (PLA_2) are enzymes that hydrolyse the *sn*-2 ester bond of phospholipids generating free fatty acids and 1-acyl-lysophospholipids (1-LPL) (Fig. 3). The PLA_2 can be divided into two major groups, secretory PLA_2 ($sPLA_2$; also known as extracellular PLA_2) and cytosolic PLA_2 ($cPLA_2$; also termed intracellular PLA_2).

The most studied mammalian PLA_2 is the type I $sPLA_2$ abundant in pancreas (Verheij *et al.*, 1981). It is also present in lung (Sakata *et al.*, 1989) and kidney (Hanasaki and Arita, 1992). This enzyme is a single polypeptide chain of 14 kDa stabilized by six disulfide bonds (Davidson and Dennis, 1990). Another $sPLA_2$ (type II $sPLA_2$) has been found in minute amounts in extracellular fluids, platelets, placenta and other

tissues (Kramer *et al.*, 1990; Seilhamer *et al.*, 1990). The type II enzyme is also a single protein with a molecular mass of 14 kDa. It has only about 37% sequence homology with type I, but contains the highly conserved amino acid residues and sequences characteristic of all $sPLA_2$s sequenced (Kramer *et al.*, 1990). This enzyme is found in several tissues such as placenta, platelets and neutrophils (Mukherjee *et al.*, 1994).

Both types of mammalian $sPLA_2$ are active at neutral to alkaline pH and require Ca^{2+} as a cofactor. Generally, other divalent cations cannot be substituted for Ca^{2+} (Verheij *et al.*, 1981). In addition, both enzymes contain His and Asp at the active site (Kramer, 1993). Considering their involvement in the AA generation from phospholipids, these phospholipases do not exhibit any preference for specific fatty acid in the *sn*-2 position of membrane phospholipids. However, they are fairly selective toward the phospholipid class, in that they favor EGP or serine glycerophospholipid (SGP) and only poorly hydrolyze CGP (Kramer *et al.*, 1989). Thus their contribution to the release of AA remains to be established. Since they are extracellular, and AA is mainly associated with the inner plasma membrane leaflet, their effects may be negligible.

Recently, several groups have characterized a cytosolic PLA_2 ($cPLA_2$) in a number of cell types (reviewed by Glaser *et al.*, 1993; Kramer, 1993; Mayer and Marshall, 1993). Like the 14 kDa $sPLA_2$, this enzyme is active at neutral pH and Ca^{2+}-dependent. However, $cPLA_2$ is distinct from $sPLA_2$ in that: 1)

it exhibits preference for AA in the *sn*-2 position of phospholipid substrates (Cordella-Miele *et al.*, 1993; Mayer and Marshall, 1993), 2) it translocates from the cytosolic to the particulate fraction in high Ca^{2+} conditions (Channon and Leslie, 1990; Kramer *et al.*, 1991), and 3) it does not contain disulfide bonds (Mukherjee *et al.*, 1994). Using cDNA cloning, the primary structure of $cPLA_2$ has been elucidated from the monoblast cell line U937 (Clark *et al.*, 1991; Sharp *et al.*, 1991). The enzyme consists of 849 amino acids with molecular mass of 85-110 kDa, contains possible phosphorylation sites for Ser/Thr and Tyr, and no apparent disulfide bonds. Southern blot analysis of human genomic DNA indicates that $cPLA_2$ is present as a single copy gene and that there are no additional genes closely related to $cPLA_2$ (Clark *et al.*, 1991). The $cPLA_2$ has no significant overall sequence homology with $sPLA_2$ or any other proteins. However, it contains a 45-amino acid domain homologous with the Ca^{2+} binding domain of Ca^{2+}-dependent PKC isoforms. An expressed fragment containing a Ca^{2+}/lipid binding domain has been shown to translocate to membranes in Ca^{2+}-dependent fashion (Clark *et al.*, 1991). However, Ca^{2+} does not appear to be an essential cofactor for catalysis. Other divalent cations can be substituted for Ca^{2+}, indicating that Ca^{2+} is required for interfacial association with the membrane but not for hydrolytic activity (Wijkander and Sundler, 1992).

One of the important aspects of the 85 kDa $cPLA_2$ is that the enzyme prefers AA in *sn*-2 position of cellular

phospholipids. With various substrates (vesicles, mixed vesicles, micelles, and *E coli*), a clear preference for *sn*-2-arachidonoyl- over *sn*-2-oleoyl-phospholipid has been shown, while *sn*-2-palmitoyl-phospholipid is a poor substrate (Clark *et al.*, 1990; Diez *et al.*, 1992).

A group of Ca^{2+}-independent $cPLA_2$s has been found in all organs tested, with the higher levels in heart, brain, lung and liver (Pierik *et al.*, 1988). In myocardial tissue, Hazen *et al.* (1991) isolated a 40 kDa isozyme which is Ca^{2+}-insensitive and prefers *sn*-2-arachidonyl-containing plasmalogen substrates, which possess an ether link on the C_1 carbon of the glycerol backbone. A considerable amount of AA is esterified at the *sn*-2 position of plasmalogens (Arthur, *et al.*, 1985). Thus, plasmalogens may be a reservoir of AA in some cell types including heart and brain.

Regulation of phospholipase A_2

It appears that multiple and complicated mechanisms of regulation of PLA_2s for AA release exist due to multiple forms of the enzyme in various cell types (Rose *et al.*, 1985; Rodorf *et al.*, 1991). Three major factors in PLA_2 regulation have been investigated: G proteins, protein kinases, and intracellular Ca^{2+} levels.

G protein regulation of PLA_2, independent of the PLC and PKC pathways has been shown (Burch, 1989; Axelrod, 1990; Murakami *et al.*, 1993). For example, α_1-adrenergic receptor

activation of thyroid cells stimulates AA liberation via a G protein without inositol phosphate formation (Burch, 1989). Bradykinin receptor stimulation also activates AA release in fibroblast cells that is independent of phosphoinositide hydrolysis and is mediated by G proteins (Axelrod, 1990). The identity of the G protein subunit involved in PLA_2 regulation and the mechanism by which G proteins activate these enzymes are still unknown. It has been suggested that G_i-like proteins may regulate receptor-coupled PLA_2, since in various cell types AA release is inhibited after treatment of cells with PTX (Burch, 1989). In the rod outer segments of the eye, the activation of PLA_2 has been associated with a G_t protein, but interestingly the $\beta\gamma$ subunit combination of the G protein rather than the α subunit was involved in the activation sequence (Axelrod *et al.*, 1988). Since many receptors that are known to activate PLA_2s are also coupled to PLC, the possibility exists that PPI breakdown products by PLC stimulation may regulate PLA_2.

Phosphorylation of PLA_2, mediated by receptor-activated kinases may be involved in the regulation of enzyme activity, since it has the possible phosphorylation sites (Clark *et al.*, 1991). The role of PKC in the regulation of $cPLA_2$ has been demonstrated, although in many cells activation of PKC is not sufficient to induce liberation of AA from cellular phospholipids (Mayer and Marshall, 1993). Activators of PKC such as the phorbol ester, TPA and DAG increase the release of

AA induced by agonists or Ca^{2+} ionophore, whereas PKC inhibitors or down-regulation of PKC by pretreatment of cells with TPA desensitize receptor-coupled AA release (Mayer and Mashall, 1993). In addition to PKC, protein kinase A and tyrosine kinase appear to be involved in the regulation of PLA_2 (Wightman *et al.*, 1982; Bonventre *et al.*, 1990). Recently, Lin *et al.* (1993) and Nemenoff *et al.* (1993) demonstrated that, in CHO and COS cells, $cPLA_2$ is a substrate for mitogen-activated protein (MAP) kinase. MAP kinase-induced phosphorylation of Ser-505, the major site of $cPLA_2$ phosphorylation by phorbol ester treatment, leads to activation of the $cPLA_2$, and replacement of this residue with Ala abolishes phosphorylation and markedly reduces agonist-stimulated AA release from transfected cells (Lin *et al.*, 1993).

Cytosolic Ca^{2+} levels have been suggested to play the primary role in the regulation of intracellular PLA_2 (Van den Bosch, 1980). In fact, the Ca^{2+} ionophore, A23187 can induce maximal release of AA from membrane phospholipids, suggesting that receptor activation is not a prerequisite for activation of PLA_2 (Glaser *et al.*, 1993). It is possible that activation of PLA_2 may be secondary to receptor-mediated stimulation of PLC leading to the formation of $I(1,4,5)P_3$ that raises intracellular Ca^{2+} (possibly together with PKC activation via DAG). Extracellular Ca^{2+} concentrations also appear to regulate PLA_2 activity and AA liberation in some cell types such as

endothelial cells (Whatley *et al.*, 1989), C62B glioma cells (Brooks *et al.*, 1989), chinese hamster ovary cells (Felder *et al.*, 1990) and MDCK-D1 cells (Insel *et al.*, 1991). Since $cPLA_2$ contains an N-terminal domain that binds to membrane vesicles in Ca^{2+}-dependent fashion (Clark *et al.*, 1991), elevated cytosolic free Ca^{2+} mobilized from intracellular stores and/or derived from extracellular source may cause translocation of the enzyme to the membranes, to stimulate AA release from membrane phospholipids.

Activity of PLA_2 in certain cells may be regulated by the synergistic actions of multiple factors. For example, Lin *et al.* (1993) proposed a scheme for phosphorylation-dependent regulation of $cPLA_2$ by G-protein and Ca^{2+} in CHO cells. When cells are stimulated by ligands, PLC is activated via a G protein, leading to generation of $I(1,4,5)P_3$ and DAG, which respectively mobilize Ca^{2+} and activate PKC. Elevated cytosolic Ca^{2+} causes the translocation of $cPLA_2$ from the cytosol to the membrane where phospholipid substrate is located (Clark et al., 1991). PKC activates MAP-kinase, and the activated MAP-kinase, in turn, phosphorylates $cPLA_2$ at Ser-505, causing an increase in $cPLA_2$ activity (Lin *et al.*, 1993). Interestingly, MAP-kinase can be also activated by certain G proteins (Alblas *et al.*, 1993; Winitz *et al.*, 1993).

Several inhibitors of 14 kDa $sPLA_2$ have been identified or designed (Mobilio and Marshall, 1989). These inhibitors of 14-kDa $sPLA_2$ do not block the activity of 85-kDa $cPLA_2$ (Mayer

and Marshall, 1993). Recently, an effective inhibitor of 85-kDa $cPLA_2$ has also been reported (Bartoli *et al.*, 1994).

Phospholipase C pathway

Phospholipase C (PLC) is a phosphodiesterase which hydrolyses the glycerophosphate bond of phospholipids to generate DAG and the water soluble head groups (Fig. 3). DAG can be cleaved to AA by subsequent lipases, DAG-lipase and monoacylglycerol lipase (MAG-lipase) (Dixon and Hokin, 1984; Konrad *et al.*, 1994).

As described in the earlier section, multiple isoforms of PLC that catalyze PPI hydrolysis have been identified and classified into 3 types, PLC-β,PLC-γ and PLC-δ. PLC is regulated by two basic mechanisms depending on isozymes; the β-isozymes can be activated by G proteins of the G_q family, while the γ-isozymes are regulated by the tyrosine kinase activity intrinsic to the receptors for several growth factors.

It is well established that Ca^{2+}-mobilizing agonists, such as muscarinic- and α_1-adrenergic ligands activate PLC and induce generation of $I(1,4,5)P_3$ and DAG in many cell types including rat submandibular gland acinar cells (Fleming *et al.*, 1987). The liberated DAG can be metabolized further in at least two pathways. First, it can be phosphorylated to phosphatidic acid (PA) by DAG-kinase. Second, DAG-lipase

(possibly together with MAG-lipase) hydrolyses DAG to AA. The mechanism for AA release by the PLC/DAG-lipase pathway was proposed by two independent groups (Rittenhouse, 1982; Majerus *et al.*, 1984). In most cells more than 50% the released AA is liberated from phosphoinositides, while other phospholipids (especially PC) contribute the remainder (Majerus *et al.*, 1984). Since PPI molecules contain AA at the *sn*-2 position, the bulk of the DAG produced by PLC contains AA which is subsequently liberated by the sequential action of DAG- and MAG-lipases.

Receptor-mediated release of AA by this pathway has been demonstrated in various cell types, including platelets (Rittenhouse, 1982; Majerus *et al.*, 1984), pancreatic minilobules (Dixon and Hokin, 1984), neutrophils (Balsinde *et al.*, 1991), alveolar macrophages (Errasfa, 1991), brain (Stronsznajder and Samochocki, 1991; Sun, 1992), endothelial cells (Whatley *et al.*, 1993), and pancreatic islets (Konrad *et al.*, 1994). In most of these studies, investigators labelled the cells with [^{3}H]AA or [^{14}C]AA, then treated them with agonists. They found increases in radioactive AA and DAG release (sometimes PA also) coupled with a reduction in the levels of PPI, but no changes in the amounts of other phospholipids.

Inhibition of AA generation in this pathway has been reported. A specific inhibitor of PLC, U73122 (1-{6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-1H-pyrrole-

2,5-dione) has been found to block $I(1,4,5)P_3$ production, Ca^{2+} increase and AA release in a number of agonist-stimulated cells, such as human platelets (Bleasdale *et al.*, 1990), erythroleukemia cells (Wu *et al.*, 1992), thyroid cells (Wang *et al.*, 1994), pancreatic acinar cells (Willems *et al.*, 1994) and β-TC3 cells (Chen and Hsu, 1995). In most studies, the inactive analogue, U73343 (1-{6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-2,5-pyrrolidine-dione)had no effects. Strosznajder and Samochocki (1992) also reported that neomycin, a putative PLC inhibitor, significantly reduced AA levels in carbachol-stimulated rat brain synaptoneurosomes.

Dixon and Hokin (1984) found that a specific inhibitor of DAG-lipase,RHC80267 [1,6-bis-(cyclohexyloximinocarbonylamino)-hexane] inhibited AA levels in caerulein-stimulated pancreatic minilobules with a corresponding increase in DAG concentration. Recently, Konrad *et al.*, (1994) demonstrated that the muscarinic agonist, carbachol increased concentrations of free AA in pancreatic islets, and that this carbachol-AA response was inhibited by RHC80267 in a dose-dependent manner. The blocker was very specific to DAG-lipase, and it did not affect other lipases including MAG-lipase (Konrad *et al.*, 1994).

PLC requires Ca^{2+} to hydrolyse PPI and an increase in free Ca^{2+} leads to a dose-dependent activation of the enzyme (Eberhard and Holz, 1988; Smith and Waite, 1992).

Phospholipase D pathway

PLD cleaves the phosphate diester bond of phospholipids, generating phosphatidic acid (PA) and the corresponding water-soluble headgroup (Fig. 3). The PA can be metabolized by a PA phosphohydrolase (PAP) to produce DAG, which in turn can be hydrolysed by a DAG lipase releasing AA as discussed in PLC pathway.

The mammalian PLD was initially characterized by Kanfer and co-workers in 1970s (Saito and Kanfer, 1975). However, the important role of the enzyme in cell regulation has been realized only fairly recently after observations that the enzyme is involved in the generation of DAG, a PKC activator. It has been suggested that many of the agonists which stimulate $PI(4,5)P_2$ hydrolysis also promote phosphatidylcholine (PC) hydrolysis in numerous cell types (reviewed by Billah and Anthes, 1990; Exton, 1990). When cells are treated with the agonists, *e.g.* carbachol and bradykinin, the typical pattern is a biphasic increase in DAG, with an initial, rapid and transient peak followed by a more slowly-developing and sustained accumulation (Exton, 1990; Thompson *et al.*, 1993; Zheng *et al.*, 1994). The first peak represents a low concentration of DAG, and is associated with increases in $I(1,4,5)P_3$ and cytosolic Ca^{2+} levels. The second phase is much greater and generated in parallel with a similar amount of choline and/or phosphocholine, indicating that the DAG is released by PC hydrolysis (Exton, 1990). Proof of PC breakdown

has been supported by an analysis of molecular species of DAG. In a number of cells stimulated with agonists, DAG formed in the first phase contains predominantly those fatty acids found in the phosphoinositide pool, stearic- and arachidonic acids, whereas the later phase contains more saturated fatty acids, typically found in PC (Kennerly, 1987; Pessin and Raben, 1989; Augert *et al.*, 1989; Horwitz, 1991; Thompson *et al.*, 1993).

The identification of phospholipases involved in PC hydrolysis has been complicated, since breakdown products of PC by PLC (DAG and phosphocholine) and by PLD (PA and choline) are rapidly interconvertible through highly active, specific kinases and phosphatases. It was initially thought that PC hydrolysis in response to agonists mainly resulted from activation of PLA_2 and PC-specific PLC since the products observed were AA and DAG. Recently, however, growing evidence indicates that the major mechanism of the second DAG generation from PC is the activation of PLD (for reviews, Billah and Anthes, 1990; Exton, 1990; 1994b). The recognition that PLD is a major enzyme involved in PC hydrolysis came from the following evidence. First, accumulation of PA frequently preceded that of DAG (Trilivas and Brown, 1989; Thompson et al., 1990; Exton, 1994b). Second, more importantly, PLD has a unique catalytic activity on PC. In the presence of alcohol such as ethanol or butanol, PLD catalyzes a transphosphatidylation reaction that transfers the phosphatidyl moiety to the alcohol, forming a

phosphatidylalcohol (Dawson, 1967). No other enzyme can catalyze this reaction. When cells are activated with agonists in the presence of ethanol, phosphatidylethanol (PEt) is formed (Kobayashi and Kanfer, 1987; Billah and Anthes, 1990). Because of the metabolic stability of PEt, this method has been applied to measure PLD activity in various cell systems (for reviews, Billah and Anthes, 1990; Shukla and Halenda, 1991; Thompson *et al.*, 1993; Exton, 1994b).

Regulation of phospholipase D

Compared with PLA_2 and PI-specific PLC, the mechanism of agonist-stimulated PLD activity on PC hydrolysis has not been clearly elucidated. One of the major difficulties in this area is that PLD has not been purified and fully characterized. However, in many cases, it is suggested that PLD activation is a downstream effect of the preliminary hydrolysis of $PI(4,5)P_2$ by PLC, which produces two second messengers: DAG, a PKC activator and $I(1,4,5)P_3$, a intracellular Ca^{2+} mobilizer (Cockcroft, 1992; Plevin *et al.*, 1992; Exton, 1994b; Guillemain and Rossignol, 1994; Zheng *et al.*, 1994). In support of this, phorbol esters, PKC activators stimulate PLD activity in most types of cells so far examined suggesting that the kinase plays an important role in activation of the enzyme (for reviews, Exton, 1990; 1994b; Billah and Anthes, 1990; Shukla and Halenda, 1991; Thompson *et al.*, 1993). In addition, inhibitors of PKC such as K252a, H-7, staurosporine,

Ro-31-8220 or sphingosine block the effects of agonists and phorbol esters. Down-regulation of PKC by prolonged TPA treatment has a similar effect in various cell types (Martinson *et al.*, 1990; Reinhold *et al.*, 1990; Cook *et al.*, 1991; Sadoshima and Izumo, 1993; Thompson *et al.*, 1993; Exton, 1994b; Plevin *et al.*, 1994). However, in many of these studies, the inhibitors caused partial or no inhibition of PLD activation by agonists even though they abolished or substantially reduced the activation by phorbol esters (Liscovitch and Amsterdam, 1989; Cook *et al.*, 1991; Kanoh *et al.*, 1992; Llahi and Fain 1992). These results indicate that PKC-independent pathway for a agonist-induced PLD activation may also operate in certain cell types.

It has been reported that activation of PLD is dependent on Ca^{2+} concentrations. PLD is stimulated by the Ca^{2+} ionophores, ionomycin and A23187 in several cell types (for reviews, Billah and Anthes, 1990; Thompson *et al.*, 1993). Chelators of extracellular Ca^{2+} (EGTA) and intracellular Ca^{2+} (BAPTA/AM) inhibit the agonist-mediated PLD activation in a number of cell types, including erythroleukemia cells (Wu *et al.*, 1992), pulmonary artery endothelial cells (Natarajan and Garcia, 1993) and parotid acini (Guillemain and Rossignol, 1994). Furthermore, two inhibitors of $PI(4,5)P_2$-PLC hydrolysis, U73122 and neomycin, reduced levels of $I(1,4,5)P_3$, Ca^{2+} mobilization and PLD activation in agonist-stimulated-erythroleukemia cells (Wu *et al.*, 1992) and -pituitary

gonadotrophs (Zheng *et al.*, 1994). These findings indicate that PLD can be activated by the receptor-mediated rise in Ca^{2+} concentration, and support the proposal that the PLD activation may be a downstream effect of $PI(4,5)P_2$-PLC hydrolysis in certain cells. However, the cellular mechanism of PLD activation by Ca^{2+} has not been clearly established. Wu *et al.*, (1992) suggested that elevated Ca^{2+} may cause the translocation of PLD from cytosol to membranes, or that the ion may bind directly to PLD, resulting in activation of the enzyme. It is also possible that Ca^{2+} activates Ca^{2+}-dependent PKC, which in turn stimulates PLD as noted above.

There is some evidence that activation of PLD is mediated by G-proteins. Addition of stable GTP analogues such as $GTP_\gamma S$ to permeabilized cells or plasma membranes caused the activation of PLD (for reviews, Billah and Anthes, 1990; Exton, 1990; Cockcroft, 1992; Thompson *et al.*, 1993). Since in many systems, PLD activation is secondary to the $PI(4,5)P_2$-PLC effect, the G_q protein coupled $PI(4,5)P_2$ is a probable effector. However, in HL60 cells metabolically inhibited by neomycin, where PLC activation was abolished, it was still possible to stimulate PLD activation with $GTP_\gamma S$ (Geny and Cockcroft, 1992), suggesting that a G-protein may be directly involved in regulating PLD. Whether a novel G-protein, discrete from G_q family or a conventional heterotrimeric G-protein is associated with PLD activation is yet to be defined. Recent results suggest that a low-molecular-weight

GTP-binding protein, ADP-ribosylating factor (ARF) may also activate PLD in HL60 cells (Brown *et al.*, 1993; Cockcroft *et al.*, 1994) and rat brain (Massenburg *et al.*, 1994). Moss and Vaughan (1993) reported that this molecule can act as an ADP-ribosylation factor for cholera toxin action on $G_{s\alpha}$. ARF has been also involved in the regulation of vesicle protein trafficking (Rothman and Orci, 1992; Khan *et al.*, 1993; Moss and Vaughan, 1993). These findings suggest that ARF-PLD activation may cause changes in the lipid composition of membranes (Khan *et al.*, 1993).

