THE REGULATION OF PHOSPHOLIPASE A_2 ACTIVITY IN MITOGEN-STIMULATED SWISS 3T3 FIBROBLASTS

A thesis presented for the degree of

Doctor of Philosophy

by

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March 1992

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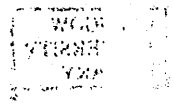
Dedicated to the memory of my Father,

James Currie



ACKNOWLEDGEMENTS

I would like to thank Professor M.D. Houslay and the late Professor R.M.S. Smellie for making available the facilities of the Department for this research work. In addition, I would like to thank management and research staff at Fisons Pharmaceutical Research and Development laboratories for the opportunity to work in their Biochemistry Department. Thanks also go to my Supervisors, Dr. Michael Wakelam and Dr. Clive Jackson for their advice and encouragement during this work. I would like to acknowledge all members of A20, past and present, for making the past three years such a memorable experience! In particular, thanks to Liz for her friendship and much appreciated moral support. Thanks also to Dr. Godfrey Smith and Dr. Karen Crichton for their expertise on the calcium experiments and a special thanks to Mrs. Sadie Brown for her patience and diligence in the typing of this thesis. Finally, I want to thank my family, in particular my Mother, whose constant love and support throughout my entire education has been the greatest contribution of all.

ABBREVIATIONS

AA	Arachidonic acid
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
sn–1,2–DAG	sn–1,2–diacylglycerol
DMSO	dimethylsulphoxide
EGTA	ethylene glycol bis (β–amino ethyl ether) tetraacetic acid
GDPβS	guanosine 5'–(2–0–thio)diphosphate
GPC	glycerophosphatidylcholine
GPI	glycerophosphatidylinositol
GTPγS	guanosine 5'–(3–0–thio) triphosphate
Hepes	4–(2–hydroxyethyl)–1–piperazine ethanesulphonic acid
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
$InsP_1$	inositol monophosphate
$InsP_2$	inositol bisphosphate
$InsP_3$	inositol trisphosphate
LTB_4	leukotriene B_4
LTC	leukotriene C
LTD	leukotriene D
LTE	leukotriene E
LysoPtdCho	lysophosphatidylcholine
LysoPtdEtn	lysophosphatidylethanolamine
LysoPtdIns	lysophosphatidylinositol
LysoPtdOH	lysophosphatidic acid
PDBu	phorbol dibutyrate
PGB_1	prostaglandin B_1
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2

$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
6–K–$PGF_{1\alpha}$	6–Keto–prostaglandin $F_{1\alpha}$
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12–myristate 13–acetate
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
PtdIns	phosphatidylinositol
PtdIns(4,5)P_2	phosphatidylinositol(4,5)bisphosphate
PtdOH	phosphatidic acid
PtdSer	phosphatidylserine

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SUMMARY

A variety of agonists that stimulate inositol phospholipid turnover also activate phospholipase A_2 (PLA_2). This study examines the possibility that bombesin, a well–characterised calcium–mobilising peptide mitogen, may fulfil this criterion. The profile of arachidonic acid (AA) release, as well as the regulatory mechanism(s) behind bombesin–stimulated PLA_2 activity, were examined. It was found that the AA released upon bombesin stimulation, was composed of two phases – an initial transient phase that is essentially independent of extracellular calcium and of PKC activation and a later, sustained phase that is absolutely dependent on extracellular calcium and shows a requirement for PKC activation. This study focusses on the first of these phases and the regulatory mechanism involved in release.

In earlier works it was considered that agonist–induced PLA_2 activation was simply the consequence of activation of phosphoinositide–hydrolysing PLC i.e. a combined effect of calcium mobilisation and PKC activation. More recently, arguments have been presented against this notion and work presented in this study strongly suggests that the calcium–mobilising peptide bombesin activates a phosphatidylcholine–specific PLA_2 via a G protein–coupled mechanism that is parallel to, but independent of, activation of phosphoinositide–hydrolysing PLC.

The AA released via this pathway was not substantially metabolised via either cyclooxygenase or lipoxygenase enzymes. This raised the possibility that the fatty acid itself might have an intracellular role. Within this context, the effect of exogenously added AA on a short–term response (intracellular calcium mobilisation) and a long–term response (DNA synthesis) was studied. The fatty acid appeared to enhance both types of response in this system. The possibility that AA may be a second messenger within these cells must therefore be considered. In this case, at least three main 'second messengers' would be generated during interaction of bombesin with its receptor, namely inositol (1,4,5) trisphosphate, sn–1,2–diacylglycerol and arachidonic acid.

CHAPTER 1

INTRODUCTION

1.1 REGULATION OF THE CELL CYCLE

The growth and division of a single cell into daughter cells comprises the basic events that constitute the cell cycle. Most animal cells *in vivo* exist in a non–proliferating or quiescent state but have the capacity to respond to extracellular signals such as growth factors, hormones and neurotransmitters by re–entering the cycle and increasing their rate of proliferation (Draetta , 1990,Lewin , 1991)

Most of the work involved in preparing for division occurs during the growth phase of the cell cycle which is termed interphase. This occurs after M phase which consists of mitosis and cytokinesis. Interphase starts with the G_1 phase in which the cells, whose biosynthetic activities have been greatly slowed during mitosis, resume a high rate of biosynthesis. This S phase begins when DNA synthesis starts and ends when the DNA content of the nucleus has doubled and the chromosomes have replicated. The cell then enters the G_2 phase, which ends when mitosis starts. Cell cycle times vary widely in higher eukaryotic cells, with most of the variability being in the length of G_1. This may be due to G_1 consisting of a series of subphases (Pardee, 1989), starting either from G_0 non–proliferating state or from the previous cell cycle. Cells *in vivo* can remain healthy for very long periods in the quiescent state (G_0). Cells in culture can also be made to enter a quiescent state. Fibroblasts in culture move out of cycle and into G_0 within 1h after being placed in suboptimal conditions such as in a low serum–containing medium. These cells can be stimulated to re–enter the cycle during G_1 by adding back serum or defined growth factors to the medium (Campisi *et al.*, 1984). It is clear that the cell cycle must be finely regulated. The parent cell must pace its division to occur exactly when its mass has doubled. Cells must also coordinate events in the cycle. Regulation may occur at two different levels – intracellular and extracellular.

Physiological control of growth initiation is external; it is created by other cells as required in a multicellular organism. External controls switch the intracellular machinery between G_0 and G_1, leading to DNA synthesis and cell

division. Once the commitment to DNA synthesis has occurred, the subsequent events are regulated intrinsically by the cell itself (Pledger *et al.*, 1978). Defined mitogens include polypeptide growth factors, hormones and various pharmacological agents. These all bind to specific, high affinity receptors, which upon occupancy, promote the generation of early signals in the membrane and cytosol which are propagated into the nucleus. These early events precede various molecular and cellular responses which all lead to a common final pathway of DNA synthesis and cell division.

Quiescent cultures of the Swiss 3T3 cells line (Todaro & Green, 1963) have provided a useful model system for studies on growth control. Studies conducted with combinations of defined growth–promoting molecules have revealed the existence of striking synergistic interactions (Rozengurt, 1982). Tumour cell lines, which are significantly less dependent on external growth factors and peptides for proliferation, often produce growth factors themselves which may act in an autocrine manner. A link between production of certain growth–promoting molecules and the expression of transformation is well illustrated in the homology between the amino acid sequence of the β chain of PDGF and the transforming protein of the simian sarcoma virus encoded by the *sis* oncogene (Doolittle *et al.*, 1983). It therefore seems likely that the signals elicited by peptides and growth factors may play a critical role in modulating normal and abnormal cell proliferation.

Internal regulation of the cell cycle was initially thought to depend on the activity of a protein termed 'maturation promoting factor' (MPF) (Murray & Kirschner, 1991). However, other studies conducted by Nurse (1990) on the activation of cell division cycle (cdc) genes, showed that one of the key proteins regulating cell cycle events is a serine/threonine protein kinase, the cdc^{2+} gene product $p34^{cdc2}$. This is thought to phosphorylate proteins involved in the control of the major events of M phase. The kinase is itself regulated by phosphorylation/

dephosphorylation reactions with $p80^{cdc25}$, the product of the $cdc25^+$ gene involved in activation. It is not entirely clear how the M phase control mechanism is coupled to other mechanisms measuring time, cell mass and growth rate, but the requirements for several signals may explain why there are a number of different elements involved in the mechanism.

1.2 EARLY MITOGEN–STIMULATED EVENTS IN QUIESCENT CELLS

1.2.1 Ion fluxes

Changes in intracellular ion composition are prominent following cellular activation by mitogens. These provide a useful tool for distinguishing responses to different types of mitogen.

(a) Changes in intracellular calcium

One of the earliest responses elicited by addition of the mitogens bombesin (Hesketh *et al.*, 1985), PDGF (Frantz, 1985) and vasopressin (Nanberg & Rozengurt, 1988) to quiescent 3T3 cells is mobilisation of Ca^{2+} from intracellular stores. The addition of these mitogens causes a transient rise in cytosolic Ca^{2+} concentration, increases Ca^{2+} efflux from cells and decreases the total Ca^{2+} content of the cell. A transient acidification also occurs as a result of the initial rise in Ca^{2+}. This may result from action of a Ca^{2+}/H^+ antiport system involved in re–uptake of Ca^{2+} into intracellular stores (Ives & Daniel, 1987). The mobilisation of intracellular Ca^{2+} has been proposed to be mediated by $Ins(1,4,5)P_3$ which is a second messenger in the action of many ligands that induce receptor–mediated inositol lipid turnover and calcium mobilisation (Berridge & Irvine, 1984).

A more sustained phase of Ca^{2+} release may occur later in response to agonist stimulation and rather than being dependent on the calcium content of intracellular stores, may be dependent on receptor–triggered calcium influx (Meldolesi *et al.*, 1991). This influx of calcium is physiologically important in a wide range of intracellular events. In many secretory cells, exocytosis induced by receptor

activation has been found to depend on influx and to be eliminated when this is blocked ((Stauderman *et al.*, 1990). Other signalling events, such as activation of intracellular phospholipases, are largely calcium dependent. In some systems, the generation of AA via PLA_2 activation may be the consequence of the prolonged influx–sustained increases of intracellular calcium rather than of the initial spike (Brooks *et al.*, 1989). Calcium influx is also important in cell growth. An increase in calcium via $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ is obligatory for normal progression to DNA synthesis after egg–fertilisation (Irvine & Moor, 1986). Calcium is centrally involved in mitosis itself, where it is essential for the disassembly process of the spindle fibres that occurs during anaphase.

The cell proliferation response induced by EGF is severely curtailed when calcium influx is inhibited by imidazole derivatives (Magni *et al.*, 1991). In addition, bombesin–induced mitogenesis in Swiss 3T3 cells is strictly dependent on medium calcium concentration. This may be partly due to the involvement of calmodulin. Chafouleas *et al.* (1982) have shown that cellular calmodulin content increases about 2–fold at late G_1 and/or early S phase of the cell cycle. It has also been reported that a calmodulin antagonist (Hidaka *et al.*, 1981) or expression of calmodulin anti–sense RNA (Rasmussen & Means, 1989) potently inhibits cell proliferation. The intracellular calcium concentration is therefore a vitally important regulatory factor during the events of the cell cycle.

(b) **Cytosolic pH changes**

Stimulation of Na+ influx and a rise in cytosolic pH of about 0.2 units represent an almost universal response that precedes metabolic activation of quiescent cells (Pouyssegur *et al.*, 1985; Grinstein *et al.*, 1989). Mitogenic agents such as purified growth factors (Moolenaar *et al.*, 1983), peptides (Hesketh *et al.*, 1988) and serum (Schuldiner & Rozengurt, 1982) have all been shown to cause a rapid rise in intracellular pH occurring within a few minutes. This alkalinisation results largely from activation of a membrane–bound amiloride sensitive Na^+/H^+ antiporter.

Activation may occur via a phosphorylation of the protein by protein kinase C (PKC) (Vara & Rozengurt, 1985). Schuldiner and Rozengurt (1982) showed that incubation of quiescent 3T3 cells with PDGF, vasopressin and insulin increased the cellular pH by 0.16 pH units as measured from the uptake of weak acids. The ability of these mitogens to cause cytoplasmic alkalinisation suggested that the activation of Na^+/H^+ exchange was a primary effect of the mitogens rather than a secondary mechanism for the extrusion of protons resulting from a growth factor induced acceleration of cellular metabolism. Prevention of mitogen–stimulated Na^+ influx and pH rise in a fibroblast mutant lacking Na^+/H^+ antiport activity (Pouyssegur *et al.*, 1984) suppressed growth factor induced DNA synthesis at neutral and acidic external pHs. More detailed studies have demonstrated that the increased intracellular pH is required throughout the G_o/G_1 period preceding DNA synthesis. When cells have reached the restriction point, they can enter and progress into S phase at a much lower pH (Pouyssegur *et al.*, 1985). Mitogen–stimulated Na^+/H^+ antiport activity is therefore critical in regulating the rate of cell progression from G_o/G_1 to S phase. In the study by Hesketh *et al.* (1988), both calcium and pH responses to mitogens in single 3T3 fibroblasts were examined. Although both changes were found necessary for progression to DNA synthesis, they were not solely sufficient to commit cells to S phase. Elevation of intracellular pH in human fibroblasts in the absence of growth factors had little effect on DNA synthesis and an increase in thymidine incorporation was only in the presence of serum (Moolenaar *et al.*, 1986).

In certain cell types, e.g. T lymphocytes, inhibition of Na^+/H^+ exchange and prevention of cytoplasmic alkalinisation by amiloride analogues did not prevent mitogen–stimulated DNA synthesis or *c–fos* expression (Grinstein *et al.*, 1989). This may indicate that intracellular pH may be regulated differently in these cells. The common underlying mechanism between cell types is likely to be an increase in the intracellular Na^+ concentration and not an alkalinisation. Some controversy surrounds the importance of the Na^+/H^+ antiporter in cell proliferation. It is almost

always stimulated early in the mitogenic response and may regulate some of the reactions that lead to proliferation. However, the involvement of other regulatory factors in this process is certainly evident and the importance attached to different signals may vary between cell types.

1.2.2 Protein phosphorylation

Another series of early events that occur in mitogen–stimulated cells during the pre–replicative phase of the cell cycle involve the regulation of various protein functions through phosphorylation/dephosphorylation reactions. There are a number of protein kinases and phosphatases important in signal transmission. Initial phosphorylations are often on tyrosine residues as with the autophosphorylation by the EGF receptor's intracellular tyrosine kinase (Yarden & Ullrich, 1988), but subsequent phosphorylations are mainly on serine and threonine residues as seen with PKC (Kikkawa *et al.*, 1989). PKC plays a pivotal role in signal transmission and has a large number of targets, most of which are yet to be determined. One important target is the Na^+/H^+ antiporter, mentioned in 1.2.1. Others may include the Ca^{2+}–transport ATPase and the Na^+/Ca^{2+} exchange protein, both of which remove calcium from the cytosol (Nishizuka, 1986). PKC also causes inhibition of receptor–mediated hydrolysis of inositol phospholipids. Such a negative feedback role of PKC may also be extended to long–term responses such as cellular proliferation. The EGF receptor can be phosphorylated by PKC, resulting in a rapid decrease in high affinity binding of EGF as well as inhibition of ligand–induced tyrosine phosphorylation (Schlessinger, 1986). Long–term phorbol myristate acetate (PMA) pre–treatment, which causes PKC depletion, relieves the cell from receptor down–regulation and may provide useful information regarding the role of PKC in the mitogenic action of an agonist. PKC also exhibits a positive forward action. The kinase may be involved in gene expression, such as induction of the IL2 receptor and some proto oncogene activation (Nishizuka, 1986). Studies on fibroblasts overproducing PKC suggest a positive action in growth regulation. Overexpression

of the β1 form of PKC leads to increased growth and colony formation in the presence of PMA (Housey *et al.*, 1988). This is probably due to increased phosphorylation of critical cellular proteins involved in the control of cellular growth and morphology. These results suggest that disturbances in the control of PKC down–regulation could accentuate the action of tumour promoters and thus potentiate the carcinogenic process.

Another kinase that is activated in response to a variety of ligands is the protein product of the c–raf–1 gene (the cellular homologue of *v–raf*, the transforming gene of the murine sarcome virus 3611 (Rapp *et al.*, 1988)), designated Raf–1 which has intrinsic kinase activity toward serine and threonine residues. Stimulation of fibroblasts with PDGF, EGF, FGF and PMA causes hyperphosphorylation of Raf–1 itself and activation of the protein (Blackshear *et al.* 1990). Whether this is a direct receptor–mediated activation via tyrosine phosphorylation remains uncertain and further understanding will require mutational analysis of the phosphorylation sites (Ping *et al.*, 1991). It is also unknown whether Raf–1 has a central or peripheral role in the signal transduction process. It certainly plays a significant role in signalling pathways emanating from the tyrosine kinase class of receptors and since Raf–1 can be indirectly activated by PMA, it may also be involved in signalling pathways from non–tyrosine kinase receptors. One of the functions of Raf–1 may be the integration of signals from a variety of sources. More specifically, it is known that constitutively activated forms of Raf–1 can induce transcription of growth factor–regulated early response genes including c–*fos* and β–actin genes (Siegfried & Ziff, 1990). Thus the ultimate role of Raf–1 may be to carry signals to specific transcription factors.

During early G_1 phase of the cell cycle, the multiple phosphorylation of the 40S ribosomal protein, S6 has been suggested to facilitate the initiation of protein synthesis (Martin–Pérez *et al.*, 1984). The S6 kinases that promote this phosphorylation have been identified as two groups –the 90kd and 70kd kinases

(Sturgill & Wu, 1991) which appear to be under separate regulation. The 90kd S6 kinases are phosphorylated *in vitro* by $p42^{mapK}$ – a MAP kinase. MAP kinases (mitogen activated protein kinases) are activated by a diverse array of mitogenic agents via threonine and tyrosine phosphorylations and have been shown to have several correlations with $p34^{cdc2}$. MAP kinases have been suggested to exert control over the G_o to G_1 transition, just as $p34^{cdc2}$ functions at G_1 to S phase and G_2 to M phase transitions. Their importance in mitogenesis has been demonstrated in cells defective in MAP kinase activation which show defective mitogenesis (L'Allemain *et al.*, 1991). No kinases capable of phosphorylation and activation of the 70kd S6 kinases have yet been identified, although possibilities include Raf–1 and the 90kd S6 kinase (Sturgill & Wu, 1991).

The cell cycle protein $p34^{cdc2}$ is also a kinase that is regulated by phosphorylation/dephosphorylation as previously discussed (see Section 1.1). Phosphorylation of the cyclin B–$p34^{cdc2}$ complex causes activation and starts the events such as chromosome condensation, nuclear membrane breakdown and reorganisation of the microtubule network that characterise mitosis.

The clear importance of phosphorylation/dephosphorylation reactions in cell cycle regulation is demonstrated by their involvement throughout various stages. The regulation of the kinases and phosphatases responsible depends on the activation of the signal transduction pathways associated with receptor activation.

1.2.3 Gene transcription

Abnormal expression of a variety of normal cellular genes (proto–oncogenes) can transform a normal cell into a tumour cell. Several of these genes encode protein products which are homologous to either a growth factor or a growth factor receptor. For example, the *sis* oncogene of the simian sarcoma virus shows striking homology with the β–chain of PDGF (Waterfield, 1983). Part of the sequence of the EGF receptor is almost identical to another oncogene, erb–B, carried by avian erythroblastosis virus (Downward, 1984). Several virally encoded transforming

proteins have normal cellular homologues that function in cell cycle regulation, suggesting that in general, proto–oncogenes might be involved in cell cycle control (Greenberg & Ziff, 1984). The c–*onc* genes, especially those encoding nuclear antigens, are differentially regulated at the transcriptional level, during cell cycle progression.

Stimulation of BALB/c–3T3 cells with defined mitogens induces a rapid, transient increase in transcription of c–*fos*, a nuclear protein, whose function, until recently, remained unknown (Greenberg & Ziff, 1984). This is followed by a more protracted elevation of c–*myc* gene expression. The increase in c–*fos* levels occurs as 3T3 cells progress from G_0 to G_1 before S phase. In similar conditions, many other c–*onc* genes including c–ki–*ras* and c–*abl* show barely detectable levels of transcription. An early response gene such as c–*fos*, whose deregulation can result in cell transformation, may be a gene that directly controls normal cell growth. The expression of c–*fos* throughout the cell cycle rather than during a specific stage, may be one mechanism of transformation.

It has been reported that expression of the c–*myc* gene introduced into 3T3 cells by gene transfer, partly substitutes for PDGF and stimulates DNA synthesis in the presence of EGF and platelet–poor plasma (Armelin *et al.*, 19). This strongly suggests that the c–*myc* gene may play a role of crucial importance in rendering cells competent for DNA synthesis.

Bombesin–stimulated induction of c–*fos* and c-*myc* has been suggested to depend on an increase in intracellular calcium and in PKC activation (Rozengurt & Sinnett–Smith, 1987). However, EGF may not depend on PtdIns(4,5)P_2 breakdown or activation of PKC to induce c–*fos* or c–*myc* expression (McCaffray *et al.*, 1987). This illustrates the fact that gene expression may be activated through various pathways.

Another of the genes that is rapidly expressed in response to mitogenic stimulation is *jun*–B. This has been shown to encode a protein related to the human

transcription factor, AP–1, the product of the protooncogene c–*jun*. It is now known that *fos* binds to the AP–1 consensus recognition sequence and this suggests there is some connection between *fos*, *jun* and AP–1. It is possible that *fos* and *jun* may constitute a class of ligands that interact with genomic receptors containing the AP–1 site, thereby modulating gene transcription. As such, they may comprise a primordial signalling system adopted for use by selected target genes in different cell types (Curran & Franza, 1988).

1.3 SIGNAL TRANSDUCTION PATHWAYS IMPLICATED IN THE CONTROL OF CELLULAR PROLIFERATION

The flow of information upon cell stimulation by a ligand can be traced through a cascade of regulatory reactions that involve a number of specific enzymes, regulatory proteins and other regulatory factors. Comparison between these regulatory cascades reveals a highly sophisticated design underlying the signal transduction systems.

Both the adenylyl cyclase and the phospholipase C–based signal cascades may be divided into three functional domains: the first, occurring at the plasma membrane, functions to convert the external stimulus into an intracellular message; the second, which is mainly cytosolic, is responsible for translating the intracellular message into regulatory activities; and the third domain contains the regulatory activities directed towards various cellular proteins to result in the appropriate cellular responses. The transducing factor in the first domain may take the form of a GTP–binding protein that couples the receptor to the transducing pathway (Gilman, 1984). This contributes to the rigorous control within the system. The minimal essential component in the second translatory domain is a second messenger activated protein. Upon binding of the second messenger, this protein becomes capable of modulating specific cellular responses. For both cAMP and DAG, regulatory activities are

performed by multifunctional protein kinases, cAMP dependent protein kinase (Walsh *et al.*, 1968) and PKC (Nishizuka, 1986).

The mechanisms responsible for terminating signal transduction are as important as those involved in activation. Starting from the membrane receptor, at least two mechanisms have been suggested that may terminate receptor function. Phosphorylation by specific kinases may render the receptor insensitive to stimulation by its agonist, or internalisation via endocytosis may cause the same effect (Sibley *et al.*, 1987). These mechanisms are the basis of the well–known phenomenon of desensitization.

For the coupling G proteins, it has been suggested that the mechanism of terminating the coupling activity is intrinsic to these proteins. G proteins are heterotrimers, consisting of an α, β and γ subunit in its inactive state, a G protein contains bound GDP on its α subunit. The exchange of GDP for GTP is part of the activation process of the G proteins. Since the proteins also express GTPase activity intrinsic to the α subunit, the active GTP–bound form is converted back to the inactive GDP–bound form by the GTPase reaction (Stryer & Bourne, 1986).

The disposal of excess second messengers is often achieved by enzymatic degradation. Thus cAMP and cGMP are hydrolysed by cyclic nucleotide phosphodiesterases, and IP_3 and DAG may be degraded by IP_3 phosphatase and DAG lipase respectively. DAG may also be cleared from the cell via DAG kinase. Examination of the regulation of the basic reactions of the signal cascades has revealed various potential means for intrasignal system fine–tuning and intersignal system cross–talk, suggesting that the various reactions involved in signal transduction form an interacting network.

1.3.1 Tyrosine kinase activity

Tyrosine phosphorylation is an important cell regulatory mechanism. The fact that many polypeptide growth factor receptors have an intrinsic tyrosine kinase activity and several protooncogene products are themselves tyrosine kinases lends

strong support for a role of tyrosine phosphorylation in cell proliferation (Hunter & Cooper, 1985). The finding that the EGF receptor contains as an integral part of its structure, a tyrosine–specific protein kinase capable of autophosphorylation (Cohen *et al.*, 1980) was followed by similar reports for insulin (Kasuga *et al.*, 1982) and PDGF (Ek *et al.*, 1982).

The identification of endogenous tyrosine kinase substrates has recently received considerable attention. Studies have shown that the stimulation of PLCγ1 by growth factors may involve tyrosine phosphorylation of the enzyme (Rhee *et al.*, 1989). In addition, interest has focussed on pp42, a serine/threonine protein kinase (MAP kinase see earlier) whose activation leads to phosphorylation of the ribosomal protein, S6 (Sturgill & Wu, 1991). More recently, Leeb–Lundberg and Song (1991) have suggested that bradykinin and bombesin stimulate tyrosine phosphorylation of a 120kd group of proteins in Swiss 3T3 cells. This is a PKC–independent signal and occurs rapidly (within 0.5min) after peptide stimulation. In contrast to EGF–promoted tyrosine phosphorylation, the peptide–promoted phosphorylation via cytosolic kinases probably depends on formation of a cellular 'second messenger' and may be related to inositol phosphate formation. It is highly likely that growth occurs as a result of interaction between the different signalling pathways.

1.3.2 Adenylyl cyclase

Another early signal that may be generated within cells in response to mitogenic stimulation is cAMP. This occurs via the stimulation of adenylyl cyclase activity by a specific G protein (G_s) linked to the receptor (Rozengurt *et al.*, 1981). The effect of cAMP on proliferation has been controversial. In 3T3 cells increased levels of cAMP have been suggested to reduce the rate of growth and proliferation (Kram *et al.*, 1973). However, another group (Rozengurt *et al.*, 1981) has found cAMP will act synergistically with growth–promoting agents to stimulate DNA synthesis in quiescent cultures of 3T3 cells. In addition, Millar and Rozengurt (1988) suggest that bombesin causes enhanced cAMP accumulation in Swiss 3T3

cells. They propose that this may be one mechanism by which bombesin may affect mitogenesis. Contrary to this, it has been found that cAMP levels drop when quiescent cells are stimulated by mitogens (Otten *et al.*, 1972).

In BALB-c-3T3 cells, raising cAMP levels has been shown to potentiate the acquisition of the competence state (O'Keefe & Pledger, 1983), but sustained elevation impedes progression into the rest of the pre–replication phase. In some 3T3 cell lines which are quiescent in the presence of insulin, elevation of cAMP initiates the pre–replication development which continues until DNA synthesis (Rozengurt & Mendoza, 1985). In other systems such as bronchial epithelial cells, cAMP has a major role in the progression into the pre-replicative phase initiated by other factors (Willey *et al.*, 1985). It has also been shown by Smets and Van Rooy (1987) that cholera toxin–treated 3T3 cells during the G_0 to G_1 transition, increased the fraction of cells responding to serum stimulation, whereas addition during late G_1 inhibited the onset of DNA synthesis. Cholera toxin causes persistent activation of G_s. Thus, the major control points in the pre–replicative phase (initiation and progression) can be dependent on cAMP augmentation.

Such opposite effects of the cAMP cascade as mentioned may be explained in a number of ways. There may be variation in the optimal concentration of cAMP required (Pawelek, 1979). Alternatively, the effect may depend on the differentiation state of the cell. Positive and negative regulatory elements have been demonstrated in the promoters of cAMP responsive genes. The accessibility of these elements in the promoter of a growth related gene could depend on the differentiation state of the cell (Dumont *et al*, 1989). Another explanatory factor may be the existence of two isozymes of cAMP dependent protein kinase. Studies have demonstrated changes in their levels in a range of human cancer cell lines (Katsaros *et al.*, 1987).

The isozymes differ in their R subunits (Hoffman *et al.*, 1975) and since both isozymes have been detected in most cells, selective activation of the enzymes may be a function of cAMP in regulating mitosis. Katsaros *et al.* (1987) have suggested the

involvement of the type II protein kinase in growth inhibition. A range of cAMP analogues were used to demonstrate inhibition of growth of human cancer cell lines. This inhibition appeared to be associated with an increase in the cellular levels of the RII subunit. Activation of this subunit by site selective analogues has also been shown to reverse the transformation of NIH–3T3 cells (Tagliaferri *et al.*, 1988). Ridgeway *et al.* (1988) have also shown that treatment of Kirsten murine sarcoma virus transformed Balb/c–3T3 cells with dibutyryl cAMP inhibits the expression of transformation related properties such as anchorage independent growth. This strongly implies that a decrease in cellular cAMP levels is associated with the loss of controlled proliferation. The importance of cAMP in the regulation of cellular proliferation is demonstrated by Vallar *et al.* (1987) in growth hormone secreting pituitary adenomas which exhibit altered G_s and increased adenylyl cyclase.

Recent studies involving mutational analysis have also proved useful in identifying the importance of cAMP. Landis *et al.* (1989) showed that constitutive production of cAMP, as a consequence of mutations in α_S, may contribute to the abnormal growth of certain pituitary tumours. More recently, Zachary *et al.* (1990) showed that replacement of glutamine–227 with leucine in the GTP–binding domain of α_S caused increased basal adenylate cyclase activity, cAMP accumulation and mitogenic sensitivity in response to forskolin. This supports a role for cAMP in the regulation of cell proliferation and suggests that alterations in the G protein can directly modify the ability of cells to respond mitogenically to extracellular factors.

1.3.3 Phosphoinositide–specific phospholipase C

One of the most widely studied signalling pathways that is activated upon mitogenic stimulation is that of phosphoinositide hydrolysis by PLC with the resultant second messengers, Ins(1,4,5)P_3 and DAG (Berridge, 1987; Whitman & Cantley, 1988). The 'bifurcating signal' hypothesis of Berridge (1983) proposes that breakdown of phosphatidylinositol (4,5) bisphosphate activates two distinct signalling pathways: production of Ins(1,4,5)P_3 triggers release of calcium from

intracellular stores (Streb *et al.*, 1983), and release of DAG activates PKC (Nishizuka, 1986).

Activation of PLC is regulated by a specific G protein (G_p) coupled to the receptor. This protein is probably the G_q/G_{11} species. Taylor *et al.* (1991) have purified an α subunit that stimulates partially purified PLC and report that this G protein specifically activates the β1 isozyme, but not the γ1 and δ1 isozymes of PLC. This G protein appears to be related to the G_q class of G protein α subunits. This observation has also been made by Smrcka *et al.* (1991).

The extremely rapid activation of PLC in response to agonists such as bombesin (Plevin *et al.*, 1990), bradykinin (Slivka & Insel, 1987) and PDGF (Blakeley *et al.*, 1989) has been suggested to be the initial signal required for activation of other enzymatic pathways such as PLA_2 (Axelrod, 1990) and PLD (Cook *et al.*, 1991). It is now recognised however, that this is not always true and that certainly for PLA_2, an independent regulatory mechanism exists (see Section 1.5). The PLC–stimulated hydrolysis of PtdIns(4,5)P_2 is however, essential in mitogenesis and this is well demonstrated in experiments where microinjection of anti–PIP_2 antibodies into NIH–3T3 cells abolished the stimulation of DNA synthesis in response to PDGF and bombesin (Matuoka *et al.*, 1988). Both Ins(1,4,5)P_3 and DAG play important roles in the regulation of proliferation.

1.3.1.(i) Inositol (1,4,5) trisphosphate

The production of more than 20 inositol phosphate isomers has been identified in agonist–stimulated cells (Majerus *et al.*, 1988). Ins(1,4,5)P_3 induced calcium release occurs within seconds (Hesketh *et al.*, 1985) of agonist addition. This has been shown to occur via an Ins(1,4,5)P_3 receptor, localised to the endoplasmic reticulum (Ross *et al.*, 1989). In addition to the rapid production of Ins(1,4,5)P_3, induction of a whole cascade of kinases and phosphatases specific for inositol phosphates occurs. $InsP_3$ kinase activation leads to Ins(1,3,4,5)P_4 production which may also be involved in the promotion of calcium–mediated responses in

several systems (Irvine & Moor, 1986). For long–term processes such as regulation of proliferation, changes in the entry of calcium into the cell may be just as important as the intracellular release, thereby requiring the involvement of multiple inositol phosphate isomers (Whitman & Cantley, 1988).

(ii) sn-1,2–diacylglycerol

DAG, the other product of PLC–mediated PtdIns(4,5)P_2 hydrolysis, unlike Ins(1,4,5)P_3, remains associated with the plasma membrane and functions as an endogenous activator of PKC. Different isozymes of PKC appear to have different regulatory requirements: activation of α, β and γ PKC by DAG appears to require calcium (Gschwendt *et al.*, 1991) whereas δ and ε that lack the calcium–binding region (Ono *et al.*, 1988) may be activated by DAG alone. There may even be, at least one, isozyme which does not require DAG for activation (Ono *et al.*, 1989). The importance of PKC has been discussed (see Section 1.2.2) and the existence of a variety of isozymes with different regulatory requirements may enhance the involvement of the enzyme in proliferation.

The generation of DAG in agonist–stimulated cells has, more recently, been shown to occur from alternative sources to PtdIns(4,5)P_2. Phosphatidylcholine has been identified as a major alternative source (Muir & Murray 1987) which may contribute to the second phase of the biphasic DAG response in bombesin–stimulated Swiss 3T3 cells (Cook & Wakelam, 1989). This has been proposed to occur as a result of PLC– (Muir & Murray, 1987) or PLD– (Cook & Wakelam, 1989) stimulated hydrolysis of the phospholipid.

As well as serving as the endogenous activator of PKC, 1–stearoyl 2–arachidonyl diacylglycerol can, when hydrolysed by diglyceride lipase, serve as a source of arachidonic acid (AA), although generation of this fatty acid is usually a result of PLA_2 activation (Axelrod, 1990).

The importance of DAG in the stimulation of cell proliferation is demonstrated by the similar actions of tumour promoting phorbol esters (Castagna *et al.*, 1982)

which mimic the DAG activation of PKC by directly binding to the enzyme, although in a sustained manner. These have proved useful in the investigation of the role PKC plays in a variety of cellular processes.

1.3.4 Receptor mediated phospholipase D activation

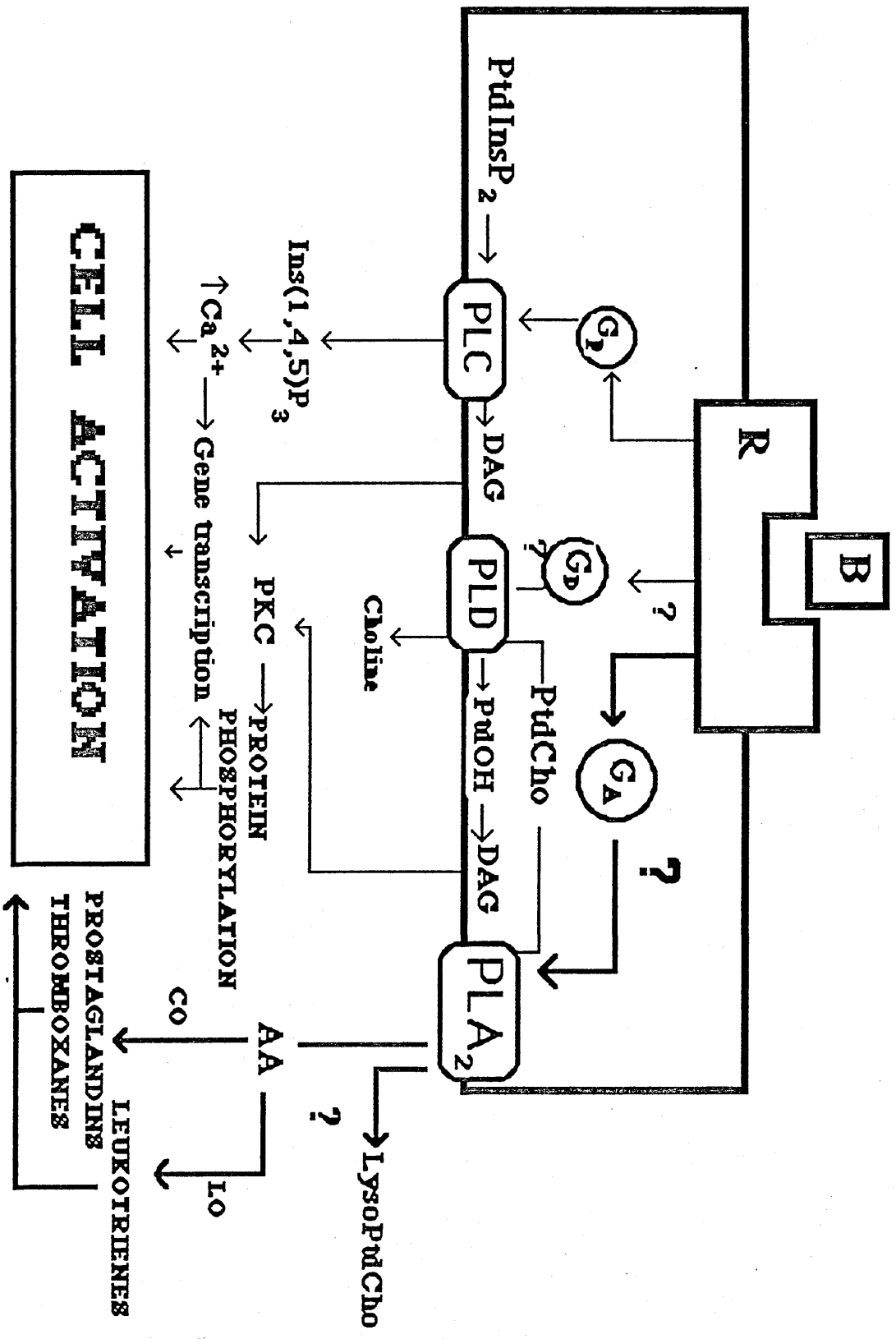
Recent evidence has suggested the involvement of PLD in signal transduction (Cook *et al.*, 1991; Thomson *et al.*, 1991). The enzyme normally hydrolyses phosphatidylcholine producing free choline and phosphatidic acid. Receptor–coupled PLD activation was first demonstrated by Billah *et al.* (1989) in granulocytes and since then has been observed in a number of cell types (Thomson *et al.*, 1991). (Martin and Michaelis, 1989) PLD activity may be dependent on both calcium levels (Billah *et al.*, 1989) and PKC activity (Cook *et al.*, 1991). This may suggest a link between PLD activation and PIP_2 hydrolysis by PLC. However, there are situations in which PLD activation seems independent of prior PLC activity. In collagen–stimulated platelets (Randall *et al.*, 1990) and in EGF–stimulated 3T3 fibroblasts (Cook & Wakelam, 1992, in press). In both situations PLD activation occurs in the absence of significant PtdIns(4,5)P_2 hydrolysis by PLC. It seems likely that PLD is under different regulation between systems.(Liscovitch 1990,Thomson *et al.* 1991)

PLD activation gives rise to two potential second messengers, DAG and phosphatidic acid (PtdOH). The PtdOH produced may be converted to DAG by phosphatidic acid phosphohydrolase, leading to higher intracellular DAG levels. The role of DAG generated from phosphatidylcholine is uncertain in terms of PKC activation. It may activate some forms of PKC. Phosphatidic acid may act as a mitogenic agent but this is also unclear. Recent observations (Bauldry *et al.*, 1991) suggest that PLD activity may be induced in virally transformed cells. This may implicate PLD in the process of transformation. In general though, the main functional role of PLD remains to be established.

1.3.5 Receptor–mediated phospholipase A_2 activation

Mitogenic stimulation of different cell types leads to activation of phospholipase A_2. This enzyme was initially thought to be regulated by prior activation of the phosphoinositide–PLC pathway (Billah, 1987). However, it is now well established that in a number of systems, PLA_2 may be independently regulated through a specific G protein (G_a) linked directly to the receptor (Axelrod *et al.*, 1988). Activation of PLA_2 results in the hydrolysis of major phospholipids generating arachidonic acid and the corresponding lysophospholipid. This production of arachidonic acid is widely used as a means of measuring PLA_2 activation. Although AA can be formed via other enzymatic pathways, for example via DAG lipase, the PLA_2 pathway forms, by far, the major route for formation.

AA can be further metabolised intracellularly via a series of enzymes, giving rise to prostaglandins, leukotrienes and epoxygenase products (Needleman *et al.*, 1986). These are discussed in Section 3.1.6. Both AA and its metabolites have been suggested to be of importance in mitogenesis. The production of AA in Swiss 3T3 cells has been proposed to stimulate DNA synthesis in response to various ligands (Gil *et al.*, 1991; Takuwa *et al.*, 1991). In addition, the fatty acid has been suggested to have a second messenger type role and to release intracellular calcium (Wolf *et al.*, 1986; Chan & Turk, 1987; Chow & Jondal, 1990), induce calcium influx (Alonso *et al*,.1990) and activate calcium extrusion (Randriamampita & Trautmann, 1990) in various cell types. It has also been suggested to activate PKC directly (in particular the γ and α isoforms) in the absence of calcium (Murakami *et al.*, 1985, 1986; Shearman *et al.*, 1991) and in a synergistic manner with DAG (Lester *et al.*, 1991). Lysophospholipids, the other products of PLA_2 hydrolysis have also been suggested to regulate PKC (Oishi *et al.*, 1988) both positively and negatively. It was suggested by Oishi *et al.* (1988) that since PLA_2 may generate two different second messengers that directly activate PKC, one of which (lysoPtdCho) having an activation potency comparable to that of DAG, coupled with


R
B
PtdInsP2
PLC
G1
DAG
Ins(1,4,5)P3
↑Ca2+
Gene transcription
PKC
PROTEIN
PHOSPHORYLATION
PLD
G?
?
Choline
PtdOH
DAG
PtdCho
GA
?
PLA2
?
LysoPtdCho
AA
CO
LO
LEUKOTRIENES
PROSTAGLANDINS
THROMBOXANES
CELL ACTIVATION

the high membrane content of PtdCho, it was conceivable that the lysoPtdCho/AA system could be at least as functionally important as the DAG system. This however, would only be true if the products of PLA_2 hydrolysis were generated at a high enough concentration. The most potent lysophospholipid in the induction of cellular proliferation is lysophosphatidic acid (LPA). Van Corven *et al.* (1989) have shown the ability of LPA to activate three separate signalling cascades and ultimately stimulate DNA synthesis. The initial mechanism underlying the action of LPA is unknown. However, it is thought to involve, in NG108 cells, regulation of a phosphatase which will dephosphorylate certain proteins phosphorylated by tyrosine kinase. In NG108 cells, the activation of $pp60^{src}$ (Moolenaar *et al.*, unpublished data) is thought to be regulated in this manner.

Activation of PLA_2, along with PLC, may constitute one of the very early mitogenic signals in stimulated cells, leading to activation of various signalling pathways and ultimately cell proliferation. As such, the comprehension of how this enzyme is distributed and regulated is of substantial importance in understanding how a quiescent cell may enter G_1 and become committed to DNA synthesis.

1.4 PHOSPHOLIPASE A_2 - A HISTORICAL PERSPECTIVE

PLA_2 is a ubiquitous enzyme found in various forms both extracellularly and intracellularly. In the gastro–intestinal tract, PLA_2 that has been secreted by the pancreas breaks down phospholipids for dietary absorption (Verheij *et al.*, 1981). However, in other mammalian tissues, AA released by PLA_2, serves as the common precursor to the leukotrienes and prostaglandins involved in inflammatory diseases (Dennis, 1987) and may itself have a messenger–type function.

1.4.1 Extracellular and secreted low molecular weight forms

PLA_2s are undoubtedly involved in modulation of membrane composition and signal transduction and their regulation is key to many aspects of homeostasis. Secreted PLA_2s have been shown to have a range of pharmacological and higher–order digestive actions (Davidson & Dennis, 1990). The vast majority of PLA_2s that

have been structurally characterised so far are extracellular, mainly isolated from the venoms of snakes. Snake venom glands are thought to have evolved from digestive glands, because of their location and morphology and their secretion of a great variety of lytic enzymes. Indeed, there is a high degree of similarity between mammalian pancreatic PLA_2 and many venom PLA_2s.

In general, mammalian extracellular PLA_2s are of low molecular weight (approximately 14kd) and can be classified into two types, group I (PLA_2–I) and group II (PLA_2–II), based on their primary structures. Several studies have implicated the correlation of PLA_2–II in the pathogenesis of inflammation (Murakami *et al.*, 1990). Glycogen–induced peritonitis in rabbits was found to be associated with high levels of soluble PLA_2 in peritoneal exudate fluid (Franson *et al.*, 1988). A similar PLA_2 was found in the ascitic fluid of rodents after intraperitoneal injection of casein or zymosan (Gans *et al.*, 1989). PLA_2 is vasoactive and proinflammatory. Intradermal injection of PLA_2 induces sustained hyperemia (Vadas *et al.*, 1981) and an acute inflammatory infiltrate (Pruzanski *et al.*, 1986). Several studies have also documented the induction of oedema in mouse footpads after injection with PLA_2 (Vishwanath *et al.*, 1988).

Group II PLA_2s also alter the function of phagocytes. Co–incubation of human neutrophils and monocytes with PLA_2 from synovial fluid results in marked superoxide generation and lysosomal enzyme release, but decreased chemostatic responsiveness (Pruzanski & Vadas, 1991). Furthermore, group II PLA_2s modulate the inflammatory response to microorganisms; PLA_2 directly, or synergistically with bacterial permeability–increasing protein, degrades the membrane phospholipids of gram–negative bacteria (Kaplan–Harris *et al.*, 1980). PLA_2 activators such as mellitin also enhance the killing of microbes.

High levels of PLA_2 activity have been found in synovial fluid from the inflamed joints of patients with rheumatoid arthritis,psoriasis and osteoarthritis (Pruzanski *et al.*, 1985). Characterisation of this PLA_2 shows it is a calcium–

dependent, neutral active 124 amino acid enzyme which has a molecular weight of 14kd and is strongly cationic with pI >10.5 (Kramer *et al.*, 1989). More recently, the structure of this enzyme has been identified (Wery *et al.*, 1991) and this may be of substantial use in future drug design. This enzyme has been suggested to be identical to PLA_2 purified from human platelets and placenta (Lai & Wada, 1988; Kramer *et al.*, 1989).

There is substantial evidence that both local and systematic inflammatory reactions may share a common repertoire of soluble mediators. Extracellular Group II PLA_2 may serve as a link joining the proximal and distal limbs of the inflammatory response. Since the early stages of inflammation are short and the stage of later effectors involves various factors, neither is easily amenable to effective therapeutic intervention. Therefore, selective inhibition of PLA_2-II may become useful to this end.

PLA_2–I is mainly secreted from the pancreas as an inactive zymogen, which is further converted into the active form by proteases and then has been thought to act as a digestive enzyme (de Haas *et al.*, 1968). However, recent studies have shown that PLA_2–I is not restricted to the pancreas. It has also be detected in rat stomach (Yasuda *et al.*, 1990) and human serum (Nishijima *et al.*, 1983). In addition, the existence of isoforms of PLA_2 in porcine pancreas has been reported (Diccianni *et al.*,1990). These are distinguished by their sensitivity to heparin. The minor isoform is insensitive to changes in pH and calcium concentration and to heparin. In tissues, heparin–resistant isoforms of PLA_2 may be important when the inhibitor heparin is abundant, such as released from mast cells at sites of degranulation. A novel effect of PLA_2–I has recently been described in Swiss 3T3 cells (Arita *et al.*, 1991) where PLA_2 was shown to cause increased proliferation, shown by increased [^{3}H]thymidine incorporation and 3–0–methyl–D–[1–^{3}H] glucose transport, via specific binding sites of approximately M_r 200,000. Pancreatic–type PLA_2, also recognised the sites and stimulated DNA synthesis. These same PLA_2–I binding

sites have also been found in some tissues of several animal species, especially in vasculature. These findings may suggest some role of PLA_2–I in vascular function, which is now being investigated. This may offer a new viewpoint on the effect of mammalian extracellular PLA_2 on cellular function.

1.4.2 The high molecular weight cytosolic PLA_2s

Owing to their relative abundance, most structural and mechanistic studies have focussed on the secreted forms of PLA_2. However, there is now good evidence that hormonally induced eicosanoid production is mediated through a cytosolic PLA_2. A number of ligands that couple to GTP–binding proteins exposed to the cytosol have been shown to stimulate PLA_2 activity (Burch *et al.*, 1986; Jelsema & Axelrod, 1987; Gupta *et al.*, 1990). Many of these ligands also mobilise calcium. This is of interest since submicromolar concentrations of calcium have been shown to cause association of a $cPLA_2$ activity with the membrane fraction (Channon & Leslie, 1990). It is unlikely that a secreted PLA_2 located in a secretory granule could be directly affected by phosphorylation or changes in calcium. In addition, the reducing environment of the cytosol would inactivate the disulphide–rich secreted PLA_2. It therefore seems likely that a cytosolic form would be a likely candidate for promotion of the PLA_2–linked intracellular signals. Recently, a number of studies have reported the existence of a high molecular weight form of the enzyme. Most of these studies have been performed on the human monocytic cell line U937. One of the earlier reports by Diez and Mong (1990) suggested that the enzyme was calcium dependent with a pH optimum of 8.0 and a molecular weight of 56kd. However, subsequent work by Kramer *et al.* (1991) suggests that the 56kd protein was not PLA_2 but a major contaminating band. Kramer *et al.* (1991) have purified the $cPLA_2$ to near homogeneity and found it to be approximately 100kd. They suggest that this form of PLA_2 is involved in the release of eicosanoids in response to ligand activation. This study was followed by molecular cloning and expression of this $cPLA_2$ in order to further understanding of the molecular structure and mechanism of

the enzyme (Sharp *et al.*, 1991). Interestingly, there was no sequence similarity between the human $cPLA_2$ and 68 known secretory PLA_2s. Similarly, computer analysis for global evolutionary relatedness between $cPLA_2$ and other known proteins has shown that none is closely related. It will therefore be of interest to determine the mechanism whereby $cPLA_2$ catalyses the same reaction as the secretory PLA_2s. Studies by Clark *et al.*(1990, 1991) have also suggested the existence of a high molecular weight $cPLA_2$ in U937 cells. They have demonstrated that an amino–terminal 140 amino acid fragment of $cPLA_2$ translocates to natural membrane vesicles in a calcium dependent fashion. This 140 amino acid domain contains a 45 amino acid region with homology to PKC, the synaptic vesicle protein p65 which is involved in fusion, the GTPase activating protein GAP, and PLC. Clark *et al.* (1991) suggest the importance of calcium in promoting the association of $cPLA_2$ with its aggregated substrate and postulate that the homology between these proteins delineates a calcium–dependent phospholipid binding motif providing a mechanism for calcium to translocate and activate cytosolic proteins.

