# ACTIVATION OF PHOSPHOLIPASE D BY THE LHRH RECEPTOR AND ASSOCIATED MECHANISMS

**by**

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I declare that the studies presented in this thesis are the result of my own independent investigation with the exception of antiphosphotyrosine immunoblotting which was carried out by Jim Simpson, [$^{125}$I] buserelin binding studies which were carried out by Rory Mitchell, transfection of COS 7 and CHO cells with cDNA which was carried out by Eve Lutz, studies on the effect of piceatannol on PKC activity which were carried out by Melanie Johnson and experiments to determine the effect of DMV on LH secretion from the anterior pituitary which were carried out by Fiona Thompson. Radioimmunossays were also carried out with the assistence of John Bennie and Sheena Carroll.

This work has not been and is not being currently submitted for candidature for any other degree.

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**ABSTRACT**

The αT3-1 gonadotroph-derived cell line was used as a model system to study intracellular signalling systems associated with the LHRH receptor, with particular interest in phospholipase D (PLD) and mechanisms involved in its regulation, such as protein kinase C (PKC) and non-receptor tyrosine kinases.

Phospholipase D activation in αT3-1 cells proceeded after a delay when stimulated by LHRH, or by the phorbol ester, phorbol 12,13-dibutyrate (PDBu). LHRH-stimulated PLD activity was fully resistant to desensitisation over a 40 min time course. The $Ca^{2+}$ ionophore, ionomycin was unable to stimulate PLD activity. LHRH-stimulated PLD activity was inhibited by PKC downregulation or by bisindolylmaleimide PKC inhibitors at an approximately 8-fold higher concentration than that seen for the PDBu-stimulated PLD activity. Another PKC inhibitor, H7, inhibited PDBu-stimulated PLD activity in a manner that indicated multiple components to the inhibition and inhibited LHRH-stimulated PLD activity with a low potency. This is consistent with the possibility that one mediator of the LHRH response is a form of PKC which is relatively resistant to certain PKC inhibitors and insensitive to phorbol esters, or that some other unknown kinase is involved. The tyrosine kinase inhibitors, lavendustin A and genistein were able to selectively inhibit the LHRH-stimulated PLD activity. Phospholipase D activation stimulated by LHRH was insensitive to pertussis toxin pretreatment, indicating that the heterotrimeric G-proteins, Gi and Go are not involved.

LHRH induced tyrosine phosphorylation of a number of proteins in αT3-1 cells; a similar phosphorylation profile was also produced by stimulation with PDBu. Tyrosine phosphorylation of proteins in αT3-1 cells

was reduced by ionomycin. Two members of the src-family of non-receptor tyrosine kinases, src and fyn, were detected at significant levels in these cells by immunoblot. Using immunoprecipitation techniques we have shown that the enzymic activity of fyn is increased within 10 min of LHRH stimulation.

The LHRH-stimulated PLD activity could not be detectably enhanced by a conditioning preincubation with LHRH, such as brings about the priming effect of LHRH ( a phenomenon of cellular sensitisation elicited by LHRH receptor activation). Removal of steroids from the growth medium severely impaired the PLD activation due to LHRH; this response was largely restored by addition of oestradiol-17β but not progesterone. This may have implications for the LHRH priming effect in vivo, which is dependent upon the level of circulating steroid hormones.

The murine LHRH receptor was transiently expressed in COS 7 cells from the cDNA in a pcDNA vector using a DEAE dextran transfection procedure. It was demonstrated that LHRH-stimulated PLD activation displayed similar sensitivity to the bisindolylmaleimide PKC inhibitor, Ro 31-8220, to that seen in αT3-1 cells. It was not however sensitive to tyrosine kinase inhibitors or pertussis toxin. Using site-directed mutagenesis of residues 87 and 318 in the transmembrane 2 and 7 domains of the LHRH receptor (which represents an inversion of the consensus motif in the G-protein coupled receptor family), it was found that mutation of Asp 318 to Asn was alone sufficient to produce a rapidly desensitising PLD response to LHRH, in contrast to the non-desensitising wild type.

**Some of the results presented in this Thesis have been published as follows:**

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# CHAPTER 1

# INTRODUCTION

## 1.1 Aims of this study

The aim of this research project has been to elucidate the mechanism whereby phospholipase D is activated by the LHRH receptor in pituitary gonadotrophs, and as far as possible to identify the intermediary steps involved. Experiments to address the involvement of protein kinase C-like enzymes and non-receptor tyrosine kinases in LHRH receptor signalling and site-directed mutagenesis to address the role of specific receptor residues in signal transduction have been key areas of research.

These signalling systems have been investigated mainly in the αT3-1 pituitary gonadotroph-derived cell line, so that a better understanding of their function may indicate what role, if any, they play in LHRH signalling and self priming in pituitary gonadotrophs (Fink, 1988). The use of a cell line has been necessary due to difficulty in studying intracellular signalling systems in a highly heterogeneous tissue, such as the anterior pituitary.

In addition to phospholipids playing a structural role in the membrane, it is increasingly clear that they act as substrates for various phospholipases, phospholipase $A_2$ ($PLA_2$), phospholipase C (PLC) and phospholipase D (PLD), which generate lipid second messengers for signal transduction in response to extracellular stimuli (Dennis *et al*, 1991). Lipid-derived second messengers derived from the hydrolysis of phosphoinositides have long been recognised as forming the basis for a major signalling pathway. More recently, it has been recognised that the hydrolysis of other phospholipids, such as phosphatidylcholine may also play a major role in intracellular signalling mechanisms (Billah and Anthes, 1990).

## 1.2 Inositol phospholipids and intracellular signal transduction

One of the earliest lipid-derived messenger pathways to be elucidated was that of the phospholipase C (PLC) mediated hydrolysis of the phosphoinositides. The phosphoinositides are a minor group of membrane phospholipids, with a myo-inositol head group that can be phosphorylated at multiple sites (Hawthorne, 1982). They form a minor component of most eukaryotic membranes. The three most predominant phosphoinositides are phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate ($PIP_2$). Collectively, these three lipids usually constitute less than 8% of the total membrane phospholipids of mammalian cells (Hawthorne, 1982).

It was demonstrated that stimulation of cell-surface receptors initiated the hydrolysis of inositol phospholipids, for example it was found that in agonist-stimulated exocrine pancreas, an increase in lipid radiolabelling was confined to two lipids, PI and phosphatidic acid (PA) (Hokin and Hokin, 1955; Hokin, 1985). It was also found that an appropriate stimulus caused a substantial decrease in the PI present in the membrane (Hokin-Neaverson, 1974). It was later realised that it was not PI that was the primary target of PLC hydrolysis, but its phosphorylated derivative, $PIP_2$ (Irvine *et al*, 1984). Recognition of the full importance of $PIP_2$ came with the observation that in vasopressin-stimulated hepatocytes, a decrease in $PIP_2$ preceded changes in PI and was largely independent of $Ca^{2+}$ (Michell and Kirk 1981). When the aqueous products of phosphoinositide hydrolysis were examined in agonist-stimulated salivary glands, it was found that inositol 1,4,5-trisphosphate ($IP_3$) formation preceded that of PI (Berridge,

1983), confirming that the primary phosphoinositide cleaved by the receptor-mediated activation of PLC was $PIP_2$.

Inositol 1,4,5-trisphosphate has been shown to be capable of acting as an intracellular messenger by releasing $Ca^{2+}$ from intracellular stores (Streb *et al*, 1983). However, not all of the intracellular $Ca^{2+}$ stores are sensitive to $IP_3$; it appears that the $IP_3$-sensitive $Ca^{2+}$ pool is found in a perinuclear location (Ross *et al*, 1989). An $IP_3$ receptor has been purified, which appears to act as a channel for $Ca^{2+}$ release upon $IP_3$ binding; thus the $IP_3$-receptor can be considered a ligand-gated ion channel (Suppattapone *et al*, 1988).

Hydrolysis of $PIP_2$ by PLC has been shown to directly produce two intracellular messengers, $IP_3$ and diacylglycerol (DAG). It has been demonstrated that increased levels of DAG, derived from the agonist-induced hydrolysis of phosphoinositides increased the affinity for $Ca^{2+}$ of the $Ca^{2+}$-activated, phospholipid-dependent Ser/Thr protein kinase, protein kinase C (PKC), thereby activating it (Kishimoto *et al*, 1980).

Phospholipase C, the enzyme responsible for the receptor mediated hydrolysis of $PIP_2$, has been shown to consist of a superfamily of enzymes. The three main subgroups are β, γ and δ (reviewed in Rhee and Choi, 1992). It has been possible to show the widespread distribution of these subgroups in various tissues by protein purification, immunological analysis, biochemical comparison and by molecular cloning of the various enzymes. It has been reported that these three subtypes of PLC are regulated by different mechanisms, for example β-type PLCs are regulated by heterotrimeric G proteins, γ-type PLCs are regulated by tyrosine phosphorylation, and there are reports that PLC-δ can be activated by free fatty acids (reviewed in Liscovitch and Cantley, 1994).

## 1.3 Receptor-mediated hydrolysis of phosphatidylcholine by phospholipases

Hydrolysis of phosphoinositides is generally an early event following stimulation, however hydrolysis of other phospholipids, particularly phosphatidylcholine, is reported to produce DAG at a later phase. The hydrolysis of PC can be catalysed by distinct species of receptor linked phospholipases, namely PC specific PLC, PLD and $PLA_2$ (reviewed in Dennis *et al*, 1991; Billah and Anthes 1990).

Phosphatidylcholine (PC) is the principal phospholipid in mammalian cells and can account for up to 50% of the total phospholipid content (White, 1973). It consists of three main forms, 1,2-diacyl-*sn*-glycero-3- phosphocholine , 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphocholine and 1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphocholine; 1,2-diacyl-*sn*-glycero-3-phosphocholine has been shown to be the predominant subclass in most mammalian cell types (Horroks and Sharma 1982).

The major pathway of PC biosynthesis is called the CDP-choline or Kennedy pathway (Kent, 1990). In this pathway there is an ATP-dependent pohsphorylation of choline. Formation of an activated intermediate, CDP-choline is then catalysed by CTP: phosphocholine cytidyltransferase. The lipid PC is then generated by the transfer of CDP-choline to 1,2-diacylglycerol (Kent, 1990).

### 1.3.1 Phospholipase D

It has been reported in several systems that the production of DAG is temporally dissociated from the generation of $IP_3$, often to the extent that DAG can be formed in the absence of $IP_3$ formation (Farese *et al*, 1985). It has also been noted that DAG can be produced in a biphasic manner, with an initial first phase of DAG production associated with $IP_3$ production. This

type of biphasic response has been observed in angiotensin II- or endothelin-stimulated vascular smooth muscle cells (Griendling *et al*, 1986; Sunako *et al*, 1989) and in vasopressin-stimulated fibroblasts and hepatocytes (Thomas *et al*, 1983; Huang and Cabot, 1990). The levels of DAG produced in the second phase are generally much larger than those in the initial phase, and can be seen parallel with the generation of similar levels of choline (Thompson *et al*, 1990). Studies of the fatty acid composition of agonist-dependent DAG production have revealed that DAG produced in the early phase contains predominantly those fatty acids found in the phosphoinositide pool (stearate, arachidonate), whereas the later phase contains more saturated fatty acids, typically found in phosphatidylcholine (Pessin and Raben, 1989; Pettit and Wakelam, 1993).

Phospholipase D has been shown to be selective for phosphatidylcholine, and to a lesser extent phosphatidylethanolamine, and in a brain microsomal assay this activity has been shown to be selective only towards the head group (i.e. choline), as hydrolysis of the phospholipid was not dependent upon the fatty acid chain length or composition (Horwitz and Davis, 1993). Much of the evidence for PLD-hydrolysis of PC has come from the measurement of hydrolysis products, and measurements of the time course of PA and DAG generation. These methods were used in order to link agonist stimulation of a $P_2$ purinergic receptor with PLD activation in endothelial cells (Martin and Michaelis, 1989). However, it is important not to overlook the fact that DAG kinase and phosphatidate phosphohydrolase (PPH) are able to interconvert DAG and PA, and that rapid interconversion of metabolites may obscure true relationships. This point is clearly demonstrated in endothelial cells, where a very high PPH activity in the particulate fraction means that it is not possible to detect any bradykinin-stimulated formation of PA (Martin and Michaelis, 1988).

Evidence for the presence of a receptor-coupled PLD has also come from the exploitation of a unique catalytic activity of the enzyme. In the presence of a primary alcohol, PLD catalyses a transphosphatidylation reaction, in which the phosphatidyl moiety is transferred to the alcohol, forming a phosphatidylalcohol (Dawson, 1967). Since this phosphatidylalcohol is stable and is not hydrolysed by PPH, this forms the basis of a selective assay for PLD, which can be used in whole cells (Pai *et al*, 1988; Randall *et al*, 1990). If a sufficiently high concentration of alcohol is used, the production of PA by PLD can be blocked, providing a means of determining the proportion of DAG generated by the PLD pathway, although the specificity of alcohols at these levels may limit their usefulness (Bonser *et al*, 1989).

Phospholipase D is an enzyme found primarily in particulate cellular fractions of mammalian cells, having been partially purified from rat brain (Chalifa *et al*, 1990) and rat neutrophils (Olsen *et al*, 1991). However a soluble form of the enzyme has also been detected in bovine tissue (Wang *et al*, 1991). Plasma membrane PLD from rat brain synaptosomes (Chalifa *et al*, 1990) has been shown to require $Ca^{2+}$, but still has considerable activity at $Ca^{2+}$ concentrations less than 1 μM. So far PLD has not been purified to homogeneity, however the studies that have been carried out on partially purified PLD suggest that different PLD isoenzymes exist that vary in their pH optima, responses to detergents and cations, subcellular distribution and substrate specificity (reviewed in Exton, 1994).

### 1.3.2 Mechanisms of receptor activation of phospholipase D

Studies of PLD in mammalian systems indicate that receptor-mediated activation occurs through multiple pathways, such as protein

kinases, $Ca^{2+}$ and multiple types of GTP-binding proteins (Billah, 1993; Exton, 1994).

In many cell types, PKC appears to be important in PLD activation, with phorbol esters being commonly used as an experimental means to stimulate PLD activity (Billah and Anthes, 1990). Strong evidence in the favour of PKC involvement in PLD regulation comes from studies in which the levels of PKC in the cells have been modified. For example, prolonged exposure to phorbol esters was shown to block PLD activation stimulated by PMA and endothelin-1 in Rat-1 fibroblasts (MacNulty *et al*, 1990) and by bradykinin in bovine pulmonary endothelial cells (Martin *et al*, 1989). In several systems, overexpression of PKC isoforms resulted in an increase in PLD activity. For example, over-expression of PKC-β1 in rat fibroblasts resulted in facilitation of the PLD activity stimulated not only by the phorbol ester PMA (Pai *et al*, 1991a), but also through receptor-activated systems such as endothelin (Pai *et al*, 1991b), and α-thrombin (Pachter *et al*, 1992). Selective inhibitors of PKC have also been used in order to determine whether PKC is involved in the activation of PLD, for example in endothelial cells, the $P_2$-purinergic receptor-stimulated activation of PLD is sensitive to the PKC inhibitor Ro 31-8220 (Purkiss and Boarder, 1992). It has also been demonstrated that phorbol ester (PMA)-stimulated PLD activity in Swiss 3T3 cells was selectively inhibited by Ro 31-8220, while bombesin-stimulated PLD activity was not significantly altered by Ro 31-8220 (Cook *et al*, 1991). The mechanism by which PKC brings about PLD activation is unclear. It may be through a direct phosphorylation of PLD, or PKC may be acting upon an intermediary regulatory protein. It has also been reported that PKC may be capable of stimulating PLD activity in the absence of ATP, suggesting the unconventional possibility that a non-phosphorylation mechanism can also be involved (Conricode *et al*, 1992). The existence of

both phosphorylating and non-phosphorylating PKC mechanisms for the agonist-induced activation of PLD could explain why inhibitors of the ATP-binding site of PKC produce variable results.

Several studies have suggested that tyrosine kinases play a role in the receptor-mediated activation of PLD, involving both receptor and non-receptor tyrosine kinases (Billah, 1993). Selective inhibitors of protein-tyrosine kinases have been shown to inhibit receptor-linked activation of PLD in many cases. For example in human neutrophils, the formyl-Met-Leu-Phe (fMLP)-stimulated activation of PLD is inhibited by the tyrosine kinase inhibitors ST271 and ST638 (Uings *et al*, 1992). Stimulation of the EGF receptor in Swiss 3T3 cells leads to the tyrosine kinase-dependent activation of PLD, in the absence of detectable PLC activity (Cook and Wakelam, 1992). The protein-tyrosine phosphatase inhibitor pervanadate has also been shown to stimulate PLD activity in HL-60 cells through an elevation in the level of tyrosine phosphorylation (Bourgoin and Grinstein, 1991). A possibly more direct stimulation of PLD activity has been observed in cells overexpressing the viral form of the non-receptor tyrosine kinase c-*src* (Song and Foster, 1993). It would therefore appear that PLD activity can be stimulated through the activation of both receptor and non-receptor tyrosine kinases.

Calcium also appears to play a critical role in PLD activation in many systems (Billah, 1993). Ionophores of $Ca^{2+}$, such as ionomycin and A 23187 have been shown to stimulate PLD in many cell types (Pai *et al*, 1988). It has also been demonstrated that removal of extra-cellular $Ca^{2+}$ can attenuate PLD activation stimulated by fMLP in HL-60 granulocytes (Pai *et al*, 1988).

It is well established that PLD can be activated by receptors which are coupled to their effector systems through heterotrimeric G proteins

(Thompson *et al*, 1993a). Many receptor-stimulated PLC and PLD systems are blocked by the microbial toxin, pertussis toxin, which acts by ADP-ribosylating the $\alpha$-subunit of the Gi and Go subtypes of heterotrimeric G proteins, thus preventing their activation (Milligan, 1988). For example in HL-60 granulocytes, the increased activity of both PLC and PLD stimulated by fMLP was blocked by pertussis toxin pre-treatment (Pai *et al*, 1988). However in hepatocytes, vasopressin-, angiotensin II- and adrenaline-stimulated PLC and PLD activations were insensitive to pertussis toxin, even though they could be activated in membrane preparations by non-hydrolyzable analogues of GTP (Bocckino *et al*, 1987). An insensitivity to pertussis toxin does not rule out G protein involvement, but simply shows that there is no Gi or Go involvement. A G protein involvement in the activation of PLD has also been extensively demonstrated by the use of the non-hydrolysable GTP analogue, GTP$\gamma$S, in permeablised cells (Anthes *et al*, 1991). It therefore appears that PLD can be activated by a variety of G protein-coupled receptors, linked to the intracellular signalling machinery by G proteins probably belonging to both Gq and Gi families.

Recently evidence has come to light that members of the family of low molecular weight G proteins related to Ras, ARF (ADP-ribosylation factor) and Rho, are closely involved in the regulation of PLD (Cockroft *et al*, 1994; Brown *et al*, 1993; Bowman *et al*, 1993). There is evidence that ARF is involved in the regulation of membrane traffic, through the formation of coated vesicles, and transport between the endoplasmic reticulum and cis-golgi (Balch *et al*, 1992). This raises the possibility that PLD may also play a role in membrane traffic and vesicle formation by changing the characteristics of membranes (reviewed in Kahn *et al*, 1993).

It is not known if other types of low molecular weight G proteins are responsible for the regulation of PLD. Low molecular weight (20-25 kDa) G

proteins related to Ras, exist as a superfamily of proteins grouped into four subfamilies: Ras, Rho, Rab and Ran (Lowy and Willumsen, 1993). ADP-ribosylation factor (ARF) is related to these other families of low molecular weight G protein, although it shares some properties with the heterotrimeric G proteins. These low molecular weight G proteins cycle between an inactive GDP-bound form and an active GTP-bound state. This exchange is normally very slow, but can be greatly accelerated by another family of proteins, the guanine-nucleotide exchange factors (GEFs) (Feig, 1994). Activity of the Ras type G proteins can also be prolonged by proteins known as guanine-nucleotide dissociation inhibitors, or GDIs (reviewed in Feig, 1994).

### 1.3.3 Phosphatidylcholine selective phospholipase C

It has been demonstrated that as well as a PLC activity being involved in the hydrolysis of inositol phospholipids, there is a form of PLC which selectively hydrolyses PC. Phosphatidylcholine has been shown to be a substrate for PLC partially purified from dog heart cytosol (Wolf and Gross, 1985), bull seminal plasma (Sheikhnejad and Srivastava, 1986) and promonocytic U937 cells (Clark *et al*, 1986). These activities have been shown to exhibit a neutral pH optima and not detectably to hydrolyse inositol phospholipids. So far PC-PLC has not been extensively purified, but its existence is expected due to the enzymic activity that produces choline phosphate from PC (Nishizuka, 1992). It is thought that PLC-mediated PC hydrolysis is responsible for the later phase of DAG production, as is also thought to be the case for PLD-mediated hydrolysis of PC. In contrast the hydrolysis of $PIP_2$ by PLC appers to be responsible for an early and transient peak of DAG (reviewed in Liscovitch, 1992).

### 1.3.4 Phospholipase $A_2$

As well as generating PA and DAG, PC hydrolysis also leads to the generation of other lipid products with distinct cellular signalling functions. Although not the focus of this study, $PLA_2$-generated second messengers represent an important component of lipid-derived cellular signalling mechanisms and should not be overlooked in the consideration of agonist-induced alteration in cellular function.

Phospholipase $A_2$ has been shown to be present as a 14 kDa secretory $PLA_2$, present in digestive enzymes and snake venom, requiring millimolar levels of $Ca^{2+}$ for activity (Davidson and Dennis, 1990). There is little evidence that this family of low molecular weight $PLA_2$ are involved in intracellular signalling. In contrast, a high molecular weight (85 kDa) cytosolic $PLA_2$ has been demonstrated to be activated in response to physiologically-relevant changes in free $Ca^{2+}$ (Gronich *et al*, 1990; Clark *et al*, 1991). Ligand-induced activation of cytosolic $PLA_2$ selectively leads to the release of arachidonic acid (AA) from the *sn*-2 position of PC or phosphatidylethanolamine (PE), while inositol phospholipids are poor substrates (Gronich *et al*, 1990; Diez and Mong, 1990). The action of $PLA_2$ produces the products AA and lysophosphatidic acid (LPA), used in the further biosynthesis of cellular regulators, such as prostaglandins, leukotrienes and platelet activating factor (PAF) (Irvine, 1982). It has been demonstrated that it is possible for AA to synergistically activate $Ca^{2+}$-dependent PKC isoforms in the presence of DAG, at near basal $Ca^{2+}$ concentrations (Shinomura *et al*, 1991; Chen and Murakami, 1992). It is also possible that LPA has signalling functions, since LPA has been shown to enhance the activity of the $\alpha$, $\beta$ and $\gamma$ isoforms of PKC in vitro (Sasaki *et al*, 1993). Lysophosphatidic acid is also known to act as an agonist for a G

protein-coupled cell surface receptor (Durieux and Lynch, 1993). It has also been reported that AA is capable of activating PLC-δ (Liscovitch and Cantley, 1994).

### 1.3.5 Phosphatidate and diacylglycerol in intracellular signalling

Several reports have suggested that the appearance of certain cellular responses are more closely associated with PA than with DAG formation. For example, in unprimed neutrophils, it is the level of PA generated by PLD rather than the level of DAG that correlates with the fMLP-stimulated respiratory burst (Koenderman *et al*, 1989; Rossi *et al*, 1990). Similar relationships have been reported for proliferation in fibroblasts (Zhang *et al*, 1990) and insulin secretion in pancreatic islets (Dunlop and Metz, 1989; Konrad *et al*, 1991). One approach that has been taken in order to determine whether PA or DAG is functionally important is the use of propranolol, which has been described as an inhibitor of PPH at high concentrations, both *in vitro* and *in vivo* (Billah and Anthes, 1990). In unprimed human neutrophils, propanolol was shown to potentiate the fMLP-stimulated respiratory burst (Rossi *et al*, 1990), however in primed neutrophils in which the PLD activity is amplified, propanolol inhibited the respiratory burst (English and Taylor, 1991); this is consistent with the hypothesis that priming changes the key regulatory second messenger from PA to DAG. Although it has been demonstrated that propanolol can be a useful experimental tool, the high concentrations required to inhibit PPH are known to affect membrane fluidity (Barret and Cullum, 1968), which may affect cellular responses. There have been several *in vitro* observations which are consistent with a second messenger role for PA. Early evidence suggested that PA acted as a $Ca^{2+}$-ionophore (Putney *et al*, 1980), although this was later attributed to contamination of samples by

oxidised lipids (Holmes and Yoss, 1983), and in A-431 carcinoma cells the capacity of PA to mobilise $Ca^{2+}$ was dependent on the generation of $IP_3$. It has been reported that PA can activate PLC in membranes and soluble extracts from platelets (Jackowski and Rock, 1989), and also activate phosphatidylinositol-4-phosphate-5-kinase in synaptosomal membranes, thereby increasing the level of $PIP_2$ (Moritz *et al*, 1992). However, these affects of PA do not account for its effects seen in the absence of PLC activity.

There is evidence that PA-sensitive protein kinase(s) exist that are able to phosphorylate multiple substrates, distinct from those of DAG-stimulated PKC (Bocckino *et al*, 1991). It seems certain that PA does have some important second messenger functions, such as the activation of phosphatidylinositol 4-phosphate kinase purified from bovine brain membranes (Moritz *et al*, 1992). It has also recently been reported that PA is able to bind to PKC ζ in a cell free system, bringing about a shift in its electrophoretic mobility and causing its activation, assessed by autophosphorylation and phosphorylation of exogenous PKC substrates (Limatola *et al*, 1994).

Several observations suggest that PC-derived DAG is not capable of activating PKC. It was demonstrated in α-thrombin stimulated fibroblasts that under conditions which stimulate the production of DAG derived from both PI snd PC hydrolysis, there was detectable activation of PKC. Whereas under conditions where α-thrombin only stimulates the hydrolysis of PC, there was no detectable activation of PKC (Leach *et al*, 1991). It has also been shown that in IgE-stimulated RBL 2H3 mast cells the PLD-mediated production of DAG is not responsible for PKC activation (Lin *et al*, 1992). However, the idea that DAG derived from PC is ineffective at activating PKC has been challenged by results which show that in α-

thrombin- and PDGF-stimulated fibroblasts the late phase of DAG production from PC hydrolysis is associated with the activation of PKC ε, but not PKC α (Ha and Exton, 1993). This study concluded that it was the absence of $Ca^{2+}$ associated with PC hydrolysis that was responsible for this differential activation. It has also been demonstrated that phagocytosis in the human neutrophil, mediated by complement receptors, is associated with phosphorylation of a PKC substrate, the myristoylated alanine-rich C-kinase substrate (MARCKS) protein, as well as DAG generation through PLD (Fallman *et al*, 1992). It appears that in some cases, PC-derived DAG may be involved in the sustained activation of PKC, as the kinetics of this DAG formation are more prolonged than that derived form $PIP_2$ hydrolysis and in the absence of a rise in cytosolic $Ca^{2+}$, PC-derived DAG may therefore be important for the activation of $Ca^{2+}$-independent PKCs such as PKC ε (Ha and Exton, 1993). Sustained PKC activation may be important in events such as proliferation, differentiation and in cellular priming events.

## 1.4 Protein kinase C

Protein kinase C (PKC) was identified by Nishizuka and co-workers in 1977 (Takai *et al*, 1977) as a proteolytically-activated protein kinase with no obvious role at the time. It was later shown to be a $Ca^{2+}$-activated, phospholipid-dependent enzyme (Takai *et al*, 1979), which has been shown to be modulated by DAG, resulting in an increased affinity for $Ca^{2+}$ (Takai *et al*, 1979; Kishimoto *et al*, 1980). Activation of PKC requires diacylglycerol with a 1,2-*sn* conformation. With respect to the side chains, unsaturated fatty acids have been shown to most effective and fatty acid chain length is not critical, although there is an optimal chain length (Mori *et al*, 1982). The consensus from a variety of studies is that protein kinase C activation occurs via a two step process. Firstly, inactive soluble PKC

(which readily reacts with MgATP) binds anionic phospholipids (especially phosphatidylserine) and $Ca^{2+}$, resulting in PKC associating with the membrane, but having a relatively low kinase activity. Diacylglycerols or phorbol esters can then induce greater PKC activity (Bazzi and Nelsestuen, 1991). The stoichiometry for the interaction of the co-factors *in vitro* is considered to be 4-10 phosphatidylserine molecules to one DAG/ phorbol ester molecule to one $Ca^{2+}$ ion for every monomeric PKC molecule (Hannun *et al*, 1986a; Hannun and Bell, 1990).

### 1.4.1 Protein kinase C subtypes

There are multiple discrete subspecies of PKC, each showing subtly different enzymic properties, differential tissue expression and specific intracellular localisation (Nishizuka, 1988). So far eleven subspecies of PKC have been identified, which can be classified into three subgroups; conventional, novel and atypical PKCs. The 4 conventional PKCs have been designated: $\alpha$, $\beta$I, $\beta$II and $\gamma$, the novel isoforms of PKC consist of $\delta$, $\varepsilon$, $\eta$, $\mu$ and $\theta$, and the atypical PKCs are $\zeta$ and $\lambda$ (Hug and Saare, 1993).

The conventional group of PKC enzymes have four conserved (C1-C4) and five variable (V1-V5) regions. The C1 domain is a putative membrane binding domain, containing two cysteine rich zinc finger motifs that bind to DAG or phorbol esters (Ono *et al*, 1989) and a pseudosubstrate sequence which inhibits the activity of the catalytic domain in the inactive state (Hug and Saare, 1993). The C2 domain is a $Ca^{2+}$-binding domain and appears to be required for the $Ca^{2+}$ sensitivity of the conventional PKCs (Ono *et al*, 1988). The C3 domain contains the catalytic site and C4 appears to be involved in substrate recognition. The novel group of PKCs lack a C2 domain and do not require $Ca^{2+}$ to be activated (reviewed in Nishizuka, 1992). The atypical group of PKCs also lack a $Ca^{2+}$ binding

domain, but unlike the conventional and novel PKCs which have two cysteine-rich zinc-finger motifs they only have one. As a consequence they are reported to be unaffected by DAG or phorbol esters. The signalling pathway activating the atypical PKCs is unknown at present, however, there is evidence that PKC ζ can be activated by cis-unsaturated fatty acids and by PA (Nakanishi and Exton, 1992; Ways *et al*, 1992; Limatola *et al*, 1994).

### 1.4.2 Activators of protein kinase C

There is good evidence that both 1,2-DAGs and tumour-promoting phorbol esters directly activate all but the atypical PKCs by binding to two DAG/phorbol ester binding domains and increasing the enzyme's affinity for $Ca^{2+}$ and/or phospholipid (Castagna *et al*, 1982; Ogita *et al*, 1992). It has been demonstrated that diacylglycerols are able to displace phorbol ester ligands from PKC, which suggests that phorbol esters and DAG share a common binding site, and that phorbol esters can activate PKC by binding at the DAG binding site (Leach *et al*, 1983).

The phorbol ester used in the present study was phorbol 12,13-dibutyrate (PDBu), which in the presence of $Ca^{2+}$ shows no marked selectivity of activation between PKCs isolated from a range of tissues. However, other PKC activators, such as mezerein and 1,2-dioctanoyl-sn-glycerol (DOG) do display a marked difference in their ability to activate certain isoforms (Evans *et al*, 1991).

### 1.4.3 Inhibitors of protein kinase C

Inhibitors of protein kinase C activity are widely used to study systems in which PKC may be involved. The mode of action of these compounds may be through interference with the $Ca^{2+}$/phospholipid interaction, DAG/phorbol ester binding or ATP or substrate binding.

Compounds preventing the interaction of $Ca^{2+}$ with PKC, such as melittin and polymyxin B (Huang, 1989) also block the activity of other $Ca^{2+}$-dependent enzymes. They are therefore not very selective PKC inhibitors and are rarely used as such. Compounds such as calphostin C and sphingosine are reported to interfere with PKC-lipid interactions. Calphostin C blocks DAG/phorbol ester binding with PKC and is reasonably selective for PKC over other protein kinases. It does however cause an irreversible inhibition of phorbol ester binding, which may limit its uses (Kobayashi *et al*, 1989). Sphingosine, an 18-carbon lipid, causes PKC inhibition by binding competitively to the DAG/$Ca^{2+}$ and phosphatidylserine binding sites. However sphingosine also has effects on biological systems which are independent of PKC, limiting its use as a selective PKC inhibitor (Hannun *et al*, 1986b). Some of the compounds that inhibit PKC by interacting with the enzyme's catalytic domain include the isoquinolinesulphonamides, such as 1-(5-isoquinolinesulphonyl)-2-methyl piperazine (H7), and substances with an indole carbazole structure, such as staurosporine, K252a and their structural congeners, the bisindolylmaleimides, such as Ro 31-8220, which has been shown to potently inhibit PKC activity at ATP levels similar to those found in whole cells (Davis *et al*, 1992), and GF 109203X (Toullec *et al*, 1991). The action of H7 on PKC is reversible and competitive with ATP (Hidaka *et al*, 1984). There is a considerable sequence homology for ATP binding sites among serine/threonine kinases. H7 therefore only displays a slight selectivity for PKC, over other kinases. Bisindolylmaleimides such as Ro 31-8220 and GF 109203X are related to the general kinase inhibitor staurosporine. Both staurosporine and K252a inhibit PKC by interacting with the catalytic domain, perhaps competing at the ATP binding site (Davis *et al*, 1992). Both of these compounds are however not highly-selective PKC inhibitors, as they are able to potently inhibit other protein kinases by the same mechanism. Analogues of

staurosporine, of the bisindolylmaleimide family of compounds, have been developed by modification of the side chains, which have a much higher selectivity for PKC over other protein kinases (Davis *et al*, 1989). Two such compounds, Ro 31-8220 (Davis *et al*, 1992), and GF 109203X (Toullec *et al*, 1991), are up to 100 times more selective for PKC compared to PKA than is staurosporine.

Certain groups of PKC inhibitors have been shown to possess partial selectivity for certain PKC isoforms. Members of the bisindolylmaleimide family of compounds have been shown to be more potent at inhibiting the $Ca^{2+}$-dependent PKC isoforms $\alpha$ and $\beta$ than the $Ca^{2+}$-independent PKC isoform $\varepsilon$ (Wilkinson *et al*, 1993). It has also been shown that a novel protein kinase inhibitor, rottlerin shows selectivity for PKC $\delta$ over other isoforms of PKC (Gschwendt *et al*, 1994). It may be possible to utilise this slight selectivity in attempting to determine which isoforms are involved in a particular biochemical pathway in cells. There is also evidence for different PKC isoforms showing varying sensitivities to H7, since an uncharacterised PKC isoform in the anterior pituitary shows an insensitivity to inhibition by H7 compared to other PKC activities isolated from this tissue (Johnson and Mitchell, 1989).

### 1.5 Non-receptor tyrosine kinases

Tyrosine phosphorylation has recently been implicated in the action of several neuropeptides which act through G protein-coupled receptors, such as bombesin, vasopressin and endothelin (Zachary *et al*, 1991). This tyrosine phosphorylation is presumed to be mediated by the action of non-receptor tyrosine kinases, since G protein-coupled receptors have no intrinsic tyrosine kinase activity. It has been observed that non-receptor tyrosine kinases increase the phosphorylation of an apparently partially

distinct spectrum of cellular proteins compared with receptor tyrosine kinases (Cantley *et al*, 1991).

### 1.5.1 Src-type tyrosine kinases

One of the main groups of non-receptor tyrosine kinase is the *src*-family, consisting of a group of nine closely related proteins: *src*, *fyn*, *lck*, *lyn*, *yes*, *hck*, *blk*, *fgr* and *yck*. These enzymes have been demonstrated to be involved with cellular transformation and in intracellular signal transduction (Cooper, 1990). In addition to a C-terminal tyrosine kinase domain, *src* family members share the conserved internal homology regions, SH2 and SH3. The SH2 domain has been shown to interact with specific phosphotyrosine residues (Songyang *et al*, 1993). It has been demonstrated that SH3 domains are able to bind to the proline-rich sequence motif X-P-X-X-P-P-P-Z-X-P, where X represents any amino acid, and Z represents a hydrophobic amino acid (Ren *et al*, 1993). It has also been suggested that SH3 domains may be important for localisation to the cytoskeleton, since SH3 containing proteins are often associated with the cytoskeleton (Musacchio *et al*, 1992). The N-terminal region of *src* kinases, which is myristoylated, appears to be primarily responsible for targeting the proteins to cellular membranes, particularly the inner surface of the plasma membrane (Resh, 1994).

There is good evidence that this *src*-family of kinases are maintained in an inactive state through phosphorylation of a tyrosine at position 527 near the C-terminus by CSK (C-terminal src kinase), which is believed to be a universal regulator of *src* family kinases (Okada *et al*, 1991). Tyrosine phosphatases are thought to be able to bring about the activation of *src*-family kinases by dephosphorylation of tyrosine 527 (Courtneidge, 1985). It is thought that the interaction of the phosphorylated tyrosine 527 with the

SH2 domain sterically impedes the catalytic domain (Koch *et al*, 1991), while the SH3 domain may also bind to the C-terminal tail or kinase domain and aid inhibition. Tyrosine phosphorylation of *src* at position 416 is thought to stabilise the catalytic domain in an active conformation, as is also the case for autophosphorylation of the cAMP-dependent protein kinase (Knighton *et al*, 1991).

It has been shown in platelets (which contain particularly high levels of c-*src*) that agonists which directly or indirectly activate PKC such as the phorbol ester PMA, vasopressin and the $Ca^{2+}$ ionophore A23187 increase the activity of the non-receptor tyrosine kinases c-*src* and c-*fyn*. Stimulation of platelets by the above pathways causes the phosphorylation of c-*src* at Ser-12, and also increases the substrate affinity of c-*src* by two to three fold (Liebenhoff *et al*, 1993). In many cell types a rise in cytosolic $Ca^{2+}$ is sufficient to elicit tyrosine phosphorylation (Vostal *et al*, 1993). However, a clear picture of how non-receptor tyrosine kinases are activated after G protein-coupled receptor activation has not yet emerged.

### 1.5.2 Focal adhesion kinase (p125 FAK)

Stimulation of G protein-coupled neuropeptide receptors has also been shown to stimulate the activity of a 125 kDa tyrosine kinase, focal adhesion kinase (FAK) (Zachary *et al*, 1992). Focal adhesion kinase is interesting in that it lacks the non-catalytic domains or motifs which are found in other receptor and non-receptor tyrosine kinases (Schaller *et al*, 1992). The catalytic domain possesses all the conserved motifs of other tyrosine kinases, however, this region is flanked by large amino- and carboxy-terminal domains lacking any significant homology to other tyrosine kinases. Focal adhesion kinase does not possess any acylation sites, which would confer membrane association, or sites for association

with other proteins, such as SH2 or SH3 domains (Cobb *et al*, 1994; Ren *et al*, 1993).

There is evidence for the constitutively active v-*src* bringing about FAK activation when expressed in NIH 3T3 cells (Guan *et al*, 1990). It is therefore possible that *src* type kinases may be responsible for tyrosine phosphorylating FAK, downstream of G protein-coupled receptor activation.

### 1.5.3 Other non-receptor tyrosine kinases

Another type of signal-activated non-receptor tyrosine kinase is p72 *syk*. This kinase possess a kinase domain, similar to other tyrosine kinases, and two SH2 domains (Taniguchi *et al*, 1991). It has also been demonstrated that *syk* can be rapidly activated following thrombin stimulation of platelets, which appears to be dependent upon $Ca^{2+}$ (Wang *et al*, 1994). It has been shown that the JAK (janus or just another kinase) family of tyrosine kinases, which lack SH2 or SH3 domains, are activated by association with cytokine receptors (Ihle *et al*, 1994). Another non-receptor tyrosine kinase c-Abl has so far not been linked with any receptor. It has however been shown to bind to actin filaments in the cytoplasm and bind to specific DNA sequences (Wang, 1993).

### 1.5.4 Inhibitors and activators of tyrosine phosphorylation

Several groups of compounds have been developed that have the ability to selectively inhibit protein-tyrosine kinases, and these have been extensively used as a method of determining the involvement of tyrosine kinases in a variety of purified and whole cell systems. A number of compounds have been particularly useful in identifying the cellular actions of protein-tyrosine kinases in response to receptor agonists.

Lavendustin A has been shown to be a potent inhibitor of tyrosine kinases and to have little effect on cAMP-dependent protein kinase or PKC

(Hsu *et al*, 1991). Lavendustin A is a competitive inhibitor with respect to ATP, but a non-competitive inhibitor with respect to a *src* peptide substrate (Hsu *et al*, 1991). Binding analysis has also revealed that lavendustin A acts as a slow, tight binding inhibitor with a two step mechanism (Hsu *et al*, 1991). Another compound of the isoflavine family, genistein has been reported to inhibit the tyrosine kinase activity of the EGF receptor tyrosine kinase, pp60 v-*src* and pp110 gag-fes *in vitro*. This inhibition was shown to be competitive with respect to ATP and non-competitive to a phosphate acceptor, histone H2B. It has also been demonstrated that genistein has scarcely any effect on the serine/ threonine specific protein kinases, cAMP-dependent protein kinase and PKC, making it an excellent (and widely used) compound for inhibiting protein-tyrosine kinases (Akiyama *et al*, 1987). Piceatannol (3,4,3', 5'-tetrahydroxy-trans-stilbene), has been previously identified as an antileukemic agent, and has been shown to act as an inhibitor of protein-tyrosine kinase activity. Piceatannol inhibits the purified thymocyte protein-tyrosine kinase, p40, by competing for the peptide or protein binding site, due to its structural similarity with tyrosine. Piceatannol has also been shown to inhibit the activity of the p56 *lck* non-receptor tyrosine kinase in LSTRA leukemic cell membranes or intact cells. Piceatannol did not detectably inhibit the activity of cAMP-dependent protein kinase (Geahlen and McLaughlin, 1989). However, it has been demonstrated that piceatannol will inhibit the $Ca^{2+}$-dependent activity of PKC from $\alpha$T3-1 cells, using the glycogen synthase (GS) peptide (100 $\mu$M) as a substrate (M. Johnson, unpublished results). The mode of action of piceatannol differs from that of the other tyrosine kinase inhibitors described above, which compete at the ATP binding site, in that piceatannol has a structural similarity with tyrosine and binds to the substrate binding site (Geahlen and McLaughlin, 1989). This mode of action is similar to that of synthetic compounds called tyrphostins which also inhibit the activity of

protein-tyrosine kinases by blocking substrate binding (Yaish *et al*, 1988; Schechter *et al*, 1989). Another compound which is structurally related to piceatannol and tyrphostins and having a similar mode of action is methyl 2,5-dihydroxycinnamate (MDC). This compound has been shown to potently inhibit the EGF-receptor tyrosine kinase, although due to its polar nature it is unsuitable for whole cell assays (Umezawa *et al*, 1990).

It has been demonstrated that peroxides of vanadate (pervanadate) are capable of stimulating the tyrosine phosphorylation of the insulin receptor in intact cell through the inhibition of tyrosine phosphatase activity rather than activation of the autokinase reaction. This effect of pervanadate was $10^2$-$10^3$ times more potent than that of vanadate (Fantus *et al*, 1989). Since pervanadate has been demonstrated to elevate the levels of phosphotyrosine in whole cells, we have also used it in this study in order to mimic the receptor activation of tyrosine kinases in $\alpha$T3-1 cells.

## 1.6 Tyrosine phosphatases

The level of tyrosine phosphorylation of cellular proteins can be controlled by the action of tyrosine kinases, which have been discussed above, and also by the action of tyrosine phosphatases. As is the case with tyrosine kinases, tyrosine phosphatases can be roughly classified into two subfamilies, receptor tyrosine phosphatases and non-receptor tyrosine phosphatases (Pot and Dixon, 1990). In common with tyrosine kinases, tyrosine phosphatases are a diverse group of enzymes with a large number of possible regulatory domains (Pot and Dixon, 1990). An example of the receptor tyrosine phosphatases is CD45, which has intracellular tandem repeats of a 200-amino acid protein-tyrosine phosphatase domain, a single membrane spanning region and an extracellular domain (Alexander, 1990). A representative of the non-receptor class of tyrosine phosphatases

is PTP 1B, which has a single copy of the conserved 200-amino acid tyrosine phosphatase domain with no transmembrane domain (Guan *et al*, 1990). The diverse structures of non-receptor tyrosine kinases suggests multiple methods of regulation (Pot and Dixon, 1990).

