LEUKOTRIENE BIOSYNTHESIS AND SECONDARY MESSENGERS IN RAT BASOPHILIC LEUKAEMIA CELLS

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by

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The most sophisticated aspect of Science is its simplicity, and the simplest aspect of Science is its unity. Is unity not the essence of Science?

S. H. I. Galadari

To my late Father who was like
"a candle giving light while it melts away"

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ABSTRACT

Previous studies have demonstrated that cyclic-AMP (cAMP) and neuropeptides activating adenyl cyclase in lungs inhibit platelet activating factor-induced (PAF-induced) and antigen-induced Leukotriene biosynthesis. The possibility exists that this effect is due to an inhibition of agonist-induced transducing events such as the phosphatidylinositol (PI) cycle, leading to inhibition of phospholipase A_2 (PLA_2) activation and arachidonic acid release.

In a study of Leukotriene biosynthesis and second messenger interactions in a model system, RBL-1 cells, the effects of a number of agonists upon arachidonic acid release and Leukotriene generation, as well as upon PI breakdown and inositol phosphate (IP) release, were investigated. Amongst these, Leukotriene D_4 (LTD_4), 5'-(N-ethyl) carboxamidoadenosine (NECA), immunological challenge and the calcium ionophore A23187 were found to induce PI breakdown, whilst A23187 induced Leukotriene biosynthesis. When LTD_4-induced and immunological challenge-induced PI breakdown and IP release were conducted in the presence of cAMP analogues, 8 bromo-cAMP (8Br-cAMP) and dibutyryl-cAMP (db-cAMP) at 100μM concentrations, marked inhibitions in IP release were observed. For the first time, it was shown that cAMP analogues markedly inhibit LTD_4-induced and immunological challenge-induced PI breakdown and IP release in RBL-1 cells. This effect does not appear to be a general response to cAMP analogues since NECA-induced IP release was unaffected by 100μM 8Br-cAMP.

Furthermore, the 5-lipoxygenase inhibitor AA861 at 100μM concentration abolished the A23187-induced IP release. The A23187-induced IP release appears to be mediated by LTD_4 (and/or other peptido-Leukotrienes) since the LTD_4 receptor antagonist, FPL-55712, at 10μM concentration also led to a significant inhibition of A23187-induced IP release. However, LTD_4, NECA and immunological challenge did not activate PLA_2 since incubation of RBL-1 cells with these agonists did not lead to any significant arachidonic acid release or Leukotriene biosynthesis.

The findings in this thesis suggest that in RBL-1 cells:

a) cAMP plays an inhibitory role upon PI-specific phospholipase C (PIC) activation and IP release, and this effect is receptor specific.

b) PLA_2 activation leads to PIC activation and IP release, mediated by Leukotrienes, indicating a modulatory role for Leukotrienes.

c) PIC activation does not lead to PLA_2 activation implying that Leukotriene biosynthesis is independent of the PI cycle.

These findings, together with others presented and discussed in this thesis, have enabled a better understanding of Leukotriene biosynthesis and second messenger interactions in RBL-1 cells, including a new appraisal of the LTD_4 signalling pathway.

1 INTRODUCTION

1.1.1 Discovery of Leukotrienes

Substances, which we now term the Leukotrienes, were discovered in the late 1930s by Feldberg and Kellaway, as smooth muscle stimulating factor released into perfusates from dog lungs during treatment with cobra venom (1). The same or similar factors were formed by perfused guinea pig lungs from animals sensitized to egg albumin, during challenge with antigen (2). Histamine, which was also released in these experiments, induced rapid, short-lasting contractions of isolated guinea pig jejunum, whereas the new factor induced contractions with a slow onset and a prolonged duration, hence termed "Slow Reacting Substance" (SRS) by Kellaway and Trethewie (2). In 1960, Brocklehurst (3) extended the name for immunologically released SRS to "Slow Reacting Substance of Anaphylaxis" (SRS-A). This designation was used until the chemical structure of SRS-A was reported (4) and the term "Leukotrienes" was adopted (5).

A great deal of interest in SRS-A followed observations by Brocklehurst (3) that exposure of lung tissue from asthmatic patients to pollen induced formation of SRS-A. SRS-A was also produced during allergen-induced constriction of bronchial rings from post mortem tissue from these patients. Moreover, human bronchial smooth muscle was very sensitive to SRS-A obtained from guinea pig lung (6). These results and the observations that IgE or human atopic sera could be used to sensitize monkey lung fragments *in vitro* to form SRS-A in response to antigen challenge (7) suggested that SRS-A mediates the bronchospasm in human asthma and anaphylaxis (for a review see 8). In addition to the airway smooth muscle stimulating activity, SRS-A also enhanced permeability of blood vessels in guinea pig skin (9). The latter effect may contribute to the development of symptoms in asthma and immunological tissue injuries.

1.1.2 Preliminary Characterization

The task of chemical characterization of SRS-A attracted the interest of several groups of investigators, including this group, for a number of years. Early experiments indicated that the factor was soluble in water, 80% methanol, or 80% ethanol but not in acetone, chloroform, and chloroform-

methanol mixtures (10,11). The solubility of SRS-A in diethyl ether was enhanced by acidification to pH 2-3, suggesting the presence of acidic groups in the molecule. Additional studies were performed with SRS formed by cat paws during perfusions with compound 48/80. Treatment of partially purified SRS with N,N'-carbodi-p-tolylimide, phenylisocyanate, iodine monobromide, potassium permanganate, or acetic anhydride abolished or reduced the biological activity (12). Based on these results the authors suggested that SRS is an unsaturated carboxylic acid containing hydroxyl groups. A more efficient purification procedure for SRS-A from human lung or rat peritoneal cavity consisted of ethanol precipitation of proteins, mild alkaline hydrolysis of phospholipids, desalting Amberlite XAD-2 chromatography, and fractionation on silicic acid chromatography (13). The purified materials were analysed by Sephadex LH-20 chromatography, polyacrylamide gel electrophoresis, and ultraviolet (uv) spectroscopy. The results suggested that SRS-A was a low molecular-weight compound with an isoelectric point of 4.7 and was absorbing uv in the regions 210-240 and 250-300 nm, although since the isolate was still grossly impure, the uv absorbance had no significance for SRS-A. Gas chromatography analyses showed that the purified preparations of SRS-A were contaminated with a number of saturated and unsaturated fatty acids. Observations that Limpet and snail arylsulphatases inactivated SRS-A and that analysis by spark source mass spectrometry showing increased abundance of phosphorus, sulphur and potassium in biologically active samples of SRS-A compared to controls, led to a suggestion that SRS-A might contain a sulphate ester group (14).

Bach and Brashler (15,16) observed that a calcium ionophore, A23187, stimulated the formation of SRS-A by rat peritoneal cells. Orange and Chang (17) and Orange *et al.* (18) reported that cysteine enhanced the immunologic release of SRS-A. Using cultured rat basophilic leukaemia cells (RBL-1 cells), Jakschik *et al.* (19) reported that arachidonic acid further stimulated ionophore A23187-induced SRS formation and that the acetylenic analogue of arachidonic acid, 5,8,11,14-eicosatetraenoic acid, had the opposite effect. The latter compound inhibits prostaglandin endoperoxide synthase, which converts arachidonic acid to prostaglandin H_2, and platelet lipoxygenase, which is now known to form a 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid from arachidonic acid. Indomethacin, a selective inhibitor of the former enzyme, had no effect on SRS formation. Jakschik *et al.* (19) also reported that radioactivity from [^{14}C]- or [^{3}H]-labelled arachidonic acid comigrated with SRS activity on two-dimentional thin-layer chromatography and on DEAE-

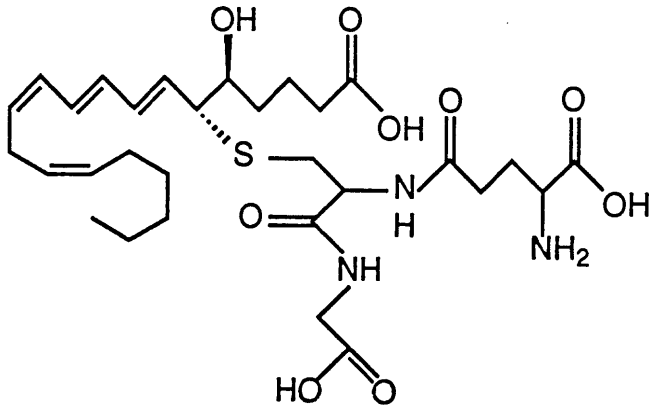
cellulose column chromatography. They suggested that SRS was a metabolite of arachidonic acid metabolism, possibly formed by a lipoxygenase pathway.

The first highly purified SRS-A preparation was obtained from independent work done in this laboratory on the structure elucidation of SRS-A, and a unique uv spectrum of this highly purified SRS-A was reported by Morris *et al.* (20) showing an absorption maximum around 280nm consistent with a triply conjugated or aromatic chromaphore. This finding together with results described above provided the starting point for the complete characterization of SRS (5) and SRS-A (4), the latter study involving the antigen challenged guinea pig lung material originally discovered by Kellaway and Trethewie (2). It was further discovered that SRS and SRS-A, although derived by different ways, were made up of the same compounds and found to consist of a novel class of peptidolipids- the peptido leukotrienes (p-Leukotrienes). The first structure reported was that of Leukotriene C_4 (LTC_4) determined as the S-glutathionyl conjugate of an arachidonic acid metabolite 5-(S)-hydroxy-6-(R)-γ-glutamyl-cysteinyl-glycinyl-7,9,11,14-eicosatetraenoic acid (21,22), as seen in Figure 1(a), in work involving partial characterization and then synthesis of an assumed structure. Concomitantly in this laboratory, both the full structure of the major biologically active species of lung derived SRS-A and of RBL-1 SRS was defined as 5-(S)-hydroxy-6-(R)-cysteinyl-glycinyl-7,9,11,14-eicosatetraenoic acid (4,23,24), later termed "Leukotriene D_4" (LTD_4) (25) shown in Figure 1(b).

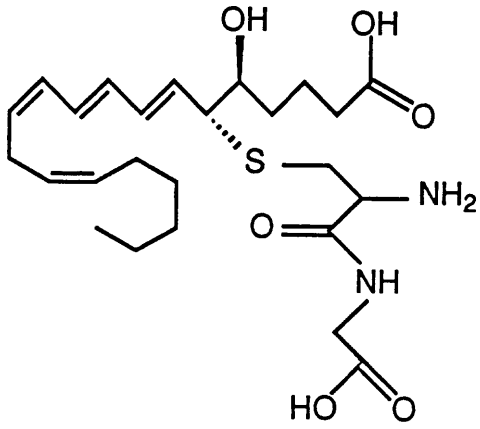
Examination of the structure of LTC_4 and LTD_4 clearly indicated that they were both formed by the same biosynthetic route: a combination of oxygenation of arachidonic acid by 5-lipoxygenase (26-29) and further metabolism by the glutathione detoxification pathway (29), involving the enzymes glutathione transferase and γ-glutamyltranspeptidase (see Section 1.3.2). Further steps in this pathway would be expected to result in the formation of a cysteinyl and an N-acetylcysteinyl Leukotriene; the former has now been reported and termed "Leukotriene E_4" (LTE_4) (30) as can be seen in Figure 1(c).

Prior to a more indepth study of Leukotriene biosynthesis, it would be appropriate to discuss the steps prior to 5-lipoxygenase action and Leukotriene generation, namely PLA_2 activation and arachidonic acid release.

a) LTC_4



b) LTD_4



c) LTE_4

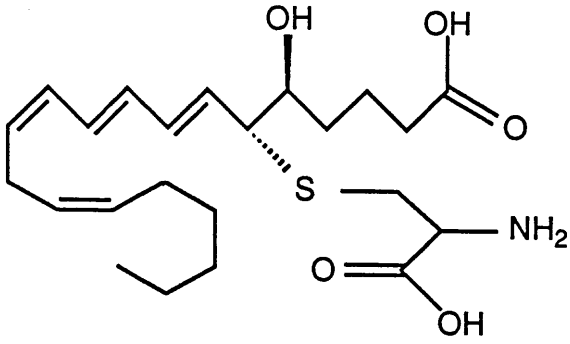


Figure 1: The chemical structure of peptido-Leukotrienes C_4, D_4 and E_4.

1.2 Phospholipase A_2 Activation and Arachidonic Acid Release

It is well established that in most tissues the synthesis of eicosanoids is limited by the availability of their common precursor, free arachidonic acid, which must be released from esterified stores in complex lipids (31-33). Liberation of arachidonic acid involves hydrolysis of an ester bond i.e lipase activity. The three essential questions concerning the control of arachidonic acid availability are:

a) Which lipases are involved?
b) How are they controlled?
c) Which substrates are hydrolysed?

The following sections will attempt to answer these questions.

1.2.1 Lipases and Their Substrates Involved in Arachidonic Acid Release

Before studying the lipases, it is important to know the main source(s) of arachidonic acid. Generally, triacylglycerol, the principal neutral storage lipid, is less rich in arachidonyl moieties than are phospholipids. Only in tissues where the mass of neutral lipids matches or exceeds that of phospholipids, such as adrenal cortex or adipose tissue, might they be expected to make a significant contribution to the free arachidonic acid pool (34).

The bulk of the arachidonic acid in mammalian cells is esterified in the fatty acyl chain of glycerophospholipids, almost exclusively in the 2 acyl-position (sn-2 position) (35). This has led to suggestions that phospholipases are responsible for controlling free arachidonic acid levels (36) and this concept is now generally accepted. However, the free fatty acid level in a tissue actually represents a balance between the liberation of the acid by hydrolysis and its re-esterification into complex lipids by acyltransferases.

Arachidonic acid is unusual amongst the major fatty acids in that the level of the free acid in the cytoplasm (37) and plasma (38) is low, disproportionately so compared with its esterified levels. It has become clear that arachidonic acid is not incorporated during *de novo* biosynthesis of glycerophospholipids (i.e by acylation of glycerol-3-phosphate or lysophosphatidic acid to yield phosphatidic acid, PA), but it enters at a later stage, by acylation of a glycero-lysophospholipid such as lysophosphatidylcholine (see 39 for a review). This reaction is catalysed by

distinct acyltransferase(s) with different specificities from those which synthesize phosphatidic acid (40,41). A decrease in the reacylation rate of arachidonic acid could cause a rapid rise in the free levels of this fatty acid (35). However, although in resting tissue acylation may control the overall levels of arachidonic acid in the cell (in equilibrium with acyl-CoA hydrolase and phospholipases), an inhibition in this process cannot account for stimulated arachidonic acid liberation in all tissues for example a stimulation of acyl transfer accompanies increased arachidonic acid liberation in platelets aggregating in response to thrombin (42) and in mitogen-stimulated lymphocyte (43). In many, and probably most tissues, stimulation of arachidonic acid liberation is most likely to be controlled by changes in phospholipase activity (31-33).

With the exception of phospholipase C (PLC) (see Section 1.4), the main route of phospholipid degradation in mammals is by deacylation catalysed by the action of phospholipase A_1 (PLA_1) or phospholipase A_2 (PLA_2) activities. Sites on phospholipids, at which various phospholipases act, are shown in Figure 2.

There are four main routes of arachidonic acid mobilization from phospholipids. These are:

1) Direct deacylation of phospholipids by PLA_2,
2) Formation of diacylglycerol (DAG) by PLC, followed by DAG lipase,
3) Formation of DAG by PLC, phosphorylation of DAG by DAG kinase to PA and then deacylation by a PA specific PLA_2,
4) Deacylation by PLA_1 followed by a lysophospholipid specific PLA_2.

These four routes are shown diagrammatically in Figure 3. As seen from Figure 3, PLA_2 activity leads to the direct release of arachidonic acid from a variety of phospholipids, resulting in the formation of a lysophospholipid and the subsequent formation of numerous biologically active arachidonate metabolites (see below). Arachidonic acid can also be generated from either phosphoinositides (35) or phosphatidylcholine (44) by prior activation of phosphoinositide-specific PLC (PIC) or PLC, respectively, which generate arachidonyl-diglyceride as seen in Figure 3. Arachidonic acid can then be liberated from the diglyceride by the action of DAG lipase (35). Alternatively, DAG may be phosphorylated by the action of DAG kinase leading to the formation of phosphatidic acid (PA), with subsequent release of arachidonic

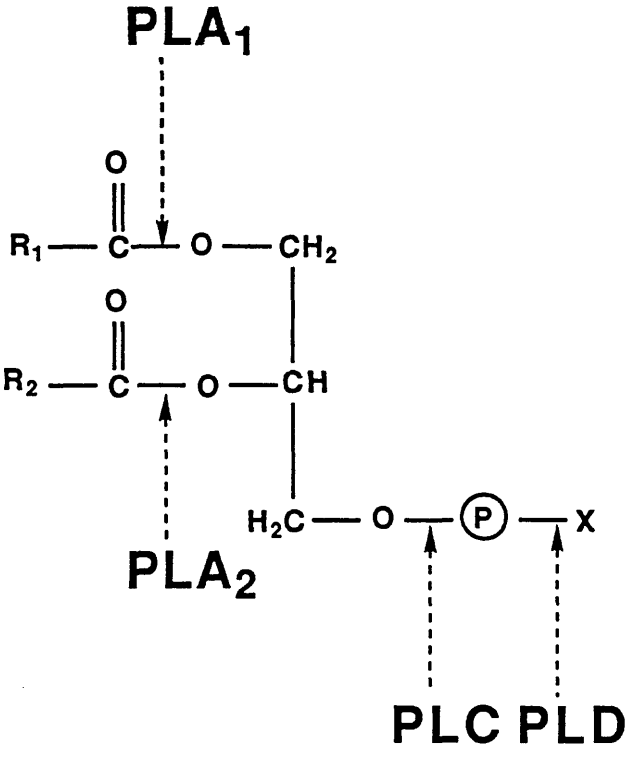


Figure 2: Sites of cleavage of phospholipids by various phospholipases. X, choline, ethanolamine or inositol; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLC, phospholipase C and PLD phospholipase D.

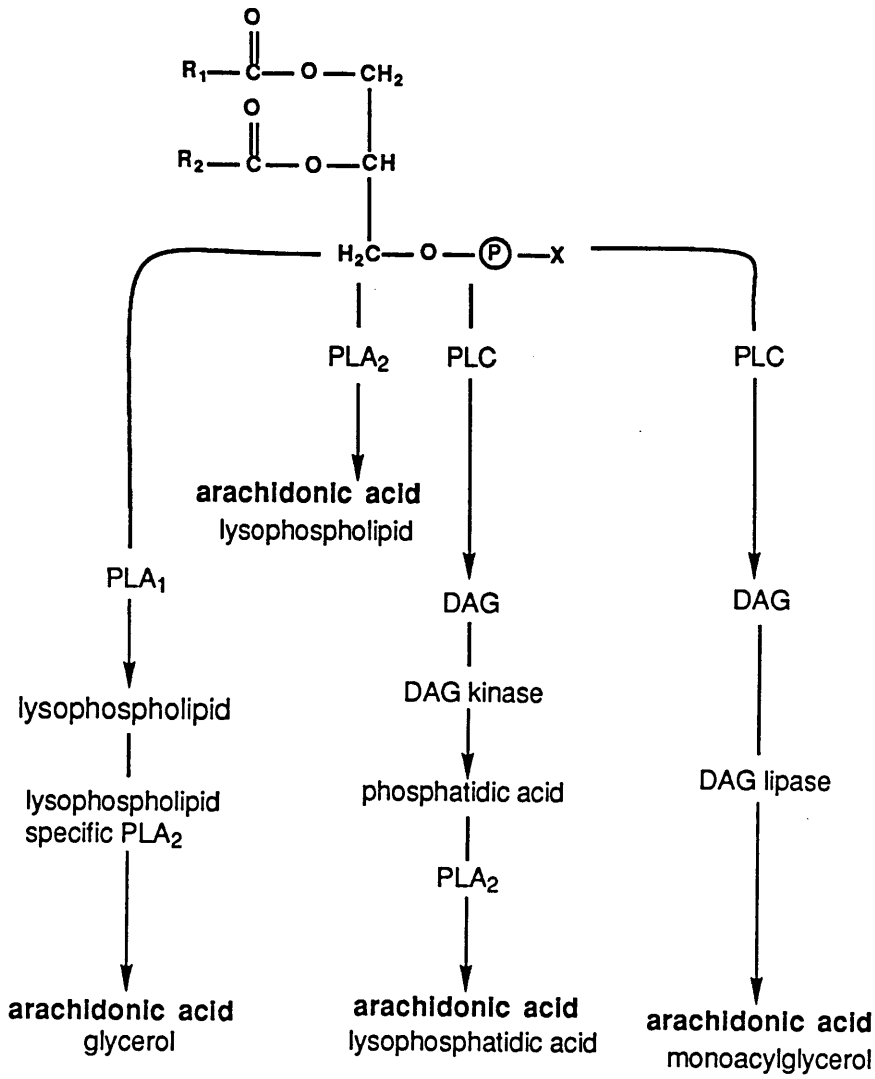


Figure 3: Pathways of arachidonic acid release from phospholipids. DAG, diacylglycerol; R_1, fatty acid; R_2 , arachidonyl group; P phosphate; X choline, inositol, or ethanolamine.

acid through the activation of PLA_2 as shown in Figure 3. PIC/PLC and DAG lipase appear to be less sensitive to calcium than PLA_2 (35).

PLA_2 is activated *in vitro* by unphysiologically high levels of Ca^{2+}, but nevertheless, it has been commonly supposed that Ca^{2+} is the major regulator of this enzyme *in vivo* (36). Some of the phospholipases appear to be coupled to specific receptors by GTP binding regulatory proteins (G-proteins, see below). Receptor-mediated release of arachidonic acid has been demonstrated in fibroblasts and platelets (45), and in neutrophils (46-48). More recently, α_1-adrenergic receptor activation in rat thyroid cell line FRTL5 (49), MDCK cells (50) and in the pineal (51) has been shown to stimulate arachidonic acid release. Similarly G-protein-induced PLA_2 activity has also been reported in dark adapted bovine rod outer segment (retinal ROS) upon exposure to light (52).

1.2.2 Modulation of PLA_2 Activity

PLA_2 activity appears to be under both inhibitory and stimulatory control by a variety of mechanisms as shown in Figure 4. PLA_2 can be regulated in a stimulatory or an inhibitory manner by G-proteins either directly or via other second messenger systems that are also coupled to G-proteins (52). In the retinal ROS, the light-induced stimulation of PLA_2 has been directly linked to the dissociated $\beta\gamma$-subunits of G_t (transducin, the G-protein involved in ROS receptor effector coupling) (53). In this system, there was also evidence for a G-protein-dependent inhibition of PLA_2. This was demonstrated by the GTP-induced inhibition of PLA_2 in both dark-adapted G_t-depleted ROS, as well as in light-activated transducin-rich ROS (53). It remains to be shown whether this inhibition of PLA_2 is linked directly to a specific G-protein. Since PLA_2 is a Ca^{2+}-dependent enzyme and will respond to increased cytosolic levels of Ca^{2+} (35), G-proteins may modulate PLA_2 activity either by opening ion channels or by IP_3-induced release of Ca^{2+} from intracellular stores subsequent to the activation of PIC. Recently, the Na^+-H^+ transport system has also been shown to modulate PLA_2 activity directly (54). Since Na^+ channels have been reported to be coupled to G-proteins in certain systems (55), this may represent another G-protein-mediated effector system that can modulate PLA_2 activity. PLA_2 activity has also been shown to be modulated by changes in cytosolic levels of cAMP (45), which may be the result of G_s-mediated stimulation of the adenyl cyclase activity, G_i-mediated inhibition of the cyclase, or G-protein-dependent increases in the cAMP

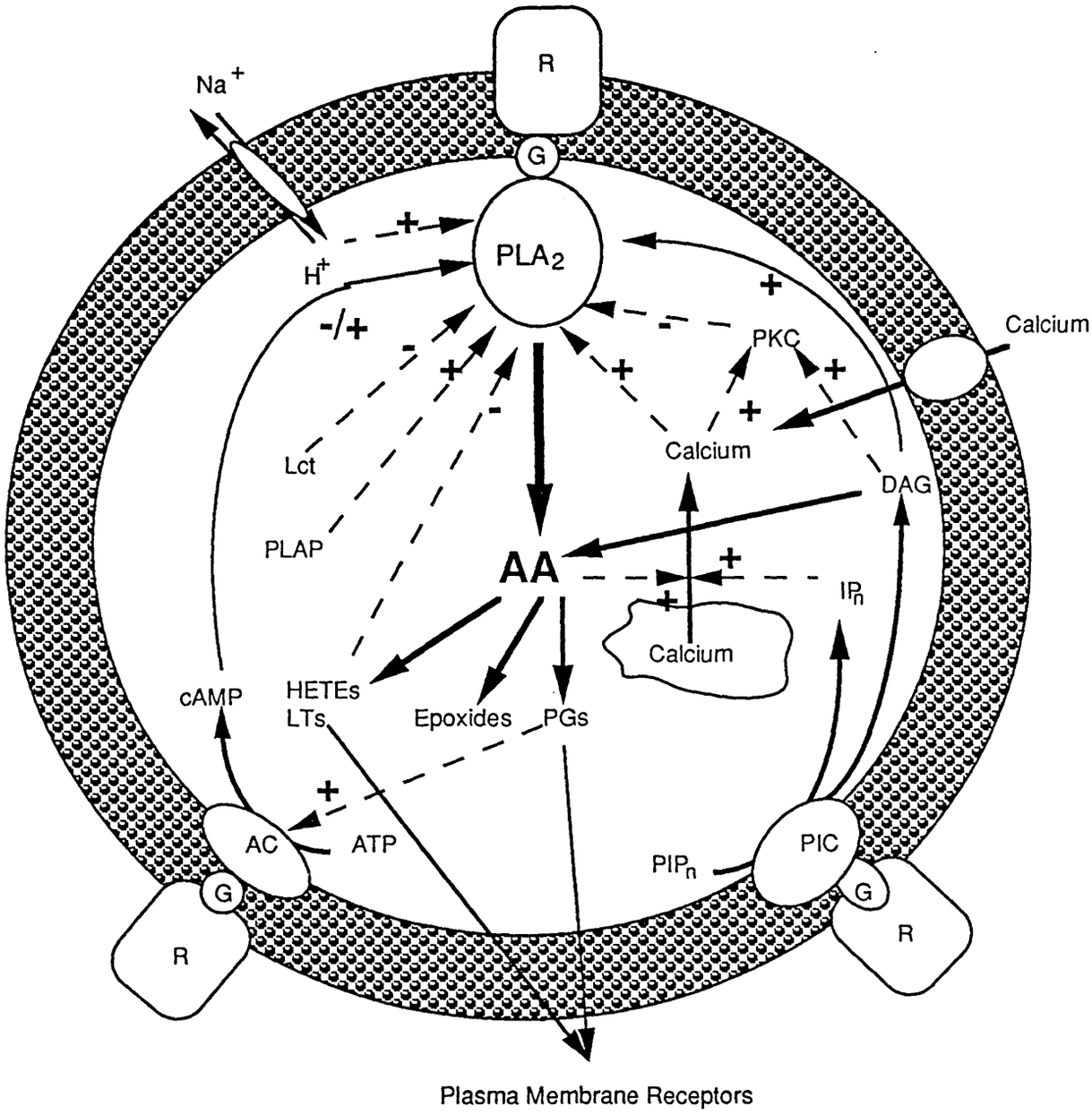


Figure 4: Phospholipase A2 stimulatory and inhibitory pathways. The biochemical pathways are discussed in the text. (+) denotes stimulation and (-) denotes inhibition. PGs, prostaglandins; HETES, hydroxyeicosatetraenoic acids; LTs, Leukotrienes, IPn, inositol phosphates (n=1 to 3); PIPn, phosphoinositides (n=0 to 2); PKC, protein kinase C; PIC, phosphoinositide-specific phospholipase C; PLA2, phospholipase A2; AC, adenyl cyclase; G, GTP-binding protein; Lct, lipocortin, a phospholipase A2 inhibiting protein; DAG, diacylglycerol; PLAP, a phospholipase A2 activating protein.

phosphodiesterase activity. Thus, a multiplicity of G-proteins appear to modulate PLA_2 activity. While some of these G-proteins may act directly to stimulate or inhibit PLA_2 activity, the enzyme may also be modulated by G-protein-dependent second messengers such as Ca^{2+}, cAMP and/or the kinases they activate (Figure 4).

Another G-protein that has been implicated in modulation of PLA_2, either directly or by way of effects on PIC or adenyl cyclase, is the 21 kDa *ras* gene product, $p21^{ras}$. *Ras*-induced metabolic alterations include increased PLA_2 and PIC activities (55), and decreased cAMP levels (56). In NIH-3T3 cells, however, Ej-*ras* transformation markedly reduces basal and growth factor stimulated PLA_2 and PIC activities (57).

In addition to modulation of PLA_2 by multiple G-proteins, either directly or indirectly, both PLA_2 stimulatory and inhibitory proteins have been described. A mellitin-like protein that stimulates PLA_2 has recently been reported (58). Lipocortins have been defined as proteins that are inducible by glucocorticoids, secreted by cells and thought to be PLA_2 inhibitors regulated by phosphorylation (27,59).

An important requirement of receptor-mediated signal transduction is its termination. In those systems in which PLA_2 activity is linked to G-proteins, this may occur by reassociation of the inactive heterotrimer following activation of the GTPase associated with the α-subunit of the G-protein. In addition to shutting off the signal at the level of the G-protein, there appear to be several other mechanisms operative either at the level of the enzyme itself or subsequent to PLA_2 activation. These include: reacylation of the lysophospholipids using the released arachidonic acid (60) and/or arachidonic acid metabolites (12-HETE) (61); feedback inhibition of PLA_2 activity by arachidonic acid or an arachidonic acid metabolite (62); reduction of cytosolic Ca^{2+} levels; and activation of protein kinases, which could turn off the systems at the level of the receptor, the G-protein(s), stimulatory or inhibitory proteins, and/or the enzyme itself.

1.3 5-Lipoxygenase and Leukotriene Biosynthesis

Arachidonic acid and related polyunsaturated fatty acids are metabolized either by cyclo-oxygenase to produce the prostaglandins (PGs) or by lipoxygenases to generate hydroperoxy derivatives. In mammalian systems the hydroperoxy fatty acids may be further metabolized to Leukotrienes (see Section 1.3.2).

1.3.1 5-Lipoxygenase and Lipoxygenase Pathways

Mammalian tissues have several lipoxygenases (e.g. 5-, 12-, 15-lipoxygenases), each with a positional and stereochemical specificity of arachidonic acid oxygenation. Some lipoxygenases are involved in the biosynthesis of bioactive eicosanoids, while others are still of unknown physiological significance. Fatty acid cyclo-oxygenase is an atypical lipoxygenase incorporating two molecules of oxygen into arachidonic acid and producing prostaglandin (PG) peroxides.

The lipoxygenases are a group of iron containing dioxygenases found throughout the animal and plant kingdoms (63). They catalyse the hydroperoxidation of polyunsaturated fatty acids that contain a penta-1,4-*cis*-diene systems (e.g linoleic acid: $C_{18:3}$, arachidonic acid: $C_{20:4}$) (64), as shown in Figure 5. The initial step of hydroperoxidation is stereospecific hydrogen abstraction from position 3 of the penta-1,4-diene system. It has been suggested that the hydrogen radical ($H^{\bullet}$) thus formed disintegrates into a proton (H^{+}), and an electron that is taken up by the non-haem iron within the lipoxygenase (Figure 5).

The intermediate fatty acid radical reacts with molecular oxygen (triplet state) to form the peroxy radical; an electron is transferred from the enzyme-bound iron to generate the peroxy anion, which is protonated to give the hydroperoxide. At the same time, a one electron shift occurs, generating the conjugated 1-*cis*,3-*trans*-diene system (65).

The formation of a conjugated 1,3-diene product from a 1,4-diene substrate is the basis of most assays for lipoxygenase activity. The 1,3-dienes, unlike their 1,4-counterparts, exhibit a strong absorbance in the ultraviolet (uv) region (λ_{max} 234nm, ε 30000). Thus formation of the hydroperoxide may conveniently be monitored by measuring the uv absorbance at 234 nm (64). The hydroperoxides, so produced, are then converted to a series of less toxic metabolites.

Although plant lipoxygenases have been known for some time, it is in the last few years that these enzymes have been characterized in mammalian tissue. In one of the first reports, Nugteren (66) demonstrated that a suspension of blood platelets contained a soluble enzyme that converted a series of polyunsaturated fatty acids to the corresponding hydroperoxy acid. As for the plant enzyme, the possession of a penta-1,4-diene system within the substrate was a prerequisite for hydroperoxidation. This mammalian enzyme was a 12-lipoxygenase; it possessed an ω 6,9,12 specificity (66).

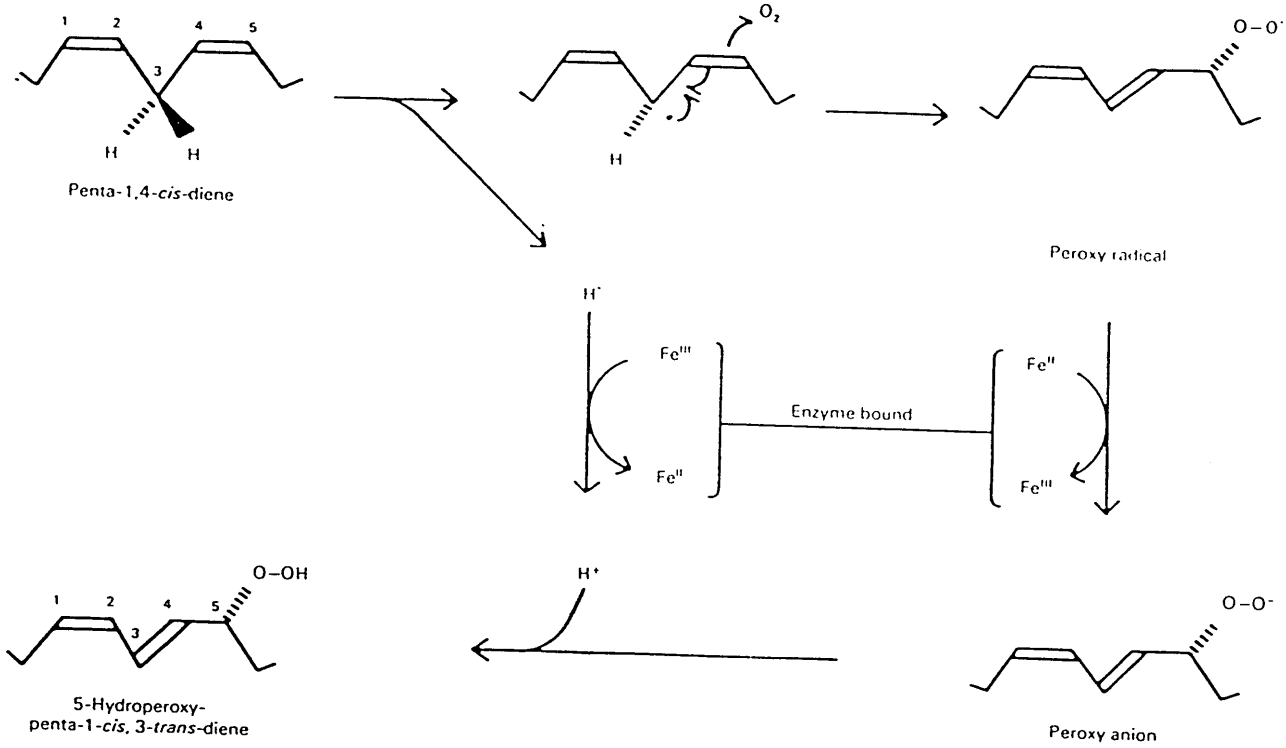


Figure 5: The mode of action of lipoxygenases.

After the demonstration of the mammalian platelet 12-lipoxygenase, Borgeat *et al.* (67) found that a suspension of rabbit polymorphonuclear leukocytes (PMNLs) could convert arachidonic acid to 5-hydroxyeicosa-6,8,11,14-tetraenoic acid (5-HETE). However, the hydroperoxy derivative was not isolated; presumably it was rapidly reduced to the alcohol by the enzymes within the PMNLs suspension. This biosynthesis could not be inhibited by indomethacin (a cyclo-oxygenase inhibitor) indicating that 5-HETE was not a product of the cyclo-oxygenase pathway. The authors proposed that PMNLs contained a novel 5-lipoxygenase acting on a penta-1,4-*cis* diene system from the carboxyl end of the acid and generating 5-HETE (67). 15-lipoxygenase activity has been reported in rabbit leukocytes and reticulocytes (68,69).

The 5-lipoxygenase enzyme has also been found in rat basophilic leukaemia (RBL) cells (70). This enzyme in RBL cells was shown to be Ca^{2+}-dependent; however, the instability of this RBL-derived enzyme hampered further purification and characterization (70). In the last two years, five groups independently succeeded in purifying mammalian 5-lipoxygenase from various sources, to near homogeneity, and the purified enzyme was found to be a peripheral cytosolic protein with a molecular weight of about 72-80 kDa (71-75). 5-lipoxygenase requires phosphatidylcholine (72) or various cofactors (74) in addition to Ca^{2+} and ATP, in millimolar concentrations. The enzyme is stabilised by the addition of EDTA, dithiothreitol, phosphatidylcholine or ferrous ion (72-75). It was also confirmed that the purified 5-lipoxygenase from mammalian tissues possessed the Leukotriene A_4 (LTA_4) synthase activity (for the conversion of 5-hydroperoxy eicosatetraenoic acid, HPETE, to LTA_4 see below) (72-75).

The roles of Ca^{2+}, ATP and other cofactors remain to be elucidated. The ED_{50} value for Ca^{2+} in the activation of the 5-lipoxygenase (LTA_4 synthase), however, is extremely high (10^{-4}-10^{-3}M), that is much higher than the physiological level in the cells (10^{-8}M), and even higher than that in the activated cells.

1.3.2 Leukotriene Biosynthesis

A key intermediate in the biosynthesis of Leukotriene C_4 (LTC_4) is *trans*-5(S),6(S)-oxido-7,9*trans*-11,14-*cis*-eicosatetraenoic acid (30). This epoxide was postulated as an intermediate in the biosynthesis of LTC_4 (5) and in the biosynthesis of 5(S),12(R)-di-hydroxy-6,8,10,14-eicosatetraenoic acid (76,77). The intermediate in leukocytes was detected by a reaction with

alcohols, which yielded 5-hydroxy-12-alkoxy-6,8,10,14-eicosatetraenoic acids. Using this trapping procedure, the half-life of the intermediate in 50% aqueous acetone solution was determined as 4 minutes at 37°C and pH 7.4. The intermediate was rapidly hydrolysed by lowering of the pH to 1. 5(S),12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid and 5,6-oxido-7,9,11,14-eicosatetraenoic acid were named Leukotrienes B_4 (LTB_4) and A_4 (LTA_4) respectively (78).

Evidence that LTA_4 is an intermediate in Leukotriene C_4 biosynthesis has been obtained by alcohol trapping (79), and enzymatic conversion of synthetic unlabelled epoxide to LTC_4 (80). The synthetic epoxide was also enzymatically transformed to LTB_4 (81). These transformations are shown in Figure 6.

A common feature among LTA_4, LTB_4, and LTC_4 is the presence of a 5(S)-oxygen substituent. Labelling experiments with $^{18}O_2$ showed that this oxygen in LTB_4 is derived from molecular oxygen (76). The same was true for 5(S)-hydroperoxy-6,8,11,14-eicosatetraenoic acid, another product of arachidonic acid metabolism in PMNLs.

One of the original structural studies on slow reacting substance (SRS) from RBL cells indicated that it was related to LTC_4 but was less polar (82). The difference was confined to the peptide part, which was identified as cysteinyl-glycine. This suggested that the compound was a metabolite of LTC_4 formed by elimination of the amino terminal glutamyl residue (82). Further evidence was obtained by treating LTC_4 with γ-glutamyltranspeptidase. This enzyme catalyses the initial step (removal of glutamic acid) in the metabolism of glutathione, glutathione disulphide, and various glutathione conjugates (82,83). The product obtained was identical to SRS from RBL cells and was named LTD_4 (25). Using inhibitors of γ-glutamyltranspeptidase, it was shown that LTC_4 is an intermediate in the formation of LTD_4 (82,84,85). Additional metabolic experiments using [5,6,8,9,11,12-$^3H_6$]-LTC_3 and guinea pig tissues showed that [^{3}H]-LTD_3 was rapidly formed by lung homogenates as shown in Figure 7 (86). This reaction was found to be inhibited by glutathione. Moreover, in the presence of glutathione, γ-glutamyltranspeptidase catalysed the addition of a γ-glutamyl residue to the amino group of [^{3}H]-LTD_3 to form [^{3}H]-LTC_3. Kinetic evidence for the formation of such an intermediate has been reported before (87). The K_m value for LTC_4 in the forward reaction catalysed by γ-glutamyltranspeptidase is approximately the same as the K_m value for hydrolysis of glutathione (about 6μM, 88,89). It is therefore likely that extracellular glutathione or glutathione disulphide will influence the

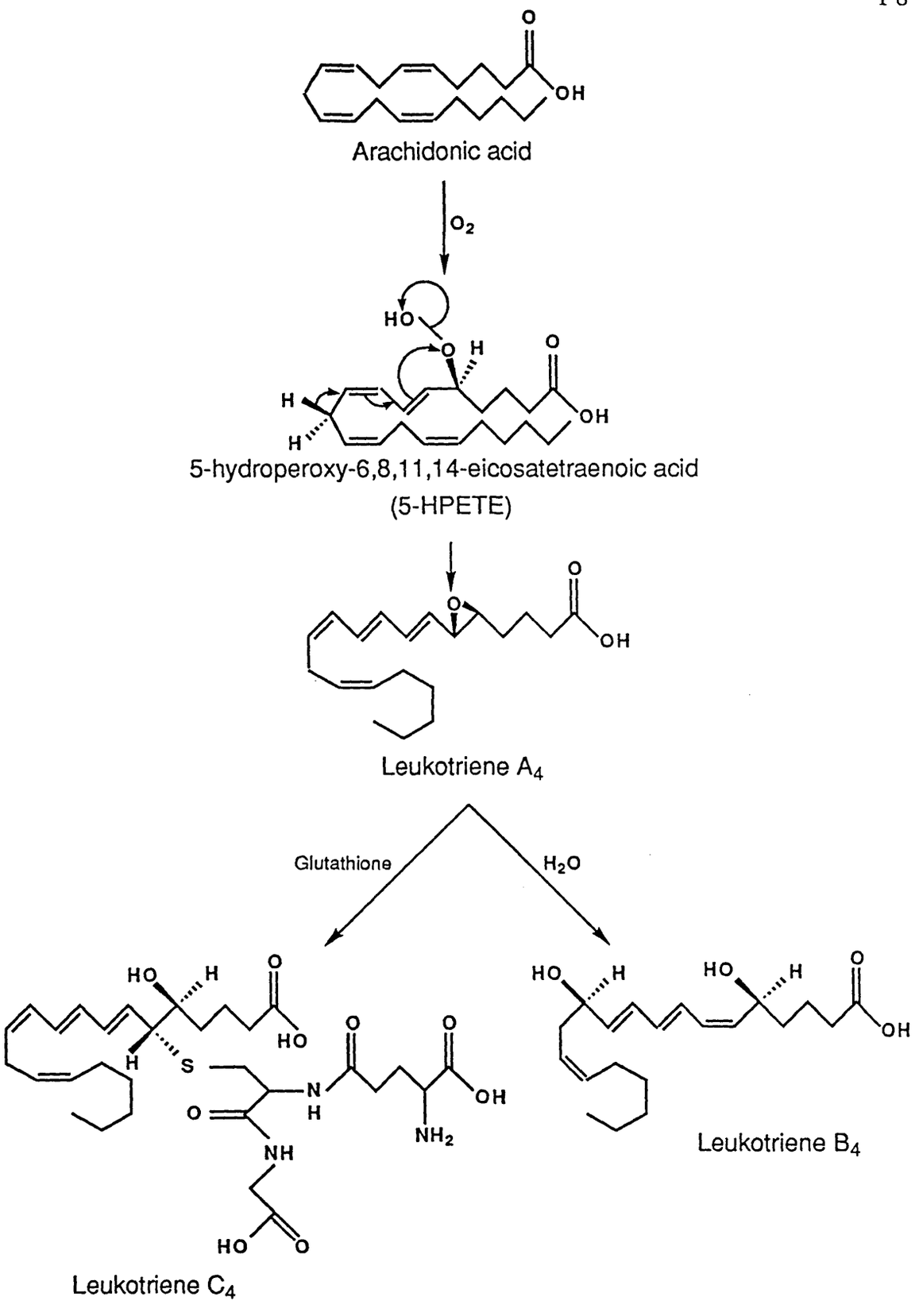


Figure 6: Transformations of LTA_4 to Leukotriene C_4 and Leukotriene B_4.

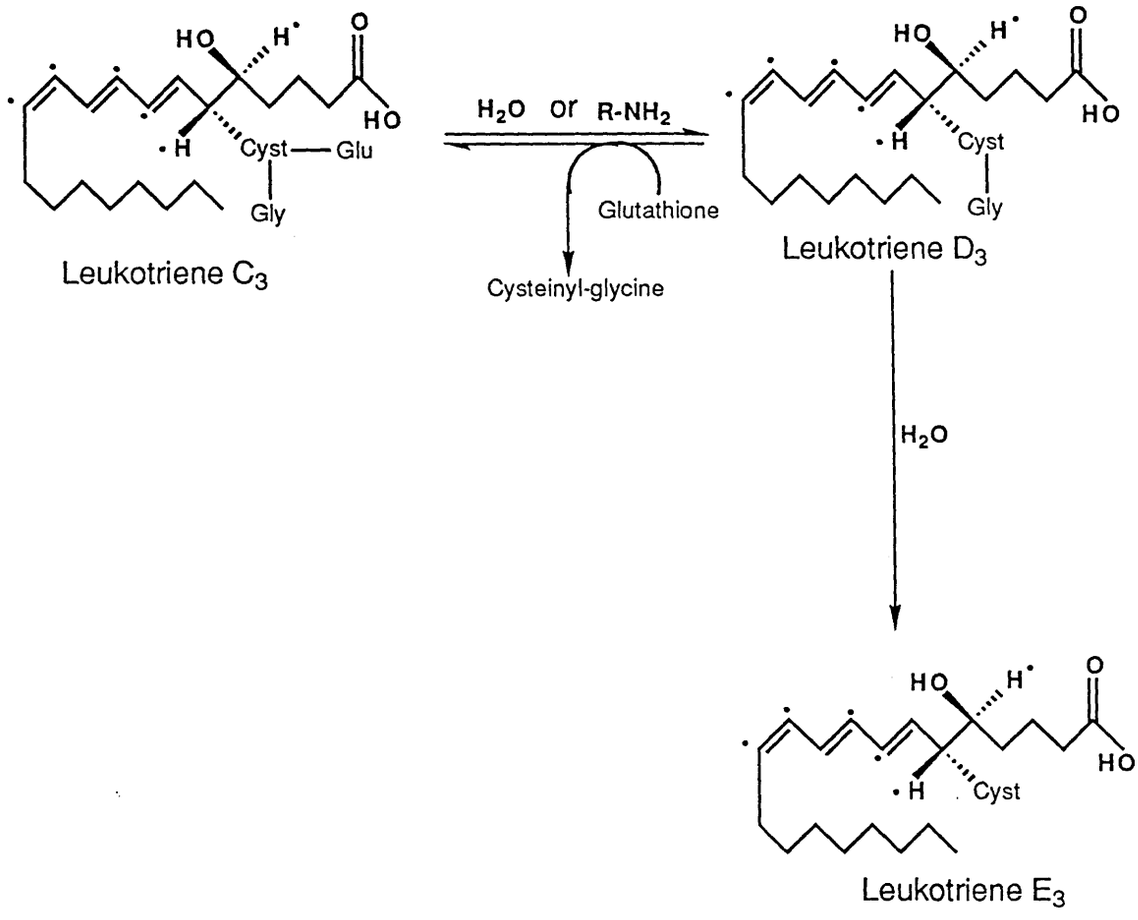


Figure 7: Metabolic experiments using [^{3}H]-Leukotriene C_3 and guinea pig tissues: The elucidation of the involvement of [^{3}H]-Leukotriene D and [^{3}H]-Leuktriene E in the biosynthesis of peptido-Leukotrienes.

metabolism of Leukotrienes by γ-glutamyltranspeptidase. [^{3}H]-LTD_3 was degraded further by hydrolysis of the peptide bond (90). The product, [^{3}H]-LTE_3, was formed rapidly by kidney and more slowly by lung and liver. The reaction is catalysed by a membrane bound dipeptidase (89-91), as shown in Figures 7 and 8. This finding is further augmented by reports of conversion of LTC to LTE (84,92). LTE can be further converted to LTF by addition of a γ-glutamyl residue to the amino group of LTE (93). However this has not been substantiated *in vivo.*

The generation of Leukotrienes has been described in a number of tissues or cell suspensions, as shown in Table 1. The Leukotrienes formed by individual cells or tissues depend on the enzymes present in the tissue and on the incubation conditions used. For example, rat basophilic leukaemic-1 (RBL-1) cells incubated with the calcium ionophore, A23187, are a rich source of LTD_4, but altered incubation conditions yield 5S,12R-*cis-trans-trans*-LTB_4 and its isomers [5S,12S-all-*trans*-LTB_4; and 5-hydroxyeicosatetraenoic acid (5-HETE)], but little LTD_4 (94,95,). Inclusion of serine borate (a γ-gultamyl transpeptidase inhibitor) in incubations of RBL-1 cells results in the generation of LTC_4 (82,85) and LTD_4 (82,86). Rat SRS-A contains LTC_4, LTD_4, and LTE_4, whereas murine mastocytoma cells lack γ-glutamyltranspeptidase and form LTC_4 (5). Note also that effluent from guinea pig lungs during antigen challenge contained appreciable amounts of LTB_4 in addition to LTD_4 (97). Guinea pig lung contains sufficient γ-glutamyltranspeptidase to convert tens of nanomoles of LTC_4 per minute to LTD_4, which probably accounts for the fact that the major biological activity of guinea pig SRS-A (assayed on guinea pig ileum) is that of LTD_4 (4,94), suggesting also that LTD_4 is the major cause of bronchoconstriction in the lung.

