Mitogenic Signalling by the Endothelin Receptor in Rat-1 Fibroblasts

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Abbreviations

ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin (fraction v)
cAMP	Adenosine 3',5'-cyclicmonophosphate
Ca^{2+}	Calcium
$[Ca^{2+}]$	Calcium concentration
CDP Cho	Cytidine 5'-diphosphocholine
Cho	Choline
ChoP	Phosphocholine
CMP PtdOH	Cytidine 5'monophosphate-phosphatidic acid
CTP	Cytidine 5'-triphosphate
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
EC_{50}	Concentration causing 50% of maximum response
EGF	Epidermal growth factor
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene glycol-bis (β-amino-ethyl ether) N,N,N',N'-tetra-acetic acid.
ET	Endothelin
FGF	Fibroblast growth factor
GroPCho	Glycerophosphocholine
GDP	Guanosine 5'-diphosphate
GDP[S] or GDPβS	Guanosine 5'-[β–thio] diphosphate
GTP	Guanosine 5'-triphosphate
GTP[S] or GTPγS	Guanosine 5'-[γ-thio] triphosphate
HBG	Hanks' buffered saline solution with 1% (w/v) BSA and 10mM glucose.
InsP	Total D-*myo*-inositol phosphates

$InsP_1$	D-*myo*-Inositol monophosphate
$Ins(1,4)P_2$	D-*myo*-Inositol 1,4-bisphosphate
$Ins(1,3,4)P_3$	D-*myo*-Inositol 1,3,4-trisphosphate
$Ins(1,4,5)P_3$	D-*myo*-Inositol 1,4,5-trisphosphate
$Ins(1,3,4,5)P_4$	D-*myo*-Inositol 1,3,4,5-tetrakisphosphate
LPA	Lysophosphatidic acid
PDBu	Phorbol 12,13-dibutyrate
PDGF	Platelet-derived growth factor
pH_i	Intracellular pH
PIC	Phosphoinositidase C
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PPH	Phosphatidate phosphohydrolase
PtdBut	Phosphatidylbutanol
PtdCho	Phosphatidylcholine
PtdEtOH	Phosphatidylethanol
PtdIns	Phosphatidylinositol
PtdIns(3)P	Phosphatidylinositol 3-phosphate
PtdIns(4)P	Phosphatidylinositol 4-phosphate
$PtdIns(3,4)P_2$	Phosphatidylinositol 3,4-bisphosphate
$PtdIns(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate
$PtdIns(3,4,5)P_3$	Phosphatidylinositol 3,4,5-trisphosphate
PtdIns-PLC	Inositol lipid-specific PLC
PtdOH	Phosphatidic acid
SS6	Sarafotoxin S6
Tris	Tris (hydroxymethyl) aminomethane

Summary

Endothelin-1 (ET-1) was found to be a complete mitogen in Rat-1 fibroblasts and therefore the signalling pathways which might mediate its action were investigated. ET-1 stimulated both sustained phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and phosphatidylcholine (PtdCho) hydrolysis. The rank order of potency for both pathways stimulated by a range of ET isopeptides was the same for both responses (ET-1 ~ ET-2 > ET-3) suggesting that the same receptor controls both signalling pathways. PtdCho hydrolysis occurred kinetically downstream of PtdIns(4,5)P_2 hydrolysis by a phospholipase D-catalysed mechanism apparently involving both protein kinase C-dependent and -independent means of activation.

The regulation of ET-1-stimulated PtdIns(4,5)P_2 hydrolysis was investigated and compared with the response to another mitogen for Rat-1 cells, lysophosphatidic acid (LPA). The ET-1-stimulated generation of Ins(1,4,5)P_3 was biphasic consisting of a transient Ca^{2+}-independent phase and a sustained Ca^{2+}-dependent phase while the response to LPA was transient. The effect of non-hydrolysable guanine nucleotide analogues on both ET-1- and LPA-stimulated inositol phosphates accumulation was investigated in permeabilized cells. GTP[S] significantly potentiated the response to LPA but had little effect on the ET-1 response. Similarly, LPA enhanced the GTP[S]-stimulated response while ET-1 had little effect. In contrast, GDP[S] inhibited the accumulation of inositol phosphates in response to stimulation with ET-1 alone or in combination with GTP[S] to similar extents. GDP[S] only inhibited the response to LPA alone to a small extent but the degree of inhibition was greater when LPA was used in combination with GTP[S]. The differences in the regulation of PtdIns(4,5)P_2 hydrolysis by ET-1 and LPA was also demonstrated by the ability of PMA to inhibit inositol phosphates accumulation in response to LPA but not ET-1 in permeabilized and intact cells.

Binding studies were performed with [^{125}I] ET-1 on membranes from Rat-1 cells to characterize the ET receptor on Rat-1 cells. [^{125}I] ET-1 bound to a single class of binding sites in a saturable manner and with high affinity (K_D ~ 2nM). The degree of dissociation of bound [^{125}I] ET-1 was very little suggesting a tight association between agonist and receptor. Competition binding experiments with ET isopeptides yielded the same rank order of potency (ET-1 ~ ET-2 > ET-3) as was obtained for PtdIns(4,5)P_2 and PtdCho hydrolysis. These results suggested an ET-1-selective receptor, perhaps similar to the previously characterised ET_A receptor subtype.

Chapter 1

Introduction

1.1. The cell cycle.

The cell cycle is the period during which a cell undergoes the events necessary for successful cell division and depending on cell type may take up to 30h to complete. It consists of a number of phases known as G_1 (the gap before DNA replication), S phase (when chromosomes are replicated), G_2 (gap after DNA replication) and M phase (when the replicated chromosomes are segregated into two daughter cells). Cell proliferation is a tightly controlled process and *in vivo* many cells are in a quiescent state termed G_0 with unreplicated DNA. Cells may enter the G_1 phase starting either from a G_0 nonproliferating quiescent state or from the previous cell cycle.

Extracellular factors in the form of growth factors or mitogenic hormones determine whether a quiescent cell will begin to proliferate and also whether a normal proliferating cell in G_1 will continue to traverse the cycle or revert to quiescence (reviewed in Pardee, 1989). After entry into S phase, however, the cell cycle events depend largely on intracellularly triggered events, and the control regulating the onset of M phase is now believed to be mainly dependent on the protein kinase product of the *cdc* 2 gene, $p34^{cdc\,2}$. In fact, activation of cdc2 kinase is now believed to be the common mechanism which regulates progression of all eukaryotic cells through the cell cycle (Draetta and Beach, 1988; reviewed in Lewin,1990; Nurse, 1990 and Draetta, 1990). Levels of $p34^{cdc\,2}$ are fairly constant throughout the cell cycle but its kinase activity fluctuates dramatically. In G1 it is unphosphorylated and inactive. As cells proceed through S phase into G2 it becomes progressively phosphorylated on threonine and tyrosine residues. Dephosphorylation of both phosphotyrosine and phosphothreonine residues causes activation of the enzyme before onset of M phase. The enzyme is also regulated by interaction with a class of proteins termed cyclins whose levels vary through the cell cycle. A number of proteins have been shown to be phosphorylated *in vitro* by cdc2 kinase, for example Histone H1, RNA polymerase and $p60^{c\text{-}src}$.

The use of clonal cell lines has greatly facilitated the analysis of the early events induced by growth factors. These cell lines can be made quiescent by either withdrawing serum growth factors from the culture medium or by allowing the cells to reach confluency so that all the available growth factors are depleted and the cells are contact inhibited. By re-adding serum or defined growth factors, quiescent cells are stimulated to re-enter the cell cycle and thus early biochemical events in response to the growth stimulus can be investigated. Regulation of cell proliferation is defective in cancer cells and therefore the identification of an altered process in a cancer or transformed cell would suggest that it is important in growth control and provide a potential site for therapeutic intervention

Multiple regulatory pathways have been proposed in several systems for the control of entry into S phase. For example, in Balb/c 3T3 mouse fibroblasts two distinctive steps have been proposed; "competence" is achieved by a brief exposure to platelet-derived growth factor (PDGF) but "progression" requires the continuous presence of epidermal growth factor (EGF) and somatomedin C or insulin (O' Keefe and Pledger, 1983). Other studies have shown that after a certain stage in G_1 cells become "committed" to division and complete the cycle in a growth factor-independent manner (Zetterberg and Larson, 1985).

1.2. Early growth factor-stimulated events.

Since the initiation of DNA synthesis is a late event, occurring 10-15h after the addition of a mitogen, attention has focused on the initial cellular responses associated with a mitogenic stimulus and their importance in the later proliferative response. It has become evident that an increasing list of growth-promoting agents acting on different types of cells elicit a common pleiotypic response (Fig. 1.1). These early growth factor-stimulated events include (1) a

rise in intracellular pH (pH_i) due to activation of the amiloride-sensitive Na^+/H^+ antiporter, (2) a transient rise in cytoplasmic Ca^{2+}, (3) the phosphorylation of a common set of proteins, (4) an increase in c-*fos* and c-*myc* mRNA (reviewed in Rozengurt 1985 and 1986).

1.2.1. pH_i changes

Stimulation of a rise in cytoplasmic pH (pH_i) has been reported to be a common response in the activation of many quiescent cell types. Growth promoting agents such as serum, endothelin, EGF, α-thrombin, insulin, bombesin, PDGF, vasopressin, alone or in combination, have all been shown to induce a rapid rise in pH_i of 0.1-0.3 pH units (Moolenaar *et al.*, 1983; Pouyssegur *et al.*, 1985; Hesketh *et al.*, 1985, 1988; Ives and Daniel, 1987; Simonson *et al.*, 1989). Mitogenic stimuli which bypass membrane receptor activation such as the Ca^{2+} ionophore A23187 and the tumour promoter phorbol myristate acetate (PMA) can also induce an increase in cytoplasmic pH (Hesketh *et al.*,1985, 1988; Ives and Daniel, 1987).

It is now clearly established that this cytoplasmic alkalinization results from growth factor activation of a membrane-bound amiloride-sensitve Na^+/H^+ antiporter which appears to result in a modification of the pH_i sensitivity of the exchanger (reviewed in Grinstein *et al.*, 1989; Barber, 1991). The internal Na^+ concentration is maintained by its efflux via the ouabain-sensitive Na^+/K^+ pump i.e. a Na^+ cycle exists (Rozengurt, 1985). Considerable information exists which suggests that the activation of the antiporter is either essential or at least permissive for the development of the proliferative response. For example, in some systems proliferation can be induced by an exogenously imposed cytoplasmic alkalinization, inhibitors of Na^+/H^+ exchange can block cell growth and under certain conditions growth is impaired in mutant cells devoid of Na^+/H^+ exchange activity (Pouyssegur *et al.*, 1985; reviewed in Grinstein, 1989). However, the importance of the antiporter to the processes leading to

cellular proliferation remains controversial and a large body of evidence indicates that stimulation of the antiporter and the associated alkalosis are not by themselves sufficient to initiate cell growth and, in addition, cell proliferation can occur in the absence of alkalinization (see Grinstein, 1989 for further discussion).

1.2.2. Intracellular Ca^{2+} changes

In addition to pH_i changes, a characteristic feature of many growth stimuli is that they induce increases in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). This was originally detected by measurement of $^{45}Ca^{2+}$ efflux; for example from Swiss 3T3 cells following stimulation with serum, vasopressin and prostaglandin $F_{2\alpha}$ (Lopez-Rivas and Rozengurt, 1983; Smith and Smith, 1984). This enhanced efflux was subsequently shown to be a consequence of stimulating the release of calcium from intracellular reservoirs; for example, stimulation of Swiss 3T3 cells with PDGF resulted in the loss of approximately half of the calcium stored intracellularly within 5min (Frantz, 1985; reviewed in Rozengurt, 1985).

The method most frequently used now to investigate Ca^{2+} responses in cells is fluorescent dye analysis using dyes such as quin-2 or fura-2. An increased fluorescence signal was seen in human fibroblasts loaded with quin-2 and stimulated with PDGF or EGF (Moolenaar *et al.*, 1984) or Swiss 3T3 cells loaded with quin-2 or fura-2 and stimulated with bombesin, PDGF, vasopressin, $PGF_{2\alpha}$, EGF, bradykinin or endothelin (Morris *et al.*,1984; Hesketh *et al.*, 1985; Ives and Daniel, 1987; Hesketh *et al.*, 1988; Takuwa *et al.*, 1989). Endothelin-stimulated $[Ca^{2+}]_i$ increases were also observed in fura-2 loaded Rat-1 cells and A10 cells (Muldoon *et al.*, 1989). Results from these and other studies also suggest that the increase in $[Ca^{2+}]_i$ is due to the influx of extracellular Ca^{2+} aswell as release from intracellular stores (Simonson *et al.*,

1989; Ohnishi *et al.*, 1989; see Meldolesi *et al.*, 1991 for further discussion of Ca^{2+} influx).

The concentration of cytosolic free calcium is clearly very important for the control of many essential cellular responses and therefore the increases in $[Ca^{2+}]_i$ observed with many growth factors and mitogenic peptides may have a role to play in their mechanism of action.

1.2.3. Protein phosphorylation

A common consequence of mitogenic stimulation by different types of growth factors is the phosphorylation of numerous proteins on serine, threonine and tyrosine residues which may have important implications for the activity of many cellular proteins. It is now becoming clear that a number of elements involved in controlling cell division are regulated by changes in phosphorylation.

As discussed above (Section 1.2.1), activation of the Na^+/H^+ antiporter resulting in cytoplasmic alkalinization is believed to have a role to play in the mitogenic response. It has now been shown by immunoprecipitation studies in hamster fibroblasts and A431 cells labelled with [^{32}P] orthophosphate that the antiporter is phosphorylated (~1mol of phosphate per mol of antiporter) in growing cells and that mitogenic stimulation of quiescent cells with EGF, thrombin, phorbol esters or serum stimulates phosphorylation of the antiporter on serine residues with a time course similar to that for the change in pH_i. It was suggested that the affinity of the antiporter for H^+ is altered by phosphorylation (Sardet *et al.*, 1990).

Similarly, phosphorylation of the 40S ribosomal protein S6 is also associated with cell activation. Stimulation of quiescent Swiss 3T3 cells with serum, EGF, $PGF_{2\alpha}$ or insulin resulted in the multiple phosphorylation of S6 at serine residues. EGF, $PGF_{2\alpha}$ and insulin each induced phosphorylation of the same subset of tryptic peptides (eight out of the eleven phosphorylated by

serum) suggesting that they share a common regulatory pathway for the phosphorylation of S6 (Martin-Perez *et al.*, 1984). Mitogen-activated S6 kinases with a specificity for S6 have now been purified from several systems including Swiss 3T3 cells (Blenis *et al.*, 1987; Jeno *et al.*, 1988, 1989). Distinct 90kD and 70kD S6 kinases have been identified which are both activated by serine/threonine phosphorylation in response to mitogens (reviewed in Sturgill and Wu, 1991). Phosphorylation of S6 may play an important role in stimulating or facilitating increased rates of initiation of protein synthesis during the early mitogenic response.

A widely studied tyrosine-phosphorylated protein is pp42 which becomes phosphorylated after stimulation of fibroblasts by a number of diverse mitogens including EGF, PDGF, PMA, thrombin and insulin-like growth factor II. It is a serine/threonine-specific protein kinase and one of two phospho forms, pp42A, has now been shown to correspond to MAP (mitogen-activated protein) kinase (Rossomondo *et al.*, 1989; reviewed in Thomas, 1992). Two forms of MAP kinase have now been purified from fibroblasts having M_r 42kD and 44kD and activation of both forms requires threonine and tyrosine phosphorylation which suggests that the enzyme could serve to integrate information from converging signal transduction pathways (Anderson *et al.*, 1990). Supporting this, in CCl39 hamster fibroblasts pertussis toxin was able to inhibit by ~75% the thrombin-induced activation of MAP kinase and the tyrosine phosphorylation of the protein, but not that induced by fibroblast growth factor (FGF), indicating that the two agonists utilise different signalling pathways to stimulate these phosphorylations and to activate the enzyme (L'Allemain *et al.*, 1991a). MAP kinase activating factors have been described in EGF-stimulated Swiss 3T3 cells and NGF-stimulated PC12 cells but it is not clear whether they are molecules that accelerate the autophosphorylation reaction of the enzyme or kinases that independently phosphorylate and activate the enzyme (Ahn *et al.*, 1991; Seger *et al.*, 1991; Gómez and Cohen, 1991; reviewed in Thomas, 1992). A

downstream function of MAP kinase may be to regulate S6 kinase as *in vitro* insulin-stimulated MAP kinase will phosphorylate and reactivate S6 kinase II (90kD) previously inactivated by phosphatase treatment (Sturgill *et al.*, 1988). In contrast, MAP kinase and 70kD S6 kinase have been shown to lie on distinct signalling pathways (Ballou *et al.*, 1991). In addition, MAP kinase has been shown to phosphorylate the c-jun protein and has been suggested to positively regulate the *trans* -acting activity of c-*jun* (Pulverer *et al.*, 1991). As a result of these studies, it has been suggested that MAP kinase has a key role in a kinase cascade involved in the control of cell proliferation and may be a critical control element for the G_0 to G_1 transition (Sturgill and Wu, 1991; L'Allemain *et al.*, 1991b).

Many growth factors have also been shown to regulate Raf-1 kinase which is a 74kD serine/threonine kinase encoded by the cellular homologue of v-*raf*, the transforming gene of mouse sarcoma virus 3611 (reviewed in Li *et al.*, 1991). Mitogenic stimulation by a number of factors increased the kinase activity of Raf-1 and was accompanied by serine/threonine and tyrosine phosphorylation of the protein (Morrison *et al.*, 1988; Blackshear *et al.*, 1990). In PDGF-treated Balb 3T3 cells, Raf-1 tyrosine phosphorylation and kinase activity were increased and the Raf-1 protein directly associated with the ligand-activated PDGF receptor. It was suggested that the PDGF receptor directly activates Raf-1 kinase by phosphorylating it on tyrosine residues (Morrison *et al.*, 1989). Raf-1 kinase has also been shown to be a substrate for MAP kinase *in vitro* (Anderson *et al.*, 1991) while Raf-1 kinase kinase activity from HIR.5 cell lysates was shown to comigrate with MAP kinase activities (Lee *et al.*, 1992). Perhaps, as has been proposed for MAP kinase, Raf-1 may be involved in integrating signals from different sources. When Raf-1 function was inhibited by expressing c-*raf*- 1 antisense RNA or kinase-defective c-*raf*-1 mutants, the proliferation of normal NIH/3T3 cells was inhibited, as was serum- and PMA-stimulated DNA replication and *raf*-transformation. Proliferation and

transformation by Ki- and Ha-*ras* oncogenes was also blocked (Kolch *et al.*, 1991). These results therefore support a role for Raf-1 in the proliferative response. In addition, microinjection of mutant c-*raf* proteins into G_0-arrested NIH/3T3 cells induced DNA synthesis and morphological transformation (Smith *et al.*, 1990).

Although this section has discussed the importance of phosphorylation reactions in the proliferative response, it is worth mentioning that dephosphorylation reactions probably also have a role to play. As was mentioned in section 1.1, cdc2 kinase is activated by dephosphorylation at threonine and tyrosine residues before onset of M phase. Thus, protein phosphatases may also have key roles in the regulation of cell function (reviewed in Tonks, 1990).

1.2.4. Transcription of immediate early genes

In addition to the events taking place in the cytosol described above, a common response to mitogenic stimuli is the transient expression of the cellular oncogenes c-*fos*, c-*myc* and c-*jun*.. These genes are transcribed in the absence of protein synthesis very early in the proliferative response (Greenberg and Ziff, 1984; Ryder and Nathan, 1988; Takuwa *et al.*, 1989; Simonson *et al.*, 1989). The enhanced expression of c-*fos* mRNA is detected first, usually within minutes of stimulation, while c-*myc* mRNA appears more slowly (Müller *et al.*, 1984; Rozengurt and Sinnett-Smith, 1987; Takuwa *et al.*, 1989).

The rapid induction of expression of these early genes after mitogenic stimulation suggests that they have a primary role in mediating the growth response. Fos has been implicated as a *trans* -acting factor that is capable of stimulating gene expression, not by directly binding to DNA, but by interaction with the sequence-specific transcription factor c-Jun/AP-1 ($pp44^{c\text{-}jun}$) which in turn recognises specific *cis* -elements (Curran and Franza, 1988; Chiu *et al.*,

1988). Fos therefore acts as a "third messenger" that is involved in the transmission of signals elicited by cytoplasmic second messengers to the transcriptional machinery in the nucleus.

1.3. Mitogenic signal transduction pathways

It is unclear what governs the mitogenic potential of an agonist in a particular cell type. However, from studying the responses of cells grown in culture to growth factors and mitogenic hormones, a number of signal transduction pathways have been implicated in the initiation of cell proliferation (Fig. 1.1). As a result growth promoting agents have been divided into two categories. One class binds to transmembrane surface receptors possessing an intrinsic tyrosine kinase, activation of which is believed to initiate a series of cellular events leading to DNA replication. The second class binds to G protein-coupled receptors that activate multiple effector systems including enzymes producing second messenger molecules e.g. adenylyl cyclase-catalysed formation of cAMP, phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate, phospholipase C- and phospholipase D-catalysed hydrolysis of phosphatidylcholine, phospholipase A_2-catalysed generation of arachidonic acid (reviewed in Pouyssegur, 1990).

1.3.1 Receptor tyrosine kinases

Growth factors belonging to this class include epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) and all bind to cell surface receptors that possess an intrinsic ligand-activated protein tyrosine kinase activity contained in the cytoplasmic domain (reviewed in Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990). Ligand binding induces receptor oligomerization and leads to activation of the

kinase function and enhanced ligand binding affinity. A common consequence of receptor activation is auto-phosphorylation. Two PDGF-dependent auto-phosphorylation sites have been identified in the human PDGF receptor; one of which, tyr-751, appears to be involved in modulating the interactions between activated receptors and cellular proteins (Kazlauskas and Cooper, 1989). EGF receptor auto-phosphorylation sites also appear to modulate the interaction of activated EGF receptors with other proteins (Margolis *et al.*, 1990). The tyrosine kinase domain is the most highly conserved portion of all receptor tyrosine kinase molecules and contains a consensus sequence that functions as part of the binding site for ATP. The kinase activity is essential for signal transduction and induction of cellular responses such as mitogensis and transformation (Escobedo *et al.*, 1988; Pandiella *et al.*,1989; Margolis *et al.*, 1990). Receptor activation triggers numerous cellular responses including stimulation of Na^+/H^+ exchange, Ca^{2+} influx, inositol lipid turnover and gene expression (Kruijer *et al.*, 1984; Hasegawa-Sasaki, 1985; Frick *et al.*, 1988; Pandiella *et al.*, 1989; see also Section 1.2.).

Several receptor tyrosine kinase substrates have been identified and include proteins which have been implicated in the control of proliferation. Both PDGF and EGF receptor activation can induce tyrosine phosphorylation of, and association with, phospholipase C-γ1 (PLCγ1), GTPase activating protein (GAP), type 1 PI kinase and Raf-1 kinase (Kaplan *et al.*, 1987; Morrison *et al.*, 1989; Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989; Kazlauskas *et al.*, 1990; Kaplan *et al.*, 1990; Todderud *et al.*, 1990; Anderson *et al.*, 1990; Ellis *et al.*, 1990; Rhee, 1991). Binding of PLC-γ1 and GAP to activated growth factor receptors may be mediated via SH2 (*src* -homology 2) domains (Anderson *et al.*, 1990). Tyrosine phosphorylation of Raf-1 and PLC-γ1 has been shown to induce their activation (Morrison *et al.*, 1990; Nishibe *et al.*, 1990) and suggests an important role for these and the other associated proteins in mediating growth factor responses.

1.3.2. Adenylyl cyclase

Adenylyl cyclase catalyses the formation of cAMP from ATP which then activates cAMP-dependent protein kinase (protein kinase A). Receptors coupling to adenylyl cyclase (e.g.ß-adrenergic) do so via guanine nucleotide binding proteins (G proteins) that mediate activation (G_s) or inhibition (G_i) of the enzyme (reviewed in Levitzki, 1990). The role of cAMP as a second messenger in mediating the mitogenic response is controversial and it has been reported to have both positive and negative effects on proliferation (reviewed in Dumont *et al.*, 1989; Wakelam, 1990)

In Swiss 3T3 cells cAMP has been shown to be a growth promoting signal; cholera toxin, at concentrations which increased cAMP, acted synergistically with serum, insulin, phorbol esters, EGF and FGF to stimulate DNA synthesis. The dose-dependency for inducing DNA synthesis and increasing cAMP were similar. Also, inhibitors of phosphodiesterase activity such as IBMX (1-methyl-3-isobutylxanthine) or Ro-20-1724 potentiated the stimulation of DNA synthesis and the increase in cAMP (Rozengurt *et al.*, 1981). PDGF which is mitogenic in Swiss 3T3 cells also stimulated an increase in cAMP by a prostaglandin E-dependent mechanism (Rozengurt *et al.*, 1983; the role of cAMP in initiating DNA synthesis in Swiss 3T3 cells is further discussed in Rozengurt, 1985). In addition, Swiss 3T3 cells expressing constitutively active $G_{s\alpha}$ showed increased mitogenic sensitivity in response to forskolin associated with enhanced intracellular cAMP accumulation (Zachary *et al.*, 1990). In *S. cerevisae* cAMP is the positive signal for growth elicited in response to a sufficient nutrient supply (Dumont *et al.*, 1989 and references therein).

In contrast to this role for cAMP, it has been demonstrated that the cAMP level is higher in dense, non-proliferating fibroblast cultures than in growing cells and that cAMP levels drop when growth arrested cells are stimulated by mitogens (Froehlich and Rachmeler, 1972; Otten *et al.*, 1972). In Rat-1

fibroblasts and human fibroblasts, LPA (lysophosphatidic acid) -induced cell proliferation appears to be mediated via an inhibition of adenylyl cyclase (van Corven *et al.*, 1989). Likewise, in 5-hydroxytryptamine-stimulated CHL fibroblasts inhibition of adenylyl cyclase appears to be important for DNA synthesis (Seuwen *et al.*, 1988) while in human fibroblasts forskolin, a potent stimulator of adenylyl cyclase, inhibited the stimulatory effect of PDGF on [^{3}H] thymidine incorporation with a dose dependency similar to that observed for cAMP formation (Heldin *et al.*, 1989).

The growth promoting and inhibiting effects of an increase in cAMP may be due to the existence of two distinct isozymes of cAMP-dependent protein kinase which differ in their regulatory subunits. Use of cAMP analogues modified at either the C-8 position or the C-6 position has suggested the involvement of the type II protein kinase in growth inhibition. Use of the analogues inhibited the growth of a range of human cancer cell lines associated with an increase in the cellular levels of the regulatory unit of the type II kinase (Katsaros *et al.*, 1987) while transformation of NIH 3T3 cells by Harvey murine sarcoma virus was reversed (Tagliaferri *et al.*, 1988). The different effects of cAMP on proliferation may also be linked to the stage in the cell cycle. In Swiss 3T3 cells exposed to cholera toxin at different stages in the cell cycle, cAMP appeared to be positively involved in the acquisition of the state of competence by quiescent cells (i.e. G_0-G_1) but antagonistic to the onset of DNA replication (i.e. G_1-S) in committed cells (Smets and van Rooy, 1987). The importance of cAMP in cell growth regulation is, however, emphasized by the discovery of an altered G_s protein in a group of human growth hormone-secreting pituitary adenomas with high intracellular cAMP levels (Vallar *et al.*, 1987).

1.3.3 Phosphoinositide hydrolysis

The phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is a major signalling pathway which has been implicated in the control of cell proliferation (reviewed in Berridge, 1987; Whitman and Cantley, 1988; Wakelam, 1990). PtdIns(4,5)P_2 is a quantitatively minor inositol lipid largely confined to the inner leaflet of the plasma membrane which is hydrolysed to yield two second messengers, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and *sn* -1,2-diacylglycerol (DAG). Ins(1,4,5)P_3 mobilizes calcium from intracellular stores thus raising the intracellular free calcium concentration ($[Ca^{2+}]_i$), and DAG activates protein kinase C (PKC) in a Ca^{2+} and phosphatidylserine-dependent manner (see section 1.4).

The role of increased $[Ca^{2+}]_i$ as an early signal in mitogenesis has already been discussed (section 1.2.2). Protein kinase C can phosphorylate many different cellular proteins. An important site of action is the Na^+/H^+ exchanger (section 1.2.1) and in mouse thymocytes and Swiss 3T3 fibroblasts PKC activation has been shown to cause an increase in intracellular pH (Hesketh *et al.*, 1985). Therefore both messengers generated by PtdIns(4,5)P_2 hydrolysis can activate ionic events which have been implicated in the control of cell growth. PKC activation and increased $[Ca^{2+}]_i$ have also been shown to be involved in the expression of c-*fos* and c-*myc* (Rozengurt and Sinnett-Smith, 1987; Kaibuchi *et al.*, 1986), genes which are considered early signals in the mitogenic response (section 1.2.4).

However, there is no unequivocal evidence for the necessity of ion fluxes or c-*fos* and c-*myc* expression for cell proliferation and other approaches have been used to determine the importance of PtdIns(4,5)P_2 hydrolysis in mitogenesis. Microinjection of anti-PtdIns(4,5)P_2 antibodies into NIH 3T3 cells abolished DNA synthesis stimulated by PDGF and bombesin suggesting a crucial role for PtdIns(4,5)P_2 hydrolysis for proliferation in response to these mitogens (Matuoka, 1988). Injection of this antibody into *ras* -, *src* - or *erb* -transformed

cells inhibited oncogene-induced cell proliferation (Fukami *et al.*, 1988). Neomycin, which has been proposed to inhibit phospholipase C activity by selectively binding polyphosphoinositides, inhibited thrombin-induced mitogensis at doses similar to those necessary to block thrombin-stimulated incorporation of ^{32}P into phosphoinositides (Carney *et al.*, 1985).

The importance of protein kinase C in the stimulation of cell proliferation is demonstrated by the fact that the enzyme is the target of action of the tumour promoting phorbol esters, e.g. PMA (Castagna *et al.*, 1982; Niedel *et al.*, 1983) and of teleocidin and debromoaplysia toxin (Fujiki *et al.*, 1984). These compounds are generally accepted to act primarily by mimicking the function of DAG and, since they are not enzymatically removed, they cause sustained activation of PKC. Further evidence for a role for PKC in growth control comes from overexpression studies in rat fibroblasts. These cell lines show morphological changes and anchorage-independent growth in the presence or absence of PMA (Housey *et al.*, 1988).

In addition to acting as a tumour promoter *in vivo* PMA can act as a mitogen or co-mitogen and mimic a number of the early events associated with mitogenic stimulation in a variety of cultured cell lines. Similarly, the calcium ionophores A23187 or ionomycin which mimic the Ca^{2+} branch of the inositol lipid signalling pathway appear to be co-mitogenic in some cell types and also appear to mimic early growth factor stimulated events (Whitman and Cantley, 1988 and references therein). The tumour promoter thapsigargin inhibits the endoplasmic reticulum Ca^{2+}-ATPase resulting in a pronounced increase in the concentration of $[Ca^{2+}]_i$ supporting the proposed role of Ca^{2+} in cell growth (Thastrup *et al.*, 1990).

Prolonged treatment of many cell types with phorbol esters such as PMA results in down-regulation of protein kinase C (Rodriguez-Pene and Rozengurt, 1984) and this technique has been used as a means of inhibiting mitogen-stimulated PKC activity. Mitogenic stimulation by vasopressin was inhibited in

Swiss 3T3 cells treated with phorbol 12,13-dibutyrate (Collins and Rozengurt, 1984). In Rat-1 cells, the ability of endothelin-1 to stimulate DNA synthesis and anchorage-independent growth was markedly reduced by the depletion of PKC induced by prolonged PMA treatment (Muldoon *et al.*, 1990).

Further evidence which supports a role for PtdIns(4,5)P_2 hydrolysis in cell proliferation is the observation that cells transformed with various oncogenes show enhanced inositol lipid turnover as measured by elevated DAG or inosiol phosphates e.g. *sis, erb* -B, *fes, fms, src, ros, abl, ras* (Fleischman *et al.*, 1986; Wakelam *et al.*, 1986; Whitman and Cantley, 1988 and references therein).

However, it is important to realise that there are many systems in which proliferation has been shown to occur in the absence of inositol lipid turnover e.g. EGF in Balb/c 3T3 cells (Besterman *et al.*, 1986) or PDGF in C3H10T1/2 fibroblasts (Hill *et al.*, 1990). In addition, for some mitogens, activation of phosphoinositide turnover elicited many of the early signals generally associated with mitogenesis but was shown to be insufficient to stimulate or maintain continuous cell proliferation (Seuwen *et al.*, 1990; Black and Wakelam, 1990). Cell growth is probably sustained by a variety of interconnected intracellular signals within which the importance of inositol lipid turnover can vary widely depending on the cell type and receptor stimulated (see Whitman and Cantley, 1988 for further discussion of correlations and non-correlations between inositol lipid turnover and the regulation of cell proliferation).

1.3.4. Phosphatidylcholine hydrolysis

A variety of mitogens have now also been shown to stimulate the hydrolysis of phosphatidylcholine (PtdCho) which may be another source of second messengers. PtdCho is hydrolysed by phospholipase C (PLC) to generate phosphocholine (PCho) and DAG and by phospholipase D (PLD)

generating choline (Cho) and phosphatidic acid (PtdOH). PtdOH can be further metabolised to DAG by phosphatidate phosphohydrolase (PPH).

As discussed already (section 1.3.3), there is strong evidence for a role for DAG-stimulated activation of PKC in mitogensis; however, there is increasing evidence for sources of the lipid other than PtdIns(4,5)P_2 breakdown. Indeed, some PKC-mediated events such as increases in pH_i (Bierman *et al.*, 1990) and phosphorylation of an 80kD phosphoprotein, the MARCKS protein (Takuwa *et al.*, 1991) persist long after Ins(1,4,5)P_3-stimulated $[Ca^{2+}]_i$ elevation has declined In Balb/c 3T3 cells microinjection of DAG but not Ins(1,4,5)P_3 stimulated mitogenesis (Suzuki-Sekimori *et al.*, 1989).

A number of oncogenes (Lacal *et al.*, 1987) and growth factors which do not stimulate inositol lipid hydrolysis are still able to increase cellular DAG content. The AA isoform of PDGF in vascular smooth muscle cells can stimulate DAG production and increase membrane associated PKC activity in the absence of Ins(1,4,5)P_3 generation (Block *et al.*, 1989; Sachinidis *et al.*,1990). Similarly, EGF stimulation of IIC9 cells resulted in increased mass of DAG in the absence of phosphoinositide hydrolysis (Wright *et al.*, 1990). In IIC9 fibroblasts stimulated with α-thrombin (Wright *et al.*, 1988) or Swiss 3T3 cells stimulated with bombesin (Cook *et al.*, 1990) there was a biphasic generation of DAG where the initial peak of DAG formation corresponded to transient Ins(1,4,5)P_3 formation followed by a sustained second phase which was kinetically dissociated from Ins(1,4,5)P_3 accumulation and lasted for up to 4h (Takuwa *et al.*, 1991). The source of this sustained generation of DAG has been ascribed to hydrolysis of PtdCho based on fatty acid composition (Pessin and Raben, 1989).

Studies in a variety of cell types have shown that PMA can stimulate DAG formation (e.g. Cook *et al.*, 1990). Phorbol ester-stimulated DAG accumulation occurs in the absence of inositol phosphate formation; in fact PMA inhibits receptor-stimulated inositol phosphates accumulation by a PKC-mediated,

negative-feedback pathway (Brown *et al.*, 1987; Cook and Wakelam, 1991a).

Such observations suggest that growth factors are able to stimulate DAG generation from a source other than inositol lipids and there has been increasing interest in the stimulated hydrolysis of PtdCho as a mitogenic signalling pathway (Cook and Wakelam, 1991b). Exogenous addition of PtdCho-hydrolysing PLC is sufficient to elicit a potent mitogenic response in Swiss 3T3 cells by a mechanism that is independent of PKC (Larrodera *et al.*, 1990). Also, cells overexpressing PKC β1 show enhanced PLD activity and this may contribute to the growth abnormalities seen in these cells (Pai *et al.*, 1991). In addition, rapid activation of PtdCho hydrolysis was observed in Ki-*ras* -transformed rat fibroblasts which also suggests a role in growth control (Lopez-Barahona *et al.*, 1990). PtdCho hydrolysis can also generate PtdOH which may itself function as a second messenger. In fact, it is now becoming clear that in some systems PLD-catalysed hydrolysis of PtdCho is a transient response (Martinson *et al.*, 1990; Plevin and Wakelam, 1992) and therefore may not be contributing significantly to sustained DAG formation suggesting that PtdOH may be the important messenger generated. PtdOH was shown to inhibit p21 ras GTPase activating protein (GAP) and stimulate a GTPase inhibiting protein which would tend to increase the proportion of RAS bound to GTP and therefore promote the biological activity of RAS. This suggests that PtdOH may be important in regulating the activity of the *ras* oncogene product (Tsai *et al.*, 1989, 1990). Both PtdOH and lyso-PtdOH can act as growth factors and have been shown to stimulate DNA synthesis in fibroblast cell lines (Moolenaar *et al.*, 1986; van Corven *et al.*, 1989; Knauss *et al.*, 1990; van Corven *et al.*, 1992). Whether PtdOH and lyso-PtdOH produced by stimulated lipid hydrolysis can actually leave the cell and act in an autocrine or paracrine manner remains to be determined.

1.4. Receptor coupled PtdIns(4,5)P_2 hydrolysis

The phosphoinositides are a small group of membrane phospholipids which are unique in that their *myo* -inositol headgroup can be phosphorylated at multiple sites. They form a minor component of most, if not all, eukaryotic membranes. The three most predominant phosphoinositides are phosphatidylinositol (PtdIns), phosphatidylinositol 4-monophosphate (PtdIns(4)P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and they interconvert via a series of successive phosphorylation and dephosphorylation reactions controlled by specific kinases and phosphatases. Binding of many agonists to their receptors leads to the activation of an inositol lipid-specific phospholipase C (PtdIns-PLC), also known as phosphoinositidase C (PIC), which catalyses the hydrolysis of inositol phospholipids to generate inositol phosphates and *sn* -1,2-diacylglycerol (DAG). Hydrolysis of PtdIns(4,5)P_2 generates a bifurcating pathway consisting of inositol 1,4,5-trisphosphate- (Ins(1,4,5)P_3) mediated release of calcium from intracellular stores and DAG activation of protein kinase C (PKC) (reviewed in Berridge, 1987a and 1987b).

1.4.1. PtdIns-PLCs

The phospholipase Cs are phosphodiesterases which hydrolyse the glycerophosphate bond of phospholipids (reviewed in Meldrum *et al.*, 1991). From purification studies, immunological characterization and molecular cloning, it has been shown that numerous PtdIns-PLCs exist in various tissues. From primary structure comparisons the superfamily of enzymes can be divided into four classes of isozymes, α, β, γ and δ, with more than one enzyme of each type. A significant sequence homology exists in two regions shared by PLC-β, -γ and -δ which suggests that they may form a catalytic domain. In addition, members of the γ class share regions of sequence homology with several other proteins in what are known as *src* -homology (SH) domains (Rhee *et al.*, 1991).

Biochemical characterization has failed to clarify the relationship between enzymes of the PLC-α class and the other PtdIns-PLC classes.

The way in which receptors couple to the different forms of PtdIns-PLC is clearly of considerable interest. As discussed previously, receptors can be divided into two general categories based on the signalling pathways they employ, i.e. G protein-coupled receptors and receptors with intrinsic tyrosine kinase activity. Agonists employing both types of receptor have been shown to stimulate PLC-catalysed hydrolysis of inositol lipids (see section 1.4.2 and 1.4.3).

G proteins, which couple seven transmembrane domain receptors to effectors, are a highly conserved family of membrane associated, heterotrimeric proteins composed of α, β and γ subunits. Agonist receptor activation stimulates the exchange of GTP for GDP bound to the α-subunit, thus causing the α-subunit with bound GTP to dissociate from βγ and enabling it to stimulate the effector enzyme e.g. PtdIns-PLC. An intrinsic GTPase activity hydrolyses GTP to GDP and as a result the α-subunit reassociates with βγ (reviewed in Gilman, 1987; Casey and Gilman, 1988).

Evidence exists that many receptors couple to PtdIns-PLC via G proteins. In some instances, coupling can be inhibited by pertussis toxin treatment which ADP-ribosylates the α-subunit of G_i, the inhibitory G protein which couples to adenylyl cyclase, leading to its inhibition. This suggested a role for a G_i-like protein in linking certain receptors to PtdIns-PLC e.g. in HL60 cells stimulated with fMet-Leu-Phe (Gierschik *et al.*, 1989). However, there are also a large number of receptor systems which operate via pertussis toxin-insensitive G proteins (reviewed in Meldrum *et al.*, 1991) designated as G_p (Cockcroft, 1987). Recently a number of new pertussis toxin-insensitive α-subunits have been identified using the polymerase chain reaction (PCR) technique (Strathmann *et al.*, 1989; reviewed in Simon *et al.*, 1991). Among these, $G_{\alpha q}$ and $G_{\alpha 11}$ are widely expressed and are highly homologous. It has been

suggested that these α-subunits may be involved in pertussis toxin-insensitive pathways coupling to PtdIns-PLC (Strathmann and Simon, 1990). Indeed, a novel 42kD protein with sequence identity with the $G_{\alpha q}$ clone was purified and shown to stimulate PtdIns-PLC in a pertussis toxin-insensitive manner (Pang and Sternweis, 1990; Taylor *et al.*, 1990; Smrcka *et al.*, 1991). It was subsequently shown in reconstitution experiments that this G protein specifically activates PLC-β1 and not the γ1 or δ1 isozymes of PtdIns-PLC (Taylor *et al.*, 1991; Martin *et al.*, 1991, Wu *et al.*, 1992).

The coupling of tyrosine kinase growth factor receptors to PtdIns-PLC has been shown to occur by alternative means (reviewed in Rhee, 1991). Studies in permeabilized cells have shown a lack of effect of guanine nucleotide analogues on growth factor-stimulated phosphoinositide hydrolysis suggesting a coupling mechanism independent of G proteins (Hasegawa-Sasaki, 1988; Cattaneo and Vincentini, 1989; Hapler *et al.*, 1990). Furthermore, as was discussed previously (setion 1.3.1), PtdIns-PLCγ has been shown to associate with and be tyrosine phosphorylated by several tyrosine kinase receptor systems (Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989). Binding of PLCγ to activated growth factor receptors may be mediated via SH2 (*src* -homology 2) domains (Anderson *et al.*, 1990) and tyrosine phosphorylation has been shown to activate PLCγ1 and result in its translocation to the plasma membrane (Nishibe *et al.*, 1990; Todderud *et al.*, 1990). There is some evidence that the GTP binding protein $p21^{ras}$ together with GAP (GTPase activating protein) may be involved in modulating PtdIns-PLCγ within the cell. Microinjection of either PLCγ or Ras protein induced DNA synthesis in NIH 3T3 cells; both responses were inhibited by co-injection of antibodies to PtdIns-PLCγ. In contrast, co-injection with a neutralizing antibody to Ras only inhibited the DNA systhesis induced by Ras and not by PLCγ (Smith *et al.*, 1990).

Thus the nature of coupling to the PLC-δ class of enzymes remains to be elucidated; perhaps it will involve a pertussis toxin-sensitive G protein.

1.4.2. $Ins(1,4,5)P_3$ and Ca^{2+}

A common feature of calcium-mobilizing agonists is that they stimulate inositol lipid turnover (reviewed in Michell, 1975; Berridge, 1987b; Berridge and Irvine, 1989). $Ins(1,4,5)P_3$ was shown to be formed rapidly as a result of stimulated $PtdIns(4,5)P_2$ hydrolysis in blowfly salivary glands stimulated with 5-hydroxytryptamine (Berridge, 1983). The first direct evidence that $Ins(1,4,5)P_3$ functioned to mobilize intracellular calcium from a non-mitochondrial store was obtained by adding purified $Ins(1,4,5)P_3$ to permeabilized pancreatic acinar cells (Streb *et al.*, 1983).

$Ins(1,4,5)P_3$ released calcium from a non-mitochondrial store which had characteristics which suggested that it was the endoplasmic reticulum (Streb *et al.*, 1984), but only part of this pool seems to be $Ins(1,4,5)P_3$-sensitive. Another candidate for the $Ins(1,4,5)P_3$-sensitive calcium pool is the calciosome, a small membrane vesicle which has some properties characteristic of the sarcoplasmic reticulum of muscle (Volpe *et al.*, 1987). Of the inositol phosphates tested, only those having phosphates on the 4- and 5-position were capable of stimulating release of calcium, $Ins(1,4,5)P_3$ being the most potent. From these studies it appeared that two phosphates on the 4- and 5-positions are essential to stimulate the release of calcium whereas the phosphate at the 1-position functioned to enhance the affinity of the molecule for its receptor (Burgess *et al.*, 1984; Irvine *et al.*, 1984). In agreement with its role as a second messenger, $Ins(1,4,5)P_3$ is generated rapidly after receptor activation, e.g. in bombesin-stimulated Swiss 3T3 cells (Cook *et al.*, 1990), and exerts its effect rapidly, e.g. in bradykinin stimulated NG115-401L cells $InsP_3$ generation and calcium release occur with similar time courses (Jackson *et al.*, 1987).

To release calcium, $Ins(1,4,5)P_3$ must bind to receptors that are linked to calcium channels connected with the $Ins(1,4,5)P_3$-sensitive calcium pool. An $Ins(1,4,5)P_3$-binding protein (Mr 260k) was isolated from rat cerebellar membranes (Supattapone *et al.*, 1988) and immunocytochemical studies using a

specific antibody on Purkinje cells revealed that this receptor was localized on the nuclear membrane and parts of the rough and smooth endoplasmic reticulum (Ross *et al.*, 1989). The cloning of the cDNA for the Ins(1,4,5)P_3 receptor from a mouse cerebellum cDNA library has been reported together with its deduced amino-acid sequence (Furuichi *et al.*, 1989). Purified Ins(1,4,5)P_3 binding protein has been reconstituted into lipid vesicles; Ins(1,4,5)P_3 and other inositol phosphates stimulated calcium flux in the vesicles with potencies and specificities that matched their calcium releasing actions in intact cells, indicating that the purified Ins(1,4,5)P_3 binding protein was a physiological receptor responsible for calcium release (Ferris *et al.*, 1989). These results also established that a single protein mediates both recognition of Ins(1,4,5)P_3 and the stimulation of calcium flux. From these studies it was also apparent that the Ins(1,4,5)P_3 receptor showed similarities with the ryanodine receptor which mediates calcium release from sarcoplasmic reticulum of skeletal muscle. Included among a number of shared features is the association of both receptors into tetrameric structures (Feruichi *et al.*, 1989; Mignery *et al.*, 1989; Takeshima *et al.*, 1989). A characteristic feature of the Ins(1,4,5)P_3 receptor is that it does not desensitize. Any decline in the release of calcium can usually be attributed either to the rapid metabolism of Ins(1,4,5)P_3 or to the transfer of the mobilized calcium to an Ins(1,4,5)P_3-insensitive pool (Stauderman, 1988; Taylor *et al.*, 1989).

Recent work has suggested that Ins(1,3,4,5)P_4, which is generated from Ins(1,4,5)P_3 via a 3-kinase activity, may also have a second messenger role. The Ca^{2+}-mediated sea urchin egg fertilisation envelope formation was shown to require microinjection of both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Irvine and Moor, 1985). Similarly, both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were required to generate a Ca^{2+}-activated K^+ current in lacrimal acinar cells (Morris *et al.*, 1987; Changya *et al.*, 1989). In addition to mobilizing intracellular calcium through Ins(1,4,5)P_3, many agonists also stimulate influx of extracellular

calcium. Although the mechanism and regulation of Ca^{2+} entry remains unclear $Ins(1,3,4,5)P_4$ has been suggested to be involved in regulating this calcium entry in the simultaneous presence of $Ins(1,4,5)P_3$ in some systems (Irvine *et al.*, 1988; Irvine, 1990). Irvine and colleagues have suggested a coupled mechanism for receptor-stimulated Ca^{2+} control in which the $Ins(1,4,5)P_3$-sensitive Ca^{2+} pool is reloaded by an $Ins(1,3,4,5)P_4$-controlled process. An $Ins(1,3,4,5)P_4$- and Ca^{2+}-sensitive Ca^{2+}- and Mn^{2+}-permeable channel has been characterized in endothelial cells which is activated by an increase in cytoplasmic Ca^{2+}. $Ins(1,3,4,5)P_4$ was shown to enhance the activity of the channel (Lückhoff and Clapham, 1992). However, there have also been reports that Ca^{2+} influx may occur independently of $Ins(1,3,4,5)P_4$ (Penner *et al.*, 1988; Bird *et al.*, 1991). Recently, a Ca^{2+} channel was characterized in mast cells which is activated by depleting intracellular Ca^{2+} stores (Hoth and Penner, 1992). In PC12 cells two independently regulated mechanisms of Ca^{2+} influx were demonstrated; one pathway was sustained by depletion of the intracellular $Ins(1,4,5)P_3$-sensitive Ca^{2+} store while the second pathway was dependent on receptor-effector coupling via G protein(s) distinct from that (those) involved in activation of PtdIns-PLC (Clementi *et al.*, 1992). A second messenger role for $Ins(1,3,4,5)P_4$ has also been proposed in inducing the sequestration of intracellular Ca^{2+} (reviewed in Boynton *et al.*, 1990).

In addition to their rapid formation, second messenger molecules also usually degrade quickly. There are a number of enzyme systems capable of removing $Ins(1,4,5)P_3$ as soon as it is produced and the $Ins(1,4,5)P_3$ generated in response to agonist stimulation is often very transient in nature (e.g. Fu *et al.*, 1988; Cook *et al.*, 1990). $Ins(1,4,5)P_3$ is metabolized by two routes involving $InsP_3$-5-phosphatase which generates $Ins(1,4)P_2$ or $InsP_3$-3-kinase, a Ca^{2+}/calmodulin-sensitive enzyme, which generates $Ins(1,3,4,5)P_4$ (reviewed in Shears, 1989; Erneux and Takazawa, 1991). Purified $InsP_3$ -3-kinase has been shown to be a substrate for protein kinase C with phosphorylation causing a

decrease in its activity (Sim *et al.*, 1990). $Ins(1,4)P_2$ does not mobilize Ca^{2+} but, as discussed above, $Ins(1,3,4,5)P_4$ may have a role in some cells in regulating Ca^{2+} influx (e.g. Cullen *et al.*, 1990). $Ins(1,3,4,5)P_4$ is a substate for the same 5-phosphatase that catalyses the dephosphorylation of $Ins(1,4,5)P_3$ and it is metabolised to the inactive $Ins(1,3,4)P_3$ (Connolly *et al.*, 1987). $Ins(1,3,4)P_3$ and $Ins(1,4)P_2$ can be further dephosphorylated by a series of enzymes to the monophosphate level. A single enzyme, an $InsP_1$ phosphatase, can then dephosphorylate different isomers of $InsP_1$ (Gee *et al.*, 1988), the final product being inositol which can be reused in inositol lipid synthesis. $InsP_1$ monophosphatase is sensitive to inhibition by lithium and this is often exploited in measurements of phosphoinositide hydrolysis as it allows the accumulation of total inositol phosphates consisting predominantly of $InsP_1$ (Berridge *et al.*, 1982). As well as these metabolic routes, levels of $Ins(1,4,5)P_3$ in response to stimulation are also subject to negative feedback inhibition via activation of protein kinase C. Swiss 3T3 cells in which protein kinase C had been down-regulated showed elevated basal and bombesin-stimulated production of inositol phosphates. Protein kinase C has been suggested to mediate this effect by uncoupling of bombesin-stimulated hydrolysis of $PtdIns(4,5)P_2$ at the level of the G protein (Brown *et al.*, 1987; Brown *et al.*, 1990).

1.4.3. DAG/Protein kinase C

Whereas $Ins(1,4,5)P_3$ is released into the cytoplasm following activation of PtdIns-PLC, the neutral lipid *sn* -1,2-diacylglycerol (DAG) remains within the plasma membrane where it functions as the physiological activator of protein kinase C (PKC) (reviewed in Nishizuka, 1984 and 1986; Ganong *et al.*, 1986). In addition to DAG the enzyme requires calcium and phosphatidylserine. DAG is believed to activate PKC by lowering the K_m of the enzyme for Ca^{2+} (Kishimoto *et al.*, 1980). Conversely, prior elevation of Ca^{2+} can enhance the

activation of PKC by DAG (Dougherty and Niedel, 1986). During the activation of PKC the enzyme appears to be translocated from the cytosol onto the membrane (Kraft and Anderson, 1983; Farrar *et al.*, 1985; Guy *et al.*, 1986; Haller *et al.*, 1990). However, this may also reflect a situation where the enzyme is plasma membrane associated and becomes more firmly bound through interaction with DAG. The importance of the DAG/PKC pathway in cell proliferation is illustrated by the fact that PKC has been identified as the cellular receptor for the tumour promoting phorbol esters (Castagna *et al.*, 1982; Niedel *et al.*, 1983; see section 1.3.3)

It is now clear that PKC is not a single enzyme but rather exists in multiple forms (Huang *et al.*, 1986; Nishizuka, 1988; Kikkawa *et al.*, 1989; Parker *et al.*, 1989). These proteins are derived both from multiple genes and from alternate splicing of single RNA transcripts, yet possess a primary structure containing conserved structural motifs with a high degree of sequence homology. There are at least seven subspecies of PKC; α, βI, βII and γ PKC were initially isolated followed by δ, ε and ζ PKC which were isolated under low stringency conditions and have a structure closely related to but distinct from the species initially described. All species are composed of a single polypeptide chain, with each in the group of α, βI, βII and γ having four conserved regions designated C1-C4. The δ, ε and ζ subspecies lack the C2 region. The conserved regions C1 and C2 appear to include the regulatory domain but as yet the sites involved in Ca^{2+}, DAG and phospholipid binding have not been identified with any certainty. The C1 domain has been shown to be important for binding phorbol esters and may therefore be the site of DAG regulation while the C2 region appears to play a role in the Ca^{2+}-sensitivity of the enzyme (Ono *et al.*, 1989). The carboxyl-terminal half containing the regions C3 and C4 appears to be the protein kinase domain as it shows sequence homology with many other protein kinases. Also, the C3 region has an ATP-binding sequence (Kikkawa *et al.*, 1989).

Using a combination of biochemical, immunological and cytochemical procedures with specific antibodies, a differential expression of PKC subspecies has been suggested (reviewed in Nishizuka, 1988; Kikkawa *et al.*, 1989). For example, γ PKC appears to be expressed solely in the brain and spinal cord and is not found in other tissues and cell types (Huang *et al.*, 1987). In contrast, α PKC is widely distributed in many tissues and cell types. In general, one cell type contains more than one subspecies of PKC.

The enzyme subspecies purified from tissues show subtle differences in their mode of activation and sensitivity to Ca^{2+}. PKC isozymes α, β and γ are markedly activated by phospholipids, DAG and Ca^{2+} while the δ, ε and ζ isozymes are activated to a lesser extent by phospholipids and DAG and are relatively Ca^{2+}-independent (reviewed in Kikkawa *et al.*, 1989; Parker *et al.*, 1989; Schaap and Parker, 1990). The activation of α–, β– and γ–PKC by DAG and phosphatidylserine could also be potentiated by unsaturated fatty acids at physiologically relevant Ca^{2+} concentrations (Shinomura *et al.*, 1991; Chen and Murakami, 1992). The PKC isozymes also show differences in their catalytic activity towards substrates. The EGF receptor of A431 cells appears to be phosphorylated most rapidly by α PKC and more slowly by γ PKC (Ido *et al.*, 1987). Also, histone III-S which is the standard PKC substrate was very poorly phosphorylated by PKC-ε compared with α, β and γ (Schaap and Parker, 1990). As was mentioned previously (section 1.3.3), prolonged PMA treatment can cause down-regulation of PKC due to an accelerated rate of degradation (Young *et al.*, 1987). However, studies have shown differences in phorbol ester induced down-regulation of PKC between cell lines, possibly reflecting different sensitivities of different PKCs to PMA (Adams and Gullick, 1989). Additionally, activation of PKC can stimulate diametrically opposed processes such as proliferation and differentiation suggesting the involvemnt of different isozymes (Berridge, 1987a).

While PKC activation appears to be a common event of mitogenic

stimulation, the targets of the enzyme are largely undetermined. PKC can phosphorylate many different proteins *in vitro* but it has been more difficult to identify substrates in intact cells. An important target appears to be the Na^+/H^+ antiporter which is responsible for cytoplasmic alkalinization (section 1.2.2). Another substrate is vinculin; phosphorylation of this protein may be involved in the cytoskeletal reorganisation which accompanies phorbol ester stimulation of cultured cells (Schliwa *et al.*, 1984). Lipocortin was also suggested to be a PKC substrate; phosphorylation has been suggested to suppress its inhibitory activity towards PLA_2 (Touqui *et al.*, 1986). Caldesmon, a protein which binds actin, calmodulin, tropomyosin and myosin, has also been shown to be phosphorylated by PKC. Since caldesmon was observed to be phosphorylated in mitotic cells at the same site phosphorylated by cdc2 kinase *in vitro* , it has been suggested that the protein may play a role in the alteration of the actin cytoskeleton at mitosis and therefore phosphorylation by PKC may also be an important regulatory mechanism (Marston and Redwood, 1991). Other possible PKC substrates relevant in the mitogenic response may be adenylyl cyclase (reviewed in Houslay, 1991), MAP kinase (Rossomondo *et al.*, 1989), Fos (Barber and Verma, 1987) and Jun (Pulverer *et al.*, 1991)

There are a number of metabolic pathways capable of removing DAG once it is formed. The action of DAG kinase results in the phosphorylation of DAG to phosphatidic acid (PtdOH) which may itself have important biological effects (section 1.3.4). PtdOH then combines with CTP to form CMP-phosphatidate which accepts a free *myo* -inositol molecule to reform PtdIns. DAG kinase may therefore have an important role in controlling the concentration of DAG and hence regulating PKC activity as well as initiating resynthesis of phosphoinsitides (Bishop *et al.*, 1986; reviewed in Kanoh *et al.*, 1990). Activation of DAG kinase appears to involve translocation from the cytosol to membranes (Maroney and Macara, 1989) and it can also be phosphorylated *in vitro* by PKC suggesting that this may have a role to play in the mechanism of

activation. DAG is also a target for hydrolysis by DAG lipase (Bishop *et al.*, 1986). This releases arachidonic acid which may itself have important biological effects or alternately be further metabolised to generate prostaglandins

1.4.4. Phosphatidylinositol 3-kinase

Recently a number of 3-phosphorylated novel inositol lipids have been identified. Type I PtdIns kinase (reviewed in Downes and Carter, 1991) was found associated with a number of tyrosine kinases including $pp60^{v\text{-}src}$, middle T/$pp60^{c\text{-}src}$ and the PDGF receptor (Kaplan *et al.*, 1987; Courtneidge and Heber, 1987; Whitman *et al.*, 1987). It was subsequently shown to make a novel inositol lipid, phosphatidylinositol 3-phosphate (Whitman *et al.*, 1988). A possible role for type I PtdIns kinase in cell proliferation is suggested by the observation that PDGF receptors that are deficient in their ability to associate with this kinase fail to stimulate growth (Coughlin *et al.*, 1989). Additionally, a novel phosphoinositide containing four phosphates, suggested to be phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), was observed in activated but not in unstimulated neutrophils (Traynor-Kaplan *et al.*, 1988), while both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 appeared following PDGF stimulation of vascular smooth muscle cells (Auger *et al.*, 1989). However, the role these lipids or their metabolites play in signal transduction pathways is unclear. PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 have been shown to be resistant to hydrolysis by several phospholipase Cs (Lips *et al.*, 1989; Serunian *et al.*, 1989) suggesting that the 3-phosphorylated lipids themselves may act as second messengers.

1.5. Receptor-stimulated phosphatidylcholine hydrolysis

In addition to PtdIns(4,5,)P_2 hydrolysis, many growth factors and hormones also stimulate the hydrolysis of another lipid, phosphatidylcholine (PtdCho) (reviewed in Löffelholz, 1989; Pelech and Vance, 1989; Exton, 1990; Billah and Anthes, 1990). PtdCho is the main phospholipid in mammalian tissues. Phosphodiesteratic cleavage of PtdCho may occur via a number of phospholipases; a phospholipase C (PLC) activity produces DAG and phosphocholine, a phospholipase D (PLD) activity generates phosphatidic acid (PtdOH) and choline and a phospholipase A_2 (PLA_2) activity generates arachidonic acid and lysophosphatidylcholine. This section will concentrate on PLC- and PLD-catalysed hydrolysis of PtdCho. In addition to hydrolysis, PLD catalyses a unique transfer reaction whereby the phosphatidyl moiety of the phospholipid substrate is transferred to primary alcohols to produce phosphatidylalcohols.