### 1.7 LHRH receptor signaling and the LHRH-self priming effect

The present study has addressed the signalling mechanisms associated with LHRH-receptor activation (specifically PLD activation and tyrosine phosphorylation), in order to elucidate the biochemical events which may underlie LHRH receptor signalling, and in particular the LHRH-self priming mechanism occurring in gonadotrophs in the anterior pituitary.

The anterior pituitary gland contains several distinct types of secretory cell, releasing at least six different circulating hormones (Denef and Andries, 1982). Luteinising hormone (LH) and follicle-stimulating hormone (FSH) are released from gonadotrophs, growth hormone (GH) from somatotrophs, prolactin from mammotrophs, adrenocorticotrophic hormone (ACTH) from corticotrophs and thyroid-stimulating hormone (TSH) is released from thyrotrophs, whilst somato-mammotrophs secrete both GH and prolactin (Denef and Andries, 1982). Release of these hormones is regulated mainly by peptides released into the hypophysial portal blood vessels from the median eminence of the hypothalamus. One such peptide is the decapeptide, luteinising hormone-releasing hormone (LHRH; pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$) which is the main physiological stimulus for the release of LH and FSH from gonadotrophs.

An aspect of particular interest in the gonadotroph is the self-priming effect of LHRH, which is the mechanism by which LHRH induces a profound increase in gonadotroph responsiveness (Aiyer *et al*, 1974a). This priming effect is an essential factor in bringing about the surge of LH release which

triggers ovulation (Fink, 1986). Priming is enabled in part by the history of exposure of the anterior pituitary to steroid hormones prior to the gonadotroph surge; that is high levels of circulating oestrogens (mainly oestradiol-17β) and low levels of progesterone (Fink, 1986; Fink, 1988). The release of LHRH from the hypothalamus is of a pulsatile nature and although the frequency of release is increased near the LH surge, the additional hormone output is too small to account for the very large increment in LH release from the pituitary (Fink, 1988). The increase in pituitary responsiveness, initiated by oestrogen, is further intensified by the release of ovarian progesterone during the early part of the LH surge (Fink, 1988). The effective role of the LHRH-priming effect is to co-ordinate the increase in LHRH release with the increased responsiveness of the pituitary, in order that both events result in a massive surge in LH release from the pituitary, and thus initiate ovulation in female mammals.

It has been reported that LHRH-induced LH release coincides with an increase in $^{45}Ca^{2+}$ efflux from pre-loaded anterior pituitary pieces (Williams, 1976). It has also been shown that LHRH can induce inositol phospholipid breakdown in dispersed anterior pituitary cells (Snyder and Bleasdale, 1982), and that this was followed by an increase in $IP_3$ and DAG accumulation (Schrey, 1985). GTP analogues have also been shown to be capable of increasing inositol phosphate production and LH release from ATP-permeablised, dispersed rat anterior pituitary cells (Andrews and Conn, 1986). It therefore seems likely that $IP_3$ production and $Ca^{2+}$ are major pathways involved in LHRH-induced LH release. The LHRH-mediated increase in intracellular $Ca^{2+}$ levels may induce LH release by modulating the activity of a number of different $Ca^{2+}$-dependent processes. One particular example is that LHRH treatment of ovariectomised rats

causes a redistribution of calmodulin from the cytosol to the plasma membrane (Conn *et al*, 1981).

LHRH receptor activation has been shown to lead to the generation of DAG (Andrews and Conn, 1986), which is known to lead to the activation of PKC (Nishizuka, 1992). LHRH can induce PKC translocation to the cell membrane, with a time-course and concentration-dependence similar to that of LHRH-induced LH release (Naor *et al*, 1985). Furthermore, DAG and phorbol esters have been shown to induce gonadotrophin release from anterior pituitary cells in culture (Conn *et al*, 1985) and anterior pituitary pieces in vitro (Turgeon and Waring, 1986; Johnson and Mitchell, 1989). Introduction of purified rat brain PKC into permeablised anterior pituitary cells also induced LH release (Naor *et al*, 1989), consistent with a role for PKC in LH release responses. It has also been demonstrated that inhibitors of protein-tyrosine kinases are able to inhibit the LHRH-stimulated release of LH from anterior pituitary pieces *in vitro* (Johnson *et al*, 1994).

It is also possible that $PLA_2$ could play a role in LHRH signalling in the pituitary. Since it has been demonstrated using cultured anterior pituitary cells, that arachidonic acid (AA) can induce LH release, and that LHRH can induce an increase in [$^3$H]-AA release from pre-labelled cells (Naor and Catt, 1981; Chang *et al*, 1986). It has also been shown that inhibitors of $PLA_2$ are able block the onset of LHRH priming, but do not effect the immediate LHRH-stimulated release of LH from the anterior pituitary. The LHRH priming effect could also be partially mimicked by a conditioning preincubation with arachidonic acid (Thompson *et al*, 1994).

It has also been demonstrated that LHRH-receptor activation can lead to the stimulation of PLD activity in the $\alpha$T3-1 pituitary gonadotroph derived cell line (Netiv *et al*, 1991), and in ovarian granulosa cells (Liscovitch and Amsterdam, 1989).

### 1.7.1 $\alpha$T3-1 cell line

Biochemical studies on pituitary cell types have been hampered by the heterogeneous nature of the tissue; gonadotrophs constitute approximately 5% of the cells in the anterior pituitary. One way of overcoming this problem has been the use of an established cell line. This has been done by producing the $\alpha$T3-1 anterior pituitary clonal cell line with the properties of gonadotrophs (Windle *et al*, 1990; Horn *et al*, 1991). The cell line was obtained by targeted tumorigenesis in transgenic mice using the SV40 Tag oncogene linked to the promoter region of the human gonadotrophin hormone $\alpha$ subunit. This cell line expresses high affinity LHRH receptors, but does not synthesise or secrete the $\beta$ subunits of LH or FSH (Windle *et al*, 1990), limiting the usefulness of this cell line as a model for studying the primed release of LH.

Despite the drawback of not being able to assay a secretory end-point in these cells, they have contributed importantly to progress in the field of LHRH signalling. Previous studies have established clearly that LHRH can stimulate PLC-mediated phosphoinositide hydrolysis, with an absence of short term homologous desensitisation (Davidson *et al*, 1994). It is also capable of mobilising calcium from intracellular and extracellular locations (Mitchell *et al*, 1988). There is now increasing evidence that activation of PLD (Netiv *et al*, 1991) and $PLA_2$ mediated pathways also occur, as levels of arachidonate metabolites are elevated in pituitary cells as a consequence of LHRH stimulation (Dan-Cohen *et al*, 1992).

The present study has examined the activation of PLD in the $\alpha$T3-1 cell line. By assessing the generation of the stable product, phosphatidylbutanol (PtdBut), that is produced by PLD in the presence of butan-1-ol, a study has also been made of the kinetics of PLD activation by LHRH and the effect of various agents on the activation process.

# CHAPTER 2

# MATERIALS AND GENERAL METHODOLOGY

## 2.1 Materials

[9,10-$^{3}$H]-Palmitate (60 Ci/ mmol) was obtained from NEN Du Pont de Nemours, Germany, Ro 31-8220 was a gift from Roche Products Ltd, Welwyn, Herts, UK. Ionomycin , phorbol 12,13-dibutyrate, LHRH and the LHRH antagonist ([Ac-D-p-Cl-Phe$^{1,2}$, D-Trp$^{3}$,D-Arg$^{6}$,D-Ala$^{10}$]-LHRH), sodium orthovanadate, chloroquine phosphate, geniticin, penicillin, streptomycin, β-estradiol, progesterone and DL-threo-dihydrosphingosine were obtained from Sigma Chemical Co, Poole, Dorset, UK. Lavendustin A, 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine (H7), genistein, methyl 2,5-dihydroxycinnamate and GF 109203X were obtained from Calbiochem-Novabiochem, Nottingham, UK. Piceatannol and N-[1-(2,3-Dioleoyloxy) propyl]-N, N, N-trimethyl ammoniummethylsulphate (DOTAP) were obtained from Boehringer-Mannheim UK, Lewes, East Sussex. DEAE-dextran was obtained from Promega. The FYN3 polyclonal rabbit antibody was obtained from Santa-Cruz Biotechnology, NBS Biologicals, Hatfield, England. The GD11 mouse monoclonal anti-src antibody and the 4G10 mouse monoclonal anti-phosphotyrosine antibody were obtained from Upstate Biotechnology Incorporated (UBI), Lake Placid. Screw top glass vials (2 ml) were obtained from Chromacol. All other laboratory reagents were AnalaR grade from BDH, Merck Ltd, Poole, Dorset, UK.

## 2.2 Data analysis

All concentration-response curves were analysed by a non-linear, error-weighted direct fitting program, modelling on Hill kinetics (P.fit, Elsevier Biosoft, Cambridge). The significance of the differance between values was determined using the Mann-Whitney U test.

### 2.3 Cell culture

All cultures were grown in a humidified atmosphere of 95% air/ 5% $CO_2$ at a constant temperature of $37^0$C, and received fresh medium every 3-4 days. αT3-1 cells were cultured in Dulbecco's modified eagle medium supplemented with 10% foetal calf serum (heat inactivated), sodium pyruvate (0.11 g/l), 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. COS 7 and CHO cells were cultured in Hams F-12 medium supplemented with 10% foetal calf serum (heat inactivated), 100 U/ml penicillin and 0.1 mg/ml streptomycin. They also received fresh medium every 3-4 days. Cell lines were split when 100% confluent and seeded at low density in fresh flasks. Cells were harvested by trypsin digestion: medium was removed and replaced with 1.5 ml of a 0.25% (v/v) trypsin solution and left for 10 min, the cells were then removed from flask by agitation with fresh growth medium. The cells were then suspended in an appropriate volume for transfer to a fresh flask, or to a 12 well culture plate for assays (1/3 of a confluent flask for each plate) and used after 2 days.

Cell lines were frozen for storage in liquid $N_2$ by resuspending harvested cells at an estimated density of $10^7$ cells/ml in 95% foetal bovine serum (heat inactivated) + 5% dimethylsulphoxide. The cell suspension was aliquoted into cryostat tubes and placed in a closed polystyrene box (room temperature). The box was then placed at $-70^0$C for 3-5 hrs before being transferred to liquid $N_2$. Frozen cells were recovered from liquid $N_2$ by thawing in water at $37^0$C, after the liquid $N_2$ had evaporated from the vial. The contents of the cryostat tube were then transferred to a flask with the appropriate volume of medium, under sterile conditions. Dimethylsulphoxide was removed after the cells were allowed 24 hr recovery, by total replacement of growth medium with fresh growth medium.

## 2.4 ANIMALS

Adult female (~200 g) COB Wistar rats were maintained under controlled lighting conditions (lights on from 05.00 to 19.00 h) and temperature (22$^{0}$C) with free access to food pellets (CRM, Labsure, Manea, Cambs, UK) and tap water.

Female rats were assessed for a minimum of two 4 day oestrous cycles (determined by vaginal lavage) before being anaesthetised with Sagatal (30 mg/kg body weight; sodium pentabarbitone; May and Baker, Dagenham, Essex) by 11 am on the day of the cycle under investigation. The cytological characteristics of the vaginal smears in 4-day cyclic rats were as follows:

Metoestrus: Large number of leucocytes, epithelial cells and cornified epithelial cells.
Dioestrus: Leucocytes, epithelial cells and cornified epithelial cells, but in smaller amounts than in metoestrus.
Pro-oestrus: Mainly nucleated epithelial cells.
Oestrus: Predominantly cornified epithelial cells.

## 2.5 Assay of phospholipase D activity

In order to label the phosphatidyl moiety of phosphatidylcholine, cells were incubated for 2 h with [$^3$H] palmitate (5 μCi/ ml, 1 ml in each well) in minimal essential medium, at 37 °C under 5% $CO_2$ /95% $O_2$. Cells were washed twice with warm MEM (37°C), containing 1% fatty acid free BSA. This was then replaced with 1 ml warm MEM containing 0.5% fatty acid-free BSA to each well. Butan-1-ol was added to each to give a concentration of 30 mM (since previous experiments in this and other laboratories have shown that this concentration of butan-1-ol provides optimal production of [$^3$H]PtdBut, without adverse effects on cell viability as

assessed by trypan blue exclusion). Various inhibitory drugs were added, immediately before adding the stimulating agent which was present for 30 min, unless otherwise stated. Reactions were terminated by removing the 1 ml of medium and adding 0.5 ml ice-cold methanol. Cells were then scraped out of wells and transferred to a 2 ml screw-top glass vial. Chloroform was added to give a chloroform/ methanol ratio of 1:1. Tubes were mixed and left for 15 min, before distilled water was added to give a chloroform/ methanol/ water ratio of 1:1:0.8. The vials were vigorously shaken and centrifuged for 5min to separate the two phases. The top aqueous phase was carefully aspirated and discarded while the lower organic phase was left in the vial and dried under vacuum in a centrifugal evaporator (V.A. Howe, Banbury, UK) for approximately 50 min at 30 °C. Once the lower organic phase had evaporated it was resuspended in 50 μl chloroform/ methanol (19:1) and then spotted onto Whatman LK5D thin layer chromatography laned plates (silica 250 μm). The plates were developed using the organic phase of a mixture: ethyl acetate/ 2,2,4-trimethylpentane/ acetic acid/ water (110: 50: 20: 100). In this system, phosphatidylbutanol ($R_f$ =0.3) is well separated from phosphatidate ($R_f$ =0.1), major phospholipids (Rf =0) and neutral lipids (Rf = 0.7-0.95). The region containing PtdBut, and corresponding to a [$^{14}$C]PtdBut standard (gift from R. Randall, Wellcome Research Laboratories, Kent, UK) was scraped off the plate and radioactivity quantified by liquid scintillation counting.

# CHAPTER 3

# INVOLVEMENT OF PROTEIN KINASE C, AND OTHER MECHANISMS IN THE ACTIVATION OF PLD

## 3.1 INTRODUCTION

There is considerable evidence that PKC plays a key role in the regulation of PLD in many systems (Thompson *et al*, 1993a). Phospholipase D activity can be stimulated in many tissues by phorbol esters (with 4β position stereospecificity), suggesting a role for PKC in activating the enzyme (Billah and Anthes, 1990). Not surprisingly, the phorbol ester-induced activation of PLD is highly sensitive to inhibitors of PKC. Interestingly, certain groups of PKC inhibitors, specifically the bisindolylmaleimides, which have been used in this study show partial selectivity for the $Ca^{2+}$-independent PKC isoforms (Wilkinson *et al*, 1993). Certain unidentified, PDBu-stimulated, PKC activities in pituitary and lung (Ison *et al*, 1993) have also been demonstrated to have a relative resistance to another PKC inhibitor 1-(5-isoquinolinyl sulphonyl)-2-methyl-piperazine (H7). It has also been postulated that this H7-resistant PKC activity plays an important role in the LHRH-priming effect (Johnson *et al*, 1992). Thus it is conceivable that the involvement of different PKC isoforms in a response, such as PLD activation, could be reflected by slightly different sensitivities to inhibitors.

Further strong evidence of the importance of PKC in the activation of PLD has been demonstrated by the over-expression of PKC α (Eldar *et al*, 1993) and β1 (Pai *et al*, 1991a) in rat fibroblasts, in which the PLD activity stimulated by the phorbol ester, phorbol 12-myristate 13-acetate (PMA), and also that mediated by receptor-activated systems such as endothelin (Pai *et al*, 1991b) or α-thrombin (Pachter *et al*, 1992) were enhanced. Another technique widely used in order to implicate PKC involvement, also used in this study, is down-regulation of PKC isoforms, brought about by prolonged preincubation with phorbol esters (McArdle and Conn, 1989).

Diacylglycerol derived from the hydrolysis of $PIP_2$ by PLC has been shown to lead to the activation of protein kinase C (Nishizuka, 1992). However, doubts have been expressed as to whether DAG resulting from PLD-mediated hydrolysis of PC can be similarly effective. Several observations have suggested that PC derived DAG is not capable of activating PKC. For example it was demonstrated that in α-thrombin stimulated fibroblasts, DAG derived from PI but not PC hydrolysis is responsible for the activation of PKC (Leach *et al*, 1991). In IgE-stimulated RBL 2H3 mast cells the PLD mediated production of DAG does not appear to be responsible for PKC activation (Lin *et al*, 1992). However, the idea that DAG derived from PC is ineffective at activating PKC has been challenged by results which show that in α-thrombin- and PDGF-stimulated fibroblasts the late phase of DAG production from PC hydrolysis is associated with the activation of PKC ε, but not PKC α; this difference was shown to be due to the absence of a $Ca^{2+}$ elevation associated with PC hydrolysis, and not due to differences in the species of DAG produced (Ha *et al*, 1993). It has also been suggested that PLD-generated DAG is responsible for a sustained activation of PKC, since the kinetics of DAG accumulation are prolonged, compared to DAG generated from $PIP_2$ by the action of PLC (Billah and Anthes, 1990).

Studies have shown that the consensus composition of the fatty acid side chains is different in DAG derived from PC compared to $PIP_2$ (Pessin and Raben, 1989; Pettit and Wakelam, 1993). This difference may conceivably influence DAG's cellular actions, such as activating PKC, perhaps making it more selective for certain PKC isoforms (Pettit and Wakelam, 1993). Nevertheless it has been demonstrated that it is the absence of a rise of $Ca^{2+}$ and not the source of DAG that is important in thrombin- and PDGF-stimulated fibroblasts (Ha and Exton, 1993). In Swiss

3T3 cells stimulated with bombesin, the fatty acid profile of DAG species produced is time-dependent, with DAG at early time points having a similar profile to that of phosphoinositides and at later time points being similar to PC (Pettit and Wakelam, 1993).

Receptors coupled to heterotrimeric G proteins are widely linked to the activation of PLD in many types of cell (Billah, 1993). A commonly employed technique for demonstrating G protein involvement has been to use the unhydrolysable, stable GTP-analogue, guanosine 5'-($\gamma$-thio)triphosphate (GTP$\gamma$S) in cell free preparations and permeablised cells (Anthes *et al*, 1991). This method has implicated an involvement of G proteins in PLD activation in many cell types, such as responses to fMLP in neutrophils (Olsen *et al*, 1991), and to endothelin in mesangial cells (Kester *et al*, 1992). In several systems, receptor-stimulated activation of PLD can be inhibited by pretreating the cells with the microbial toxin, pertussis toxin, which ADP-ribosylates the $\alpha$ subunit of G-proteins of the Gi and Go families, thus preventing their activation (Milligan, 1988). In fMLP-stimulated HL-60 granulocytes the activation of both PLC- and PLD was blocked by pertussis toxin pre-treatment (Pai *et al*, 1988). However in hepatocytes, the activation of PLC and PLD by vasopressin-, angiotensin II- and adrenaline were insensitive to pertussis toxin, even though these enzymes can be activated in membrane preparations by non-hydrolyzable analogues of GTP (Bockino *et al*, 1987). An insensitivity to pertussis toxin does not rule out G protein involvement, but simply shows that there is no Gi or Go involvement. It therefore appears that PLD can be activated by a variety of G protein-coupled receptors, linked to the intracellular signalling machinary by both Gq and Gi G proteins.

As well as heterotrimeric G proteins being involved in the receptor activation of PLD, members of a group of low molecular weight G proteins,

ARF and Rho have also been implicated in PLD activation (Brown *et al*, 1993; Cockroft *et al*, 1994; Bowman *et al*, 1993). It has been demonstrated that ARF plays a role in vesicle traffic and is a component of the coat of golgi-derived coated vesicles (Serafini *et al*, 1991). In cell free PLD assays carried out in HL-60 cells a cytosolic factor is required for PLD activity. Activity can be reconstituted in cytosol depleted cells by replacing cytosol, and the required cytosolic factors appear to be ARF1 and ARF3 (Cockroft *et al*, 1994). Activation of PLD was dependent on the concentration of ARF protein, indicating a stoichiometric, rather than a catalytic activation of PLD. Activation of PLD by ARF was observed at 100 nM $Ca^{2+}$, however this was greatly enhanced at 10 μM $Ca^{2+}$ (Cockroft *et al*, 1994). The guanine nucleotide exchange factor protein for ARF (ARF-GEF) has been shown to be a target for an inhibitor of vesicle transport, brefeldin A (Donaldson *et al*, 1992). This raises the possibility that brefeldin A could be used to assess whether PLD is activated by ARF in whole cell systems.

Intracellular $Ca^{2+}$ levels appear to be important in the regulation of PLD in some systems. In a wide variety of studies it has been demonstrated that depletion of intracellular and/or extracellular $Ca^{2+}$ inhibits the activation of PLD (Purkiss and Boarder, 1992; Billah *et al*, 1989; Bockino *et al*, 1987; Cook *et al*, 1991), although this technique has not been used in this study. Calcium ionophores are also widely used in order to raise the cytoplasmic $Ca^{2+}$ concentration, and in several cell types they have been shown to stimulate the activation of PLD (Billah *et al*, 1989; Bockino *et al*, 1987; Van Blitterswijk *et al*, 1991).

The kinetics of PLD activation can vary considerably, depending on cell type and receptor pathway. The time course for PLD activation has been shown to vary from ~5 sec, followed by rapid desensitisation (Briscoe *et al*, 1994), to a substantial delay after receptor activation (5-10 min),

followed by a very slow desensitisation (Netiv *et al*, 1991). It could be postulated that the time course of PLD activity will be closely related to the cellular functions of PA and DAG. It may also reflect the action of individual isoforms of PLD.

## 3.2 METHODS

### 3.2.1 Phospholipase D assay

Assays for phospholipase D were carried out using the standard protocol, as described in section 2.5.

## 3.3 RESULTS

### 3.3.1 PLD kinetics and activators

The production of [$^3$H] phosphatidylbutanol (PtdBut) in $\alpha$T3-1 cells incubated with LHRH for 30 min showed a marked increase over the very low levels in control cells (approximately 60 000 dpm in stimulated cells compared to approximately 3000 dpm in control cells) and clear concentration-dependence (Figure 3.1). Under these conditions, the $EC_{50}$ for PLD activation by LHRH was $16 \pm 5$ nM and reached a maximum by approximately 100 nM. A concentration of 100 nM LHRH was therefore used in most of the other experiments in this study, in order to elicit a large (but not supra-maximal) PLD response.

The time course (up to 40 min) of [$^3$H] PtdBut accumulation elicited by LHRH (100 nM) or PDBu (1 $\mu$M) is shown in Figure 3.2. In the LHRH-stimulated cells there was a lag of between 5 and 10 min before [$^3$H] PtdBut production could be detected. The rate of [$^3$H] PtdBut accumulation appeared to be approximately constant between 5 and 40 min. In PDBu-stimulated cells there was an even longer lag of between 10 and 15 min before [$^3$H] PtdBut levels were detectable. The activation of PLD by PDBu appeared to occur at a rate comparable to the LHRH stimulated activity only

for around 10 min before the rate of [$^3$H] PtdBut accumulation declined. Thus at all times, especially the longer time points, PDBu (1 μM) caused a much lower level of [$^3$H] PtdBut accumulation than LHRH (100 nM).

The LHRH receptor antagonist [Ac-D-p-Cl-Phe$^{1,2}$,D-Trp$^3$,D-Arg$^6$,D-Ala$^{10}$]-LHRH (Neckola *et al*, 1982) (1 μM),completely prevented activation of PLD by LHRH (100 nM), whilst the antagonist itself caused no detectable activation of PLD (Table 3.1). The biologically-inactive phorbol ester isomer, 4α-phorbol 12,13-dibutyrate (4α-PDBu, 1 μM) elicited no increase in PLD activity at a concentration of 1 μM. The $Ca^{2+}$ ionophore, ionomycin (30 μM) caused no significant activation of PLD by itself, although it did slightly augment the PDBu-stimulated PLD activity (Table 3.1). A combination of LHRH (100 nM) and PDBu (1 μM) elicited approximately additive levels of [$^3$H] PtdBut production (Table 3.1), even though the large accumulation due to 100 nM LHRH was maximal on its own concentration-response curve. To address the possibility that the lag in [$^3$H] PtdBut production was due to a requirement for protein synthesis in the mechanism of PLD activation, experiments were carried out in the presence of cycloheximide (30 μM), but this clearly had no effect on LHRH-induced PLD activity (Table 3.1).

Sphingosine and its metabolite, sphingosine-1-phosphate have been reported to increase phosphatidic acid levels by PLD activation (Desai *et al*, 1992). DL-threo-dihydrosphingosine (30 μM) did cause a slight activation of PLD, 12 ± 2.5 % of that observed for LHRH (100 nM) stimulation (Table 3.1).

### 3.3.2 PKC involvement

Two approaches were used to assess the role of PKC in LHRH-induced PLD activation; firstly by employing a number of selective PKC

inhibitors and secondly, down-regulation of PKCs in $\alpha$T3-1 cells by prolonged preincubation with a phorbol ester prior to LHRH stimulation (McArdle and Conn, 1989). Over a 30 min incubation with LHRH (100 nM), the selective bisindolylmaleimide PKC inhibitor Ro 31-8220 (Davis *et al*, 1992) completely inhibited the evoked PLD activity with an $IC_{50}$ of 460 $\pm$ 180 nM (Figure 3.3). In cells stimulated for 30 min with PDBu (1 $\mu$M), Ro 31-8220 again completely inhibited PLD activity but with an $IC_{50}$ of 62 $\pm$ 30 nM (Figure 3.3). This represents an approximately eight fold greater potency of Ro 31-8220 when PDBu was used as a stimulus instead of LHRH. Another highly-selective bisindolylmaleimide PKC inhibitor GF 109203X (Toullec *et al*, 1991) completely inhibited the PLD activity stimulated by LHRH (100 nM), with an $IC_{50}$ of 1.0 $\pm$ 0.2 $\mu$M (Figure 3.4). PDBu-stimulated PLD activity was more potently inhibited by GF 109203X, with an $IC_{50}$ of 161 $\pm$ 19 nM (Figure 3.4).

A PKC inhibitor of another structural class, 1-(5-isoquinolinyl sulphonyl)-2-methyl-piperazine (H7) (Hidaka *et al*, 1984) inhibited LHRH-stimulated activation of PLD with an $IC_{50}$ of 232 $\pm$ 25 $\mu$M (Figure 3.5). The slope factor of the concentration-inhibition curve (1.27 $\pm$ 0.15) was consistent with a single component to the inhibition. However in cells stimulated with PDBu (1 $\mu$M) (Figure 3.5), H7 inhibited the response over a very wide concentration range (slope factor = 0.56 $\pm$ 0.05). This is consistent with multiple components being involved in the inhibition. The concentration-inhibition curve gave a much better fit to a 2 component Hill equation than to a one site model with component parameters of $IC_{50}$(i) = 12.5 $\pm$ 1.9 $\mu$M (45% of sites) and $IC_{50}$(ii) = 635 $\pm$ 85 $\mu$M (55% of sites).

Down-regulation of PKC isoforms using 300 nM PDBu caused no significant reduction of the LHRH-stimulated PLD activity after 1 hr. However after 16 hrs it resulted in a 74 $\pm$ 3% reduction in the LHRH-

stimulated PLD activity, and after 24 hrs of phorbol treatment the LHRH-stimulated activation of PLD was attenuated by 82 ± 9% (Figure 3.6).

### 3.3.3 G-protein involvement

The LHRH-stimulated activation of PLD was insensitive to pertussis toxin up to a concentration of 300 ng/ml, the highest concentration used. The αT3-1 cells were pretreated with pertussis toxin for approximately 16 hrs prior to the assay, which represents a fully adequate time for the inactivation of any G-proteins sensitive to ADP-ribosylation by pertussis toxin (Milligan, 1988).

Brefeldin A, a compound which has been demonstrated to inhibit the guanine nucleotide exchange on the low molecular weight G protein ARF (Donaldson *et al*, 1992), inhibited the LHRH-stimulated PLD activity with an $IC_{50}$ of 320 ± 115 μM (Figure 3.7), however this inhibition was not complete by 1 mM brefeldin A, the highest concentration used.

## 3.4 DISCUSSION

Despite being clearly coupled via G proteins to phosphoinositide hydrolysis (Snyder and Bleasdale, 1982; Schrey, 1985; Anderson *et al*, 1992, Davidson *et al*, 1994), the LHRH receptor is an atypical member of the family of such receptors since it lacks an extended C-terminal tail (Tsutumi *et al*, 1992). There is evidence that phosphorylation of the C-terminal tail of several G protein-coupled receptors contributes importantly to receptor desensitisation (Probst *et al*, 1992). Therefore the lack of a C-terminal tail on the LHRH receptor may contribute to the complete lack of desensitisation seen in the PLD response to LHRH over the 40 min time course used in this study (Figure 3.2). Desensitisation may also be altered by virtue of the unusual sequence differences in consensus sequences of

the transmembrane 2 and transmembrane 7 domains (Zhou *et al*, 1994); this topic will be discussed further in Chapter 6.

LHRH-stimulated activation of PLD proceeded after a lag of about 5 min (Figure 3.2). This result is similar to that of a previous study carried out on $\alpha$T3-1 cells, in which it was proposed that PLD activation required a particularly high receptor occupancy, thus dictating a lag before PLD activity could be detected (Netiv *et al*, 1991). However, the observation of an even more pronounced lag in activation by PDBu suggests that the delay is mainly due to some factor other than the rate of receptor occupancy by an agonist. The experiments with cycloheximide also indicate that the lag in activation of PLD by LHRH was not due to the synthesis of an intermediary activating protein.

It is unclear why the mechanism through which PDBu causes activation of PLD becomes so markedly attenuated after 10 min of detectable activity. It is known that even quite short incubations of cells (including $\alpha$T3-1 cells) with high concentrations of phorbol esters can lead to down-regulation of many PKC isoforms (Johnson *et al*, 1993a), and as has been previously shown, phorbol esters will have a more profound down-regulatory effect on PKC than receptor agonists (Lutz *et al*, 1993). However, a 1 hr preincubation with PDBu (300 nM) did not result in any detectable reduction of the LHRH-stimulated activation of PLD (Figure 3.6). The prolonged delay before PDBu caused PLD activation compared to LHRH is also unexplained. It is of course possible that PDBu is activating isoforms of PKC not normally involved in the LHRH receptor activation of PLD, or that a different isoform of PLD may be activated through the PDBu-stimulated pathway. The additive effect of LHRH and PDBu on the PLD response supports the theory that these two stimuli do use different

biochemical pathways at least to some degree in order to bring about the activation of PLD.

Calcium ionophores are in general good activators of PLD (Thompson *et al*, 1991). However, increasing the concentration of intracellular calcium with ionomycin, which has been reported to release $Ca^{2+}$ from intracellular stores (Morgan and Jacob, 1994), was not sufficient by itself to activate PLD in this study. Furthermore ionomycin was not able to potentiate to any great extent the PLD response elicited by PDBu. Both $\alpha$ and $\beta$ isoforms of PKC have been implicated in PLD activation in different systems. It was demonstrated in membane preparations from Chinese hamster lung fibroblasts that addition of purified PKC $\alpha$, and to a lesser extent PKC $\beta$ stimulates PMA and $Ca^{2+}$-induced PLD activity (Conricode *et al*, 1994). Overexpression of PKC $\beta$ in fibroblasts has also been shown to lead to an elevation in the phorbol-stimulated PLD activity (Eldar *et al*, 1993; Pai *et al*, 1991a). It is therefore entirely possible that $Ca^{2+}$-dependent PKCs are also involved here, since it is known that LHRH receptor activation increases intracellular $Ca^{2+}$ levels within the cells (Limor *et al*, 1987; Anderson *et al*, 1992), which could contribute to activation of such $Ca^{2+}$-dependent PKC isoforms. Receptor systems that activate PLD in a PKC-independent manner, such as the angiotensin II-stimulated activation of PLD in vascular smooth muscle cells have been shown to be blocked by chelation of intracellular and/or extracellular $Ca^{2+}$ (Lassegue *et al*, 1991; Billah *et al*, 1989), whereas the activation of PLD in $\alpha$T3-1 cells appears to be highly dependent on PKC. Interestingly a report has described the inhibition by $Ca^{2+}$ of guanosine 5'-0-(3-thiotriphosphate)-induced PLD activation in permeablised NG 108-15 neuroblastoma X glioma cells (Liscovitch and Eli, 1991).

Sphingosine has previously been reported to be an inhibitor of PKC (Hannun and Bell, 1990). Further studies have also demonstrated that sphingosine has complex biological effects, many of which seem to be independent of PKC (Faucher *et al*, 1988), such as the activation of sphingosine-activated protein kinase (Pushkareva *et al*, 1993), illustrating the complex modulation of signalling systems by these lipids. It has also recently been reported that sphingosine, and its metabolite, sphingosine 1-phosphate are able to stimulate PLD activity (Desai *et al*, 1992). It was also found that sphingosine caused a modest increase in PLD activity in this study. It is therefore possible that sphingosine and sphingosine 1-phosphate produced within the cell are able inhibit the activity of PKC, while at the same time increasing the activity of PLD. This might be important if DAG and PA were needed for cellular functions not involving PKC.

The present results provide strong evidence for an obligatory role of PKC in the LHRH-stimulated activation of PLD, since this response was greatly attenuated by PKC down-regulation and fully blocked by selective PKC inhibitors. The bisindolylmaleimide PKC inhibitors Ro 31-8220 and GF 109203X were about eight times more potent against PDBu-stimulated PLD activation than against LHRH-stimulated PLD activation. Since all phorbol ester-sensitive PKC isoforms are likely to be strongly activated by the relatively high concentration of PDBu used (1 $\mu$M), it is possible that a form of PKC relatively resistant to Ro 31-8220 may be activated in response to LHRH, in contrast to the consensus of those, more sensitive, isoforms activated by PDBu. It has been reported that a bisindolylmaleimide related to Ro 31-8220 shows selectivity for the $Ca^{2+}$-dependent PKCs compared to PKC $\varepsilon$ (Wilkinson *et al*, 1993). It has been demonstrated that $\alpha$T3-1 cells express the PKC isoforms $\alpha$, $\varepsilon$ and $\zeta$ at high levels (Johnson *et al*, 1993b). Interestingly, the atypical $Ca^{2+}$-independent isoform, PKC-$\zeta$ only has one

cysteine-rich zinc finger domain, and is therefore not activated by phorbol esters which require two zinc finger domains. this PKC isoform also shows a relative resistance to bisindolylmaleimides and related indolocarbazoles compared to other PKC isoforms (Martiny-Baron *et al*, 1993). It is therefore possible that PKC $\zeta$, or a related species may be involved in the LHRH-stimulated PLD pathway, since this response is less sensitive to bisindolylmaleimides than the PDBu-stimulated response. The relatively high potency of Ro 31-8220 and GF 109203X on LHRH responses, compared to other studies in whole cells does however suggest that some form of PKC is involved in PLD activation. For example Ro 31-8220 was able to attenuate the LHRH-stimulated PLD activity at a two-fold lower concentration than that seen for PMA-stimulated PLD activity in Swiss 3T3 cells (Cook *et al*, 1991). It has also been demonstrated that Ro 31-8220 potently inhibits the $P_2$ receptor- or PMA-stimulated activation of PLD in endothelial cells at a concentration of less than 1$\mu$M (Purkiss and Boarder, 1992). The other bisindolylmaleimide PKC inhibitor used in this study, GF 109203X, has been shown to inhibit platelet aggregation stimulated by $\alpha$-thrombin and collagen with $IC_{50}$s of 875 $\pm$ 216 and 760 $\pm$ 250 nM respectively, and to inhibit the phosphorylation of major cellular substrates of PKC (Toullec *et al*, 1991). The concentrations required in all of the above studies in whole cells compare favourably with our own results for the bisindolylmaleimide inhibition of LHRH- and phorbol-stimulated PLD activity, although it is of course possible that an unknown kinase with some sensitivity to bisindolylmaleimides (other than a PKC isoform) is mediating the LHRH-stimulated activation of PLD.

Another PKC inhibitor, H7, also inhibited both the LHRH- and PDBu-induced increases in PLD activity. LHRH-stimulated PLD activity was inhibited in a monophasic fashion, suggesting either that only one PKC

isoform was involved, or that all those involved had the same low sensitivity to H7. Compared to the potency of inhibition of various PKC isoforms by H7 in cell-free assays, the LHRH-induced PLD activity was markedly insensitive to H7 (Schapp and Parker, 1990). However, H7 competes for ATP binding, and in a whole cell system it has been shown that H7 blocks the release of LH from LHRH-stimulated anterior pituitaries with an $IC_{50}$ of 71 ± 13 μM (Johnson *et al*, 1992), while the H7 inhibits the PDBu-induced release of LH from anterior pituitaries *in vitro* with an $IC_{50}$ of 1.7 ± 1.5 μM (Thompson *et al*, 1993b). It has also been reported that there is a distinct H7-resistant form of PKC in the rat anterior pituitary gland, that may represent either a novel or modified PKC isoform, although it is clear that this species is phorbol ester-activated and can be clearly separated from PKC-ζ by ion-exchange and hydroxyapatite chromatography (Ison *et al*, 1993).

There appear to be multiple components to the inhibition by H7 of PDBu-evoked PLD activity. Interestingly this suggests that PLD activation by stimulation with phorbol esters involves more than one isoform of PKC, and whilst it has already been shown using overexpression studies that the $Ca^{2+}$-dependent PKC isoforms α and β1 can participate in PLD activation (Eldar *et al*, 1993; Pai *et al*, 1991a), it now seems likely that further species of PKC may also be able to contribute.

The fact that compounds which inhibit the activity of PKC by competing with the ATP-binding site are capable of inhibiting the LHRH- and PDBu-stimulated PLD activity suggests that the mechanism by which PKC brings about PLD activation involves protein phosphorylation. This contrasts with one study which concluded that PKC activates PLD though a phosphorylation-independent mechanism, since there was no ATP requirement (Conricode *et al*, 1992).

PDBu-stimulated activation of PLD activity was almost completly desensitised over 16 hrs exposure to PDBu, consistent with the activator-induced down-regulation of PDBu-sensitive PKC isoforms (Hug and Saare, 1993), which has been reported to result in a faster down-regulation of the $Ca^{2+}$-dependent PKC $\alpha$, compared to the $Ca^{2+}$-independent PKC $\varepsilon$ (Huwiler *et al*, 1991). In contrast, although apparently blocked by highly-selective PKC inhibitors, LHRH-induced PLD activation showed no desensitisation through 40 min, thus suggesting that a PKC isoform resistant to down-regulation (perhaps $\zeta$) was playing a significant role, in contrast to the PDBu-stimulated PLD activation. However, it may be that other, more complex mechanisms may be responsible for the results observed. In fact the loss of the vast majority of LHRH-induced PLD activation following down-regulation of PDBu-sensitive PKCs may count against the major involvement of a PDBu-insensitive PKC such as $\zeta$, since it has been observed that PKC $\zeta$ was not down-regulated as a result of long term phorbol ester treatment (Ways *et al*, 1992; Chen and Murakami, 1992). However there appears to be some controversy as to whether PKC $\zeta$ can be down-regulated by phorbol ester treatment, since it has been reported PKC $\zeta$ translocates to the membrane as a result of PMA treatment in human platelets (Crabos *et al*, 1992) and has been shown to be down-regulated as a result of PDBu-treatment in $\alpha$T3-1 cells (Johnson *et al*, 1993a). Nevertheless, since the down-regulation protocol involves intense activation of PDBu-sensitive isoforms in the initial stages (which could lead to profound secondary effects within the cell), the interpretation of the down-regulation result must require a degree of caution. It has also been reported that other proteins have Cys-rich motifs, responsible for phorbol binding, such as Raf, myosin heavy chain kinase, DAG kinases, and the low molecular weight G-proteins Vav and n-chimerin (reviewed in Liscovitch

and Cantley, 1994). It is therefore likely that these, and other proteins will be affected by long-term phorbol ester treatment.

LHRH-stimulated PLD activation was insensitive to inhibition by pertussis toxin (up to 300 ng/ml), which would suggest that the G-protein involved is not a member of the Gi or Go family which are sensitive to pertussis toxin (Milligan, 1988). This result is similar to recent evidence that inositol phosphate production in $\alpha$T3-1 cells is also insensitive to pertussis toxin, where it was postulated that the G protein involved was a member of the Gq/$G_{11}$ family (Anderson *et al*, 1992). It therefore seems likely that the activation of both PLC and PLD are linked to the LHRH receptor in the intracellular signalling pathway by a pertussis toxin-insensitive G protein, possibly Gq. Although the involvement of both PKC (Chapter 3) and tyrosine kinases (Chapter 4) in the activation of LHRH-stimulated PLD suggests that there is no direct interaction between the subunits of heterotrimeric G proteins and PLD.

Brefeldin A, an inhibitor of vesicle transport in the golgi, was clearly able to inhibit LHRH-stimulated PLD activation, although at a slightly higher concentration than that used to prevent the formation of coated vesicles on golgi cisternae in a cell free system, which was 150 µM (Orci *et al*, 1991). The cellular target of brefeldin A appears to be the guanosine nucleotide exchange factor for the low molecular weight G-protein ARF (Donaldson *et al*, 1992). It has recently been reported that ARF is able to activate PLD in a GTP-dependent manner in HL60 cells (Cockroft *et al*, 1994; Brown *et al*, 1993). These results raise the possibility that PLD may be involved in vesicle formation within the cell, possibly by the formation of an area of polar phospholipids on the membrane, or by the generation of lipid second messengers which control other components of the veisicle formation or fusion mechanism. This pathway also demonstrates a possible means by

which the vesicle formation machinery could be under direct receptor-linked second messenger control. It has also been postulated that another low molecular weight G-protein, Rho, may also be involved in the activation of PLD in neutrophils, since the Rho-GDP dissociation inhibitor (Rho-GDI), a regulatory protein that specifically inhibits the functions of the Rho family of small G-proteins was able to inhibit GTP$\gamma$S-stimulated PLD activity (Bowman *et al*, 1993). It is possible that the low molecular weight G protein ARF may be involved as a direct activator of PLD in the LHRH-stimulated activation of PLD, downstream of other components in the activation pathway. It must however be noted that the use of brefeldin A is not proof of the involvement of ARF, and can only be used as a pointer.

The results presented in this chapter provide strong evidence for an obligatory involvement of PKC (possibly a phorbol ester insensitive isoform, such as PKC $\zeta$) in the LHRH-stimulated activation of PLD at some point in the signalling pathway. However, this does not mean that PKC brings about PLD activation through a direct phosphorylation, since there appear to be other components to the activation process downstream of PKC, such as tyrosine kinase (Chapter 4) and possibly ARF. It is apparent that the LHRH receptor is coupled to the downstream signalling machinery, in the case of PLD activation through a pertussis toxin-insensitive G-protein, possibly a member of the Gq family. It is also possible that a low molecular weight G-protein, such as ARF is involved in the LHRH-mediated activation of PLD, since an inhibitor of the GTP-exchange factor for ARF, brefeldin A, is able to attenuate the LHRH-stimulated activation of PLD. It is interesting that additivity is observed in the PLD response when stimulated by both LHRH and PDBu; this suggests that these two stimuli activate PLD through different pathways, further consistent with the possible involvement of a

phorbol ester-unresponsive PKC isoform in the LHRH-stimulated pathway.

**Table 3.1**

**Compounds effecting the activity of PLD in αT3-1 cells**

Effect of various compounds on the accumulation of [$^3$H] PtdBut in αT3-1 cells over a 30 min time course from three separate experiments. Values are expressed as a % of the LHRH (100 nM)-stimulated PLD activity, with the basal level of [$^3$H] PtdBut accumulation (~3000 dpm) subtracted, and are means ± SEM from 4-8 separate determinations.