In addition to 5-lipoxygenase, preparations of human leukocytes contain enzymes that catalyse the introduction of oxygen into arachidonic acid at C-12 and C-15. Under appropriate conditions this can lead to the formation of novel Leukotrienes such as 14,15-LTB_4 and 14,15-LTC_4 (98). The biological actions of these Leukotrienes have not yet been determined.

As mentioned above, there is some variation in the peptido-leukotrienes present in different SRS and SRS-As. However, Ohnishi *et al.* (99) raised another possibility when they identified the SRS produced from rat peritoneal cells as a Leukotriene, with a sulphone rather than sulphide linkage at C-6. This finding suggested that sulphones as well as sulphides may be constituents of SRS-A. Leukotriene sulphones have recently been synthesized chemically (100) and have been shown to possess biological

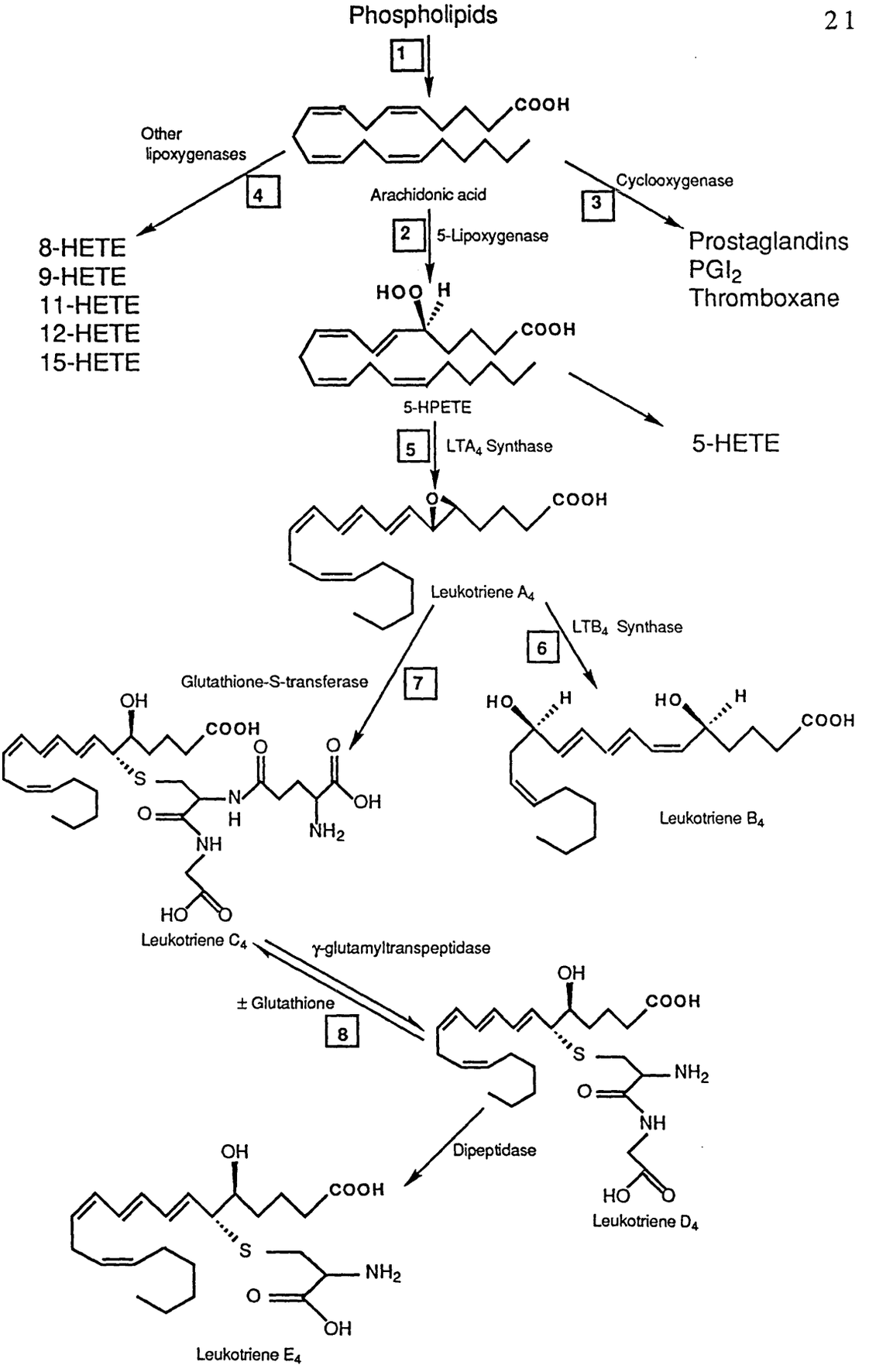


Figure 8: Summary of the 5-lipoxygenase pathway of arachidonic acid metabolism and Leukotriene biosynthesis.

Table 1: The occurrence of Leukotrienes

Source	LTA_4	LTB_4	LTC_4	LTD_4	LTE_4
Body Fluids or Secretions					
Cystic Fibrosis sputum		+	+		
Rheumatoid synovial fluid		+			
Cells					
Leukocytes					
Basophilic Leukaemia	+	+	+	+	+
Eosinophilic		+	+	+	
Polymorphonuclear	+	+	+		
Macrophages			+	+	
Mastocytoma cells	+		+		
Peritoneal cells (mixed)			+	+	+
Tissues					
Lung			+	+	+
Paws				+	+
Spleen			+		

activity that closely resembles that of the Leukotriene sulphides and that is antagonised by FPL-55712 (101).

Finally, Figure 9 summarizes the 5-lipoxygenase metabolic pathway involved in Leukotriene biosynthesis.

1.3.3 **Biological Effects of Leukotrienes**

The isolation, characterization and chemical synthesis of various Leukotrienes have stimulated renewed interest in the pharmacological and physiological properties of these compounds. There have been more than a hundred original published reports with respect to biological actions of this class of arachidonic acid metabolites and for a detailed coverage of this aspect of Leukotriene biosynthesis references 102 and 103 would be most useful.

Leukotrienes have potent biological actions that may be divided into two groups: those involving contraction of smooth muscle (as exhibited by the peptido-Leukotrienes) and those involving chemotaxis (such as LTB_4 and its isomers).

The C-6 amino acid-substituted Leukotrienes have potent actions on a variety of smooth muscle preparations, whereas LTB_4 is a potent chemokinetic and chemotactic agent that has relatively little smooth muscle-stimulating action. The action of Leukotrienes occurs at various receptor sites in tissues. Also, as shown by different pharmacological actions and experiments in which tissues were made tachyphylactic to Leukotrienes, the receptors occupied by LTB_4 clearly differ from those for LTC_4, LTD_4, and LTE_4 (104,105). These receptors have been partially characterized by the use of FPL-55712, the antagonist used most regularly in this respect (106,109). The following sections describe the main sites at which Leukotrienes act.

I) Effects of Leukotrienes on Gastrointestinal Smooth Muscle

For many years guinea pig ileum *in vitro* was used to assay SRS-A (110), and LTC_4, LTD_4, and LTE_4 are more active than histamine on this preparation by more than three orders of magnitude. The relative order of potency is $LTD_4 > LTC_4 > LTE_4$ (111). Guinea pig ileum is sensitive to Leukotrienes and responds to picomole doses.

Rat stomach strip (112), guinea pig uterus (113), and guinea pig gallbladder strips (114) are also contracted by Leukotrienes but tend to be

less sensitive than guinea pig ileum. These contractile responses are all antagonised by the Leukotriene antagonist, FPL-55712.

II) Effects of Leukotrienes in Airways

The effects of Leukotrienes on respiratory functions have attracted special interest because of the proposed mediator role for Leukotrienes in asthma. LTC_4, LTD_4, and LTE_4 are potent stimulators of airway smooth muscles from different species (106,115-117). In general, prepheral airways are more sensitive than central airways. The Leukotriene concentrations required to increase the resting tension of guinea pig lung and trachea strips by 100% (EC_{100}) are 0.6 and 50nM respectively. These contractile responses to Leukotrienes were reversed by FPL-55712 (118). Investigations on structure-activity relations for Leukotriene C and D analogues on guinea pig airways have indicated that the position of the peptide substituent (C-6) is important for maximal activity (119-122), and partial saturation of the triene reduces the smooth muscle stimulating activity (119). Free amino and carboxyl groups of the peptide are also essential, but derivitization of the arachidonyl carboxyl group does not lead to loss of biological activity (120). The stereochemistry at C-5 and C-6 is critical for respiratory and intestinal smooth muscle stimulating activity (119-122).

III) Microvascular Effects of Leukotrienes

In addition to their respiratory effects, Leukotrienes are known to increase vascular permeability (9). Leukotrienes C_4 and D_4 in doses as low as 100 femtomoles induce leakage of Evans blue (a dye used to examine microvascular permeability) in guinea pig skin (115). Additional experiments have been performed using the hamster cheek pouch model (123). Leukotrienes C_4 and D_4 at 10^{-10} to 10^{-8} M concentrations induce transient constriction of arterioles and especially terminal arterioles, followed by leakage of circulating fluorescein isothiocyanate-conjugated dextran into the perivascular space around postcapillary venules (124). Histamine produces similar effects on capillary permeability at three to four orders of magnitude greater concentrations but with no vasoconstrictor activity (124). LTB_4 has no direct effect on vascular permeability but it induces adhesion of leukocytes to the endothelial cells of postcapillary venules (124) and subsequently leukocyte-dependent increases in vascular permeability.

IV) Effects of Leukotrienes on The Cardiovascular System

Leukotrienes C_4 and D_4 have potent effects in the cardiovascular system (103). There are species differences in these actions; sometimes Leukotrienes invoke release of cyclo-oxygenase products, and their actions differ according to the route of administration. When given intravenously in the guinea pig, LTC_4 and LTD_4 cause an initial hypertensive response, which is probably reflex in origin and caused by bronchoconstriction, followed by long-lasting hypotension (111). When Leukotrienes are given intra-arterially, the hypotension is more prolonged, whereas the hypertensive phase is less marked. Cyclo-oxygenase products are probably involved in this response: indomethacin inhibits the hypertension and shortens the duration of the hypotension.

Leukotrienes have actions on the cardiac function and are potent vasoconstrictors in the coronary circulation *in vitro* and *in vivo.* As with other actions of Leukotrienes, species differences occur. In guinea pig isolated perfused hearts, at constant pressure, LTC_4 and LTD_4 cause marked reduction in coronary flow (110,125). The reduction in flow is due to a direct vasoconstrictor action in the coronary circulation, because neither LTC_4 nor LTD_4 have any direct action on spontaneously beating atria or driven ventricular strips (125). In rat hearts (perfused under constant pressure), LTD_4 causes a similar reduction in flow but reduces the spontaneous heart rate to a greater extent than contractility, indicating an action on conductivity in this species (126). LTE_4 has similar actions in guinea pig and rat hearts but is 10-15 times less active (126). In rabbit and cat isolated hearts, LTC_4 and LTD_4 also have potent actions in reducing coronary flow (126). However, LTB_4, even at high doses, has no detectable action in perfused hearts of any species. The action of Leukotrienes in guinea pig and rat hearts are antagonised by FPL-55712.

V) Role of Leukotrienes in The Central Nervous System

Recent findings demonstrate formation of Leukotrienes in the central nervous system (CNS). Various groups have demonstrated the production of LTC_4, LTD_4, and LTE_4 in rat and gerbil brain tissue (127).

Stimulation of sliced rat brain tissue with calcium ionophore, A23187, and arachidonic acid lead to the formation of LTC_4, LTD_4, and LTE_4. Moreover, LTB_4 and various monohydroxyeicosatetraenoic acids (5-HETE, 9-

HETE, 11-HETE, 12-HETE, and 15-HETE) have been isolated and identified from incubations of chopped rat brain tissue with A23187 (128). The formation of 11-HETE is inhibited by indomethacin, indicating that this compound is formed as a by-product via the cyclo-oxygenase pathway (96). The isolation of 9-HETE suggests the presence of a novel lipoxygenase activity in the brain, since formation of this compound has not been reported to occur in other mammalian tissue. A23187 causes a dose-dependent stimulation of LTB_4 and LTC_4 biosynthesis (128). Regional distribution studies have indicated the highest production of LTC_4 in the hypothalamus and median eminence, whereas the formation in cerebellum and brain cortex were found to be low (128).

Immunohistochemical analysis, with the indirect immunofluorescence technique, have demonstrated LTC_4-immunoreactive fibres in the median eminence, the fibres terminating close to the portal vessels (128). In addition, single fibres with the same appearance have been observed in other brain regions, including many areas in the hypothalamus. The distribution pattern of LTC_4-positive fibres in the median eminence is similar to the pattern of leutenising hormone-releasing hormone- (LHRH) immunoreactive fibres (129). Furthermore, elution-restaining experiments have shown that a large portion of LTC_4- and LHRH-positive neurofibres are identical. This indicates that the two compounds are present in the same neurons. Taken together, these findings demonstrate a complex relation between LTC_4 and LHRH and gives further evidence for a physiological role of LTC_4 in the regulation of LH secretion.

A putative role for Leukotrienes as neuromodulators was suggested by the finding that LTC_4 and LTD_4 cause excitation of cerebellar Purkinje neurons (130). Further evidence for such a role was obtained by the findings of LTC_4 binding sites in the rat and guinea pig brain (131). Very weak or no binding was observed for LTB_4, LTD_4, or LTE_4, indicating that LTC_4 may be the active metabolite in the brain.

Finally, a direct connection between neuropeptides and Leukotriene biosynthesis and therefore a possible neuro-immune interaction has been shown from work in this laboratory (238,239,308,404).

1.4 Phosphoinositide-Specific Phospholipase C Activation, Phosphoinositide Breakdown and Signal Transduction

A wide variety of receptors for hormones, neurotransmitters and growth factors control cell function via the GTP-dependent activation of phosphoinositide specific phospholipase C (PIC). The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) following receptor-dependent activation of PIC simultaneously generates two powerful intracellular signal molecules: inositol 1,4,5-trisphosphate (IP_3), which stimulates Ca^{2+} release from intracellular stores; and diacylglycerol (DAG), which activates protein kinase C (PKC) (reviewed in 132). Activation of PIC by hormones, neurotrasmitters and growth factors is, in many ways, analogous to the hormonal regulation of adenyl cyclase, but the molecular and mechanistic details of the former are incompletely understood. The following sections attempt to review some aspects of this new and growing signalling pathway.

1.4.1 Role of GTP-Binding Regulatory Proteins

GTP-binding regulatory proteins (G-proteins) are a family of membrane associated proteins composed of α, ß and γ subunits. The α subunit binds guanine nucleotides, and α, ß, γ subunits are always tightly associated (133). There are a number of different G-proteins reported and these are most readily distinguished by their α subunits, though there are also more subtle structural and functional differences in some ß and γ subunits (134). Activation of G-proteins requires their association with a membrane, plasma membrane in most cases but intracellular retinal disc membranes in photoreceptors. Attachment of the α subunit to the cytoplasmic face of the membrane may be achieved by the ßγ complex (135) or by fatty acids covalently linked to the N-termini of some α subunits (136). For α_s (α subunit of G-protein involved in the activation of adenyl cyclase), which lacks covalently bound lipid, the effector itself, adenyl cyclase, may also provide an anchor to the plasma membrane (137). The nature of the attachment is important because G-protein activation almost certainly involves dissociation of the α subunit from the ßγ subunits, and if the latter provide the only membrane attachment the active α subunit would leave the membrane.

The features common to each of the G-protein-linked signalling pathways are shown in Figure 9. GDP bound to the G-protein α subunit normally dissociates only slowly, $t_{1/2}$ of 1-5 minutes for G_s and hours for

transducin (G_t, G-protein involved in "phototransduction") (138), but the concerted actions of intracellular Mg^{2+} and activated receptors catalyse G-protein activation by increasing the rate of GDP dissociation from the α subunit and its replacement by GTP. A useful pharmacological tool is the AlF_4^- complex which circumvents the need for GDP dissociation by binding alongside GDP and mimicking the terminal phosphate group of GTP, thereby promoting G-protein activation (139).

When an agonist has diffused to within a few molecule diameters of the receptor, the two begin to interact and part of the free energy of binding is used to deform the protein (140) and switch it to an "active" conformation, but little is known of the structural changes that accompany receptor activation. Receptor interactions, following receptor ligand binding, with G-proteins are summarised in Figure 10. The activated receptor has high affinity for a conformation of the G-protein in which its α and βγ subunits are associated and the single guanine nucleotide-binding site of the α subunit is empty (141). Activated receptors therefore do more than promote release of bound GDP, they also hold open the guanine nucleotide-binding site (142). Although only the α subunit undergoes guanine nucleotide exchange, the βγ complex plays an essential role in presenting it to the receptor; without it there is little effect of receptors on the α subunit (see Figure 10) (143). The ternary complex of agonist, receptor and G-protein is normally transitory because a guanine nucleotide binds within milliseconds to the α subunit, the affinity of the receptor for the G-protein is decreased and the two dissociate (144). However, under experimental conditions, where guanine nucleotides can be omitted, the association between the proteins is long-lived and can be easily measured.

G-protein α subunits contain sites for covalent modification by bacterial toxins that catalyse NAD-dependent ADP-ribosyltransferase reactions. G_s and G_t serve as substrates for cholera toxin-catalysed ADP-ribosylation on an internal arginine residue. This modification results in the persistent activation of these proteins. G_o (G-protein involved in the control of voltage dependent calcium channels), the G_{ts}, and the G_{is} can be ADP-ribosylated by pertussis toxin on a cysteine residue near the carboxyl terminus. This modification interferes with the interaction between the G-protein and receptors. $G_o\alpha$ and $G_i\alpha$ (but not $G_s\alpha$ or $G_t\alpha$) contain N-linked myristate (136), presumably on the amino-terminal glycine residue. The significance of this covalent modification is not clear; however, since the primary sequences of the α subunits do not

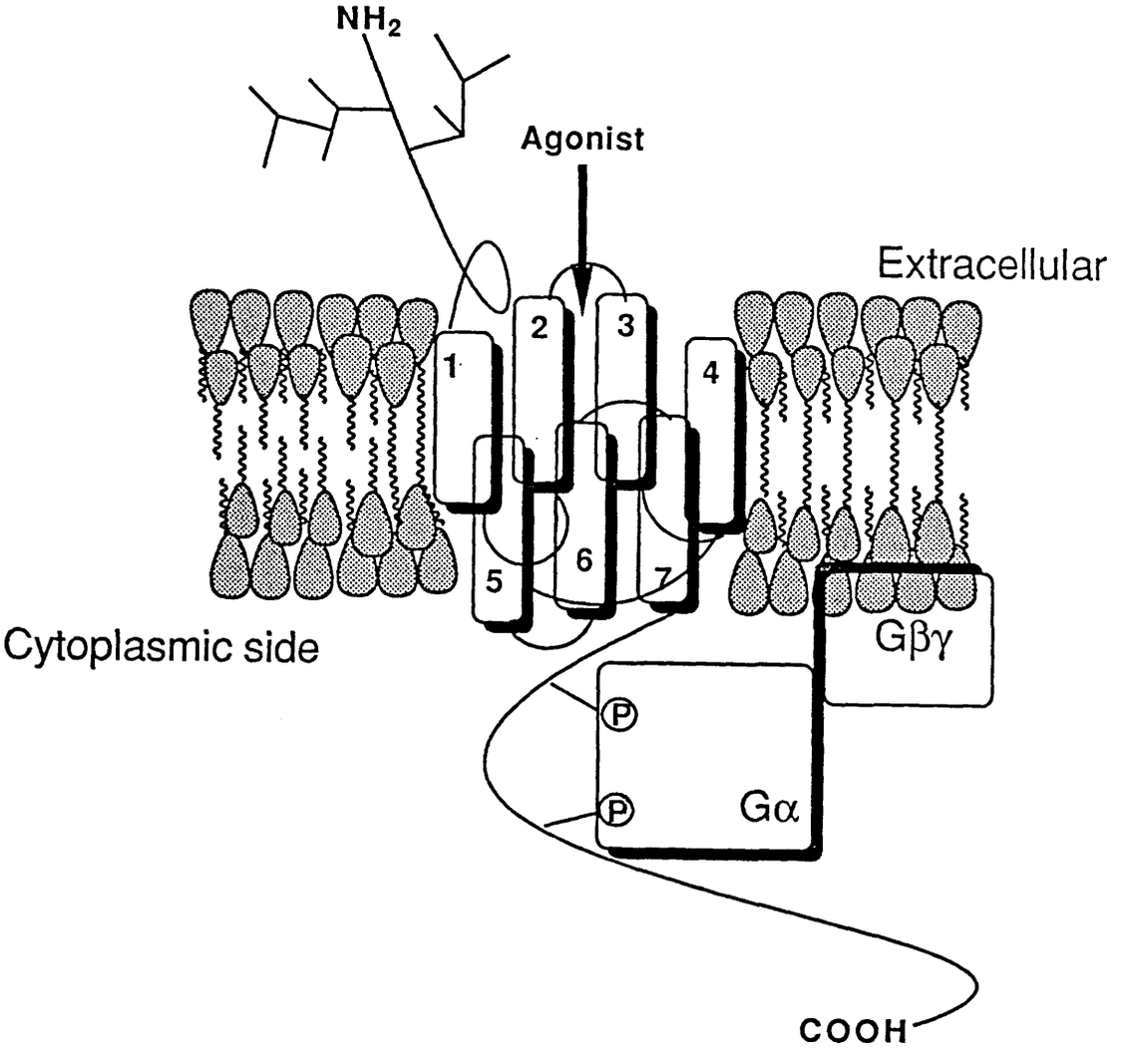


Figure 9: The model illustrating some of the features predicted to be shared by all receptors that interact directly with GTP-binding proteins.

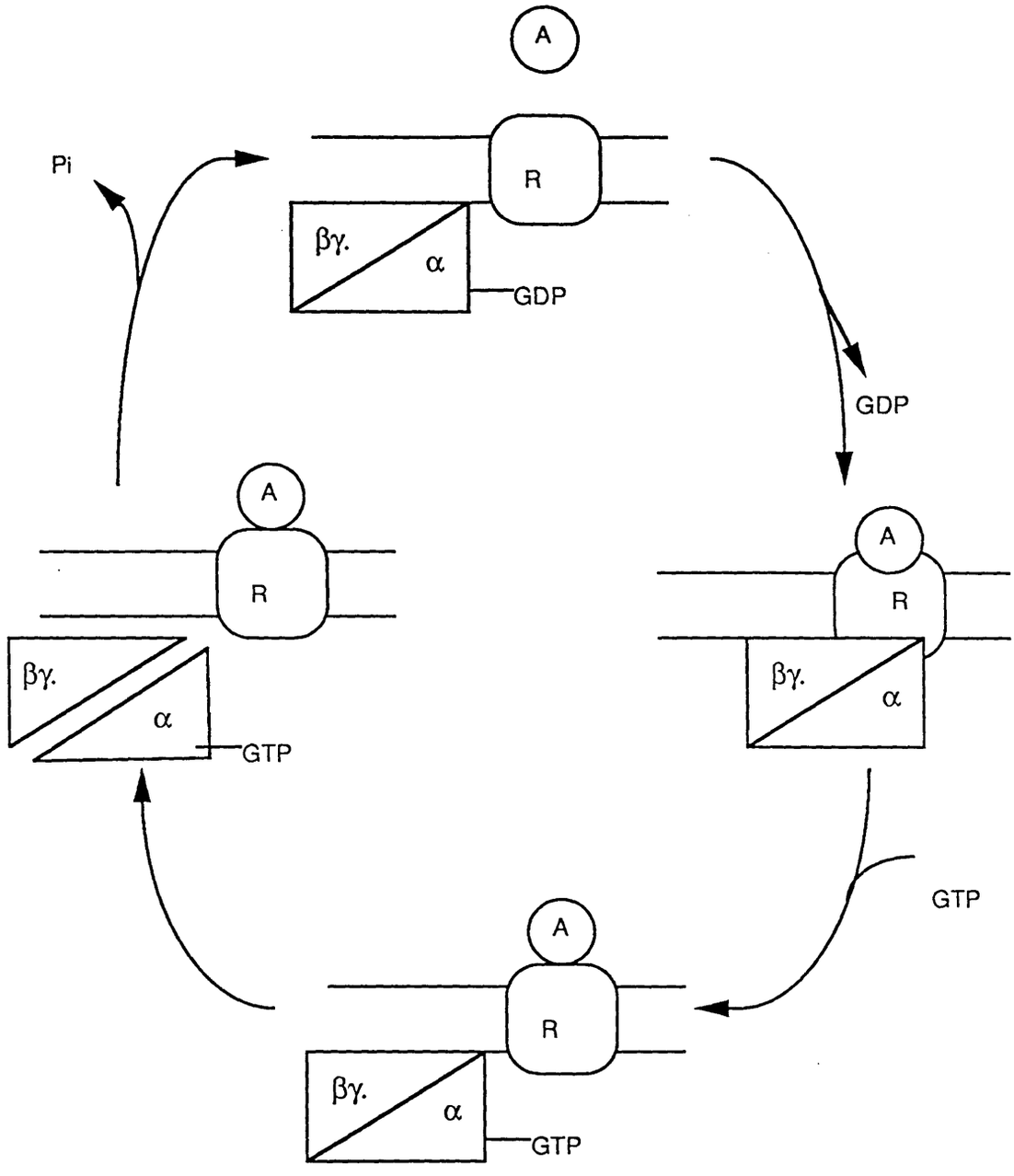


Figure 10: Receptor interactions with G-proteins:
A simplified scheme showing the coupling of the receptor (R) and G-protein ($\alpha\beta\gamma$) cycles. At rest the GTPase activity is at least ten fold greater than the rate of guanine nucleotide exchange; very little G-protein is therefore active in the active GTP-bound form. Occupied receptors catalytically activate the G-protein by increasing the rate of guanine nucleotide exchange without affecting the intrinsic GTPase activity, and therefore increase the fraction of G-protein in the active form. This G-protein cycle is coupled to the receptor cycle in which the receptor switches between conformations with high and low affinity for its agonist (A).

predict any hydrophobic (transmembrane) domain, the myristate may serve to target the proteins to the membrane.

1.4.2 G-Protein Dependent Regulation of Phosphoinositide-specific Phospholipase C

Definitive evidence that a G-protein participates in agonist stimulated PIP_2 hydrolysis was provided by Cockcroft and Gomperts (145) and by Litosch *et al.* (146), who found that guanine nucleotide analogues specifically stimulated the breakdown of endogenous phosphoinositides (PI) in the membranes of human neutrophils and blowfly salivary glands, respectively. Similar observations have been made in a large number of membrane preparations and include the important conclusion that agonist responses in these broken cell systems are largely, although not always entirely, guanine nucleotide dependent (see 147 for a study involving muscarinic receptors).

The G-proteins implicated in the activation of PIC by agonists, G_pS, have generally been assumed to be structurally and functionally analogous to the G-proteins that regulate adenyl cyclase (G_i and G_s) and cGMP phosphodiesterase (G_t). These proteins, which have been purified to homogeneity and characterized in functional reconstitution assays, are heterotrimers. Their α subunits possess a guanine nucleotide binding site, a high affinity GTPase and are the substrates for ADP-ribosylation by bacterial toxins. They bind GDP in the resting state and the agonist-receptor complex functions by promoting the exchange of GDP for GTP and the dissociation of the α subunit, to which GTP is bound (α^{GTP}), from the $\beta\gamma$ complex. In the majority of definitive studies α^{GTP} has been shown to be the active G-protein species responsible for effector regulation. Its inactivation is achieved by the intrinsic GTPase, which converts bound GTP to GDP, followed by reassociation with $\beta\gamma$ complex.

In most of the reported studies agonist-stimulated inositol phospholipid hydrolysis in membrane preparations is very much reduced by comparison with the responses of intact cells. By contrast, Turkey erythrocyte membranes prepared from [^{3}H]-inositol-labelled cells respond to guanine nucleotides with prolonged accumulation of inositol phosphates (IPs) (148). The cells are endowed with P_{2y} purinoceptors which remain efficiently coupled to PIC in the membrane preparation and membrane responses to the specific P_{2y} agonist 2-thiomethyl ATP are absolutely dependent on the presence of guanine nucleotides (149). These membranes have, therefore, been used to carry out

a detailed analysis of the mechanism of P_{2y} and G-protein-dependent activation of PIC.

During the preparation of this thesis it has been suggested that the activation of Turkey erythrocyte PIC by non-hydrolysable analogues of GTP, such as GTPγS, follows first order kinetics and the rate of activation is dramatically accelerated by purinergic agonists (150). This suggests that agonist-occupied P_{2y} receptors function by accelerating a guanine nucleotide exchange reaction, presumably on G_p. Further analogy with known G-proteins is suggested by the observations that non-hydrolysable GTP analogues consistently generate a higher level of PIC activity than does GTP itself and that the effects of GTP analogues are reversed by GDP or GDPßS. Inactivation of Turkey erythrocyte PIC by the addition of excess GDPßS to membranes that had been preactivated with different GTP analogues revealed that inactivation was very rapid when GTP itself was used to activate, but was extremely slow for non-hydrolysable analogues, particularly so for GTPγS. Taken together these results suggest that an intrinsic GTPase participates in the inactivation of G_p.

As mentioned above, adenyl cyclase is controlled by both a stimulatory (G_s) and an inhibitory G-protein (G_i). All the evidence pointed above to G_p being stimulatory, but there are suggestions that there may be an inhibitory pathway responsible for switching off the hydrolysis of inositol lipids. An inhibitory pathway in the hippocampal neurons was proposed on the basis that acidic amino acids strongly suppress the formation of inositol phosphates induced by carbachol or histamine (151). Another example is found in the pituitary, where the release of hormones is inhibited by dopamine acting on D_2-receptors. While much of this inhibition probably occurs by lowering the level of cAMP, dopamine may also act by inhibiting the breakdown of inositol lipids (152). Dopamine is able to suppress the stimulatory effect of angiotensin II on both prolactin secretion and the formation of inositol phosphates (152). Although these examples suggest the existence of an inhibitory pathway, more biochemical information is required before one can conclude that this pathway is mediated through a G-protein.

1.4.3 Phosphoinositides and Inositol Phosphates

Receptor-stimulated phosphatidylinositol (PI) metabolism was discovered by Hokin and Hokin in 1953 (153) and substantially characterized by the same workers during the following few years (154). However, the

function of this response remained obscure until Michell (155) realized that it might in some way be responsible for the receptor-stimulated elevation in the cytoplasmic concentration of Ca^{2+} that is a major intracellular trigger to the physiological responses of the target cells, from rapid contraction or secretion through to the initiation of cell proliferation. In 1979, Nishizuka and co-workers discovered that 1,2-diacylglycerol (DAG) released during receptor-activated inositol lipid hydrolysis could serve as a second messenger activating protein kinase C (PKC), an observation that transformed inositol lipid hydrolysis into a signalling reaction responsible for the generation of at least two intracellular signals (156). Finally, in recent years, it has been recognised that phosphatidylinositol 4,5-bisphosphate (PIP_2) is the major or only lipid hydrolysed as a direct result of receptor activation (157) and that inositol 1,4,5-trisphosphate (IP_3) liberated by this reaction causes the release of Ca^{2+} from an intracellular pool into the cytosol (158). Thus, it is now possible to conclude that many receptors cause the activation of phosphoinositide-specific phospholipase C (PIC) which catalysed PIP_2 hydrolysis, directly yielding two cellular messengers, DAG and IP_3 and indirectly causing an increase in the cytosolic concentration of a third cellular messenger, calcium ions. There have been recent reviews on this new and exciting topic (158-160) and we shall study this pathway in more depth in the following sections.

I) Inositol Lipid Metabolism

Compared to all the other phospholipids in the membrane, Phosphatidylinositol (PI) is unique in that it can be further phosphorylated, by a PI kinase, which transfers a phosphate from ATP onto the 4-position of the inositol headgroup to give phosphatidylinositol 4-phosphate (PIP). A separate PIP kinase adds another phosphate to the 5-position to give the phosphatidylinositol 4,5-bisphosphate (PIP_2), which is the substrate used by the receptor mechanism to generate IP_3 as seen in Figure 11. Only a small proportion (10-20%) of the inositol lipid pool is involved in signalling (161-164). Further evidence for separate pools has come from experiments with Mn^{2+}, which stimulates an enzyme reaction leading to inositol headgroup exchange. This increases the incorporation of [^{3}H]-inositol into a PI pool that is insensitive to agonists (165,166). Presumably Mn^{2+} stimulates incorporation of inositol into the agonist-insensitive pool, as shown in Figure 12.

The agonist-sensitive pool seems to be confined to the plasma membrane (164), which is where most of the PIP_2 is thought to reside (132).

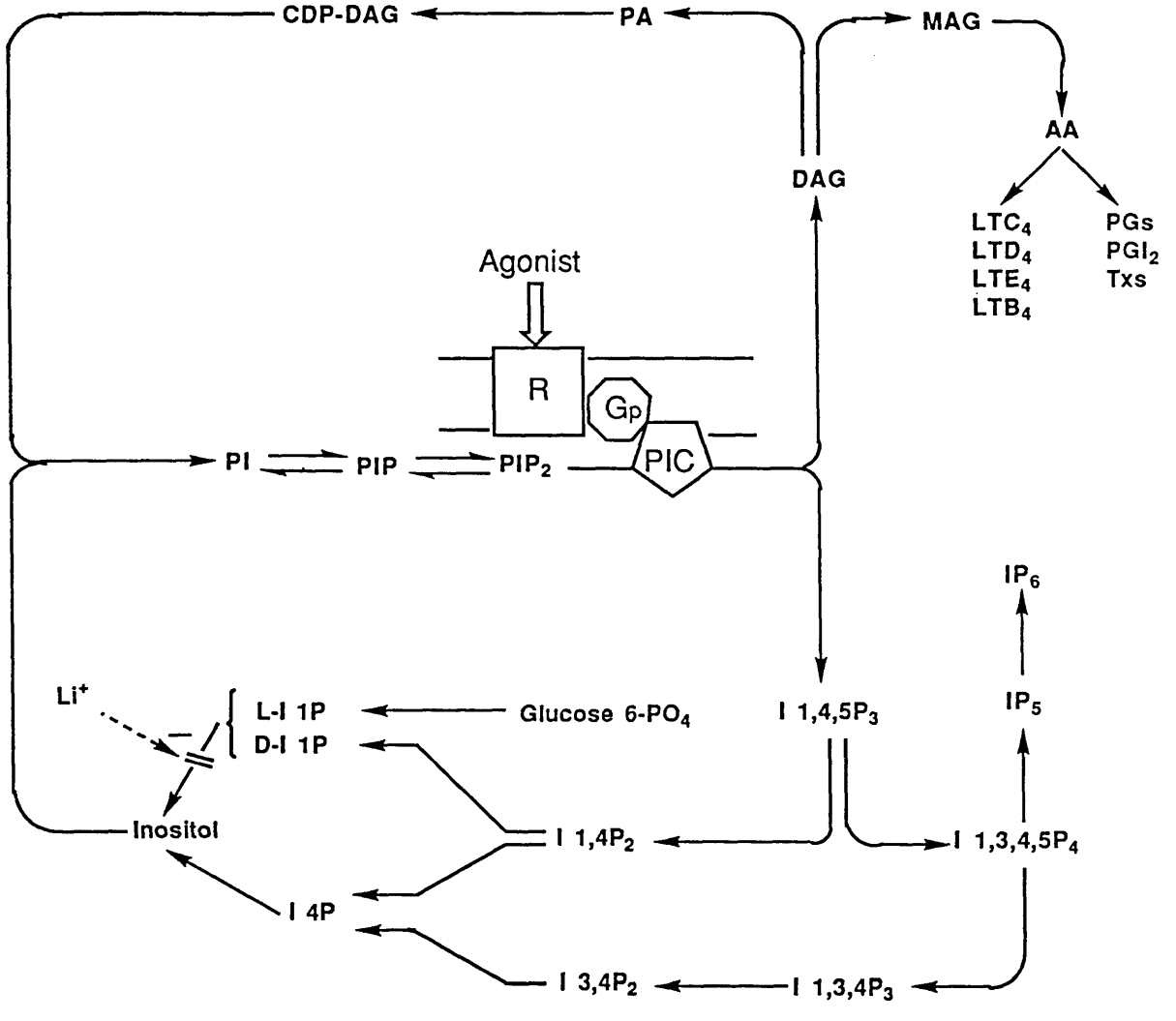


Figure 11: Receptor mediated inositol phosphate release from inositol lipids. When an agonist binds its receptor (R), it activates a GTP-binding protein (Gp), which then binds GTP as part of an on-reaction leading to the stimulation of PI-specific phospholipase C (PIC). This intum leads to the generation of the two second messengers IP_3 and DAG both of which can be metabolized via two separate pathways, IP_3 leading to inositol polyphsphates and DAG leading to the generation of AA and the eicosanoids. For more detail and abbreviations see text.

Even the PIP_2 within the plasma membrane, however, may not be a homogeneous pool (167,168). In WRK-1 cells it has been estimated that only 60% of the PIP_2 is available for signalling, while the remainder is associated with the receptor-insensitive pool and remains stable for many hours (167). The PIP_2 in erythrocytes is similarly compartmentalized with a large metabolically inactive pool somehow kept apart from a smaller pool, which is turning over rapidly (168). It is difficult to imagine how PIP_2 might be compartmentalized within the plasma membrane, but a recent observation that polyphosphoinositides might be covalently linked to myelin basic protein may explain how these lipids could be removed from the metabolic pool (169). There are clear indications, therefore, that the pool of PIP_2 available to the receptor may be a lot smaller than hitherto suspected.

The fact that IP_3 is produced in many different cells provides unequivocal evidence that PIP_2 is one of the lipids used by the receptor mechanism. There have been suggestions that the other two inositol lipids may also be used under certain circumstances, or by certain agonists. When platelets respond to thrombin, it has been argued that PIP_2 is used at the beginning and is followed later by a calcium-dependent hydrolysis of PI (170). Vascular smooth muscle is also thought to switch over to hydrolysing PI during sustained stimulation (171). Since PI and PIP would release inositol phosphates that do not mobilize calcium, this would provide a mechanism for biasing the signal pathway towards PKC and away from calcium. The possibility that the other inositol lipids might be hydrolysed during signalling must not be ignored, but here PIP_2 will be considered as the precursor used to translate incoming signals into second messengers.

An increased hydrolysis of PIP_2 to give 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) has been measured in a number of stimuli including neurotransmitters, releasing factors, hormones, growth factors, fertilization and even light (132,158-160). Consistent with its role as a second messenger, the increase in the level of IP_3 was found to precede the onset of calcium-dependent events in blowfly salivary gland (172) and in neutrophils (173). Measurements of intracellular calcium in different cell types have revealed that the increase in IP_3 either preceded (174,175) or coincided with the onset of the calcium signal (176-180). This evidence is consistent with the hypothesis that external signals act through a receptor mechanism to produce IP_3, which then mobilizes calcium.

As shown in Figure 11, the transduction unit within the plasma membrane consists of three main components:

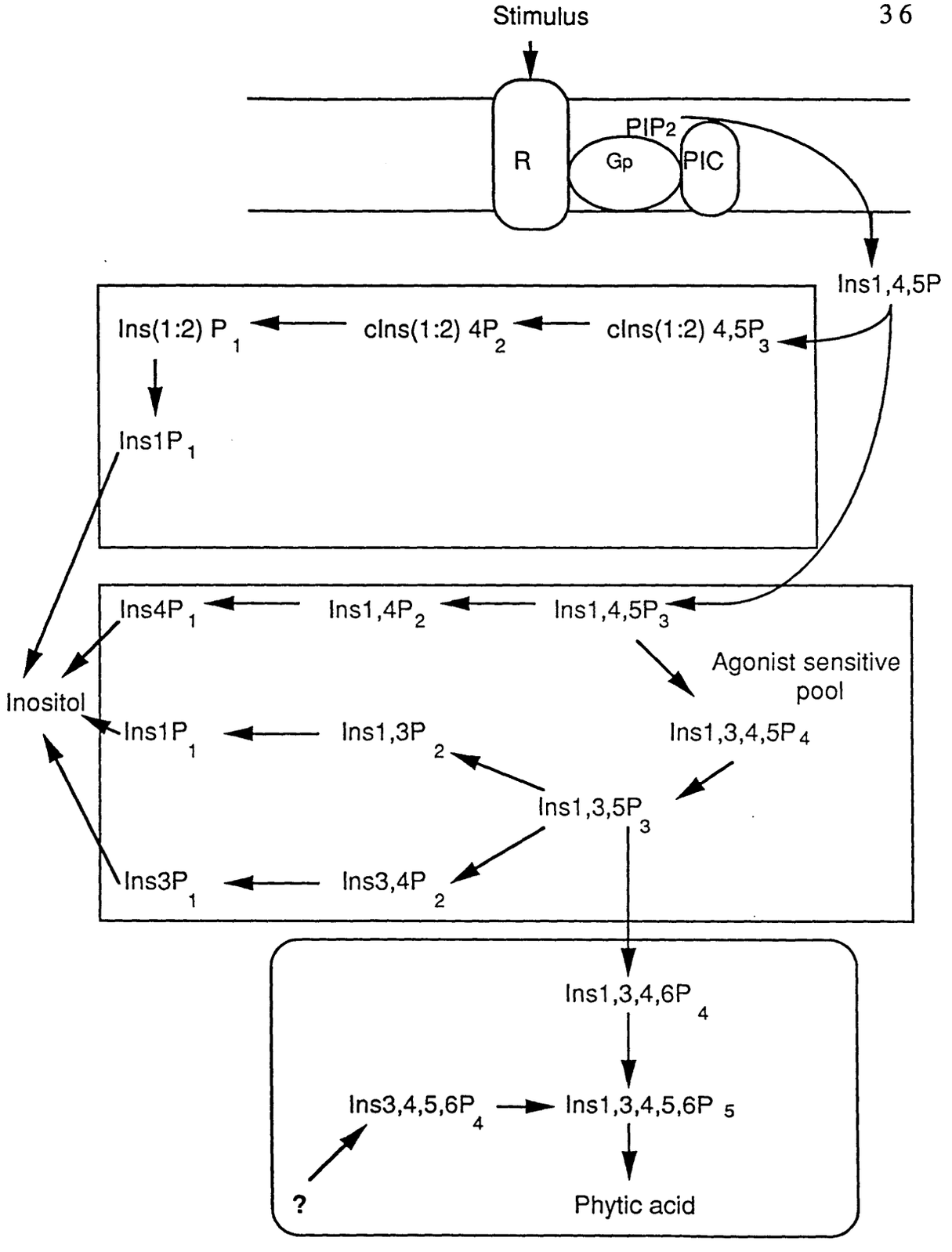


Figure 12: Agonist sensitive and insensitive inositol phosphate pools.

1) A receptor (R) that detects the incoming signal
2) A G-protein (Gp)that serves to couple the receptor to the third component
3) The phosphoinositide-specific PLC (PIC) responsible for cleaving the lipid precursor.

PIC cleaves the phosphodiester bond between the glycerol backbone and the phosphate group (see Figure 2), which requires the addition of a hydroxyl group. This group can enter as a free OH, causing the release of IP_3, or the enzyme may use the resident hydroxyl located on the 2-position of the inositol ring, thus leading to the formation of inositol 1:2-cyclic 4,5-trisphosphate (cIns 1:2,4,5P_3) as shown in Figure 12. The formation of cIns 1:2,4,5P_3 has been demonstrated *in vitro* following incubation of PIP_2 with purified PIC (181). There also is a report that cIns 1:2,4,5P_3 exists in blood platelets, and that its concentration rises in response to thrombin (182), but others have failed to find this cyclic form (183). Considering the uncertainty and lack of established messenger function surrounding cIPs, only the non-cyclic isomers will be discussed here (for more detail on the cIPs refer to 160)

II) Metabolism of Inositol Phosphates

Both of the second messengers formed by the hydrolysis of PIP_2 can be metabolized via two separate pathways. As mentioned in Section 1.2.1, it has been known for some time that DAG can either be phosphorylated to phosphatidic acid by a DAG kinase or it can be hydrolysed to release arachidonic acid. Since the latter is the precursor of the eicosanoids (Leukotrienes, prostaglandins and thromboxanes), the primary second messenger DAG can give rise to additional messengers. As can be seen in Figure 11, IP_3 can also be metabolized via two separate pathways: either it can be dephosphorylated sequentially via phosphomonoesterases to free inositol or it can enter an inositol tris/tetrakis pathway to form some newly identified inositol polyphosphates, which may have messenger functions.

The first enzyme in the dephosphorylation pathway of IP_3 metabolism is IP_3 5-phosphatase, which has a somewhat precise positional selectivity in that it specifically removes the phosphate at the 5-position to give inositol 1,4 di-phosphate (IP_2) (184-186). Although the enzyme is predominantly particulate and associated with the plasma membrane (184-188), the enzyme found in platelets is soluble (186). This trisphosphatase is of key importance in that it terminates the second messenger action of IP_3 because the product IP_2 is

incapable of releasing Ca^{2+}. The enzyme is particularly sensitive to divalent cations in that it has an absolute requirement for Mg^{2+} and is markedly inhibited by silver, zinc, and cobolt (184,185). Calcium inhibits the enzyme in platelets (186), but activates that from macrophages (189) and smooth muscle (190). The hydrolysis of IP_3 is insensitive to lithium (196-199), but is blocked by spermine (199) and by 2,3-diphosphoglyceric acid (184,185,191).

The next enzyme in the pathway is an inositol bisphosphatase with less precise positional selectivity in that it hydrolyses IP_2 to both Ins 1-phosphate and Ins 4-phosphate (185). The final step is to remove the phosphate from the two IP_1 isomers through inositol monophosphatase(s). Most of the inositol phosphates within the cell are in the D-form, but there is a measurable quantity of L-Ins 1-phosphate that originates from the isomerization of glucose 6-phosphate by an L-myo-inositol-1-phosphate synthase (192). A myo-inositol-1-phosphatase can hydrolyse both D-Ins 1-phosphate and L-Ins 1-phosphate (193), but is incapable of dephosphorylating Ins 2-phosphate, so the metabolic route for this isomer is unknown. The Ins 1-phosphatase is inhibited by lithium with a K_i value of approximately 1mM (193,194), which is somewhat lower than that for the bisphosphatase (185).

The alternative pathway begins with a newly discovered IP_3 3-kinase that phosphorylates IP_3 to inositol 1,3,4,5-tetrakisphosphate (IP_4) (195). The kinase is a soluble enzyme first described in brain (195), but is apparently ubiquitous as it has been found in liver (196,197), parotid (197), human T cells (198), and RINm5F cells (199). The kinase is a Mg^{2+}-requiring soluble enzyme, which transfers a phosphate from ATP specifically to the 3-position of IP_3 to form IP_4. The significance of the latter is that it is the precursor of inositol 1,3,4-trisphosphate (Ins1,3,4-P_3) as seen in Figure 12 (200). This new isomer was first described in parotid gland (201), and has since been found in many other cells (196,197,202-204).

A characteristic feature of this new Ins1,3,4-P_3 isomer is that it has a somewhat slower turnover than IP_3. In response to agonists, IP_3 builds up immediately, whereas there is a delay before Ins1,3,4-P_3 begins to rise (196,197,199,205). When agonists are withdrawn, the IP_3 isomer decays rapidly, and on the basis of *in vitro* experiments it has been estimated to have a half-life of approximately four seconds (185). On the other hand, the Ins 1,3,4,-P_3 isomer has much longer half-life (202). The latter is derived from IP_4 by the specific removal of the 5-phosphate by an enzyme that is similar, if not identical, to the inositol trisphosphatase discussed earlier (200). The delay in the appearance of Ins 1,3,4-P_3 may depend on the time taken for IP_3 to pass

through this tris/tetrakis pathway, during which it undergoes a two-step isomerization using IP_4 as an intermediary as shown in Figure 12. The latter may also be the precursor for the formation of Ins P_5 and Ins P_6, which have been identified in various cells (204).

Like IP_3, the new Ins 1,3,4-P_3 isomer is dephosphorylated through a sequence of dephosphorylation reactions that are beginning to be defined. Preliminary evidence suggests that one pathway depends on removal of the phosphate on the 1-position to give Ins 3,4-P_2, which is then dephosphorylated to either Ins 3-P or Ins 4-P. The latter is most likely since Ins 4-P has already been identified in brain (206). When cells are incubated in the presence of 10mM Li^+ there is a selective enhancement of Ins 1,3,4-P_3 but no change in IP_3 (197,203).