PtdCho hydrolysis was first reported in response to phorbol esters and was detected as a stimulated release of labelled choline and phosphocholine from mouse embryo fibroblasts (Mufson *et al.*, 1981). Hormone-stimulated PtdCho hydrolysis had been suggested by the kinetics of DAG accumulation in several cell types and the fatty acid composition of the DAG formed, both of which indicated lipid sources in addition to PtdIns(4,5)P_2 (see section 1.3.4). Quantitative discrepancies between PtdIns(4,5)P_2 hydrolysed and DAG formed were also observed (Pickford *et al.*, 1987). Evidence for PtdCho hydrolysis arose from a number of studies using [^{3}H] choline isotopic labelling which showed increased release of labelled choline and/or phosphocholine in response to agonist stimulation (e.g.Besterman *et al.*, 1986; Muir and Murray, 1987; Price *et al.*, 1989; Cook and Wakelam, 1989)

1.5.1. Distinguishing between PLC- and PLD-catalysed PtdCho hydrolysis

In order to distinguish between agonist-stimulated PLC- and PLD-catalysed hydrolysis of PtdCho, kinetic analysis of product formation, i.e. choline versus phosphocholine and PtdOH versus DAG, has been used. In endothelial cells stimulated with ATP an increase in [^{3}H] choline was detected in the absence of any change in [^{3}H] phosphocholine suggesting a PLD pathway (Martin and Michaelis, 1989). Carbachol stimulated [^{3}H] PtdOH formation more rapidly than DAG in 1321 N1 astrocytoma cells also suggesting a PLD catalysed hydrolysis (Martinson *et al.*,1989). The concurrent formation of DAG and phosphocholine observed in 3T3 L1 cells (Besterman, 1986) and A10 cells (Grillone *et al.*, 1988) was suggested to be indicative of a PLC-mediated breakdown of PtdCho .

However, the products of PLC and PLD are rapidly interconvertible by the activity of specific kinases and phosphatases and therefore conclusions from these types of isotopic labelling experiments can only be tentative. A novel approach labels endogenous phospholipids selectively with ^{32}P without labelling cellular ATP. In such a system [^{32}P] PtdOH or [^{32}P] phosphocholine must be formed from [^{32}P] phospholipids exclusively by PLD or PLC respectively and not by kinases. HL-60 cells labelled with [^{32}P] lysoPtdCho and stimulated with formyl-Met-Leu-Phe (fMLP), PMA, OAG or A23187 showed increased [^{32}P] PtdOH indicating conclusively a PLD-catalysed hydrolysis of PtdCho (Pai *et al.*, 1988a; Billah *et al.*, 1989).

Definitive assignment of PLD activity can also be achieved in the transphosphatidylation reaction of PLD by using primary alcohols to give rise to the corresponding phosphatidylalcohol. These are not substrates for further metabolism and therefore their accumulation can be used to conclusively detect PLD activity. In cultured endothelial cells ATP-dependent [^{14}C] phosphatidylethanol (PtdEtOH) formation increased at the expense of [^{14}C]

PtdOH with increasing EtOH concentration. Also, the time course of [^{14}C] PtdEtOH synthesis stimulated by ATP in the presence of EtOH paralleled that of [^{14}C] PtdOH formation (Martin and Michaelis, 1989). In HL-60 cells stimulated with fMLP [^{32}P] PtdEtOH formation paralleled that of [^{32}P] PtdOH with respect to time course, fMLP concentration, inhibition by a specific fMLP antagonist and calcium concentration (Pai *et al.*, 1988a). Similar results have also been demonstrated in many other systems supporting a role for PLD-catalysed PtdCho hydrolysis (e.g. Billah *et al.*, 1989; Bonser *et al.*, 1989) and suggest that it is a more widespread mechanism of PtdCho hydrolysis than the PLC-mediated pathway.

1.5.2. Regulation of agonist-stimulated PtdCho hydrolysis

Most agonists that cause PtdCho hydrolysis also stimulate PtdIns(4,5)P_2 breakdown (e.g. Cook and Wakelam, 1991a) resulting in the mobilization of intracellular calcium and activation of PKC (section 1.4). Indeed, PMA which activates PKC and the calcium ionophore A23187 act synergistically in HL-60 cells to activate PLD (Billah *et al.*, 1989) suggesting that prior PtdIns(4,5)P_2 hydrolysis is necessary for subsequent PtdCho breakdown. Tumour promoting phorbol esters and exogenous DAGs promote PtdCho hydrolysis in many cell types (e.g. Billah *et al.*, 1989; Liscovitch, 1989; Huang and Cabot, 1990a) while inactive phorbols are without effect (e.g. Besterman *et al.*, 1986). Further support for a role for PKC-dependent activation of PtdCho hydrolysis comes from observations with PKC inhibitors and down-regulation of PKC by prolonged PMA treatment; both treatments can block the responses to agonists and phorbol esters. In bombesin-stimulated Swiss 3T3 cells, PtdCho hydrolysis is kinetically downstream of the first phase of DAG/Ins(1,4,5)P_3 formation and is abolished in cells which have been depleted of PKC activity by chronic PMA treatment (Cook and Wakelam, 1989; Cook *et al.*, 1990). Similarly in

carbachol-stimulated 1321N1 astrocytoma cells down-regulation of PKC blocks the ability of carbachol to stimulate choline generation (Martinson *et al.*, 1989) while PtdEtOH formation in response to PMA in NG108-15 cells was completely abolished in PKC-down-regulated cells (Liscovitch, 1989). Use of PKC inhibitors such as staurosporine produces similar effects; for example, in REF52 cells the inclusion of staurosporine blocked vasopressin-induced [^{3}H] PtdEtOH formation (Huang and Cabot, 1990b), and in HL-60 cells the PKC inhibitor K252a partially inhibited PMA-induced [^{32}P] PtdEtOH formation (Billah *et al.*, 1989). Finally, overexpression of PKC β1 enhances PLD activity and DAG formation in PMA-stimulated rat fibroblasts (Pai *et al.*, 1991).

Despite the considerable evidence that prior PtdIns(4,5)P_2 hydrolysis is necessary for PtdCho breakdown, there are several systems in which this does not seem to be the case. Certain agonists stimulate the generation of choline metabolites in the absence of inositol lipid hydrolysis; e.g. in IIC9 cells, EGF stimulated an increase in [^{3}H] choline metabolites without any significant change in inositol phosphates (Wright *et al.*, 1990). In Swiss 3T3 cells EGF stimulated phosphatidylbutanol formation in the presence of 0.3% (v/v) butanol without stimulating inositol phosphates accumulation (Cook and Wakelam, 1992). In MDCK-D1 cells, α1-adrenergic receptor activation promoted PtdCho hydrolysis more rapidly than PtdIns(4,5)P_2 breakdown and was unaffected by the presence of neomycin, an inhibitor of PtdIns(4,5)P_2 hydrolysis, and the PKC inhibitors sphingosine and H7 (Slivka *et al.*, 1988). In addition, in Jurkat cells, a human T cell line, interleukin-1 stimulated rapid DAG and phosphocholine production from PtdCho in the absence of inositol lipid turnover (Rosoff *et al.*, 1988). Also, in neutrophils stimulated with fMet-Leu-Phe (fMLP) in the absence of cytochalasin B, PtdIns(4,5)P_2 was maximally hydrolysed (Truett *et al.*, 1988) with minimal stimulation of PLD (Billah *et al.*, 1989).

There is evidence that receptor-mediated Ca^{2+} flux may also be involved in activating PtdCho hydrolysis, although it varies markedly between cell types.

The calcium ionophore A23187 has been shown to stimulate PLD activity in several cell types including HL-60 granulocytes (Billah *et al.*, 1989) and human polymorphonuclear leukocytes (Agwu *et al.*, 1989). In HL-60 granulocytes fMLP-stimulated PLD activity was dependent on extracellular Ca^{2+} (Pai *et al.*, 1988) while in human platelets A23187 was able to stimulate greater increases in PtdOH than PDBu (phorbol 12,13-dibutyrate) (Huang *et al.*, 1991). However, in Swiss 3T3 cells the role of Ca^{2+} appears to be subordinate to that of PKC as even A23187-stimulated PLD activity was inhibited by the PKC inhibitor Ro-31-8220 (Cook *et al.*, 1991c).

A role for a guanine nucleotide-binding protein (G protein) in regulating PtdCho hydrolysis has been suggested from experiments using pertussis toxin and non-hydrolysable analogues of GTP such as GTPγS (guanosine 5'-[γ-thio] triphosphate). Incubation of rat liver plasma membranes (Bocckino *et al.*, 1987; Irving and Exton, 1987; Hurst *et al.*, 1990) and permeabilized endothelial cells (Martin and Michaelis, 1989) with GTPγS has indicated G protein involvement in the activation of a PLC and/or PLD whose activities are regulated by P_2 purinergic agonists. The non-hydrolysable GDP analogue, GDPβS (guanosine 5'-[β-thio] triphosphate), inhibited GTPγS stimulation and the response was pertussis toxin-insensitive. Similarly, GTPγS stimulated [^{14}C] PtdEtOH formation in homogenates from HL-60 cells (Anthes *et al.*, 1989) while the fMLP-stimulated response in human neutrophils was pertussis toxin-senstitive suggesting the involvement of a G_i-like guanine nucleotide-binding protein (Agwu *et al.*, 1989). Direct activation of G proteins using aluminium fluoride can also stimulate PLD activity, for example in synaptosomes (Qian and Drewes, 1989) and Swiss 3T3 cells (Diaz-Meco *et al.*, 1989). However, while there is evidence for a guanine nucleotide-stimulated PLD activity, it remains to be established that it is a direct receptor-G protein-PLD activation and not a PLD activity regulated downstream of another G protein-coupled signalling pathway such as PtdIns(4,5)P_2 hydrolysis. For example, in platelet membranes PMA

pretreatment and the presence of Ca^{2+} enhanced the GTPγS-stimulated PLD activity suggesting that PLD is activated secondarily to PtdIns-PLC (van der Meulen and Haslam, 1990).

1.6. Endothelin

Since the discovery of endothelin-1 (ET-1), the most potent vasoconstrictor known, there has been extensive research carried out to determine the physiological and pathological significance of this novel peptide. The following sections review what is currently known about the structure, biosynthesis and mechanism of action of the endothelins.

1.6.1. Structure and biosynthesis

The endothelins are a family of 21-amino acid peptides. ET-1 was originally isolated by Yanagisawa and colleagues from medium conditioned by porcine endothelial cells (Yanagisawa *et al.*, 1988a). The corresponding cDNA was also isolated and sequenced and indicated that the 21-amino acid biologically active mature peptide was synthesised as a 203 amino acid preprotein precursor. A 39-amino acid residue intermediate, termed "big endothelin", is generated from preproendothelin by proteolytic cleavages at paired basic residues. Mature ET-1 is then produced through an unusual, previously unknown, processing between Trp^{21}-Val^{22} by a putative "endothelin converting enzyme" (Yanagisawa *et al.*, 1988a; Sawamura *et al.*, 1989). Conversion of big ET-1 to ET-1 is essential for bioactivity; the vasoconstrictor activity of big ET-1 is about 100-fold lower than that of ET-1 (Kimura *et al.*, 1989) and therefore the converting enzyme may be an important target for pharmacological control of ET-1 release.

Cloning and sequence analysis have now shown that there are in fact three ET-related genes in human, porcine and rat genomes which encode very similar

peptides known as ET-1, ET-2 and ET-3 (Itoh *et al.*, 1988; Yanagisawa *et al.*, 1988b; Inoue *et al.*, 1989a). The existence of another member of this family, the vasoactive intestinal contractor (VIC) peptide, was reported in mouse intestine (Saida *et al.*, 1989) but this peptide has now been shown to be identical to rat ET-2 (Bloch *et al.*, 1991). Human and porcine ET-1 have identical sequences. Human and mouse/rat ET-2 (VIC) differ from one another by one amino acid and differ from ET-1 by two and three amino acids, respectively. Human and rat ET-3 have identical sequences but differ from ET-1 by six amino acids. As illustrated in Fig.1.2, ET isopeptides are highly homologous and share a common design including, (i) two disulfide bonds (Cys^{1}-Cys^{15} and Cys^{3}-Cys^{11}), (ii) a cluster of polar charged side chains residing within a hairpin loop (residues 8-10), and (iii) a hydrophobic C-terminus (residues 16-21) containing the aromatic indole side chain at Trp^{21}. Experiments with synthetic derivatives of ET-1 demonstrated that these features are essential for the expression of complete biological activity (Kimura *et al.*, 1988).

ET-1 also shows remarkable sequence similarity to a family of 21 residue toxins from the venom of the Israeli burrowing asp, *Atractaspis engaddensis*, known as the sarafotoxins S6 (Kloog *et al.*, 1988; Kloog and Sokolovsky, 1989). It therefore seems likely that the two peptide families share a common evolutionary origin. The high degree of sequence homology between the ET isopeptides and between ETs and sarafotoxins suggests that the ET genes have evolved under strong evolutionary pressure to conserve the structure and function of mature ET peptides.

ET isopeptides are differentially expressed in specific tissues suggesting that tissue -specific factors exist to control the rate of ET gene expression. Northern blot analysis of porcine tissues with cloned prepro-ET-1 cDNA as a probe showed that the mRNA was expressed in cultured aortic cells and also in aortic endothelium *in vivo* (Yanagisawa *et al.*, 1988a). ET-3 mRNA has been detected in human fetal lung, pancreas, spleen and a small amount in kidney but

not in cultured human umbilical vein endothelial calls (HUVEC). In contrast, ET-1 mRNA was found in human fetal lung, pancreas, spleen, low levels in fetal atrium and ventricle and high levels in HUVEC (Bloch *et al.*, 1989). *In situ* hybridization studies have also been used to analyse several rat tissues. ET-3 was predominantly expressed in adult kidney, eye and brain, whereas both ET-3 and ET-1 were abundantly expressed in the lung (MacCumber *et al.*, 1989). Another report (Nunez *et al.*, 1990) has demonstrated the expression of ET-1 mRNA in porcine aortic endothelial cells, all heart chambers, adrenals and kidney, aswell as human cardiac ventricular tissue, lung and cultured umbilical endothelial cells. Using *in situ* hybridization and immunocytochemistry, neurones of the human spinal cord, human dorsal root ganglia (Giaid *et al.*, 1989, Lee *et al.*,1990) and paraventricular and supraoptic neurones in the porcine and rat posterior pituitary (Yoshizawa *et al.*, 1990) have also been found to contain measureable ET-1 mRNA suggesting a neuromodulator / neurotransmitter role for ET. ET-2 mRNA was detected in mouse intestine (Saida *et al.*,1989), the HTB119 cell line (derived from a human small cell lung carcinoma) and in the COS-7 cell line derived from monkey kidney (Bloche *et al.*, 1991). Low levels of ET-2 mRNA were also detected in fetal human kidney but not in HUVEC or in fetal lung, spleen, liver or adrenal gland (Bloch *et al.*, 1991). The tissue distribution of gene expression is therefore distinct for each of the ET genes. In particular, neither the ET-2 or ET-3 gene is expressed in HUVEC whereas the ET-1 gene is expressed at high levels suggesting that these genes have biological roles outside the vasculature. Additionally, the cDNAs for two different ET receptors have recently been cloned and shown to encode an ET-1 selective and nonselective receptor, with only the selective receptor being expressed in aorta while the nonselective is not (Sakurai *et al.*, 1990; Arai *et al.*, 1990).

The induction of ET-1 mRNA and the rate of peptide release have been found to be increased by a growing number of agents and mechanical stimuli

including thrombin, transforming growth factor β (TGFβ), angiotensin II, vasopressin, hemodynamic shear stress, interleukin 1, phorbol esters and calcium ionophores (Yanagisawa *et al.*, 1988a and 1989 ; reviewed in Yanagisawa and Masaki, 1989a and 1989b). Some of these inducers are known to stimulate inositol lipid turnover resulting in increases in $[Ca^{2+}]_i$ and activation of protein kinase C, which may exert effects at the level of gene transcription and/or translation. The increase in mRNA induced by agents such as phorbol esters and TGF β has been suggested to result from increased transcription of the ET-1 gene possibly via interaction with *cis* -nucleotide sequences (Kurihara *et al.*, 1989; Inoue *et al.*, 1989b). The level of ET-1 mRNA may be controlled not only by transcriptional regulation but also by post-transcriptional regulation of mRNA degradation. Half-life studies have revealed that ET-1 mRNA is extremely labile, having an intracellular half-life of about 15min (Yanagisawa *et al.*, 1989; Inoue *et al.*, 1989b).

Plasma levels of ET-1 are extremely low in healthy volunteers (e.g. Ando *et al.*, 1989) and intravenously injected labelled ET-1 was quickly eliminated from the blood stream of the rat (Anggård *et al.*, 1989; Shiba *et al.*, 1989). It therefore seems likely that ET-1 acts as an autocrine or paracrine signal, being produced in epithelial or endothelial cells and acting on adjacent smooth muscle, rather than a circulating hormone (e.g. De Nucci *et al.*,1988; MacCumber *et al.*, 1990).

1.6.2. Biological effects

ET-1 has a wide range of biological actions in diverse tissues and species (reviewed in Yanagisawa and Masaki, 1989b; Anggård *et al.*, 1990; Simonson and Dunn, 1991). ET-1 is the most potent mammalian vasoconstrictor peptide known and has a very strong and sustained constrictor activity *in vitro* and pressor activity *in vivo*. Constriction of isolated arterial strips is long lasting and

extremely difficult to wash out (Yanagisawa *et al.*, 1988a). Intravenous bolus injection of ET-1 caused a transient, dose-related depressor response followed by a sustained, dose-dependent rise in arterial pressure both in anesthetized rats (Yanagisawa *et al.*, 1988a; DeNucci *et al.*, 1988; Inoue *et al.*, 1989a; Wright and Fozard, 1988) and in intact, conscious rats (Yanagisawa *et al.*, 1988b). The initial transient depressor response may be due to the ability of ET-1 to stimulate the release of prostaglandins and/or endothelium-derived relaxing factor (EDRF) from perfused tissues. ET-3 is a weaker vasoconstrictor than ET-1 or ET-2 but produces a significantly greater initial depressor effect (Inoue *et al.*, 1989a). Other cardiovascular effects of ET-1 include positive inotropic (i.e. increase in contractile tension) and chronotropic (i.e. increase in the rate of contraction) effects on myocardium (Ishikawa *et al.*, 1988a, 1988b). ET-1 is also a potent renal peptide with numerous effects including increased renal vascular resistance, decreased glomerular filtration rate and renal plasma flow (King *et al.*, 1989) while in liver ET-1 was found to produce a sustained increase in portal vein pressure, an increase in glycogenolysis and alterations in hepatic oxygen consumption (Ganchi *et al.*, 1990).

ET also contracts numerous nonvascular smooth muscle preparations including intestinal, tracheal, lung and uterine. Both ET-1 and ET-3 caused contraction of rat stomach strips, rat colon and guinea pig ileum (Takahashi *et al.*, 1990). ET-1 produced concentration-dependent contraction of airway smooth muscle preparations isolated from humans, guinea pigs, rats and mice (Henry *et al.*, 1990) and ET-1 and ET-2 were equally efficacious at contracting human isolated bronchial tissue (McKay *et al.*, 1991). ET-1 both contracted and restored rhythmicity to the rhythmically quiescent rat uterus (Borges *et al.*, 1989).

ET-1 also has many neuroendocrine effects. In rat isolated glomerular preparations, ET-1 inhibited renin release (Takagi *et al.*, 1988). Infusion into dogs caused a rapid increase in the plasma level of atrial natriuretic factor, renin,

aldosterone, vasopressin and circulating catecholamines (Goetz *et al.*, 1988; Miller *et al.*, 1989) while ET-1 stimulated angiotensin I to angiotensin II conversion in cultured pulmonary artery endothelial cells (Kawaguchi *et al.*, 1990). ET-1 also appears to regulate synaptic transmission in both central and peripheral nervous systems by acting as a neuromodulater and perhaps even as a neurotransmitter (Giaid *et al.*, 1989; MacCumber *et al.*, 1990; Yoshizawa *et al.*, 1990; Stojilkovic, 1990).

A very interesting role of ET-1, and the subject of this thesis, is its action as a mitogen. This has been shown in vascular smooth muscle cells (Komuro *et al.*, 1988; Nakaki *et al.*, 1989), vascular endothelial cells (Takagi *et al.*,1990), fibroblasts (Brown and Littlewood, 1989; Muldoon *et al.*, 1990; Takuwa *et al.*, 1989), C_6 glioma cells (MacCumber *et al.*, 1990; Zhang *et al.*,1991), mesangial cells (Simonson *et al.*, 1989) and human cancer cell lines (Shichiri *et al.*, 1991).

Due to the wide variety of biological actions now found for ET-1 there is considerable interest in its role in the pathogenesis of cardiovascular, renal and pulmonary disease (reviewed in Yanagisawa and Masaki, 1989b; Nayler, 1990; Webb, 1991; Rubanyi and Parker Botelho, 1991).

1.6.3. Mechanism of action

Specific high-affinity binding sites for ET-1 have been demonstrated in many tissues in agreement with the wide ranging pharmacological effects of the peptide. High-affinity, saturable binding sites have also been characterized in intact cells and in membrane preparations from vascular and nonvascular smooth muscle, fibroblasts, glomerular mesangial cells and neurons (reviewed in Yanagisawa and Masaki, 1989b; Simonson and Dunn, 1990a). Most recently there have been two cDNA clones for ET receptors isolated from a rat and bovine lung cDNA library that predict typical G protein-coupled receptors (Sakurai *et al.*, 1990; Arai *et al.*, 1990) and an ET receptor was purified from

bovine lung (Kozuka *et al.*, 1991).

The mechanism of action of the ETs is unknown but ET receptor-binding is known to activate a number of signal transduction pathways. Inositol lipid hydrolysis resulting in the activation of protein kinase C and increased $[Ca^{2+}]_i$ has been demonstrated in many systems as well as activation of the arachidonic acid cascade (reviewed in Simonson and Dunn 1990a, 1990b). The aim of the present study was to investigate the signalling pathways mediating the mitogenic action of ET-1 in Rat-1 cells, a rat embryo fibroblast cell line. PtdCho and PtdIns(4,5)P_2 hydrolysis were investigated with special emphasis on their mechanisms of regulation.

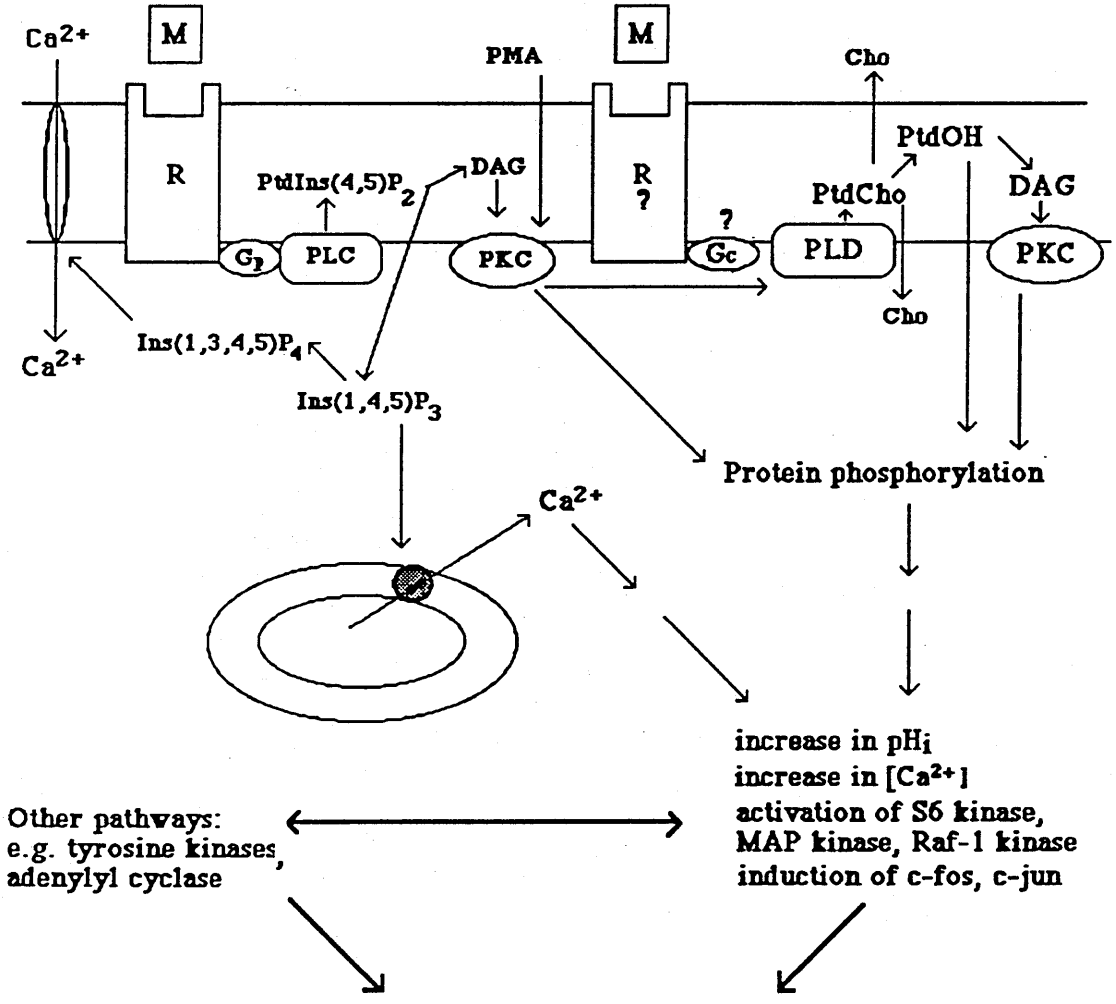
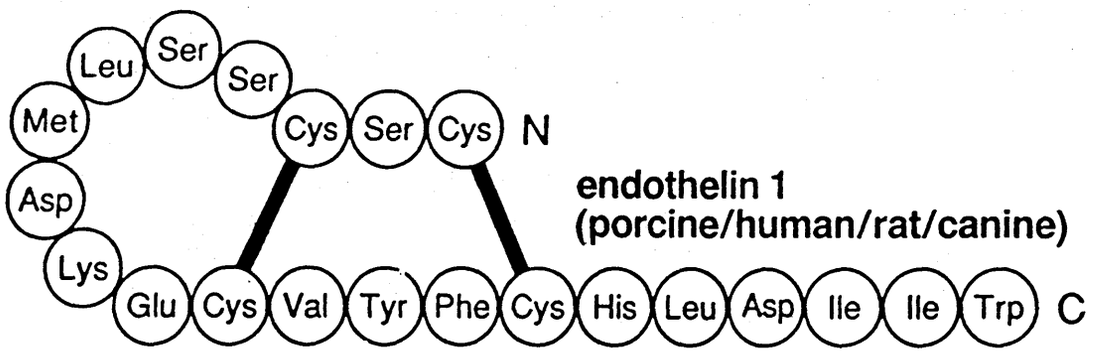
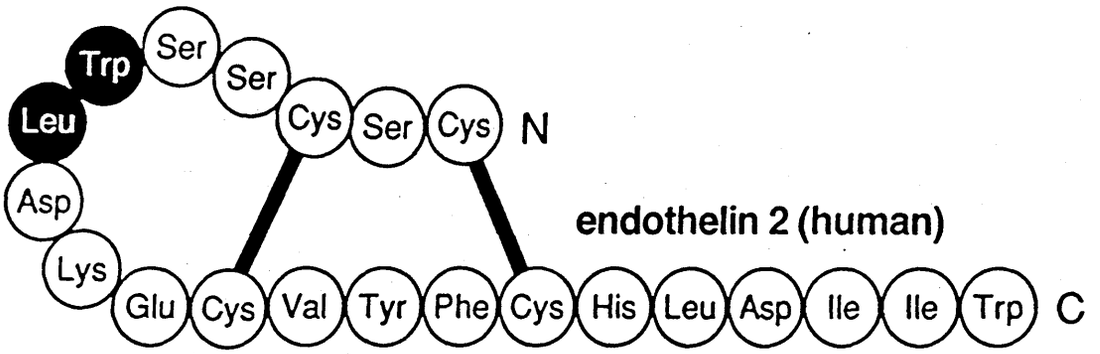

Ca2+
M
R
PtdIns(4,5)P2
DAG
PMA
M
R
?
Cho
PtdOH
PtdCho
DAG
?
Gp
PLC
PKC
Gc
PLD
PKC
Cho
Ca2+
Ins(1,3,4,5)P4
Ins(1,4,5)P3
Protein phosphorylation
Ca2+
increase in pHi
increase in [Ca2+]
activation of S6 kinase,
MAP kinase, Raf-1 kinase
induction of c-fos, c-jun
Other pathways:
e.g. tyrosine kinases,
adenylyl cyclase
PROTEIN SYNTHESIS
AND
DNA SYNTHESIS

Fig. 1.1 Schematic representation of the role of PtdIns(4,5)P_2 and PtdCho hydrolysis in stimulating early mitogenic signals.

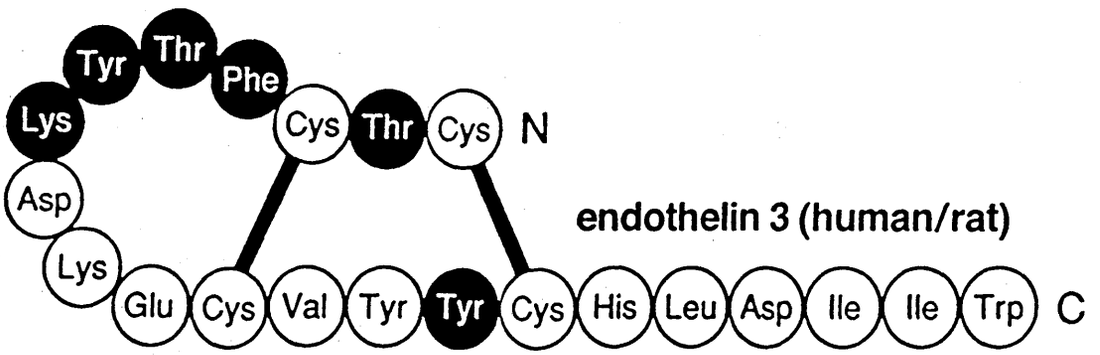
(M is a mitogen acting through its receptor, R.)



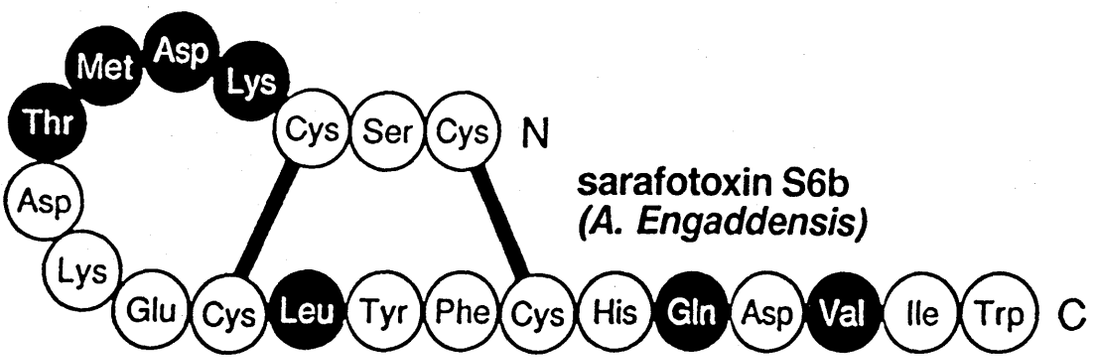
endothelin 1
(porcine/human/rat/canine)



endothelin 2 (human)



endothelin 3 (human/rat)



sarafotoxin S6b
(A. Engaddensis)

Fig. 1.2 Structures of the endothelins and sarafotoxin.

Filled circles indicate residues that differ from the ET-1 sequence. (Taken from Sakurai *et al.*, 1992).

Chapter 2

Materials & Methods

2.1 Cell lines and materials.

2.1.1 Cell lines.

Rat-1 fibroblasts were kindly donated by Dr. J. Wyke, Beatson Institute for Cancer Research, Glasgow.

2.1.2 Materials.

The following is a list of the sources for materials used during the course of this research:

Aldrich Chemical Company Ltd, Gillingham, England.

2,2,4-trimethylpentane (iso-octane).

Amersham International plc, Amersham, Buckinghamshire, England.

[*methyl*-^{3}H] Choline chloride (specific activity 75-85 Ci/mmol),

(3-[^{125}I]*iodotyrosyl*) Endothelin-1 (specific activity ~2000 Ci/mmol)

[2-^{3}H]*myo*-inositol (specific activity 10-20 Ci/mmol),

[2-^{3}H] D-*myo*-inositol(1,4,5) trisphosphate (specific activity 20-60 Ci/mmol),

[9,10(n)-^{3}H] Palmitic acid (specific activity 40-60 Ci/mmol),

Phosporyl[*methyl*-^{14}C]choline, ammonium salt (specific activity 50-60 mCi/mmol),

[*methyl*-^{3}H] Thymidine (specific activity 25 Ci/mmol)

[20(n)-^{3}H] Phorbol-12,13-dibutyrate (specific activity 10-20 Ci/mmol).

Glycerophospho[*N-methyl*-^{3}H]choline (GroPCho) had previously been prepared from [^{3}H] PtdCho by transacylation with monoethylamine according to the method of Clarke & Dawson (1981).

BDH Chemical Company, Poole, England.

Ammonium formate, butan-1-ol, calcium chloride, EDTA, ethanol, Hepes,

orthophosphoric acid, potassium di-hydrogen phosphate, perchloric acid, sodium hydroxide, sodium carbonate, trichloroacetic acid and universal indicator.

Biogenesis Ltd. (formerly Biomedical Technologies Inc.), Bournemouth, England.

Epidermal growth factor (receptor grade) purified from mouse submaxillary glands.

BioMac, University of Glasgow, Glasgow, Scotland, U.K.

Endothelin-1, -2 and -3.

Boehringer (UK) Ltd, Lewes, England.

ATP, dithiothreitol and Tris.

Cambridge Research Biomedicals, Cambridge, England.

Bombesin, [Arg^8]vasopressin.

Fisons Scientific Apparatus,

Acetic acid (glacial), chloroform, dimethyl sulphoxide, ethyl acetate, methanol, magnesium chloride, potassiunm chloride, Folin and Ciocalteu's phenol reagent.

Formachem (Research International)plc, Strathaven, Scotland.

D-glucose, sodium hydrogen carbonate

Gibco, Paisley, Scotland.

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

Koch-Light Ltd, Suffolk, England.

Magnesium sulphate.

May and Baker, Dagenham, England.

Formic acid, hydrochloric acid and sodium tetraborate.

Riedel-DeHaenAg Seelze-Hannover, Germany.

Sodium di-hydrogen carbonate.

Roche (UK) Ltd, Welwyn Garden City, England.

The provision of the drug Ro-31-8220, a PKC inhibitor, by Dr. P. D. Davis is gratefully acknowledged.

Wellcome Diagnostics, Beckenham, England.

Streptolysin-O.

Whatman Ltd, Maidstone, England.

LK5DF glass backed thin layer chromatography plates, pre-laned with pre-adsorpbent strip.

All other chemicals including ion-exchange resins, phorbol esters and prostaglandins were obtained from the Sigma Chemical Company, Poole, England.

2.2 Buffer composition.

Phosphate buffered saline (PBS)-Calcium and magnesium free

146mM sodium chloride, 5.4mM potassium chloride, 9.6mM di-sodium

hydrogen orthophosphate, 1.5mM potassium di-hydrogen orthophosphate.

When freshly prepared PBS was between pH 7.2-7.4.

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 pH 7.4 and filtered before aliquoting through a sterile 0.22μm membrane (Flow pore D).

Hanks buffered saline (Hanks).

1.26mM calcium chloride, 0.5mM magnesium chloride, 0.4mM magnesium sulphate, 5.37 mM potassium chloride, 137mM sodium chloride, 4.2mM sodium hydrogen carbonate, 0.35mM sodium di-hydrogen phosphate.

When freshly prepared the pH was between 7.2 - 7.4.

Hanks buffered saline with glucose and BSA (HBG).

Buffer prepared as above containing 10mM D-glucose and 1% (w/v) BSA (fraction V) and pH adjusted to 7.4.

"Low Calcium" Hanks buffered saline.

0.31mm calcium chloride, 0.5mM magnesium chloride, 0.4mm magnesium sulphate, 5.37mM potassium chloride, 137mM sodium chloride, 4.2mM sodium hydrogen carbonate, 0.35mM sodium di-hydrogen phosphate, 0.33mM EGTA.

pH adjusted to 7.4 using 1M sodium hydroxide.

The free [Ca^{2+}] of this buffer was 1μM.

Permeabilisation buffer.

120mM potassium chloride, 20mM Hepes, 6mM magnesium chloride,

2mM potassium dihydrogen orthophosphate, 2mM ATP, 0.1mM EGTA, 1mg/ml BSA (fraction V).

pH adjusted to 7.4 using 1M KOH.

Binding buffer for membranes.

10mM Tris, 5mM magnesium chloride, 3mM EDTA, 1mM EGTA.

pH adjusted to 7.4 using 1M HCL.

Binding buffer for whole cells.

Hanks buffered saline containing 0.05% (w/v) BSA, 10mM Hepes, 10mM glucose.

pH adjusted to 7.4.

All buffers were made using distilled water and stored at $4^{o}C$.

2.3 Ion exchange resins.

2.3.1 Preparation of Dowex-formate.

Dowex 1x8-200 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 wash was repeated twice. The Dowex was transferred to a scintered 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 50 volumes of water until the pH of the slurry was constant at approximately 5.5. Following each preparation of Dowex formate it was characterised by performing ml by ml elutions of a mixture of [^{3}H]Ins(1)P, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3.

2.3.2 Preparation of Dowex-50W-H^+.

Dowex-50W-H^+, strongly acidic cation exchange resin, 8% cross-linked, with a dry mesh of 200-400, was prepared as described below. Dowex was washed three times in three volumes of distilled water, allowing the resin to settle and discarding the 'fines' each time. The Dowex was then washed three times with three volumes of 1M HCl accompanied by gentle stirring with a plastic rod. Washings were discarded each time. Finally, the Dowex was washed four or five times with three volumes of distilled water until the pH of the wash was constant at about pH 5.5.

Each newly prepared batch of Dowex was characterised by performing an elution profile of a mixture of radiolabelled 'standards' of GroPCho, ChoP and Cho added to a Rat-1 cell extract.

2.4 Culture of Rat-1 fibroblast cells.

Rat-1 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine (2mM), penicillin/streptomycin (100 IU/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 pen/strep. Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. Except for experimental purposes cells were grown in 75 cm^2 culture flasks and were

passaged when sub-confluent. Every two or three weeks a single flask was allowed to grow to confluency to ensure that cells were contact inhibiting and were not exhibiting aberrant morphology. Cells which exhibited transformed morphology, showed reduced responsiveness to given agonists or reached passage numbers higher than 30 were discarded.

2.4.1 Cell passage.

Cells were passaged at a ratio of 1 to 10 according to the following general protocol.

(1) Medium was aspirated and replaced with 3ml of sterile trypsin solution per 75 cm^2 flask.

(2) Flasks were returned to the incubator for 2-3 minutes until cells were detaching from the flask bottom. Trypsin activity was inhibited by addition of 7ml of complete medium.

(3) After transfer to a sterile tube, the cell suspension was centrifuged at 800g for 3 minutes and the supernatant discarded.

(4) Cells were resuspended in complete medium and aliquoted into new sterile flasks containing 10ml of fresh medium. Flasks were rocked gently to ensure even distribution of cells across the flask bottom and returned to the incubator.

2.4.2 Cryogenic preservation of cell lines.

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

(1) Cells were trypsinized as outlined above (cell passage 1-3)

(2) Cells were 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.

(3) Cells were slow frozen for 24 hours at -80°C before transfer to a vat of liquid N_2.

To bring cells up from storage, vials were removed from liquid N_2 and thawed rapidly in a water bath at 37°C. Each aliquot of cells was plated out in 3ml of fresh medium in a 25cm^2 flask and left to attach overnight. The next day the medium and suspended dead cells were removed and the surviving cells were fed normally.

2.5 Measurement of [^{3}H] thymidine incorporation.

Rat-1 cells were plated in 24-well plates at a density of ~1,000 cells per well in DMEM containing 10% (v/v) newborn calf serum. After 24h the medium was changed to DMEM containing 1% (v/v) newborn calf serum and the cells grown to quiescence over 2 days. The cells were then washed in serum-free DMEM and incubated in serum-free DMEM containing 1μCi/ml [^{3}H] thymidine and the agonists of choice for 24h. At the end of the labelling time the medium was removed and the monolayers washed twice with 0.5ml of Hanks followed by three 0.5ml washes with 5% (w/v) TCA and two 0.5ml washes with ethanol. The cells were solubilised in 0.5ml 0.3M NaOH and the samples transferred to 5ml insert vials. Optiphase Hi-safe scintillation fluid was added and the radioactivity associated with [^{3}H] thymidine counted.

2.6 Measurement of stimulated inositol phosphate formation.

2.6.1 Preparation of samples for [^{3}H] inositol phosphate measurement.

After plating on 24 well plates (~2,000 cells/well) in DMEM + 10%

newborn calf serum for 24 hours, Rat-1 cells were grown in 0.5ml of inositol-free DMEM containing 1% dialysed newborn calf serum with 1μCi/ml of [2-^{3}H]*myo*-inositol for 48 hours, by which time the cells were confluent, quiescent and labelled to isotopic equilibrium. Labelling medium was aspirated and cells were washed twice with 0.5ml of HBG (pH 7.4) at 37°C and then incubated for 10min with HBG containing 10mM LiCl (HBG/LiCl). Finally, cells were stimulated with 150μl of HBG/LiCl containing the test reagent at the final concentrations and for the times indicated.

Incubations were terminated by direct addition of 500 μl of ice cold methanol. After scraping the cell debris and transferring to a 5ml insert vial, each well was washed with a further 200 μl of methanol and the two washes pooled. Phospholipids were extracted from the cell debris by addition of 350 μl of chloroform followed by vortexing and standing on ice for 30 minutes or at 4°C overnight. After addition of 500μl of distilled water and 350μl of chloroform samples were centrifuged at 14,000g for 3 minutes to split the phases and 800μl of the upper aqueous/methanolic phase was transferred to a 5 ml insert vial for measurement of total inositol phosphates. In addition, an aliquot of the chloroform phase was removed, dried down in an insert vial and, after addition of 4ml of Optiphase Hi-safe scintillation fluid, the radioactivity in the total inositol lipids determined by scintillation counting.

2.6.2 Assay of total inositol phosphates.

Total inositol phosphates were assayed by accumulation in the presence of 10mM LiCl essentially by the method of Berridge *et al.* (1982). Samples (800μl) were added to insert vials containing 500μl Dowex formate (1x8 200-400 mesh), followed by 3 ml of distilled water to mix. After the Dowex had settled the supernatant was aspirated. The removal of glycerophosphoinositides was achieved by two washes with 3 ml of 60 mM ammonium formate/5mM

sodium tetraborate followed by a further 3 ml wash with water. Finally total inositol phosphates (mainly $InsP_1$ and $InsP_2$) were eluted by a 1ml wash of 1M ammonium formate/ 0.1M formic acid. The supernatant was removed to an insert vial and mixed with Optiphase Hi-safe scintillation fluid. The radioactivity was estimated by liquid scintillation counting.

2.6.3 Preparation of samples for analysis of individual inositol phosphate fractions.

Rat-1 cells were plated in 75cm^2 flasks in DMEM containing 10% newborn calf serum. After 48h, the medium was changed to inositol-free DMEM containing 1% dialysed newborn calf serum and 8μCi/ml [2-^{3}H] *myo*-inositol for 48h. Cells were harvested by scraping, preincubated for 45min in the conditioned DMEM and then washed twice in HBG. Aliquots (50μl) of resuspended cells (approx. 2x10^4 cells/aliquot) were incubated with agonist or vehicle in assay buffer (250μl final volume) at 37^{o}C at the final concentration and for the times indicated. The reaction was terminated by the additin of 50μl 10% (v/v) perchloric acid and the samples extracted on ice for 20min. The samples were then neutralized by the addition of 1.5M KOH/60mM Hepes in the presence of Universal Indicator and the precipitated potassium perchlorate removed by centrifugation at 14,000g for 3min at 4^{o}C. The supernatants were analysed by ion-exchange chromatography on Dowex 1x8 formate columns.

2.6.4 Assay of individual inositol phosphate fractions.

Neutralized supernatants were diluted in 5ml of 5mM sodium tetraborate/ 0.5mM-EDTA, pH 6.7 and applied to 1 ml columns of Dowex formate (1x8; 200-400 mesh) prepared in glass wool plugged pasteur pipettes. The columns were then washed with 10 ml of water followed by 14ml of 60 mM ammonium

formate/ 5m-sodium tetraborate to remove free inositol and glycerophosphoinositides, respectively. Inositol mono-, bis-, tris-, and tetrakisphosphates were eluted with sequential 18 ml washes of 0.1M-formic acid containing 0.2M-, 0.4M-, 0.8M- and 1.2M- ammonium formate respectively. Aliquots of each fraction were mixed with Optiphase Hi-safe scintillant and the radioactivity determined by liquid scintillation counting.

2.7 Measurement of stimulated $Ins(1,4,5)P_3$ mass formation.

2.7.1 Preparation of samples for measurement of $Ins(1,4,5)P_3$ mass.

Samples were prepared from cells harvested from flasks or attached to plastic wells.

Rat-1 cells grown to confluency in $75cm^2$ flasks were harvested by scraping into their culture medium. After a 45min incubation time at 37^oC (this incubation period has been shown to allow full recovery of $Ins(1,4,5)P_3$ levels to basal) the cells were spun down and washed twice in HBG (pH 7.4). The cells were then resuspended in HBG and incubated at 37^oC for 10min. Aliquots (50µl) containing approximtely 10^6 cells were stimulated with 25µl of agonist at 37^oC in plastic polytubes and the reactions terminated by the addition of 25µl of ice-cold 10% (v/v) perchloric acid. The samples were extracted for 20min on ice and neutralized with 1.5M KOH/60mM Hepes containing Universal Indicator. The cell debris and precipitated potassium perchlorate were removed by centrifugation at 14,000g for 3min at 4^oC. A 25µl aliquot of the supernatant was removed for assay of $Ins(1,4,5)P_3$ mass.

Alternatively, Rat-1 cells were grown to confluency in 12-well plates. The medium was removed and the monolayers washed twice with 0.5ml HBG and

incubated with 1ml of HBG for 20min at 37°C. The cells were then stimulated with 175µl of agonist in HBG at 37°C at the concentrations and for the times indicated. Incubations were terminated by the addition of 25µl of ice-cold 16% (v/v) perchloric acid followed by extraction on ice for 20min. Samples were neutralized with 1.5M KOH/ 60mM Hepes containing Universal Indicator. The cells were then scraped and transferred to eppendorf tubes. The cell debris and precipitated potassium perchlorate were removed by centrifugation at 14,000g for 5min at 4°C. The supernatant (200µl) was removed for assay of $Ins(1,4,5)P_3$ mass.

2.7.2 Assay of $Ins(1,4,5)P_3$ mass.

Samples prepared as described were assayed for $Ins(1,4,5)P_3$ mass by the competitive binding assay of Palmer *et al.* (1989). The assay employs a bovine adrenocortical microsomal preparation which possesses a single population of specific high affinity binding sites for $Ins(1,4,5)P_3$.

Aliquots (25µl) of adrenal cortex preparation (20mg/ml of protein) were incubated with 200µl of sample or $Ins(1,4,5)P_3$ standard, 100µl of binding buffer (100mM Tris, 4mM EDTA, 4mM EGTA, 4mg/ml BSA; pH 8.5), 25µl water and 50µl of [^{3}H] $Ins(1,4,5)P_3$ (approx. 3,000 cpm) on ice for 20min. Nonspecific binding was determined in the presence of 1µM $Ins(1,4,5)P_3$. Incubations were teminated by centrifugation at 12,000rpm for 3min at 4°C and the supernatant then aspirated. The radioactivity bound to the pellet was determined by liquid scintillation counting after solubilisation in Hi-Safe scintillant. The use of a standard curve of displacement of [^{3}H] $Ins(1,4,5)P_3$ by unlabelled $Ins(1,4,5)P_3$with each assay allowed estimation of $Ins(1,4,5)P_3$ mass in cell extracts.

2.8 Measurement of stimulated PIP_2 mass hydrolysis.

Rat-1 cells were grown to confluence and quiescence in 24-well plates. The medium was removed and the cells washed twice with 0.5ml HBG (pH 7.4) and incubated with HBG for 20min. Stimulation was then carried out with 150µl of agonist in HBG at the concentrations and for the times indicated at 37°C and the reaction terminated by the addition of 100µl of ice-cold 20% (w/v) TCA. The cells in each well were scraped and transferred to eppendorf tubes. The samples were spun at 14,000g for 2min at 4°C and the supernatant removed. The wells were then washed with 500µl of 5% (w/v) TCA/ 1mM EDTA and this used to resuspend the cell pellet. These samples were then spun at 14,000g for 2min at 4°C and the supernatant removed. The pellet was washed with 500µl water ,spun as before and the supernatant removed. Chloroform/ methanol/ 12MHCL (40 : 80 : 1, by volume) (500µl) was added to the pellet and the samples left at room temperature for 10-20min. Then, 165µl chloroform and 300µl 0.1M HCL was added, the samples left for a further 10min and finally spun at 14,000g for 2min. An aliquot (250µl) of the lower phase was taken and dried down *in vacuo*.

The dried lipid extract was deacylated and deglycerated by the addition of 250µl of 1M KOH and incubation of the samples at 100°C for 30min. After placing on ice for 5min, the samples were spun at 14,000g for 2min and then passed through a 0.5ml (50% w/v slurry) of Dowex 50 (H^+, 200-400 mesh) column. The columns were washed with 3 x 0.75ml water. The resulting 2.5ml sample was washed with 3ml butanol : petroleum ether (5:1; v/v). The upper phase was aspirated and the sample washed with a further 2ml of the same solvent. The upper phase was once again aspirated and discarded. An aliquot of the lower phase (1ml) was dried down, redissolved in water and assayed using the $Ins(1,4,5)P_3$ mass assay described above.

2.9 Assay of PtdCho hydrolysis.

2.9.1 Preparation of samples for mesurement of PtdCho breakdown.

Rat-1 cells were plated at a density of approximately 2,000 cells/ml on 24 well plates and grown in DMEM + 10% newborn calf serum for 24 hours. They were then labelled with 5μCi/ml [^{3}H] choline chloride in DMEM + 1% newborn calf serum for 48 hours by which time they were confluent and quiescent. Following removal of the labelling medium, the cells were washed twice with 0.5ml HBG (pH 7.4) and then preincubated with 0.5ml HBG for 30min.. Finally, cells were incubated in 150μl of HBG containing the given concentration of test reagent for the required time.

For experiments where intracellular or cell associated choline metabolites were studied, incubations were terminated by aspiration of the test solution and addition of 500μl of ice cold methanol. For experiments where intracellular and extracellular metabolites were studied incubations were terminated by addititon of 500μl of ice cold methanol to the incubation buffer. Cell debris was scraped, transferred to a 5ml insert vial and pooled with a further 200μl methanol wash. Phospholipids were extracted by addition of 350μl chloroform followed by vortexing. After standing on ice for 1hour or at 4^{o}C overnight, a further 350μl of chloroform and 500μl of water was added and the phases were split by centrifuging at 14,000g for 3 minutes. A 1ml sample of the aqueous methanolic phase was taken for separation of the major water soluble metabolites of choline by cation exchange chromatography.

2.9.2 Separation of the major water soluble metabolites of choline.

The 1ml aqueous methanolic samples from experiments were loaded on to

1ml Dowex-50W-H^+ columns prepared in glass wool plugged pasteur pipettes. The flow through and a further 8 ml water wash were pooled and collected as the glycerophosphocholine fraction. A further wash with 20 ml of water was collected as the phosphocholine fraction, whilst a final wash with 20 ml of 1M HCl eluted off the choline fraction. Two ml aliquots of each fraction were mixed with Optiphase Hi-safe scintillant and radioactivity determined by liquid scintillation counting.

2.9.3 Analysis of [^{3}H] choline containing lipids.

[^{3}H]choline labelled Rat-1 cells were stimulated with agonist or vehicle as described above. Following extraction in chloroform and methanol as described above, the aqueous methanolic phase was removed and the chloroform phase dried *in vacuo*. Choline-containing lipids were re-dissolved in 100µl of chloroform/methanol (19/1; v/v) and half the sample was applied to silica gel 60 t.l.c. plates. The t.l.c. plates were developed fully in a lined, equilibrated tank in a solvent of chloroform:methanol:acetic acid:water (25:15:4:2, v/v) (Skipski *et al.*, 1964). PtdCho and sphingomyelin were identified by their R_f values and co-migration with authentic standards. After excision of the appropriate spot the radioactivity in PtdCho was determined by scintillation counting using 4ml of Optiphase Hi-safe.

2.10 Measurement of Phospholipase D transferase activities in whole cells.

2.10.1 Assay of PtdBut formation in [^{3}H] palmitate labelled Rat-1 cells.

PLD catalysed phosphatidyltransferase activity was assayed by measuring

the incorporation of butan-1-ol into phosphatidyl moieties.

Rat-1 cells were sub-cultured into 24-well culture plates and grown in DMEM + 10% newborn calf serum until approximately 70% confluent. The growth media was then replaced with 0.5ml of DMEM + 1% newborn calf serum containing 4 μCi/ml of [^{3}H]palmitic acid and the cells were labelled for approximately 48 hours. For experimental purposes the labelling medium was removed by aspiration and cells were washed twice with 0.5ml HBG (pH 7.4) followed by a preincubation for 20min with 0.5ml HBG. For kinetic studies cells were incubated for a further 5 minutes in 0.5ml of HBG containing 30 mM butan-1-ol (0.3%, v/v) at 37^o C prior to addition of agonists. Finally cells were incubated in HBG + 30mM butan-1-ol + the required concentration of test reagent for the indicated time. For dose dependency studies there was no pre-incubation with butan-1-ol and the appropriate concentration of test reagent in HBG + 30mM butan-1-ol was added directly to the cells for 15 minutes. Incubations were terminated by aspiration of the incubation medium followed by the addition of 0.5ml of ice-cold methanol. Cell debris was scraped off the plate surface and transferred to screw-top glass tubes together with a further 0.2ml of methanol wash. Phospholipids in these pooled scrapings were extracted by the addition of 0.7ml of chloroform, vigorous vortexing and standing at room temperature for 15 minutes. After addition of 0.6ml of water the samples were centrifuged for 5 minutes at 14,000g, the upper aqueous/methanolic phase discarded and the chloroform phase dried *in vacuo*. Samples were routinely analysed by thin layer chromatography immediately.

2.10.2 Isolation and Identification of Phosphatidylbutanol.

Dried chloroform extracts were re-dissolved in 50μl of chloroform/methanol (19/1) with vigorous vortexing and applied to Whatman LK5DF, 20cm x 20cm, pre-laned silica gel thin layer chromatography plates.

Once the samples had dried, the plates were fully developed once in the organic phase of 2,2,4-trimethylpentane:ethyl acetate:acetic acid:water (50/110/20/100, v/v) under equilibrium conditions in an un-lined chromatography tank.

After drying, plate lanes were divided into 0.5cm strips, the silica gel scraped into scintillation vials and radioactivity counted in 4ml of Optiphase Hi-safe scintillant. The band of radioactivity corresponding to PtdBut was identified by the following criteria:-

(a) its co-migration with an authentic [^{14}C]PtdBut standard developed on the same t.l.c. plate,

(b) its unique appearance in the presence of butan-1-ol in response to a suitable stimulus,

(c) its dose dependent appearance with increasing butan-1-ol concentration up to 100mM in response to a suitable stimulus.

2.11 Measurement of [^{3}H] PDBu binding to intact cells.

Rat-1 cells in 24-well plates were grown to confluency. The medium was removed and the monolayers washed twice with 0.5ml HBG (pH 7.4). The cells were then incubated with 150µl of 100nM [^{3}H] PDBu in HBG for 20min. Specific binding was defined by the inclusion of 20µM unlabelled PDBu. The binding reaction was terminated by aspirating the incubation medium and washing the cells three times with 0.5ml of ice-cold HBG. The cells were solubilised with 0.5ml of 0.5M NaOH/ 0.1% (v/v) Triton x-100 and bound radioactivity was determined by scintillation counting.

2.12 [^{125}I] Endothelin binding studies.

2.12.1 Preparation of membranes from Rat-1 cells.

Rat-1 cells were grown in 75cm^2 flasks or glass burlers to confluency and then harvested by scraping from flasks or trysinisation from burlers. After a 45min incubation at 37^{o}C to allow recovery from the physical perturbation caused by harvesting, the cells were spun at 800g for 5min. The supernatant was removed and the pellet washed twice in PBS. The final pellet was sometimes stored at -80^{o}C until needed.

For the preparation of membranes, the cells were resuspended in 5ml of 10mM Tris/ 0.1mM EDTA (pH 7.4) and homogenised with 25 strokes of a Potter homogeniser. The homogenate was spun at 500g for 10min in a Beckman L5-50B centrifuge with a Ti 50 roter and the supernatant taken and spun at 48,000g for 10min. The resulting pellet was resuspended in 5ml 10mM Tris/ 0.1mM EDTA and spun again at 48,000g for 10min. The final pellet was resuspended in 1ml of 10mM Tris/0.1mM EDTA (pH 7.4) and stored in 100µl aliquots at -80^{o}C.

2.12.2 Measurement of [^{125}I] ET-1 binding to Rat-1 membranes.

Rat-1 membranes (100µl; 25µg protein) were incubated with 100µl binding buffer (10mM Tris, 5mM $MgCl_2$, 3mM EDTA, 1mM EGTA; pH 7.4) and 50µl [^{125}I] ET-1 (~88,000 d.p.m.) at 25^{o}C for 30min to reach equilibrium binding. Excess unlabelled ET-1 (1µM) was included to determine nonspecific binding. Binding was terminated by separating bound ligand from free ligand by vacuum filtration through GF/C filters. The filters were washed three times with 4ml of ice-cold binding buffer and then counted in a gamma radiation counter.

2.13 Measurement of intracellular calcium in Rat-1 cells

Rat-1 cells were grown on glass coverslips. The confluent monolayers were washed in HBG (pH 7.4) and incubated in HBG containing 5μM Indo-1 AM, 0.125% (w/v) plurionic acid, 0.5% (v/v) DMSO for 20min 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 1.5 ml/min (35-37^{o}C) and illuminated at 360nm. Light emitted from the cells with a wavelength 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 3 Hz. 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 $[Ca^{2+}]_i$ from cells loaded with AM esters of Ca^{2+} indicators the experimental records are presented in terms of raw fluorescence data.

2.14 Permeabilization of Rat-1 cells.

2.14.1 Electropermeabilization.

Rat-1 cells were grown to confluency in 75cm^2 flasks and then harvested by scraping into their culture medium. After a 45min incubation at 37^{o}C the cells were centrifuged at 800g for 3min and the pellet washed twice in permeabilization buffer (pH 7.4). The cells were then resuspended in permeabilization buffer and exposed to six discharges at 1 sec intervals of a 3μF capacitor with a field strength of 2kV/cm.

2.14.2 Permeabilization of cells using streptolysin-O.

Rat-1 cells were grown to confluency and quiescence on 24-well plates. The monolayers were washed three times with 0.5ml of HBG (pH 7.4) and incubated with 0.5ml of permeabilization buffer containing 0.6 units/ml of streptolysin-O for 5min. The cells were then washed with 0.5ml of permeabilization buffer for 15min.

2.15 Lowry protein assay. (Lowry *et al.*, 1951)

Aliquots of sample (2,4,6,8µl) were incubated with 1ml of reaction mixture (1% $CuSO_4$, 2% sodium potassium tartrate and 2% sodium carbonate in 0.1M NaOH) for 10min at room temperature. Then, 100µl of dilute Folin & Ciocalteu's phenol reagent (1:1 with water) was added and the samples incubated for a further 20min at room temperature. Finally, absorbance was measured at 750nm. Protein concentration was calculated from a standard curve constructed using a 1mg/ml solution of BSA.

2.15 Analysis and presentation of results.

Unless otherwise stated all experiments were performed at least three times and each data point represents the mean ± standard deviation (S.D.) of triplicate determinations. Statistical significance was estimated by a paired Students 't' test on an Apple Macintosh Stat-Works program.

EC_{50} and IC_{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 presented as simple line graphs from the Apple Macintosh Cricket Graph program.

Chapter 3

Signal transduction pathways stimulated by endothelin

3.1. Introduction

Although the wide range of biological actions of the endothelins (ETs) is now appreciated (section 1.6.2), little is known about their mechanism of action. The original report describing ET-1-stimulated vasoconstriction demonstrated that extracellular calcium was needed for contraction and that vasoconstriction by ET-1 was inhibited by nicardipine suggesting that influx of extracellular calcium was required for the action of the peptide (Yanagisawa *et al.*, 1988a). This led the authors to suggest that ET-1 might be acting directly on membrane calcium ion L-channels. Later work confirmed the importance of extracellular calcium both in ET-1 induced contraction (Marsault *et al.*, 1990; Miyauchi *et al.*, 1990) and in other responses such as mitogenesis (Nakaka *et al.*, 1989; Shichiri *et al.*, 1991) but showed that ET-1 does not act directly on Ca^{2+} channels (Sugiura *et al.*, 1989a; Badr *et al.*, 1989).

It is now widely accepted that ET-1 binds to a specific membrane receptor leading to the activation of a number of signal transduction pathways including polyphosphoinositide hydrolysis and activation of the arachidonic acid cascade (reviewed in Simonson and Dunn, 1990a, b). The activity of PtdIns-PLC, as measured by the generation of inositol phosphates, increases rapidly after stimulation with ET-1. ET-1 stimulated a dose-dependent accumulation of inositol phosphates in fibroblasts (Muldoon *et al.*, 1989), vascular smooth muscle cells (Araki *et al.*, 1989; Resink *et al.*, 1988), mesangial cells (Simonson *et al.*,.1989), endothelial cells (Vigne *et al.*, 1990a), adrenal glomerulosa cells (Woodcock *et al.*, 1990) and in both neurons and glial cells (Lin *et al.*, 1990). The closely related peptide sarafotoxin S6b (SS6) could also stimulate phosphoinositide turnover in rat heart and brain (Kloog *et al.*, 1988) and neurally-related cell cultures (Lin *et al.*, 1990) suggesting that the endothelins and sarafotoxins share the same signalling pathways. The initial inositol phosphate product of PtdIns-PLC-catalysed hydrolysis of PtdIns(4,5)P_2 is Ins(1,4,5)P_3 and its formation has been demonstrated as early as 5sec after

stimulation with ET-1 in adrenal glomerulosa cells (Woodcock *et al.*, 1990) and 10sec in rat mesangial cells (Simonson *et al.*, 1989). Rapid generation of $InsP_3$ (consisting of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$) has also been observed in vascular smooth muscle cells (Resink *et al.*, 1990a), fibroblasts (Takuwa *et al.*, 1989) and endothelial cells (Vigne *et al.*, 1990a).