1.5 REGULATION OF PLA_2

A problem in attempting to identify how PLA_2 is regulated exists due to the multiple forms of the enzyme that are present in mammalian systems. Forms may vary between cell types as well as within the same cell. For example, in sheep platelets there appear to be at least two isoforms of PLA_2 which hydrolyse PtdCho and PtdEtn respectively. The isoforms also differ in their sensitivity to calcium, with the PtdEtn–specific form being calcium insensitive (Ballou *et al.*, 1986).

In general, cPLA2 may be activated via the second messengers generated by the PtdIns(4,5)P_2–PLC pathway i.e. calcium, DAG (directly or via PKC activation) or it may be activated directly via a specific G protein.

1.5.1 Role of calcium in PLA_2 activation

In many cell types, PLA_2 activation is closely linked to the occupancy of calcium–mobilising receptors (Lapetina, 1984). In rat platelets (Nakashima *et al.*, 1989) [^{3}H]AA release in response to agonist stimulation was markedly decreased in the absence of extracellular calcium or in the presence of the intracellular calcium chelator, quin 2. Thus, the rise in intracellular calcium was suggested to play a primary role in PLA_2 activation. In addition, Whatley *et al.* (1989) have demonstrated that PAF production via PLA_2 in endothelial cells is dependent upon increases in intracellular calcium that are mediated by hormone–induced activation of a calcium channel. The dependence of PLA_2 activation on prior polyphosphoinositide hydrolysis by PLC has been suggested in a number of reports (Meade *et al.*, 1986; Tamiya–Koizumi)and had become a fairly widespread model until recently when various groups showed that Ins(1,4,5)P_3 production and AA release could be dissociated from one another (Slivka & Insel, 1988; Burch & Axelrod, 1987) and indeed AA release could occur in the absence of inositol phospholipid turnover (Gil *et al.*, 1991; Kanterman *et al.*, 1990).

The possibility that PLA_2 activation might also involve calcium–independent mechanisms has been raised by several studies. A phosphatidylethanolamine–specific PLA_2 purified from human platelets does not require calcium for activity (Ballou *et al.*, 1986). In addition, in HL60 cell homogenates, a PLA_2 has been reported which degrades phosphatidylcholine in the absence of free calcium (Billah *et al.*, 1986). More recently, Mizuno *et al.* (1991) have suggested the existence of a phosphatidylcholine–specific PLA_2 that is calcium independent in rat parotid gland. The situation also exists in which both calcium–dependent and –independent pathways for PLA_2 activation exist in the same cell (Buckley *et al.*, 1991). Bradykinin was shown to rapidly stimulate AA release in endothelial cells and this was closely coupled to changes in calcium concentration. However, it was also shown in the same cells that AlF_4^-–stimulated AA release did not require the presence

PAF; Platelet Activating Factor

of extracellular calcium or the mobilisation of intracellular calcium which was in distinct contrast to the bradykinin response. This, therefore, suggests the existence of multiple pathways of PLA_2 activation within the same cell type and the possible existence of more than one PLA_2 isoform.

1.5.2 Role of PKC in PLA_2 activation

Many of the calcium–dependent PLA_2 activities such as those previously described also show a requirement for PKC activation (Whatley *et al.*, 1989; Tao *et al.*, 1989). In addition, PLA_2 can be activated by a PKC dependent mechanism which is not dependent on influx of extracellular calcium (Ho *et al.*, 1987) or the mobilisation of intracellular calcium (Carter *et al.*, 1989) and may be due to enhanced sensitivity of PLA_2 to calcium. The fact that PKC may be a regulatory factor for Na^+/H^+ exchange may be important for its role in AA release (Sweatt *et al.*, 1986; Akiba *et al.*, 1989). In rabbit platelets intracellular alkalinisation was important for the enhancement of AA release by PKC and when extracellular Na^+ was replaced with N–methyl–D–glucamine, the enhancement was reduced by approximately 50%. PKC has also been suggested to modulate prostaglandin synthesis in mouse peritoneal macrophages (Pfannkuche *et al.*, 1989) and this has been proposed to occur by modulation of AA reacylation via phosphorylation of a regulatory protein linked to acyltransferase activity. It therefore appears that PKC may be involved at various levels in the regulation of AA release. Indeed in the alveolar macrophage, Sporn *et al.* (1990) have demonstrated the existence of PKC–dependent and –independent AA release in response to different stimuli.

There have been some reports suggesting that activators of PKC i.e. diacylglycerols, also cause activation of PLA_2 but independently of PKC activation (Kramer *et al.*, 1987; Burch, 1988). Kramer *et al.* (1987) showed that DAGs are able to stimulate soluble PLA_2 with sonicated arachidonyl–PC as substrate. This effect appears to be far greater for intracellular PLA_2s and may occur directly via conformational changes at the level of PLA_2 itself (Burch, 1988). The study by

Burch correlated DAG activation of PLA_2 to a biological effect, potentiation of PGE_2 synthesis in response to receptor agonists. This action of DAG may be physiologically relevant in that DAG, newly synthesised in response to a variety of agonists, may activate or potentiate PLA_2 activity.

1.5.3 Endogenous PLA_2 inhibition by lipocortins

Lipocortins have been proposed to be important in *in vivo* antiinflammatory reactions due to the fact that purified proteins in this family added to prelabelled cells appear to decrease the release of [^{3}H]AA or thromboxane in response to stimuli (Cirino *et al.*, 1987). Identification of these proteins revealed their similarity to two protein tyrosine kinase substrates, p35 and p36, also termed calpactins. The p35 protein, which is a substrate of the EGF receptor tyrosine kinase (Fava & Cohen, 1984) and is also known as calpactin II, was found to be identical to lipocortin I. Furthermore, this protein was 50% identical to the p36 protein, which is a major substrate of the viral oncogene protein tyrosine kinase $pp60^{v-src}$ (Gould *et al.*, 1986) and is itself the same as lipocortin II and calpactin I. This relatedness appeared to link oncogenesis and growth regulation with a protein which was involved in the regulation of eicosanoid synthesis. Additional members of this protein family have since been found (Pepinsky *et al.*, 1988) and there are now six lipocortins. The stimulation of lipocortin synthesis has been shown to occur in response to glucocorticoids, causing inhibition of PLA_2. However, it has not been concluded that these proteins are the primary agents of the antiinflammatory actions observed. Problems have arisen in comparing *in vitro* and *in vivo* studies. Purified 'lipocortin' inhibits stimulated AA release but not basal release (Fradin, 1988) leaving open the possible intervention of other mechanisms. There is also controversy over the question of whether any of the recombinant lipocortins are actually induced by glucocorticoids.

It was proposed that lipocortin action was regulated by phosphorylation and inactivation (Davidson & Dennis, 1989). However, the relationship of this

phosphorylation to eicosanoid biosynthesis has not been proven and is likely to be complex if it does exist. Experiments claiming to show lipocortin phosphorylation and inactivation have often proven inadequate (Hirata *et al.*, 1984) with no added calcium present which is necessary for the reactions to proceed. Another type of mechanism proposed for lipocortin action involves the 'substrate depletion model' (Davidson & Dennis, 1989). This involves binding of lipocortin to the phospholipid substrate, preventing access to the phospholipase. Support for this model comes from studies in which inhibition was overcome by raising the substrate concentration in amounts consistent with overcoming depletion of the substrate by lipocortins used in the assays (Aarsman *et al.*, 1987). In addition, no binding of inhibitor protein to PLA_2 was detected, but extensive binding to substrate was seen. It therefore, seems that the action of lipocortins on PLA_2 must be studied within a broader perspective than previously. They may be connected to growth regulation and oncogenesis, or may play a role in inflammation, but there is not sufficient evidence to say they are direct regulators of PLA_2.

PLA_2 is obviously an attractive therapeutic target in the development of antiinflammatory compounds and constant research goes into the production of effective PLA_2 inhibitors. Compounds which have been identified as inhibitors of PLA_2 include bromophenacylbromide, mepacrine and manoalide. BPB covalently modifies essential histidine residues associated with the catalytic site on the enzyme (Kyger *et al.*, 1984). However, it inactivates a wide variety of other enzymes through similar mechanisms. Mepacrine has been shown to inhibit PLA_2 activity *in situ* but not *in vitro* by altering calcium availability to the enzyme and possibly by modifying the structure of membrane phospholipids (Disc *et al.*, 1982). Manoalide has been shown to potently inactivate bee and cobra venom PLA_2 with IC_{50} values of 0.05 and 2μM respectively(Lombardo and Dennis, 1985; Glaser *et al.*, 1986), much lower than that required of BPB and mepacrine. Other work has shown manoalide to inhibit AA release from polymorphonuclear leukocytes and macrophages in response

to A23187 stimulation (Meade *et al.*, 1986; Mayer *et al.*, 1988). The mechanism of inactivation has been suggested to occur via structural modification of specific lysine residues near the active site of the enzyme (Lombardo and Dennis, 1985). However, again this inhibitor appears relatively non–specific since it has been reported to interact with other phospholipases. Another potential inhibitor of PLA_2 is aristolochic acid (Rosenthal *et al* 1989) which has been reported to interact directly but non–covalently with isolated PLA_2s, inducing a change in secondary structure as measured by alterations in the alpha–helical content of the protein. It has been suggested that the lack of attenuated effects of aristolochic acid in biological systems may imply that this agent retains its specificity for PLA_2 *in situ.* However, this requires further investigation and as yet, a specific inhibitor for PLA_2 has not been found.

1.5.4 Receptor regulation via specific G proteins

A family of guanine nucleotide binding proteins have been shown to function in receptor–linked signal transduction, coupling receptor activation to modulation of effector systems such as adenylyl cyclase and phospholipase C. These G proteins exhibit sensitivity to certain toxins and this has proved a useful tool in the identification procedure. Cholera and pertussis toxins stoichiometrically ADP–ribosylate specific G proteins, altering their capacity to function in signal transduction (Gilman, 1984). Pertussis toxin interacts with $G_{\alpha i}$, stabilising its inactive form and blocking its function whereas cholera toxin has been shown to stabilise the active state of stimulatory G proteins ($G_{\alpha s}$) and perpetuate their functions. Toxin sensitivity has also been shown to exist for G proteins that regulate phospholipase C in some but not all cell types and this has proved useful in studies where the dissociation of PLC activity from other events has been studied.

The activation of PLA_2 by a specific G protein has now been demonstrated in a number of systems. Burch and Axelrod (1987) showed that bradykinin receptors in Swiss 3T3 cells are coupled by G proteins to both PLC and PLA_2. Both AA release and $Ins(1,4,5)P_3$ production were affected by addition of non–hydrolysable

analogues of GTP and GDP to permeabilised cells. It was possible, however, to dissociate the pathways through use of phorbol ester pretreatment which inhibited Ins(1,4,5)P_3 production but was without effect on AA release, and use of cycloheximide which inhibited PGE_2 synthesis possibly by inhibiting the synthesis of a PLA_2 activating protein but not Ins(1,4,5)P_3 production. The pathways thus seem to be independently regulated, possibly via separate G proteins. This phenomenon has also been demonstrated in neutrophils (Cockcroft & Stutchfield, 1989), MDCK cells (Slivka & Insel, 1988) and human platelets (Nakashima *et al.*, 1987). Further support for a direct activation of PLA_2 by a G protein has come from work on rod outer segments of bovine retina (Jelsema, 1987) in which it was shown that light activated PLA_2 through the action of the G protein transducin. Additional studies (Jelsema & Axelrod, 1987) examined the effect of the α and $\beta\gamma$ subunits of transducin on PLA_2 activity. The α subunit caused a slight increase in enzyme activity. In contrast, the $\beta\gamma$ subunit markedly stimulated PLA_2. Addition of equimolar amounts of α– and $\beta\gamma$–subunits caused an inhibition of PLA_2 activity in the dark–adapted rod outer segments probably due to the reassociation of the $\alpha\beta\gamma$ heterotrimer. It remains to be established whether the $\beta\gamma$–induced increase in PLA_2 occurs by direct stimulation of the enzyme or by inactivation of a PLA_2 inhibitor. Further evidence for the involvement of the $\beta\gamma$ subunit in PLA_2 activation has come from Kim *et al.* (1989) who have shown that $\beta\gamma$ activates the cardiac muscarinic K^+ channel by stimulating the production of lipoxygenase–derived second messengers. An antibody against PLA_2 was shown to prevent $\beta\gamma$ from opening K^+ channels. In addition, the lipoxygenase–blocking drug, NDGA, prevented K^+ channel opening in response to both $\beta\gamma$ and AA. Codina *et al.* (1987), however, have firmly challenged the argument that $\beta\gamma$ can open K^+ channels and have strengthened the case that α_i is responsible by showing that K^+ channels are opened by recombinant α_i protein made in bacteria and that a monoclonal anti–α_i antibody blocks G protein–mediated opening of the channels.

The number of β and γ subunits identified to date is far fewer than for the α subunits (Schmidt & Neer, 1991). Three different βs and three γs have been described, and these different subunits may form dimers with distinct functional characteristics. Both transducin βγ and brain βγ have equivalent abilities to couple transducin to rhodopsin in a reconstituted system (Kanaho *et al.*, 1984). However, the relative ability of these two dimers to inhibit α_s activation of adenylate cyclase is different (Casey *et al.*, 1989). Differences have also been noticed in the ability of βγ dimers to activate PLA_2. Brain βγ will stimulated PLA_2 activity in atrial membranes but transducin βγ is much less effective (Kim *et al.*, 1989). In these studies, the β subunit was predominantly β_1, therefore, the functional differences may depend on the γ subunit. The significance of this subunit in the regulation of signalling paths is under further investigation (Schmidt & Neer, 1991) but remains highly controversial, for several reasons. It remains to be demonstrated in the majority of cases whether the βγ preparations used are at all contaminated with the α subunit. This would obviously weaken the argument that βγ is the active subunit. The proposal that βγ activates PLA_2 is also open to criticism since many situations exist in stimulated cells where the heterotrimeric G protein has dissociated, and the α subunit e.g. α_s activates a signalling pathway e.g. adenylyl cyclase/cAMP, leaving free βγ subunits that should, according to the theory, stimulate PLA_2 activity. This is not the case.

The identification of the G protein involved in PLA_2 regulation remains unknown. However, several possibilities have been suggested. The possibility has been considered that the G protein involved is a *ras* proto–oncogene. The mammalian *ras* genes H–*ras*, K–*ras* and N–*ras* encode small molecular weight G proteins normally involved in cellular regulation that are activated by oncogenic mutation. Microinjection of the H–*ras* oncogene into fibroblasts (Bar–Sagi & Feramisco, 1986) had no effect on inositol phospholipid metabolism but activated PLA_2. This finding was consistent with the normal H–*ras* protein being responsible for control of AA production. In contrast to this, Wakelam *et al.* (1986) have

suggested that the *ras* proteins are responsible for activation of inositol phospholipid breakdown and could therefore be forms of G_p. These discrepancies have been explained by Burgoyne *et al.* (1987) to be due perhaps to the fact that AA, produced by *ras* activation of PLA_2 may stimulate inositol phosphate production (Zeitler & Handwerger, 1985). More recently, however, it has become clear that *ras* proteins do not directly control the activity of PLC or PLA_2 since the introduction of compounds designed to duplicate the enzymic activity could not produce DNA synthesis in cells injected with an anti–*ras* antibody (Yu *et al.*, 1988). It seems more likely that any increased PLA_2 activity in *ras*–transformed cells is due to an indirect effect of other signalling pathways activated by *ras*–transformation e.g. increased DAG production and increased PtdCho breakdown (Grand & Owen, 1991).

Several recent studies have postulated on the identification of the G protein involved in PLA_2 activation in certain systems. Gupta *et al.* (1990) have suggested that the stimulation of PLA_2 by thrombin and type 2 (P_2)–purinergic receptor agonists in Chinese hamster ovary cells is mediated by G_i. To decipher α chain regulatory regions responsible for control of PLA_2, chimeric cDNAs were constructed in which different lengths of the α subunit of G_s were replaced with the corresponding sequence of the $G_i\alpha$ subunit (α_{i2}). When a carboxyl–terminal chimera, which has the last 38 amino acids of α_s substituted with the last 36 residues of α_{i2}, was expressed in the cells, the receptor–stimulated PLA_2 activity was inhibited, although the chimera could still activate adenylate cyclase. It was thus proposed that the last 36 amino acids of α_{i2} are critical for G protein regulation of PLA_2. This type of study offers distinct advantages over pertussis toxin treatment of cells because of better specificity. Specific α chain sequences may be used to selectively inhibit pathways regulated by different G proteins. The inhibition of PLA_2 described in this study may be due to direct interaction with PLA_2 or a regulatory peptide required for G_i regulation of the enzyme. If G_i does regulate PLA_2 then it would be expected that pertussis toxin would inhibit all activation.

A study by Cantiello *et al.* (1990) has suggested that activation of Na^+ channels in epithelial cells is mediated by $G_{\alpha i3}$ stimulation of PLA_2 activity. Picomolar concentrations of α_{i3} were capable of stimulating PLA_2 activity, and allowing lipoxygenase–mediated Na^+ channel activation. Further evidence for involvement of a G_i in PLA_2 regulation has been suggested by Lowndes *et al.* (1991). Expression of the GTPase–deficient G_{i2} α subunit oncogene *gip* 2 in Chinese hamster ovary cells altered the regulation of PLA_2 to different extents depending on the agonist used, causing differing degrees of attenuation. This study showed additional evidence for involvement of G_{i2} in PLA_2 activation.

Another suggestion that has been made in G protein regulation of PLA_2 is that dual regulation may exist. Burch *et al.* (1987) suggested that PLA_2 may be subject to regulation by both stimulatory and inhibitory G proteins. This conclusion was based on the sensitivity of PGE_2 synthesis to both cholera and pertussis toxins. Ideally reconstitution experiments will be required to clarify whether G_i, G_o or G_q proteins are involved. In addition, experiments using chimeras such as those previously described in different cell types would be informative.

1.6 PATHWAYS OF ARACHIDONIC ACID METABOLISM

Arachidonic acid may be metabolised via three main pathways. These are (i) the cyclooxygenase pathway, producing prostanoids, which include non–prostanoic derivatives like thromboxanes in addition to true prostaglandins (Smith, 1989), (ii) various lipoxygenase products (Needleman *et al.*, 1986) and (iii) NADPH–dependent cytochrome p–450 epoxygenase metabolites (Snyder *et al.*, 1983).

1.6.1 Cyclooxygenase

Conversion of AA to the prostaglandin endoperoxide PGH_2 is mediated by PGG/H synthase, an integral membrane protein found in the endoplasmic reticulum of prostanoid forming cells (DeWitt *et al.*, 1981). This enzyme has two distinct catalytic activities, a bis–oxygenase (cyclooxygenase) involved in PGG_2 formation

and a hydroperoxidase mediating a net two–electron reduction of the 15–hydroperoxyl group of PGG_2 to yield PGH_2 (Ohki *et al.*, 1979). The cyclooxygenase is specifically inhibited by aspirin and related non–steroidal anti–inflammatory drugs. Aspirin causes O–acetylation at a serine residue located 70 amino acids from the C–terminus (DeWitt & Smith, 1988). Indomethacin, meclofenamate and flurbiprofen also cause irreversible inactivation of cyclooxygenase activity but apparently without covalent modification of the enzyme (Rome & Lands, 1975).

The level of PGG/H synthase has been shown to be influenced in various cell systems by steroids, growth factors and tumour promoters (Smith, 1989), suggesting that regulation of the level of the enzyme is an important factor in regulating prostanoid formation. The biologically active eicosanoids, which are considered to be PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2, are synthesised in a cell–specific manner from PGH_2. In comparison to PGG/H synthase, relatively little is known about the enzymes which catalyse PGH_2 metabolism. Both PGI_2 synthase (DeWitt & Smith, 1983) and TxA_2 synthase (Shen & Tai, 1986) have been purified to homogeneity and both appear to be related membrane–bound haemoproteins with a cytochrome p–450 chromophore. It seems likely that these enzymes, along with other PGH_2 metabolising enzymes are regulated coordinately with PGG/H synthase.

PGE_2 synthesis requires reduced glutathione. The formation of PGE_2 is catalysed by several distinct membrane–bound PGH–PGE isomerases which lack glutathione S–transferase activity (Smith, 1989), as well as by several subtypes of soluble glutathione S–transferase. The question of which of these enzymes is important in PGE_2 synthesis *in vivo* is unresolved. Several, but not all, soluble proteins with PGH–PGD isomerase activity have been purified (Tachibana *et al.*, 1987).

Eicosanoids are lipophilic substances and are believed to be easily capable of crossing plasma membranes and generating effects in neighbouring cells.

Prostaglandins are thought to have individual cell surface receptors (Schafer *et al.*, 1979) through which they exert these effects. A common mechanism of action of several prostaglandins like PGD_2, PGE_2 and PGI_2 is to alter cell cAMP via adenylyl cyclase stimulation, and to subsequently alter AA release at the PLA_2 level, depending on the target cell. This has been demonstrated in human platelets (Gorman, 1977) and since cAMP is a potent inhibitor of AA release in platelets, PGD_2 and PGI_2 will thus inhibit eicosanoid synthesis. The physiological relevance of this inhibition by PGD_2 is reinforced in considering that this is the main cyclooxygenase product of mast cells.

In addition to stimulating cAMP, prostaglandins have also been shown to cause an increase in intracellular calcium which may occur via increased PtdIns(4,5)P_2 turnover (Macphee *et al.*, 1984). Based on measurements of the effects of prostaglandins on second messenger levels, Muallem *et al.* (1989) classified the receptors into three classes: a PLC/Ca^{2+}–linked receptor accepting $PGF_{2\alpha}$, PGD_2 and TxB_2; a PLC/Ca^{2+}–linked receptor accepting PGE_2, PGE_1, PGA_2, PGI_2 and 6–oxo–$PGF_{1\alpha}$; and an adenylyl cyclase–linked receptor accepting PGE_2, PGE_1, PGA_2 and PGI_2. This classification may however be somewhat limited given that other signalling molecules have been shown to be affected by prostaglandins in addition to those mentioned. $PGF_{2\alpha}$ and PGB_2 have been shown to induce a PKC–dependent contraction of rat aorta by a mechanism that does not depend on phosphoinositide hydrolysis (Heaslip & Sickels, 1989). It was suggested that these prostaglandins may activate a DAG–generating signal–transduction pathway independent of phosphoinositide hydrolysis, possibly by stimulating PLC–dependent hydrolysis of phosphatidylcholine. Another potential pathway that may be regulated by prostaglandins is that of AA mobilisation itself. Rosenthal and Franson (1989) have suggested that PGB_1 is capable of inhibiting agonist–stimulated AA release in both human neutrophils and endothelial cells. The effect was rapid, dose–dependent and resulted in approximately 95% inhibition of A23187–stimulated

release. This effect appeared to be at the level of PLA_2 itself. However, further studies are required to determine whether PGB_1 also inhibits other paths of receptor–mediated signal transduction. The possibility may exist that feed–back loops between prostaglandins and PLA_2 regulation are present in certain systems.

1.6.2 Lipoxygenase

Leukotrienes and related mono– di– and tri–hydroxy fatty acid products are formed via lipoxygenase pathways. There are three different mammalian lipoxygenases which catalyse the insertion of oxygen into AA at positions 5, 12 or 15. The initial of these reactions is a hydroperoxyeicosatetraenoic acid (i.e. 5–, 12– or 15HPETE) which can then be reduced to the corresponding hydroxyeicosatetraenoic acid (i.e. 5–, 12– or 15–HETE). The 12–lipoxygenase is present in platelets (Nugteren, 1982) and leukocytes (Yokayama *et al.*, 1986) but the physiological function of 12—HETE is unclear. The 15–lipoxygenase is found in leukocytes (Narumiya & Salman, 1982) and a variety of other cells but is not always expressed. Many biological actions of 15–HETE and other 15–lipoxygenase products have been described *in vitro*. Wallukat *et al.* (1991) studied the influence of lipoxygenase products on the β–adrenergic receptor of rat heart myocytes and found that 15–HETE at 10^{-10}M induced supersensitivity of the receptor to its agonists. In pulmonary tissues, 15–HETE is a potent proinflammatory metabolite. In cultured human airways, mucus glucoprotein release was stimulated by 1μM 15–HETE (Marom *et al.*, 1983). 15–HETE at <10μM also stimulates angiogenesis. This effect is due to stimulation of both migration and proliferation of endothelial cells. In fetal bovine aortic endothelial cells, stimulation of cell proliferation and DNA synthesis by 15–HETE has been suggested to occur via inhibition of DAG kinase, leading to an increase in cellular DAG (Setty *et al.*, 1987). There appear to be regulatory relationships between the various lipoxygenase pathways. For example, 15–HETE (6μM) is a potent inhibitor of both 5– and 12–lipoxygenase (Vanderhoek *et al.*, 1980). Moreover, 15–lipoxygenase can inactivate the peptidoleukotrienes by

15–lipoxygenation (Sirois, 1979). Thus, 15–lipoxygenases appear to be involved in antiinflammatory as well as proinflammatory reactions.

Another group of 15–lipoxygenase metabolites are the lipoxins (Rokach & Fitzsimmons, 1988), which are products of multiple oxygenations of AA. A variety of biological effects of lipoxins have been reported such as inhibition of killer cell toxicity, modulation of leukocyte chemotoxis, vasodilation, smooth muscle contraction in lung tissue, activation of protein kinases and inhibition of the phosphatidylinositol cycle (Samuelson, 1987). The large variety of effects of 15–lipoxygenase still require considerable investigation but it is thought that these enzymes may have a major function in the oxidative modification of lipoproteins as an early event in the pathogenesis of inflammatory states such as atherosclerosis.

The 5–lipoxygenase pathway is involved in leukotriene formation. Thus 5–HETE can be converted by removal of water to an acid–labile 5,6–epoxide containing a conjugated triene structure, leukotriene A_4 (LTA_4). This can be converted to LTB_4 by net addition of water via LTB_4 hydrolase or to LTC_4 by addition of a glutathionyl group at C–6 by the action of glutathione 5–transferase. Leukotrienes containing peptides or amino acids at C–6 are termed peptidoleukotrienes. LTC_4 can be cleaved by γ–glutamyl–transpeptidase to produce LTD_4 which can be further metabolised to LTE_4 by a dipeptidase (Smith, 1989). Most attention has focussed on LTB_4 which has been shown to be an important modulator of many immune cell functions either as an internal messenger or as an external signal, linking inflammatory cells to lymphocytes. LTB_4 is one of the most powerful chemokinetic and chemotactic agents and it can induce neutrophil aggregation, degranulation and enhanced binding to endothelial cells (Rola–Pleszczynski, , 1989). It has also been shown to induce cation fluxes and increase cytoplasmic calcium via activation of phosphoinositide hydrolysis (Mong *et al.*, 1986). High– and low–affinity LTB_4 receptors have been identified and signal transduction via the high–affinity form has been suggested to involve a G protein (Mong *et al.*, 1986). The mechanism underlying 5–lipoxygenase

activation has recently been proposed to involve a specific activating protein termed 5–lipoxygenase activating protein or FLAP (Dixon *et al.*, 1990). This was discovered through the use of a compound MK–886 which blocks the synthesis of leukotrienes in intact activated leukocytes. The mechanism by which FLAP activates 5–lipoxygenase is not known. The authors have suggested that since the translocation of the enzyme to the membrane after A23187 activation is blocked by MK–886, FLAP could be a membrane anchor for 5–lipoxygenase. According to this model, a stable complex would be required to form at the membrane between activated 5–lipoxygenase, FLAP and possibly other components. The formation of this complex could regulate the interaction of 5–lipoxygenase with AA. It has been suggested that this type of mechanism may be fairly widespread for regulating enzymes that act upon membrane–associated substrates.

The leukotriene LTD_4 also appears to act via specific receptors that are coupled to at least two separate G proteins with different toxin sensitivities. This leukotriene has also been shown to stimulate signalling pathways such as PI–PLC and a receptor operated Ca^{2+} channel (Crooke *et al*,. 1989). LTD_4 has also been suggested to indirectly activate PLA_2 via production of endogenous activating proteins (PLAPs). The LTD_4 transduction pathway seems to be regulated at the receptor level by both homologous and heterologous desensitization via PLC. There appear to be differences in the mechanisms of action and regulation of the different leukotrienes and this is an area into which future studies should be directed.

1.6.3 Epoxygenase

The cytochrome p450 enzymes are widely distributed in animal tissues, plants and microorganisms and appear to be a multigene family whose main function is the monooxygenation of lipophilic substances. Oxidation of AA by cytochrome p450 results in the formation of eicosatetraenoic acids (Needleman *et al.*, 1986). In addition both cyclooxygenase and lipoxygenase products can be metabolised via this route to form epoxygenase products.

Various biological effects of these products have been observed. Incubations of anterior pituitary cells with synthetically produced epoxygenase metabolites of AA caused an increase in the release of luteinising hormone (Needleman, 1986). The most potent metabolite was 5(6)epoxyeicosatetranenoic acid with a maximal response at 1μM. The specificity of this response is however questionable and it may be due to the stimulation of the formation of a cyclooxygenase metabolite. In a mouse pituitary cell line (AtT–20), inhibitors of cytochrome p450 enzymes were shown to suppress secretogogue–induced ACTH release, suggesting a role for epoxides in pituitary function. The cytochrome p450 pathway may also have a role in renal function. It has been reported that 5(6) epoxyeicosatetraenoic acid or one of its metabolites inhibits sodium absorption and potassium secretion in rabbit cortical collecting tubule. In addition, vascular Na^+/K^+ ATPase activity is inhibited by these products in the medullary thick ascending limb of loop of Henlé (Needleman, 1986). More recently, it has been suggested that cytochrome p450 products are endogenous modulators of the growth response in rat mesangial cells (Sellmayer *et al.*, 1991). Inhibition of cyclooxygenase or lipoxygenase alone had no effect on cell growth caused by EGF, PMA and vasopressin. Stimulation with these substances followed by GC–MS analysis revealed an epoxyeicosatetraenoic acid–like substance suggesting these compounds may be important mediators of the growth response to mitogenic agents. This represents a novel role for these products although the underlying mechanism has yet to be elucidated.

It seems clear that the eicosanoids from each of the three groups have wide–ranging effects upon the intra– and inter–signalling networks. Therefore, in studies examining the role of PLA_2 in signal transmission, it will be important to consider not only the immediate product of phospholipid hydrolysis, AA, but also the potentially important metabolites of this fatty acid.

1.7 BOMBESIN - A MITOGENIC PEPTIDE FOR SWISS 3T3 CELLS

Bombesin is a tetradecapeptide isolated from frog skin and has a wide range of biological activities. Bombesin–like peptides such as gastrin release peptide (GRP) and neuromedin B (NMB) are widely distributed in mammals and have diverse functions including neurotransmission in the central nervous system (Minamino *et al.*, 1985) and stimulation of exocrine secretion in the pancreas and gut (Knigge *et al.*, 1984). They are potent mitogens *in vitro* for Swiss 3T3 cells (Takuwa *et al.*, 1991) and have been proposed to act as growth factors for human fetal lung (Spindel *et al.*, 1987) and human small cell lung cancer (Cuttitta *et al.*, 1985).

The effects of bombesin appear to be exerted upon its binding to a single class of high affinity cell surface receptors which have now been well characterised (Brown *et al.*, 1988; Sinnett–Smith *et al.*, 1988; Battey *et al.*, 1991). This receptor is a member of the G protein–coupled receptor superfamily with seven hydrophobic transmembrane domains. Evidence for the existence of bombesin receptor subtypes was put forward by Schrenck *et al.* (1989) and Shapiro *et al.* (1991). There appear to be to date three subtypes of the receptor present in distinct tissues. Oesophageal smooth muscle expresses an NMB preferring receptor type, while pancreatic acinar cells and Swiss 3T3 cells possess a GRP preferring receptor type. The latter two subtypes were distinguished by their differences in sensitivity to a specific GRP receptor antagonist (Shapiro *et al.*, 1991). As yet,. there is no evidence of bombesin receptor subtypes existing within the same cell.

The bombesin receptor in Swiss 3T3 cells is linked to a pertussis toxin insensitive G protein which mediates activation of PLC (Fischer & Schonbrunn, 1988), the exact identity of which remains unclear. However, as mentioned earlier, it may be a member of the G_q/G_{11} species.

Bombesin acts as a typical Ca^{2+} messenger system hormone. Takuwa *et al.* (1987) have demonstrated that it has three measurable effects on cellular Ca^{2+} metabolism: a transient stimulation of radiocalcium efflux from [$^{45}Ca^{2+}$]–prelabelled cells; a transient rise in the intracellular calcium concentration and a rapid and sustained increase in calcium influx rate. These results imply that bombesin stimulates the cycling of calcium across the plasma membrane. The transient rise in Ins(1,4,5)P_3 in response to bombesin has been well characterised (Takuwa *et al.*, 1987; Cook *et al.*, 1990; Plevin *et al.*, 1990) as has the biphasic DAG response (Cook *et al.*, 1990). This increase in DAG leads to activation of PKC in Swiss 3T3 cells (Erusalimsky *et al.*, 1988). The source of bombesin–stimulated DAG formation may comprise more than one phospholipid. It is thought that the initial phase of DAG that corresponds with Ins(1,4,5)P_3 is generated from PLC–stimulated hydrolysis of PtdIns(4,5)P_2. However, the second phase has been proposed to come from hydrolysis of PtdCho, either via PLC (Muir & Murray, 1987) or PLD (Cook & Wakelam, 1989). Thus, bombesin may stimulate the turnover of more than one phospholipid type. Activation of PKC by bombesin may be important in the peptide's ability to stimulate protein synthesis since PKC has been suggested to regulate certain eukaryotic initiation factors via phosphorylation as mentioned in Section 1.2.2 (Morley & Trough, 1989). In Swiss 3T3 cells, PKC–α appears to be the dominant isoform and is active for a prolonged period of time in the face of limited calcium influx as far as the level of DAG remains elevated (Takuwa *et al.*, 1991). It is possible, however, that other calcium–independent PKC isoforms such as δ and ς might be involved in the sustained phase of bombesin action. However, this remains to be investigated.

Bombesin has also been shown to cause increased expression of the cellular oncogenes c–*fos* and c–*myc* (Bravo *et al.*, 1987). Prolonged treatment of the cells with PMA, which causes a selective decrease in PKC activity, partially inhibited the

induction of c–*fos* and c–*myc* expression. This suggests a partial role of PKC in protooncogene induction by bombesin.

As mentioned earlier (see Section 1.3.2), the question of whether bombesin enhances cAMP accumulation in Swiss 3T3 cells has been one of controversy. Millar and Rozengurt (1988) suggested that bombesin, in the presence of either forskolin or cholera toxin, caused a 4–6 fold enhancement of cAMP. Other groups however, (Fischer & Schonbrunn, 1988; Takuwa *et al.*, 1991) have failed to detect any increase in cAMP levels in response to the peptide.

The possibility that bombesin may induce an increase in tyrosine phosphorylation was suggested in Section 1.3.1. The identity of the phosphorylated proteins remains uncertain but since bradykinin and vasopressin also stimulated their phosphorylation, they may be important in the cellular signalling for a number of agonists. Previous studies examining the effect of bombesin on tyrosine phosphorylation include Cirrillo *et al.* (1986) who showed an increase in tyrosine phosphorylation of a 115Kd protein species in 3T3 cells. However, Isacke *et al.* (1986) were unable to repeat this effect. The possibility does, however, remain that this may represent another, potentially important, signalling event that comprises part of the mitogenic effect characteristic of bombesin.

1.8 AIMS

Previous studies of bombesin–stimulated Swiss 3T3 cells have focussed on the activation of PLC, examining Ins(1,4,5)P_3 and DAG production. In an effort to extend the knowledge of how bombesin exerts its mitogenic effect, this study examines the possibility that PLA_2 activation may be an important effect of bombesin stimulation. The main aims of the study were:-

1. To characterise the kinetics and composition of the PLA_2 response,
2. To identify possible regulatory factors for this enzyme,

3. To determine the mechanism behind initial PLA_2 activation and the relationship between activation of PLA_2 and PLC in response to bombesin stimulation,
4. To ascertain whether the products of PLA_2 hydrolysis have a role to play within the cell.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 CELL LINES AND MATERIALS

2.1.1 Cell Line

Swiss mouse 3T3 fibroblasts

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

2.1.2 Materials

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

Amersham International plc, Buckinghamshire, England

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

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

[methyl–^{3}H]choline chloride (75.9Ci/mmol)

[^{3}H]15–HETE assay kit

[2–^{3}H]myo–inositol (10–20Ci/mmol)

[20(n)–^{3}H]phorbol dibutyrate (10Ci/mmol)

[5,6(n)–^{3}H]prostaglandin E_2 (140Ci/mmol)

[U–^{14}C]sucrose (460mCi/mmol)

[methyl–^{3}H]thymidine (25Ci/mmol)

Beckman RIIC Ltd., High Wycombe, England

Ultrasphere ODS column

BDH Chemical Co., Poole, England

Ammonium formate, EDTA, Gelatin, Hepes, Orthophosphoric acid, perchloric acid, petroleum ether (60–80°), potassium dihydrogen phosphate, sodium carbonate, sodium hydrogen orthophosphate, sodium hydroxide, trichloroacetic acid, Universal indicator.

Biogenesis Ltd., Bournemouth, England

Epidermal Growth Factor (receptor grade) purified from mouse submaxillary glands.

Boehringer (UK) Ltd., Lewes, England

ATP

GDPβS

GTPγS

Cambridge Research Biomedicals, Cambridge, England

Bombesin

DuPont NEN Research Products, Stevenage, England

[^{3}H]water (1mCi/g)

FSA Lab Supplies, Loughborough, England

Chloroform, diethyl ether, ethanol, ethyl acetate, methanol, sap-pak columns

Gibco, Paisley, Scotland

Dulbeccos modified essential medium (DMEM 10x), gentamycin,, glutamine (100x), penicillin/streptomycin, sodium bicarbonate (7.5%)

Koch–Light Ltd., Suffolk, England

Calcium chloride, dimethyl sulphoxide (DMSO), magnesium sulphate, potassium chloride

May & Baker, Dagenham, England

Acetic acid, formic acid, hydrochloric acid, sodium tetraborate

Merck, Essex, England

Lichrosorb C–18 column

National Diagnostics, Aylesbury, England

Ecoscint

Porton Products, Porton Down, Salisbury, England

Pertussis toxin

Wellcome Diagnostics, Dartford, Kent, England

Streptolysin – O

Whatman Ltd., Maidstone, England

t.l.c. plates

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

Soyabean 15–lipoxygenase (Spec.ac.90u/μg) was kindly donated by Dr. C.C.Hallam, Fisons Pharmaceuticals.

2.2 BUFFER COMPOSITION

Phosphate buffered saline (PBS)

146mM sodium chloride, 5.4mM potassium chloride, 9.6mM di-sodium hydrogen orthophosphate, 1.5mM potassium di–hydrogen orthophosphate.

When freshly prepared, PBS was adjusted to pH7.2–7.4 if necessary with 1M sodium hydroxide and/or 1M hydrochloric acid.

Sterile trypsin solution for cell passage

Trypsin was prepared as a solution of 0.1% (w/v) trypsin, 0.025% (w/v) EDTA and 10mM glucose in PBS at pH7.4 and filtered before aliquoting through a sterile 0.22μm membrane (Flow pore D).

Hank's buffered saline (Hanks)

1.26mM calcium chloride, 0.5mM magnesium chloride, 0.9mM magnesium sulphate, 5.37mM potassium chloride, 137mM sodium chloride, 4.2mM sodium hydrogen carbonate, 0.35mM sodium dihydrogen phosphate.

When freshly prepared, the pH was between 7.2–7.4.

Hank's buffered saline with glucose and BSA (HBG)

Hanks was prepared as above and 10mM D–glucose and 1% (w/v) BSA (fraction V) added.

When freshly prepared, the pH was adjusted to 7.4 as for PBS.

Permeabilisation buffer

20mM Hepes, 120mM potassium chloride, 6mM magnesium chloride, 2mM potassium di–hydrogen orthophosphate, 0.1mM EGTA, 61μM calcium chloride (0.5μM calcium).

When freshly prepared, the pH was adjusted to 7.5 with 1M potassium hydroxide. When using, 2.5MM ATP and 1mg/ml BSA were added.

2.3 ION EXCHANGE RESIN

Preparation of Dowex formate

Dowex 1 x 8–200 chloride form, strongly basic anion exchange resin, 8% cross-linked with a dry mesh of 100–200 was treated as described below to obtain the formate form for separation of inositol phosphates. A known packed volume of Dowex was washed with distilled water, left to settle and the 'fines' discarded. This was repeated twice. The Dowex was transferred to a sintered glass funnel and washed with 20 volumes of 2M sodium hydroxide. The Dowex was then washed with 10 volumes of water followed by 5 volumes of 1M formic acid. Finally, the Dowex was washed with water until the pH of the slurry was constant at ~5.5. Following each preparation of Dowex formate, it was characterised by performing ml by ml elutions of a mixture of [^{3}H]inositol (1) monophosphate, [^{3}H]inositol (1,4) bisphosphate, [^{3}H]inositol (1,4,5) trisphosphate.

2.4 CULTURE OF SWISS 3T3 MOUSE FIBROBLAST CELLS

Swiss 3T3 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine (2mM), penicillin/streptomycin (100IU/ml) and newborn calf serum (10%). Medium was changed every two days as it became acidified and depleted of serum. To prevent selection of resistant strains of bacteria, gentamycin was used in rotation with penicillin/streptomycin. Cells were kept in a humidified atmosphere of 5% CO_2 at 37°C. Except for experimental

dishes, cells were grown in 25 or 75cm^2 culture flasks and were passaged when 80% confluent. Cells were checked regularly for their ability to contact inhibit when 100% confluent. Cells which exhibited transformed morphology, showed reduced responsiveness to given agonists or reached passage numbers higher than 40, were discarded.

Cell Passage

Cells were passaged at a ratio of 1 to 5 according to the following protocol:

The medium was aspirated and replaced with 3ml of sterile trypsin solution per 75cm^2 flask. Flasks were returned to the incubator for 2–3 minutes until cells were detached from the flask bottom. Trypsin activity was inhibited by addition of 6ml of medium. The cells were then suspended by aspiration to ensure maximal dispersal.

After transfer to a sterile tube, the cell suspension was centrifuged at 1000 rpm for 5 minutes and the supernatant discarded. Cells were then resuspended in complete medium and aliquoted into new sterile flasks containing 9ml of fresh medium. The flasks were rocked gently to ensure even distribution of the cells across the bottom, and then returned to the incubator.

Cryogenic preservation of cell lines

Cell lines were stored in liquid nitrogen according to the following protocol:

Cells were trypsinised as outlined. They were then resuspended in DMEM containing 20% (v/v) newborn calf serum and 8% (v/v) DMSO at a density of approximately 10^6 cells per ml of freezing medium and aliquoted into freezing vials.

Cells were frozen slowly for 24h at –80°C before transfer to a vat of liquid nitrogen. To bring cells up from frozen, vials were removed from liquid nitrogen and thawed rapidly in a water bath at 37°C. Each aliquot of cells was plated out in

9ml fresh medium and left to attach overnight. The following day, the medium and suspended dead cells were removed and the surviving cells were fed normally.

2.5 THE RADIOLABELLING AND ANALYSIS OF PHOSPHOLIPIDS

2.5.1 Cell incubation and lipid extraction

Swiss 3T3 cells were seeded onto 6–well plates and grown in DMEM containing 10% newborn calf serum for 48h until approximately 90% confluent. The medium was then changed to one containing 1μCi/ml [5,6,8,9,11,12,14,15 – ^{3}H]arachidonic acid (150–230Ci/mmol) for 12, 24, 36, 48, 60 and 72h. The plates were then transferred to a heated aluminium block plate holder. The incubation medium was removed and the monolayers washed three times with 0.5ml HBG for a total of 45 minutes. Ice-cold methanol (1ml) was then added to each well immediately upon aspiration of the HBG and the contents were scraped with a wax–filled pipette tip and transferred to a polypropylene tube. Each well was washed further with an additional 0.5ml ice-cold methanol and this was combined with the previously scraped samples. Chloroform (0.75ml) was added to each tube, the contents thoroughly mixed, and samples allowed to extract for at least 30 minutes at 4°C Following this, 0.75ml each of chloroform and water were added to every sample, tubes vortex–mixed and samples left to stand to allow separation of the phases. Finally, 1ml of the lower, organic phase was transferred to a 2ml glass trident vial. The samples were then dried under vacuum in a centrifugal evaporator. Lipids were dissolved in chloroform:methanol (19:1 v/v) and used for thin layer chromatography.

2.5.2 Thin layer chromatography

A 20 x 20cm silica gel 60 plate was soaked in 1mM EDTA and allowed to dry at room temperature. The plate was activated by incubation at 120°C for 1h before use. Samples, prepared as described, and standard lipids were spotted onto the plate in a volume of 30µl. The plates were developed in a solvent of chloroform:methanol:glacial acetic acid:water (75:45:3:1 v/v). Plates were then allowed to dry in a fume cupboard and the standards located by exposure to iodine vapour in an enclosed chamber. By reference to the migration of standards, the phospholipids of interest were marked, the silica removed from the plate with a razor blade and transferred to a scintillation vial. 3ml of scintillation fluid (optiphase 3) was added to each vial, the contents mixed and the radioactivity determined by scintillation counting. Standard Rf values on this system were: sphingomyelin – 0.075; phosphatidylcholine – 0.125; phosphatidylinositol – 0.48; phosphatidylethanolamine – 0.56; phosphatidylserine – 0.825; phosphatidic acid – 0.91.

2.6 MEASUREMENT OF ARACHIDONIC ACID GENERATION

2.6.1 Preparation of samples

Cells were seeded onto 24–well plates and grown in DMEM containing 10% NCS until approximately 90% confluent when the medium was removed, and replaced with medium containing 0.5µCi/ml [^{3}H] arachidonic acid for 24h. Plates were then transferred to an aluminium plate warmer at 37°C. The labelling medium was removed and cells washed three times in 0.5ml HBG for a total of 45 minutes. The cells were stimulated by addition of the appropriate agonist in 200µl HBG for the required time. To determine basal levels of arachidonic acid, the appropriate wells were treated with HBG alone for the equivalent times.

When measuring extracellular arachidonic acid release, the incubation medium was removed and transferred to an Eppendorf tube. This was centrifuged

for 5 minutes at 1000 rpm and an aliquot of 200µl was counted for radioactivity. When examining intracellular liberation, the medium was aspirated and 1ml of ice-cold methanol, containing 15µl of glacial acetic acid, was added to each well. The plates were left on ice for 20–30 minutes. The cell debris was then scraped from each well and transferred to a polypropylene tube. Each well was further washed with an additional 0.5ml methanol and transferred to the appropriate tubes. Chloroform (0.75ml) was then added to each tube, the samples mixed and left to extract at 4°C for at least 30 minutes. Phases were split by the addition of 0.75ml each of chloroform and water followed by a vortex mix and short centrifugation (1 min) at 800g. The upper phase was aspirated and 1ml of the lower phase transferred to a glass vial and dried under nitrogen. At this point, samples were prepared for either thin layer chromatography or silicic acid column chromatography. For the former, samples were dissolved in 30µl of chloroform:methanol (2:1 v/v). For the latter, samples were dissolved in 1ml of petroleum ether (60–80°) containing 4% (v/v) diethyl ether.

2.6.2 Thin layer chromatography

A 20 x 20cm silica gel 60 plate was activated at 120°C for 1h prior to use. Samples, prepared as described, and standard arachidonic acid were spotted onto the plate and developed with the solvent system obtained from the upper phase of ethyl acetate:acetic acid:2,2,4–trimethylpentane:water (45:10:25:50 v/v/v/v). Plates were then allowed to air–dry in a fume cupboard and standards located either by radioactivity counting (if radiolabelled standard) or exposure to iodine vapour in an enclosed chamber (if unlabelled). By reference to the migration of standards, the arachidonic acid was marked, scraped from the plate with a razor blade, and transferred to a scintillation vial to which was added 4ml of scintillation fluid. The contents were mixed and radioactivity determined by scintillation counting.

2.6.3 Silicic acid column chromatography

Silicic acid columns were prepared by adding 0.5g silicic acid to glass wool plugged Pasteur pipettes. The columns were supported in a metal rack and each washed with 10ml petroleum ether (60–80°) before use. The samples, dissolved in 1ml petroleum ether/diethyl ether (96/4 v/v) were applied to the columns and the solvent allowed to run to waste. The columns were then washed with 3ml petroleum ether/diethyl ether (96/4 v/v). The arachidonic acid–containing fraction was collected in a scintillation vial by eluting with 3ml diethyl ether. Scintillation fluid (10ml) was added to this and the radioactivity determined by liquid scintillation spectrometry.

2.7 MEASUREMENT OF LYSOPHOSPHOLIPID GENERATION

2.7.1 Preparation of cells

Swiss 3T3 cells were cultured in 24–well plates and labelled with 2μCi/ml [^{3}H]choline chloride for 48h. The cell density at the onset of labelling was such that at the end of the labelling period the cultures were both confluent and quiescent. Plates were transferred to a heated aluminium block and the monolayers washed, as before, with HBG. Cells were then stimulated for the appropriate times by the addition of 200μl of agonist in HBG. The medium was aspirated and reactions terminated by the immediate addition of 1ml of ice–cold methanol containing carrier lysophosphatidylcholine, lysophosphatidylethanolamine and lysophosphatidylinositol at 1mg/ml. Phospholipids and lysophospholipids were extracted as described in Section 2.6.1 and the lower phase dried under vacuum. The lipids were dissolved in 30μl of chloroform:methanol (2:1 v/v), ready for thin layer chromatography.

2.7.2 Thin layer chromatography

An EDTA–treated silica gel 60 plate was activated at 120°C for 1h. Samples and standard lipids were spotted in 30µl amounts and the plate developed for approximately 90 minutes in a tank pre-equilibrated with a solvent system of chloroform:methanol:glacial acetic acid:water (50:30:8:3 v/v/v/v). The plate was air-dried in a fume cupboard and the standards located by exposure to iodine vapour. By reference to the migration of standards, lysophosphatidylcholine and other lysolipids were scraped from the plate and transferred to a scintillation vial containing 4ml scintillation fluid. The radioactivity was determined by scintillation counting.

Standard Rf values on this system are: phosphatidylcholine – 0.312; phosphatidylethanolamine – 0.79; phosphatidylserine – 0.8; phosphatidic acid – 0.825; lysophosphatidic acid – 0.6; lysophosphatidylcholine – 0.106; lysophosphatidylethanolamine – 0.52; lysophosphatidylinositol – 0.4; lysophosphatidylserine – 0.306.

