Phospholipid signalling pathways in *ras* transformed fibroblasts

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This thesis is submitted for the degree of

Doctor of Philosophy

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April, 1990

Page

List of Figures

List of Tables

Abbreviations

All abbreviations used were those recommended by the Biochemical Journal including:

Cho	choline
ChoP	choline phosphate
DAG	sn 1,2-diacylglycerol
EGF	epidermal growth factor
GAP	GTPase activating protein
GroPtdIns	glycerophosphatidylinositol
GroPtdInsP	glycerophosphatidylinositol monophosphate
$GroPtdInsP_2$	glycerophosphatidylinositol bisphosphate
GroPCho	glycerophosphocholine
Ins(1)P	inositol 1 monophosphate
Ins(4)P	inositol 4 monophosphate
$Ins(1,4)P_2$	inositol 1,4 bisphosphate
$Ins(1,3)P_2$	inositol 1,3 bisphosphate
$Ins(3,4)P_2$	inositol 3,4 bisphosphate
$Ins(1,4,5)P_3$	inositol 1,4,5 trisphosphate
$Ins(1,3,4)P_3$	inositol 1,3,4 trisphosphate
$Ins(1,3,4,5)P_4$	inositol 1,3,4,5 tetrakisphosphate
$NaDodSO_4$	sodium dodecyl sulphate
PDBu	phorbol 12, 13 dibutyrate
PDGF	platelet derived growth factor
$PGF_{2\alpha}$	prostaglandin$F_{2\alpha}$
PIC	phosphoinositide specific phospholipase C
PKC	protein kinase C
PLD	phospholipase D
PtdCho	phosphatidylcholine
PtdIns	phosphatidylinositol

PtdIns(4)P	phosphatidylinositol 4 phosphate
$PtdIns(4,5)P_2$	phosphatidylinositol 4,5 bisphosphate
TPA	12-O-tetradecanoyl phorbol 13-acetate

Acknowledgements

For advice during this project and for critical discussion during the writing of the thesis my thanks to Dr. M.J.O. Wakelam.

I would like to thank the late Professor R.M.S. Smellie and Professor M.D. Houslay for use of the facilities of the Biochemistry Department for this research. I acknowledge receipt of funding from the Cancer Research Campaign.

For the excellent typing of this thesis and much needed moral support I must extend my thanks to Mrs Sadie Brown. My thanks are also extended to friends and colleagues past and present in the Molecular Pharmacology Group for their good company and helpful discussion. Special thanks go to Simon Cook for help in preparing figures for Chapters 1 and 2.

SUMMARY

The aim of the work described in this thesis was to investigate the effects of over-expression of normal ras proteins upon lipid signalling pathways in cultured NIH-3T3 fibroblasts. The ras oncogene family has been implicated in the growth and development of a high percentage of human tumours, but despite extensive research the mechanism of action of ras is still unknown. It has been proposed that $p21^{ras}$ proteins function in a G-protein-like manner mediating the effects of certain growth factors, by coupling their receptors to the stimulation of PIC.

To study the role of $p21^{ras}$ in regulating second messengers as part of a signal transduction mechanism, NIH-3T3 fibroblasts, transformed by constitutive over-expression of the c-Ha-ras-1, c-Ki-ras-2 or N-ras genes, were used. The study also utilised a clone of NIH-3T3 cells which over-expressed the $p21^{Ha\text{-}ras}$ proto-oncogene in the presence of a glucocorticoid inducer; this allowed the study of short term transformation by the ras proto-oncogene. Wild-type NIH-3T3 fibroblasts which express Ha-, Ki- or N-ras at very low levels were used as a control.

Data obtained from these studies indicate clearly that over-expression of $p21^{ras}$ in NIH-3T3 cells is associated with increased cell proliferation and transformation. The results suggest that $p21^{ras}$ can function at a post receptor level to enhance growth factor stimulated inositol phosphate generation, without causing a change in the basal levels of inositol phosphates. This data supports the proposal that $p21^{ras}$ is coupled to PtdIns(4,5)P_2 hydrolysis and demonstrates that over-expression of normal $p21^{ras}$ proteins causes an amplification of growth factor stimulated inositol phosphate generation which may act as an initial signal for cell proliferation.

NIH-3T3 cells transformed by over-expression of ras genes demonstrated reduced $PGF_{2\alpha}$-stimulated inositol phosphate generation. The ras-induced desensitization to $PGF_{2\alpha}$ is not due to any reduction in receptor number, or change in Ka or Kd and thus would appear to be due to a reduced coupling of the agonist receptor complex to PIC. The desensitization of $PGF_{2\alpha}$ induced inositol phospholipid turnover is associated with proliferative state, since the desensitization is observed both in the transformed cells and in rapidly proliferating, non-contact inhibited NIH-3T3 cells. PKC catalysed phosphorylation of the $PGF_{2\alpha}$ receptors appears to be the most likely explanation of this phenomenon as NIH-3T3 cells incubated in the presence of TPA show a reduced response to $PGF_{2\alpha}$. PKC catalysed phosphorylation of receptors may play a crucial role in a negative feedback regulation of the Ca^{2+} mobilising pathway.

This led to an investigation of the role of inositol phospholipid turnover in the stimulation of cell proliferation. The results indicate that, in some cell lines breakdown of inositol phospholipids is either, not obligatory for the onset of mitosis, or in itself not a complete signal.

The effect of over-expression of normal Ha-, Ki- and N-ras genes in NIH-3T3 cells, upon both stimulated and basal PtdCho metabolism was also examined. The data presented in this section of the work suggests that stimulation of PKC either directly (e.g. by TPA) or indirectly, via activation of $PtdIns(4,5)P_2$ hydrolysis (e.g. by $PGF_{2\alpha}$ and serum) may stimulate breakdown of PtdCho by PLD. The results show that in ras transformed cells PtdCho turnover is increased apparently both by PLD and phospholipase A_2 pathways.

The activation of PKC in NIH-3T3 cells may lead to both the desensitization of PtdIns(4,5)P_2 hydrolysis and to the stimulation of PtdCho breakdown, thus potentiating its own activation whilst reducing the increase in intracellular free Ca^{2+} concentration. It is possible that the stimulation of PtdCho breakdown in response to $PGF_{2\alpha}$ is activated by the increase in PKC activity as a consequence of prior PtdIns(4,5)P_2 hydrolysis although a direct receptor activated effect cannot be ruled out. It appears that over-expression of $p21^{\underline{ras}}$ may induce rapid proliferation and hence transformation of fibroblasts by the modulation of second messenger production via both PtdIns(4,5)P_2 and PtdCho hydrolysis.

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

1.1 The cell cycle

Cells grow by progressing through the phases of the cell cycle: G_1, S, G_2 and M. The replication of DNA and the synthesis of the histone proteins occurs only in the DNA-synthesis, S phase of interphase. The S phase is preceded and followed by two growth phases G_1 and G_2, respectively, during which there is no net synthesis of DNA. In the G_2 phase, a cell contains two copies of each chromosome present in the G_1 phase. Throughout interphase there is continued cellular growth and synthesis of other cellular macromolecules such as RNA, protein and membranes. The cell finally divides during M, the mitosis phase.

Different mammalian cells and cells in culture have similar durations for S, G_2 and M about 7, 3 and 1h, respectively (Fawcett, 1981). The G_1 phase can be as short as 2 to 3h and as long as several days. Cells grown in culture can be made to enter a quiescent or non-proliferating phase (G_o) by either withdrawing serum growth factors from the culture medium, or by allowing the cells to reach confluency so that all the available growth factors are depleted and the cells are contact inhibited. Withdrawing growth factors from cultures in exponential growth, does not stop proliferation immediately. Those cells in or beyond late G_1, are committed to division and complete the cycle (Zetterberg & Larson, 1985). When these progeny enter early G_1 they and the other cells in the culture progress no further and the culture, therefore, then consists solely of quiescent G_o cells. Quiescent cells have the potential to re-enter the cell cycle at the G_1 stage and divide in response to appropriate mitogenic signals (i.e. addition of serum or defined growth factors).

Two major pathways have been implicated in the control of cell proliferation. Certain growth factors, of which epidermal growth factor (EGF) is a classic example, act on receptors which have an intrinsic protein tyrosine kinase activity. The phosphorylation of certain proteins on tyrosine residues is thought to be a stimulus which leads to DNA synthesis. The alternative pathway, which is the subject of this introduction is utilised by those growth factors which stimulate the hydrolysis of inositol phospholipids. Initial mitogen-induced biochemical changes are discussed in the following sections.

1.2 Mitogen induced ion changes

One of the initial events following growth factor stimulation of quiescent fibroblasts is an increase in the activity of the amiloride-sensitive Na^+/H^+ antiporter, implicating cytoplasmic pH as a potential 'messenger' (Moolenaar et al., 1983). Activation of Na^+/H^+ exchange may bring about the elevation of intracellular pH (by approximately 0.2 units) thought necessary for DNA synthesis to occur (Schuldiner & Rozengurt, 1982). The internal Na^+ concentration is maintained by its efflux via the ouabain-sensitive Na^+/K^+ pump. Mitogen induced increases in pH have been detected in several cell types, for example, human fibroblasts (Moolenaar et al., 1983), Swiss 3T3 fibroblasts (Ives & Daniel, 1987) and mouse thymocytes (Hesketh et al., 1985). Hesketh et al. (1985) demonstrated that mitogen stimuli capable of bypassing receptors, the Ca^{2+} ionophore A23187 and the protein kinase C(PKC) activating phorbol ester 12-O-tetradecanoyl phorbol 13-acetate (TPA), can also induce an increase in cytoplasmic pH. Owen et al. (1989) demonstrated that NIH-3T3 cells use both an inositol trisphosphate, Ca^{2+}-mediated pathway and a PKC-dependent pathway to regulate Na^+-H^+ exchange

and suggested that one pathway is not more important than the other. Ives and Daniel (1987), however, have shown that mitogen-induced alkalinisation of the cell by activation of the Na^+/H^+ antiporter is independent of changes in intracellular Ca^{2+}. It has not yet been determined, however, whether the raised pH values associated with exponentially growing cells is a regulatory signal or if it is a consequence of cell proliferation.