The other product of $PtdIns(4,5)P_2$ breakdown is *sn*-1,2-diacylglycerol (DAG) and elevated DAG levels in response to ET-1 stimulation have been reported in several systems. A biphasic increase in DAG that is sustained for 20min or more was observed in response to ET-1 in vascular smooth muscle cells (Lee *et al.*, 1989; Griendling *et al.*, 1989) and C6 glioma cells (Zhang *et al.*, 1991). Sustained DAG formation was also observed in fibroblasts (Muldoon *et al.*, 1989; Takuwa *et al.*, 1989). DAG is the main physiological activator of protein kinase C (PKC) and increased PKC activity induced by ET-1 has been demonstrated by different approaches. In Swiss 3T3 cells stimulation with ET-1 increased the incorporation of ^{32}P into an 80kDa protein which is a PKC substrate (Takuwa *et al.*, 1989) while in vascular smooth muscle cells PKC activity (measured as histone H1 phosphorylation) was translocated to the plasma membrane in response to ET-1 stimulation (Lee *et al.*, 1989).

$Ins(1,4,5)P_3$ mobilizes calcium from intracellular stores and much attention has been paid to calcium signalling by the ET peptides. Ca^{2+} mobilization in response to ET-1 is typically biphasic consisting of a rapid transient increase followed by a smaller but sustained increase. This type of Ca^{2+} signal has been observed in Swiss 3T3 fibroblasts (Takuwa *et al.*, 1989; Ohnishi *et al.*, 1989), rat and human glomerulosa mesangial cells (Simonson *et al.*, 1990, Simonson and Dunn, 1991b), vascular smooth muscle cells (Hirata *et al.*, 1988; Simpson and Ashley, 1989; Marsden *et al.*, 1989; Kai *et al.*, 1989; Wallnöfer *et al.*, 1989), endothelial cells (Vigne *et al.*, 1990b) and atrial cells (Vigne *et al.*, 1990a). Generally, both intracellular release and extracellular influx of Ca^{2+} contribute to the transient phase, whereas influx is mainly

responsible for the sustained phase. In some cases the influx of Ca^{2+} is sensitive to inhibition by dihydropyridine (DHP) and other voltage-sensitive L-channel blockers but in other systems it is DHP-insensitive suggesting that influx occurs via some type of receptor- or second-messenger-operated channels.

Although most attention has focused on the hydrolysis of PtdIns(4,5)P_2 as a cellular signalling pathway for ET-1, there have been reports in some systems of other second-messenger-generating pathways. ET-1 stimulated the release of prostacyclin and thromboxane A_2 from pig or rat isolated lungs, suggesting activation of phospholipase A_2 (PLA_2) (De Nucci *et al.*, 1988). PLA_2 activation was also demonstrated in vascular smooth muscle cells by the ET-induced extracellular release of arachidonic acid (AA) derived from PtdCho and PtdIns (Resink *et al.*, 1989; Resink *et al.*, 1990a; Reynolds *et al.*, 1989). Similarly, ET-1 stimulated AA release and prostaglandin $F_{2\alpha}$ and thromboxane B_2 synthesis in rat mesangial cells (Simonson and Dunn, 1990c). In vascular smooth muscle cells PLA_2 activity was proposed to occur independently of PtdIns(4,5)P_2 hydrolysis due to the lack of inhibition of the AA response by neomycin (Resink *et al.*, 1990a) or PMA (Reynolds *et al.*, 1989) treatment.

Studies in C6 glioma cells and vascular smooth muscle cells have demonstrated ET-stimulated PtdCho hydrolysis measured as increased levels of choline and choline phosphate (Zhang *et al.*, 1991; Resink *et al.*, 1990a). In Swiss 3T3 fibroblasts ET-1 caused a decrease in cAMP levels (Takuwa *et al.*, 1989) while in rat nephron segments ET inhibited vasopressin-dependent cAMP accumulation (Tomita *et al.*, 1990). In contrast in rat glomerulosa mesangial cells ET-1 potentiated β-adrenergic-mediated cAMP accumulation by a prostaglandin E_2-dependent mechanism (Simonson and Dunn, 1990c). Finally, in a neuronal cell line ET-1 stimulated a rise in cGMP indicating activation of guanylate cyclase (Reiser, 1990).

ET-1, therefore, appears to interact with a number of signal transduction

pathways either directly or indirectly. In this work the second messenger pathways mediating the mitogenic effect of ET-1 in Rat-1 cells were investigated. Attention was focussed on PtdIns(4,5)P_2 and PtdCho hydrolysis as both these pathways generate second messengers with potentially important roles in the proliferative response (section 1.3.3 and 1.3.4)

3.2. ET-1 is a complete mitogen in Rat-1 cells

When proliferation was measured as an increase in the incorporation of [^{3}H]thymidine into DNA, ET-1 was found to be a complete mitogen for Rat-1 cells. Although not as potent as 10% serum (5.7 ± 1.8 fold increase, n=24), a maximum dose of ET-1 (100nM) stimulated a 2.2 ± 0.5 (n=28) fold increase in [^{3}H]thymidine incorporation in quiescent cells in serum-free medium in the absence of any other added growth factors (Fig. 3.1). The other ET peptides, ET-2 (100nM) and ET-3 (300nM), stimulated [^{3}H]thymidine incorporation to a similar extent (2.2 ± 0.5 fold for ET-2; 1.8 ± 0.3 fold for ET-3; n=4) and the phospholipid lysophosphatidic acid (LPA) was also equally effective (1.9 ± 0.3 fold; n=20). Bombesin, however, did not significantly increase [^{3}H]thymidine incorporation (1.0 ± 0.2 fold; n=7). The response to ET-1 was dose-dependent (Fig. 3.2) with a maximum response at about 10nM and with an EC_{50} value of 2.4 ± 1.3nM (n=3).

Long term PMA treatment has been reported to down-regulate PKC activity (Rodriguez-Pena and Rozengurt, 1984). In Rat-1 cells pretreated for 48h with 400nM PMA the incorporation of [^{3}H]thymidine in response to ET-1 was inhibited to a variable extent. A significant inhibition ($p < 0.05$) was observed in four out of a total of six experiments (69 ± 24% inhibition, n=4) (Fig. 3.3a). In the remaining two experiments PMA pretreatment had no effect. The variable results obtained in cells treated with PMA may be due to the

incomplete down-regulation of protein kinase C activity (see section 3.7). The same PMA treatment did not significantly affect the response to serum (Fig. 3.3a) or LPA (results from a typical experiment : -PMA, 194 ± 9% of basal with LPA; +PMA, 212 ± 23% of basal with LPA,; n=5) in five out of six experiments. Chronic PMA pretreatment did not affect the basal incorporation of [^{3}H] thymidine (-PMA, 1547 ± 61 d.p.m.; +PMA, 1601 ± 135 d.p.m.; n=6).

The effect of pertussis toxin pretreatment was also investigated. Pertussis toxin ADP-ribosylates the α-subunit of G_i and G_O and thus inhibits productive coupling to the effector system (Reisine, 1990). The incorporation of [^{3}H] thymidine in response to ET-1 was however pertussis toxin-insensitive (Fig. 3.3b) suggesting that coupling of the ET receptor to mitogenic signalling pathways does not involve G_i -like G proteins. The mitogenic response to LPA in Rat-1 fibroblasts has been reported to be pertussis toxin-sensitive (van Corven, 1989); however, in the work presented here the incorporation of [^{3}H] thymidine in response to LPA was only inhibited by pertussis toxin in one experiment and otherwise was found to be insensitive to the toxin pretreatment (results from a typical experiment : -PTox, 163 ± 6% of basal with LPA; +PTox, 183 ± 7% of basal with LPA; n=3).

3.3. ETs stimulate PtdIns(4,5)P_2 hydrolysis

PtdIns(4,5)P_2 hydrolysis was measured by the generation of the water-soluble inositol phosphate products. ET-1 (100nM) stimulated a rapid increase in the mass of Ins(1,4,5)P_3 which peaked at 5-10sec with a 7-10-fold increase in stimulated levels over control (results from a typical experiment : 0.8 ± 0.4 pmol in control; 7.9 ± 0.9 pmol with ET-1; n=6) and then declined towards basal levels (Fig 3.4). However, levels remained elevated 2-4 fold above controls at 10min (results from a typical experiment : 1.3 ± 0.2 pmol in control;

4.2 ± 0.7 pmol with ET-1; n=4).

ET-1 stimulated the accumulation of [^{3}H] inositol phosphates (InsP) in the presence of 10mM LiCl after a lag time of about 1min; this is probably because lithium exerts its effect downstream of PtdIns(4,5)P_2 hydrolysis by inhibiting the metabolism of inositol phosphates at the level of the Ins(1,4)P_2/Ins(1,3,4)P_3 1-phosphatase and inositol monophosphatase (Nahorski *et al.*, 1991). The accumulation of inositol phosphates in response to ET-1 was biphasic with an initial rapid increase up to 2-5min after which levels continued to increase at a slower rate in a linear manner up to 30-40min (Fig. 3.5). Subsequent measurements were carried out at 20min, a time at which ET-1 stimulated an approximately 4-6-fold increase in InsP over controls (results from a typical experiment : 1156 ± 75 d.p.m. in control; 6122 ± 332 d.p.m. with 100nM ET-1; n=3). The accumulation of [^{3}H] InsP in response to ET-1 was dose-dependent with a maximum increase at approximately 10nM and an EC_{50} value of 2.0 ± 1.0nM (n=5) (Fig. 3.6). ET-2 and ET-3 also stimulated the accumulation of [^{3}H] InsP in a time-dependent manner (Fig. 3.7) with a similar 4-6-fold increase over controls at 20min (results from a typical experiment : 4758± 1120 d.p.m.in control, 21032 ± 74 d.p.m. with 100nM ET-2, 18580 ± 1498 d.p.m. with 300nM ET-3). Pharmacological characterization of [^{3}H] InsP accumulation using ET-2, ET-3 and sarafotoxin S6b (SS6) yielded EC_{50} values of 1.2 ± 0.3nM, 54 ± 18nM and 1.7 ± 0nM respectively (n=3) (Fig. 3.8) thus giving a rank order of potency of ET-1 = ET-2 = SS6 > ET-3.

3.4 ETs stimulate PtdCho hydrolysis

PtdCho hydrolysis was measured by the generation of the water soluble choline products in [^{3}H] choline-labelled cells. The generation of [^{3}H] choline in response to a maximum dose of ET-1 (100nM) was also very rapid. There was a significant increase ($p < 0.05$) in both intracellular and total (intracellular +

extracellular) choline after 10sec which had at least doubled by 1min (Fig. 3.9a, c). Thereafter, the rate of production of total choline decreased and by 5min any further increase was paralleled by increased basals. Both stimulated and basal levels of total choline reached steady state after about 30min (Fig. 3.9b). The kinetics of intracellular choline changes were somewhat different, peaking at about 5min and remaining elevated for a further 5-10min before decreasing and returning to near-basal levels at 60min (Fig. 3.9c). The maximum obtainable increase in both intracellular and total choline levels in the presence of ET-1 over controls varied between 2-5 fold. The greater increase in basal values when total choline was measured rather than intracellular is probably due to the accumulation of choline in the extracellular medium where it is not susceptible to metabolism.

The generation of [^{3}H] choline in response to ET-1 was also dose-dependent with a maximum response at about 10nM and an EC_{50} value of 0.8 ± 0.4nM (n=8), which is very close to that for stimulated [^{3}H] InsP accumulation (Fig. 3.10). ET-2 and ET-3 were also effective at stimulating choline generation (Fig. 3.11). [^{3}H] Choline levels increased up to about 5min with 3-5-fold increases over controls and any subsequent increases were paralleled by increased basal values. The dose-dependencies were very similar to that for InsP accumulation (Fig. 3.12). EC_{50} values for ET-2, ET-3 and SS6 of 0.7 ± 0.2nM, 56 ± 18nM and 1.2 ± 0.2 nM (n=4) respectively were obtained, yielding the same rank order of potency ET-1 = ET-2 = SS6 > ET-3.

3.5 ET-1 stimulates PtdCho synthesis

In addition to measuring choline generation in response to ET-1 stimulation, choline phosphate levels were also determined since this metabolite may also be a product of PtdCho hydrolysis. There was no significant change in [^{3}H] choline phosphate content untill approximately 30min after stimulation

with ET-1 when a decrease of 15-30% below controls was observed (Fig. 3.13). In order to determine whether this might be due to stimulated incorporation into parent lipid, PtdCho synthesis in response to ET-1 stimulation was investigated. The increased incorporation of [^{3}H] choline into PtdCho was evident as early as 5min after stimulation and increased up to 20-30min, when it was paralleled by the basal increase (Fig. 3.14). The maximum response was 150-180% of the basal (results from a typical experiment; 5097 ± 861 d.p.m. in control; 8357 ± 453 d.p.m. with ET-1; 20min stimulation, n=3). [^{3}H] Glycerophosphocholine generation did not change in response to ET-1 stimulation up to 60min (results from a typical experiment : 9000 ± 1928 d.p.m. in control; 8738 ± 2154 d.p.m.with ET-1).

3.6. PMA-stimulated PtdCho hydrolysis

The PKC-activating phorbol esters have been shown to stimulate PtdCho hydrolysis in many systems (see section 1.5.2). The ability of PMA to induce choline generation was therefore investigated in Rat-1 cells. The generation of [^{3}H] choline in response to PMA was time- and dose-dependent. PMA stimulated choline generation after a lag time of 1-2min and levels increased rapidly up to approximately 5min when the rate decreased and by 15min the stimulated increase was paralleled by the basal increase (Fig. 3.15). Similar to ET-1, PMA also caused a decrease in choline phosphate levels after 30-40min (results from a typical experiment : 3444 ± 102 d.p.m. in control; 2600 ± 142 d.p.m. with 300nM PMA; 2747 ± 96 d.p.m. with 100nM ET-1; 45min stimulation).

The EC_{50} value for PMA-induced choline generation was 27 ± 14nM (n=3) (Fig. 3.16). β-phorbol (4β,9α,12β,13α,20-Pentahydroxytiglia-1,6-dien-3-one) which is inactive at stimulating protein kinase C gave no response

(results from a typical experiment : 708 ±35 d.p.m. in control; 750 ± 51 d.p.m. with 300nM β-phorbol; 2954 ± 257 d.p.m. with 300nM PMA; 10min stimulation). The magnitude of the PMA response was, however, generally smaller than that obtained with ET-1 (results from a typical experiment : 1106 ± 306 d.p.m. in control; 2130 ± 38 d.p.m. with 300nM PMA; 3145 ± 248 d.p.m. with 100nM ET-1; 10min stimulation, n=4).

3.7. The role of PKC in ET-1-stimulated PtdCho hydrolysis

The ability of PMA to stimulate [^{3}H] choline generation suggested a role for PKC in activating PtdCho hydrolysis. In order to investigate the role of PKC in mediating ET-1-stimulated PtdCho breakdown Rat-1 cells were pretreated for 48h prior to stimulation with 400nM PMA to down-regulate PKC. The effect of chronic PMA treatment on PKC activity was assessed by measuring the binding of [^{3}H] PDBu (Phorbol 12,13-dibutyrate) to intact Rat-1 cells. Specific [^{3}H] PDBu binding was time-dependent reaching equilibrium after 20-25min (Fig. 17a) and saturable with a K_D ~ 35nM (Fig. 17b). Pretreatment of Rat-1 cells with 400nM PMA for 48h reduced specific binding to 30-40% of that in control cells. Binding of [^{3}H] PDBu to cells treated for an equivalent length of time with 400nM β-phorbol or vehicle was the same as in control cells (Fig. 3.18).

Chronic PMA pretreatment totally abolished [^{3}H] choline generation in response to ET-1 and PMA (Table 3.1). In control cells which had been treated with the inactive β-phorbol for an equivalent length of time the stimulated generation of choline was as observed in untreated cells (results from a typical experiment : 334 ± 21% of basal in control cells, 320 ± 30% of basal in β-phorbol-treated cells; 10min stimulation with 100nM ET-1). Although ET-1 stimulated a larger increase in choline generation than PMA, the response was also completely inhibited by chronic PMA treatment.

The effect of long term PMA pretreatment on InsP accumulation was also examined. ET-1-stimulated [^{3}H] InsP generation remained the same in PKC-down-regulated cells indicating that coupling of the ET-1 receptor to the phosphoinositide system was still intact and unaffected by prolonged PMA treatment (Table 3.1)

3.8. ET-1 and PMA stimulate PtdCho hydrolysis by a phospholipase D-catalysed mechanism

ET-1 stimulated choline generation in the absence of any change in choline phosphate suggesting a phospholipase D- (PLD) catalysed hydrolysis of PtdCho. In order to more conclusively define a PLD-mediated pathway the ability of ET-1 to stimulate the phosphatidyltransferase activity of PLD was examined. The generation of phosphatidylalcohols is an unequivocal marker for PLD activity since phosphatidylalcohols can not be formed by alternative pathways (Pai *et al.*, 1988b). In the presence of 0.3% (v/v) butan-1-ol in [^{3}H] palmitic acid-labelled cells, ET-1 stimulated the rapid generation of [^{3}H] phosphatidylbutanol (PtdBut) with a significant increase ($p < 0.05$) above control levels after 10sec. PtdBut levels continued to rise up to 5min although the rate of increase was slower after 2min (Fig. 3.19a). At about 5min a maximum increase of approximately 8-fold was observed which remained unchanged for up to 30min. When a submaximal concentration of ET-1 (1nM) was used the initial rate of accumulation of PtdBut was slower but the response still levelled out at 5min (Fig. 3.19b) suggesting it was a desensitization and not simply a situation where the [^{3}H] label had become rate limiting. The generation of [^{3}H] PtdBut was dose-dependent (Fig. 3.20) with a maximum response at about 10nM and an EC_{50} value of 2.6 ± 1.5nM (n=3). This is similar to the EC_{50} value for ET-1-stimulated choline generation (0.8 ± 0.4nM).

In keeping with its ability to stimulate choline generation PMA also

induced the formation of [^{3}H] PtdBut in the presence of 0.3% (v/v) butan-1-ol in [^{3}H] palmitic acid-labelled cells. [^{3}H] PtdBut was generated with a lag time of approximately 2min following stimulation with 300nM PMA similar to choline generation, but the response was more sustained than that to ET-1 and lasted for up to 10-15min before levelling off (Fig. 3.21). The maximum response obtained was about 5-fold and in most experiments it was smaller than the increase obtained with ET-1 stimulation (results from a typical experiment : 2183 ± 107 d.p.m. in control; 10316 ± 354 d.p.m. with 300nM PMA; 17250 ± 2006 d.p.m. with 100nM ET-1; n=3).

3.9. Regulation of ET-1- and PMA-stimulated PLD activity by PKC and Ca^{2+} influx.

The ability of long-term PMA treatment to abolish the generation of choline in response to ET-1 and PMA suggested a role for PKC in regulating PLD activity. This was further investigated by examining the effect of the PKC inhibitor Ro-31-8220, a staurosporine analog (Davis *et al.*, 1989), on the stimulated generation of [^{3}H] PtdBut. Pretreatment of [^{3}H] palmitic acid-labelled Rat-1 cells with increasing concentrations of Ro-31-8220 for 10min resulted in the dose-dependent inhibition of PtdBut accumulation in response to both PMA and ET-1 (Fig. 3.22) thus confirming the choline results from the PKC-down-regulated cells. At 10μM, a concentration at which it is considered a specific PKC inhibitor (Davis *et al.*, 1989), Ro-31-8220 inhibited the PMA response by about 80% (results from a typical experiment : 3229 ± 1097 d.p.m. in control; 16825 ± 3871 d.p.m. with 300nM PMA; 5629 ± 639 d.p.m. with 300nM PMA in cells pretreated with 10μM Ro-31-8220; 15min stimulation, n=3). In contrast, at the same concentration, Ro-31-8220 only inhibited the ET-1 response by approximately 40% (results from a typical experiment : 1309 ± 69 d.p.m. in control; 8870 ± 2917 d.p.m. with ET-1; 5899 ± 710 d.p.m. with ET-

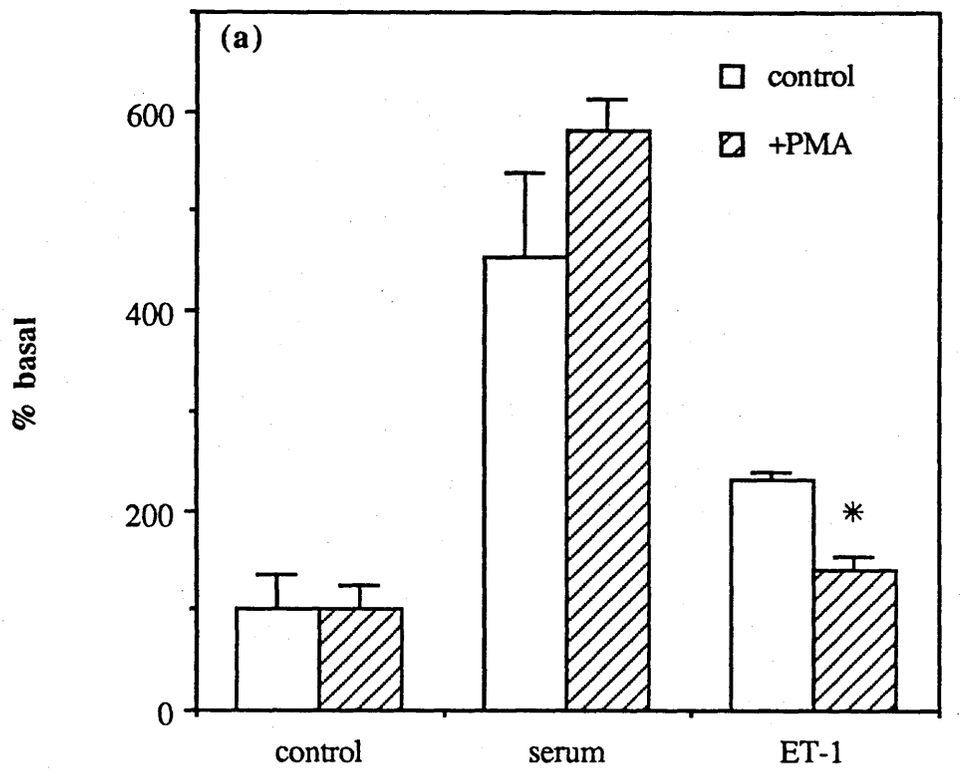
1 in cells pretreated with 10μM Ro-31-8220; 10min stimulation, n=4). The ET-1 response could be further inhibited by higher concentrations of Ro-31-8220 but at these concentrations the effect of the inhibitor becomes non-specific (Davis *et al.*, 1989).

Since Ca^{2+} influx has also been reported to have a role to play in the activation of PLD in some systems (section 1.5.2) the effect of lowering the external [Ca^{2+}] on ET-1-stimulated PtdCho hydrolysis was investigated. Cells were preincubated for 15min prior to stimulation in a low [Ca^{2+}] buffer with added EGTA (0.33mM) to reduce the free [Ca^{2+}] to 1μM and then stimulated under the same conditions. This treatment inhibited both ET-1-induced formation of choline in [^{3}H] choline-labelled cells (53 ± 8% inhibition, n=4) and PtdBut in [^{3}H] palmitic acid-labelled cells (57 ± 9% inhibition, n=4) but had no effect on the PMA-stimulated response (Table 3.2). ET-1-stimulated PtdBut formation was equally inhibited by low Ca^{2+} conditions and Ro-31-8220 (10μM) pretreatment and the two treatments combined produced the same degree of inhibition as each on its own suggesting a similar site of action (57 ±9% inhibition in low [Ca^{2+}], 49 ± 14% inhibition by Ro-31-8220, 61 ± 14% inhibition by Ro-31-8220 in low [Ca^{2+}]; n=3-4). Basal values of choline generation and PtdBut accumulation were unaffected by reducing the [Ca^{2+}]. Low Ca^{2+} conditions did not inhibit ET-1-stimulated InsP accumulation; the basal accumulation of [^{3}H] InsP was decreased but the ET-1-stimulated response was increased by 3-4 fold when the external [Ca^{2+}] was reduced to 1μM .(Table 3.2)

Since these results suggested a role for Ca^{2+} influx in activating PLD, the ability of the Ca^{2+} ionophore A23187 to stimulate PtdCho hydrolysis was examined (Table 3.2). A23187 (5μM) stimulated the formation of PtdBut although the response was smaller than that obtained with ET-1 (4.2 ± 0.8 fold increase over controls, n=4) . The response to the ionophore could be totally inhibited by reducing the external [Ca^{2+}] to 1μM but was only inhibited to a

small extent (24 ± 12%, n=3) by pretreatment with 10μM Ro-31-8220.

[^{3}H] PtdBut accumulation in response to ET-1, PMA and A23187 in combination with each other was also investigated (Table 3.3). PMA and A23187 together produced an additive response of similar magnitude to that obtained with ET-1. A23187 had no effect on the ET-1-stimulated PtdBut formation but PMA was synergistic and increased the ET-1 response by about 2-fold (2.0 ± 0.3 fold, n=3). The response to PMA and ET-1 in combination was greater than to PMA and A23187 in combination. The response to PMA and ET-1 in combination was not further increased by including A23187.


(a)
control
+PMA
600
400
200
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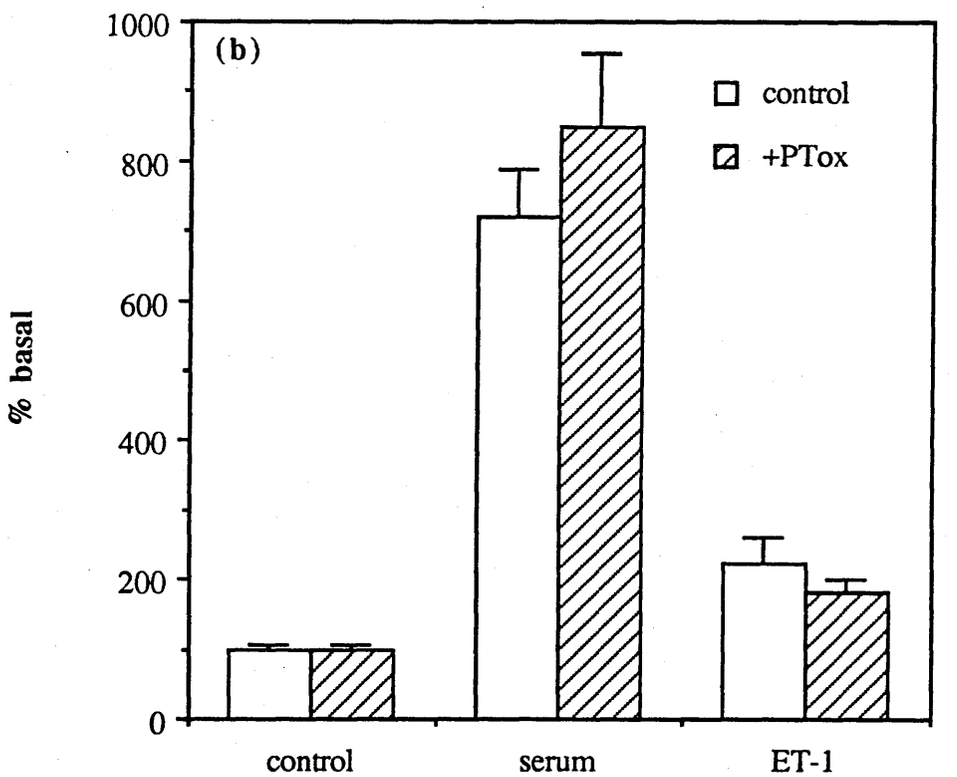
(b)
control
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% basal
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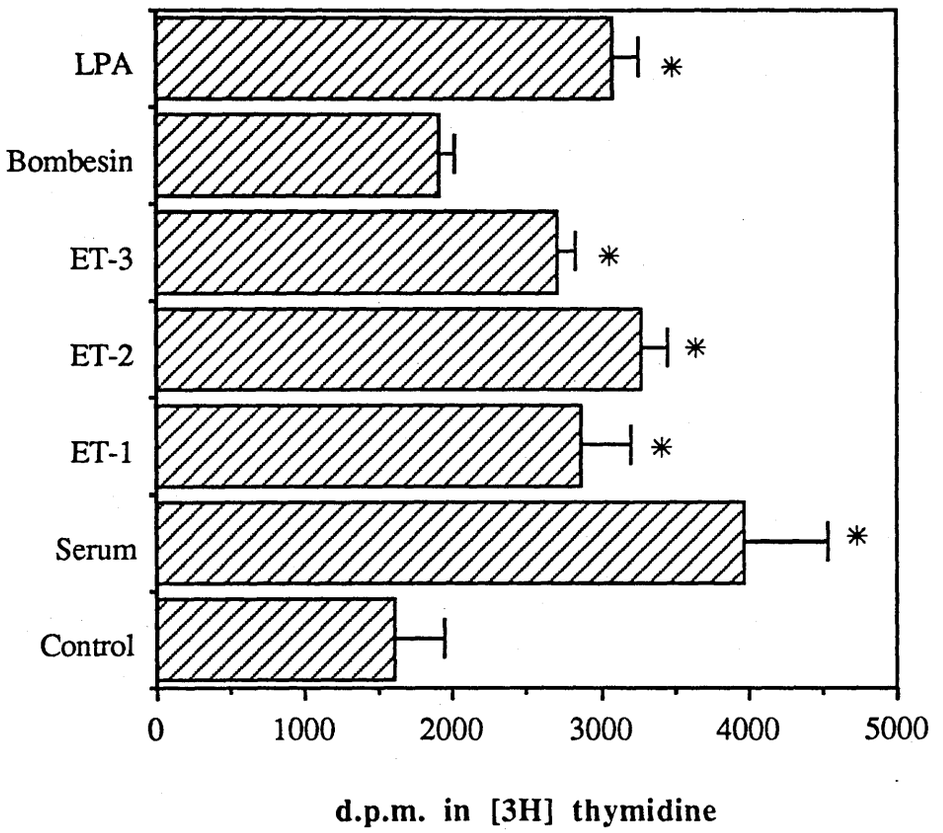
ET-1

Fig. 3.1 Mitogen-stimulated [^{3}H]thymidine incorporation in Rat-1 cells.

Rat-1 cells were grown to quiescence in DMEM +0.5% serum for 48h and then incubated in serum-free medium with the indicated agonist and 1μCi/ml [^{3}H]thymidine for a further 24h. Concentrations of agonists used were as follows : 10% serum, 100nM ET-1, 100nM ET-2, 300nM ET-3, 100nM bombesin, 100μM LPA. The incorporation of [^{3}H]thymidine was measured as described in section 2.5. Results are expressed as means ± S.D. from a typical experiment where n=4. Basal value = 1604 ± 339 d.p.m. ✳, $p < 0.05$.

Fig. 3.2 Dose-dependence of ET-1-stimulated [^{3}H]thymidine incorporation.

Rat-1 cells were grown to quiescence by incubating in the presence of 0.5% serum for 48h. The cells were stimulated with increasing concentrations of ET-1 in serum-free medium containing 1μCi/ml [^{3}H]thymidine for 24h and the radioactivity incorporated was then measured as described in section 2.5. Results are expressed as means ± S.D. from a typical experiment where n=3. Basal value = 1418 ± 178 d.p.m.


LPA
Bombesin
ET-3
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Serum
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1000
2000
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4000
5000
d.p.m. in [3H] thymidine

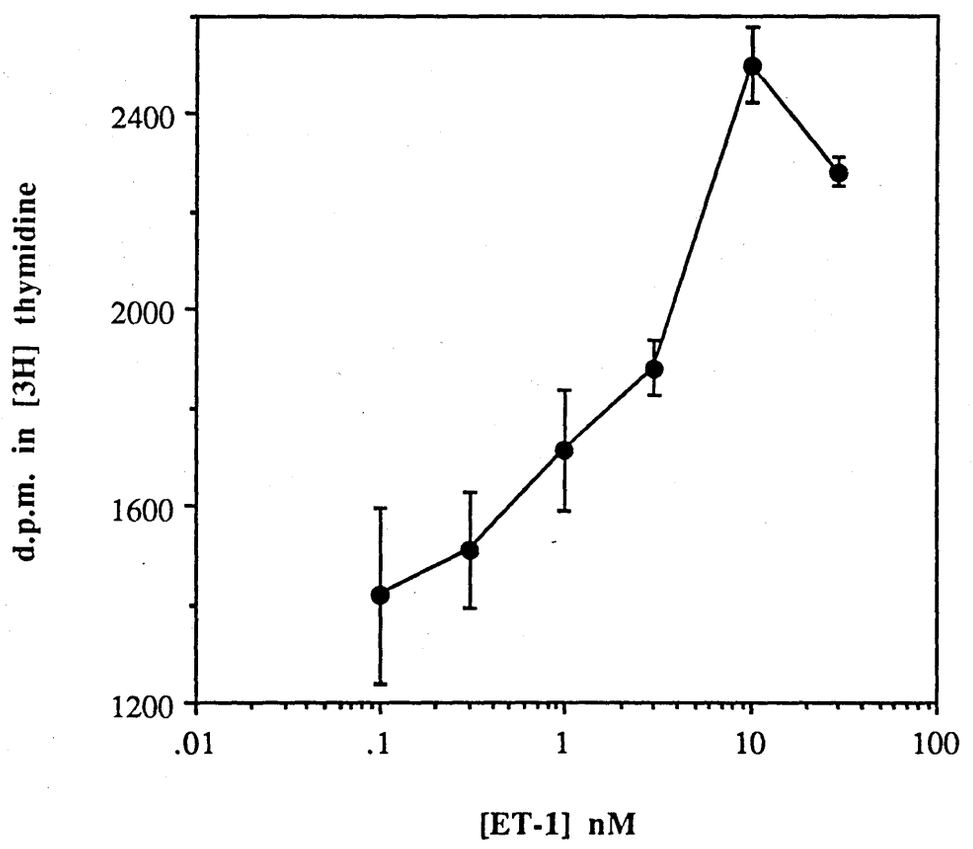

d.p.m. in [3H] thymidine
2400
2000
1600
1200
.01
.1
1
10
100
[ET-1] nM

Fig. 3.3 Mitogen-stimulated [^{3}H] thymidine incorporation in PMA- and pertussis toxin-treated cells.

Rat-1 cells were grown to quiescence in medium containing 0.5% serum for 48h. For the final (a) 48h (400nM PMA or vehicle) or (b) 16h (100ng/ml pertussis toxin) the cells were pretreated with the stated agent. Stimulation was then carried out with the indicated agonist (10% serum, 100nM ET-1) in serum-free medium containing 1μCi/ml [^{3}H]thymidine for 24h. The incorporated radioactivity was measured as outlined in section 2.5. Results are expressed as means ± S.D. from a typical experiment where n=3-6. Basal values (d.p.m.) = (a) -PMA, 2190 ± 738; +PMA, 1791 ± 453, (b) -PTox, 1660 ± 109; +PTox, 1628 ± 112. ✳, $p < 0.05$.

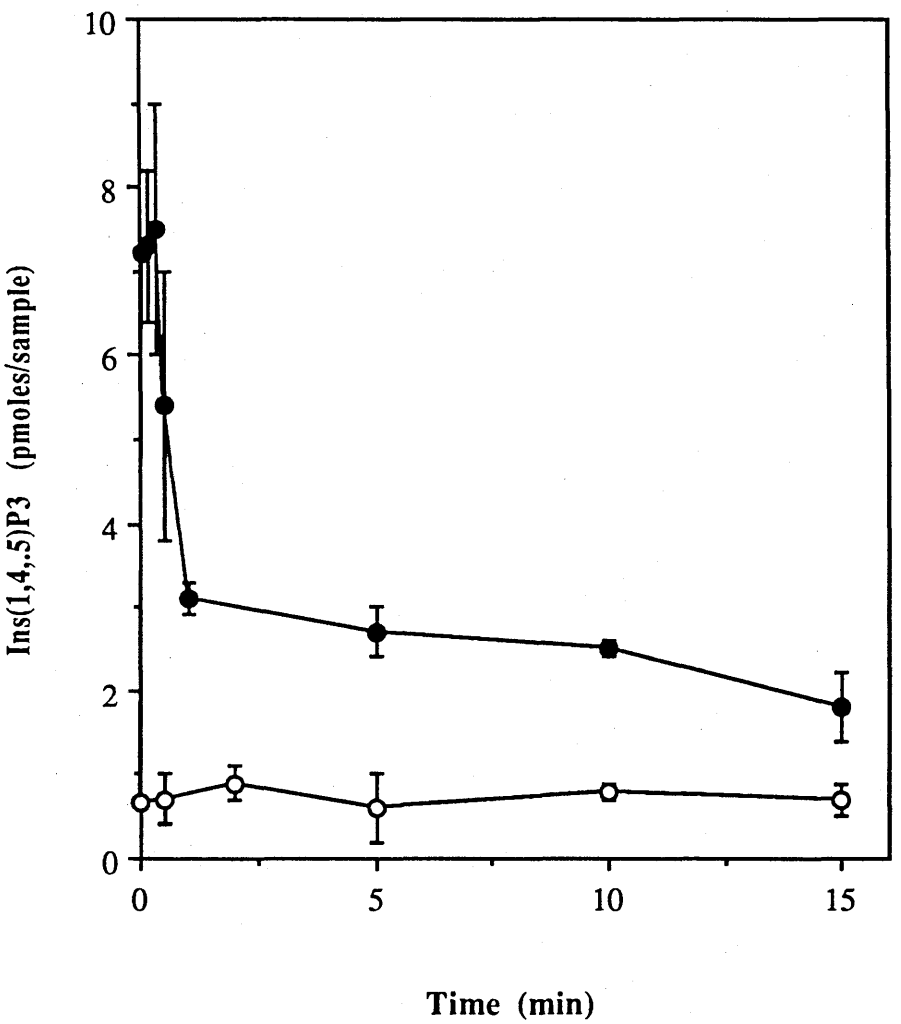
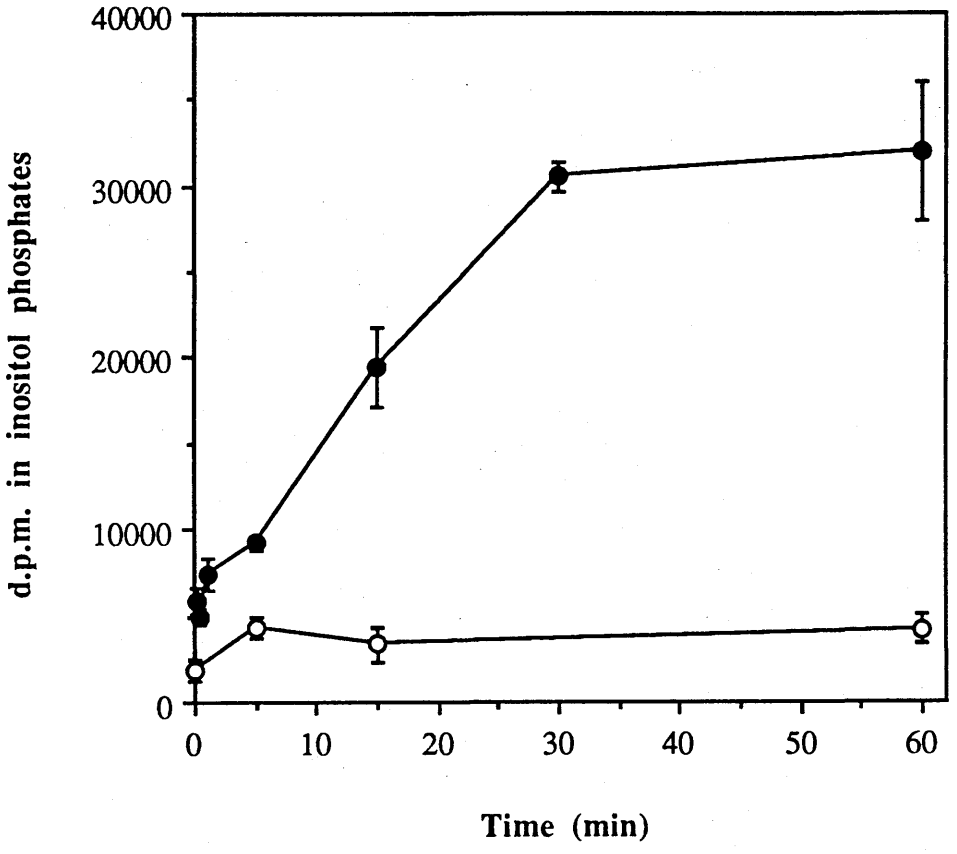

10
8
6
4
2
0
0
5
10
15
Ins(1,4,,5)P3 (pmoles/sample)
Time (min)

Fig. 3.4 Time course of ET-1-stimulated Ins(1,4,5)P_3 mass generation.

Rat-1 cells were grown to confluency and harvested as described in section 2.7.1. Stimulation was carried out with 100nM ET-1 for the stated times and Ins(1,4,5)P_3 levels were measured by the mass assay of Palmer *et al.*, 1989 (section 2.7.2). The results are expressed as means ± S.D. from a typical experiment where n=6. O, control cells; ●, ET-1-stimulated cells.


40000
30000
20000
10000
0
d.p.m. in inositol phosphates
0
10
20
30
40
50
60
Time (min)

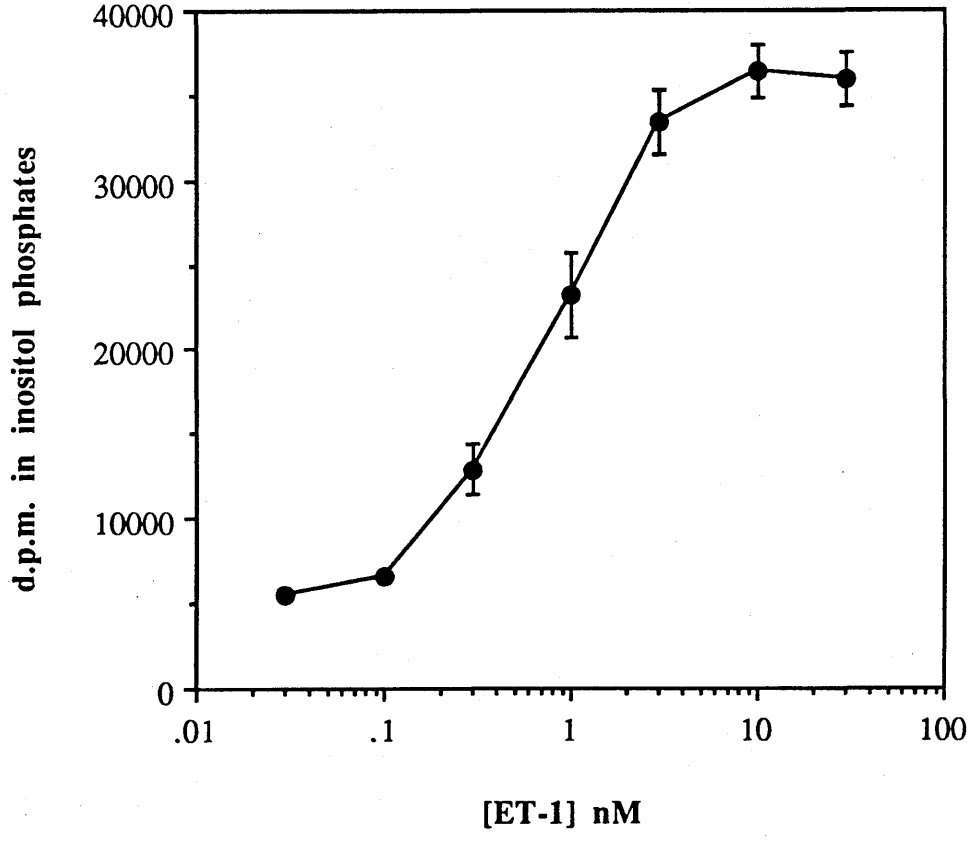
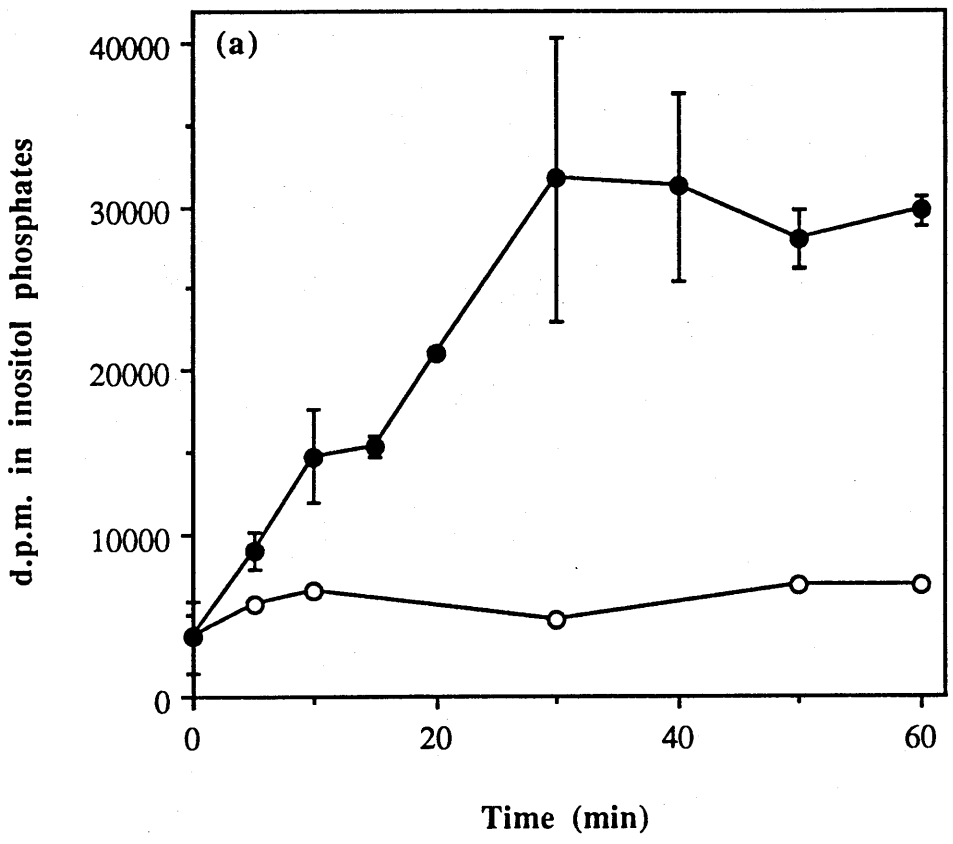

40000
30000
20000
10000
0
d.p.m. in inositol phosphates
.01
.1
1
10
100
[ET-1] nM

Fig. 3.5 Time course of ET-1-stimulated [^{3}H] inositol phosphates accumulation.

[^{3}H] Inositol-labelled Rat-1 cells (1μCi/ml for 48h) were stimulated with 100nM ET-1 for the stated times in the presence of 10mM LiCl. The radioactivity associated with total [^{3}H] inositol phosphates was determined as described in section 2.6.2. The results are expressed as means ± S.D. from a typical experiment where n=3. O, control cells; ●, ET-1-stimulated cells.

Fig. 3.6 Dose-dependence of ET-1-stimulated [^{3}H] inositol phosphates accumulation.

[^{3}H] Inositol-labelled Rat-1 cells (1μCi/ml for 48h) were stimulated with increasing concentrations of ET-1 for 20min in the presence of 10mM LiCl and the radioactivity associated with [^{3}H] inositol phosphates was measured as described in section 2.6.2. The results are expressed as means ± S.D. from a typical experiment where n=5. Basal value = 5464 ± 157 d.p.m.


(a)
40000
30000
20000
10000
0
0
20
40
60
d.p.m. in inositol phosphates
Time (min)

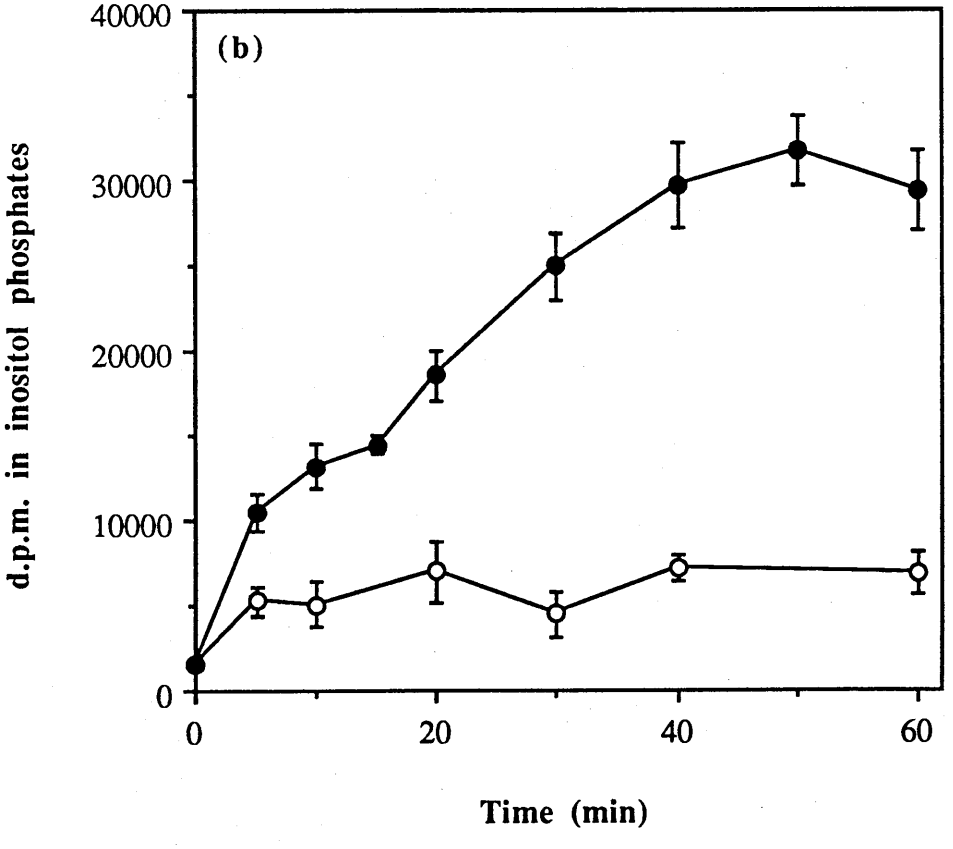
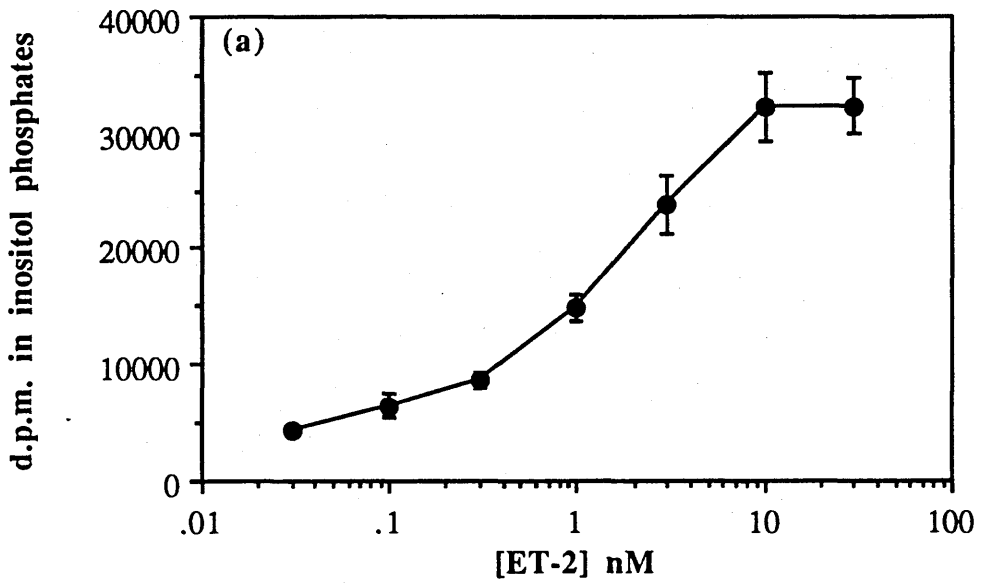
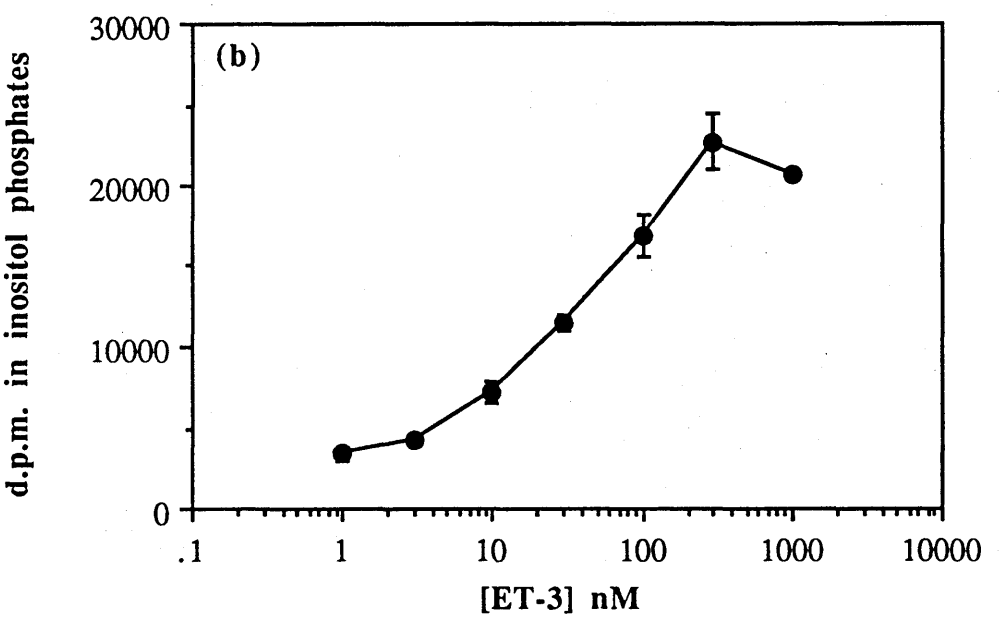

(b)
40000
30000
20000
10000
0
0
20
40
60
d.p.m. in inositol phosphates
Time (min)

Fig. 3.7 Time course of ET-2- and ET-3-stimulated [^{3}H] inositol phosphates accumulation.

[^{3}H] Inositol-labelled Rat-1 cells (1μCi/ml for 48h) were stimulated with (a) 100nM ET-2 or (b) 300nM ET-3 for the stated times in the presence of 10mM LiCl. The radioactivity associated with total [^{3}H] inositol phosphates was measured as described in section 2.6.2. The results are expressed as means ± S.D from a typical experiment where n=2. O, control cells; ● (a) ET-2 - or (b) ET-3-stimulated cells.


(a)
d.p.m. in inositol phosphates
40000
30000
20000
10000
0
.01
.1
1
10
100
[ET-2] nM


(b)
d.p.m. in inositol phosphates
30000
20000
10000
0
.1
1
10
100
1000
10000
[ET-3] nM

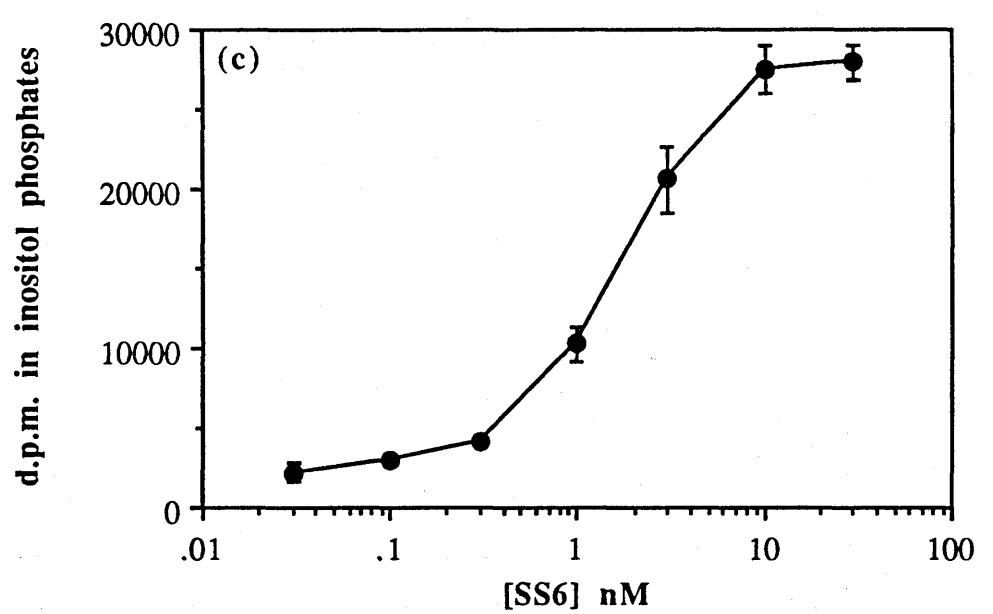
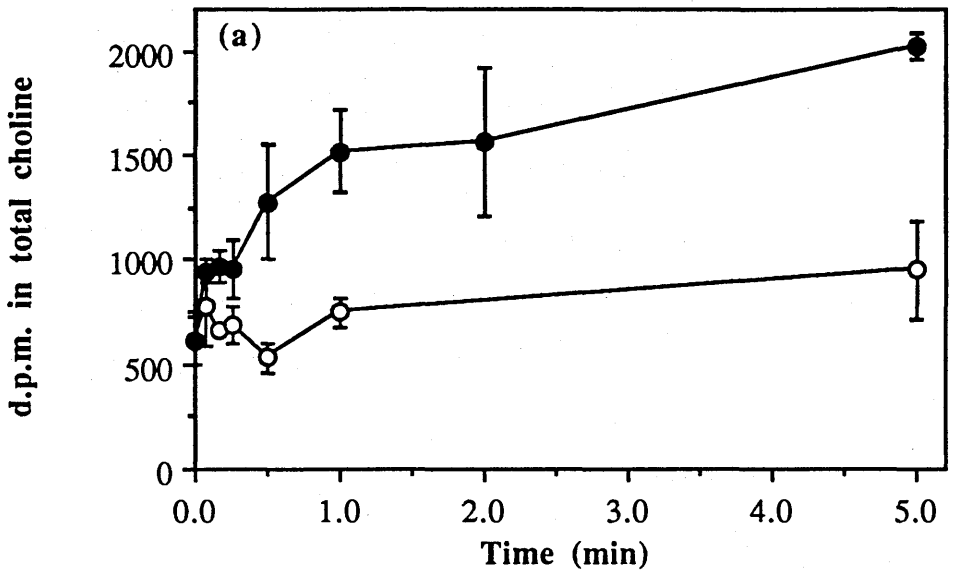
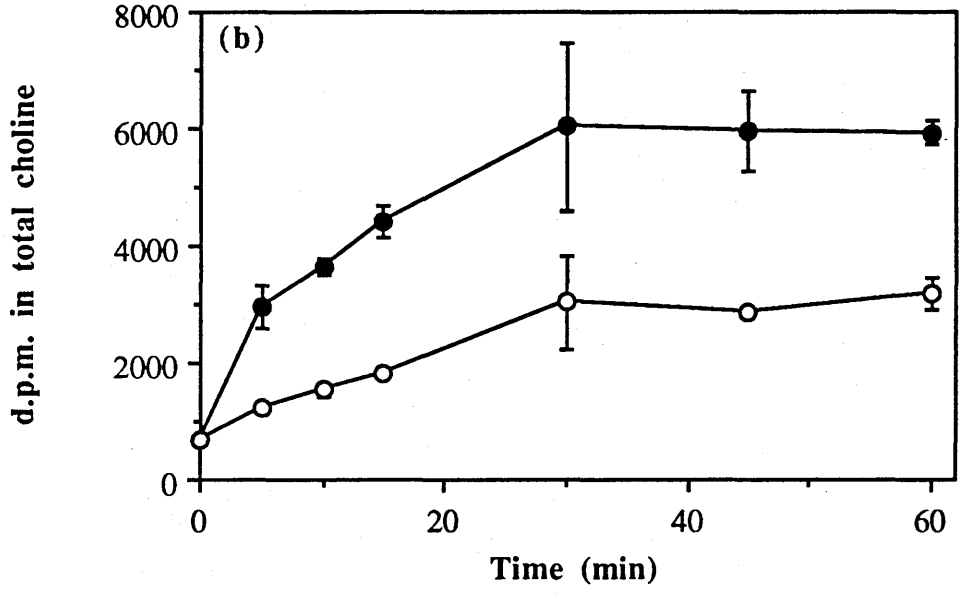

(c)
d.p.m. in inositol phosphates
30000
20000
10000
0
.01
.1
1
10
100
[SS6] nM

Fig. 3.8 Dose-dependence of ET-2-, ET-3- and SS6-stimulated [^{3}H] inositol phosphates accumulation.

[^{3}H] Inositol-labelled Rat-1 cells (1μCi/ml for 48h) were stimulated with increasing concentrations of (a) ET-2, (b) ET-3 and (c) SS6 for 20min in the presence of 10mM LiCl and the radioactivity associated with total [^{3}H] inositol phosphates determined as outlined in section 2.6.2. The results are expressed as means ± S.D. from typical experiments where n=3. Basal values (d.p.m.) = (a) 4272 ± 76, (b) 3709 ± 437, (c) 2173 ± 585.