Whenever one has such a bifurcation point in a metabolic pathway, it is of interest to determine whether there is some control over the proportion of IP_3 that is channelled down each pathway. There are indications that the DAG/PKC pathway may act to stimulate the hydrolysis of IP_3 (207,208), whereas the increase in intracellular Ca^{2+} may stimulate the kinase in RINm5F (199) and HL 60 cells (209), thus leading to an enhanced phosphorylation of IP_3 to IP_4. This activation of both metabolic pathways may explain why the increase in IP_3 formation that occurs following stimulation is so short-lived. Activation of the kinase by Ca^{2+} will ensure the conversion of IP_3 to IP_4, both of which may perform specific messenger functions within the cell (200,201).

1.4.4 Activation of Protein Kinase C by Diacylglycerol and Calcium

The neutral DAG that remains within the plane of the plasma membrane functions as a second messenger by activating PKC (153,159). In addition to DAG, the enzyme requires Ca^{2+} and phosphatidylserine. When cells are activated there is usually an increase in Ca^{2+}, which may contribute to the activation of PKC perhaps by increasing the binding affinity of the enzyme for DAG (210,211). Experimentally the enzyme can be activated in intact cells either by adding a monoacyl-derivative of DAG such as 1-oleoyl-2 acetylglycerol (OAG) or by means of phorbol esters (156,159,212). In some cells, such as the adrenal medulla, PKC may be activated solely through Ca^{2+} entering via voltage-dependent calcium channels (213). In most cells, however, where both limbs of the signal pathway are activated, DAG and

Ca^{2+} probably act synergistically to stimulate the enzyme. An important aspect of the activation process appears to be a physical translocation of the enzyme from the cytosol into the membrane, which might be the role of Ca^{2+} (214,215). This Ca^{2+}-mediated translocation into the membrane may then serve to 'prime' the enzyme to the stimulatory action of DAG. A role for Ca^{2+} in activating PKC may be particularly relevant to the feedback interactions discussed later.

1.4.5 Inositol 1,4,5-Trisphosphate and Calcium Signalling Pathway

For small cells with a large surface-to-volume ratio, entry of Ca^{2+} across the plasma membrane, usually through voltage-dependent calcium channels, is sufficient for cell activation. There is no evidence for any role for inositol lipid hydrolysis in order to open such voltage-dependent calcium channels. This hydrolysis of inositol lipid is mainly confined to the action of calcium-mobilizing agonists, which bind to specific cell-surface receptors and gain access to both internal and external sources of calcium. The response to stimulation by calcium-mobilizing agonists is a release of internal calcium, termed Phase I, which is soon followed by entry of Ca^{2+} across the plasma membrane, termed Phase II. Much of the Ca^{2+} mobilized from the internal store during Phase I is pumped out of the cell (216), and/or pumped into an agonist insensitive intracellular pool, resulting in the Ca^{2+} content of the cell declining by as much as 50% (217-219). Removing external Ca^{2+} or adding Ca^{2+} entry antagonists usually has no effect on Phase I, but severely curtails Phase II (179,210,220). During Phase I, the level of intracellular Ca^{2+} ($[Ca^{2+}]_i$) increases very fast, reaching a maximum value within seconds. This high level is usually not maintained but decays back close to the resting level through the action of various negative feedback pathways that cooperate to curtail the calcium signal.

Of all the inositol phosphates that have been identified in cells, IP_3 is the only one, so far, for which a clear second messenger role has been identified: it functions to release Ca^{2+} from the endoplasmic reticulum (ER). Most attention has focused on its role in stimulating the release of Ca^{2+} during cell activation, but of equal importance might be a role for Ins1,3,4-P_3 in regulating the resting or basal level of Ca^{2+} (221). Small variations in the endogenous turnover of inositol lipid may serve to adjust the resting level of IP_3 and hence Ca^{2+}. Even though activation of muscarinic receptors in

adrenal chromaffin cells increase the levels of IP_3 and Ca^{2+}, there is no secretion, although responsiveness to nicotinic receptors is enhanced (222), apparently because of an increase in the resting level of Ca^{2+}. Such alterations in responsiveness to incoming signals by resetting the resting level of Ca^{2+} could be particularly important in modulating synaptic transmission in the nervous system.

The first direct evidence that IP_3 functioned to mobilize intracellular Ca^{2+} was obtained using permeabilized pancreatic cells (223). By incubating pancreatic cells in a low-calcium medium, the plasma membrane became leaky, thus allowing access to the internal membrane systems that sequester Ca^{2+}. Using a Ca^{2+} electrode it was possible to monitor the uptake of Ca^{2+} into the ER and its subsequent release following the addition of IP_3 (223). Similar observations have now been reported for many other permeabilized cells (160). Based on all these studies, a detailed picture is beginning to emerge concerning this IP_3-sensitive intracellular Ca^{2+} store. The identity of the store was initially based on the use of metabolic inhibitors, but subsequent cell fractionation experiments have confirmed that IP_3 releases Ca^{2+} from a membrane fraction derived solely from ER (240-244). However, there is some degree of variability concerning the size of the IP_3-sensitive pool (160).

In order to release Ca^{2+}, IP_3 acts through a specific receptor, which may either be connected to or an integral part of the putative calcium channel. Of the inositol phosphates tested, only those having phosphates on the 4- and 5-positions are capable of stimulating release (227), and their order of potencies are IP_3 > glycerophosphoinositol 4,5-P_2 = Ins 2,4,5-P_3 > Ins 4,5-P_2. This sequence suggests that the two phosphates on the 4- and 5-positions are essential to stimulate the release of Ca^{2+}, whereas the phosphate on the opposite side of the molecule (1-position) functions to enhance the affinity of the molecule for the receptor. The product of IP_3 hydrolysis is Ins 1,4-P_2, which does not release Ca^{2+} (228,229), nor do a number of other compounds including Ins 1-P, Ins 2-P, Ins P_6, cIns 1:2P,2,3-diphosphoglyceric acid, fructose 1,6-bisphosphate, and myo-inositol.

The first steps towards the isolation and characterization of the putative receptor were the identification of specific binding sites for IP_3 on various cells (195,196,230) and the development of a specific affinity label (197). Binding sites could also be demonstrated on permeabilized liver cells and neutrophils (196). The ability of different inositol phosphates to displace the [^{32}P]-IP_3 showed exactly the same specificity as for the release of Ca^{2+} described above i.e. IP_3 > Ins 2,4,5-P_3 > Ins 4,5-P_2.

An arylazide derivative of IP_3 has been developed as a photoaffinity label of the receptor (197). When added to permeabilized cells and activated by light this label causes specific inhibition of IP_3-induced Ca^{2+} release. This irreversible inhibition was prevented if the cells were incubated with a 10-fold excess of unmodified IP_3. The development of this affinity label may provide a first step in the isolation of IP_3 receptor.

During Phase II, when the cell switches over to using extracellular Ca^{2+}, it is necessary to account for the enhanced entry of Ca^{2+} induced by agonists. The term receptor-operated calcium channels was introduced in order to distinguish it from Ca^{2+} entry mediated by voltage-dependent calcium channels. However, such receptor-operated calcium channels have never been detected in patch-clamp experiments, suggesting that a simple model of a channel being opened by the binding of an agonist may not be applicable. A number of hypotheses have been advanced to explain how the entry of external Ca^{2+} may depend on the hydrolysis of inositol lipid.

One possibility is that the receptor mechanism may generate its own channel in the form of phosphatidic acid (204). The idea is that the DAG formed by the hydrolysis of PIP_2 is phosphorylated to phosphatidic acid, which then acts as Ca^{2+} ionophore before it is resynthesized back to PI. Apart from the fact that it is formed during stimulation, the main evidence implicating an ionophoric role for phosphatidic acid is based on the fact that the addition of this lipid can stimulate various cells (204). When tested on artificial membranes, however, phosphatidic acid failed to increase the flux of Ca^{2+} (270), which seemed to argue that it may not act simply as a Ca^{2+} ionophore. Recent studies on human A431 carcinoma cells reveal that phosphatidic acid increases intracellular Ca^{2+} by stimulating the formation of IP_3 (199). Instead of acting as an ionophore, phosphatidic acid mimicked the action of other growth factors by stimulating the hydrolysis of PIP_2 (199).

The other hypotheses for linking inositol lipids to Ca^{2+} entry across the plasma membrane are both centred around a role for inositol polyphosphates. There apparently is no direct role for IP_3 in regulating Ca^{2+} entry across the plasma membrane; this second messenger had no effect on Ca^{2+} release from plasma membrane vesicles (200). However, there is preliminary evidence that IP_4 may function to control Ca^{2+} entry across the plasma membrane (231). The idea is that IP_3 would release Ca^{2+} from the ER during Phase I and then would be converted into IP_4, which would function to control Ca^{2+} entry across the plasma membrane during Phase II. This is portrayed diagrammatically in Figure 13. Since the conversion of IP_3 to IP_4 is enhanced

by Ca^{2+} (177,185), stimulation of the Ca^{2+} influx by IP_4 would be an example of Ca^{2+}-induced Ca^{2+} entry. The first direct evidence that IP_4 acts to promote Ca^{2+} entry has been obtained by injecting this molecule into eggs of the sea urchin *Lytechninus varriegates* . Injection of submicromolar concentrations of IP_4 caused an immediate elevation of the fertilization envelope, which required the presence of extracellular Ca^{2+}. In addition, the action of IP_4 was dependent on the coinjection of IP_3, which is known to mobilize intracellular Ca^{2+} (227). In some way the action of IP_4 seems to require the prior emptying of intracellular Ca^{2+} stores. Such a mechanism contains the main element of another hypothesis concentrating the way in which inositol polyphosphates might act to regulate Ca^{2+} (see Figure 13) (203).

A possible role for IP_4 in promoting Ca^{2+} entry was mentioned above, but a function for Ins 1,3,4-P_3 has yet to be found. Since the cell invests energy to convert IP_3 to the new inositol polyphosphates, it is reasonable to suspect that they might have some second messenger role (179,180). Recent experiments have shown that Ins 1,3,4-P_3 will release Ca^{2+} from permeabilized Swiss 3T3 cells but with much lower efficacy (R.F Irvine unpublished data). However, the concentration of Ins 1,3,4-P_3 is known to rise above that of IP_3, so it is possible that the former may function to keep the internal pools of Ca^{2+} discharged during Phase II, after the level of IP_3 has returned close to its basal level.

1.4.6 Dual Signal Hypothesis of Cell Activation

The hallmark of the inositol lipid signalling system is that there is a bifurcation in the flow of information. The dual signal hypothesis concerns the way in which the IP_3/Ca^{2+} and DAG/PKC signal pathways cooperate with each other to control a whole host of cellular processes. In many cells the two pathways appear to act synergistically (153,159). The existence of a synergistic interaction was demonstrated by using pharmacological agents capable of stimulating each pathway independently of the other. Calcium ionophores (A23187 or ionomycin) were used to raise intracellular Ca^{2+}, whereas phorbol esters or OAG could mimic the stimulatory effect of DAG on PKC. By combining a threshold concentration of an ionophore with a threshold concentration of an activator of PKC, it is possible to activate many different cellular responses, including secretion of enzymes, hormones, neurotransmitters, muscle contraction, and DNA synthesis. While such evidence suggests roles for both pathways, it provides no indication of their

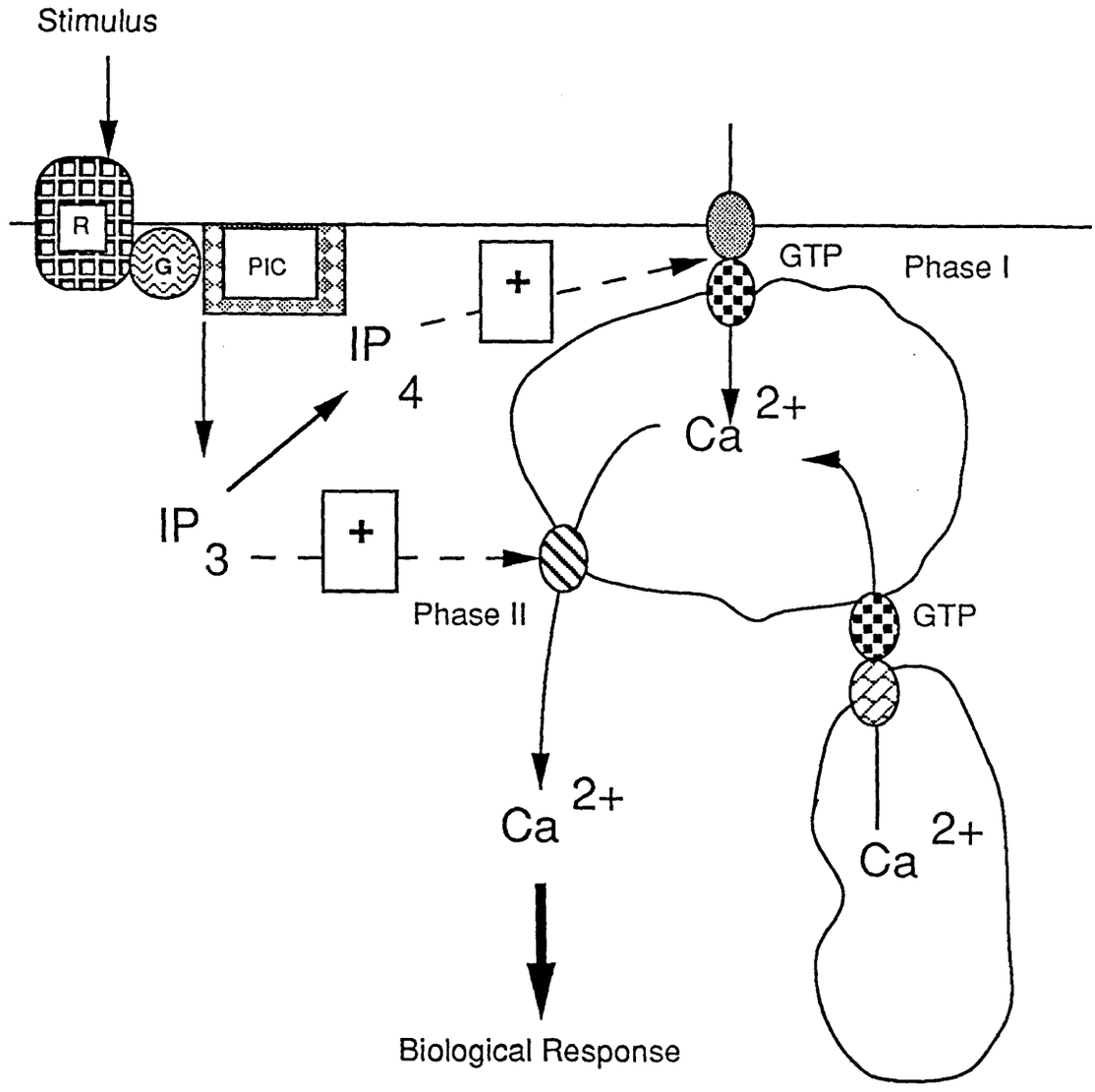


Figure 13: The proposed role of IP_3 and IP_4 in regulating calcium mobilization from intracellular as well as extracellular calcium pools.

relative contributions to the final response. The dual signal hypothesis outlined in Figure 14 integrates information from many different cell types and summarizes how both limbs of the signal pathway function in the control of cellular activity: It is proposed that the IP_3/Ca^{2+} pathway plays a major and direct role in initiating cellular responses. The DAG/PKC pathway may also contribute directly to the final response, but its predominant role appears to be as a modulator of either the Ca^{2+}-signalling pathway (homologous interactions) or of other signal pathways (heterologous interactions).

The view that IP_3 is the primary second messenger in initiating final responses is based mainly on the fact that it controls the calcium intracellular level, $[Ca^{2+}]_i$, which has been recognised as a major second messenger for stimulating a whole range of cellular processes such as contraction, secretion, and metabolism. Secondly, IP_3 can trigger a number of complex physiological processes when added to permeabilized cells or when injected into intact cells (160).

1.4.7 Modulatory Role of Diacylglycerol/Protein Kinase C Pathway

Once PKC has been activated through the concerted action of DAG and Ca^{2+}, it begins to phosphorylate specific proteins that are thought to contribute to the final response. Although PKC can phosphorylate a large number of proteins when studied *in vitro*, it has proved extraordinarily difficult to identify the function of the smaller number of proteins that are phosphorylated in intact cells. Some of the identified substances include vinculin, the epidermal growth factor (EGF) receptor, glycogen synthase, and lipocortin. Phosphorylation of the EGF receptor results in a decrease in both the affinity of the receptor for EGF and its tyrosine kinase activity, which is a good example of the modulatory action of PKC.

As more is learnt about PKC, it becomes apparent that many of its functions are connected with: a) modulating processes within the inositol lipid pathway (homologous interactions) or b) those connected with other signal pathways, particularly the cAMP system (heterologous interactions). These actions will be addressed below.

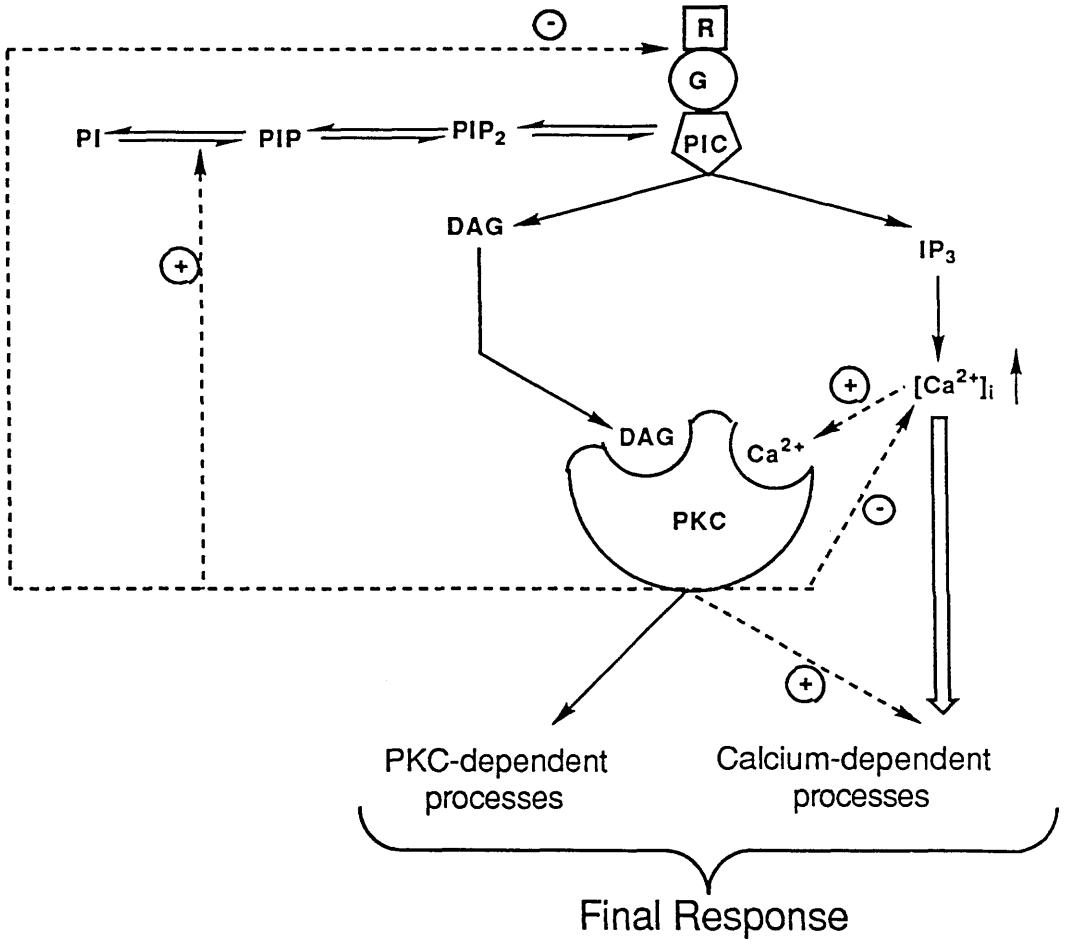


Figure 14: Dual signal hypothesis. The dashed lines represent the homologous interactions operating between the IP_3/Ca^{2+} and DAG/PKC pathways. See text for more detail and abbreviations.

a) Homologous Interactions Related to Calcium Signalling

The mechanisms responsible for increasing the $[Ca^{2+}]_i$ are under stringent negative feedback control. In order to respond rapidly to external stimuli, there usually is an explosive increase in $[Ca^{2+}]_i$ during Phase I far exceeding that necessary to activate the cell. If unchecked, the Ca^{2+} level may rise to levels that would damage the cell, which may explain the negative feedback interactions, shown in Figure 14, that cooperate to curtail the increase in $[Ca^{2+}]_i$ levels, especially when cells are strongly stimulated.

Of particular importance with regard to curbing increases in $[Ca^{2+}]_i$ levels are two negative feedback loops where PKC acts both to inhibit the hydrolysis of PIP_2, and to reduce intracellular Ca^{2+} by stimulating Ca^{2+} pumps. PKC is also at the heart of the positive feedback interactions in that its activity is enhanced by Ca^{2+} and it also seems to be responsible for stimulating the kinases that form PIP_2 (see Figure 14). PKC occupies a pivotal position, because it can either enhance or inhibit Ca^{2+} signalling; its net effect will depend on the degree to which these feedback mechanisms are expressed in each particular cell. For example, activation of PKC with phorbol esters enhances the contractility of certain smooth muscle cells but relaxes others (210). Within the same muscle, agonist-dependent contractions were reduced, presumably due to the negative feedback effect on PIP_2 hydrolysis, whereas potassium-induced contractions were enhanced (209). From the large repertoire of second messenger interactions at its disposal, each cell selects those that enable it to respond appropriately to external signals.

Stimulation of PIP_2 Formation: When cells are stimulated, especially with a high concentration of agonist, there initially is a fall in the level of PIP_2, but the level is soon restored to and sometimes exceeds the resting level. This increase in PIP_2 formation might be a consequence of a positive feedback effect operated through PKC because similar increases can be obtained when certain cells are stimulated with phorbol esters (210). In the case of thymocytes, the increase in PIP_2 labelling brought about by phorbol esters resulted in an increased formation of IP_3 when cells were subsequently challenged with concanavalin A (210). The DAG/PKC limb of the bifurcating signal pathway may thus have an important effect on inositol lipid metabolism by enhancing the formation of the PIP_2 used by the receptor mechanism.

Inhibition of Calcium Signalling: An important action of DAG/PKC pathway is to inhibit Ca^{2+} signalling. The basic observation is that pretreatment with phorbol esters reduces agonist-induced increases in $[Ca^{2+}]_i$

level in rat basophilic leukaemia cells (211), blood platelets (212), liver (213), Swiss 3T3 cells (214), neutrophils (215), vascular smooth muscle (216), adrenal glomerulosa cells (217), WRK-1 cells (218), and human A431 cells (221). The DAG/PKC pathway can modulate Ca^{2+} signalling either by inhibiting the hydrolysis of PIP_2, thus preventing the generation of IP_3, or by activating the Ca^{2+} pumps that remove Ca^{2+} from the cytosol. When cells are pretreated with a phorbol ester or OAG to activate PKC, there is a marked reduction in agonist-induced PIP_2 hydrolysis and inositol phosphate formation in cultured human 1321N1 astrocytoma cells (220), hippocampal slices (221), blood platelets (212,218,222), vascular smooth muscle (216), WRK-1 cells (218) and adrenal glomerulosa cells (217). There are a number of sites where the DAG/PKC pathway could act to reduce the agonist-dependent hydrolysis of PIP_2. In liver, which has a number of receptors acting through the inositol lipid pathway, phorbol esters seem to exert a selective inhibition of α_1-receptors, while the effects of vasopressin and angiotensin II were relatively unimpaired (213). A similar situation exists in Swiss 3T3 cells where a phorbol ester inhibited inositol phosphate formation induced by bombesin but not that of platelet derived growth factor (PDGF) (223). Such selective actions suggest that the DAG/PKC pathway may exert its negative feedback effect by phosphorylating receptors rather than G_p or PIC. In cultured smooth muscle cells, the phorbol ester-induced inhibitory effect on PI metabolism mediated by α_1-receptor agonist was associated with a rapid ($t_{1/2}$ of 2 minutes) increase in the phosphorylation of the α_1-adrenergic receptor (232).

b) Heterologous Interactions Related to Cyclic Nucleotides

In addition to the interactions operating within the inositol lipid pathway, there are reciprocal interactions with other second messenger pathways, particularly the cyclic nucleotides (156,159). There are numerous examples where the stimulation of receptors that hydrolyse inositol lipids strongly potentiates those receptors that form cyclic-AMP (cAMP). For example, the production of cAMP in the pineal by ß-receptors is enhanced by activation of α_1-receptors (233). Similarly, adenosine- or VIP-induced accumulation of cAMP in brain is increased by the simultaneous activation of α_1-, $5HT_2$-, or H_1-receptors, all of which stimulate inositol lipid hydrolysis (234). This enhancement of cAMP formation appears to be mediated through the DAG/PKC pathway because phorbol esters augmented the accumulation of cAMP by ß-receptors in the pineal gland (233), frog erythrocytes (235), and

S49 lymphoma cells (236). The increase in cAMP induced by adenosine in brain is also augmented by phorbol esters (224). Just where PKC acts is unclear, but the evidence suggests that it may facilitate the interaction between G_S and adenyl cyclase (236). In platelets, PKC phosphorylates G_i, so reducing the activity of the pathway, which inhibits adenyl cyclase while having no effect on the stimulatory pathway (225).

Having reviewed Leukotriene biosynthesis and the inositol phosphate signalling pathway, we shall now proceed to determine their relevance in RBL-1 cells.

1.5 Leukotriene Biosynthesis and Second Messenger Interactions: Implications in RBL-1 cells

Preliminary work carried out in this laboratory provided evidence for the existence of a control mechanism of Leukotriene biosynthesis involving neuropeptides. In this work it was shown that vasoactive intestinal polypeptide (VIP) was capable of inhibiting platelet activating factor (PAF) - stimulated peptido-Leukotriene biosynthesis (237). This finding was further substantiated and extended to a preliminary study of the mechanisms by which VIP exerted its inhibitory effect upon PAF-induced Leukotriene generation (238). This was done by using rat and guinea pig chopped lung preparations, and it was found that a variety of VIP related neuropeptides and calcitonin gene related peptide (CGRP) were capable of exerting this inhibitory effect (239). This negative (inhibitory) control was thought to be receptor mediated and may have occured by cAMP mediated processes. These investigations led to a model being proposed that VIP and other substances capable of activating adenyl cyclase might exert a broad bidirectional control on the actions of agonists such as PAF and bradykinin, which act through PI signalling pathway (240-242). This model is represented in Figure 15, and as can be seen, a number of secondary messengers are involved in this hypothetical model. The three enzyme-mediated signalling pathways implicated in this model: adenyl cyclase (AC), phospholipase A_2 (PLA_2) and phosphoinositide-specific phospholipase C (PIC), are of particular interest as these are involved in a large number of cellular responses.

To study this cAMP-mediated inhibitory process, as well as other possible secondary messenger interactions, it was thought necessary to use a much-less complex system than that of the chopped lung used extensively in previous investigations in this laboratory. In the work presented in this thesis, a

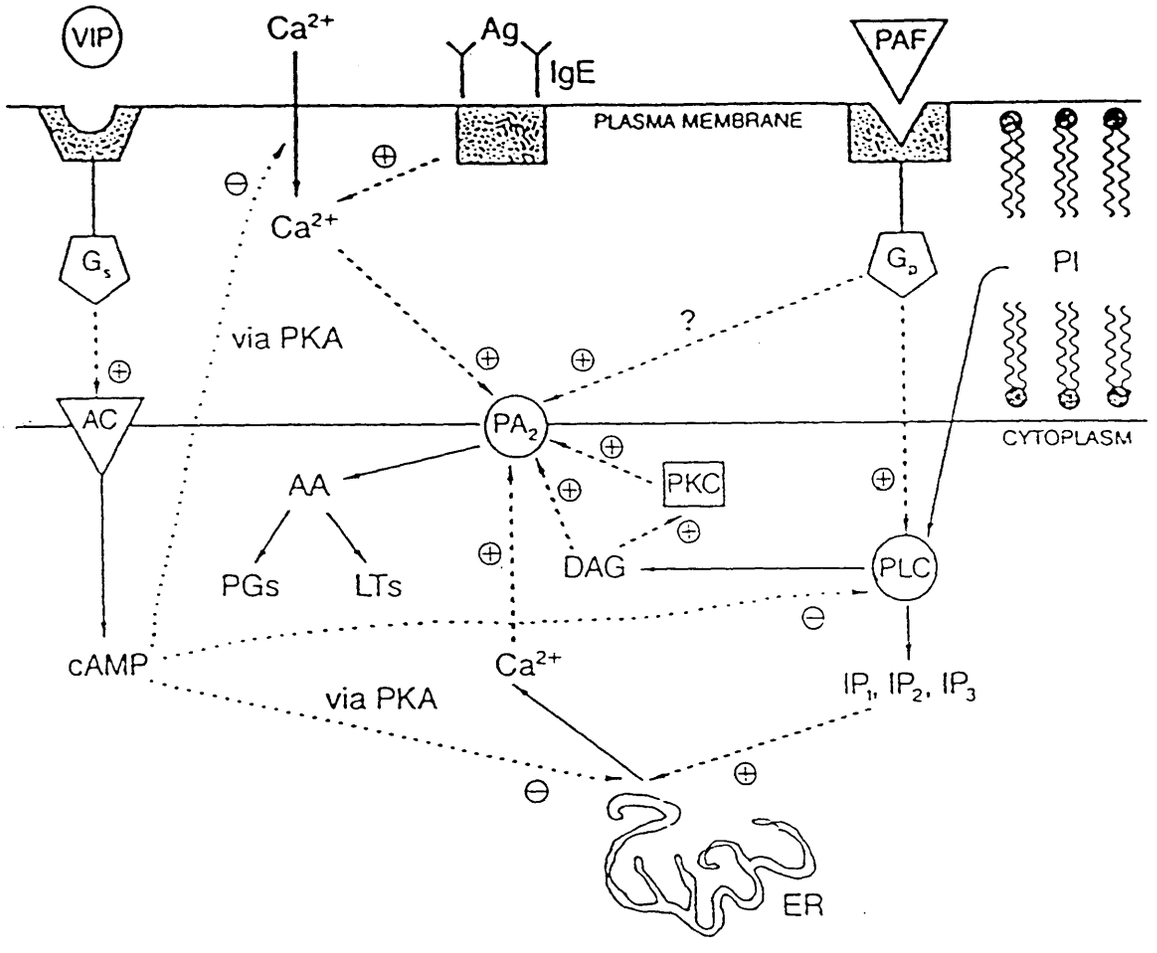


Figure 15: Proposed mechanism for the inhibition of Leukotriene (LT) biosynthesis by neuropeptides. Agonist-induced activation of adenyl cyclase (AC) via a specific GTP-binding protein (G_s), leads to increased intracellular cAMP levels. cAMP then inactivates phospholipase C (PLC), opposing the agonist-induced, G-protein-mediated production of inositol phosphates (IP_1, IP_2 and IP_3) and diacyl glycerol (DAG). This inhibits the putative activation by PLC and protein kinase C (PKC) of phospholipase A_2 (PA_2), the enzyme responsible for arachidonic acid (AA) liberation from membrane phospholipids. VIP, CGRP and some β-adrenoceptor agonists (terbutalin, salbutamol and fenoterol) inhibit IgG- and IgE-mediated Leukotriene biosynthesis, probably by counteracting calcium mobilization via protein kinase A (PKA), since the activity of PA_2 is strongly dependent on calcium. PI, phosphatidylinositol; PGs, prostaglandins.

rat basophilic leukaemic cell line (RBL-1) has been used as a model system, since from earlier studies on the structure elucidation of slow reacting substance of anaphylaxis (SRS-A) in this laboratory (24), it was demonstrated that these cells possess the necessary machinery for Leukotriene biosynthesis. This cell line was generated in rats by using the chemical carcinogen β-chlorethylamine and was subsequently established as the RBL-1 cell line (243). Furthermore, the RBL-1 cell line has been important for the physicochemical characterization of the IgE receptor, as well as, for studying the steps of the activation-secretion responses (244).

In this thesis, firstly, the effects of a number of inflammatory mediators were therefore studied in the RBL-1 cell line: PAF, and the peptides bradykinin and fMLP were chosen initially because they had been shown to stimulate peptido-Leukotriene (p-Leukotriene) generation in rat chopped lung preparations in our earlier work (246). fMLP has also been independently shown to lead to release of Leukotriene B_4 as well as p-Leukotrienes, following stimulation of reperfused rabbit hearts *in vitro* after myocardial infarction (245). It has also been found to induce arachidonic acid release and 5-Lipoxygenase metabolite formation in neutrophils (247). Bradykinin plays an important role in the generation of the inflammatory response. It has been found to activate phospholipase A_2 (PLA_2) and arachidonic acid release (163), and is amongst the most potent activators of prostaglandin release in a number of tissues (for a review see 248). PAF (1-O-alkyl-2-acetyl-sn glyceryl-3-phosphoryl choline) was first derived from rabbit basophils (249) and is formed by acetylation in the sn-2 position of lysophospholipids. It causes vasoconstriction and oedema in isolated perfused rat lungs accompanied by Leukotriene C_4 (LTC_4) and Leukotriene D_4 (LTD_4) formation, and this effect is blocked by diethylcarbamazine, an inhibitor of Leukotriene biosynthesis (250). Furthermore, it is known to promote Ca^{2+}-dependent platelet aggregation, serotonin secretion, thromboxane production (251) and has been shown to induce Leukotriene biosynthesis in neutrophils as well as lung tissue (252), Moreover, Motasim-Billah and Lapetina have shown that PAF stimulates PI breakdown. They showed that stimulation of horse platelets (pre-labelled with ^{32}P) with PAF for 5 seconds leads to a 50% loss of [^{32}P]-PIP_2 (253), and hence PAF was studied in the RBL-1 experiments described below.

Mellitin is a major constituent of bee venom and is a member of the class of amphiphilic venom peptides with five positively charged amino acids. It has been shown to trigger mast cells and cause histamine release as well as activating phospholipase A_2 (PLA_2) (254), and was thus also a candidate for

study here with respect to arachidonic acid release and p-Leukotriene biosynthesis. Arachidonic acid release and Leukotriene biosynthesis can also be mediated by antigen challenge *in vivo*. *In vitro* antigen challenge of RBL cells (2H3 subclone), involving pre-incubation of inflammatory cells in culture with IgE then challenge with antigen, has been recently reported to cause histamine and arachidonic acid release (255). These responses to antigen challenge have also been reported in mast cells and human basophils (256). Compound 48/80 is a mixed polymer of p-methoxy-N-methyl-phenylethylamine crosslinked by formaldehyde. It was first described as a histamine releaser in 1951 (257), and has been reported to mediate arachidonic acid release and prostaglandin biosynthesis (247), hence it was decided to test its effect on the RBL-1 cell line.

It has also been reported that fMLP receptors in polymorphonuclear leukocyte (PMNL) membranes are coupled to PI breakdown through a pertussis toxin-sensitive guanine nucleotide regulatory protein (G-protein) (258). Occupancy of this fMLP receptor leads to the rapid hydrolysis of PIP_2 in PMNL cells and in differentiated HL 60 cells (259).

Mantyh et al. (260) have suggested that substance P receptors are linked to PI turnover in rat neuronal tissues. Very recently, substance P has also been shown to induce IP_3 and inositol (1,3,4,5)-tetrakisphosphate (IP_4) formation in rat parotid acinar cells (261). Substance P meets many of the requirements for the mediation of the local inflammatory response. Apart from being widely distributed in primary sensory neurons, from where it is released during antidromic stimulation, it produces vasodilation and vascular permeability, endothelial adherence of PMNs and monocytes and degranulation of mast cells (262).

Histamine induces PI hydrolysis in the central nervous system (CNS), neuroblastoma and astrocytoma cells. There are three subtypes of histamine receptors, H_1, H_2 and H_3 and the PI response appears to be mediated through a H_1 receptor subtype. The stimulatory effect of histamine can be blocked by inclusion of pyrilamine, a H_1 antagonist, whereas the H_2 antagonist cimetidine is ineffective (263). Furthermore, the magnitude of histamine-stimulated PI turnover mirrors that of the density of H_1 receptors on membranes (264,265). Histamine has a number of actions mediated via the H_1, H_2 and H_3 receptor subtypes, such as, smooth muscle contraction, increased vascular permeability, neural reflexes and mucus secretion, and its sources are mast cells and basophil granules (266).

Therefore, the effect of these agonists upon the phosphatidylinositol pathway was also studied.

Evidence derived from several different secretory systems confirms the belief that a bioamplification mechanism involving PIC and PLA_2 interactions exists. The fact that separate pathways appear to be involved in the activation of PIC and PLA_2 does not obviate the possibility that the actions of these two enzyme systems are somehow linked. Arachidonic acid and its metabolites are capable of stimulating PIC activation and PI breakdown, IP release, increasing Ca^{2+} availability, and promoting secretion in various tissues (267). Arachidonic acid and/or its metabolites may therefore act as feedback modulators of PIC.

During the course of this study, answers were sought to the following questions:

1) Does cAMP play any modulatory role on Leukotriene generation and PI breakdown?
2) Does PLA_2 activation lead to PIC activation and PI breakdown?
3) Does PIC stimulation induce PLA_2 activation and Leukotriene generation?

These questions were approached by different experimental strategies including:

a) Measurement of Leukotrienes by high sensitivity radioimmunoassay,
b) The use of labelled arachidonic acid precursor
and c) The use of labelled Inositol precursor.

2 EXPERIMENTAL

2.1 Outline of Experimental Procedure

During the course of this project the effect of various inflammatory mediators on Leukotriene generation and inositol phosphate (IP) formation has been studied. The effect of cAMP analogues on Leukotriene generation and IP formation were also investigated. Figures 16-18 illustrate the basic experimental flow diagram; a more concise explanation of experimental methodology will be given under appropriate headings.

2.2 Cell Culture of RBL-1 Cells

The RBL-1 cell line used in this study was initially purchased from Flow Laboratories, UK. Cells were then grown in suspension cultures from which a frozen stock was set up and every 2-3 months a new batch of cells was thawed and used for the continuous culture that our work required.

RBL-1 cells were grown as 500-1000 ml spinner cultures in Minimum Essential Medium with Earls' salts (EMEM) supplemented with 2mM L-glutamine, 6μg gentamycin (or 10000 units of penicillin/streptomycin solution) and 10% foetal calf serum. The cells grow well when supplemented with 10% foetal calf serum; 5% foetal calf serum was tried but cell viability was not as good as when 10% foetal calf serum was used. RBL-1 cells can be grown in suspension or as a monolayer. Always two flasks of stock were kept in culture to act as back-up in case of spinner fault or infection. The cells were allowed to go through a prolonged stationary phase and were fed near the time that they reach their critical density, unless otherwise stated. Refeeding was carried out by spinning down (at about 1500 rpm) and resuspending in fresh growth medium at approximately $3x10^5$ cells per ml. The cells go through a lag phase of about 12 hours (no change in cell number), then multiply rapidly for 24-48 hours, reaching a critical density of $1.5\text{-}2x10^6$ cells/ml. They can be maintained at this density with 80% viability up to 72 hours.

In flasks the cell viability remains at about 75% and the cell density at approximately $5x10^5\text{-}1x10^6$ cells/ml. They never reach a true stationary phase i.e. a state where most (80-90%) of cells in the culture are in the G-1 phase of growth. The cultures were examined twice daily.

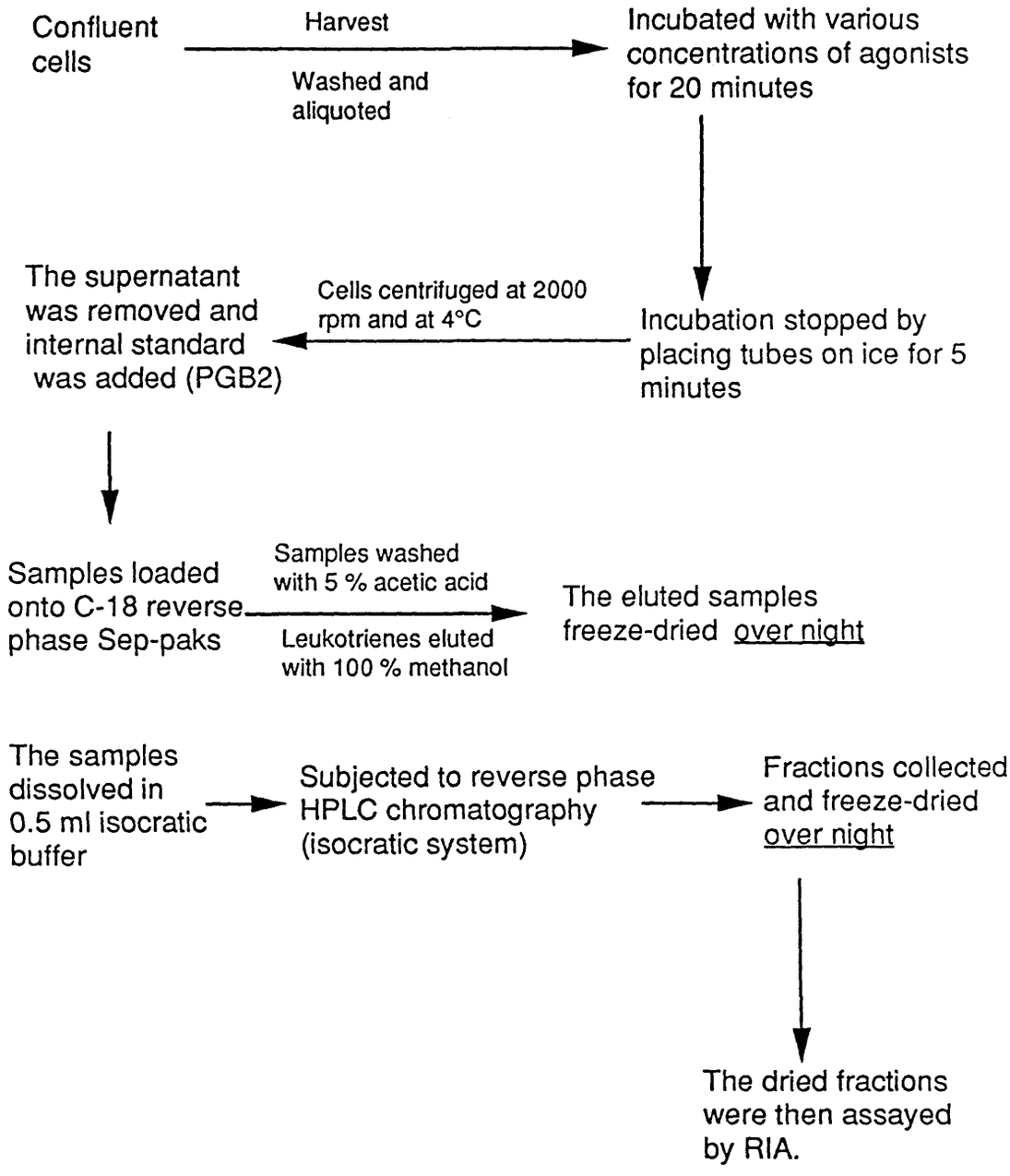


Figure 16: An outline of the experimental procedure used for peptido-Leukotriene measurement.

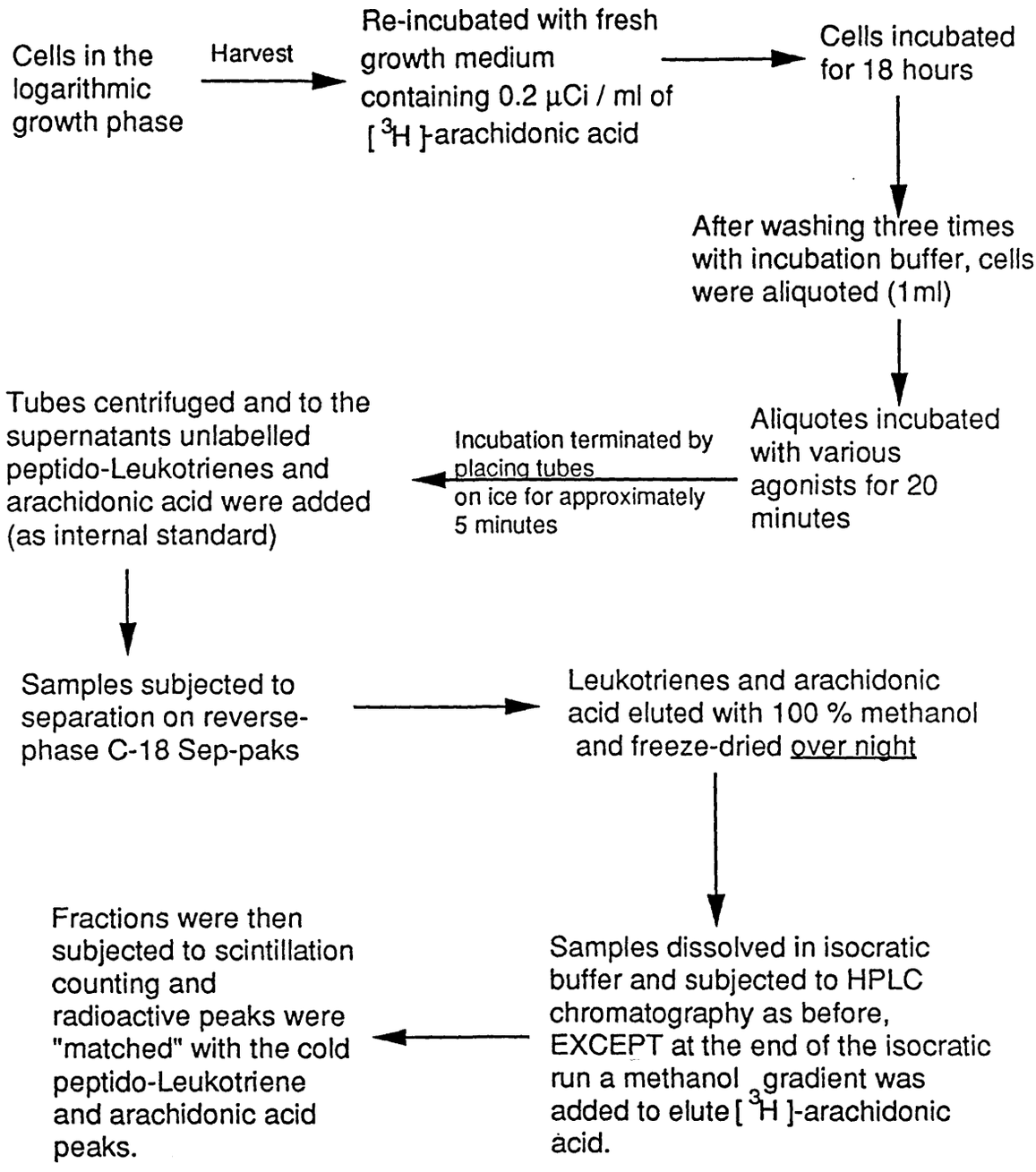


Figure 17: An outline of the experimental procedure used for the measurement of labelled arachidonic acid and Leukotrienes.

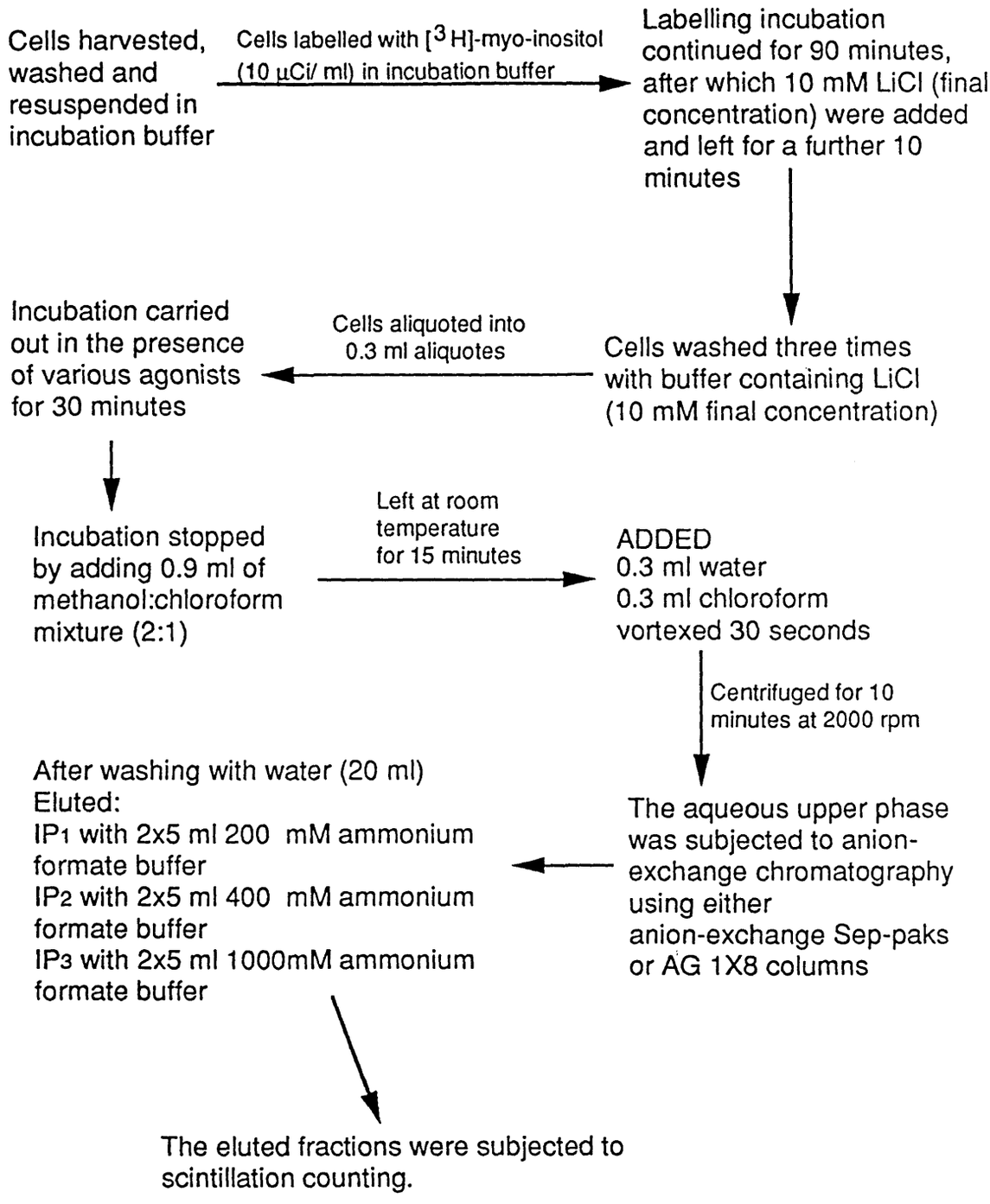


Figure 18: An outline of the experimental procedure used in inositol polyphosphate measurements.