Tyrosine phosphorylation appears to be involved in PLD activation. Receptors possessing intrinsic tyrosine kinase activity, such as those for EGF and PDGF can stimulate PLD in fibroblasts (Ben-Av and Liscovitch, 1989; Fisher *et al.*, 1991; Cook and Wakelam, 1992). Pretreatment of fibroblasts with tyrosine kinase inhibitors, such as AG18 and ST638, reduced both tyrosine phosphorylation and PLD activity (Bourgoin and Grinstein, 1992; Cook and Wakelam, 1992), while the phosphoprotein phosphatase inhibitor, pervanadate, increased tyrosine phosphorylation and PLD in fibroblasts (Bourgoin and Grinstein, 1992) and neutrophils (Uings *et al.*, 1992). The tyrosine phosphorylation-stimulated PLD activation was not influenced by a DAG kinase inhibitor, R590122 (Bourgoin and Grinstein, 1992) or a PKC inhibitor, Ro-31-8220 (Cook and Wakelam, 1992), suggesting that this PLD activation was not dependent on PKC activation. However, the mechanism of the

tyrosine kinase activity on PLD stimulation remains unknown.

Although membrane PC contains a lower percentage of arachidonic acid at the *sn*-2 position than do PPIs (Ojima-Uchiyama *et al.*, 1988), the lipid appears to be a potential source of free AA since PC is the major phospholipid in cell membranes. As noted above, the simplest route of AA release is its direct cleavage from the *sn*-2 position by a action of PLA_2. Another possible pathway of AA liberation is mediated by sequential actions of PLD/PAP/DAG-lipase. This pathway has been described in mast cells (Lin *et al.*, 1991; Ishimoto *et al.*, 1994) and endothelial cells (Mattila *et al.*, 1993). In endothelial membranes, Mattila *et al.* (1993) found that interferon-γ (INF-γ) stimulated AA release. AA liberation by INF-γ was not modified by blockers of the PLA_2 pathway (bromophenacyl bromide) or the PLC pathway (neomycin). However, the PAP inhibitor, propranolol, inhibited the release of DAG and additional AA from PA derived from PC by PLD hydrolysis. Ishimoto *et al.* (1994) also reported that treatment of mast cells with ionomycin stimulated AA release, and that this effect was partially blocked by bromophenacyl bromide. Addition of ethanol to the cells reduced the ionomycin-AA response to 40% of control levels with the elevation of PEt formation. Propranolol inhibited AA liberation, but increased PA accumulation in dose-dependent manner (Ishimoto *et al.*, 1994). These results indicate that, at least in part, AA liberation is mediated by the PLD pathway in certain cell types.

Aims of the Present Study

There is accumulating evidence that arachidonic acid may act as a second messenger that regulates key cellular functions including the phosphoinositide effect. Free AA can be liberated from membrane phospholipids by the receptor-mediated actions of phospholipases and other enzymes. Recent results suggest that PLD may be involved in the production of lipid signalling molecules including AA in a number of cell types. Since nothing is known about the role of AA in signal transduction in submandibular glands, the present study was undertaken. The specific aims were (1) to examine the regulation of the phosphoinositide cycle activity by AA and to determine the mechanism of action of its regulatory effects, (2) to investigate the major membrane phospholipid sources of AA and establish the agonist-coupled signalling pathways leading to the release of the fatty acid.

CHAPTER II

REGULATION OF THE PHOSPHOINOSITIDE SIGNAL TRANSDUCTION PATHWAY BY ARACHIDONIC ACID IN RAT SUBMANDIBULAR ACINAR CELLS[1]

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Abstract

Modulation of the phosphoinositide signal transduction pathway by arachidonic acid (AA) in collagenase-dispersed rat submandibular acinar cells was investigated. The muscarinic agonist, carbachol, stimulated PI(4,5)P_2 hydrolysis and the generation of I(1,4,5)P_3 to five-fold the control levels. This response was inhibited by 75% on pretreatment of cells with AA. The AA inhibitory effect was not duplicated by a range of prostaglandins and leukotrienes and was not reversed by blockers of the cyclooxygenase and lipoxygenase synthetic pathways, indicating that AA action was not mediated by eicosanoid metabolites. Additional experiments confirmed that the enzyme, protein kinase C, was also not a mediator of the AA effect. Arachidonic acid did not affect the uptake of radioactive inositol into acinar cells, but it did inhibit the incorporation of inositol into inositol phospholipids of the phosphoinositide cycle. In studies on inositol phospholipid turnover, AA alone reduced the level of PI(4,5)P_2 but not of PI(4)P or PI. Under conditions of phosphoinositide cycle stimulation with carbachol, AA significantly lowered PI(4,5)P_2 and PI(4)P but not PI. These findings suggest that arachidonic acid may regulate the phosphoinositide response by inhibiting the synthetic phase of the cycle at a locus distal to PI generation.

Introduction

Arachidonic acid (AA) is the precursor of a range of metabolites (*e.g.*, prostaglandins, thromboxanes, leukotrienes), known collectively as eicosanoids. These have widespread regulatory roles in many physiological processes of the cardiovascular, immune, and reproductive systems and others (Shimizu and Wolfe, 1990). It has recently become apparent that not all actions of AA are mediated by its eicosanoid products and that the acid itself may act as a second messenger that modulates key cell regulators such as cytosolic calcium levels and ion channel activity (Wolf *et al.*, 1986; Naccache *et al.*, 1989). The sources of liberated AA are membrane phospholipids, from which the acid may be released directly by the action of phospholipase A_2, or indirectly, after initial hydrolysis of the lipids by phospholipase C (PLC) or phospholipase D (Axelrod *et al.*, 1988; Dennis *et al.*, 1991).

Earlier work in our laboratory (Fleming *et al.*, 1987) showed that calcium-mobilizing agonists, such as muscarinic- and α_1-adrenergic ligands, regulated exocrine responses in submandibular gland acinar cells by stimulating the phosphoinositide signal transduction pathway. These agents activate PLC and cause the hydrolysis of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] with the release of the second messenger products inositol 1,4,5-trisphosphate [$I(1,4,5)P_3$]

and diacylglycerol (DAG). It has been demonstrated that the phosphoinositide effect can be modulated by AA in a number of cell types, including placental cells (Zeitler and Handwerger, 1985) and platelets (Fan *et al.*, 1990). Since AA liberation could be secondary to PLC action, it has been proposed that the AA effect on phosphoinositide metabolism represents a negative feedback regulatory mechanism (Chaudhry *et al.*, 1989).

Since nothing is known about the potential regulatory actions of AA on signal transduction in mucin-secreting salivary glands, the present study was undertaken to determine whether the fatty acid might regulate the phosphoinositide response in rat submandibular acinar cells.

Materials and methods

Materials

Nembutal (Pentobarbital sodium) was supplied by Abbot Labs. (Toronto, Ontario). Purified collagenase, CLSPA grade, was obtained from Worthington (Freehold, NJ). Hank's Balanced Salt Solution (HBSS), HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer and minimal essential medium (MEM) amino acid were purchased from Gibco Canada Inc. (Burlington, Ontario). *Myo*-[2-^{3}H]inositol ([^{3}H]inositol), adenosine 5'-[γ-^{32}P]-triphosphate ([^{32}P]ATP) and the protein kinase C (PKC) assay kit were products of the Amersham Corp. (Arlington Heights, IL). Arachidonic acid and other fatty acids were provided by Nucheck Prep (Elysian, MN). BW755C (3-amino-1-(-3 trifluromethyl phenyl)-2-pyrazoline) was a gift from Burroughs Welcome Inc. (Kirkland, Quebec). Ecolume scintillation cocktail solution was obtained from ICN Biomedicals Canada Ltd (Mississauga, Ontario). All other reagents were from Sigma (St. Louis, MO).

Preparation of cells

Male Sprague-Dawley rats (250-300 g body weight) were subjected to anaesthesia by an injection of Nembutal (100 mg/kg body weight) and blood was drained via the vena cava. The submandibular glands were removed, chopped into small pieces and pooled, then the acinar cells were prepared by

enzymatic dissociation as described previously (Fleming *et al.*, 1987). The fragments were incubated at 37° C for 60 min in 12.5 ml/rat of modified Ca^{2+}-free HBSS, containing 500 U purified collagenase, 12.5 mg hyaluronidase, 0.2% bovine serum albumin (BSA), 4.8 mM $NaHCO_3$, 5 mM β-hydroxybutyrate, 5 mM inosine, 0.5 mM adenine, 9 mM KCl. The cell suspensions were supplemented with 2% MEM amino acids (by vol), buffered to pH 7.2 with 15 mM HEPES and maintained under atmospheric conditions. The tissues were sheared mechanically by repeated pipeting at 15 min intervals during the incubation and the preparation was filtered through a nylon mesh (320 μm). The resulting dispersed cells were washed twice in fresh culture medium containing Ca^{2+} (1.3 mM) and resuspended at a concentration of 80 mg wet weight *per* ml for experimental treatment.

Phosphoinositide hydrolysis studies

For radiolabelling, the cell:volume ratio was increased to 300 mg cell wet weight/ml of culture medium/17 μCi *myo*-[2-^{3}H]inositol and the preparations were incubated for 60 min at 37° C. The cells were washed once in fresh BSA-free culture medium containing 5 mM inositol, twice in BSA- and inositol-free medium, then resuspended in 1 ml aliquots for experimental treatment.

Cells were exposed to AA (1 nM - 100 μM, prepared at a stock concentration of 10 mM in ethanol) for five min, then to

carbachol (100 μM) for an additional five min. Reactions were terminated with a final concentration of 10% ice-cold trichloroacetic acid (TCA). After centrifugation at 2,000 *g* for 20 min, the supernatant fluid was neutralized by 5 M NaOH, and the water-soluble, radiolabelled inositol phosphates were separated by anion exchange chromatography as follows (Fleming *et al.*, 1987). The neutralized supernatant mixture was loaded on to a 1.5 x 12 cm Econo-Pac column (Bio Rad) which contained 1.5 g of Bio Rad AG 1- X8 resin (100-200 mesh, formate form). Free inositol and glycerophosphoinositol were eluted with 15 ml of water and 20 ml of 5 mM disodium tetraborate/60 mM sodium formate, respectively. Inositol 4-phosphate [I(4)P]; inositol 1,4-bisphosphate [I(1,4)P_2] and I(1,4,5)P_3 were then sequentially eluted with 15 ml of the respective buffers, 0.1 M formic acid/0.2 M ammonium formate; 0.1 M formic acid/0.4 M ammonium formate; 0.1 M formic acid/1.0 M ammonium formate. Two ml samples of the eluate were combined with 10 ml Ecolume, mixed vigorously, then counted [^{3}H] by Beckman LS 5801 β-counter.

For determination whether AA modifications of the phosphoinositide effect were mediated by eicosanoid products, two additional series of experiments were carried out. In the first, cells were incubated with prostaglandins of the 2-series, or leukotrienes, (1 nM - 1 μM) before carbachol treatment. In the second series of experiments, cells were exposed to indomethacin (10 μM), a blocker of the

cyclooxygenase (prostaglandin) pathway; or to nordihydroguaiaretic acid (NDGA, 10 μM), a blocker of the lipoxygenase (leukotriene) pathway; or to BW755C (10 μM), a blocker of both pathways (Higgs and Vane, 1983) for 10 min, then to AA and carbachol as described above. The effects of these treatments on I(1,4,5)P_3 levels were compared.

For comparison with the AA effect, the actions of the following four saturated and unsaturated fatty acids on the carbachol-I(1,4,5)P_3 response were assessed: stearic (18:0), linoleic (18:2ω6), arachidic (20:0) and dihomo-γ-linolenic (20:3ω6).

Protein kinase C assay

It has been demonstrated that, in some cell types, arachidonic acid stimulates the enzyme protein kinase C (Murakami and Routtenberg, 1985; Dell and Severson, 1989; Fan *et al.*, 1990). Experiments were therefore carried out to determine whether AA modulation of the phosphoinositide effect might be mediated by PKC in submandibular cells.

PKC activation is characterized by the enzyme's translocation from cytosol to membranes. We have shown that phorbol ester activators deplete cytosolic- and enhance membrane-bound PKC in submandibular cells (Fleming *et al.*, 1992). Cytosolic PKC was therefore measured by using an assay kit (Amersham) with a high-affinity synthetic substrate. The PKC activator was the phorbol ester, 12-*O*-

tetradecanoylphorbol-13-acetate (TPA, 3 μM) and assay buffer also contained the cofactors Lα-phosphatidyl-L-serine (8 mol %) and Ca^{2+} (12 mM) in 50 mM Tris/HCl.

Collagenase-dispersed cells were treated with TPA (3 μM) or AA (100 μM) for 15 min at 37° C. The cells were then centrifuged at 200 *g* for 30 s and the supernatant culture medium discarded. The cell pellets were sonicated in ice-cold sample buffer (50 mM Tris/HCl, 5 mM EDTA, 10 mM EGTA, 0.3% β-mercaptoethanol, 2 μg/ml leupeptin and 50 μg/ml phenylmethylsulphonyl fluoride) for 7 s at 10% power (Microson Ultrasonic cell disrupter, XL 2005, 1/8" microprobe; Heat Systems Inc., Farmingdale, NY), then centrifuged at 15,000 *g* for 10 min at 4° C. The clear supernatant was diluted to 16 μg cell protein/ml and assayed for PKC as the cytosolic solution.

Protein kinase C remaining in the cytosol was activated in 25 μl of activator buffer, containing fresh TPA, and 25 μl [^{32}P]ATP (0.25 μCi) and incubated for 15 min at 25° C. The reaction was halted with 100 μl acidic stop reagent. ^{32}P-phosphorylated substrate in 125 μl aliquots was localized on binding papers which were washed three times in 5% acetic acid. The papers were placed in vials with 6.5 ml scintillation fluid and [^{32}P] activity was measured by scintillation counting.

Inositol incorporation and phosphoinositide turnover studies

The effect of AA on the uptake of inositol into submandibular

cells was examined. Cells were exposed to AA (100 μM) or left as controls in medium containing 17 μCi *myo*-[2-^{3}H]inositol *per* ml. Over a 60-min time course, 1-ml samples were taken, washed three times in phosphate-buffered saline and centrifuged at 200 *g* for three min. Total inositol in the pellets was measured by scintillation counting.

In other experiments, the effect of AA on the incorporation of inositol into phosphoinositides was examined. Cells were labelled with *myo*-[2-^{3}H]inositol as before for 60 min, in the presence or absence of AA. Samples were precipitated with cold 10% TCA, then washed once with 1 ml of 5% TCA containing 1 mM EDTA, and once with 1 ml H_2O. Lipid in the precipitate was extracted twice with 1.5 ml of a mixture of chloroform, methanol and HCl (100:100:1, by vol) and once with 1.0 ml of a mixture of chloroform, methanol and HCl (200:100:1, by vol). The three extracts were combined, and 1.1 ml of 0.1 N HCl and 1.5 ml chloroform were added to produce a biphasic separation. The organic (lower) fraction containing phosphoinositides was dried under a stream of N_2 and its lipids deacylated to produce their water-soluble glycerophosphoesters as follows (Creba *et al.*, 1983).

The dried lipid extracts were reconstituted in 1 ml of chloroform, then 0.2 ml of methanol and 0.2 ml of 1 M NaOH in methanol/water (19:1, by vol) were added. The mixture was left 20 min at room temperature before the addition of 1 ml of chloroform, 0.6 ml of methanol and 0.6 ml of water. The

samples were mixed completely and centrifuged at 1,000 *g* for 10 min. Of the aqueous (upper) phase, 1 ml was collected and neutralized with boric acid. The water-soluble glycerophospho derivatives of PI, PI(4)P, and PI(4,5)P_2 were separated according to the method of Creba *et al*. (1983) with minor modification.

The mixture of deacylated phospholipids was loaded on to an ion exchange chromatography column as described above. After washing the column with 20 ml of water, glycerophosphatidylinositol (from PI) was eluted with 20 ml of 5 mM disodium tetraborate/0.18 M ammonium formate. The glycerophospho derivatives of PI(4)P and PI(4,5)P_2 were then sequentially eluted with 20 ml of the respective buffers, 0.1 M formic acid/0.3 M ammonium formate and 0.1 M formic acid/0.75 M ammonium formate. Three ml samples of the eluates were combined with 15 ml Ecolume, mixed thoroughly, and quantitated by [^{3}H] counting in a Beckman LS 5801 β-counter.

Experiments were also carried out to determine the effect of AA in the presence or absence of carbachol, on the turnover of phosphoinositides in cells labelled to equilibrium with [^{3}H]inositol. Cells were labelled for 60 min with *myo*-[2-^{3}H]inositol as above, then treated with AA (100 μM) alone for five min, carbachol alone (100 μM) for five min or AA for five min followed by carbachol for five min. The cells were then precipitated with TCA and the phosphoinositides deacylated and quantified as above.

In all experiments, AA and eicosanoid derivatives were prepared as concentrated solutions in ethanol. The ethanol concentration in culture medium was never more than 1%, and this vehicle was routinely added to control preparations.

Statistical analysis

Results were examined statistically by analysis of variance and Duncan's multiple range test. Values of $p < 0.05$ were considered significant.

Results

Phosphoinositide hydrolysis studies

Carbachol stimulated the generation of $I(1,4,5)P_3$ to five-fold the control level and of $I(1,4)P_2$ to 3.5-fold the control level in submandibular cells (Fig. 4). Preincubation of cells with AA inhibited the carbachol-$I(1,4,5)P_3$ response to 25% of its normal value ($p < 0.01$). A small reduction by AA of carbachol-induced levels of $I(1,4)P_2$ was also suggested but this was not statistically significant ($p > 0.05$). No changes from control values of I(4)P were observed with any of the treatments, and AA alone had no effect on inositol phosphates. Inhibition of carbachol-induced $I(1,4,5)P_3$ was not observed at AA concentrations lower than 100 μM. Assessment of cell viability by trypan blue exclusion confirmed that the AA effect was not due to membrane disruption.

Treatment of cells with indomethacin, NDGA, or BW755C before AA plus carbachol exposure did not reverse the AA inhibitory effect on carbachol-stimulated $I(1,4,5)P_3$ (Fig. 5). These blockers alone had no effect on phosphoinositide hydrolysis (data not shown). Furthermore, prostaglandins A_2, D_2, E_2 and $F_{2\alpha}$, and leukotrienes B_4, C_4 and D_4 in concentrations up to 1 μM did not mimic AA in reducing the carbachol-$I(1,4,5)P_3$ response (Fig. 6). Again, these agents alone had no effect on inositol phosphate levels (data not shown).

In studies with other fatty acids, the unsaturated

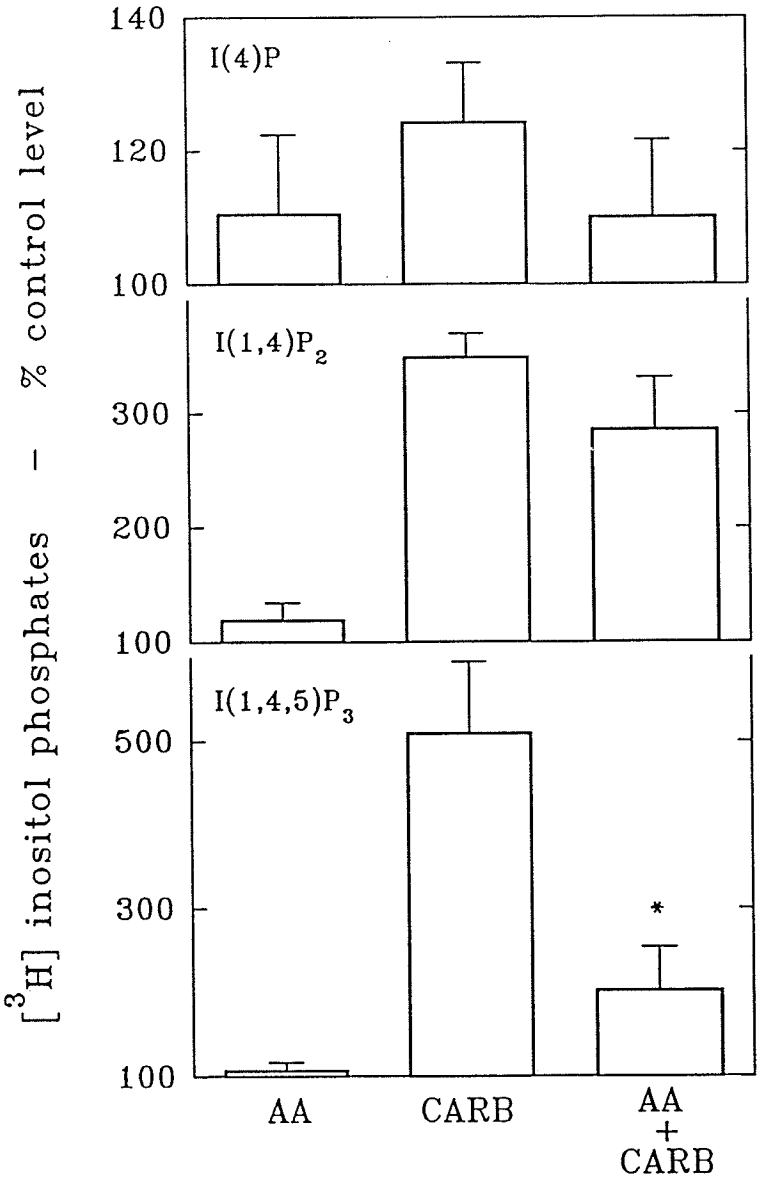

Fig. 4. Effects of arachidonic acid, carbachol or a combination of the two, on radiolabelled inositol phosphates in rat submandibular acinar cells.

Dispersed acinar cells were incubated for 60 min with *myo*-[2-^{3}H]inositol then exposed to arachidonic acid (AA, 100 μM, five min) or to carbachol (CARB, 100 μM, five min) or to AA (five min) followed by carbachol (five min). Inositol phosphates were extracted and quantified by anion exchange chromatography and scintillation counting as described in Materials and methods. Values are means ± SEM, n = 11, expressed as a percentage of control levels. *$p < 0.01$ compared with control response of 100%. Typical average observed counts for carbachol-treated samples in a single experiment were I(4)P, 15,975 dpm; I(1,4)P_2, 8,758 dpm; and I(1,4,5)P_3 3,683 dpm *per* replicate containing 8 mg cell protein.