(a)
d.p.m. in total choline
2000
1500
1000
500
0
0.0
1.0
2.0
3.0
4.0
5.0
Time (min)


(b)
d.p.m. in total choline
8000
6000
4000
2000
0
0
20
40
60
Time (min)

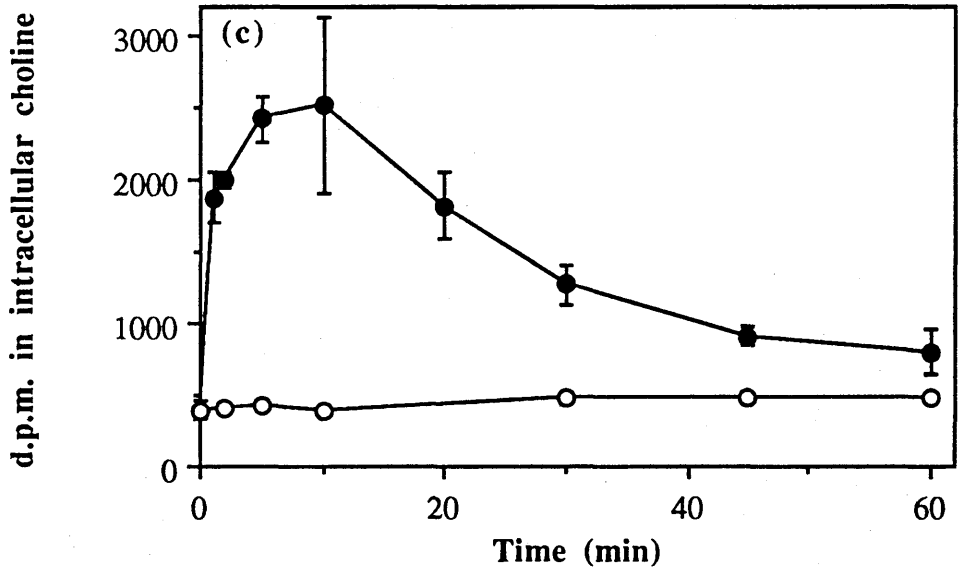

(c)
d.p.m. in intracellular choline
3000
2000
1000
0
0
20
40
60
Time (min)

Fig. 3.9 Time course of ET-1-stimulated changes in intracellular and total [^{3}H] choline.

Rat-1 cells were labelled with 5μCi/ml [^{3}H] choline for 48h and then stimulated with 100nM ET-1 for the indicated times. The choline-containing fractions were separated as described in section 2.9.2 and the radioactivity associated with (a), (b) total or (c) intracellular [^{3}H] choline was determined. The results are expressed as means ± S.D. from typical experiments where n=3-5. O, control cells; ●, ET-1-stimulated cells.

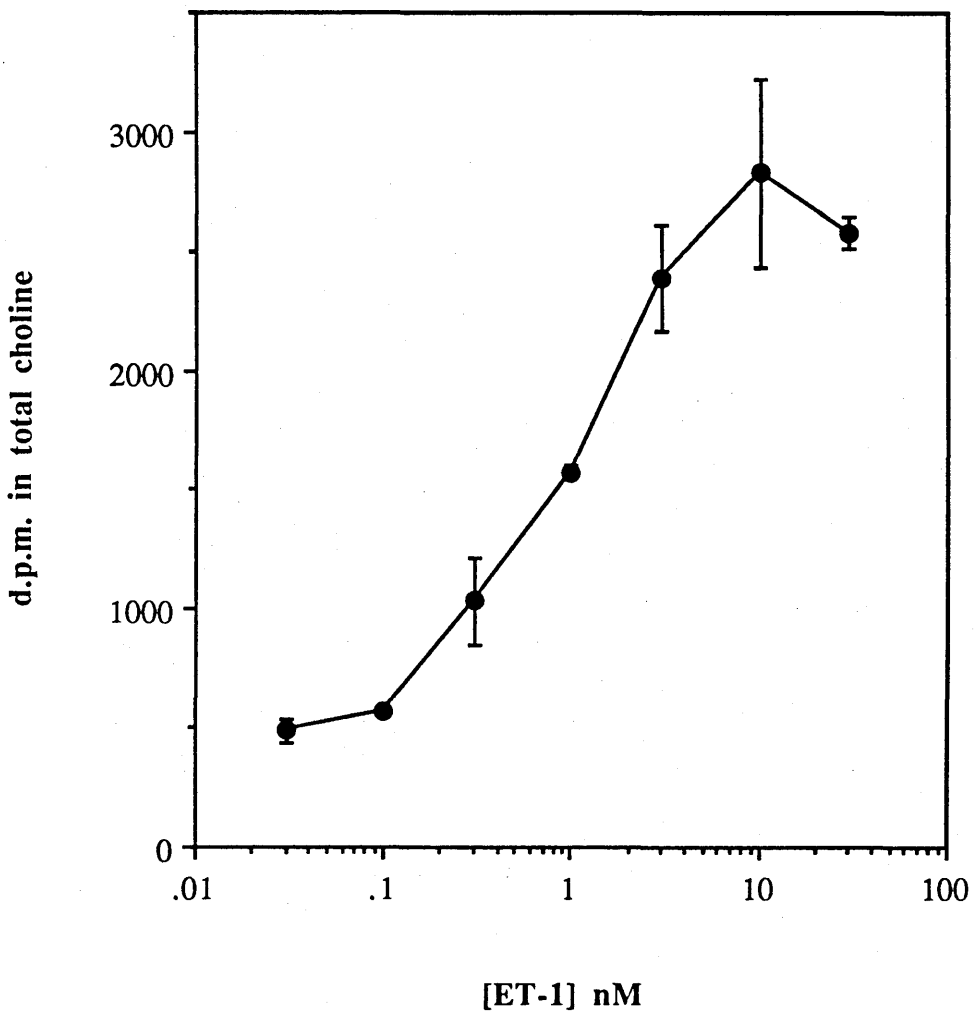
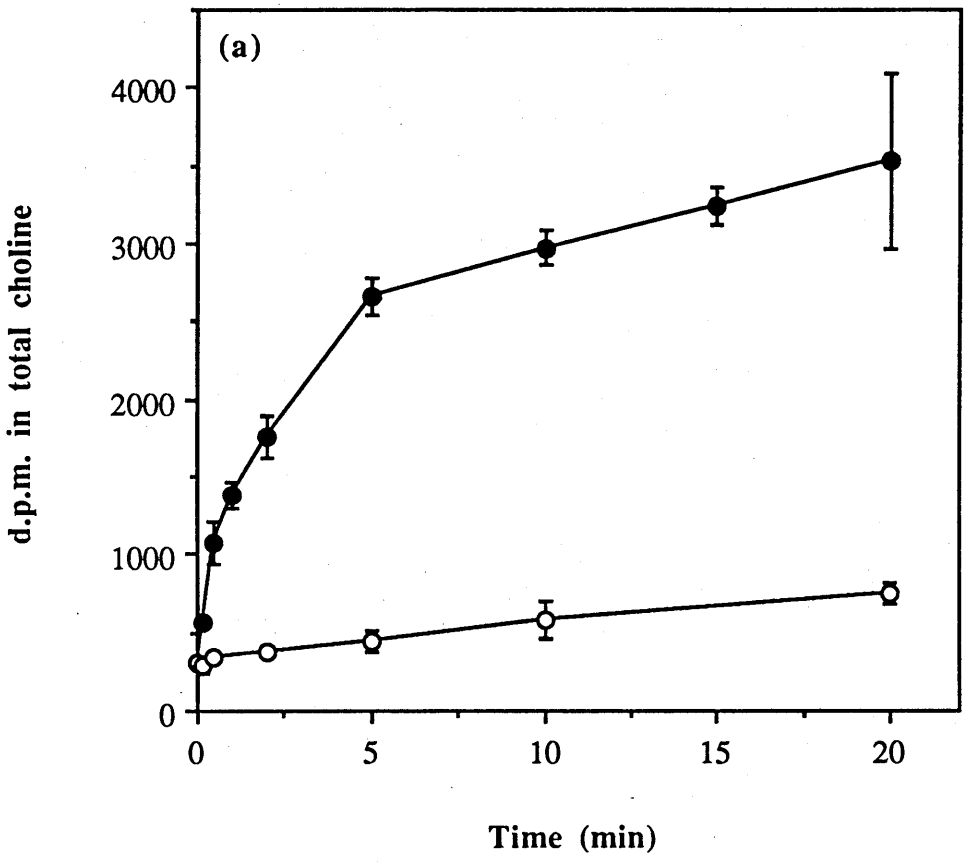

d.p.m. in total choline
3000
2000
1000
0
.01
.1
1
10
100
[ET-1] nM

Fig. 3.10 Dose-dependence of ET-1-stimulated [^{3}H] choline generation.

Rat-1 cells prelabelled for 48h with 5μCi/ml [^{3}H] choline were stimulated with increasing concentrations of ET-1 for 10min and the radioactivity associated with total [^{3}H] choline determined as described in section 2.9.2. The results are expressed as means ± S.D. from a typical experiment where n=8. Basal value = 530 ± 95 d.p.m.


(a)
4000
3000
2000
1000
0
0
5
10
15
20
d.p.m. in total choline
Time (min)

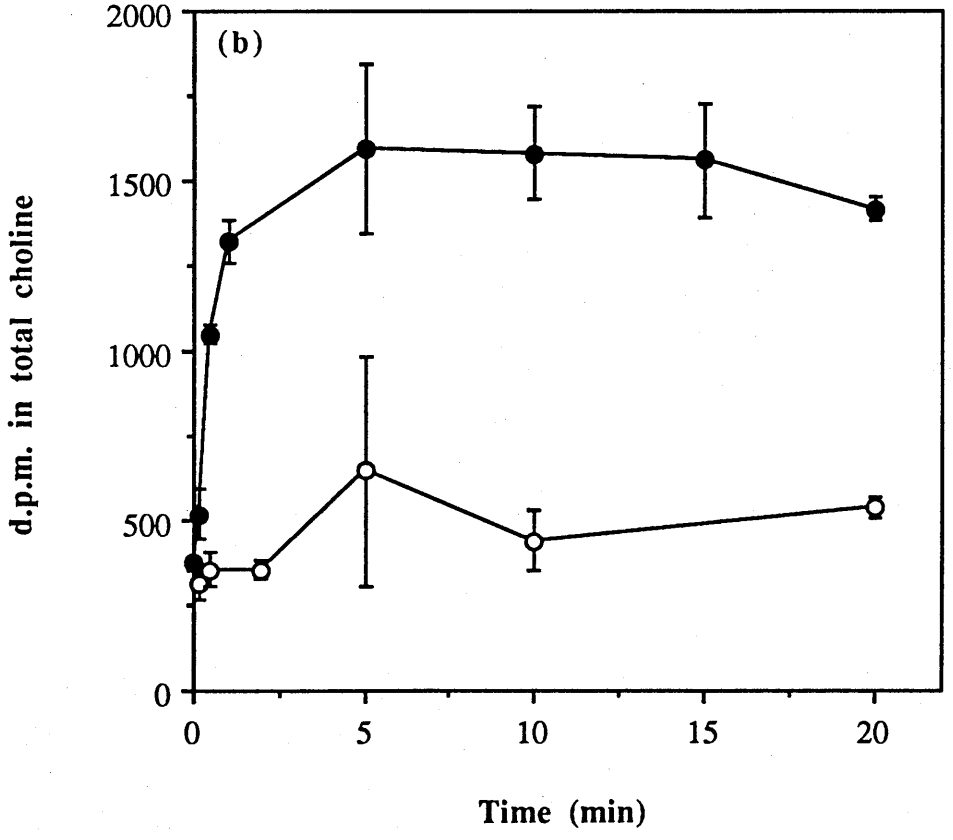
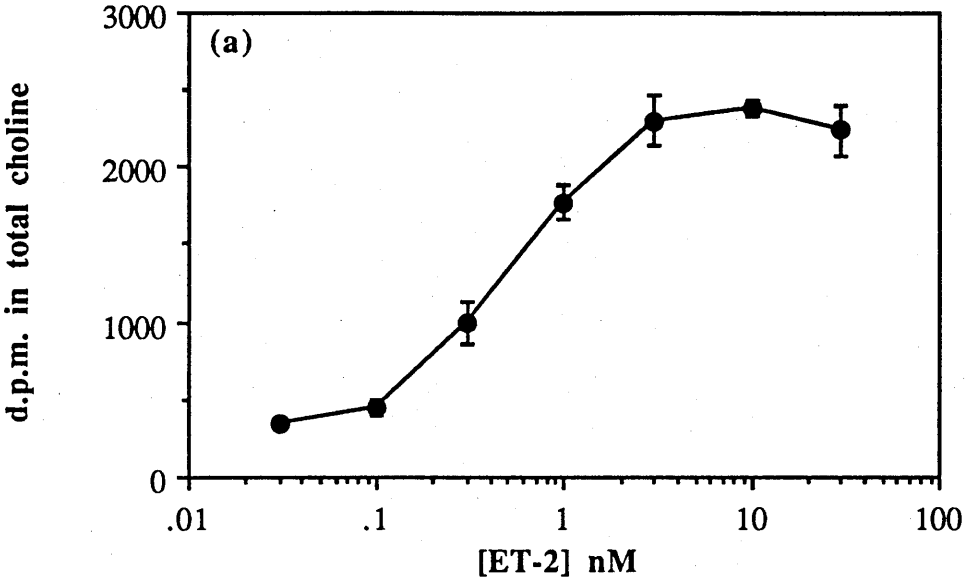
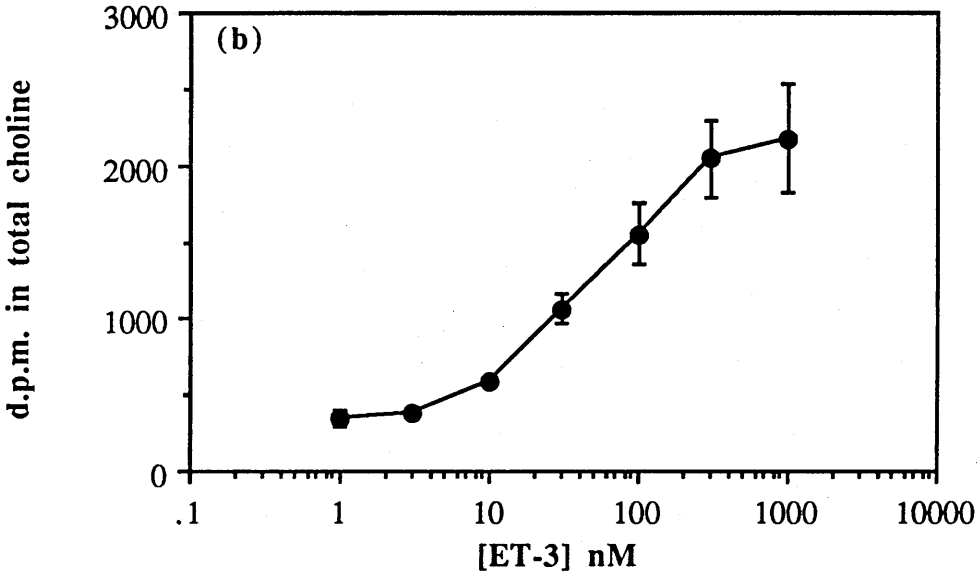

(b)
2000
1500
1000
500
0
0
5
10
15
20
d.p.m. in total choline
Time (min)

Fig. 3.11 Time course of ET-2- and ET-3-stimulated changes in total [^{3}H] choline.

[^{3}H] Choline-labelled Rat-1 cells (5μCi/ml for 48h) were stimulated with (a) 100nM ET-2 or (b) 300nM ET-3 for the stated times and the radioactivity associated with total [^{3}H] choline measured as described in section 2.9.2. The results are expressed as means ± S.D. O, control cells; ●, (a) ET-2-, (b) ET-3-stimulated cells.


(a)
3000
2000
1000
0
.01
.1
1
10
100
[ET-2] nM
d.p.m. in total choline


(b)
3000
2000
1000
0
.1
1
10
100
1000
10000
[ET-3] nM
d.p.m. in total choline

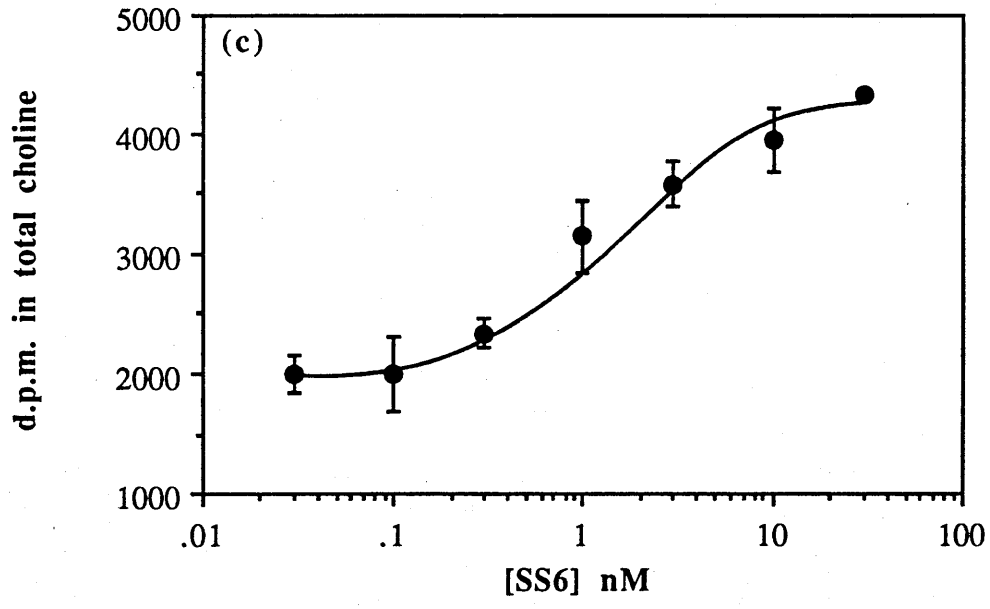
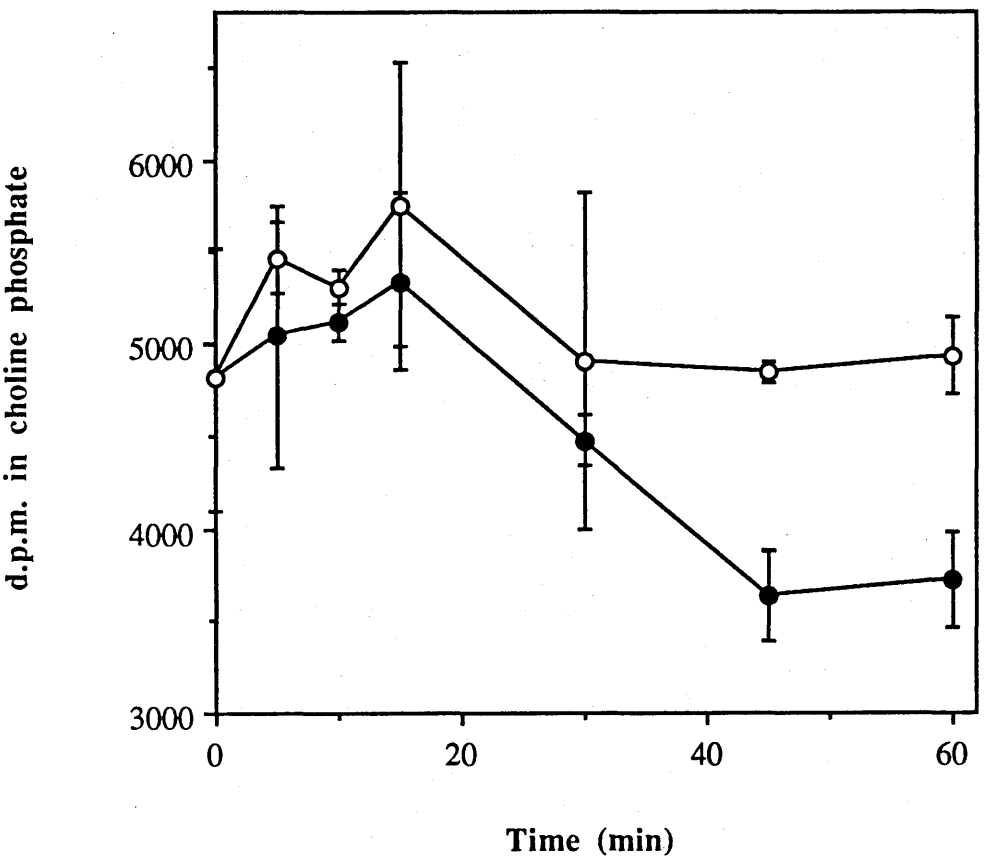

(c)
5000
4000
3000
2000
1000
.01
.1
1
10
100
[SS6] nM
d.p.m. in total choline

Fig. 3.12 Dose-dependence of ET-2-, ET-3- and SS6-stimulated [^{3}H] choline generation.

[^{3}H] Choline-labelled Rat-1 cells (5μCi/ml for 48h) were stimulated with increasing concentrations of (a) ET-2, (b) ET-3 and (c) SS6 for 10min and the radioactivity associated with total [^{3}H] choline determined as described in section 2.9.2. The results are expressed as means ± S.D. from a typical experiment where n=4. Basal values (d.p.m.) = (a), 369 ± 85, (b), 397 ± 35, (c) 1646 ± 138.


d.p.m. in choline phosphate
6000
5000
4000
3000
0
20
40
60
Time (min)

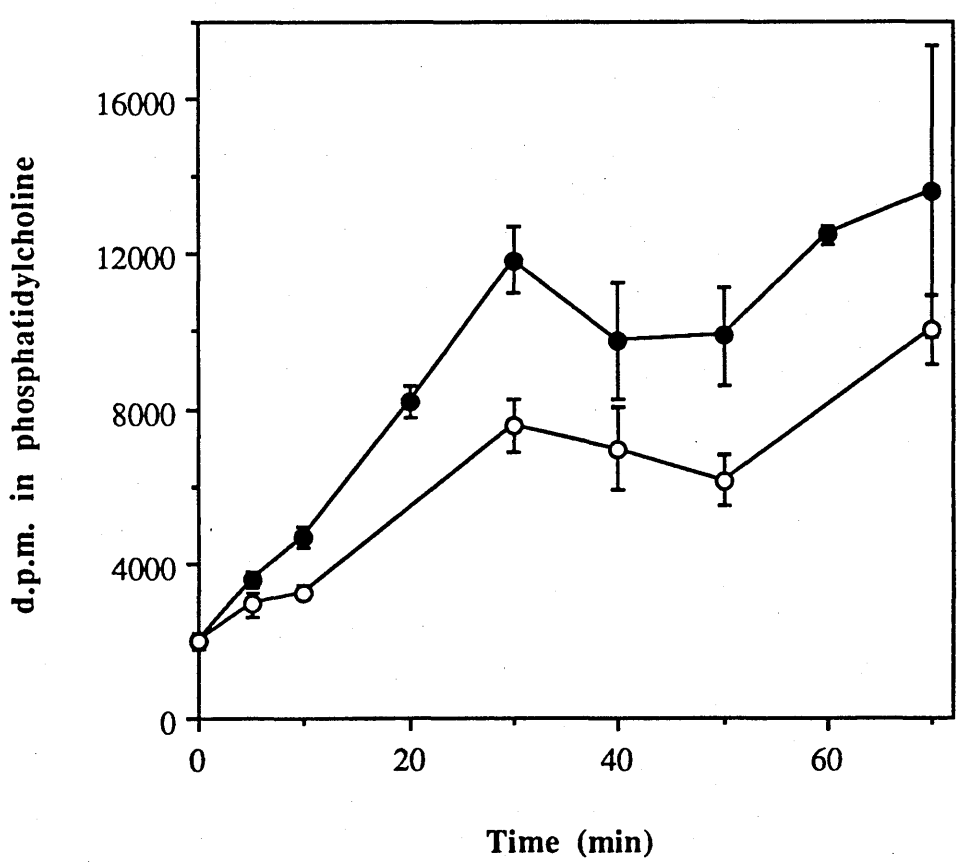
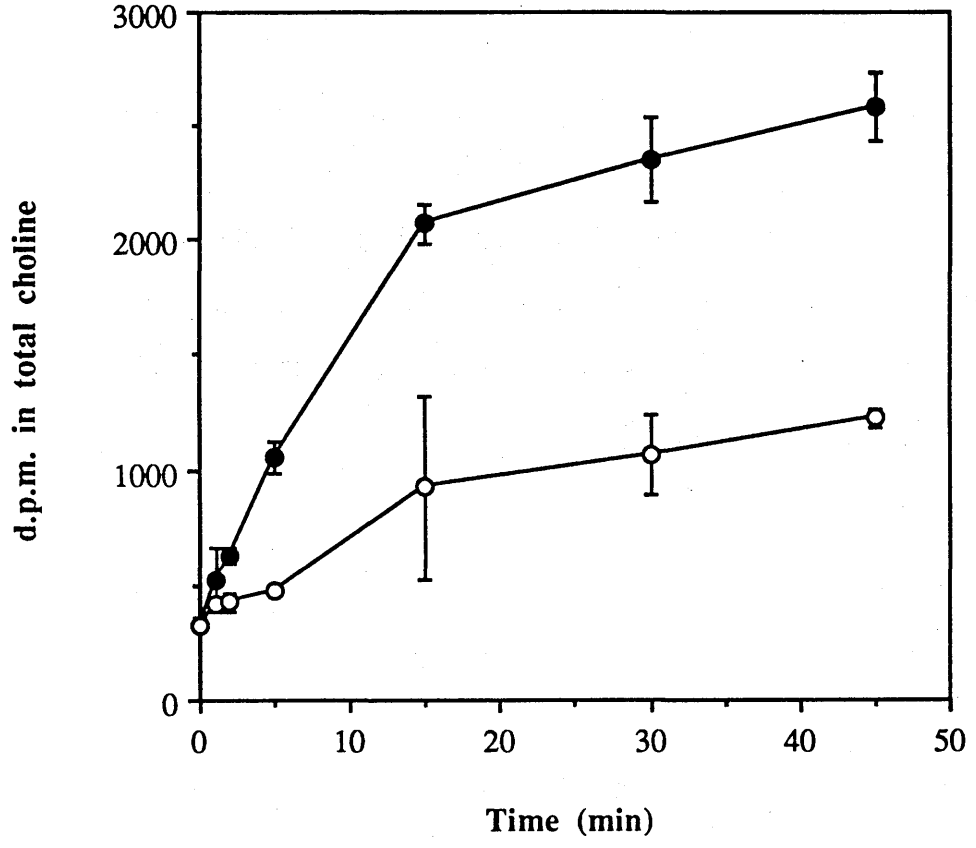

d.p.m. in phosphatidylcholine
16000
12000
8000
4000
0
0
20
40
60
Time (min)

Fig. 3.13 Time course of ET-1-stimulated decrease in [^{3}H] choline phosphate formation.

Rat-1 cells were labelled for 48h with 5μCi/ml [^{3}H] choline and then stimulated with 100nM ET-1 for the stated times. Choline containing fractions were separated as described in section 2.9.2 and the radioactivity associated with [^{3}H] choline phosphate determined. Results are expressed as means ± S.D. from a typical experiment where n=5. O, control cells; ●, ET-1-stimulated cells.

Fig. 3.14 Time course of ET-1-stimulated increase in [^{3}H] PtdCho synthesis.

Rat-1 cells were grown to confluency and quiescence and then preincubated with 28μM choline chloride and 5μCi/ml of [^{3}H] choline for 30min. Stimulation was carried out with 100nM ET-1 for the stated times and the samples were extracted as described in section 2.9.1. The lipid phases were dried down, redissolved in chloroform/methanol (19:1, v/v) and separated by t.l.c. as described in section 2.9.3. Results are expressed as means ± S.D. from a typical experiment where n=3. O, control cells; ●, ET-1-stimulated cells.


3000
2000
1000
0
0
10
20
30
40
50
d.p.m. in total choline
Time (min)

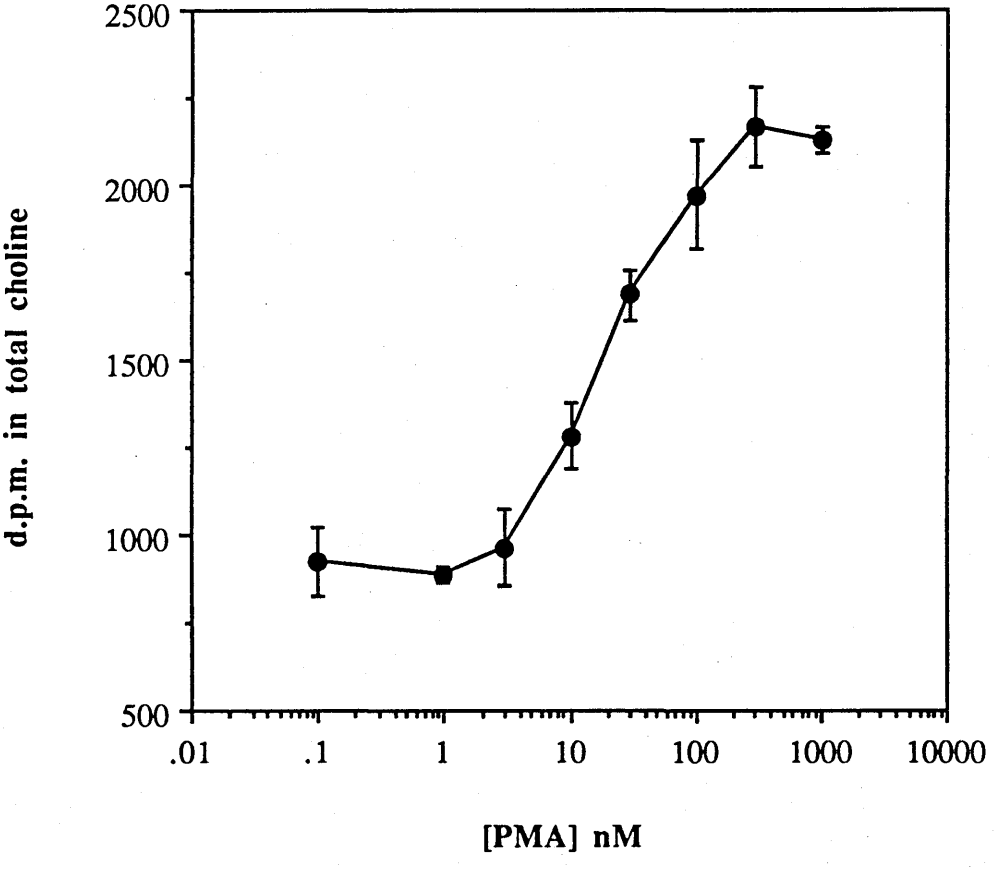

2500
2000
1500
1000
500
.01
.1
1
10
100
1000
10000
d.p.m. in total choline
[PMA] nM

Fig. 3.15 Time course of PMA-stimulated [^{3}H] choline generation.

[^{3}H] Choline-labelled Rat-1 cells (5μCi/ml for 48h) were stimulated with 300nM PMA for the indicated times. The choline-containing fractions were separated as described in section 2.9.2 and radioactivity associated with total [^{3}H] choline determined. The results are expressed as means ± S.D. from a typical experiment where n=3. O, control cells stimulated with vehicle; ●, PMA-stimulated cells.

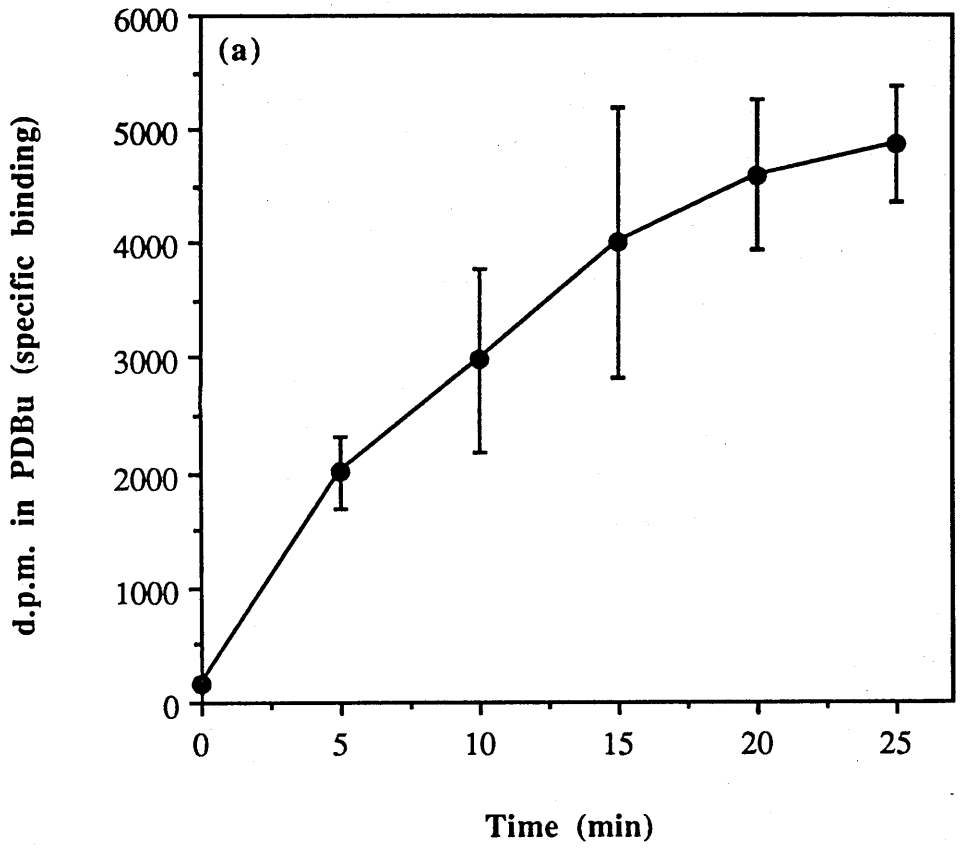
Fig. 3.16 Dose-dependence of PMA-stimulated [^{3}H] choline generation.

[^{3}H] Choline-labelled Rat-1 cells (5μCi/ml for 48h) were stimulated with increasing concentrations of PMA for 10min and the radioactivity associated with total [^{3}H] choline was measured as outlined in section 2.9.2. Results are expressed as means ± S.D. from a typical experiment where n=3. Basal value = 925 ± 100 d.p.m.

Table 3.1 ET-1-stimulated [^{3}H] choline generation and [^{3}H] inositol phosphates accumulation in control and PKC-down-regulated cells.

Rat-1 cells were labelled with [^{3}H] choline (5μCi/ml) or [^{3}H] inositol (1μCi/ml) for 48h in the presence of PMA (400nM) or the inactive β-phorbol (400nM). Stimulation was carried out with 100nM ET-1 or 300nM PMA and the radioactivity associated with total [^{3}H] choline or [^{3}H] inositol phosphates was measured as described in sections 2.9.2 and 2.6.2 respectively.. Results are expressed as means ± S.D. from a typical experiment where n=3.

	+PMA	+β-phorbol
	[^{3}H] Choline generation (d.p.m.)	
10min Control	488±133	521±58
10min ET-1	433±19	1667±158
10min PMA	477±67	1242±154
	[^{3}H] Inositol phosphates accumulation (d.p.m.)	
20min Control	11352±4035	15716±2033
20min ET-1	37812±5679	49151±5687


(a)
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
d.p.m. in PDBu (specific binding)
Time (min)

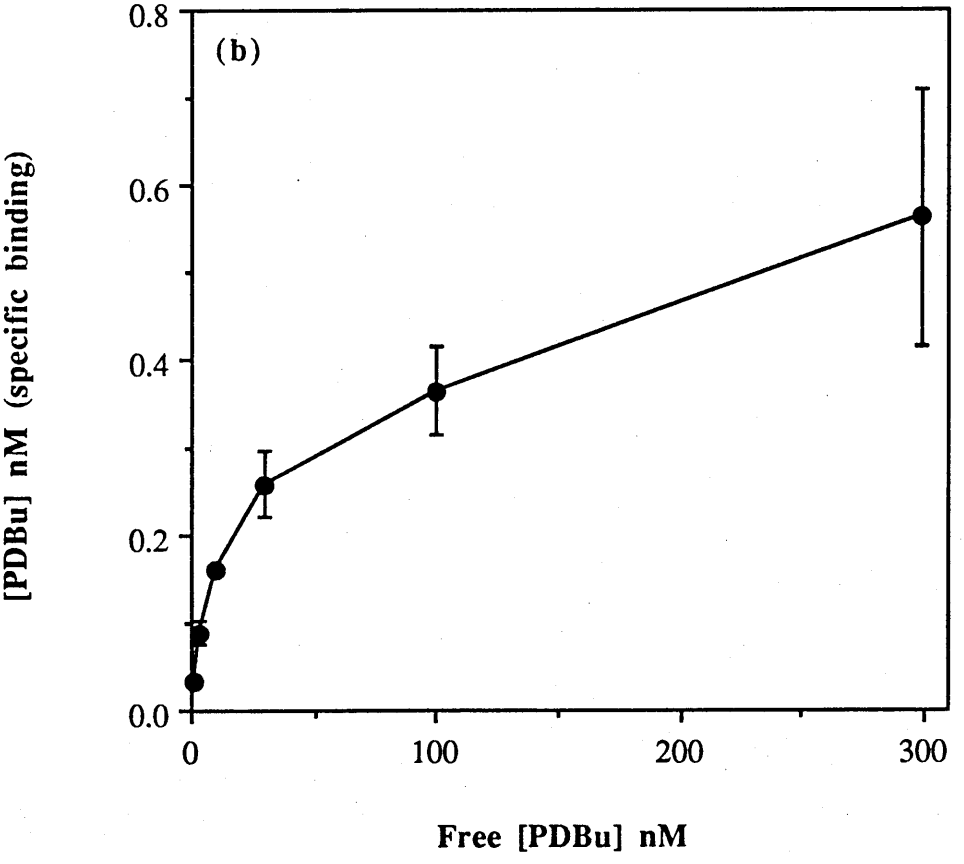

(b)
0.8
0.6
0.4
0.2
0.0
0
100
200
300
[PDBu] nM (specific binding)
Free [PDBu] nM

Fig. 3.17 Time- and dose-dependence of [^{3}H] PDBu binding.

Rat-1 cells were grown to confluency and quiescence and then incubated with (a) 100nM [^{3}H] PDBu ± 25μM unlabelled PDBu for the indicated times or (b) increasing concentrations of [^{3}H] PDBu ± 25μM unlabelled PDBu for 20min. The amount of specific [^{3}H] PDBu binding was determined as described in section 2.11. Results are expressed as means ± S.D. from a typical experiment where n=3.

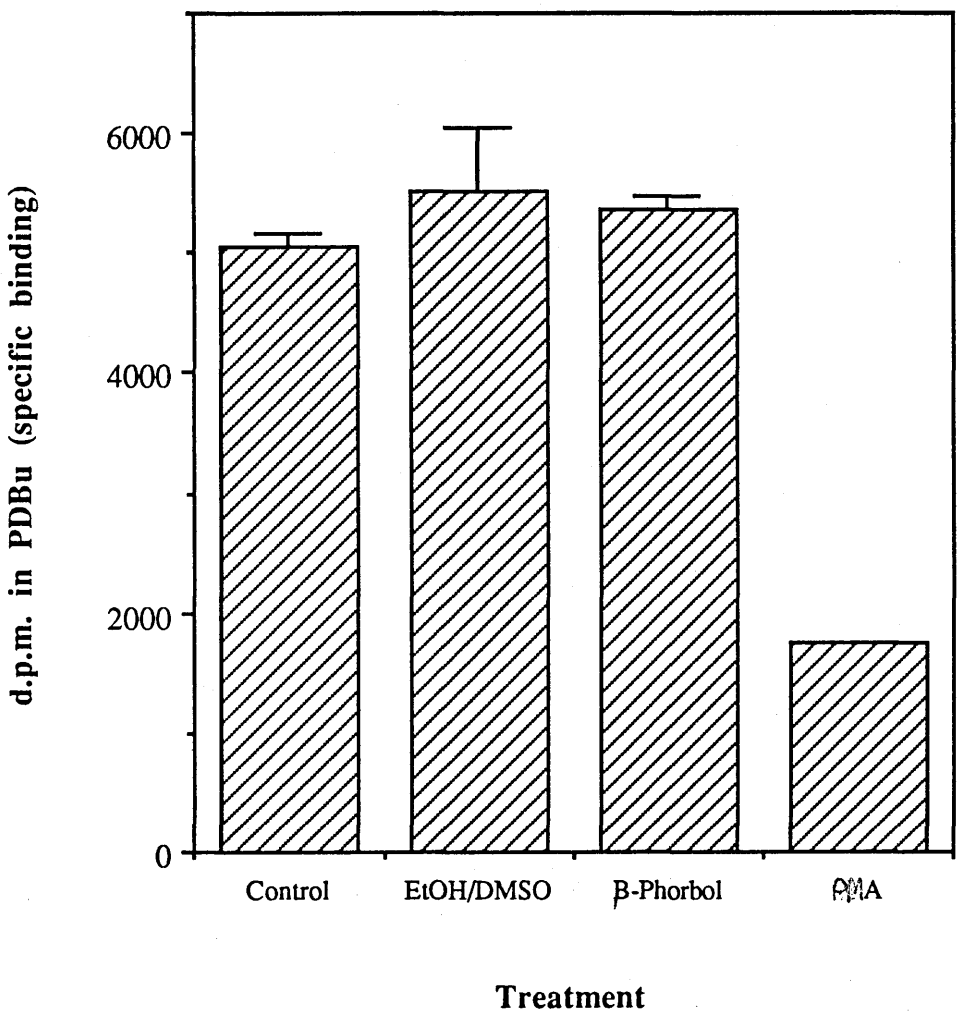
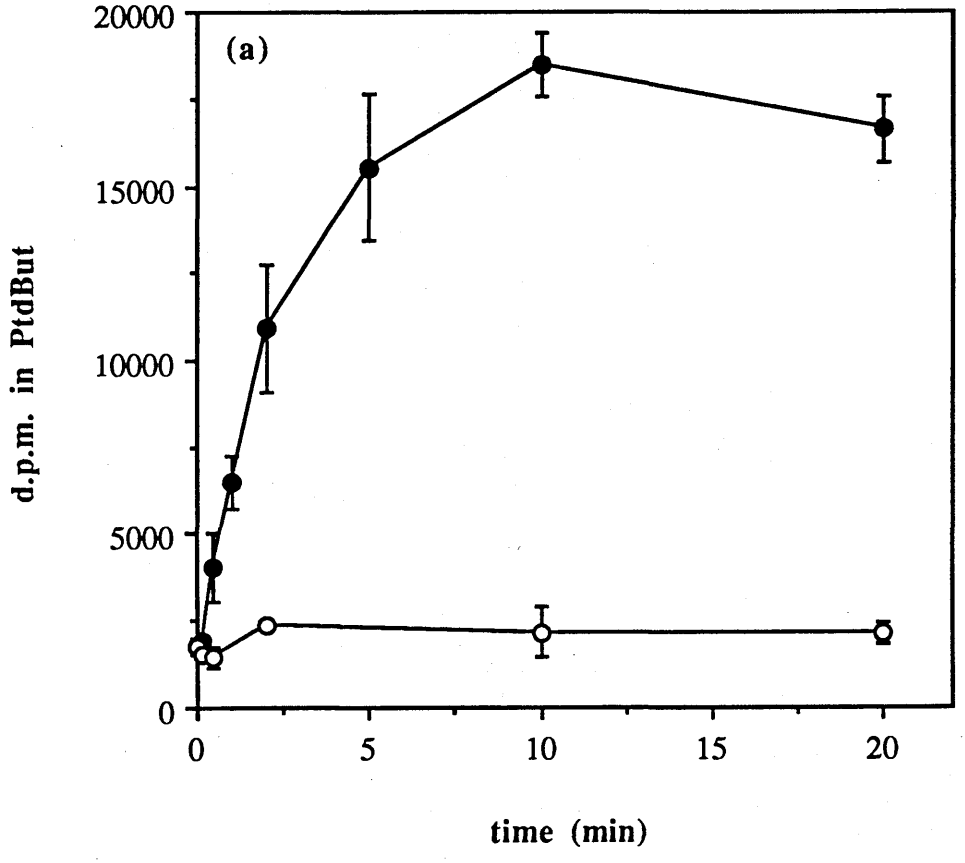

d.p.m. in PDBu (specific binding)
6000
4000
2000
0
Control
EtOH/DMSO
β-Phorbol
PMA
Treatment

Fig. 3.18 Binding of [^{3}H] PDBu in PKC-down-regulated cells.

Rat-1 cells were treated for 48h with 400nM PMA, 400nM β-phorbol, DMSO/EtOH solvent control or medium control. Cells were then incubated with 100nM [^{3}H] PDBu ± 25μM unlabelled PDBu for 20min and the amount of specific [^{3}H] PDBu binding measured as described in section 2.11. Results are expressed as means ± S.D. from a typical experiment where n=3.


(a)
20000
15000
10000
5000
0
0
5
10
15
20
d.p.m. in PtdBut
time (min)

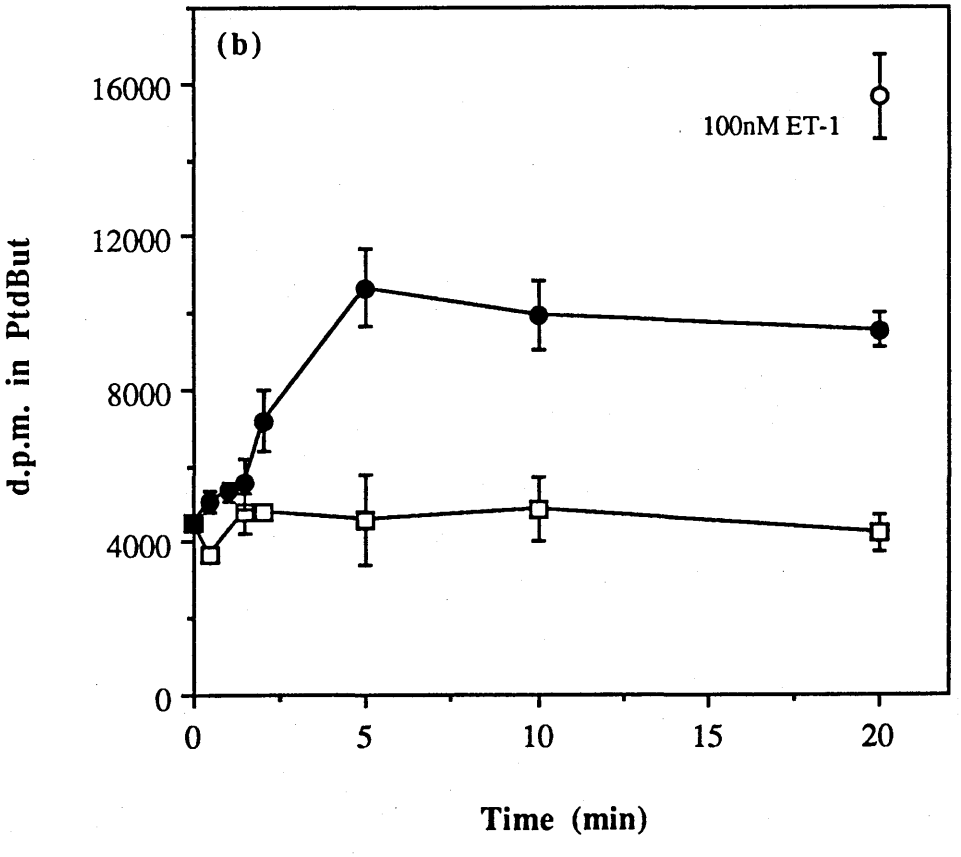

(b)
100nM ET-1
16000
12000
8000
4000
0
0
5
10
15
20
d.p.m. in PtdBut
Time (min)

Fig. 3.19 Time course of ET-1-stimulated [^{3}H] PtdBut formation.

[^{3}H] Palmitic acid-labelled cells (4μCi/ml for 48h) were stimulated with (a) 100nM or (b) 1nM ET-1 in the presence of 0.3% (v/v) butanol for the indicated times and the formation of [^{3}H] PtdBut was determined as described in section 2.10. Results are expressed as means ± S.D. from typical experiments where n=3. O, control cells; ●, ET-1-stimulated cells.

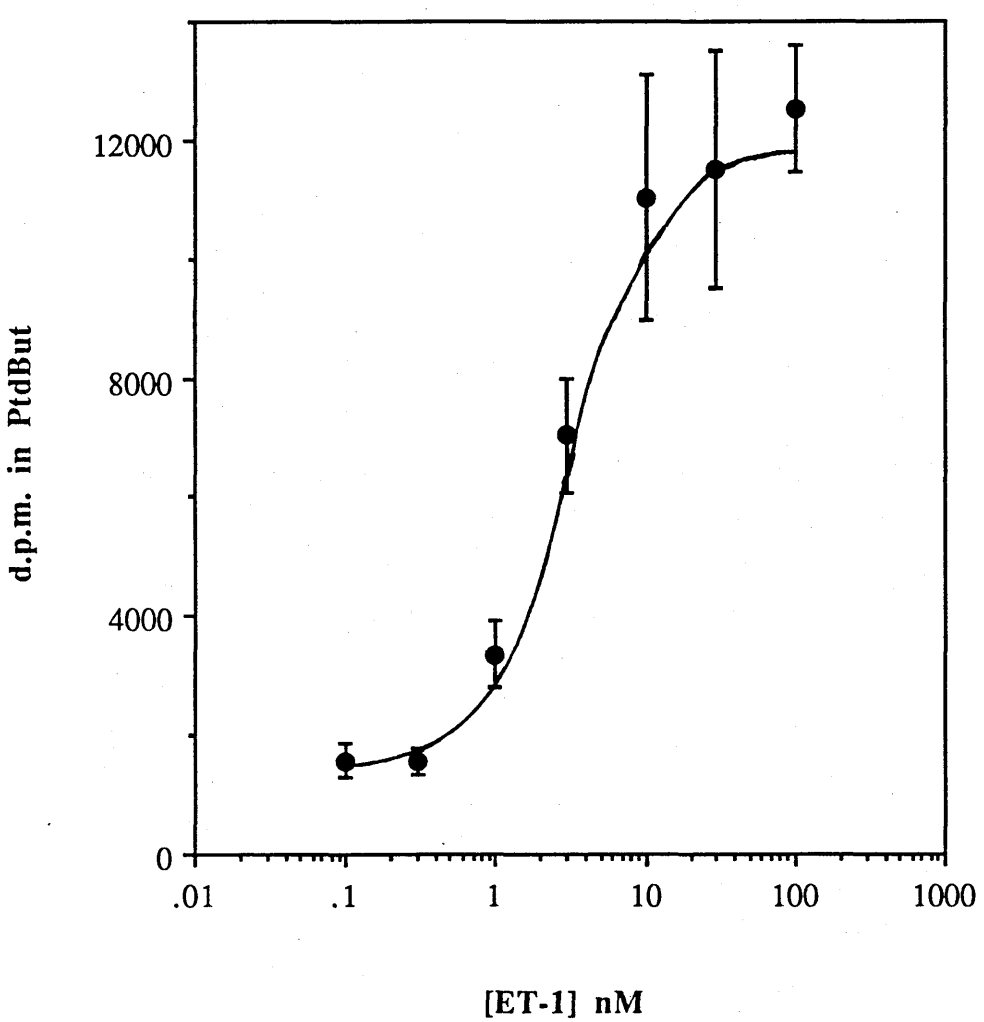

12000
8000
4000
0
d.p.m. in PtdBut
.01
.1
1
10
100
1000
[ET-1] nM

Fig. 3.20 Dose-dependence of ET-1-stimulated [^{3}H] PtdBut formation.

[^{3}H] Palmitic acid-labelled Rat-1 cells (4μCi/ml for 48h) were stimulated with increasing concentrations of ET-1 in the presence of 0.3% (v/v) butanol for 10min and the formation of [^{3}H] PtdBut was measured as described in section 2.10. Results are expressed as means ± S.D. from a typical experiment where n=3. Basal value = 1572 ± 284 d.p.m.

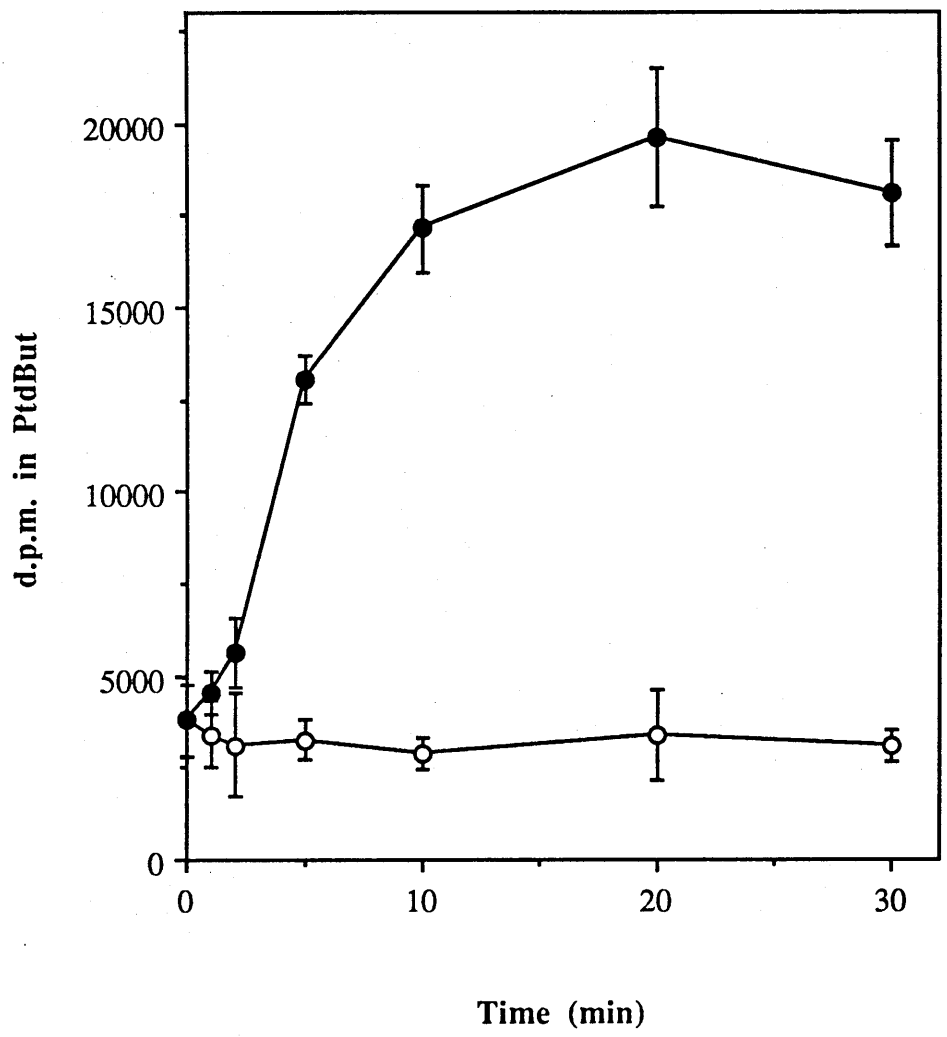
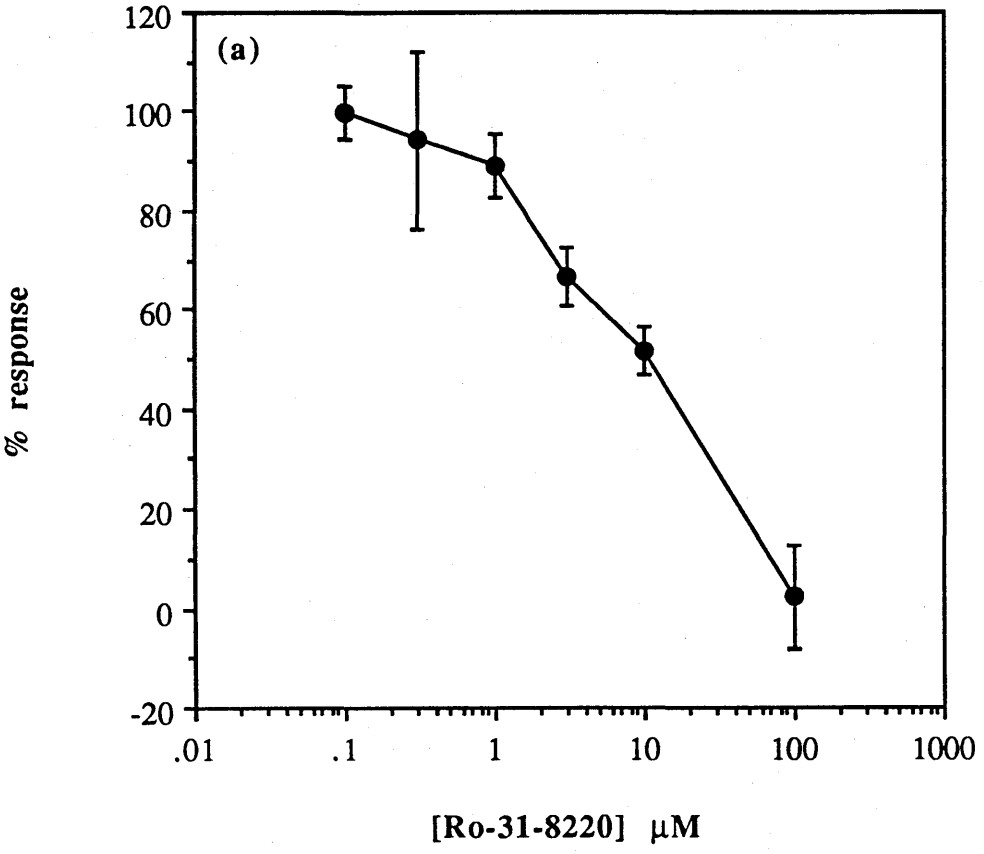

20000
15000
10000
5000
0
0
10
20
30
d.p.m. in PtdBut
Time (min)

Fig. 3.21 Time course of PMA-stimulated [^{3}H] PtdBut formation.

[^{3}H] Palmitic acid-labelled Rat-1 cells (4μCi/ml for 48h) were stimulated with 300nM PMA in the presence of 0.3% (v/v) butanol for the indicated times and [^{3}H] PtdBut formation was determined as described in section 2.10. Results are expressed as means ± S.D. from a typical experiment where n=3. O, control cells; ●, PMA-stimulated cells.


(a)
120
100
80
60
40
20
0
-20
% response
.01
.1
1
10
100
1000
[Ro-31-8220] μM

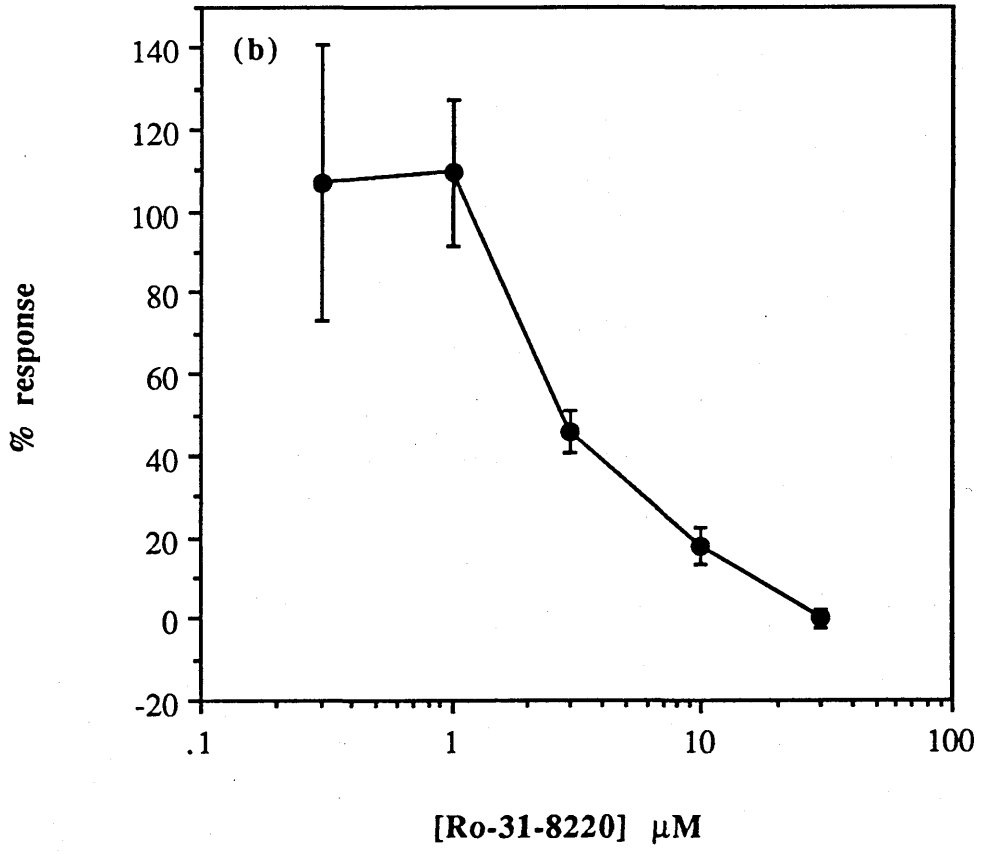

(b)
140
120
100
80
60
40
20
0
-20
% response
.1
1
10
100
[Ro-31-8220] μM

Fig. 3.22 Effect of Ro-31-8220 on ET-1- and PMA-stimulated PLD activity.

[^{3}H] Palmitic acid-labelled Rat-1 cells (4μCi/ml for 48h) were preincubated with increasing concentrations of Ro-31-8220 for 10min and then stimulated with (a) 100nM ET-1 for 10min or (b) 300nM PMA for 15min. [^{3}H] PtdBut formation was determined as described in section 2.10. Results are expressed as means ± S.D. from typical experiments where n=3-4. control values (d.p.m.) = (a) basal, 2001 ± 257; ET- stimulated, 7076 ± 2046, (b), basal, 3229 ± 1097; PMA-stimulated, 16825 ± 3871.

Table 3.2. Role of extracellular Ca^{2+} in agonist-stimulated PtdCho and PtdIns(4,5)P_2 hydrolysis.