**Table 3.1**

| Induced Response | dpm x $10^3$ (mean % response to LHRH in parentheses) |
|---|---|
| **a** | |
| LHRH (100 nM) | 61 ± 5.5 (100 ± 9) |
| LHRH antagonist (1 μM) | 0.6 ± 1.2 (-1 ± 2) |
| LHRH (100 nM) + LHRH antagonist (1 μM) | 1.8 ± 1.2 (1 ± 2) |
| **b** | |
| LHRH (100 nM) | 61.2 ± 6.2 (100 ± 10) |
| 4β-PDBu (1 μM) | 33 ± 4.8 (54 ± 4) |
| 4α-PDBu (1 μM) | 1.2 ± 1.2 (0 ± 2) |
| LHRH (100 nM) + 4β-PDBu (1 μM) | 79.1± 5.5 (131 ± 9) |
| Ionomycin (30 μM) | 1.8 ± 3.8 (1 ± 2.1) |
| 4β-PDBu (1 μM) + ionomycin (30 μM) | 39 ± 2.4(64 ± 4) |
| **c** | |
| LHRH (100 nM) | 61 ± 6 (100 ± 10) |
| LHRH (100 nM) + cycloheximide (30 μM) | 60 ± 2.4 (98 ± 4) |
| DL-threo-dihydrosphingosine (30 μM) | 7.3 ± 1.5 (12 ± 2.5) |

## Figure 3.1

### Concentration response curve for LHRH-stimulated activation in $\alpha$T3-1 cells

Concentration-response curve for LHRH-stimulated activation of PLD (ie [$^{3}$H]PtdBut accumulation) over a 30 min incubation with the basal value subtracted. Values are means ± SEM from 4-8 separate determinations.

**Figure 3.1**

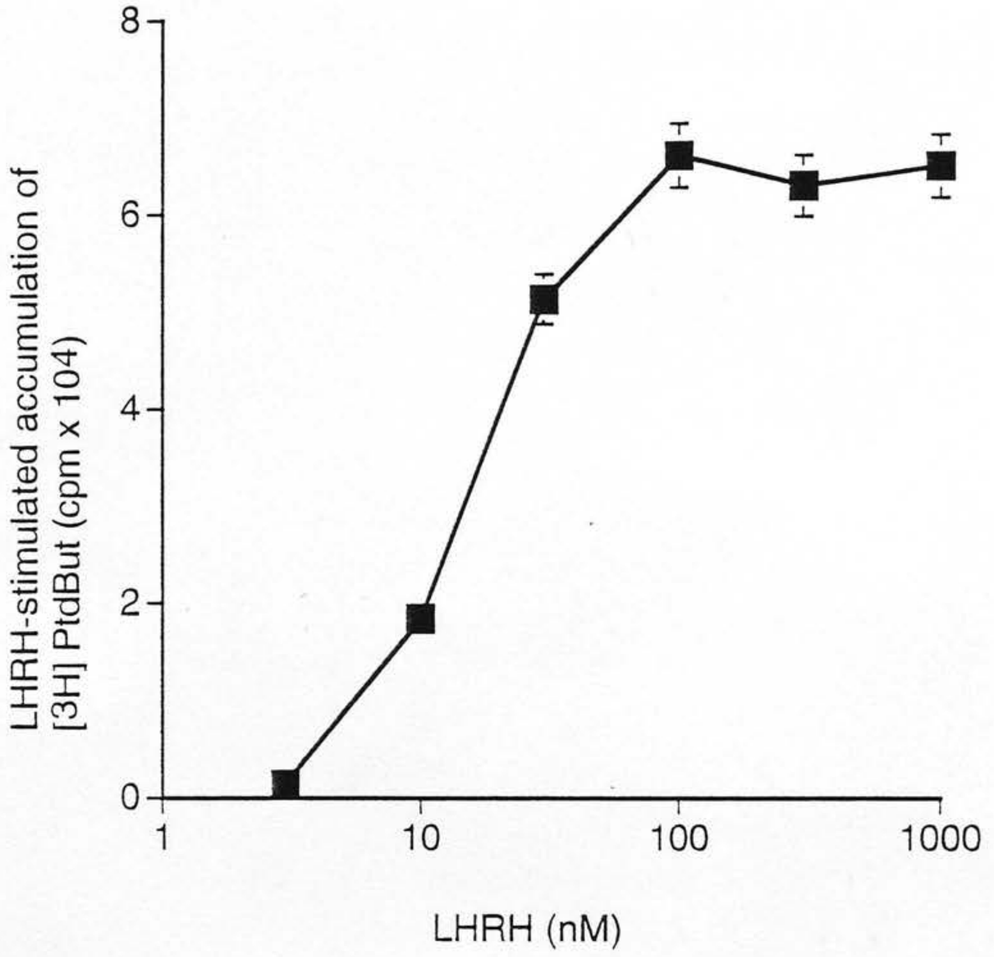
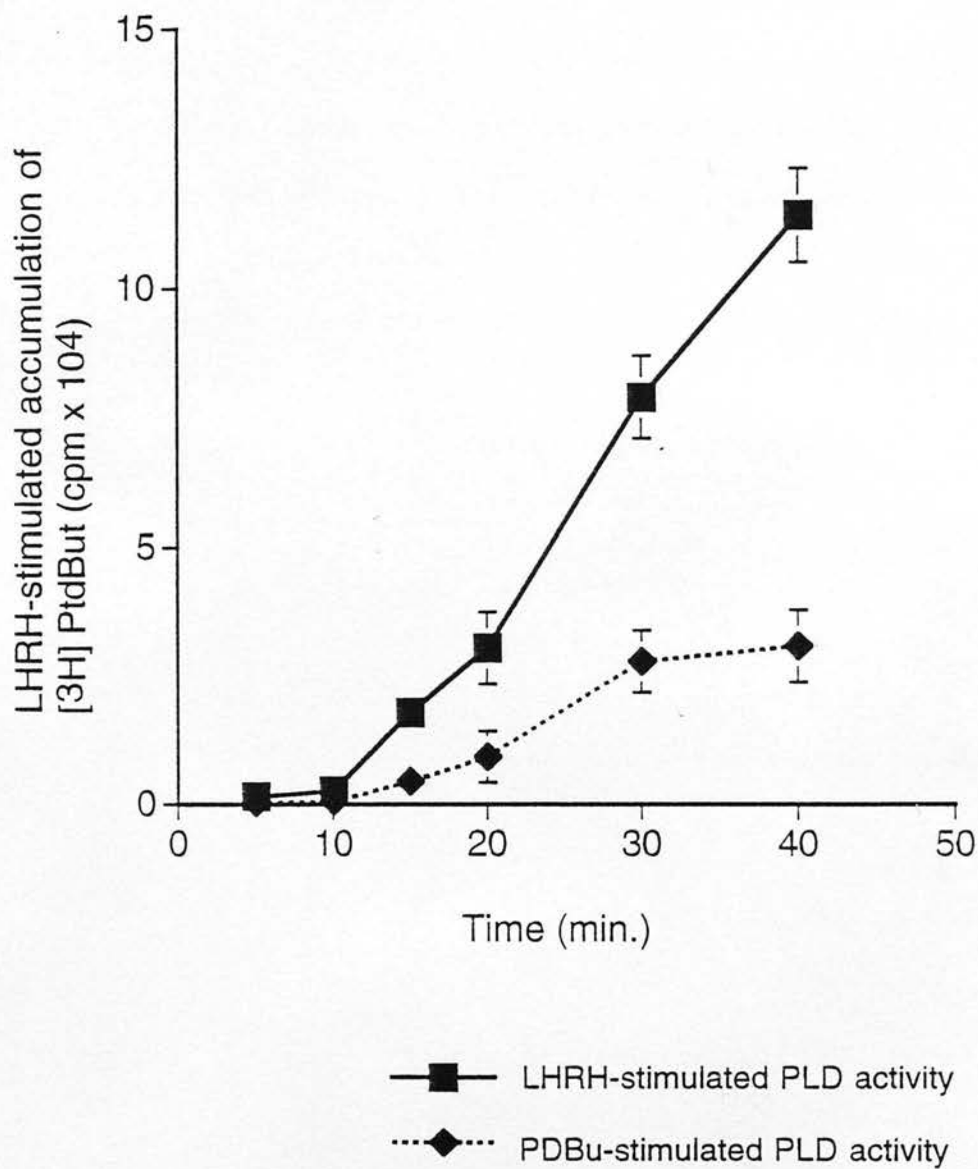


**Figure 3.2**

**Time course of LHRH- and PDBu-stimulated activation of PLD in $\alpha$T3-1 cells**

Time course of PLD activation by LHRH (100 nM) and PDBu (1 $\mu$M) with the basal values subtracted. Values are means ± SEM from 4-8 separate determinations.

**Figure 3.2**



## Figure 3.3

**Concentration response curve for the inhibition of LHRH-and PDBu-stimulated PLD activity by Ro 31-8220 in αT3-1 cells**

The effect of the protein kinase C inhibitor Ro 31-8220 on stimulus-induced PLD activation. Concentration-dependent inhibition of responses to LHRH (100 nM)and and to PDBu (1 μM) with the basal values subtracted. Stimulation was for 30 min, and values are means ± SEM from 4-8 separate determinations. Values above and including 0.01 μM Ro 31-8220 for LHRH-stimulated PLD activity and above and including 0.03 μM Ro 31-8220 for PDBu-stimulated PLD activity are significantly different from the stimulus-induced PLD activity ($p<0.05$, Mann-Whitney U test). There was no effect of Ro 31-8220 on the basal accumulation of [$^{3}$H] PtdBut.

**Figure 3.3**

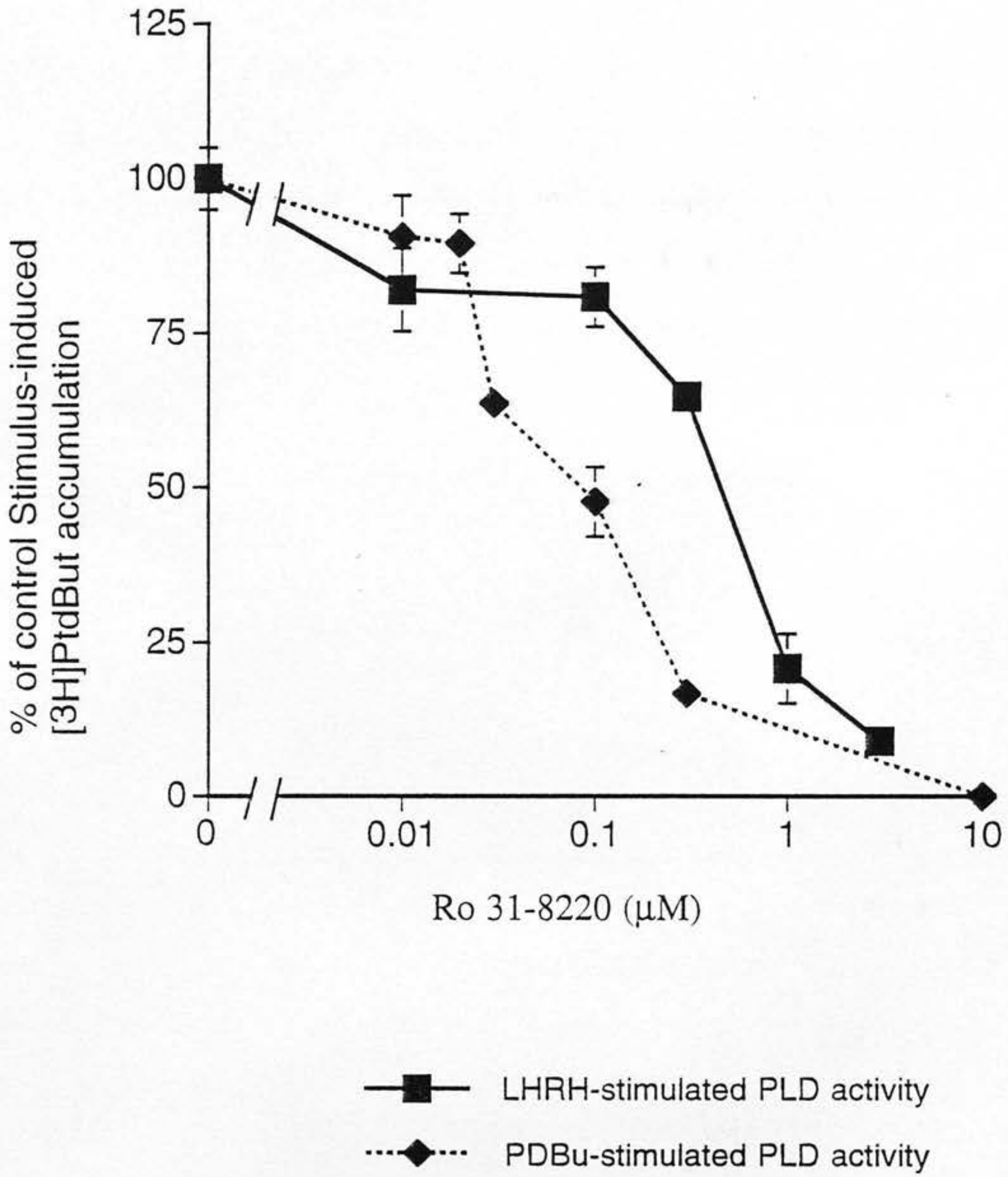


**Figure 3.4**

**Concentration response curve for the inhibition of LHRH- and PDBu-stimulated PLD activity by GF 109203X in αT3-1 cells**

The effect of the protein kinase C inhibitor GF 109203X on stimulus-induced PLD activation. Concentration-dependent inhibition of responses to LHRH (100 nM)and and to PDBu (1 μM), with basal values subtracted. Stimulation was for 30 min, and values are means ± SEM from 4-8 separate determinations. Values above and including 0.3 μM GF 109203X for LHRH-stimulated PLD activity and 0.03 μM GF 109203X for PDBu-stimulated PLD activity are significantly different from the stimulus-induced PLD activity ($p<0.05$, Mann-Whitney U test). There was no effect of GF 109203X on the basal accumulation of [$^3$H] PtdBut.

**Figure 3.4**

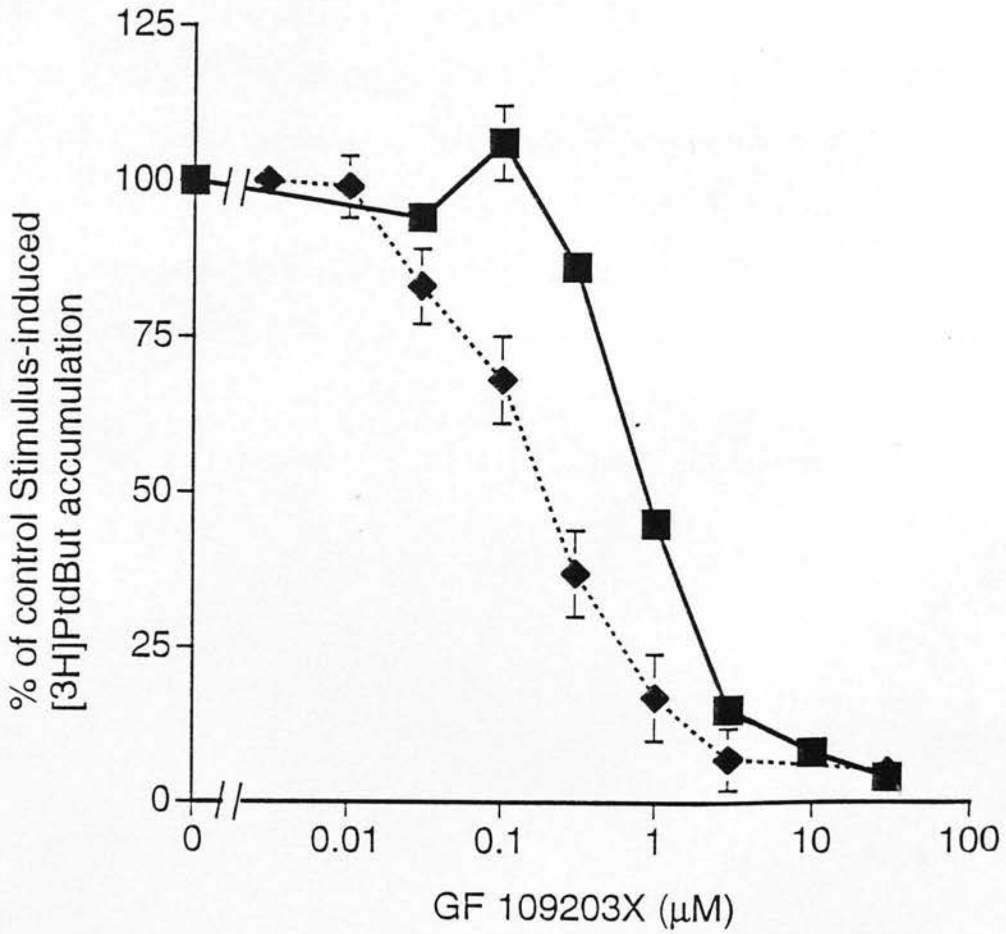
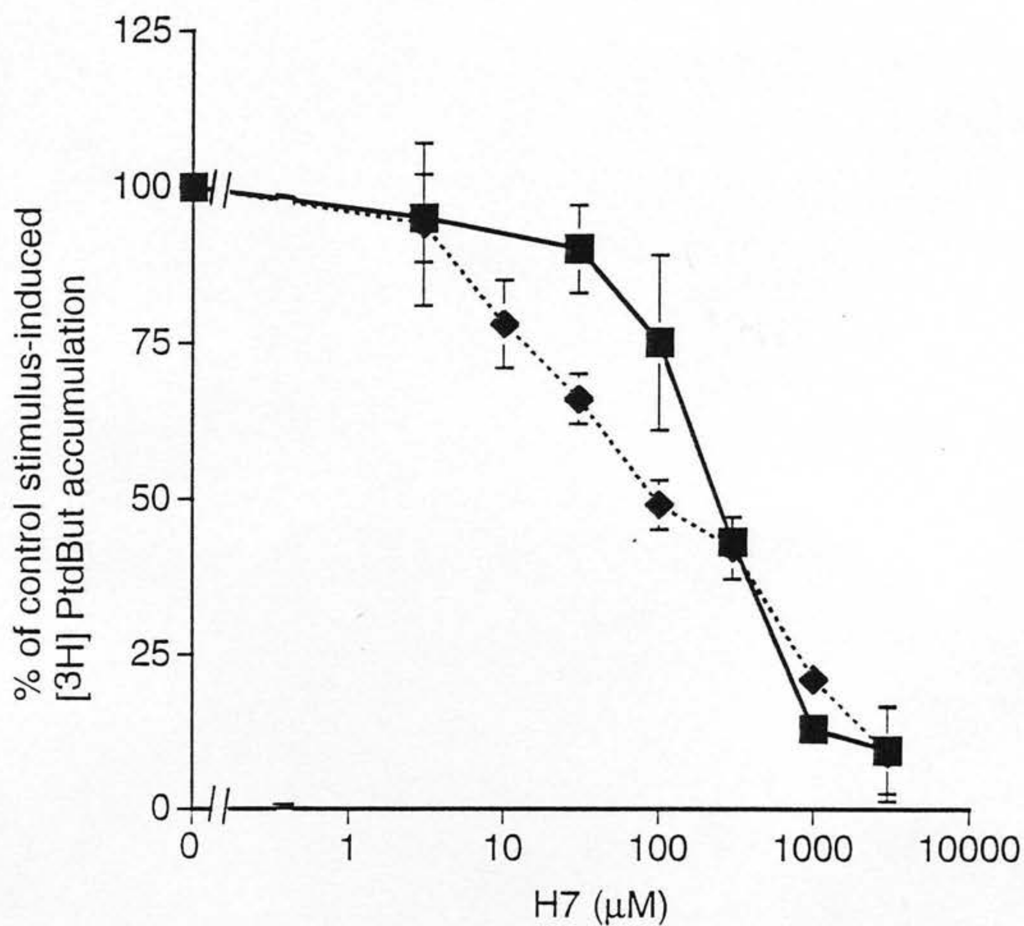


**Figure 3.5**

**concentration response curve for the inhibition of LHRH- and PDBu-stimulated PLD acrivity by H7 in αT3-1 cells**

The effect of the protein kinase C inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine H7 on stimulus-induced PLD activation. Concentration-dependent inhibition of responses to LHRH (100 nM)and and to PDBu (1 μM). Stimulation was for 30 min, and values are means ± SEM from 4-8 separate determinations. Values above 10 μM H7 for the LHRH-stimulated PLD activity and 100 μM for the PDBu-stimulated PLD activity are significantly different from the stimulus-induced PLD activity ($p<0.05$, Mann-Whitney U test). Curve fitting analysis indicated an unusually low Hill coefficient (0.56 ± 0.05; consistent with the contribution of multiple components) in the case of H7 inhibition of PDBu-stimulated PLD activity but not for LHRH-stimulated PLD activity. There was no effect of H7 on the basal accumulation of [$^3$H] PtdBut.

**Figure 3.5**




LHRH-stimulated PLD activity

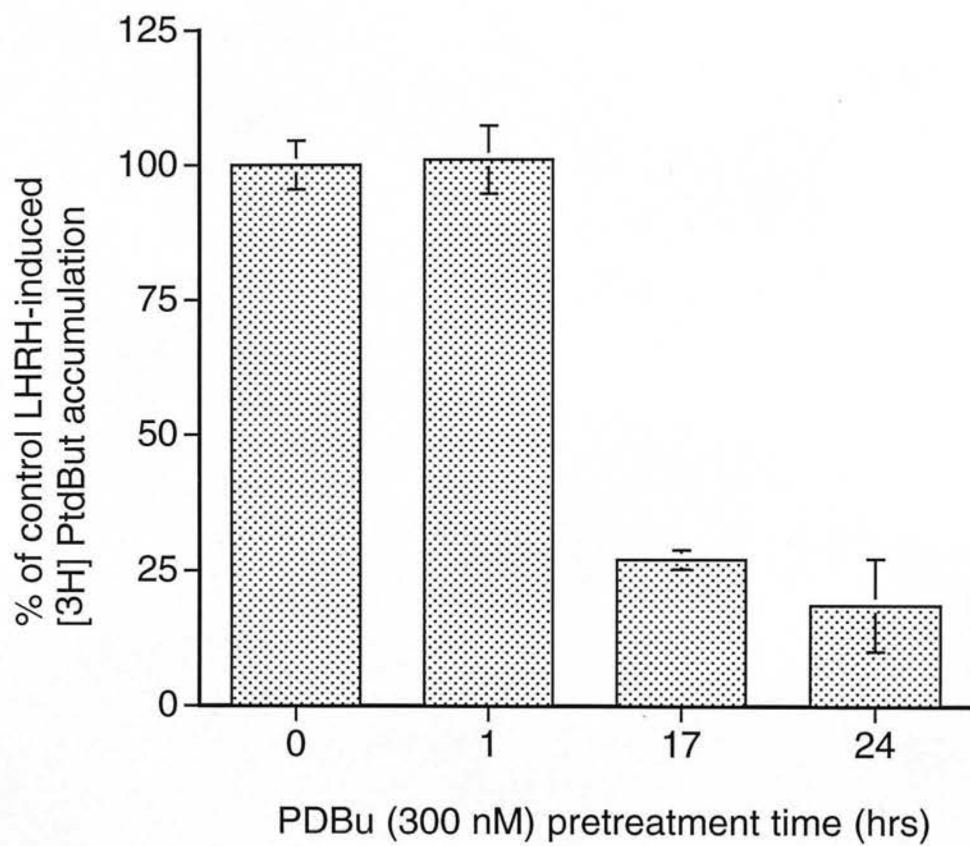
PDBu-stimulated PLD activity

**Figure 3.6**

**LHRH-stimulated activation of PLD in αT3-1 cells treated for varying periods of time with PDBu (300 nM)**

LHRH-stimulated activation of PLD in αT3-1 cells treated with the phorbol ester PDBu (300 nM) for different lengths of time over a 24 hr period. LHRH (100 nM) stimulation of PLD activity was for 30 min, and values are means ± SEM from 4-8 separate determinations with the basal levels of [$^{3}$H]PtdBut accumulation subtracted.

**Figure 3.6**

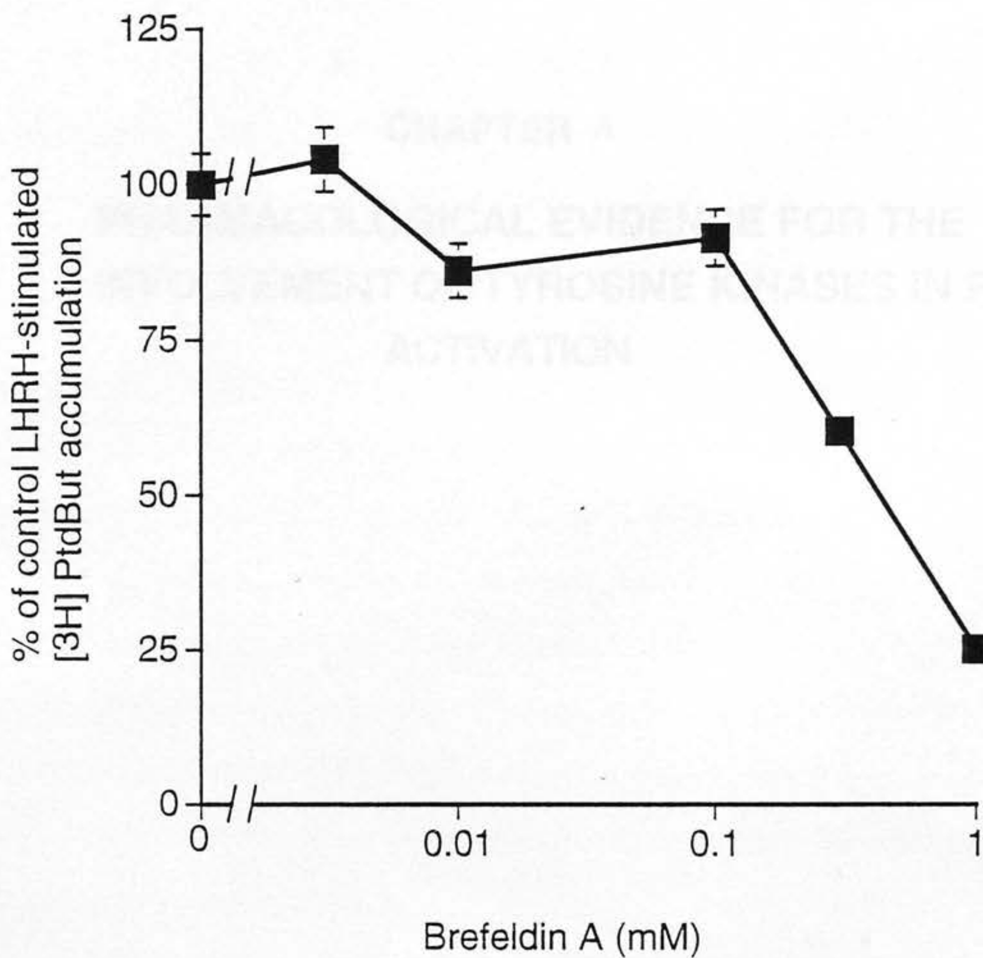


## Figure 3.7

**Concentration response curve for the inhibition of LHRH-stimulated PLD activity by brefeldin A**

Inhibition of LHRH-stimulated PLD activity by an inhibitor of ARF guanine nucleotide exchange factor, brefeldin A. Stimulation was for 30 min, and values are means ± SEM from 4-8 separate determinations with the basal level of $[^3H]$PtdBut accumulation subtracted.

**Figure 3.7**



# CHAPTER 4

# PHARMACOLOGICAL EVIDENCE FOR THE INVOLVEMENT OF TYROSINE KINASES IN PLD ACTIVATION

## 4.1 INTRODUCTION

It is becoming increasingly clear that tyrosine kinases are involved in the receptor linked activation of phospholipase D in many cell types, including those receptors acting through G protein-coupled receptors, as well as receptors with intrinsic tyrosine kinase activity (Thompson *et al*, 1993a). It has been demonstrated that tyrosine phosphorylation is involved in fMLP-, platelet activating factor (PAF)- and leukotriene-stimulated activation of PLD in human neutrophils, and that this receptor stimulated PLD activity can be attenuated by the tyrosine kinase inhibitors, ST271, ST638 and erbstatin, although they had no effect on the phorbol ester-stimulated PLD activity. In parallel it was also demonstrated that tyrosine phosphorylation was not involved in the fMLP-stimulated activation of PLC (Uings *et al*, 1992). In another example of a G protein -coupled system, it was demonstrated that in cells derived from vascular smooth muscle, endothelin-1 was able to stimulate PLD activity through a PKC- and tyrosine kinase-dependent mechanism, and that the PMA-stimulated PLD activity was insensitive to tyrosine kinase inhibitors (Wilkes *et al*, 1993). Tyrosine kinase inhibitors have also been shown to inhibit the EGF-stimulated activation of PLD in Swiss 3T3 cells, indicating that receptors with intrinsic tyrosine kinase activity can also couple to PLD activation (Cook and Wakelam, 1992).

The level of tyrosine phosphorylation of cellular proteins can be controlled by the action of tyrosine kinases, and by the action of tyrosine phosphatases, which remove phosphate groups from phosphotyrosine residues (Pot and DIxon, 1990). Cell permeable peroxides of vanadate are capable of inhibiting the activity of tyrosine phosphatases, thus increasing the level of tyrosine phosphorylation within the cell and enhancing the effect of tyrosine kinases (Grinstein *et al*, 1990). This effect of pervanadate

has been reported to be $10^2$-$10^3$ times more potent than that of vanadate in cell free assays (Fantus *et al*, 1989). It was demonstrated in HL-60 cells that peroxides of vanadate were able to stimulate the activation of PLD, and that this PLD stimulation can be blocked by selective inhibitors of tyrosine kinases (Bourgoin and Grinstein, 1991). Strong evidence for the involvement of a non-receptor tyrosine kinase in the activation of PLD has also been demonstrated by an increase in PLD activity in BALB/c 3T3 cells transformed by constitutively active v-*src*. The evoked PLD activity in this system has been shown to be independent of PKC and distinct from the PLD activity stimulated by phorbol esters (Song and Foster, 1993). However, the results of this study do not necessarily mean that the physiologically relevant activation of PLD is caused by c-*src* or other *src*-type kinases in normal cells.

In the present study we have assessed the effects of a number of selective tyrosine kinase inhibitors on both the LHRH receptor-stimulated, and the phorbol ester-stimulated PLD activity. We have also used the tyrosine phosphatase inhibitor pervanadate in order to bring about a more direct elevation of tyrosine phosphorylation by the inhibition of tyrosine phosphatases (Grinstein *et al*, 1990).

## 4.2 METHODS

### 4.2.1 Phospholipase D assay

Assays for phospholipase D were carried out using the standard protocol, as described in section 2.5.

### 4.2.2 Preparation of Pervanadate

Pervanadate was prepared as previously described, with minor modifications (Fantus *et al*, 1989). $Na_3VO_4$ was added to divalent cation-free Earle's balanced salt solution containing HEPES (30 mM) to give a

final concentration of 5 mM. The pH was adjusted to 7.4 with NaOH, then $H_2O_2$ (1 M) was added to give a concentration of 1 mM and the solution was left for 15 min before 4 mg of catalase was added, in order to remove excess $H_2O_2$. The solution remained clear throughout the above procedure.

## 4.3 RESULTS

In order to assess whether tyrosine phosphorylation was involved in LHRH-stimulated activation of PLD, a number of compounds were used which are known to inhibit or enhance cellular tyrosine phosphorylation.

Over a 30 min stimulation with LHRH (100 nM) the highly-selective tyrosine kinase inhibitor lavendustin A, which has been demonstrated to be a competitor with respect to ATP, but a non-competitive inhibitor with respect to a *src*-peptide substrate (Hsu *et al*, 1991). Binding analysis has also revealed that lavendustin A acts as a slow, tight binding inhibitor with a two step mechanism (Hsu *et al*, 1991). Lavendustin A inhibited approximately 60% of the LHRH-stimulated PLD activity with an $IC_{50}$ of 133 $\pm$ 17 nM (Figure 4.1). Even at concentrations up to 20 $\mu$M, lavendustin A caused no further inhibition of PLD. In contrast lavendustin A had no effect on PLD activity elicited by the PKC activator PDBu (1 $\mu$M) over a 30 min stimulation (Figure 4.1). The inactive isomer of lavendustin A, lavendustin B (Hsu *et al*, 1991), had no detectable effect on the LHRH-stimulated PLD activity at a concentration of 10 $\mu$M.

Another tyrosine kinase inhibitor investigated was piceatannol. This compound has been shown to inhibit the purified thymocyte protein-tyrosine kinase, p40, by competing for the peptide or protein binding site (Geahlen and McLaughlin, 1989). It has been shown that piceatannol does not inhibit the activity of cAMP-dependent protein kinase (Geahlen and McLaughlin, 1989). However, it has also been demonstrated that

piceatannol will inhibit the $Ca^{2+}$-dependent activity of PKC from $\alpha$T3-1 cells, using glutamate synthase (GS)-peptide (100 $\mu$M) as a substrate with an $IC_{50}$ of 163 $\pm$ 12 $\mu$M (M. Johnson, unpublished results). In this study piceatannol inhibited the LHRH-stimulated activation of PLD with an $IC_{50}$ of 35 $\pm$ 5 $\mu$M. Piceatannol also caused inhibition (albeit with a somewhat lower potency) of PDBu-stimulated PLD activity, with an $IC_{50}$ of 155 $\pm$ 50 $\mu$M.

Genistein is a further tyrosine kinase inhibitor investigated here, which has been shown to be competitive inhibitor with respect to ATP and non-competitive to a phosphate acceptor, histone H2B (Akiyama *et al*, 1987). These authors also demonstrated that genistein has scarcely any effect on the serine/ threonine specific protein kinases, cAMP-dependent protein kinase and PKC. Genistein inhibited the LHRH (100 nM)-stimulated activation of PLD with an $IC_{50}$ of 92 $\pm$ 34 $\mu$M (Figure 4.2), whilst causing no significant inhibition of PDBu (1 $\mu$M)-stimulated PLD activation up to 300 $\mu$M (Figure 4.2). Although the above tyrosine kinase inhibitors were able to inhibit the LHRH-stimulated PLD activity, another tyrosine kinase inhibitor MDC was unable to inhibit the LHRH- or PDBu-stimulated PLD (results not shown) at 1 mM, the highest concentration used. It was not assessed whether MDC was able to enter the cells here, although MDC is a polar compound and its anticipated low ability to enter cells may explain this result (Umezawa *et al*, 1990).

The tyrosine phosphatase inhibitor pervanadate (1 mM) was able to stimulate PLD activity over a 30 min period (Table 4.1) to a level similar to that evoked by PDBu (1 $\mu$M), whilst pervanadate and PDBu had an additive effect on [$^3$H] PtdBut accumulation (Table 4.1). The $Ca^{2+}$ ionophore ionomycin which has been reported to release $Ca^{2+}$ from intracellular stores (Morgan and Jacob, 1994), markedly attenuated the PLD response elicited by pervanadate (Table 4.1). In order to determine whether PKC was

involved in the pervanadate-stimulated pathway of PLD activation (i.e. downstream of tyrosine kinase activation), we used the selective PKC inhibitor Ro 31-8220 (Davis *et al*, 1992), which had no detectable effect on pervanadate-induced PLD activation (Table 4.1), in contrast to its potent inhibition of the corresponding response to LHRH (Chapter 3).

## 4.4 DISCUSSION

The present results strongly suggest the involvement of tyrosine kinases in the activation of PLD by LHRH, in addition to the previous evidence for an involvement of PKC in the same mechanism (Chapter 3).

Lavendustin A was able to inhibit with high potency the majority of the LHRH-induced PLD response, leaving a residual PLD activity that was unaffected by further elevating the lavendustin A concentration. The portion of the LHRH-induced response insensitive to lavendustin A may represent a separate pathway of PLD activation. Lavendustin A had no effect on the PDBu-activated PLD activity, which raises the possibility that PDBu utilises the same pathway as the lavendustin A-insensitive portion of the LHRH response, or a totally separate pathway to that involving tyrosine phosphorylation. Both LHRH and PDBu responses were fully sensitive to PKC inhibitors (albeit with somewhat different potencies), which is consistent with the idea that the lavendustin A-sensitive, presumably PDBu-insensitive component of LHRH action may be mediated by a PDBu-insensitive PKC isoform, such as PKC ζ. Alternatively, the incomplete inhibition of the LHRH-stimulated PLD activity by lavendustin A may be due to the slow initial binding of the inhibitor to its target (Hsu *et al*, 1991).

Another tyrosine kinase inhibitor, piceatannol was able to completely inhibit the LHRH-evoked PLD activity, and was also able to inhibit the PDBu-stimulated activation of PLD at a slightly higher concentration.

However this concentration was very similar to that required for inhibition of $Ca^{2+}$-dependent PKC activity by piceatannol in αT3-1 cells (M. Johnson; unpublished results), and may therefore represent the inhibition of PKC stimulated by piceatannol. Care should be taken in the interpretation of the piceatannol results, since piceatannol is reported to block the substrate binding site (Geahlen and McLaughlin, 1989), therefore the inhibition may be dependent on the substrate as well as the kinase. The tyrosine kinase inhibitor genistein displayed greater than 10 fold selectivity towards LHRH-stimulated PLD activity, compared to the PDBu-induced response, indicating that any inhibition of the PDBu-stimulated PLD activity was probably non-specific, possibly involving the inhibition of PKC or other Ser/Thr protein kinases. Another tyrosine kinase inhibitor MDC, did not cause any detectable inhibition of the LHRH- or PDBu-stimulated activation of PLD. It has been reported that this compound is poorly able to enter cells, due to its polar nature, making it unsuitable for whole cell assays (Umezawa *et al*, 1990). However, these results do indicate that the conditions used in the PLD assay do not disrupt the integrity of the cells.

These results with tyrosine kinase inhibitors on the LHRH- and PDBu-stimulated PLD activity are wholly consistent with the idea that the mechanism for the LHRH-stimulated activation of PLD involves a tyrosine kinase, and since piceatannol and genistein were able to completely inhibit the LHRH-stimulated PLD activity, it is likely that tyrosine phosphorylation is an obligate requirement for PLD activation by LHRH in this system.

The PDBu-evoked PLD activity was only inhibited at higher doses of some of the tyrosine kinase inhibitors, indicating the probable lack of involvement of tyrosine kinases using this mechanism. This information further reinforces the evidence from studies with PKC inhibitors (Chapter 3) that LHRH and PDBu stimulate PLD activation using independent

mechanisms in αT3-1 cells, as has been proposed in previous studies for the activation of PLD by G protein-coupled receptors (Uings *et al*, 1992; Wilkes *et al*, 1993). However, we have observed that PDBu is able to elicit tyrosine phosphorylation in αT3-1 cells through an unidentified mechanism (see Chapter 5).

In the present study, pervanadate (1 mM) caused a marked stimulation of PLD activity, further supporting the evidence that tyrosine phosphorylation participates in the pathway of activation of PLD by LHRH. The pervanadate-stimulated activation of PLD was unaffected by the PKC inhibitor Ro 31-8220, indicating that the tyrosine kinase in the LHRH-stimulated PLD activation pathway, which has its effect enhanced by pervanadate, is downstream of PKC, since Ro 31-8220 has no effect on this activity. The additive effect on [$^3$H]PtdBut accumulation of co-stimulation with pervanadate and PDBu points towards these two stimuli leading to the activation of PLD through two separate pathways, although the level of PLD activation with pervanadate and PDBu co-stimulation was not as high as the LHRH-stimulated PLD activity. If PDBu were activating the PKC upstream of the tyrosine kinase involved in the LHRH-stimulated activation of PLD, it would be expected that the PDBu-stimulated PLD activity would be attenuated by protein-tyrosine kinase inhibitors with the same potency as for the LHRH-stimulated response. The results presented in this Chapter demonstrate that this is clearly not the case, lending further support to the theory that the PKC involved in the LHRH-stimulated activation of PLD is a species not activated by phorbol esters, such as PKC ζ.

Ionomycin markedly reduced the PLD activation elicited by pervanadate, as indeed it prevented the LHRH agonist-induced tyrosine phosphorylation of αT3-1 cellular proteins (Chapter 5). This is consistent with the theory that $Ca^{2+}$ is capable of inducing activation of tyrosine

phosphatases, possibly similar to PTP 1B which is a widespread tyrosine phosphatase that is activated through the cleavage of its membrane localisation domain by the $Ca^{2+}$-activated protease calpain (Frangioni *et al*, 1993). Another phosphatase which has been shown to have some protein-tyrosine phosphatase activity, calcineurin, can be activated by the $Ca^{2+}$-binding protein, calmodulin (Pallen and Wang, 1985). It is unlikely that these effects of ionomycin are the result of any cytotoxic effects, since ionomycin was unable to significantly effect the PDBu-stimulated activation of PLD in αT3-1 cells (Chapter 3). The effects of $Ca^{2+}$ on tyrosine phosphorylation may depend on cell type since an increase in intracellular $Ca^{2+}$ has been reported to increase tyrosine kinase activity in angiotensin-II stimulated GN4 liver epithelial cells (Huckle *et al*, 1992). In Swiss 3T3 cells, depletion of intracellular $Ca^{2+}$ did not affect lysophosphatidic acid (LPA)-induced tyrosine phosphorylation (Seufferlein and Rozengurt, 1994). In contrast, PKC rather than $Ca^{2+}$ appears to be a necessary (though not solely sufficient) intermediary in endothelin-stimulated tyrosine phosphorylation and mitogenesis in kidney glomerular mesangial cells (Simonson and Hermar, 1993). Interestingly, elevation of $Ca^{2+}$ levels in epidermal keratinocytes leads to activation of the non-receptor tyrosine kinase c-*src*, through a mechanism involving tyrosine dephosphorylation (consistent with $Ca^{2+}$-activation of a tyrosine phosphatase) but leads concomitantly to the inactivation of the related c-*yes* tyrosine kinase activity (Zhao *et al*, 1992).

It was not within the scope of this study to evaluate whether tyrosine kinases directly phosphorylate PLD in response to LHRH stimulation, since PLD has not been isolated, and there are no specific antibodies which are able to recognise PLD. However, one way this might be done would be to immunoprecipitate cellular proteins from αT3-1 cells, pre-stimulated with

LHRH, with an anti-phosphotyrosine antibody and assay the immunoprecipitated proteins for PLD activity. This experiment might determine whether PLD is directly tyrosine phosphorylated in response to LHRH. It would not however determine which tyrosine kinase was involved and it is possible that any immunoprecipitated PLD would not be tyrosine phosphorylated itself, but bound to tyrosine phosphorylated regulatory proteins.

It has been shown in platelets, which contain particularly high levels of c-*src*, that agonists directly or indirectly activating PKC, such as the phorbol ester, phorbol 12-myristate 13-acetate (PMA), vasopressin and the $Ca^{2+}$ ionophore A23187 increased the activity of the non-receptor tyrosine kinases c-*src* and c-*fyn*. Stimulation of platelets by the above pathways causes the phosphorylation of Ser-12 on c-*src*, which is a potential phosphorylation site for PKC, and consequently increases the substrate affinity of c-*src* by two to three fold (Liebenhoff *et al*, 1993). Similarly another member of the *src* family of tyrosine kinases *fgr*, has been shown to be activated in response to fMLP-stimulation in neutrophils (Gutkind and Robbins, 1989), and c-*src* can also be activated as a result of endothelin-1 receptor activation in mesangial cells (Simonson and Hermar, 1993). Phospholipase D activity has been shown to be elevated in BALB/c 3T3 cells transformed by v-*src*, although this activation was shown to independent of PKC (Song and Foster, 1993). As *src*-type kinases have been shown to be activated through G protein-coupled receptors in previous studies, and also to bring about PLD activation, a member of the *src* family of non-receptor tyrosine kinases is currently the most likely candidate for the tyrosine kinase participating in LHRH receptor signalling.