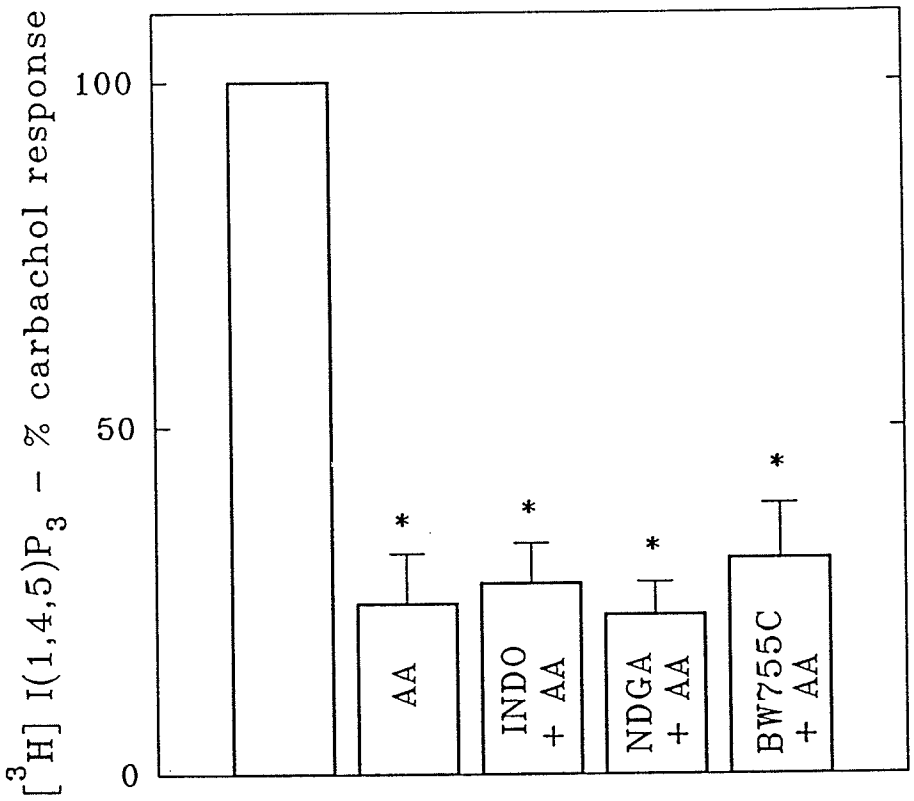

Fig. 5. Lack of effect of blockers of eicosanoid synthesis on the arachidonic acid inhibition of carbachol-stimulated $I(1,4,5)P_3$ in rat submandibular acinar cells.

Cells were preincubated with indomethacin (INDO, 10 μM), NDGA (10 μM) or BW755C (10 μM), for five min, then exposed to AA plus carbachol or to carbachol alone (100% response, first column) as in Fig. 4. Means ± SEM, n = 3. *$p < 0.01$ compared with carbachol response.

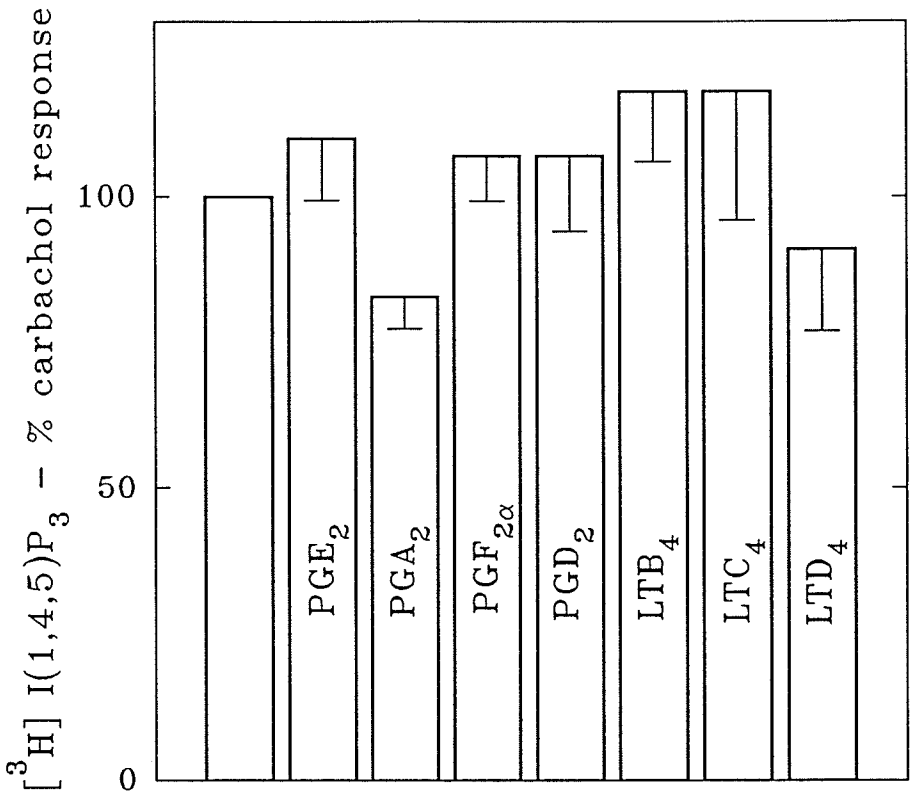

Fig. 6. Lack of effect of prostaglandins and leukotrienes on the carbachol-I(1,4,5)P_3 response in rat submandibular acinar cells.

Cells were exposed to a range of prostaglandins (PG) or leukotrienes (LT) (all 1 μM) for five min, followed by carbachol (100 μM) for five min; or to carbachol alone (first column, 100% response) for five min. I(1,4,5)P_3 levels were measured as in Fig. 4. Means ± SEM, n = 3. Lower concentrations of eicosanoids (1 - 100 nM) were also without effect.

species, γ-linolenic acid (20:3ω6) and linoleic acid (18:2ω6), duplicated the AA effect by inhibiting carbachol-induced I(1,4,5)P_3 to 49% and 37% of the normal carbachol response, respectively (Fig. 7). The saturated stearic (18:0)- and arachidic (20:0) acids, however, did not reduce the carbachol-I(1,4,5)P_3 response.

Protein kinase C activation

The phorbol ester, TPA, as a positive control, caused a reduction of 40% in the cytosolic level of PKC (Fig. 8), consistent with the enzyme's activation/translocation to the membrane compartment, as demonstrated in an earlier study in our laboratory (Fleming *et al.*, 1992). Arachidonic acid had no effect on PKC activation, indicating that its modulation of the phosphoinositide effect was not mediated by the enzyme.

Inositol incorporation and phosphoinositide turnover studies

Experiments on the uptake of radiolabelled inositol into submandibular cells showed that equilibrium conditions were reached by 60 min and that AA had no effect on either the magnitude or kinetics of uptake (Fig. 9).

Arachidonic acid did, however, inhibit the incorporation of inositol into phosphoinositides (Fig. 10; Table 1). For example, at 40 and 60 min, the respective radiolabelled PI(4,5)P_2 levels in AA-exposed cells were 45 and 65% of the corresponding control values (Table 1). [^{3}H]inositol-labelled

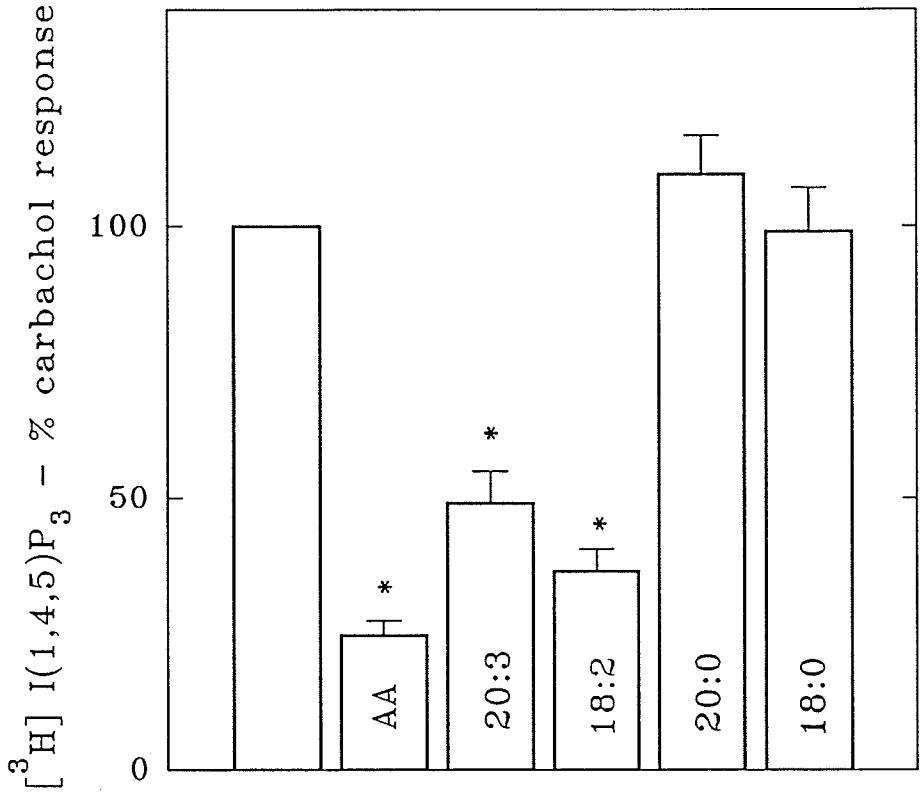

Fig. 7. Effect of saturated and unsaturated fatty acids on the carbachol-I(1,4,5)P_3 response in rat submandibular acinar cells.

Cells were exposed to saturated (20:0; 18:0) or unsaturated (18:2; 20:3; 20:4 (AA)) fatty acids (100 μM) for five min, followed by carbachol (100 μM) for five min; or to carbachol alone (first column, 100% response) for five min. I(1,4,5)P_3 was quantified as in Fig. 4. Means ± SEM, n = 3. *$p < 0.01$ compared with carbachol response.

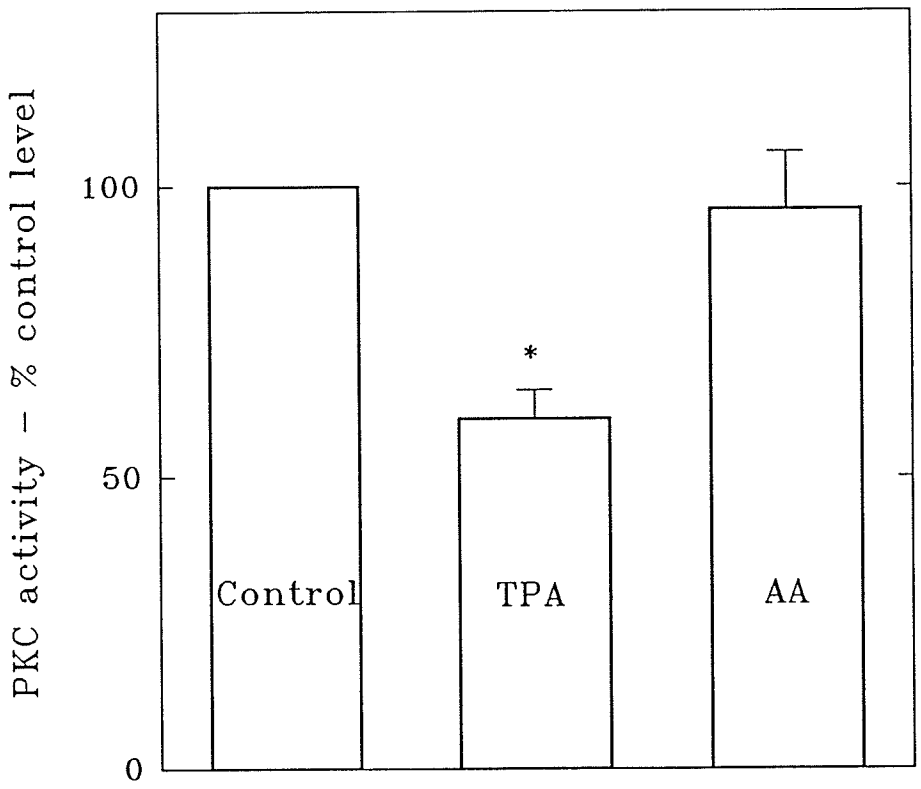

Fig. 8. Effect of the phorbol ester, TPA or arachidonic acid in lowering the cytosolic content of protein kinase C (PKC) in rat submandibular acinar cells.

Cells were exposed to TPA (3 μM) or AA (100 μM) or left as controls, for 15 min. The cells were then washed and assayed for PKC activity remaining in the cytosol by use of an assay kit (Amersham) as described in Materials and methods. Means ± SEM, n = 4. *p < 0.01 compared with control level.

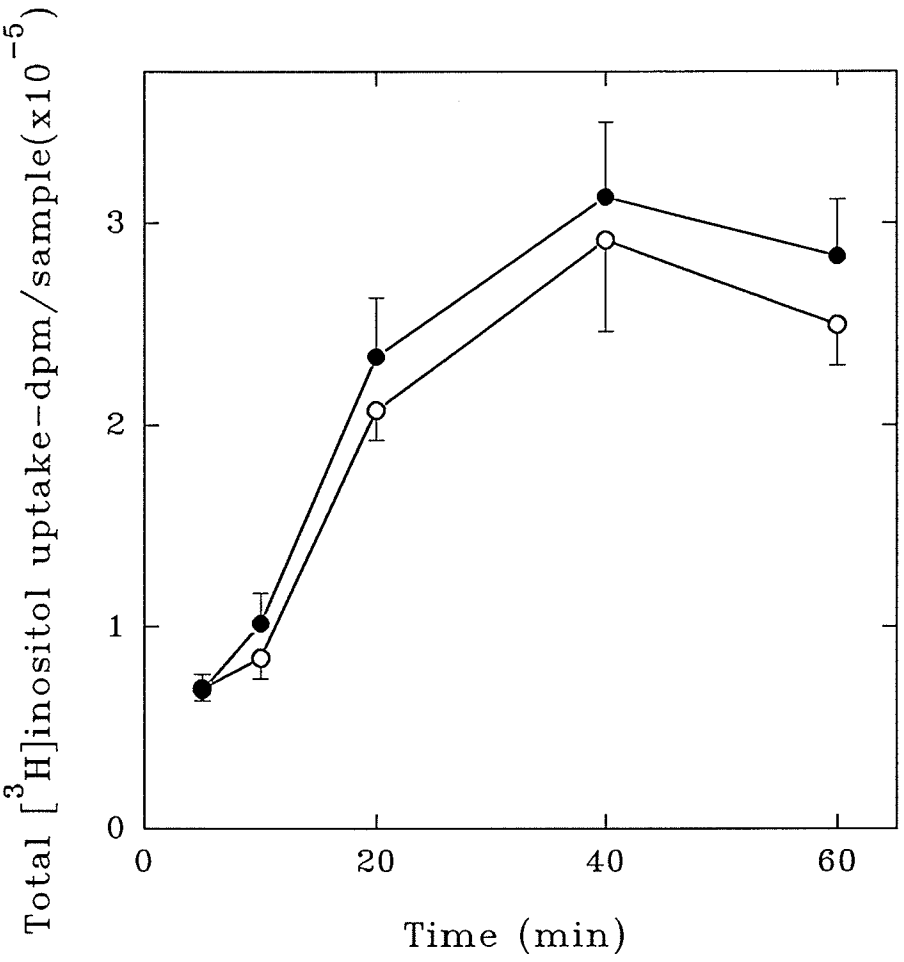

Fig. 9. Uptake of inositol into dispersed rat submandibular acinar cells in the presence or absence of arachidonic acid.

Cells were incubated with *myo*-[2-^{3}H]inositol (17 μCi/ml) in the absence (○) or presence (●) of AA (100 μM) over a 60-minute time course. One ml aliquots were sampled at the times indicated, the cells washed, and their total radiolabelled inositol content (free inositol, phosphoinositides and inositol phosphates) measured by scintillation counting. Means ± SEM of triplicate samples in a single experiment.

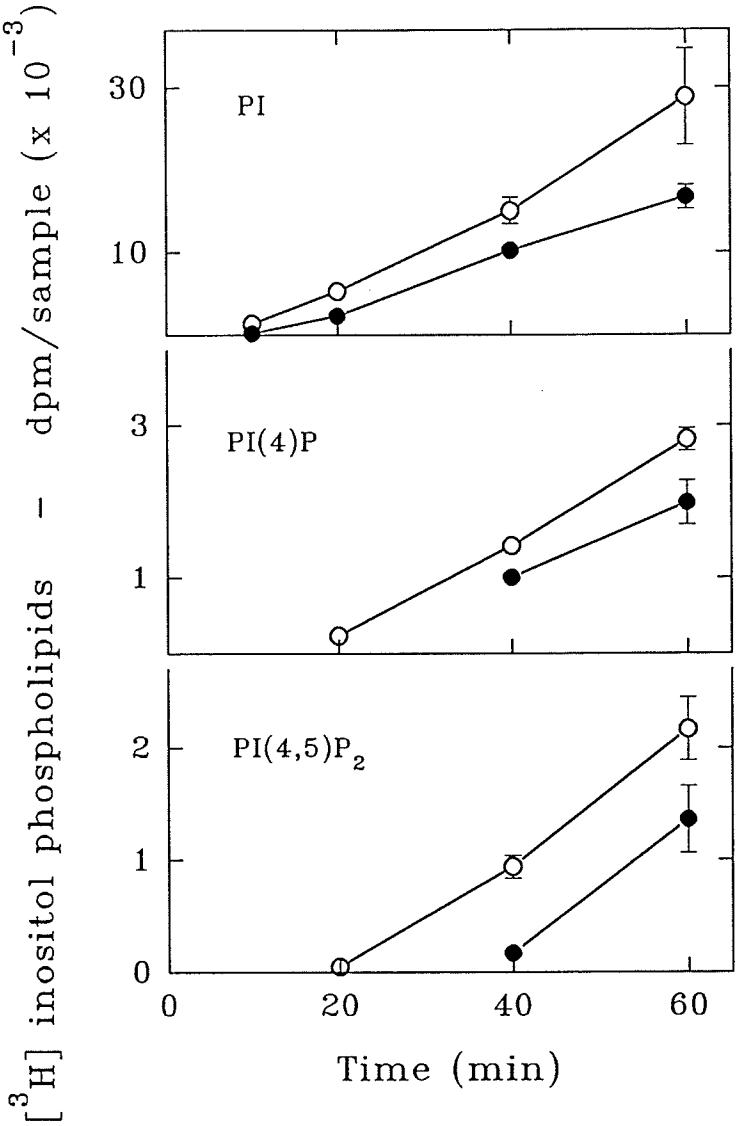

Fig. 10. Incorporation of inositol into submandibular acinar cell phosphoinositides in the presence or absence of arachidonic acid.

Cells were incubated with *myo*-[2-^{3}H]inositol (17 μCi/ml) in the absence (O) or presence (●) of AA (100 μM) over a 60 minute time course. Phosphoinositides were localized in the organic phase of a biphasic extract and deacylated to form their water-soluble glycerophospho derivatives, which were then separated by anion exchange chromatography as described in Materials and methods. Values are the averages of two replicates from a single experiment that was carried out four times with the same result. The bars show the range of values for each time point.

Table 1

Effect of arachidonic acid on the incorporation of inositol into phosphoinositides over 60 minutes in rat submandibular acinar cells

Time (min)	*Myo*-[2-^{3}H]inositol in phosphoinositides in cells exposed to arachidonic acid, percent of control levels		
	PI	PI(4)P	PI(4,5)P_2
10	21 ± 1		
20	37 ± 3		
40	63 ± 5	60 ± 8	45 ± 17
60	79 ± 9	73 ± 3	65 ± 6

All values are significantly reduced compared with the corresponding control response ($p < 0.05$).

Dispersed rat submandibular acinar cells were incubated with *myo*-[2-^{3}H]inositol (17 μCi/ml) in the presence or absence of AA (100 μM) for 60 min. Phosphoinositides were separated and quantified by scintillation counting as described in Materials and methods. Values are means ± SEM calculated from four separate experiments with duplicate samples in each.

PI(4)P and PI(4,5)P_2 were not detected before 20 min in controls and 40 min in AA-treated cells.

In further studies, the effect of AA on phosphoinositide turnover was examined in cells prelabelled to equilibrium with [^{3}H]inositol and activated with carbachol to stimulate the phosphoinositide cycle (Table 2). Carbachol reduced PI(4,5)P_2 levels to 49% of the control value but had no significant effect on PI or PI(4)P. Pre-treatment of cells with AA before carbachol, further reduced the PI(4,5)P_2 response to 26% of that in controls. Cells treated with a combination of AA and carbachol also displayed significantly lower levels of PI(4)P than did those exposed to either AA or carbachol alone. AA alone produced a small but significant ($p < 0.05$) reduction in labelled PI(4,5)P_2 but had no effect on PI or PI(4)P.

Table 2

Effect of arachidonic acid, carbachol, or a combination of both on inositol phospholipids in rat submandibular acinar cells

	Myo-[2-^{3}H]inositol in phosphoinositides, percent of control levels		
Treatment	PI	PI(4)P	PI(4,5)P_2
Control	100	100	100
AA	107 ± 9	85 ± 8	81 ± 5*
Carbachol	88 ± 7	86 ± 5	49 ± 6*
AA + Carbachol	89 ± 4	59 ± 9*+	26 ± 6*+

*significantly different from control, $p < 0.05$. +significantly different from carbachol response, $p < 0.05$

Dispersed rat submandibular acinar cells were labelled for 60 min with *myo*-[2-^{3}H]inositol, then treated with AA (100 μM, five min), carbachol (100 μM, five min) or AA (five min) followed by carbachol (five min). Inositol phospholipids were extracted, deacylated and quantified as described in Materials and methods. Values are means ± SEM, n = 3.

Discussion

Previous work in our laboratory showed that Ca^{2+}-mobilizing agonists of three classes activated the phosphoinositide signal transduction pathway in rat submandibular acinar cells, to cause hydrolysis of $PI(4,5)P_2$ and generation of $I(1,4,5)P_3$ (Fleming *et al.*, 1987). There is growing evidence that agonists may also stimulate the release of arachidonic acid secondary to the PLC-$PI(4,5)P_2$ effect, or *via* phospholipase A_2 hydrolysis of non-inositol phospholipids, and that liberated AA may act as a feedback regulator of the phosphoinositide cycle (Irvine *et al.*, 1979; Zeitler and Handwerger, 1985; Chaudhry *et al.*, 1989; Murphy and Welk, 1989; Maruyama, 1990). The nature of the AA regulatory response, however, is not consistent among different cell types. For example, AA has been reported to stimulate phosphoinositide cycle activity in placental cells (Zeitler and Handwerger, 1985), but to inhibit the phosphoinositide effect in pancreatic cells (Chaudhry *et al.*, 1987, 1989; Maruyama, 1990).