2.3 Leukotriene Separation and Purification

SRS-A purification was originally based on ethanol precipitation of contaminating proteins from perfusates (3) or lyophilisation of the perfusate followed by extraction with 80% ethanol and finally ether extraction (11). By the early 1970's, Orange *et al.* (13), introduced a chromatographic step with Amberlite XAD resin after ethanol extraction of SRS-A to remove more polar contaminants such as histamine. Towards the end of the 1970's Morris *et al.* (268) reported another extraction procedure consisting of charcoal adsorption, elution with 80% ethanol followed by gel filtration on Sephadex G-15, and reverse phase HPLC on ODS (octadecyl silica) columns.

These methods of extractions were long and tedious and no valid and rapid alternative existed until the 1980's. In 1980 Powel W.S. (269) reported the use of either ODS prepared cartridges or silica in prostaglandin extraction and in 1980 Morris *et al.* (24,94), published the use of Sep-paks in SRS-A extraction.

2.3.1 Sep-Pak Extraction of Leukotrienes

In this study C-18 Sep-paks were used, rather than silica Sep-paks, so that the incubation fluids could be loaded directly onto the cartridges (silica Sep-paks require the samples to be in an organic hydrophobic solvent). In contrast to the previously reported data (270), recoveries of [^{3}H]-LTD_4 (NEN., UK., 39 Ci/mmol) added to incubation buffer were found to be consistently greater than 85% when methanol was used as the eluant.

2.3.2 Protocol for C-18 Sep-Pak Extraction

The C-18 Sep-paks were first primed using 3ml of HPLC grade methanol and then equilibrated with 5ml of 5% acetic acid (Analar). The incubation supernatants, to which 100µg of the internal standard prostaglandin B_2 (PGB_2) and approximately 100 cpm of [^{3}H]-Leukotrienes (LTC_4, D_4, E_4) were added, were carefully loaded onto their respective cartridge (one at a time) from a plastic syringe making sure no air was pushed through the cartridge at any stage of the extraction. Each Sep-pak was then washed with 5ml of 5% acetic acid, so as to remove salts and polar substances. The Leukotrienes were then eluted with 3ml of methanol. The

eluates were collected in 10ml plastic tubes, which were kept on ice, and lyophilised under vacuum.

2.3.3 HPLC Separation of Leukotrienes

The use of high performance liquid chromatography (HPLC) in peptide separations was introduced due to advantages it had over traditional chromatographic methods, some of which include:

a) High degree of resolution due to enhanced number of theoretical plates per unit length of column,
b) Large number of applications with the ability to use normal phase, reverse phase, ion-exchange and gel filtration columns,
c) Rapidity of the running time.

The application of HPLC to the purification of SRS-A was introduced for the first time in 1978, when Morris *et al.* published its use in the first absolute purification of SRS-A, in what was to be a decisive step in the characterization of the structure of SRS-A (20). Both from this laboratory and from other laboratories different protocols were published including various solvent systems and gradient or isocratic elutions (25,268,270,271).

Prior to the current study an isocratic HPLC protocol had been set up in this laboratory that was rapid enough to run up to 12 samples a day and have a reasonable separation of the peptido-Leukotrienes (p-Leukotrienes). This involved the use of 3µm ODS Nucleosil column (4.6mm x 15cm) which due to the smaller diameter of the ODS particles and hence a high number of theoretical plates/unit length of column, would provide better resolution/duration of analysis ratio compared to other columns. The mobile phase consisted of CH_3OH : H_2O : CH_3COOH : NH_4OH (67 : 33 : 0.07 : 0.1, pH 6.2). The drawback with this system was that the separation of Leukotriene D_4 (LTD_4) from LTB_4 was quite poor, as shown in Figure 19(a). Therefore in the work presented here an attempt was made to enhance the separation between LTD_4/LTB_4 and Leukotriene E_4 (LTE_4). The most logical approach would have been the use of a gradient elution which not only gives a better separation but also better reproducibility of the runs. However, in an isocratic system the retention times with respect to the internal standard are reproducible and the rapidity of the runs made it still more favourable than the gradient elution method. Having decided to use an isocratic elution, an attempt was made to enhance the separation between LTD_4 and LTB_4 by

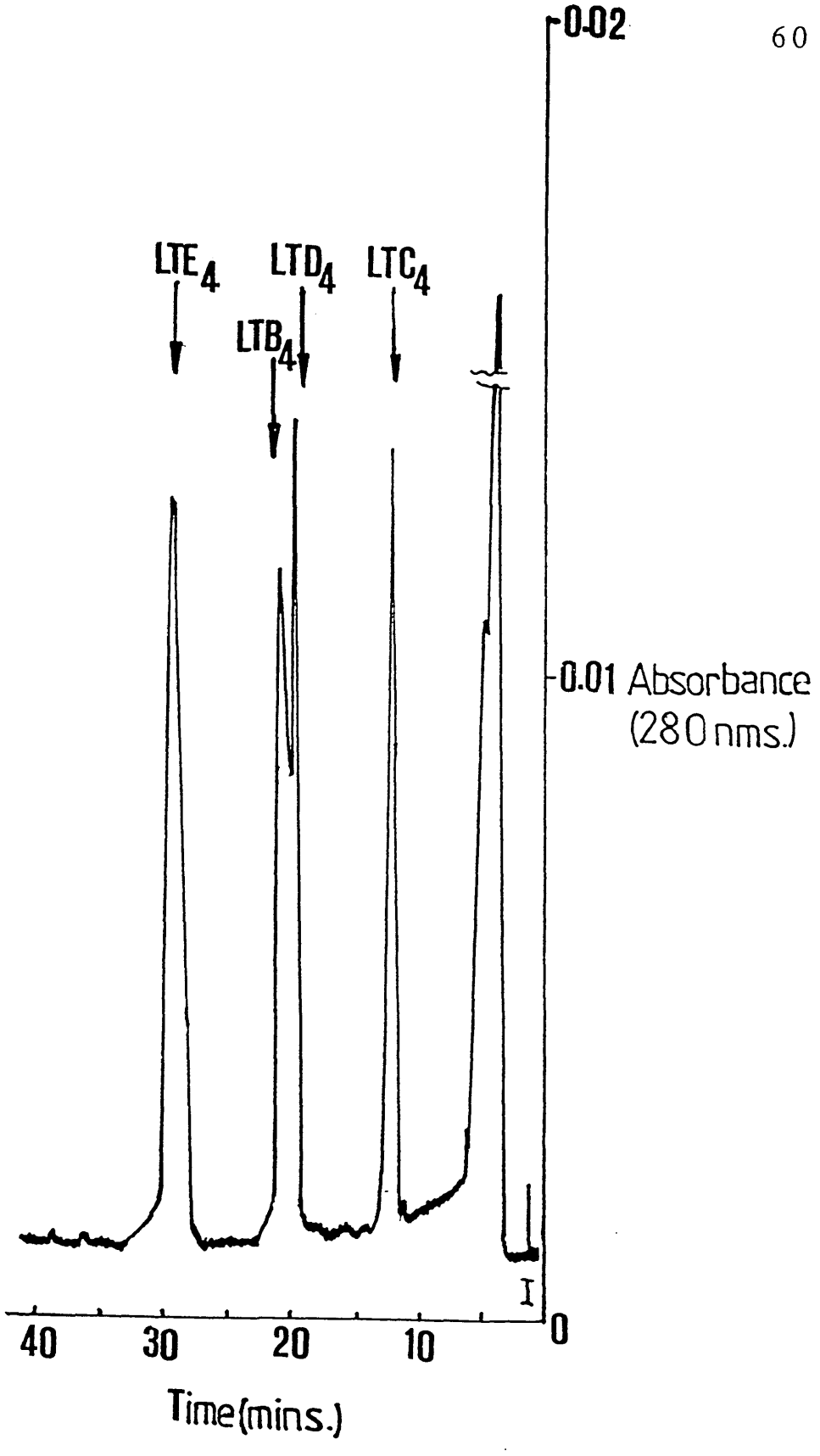


Figure 19(a): Absorbance (280 nm) profile of standard peptido-Leukotrienes using the 3µm (4.6mm x 15cm) Nucleosil column. As can be seen LTB_4 and LTD_4 separations are poor. I, injection.

using different solvent ratios and different pH adjustments as well as different column temperatures but no satisfactory improvement in the separation was established. Then, it was decided to use a 5μm Nucleosil column with the dimensions of 4.6mm x 25cm. It was found that at a flow rate of 0.7 ml/min and using the same mobile phase as before, we were able to achieve a much better separation between LTD_4 and LTB_4 as well as LTE_4, as shown in Figure. 19(b), and the whole run could be completed in 40 minutes; the method was modified by the addition of 100 cpm of labelled peptido-Leukotrienes (p-Leukotrienes) so that those fractions corresponding to the elution of radioactivity would be taken for quantification with RIA (see Section 2.3.6). Figure 19(c) illustrates a typical HPLC profile when RBL-1 cells were incubated with agonist.

During the preparation of this thesis a new Nucleosil ODS column (3μm, 4.6mm x 25cm) became available which had the advantage of having a greater number of theoretical plates/unit length than the 5μm, 4.6mm x 25cm ODS column, and therefore even a better resolution of the two peaks than with the 5μm column. However, the separation was not improved greatly and therefore it was decided to use the 5μm ODS (4.6mm x 25cm) column. It should be added that the 3μm column was extremely good in the separation of the hydrophobic compounds which were eluted with pure methanol and hence would be of immense value in the separation of more hydrophobic compounds than p-Leukotrienes. The HPLC system consisted of: Kontron liquid chromatography pump model 410 and a UV. detector (Waters Ass., model 480) monitoring at 280nm (unless otherwise stated). The average recoveries of p-Leukotrienes using this HPLC condition was determined by the use of [^{3}H]-LTD_4 elution and was found to be greater than 90%.

2.3.4 Protocol for HPLC Separation of Leukotrienes

The lyophilised samples that were eluted from C-18 Sep-paks were dissolved in 0.5ml of isocratic solvent, centrifuged and injected onto the column (flow rate was 0.7 ml/min). Fractions were collected and half were kept at -20°C. To the other half 4.5 ml of Aquassure scintillation fluid was added and counted on a Kontron scintillation counter. Those fractions corresponding to peaks of radioactivity were taken from the -20°C freezer and lyophilised ready for RIA.

In the study where RBL-1 cells were labelled with [^{3}H]-arachidonic acid, the Sep-pak extraction and HPLC was as described above except that, prior to

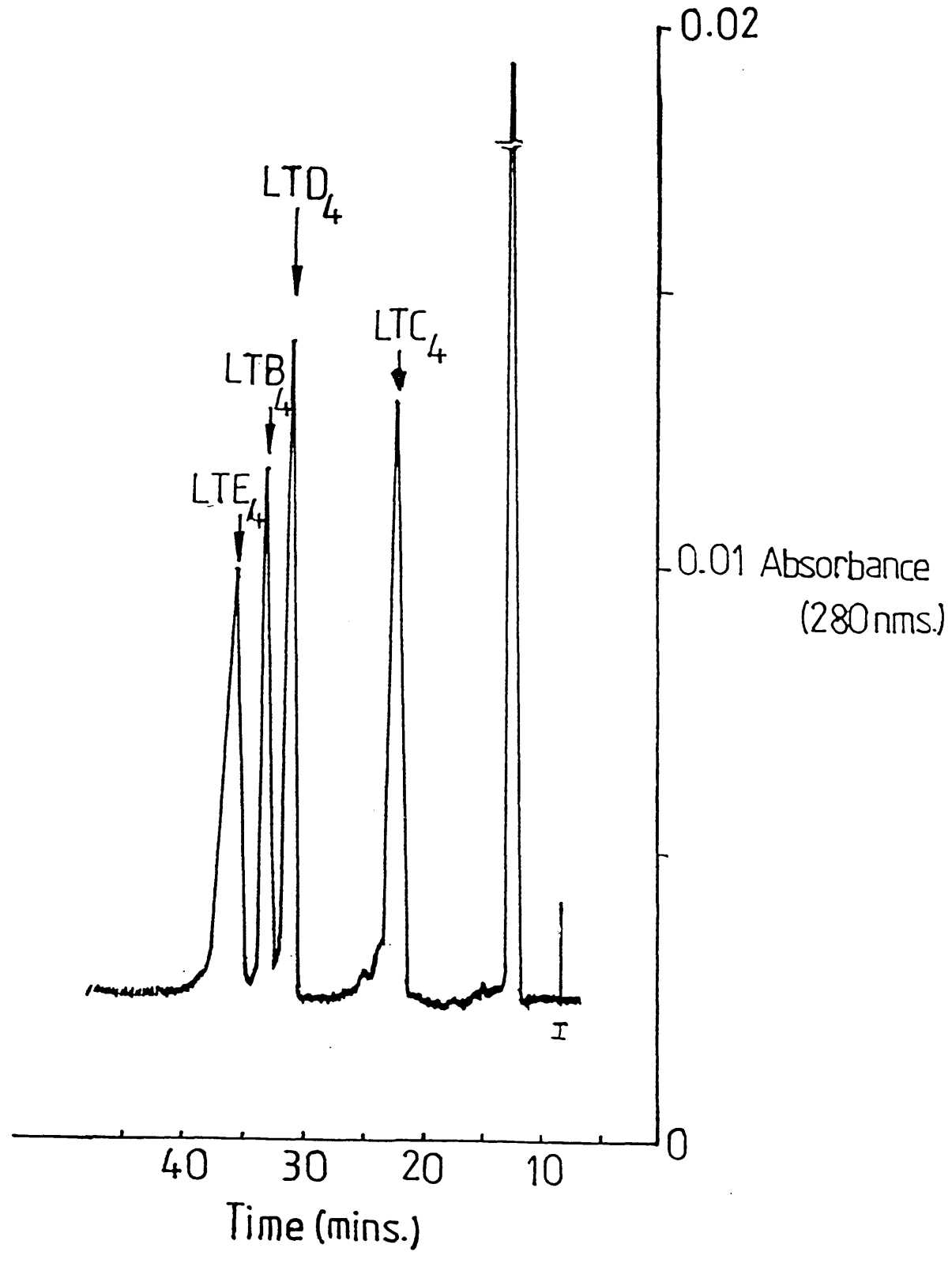


Figure 19(b): The absorbance (280) profile of standard peptido-Leukotrienes using the 5µm (4.6mm x 25cm) Nucleosil column. Note the highly improved separation between LTB_4 and LTD_4

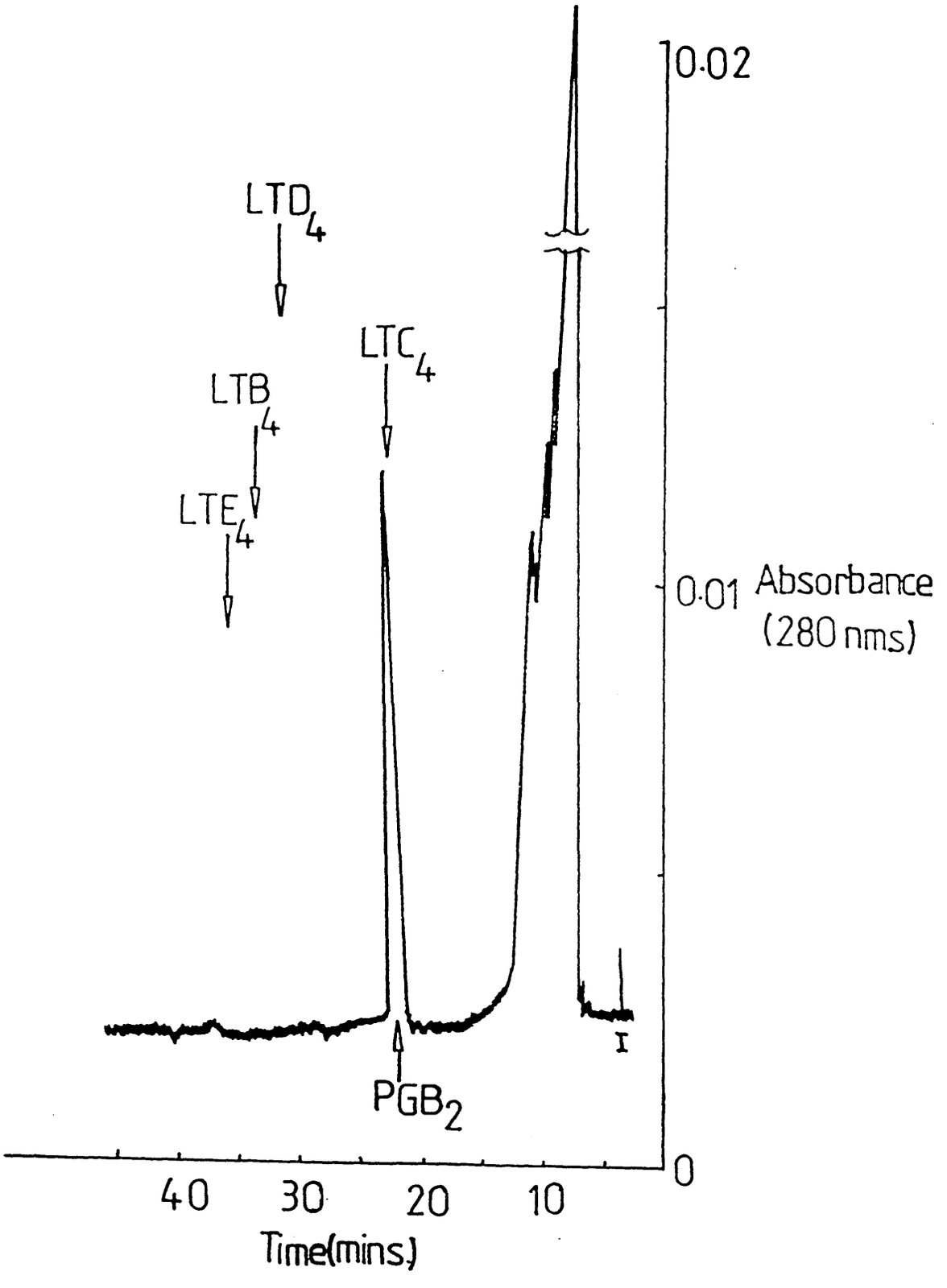


Figure 19(c): A typical HPLC profile obtained when RBL-1 cells are incubated with agonist. Arrows show the retention times of standard peptido-Leukotrienes.

Sep-pak extraction, unlabelled arachidonic acid and Leukotrienes were added to the supernatants, and at the end of the HPLC run a methanol wash gradient was added to elute arachidonic acid monitoring the absorbance at 205nm. Scintillation fluid (Aquassure, NEN.), 4.5ml, was added to the collected fractions, which were then subjected to scintillation counting (all the fractions were counted).

Blank runs were carried out prior to injection of samples, to curtail the column "memory" effect whereby some of the Leukotrienes are left on the column and not eluted. This was particularly evident when calcium ionophore, A23187, was used to stimulate Leukotriene generation and large amounts of Leukotrienes were produced.

2.3.5 Radioimmunoassay of Leukotrienes

The amounts (except with Ca^{2+} ionophore A23187) of Leukotrienes in the samples were so small that a uv trace could not be observed. A more accurate way of measuring Leukotriene biosynthesis than by uv detection was therefore required. The classical method used for quantification of SRS-A was the use of guinea pig ileum bioassay. However, the drawbacks to this classical method are:

a) The low detectability (10pmol at best) and
b) The lack of sensitivity to LTC_4 and LTE_4 (30).

In the present study the amounts of Leukotrienes generated (except with A23187) were too low for detection by guinea pig ileum bioassay. By far the more sensitive method is RIA. In this laboratory antisera were raised against thyroglobulin-conjugated LTD_4 (273). The resulting RIA was more sensitive than those previously reported (334). The preparation of the immunogen and the immunization of the animals, as well as the development of the assay protocol, have been reported previously (273).

The antiserum was re-characterized for the present study, and it was found to cross react with LTC_4, LTE_4 and LTB_4 (60%, 40% and 6% respectively) as shown in Figure 20 (a-c), but not with PGB_2, PGE_2 or glutathione. The detection limit for the antiserum was <10fmols and the detection at 50% binding was 100fmols of LTD_4.

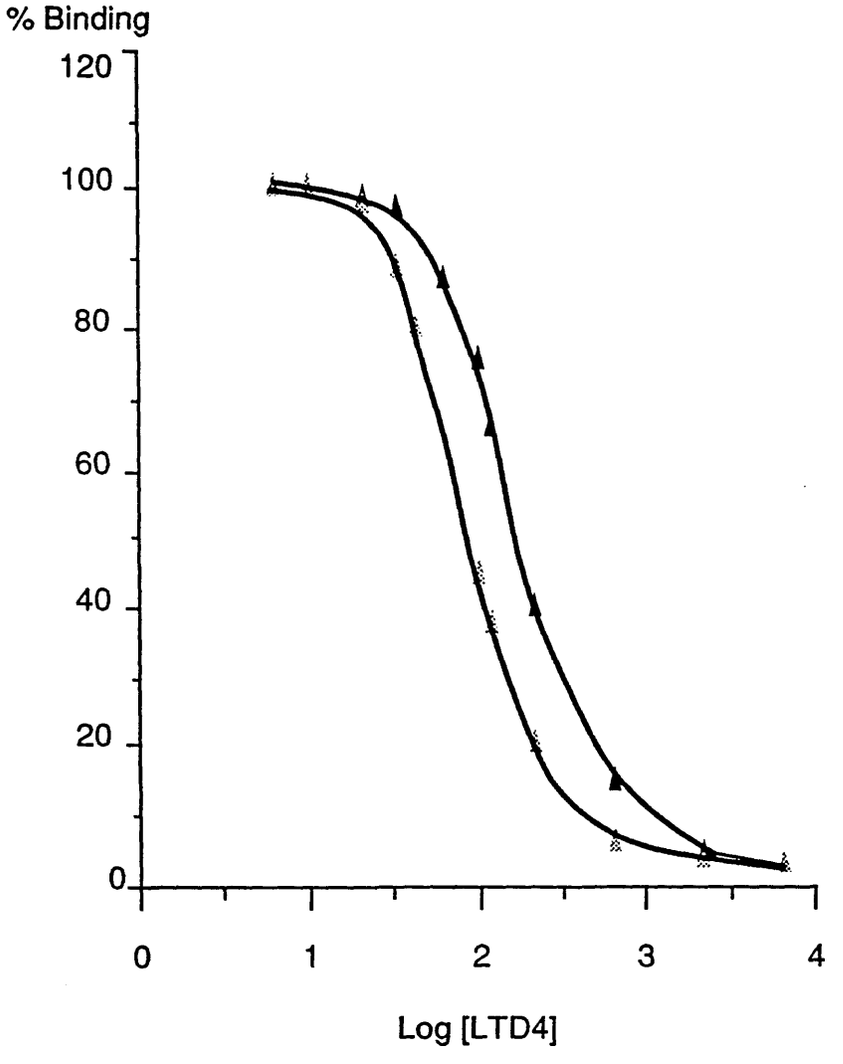


Figure 20(a): Binding curves for LTD4 and LTC4 obtained using the antiserum "Emma".

% LTC4 Binding ▲ % LTD4 Binding ▲

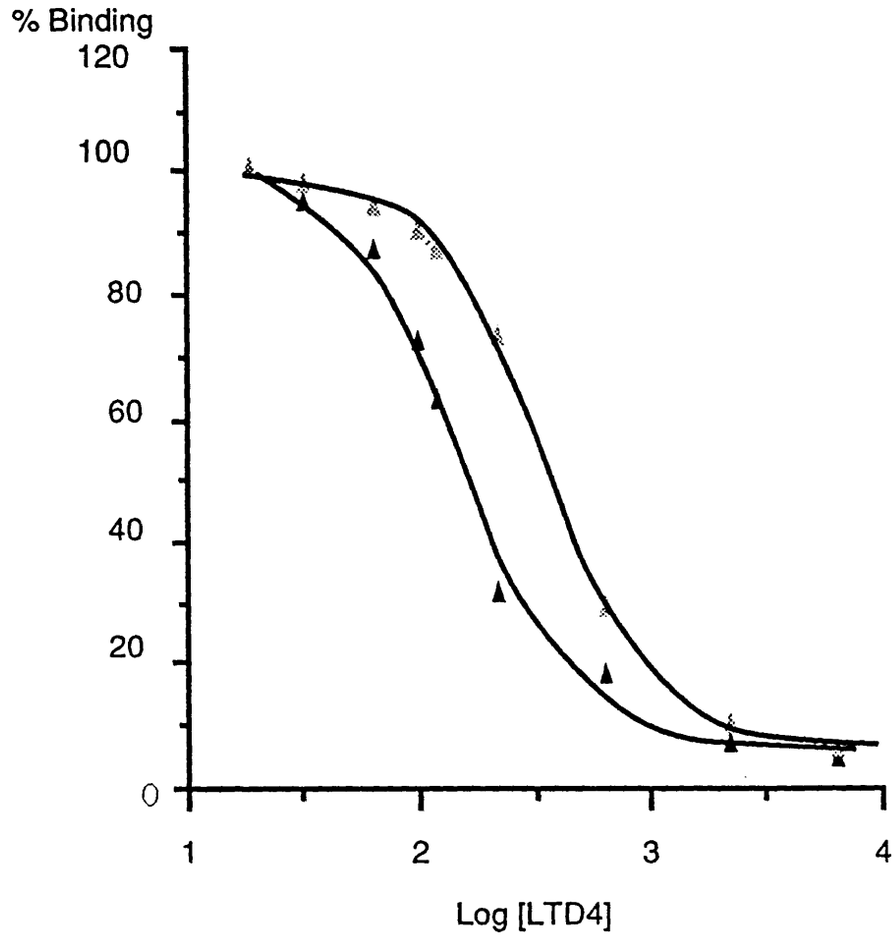


Figure 20(b): Binding curves for LTD4 and LTE4 obtained using the antiserum "Emma".
% LTD4 Binding ▲
% LTE4 Binding

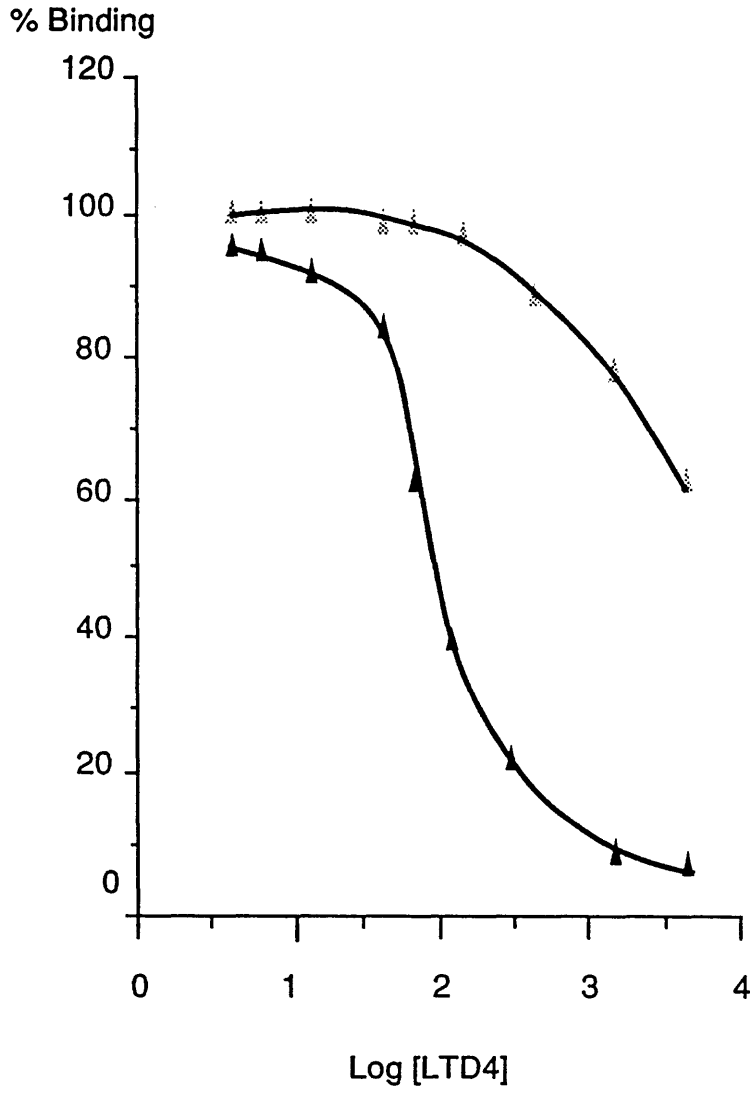


Figure 20(c): Binding curves for LTD4 and LTB4 obtained using the antiserum "Emma".

% LTB4 Binding

% LTD4 Binding

2.3.6 Protocol for Radioimmunoassay of Leukotrienes

The standard curve was established with serial dilutions of synthetic LTD_4, taking 50µl of each dilution into plastic RIA tubes (in triplicate), to give final concentrations in the range of 5-7500fmols in assay buffer (phosphate buffered saline containing 0.25% gelatin, pH 7.4). The lyophilised HPLC fractions were dissolved in 50µl of assay buffer. The antiserum was diluted to $1.2x10^3$ in assay buffer and 50µl were added to the samples and standards. The tubes were then mixed and allowed to pre-incubate for 25 minutes at room temperature. The label, [^{3}H]-LTD_4 (NEN., UK, 6000 dpm, 75fmols), in 50µl buffer was added to all the tubes and left for 90 minutes to incubate at room temperature. The unbound label was then precipitated by adding 0.5ml of charcoal mixture (1g Norit. X charcoal (BDH., UK)) and 0.1g of dextran (Sigma, UK, in 250ml of assay buffer kept at 4°C) and immediately centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatants were then transferred to scintillation vials and 4.5ml of scintillation liquid added. The samples were counted on a Kontron scintillation counter (efficiency of 40%). The amounts of p-Leukotrienes were then determined from the binding curve of LTD_4 and from cross reactivity factors described above.

Figure 21 illustrates the effect of the addition of approximately 100 cpm of [^{3}H]-LTD_4 to the standard tubes (to compare the effect of adding 100 cpm to the sample tubes prior to Sep-pak and HPLC) and as can be seen only a small effect was observed with respect to the standard curve without the addition of 100 cpm. In every standard curve 100 cpm were then added to overcome the effect of 100 cpm in the sample tubes.

2.3.7 Determination of Leukotriene Generation

The confluent cells were harvested and washed twice, by centrifugation at 1500 rpm for 5 minutes at 4°C, with the incubation buffer (containing: NaCl 150mM, KCl 3.7mM, Na_2HPO_4 3mM, KH_2PO_4 3.5mM, $CaCl_2$ 0.9mM, D-glucose 5.6mM, 0.1% BSA and 10000 units/l of Heparin, pH 7.0). The pellet was then resuspended in buffer to give a final concentration of 0.5-$1.0x10^7$cells/ml, and divided into 3ml aliquots. The tubes were then left to equilibrate for 15 minutes at 37°C after which the agonist and/or cAMP analogue was added. The incubation was continued for 20 minutes after which the cells were put on ice. The tubes were then centrifuged at 1500 rpm for 10 minutes at 4°C and the supernatants transferred into fresh tubes. The

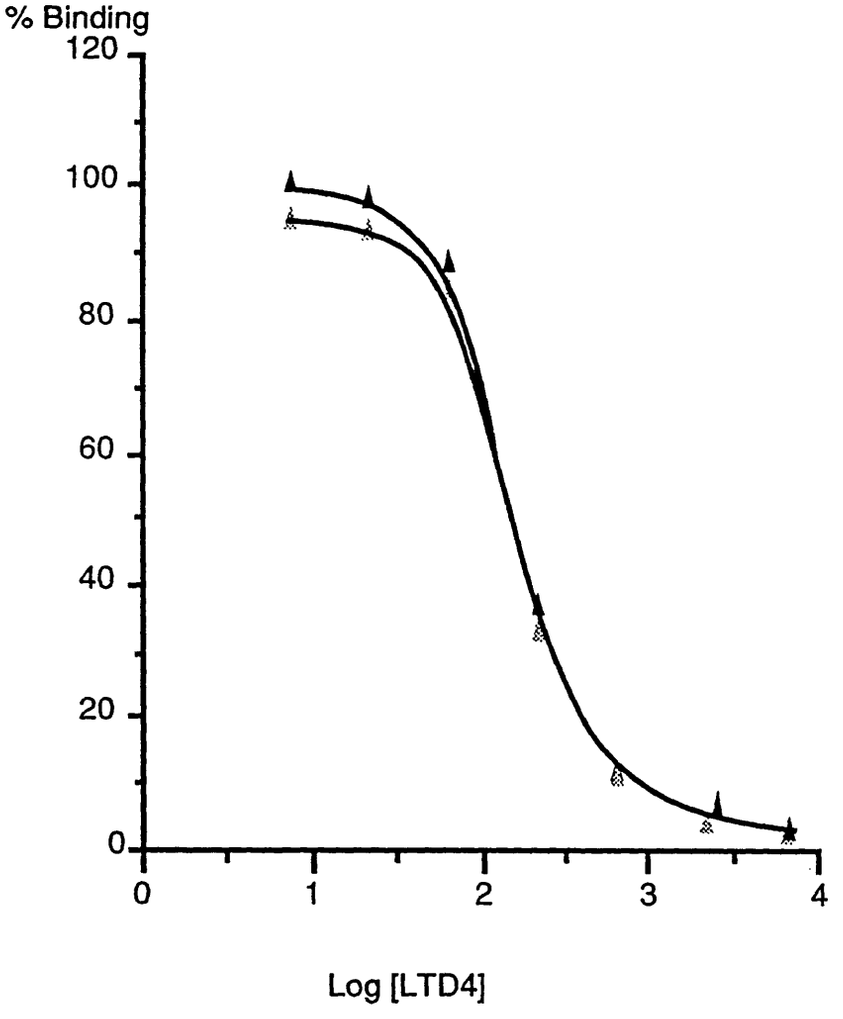


Figure 21: LTD4 binding curves in the presence and absence of 100 cpm [^{3}H]-LTD4.

▲ % LTD4 Binding plus 100 cpm LTD4

▲ % LTD4 Binding

internal standard, PGB_2, and 100 cpm of labelled p-Leukotrienes was added to the samples, which were then subjected to Sep-pak extraction and HPLC followed by RIA (as described above).

2.3.8 Determination of Labelled Arachidonic Acid Release and Leukotriene Generation

The cells were harvested after 48 hours in culture (in the logarithmic phase of growth) by centrifugation (as described above). The pellet was resuspended in fresh growth medium containing 0.2μCi/ml of [^{3}H]-arachidonic acid (NEN., UK) and transferred to a large (175cm^2) tissue culture flask and incubated for 16 hours. The cells were then harvested and washed three times in the incubation buffer (as described above). The pellet was then resuspended to give a final concentration of 0.5-1.0x10^{7}cells/ml, and divided into 1ml aliquots. The tubes were left to equilibrate for 15 minutes at 37°C before starting the incubation. The agonists and/or cAMP analogue were added and the incubation was continued for 20 minutes after which the samples were placed on ice and then centrifuged (as described above). The supernatants were transferred to fresh tubes to which standard Leukotrienes and arachidonic acid were added. The samples were then Sep-paked and lyophilised under vacuum ready for HPLC analysis (as described above).

2.4 Separation of Inositol Phosphates

Inositol polyphosphates were initially separated by the use of Dowex anion-exchange chromatography (274). This involved the mixing of Dowex-1 resin (100-200 mesh, Cl^- form) with 2M NaOH to get to the OH^- form, then with 1M formic acid to convert the OH^- form to the formate form. Then 0.5ml of a 50% (wt/vol) slurry of Dowex-1 was poured into Pasteur pipettes, plugged with glass wool, and equilibrated with distilled water to get the pH near to neutrality. Then the aqueous sample was applied and elution carried out under gravity with different concentrations of ammonium formate buffer to elute the inositol polyphosphates. This method was very slow and tedious, therefore very few samples could be handled at a time. This cumbersome technique was eliminated with the introduction of HPLC to separate inositol phosphates (IPs) (275). This method consisted of a Partisil 10-SAX column and a gradient elution using ammonium formate/0.7M phosphate buffer. Although using HPLC to separate IPs is more reproducible and the various isomers can be

separated (276), it has the drawback of requiring long running times and long periods for the re-equilibration of the column, therefore reducing the number of samples that were possible to analyse at a time. In our work we wanted to study IP turnover qualitatively without the need to separate the various isomers and cyclic-IPs, and process as many samples at a time as possible. Therefore, we decided to try Sep-pak anion-exchange cartridges (Accell QMA, Waters Assc.) which would facilitate rapid and qualitative separation of IPs. From our preliminary work we found a recovery of more than 85% and a reasonable separation of IPs using different concentrations of ammonium formate buffer.

2.4.1 Protocol for Anion-Exchange Sep-Pak of Inositol Phosphates

The Sep-paks were first primed using 3ml of methanol and then washed with 10ml of deionised water. Each sample was loaded onto a Sep-pak and allowed to elute under gravity. Then it was washed with 20ml of deionised water after which IP_1 was eluted with two 5ml washes using 0.2M ammonium formate/0.1M formic acid. IP_2 was then eluted with two 5ml washes with 0.4M ammonium formate/0.1M formic acid. Finally IP_3 was eluted with two 5ml washes with 1M ammonium formate/0.1M formic acid (Under these conditions it is expected that all isomers of IPs would be eluted). To each eluate 15ml of Aquassure scintillation fluid (NEN, UK) was added and after mixing were counted on a Kontron scintillation counter with an efficiency of 40%.

During the preparation of this thesis pre-packed columns of AG1-X8 200-400 mesh (strongly basic anion exchanger with quaternary ammonium functional groups attached to the styrene divinylbenzene copolymer lattice, BioRad, UK) resin already in the formate form, became available, and after modification to enhance elution times, hence increase rapidity without loss of yields or separation power, was used for the remainder of the study. The protocol was the same as that for Sep-pak cartridges except that no methanol was used; instead priming included deionised water. Elutions with standard [^{3}H]-IPs showed extremely good recoveries (greater than 90%) and more reproducibility than that achieved with Sep-pak cartridges (Accell QMA. Waters Assc).

2.4.2 Determination of Inositol Phosphate Formation

Confluent cells were harvested and washed twice (as described before) with incubation buffer (containing: NaCl 118mM, KCl 4.6mM, $MgSO_4$ $7H_2O$ 1.1mM, $NaHCO_3$ 24mM, KH_2PO_4 1mM, D-glucose 11.1mM, HEPES 5mM and 0.1% BSA pH 7.4), reported previously by Sarau *et al.* (277). The pellet was resuspended in incubation buffer containing 10μCi/ml of [^{3}H]-myo-inositol and transferred to a 2mm petri dish and left to incubate at 37°C in a CO_2 incubator for 90 minutes after which 10mM LiCl was added and left for a further 10 minutes The cells were then centrifuged, washed three times to remove excess free label and finally the pellet was resuspended in the incubation buffer containing 10mM LiCl to give a final concentration of 0.5-1.0x10^{8}cells/ml, and divided into 300μl aliquots and left to equilibrate for 15 minutes at 37°C before incubation with agonists. To study the effect of cAMP on inositol phosphate (IP) accumulation, cAMP analogues were added. In all cases the incubation with agonists was continued for 30 minutes (unless otherwise stated) after which the stimulation was stopped by adding 900μl of methanol/chloroform mixture (1:2) and leaving the mixture at room temperature for 15 minutes. Finally 300μl of chloroform and 300μl of deionised water were added, the mixtures were vortexed for 30 seconds and the two phases were separated by centrifugation at 3000 rpm. The upper aqueous phase was then loaded on to anion-exchange Sep-paks or AG1-X8 columns (as described above).

2.5 Preparation of Membrane-Enriched Fraction

RBL-1 cells were harvested by centrifugation at 2000 rpm for 10 minutes at 4°C. Cells were washed twice with 50mM sodium phosphate buffer (pH 7.0) containing 1mM EDTA and 0.1% gelatin (buffer A). Cells were resuspended in buffer A at 2x10^7 cells/ml and disrupted by nitrogen cavitation with a Par bomb at 750 p.s.i. for 10 minutes at 0°C. The broken cell preparation was centrifuged at 1000 x g for 10 minutes at 0°C. The supernatant was separated and centrifuged at 100000 x g for 60 minutes. The high speed pellet was resuspended in 10mM Tris-HCl (pH 7.5 at room temperature; buffer B) containing 10% (w/v) sucrose. The suspension (15ml) was carefully layered onto 15ml of buffer B containing 40% (w/v) sucrose in nitrocellulose centrifuge tubes. The tubes were centrifuged at 100000 x g for 60 minutes in an AW-27 swinging bucket rotor. Membranes that settled at the

boundary layer were collected and diluted with 5 volumes of 50mM Tris-HCl (pH 7.5 at 25°C; buffer C) and centrifuged at 50000 x g for 30 minutes. Pellets were washed by resuspension and centrifugation in equal volume of buffer C. The membrane suspension was rapidly frozen in liquid nitrogen and stored at -80°C.

2.5.1 Radioligand Binding Assay

[^{3}H]-LTD_4 binding assays were performed in a total volume of 200µl in tissue culture minisorb tubes. Samples contained (unless otherwise stated), buffer C containing 10mM $MgCl_2$, 10mM $CaCl_2$, and [^{3}H]-LTD_4 (1nM) in the presence or absence of 0.1µM unlabelled LTD_4. Equilibrium of [^{3}H]-LTD_4 binding was reached with incubation for 40 minutes at room temperature. The free and bound ligand were separated after diluting samples with 5ml of buffer D (25mM Tris-HCl buffer pH 7.5 at 25°C) at 0°C and rapidly filtered through Whatman GF/C filters. The filters were rapidly washed with 15ml of buffer D and then placed in scintillation vials with 10ml Aquassure (NEN) and the radioactivity determined by scintillation counting. All assays were carried out in triplicates and binding data was presented as specific [^{3}H]-LTD_4 binding which was defined as the difference between the binding in the presence and absence of 0.1µM unlabelled LTD_4. At 0.1nM and 1nM [^{3}H]-LTD_4 concentration the specific binding accounted for approximately 70% of the total binding.

2.6 Statistical Analysis

All experiments were conducted in triplicates at least, and results are reported as means ± s.e of the means. The logarithms of the data (reported as % control) were compared using unpaired students' t test and with a level of significance corresponding to $p<0.05$, or as otherwise stated.

3 RESULTS

3.1 Effect of Ionophore and Inflammatory Mediators on Leukotriene Biosynthesis

3.1.1 *Introduction*

A number of inflammatory mediators have been reported to induce Leukotriene biosynthesis in various physiological systems, for example perfused lungs. Voelkel et al. (250) utilized guinea pig ileum, the classical tissue used for the bioassay of SRS-A, in order to measure Leukotriene biosynthesis. However, in this type of study it is only possible to correlate the agonist-induced guinea pig ileum contraction to total p-Leukotriene biosynthesis (even when utilizing a specific antagonist such as FPL-55712), and at its most sensitive, could detect 10 picomoles of total Leukotrienes. A better method of detection of p-Leukotriene generation is to attempt measurement of individual p-Leukotrienes (LTC_4, LTD_4, LTE_4), and at a higher sensitivity than that attainable by bioassay. The combined use of HPLC-RIA provides for both pure samples of individual p-Leukotrienes and their sensitive detection, since the antisera raised in the laboratory for this work have allowed a detection limit of less than 10 femtomoles of LTD_4 (see Materials and Methods).

Immunological stimulation of Leukotriene generation was first discovered by Kellaway and Trethewie in 1940 (2). They found that a factor was formed in perfused guinea pig lungs when animals previously sensitized to egg albumin were subsequently challenged with this antigen. Ishizaka *et al.* have recently demonstrated that IgE or human atopic sera can be used to sensitize monkey lung fragments *in vitro* to form SRS-A in response to antigen challenge (7).

The results of studies on the RBL-1 cell model system in this laboratory, using potential agonists, as well as immunological stimulation, are presented below.

3.1.2 Effect of Inflammatory Mediators

The inflammatory mediators tested were fMLP, bradykinin, PAF, compound 48/80 and mellitin, compounds which had not been studied

previously in RBL-1 cells as potential candidates for agonist activity, inducing Leukotriene generation. It was anticipated that the data presented would then facilitate the study of the modulation of p-Leukotriene biosynthesis by second messengers in RBL-1 cells and the possible role that cyclic AMP (cAMP) may play in this modulation.

The basal unstimulated levels of p-Leukotrienes in RBL-1 cells were found to be; 70±50, 80±30, 260±110 and 410±150 femtomoles per 10^7 cells/ml for LTC_4, LTD_4, LTE_4 and total p-Leukotrienes respectively as seen in Table 2(a) (means±s.e. of the means, n=6). In separate and triplicate experiments RBL-1 cells at a cell count of approximately 10^7cells/ml were incubated with: PAF, bradykinin, fMLP and compound 48/80 at 10μM concentrations and mellitin at 3μM concentration. Variability in cell/ml of stock solution was determined and the data corrected to 10^7cells/ml. Surprisingly, none of the agonists produced a significant increase in the level of p-Leukotrienes observed in Table 2(b) when compared to the unstimulated basal p-Leukotriene levels. The optimal concentration for Leukotriene generation in rat and guinea pig lung tissue for PAF, bradykinin and fMLP has been shown previously to be 10μM (245,258). This optimal concentration is similar to that reported for bradykinin-induced prostaglandin I_2 (PGI_2) release in vascular endothelial cells and in guinea pig lungs (277) and for fMLP-induced Leukotriene B_4 (LTB_4) production in rabbit colons (279). These findings have important repercussions for the classification of receptors and/or secondary events in basophils, insofar as receptors for those mediators tested are presumably not present in RBL-1 cells, or at least not coupled to p-Leukotriene generation.

3.1.3 Immunological Stimulation of Leukotriene Biosynthesis

Recently, it has been reported that human basophils are capable of releasing approximately 90 picomoles of LTC_4 per μg of histamine release when challenged with anti-IgE antibody, although variations of up to 1000 fold were observed from donor to donor (280). It was claimed that purified human basophils generated only LTC_4, and that conversion to LTE_4 is possible in impure preparations. Samples were analysed by a strategy similar to that developed in this laboratory (273), involving the use of HPLC and RIA using antisera raised against LTC_4.

Experiments were initiated to test whether immunological challenge (*in vitro*) would lead to p-Leukotriene biosynthesis in RBL-1 cells. It is well

documented that RBL-1 cells do possess IgE receptors ($F_{C\varepsilon}$ receptors with high affinity for IgE) (281,282), and hence it was decided to prime the cells with IgE and then to crosslink the IgE receptors using antigen (Human Serum Albumin conjugated to dinitrophenol hapten, HSA-DNP) since IgE receptor activation occurs by receptor aggregation (achieved by receptor crosslinkers) not by ligand binding.

When RBL-1 cells, primed with 0.5µg/ml of IgE raised against DNP hapten for 16 hours, were incubated with 20ng of DNP-HSA conjugate for 20 minutes at 37°C, no significant release of p-Leukotrienes, measured by HPLC-RIA, was observed. It can be seen from Table 2(c) that immunological stimulation leads to the generation of 65±13, 88±12, 207±17 and 360±17 femtomoles per 10^7 cells/ml of LTC_4, D_4, E_4 and total Leukotrienes respectively, and these levels are similar to the control (basal).

3.1.4 Effect of Ionophore on Leukotriene Biosynthesis

The calcium ionophore A23187 is an antibiotic that possesses weak *in vitro* activity against gram-positive organisms. It also greatly increases the ability of divalent cations to cross biological membranes, giving it the properties of an ionophore. A23187 specifically forms stable complexes consisting of two molecules of A23187 per divalent cation complexed. The order of ion specificity observed for A23187 is: $Mn^{2+} > Ca^{2+} = Mg^{2+} >> Sr^{2+}$.

Ionophore has been used previously to induce Leukotriene biosynthesis in RBL-1 cells, including the original work in the structural elucidation of SRS and SRS-A carried out in this laboratory (20,24). However, the source of the original RBL-1 cell line was different, and the effect of the calcium ionophore A23187 was therefore tested on the RBL-1 cell line used in this thesis, to see if the current cell line did indeed have the "machinery" to produce Leukotrienes. When RBL-1 cells at a concentration of 10^7 cells/ml were incubated with A23187 at 10µM and at 37°C for 20 minutes, a considerable Leukotriene generation was observed as seen in Table 2(d). It can be seen from Table 2(d) that the current RBL-1 cell line possesses the required enzymes for p-Leukotriene biosynthesis, and that this cell line is able to produce approximately 50 picomoles of total p-Leukotrienes per 10^7 cells/ml when incubated with 10µM A23187.