Low molecular weight G proteins such as Ras, Rho and ARF have been shown to be regulated by another group of proteins which change the

G proteins between an active, GTP-bound state and an inactive GDP-bound state (Barbacid, 1987). Agonists which stimulate G protein-coupled receptors, such as thrombin and LPA have been shown to lead to the activation of Ras through a process that involves a pertussis toxin-sensitive heterotrimeric G-protein, and which can also be blocked by the tyrosine kinase inhibitor, genistein (Van Corven *et al*, 1993). It has also been reported that proteins which stimulate the intrinsic GTPase activity of Ras by up to 100-fold, Ras GTPase activating proteins (Ras-GAP), can act as a substrate for v-*src* (Park and Jove, 1993). Another protein involved in the regulation of Ras-type low molecular weight G-proteins is Vav, a guanosine nucleotide releasing factor, which mediates the exchange of GDP for GTP, thus activating Ras-type G-proteins (Downard, 1992). It has been shown that Vav shows increased activity when tyrosine phosphorylated, and is also a substrate for the *src*-type non-receptor tyrosine kinase p56 *lck* (Gulbins *et al*, 1993). A non-receptor tyrosine kinase has also been shown to inhibit the GTPase activity of another low molecular weight G-protein, p21 cdc 42, which is related to Rho (Manser *et al*, 1993). It therefore appears that the activity of low molecular weight G proteins can be both positively and negatively controlled by the action of non-receptor tyrosine kinases. Both Ras-GAP and Vav have SH2 and SH3 domains, which mediate protein-protein interactions (Vogel *et al*, 1988; Bustelo *et al*, 1992; Songyang *et al*, 1993; Ren *et al*, 1993). It is interesting that GTP-exchange on low molecular weight G proteins can be regulated by non-receptor tyrosine kinases, since low molecular weight G proteins have also been implicated in the regulation of PLD in several systems (Cockroft *et al*, 1994; Brown *et al*, 1993; Bowman *et al*, 1993). It has also been demonstrated here that an inhibitor of ARF guanine nucleotide exchange factor, brefeldin A is able to inhibit the LHRH-stimulated activation of PLD in $\alpha$T3-1 cells

(Chapter 3). The exact mechanism whereby tyrosine kinases regulate PLD is not clear at present, however, in the case of LHRH-stimulated PLD, it is possible that a non-receptor tyrosine kinase modulates the activity of a low molecular weight G protein, which in turn activates PLD.

It is difficult to conclude exactly how non-receptor tyrosine kinases fit into the PLD activation pathway stimulated by LHRH, although there is a clear case for their involvement. It can be ascertained from the results presented in this Chapter that there is no pervanadate-enhanced tyrosine kinase activity upstream of PKC involved in the activation of PLD. A possible explanation of the results is that a non-receptor tyrosine kinase is involved downstream of PKC. This may be similar to a proposed mechanism in thrombin-stimulated human platelets, whereby PKC phosphorylation of *src* has been proposed to increase the substrate affinity of *src* (Liebenhoff *et al*, 1993). Another possibility is that the tyrosine kinase involvement in LHRH-stimulated PLD activation is independent of PKC, and that both the tyrosine kinase pathway and PKC are needed in order to fully activate PLD. The fact that PKC inhibitors and the tyrosine kinase inhibitors, genistein and piceatannol totally inhibit LHRH-stimulated PLD activation is consistent with the idea that both tyrosine kinases and PKC are involved in the same pathway, and that the non-receptor tyrosine kinase is downstream of PKC.

**Table 4.1**

**Modulators of tyrosine phosphorylation, PKC and $Ca^{2+}$-mobilisation on PLD activity in $\alpha$T3-1 cells**

Effect of various compounds on PLD activity in $\alpha$T3-1 cells. Time course of the assay was 30 min in each case. Values are expressed as a % of the LHRH (100 nM)-stimulated PLD activity, with the basal level of [$^{3}$H]PtdBut accumulation subtracted, and are means $\pm$ SEM from 4-8 separate determinations.

**Table 4.1**

| Induced Response | dpm x $10^3$ (mean % of response to LHRH in parentheses) |
|---|---|
| LHRH (100 nM) | 61 ± 5.5 (100 ± 9) |
| Pervanadate (1 mM) | 13.4 ± 2.4 (22 ± 4) |
| 4β-PDBu (1 μM) | 33 ± 2.4 (54 ± 4) |
| Ionomycin (30 μM) | 0.6 ± 1.2 (1 ± 2) |
| Pervanadate (1 mM) + ionomycin (30 μM) | 4.3 ± 1.8 (7 ± 3) |
| Pervanadate (1 mM) + 4β-PDBu (1 μM) | 47.6 ± 4.2 (78 ± 7) |
| Pervanadate (1 mM) + Ro 31-8220 (1 μM) | 12.8 ± 2.4 (21 ± 4) |

**Figure 4.1**

**Concentration response curve for the inhibition of LHRH- and PDBu-stimulated PLD activity by lavendustin A in $\alpha$T3-1 cells**

The effect of the tyrosine kinase inhibitor lavendustin A on stimulus-induced PLD activation. Concentration-dependent inhibition of responses to LHRH (100 nM)and PDBu (1 $\mu$M). Stimulation was for 30 min, and values are means $\pm$ SEM from 4-8 separate determinations. Values for LHRH-stimulated PLD activity are significantly different form the LHRH-stimulated response above 0.1 $\mu$M lavendustin A ($p<0.05$; Mann Whitney U test).

**Figure 4.1**

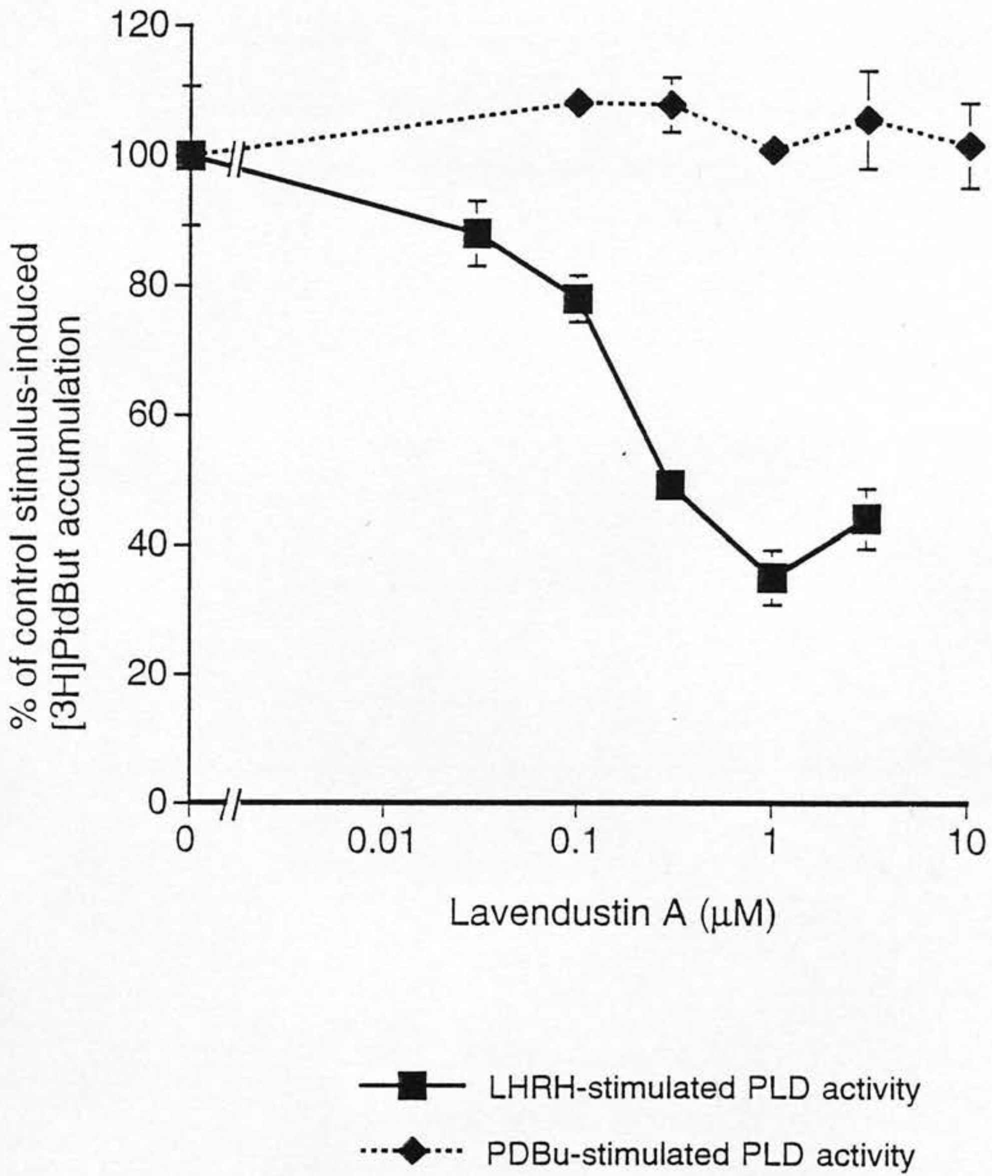
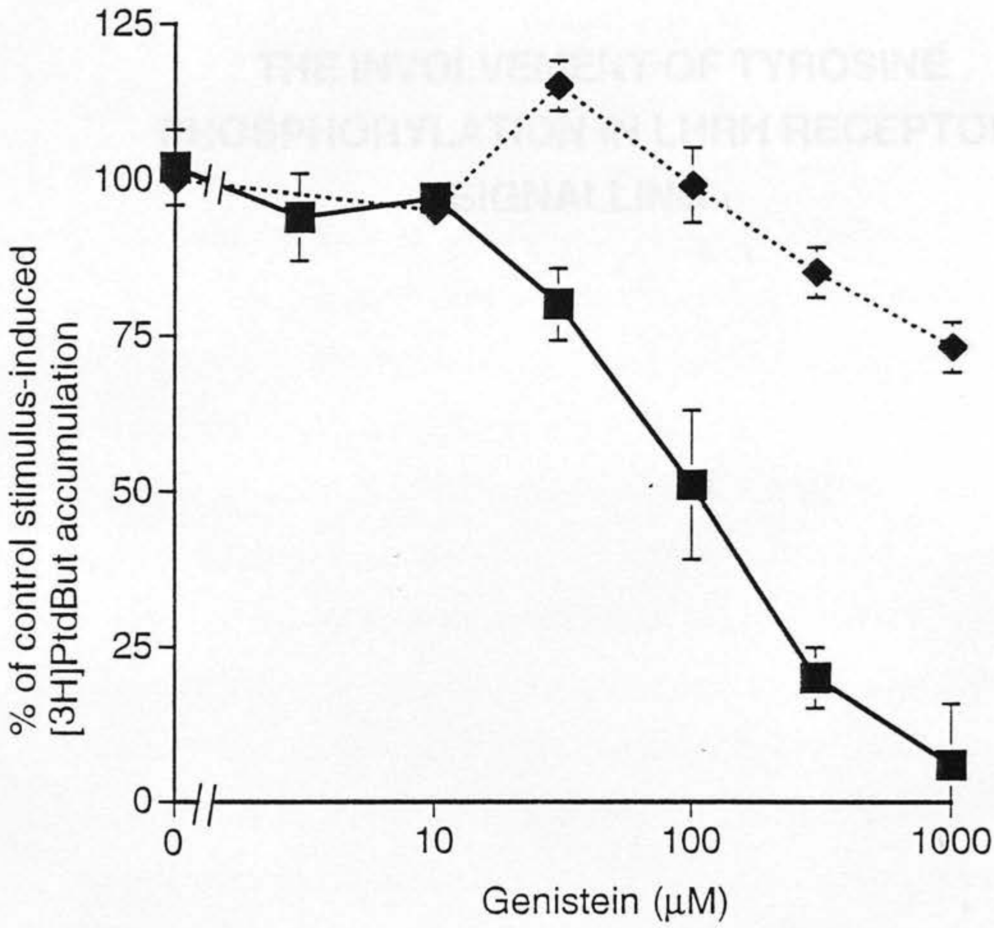


Figure 4.2

**Concentration response curve for the inhibition of LHRH- and PDBu-stimulated PLD activity by genistein in αT3-1 cells**

The effect of the tyrosine kinase inhibitor genistein on stimulus-induced PLD activation. Concentration-dependent inhibition of responses to LHRH (100 nM)and PDBu (1 μM). Stimulation was for 30 min, and values are means ± SEM from 4-8 separate determinations. Values above 30μM genistein for the LHRH-stimulated PLD activity and 300 μM genistein for the PDBu-stimulated PLD activity are significantly different from the stimulus-induced PLD activity ($p<0.05$; Mann Whitney U test).

**Figure 4.2**




LHRH-stimulated PLD activity

PDBu-stimulated PLD activity

# CHAPTER 5

# THE INVOLVEMENT OF TYROSINE PHOSPHORYLATION IN LHRH RECEPTOR SIGNALLING

## 5.1 INTRODUCTION

There is considerable evidence that stimulation of cells with compounds through G protein-coupled receptors linked to phosphoinositide hydrolysis, such as bombesin, vasopressin, endothelin, lysophosphatidic acid, bradykinin and thrombin (as is also the case with LHRH), can lead to the tyrosine phosphorylation of cellular proteins through the activation of non-receptor tyrosine kinases (Zachary *et al*, 1992; Seufferlein and Rozengurt, 1994; Offermanns *et al*, 1993; Hordijk *et al*, 1994; Lundberg and Song, 1991; Force *et al*, 1991). Receptor-stimulated tyrosine phosphorylation is more commonly associated with receptor tyrosine kinases, such as growth factor receptors, which have intrinsic tyrosine kinase activity in their cytoplasmic domains, although activation of receptor tyrosine kinases has also been shown to activate *src*-type non-receptor tyrosine kinases (Kypta *et al*, 1990). Tyrosine phosphorylation has also been reported upon activation of T-cell receptors, which also have no intrinsic protein-tyrosine kinase activity (Wang *et al*, 1994). Members of the *src*-family of non-receptor tyrosine kinases are reported to be activated following association with T-cell receptors, and this tyrosine kinase activation can in turn result in the activation of PLC-$\gamma$ (reviewed in Mustelin and Burn, 1993). However, no such direct interaction between non-receptor tyrosine kinases and G-protein coupled receptors has been reported.

The level of tyrosine phosphorylation within cells can be regulated by the activity of protein tyrosine phosphatases, as well as tyrosine kinases, and these can be roughly divided into two families, receptor tyrosine phosphatases and non-receptor tyrosine phosphatases. Non-receptor tyrosine phosphatases represent a diverse group of enzymes, which are potentially regulated by intracellular signalling mechanisms. For example,

it has recently been demonstrated that a ubiquitously expressed tyrosine phosphatase, PTP-PEST, can be inactivated as a result of phosphorylation by cAMP-dependent protein kinase and PKC (Garton and Tonks, 1994). Another abundant non-receptor tyrosine phosphatase, PTP-1B, is normally localised to the cytoplasmic face of the endoplasmic reticulum by a C-terminal targeting sequence. It has been found that the $Ca^{2+}$-activated phosphatase, calpain, is able to cleave the C-terminal membrane targeting sequence of PTP-1B, resulting in a 2-fold increase in the activity of PTP-1B and resulting in its subcellular relocation, probably bringing it into close proximity with its substrates (Frangioni *et al*, 1993). Another Ser/Thr and tyrosine phosphatase, calcineurin (PP2B), can be activated in a $Ca^{2+}$-dependent manner after its association with calmodulin, a major intracellular receptor for $Ca^{2+}$ ions (Pallen and Wang, 1985). It is possible that there may be important messenger activated tyrosine phosphatases as well as tyrosine kinases in αT3-1 cells, which play a role in regulating the level of tyrosine phosphorylation.

It has been demonstrated that agonists which activate PKC, either directly or indirectly in platelets can increase the substrate affinity of the c-*src* non-receptor tyrosine kinase (Liebenhoff *et al*, 1993). Stimulation of G protein-linked systems with the non-hydrolysable analogue of GTP, GTPγS, has also been reported to stimulate tyrosine phosphorylation (Nasmith *et al*, 1989). Similarly another member of the *src* family of tyrosine kinases, *fgr*, has been shown to be activated in response to fMLP-stimulation in neutrophils (Gutkind and Robbins, 1989). There is also evidence that c-*src* can be activated as a result of endothelin-1 receptor activation in mesangial cells (Simonson and Hermar, 1993), and by LPA receptor stimulation in NIE 115 neuroblastoma cells (Jalink *et al*, 1989). It has recently been reported that one of the potential substrates of c-*src* and to a lesser extent *fyn in vitro*,

is the activated form of the $Ca^{2+}$-independent PKC δ from porcine spleen. This tyrosine phosphorylation increased the specificity of PKC δ for certain substrates (Gschwendt *et al*, 1994).

Bombesin, vasopressin and endothelin have been shown to stimulate the rapid tyrosine phosphorylation of the tyrosine kinase p125 focal adhesion kinase (FAK) in Swiss 3T3 cells (Zachary *et al*, 1991). All of these neuropeptides have receptors that signal via heterotrimeric G proteins, and have seven hydrophobic transmembrane domains. It has been demonstrated that neuropeptide receptor stimulation often results in the rapid tyrosine phosphorylation of a group of proteins of 110-130 kDa, as well as minor components of 90 and 70-80 kDa (Zachary *et al*, 1991). Following neuropeptide receptor stimulation, there has also been shown to be phosphorylation of a *src* transformation-associated substrate, p130, possibly FAK, or other target proteins of a similar molecular weight (Schaller *et al*, 1992). There is also strong evidence that v-*src* is able to phosphorylate p125 FAK (Schaller *et al*, 1992), and that a stable association can be formed between p125 FAK and two *src*-type tyrosine kinases, *src* and *fyn* (Cobb *et al*, 1994). This raises the possibility that cellular *src*-type tyrosine kinases are also involved in the regulation of p125 FAK in other signalling systems activated by G protein-coupled receptors. It has been reported that FAK may be an important early step in intracellular signalling pathways, triggered in response to cell interactions with the extracellular matrix (Hanks *et al*, 1992). Focal adhesion kinase has also been reported to phosphorylate components of the cytoskeleton in response to integrin activation (Schaller *et al*, 1992).

In studying non-receptor tyrosine kinases associated with the LHRH signalling pathway we have concentrated on the *src*-type tyrosine kinases, since these were the most highly characterised at the time of starting the

study, and have also been implicated in the activation of PLD (see chapter 4). We have used variety of immunoblot and immunoprecipitation techniques in order to investigate the LHRH- and PKC-induced tyrosine phosphorylation of cellular proteins in the αT3-1 cell line.

## 5.2 METHODS

### 5.2.1 SDS-polyacrylamide gel electrophoresis and immunoblotting with Phast system

Confluent cultures of αT3-1 cells were incubated with the LHRH receptor agonist buserelin (100 nM; [D-Ser(tBu)$^6$, DesGly$^{10}$] LHRH ethylamide), 300 nM PDBu, 30 μM ionomycin or saline (control). Reactions were terminated after 20 min by scraping cells into a buffer containing 20% (w/v) sodium dodecyl sulphate and 5% (v/v) 2-mercaptoethanol, then heating to 100°C for 5 min. Cellular proteins were separated on 7.5% homogeneous microgels which were run on a PhastSystem electrophoresis apparatus (Pharmacia, Milton Keynes, UK), then electroblotted onto Immobilon-P polyvinylidene membranes (Millipore). For the antiphosphotyrosine blots the Immobilon was then probed with the 4G10 mouse monoclonal antibody (UBI), which recognises phosphotyrosine, or with the 4G10 antibody blocked with o-phospho-L-tyrosine (1 mM) (Sigma). A horseradish peroxidase (HRP)-labelled secondary antibody (Scottish Antibody Production Unit) was used to bind to the primary 4G10 antibody. Enhanced chemiluminescence (ECL; Amersham, UK) was employed to visualise antibody binding; this method uses the principal of the conversion of a non-luminescent chemical to a highly luminescent one by horseradish peroxidase bound to the secondary antibody. This chemiluminescence can then be detected on photographic film.

For the immunodetection of *fyn*, the Immobilon was probed with the FYN3 rabbit polyclonal antibody (Santa Cruz), or with antibody blocked with the peptide used to generate the antibody. A secondary HRP-linked anti-rabbit antibody was then used to bind to the primary antibody; antibody binding was then visualised by ECL-detection. For immunodetection of *src*, the Immobilon was probed with a mouse monoclonal antibody (GD 11) (UBI). A secondary HRP-linked anti-mouse antibody was then used to bind to the primary antibody; antibody binding was visualised with the ECL-detection system.

### 5.2.2 Tyrosine kinase immunoprecipitation and kinase assay

αT3-1 cells were grown to confluency in tissue culture dishes (10 cm diameter), and were then quiesced using serum-free medium for approximately 24 hrs prior to the assay. The cells were then stimulated with LHRH (100 nM) for 10 min, while control cells were left unstimulated. After this stimulation the cells were scraped in 1.8 ml ice-cold phosphate buffered saline (PBS), plus protease inhibitors (aprotinin (50 μg/ml), leupeptin (200 μg/ml and PMSF (2 mM)) and orthovanadate (100 μM). The cell debris was pelleted by centrifugation and resuspended in 1 ml NP-40 lysis buffer (see below). This was then left to stand on ice for 20 min.

Non-solubilised debris was then pelleted by spinning at 14000 g in a benchtop centrifuge for 15 min at 4°C, the pellet was then discarded. The supernatant was incubated, with mixing, with protein A-sepharose (100 μl) at 4°C for 2 hrs in order to clear the supernatant of any proteins non-specifically binding to protein A-sepharose. The protein A-sepharose was then pelleted for 15 min at 4°C. The pellet was then discarded and the supernatant transferred to a fresh tube. Antibody (FYN3; 10 μg/ml) and

protein A-sepharose were then added to the supernatant and incubated for 2 hrs at 4°C. At the end of this incubation the protein A-sepharose-antibody-protein complex was pelleted (4°C), and the supernatant discarded. The pellet was then washed three times in NP 40 lysis buffer plus inhibitors, then once with Tris-HCl (50 mM) pH 7.2, NaCl (150 mM).

NP-40 Lysis Buffer:
5 mM Tris-HCl pH 8.0
150 mM NaCl
1% NP-40
$Na_3VO_4$ (200 μM)
leupeptin (200 μg/ml)
aprotinin (50 μg/ml)
PMSF (2 mM)

Inhibitors were added fresh daily from stock solutions. PMSF was added immediately prior to assay, as it is extremely unstable.

Kinase assay

The tyrosine kinase substrate used in the assay of tyrosine kinase activity was acid-denatured enolase (200 μg/ml) (Cooper, 1984).

Reaction Buffer:
HEPES pH 7.5 (20 mM)
$MgCl_2$ (10 mM)
$MnCl_2$ (10 mM)
$Na_3VO_4$ (100 μM)
Leupeptin (200 μg/ml)
Aprotinin (50 μg/ml)

The immunoprecipitate was resuspended in 100 μl reaction buffer. Reaction was started by the addition of 20 μl ATP solution (5 μM ATP + [$\gamma^{33}$P] ATP (5μCi)) to the reaction buffer and immunoprecipitate. The time course of the kinase assay was 5 min (37 °C), and was terminated by the addition of ice cold Tris-HCl pH 7.5 (50 mM), NaCl (150 mM). This was then centrifuged for 15 min (4 °C) in order to pellet the protein A-sepharose. The supernatant containing the enolase was then boiled in

SDS-PAGE sample buffer and the samples run on 7.5% homogenous polyacrylamide gels, with enolase running as a broad band at approximately 50 kDa. The phosphorylation of the enolase substrate was assessed by the use of a phosphorimager.

### 5.2.3 Resolution of products from the immunoprecipitation-kinase assay by SDS-polyacrylamide gel electrophoresis

Polyacrylamide gels (7.5%) were prepared in a BioRad gel former (20 cm x 20 cm x 1.5 mm) according to manufacturers instructions and allowed to set for 45-60 min with a layer of iso-butyl alcohol on top to level the surface and exclude air. The top of the gel was then extensively washed and the inner surface of the glass plates dried with filter paper. The stacking gel was prepared according to the manufacturers instructions and poured on top of the separating gel. Well formers were carefully placed in top of the stacking gel, being careful to exclude air bubbles. The gel was then allowed to set for 35-45 min. Well formers were carefully removed and the wells carefully rinsed out with distilled water. Gels were then clipped into a BioRad gel tank and the upper and lower reservoirs filled with tank buffer.

Previously prepared protein samples were then thawed out and warmed up in order to aid pippeting. 150 μl of protein sample was added to each well, an equal volume of 1x SDS sample buffer was added to marker lanes which had concentrated radiolabeled protein standards (Rainbow markers, Amersham). A voltage was then applied and the gels run to the bottom (2 hrs at 25 mA for the stacking gel, then 3 hrs at 40 mA for the running gel).

<u>Tank buffer:</u> 25 mM Tris base, pH 6.8

192 mM glycine
0.1% SDS

## 5.3 RESULTS

### 5.3.1 Anti-phosphotyrosine immunoblotting

In order to determine whether protein-tyrosine phosphorylation occurs in αT3-1 cells in response to LHRH stimulation (100 nM), cellular proteins were separated using SDS-PAGE on a 7.5% homogeneous polyacrylamide gel (section 5.2.1) with an antibody which recognised phosphotyrosine (4G-10). The LHRH-receptor agonist, buserelin was observed to induce tyrosine phosphorylation on many proteins of molecular mass ~60 to >170 kDa, but especially notable was a broad band at around 128 kDa and a diffuse band at around 76 kDa (Figure 5.1). A time course of tyrosine phosphorylation in response to buserelin and PDBu was assessed (Figure 5.2), with time points at 3, 10, and 60 min in order to determine at what point the tyrosine phosphorylation of cellular proteins was maximal. It would appear that the protein-tyrosine phosphorylation induced by the LHRH receptor agonist buserelin was maximal by the 10 min minute time point, and did not appear to have reduced by 60 min (Figure 5.2). By varying exposure conditions during development of the immunoblot reaction, it could be seen that each of these bands consisted of a minimum of two proteins (Figure 5.1). Similar phosphorylations of protein-tyrosine were also evoked with the activator of PKC, PDBu (1 μM) (Figure 5.1). The $Ca^{2+}$-ionophore, ionomycin(30 μM), reduced the level of tyrosine phosphorylation in all the target proteins below that observed in unstimulated αT3-1 cells.

### 5.3.2 Immunodetection of src-family tyrosine kinases

It is apparent that the tyrosine kinase or kinases activated by LHRH receptor stimulation are non-receptor tyrosine kinases, since the LHRH receptor has no intrinsic tyrosine kinase activity (Tsutsumi *et al*, 1992). Although other families of non-receptor tyrosine kinases exist, what little evidence is available suggests that *src*-family tyrosine kinases may well be activated downstream of G protein-coupled receptors (Liebenhoff *et al*, 1993). Therefore we have concentrated here on the *src* family of tyrosine kinases.

Cellular proteins were separated by SDS-PAGE on 7.5% homogenous polyacrylamide gels and blotted onto Immobilon-P membranes (Millipore) for immunodetection. In αT3-1 cells, two of the *src* family of non-receptor tyrosine kinases were detectable with the antibodies used. Using the mouse monoclonal antibody to src (GD 11), and an HRP-linked secondary anti-mouse antibody, a single band was detected at approximately 60 kDa, using the ECL detection system (Amersham) (Figure 5.3). The rabbit affinity-purified polyclonal antibody FYN3 (Santa Cruz), with a secondary HRP-liked anti-rabbit antibody and using the ECL detection system, detected fyn as single specific band at approximately 60 kDa, non-specific antibody binding was detected by blocking the FYN3 antibody with the peptide used to generate the antibody prior incubating with the Immobilon membrane (figure 5.3). Other members of the *src* family of non-receptor tyrosine kinases; *lyn*, *yes*, *hck*, and *fgr* were not detectable in αT3-1 cells using the technique described in the methods section, although *src*, *fyn*, *lyn*, *yes*, *hck* and *fgr* were detectable by the same technique, using the same antibodies in human neutrophils (results not shown). This provides a positive control with which to compare the

negative results in the αT3-1 cells. All of the above polyclonal antibodies to the *src*-type kinases, except the *src* antibody, GD 11, which is a monoclonal antibody, were raised against peptides corresponding to the N-terminal region of the protein, which has been reported to be highly variable between different members of the *src*-type tyrosine kinase family (Cooper and Howell, 1993). It was reported in the product literature for the above antibodies that there is no detectable cross-reactivity with other members of the *src* family of tyrosine kinases.

### 5.3.3 Fyn immunoprecipitation

Confluent 10 cm diameter dishes of αT3-1 cells were stimulated for 10 min with LHRH (100 nM) in order to attempt to bring about activation of p59 *fyn*. An immunoprecipitation was then carried out as described in section 5.2.2, using the FYN 3 antibody (10 μg/ml) in order to recover p59 *fyn* in a state where its activity could be assessed in a partially-purified state, free of other tyrosine kinases which may have a high constitutive level of activity, or which may also be activated by LHRH. Stimulation of αT3-1 cells for 10 min with LHRH (100 nM) resulted in approximately a 50% increase in [$^{33}$P] incorporation into an acid-denatured enolase substrate (Figure 5.4). It has been demonstrated that enolase can be phosphorylated on a single tyrosine and is an effective substrate for pp60 v-*src*; the extent of enolase phosphorylation by tyrosine kinases *in vitro* can be increased by a brief prior exposure of the substrate to pH 4 (Cooper *et al*, 1984).

## 5.4 DISCUSSION

Since many of the tyrosine phosphorylations induced here by LHRH could also be elicited by PDBu (Figure 5.1), it is possible (if there is only one tyrosine kinase step involved) that LHRH and PDBu are both able to activate this component of the signalling mechanism. The protein-tyrosine

phosphorylations induced in αT3-1 cells by the LHRH-receptor agonist, buserelin, appear remarkably similar to those induced by lysophosphatidic acid (LPA), bombesin and endothelin in Swiss 3T3 cells and in Rat-1 fibroblasts, with proteins of 70-80 kDa and 110-130 kDa being the main phosphorylation targets (Seufferlein and Rozengurt, 1994). In each of the above cases, as well as in the present study, the administration of a phorbol ester appears to induce protein-tyrosine phosphorylations similar to those observed with the receptor ligands, indicating that PKC may be an important regulator of intracellular tyrosine phosphorylation. It is not known if any if the proteins phosphorylated in response to LHRH are the tyrosine kinase p125 FAK. In Swiss 3T3 cells, stimulation of FAK activity can be induced by direct activation of PKC with PDBu (Sinnett-Smith *et al*, 1993). However, evidence has been put forward that bombesin stimulation leads to the activation of FAK through a PKC-independent pathway, and that the integrity of the cytoskeleton is essential for both the PKC-dependent and independent activation of FAK (Sinnett-Smith *et al*, 1993). It is therefore possible that in the present study, the similar tyrosine phosphorylation induced by PDBu and LHRH could involve different signalling pathways. Indeed, it has recently been demonstrated that the PDBu-stimulated tyrosine phosphorylation of proteins in αT3-1 cells is almost completely blocked by the protein kinase C inhibitor GF 109203X (3 μM), while LHRH-stimulated tyrosine phosphorylation is only partially blocked by GF 109203X (3 mM) (Johnson *et al*, 1994). This suggests that the PDBu-stimulated tyrosine phosphorylation in αT3-1 cells is dependent upon PKC, while the LHRH-stimulated tyrosine phosphorylation is only partially dependent upon PKC.

Only two of the *src* family of non-receptor tyrosine kinases were detectable using immunoblotting techniques in αT3-1 cells. We were also

able to confirm that *lyn*, *yes*, *hck*, and *fgr* were not detectable with the antibodies used, although the sensitivity of the technique does not exclude their presence at very low levels. All of the above *src*-type kinases were detectable using these antibodies in human neutrophils, which were used as a positive control. It has been reported that there are central nervous system (CNS) and peripheral forms of c-*src*, and that the CNS form of c-*src* has an increased kinase activity, and contains six additional amino acids in its regulatory domain, generated by alternative splicing (Brugge *et al*, 1985). While both forms of c-*src* are expressed in the CNS, only the unmodified form of c-*src* can be detected in peripheral tissues (Brugge *et al*, 1985). It has also been reported that there are two different forms of *fyn* mRNA that arise from the mutually exclusive splicing of alternative seventh exons; one of these products is found at high levels in the brain (Cooke *et al*, 1991), the other form is preferentially expressed in T lymphocytes (Cooke and Purlmutter, 1989). The presence of *src* and *fyn* together in the same cell has also been noted in platelets, in which the level of fyn is approximately 5-10 fold lower than that of *src* (Liebenhoff *et al*, 1993). This does not necessarily imply that *fyn* only plays a minor role in tyrosine phosphorylation events. Indeed, it has been demonstrated that both *src* and *fyn* are potentially phosphorylated by PKC in response to thrombin stimulation, leading to an increase in substrate affinity (Liebenhoff *et al*, 1993). This step could also be crucial to the mechanism whereby LHRH- and PDBu-stimulated PKC activity in $\alpha$T3-1 cells increases the level of tyrosine phosphorylation of cellular proteins. It is also possible that *src*-type tyrosine kinases may then activate FAK, or other related tyrosine kinases.

When *fyn* was partially purified from $\alpha$T3-1 cells by immunoprecipitation, using the FYN3 polyclonal antibody (section 5.3.3), it was found that prior stimulation of the cells with LHRH for 10 min increased

the ability of such immunoprecipitates to bring about phosphorylation of a denatured enolase substrate by approximately 50%. This indicates that this non-receptor tyrosine kinase may be responsible for some of the tyrosine phosphorylation resulting from LHRH-receptor stimulation. It was not possible to assess whether *src* was similarly stimulated in response to LHRH, although it is conceivable that *src* will also be activated by LHRH, although redundancy of function between *src* and *fyn* has not been ascertained in this system. However it has been demonstrated that long term potentiation (LTP) is impaired in *fyn* mutant mice, while *src*, *yes* or *abl* mutants show no impairment in LTP (Grant *et al*, 1992). It is not possible to conclude from the increased phosphorylation of enolase by immunoprecipitated *fyn* whether LHRH-stimulation results in an increase in kinase activity or in an increase in substrate affinity.

A potential target for non-receptor tyrosine kinases such as *src* or FAK are regulatory proteins associated with low molecular weight G proteins, which have been shown to activate another important signalling enzyme investigated in this study, PLD (discussed in Chapter 3). For example, in B cells, activation of the B cell receptor, possessing no intrinsic tyrosine kinase activity, has been shown to stimulate the activity of *src*-type kinases. These kinases have also been shown to phosphorylate the Grb2 adapter protein, which can then interact with Sos, the guanine nucleotide exchange factor for the low molecular weight G protein Ras (Smit *et al*, 1994). It has also been reported that Vav, a protein with homology to guanine nucleotide releasing factors that activate Ras is phosphorylated by p56 *lck*, a *src*-type non-receptor tyrosine kinase, activated by the T cell receptor. This is reported to inhibit the activity of Vav and thus maintain the low molecular G-protein in an active state (Gulbins *et al*, 1993). A non-receptor tyrosine kinase has also been shown to inhibit the GTPase activity

of another low molecular weight G protein, p21 cdc 42, related to Rho (Manser *et al*, 1993).

In platelets (which contain particularly high levels of c-*src*), agonists that directly or indirectly activate PKC such as the phorbol ester PMA, vasopressin and the $Ca^{2+}$ ionophore A23187 have been shown to bring about an increase in the activity of the non-receptor tyrosine kinases c-*src* and c-*fyn*. Stimulation of platelets by the above pathways can lead to the phosphorylation of Ser-12 on c-*src*, which is thought to be responsible for an increase in the substrate affinity of c-*src* by two to three fold (Liebenhoff *et al*, 1993). It is therefore conceivable that *src*-type non-receptor tyrosine kinases are activated by the same pathway in αT3-1 cells stimulated with LHRH. This would also explain how PDBu is capable of stimulating tyrosine phosphorylation in a similar fashion to the receptor stimulation.

A feature of *src*-type non-receptor tyrosine kinases is that they have SH2 and SH3 domains which allow them to interact with other proteins involved in the intracellular signalling mechanism. For example it has recently been demonstrated that both *src* and *fyn* are able to form a stable association with p125 FAK, through interaction of the SH2 domain on *src* and *fyn* with phosphotyrosine residues on FAK (Cobb *et al*, 1994). It has also been demonstrated that the SH3 domain of *fyn* can mediate binding to phosphatidylinositol 3-kinase in T cells, which is thought to play an important role in T cell signalling (Prasad *et al*, 1993). It is known that SH3 domains are able to bind to the proline-rich sequence motif X-P-X-X-P-P-P-Z-X-P, where X represents any amino acid, and Z represents a hydrophobic amino acid (Ren *et al*, 1993). It has also been suggested that SH3 domains may be important for localisation to the cytoskeleton, since SH3 containing proteins are often associated with the cytoskeleton (Musacchio *et al*, 1992).

As well as enzymes mediating protein-tyrosine phosphorylation, tyrosine dephosphorylation is also an important event in intracellular signal transduction pathways. It is apparent from the αT3-1 cells treated with the $Ca^{2+}$-ionophore ionomycin, that an increase in intracellular $Ca^{2+}$ levels reduced the level of phosphotyrosine seen on intracellular proteins. It is likely that this has occurred through the activation of a $Ca^{2+}$-activated tyrosine phosphatase, such as PTP-1B, which can be activated by cleavage of its C-terminal membrane localisation domain by the $Ca^{2+}$-activated protease, calpain (Frangioni *et al*, 1993). Another possibility is the $Ca^{2+}$/calmodulin-dependent phosphatase, calcineurin, which has some tyrosine phosphatase activity (Pallen and Wang, 1985), or another related tyrosine phosphatase. It has also been demonstrated that in pancreatic acini, protein tyrosine dephosphorylation (probably mediated by PTP 1) is involved in the $Ca^{2+}$-dependent secretion of amylase, suggesting that $Ca^{2+}$ is responsible for activating a tyrosine phosphatase in this case as well (Jena *et al*, 1991).

So in conclusion, both LHRH and PDBu appear to capable of inducing tyrosine phosphorylation in αT3-1 cells, making it likely that the tyrosine kinase involved in LHRH-signalling is downstream of PKC. The major LHRH- and PDBu-induced tyrosine phosphorylations are seen on proteins of 70-80 kDa and 110-130 kDa. It is also apparent that raising the intracellular $Ca^{2+}$ concentration with ionomycin reduces the level of tyrosine phosphorylation in αT3-1 cells, probably through the activation of an unidentified $Ca^{2+}$-stimulated tyrosine phosphatase. There were two members of the *src*-family of tyrosine kinases detectable by immunoblotting in αT3-1 cells; *src* and *fyn*. Using immunoprecipitation to partially purify *fyn* we found that LHRH-stimulation increased the phosphorylation by anti-*fyn* immunoprecipitates of an exogenous enolase substrate.

**Figure 5.1**

**Anti-phosphotyrosine immunoblots of buserelin, PDBu and ionomycin treated αT3-1 cells**

Anti-phosphotyrosine immunoblots of αT3-1 cells from 2 separate experiments, using the 4G10 anti-phosphotyrosine mouse monoclonal antibody. Lanes 1 and 8, control (saline); 3 and 6, the LHRH receptor agonist buserelin ([D-Ser(tBu)$^{6}$, DesGly$^{10}$] LHRH ethylamide; 100 nM); lane 2, PDBu (300 nM); lane 7, ionomycin (30 μM). Lanes 4 and 5 are buserelin-treated cells immunostained with phosphotyrosine-blocked antibody, in order to detect non-specific antibody binding. The incubation time of the cells in all conditions before solublisation was 20 min. 2.5 μg of protein was loaded per lane, determined by Bradford method (Pierce).

Figure 5.1

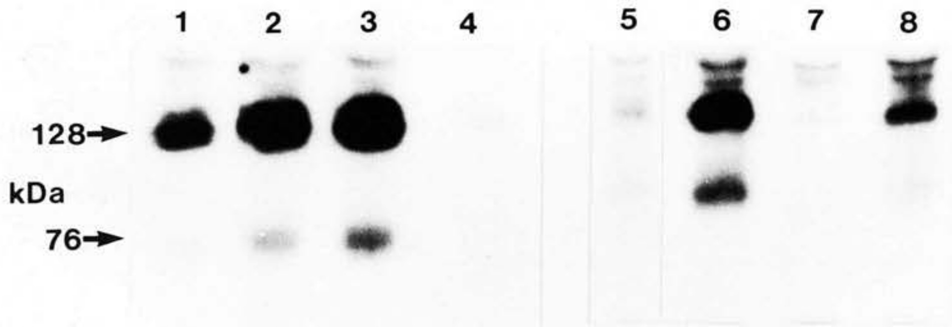


**Figure 5.2**

**Time course of buserelin-stimulated tyrosine phosphorylation in $\alpha$T3-1 cells**

Time course of protein tyrosine phosphorylation induced by the LHRH receptor agonist buserelin ([D-Ser(tBu)$^{6}$, DesGly$^{10}$] LHRH ethylamide; 100 nM) assessed by anti-phosphotyrosine immunoblotting, over two separate experiments, which compare the tyrosine phosphorylations induced at 3 and 10 min, and at 10 and 60 min.

Figure 5.2

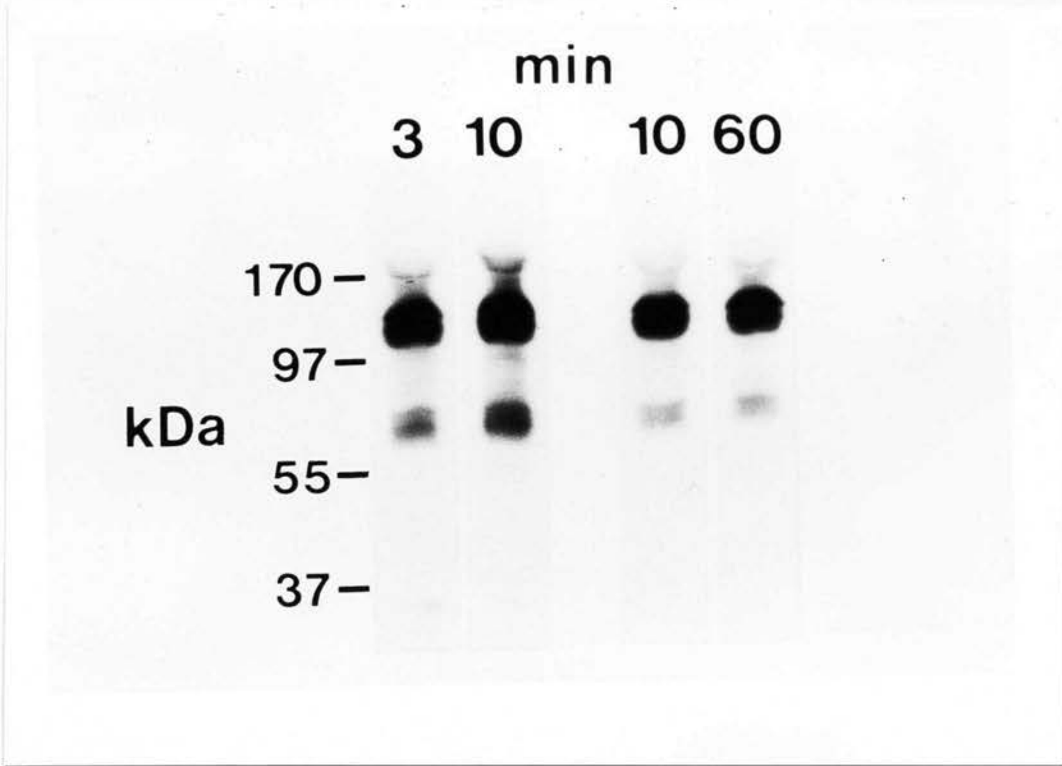


**Figure 5.3**

**Immunoblot of the non-receptor tyrosine kinases src and fyn in $\alpha$T3-1 cells**

Immunoblot of protein samples from $\alpha$T3-1 cells. src was detected with the monoclonal mouse antibody GD 11 (UBI) (lanes 1 and 2). Fyn was detected with the FYN3 rabbit polyclonal antibody (Santa Cruz Biotechnology) (lanes 3 and 4). In order to detect non-specific binding associated with the FYN3 antibody, the antibody was blocked with the peptide used to produce the antibody before incubating with the blots (lane 5). Antibody binding was visualised using a peroxidase-linked secondary antibody and chemiluminescence detection system (ECL; Amersham).