In the present study, arachidonate had an inhibitory effect on the phosphoinositide cycle under conditions of cycle activation with carbachol. This finding is consistent with those of Chaudhry *et al.* (1987) on pancreatic acini. However, while these workers demonstrated a concentration-dependent inhibitory response with AA, only an arachidonate concentration of 100 μM was effective in reducing

phosphoinositides in the present study. This may reflect different sensitivities to AA in the two experimental models, or indicate that AA was bound/inactivated by some component(s) of the culture medium or cell glycocalyx of the submandibular preparations. Arachidonate reduced the carbachol-I(1,4,5)P_3 response to about a quarter of its normal magnitude, but had no effect on inositol phosphate levels in resting, non-agonist-stimulated cells (Fig. 4). Since AA is readily oxidized to its eicosanoid metabolites, which may act as intracellular second messengers (Piomelli and Greengard, 1990; Shimizu and Wolfe, 1990), the AA inhibition of carbachol-induced I(1,4,5)P_3 was re-examined in the presence of blockers of the cyclooxygenase-prostaglandin pathway (indomethacin, BW755C) or of the lipoxygenase-leukotriene pathway (NDGA, BW755C). None of these agents reversed the AA inhibitory effect (Fig. 5). Moreover, prostaglandins A_2, E_2, D_2 and $F_{2\alpha}$, and leukotrienes B_4, C_4 and D_4 did not duplicate the AA effect in lowering carbachol-enhanced I(1,4,5)P_3 levels. These findings are consistent with earlier observations on pancreatic cells (Chaudhry *et al.*, 1989; Maruyama, 1990) that AA modulation of the phosphoinositide cycle is not mediated by eicosanoids.

Another potential mediator of arachidonate action is the enzyme, protein kinase C (PKC). Arachidonic acid activates PKC purified from bovine aorta (Dell and Severson, 1989) and rat brain (Murakami and Routtenberg, 1985) and also stimulates enzyme activity in intact platelets (Fan *et al.*, 1990). It has

been demonstrated that PKC, in turn, inhibits phosphoinositide turnover in many cell types, including neutrophils (Kato *et al.*, 1986) and insulinoma cells (Yamatani *et al.*, 1990). We have previously found that PKC activation in rat submandibular cells, as in many others, is characterized by a translocation of enzyme from cytosol to membranes (Fleming *et al.*, 1992). In the present study, while the positive control, TPA, caused a 40% reduction in cytosolic PKC, AA had no effect on enzyme translocation. This result suggests that AA effects on phosphoinositide metabolism in the submandibular model are independent of PKC mediation, although arachidonate action on kinase already bound to the plasma membrane cannot be ruled out.

Our findings that other unsaturated long-chain fatty acids duplicated the inhibitory effect of AA on carbachol-enhanced $I(1,4,5)P_3$ are also compatible with the earlier observations of Chaudhry *et al.* (1989) on pancreatic cells. This suggests that the presence of unsaturated bonds, rather than the absolute chain length of the fatty acids, may be the key factor in the $I(1,4,5)P_3$ inhibition effect. The physiological significance of this is not obvious, since little is known about the availability of unsaturated fatty acids other than arachidonate, their specific lipid sources and their mechanisms of release. Since both 18:2ω6 and 20:3ω6 can act as precursors of AA, it is possible that their effects may be due to AA synthesis. The lack of effect of saturated

fatty acids and the observation that cells continued to exclude trypan blue after treatment confirmed that the actions of AA and the other unsaturated species were not due to non-specific membrane perturbation by fatty acids.

The possible site of regulation by AA was investigated in experiments on inositol uptake and incorporation into phosphoinositides and on phosphoinositide cycle turnover under different conditions. In simple uptake studies, it was found that AA had no effect on the total amount of [^{3}H]inositol entering the cells over a time course that reached equilibrium between 40 and 60 min (Fig. 9). However, the incorporation of radioactive inositol into PI, PI(4)P and PI(4,5)P_2 was impaired in the presence of AA (Fig. 10; Table 1). This finding suggested an overall inhibition of synthesis of phosphoinositides but did not indicate the locus of inhibition, since a block at any position in the cycle could cause a reduction by feedback control. Turnover of phosphoinositides was therefore examined in cells that had been prelabelled to equilibrium with [^{3}H]inositol, then exposed to carbachol to activate the phosphoinositide effect. Arachidonate caused a significant decrease in PI(4,5)P_2 but not PI(4)P or PI in cells in the resting state (Table 2). When cells were stimulated with carbachol in the absence of arachidonate, PI(4,5)P_2 levels decreased, consistent with the agonist's effect of increasing I(1,4,5)P_3 (Fig. 4). However, when both AA and carbachol were present, the decrease in

$PI(4,5)P_2$ was significantly greater and a reduction of PI(4)P levels was now also detected. This suggests that the AA effect of depressing the carbachol-$I(1,4,5)P_3$ response was not due to the acid's inhibition of phospholipase C (PLC), since impaired PLC activity would be expected to enhance, not lower, $PI(4,5)P_2$ levels. In light of the inositol incorporation studies (Fig. 10), it is likely, therefore, that the AA effect on the phosphoinositide cycle is exerted in the phospholipid synthetic phase. Moreover, since no treatment lowered PI levels (Table 2), we propose that the regulatory site lies distal to PI synthesis but proximal to $PI(4,5)P_2$ synthesis.

Chaudhry *et al.* (1989) also showed that AA decreased $PI(4,5)P_2$ levels and, in addition, depleted cell ATP to 60% of the normal value in pancreatic cells. They proposed that reduced $PI(4,5)P_2$ could therefore result from a decreased availability of ATP as the donor of γ phosphate in the phosphorylation of PI to PI(4)P and of PI(4)P to $PI(4,5)P_2$ by the respective enzymes PI 4-kinase and PI(4)P 5-kinase. It is possible that such a mechanism may also operate in rat submandibular acinar cells. However, a general reduction in ATP levels would be expected to have widespread physiological effects, involving kinases, on many synthetic pathways, unless the targeted ATP were compartmentalized in a phosphoinositide-specific pool.

Another potential mechanism for the AA regulatory effect would be the fatty acid's direct inhibition of PI 4-kinase

and/or PI(4)P 5-kinase. We are currently investigating this possibility by direct assay of the kinases extracted from membranes of control and arachidonate-treated cells and by examining the effect of AA on the kinetics of purified kinase activity in cell-free systems.

We recently observed (unpublished) that agonists of two different classes, carbachol (muscarinic cholinergic) and methoxamine (α_1-adrenergic), stimulated the liberation of arachidonic acid in submandibular cells, although the specific phospholipid precursor has not been identified. Earlier work in our laboratory showed that the same agonists activated the phosphoinositide effect in the model (Fleming *et al.*, 1987). These findings, taken together with those of the present study, suggest that stimulation of phosphoinositide hydrolysis in submandibular cells is accompanied by a release of arachidonic acid, which acts as a negative feedback inhibitor of phosphoinositide cycle activity by regulating the synthesis of inositol phospholipids.

CHAPTER III

REGULATION OF PHOSPHATIDYLINOSITOL KINASES BY ARACHIDONIC ACID IN RAT SUBMANDIBULAR GLAND CELLS[2]

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[2]From a published article: **Chung HC, Fleming N** (1995) Regulation of phosphatidylinositol kinases by arachidonic acid in rat submandibular gland cells. *Eur J Physiol* 429:789-796.

Abstract

Phosphoinositide kinases were characterized in membrane extracts of rat submandibular gland cells. Both phosphatidylinositol (PI) 4-kinase and phosphatidylinositol-4-phosphate [PI(4)P] 5-kinase phosphorylated endogenous substrates in reactions that were linear for up to five min, were activated by Mg^{2+} and showed maximal activity around neutral pH. PI 4-kinase was stimulated by Triton X-100 at an optimal concentration of 0.22% but the detergent had an inhibitory effect on PI(4)P 5-kinase. Arachidonic acid (AA), at concentrations greater than 100 μM, inhibited the activity of both enzymes in a dose-dependent manner. The inhibitory effect was replicated by other unsaturated fatty acids, but not by a saturated fatty acid, of the *sn*-20 series. The nature of AA inhibition of the kinases was examined in enzyme kinetic studies with exogenous phosphoinositide and adenosine 5′-triphosphate (ATP) substrates. Lineweaver-Burk plots of PI 4-kinase activity showed that AA had no effect on the apparent K_m for either PI or ATP, but that the fatty acid significantly reduced V_{max}(PI) from 331 to 177 pmol.mg^{-1}.min^{-1} and V_{max}(ATP) from 173 to 59 pmol.mg^{-1}.min^{-1}. This inhibitory action was consistent on PI(4)P 5-kinase kinetics, where again, AA did not alter apparent K_m values but lowered V_{max} for both PI(4)P and ATP by around 50%. Since the combination of a reduced V_{max} and an unchanged K_m indicates noncompetitive enzyme

inhibition, it is proposed that AA regulates phosphoinositide cycle activity in submandibular gland cells by acting as a noncompetitive inhibitor of PI 4-kinase and PI(4)P 5-kinase.

Introduction

Agonists of several different classes (*e.g.*, muscarinic, α_1-adrenergic, tachykinin) control exocrine responses in mucous acinar cells of the rat submandibular gland by stimulating receptors coupled to the phosphoinositide signal transduction pathway (Fleming *et al.*, 1987; 1992). Receptor occupation activates phospholipase C, which catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] to release inositol 1,4,5-trisphosphate [$I(1,4,5)P_3$] and 1,2-diacylglycerol (DAG). These second messengers respectively mobilize Ca^{2+} from endoplasmic reticulum stores and activate the enzyme protein kinase C (PKC), which, in turn, modulate many physiological functions of the cell (Nishizuka, 1992). $PI(4,5)P_2$ is rich in arachidonic acid (AA), the precursor of a range of bioactive eicosanoid products, esterified at the *sn*-2 position on the glycerol backbone (Dennis *et al.*, 1991). Free AA may be released from inositol phospholipids directly by phospholipase A_2 (PLA_2) action, or it may be cleaved from the phosphoinositide breakdown products, DAG or phosphatidic acid, after initial phospholipid hydrolysis by phospholipase C (PLC) or phospholipase D (PLD) (Dennis *et al.*, 1991; Liscovitch, 1992). It has been shown that AA modifies the phosphoinositide effect in several cell types, including platelets (Fan *et al.*, 1990), pancreatic acinar cells (Chaudhry *et al.*, 1987) and placental cells (Zeitler and

Handwerger, 1985). These observations have led to the proposal that the phosphoinositide-derived AA may act as a negative feedback regulator of the phosphoinositide cycle (Chaudhry *et al.*, 1987).

Consistent with this suggestion, in an earlier study, we found that AA had an overall inhibitory effect on phosphoinositide cycle turnover in carbachol-stimulated cells of the rat submandibular glands (Chung and Fleming, 1992). More specifically, the fatty acid lowered levels of phosphatidylinositol 4-phosphate [PI(4)P] and PI(4,5)P_2, but not of PI in the activated cycle. Additional observations indicated that the AA effect was not mediated by PLC activity (Chung and Fleming, 1992). Therefore, it was suggested that the site of regulatory interaction was in the phospholipid synthetic phase of the cycle, distal to phosphoinositide synthesis. It was further speculated that possible targets for AA inhibition were one or both of the kinases that regulate the reactions which sequentially phosphorylate PI to PI(4)P to PI(4,5)P_2, *i.e.*, the respective enzymes PI 4-kinase and PI(4)P 5-kinase.

The present study was undertaken to characterize these kinase reactions more precisely in membrane preparations from submandibular gland cells and to examine the nature of the potential AA inhibition of kinase activity. Enzyme kinetic studies show that AA acts directly as a noncompetitive inhibitor of both kinases and suggest that this is its mechanism of action in regulation of the phosphoinositide effect.

Materials and methods

Materials

Triton X-100 and a protein assay kit (bovine serum albumin standard) were obtained from Bio-Rad Labs (Mississauga, Ontario). HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer was a product of Gibco Canada (Burlington, Ontario). Adenosine 5′-[γ-^{32}P]-triphosphate ([^{32}P]ATP, specific activity 3,000 Ci/mmol) was supplied by the Amersham Corporation (Arlington Heights, IL). [Inositol-2-^{3}H(N)]-phosphatidylinositol-4-phosphate, *i.e.*, [^{3}H]PI(4)P, specific activity 2-10 Ci/mmol) was provided by Du Pont-New England Nuclear (Wilmington, DE). AA and other fatty acids were obtained from Nucheck Prep (Elysian, MN). Kodak X-Omat AR X-ray film was supplied by Picker International (Highland Heights, OH). HP-KF silica gel high performance thin layer chromatography (TLC) plates were supplied by Whatman International (Maidstone, Kent, UK). Phosphoinositide markers for TLC and all other chemicals used were from Sigma (St. Louis, MO).

Membrane extraction

Membrane extracts of the pooled submandibular glands of two to four male Sprague-Dawley rats (250-300 *g* body weight) were prepared as described by Smith and Chang (1989). All procedures were carried out at 4° C. Briefly, the glands were

chopped into small pieces and homogenized in a ground glass homogenizer in 4 volumes of 0.32 M sucrose in 10 mM HEPES buffer, pH 7.4. The preparation was centrifuged at 200 *g* for 10 min, the pellet re-homogenized and again centrifuged at 200 *g* for 10 min. The two supernatants were combined and centrifuged at 48,000 *g* for 30 min. The resulting membrane-rich pellet was extracted with 0.2% Triton X-100 in a 0.25 M sucrose/25 mM HEPES buffer pH 7.4 and centrifuged at 48,000 *g* for 30 min. The clear supernatant, usually diluted tenfold to a final Triton X-100 concentration of 0.02%, was used as a source of both phosphoinositide kinases and endogenous phosphoinositide substrates in subsequent studies on enzyme activity. Extractions were freshly prepared for each experiment and standardized according to protein content, measured by the method of Bradford (1976) (Bio-Rad kit, bovine serum albumin standard).

Assay of phosphatidylinositol 4-kinase and phosphatidylinositol 4-phosphate 5-kinase

Enzyme activities were assayed by measuring the phosphorylation of the substrates PI and PI(4)P to the respective products PI(4)P and PI(4,5)P_2, which were quantitated by the transfer of [^{32}P] from radiolabelled ATP. Studies on the phosphorylation of both endogenously and exogenously-added substrates were carried out. In initial experiments, the kinase reactions were characterized for

optimal conditions with respect to ATP, $MgCl_2$ and Triton X-100 concentrations, as well as pH. Therefore, each of these parameters was varied in turn in the following assay procedure (endogenous substrates). Submandibular membrane extracts (100 μg protein) were preincubated for 10 min at 30° C in 90 μl buffer containing 40 mM HEPES, 3 mM dithiothreitol, 10-50 mM $MgCl_2$, 2 mM ethylenebis(oxonitrilo)tetraacetate (EGTA), 0.02-0.42% Triton X-100, pH 6.4-8.4 (Mesaeli *et al.*, 1992). In experiments investigating the effect of Triton X-100 on enzyme activity, 100 μM exogenous PI or PI(4)P were added to the assay mixture to approximate the concentrations of these substrates used in subsequent enzyme kinetics studies. Phosphorylation was initiated by the addition of 10 μl [^{32}P]ATP (0.3 mM, 4 μCi). Preliminary experiments showed that phosphorylation reactions with endogenous substrates were linear for up to five min. Therefore, reactions were stopped at three min by the addition of 1.5 ml of a mixture of chloroform and methanol (1:2, by vol).

Phospholipids were extracted by the sequential addition of 0.5 ml 2.4 N HCl and 0.5 ml chloroform. The mixture was centrifuged at 1,000 *g* for 10 min to produce a sharp biphasic separation and the lower (chloroform) phase removed. The upper phase was re-extracted with 1 ml chloroform and the mixture centrifuged again. The two lower phases were combined, washed with 2 ml of a mixture of methanol and 1 N HCl (1:1, by vol) and centrifuged at 1,000 *g*. The lower phase was removed,

evaporated under N_2 and the phospholipid-containing residue solubilized in 100 μl of a mixture of chloroform, methanol and water (75:25:2, by vol). The samples were applied to high performance TLC plates which had been impregnated with 1% potassium oxalate in methanol and water (2:3, by vol) and activated at 110°C for 1 h (Mesaeli *et al.*, 1992). Chromatograms were developed at room temperature in a solvent system of chloroform, acetone, methanol, acetic acid and water (40:15:13:12:8, by vol) as described by Jolles *et al.* (1981). [^{32}P]-labelled phospholipid spots were visualized by overnight autoradiography on Kodak X-Omat AR X-ray film, and radiolabelled PI(4)P and PI(4,5)P_2 were identified by comparison of their relative mobility to purified standard markers [R_f values, PI(4)P = 0.27; PI(4,5)P_2 = 0.19]. Labelled phosphoinositides were scraped from the TLC plates, mixed with 5 ml of Ecolume and quantitated by scintillation counting as described previously (Chung and Fleming, 1992).

The effect of a range of concentrations of AA and other saturated and unsaturated fatty acids of the *sn*-20 series on the phosphorylation of endogenous PI and PI(4)P was examined under the optimal conditions derived empirically as above. In these studies, the membrane extracts were exposed to fatty acid for five min before the initiation of phosphorylation by the addition of [^{32}P]ATP. Acids were solubilized in dimethylsulfoxide (DMSO) which was routinely added to controls at the same concentration (never more than 0.5%).

Radiolabelled phosphoinositides were extracted and quantitated as described above.

Enzyme kinetics

The nature of AA inhibition of PI kinases was investigated in kinetic studies in which a range of concentrations of exogenous phosphoinositide and ATP substrates were used. Membrane preparations were preincubated with 200 μM AA for five min or left untreated as controls. For the PI 4-kinase assay, extracts in the same assay buffer as above, with the Triton X-100 concentration adjusted to 0.22%, were mixed with concentrations of between 0 μM and 400 μM PI plus a near-saturating fixed concentration of 300 μM [^{32}P]ATP for three min. Phosphorylation was again measured by radiolabelled PI(4)P product formation. Data were corrected for the effects of endogenous substrate by subtracting the value obtained in the absence of exogenous PI. Lineweaver-Burk plots were constructed and the apparent K_m and V_{max} of PI 4-kinase for PI calculated. In a second series of experiments, K_m and V_{max} for ATP were similarly determined by using a range of ATP concentrations (0.05-2 mM) and a fixed concentration of 100 μM PI in reaction mixtures.

The same approach was taken in studies on PI(4)P 5-kinase. K_m and V_{max} were again determined for PI(4)P [PI(4)P concentration range 0-100 μM; ATP 300 μM] and for ATP [ATP 0.05-2 mM; PI(4)P 100 μM] in the presence or absence of AA. In

these experiments Triton X-100 was used at a final concentration of 0.02%.

Statistical analysis

Results were examined statistically by two-way analysis of variance and means were compared by Duncan's multiple range test or the Student *t*-test. Values of $p < 0.05$ were considered significant.

Results

Characterization of phosphoinositide kinase reactions

Both phosphoinositide kinases phosphorylated their endogenous substrates to the corresponding radiolabelled products in submandibular cell membrane extracts when incubated with [^{32}P]ATP (Fig. 11). Reactions were linear for up to five min. In subsequent experiments, therefore, phosphorylations were terminated after three min to ensure constant rate synthesis. Preliminary experiments (not shown) confirmed that, as in other systems (Collins and Wells, 1983; Mesaeli *et al.*, 1992), both enzymes displayed maximal activity at pH 7.4. Assay buffer systems were thus standardized to optimal conditions at this pH.

Kinase dependence on Mg^{2+} concentration was also investigated. A Mg^{2+} content of 10 mM produced maximal activity in both kinases (Fig. 12). Higher Mg^{2+} levels appeared to inhibit PI 4-kinase but not PI(4)P 5-kinase.

Since phosphoinositide kinases are detergent-sensitive (Carpenter and Cantley, 1990; Pike, 1992), the effect of a range of concentrations of Triton X-100 on phosphorylation reactions was tested. Preliminary observations with endogenous substrate suggested that PI 4-kinase activity was enhanced by Triton X-100 but that PI(4)P 5-kinase activity was depressed. This was confirmed in additional studies in which 100 μmol of exogenous substrate, either PI or PI(4)P, was added to

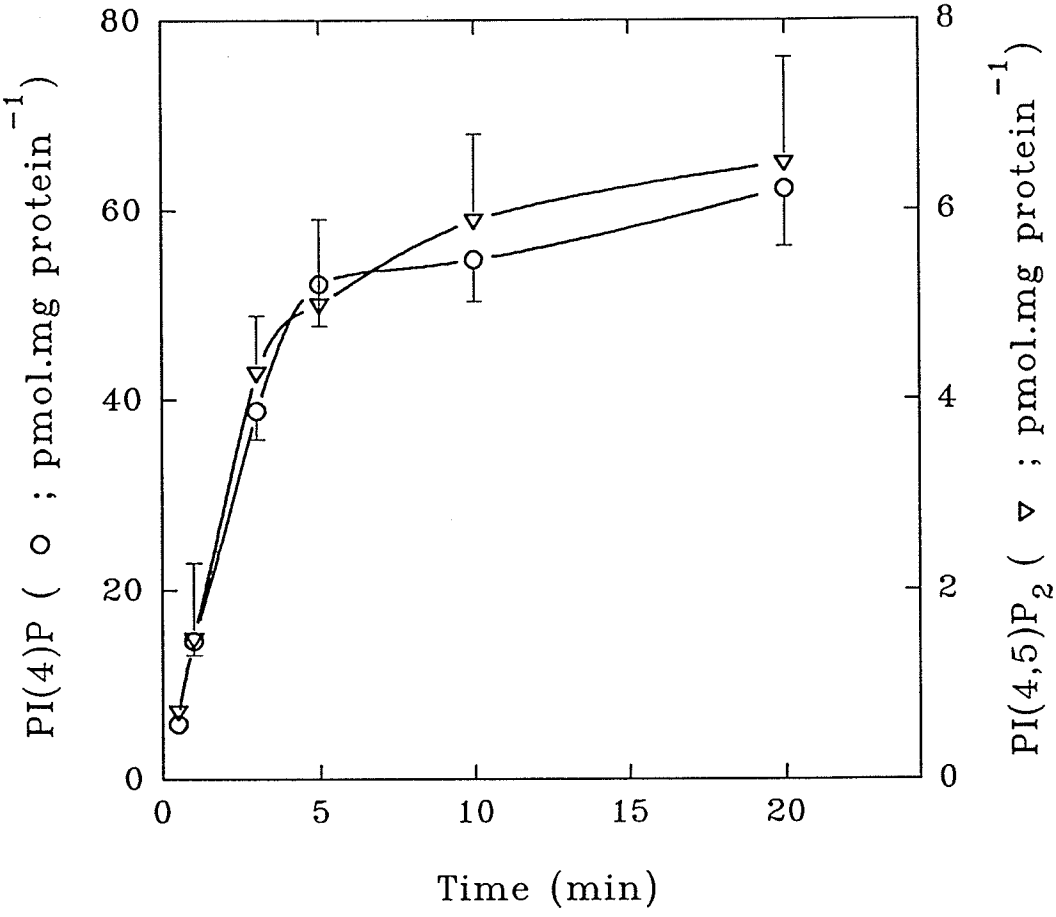

Fig. 11. Time course of the activities of phosphatidylinositol 4-kinase and PI(4)P 5-kinase from endogenous substrate in membranes of rat submandibular gland cells.