It should be added that in earlier work on Leukotriene structure elucidation (24,25,283), cells were pre-incubated with L-cysteine and arachidonic acid for short periods to enhance Leukotriene production.

	Agonist	LTC_4	LTD_4	LTE_4	TOTAL
			fmoles $(10^7$ cells/ml$)^{-1}$		
(a)	**Control** (n=6)	70±50	80±30	260±110	410±150
(b)	**PAF** 10μM (n=3)	78±11	N.D	380±65	458±10
	Mellitin 3μM (n=3)	80±60	31±16	155±50	266±83
	Bradykinin 10μM (n=3)	90±31	N.D	277±101	375±136
	Compound-48/8010μM (n=3)	72±20	70±15	256±17	398±119
	fMLP 10μM (n=3)	65±30	N.D	236±23	301±60
(c)	**Challenge with DNP-HSA** 20ng (n=3)	65±13	88±12	207±17	360±17
(d)	**A23187** 10μM (n=6)	15600±420	10100±260	27300±760	53000±2378

Table 2: Effect of various agonists upon peptido-Leukotriene (p-LT) generation in RBL-1 cells. Data expressed as means±s. e. of the means; N.D. , not detectable.

However, it was decided not to carry out the preincubation with L-cysteine and arachidonic acid, so as to interfere as little as possible with the normal cellular functions, since the objective was to study the signalling pathway(s) involved.

These data prove that the current RBL-1 cell line is viable and fully capable of biosynthesis of p-Leukotrienes, and therefore discount cell viability problems leading to the lack of agonist activity observed with the other compounds studied earlier, shown in Table 2(b) and (c).

3.1.5 Effect of PMA on Leukotriene Biosynthesis

Protein kinase C (PKC) is an enzyme that is activated when receptor-mediated hydrolysis of inositol phospholipid occurs and inositol polyphosphates and 1,2-diacylglycerol (DAG) are generated. PKC requires calcium and phosphatidylserine for its activation (284) and this is achieved by DAG which dramatically increases the affinity of this enzyme for Ca^{2+} thereby rendering it fully active without a net enhancement in intracellular calcium concentration, $[Ca^{2+}]_i$ (285).

PKC is implicated in transmembrane signalling in that it has been shown to relay information from a variety of extracellular signals across the membrane to regulate many calcium dependent processes. The role of PKC in stimulus-response coupling was first demonstrated in 1982 for the release of 5-hydroxy tryptamine (5-HT) from platelets (286), and subsequently shown for the release, secretion and exocytosis of cellular constituents from a variety of endocrine and exocrine tissues and for the activation of many other cellular functions such as histamine release in basophils (211) and in mast cells (287).

4β-phorbol 12-myristate 13-acetate (PMA) is a membrane permeable phorbol ester which activates PKC and has recently been widely used to study the effects of PKC activation. The addition of PMA to resting cells is believed to result in the translocation of PKC from the cytosol to the intracellular membranes, which are rich in phosphatidylserine and Ca^{2+}, leading to the subsequent induction of its kinase activity (288,289). In view of these findings, it was important to examine whether PKC stimulation alone would be a necessary or sufficient condition for p-Leukotriene biosynthesis in RBL-1 cells, and these experiments are summarised in Table 3.

RBL-1 cells at a cell density of 10^7 cells/ml were incubated with PMA at a range of concentrations from 0.01-1μM. Table 3 shows the effect of PMA on p-Leukotriene biosynthesis in RBL-1 cells. It can be clearly seen that PMA at concentrations in the range of 0.01-1μM does not lead to asignificant

Agonist	LTC_4	LTD_4	LTE_4	TOTAL
		fmoles $(10^7$ cells/ml$)^{-1}$		
Control (n=3)	88±33	29±8	153±20	271±38
PMA 0.01μM (n=3)	103±11	59±6	127±1	289±15
PMA 0.1μM (n=3)	93±5	N.D	105±4	198±8
PMA 1.0μM (n=3)	55±14	98±7	142±3	295±8

Table 3: Effect of the phorbol ester, PMA, upon peptido-Leukotriene (p-LT) biosynthesis in RBL-1 cells. Data expressed as means±s. e. of the means; N.D. , not detectable.

enhancement, with respect to control, in the biosynthesis of p-Leukotrienes in RBL-1 cells.

3.1.6 **Discussion**

The lack of effect demonstrated in the fifteen experiments summarized in Table 2(b) for the inflammatory mediators, which are known secretagogues of Leukotrienes in other systems (239,240,247,252), implies that this clone of RBL-1 cells may not possess any receptors and/or coupling to PLA_2 activation, for these agonists. When immunological stimulation was conducted in RBL-1 cells (Table 2(c)), no significant effect was observed with respect to p-Leukotriene biosynthesis, thus suggesting that the anticipated IgE receptor crosslinking either does not take place or does not lead to Leukotriene generation; in later work presented in Section 3.6 this ambiguity is resolved. In contrast to these findings, in recent independent studies others have proposed that IgE receptor crosslinking does lead to general Leukotriene generation in RBL-2H3 cells, a sub-clone of RBL cells (290,291) (although the quantitive amounts are very small) and to LTC_4 biosynthesis in human basophils and mast cells (256). Nevertheless, the data presented here on RBL-1 cells have now been independently confirmed by B. A. Jakschik and C. Wong (personal communication June 1990, when these results were presented by the author at the Seventh International Conference on Prostaglandins and Related Compounds in Florence (402)).

Calcium ionophore, A23187, which allows Ca^{2+} entry into the cells from the extracellular medium as well as mobilization of Ca^{2+} from intracellular pools, did lead to a very significant p-Leukotriene generation. The enhanced $[Ca^{2+}]_i$ is clearly sufficient to activate PLA_2, followed by arachidonic acid release and its metabolism by the 5-Lipoxygenase pathway to p-Leukotrienes.

When RBL-1 cells were incubated with PMA alone, no significant stimulatory effect was detected with respect to p-Leukotriene generation as can be seen in Table 3. This finding implies that PKC activation alone is not enough to elicit an enhancement in p-Leukotriene biosynthesis.

Recent reports have indicated that PMA enhances the response to A23187 and/or agonist stimulation in a number of cells including; platelets (292), macrophages (293), polymorphonuclear leukocytes (PMNL) (294), and basophils (295). Recently, it has been suggested that PMA enhances A23187-induced LTB_4 as well as PAF generation in human neutrophils (296). It is

suggested that PKC activation is required to couple an A23187-induced rise in $[Ca^{2+}]_i$ to the generation of LTB_4 and PAF (296). However, PMA alone does not stimulate LTB_4 synthesis in human PMNLs , nor LTC_4 synthesis in mouse peritoneal macrophages (293).

It is important to note that while PKC activation in response to other stimulants does play an important role in Leukotriene generation (296), it was found here that PMA-mediated PKC activation alone is not a sufficient condition for Leukotriene generation as shown in Table 3. This is in agreement with independent work previously reported (293). McIntyre *et al.* (296) also report that, although in human neutrophils PKC activation in conjunction with other stimulants plays an important role, PKC activation alone is not sufficient to induce LTB_4 and PAF formation. Our data corroborates this, as PMA alone did not cause any significant p-Leukotriene generation. This lack of stimulatory effect by PMA in RBL-1 cells is not likely to be due to a lack of effect on PKC itself, since during the preparation for this thesis, it has been shown that PMA does lead to PKC translocation from the cytosolic to the particulate fraction and hence its activation in RBL-1 cells (297).

Our data allow a proposal that if PKC plays a role in Leukotriene biosynthesis then it is only in synergism with other cellular responses such as Ca^{2+} influx and enhanced $[Ca^{2+}]_i$ levels in RBL-1 cells. This synergism could be the prerequisite to couple a rise in $[Ca^{2+}]_i$ to Leukotriene production. This coupling could be at the level of the initial reaction in the generation of these mediators, a phospholipase A_2 (PLA_2) like activity that mobilizes arachidonic acid from the sn-2 position of phospholipids in the cell membranes.

3.2 Effect of Ionophore, NECA and Inflammatory Mediators on Labelled Arachidonic Acid Release and Leukotriene Generation

3.2.1 *Introduction*

In view of the findings in the previous section, it was important to examine earlier stages in the p-Leukotriene biosynthetic pathway, in particular the verification of arachidonic acid release itself in case this was indeed released by the agonists used, but directed down competing metabolic pathways, not leading to the p-Leukotrienes. Arachidonic acid released from phospholipids could undergo two possible metabolic routes: metabolism through the cyclo-oxygenase pathway, which would then in turn lead to

prostaglandins, thromboxane and prostacyclin biosynthesis, and/or metabolism through the lipoxygenase pathway, and in the case of 5-lipoxygenase, the generation of 5-hydroxyeicosatetraenoic acid (5-HETE), p-Leukotrienes and/or LTB_4. These two pathways could be competing with one another for the metabolism of arachidonic acid following cellular stimulation, or arachidonic acid could be preferentially metabolized by one pathway rather than the other. It has also been reported that prostaglandins have an inhibitory effect upon Leukotriene biosynthesis and that Leukotrienes have a stimulatory effect upon prostaglandin generation (298). This, in either case, would lead to reduction in p-Leukotrienes since if arachidonic acid release is channelled into the cyclo-oxygenase pathway less of it would be available for p-Leukotriene generation, and on the other hand if arachidonic acid is utilised by the lipoxygenase pathway, the level of p-Leukotrienes would diminish very quickly since the Leukotrienes generated would enhance prostaglandin formation and hence reduction in Leukotrienes.

In addition to these points, it should be mentioned that there are two other lipoxygenases beside the 5-lipoxygenase; the 12- and 15-lipoxygenases, and arachidonic acid metabolism may be channelled with preference to these enzymes under certain conditions leading to the generation of 12- and/or 15-hydroperoxyeicosatetraenoic acid (12- and 15-HPETE) both of which lead to inhibition of 5-lipoxygenase (299). Therefore, in addition to measurement of Leukotriene generation, proof and measurement of arachidonic acid release following agonist stimulation is important to the study of the effect of these agonists in RBL-1 cells. It was also felt that an alternative technique for visualising the production of Leukotrienes would provide effective and independent corroboration of the data. The utilisation of [^{3}H]-arachidonic acid was used to achieve these objectives.

3.2.2 Effect of Inflammatory Mediators on Labelled Arachidonic Acid Release and Leukotriene Generation

In the previous section experiments to see the effect of a number of inflammatory mediators upon p-Leukotriene generation were performed by measuring the end-products (p-Leukotrienes). In view of the lack of effect by these mediators, with the exception of A23187, it was decided to test whether these mediators would affect metabolic steps prior to p-Leukotriene generation, namely arachidonic acid release or LTB_4 formation. Our

methodology was such that any effect upon arachidonic acid, p-Leukotriene or LTB_4 biosynthesis would be observed

It was also decided to investigate whether LTD_4 itself has any effect on p-Leukotriene generation in the RBL-1 cell model system. It has been previously reported that LTD_4 treatment of bovine aortic endothelial cells and murine smooth muscle cells in culture lead to an increase in PLA_2 activity (300). This LTD_4 treatment resulted in a dose-dependent enhancement in PLA_2 activity with phosphatidylcholine as a substrate. The induction of PLA_2 activity occured prior to the appearance of prostanoids in the media (300). Moreover, it is a well established biochemical concept that in some metabolic pathways, the product of a particular route is able to induce its own generation (positive feedback control), or conversely inhibit its generation, (negative feedback control) (301).

The effect, individually, of bradykinin, PAF, LTD_4, fMLP and mellitin on [^{3}H]-arachidonic acid and [^{3}H]-Leukotriene biosynthesis was investigated by addition of between 3 and 10μM concentrations of agonist to approximately 10^7 cells/ml RBL-1 cells, which had been prelabelled with 0.2 μCi/ml [^{3}H]-arachidonic acid for 16 hours in the logarithmic phase of growth. The counts released (arachidonic acid) or incorporated (Leukotrienes) are expressed as percentage of control/basal levels. Table 4(a) illustrates the data obtained when different agonists are used to induce [^{3}H]-arachidonic acid release and [^{3}H]-Leukotriene generation. In triplicate and separate experiments, bradykinin, PAF and fMLP at 10μM concentration, LTD_4 at 1μM concentration and mellitin at 3μM concentration were incubated with RBL-1 cells, pre-labelled with [^{3}H]-arachidonic acid. From Table 4(a), it can be seen that these mediators and agonists do not induce release or biosynthesis of significant amounts of any of the possible p-[^{3}H]-Leukotrienes or indeed [^{3}H]-arachidonic acid itself.

Furthermore, as shown in Table 4(a), LTD_4 did not induce any significant generation of [^{3}H]-Leukotrienes, a surprising result since LTD_4 receptors have recently been reported to be present on RBL-1 cell membranes (302). These receptors have been characterized by using radioligand binding assay. It has been found that [^{3}H]-LTD_4 binding to its receptor on RBL-1 cell membranes is stereoselective, specific and saturable. Furthermore, this was confirmed for this thesis work in our own RBL-1 cell line in radioligand binding assays in which greater than 70% specific binding was found. Sarau *et al.* (277) have reported that LTD_4 receptors on RBL-1 cell membranes are coupled to phosphoinositide-specific phospholipase-C (PIC),

with which the data presented in this thesis are independently in agreement (see Section 3.3.3). However, importantly, the data in Table 4(a) show that LTD_4 receptor binding does not lead to arachidonic acid release and subsequent potentiation of p-Leukotriene generation, thus suggesting that, unlike bovine endothelial cells and murine smooth muscle cells (300), LTD_4 receptors on RBL-1 cell membranes are not coupled to PLA_2 activation. This novel finding is discussed later, in relation to other results in this thesis.

When RBL-1 cells, primed with IgE raised against DNP hapten and labelled with [^{3}H]-arachidonic acid (0.2μCi/ml) for 16 hours in the logarithmic phase of growth, were challenged with HSA-DNP conjugate (20ng) for 20 minutes at 37°C, in agreement with RIA-HPLC data (Section 3.1.3), no significant increase in the level of p-[^{3}H]-Leukotrienes or indeed [^{3}H]-arachidonic acid itself were detected, as seen in Table 4(a). Examination of the ultra violet (uv) traces, together with scintillation counting across the LTB_4 elution position, also failed to detect any LTB_4 generation when the inflammatory agonists shown in the Table were used as secretagogues in RBL-1 cells.

3.2.3 Effect of NECA on Labelled Arachidonic Acid Release and Leukotriene Generation

It has been known for sometime that adenosine profoundly alters degranulation and mediator release in basophils (303). However, the effects of adenosine or its analogues appear to be complicated; one report claims that adenosine inhibits degranulation by a mechanism that may be mediated via A_2 adenosine receptor subtypes linked to adenyl cyclase (304), while other reports claim that adenosine enhances IgE- and A23187-induced degranulation by a mechanism independent of enhanced adenyl cyclase activity (305,306).

In view of the modulatory role proposed for adenosine and its analogues and the contradictory work reported above, it was decided to investigate the role that adenosine might play in arachidonic acid release and Leukotriene biosynthesis in RBL-1 cells. For this investigation, the compound 5'-(N-ethyl) carboxamidoadenosine (NECA), a metabolically stable adenosine analogue was used. This compound shows greater affinity for adenosine A_2 receptors than adenosine itself and mimics adenosine's biological effects (307). RBL-1 cells were pre-labelled with [^{3}H]-arachidonic acid at 0.2μCi/ml concentration for 16 hours and at 37°C. The cells were then centrifuged and

	Agonist	LTC_4	LTD_4	LTE_4	Total pLT	LTB_4	AA
				% of control			
(a)	**Bradykinin** 10μM (n=3)	113±4	90±5	65±4	89±4	N.D	81±3
	PAF 10μM (n=3)	94±3	102±4	158±5	121±3	N.D	88±3
	LTD_4 1μM (n=4)	136±4	148±5	77±5	120±4	N.D	85±2
	fMLP 10μM (n=4)	109±5	78±4	116±8	101±6	N.D	87±4
	Mellitin 3μM (n=3)	121±2	68±4	148±8	112±5	N.D	95±2
	Challenge with DNP-HSA 20ng (n=4)	105±3	N.D	104±1	103±1	N.D	114±8
(b)	**NECA** 10μM (n=3)	105±11	104±10	118±7	109±1	N.D	102±13
(c)	**A23187** 10μM (n=6)	802±223	1763±489	414±95	335±75	304±4	829±221

Table 4: The effect of various agonists on [^{3}H]-arachidonic acid (AA) release and [^{3}H]-Leukotriene (LT) generation in RBL-1 cells. pLT, peptido-Leukotriene. Data expressed as means±s. e. of the means; N.D. , not detectable.

washed to remove excess [^{3}H]-arachidonic acid not incorporated into the phospholipids of the cells. After aliquoting into 1ml fractions the cells were incubated with NECA at 10μM concentration for 20 minutes at 37°C. Table 4(b) illustrates the effect of NECA upon [^{3}H]-arachidonic acid and [^{3}H]-Leukotriene biosynthesis in RBL-1 cells. It can be observed from Table 4(b) that 10μM NECA when incubated with RBL-1 cells does not lead to any statistically significant [^{3}H]-arachidonic acid release, nor does it lead to any statistically significant [^{3}H]-p-Leukotriene generation.

3.2.4 Effect of Ionophore on Labelled Arachidonic Acid Release and Leukotriene Generation

As mentioned earlier, calcium ionophore, A23187, has been used previously to induce p-Leukotriene generation. Its effect relies upon an enhancement in $[Ca^{2+}]_i$ level, which then in turn leads to PLA_2 activation and arachidonic acid release from phospholipids in the membranes, with further metabolism of arachidonic acid by the 5-lipoxygenase pathway to Leukotrienes. In effect, this method of Leukotriene generation by-passes receptor mediated PLA_2 activation and leads to direct activation of the enzyme.

When the Ca^{2+} ionophore A23187, at 1μM and 10μM concentrations, was incubated with RBL-1 cells pre-labelled with [^{3}H]-arachidonic acid (0.2μCi/ml), in agreement with the HPLC-RIA data (Section 3.1.4), a significant increase in both [^{3}H]-arachidonic acid and [^{3}H]-Leukotriene generation was observed as seen in Table 4(c) and Table 5.

Previous work from this laboratory had shown that cAMP inhibited PAF-induced p-Leukotriene generation in chopped rat lung preparations (308). An attempt was made therefore, to investigate the role of the cAMP analogue on A23187-induced arachidonic acid and Leukotriene biosynthesis by using membrane permeable dibutyryl-cAMP (db-cAMP). When A23187 at 1μM concentration was incubated with RBL-1 cells, pre-labelled with [^{3}H]-arachidonic acid (0.2μCi/ml) in the presence of 100μM db-cAMP, a small but statistically significant inhibition (20%, $p<0.05$, $n=6$) of [^{3}H]-LTC_4 level was observed, but no statistically significant effect could be detected for [^{3}H]-LTD_4, [^{3}H]-LTB_4, [^{3}H]-LTE_4 and [^{3}H]-arachidonic acid biosynthesis, and these data are shown in Table 5.

Agonist	LTC_4	LTD_4	LTE_4	LTB_4	total LT	AA
			% of control			
A23187 1μM (n=5)	302±11	916±358	352±62	547±163	529±149	210±43
A23187 1μM+ **db-cAMP** 100μM (n=6)	243±11*	621±184	333±62	689±189	472±112	212±36

Table 5: The effect of dibutyryl-cyclic AMP on ionophore-induced [^{3}H]-arachidonic acid (AA) release and [^{3}H]-Leukotriene (LT) generation in RBL-cells. Data expressed as means±s. e. of the means, (* $p<0.05$).

3.2.5 **Discussion**

Inflammatory mediators, such as those used in these investigations, have been reported to induce Leukotriene biosynthesis in various physiological systems (250). The mediators tested here have not previously been used in studies with RBL-1 cells as a model system. When compared with basal unstimulated Leukotriene levels, these mediators, with the exception of A23187, did not lead to any significant increase in [^{3}H]-Leukotriene biosynthesis, nor to an enhancement in [^{3}H]-arachidonic acid release. These data, together with the HPLC-RIA data (Section 3.1.2) support the hypothesis made here that the mediators tested do not possess PLA_2 coupled-receptors on RBL-1 cell membranes.

LTD_4 has been reported to induce PLA_2 activity and prostanoid biosynthesis in a variety of cells and tissues (309-311), including bovine aortic endothelial cells and murine smooth muscle cells in culture (300). Also the presence of LTD_4 receptors on RBL-1 cell membranes has been reported previously (302). We therefore examined the effect of LTD_4 upon Leukotriene biosynthesis in RBL-1 cells. Our data shown in Table 4 are in contradiction to the findings mentioned above since we were unable to show any significant increase in the [^{3}H]-Leukotrienes or in [^{3}H]-arachidonic acid level, hence implying a lack of PLA_2 activation following LTD_4 receptor binding in RBL-1 cells.

In view of this anomalous finding, it was important to demonstrate the presence or absence of LTD_4 receptors in the RBL-1 cell line used in this work. Radioligand binding assays were carried out and the data produced showed greater than 70% specific binding of [^{3}H]-LTD_4. It is possible to conclude therefore, that the coupling of the LTD_4 receptor to PLA_2 is absent in RBL-1 cells.

When NECA was used as agonist, no significant release of [^{3}H]-arachidonic acid nor an enhancement in [^{3}H]-Leukotriene biosynthesis was observed, as can be seen in Table 4(b). The increase in arachidonic acid release reported to be mediated by NECA (305,306) (independent of adenyl cyclase) was in synergism with IgE- and A23187-mediated responses. However, in the study presented here, it was decided to clarify an already complex picture by using NECA on its own. The results show that NECA alone does not activate PLA_2 in RBL-1 cells.

The Ca^{2+} ionophore, A23187, in agreement with the HPLC-RIA data (Section 3.1.4), did lead to a significant enhancement in [^{3}H]-arachidonic acid

and [^{3}H]-Leukotriene biosynthesis as shown in Table 4(c). A23187 is a non-specific secretagogue which leads to increased $[Ca^{2+}]_i$ level by mobilization of Ca^{2+} from intracellular pools as well as facilitating Ca^{2+} entry from the extracellular medium. Therefore, if $[Ca^{2+}]_i$ is raised sufficiently to cause PLA_2 activation (since increased Ca^{2+} activates the enzyme), this would result in the release of [^{3}H]-arachidonic acid and [^{3}H]-Leukotriene generation, as observed here.

Although a small but statistically significant inhibition ($p<0.05$, $n=6$) of [^{3}H]-LTC_4 level was observed when RBL-1 cells, pre-labelled with [^{3}H]-arachidonic acid, were incubated with A23187 at 1μM concentration in the presence of 100μM db-cAMP, no effect could be detected for [^{3}H]-LTD_4, [^{3}H]-LTB_4, [^{3}H]-LTE_4 and [^{3}H]-arachidonic acid biosynthesis in the medium, as seen in Table 5. Previous work from this laboratory had shown that cAMP inhibits PAF-induced p-Leukotriene generation in chopped rat lung preparations (308). Unlike the present results in RBL-1 cells, in rat chopped lungs, cAMP at 300nM leads to a statistically significant inhibition of PAF-mediated generation of LTC_4, LTD_4, LTE_4 and total Leukotrienes (308). One possible explanation for this lack of effect in RBL-1 cells could be that A23187 by-passes receptor-mediated activation of PLA_2 causing direct activation of PLA_2, and the site of action of cAMP could well be prior to Ca^{2+}-mediated PLA_2 stimulation.

3.3 The Effect of Inflammatory Mediators on Labelled Inositol Phosphate Formation

3.3.1 *Introduction*

Phosphatidylinositol (4,5)-bisphosphate (PIP_2) hydrolysis by phosphatidylinositol-specific phospholipase C (PIC) leads to the formation of two second messenger molecules; inositol (1,4,5)-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), which constitute a bifurcating signal pathway which is a central component in the control mechanisms of many different cells (160). When PIP_2 is hydrolysed following receptor activation, IP_3 is released into the cytosol, leaving DAG within the plane of the membrane. The primary function of IP_3 is to mobilize calcium from intracellular stores (158,312) to constitute an IP_3/Ca^{2+} pathway, whereas the other limb is controlled through DAG, which stimulates PKC (156) to form the DAG/PKC pathway. Together the two limbs of this bifurcating signal pathway provide an exceptionally versatile signalling

mechanism, which has been adapted to control short-term cellular responses such as contraction, secretion and metabolism, and may also play a role in long-term events such as growth and perhaps even information storage in the brain (160).

Having studied PLA_2 activation by various agents and the effect of cAMP upon this activation, it was decided to study the effect of these mediators on PIC activation and its signalling pathway.

3.3.2 Agonist-Induced Phosphatidylinositol Breakdown and Inositol Phosphate Formation

RBL-1 cells were first incubated with [^{3}H]-myo inositol, 10μCi/ml, for 90 minutes and at 37°C, after which the labelling incubation was carried out for a further 10 minutes in the presence of 10mM final concentration of LiCl. In separate and triplicate experiments the following agonists were then incubated with the pre-labelled RBL-1 cells in incubation buffer (containing LiCl at a final concentration of 10mM): fMLP, bradykinin and PAF at 1 and 10μM concentrations, histamine at 5 and 50μM concentrations, substance P at 0.6 and 6μM concentrations, and mellitin at 0.3 and 3μM concentrations. The concentrations used in these experiments were in accordance with those recently reported to cause PI breakdown and IP release in other systems (313-321). The inositol phosphates were separated using anion exchange Sep-pak cartridges or BioRad AG1X-8 pre-packed columns (See Experimental Section). IP_1, IP_2 and IP_3 were eluted by using increasing concentration of ammonium formate buffer. It should be noted that the various IP isomers are not separated by using this methodology. As can be seen in Figures 22 and 23, no substantial inositol polyphosphates were released following RBL-1 cell activation with these inflammatory agonists.

The inflammatory mediators that were used in the investigation of PLA_2 activation and Leukotriene formation (see Sections 3.1.2 and 3.2.2) have been reported to possess receptors that are linked to PI turnover. Bradykinin induces a rapid release of IP_3 from a mouse neuroblastoma hybrid cell line (322) and rat PC-12 cells (323) following receptor binding.

It has also been reported that fMLP receptors in polymorphonuclear leukocyte (PMNL) membranes are coupled to PI breakdown through a pertussis toxin-sensitive guanine nucleotide regulatory protein (G-protein) (321). Occupancy of this fMLP receptor leads to the rapid hydrolysis of PIP_2 in PMNL cells (322,324) and in differentiated HL 60 cells (259).

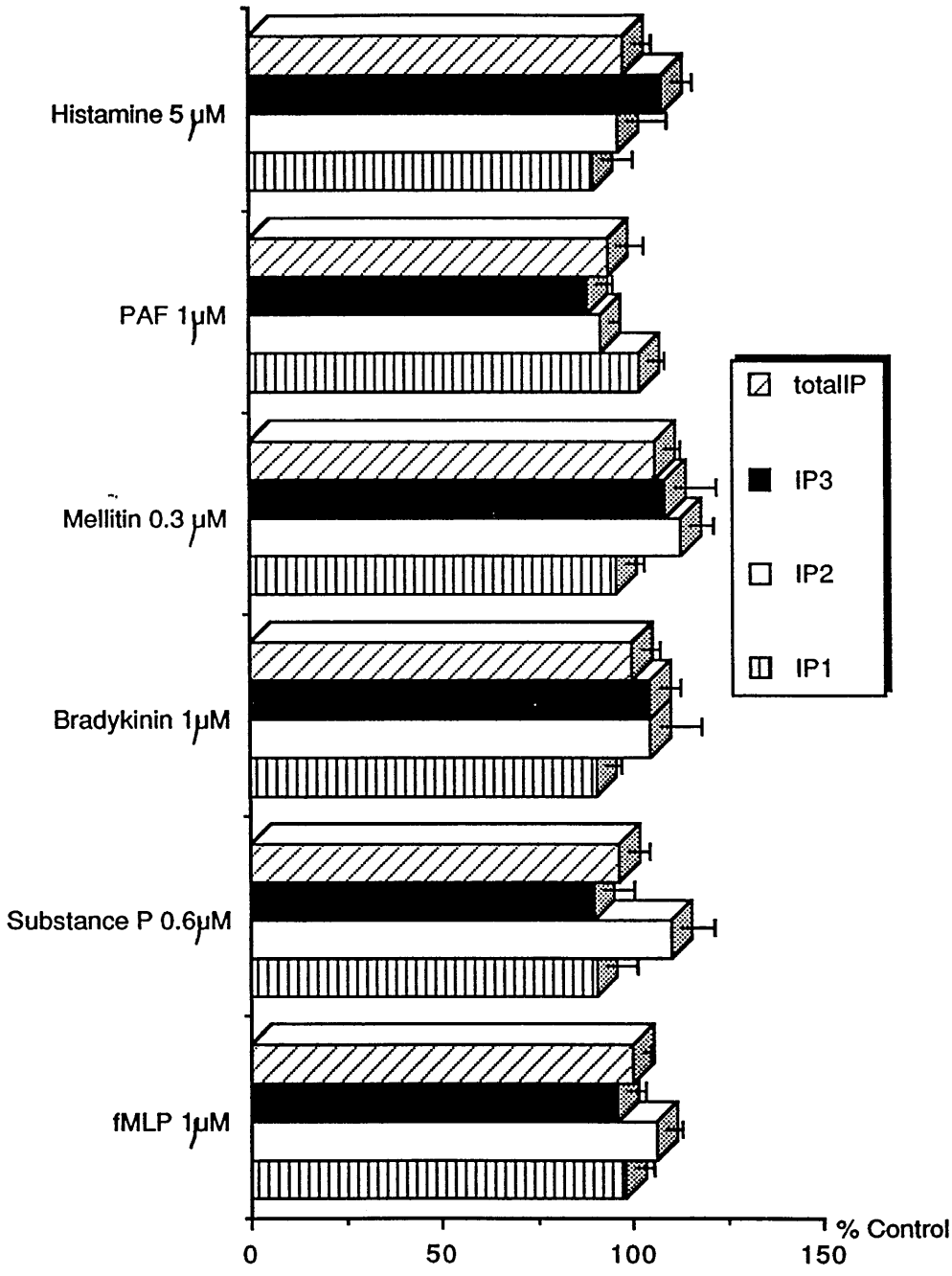


Figure 22: Effect of various agonists on IP release.
Data expressed as means±s.e. of the means (n=3).

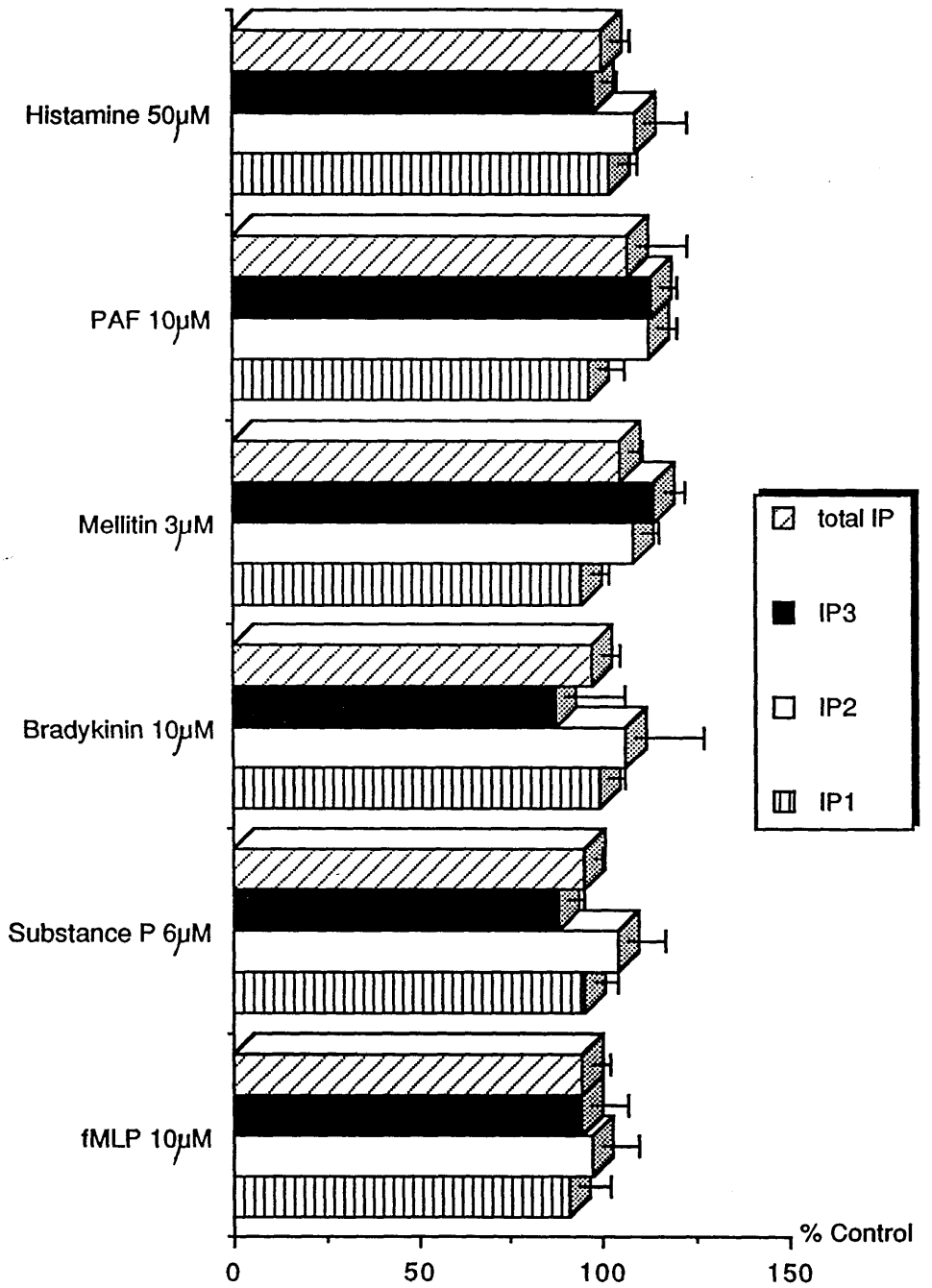


Figure 23: The effect of various agonists on IP release.
Data expressed as means±s.e. of the means (n=3).

Mantyh et al. (260) have suggested that substance P receptors are linked to PI turnover in rat neuronal tissues. Very recently substance P has also been shown to induce IP_3 and inositol (1,3,4,5)-tetrakisphosphate (IP_4) formation in rat parotid acinar cells (261). Substance P meets many of the requirements for the mediation of the local inflammatory response. Apart from being widely distributed in primary sensory neurons, from where it is released during antidromic stimulation, it produces vasodilation and vascular permeability, endothelial adherence of PMNs and monocytes and degranulation of mast cells (262).

Histamine induces PI hydrolysis in the central nervous system (CNS), neuroblastoma and astrocytoma cells. There are three subtypes of histamine receptors, H_1,H_2, and H_3 and the PI response appears to be mediated through a H_1 receptor subtype. The stimulatory effect of histamine can be blocked by inclusion of pyrilamine, a H_1 antagonist, whereas the H_2 antagonist cimetidine is ineffective (91). Furthermore, the magnitude of histamine-stimulated PI turnover mirrors that of the density of H_1 receptors on membranes (264,265). Histamine has a number of actions mediated via the H_1, H_2 and H_3 receptor subtypes, such as, smooth muscle contraction, increased vascular permeability, neural reflexes and mucus secretion, and its sources are mast cells and basophil granules (266).

Motasim-Billah and Lapetina have shown that PAF stimulates PI breakdown. They showed that stimulation of horse platelets (pre-labelled with ^{32}P) with PAF for 5 seconds led to a 50% loss of [^{32}P]-PIP_2 (253).

The mediators used have been shown to possess inflammatory activity (see Section 3.1.2), and as mentioned above, they also lead to PIC activation, PIP_2 hydrolysis and IP_3 formation. It is therefore of interest that the incubation of RBL-1 cells with these mediators at two different concentrations did not induce any significant IP release in RBL-1 cells, as shown in Figures 22 and 23, and these data again suggest the lack of mediator receptors in these cells.

3.3.3 LTD_4-Induced Phosphatidylinositol Breakdown and Inositol Phosphate Formation

RBL-1 cells do possess LTD_4 receptors on their membranes as demonstrated in this thesis and independently elsewhere (277). These receptors are coupled to PIC activation and PI hydrolysis, as shown by Sarau *et al.* during the characterization of LTD_4 receptors on RBL-1 cell membranes in 1987 (277). These authors observed that when [^3H]-myo-inositol labelled

cells were treated with LTD_4, a rapid hydrolysis of PI lipids occured and IP_3 formed within 1 minute. LTD_4 also induced formation of IP_1 and IP_2.

When pre-labelled RBL-1 cells with [^{3}H]-myo-inositol (10μCi/ml) were incubated with 1μM LTD_4 at 37°C for 30 minutes, in agreement with previously reported findings (277), a significant release of IP_1, IP_2, IP_3 and total IP was observed as seen in Figure 24. This finding is interesting since LTD_4 did not previously activate PLA_2 and hence did not lead to arachidonic acid release (see Section 3.2.2), and yet here LTD_4 activated PIC and caused a significant increase in phosphatidylinositol lipid breakdown and inositol polyphosphate release in the data shown in Figure 24.

It has been determined that the kinetic responses of LTD_4-induced PI hydrolysis and IP formation are consistent with the currently accepted mechanism of receptor-mediated PI turnover (277). Hence, agonist bound to its receptor activates G-protein coupled to PIC and then PIC hydrolyses PIP_2 to generate the two intracellular messengers, DAG and IP_3 (277,312). The EC_{50} of LTD_4 was found to be 5±4nM and it was observed that the maximal stimulation occured at 1μM LTD_4 concentration (277). It has also very recently been established that pretreatment of RBL-1 cells with pertussis toxin for 2.5 hours at 37°C, a protein exotoxin produced by *Bordetella pertussis* which ADP-ribosylates G-proteins (325,326), does not affect LTD_4-induced PI breakdown. This suggests that G_i- or G_o-like proteins, GTP-binding regulatory proteins responsible for inhibition of the adenyl cyclase signalling and that responsible for the inhibition of voltage sensitive Ca^{2+} channels respectively, are not involved in the receptor-mediated signal transduction process in RBL-1 cells (277).

3.3.4 Effect of Adenyl Cyclase Activators and cAMP Analogues on LTD_4-Induced Phosphatidylinositol Breakdown

It is now documented that addition of cAMP inhibits PI turnover in various tissues, for example platelets (327,328), neuro tumor cells (329) and neutrophils (330). It has also been demonstrated in several cell systems (e.g. erythrocytes, neutrophils and mast cells) that cAMP inhibits PI kinase, the enzyme that phosphorylates PI to phosphatidylinositol 4-phosphate (PIP) which is further phosphorylated to PIP_2, in lysed cells (330-332). Moreover, evidence also exists that suggests that treatment of intact cells with agents which elevate cAMP, inhibit PI kinase (333). In view of these findings it was decided to examine the effect of agents capable of activating adenyl cyclase,

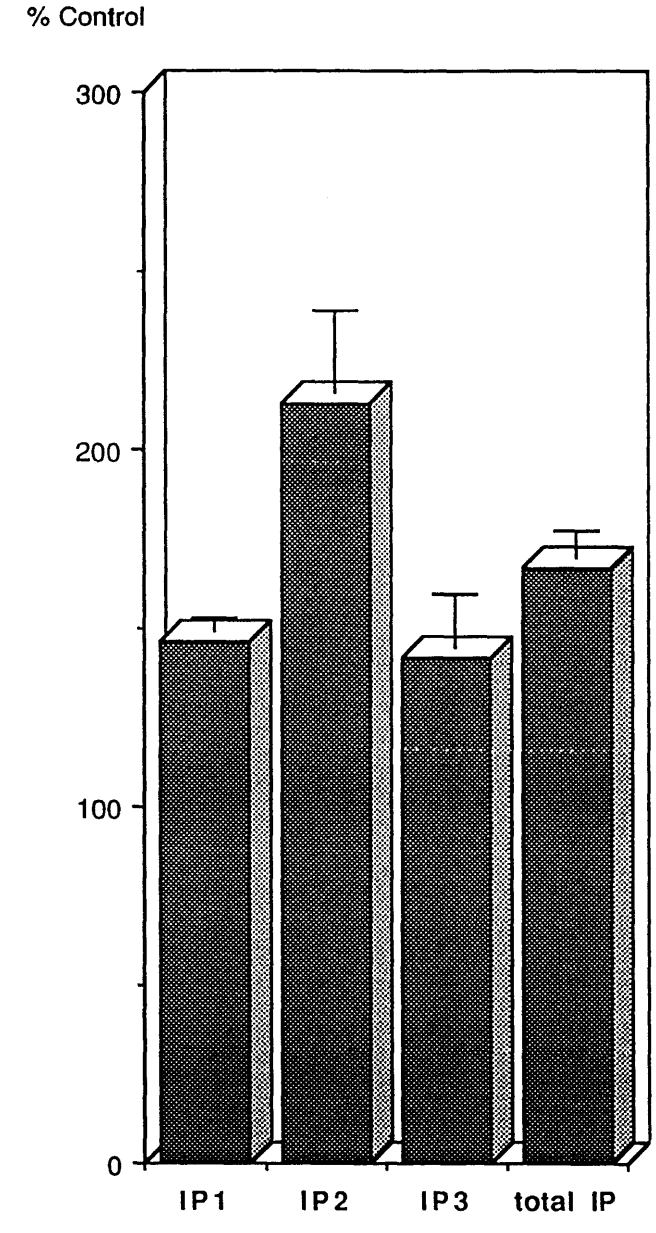


Figure 24: Effect of 1μM LTD4 upon inositol polyphosphate release (n=12). Data expressed as means±s.e. of the means.

and the effect of 8Br-cAMP, a stable cAMP analogue (simulating the effect of cAMP), on phosphoinositide turnover in RBL-1 cells.

PGE_2 and PGI_2 are among the most potent stimulators of adenyl cyclase (334,335). Forskolin, a naturally occuring diterpene isolated from *Coleus forskohlii*, has also been shown to stimulate adenyl cyclase *in vitro* and *in vivo* resulting in increased intracellular cAMP levels (see reference 110 for a review). The effect of PGI_2, PGE_2 and forskolin were therefore tested on LTD_4-induced phosphoinositide hydrolysis.

In separate experiments RBL-1 cells, pre-labelled with [^{3}H]-myo-inositol (10μCi/ml), were incubated with: PGI_2 at 10μM concentration, PGE_2 at 6μM concentration and forskolin at 100μM concentration for 2 minutes prior to addition of LTD_4. After the addition of 1μM LTD_4, the incubation was carried out for 30 minutes. In control tubes the appropriate volume of vehicle (solvent in which agonist/adenyl cyclase activators were dissolved) was added to ensure similar treatment to the tubes containing the actual compounds. The inositol polyphosphates were separated as described above. As can be seen from Figures 25 and 26, PGI_2 and PGE_2 at 10μM and 6μM concentrations respectively, showed no statistically significant decrease in LTD_4-induced IP release at the concentrations used. The lack of effect by PGE_2 and PGI_2 could be due to absence of receptors for these agents that are coupled to the adenyl cyclase signalling pathway on RBL-1 cell membranes. Similarly, forskolin at 100μM did not show any reduction in LTD_4-induced IP release as seen in Figure 27. During the work in preparation for this thesis forskolin has been reported to have non-specific effects on cellular responses (336). Therefore, it was felt that membrane permeable 8Br-cAMP and db-cAMP are more pertinent to correlate to cAMP effects on phosphoinositide hydrolysis in RBL-1 cells.

In different experiments, RBL-1 cells which had been previously labelled with [^{3}H]-myo-inositol (10μCi/ml) were incubated with either 8Br-cAMP at 10 and 100μM concentrations, or with db-cAMP at 100μM concentration for 2 minutes prior to LTD_4 stimulation. LTD_4 incubation was carried out as described previously and the inositol polyphosphates were separated and measured by using anion exchange chromatography and scintillation counting. When LTD_4 stimulation was carried out in the presence of 10μM 8Br-cAMP, IP_3 and total IP release were significantly inhibited ($p<0.001$ and $p<0.05$ respectively) as shown in Figure 28. When the concentration of 8Br-cAMP was increased to 100μM, again LTD_4-induced IP release was inhibited. From Figure 29 it can be observed that 100μM 8Br-

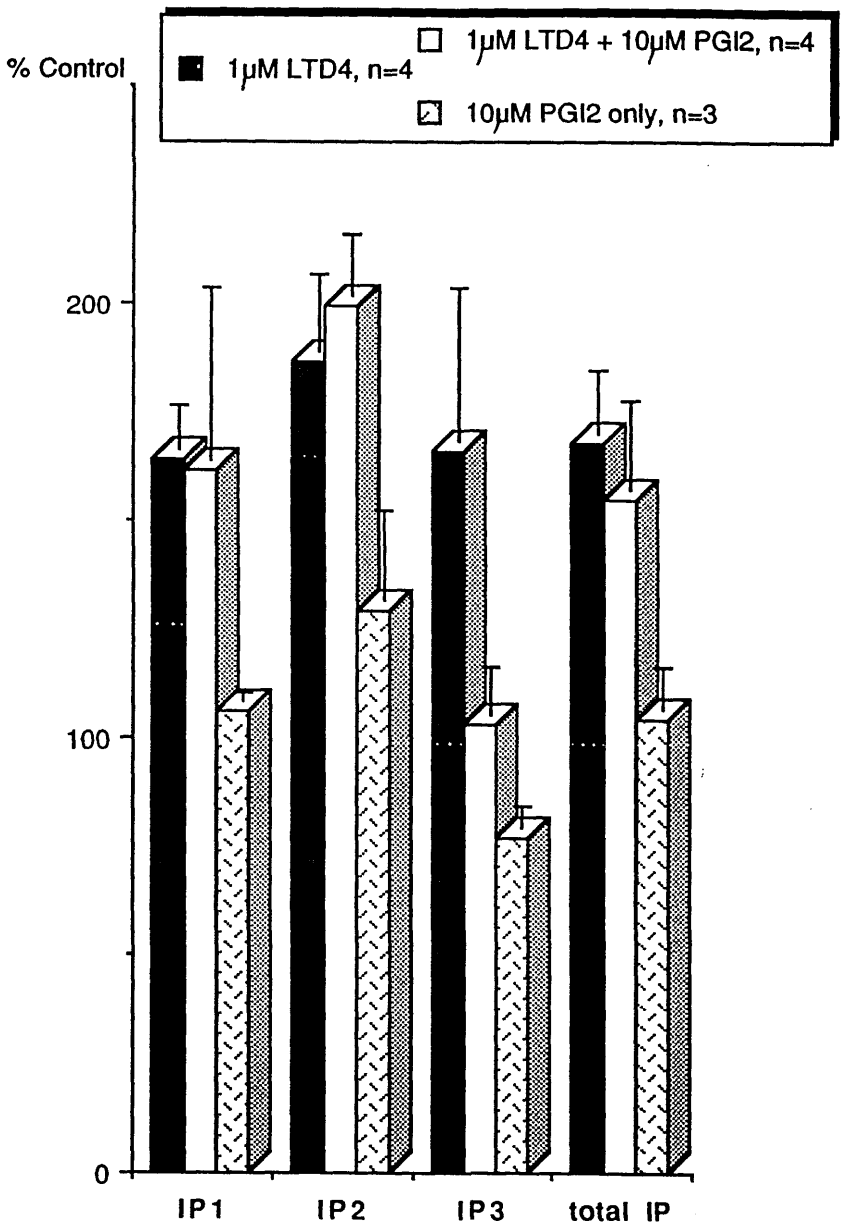


Figure 25: Effect of prostaglandin I2 on Leukotriene D4-induced inositol polyphosphate release. Data expressed as means±s.e. of the means.

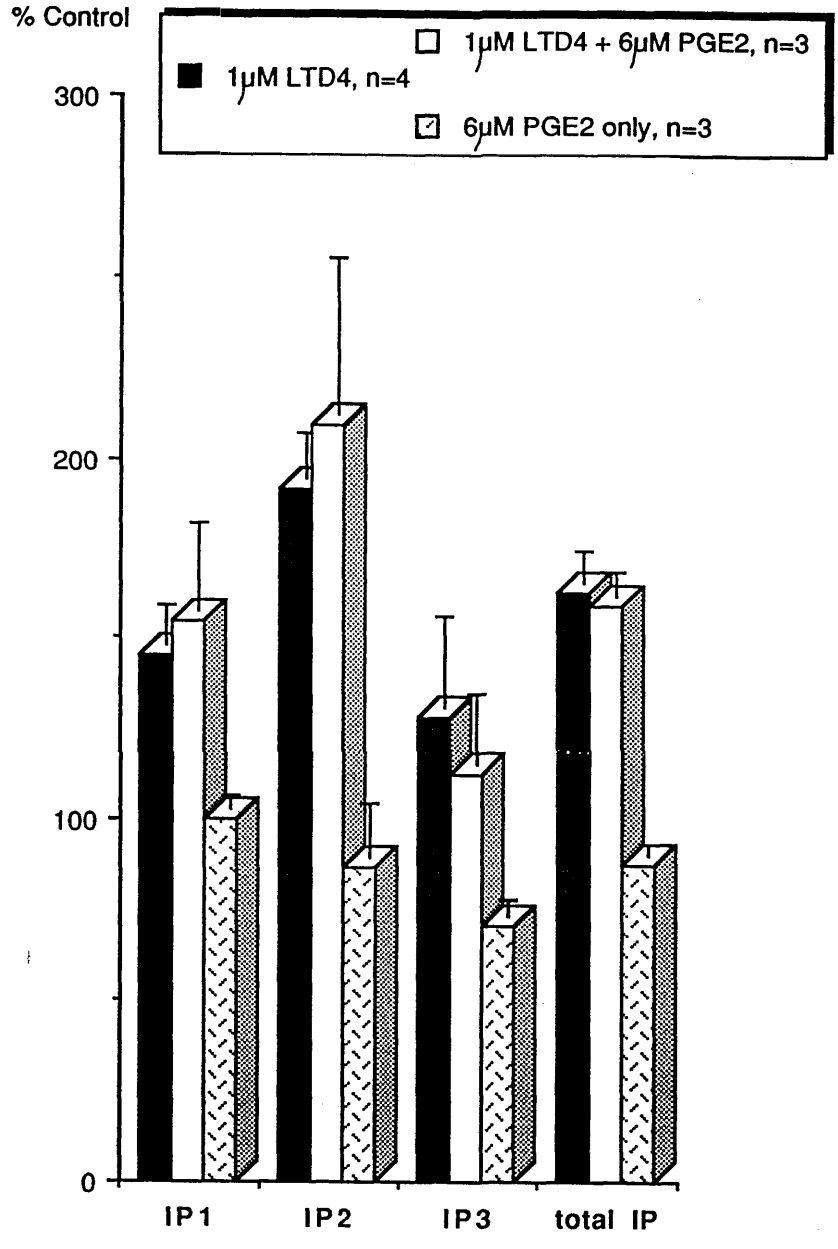


Figure 26: Effect of prostaglandin E2 on Leukotriene D4-induced inositol polyphosphate release. Data expressed as means±s.e. of the means.