Figure 5.3

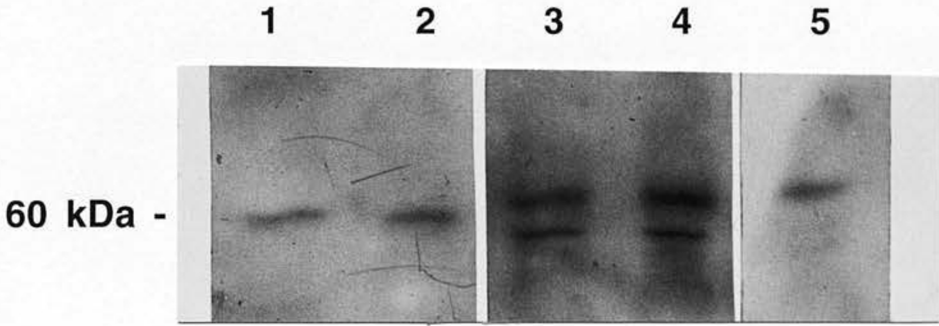
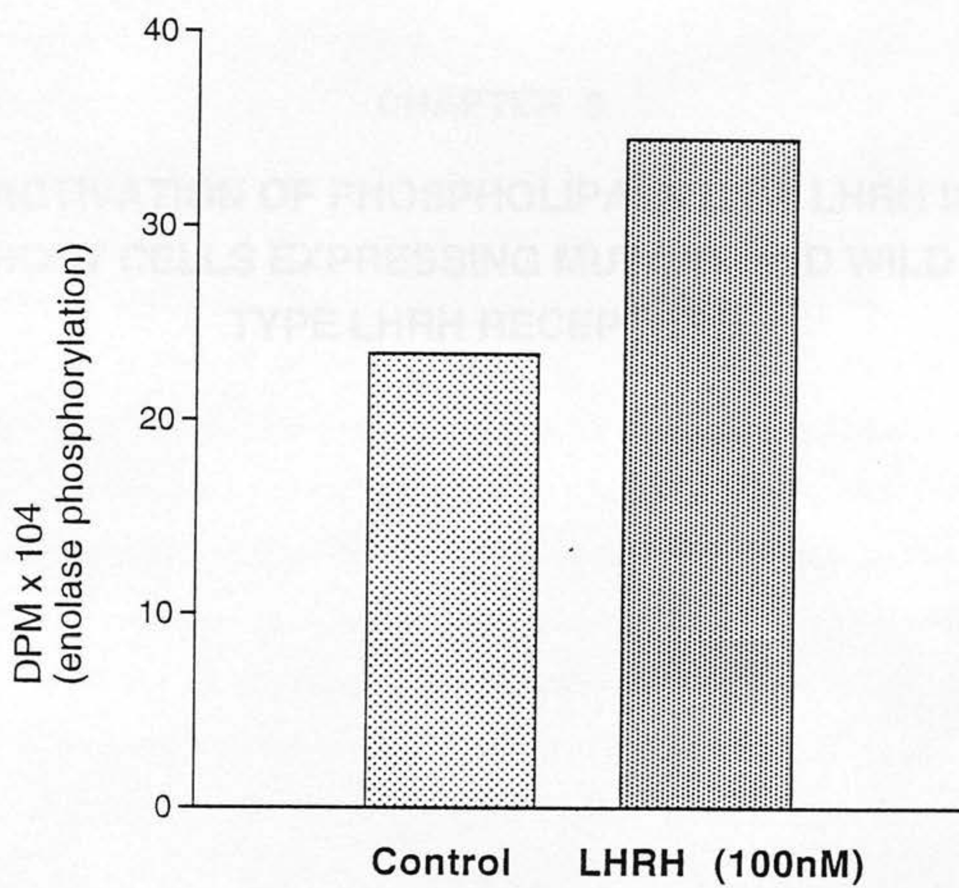


**Figure 5.4**

**Phosphorylation of an exogenous substrate by partially purified fyn**

Phosphorylation of an acid-denatured enolase substrate by the fyn non-receptor tyrosine kinase, immunoprecipitated using FYN3 polyclonal rabbit antibody (Santa Cruz). αT3-1 cells were either stimulated with LHRH (100 nM) for 10 min prior to lysis, or they were left unstimulated (n = 1). Phosphorylation of acid-denatured enolase was quantified using a BioRad phosphorimager.

**Figure 5.4**



# CHAPTER 6

# ACTIVATION OF PHOSPHOLIPASE D BY LHRH IN HOST CELLS EXPRESSING MUTANT AND WILD TYPE LHRH RECEPTORS

## 6.1 INTRODUCTION

The main experimental model used in this study has been the activation of PLD in αT3-1 cells stimulated with LHRH. Studies have also been carried out to determine whether the LHRH receptor will bring about the activation of PLD though the same pathway when transfected into COS 7 and CHO cells. Using site-directed mutagenesis of the LHRH receptor a preliminary examination has been made of the importance of certain domains of the receptor protein in mediating PLD activation.

It is becoming increasingly apparent that distinct cytoplasmic and transmembrane domains of G protein-coupled receptors have distinct cellular functions. For example, in the β2 adrenergic receptor, phosphorylation of serine and threonine residues in the C-terminal cytoplasmic tail by the β adrenergic receptor kinase results in receptor desensitisation (Hausdorff *et al*, 1991). It has also been demonstrated that ionic interactions between residues of different transmembrane domains on G protein-coupled receptors are critical for their structure and function (Cohen *et al*, 1992; Khorana, 1992). The intracellular cytoplasmic loops of G-protein coupled receptors are also important for their function, as has been demonstrated with G protein interactions with the 3rd intracellular loop (Cheung *et al*, 1992). A characteristic shared by most G protein-coupled receptors is that continued stimulation results in their activity becoming rapidly attenuated, referred to as receptor desensitisation (reviewed in Wilson and Applebury, 1993). Receptor desensitisation has been shown to be dependent on the actions of receptor kinases, homologous to the β-adrenergic receptor kinase, which phosphorylate activated receptors primarily on the receptors carboxyl-terminal tail, and possibly also in the 3rd intracellular loop of the muscarinic $M_1$ receptor (Palczewski and Benovic, 1991; Lameh *et al*, 1992). It is also possible that

PKC and cAMP dependent protein kinase also play a role in receptor desensitisation (Lohse, 1993). Other proteins called arrestins are also responsible for the attenuation of G protein-coupled receptor activity. It has been shown that arrestins are able to bind to the activated and phosphorylated receptors, terminating receptor activity by blocking the sites of interaction with G proteins (Wilson and Applebury, 1993).

Hydrophobicity analysis of the LHRH-receptor sequence has demonstrated seven stretches of highly hydrophobic amino acids with 20-30% sequence similarity to other G protein-coupled receptors, with the highest degree of homology to the interleukin-8 receptor (Tsutsumi *et al*, 1992). Several features of the LHRH receptor are however unusual. The LHRH receptor is the smallest characterised member of the G protein linked receptor superfamily, and unlike any other G protein-coupled receptor it lacks a polar C-terminal cytoplasmic tail. Phosphorylation of the C-terminal tail has been shown to contribute to desensitisation of several G protein-coupled receptors (Probst *et al*, 1992). The first cytoplasmic loop of the LHRH receptor is also longer than in any other G protein-coupled receptor (Tsutsumi *et al*, 1992). Another important feature of the LHRH receptor is the substitution of serine for the conserved tyrosine, located adjacent to the transmembrane 3 domain, creating a potential, but not ideal phosphorylation site in a domain that has been shown to be important in the signal transduction mechanisms of other G protein-coupled receptors (Tsutsumi *et al*, 1992).

An unusual feature of the LHRH receptor, likely to be important for receptor function are two residues, one is asparagine (Asn) 87 in the transmembrane 2 domain (TM 2), the other is aspartate (Asp) 318 in the transmembrane 7 domain (TM 7) (Zhou *et al*, 1994). In most other G protein linked-receptors, these residues are reversed (Probst *et al*, 1992).

Interestingly, most G protein-coupled receptors show a relatively rapid desensitisation of their inositol phosphate responses (Probst *et al*, 1992), in contrast to the LHRH receptor which is remarkably resistant to this phenomenon (Davidson *et al*, 1994; R. Mitchell, unpublished observations). Murine LHRH receptor mutants generated by site-directed mutagenesis and transiently transfected into COS 7 cells have been used in order to assess whether the kinetics of PLD activation, or the intracellular signalling pathways leading to PLD activation are affected. The mutant receptors used had Asp 318 changed to Asn (mutant 99), and Asn 87 changed to Asp as well as Asp 318 changed to Asn (mutant 101), which is the consensus found in virtually all other characterised G protein-coupled receptors. It has been reported that the 99 mutant can bind LHRH receptor ligands to approximately 70% of the level of the wild type receptor, while the 101 mutant binds LHRH receptor ligands to about 40% of the capacity of the wild type receptor (Zhou *et al*, 1994). It has also been reported that LHRH-induced inositol phosphate production is greatly reduced in both the 99 and 101 mutants (Zhou *et al*, 1994). It was also been found that the inositol phosphate production in both of the above mutants transfected into COS 7 cells appears to be non-desensitising, as observed with the wild-type receptor (R. Mitchell, unpublished observations).

Another aim of this study was to establish whether PKC and tyrosine kinases are involved in the LHRH-stimulated activation of PLD in CHO cells stably transfected with the wild-type LHRH receptor, using inhibitors of PKC and of protein-tyrosine kinases.

## 6.2 METHODS

### 6.2.1 Phospholipase D assay

Assays for phospholipase D were carried out using the standard protocol, as described in section 2.5.

### 6.2.2 Transfection of COS 7 cells with receptor cDNA

COS 7 cells were grown as described in Chapter 2. For transfection, cells were washed twice with OptiMEM (Gibco) supplemented with 100 U/ml each of streptomycin and penicillin at 37°C before exposure to the transfecting medium for 4 hr. The transfecting medium consisted of OptiMEM, penicillin/ streptomycin (100 U/ml each), 400 µg/ml DEAE dextran (Promega), 100 µM chloroquine phosphate (Sigma) and 20 µg plasmid per 75 $cm^2$ flask. This was then replaced with 10% DMSO in PBS for 2 min, then DMEM/ 2% UltraSer G/ penicillin/ streptomycin. Cells were then grown for 24 hr before being trypsinised and replated into 12 well plates. Phospholipase D assays were then carried out 48 hr later as described in Chapter 2.

<u>DEAE-Dextran mediated transfection</u>

This is an efficient technique for introducing DNA into cultured cells. In general DEAE-dextran mediated transfection is successful for transient, but not stable transfection of cells (Gluzman, 1981). The transfection efficiency can be increased by adding DMSO (10%) to the cells for a short period. The exact mechanism through which it acts is unknown, but it may modify membrane structure to enhance the uptake of DNA. Chloroquine is applied to cells simultaneously with DNA, and appears to enhance transfection by binding to DNA and inhibiting intracellular DNA degradation by lysosomes.

### 6.2.3 Transfection of CHO cells with receptor cDNA

Cells for transfection were cultured in Hams F12 medium with 10 % foetal calf serum, supplemented with 100 U/ml each of streptomycin and penicillin at 37°C until they were 60-80% confluent in cell culture dishes (10 cm diameter). The cells were incubated overnight in the transfection medium (OptiMEM, penicillin/streptomycin (100 U/ml each), DOTAP (Boehringer Mannheim), 20 µg pcDNA with neomycin resistance gene per dish). Next day the medium was changed for Hams F12 with 2% Ultra Sera G and penicillin/streptomycin (100 U/ml each). The following day the medium was changed again, to the same as above, plus the neomycin analogue geniticin (500 µg/ml). Selection of cells took place 1 month later by placing cloning rings over individual colonies in order to remove and re-plate them. Selection of cells expressing functional LHRH receptors was carried out by equilibrium binding of [$^{125}$I] buserelin (an LHRH-receptor agonist), according to previously described procedures (Mitchell *et al*, 1988).

<u>DOTAP Transfection-reagent</u>

The transfection reagent DOTAP (N-[1-(2,3-dioleoyloxy) propyl]-N, N, N-trimethyl-ammonium\methylsulphate) is suitable for the transfection of DNA into mammalian cells for stable or transient gene expression. Mixing DOTAP with DNA results in spontaneously formed stable complexes, which can be added directly to serum-containing tissue culture medium. The DNA/DOTAP complexes fuse directly with the cell surface, releasing DNA into the cytoplasm. This method of transfection is also reported not to be cytotoxic (Stamatatos *et al*, 1988; Leventis and Silvius, 1990).

## 6.3 RESULTS

Levels of buserelin binding were determined by [$^{125}$I] buserelin binding (Mitchell *et al*, 1988). The Kd for [$^{125}$I] buserelin binding to wild type, 99 mutant and 101 mutant receptors in COS 7 cells were 1.03 nM, 0.89 nM and 0.79 nM respectively. The $B_{max}$ values for [$^{125}$I] buserelin binding to wild type, 99 mutant and 101 mutant receptors in COS 7 cells were 506, 263 and 141 fmol/mg protein respectively ([$^{125}$I] buserelin binding expriments were carried out by R. Mitchell).

It is apparent from the time course of LHRH- (100 nM) stimulated PLD activation in COS 7 cells transiently transfected with the wild-type LHRH receptor that activation of PLD proceeds without any detectable delay (Figure 6.1), which was seen in the case of LHRH-stimulated PLD activity in $\alpha$T3-1 cells (Chapter 3). The LHRH-stimulated PLD activity in COS 7 cells transfected with the wild type LHRH receptor does not appear to desensitise within 60 min (Figure 6.1), which is also the case for the LHRH-stimulated PLD activation in $\alpha$T3-1 cells (Chapter 3).

In COS 7 cells transiently transfected with the 99 and 101 mutant LHRH receptors, LHRH-stimulation of PLD activity initially occurs at a higher rate than is the case for the wild type receptor in COS 7 cells, although the LHRH-stimulated PLD activity appears to be fully desensitised within 5 min (Figure 6.1); no time points less than 5 min were studied. It should be remembered that figure 6.1 represents an accumulation of a stable product (phosphatidylbutanol), therefore the stable plateau observed with the 99 and 101 mutant LHRH receptors represents a lack of further detectable PLD activity.

In order to assess whether PKC is involved in the LHRH-stimulated activation of PLD in CHO cells stably expressing the wild type LHRH

receptor (Chapter 6.2), we used the selective bisindolylmaleimide PKC inhibitor Ro 31-8220 (Davis *et al*, 1992). It was found that Ro 31-8220 completely inhibited the LHRH-stimulated PLD activity with an $IC_{50}$ of 670 +/- 160 nM (Figure 6.2). This was very similar to Ro 31-8220 inhibition of LHRH-stimulated PLD activity in αT3-1 cells, which was 460 ± 180 nM (see Chapter 3).

In order to assess whether non-receptor tyrosine kinases are involved in LHRH receptor activation of PLD in transfected CHO cells in a similar manner to that seen in αT3-1 cells, we used the tyrosine kinase inhibitors, lavendustin A (Hsu *et al*, 1991) and genistein (Akiyama *et al*, 1987). Neither of these compounds had any effect on the LHRH-stimulated PLD activity in CHO cells stably transfected with the wild type LHRH receptor. Lavendustin A was used at 3 μM and genistein was used at 100 μM. These concentrations are well above the $IC_{50}$s observed for the inhibition of LHRH stimulated PLD activity in αT3-1 cells (see Chapter 4). Pertussis toxin (100 ng/ml) did not cause any significant attenuation of the LHRH-stimulated activation of PLD in CHO cells stably transfected with the wild type LHRH receptor (as was also the case in LHRH-stimulated PLD activity in αT3-1 cells (Chapter 3), indicating that the G proteins Gi and Go are not involved in coupling the LHRH receptor to PLD activation.

## 6.4 DISCUSSION

The results presented above indicate that both the single mutation of Asp 318 to Asn (99 mutant) and the double mutation of Asp 318 to Asn and Asn 87 to Asp (101 mutant) result in the reversion of the non-desensitising LHRH-stimulated PLD response seen with the wild type receptor in both COS 7 cells and αT3-1 cells, to a desensitising response, characteristic of other G protein-coupled receptors, which have the same TM 2 Asp and TM

7 Asn as the double mutant (Probst *et al*, 1992). The wild type murine LHRH receptor displayed a complete lack of desensitisation in the LHRH-stimulated PLD response over a 60 min time course, which may be functionally significant for the cell. This is in contrast to the LHRH-stimulated production of inositol phosphates in COS 7 cells transfected with the 99 and 101 mutant LHRH receptors, in which there was no detectable desensitisation, although the levels of inositol phosphates produced are lower than that observed with the wild-type receptor (R. Mitchell, unpublished observations). These results raise the possibility that the activation of PLC and PLD are not coupled to the LHRH receptor by the same mechanism, possibly involving different G proteins. A similar non-desensitising response has recently been reported for inositol phosphate production stimulated by the murine LHRH receptor in $\alpha$T3-1 cells (Davidson *et al*, 1994). Furthermore in $GH_3$ cells transfected with LHRH-receptor cDNA the hydrolysis of inositol phospholipids was also non-desensitising (Davidson *et al*, 1994). The absence of desensitisation in both cell types in this, and the present study suggests that the lack of desensitisation is likely to be due to an intrinsic property of the receptor.

It is interesting that in most other G protein-coupled receptors, in which the TM 2 Asp and TM 7 Asn are conserved, agonist-induced enzyme activation is rapidly desensitised (Probst *et al*, 1992). In contrast, in the LHRH receptor there appears to be a natural reciprocal mutation between the transmembrane 2 and 7 domains(Zhou *et al*, 1994). It is possible that one or both of these residues is responsible for the lack of desensitisation observed in LHRH-stimulated PLD activity, or that these residues may interact with other loci on the receptor to influence desensitisation, since mutating these residues back to the consensus seen in other G protein-coupled receptors resulted in the LHRH-stimulated PLD activity reverting to

a desensitising response. It has also been demonstrated that it is likely that these two residues lie in close proximity when the receptor is inserted in the membrane, and that there may be hydrogen bonding between them (Zhou *et al*, 1994). It has been postulated that the mutation of Asp 318 to Asn is able to maintain the hydrogen bonding between the two residues, and that Asn is able to act as both a hydrogen bond acceptor and donor in this site (Zhou *et al*, 1994). It is therefore likely that in both the single and double mutant the structural integrity of the receptor is maintained, with the function of the natural reciprocal mutation being lost, resulting in a desensitisation of the PLD response following LHRH-stimulation. The fact that a single mutation of Asp 318 to Asn has the same result as the double mutation in reverting LHRH-stimulated PLD activity to a desensitising response suggests that this residue in TM 7 plays the more important role in the non-desensitising response, or interacts with other important loci on the receptor. It has been reported that a single mutation of Asn 87 to Asp results in a complete loss of agonist binding, suggesting that this mutation results in loss of hydrogen binding between TM 2 and TM 7, leading to the loss of a functional structure and agonist binding (Zhou *et al*, 1994). A previous study carried out in COS-1 cells implicated the Asp 318 residue of the rat LHRH receptor in receptor coupling to the hydrolysis of inositol phospholipids, since mutation of Asp 318 to Asn resulted in a loss of IP hydrolysis as a result of LHRH-receptor stimulation (Cook *et al*, 1994). However, other studies have demonstrated that this mutant receptor is capable of stimulating inositol phosphate production (Zhou *et al*, 1994; R. Mitchell, unpublished observations). The Asp 318 may be located very close to the end of the TM 7 domain and may therefore be able to interact directly with cytoplasmic proteins, or to influence residues which do. It is therefore possible that mutating Asp 318 to Asn allows the receptor to

interact with intracellular components normally involved in bringing about receptor desensitisation, as is the case with other G protein-coupled receptors. Another study has been carried out using site-directed mutagenesis of the conserved Asn in TM 7 and Asp in TM 2 in the $5HT_{1A}$ receptor (Chanda *et al*, 1993). It was found that mutation of Asn 396 in the TM 7 domain to Ala, Phe or Val resulted in a loss of agonist binding. In contrast mutation of Asn 396 to Gln did not effect agonist binding. It is therefore possible that the mutation to Gln retains hydrogen bonding with another domain, possibly TM 2 as has been previously postulated (Zhou *et al*, 1994), while this interaction is disrupted by the other mutations.

It is clear that the initial rate of LHRH-stimulated PLD activation was lower in the wild type LHRH receptor than with the mutant LHRH receptors. Therefore, it may be that Asp in position 318 is less efficient at coupling to intracellular components involved in PLD activation than with Asn in position 318, or the interaction of this residue with other loci on the receptor may be altered, resulting in a lower initial rate of LHRH-stimulated PLD activation.

Although it was demonstrated in this study that residues in the transmembrane 2 and 7 domains are important factors in defining the LHRH-stimulated PLD activity as a non-desensitising response, it is still possible that other factors, such as the lack of a C-terminal tail (Tsutsumi *et al*, 1992) are also important in this effect, since it has been demonstrated that phosphorylation of the C-terminal tail can be an important factor in receptor desensitisation (Probst *et al*, 1992).

It is interesting that the LHRH-stimulated PLD activity in COS 7 cells transfected with the wild type LHRH receptor proceeds without a detectable delay in the formation of PtdBut, while in αT3-1 cells there was a significant delay in LHRH-stimulated PLD activation (Chapter 3). It therefore seems

likely that the delay in PLD activation may be due to intracellular components in the signalling pathway and not as a result of any function of the receptor, since no delay was observed in COS 7 cells. It has already been demonstrated that the delay observed in αT3-1 cells was not due to a necessity for protein synthesis (Chapter 3).

It was also demonstrated in this study that the selective PKC inhibitor, Ro 31-8220 was able to inhibit the LHRH-stimulated PLD activity in CHO cells stably expressing the LHRH receptor at a very similar concentration to that seen for LHRH-stimulated PLD activity in αT3-1 cells (Chapter 3). It is therefore possible that the PKC(s) involved in the LHRH-stimulated activation of PLD in CHO cells are the same or similar to those in αT3-1 cells, which shows an approximately 8-fold lower sensitivity to Ro 31-8220 than the PDBu-stimulated PLD activity in αT3-1 cells (see Chapter 3). This concentration of Ro 31-8220 represents a potent inhibition of PKC; the selectivity of the bisindolylmaleimide PKC inhibitors on the responses observed in the present study is discussed in greater depth in Chapter 3. From immunoblot analysis it has been shown that CHO cells contain a high level of PKC ζ, and also PKC α, although it has not been able to rule out the presence of other isoforms (J. Simpson, unpublished results).

It would appear that non-receptor tyrosine kinases are not involved in the activation of LHRH-stimulated activation of PLD in CHO cells transfected with the wild type LHRH receptor, as they are in αT3-1 cells (Chapter 4). Neither lavendustin A or genistein had any detectable effect on the LHRH-stimulated activation of PLD. Since it is not known exactly which non-receptor tyrosine kinase is involved in the LHRH-stimulated PLD activation in αT3-1 cells, it has not been possible to ascertain whether CHO cells contain the kinase or kinases involved.

The absense of desensitisation of second messenger systems in response to LHRH-receptor desensitisation may not be out of keeping with the pulsatile nature of LHRH release *in vivo* (Fink, 1988). The physiological response may be frequency modulated, and the receptor could function as an amplifier of the frequency-encoded signal. In fact, it may be innapropriate for a frequency-modulated sytem to display desensitisation over a time-frame which is short compared with the duration of the LHRH-pulse, since this would limit the duration of the pulse. It is therefore possible that the LHRH-receptor has evolved to be maximally active over a period of hours prior to ovulation, when a rapidly desensitised response would be inappropriate.

In conclusion, it would appear that the natural reciprocal mutation between the TM 2 and TM 7 domains and in particular Asp 318 in TM 7 is significantly responsible for the lack of desensitisation in the LHRH-stimulated PLD activity. Mutating Asp 318 to Asn, which is the residue found in this position in most other G protein-coupled receptors (Probst *et al*, 1992), resulted in the LHRH-stimulated PLD activity having a higher initial rate, followed by complete desensitisation within 5 min. A double mutation of Asp 318 to Asn in TM7 as well as Asn 87 to Asp in TM 2, which is reported to interact though hydrogen bonding with Asp 318 in TM 7 (Zhou *et al*, 1994) had the same result as the single mutation. It appears that PKC is involved in the LHRH-stimulated activation of PLD in CHO cells stably expressing the wild type LHRH receptor, since the sensitivity to Ro 31-8220 is similar to that of LHRH-stimulated PLD activation in $\alpha$T3-1 cells. The wild type LHRH receptor-stimulated PLD activity in transfected CHO cells was unaffected by tyrosine kinase inhibitors, or by pertussis toxin.

**Figure 6.1**

**Time course of LHRH-stimulated PLD activation in COS 7 cells transiently transfected with LHRH receptor cDNA**

Time course of [$^3$H] PtdBut accumulation in COS 7 cells stimulated with LHRH (100 nM). The cells were transiently transfected with cDNA for the wild-type, 99 mutant and 101 mutant receptors. All values are means ± SEM from 4-8 separate determinations with the basal level of [$^3$H]PtdBut accumulation subtracted. Basal values were not significantly different between the three clones, and did not significantly change over the time course.

**Figure 6.1**

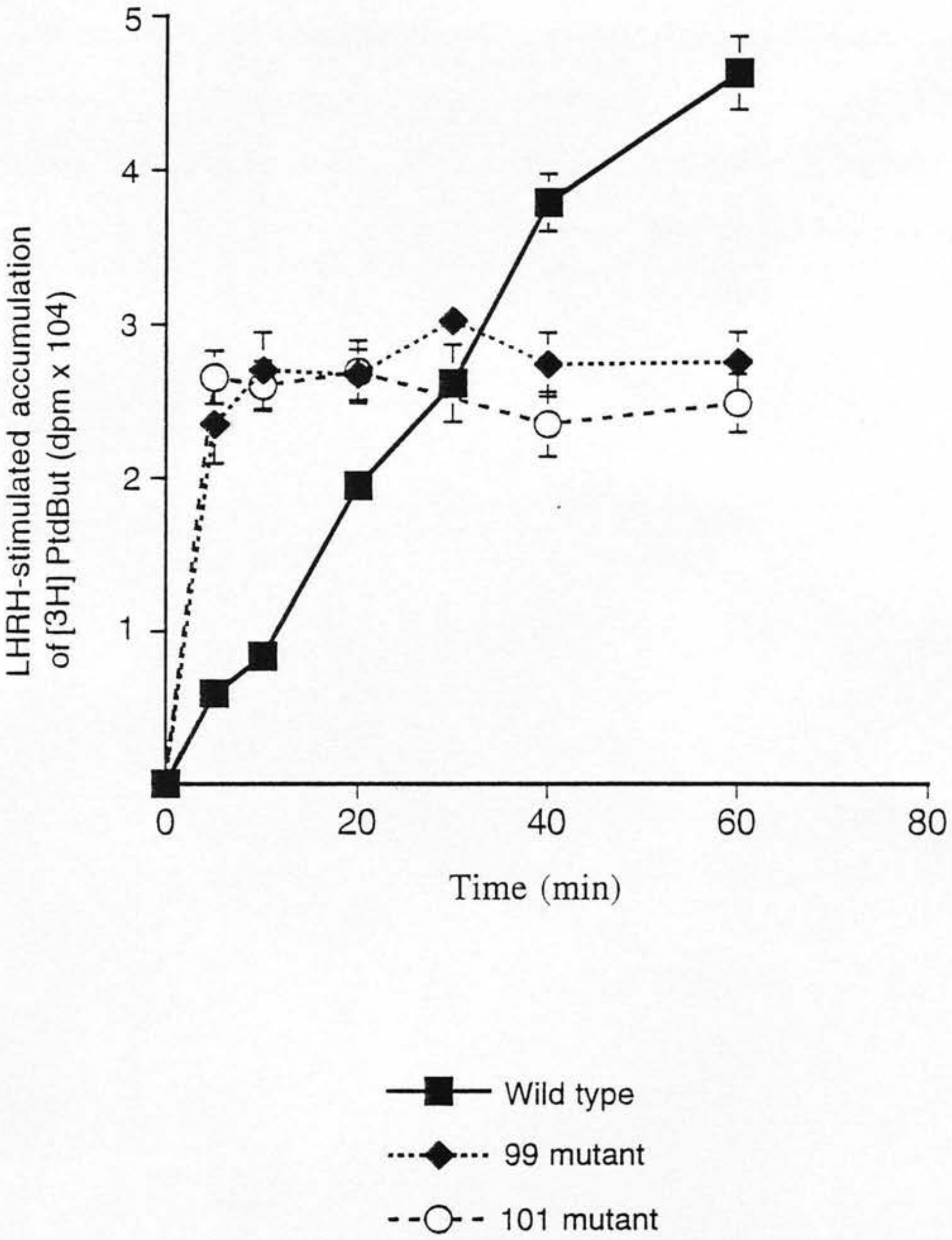
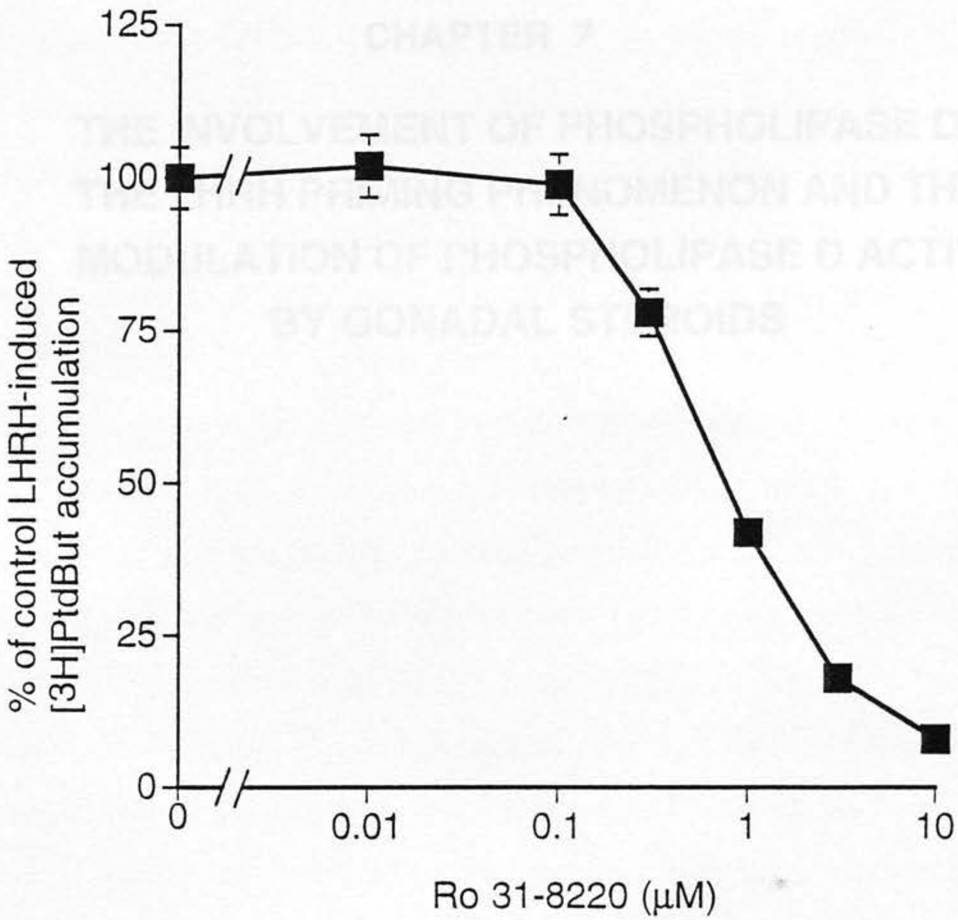


**Figure 6.2**

**Concentration response curve for the inhibition of LHRH-stimulated PLD activity by Ro 31-8220 in COS 7 cells transiently transfected with wild type LHRH receptor cDNA**

The effect of the protein kinase C inhibitor Ro 31-8220 on the LHRH- (100 nM) stimulated activation of PLD on CHO cells stably transfected with cDNA for the wild-type LHRH receptor. Stimulation was for 30 min, and values are means ± SEM from 4-8 separate determinations with the basal level of [$^{3}$H]PtdBut accumulation subtracted. There was no effect of Ro 31-8220 on the basal PLD activity

**Figure 6.2**



# CHAPTER 7

# THE INVOLVEMENT OF PHOSPHOLIPASE D IN THE LHRH PRIMING PHENOMENON AND THE MODULATION OF PHOSPHOLIPASE D ACTIVATION BY GONADAL STEROIDS

## 7.1 INTRODUCTION

One of the key reasons for investigating LHRH-activated intracellular signalling mechanisms in the αT3-1 cell line has been to try to derive insights into mechanisms underlying the LHRH-priming phenomenon. This is a unique mechanism in pituitary gonadotrophs, in which LHRH significantly increases the responsiveness of gonadotrophs to itself; prior exposure of gonadotrophs to LHRH leads to a substantial augmentation of the LH and FSH release that is caused by a second exposure to LHRH (Aiyer and Fink, 1974b). The role of the LHRH priming effect would appear to be to co-ordinate the surge of LHRH and the increase in pituitary responsiveness to LHRH so that both events reach a peak simultaneously, and as a consequence produce a massive surge of LH release from gonadotrophs in the anterior pituitary (Fink, 1988). It has also been demonstrated that this priming effect is enabled in part by the history of exposure of the anterior pituitary to steroid hormones. The plasma concentrations of gonadotrophins are maintained at relatively low levels for most of the oestrous cycle by the negative feedback of low concentrations of oestrogen and high plasma concentrations of progesterone acting on the hypothalamus and the anterior pituitary (Fink, 1988). The LH surge is preceded by a surge of oestrogen and is accompanied and further intensified by a significant increase in the plasma concentration of progesterone (Fink, 1988). This priming effect of LHRH is also dependent upon protein synthesis, demonstrated by the ability of the inhibitor of protein synthesis, cycloheximide, to block the initiation of the LHRH-priming effect. The priming effect is also dependent upon the integrity of microfilaments (Fink, 1988).

Several intracellular signalling mechanisms have been shown to be important in the LHRH priming effect. For example, it has been

demonstrated in the anterior pituitary that prior exposure to LHRH resulted in an enhanced LHRH-induced production of inositol phosphates, as well as an enhanced release of $Ca^{2+}$ from intracellular stores (Mitchell *et al*, 1988). As well as this elevation in the level of inositol phosphates there will also be an increase in DAG production, which could lead to an enhanced activation of PKC. Indeed it has been demonstrated that inhibitors of PKC are able to inhibit the onset of priming, and in particular a partially H7-insensitive isoform of PKC may be involved in the priming effect (Johnson *et al*, 1992). The phospholipase, $PLA_2$, also appears to be specifically involved in the LHRH priming phenomenon, since inhibitors of $PLA_2$ are able block the onset of LHRH priming, but do not effect the immediate LHRH-stimulated release of LH from the anterior pituitary. The LHRH priming effect can also be partially mimicked by a conditioning preincubation with arachidonic acid (Thompson *et al*, 1994). An enzyme which is reported to phosphorylate $PLA_2$, mitogen activated protein kinase (MAPK) (Lin *et al*, 1993) is also thought to play some role in the LHRH priming effect, since it has been shown to be activated by LHRH in a concentration dependent manner after 5-10 min, continuing up to 60 min after stimulation, and has the highest activity in pro- and di-oestrus, when priming occurs (Mitchell *et al*, 1994). Elevation of cAMP levels, and the activation of cAMP-dependent protein kinase may also additionally be important in the LHRH priming effect (Turgeon and Waring, 1994). Tyrosine phosphorylation has also been demonstrated to be important in the release of LH from the anterior pituitary in response to LHRH, although not specifically in the primed release, by the use of protein tyrosine kinase inhibitors (Johnson *et al*, 1994).

It is possible that PA or DAG produced by the action of PLD on PC might somehow be involved in increasing the responsiveness of pituitary

gonadotrophs to LHRH by activating the PKC or PKC-like kinases which have been shown to play a key role in priming (Johnson *et al*, 1992). Indeed, it was recently demonstrated that PA is capable of strongly activating PKC $\zeta$ in a cell free system (Limatola *et al*, 1994). In the present study it has been shown that the LHRH-stimulated activation of PLD appears to be non-desensitising over a time course of 40 min (Chapter 3), which is similar to the time course needed to elicit the LHRH priming phenomenon. Phospholipase D has previously been closely linked to the control of cellular events, such as mitogenesis (reviewed in Boarder, 1994 and Thompson *et al*, 1993a), and also in neutrophil priming, which results in the enhanced production of superoxide (Babior, 1984). Phospholipase D activity in resting, or unprimed neutrophils is poorly stimulated by fMLP, but in neutrophils that have been primed by pretreatment with granulocyte-macrophage colony stimulating factor, platelet activating factor, or cytochalasin B, there is a 5-10 fold higher responsiveness observed in the fMLP-stimulated activation of PLD (Mullmann *et al*, 1990; Anthes *et al*, 1991). Preliminary experiments indicated that it was not going to be possible to assess whether there is any involvement of PLD in LHRH-priming in anterior pituitary tissue, due to variability of the signal, presumably as a result of the heterogeneous nature of the tissue. Therefore attempts have been made to establish in the $\alpha$T3-1 cell line whether a conditioning preincubation with LHRH will result in an augmented PLD response upon subsequent LHRH exposure. It has been demonstrated that this type of initial LHRH-stimulus, followed by a second LHRH stimulus is capable of bringing about a significant augmentation in the release of LH from hemisected pituitaries *in vitro* (Fink,1986). The release of LH cannot be measured from the $\alpha$T3-1 cell line, since it does not synthesise or secrete the $\beta$ subunit of LH (Windle *et al*, 1990).

Another area of particular interest has been the role of steroid hormones such as oestrogen and progesterone in modulating LHRH-stimulated PLD activity in αT3-1 cells, since they have been shown to play such a critical role in the control of the LHRH-priming effect *in vivo* (Fink, 1988). Oestrogen and progesterone bind to nuclear receptors, which act as trans-acting transcription factors (reviewed in Weisz and Bresciani, 1993). These steroid receptors are capable of binding to specific cis-acting enhancer elements on the DNA, usually located within the 5'-flanking regions of the target genes. Oestrogen has also been reported to regulate gene expression by influencing mRNA stability or via interaction with transcription regulation factors (Weisz and Bresciani, 1993).

Steroids have also been shown to modulate the activity and expression of enzymes involved in intracellular signalling pathways. For example, it has been demonstrated that oestrogen can modulate PKC levels in pituitary cells, by regulating the mRNA levels of $Ca^{2+}$-dependent and -independent forms of PKC (Maeda and Lloyd, 1993). It has also been observed that in human endometrial fibroblasts, PLC activity can be enhanced by oestrogen in a $Ca^{2+}$-dependent manner (Imai *et al*, 1990), and that oestrogen can regulate the expression of a secretory form of $PLA_2$ in the quail oviduct (Fayard *et al*, 1994). The level of the heterotrimeric G protein, $G_{i2}$, which inhibits the activity of adenylate cyclase has been shown to be upregulated during gestation in the rat by the high levels of oestrogen (Tanfin *et al*, 1991). Progesterone independent trans-activation of the progesterone receptor has also been reported to occur as a result of LHRH-priming; this is suggested to be as a result of cAMP-dependent protein kinase activation, following an elevation of cAMP levels associated with LHRH receptor activation (Turgeon and Waring, 1994).

## 7.2 METHODS

### 7.2.1 Phospholipase D assay

Assays for phospholipase D were carried out using the standard protocol, as described in section 2.4.

### 7.2.2 Pre-stimulating αT3-1 cells with LHRH

Quiescent αT3-1 cells were pre-labelled and washed as described in section 2.4. They were then stimulated with 10 nM LHRH in the absence of butan-1-ol, in order to stimulate PLD without forming stable phosphatidylbutanol, for 15 min and then washed again as previously described (section 2.4). After a period of 1 hr they were stimulated again with 10 nM LHRH for a period of 30 min in the presence of butan-1-ol (30 mM). The remainder of the assay was as described in section 2.4. Controls were carried out with no pre-stimulus in order to determine whether an increment in the LHRH-stimulated PLD response had occurred as a consequence of a prior LHRH stimulus (i.e. was there evidence for priming of the PLD response ?).

### 7.2.3 Steroid hormone treatment of αT3-1 cells

αT3-1 cells were plated out in 12 well culture plates (Costar) and grown for approximately 24 hrs, as described in section 2.3. The growth medium was then changed for DMEM with no phenol red, 10% steroid free (charcoal stripped) foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin, approximately 48 hrs prior to the assay. In order to determine the effect on LHRH-stimulated PLD of specific steroid hormones, oestrogen (1 nM) was added to the growth medium 16 hrs prior to the assay and progesterone (200 nM) was added 3 hrs prior to the assay. It has been demonstrated that the growth rate of αT3-1 cells grown in steroid stripped

growth medium is not reduced, as assessed by 5-bromo-2'-deoxyuridine incorporation (M. Johnson, unpublished observations).

### 7.2.4 Hormone secretion assay

Female COB Wistar rats were maintained as described in section 2.4. Female rats were killed at approximately 13.00 h on pro-oestrus (assessed as described in section 2.4). Pituitary glands were removed and the anterior lobes separated from the posterior pituitary. The anterior lobes were then bisected. These hemipituitaries were allocated one/flask, such that hemipituitaries from the same rat were in different treatment groups.

The hemipituitaries were then incubated in 2 ml of pre-warmed and gassed (95% $O_2$/5% $CO_2$) HEPES-buffered minimal essential medium (MEM) with Earle's salts at 37$^oC$ in a shaking water bath under an atmosphere of 95% $O_2$/5% $CO_2$. The hemipituitaries were then washed in 2 ml of fresh medium for approximately 15 min after which the medium was changed before adding a stimulus of LHRH (1 nM) and the drug to be studied. All the medium was removed from the flasks and then replaced along with the drug and LHRH at the end of the 1st, 2nd and 3rd hour. The medium removed at the end of each hour was stored at -20$^oC$, until assayed for LH by radioimmunoassay (Daane and Parlow, 1971).

## 7.3 RESULTS

It was set out to deduce whether or not the LHRH-stimulated PLD activity could be enhanced by an initial exposure to LHRH, as has been shown to occur with the release of LH from the pituitary (Fink, 1986). The effect of the removal of steroid hormones from the growth medium on the LHRH-stimulated activation of PLD, and their subsequent readdition was also studied.

An LHRH (10 nM) pre-stimulus (15 min), in the absence of butan-1-ol (to ensure that no PtdBut is formed), was applied to the αT3-1 cells, 1 hr prior to the cells being stimulated with LHRH (10 nM) for 30 min, in the presence of butanol (see section 7.2.2). The level of LHRH-stimulated PLD activity in cells which received a pre-stimulation was 95 ± 5% of the LHRH-stimulated PLD activity in cells which were not pre-stimulated (Figure 7.1). An LHRH concentration of 10 nM was used for both the pre-stimulus and the stimulus in order to ensure that the LHRH-stimulated PLD response was not maximal, in which case it might not have been possible to observe any further augmentation of the LHRH-stimulated activity that might have occurred. No change was observed in the basal PLD activity in cells receiving a pre-stimulus, but no further stimulus, compared to cells receiving no prestimulus with LHRH.

In order to determine whether or not oestrogen plays a role in modulating LHRH-stimulated PLD activity, αT3-1 cells were grown in a steroid-free growth medium. To determine the effect of oestrogen alone on the LHRH-stimulated PLD activity, oestrogen was added back to the growth medium approximately 16 hrs prior to the assay (see section 7.2.3 for experimental details). The LHRH-stimulated PLD activity in the cells grown in steroid-free growth medium was taken as the basal level (Figure 7.2). In the cells which had oestrogen (1 nM) added back 16 hrs prior to the assay, LHRH-stimulated PLD activity was 132 ± 6% of that seen in αT3-1 cells grown in steroid stripped growth medium, while the LHRH-stimulated PLD activity in cells grown in normal growth medium was 125 ± 5% of the basal level (Figure 7.2). The LHRH-stimulated PLD activities in αT3-1 cells grown in normal growth medium, and with the readdition of oestrogen are significantly different from the LHRH-stimulated PLD activity in cells grown in steroid stripped medium ($p<0.05$; Mann Whitney U-test).

In order to determine the involvement of progesterone in the regulation of LHRH-stimulated PLD activity, we cultured αT3-1 cells in the absence of steroid hormones, as described in section 2.3, then added progesterone (200 nM) back to the growth medium approximately 3 hrs prior to the assay. In cells pre-treated with progesterone the LHRH-stimulated PLD activity was 110 ± 5% of that seen in cells grown in the absence of steroid hormones (Figure 7.2). In αT3-1 cells pretreated with both oestrogen (1 nM) and progesterone (200 nM), the LHRH-stimulated PLD activity was 129 ± 4% of that seen in cells grown in steroid free growth medium (Figure 7.2), which is significantly different from cells grown in steroid-stripped medium ($p<0.05$; Mann Whitney U-test). It therefore seems that oestrogen is needed for maximal PLD activation by LHRH. There was no detectable difference in the basal PLD activity between cells grown in normal growth medium, in steroid-stripped medium or in cells grown in stripped medium with the readdition of oestrogen or progesterone.