Perona and Serrano (1988) transfected fibroblasts with the yeast plasma membrane H^+-ATPase; an H^+-pumping ATPase which regulates intracellular pH in fungi and plants. The resulting fibroblasts demonstrated a sustained increase in intracellular pH and acquired the growth characteristics of transformed cells. This experimental technique bypassed the requirement for growth factor stimulation and demonstrated that sustained elevation of intracellular pH directly influences cell proliferation.

However, early studies on pH modulation utilised a HCO_3^--free buffer. Ganz et al. (1989) have now demonstrated that a range of growth factors stimulate mitosis in a HCO_3^--containing buffer with no subsequent change in pH. As the Na^+/H^+ antiporter is active under these conditions, this demonstrated that cytosolic pH is tightly regulated and suggests that it is the influx in Na^+ ions rather than a change in intracellular pH which is important in mitogenesis.

The major ionic change in mitosis is in intracellular Ca^{2+} concentration. Using Quin 2 fluorescence, Hesketh et al. (1985) demonstrated that vasopressin and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in Swiss 3T3 cells and concanavalin A (Con A) in thymocytes caused a rapid, transient increase in intracellular Ca^{2+} concentration which was only partly sensitive to the removal of external Ca^{2+}. The Con A induced Ca^{2+} concentration

increase in quiescent thymocytes declined following the achievement of the peak value of about 250nM (from 100nM in the control) to a new plateau of about 150nM. After removal of external Ca^{2+} by the addition of EGTA to the medium, Con A induced a smaller transient signal of 150nM which returned to resting level within 8 minutes. EGF, however, induced a slower rise in internal Ca^{2+} concentration which peaked after 2 minutes and was absolutely dependent upon the presence of external Ca^{2+}. Blakely et al. (1989) demonstrated that in Swiss 3T3 cells bombesin-stimulated efflux of $^{45}Ca^{2+}$ was independent of extracellular Ca^{2+}, but that platelet derived growth factor (PDGF)-stimulated efflux was markedly inhibited by chelation of external Ca^{2+} by EGTA. Thus two different mechanisms of increasing $[Ca^{2+}]_{int.}$ appear to be operating, one involving release of internal stores of Ca^{2+} and the other involving Ca^{2+} entry (see Section 1.5).

1.3 Stimulation of second messenger metabolism

Many growth factors, for example bombesin, bind to specific cell surface receptors which are linked via a GTP-binding protein (G-protein) to the stimulation of a phosphoinositide-specific phospholipase C (PIC). On stimulation PIC catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), a lipid located in the inner leaflet of the plasma membrane, to generate two second messenger molecules, sn-1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3). Both DAG and Ins(1,4,5)P_3 are part of a signal transduction mechanism which controls a variety of cellular processes including secretion, metabolism, contraction, neural activity and cell proliferation (Downes & Michell, 1985; Berridge & Irvine, 1989). DAG activates PKC which leads to the phosphorylation of a range of cellular proteins, including the Na^+/H^+ antiporter (Hesketh et al., 1985). Specific binding of

$Ins(1,4,5)P_3$ to receptors on the smooth endoplasmic reticulum (Spat et al., 1986) causes a transient efflux of Ca^{2+} which elicits a variety of secretory, metabolic and mitogenic processes. Receptors which do not mobilise intracellular Ca^{2+} do not stimulate inositol phospholipid catabolism.

1.3.1 The inositol phospholipid pathway

Figure 1.1 shows the routes of metabolism of compounds containing inositol and phosphate.

Synthesis of phosphatidylinositol (PtdIns; reaction 1,2 & 3, Fig. 1.1)

Phosphorylation of DAG by DAG kinase results in the formation of phosphatidic acid (reaction 1), which subsequently reacts with CTP to form CDP-diacylglycerol, catalysed by phosphatidate cytidyltransferase (reaction 2). Inositol is added to CDP-diacylglycerol, catalysed by CDP-diacylglycerol: inositol phosphatidyl-transferase, to form PtdIns (reaction 3).

Synthesis of polyphosphoinositides (reactions 4 & 5, Fig.1.1)

PtdIns is phosphorylated at the 4 position on the inositol ring by an ATP-requiring PtdIns-4-kinase (reaction 4) to form phosphatidylinositol 4-phosphate (PtdIns(4)P). This is in turn phosphorylated on the 5-position by PtdIns(4)P-5-kinase (reaction 5) to generate $PtdIns(4,5)P_2$.

Degradation of inositol phospholipids (reactions 6,7 & 8, Fig.1.1)

The pathways of degradation of inositol phospholipids are the dephosphorylation of $PtdIns(4,5)P_2$ by the sequential action of $PtdIns(4,5)P_2$ 5-phosphomonoesterase (reaction 6) and a PtdIns(4)P 4-phosphomonoesterase (reaction 7) and removal of the entire inositol phosphate head group by phosphodiesterase enzymes (reaction 8).

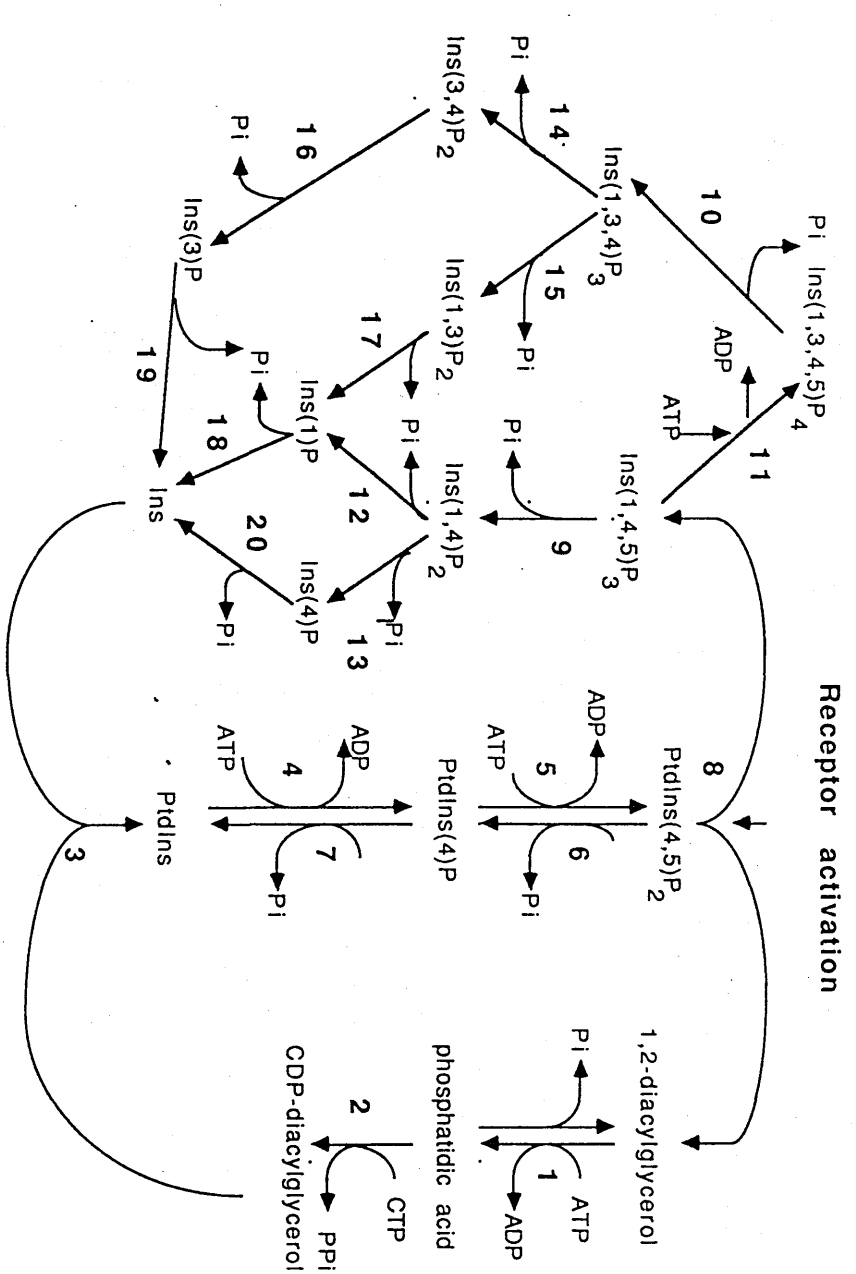


Figure 1.1 Pathways of inositol lipid metabolism

Hydrolysis by phospholipase C (reaction 8, Fig.1.1)

PIC occupies a central position in the signal transduction pathway, since it catalyses the hydrolysis of PtdIns(4,5)P_2 to generate the two second messenger molecules Ins(1,4,5)P_3 and DAG (Berridge & Irvine, 1984; Majerus et al., 1986). A cytosolic PIC was first detected in pancreas (Dawson, 1959), but has since been found in all mammalian cells examined in addition to higher plants and some bacteria (Irvine et al., 1980; Irvine, 1982; Shukla, 1982). Recent isolation, purification and determination of the nucleotide sequences encoding phospholipase C proteins have demonstrated that PIC is not a single protein but, a large family of potentially diverse proteins. These enzymes derived from a variety of tissues, have molecular masses ranging from 56kDa to 154kDa, and exist both as soluble and membrane-associated proteins (Boyer et al., 1989).

Examples of PIC enzymes purified are the three isoenzymes from bovine brain. These have molecular weights of 150kDa, 145kDa and 85kDa (Ryu et al., 1987); a 65kDa form from bovine brain membrane (Katan & Parker, 1987); a 62kDa form from pig uterus (Bennett & Crooke, 1987) and an 88kDa form from bovine brain cytosol (Rebecchi & Rosen, 1987). The relationship between these multiple forms is not clear.