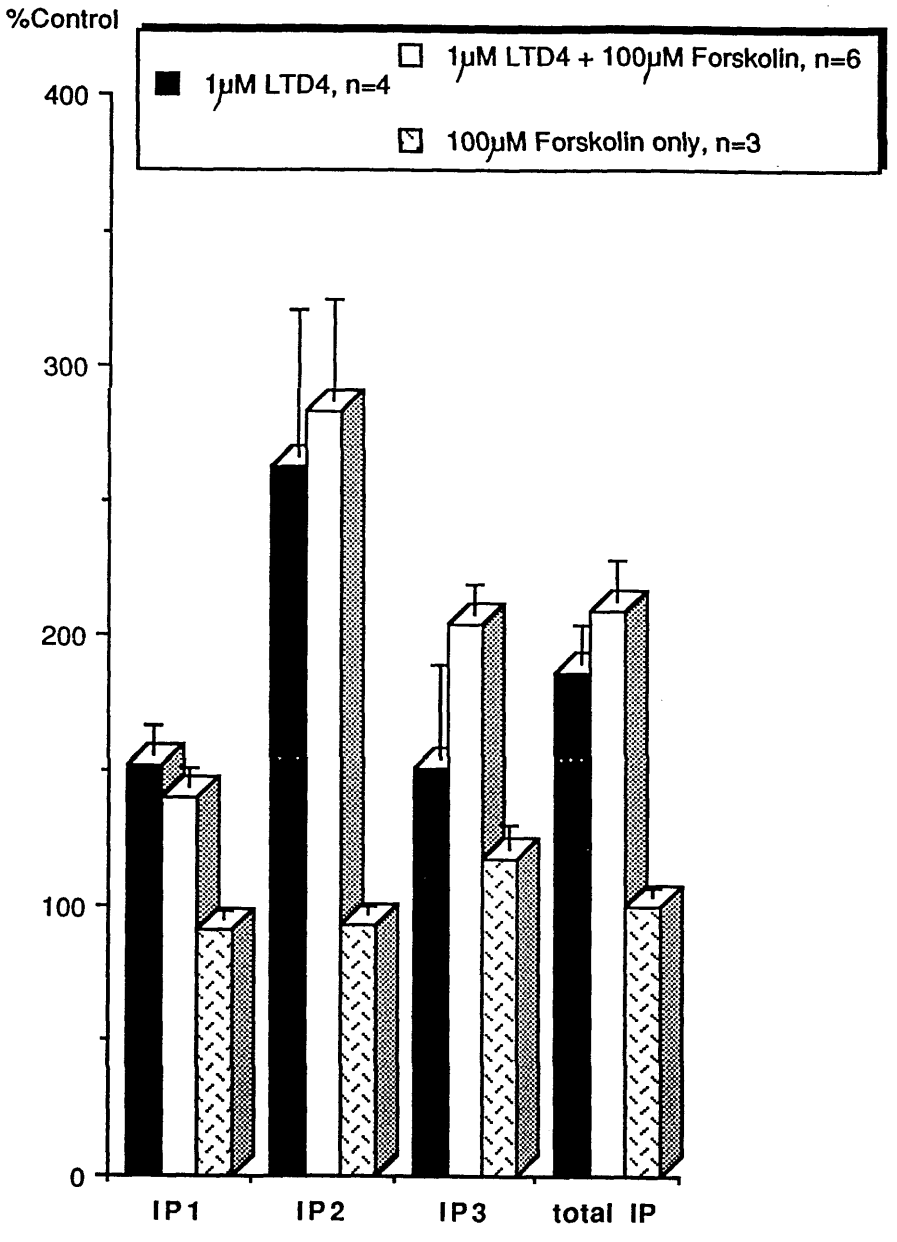


Figure 27: Effect of forskolin on Leukotriene D4-induced inositol polyphosphate release. Data expressed as means±s.e. of the means.

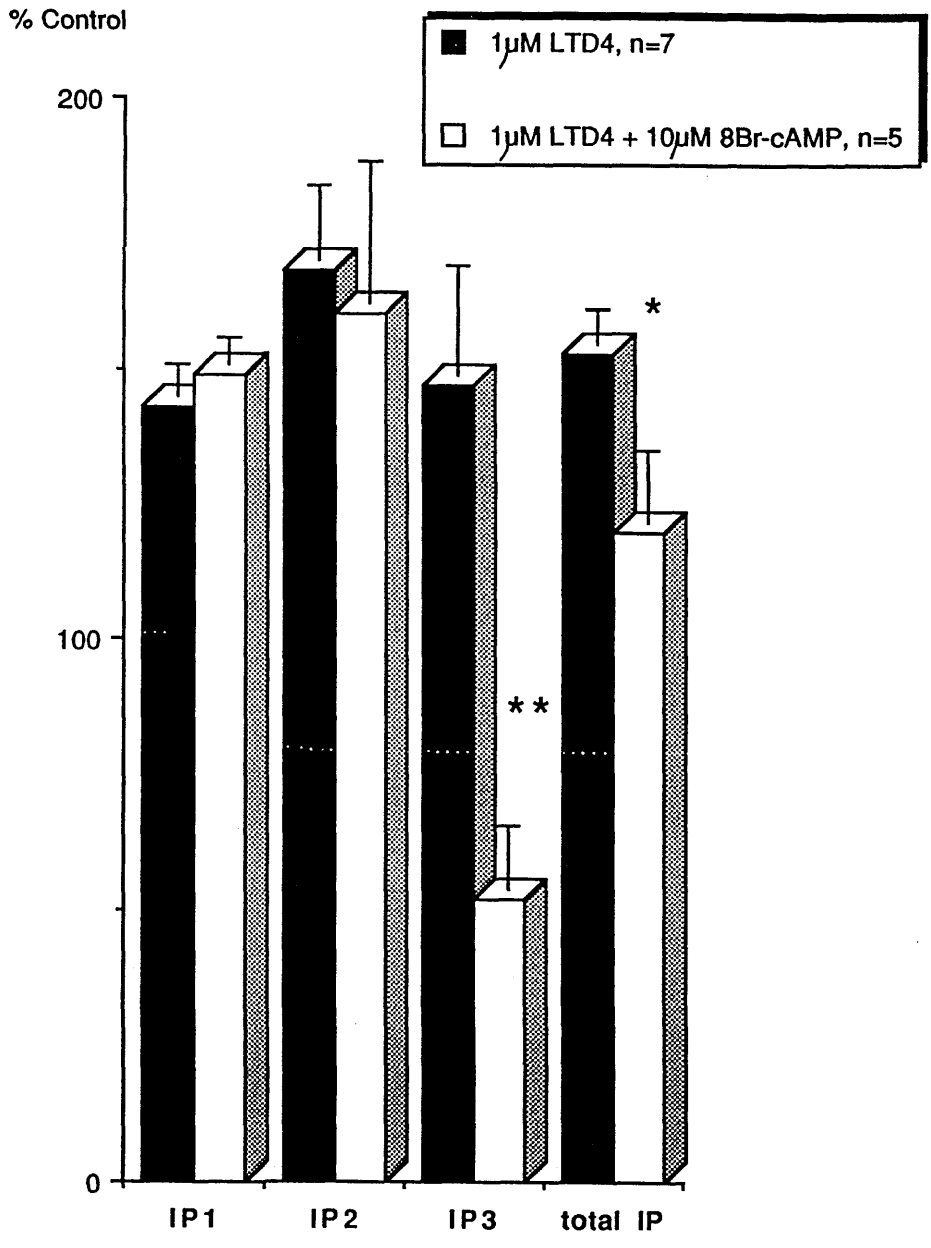


Figure 28: The effect of 10μM 8bromo-cyclic AMP on Leukotriene D4-induced inositol polyphosphate release. Data expressed as means±s.e. of the means, (* $p<0.05$, ** $p<0.001$).

cAMP leads to a statistically significantly inhibition of IP_1 ($p<0.01$), IP_3 ($p<0.01$) and total IP ($p<0.001$) release. Similarly, when LTD_4 incubation was carried out in the presence of 100μM db-cAMP a statistically significant inhibition of total IPs ($p<0.05$) was observed as seen in Figure 30. Both cAMP analogues inhibited total IP release, although the effect was more marked with 8Br-cAMP ($p<0.001$) than with db-cAMP ($p<0.05$).

To test whether this inhibitory effect of cAMP analogues was mediated via protein kinase A (PKA), the cAMP-dependent protein kinase, 8Br-cAMP-mediated inhibition of LTD_4-induced IP release was carried out in the presence of PKA inhibitor at a concentration of 20μM. It can be seen from Figure 31 that PKA inhibitor does not alleviate 8Br-cAMP-mediated inhibition of LTD_4-induced IP release significantly.

3.3.5 **Discussion**

The last few years have seen considerable progress in our appreciation of the pivotal role that inositol phospholipids perform in intracellular signalling processes. It is now well accepted that stimulation of inositol phospholipid metabolism by agonists causes the hydrolysis of PIP_2, by PIC, to produce two intracellular messengers, DAG and IP_3 (158,337). DAG interacts with PKC causing its translocation to the membrane domain and its activation and hence phosphorylation of various target proteins within the cell (283). IP_3, however, binds to a membrane-bound intracellular receptor which is believed to reside on the membranes of the smooth endoplasmic reticulum (196). Occupancy of this receptor releases calcium into the cytosol which may, however, involve a G-protein (338). The rise in $[Ca^{2+}]_i$ triggered by this route is transient, but undoubtedly suffices to elicit the rapid changes that calcium dependent processes undergo. There are two points at which the phosphatidylinositol (PI) cycle could affect Leukotriene biosynthesis. The enhanced $[Ca^{2+}]_i$ brought about by IP_3 could lead to PLA_2 activation and arachidonic acid release, which then could undergo 5-Lipoxygenase metabolism to generate Leukotrienes. The other point involves further metabolism of DAG by DAG lipase, which releases arachidonic acid from the sn-2 position of DAG with the concomitant formation of monoacylglycerol (see Section 1.2.1 and Figure 3) (339). The free arachidonic acid could then undergo metabolism via the 5-lipoxygenase pathway to yield Leukotrienes.

The inflammatory agonists which were tested for activation of PIC in RBL-1 cells, have been shown to act through the phosphoinositide signalling

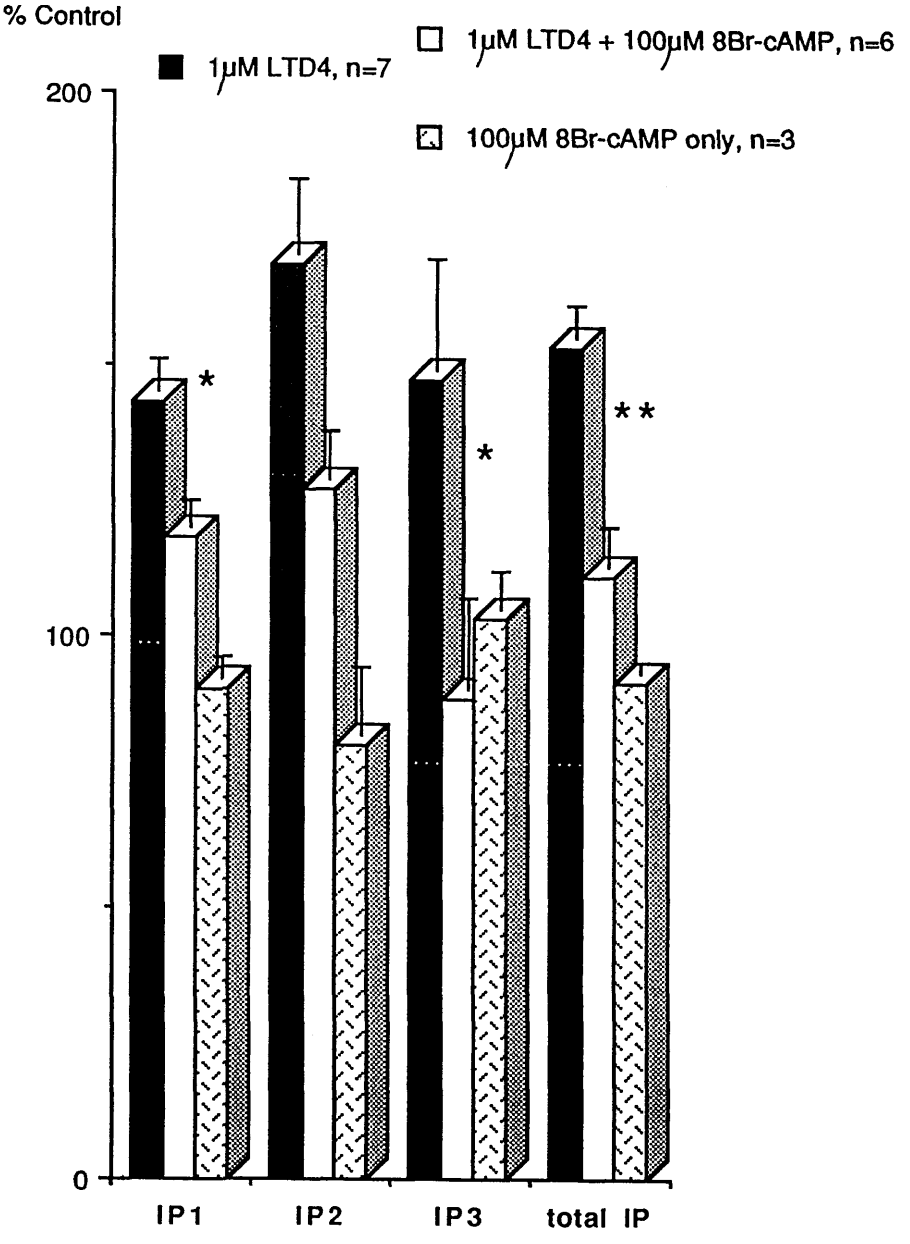


Figure 29: The effect of 100μM 8bromo-cyclic AMP on Leukotriene D4-induced inositol polyphosphate release. Data expressed as means±s.e. of the means (* $p<0.01$, ** $p<0.001$).

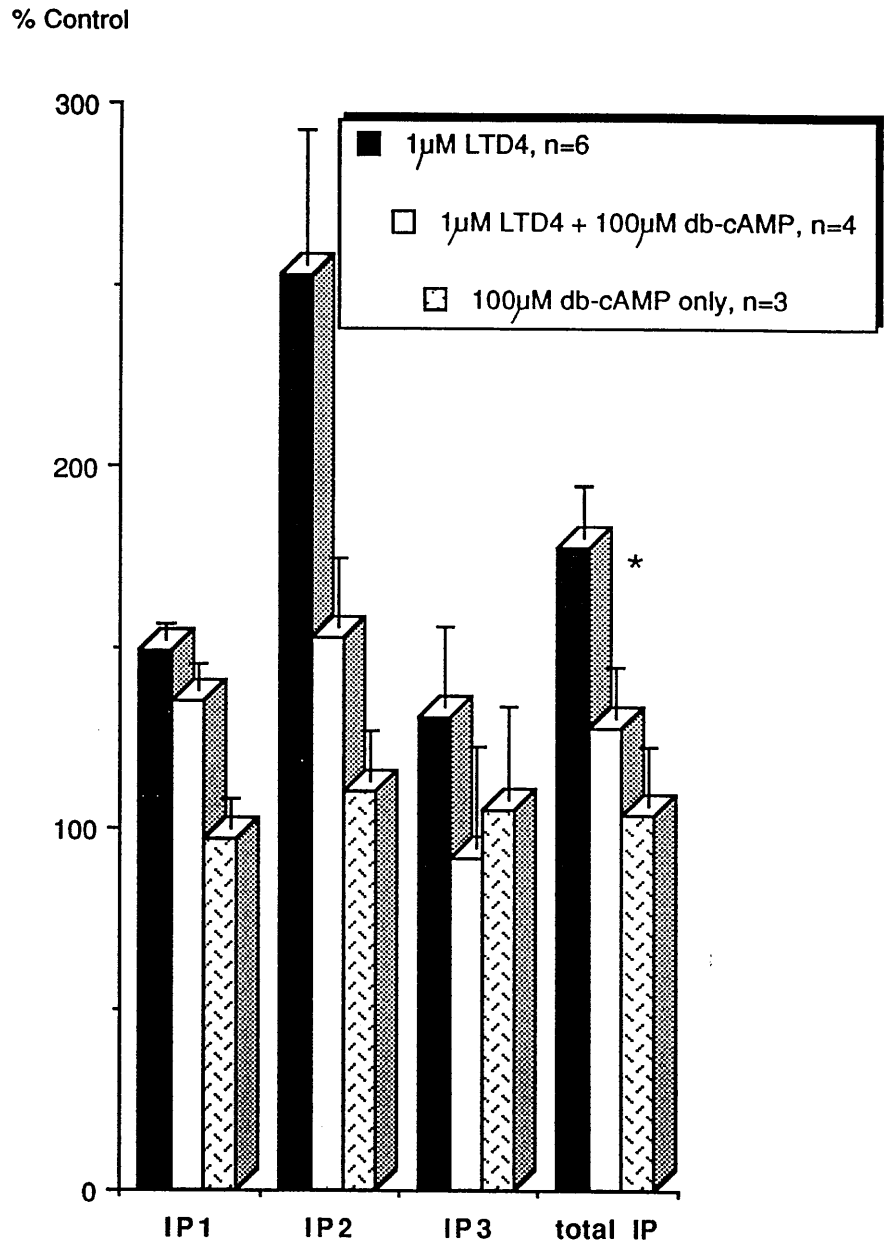


Figure 30: The effect of dibutyryl-cyclic AMP on Leukotriene D4-induced inositol polyphosphate release. Data expressed as means±s.e. of the means, (* $p<0.05$).

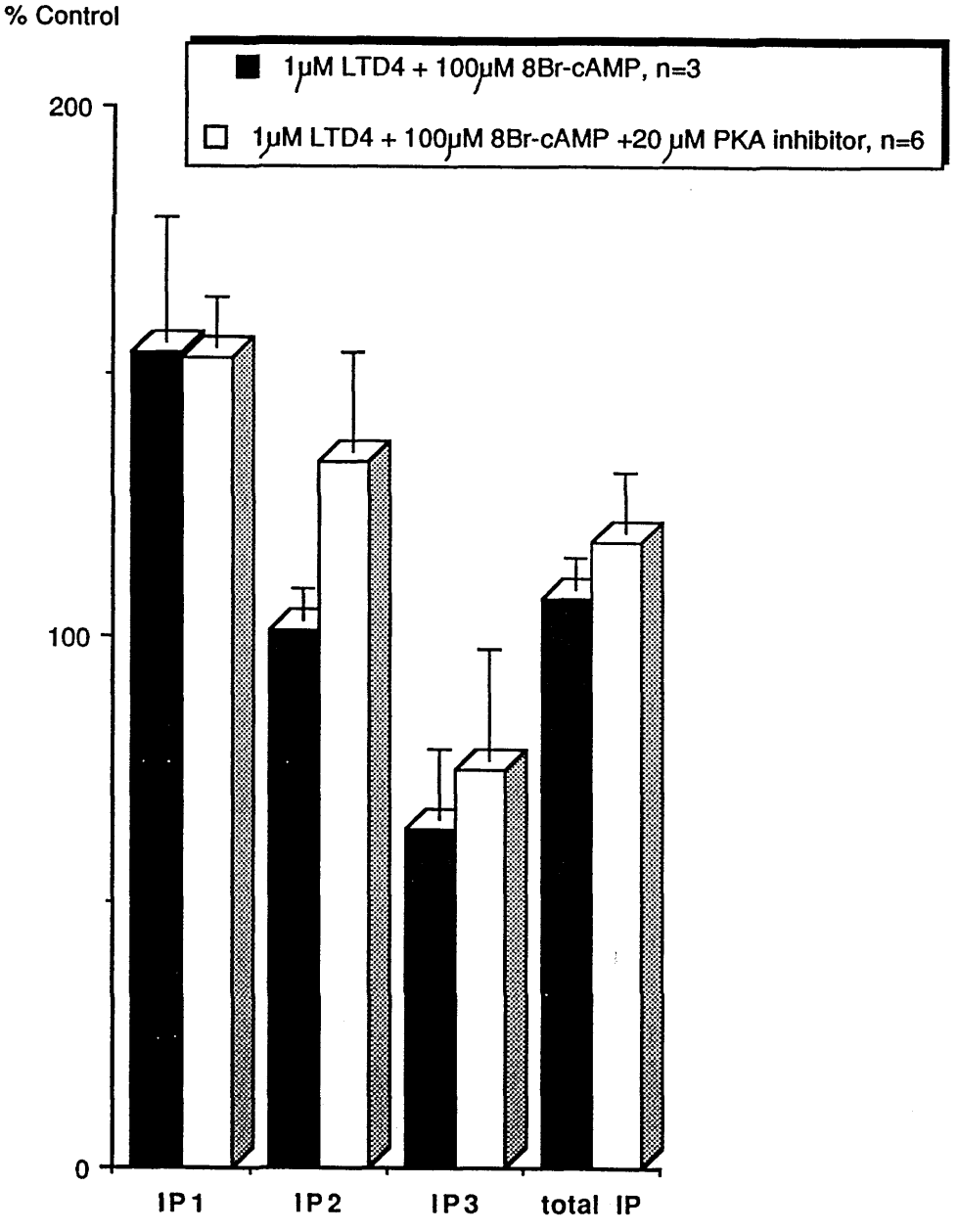


Figure 31: The effect of protein kinase A inhibitor on 8bromo-cyclic AMP-mediated inhibition of Leukotriene D4-induced inositol polyphosphate release. Data expressed as means±s.e. of the means.

pathway in other systems (317-320). These agonists (fMLP, substance P, PAF, histamine, bradykinin and mellitin) were used here at two different concentrations, and at both of these concentrations no significant amounts of IP_1, IP_2, IP_3 and total IP were found (Figures 22 and 23). The lack of effect shown by these agonists would imply that receptors for these mediators are not present on RBL-1 cell membranes. This is further supported by the lack of stimulatory effect by these mediators upon PLA_2 and Leukotriene biosynthesis observed in Sections 3.1.2 and 3.2.2. RBL-1 cells do produce histamine and PAF, both of which did not lead to any PIC activation. However, a conclusive finding with respect to the presence/absence of receptors for these agonists in the RBL-1 cell model can only be reached if radioligand binding assays are performed for each agonist on the RBL-1 cell membranes, for which there was insufficient time in this thesis program.

When RBL-1 cells were incubated in the presence of 1μM LTD_4, in agreement with independent findings (277), we observed a significant release of [^{3}H]-IP_1,-IP_2,-IP_3 and -total IP, as can be seen in Figure 24. LTD_4 receptors on RBL-1 cell membranes are clearly coupled to PIC activation, and receptor activation by LTD_4 binding is thus shown to lead to phosphoinositide breakdown and inositol polyphosphate (IP) formation.

Receptor activation of adenyl cyclase and the concomitant enhancement of intracellular cAMP levels by PGE_2, PGI_2 and forskolin have been previously reported (334,335,340). However, pre-incubation of RBL-1 cells with PGI_2 and PGE_2 did not have any effect upon LTD_4-induced phosphoinositide hydrolysis shown in Figures 25 and 26. This lack of effect could be due to the absence of receptors for these agonists on RBL-1 cell membranes; no reports indicating the presence of these receptors on RBL-1 cell membranes were subsequently found in the literature. Again ligand binding assays could be performed to be certain of this.

Forskolin, which is believed to interact with and activate adenyl cyclase directly (340), did not lead to any significant inhibition of LTD_4-induced phosphoinositide breakdown in the data shown in Figure 27. When RBL-1 cells were incubated with forskolin alone at 100μM concentration, no change in the level of IPs were observed with respect to control (Figure 27). During the work in preparation for this thesis, it has been suggested that forskolin is not a specific adenyl cyclase activator, and that it has multiple sites of action (for a review see 336). It is therefore possible that forskolin is modulating some other site independent of adenyl cyclase. We therefore decided to use membrane permeable cAMP analogues to study cAMP-mediated responses on LTD_4-

induced phosphoinositide hydrolysis, and 8Br-cAMP and db-cAMP were chosen.

It can be observed from Figure 28 that 8Br-cAMP at a concentration of 10μM leads to a statistically significant inhibition of IP_3 ($p<0.001$) and total IPs ($p<0.05$). Similarly, 8Br-cAMP at 100μM concentration led to a statistically significant inhibition of IP_1 ($p<0.01$), IP_3 ($p<0.01$) and total IPs ($p<0.001$) while 100μM db-cAMP led to a statistically significant inhibition of total IPs ($p<0.05$) as can be seen in Figures 29 and 30, thus demonstrating that elevation of the $[cAMP]_i$ level, simulated by having high concentrations of cAMP analogues, counteracts PIC activation in RBL-1 cells. db-cAMP is known to be less stable than 8Br-cAMP and that it degrades to butyryl-cAMP and butyrate which could explain why it was not as potent as 8Br-cAMP in the data shown in Figure 30. For the first time we have shown that cAMP analogues inhibit LTD_4-induced phosphoinositide hydrolysis in RBL-1 cells, thus extending to RBL-1 cells the types of systems where elevated intracellular cAMP levels counteract PIC activation.

It is well documented that cAMP is an important inhibitory regulator of platelet function, which antagonises Ca^{2+}-dependent cellular processes (328,341) and PIC-mediated membrane PIP_2 hydrolysis in platelets and in other tissues (240,327-330,342). These findings, together with the data determined here, suggest a negative modulatory role for cAMP upon the phosphoinositide signalling system, at least on LTD_4-induced phosphoinositide breakdown in RBL-1 cells. The exact mechanism by which cAMP blocks phosphoinositide turnover is unknown. In addition to the effect of cAMP upon PI kinase, an inhibition of PIC by cAMP has been proposed (95,105), although no direct effect by cAMP upon purified PIC, as assayed *in vitro*, has been shown.

Alternatively, it has been suggested that cAMP may act by preventing the rise in $[Ca^{2+}]_i$ necessary for a response to stimulation (343). Also, it has been reported that the increase in cAMP prevents the agonist-induced activation of PIC and that this is mediated by protein kinase A (the cAMP dependent protein kinase, PKA), but its mechanism is poorly understood (253,344,345). Figure 31 illustrates the effect of PKA inhibitor upon 8Br-cAMP-induced inhibition of LTD_4-stimulated IP release. As can be seen, PKA inhibitor does not significantly alleviate the inhibition of LTD_4-induced IP release caused by 100μM 8Br-cAMP. This could be due to PKA inhibitor, a polypeptide, not being able to enter the intact cell and therefore not able to relieve the kinase of its possible inhibitory action. The inhibitory effect of PKA

inhibitor has only been shown in lysed cell preparations (346). Other possibilities include inhibition of PI kinase (330-332), therefore affecting conversion of PI to PIP and PIP_2, and inhibition of the rise in the $[Ca^{2+}]_i$ level necessary for a stimulatory response (343).

3.4 The Effect of Ionophore on Labelled Inositol Phosphate Formation

3.4.1 *Introduction*

The calcium ionophore A23187 has been used widely to induce calcium-dependent secretion from cells on the assumption that permeabilization of the cell membranes to Ca^{2+} ions leads to a rise in $[Ca^{2+}]_i$, which in turn serves as a signal for secretion. In this way, events that precede mobilization of Ca^{2+} ions via receptor stimulation are by-passed. One such event is thought to be the rapid hydrolysis of membrane inositol phospholipids to generate IP_3 and DAG. It has been suggested that A23187 causes a small but significant stimulation of phosphoinositide breakdown in RBL-2H3 cells (165,347), and that the exocytosis in response to A23187 is closely related to this hydrolysis of inositol phospholipids (347). However, in contradiction with the above observation, it has been reported that A23187 has no effect at all on inositol phospholipid hydrolysis in PC-12 cells (350), suggesting that A23187-induced phosphoinositide breakdown is tissue specific.

In view of the stimulatory effect of A23187 upon phosphoinositide breakdown in RBL-2H3 cells, it was decided to investigate the effect of A23187 on phosphoinositide breakdown in RBL-1 cells.

3.4.2 Ionophore-Induced Phosphatidylinositol Breakdown and Inositol Phosphate Release

When RBL-1 cells, pre-labelled with $[^3H]$-myo-inositol, were incubated with A23187 at 10μM concentration and for 30 minutes, as can be seen in Figure 32, a small increase in labelled IP_1 and IP_3 with respect to control was observed. However, when the incubation time and the A23187 concentration were increased to 45 minutes, and 20μM concentration respectively, a much higher and more significant effect was seen on IP_1, IP_2, IP_3, and total IP as shown in Figure 32. The cells were tested for A23187 toxicity at 20μM and 40μM ionophore concentration, and at both concentrations the cells were

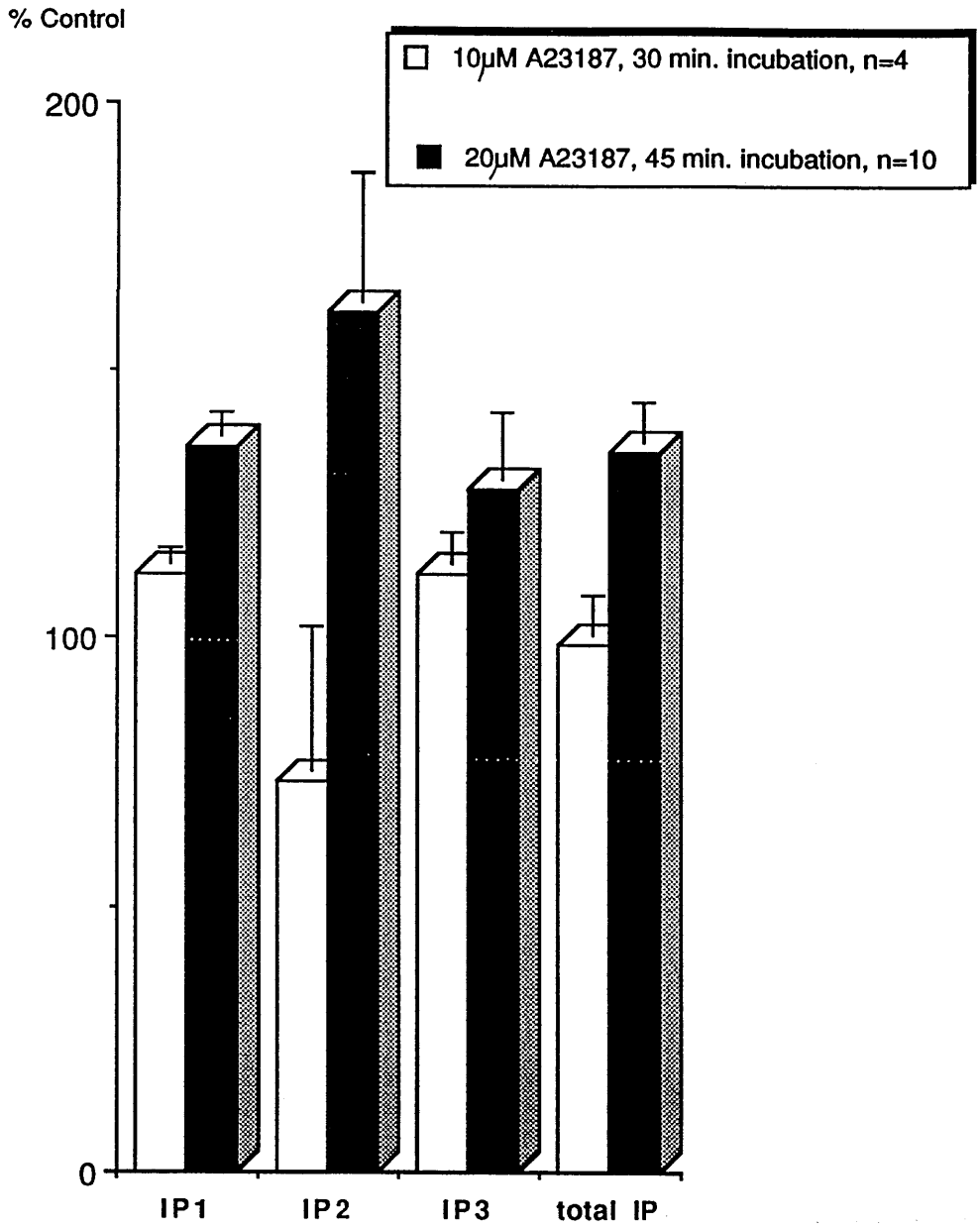


Figure 32: Ionophore-induced inositol polyphosphate release. Data expressed as means±s.e. of the means.

found to be more than 80% viable (normal viability) up to 1.5 hours after A23187 addition (using the trypan blue dye-exclusion test).

In view of the inhibitory effect of 8Br-cAMP on LTD_4-induced IP release, the effect of 8Br-cAMP on A23187-induced IP release was tested. RBL-1 cells, pre-labelled with [^{3}H]-myo-inositol, were incubated with A23187 at 20μM concentration and for 45 minutes in the presence of 100μM 8Br-cAMP. Some inhibition was observed as seen in Figure 33, but this inhibitory effect was not statistically significant.

3.4.3 Effect of 5-Lipoxygenase Inhibitor on Ionophore-Induced Inositol Phosphate Release

From Figure 32, it can be seen that 10μM A23187 leads to a small release of IPs, but when the A23187 concentration and incubation time are increased to 20μM and 45 minutes respectively, a significant release of IPs is observed. This implies that the ionophore-induced PI lipid turnover could be mediated via a process other than just an increase in $[Ca^{2+}]_i$ level in RBL-1 cells, since it has been shown that (a) the threshold concentration for stimulation of PI lipid breakdown by A23187 was approximately 0.1μM, and (b) that 0.1-1μM A23187 was sufficient to increase $[Ca^{2+}]_i$ level to 1μM, a concentration high enough to cause activation of PIC (347). But, a significant IP release is observed only when the A23187 concentration was increased to 200 fold that of the reported threshold concentration, and the incubation time to 45 minutes, in RBL-1 cells. We therefore postulated that the observed A23187-induced PI lipid breakdown in RBL-1 cells was not merely due to increased $[Ca^{2+}]_i$ level, but to some other intracellular signal. An experiment to test this postulate was therefore designed.

It was seen in Sections 3.1.4 and 3.2.4 that A23187 at a concentration of 10μM leads to a significant increase in Leukotriene generation and we have shown in Section 3.3.3 that 1μM LTD_4 was sufficient to induce PI lipid hydrolysis and IP release. Therefore, it was reasonable to deduce that the intracellular signal deduced above could very well be a metabolite of the 5-lipoxygenase pathway, such as LTD_4, since the A23187-induced $[Ca^{2+}]_i$ level (>1μM) is high enough to induce PLA_2 activation, arachidonic acid release and Leukotriene generation. Hence, RBL-1 cells were incubated with compound AA861 prior to A23187 stimulation. AA861, a derivative of benzoquinone, selectively inhibits the 5-lipoxygenase enzyme (349).

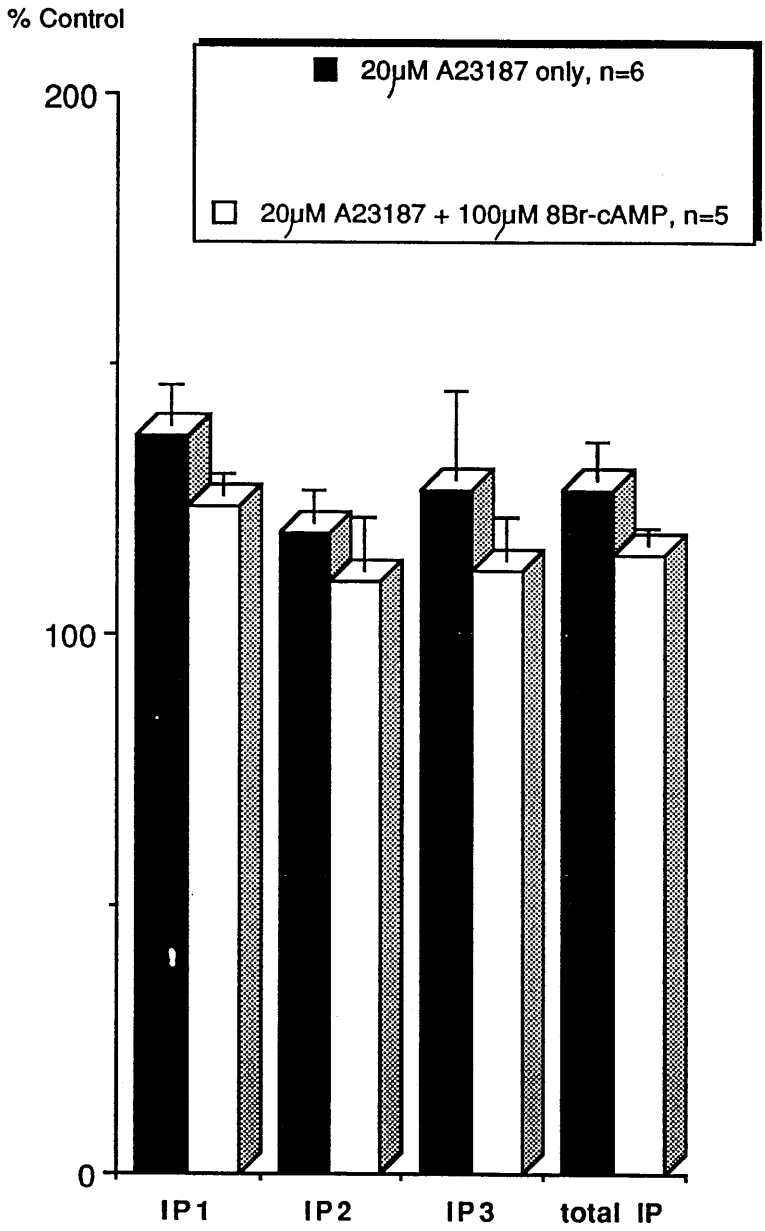


Figure 33: The effect of 8bromo-cyclic AMP on ionophore-induced inositol polyphosphate release. Data expressed as means±s.e. of the means.

RBL-1 cells, pre-labelled as described previously, with [^{3}H]-myo-inositol at a radioactivity concentration of 10μCi/ml were incubated with the 5-lipoxygenase inhibitor AA861 at a concentration of 100μM and a pre-incubation time of five minutes. After the five minute pre-incubation with AA861, A23187 at 20μM concentration was added and the incubation was continued for 45 minutes at 37°C. The various IPs were separated as described previously, and the data is reported in Figure 34. Clearly, it can be seen from Figure 34 that AA861 at a 100μM concentration inhibits A23187-induced IP release. AA861 causes a statistically significant inhibition of IP_1 ($p<0.02$), IP_3 ($p<0.05$) and total IP ($p<0.05$).

3.4.4 Effect of LTD_4 Antagonist on Ionophore-Induced Inositol Phosphate Formation

Having shown that A23187-induced PI lipid turnover is mediated by a 5-lipoxygenase metabolite(s), it was decided to address the question, whether p-Leukotrienes are acting as the intracellular signal for A23187-induced PI lipid turnover, since the earlier experiments had shown that LTD_4 receptor activation leads to PI lipid turnover in RBL-1 cells (Section 3.3.3). To carry out our investigation, FPL-55712 (Sodium-7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxyperoxy]-4-oxo-8-benzopyran-2-carboxylate) was used, which had been shown to be a selective antagonist of SRS-A as early as 1973 (118), and was therefore the first Leukotriene receptor antagonist. In general, FPL-55712 has been shown to antagonise the actions of p-Leukotrienes, showing comparable potencies as a competitive antagonist against LTC_4, LTD_4 and LTE_4 activity (350). It has also been shown to potently inhibit the LTD_4-induced bronchoconstrictor response in monkeys (251).

Therefore, RBL-1cells at a cell number of 10^8 cells/ml, pre-labelled with 10μCi/ml of [^{3}H]-myo-inositol, were pre-incubated for 2 minutes with FPL-55712 at 10μM. Then, A23187 at 20μM concentration was added and the incubation was carried out for 45 minutes. A marked inhibition of A23187-induced IP release was observed, as seen in Figure 35. FPL-55712 at 10μM concentration shows a statistically significant inhibition of IP_1 and total IP release ($p<0.001$ and $p<0.02$ $n=4$, respectively). Incubation of RBL-1 cells with FPL-55712 alone, at 10μM concentration and for 45 minutes had no effect at all upon basal IP levels as shown in Figure 35.

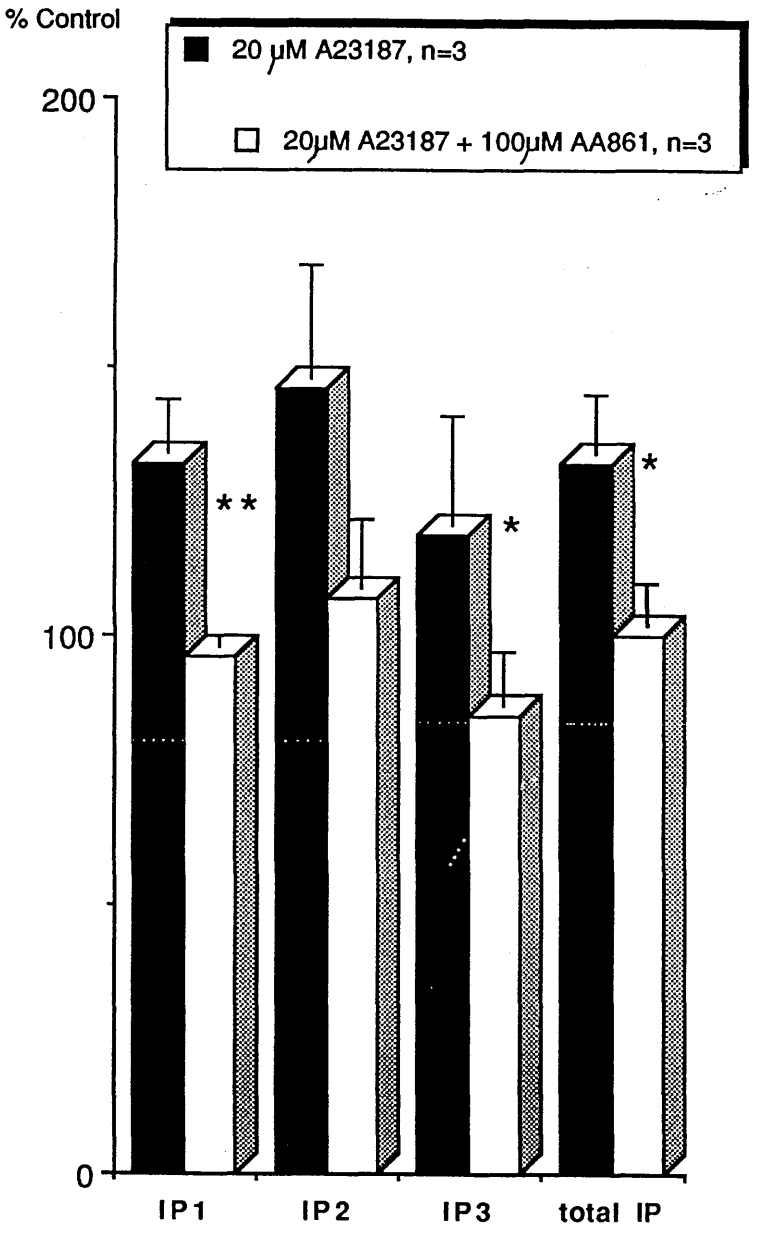


Figure 34: The effect of 5-lipoxygenase inhibitor, AA861, on ionophore-induced inositol polyphosphate release. Data expressed as means±s.e. of the means, (* $p<0.05$, ** $p<0.02$).

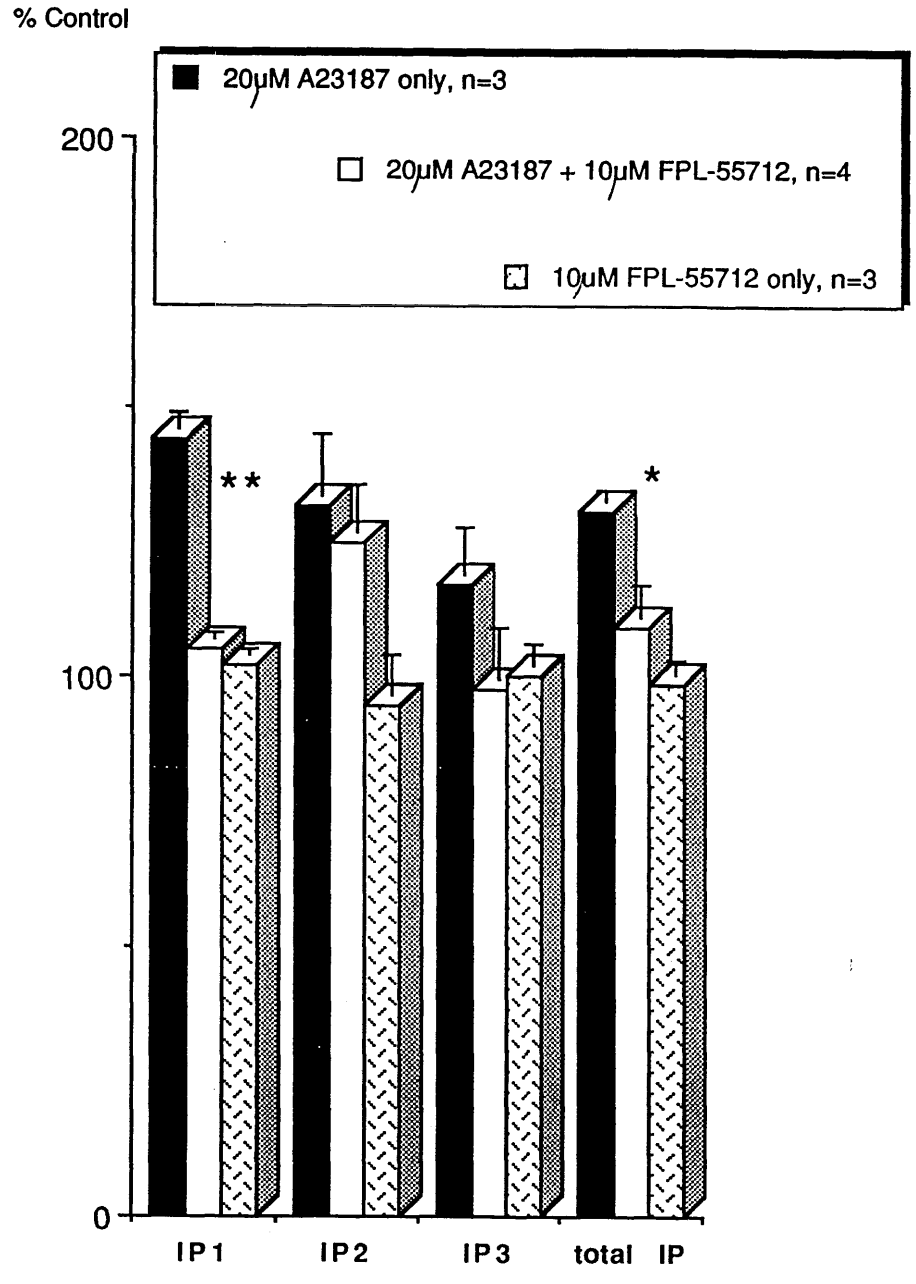


Figure 35: The effect of LTD4 receptor antagonist, FPL-55712, on ionophore-induced inositol polyphosphate release (* $p<0.02$, ** $p<0.001$).

Finally, RBL-1 cells were incubated with LTD_4 in the presence of FPL-55712 to test the antagonistic effect of FPL-55712 upon LTD_4-induced IP release, this is shown in Figure 36.