2.8 MEASUREMENT OF ARACHIDONIC ACID METABOLITES

2.8.1 Cyclooxygenase products

(i) Preparation of samples

[^{3}H] arachidonate labelled cells were washed and stimulated as previously described (see Section 2.6.1). Reactions were terminated by the addition of 1ml of methanol containing PGE_2, 6–keto $PGF_{1\alpha}$, PGD_2 and $PGF_{2\alpha}$ (2µg carrier/ml methanol). The contents of each well were scraped and transferred to small centrifuge tubes which were kept on ice for 20–30 minutes and then centrifuged at 1000g for 15 minutes at 4°C. 0.8ml of the supernatant was transferred to a glass vial for hplc analysis. This was acidified to pH3.0 by the addition of 0.2ml sodium citrate (0.1M)/citric acid (0.1M) solution, pH3.0. An additional 0.2ml orthophosphoric acid, pH3.0, was added to maintain the correct aqueous:organic ratio for hplc. The total volume for hplc was 1.2ml.

(ii) High performance liquid chromatography

1ml samples were injected onto a 30cm x 0.39cm μ–Bondapak C–18 reverse phase column and eluted with 32% acetonitrile/68% orthophosphoric acid pH3.0, at 1.0ml/min for 30 minutes. The column was then flushed with 95% acetonitrile/5% orthophosphoric acid, pH3.0 at 2.0ml/min for 3.5 minutes and the run was completed with 32% acetonitrile/68% orthophosphoric acid, pH3.0, at 2.0ml/min for 3.5 minutes. Fractions of 0.5ml were collected in scintillation vials to which 4.5ml of scintillation fluid was added and the radioactivity determined by liquid scintillation spectrometry. Arachidonic acid and prostaglandins were detected by comparison with the elution profiles of standards.

Standards were prepared for chromatography by dissolving in acetonitrile 32%/orthophosphoric acid, pH3.0, 68%. These were detected by U.V. absorption at 216nm. The retention times were as follows: PGE_2 – 21.35 minutes; PGD_2 – 25.14 minutes; $PGF_{2\alpha}$ – 18.08 minutes; 6–keto $PGF_{1\alpha}$ – 25.64 minutes; AA – 44.10 minutes.

2.8.2 Lipoxygenase products

Two different protocols were used for the measurement of these products.

(i) Protocol 1

Preparation of samples

[^{3}H] arachidonate labelled cells were washed and stimulated as previously described (see Section 2.6.1). Reactions were terminated by addition of 1ml methanol containing 5–HETE and LTB_4 (2μg carrier/ml methanol). The methanol content of the stopped reactions was increased to 70% and the samples kept at –20°C for at least 20 minutes to precipitate excess protein. After scraping and centrifugation at 1000g for 15 minutes at 4°C, the supernatant was removed for analysis. The methanol content of the samples was reduced to 50% with water prior to analysis.

High performance liquid chromatography

Injections of 1ml were made onto a 25cm x 0.39cm Lichrosorb C–18 column using an autosampler. Products were eluted with methanol 68.7%/(methanol 20%/ammonium acetate (0.5%) 80%) 31.3% at 2.0ml/min for 12 minutes. This was followed by an increase in the methanol fraction to 93.7% at 2.0ml/min for 6.5 minutes. The run was completed by decreasing the methanol to 50% at 2.0ml/min for 3 minutes. Samples were compared with the elution profiles of standards which were run using a radiochemical detector. The retention times were as follows: LTB_4 – 5.2 minutes; 5–HETE – 11.9 minutes and AA – 18.85 minutes.

(ii) Protocol 2

Preparation of samples

[^{3}H] arachidonate labelled cells were washed and stimulated for the required times with the appropriate agonists. The supernatant was removed and PGB_2 (100ng/ml), prepared in ethanol, was added to each sample as an internal standard. This was then acidified to pH3 – 3.5 with 1M hydrochloric acid. At this point the sample was ready to apply to C–18 Sep-pak columns. These columns were washed with 10ml methanol, 10ml deionised water (pH3.0) and 10ml deionised water prior to addition of the sample. This was applied via a syringe attached to the top of the column. This was then washed with a further 20ml water and leukotrienes were eluted with 10ml ethyl acetate. After removing any residual water from the samples, they were dried under a flow of nitrogen and re-dissolved in 100-µl of hplc solvent (see below) ready for injection.

High performance liquid chromatography

Samples (25µl) were injected onto a reverse phase 5µm ultrasphere ODS column (4.6mm i.d. x 25cm) for separation of products. The solvent system used consisted of de-ionised water:methanol:acetonitrile:acetic acid (45:30:25:0.05 v/v/v/v) pH5.7 and methanol. Products were eluted by increasing the concentration of methanol linearly over a period of 40 minutes until 100% methanol was obtained.

The flow rate was 1ml/min and products were detected at 270nm. This concentration of methanol was retained for 10 minutes at a flow rate of 1.5ml/min. The concentration of methanol was then decreased over a period of 1 minute at 1.5ml/min until the starting concentrations were re-achieved. This was retained for 9 minutes at a flow rate of 0.6ml/min. Identification of leukotrienes was achieved by reference to authentic standards run using the same hplc system.

2.9 PERMEABILISATION OF SWISS 3T3 CELLS

2.9.1 Electroporation

Monolayers of Swiss 3T3 fibroblasts were grown in 75cm^2 flasks. Cells were prelabelled with either [^{3}H] inositol (5μCi/ml) for 48h or [^{3}H] arachidonic acid (1μCi/ml) for 24h. After the labelling period, cells were harvested mechanically with a cell scraper, incubated for 45 minutes in prelabelling medium, washed twice by centrifugation (800g for 2 minutes) and resuspended in a high potassium incubation buffer. The final cell suspension (6 x 10^6 cells/3ml buffer) was then exposed to four discharges of a 3μF capacitor with a field strength of 2KV/cm spaced by 2 second intervals. Approximately 90–95% of the fibroblasts were found to be permeable to ethidium bromide after this procedure.

For arachidonic acid or inositol phosphate measurements, 50μl of permeabilised cells (10^4 cells) were incubated with agonists in incubation buffer in a final volume of 250μl. After the appropriate times, the reactions were terminated either with 250μl methanol (for arachidonic acid measurements) or 250μl perchloric acid (for inositol phosphate measurements) and the products measured as described above.

2.9.2 Streptolysin–O permeabilisation

(i) Determination of cell permeabilisation

(a) Entry of fluorescent dye

Swiss 3T3 cells were scraped from a 75cm^2 flask and transferred to a centrifuge tube kept at 37°C for 45 minutes to allow recovery. Cells were then washed three times in HBG and finally resuspended in permeabilisation buffer containing streptolysin–O (0.6U/ml) and the fluorescent stain bizbenzimide (10μg/ml). After 5 minutes, the cells were transferred to a slide and examined for fluorescence.

(b) Assay for lactate dehydrogenase activity

Confluent Swiss 3T3 cells grown on 6 well plates were washed three times with HBG and then exposed for 5 minutes to various concentrations of streptolysin–O prepared in permeabilisation buffer. The incubation medium was removed and transferred to Eppendorfs. These were centrifuged for 5 minutes to remove any proteins that could interfere with results.

The equilibrium constant of the LDH reaction is K = 2.7 x 10" mol/l (30°C); it strongly favours formation of lactate and NAD.

Therefore, the measurement has to be carried out in an alkaline medium with relatively high concentrations of lactate and NAD to obtain reasonable reactivity velocities for accurate photometric readings.

The readings were carried out on a spectrophotometer under the following conditions: Wavelength – 339nm; light path – 10mm; absorbance – 0.1; temperature – 30°C.

The following solutions were pipetted successively into a cuvette: 0.10ml of β–NAD (172mmol/l) and 2.40ml of Tris/L–lactate (Tris, 112mmol/l, pH9.30, 25°C; L-lactate 56mmol/l; potassium chloride, 170mmol/l). This was thoroughly mixed with a plastic spatula and placed in the spectrophotometer for 5 minutes or until

the desired temperature was reached. At this point, 150µl of the sample was added and mixed. After 30 seconds, the reading was taken over a period of 5 minutes.

(ii) Protocol for permeabilisation

Swiss 3T3 cells were grown on 24–well plates in DMEM containing 10% newborn calf serum. Cells were prelabelled with the appropriate radioisotopes for the relevant length of time. After labelling, the monolayers were washed in 250µl HBG for 45 minutes. Cells were permeabilised by incubation with 500µl streptolysin–O (0.6U/ml) in permeabilisation buffer for 5 minutes. This was followed by a further 15 minutes wash in HBG and then stimulation with the appropriate agonist in 200µl permeabilisation buffer. Reactions were terminated by the addition of 200µl ice–cold methanol (for arachidonic acid measurements) or 200µl of 10% perchloric acid (for inositol phosphate measurements) and the products measured as described above.

Approximately 90–95% of the fibroblasts were found to be permeable to bizbenzimide after treatment with streptolysin–O. In addition, 0.6U/ml of streptolysin–O gave maximal release of lactate dehydrogenase from these cells.

2.10 MEASUREMENT OF STIMULATED INOSITOL PHOSPHATE FORMATION

2.10.1 Incubation of cells with test reagents

After seeding on 24–well plates in DMEM containing 10% NCS for 24h, cells were grown in 0.5ml of inositol–free DMEM containing 1% glutamine and 1% dialysed calf serum with 5µCi/ml of [2–^{3}H]myoinositol for 48h, by which time the cells were confluent, quiescent and labelled to isotopic equilibrium. Labelling medium was then aspirated and cells washed three times in HBG for 45 minutes. The cells were next incubated with 200µl of incubation buffer containing the test reagent at the required concentration for the appropriate times.

2.10.2 Assay of individual inositol phosphate fractions

Incubations were terminated by addition of 200µl of ice–cold 10% perchloric acid. Inositol phosphates were extracted on ice for at least 30 minutes, followed by scraping and removal of the cell debris into a plastic Eppendorf tube. The 24–well plates were washed with a further 200µl of perchloric acid for 5 minutes at 4°C. The supernatants were removed to a second tube, neutralised by the addition of 1.5M potassium hydroxide/60mM Hepes in the presence of universal indicator and the precipitated potassium perchlorate removed by centrifugation at 14,000g for 5 minutes at 4°C. The supernatants were removed and made up to 4ml with 5mM sodium tetraborate/0.5mM EDTA, pH6.7.

Individual inositol phosphates were separated by batch elution from 1ml Dowex formate columns in glass wool-plugged Pasteur pipettes. Samples were loaded onto individual columns and washed with 10ml water. This was followed by 14ml of 60mM ammonium formate/5mM sodium tetraborate which removed glycerophosphoinositides. Elution of inositol monophosphate (IP) was achieved with 16ml of 0.2M ammonium formate/0.1M formic acid. Inositol bisphosphate (IP_2) was eluted with 16ml of 0.4M ammonium formate/0.1M formic acid and a further 16ml of 0.8M ammonium formate/0.1M formic acid was used to elute inositol trisphosphate (IP_3). To determine the amounts of each inositol phosphate, 3ml from the IP_1 fraction, 4ml from the IP_2 fraction and 5ml from the IP_3 fraction were added to 15ml of scintillation fluid and the radioactivity determined by liquid scintillation spectrometry.

2.10.3 Assay of total inositol phosphates

Total inositol phosphates were assayed by accumulation in the presence of 10mM lithium chloride essentially by the method of Berridge *et al* (1982). Dowex formate (1 x 8 200–400 mesh) 500µl, was added to samples, followed by 3ml of distilled water. After the Dowex had settled, the supernatant was aspirated. The removal of glycerophosphoinositides was achieved by washing with 3ml of 60mM

ammonium formate/5mM sodium tetraborate followed by a further 3ml wash with water.

Total inositol phosphates (mainly IP_1 and IP_2) were eluted with 1ml of 1M ammonium formate/0.1M formic acid. 0.8ml of the supernatant was removed and added to 5ml of scintillation fluid. The radioactivity was determined by liquid scintillation counting.

2.11 QUANTITATION OF THE ARACHIDONIC ACID CONCENTRATION PRODUCED IN STIMULATED CELLS

The amount of arachidonic acid produced in stimulated cells was measured via its conversion to 15–hydroxyeicosatetraenoic acid (15–HETE) using soyabean 15–lipoxygenase. This was converted to a concentration by measuring the intracellular volume of Swiss 3T3 fibroblasts (see 2.12).

(i) Preparation of samples

Swiss 3T3 cells were seeded onto 6– or 24–well plates and grown to quiescence in DMEM containing 10% newborn calf serum. Plates were then washed in 0.5ml HBG for 45 minutes. This was followed by stimulation with agonists for 20 seconds (a point at which the intracellular early phase of arachidonic acid release should be maximal). The reaction was terminated by the addition of 1ml of methanol. Arachidonic acid was extracted and separated on silicic acid columns as previously described. Once eluted from the columns in diethyl ether the samples were dried down and re-dissolved in 20µl ethanol.

(ii) Enzyme assay with soyabean 15–lipoxygenase

The stock enzyme (specific activity 90U/µl) was prepared in 1M ammonium sulphate/0.05M sodium phosphate, pH8.0. For use in experiments, this was diluted to 10pM in 0.1M phosphate buffer, pH7.4.

Reactions were performed in a heated water bath at 37°C. For incubation, 5µl of enzyme was added to a polypropylene tube containing 29µl

phosphate buffer and 6µl of either the sample or standard arachidonic acid (this was used in the range 0.08ng/ml – 3.125ng/ml). The reaction was allowed to proceed for 30 minutes and stopped by the addition of 5µl nordihydroguaiaretic acid (NDGA) 920µM and 5µl of sodium borohydride (50mg/ml).

(iii) HPLC for arachidonic acid and 15–HETE detection

It is possible to check the efficiency of the enzyme in converting arachidonic acid to 15–HETE by separating these components on hplc.

Samples, containing [^{3}H] arachidonic acid (0.6µCi), were treated as above and were prepared in 50% methanol/50% ammonium acetate (0.5%).

Injections of 25µl were made onto a lichrosorb C–18 column (25cm x 0.39cm). Using a 2ml/min flow rate, products were eluted with methanol 62.5%/acetonitrile 37.5% for 5 minutes. This was followed by an increase in the methanol fraction to 75% for 10 minutes and a further increase to 93.7% for 8.5 min. The run was completed by decreasing the methanol to 43.7% for 15 minutes.

Elution profiles of the samples were compared with those of standards which were determined using a radiochemical detector. The retention times were: 15–HETE – 6.2 minutes and AA – 11.4 minutes.

(iv) 15–HETE assay system

This assay was used as a means of directly measuring the amount of 15–HETE formed in samples treated with the 15–lipoxygenase enzyme. The assay was based on the competition between unlabelled 15–HETE in the sample or standard and a fixed quantity of tritium labelled 15–HETE for binding to a limited quantity of an antibody which has high specificity and affinity for 15–HETE. The amount of radioactive ligand bound by the antibody was inversely proportional to the concentration of added non–radioactive ligand.

Samples were assayed as described using instructions obtained with a [^{3}H] 15–HETE assay kit.

2.12 DETERMINATION OF INTRACELLULAR VOLUME

Swiss 3T3 cells were seeded onto a 24–well plate and grown in DMEM containing 10% NCS until confluent and quiescent. The incubation medium was then aspirated and cells washed in 1ml of HBG for 30 minutes at 37°C. After this period, the medium was aspirated and cells incubated with 0.25ml of HBG containing 0.2μCi/ml [^{3}H] water and 0.04μCi/ml [^{14}C] sucrose, for 30 minutes. The medium was aspirated and 0.5ml of 1M sodium hydroxide added to each well to solubilise the cells. A sample of 0.4ml was then taken from each well and transferred to a scintillation vial. 12ml of scintillation fluid was added and the radioactivity determined by scintillation counting. Essentially, the [^{14}C] sucrose was assumed to represent contamination by the extracellular medium and [^{3}H] water, after correction for contamination by the extracellular medium, was assumed to be intracellular.

2.13 DETERMINATION OF THYMIDINE INCORPORATION IN SWISS 3T3 CELLS

For measurement of DNA synthesis, cells were grown to confluence and quiescence in DMEM containing 10% NCS. The medium was then changed to one containing 0.5% NCS for 24h. Additions of mitogens were made at the concentrations indicated in serum–free DMEM containing 1μCi/ml of [^{3}H] thymidine and the cells were incubated for 24h. The medium was then aspirated and the monolayers were washed twice with 0.5ml HBG, three times with 0.5ml ice–cold 5% (v/v) trichloroacetic acid and twice with 0.5ml ice–cold ethanol. The cells were then solubilised in 1ml of 0.3M sodium hydroxide and this was transferred to a vial containing 0.2ml of 1.5M hydrochloric acid. The radioactivity was determined by scintillation counting in 5ml scintillation fluid.

2.14 PHORBOL DIBUTYRATE BINDING ASSAY

Swiss 3T3 cells were seeded onto 24–well plates and grown to quiescence in DMEM containing 10% newborn calf serum. The medium was aspirated and the monolayer washed twice with 0.5ml of PBS at 37°C. Cells were then incubated for 15 minutes with 150µl of assay buffer (PBS + 0.1%(w/v) BSA) containing 10–100nM [^{3}H] phorbol dibutyrate (specific radioactivity 25Ci/mmol) ± 200–fold excess unlabelled phorbol dibutyrate for non–specific binding. Cells were then rapidly washed three times with 0.5ml of ice–cold assay buffer and solubilised with 0.5ml of 0.5M sodium hydroxide/0.1% v/v Triton X–100. the solubilised cells were transferred to vials and assayed for radioactivity in 4ml of scintillation fluid.

Three wells were left in each 24–well plate for cell counting.

2.15 INTRACELLULAR CALCIUM MEASUREMENTS

2.15.1 Preparation of cells

Glass coverslips which had been sterilised were coated on one side with 0.3% of gelatin. This was allowed to dry under sterile conditions and the coverslips were placed in a 9cm petri dish (four coverslips per dish). Swiss 3T3 cells were seeded onto the coverslips and were grown in DMEM containing 10% newborn calf serum until confluent and quiescent.

2.15.2 Calcium measurements

The confluent monolayers were washed in HBG and incubated with HBG containing 5µM indo–1 AM, 0.125% (w/v) plurionic acid, 0.5% (v/v) DMSO for 45 minutes at room temperature. Coverslips were placed in a perspex bath which was fixed to the stage of a Nikon Diaphot inverted microscope. The cells were perfused with HBG with or without additions at 1.5ml/min (35–37°C) and illuminated at 360nm. Light emitted from the cells with a wave-length greater than 385nm was directed at a 460nm dichroic mirror which sent longer wavelength light via a 495nm barrier filter to a photomultiplier. Shorter wavelengths were reflected by the dichroic

mirror and sent via a 405nm barrier filter to a second photomultiplier. The signals from the two photomultipliers were divided using analogue circuitry and a signal representing the ratio filtered at 3Hz. No correction was made for cell autofluorescence which was less than 5% of a typical indo loaded preparation. Due to the difficulties associated with absolute calibrations for intracellular calcium concentrations from cells loaded with AM esters of calcium indicators (Highsmith *et. al.*, 1986) the experimental records are presented in terms of raw fluorescence data.

ANALYSIS AND PRESENTATION OF RESULTS

Unless otherwise stated, all experiments were performed at least three times and each data point represents the mean ± S.D. of triplicate determinations. The statistical significance was estimated by a paired students 't' test on an Apple Macintosh stat–works programme and significance was generally taken as values of $p<0.05$. EC_{50} values were calculated from dose-response curves fitted to a logistic equation (non-linear regression analysis) as defined by DeLean *et. al.*, 1980. However, for presentation purposes, dose–response curves were shown as simple line graphs from the Apple Macintosh Cricket Graph programme.

CHAPTER 3

THE MECHANISMS OF ARACHIDONIC ACID RELEASE IN BOMBESIN-STIMULATED SWISS 3T3 FIBROBLASTS

INTRODUCTION

The results presented in this chapter form a general introduction and basis for subsequent studies within this research project. The quintessential features of this chapter are firstly, the definition of the profile of the response to bombesin and, secondly, a general indication of the regulatory requirements of this response. Having determined this, subsequent chapters attempt to analyse certain aspects of the response in more detail.

Elucidation of the molecular mechanisms leading to cellular proliferation requires the identification and characterisation of the signalling pathways that, when activated, induce a mitogenic response. In considering the mitogenic action of bombesin, various effects attributed to this peptide may play important roles, such as the rapid increase in intracellular calcium via Ins(1,4,5)P_3 production (Blakely *et al.* 1989; Cook *et al.*, 1990), activation of PKC (Erusalimsky *et al.*, 1988), stimulation of the Na^+/H^+ antiport system (Mendoza *et al.*, 1986) and induction of the cellular oncogenes c–*fos* and c–*myc* (Bravo *et al*, 1987). Of these effects, probably the best characterised is that of the activation of phosphoinositide–specific PLC. The consequent production of Ins(1,4,5)P_3 and sn–1,2–DAG has been well studied in this system and the former has been shown to be a transient process that is rapidly desensitised (Brown *et al.*, 1987). Another enzyme suggested to be activated in this system in response to bombesin is PLD. Cook and Wakelam (1989) have proposed the existence of bombesin–stimulated PtdCho hydrolysis via PLD. They suggest that this contributes to the elevation of cellular DAG in the absence of significantly amplified PtdIns (4,5)P_2 breakdown, and that this event may be relevant to the activation of PKC in long–term cellular responses, since the agonist–stimulated PtdIns (4,5)P_2 hydrolysis is rapidly desensitised. Therefore, the interaction of both PLC and PLD signalling pathways may be important in bombesin–stimulated mitogenesis.

The possibility that the mitogenic action of bombesin may involve stimulation of another pathway, namely AA release via PLA_2 activation, has received relatively little attention. Recently, evidence has indicated that the liberation of AA and its possible conversion to cyclooxygenase metabolites constitutes one of the early mitogenic signals induced by bombesin in 3T3 cells (Millar & Rozengurt, 1990). It is also noteworthy that the PDGF AA and BB homodimers that stimulate re-initiation of DNA synthesis in the absence of other growth promoting factors, induce a large and sustained release of AA in Swiss 3T3 cells (Mehmet *et al.*, 1990). Recent results obtained with mastoparan (Gil *et al.*, 1990) also support the conclusion that AA release contributes to mitogenic signal transduction in this cell line. Mastoparan stimulated AA release in the absence of inositol phosphate accumulation or PKC activation and the results strongly suggest direct involvement of the AA signalling pathway in mastoparan–stimulated DNA synthesis.

Given the potential importance of the pathway in the mitogenic process in this system, the present work was undertaken to clarify the regulatory mechanisms controlling AA release in bombesin–stimulated 3T3 cells and to potentially identify the role played by the pathway in signal transmission. Given the extensive amount of work already performed on this system with regard to the bombesin receptor (Brown *et al.*, 1988; Battey *et al.*, 1991) and how it couples to different signalling pathways within these cells (Brown *et al.*, 1987; Takuwa *et al.*, 1987; Cook & Wakelam 1989; Plevin *et al.*, 1990), it may be possible to relate activation of AA release to other established effects of bombesin stimulation.

RESULTS

The incorporation of AA into cellular phospholipids was examined over a 24h labelling period. This gave an indication of the optimal labelling time for these cells and also, as has been examined in Chapter 4, which phospholipids may be potential sources of the AA released in bombesin–stimulated cells. Approximately 70% of the radiolabel was incorporated into cellular phospholipids. Figure 3.1 shows that the label incorporated most readily into PtdCho (48%) and PtdEtn (26%) and to a lesser extent into PtdIns (10%) and PtdOH (12%). Little AA was found in PtdSer and sphingomyelin (0.7% and 2.4% respectively). The optimal labelling time was approximately 20h. In all subsequent experiments, cells were labelled for 24h prior to agonist stimulation.

Addition of bombesin to Swiss 3T3 cells caused the release of AA from prelabelled cells. Figure 3.2 shows the timecourse of release over 40 min. Bombesin stimulated a rapid increase in free AA within 1 min (2.7–fold stimulation). This was a transient response since AA levels returned to basal by 5 min. However, there was evidence for a later, sustained phase of release occurring after 10 min stimulation, with levels reaching 11.1–fold after 40 min. This response was dose-dependent with an EC_{50} value of 3.0 ± 0.4nM as shown in Figure 3.3.

To determine whether the initial phase of this response was due to activation of PLA_2, the formation of lysophospholipids was examined. Since PLA_2 hydrolysis of phospholipids will result in the formation of both AA and a lysophospholipid, it is essential that both products are measured in the same experiment. Measurements were therefore conducted in cells double–labelled with [^{14}-C] AA and [^{3}H] choline so that formation of both products could be determined over the same timecourse. Upon bombesin stimulation, there was no apparent formation of LysoPtdEtn, LysoPtdIns or LysoPtdOH over a 10 min period (Fig.3.4a). However, a 1.7–fold increase in LysoPtdCho levels was observed after 1 min stimulation with bombesin (Fig.3.4b). This was shown to be significant when analysed by a one–tailed t–test.

For LysoPtdCho p=0.005 whereas for LysoPtdEtn, LysoPtdIns and LysoPtdOH p=0.32, 0.28 and 0.36 respectively. The levels of LysoPtdCho subsequently decreased but values remained above basal for at least 10 min. A more detailed profile of this response is shown in Chapter 4 where the initial phase of AA release is examined in greater depth.

As previously described, PLA_2 activation has been suggested to be under regulation by PLC in some systems. However, in other cases, the enzymes are independently controlled. In some cell types, such as neutrophils, this can be demonstrated through differential susceptibility of the GTP–binding proteins involved in each pathway to bacterial toxins (pertussis toxin and cholera toxin) (Cockcroft & Stutchfield, 1989). In the present system, the bombesin receptor is known to couple to the PLC pathway via a pertussis toxin insensitive G protein (Fischer & Schonbrunn, 1988). It was therefore decided to examine whether AA release, as shown here, was sensitive to toxin treatment. This was seen as a potential means of discriminating between the PLA_2 and PLC pathways. Experiments suggested that the putative G protein involved in stimulation of this pathway was also insensitive to pertussis toxin, since AA release showed a similar fold increase in toxin–treated and untreated cells (2.8–fold and 3–fold respectively). The toxin did, however, appear to stimulate the basal release of [^{3}H] AA in control cells (Fig.3.5). Since pertussis toxin could not be used as a tool to distinguish between pathways in this system, other methods of discrimination were tested.

Short–term pretreatment with PMA can be used to potentially dissociate the activation of PLA_2 from PLC. A 15 min pretreatment with PMA will abolish bombesin–stimulated PLC–catalysed PtdIns (4,5)P_2 hydrolysis by a mechanism involving PKC–mediated negative feedback (Brown *et al.*, 1987). However, the same treatment in this system caused significant enhancement (p=0.009) of AA release by 5 minutes (Fig.3.6). This result suggested that bombesin–stimulated AA release was

not dependent on prior PtdIns (4,5)P_2 hydrolysis by PLC, since treatment that abolished the latter, potentiated the former.

Additional experiments were performed to determine the interrelationships, if any, between the PLC– and PLA_2–catalysed pathways. Dependence of the AA response upon the PKC activity or increased calcium levels may suggest that prior activation of phosphoinositide hydrolysis by PLC is required. By studying the effects of altering the levels of PKC and extracellular calcium concentration on the AA response, the importance of these factors was established.

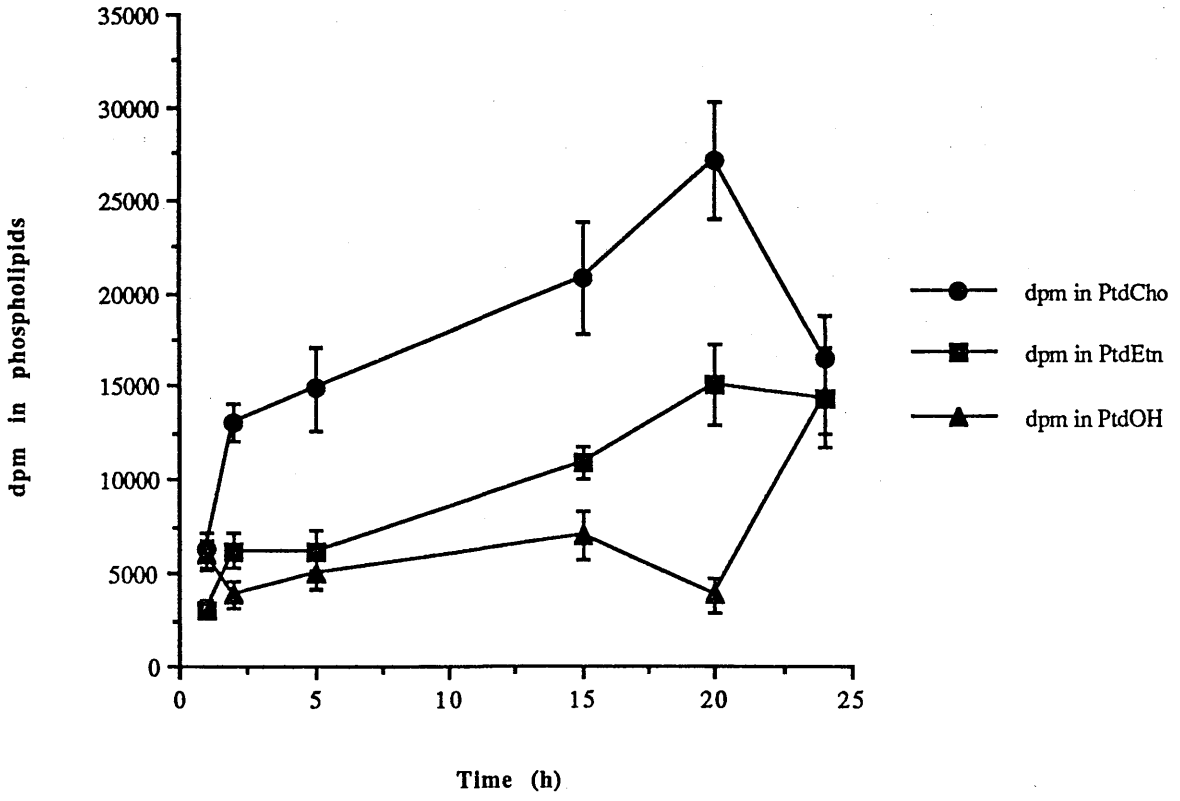
The Swiss 3T3 cell is fairly unusual in that its levels of PKC can be completely down–regulated after a 48h pretreatment with phorbol ester (Adams & Gullick, 1989). As a check that this treatment was effective, phorbol dibutyrate binding experiments were performed as a means of measuring the levels of PKC in PMA–treated and untreated cells (Fig.3.7). This data clearly showed that after long–term PMA pretreatment there was virtually no PKC activity remaining within the cells (PMA pretreated cells contained $3.2 \pm 2.8\%$ of the PKC levels found in controls). The AA response to bombesin was studied in cells that had received a 48h pretreatment with 300nM PMA. Figure 3.8 shows that the rate and extent of the response are both essentially the same in control and down–regulated cells (3.1–fold in control cells c.f. 2.8–fold in PMA–treated cells). Further experiments were performed using the PKC inhibitor staurosporine. A 15 min pretreatment of 3T3 cells with staurosporine had no significant effect on AA release for up to 10 min of stimulation with bombesin (3.2–fold in control cells c.f. 3.3–fold in staurosporine–treated cells). Taken together, these data suggest that the initial phase of bombesin–stimulated AA release is not dependent on PKC activation.

Another potential regulatory factor of PLA_2 is calcium. In various cell types, calcium mobilising agonists are reported to liberate AA and its metabolites in an extracellular calcium–dependent manner (Ho & Klein, 1987; Conklin *et al.*, 1988; Felder *et al.*, 1990). However, other studies suggest the existence of a calcium

independent component (Bonventre & Swidler, 1988; Coyne *et al.*, 1989; Takuwa *et al.*, 1991). In the present study, AA release was examined over 40 min under conditions where extracellular calcium was buffered to 150nM using 0.3mM EGTA. This was compared with the AA response under normal conditions, i.e. extracellular calcium concentration of 1.26mM. As shown in Figure 3.10, bombesin–stimulated [^{3}H] AA release occurred rapidly and to a similar extent regardless of the extracellular calcium concentration (2.7–fold in normal calcium c.f. 2.9–fold in low calcium). However, the second sustained phase of release was differently affected. After 10 min stimulation at 1.26mM calcium, [^{3}H] AA release continued to increase with time up to at least 40 min. In contrast, when cells were treated with 150nM calcium, this increase was abolished. These observations support the conclusion that the effect of bombesin on AA release is temporally composed of two distinct phases i.e. the first phase (within 10 min) which is largely independent of the extracellular calcium concentration and the second sustained phase (after 10 min) which is totally dependent on the extracellular calcium concentration.

Figure 3.1 Incorporation of [^{3}H] arachidonate into membrane phospholipids

[^{3}H] arachidonate was added to Swiss 3T3 cells for the times indicated and incorporation into individual phospholipids was examined using thin layer chromatography as described. Results are presented as the mean ± S.D. from a typical experiment where n=3.


35000
30000
25000
20000
15000
10000
5000
0
dpm in phospholipids
0
5
10
15
20
25
Time (h)
dpm in PtdCho
dpm in PtdEtn
dpm in PtdOH

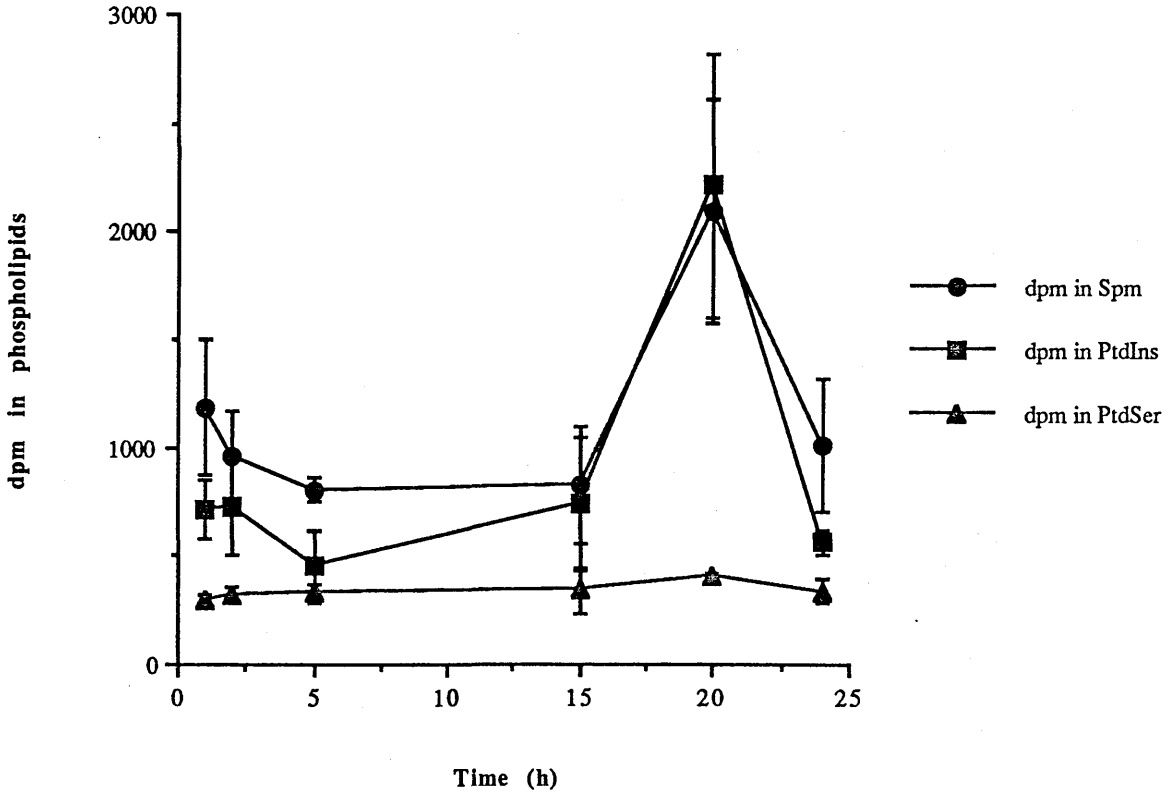

3000
2000
1000
0
dpm in phospholipids
0
5
10
15
20
25
Time (h)
dpm in Spm
dpm in PtdIns
dpm in PtdSer

Figure 3.2 Timecourse of bombesin-stimulated arachidonic acid release in Swiss 3T3 cells.

Cells were treated as described and stimulated with either HBG (O) or 617nM bombesin in HBG (●) for the times indicated. Total arachidonate release was measured as described. Results are representative of means ± S.D. where n=3.

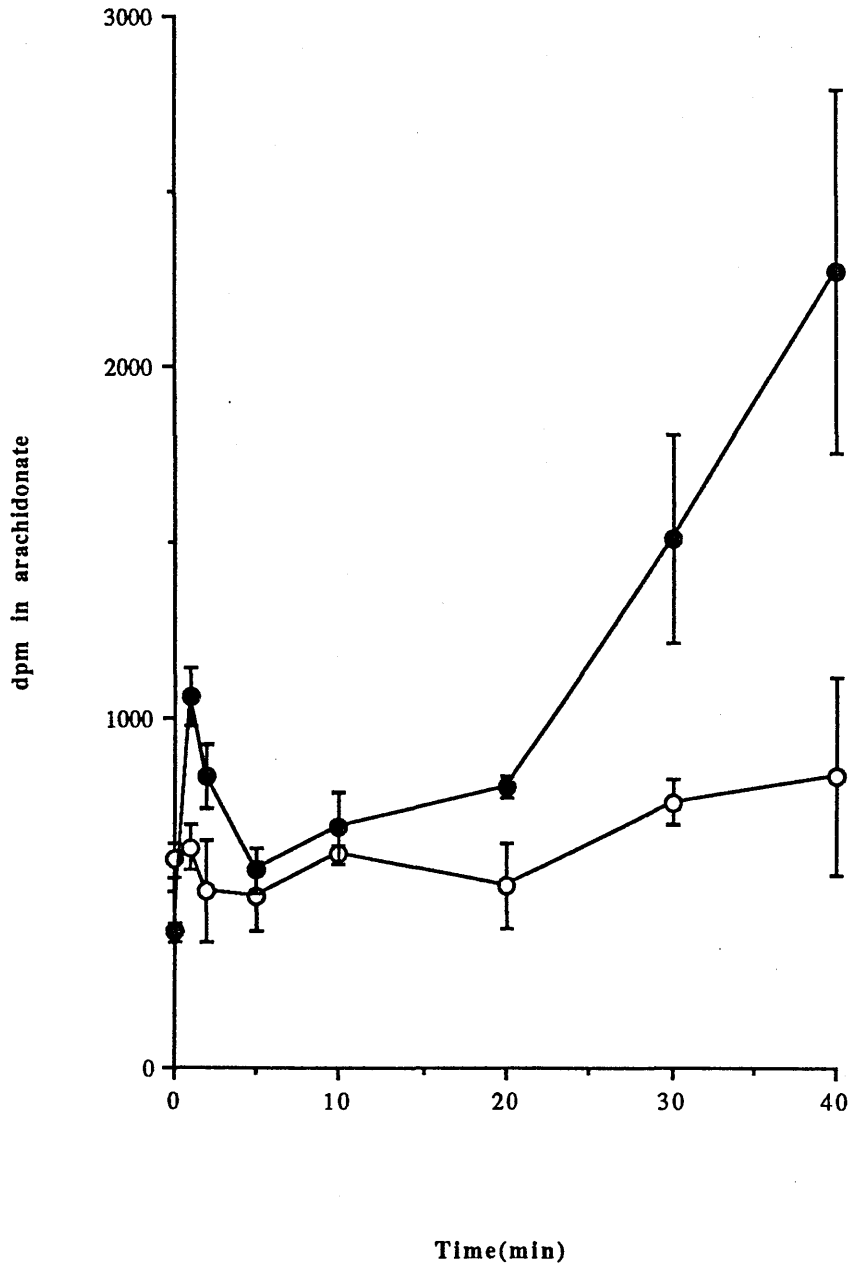

3000
2000
1000
0
dpm in arachidonate
0
10
20
30
40
Time(min)

Figure 3.3 Dose response of arachidonic acid release to bombesin in Swiss 3T3 cells

Cells were stimulated with increasing concentrations of bombesin for 30 min and [^{3}H] arachidonate release measured as described. Results are presented as the mean ± S.D. from a single typical experiment where n=3.

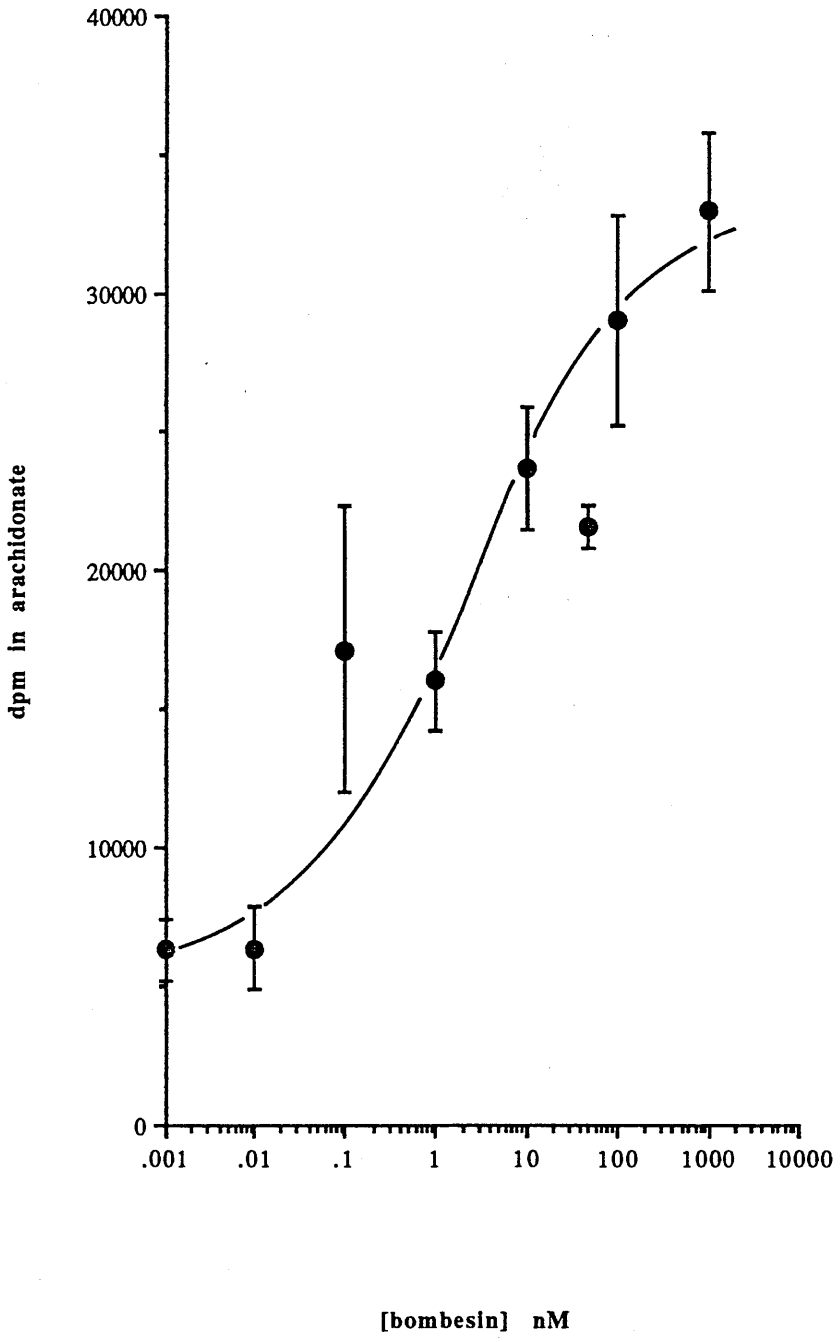
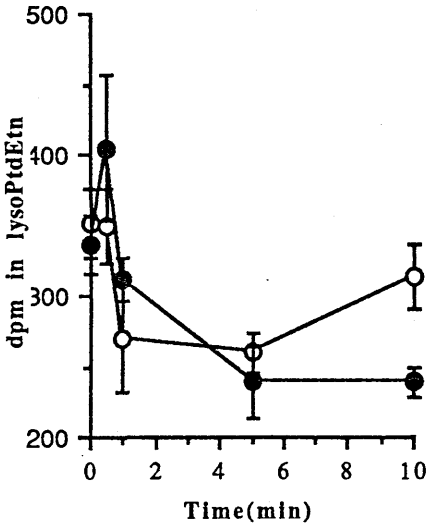
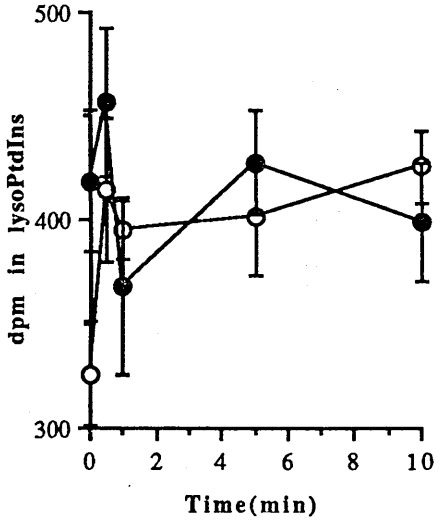
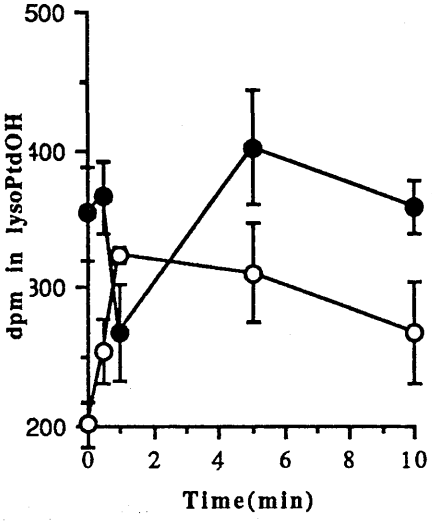

40000
30000
20000
10000
0
dpm in arachidonate
.001
.01
.1
1
10
100
1000
10000
[bombesin] nM

Figure 3.4 Bombesin–stimulated lysophospholipid production in Swiss 3T3 cells

Cells were stimulated for the indicated times with 617nM bombesin (●) or HBG (O) (a) LyosPtdEtn, LysoPtdIns and LysoPtdOH and (b) LysoPtdCho production were measured using thin layer chromatography. Results are presented as the mean ± S.D. from a single typical experiment where n=3.


500
400
300
200
dpm in lysoPtdEtn
0
2
4
6
8
10
Time(min)


500
400
300
dpm in lysoPtdIns
0
2
4
6
8
10
Time(min)


500
400
300
200
dpm in lysoPtdOH
0
2
4
6
8
10
Time(min)

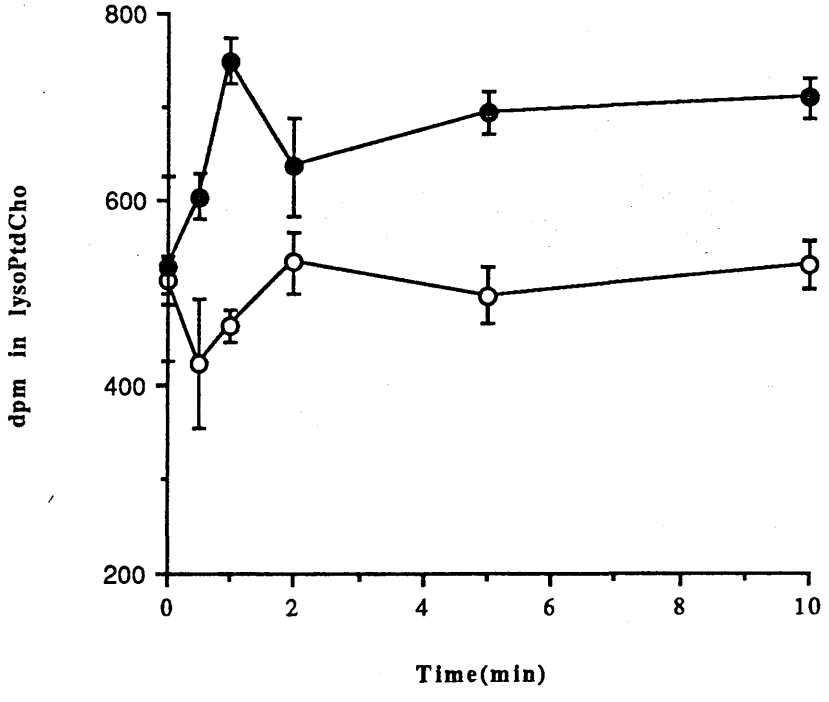

800
600
400
200
dpm in lysoPtdCho
0
2
4
6
8
10
Time(min)

Figure 3.5 Effect of pertussis toxin pretreatment on bombesin–stimulated arachidonate release.

Cells were pretreated for 16h with either pertussis toxin (100ng/ml)(□,■)or incubation medium(○,●)prior to stimulation with 617nM bombesin for the indicated times. Arachidonate release was measured as previously described. Results are presented as the means ± S.D. from a single typical experiment where n=3.