When soluble PIC activity was assessed using pure lipid substrates all three inositol phospholipids were hydrolysed with equal facility. Under in vitro conditions cytosolic PICs from brain showed considerable activity at 1uM Ca^{2+} when PtdIns(4,5)P_2 was presented as a component of a lipid mixture such as would be present in the inner bilayer of intact cells; hydrolysis of PtdIns(4,5)P_2 was about 100 times more rapid than that of PtdIns (Irvine et al., 1984a). However, at the higher

ionic strength pertaining *in vivo* in the presence of Mg^{2+}, the enzyme shows no activity below 100uM Ca^{2+}, and is maximal at 1mM Ca^{2+}. When PtdIns(4,5)P_2 is presented in a non-bilayer configuration, the enzyme catalyses the hydrolysis of PtdIns(4,5)P_2 under ionic conditions similar to those *in vivo*. From the basic characteristics of the soluble PIC, it has been suggested that on receptor activation, a change in the presentation of the substrate by perturbation of the lipid bilayer could control enzyme activity (Irvine *et al.*, 1984a).

For a reaction controlled by receptors, Irvine and Dawson (1978) proposed that an alternative possibility would be for the occupied receptor to interact with a plasma membrane-bound PIC. The first unequivocal demonstration for a membrane-bound PIC came from work with human erythrocytes (Downes & Michell, 1982). Later evidence for a membrane-bound PIC was found in synaptosomes (Van Rooijen *et al.*, 1983), neutrophils (Cockcroft *et al.*, 1984) and liver (Seyfred & Wells, 1984). However, the enzyme is probably only loosely membrane-associated and not an integral protein.

Occupancy of EGF and PDGF receptors stimulates their tyrosine kinase activity and recent evidence has suggested that at least one isoenzyme of PIC (PIC-γ) is a substrate for the tyrosine kinase activity of growth factor receptors known to stimulate PtdIns(4,5)P_2 hydrolysis (Meisenhelder *et al.*, 1989).

Metabolism of inositol phosphates

Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 5-phosphatase (reactions 9 & 10, Fig.1.1)

The first step in the catabolism of Ins(1,4,5)P_3 and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) is removal of the 5-phosphate (Batty *et al.*, 1985) to yield inositol 1,4-bisphosphate (Ins(1,4)P_2) (reaction 9) and inositol

1,3,4-trisphosphate ($Ins(1,3,4)P_3$) (reaction 10), respectively. These reactions appear to be catalysed by the same Mg^{2+}-dependent 5-phosphatase (Connolly et al., 1987).

In permeabilised hepatocytes Joseph et al. (1987) observed that $Ins(1,4,5)P_3$ hydrolysis was inhibited by $Ins(1,3,4,5)P_4$ implying that, in these cells at least, there is competition of both substrates for a single phosphatase. This may have the effect of extending the intracellular half-life of $Ins(1,4,5)P_3$ and thus prolong Ca^{2+} mobilisation.

Ins(1,4,5)P_3-3-kinase (reaction 11, Fig.1.1)

The ATP-dependent kinase, $Ins(1,4,5)P_3$-3-kinase which converts $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$ (reaction 11) probably plays a key role in regulating $Ins(1,4,5)P_3$ metabolism. The $Ins(1,4,5)P_3$-3-kinase has an approximate 10-fold higher affinity for $Ins(1,4,5)P_3$ than the 5-phosphatase and is in some cells activated by Ca^{2+}-calmodulin (Biden et al., 1987; Morris et al., 1987a). Thus it is possible that this enzyme may be regulated by receptor-mediated changes in cytosolic [Ca^{2+}] which would result in a feed-forward mechanism promoting $Ins(1,3,4,5)P_4$ production (the possible functions of $Ins(1,3,4,5)P_4$ are discussed in Section 1.5.1).

Ins(1,4)P_2 4-phosphatase (reaction 12, Fig.1.1);

Ins(1,4)P_2 and Ins(1,3,4)P_3 1-phosphatase (reactions 13 & 14, Fig.1.1)

$Ins(1,4)P_2$ can be metabolised in vitro to either inositol 1-monophosphate (Ins(1)P) (reaction 12) or inositol 4-monophosphate (Ins(4)P) (reaction 13) by enzymes which may (Storey et al., 1984) or may not (Ackerman et al., 1987) exhibit differential sensitivity to Li^+. Evidence from many cell types now indicates that less than 5% of $Ins(1,4)P_2$ dephosphorylation is performed by a

4-phosphatase (Morris et al., 1988b; Balla et al., 1988). $Ins(1,4)P_2$ and $Ins(1,3,4)P_3$ are hydrolysed to Ins(4)P (reaction 13) and inositol 3,4-bisphosphate ($Ins(3,4)P_2$) (reaction 14), respectively by the same inositol 1-phosphatase (Inhorn & Majerus, 1987).

Ins(1,3,4)P_3 4-phosphatase (reaction 15, Fig.1.1) and Ins(3,4)P_2 4-phosphatase (reaction 16, Fig.1.1)

Both $Ins(1,3,4)P_3$ and $Ins(3,4)P_2$ can be hydrolysed by a 4-phosphatase (Shears et al., 1987a; Inhorn & Majerus, 1988), but it has not yet been determined if these reactions are catalysed by a single enzyme. These phosphatase activities are both Mg^{2+} independent, which is unusual amongst the inositol phosphatases with the exception of the $Ins(1,3)P_2$ 3-phosphatase. Inhorn and Majerus (1988) exploited the Mg^{2+} independence of the $Ins(1,3,4)P_3$ 4-phosphatase to determine the contribution this enzyme made to $Ins(1,3,4)P_3$ metabolism. $Ins(1,3,4)P_3$ dephosphorylation in homogenates of a number of tissues was assayed in the presence and absence of Mg^{2+} and in the brain the 4-phosphatase accounted for 72% of the total. In every other tissue analysed, the 4-phosphatase route was a minor pathway ($<$20% of total).

Ins(1,3)P_2 3-phosphatase (reaction 17, Fig.1.1).

The 3-phosphatase hydrolysis of $Ins(1,3)P_2$ has been characterised using ^{32}P-labelled substrates (Bansal et al., 1987) and is Mg^{2+}-independent.

Inositol monophosphatase (reactions 18,19 & 20, Fig.1.1)

It has now been proven that a single enzyme dephosphorylates Ins(1)P (reaction 18), inositol 3-monophosphate (Ins(3)P) (reaction 19) and Ins(4)P (reaction 20) (Gee et al., 1988).

1.4 Signal transduction by mammalian G-proteins

Although it appears that a G-protein is involved in control of PIC, this protein has not been identified. G-proteins transduce a diverse series of extracellular signals by regulating a number of effector systems which are either ion channels or enzymes able to modulate the rate of synthesis of intracellular second messengers.

The G-protein family includes several well-characterised members, each associated with the plasma membrane, for example:- (a) G_s, which mediates hormonal stimulation of adenylate cyclase (Gilman, 1984); (b) G_i, which mediates hormonal inhibition of adenylate cyclase (Gilman, 1984) and (in some cells) stimulation of PtdIns(4,5)P_2 hydrolysis (Gierschik & Jakobs, 1987); (c) G_o, which mediates receptor regulation of certain classes of Ca^{2+} channels (Hescheler *et al.*, 1987); (d) TD1 and TD2 which activate cGMP phosphodiesterase in retinal rods and cones respectively (Stryer, 1986).

Identified G-proteins consist of an alpha, beta and gamma subunit heterotrimer. In the resting state the G-protein exists in the holomeric form with GDP bound to the nucleotide binding site of the alpha subunit. Hormone-receptor complexes activate specific G-proteins by enhancing the rate of release of GDP, the rate limiting step in G-protein activation/deactivation, with the released GDP being exchanged for GTP (Gilman, 1987). Binding of GTP to the G-protein alpha subunit causes it to dissociate from the beta/gamma dimer, thereby generating two potentially active subunits, alpha-GTP and beta/gamma, which are then able to interact with the catalytic moiety of a particular second messenger generating system and alter the rate of synthesis of a second messenger. The alpha subunits also possess a site for NAD^+-dependent ADP-ribosylation by bacterial toxins (see Section

1.4.1.4). Hydrolysis of the terminal phosphate of the bound GTP by the intrinsic GTPase activity deactivates the alpha subunit and in this GDP-bound form it reassociates with beta/gamma subunits restoring the G-protein to the deactivated state.

1.4.1 G-protein coupling in the inositol phospholipid pathway

The first indication that a G-protein was involved in the stimulation of PtdIns(4,5)P_2 hydrolysis came from studies of Goodhart et al. (1982) who demonstrated a shift in the binding curve for α_1-adrenergic receptors when poorly hydrolysed GTP analogues were added to membrane preparations of hepatocytes. There is now an increasing amount of evidence supporting involvement of G-proteins in receptor stimulated PtdIns(4,5)P_2 hydrolysis. This is summarised briefly as follows:

1.4.1.1 Activation of PIC by GTP and its poorly hydrolysed analogues

Litosch et al. (1985) found that a crude plasma membrane preparation of blowfly salivary glands produced Ins (1,4,5)P_3 in response to 5-hydroxytryptamine, only in the presence of GTP. This work has been extended to demonstrate that activation of PIC by GTP and its poorly hydrolysed analogues, can stimulate the hydrolysis of PtdIns(4,5)P_2 in the absence of agonists, in neutrophil plasma membranes (Cockcroft & Gomperts, 1985), pancreatic cells (Merritt et al., 1986) and rat hepatocytes (Wallace & Fain, 1985; Uhing et al., 1986). These results suggest a regulatory role for a G-protein in the inositol phospholipid signalling pathway.