Membrane extracts in 0.02% Triton X-100 were incubated for up to 20 min with [^{32}P]ATP as described in Materials and methods. Radiolabelled phosphoinositides were extracted with a mixture of chloroform, methanol and HCl, separated by thin layer chromatography (TLC) and quantitated by scintillation counting. Values are means ± SEM, n = 5.

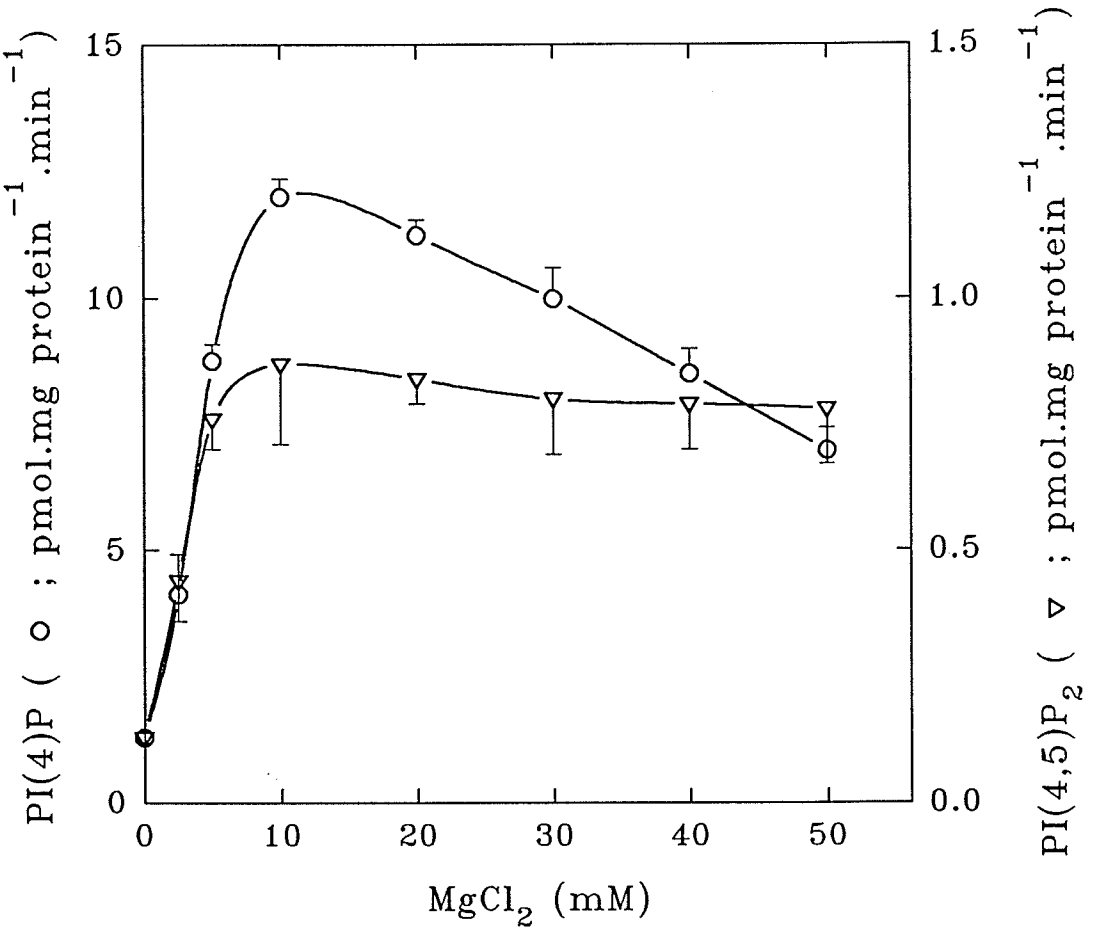

Fig. 12. Effect of Mg^{2+} concentration on PI 4-kinase and PI(4)P 5-kinase activities in rat submandibular cell membranes.

Different concentrations of $MgCl_2$ were added to membrane preparations (0.02% Triton X-100) in the presence of [^{32}P]ATP and the phosphorylation of endogenous substrates was allowed to proceed for three min. Radiolabelled phosphoinositides were separated and quantitated as described for Fig. 11. Means ± SEM, n = 4.

stimulate phosphorylation rates and amplify variations caused by the detergent (Fig. 13). PI 4-kinase activity was maximal at 0.22% Triton X-100 and remained so up to 0.4% detergent. PI(4)P 5-kinase activity was maximal at 0.02% Triton X-100 but was inhibited in a concentration-dependent manner by a higher detergent content (Fig. 13). Therefore, in subsequent enzyme kinetic studies with added exogenous substrates, PI 4-kinase was assayed in buffer containing 0.22% Triton X-100, while PI(4)P 5-kinase was assayed in 0.02% detergent. In experiments investigating the effect of fatty acids on kinase activity in endogenous enzyme/substrate preparations, Triton X-100 was adjusted to a compromise value of 0.12%, so that the sequential phosphorylation reactions of both enzymes could be followed in the same experimental samples.

Effects of arachidonic acid on phosphoinositide kinases

AA inhibited both PI 4-kinase and PI(4)P 5-kinase in a dose dependent manner (Fig. 14). The inhibitory effect on either enzyme was not evident at AA concentrations of less than 100 μM and was maximal at the highest dose tested, *i*.e. 1 mM. For comparison, the effects of other fatty acids (100 μM) of the *sn*-20 series on kinase activity were examined. The saturated species, arachidic acid (20:0) did not inhibit the enzymes but three unsaturated acids, *i*.e. 11-eicosaenoic (20:1), 11-14 eicosadienoic (20:2) and 8-11-14 eicosatrienoic (homogamma linolenic, 20:3) all significantly reduced product formation

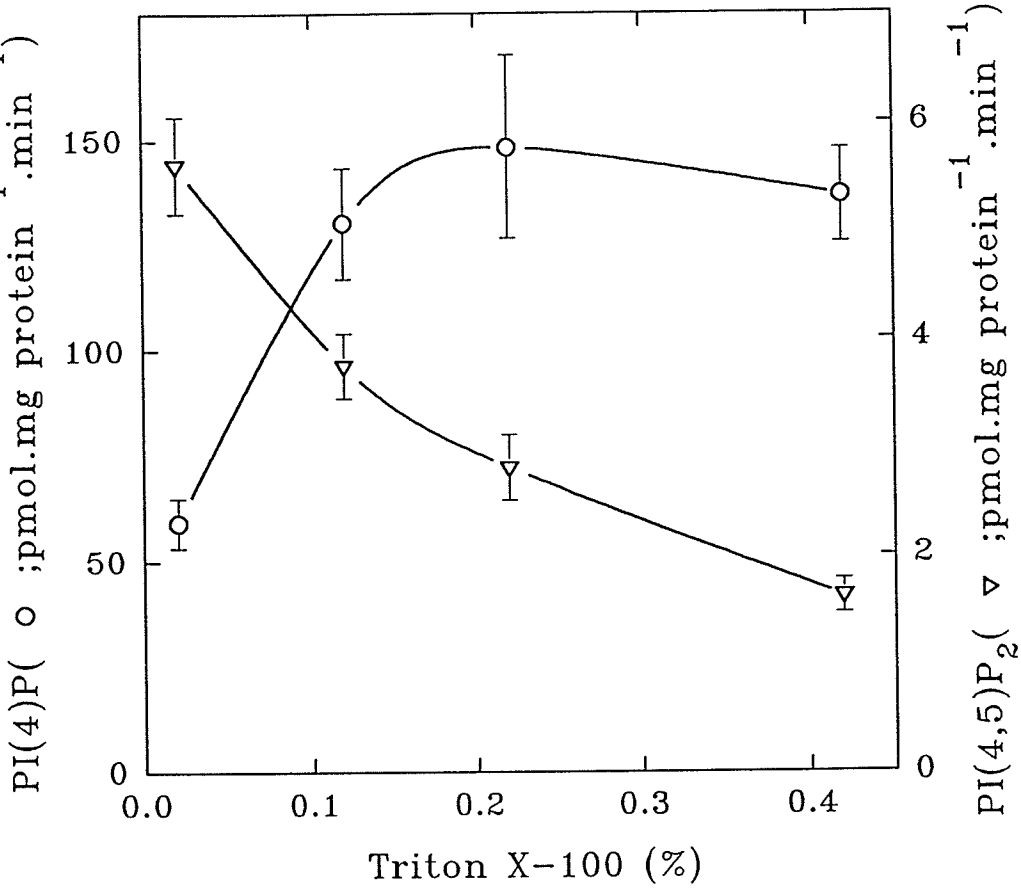

Fig. 13. Effect of Triton X-100 concentration on PI 4-kinase and PI(4) 5-kinase activities in rat submandibular gland membranes.

Triton X-100 at the final concentrations shown was added to membrane preparations in the presence of [^{32}P]ATP plus 100 μM exogenous PI or PI(4)P substrate. Phosphorylation reactions were allowed to proceed for three min. Radiolabelled phosphoinositides were separated and quantitated as described for Fig. 11. Means ± SEM, n = 4.

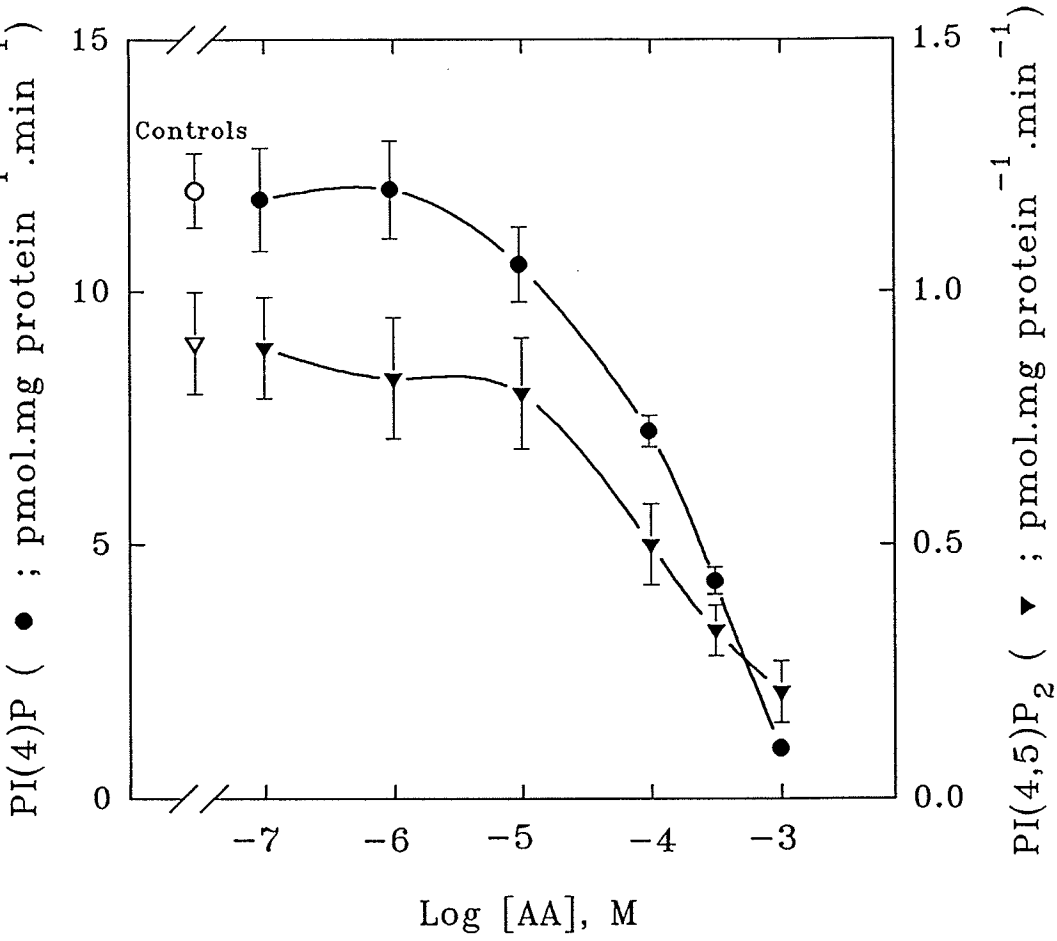

Fig. 14. Concentration response of arachidonic acid (AA) inhibition of PI(4)P and PI(4,5)P_2 formation in rat submandibular gland membranes.

Membrane extracts in 0.02 % Triton X-100 were preincubated for five min with the concentrations of AA shown, then phosphorylation of endogenous substrates in 100 μg membrane protein was initiated by the addition of [^{32}P]ATP. Radiolabelled phosphoinositides were separated and quantitated as described in Fig. 11. Means ± SEM, n = 4.

by both kinases (Fig. 15).

Kinetic studies on phosphoinositide kinases

Lineweaver-Burk plots were constructed for both enzymes and the effects of AA on K_m and V_{max} for PI and PI(4)P substrates and for ATP were determined. For PI 4-kinase, the K_m value for PI was 167 ± 23 μmol/l (mean ± SEM) and for ATP it was 260 ± 17 μmol/l (Fig. 16a,b). AA had no effect on these K_m values, but did significantly reduce V_{max} for both PI (from 331 ± 25 to 177 ± 18 $pmol.min^{-1}.mg^{-1}$ protein) and ATP (from 173 ± 20 to 59 ± 6 $pmol.min^{-1}.mg^{-1}$ protein) (Fig. 16a,b). This effect was duplicated for PI(4)P 5-kinase. Again AA did not change the Michaelis-Menten constants for PI(4)P or ATP, but significantly lowered V_{max} for PI(4)P (6.5 ± 0.6 to 3.5 ± 0.3 $pmol.min^{-1}.mg^{-1}$ protein) and ATP (5.2 ± 0.4 to 2.7 ± 0.3 $pmol.min^{-1}.mg^{-1}$ protein) (Fig. 17a,b).

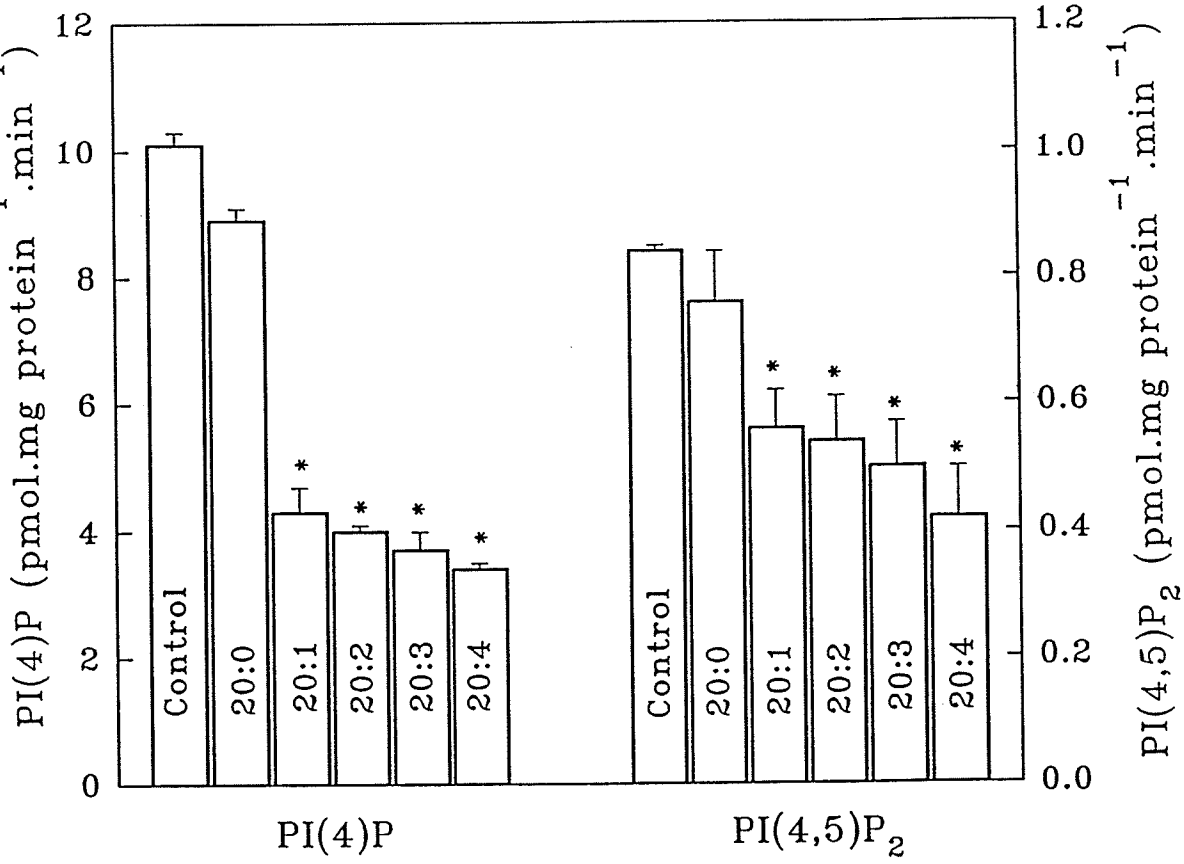

Fig. 15. Effect of fatty acids of the *sn*-20 series on PI(4)P and PI(4,5)P_2 formation in rat submandibular gland membranes.

Membranes were preincubated for five min with the acids shown (100 μM concentration). Phosphorylation of endogenous substrates in 100 μg membrane protein was initiated by the addition of [^{32}P]ATP, and was allowed to proceed for three min. Labelled phosphoinositides were separated by TLC and quantitated by scintillation counting. The saturated acid, 20:0, had no effect on phosphorylation, while all the unsaturated species significantly (*) inhibited synthesis of both phosphoinositide products. A compromise concentration of 0.12% Triton X-100 was used to permit the assay of reasonable levels of both kinases in the same samples. Means ± SEM, n = 4.

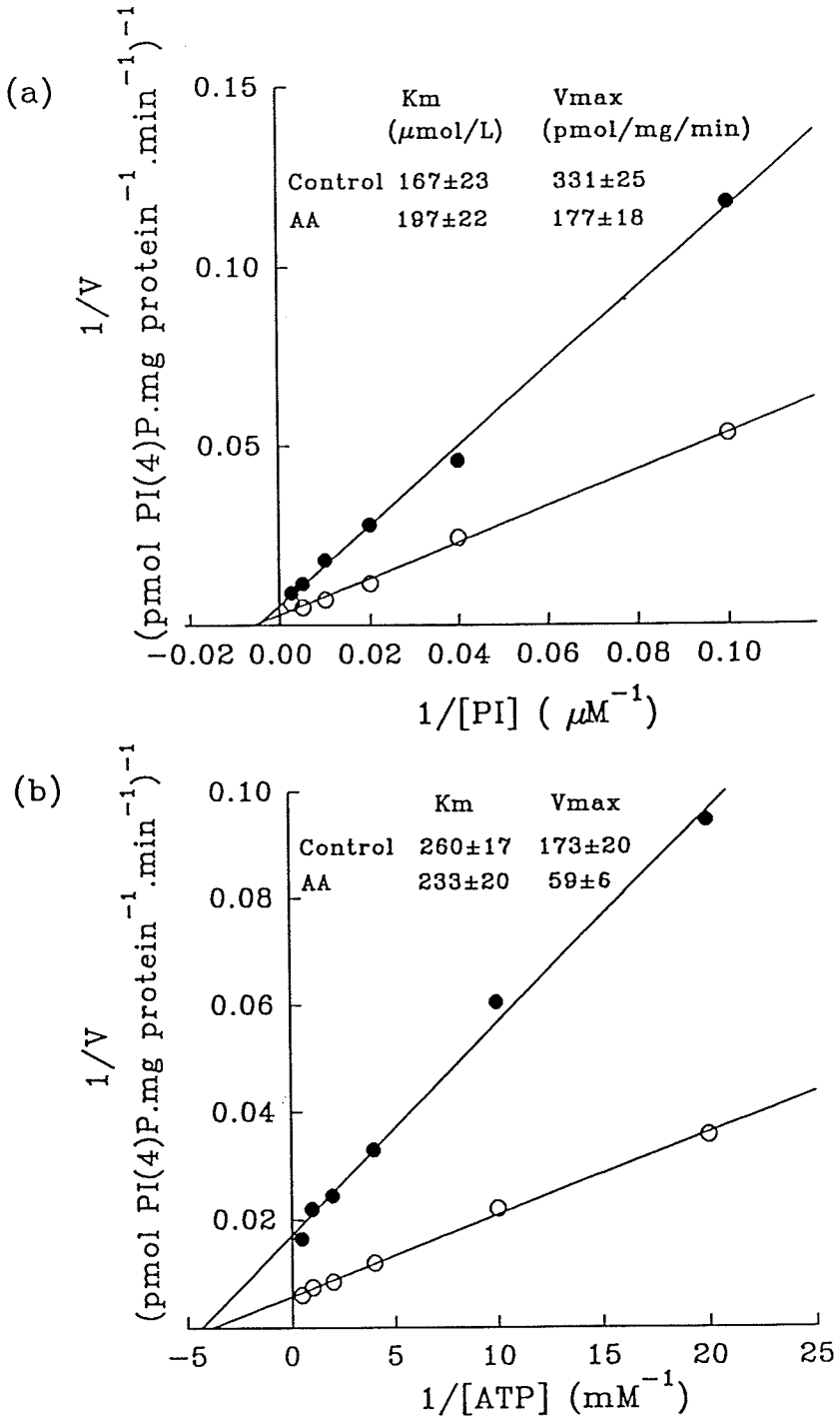

Fig. 16. Effect of AA on the kinetics of PI 4-kinase in rat submandibular gland membranes.

Membrane preparations were preincubated in the absence (o) or presence (•) of 200 μM AA for five min. PI 4-kinase activity was then assayed in a 0.22% Triton X-100 buffer over a three-min period as described in Materials and methods. Lineweaver-Burk plots are shown for a range of concentrations of exogenous PI (**a**) and ATP (**b**). Points on the graphs are from a single representative experiment. V_{max} and apparent K_m values are means ± SEM, n = 4.