In order to assess whether PLD plays a role in the release of LH from the anterior gland *in vitro*, we have used the primary alcohol, butan-1-ol, which at high concentrations will lead to the production of phosphatidylbutanol, instead of PA from the hydrolysis of PC by PLD, and has been used as a pharmacological inhibitor of PLD responses in neutrophils (Bonser *et al*, 1989). We have also used demethoxyviridin (DMV), a derivative of the fungal metabolite wortmannin, which has been reported to inhibit PLD activity in the human neutrophil at a site between the activated receptor and PLD (Bonser *et al*, 1991). It is apparent that butan-1-ol was able to inhibit both the initial and the primed release of LH from the anterior pituitary, stimulated by LHRH (1 nM), while not affecting the basal release (Figure 7.3). It also appears that DMV also had a similar effect on both the initial and primed release of LHRH, with no apparent effect on the

basal release (Figure 7.4) (experiment with DMV carried out by F.J. Thompson).

## 7.4 DISCUSSION

It is apparent that with the conditions used this study (i.e. pretreating αT3-1 cells with LHRH (10 nM) 1 hour prior to a further LHRH (10 nM) stimulus) there was no detectable enhancement of the LHRH-stimulated activation of PLD. If PLD were closely involved in controlling the LHRH priming effect, it is conceivable that the LHRH-stimulated PLD activity would be enhanced by a previous exposure to LHRH, since it has already been shown that there is a facilitation of the LHRH-stimulated activation of PLC in pro-oestrous anterior pituitaries by conditions which lead to the primed release of LH (Mitchell *et al*, 1988). This increased PLC activity may then lead to an increase in PKC activity. An enhanced production of PA and DAG derived from the hydrolysis of PC by PLD might also activate kinases, such as PKC, involved in modulating the priming response, since it has been demonstrated that an unidentified H7-resistant isoform of PKC, or a PKC-like enzyme is closely involved in the LHRH-priming effect (Johnson *et al*, 1992). An enhanced stimulation of PLD as well as PLC by LHRH during the priming phenomenon might cause a massive stimulation of PKC, while the activation of PLD would not result in the release of $Ca^{2+}$ from intracellular stores. The fact that the LHRH-stimulated PLD activity was not enhanced by the conditions used in this study does not necessarily mean that PLD has no role to play in the LHRH priming effect, only that a potentiation of the PLD response does not appear to be mediated directly by LHRH, and is probably not augmented in the same way as PLC.

It has been previously noted that there is ovarian steroid-dependent modulation of pituitary responsiveness to different hypothalamic factors,

such as somatostatin, dopamine, LHRH and TRH (Drouva *et al*, 1988; Aiyer and Fink, 1974). It has also been shown that there is an increase in PKC activity in the pituitary following exposure to oestrogen (Thomson *et al*, 1993b), and that this increase in PKC activity is not mediated by oestrogen-evoked changes in hypothalamic secretion of LHRH, TRH, dopamine or somatostatin, which might have indirectly triggered enzyme activation (Drouva *et al*, 1990). It is significant that the LHRH-stimulated PLD activity is lower in αT3-1 cells grown in the absence of steroid hormones and that the full response can be regained by the readdition of oestrogen, but not progesterone to the growth medium. One possibility is that the level of expression of PKC, which is required for LHRH-induced PLD activation is normally under steroidal control, and is thus reduced in cells grown in the absence of steroids. It has been reported that oestrogen treatment of normal rat pituitary tissue and pituitary tumours can increase mRNA levels of the PKC isoforms, α, β, δ, ε and ζ (Maeda and Lloyd, 1993). It has been demonstrated by immunodetection that αT3-1 cells express the PKC isoforms α, ε and ζ at high levels (Johnson *et al*, 1993), therefore it is possible that one, or all of these isoforms could be modulated by the level of oestrogen in the growth medium, as has already been demonstrated in the anterior pituitary (Maeda and Lloyd, 1993). It is also possible that other components of the intracellular signalling pathway, as well as PKC, are regulated by steroids. Previous studies have demonstrated that oestrogen is capable of increasing the apparent activity of phospholipases, for example the PLC activity in human endometrial fibroblasts was apparently enhanced by oestrogen in a $Ca^{2+}$-dependent manner (Imai *et al*, 1990). It has also been shown that the LHRH-stimulated release of AA from the anterior pituitary is enhanced in pro-oestrus, and that the LHRH-stimulated release of AA is higher in ovariectomized/oestradiol-17β-replaced rats,

compared with ovariectomized rats (Thomson *et al*, 1994). Furthermore the level of expression of a secretory form of $PLA_2$ appears to be regulated by oestrogen in the quail oviduct, this expression of $PLA_2$ also appears to correlate with the proliferation of oviduct cells (Fayard *et al*, 1994). The question of PLD expression being increased by prior exposure to oestrogen cannot be approached at the present time, due to the lack of mRNA and protein sequence information for mammalian PLD. An observation has also been made that a high dose of oestrogen (10 nM) can stimulate a rapid (maximal in 10 sec) activation of a *src*-type non-receptor tyrosine kinase in human breast cancer MCF-7 cells under conditions of oestradiol-stimulated cell growth, suggesting a possible non-genomic effect. This effect was also blocked by anti-oestrogens, suggesting that this was a specific effect (Migliaccio *et al*, 1993).

It is clearly not possible to assess from the results presented here the mechanism by which oestrogen enhances the LHRH-stimulated PLD activity, or which components of the signalling pathway it is affecting. Studying the level of expression of components of the signalling pathway as a result of steroid removal might provide some insights into the mechanism involved. However, if the effect of oestrogen is the same as that observed in most other systems, it is likely to be a consequence of increased expression of critical proteins. It is a possibility that the level of PLD expression, or components of its regulatory mechanism, are modulated with respect to the concentration of circulating oestrogens on different days of the oestrous cycle. It would therefore be possible that an increased level of LHRH-stimulated PLD activity might play a role in altering the level of gonadotroph responsiveness on different days of the oestrous cycle, with the highest level in pro-oestrus, when LHRH priming occurs and the level of cell responsiveness is at its highest. If this theory is correct,

oestrogen would most likely have a long term genomic effect. We have attempted to determine whether PLD activity in the anterior pituitary, labelled in vitro with [$^3$H] palmitate for 2 hrs before being stimulated for 30 min with LHRH (100 nM), is higher on any particular day of the oestrous cycle in female COB wistar rats. However, the high degree of variability in the LHRH-stimulated PLD activity between animals made it impossible for any conclusions to be drawn (results not shown).

In order to try and assess whether PA or DAG produced by the action of PLD on PC are important for the initial or primed release of LH from the anterior pituitary in response to LHRH, high concentrations of butan-1-ol were used in order to prevent bioactive PA being formed due to the formation of PtdBut by a transphosphatidylation reaction (Dawson, 1967). However it appears that butanol affected both the initial and the primed release of LH stimulated by LHRH (Figure 7.3). It may be that PLD is important in the LHRH-stimulated release of LH from pituitary gonadotrophs, since it has already been postulated that PLD may play a role in regulating vesicle fusion, possibly by creating an area of charged phospholipids on the inner surface of the cell membrane and enabling vesicle fusion (reviewed in Kahn *et al*, 1993). It is also possible that the high concentration of butanol is affecting the cell membranes or cytoskeleton, thus attenuating the LHRH-stimulated release of LH. Demethoxyviridin, a derivative of the fungal metabolite wortmannin, which has been reported to inhibit PLD activity in the human neutrophil at a site between the activated receptor and PLD (Bonser *et al*, 1991), inhibited both the initial and primed LHRH-induced release of LH from the anterior pituitary, without affecting the basal release. This is similar to the effect of butanol on LHRH-induced LH release, further implicating PLD in the regulated release of LH from pituitary gonadotrophs, but not specifically in

the primed release of LH, as has been demonstrated with $PLA_2$ (Thomson *et al*, 1994), and to certain extent PLC (Mitchell *et al*, 1988).

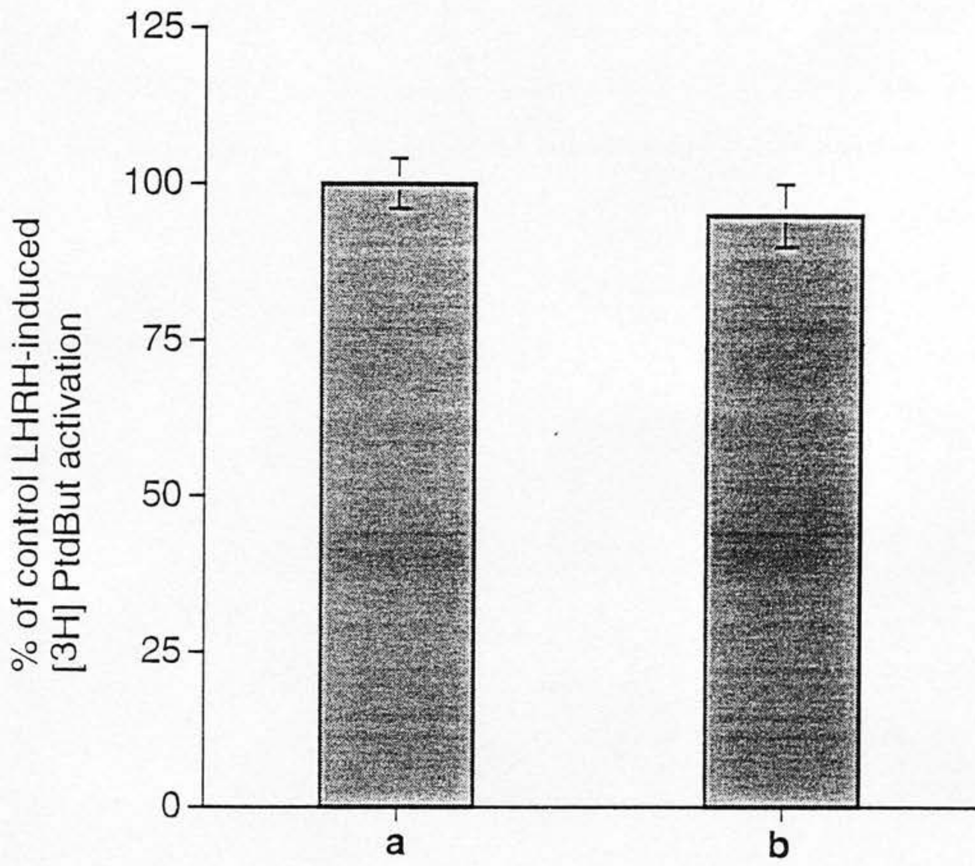
So in conclusion, it would appear that under the conditions investigated, the LHRH-stimulated activation of PLD was not enhanced by a previous LHRH stimulus in $\alpha$T3-1 cells. However, LHRH-stimulated PLD activity was reduced in $\alpha$T3-1 cells grown in the absence of steroid hormones, and readdition of oestrogen resulted in a recovery of the LHRH-stimulated PLD response. Although the mechanism by which oestrogen modulates PLD activity is not known, it is possible that the level of expression of components of the signalling machinery, such as PKC or PLD are regulated by oestrogen. It is also possible that oestrogen-enhanced expression of key signalling proteins plays an important role in the increased level of responsiveness observed in the LHRH priming effect in pro-oestrus pituitaries (Fink, 1988). It also appears that PLD is important in the regulated release of LH from pituitary gonadotrophes, since both high levels of butanol and DMV are able to attenuate the initial and primed release of LH from hemisected pituitaries *in vitro*. It may therefore be that PLD is required as part of regulated secretory mechanism of pituitary gonadotrophs, and that the LHRH-stimulated activation of PLD can be augmented by high circulating levels of oestrogen at pro-oestrous as part of a general priming of the regulated secretory mechanism.

**Figure 7.1**

**LHRH-stimulated activation of PLD in αT3-1 cells following an LHRH prestimulus**

10 nM LHRH-stimulated activation of PLD over 30 min in αT3-1 cells pretreated with LHRH (10 nM) for 15 min, 1 hr prior to the assay **(a)**. This is compared to αT3-1 cells which did not receive a prior LHRH stimulus, but were stimulated with LHRH (10 nM) for 30 min **(b)**. Values are expressed as a % of the PLD activity in cells which were not prestimulated, with the basal level of [$^3$H] PtdBut subtracted, and are means ± SEM from 4-6 separate determinations. The basal level of [$^3$H] PtdBut accumulation in these experiments was approximately 2000 DPM, while in cells stimulated with LHRH (10 nM) for 30 min it was 20000-30000 DPM.

**Figure 7.1**



## Figure 7.2

**Effect of steroid hormones on LHRH-stimulated activation of PLD in αT3-1 cells**

LHRH- (100 nM) stimulated activation of PLD in αT3-1 cells (over 30 min) grown in the absence of steroid hormones, or with the readdition of oestrogen (1 nM) 16 hrs prior to the assay, progesterone (200 nM) 3 hrs prior to the assay, or both oestrogen (1 nM) and progesterone (200 nM). The LHRH (100 nM) stimulated PLD activity in αT3-1 cells grown in normal growth medium (section 2.3) is also shown. All results are expressed as a % of the LHRH-stimulated PLD activity in αT3-1 cells grown in the absence of steroid hormones, and are means ± SEM of 4-6 separate determinations (*$p<0.05$). The basal levels of PLD were constant for all of the conditions used. The basal level of [$^{3}$H] PtdBut accumulation in these experiments was approximately 2000 DPM, while in cells stimulated with LHRH (100 nM) for 30 min it was 60000-70000 DPM.

**Figure 7.2**

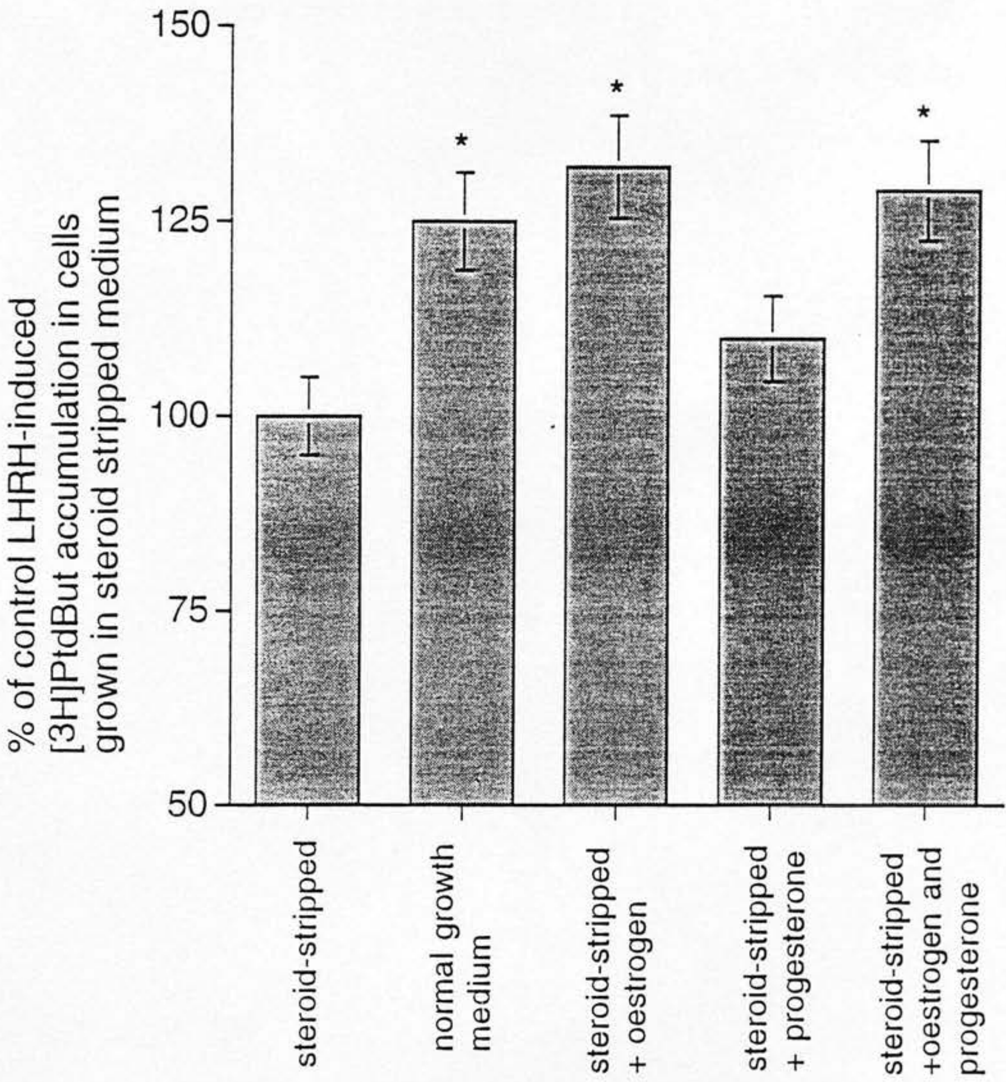
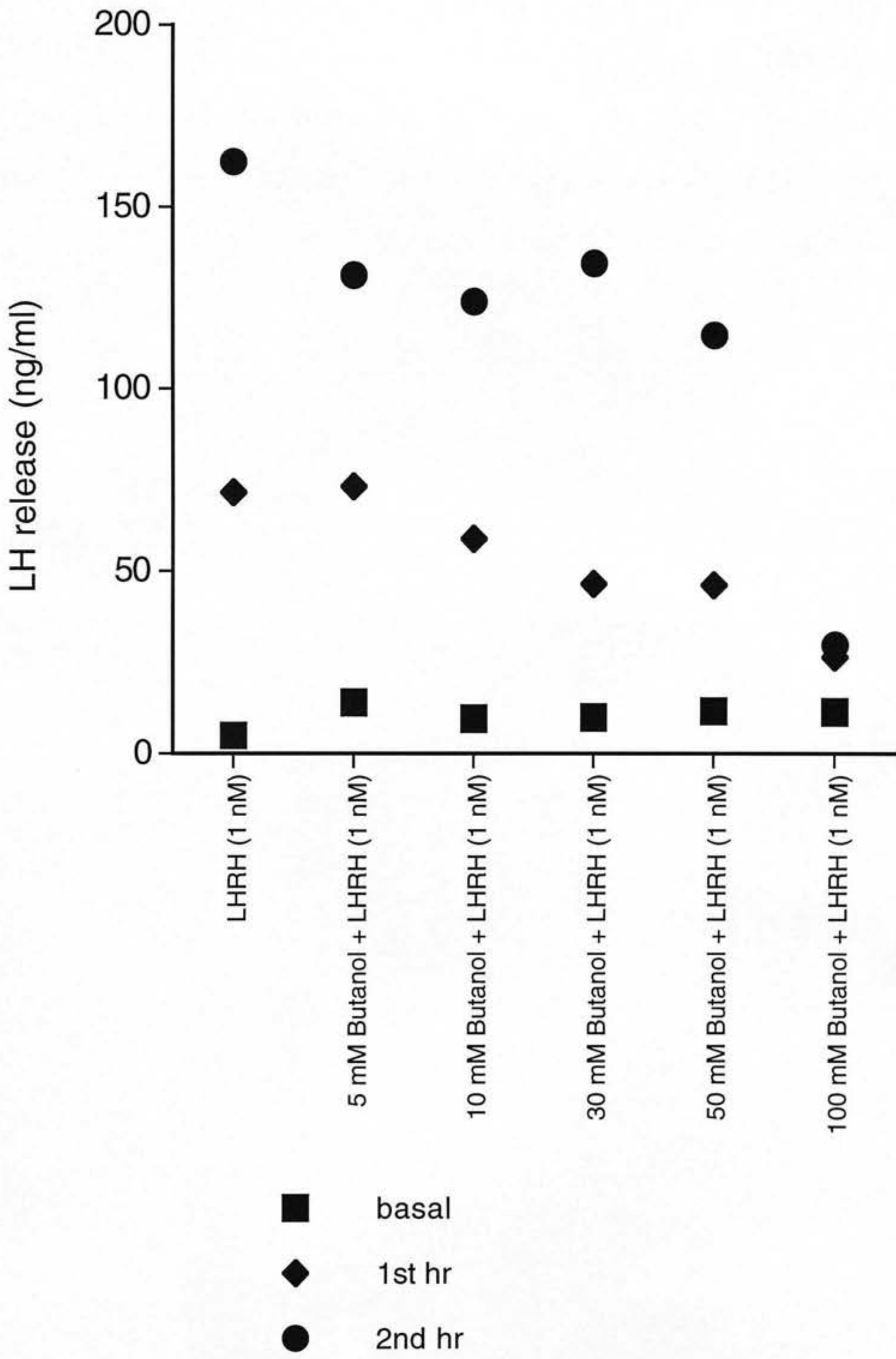


Figure 7.3

**The effect of butan-1-ol on LHRH-induced LH release from pro-oestrous rat hemipituitaries**

Control treatments were incubated for consecutive hours in the presence of medium only (basal), followed by consecutive periods (1st and 2nd hr) in the presence of LHRH (1 nM). Different concentrations of butan-1-ol were used in order to assess the effect on the basal, 1st and 2nd hours of LH release. Points are the average release from 2 determinations, which varied by less than 10 %.

**Figure 7.3**

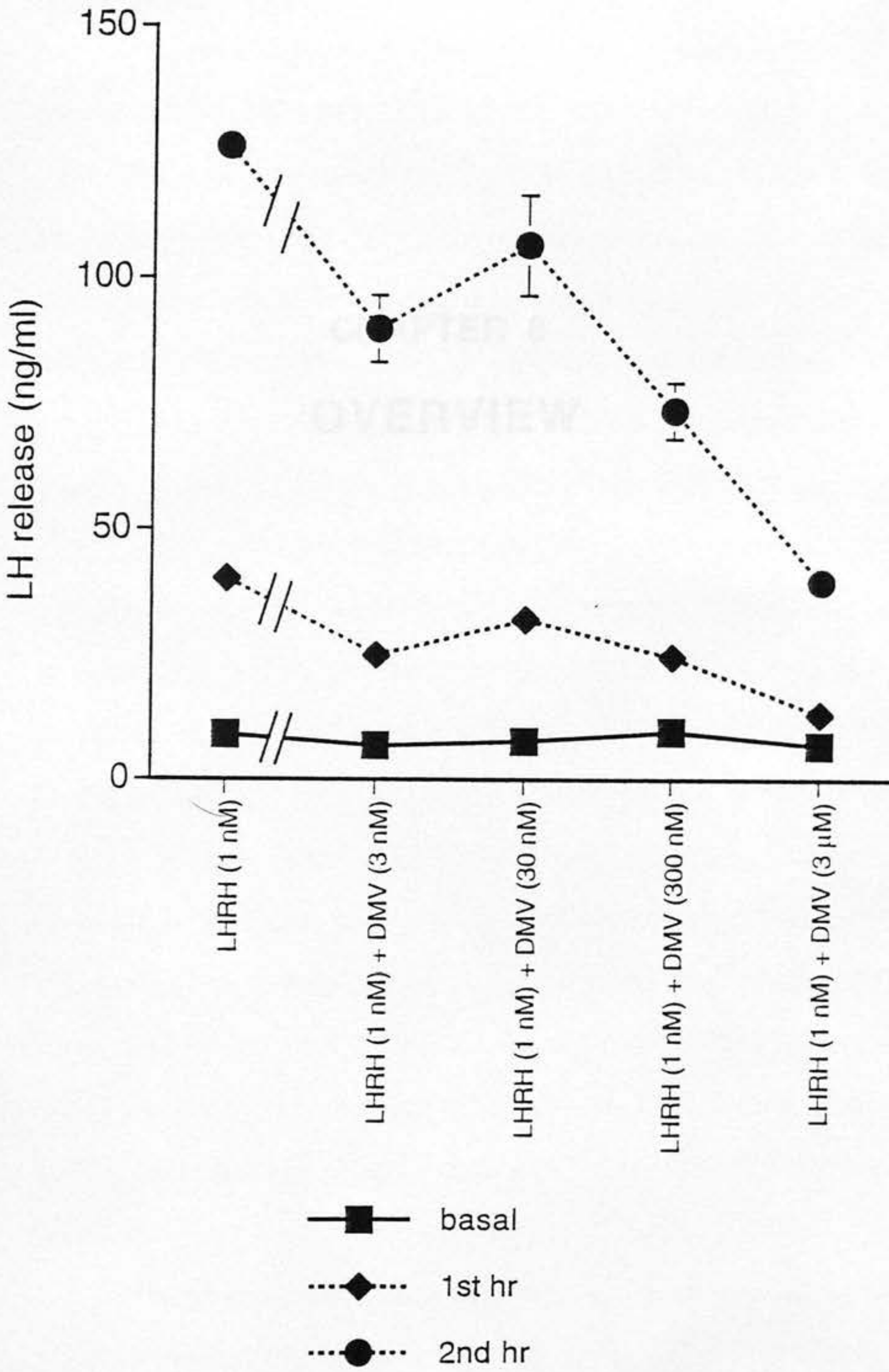


## Figure 7.4

**The effect of demethoxyviridin (DMV) on LHRH-induced LH release from pro-oestrous rat hemipituitaries**

Control treatments were incubated for consecutive hours in the presence of medium only (basal) followed by consecutive periods (1st and 2nd hr) in the presence of LHRH (1 nM). A concentration range of DMV was used in order to assess the effect on the basal, 1st and 2nd hours of LH release. Data are means ± SEM for 4 determinations.

**Figure 7.4**



# CHAPTER 8

# OVERVIEW

The present studies show that the activation of PLD as a result of LHRH receptor-stimulation in αT3-1 cells proceeds after a delay of 5-10 min, and does not desensitise within 40 min. It was also found that the PDBu-stimulated activation of PLD proceeded after a similar delay, but then appeared to desensitise over a period of 10-15 min.

The results presented provide strong evidence for an obligatory involvement of PKC in the LHRH-stimulated activation of PLD in αT3-1 cells, with a slightly lower sensitivity to bisindolylmaleimide PKC inhibitors than seen for the inhibition of the PDBu-stimulated PLD activity in the same cell type, indicating the possible involvement of a phorbol-insensitive isoform of PKC, such as PKC ζ. This does not signify that PKC necessarily brings about PLD activation through a direct phosphorylation, indeed it is likely that there are other components to the activation process downstream of PKC. It is apparent that the LHRH receptor is coupled to the downstream signalling machinery, leading to PLD activation, through a pertussis toxin insensitive G protein, possibly a member of the Gq family of heterotrimeric G proteins (Milligan, 1988). It is also possible that a low molecular weight G protein, such as ARF may also be involved in the LHRH-mediated activation of PLD, since an inhibitor of the ARF guanine nucleotide exchange factor, brefeldin A, was able to attenuate LHRH-stimulated PLD activity. It is interesting that additivity was observed in the PLD responses when stimulated by both LHRH and PDBu, suggesting that these two stimuli are able to activate PLD through different pathways, further implicating a PKC isoform which is not phorbol ester-activated in the LHRH-stimulated pathway.

There appears to be an involvement of non-receptor tyrosine kinases in the activation of PLD stimulated by LHRH, since this response was potently blocked by inhibitors of protein-tyrosine kinases. In contrast PDBu-

stimulated PLD activity was not blocked by tyrosine kinase inhibitors at equivalent low concentrations. There was no apparent inhibition of the pervanadate-stimulated PLD activity by selective inhibitors of PKC; a possible explanation being that a non-receptor tyrosine kinase is involved downstream of PKC, and that PKC is able to bring about the activation of the tyrosine kinase, as has been proposed in the case in thrombin-stimulated human platelets, where PKC phosphorylation of c-*src* has been proposed to increase the substrate affinity of c-*src* (Liebenhoff *et al*, 1993). The fact that PKC inhibitors and the tyrosine kinase inhibitors, genistein and piceatannol totally inhibited the LHRH-stimulated PLD activity does indicate, although not conclusively, that tyrosine kinases and PKC are involved in the same pathway, with the non-receptor tyrosine kinase being downstream of PKC. It is possible that a low molecular weight G-protein might also be activated by non-receptor tyrosine kinases in $\alpha$T3-1 cells, as this has been previously demonstrated in other systems, possibly through the modulation of activating or inhibitory proteins (Downard, 1992; Manser *et al*, 1993, Gulbins *et al*, 1993). Low molecular weight G proteins have also been shown to be closely involved in the activation of PLD (Cockroft *et al*, 1994; Brown *et al*, 1993; Bowman *et al*, 1993), as well as possibly being involved in the activation of PLD in LHRH-stimulated $\alpha$T3-1 cells. Therefore it is possible that in $\alpha$T3-1 cells stimulated by LHRH, a phorbol ester-insensitive isoform of PKC is activated, which can then activate a non-receptor tyrosine kinase, which in turn activates a low molecular weight G protein, which then may be able to bring about the activation of PLD (Figure 8.1).

Both LHRH and PDBu appear to capable of inducing tyrosine phosphorylation in $\alpha$T3-1 cells, further indicative of there being a tyrosine kinase involved in LHRH-signalling mechanisms, downstream of PKC. It

was also demonstrated in this study that PDBu-stimulation of PLD activity was not attenuated by protein-tyrosine kinase inhibitors, in comparison to the LHRH-stimulated activation of PLD, suggesting that the PKC upstream of tyrosine kinase in the LHRH-stimulated pathway is not activated by phorbol esters. The major LHRH- and PDBu-induced tyrosine phosphorylations are seen on proteins of 70-80 kDa and 110-130 kDa. It has been demonstrated that the PDBu-induced tyrosine phosphorylations are almost totally blocked by the PKC inhibitor GF 109203X, while the tyrosine phosphorylation induced by LHRH can only be partially blocked by this PKC inhibitor (Johnson *et al*, 1994). This would suggest that not all the tyrosine phosphorylation induced by LHRH is dependent upon the activation of PKC. Raising the intracellular $Ca^{2+}$ concentration with the $Ca^{2+}$ ionophore ionomycin, reduced the level of tyrosine phosphorylation in $\alpha$T3-1 cells, probably through the activation of an unidentified $Ca^{2+}$-stimulated tyrosine phosphatase.

It was shown that there are two members of the *src*-family of non-receptor tyrosine kinases detectable by western blotting in $\alpha$T3-1 cells; *src* and *fyn*. Using immunoprecipitation to partially purify *fyn*, it was demonstrated that prior LHRH-stimulation increased the phosphorylation of an exogenous denatured enolase substrate. It is possible that *src* and *fyn* may be involved in the LHRH-stimulated activation of PLD, although we have not studied the activation of any other non-receptor tyrosine kinase by LHRH.

A natural reciprocal mutation appears to be present between the TM 2 and TM 7 domains of the LHRH receptor (compared to the majority of other G protein-coupled receptors). Residue Asp 318 in TM 7, which is replaced by an Asn in this position in most other G protein-coupled receptors (Probst *et al*, 1992), appears to be an important factor in the lack

of desensitisation of the LHRH-stimulated activation of PLD. Mutating Asp 318 to Asn and transient expression of this mutant cDNA in COS 7 cells, resulted in the LHRH-stimulated PLD activity having a higher initial rate, followed by complete desensitisation within 5 min. A double mutation of Asp 318 to Asn and Asn 87 to Asp in TM 2, which is reported to interact though hydrogen bonding with Asp 318 in TM 7 (Zhou *et al*, 1994), had the same result as the single mutation. It therefore appears that Asp 318 in TM 7 is an important factor in the lack of desensitisation observed in the LHRH-stimulated activation of PLD, although the mutation of Asp 318 to Asn did not effect the non-desensitising hydrolysis of inositol phospholipids in the same cell type (R. Mitchell, unpublished observation).

Protein kinase C appears to be involved in the LHRH-stimulated activation of PLD in CHO cells stably transfected with the wild type LHRH receptor, with a similar sensitivity to Ro 31-8220 as was observed for the LHRH-stimulated activation of PLD in αT3-1 cells. The wild type LHRH receptor stimulated activation of PLD in CHO cells was unaffected by pertussis toxin, or by tyrosine kinase inhibitors, suggesting that tyrosine phosphorylation is not important for the activation of PLD by this route in CHO cells.

It would appear that LHRH-stimulated PLD activity cannot be enhanced by a previous LHRH stimulus in αT3-1 cells, as is the case for the primed release of LH from the anterior pituitary (Aiyer *et al*, 1974b). However it was demonstrated that LHRH-stimulated PLD activity was reduced in αT3-1 cells grown in the absence of steroid hormones, and that the re-addition of oestrogen brought about a recovery in the LHRH-stimulated PLD response. Although the mechanism by which oestrogen modulates PLD activity is not known, it is possible that the level of expression of components of the signalling machinery, such as PKC, or

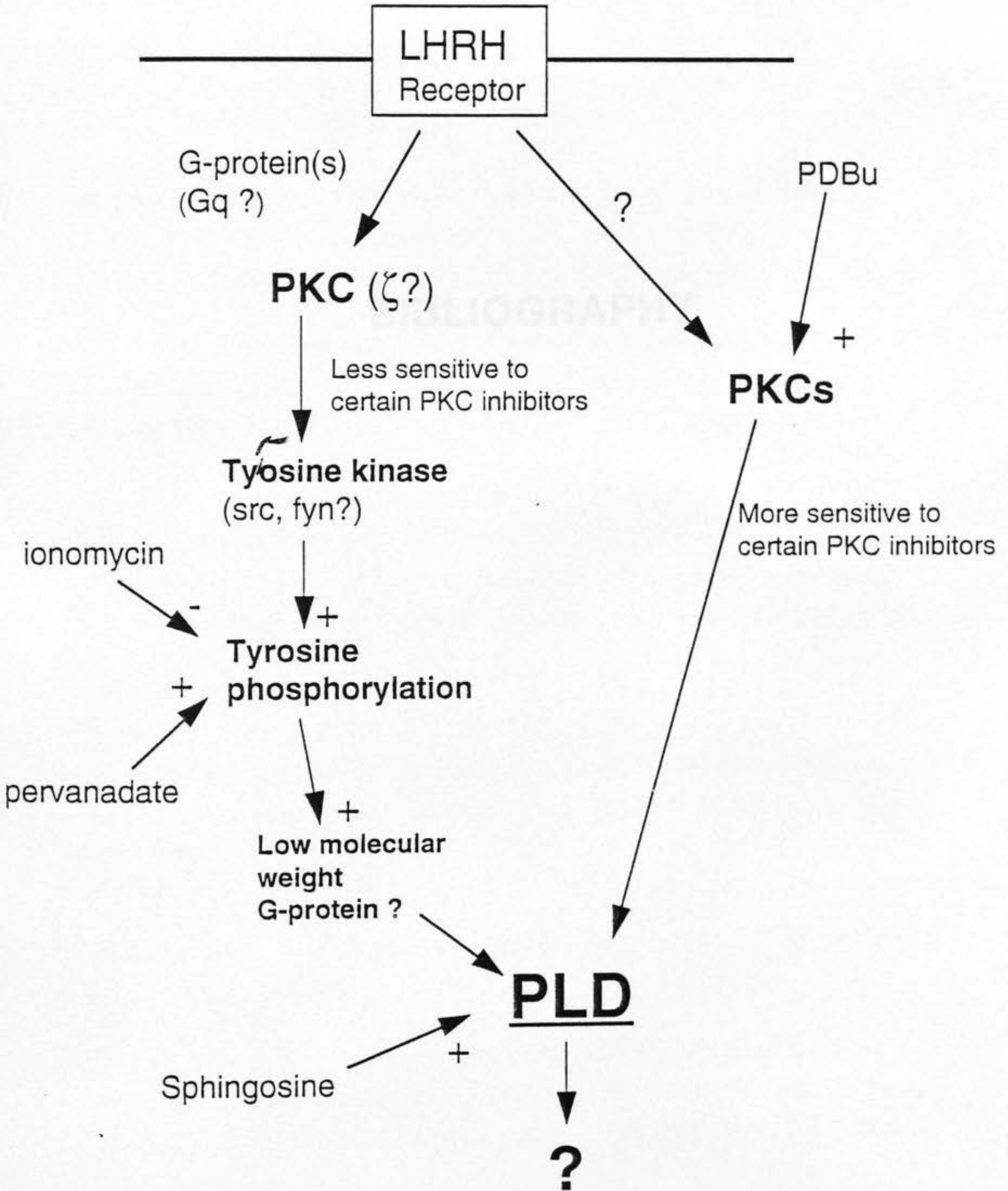
PLD itself are regulated by oestrogen. It may be that oestrogen-enhanced expression of components involved in the activation of PLD plays an important role in the increased level of responsiveness observed in the LHRH priming effect in pro-oestrous pituitaries, which appears to be dependent on an increase in the level of circulating oestrogen prior to the surge in LH release (Fink, 1988). Phospholipase D does not appear to be specifically involved in the primed release of LH from the anterior pituitary, since both butanol and DMV were able to inhibit the initial and primed release of LH in response to LHRH *in vitro.*

The present study has examined the mechanism of PLD activation via the LHRH, G protein-coupled receptor, in a cell line derived from pituitary gonadotrophs. The exact physiological role of PLD in this and similar systems will be difficult to asertain until PLD has been characterised at molecular level, and the development of selective pharmacological and molecular probes has occured.

Figure 8.1

**Overview of the probable mechanism of activation of PLD by LHRH in the αT3-1 cell line**

**Figure 8.1**



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# Dual involvement of protein kinase C and tyrosine phosphorylation in LHRH-induced activation of phospholipase D in αT3-1 cells

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SHORT TITLE: Protein kinase C and tyrosine kinases in phospholipase D activation

## SYNOPSIS

The mechanisms of phospholipase D (PLD) activation by the luteinising hormone-releasing hormone (LHRH) receptor were investigated in the αT3-1 gonadotroph cell line. After a 5-10 min delay, LHRH-induced activation of PLD was detectable and continued unattenuated for up to 40 min. Phospholipase D activation by 4β phorbol-12,13-dibutyrate (PDBu) also appeared only after a lag, but thereafter was attenuated within 10 min. LHRH- and PDBu-induced PLD activation was inhibited by down-regulation of protein kinase C (PKC) and by PKC inhibitors. However, Ro 31-8220, GF 109203X and H7 showed lower potency on LHRH than on PDBu responses. Effects of the tyrosine kinase inhibitors lavendustin A, genistein and piceatannol implicated tyrosine phosphorylation in the PLD response to LHRH but not PDBu. Furthermore, the tyrosine phosphatase inhibitor pervanadate increased PLD activity. This response was, however, unaffected by Ro 31-8220 (suggesting that PKC was not downstream of tyrosine phosphorylation in the mechanism), but was attenuated by ionomycin. Anti-phosphotyrosine immunoblots revealed both LHRH- and PDBu-induced tyrosine phosphorylation of a number of proteins, with bands at around 76 and 128 kDa being particularly prominent. Ionomycin reduced the level of tyrosine phosphorylation below that seen in unstimulated cells. LHRH-induced activation of PLD in αT3-1 cells thus appears to involve both tyrosine kinases and PKC; distinct isoforms of PKC acting at separate sites may contribute to receptor-induced PLD activation.

## INTRODUCTION

Phospholipase D (PLD) is a phospholipid-hydrolysing enzyme (EC 3.1.4.4.) whose main substrate is phosphatidylcholine (PtdCho),the principal phospholipid in mammalian cells[1, 2, 3, 4]. The products of the action of PLD on PtdCho are phosphatidic acid (PtdOH) and choline[4]. Phosphatidic acid can be further hydrolysed to diacylglycerol (DAG) by the enzyme phosphatidate phosphohydrolase (EC 3.1.3.4.) [2].

Diacylglycerol (DAG) derived from the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) by phospholipase C (PLC) can lead to the activation of protein kinase C (PKC) [4, 5, 6], although it is not entirely certain that DAG resulting from PLD-mediated hydrolysis of PtdCho is similarly effective. Nevertheless it has been suggested that PLD-generated DAG may be responsible for a sustained activation of PKC, since the kinetics of such DAG accumulation are prolonged, compared to DAG generated from $PtdInsP_2$ by the action of PLC [4]. Since the consensus composition of the fatty acid side chains is different in DAG derived from PtdCho compared to $PtdInsP_2$ [7] this may conceivably influence its effectiveness at activating PKC, perhaps making it more selective for certain PKC isoforms.

There is evidence from several cell types that G-proteins [4, 8], PKC [4, 8, 9], $Ca^{2+}$ [4, 10, 11] and tyrosine phosphorylation [4, 10, 12, 13] can be involved in the receptor-linked activation of PLD. This may indicate a general versatility of the enzyme, in being able to be activated by a large number of biochemical signalling pathways, or it is possible that multiple PLD isoforms exist, each activated by a different pathway.

In the present study we have investigated the activation of PLD in the $\alpha$T3-1 cell line, which is a clonal pituitary gonadotroph cell line that was obtained by targeted oncogenesis in transgenic mice [14]. By assessing the generation of the stable product, phosphatidylbutanol (PtdBut), that is produced by PLD in the presence of butan-1-ol, we have been able to study the kinetics of PLD activation by luteinising hormone-releasing hormone (LHRH) and the effect of various pharmacological agents on the activation process. Previous studies have established clearly that LHRH can activate PLC-mediated phosphoinositide hydrolysis and mobilise calcium from intracellular and extracellular locations [15, 16], whilst there is now increasing evidence that activation of both phospholipase $A_2$ ($PLA_2$) and phospholipase D

(PLD) mediated pathways can also occur in pituitary cells as a consequence of LHRH stimulation [9, 17].

## EXPERIMENTAL

### Materials

[9,10-$^3$H]-Palmitate (60 Ci/ mmol) was obtained from NEN Du Pont de Nemours, Germany. Ro 31-8220 was a gift from Roche Products Ltd, Welwyn, Herts, UK. Ionomycin, 4β-phorbol 12,13-dibutyrate (PDBu), LHRH and the LHRH antagonist ([Ac-D-p-Cl-Phe$^{1,2}$, D-Trp$^3$,D-Arg$^6$,D-Ala$^{10}$]-LHRH) were obtained from Sigma Chemical Co, Poole, Dorset, UK. The LHRH receptor agonist, buserelin, ([D-Ser(tBu)$^6$, DesGly$^{10}$]-LHRH ethylamide) was obtained from Hoechst, Milton Keynes, Bucks. Lavendustin A, 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine (H7), genistein and GF 109203X were obtained from Calbiochem-Novabiochem, Nottingham, UK. Piceatannol was obtained from Boehringer-Mannheim UK, Lewes, East Sussex. All other laboratory reagents were Analar grade from BDH, Merck Ltd, Poole, Dorset, UK.

### Cell Culture

The αT3-1 cells were cultured in Dulbecco's modified Eagle medium with 0.11 g/l Na pyruvate (pyruvate-DMEM; Gibco BRL, Paisley, Scotland, UK), containing 10% foetal calf serum, penicillin/ streptomycin (100 U/ ml each) and 2 mM L-glutamine [14]. The cells were quiesced in serum-free pyruvate-DMEM for approximately 24 hours prior to the experiments. Phospholipase D assays were carried out in 12 well tissue culture plates (Costar).

### Assay of Phospholipase D Activity

In order to label the phosphatidyl moiety of PtdCho, cells were incubated for 2 h with [$^3$H] palmitate (5 μCi/ ml, 1 ml in each well) in minimal essential medium (MEM; Gibco BRL), at 37°C under 5% $CO_2$ /95% $O_2$. Cells were washed twice with warm MEM containing 1% fatty acid free BSA (Sigma), then 1 ml warm MEM containing 0.5% fatty acid-free BSA was added to each well. Butan-1-ol was added to each to give a concentration of 30 mM (since previous experiments in this and other laboratories have shown that this concentration of butan-1-ol provides optimal production of [$^3$H]PtdBut, without adverse effects on cell viability

as assessed by trypan blue exclusion [18]). Various inhibitory drugs were added, immediately before adding the stimulating agent which was present for 30 min, unless otherwise stated. Reactions were terminated by removal of the 1 ml of medium and addition of 0.5 ml ice-cold methanol. Cells were then scraped out of wells and transferred to a 2 ml screw-top glass vial (Chromacol Ltd, Mundells Industrial Centre, Welwyn Garden City, Herts, UK). Chloroform was added to give a chloroform/ methanol ratio of 1:1. Tubes were mixed and left for 15 min, before distilled water was added to give a chloroform/ methanol/ water ratio of 1:1:0.8. The vials were shaken vigorously and centrifuged for 5 min to separate the two phases. The top aqueous phase was carefully aspirated and discarded while the lower organic phase was left in the vial and dried under vacuum in a centrifugal evaporator (Gyrovap; V.A. Howe, Banbury, UK) for approximately 50 min at 30°C. Once the lower organic phase had evaporated the residue was resuspended in 50 µl chloroform/ methanol (19:1) and then spotted onto Whatman LK5D thin layer chromatography plates with pre-scored lanes (silica 250 µm). The plates were developed using the organic phase of a mixture: ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100). In this system, PtdBut ($R_f \approx 0.3$) is well separated from PtdOH ($R_f = 0.1$), major phospholipids ($R_f = 0$) and neutral lipids ($R_f = 0.7$-$0.95$). The region containing PtdBut, and corresponding to a [$^{14}$C]PtdBut standard (gift from R. Randall, Wellcome Research Laboratories, Kent, UK) was scraped off the plate and counted by liquid scintillation counting.