1.4.1.2 GTP and its poorly hydrolysed analogues decrease the affinity of receptors for their agonists

Examples of GTP induced reduction in agonist affinity are the hepatocyte vasopressin receptor (Cantou et al., 1980), the f-Met-leu-Phe receptor of neutrophil membranes (Koo et al., 1983) and the substance P receptor in submaxillary glands (Bœhouth & Musacchio, 1985).

1.4.1.3 Potentiation of receptor-stimulated events by GTP and its poorly hydrolysed analogues

Haslam and Davidson (1984) demonstrated that a poorly hydrolysed analogue of GTP (GTP S) stimulated DAG formation from permeabilised platelets. Poorly hydrolysed GTP analogues have been shown to stimulate Ca^{2+} mobilisation in mast cells (Gomperts, 1983) and to activate Limulus photoreceptors on microinjection (Bolsover & Brown, 1982). Poorly hydrolysed GTP analogues have also been shown to stimulate Ins(1,4,5)P_3 generation and synergistically augment the effect of Ca^{2+}-mobilising receptor agonists in permeabilised platelets (Baldessare & Fisher, 1986).

Harden et al. (1988) demonstrated that addition of GTP S to turkey erythrocyte membranes enhanced PtdIns(4,5)P_2 hydrolysis resulting in the rate of generation of inositol bis-, tris- and tetrakis-phosphate to be increased 20-50 fold compared to basal. The P_2-receptor agonist 2-methylthioadenosine triphosphate greatly increased inositol phosphate generation and decreased [^{3}H]PtdIns(4)P and [^{3}H]PtdIns(4,5)P_2 in the presence of a low (0.1uM) concentration of GTP S. This work represents a major step forward as turkey erythrocyte membranes stimulated through P_{2y} receptors are the best isolated membrane system yet identified for analysis of receptor-PIC coupling. These data further suggest that a G-protein is involved in the coupling of certain receptors to PIC.

1.4.1.4 Modulation of receptor-stimulated events by bacterial toxins

Pertussis toxin and cholera toxin (from Bordetella pertussis and Vibrio cholerae, respectively) catalyse the transfer of an ADP-ribose group from NAD^+ to the alpha subunit of particular G-proteins and have been a major tool in the isolation and preliminary identification of G-proteins. Each toxin has a

similar subunit structure consisting of an A (active) subunit and a more complex B (binding) subunit. In each case the B subunit binds to a cell surface receptor and thereby allows the A subunit to enter the cell. Once inside the cell, the A subunit of cholera toxin catalyses ADP-ribosylation of the alpha subunit of G_s, inhibits its intrinsic GTPase activity, and thereby leads to persistent activation of adenylate cyclase. The alpha subunit of G_i is a substrate for ADP-ribosylation by the A subunit of pertussis toxin, and this prevents its interaction with receptor agonists. However, attempts to identify a G-protein essential to PIC activation by this method have been confusing.

In some cell types a pertussis-toxin-sensitive G-protein appears to be involved, for example in mast cells, macrophages, neutrophils and lymphocytes, receptor coupling to PIC is attenuated by pre-treatment of the cells with pertussis toxin (Cockcroft, 1987). Pre-treatment of cells such as neutrophils (Verghese et al., 1985) and CCL39 fibroblasts (Paris & Pouyssegur, 1986) with pertussis toxin resulted in the ADP-ribosylation of a 40kDa G-protein and approximately 50% inhibition of agonist-stimulated PtdIns(4,5)P_2 hydrolysis. Pertussis toxin is without effect on PIC activation in other cells, including GH_3 cells (Martin et al., 1986), pancreas, liver, heart, adipocyte cells (Cockcroft, 1987) and skeletal myoblasts (Gardner et al., 1989).

Low and Hughes (1987) suggested the existence of a novel cholera-toxin sensitive G-protein involved in the coupling of receptors to PIC in pituitary clonal cells, since pre-treatment of these cells with cholera toxin inhibited certain agonist stimulated inositol phosphate generation. A similar inhibition of inositol phospholipid metabolism was observed when L6 cells pre-treated with cholera toxin were stimulated with vasopressin. However, this

effect has been demonstrated to be cAMP mediated and to be a consequence of a reduction in agonist binding sites (Gardner et al., 1989).

As bacterial toxins are of limited use in identifying a G-protein essential to PIC activation more selective probes were required. In the cases where a pertussis-toxin-sensitive G-protein appears to be involved, the G-protein is likely to be one of G_i1, G_i2, G_i3 or G_o. As the alpha-subunits of each of these G-proteins are extremely similar, the use of polyclonal antisera against purified G-protein fractions, while providing more information, did not allow an unequivocable identification of the individual species. Mitchell et al. (1989) used a series of highly selective anti-peptide antisera against sequences which are unique to the various 'G_i-like' G-proteins together with oligonucleotide probes to the mRNAs to assess the expression of G-proteins in membranes of rat adipocytes. They were able to detect each of the specific alpha subunits of G_i1, G_i2, G_i3 and G_o. The availability of such highly selective probes will allow detection of these G-proteins in other systems and should now enable greater progress to be made in assigning a role to each of these G-proteins.

In the majority of cells, pre-treatment with either pertussis toxin or with cholera toxin does not modify agonist generation of inositol phosphates, and no convenient probe is available to identify the relevant G-protein. It is clear that none of the well-characterised G-proteins can be universally responsible for mediating receptor-PIC coupling. The identity of this G-protein (G_p) and the demonstration of its action in a reconstituted system has yet to be achieved.

1.5 The function of the second messengers

The importance of the inositol phospholipid pathway in the activation of mitosis is demonstrated by experiments where microinjection of anti-PtdIns(4,5)P_2 antibodies into NIH-3T3 cells abolished the stimulation of DNA synthesis in response to PDGF and bombesin (Matuoka et al., 1988).

The hydrolysis of PtdIns(4,5)P_2 represents a bifurcation in the signal pathway because both Ins(1,4,5)P_3 and DAG function as second messengers, controlling the two major ionic events which occur prior to the onset of DNA synthesis. Ins(1,4,5)P_3 regulates intracellular Ca^{2+}, whereas the DAG/PKC limb activates the membrane-bound Na^+/H^+ antiporter.

1.5.1 Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4

A characteristic feature of many growth stimuli is that they induce a dramatic increase in the intracellular level of Ca^{2+}. Evidence for Ins(1,4,5)P_3 being the Ca^{2+} mobilising second messenger came from Streb et al. (1983) who first demonstrated that Ins(1,4,5)P_3 applied to permeabilised pancreatic accinar cells caused a rapid release of Ca^{2+} from a vesicular store, thought to be the smooth endoplasmic reticulum. These studies have been repeated on many cell types (including insulinoma, GH_3 and Swiss 3T3 cell lines, hepatocytes and artery smooth muscle cells) and have all shown rapid release of a fraction of the sequestered Ca^{2+} by Ins(1,4,5)P_3, with a half maximal response in the range 0.2-0.8uM (see Berridge & Irvine, 1984). The receptor to Ins(1,4,5)P_3 has been characterised by using labelled Ins(1,4,5)P_3 (Guillemette et al., 1988) and purified from brain homogenates (Supattapone et al., 1988). Using antibodies directed against a purified Ins(1,4,5)P_3-receptor glycoprotein in electron microscope immunocytochemical studies of rat cerebellar Purkinje

cells, Ross et al. (1989) demonstrated that the Ins(1,4,5)P_3 receptor is localised to endoplasmic reticulum, including portions of the rough endoplasmic reticulum and possibly other smooth surfaced structures but not mitochondria or the cell membrane. Furuichi et al. (1989) have now cloned and expressed a functional P_{400} protein from cerebellar Purkinje neurons and have demonstrated that this protein is a receptor for Ins(1,4,5)P_3. Occupancy of these receptors stimulates the release of Ca^{2+} into the cytosol from an Ins(1,4,5)P_3-sensitive Ca^{2+} pool via a mechanism which does not require a G-protein (Palmer & Wakelam, 1989). The vicinal phosphates at the 4,5-positions on the inositol ring are essential for mobilising Ca^{2+} whereas the phosphate at the 1-position enhances the affinity of the molecule for its receptor. Three molecules of Ins(1,4,5)P_3 are required to open one Ca^{2+}-channel (Meyer et al., 1988). A characteristic feature of the Ins(1,4,5)P_3 receptor is that it does not desensitize. Any decline in the release of Ca^{2+} can usually be attributed either to the rapid metabolism of Ins(1,4,5)P_3 or the transfer of the mobilised Ca^{2+} to an Ins(1,4,5)P_3-insensitive pool.

Ins(1,4,5)P_3 possesses the characteristics of a classical second messenger in that it is produced, degraded and brings about its effect rapidly at very low concentrations ($<$1uM). Jackson et al. (1987) have demonstrated similar time-courses for Ins(1,4,5)P_3 production and the increase in Ca^{2+} concentration with Ins(1,4,5)P_3-induced Ca^{2+} release occurring within 2 secs. Kirk et al. (1987) have estimated the half life of Ins(1,4,5)P_3 in liver cells to be 1.2 secs.