3.4.5 **Discussion**

Initial studies with A23187 by Foreman et al. (351) provided the first convincing evidence that recruitment of Ca^{2+} ions can stimulate exocytosis in mast cells. It has been widely assumed since then, that calcium ionophores intercalate in membrane lipids, and act as diffusible calcium carriers that permit redistribution of Ca^{2+} ions as dictated by the concentration gradients of free Ca^{2+} ions. The increase in $[Ca^{2+}]_i$ could thus be attributed to enhanced permeability of the plasma membrane to high concentrations of external Ca^{2+} ions and to the release of calcium from mitochondrial and other membrane-bound calcium stores. In this way, events that precede mobilization of Ca^{2+} ions via receptor stimulation are by-passed. Although, this model is probably overly simplistic in view of the lack of a calcium response in ATP-depleted cells (347), calcium ionophores have been used extensively to stimulate Ca^{2+} dependent cellular processes. It is believed that Ca^{2+} ionophores lead to Ca^{2+} mobilization from both external environment and intracellular Ca^{2+} pools.

Previous studies with A23187 in a variety of cells have produced contrary results with respect to A23187-induced PI hydrolysis (350). When, in studies elsewhere, A23187 did not seem to have any effect, both radiolabelled inositol lipids and water soluble hydrolysis products were estimated; it was apparent that the ionophore does stimulate hydrolysis of inositol lipids (347). Stimulation by A23187 has been reported in some cases to lead to the formation of just inositol mono- and bisphosphate (352) and in other cases to IP_1, IP_2 and IP_3 (347). Moreover, it has been reported in RBL-2H3 cells that 0.1μM of A23187 stimulates the hydrolysis of PI lipids, and at this A23187 concentration, the $[Ca^{2+}]_i$ level was estimated to be greater than 1μM (347). This would suggest that the hydrolysis of PI lipids could be due to the great enhancement in the $[Ca^{2+}]_i$ level.

In the studies presented in this thesis, it was found that the effect of A23187 on IP release in RBL-1 cells was apparent and significant only using a high concentration (20μM), and an incubation time longer than that used for LTD_4-induced IP release (45 minutes). Under these conditions the A23187-induced PI breakdown reach a marked extent as seen in Figure 32. However,

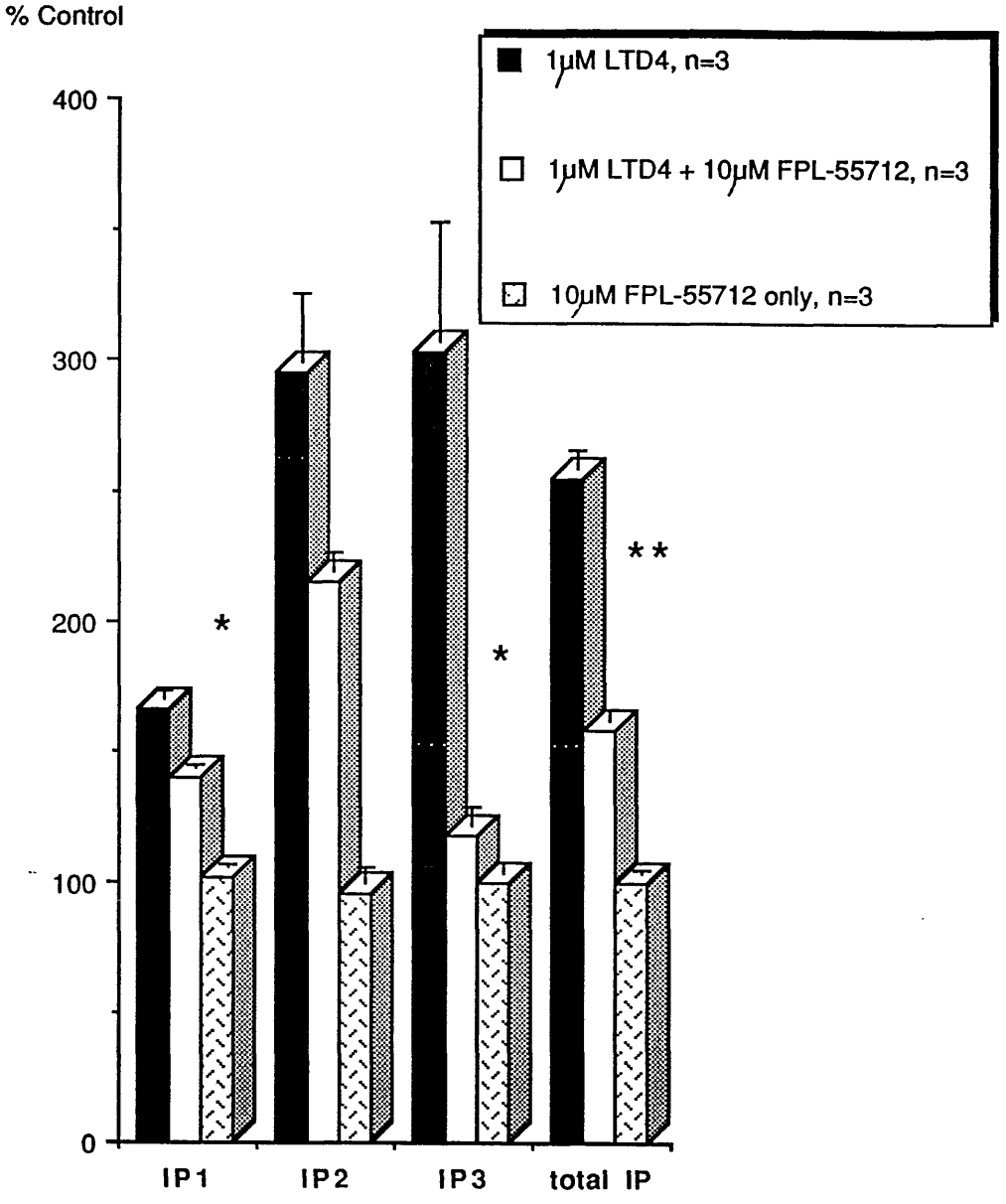


Figure 36: The effect of Leukotriene D4 receptor antagonist, FPL-55712, on Leukotriene D4-induced inositol polyphosphate release.
Data expressed as means±s.e. of the means, (* $p<0.02$, ** $p<0.001$).

10μM ionophore concentration and a 20 minutes incubation time are the well established conditions for a maximal effect of A23187 on Leukotriene biosynthesis in RBL-1 cells (94). This suggests that the effect of A23187 on IP formation might be due to, firstly, activation of PLA_2 and arachidonic acid release from phospholipids, and secondly, biosynthesis of LTD_4. The data presented in Section 3.3.3, Figure 24, provide convincing evidence that if formed, LTD_4 leads to PI lipid hydrolysis.

In order to substantiate this hypothesis, the incubation was conducted in the presence of a 5-lipoxygenase inhibitor, AA861, and indeed it was observed that the latter significantly reduced A23187-induced IP_1 ($p<0.02$), IP_3, ($p<0.05$) and total IP ($p<0.05$) formation as seen in Figure 34, thus showing that the effect of A23187 upon PI breakdown and IP formation is most probably mediated on the A23187-induced IP formation by 5-lipoxygenase metabolites. In Section 3.3.3 it was demonstrated that LTD_4 is active in inducing PI breakdown in RBL-1 cells and thus experiments were then designed to see the effect of the LTD_4 antagonist FPL-55712 on A23187-induced IP release. At 10μM concentration, FPL-55712 virtually abolished A23187-induced IP_1 release and significantly reduced total IP formation, as shown in Figure 35. This implies that the effect of A23187 upon IP release is mediated by LTD_4 and/or the other p-Leukotrienes. To substantiate the antagonistic effect of FPL-55712 on LTD_4 receptors in RBL-1 cells, the cells, pre-labelled with [^{3}H]-myo-inositol, were incubated with 10μM FPL-55712 for 2 minutes prior to the addition of 1μM LTD_4. The incubation was then continued for 30 minutes, and IP release was measured as previously described. Figure 36 illustrates the inhibitory effect of 10μM FPL-55712 on LTD_4-induced IP release. As can be seen from Figure 36, FPL-55712 leads to a statistically significant inhibition of IP_1 ($p<0.02$), IP_3 ($p<0.02$) and total IP ($p<0.001$).

Although 8Br-cAMP was shown to significantly reduce LTD_4-induced IP formation, it did not show any statistically significant effect on A23187-induced IP formation. This could be due to the enhanced intracellular Ca^{2+} concentration, brought about by A23187, having an opposing effect upon the 8Br-cAMP-mediated inhibition.

3.5 Adenosine Receptor-Induced Phosphatidylinositol Hydrolysis

3.5.1 *Introduction*

A modulatory role for adenosine in mast cells was first reported by Maquardt *et al.* when they observed that adenosine enhanced A23187-induced histamine release from rat peritoneal mast cells (353). Subsequent studies have confirmed this modulatory role of adenosine when immunological activation was used (354). Furthermore, in human basophils, adenosine receptor activation is known to modulate mediator release (303).

Recently, it has been shown that there are two pharmacologically characterized purinergic binding sites; P_1- at which adenosine and AMP act and are linked to the adenyl cyclase effector system, and P_2- at which ATP and ADP act and are linked to the PIC effector system (355-358). Adenosine receptors (P_1-purinergic receptors) of which there are two major subclasses, A_1 and A_2, are ubiquitously distributed in tissues and cultured cell lines (359). These receptors are further distinguished by their different affinities for various metabolically stable analogues of adenosine. A rank order of potency or binding affinity of NECA > adenosine > R-PIA (R-phenylisopropyladenosine) is characteristic of an interaction with the A_2 receptor subtype, and the reverse order implies interaction with the A_1 receptor subtype (307). The A_1 receptor subtype has been implicated in the inhibition of adenyl cyclase and reduction in Ca^{2+} channel activity while the A_2 subtype is reported to mediate adenyl cyclase activation and an increase in cAMP level (360).

Basophils, as mentioned above, possess surface receptors for adenosine that can influence the secretory response to immunological and non-immunological stimuli. Adenosine analogues suppress the release of inflammatory mediators from blood basophils by interacting with both A_2 receptor subtypes on the cell membrane and intracellular purinergic sites. This suppression is associated with an increase in the cAMP level (361,362).

In view of this finding and the modulatory role that adenosine plays in the inflammatory response, it was decided to investigate the effect of the metabolically stable and more potent adenosine analogue, NECA, as this had not been previously studied, in RBL-1 cells.

3.5.2 NECA-Induced Phosphatidylinositol Hydrolysis

During the course of this thesis work, it has been suggested by Hydar Ali *et al.* (363) that a category of adenosine receptors exist that are distinctly different from the A_1/A_2 adenosine receptor subtypes, or from the P_2-purinergic receptors (364). Unlike A_1/A_2 adenosine receptor subtypes, the adenosine receptors in an RBL-2H3 clone have the characteristics of being coupled by a G-protein, similar to G_t (transducin, the G-protein involved in phototransmission signalling in rod outer segments of the eye), to PIC, rather than by G_i (inhibitory) or G_s (stimulatory) G-proteins to adenyl cyclase. Furthermore, the conventional antagonist of A_1/A_2 adenosine receptor subtypes, theophyline, was reported not to block the responses of RBL-2H3 cells to NECA, and although 8-phenyltheophyline does block the responses to NECA, it does so in a non specific and nonselective manner (363). We tried to investigate the presence of such adenosine receptors in our test model, the RBL-1 clone using NECA.

RBL-1 cells, pre-labelled with [^{3}H]-myo-inositol (10μCi/ml), were subjected to a time course incubation with NECA at 10μM concentration up to 20 minutes to see whether the IP release in RBL-1 cells confirmed the pattern observed in an RBL-2H3 clone by Hydar Ali *et al.* (363). Following incubations with 10μM NECA, the IPs released were measured at 0, 2, 5, 10, and 20 minutes and they were separated using anion-exchange chromatography as described previously. [^{3}H]-IPs were detected using scintillation counting. In this time course experiment, NECA at 10μM concentration, produced an enhancement in IP levels over a period of 20 minutes as shown in Figure 37. IP_3 is significantly released and reaches 100% change with respect to basal level within 2 minutes of incubation of RBL-1 cells with NECA, reaching a plateau at 20 minutes as seen in Figure 37. This is in disagreement with previous findings (363), whereby both inositol (1,4,5)P_3 and inositol (1,3,4)P_3 enhancement return to basal levels within 10 minutes.

IP_2 release, in agreement with Hydar Ali (363), reaches a plateau at 2.5 minutes. However, in the experiments reported here, IP_2 formation remains at this peak for up to 10 minutes after which the IP_2 level increases two fold up to 20 minutes as seen in Figure 37. This latter increase in IP_2 is again in contradiction with published data in RBL-2H3 cells (363), whereby the IP_2 level was reported to decline following the peak at 2.5 minutes, reaching basal levels within 20 minutes. Also contrary to Hydar Ali *et al.* (363), we could not observe an IP_1 peak at 2.5 minutes. In fact, no enhancement in IP_1 release at

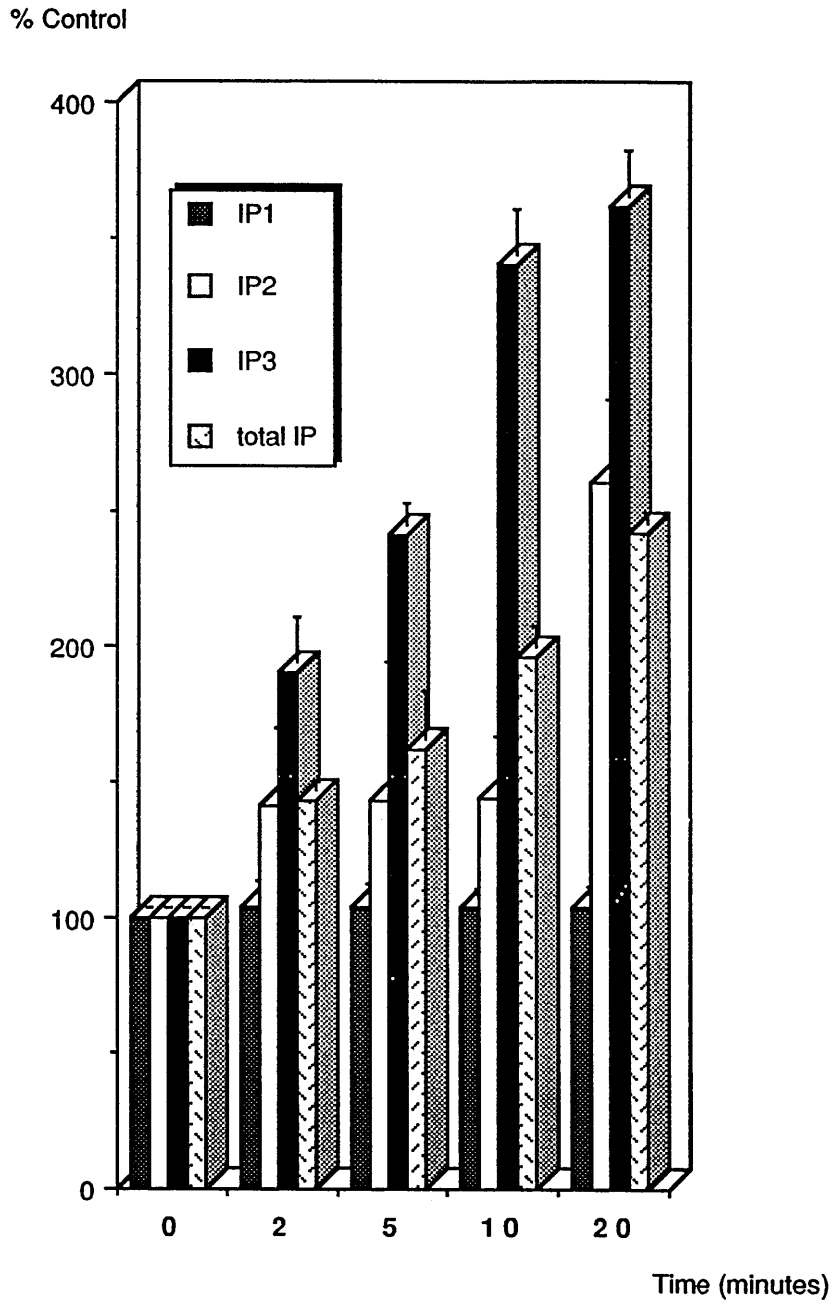


Figure 37: Time course of NECA-induced inositol polyphosphate release (n=3). Data expressed as means±s.e. of the means.

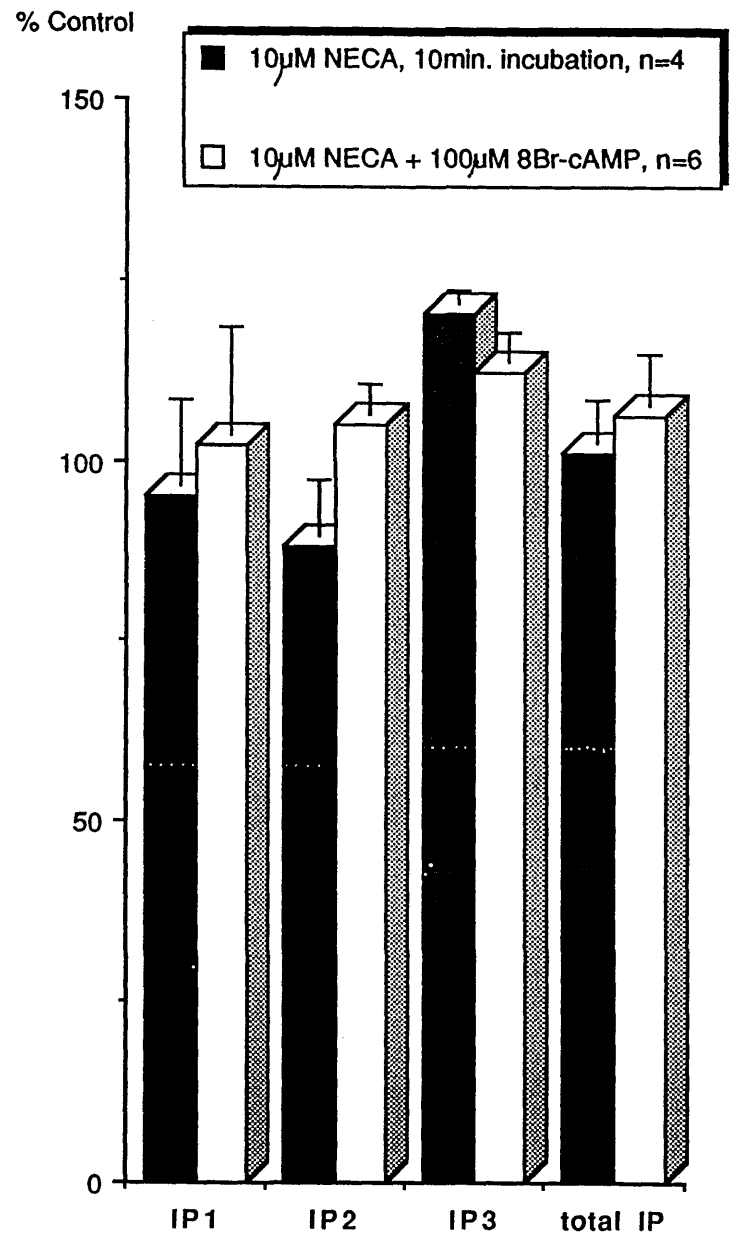


Figure 38: The effect of 8bromo-cyclic AMP on NECA-induced inositol polyphosphate release. Data expressed as means±s.e. of the means.

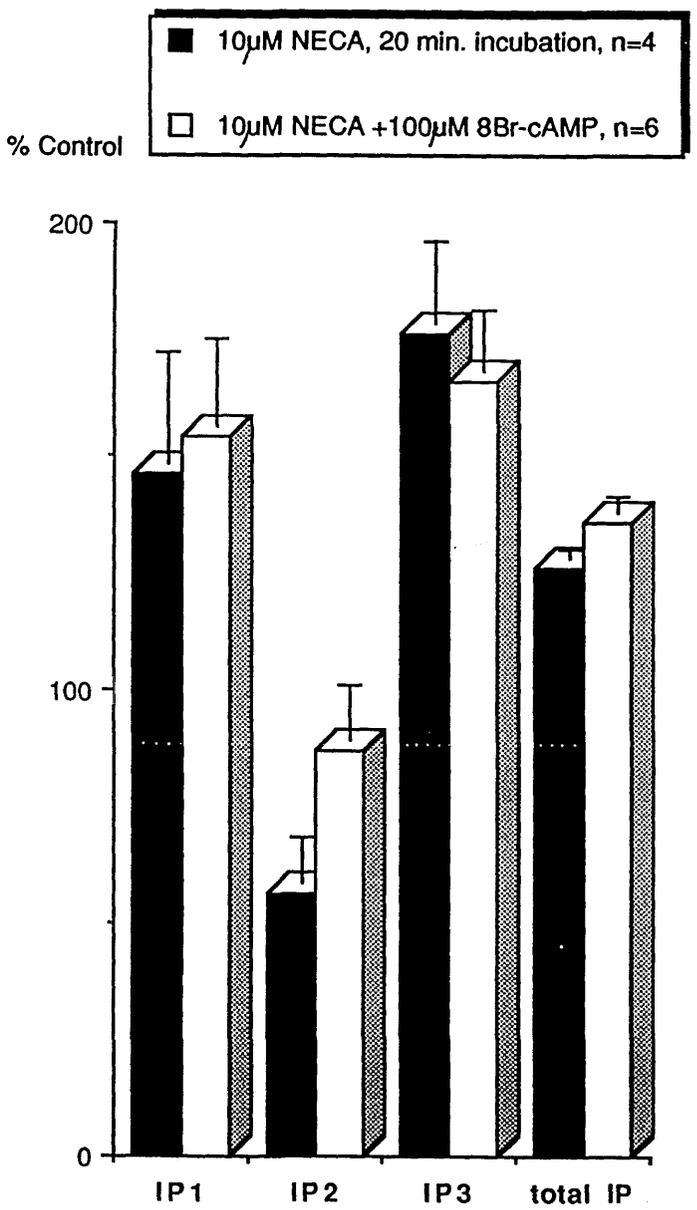


Figure 39: The effect of 8bromo-cyclic AMP on NECA-induced inositol polyphosphate release. Data expressed as means±s.e. of the means.

all was observed when RBL-1 cells were stimulated with NECA over the 20 minute experimental period as shown in Figure 37. Total IPs showed a continual enhancement in the IP level during the 20 minutes incubation time as shown in Figure 37. Furthermore, it can also be seen from Figure 37 that neither the IP_2 level, nor the IP_3 level declined over the experimental incubation time.

3.5.3 Effect of 8Br-cAMP on NECA-Induced Phosphatidylinositol Hydrolysis

LTD_4-induced IP release was shown in Section 3.3.4 to be inhibited when RBL-1 cells were incubated in the presence of 8Br-cAMP. To see whether 8Br-cAMP would have any effect upon NECA-induced IP release, it was decided to incubate RBL-1 cells, pre-labelled with 10μCi/ml of [^{3}H]-myo-inositol, in different experiments with 10μM NECA and in the presence of 8Br-cAMP at 100μM concentration for 10 and 20 minutes respectively. The data in Figures 38 and 39 illustrate that 8Br-cAMP does not have an overall consistent effect on NECA-induced IP release at either 10 or 20 minute incubation times in RBL-1 cells.

3.5.4 Discussion

It has been previously shown that adenosine attenuates mediator release in human basophils (303). This has been attributed to the presence of two subclasses of adenosine receptors, A_1 and A_2, which are linked to the adenyl cyclase effector system (one inhibitory and the other stimulatory) (see 360 for a review). Although there has been no direct demonstration that adenosine receptors are coupled with an effector system other than adenyl cyclase, during the work for this thesis, it has been independently reported that RBL-2H3 cells respond to adenosine receptor activation by a stable analogue of adenosine, NECA, that is more potent than adenosine as an agonist. This response involves PIC activation, a transient increase in IP levels and a substantial, but transient, increase in the $[Ca^{2+}]_i$ level by cholera- and pertussis-toxin-sensitive mechanisms that did not involve cAMP (363,365,367,368). NECA has been shown previously to be a selective A_2 antagonist (368), and the discovery that it induces PIC activation is quiet interesting, as it implies that there might be a novel subtype of adenosine

membrane receptor, at which NECA acts, and which is coupled to the PIC signalling system.

The stimulation of PI hydrolysis by NECA in washed, permeabilized RBL-2H3 cells is reported to be blocked by both cholera toxin and pertussis toxin (363). This implies that the NECA-sensitive receptor activated PIC via a GTP binding protein (G-protein). Therefore, unlike the A_1/A_2 adenosine receptor subtypes, the adenosine receptors in RBL cells have the characteristics of being coupled, by a G_t-like G-protein (i.e. cholera and pertussis toxin sensitive), to PIC, rather than by G_s or G_i to adenyl cyclase. Furthermore, it has been shown in RBL-2H3 cells that the conventional antagonists of A_1/A_2 adenosine receptor subtypes either do not block the responses to NECA (e.g theophyline) or do so in a non-selective manner (e.g 8-phenyltheophyline and 8-p-sulphophenyltheophyline) (171).

The possibility that the agonist acted at an intracellular purinergic site (361,362,370) is highly unlikely since, as mentioned before, NECA is not membrane permeable, and therefore must act at a plasma membrane binding site, and is not transported into the cells by adenosine uptake mechanisms (364). When RBL-1 cells were subjected to stimulation with NECA, a metabolically stable adenosine analogue, over a time period of 20 minutes, a significant release in IPs was observed as shown in Figure 37. The optimal incubation time for IP release during the experimental period was at 10 and 20 minutes. The data shown in Figure 37 indicate that in RBL-1 cells, NECA also induces PIC activation and PI hydrolysis. Our finding is in agreement with previous reports (363,365,366) and the presence of a novel subtype of adenosine receptor in RBL-1 cells is therefore suggested, although a radioligand binding assay is necessary for conclusive evidence. This novel receptor subtype has been distinguished from the putative A_3 adenosine receptor subtype (371). We do not, however, dismiss the possibility that different subtypes of adenosine receptors couple with different effector systems other than adenyl cyclase and PIC.

It was also interesting to find that when RBL-1 cells were incubated with NECA, at 10μM concentration and for 10 or 20 minutes, in the presence of 100μM 8Br-cAMP, the latter did not appear to have an overall consistent or significant effect in inhibiting NECA-induced PI hydrolysis as can be seen in Figures 38 and 39. This could be due to the fact that the NECA binding site is coupled to a G-protein which is not responsive to cAMP-mediated mechanisms. It has been shown (see Sections 3.5.2 and 3.6.2) that Antigen- and NECA-induced PIC activation are mediated by different mechanisms (e.g

different G-proteins) and that LTD_4 can interact with at least two G-proteins, pertussis toxin sensitive and pertussis toxin insensitive proteins depending on cell type (372) and the state of differentiation of the cells (373). This could explain why PIC activation by these agents, unlike NECA, was inhibited with 8Br-cAMP.

3.6 Immunological Challenge and Phosphatidylinositol Breakdown

3.6.1 *Introduction*

Tissue mast cells and blood basophils play an important role in inflammation and the allergic response (266), and they are important sources of inflammatory mediators. They can be activated by immune reactions, both directly via sensitization with IgE, and indirectly via the anaphlatoxins C3a and C5a. A characteristic of mast cells and basophils is the presence of $F_{c\varepsilon}$ receptors (IgE receptors) which bind IgE with high affinity, $K_a > 10^{10}M^{-1}$ (374). The IgE is bound through the F_c-portion of the molecule with the F_{ab} region available for interaction with antigen. Thus, with IgE bound to the cell surface, the activation of the cell acquires antigenic specificity. Activation is due to the bridging of the IgE-molecules by antigen (375), and cell activation requires the bridging of very few of the IgE molecules on the cell surface.

The bridging of IgE molecules on the cell membrane bound to the IgE F_c receptor results in receptor aggregation, which brings about a number of biochemical changes in mast cells and basophils. These changes include; phospholipid methylation (376,377), a rapid rise and fall in cAMP within 2 minutes (378) (although, not in RBL cells (379)), depolarization of plasma membrane potential (380), hydrolysis of membrane inositol phospholipids and enhancement of $[Ca^{2+}]_i$ (380,381). Previous studies have shown that activation of RBL cells by aggregating the IgE molecules bound to the IgE receptor, increases the level of IPs within 10-40 seconds (165), and that stimulation of hydrolysis of inositol phospholipids is dependent on the extent and rate of IgE receptor aggregation on the plasma membrane (382). Experiments with pre-formed oligomers of IgE have shown an excellent correlation between the amount of oligomer bound (and therefore of receptors aggregated) and the rise in the IPs (383).

3.6.2 IgE-Induced Phosphatidylinositol Breakdown and Inositol Phosphate Formation

In view of the stimulatory effect of IgE receptor crosslinking upon inositol phospholipid breakdown mentioned above, it was important to examine whether IgE receptor crosslinking leads to PI lipid turnover in RBL-1 cells. In this type of experiment, the RBL cells have to be first primed by incubating with IgE, after which aggregation of IgE receptor, through crosslinking the IgE molecules bound to the IgE receptor can be induced (i.e challenge). There are various aggregating agents that have been used in cell culture systems, such as binding of receptors with IgE raised against BSA-DNP hapten (or any antigen) and adding the hapten (antigen) after IgE binding, antibodies raised against IgE receptor itself, using IgE dimers or trimers etc.. Basophils have been shown to be activated by bivalent anti-IgE-receptor antibody which cross-bridges the IgE-receptor (384).

RBL-1 cells were first primed with 0.5μg/ml of rat IgE for 16 hours to ensure sufficient IgE molecules bound to the IgE receptor. The cells, pre-labelled with [^{3}H]-myo-inositol at 10μCi/ml, were then, in different experiments, incubated with 1μg and 10μg of anti-IgE antibodies (termed antigen, Ag, in Figures 40-42). The incubation was carried out for 30 minutes and under the same conditions as described previously (see Section 3.3.3). The IPs were separated by anion-exchange chromatography and measured using scintillation counting. As can be seen in Figure 40, at both antigen concentrations, 1μg and 10μg, IgE receptor aggregation achieved using IgE antibodies did lead to significant IP release.

A different experimental strategy from above with respect to IgE receptor crosslinking was then followed. Here, RBL-1 cells were primed with 0.5μg/ml of rat IgE raised against DNP hapten for 16 hours. Then the cells, pre-labelled with [^{3}H]-myo-inositol, were treated as described for antigen above. RBL-1 cells were incubated with dinitrophenol (DNP) conjugated to human serum albumin (HSA) at 20ng to lead to receptor crosslinking and hence cell activation. As shown in Figure 43, 20ng of HSA-DNP did lead to significant IP release, although not to the same extent as that seen with anti-IgE antibodies (Ag). This might be due to the lower amount of HSA-DNP i.e. 20ng compared to 1 and 10μgs of Ag used respectively.

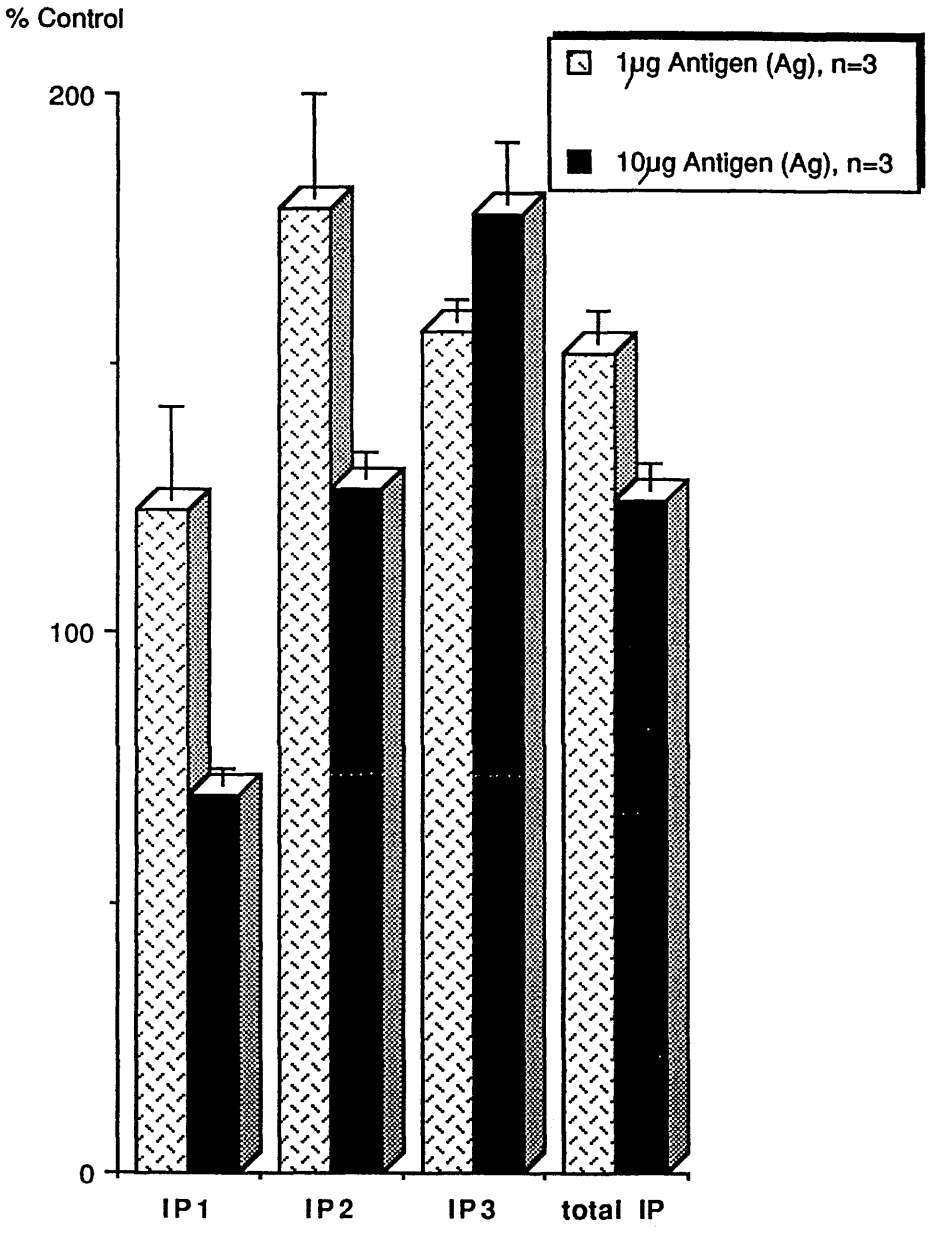


Figure 40: Immunologically-inudced inositol polyphosphate release. Data expressed as means±s.e. of the means.

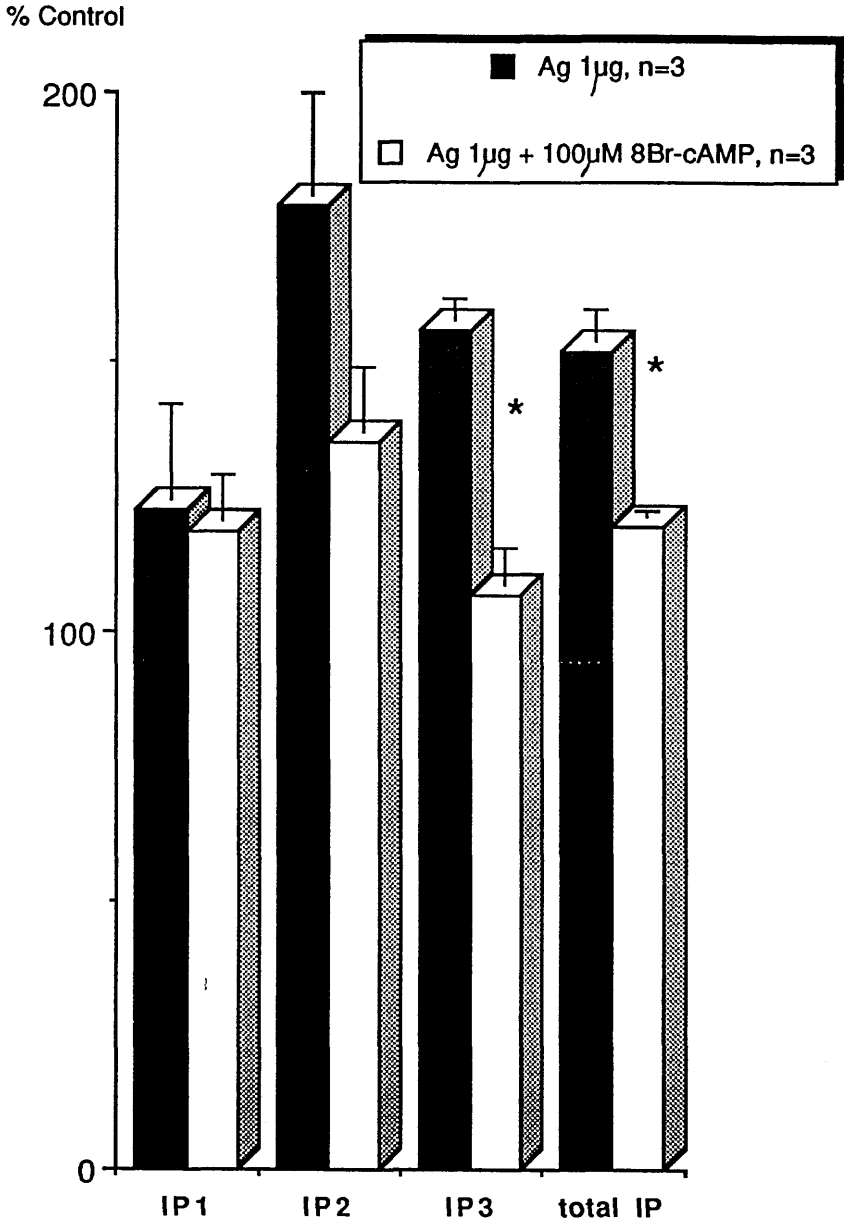


Figure 41: The effect of 8bromo-cyclic AMP on immunologically-induced inositol polyphosphate release. Data expressed as means±s.e. of the means, (* $p<0.02$).

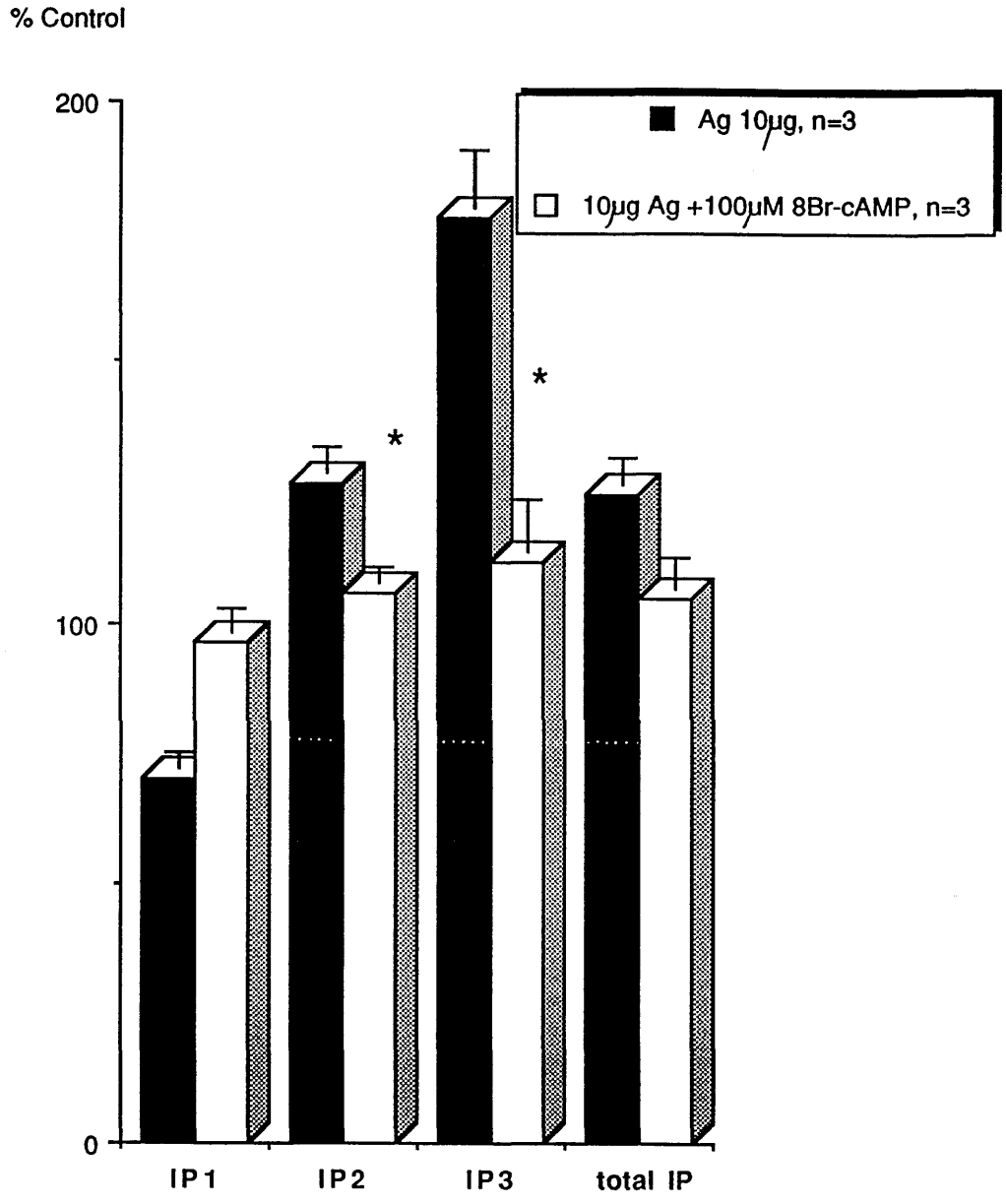


Figure 42: The effect of 8bromo-cyclic AMP on immunologically-induced inositol polyphosphate release. Data expressed as means±s.e. of the means, (* $p<0.02$).

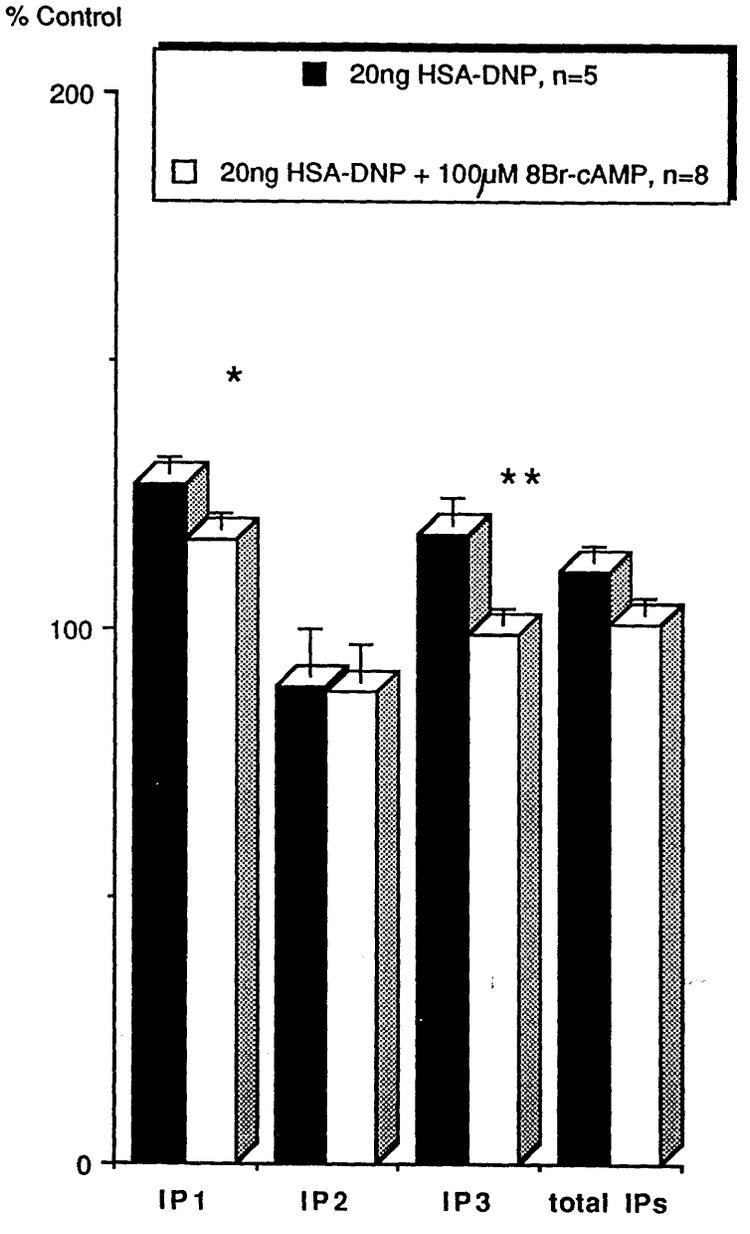


Figure 43: The effect of 8bromo-cyclic AMP on immunologically-induced inositol polyphosphate release. Data expressed as means±s.e. of the means, (* $p<0.05$, ** $p<0.02$).

3.6.3 Effect of 8Br-cAMP on IgE-Induced Phosphatidylinositol Hydrolysis

In view of the modulatory role found with 8Br-cAMP on LTD_4-induced IP release, shown in Section 3.3.4, it was decided to examine its effect on immunologically-induced IP release in RBL-1 cells.

RBL-1 cells were primed with 0.5μg/ml of IgE for 16 hours and then labelled with [^{3}H]-myo-inositol at 10μCi/ml. In different experiments, as described above, the cells were then activated using anti-IgE antibodies (Ag) at 1 and 10μg in the presence of 8Br-cAMP at 100μM concentration. As can be seen from Figures 41 and 42, 8Br-cAMP inhibits Ag-induced IP release. A statistically significant inhibition was observed for IP_3 ($p<0.02$) and total IPs ($p<0.02$) when 1μg of Ag was used as shown in Figure 41. When 10μg of Ag was used to induce IP release, again a 100μM concentration of 8Br-cAMP led to inhibition of Ag-induced IP release. As seen in Figure 42, a statistically significant inhibition of IP_2 ($p<0.02$) and IP_3 ($p<0.02$) was observed.

Similarly, when RBL-1 cells, primed with IgE raised against DNP hapten and pre-labelled with [^{3}H]-myo-inositol, were incubated with 20ng of HSA-DNP in the presence of 100μM 8Br-cAMP, an inhibition in IP release was observed as shown in Figure 43. A statistically significant inhibition of IP_1 ($p<0.05$) and IP_3 ($p>0.02$) was seen.

3.6.4 Discussion

Mast cells, basophils and their transformed analogue RBL cells, possess F_C receptors with high affinity for monomeric IgE ($F_{C\varepsilon}$) on their plasma membranes (for a review see 385). Cellular activation occurs when these receptors are aggregated and form a cluster. The aggregation of just a small fraction (10%) of IgE receptors by antigen-IgE complexes (382,386,390) is sufficient to induce maximal rates of secretion of histamine by a Ca^{2+}-dependent process of exocytosis in RBL cells (385). Antigen-stimulation of RBL-2H3 cells also leads to PI hydrolysis, transient increase in $[Ca^{2+}]_i$, a gradual enhancement in intracellular pH (pH_i) and activation of PKC (390). This process of PIC activation and PI hydrolysis is pertussis toxin and cholera toxin resistant, but the antigen-induced Ca^{2+} influx pathway is cholera toxin sensitive (363). One consistent observation in RBL-2H3 cells has been that all responses to antigen were highly dependent upon high concentrations of external Ca^{2+} (157,390-392) and influx of Ca^{2+} ions (390,394); accordingly,

blockade of influx with La^{3+} leads to rapid abrogation of the Ca^{2+} signal, secretion and a partial suppression of the hydrolysis of the inositol phospholipids (390). The influx of Ca^{2+} provides a necessary amplification of all stimulatory signals for secretion (395).

In the work presented here, using anti IgE antibody at 1μg and 10μg to crosslink IgE receptors, we observed an enhancement in PI hydrolysis at both Ag concentrations as shown in Figure 40. Hydar Ali *et al*, have during the course of this thesis independently reported findings which are in agreement with our results, using HSA-DNP conjugate to induce receptor aggregation and PI breakdown in RBL-2H3 cells (396). When RBL-1 cells primed with IgE raised against DNP hapten were incubated with DNP-HSA, it was found that this leads to PI hydrolysis as can be seen in Figure 43, although not to the same extent as that observed above for Ag. This lower potency in PI hydrolysis by HSA-DNP is probably due to the low amounts of antigen used, 20ng compared to 1 and 10μgs used above.

From the data for immunologically induced IP release seen in Figures 40 and 43, it can be seen that there are variations in the breakdown of IPs. This could be due to the lower degree of separation of IPs when using Dowex AG1X8/Sep-pak columns as opposed to HPLC separations, or the degree of direct hydrolysis of precursor phospholipids: i.e $PIP_2 \Rightarrow IP_3$, $PIP \Rightarrow IP_2$ and $PI \Rightarrow IP_1$. In streptolysin permeabilized RBL-2H3 cells, it has been reported that both PIP_2 and PIP might be substrates for PIC (396). This has also been reported in platelets (397) but not in other cells (398). The direct derivation of IP_1 from PI has also been reported in RBL cells (399,400). However, in the intact cell the various IPs are derived from IP, but it is the kinetics of the initial response that varies with the type of stimulant used (399). Therefore, it is a possibility that the variation is due to differences in rates of aggregation of IgE receptors. When aggregation is slow the increase in IP_3 is eclipsed by its rapid conversion to other inositol phosphates; conversely, when aggregation is fast , the IP_3 level would be higher as the rate of its formation is greater than the rate of its degradation. This is inherent in the requirement of IgE receptor aggregation (unlike receptor-ligand binding-induced PI hydrolysis) prior to PIC activation and PI hydrolysis.