**Preparation of Pervanadate**

Sodium orthovanadate was added to divalent cation-free Earle's balanced salt solution containing HEPES (30 mM) to a final concentration of 5 mM. The pH was adjusted to 7.4 with NaOH, then $H_2O_2$ (1 M) was added to give a concentration of 1 mM and the solution was then left for 15 min before 4 mg of catalase (Sigma) was added to remove excess $H_2O_2$. The solution remained clear throughout the above procedure.

**Antiphosphotyrosine Immunoblotting**

Confluent cultures of αT3-1 cells were incubated with the LHRH receptor agonist buserelin (100 nM; [D-Ser(tBu)$^6$, DesGly$^{10}$] LHRH ethylamide), 300 nM PDBu, 30 µM ionomycin or saline (control). Reactions were terminated after 20 min by scraping cells into a buffer

containing 20% (w/v) sodium dodecyl sulphate and 5% (v/v) 2-mercaptoethanol, then heating to 100°C for 5 min. Cellular proteins were separated on 7.5% homogeneous microgels which were run on a PhastSystem electrophoresis apparatus (Pharmacia, Milton Keynes, Bucks, UK), then electroblotted onto Immobilon-P membranes (Millipore, Watford, Herts, UK). The membranes were then probed with the 4G10 monoclonal antibody (Upstate Biotechnology Incorporated, TCS Biologicals, Botolph Claydon, Bucks, UK), which recognises phosphotyrosine, or with antibody blocked with 1 mM o-phospho-L-tyrosine (Sigma). An HRP-labelled secondary antibody (Scottish Antibody Production Unit, Carluke, Lanarks, UK) and enhanced chemiluminescence (ECL; Amersham International plc, Little Chalfont, Bucks, UK) were employed to visualise antibody binding.

**Data analysis**

All concentration-response curves were analysed by a non-linear, error-weighted direct fitting program, modelling on Hill kinetics (P.fit, Elsevier Biosoft, Cambridge).

## RESULTS

The production of [$^3$H]PtdBut in $\alpha$T3-1 cells incubated with LHRH for 30 min showed a marked increase over the very low levels in control cells and clear concentration dependence (Figure 1a). Under these conditions, the $EC_{50}$ for PLD activation by LHRH was 16 ± 5 nM and reached a maximum by approximately 100 nM. A concentration of 100 nM LHRH was therefore used in most of the other experiments in this study, in order to elicit a large (but not supra maximal) PLD response.

The time course (up to 40 min) of [$^3$H]PtdBut accumulation elicited by LHRH (100 nM) or PDBu (1 $\mu$M) is shown in Figure 1b. In the LHRH-stimulated cells there was a lag of between 5 and 10 min before [$^3$H]PtdBut production could be detected. The rate of [$^3$H]PtdBut accumulation appeared to be approximately constant between 5 and 40 min. In PDBu-stimulated cells there was an even longer lag of between 10 and 15 min before [$^3$H]PtdBut levels were detectable. The activation of PLD by PDBu appeared to occur at a rate comparable to the LHRH-stimulated activity only for around 10 min before the rate of [$^3$H]PtdBut accumulation declined. Thus at all times, especially the longer time points, PDBu (1 $\mu$M) caused a much lower level of [$^3$H]PtdBut accumulation than LHRH (100 nM).

The LHRH receptor antagonist [Ac-D-p-Cl-Phe$^{1,2}$,D-Trp$^{3}$,D-Arg$^{6}$,D-Ala$^{10}$]-LHRH (1 $\mu$M) [19], completely prevented activation of PLD by LHRH (100 nM); whilst the antagonist itself caused no detectable activation of PLD. The biologically-inactive phorbol ester isomer, 4$\alpha$-phorbol 12,13-dibutyrate (4$\alpha$-PDBu, 1 $\mu$M) [20] elicited no increase in PLD activity at a concentration of 1 $\mu$M. The $Ca^{2+}$ ionophore, ionomycin [21] (30 $\mu$M) caused no significant activation of PLD by itself, although it did slightly augment the PDBu-stimulated PLD activity (Table 1). A combination of LHRH (100 nM) and PDBu (1 $\mu$M) elicited approximately additive levels of [$^{3}$H]PtdBut production (Table 1), even though the large accumulation due to 100 nM LHRH was maximal on its own concentration-response curve. To address the possibility that the lag in [$^{3}$H]PtdBut production was due to a requirement for protein synthesis in the mechanism of PLD activation, experiments were carried out in the presence of cycloheximide (30 $\mu$M), but this clearly had no effect on LHRH-induced PLD activity (Table 1).

In order to assess the role of PKC in LHRH-induced PLD activation, we utilised two approaches; firstly employing a number of selective PKC inhibitors and secondly, down-regulating the PKCs in $\alpha$T3-1 cells by prolonged preincubation with a phorbol ester [22] prior to LHRH stimulation. Over a 30 min incubation with LHRH (100 nM), the selective bisindolylmaleimide PKC inhibitor Ro 31-8220 [23] completely inhibited the evoked PLD activity with an $IC_{50}$ of 460$\pm$180 nM (Figure 2a). In cells stimulated for 30 min with PDBu (1 $\mu$M), Ro 31-8220 again completely inhibited PLD activity but with an $IC_{50}$ of 62$\pm$30 nM (Figure 2b). Another highly-selective bisindolylmaleimide PKC inhibitor GF 109203X completely inhibited PLD activity stimulated by LHRH (100 nM), with an $IC_{50}$ of 1.0 $\pm$ 0.2 $\mu$M (Figure 2c), PDBu-induced PLD activity was more potently inhibited by GF 109203 X, with an $IC_{50}$ of 161 $\pm$ 19 nM (Figure 2d). This represents an approximately eight fold greater potency of Ro 31-8220 and GF 109203 X when PDBu was used as a stimulus instead of LHRH. A PKC inhibitor of another structural class, 1-(5-isoquinolinyl sulphonyl)-2-methyl-piperazine (H7) [24] inhibited LHRH induced PLD activation with an $IC_{50}$ of 232 $\pm$ 25 nM (Figure 2e). The slope factor of the concentration-inhibition curve (1.27 $\pm$ 0.15) was consistent with a single component to the inhibition. However in cells stimulated with PDBu (1 $\mu$M) (Figure 2f), H7 inhibited the response over a very wide concentration range (slope factor = 0.56 $\pm$ 0.05). This is consistent with multiple components being involved in the inhibition. The

concentration-inhibition curve gave a much better fit to a 2 component Hill equation than to a one site model with component parameters of $IC_{50}(i) = 12.5 \pm 1.9$ μM (45% of sites) and $IC_{50}(ii) = 635 \pm 85$ μM (55% of sites). Down-regulation of PKC isoforms over a 24 hour period using 300 nM PDBu resulted in a major attenuation of the LHRH- and PDBu- induced PLD activation (82 ± 9% and 72 ± 5%, n=4), no change was seen in the basal [$^3$H]PtdBut accumulation.

In order to assess whether tyrosine phosphorylation was involved in LHRH signalling and in PLD activation in this pathway, we used a number of compounds known to inhibit or enhance cellular tyrosine phosphorylation and employed anti-phosphotyrosine immunoblotting to determine tyrosine phosphorylation of cellular proteins in response to stimulation. Over a 30 min stimulation with LHRH (100 nM) the highly selective tyrosine kinase inhibitor lavendustin A [25] inhibited approximately 60% of the PLD response with an $IC_{50}$ of 133 ± 17 nM (Figure 3a). Even at concentrations up to 20 μM, lavendustin A caused no further inhibition of PLD. Lavendustin A had no effect on PLD activity elicited by 30 min stimulation with PDBu (1 μM) (Figure 3b). Another tyrosine kinase inhibitor, piceatannol [26] also inhibited LHRH-induced PLD activity with an $IC_{50}$ of 35 ± 5 μM (Figure 3c). Piceatannol also caused some inhibition, albeit with lower potency, of PDBu-stimulated PLD activity, with an $IC_{50}$ of 155 ± 50 μM (Figure 3d). A further tyrosine kinase inhibitor, genistein [27] inhibited the LHRH-induced PLD activity with an $IC_{50}$ of 92 ± 34 μM (Figure 3e), whilst causing only minor inhibition of PDBu-induced PLD activity (Figure 3f).

Pervanadate, the peroxide of the tyrosine phosphatase inhibitor vanadate, is reported to elevate levels of tyrosine phosphorylation, presumably by inhibiting tyrosine phosphatases and unlike vanadate is cell-permeable allowing it to be used in whole cell assays [28, 29]. Pervanadate (1 mM) was able to induce PLD activation over a 30 min period to a level similar to that evoked by PDBu (1 μM). Pervanadate and PDBu had an additive effect on [$^3$H]PtdBut accumulation, whereas ionomycin substantially blocked the PLD response elicited by pervanadate. The PKC inhibitor Ro 31-8220 (1 μM) was ineffective at blocking pervanadate-induced PLD activity (Table 1).

When protein-tyrosine phosphorylation in αT3-1 cells was investigated by immunoblotting with an antibody which recognised phosphotyrosine, the LHRH-receptor

agonist, buserelin ([D-Ser(tBu)$^6$,DesGly$^{10}$]-LHRH ethylamide), was observed to induce tyrosine phosphorylation on many proteins of molecular mass ~60 to >170 kDa, but especially notable was a broad band at around 128 kDa and a diffuse band at around 76 kDa (Figure 4) whose phosphorylations were maximal within 10 min. By varying exposure conditions during development of the immunoblot reaction, it could be seen that each of these bands consisted of at least 2 proteins. Similar phosphorylations of protein-tyrosine were also evident with PDBu (Figure 4), but 30 μM ionomycin reduced the level of phosphorylation below that observed in control cells, indicating that the effects of buserelin and PDBu were not mediated by alterations in intracellular $Ca^{2+}$.

**DISCUSSION**

The results of the present study indicate that LHRH brings about the activation of PLD in αT3-1 cells by a process involving both PKC and a tyrosine kinase, that does not require protein synthesis, and cannot be mimicked to any significant degree simply by elevation of cytosolic $Ca^{2+}$ levels.

Despite being clearly coupled via G-proteins to phosphoinositide hydrolysis, the LHRH receptor is an atypical member of the family of such receptors since it lacks an extended C-terminus [30]. There is evidence that phosphorylation of the C-terminal tail of several G-protein linked receptors contributes importantly to receptor desensitisation [31]. Therefore the lack of a C-terminal tail on the LHRH receptor may be an important factor in the complete lack of desensitisation seen in the PLD response to LHRH over the 40 min time course used in this study (Figure 1b).

LHRH-induced PLD activation proceeded after a lag of about 5 min (Figure 1b). This result is similar to that of a previous study carried out on αT3-1 cells, in which it was proposed that PLD activation required a particularly high receptor occupancy; dictating a lag before PLD activity could be detected [32]. However our observation of an even more pronounced lag in activation by PDBu suggests that the delay is mainly due to some factor other than the rate of receptor occupation by an agonist. Our experiments with cycloheximide indicate that the lag in activation of PLD by LHRH is not due to the synthesis of an intermediary activating protein.

It is unclear why the mechanism through which PDBu causes activation of PLD becomes so markedly attenuated after 10 min of activity, although it is known that even quite short incubations of cells (including $\alpha$T3-1 cells) with high concentrations of phorbol esters lead to down-regulation of many PKC isoforms [33], and as has been previously shown, phorbol esters will have a more profound down-regulatory effect than agonists of cell surface receptors [34]. The delay before PDBu caused PLD activation compared to LHRH is also unexplained. It is of course possible that PDBu is activating isoforms of PKC not normally involved in the LHRH receptor activation of PLD, or that a different isoform of PLD may be activated through the PDBu-stimulated pathway. The additive effect of LHRH and PDBu on the PLD response supports the theory that these two stimuli do use different biochemical pathways, at least to some degree, in order to bring about the activation of PLD.

Increasing the concentration of intracellular calcium with ionomycin was not sufficient by itself to activate PLD. Furthermore ionomycin was not able to potentiate to any great extent the PLD response elicited by PDBu. Both $\alpha$ and $\beta_1$ isoforms of PKC have been implicated in PLD activation in different systems [35, 36, 37], so it is entirely possible that they are also involved here, as LHRH receptor activation increases intracellular calcium levels within the cells [16, 38] which could contribute to activation of such $Ca^{2+}$-dependent PKC isoforms. Some studies on PLD have concluded that $Ca^{2+}$ is essential for PLD activation [10], but interestingly a recent report has described inhibition by $Ca^{2+}$ of guanosine 5'-0-(3-thiotriphosphate)-induced PLD activation in permeabilised NG 108-15 neuroblastoma X glioma cells [39].

The present results provide strong evidence for an obligatory role of PKC in LHRH-induced activation of PLD, since this response was greatly attenuated by PKC down-regulation and fully blocked by selective PKC inhibitors. The bisindolylmaleimide PKC inhibitor Ro 31-8220 was about eight times more potent against PDBu-induced PLD activation than against LHRH-induced PLD activation. Similar results have been reported for bombesin-induced PLD activation in Swiss 3T3 cells [9]. Since all phorbol ester-sensitive PKC isoforms are likely to be activated by the relatively high concentration of PDBu used (1 µM), it is possible that a form of PKC relatively resistant to Ro 31-8220 may be activated in response to LHRH, compared to the consensus of those activated by PDBu. It has been

reported that a bisindolylmaleimide related to Ro 31-8220 shows selectivity for the $Ca^{2+}$-dependent PKCs compared to PKC ε [40]. We have shown previously that αT3-1 cells express α, ε and ζ isoforms at high levels [41]. Evidence has been provided both for and against a role for PKC ε in receptor-evoked PLD activation [42, 43] Interestingly, the atypical $Ca^{2+}$-independent isoform, PKC ζ does not have a phorbol ester binding domain; and is not activated by phorbol esters , it also shows a relative resistance to bisindolylmaleimides and related indolocarbazoles compared to other PKC isoforms [44]. Whilst the relatively high potency of Ro 31-8220 and GF 109203X on LHRH responses does suggest that some form of PKC is involved, it is of course possible that an unknown kinase (other than a PKC isoform) with some sensitivity to bisindolylmaleimides is mediating LHRH responses.

Another PKC inhibitor H7 also inhibited both the LHRH and PDBu-induced increases in PLD activity. LHRH-induced PLD activity was inhibited in a monophasic fashion, suggesting either that only one PKC isoform is involved, or that all the ones that are involved have the same low sensitivity to H7. Compared to the potency of inhibition of various PKC isoforms by H7 in cell-free assays, the LHRH-induced PLD activity and one of the two components to PDBu-induced PLD activity were insensitive to H7; in particular PKCs α–γ and ε are known to be sensitive to inhibition by H7 [45]. It has been reported that there is a distinct H7-resistant form of PKC in the rat anterior pituitary gland, that may represent either a novel or modified PKC isoform, although it is clear that this species is phorbol ester-activated and can be clearly separated from PKC-ζ by ion-exchange and hydroxyapatite chromatography [46]. It is a possibility that this species of PKC is involved in the pathway of PLD activation by LHRH. There appear to be multiple components to the inhibition by H7 of PDBu-evoked PLD activity. Interestingly this suggests that PLD activation by stimulation with phorbol esters involves more than one isoform of PKC, and whilst it has already been shown using overexpression studies that the $Ca^{2+}$-dependent PKC isoforms α and β1 are potentially involved in PLD activation [36, 37] it now seems likely that further species of PKC can also contribute.

As well as PKC, our results strongly suggest the involvement of tyrosine kinases in the activation of PLD by LHRH. Similar results indicating the concurrent involvement of PKC and tyrosine kinases in receptor activation of PLD have recently been reported in the response to

endothelin-1 of A10 vascular smooth muscle cells [13]. In our experiments lavendustin A was able to inhibit with high potency the majority of the LHRH-induced PLD response, leaving a residual PLD activity that was unaffected by further elevating the lavendustin A concentration. The portion of the LHRH-induced response insensitive to lavendustin A may represent a separate pathway of PLD activation. Lavendustin A had no effect on the PDBu-induced PLD activity; raising the possibility that PDBu utilises the same pathway as the lavendustin A-insensitive portion of the LHRH response. However both LHRH and PDBu responses were fully sensitive to PKC inhibitors; consistent with the idea that the lavendustin A-sensitive, presumably PDBu-insensitive component of LHRH action may be mediated by a PDBu-insensitive PKC isoform, such as $\zeta$. In a mixed micelle assay of PKC activity on isoforms from midbrain cytosol, there was no evidence that lavendustin A (10 $\mu$M) could inhibit either PDBu-evoked or basal activity which appears to include PKC $\zeta$ under these conditions [41; our own unpublished observations]. Another tyrosine kinase inhibitor, piceatannol was able to inhibit completely the LHRH-evoked PLD activity, but also affected PDBu-induced PLD activity at a much higher concentration, so its additional effect may therefore be non-specific. The tyrosine kinase inhibitor genistein also displayed greater than 10 fold selectivity towards LHRH-induced PLD activation, compared to the PDBu-induced response, where only minor inhibition was observed at the highest concentration tested.

The peroxides of vanadate have been shown to be potent enhancers of tyrosine phosphorylation in intact cells, and in this study pervanadate (1 mM) caused marked stimulation of PLD activity, supporting the evidence that tyrosine phosphorylation participates in the pathway of PLD activation in $\alpha$T3-1 cells. Pervanadate-induced PLD activation was unaffected by the PKC inhibitor Ro 31-8220, making it unlikely that the tyrosine kinase step can be upstream of PKC in the activation pathway of PLD activation. The additive effect on [$^3$H]PtdBut accumulation of co-stimulation with pervanadate and PDBu points towards a necessity for both a tyrosine kinase and PKC for maximal activation of PLD. Interestingly, ionomycin drastically reduced the PLD activation elicited by pervanadate, as indeed it prevented LHRH agonist-induced tyrosine phosphorylation of $\alpha$T3-1 cell proteins (Figure 4), consistent with the $Ca^{2+}$-induced activation of tyrosine phosphatases. The effects of $Ca^{2+}$ on tyrosine phosphorylation may depend on cell type since an increase in intracellular $Ca^{2+}$ has

been reported to increase tyrosine kinase activity in angiotensin-II stimulation of GN4 liver epithelial cells [47], whereas in Swiss 3T3 cells depletion of intracellular $Ca^{2+}$ did not affect lysophosphatidic acid (LPA)-induced tyrosine phosphorylation [48]. In contrast, PKC rather than $Ca^{2+}$ appears to be a necessary (though not solely sufficient) intermediary in endothelin-1 stimulated tyrosine phosphorylation and mitogenesis in kidney glomerular mesangial cells [49]. Interestingly, elevation of $Ca^{2+}$ levels in epidermal keratinocytes leads to activation of c-*src* through a mechanism involving tyrosine dephosphorylation (consistent with $Ca^{2+}$-activation of a tyrosine phosphatase) but leads concomitantly to the inactivation of the related c-*yes* tyrosine kinase activity [50].

Since many of the tyrosine phosphorylations induced here by LHRH could also be elicited by PDBu (Figure 4), it is possible (if there is only one tyrosine kinase step involved) that LHRH and PDBu can both activate this component. The protein-tyrosine phosphorylations induced in αT3-1 cells by the LHRH-receptor agonist, buserelin, appear remarkably similar to those induced by lysophosphatidic acid (LPA), bombesin and endothelin in Swiss 3T3 cells and in Rat-1 fibroblasts, with proteins of 70-80 kDa and 110-130 kDa being the main phosphorylation targets [48, 51, 52]. In each of the above cases, as well as in the present study, the administration of a phorbol ester appears to induce protein-tyrosine phosphorylations similar to those observed with the ligands. In Swiss 3T3 cells however, it seems unlikely that any typical PKC directly mediates the receptor-induced tyrosine phosphorylation since neither prolonged exposure to phorbol ester (which induces PKC downregulation) nor administration of GF109203X were able to inhibit this response [48]. PDBu-induced activation of PLD was largely unaffected by tyrosine kinase inhibitors, suggesting that although PKC activation of tyrosine phosphorylation clearly can occur, PDBu-induced activation of PLD does not rely on an intermediary tyrosine kinase, but occurs in parallel through a direct route.

PDBu-induced activation of PLD rapidly becomes desensitised, consistent with the activator-induced down-regulation of PDBu-sensitive PKC isoforms [53]. In contrast, although blocked by selective PKC inhibitors, LHRH-induced PLD activation showed no desensitisation through 40 min; suggesting that a PKC isoform resistant to down-regulation (perhaps ζ) was involved. It is thus feasible that a tyrosine kinase (activated by the LHRH

receptor through PKC ζ-like species, and perhaps additionally by PDBu-sensitive PKCs such as α and β) can lead to PLD activation. Agonist activation of the LHRH receptor may lead to activation of the former pathway (through a PKC ζ-like species), whereas PDBu stimulation is likely to lead largely to the triggering of the latter pathway. It is of course possible that other more complex mechanisms may be responsible for the results observed, in fact the loss of the vast majority of LHRH-induced PLD activation following down-regulation of PDBu-sensitive PKCs may count against the major involvement of a PDBu-insensitive PKC such as ζ. Nevertheless, since the PKC down-regulation protocol involves intense activation of PDBu-sensitive isoforms in the initial stages which could lead to profound secondary effects within the cell, the interpretation of the down-regulation result must require a degree of caution.

The question remains as to which tyrosine kinase or kinases are involved in the LHRH receptor activation of PLD. Since the LHRH receptor is a classical G-protein linked receptor, with no tyrosine kinase domain, downstream involvement of non-receptor tyrosine kinases clearly must occur. It has been reported that agonists that directly or indirectly activate PKC in platelets increase the activity of the c-*src* non-receptor tyrosine kinase [54] . Similarly another member of the *src* family of tyrosine kinases *fgr*, is activated in response to fMLP-stimulation in neutrophils [55], and c-*src* is also activated as a result of endothelin-1 receptor activation in mesangial cells [49] and of lysophosphatidic acid receptor stimulation in N1E 115 neuroblastoma cells [56]. Phospholipase D activity was shown to be elevated in BALB/c 3T3 cells transformed by v-*src* (a constitutively active form of c-*src*) [57 ]. As *src* has been shown to be activated by G-protein linked receptors in previous studies, and also to bring about PLD activation, a member of the *src* family of non-receptor tyrosine kinases is currently the most likely candidate for the tyrosine kinase participating here in LHRH receptor signalling.

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# FIGURE LEGENDS

## Figure 1

**(a)** Concentration-response curve for LHRH-induced activation of PLD (ie [$^3$H]PtdBut accumulation) over a 30 min incubation. **(b)** Time course of PLD activation by LHRH (100 nM) and PDBu (1 μM). Values are means ± SEM from 4-8 separate determinations. Typical basal dpm/ assay are 1000-2000, with no increase in this basal activity over the time course. Error bars on some of the points are obscured by the point symbol.

## Figure 2

The effects of protein kinase C inhibitors on stimulus-induced PLD activation. Concentration-dependent inhibition of responses to LHRH (100 nM; in a, c and e) and to PDBu (1 μM; in b, d and f) is shown for PKC inhibitors Ro 31-8220 (a, b), GF 109203X (c, d), and 1-(5-isoquinolinesulphonyl)-2-methylpiperazine H7 (e, f). Values are means ± SEM from 4-8 separate determinations. Curve fitting analysis indicated an unusually low Hill coefficient (0.56 ± 0.05; consistent with the contribution of multiple components) in the case of H7 inhibition of PDBu-evoked PLD activity but not in other cases. Error bars on some of the points are obscured by the point symbol.

## Figure 3

The effects of tyrosine kinase inhibitors on stimulus-induced PLD activation. Concentration-dependent inhibition of responses to LHRH (100 nM; in a, c and e) and PDBu (1 μM; in b, d and f) is shown for tyrosine kinase inhibitors, lavendustin A (a, b), piceatannol (c, d) and genistein (e, f). Values are the means ± SEM from 4-8 separate determinations. Error bars on some of the points are obscured by the point symbol.

## Figure 4

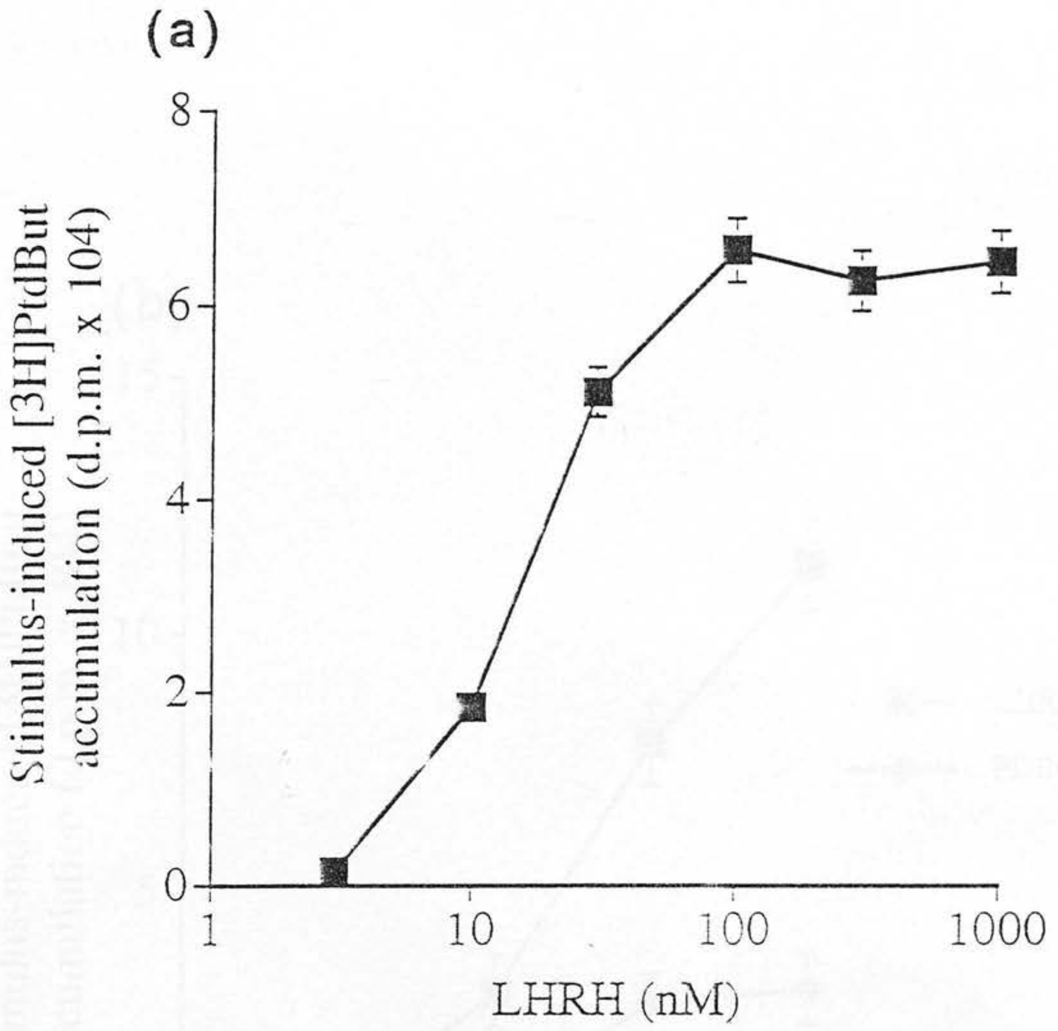
Anti-phosphotyrosine immunoblots of αT3-1 cells, using the 4G10 anti-phosphotyrosine antibody. Lanes 1 and 8, control (saline); 3 and 6, the LHRH receptor agonist buserelin ([D-Ser(tBu)$^6$, DesGly$^{10}$] LHRH ethylamide; 100 nM); 2, PDBu (300 nM); 7, ionomycin (30 μM); 4 and 5, buserelin-treated cells immunostained with phosphotyrosine-blocked antibody. Results are typical of at least 4 independent experiments.

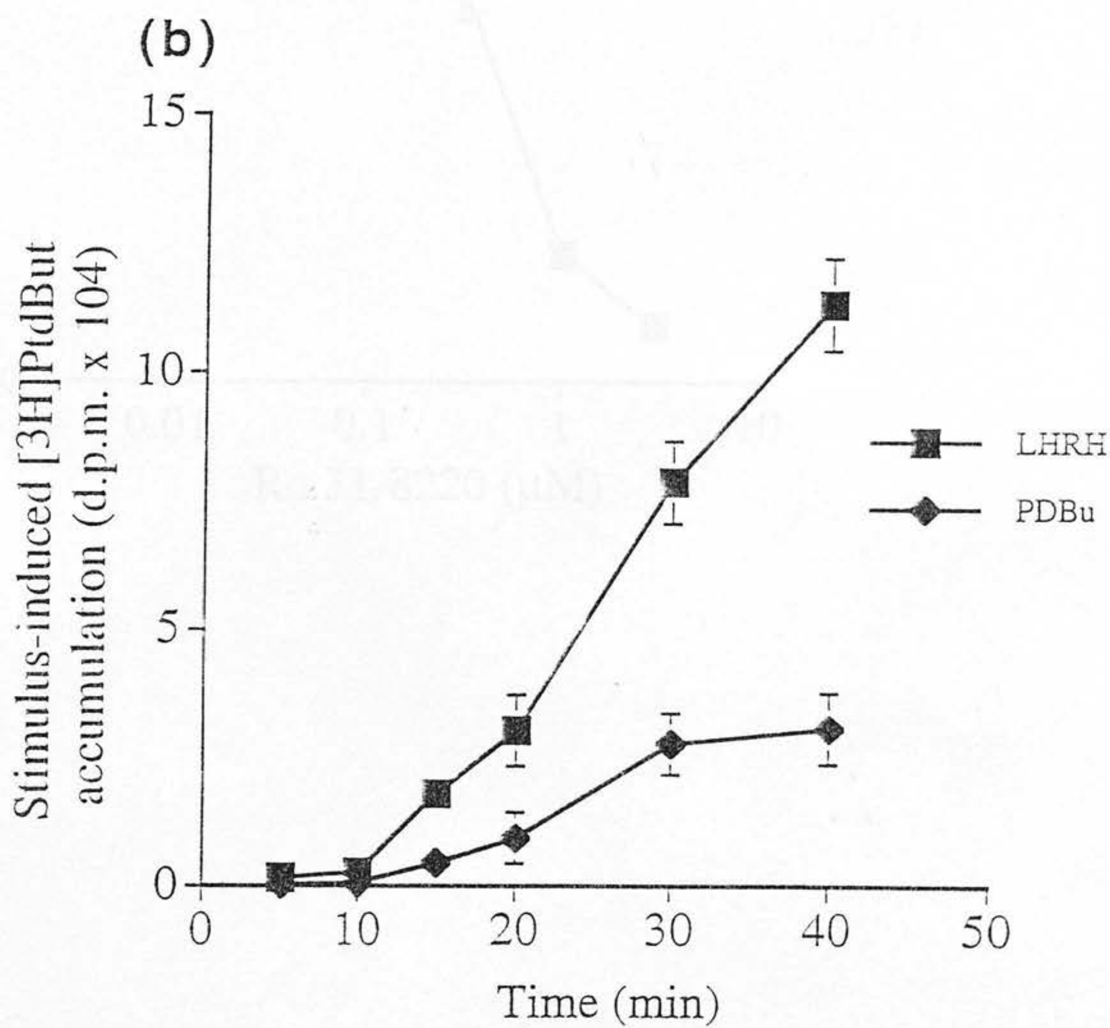
TABLE 1

| Condition | % of control LHRH-induced [$^3$H]PtdBut accumulation |
|---|---|
| LHRH (100 nM) | 100 ± 6 (8) |
| LHRH antagonist (1 μM) | 0 ± 1 (4) |
| LHRH (100 nM) + LHRH-antagonist (1 μM) | 1 ± 0.5 (4) |
| 4β-PDBu (1 μM) | 54 ± 4 (8) |
| 4α-PDBu (1 μM) | 0 ± 1 (4) |
| Ionomycin (30 μM) | 1 ± 0.4 (4) |
| Pervanadate (1 mM) | 22 ± 4 (8) |
| LHRH (100 nM) + 4β-PDBu (1 μM) | 131 ± 9 (4) |
| LHRH (100 nM) + cycloheximide (30 μM) | 98 ± 4 (4) |
| 4β-PDBu (1 μM) + ionomycin (30 μM) | 64 ± 4 (4) |
| Pervanadate (1 mM) + ionomycin (30 μM) | 7 ± 3 (4) |
| Pervanadate (1 mM) + 4β-PDBu (1 μM) | 78 ± 7 (4) |
| Pervanadate (1 mM) + Ro 31-8220 (1 μM) | 21 ± 4 (4) |

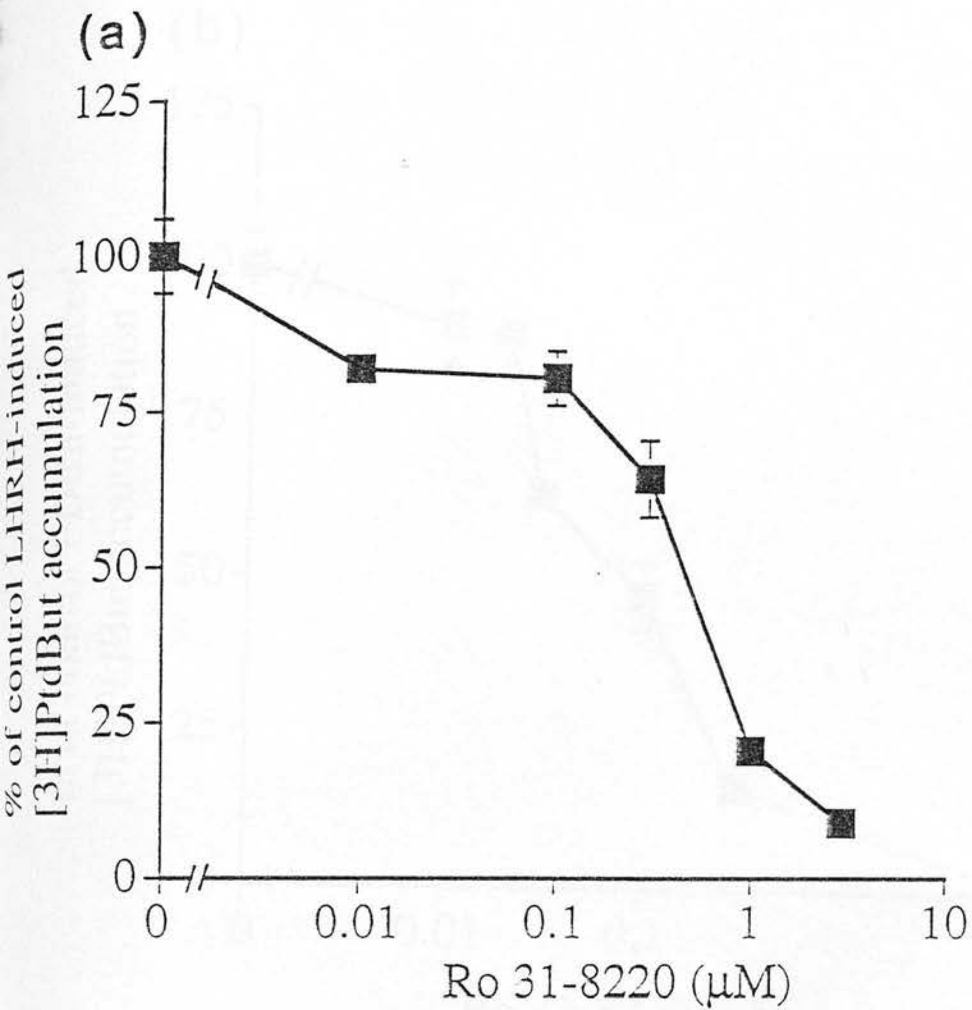
Effects of agents modifying various cellular signalling pathways on [$^3$H]PtdBut accumulation in αT3-1 cells. Values are the means ± SEM from the number of experiments indicated in parentheses. Neither cycloheximide nor Ro 31-8220 had any effect on unstimulated [$^3$H]PtdBut accumulation.

FIGURE 1





(b)
15
10
5
0
Stimulus-induced [3H]PtdBut accumulation (d.p.m. x 104)
0
10
20
30
40
50
Time (min)
LHRH
PDBu



(a)
% of control LHRH-induced [3H]PtdBut accumulation
125
100
75
50
25
0
0
0.01
0.1
1
10
Ro 31-8220 (μM)

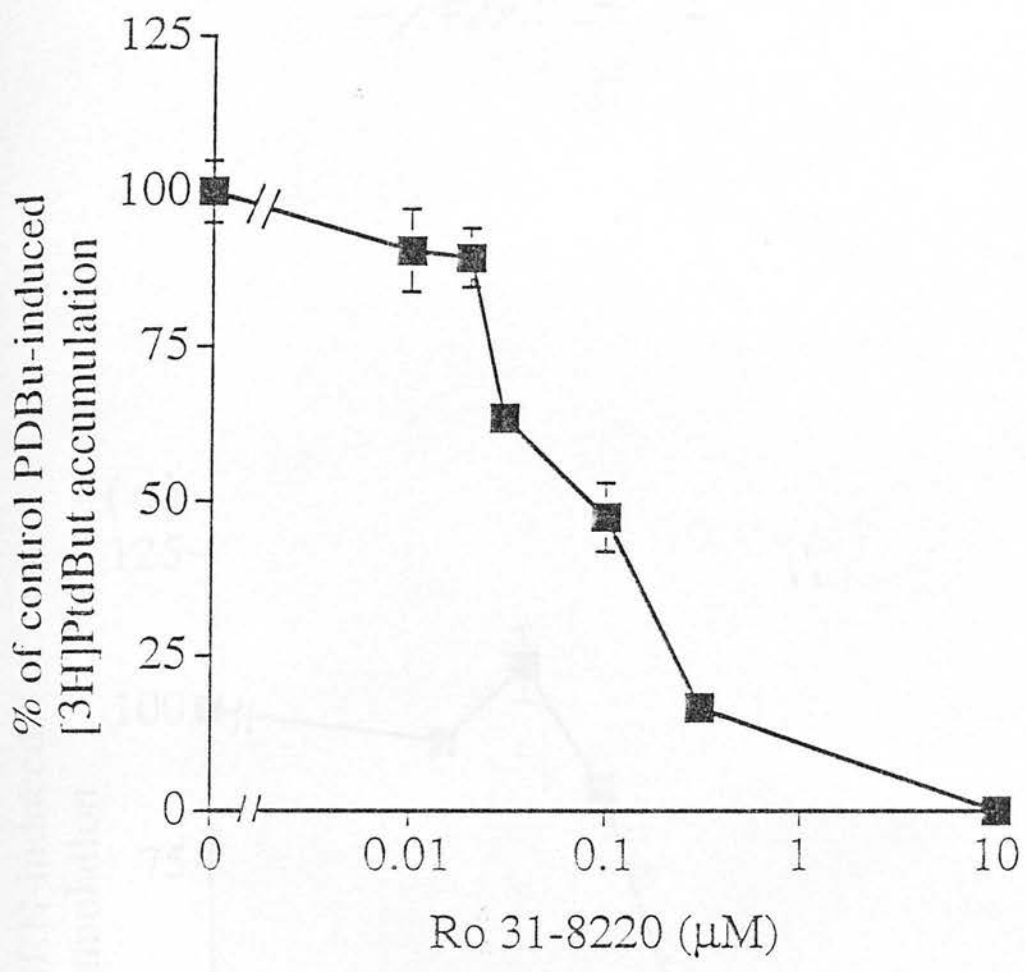

(b)
125
100
75
50
25
0
% of control PDBu-induced
[3H]PtdBut accumulation
0
0.01
0.1
1
10
Ro 31-8220 (μM)