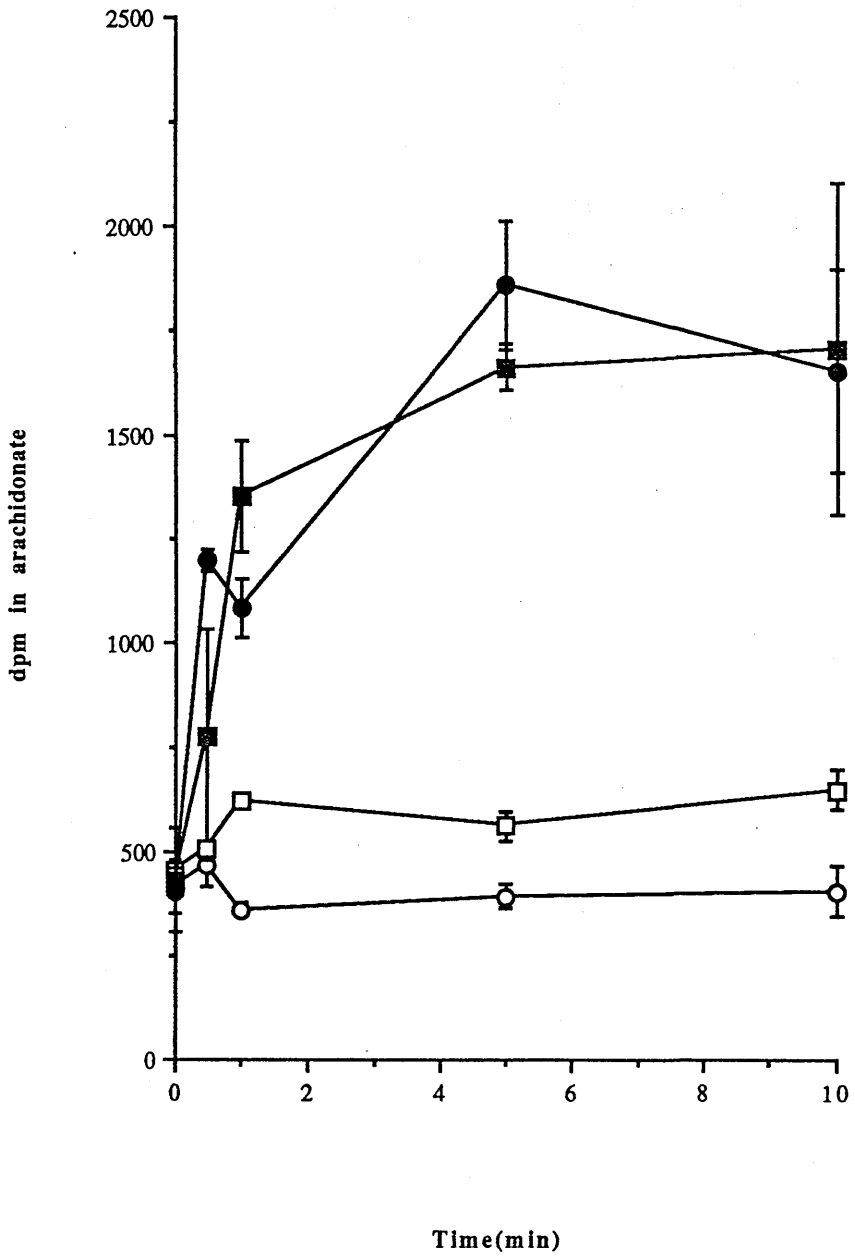

2500
2000
1500
1000
500
0
dpm in arachidonate
0
2
4
6
8
10
Time(min)

Figure 3.6 Effect of short-term PMA pretreatment on bombesin-stimulated arachidonate release

Cells treated as described were pretreated for 15 min with either 300nM PMA (■) or HBG (●). The arachidonate released in response to bombesin stimulation was measured as described and compared with unstimulated values (O). Results are presented as the mean ± S.D. from a single typical experiment where n=3.

N.B. In the presence of PMA,basal values were slightly increased.

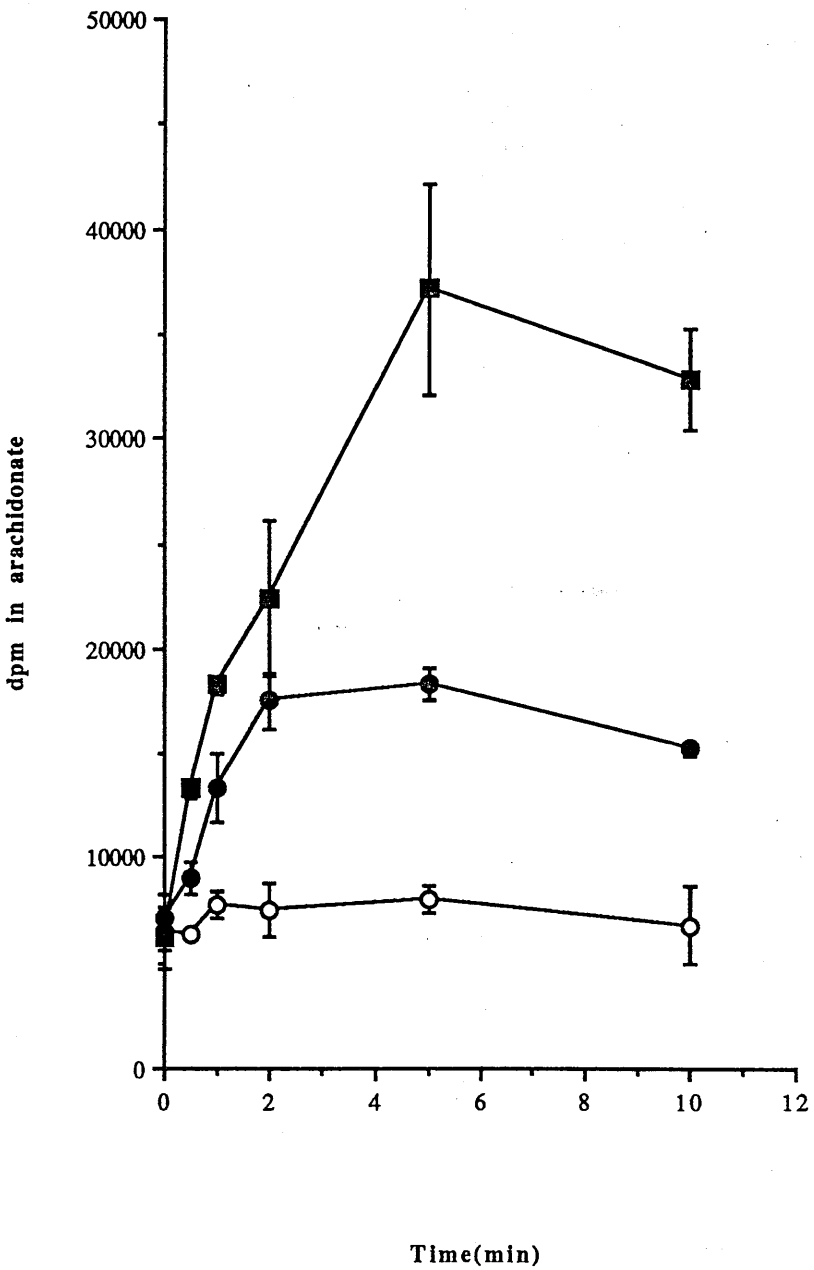

50000
40000
30000
20000
10000
0
dpm in arachidonate
0
2
4
6
8
10
12
Time(min)

Figure 3.7 Differences in phorbol dibutyrate binding in control and PKC down-regulated cells.

Swiss 3T3 cells were treated for 48h with either incubation medium, 300nM TPA, β-phorbol, or DMSO. Phorbol dibutyrate binding was measured as described. Results are presented as the mean ± S.D. from a single typical experiment where n=3.

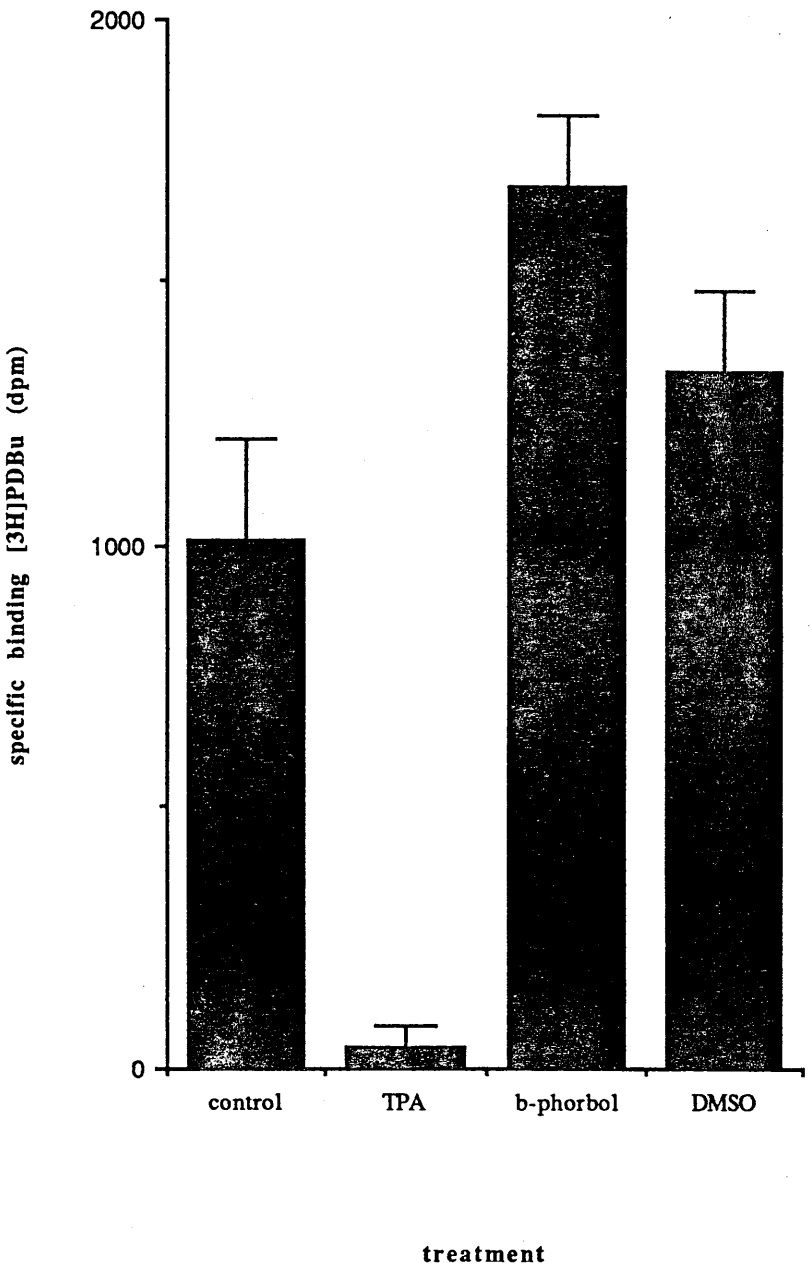

2000
1000
0
specific binding [3H]PDBu (dpm)
control
TPA
b-phorbol
DMSO
treatment

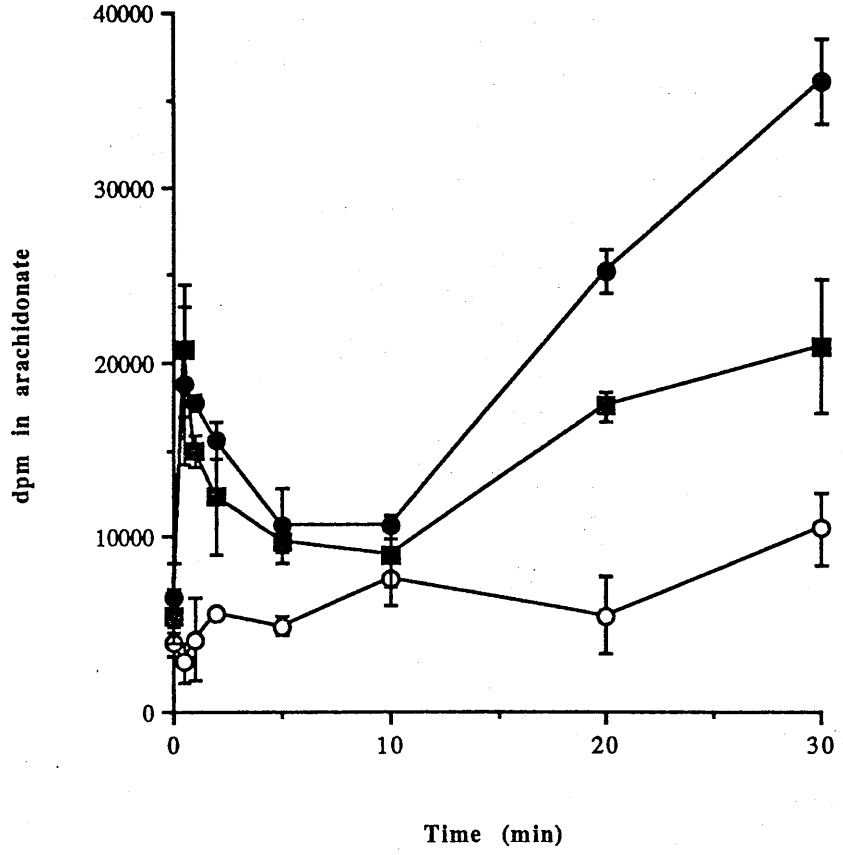
Figure 3.8 Effect of long-term PMA pretreatment on bombesin-stimulated arachidonate release

Swiss 3T3 cells were treated for 48h with either 300nM PMA (■) or incubation medium (●) Cells were then stimulated with 617nM bombesin for the times indicated and values compared with basal levels (O). Results are presented as the mean ± S.D. from a typical experiment where n=3.

Figure 3.9 Effect of staurosporine treatment on bombesin-stimulated arachidonate release

Swiss 3T3 cells were pretreated for 15 min with either 10μM staurosporine (▬) or incubation medium (◓). Cells were then stimulated with 617nM bombesin and arachidonic acid release measured. These values were compared with those obtained in the presence of HBG alone (O).
Results are presented as the mean ± S.D. from a single typical experiment where n=3.

N.B. Values obtained after 20 minutes in the presence of PMA or staurosporine are significantly different from controls ($p < 0.05$)


40000
30000
20000
10000
0
dpm in arachidonate
0
10
20
30
Time (min)

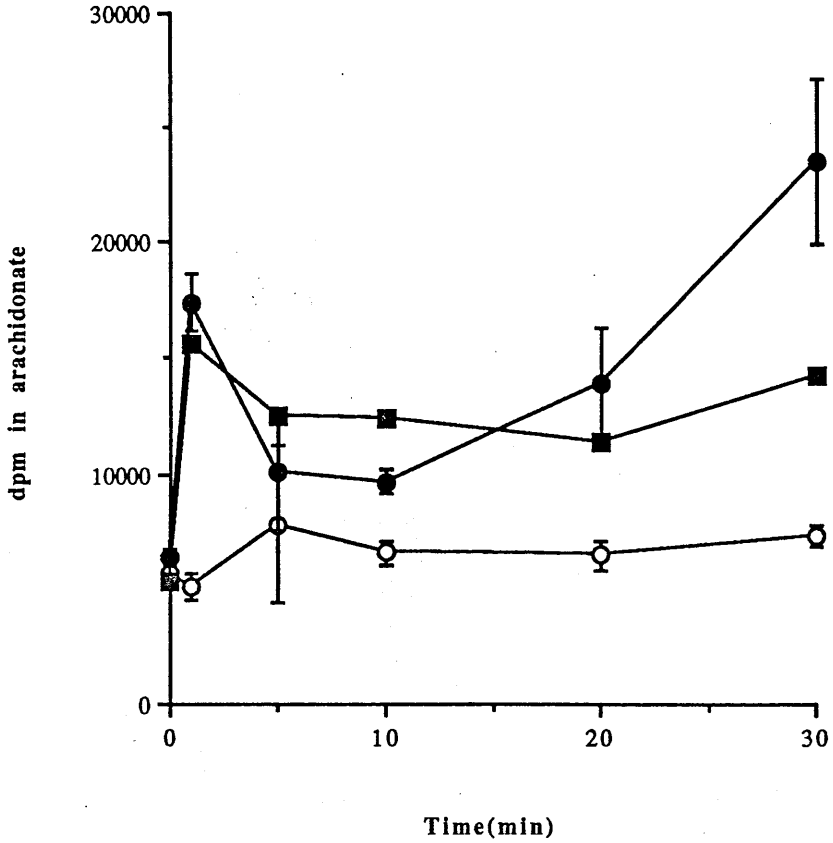
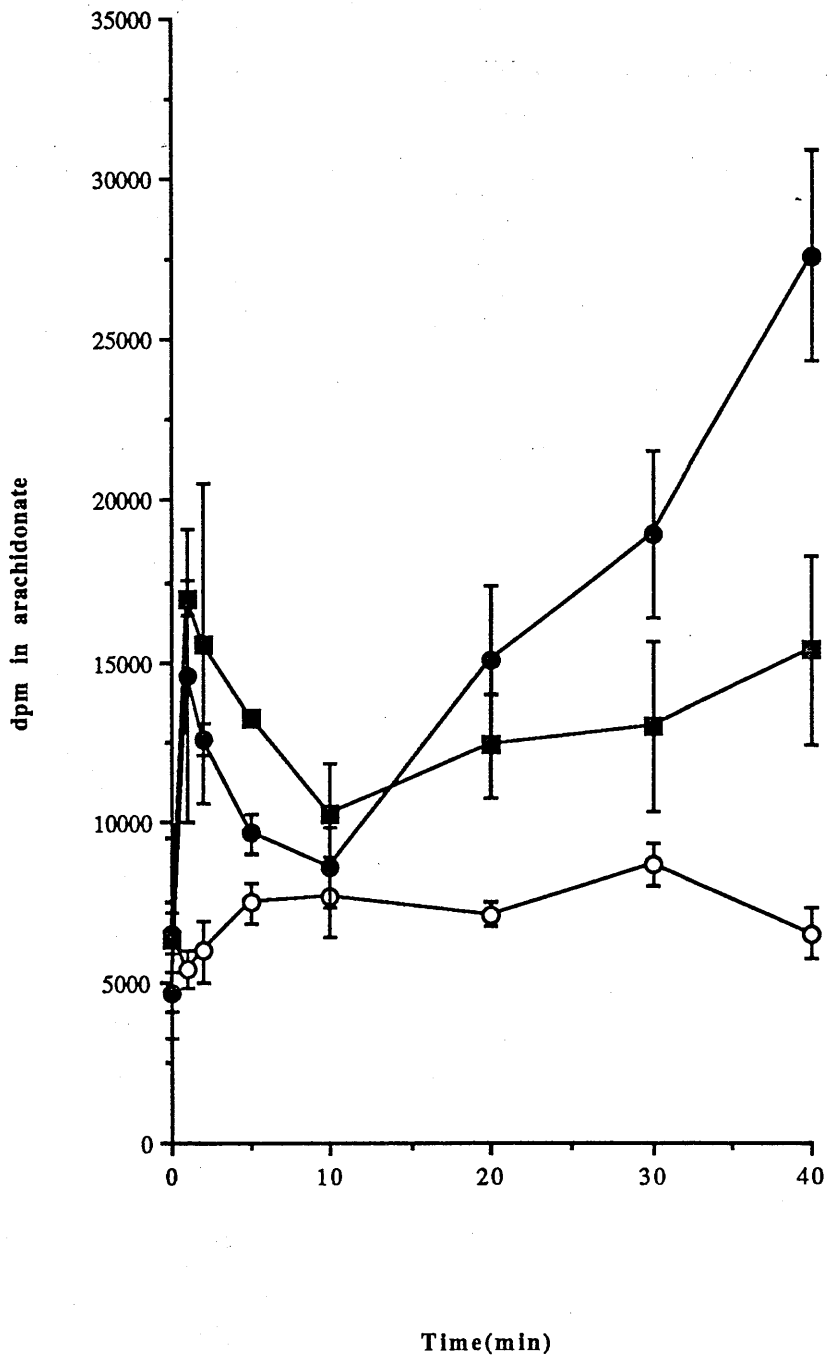

30000
20000
10000
0
dpm in arachidonate
0
10
20
30
Time(min)

Figure 3.10 Effect of reducing extracellular calcium on bombesin–stimulated arachidonate release

[3H] arachidonate labelled cells were stimulated with 617nM bombesin in medium containing 1mM calcium (●) or medium in which the calcium had been buffered to 150nM with EGTA (■). Arachidonate was measured as described. Results are expressed as means ± S.D. from a typical experiment where n=3.

N.B. Values obtained after 20 minutes in medium containing 150nM calcium are significantly different from controls ($p < 0.05$)


35000
30000
25000
20000
15000
10000
5000
0
dpm in arachidonate
0
10
20
30
40
Time(min)

DISCUSSION

The results presented in this chapter demonstrate that bombesin induces a biphasic increase in AA liberation with the initial phase probably regulated by PLA_2. Since neither the formation of lyso–phospholipids nor the effect of using putative PLA_2 inhibitors has been studied at later time points, it is difficult to tell whether the second phase of release is also due to a PLA_2 activity or whether a separate pathway (e.g. DAG lipase) is involved. Given the elevated levels of DAG at later time points in response to bombesin, the latter pathway could potentially be responsible for increased AA production. In the recent report by Takuwa *et al.* (1991), which has subsequently shown the existence of a biphasic AA response to bombesin, the proposal is made that both phases are mediated by PLA_2 since mepacrine inhibits each to a similar extent. However, the specificity of mepacrine, as with many putative PLA_2 inhibitors, is questionable. Is therefore remains possible that an additional pathway is involved.

The first transient phase of AA release occurs within 1 min of stimulation with bombesin. The precise kinetics of the response have not been characterised in this chapter (but see Chapter 4). This may be of importance in determining whether a direct receptor–mediated effect is involved i.e. receptor–mediated PLA_2 activation.

Since bombesin stimulates the rapid hydrolysis of PtdIns $(4,5)P_2$ by PLC, it seemed possible that the resultant combination of calcium mobilisation and PKC activation could regulate the AA released by bombesin. However, experiments using PKC down–regulated cells, strongly suggest that PKC activation is not necessary for the initial phase of the AA response.

The effect of reducing extracellular calcium showed that the initial phase did not require bombesin stimulated entry of extracellular calcium. The response occurred to the same extent regardless of the calcium concentration in the medium. This, together with the lack of involvement of PKC, suggests that the first phase of release may be regulated by a PLA_2 that is activated independently of PLC. This

possibility is strengthened by the data shown in Figure 3.6 for PMA treated cells. This effect with PMA has been observed in other systems leading to the theory that receptor–mediated phosphoinositide hydrolysis and AA release can occur in a non–interdependent manner via parallel activation of PLA_2 and PLC (Burch & Axelrod, 1987; Van Corven *et al.*, 1989; Cockcroft & Stutchfield, 1989).

The second, sustained phase of AA release showed completely different characteristics to that of the first. It appeared to be absolutely dependent upon extracellular calcium as the increase in AA release was completely abolished in medium where the calcium had been reduced to 150nM. This effect has subsequently also been observed by Takuwa *et al.* (1991). These authors also showed that if the medium calcium was restored to 2mM after 30 min in cultures treated with low calcium conditions, [^{3}H] AA release immediately increased at a constant rate during the second phase. Thus, the calcium dependent process underlying the second phase response may be readily reversible even after a prolonged calcium depletion.

These observations may reflect the involvement of a PLA_2 with a different sensitivity to calcium. Perhaps the second phase is dependent upon stimulated calcium entry. Ballou *et al.* (1986) showed the existence of two different substrate–specific forms of the enzyme in platelets: one for PtdCho, which was calcium dependent and another for PtdEtn, which was calcium independent. In Swiss 3T3 cells, a small loss of AA from PtdEtn at later times has been observed. However, the significance of this is uncertain. Alternatively, a different enzyme such as DAG lipase may be involved in the second phase.

The role of PKC was explored up to 30 min stimulation in the second phase (c.f. 40 min for Ca^{2+} effects), An inhibitory effect was detected after 20 min in cells where PKC had been down–regulated or inhibited. This may reflect the existence of a PLA_2 isozyme with a PKC dependency. In addition, these results may suggest that PKC activation of PLA_2 is dependent on calcium.

The mechanism behind PKC activation of PLA_2 is unclear. It was previously proposed that PKC might phosphorylate and inactivate the physiological inhibitors of PLA_2 known as lipocortins (Hirata *et al.*, 1984). However, this has since proved inaccurate. More recently, it was suggested by Akiba *et al.* (1989) that PKC may enhance AA liberation by stimulation of Na^+/H^+ exchange which may modulate PLA_2. This type of mechanism may be important in the second phase of AA release. Similar observations by Takuwa *et al.* (1991) concerning PKC, strengthen the possibility that the second phase of release is under different regulation from the first, and lend further credence to the possible involvement of different isoforms of PLA_2 in each of the phases. The existence of isoforms that have different calcium sensitivities has already been mentioned. Isoforms of PLA_2 have also been distinguished by differences in substrate specificity (Kramer *et al.*, 1989) by the pH optimum of catalytic activity (Ballou *et al.*, 1986) and in electrophoretic mobility (Moreno *et al.*, 1988). The physiological relevance of isoforms is not known. Potentially different substrate specific forms of PLA_2 may provide a rational basis for some apparently conflicting observations concerning the phospholipid source and pathway involved in AA release in certain cell types. This may certainly be true in the present system. Under different stimulatory conditions, the isoforms of PLA_2 may be differentially regulated providing more potential variability in the AA response generated. In addition, the potential for control of AA release may be greatly increased under these circumstances where a number of regulatory factors are involved.

CHAPTER 4

BOMBESIN STIMULATES THE RAPID ACTIVATION OF PHOSPHOLIPASE A_2 CATALYSED PHOSPHATIDYLCHOLINE HYDROLYSIS IN SWISS 3T3 CELLS

INTRODUCTION

Data presented in the previous chapter showed the novel biphasic nature of AA generation in response to bombesin in 3T3 cells and indicated the potential for there being two separate mechanisms regulating the initial transient phase of release and the later sustained phase. The initial phase of AA generation may be regulated by the early activation of a PLA_2. The work presented in this chapter attempts to strengthen the evidence for PLA_2 involvement in this response and relate activation of this enzyme to that of PLC, dissociating between the likelihood of a receptor–mediated process involved in PLA_2 activation and the alternative, prior involvement of PLC.

In Chapter 3, the AA measured was total release both within and from the cell, i.e. there was no discrimination between intra– and extracellular AA. Many previous studies examining agonist induced AA release have often monitored the levels in the extracellular medium (Jaffe *et al.*, 1987, Resink *et al.*, 1987). However, changes in extracellular levels of AA are unlikely to sensitively and accurately reflect the time course of AA changes inside the cell. The AA measured in this chapter comprises only intracellular liberation and, as such, clarifies the kinetics and extent of release for the initial phase. Previously, the earliest time point measured was 30 sec. This is a relatively long period of stimulation by which time the initial intracellular phase could be declining or even finished. By examining shorter stimulation times, it is possible to clarify the kinetics of release and to dissociate between bombesin–stimulated AA release and $InsP_3$ production.

One of the main problems associated with attempting to measure potentially rapid yet small changes in AA in stimulated cells is the lack of efficient separation of the fatty acid in some thin layer chromatography systems. This was overcome in the present chapter by using silicic acid column chromatography (see Chapter 2) in which AA can be eluted with a high (approximately 90%) recovery rate from samples with greater consistency.

The question of the phospholipid substrate specificity of the enzyme involved in the initial phase of release is also addressed in the present chapter. This gives additional information regarding phospholipid turnover involvement in signal transmission in this system.

RESULTS

The method of silicic acid column chromatography was widely used in this chapter for measurement of AA liberation. As such, it was important to characterise the elution profile of AA from these columns using a solvent system of petroleum ether (60–80°) with an increasing concentration (up to 4%) of diethyl ether. A step–by–step elution profile is shown in Figure 4.1 with the AA being eluted from the column in 4% diethyl ether. Methanol could be added after the diethyl ether to elute any phospholipids in the samples. Any remaining free AA was usually associated with this eluant. Various attempts were made to increase total AA recovery by using different percentages of diethyl ether in methanol before using methanol alone. This, however, made little difference and recovery remained approximately 90%. Columns were used as described in Chapter 2.

Addition of bombesin to Swiss 3T3 cells caused the rapid release of AA from prelabelled cells (Chapter 3). To determine the kinetics of AA liberation in response to bombesin, measurements of intracellular AA were made over a timecourse with short stimulation periods. AA was significantly elevated after a 2 sec stimulation, maximal between 10–30 sec and had returned to basal by 5 min (Fig.4.2). In the experiment shown in Figure 4.2, the maximum increase in AA release was 1.3–fold after 10 sec; the mean increase in release over 8 independent experiments was 3.4 ± 0.4–fold. Figure 4.3 shows that the response was dose–dependent with an EC_{50} value of 2.7 ± 0.6nM.

The phospholipid source of the AA was examined by determining release of the fatty acid from various phospholipids. Figure 4.4 shows that AA was incorporated into PtdCho, PtdIns, PtdEtn and PtdOH with virtually no label being detected in other phospholipids (see Chapter 3). No attempt was made to resolve the inositol phospholipids. The timecourse shown in the figure suggests that bombesin stimulated the release of [^{3}H]AA only from PtdCho and that this was a very rapid event, evident between 2–5 sec and significant after 10 sec ($p=0.013$). The dpm

released for PtdCho was approximately equal to the amount of free AA measured. In a representative experiment, after a 5 sec stimulation, 4783 dpm was lost from PtdCho and 4977 dpm found in free AA. Stimulation with the agonist resulted in a significant increase in [^{3}H]AA–labelled PtdOH ($p=0.006$ after 30 sec). This could be due to either bombesin–stimulated PLC catalysed PtdIns (4,5)P_2 hydrolysis followed by the phosphorylation of DAG by diacylglycerol kinase, or PLD catalysed PtdCho breakdown, both of which have been demonstrated in Swiss 3T3 cells (Cook & Wakelam, 1989; Cook *et al.*, 1990).

To assess whether PLA_2 activation might provide the mechanism for mobilisation of AA in response to bombesin, it was necessary to show that both AA release and lysoPtdCho production occurred over the same timecourse. As described in Chapter 3, to achieve this, cells were labelled with both [^{14}C]AA and [^{3}H]choline. Bombesin stimulated lysoPtd[^{3}H]Cho as determined by thin layer chromatography showed a very rapid increase after 2 sec (almost 3–fold) with peak production after 5–10 sec (Fig.4.4b). The timecourse of loss of [^{14}C]AA label from PtdCho was the same as that for lysoPtdCho generation (Fig.4.5).

The incubation medium in which the above experiments were performed, contained calcium at a concentration of 1.26mM. To determine whether bombesin–stimulated AA production required the influx of calcium, which then activated PLA_2, the effect of reducing extracellular calcium to approximately the intracellular concentration (150nM) on bombesin–stimulated AA release was examined (Fig.4.6). Inclusion of EGTA (0.33mM) in the Hanks buffered saline had no significant effect on AA release (2.9–fold stimulation in normal [Ca^{2+}] c.f. 2.8–fold in low [Ca^{2+}] after 10 sec; values are means obtained from 3 separate experiments). Whilst ruling out a role for Ca^{2+} influx in bombesin–stimulated AA release, these experiments cannot address the possibility that bombesin–stimulated release of intracellular stored calcium could be involved in activation of PLA_2. Swiss 3T3 cells were therefore treated with thapsigargin at a concentration reported to rapidly elevate intracellular calcium levels as

a consequence of inhibition of the endoplasmic reticulum Ca^{2+}–ATPase (Thastrup *et al.*, 1990). No effect of the tumour promoter was observed upon AA release in control cells. Thapsigargin did potentiate the response to bombesin, but only after a total incubation time of greater than one minute (Fig.4.7). Experiments were, therefore, performed to check that thapsigargin had indeed stimulated an increase in intracellular calcium. Figure 4.8 shows that stimulation of Swiss 3T3 cells with bombesin or thapsigargin caused an increase in intracellular calcium. However, there were clear differences between the two responses. 100nM bombesin stimulated an almost immediate increase in calcium which reached 10% of peak after 0.42 ± 0.08 sec and maximum intracellular calcium release was achieved after 6.0 ± 2.6 sec (mean of 3 experiments), 1μM thapsigargin, on the other hand, only stimulated an increase in intracellular calcium after 6 sec with 10% of peak being reached after 6.4 ± 1.25 sec and maximal after 22 ± 2.6 sec (mean of 3 experiments).

As was shown in Chapter 3, the release of AA in response to bombesin at later time points (longer than 10 min) is sensitive to calcium depletion, with the stimulated release being reduced by at least 50% after 30 min in low Ca^{2+}. This sensitivity to calcium has been seen in previous studies in other cell types (Kanterman *et al.*, 1990), which have examined release of AA only at later time points and have only taken eicosanoid release from cells into account. As previously mentioned, the possibility of different mechanisms of AA release, with different regulatory requirements, existing within the same cell is an important consideration that has been ignored in many previous studies.

PLA_2 has been reported to be regulated by PKC activity in several systems (Pfannkuche *et al.*, 1989). To explore the possibility that this very rapid release of AA was dependent upon prior activation of PLA_2 by PKC, the enzyme was either down–regulated by chronic PMA pretreatment, or inhibited by a 15 min pretreatment with staurosporine (10^{-6}M). Cells pretreated with PMA, or its inactive analogue

β–phorbol, showed no differences in bombesin–stimulated AA release at the early time points measured (Fig.4.9). These results are supported by data obtained with cells pretreated with the PKC inhibitor, staurosporine (Fig.4.10) where no difference was observed in bombesin–stimulated AA release measured between untreated cells and cells treated with the inhibitor.

Figure 4.1 Arachidonate elution profile from silicic acid columns

[^{3}H]AA (0.1μCi) prepared in 1ml petroleum ether/4% diethyl ether was added to petroleum ether–washed columns and eluted in step–by–step additions as shown. The results are from a typical experiment where n=3.

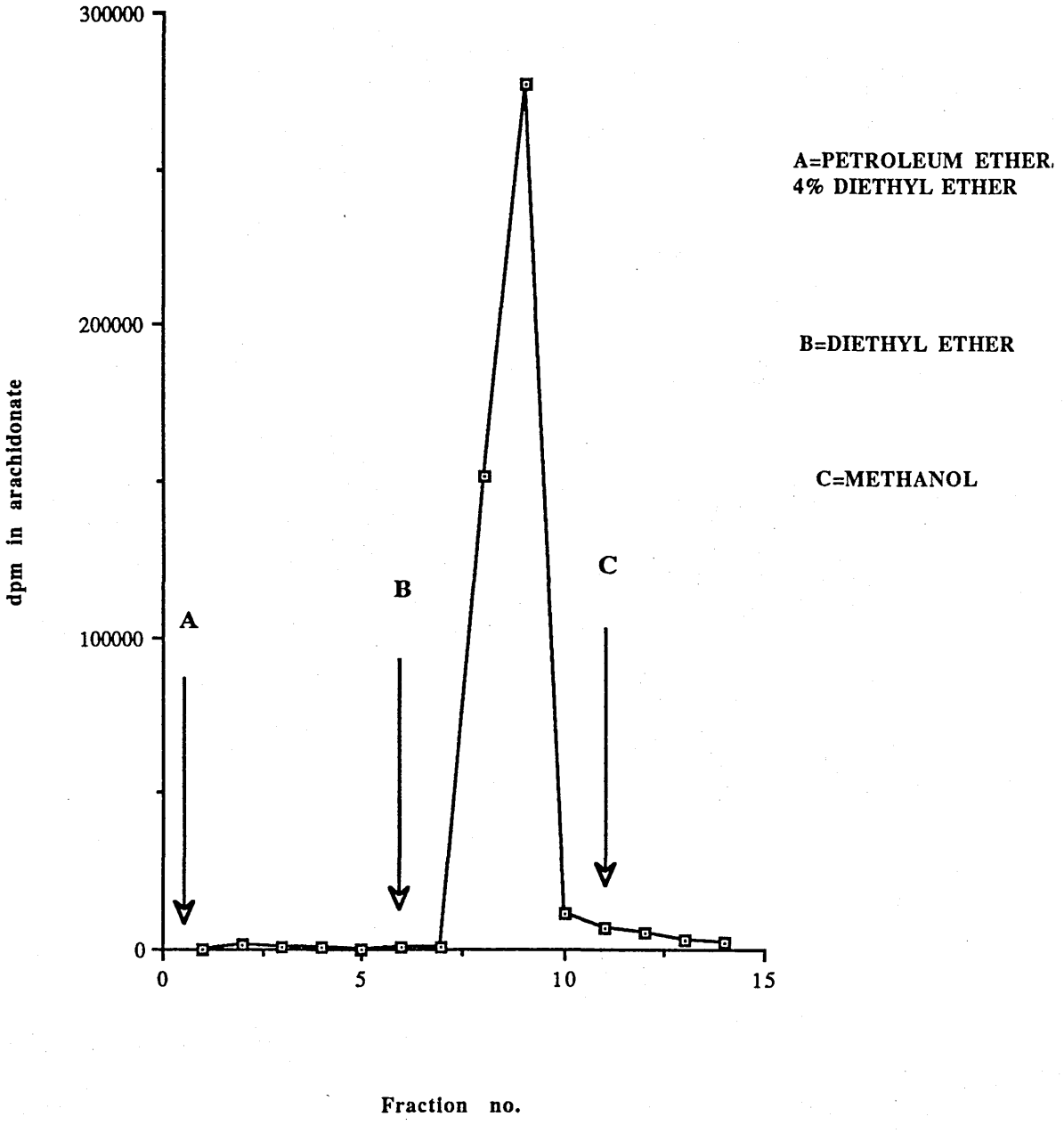

300000
200000
100000
0
dpm in arachidonate
A
B
C
0
5
10
15
Fraction no.
A=PETROLEUM ETHER.
4% DIETHYL ETHER
B=DIETHYL ETHER
C=METHANOL

Figure 4.2 Timecourse of bombesin-stimulated arachidonate generation

Swiss 3T3 cells, treated as described in the Materials and Methods chapter, were radiolabelled and stimulated with 617nM bombesin (●) or treated with HBG (O) for the stated times. AA was measured using silicic acid column chromatography as described. the results are expressed as means ± S.D. from a single typical experiment where n=3.

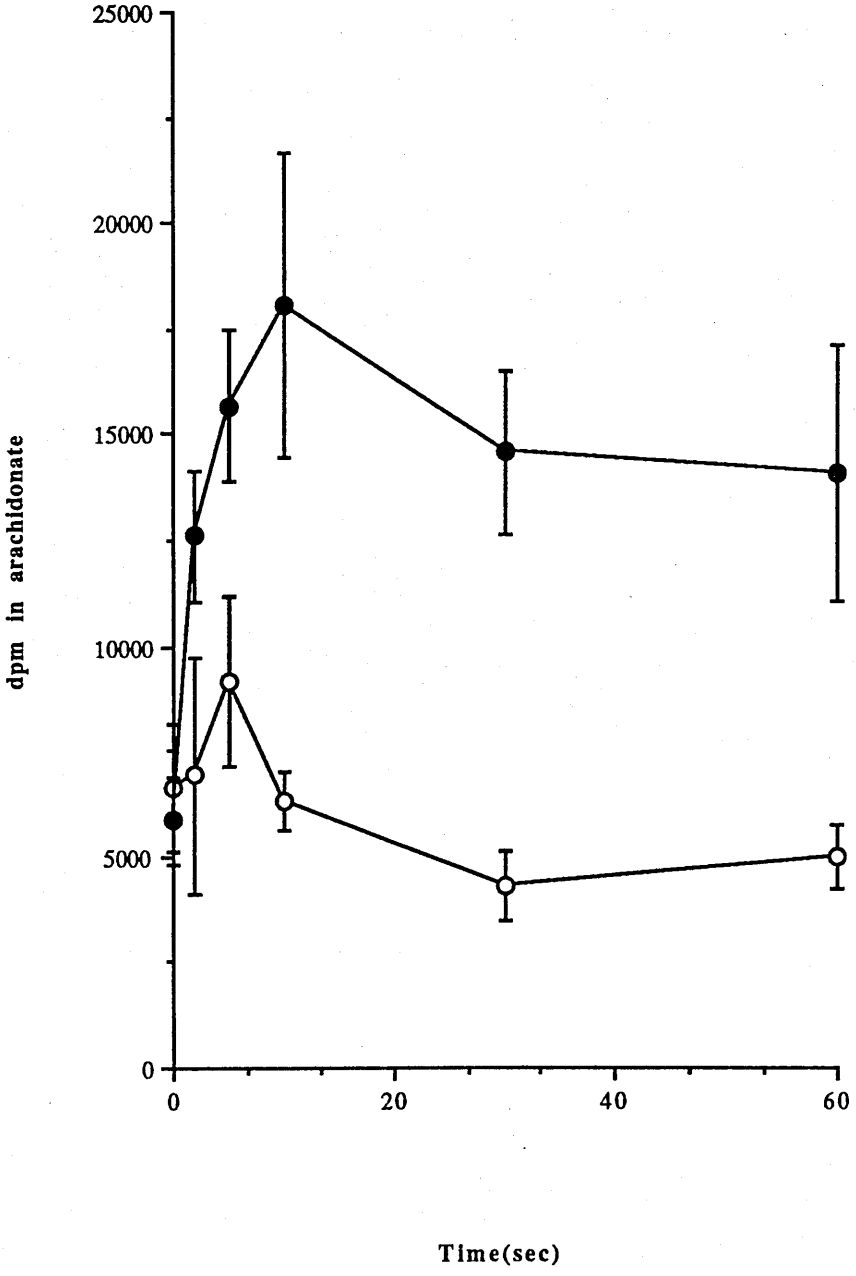

25000
20000
15000
10000
5000
0
dpm in arachidonate
0
20
40
60
Time(sec)

Figure 4.3 Dose–dependence of bombesin–stimulated arachidonate generation

[^{3}H]arachidonate labelled Swiss 3T3 cells were stimulated with increasing concentrations of bombesin for 20 sec. The results are expressed as means ± S.D. from a single typical experiment where n=3.

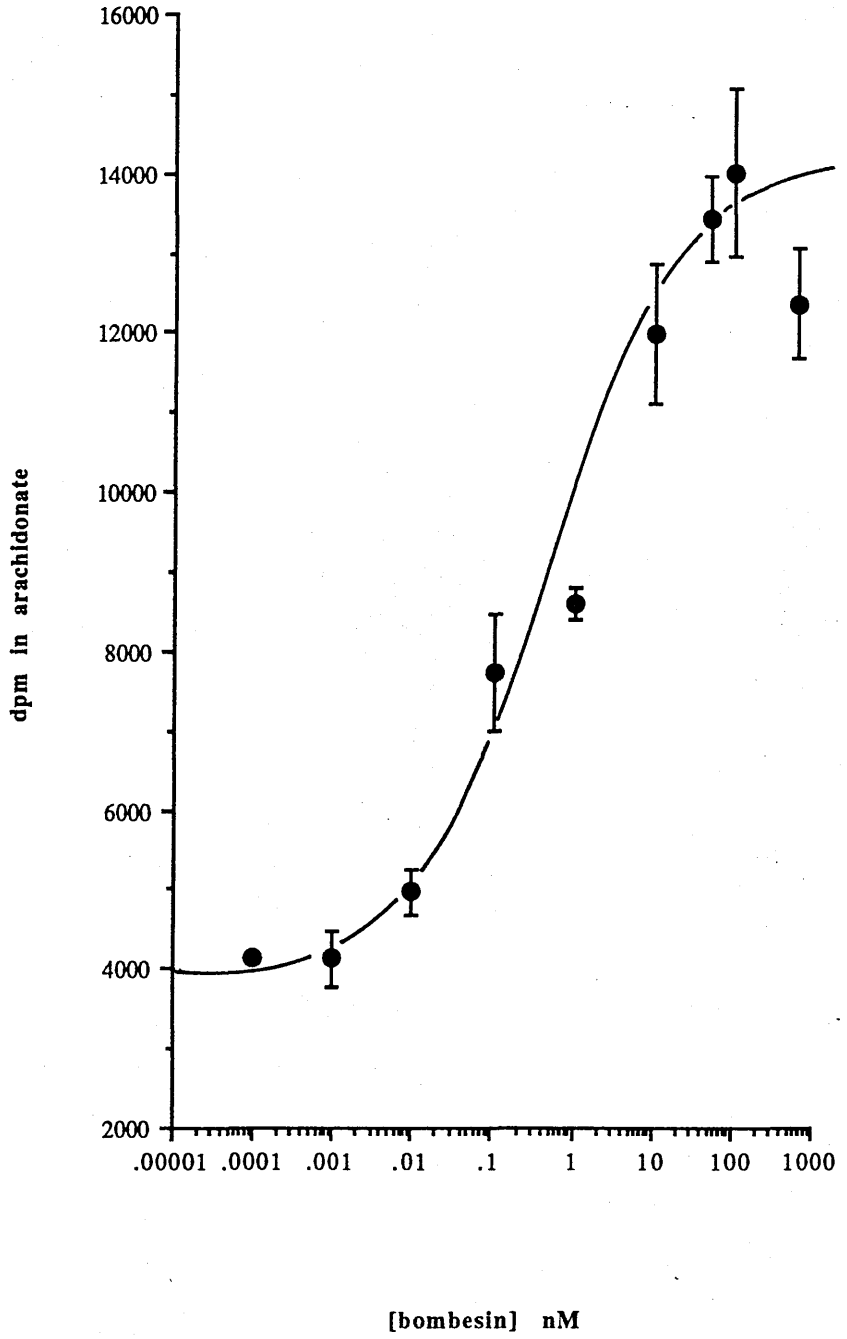
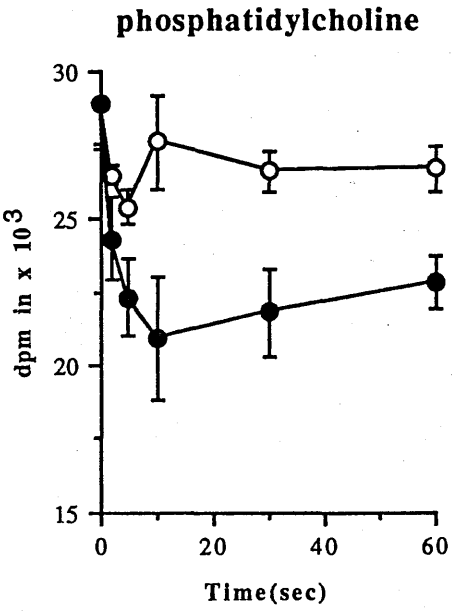
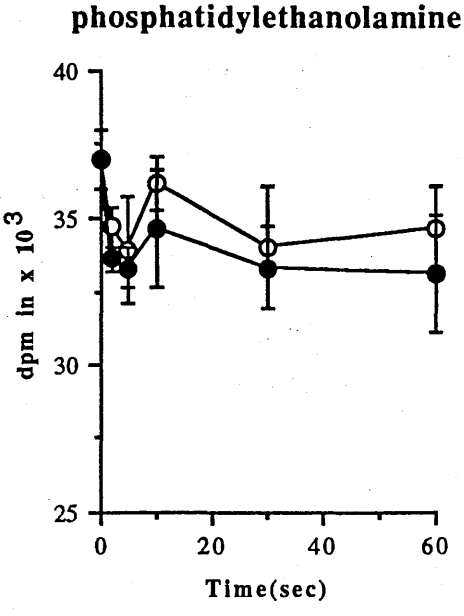
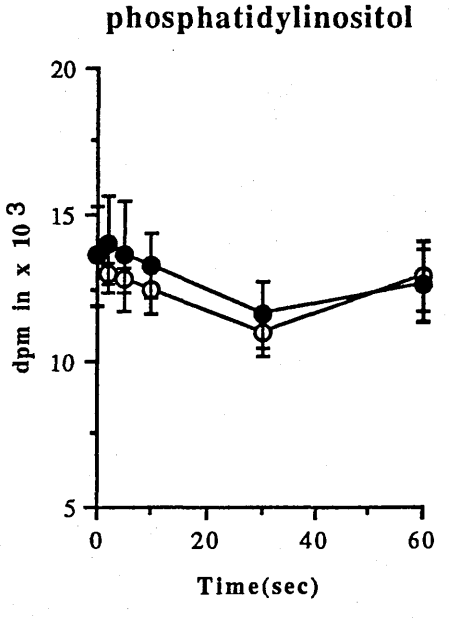

16000
14000
12000
10000
8000
6000
4000
2000
dpm in arachidonate
.00001 .0001 .001 .01 .1 1 10 100 1000
[bombesin] nM

Figure 4.4 Bombesin-stimulated changes in the [^{3}H]arachidonate labelling of Swiss 3T3 cells

Swiss 3T3 cells, labelled with [^{3}H]arachidonate, were incubated with either HBG (O) or with 617nM bombesin (●) for the required times. Cellular phospholipids were extracted as described. Changes in the arachidonate content of PtdCho, PtdEtn, PtdIns and PtdOH in response to this treatment were detected using thin layer chromatography. Results are expressed as means ± S.D. of 4 experiments in which n=3 in each case.


phosphatidylcholine
30
25
20
15
dpm in x 10^3
0
20
40
60
Time(sec)


phosphatidylethanolamine
40
35
30
25
dpm in x 10^3
0
20
40
60
Time(sec)


phosphatidylinositol
20
15
10
5
dpm in x 10^3
0
20
40
60
Time(sec)

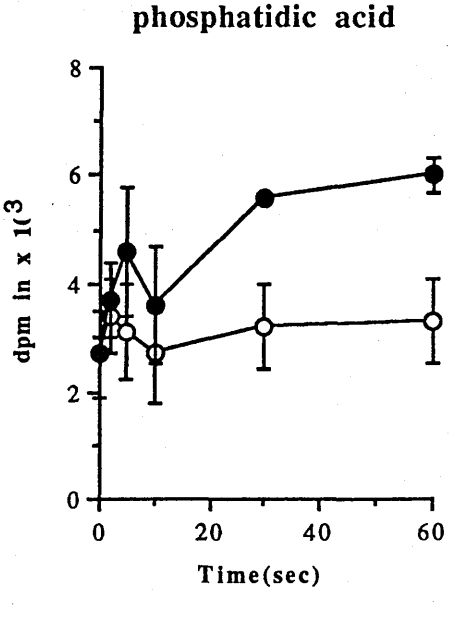

phosphatidic acid
8
6
4
2
0
dpm in x 10^3
0
20
40
60
Time(sec)

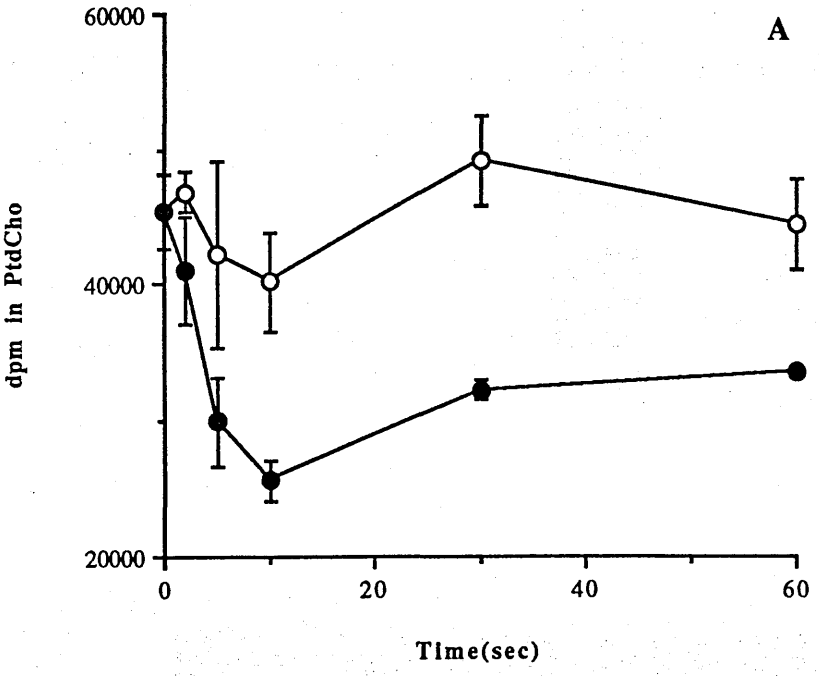
Figure 4.5 Bombesin–stimulated arachidonic acid release from phosphatidylcholine and lysophosphatidylcholine formation

Cells were double labelled with [^{3}H]choline and [^{14}C]arachidonate as described. PtdCho and LysoPtdCho were detected using thin layer chromatography.