As with LTD_4-induced IP formation, IgE receptor crosslinking-mediated PI breakdown and IP formation, achieved by two different experimental strategies, was inhibited by the cAMP analogue, 8Br-cAMP at 100μM concentration as shown in Figures 41-43. The effect of 8Br-cAMP reported here further substantiates the possible inhibitory role of cAMP on PI hydrolysis

in RBL-1 cells. The antigen-induced PIC activation must be similar to LTD_4-induced PI hydrolysis in that similar processes are activated following ligand-receptor binding/receptor activation. If current concepts of signal transduction across the plasma membrane are correct, the only difference between the stimulation with antigen and with NECA is in receptors, and the G-proteins that couple these receptors to PIC. The lack of effect by 8Br-cAMP upon NECA-mediated PI hydrolysis (unlike LTD_4- and antigen induced-PI hydrolysis) indeed highlights this point and further suggests that cAMP, probably by acting through cAMP-dependent protein kinase (PKA), phosphorylates either receptor or G-protein to propagate its inhibitory role.

4 DISCUSSION AND CONCLUSION: PERSPECTIVE ON LEUKOTRIENE GENERATION AND SECONDARY MESSENGER INTERACTIONS IN RBL-1 CELLS.

The experiments presented in this thesis examine the interactions between second messengers and Leukotriene biosynthesis (401,402). As was mentioned earlier in Section 1.5, previous studies carried out in this laboratory had shown that Leukotriene generation induced by agonists acting through the phosphoinositide (PI) signalling pathway was modulated by agents capable of inducing adenyl cyclase activation. This modulatory effect was therefore hypothesized to be mediated by interaction between cAMP and the PI signalling pathway. Here, in the light of our findings in RBL-1 cells, these interactions between second messengers as well as interactions between phosphoinositide-specific phospholipase C and phospholipase A_2 are discussed. Leukotriene D_4 (LTD_4) receptor signal transduction is also reviewed with reference to the results presented both here and in other laboratories during the course of this thesis.

4.1 The Effect of Dibutyryl-Cyclic AMP upon Arachidonic Acid Release and Leukotriene Generation

Cyclic-AMP (cAMP), since its discovery more than 30 years ago, has had a decisive role in our understanding of the transmembrane signalling system. The cAMP level is under control by activation/inhibition of adenyl cyclase which is mediated by GTP binding regulatory proteins, G_S/G_i for activation and inhibition respectively and the rate of cAMP removal by cAMP phosphodiesterase (403). Once the cAMP level is enhanced a number of events take place such as protein phosphorylation mediated by cAMP dependent protein kinase (PKA).

An interaction between enhanced intracellular cAMP levels, $[cAMP]_i$, and Leukotriene generation was first observed from work carried out in this laboratory, when it was shown that vasoactive intestinal polypeptide (VIP), known as a potent activator of adenyl cyclase in the airways, was shown to inhibit the agonist-induced Leukotriene generation in rat lung tissue (237,238). This effect of VIP was then extended to other neuropeptides (404).

In view of these findings the effect of dibutyryl-cAMP (db-cAMP) on calcium ionophore-induced arachidonic acid release and Leukotriene

generation was examined. As can be observed from Table 5, a small but statistically significant inhibition of Leukotriene C_4 (LTC_4) was seen. The effect of db-cAMP at 100μM concentration was not as prominent with respect to Leukotriene B_4 (LTB_4), Leukotriene D_4 (LTD_4), Leukotriene E_4 (LTE_4), total Leukotrienes and arachidonic acid. This is in agreement with findings in guinea pig lungs, where agents capable of increasing cAMP levels were not able to inhibit ionophore-induced LTD_4 generation (405). One possible explanation for this lack of effect could be the fact that A23187 by-passes receptor-mediated activation of Phospholipase A_2 (PLA_2), directly activating PLA_2, and the site of action of cAMP could well be prior to Ca^{2+}-mediated PLA_2 stimulation. However, in rat chopped lungs, cAMP at 300nM showed a statistically significant inhibition of PAF-mediated generation of LTC_4, LTD_4, LTE_4 and total Leukotrienes (308). Moreover, several recent reports on the inhibition of PLA_2 activity by substances capable of raising the intracellular cAMP concentration have appeared in the literature. Adenosine inhibits IgE-induced Leukotriene or prostaglandin D_2 (PGD_2) generation in human lungs (406); tabutaline and salbutamol decreased IgG-induced, but not ionophore-induced, LTD_4 generation in guinea pig lungs (405).

Besides the stimulatory effect of increased $[Ca^{2+}]_i$ levels upon PLA_2 activation, the enzyme appears to be under both inhibitory and stimulatory control by a variety of mechanisms (see Section 1.2.2 and Figure 4). One such regulatory mechanism involves G-proteins either directly or via other second messenger systems that are coupled to G-proteins (53). The multiplicity of G-proteins involved in the regulation of PLA_2 is further demonstrated by the different toxin sensitivities of the enzyme observed in various systems. In rod outer segment (ROS) membranes, for example, light- and GTPγS-stimulated PLA_2 is inhibited by both pertussis and cholera toxin, whereas in the RAW macrophages, both toxins stimulate PLA_2. Enhanced $[cAMP]_i$ levels could have a modulatory effect by either interacting with some of these PLA_2 regulatory mechanisms directly, or indirectly, by interacting through the action of cAMP-dependent protein kinase (PKA) which could lead to a number of phosphorylations of target proteins such as G-proteins, phospholipase activating protein (PLAP), or indeed lipocortins as these have been shown to be regulated by phosphorylation (27,59). Alternatively, cAMP could regulate receptor-mediated calcium fluxes and stimulate the storage of calcium (i.e. uptake of calcium into IP_3-insensitive calcium pools) thereby diminishing calcium availability for PLA_2 activation.

Furthermore, there is a possibility that this action of enhanced cAMP level is regulated by the products of PLA_2 activation that are known inhibitors (Leukotrienes) or activators (PGE_2, PGI_2, PGD_2) of adenyl cyclase activity. For example, in the aplysia nervous system FMRF-NH_2, by acting through 12-lipoxygenase metabolites can re-open 5-Hydroxytryptamine-induced (through cAMP) K^+ channel closure (407). However, these points are speculative and a receptor-mediated arachidonic acid release and Leukotriene generation is required to elucidate possible site(s) of action of enhanced $[cAMP]_i$ levels in RBL-1 cells. From the data presented in Table 5 of this thesis, it can nevertheless be concluded that possible sites of cAMP-mediated inhibitory responses with respect to PLA_2 activity are prior to PLA_2 activation by Ca^{2+}.

4.2 Effect of cAMP Analogues on Inositol Phospholipid Breakdown and Inositol Phosphate Release

In addition to the interactions operating within the inositol phospholipid pathway, there are reciprocal interactions with other second messenger pathways, particularly the cyclic nucleotides (156,159).

In this study, the effect of cAMP analogues were examined by three different PIC stimulatory agonists in RBL-1 cells: LTD_4, immunological challenge and NECA (see Sections 3.3.4, 3.5.3, 3.6.3). LTD_4- and immunologically-induced PIC activation and IP release are brought about by two different mechanisms; LTD_4 involves ligand binding while immunological challenge involves receptor crosslinking for activation to take place. When LTD_4 is used as an agonist, it can be seen from Figure 28 that 8Br-cAMP at a concentration of 10μM leads to a statistically significant inhibition of IP_3 and total IPs. Similarly, 8Br-cAMP at 100μM concentration leads to a statistically significant inhibition of IP_1 and total IPs while 100μM db-cAMP leads to a statistically significant inhibition of total IPs as can be seen in Figures 29 and 30, thus demonstrating that elevation of the $[cAMP]_i$ level, simulated by having high concentrations of cAMP analogues, counteracts PIC activation in RBL-1 cells. Db-cAMP is known to be less stable than 8Br-cAMP and there is evidence that it degrades to butyryl-cAMP and butyrate which could explain why it was not as potent as 8Br-cAMP in the data shown in Figure 30.

For the first time we have shown that cAMP analogues inhibit LTD_4-induced phosphoinositide hydrolysis in RBL-1 cells, thus extending to RBL-1 cells the types of systems where elevated intracellular cAMP levels counteract PIC activation

Furthermore, as with LTD_4-induced IP formation, IgE receptor crosslinking-mediated PI breakdown and IP formation, achieved by two different experimental strategies, was inhibited by the cAMP analogue, 8Br-cAMP, at 100μM concentration as shown in Figures 41-43 of this thesis. The reciprocal relationship between cyclic nucleotide and inositol phospholipid metabolism has not received much attention except in a few cell types such as platelets. Thrombin-induced platelet responses such as the increase in intracellular Ca^{2+}, phosphorylation of the 40kD protein, aggregation, and the release of 5-HT are all inhibited by agents that increase cAMP (408-410). Moreover, the inhibition of thrombin-induced PIC activation by cAMP has also very recently been observed in the human erythroleukaemia (HEL) cells (411). These findings, together with the data determined in this thesis, suggest a negative modulatory role for cAMP upon the phosphoinositide signalling system, at least on LTD_4- and immunological challenge-induced phosphoinositide breakdown in RBL-1 cells. However, it is interesting to find that when RBL-1 cells are incubated with NECA, at 10μM concentration and for 10 or 20 minutes, in the presence of 100μM 8Br-cAMP, the latter does not appear to have an overall consistent or significant effect in inhibiting NECA-induced PI hydrolysis as can be seen in Figures 38 and 39. This implies that this inhibitory effect of cAMP observed with LTD_4- and IgE-induced IP release, is not a general inhibitory effect and is applicable only to certain receptor-effector signalling pathways.

The antigen-induced PIC activation must be similar to LTD_4-induced PI hydrolysis in that similar processes are activated following ligand-receptor binding/receptor activation. However, a notable difference between IgE receptor mediated and LTD_4-induced PI hydrolysis is that one type of stimulation requires aggregation of receptors and the other requires the binding of LTD_4 to a receptor. The former process is presumably dependent on the kinetics of receptor aggregation, whilst the latter is dependent on ligand binding. Furthermore, IgE receptors need to be primed with IgE before any aggregation and hence receptor activation can take place (unless aggregation is achieved by using anti-receptor antibodies). Studies involving oxidative iodination of RBL cells with ^{125}I and then reaction with ^{131}I-IgE have led to a better understanding of IgE receptor turnover (see 385 for a review). Unliganded receptors are endocytosed and degraded with a half life of 8-12 hours (385). However, in the presence of IgE, degradation is slower. In agreement with radiolabelled IgE experiments, it has been shown that when examined by electron microscopy and autoradiography, the retained

radioactivity is seen on the surface of the cell only (385). One possible explanation for this is that liganded receptors are not endocytosed, or if endocytosed, not degraded. Somehow the bound IgE protects the receptors from proteases in the lysosomes, due perhaps to conformational changes. IgE receptor mediated PIC activation and inositol lipid hydrolysis is very likely mediated via G-proteins. Furthermore, this PIC activation appears to be mediated by a toxin resistant G-protein, since its activation is not impaired in cholera toxin- or pertussis toxin-treated cells, but the activation is suppressed in a concentration dependent manner by GDPβS (389).

If current concepts of signal transduction across the plasma membrane are correct, the only difference between the stimulation with antigen and with NECA is in receptors, and the G-proteins that couple these receptors to PIC. The lack of effect by 8Br-cAMP upon NECA-mediated PI hydrolysis (unlike LTD_4- and antigen induced-PI hydrolysis) indeed highlights and confirms this point and further suggests that cAMP, probably by acting through cAMP-dependent protein kinase (PKA), phosphorylates either the receptor or the G-protein coupled to the receptor to propagate its inhibitory role.

Although, previously it has been shown that NECA is a selective antagonist of the A_2 adenosine receptor subclass (368), which is coupled to the adenyl cyclase effector pathway, its stimulatory effect upon PIC and the hydrolysis of inositol phospholipids imply that there might be a novel subtype of adenosine membrane receptor, at which NECA acts, and which is coupled to the PIC effector pathway. Inositol phospholipid hydrolysis mediated by NECA in washed, permeabilized RBL-2H3 cells is blocked by both cholera toxin and pertussis toxin (363). This implies that the NECA-sensitive receptor activates PIC via a G-protein. Therefore, unlike the A_1/A_2 adenosine receptor subtypes, the putative adenosine receptor in RBL cells coupled to PIC have the characteristics of being coupled to a G-protein similar to G_t (transducin) which is both cholera toxin and pertussis toxin sensitive. LTD_4, on the other hand can interact with at least two G-proteins, pertussis toxin sensitive and pertussis toxin insensitive, depending on cell type and the state of differentiation of the cell (373), although in the case of RBL-1 cells the G-protein coupling LTD_4 receptor to PIC is apparently pertussis toxin insensitive (412). From these observations it is apparent that the G-proteins involved in receptor/effector coupling are different for these agonists, since LTD_4-mediated (possessing pertussis toxin-insensitive G-proteins coupled to its receptor) and IgE-mediated (possessing pertussis toxin insensitive G-proteins coupled to its receptor) PI hydrolysis is inhibited by incubation in the presence

of 100μM concentration of 8Br-cAMP, as seen in Figures 29 and 41-43 of this thesis, but not NECA-mediated (possessing both cholera toxin- and pertussis toxin-sensitive G-proteins coupled to its putative receptor) PI hydrolysis as seen in Figures 38 and 39 of this thesis.

The exact mechanism of this inhibitory action of cAMP (simulated through the action of 8Br-cAMP) has not been established. It has been reported that the increase in cAMP prevents the agonist-induced activation of PIC and that this is mediated by PKA, but its mechanism is poorly understood (253,344,345). Figure 31 illustrates the effect of PKA inhibitor upon 8Br-cAMP-induced inhibition of LTD_4-stimulated IP release. As can be seen, PKA inhibitor does not significantly alleviate inhibition of LTD_4-induced IP release caused by 100μM 8Br-cAMP. This could be due to PKA inhibitor, a polypeptide, not being able to enter the intact cell and therefore being unable to relieve the kinase of its possible inhibitory action. The inhibitory effect of PKA inhibitor has only been shown in lysed cell preparations (346). In human platelets and in the human erythroleukaemia cell line, HEL, a specific protein is phosphorylated by the action of cAMP. This is a 22 kD protein that binds [α-^{32}P] GTP on nitrocellulose blots and displays strong cross reactivity with the monoclonal antibody M90, which recognises an epitope between amino acids 107 and 130 of the H-ras p21 protein, which is one of the major GTP binding regions of the protein (413). Elevation of intracellular cAMP levels in platelets and HEL cells by the prostacyclin analogue Iloprost results in a shift of the apparent molecular mass of the M90 immunoreactive band from 22kD to 24kD. This shift correlates with translocation of the modified 22kD protein from a particulate to a cytosolic fraction (413). It is proposed that these changes in the mobility of the 22kD protein are the result of a covalent modification of the protein such as phosphorylation. Indeed, the treatment of ^{32}P-labelled platelets with Iloprost results in the phosphorylation of a 22kD to 24kD GTP-binding protein by PKA (413). This phosphorylated protein can be immunoprecipitated with M90 (413), suggesting that the 22kD translocated protein and the phosphorylated protein are identical.

Other possible mechanisms include inhibition of phosphatidylinositol kinase (101,331,332), and therefore affecting conversion of PI to PIP and PIP_2, or inhibition of the rise in $[Ca^{2+}]_i$ levels, by enhansing Ca^{2+} uptake into IP_3 -insensitive Ca^{2+} pools and/or decreasing Ca^{2+} influx, necessary for a stimulatory response (343). Recently, there have been a number of reports indicating that mammalian cells contain at least five immunologically distinct phospholipase C enzymes that appear to be separate gene products (for a

review see 414). These PIC isozymes possess limited overall sequence similarity and thus may be regulated by different means and hence be substrates for the inhibitory action of cAMP. It has been shown very recently that in rat glioma cells, cAMP elevating agents lead to PICγ phosphorylation and decrease IP formation induced by noradrenaline, an agonist thought to interact with PIC via G-proteins (415). Similarly, cAMP elevating agents induce the phosphorylation of a specific serine residue in PICτ and of a 76kD protein co-precipitated by anti-PICτ monoclonal antibodies in BALB/c-3T3 cells (416). However, very recently it has been reported that immuno-precipitation of a PICδ isozyme in the neuro tumor cell line NCB-20, failed to show ^{32}P orthophosphate labelled PICδ isozyme following treatment with either forskolin or db-cAMP (417). This suggests that PIC, at least PICδ isozyme, might not be the target for cAMP-dependent protein kinase-mediated phosphorylation and nor is PICβ (418).

Both GTPγS- and NaF-induced IP release from NCB-20 cell membranes, previously labelled with [^{3}H]-myo-inositol, were blocked by pretreatment of the cells with cAMP-dependent protein kinase (PKA) activators, suggesting a GTP-binding protein as the target for PKA-mediated phosphorylation. In the NCB-20 cells it is proposed that the G-protein responsible for PIC activation (G_p) is uniquely sensitive to PKA-mediated phosphorylation and that phosphorylation inhibits stimulus-secretion coupling in these cells. Two possible G-proteins could be involved in coupling the bradykinin receptor to PIC in the NCB-20 cell line. One a substrate for pertussis toxin and the other for PKA (417). Therefore, it appears that down regulation of PIC by PKA may occur through a family of related small molecular weight G-proteins. A possible G-protein site of action for cAMP-mediated inhibition of IP release is further substantiated from the finding in this thesis that LTD_4- and immunological challenge-induced IP release (with G-proteins insensitive to pertussis toxin) were inhibited by 8Br-cAMP, while NECA-induced IP release (with G-proteins sensitive to both pertussis and cholera toxins) was not inhibited. Figure 44 summarises the effect of 8Br-cAMP on LTD_4-induced and immunological challenge-induced IP release in RBL-1 cells and its possible sites of action.

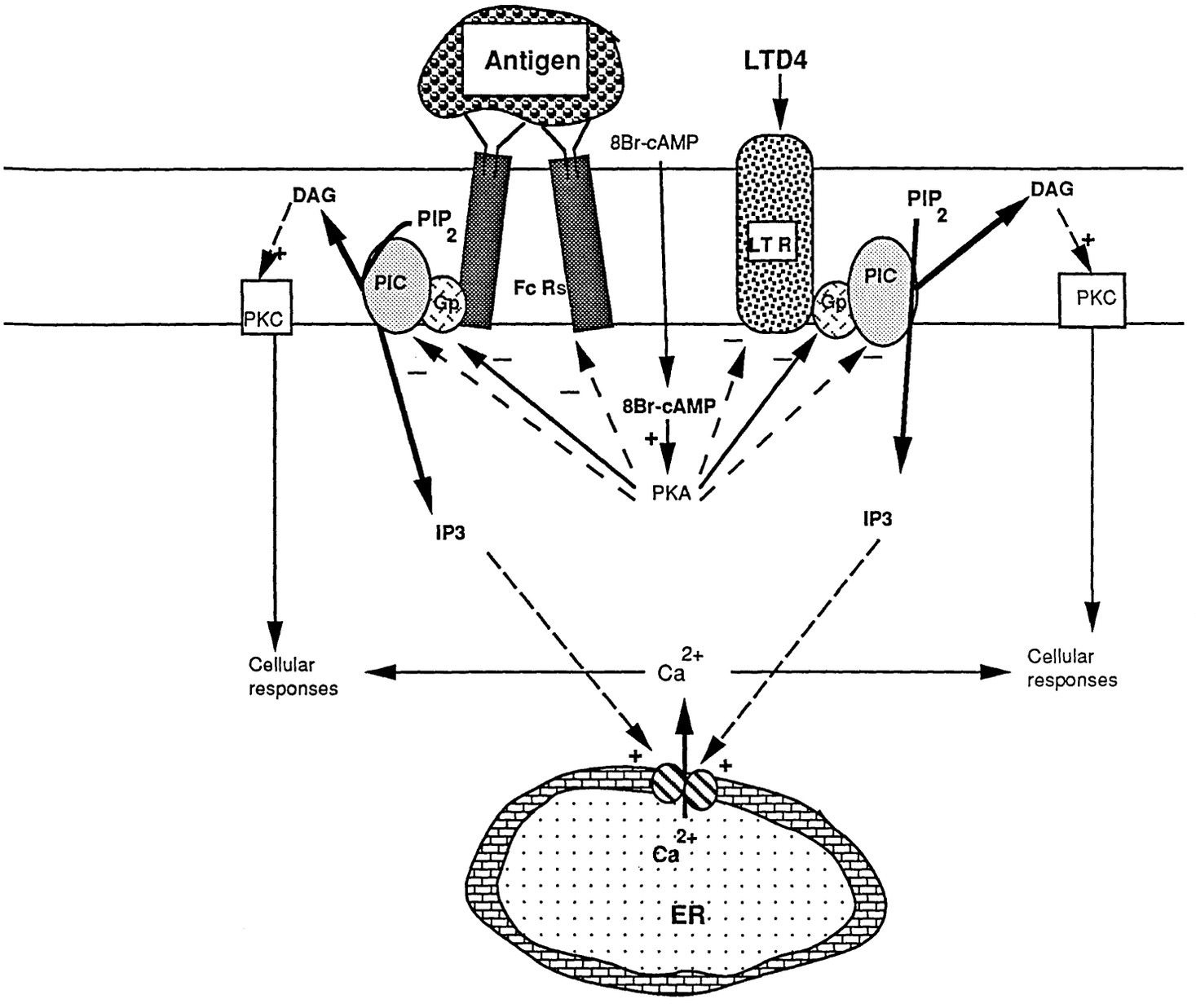


Figure 44: The effect of 8Br-cAMP upon LTD4- and immunologically-induced IP release in RBL-1 cells. For abbreviations see text.

4.3 Phospholipase A_2 and Phospholipase C Activation: Possible Interactions

In a variety of target cells various classes of agonists can alter phospholipid metabolism through two pathways: receptor-mediated activation of PLA_2 and PIC. The activation of PLA_2 leads to the release of arachidonic acid and the subsequent conversion of arachidonic acid by cyclo-oxygenase and lipoxygenase(s) enzymes to a number of biologically active metabolites (see Section 1.2), some of which have very recently been reported to act as second messengers (for a review see 419). Receptor-mediated activation of PIC promotes hydrolysis of inositol lipids and generation of yet another set of second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (see Section 1.4).

However, an unresolved question regarding receptor-mediated alterations in phospholipid metabolism, with concomitant generation of Leukotrienes, prostaglandins and inositol polyphosphates, concerns the order of these two enzymatic reactions mentioned above. Receptor-mediated activation of PLA_2 and PIC could occur in 3 possible manners:

a) PIC activation leading to PLA_2 activation:

$$PIC \Rightarrow PLA_2$$

b) PLA_2 activation leading to PIC activation:

$$PLA_2 \Rightarrow PIC$$

c) Activation occuring in a parallel manner:

$$PIC \Rightarrow$$
$$PLA_2 \Rightarrow$$

Considering the sequential activation hypothesis (i.e a and b above), one could speculate that activation of one enzymatic pathway would be a prerequisite for activation of the other. For example, the DAG generated by PIC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) might be hydrolysed by a DAG lipase to release arachidonic acid or the enhanced intracellular calcium concentration ($[Ca^{2+}]_i$) brought about by IP_3-mediated Ca^{2+} mobilization would cause PLA_2 activation. The parallel regulation (i.e c above) implies that the two enzymes are activated independently of each other such that production of IP_3 and DAG is not required for PLA_2 activation and that PLA_2 activation is not required for PIC stimulation.

There have been a number of attempts to resolve this question. Several investigators initially proposed that the sequential pathway is the means by

which agonist receptors lead to release and metabolism of arachidonic acid (a, above) and it has often been postulated that activation of PLA_2 is the consequence of PIC-induced hydrolysis of PIP_2 (339,420,421). This postulate seems attractive in view of the fact that IP_3 can mobilize calcium and that most, although not all, PLA_2 activities are stimulated by calcium (36). However, when arachidonic acid metabolites were recognised as possible transducers of receptor signalling pathways, the possibility of direct stimulation of PLA_2 by some agonists were noted (422). Recently, several studies have been conducted in various systems whereby it has been shown that either arachidonic acid release is mediated primarily by PLA_2 activation and only slightly by PIC activation, as in for example platelets (423,424) or PLA_2 activation and arachidonic acid release is totally independent of PIC activation, as has been shown in porcine aortic endothelial cells (314), human neutrophils and differentiated HL60 cells (425) and in hippocampal neurons (426).

Evidence presented in this thesis has been obtained for PLA_2-induced PIC activation for the first time in RBL-1 cells by using the calcium ionophore A23187. This calcium ionophore has been used widely to induce Ca^{2+}-dependent cellular functions, such as secretion, on the assumption that permeabilization of the cell membranes to Ca^{2+} ions leads to a rise in the level of $[Ca^{2+}]_i$, which in turn serves as a signal for secretion. In this way, events that precede mobilization of Ca^{2+} ions via receptor stimulation are by-passed. One such event is thought to be the rapid hydrolysis of membrane inositol phospholipids to generate IP_3 and DAG (165,347). Recently it has been reported in RBL-2H3 cells that the threshold concentration of A23187 for stimulation of the hydrolysis of PI lipids appeared to be 0.1μM, and at this A23187 concentration, the $[Ca^{2+}]_i$ level was estimated to be greater than 1μM (347). This would suggest that the hydrolysis of PI lipids could be due to the great enhancement in $[Ca^{2+}]_i$ level, which in turn leads to PIC activation and IP release (425). Therefore, Ca^{2+}-mediated PIC activation may represent a positive feedback mechanism for Ca^{2+}-induced responses.

In the studies presented in this thesis, it was found that the effect of A23187 on IP release in RBL-1 cells was apparent and significant only when using a high concentration (20μM), and an incubation time longer than that used for LTD_4-induced IP release (45 minutes). Under these conditions the A23187-induced PI breakdown reach a marked extent as seen in Figure 32. However, 10μM ionophore concentration and a 20 minutes incubation time are the well established conditions for a maximal effect of A23187 on

Leukotriene biosynthesis in RBL-1 cells, as shown in Section 3.2.4 and Table 4(e) of this thesis (see also (24)). This suggested that the effect of A23187 on IP formation might be due to, firstly, activation of PLA_2 and AA release from phospholipids, and secondly, biosynthesis of LTD_4. The data presented in Section 3.3.3, Figure 24, provides convincing evidence that if formed, LTD_4 leads to PI lipid hydrolysis.

In order to substantiate this hypothesis, the incubation was conducted in the presence of a 5-lipoxygenase inhibitor, AA861, and it was observed that the latter significantly reduced A23187-induced IP_1, IP_3 and total IP formation as seen in Figure 34, thus showing that the effect of A23187 upon PI breakdown and IP formation is most probably mediated by 5-lipoxygenase metabolites. In Section 3.3.3 it was demonstrated that LTD_4 is active in inducing PI breakdown in RBL-1 cells and thus experiments were then designed to see the effect of the LTD_4 antagonist FPL-55712 on A23187-induced IP release. At 10μM concentration FPL-55712 virtually abolished A23187-induced IP_1 release and significantly reduced total IP formation, as shown in Figure 35, thus implying that this effect of A23187 upon IP release is mediated by LTD_4 and/or the other p-Leukotrienes. To substantiate the antagonistic effect of FPL-55712 on the LTD_4 receptor in RBL-1 cells, the cells, pre-labelled with [^{3}H]-myo-inositol, were incubated with 10μM FPL-55712 for 2 minutes prior to the addition of 1μM LTD_4. Figure 36 illustrates the inhibitory effect of 10μM FPL-55712 on LTD_4-induced IP release. As can be seen from Figure 36, FPL-55712 leads to a statistically significant inhibition of IP_1 ($p<0.02$), IP_3 ($p<0.02$) and total IP ($p<0.001$). Therefore, it can be concluded that in RBL-1 cells PLA_2 activation leads to PIC activation and that this effect of PLA_2 is mediated via a metabolite(s) of the 5-lipoxygenase pathway of arachidonic acid metabolism, most probably LTD_4 (and/or other p-Leukotrienes).

It has been recently suggested that arachidonic acid and/or its metabolites may act as feedback modulators of the PIC pathway (267). It is suggested that PLA_2 activation, achieved by enhancement in $[Ca^{2+}]_i$ or extracellular Ca^{2+} influx or protein kinase C-mediated protein phosphorylation, leads to arachidonic acid release and formation of its metabolites. In turn these metabolites participate in a positive or negative feedback mechanism to mediate further changes in the PIC pathway and Ca^{2+} release. However, in RBL-1 cells we observe that agents capable of inducing PIC activation and inositol phosphate (IP) release, such as LTD_4, NECA and Immunological challenge, do not lead to arachidonic acid release

and Leukotriene generation (see Table 4(a) and 4(b)). Furthermore, PKC activation alone, achieved by incubating RBL-1 cells with PMA, does not lead to Leukotriene generation as shown in Table 3 of this thesis. This is in agreement with studies involving LTB_4 generation in human polymorphonuclear leukocytes (PMNLs) and that of LTC_4 generation in mouse peritoneal macrophages (293,427). Recently, PKC has been implicated in the biosynthesis of PAF and LTB_4 in human neutrophils (296). In this study the role of PKC was investigated by using the phorbol ester 4β–phorbol 12-myristate 13-acetate (PMA) to activate PKC, and long chain amines such as sphingosine, stearylamine and palmitoylamine which have been shown to inhibit PKC-mediated activation of the respiratory burst in the neutrophil (428). It was found that PMA enhanced A23187-induced PAF and LTB_4 generation and it was also observed that the long chain amines inhibited A23187-induced PAF and LTB_4 formation. It was therefore concluded that PKC activation was required to couple a rise in the intracellular Ca^{2+} concentration brought about by A23187 to PAF and LTB_4 generation. Our data allow a proposal that if PKC plays a role in PLA_2, arachidonic acid release and Leukotriene generation, then it is only in synergism with other cellular responses such as Ca^{2+} influx and enhanced intracellular Ca^{2+} concentrations in RBL-1 cells. This synergism could be a prerequisite to couple a rise in intracellular Ca^{2+} concentration to Leukotriene production. This coupling could be at the level of the initial reaction in the generation of these mediators, a PLA_2-like activity that mobilizes arachidonic acid from the sn-2 position of phospholipids in the cell membranes. Therefore it appears that PIC does not modulate PLA_2 activity, and Leukotriene biosynthesis is not dependent upon IP release in RBL-1 cells. This is in agreement with recent findings in porcine endothelial cells (314), human neutrophils, differentiated HL60 cells (425) and hippocampal neurons (426). Therefore, we propose that the PLA_2/PIC interactions in these cells are as shown in Figure 45.

The lack of effect by these agonists (LTD_4, NECA and Immunological challenge) upon PLA_2 activity could be due to the absence of a PLA_2-coupled effector system since they do activate PIC via a receptor-mediated pathway. Furthermore, there is evidence that PLA_2 and PIC are activated by distinct GTP-binding proteins in some cells, for example in FRTL-5 rat thyroid cells in response to α_1-adrenergic stimulation (429) and human platelets (430). It could well be that G-protein coupled to PLA_2 is absent with respect to these agonists since both their receptors and PLA_2 are present in RBL-1 cells. G-protein-mediated regulation of PLA_2 has been suggested in FRTL-5 cells

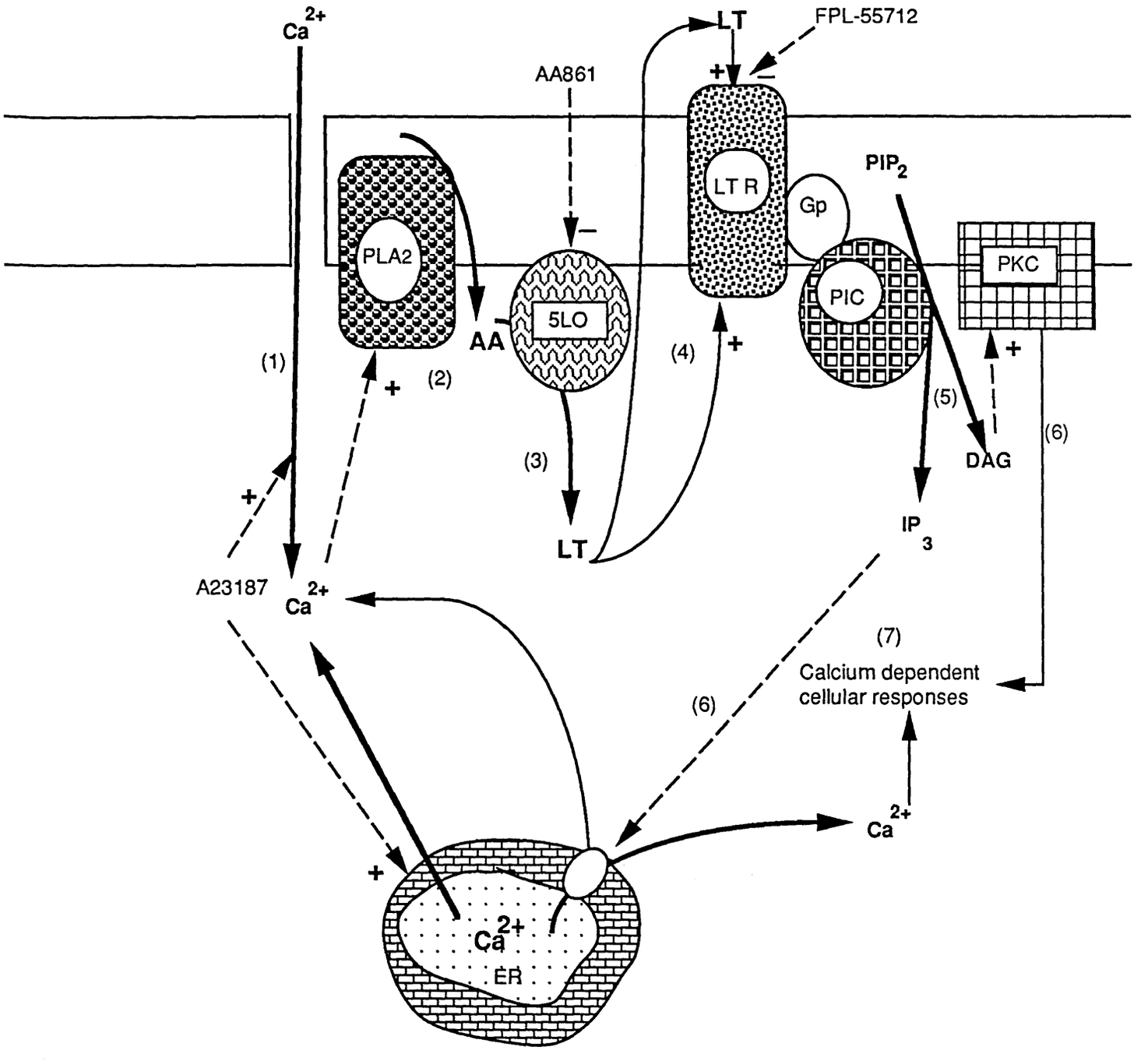


Figure 45: Interactions between PLA2 and PIC in RBL-1 cells. For abbreviations see text.

(429), Swiss 3T3 fibroblasts (431), rod outer segments (52), neutrophils (52,432), mast cells (433) and *Aplysia* sensory neurons (436), and the putative G-protein has been designated G_a (429). Unlike G_p (the G-protein involved in PIC regulation) which is a substrate for pertussis toxin in some cell types only (398,435). G_a appears to be a substrate for pertussis toxin in all cell types that have been investigated (429,431,434) including mast cells (436). However, more experiments, using stable GTP analogues and membrane preparations, are needed to substantiate the hypothesis that the G-protein coupled to PLA_2 is absent with respect to the agonists mentioned above.

When combining data from our previous study with that presented in this thesis, one is able to propose a general scheme with respect to possible secondary messenger interactions, as shown in Figure 46.

4.4 LTD_4 Receptor Signalling Pathway: A new Appraisal

From the work done in preparation for this thesis, it was observed that specific LTD_4 binding sites are present in RBL-1 cells (see Experimental Section) and LTD_4 binding to these sites leads to PIC activation and IP release (see Section 3.3.3). A model for the signal transduction processes of LTD_4 receptors has recently been presented (for a review see 437). When activated by LTD_4, the LTD_4 receptor can interact with at least two G-proteins (pertussis toxin sensitive and pertussis toxin insensitive) which act as transducers to a number of signalling systems, including a G-protein activated PIC, a receptor operated calcium channel and an as yet unidentified process which appears to be independent of IP_3 release, shown as **"(?)"** in Figure 47 (437). As can be seen from Figure 47, several second messengers result from these interactions which lead to PKC-induced topoisomerase I phosphorylation, and its activation, enhanced transcription of a gene for a protein that activates a phosphatidylcholine (PC) specific PLA_2, phospholipase activating protein (PLAP) (437). PLAP increases PLA_2 activity either through direct interactions with the enzyme, the substrate or an inhibitor of the enzyme. PLA_2 activation in turn leads to release of arachidonic acid and Leukotriene biosynthesis (and/or prostaglandin biosynthesis).

Data obtained in this thesis using the RBL-1 cell line as model system have shown that LTD_4 receptor activation does not induce PLA_2 activation, arachidonic acid release and Leukotriene biosynthesis as seen in Table 3 (a),

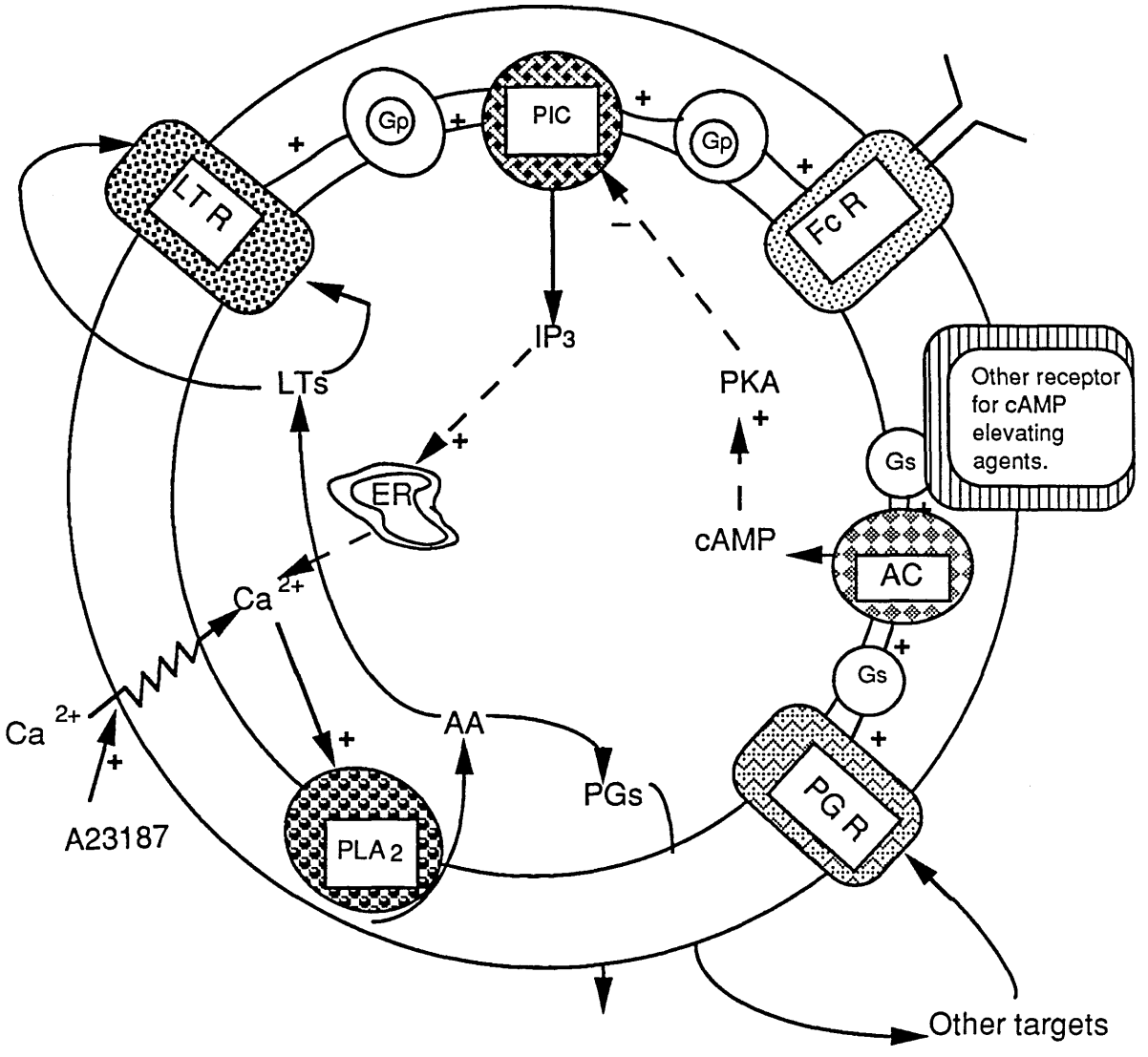


Figure 46: Net work of possible second messenger interactions. Strong calcium influx or antigen challenge may produce local levels of Leukotrienes high enough to activate phosphoinositide breakdown and trigger cellular responses. Increase in the level of intracellular cAMP, brought about by agents capable of activating adenyl cyclase, would then lead to attenuation of this and/or other Leukotriene-mediated cellular response. For more detail and abbreviations see text.

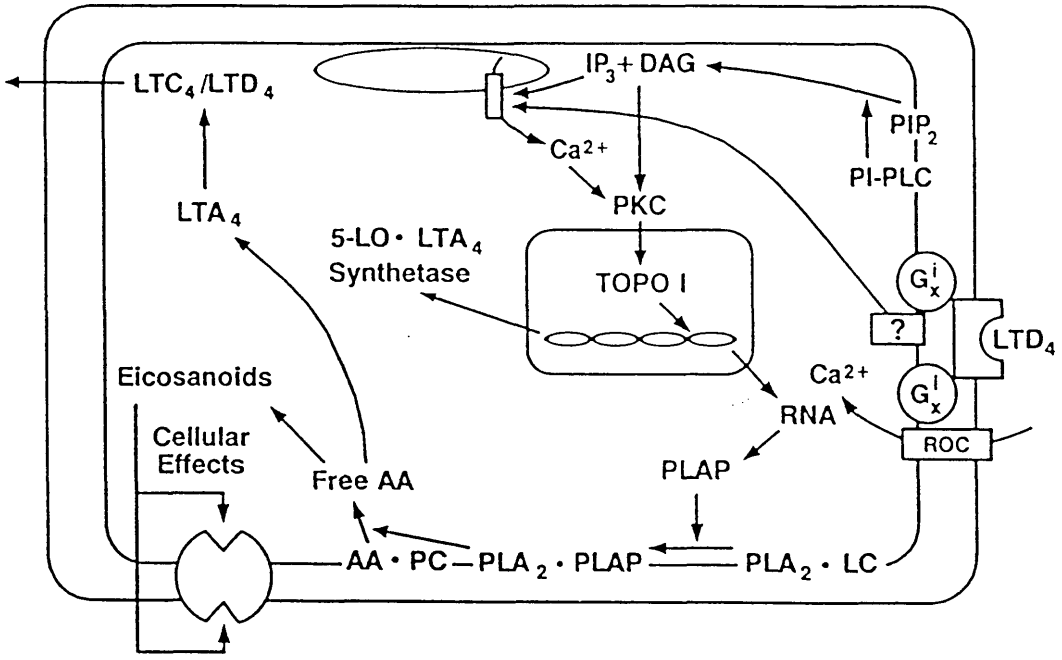


Figure 47: Model of LTD_4 signal transduction. $G_{i,}$ inhibitory guanine nucleotide binding protein, G_x, unspecified guanine nucleotide binding protein; PI-PLC, phosphoinositide-specific phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; PKC, protein kinase C; Topo I, topoisomerase I; PLAP, phospholipase activating protein; PLA_2, phospholipase A_2; AA, arachidonic acid; 5-LO, 5-lipoxygenase; LC, lipocortin; PC, phsophatidylcholine; ROC, receptor operated channel. (taken from Crooke *et al.* (439))

thus indicating that the second phase of the signalling process, proposed by Crooke *et al.* (439), is absent in RBL-1 cells. This, as mentioned above, could be due to the absence of the coupling between the LTD_4 receptor and the PLA_2 effector system. However, in the model proposed here, the LTD_4 receptor is not directly coupled to PLA_2 via G-protein, but instead LTD_4 receptor-induced PLA_2 activation is mediated by topoisomerase I phosphorylation, DNA transcription and PLAP synthesis. Therefore, if this model for LTD_4 signal transduction is correct, PKC-induced topoisomerase I phosphorylation, DNA transcription and PLAP synthesis must be absent in RBL-1 cells which would account for the lack of effect observed by LTD_4 upon arachidonic acid release and Leukotriene biosynthesis in these cells as shown in Table 4(a) of this thesis. However, this has to be substantiated by further experiments to test whether LTD_4 receptor activation would lead to topoisomerase I phosphorylation and whether this is inhibited by LTD_4 antagonists in RBL-1 cells. Furthermore, the effect of PKC inhibitor, staurosporine, upon this phosphorylation in RBL-1 cells could be examined. The effect of PKC-induced topoisomerase I activation has been shown very recently in a human monocytic cell line, U937, where LTD_4 receptor activation does lead to PLA_2 activation (438).

Studies designed to determine the identity of the IP_3-independent process mentioned above (see **"?"** in Figure 47) have resulted in the demonstration that LTD_4 induces the production of inositol 1,3,4,5-tetrakisphosphate (IP_4) which can not be accounted for by metabolism of IP_3 (439). The production of IP_4 is receptor-mediated, partially inhibited by pertussis toxin and blocked by antagonists of the LTD_4 receptor. The tentative conclusion one can draw from these findings is that LTD_4 receptors are coupled through pertussis toxin-sensitive and -insensitive G-proteins to a phosphatidylinositol 3,4,5-trisphosphate-specific phospholipase C (PIP_3-PIC) and produces IP_4 directly via this mechanism. Furthermore, LTD_4 induces a rapid and relatively transient enhancement in PIP_3 levels in the membrane. This increase is entirely blocked by pretreatment with pertussis toxin. It is found that the increase in PIP_3 content is receptor-mediated and blocked by LTD_4 antagonists (439). This occurs in the presence or absence of extracellular Ca^{2+}. Thus, PIP_3 levels are increased with increasing amounts of IP_4 in a receptor-mediated fashion. The precise mechanisms accounting for the enhancement in PIP_3 are not delineated as yet, nor is the role of enhancement in PIP_3 levels in signal transduction. However, the possibility

Agonist	IP release	LT generation	[^{3}H]-LT & -AA measurement
A23187	(+)	(+)	(+)
LTD_4	(+)	**N.D**	(-)
IgE receptor activation	(+)	(-)	(-)
fMLP	(-)	(-)	(-)
Bradykinin	(-)	(-)	(-)
PAF	(-)	(-)	(-)
Substance P	(-)	**N.D**	**N.D**
Histamine	(-)	**N.D**	**N.D**
Mellitin	**N.D**	(-)	(-)
Compound 44/80	**N.D**	(-)	(-)

Table 6: Summary of the effects of various agonists upon the two phospholipases: phosphoinositide-specific phospholipase C and phospholipase A_2, in RBL-1 cells. **(+)** represents an enhancement on biosynthesis/release. **(-)** represents a lack of effect on biosynthesis/release. **N.D** implies not determined. IP = inositol polyphosphate, AA = arachidonic acid and LT = Leukotriene.

that the increase in IP_4 might result from the increase in substrate (PIP_3) for a PIC can not be ruled out.

Finally, Table 6 summarises the effects of various agonists upon the two phospholipases: PLA_2 (arachidonic acid release and Leukotriene generation) and PIC (IP release). A number of agonists, previously described as acting through either the PIC signalling pathway and/or PLA_2-mediated arachidonic acid cascade, did not induce arachidonic acid release and Leukotriene generation (measure of PLA_2 activation), nor IP release (measure of PIC activation). These findings could have important repercussions for the classification of receptors and/or secondary events in basophils, insofar as receptors for those agonists tested are presumably not present in RBL-1 cells, or at least not coupled to Leukotriene generation and/or IP release and evidence to the contrary was not subsequently found in the literature. However, receptor radioligand binding assays should be performed in the future to investigate this hypothesis.

The mechanisms involved in secondary messenger interactions are already complex, but in conclusion, the present study has demonstrated that amongst other findings, in RBL-1 cells:

a) Cyclic-AMP has a modulatory role on phosphoinositide breakdown and IP release.

b) Phospholipase A_2 activation does lead to phosphoinositide-specific phospholipase C activation and IP release.

c) Phosphoinositide-specific phospholipase C stimulation does not induce Phospholipase A_2 activation, arachidonic acid release and Leukotriene generation.

These are novel findings for second messenger events in RBL-1 cells and they complement and expand other data recently reported in the literature.

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List of Publications Related to the Work Described in this Thesis

1) Di Marzo V, Tippins JR, Galadari SHI and Morris HR: Neuropeptides (1988) 11, 169-172.

2) Galadari SHI, Morris HR and Di Marzo V: Biochem. Int. (1990) 22, 379-386.

3) Galadari SHI, Morris HR and Di Marzo V: Abstract, 37, Presented at the Seventh International Conference on Prostaglandins and Related Compounds, Florence (Italy), May 28-June 1, 1990.

4) Di Marzo V, Galadari SHI and Morris HR: Abstract, 94, Presented at the 18thAharonkatzir-Katchalsky Conference on "From Receptor to Cell Response, Physiology and Pharmacology, S Maria Imbaro, Chieti, Italy, September 9-14, 1990.

5) Galadari SHI, Morris HR and Di Marzo V Biochem. Biophys. Acta. (1991), submitted.

6) Di Marzo V, Galadari SHI, Tippins JR and Morris HR: Life Sciences (1991), in press.