FIGURE 2

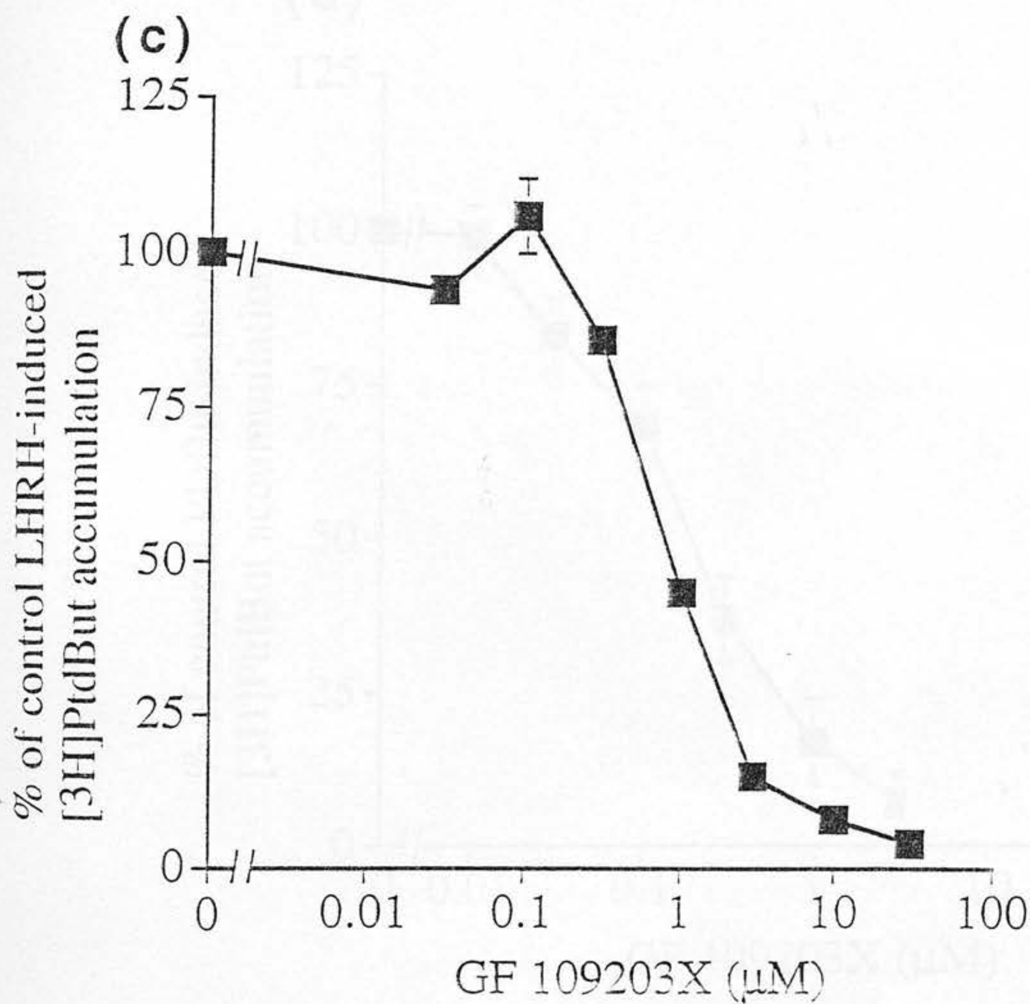


FIGURE 2

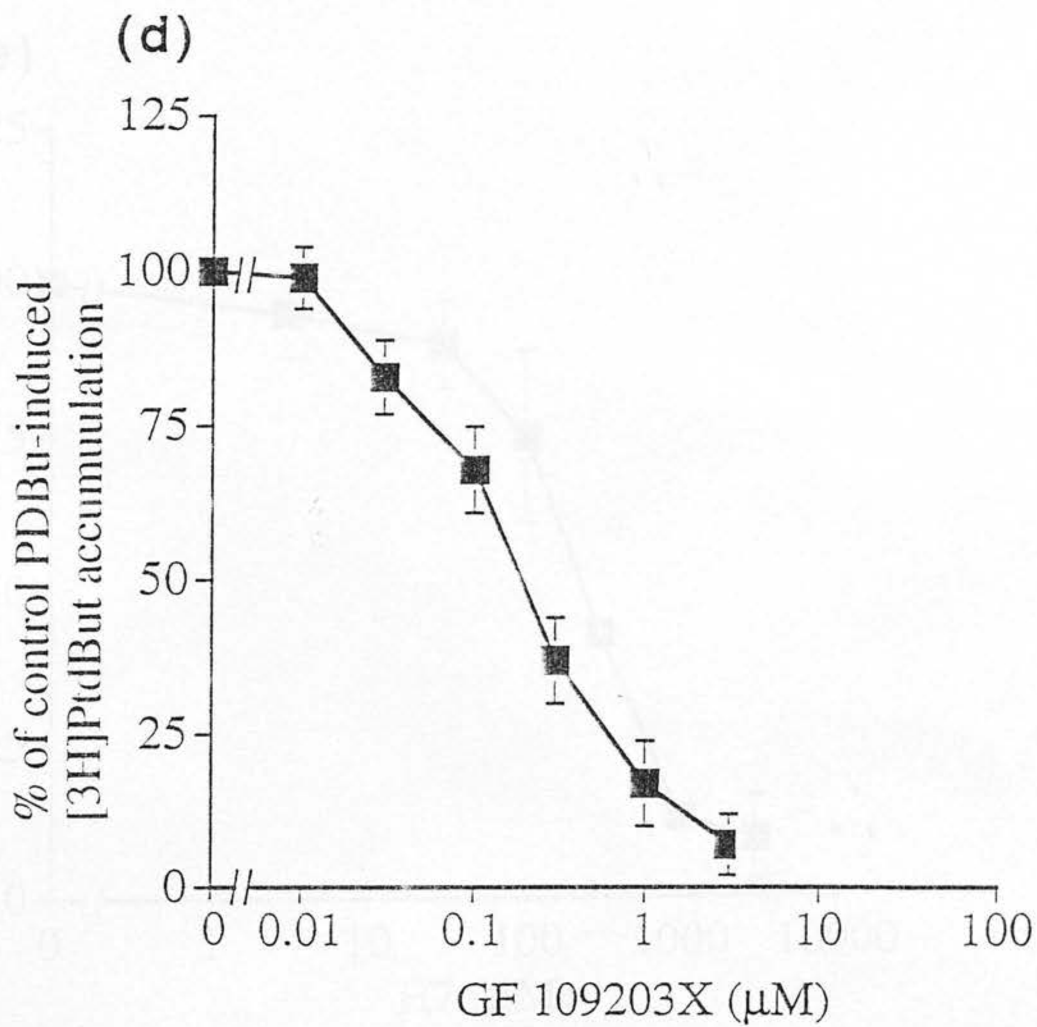


FIGURE 2

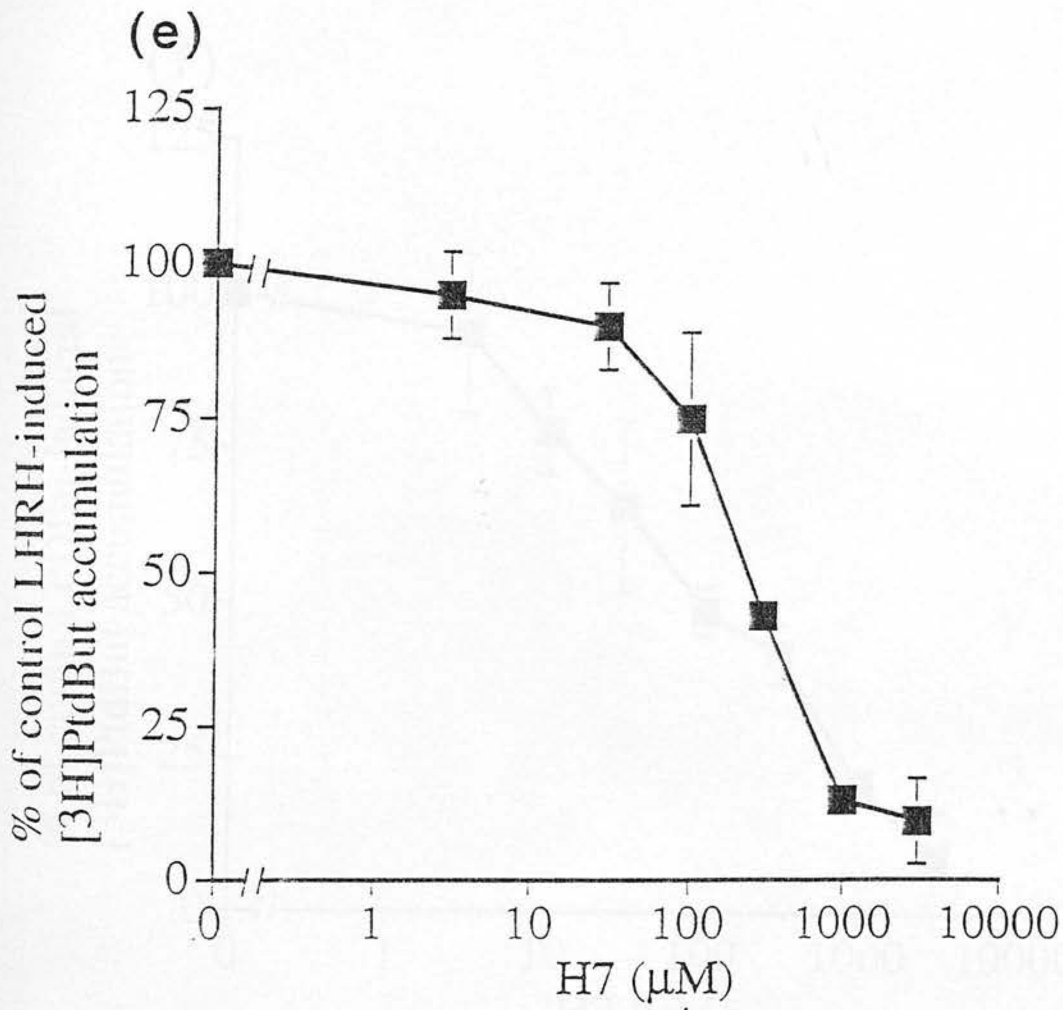


FIGURE 2

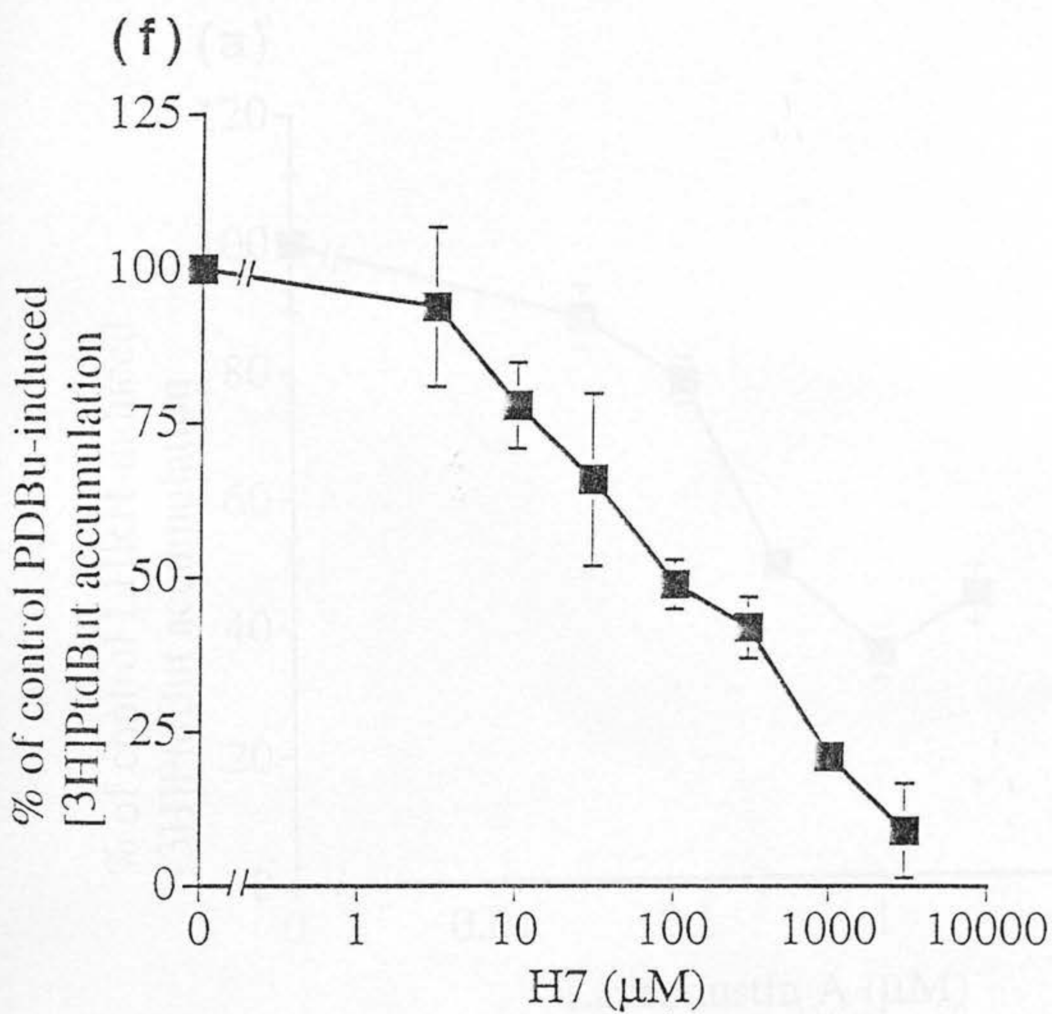


FIGURE 3

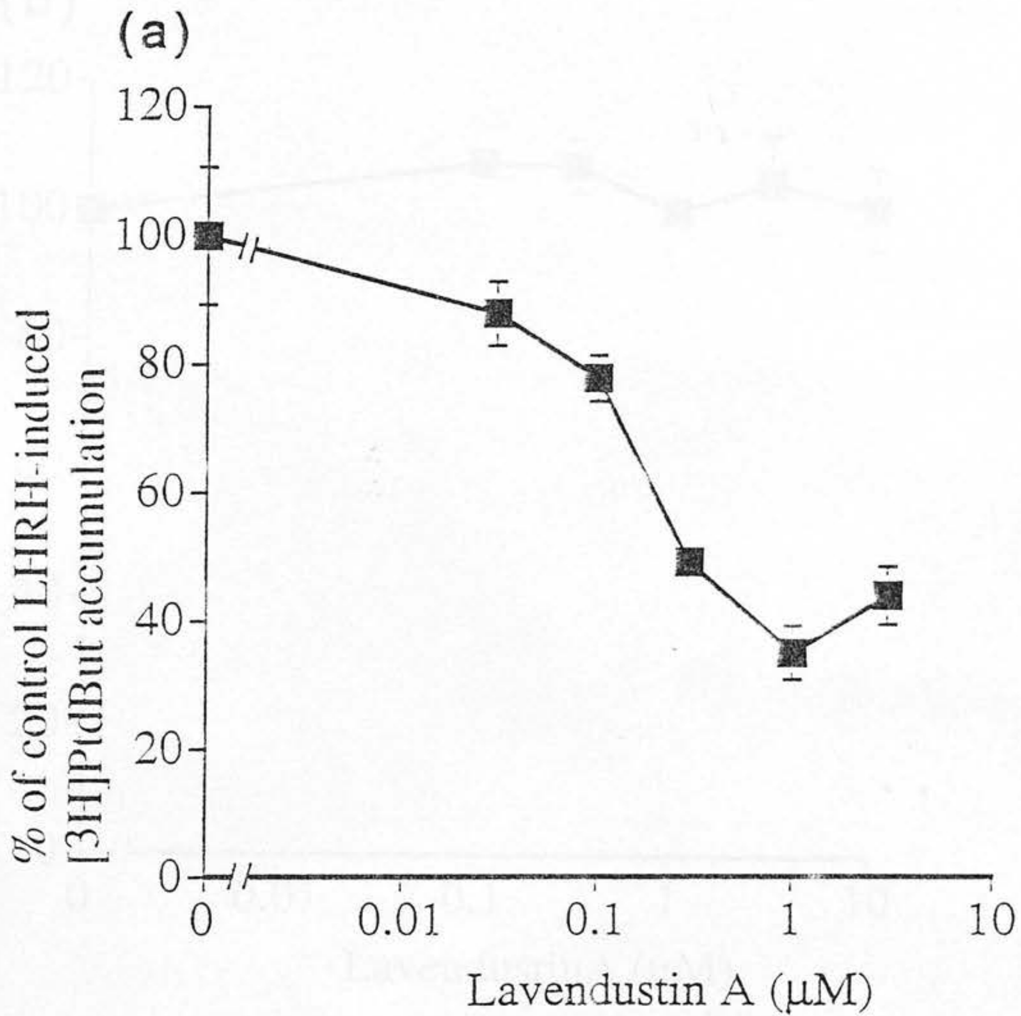


FIGURE 5

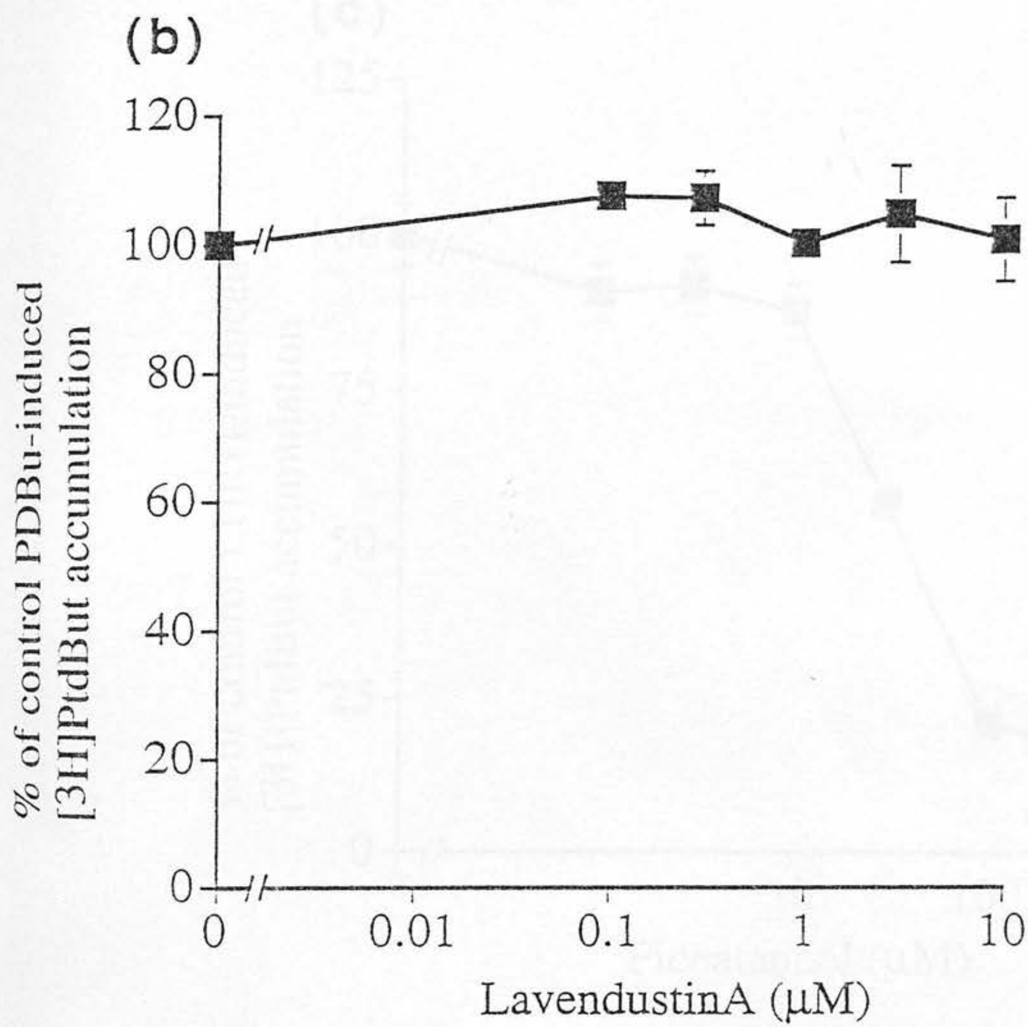


FIGURE 3

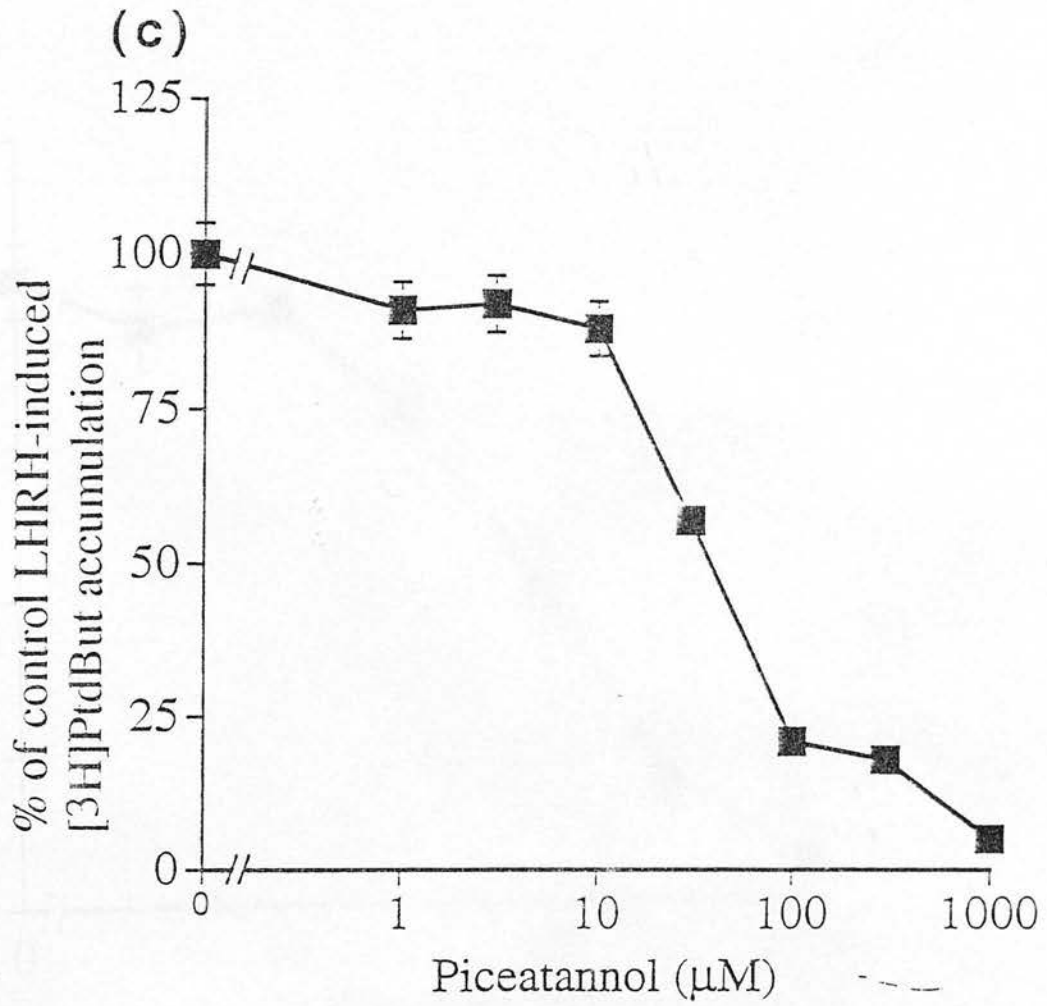


FIGURE 3

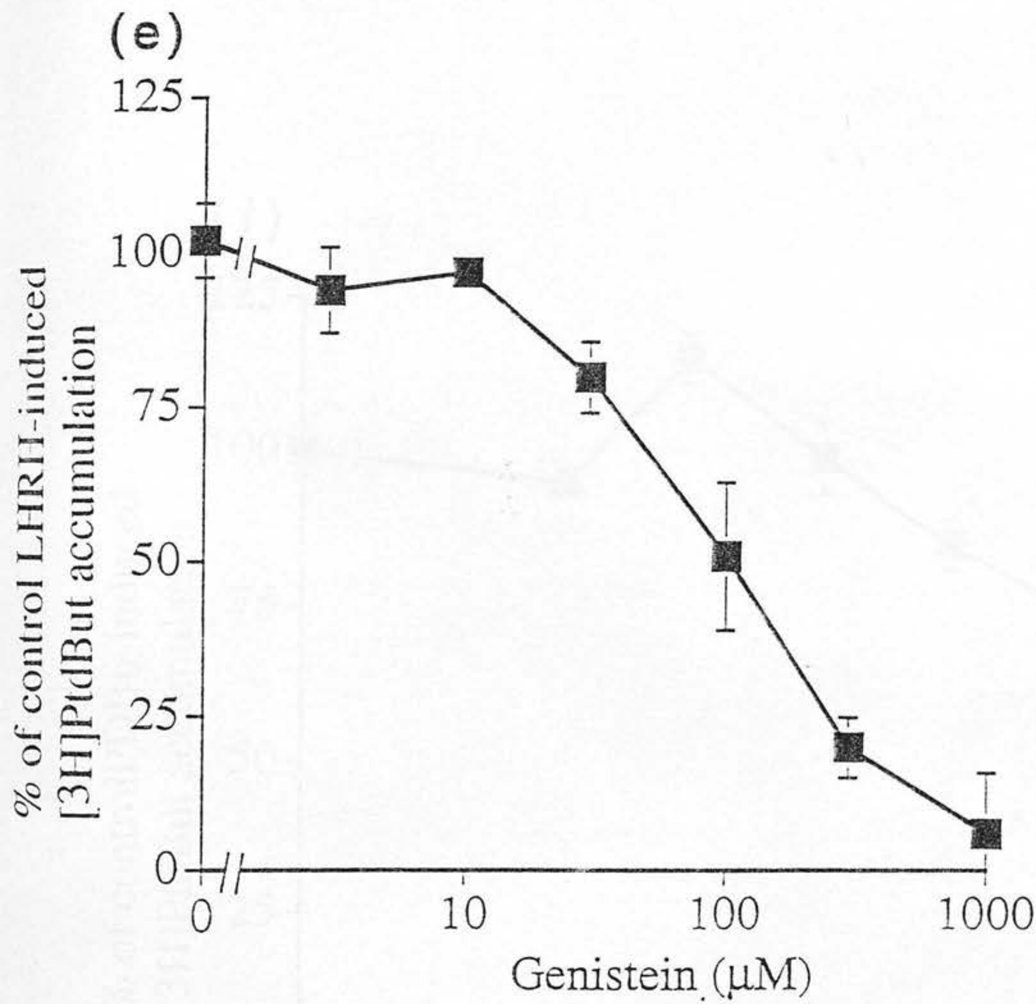


FIGURE 3

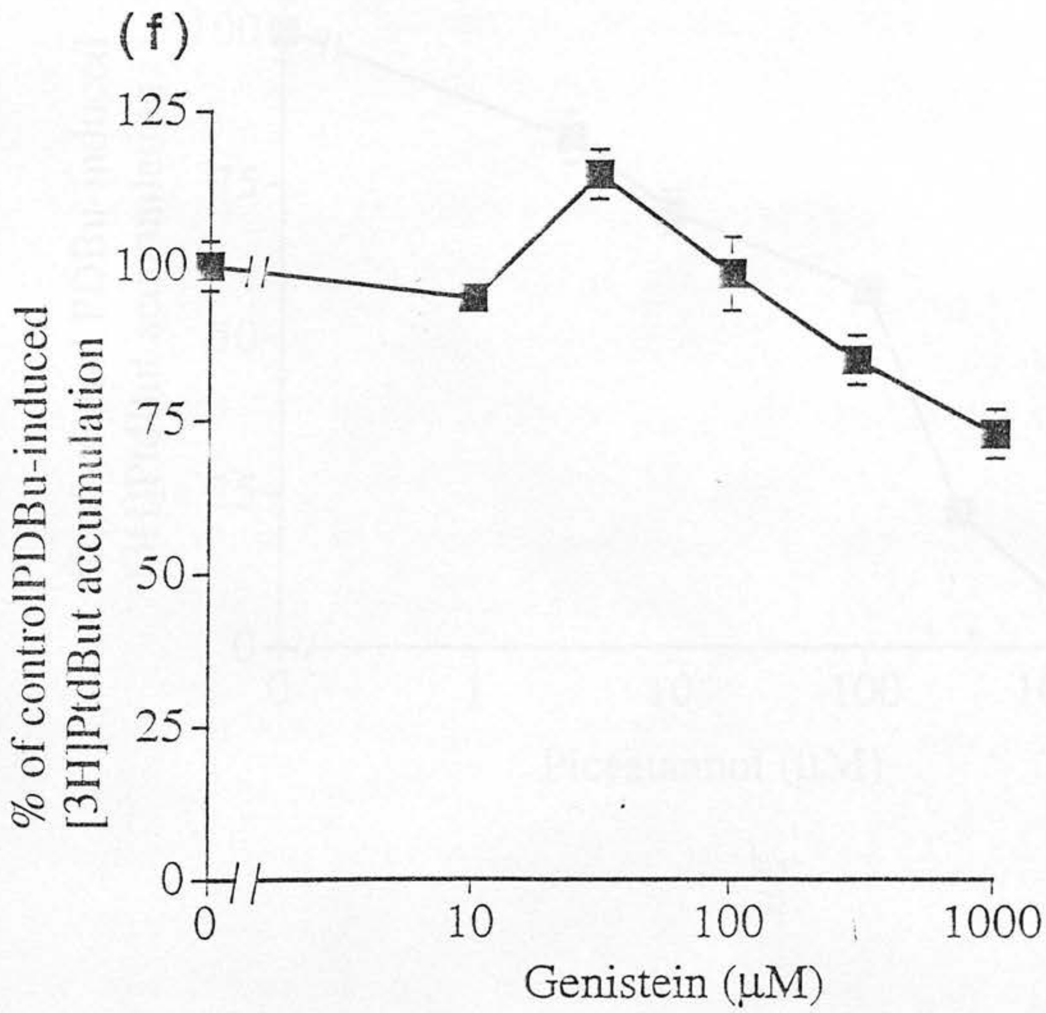
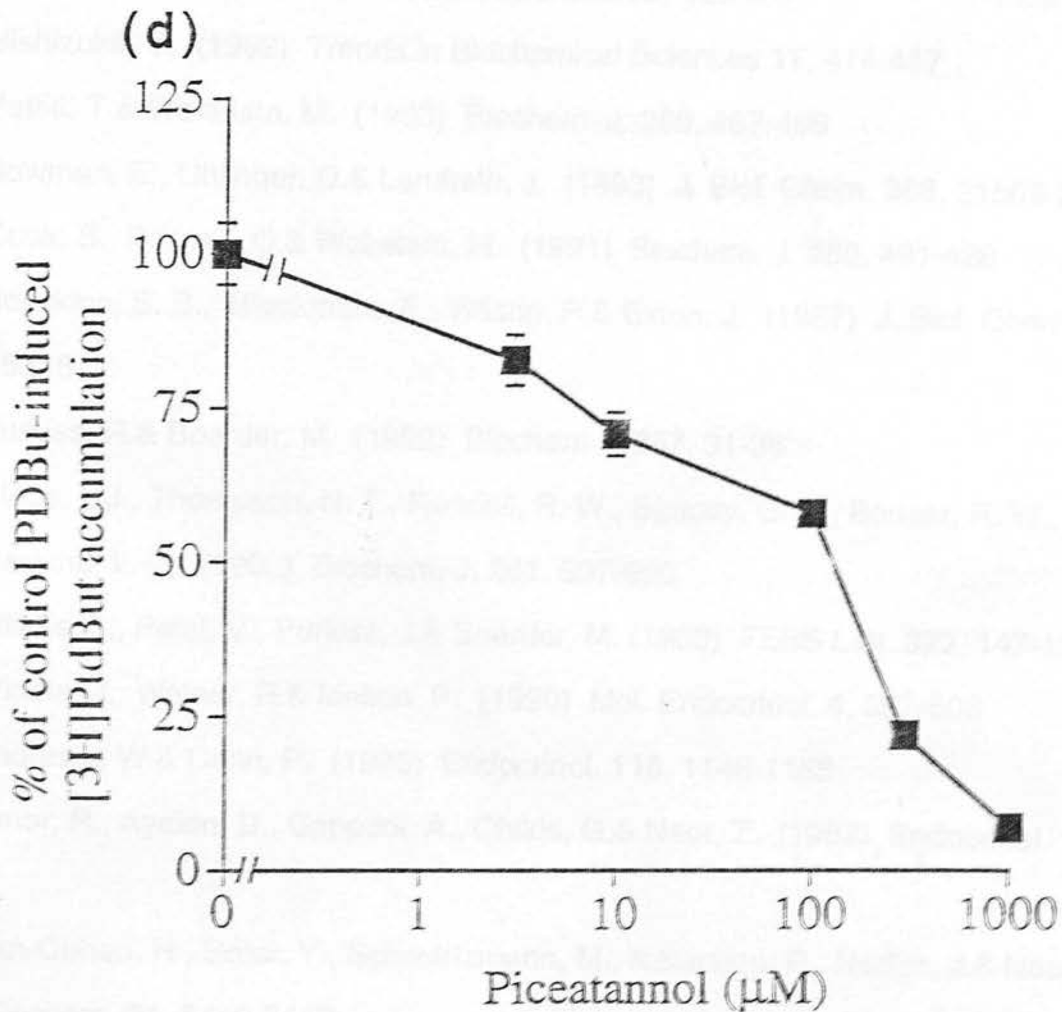


FIGURE 3



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# INVOLVEMENT OF A NOVEL FORM OF PKC AND TYROSINE KINASE IN THE ACTIVATION OF PLD BY LHRH,

Myles Fennell, James Simpson, Rory Mitchell, Neil Thompson* and Lawrie Garland*, MRC Brain Metabolism Unit, 1 George Square, Edinburgh and *Wellcome Research Laboratories, Langley Court, Beckenham, U.K.

The αT3-1 gonadotrope cell line was used to study signalling pathways associated with the LHRH receptor, including phospholipase D (PLD) and the *src* family of tyrosine kinases. [$^3$H]palmitate was used to label membrane phospholipids and cells were incubated with butan-1-ol (30 mM) in order to assay PLD activity as production of [$^3$H]phosphatidylbutanol (PtdBut). PLD activation by LHRH (100 nM) proceeded with a lag of about 5-10 min. After this the rate was approximately constant up to 40 min, which was the longest time point examined. In phorbol 12,13-dibutyrate (PDBu) (1 μM)-stimulated cells, activation of PLD occured after a delay of 10-15 min, but had virtually stopped by 25 min. After a 30 min stimulation the accumulation of PtdBut was approximately 10-fold and 4-fold over basal for LHRH and PDBu respectively. Ionomycin (30 μM) did not activate PLD. Ro 31-8220 (a selective PKC inhibitor) inhibited PLD activity elicited by 30 min incubations with PDBu or LHRH, displaying $IC_{50}$s of 62 ± 30 nM and 460 ± 180 nM respectively. H7 inhibited PDBu-induced PLD activity in a manner that indicated there are multiple components to the inhibition. LHRH-induced PLD activation was inhibited with low potency by H7 with an $IC_{50}$ of 240 ± 14 μM. Another PKC inhibitor GF 109203X also inhibited the LHRH-stimulated PLD activity with an $IC_{50}$ of 1 ± 0.1 μM. The tyrosine kinase inhibitor lavendustin A inhibited approximately 70% of the PLD response to LHRH with an $IC_{50}$ of 133 ± 17 nM. Lavendustin A (0.1-3 μM) had no effect on the response elicited by PDBu. Another tyrosine kinase inhibitor piceatannol also caused partial inhibition of LHRH-stimulated PLD at concentrations up to 100 μM. In addition, the tyrosine phosphatase inhibitor, pervanadate (1 mM) caused significant activation of PLD. LHRH was also shown by antiphosphotyrosine immunoblots to induce tyrosine phosphorylation of numerous proteins of molecular mass 63 to >170 kDa. A number of these phosphorylations were also elicited by PDBu but not by ionomycin. Phosphorylation of proteins >170 kDa increased continuously over 3-60 min, whereas other phosphorylations were maximal at 10 min. Immunoblotting experiments showed that αT3-1 cells contain *src* and *fyn* (but not *fgr, hck, lyn* or *yes*) tyrosine kinases and preliminary results using an immunoprecipitation/kinase assay have shown that *fyn* is activated by LHRH (100 nM) within 10 min.

Characteristics of phospholipase D activation by LHRH in the $\alpha$T3-1 gonadotrope-derived cell line

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The mouse gonadotrope-derived clonal cell line, $\alpha$T3-1 (Windle *et al,* 1990) was used as a model system to study receptor activation of phospholipase D (PLD). Induction of PLD activity in $\alpha$T3-1 cells was assessed in response to phorbol 12,13-dibutyrate (PDBu; 1 $\mu$M) or LHRH (100 nM). Cells were preincubated with 5 $\mu$Ci/ml [9,10 $^3$H] palmitate for 2 h, to allow labelling of membrane phospholipids. Butan-1-ol (30 mM) was used as the nucleophilic acceptor in the transphosphatidylation reaction carried out by PLD (Pai *et al,* 1988) and the stable reaction product phosphatidylbutanol (PtdBut) was then separated by thin layer chromatography before the label incorporated was quantified by scintillation counting.

Time course studies carried out with quiescent $\alpha$T3-1 cells demonstrated significant accumulation of PtdBut only after 10 or more min stimulation with LHRH. From 10 min, the rate of PtdBut accumulation in response to LHRH was approximately constant, up to 40 min (the longest time assessed). In PDBu-stimulated $\alpha$T3-1 cells, there was no detectable PtdBut accumulation until 15 min and it was 20 min before accumulation approached the level of PtdBut achieved with LHRH at 10 min. After 20 min, the rate of PDBu-stimulated PtdBut accumulation reduced and by 40 min was minimal. At 30 min the stimulus-induced formation of PtdBut was approximately 10-fold and 4-fold over basal for LHRH and PDBu respectively. Basal values ranged from 900-1500 dpm per assay. The delay in LHRH receptor-induced formation of PtdBut is consistent with previous observations on Ptdethanol formation (Netiv *et al,* 1991). The protein kinase C inhibitor Ro 31-8220 (Davis *et al,* 1989) completely inhibited the PtdBut formation elicited by 30 min incubations with PDBu or LHRH with $IC_{50}$s of 62 $\pm$ 30 nM and 460 $\pm$ 180 nM respectively. The highly-selective tyrosine kinase inhibitor lavendustin A (Hsu *et al,* 1991) inhibited approximately 70% of the response to LHRH with an $IC_{50}$ of 133 $\pm$ 17 nM. Lavendustin A (0.1 - 3 $\mu$M) had no effect on the response elicited by PDBu. In addition, the tyrosine phosphatase inhibitor, pervanadate (1 mM; Uings *et al,* 1992) caused PtdBut formation to approximately 5-fold of basal levels after 30 min incubation.

To assess whether LHRH could induce tyrosine phosphorylation of proteins, quiescent cells were incubated for 3 - 60 min with the LHRH agonist [D-Ser(tBu)$^6$, DesGly$^{10}$]LHRH ethylamide (buserelin; 100 nM), and cell extracts separated on 7.5% homogeneous gels by SDS-PAGE (PhastSystem, Pharmacia). Western blots of the gels on polyvinylidene difluoride membranes were probed with a mouse monoclonal anti-phosphotyrosine antibody (4G10, UBI) with or without 1 mM phosphotyrosine. Compared with saline-treated controls, buserelin induced numerous tyrosine phosphorylations on proteins of molecular mass 63 to > 170 kDa, but especially on 66, 76 and 119 kDa proteins. Phosphorylation of proteins > 170 kDa increased continuously over 3 - 60 min whereas other phosphorylations were maximal at 10 min. These results suggest that both PKC and a tyrosine kinase are involved in PLD activation by the LHRH receptor.

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Windle, J.J., Weiner, R.I. & Mellon, P.M. (1990) *Mol. Endocrinol.* 4, 587-603.

UNCONVENTIONAL SIGNALLING BY THE LHRH RECEPTOR. R. Mitchell, M.S. Johnson, J. Simpson, P.J. Sim, W.B. Wolbers, M. Fennell and A.J. Ison. MRC Brain Metabolism Unit, University Department of Pharmacology, 1 George Square, Edinburgh EH8 9JZ, Scotland

The LHRH receptor is a member of the family of G protein-linked receptors coupled to hydrolysis of phosphoinositides. The dependence of LHRH-induced gonadotrophin secretion upon phospholipase C activity has however been questioned. In view of the unique ability of the LHRH receptor to elicit the phenomenon of self-priming, we have sought to define unconventional signals emanating from this receptor that may not be generated by other members of the receptor family.

The phenomenon of LHRH self-priming is dependent upon an apparently novel species of PKC (with a pituitary-selective distribution) that we have partially purified and characterised. Amongst the cellular targets of a PKC with these properties are $PLA_2$ and PLD.

Signalling by means of tyrosine kinase and MAP kinase cascades is classically associated with growth factor receptors. Nevertheless we have shown that each of these is strongly activated by LHRH both in normal pituitary tissue and in the $\alpha$T3-1 gonadotroph cell line. LHRH-induced production of phosphotyrosine-immunoreactive proteins is mimicked in part by phorbol esters but not by ionomycin and is essential for LHRH-induced PLD activation and LH secretion. LHRH-induced MAP kinase activation correlates strongly with the ability of gonadotrophs to demonstrate self-priming and occurs through a PKC-dependent rather than $Ca^{2+}$-dependent or tyrosine kinase-dependent mechanism. Since MAP kinase is involved in the regulation of transcription, translation and cytoskeletal organisation, it is a strong candidate for a central role in the self-priming phenomenon which is reliant on the rapid induction of protein synthesis and cytoskeletal changes.

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12 W

SYNERGISTIC ACTIVATION OF PHOSPHOLIPASE D (PLD) IN PERMEABILIZED PLATELETS BY GTP[S] AND PHORBOL ESTER OR BY GTP[S] AND $Ca^{2+}$; CLOSE CORRELATION WITH SECRETION.
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Human platelets were labelled with [$^3$H]arachidonate and [$^{14}$C]serotonin (5-HT), electropermeabilized and incubated with GTP[S] and/or phorbol 12-myristate 13-acetate (PMA) in the absence or presence of $Ca^{2+}$ (pCa > 9 or 6). PLD activity was determined from the accumulation of [$^3$H]phosphatidate (PA) and [$^3$H]phosphatidylethanol (PEt) in incubations without and with ethanol, respectively, and secretion from the release of 5-HT. At pCa > 9, GTP[S] and PMA acted synergistically to stimulate the formation of either PA or PEt, but had little or no effect on [$^3$H]diacylglycerol (DAG) levels. Secretion correlated with PA or PEt formation and was partially inhibited by ethanol. BAPTA, a known inhibitor of $Ca^{2+}$-independent secretion from permeabilized cells, suppressed PLD activity. At pCa 6, GTP[S] alone greatly stimulated PA, PEt and DAG formation; PMA had no effect on PA or PEt accumulation but inhibited that of DAG. These increases in PA were largely attributable to PLD activity and those in DAG to the action of phospholipase C. Secretion correlated with PA and not DAG levels. The results indicate that in platelets, a G protein, protein kinase C and $Ca^{2+}$ all contribute to the activation of PLD, and suggest that PA generated by PLD may act directly or indirectly to promote secretion. (Supported by MRC Grant MT-5626)

13 W

LHRH ACTIVATES PHOSPHOLIPASE D BY A PKC-INDEPENDENT MECHANISM IN αT3-1 CELLS.
M. Fennell, E. Lutz, R. Mitchell, L. Garland*, MRC Brain Metabolism Unit, 1 George Square, Edinburgh, *Wellcome Research Laboratories, Beckenham.

The gonadotrophe-derived cell line, αT3-1, was used as a model system to study signalling mechanisms in pituitary gonadotrophes. The induction of phospholipase D (PLD) activity in αT3-1 cells in response to phorbol 12,13-dibutyrate (PDBu) or LHRH was assessed. Cells were preincubated with 5 μCi/ml [9,10-$^3$H] palmitate for 2 hours. Butan-1-ol was used as the nucleophilic acceptor in the transphosphatidylation reaction carried out by PLD. Phosphatidylbutanol was separated by TLC and quantified by scintillation counting. 100 nM LHRH and 1 μM PDBu caused a similar level of PLD activation in αT3-1 cells. The selective PKC inhibitor Ro31-8220 was used to determine the involvement of PKC in PLD activation induced by PDBu and LHRH. Ro31-8220 caused complete inhibition of PDBu stimulated PLD activity in αT3-1 cells with an $IC_{50}$ of 500 ± 80 nM, similar to the $IC_{50}$ for Ro31-8220 on PKC activity in mixed-micelle histone IIIS phosphorylation assays ($IC_{50}$ of 210 ± 20 nM). However, in LHRH-stimulated αT3-1 cells, Ro31-8220 (10 nM - 10 μM) had no effect on a major proportion of the induced PLD activity. It would appear that LHRH is activating PLD (at least in part) via a PKC-independent mechanism in αT3 cells.

4 W

DIFFERENTIAL TEMPORAL DEPENDENCY OF THE IGE-DEPENDENT DIACYLGLYCEROL (DAG) PRODUCTION ON TYROSINE KINASE (TK) ACTIVATION IN RBL 2H3 MAST CELLS.,
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FcERI aggregation on the surface of mast cells results in a biphasic increase in phosphatidic acid (PA) and DAG production leading to protein kinase C activation and degranulation. To disect out specific signalling mechanisms, chimeric human FcERIα receptors (see related abs.) were stably transfected into RBL 2H3 cells. While aggregation of the wild type receptor leads to both phases of DAG production and TK activation, at least two cell lines (8HA3 and 3/4A3) transfected with chimeric receptors produced the initial phase of DAG production in the absence of TK activity. To support these findings, sensitized RBL 2H3 cells were triggered with TNP-OVA (10ng/ml) in the absence or presence of a TK inhibitor, tyrophostin #8 (TKI). TKI inhibited IgE-dependent phosphatidylcholine-specific phospholipase D (PC-PLD) activation and the second, but not the initial, increase in DAG production. Taken together, the above results demonstrate that although the initial increase in PA and DAG production is independent of TK activation, the secondary increase in PA and DAG production, which is dependent on PC-PLD activation, is directly influenced by TK activation.

Receptor Transduction Mechanisms as targets for new drugs, London, September 1992, P10.

# PROTEIN KINASE C AND A TYROSINE KINASE ARE INVOLVED IN PHOSPHOLIPASE D ACTIVATION BY THE LHRH RECEPTOR IN αT3-1 CELLS

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The gonadotroph -derived cell line, αT3-1 was used as a model system to study signalling mechanisms in pituitary gonadotrophs. The induction of phospholipase D (PLD) activity in αT3-1 cells in response to 1μM phorbol 12,13 -dibutyrate or 100nM LHRH was assessed. Cells were preincubated with 5μCi/ ml [9,10 -$^{3}$H] palmitate for 2 hours. Butan-1-ol was used as a nucleophilic acceptor in the transphosphatidylation reaction carried out by PLD. Phosphatidylbutanol was separated by TLC and quantified by scintillation counting.

In quiescent αT3-1 cells the protein kinase C (PKC) inhibitor Ro 31-8220 completely inhibited the PLD activity elicited by PDBu or LHRH, with $IC_{50}$s of 62±68 nM and 460±180 nM respectively. In quiescent αT3-1 cells the highly selective tyrosine kinase inhibitor Lavendustin A inhibited LHRH -induced PLD activity down to about 35% of its maximum with an $IC_{50}$ of 133±17 nM. When PLD activity was elicited by PDBu, Lavendustin A had no inhibitory effect. These results suggest that PKC and a tyrosine kinase are both involved in PLD activation via the LHRH receptor, with the tyrosine kinase step coming before PKC in the activation pathway.

In contrast, in growing αT3-1 cells Ro 31-8220 only partially inhibited LHRH stimulated PLD activity. It therefore seems likely that another (PKC-independent ) pathway can participate in LHRH -induced PLD activation.