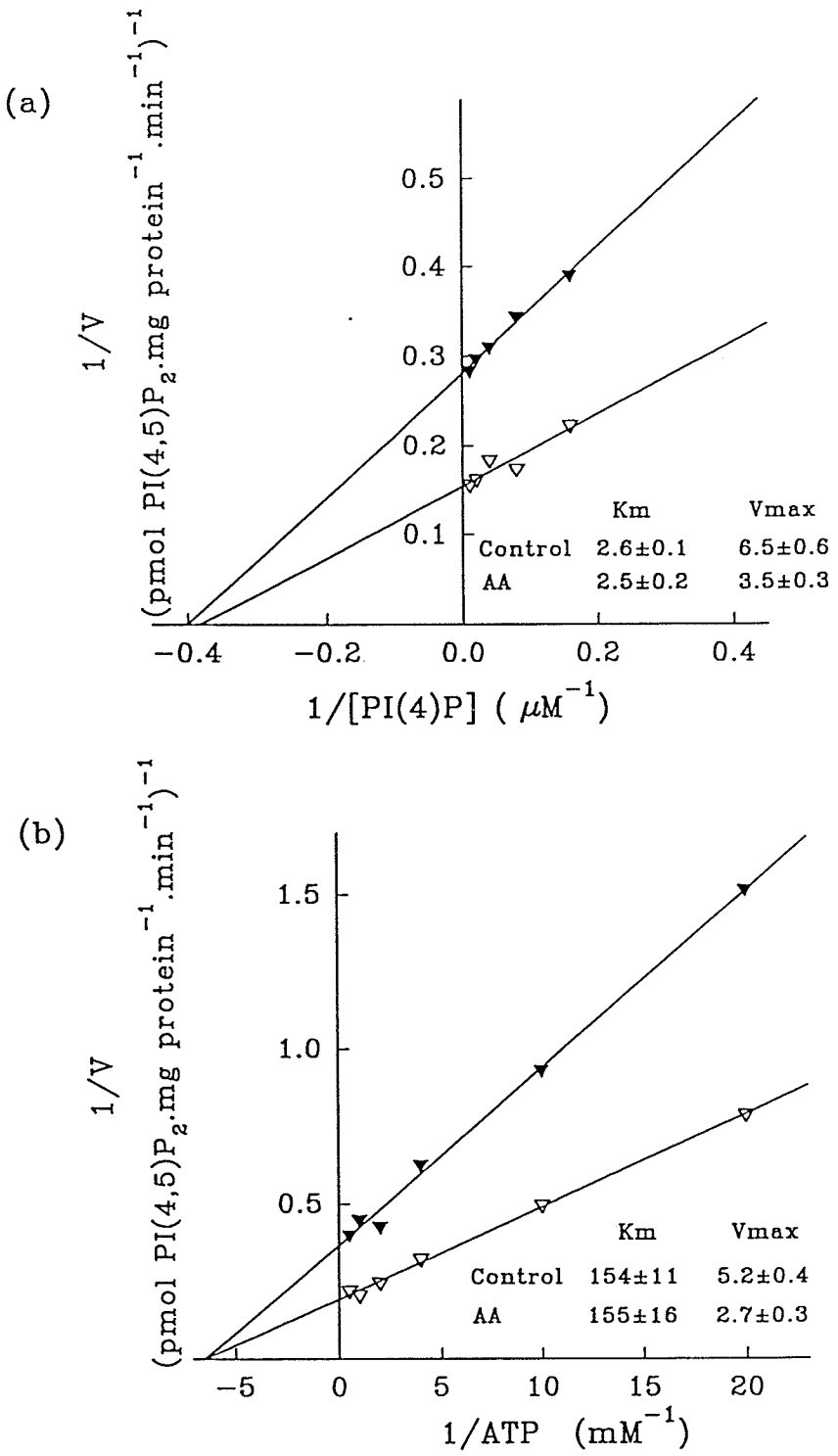

Fig. 17. Effect of AA on the kinetics of PI(4)P 5-kinase in rat submandibular gland membranes.

Membrane preparations were preincubated in the absence (▽) or presence (▼) of 200 μM AA for five min. PI(4)P 5-kinase activity was then assayed over a three-min period in 0.02% Triton X-100 buffer as described in Materials and methods. Lineweaver-Burk plots are shown for a range of concentrations of exogenous PI(4)P (**a**) and ATP(**b**). Points on the graph are from a single representative experiment. V_{max} and apparent K_m values are means ± SEM, n = 4. Units are the same as in Fig. 16 (a).

Discussion

In earlier work we described an inhibitory effect of AA on phosphoinositide cycle activity in rat submandibular gland cells and verified that this effect was not mediated by cyclooxygenase or lipoxygenase oxidation products (prostaglandins or leukotrienes) (Chung and Fleming, 1992). Levels of phosphoinositides were particularly depressed by the acid in preparations that were treated with the PI-coupled muscarinic agonist, carbachol (Chung and Fleming, 1992). We speculated that potential targets for AA action were the kinases that regulate inositol phospholipid synthesis (Chung and Fleming, 1992). In the present study we have characterized the reactions of both PI 4-kinase and PI(4)P 5-kinase in membrane extracts of submandibular glands, demonstrated a direct inhibitory effect on each by AA and identified the nature of the interaction between the fatty acid and enzyme.

As in other models (Mesaeli *et al.*, 1992; Conway *et al.*, 1993), the phosphoinositide kinases phosphorylated endogenous substrate to the appropriate product upon the addition of radiolabelled ATP. Characteristics of enzyme action were consistent with those described for PI kinases in preparations of rat sarcolemma (Mesaeli *et al.*, 1992), rat liver lysosomes (Collins and Wells, 1983) and bovine adrenal medulla (Husebye *et al.*, 1990). Both reactions were Mg^{2+} dependent, with an optimal ion concentration of 10 mM and both displayed maximal

activity around physiological pH.

Phosphoinositide kinases have been characterized by their responses to nonionic detergent treatment. PI 4-kinase (type 2 PI kinase), which comprises up to 95% of total PI kinase activity in cells (Pike, 1992), is activated by detergent, whereas PI 3-kinase (type 1 PI kinase), a species contributing only around 2% of total activity, is inhibited by detergent (Carpenter and Cantley, 1990; Downes and MacPhee, 1990; Pike, 1992). The type 1 enzyme phosphorylates the inositol ring at the D-3 position to produce PI(3)P, which is not an intermediate of the phosphoinositide cycle and is not hydrolysed by phospholipase C (Downes and MacPhee, 1990; Carpenter and Cantley, 1990; Mesaeli *et al.*, 1992). Given the relative abundance of type 2 over type 1 PI kinase in cells and our observation that PI kinase examined in the present study was activated by Triton X-100, it is highly likely that the species analyzed in submandibular extracts was PI 4-kinase. This would be consistent with studies on PI kinase in rat heart sarcolemma (Mesaeli *et al.*, 1992) and rat pancreatic acinar cell membranes (Conway *et al.*, 1993), in which type 2 enzyme activity predominated in membrane extracts. Our finding that the PIP kinase activity examined in the present study was lowered by Triton X-100 further indicates that the enzyme under investigation was indeed the PI(4)P 5-kinase species, as the same enzyme was shown to be inhibited by detergent in cardiac membranes (Kasinathan *et al.*, 1989; Wolf, 1990).

AA inhibited product formation from endogenous substrates for both enzymes in a dose-dependent way. The fatty acid had no effect at concentrations under 100 μM and showed a maximal inhibitory effect for both kinases at 1 mM. While effective doses of AA appear to be non-physiological, high concentrations of the acid must often be used *in vitro* to produce biological effects. This is probably a reflection of its rapid acylation to membrane phospho- and neutral lipids, non-specific binding to other membrane and cytosolic components and oxidative degradation. Concentrations used in the present work are of the same order as those required to inhibit PI kinase in platelet membranes [500 μg/ml (approximately 1.64 mM)], (Kanoh *et al.*, 1990) and a Ca^{2+}/calmodulin-dependent protein kinase II in rat brain cortex [100 μM, (Piomelli *et al.*, 1989)]. Further studies with unsaturated and saturated fatty acids of the *sn*-20 series indicated that only the unsaturated species duplicated the AA effect in inhibiting the phosphoinositide cycle kinases. Although these studies were carried out in compromise concentrations of Triton X-100, that were not optimal for either enzyme, significant reductions in the activities of both were observed. This suggests that at least one double bond, possibly involved in fatty acid-kinase binding, is a requirement for inhibitory activity. These findings are consistent with our earlier observations that unsaturated fatty acids inhibit the phosphoinositide cycle in

submandibular gland cells (Chung and Fleming, 1992) and with those of Chaudhry *et al.* (1987), who first noted the effect in pancreatic acini. Apart from AA, the unsaturated *sn*-20 acids examined are not present in cells in significant amounts, and are simply used here as probes of the regulatory mechanism.

Additional experiments confirmed that AA inhibition of PI(4)P and PI(4,5)P_2 formation was not due to the fatty acid's stimulation of the phosphomonoesterases catalyzing the reverse reactions that convert PI(4,5)P_2 to PI(4)P to PI in the cycle. Addition of up to 200 μM inositol-radiolabelled PI(4,5)P_2 to membrane extracts produced increased levels of PI(4)P and PI, but AA did not further enhance this response (results not shown).

The mechanism of the inhibitory action of AA on kinase activity was further investigated by examining the effect of AA on enzyme kinetics with respect to both inositol phospholipid and ATP substrates. For PI 4-kinase, AA had no effect on the apparent K_m(PI) but did significantly reduce V_{max}(PI) to about half the control value. Kinetics were modified in the same way by AA relative to ATP hydrolysis. Again K_m(ATP) did not change, while V_{max}(ATP) was lowered by AA to around a third of normal. This effect of AA was reproduced in kinetic studies on PI(4)P 5-kinase, where V_{max} for both PI(4)P and ATP was significantly reduced, but the apparent K_m value for these substrates remained unchanged. This combination of reduced V_{max} and unchanged K_m is a classical

indicator of non-competitive enzyme inhibition. Therefore, we propose that AA acts as a noncompetitive inhibitor of both PI 4-kinase and PI(4)P 5-kinase and, thus, exerts its effect by direct binding of these enzymes at a locus other than the substrate binding site.

Kinetic analysis of enzymes acting on phospholipid substrates in mixed micellar preparations can be complicated by complex binding behaviour at the water/lipid interface. For example, an enzyme may bind to a substrate molecule in the micelle surface, or bind to the micelle non-specifically, leading to the phenomenon of substrate surface dilution (Deems *et al.*, 1975). An additional possibility is the two-stage binding of enzyme to substrate in the dual phospholipid model proposed for the action of PLA_2 on phosphatidylcholine (PC) or phosphatidylethanolamine (Hendrickson and Dennis, 1984). Binding characteristics may become increasingly complex with variations in detergent, or fatty acid content of kinetic assay buffers. Thus, in the present study, non-specific effects of surface dilution, and fatty acid or detergent concentration cannot be ruled out. However, the micellar/substrate binding behaviour of phosphoinositide kinases, acting to add phosphate (*i.e. P*) groups to a polar, hydrophilic inositol head group projecting into an aqueous environment, may be somewhat simpler than that of a PLA_2 targeted to an internalized fatty acid/glycerol ester bond in a hydrophobic milieu. Moreover, classical Lineweaver-Burk

double reciprocal plots were obtained under conditions where AA and Triton X-100 were held constant for each kinase, and only substrate concentrations were varied. We believe, therefore, that the AA effects on kinase activity are likely to represent a true non-competitive enzyme inhibition. This may not be the only mechanism of regulation of the phosphoinositide cycle by AA. Chaudhry *et al*. (1987; 1988) showed that AA inhibited PI(4,5)P_2 synthesis in pancreatic acinar cells and suggested that the fatty acid's action might be related to its demonstrated capacity to increase oxygen consumption and/or deplete ATP levels. We have confirmed that AA reduces ATP in the submandibular model and also acts as an inhibitor of protein synthesis (Fleming and Mellow, 1995). AA may therefore function as a regulator of different physiological effects by a range of mechanisms that act independently, or in concert.

Kinases of the phosphoinositide cycle are thought to be regulated by several classes of bioactive molecules including cyclic nucleotides, growth factors and cytokines (Carpenter and Cantley, 1990; Downes and MacPhee, 1990; Pike, 1992). Some of the proposed regulators such as PKC and GTP-binding G regulatory proteins, are agents associated with the phosphoinositide signal transduction pathway. Conway *et al* (1993) showed that PI 4-kinase in rat pancreatic acini was stimulated by PKC, an enzyme activated by DAG, which is produced on the hydrolysis of PI(4,5)P_2 by PLC. Therefore,

they proposed a positive feedback regulation of PI 4-kinase by PKC activated by the phosphoinositide breakdown. Phosphoinositides are rich in AA, esterified at the *sn*-2 position of the glycerol backbone. Modulation of the phosphoinositide cycle by AA may then also represent a feedback regulatory mechanism, as suggested by Chaudhry *et al.* (1988) after observing an AA induced inhibition of $PI(4,5)P_2$ synthesis in pancreatic cells. Such a mechanism would require the cleavage of AA either directly from phosphoinositides, or from a phosphoinositide hydrolysis product, such as DAG. Direct, PLA_2-catalysed release of AA from inositol phospholipids has been demonstrated in pancreatic acinar cells treated with Δ^9-tetrahydrocannabinol (Chaudhry *et al.*, 1988) and in carbachol-stimulated lipid extracts of rat brain cortex synaptoneurosomes (Strosznajder and Samochocki, 1992). A more indirect route of AA release was proposed by Dixon and Hokin (1984) who presented evidence that exposure of pancreatic lobules to caerulein stimulated the PLC hydrolysis of phosphoinositide to produce DAG as the source of liberated AA.

Non-inositol phospholipids, particularly PC, may also be significant sources of regulatory AA. Again, AA may be released from PC by the action of PLA_2, as described for the muscarinic-agonist-induced insulin secretion pathway in pancreatic islets (Konrad *et al.*, 1992), or from PC derivatives after initial hydrolysis of the phospholipid by PC-specific PLC (Rubin *et al.*, 1992) or PLD (Exton, 1990). PLD

may not be directly coupled to agonist receptors, but may be stimulated by the enzyme PKC, which, itself, is activated by DAG generated in PLC phospholipid hydrolysis (Exton, 1990). In earlier work we found that carbachol stimulated PLC cleavage of $PI(4,5)P_2$ in rat submandibular gland cells (Fleming *et al.*, 1987; Chung and Fleming, 1992). We have recently found that the same muscarinic agonist also elevates free AA in the model (unpublished observation). The specific phospholipid source of AA and its receptor-coupled enzymatic cleavage pathways remain to be identified.

CHAPTER IV

MUSCARINIC REGULATION OF PHOSPHOLIPASE D AND ITS ROLE IN ARACHIDONIC ACID RELEASE IN RAT SUBMANDIBULAR ACINAR CELLS[3]

[3]From: **Chung HC, Fleming N** (1995) Muscarinic regulation of phospholipase D and its role in arachidonic acid release in rat submandibular acinar cells. *Eur J Physiol* (in press).

Abstract

The characteristics of muscarinic cholinergic-induced phospholipase D (PLD) activation and the involvement of the enzyme in the release of arachidonic acid (AA) were examined in rat submandibular acinar cells. Carbachol produced a dose-response activation of PLD to around five fold control values at 100 μM agonist concentration. This was associated with the appearance of free choline, phosphatidic acid (PA) and arachidonic acid, indicating that the PLD substrate was phosphatidylcholine (PC). The carbachol response was inhibited by 60% by U73122, a blocker of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2]-specific phospholipase C (PLC), suggesting that PC-PLD cleavage was, at least in part, secondary to agonist-coupled PI(4,5)P_2-PLC hydrolysis. Consistent with this, PLD was also activated to levels comparable to those induced by carbachol, by the phorbol ester, TPA, and the Ca^{2+}-mobilizer, thapsigargin, two agents that respectively mimic the diacylglycerol (DAG) activation of protein kinase C (PKC) and the I(1,4,5)P_3 elevation of cytosolic Ca^{2+} in the phosphoinositide effect. The cell permeant Ca^{2+} chelator, BAPTA/AM abolished the thapsigargin-induced activation of PLD and inhibited the carbachol- and TPA-PLD responses by 60%. The PKC inhibitor, Ro-31-8220, also inhibited the carbachol- and TPA-PLD responses to a level of approximately double control values, but had no effect on the

thapsigargin induced elevation of PLD. A role for both the PKC-associated and Ca^{2+}-mobilizing arms of the $PI(4,5)P_2$-PLC pathway in PLD regulation is suggested. Pretreatment of cells with the PA-phosphohydrolase blocker, propranolol, significantly enhanced the carbachol-induced elevation of PA, but decreased agonist-stimulated DAG and AA, indicating that PC was the likely source of AA. We therefore propose that, in submandibular mucous acinar cells, muscarinic activation of $PI(4,5)P_2$-PLC pathway regulates PC-PLD through both the PKC- and Ca^{2+}-mobilizing arms of the phosphoinositide response, and that diacylglycerol, derived from PC via phosphatidic acid, is a source of free arachidonic acid.

Introduction

Arachidonic acid (AA; 20:4ω6) is an essential fatty acid esterified at the *sn*-2 position of membrane phospholipids. It is the precursor of a range of bioactive molecules, collectively termed eicosanoids, including prostaglandins, leukotrienes and thromboxanes. It is increasingly evident that AA is an important signalling molecule in its own right, with regulatory functions that are independent of its conversion to eicosanoid metabolites (Graber *et al.*, 1994). In earlier work on rat submandibular acinar cells, we found that AA inhibited phosphoinositide cycle activity by a mechanism that was not blocked by inhibitors of eicosanoid synthesis and was not duplicated by a range of prostaglandins and leukotrienes (Chung and Fleming, 1992). Subsequent enzyme kinetics studies on phosphoinositide cycle enzymes revealed that AA significantly reduced V_{max} but had no effect on the apparent K_m of phosphatidylinositol (PI) 4-kinase and phosphatidylinositol 4-phosphate [PI(4)P] 5-kinase, for both phosphoinositide- and ATP substrates (Chung and Fleming, 1995). It was thus proposed that arachidonate acts as a noncompetitive inhibitor of these kinases in submandibular cells (Chung and Fleming, 1995). Later studies on the same model showed that AA also regulated protein synthesis, ATP levels, endoplasmic reticulum-Ca^{2+} efflux and mucin secretion (Fleming and Mellow, 1995), and established a central role for the fatty acid as a multipotent

regulator of cell function.

The observation that the muscarinic agonist, carbachol, stimulated AA release in submandibular cells (unpublished) caused us to examine the possible phospholipid source of the acid and the characteristics of its enzymatic cleavage pathways. Preliminary studies showed that carbachol did not stimulate the release of AA directly from phosphoinositides or phosphatidylcholine (PC) by the direct action of phospholipase A_2 acting at the *sn*-2 position (assessed by measuring the appropriate lysophospho- derivatives). We therefore turned our attention to a second pathway in which the initial step is the agonist-coupled hydrolysis of PC by the enzyme, phospholipase D (PLD) (Billah and Anthes, 1990; Cockcroft, 1992; Exton, 1994b). This cleavage releases phosphatidic acid (PA), which is converted to diacylglycerol (DAG), a potential source of AA by phosphatidate phosphohydrolase (PAP) (Ishimoto *et al.*, 1994). Agonist stimulation of PC-PLD has been demonstrated in a number of cell types including vascular smooth muscle cells (Plevin *et al.*, 1992; 1994), HL60 cells (Geny and Cockcroft, 1992), Swiss 3T3 cells (Cook *et al.*, 1991), neutrophils (Cockcroft, 1992) and parotid cells (Guillemain and Rossignol, 1994). In many cases, PC-PLD activation appears to be a secondary effect to the preliminary hydrolysis of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] by a $PI(4,5)P_2$-specific phospholipase C (PLC) (Cockcroft, 1992; Exton, 1994b; Plevin *et al.*, 1992; Guillemain and Rossignol,

1994). In support of this, PLD can be stimulated by phorbol ester probes, which mimic PI(4,5)P_2-derived DAG in their capacity to activate protein kinase C (PKC), and so, PKC is a probable mediator of the PI(4,5)P_2-PLC pathway regulation of PC-PLD (Plevin *et al.*, 1992; 1994; Conricode *et al.*, 1994). We have previously established that muscarinic stimulation activated the PI(4,5)P_2 effect in submandibular cells (Fleming *et al.*, 1987; 1989; Chung and Fleming, 1992), and showed that PKC had a central role in the phosphoinositide regulation of the exocrine response (Fleming *et al.*, 1992). We were therefore prompted to undertake the present study on agonist-coupled PLD activation in the submandibular model, to examine the possible involvement of the PI(4,5)P_2-PLC pathway in this, and to investigate the role of PC as a source of arachidonic acid.

Evidence is presented that carbachol stimulates PC-PLD in submandibular cells, that enzyme activation is secondary to the PI(4,5)P_2-PLC effect, and that both the PKC-activating and calcium-mobilizing arms of the phosphoinositide response are involved in PLD regulation. It is further indicated that DAG, derived from PC is a source of free arachidonate.

Materials and methods

Materials

[5,6,8,9,11,12,14,15-^{3}H]arachidonic acid ([^{3}H]AA, specific activity 209 Ci/mmol), [methyl-^{3}H]choline chloride ([^{3}H]choline, specific activity 75.9 Ci/mmol) and *myo*-[2-^{3}H]inositol ([^{3}H]inositol, specific activity 17.8 Ci/mmol) were products of the Amersham Corp. (Arlington Heights, IL). Silica gel-coated thin layer chromatography plates were from Whatman International Ltd. (Maidstone, Kent, UK). The phosphatidylethanol (PEt) standard was supplied by Avanti Polar-lipids Inc. (Alabaster, AL). BAPTA/AM ([1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid tetra-(acetoxymethyl)-ester] and thapsigargin were provided by Calbiochem (La Jolla, CA). U73122 (1-{6-[(17ß-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-1H-pyrrole-2,5-dione) was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Ro-31-8220 (3-{1-[3-(amidinothio)propyl]-3-indolyl}-4-(1-methyl-3-indolyl)-1H-pyrrole 2,5-dione) was a gift from Roche Products Ltd. (Welwyn Garden City, Herts, UK). All other chemicals used were purchased from Sigma (St. Louis, MO).

Preparation and radiolabelling of cells

Acinar cells, prepared as described in Chapter II, were suspended in 6 ml of albumin-free culture medium, at a

cell:volume ratio of 200 mg wet weight per ml, and cell lipids radiolabelled by incubation with 10 μCi of [^{3}H]AA for 90 min. Preparations were then washed in three changes of fresh culture medium and resuspended in 1 ml aliquots containing 60 mg wet weight of cells for experimental treatment. In choline release studies, 6 ml of cell suspension were incubated for 60 min with 10 μCi of [^{3}H]choline, washed and resuspended as before. In experiments on PI(4,5)P_2 hydrolysis, cells were radiolabelled with 100 μCi of [^{3}H]inositol, washed and resuspended as described previously (Chung and Fleming., 1992).