(a) [^{14}C]arachidonate release from PtdCho in response to bombesin 617nM (●)

(b) LysoPtd[^{3}H]Cho formation in response to bombesin (617nM) (●) or HBG (O).

Results are expressed as means ± S.D. from a typical experiment where n=3.


A
60000
40000
20000
dpm in PtdCho
0
20
40
60
Time(sec)

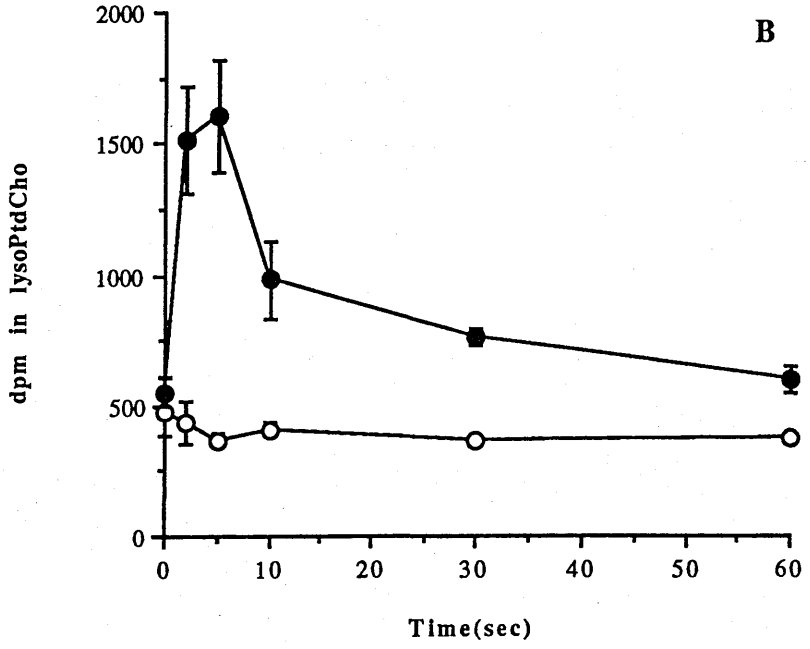

B
2000
1500
1000
500
0
dpm in lysoPtdCho
0
10
20
30
40
50
60
Time(sec)

Figure 4.6 Effect of depleting extracellular calcium on bombesin–stimulated arachidonate release

[^{3}H]arachidonate labelled cells were washed and stimulated with 617nM bombesin in medium containing 1mM calcium (O) or medium in which the calcium had been buffered to 150nM with EGTA (●). Arachidonate was measured as described. Results are expressed as means ± S.D. from a typical experiment where n=3.

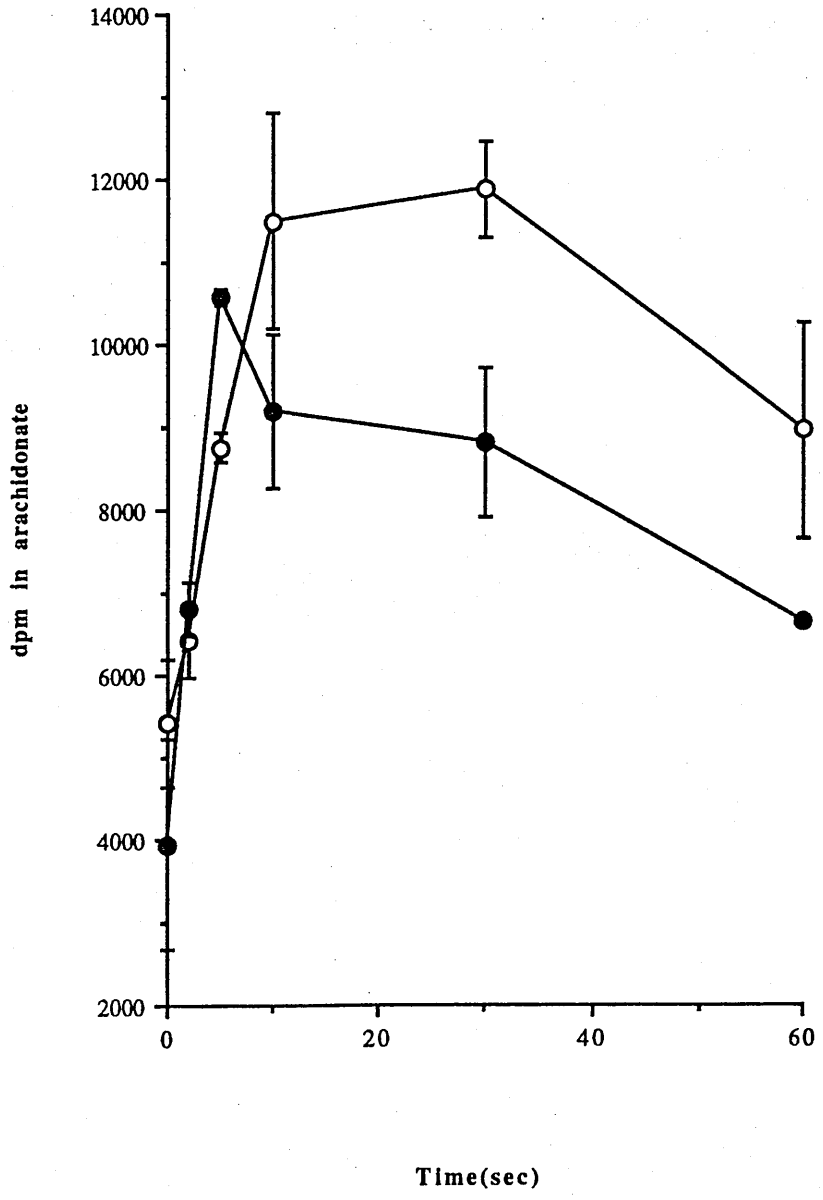

14000
12000
10000
8000
6000
4000
2000
0
20
40
60
dpm in arachidonate
Time(sec)

Figure 4.7 Effect of thapsigargin upon arachidonate release

Swiss 3T3 cells, labelled with [^{3}H]arachidonate, were washed in HBG and then the appropriate samples were preincubated with (□, ■) or without (O, ●) thapsigargin (1μM) for the indicated times. The cells were then stimulated for 20 sec with (●, ■) or without (○, □) bombesin (617nM) and arachidonate generation determined using silicic acid column chromatography. Results are expressed as means ± S.D. from a single typical experiment where n=3.

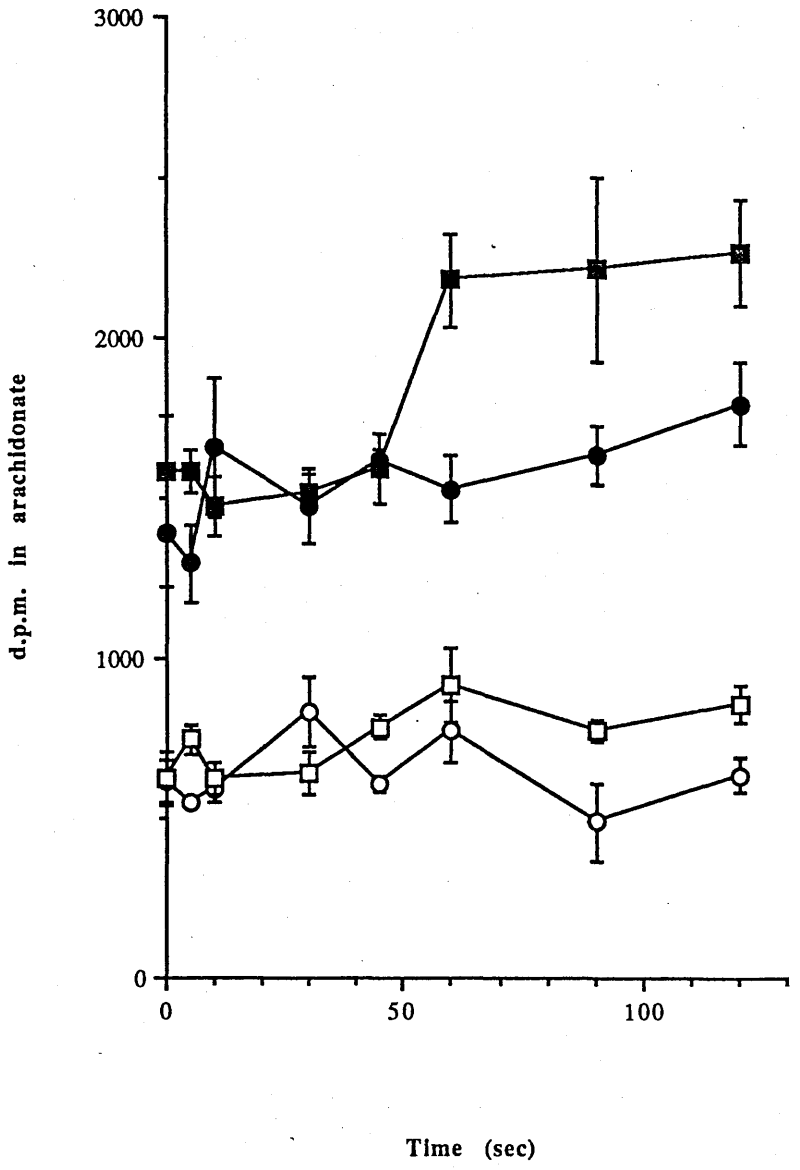
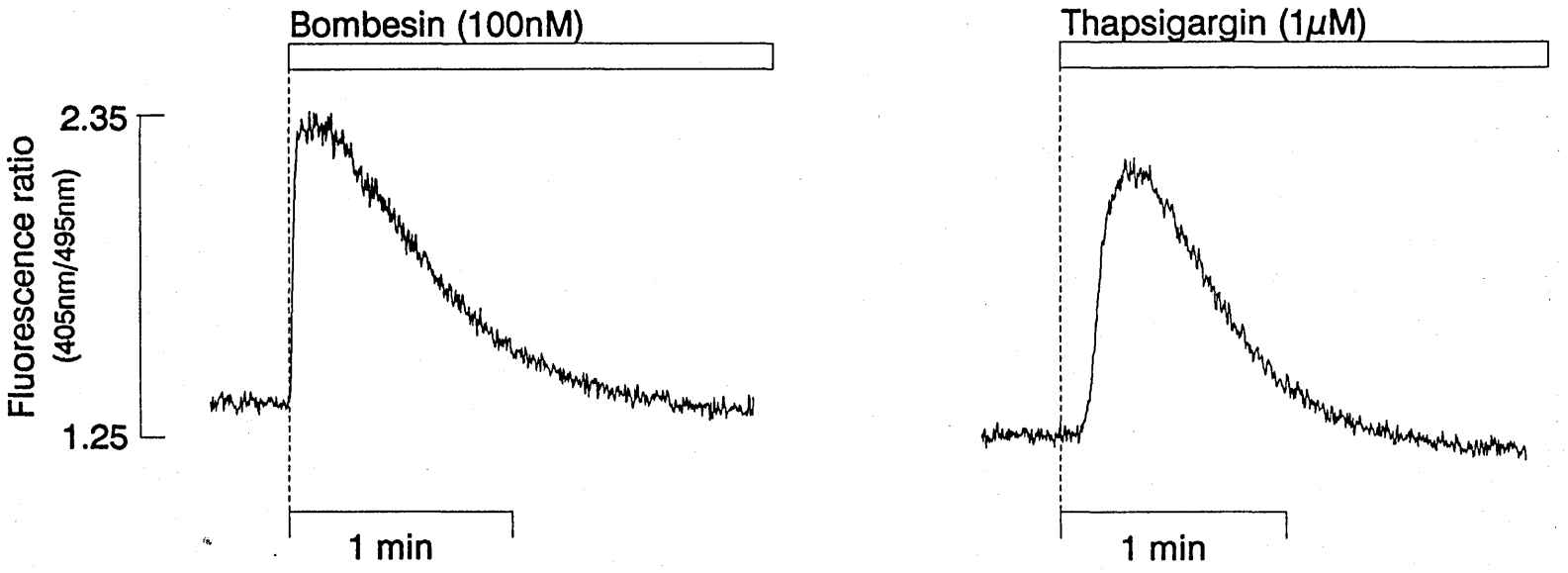

3000
2000
1000
0
0
50
100
d.p.m. in arachidonate
Time (sec)

Figure 4.8 Stimulation of changes in intracellular calcium by bombesin and thapsigargin

Swiss 3T3 cells, grown on gelatin coated coverslips were loaded with Indo–1–AM and stimulated with either 100nM bombesin or 1μM thapsigargin. Changes in $[Ca^{2+}]$ as indicated by the fluorescence ratio were measured as described in the Methods section. The results are from a single experiment typical of three.


Bombesin (100nM)
Thapsigargin (1μM)
Fluorescence ratio
(405nm/495nm)
2.35
1.25
1 min
1 min

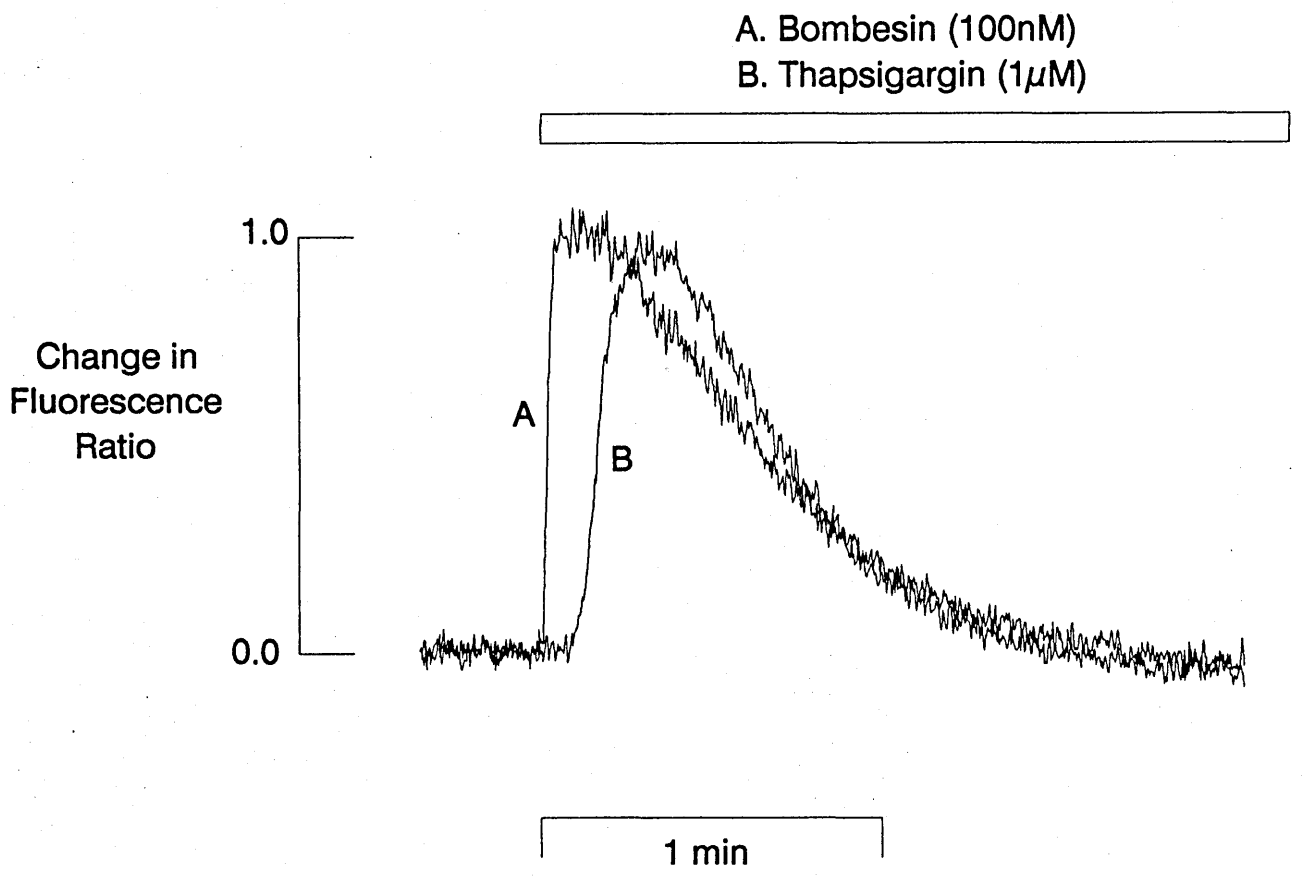

A. Bombesin (100nM)
B. Thapsigargin (1μM)
1.0
0.0
Change in
Fluorescence
Ratio
A
B
1 min

Figure 4.9 Effect of long–term PMA pretreatment upon bombesin–stimulated arachidonate release

Swiss 3T3 cells were incubated with 300nM PMA for 48h prior to the experiment. Controls involved cells treated with 300nM β–phorbol, DMSO or ethanol. Stimulation with bombesin (617nM) was performed as previously. Cells treated with PMA (●) are compared with untreated cells (O). Results are expressed as means ± S.D. from a single typical experiment where n=3.

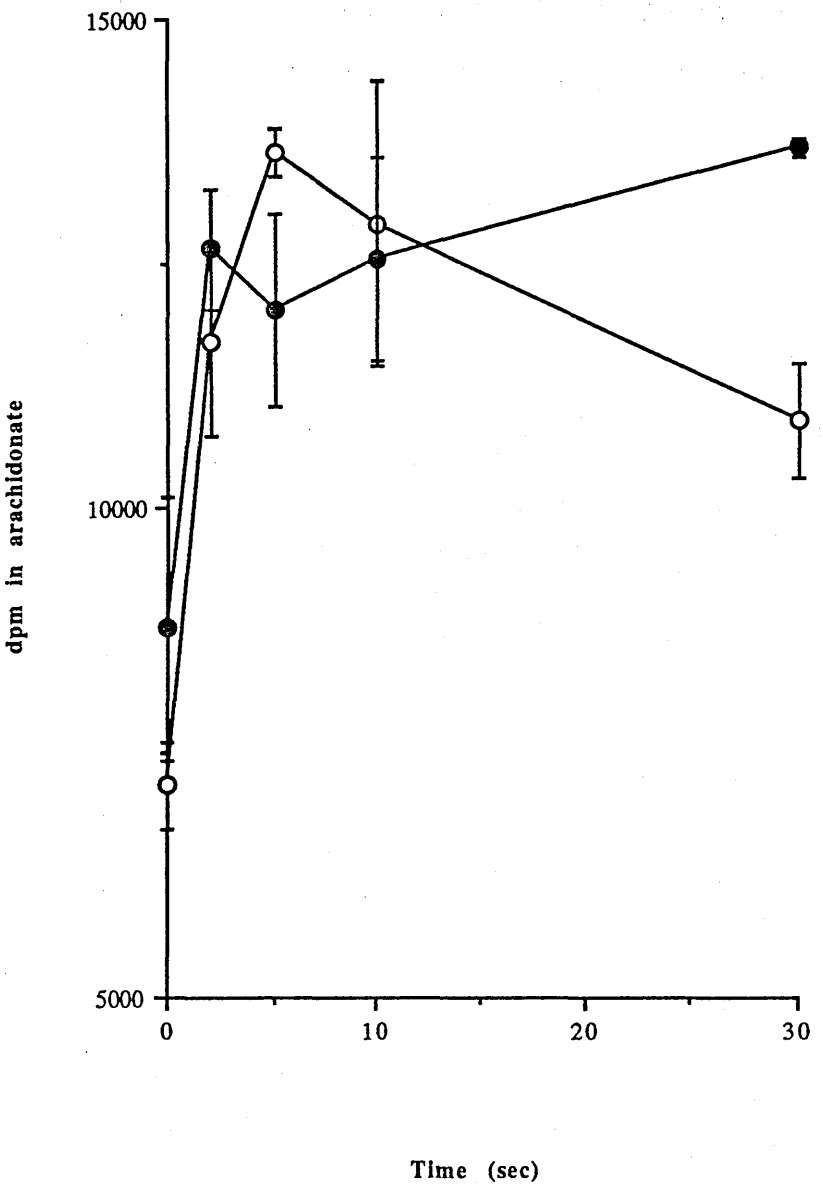
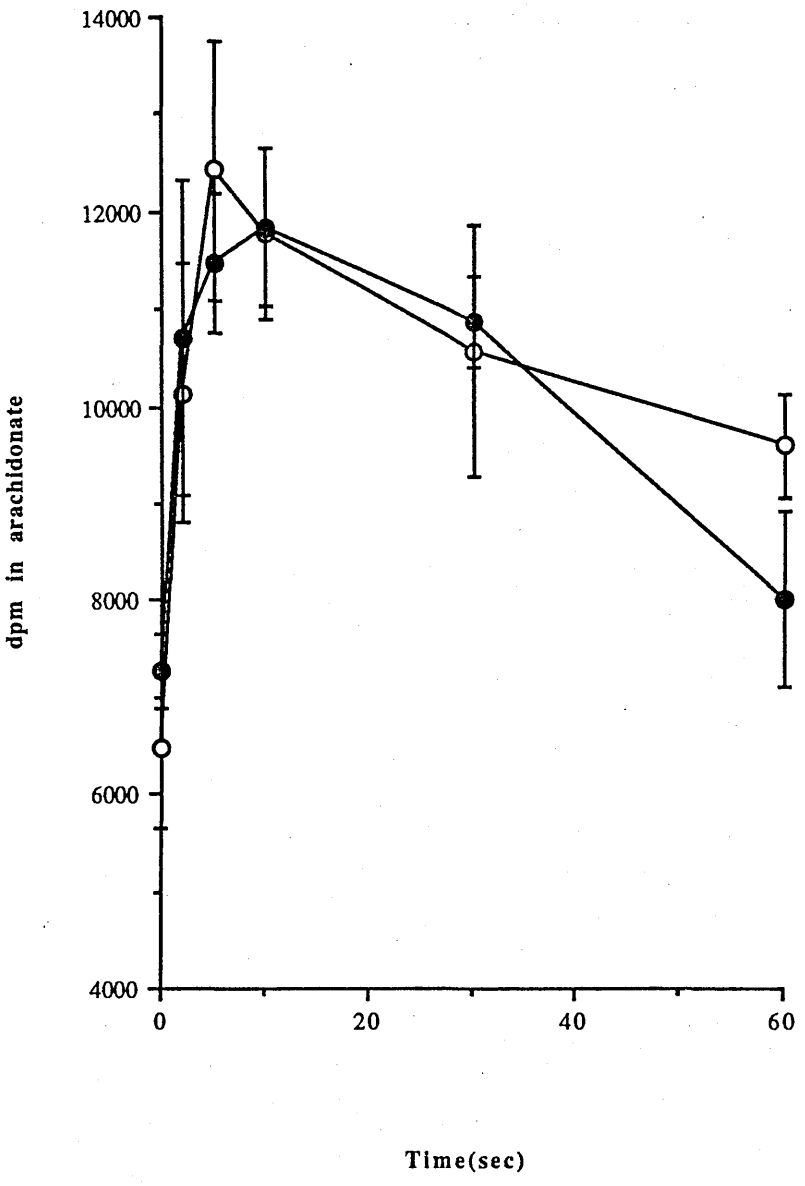

15000
10000
5000
0
10
20
30
dpm in arachidonate
Time (sec)

Figure 4.10 Effect of staurosporine treatment on bombesin–stimulated arachidonate release

Swiss 3T3 cells, labelled with [^{3}H]arachidonate, were incubated with staurosporine (10^{-6}M) for 15 min prior to stimulation with 617nM bombesin for 20 sec. Arachidonate release was measured in cells pretreated with staurosporine (●) and in untreated cells (O). Results are expressed as means ± S.D. from a single typical experiment where n=3.


14000
12000
10000
8000
6000
4000
dpm in arachidonate
0
20
40
60
Time(sec)

DISCUSSION

The results presented in this chapter were mainly derived using silicic acid column chromatography. The step–by–step elution profile shown in Figure 4.1 illustrates that AA is eluted during the addition of diethyl ether. This method was improved by using batch elution with the AA–containing fraction collected in 3ml of diethyl ether. The 'purity' of this fraction was checked by running it on a thin layer chromatography system and scraping the entire lane. This method proved to be more sensitive and convenient for detecting free AA than other tested thin layer chromatography methods.

Results using this technique demonstrate that bombesin stimulates the rapid generation of AA in Swiss 3T3 cells (Fig.4.2). Examination of the cellular phospholipids demonstrated that the fatty acid was derived only from PtdCho (Figs.4.4 & 4.5) and that there was a corresponding increase in LysoPtdCho which paralleled the decrease in [^{3}H]AA labelling of PtdCho. These experiments thus demonstrate that a phospholipase of the A class, presumably PLA_2, with a substrate specificity for PtdCho is stimulated in Swiss 3T3 cells by bombesin. A PtdCho–specific PLA_2 has been reported in human platelets (Ballou *et al.*, 1986), whilst a 110Kd PLA_2 which is activated by physiologically relevant increases in intracellular calcium (Clark *et al.*, 1990; Gronich *et al.*, 1990) and by PMA or hormonal treatment (Gronich *et al.*, 1988) has been suggested to be present in U937 cells. However, this enzyme species does not show specificity for PtdCho over PtdEtn. A recent study by Clark *et al.* (1990) has reported the cloning and expression of a cDNA encoding an 85Kd cytosolic PLA_2 from U937 cells. This preferentially hydrolyses AA–containing PtdCho rather than PtdCho with other fatty acids in the sn–2 position. Although at present it is uncertain whether this PLA_2 distinguishes between different classes of phospholipids in a natural membrane, it has been suggested that it may be important in initiating the synthesis of the inflammatory mediator, Platelet Activating Factor (PAF), since studies have shown that the most likely phospholipid precursor for PAF is an

arachidonyl PtdCho (Suga *et al.*, 1990). Clark *et al.* (1991) suggest that future studies will involve the determination of the head group specificity of this cytosolic PLA_2 when incubated with natural membranes. The existence of enzymes which exhibit different substrate specificities may increase the scope with which a cell can respond to stimulation and will thus lead to increased diversity in the overall response.

Whilst the generation of AA was sustained over a 1 min period (Fig.4.2), that of LysoPtdCho was more transitory (Fig.4.5). Low levels of LysoPtdCho were also observed in Chapter 3 and are not surprising since its detergent properties would be harmful to cell membranes. The rapid turnover of the lysophospholipid may be mediated through combinations of deacylation, reacylation or transacylation. In some cells, lysophospholipase activity has been shown to be greater than phospholipase activity, which accounts for a relatively low concentration of lysophospholipid even in the presence of a raised PLA_2 activity (Meade *et al.*, 1986). In bombesin–stimulated Swiss 3T3 cells, there is no detectable increase in glycerophosphocholine (Cook & Wakelam, 1989), suggesting LysoPtdCho is not further degraded but, rather, reacylated in these cells.

A stimulation of AA release by PLA_2 activation has been observed in many systems in response to a range of agonists (Axelrod *et al.*, 1988). However, the increase in fatty acid release has generally been detected after at least 1 min. As can be seen from Figure 4.2, bombesin–stimulated AA release was observed after 2 sec. This result would suggest that the activation of PLA_2 is closely coupled to receptor occupation.

The rapidity of AA release compared to reports in the literature was probably due to the work reported here involving the measurement of cell associated rather than incubation medium AA. Measurement of the release of AA into the extracellular medium, as in Chapter 3, does not give an accurate determination of the kinetics of phospholipase activation. For this, it is essential that intracellular, or cell associated AA is measured.

Agonist–stimulated PLA_2 activity has been proposed to be a consequence of an increase either in intracellular free calcium concentration (Kanterman *et.al.*, 1990) or in PKC activity (Pfannkuche *et.al.*, 1989) resulting from prior stimulated PtdIns $(4,5)P_2$ breakdown. Whilst the direct relationship between PLC and PLA_2 has not been investigated in this work, the rapid generation of AA (Fig.4.2) is at least as fast as that of sn–1,2–DAG and Ins $(1,4,5)P_3$ formation previously observed in the Swiss 3T3 cell in response to bombesin stimulation (Cook *et.al.*, 1990). This observation would question a role for PtdIns $(4,5)P_2$ hydrolysis in stimulating PLA_2. However, in order to address this possibility in more detail the effects of modulating PKC activity and intracellular calcium concentration upon bombesin–stimulated AA generation were examined.

Incubation of Swiss 3T3 cells in a Ca^{2+} EGTA buffer, in which the free calcium concentration was maintained at 150nM, was without effect upon bombesin–stimulated AA release (Fig.4.6). Consequently, agonist–stimulated entry of the ion is not required for the rapid activation of PLA_2. In order to examine if a change in intracellular calcium levels, as a results of $Ins(1,4,5)P_3$–stimulated release, had a role in bombesin–stimulated AA release, the cells were stimulated with thapsigargin. This has been shown to raise the intracellular calcium concentration independently of phosphoinositide metabolism and PKC activation (Jackson *et.al.*, 1988). This effect has been attributed to inhibition of the endoplasmic reticulum Ca^{2+}–ATPase (Thastrup *et.al.*, 1990). Thapsigargin increased intracellular calcium in Swiss 3T3 cells (Fig.4.8) reaching 10% of peak after about 6 sec. However, as shown in Figure 4.7, thapsigargin was unable to stimulate PLA_2 activity. The tumour promoter was able to potentiate bombesin–stimulated AA release, but only after a total of 80 sec exposure, suggesting that an increase in intracellular calcium was not the mechanism whereby bombesin stimulated rapid i.e. within 2 sec generation of AA. Additionally, experiments where cells were treated with PMA for 15 min prior to agonist addition showed inhibition of bombesin–stimulated Ins $(1,4,5)P_3$ formation (Plevin *et.al.*,

1990), yet this treatment resulted in the potentiation of bombesin–stimulated AA generation (see Chapter 3, Fig.3.6). Together, these results strongly suggest that an Ins (1,4,5)P_3 stimulated intracellular increase in calcium concentration is not essential for rapid stimulation by bombesin of PLA_2 activity.

Experiments performed on cells in which PKC was either down–regulated by pretreatment with PMA for 48h, or inhibited by staurosporine, showed no significant difference in the bombesin–stimulated rapid release of AA compared with control cells (Figs.4.9, 4.10). Thus, there does not appear to be a role for PKC activation in the rapid stimulation of PLA_2, lending further support to the proposal that initial activation of the enzyme is a response to receptor occupation rather than being subject to regulation by other signals. This suggestion is supported by the finding that the EC_{50} for bombesin–stimulated AA generation, 2.7± 0.6nM (Fig.4.3), is very similar to those found for the stimulation of Ins (1,4,5)P_3 and DAG production (Cook *et.al.*, 1990) and choline generation (Cook & Wakelam, 1989) in the same cells.

If the activation of PLA_2 is coupled directly to receptor occupation, it is likely that a G–protein may mediate this interaction. The bombesin receptor has been shown to couple to at least one G–protein which is insensitive to cholera toxin and pertussis toxin (Fischer & Schonbrunn, 1988) and to couple the receptor to PLC (Plevin *et.all.*, 1990). G–protein regulation of PLA_2 has been suggested in a variety of cell types (Cockcroft & Stutchfield, 1989; Burch & Axelrod, 1987) although the G–proteins involved have not yet been identified. Since the EC_{50} values for bombesin–stimulated AA release (Fig.4.3), Ins (1,4,5)P3 and DAG production (Cook *et.al.*, 1990) and choline generation (Cook & Wakelam, 1989) are almost identical, stimulation of all three phospholipases (A_2, C and D) may occur through the activation of the same receptor. Whether this involves three separate G–proteins coupled to a single receptor, or whether isotypes of the bombesin receptor, with identical agonist affinities exist is uncertain, though none of these responses are sensitive to pertussis or cholera toxins. The identity of the putative G–protein(s) involved remains open to

speculation. However, the recent evidence suggesting the existence of a novel family of G proteins (G_q) increases the potential for involvement in numerous signalling pathways. A variety of roles have been suggested for this family including ion channel activation, PLA_2 activation (Strathmann & Simon, 1990) and PLC–β1 activation (Taylor *et al.*, 1991). It is possible that one or several of this G–protein family could be instrumental in coupling the bombesin receptor to the signalling systems mentioned. However, there is, as yet, no direct evidence for this.

The alternative suggestion, that there may be subtypes of the bombesin receptor, is also highly speculative. The receptor has been purified and cloned from Swiss 3T3 cells (Battey *et al.*, 1991) with no evidence for different subtypes existing within the same cell. However, there do appear to be differences in receptor types between systems (Van Schrenk *et al.*, 1989 and see Introduction) with different binding affinities for gastrin releasing peptide (GRP) and neuromedin B (NMB). Whether these subtypes would exhibit different coupling mechanisms to and regulatory requirements for the signalling pathways involved remains to be established.

CHAPTER 5

BOMBESIN STIMULATES THE RELEASE OF ARACHIDONATE FROM PERMEABILISED CELLS IN A GTP-DEPENDENT MANNER

INTRODUCTION

The ability of neurotransmitters, hormones and growth factors to bind to specific cell–surface receptors and elicit their effects via GTP–binding proteins is considerably widespread (see Chapter 1). Probably the most widely studied system in which involvement of a G protein has been implicated is the regulation of phosphoinositide hydrolysis by a PtdIns (4,5)P_2–specific PLC (Cockroft, 1987). Some of the most convincing evidence for this involves the capacity of GTP and its non–hydrolysable analogues to enhance the agonist–mediated accumulation of the products of hydrolysis in permeabilised cells. The ability to block this response in some, but not all, cell types by treatment of the cells with pertussis toxin suggests that there may be cell–specific G proteins involved.

In 3T3 cells, the bombesin receptor has been suggested to interact with specific GTP–binding proteins. In one case, but only when excessively over–expressed, the product of the N–ras gene (Wakelam *et al.*, 1986) and in another, a pertussis toxin substrate (Letteria *et al.*, 1986). More recently, Fischer & Schonbrunn (1988) demonstrated that exposure of Swiss 3T3 cells to pertussis toxin or cholera toxin did not affect either the affinity of membrane bombesin receptors for agonists or guanine nucleotide regulation of receptor binding. There appear to be multiple G proteins involved in the coupling of receptors to the regulation of PtdInsP_2 turnover and the latter report suggests that the G protein coupled to the bombesin receptor belongs to the pertussis toxin insensitive group.

That PLC activity in Swiss 3T3 cells is regulated by a specific G protein is well established, although the nature of the protein involved remains to be absolutely elucidated. A recent study by Taylor *et al.* (1991) has shown that members of the G_{11} class of α subunits are involved in PLC–β1 regulation. Others have also observed stimulation of partially purified PLC activity by purified brain G_q in the presence of fluoroaluminate (Smrcka *et al.*, 1991). Both PLC and G_q have widespread tissue distribution (Rhee *et al.*, 1989; Strathmann & Simon, 1990) so the pathway may be common to several eukaryotic tissues and species.

Indeed the G_q/G_{11} species has been detected in Swiss 3T3 cells (Kaur & Wakelam, unpublished data).

Involvement of G proteins in receptor stimulated PLA_2 activity has been suggested in a number of cell types (Murayama & Ui, 1985; Burch *et al.*, 1986; Burch & Axelrod, 1987; Teitelbaum, 1990). Unfortunately, research into this area has been hampered by the long–standing dogma that PLA_2 was activated only as a result of prior PLC activation (Axelrod *et al.*, 1988). Direct G protein regulation was thought to intervene only at the site of receptor–linked PLC stimulation. However, this has now been questioned. Evidence supporting a role for G proteins in PLA_2 activation ranges from stimulation of the enzyme by guanine nucleotides in 3T3 cells (Burch & Axelrod, 1987) and permeabilised rat basophilic leukaemia cells (Narasimhan *et al.*, 1990) to differential susceptibility to inhibition by pertussis toxin in neutrophils (Cockroft & Stutchfield, 1989). Although a range of G proteins have been characterised, the G proteins that are coupled to PLA_2 remain to be identified. Cholera toxin stimulates $G_{S\alpha}$ but has been shown to either stimulate (Burch *et al.*, 1988) or inhibit (Jelsema, 1987) PLA_2, depending on the receptor or cell type investigated. Pertussis toxin inhibits the functioning of G proteins of the i and o series (Gilman, 1987) and transducin (Stryer, 1986) but has been shown to inhibit or to stimulate PLA_2 (Burch *et al.*, 1988). It appears that there are multiple G proteins that are linked to PLA_2 activation. There may be several PLA_2 isozymes that have different calcium requirements, pH optima and intracellular locations and may have the ability to translocate from cytosol to membrane upon activation. (Clark *et al.*,1991). Some G proteins may directly couple a receptor to an effector PLA_2. However, others may modulate PLA_2 activity indirectly by stimulating calcium channel opening or by stimulating Ins (1,4,5)P_3–induced release of calcium from intracellular stores. It has even been suggested that PLA_2 may be under dual regulation by inhibitory and stimulatory G proteins, as for adenylate cyclase (Burch *et al.*, 1988).

The $\beta\gamma$ subunit of certain G proteins has also been implicated in the activation of PLA_2 (Jelsema & Axelrod, 1987; Kim *et al.*, 1989). This potentially novel mechanism has raised considerable controversy. Whether the $\beta\gamma$ subunit is active upon dissociation from α has yet to be convincingly demonstrated. However, this does raise the possibility of more extensive variation in the G protein regulation of PLA_2 and PLC. It also raises the possibility of both phospholipases being regulated by the same G protein but different subunits which would explain a number of observations concerning phospholipase activation in the same cell type.

Work presented in this chapter attempts to demonstrate the involvement of a GTP–binding protein in the initial AA response to bombesin described in Chapter 4. In addition, the correlation between this putative G protein and that involved in bombesin–stimulated PLC activation is also explored, with regard to further dissociating between the two pathways.

RESULTS

In order to examine whether analogues of GTP have an effect on the AA response to bombesin, an effective permeabilised cell system had to be established. Previous work in Swiss 3T3 cells had shown the effective use of electroporation in examining the stimulation of $InsP_3$ formation by bombesin (Plevin *et al.*, 1990). It was therefore decided to use this method for examining the generation of AA. Figure 5.1 shows a timecourse of AA release in response to bombesin, GTPγS and the two agents together. This represents a typical experiment where n=6. No AA release was detected in response to any of the stimuli tested over a period of 10 min, by which time the first phase of release is over in whole cells. Electroporation involves using cells in suspension whereas all previous experiments were performed on a confluent monolayer. This lack of response may be due to increased basal rates of AA release in the suspended cells, making stimulated levels appear negligible. Conceivably, detaching cells from dishes with a calcium–free enzyme–containing medium or by scraping with a rubber policeman (both of which were employed here), followed by several cycles of centrifugation and suspension of cells may damage the surface of the cells such that non–specific leakage of cellular metabolites may occur affecting the coupling between membrane receptors and the effector system under study. Thus, employment of the cell monolayer may be indispensible for studying the small effects of agonists on AA release in permeabilised Swiss 3T3 cells.

Various methods are available for permeabilising cells grown as a monolayer, which involve addition of either a detergent (Martin & Michaelis, 1990) or a bacterial toxin (Howell & Gomperts, 1987) in a suitable permeabilisation buffer to the cells. This eliminates any mechanical disruption and damage to the monolayer. It was decided to examine the effects of streptolysin-O on Swiss 3T3 cells. This bacterial toxin has been used to permeabilise a variety of cell types (Howell & Gomperts, 1987; Stutchfield & Cockroft, 1988) and will generate lesions in cholesterol–containing membranes of up to 12nm. These lesions are

small enough not to allow toxin entry and permeabilisation of intracellular membranes but are large enough to allow access of nucleotides for studies such as those described here.

Experiments were performed initially to check that streptolysin–O caused sufficient permeabilisation of Swiss 3T3 cells. The release of the cytosolic protein lactate dehydrogenase (LDH) was monitored in streptolysin–O treated cells. Figure 5.2 shows the relationship between increasing concentrations of the toxin and the activity of LDH in the medium. Maximal LDH activity was achieved at 0.6U/ml streptolysin–O. This is slightly higher than the concentration quoted by Howell & Gomperts (1987) of 0.4U/ml, and was the concentration used in subsequent experiments. This concentration caused approximately 100% permeabilisation of 3T3 cells as examined by entry of the fluorescent dye, bisbenzimide.

It was then essential to determine the response of the cells to bombesin and to different analogues of GTP. Initially, the generation of $InsP_3$ was examined in streptolysin–O permeabilised cells. Figure 5.3 shows a timecourse of $InsP_3$ production in response to bombesin and GTPγS. The response to bombesin is slow and small as is that to GTPγS alone. The response to GTPγS shows an obvious lag time before onset of $InsP_3$ release. Under normal resting conditions, the rate of exchange of GDP for GTP is very slow. This rate is accelerated by the addition of an agonist. Thus, when both bombesin and GTPγS were added together, there was a significant potentiation of the response. In addition, the response was maintained at potentiated levels for up to 1 min and possibly longer. A similar potentiation was observed for $InsP_2$ formation and for $InsP_1$, although to a smaller extent. These results are comparable with those obtained in electropermeabilised cells. The fold–stimulation achieved with bombesin alone and with bombesin and GTPγS together in streptolysin–O permeabilised cells was almost identical to that seen in electropermeabilised Swiss 3T3 cells. Figure 5.4 provides additional evidence that the inositol phosphate response remains intact in

streptolysin–O permeabilised cells. Figure 5.4(a) shows a dose–response curve demonstrating the effect of GTPγS on inositol phosphate production in bombesin–stimulated cells. Significant potentiation (p=0.012) was observed in the presence of GTPγS. The EC_{50} value for bombesin was slightly decreased by the presence of GTPγS (control = 1.2 ± 0.3nM; +GTPγS = 0.3 ± 0.18nM). The EC_{50} for the peptide decreased only marginally after streptolysin–O treatment (intact cells = 1.65 ± 0.4nM) suggesting receptor integrity had been maintained. In addition, the response was significantly (p=0.009) decreased by pre–incubation of the permeabilised cells with GDPβS at millimolar concentrations (Fig.5.4b)

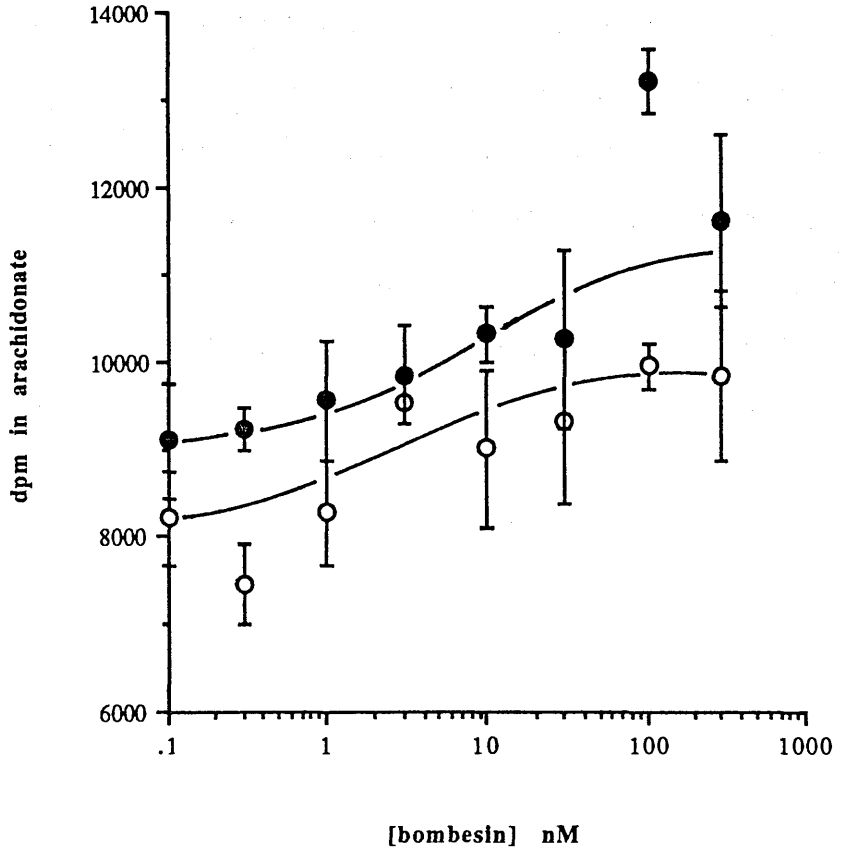
Similar experiments were performed to examine the effects of these non–hydrolysable analogues of GTP on AA generation. GTPγS, in the presence of bombesin, stimulated AA release in this system, whereas bombesin alone stimulated negligible release. The small level of release observed was probably due to some residual GTP remaining within the cell (Fig.5.5). This finding is similar to the results presented for $InsP_3$ and is consistent with a requirement for GTP to allow the receptor to transduce its message through G protein coupling to PLA_2. Figure 5.6a shows a dose–response curve demonstrating the effect of GTPγS on AA release. The EC_{50} value for the peptide in permeabilised cells is similar (3.2 ± 0.8nM) to that in whole cells. Figure 5.6b shows that 1mM GDPβS pretreatment inhibits the AA response and, as shown in Figure 5.7, totally inhibits the potentiation of the response achieved with GTPγS. These results are also consistent with the previous suggestion that a GTP–binding regulatory protein is involved in the coupling of bombesin receptors to PLA_2.

In a further effort to discriminate between the regulation of PLC and PLA_2 activity, the aminoglycoside antibiotic neomycin was used. In all experiments this reduced bombesin–stimulated $InsP_3$ production, though to varying extents (12% – 89% of normal levels). Figure 5.8a shows this effect, presented as a mean of five experiments where inhibition was greater than 50%. In these experiments, AA release was unaffected (Fig.5.8b). These

studies indicate that bombesin–stimulated AA release is not a consequence of prior phosphoinositide hydrolysis.

Figure 5.1 Lack of bombesin–stimulated arachidonate release in electropermeabilised Swiss 3T3 cells

Swiss 3T3 cells were treated as described in the Materials and Methods section and were stimulated with (a) increasing concentrations of bombesin with GTPγS ● (30μM), (b) HBG (O), bombesin (30nM) (●), GTPγS (30μM) (■) or both (▲) for the times indicated. Each point represents the mean ± S.D. of triplicate determinations from a single experiment representative of two others.


14000
12000
10000
8000
6000
dpm in arachidonate
.1
1
10
100
1000
[bombesin] nM

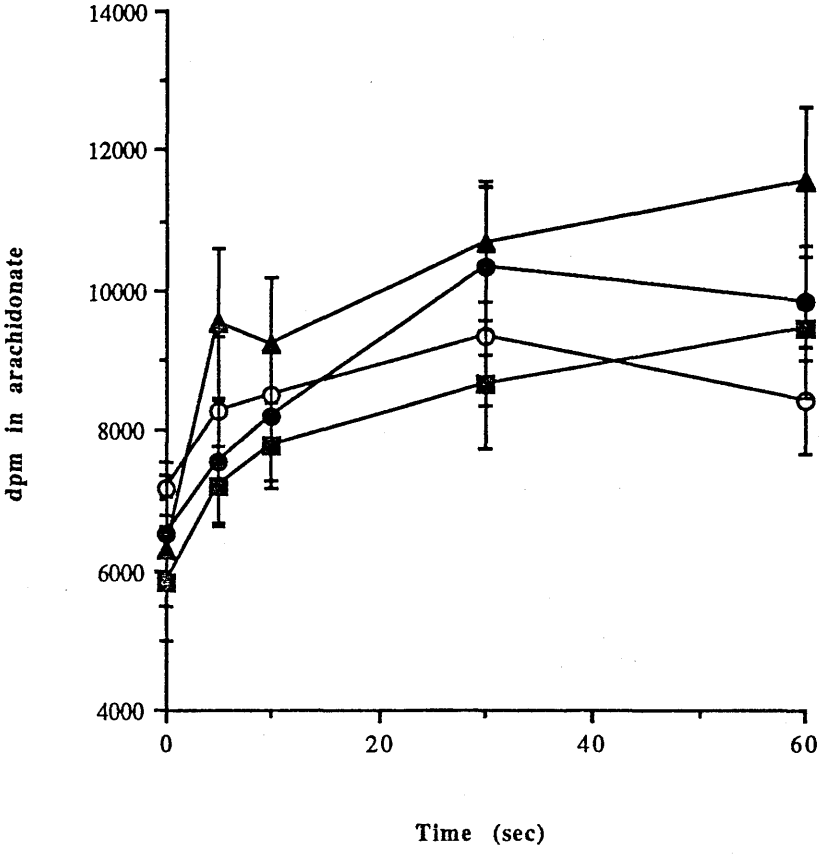

14000
12000
10000
8000
6000
4000
dpm in arachidonate
0
20
40
60
Time (sec)

Figure 5.2 Release of lactate dehydrogenase from Swiss 3T3 cells permeabilised with streptolysin–O

Swiss 3T3 cells were treated with increasing concentrations of streptolysin–O and the amount of lactate dehydrogenase released into the medium was measured as described. Results are representative of three experiments.