Cells responding to Ca^{2+}-mobilising agonists that act through the hydrolysis of PtdIns(4,5)P_2, draw upon both intracellular and extracellular sources of Ca^{2+}. The Ca^{2+}

mobilised from intracellular stores appears to be derived from the $Ins(1,4,5)P_3$-sensitive non-mitochondrial pool. Only part of this intracellular pool seems to be $Ins(1,4,5)P_3$-sensitive, with $Ins(1,4,5)P_3$ stimulating the release of approximately 30-50% of the Ca^{2+} taken up by the non-mitochondrial store, the remainder can be released by Ca^{2+} ionophores. This suggests that separate mechanisms exist for Ca^{2+} uptake and release from vesicular stores. The anatomical location and identity of the $Ins(1,4,5)P_3$-sensitive and -insensitive pools are uncertain. The hypothesis that the endoplasmic reticulum is the $Ins(1,4,5)P_3$-sensitive Ca^{2+} pool has recently been challenged. Volpe et al. (1988) have shown that the calcium binding protein calsequestrin is present within small membrane vesicles termed calciosomes. These vesicles which are found in non-muscle cells, have some properties characteristic of the sarcoplasmic reticulum of muscle. It is still not clear how calciosomes are related to the $Ins(1,4,5)P_3$-sensitive or -insensitive stores. It is not known whether or not the $Ins(1,4,5)P_3$-insensitive pool can contribute to Ca^{2+} signals initiated by $Ins(1,4,5)P_3$. Studies on permeabilised cells have demonstrated that GTP can release Ca^{2+} from both pools independently of $Ins(1,4,5)P_3$ (Chueh & Gill, 1986) and that this release can be blocked by treatments that have no effect on the $Ins(1,4,5)P_3$-sensitive system. With the relative constant levels of GTP in cells, the physiological significance of GTP-induced calcium release in intact cells is doubtful. It has, however, been suggested that GTP may somehow enlarge the $Ins(1,4,5)P_3$-sensitive pool at the expense of the $Ins(1,4,5)P_3$-insensitive pool (Ghosh et al., 1989) and this process may be controlled by $Ins(1,3,4,5)P_4$ (Irvine et al., 1988). The $Ins(1,4,5)P_3$-insensitive pool may also help to

amplify and propagate the calcium signal derived from the $Ins(1,4,5)P_3$-sensitive store by a mechanism of calcium-induced calcium release.

Receptor-stimulated changes in intracellular Ca^{2+} demonstrates two components; a large initial, but transient increase in intracellular Ca^{2+} dependent on $Ins(1,4,5)P_3$-mediated Ca^{2+} release from the internal pool and a delayed phase more dependent on Ca^{2+} influx (Putney, 1986). Both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ have been implicated in controlling this slow entry of external calcium. An association between the $Ins(1,4,5)P_3$-sensitive pool and the plasma membrane is implicated in several models proposed to account for the sustained influx of Ca^{2+} in agonist-stimulated cells, and for the reloading phase of the Ca^{2+} pool after agonist stimulation. In the capacitative model proposed by Putney (1986), Ca^{2+} enters the cell through the $Ins(1,4,5)P_3$-sensitive Ca^{2+} pool; $Ins(1,4,5)P_3$ controls a channel in an endomembraneous compartment which indirectly regulates calcium entry across the plasma membrane through the operation of a negative-feedback loop. $Ins(1,3,4,5)P_4$ at low concentrations increases intracellular Ca^{2+} concentrations provided that a calcium-mobilising inositol tris-phosphate is present (Irvine & Moor, 1986; Irvine, 1989). Irvine and Moor (1986) demonstrated that Ca^{2+}-mediated sea urchin egg fertilisation envelope formation required the microinjection of both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$. This was demonstrated by making use of $Ins(2,4,5)P_3$ which can act at the $Ins(1,4,5)P_3$ receptor to stimulate the release of Ca^{2+}, but unlike $Ins(1,4,5)P_3$, is not a substrate for $Ins(1,4,5)P_3$-3-kinase and thus cannot be used to generate $Ins(1,3,4,5)P_4$ in the cell. Irvine et al. (1988) proposed an extension of Putney's capacitative

model to include a role for $Ins(1,3,4,5)P_4$ suggesting that $Ins(1,3,4,5)P_4$ controls the transfer of Ca^{2+} between intracellular pools. The contribution of $Ins(1,3,4,5)P_4$ to intracellular Ca^{2+} mobilisation is as yet unknown.

1.5.2 The DAG/PKC limb of the pathway

PKC is not a single enzyme but exists as a large family of multiple isoforms with closely related structures (Nishizuka, 1988), that may have a distinct tissue expression and intracellular localisation. Molecular cloning techniques have identified at least seven isoforms within a given species (α, βI, βII, γ, δ, ε, ζ). The general structure of PKC isoforms consists of a single polypeptide chain with four conserved (C) regions and five variable (V) regions. PKCs α, βI, βII and γ show similar effector dependence and substrate specificity, although there are relative differences that are likely to affect cellular responses. The apparent K_m values for ATP and Mg^{2+} and the K_a values for phosphatidylserine are similar for PKC, α, βI, βII and γ. PKC α, βI, βII and γ isoforms are all dependent upon DAG or phorbol ester for activation (Huang *et al.*, 1988). However, under different experimental conditions there is a variation in the response of PKC isoforms to DAG and phorbol ester implying that there may be differences between the isoforms in the binding of these effectors. *In vivo* this may depend upon the composition of the membranes with which the PKCs interact.

Recent work by Nishizuka's laboratory has indicated that the acyl chain structures of the DAGs which activate the multiple forms of PKC may be different (Nishizuka, 1988). There may be other mechanisms to activate PKC; *in vitro* the γ-isoform (major form in brain) has been shown to be activated by oleic acid, arachidonic acid and the arachidonate-derived eicosanoid lipoxin A. The lack of a requirement for Ca^{2+} or DAG in this activation

implies additional mechanisms of PKC regulation that are not necessarily linked to inositol phospholipid turnover. The nature of the activation by fatty acids is complex. However, Seifert et al. (1988) have shown (on a mixture of PKC isoforms) that they may act simply to provide a lipid environment by substitution for phosphatidylserine. Some substrates can be phosphorylated in a Ca^{2+}-independent manner, the implication being that the absolute requirement of Ca^{2+} in vivo in the activation of PKC isoforms may depend upon the particular substrate. The Ca^{2+} dependence of the PKC isoforms appears to be complex as different research groups have obtained different K_a values for Ca^{2+} with the PKC α, βI, βII and γ isoforms e.g. 200nM (Jaken & Kiley, 1987) to 500nM (Huang et al., 1988). This covers a range from near-resting to near-maximally stimulated levels for most cells. Thus mobilisation of Ca^{2+} would be likely to influence the extent to which these PKC isoforms can be activated in response to DAG, at least with respect to substrates that are 'Ca^{2+}-dependent' in their phosphorylation.

PKCs δ, ε, and ζ have only been identified through cDNA cloning (e.g. Schaap et al., 1989) and much less is known about their kinetic and physical properties. Schaap et al. (1989) have shown that both the phorbol binding and kinase activities of PKC-ε are independent of Ca^{2+}.

The pleiotropic effects of PKC in cell regulation may be shared by the various isoforms, which might control distinct signalling pathways, and/or transduce specific extracellular signals.

DAG generated as a result of mitogen-stimulated PtdIns(4,5)P_2 hydrolysis is the physiological activator of the Ca^{2+}- and phospholipid-dependent (particularly phosphatidylserine) PKC (Nishizuka, 1984). In keeping with its role as a second messenger, very rapid increases in the level of DAG have been

measured upon stimulation (Wright et al., 1988; Cook et al., 1989). The DAG is present in membranes only transiently; within a minute of formation DAG is rapidly removed by re-cycling into PtdIns or by lipases which de-acylate it to generate arachidonic acid for synthesis of prostaglandins, thromboxanes and leukotriens (Irvine, 1982) which may have effects on DNA synthesis. With the rapid metabolism of DAG, PKC is active for only a short time after stimulation of the receptor. However, the consequence of this enzyme activation may persist for a long time depending on the biological stability of the phosphate that is covalently attached to each substrate protein molecule. It has been proposed that upon generation of DAG, PKC is translocated from the cytosol (where it is normally inactive) to the membrane (see e.g. Farrar et al., 1985) where it can actively phosphorylate its substrates on selected threonine and serine residues. However, the precise intracellular topography of PKC is unknown since the enzyme is usually extracted in the presence of high concentrations of a Ca^{2+} chelator to prevent proteolysis by the Ca^{2+} dependent protease, calpain. Recent immunocytochemical analysis using monoclonal antibodies against PKC indicates that intracellular localisation of this enzyme varies with cell types. Therefore, more detailed studies of the precise location of the interaction of the Ca^{2+}/phospholipid dependent PKC with DAG are required and it seems more likely that PKC is plasma membrane associated and simply more firmly bound as a result of its interaction with DAG. Following proteolysis by calpain, two fragments are obtained; a 51kDa catalytically active domain and a 26kDa hydrophobic domain which encompasses the regulatory and membrane binding regions.

DAG molecules with an sn,1,2 configuration with various fatty acids of different chain length are capable of activating PKC,

with those having an unsaturated fatty acid at either position 1 or 2 being most active (Mori et al., 1982). It is postulated that one molecule of DAG can activate one molecule of PKC in the presence of greater than four but less than ten molecules of phosphatidylserine. The effect of DAG is to increase the apparent affinity of PKC for Ca^{2+} (Kishimoto et al., 1980). However, prior elevation of intracellular Ca^{2+} can augment the activation by DAG (Dougherty & Niedel, 1986). The fact that PKC is Ca^{2+} dependent demonstrates the interaction of the two limbs of the phospholipid signalling pathway i.e. the effect of Ca^{2+} and DAG on PKC.