Exposure of dispersed cells to test substances

Optimal concentrations of agonist, probes and blockers were established in preliminary studies. In one series of experiments, the effects of a range of concentrations (1 μM - 1 mM) of carbachol on PC-PLD was examined. Cells labelled with [^{3}H]AA were preincubated with 1% ethanol for five min, then incubated for 10 min with carbachol. Controls were also preincubated with ethanol, but were not treated with carbachol. Reactions were terminated by the addition of 3 ml of a mixture of chloroform, methanol and HCl (100:200:1, by vol). Cell lipids were extracted in the organic phase as described previously (Chung and Fleming, 1995) and PLD activity was measured by the transphosphatidylation reaction as described below. In other experiments, carbachol (100 μM)-

induced release of choline from [^{3}H]choline labelled lipids was measured over 10 min. In this case free labelled choline in the aqueous phase of chloroform and methanol extract after agonist treatment was measured as described below.

In additional studies the effect of the PI(4,5)P_2-PLC blocker, U73122 (Wu *et al.*, 1992; Wang *et al.*, 1994), or its inactive analogue, U73343, on PLD activation was assessed. Cells were exposed to 10 μM U73122 in a final concentration of 1% dimethyl sulphoxide (DMSO) for 15 min then treated with carbachol as above. Controls included DMSO but no U73122. PLD activity was measured as before. The capacity of U73122 to inhibit PI(4,5)P_2 hydrolysis over a 10 min period was measured by its effect on the production of [^{3}H]-I(1,4,5)P_3, [^{3}H]-I(1,4)P_2 and [^{3}H]-I(4)P in carbachol-treated cells prelabelled with [^{3}H]inositol. Inositol phosphates were extracted by trichloroacetic acid precipitation, separated by anion exchange chromatography and quantitated by scintillation counting as described before (Fleming *et al.*, 1987).

In other experiments the effects of the phorbol ester, TPA (1 μM), and the cytosolic Ca^{2+}-elevator, thapsigargin (Gericke *et al.*, 1993) (2 μM), on PLD activity were compared with the carbachol-PLD stimulating effect. In these studies, cells were also pretreated with Ro-31-8220 (10 μM), an inhibitor of PKC activity (Nixon *et al.*, 1992), or BAPTA/AM (100 μM), a cell permeant Ca^{2+} chelator (Natarajan and Garcia, 1993) for 15 min, before treatment with carbachol, TPA or

thapsigargin. TPA, thapsigargin, Ro-31-8220 and BAPTA/AM were solubilized in 1% (final) DMSO, and this solvent was included in controls at the same concentration. PLD activity was again measured.

In a final series of experiments, cells were radiolabelled with [^{3}H]AA, preincubated in the presence or absence of the PAP blocker, propranolol (Jamal *et al.*, 1991) (100 μM) for 15 min, then treated with carbachol for 10 min and extracted with chloroform/methanol. [^{3}H]-labelled phosphatidic acid, diacylglycerol and free AA in the organic phase were separated by thin layer chromatography (TLC) and quantitated by scintillation counting as described below.

Assays

PLD was assayed by measuring the formation of [^{3}H]phosphatidylethanol (PEt) in the PLD-specific transphosphatidylation reaction (Dawson, 1967; Kobayashi and Kanfer, 1987). In this reaction, PEt is produced at the expense of PA in the presence of ethanol. The extracted organic phase of cells incubated with 1% ethanol was dried under N_2, resolubilized in 100 μl of a mixture of chloroform, methanol and H_2O (75:25:2, by vol), and spotted on to silica gel TLC plates which had been activated at 110° C for 1 hour. The chromatograms were developed at room temperature in the organic phase of ethyl acetate, 2,2,4-trimethyl pentane, acetic acid and H_2O (13:2:3:10, by vol) (Pfeilschifter and

Merriweather, 1993). Lipids were visualized by iodine vapour, and PEt identified by reference to a purified standard (R_f = 0.34). [^{3}H]choline was measured in the aqueous phase of cell extracts. The upper, aqueous phase was evaporated, resolubilized in 50 μl of 50% ethanol and applied to TLC plates. The plates were developed in a solvent system of methanol, 0.5% NaCl and NH_4OH (10:10:1, by vol) (Martinson *et al.*, 1989). Radiolabelled choline was detected at R_f = 0.11.

[^{3}H]PA was measured in the organic phase of cell extracts by TLC (R_f = 0.25) in the same solvent system used for PEt (above). [^{3}H]DAG and [^{3}H]AA in the extracted organic phase were separated by TLC in a solvent system of diethyl ether, petroleum ether and acetic acid (50:50:1, by vol, empirically derived) (DAG R_f = 0.27; AA R_f = 0.48).

In all cases, TLC-separated radiolabelled species were scraped from the TLC plates and quantitated by scintillation counting as described in chapter III.

Statistical analysis

The data were analyzed statistically by two-way analysis of variance. Means were compared by Duncan's multiple range test or the Student t-test. Values of $p<0.05$ were considered significant.

Results

Activation of phospholipase D by carbachol

Carbachol stimulated the activation of PLD, assessed by [^{3}H]PEt generation in the transphosphatidylation reaction, in a concentration-dependent manner (Fig. 18). The maximal response of approximately five fold control values was reached at an agonist concentration of 100 μM. The carbachol effect was abolished by preincubation of submandibular cells with 10 μM atropine for 10 min before treatment (data not shown). Over the same 10 min time period, carbachol also enhanced the release of free [^{3}H]choline to 160% control values ($p < 0.05$), providing additional proof that the substrate for PLD action was PC (Fig. 18, inset). The difference in magnitude of the carbachol elevation of PEt levels (470% control) and its stimulation of free choline (160% control), is a probable reflection of the relative stabilities of these products. PEt is non-physiological and is not further metabolized in the cell, whereas choline is rapidly incorporated into synthetic pathways, including the resynthesis of PC.

Involvement of the phosphatidylinositol 4,5-bisphosphate-phospholipase C hydrolysis pathway in phospholipase D activation

The carbachol activation of PLD was re-examined under conditions in which the hydrolysis of PI(4,5)P_2 by PLC was

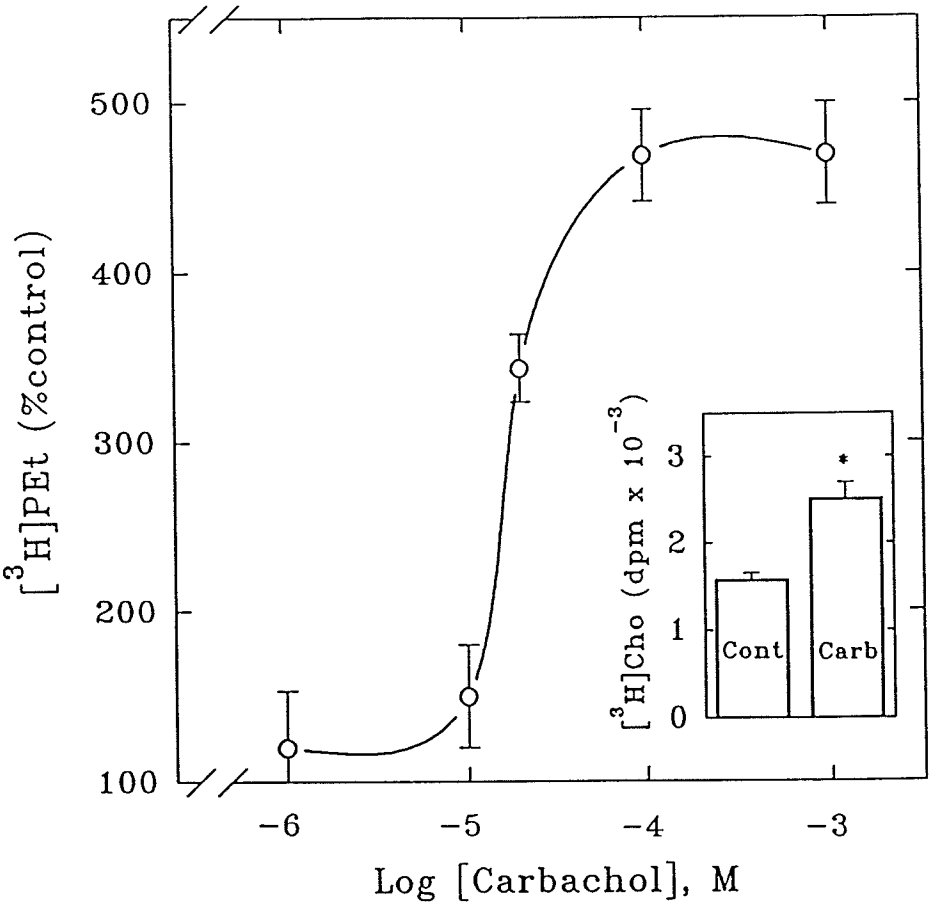

Fig. 18. Effect of carbachol concentration on PLD activation ([^{3}H]PEt accumulation) in rat submandibular acinar cells.

[^{3}H]arachidonic acid-labelled acinar cells were preincubated with 1% ethanol for five min, then treated with carbachol for 10 min. Radiolabelled PEt was extracted with a mixture of chloroform, methanol and HCl, separated by TLC and quantitated by scintillation counting as described in Materials and methods. Values are means ± SEM , n = 4. The average basal value for unstimulated controls was 655 d.p.m. [^{3}H]PEt.

Inset: The effect of carbachol on choline release in submandibular cells. Cells prelabelled with [^{3}H]choline were incubated with or without 100 μM carbachol for 10 min. Radiolabelled choline was extracted, separated by TLC and quantitated by scintillation counting as described in Materials and methods. Values are means ± SEM, n = 5.
$^*p < 0.05$ compared with control value.

inhibited. Preincubation of cells with an optimal concentration of the aminosteroid PLC inhibitor, U73122 (10 μM), for 15 min reduced the subsequent carbachol activation of PLD to 40% of the normal stimulated value (Fig. 19). The inactive analogue, U73343, had no effect on the carbachol response (data not shown). The inhibitory effect of U73122 on the PLC pathway was confirmed by measuring phosphoinositide hydrolysis in [^{3}H]inositol labelled cells exposed to a range of concentrations of the blocker. U73122, at an optimal concentration of 10 μM, inhibited the production of [^{3}H]-I(1,4,5)P_3, [^{3}H]-I(1,4)P_2 and [^{3}H]-I(4)P by 90% (Fig. 19, inset). These findings indicate that inhibition of PI(4,5)P_2-PLC significantly reduces, but does not completely abolish the carbachol activation of PLD.

Possible regulation of PLD by both the DAG-PKC and the Ca^{2+}-mobilizing arms of the PLC pathway was investigated by using appropriate probes and blockers. The phorbol ester, TPA, a PKC activator, stimulated PLD to around seven fold control values, a level greater than that produced by carbachol (Fig. 20). It is likely that this response reflects a sustained action of the stable TPA, compared with the more transient effects of carbachol-induced metabolites (DAG/Ca^{2+}) in enzyme activation. The phorbol-12,13-didecanoate (4-α-PDD), which does not activate PKC, had no effect on PLD activity (data not shown). The TPA elevation of PLD activity was reduced to 16% of the normal phorbol ester response by the PKC inhibitor, Ro-

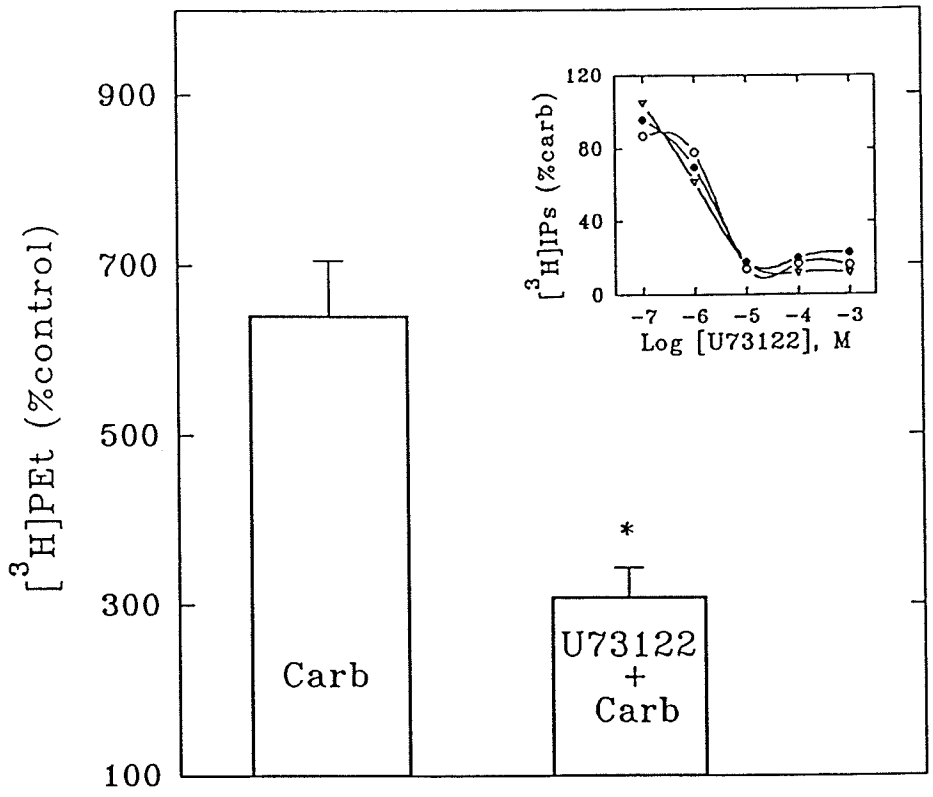

Fig. 19. The effect of U73122, a phospholipase C inhibitor on carbachol-stimulated phospholipase D in rat submandibular acinar cells.

Cells prelabelled with [^{3}H]AA were preincubated with or without 10 μM U73122 for 15 min, then treated with 100 μM carbachol plus 1% ethanol for 10 min. Radiolabelled PEt was separated and quantitated as described for Fig. 18. Values are means ± SEM, n = 4. The average basal value for unstimulated controls was 612 d.p.m. [^{3}H]PEt. $^*p < 0.01$ compared with the carbachol response.

Inset: The effect of U73122 concentration on the carbachol-induced production of inositol phosphates in submandibular acini. Cells prelabelled with [^{3}H]inositol were preincubated with or without 10 μM U73122 for 15 min, then exposed to 100 μM carbachol for 10 min. [^{3}H]-I(1,4,5)P_3 (∇), [^{3}H]-I(1,4)P_2 ($\bullet$), and [^{3}H]-I(4)P(O), were extracted and quantitated by anion exchange chromatography and scintillation counting as described in Materials and methods. Values, expressed as a percentage of the carbachol response for each inositol phosphate, are means, n = 4. The SEMs (never more than 13%), are omitted for clarity. Average carbachol-stimulated values were :[^{3}H]-I(1,4,5)P_3, 3130 d.p.m.; [^{3}H]-I(1,4)P_2, 6791 d.p.m.; [^{3}H]-I(4)P, 13312 d.p.m.

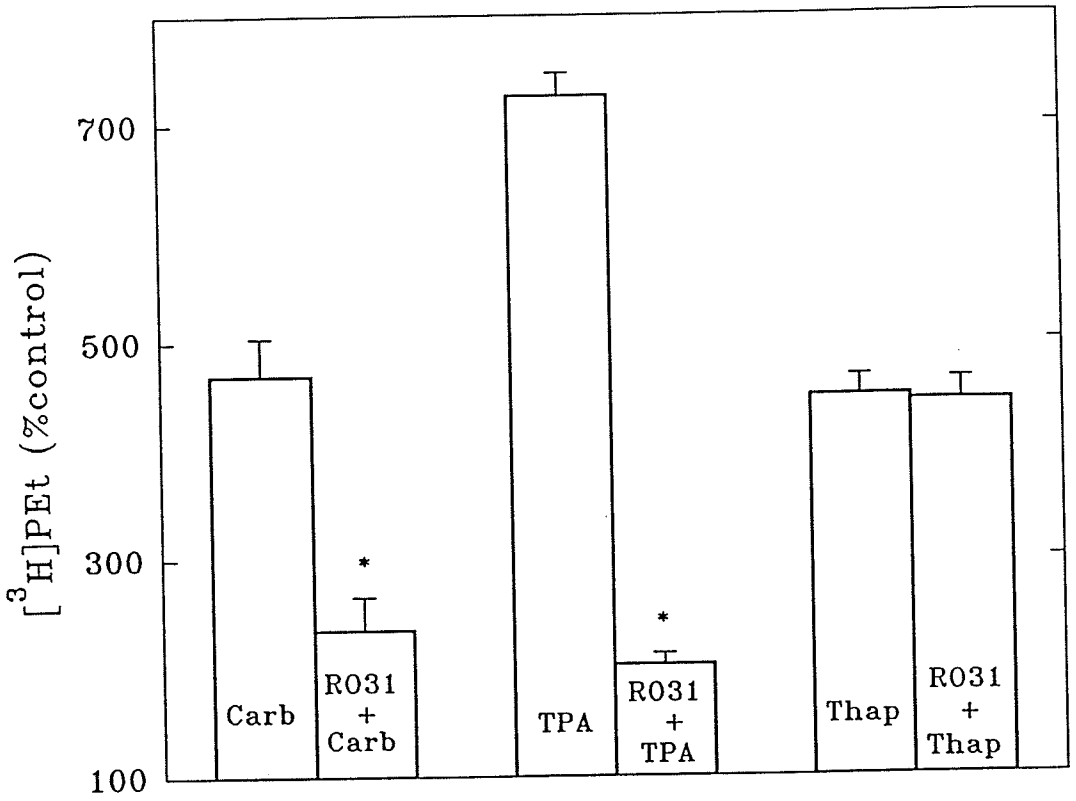

Fig. 20. The effect of Ro-31-8220, a protein kinase C (PKC) inhibitor on phospholipase D in rat submandibular acinar cells.

Cells prelabelled with [^{3}H]AA were preincubated in the presence or absence of 10 μM Ro-31-8220 (RO31) for 15 min, then treated with 100 μM carbachol, or 1 μM TPA, or 2 μM thapsigargin (Thap) plus 1% ethanol for 10 min. Radiolabelled PEt was separated and quantitated as described for Fig. 18. Values are means ± SEM, n = 4. The average basal value for unstimulated controls was 706 d.p.m. [^{3}H]PEt. $^*p < 0.01$ compared with the corresponding carbachol or TPA response.

31-8220. Carbachol activation of PLD was also inhibited by Ro-31-8220 to about the same absolute level of approximately two fold control values (Fig. 20). Thapsigargin (2 μM), an agent that elevates cytosolic Ca^{2+} by inhibition of influx-directed endoplasmic reticulum Ca^{2+}-ATPase, activated PLD to four fold control levels. This response was not inhibited by Ro-31-8220 (Fig. 20). Taken together, these findings suggest that both PKC and cytosolic Ca^{2+}-levels regulate PLD in submandibular cells. Additional experiments with the cell permeant Ca^{2+} chelator, BAPTA/AM, supported this idea. As expected, preincubation of cells with 100 μM BAPTA/AM completely inhibited the thapsigargin-induced activation of PLD (Fig. 21). The chelator also inhibited the carbachol-PLD effect by 76% and the TPA-PLD effect by 62% (Fig. 21). This latter observation most likely indicates a calcium-requirement of PKC for full functional activity, though TPA activation of PLD was not completely blocked by BAPTA/AM treatment.

Carbachol-induced release of phosphatidylcholine metabolites

Carbachol effects on the production of radiolabelled PA, DAG and AA in [^{3}H]AA-incubated cells were measured. The agonist stimulated PA levels to around four times control values and also produced smaller, but statistically significant increases in DAG [150 ± 9 % (mean ± SEM), of controls)] and AA [144 ± 8 % (mean ± SEM), of controls)] (both $p < 0.05$) (Fig. 22). The lower levels of DAG and AA are again probably due to the rapid

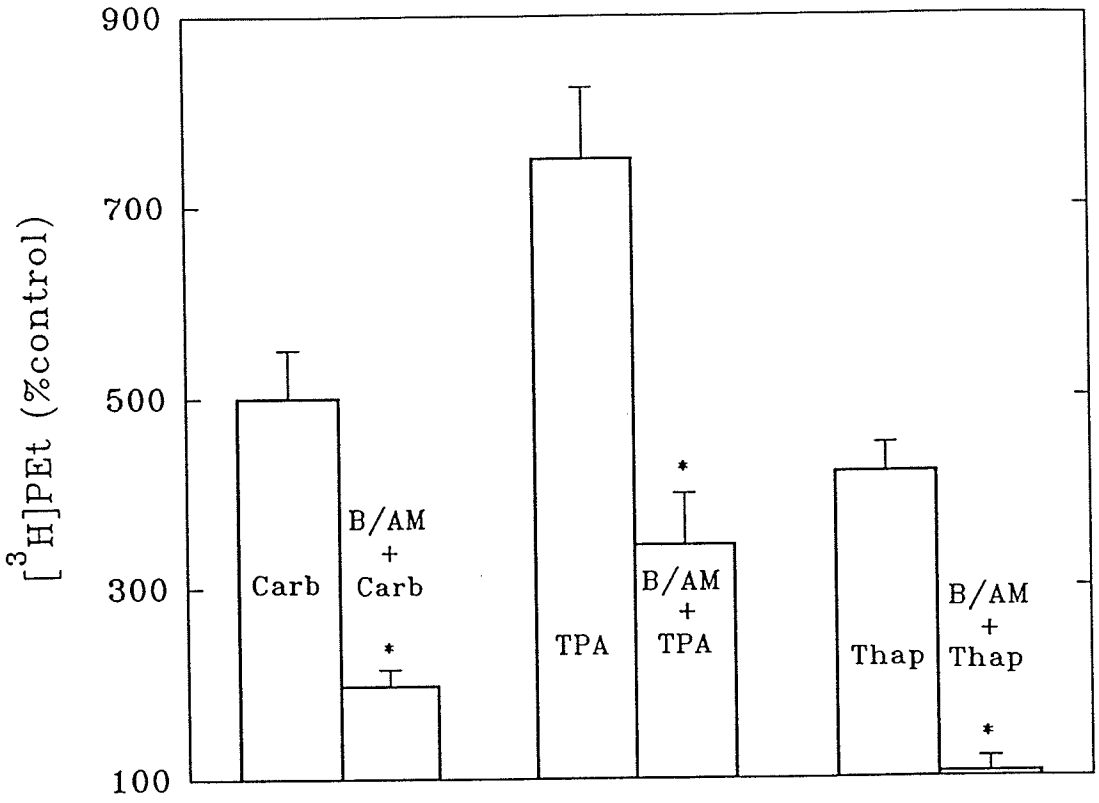

Fig. 21. The effect of BAPTA/AM, a cell-permeant Ca^{2+} chelator on phospholipase D in rat submandibular acinar cells.

Cells prelabelled with [^{3}H]AA were preincubated in the presence or absence of 100 μM BAPTA/AM (B/AM) for 15 min, then treated with 100 μM carbachol, or 1 μM TPA, or 2 μM thapsigargin (Thap) plus 1% ethanol for 10 min. Radiolabelled PEt was separated and quantitated as described for Fig. 18. Values are means ± SEM, n = 4. The average basal value for unstimulated controls was 683 d.p.m. [^{3}H]PEt. $^*p < 0.01$ compared with the corresponding carbachol, TPA, or thapsigargin response.