Rat-1 cells prelabelled for 48h with 4μCi/ml [^{3}H] palmitic acid were preincubated for 15min under normal [Ca^{2+}] conditions (1.26mM) or with 0.33mM added EGTA to reduce extracellular [Ca^{2+}] to 1μM. Stimulation was carried out with 100nM ET-1, 300nM PMA and 5μM A23187 for 10min (total [^{3}H] choline and [^{3}H] PtdBut formation) or 20min ([^{3}H] InsP accumulation). Where used, Ro-31-8220 was added at 10μM 10min prior to stimulation. [^{3}H] Choline generation and [^{3}H] PtdBut and [^{3}H] InsP accumulation were measured as described in sections 2.6.2, 2.9.2 and 2.10. Results are expressed as means ± S.D. from typical experiments where n=3.

	Control	+EGTA
	[^{3}H] Choline (d.p.m.)	
Control	805±265	576±135
ET-1	2651±287	1187±57
	[^{3}H] PtdBut (d.p.m.)	
Control	2445±524	2325±674
ET-1	26437±4652	13227±3641
ET-1+Ro-31-8220	12243±1640	10467±647
PMA	15413±3181	16420±2688
A23187	12209±961	2813±269
A23187+Ro-31-8220	8693±569	3991±426
	[^{3}H] InsP (d.p.m.)	
Control	3174±321	541±125
ET-1	13215±2495	7623±776

Table 3.3. Effect of A23187 and PMA on ET-1-stimulated [^{3}H] PtdBut formation.

Rat-1 cells were labelled for 48h with 4μCi/ml [^{3}H] palmitic acid and then stimulated with ET-1 (100nM), A23187 (5μM) and PMA (300nM) alone and in combination with each other in the presence of 0.3% (v/v) butanol for 10min. [^{3}H] PtdBut formation was measured as outlined in section 2.9.2. Results are expressed as means ± S.D. from a typical experiment where n=3.

	d.p.m. in [^{3}H] PtdBut
Control	3554 ± 386
ET-1	30948 ± 1677
A23187	17360 ± 999
PMA	15891 ± 1652
A23187 + PMA	37212 ± 1652
ET-1 + A23187	32752 ± 4167
ET-1 + PMA	54897 ± 3979
ET-1 + A23187 + PMA	57724 ± 14311

3.10 Discussion

Since the discovery of ET-1 (Yanagisawa *et al.*, 1988a) many investigators have sought to determine the second messenger-generating pathways which may mediate its mechanism of action. So far most attention has focused on the hydrolysis of PtdIns(4,5)P_2 as a means of generating intracellular signals. However, there is accumulating evidence from other systems for agonist-induced PtdCho hydrolysis and many hormones and growth factors have now been shown to stimulate PtdCho hydrolysis in addition to PtdIns(4,5)P_2 breakdown (section 1.5). PtdCho hydrolysis was of interest when investigating the signalling pathways with which the ET-1 receptor interacts as it could potentially generate another source of DAG or alternatively yield phosphatidic acid (PtdOH) which has also been implicated as an important signalling mediator (section 1.3.4 and section 6.1).

ET-1 has been shown to stimulate proliferation of a number of cultured cell lines (Komuro *et al.*, 1988; Simonson *et al.*, 1989; Nakaki *et al.*, 1989; Brown and Littlewood, 1989; Takuwa *et al.*, 1989; MacCumber *et al.*, 1990; Shichiri *et al.*, 1991; Yada *et al.*, 1991) and in this study the three ET peptides were shown to be mitogens for Rat-1 cells (Fig. 3.1). ET-1 dose-dependently increased the incorporation of [^{3}H] thymidine to a maximum of 2-fold above control in the absence of any other growth factors indicating that it is a complete mitogen. While this work was in progress Muldoon *et al.* (1990) also reported that ET-1 stimulated DNA synthesis in Rat-1 cells thus supporting the results presented here. Further characterization of ET-induced [^{3}H] thymidine incorporation showed that in some experiments the response was sensitive to chronic (400nM, 48h) PMA-pretreatment (Fig. 3.3a). Since such exposure to PMA has been reported to down-regulate PKC activity (Rodriguez-Pena and Rozengurt, 1984) these results suggested an important role for PKC in the proliferative response to ET-1. [^{3}H] PDBu binding studies demonstrated a large decrease in binding in cells pretreated with PMA (Fig. 3.18); however, binding

was never totally inhibited suggesting residual PKC activity. This may reflect the presence of different isozymes of PKC with different sensitivities to PMA-induced down-regulation as has been suggested previously (Adams and Gullick, 1989). This may explain why PMA pretreatment did not always inhibit ET-1-stimulated [^{3}H] thymidine incorporation. Similar PMA treatment has been shown to inhibit ET-1-induced DNA synthesis and anchorage-independent growth also in Rat-1 cells (Muldoon *et al.*, 1990) while long-term PDBu pretreatment inhibited [^{3}H] thymidine incorporation in response to ET-1 in Swiss 3T3 fibroblasts (Takuwa *et al.*, 1989). ET-1-induced [^{3}H] thymidine incorporation was pertussis toxin-insensitive suggesting the involvement of pertussis toxin-insenstive signalling pathways, i.e. signalling pathways not involving interaction with G_i -like G proteins. A number of novel G proteins have now been identified which include pertussis toxin-insensitive proteins (see section 1.4.1)

Since ET-1 was shown to be mitogenic for Rat-1 cells this was therefore a relevant model system in which to investigate further the signal transduction pathways stimulated by this peptide which might be important in its mechanism of action. A previous report demonstrated PtdIns(4,5)P_2 hydrolysis stimulated by ET-1 in Rat-1 cells (Muldoon *et al.*, 1989) and results presented here confirm this (Fig. 3.4). However, previous studies have employed [^{3}H] inositol-labelled cells and have generally measured InsP_3 generation in the presence of LiCl, often with no attempt to separate the different isomers. In this work the first mass measurements of Ins(1,4,5)P_3 generation in response to ET-1 stimulation in any system are presented. The magnitude of the response observed was much greater than that reported previously (Muldoon *et al.*, 1989) being approximately 10-fold above controls at 5-10sec, probably due to the greater sensitivity of the Ins(1,4,5)P_3 assay. The Ins(1,4,5)P_3 response was very rapid peaking at 5-10sec and then returned towards basal levels after 30sec. It was however still elevated above control levels at 15min. This contrasts with

studies using bombesin-stimulated Swiss 3T3 cells in which the Ins(1,4,5)P_3 signal was completely desensitized by 30sec (Cook *et al.*, 1990) or bradykinin-stimulated NIH 3T3 fibroblasts and NG108-15 cells (Fu *et al.*, 1988), possibly reflecting differences in the mechanism of action of the peptides. However, a similar biphasic Ins(1,4,5)P_3 response involving a transient peak and then a sustained phase was observed with the nonpeptide, muscarinic receptor agonist carbachol in human neuroblastoma cells (Lambert *et al.*, 1991). The sustained phase of the ET-1 response, which suggests sustained PtdIns(4,5)P_2 hydrolysis (see chapter 4, Fig. 4.4), obviously has important implications for both the intracellular free Ca^{2+} concentration and DAG levels which may in turn be important in the mitogenic activity of ET-1 in Rat-1 cells. When [^{3}H] inositol-labelled cells were utilized, ET-1 stimulated a biphasic increase in [^{3}H] InsP in the presence of 10mM LiCl consisting of an initial rapid phase followed by a slower linear increase up to 30-40min (Fig. 3.5). The plateau observed after 40min may reflect desensitization of the response or, alternatively, a situation were [^{3}H] label has become limiting. This assay was used to examine the dose-dependency of ET-1-stimulated PtdIns(4,5)P_2 hydrolysis; ET-1 stimulated InsP accumulation with a maximum response at 10nM and an EC_{50} value of about 2nM (Fig. 3.6). This value is similar to that obtained for ET-1-stimulated [^{3}H] thymidine incorporation (Fig. 3.2, ~2nM) and also to the EC_{50} values reported in the literature for ET-1-stimulated InsP generation, DAG formation, arachidonic acid release and intracellular Ca^{2+} increases in vascular smooth muscle cells (Marsden *et al.*, 1989; Van Renterghem *et al.*, 1988; Resink *et al.*, 1990; Griendling *et al.*, 1989; Araki *et al.*, 1989) and the K_d for ET-1 binding to Swiss 3T3 fibroblasts (Fabregat and Rozengurt, 1990).

In common with many other mitogenic peptides ET-1 stimulated PtdCho hydrolysis in addition to PtdIns(4,5)P_2 breakdown (Fig. 3.9). The response was rapid with an increase in [^{3}H] choline as early as 10sec after stimulation and dose-dependent (Fig. 3.10) with an EC_{50} value comparable with that obtained

for InsP accumulation (2.0 ± 1.0nM for InsP accumulation; 0.8 ± 0.4nM for choline generation). There was no change in [^{3}H] choline phosphate levels until approximately 30-40min after stimulation with ET-1 when about a 30% decrease below controls was observed (Fig. 3.13). This may be the result of incorporation into lipid due to the activation of the PtdCho synthetic pathway since increased PtdCho synthesis was detected as early as 5min after agonist stimulation (Fig. 3.14). In fact, there may be generation of choline phosphate at these earlier time points due to choline kinase or PtdCho-specific phospholipase C activity but this may not be detected due to rapid reincorporation into the parent lipid. Price *et al.* (1989) have reported stimulated PtdCho synthesis in Swiss 3T3 cells in response to a number of growth factors but their observations were made only after a 30min stimulation.

The other ET peptides were also effective at stimulating both InsP accumulation and choline generation to a similar extent as ET-1(Fig. 3.7 and 3.11) and a comparison of their dose-dependencies with ET-1 and the related peptide toxin, SS6, yielded the same rank order of potency i.e. ET-1 = ET-2 = SS6 > ET-3 for both responses, with each peptide having a similar EC_{50} value for both responses (~1-2nM for ET-1, ET-2 and SS6; ~50nM for ET-3). These results therefore suggest that the two second messenger-generating pathways are controlled by activation of the same receptor subtype.

PtdCho hydrolysis in response to ET-1 has been reported for vascular smooth muscle cells (Resink *et al.*, 1989; 1990) but the authors did not investigate the nature of the water-soluble choline products. Another study in C6 glioma cells (Zhang *et al.*, 1991) demonstrated choline and choline phosphate generation in response to ET-1 stimulation but did not address the mechanism of PtdCho hydrolysis i.e. whether it was catalysed by a PLC or a PLD activity. In the work presented here the hydrolysis of PtdCho was observed as an early 10sec increase in [^{3}H] choline with no significant change in [^{3}H] choline phosphate up to 30min. These results suggested that ET-1-

stimulated breakdown of PtdCho in Rat-1 cells occurred via a PLD-catalysed mechanism. This was confirmed by the ability of ET-1 to stimulate the formation of [^{3}H] PtdBut in the presence of 0.3% butanol, as phosphatidyltransferase activity is an unequivocal marker for PLD activity (Pai *et al.*, 1988b). The kinetics of PtdBut formation in response to ET-1 were similar to choline generation with significantly elevated levels after 10sec which increased up to about 5min (Fig. 3.19). The response was dose-dependent (Fig. 3.20) with an EC_{50} value of ~2-3nM which is similar to that for both InsP and choline generation. Recently, ET-1 has also been shown to activate PLD in Rat-6 cells measured as the formation of phosphatidylethanol (Pai *et al.*, 1991b).

PtdCho hydrolysis is frequently proposed as an alternative source of DAG to maintain DAG levels once PtdIns(4,5)P_2 hydrolysis has been desensitized. Indeed, Griendling *et al.* (1989) observed a biphasic accumulation of DAG in response to ET-1 in rat aortic vascular smooth muscle cells which was sustained for at least 20min while $InsP_3$ generation in the same cells was transient and had returned to basal levels by 5min (Sugiura *et al.*, 1989b). Similarly, in C6 glioma cells the ET-1-stimulated increase in DAG was sustained for at least 15min while Ins(1,4,5)P_3 formation had returned to resting levels at 3min (Zhang *et al.*, 1991). Although transient $InsP_3$ generation does not necessarily imply transient PtdIns(4,5)P_2 hydrolysis, these reports might suggest other sources of DAG. DAG accumulation in Rat-1 cells is also a sustained process and levels were still elevated 2-fold above controls even after 24h (Muldoon *et al.*, 1990). However, the results presented here suggest that PtdCho hydrolysis could only contribute an early transient role to this DAG accumulation since the PLD response levels off after approximately 5min stimulation with ET-1. In addition, intracellular choline levels were only maintained for 10-15 min and then began to decrease. Similar transient activation of PLD has also been observed in bombesin-stimulated Swiss 3T3 fibroblasts (Cook *et al.*, 1991c; C. Briscoe, personal communication), vasopressin-stimulated A10 smooth muscle

cells (Plevin and Wakelam,1992) and carbachol-stimulated 1321N1 cells (Martinson *et al.*, 1990). The possibility exists that the phosphatidic acid which is the initial product of PLD-catalysed PtdCho hydrolysis is only slowly converted to DAG as was suggested for carbachol-stimulated 1321N1 cells (Martinson *et al.*, 1990). However, PtdIns(4,5)P_2 is more likely to contribute significantly to prolonged DAG formation in Rat-1 cells due to the sustained nature of Ins(1,4,5)P_3 generation and the prolonged accumulation of InsP. This therefore raises the possibility that PtdOH itself functions as a second messenger (this is discussed further in chapter 6).

PtdCho hydrolysis in Rat-1 cells could also be stimulated by the PKC-activating phorbol ester PMA (Fig. 3.15 and 3.21) suggesting that activation of PKC in these cells can lead to breakdown of PtdCho. This is supported by the inability of the inactive ß-phorbol to elicit a response. In order to investigate further the role of PKC in stimulating PLD activity, cells were treated with 400nM PMA for 48h prior to stimulation since this treatment has been reported to down-regulate PKC (Rodriguez-Pena and Rozengurt, 1984). The PMA- and ET-1-stimulated generation of choline was abolished in the down-regulated cells but not in cells which had been treated with the inactive control, ß-phorbol (Table 3.1). These results imply that stimulation of PLD activity may be secondary to the activation of PKC, for example as a result of PtdIns(4,5)P_2 hydrolysis. This is supported by comparing the kinetics of Ins(1,4,5)P_3 and choline generation as the former appears to precede the later. The loss in PKC activity was confirmed by [^{3}H] PDBu binding; specific binding of [^{3}H] PDBu was decreased in PMA-treated cells but remained the same as controls in ß-phorbol-treated cells. Long term PMA pretreatment did not affect the ability of ET-1 to stimulate inositol phosphates accumulation suggesting that it had not affected events upstream from PKC such as receptor function or coupling to the inositol phosphate pathway. This contrasts with the effect of phorbol ester treatment in human vascular smooth muscle cells where pretreatment of the cells

for 24h with PMA resulted in decreased ET-1 binding and inhibited both the inositol phosphates and DAG responses to ET-1 (Resink *et al.*, 1990b) or in rat vascular smooth muscle cells where preexposure for 18h with PDBu resulted in a decrease of ET-1 binding sites (Roubert *et al.*, 1989). It is noteworthy that in the Rat-1 cells the magnitude of the choline and PtdBut responses stimulated by PMA were usually smaller than that obtained with ET-1. Also, although PMA pretreatment totally abolished the ET-1-stimulated choline response, not all the PKC activity as defined by [^{3}H] PDBu binding was down regulated, though it may be that the residual activity is incapable of stimulating PLD. Billah and Anthes (1990) have suggested that PMA may directly interact with PLD in the same way as it does with PKC and therefore it may be possible that the inhibition of ET-1-stimulated choline generation is due to PMA-induced down-regulation of PLD aswell as PKC. In lymphocytes there is evidence for protein kinase C-independent activation of PLD by phorbol esters (Cao *et al.*, 1990) while in HL-60 granulocytes the PKC inhibitor K252a inhibited PMA-induced protein phosphorylation but caused only partial inhibition of PLD activation (Billah *et al.*, 1989a) suggesting that PKC does not solely mediate the action of PMA. Therefore, these results may suggest two possible mechanisms of ET-1-stimulated PLD activation, a PKC-dependent and -independent pathway. This is supported by studies investigating the ability of the PKC inhibitor, Ro-31-8220, to inhibit PMA- and ET-1-stimulated PtdBut formation. Used at a concentration (10μM) at which it is considered a selective PKC inhibitor, Ro-31-8220 inhibited PMA-stimulated PLD activity by about 80% which is consistent with a predominantly PKC-mediated activation of PLD by PMA. In contrast, at the same concentration, ET-1-stimulated PLD activity was only inhibited by approximately 40%. Although the involvement of distinct isoforms of PKC with different sensitivities to PMA induced down-regulation and inhibition by Ro-31-8220 is possible, these results suggest an additional PKC-independent pathway of PLD activation. Similar results have also been reported for

bombesin-stimulated Swiss 3T3 fibroblasts (Cook *et al.*, 1991). Likewise, the PKC inhibitors sphingosine (20μM) and H7 (100μM) had no effect on epinephrine-stimulated choline phosphate generation in MDCK-D1 cells (Slivka *et al.*, 1988).

In some systems Ca^{2+} has also been proposed to have a role in regulating PLD activity. When ET-1-stimulated choline and PtdBut formation was examined under conditions where the extracellular [Ca^{2+}] was buffered at 1μM both responses were inhibited by 40-50% (Table 3.2). Reducing the [Ca^{2+}] further (100nM) did not produce any further inhibition. In contrast PMA-stimulated PLD activity was unaffected which is consistent with its predominantly PKC-mediated mechanism of action. Consistent with the ability of Ca^{2+} influx to activate PLD, the Ca^{2+} ionophore A23187 stimulated PtdBut formation by a mechanism that was totally dependent on the extracellular [Ca^{2+}]. The effect of the PKC inhibitor Ro-31-8220 was also investigated under normal and low Ca^{2+} conditions. Ro-31-8220 inhibited ET-1-stimulated PLD activity to a similar extent as EGTA treatment but both treatments combined did not produce any further inhibition. This suggests a similar site of action and may indicate that Ca^{2+} is required as a cofactor for PKC activation in addition to acting as a primary activator of PLD. The importance of Ca^{2+} as a cofactor for PKC activity is also suggested by the increased InsP response to ET-1 under low [Ca^{2+}] conditions, possibly due to the removal of negative feedback inhibition mediated by PKC. Ro-31-8220 inhibited the A23187-stimulated response by approximately 25% supporting the possibility that the role of Ca^{2+} influx is partly to act as a cofactor for PKC activation but also demonstrating that it can stimulate PLD activity in a PKC-independent manner. Similar results have also been reported for bombesin-stimulated Swiss 3T3 cells (Cook *et al.*, 1991) although in that case EGTA and Ro-31-8220 together produced a greater inhibition than either alone but it was still less than additive.

The regulation of PLD activity by ET-1 stimulation was further

investigated by additivity experiments with A23187 and PMA. A23187 and PMA together gave an additive or greater than additive response approximately of the same magnitude as that stimulated by ET-1 suggesting that Ca^{2+} influx and PKC activation can regulate PLD by different mechanisms in Rat-1 cells. A23187 in combination with ET-1 did not produce a greater response than ET-1 alone suggesting that they share a similar pathway of PLD activation and therefore implying that the ET-stimulated response is partly mediated by influx of Ca^{2+}. ET-1 and PMA together were synergistic and produced a greater response than PMA + A23187. Since experiments using cells where PKC activity had been down-regulated by PMA pretreatment or inhibited by Ro-31-8220 suggested that PLD activation by both agonists involved PKC, the synergism observed when both were used together may indicate that ET-1 and PMA activate different isoforms of PKC and possibly different isoforms of PLD. Also, it appears that ET-1 regulates PLD by another unidentified pathway as evidenced by the phosphatidyltransferase activity still measured in the presence of Ro-31-8220 in low Ca^{2+} conditions. In rat macrophages, colony stimulating factor-1 (a receptor tyrosine kinase agonist) activation of PtdCho-specific phospholipase C required tyrosine phosphorylation (Ghosh Choudhury *et al.*, 1991). Similarly, in human neutrophils, PLD activity stimulated by fMet-Leu-Phe, platelet-activating factor and leukotriene B_4 was inhibited by tyrosine kinase inhibitors which did not inhibit PLD *per se* (Uings *et al.*, 1992). The fMet-Leu-Phe receptor does not have a tyrosine kinase activity; therefore, it is likely that a non-receptor tyrosine kinase(s) is responsible for the activation of PLD by fMet-Leu-Phe in the neutrophil. ET-1 has been shown to stimulate tyrosine phosphorylation of a number of proteins in rat mesangial cells (Force *et al.*, 1991), Swiss 3T3 cells (Zachary *et al.*, 1991) and Rat-1 cells (R. Plevin, personal communication) and it is therefore tempting to speculate that tyrosine phosphorylation may have a role to play in ET-1-stimulated PLD regulation.

In summary therefore, this chapter shows that ET-1 is a complete mitogen

in Rat-1 cells and effective in stimulating both the PtdIns(4,5)P_2 and PtdCho second messenger-generating pathways. Pharmacological characterization of both responses using ET-1 and the analogous peptides ET-2, ET-3 and SS6 suggested that both pathways are controlled by the same receptor. PtdCho hydrolysis appears to take place kinetically downstream from Ins(1,4,5)P_3 generation by a PLD-catalysed mechanism that involves both PKC-dependent and independent pathways. This would generate PtOH which may itself be an important second messenger thus generating a greater diversity of potential mitogenic mediators.or, alternatively, it may be further metabolized to DAG by phosphatidate phosphohydrolase.

Chapter 4

Regulation of ET-1-stimulated Ins(1,4,5)P_3 formation; comparison with LPA

4.1. Introduction.

It is now generally accepted that many receptors linked to the phospholipase C-catalysed hydrolysis of PtdIns(4,5)P_2 do so by interaction with a G protein referred to as G_p (reviewed in Cockcroft, 1987; Fain *et al.*, 1988; Boyer *et al.*, 1989a; Litosch, 1990). Although as yet the system is not as well defined as agonist-stimulated adenylyl cyclase or rhodopsin activation, a number of approaches have been used to investigate the involvement of a G protein in coupling receptors to polyphosphoinositide hydrolysis.

Studies into the adenylyl cyclase system have shown that the interaction of a receptor with a G protein following stimulation with an appropriate agonist results in the dissociation of GDP and the subsequent binding of GTP to the G protein. A consequence of this is that the affinity of the receptor for the agonist is decreased (Casey and Gilman, 1988). The effects of guanine nucleotides on the binding of hormones and growth factors which stimulate PtdIns(4,5)P_2 breakdown have therefore been studied to investigate for the involvement of a G protein. Receptors exist as high and low affinity components and the efficacies of agonists acting through G protein-coupled receptors to stimulate polyphosphoinositide hydrolysis have been correlated with their capacity to form a GTP-sensitive high affinity binding state (Evans *et al.*, 1985; Taylor and Merritt, 1986). Guanosine 5'-[βγ-imido] triphosphate (Gpp[NH]p), a non-hydrolysable analogue of GTP, decreased the affinity of [^{3}H] vasopressin for its receptor on hepatic plasma membranes which was reflected by an increase in its rate of dissociation (Bojanic and Fain, 1986). In permeabilized human neuroblastoma cells the binding affinity of carbachol was significantly reduced by GppNHp (Wojcikiewicz *et al.*, 1990). Guanine nucleotides have also been shown to decrease the binding of other hormones that stimulate PtdIns(4,5)P_2 breakdown (reviewed in Fain *et al.*, 1988). In order to recycle back to an inactive state an intrinsic GTPase activity hydrolyses the bound GTP to GDP which promotes reassociation of the α-subunit with βγ. Activation of GTPase

activity has been observed in vasopressin-stimulated hepatic plasma membrane preparations and GTPase activity migrated with bound [^{3}H] vasopressin when solubilized membranes were subjected to sucrose gradient centrifugation (Fitzgerald *et al.*, 1986).

More direct evidence for G protein regulation of PtdIns-PLC has come from reports of the ability of fluoride and stable analogues of GTP to stimulate polyphosphoinositide turnover. Activation by fluoride is caused by interaction of aluminium fluoride (AlF_4^-) with bound GDP so that it mimics the role of the γ phosphate of GTP (Bigay *et al.*, 1985). AlF_4^- and GTP[S] (guanosine 5'-[γ-thio] triphosphate) stimulated $InsP_3$ production in hepatocyte membranes in a manner that could be inhibited by GDP[S] (guanosine 5'-[β-thio] diphosphate) (Cockcroft and Taylor, 1987). Similarly, in plasma membrane preparations from human neutrophils GTP[S] stimulated the breakdown of polyphosphoinositides (Cockcroft and Gomperts, 1985) and in turkey erythrocyte membranes GTP[S]-induced inositol phosphates accumulation was accompanied by a corresponding decrease in polyphosphoinositides (Harden *et al.*, 1988). Guanine nucleotides can potentiate hormone effects on PtdIns-PLC activity. GTP[S] potentiated bombesin-stimulated $InsP_3$ generation in permeabilized Swiss 3T3 cells while GDP[S] had an inhibitory effect (Cattaneo and Vincentini, 1989; Plevin *et al.*, 1990). Similar effects were also reported for angiotensin II-stimulated membranes from rat mesangial cells (Pfeilschifter and Bauer, 1987) and carbachol- and histamine-stimulated rat brain cortical membranes (Claro *et al.*, 1989). A requirement for guanine nucleotides for receptor coupling to PtdIns-PLC is also supported by the observations that purinergic agonist-stimulated inositol phosphates accumulation in turkey erythrocyte membranes (Boyer *et al.*, 1989b) and carbachol-stimulated inositol phosphates accumulation in membranes from astrocytoma cells (Orellana *et al,* 1987) occurred only in the presence of added guanine nucleotides.

The rate-limiting step in the activation process is thought to be the dissociation of GDP from the G protein and agonist-receptor occupation is believed to accelerate GTP/GDP exchange thus enhancing the onset of PtdIns(4,5)P_2 hydrolysis. In turkey erythrocyte membranes activation of polyphosphoinositide turnover by GTP[S] occurred with a considerable lag time which was decreased in a concentration-dependent manner by a P_{2y}-purinergic agonist. Increasing concentrations of agonist also increased the rate constant in a saturable manner indicating that the rate of activation was increased by receptor stimulation (Boyer *et al.*, 1989b). Plevin *et al.*. (1990) also reported similar effects with bombesin in Swiss 3T3 cells where a potentiating effect with GTP[S] on $InsP_3$ generation was observed at early time points after stimulation when GTP[S] on its own had no effect.

The β_2-adrenergic receptor which couples to adenylyl cyclase can be uncoupled from G protein activation by phosphorylation of the receptor via the β-adrenergic receptor kinase (βARK) (reviewed in Palczewski and Benovic, 1991). Similarly, many receptors which couple to the inositol lipid signalling pathway via a G protein also seem to be regulated by phosphorylation and a feature of many agonists which stimulate polyphosphoinositide hydrolysis is the sensitivity of the response to inhibition by PKC-activating phorbol esters such as PMA. PMA pretreatment for 15min inhibited bombesin-stimulated inositol phosphates accumulation in Swiss 3T3 cells with no effect on bombesin binding. Supporting a role for PKC in mediating a negative-feedback pathway was the observation that in cells where PKC had been down-regulated, basal and bombesin-stimulated production of inositol phosphates were elevated (Brown *et al.*, 1987). The third cytoplasmic loop of the bombesin receptor, which is the proposed site involved in the receptor/G protein interaction (Kobilka *et al.*, 1988), has serine residues which potentially could be phosphorylated by serine/threonine protein kinases (Battey *et al.*, 1991), suggesting that receptor function may be regulated by protein kinases. Such inhibition of

polyphosphoinositide turnover by short exposures to PMA seems to be a general feature of agonists whose receptors couple to PtdIns-PLC by a G protein interaction, for example: carbachol-stimulated astrocytoma cell membranes, angiotensin II-stimulated mesangial cell membranes, f-Met-Leu-Phe-stimulated polymorphonuclear leukocytes and HL60 cells, vasopressin-stimulated Swiss 3T3 cells, angiotensin II- and thrombin-stimulated vascular smooth muscle cells, bombesin-stimulated Swiss 3T3 cells (Orellana *et al.*, 1987; Pfeilschifter and Bauer, 1987; Smith *et al.*, 1987; Cockcroft and Stutchfield, 1989; Blakeley *et al.*, 1989; Kawahara *et al.*, 1988; Huang and Ives, 1989; Cook and Wakelam, 1991a). In membranes from astrocytoma cells the addition of purified PKC mimicked the ability of PMA pretreatment to inhibit GTP[S]-stimulated $InsP_3$ production (Orellana *et al.*, 1987) while in membrane preparations from mesangial cells angiotensin II+GTP[S]-stimulated $InsP_3$ generation was similarly inhibited with OAG or PMA pretreatment (Pfeilschifter and Bauer, 1987). In contrast PDGF, which mediates its effects via a receptor with intrinsic tyrosine kinase activity, stimulated polyphosphoinositide turnover in a manner which was insensitive to PMA inhibition (Kawahara *et al.*, 1988; Blakeley *et al.*, 1989; Huang and Ives, 1989). PKC-mediated inhibition of agonist-stimulated polyphosphoinositide turnover is thought to be due to impaired coupling of the receptor to PtdIns-PLC caused by PKC-mediated phosphorylation at several sites, possibly including the G protein itself. Both receptor-G protein coupling (Pfeilschifter and Bauer, 1987; Plevin *et al.*, 1990) and G protein-PtdIns-PLC coupling (Orellana *et al.*, 1987; Smith *et al.*, 1987) have been proposed as sites of inhibition as well as PtdIns-PLC itself (Geny *et al.*, 1989; Ryu *et al.*, 1990).

Heterogeneity in the G proteins (G_p) coupling receptors to PtdIns-PLC was suggested by the ability of pertussis toxin to inhibit the response in some systems but not in others (reviewed in Fain *et al.*, 1988; Cockcroft, 1987). In HL60 cells f-Met-Leu-Phe-stimulated polyphosphoinositide breakdown was pertussis toxin-sensitive and in membranes from these cells the formyl peptide

receptor was shown to functionally interact with both G_{i2} and G_{i3} (Gierschik *et al.*, 1989). Similarly, the f-Met-Leu-Phe-stimulated response in polymorphonuclear leukocytes was also pertussis toxin-sensitive (Smith *et al.*, 1987). Prior treatment with pertussis toxin had little or no effect on inositol phosphates accumulation in response to vasopressin+GTP[S] in hepatocyte plasma membranes (Uhing *et al.*, 1986), $PGF_{2\alpha}$, bombesin and vasopressin in Swiss 3T3 cells (Taylor *et al.*, 1988), bradykinin in NG108-15 cells (Fu *et al.*, 1988) or angiotensin II in rat liver epithelial cells (Hepler *et al.*, 1990) therefore arguing against a major role for a pertussis toxin substrate in coupling any of these agonist receptors to PtdIns-PLC. A number of pertussis toxin-insensitive G proteins have now been identified (section 1.4.1). Among these G_q and G_{11} are widely expressed and highly homologous. In reconstitution experiments $G\alpha_q$ was shown to specifically activate PLC-β1 (Martin *et al.*, 1991; Taylor *et al.*, 1991; Wu *et al.*, 1992).

Increasing interest is being shown in the receptor-G protein-effector interactions for two important mitogens, ET-1 and lysophosphatidic acid (LPA). Both ET-1 and LPA have been reported to stimulate inositol phosphates accumulation which was dependent on the presence of GTP[S] in A10 membranes and permeabilized Rat-1 cells respectively (Takuwa *et al.*, 1990; van Corven *et al.*, 1989) while in permeabilized rat mesangial cells ET-1-stimulated inositol phosphates accumulation was potentiated in the presence of GTP[S] (Thomas *et al.*, 1991). Having established that ET-1 potently stimulated the hydrolysis of PtdIns(4,5)P_2 in Rat-1 cells (see section 3.3) it was therefore of interest to characterize the nature of the receptor-G protein-PtdIns-PLC interaction involved in this system using the criteria discussed above. Since LPA has already been reported to stimulate polyphosphoinositide hydrolysis in a GTP-dependent manner in these cells a more rigorous investigation of the nature of the G protein interaction using the same criteria could reveal important differences in the way these agonists function as mitogens. LPA has been

suggested to exert its mitogenic effect in Rat-1 cells via the inhibition of adenylyl cyclase (van Corven *et al.*, 1989) but ET-1 has no effect on adenylyl cyclase activity in these cells (F. McKenzie and E. Adie, personal communication) and therefore must have a different mode of action. In the studies presented here a comparison of the characteristics of ET-1- and LPA-stimulated PtdIns(4,5)P_2 hydrolysis and Ins(1,4,5)P_3 formation in both intact and electropermeabilized Rat-1 cells is presented.

4.2. Comparison of PtdIns(4,5)P_2 hydrolysis stimulated by ET-1 and LPA in intact cells.

In Rat-1 cells LPA stimulated the accumulation of [^{3}H] inositol phosphates in a dose-dependent manner with a maximum increase at about 10μM and an EC_{50} value of 3.0 ± 2.3 μM (n=7) (Fig. 4.1). LPA is therefore a far less potent agonist at stimulating polyphosphoinositide hydrolysis than ET-1 (EC_{50} ~2nM). The maximum response to LPA was also smaller than that obtained with ET-1 with a 2-3 fold increase in [^{3}H] inositol phosphates accumulation over controls after 20min. When the formation of Ins(1,4,5)P_3 mass stimulated by a maximum concentration of LPA (100μM) was measured further differences to the ET-1-stimulated response were observed. LPA stimulated the rapid generation of Ins(1,4,5)P_3 with a peak response at about 10s which was 6-10 fold (n=5) above basal values (results from a typical experiment : 2.94 ± 0.85 pmol in control; 21.2 ± 3.3 pmol with 100μM LPA; n=5) (Fig. 4.2). Stimulated levels then quickly declined to basal and by 2min were not significantly different from control values (results from a typical experiment : 3.26 ± 0.26 pmol in control; 4.10 ± 0.42 pmol with 100μM LPA; n=5). This is in contrast with ET-1-stimulated formation of Ins(1,4,5)P_3 where stimulated levels were maintained 2-4 fold above controls up to 10min (Fig. 4.3). The sustained phase of ET-1-

stimulated Ins(1,4,5)P_3 generation was dependent on the extracellular [Ca^{2+}] and could be abolished by including 0.33mM EGTA to reduce the extracellular [Ca^{2+}] to 1μM (Fig. 4.3a). Furthermore, if Ca^{2+} was re-added once stimulated levels had returned to basal, a further 3-4 fold increase in Ins(1,4,5)P_3 was stimulated ; this response had returned to basal after about 2 min (Fig. 4.3b). Thus ET-1 appears to stimulate an additional Ca^{2+}-dependent pathway of polyphosphoinositide hydrolysis compared with LPA.

Since these differences in Ins(1,4,5)P_3 formation in response to LPA and ET-1 suggested differences in the rates of Ins(1,4,5)P_3 degradation or the hydrolysis of PtdIns(4,5)P_2 , mass levels of this lipid were measured following stimulation with both agonists (Fig. 4.4). LPA stimulated a rapid decrease in the mass of PtdIns(4,5)P_2 with a maximum reduction of 40-50% in lipid levels after 10s (results from a typical experiment : 9.5 ± 1.8 pmol in control; 4.8 ± 0.5 pmol with 100μM LPA; n=4). However, in parallel with Ins(1,4,5)P_3 formation, the response was transient and lipid levels had returned to control values within 2min after stimulation with LPA and, in some experiments, subsequently increased above control values before returning to basal (8.5 ± 0.6 pmol in control; 11.0 ± 0.6 pmol with 100μM LPA; 5min stimulation; n=2). This is probably due to the time it takes to regulate the activity of the enzymes responsible for re-synthesizing PtdIns(4,5)P_2 once lipid levels have returned to control values. ET-1also stimulated a rapid decrease in PtdIns(4,5)P_2 mass but a maximum reduction was only observed after nearly 1min following stimulation when a 70-85% reduction in stimulated levels below control was observed (results from a typical experiment : 8.6 ± 1.0 pmol in control; 1.5 ± 0.3 pmol with 100nM ET-1; n=4) which is greater than the decrease obtained with LPA. The stimulated decrease was also more sustained than that obtained with LPA thus paralleling the formation of Ins(1,4,5)P_3 and stimulated levels only returned to control values after about 20min.

The inositol phosphates responses to LPA and ET-1 in intact cells were further characterized by investigating the sensitivity to inhibition by short pretreatments with PMA (Fig. 4.5). Following a 15min preincubation with 300nM PMA, dose-dependent [^{3}H] inositol phosphates accumulation stimulated by LPA was decreased at all concentrations of agonist and the inhibition was 60-90% at a maximum dose of LPA (Fig. 4.5b). In contrast, the response to ET-1 was unaffected by PMA pretreatment (Fig. 4.5a). Increased concentrations of PMA up to 1μM or a prolonged preincubation time up to 60min were also without any effect on the ET-1-stimulated accumulation of inositol phosphates (379 ± 56% of basal with ET-1 in control cells, 446 ± 14% of basal with ET-1 in cells pretreated with 1μM for 15min; 310 ± 20% with ET-1 in control cells, 360 ± 20% with ET-1 in cells pretreated with 300nM PMA for 60min). When cells were exposed to 100ng/ml pertussis toxin for 18-20h prior to stimulation this treatment had little effect on subsequent inositol phosphates accumulation in response to either LPA or ET-1 (Fig. 4.6). Pertussis toxin has been shown to ribosylate a ~40kD substrate in Rat-1 cells transfected with the α_2 -C10 adrenergic receptor (Milligan *et al.*, 1991).

4.3. Differences in Ca^{2+} mobilization stimulated by ET-1 and LPA.

Since the primary function of Ins(1,4,5)P_3 is to release stored Ca^{2+}, intracellular Ca^{2+} mobilization in response to both ET-1 and LPA was also measured . Changes in intracellular Ca^{2+} levels were measured by the fluorescence emitted from cells loaded with the Ca^{2+} indicator Indo-1 AM. Due to the difficulty in calibrating the fluorescence emitted as a Ca^{2+} concentration, the results are presented as fluorescence data. Both ET-1 and LPA stimulated a biphasic increase in intracellular Ca^{2+} levels consisting of a rapid transient phase and a second smaller but sustained phase (Fig. 4.7). However, in LPA-stimulated cells the level of Ca^{2+} immediately returned to basal if the cells were

washed with buffer and a rechallenge with LPA could again elicit a biphasic response similar to the first one recorded (Fig. 4.7b). In contrast, Ca^{2+} levels in response to ET-1 remained high despite washing with buffer and a rechallenge with the peptide could not elicit any further increases (Fig. 4.7a). For both agonists the initial peak increase in intracellular Ca^{2+} was independent of the extracellular [Ca^{2+}]; however, if EGTA was included to buffer the extracellular [Ca^{2+}] to 1μM the sustained phase of the response was abolished (Fig. 4.8). Re-adding a 1.26mM Ca^{2+}-containing buffer elicited a second, sustained increase in the level of Ca^{2+} similar to the second phase of the biphasic response, suggesting that both ET-1 and LPA increase the intracellular [Ca^{2+}] by release from intracellular stores and entry of external Ca^{2+}. In some experiments changing to a 1μM Ca^{2+} buffer in the absence of agonist resulted in an increase in intracellular Ca^{2+} (e.g. Fig. 4.8a), but the significance and mechanism of this is unclear.

4.4. The role of guanine nucleotides in ET-1- and LPA-stimulated inositol phosphates accumulation.

In order to investigate potential differences in the interaction of both LPA and ET-1 receptors with the inositol lipid signalling pathway experiments were performed using electropermeabilized cells. Electropermeabilization was carried out by exposing cells in suspension to six discharges of a 3 μF capacitor with a field strength of 2kV/cm; approximately 95% of cells were found to be permeable to Hoescht stain following this procedure (Fig. 4.9).

The involvement of a G protein in receptor-effector coupling was assessed by studying the effect of the non-hydrolysable GTP analogue, GTP[S], on [^{3}H] inositol phosphates accumulation. In permeabilized cells LPA gave a maximum response 2-3-fold over basal values, but the addition of 30μM GTP[S] clearly potentiated LPA- stimulated inositol phosphates accumulation at all concentrations of the agonist employed (results from a typical experiment : 1495

± 183 d.p.m. in control; 2320 ± 66 d.p.m. with 100μM LPA; 2596 ± 143 d.p.m. with 30μM GTP[S]; 5356 ± 206 d.p.m. with LPA + GTP[S]) (Fig. 4.10b). Potentiation of the response was specific for non-hydrolysable analogues of GTP; Gpp[NH]p was less effective than GTP[S] (54 ± 3 % of the GTP[S] response, n=2) whereas GTP, GDP, GMP and ATP were without effect (n=3). In contrast, GTP[S] had little effect on ET-1-stimulated [^{3}H] inositol phosphates accumulation in permeabilized cells. ET-1 alone stimulated a maximum response which was not significantly enhanced by the addition of 30μM GTP[S] (Fig. 4.10a). In subsequent experiments examining nucleotide effects on ET-1-stimulated inositol phosphates accumulation, the peptide was used at a submaximum concentration (0.3nM or 1nM) to ensure that the system was not being stimulated to its full capacity by the peptide alone

The effect of LPA and ET-1 on the dose-dependent accumulation of [^{3}H] inositol phosphates in response to GTP[S] in permeabilized cells was also investigated. GTP[S] stimulated a maximum increase of about 2-fold above basal and the response was substantially increased at all concentrations of the nucleotide by a maximum concentration of LPA (results from a typical experiment : 1323 ± 81 d.p.m. in control; 2206 ± 102 d.p.m. with 100μM GTP[S]; 2187 ± 120 d.p.m. with 30μM LPA; 4455 ± 506 d.p.m. with GTP[S] + LPA). LPA also shifted the EC_{50} value for the nucleotide to the left (-LPA, 86.9 ± 15.6 μM; +LPA, 31.2 ± 8.6 μM; n=3) (Fig. 4.11b). In contrast, ET-1 had little effect on the magnitude or EC_{50} value of the GTP[S] response (Fig. 4.11a). Similar experiments were also carried out in cells which had been permeabilized by streptolysin-O treatment. Cells permeabilized with streptolysin-O were grown as monolayers while cell suspensions were used for electropermeabilization. In agreement with the results obtained in electropermeabilized cells, the GTP[S] response was also not affected by ET-1 in toxin-permeabilized cells (results from a typical experiment : 963 ± 22 d.p.m. in control; 2378 ± 262 d.p.m. with 10μM GTP[S]; 3640 ± 419 with 1nM ET-1;

5529 ± 761 d.p.m. with GTP[S] + ET-1; n=3). The GTP[S]-stimulated accumulation of inositol phosphates was potentiated by LPA but the increase was not as great as that obtained in electropermeabilized cells (results from a typical experiment : 311 ± 60 d.p.m. in control; 1172 ± 193 d.p.m. with 10μM GTP[S]; 773 ± 83 d.p.m. with 30μM LPA; 2306 ± 97 d.p.m. with GTP[S] + LPA). Therefore, electroporation was used as the preferred method for permeabilization.

4.5. Effect of GTP[S] on the kinetics of $InsP_3$ formation in response to ET-1 and LPA in permeabilized cells.

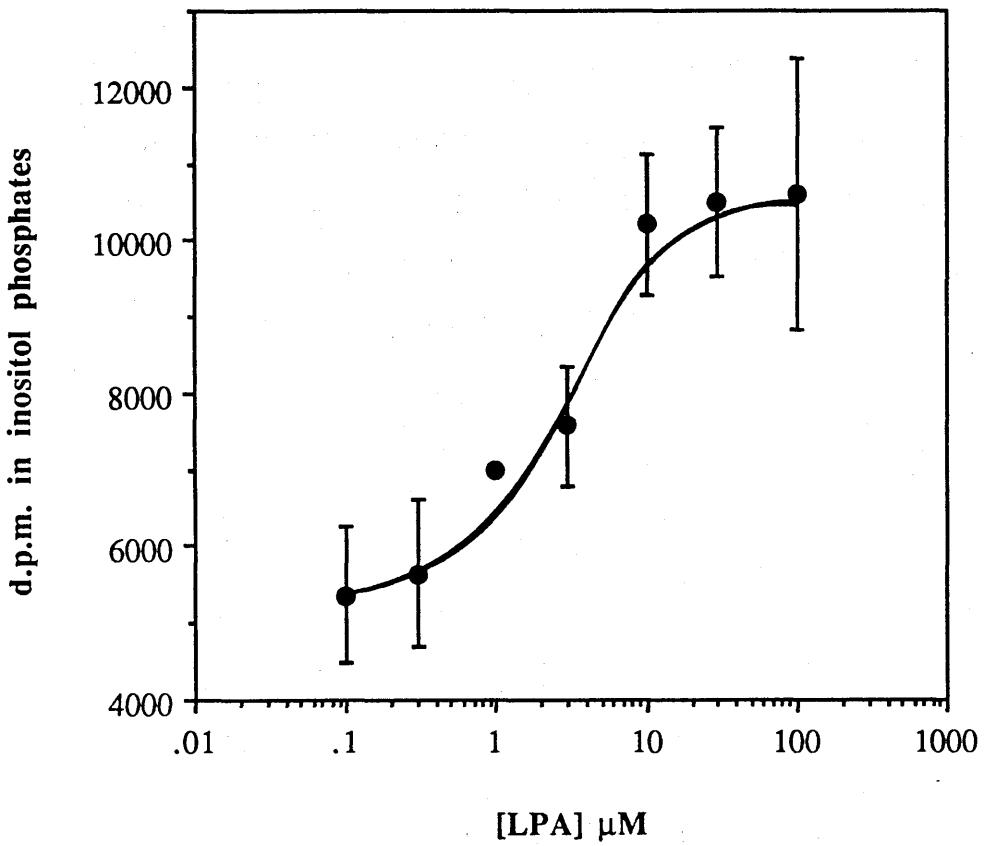
For G protein-coupled receptors, agonist-receptor occupation is thought to accelerate GTP/GDP exchange and thus enhance the onset of PtdIns(4,5)P_2 hydrolysis. In permeabilized Rat-1 cells GTP[S] alone stimulated the formation of [^{3}H] $InsP_3$ but only after a lag time of about 30s (Fig. 4.12). However, in the presence of LPA (30μM), a potentiating effect of GTP[S] was observed as early as 10s after stimulation indicating that the lag time for the onset of the nucleotide response had been decreased by LPA (Fig. 4.12a). A similar effect was observed for the kinetics of [^{3}H] $InsP_2$ formation (results from a typical experiment : 2004 ± 132 d.p.m. in control; 2128 ± 156 d.p.m. with GTP[S]; 4928 ±116 d.p.m. with LPA; 6912 ± 180 d.p.m. with LPA + GTP[S]; 10s stimulation). However, $InsP_3$ generation in response to a maximum concentration of ET-1 (10nM) was not enhanced by GTP[S] and the agonist did not reduce the lag time for the onset of a GTP[S] response (Fig. 4.12b). At submaximal concentrations (1nM) of ET-1 a potentiating effect with GTP[S] was observed and the presence of ET-1 reduced the lag time for the onset of the GTP[S] effect (Fig. 4.12c). The ET-1-stimulated generation of [^{3}H] $InsP_3$ was increased about 2-fold in the presence of GTP[S] as early as 10sec following stimulation with a submaximum concentration of the peptide. The sustained generation of [^{3}H] $InsP_3$ stimulated by 1nM ET-1 compared with the transient

response stimulated by a maximum concentration probably reflects differences in the activation of the kinases and phosphatases responsible for $InsP_3$ metabolism.

4.6. Effect of GDP[S] and PMA pretreatment on ET-1 and LPA-stimulated inositol phosphates accumulation in permeabilized cells.

Despite the lack of potentiation of ET-1-stimulated inositol phosphates accumulation by GTP[S] when a maximum concentration of agonist was used, the ability of submaximal concentrations of the peptide to reduce the lag time for the onset of GTP[S]-stimulated [^{3}H] $InsP_3$ generation suggested a role for a G protein in coupling the receptor to PtdIns-PLC. In order to investigate this further the effect of the nonhydrolysable GDP analogue, GDP[S], on agonist - stimulated [^{3}H] inositol phosphates accumulation was studied. Following a preincubation with 1mM GDP[S] the responses to both ET-1 and LPA, alone and in combination with GTP[S], were inhibited (Fig. 4.13). GDP[S] was equally effective against ET-1 in the presence or absence of GTP[S] inhibiting inositol phosphates accumulation by 40-55% (n=5). However, although the response to LPA in combination with GDP[S] was substantially inhibited by ~50%, GDP[S] had little effect on stimulation by LPA alone (10-20%, n=5).

Since short PMA pretreatment was shown to inhibit the LPA-stimulated inositol phosphates accumulation in intact cells but to have no effect on the ET-1 response, similar experiments were carried out in permeabilized cells (Fig. 4.14). Following preincubation with 300nM PMA for 15min the accumulation of [^{3}H] inositol phosphates stimulated by LPA was decreased by about 50%. The response to GTP[S] was not significantly affected and, in agreement with the results obtained from intact cells, stimulation by ET-1 was also not inhibited (results from a typical experiment : 151± 11% basal with GTP[S]; 138 ± 3% basal with GTP[S] + PMA; 232 ± 14% basal with ET-1; 257 ± 6% basal. with ET-1 + PMA).


d.p.m. in inositol phosphates
12000
10000
8000
6000
4000
.01
.1
1
10
100
1000
[LPA] μM

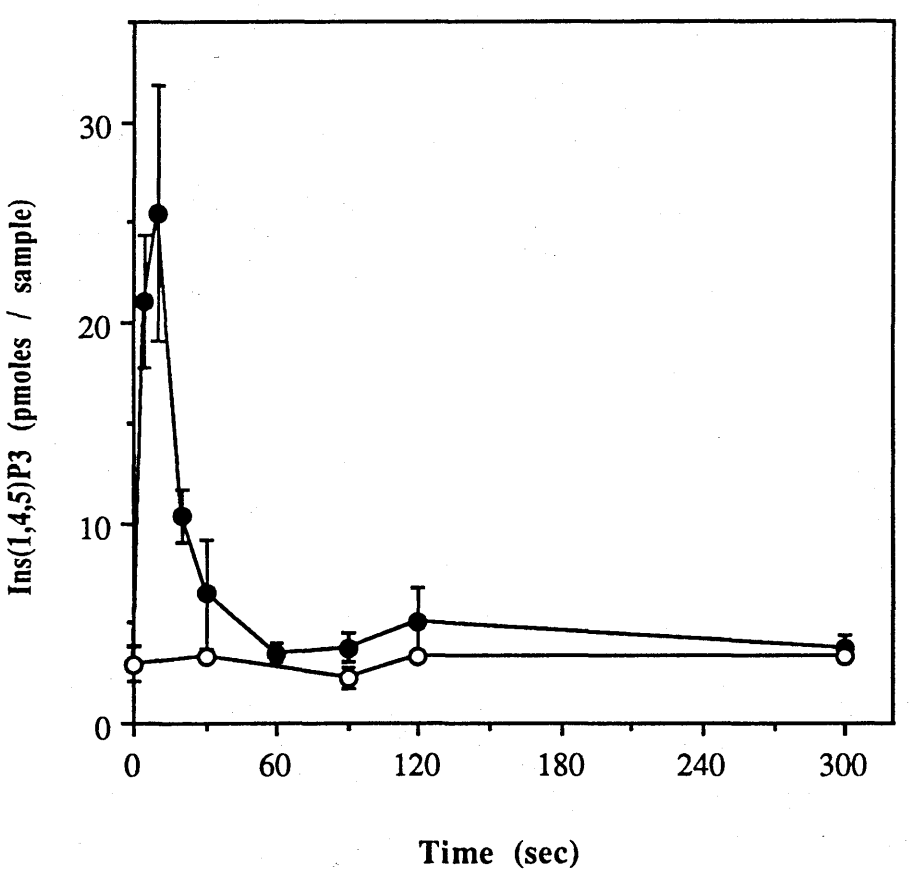
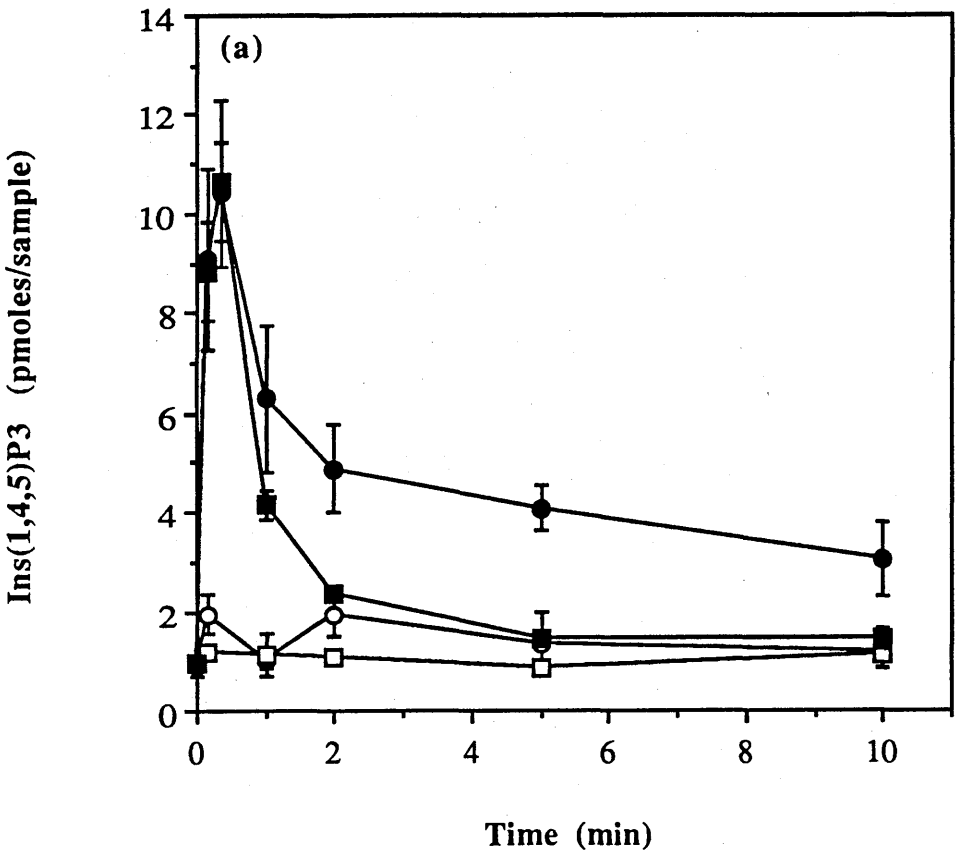

Ins(1,4,5)P3 (pmoles / sample)
30
20
10
0
0
60
120
180
240
300
Time (sec)

Fig. 4.1 Dose dependence of LPA-stimulated inositol phosphates accumulation.

Rat-1 cells labelled for 48h with 1μCi/ml [^{3}H] inositol were stimulated with increasing concentrations of LPA for 20min in the presence of 10mM LiCl and the radioactivity associated with total inositol phosphates was measured as described in section 2.6.2. The results are expressed as means ± S.D. from a typical experiment where n=7. The basal value was 5363 ± 880 d.p.m.

Fig. 4.2 Time course of LPA-stimulated Ins(1,4,5)P_3 mass formation.

Rat-1 cells were grown to confluency and quiescence. Stimulation was then carried out with 100μM LPA (●) or vehicle (O) for the indicated times and Ins(1,4,5)P_3 levels were measured by mass assay (section 2.7). Each point represents the mean ± S.D. of triplicate determinations from a typical experiment where n=5.


(a)
Ins(1,4,5)P3 (pmoles/sample)
14
12
10
8
6
4
2
0
0
2
4
6
8
10
Time (min)

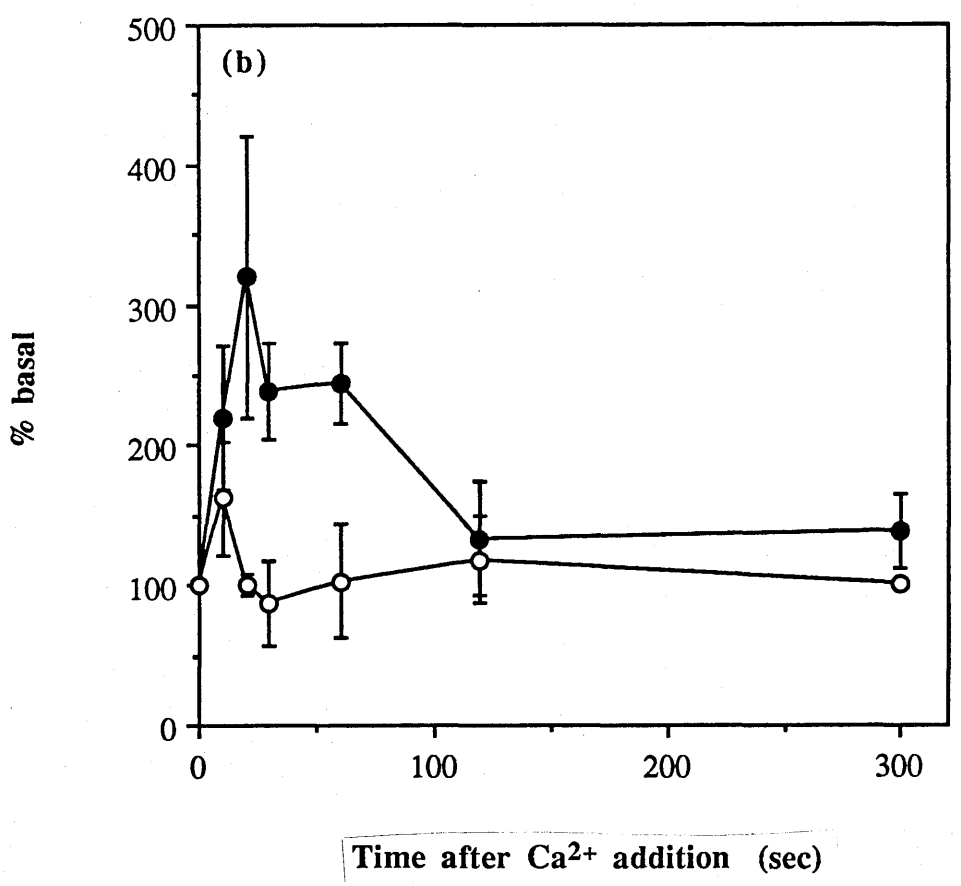
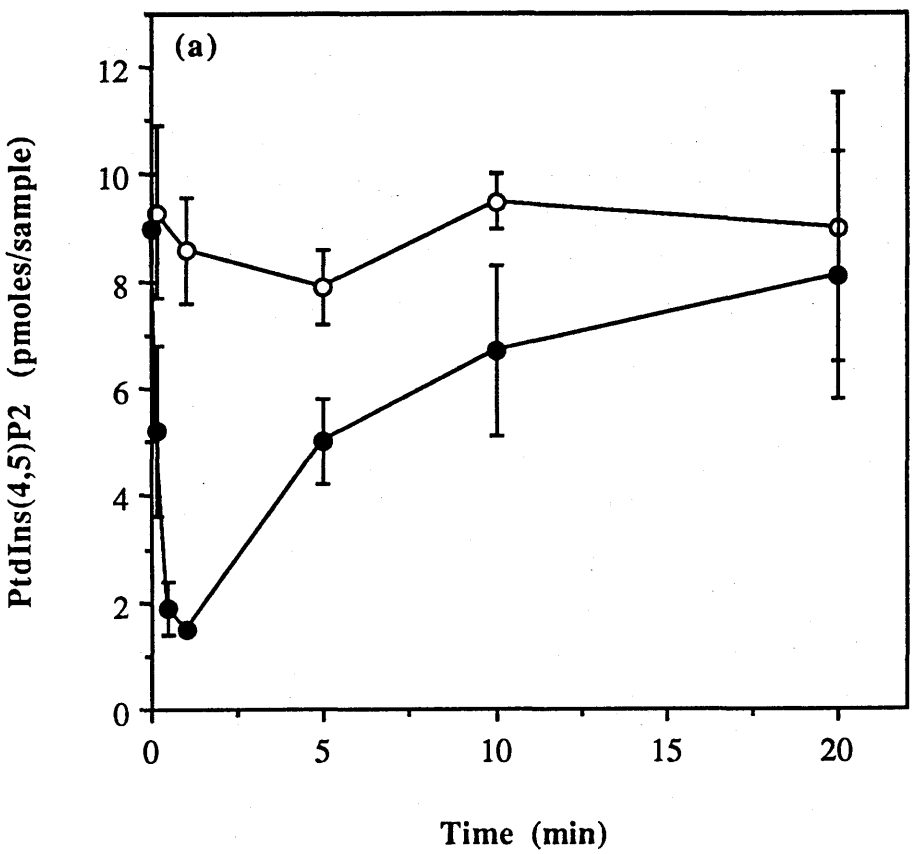

(b)
% basal
500
400
300
200
100
0
0
100
200
300
Time after Ca2+ addition (sec)

Fig. 4.3 Ca^{2+}-dependency of ET-1-stimulated $Ins(1,4,5)P_3$ mass formation.

(a) Rat-1 cells were grown to confluency and quiescence. Cells were then preincubated in normal HBG containing 1.26mM Ca^{2+} (O, ●) or HBG low in Ca^{2+} containing 0.33mM EGTA to give a 1μM free [Ca^{2+}] (□, ■) for 20min and then stimulated with vehicle (O, □) or 100nM ET-1 (●, ■) in the same buffers. In (b) a 1.26mM Ca^{2+}-containing buffer was added after a 5min preincubation with 100nM ET-1 in low Ca^{2+} buffer. $Ins(1,4,5)P_3$ levels were measured as described previously (section 2.7). O, 1μM Ca^{2+}; ●, 1.26mM Ca^{2+}.

Results are expressed as means ± S.D. from typical experiments where n=3-6.