Mitogenesis only occurs if there is persistant activation of PKC, which requires a maintained increase in cellular DAG levels, and it is important to note that the inositol phospholipid pathway is not the only stimulated source of DAG in cells (See Fig.1.2). Many agonists that stimulate PtdIns(4,5)P_2 metabolism have now also been shown to activate the hydrolysis of phosphatidylcholine (PtdCho) in various cell types (see Pelech & Vance, 1989 for review). The phorbol ester TPA can itself stimulate PtdCho breakdown (Grove & Schimmel, 1982; Muir & Murray, 1987). This suggests that stimulation of PKC by DAG could lead to further generation of DAG by activating PtdCho breakdown. Mitogen-stimulated PtdIns(4,5)P_2 hydrolysis has been demonstrated to be a transient effect which is desensitised within a few minutes (Brown et al., 1987). However, a detailed examination of Ins(1,4,5)P_3 and sn-1, 2-diacylglycerol levels within bombesin stimulated Swiss 3T3 cells has demonstrated that the DAG level remained elevated for at least 60 min whilst the Ins(1,4,5)P_3 had returned to basal levels within 5 min (Cook et al., 1989). The finding that inositol phospholipids are not the sole source of DAG for PKC activation and the fact that particular isoforms may be specific for particular types of DAG supports the

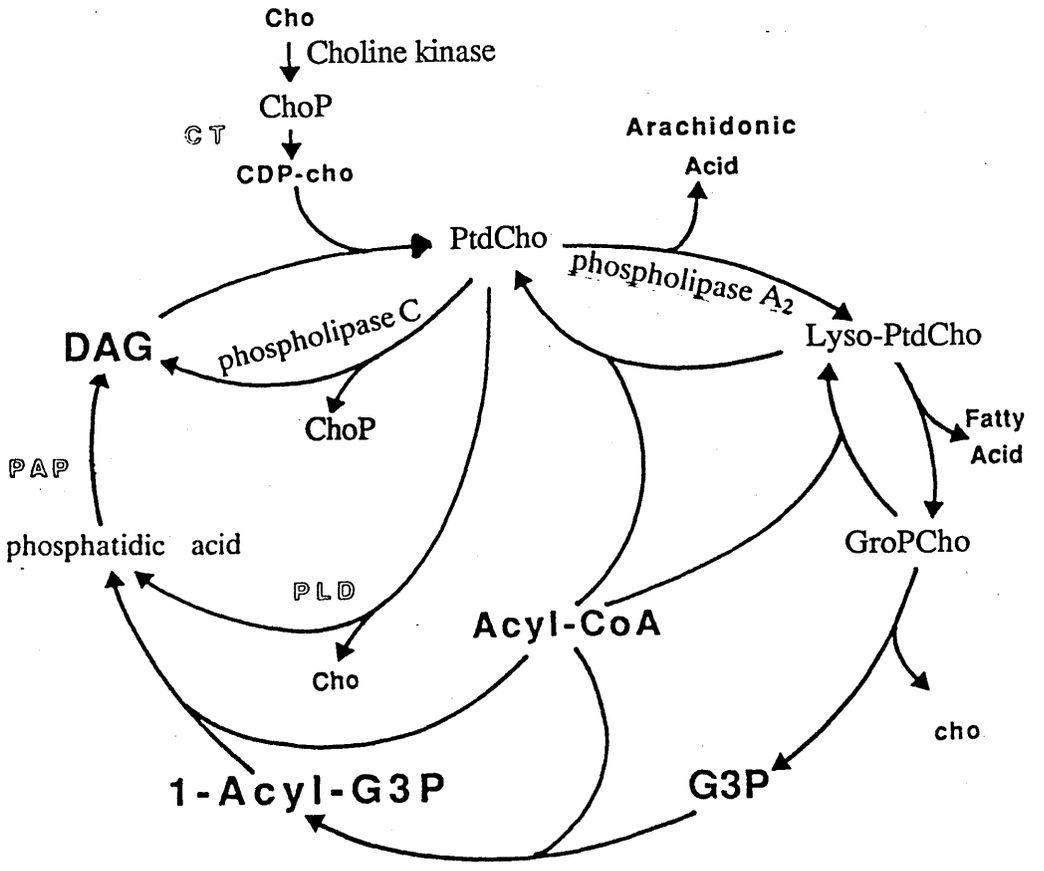


Figure 1.2 Pathways of Phosphatidylcholine metabolism

The figure shows the PtdCho cycles for generation of DAG. The DAG can be generated directly via PtdCho specific phospholipase C or by the action of PLD to yield phosphatidic acid, which is cleaved to DAG by phosphatidic acid phosphohydrolase (PAP). PtdCho can also be hydrolysed by phospholipase A_2 to yield Lyso-PtdCho and arachidonic acid. Lyso-PtdCho may be re-esterified to PtdCho or catabolized to GroPCho, which can be further degraded to glycerol 3-phosphate (G3P) and Cho. G3P can be converted back to DAG via phosphatidic acid synthesis. Phosphorylation of Cho by choline kinase results in the formation of ChoP, which subsequently reacts with CTP to form CDP-Cho, catalysed by CTP : ChoP cytidylyltransferase (CT). DAG can react with CDP-Cho to complete another PtdCho turnover cycle.

possibility that the various PKC isoforms relate to different signalling pathways.

PKC is a ubiquitous enzyme which plays a pivotal role in cell adjustment to the environment. The importance of PKC in the stimulation of cell proliferation is demonstrated by the fact that tumour-promoting phorbol esters, when intercalated into the cell membrane, may substitute for DAG and permanently activate PKC, since they bind to the regulatory domain of the enzyme. A further indication that PKC is involved in cell growth is the observation that the anti-neoplastic lipoidal amine CP-46, 665-1 is a potent inhibitor of this enzyme (Shoji et al., 1985). While there is convincing evidence for the involvement of PKC in the control of cell proliferation, the targets of the enzyme remain mostly undetermined. An important site of action of PKC is the activation of the Na^+/H^+ antiporter (see Section 1.2). Vinculin is also phosphorylated in vivo (Werth & Pastan, 1984) and it is possible that this phosphorylation is involved in cytoskeletal reorganisation, an event stimulated by tumour promoting phorbol esters (Schliwa et al., 1984).

PKC activation exerts both positive and negative effects on cellular events; one function of PKC appears to be intimately related to negative feedback control of cell surface receptors coupled to PtdIns(4,5)P_2 hydrolysis and also receptors of other signalling systems. Phosphorylation of adrenergic receptors by PKC and/or protein kinase A (mediator of cAMP action) is thought to play an important role in the modulation of adrenergic transmission. Leeb-Lundberg et al. (1986) showed that agonist-mediated desensitization of the α_1-adrenergic receptor (AR), coupled to PtdIns(4,5)P_2 hydrolysis, is associated with increased phosphorylation of α_1-AR. Stimulation of DDT_1 MF-2 cells

(smooth muscle cells) with bradykinin, lead to desensitization and phosphorylation of the α_1-AR. Phorbol esters, which activate PKC, also promote phosphorylation of the α_1-AR and its uncoupling from stimulation of PtdIns(4,5)P_2 hydrolysis in DDT_1 MF-2 cells (Leeb-Lundberg et al., 1985). Bouvier et al. (1987) demonstrated that α_1- and β_2-AR purified from DDT_1 MF-2 cells and hamster lungs respectively, can be directly phosphorylated in vitro by both protein kinase A and PKC. Although protein kinase A phosphorylates the α_1-AR, β_2-AR agonists do not promote this reaction (Bouvier et al., 1987). The functional significance of this phosphorylation is unknown. Phorbol esters promote phosphorylation of β_2-AR in conjunction with modulation of β_2-agonist-stimulated adenylate cyclase activity in avain erythrocytes (Kelleher et al., 1984). This suggests that PKC activated by PtdIns(4,5)P_2 hydrolysis, stimulated by various receptors, may also contribute to some forms of heterologous phosphorylation and desensitization of the β_2-AR. Both sensitization (e.g. Sugden et al., 1985) and desensitization (e.g. Kelleher et al., 1984) of the adenylate cyclase system have been reported following phorbol ester treatment. Both α_1-AR and β_2-AR are substrates for protein kinase A and PKC. However, agonist occupancy of the two receptors facilitates their phosphorylation only by the protein kinase coupled to their own signal transduction pathway. The phosphorylation occurs almost exclusively on serine residues and amounts to 2-3 moles phosphate/mol receptor. Phosphorylation of the α_1-AR by PKC or phosphorylation of the β_2-AR by protein kinase A is without effect on its agonist binding properties (Sibley et al., 1987).

These observations support the notion that PKC contributes to the desensitization of the α_1- and β_2-adrenergic

transduction pathways respectively. Negative feedback control by PKC also extends to the EGF receptor, where PKC phosphorylates the receptor (Hunter et al., 1984) inhibiting receptor tyrosine kinase activity (Downward et al., 1985) and decreasing the apparent binding affinity of the receptor for EGF (e.g. Brown et al., 1984).

1.5.3 Interaction between $Ins(1,4,5)P_3/Ca^{2+}$ and DAG/PKC pathways

The two limbs of the inositol phospholipid signal transduction pathway may act separately to produce their individual physiological responses, but they can also act synergistically. The cooperative effect of both signal pathways has been shown by the use of Ca^{2+} ionophores and phorbol esters to activate cellular processes normally activated by an agonist (Berridge, 1987b). Nishizuka (1984) demonstrated that, in platelets, the combination of a phorbol ester and a Ca^{2+} ionophore induces a maximal secretion of 5-hydroxytryptamine at concentrations that have no effect when each drug is administered alone. Combination of a Ca^{2+} ionophore and phorobol ester have since been shown to activate many different cellular processes, including DNA synthesis and cell proliferation (see Berridge, 1987b). The relative importance of each pathway may also vary with time, in that calcium may be responsible for initiation whereas DAG may be more important in maintaining a physiological response. The interaction between Ca^{2+} and PKC can be demonstrated by their effects on the Na^+/H^+ antiporter. Owen et al. (1989) demonstrated that NIH-3T3 cells use both an inositol trisphosphate, Ca^{2+}-mediated pathway and a PKC-dependent pathway to regulate Na^+/H^+ exchange. In Swiss 3T3 cells, PDGF and bombesin caused a rapid cytoplasmic acidification, due to increased cytoplasmic Ca^{2+}, followed by a slower alkalinisation (Ives & Daniel, 1987). Protons are generated by cellular

metabolism which is enhanced by growth factors and it has been shown that an increase in the internal concentration of H^+ can activate the Na^+/H^+ antiporter of plasma membrane vesicles of renal proximal tubule cells (Aaronson et al., 1982) and intact human fibroblasts (Moolenaar et al., 1982) in a cooperative fashion. However, it has been demonstrated that mitogen-induced alkalinisation of cells by activation of the Na^+/H^+ antiporter is not affected directly by Ca^{2+} or Ca^{2+}/Calmodulin changes (Ives & Daniel, 1987; Mitsuhashi & Ives, 1988), but reduced intracellular Ca^{2+} will reduce growth factor induced activation of the Na^+/H^+ antiporter in vascular smooth muscle cells (Mitsuhashi & Ives, 1988). This is most likely due to the fact that PKC is a Ca^{2+} dependent enzyme and only when there is sufficient Ca^{2+} will PKC activate the antiporter via a phosphorylation reaction. Transformed cells have the capacity to grow in lower concentrations of Ca^{2+}, which may mean either that they have elevated levels of Ca^{2+} or that their responsiveness to Ca^{2+} has been enhanced.