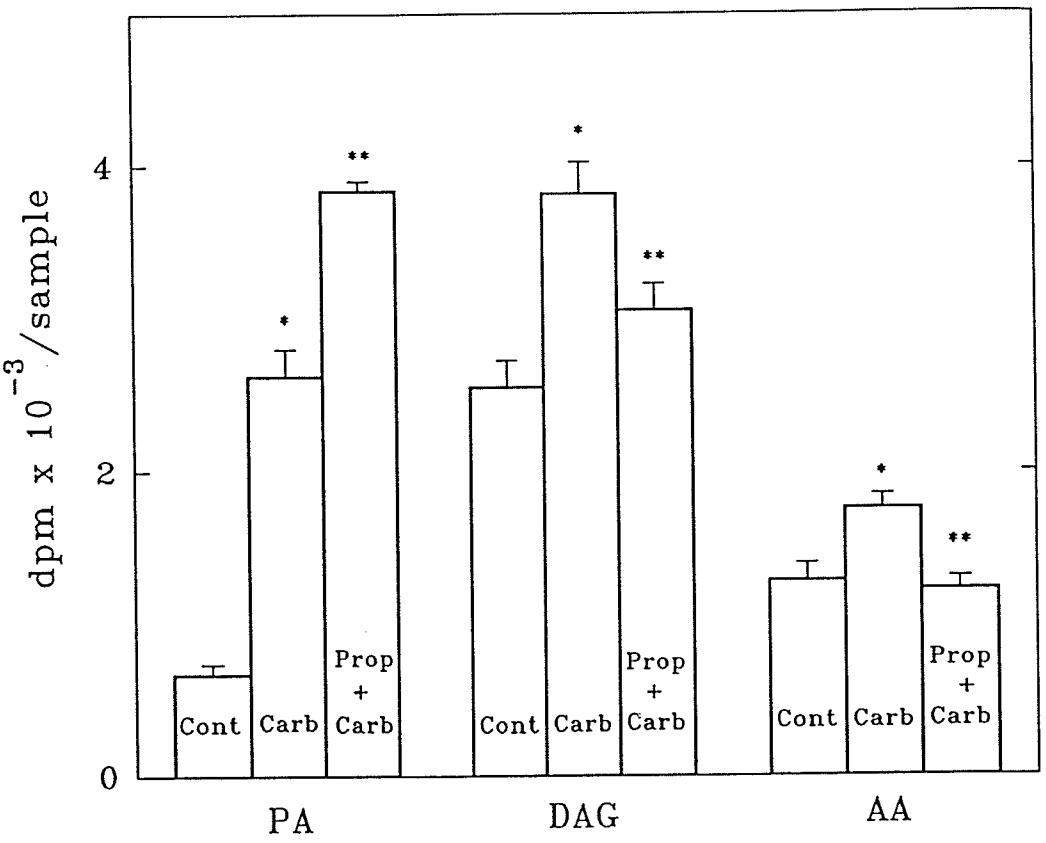

Fig. 22. The effect of carbachol on production of phosphatidic acid (PA), diacylglycerol (DAG) and arachidonic acid (AA) in rat submandibular acinar cells.

Cells prelabelled with [^{3}H]AA were incubated with or without 100 μM carbachol for 10 min. Radiolabelled PA, DAG and AA were extracted with a mixture of chloroform, methanol and HCl, separated by TLC and quantitated by scintillation counting as described in Materials and methods. Other preparations were treated with 100 μM propranolol, a PAP inhibitor, for 15 min before carbachol exposure, and PA, DAG and AA measured as before. Values are means ± SEM, n = 9. Propranolol alone had no effect on control values (not shown). $^{*}p < 0.05$ compared with the control value; $^{**}p < 0.05$ compared with the carbachol response.

metabolism of these products. Preincubation of cells with 100 μM propranolol, an inhibitor of PAP, the enzyme that converts PA to DAG, increased the carbachol-induced PA to six fold control values, but significantly reduced DAG and AA ($p < 0.05$) (Fig. 22). It was noted that the total carbachol-induced PA-[^{3}H] d.p.m. gained by propranolol treatment (average 1212 d.p.m.) were equal to the combined total of those lost from DAG and AA (average 1289 d.p.m.) on propranolol treatment (Fig. 22). These results are compatible with the idea that the source of AA released by carbachol in submandibular glands is DAG, derived from PA which is produced by the hydrolysis of PC by PLD.

Discussion

Activation of PLD hydrolysis of PC by a range of agonists has been described in a number of cell types (for reviews, see Billah and Anthes, 1990; Cockcroft, 1992; Thompson *et al.*, 1993; Exton, 1994b). In many cases, there is evidence that activation of PLD is secondary to the hydrolysis of phosphoinositides by PLC. The $PI(4,5)P_2$-PLC signal transduction pathway is characterized by the production of two signalling molecules - $I(1,4,5)P_3$, which mobilizes Ca^{2+} from endoplasmic reticulum stores, and DAG which stimulates the enzyme PKC to regulate many cell processes. This DAG effect is mimicked by phorbol esters, which are thus commonly used as probes of the DAG/PKC arm of the $PI(4,5)P_2$-PLC pathway (Cockcroft, 1992; Exton, 1994b). PLD can be activated by $PI(4,5)P_2$-coupled agonists such as bombesin in Swiss 3T3 cells (Cook *et al.*, 1991) and vasopressin in vascular smooth muscle cells (Plevin *et al.*, 1992). Phorbol ester activation of PLD has also been demonstrated in the same models as well as in renal mesangial cells (Pfeilschifter and Merriweather, 1993), fibroblasts (Conricode *et al.*, 1992; 1994) and others. Muscarinic activation of PC-PLD has been reported in astrocytoma cells (Martinson *et al.*, 1989), Chinese hamster lung fibroblasts (McKenzie *et al.*, 1992) and rat parotid gland (Guillemain and Rossignol, 1994). Cleavage of PC by PLD may be the first stage in the release of free arachidonic acid

(Matilla *et al.*, 1993; Ishimoto *et al.*, 1994), a molecule with regulatory functions on several cell processes (Graber *et al.*, 1994), including those of protein synthesis and secretion in submandibular acinar cells (Fleming and Mellow, 1995).

We have demonstrated in earlier work on the submandibular model that muscarinic stimulation activates the $PI(4,5)P_2$ pathway (Fleming *et al.*, 1987; Chung and Fleming, 1992), and elevates free AA (unpublished). In the present study, we therefore sought to establish a link between these observations by investigating whether the muscarinic agonist, carbachol, activated PC-PLD in an AA release mechanism, and whether this was secondary to the agonist-coupled $PI(4,5)P_2$ effect. PLD activation was assessed by the enzyme-specific transphosphatidylation reaction in which the stable product, PEt, is produced in the presence of ethanol (Exton, 1994b).

Carbachol stimulated PEt production in a concentration-dependent response and also elevated free choline, providing additional evidence that PC was the substrate for PLD action. The mediation of the $PI(4,5)P_2$-PLC pathway was indicated by the observation that treatment of cells with the PLC pathway inhibitor U73122, reduced the carbachol-PEt effect by 60%. Our results are thus consistent with those of others who found that PLD activation was secondary to $PI(4,5)P_2$ hydrolysis in several cell types (Cook *et al.*, 1991; Plevin *et al.*, 1994; Guillemain and Rossignol, 1994; Exton, 1994b). The bifurcating $PI(4,5)P_2$ pathway activates PKC and elevates cytosolic Ca^{2+} and

there is evidence that either or both of these signals can regulate PLD activity. For example, Natarajan and Garcia (1993) found that both PKC and Ca^{2+} activated PLD in bovine pulmonary artery endothelium. Guillemain and Rossignol (1994) showed that, in parotid acinar cells, muscarinic agonist stimulation of PLD was calcium-dependent, while phorbol ester activation of the enzyme was calcium-independent.

In the present study we attempted to demonstrate the nature of the $PI(4,5)P_2$ pathway regulation of PLD by manipulating cytosolic Ca^{2+} levels and PKC activity. PLD was activated by carbachol, TPA and the calcium mobilizer, thapsigargin. The TPA effect was reduced by 84% by the PKC inhibitor, Ro-31-8220, which also partially lowered the carbachol response but had no effect on thapsigargin-induced PLD activity. These findings suggest that PKC and Ca^{2+} may both regulate PLD in submandibular cells. Our finding that agonist-stimulated PLD could be only partially blocked by the PKC inhibitor is consistent with the observations of Cook *et al.* (1991) on Swiss 3T3 cells. In additional experiments in the present study, the PLD activating effects of carbachol, TPA and thapsigargin were examined in the presence of the Ca^{2+} chelator, BAPTA/AM. Pretreatment of cells with BAPTA/AM abolished the thapsigargin response but only partially inhibited both the carbachol- and TPA-PLD effects. Considering the results of these Ro-31-8220 and BAPTA/AM inhibition experiments together, it is suggested that carbachol activates

PLD by mechanisms involving both the PKC and Ca^{2+}-mobilizing arms of the PI(4,5)P_2-PLC pathway. It is further indicated that, while Ca^{2+} and PKC may act independently, a significant component of the PKC effect is calcium dependent. The involvement of calcium in the agonist- or PKC-regulation of PLD in different models is inconsistent. Pfeilschifter and Merriweather (1993) showed that, in renal mesangial cells, elevation of cytosolic Ca^{2+} by ionophore A23187 or thapsigargin produced a small increase in PLD activation. However, chelation of free Ca^{2+} with Quin 2 did not lower the ATP- or UTP-induced activation of PLD. Plevin *et al.* (1992), found that the PI(4,5)P_2-coupled agonist, vasopressin, stimulated PLD activation in A10 vascular smooth muscle cells by a mechanism that was completely blocked by Ro-31-8220 treatment, suggesting the absence of an independent stimulatory effect of the Ca^{2+}- leg of the PLC pathway, but not ruling out a PKC-calcium dependency. The partial involvement of extracellular calcium in the vasopressin activation of PLD, however, was reported in the same study. In parotid cells, depletion of extracellular calcium did not affect PLD activation by phorbol esters, but inhibited PLD stimulation by receptor-linked agonists (Guillemain and Rossignol, 1994).

In the present study, the indication that a component of the PKC effect is calcium-dependent could reflect the involvement of calcium-dependent and calcium-independent PKC isoforms in PLD activation. In this respect, the Ca^{2+}-dependent

isoform, PKC-α, has been implicated in PLD stimulation in Swiss 3T3 cells (Eldar *et al.*, 1994), hamster lung fibroblasts (Conricode *et al.*, 1994) and Madin-Darby canine kidney cells (Balboa *et al.*, 1994). In rat glomerular mesangial cells, however, Pfeilschrifter and Merriweather (1993) reported that PKC-ϵ mediated the ATP and UTP activation of PLD. In a recent study in our own laboratory we showed, by using PKC-isoform specific antibodies, that TPA activated both the α and ϵ isoforms, but that carbachol activated only PKC-ϵ (Fleming and Mellow, submitted for publication). In the present study, the TPA activation of PLD was greater than the carbachol stimulation of the enzyme, but both were reduced to the same absolute level of activity by Ro-31-8220, which is believed to be a preferential inhibitor of the PKC-α isoform (Wilkinson *et al.*, 1993). It is therefore an attractive speculation that the PKC arm of the carbachol response may be associated with the PKC-ϵ isoform, while TPA may activate PLD via both PKC-α and ϵ isoforms. Since PKC-ϵ is classified as a calcium independent form of the enzyme, this idea is supported by residual PLD-stimulating activity in carbachol- and TPA treated cells after BAPTA/AM exposure.

The fact that carbachol activation of PLD could not be completely blocked by U73122 suggests that there are other, PLC-independent, agonist-coupled routes which lead to its activation. There is evidence that activation of the enzyme is mediated by GTP-binding regulatory G proteins, discrete from

G_q of the $PI(4,5)P_2$ pathway (Cockcroft, 1992; Geny and Cockcroft, 1992; Exton, 1994b). For example, Geny and Cockcroft (1992) found that in HL60 cells, $GTP_\gamma S$ induced sustained activation of PLD which was not reduced by PKC inhibition. Recent results suggest that ADP-ribosylating factor (ARF), a member of the low-molecular-weight GTP-binding protein family may also activate PLD (Cockcroft *et al.*, 1994; Exton, 1994b). These mechanisms are currently under investigation in the submandibular model.

Since PC is the major phospholipid in cell membranes it is a potential source of arachidonate, the precursor of the eicosanoids and an important regulatory molecule in its own right. The simplest route of AA release is its direct cleavage from the *sn*-2 position by phospholipase A_2 (PLA_2) (Cockcroft, 1992). However, in unpublished studies we have not observed agonist-induced PI- or PC-PLA_2 activity in submandibular cells. Another possible pathway is the cleavage of PC by PLD to produce phosphatidic acid, which is metabolized by PA-phosphohydrolase (PAP) to DAG, which is in turn hydrolysed by DAG lipase to release AA. This pathway has been described in mast cells (Ishimoto *et al.*, 1994) and endothelial cells (Mattila *et al.*, 1993) among others, and was investigated in the present study. Carbachol stimulated the release of PA to around four times control levels, and this was accompanied by smaller, but significant increases in DAG and AA. When cells were pretreated with propranolol, an inhibitor of PAP, the PA

levels were significantly increased and the DAG and AA levels decreased. Moreover, the gain in PA-[^{3}H]-AA counts exactly matched the total loss in DAG-[^{3}H]-AA plus [^{3}H]-AA counts. These observations indicate that carbachol enhancement of AA levels reflects the release of the fatty acid from DAG, derived from PC.

In summary, the present study provides evidence that in submandibular acinar cells, muscarinic stimulation causes the activation of PC-PLD via the PI(4,5)P_2-PLC pathway, and that both the protein kinase C- and Ca^{2+}-mobilizing arms of the pathway are involved in PLD regulation. Furthermore, the phosphatidic acid produced by PC cleavage is converted to diacylglycerol, which is a source of free arachidonate in the cells. This pathway of AA release is summarized in Fig. 23. Since both carbachol (Fleming *et al.*, 1987) and AA (Fleming and Mellow, 1995) stimulate mucin release in the model, it is possible that the muscarinic-induced PLD pathway has a coupling function in the exocrine response. Agonist activation of PLD may also play a role in cholinergic stimulation of fluid secretion in salivary glands, but this is not yet been investigated. Earlier work in our laboratory showed that AA occupies a central position as a signalling molecule in the submandibular gland, with a role in the regulation of protein synthesis/secretion, phosphoinositide cycle activity, calcium fluxes and ATP levels (Chung and Fleming, 1992; 1995; Fleming and Mellow, 1995). Control of PLD activity is now here identified as an important factor in regulating the availability of arachidonic acid.

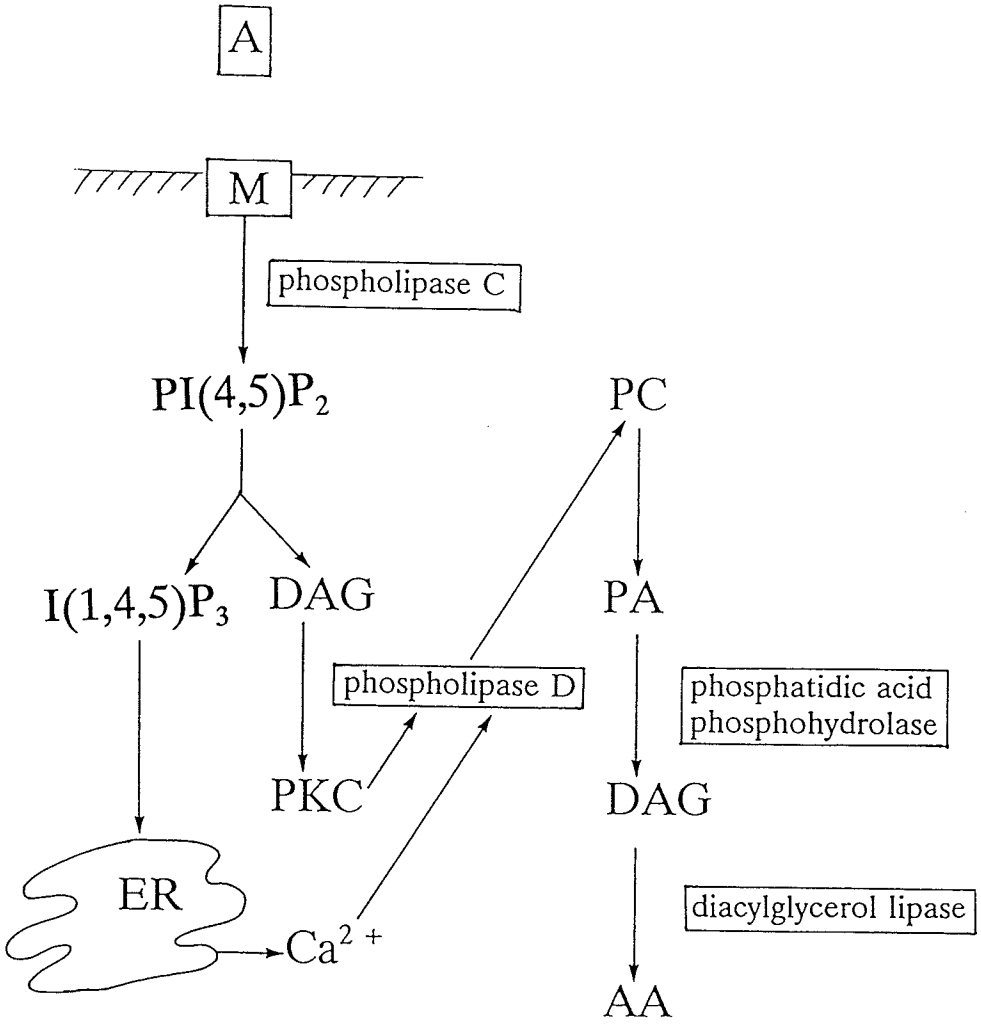

Fig. 23. Proposed pathway for muscarinic agonist-induced release of Arachidonic acid from phosphatidylcholine in rat submandibular acinar cells.

See text for details. Abbreviations: A, muscarinic agonist; M, muscarinic receptor; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; I(1,4,5)P3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; ER, endoplasmic reticulum; PKC, protein kinase C; PC, phosphatidylcholine; PA, phosphatidic acid; AA, arachidonic acid.

CHAPTER V

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The studies described in this thesis were undertaken to examine the role of arachidonic acid in the regulation of agonist-coupled signal transduction in rat submandibular glands. Our early approaches focused on the phosphoinositide cycle and the question specifically addressed was whether AA regulated cycle activity, and if so, whether this represented a feedback control mechanism by the endogenous fatty acid derived from phosphoinositides. Our experiments confirmed that AA did inhibit the muscarinic agonist-stimulated phosphoinositide cycle and, furthermore, showed for the first time that the mechanism of AA action was that of noncompetitive inhibition of both PI 4-kinase and PI(4)P 5-kinase in the synthetic phase of the cycle. Further studies indicated, however, that membrane phosphoinositides were not the probable phospholipid source of AA, at least via phospholipase A_2 action, released by carbachol treatment. Our attention therefore turned to a second possible donor phospholipid, phosphatidylcholine. Strong evidence was generated that a preliminary step in AA release was the carbachol-induced activation of PC-specific phospholipase D. It was further shown that the phosphatidic acid generated by this hydrolysis was rapidly converted into diacylglycerol, from which AA was cleaved by the enzyme DAG lipase. Additional experiments revealed that the PC-PLD reaction was secondary to the agonist-coupled hydrolysis of $PI(4,5)P_2$ and that both the $I(1,4,5)P_3/Ca^{2+}$ and the DAG/PKC arms of the phosphoinositide

effect were involved in PLD activation.

We thus propose that muscarinic stimulation releases AA that acts as a feedback inhibitor of phosphoinositide cycle activity. However, this feedback is indirect, since AA is not derived from the phosphoinositides themselves, but from PC, the hydrolysis of which is downstream to the initial $PI(4,5)P_2$-phospholipase C effect. A central regulatory role for AA in a major signal transduction pathway in submandibular gland cells is thus established, and a major phospholipid source (PC) of free arachidonate identified. Our observation that the carbachol activation of PC-PLD could not be completely blocked by inhibition of the phosphoinositide effect, leads to the idea that muscarinic receptors may also be coupled to PLD by alternative and more direct pathways that are independent of the $PI(4,5)P_2$ effect.

Investigation of such additional coupling pathways will form the future work of this laboratory. One possible mechanism is the direct linkage of the receptor to a putative G regulatory protein (termed G_e; Cockcroft, 1992) which is discrete from G_q of the $PI(4,5)P_2$ system, and is coupled to PLD. Experiments with NaF- and guanine nucleotide activators of G proteins, under conditions of G_q inactivation are now under way. A second possible pathway is one involving the ADP-ribosylating factor (ARF). ARF belongs to a subfamily of the *ras* superfamily of small, monomeric, GTP-binding proteins that are distinct from the heterotrimeric G regulatory proteins. A

role for ARF in vesicle transport and membrane fusion has been proposed, but only recently has evidence been generated to suggest that the protein may activate PLD (Brown *et al.*, 1993; Cockcroft *et al.*, 1994; Massenburg *et al.*, 1994; Boman and Kahn, 1995). A possible receptor-coupled ARF pathway leading to PC cleavage will thus be investigated in this laboratory by using a range of techniques centered around a cell free ARF-PLD activation assay.

Our experiments on the PI(4,5)P_2-PC-PLD activation sequence showed that the DAG arm of the phosphoinositide effect stimulated PLD. PLD cleavage of PC to PA to DAG therefore maintains DAG levels so that the possibility exists that this pathway forms a positive, reinforcement loop, providing fresh DAG to sustain the PLD effect. It is not known whether PC-derived DAG can stimulate PLD in the same way as PI(4,5)P_2-DAG, what the important fatty acid differences between the species might be and whether they form different metabolic pools. These parameters will also be examined in the submandibular model. Such investigations should clarify the central position of PLD and its mechanisms of activation in the release of regulatory arachidonic acid.

Other studies in this laboratory, outside the scope of this thesis, have identified several additional important functions of AA in the control of many key physiological processes in submandibular glands. AA inhibits nucleic acid- and protein synthesis and ATP levels, but stimulates Ca^{2+}

efflux from the endoplasmic reticulum and provokes mucin secretion (Fleming and Mellow, 1995). These findings, taken together with those discussed above in the body of the thesis, clearly establish a central position for arachidonate as a multipotent regulator of submandibular gland physiology. They also provide the groundwork for more in-depth investigations on the nature of AA regulatory effects on the various systems examined, and the potential arachidonate-related interactions between synthetic and secretory pathways in salivary glands.

CHAPTER VI

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