(a)
PtdIns(4,5)P2 (pmoles/sample)
12
10
8
6
4
2
0
0
5
10
15
20
Time (min)

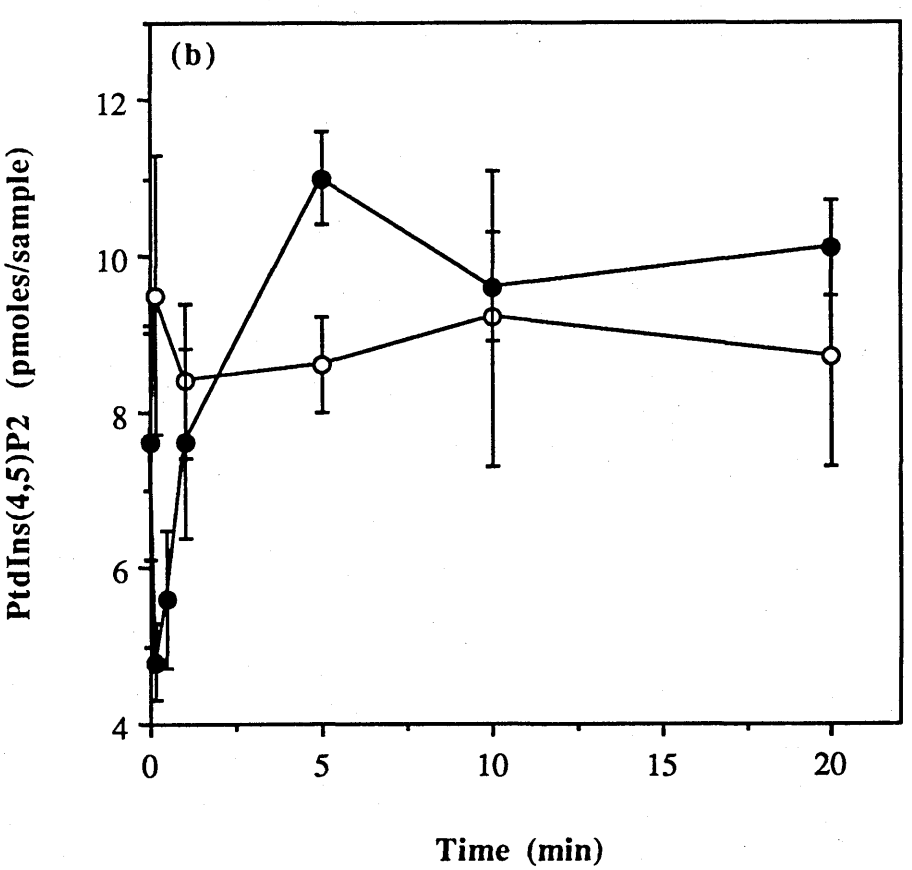
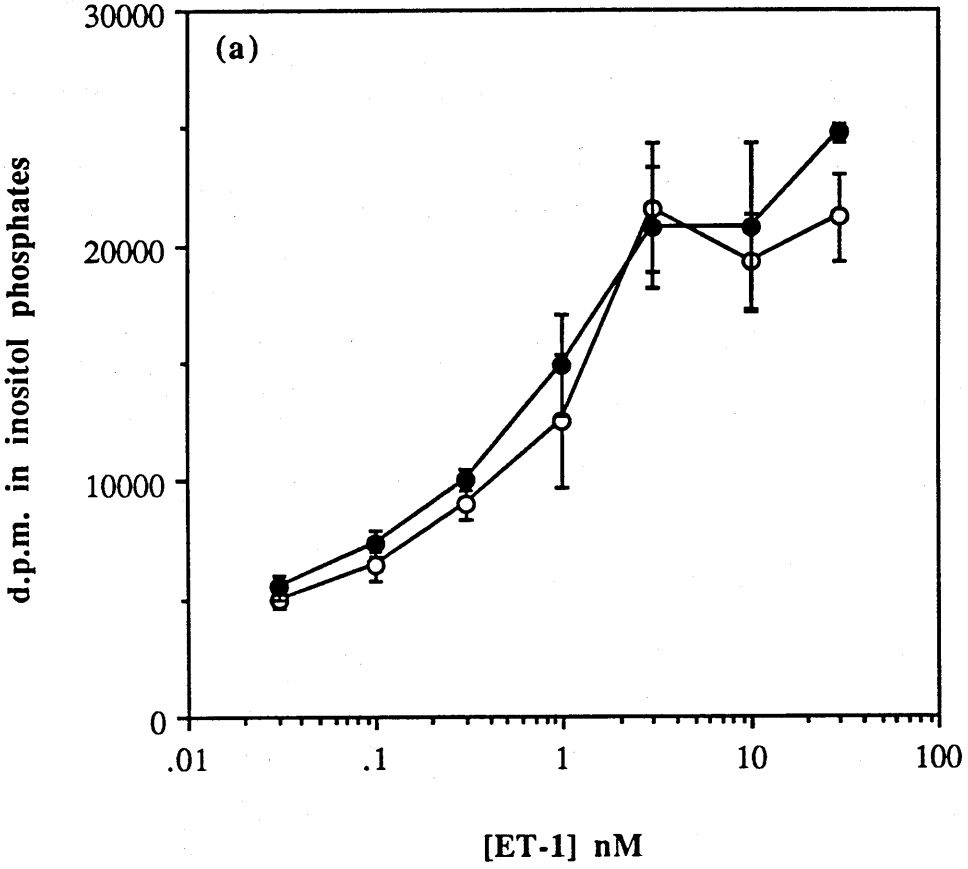

(b)
PtdIns(4,5)P2 (pmoles/sample)
12
10
8
6
4
0
5
10
15
20
Time (min)

Fig. 4.4 Time course of ET-1- and LPA-stimulated PtdIns(4,5)P_2 breakdown.

Rat-1 cells were grown to confluency and quiescence. Stimulation was then carried out with (a) 100nM ET-1 or (b) 100μM LPA for the stated times. Samples were deacylated and deglycerated as described in section 2.8 and PtdIns(4,5)P_2 mass levels determined by measuring the mass of Ins(1,4,5)P_3. Results are expressed as means ± S.D. from a typical experiment where n=3. (O), basal levels; (●), stimulated levels.


(a)
30000
20000
10000
0
.01
.1
1
10
100
d.p.m. in inositol phosphates
[ET-1] nM

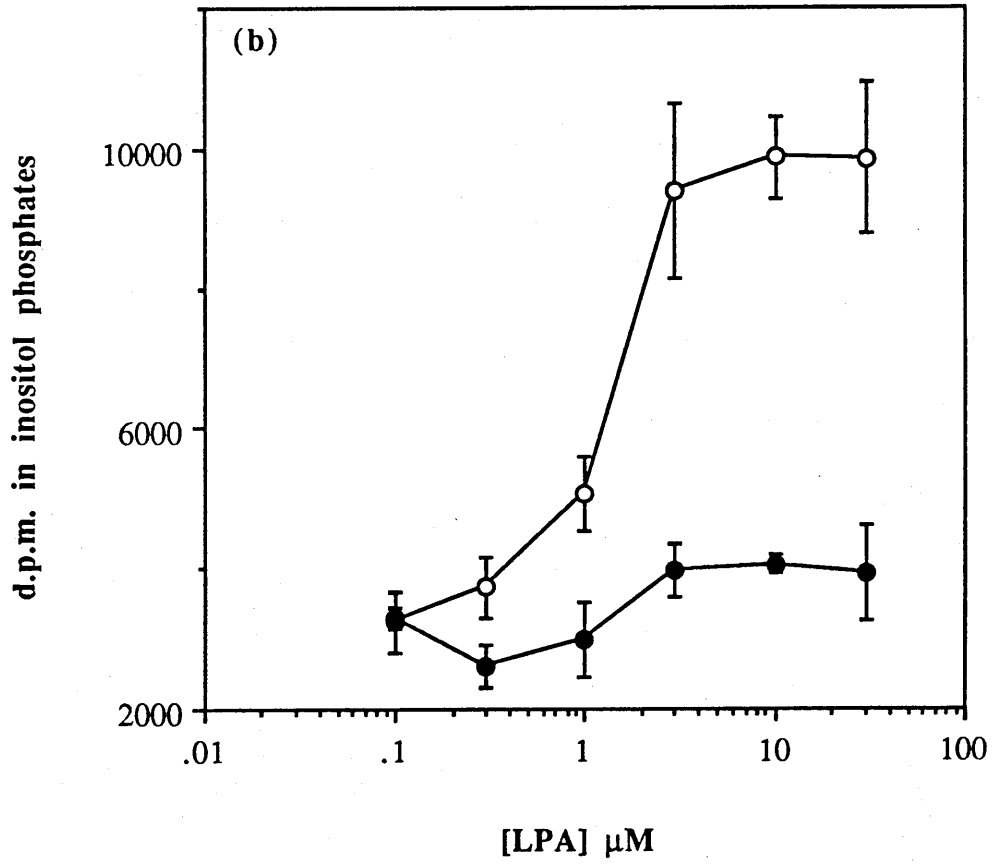

(b)
10000
6000
2000
.01
.1
1
10
100
d.p.m. in inositol phosphates
[LPA] μM

Fig. 4.5 Effect of PMA on ET-1 and LPA dose-dependent stimulation of [^{3}H] inositol phosphates accumulation.

Rat-1 cells labelled for 48h with 1μCi/ml [^{3}H] inositol were preincubated with vehicle (O) or 300nM PMA (●) for 15min prior to incubation for a further 20min with increasing concentrations of (a) ET-1 or (b) LPA. Samples were assayed for inositol phosphates as described in section 2.6.2. Results are expressed as the mean ± S.D. of triplicate determinations from a typical experiment where n=3. Basal values were (d.p.m.) (a) -PMA, 4954 ± 323; +PMA, 5491 ± 483; (b) -PMA, 2778 ± 192; +PMA, 2232 ± 315.

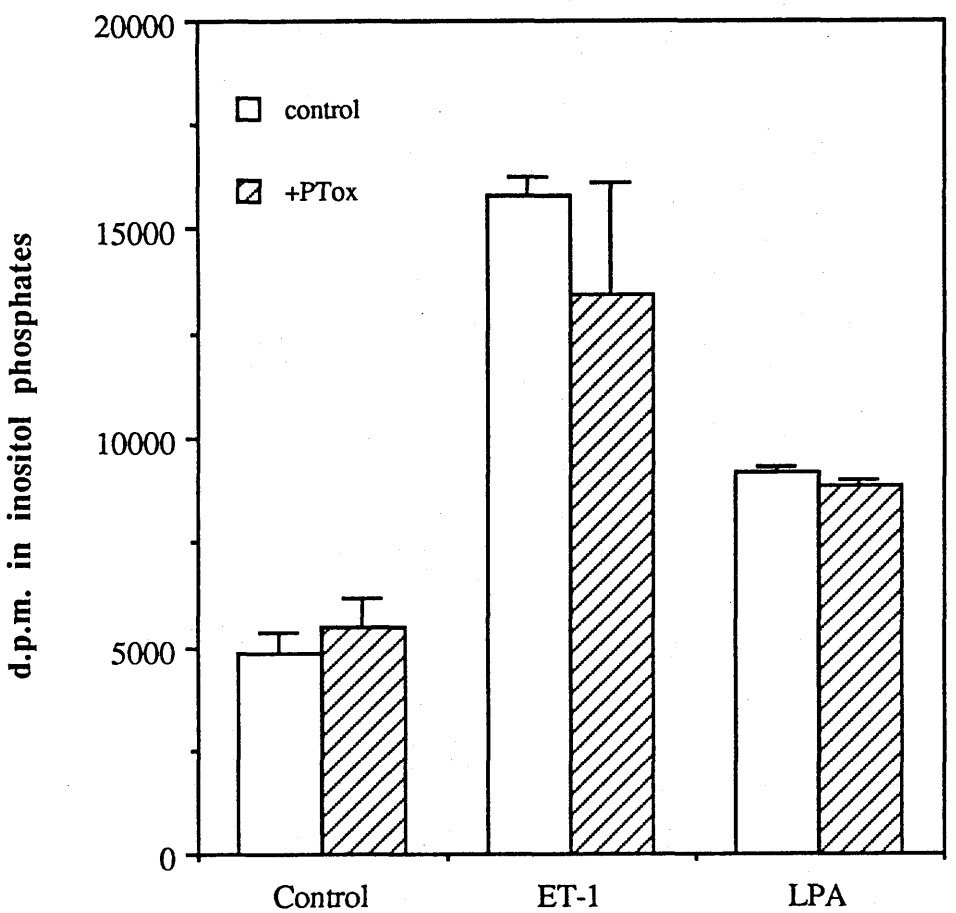
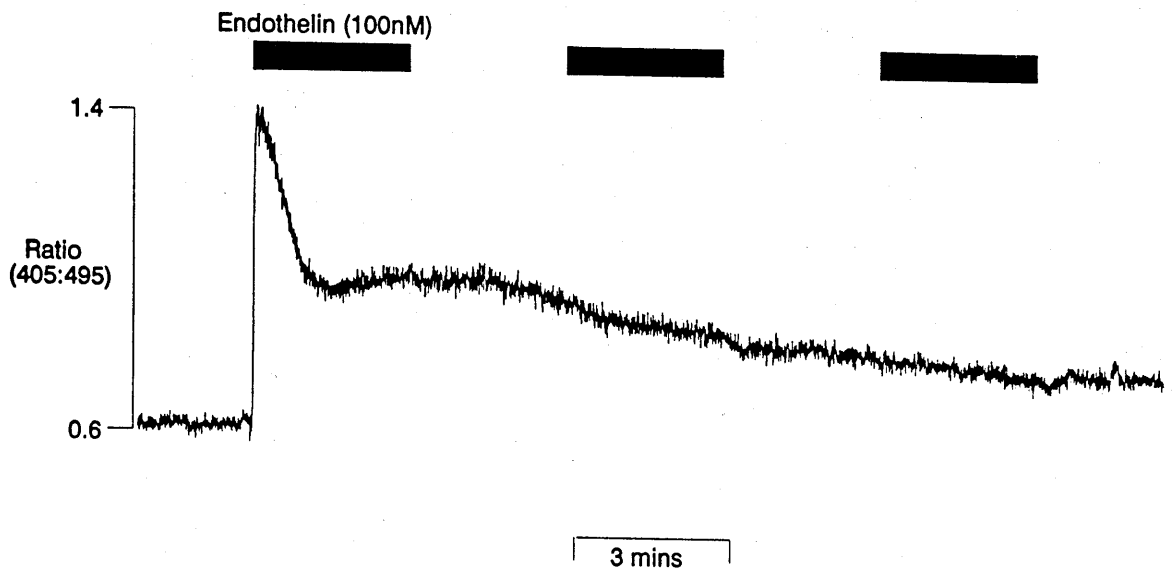

20000
15000
10000
5000
0
control
+PTox
d.p.m. in inositol phosphates
Control
ET-1
LPA

Fig. 4.6 Effect of pertussis toxin treatment on ET-1- and LPA-stimulated [^{3}H] inositol phosphates accumulation.

Rat-1 cells were labelled for 48h with 1μCi/ml [^{3}H] inositol and for the final 18-20h 100ng/ml pertussis toxin was included. Stimulation was carried out with 100nM ET-1 or 100μM LPA for 20min and [^{3}H] inositol phosphates were assayed as outlined in section 2.6.2. The results are expressed as means ± S.D. from a typical experiment where n=5. Basal values were (d.p.m.) : - PTox,4852 ± 480; +PTox, 5508 ± 676.


Endothelin (100nM)
1.4
Ratio
(405:495)
0.6
3 mins

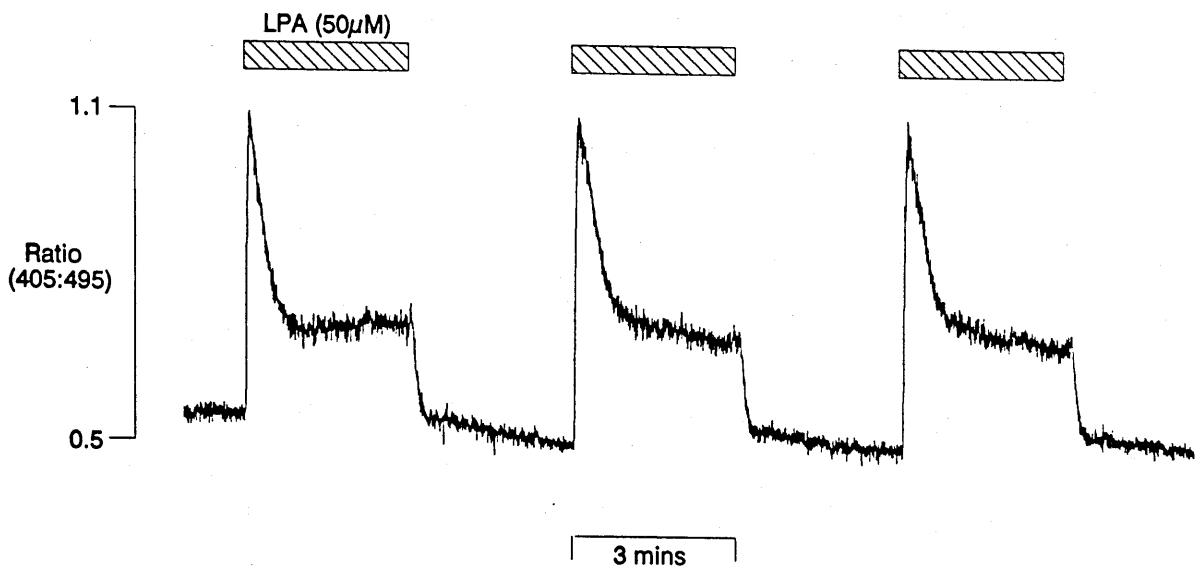
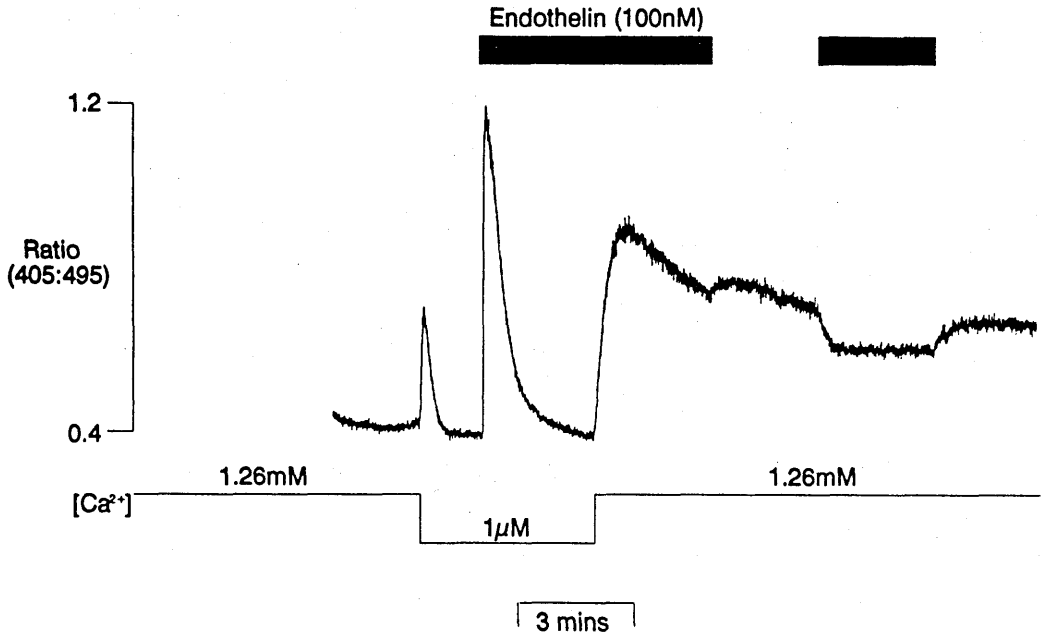

LPA (50μM)
1.1
Ratio
(405:495)
0.5
3 mins

Fig. 4.7 Time course of ET-1- and LPA-stimulated changes in intracellular Ca^{2+} levels.

Rat-1 cells were grown to confluency on glass coverslips and loaded with 5μM Indo-1 AM for 20min at room temperature. The cells were then perfused with HBG containing agonist (a)100nM ET-1 or (b)100μM LPA, and the emitted fluorescence measured as described in section 2.13. The results are expressed as the ratio of the light emitted at 405nM and 495nM from a typical experiment where n=3.

Shaded boxes indicate times when cells were exposed to agonist; during the remaining time cells were washed with buffer.


Endothelin (100nM)
1.2
Ratio
(405:495)
0.4
1.26mM
1.26mM
[Ca²⁺]
1μM
3 mins

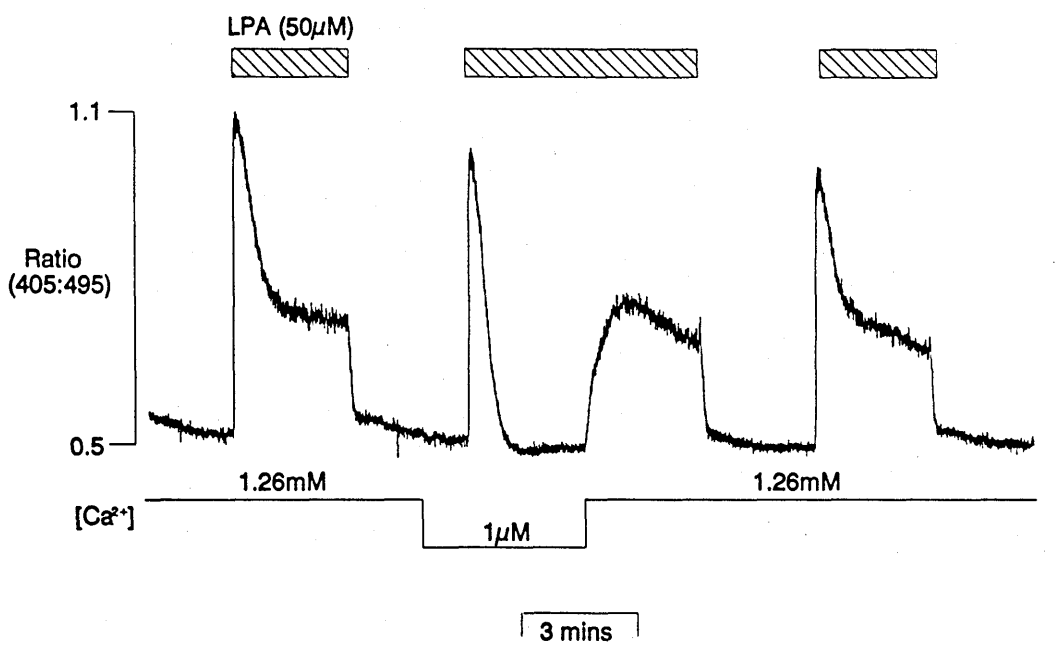
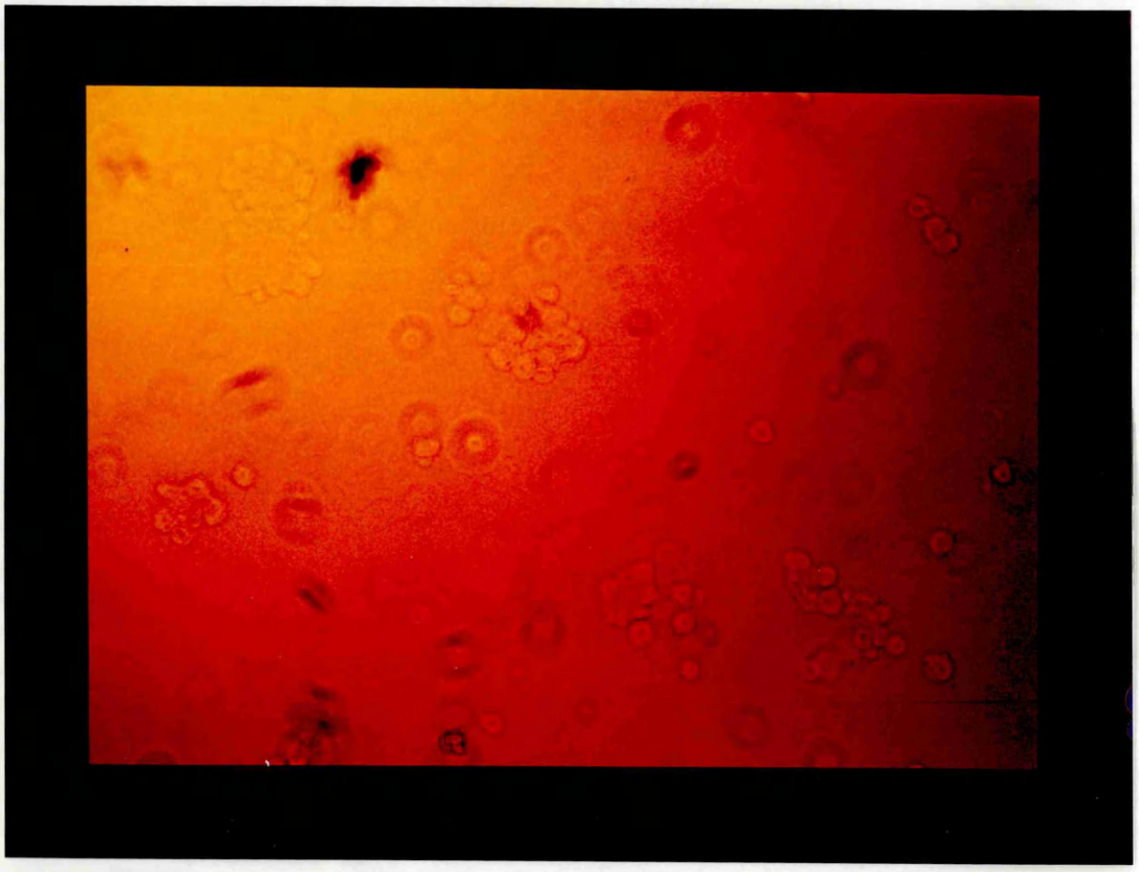

LPA (50μM)
1.1
Ratio
(405:495)
0.5
1.26mM
1.26mM
[Ca²⁺]
1μM
3 mins

Fig. 4.8 Contribution of release of intracellularly stored Ca^{2+} and the influx of extracellular Ca^{2+} to the ET-1- and LPA-induced changes in intracellular free Ca^{2+} levels.

Cells were grown to confluency on glass coverslips and loaded with 5μM Indo-1 AM for 20min at room temperature. The cells were perfused with (a) 100nM ET-1 or (b) 100μM LPA in normal HBG (1.26mM Ca^{2+}) or low Ca^{2+} HBG containing 0.33mM EGTA (1μM free Ca^{2+}) and the emitted fluoresence measured as described in section 2.13. The results are expressed as the ratio of the light emitted at 405nM and 495nM from a typical experiment where n=3. Shaded boxes indicate times when the cells were exposed to agonist; during the remaining time cells were washed with buffer.



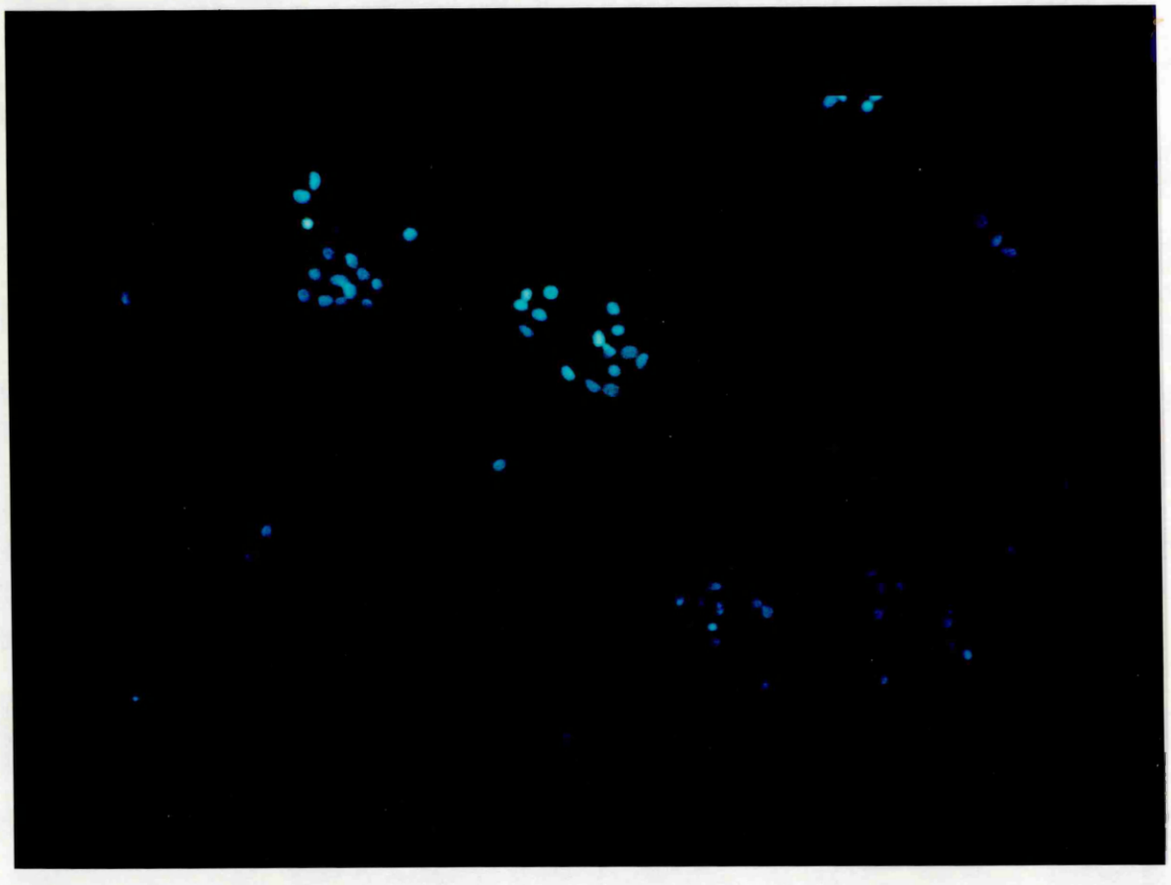
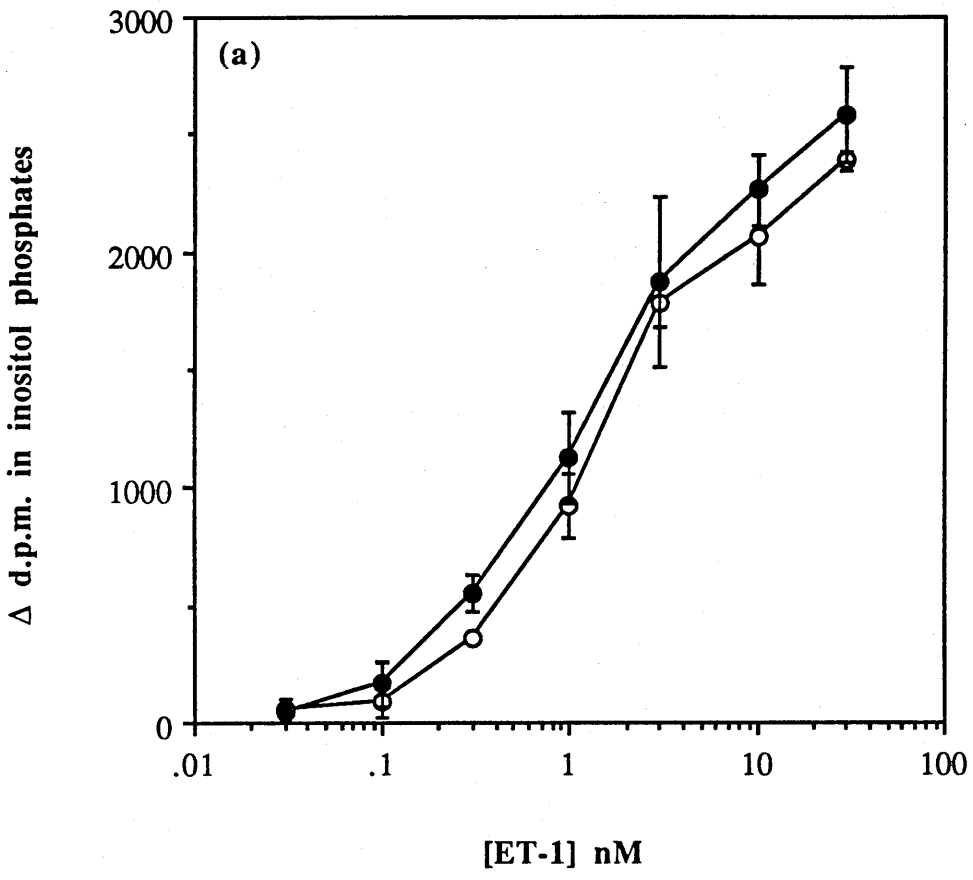


Fig. 4.9 Permeabilization of Rat-1 cells by electroporation.

Rat-1 cells in suspension were exposed to six discharges at 1sec intervals of a 3μF capacitor with a field strength of 2 kV/cm. Hoechst reagent (1mg/ml) was added to a sample of the cell suspension and the cells viewed under UV light for uptake of the dye. The photographs show permeabilized cells (a) under normal light, (b) under UV light.


(a)
3000
2000
1000
0
.01
.1
1
10
100
Δ d.p.m. in inositol phosphates
[ET-1] nM

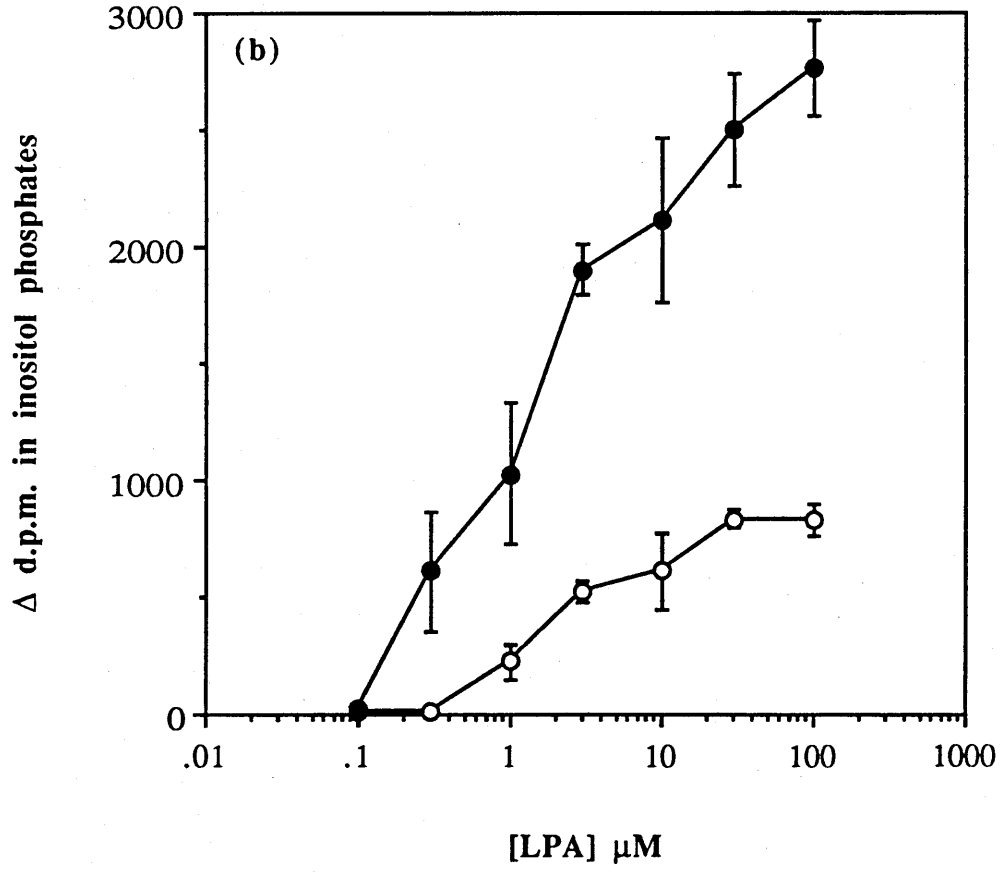
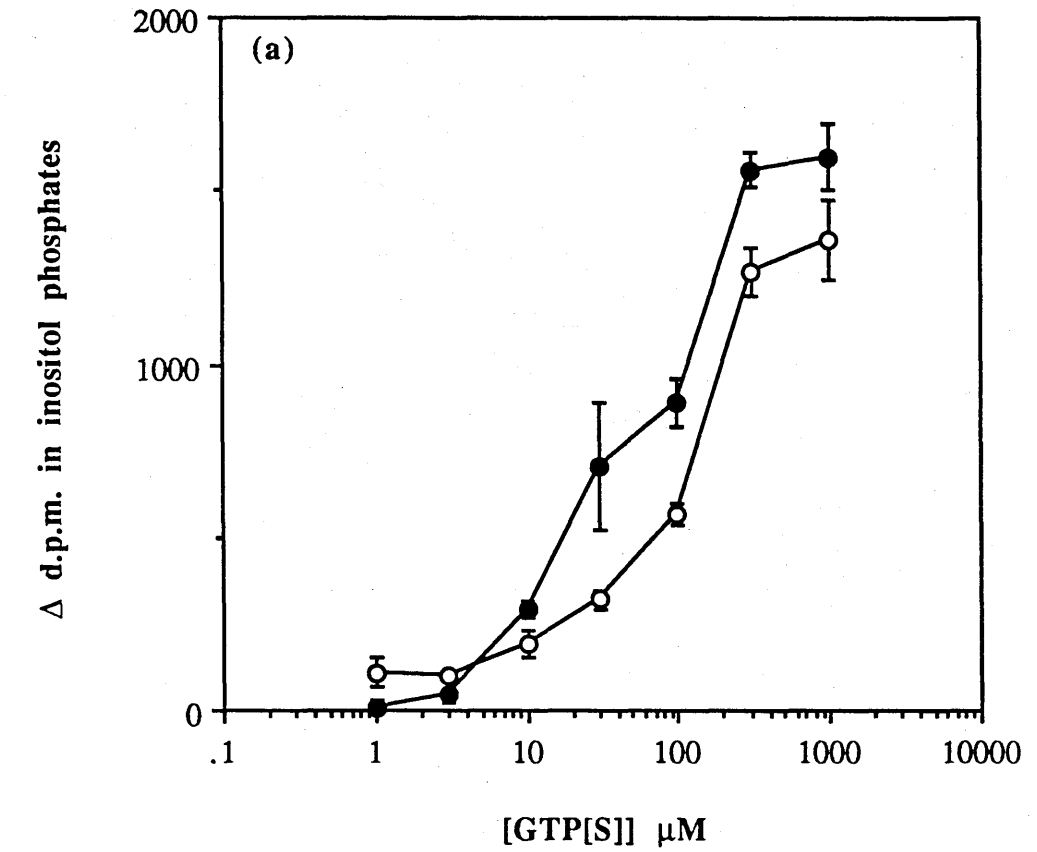

(b)
3000
2000
1000
0
.01
.1
1
10
100
1000
Δ d.p.m. in inositol phosphates
[LPA] μM

Fig. 4.10 Effect of GTP[S] on ET-1- and LPA-stimulated [^{3}H] inositol phosphates accumulation in permeabilized cells.

Rat-1 cells prelabelled for 48h with 1μCi/ml [^{3}H] inositol and then permeabilized were incubated with increasing concentrations of (a) ET-1 and (b) LPA in the presence (●) or absence (O) of 30μM GTP[S] for 5min. Results are expressed as the d.p.m. in [^{3}H] inositol phosphates due to agonist stimulation minus the basal d.p.m. Each point is the mean ± S.D. from triplicate determinations of a typical experiment where n=3-5. Basal values were (d.p.m.) (a) -GTP[S], 456 ± 13; +GTP[S], 734 ± 53; (b) -GTP[S], 1495 ± 83; +GTP[S], 2596 ± 143.


(a)
2000
1000
0
Δ d.p.m. in inositol phosphates
.1
1
10
100
1000
10000
[GTP[S]] μM

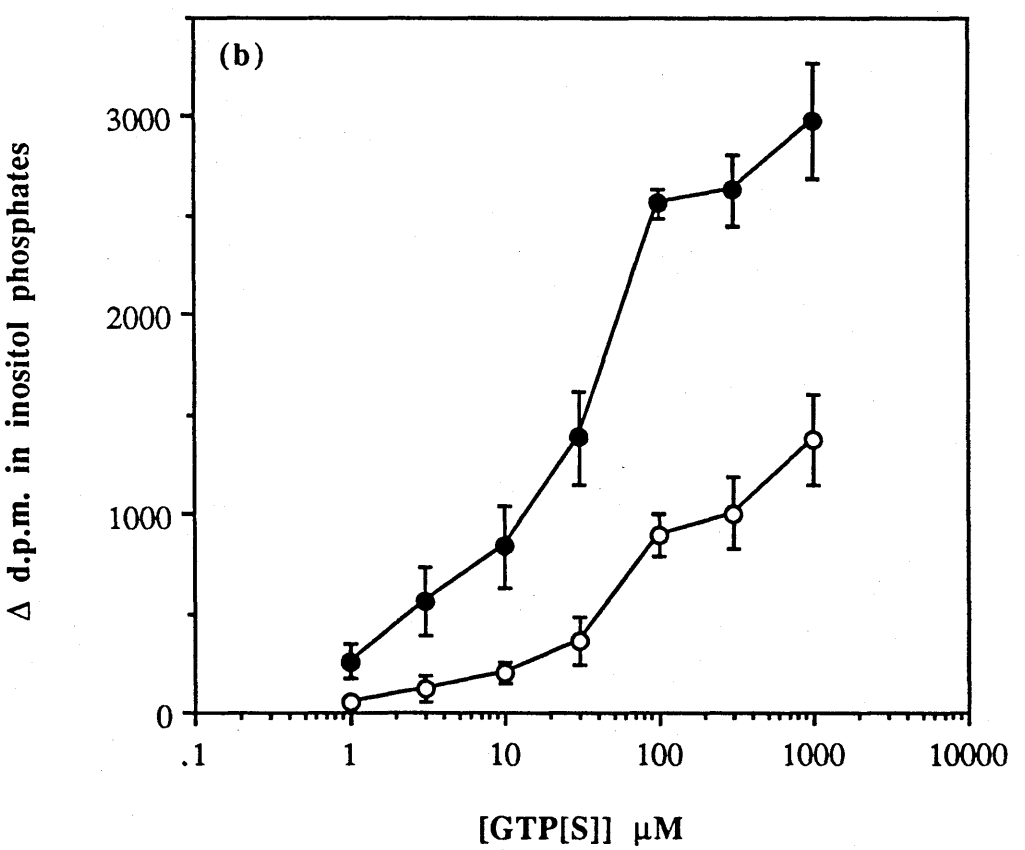

(b)
3000
2000
1000
0
Δ d.p.m. in inositol phosphates
.1
1
10
100
1000
10000
[GTP[S]] μM

Fig. 4.11 Effect of ET-1 and LPA on dose-dependent GTP[S]-stimulated [^{3}H] inositol phosphates accumulation in permeabilized cells.

Rat-1 cells prelabelled for 48h with 1μCi/ml [^{3}H] inositol and then permeabilized were incubated with increasing concentrations of GTP[S] in the presence (●) or absence (O) of (a) 0.3nM ET-1 or (b) 30μM LPA for 5min. Results are expressed as the d.p.m. in [^{3}H] inositol phosphates due to agonist stimulation minus the basal d.p.m. Each point is the mean ± S.D. of triplicate determinations from a typical experiment where n=3. Basal values were (d.p.m.) : (a) -ET-1, 473 ± 60; +ET-1, 1958 ± 48; (b) -LPA, 1312 ± 81; +LPA, 2187 ± 120.

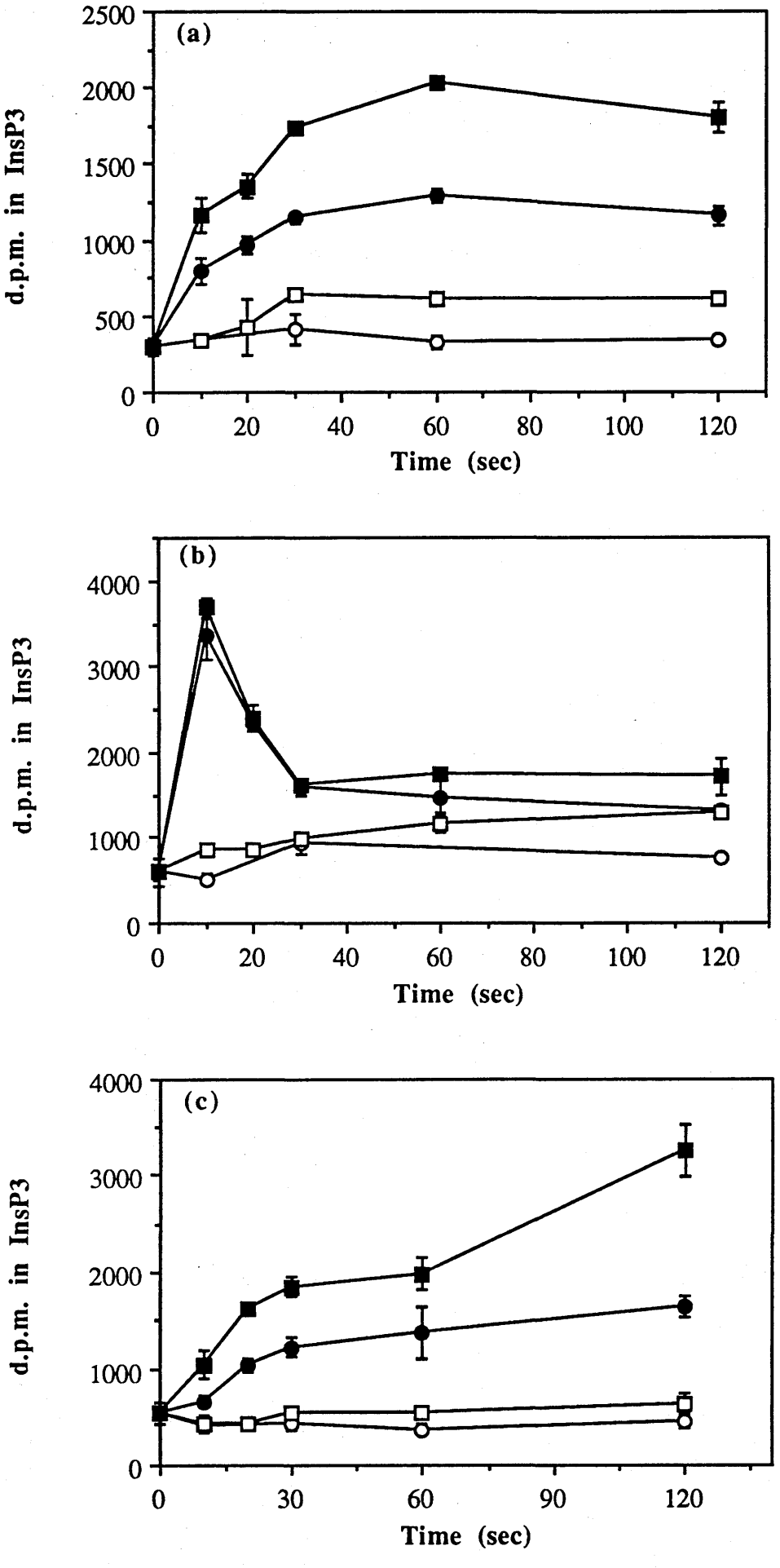
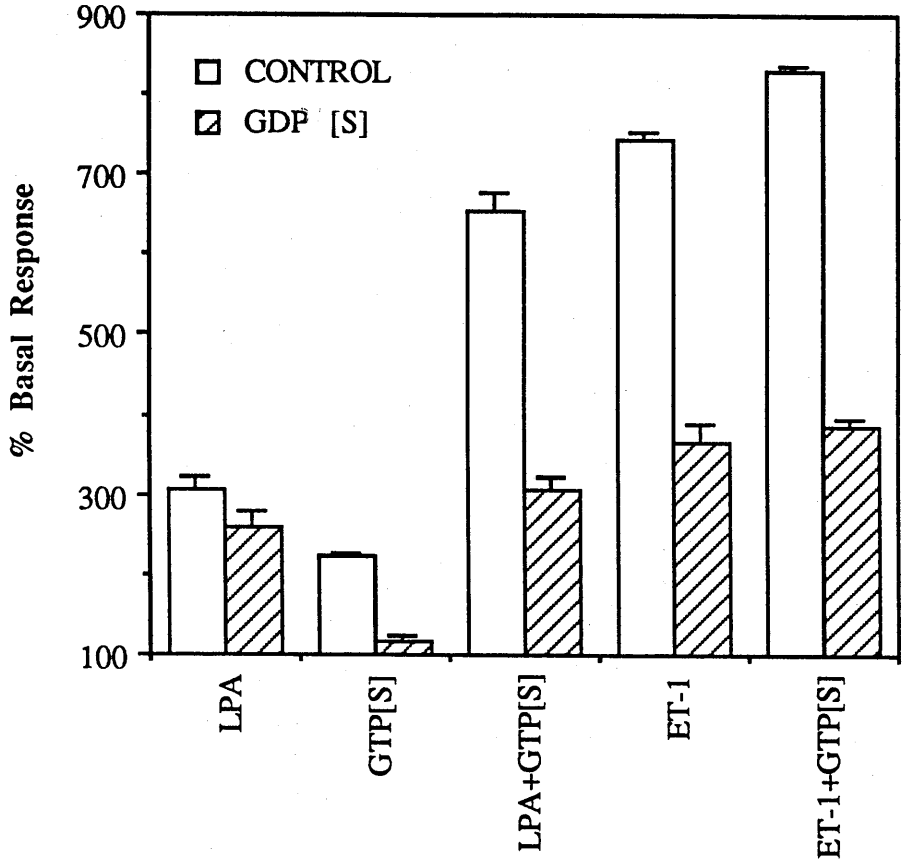

(a)
d.p.m. in InsP3
2500
2000
1500
1000
500
0
0
20
40
60
80
100
120
Time (sec)
(b)
d.p.m. in InsP3
4000
3000
2000
1000
0
0
20
40
60
80
100
120
Time (sec)
(c)
d.p.m. in InsP3
4000
3000
2000
1000
0
0
30
60
90
120
Time (sec)

Fig. 4.12 Kinetics of agonist- and GTP[S]-stimulated [^{3}H] $InsP_3$ formation in permeabilized cells.

Rat-1 cells prelabelled for 48h with 8μCi/ml [^{3}H] inositol and then electroporated were stimulated with (a) 30μM LPA, (b) 10nM ET-1 or (c) 1nM ET-1 in the presence or absence of 200μM GTP[S] for the indicated times. (O), vehicle; (❑), GTP[S]; (●), agonist; (■) agonist + GTP[S]. Results are expressed as means ± S.D. from a typical experiment where n=3.


900
700
500
300
100
% Basal Response
CONTROL
GDP [S]
LPA
GTP[S]
LPA+GTP[S]
ET-1
ET-1+GTP[S]

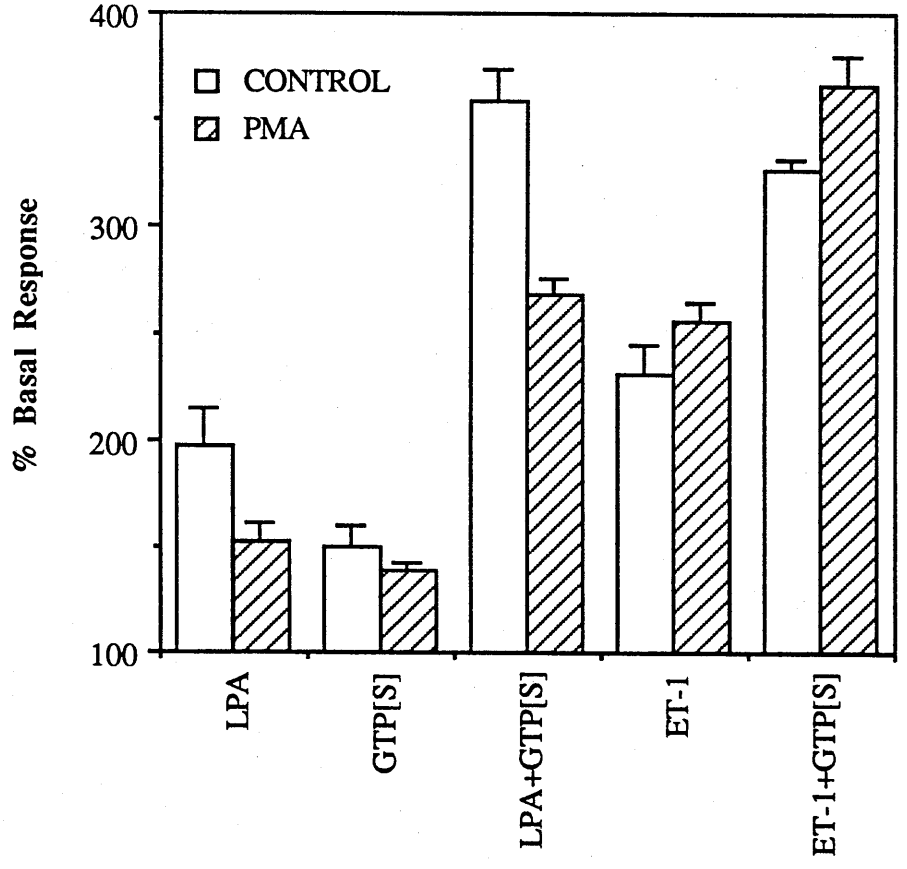

400
300
200
100
% Basal Response
CONTROL
PMA
LPA
GTP[S]
LPA+GTP[S]
ET-1
ET-1+GTP[S]

Fig. 4.13 Effect of GDP[S] on agonist- and GTP[S]-stimulated [^{3}H] inositol phosphates accumulation in permeabilized cells.

[^{3}H] Inositol-labelled cells (1μCi/ml for 48h) which had been electropermeabilized were preincubated with vehicle or 1mM GDP[S] for 5min prior to stimulation for 5min with LPA (30μM), ET-1 (0.3nM) and GTP[S] (30μM), alone and in combination. Basal values were (d.p.m.) : control, 1511 ± 93; +GDP[S], 717 ± 21. Results are expressed as the mean ± S.D. of triplicate determinations from a typical experiment where n=5.

Fig. 4.14 Effect of PMA on agonist- and GTP[S]-stimulated [^{3}H] inositol phosphates accumulation in permeabilized cells.

[^{3}H] Inositol-labelled cells (1μCi/ml for 48h) were preincubated for 15min with vehicle or 300nM PMA and then permeabilized by electroporation. Stimulation was carried out for 5min with 30μM LPA, 0.3nM ET-1 or 30μM GTP[S], alone and in combination. Basal values were (d.p.m.) : control, 629 ± 83; +PMA, 474 ± 14. Results are expressed as means ± S.D. from a typical experiment where n=3.

4.7. Discussion.

The previous chapter demonstrated that ET-1 could stimulate both PtdIns(4,5)P_2 and PtdCho hydrolysis; both these pathways generate DAG and thus could activate protein kinase C. Indeed, ET-1-stimulated DNA synthesis appears to be dependent on protein kinase C activity to some extent (Fig. 3.3; Muldoon *et al.*, 1990). In some systems a decrease in cAMP seems to be important for stimulating cell proliferation and this has been proposed to be the mechanism underlying the mitogenic response to LPA in Rat-1 cells based on the pertussis toxin-sensitivity of LPA-stimulated DNA synthesis and adenylyl cyclase activity (van Corven *et al.*, 1989). In contrast, the results presented here show that both LPA- and ET-1-stimulated [^{3}H] thymidine incorporation were pertussis toxin-insensitive arguing against a role for the inhibition of adenylyl cyclase in the mitogenic response to both these agonists. Although ET-1 has been shown to have no effect on adenylyl cyclase activity in Rat-1 cells, an inhibition was observed in response to LPA (F. McKenzie and E. Adie, unpublished observations). Since this suggested differences in the signalling pathways stimulated by ET-1 and LPA, the regulation of PtdIns(4,5)P_2 hydrolysis in response to both mitogens was investigated and compared in an attempt to gain further insight into the mechanism of ET-1-induced mitogensis in Rat-1 cells.

The accumulation of inositol phosphates in response to LPA in intact cells was considerably less than that observed with ET-1 with about a 2-3-fold increase above basal, in agreement with results reported elsewhere (van Corven *et al.*, 1992). The generation of Ins(1,4,5)P_3 mass was transient which was reflected in the transient decrease in PtdIns(4,5)P_2 mass (Fig.4.4). This was in contrast to the ET-1-stimulated response which consisted of an initial peak of Ins(1,4,5)P_3 generated independently of the external [Ca^{2+}] and a second sustained Ca^{2+}-dependent phase. Similar results have also been reported for carbachol-stimulated human neuroblastoma cells where the inclusion of EGTA

significantly reduced the plateau phase of a biphasic Ins(1,4,5)P_3 response (Lambert *et al.*, 1991). In fact, in the Rat-1 cells, re-addition of Ca^{2+} (1.26mM) once stimulated levels had returned to basal could elicit another phase of Ins(1,4,5)P_3 generation (Fig.4.3). Thus, ET-1 appears to activate an additional Ca^{2+}-dependent pathway of Ins(1,4,5)P_3 generation compared with LPA. The sustained nature of the Ins(1,4,5)P_3 response was reflected in the sustained hydrolysis of PtdIns(4,5)P_2. Although these results suggest that ET-1 and LPA stimulate the hydrolysis of PtdIns(4,5)P_2 to different extents, it is important to realise that mass measurements do not necessarily reflect the flux through the pathway. Indeed, at any time the mass of PtdIns(4,5)P_2 and Ins(1,4,5)P_3 reflect both the rate of synthesis and degradation. Although LPA-stimulated levels appear to return to control values this may simply reflect a steady state in which rates of formation and degration are balanced. However, the results do imply differences in the regulation of PtdIns(4,5)P_2 hydrolysis by LPA and ET-1.

The generation of Ins(1,4,5)P_3 almost certainly results in the release of Ca^{2+} from intracellular stores and therefore changes in intracellular Ca^{2+} levels in response to both LPA and ET-1 were compared in Indo-1-loaded cells. Both agonists stimulated a biphasic increase in intracellular [Ca^{2+}] reflecting initial mobilization from intracellular stores and subsequent entry of external Ca^{2+}. Similar patterns of Ca^{2+} mobilization in response to ET-1-stimulation have been observed in other systems including fura-2-loaded rat mesangial cells (Simonson and Dunn, 1991b) and Swiss 3T3 cells (Takuwa *et al.*, 1989). Chan and Greenberg (1991) used the inhibitor SK&F 96365 to provide evidence of receptor-mediated Ca^{2+} entry in response to ET-1 in NG108-15 cells while Mn^{2+}-quenching of Indo-1 fluorescence was taken as evidence of Ca^{2+} entry in LPA-stimulated human fibroblasts (Jalink *et al.*, 1990). Although both LPA and ET-1 stimulated a sustained phase of Ca^{2+} increase in Rat-1 cells, the LPA response could be washed out while the ET-1 response remained high. In

addition, the LPA-mediated Ca^{2+} mobilization was not desensitized and further challenges with the agonist could elicit an additional response. This is unlike the LPA-stimulated increase in Ca^{2+} in human fibroblasts which showed homologous desensitization and may therefore involve different regulatory pathways or a different receptor (Jalink *et al.*, 1990). The ET-1-stimulated response in Rat-1 cells is, however, insensitive to further challenges with the peptide in agreement with the homologous desensitization of Ca^{2+} mobilization reported for ET-1 in Swiss 3T3 cells (Fabregat and Rozengurt, 1990). The relationship between the second phase of ET-1-stimulated $Ins(1,4,5)P_3$ generation and the second phase of the Ca^{2+} increase is however unclear. Both ET-1 and LPA stimulated biphasic Ca^{2+} responses which were reduced to a single transient phase in the presence of EGTA, while re-addition of a normal (1.26mM) external [Ca^{2+}] could elicit a second phase. Clearly, however, only ET-1 stimulated a sustained, Ca^{2+}-dependent phase of $Ins(1,4,5)P_3$ generation. This, therefore, suggests that the second phase of Ca^{2+} mobilization is not dependent on $Ins(1,4,5)P_3$ generation. In addition, since both agonists stimulated Ca^{2+} entry this explanation is not sufficient to account for the sustained phase of $Ins(1,4,5)P_3$ in response to ET-1 stimulation and suggests the involvement of an additional component. The two phases of $Ins(1,4,5)P_3$ generation may reflect activation of different PtdIns-PLCs. The pertussis toxin-insensitive G protein, G_q, has been shown to activate phospholipase $C\beta_1$ while phospholipase $C\gamma$ has been shown to be activated by tyrosine phosphorylation (section 1.4.1). The possible role of phospholipase $C\gamma$ in the secondary phase of $Ins(1,4,5)P_3$ generation is discussed further in chapter 6. The inability to wash out the ET-1-stimulated Ca^{2+} response may be due to the tight association between the peptide and its receptor and will be discussed further in chapter 5.