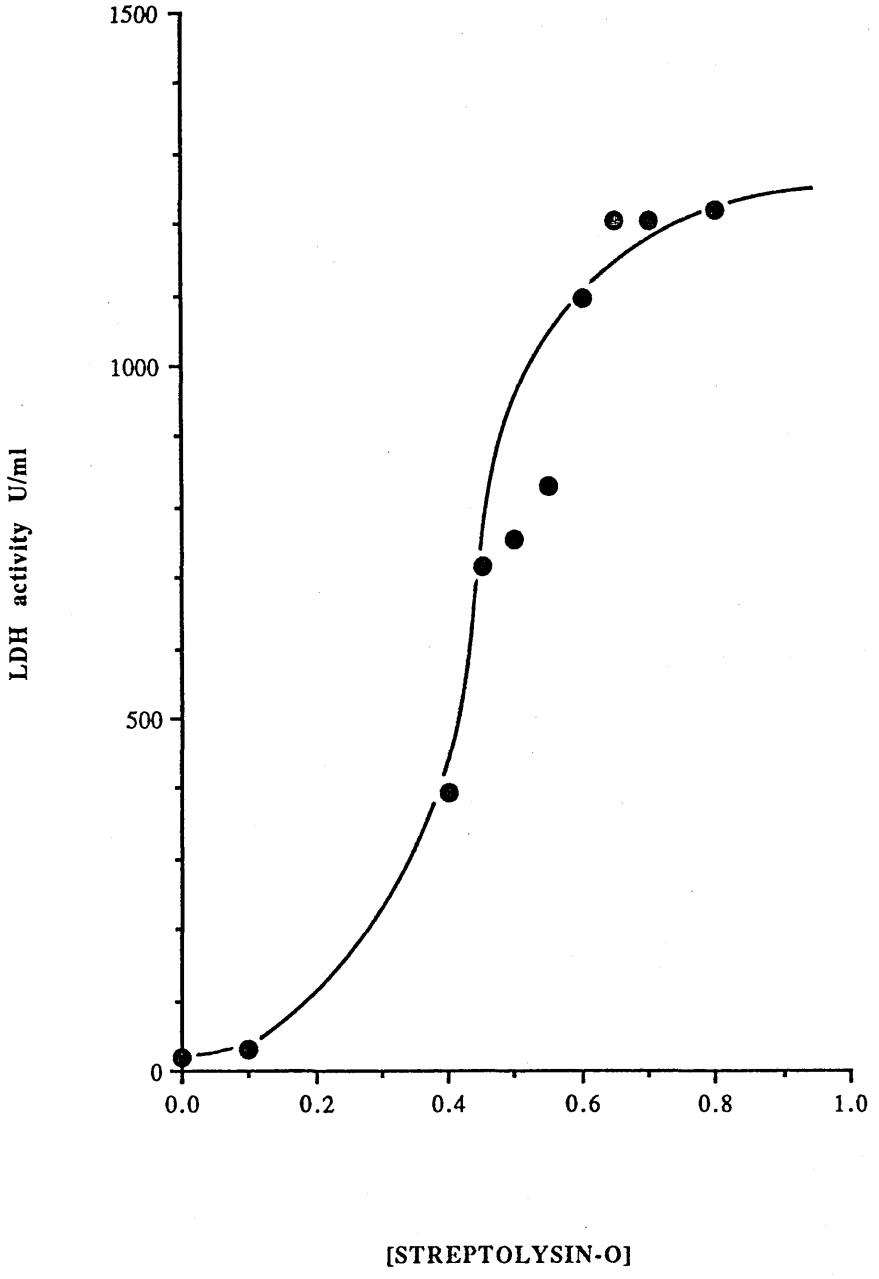
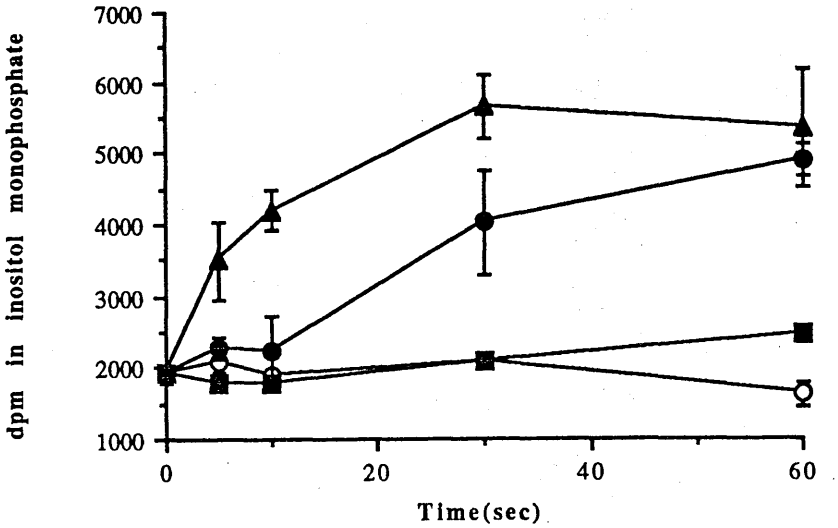
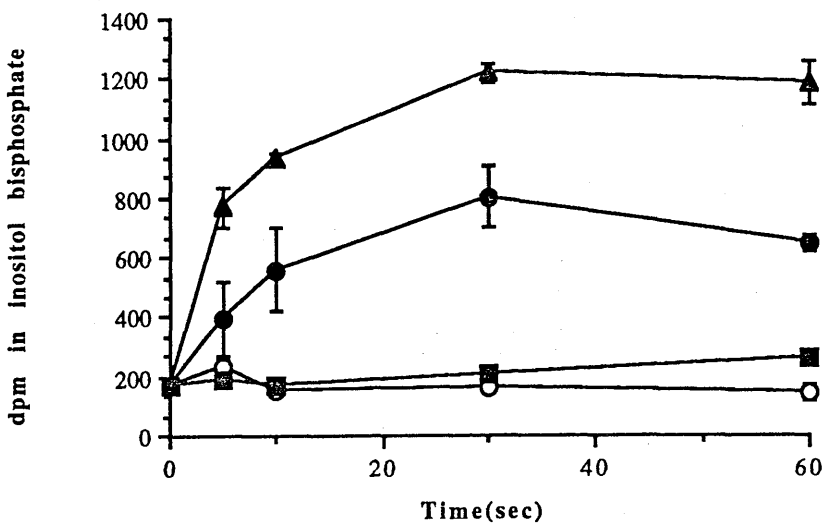

1500
1000
500
0
0.0
0.2
0.4
0.6
0.8
1.0
LDH activity U/ml
[STREPTOLYSIN-O]

Figure 5.3 **Kinetics of bombesin and GTPγS–stimulated [^{3}H]inositol phosphate formation in streptolysin–O permeabilised Swiss 3T3 cells**

Cells treated as described in the Materials and Methods section were incubated with HBG (O), 30nM bombesin (●), 30μM GTPγS (■) or both (▲) for the times indicated (a) InsP, (b) $InsP_2$ and (c) $InsP_3$ formation was measured. Each point represents the means± S.D. from a single typical experiment where n=3.

N.B. The response is significantly potentiated by GTP by 5 seconds stimulation with bombesin.


dpm in inositol monophosphate
7000
6000
5000
4000
3000
2000
1000
0
20
40
60
Time(sec)


dpm in inositol bisphosphate
1400
1200
1000
800
600
400
200
0
0
20
40
60
Time(sec)

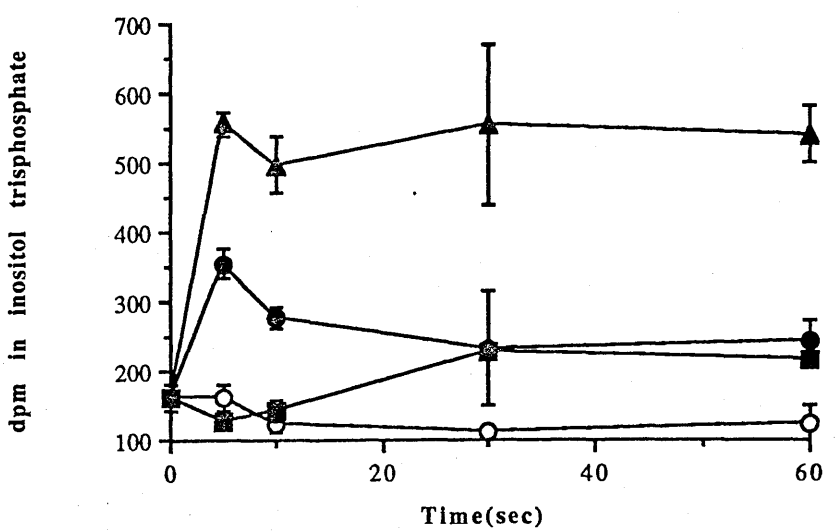
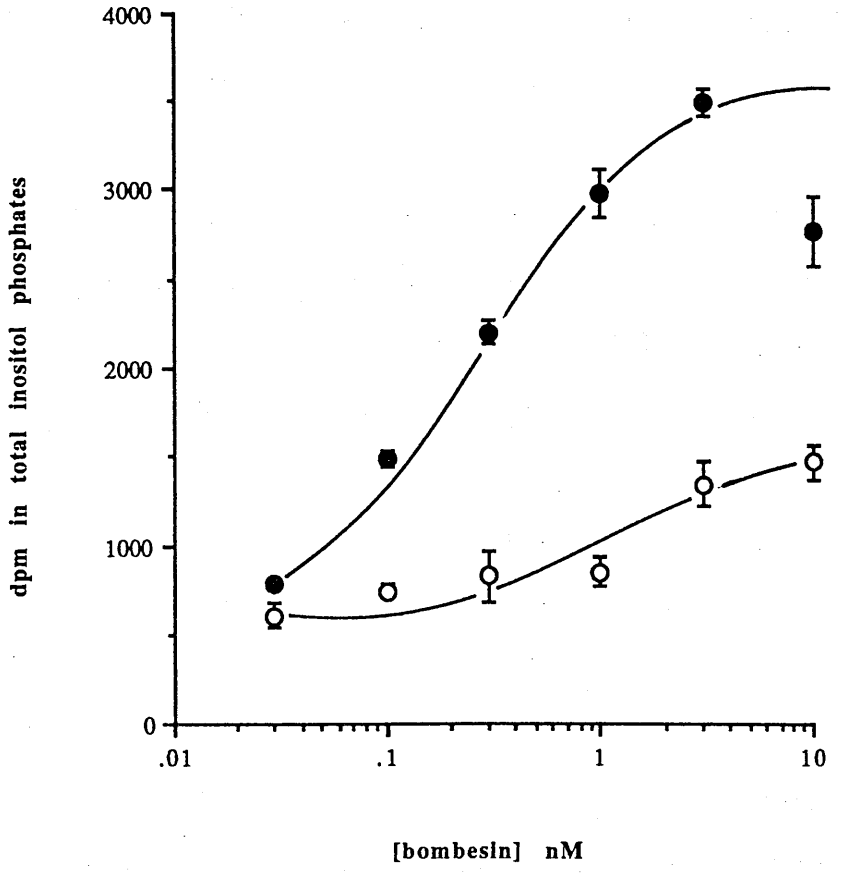

dpm in inositol trisphosphate
700
600
500
400
300
200
100
0
20
40
60
Time(sec)

Figure 5.4 (a) Effect of GTPγS on bombesin-stimulated accumulation of total [^{3}H]inositol phosphates in permeabilised Swiss 3T3 cells

Cells treated as described were incubated with increasing concentrations of bombesin in the absence (O) or presence (●) of 30μM GTPγS for 30 min at 30°C in incubation buffer containing 10mM LiCl. Each point represents the mean ± S.D. of triplicate determinations from a single experiment representative of three others.

Figure 5.4(b) GDPβS inhibition of bombesin-stimulated accumulation of total [^{3}H]inositol phosphates in permeabilised Swiss 3T3 cells

[^{3}H]inositol labelled cells were incubated with increasing concentrations of bombesin in the absence (O) or presence (●) of 2mM GDPβS in a buffer containing 10mM LiCl. Each point represents the mean ± S.D. from a single typical experiment where n=3.


dpm in total inositol phosphates
4000
3000
2000
1000
0
.01
.1
1
10
[bombesin] nM

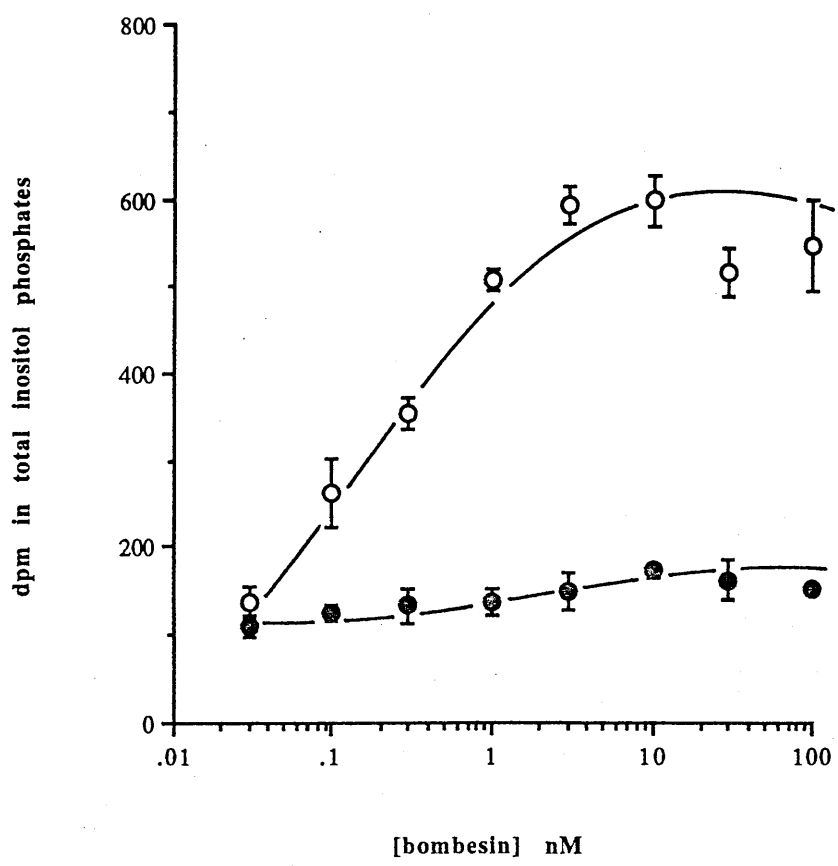

dpm in total inositol phosphates
800
600
400
200
0
.01
.1
1
10
100
[bombesin] nM

Figure 5.5 **Kinetics of bombesin– and GTPγS–stimulated [^{3}H]arachidonate release in streptolysin–O permeabilised cells**

Cells treated as described were incubated with HBG (O), 30nM bombesin (●), 30μM GTPγS (■) or both (▲) for the times indicated and AA release measured. Results represent the mean ± S.D. from a single typical experiment where n=3.

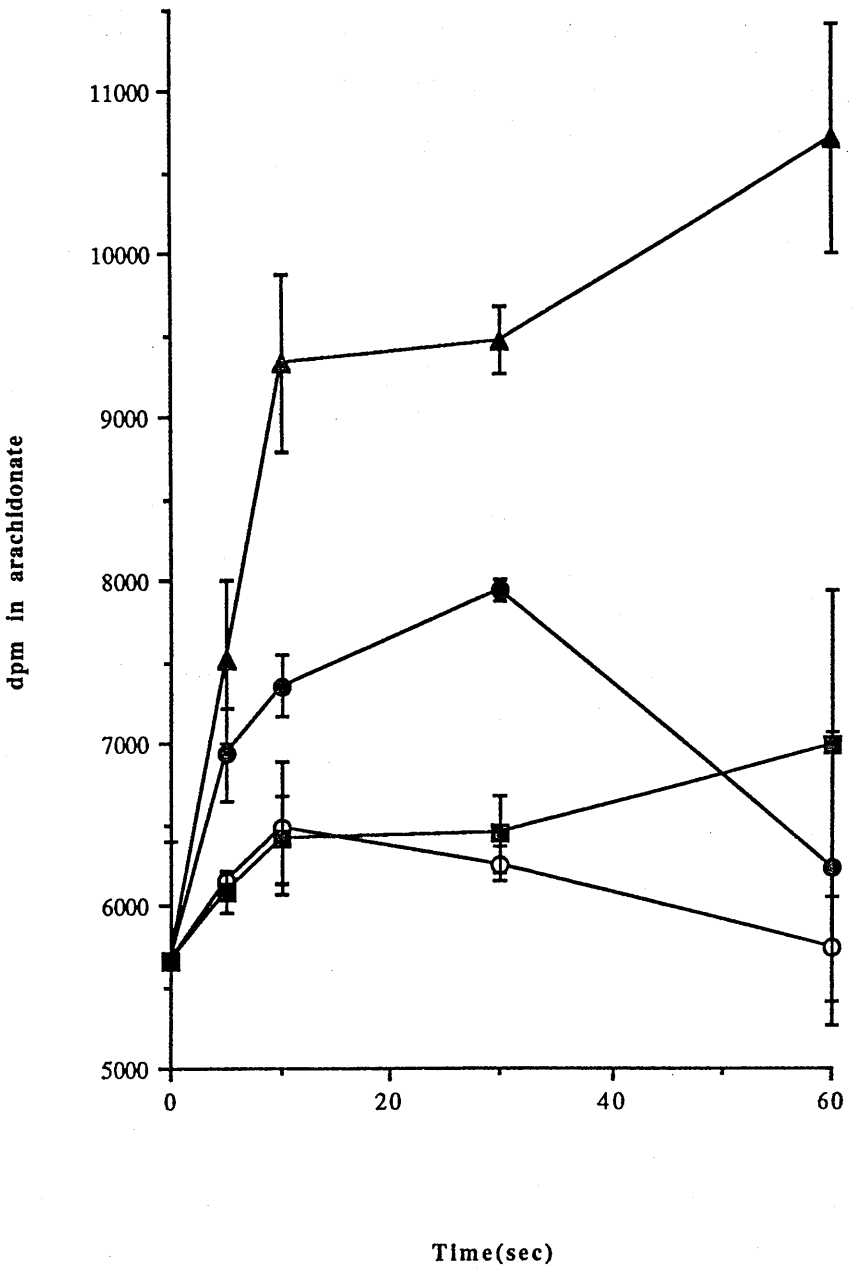
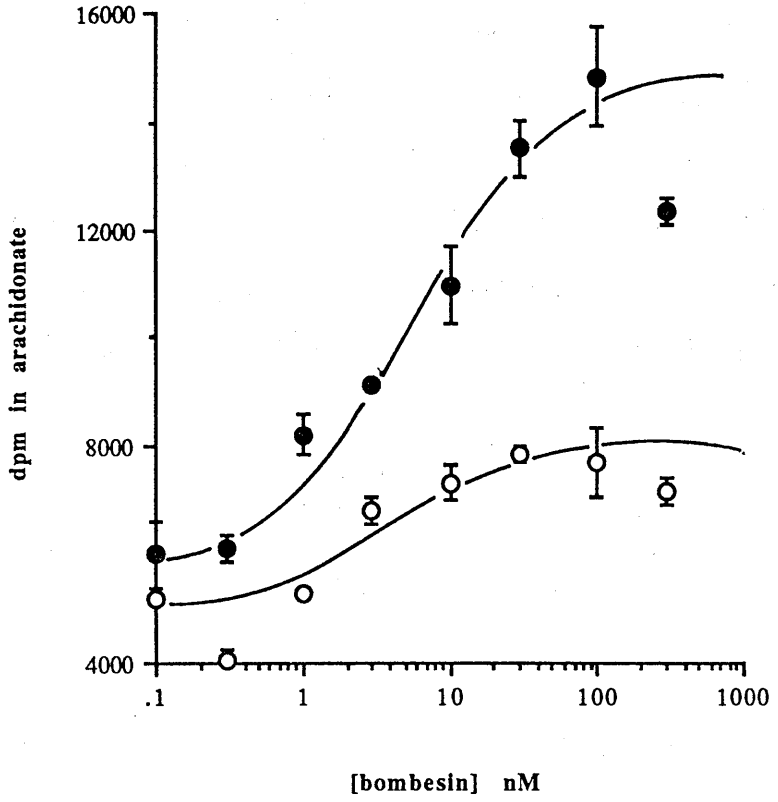

11000
10000
9000
8000
7000
6000
5000
0
20
40
60
dpm in arachidonate
Time(sec)

Figure 5.6(a) Effect of GTPγS on bombesin-stimulated [^{3}H]arachidonate release in permeabilised Swiss 3T3 cells

Cells treated as described were incubated with increasing concentrations of bombesin in the absence (O) or presence (●) of GTPγS for 30 sec. Each point represents the means ± S.D. from a single typical experiment where n=3.

Figure 5.6(b) GDPβS inhibition of bombesin-stimulated [^{3}H]arachidonate release in permeabilised Swiss 3T3 cells

[^{3}H]arachidonate labelled cells pretreated for 10 min with either 2mM GDPβS (◒) or incubation buffer (◠) were incubated with increasing concentrations of bombesin for 30 sec. Each point represents the mean ± S.D. from a single typical experiment where n=3.


16000
12000
8000
4000
dpm in arachidonate
.1
1
10
100
1000
[bombesin] nM

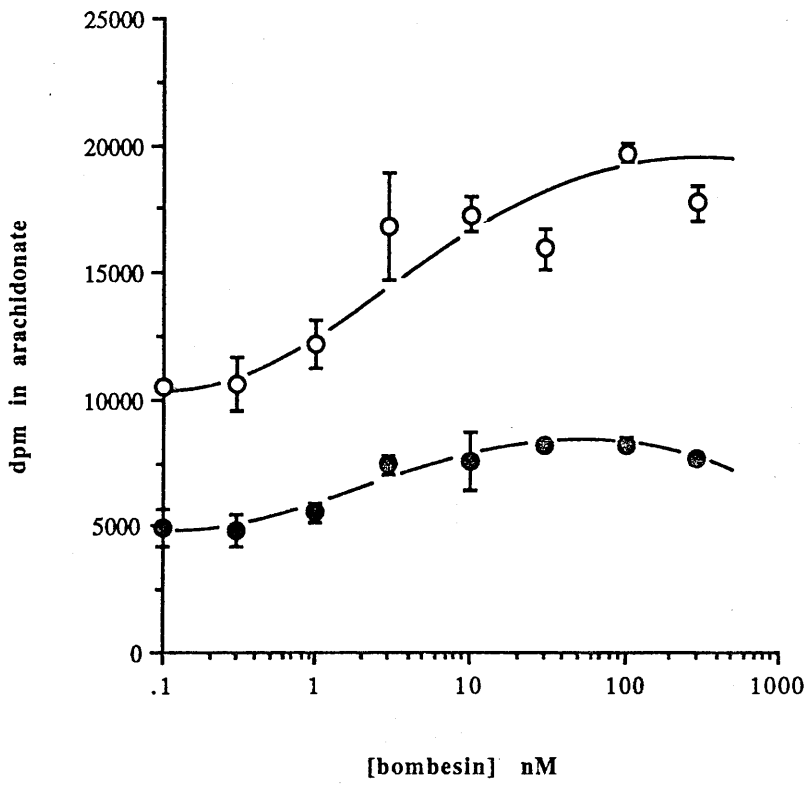

25000
20000
15000
10000
5000
0
dpm in arachidonate
.1
1
10
100
1000
[bombesin] nM

Figure 5.7 Effect of GDPβS pretreatment on bombesin and GTPγS stimulated arachidonate release in permeabilised cells

Cells treated as described were pretreated for 10 min with either 2mM GDPβS (⊘) or incubation buffer (●) and then stimulated with either bombesin, GTPγS or both for the indicated times. Each point represents the mean ± S.D. from a single typical experiment where n=3.

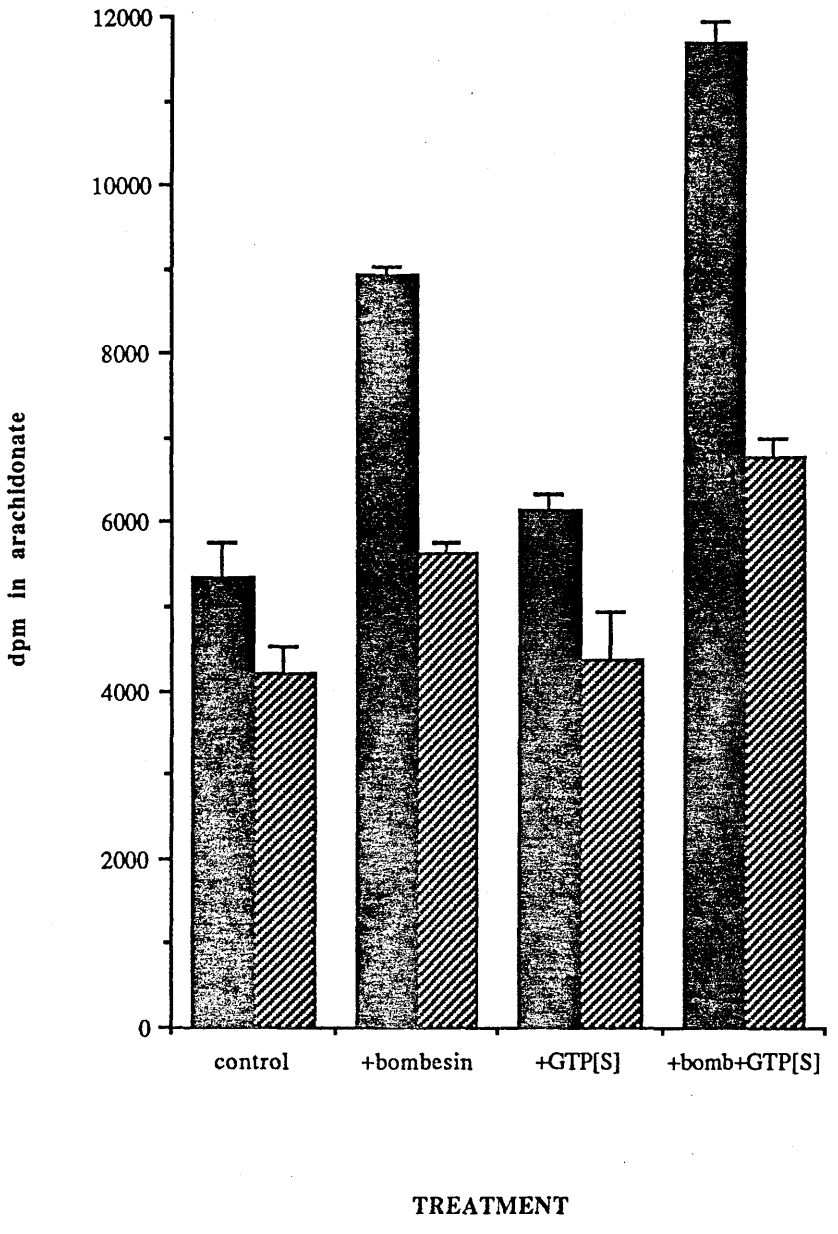
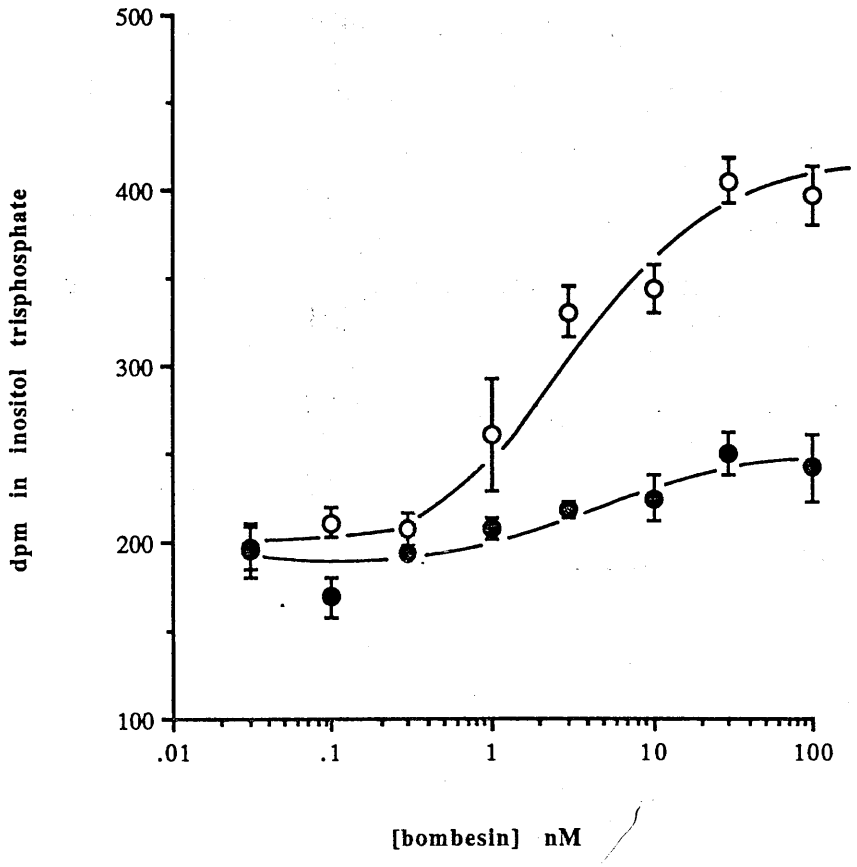
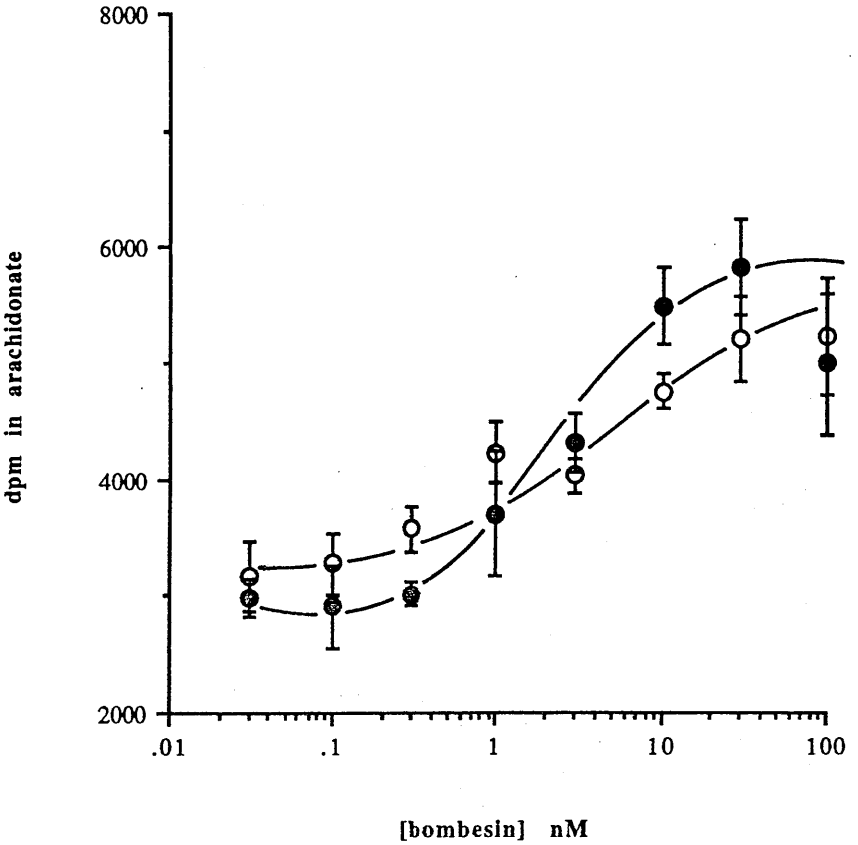

12000
10000
8000
6000
4000
2000
0
dpm in arachidonate
control
+bombesin
+GTP[S]
+bomb+GTP[S]
TREATMENT

Figure 5.8 Differential effects of neomycin on bombesin–stimulated $InsP_3$ production and arachidonate release in permeabilised Swiss 3T3 cells

Cells treated as described were pretreated for 10 min with 2mM neomycin (●) or incubation buffer (○). They were then stimulated with increasing concentrations of bombesin and either (a) $InsP_3$ production or (b) arachidonate release were measured. Results represent means ± S.D. from a single typical experiment where n=3.

N.B. For (a) experiments were not used where inhibition was below 50%.


dpm in inositol trisphosphate
500
400
300
200
100
.01
.1
1
10
100
[bombesin] nM


dpm in arachidonate
8000
6000
4000
2000
.01
.1
1
10
100
[bombesin] nM

DISCUSSION

The results presented in this chapter clearly indicate that rapid bombesin–stimulated AA release is totally dependent on the presence of GTP in the system and strongly suggest the involvement of a G protein, coupling receptor stimulation to PLA_2 activation. Previous reports in other systems have suggested a similar scenario (Teitelbaum, 1990; Narasimhan *et al.*, 1990; Kajiyama *et al.*, 1990). The kinetics of AA generation shown in Figure 5.6 are very similar to those of $InsP_3$ in Figure 5.4. The lag time observed with GTPγS alone for both $InsP_3$ production and AA release is significantly decreased in both cases by bombesin receptor occupation. In addition, both responses to bombesin are potentiated at the early time points tested in the absence of a response to GTPγS alone. These observations suggest that the dissociation of bound GDP is the rate–limiting step in the activation of the G protein and also that agonist receptor stimulation significantly enhances the exchange of GTP for GDP. This phenomenon has been described by Plevin *et al* . (1990) amongst others for $InsP_3$ production.

In various systems, receptor–mediated PLA_2 activity has been distinguished from PLC activation through differential susceptibility to pertussis toxin treatment (Teitelbaum, 1990; Cockcroft & Stutchfield, 1989; Jelsema 1987). However, both the PLC and PLA_2 responses in bombesin–stimulated Swiss 3T3 cells are pertussis toxin insensitive. In order to distinguish these pathways, alternative methods have to be used. One means of discrimination has been described in this chapter and involves inhibition of the phosphoinositide–PLC pathway using neomycin. These results (Fig.5.9) have demonstrated the apparent independence of the PLA_2 pathway from prior PLC activation in bombesin–stimulated Swiss 3T3 cells. This conclusion is strengthened by the results presented in Chapter 3, where short–term PMA pretreatment causes inhibition of PLC–mediated $InsP_3$ production but stimulation of PLA_2–mediated AA release. Overall, these results strongly implicate the involvement of a G protein in the regulation of PLA_2 in this system. In addition, this protein may be distinct from that which regulates phosphoinositide

specific PLC activity in the same system. No attempt has been made in this study to identify the G protein that may regulate PLA_2 because of the extreme diversity of G proteins.

In systems where G protein–mediated regulation of PLA_2 has been suggested, the putative G protein has been designated G_a (Burch *et al.*, 1986). However, the identity of this putative G protein remains to be determined. This particular area of research has encountered considerable controversy, not only concerning the identity of the protein but also, which subunit of the G protein is involved. Jelsema & Axelrod (1987) and Clapham & Neer (1988) have both presented evidence that the $\beta\gamma$ subunit will activate PLA_2 in the systems under study. However, various questions have been raised by Birnbaumer (1987) concerning the purity of the $\beta\gamma$'s used and the limited range of α subunits tested in these studies. These suspicions are apparently well–founded, since Jelsema & Axelrod do state that their $\beta\gamma$ preparations contain a small level of intrinsic PLA_2 activity which is obtained during the purification procedure. However, more recent findings by Kim *et al.* (1989) suggest the existence of a novel G protein dependent signalling pathway, mediated by $\beta\gamma$, causing stimulation of PLA_2 activity and metabolism of the AA produced via lipoxygenase enzymes. These metabolites are suggested to be involved in activation of the cardiac muscarinic K^+ channel. They suggest that several lipoxygenase products activate the channel through a less specific mechanism than receptor coupling, such as release of the G protein α subunit or direct channel activation. There is evidence to suggest the existence of multiple forms of β and γ (Schmidt & Neer, 1991). This may allow dimer formation with distinct functional characteristics. Differences have been noted in the relative ability of $\beta\gamma$ dimers to activate PLA_2. Brain $\beta\gamma$ will stimulate PLA_2 activity in artial membranes, but $\beta\gamma$ from transducin is much less effective (Logothetis *et al.*, 1987; Kim *et al.*, 1989). In these studies the β subunit was predominantly $\beta 1$. Therefore, these functional differences may be due to the γ subunit. Involvement of the $\beta\gamma$ subunit in Swiss 3T3 PLA_2 activity could be

checked using reconstitution experiments and possibility anti–$\beta\gamma$ antibodies. However, these may be hindered by potentially incorrect $\beta\gamma$ combinations and impurity of the preparations.

Over the past few years it has been found that the family of genes encoding the α subunits of G proteins is larger than previously thought. Strathmann & Simon (1990) have proposed the existence of a distinct class of α subunits ($G_{\alpha q}$) evident in vertebrates and invertebrates. These show less than 50% amino acid sequence identity with any of the other α subunits and are not substrates for pertussis toxin modification. It has been suggested that these novel G proteins may account for some of the less well understood activities attributed to G proteins. One such role may be that of regulating PLA_2. A recent report by Smrcka *et al.* (1991) suggests that phosphoinositide–specific PLC is regulated by G_q. In a more detailed study by Taylor *et al.* (1991), the isozymes of PLC were investigated with respect to regulation by G_q. They reported that this G protein specifically activates the β1 isozyme, but not the γ1 or δ1 isozyme of PLC. The possibility is also addressed that other phospholipases may be regulated by this family of G proteins. As Strathmann & Simon (1990) suggest, the possibility of different combinations of α, β and γ subunits may allow a cell to 'fine tune' G protein function to the specific requirements of a particular signalling pathway. Whether a form of $G_{\alpha q}$ is responsible for regulating PLA_2 activity awaits definitive evidence in the form of reconstitution experiments of G_q with specific receptors and the development of specific antibodies to these proteins.

Other G_α subunits have been proposed to activate PLA_2 in different systems. Cantiello *et al.* (1990) suggest $G_{\alpha i-3}$ regulates epithelial Na^+ channels by PLA_2 activation. Kajiyama *et al.* (1990) propose the involvement of different G proteins, G_i– and G_o–like proteins in the coupling of α_2–adrenergic and thrombin receptors respectively to PLA_2 in rabbit platelets. A detailed study by Gupta *et al.* (1990) examined the regulatory regions within the α chains responsible for control

of PLA_2. Different chimaeric cDNAs were constructed in which certain lengths of the α subunit of G_s (α_s) were replaced with the corresponding sequence of G_i, (α_{i-2}). They proposed that the last 36 amino acids of α_{i-2} are a critical domain for G protein regulation of PLA_2. However, G_i proteins are substrates for pertussis toxin and this cannot be the coupling protein in Swiss 3T3 cells.

It appears likely that the specificity of the G protein regulating PLA_2 may vary according to the system studied. In Swiss 3T3 cells, G protein regulation of PLA_2 has already been proposed (Burch & Axelrod, 1987). However, the identity of the protein requires more detailed investigation. Expression of the relevant cDNAs in Swiss 3T3 cells could resolve which G protein(s) is/are involved. Such an approach, involving PLA_2 co–expression could show how disruption of this signalling pathway, perhaps by constitutive G protein activation or over–expression, may lead to disruption of cell function. Alternatively, the opposite approach of removal of the cytosolic PLA_2 and/or specific G proteins using anti-sense RNA may be more effective in demonstrating the same effect. These types of study would help in implicating PLA_2 involvement in certain areas of signal transduction.

A final consideration that is of equal importance for future investigation, is identification of how this signalling pathway is turned off. This is important given the transient nature of the initial response. Whether this occurs at the level of the G protein (either a separate inhibitory G protein, allowing dual regulation of PLA_2, or phosphorylation and inhibition of the stimulatory G protein) or at the level of PLA_2 itself is unclear. Preliminary studies by Lowndes *et al.* (1991) have indicated that PLA_2 is phosphorylated in Chinese hamster ovary cells. They suggest that this may be a mechanism for attenuation of the signal and are currently attempting to identify regulatory phosphorylation sites within the PLA_2 polypeptide by proteolytic phosphopeptide mapping. They predict that the attenuation of PLA_2 stimulation is a compensatory mechanism resulting from constitutive activation of

certain G protein regulated signalling pathways. However, phosphorylation as a desensitization mechanism is an alternative possibility.

Cumulatively, results presented in this chapter, together with data obtained in other systems, indicate that G protein–coupled PLA_2 and PLC signals are under tight control. This feature will allow for distinct responses to be obtained in certain systems and will prevent hyperactivation of these second messenger pathways. Given the number of G proteins implicated in PLA_2 activation, the deviation in responses between systems should be considerable. It may even be possible in certain systems, contrary to long–standing belief, that PLC activation is dependent on prior receptor–mediated PLA_2 activation.

CHAPTER 6

BOMBESIN DOES NOT SIGNIFICANTLY STIMULATE THE METABOLISM OF ARACHIDONIC ACID VIA CYCLOOXYGENASE OR LIPOXYGENASE PATHWAYS

INTRODUCTION

When examining bombesin–stimulated AA release in Swiss 3T3 cells, it is important to consider the actual composition of the response. In mitogen–stimulated cells, AA may not necessarily be the final product of PLA_2 hydrolysis since it may be further metabolised into various biologically active eicosanoids via cyclooxygenase and lipoxygenase pathways. In some cases, the determination of whether AA is liberated faster than would be expected from its esterified levels is complicated because a substantial proportion is further metabolised to eicosanoids. For example, Irvine *et al.* (1982) have shown that in platelets after 10 sec stimulation only 20–25% of [^{3}H]AA liberated was still the free acid, the rest having been converted to products of cyclooxygenase and lipoxygenase enzymes.

Prostaglandins and leukotrienes, which are products of the cyclooxygenase and lipoxygenase pathways respectively, can be considered to be autocrine or paracrine hormones which bind to specific receptors. Their actions depend on cell type, location, metabolic activity of the cell and the simultaneous actions on the cell of other humoral factors. It is clear that the same eicosanoids can trigger opposite metabolic or physiological effects in different types of cells (Needleman *et al*, 1986).

In Swiss 3T3 cells, where prostaglandins are apparently required for replication, PGG/H synthase gene expression is induced by serum growth factors (DeWitt *et al.*, 1983). Induction is relatively rapid, occurring at the time of induction of immediate early response genes such as c–*fos* and c–*myc*. Thus, the PGG/H synthase gene appears to be an important cell–cycle regulated gene. In studies by Burch and Axelrod (1987), PGE_2 production was demonstrated in Swiss 3T3 cells in response to bradykinin stimulation. An immediate effect of this stimulus must be an increase in the concentration of free AA in the vicinity of the PGG/H synthase, resulting in metabolism of the free fatty acid. Other studies in Swiss 3T3 cells have also shown PGE_2 production in response to different agonists (Burch *et al.*, 1988).

Evidence for the synthesis of lipoxygenase products in Swiss 3T3 cells is much less apparent. Leukotrienes and related mono–, di– and tri–hydroxy fatty acid products are formed via lipoxygenase pathways. The 5–lipoxygenase pathway has received most attention since this is the one involved in leukotriene formation. These substances, in particular LTB_4, appear to be important modulators of many immune cell functions and are also known protagonists of numerous inflammatory processes. As such, the large majority of studies have been performed on inflammatory cells. It was therefore of interest in the present study to examine whether bombesin could stimulate the metabolism of AA via either the cyclooxygenase or lipoxygenase pathways.

The present chapter focusses on this question and examines the effect of bombesin stimulation on the production of a range of eicosanoids which may be potential metabolites of the free AA released in response to the peptide.

RESULTS

The stimulation of AA metabolism via cyclooxygenase enzymes in response to challenge with bombesin was studied initially. The range of prostaglandins examined in this study included PGE_2, PGD_2, $PGF_{2\alpha}$ and 6–keto–$PGF_{1\alpha}$. Figure 6.1 shows an hplc trace comparing the profile of prostaglandin standards with the profile of samples that had been stimulated with 617nM bombesin for 1h. The only product formed in response to stimulation was PGE_2. Table 6.1 shows that there is no significant production of any other prostaglandins at earlier time points of stimulation. Since the samples analysed contained HBG, it seemed possible that this might have affected results. The recoveries of standard [^{3}H]prostaglandins which were prepared in HBG, were checked accordingly. These were found to be between 86% and 90% on a regular basis. Therefore, sample preparation in this buffer did not hinder detection of cyclooxygenase products.

Figure 6.2 illustrates the proportion of the AA response that is composed of PGE_2. The profiles examined at various times over a 2h period, clearly show that the majority of the response appeared to be composed of the free fatty acid itself and only a very small amount (approximately 4% of total) was metabolised to PGE_2. In addition, the data in this figure indicate that the AA response reached a maximum between 60–90 min then declined. This feature can also be seen in Figure 6.3 which shows the release of AA and production of PGE_2 over a period of 4h stimulation with bombesin. PGE_2 production appeared to increase very slowly over this timecourse and even after 4h, still comprised only approximately 5% of the arachidonate response (see Table 6.2).

Since bombesin–stimulated AA generation is composed of two phases, it was of interest to check how early PGE_2 could be detected upon stimulation with the peptide. Figure 6.4 shows that no significant PGE_2 production occurred until after 10 min stimulation with bombesin when it increased to 2.3–fold over basal values. this strongly suggests that the first phase of the AA response to bombesin is not

metabolised via the cyclooxygenase pathway and may imply that it is composed entirely of AA itself. However, in order to strengthen this proposal, it was essential to examine the possibility that the AA may be metabolised via the lipoxygenase pathway.

Two different hplc separation methods were used to measure lipoxygenase products (see Chapter 2). Figure 6.5 shows a typical chromatogram of standards for LTB_4 and 5–HETE at early time points which correspond to the first phase of AA release.

In order to determine whether Swiss 3T3 cells were capable of producing lipoxygenase metabolites, cells were stimulated with the calcium ionophore, A23187 in parallel with bombesin and a larger range of lipoxygenase products examined. Extraction and subsequent hplc of the lipoxygenase products, after ionophore stimulation of the cell monolayer, gave a typical chromatogram as shown in Figure 6.7 using the water/methanol/acetonitrile/acetic acid solvent system which is changed to 100% methanol using a linear gradient over 40 min (see Chapter 2). Whilst variations in relative peak size were observed between different samples, ionophore stimulation resulted in the production of LTB_4 and a 5,6–diHETE. When cells were stimulated for the same period (i.e. 10 min) with bombesin, there was no obvious production of any of the lipoxygenase metabolites examined (Fig.6.8).

To determine whether any HETES were produced in response to stimulation with ionophore or bombesin challenge, a gradient solvent system was used upon the hplc (Fig.6.9) since HETES are only eluted with a very long retention time when the solvent system is run isocratically. The HETES were eluted with retention times only slightly greater than those of the leukotrienes. As can be seen from Figure 6.9, neither ionophore nor bombesin stimulated 5–, 12– or 15–HETE production. No examination of the peptido–leukotrienes (LTC, LTD and LTE) was performed in this study. However, hplc runs were completed at 280-nm and 310nm, wavelengths at which peptido–leukotrienes and conjugated tetraenes, such as the

lipoxins, would be detected. There were no apparent peaks in response to either stimulus at these wavelengths.

Since no lipoxygenase products were detected upon bombesin stimulation, cells were challenged with bombesin in the presence of 20μM AA. This caused a slight increase in the production of HETES, in particular 15–HETE, demonstrating that these compounds can be derived from this fatty acid and suggesting that bombesin alone may not cause a sufficient increase in free AA for HETES to be formed in this cell type.

Overall, these results illustrate that the AA response to bombesin is almost entirely composed of the free fatty acid itself. No metabolites were detected during the first phase of release and only approximately 5% of the second phase was metabolised to PGE_2. although other undefined routes of metabolism or lipid resynthesis may of course occur.

Figure 6.1 Bombesin-stimulated prostaglandin production in Swiss 3T3 cells

A representative hplc chromatogram of (a) prostaglandin standards and (b) prostaglandin production after 1h stimulation with 617nM bombesin. Isocratic solvent system; 32% acetonitrile/68% orthophosphoric acid, pH3.0. Flow rate; 1.0ml/min. Detection at 216nm.

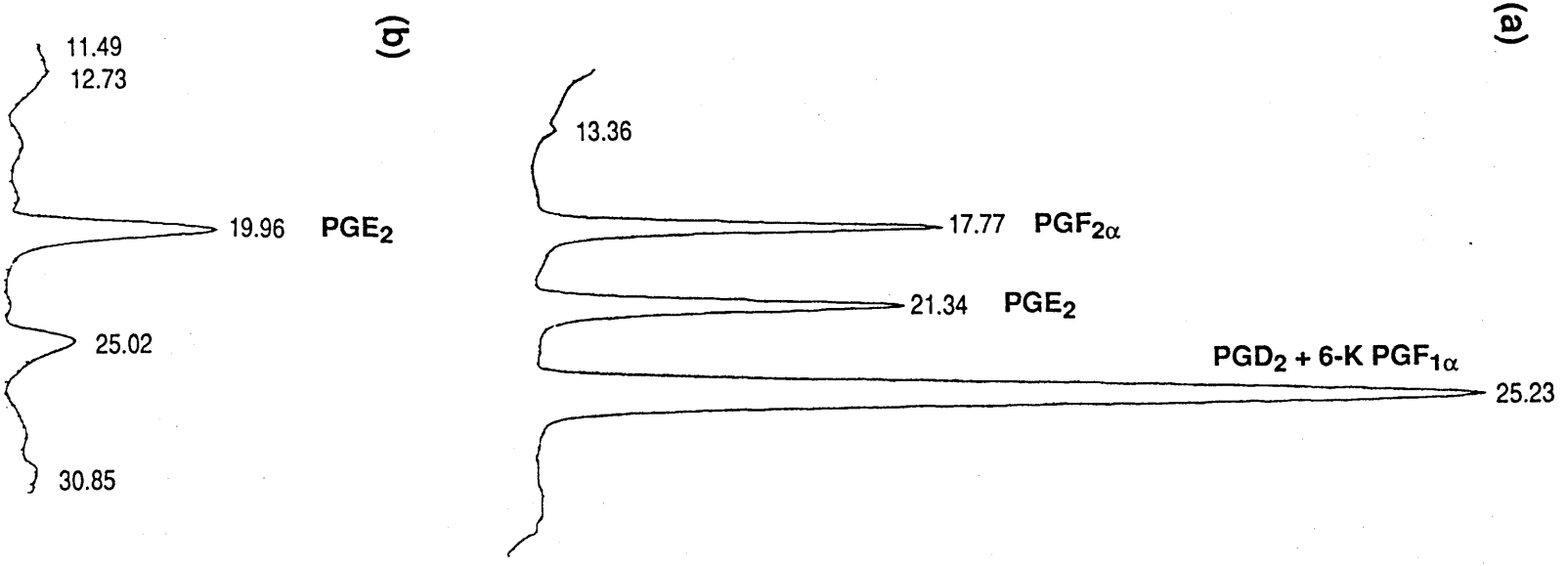

(b)
11.49
12.73
19.96
PGE2
25.02
30.85
(a)
13.36
17.77
PGF2α
21.34
PGE2
PGD2 + 6-K PGF1α
25.23

Table 6.1 The effect of bombesin on prostaglandin levels

[^{3}H]AA–labelled Swiss 3T3 cells were stimulated with bombesin (617nM) for the times indicated and prostaglandin production determined by counting the radioactivity associated with 0.5ml fractions eluted from hplc. Results are expressed as means ± S.D. from a single typical experiment where n=3.

Time(min)	dpm in PGE_2	dpm in 6-K-PGF	dpm in $PGF_{2\alpha}$	dpm in PGD_2
0	1021±185	287±41	318±19	395±22
5	968±96	294±36	289±16	387±51
10	1312±110	276±6	257±34	408±16
30	1810±98	321±18	308±18	482±35
45	2210±44	267±37	359±28	443±19
60	2365±69	383±27	351±16	476±43

Figure 6.2 Comparison of bombesin-stimulated arachidonate release and PGE_2 production

A representative hplc trace showing the radioactivity associated with AA and PGE_2 upon bombesin stimulation for the times indicated.