Growth factors such as PDGF stimulate the rapid transcription of oncogenes such as c-myc (Kaibuchi et al., 1986) and c-fos (Grinstein et al., 1988). Their expression can also be induced by stimulating cells either with a phorbol ester (Kelly et al., 1983) or with a Ca^{2+} ionophore (Greenberg & Ziff, 1984). The fact that myc and fos are oncoproteins capable of cell transformation provides further evidence to support the idea that the inositol lipid signal pathway plays a significant role in controlling cell proliferation.

1.6 The involvement of oncogenes in signal transduction

As discussed in the previous sections, the actions of certain growth factors are mediated via the activation of the inositol phospholipid signal transduction pathway. Therefore, loss of normal regulation of the pathway may lead to a loss in regulation of mitogenesis and conceivably to the uncontrolled growth and structural transformation typical of cancer. About 25 transforming genes or oncogenes have been identified and it is thought that certain oncogenes subvert the normal functioning of the inositol phospholipid pathway to bring about uncontrolled cellular growth. The identification of the proteins encoded by oncogenes and an investigation into what functions they serve will enable a greater understanding of the molecular events that convert normal cells to cancer cells. This section examines the evidence for the effects of oncogene products upon signal transduction.

1.6.1 Autocrine stimulation of growth

Mammalian cell growth is under three types of control: endocrine, paracrine and autocrine. These can be exerted either alone or in combination with each other. In endocrine control, hormones are released from endocrine organs such as the pituitary gland and carried by the blood to target receptors some distance from the endocrine organ. Paracrine control is exerted by one cell on adjacent cells by the release of growth factors. For example, in response to wounding, platelets release PDGF which acts on receptors of neighbouring epithelial cells and fibroblasts to stimulate cell proliferation and wound healing.

In autocrine control, cells respond to substances that they themselves release. Autocrine signalling is usually confined to pathological conditions. Certain tumour cells, for instance, synthesise and release growth factors that are required for normal

cellular growth and division, but these growth factors stimulate the uncontrolled proliferation of the tumour cell itself, as well as adjacent normal cells, in a paracrine fashion and cause a tumour to form.

It has been demonstrated that multiple growth factors are required for the maximum stimulation of proliferation (Pledger et al., 1978; Leof et al., 1982). Unless the cells are neoplastically transformed, more than one growth factor supplement is necessary for growth (Barnes & Sato, 1980). Van Zoelen et al. (1988) demonstrated that in normal rat kidney (NRK) cells none of the growth factors tested (EGF, type β transforming growth factor (TGF-β), PDGF and retinoic acid) was able to induce loss of density-dependent inhibition of growth by itself, but a strong synergism was observed when combinations of three of the above four growth factors were tested. Therefore, exposure of a cell to one growth factor can lower the threshold for mitogenicity of a second growth factor (Wharton et al., 1983). Moreover, growth factors operate at different points of the cell cycle. For example, transient treatment of fibroblasts with PDGF will induce a stable state 'competence' whereby cells are responsive to other circulating plasma-derived factors (O'Keefe & Pledger, 1983).

The most commonly produced autocrines by tumour cells include type α transforming growth factor (TGF-α), peptides related to PDGF, bombesin and TGF-β. Loss of requirement for specific growth factors is a common property of many types of cancer cell (Kaplan et al., 1982) and may be mediated by (a) the activation of autocrine growth factor synthesis , (b) synthesis of an altered growth factor receptor, or (c) activation of a post-receptor pathway that bypasses the growth factor receptor requirement.

1.6.1.1 Activation of autocrine growth factor synthesis

PDGF is a polypeptide sufficient in some cells to induce both DNA synthesis and cell division. It is thought that most transformed mesenchymal cells produce PDGF or PDGF-like molecules (Shipley et al., 1985). The first demonstration of a direct involvement of an oncogene product with the inositol phospholipid pathway came from the observation that the sequence of the protein encoded by the sis oncogene is almost identical with the N-terminal 109 amino acid residues of the β-chain of PDGF.

Expression of this oncogene can cause increased stimulation of hydrolysis of PtdIns(4,5)P_2 via PDGF receptors and provides direct evidence for the role of polypeptide growth factors in cellular transformation (Leal et al., 1985).

TGF-α is a secreted polypeptide that was discovered in the medium of certain retrovirus-transformed fibroblasts (Delarco & Todaro, 1978). Expression of the TGF-α gene has been demonstrated in a variety of tumours which are mostly carcinomas. The consistent expression of the TGF-α gene in squamous cell carcinomas and renal carcinomas is frequently accompanied by elevated EGF receptor expression. The effects of TGF-α are mediated through the EGF receptor, but the release and action of TGF-α and its relationship to transformation is unclear.

Experiments with cells expressing temperature sensitive mutant rodent sarcoma viruses demonstrate the relationship between the release of TGF-α and transformation. In these experiments, TGF-α was not released into the medium unless cells were grown at a temperature permissive for transformation. Thus, the strict dependency of TGF-α secretion on the transformed state of these sarcoma cells suggests that viral oncogene products such as p37^{mos} and p21$^{ki\text{-}ras}$ control TGF-α synthesis at either the

transcriptional or translational level (Ozanne et al., 1980; Delarco et al., 1981; Kaplan et al., 1982). These observations, together with the fact that TGF-α and -β cause the induction of reversible phenotypic transformation of NRK fibroblasts, have reinforced the belief that TGF-α synthesis is associated with malignant transformation.

TGF-β (Anzano et al., 1985) and PDGF-like molecules (Bowen-Pope et al., 1984) are also released from rodent cells transformed by Moloney, Harvey or Kirsten viruses. The PDGF-like molecules bind to the PDGF receptor(s) and activate both PtdIns(4,5)P_2 hydrolysis and the receptor associated tyrosine kinase.

The mode of action of TGF-β is however less clear. It has been demonstrated both to stimulate the growth of fibroblasts (Tucker et al., 1983) and to inhibit cell proliferation of, for example, normal and certain tumour-derived epithelial cell lines (Tucker et al., 1984). The expression of these two activities is modulated by other growth factors and is not solely dependent on cell type or conditions of anchorage-dependent versus anchorage-independent growth. In myc transfected rat fibroblasts TGF-β can stimulate anchorage-independent growth in the presence of PDGF, but it is anti-proliferative in the presence of EGF (Roberts et al., 1985). The effect of TGF-β upon cellular signalling is unclear although a recent report has suggested that it may activate PKC by an unidentified mechanism in immature rat brain (Markovac & Goldstein, 1988). This effect upon PKC could explain the inhibition of EGF-stimulated growth through a mechanism involving phosphorylation and transmodulation of the EGF receptor.

The importance of autocrines in maintaining the malignant state is demonstrated by the use of the polyanionic drug suramin.

This drug has been shown to block the binding of EGF, TGF-α and PDGF to their receptors. It also causes dissociation of these growth factors prebound to their receptors (Betsholtz et al., 1986). Suramin was used to demonstrate that growth and transformation of fibroblasts by the sis oncogene is dependent upon growth factor-receptor interaction, whereas transformation by viral ras is not.

The inhibition of growth of cancer cells by a receptor antagonist has also been accomplished with small-cell lung carcinoma cells in culture. These 'oat cell' cancers secrete gastrin-releasing peptide which is thought to be the mammalian equivalent of the amphibian peptide bombesin (Moody et al., 1981). This peptide is a potent mitogen and stimulates PtdIns(4,5)P_2 hydrolysis in both Swiss 3T3 and NIH-3T3 cells (Brown et al., 1984); Wakelam et al., 1986). A monoclonal antibody raised against bombesin inhibits the growth of 'oat cell' cultures and the development of tumours in nude mice can be prevented by injection of this antibody (Cuttitta et al., 1985).

1.6.1.2 Oncogene products as receptors

Cells can be transformed by increasing receptor number or by modifying existing receptors. Some oncogene products have been found to be cell surface receptors or an altered form of the receptor.

Activation of receptors can sometimes occur in the absence of growth factors. Sequence homology between the v-erb-B gene product and the cellular receptor for EGF (Downward et al., 1984) suggests that the erb-B product resembles a truncated EGF receptor, which lacks the EGF binding and autophosphorylation domains. The tyrosine kinase domain is intact and seems integral to cell transformation (Livneh et al., 1986). The erb-B product may mimic

the receptor's activity without the appropriate signal from EGF.

Evidence confirming this came from experiments involving the integration of an intact ALV genome into the c-erb-B locus in a manner that would lead to the over-expression of a truncated EGF receptor under control of the introduced ALV promoter (Raines et al., 1985). Cells expressing a truncated growth factor receptor might be constitutively activated to generate mitogenic signals, independent of its ligand.

The protein product of the c-fms oncogene has been identified as the cell surface receptor for the heomatopoietic stem cell growth factor CSF-1 (Sherr et al., 1985); the v-fms oncogene may encode an altered form of the receptor.