Comparison of ET-1- and LPA-stimulated responses in intact cells therefore clearly showed differences between the effects of these two mitogens. Sustained $Ins(1,4,5)P_3$ formation is not usually associated with peptide

receptors linked to the inositol phosphates pathway but rather is rapidly desensitized as with the LPA response. Thus, in bombesin-stimulated Swiss 3T3 cells Ins(1,4,5)P_3 generation had returned to basal by 30s (Cook *et al.*, 1990) while in NIH3T3 and NG108-15 cells the bradykinin-stimulated increase in Ins(1,4,5)P_3 mass was also transient (Fu *et al.*, 1988). Similarly, in fMet-Leu-Phe-stimulated neutrophils Ins(1,4,5)P_3 levels determined by both HPLC and competition binding studies had returned to basal by 2min (Bradford and Rubin, 1986). In contrast, in adrenal glomerulosa cells angiotensin II stimulated a biphasic increase in [^{3}H] Ins(1,4,5)P_3 generation and the secondary increase was sustained for up to 30min (Balla *et al.*, 1988). It is not possible to compare the action of ET-1 in Rat-1 cells with that in other systems since previous studies measured the kinetics of labelled InsP_3 generation without separating the different isomers. Since the sustained phase of Ins(1,4,5)P_3 does not appear to be required for Ca^{2+} mobilization this therefore raises the question of why it is generated. It may simply reflect sustained PtdIns(4,5)P_2 hydrolysis where DAG is the important mediator produced. Alternatively, it may be further metabolized to Ins(1,3,4,5)P_4 which may then have a second messenger role; for example in regulating Ca^{2+} influx as has been suggested in other systems (see section 1.4.2). Another characteristic of the ET-1 response which distinguished it from other agonists which act through G protein-coupled receptors was the insensitivity of inositol phosphates accumulation to a short PMA pretreatment. This appears to be a feature of the response in Rat-1 cells since ET-1-stimulated inositol phosphates formation in other systems has been reported to be inhibited by PMA pretreatments of 10-15min with inhibition of up to 80% (Reynolds *et al.*, 1989; Araki *et al.*, 1989; Galron *et al.*, 1990; Resink *et al.*, 1990). In contrast, preincubation with PMA markedly decreased the subsequent LPA-stimulated inositol phosphates response in a manner characteristic for an agonist acting through a receptor linked to PtdIns-PLC via a G protein. It is worth mentioning again that the site of protein kinase C-mediated inhibition varies

among cell types and agonists used (section 4.1). However, G protein-receptor kinases have been identified involved in the desensitization of adenylyl cyclase responses, e.g. βARK (reviewed in Palczewski and Benovic, 1991), and it is possible that similar enzymes may exist involved in desensitizing the inositol lipid pathway.

The receptor-G protein-effector interaction for both ET-1 and LPA was further investigated in permeabilized Rat-1 cells in combination with guanine nucleotide analogues. In permeabilized cells the characteristics of LPA-stimulated inositol phosphates formation in the presence of guanine nucleotides agreed with the criteria expected for an agonist acting through a G protein-coupled receptor. LPA-stimulated inositol phosphates accumulation was enhanced by GTP[S] in agreement with the findings of van Corven *et al*. (1989). The results presented here also demonstrate that LPA enhanced the GTP[S] response and that the presence of LPA decreased the EC_{50} value for GTP[S]-stimulated inositol phosphates accumulation. Similar results have also been reported in other systems where G protein interactions have been suggested, for example histamine- and carbachol-stimulated rat brain cortical membranes and $P_{2\gamma}$ purinergic receptor activation in turkey erythrocytes (Claro *et al*., 1989; Boyer *et al*, 1989b). In addition, kinetic studies demonstrated that LPA decreased the lag time for the onset of the GTP[S] response suggesting that LPA accelerates GTP/GDP exchange, an essential criterion for defining the involvement of a G protein in coupling a receptor to a second messenger pathway (Boyer *et al*, 1989b). Consistent with this finding GDP[S], which maintains the G protein in an inactive state, inhibited the potentiation of the LPA response by GTP[S] although it had little effect on the LPA response alone. The PMA inhibition studies carried out in intact cells were extended to permeabilized cells with similar results; the response to LPA in the presence or absence of GTP[S] was inhibited by a similar amount. The site of protein kinase C-mediated inhibition appeared to be upstream of the G protein-PtdIns-PLC

interaction since the response to GTP[S] alone was unaffected by PMA pretreatment. Although lack of a suitable labelled ligand prevented any investigation of the effect of protein kinase C on agonist affinity, modifications of the receptor or the G protein may be the site of protein kinase C-mediated inhibition causing impaired coupling without affecting the function of either receptor or G protein. Similar results have also been reported in other systems including bombesin-stimulated permeabilized Swiss 3T3 cells (Plevin *et al .*, 1990) and angiotensin II-stimulated rat mesangial cell membranes (Pfeilschifter and Bauer, 1987). As yet a receptor for LPA has not been identified. However, the results presented here demonstrating the rapid and transient nature of LPA-stimulated Ins(1,4,5)P_3 generation and the effect of guanine nucleotide analogues on second messenger formation are consistent with the existence of a specific LPA receptor which interacts with PtdIns-PLC via a G protein.

As with intact cells, ET-1-stimulated responses in permeabilized cells showed a number of differences compared with LPA and did not conform to all the criteria expected for an agonist binding to a receptor linked to a G protein. GTP[S] did not potentiate the ET-1-stimulated accumulation of inositol phosphates and the peptide had little effect on the nucleotide response. This is in contrast with reports of ET-1 responses in other systems. In A10 smooth muscle cell membranes and permeabilized rat mesangial cells GTP[S] potentiated ET-1-stimulated inositol phosphates formation (Takuwa *et al .*, 1990; Thomas *et al .*, 1991). However, in the Rat-1 cells GDP[S] substantially inhibited the response to ET-1 alone and in combination with GTP[S]. The different effects seen with GDP[S] on ET-1- and LPA-stimulated inositol phosphates accumulation may reflect differences in the ability of these agonists to stimulate guanine nucleotide exchange. Since GDP[S] inhibits both responses in the absence of GTP[S], this suggests that residual GTP remaining after permeabilization can support a response stimulated by both LPA and ET-1. However, the greater inhibition seen with ET-1 may be due to the greater

efficacy of ET-1 to stimulate guanine nucleotide exchange. Measurements of GTPase activity might give some indication of the relative abilities of ET-1 and LPA to stimulate GTP/GDP exchange. Despite the inability of a maximum concentration of ET-1 to accelerate GTP/GDP exchange as measured by the kinetics of agonist- and nucleotide-stimulated $InsP_3$ generation, a submaximum concentration of the peptide did reduce the lag time for the onset of the nucleotide response. This is probably because there is a limit to the extent to which agonists can stimulate polyphosphoinositide turnover governed by the activity of PtdIns-PLC and the availability of substrate. Since the efficacy of ET-1 to stimulate inositol phosphates accumulation is greater than LPA, a maximum concentration of ET-1 may already reach this limit and therefore an effect with GTP[S] can only be detected with a submaximum concentration. Taken together these results suggest a G protein involvement in the ET-1-stimulated response but the nature of the receptor-G protein interaction for the peptide may be different from the accepted model. This is confirmed by the lack of effect of PMA in permeabilized cells as well as in intact cells.

Due to the lack of effect of GTP[S] on the ET-1 response in electropermeabilized cells, similar experiments were performed with cells permeabilized with streptolysin-O in case electroporation hadn't allowed for full equilibration of nucleotide pools within the cells. LPA could stimulate inositol phosphates accumulation in the absence of added GTP[S] suggesting that there was enough residual GTP following electroporation to sustain a response. However, in streptolysin-O-permeabilized cells essentially the same results were obtained but the potentiation of the LPA response was not as great as that observed in electropermeabilized cells suggesting that the latter method allowed better equilibration of nucleotide pools. Indeed, the LPA-stimulated response was generally greater in toxin-permeabilized cells compared with electroporated cells supporting this hypothesis.

Despite possible artifacts with the permeabilization method the results still suggest differences in the G protein input required for LPA and ET-1 responses. The ET-1-stimulated hydrolysis of PtdIns(4,5)P_2 may involve two mechanisms only one of which requires a receptor-G protein interaction. This could explain why an effect with GTP[S] was only observed when [^{3}H] InsP_3 generation was measured at early time points (as against total inositol phosphates acumulation after 5min) in response to a submaximum concentration of ET-1; a possible candidate responsible for Ins(1,4,5)P_3 generated at later times could be phospholipase Cγ which is activated by tyrosine phosphorylation. The proposal that there are two pathways of PtdIns(4,5)P_2 hydrolysis stimulated by ET-1 is supported by the Ca^{2+} sensitivity of the second phase of Ins(1,4,5)P_3 generation. As discussed previously the simple model of Ca^{2+} influx stimulating PtdIns(4,5)P_2 breakdown is inadequate since LPA also stimulates Ca^{2+} entry. The nature of receptor-G protein coupling for ET-1 and LPA also appears to be different due to the different effects obtained with PMA in both intact and permeabilized cells. The inability of PMA to inhibit the ET-1-stimulated response may suggest that the ET-1 receptor exists in a precoupled state with the G protein and hence the site for protein kinase C-mediated inhibition is masked. Alternatively, if ET-1-stimulated Ins(1,4,5)P_3 generation involves two pathways then it is possible that only the earlier pathway is PMA-sensitive so that when PMA-sensitivity is investigated at later times the inhibitory effect of PMA is no longer detected. Finally, ET-1 may interact with the G protein itself in a manner similar to that described for certain venoms, toxins and neuropeptides such as mastoparan and bradykinin (Mousli *et al* ., 1990).

Although the nature of the interaction of the ET-1 receptor with the inositol phosphate signalling pathway remains unclear, the unusual characteristics of the ET-1-stimulated response may be significant in relation to its physiological effect. The novel receptor-G protein interaction together with a second pathway may underlie the sustained PtdIns(4,5)P_2 hydrolysis in

response to ET-1. This clearly has important implications for the level of DAG within the cell and hence activity of protein kinase C which may in turn be important for the mitogenic respone to the peptide.

Chapter 5

Characterization of binding sites for [^{125}I] ET-1 on Rat-1 cell membranes.

5.1 Introduction.

The distribution of ET receptors has been mapped using radiolabelled ETs and autoradiography, and saturable, specific binding sites for [^{125}I] ETs have been identified in numerous tissues including lung, kidney, heart, intestine, adrenal gland, eye and nervous system (Koseki *et al.*, 1989; reviewed in Simonson and Dunn, 1990a). Studies *in vitro* using intact cells and membrane preparations have also characterized specific, high affinity and saturable binding which was not displaced by other vasoactive peptides or Ca^{2+} channel antagonists suggesting the presence of a specific ET receptor(s) (Kanse *et al.*, 1989; Sugiura *et al.*, 1989; Badr *et al.*, 1989). ET-1 had originally been suggested to be an endogenous agonist for L-type Ca^{2+} channels due to its sequence similarity with biological toxins that are known to interact with ion channels and the ability of the L-channel blocker, dihydropyridine, to inhibit ET-1-induced contraction (Yangisawa *et al.*, 1988a), but this is now clearly not the case. In general, the dissociation of bound [^{125}I] ETs was extremely slow suggesting a tight association between agonist and receptor (Hirata *et al.*, 1988; Kanse *et al.*, 1989; Serradeil-Le Gal *et al.*, 1991). Competition binding studies using a variety of ET and sarafotoxin (SS6) analogs in different tissues or cell types suggested the existence of multiple ET receptors with different affinities for the ET isopeptides (Kloog *et al.*, 1989; Watanabe *et al.*, 1989; Martin *et al.*, 1990b). Cross-linking studies also supported the existence of multiple receptor subtypes and proteins with a range of molecular weights have been reported: 73kD and 60kD (Martin *et al.*, 1990b); 58kD and 34kD in rat mesangial cells (Sugiura *et al.*, 1989); 53kD, 46kD and 34kD in chick cardiac membranes (Watanabe *et al.*, 1989); 34kD and 52kD in bovine lung plasma membranes (Hagiwara *et al.*, 1990); 53kd and 38kD in rat brain membranes (Ambar *et al.*, 1990). The ET binding sites/receptors characterized can be divided into three groups ; those that bind ET-1 and ET-2 with higher affinity than ET-3, those

that bind ET-1, ET-2 and ET-3 with equal affinity and those that show highest affinity for ET-3.

Most recently the cloning and sequencing of two different ET receptors termed ET_A and ET_B has been reported (Arai *et al.*, 1990; Sekurai *et al.*, 1990; reviewed in Sekurai *et al.*, 1992). The ET_A receptor (M_r = 48.5kD) was cloned from bovine lung and showed highest selectivity to ET-1 in COS cells transfected with the cloned cDNA while the ET_B receptor (M_r = 46.9kD) cloned from rat lung encoded a non-isopeptide-selective receptor subtype with equal affinity for ET-1, ET-2 and ET-3. Interestingly, the ET_A receptor mRNA was detected in vascular smooth muscle while the ET_B receptor was not. Both receptors contained seven membrane-spanning domains similar to other G protein-coupled receptors. Both receptors also contained potential N-glycosylation sites within the N-terminal region and serine residues in the third cytoplasmic loop and cytoplasmic C-terminal tail which may be phosphorylated by serine/threonine kinases and therefore are potential target sites for receptor regulation. The cloning of ET receptors from other sources has now also been reported including the ET_B receptor from human liver and jejunum (Nakamuta *et al.*, 1991; Sakamoto *et al.*, 1991), the ET_A and ET_B receptors from human placenta (Cyr *et al.*, 1991; Ogawa *et al.*, 1991; Hosada *et al.*, 1991; Adachi *et al.*, 1991), the ET_A receptor from a rat heart cell line (Lin *et al.*, 1991) and the ET_B receptor from bovine lung (Saito *et al.*, 1991). The ET_B receptor has also been purified from bovine lung (Kozuka *et al.*, 1991). There is a high degree of homology between the receptor subtypes cloned from the different species.

ET-1 was shown to stimulate PtdIns(4,5)P_2 hydrolysis in Rat-1 cells but the nature of the interaction of the ET-1 receptor with the inositol lipid signalling pathway appeared to be different to other receptors which couple to PtdIns-PLC via a G protein (Chapter 4). A preliminary characterization of the ET-1 receptor on Rat-1 cells was therefore carried out by examining the

binding of [^{125}I] ET-1 to membranes from these cells in an attempt to further clarify the unusual characteristics of the ET-1-stimulated inositol phosphates response.

5.2 Time course of [^{125}I] ET-1 binding to Rat-1 cell membranes

At 25° C specific binding of [^{125}I] ET-1 to membranes from Rat-1 cells increased in a time-dependent manner and reached equilibrium after 15-20min (Fig. 5.1). Equilibrium binding experiments were therefore carried out for 30min at 25° C. Binding increased linearly with protein concentration up to 30-40μg of membrane protein (Fig. 5.2) and 25μg of membrane protein was therefore used in subsequent experiments. The total radioactivity bound was ~10% of the amount added and nonspecific binding was ~ 30% of total binding. There was no difference in binding whether fresh or freeze-thawed membranes were used or whether membranes were prepared from scraped or trypsinized cells.

5.3 Saturation binding of [^{125}I] ET-1 to membranes.

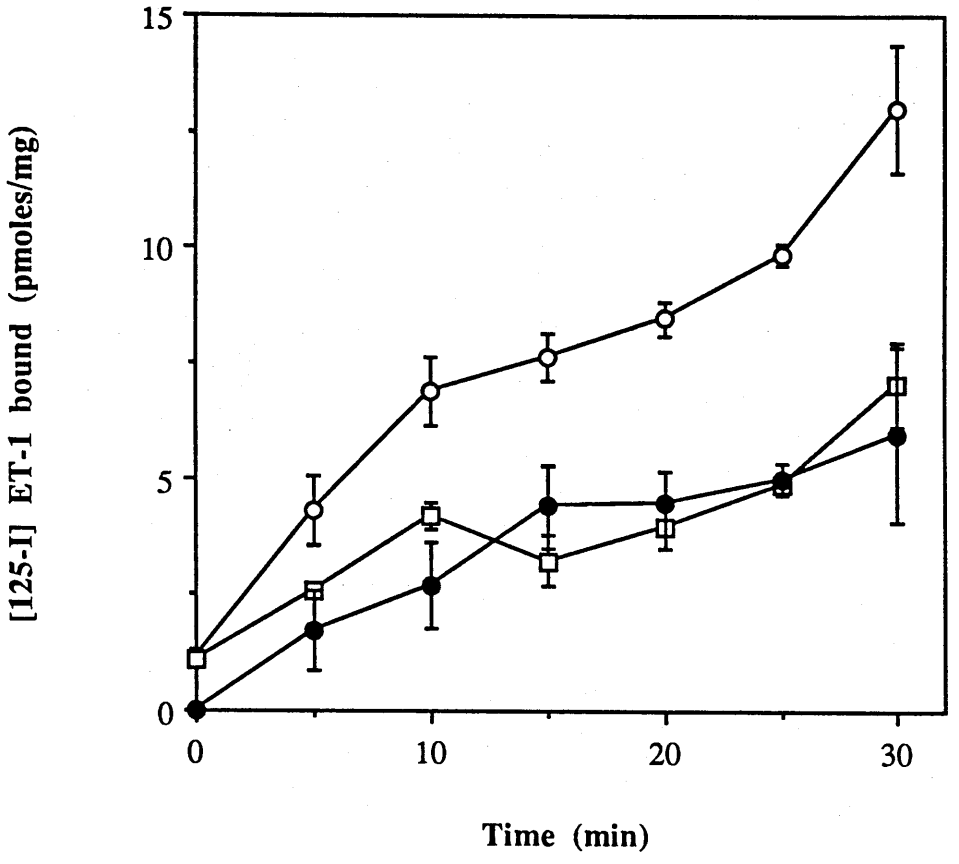
Binding of [^{125}I] ET-1 to Rat-1 cell membranes under equilibrium conditions was saturable and showed high affinity (Fig. 5.3). When the data was fitted to a rectangular hyperbola ($y = A\,x / B + x$ where A = Bmax and B = K_D) by computer-assisted non-linear regression analysis the Bmax was found to be 3.7 ± 1.1 pmoles bound ET-1 / mg of protein and the K_D value was 2.7 ± 1.3 nM (Fig. 5.3a). Scatchard analysis (Fig. 5.3b) showed that there was a single class of binding sites and yielded similar apparent Bmax (4.0 ± 1.2 pmoles ET-1 bound / mg of protein, n=4) and K_D (3.5 ± 0.7 nM) values as were obtained from curve fitting. The latter method is however more accurate for calculating saturation binding parameters as scatchard

analysis involves manipulation of the data such that there are errors in both the x- and y-axis directions.

Dissociation of bound ligand was determined by addition of 1μM unlabelled ET-1 once equilibrium binding had been reached and was found to be minimal (Fig. 5.4). Only 10-20% of bound [^{125}I] ET-1 had dissociated up to 2h after addition of excess unlabelled ET-1. Guanine nucleotide analogs had no effect on binding (Fig. 5.5). When membranes were preincubated for 10min with increasing concentrations of GTP[S] or GDP[S] prior to addition of [^{125}I] ET-1, binding was the same as in control membranes. When lower concentrations of ET-1 (1nM) or shorter incubation times (1, 2 and 5min) were used GTP[S] still had no effect.

5.4 Competition binding with ET isopeptides.

The ability of ET-1, ET-2 and ET-3 to displace [^{125}I] ET-1 binding was investigated by including increasing concentrations of each isopeptide in binding assays containing 2nM (~ K_D value) [^{125}I] ET-1 (Fig. 5.6). Each isopeptide competed with labelled ET-1 for binding and yielded IC_{50} values as follows : ET-1, 4.2 ± 3.1 nM; ET-2, 5.7 ± 3.1 nM; ET-3, 112 ± 60 nM. The corresponding K_i values, calculated from the Cheng and Prusoff equation $K_i = IC_{50} / 1 + ([ligand]/ K_D)$, were : ET-1, 2.4 ± 1.8 nM; ET-2, 3.4 ± 1.9 nM; ET-3, 58 ± 28 nM thus giving a rank order of potency for displacing [^{125}I] ET-1 binding of ET-1 ~ ET-2 > ET-3. The competition curves had slopes of approximately -1 which is consistent with [^{125}I] ET-1 and the competing unlabelled ET isopeptides interacting with a single receptor population by a reversible bimolecular reaction which obeys mass action law.


15
10
5
0
0
10
20
30
[125-I] ET-1 bound (pmoles/mg)
Time (min)

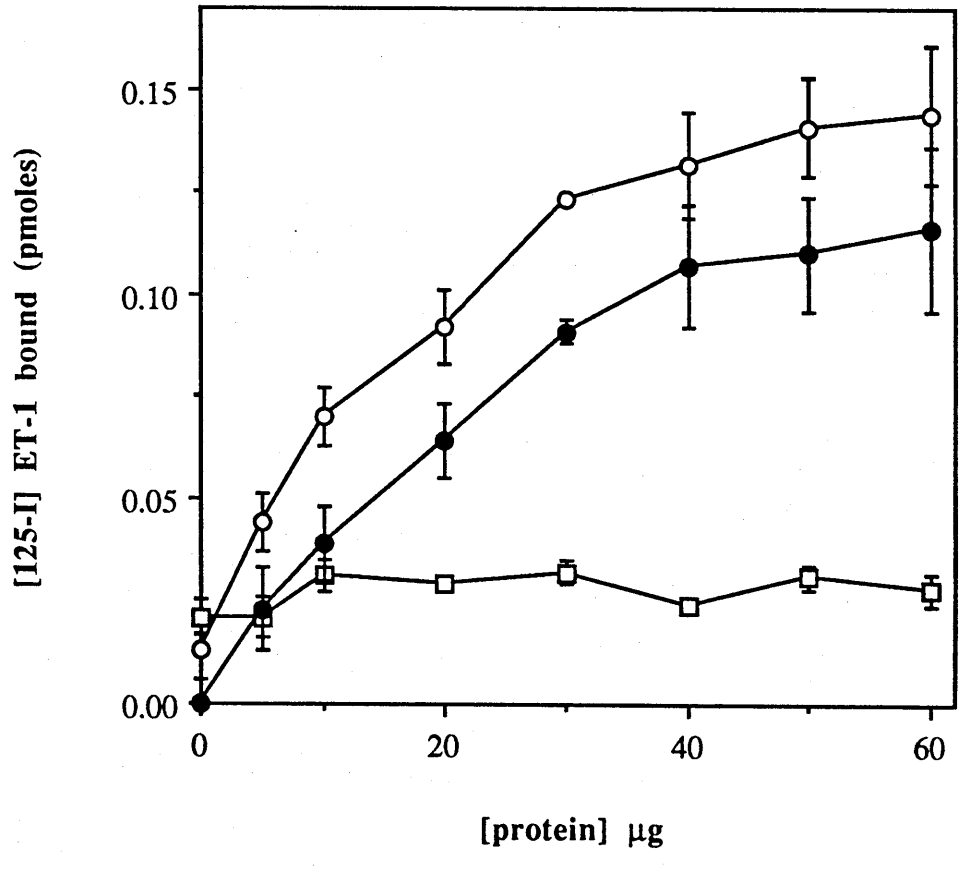
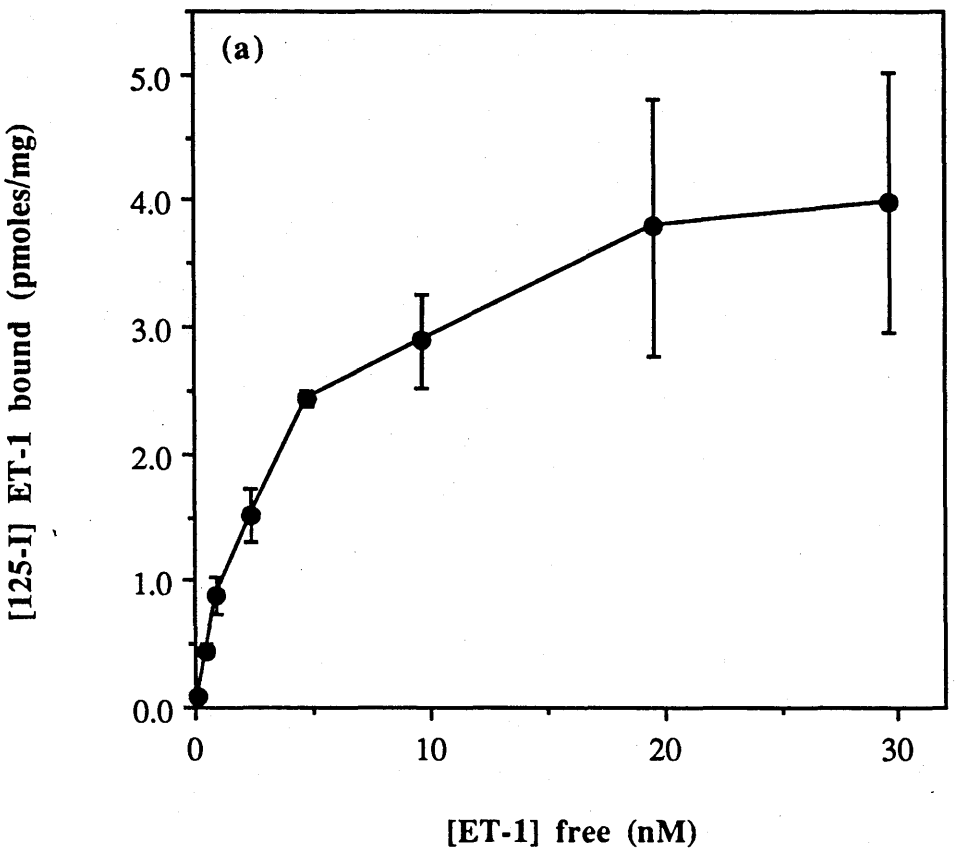

0.15
0.10
0.05
0.00
0
20
40
60
[125-I] ET-1 bound (pmoles)
[protein] µg

Fig. 5.1 Time course of [^{125}I] ET-1 binding to Rat-1 cell membranes.

Membranes (25μg) were incubated with 10nM [^{125}I] ET-1 at 25 °C for the indicated times. An excess of unlabelled ET-1 (1μM) was included to determine nonspecific binding. Bound ligand was separated from free ligand by vacuum filtration through GF/C filters. Results are expressed as the mean ± S.D. of triplicate determinations from a typical experiment where n=4. (O). total binding; (□), nonspecific binding; (●), specific binding.

Fig. 5.2 Effect of protein concentration on [^{125}I] ET-1 binding to membranes.

Membranes with increasing protein concentration were incubated with 10nM [^{125}I] ET-1 ± 1μM unlabelled ET-1 at 25 °C for 30min. Bound ligand was separated from free ligand by vacuum filtration through GF/C filters. Results are expressed as means ± S.D. from a typical experiment where n=3. (O), total binding; (□), nonspecific binding; (●), specific binding.


(a)
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
[125-I] ET-1 bound (pmoles/mg)
[ET-1] free (nM)

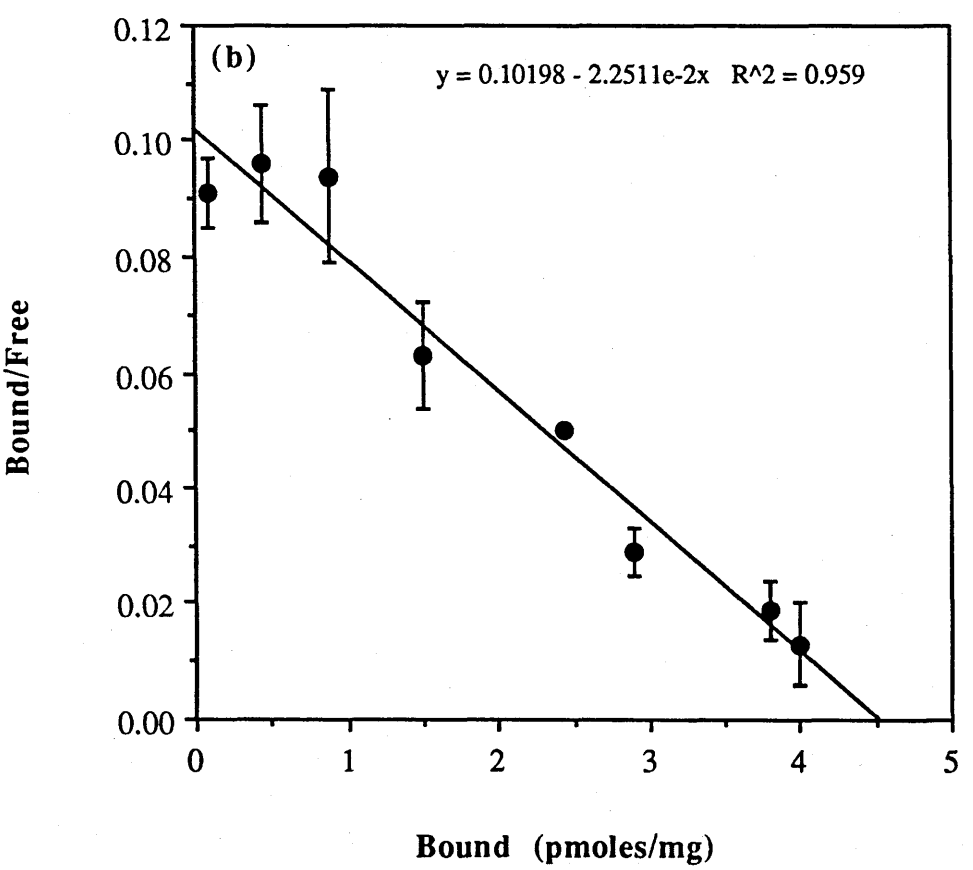

(b)
y = 0.10198 - 2.2511e-2x R^2 = 0.959
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
1
2
3
4
5
Bound/Free
Bound (pmoles/mg)

Fig. 5.3 Saturation binding of [^{125}I] ET-1 to membranes.

Membranes (25μg) were incubated with increasing concentrations of labelled ET-1 for 30min at 25 °C. Nonspecific binding was determined by including 1μM unlabelled ET-1. The data was analysed by (a) computer-assisted non-linear regression analysis and (b) scatchard analysis. Results are expressed as means ± S.D. from a typical experiment where n=4.

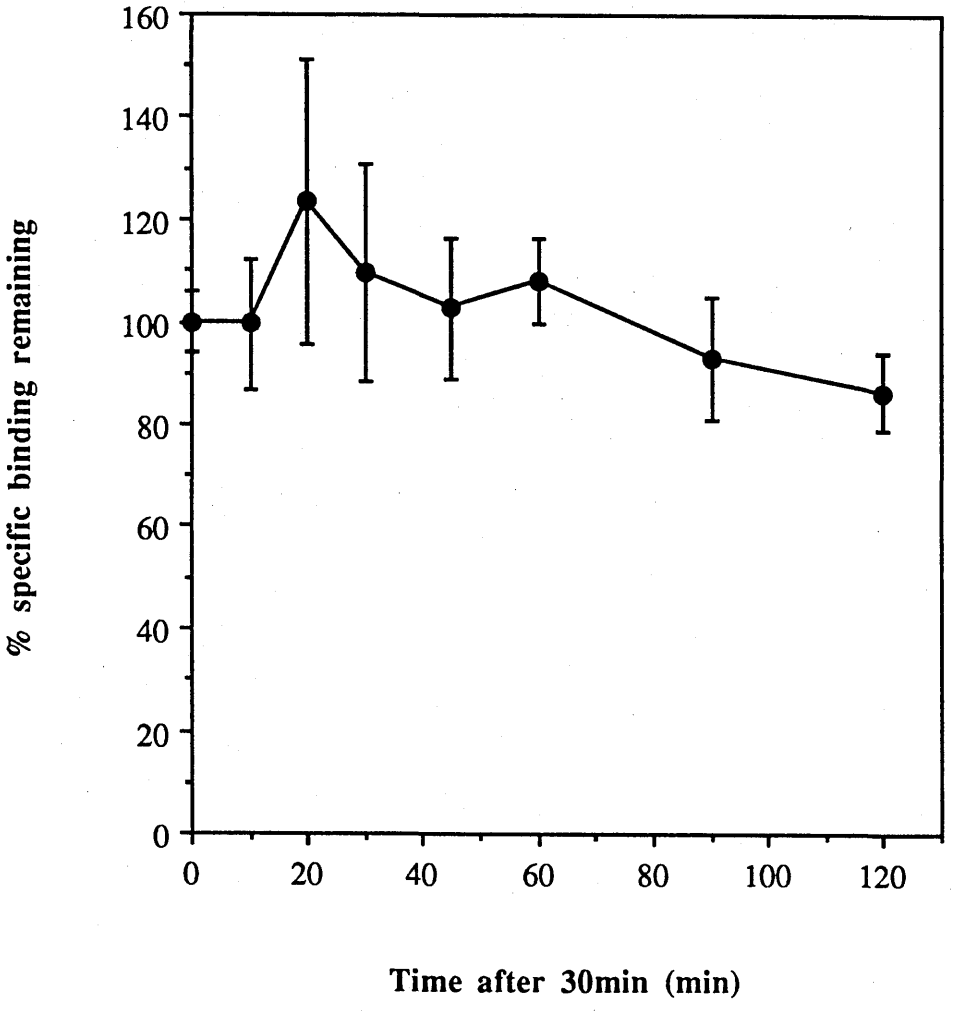
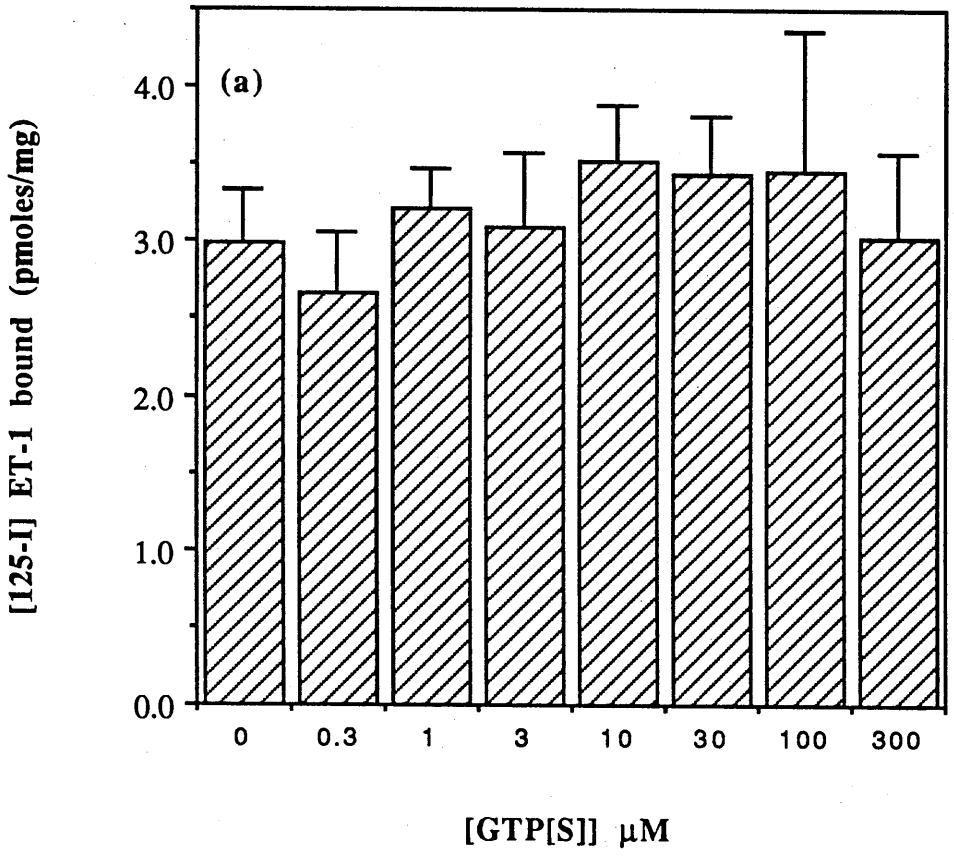

% specific binding remaining
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
Time after 30min (min)

Fig. 5.4 Time course of dissociation of bound [^{125}I] ET-1 from membranes.

Membranes (25μg) were incubated with 10nM [^{125}I] ET-1 ± 1μM unlabelled ET-1 for 30min at 25 °C. 1μM unlabelled ET-1 was then added for the indicated times. Bound and free ligand were separated by vacuum filtration through GF/C filters. Results are expressed as the mean ± S.D. of triplicate determinations from a typical experiment where n=3.


(a)
4.0
3.0
2.0
1.0
0.0
0
0.3
1
3
10
30
100
300
[125-I] ET-1 bound (pmoles/mg)
[GTP[S]] μM

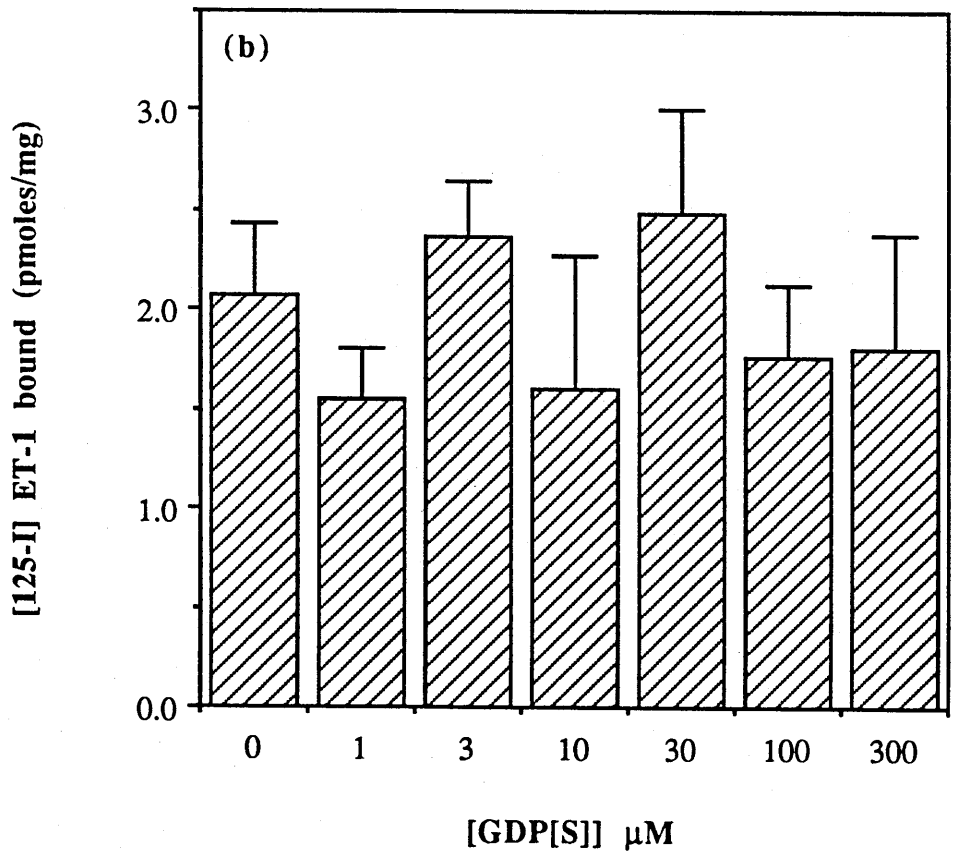

(b)
3.0
2.0
1.0
0.0
0
1
3
10
30
100
300
[125-I] ET-1 bound (pmoles/mg)
[GDP[S]] μM

Fig. 5.5 Effect of guanine nucleotides on [^{125}I] ET-1 binding to membranes.

Membranes (25μg) were preincubated with increasing concentrations of (a) GTP[S] or (b) GDP[S] for 10min at 25 °C and then incubated for a further 30min with 10nM [^{125}I] ET-1. Nonspecific binding was determined by including 1μM unlabelled ET-1. Results are expressed as means ± S.D. from typical experiments where n=3-4.

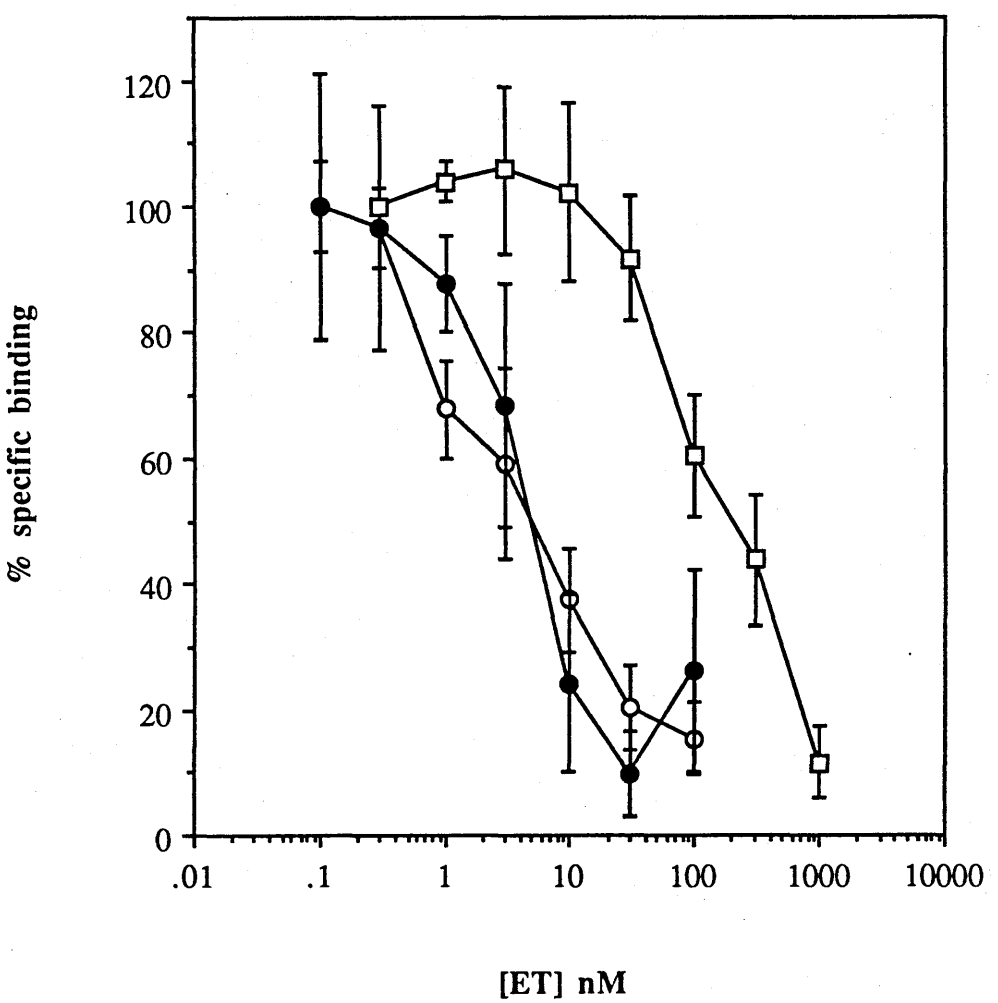

% specific binding
120
100
80
60
40
20
0
.01
.1
1
10
100
1000
10000
[ET] nM

Fig. 5.6 Competitive inhibition of [^{125}I] ET-1 binding by ET isopeptides.

Membranes (25μg) were incubated with 10nM [^{125}I] ET-1 ± 1μM unlabelled ET-1 for 30min at 25 °C in the presence of increasing concentrations of unlabelled ET-1 (O), ET-2 (●) and ET-3 (□). Bound and free ligand were separated by vacuum filtration. Results are expressed as means ± S.D. from a typical experiment where n=3.

5.5 Discussion

These studies demonstrated that [^{125}I] ET-1 binds to a single class of high affinity sites on Rat-1 cell membranes in a saturable manner. The K_D value obtained (2.7 ± 1.3 nM) is similar to the values reported for [^{125}I] ET-1 binding to Swiss 3T3 cells (Fabregat and Rozengurt, 1990), brain capillary endothelial cells (Vigne *et al.*, 1990) and human melanocytes (Yada *et al.*, 1991) although it is higher than the K_D reported for [^{125}I] ET-1 binding to vascular smooth muscle cells (Hirata *et al.*, 1988). The K_D is also very similar to the EC_{50} values (~2nM) for ET-1-stimulated inositol phosphates accumulation, choline generation and PtdBut accumulation in Rat-1 cells (Chapter 3).

There is an extremely tight association between the peptide and its receptor with very little dissociation even after 2h washing with excess unlabelled ET-1. This is in agreement with studies in other systems (Hirata *et al.*, 1988; Kanse *et al.*, 1989; Marin *et al.*, 1991; Serradeil-Le Gal *et al.*, 1991). The slow dissociation of ET-1 from its receptor almost certainly explains why the ET-1-stimulated increase in intracellular Ca^{2+} levels cannot be washed out compared with the LPA response which is easily washed out (section 4.3). The tight association between ligand and receptor may also be important for the sustained nature of ET-1-stimulated PtdIns(4,5)P_2 hydrolysis. It does not, however, appear to explain the unusual characteristics of ET-1-stimulated inositol phosphates accumulation (chapter 4) since in other systems where the dissociation of ET-1 from its receptor has been shown to be slow the inositol phosphates responses have been characteristic of G protein-coupled receptors in terms of their sensitivity to PMA and GTP[S] (Baldi and Dunn, 1990; Thomas *et al.*, 1991; Takuwa *et al.*, 1990; Resink *et al.*, 1990, Hirata *et al.*, 1988; Reynolds *et al.*, 1989).

As discussed previously (chapter 4), receptors which couple to G proteins generally show an apparent decrease in affinity for their agonist in the

presence of GTP[S]. In bovine endothelial cell membranes GTP[S] inhibited the binding of both [^{125}I] ET-1 and ET-3 in a dose-dependent manner (Eguchi *et al.*, 1991) and, similarly, in vascular smooth muscle cell membranes ET-1 binding was also inhibited by GTP[S] (Takuwa *et al.*, 1990). However, GTP[S] and GDP[S] had no effect on [^{125}I] ET-1 binding in the studies presented here. Similar results have also been reported for ET-1 binding studies in porcine aortic and rat lung membranes (Kanse *et al.*, 1989) and rat liver plasma membranes (Serradeil-Le Gal *et al.*, 1991) and also for sarafotoxin S6b binding to rat atrial membranes (Kloog *et al.*, 1988). The capacity of agonists to form a guanine nucleotide-sensitive high affinity binding complex has been correlated with their efficacy for stimulating polyphosphoinositide breakdown. Although ET-1 appeared to be very efficacious at stimulating Ins(1,4,5)P_3 generation and inositol phosphates accumulation, GTP[S] clearly had no effect on [^{125}I] ET-1 binding to membranes. This may reflect an unusual interaction of the ET-1 receptor with a G protein or, alternatively, it may suggest that only a small part of the ET-1 inositol phosphates response is generated by a G protein-regulated PtdIns-PLC activity. Both these proposals have previously been suggested by the characteristics of ET-1-stimulated inositol phosphates generation (chapter 4).

Competition binding studies yielded a rank order of potency of ET-1 ~ ET-2 > ET-3 in displacing [^{125}I] ET-1 binding. The K_i values obtained (~ 2-4nM for ET-1 and ET-2; ~ 60nM for ET-3) are similar to the EC_{50} values for inositol phosphates accumulation and choline generation in response to each of the isopeptides (~ 2nM for ET-1 and ET-2; ~ 50nM for ET-3).

Although the characteristics of the binding site on Rat-1 cell membranes suggests an ET_A type receptor with highest affinity for ET-1, this receptor has been shown to have seven membrane spanning domains and putative sites for protein kinase C-mediated phosphorylation consistent with a receptor which is coupled to a G protein in the accepted manner (Arai *et al.*, 1990). Since the

characteristics of ET-1-stimulated inositol phosphates accumulation in Rat-1 cells do not corresponsd to these structural observations, this may suggest the presence of another subtype of ET receptor on Rat-1 cells with a different receptor-G protein interaction. It is worth noting that the nonselective ET receptor subtype contains unusual sequences within the third cytoplasmic loop (Sekurai *et al.*, 1990) which is the putative site of the G protein interaction suggesting that not all subtypes of the ET receptor may interact with G proteins in the same way. Alternatively, an ET_A -like receptor may control both G protein-regulated and non-G protein-regulated PtdIns-PLC isozymes.

Chapter 6

Conclusions

6.1 Conclusions.

The object of the work presented here was to investigate the signal transduction pathways stimulated by ET-1 in Rat-1 cells which might be important in the mitogenic response to the peptide. ET-1-stimulated DNA synthesis was reported to be dependent on protein kinase C activity (Muldoon *et al.*, 1990). The inhibitory effect of chronic PMA treatment, which down-regulates protein kinase C, on [^{3}H] thymidine incorporation into DNA in response to ET-1 confirmed this (chapter 3) and therefore suggests that stimulation of cell proliferation by the peptide is at least partly dependent on activation of one or more isozymes of protein kinase C. Generation of DAG would therefore appear to have a central role since it is the physiological activator of the enzyme. Sustained elevation of DAG levels in response to ET-1 stimulation in Rat-1 cells has been reported (Muldoon *et al.*, 1990; P. Kaur, personal communication) and both PtdIns(4,5)P_2 and PtdCho hydrolysis could be potential sources of this second messenger. In other cell types stimulated Ca^{2+} influx has also been implicated in the proliferative response to ET-1 based on the inhibitory effects of Ca^{2+} L-channel blockers such as nifedipene (Nakaki *et al.*, 1989; Shichiri *et al.*, 1991). Similarly, a role for Ca^{2+} influx has also been suggested in bombesin-stimulated [^{3}H] thymidine incorporation into DNA in Swiss 3T3 cells (Takuwa *et al.*, 1991). Ca^{2+} influx has been proposed to occur by several different mechanisms including direct receptor activation of Ca^{2+} channels or activation by second messengers generated as a result of PtdIns(4,5)P_2 hydrolysis (see section 1.4.2).

The results presented in this thesis provide evidence for ET-1-stimulated PtdIns(4,5)P_2 hydrolysis by a PtdIns-PLC and PtdCho hydrolysis via PLD in Rat-1 cells. Mass measurement of Ins(1,4,5)P_3 and PtdIns(4,5)P_2 levels suggested sustained hydrolysis of PtdIns(4,5)P_2 in response to ET-1. Clearly, this may result in the prolonged generation of DAG which has important implications for the activation of protein kinase C. Measurement of

intracellular Ca^{2+} changes demonstrated two phases of Ca^{2+} mobilization following stimulation with ET-1; the initial increase is probably due to $Ins(1,4,5)P_3$-mediated release from intracellular stores while the scond phase is dependent on Ca^{2+} influx (chapter 4). As discussed earlier, Ca^{2+} influx has been implicated in the mitogenic response to ET-1 in other cell types and may also contribute to the response in Rat-1 cells. Measurement of DNA synthesis and cell division in low Ca^{2+} buffers or in the presence of Ca^{2+} channel blockers would provide more evidence on the potential role of Ca^{2+} influx in the initiation of cell proliferation by ET-1. A comparison of the changes in $Ins(1,4,5)P_3$ levels and intracellular $[Ca^{2+}]$ in response to ET-1 and LPA suggested that the second phase of Ca^{2+} mobilization was not dependent on the generation of $Ins(1,4,5)P_3$ and therefore questions the role of the sustained formation of this second messenger. In some systems $Ins(1,3,4,5)P_4$, which is generated from $Ins(1,4,5)P_3$ by an $InsP_3$-3-kinase, has been implicated in stimulating Ca^{2+} influx (see section 1.4.2); measurements of $Ins(1,3,4,5)P_4$ levels following stimulation with ET-1 might therefore provide more information on the significance of sustained $Ins(1,4,5)P_3$ generation.

PtdCho hydrolysis is increasingly being recognized as an important second messenger generating pathway; in particular, it is often put forward as an additional source of DAG. ET-1 stimulated PtdCho hydrolysis in Rat-1 cells by a PLD-catalysed mechanism (chapter 3). Although this appeared to be a transient response based on the accumulation of PtdBut, the generation of choline was more sustained suggesting another pathway of PtdCho breakdown. This could involve a PtdCho-specific PLC. The products of such a hydrolysis would be DAG and phosphocholine. The inability to detect any increase in phosphocholine levels following ET-1 stimulation may be due to the rapid resynthesis of the parent lipid (chapter 3) or further metabolism to choline by a phosphatase.

In many systems where prolonged DAG formation has been observed $Ins(1,4,5)P_3$ generation has been transient so that PtdCho hydrolysis has been considered as an alternative source to maintain DAG levels at later times (Wright *et al.*, 1988; Pessin and Raben, 1989; Matozaki and Williams, 1989; Huang and Cabot, 1990). *In vitro* experiments have shown that protein kinase C activity can be stimulated by molecular species of DAG identical to those generated by PtdCho hydrolysis (Go *et al.*, 1987) while α_1-adrenergic receptor stimulation in MDCK cells has been shown to activate protein kinase C under conditions when polyphosphoinositide hydrolysis was inhibited (Slivka *et al.*, 1988). Studies such as these suggest an important role for PtdCho hydrolysis in maintaining elevated DAG levels and thus prolonging protein kinase C activation. However, since in Rat-1 cells $PtdIns(4,5)P_2$ hydrolysis stimulated by ET-1 is sustained and could therefore maintain elevated DAG levels, this questions the role of PtdCho breakdown. Indeed, PtdCho hydrolysis by a PLD-catalysed pathway yields phosphatidic acid which must be further metabolized by phosphatidate phosphohydrolase to generate DAG. $PtdIns(4,5)P_2$ hydrolysis is therefore a more direct source of DAG. In addition, there have been some reports where protein kinase C activation has been dissociated from PtdCho-derived DAG. In IIC9 fibroblasts low concentrations of thrombin which stimulated PtdCho hydrolysis but not $PtdIns(4,5)P_2$ breakdown did not result in the activation of protein kinase C despite the generation of DAG from PtdCho (Leach *et al.*, 1991). Similarly, thyrotropin-releasing hormone treatment of GH_3 cells resulted in a rapid but transient activation of protein kinase C although the increase in DAG was sustained (Martin *et al.*, 1990b). In addition, the mitogenic response of Swiss 3T3 fibroblasts to exogenous PtdCho-hydrolysing phospholipase C was independent of protein kinase C (Larrodera *et al.*, 1990). These studies question the relevance of DAG derived from PtdCho as an activator of protein kinase C in the systems mentioned.

However, it is also important to realize the difficulties in fully measuring protein kinase C activity. The studies by Martin *et al.* (1990) measured the activation of protein kinase C by the translocation of the enzyme to the membrane. However, other studies have shown that redistributuion of protein kinase C can be dissociated from phosphorylation of its substrates (Trilivas *et al.*, 1991). But even where phosphorylation of substrates is measured as was done by Leach *et al.* (1991) interpretation is also not clear cut since different protein kinase C isozymes may have different substrate specificities (reviewed in Kikkawa *et al.*, 1989). In order to conclusively determine the sources of DAG in response to ET-1 stimulation in Rat-1 cells it would be necessary to determine the molecular species of DAGs formed and compare them with the molecular species contained in cellular phospholipids as has been done in other systems (Pessin and Raben, 1989; Divecha *et al.*, 1991). Different isozymes of protein kinase C show differences in their regulation by phospholipid metabolites and in their substrate specificities and therefore the generation of DAGs with different molecular structures may provide for a greater diversity in controlling cellular processes dependent on protein kinase C.

It is possible that DAG is not always the relevant mediator generated as a result of PtdCho hydrolysis. The results presented in chapter 3 provide evidence for PLD-catalysed hydrolysis of PtdCho which would yield phosphatidic acid (PtdOH) as the initial product rather than DAG. There is increasing evidence for a second messenger role for PtdOH. In A431 carcinoma cells and mesangial cells PtdOH increased polyphosphoinositide turnover, raised intracellular [Ca^{2+}], induced expression of c-*fos* and c-*myc* and stimulated DNA synthesis (Moolenaar *et al.*, 1986; Knauss *et al.*, 1990). In Swiss 3T3 cells PtdOH inhibited adenylyl cyclase (Murayama and Ui, 1987) while in neuroblastoma cells cGMP levels were elevated (Ohsako and Deguchi, 1981). Most recently, PtdOH-dependent protein phosphorylation has been demonstrated in soluble extracts from rat liver, brain, lung and testis.

The profiles of proteins phosphorylated were distinct from those phosphorylated in response to protein kinae C activation (Bocckina *et al.*, 1991) suggesting a PtdOH-dependent protein kinase. The ability of PtdOH to stimulate DNA synthesis is of particular interest due to the mitogenic response to ET-1 in Rat-1 cells. Thus the stimulation of PtdCho hydrolysis via phospholipase D by ET-1 in Rat-1 cells may generate PtdOH as another mediator in addition to the Ins(1,4,5)P_3 and DAG generated from PtdIns(4,5)P_2 breakdown. In addition, results presented here and previous reports (van Corven *et al.*, 1989; Jalink *et al.*, 1990; van Corven *et al.*, 1992) also provide evidence for LPA as a mitogenic stimulus for cells although the physiological relevance of this is not known. Since most studies have investigated the effect of exogenously added PtdOH or LPA it is not clear whether PtdOH or LPA produced as a result of stimulated phospholipid turnover exert their effects within the cell or are released to act in an autocrine or paracrine manner.

Although the work presented here has focussed on PtdIns(4,5)P_2 and PtdCho hydrolysis as potential mitogenic signalling pathways mediating the action of ET-1 in Rat-1 cells it is highly likely that other pathways are also involved. In vascular smooth muscle cells and rat mesangial cells ET-1 has been shown to activate PLA_2 resulting in the release of arachidonic acid (AA) (Resink *et al.*, 1989a; Resink *et al.*, 1990; Reynolds *et al.*, 1989; Simonson and Dunn, 1990c) and preliminary experiments suggest that ET-1 also stimulates AA release in Rat-1 cells (S. Currie and A. Stewart, personal communication). AA is increasingly being recognized as a potentially important second messenger molecule and has been reported to activate protein kinase C (α, β and γ isozymes to different extents) and PtdIns-PLC and to mimic the action of $InsP_3$ on Ca^{2+} mobilization (reviewed in Naor, 1991; Shinomura *et al.*, 1991; Chen and Murakami, 1992). A mitogenic role has also been proposed for AA and it has been shown to stimulate [^{3}H] thymidine

incorporation into DNA in the presence of insulin in Swiss 3T3 cells (Takuwa *et al.*, 1988; Gil *et al.*, 1991). Activation of AA release via PLA_2 or another enzymic pathway may therefore contribute in eliciting a mitogenic response to ET-1 in Rat-1 cells.

In addition to release of AA, agonist stimulation may also result in the release of other unsaturated fatty acids such as oleic acid. Indeed, ET-1 can stimulate release of oleic acid in Rat-1 cells (A. Stewart, personal communication). This is of relevance due to the reported ability of oleic acid to activate protein kinase C (El Touny *et al.*, 1990; Shinomura *et al.*, 1991) and phospholipase D *in vitro* (Chalifour and Kanfer, 1982). Results from experiments investigating the regulation of phospholipase D by ET-1 stimulation suggested the involvement of a pathway in addition to protein kinase activation and Ca^{2+} influx (chapter 3) and this could potentially be achieved by the stimulated release of oleic acid.

Tyrosine kinase activity is recognized to be important for the mitogenic response to growth factors such as PDGF and EGF (Escobedo and Williams, 1988; Escobedo *et al.*, 1988; Pandiella *et al.*, 1989). In addition, a number of proteins which have been implicated in the control of cell proliferation are regulated by phosphorylation at tyrosine residues e.g. cdc2 kinase (see section 1.1), MAP kinase and Raf-1 kinase (see section 1.2.3). Phosphorylation of proteins on tyrosine residues has now been demonstrated in response to stimulation by a number of mitogenic peptides whose receptors do not possess intrinsic tyrosine kinase activity. In rat mesangial cells ET-1 rapidly enhanced tyrosine phosphorylation of a number of proteins which were also phosphorylated in response to EGF suggesting that these agents share a common signalling pathway (Force *et al.*, 1991). Similarly, in Swiss 3T3 cells ET-2 stimulated rapid tyrosine phosphorylation of a set of substrates also phosphorylated in response to bombesin and vasopressin (Zachary *et al.*, 1991a, 1991b). Agonists have frequently been classified into different

groups, those whose receptors have intrinsic tyrosine kinase activity and those which couple to effector systems via G proteins. The above reports, however, suggest an interaction between G protein-coupled systems and tyrosine kinases. This is supported by some reports of G protein involvement in the transduction of signals by receptor tyrosine kinases (reviewed in Ives, 1991). In permeabilized vascular smooth muscle cells GTP[S] potentiated PDGF-stimulated $InsP_3$ generation (Huang and Ives, 1989) while EGF-stimulated proliferation in a human breast cancer cell line and EGF-stimulated $Ins(1,4,5)P_3$ generation in hepatocytes were inhibited by pertussis toxin (Church and Buick, 1988; Johnson and Garrison, 1987). Also, in rat macrophages colony stimulating factor 1-stimulated phosphatidylcholine-specific phospholipase C activity was reduced by pertussis toxin and enhanced by GTP[S] (Ghosh Choudhury, 1991).

Preliminary studies indicate that ET-1 can stimulate tyrosine phosphorylation of a number of proteins in Rat-1 cells (R. Plevin and M. Saville, personal communication). Since the ET receptors characterized so far contain seven membrane-spanning domains similar to other G protein-coupled receptors, this suggests the activation of a non-receptor tyrosine kinase which may constitute an additional mitogenic signalling pathway for ET-1. A common substrate of EGF and PDGF receptor tyrosine kinases is phospholipase Cγ (Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989; Nishibe *et al.*, 1990). $PtdIns(4,5)P_2$ hydrolysis in response to PDGF in Balb c/3T3 cells and Swiss 3T3 cells has been shown to be sustained (Fukami and Takenawa, 1989; Plevin *et al.*, 1991) and is therefore similar to the response to ET-1 in Rat-1 cells. The results presented here for ET-1 suggested two pathways of $PtdIns(4,5)P_2$ hydrolysis, a Ca^{2+}-independent and a Ca^{2+}-dependent pathway (chapter 4). It is therefore tempting to speculate that in addition to stimulating a Ca^{2+}-independent pathway of polyphosphoinositide turnover involving a G protein ET-1 might also stimulate PLCγ via the activation of a tyrosine kinase

in a Ca^{2+}-dependent manner. Since activation of PLCγ is believed to involve tyrosine phosphorylation rather than interaction with a G protein this hypothesis could also account for the unusual characteristics of the ET-1-stimulated inositol phosphates response regarding the effects of guanine nucleotide analogs and PMA (chapter 4). Tyrosine phosphorylation of PLCγ has been demonstrated in response to stimulation by agonists which exert their effects via receptors which do not possess intrinsic tyrosine kinase activity; for example, IgE stimulation of rat basophilic leukemia cells (Park *et al.*, 1991). It would therefore be of interest to investigate the effect of selective tyrosine kinase inhibitors such as genistein (Akiyama *et al.*, 1987) or phosphotyrosine phosphatase inhibitors on ET-1-stimulated $Ins(1,4,5)P_3$ generation and DNA synthesis in Rat-1 cells. Using this approach, tyrosine phosphorylation has been implicated in the activation of phospholipase D in response to fMet-Leu-Phe, platelet-activating factor and leukotriene B_4 in human neutrophils (Uings *et al.*, 1992). An alternative pathway which might explain the Ca^{2+} dependency of the second phase of ET-1-stimulated $Ins(1,4,5)P_3$ generation and the lack of effect of GTP[S] and PMA on inositol phosphates accumulation is that the influx of Ca^{2+} in response to the peptide activates a particular species of PtdIns-PLC. Arguing against this, however, is the observation that LPA also appears to stimulate Ca^{2+} influx, yet responses to this lipid are typical of an agonist acting through a G protein-coupled receptor.