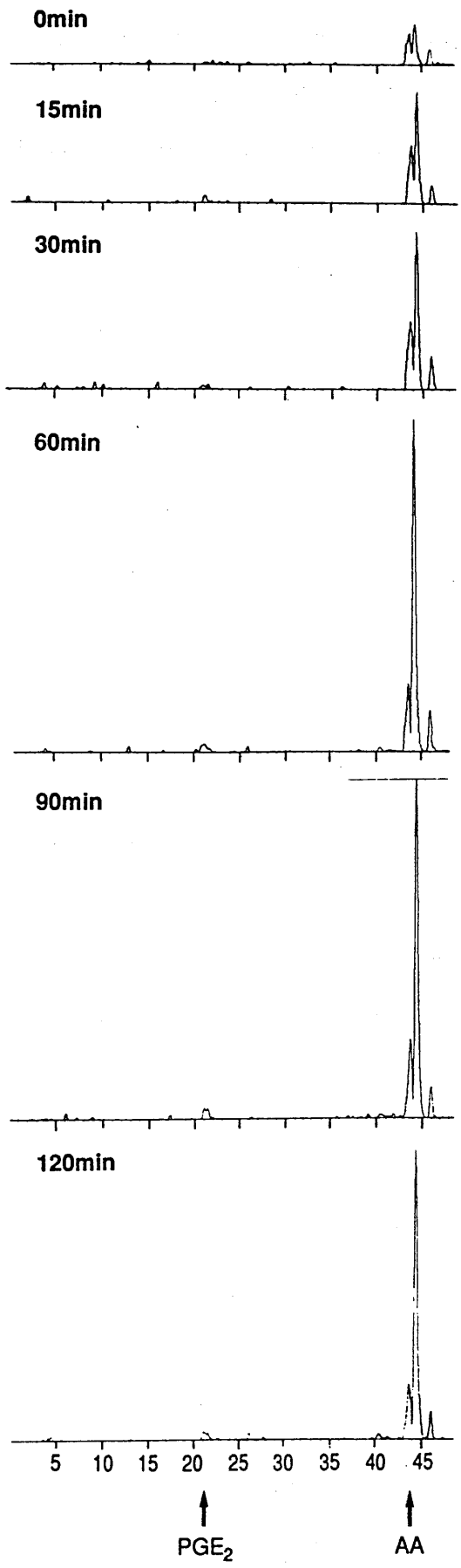

0min
15min
30min
60min
90min
120min
5
10
15
20
25
30
35
40
45
PGE2
AA

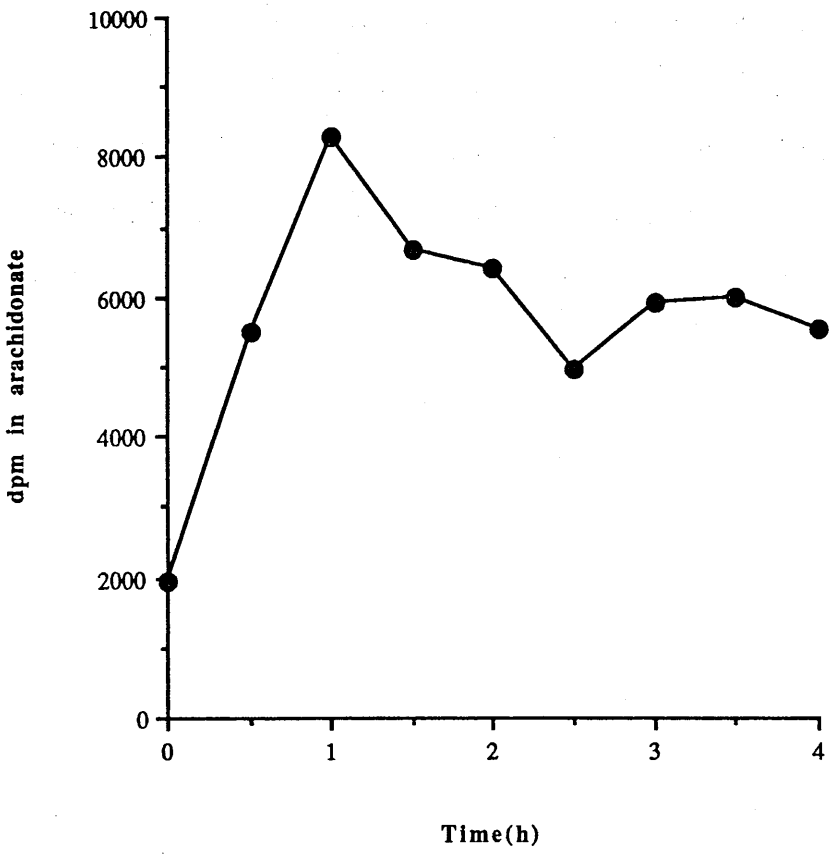
Table 6.2 Stimulation of PGE_2 production by bombesin

These data numerically depict the fold–stimulation of PGE_2 over a 4h period in response to 617nM bombesin. Results are from a single typical experiment where n=3.

Time(h)	dpm in AA	dpm in PGE_2
0	1949 ± 325	155 ± 18
0.5	5506 ± 779	314 ± 95
1	8267 ± 1154	496 ± 66
1.5	6663 ± 1532	401 ± 37
2	6420 ± 955	408 ± 103
2.5	5968 ± 824	497 ± 52
3	5931 ± 1422	502 ± 25
3.5	5998 ± 1012	432 ± 34
4	5552 ± 653	503 ± 42

Figure 6.3 Long–term timecourse of arachidonate release and PGE_2 production in response to bombesin

[^{3}H]AA–labelled Swiss 3T3 cells were stimulated with 617nM bombesin and the radioactivity associated with PGE_2 and AA was measured in 0.5ml fractions eluted from hplc. This is a single typical experiment representative of two others.


10000
8000
6000
4000
2000
0
dpm in arachidonate
0
1
2
3
4
Time(h)

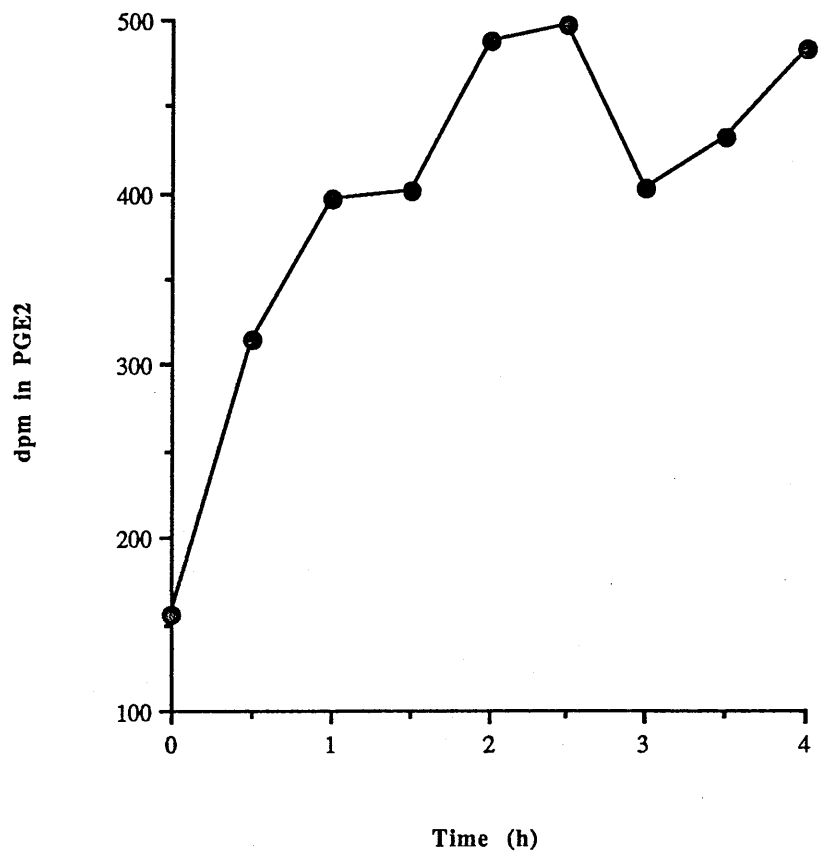
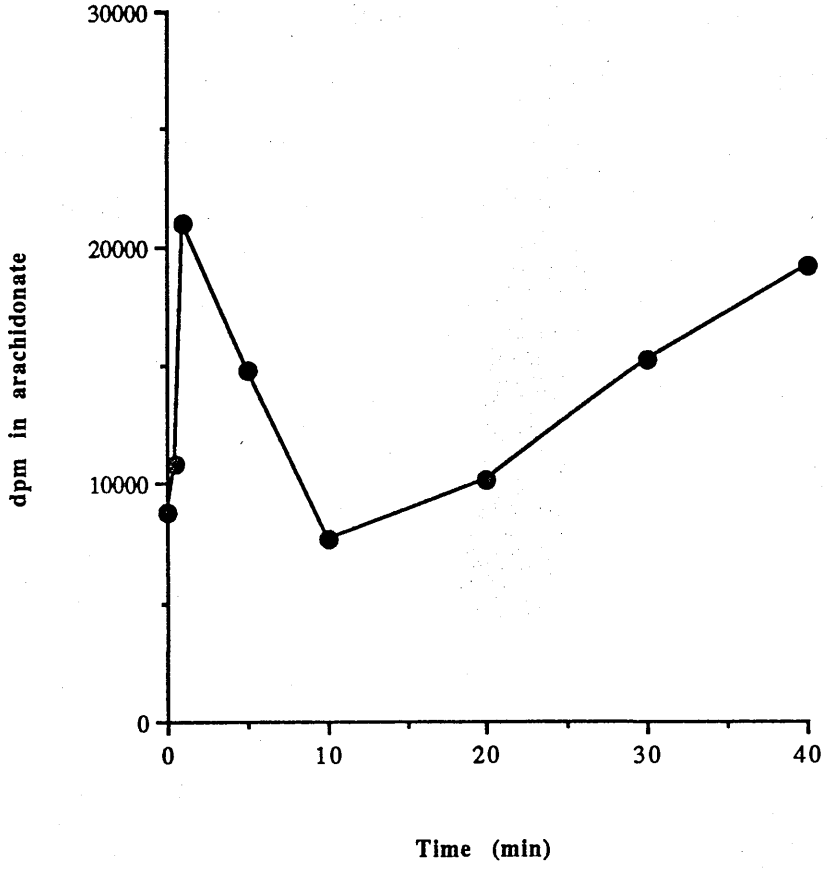

500
400
300
200
100
dpm in PGE2
0
1
2
3
4
Time (h)

Figure 6.4 Short–term timecourse of arachidonate release and PGE_2 production in response to bombesin

Swiss 3T3 cells were stimulated with 617nM bombesin and the release of AA and PGE_2 production were measured over a period of 40 min. This is a single typical experiment representative of two others.


30000
20000
10000
0
dpm in arachidonate
0
10
20
30
40
Time (min)

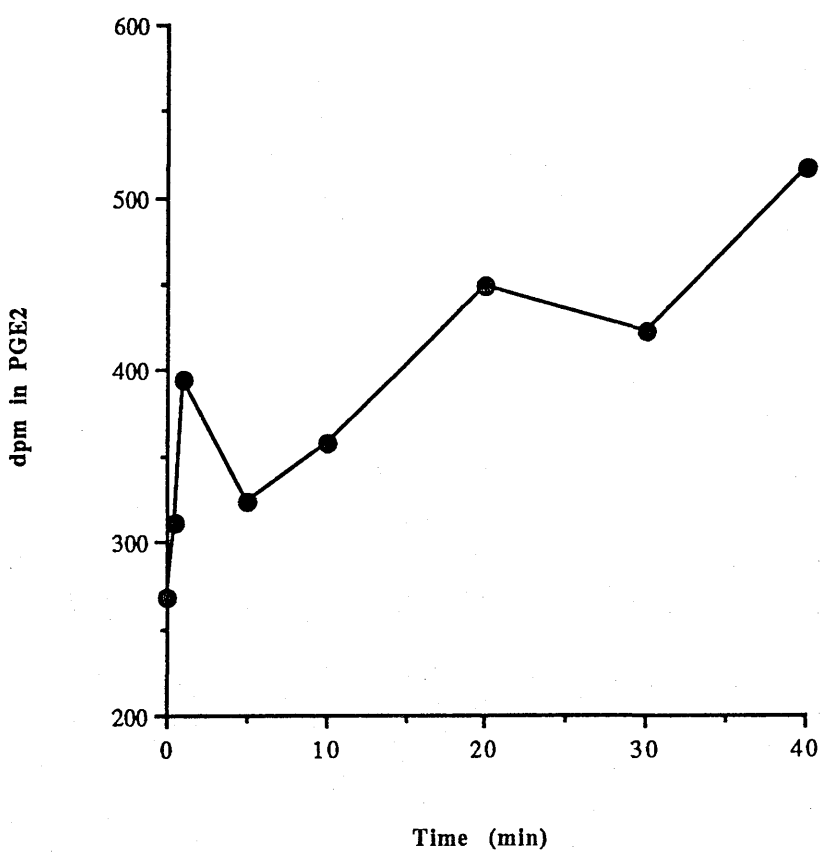

600
500
400
300
200
dpm in PGE2
0
10
20
30
40
Time (min)

Figure 6.5 Effect of bombesin stimulation on the levels of LTB_4 and 5–HETE

(a) A representative hplc chromatogram of LTB_4 and 5–HETE standards and (b) the effect of bombesin stimulation on levels of these products, over a period of 1½h. Isocratic solvent system; methanol 68.7%/(methanol 20%/0.5% ammonium acetate 80%) 31.3%. Flow rate 2.0ml/min. Detection at 270nm.

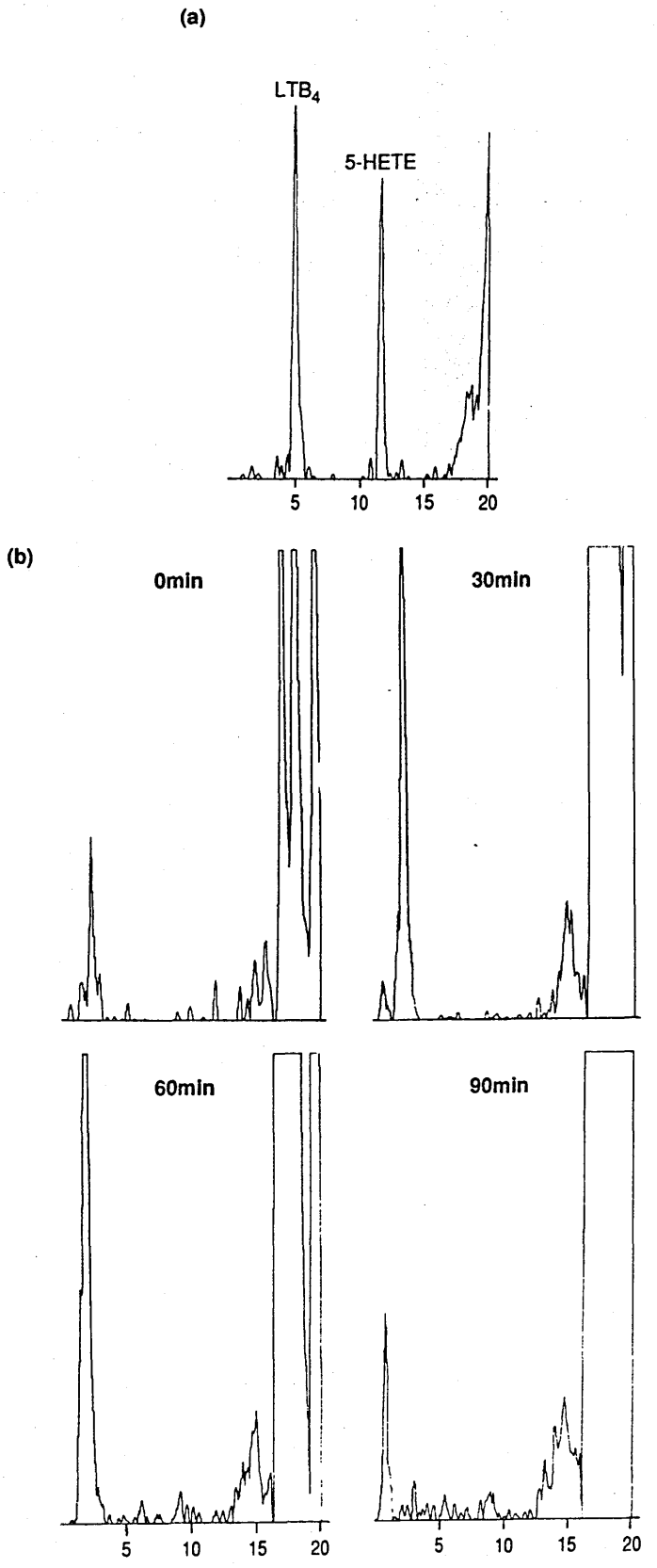

(a)
LTB4
5-HETE
5
10
15
20
(b)
0min
30min
60min
90min
5
10
15
20
5
10
15
20

Figure 6.6 The effect of short–term stimulation with bombesin on the levels of LTB_4 and 5–HETE

Swiss 3T3 cells were stimulated over a shorter timecourse with 617nM bombesin and the production of LTB_4 and 5–HETE measured. Results are from a typical experiment representative of two others.

Time(min)	dpm in AA	fold inc.	dpm in LTB_4	fold inc.	dpm in 5-HETE	fold inc.
0	28022	-	182	-	251	-
1	36429	1.3	139	-	264	-
5	54371	1.9	209	-	253	-
10	66895	2.4	216	-	303	1.2
30	128482	4.6	235	1.2	238	-

Values for LTB_4 and 5-HETE in stimulated cells were not significantly different from those in control samples as judged by the students t-test over three independent experiments ($p > 0.05$)

Figure 6.7 Effect of ionophore stimulation on LTB_4 production

A typical hplc chromatogram produced by calcium ionophore challenged Swiss 3T3 cells (10μM for 10 min at 37°C) after sep–pak extraction and hplc using a linear solvent gradient changing from 100% water/methanol/acetonitrile/acetic acid (45:30:25:0.05 v/v, pH5.7) to 100% methanol in 40 min with a flow rate of 0.6ml/min and detection at 270nm (n=3).

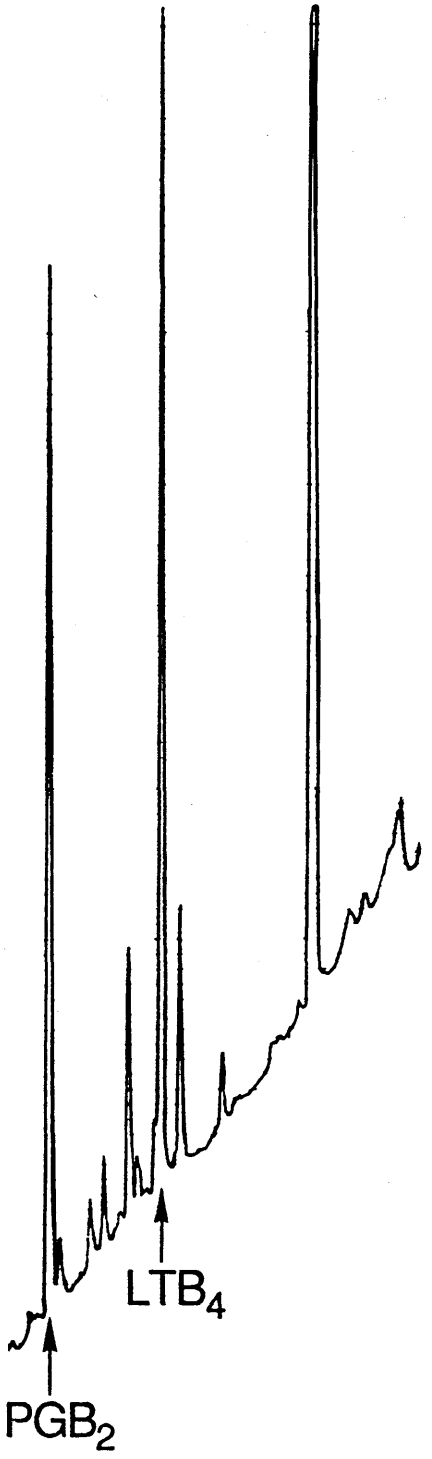

LTB4
PGB2

Figure 6.8 Effect of bombesin stimulation on LTB_4 production

A typical chromatogram produced by bombesin challenged Swiss 3T3 cells (617nM for 10 min) after sep–pak extraction and hplc using the solvent system described for the previous experiment (n=3).

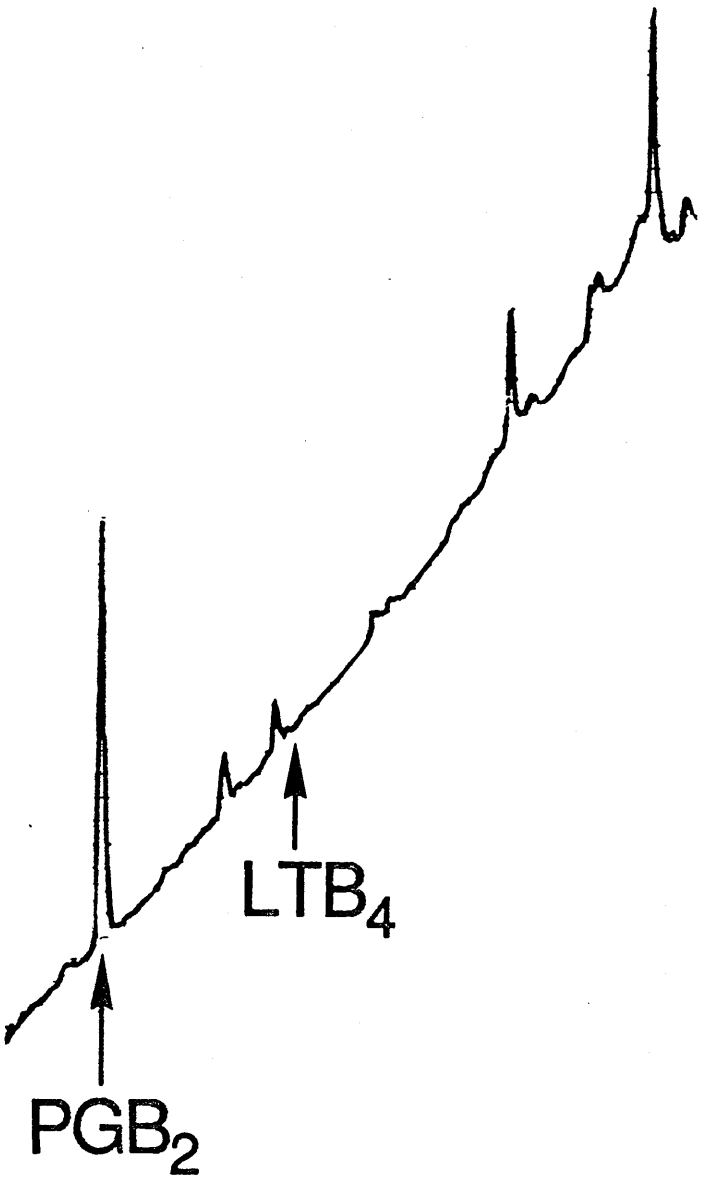

LTB4
PGB2

Figure 6.9 Effect of bombesin and ionophore stimulation on 5–, 12– and 15–HETE production.

Swiss 3T3 cells were stimulated for 10 min with either (a) bombesin (617nM) or (b) ionophore (10μM). HETE production was determined using the solvent system previously described with detection at 235nm.

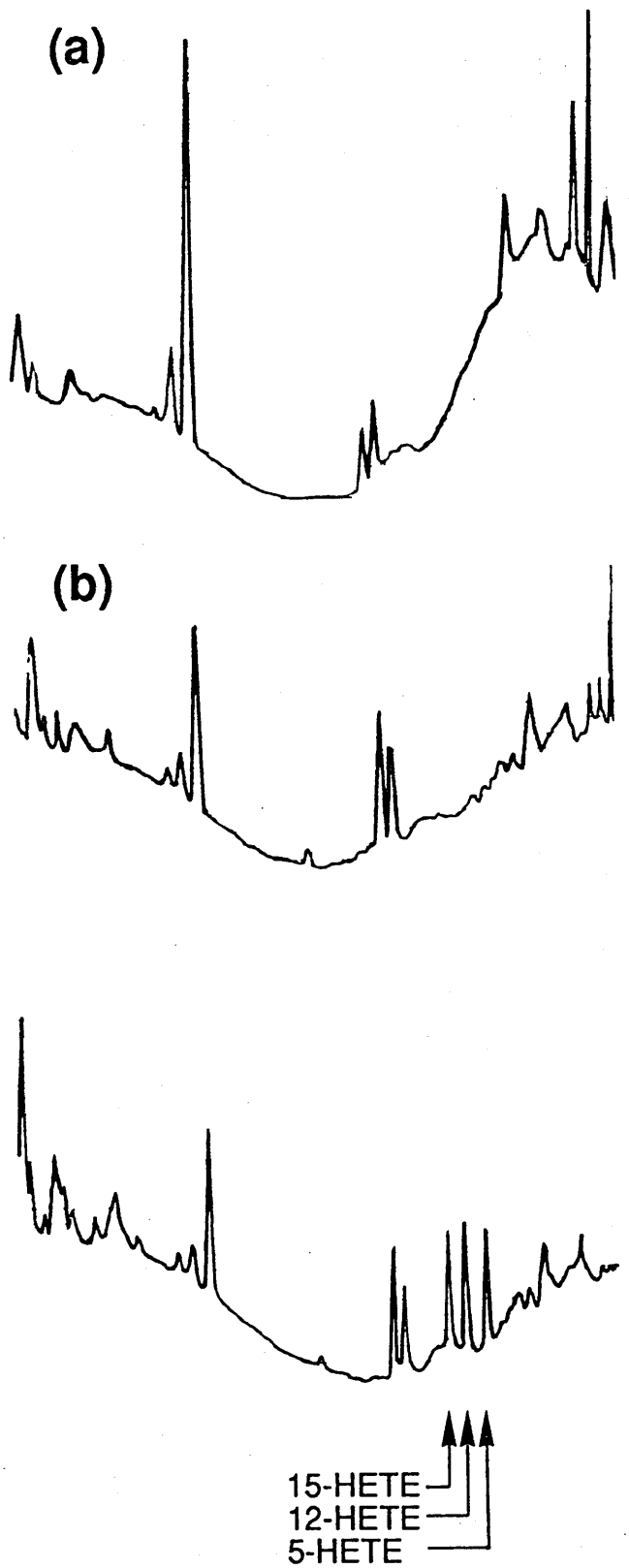
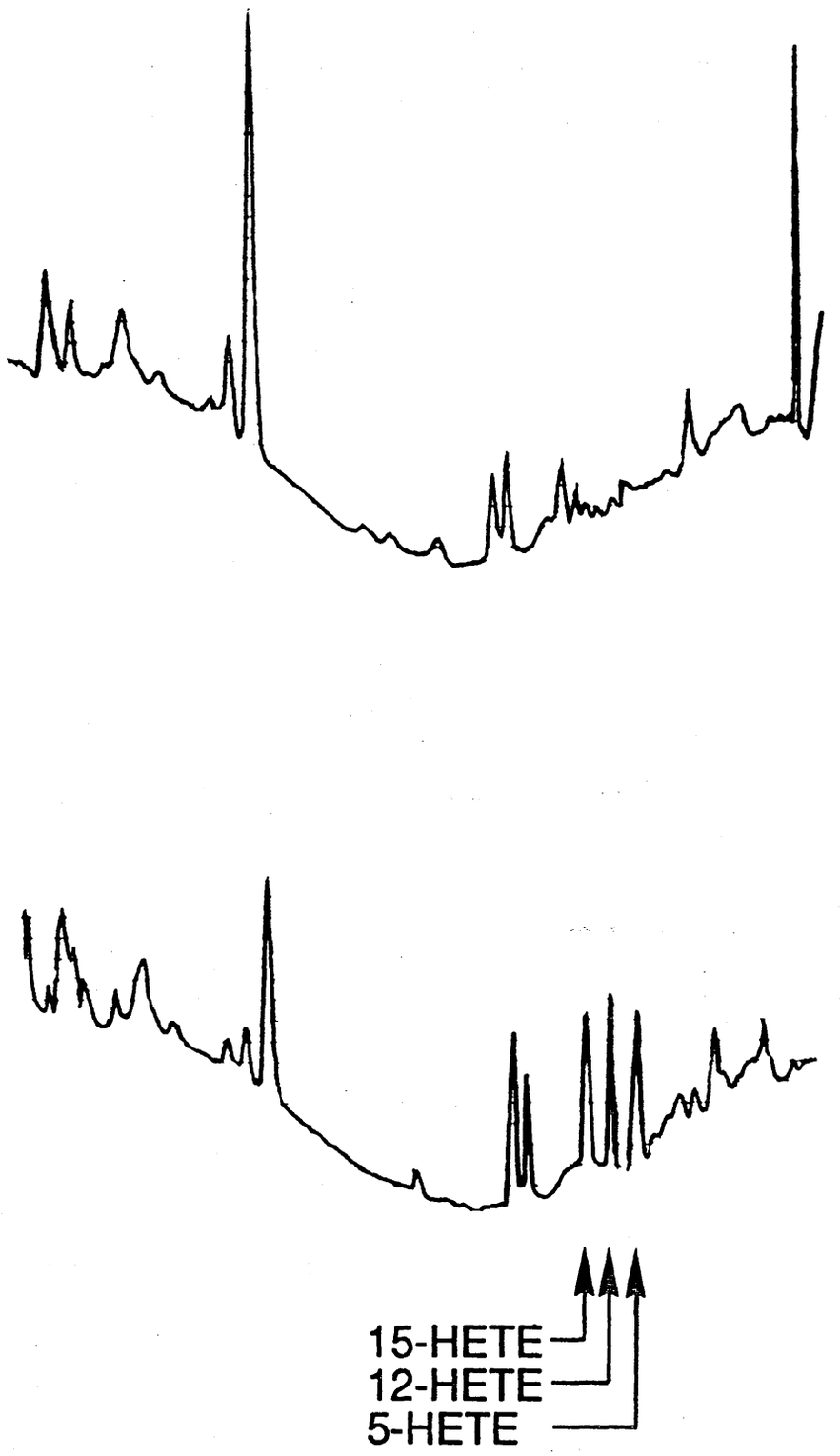

(a)
(b)
15-HETE
12-HETE
5-HETE

Figure 6.10 Effect of arachidonate on bombesin stimulated HETE production

Swiss 3T3 cells were stimulated for 10 min with 617nM bombesin with 20μM AA added. HETE production was determined as described (n=3).


15-HETE
12-HETE
5-HETE

DISCUSSION

In the Swiss 3T3 cell system, hplc analysis has shown that bombesin does not stimulate the metabolism of rapidly generated AA via any of the pathways tested. However, a small, slow increase in PGE_2 production was detected during the second phase of AA liberation. Other methods of measuring metabolites were not tested. Gas–liquid chromatography–mass spectrometry (GC–MS) techniques are sensitive and reliable but very expensive and therefore, limited. Radioimmunoassays are lower in cost, rapid and possess the sensitivity and specificity to quantitate metabolites derivatised from endogenous AA. However, antibodies to all the known AA metabolites are not readily available, limiting the technique. The hplc methods used in this study were well established and gave highly reliable information.

The metabolism of AA via the cyclooxygenase pathway has previously been studied in this system and results have suggested that PGE_2 is the main metabolite formed (Burch & Axelrod, 1987; Millar & Rozengurt, 1990). However, detailed timecourses were not performed and the possibility of other cyclooxygenase metabolites or lipoxygenase metabolites being produced was apparently not considered. The potential of bombesin to stimulate lipoxygenase activity in Swiss 3T3 cells has not been examined previously. Results obtained here suggest that the peptide does not stimulate activation of either HETE or leukotriene production in this system. However, use of a powerful stimulus such as the calcium ionophore A23187 provides information about the maximum capacity of these cells to produce hydroxy fatty acids and leukotrienes and illustrates that they have the synthetic capability. Therefore, the lipoxygenase pathway may be potentially important in the action of some agonists on these cells and this may increase the diversity in the cellular arachidonate response between different stimuli.

The limited production of PGE_2 in response to bombesin illustrated in this chapter contrasts with results obtained by Millar & Rozengurt (1988, 1990), where

PGE_2 comprises a substantial proportion of the AA response to this stimulus. It also questions their theory that bombesin stimulates the accumulation of cAMP via a cyclooxygenase–dependent mechanism. They have demonstrated that treatment with indomethacin will partially inhibit the cAMP response. However, they also suggest that PKC may have an additional involvement in this response. The issue remains controversial since other groups have been unable to demonstrate this cyclooxygenase–dependent cAMP accumulation (Murayama & Ui, 1985). In the present study, although the PGE_2 response measured is small, it may still be sufficient to exert important effects within the cell either alone or in cooperation with other factors. Recent evidence has indicated that the liberation of AA and its possible conversion to eicosanoids constitutes one of the early mitogenic signals induced by bombesin in 3T3 cells (Takuwa *et al.*, 1988, 1991; Gil *et al.*, 1991). The present study did not measure the concentration of PGE_2 formed.

The general lack of metabolism of AA in response to bombesin stimulation presents an important feature of the pathway in this system. The question that arises from this concerns the turnover of AA during both phases of the response. If the fatty acid is not metabolised via either cyclooxygenase or lipoxygenase enzymes, how do its levels decline so rapidly during the initial phase? In addition, during the second phase, AA is only metabolised to a limited extent – what eventually causes the response to decline? It is also of interest why the AA remains unmetabolised. This may indicate that the fatty acid itself plays some important intracellular role, a subject which is addressed in detail in Chapter 7.

The possible mechanisms behind turnover of AA in this system deserve consideration. One primary route by which fatty acid turnover can occur is via the deacylation – reacylation or Lands pathway. In this, AA is cleaved by PLA_2 creating a lysophospholipid which is rapidly re–esterified with another fatty acid by acyl coenzyme A:lysophosphatide acyltransferase. The free AA may be re–incorporated into phospholipids via an acyltransferase. Questions exist concerning

the number of different acyltransferase enzymes within cells. For example, in platelets Inoue *et al.* (1984) have suggested the existence of separate 1–acyl GPI and 1–acyl GPC specific acyltransferases. This may also be true for Swiss 3T3 cells and free AA could be re–incorporated into phospholipids in this manner.

Direct transfer of AA between phospholipid subclasses by transacylases has also been shown to occur in a Co–A dependent or independent manner. This has been demonstrated between intact phospholipids and lysophospholipids (Malone *et al.*, 1985) as well as between two intact phospholipids. The majority of studies examining this type of mechanism have been performed on cells of the immune system. However, this type of response may also exist in other cell types. In Swiss 3T3 cells, this mechanism may not be important in the initial AA response but may affect the eventual availability of AA–containing phospholipids for PLA_2 hydrolysis during the secondary phase. Additional work has indicated that AA is initially incorporated into the nuclear envelope and then shunted in a time–dependent fashion into other cellular compartments thus curtailing its availability for release (Caprioti *et al.*, 1988). It is possible that one primary role of the transacylase enzymes is to limit the availability of AA for release by moving it from the site of hydrolysis to other intracellular locations. This type of mechanism may lead to a slow decline in free AA as in the secondary phase of AA observed in this system. It is likely that the decrease in AA release in response to bombesin may be due to a combination of reacylation and transacylation, particularly during the first phase of release. The mechanism behind the decline of the second phase may include other factors which will switch off PLA_2 activation after a certain period. This may involve either a negative feedback mechanism exerted via AA itself or possibly the activation of a lipocortin–type factor, causing inhibition of the PLA_2 response. This does not, however, preclude the concept that reacylation and transacylation may also be important in this latter phase. It has previously been shown that bombesin–stimulated PLC activation is subject to desensitization which may occur at the level

of receptor or G–protein (Plevin *et al.*, 1990). This may also occur with bombesin–stimulated PLA_2 activity and may be responsible for switching off the second phase of AA release.

The precise nature behind AA turnover in the absence of its metabolism, as seen in this system, is dependent upon a complete understanding of the exact role of various pathways involved in the regulation of phospholipid fatty acid remodelling and fatty acid relocation. Until then, it is only possible to speculate on how this occurs in bombesin–stimulated 3T3 cells.

CHAPTER 7

IS ARACHIDONIC ACID A POTENTIAL SECOND MESSENGER IN SWISS 3T3 CELLS?

INTRODUCTION

Results presented in the previous chapters have indicated that the AA response to bombesin in Swiss 3T3 cells is unusual for a number of reasons: the fatty acid is released rapidly from its phospholipid precursor with a timecourse comparable to that observed for Ins(1,4,5)P_3 generation; the biphasic nature of the response provokes questions concerning the mechanism(s) behind release; and the lack of metabolism of AA while in the 'free liberated fatty acid' state presents the possibility that AA itself may have some important role within the cell. With regard to this, it has been proposed that AA can act as a second messenger (Abramson *et al.*, 1991) because it can exert direct effects on signal transduction. These responses are elicited by AA itself and not its metabolites.

Second messengers may exert their effects by activating an appropriate protein kinase. Previous reports have suggested that AA can activate certain forms of PKC (Murakami & Routtenberg, 1985; Nishizuka, 1988 and Shearman *et al.*, 1989). It also appears that AA can exert important effects on intracellular calcium in a variety of systems (Chan & Turk, 1987; Chow & Jondal, 1990 and Randriamampita & Trautmann, 1990) and that this may occur independently of PKC activation (Alonso *et al.*, 1990). This points to the possibility of AA having a similar role to Ins(1,4,5)P_3 in stimulating intracellular calcium release. Chow and Jondal (1990) have suggested that AA stimulates an increase in cytosolic calcium in T cells by mobilising the Ins(1,4,5)P_3–sensitive pool through a mechanism independent of phosphoinositide turnover. This may suggest a separate yet cooperative pathway with Ins(1,4,5)P_3 in the regulation of calcium release.

If AA is to be assigned a second messenger role, it must exert biological effects at appropriate concentrations. In this chapter, where the potential role of the first phase of AA released upon bombesin stimulation was examined, the concentration of AA produced during this initial phase of release had to be determined. Since the amount of AA liberated from phospholipids is quite small, in order to accurately quantitate the mass of AA released from cells using standard

extraction techniques followed by gas liquid chromatography, large numbers of cells would be required. For this reason, an alternative method was used (see Results).

The work presented in this chapter examines the possibility that the AA released in response to bombesin stimulation may be involved in regulating both short– and long–term signalling events in Swiss 3T3 cells. The possible importance of this pathway as a separate entity and also its potential to interact with other transduction pathways is considered.

RESULTS

The amount of free AA generated in response to bombesin stimulation was measured using the 15–HETE assay system described in Chapter 2. This involves synthetically converting the AA to 15–HETE using soyabean 15–lipoxygenase and measuring the 15–HETE produced with a radioimmunoassay kit. The original amount of AA was calculated based on the efficiency of conversion of the enzyme. An effective separation system was required for identification of both AA and 15–HETE. The methods tested involved bond–elut columns with a solvent system of petroleum ether/diethyl ether (9:1 v/v) and methanol for recovery of AA and 15–HETE respectively, and also sep–pak C–18 columns using a range of ethanol concentrations for elution. Neither of these methods gave satisfactory recovery of 15–HETE. Therefore, hplc was used with a lichrosorb C–18 column and a solvent system comprising an increasing gradient of methanol against ammonium acetate (0.5%). This proved reliable for separation and identification of both AA and 15–HETE. It was also possible, from hplc traces, to calculate the percentage conversion of AA to 15–HETE by the 15–lipoxygenase in the relevant experiments. Using this system, the retention times for AA and 15–HETE were 11.4 min and 6.2 min respectively. This is shown in Figure 7.1(a).

Soyabean 15–lipoxygenase was used to convert the AA produced by bombesin stimulation to 15–HETE for radioimmunoassay. Preliminary experiments were conducted prior to this to obtain the optimal conditions for conversion. These were performed using standard [^{3}H]AA (spec.ac. 200Ci/mmol). 0.6μCi of this was used for reaction with various concentrations of the enzyme ranging from 1pM to 1nM in 0.1M borate buffer, pH9.0 for 90 min. The reducing agent sodium borohydride was included to prevent HPETE formation. A concentration of 10pM gave an optimum conversion rate – 89% of AA was converted to 15–HETE over the time tested. Variations in time and concentrations did not improve on this. In addition, antibody binding in the radioimmunoassay was not affected at this concentration of enzyme whereas at

concentrations between 50pM and 1nM, it was substantially reduced. Hplc traces demonstrating the enzyme catalysed conversion of AA to 15–HETE are shown in Figures 7.1(b) and (c). Higher enzyme concentrations (>100pM) resulted in the formation of two DHPETES – 15,15 DHPETE and 8,15 DHPETE with no 15–HETE production and were therefore, unsuitable for this assay.

Having established the conditions for the enzyme reaction using standards, experimental samples that had been stimulated with bombesin for 30 sec were prepared as described in Chapter 2. Since the experiment involved an overnight incubation, standards using [^{3}H]AA were prepared alongside samples to control for the conversion to 15–HETE. The hplc trace shown in Figure 7.2 is for the conversion achieved during this experiment (approx. 85% of AA converted). Figure 7.3 shows the standard curves obtained with 15–HETE from the radioimmunoassay kit and with that from the samples tested. The actual values for AA are shown in the table that forms part of Figure 7.3. These show that the resting level of AA was approximately 340pg/10^4 cells and this increased to approximately 1.6ng/10^4 cells in bombesin–stimulated samples.

In order to calculate the concentration of AA produced upon stimulation, it is necessary to determine the cell volume. This was achieved using the method described in Chapter 2. The mean cell volume obtained from 18 samples was 155µl/10^6 cells. Using this value, basal concentrations of AA were calculated to be $0.85 \pm 0.32\mu M$ and stimulated values were $4.1 \pm 2.8\mu M$. This is an increase of 4.7–fold upon agonist stimulation which correlates well with results obtained in Chapter 4. It is worth noting that these concentrations are very similar to those obtained for the second messenger Ins(1,4,5)P_3 in a study by Cook *et al* (1990). These values relate to the first phase of AA release in response to bombesin stimulation. The second phase of release appears to be of greater magnitude and, as a result, will probably induce a greater increase in AA concentration.

As previously mentioned, AA may be involved in both long–term and short–term intracellular effects. The present study has examined both of these

possibilities. AA may play a role in mitogenesis, therefore, the effect of the fatty acid on DNA synthesis was examined. This may reflect its importance in long–term cellular events. In addition, its involvement in short–term responses was studied by examining the effect of exogenously added AA on intracellular calcium levels.

Initially, the ability of AA to act as a co–mitogen was studied. AA has previously been shown to stimulate DNA synthesis in Swiss 3T3 cells (Takuwa *et al.*, 1988) and this has been suggested to occur via PKC activation. In the present study, the effect of exogenously added AA on both insulin–stimulated and EGF–stimulated [^{3}H]thymidine incorporation was examined. As shown in Figure 7.4, treatment of cells with 30μM AA alone had no effect. Insulin (0.2μM) and EGF (10nM) also promoted little thymidine incorporation. However, addition of 30μM AA together with these agonists, enhanced the incorporation of [^{3}H]thymidine achieved. Figure 7.5 shows the effect of increasing concentrations of AA on EGF–stimulated thymidine incorporation. The increase in response is evident at 10μM AA and maximal between 30–100μM AA, concentrations which may correspond to those obtained in the second phase of bombesin–stimulated AA release. It has previously been shown by Takuwa *et al.* (1988) that other polyunsaturated fatty acids such as oleic acid and linoleic acid are ineffective in stimulating thymidine incorporation. Therefore, this effect may be specific for AA.

To examine a role for AA in a short–term response, the effect of exogenously added AA on intracellular calcium levels was investigated. Cells were loaded with the calcium indicator dye, Indo–1–AM, as described and stimulated with various concentrations of AA ranging from 10μM to 100μM, the lower value being very close to that generated during the first phase of release in response to bombesin stimulation (see previously). Figure 7.6(a) shows that 10μM AA is capable of generating an increase in the intracellular calcium concentration and may imply that the initial phase of bombesin–stimulated AA release plays such a role. The responses obtained were very variable between experiments as illustrated in

Figure 7.6(b) where a single dose of 50μM AA was added to different cell populations. The time taken for calcium to peak ranged from 5 sec to 3 min after addition of AA. This may illustrate differences in the sensitivity of calcium stores to AA between populations or may reflect differences in the time of entry of the fatty acid. The duration of the response varied between that of a transient peak, similar to the bombesin–stimulated changes in calcium, and a more sustained increase. The effect on intracellular calcium due to AA was dose–dependent as shown in Figure 7.7. An obvious problem with these experiments is the inability to mimic exactly the changes in the intracellular concentration of AA induced by an agonist. The increase in calcium levels in response to AA within Swiss 3T3 cells may not be dependent on the steady state concentration of AA within the cells, but rather on the rate of rise of the intracellular AA concentration which is rapid as illustrated in Chapter 4. This is a problem when using exogenous AA which would enter the cell more slowly than required and may be metabolised quickly. Figure 7.8 illustrates (a) the effect of AA (30μM) pretreatment on bombesin–stimulated increases in calcium and (b) the effect of bombesin (10nM) pretreatment on AA–stimulated increases in intracellular calcium. Addition of 100μM AA for 2 min prior to bombesin stimulation caused a slight decrease in the bombesin–stimulated increase in calcium levels. This could be due to AA causing calcium release from Ins(1,4,5)P_3 regulated stores, which would be reflected in the bombesin response, making it slightly smaller. It may even be possible that AA – induced calcium release from an Ins(1,4,5)P_3–insensitive store affects the bombesin response in some manner. The effect of bombesin pretreatment followed by AA is shown in Figure 7.8(b). The AA–stimulated change in intracellular calcium concentration is almost completely abolished in cells that have already received bombesin treatment. (although in view of the marked variability of AA responses alone,this should be interpreted with caution). This may reflect the fact that the intracellular AA levels generated by bombesin have already promoted an increase in the intracellular calcium concentration and are still sufficiently high that further treatment with AA has no effect. Alternatively,the AA-sensitive stores may not

have been given sufficient time to refill after the challenge with bombesin. It is not possible to tell from these experiments whether AA releases calcium from Ins(1,4,5)P_3–sensitive stores, or from completely separate stores. More sensitive measurements, involving single cell studies, would be required to answer this.

Since increases in the intracellular calcium concentration are usually associated with formation of the second messenger Ins(1,4,5)P_3, it was important to consider in the present study whether exogenously added AA caused an increase in phosphoinositide hydrolysis, leading to Ins(1,4,5)P_3 formation. This could explain the observations achieved. The effect of increasing concentrations of AA on total inositol phosphate production was therefore examined (Fig.7.9) and it was found that concentrations of 30μM and above caused stimulation of inositol phosphate production, although even at 100μM AA there was only a 1.6–fold increase.

Figure 7.1 Conversion of arachidonate to 15–HETE using soyabean 15–lipoxygenase

(a) a representative hplc chromatogram of AA and 15–HETE standards, (b) a chromatogram of AA before enzyme treatment and (c) the same sample after a 90 min treatment with 10pM 15–lipoxygenase in 0.1M phosphate buffer, pH7.4. Isocratic solvent system; methanol 68.7% (methanol 20%/0.5% ammonium acetate 80%) 31.3%. Flow rate 2ml/min.

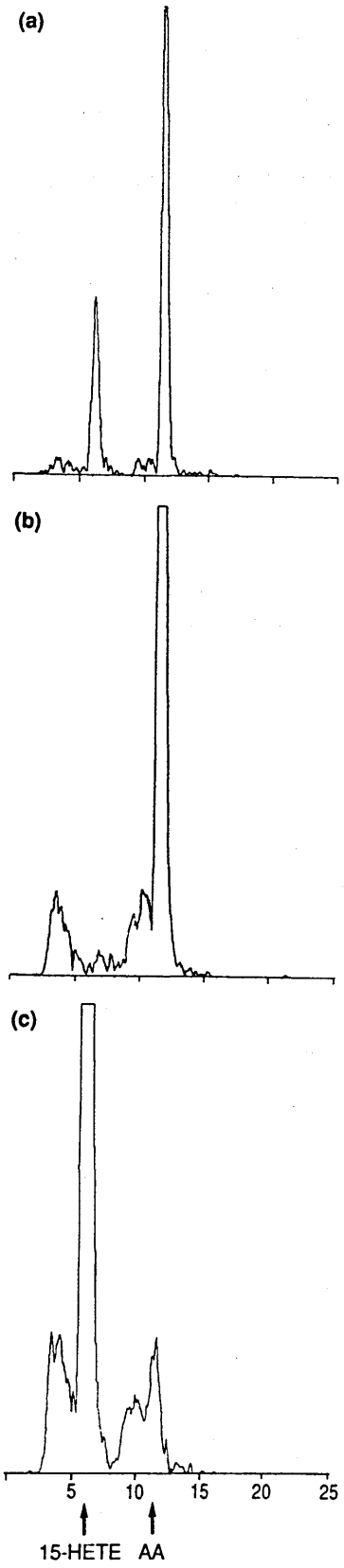

(a)
(b)
(c)
5
10
15
20
25
15-HETE
AA

Figure 7.2 Conversion of the arachidonate produced in bombesin–stimulated 3T3 cells to 15–HETE

A typical hplc chromatogram of AA and 15–HETE present in samples stimulated with 617nM bombesin for 30 sec and treated with 15–lipoxygenase as described in Materials and Methods section. Results are from a single typical experiment where n=3.

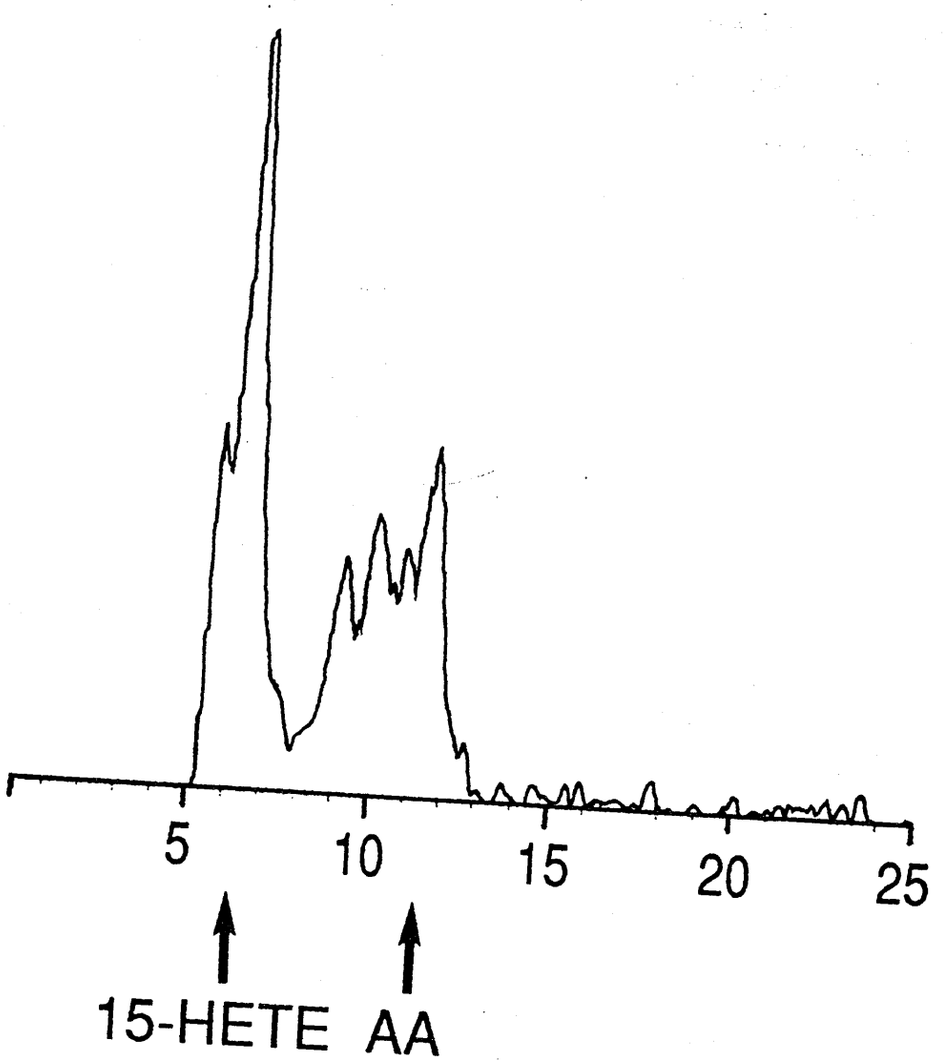

5
10
15
20
25
15-HETE
AA

Figure 7.3 Standard curve for 15–HETE produced in 15–lipoxygenase treated samples

A typical standard curve for 15–HETE standards (8–312.5pg/tube) (●) and 15–HETE produced in lipoxygenase treated samples (▲) as measured using a 15–HETE radioimmunoassay. Results are from a single typical experiment where n=3.