Results presented in chapter 6 provide a preliminary characterization of the ET receptor on Rat-1 cells. Although the order of potency of ET isopeptides in competing for bound, radiolabeled ET-1 suggested an ET-1-selective receptor, the lack of effect of GTP[S] on the binding of the peptide may distinguish the binding site on Rat-1 cells from the previously identified ET_A receptor which has the structure expected of a G protein-coupled receptor (Arai *et al.*, 1990). The inability of guanine nucleotides to affect ET-1 binding may simply be due to an unusual interaction of the ET receptor with a G

protein; alternatively, it may suggest the existence of another type of ET receptor.

Mitogenesis is clearly a complex event involving the interaction of many different signalling pathways. This work has investigated the regulation of PtdIns(4,5)P_2 and PtdCho hydrolysis by ET-1 in Rat-1 cells. Both these pathways can give rise to a number of second messengers which might initiate or facilitate the onset of cell proliferation. Given the existence of numerous species of protein kinase C and PtdIns-PLC (and possibly also PLD although as yet none have been completely purified) and the probable involvement of other signal transduction pathways such as activation of PLA_2 or non-receptor tyrosine kinases, there is clearly considerable potential for great diversity in the regulation of cellular processes.

Chapter 7

References

Adachi, M., Yang, Y., Furuichi, Y. and Miyamoto, C. (1991) Biochem. Biophys. Res. Commun. **180**, 1265-1272.

Adams, J.C. and Gullick, W.J. (1989) Biochem. J. **257**, 905-911.

Agwu, D.E., McPhail, L.C., Chabot, M.C., Daniel, L.W., Wykle, R.L., and McCall, C.E. (1989) J.Biol. Chem. **264**, 1405-1413.

Ahn, N.G., Seger, R., Bratlien, R.L., Diltz, C.D., Tonks, N.K. and Krebs, E.G. (1991) J. Biol. Chem. **266**, 4220-4227.

Akiyama, T., Ishida, I., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. and Fukami, Y. (1987) J. Biol. Chem. **262**, 5592-5595.

Ambar, I., Kloog, Y. and Sokolovsky, M. (1990) Biochemistry **29**, 6415-6418.

Anderson, D., Koch, C.A., Grey, L., Ellis, C., Moran, M.F. and Pawson, T. (1990) Science **250**, 979-982.

Anderson, N.G., Maller, J.L., Tonks, N.K. and Sturgill, T.W. (1990) Nature **343**, 651-653.

Anderson, N.G., Li, P., Marsden, L.A., Williams, N., Roberts, T.M. and Sturgill,T.W. (1991) Biochem. J. **277**, 573-576.

Ando, K., Hirata, Y., Shichiri, M., Emori, T. and Marumo, F. (1989) FEBS Lett. **1,2**, 164-166.

Anggard, E.E., Galton, S., Rae, G., Thomas, R., McLoughlin, L., De Nucci, F.and Vane, J.R. (1989) J Cardiovasc. Pharmacol. **13(Suppl. 5)**, S46-S49.

Anggard, E.E., Botting, R.M. and Vane, J.R. (1990) Bloob Vessels **27**, 269-281.

Anthes, J.C., Eckel, S., Siegel, M.I., Egan, R.W. and Billah, M.M. (1989) Biochem. Biophys. Res. Commun. **163**, 657-664.

Arai, H., Hori, S., Aramori, I., Ohkubo, H. and Nakanishi, S. (1990) Nature **348**, 730-732.

Araki, S., Kawahara, Y., Kariya, Ki, Sunako, M., Fukuzaki, H. and Takai, Y. (1989) Biochem. Biophys. Res. Commun. **159**, 1072-1079.

Auger, K.R., Serunian, L.A., Soltoff, S.P. Libby, P. and Cantley, L.C. (1989) Cell **57**, 167-175.

Badr, K.F., Munger, K.A., Sugiura, M., Snajdar, R.M., Schwartzberg, M. and Inagami, T. (1989) Biochem. Biophys. Res. Commun. **161**, 776-781.

Balla, T., Baukal, A.J., Guillemette, G. and Catt, K.J. (1988) J. Biol. Chem. **263**, 4083-4091.

Ballou, L.M., Luther, H. and Thomas, G. (1991) Nature **349**, 348-350.

Barber, J.R. and Verma, I.M. (1987) Mol. Cell. Biol. **7**, 2201-2211.

Barber, D.L. (1991) Cell. Signall. **3**, 387-397.

Battey, J.F., Way, J.M., Corjay, M.H., Shapira, H., Kusano, K., Harkins, R., Wu, J.M., Slattery, T., Mann, E. and Feldman, R.I. (1991) Proc. Natl. Acad. Sci. USA **88**, 395-399.

Berridge, M.J., Downes, C.P. and Hanley, M.R. (1982) Biochem. J. **206**, 587-595.

Berridge, M.J.(1983) Biochem. J. **212**, 849-858.

Berridge, M.J.(1987a) Biochem. Biophys. Acta **907**, 33-45.

Berridge, M.J. (1987b) Ann. Rev. Biochem. **56**, 159-193.

Berridge, M.J. and Irvine, R.F. (1989) Nature **341**, 197-205.

Besterman, J.M., Watson, S.P. and Cuatrecasas, P. (1986a) J. Biol. Chem. **261**, 723-727.

Besterman, J.M., Duronio, V. and Cuatrecasas, P. (1986b) Proc. Natl. Acad. Sci. USA.**83**, 6785-6789.

Bierman, A.J., Koenderman, L., Tool, A.J. and De Laat, S.W. (1990) J. Cell. Physiol. **142**, 441-448.

Billah, M.M., Pai, J., Mullmann, T.J., Egan, R.W. and Siegel, M.I. (1989a) J. Biol. Chem. **264**, 9069-9076.

Billah, M.M., Eckel, S., Mullmann, T.J., Egan, R.W. and Siegel, M.I. (1989b) J. Biol. Chem. **264**, 17069-17097.

Billah, M.M. and Anthes, J.C. (1990) Biochem. J. **269**, 281-291.

Bird, G. St. J., Rossier, M.F., Hughes, A.R., Shears, S.B., Armstrong, D.L. and Putney, J.W. (1991) Nature **352**, 162-165.

Bishop, W.R., Ganong, B.R. and Bell, R.M. (1986) J. Biol. Chem. **261**, 6993-7000.

Black, F.M. and Wakelam, M.J.O. (1990) Biochem. J. **266**, 661-667.

Blackshear, P.J., McNeill Haupt, D., App, H. and Rapp, U.R. (1990) J. Biol. Chem. **265**, 12131-12134.

Blakeley, D.M., Corps, A.N. and Brown, K.D. (1989) Biochem. J. **258**, 177-185.

Blenis, J., Kuo, C.j. and Erikson, R.L. (1987) J. Biol. Chem. **262**, 14373-14376.

Bloch, K.D., Eddy, R.L., Shows, T.B. and Quertermous, T. (1989) J. Biol. Chem **264**, 18156-18161.

Bloch, K.D., Hong, C., Eddy, R.L., Shows, T.B. and Quertermous, T. (1991) Genomics **10**, 236-242.

Block, L.H., Emmons, L.R., Vogt, E., Sachinidis, A., Vetter, W. and Hoppe, J. (1989) Proc. Natl. Acad. Sci. USA **86**, 2388-2392.

Bocckino, S.B., Blackmore, P.F., Wilson, P.B. and Exton, J.H. (1987) J. Biol. Chem. **262**, 15309-15315.

Bojanic, D. and Fain, J.N. (1986) Biochem. J. **240**, 361-365.

Bonser, R.W., Thompson, N.T., Randell, R.W. and Garland, L.G. (1989) Biochem. J. **264**, 617-620.

Borges, R., Von Grafenstein, H. and Knight, D.E. (1989) Eur. J. Pharmacol. **165**, 223-230.

Boulton, T.G., Yancopoulos, G.D., Gregory, J.S., Slaughter, C., Moomaw. C., Hsu, J. and Cobb, M.H. (1990) Science **249**, 64-67.

Boyer, J.L., Hepler, J.R. and Harden, T.K. (1989a) TiPS **10**, 360-364.

Boyer, J.L., Downes, C.P. and Harden, T.K. (1989b) J. Biol. Chem. **264**,884-890.

Boynton, A.L., Dean, N.M. and Hill, T.D. (1990) Biochem. Pharmacol. **40**, 1933- 1939.

Bradford, P.G. and Rubin, R.P. (1986) J. Biol. Chem. **261**, 15644-15647.

Brown, K.D., Blakeley, D.M., Hamon, M.H., Laurie, M.S. and Corps, A.N. (1987) Biochem. J. **245**, 631-639.

Brown, K.D. and Littlewood, C.J. (1989) Biochem. J. **263**, 977-980.

Brown, K.D., Littlewood, C.J. and Blakeley, D.M. (1990) Biochem. J. **270**, 557-560.

Burgess, G.M., Irvine, R.F., Berridge, M.J., McKinney, J.S. and Putney, J.W. (1984) Biochem. J **224**, 741-746.

Cao, Y.Z., Reddy, C.C. and Mastro, A.M. (1990) Biochem. Biophys. Res. Commun. **171**, 955-962.

Carney, D.H., Scott, D.L., Gordon, E.A. and LaBelle, E.F. (1985) Cell **42**, 479-488.

Casey, P.J. and Gilman, A.G. (1988) J. Biol. Chem. **263**, 2577-2580.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. **257**, 7847-7851.

Cattaneo, M.G. and Vincentini, L.M. (1989) Biochem. J. **262**, 665-668.

Chalifour, R. and Kanfer, J.N. (1982) J. Neurochem. **39**, 299-305.

Chan, J. and Greenberg, D.A. (1991) Biochem. Biophys. Res. Commun. **177**, 1141-1146.

Changya, L., Gallacher, D.V., Irvine, R.F., Potter, B.V.L. and Petersen, O.H. (1989) J. Memb. Biol. **109**, 85-93.

Chen, S.G. and Murakami, K. (1992) Biochem. J. **282**, 33-39.

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T. and Karin, M. (1988) Cell **54**, 541-552.

Church, J.G. and Buick, R.N. (1988) J. Biol. Chem. **263**, 4242-4246.

Claro, E., Garcia, A. and Picatoste, F. (1989) Biochem. J. **261**, 29-35.

Clementi, E., Scheer, H., Zacchetti, D., Fasolato, C., Pozzan, T. and Meldolesi, J. (1992) J. Biol. Chem. **267**, 2164-2172.

Cockcroft, S. and Gomperts, B.D. (1985) Nature **314**, 534-536.

Cockcroft, S. (1987) TIBS **12**, 75-78.

Cockcroft, S. and Taylor, J.A. (1987) Biochem. J. **241**, 409-414.

Cockcroft, S. and SDtutchfield, J. (1989) Biochem. J. **263**, 715-723.

Collins, M.K.L. and Rozengurt, E. (1984) J. Cell. Physiol. **118**, 133-142.

Connolly, T.M., Bansal, V.S., Bross, T.E., Irvine, R.F. and Majerus, P.W. (1987) J. Biol. Chem. **262**, 2146-2149.

Cook, S.J. and Wakelam, M.J.O. (1989) Biochem. J. **263**, 581-587.

Cook, S.J., Palmer, S., Plevin, R. and Wakelam, M.J.O. (1990) Biochem. J. **265**, 617-620.

Cook, S.J. and Wakelam, M.J.O. (1991a) Biochem. Biophys. Acta **1092**, 265-272.

Cook, S.J. and Wakelam, M.J.O. (1991b) Cell. Signal. **3**, 273-282.

Cook, S.J., Briscoe, C.P. and Wakelam, M.J.O. (1991c) Biochem. J. **280**, 431-438.

Cook, S.J. and Wakelam, M.J.O. (1992) Biochem. J. in press.

Coughlin, S.R., Escobedo, J.A. and Williams, L.T. (1989) Science **243**, 1191-1194.

Courtneidge, S.A. and Heber, A. (1987) Cell **50**, 1031-1037.

Cullen, P.J., Irvine, R.F. and Dawson, A.P. (1990) Biochem. J. **271**, 549-553.

Curran, T. and Franza, B.R. (1988) Cell **55**, 395-397.

Cyr, C., Huebner, K., Druck, T. and Kris, R. (1991) Biochem. Biophys. Res. Commun **181**, 184-190.

Davis, P.D., Hill, C.H., Keech, E., Lawton, G., Nixon, J.S., Sedgwick, A.D., Wadsworth, J., Westmacott, D. and Wilkinson, S.E. (1989) FEBS Lett. **259**, 61-63.

De Lean, A., Munson, P.J. and Rodbard, D. (1980) **235**, E97-E102.

De Nucci, G., Thomas, R., D'Orleans-Juste, P., Antunes, E., Walder, C., Warner, T. and Vane, J. (1988) Proc. Natl. Acad. Sci. USA **85**, 9797-9800.

Diaz-Meco, M.T., Larrodera, P., Lopez-Barahona, M., Cornet, M.E., Barreno, P.G. and Moscat, J. (1989) Biochem. J. **263**, 115-120.

Divecha, N., Lander, D.J., Scott, T.W. and Irvine, R.F. (1991) Biochim. Biophys. Acta **1093**, 184-188.

Dougherty, R.W. and Niedel, J.E. (1986) J. Biol. Chem. **261**, 4097-4100.

Downes, C.P. and Carter, A.N. (1991) Cell. Signall. **3**, 501-513.

Draetta, G. (1990) TIBS **15**, 378-383.

Draetta, G. and Beach, D. (1988) Cell **54**, 17-26.

Dumont, J.E., Jauniaux, J. and Roger, P.P. (1989) TIBS **14**, 67-71.

Eguchi, S., ZKozuka, M., Hirose, S., Ito, T., Hirata, Y., Marumo, F. and Hagiwara, H. (1991) Biochem. Biophys. Res. Commun. **174**, 1343-1346.

El Touny, S., Khan, W. and Hannun, Y. (1990) J. Biol. Chem. **265**, 16437-16443.

Ellis, C., Moran, M., McCormick, F. and Pawson, T. (1990) Nature **343**, 377-381.

Erneux, C. and Takazawa, K. (1991) TiPS **12**, 174-176.

Escobedo, J.A. and Williams, L.T. (1988) Nature **335**, 85-87.

Escobedo, J.A., Barr, P.J. and Williams, L.T. (1988) Mol. Cell. Biol. **8**, 5126-5131.

Evans, T., Hepler, J.R., Masters, S.B., Brown, J.H., Harden, T.K. (1985) Biochem. J. **232**, 751-757.

Exton, J.H. (1990) J. Biol. Chem. **265**, 1-4.

Fabregat, I. and Rozengurt, E. (1990) Biochem. Biophys. Res. Commun. **167**, 161-167.

Fain, J.N., Wallace, M.A. and Wojcikiewicz, R.J.H. (1988) FASEB J. **2**, 2569-2574.

Farrar, W.L., Thomas, T.P. and Anderson, W.B. (1985) Nature **315**, 235-237.

Ferris, C.D., Huganir, R.L., Supattapone, S. and Snyder, S.H. (1989) Nature **342**, 87-89.

Fitzgerald, T.J., Uhing, R.J. and Exton, J.H. (1986) J. Biol. Chem. **261**, 16871-16877.

Fleischman, L.F., Chahwala, S.B. and Cantley, L. (1986) Science **231**, 407-410.

Force, T., Kyriakis, J.M., Avruch, J. and Bonventre, J.V. (1991) J. Biol. Chem. **266**, 8650-8656.

Frantz, C.N. (1985) Exptl. Cell Res. **158**, 287-300.

Frick, K.K., Womer, R.B. and Scher, C.D. (1988) J. Biol. Chem. **263**, 2948-2952.

Froehlich, J.E. and Rachmeler, M. (1972) J. Cell Biol. **55**, 19-31.

Fu, T., Okano, Y. and Nozawa, Y. (1988) Biochem. Biophys. Res. Commun. **157**, 1429-1435.

Fujiki, H., Tanaka, Y., Miyake, R., Kikkawa, U., Nishizuka, Y. and Sugimura, T. (1984) Biochem. Biophys. Res. Commun. **120**, 330-343.

Fukami, K., Matsuoka, K., Nakanishi, O., Yamakawa, A., Kawai, S. and Takenawa, T. (1988) Proc. Natl. Acad. Sci. USA **85**, 9057-9061.

Fukami, K. and Takenawa, T. (1988) J. Biol. Chem. **264**, 14985-14989.

Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989) Nature **342**, 32-38.

Galron, R., Bdolah, A., Kloog, Y. and Sokolovsky, M. (1990) Biochem. Biophys. Res. Commun. **171**, 949-954.

Gandhi, C.R., Stephenson, K. and Olson, M.S. (1990) J. Biol. Chem. **265**, 17432-17435.

Ganong, B.R., Loomis, C.R., Hannun, Y.A. and Bell, R.M. (1986) Proc. Natl. Acad. Sci. USA **83**, 1184-1188.

Gee, N.S., Ragan, C.I., Watling, K.J., Aspley, S., Jackson, R.G., Reid, G.G., Gani, D. and Shute, J.K. (1988) Biochem. J. **249**, 883-889.

Geny, B., Stutchfield, J. and Cockcroft, S. (1989) Cell. Signal. **1**, 165-172.

Ghosh Choudhury, G., Sylvia, V.L. and Sakaguchi, A.Y. (1991) J. Biol. Chem. **266**, 23147-23151.

Giaid, A., Gibson, S.J., Ibrahim, N.B.N., Legon, S., Bloom, S.R., Yanagisawa, M., Masaki, T., Varndell, I.M. and Pdak, J.M. (1989) Proc. Natl. Acad. Sci. USA **86**, 7634-7638.

Gierschik, P., Sidiropoulos, D. and Jakobs, K.H. (1989) J. Biol. Chem. **264**, 21470-21473.

Gil, J., Higgins, T. and Rozengurt, E. (1991) J. Cell Biol. **113**, 943-950.

Gilman, A.G. (1987) Ann. Rev. Biochem. **56**, 615-649.

Go, M., Sekiguchi, K., Nomura, H., Kikkawa, U. and Nishizuka, Y. (1987) Biochem. Biophys. Res. Commun. **144**, 598-605.

Goetz, K.L., Wang, B.C., Madwed, J.B., Zhu, J.L. and Leadley, R.J. (1988) Am. J. Physiol. **255**, R1064-R1068.

Gómez, N. and Cohen, P. (1991) Nature **353**, 170-173.

Greenberg, M.E. and Ziff, E.B. (1984) Nature **311**,433-438.

Griendling, K.K., Tsuda, T. and Alexander, R.W. (1989) J. Biol. Chem. **264**, 8237-8240.

Grillone, L.R., Clark, M.A., Godfrey, R.W., Stassen, F. and Crooke, S.T. (1988) J. Biol. Chem. **263**, 2658-2663.

Grinstein, S., Rotin, D. and Mason, M.J. (1989) Biochem. Biophys. Acta **988**, 73-97.

Guy, G.R., Gordon, J., Walker, L., Michell, R.H. and Brown, G. (1986) Biochem. Biophys. Res. Commun. **135**, 146-153.

Hagiwara, H., Kozuka, M., Eguchi, S., Shibabe, S., Ito, T. and Hirose, S. (1990) Biochem. Biophys. Res. Commun. **172**, 576-581

Halter, H., Smallwood, J.I. and Rasmussen, H. (1990) Biochem. J. **270**, 375-381.

Harden, T.K., Hawkins, P.T., Stephens, L.and Boyer, J.L. (1988) Biochem. J. **252**, 583-593.

Hasegawa-Sasaki, H. (1985) Biochem. J. **232**, 99-109.

Hasegawa-Sasaki, H., Lutz, F. and Sasaki, T. (1988) J. Biol. Chem. **263**, 12970-12976.

Heldin, N., Paulsson, Y., Forsberg, K., Heldin, C. and Westermark, B. (1989) J. Cell. Physiol. **138**, 17-23.

Henry, P.J., Rigby, P.J., Self, G.J., Preuss, J.M. and Goldie, R.G. (1990) Br. J. Pharmacol. **100**, 786-792.

Hepler, J.R., Jeffs, R.A., Huckle, W.R., Outlaw, H.E., Rhee, S.G., Earp, H.S. and Harden, T.K. (1990) Biochem. J. **270**, 337-344.

Hesketh, T.R., Moore, J.P., Morris, J.D.H., Taylor, M.V., Rogers, J., Smith, G.A. and Metcalfe, J.C. (1985) Nature **313**, 481-484.

Hesketh, T.R., Morris, J.D.H., Moore, J.P. and Metcalfe, J.C. (1988) J. Biol. Chem. **263**, 11879-11886.

Hill, T.D., Dean, N.M., Mordan, L.J., Lau, A.F., Kanemitsu, Y. and Boynton, A.L. (1990) Science **248**, 1660-1663.

Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T.X., Kumagai,S., Nakajima, K. andSakakibara, S. (1988) Biochem. Biophys. Res. Commun. **154**, 868-875.

Hosoda, K., Nakao, K., Arai, H., Suga, S., Ogawa, Y., Mukoyama, M., Shirakami, G., Saito, Y., Nakanishi, S. and Imura, H. (1991) FEBS Lett. **287**, 23-26.

Hoth, M. and Penner, R. (1992) Nature **355**, 353-356.

Housey, G.M., Johnsin, M.D., Hsiao, W.L.W., O'Brien, C.A., Murphey, J.P., Kirshmeir, P. and Weinstein, I.B. (1988) Cell **52**, 343-354.

Huang, K., Nakabayashi, H. and Huang, F. L. (1986) Proc. Natl. Acad. Sci. USA **83**, 8535-8539.

Huang, F.L., Yoshida, Y., Nakabayashi, H. and Huang, K. (1987) J. Biol. Chem. **262**, 15714-15720.

Huang, C. and Ives, H.E. (1989) J. Biol. Chem. **264**, 4391-4397.

Huang, C. and Cabot, M.C. (1990a) J. Biol. Chem. **265**, 14858-14863.

Huang, C. and Cabot, M.C. (1990b) J. Biol. Chem. **265**, 17468-17473.

Huang, R., Kucera, G.L. and Rittenhouse, S.E. (1991) J. Biol. Chem. **266**, 1652-1655.

Hurst, K.M., Hughes, B.P. and Barritt, G.J. (1990) Biochem. J. **272**, 749-753.

Ido, M., Sekiguchi, K., Kikkawa, V. and Nishizuka, Y. (1987) FEBS Lett. **219**, 215-218.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (1989a) Proc. Natl. Acad. Sci. USA **86**, 2863-2867.

Inoue, A., Yanagisawa, M., Takuwa, Y., Mitsui, Y., Kobayashi, M. and Masaki (1989b) J. Biol. Chem. **264**, 14954-14959.

Irvine, R.F., Brown, K.D. and Berridge, M.J. (1984) Biochem. J. **222**, 269-272.

Irvine, R.F. and Moor, R.M. (1986) Biochem J. **240**, 917-920.

Irvine, R.F., Moor, R.M., Pollock, W.K., Smith, P.H. and Wreggett, K.A. (1988) Phil. Trans. R. Soc. Lond. **B 320**, 281-298.

Irvine, R.F. (1990) FEBS Lett. **263**, 5-9.

Irving, H.R. and Exton, J.H. (1987) J. Biol. Chem. **262**, 3440-3443.

Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K. and Masaki, T. (1988a) Am. J. Physiol. **155**, H970-H973.

Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K. and Masaki, T. (1988b) Pflugers Arch. **413**, 108-110.

Ives, H.E. and Daniel, T.O. (1987) Proc. Natl. Acad. Sci. USA **84**, 1950-1954.

Ives, H.E. (1991) Cell. Signall. **3**, 491-499.

Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsui, Y., Onda, H., Fujino, M. and Masaki, T. (1988) FEBS Lett. **231**, 440-444.

Jackson, T.R., Hallam, T.J., Downes, C.P. and Hanlet, M.R. (1987) EMBO J. **6**, 49-54.

Jalink, K., van Corven, E.J. and Moolenaar, W.H. (1990) J. Biol. Chem. **265**, 12232-12239.

Jeno, P., Ballou, L.M., Novak-Hofer, I.and Thomas, G. (1988) Proc. Natl. Acad. Sci. USA **85**, 406-410.

Jeno, P., Jaggi, N., Luther, H., Siegmann, M. and Thomas, G. (1989) J. Biol. Chem. **262**, 1293-1297.

Johnson, R.M. and Garrison, J.C. (1987) J. Biol. Chem. **262**, 17285-17293.

Kai, H., Kanaida, H. and Nakamura, M. (1989) Biochem. Biophys. Res. Commun. **158**, 235-243.

Kaibuchi, K., Tsuda, T., Kikuchi, A., Tanimoto, T., Yamashita, T. and Takai, Y. (1986) J. Biol. Chem. **261**, 1187-1192.

Kanoh, H., Yamada, K. and Sakane, F. (1990) TIBS **15**, 47-50.

Kanse, S.M., Ghatei, M.A. and Bloom, S.R. (1989) Eur. J. Biochem. **182**, 175-179.

Kaplan, D.R., Whitman, M., Schaffhausen, B., Pallas, D.C., White, M., Cantley, L. and Roberts, T.M. (1987) Cell **50**, 1021-1029.

Kaplan, D.R., Morrison, D.K., Wong, G., McCormick, F. and Williams, L.T. (1990) Cell **61**, 125-133.

Katsaros, D., Tortora, G., Tagliaferri, P., Clair, T., Ally, S., Neckers, L., Robins, R.K. and Cho-Chung, Y.S. (1987) FEBS Lett. **223**, 97-103.

Kawaguchi, H., Sawa, H. and Yasuda, H. (1990) J. Mol. Cell. Cardiol. **22**, 839-842.

Kawahara, Y., Kariya, K., Araki, S., Fukuzaki, H. and Takai, Y. (1988) Biochem. Biophys. Res. Commun. **156**, 846-854.

Kazlauskas, A. and Cooper, J.A. (1989) Cell **58**, 1121-1133.

Kazlauskas, A., Ellis, C., Pawson, T. and Cooper, J.A. (1990), Science **247**, 1578-1581.

Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989) Ann. Rev. Biochem. **58**, 31-44.

Kimura, S., Yoshitoshi, K., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1988) Biochem. Biophys. Res. Commun. **156**, 1182- 1186.

Kimura. S., Kasuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1989) J. Cardiovasc. Pharmacol. **13**, S5-S7.

King, A.J., Brenner, B.M. and Anderson, S. (1989) Am. J. Physiol. **256**, F1051-F1058.

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) J. Biol. Chem. **255**, 2273-2276.

Kloog, Y., Ambar, I., Sokolovsky, M., Kochua, E., Wollberg, Z. and Bdolah, A. (1988) Science **242**, 268-270.

Kloog, Y. and Sokolovsky, M. (1989) TiPS **10**, 212-214.

Kloog, Y., Bousso-Mittler, D., Bdolah, A. and Sokolovsky, M. (1989) FEBS Lett. **253**, 199-202.

Knauss, T.C., Jaffer, F.E. and Abboud, H.E. (1990) J. Biol. Chem. **265**, 14457-14463.

Kolch, W., Heidecker, G., Lloyd, P. and Rapp, U.R. (1991) Nature **349**, 426-428.

Komuro, I., Kurihara, H., Sugiyawa, T., Takaku, F. and Yazaki, Y. (1988) FEBS Lett. **238**, 249-252.

Koseki, C., Imai, M., Hirata, Y., Yanagisawa, M. and Masaki, T. (1989) Am. J. Physiol. **256**, R858-R866.

Kozuka, M., Ito, T., Hirose, S., Lodhi, K.M. and Hagiwara, H. (1991) J. Biol. Chem. **266**, 16892-16896.

Kraft, A.S. and Anderson, W.B. (1983) Nature **301**, 621-623.

Kruijer, W., Cooper, J.A., Hunter, T. and Verma, I.M. (1984) Nature **312**, 711-720.

Kurihara, H., Yoshizumi, M., Sugiyama, T., Takaku, F., Yanagisawa, M., Masaki, T., Hamaoki, M., Kato, H. and Yazaki, Y. (1989) Biochem. Biophys. Res. Commun. **159**, 1435-1440.

Lacal, J.C., Moscat, J. and Aaronson, S.A. (1987) Nature **330**, 269-272.

L'Allemain, G., Pouyssegur, J and Weber, M.J. (1991a) Cell Reg. **2**, 675-684.

L'Allemain, G., Sturgill, T.W. and Weber, M.J. (1991b) Mol. Cell. Biol. **11**, 1002-1008.

Lambert, D.G., Challis, R.A.J. and Nahorski, S.R. (1991) Biochem. J. **273**, 791-794.

Larrodera, P., Cornet, M.E., Diaz-Meco, M.T., Lopez-Barahona, M., Diaz-Laviada, I., Guddal, P.H., Johansen, T. and Moscat, J. (1990) Cell **61**, 1113-1120.

Leach, K.L., Ruff, V.A., Wright, T.M., Pessin, M.S. and Raben, D.M. (1991) J. Biol. Chem. **266**, 3215-3221.

Lee, T., Chao, T., Hu, K. and King, G.L. (1989) Biochem. Biophys. Res. Commun. **162**, 381-386.

Lee, M., de la Monte, S., Ng, S., Bloch, K.D. and Quertermous, T. (1990) J. Clin. Invest. **86**, 141-147.

Lee, R., Cobb, M.H. and Blachshear, P.J. (1992) J. Biol. Chem. **267**, 1088-1092.

Lewin, B. (1990) Cell **61**, 743-752.

Levitzki, A. in "G Proteins as Mediators of Cellular Signalling Processes" (Eds, Houslay, M.D.& Milligan, G), pp1-14 John Wiley & Sons Ltd., England.

Li, P., Wood, K., Mamon, H., Haser, W. and Roberts, T. (1991) Cell **64**, 479-482.

Lin, W., Lee, C.Y. and Chuang, D. (1990) Biochem. Biophys. Res. Commun. **168**, 512-519.

Lips, D.L., Makerus, P.W., Gorga, F.R., Young, A.T. and Benjamin, T.L. (1989) J. Biol. Chem. **264**, 8759-8763.

Liscovitch, M. (1989) J. Biol. Chem. **264**, 1450-1456.

Litosch, I (1990) in "G Proteins as Mediators of Cellular Signalling Processes" (Eds., Houslay, M.D. & Milligan, G.), pp151-171, John Wiley & Sons Ltd., England.

Löffelholz, K. (1989) Biochem. Pharmacol. **38**, 1543-1549.

Lopez-Barahona, M., Kaplan, P.L., Cornet, M.E., Diaz-Meco, M.T.,

Larrodera, P., Diaz-Laviada, I., Municio, A.M. and Moscat, J. (1990) J. Biol. Chem. **265**, 9022-9026.

Lopez-Rivas, A. and Rozengurt, E. (1983) Biochem. Biophys. Res. Commun. **114**, 240-247.

Lowry, O.H., Rosenburgh, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193**, 265-275.

Lückhoff, A. and Clapham, D.E. (1992) Nature **355**, 356-358.

MacCumber, M.W., Ross, C.A., Glser, B.M. and Snyder, S.H. (1989) Proc. Natl. Acad. Sci. USA **86**, 7285-7289.

MacCumber, M.W., Ross, C.A. and Snyder, S.H. (1990) Proc. Natl. Acad. Sci. USA **87**, 2359-2363.

Margolis, B., Rhee, S.G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A. and Schlessinger, J. (1989) Cell **57**, 1101-1107.

Margolis, B., Bellot, F., Honegger, A.M., Ullrich, A., Schlessinger, J. and Zilberstein, A. (1990) Mol. Cell. Biol. **10**, 435-441.

Marin, P., Delumeau, J.C., Durieu-Trautmann, U., Le Nguyen, D., Premont, J., Strosberg, A.D. and Couraud, P.O. (1991) J. Neurochem. **56**, 1270-1275.

Maroney, A.C. and Macara, I.G. (1989) J. Biol. Chem. **264**, 2537-2544.

Marsault, R., Vigne, P. and Frelin, C. (1990) Bichem. Biophys. Res. Commun. **171**, 301-305. Marsden, P.A., Danthuluri, N.R., Brenner, B.M., Ballermann, B.J. and Brock, T.A. (1989) Biochem. Biophys. Res. Commun. **158**, 86-93.

Marston, S.B. and Redwood, C.S. (1991) Biochem. J. **279**, 1-16.

Martin, T.W. and Michaelis, K. (1989) J. Biol. Chem. **264**, 8847-8856.

Martin, E.R., Brenner, B.M. and Ballermann, B.J. (1990a) J. Biol. Chem. **265**, 14044-14049.

Martin, T.F.J., Hsieh, K.P. and Porter, B.W. (1990b) J. Biol. Chem. **265**, 7623-7631.

Martin, T.F.J., Lewis, J.E. and Kowalchyk, J.A. (1991) Biochem. J. **280**, 753-760.

Martin-Perez, J., Siegmann. M. and Thomas, G. (1984) Cell **36**, 287-294.

Martinson, E.A., Goldstein, D. and Heller Brown, J. (1989) J. Biol. Chem. **264**, 14748-14754.

Martinson, E.A., Trilivas, I. and Heller Brown, J. (1990) J. Biol. Chem. **265**, 22282-22287.

Matozaki, T. and Williams, J.A. (1989) J. Biol. Chem. **264**, 14729-14734.

Matuoka, K., Fukami, K., Nakanishi, O., Kawai, S. and Taenawa, T. (1988) Science **239**, 640-643.

McKay, K.O., Black, J.L. and Armour, C.L. (1991) Br. J. Pharmacol. **102**, 422-428.

Meisenhelder, J., Suh, P., Rhee, S.G. and Hunter, T. (1989) Cell **57**, 1109-1122.

Meldolesi, J., Pandiella, A., Valler, L., Madeddu, L., Malgaroli, A., Fasolato, C. and Pozzan, T. in "Transmembrane Signalling, Intracellular Messengers and Implications for Drug Development " (Ed , S. Nahorski) John Wiley & Sons, Chichester.

Meldolesi, J., Clementi, E., Fasolato, C., Zacchetti, D. and Pozzan, T. (1991) TiPS **12**, 289-292.

Meldrum, E., Parker, P.J. and Carozzi, A. (1991) Biochim. Biophys. Acta **1092**, 49-71.

Michell, R.H. (1975) Biochim. Biophys. Acta **415**, 81-147.

Mignery, G.A., Sudhof, T.C., Takei, K. and De Camilli, P. (1989) Nature **342**, 192-195.

Miller, W.L., Redfield, M.M. and Burnett, J.C. (1989) J. Clin. Invest. **83**, 317-320.

Milligan, G., Carr, C., Gould, G.W., Mullaney, I. and Lavan, B.E. (1991) J. Biol. Chem. **266**, 6447-6455.

Miyauchi, T., Tomobe, Y., Shiba, R., Ishikawa, T., Yanagisawa, M., Kimura, S., Sugishita, Y., Ito, I., Goto, K. and Masaki, T. (1990) Circulation **81**, 1874-1880.

Moolenaar, W.H., Tsien, R.Y., van der Saag, P.T. and de Laat, S.W. (1983) Nature **304**, 645-648.

Moolenaar, W.H., Tertoolen, L.G.J. and de Laat, S.W. (1984) J. Biol. Chem **259**, 8066-8069.

Moolenaar, W.H., Kruijer, W., Tilly, B.C., Verlaan, I., Bierman, A.J. and de Laat, S.W. (1986) Nature **323**, 171-173.

Morris, J.D.H., Metcalfe, J.C., Smith, G.A., Hesketh, T.R. and Taylor, M.V. (1984) FEBS Lett. **169**, 189-193.

Morris, A.P., Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1987) Nature **330**, 653-655.

Morrison, D.K., Kaplan, D.R., Rapp, U. and Roberts, T.M. (1988) Proc. Natl. Acad. Sci. USA **85**, 8855-8859.

Morrison, D.K., Kaplan, D.R., Escobedo, J.A., Rapp, U.R., Roberts, T.M. and Williams, L.T. (1989) Cell **58**, 649-657.

Mousli, M., Bueb, J., Bronner, C., Rouot, B. and Landry, Y. (1990) TiPS **11**, 358-362.

Mufson, R.A., Okin, E. and Weinstein, I.B. (1981) Carcinogenesis **2**, 1095-1102.

Muir, J.G. and Murray, A.W. (1987) J. Cell. Physiol. **130**, 382-391.

Muldoon, L.L., Rodland, K.D., Forsythe, M.L. and Magun, B.E. (1989) J. Biol. Chem. **264**, 8529-8536.

Muldoon, L.L., Pribnow, D., Rodland, K.D. and Magun, B.E. (1990) Cell Regulation **1**, 379-390.

Müller, R., Bravo, R., Burckhardt, J., Curran, T. (1984) Nature **312**, 716-720.

Murayama, T. and Ui, M. (1987) J. Biol. Chem. **262**, 5522-5529.

Nahorski, S.R., Ragan, I. and Challis, R.A.J. (1991) TiPS **12**, 297-303.

Nakaki, T., Nakayama, M., Yamamoto, S. and Kato, R. (1989) Biochem. Biophys. Res. Commun. **158**, 880-883.

Nakamuta, M., Takayanagi, R., Sakai, Y., Sakamoto, S., Hagiwara, H., Mizuno, T., Saito, Y., Hirose, S., Yamamoto, M. and Nawata, H. (1991) Biochem. Biophys. Res. Commun. **177**, 34-39.

Naor, Z, (1991) Mol. Cell. Endocrinol. **80**, C181-C186.

Naor, Z., Shearman, M.S., Kishimoto, A. and Nishizuka, Y. (1988) Mol. Endocrin. **2**, 1043-1048.

Nayler, W.G. (1990) TiPS **11**, 96-99.

Niedel, J.E., Kuhn, L.J. and Vandenbark, G.R. (1983) Proc. Natl. Acad. Sci. USA **80**, 36-40.

Nishibe, S., Wahl, M.I., Hernandez-Sotomayor, S.M.T., Tonks, N.K., Rhee, S.G. and Carpenter, G. (1990) Science **250**, 1253-1256.

Nishizuka, Y. (1984) Nature **308**, 693-698.

Nishizuka, Y. (1986) Science **233**, 305-312.

Nishizuka, Y. (1988) Nature **334**, 661-665.

Nunez, D.J.R., Brown, M.J., Davenport, A.P., Neylon, C.B., Schofield, J.P. and Wyse, R.K. (1990) J. Clin. Invest. **85**, 1537-1541.

Nurse, P. (1990) Nature **344**, 503-508.

Ogawa, Y., Nakao, K., Arai, H., Nakagawa, O., Hosada, K., Suga, S., Nakanishi, S. and Imura, H. (1991) Biochem. Biophys. Res. Commun. **178**, 248-255.

Ohnishi, A., Yamaguchi, K., Kusuhara, M., Abe, K. and Kimura, S. (1989) Biochem. Biophys. Res. Commun. **161**, 489-495.

Ohsako, S. and Deguchi, T. (1981) **256**, 10945-10948.

O'Keefe, E.J. and Pledger, W.J. (1983) Mol. Cell. Endocrin. **31**, 167-186.

Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U. and Nishizuka, Y.(1989) Proc. Natl. Acad. Sci. USA **86**, 4868-4871.

Orellana, S., Solski, P.A. and Brown, J.H. (1987) J. Biol. Chem. **262**, 1638-1643.

Otten, J., Johson, G.S. and Pastan, I. (1972) J. Biol. Chem. **247**, 7082-7087.

Pai, J., Siegel, M.I., Egan, R.W. and Billah, M.M. (1988a) J. Biol. Chem. **263**, 12472-12477.

Pai, J., Siegel, M.I., Egan, R.W. and Billah, M.M. (1988b) Biochem. Biophys. Res. Commun. **150**, 355-364.

Pai, J., Pachter, J.A., Weinstein, I.B. and Bishop, W.R. (1991) Proc. Natl. Acad. Sci. **88**, 598-602.

Pai, J., Dobek, E.A. and Bishop, W.R. (1991) Cell. Regulation **2**, 897-903.

Palczewski, K. and Benovic, J.L. (1991) TIBS **16**, 387-391.

Palmer, S., Hughes, K.T., Lee, D.Y. and Wakelam, M.J.O. (1989) Cell. Signall. **1**, 147-156.

Pandiella, A., Beguinot, L., Vicentini, L.M. and Meldolesi, J. (1989) TiPS **10**, 411-414.

Pang, I. and Sternweis, P.C. (1990) J. Biol. Chem. **265**, 18707-18712.

Pardee, A.B. (1989) Science **246**, 603-608.

Park, D.J., Min, H.K. and Rhee, S.G. (1991) J. Biol. Chem. **266**, 24237-24240.

Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, C., Schaap, D., Stabel, S. and Webster, C. (1989) Mol. Cell. Endocrin. **65**, 1-11.

Pelech, S.L. and Vance, D.E. (1989) TIBS **14**, 28-30.

Penner, R., Matthews, G. and Neher, E. (1988) Nature **334**, 499-504.

Pessin, M.S. and Raben, D.M. (1989) J. Biol. Chem. **264**, 8729-8738.

Pfeilschifter, J. and Bauer, C. (1987) Biochem. J. **248**, 209-215.

Pickford, L.B., Polverino, A.J. and Barritt, G.J. (1987) Biochem. J. **245**, 211-216.

Plevin, R., Palmer, S., Gardner, S.D. and Wakelam, M.J.O. (1990) Biochem. J. **268**, 605-610.

Plevin, R., Cook, S.J., Palmer, S. and Wakelam, M.J.O. (1991) Biochem. J. **279**, 559-565.

Plevin, R. and Wakelam, M.J.O. (1992) Biochem. J. in press.

Pouysségur, J., Franchi, A., L'Allemain, G. and Paris, S. (1985) FEBS Lett. **190**, 115-119.

Pouysségur, J. (1985) TIBS **10**, 453-455.

Pouysségur, J., Kahan, C., Magnaldo, I. and Seuwen, K. (1990) in "Transmembrane Signalling, Intracellular Messengers and Implications for Drug Development.", (Ed. S.R. Nahorski), John Wiley & Sons Ltd., Chichester.

Price, B.D., Morris, J.D.H. and Hall, A. (1989) Biochem J. **264**, 509-515.

Pulverer, B.J., Kyriakis, J.M., Avruh, J., Nikolakiki, E. and Woodgett, J.R. (1991) Nature **353**, 670-674.

Qian, Z. and Drewes, L.R. (1989) J. Biol. Chem. **264**, 21720-21724.

Reiser, G. (1990) Br. J. Pharmacol. **101**, 722-726.

Reisine, T. (1990) Biochem. Pharmacol. **39**, 1499-1504.

Resink, T.J., Scott-Burden, T. and Bühler, F.R. (1988) Biochem. Biophys. Res. Commun. **157**, 1360-1363.

Resink, T.J., Scott-Burden, T. and Bühler, F.R. (1989) Biochem. Biophys. Res. Commun. **158**, 279-286.

Resink, T.J., Scott-Burden, T. and Bühler, F.R. (199a) Eur. J. Biochem. **189**, 415-421.

Resink, T,J,, Scott-Burden, T., Weber, E. and Bühler, F.R. (1990b) Biochem. Biophys. Res. Commun. **166**, 1213-1219.

Reynolds, E.E., Mok, L.L.S. and Kurokawa, S. (1989) Biochem. Biophys. Res. Commun. **160**, 868-873.

Rhee, S.G. (1991) TIBS **16**, 297-301.

Rodriguez-Pena, A. and Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. **120**, 1053-1059.

Ross, C.A., Meldolesi, J., Milner, T.A., Satoh, T., Supattapone, S. and Snyder, S.H. (1989) Nature **339**, 468-470.

Rossoff, P.M., Savage, N. and Dinarello, C.A. (1988) Cell **54**, 73-81.

Rossomando, A.J., Payne, D.M., Weber, M.J. and Sturgill, T.W. (1989) Proc. Natl. Acad. Sci. USA **86**, 6940-6943.

Roubert, P., Gillard, V., Plas, P., Guillon, J., Chabrier, P. and Braquet, P. (1989) Biochem. Biophys. Res. Commun. **164**, 809-815.

Rozengurt, E., Legg, A., Strang, G. and Courtenay-Luck, N. (1981) Proc. Natl. Acad. Sci. USA **78**, 4392-4396.

Rozengurt, E., Stroobant, P., Waterfield, M.D., Deuel, T.F. and Keehan, M. (1983) Cell **34**, 265-272.

Rozengurt, E. (1985) in "Molecular Aspects of Cell Regulation", Vol. 4, "Molecular Aspects of Transmembrane Signalling" (Eds., Cohen, P. & Houslay , M.D.) pp429-452. Elsevier, New York .

Rozengurt, E. (1986) Science **234**, 161-166.

Rozengurt, E. and Sinnett-Smith, J.W. (1987) J. Cell. Physiol. **131**, 218-225.

Rubanyi, G.M. and Parker Botelho, L.H. (1991) FASEB J. **5**, 2713-2720.

Ryder, K. and Nathans, D. (1988) Proc. Natl. Acad. Sci. USA **85**, 8464-8467.

Ryu, S.H., Kim, U.H., Wahl, M.I., Brown, A.B., Carpenter, G., Huang, K.P. and Rhee, S.G. (1990) J. Biol. Chem. **265**, 17941-17945.

Sachinidis, A., Locher, R., Vetter, W., Tatje, D. and Hoppe, J. (1990) J. Biol. Chem. **265**, 10238-10243.

Saida, K., Mitsui, Y. and Ishida, N. (1989) J. Biol. Chem. **264**, 14613-14616.

Saito, Y., Mizuno, T., Itakura, M., Suzuki, Y., Ito, T., Hagiwara, H. and Hirose, S. (1991) J. Biol. Chem. **266**, 23433-23437.

Sakamoto, A., Yanagisawa, M., Sakurai, T., Takuwa, Y., Yanagisawa, M. and Masaki, T. (1991) Biochem. Biophys. Res. Commun. **178**, 656-663.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) Nature **348**, 732-735.Sakurai, T., Yanagisawa, M. and Masaki, T. (1992) TiPS **13**, 103-108.

Sardet, C., Counillon, L., Franchi, A. and Pouysségur, J. (1990) Science **247**, 723-726.

Sawamura, T., Kimura, S., Shinmi, O., Sugita, Y., Yanagisawa, M. and Masaki, T. (1989) Biochem. Biophys. Res. Commun. **162**, 1287-1294.

Schaap, D. and Parker, P.J. (1990) J. Biol. Chem. **265**, 7301-7307.

Schliwa, M., Nakamura, T., Porter, K.R. and Euteneuer, U. (1984) J. Cell. Biol. **199**, 1045-1059.

Seger, R., Ahn, N.G., Boulton, T.G., Yancopoulos, G.D., Panayotatos, N., Radziejewska, E., Ericsson, L., Bratlien, R.L., Cobb, M.H. and Krebs, E.G. (1991) Proc. Natl. Acad. Sci. USA **88**, 6142-6146.

Serradeil-Le Gal, C., Jouneaux, C., Sanchez-Bueno, A., Raufaste, D., Roche, B., Préaux, A.M., Maffrand, J.P., Cobbold, P.H., Hanoune, J. and Lotersztajn, S. (1991) J. Clin. Invest. **87**, 133-138.

Serunian, L.A., Haber, M.T., Fukui, T., Kim, J.W., Rhee, S.G., Lowenstein, J.M. and Cantley, L.C. (1989) J. Biol. Chem. **264**, 17809-17815.

Seuwen, K., Magnaldo, I. and Pouysségur, J. (1988) Nature **335**, 254-256.

Seuwen, K., Kahan, C., Hartmann, T. and Pouysségur, J. (1990) J. Biol. Chem. **265**, 22292-22299.

Shears, S.B. (1989) Biochem. J. **260**, 313-324.

Shiba, R., Yanagisawa, M., Miyauchi, T., Ishii, J.Y., Kimura, S., Muchiyama, Y., Masaki, T.and Goto, K. (1989) J.

Cardiovasc.Pharmacol. **13(Suppl. 5)**, S98-S101.

Shichiri, M., Hirata, Y., Nakajima, T., Ando, K., Imai, T., Yanagisawa, M., Masaki, T. and Marumo, F. (1991) J. Clin. Invest. **87**, 1867-1871.

Shinomura, T., Asaoka, Y., Oka, M., Yosida, K. and Nishizuka, Y. (1991) Proc. Natl. Acad. Sci. USA **88**, 5149-5153.

Sim. S.S., Kim, J.W. and Rhee, S.G. (1990) J. Biol. Chem. **265**, 10367-10372.

Simon, M.I., Strathmann, M.P. and Gautam, N. (1991) Science **252**, 802-808.

Simonson, M.S., Wann, S., Mené, P., Dubyak, G.R., Kester, M., Nakazato, Y., Sedor, J.R. and Dunn, M.J. (1989) J. Clin. Invest. **83**, 708-712.

Simonson, M.S., Osanai, T. and Dunn, M.J. (1990) Biochim. Biophys. Acta **1055**, 63-68.

Simonson, M.S. and Dunn, M.J. (1990a) FASEB J. **4**, 2980-3000.

Simonson, M.S. and Dunn, M.J. (1990b) Hypertension **15(Suppl. 1)**, I 5-I 12

Simonson, M.S. and Dunn, M.J. (1990c) J. Clin. Invest. **85**, 790-797.

Simonson, M.S. and Dunn, M.J. (1991a) Hypertension **17**, 856-863.

Simonson, M.S. and Dunn, M.J. (1991b) Exptl. Cell Res. **192**, 148-156.

Simpson, A.W.M. and Ashley, C.C. (1989) Biochem Biophys. Res. Commun. **163**, 1223-1229.

Slivka, S.R., Meier, K.E. and Insel, P.A. (1988) J. Biol. Chem. **263**, 12242-12246.

Smets, L.A. and van Rooy, H. (19870 J. Cell. Physiol. **133**, 395-399.

Smith, J.B. and Smith, L. (1984) Biochem. Biophys. Res. Commun. **123**, 803-809.

Smith, C.D., Uhing, R.J. and Snyderman, R. (1987) J. Biol.Chem. **262** 6121-6127.

Smith, M.R., Heidecker, G., Rapp, U.R. and Kung, H. (1990a) Mol. Cell. Biol. **10**, 3828-3833.

Smith, M.R., Liu, Y., Kim, H., Rhee, S.G. and Kung, H. (1990b) Science **247**, 1074-1077.

Smrcka, A.V., Hepler, J.R., Brown, K.D. and Sternweis, P.C. (1991) Science **251**, 804-807.

Staudermann, K.A., Harris, G.D. and Lovenberg, W. (1988) Biochem J, **255**, 677-683.

Stojilkovic, S.S., Merelli, F., Iida, T., Krsmanovic, L.Z. and Catt, K.J. (1990) Science **248**, 1663-1666.

Strathmann, M., Wilkie, T. and Simon, M.I. (1989) Proc. Natl. Acad. Sci. USA **86**, 7407-7409.

Strathmann, M. and Simon, M.I. (1990) Proc. Natl. Acad. Sci. USA **87**, 9113-9117.

Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Nature **306**, 67-69.

Streb, H., Bayerdörffer, E., Haase, W., Irvine, R.F. and Schulz, I. (1984) J. Memb. Biol. **81**, 241-253.

Sturgill, T.W., Ray, L.B., Erikson, E. and Maller, J.L. (1988) Nature **334**, 715-718.

Sturgill, T.W. and Wu, J. (1991) Biochim. Biophys. Acta **1092**, 350-357.

Sugiura, M., Snajdar, R.M., Schwartzberg, M., Badr, K.F. and Inagami, T. (1989a) Biochem. Biophys. Res. Commun. **162**, 1396-1401.

Sugiura, M., Inagami, T., Hare, G.M.T. and Johns, J.A. (1989b) Biochem. Biophys. Res. Commun. **158**, 170-176.

Supattapone, S., Worley, P.F., Baraban, J.M. and Snyder, S.H. (1988) J. Biol. Chem. **263**, 1530-1534.

Suzuki-Sekimori, R., Matuoka, K., Nagai, Y. and Takenawa, T. (1989) J. Cell. Physiol. **140**, 432-438.

Takagi, M., Matsuoka, H., Atarashi, K. and Yagi, S. (1988) Biochem. Biophys. Res. Commun. **157**, 1164-1168.

Takagi, Y., Fukase, M., Takata, S., Yoshimi, H., Tokunaga, O. and Fujita, T. (1990) Biochem. Biophys. Res. Commun. **168**, 537-543.

Takahashi, K., Jonews, P.M., Kanse, S.M., Lam, H., Spokes, R.A., Ghatei, M.A. and Bloom, S.R. (1990) Gastroenterology **99**, 1660-1667.

Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Veda, M., Hanaoka, M., Hirose, T.and Numa, S. (1989) Nature **339**, 439-445.

Takuwa, N., Takuwa, Y. and Rasmussen, H. (1988) J. Biol. Chem. **263**, 9738-9745.

Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K. and Masaki, T. (1989) J. Biol. Chem. **264**, 7856-7861.

Takuwa, Y., Kasuya, Y., Takuwa, N., Kudo, M., Yanagisawa, M., Goto, K., Masaki, T. and Yamashita, K. (1990) J. Clin. Invest. **85**, 653-658.

Takuwa, N., Iwamoto, A., Kumada, M., Yamashita, K. and Takuwa, Y. (1991) J. Biol. Chem. **266**, 1403-1409.

Tagliaferri, P., Katsaros, D., Clair, T., Neckers, L., Robins, R.K. and Cho-Chung, Y.S. (1988) J. Biol. Chem. **263**, 409-416.

Taylor, C.W. and Merritt, J.E. (1986) TiPS **7**, 238-242.

Taylor, C.W., Blakeley, D.M., Corps, A.N., Berridge, M.J. and Brown, K.D. (1988) Biochem. J. **249**, 917-920.

Taylor, C.W., Berridge, M.J., Cooke, A.M. and Potter, B.V.L. (1989) Biochem. J. **259**, 645-650.

Taylor, S.J., Smith, J.A. and Exton, J.H. (1990) J. Biol. Chem. **265**, 17150-17156.

Taylor, S.J., Chae, H.Z., Rhee, S.G. and Exton, J.H. (1991) Nature **350**, 516-518.

Thastrup, O., Cullen, P.J., Drøbak, B.K., Hanley, M.R. and Dawson, A.P. (1990) Proc. Natl. Acad. Sci. USA **87**, 2466-2470.

Thomas, C.P., Kester, M. and Dunn, M.J. (1991) Am. J. Physiol. **260**, F347-F352.

Thomas, G. (1992) Cell **68**, 3-6.

Todderud, G., Wahl, M.I., Rhee, S.G. and Carpenter, G. (1990) Science **249**, 296-298.

Tomita, K., Nonoguchi, H. and Marumo, F. (1990) J. Clin. Invest. **85**, 2014-2018.

Tonks, N.K. (1990) Current Opinions in Cell Biol. **2**, 1114-1124.

Touqui, L., Rothhut, B., Shaw, A.M., Fradin, A., Vargaftig, B.B. and Russo-Marie, F. (1986) Nature **321**, 177-180.

Traynor-Kaplan, A.E., Harris, A.L., Thompson, B.L., Taylor, P. and Sklar, L.A. (1988) Nature **334**, 353-356.

Trilivas, I., McDonough, P.M. and Brown, J.H. (1991) J. Biol. Chem. **266**, 8431-8438.

Truett, A.P., Verghese, M.W., Dillon, S.B. and Snyderman, R. (1988) Proc. Natl. Acad.Sci. USA **85**, 1549-1553.

Tsai, M., Yu, C., Wei, F. and Stacey, D.W. (1989) Science **243**, 522-526.

Tsai, M., Yu, C. and Stacey D.W. (1990) Science **250**, 982-985.

Uhing, R.J., Prpic, V., Jiang, H. and Exton, J.H. (1986) J. Biol. Chem. **261**, 2140-2146.

Uings, I.J., Thompson, N.T., Randall, R.W., Spacey, G.D., Bonser, R.W., Hudson, A.T. and Garland, L.G. (1992) Biochem. J. **281**, 597-600.

Ullrich, A. and Schlessinger, J. (1990) Cell **61**, 203-212.

Vallar, L., Spada, A. and Giannattasio, G. (1987) Nature **330**, 566-568.

van Corven, E.J., Groenink, A., Jalink, K., Eichholtz, T. and Moolenaar, W.H. (1989) Cell **59**, 45-54.

van Corven, E.J., van Rijswijk, A., Jalink, K., van der Band, R.L., van Blitterswijk, W.J. and Moolenaar, W.H. (1992) Biochem. J. **281**, 163-169.

van der Meulen, J. and Haslam, R.J. (1990) Biochem. J. **271**, 693-700.

van Renterghen, C., Vigne, P., Barhanin, J., Schmid-Alliana, A., Frelin, C. and Lazdunski, M. (1988) Biochem. Biophys. Res. Commun. **157**, 977-985.

Vigne, P., Marsault, R., Breittmayer, J.P. and Frelin, C. (1990a) Biochem. J. **266**, 415-420.

Vigne, P., Breittmayer, J.P., Marsault, R. and Frelin, C. (1990b) J. Biol. Chem. **265**, 6782-6787.

Volpe, P., Krause, K., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J. and Lew, D.P. (1988) Proc. Natl. Acad. Sci. USA **85**, 1091-1095.

Wahl, M.I., Olashaw, N.E., Nishibe, S., Rhee, S.G., Pledger, W.J. and Carpenter, G. (1989) Mol. Cell. Biol. **9**, 2934-2943.

Wakelam, M.J.O., Davies, S.A., Houslay, M.D., McKay, I., Marshall, C.J. and Hall, A. (1986) Nature **323**, 173-176.

Wakelam, M.J.O. (1990) in "Handbook of Experimental Pharmacology", Vol. 94 / II (Eds., Cooper, C.S. & Grover, P.L.), Springer-Verlag, Berlin Heidelberg.

Wallnöffer, A., Weir, S., Rüegg, U. and Cauvin, C. (1989) Cardiovasc. Pharmacol. **13(Suppl. 5)**, S23-S31.

Watanabe, H., Miyazaki, H., Kondoh, M., Masuda, Y., Kimura, S., Yanagisawa, M., Masaki, T. and Murakami, K. (1989) Biochem. Biophys. Res. Commun. **161**, 1252-1259.

Webb, D.J. (1991) TiPS **12**, 43-46.

Whitman, M., Kaplan, D., Roberts, T. and Cantley, L. (1987) Biochem. J. **247**, 165-174.

Whitman, M. and Cantley, L. (1988) Biochim. Biophys. Acta **948**, 327-344.

Whitman, M., Downes, C.P., Keeler, M., Keller, T. and Cantley, L. (1988(Nature **332**, 644-646.

Wojcikiewicz, R.J.H., Lambert, D.G. and Nahorski, S.R. (1990) J. Neurochem. **54**, 676-685.

Woodcock, E.A., Little, P.J. and Tanner, J.K. (1990) Biochem. J. **271**, 791-796.

Wright, C.E. and Fozzard, J.R. (1988) Eur. J. Pharmacol. **155**, 201-203.

Wright, T.M., Rangan, L.A., Shin, H.S. and Raben, D.M. (1988) J. Biol. Chem. **263**, 9374-9380.

Wright, T.M., Shin, H.S. and Raben, D.M. (1990) Biochem. J. **267**, 501-507.

Wu, D., Lee, C.H., Rhee, S.G. and Simon, M.I. (1992) J. Biol. Chem. **267**, 1811-1817.

Yada, Y., Higuchi, K. and Imokawa, G. (1991) J. Biol. Chem. **266**, 18352-18357.

Yanagisawa, M., Kuriharo, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988a) Nature **332**, 411-415.

Yanagisawa, M., Inoue, A., Ishikawa, T., Kasuya, Y., Kimura, S., Kumagaye, S., Nakajima, K., Watanabe, T., Sakakibara, S., Goto, K. and Masaki, T. (1988b) Proc. Natl. Acad. Sci. USA **85**, 6964-6967.

Yanagisawa, M. and Masaki, T. (1989a) TiPS **10**, 374-378.

Yanagisawa, M. and Masaki, T. (1989b) Biochem. Pharmacol. **38**, 1877-1883.

Yanagisawa, M., Inoue, A., Takuaw, Y., Mitsui, Y., Kobayashi, M.and Masaki, T. (1989) J. Cardiovasc. Pharmacol. **13(Suppl. 5)**, S13-S17.

Yarden, Y. and Ullrich, A. (1988) Ann. Rev. Biochem. **57**, 443-478.

Yoshizawa, T., Shinmi, O., Giaid, A., Yanagisawa, M., Gibson, S.J., Kimura, S., Uchiyama, Y., Polak, J.M., Masaki, T. and Kanazawa, I. (1990) Science **247**, 462-464.

Young, S., Parker, P.J., Ullrich, A. and Stabel, S. (1987) Biochem. J. **244**, 775-779.

Zachary, I., Gil, J., Lehman, W., Sinnett-Smith, J. and Rozengurt, E. (1991a) Proc. Natl. Acad. Sci. USA **88**, 4577-4581.

Zachary, I., Sinnett-Smith, J. and Rozengurt, E. (1991b) J. Biol. Chem. **266**, 24126-24133.