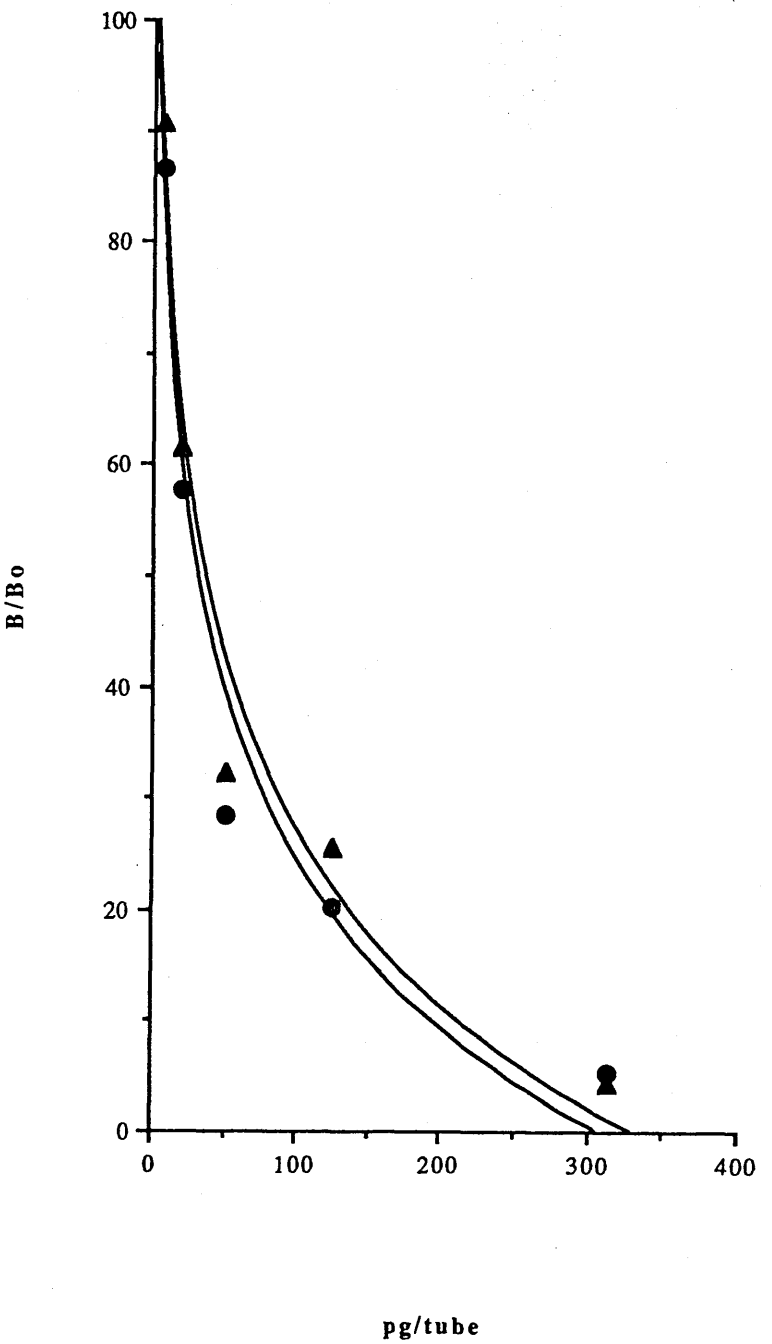

100
80
60
40
20
0
B/Bo
0
100
200
300
400
pg/tube

Table 7.1 Differences in 15–HETE production between bombesin–stimulated and control samples

Values obtained from the standard curve are shown for samples diluted 2–fold to 16–fold in both control and bombesin–stimulated cells. Values are expressed as ng/10^4 cells. These values have been corrected to allow for the percentage conversion achieved. This is a single typical experiment representative of two others.

Sample	$\%B_0/TC$	$\%B/B_0$	pg/tube	$ng/10^4$cells
TC				
NSB				
B_0	35.4			
control		-	-	-
bombesin		-	-	-
control(~2)		-	-	-
bombesin(~2)		-	-	-
control(~4)		27.1	72	0.28
bombesin(~4)		1.5	600	2.4
control(~8)		37.2	45	0.36
bombesin(~8)		5.9	250	2.0
control(~16)		71.0	23	0.37
bombesin(~16)		23.3	75	0.9

Figure 7.4 Effect of arachidonate on EGF–stimulated and insulin–stimulated [^{3}H]thymidine incorporation

Confluent Swiss 3T3 cells were incubated with 0.5μCi/ml [^{3}H]thymidine and the indicated agonist for 24h. The effect on thymidine incorporation is shown. Results are expressed as means ± S.D. from a single typical experiment where n=3.

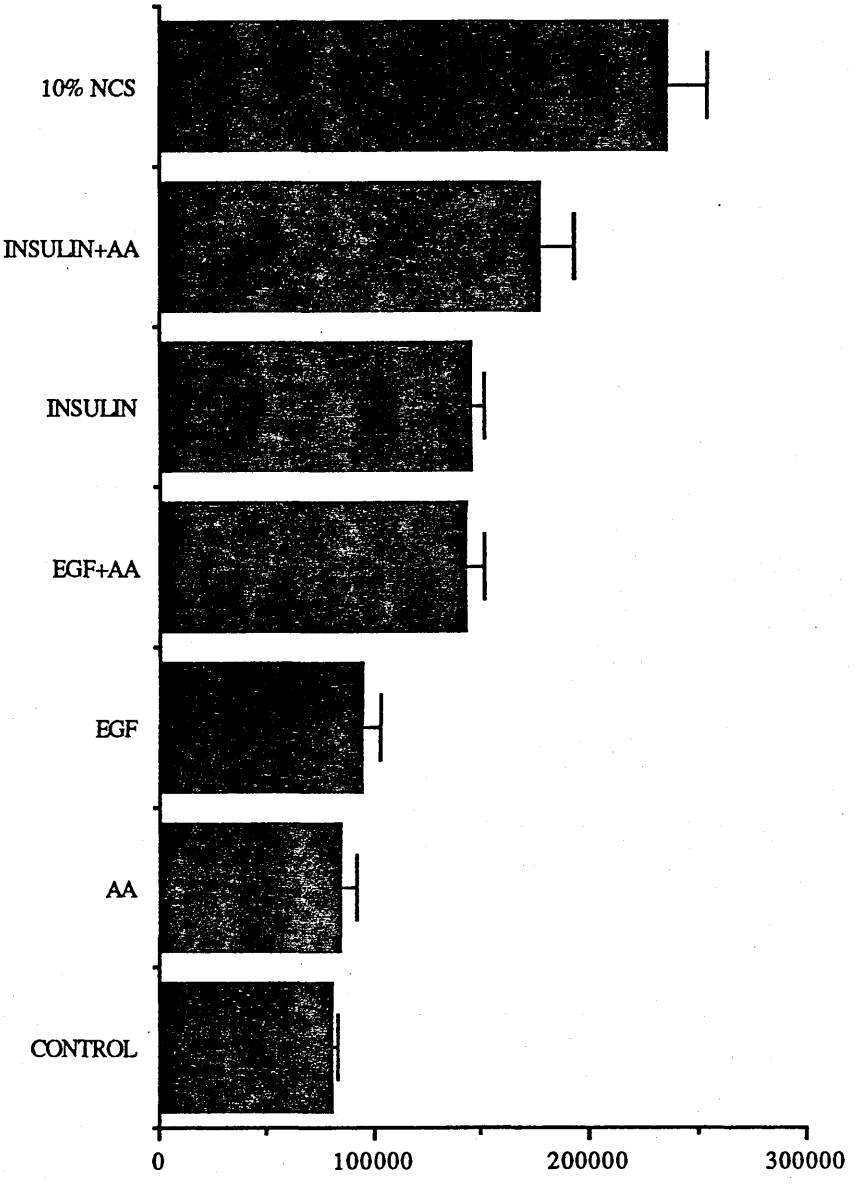

10% NCS
INSULIN+AA
INSULIN
EGF+AA
EGF
AA
CONTROL
0
100000
200000
300000
dpm in Thymidine

Figure 7.5 Dose–dependence of the effect of arachidonate on EGF–stimulated [^{3}H]thymidine incorporation

[^{3}H]thymidine labelled cells were incubated with EGF(10nM) and increasing concentrations of AA for 24h. The results are expressed as means ± S.D. from a single typical experiment where n=3.

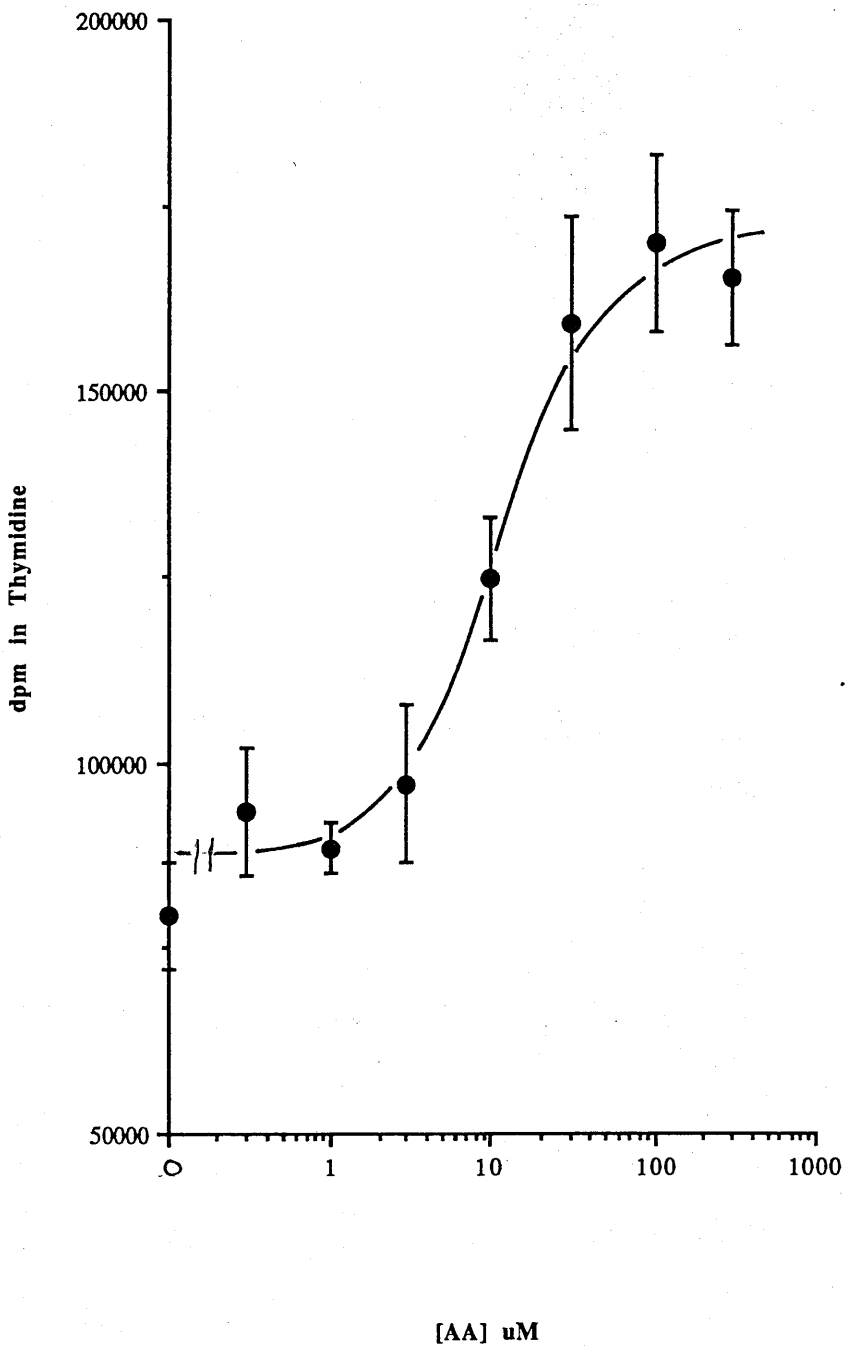
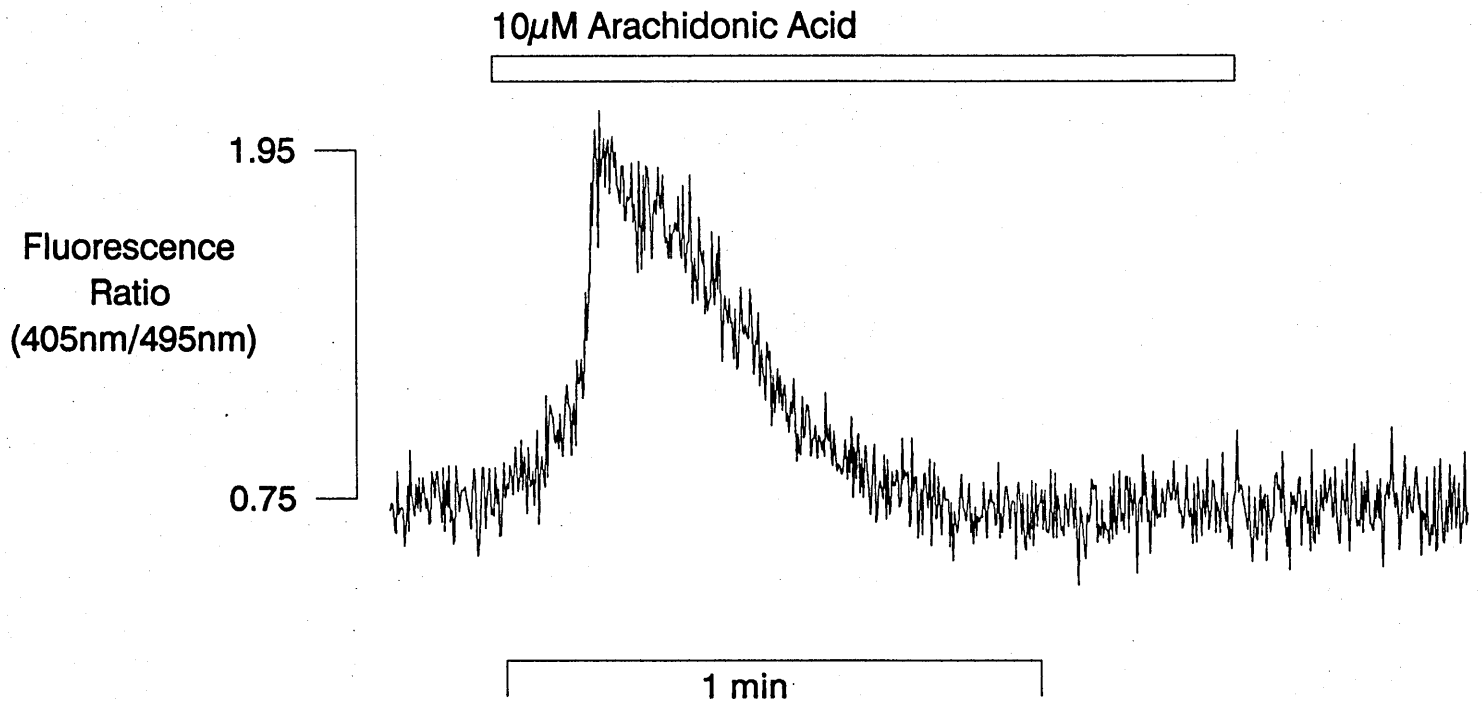

200000
150000
100000
50000
dpm in Thymidine
0
1
10
100
1000
[AA] uM

Figure 7.6 Variations in arachidonate–stimulated increases in intracellular calcium concentration

Swiss 3T3 cells, grown on glass coverslips, were loaded with 5μM Indo-1–AM and stimulated with (a) 10μM, (b) 50μM AA. changes in calcium, as indicated by the fluorescence ratio, were measured as described in the Methods section. Results are from single experiments typical of three.

N.B. The limited solubility in aqueous solutions towards the higher range of AA concentrations tested suggests that results with such concentrations should be interpreted with caution.


10μM Arachidonic Acid
1.95
Fluorescence
Ratio
(405nm/495nm)
0.75
1 min

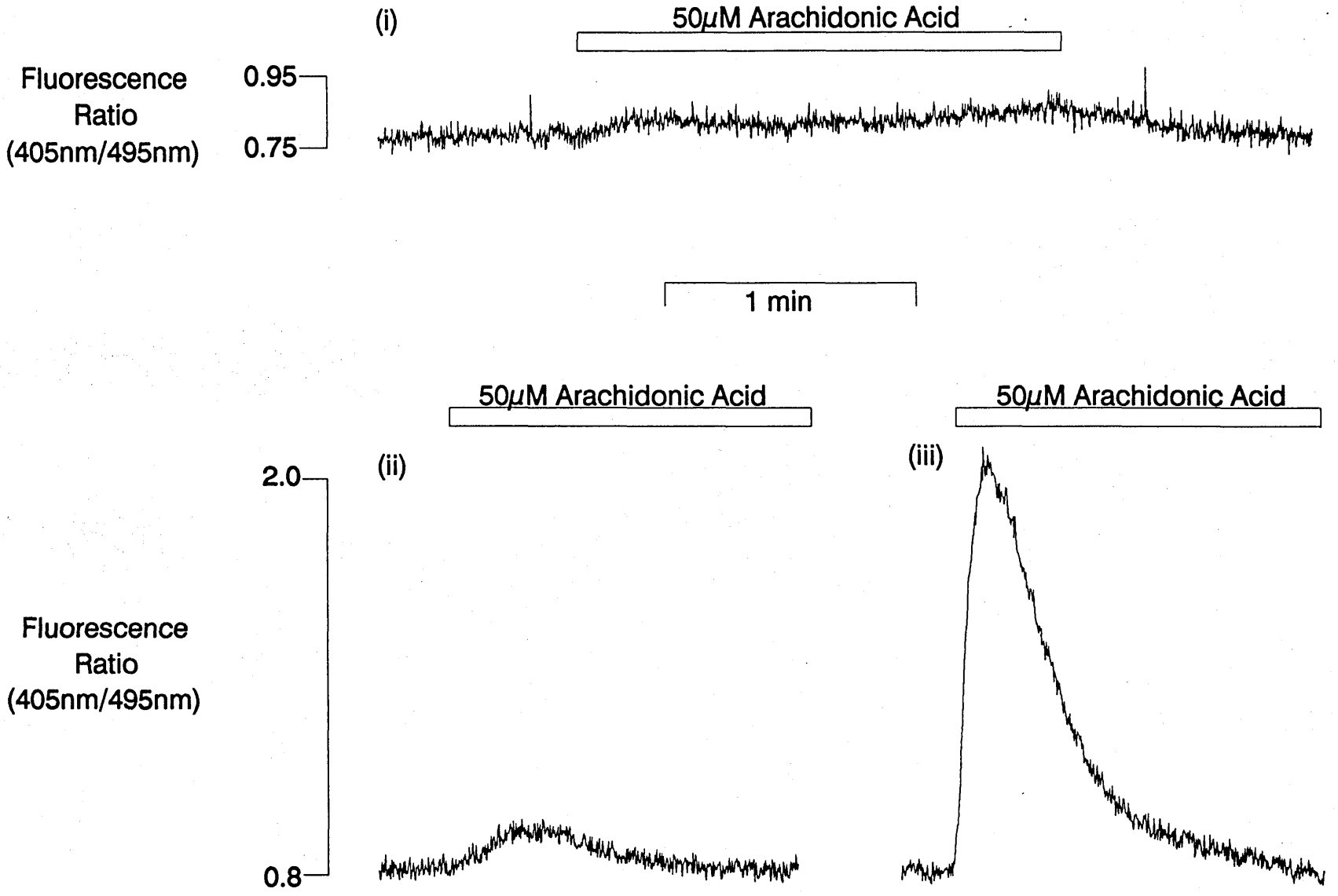

(i)
50μM Arachidonic Acid
Fluorescence
Ratio
(405nm/495nm)
0.95
0.75
1 min
50μM Arachidonic Acid
50μM Arachidonic Acid
(ii)
(iii)
2.0
0.8
Fluorescence
Ratio
(405nm/495nm)

Figure 7.7 Dose–dependence of arachidonate–stimulated changes in intracellular calcium

3T3 cells, loaded with 5μM Indo–1 were stimulated with increasing concentrations of AA. Changes in calcium were monitored as described in the Methods section. Results are from single experiments typical of three.

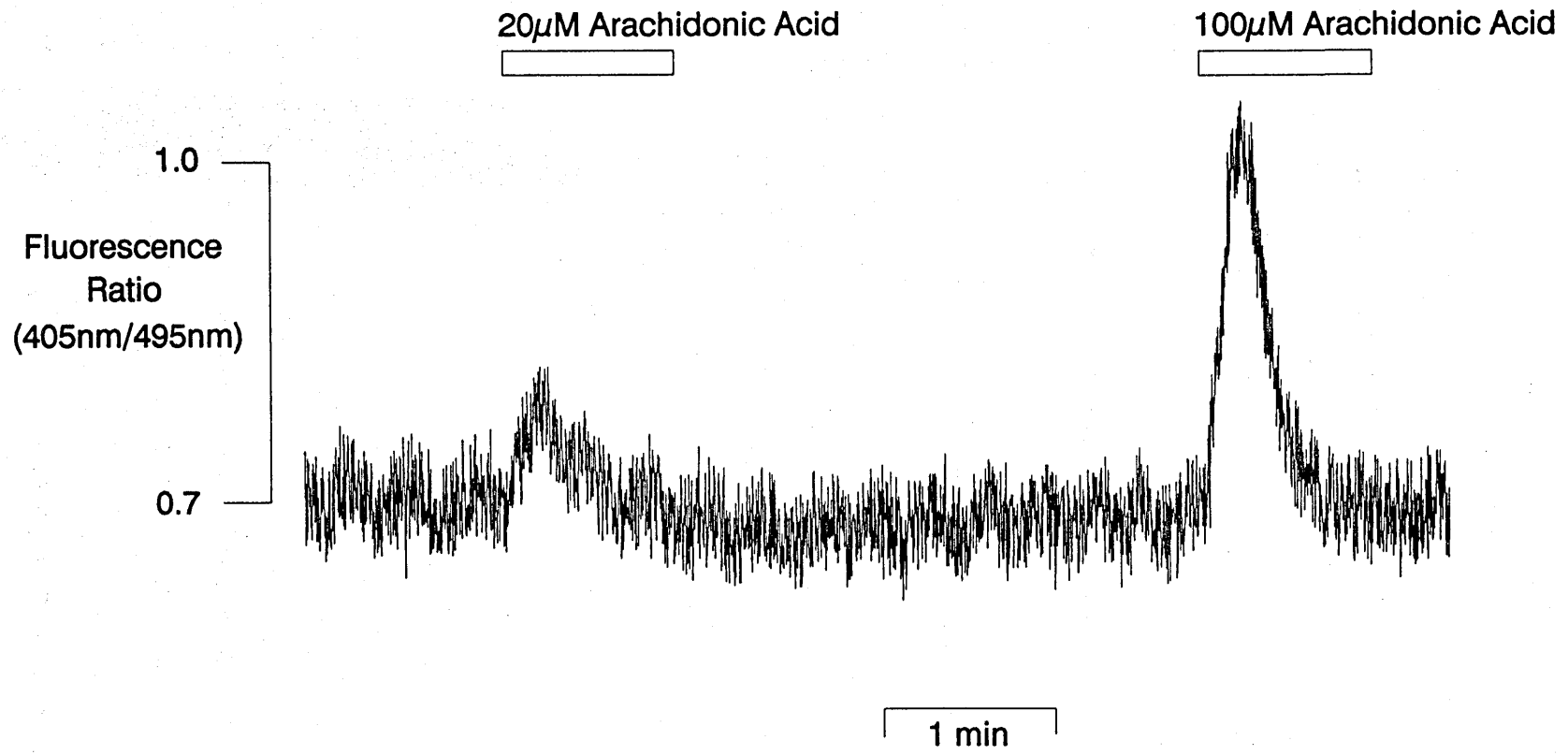
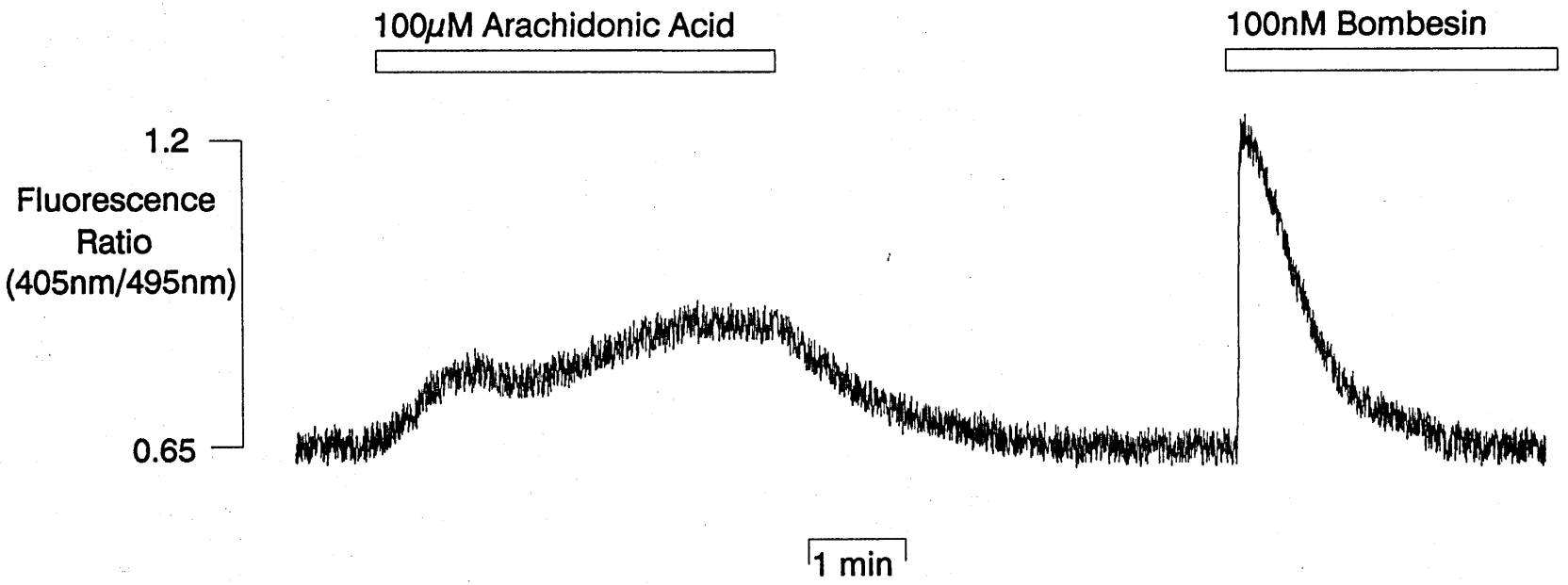

20μM Arachidonic Acid
100μM Arachidonic Acid
1.0
Fluorescence
Ratio
(405nm/495nm)
0.7
1 min

Figure 7.8(a) Effect of arachidonate pretreatment on bombesin–stimulated calcium release

Swiss 3T3 cells, loaded with 5μM Indo–1, were stimulated with 100μM AA then washed in HBG for 3 min and stimulated with 100nM bombesin. Changes in intracellular calcium were measured as described in the Methods section. Results are from single experiments typical of three.

Figure 7.8(b) Effect of bombesin pretreatment on arachidonate–stimulated calcium release

Swiss 3T3 cells, loaded with 5μM Indo–1–AM, were stimulated with 100nM bombesin then washed for 3 min in HBG. This was followed by stimulation with 100μM AA. Changes in intracellular calcium were measured as described. Results are from single experiments typical of three.


100μM Arachidonic Acid
100nM Bombesin
1.2
Fluorescence
Ratio
(405nm/495nm)
0.65
1 min

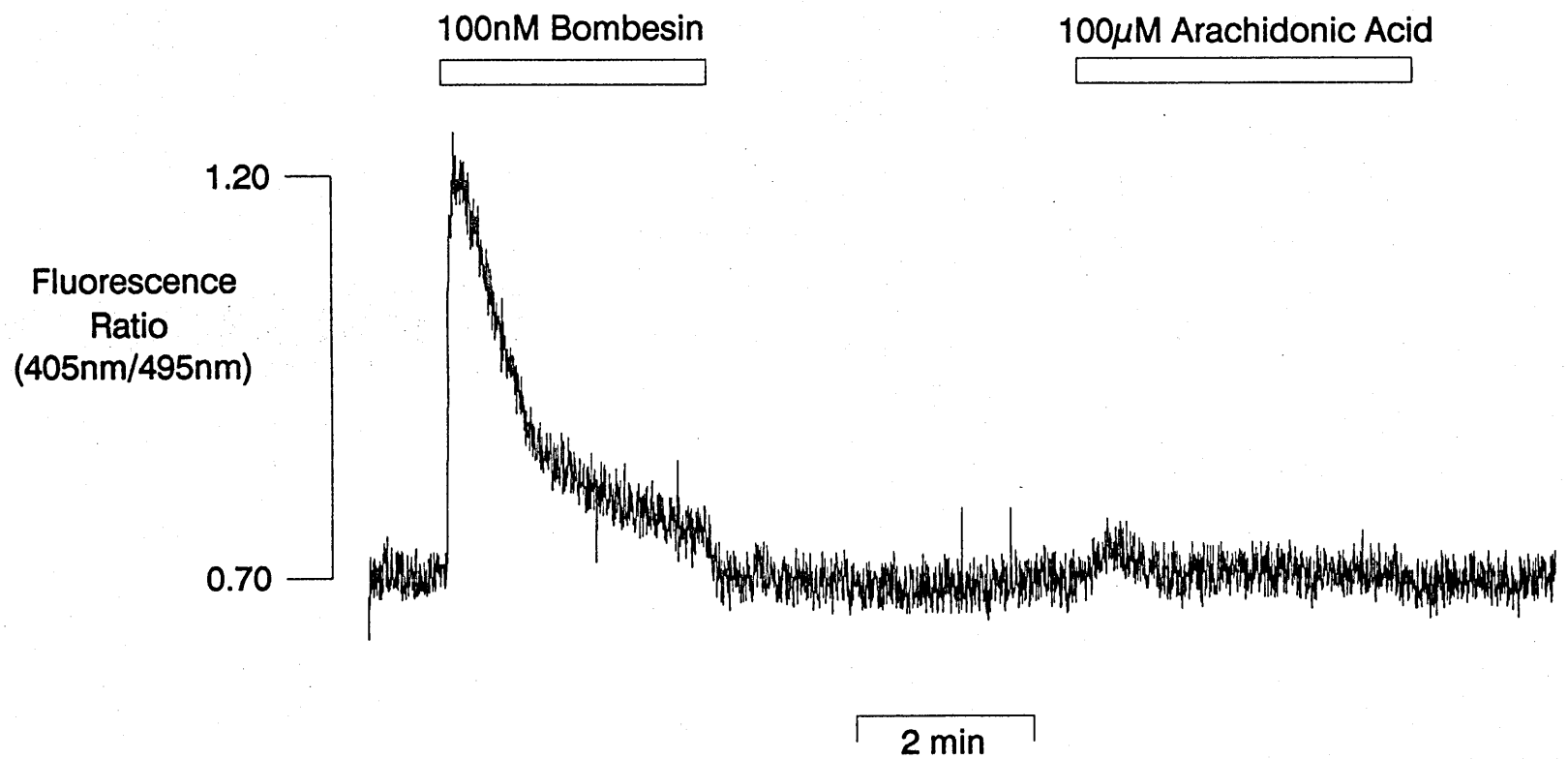
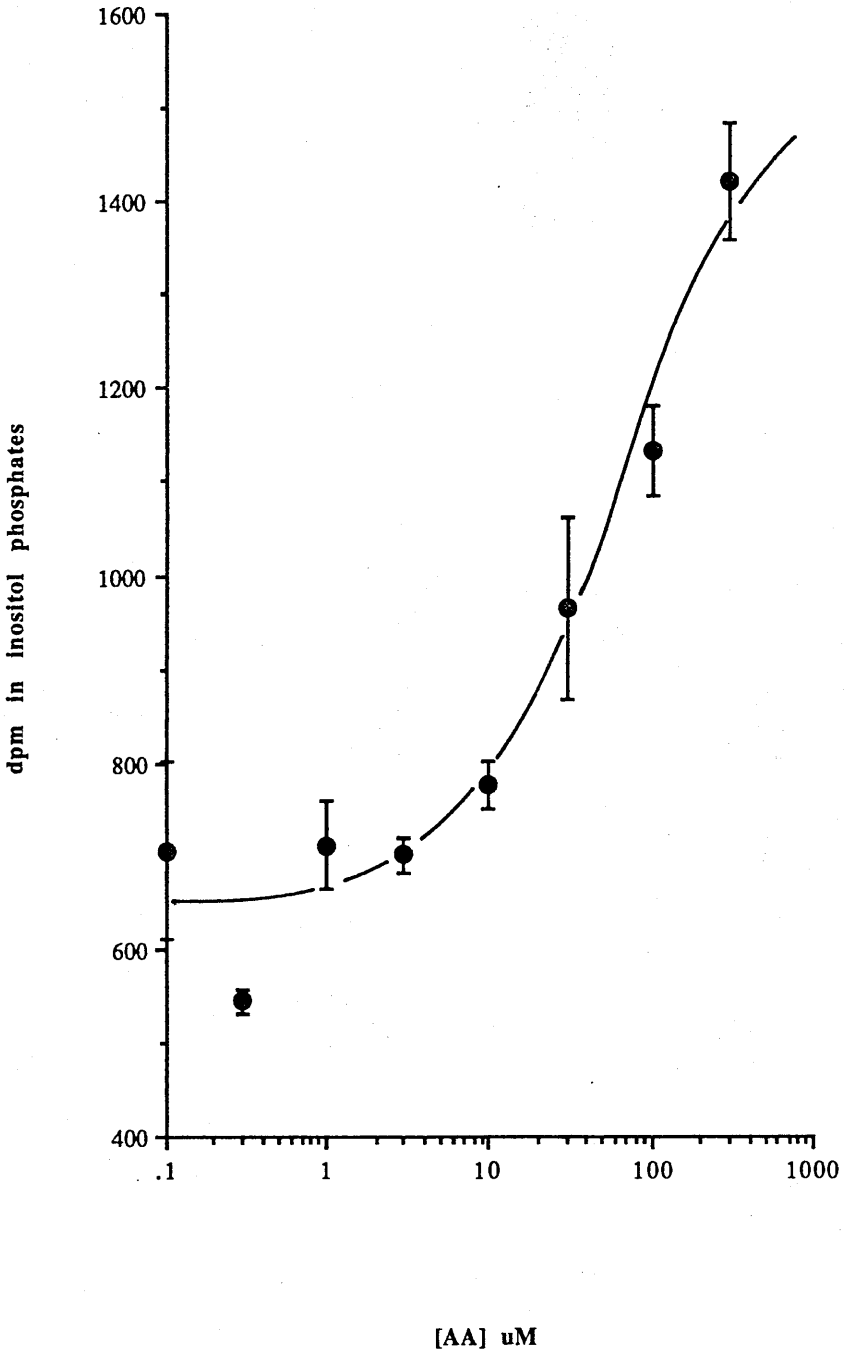

100nM Bombesin
100μM Arachidonic Acid
1.20
Fluorescence
Ratio
(405nm/495nm)
0.70
2 min

Figure 7.9 Effect of increasing concentrations of arachidonate on total inositol phosphate production

[^{3}H]inositol labelled cells were incubated with increasing concentrations of AA in a buffer containing 10mM LiCl. Each point represents the mean ± S.D. of triplicate determinations from a single typical experiment where n=3.


1600
1400
1200
1000
800
600
400
dpm in inositol phosphates
.1
1
10
100
1000
[AA] uM

DISCUSSION

It is becoming increasingly obvious that the term 'second messenger' may encompass a wider range of substances than previously thought. Results presented in this chapter suggest that AA, produced in response to bombesin stimulation, may play such a role in Swiss 3T3 cells. The concentration of AA produced during the first phase of release is in the low micromolar range. This is similar to the concentration of Ins (1,4,5)P_3 required to stimulate an increase in the intracellular calcium concentration in Swiss 3T3 cells.

AA can be formed at an 'upstream' or 'downstream' site. The first site is directly linked to a ligand–receptor – G protein complex as described by Burch *et al.* (1986). The second site is distal to the receptor complex and requires elevation of intracellular calcium and activation of PKC (Irvine, 1982). Both types of AA release are evident in the bombesin–stimulated Swiss 3T3 cell and may contribute to AA playing the role of a multifunctional compound. In this context, AA appears to be involved in exerting both long– and short–term cellular responses, namely DNA synthesis and intracellular calcium mobilisation. The apparent concentration of AA produced in response to bombesin during both phases may be sufficient for each of these responses to occur.

From results obtained in the present study (Figs. 7.4 & 7.5), it appears that the mitogenic action of bombesin is partly due to the release of AA which may stimulate DNA synthesis in a cooperative manner with other intracellular signalling pathways. This has been suggested in other studies on Swiss 3T3 cells (Gil *et al.*, 1991; Takuwa *et al.*, 1991) and indicates the potential importance of this pathway in cell proliferation. However, it is difficult to directly relate results obtained with exogenously added AA to those achieved with intracellularly–generated AA. Addition of AA to the cells may cause general membrane perturbation and increased permeability which may affect the interpretation of results. Although this factor was not examined in the present study, previous work by Chow & Jondal (1990)

has shown that concentrations of up to 50μM AA did not affect membrane integrity of T cells as judged by ^{51}Cr release.

The ability of AA to cause intracellular calcium mobilisation (Fig.7.6) may be a more general phenomenon than initially thought since similar effects have been reported in other systems (Wolf *et al.*, 1986; Chow & Jondal, 1990). The calcium mobilisation observed here may be an intrinsic activity of the fatty acid itself. However, to rule out the possibility that AA metabolites are involved, inclusion of a cyclooxygenase inhibitor such as indomethacin would be useful. Hokin (1985) has suggested that AA itself stimulates an increase in intracellular calcium based on the large amounts of unmetabolised AA and the relatively small amounts of AA metabolites released by other stimuli in other systems. The possibility exists, however, that the increase in intracellular calcium observed is due to entry of exogenous AA rather than mobilisation of intracellular AA. As stated previously, this is a problem when attempting to mimic intracellular events with exogenously added substances.

The mechanism by which AA induces an increase in the intracellular calcium concentration in 3T3 cells is unclear. Various possibilities exist, AA may interact directly with the endoplasmic reticulum membrane and induce calcium permeability. Alternatively, it may act on the calcium sequestration or release mechanism, thus disrupting the balance between the rates of uptake and leakage of calcium across the endoplasmic reticulum membrane. This cycling of calcium is a rapid process and disturbing the equilibrium via inhibition of the calcium pump could elevate intracellular calcium. Whether the calcium released in this system by AA is from an $Ins(1,4,5)P_3$–sensitive pool is not known, although this possibility has been suggested by Chow and Jondal (1990) in T cells. An obvious possibility is that AA causes calcium release indirectly by stimulating production of $Ins(1,4,5)P_3$. Although exogenously added AA causes a slight increase in phosphoinositide hydrolysis (Fig.7.9), it is possible to dissociate this from involvement in AA–induced increases in intracellular calcium by stating two important observations

from the present studies. At concentrations which do not stimulate phosphoinositide hydrolysis i.e. <30μM, an increase in intracellular calcium is still observed with AA. Furthermore, the maximum degree of stimulation of phosphoinositide hydrolysis achieved with AA is smaller than that achieved with 2nM bombesin which stimulates a smaller increase in intracellular calcium levels than that achieved with 50μM AA.

It is conceivable that AA may promote calcium release from, as yet, unidentified stores. The possibility exists that different types of rapidly exchanging calcium stores exist with different release mechanisms and possibly different molecular components. In PC12 cells, two different mechanisms have been suggested to co–exist in the same store (Meldolesi *et al.*, 1991). Use of antibodies against the Ins(1,4,5)P_3 receptor has indicated the existence of novel structures termed calciosomes which exclude certain components of the endoplasmic reticulum and may be an important source of alternative calcium stores. Two situations may exist, one in which there are areas of the cell containing calcium stores that could respond exclusively to AA and act independently or cooperatively with the Ins(1,4,5)P_3–sensitive stores in response to particular stimuli; the other in which one type of store responds to both AA and Ins(1,4,5)P_3 depending on the stimulus. This theory lends credence to the possibility that different agonists may promote calcium release via different mechanisms and may not depend entirely on Ins(1,4,5)P_3 production. A situation may then be envisaged in which a ligand will induce AA release without enhancing phosphoinositide turnover and still activate similar intracellular pathways (calcium release and PKC activation). This has already been demonstrated in neutrophils stimulated with LTB_4 (Volpi *et al.*, 1984) and in Swiss 3T3 cells stimulated with mastoparan (Gil *et al.*, 1991).

In releasing calcium, AA may have a positive effect on the biochemical pathway responsible for its own formation. The second phase of AA release is calcium–dependent and may be sensitive to the calcium released during the first

phase. This type of mechanism may allow AA to propagate the initial bombesin signal and highlights the possibility of an 'AA cycle' existing in the cell.

The possibility that AA may activate PKC in Swiss 3T3 cells has not been examined in the present study. It is known that Swiss 3T3 cells contain the α–iso form of PKC although the existence of other isoforms is uncertain. A major obstacle to assignments of a physiological role for AA in PKC activation is the very high concentration needed for enzyme activation (Naor, 1991). However, the β and γ isoforms at least, may be activated by more physiological concentrations of the fatty acid. The possibility exists that AA may act independently or in cooperation with DAG in promoting PKC activation in 3T3 cells. This has been suggested in other systems (Shearman *et al.*, 1991; Lester *et al.*, 1991) and may be accomplished either via modification of the DAG binding site or by alteration of the physical properties of the membrane such that more of the enzyme is activated. These studies indicate that PKC is under more widespread regulation than previously recognised.

The inclusion of AA as a second messenger in Swiss 3T3 cells may have certain advantages for the cell. It will increase the cells options in the way it responds to a particular agonist and will also allow an increased potential for control over a particular response. Both of these facts may be of extreme importance not only in the normal functioning of the cell but also in the behaviour of the cell in abnormal or diseased states.

CHAPTER 8
CONCLUSIONS

CONCLUSIONS

Contrary to long–standing belief, the present study demonstrates that PLA_2 activation may occur independently and in parallel with PLC and may be a signalling pathway generating the 'second messenger' AA. This may act independently or in cooperation with the more well established messengers $Ins(1,4,5)P_3$ and DAG to promote, for example, mitogenesis. Results presented in Chapter 7 suggest that AA is present in micromolar levels in stimulated cells. This is sufficient to promote an increase in the intracellular calcium concentration and may possibly exert other intracellular effects yet to be examined. The lack of metabolism of AA at early time points of bombesin stimulation in 3T3 cells suggests that any rapid intracellular effects will be due to the fatty acid itself. Many previous studies have shown little scope when examining this pathway and have avoided detailed studies on the timing of AA release and the potential for metabolism in agonist–stimulated cells (Burch & Axelrod, 1987; Slivka & Insel, 1988; Miller & Rozengurt, 1990). The studies presented here suggest that in response to bombesin, free AA can be generated intracellularly at least as fast as $Ins(1,4,5)P_3$ and DAG. In addition, this AA is not metabolised. Information presented in Chapter 6 addresses the capacity of 3T3 cells to metabolise AA. PGE_2 can be produced after longer (> 10 min) stimulation with bombesin and may therefore play a role at later stages of the response. No lipoxygenase products are formed in response to bombesin. However, this may be due to limitations of the agonist rather than of the cell since information presented in Chapter 6 suggests 3T3 cells possess lipoxygenase enzymes which are, nevertheless,, substantially less active than those in inflammatory cell types. It is highly possible though that these enzymes can be activated in 3T3 cells via certain agonists whose physiological effects may require stimulation of this metabolism. The potential of the PLA_2/AA pathway to generate an array of different metabolites offers huge scope for different intracellular actions in various cell types. In 3T3 cells this may even be extended to

differences in metabolite production between phases of release within the same cell, perhaps to different agonists.

This diversity of actions offered by the PLA_2/AA pathway may be further increased when considering the potential for interaction with other transduction pathways stimulated by bombesin. The pattern and timing of these early signals may be important in the generation of later events. The degree of interdependence between PLA_2, PLC and PLD may vary between cell types and agonists and may indicate important differences in long–term responses. A hierarchy of importance in the timing of activation of certain pathways may exist. For example, in some situations PLC would have to be activated first, leading to subsequent PLA_2 and PLD activation. In contrast, some agonists may activate PLA_2, PLC and PLD independently yet in parallel. This may require increased regulation in the coupling mechanism between the receptor and effector systems. Another scenario exists whereby PLA_2 may be activated initially, possibly leading to PLC and/or PLD activation. Systems are now being identified in which particular agonists stimulate AA release but not $Ins(1,4,5)P_3$ production and still generate a mitogenic response (Kaya *et al.*, 1987; Gil *et al.*, 1991; Stewart & Wakelam, unpublished data). It therefore appears that different degrees of emphasis may be placed on PLA_2 activation depending on the situation. The regulatory factors important in activation may also differ between agonist and/or cell type. Chapters 3,4 and 5 examined several of these factors in 3T3 cells.

The biphasic nature of the AA response discussed in Chapter 3 generates questions regarding the underlying mechanism and relative importance of each phase. The initial phase appears to depend on activation of a PtdCho–specific PLA_2 which may be under direct receptor–mediated activation via a specific G protein (see Chapters 4 and 5). The identity of the PLA_2 and G protein involved is not known, but it is possible to speculate on the existence of a high molecular weight cytosolic form of the enzyme such as that reported by Clark *et al.* (1991) and Kramer *et al.* (1991). The unavailability of antibodies to this enzyme until

recently has prevented identification of $cPLA_2$ in this study. However, this would be of high priority in future work. On this note, Leslie(unpublished data) has identified at least one form of $cPLA_2$ in Swiss 3T3 cells. Whether the enzyme is purely cytosolic or whether it may interact with the cytoskeleton remains to be confirmed.

Studies described in Chapter 5 performed on permeabilised 3T3 cells suggest the involvement of a G protein in PLA_2 activation. As previously mentioned, the identity of this protein and its relationship to the G protein coupled to PLC in this system remains unknown. Experiments using antibodies to G_p may provide information regarding the latter. This type of experiment would be of importance in strengthening the argument that PLA_2 and PLC are independently regulated in bombesin–stimulated 3T3 cells. The lack of effect of calcium on the initial phase of AA release suggests an enhanced sensitivity of the PLA_2 involved to calcium. This is an important observation since PLA_2 has been characterised as a calcium–dependent enzyme. Perhaps direct regulation via a specific G protein, as in the first phase of bombesin–stimulated AA release, confers an increase in sensitivity upon PLA_2, making it less dependent on, or independent of regulatory factors that may be more important at later stages of the response. Certain regulatory factors, such as calcium in this case, may be necessary for prolonging or sustaining the response rather than for its initiation. This may be analogous to the activation of PKC whereby DAG increases the affinity of the enzyme for calcium and PtdSer.

The strikingly different regulatory requirements of the two phases of AA release as described in this study, raises the possibility that isoforms of PLA_2 may exist in Swiss 3T3 cells. This has already been suggested in other systems (see Chapter 3) where differences in substrate specificity and regulatory requirements exist. In Swiss 3T3 cells, it is not certain whether different phospholipid substrates are required for the two phases. Detailed examination of the changes in

AA content of all the major phospholipids after bombesin stimulation would be required for the second phase of release before drawing any conclusions.

In the present system, it would be of interest to investigate the effect of abolishing one of the phases of release (the first phase) to provide an insight into how the two phases interact and whether there is any degree of interdependence. On this note, if isoforms of PLA_2 are present, a complete understanding of their respective biological functions is contingent on the development of isoform–specific inhibitors and rapid methods of separation. The ability to control which forms of the enzyme are activated would enable a better understanding of the importance of different phases of AA release, such as those described here. It is interesting to consider why the cell produces this biphasic response, possibly with the involvement of different PLA_2 isozymes. It is possible, as mentioned earlier, that the pattern of early signals is important in leading to later cellular effects i.e. the early AA response to bombesin may be essential for the secondary phase to occur. In addition, the different phases may regulate separate events within the cell or perhaps the secondary phase is simply an amplification of the first, leading to a higher, sustained level of free AA available to the cell. Multiple pathways of PLA_2 activation within a cell which may operate in a parallel or sequential manner may prove to be a more widely–used mechanism important in cellular proliferation.

Perhaps the normal functioning as well as the potency of a mitogen depends on its ability to stimulate both PLA_2 and PLC activities – two potentially independent pathways which, in this system, may or may not only act to amplify one another but may also be capable of a less potent effect individually. In this context, the 'messenger' type responses induced by AA in the Swiss 3T3 cell are subject to extensive cross–talk from other transduction pathways. For example, bombesin–stimulated calcium mobilisation may occur via products of both PLA_2 and PLC activation, promoting release from similar or separate pools. It is also possible that bombesin–stimulated PKC activation may be subject to regulation by products of PLA_2, PLC and PLD hydrolysis. The effect of AA on PKC activity in

3T3 cells has yet to be demonstrated. However, it is possible that DAG, produced from different phospholipid substrates via PLC and PLD, may act in synergy with AA as previously described (see Introduction and Chapter 7) to regulate the activity of different isoforms of PKC.

The mechanism underlying agonist activation of PLA_2 obviously varies between cell types. However, the possibility must be considered that ligand stimulation of certain receptors may be linked directly to PLA_2 activation causing generation of the 'second messenger' AA. This may prove to be a multi–faceted messenger and could perhaps even be involved in regulation of PLA_2 activity itself. For example, in the bombesin–stimulated 3T3 cell, AA released in the first phase may be involved in regulation of the second phase of release. These suggestions may also be relevant to other cellular systems since the increased importance of the PLA_2/AA pathway in mitogenesis, as suggested here, could prove to be more widespread. As such, the present emphasis on PLC as the major effector in generation of intracellular signals could shift and lead to a re–evaluation of the importance attached to other signalling pathways in regulating cellular proliferation.

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