1.6.1.3 Activation of post receptor pathways that bypass the requirement for growth factors

Many oncogenes exert their effects via tyrosine phosphorylation. However, the major substrates for these kinases remain to be identified. Some of the src-related proto-oncogenes may encode enzymes involved in the increased intracellular formation of Ins(1,4,5)P_3 and DAG (Berridge & Irvine, 1984). Treatment of fibroblasts with PDGF causes a 40-fold increase in c-myc mRNA levels within 2h (Kelly et al., 1983) and a similar increase in c-fos mRNA levels within 45 min (Greenberg & Ziff, 1984). Recent evidence has implicated the expression of c-myc and c-fos in the control of DNA synthesis as they bind to DNA at specific sites (Heldin et al., 1987). These oncogenes are co-expressed with other oncogene products, for example, c-myc and the ras oncogene are co-expressed in transformed primary and secondary rat embryo cells (Land et al., 1983). Thompson et al. (1989) introduced myc and ras oncogenes via infection with recombinant retroviruses into controlled, but small, numbers of cells from murine urogenital sinus with subsequent

reconstitution of these cells into differentiated prostate *in vivo*. When *ras* and *myc* oncogenes were introduced alone, alterations resembling different types of premalignant lesions resulted. However, when introduced together agressively growing tumours of various cell types occurred, even when less than 100 out of 1.5×10^6 cells initially became infected. This indicates that oncogene co-operativity is a necessary event for cell transformation in some systems. The existence of oncogene products as either growth factors, growth factor receptors and the post-receptor machinery (i.e. $p21^{ras}$, $p55^{c\text{-}fos}$, $p75^{c\text{-}myb}$ and $p58^{c\text{-}myc}$) indicates that the growth factor regulatory pathways are crucial in the development and maintenance of tumourogenesis and indicates the importance of tight regulation of these pathways in normal cell growth.

1.6.2 Negative regulators of cell growth

Malignant transformation may be the result not only of excessive production, expression and action of positive autocrine growth factors, but also of the failure to express or respond to specific negative regulators of cell growth.

TGF-β is a growth regulator that may function in an autocrine manner and it is bifunctional having both stimulatory and inhibitory growth regulatory activities depending on the cell type (Roberts *et al.*, 1985). TGF-β has been shown to inhibit the proliferation of MCF-7 breast cancer cells (Sporn *et al.*, 1987) and has been demonstrated to inhibit the growth of NIH-3T3 cells (Liboi *et al.*, 1988). Sporn and Roberts (1985) proposed that TGF-β could be oncogenic 'by defect' if a decrease in its expression or activity were to release cells from proliferative constraints that TGF-β may normally impose.

A fibroblast growth regulator (FGR) has been isolated from medium conditioned by exposure to density-inhibited Swiss-3T3 cells that will inhibit growth in an autocrine manner. A rat antibody (2A4) specifically bound FGR and was found to neutralise the growth inhibitory effect of FGR in a concentration-dependent fashion (Hsu et al., 1984).

Other growth inhibitors have been partially purified and characterised. These include (i) a glycopeptide from bovine cerebral cortex cells, that inhibits protein synthesis and cell growth of normal but not transformed cells (Kinders & Johnson, 1982); (ii) a heparin-like molecule, produced by cultured endothelial cells, that inhibits the growth of smooth muscle cells (Willems et al., 1982) and (iii) a lipid molecule on the plasma membrane of lymphoid tumour cells (Stallcup et al., 1984).

These growth inhibitors may act by binding to cell surface receptors triggering an intracellular signal that regulates the initiation of DNA synthesis and cell division. Alternatively, the growth inhibitor may modulate/counteract the mitogenic action of growth factors by altering the concentration of free growth factor available for interaction with its specific growth factor receptor.

1.7 The involvement of the ras gene products in signal transduction

Three functional ras genes are found in the human genome, c-Ha-ras-1, C-Ki-ras-2 and c-N-ras, and all are expressed at low levels in most cells (Hall et al., 1983). Their classification depends on the extent of homology with the oncogenes of the Harvey and Kirsten sarcoma viruses, and with the transforming gene first encountered in the SK-N-SH neuroblastoma cell line. Around 10-20% of human tumours have been shown to contain ras genes, with the majority of these being mutated genes. Approximately 1% have been

detected as having amplified normal cellular genes (proto-oncogenes) (Barbacid, 1987).

Each *ras* gene encodes a similar 21kDa protein, termed $p21^{ras}$. The *ras* proteins contain 189 amino acids (188 in the case of Ki-*ras*-2) and are identical for the first 85 amino acid residues. The next 80 amino acid N-terminal residues are highly conserved being approximately 85% homologous. In contrast, the final C-terminal amino acids share little homology, except for the last four amino acids where a conserved cysteine residue is found at position 186. Cysteine 186 is post-translationally palmitoylated, and this acylation is essential for association of the $p21^{ras}$ proteins with the plasma membrane and their function. Mutation of cysteine 186 to serine has been shown to abolish acylation, membrane binding and transformation (Willumsen *et al.*, 1984). Magee *et al.* (1987) proposed that an active acylation-deacylation cycle exists and suggested that *in vivo* acylation may act transiently during signalling to modify the interaction of $p21^{N\text{-}ras}$ with the membrane. Alternatively, interaction with other components of a signal-transducing mechanism without actual dissociation from the membrane could be modified.

The precise function of $p21^{ras}$ is still unclear, although evidence exists for a role in signal transduction pathways (Feramisco & Gross, 1984; Wakelam *et al.*, 1986; Fleischman *et al.*, 1986; Lloyd *et al.*, 1989). Their biochemical properties are similar to the properties of the alpha subunit of the G-proteins. Both $p21^{ras}$ and the alpha subunit of G-proteins bind guanine nucleotides (GTP and GDP) (Willingham *et al.*, 1980) and display an intrinsic GTPase activity at similar rates (e.g. Gibbs *et al.*, 1984). Both are attached to the inner leaflet of the plasma membrane and also share some sequence homology.

The binding of GTP appears to be crucial for the ability of p21^{ras} to promote cell proliferation and malignant transformation. Microinjection of antibodies directed against a putative GTP binding domain of p21 inhibits GTP binding and reverses the malignant phenotype of NIH-3T3 cells transformed by ras oncogenes (Feramisco et al., 1985). Many mutations are known to activate the oncogenic potential of p21^{ras} by causing severely impaired GTPase activity; these include any change at residue 12 except proline, threonine at residue 59 and lysine, leucine or arginine at position 61 (Gibbs et al., 1985). Amino acid sequences show that the glycine at the position corresponding to residue 12 of c-Ha-ras is conserved in p21^{ras}, G_{t1}, G_{t2} and the alpha subunit of G_s, supporting the idea that the two sets of proteins are functionally homologous.

Lacal et al. (1986), in a series of deletion experiments using p21$^{Ha\text{-}ras}$, demonstrated that regions 5-23 and 152-165 were absolutely required both for GTP binding and cell transformation.

The transforming mutants of ras genes are characterised by having a greatly reduced GTPase activity and this led to the proposal that transformation by p21^{ras} is the result of abnormal levels of p21^{ras} in an active, GTP bound state. However, analysis of GTPase activities associated with a large number of p21 mutants failed to reveal a quantitative relationship between GTPase activity and transforming activity. A cytosolic protein, GTPase activating protein (GAP), which has been found in all cells examined, stimulates the GTPase activity of normal glycine 12-p21$^{N\text{-}ras}$, but is without effect upon its transforming mutants aspartic 12 and valine 12-p21$^{N\text{-}ras}$ (Trahey & McCormick, 1987). It appears that the major effect of position 12 mutations is to prevent this protein from stimulating p21-GTPase activity, thereby

allowing these mutants to remain in the active GTP-bound state. GAP has been purified from the cytosolic fraction of bovine brain and human placenta and the complementary DNA cloned (Gibbs et al., 1988; Vogel et al., 1988). GAP is a 125kDa monomeric polypeptide that catalytically stimulates normal ras GTPase activity 100-fold. The GTP complexes, but not the GDP complexes, of normal and oncogenic ras proteins bind to GAP with micromolar affinities (Vogel et al., 1988). The oncogenicity of mutant $p21^{ras}$ may be due to the conformational change induced at the mutation site which prevent GAP binding, thus leading to persistent GTP binding to the ras protein. The GAP protein is thought to act at a region between amino acids 30-40 on $p21^{ras}$ as this interaction of GAP at this 'effector site' is attenuated in $p21^{ras}$ with effector site mutations (Cales et al., 1988; Adari et al., 1988). These results strongly implicate that GAP is a candidate for a ras effector protein, and functions downstream in a signal transduction pathway. Regions of GAP share amino acid similarity with the non-catalytic domain of adenylate cyclase from the yeast Saccharomyces cerevisiae. This might explain the paradox that ras proteins from mammalian and yeast cells can function in heterologous systems through different effectors (DeFeo-Jones, 1985). If the sequence similarity is functionally significant, then GAP might act as an intermediate between $p21^{ras}$ and the catalytic domain of some effector. The presence within GAP of sequences conserved among the non-receptor tyrosine kinases, the crk oncogene product and phospholipase C-148 raises the possibility that GAP shares certain of the functional, perhaps regulatory, properties of these effector molecules.

The role of $p21^{ras}$ in cell proliferation, transformation and differentiation is not yet understood. Oncogenic $p21^{ras}$, but

not normal p21^{ras} proteins, can induce terminal differentiation of a rat pheochromocytoma cell line (Bar-Sagi & Feramisco, 1985). Both oncogenic p21^{ras} and nerve growth factors induce differentiation after a lag of 15-24h. Therefore it is possible that ras oncogenes may switch on the same signal transduction pathway used by nerve growth factors. Such a conclusion is supported by the observation that the anti-ras antibody (Y13-259) inhibits neurite formation induced by nerve growth factors (Hagag et al., 1986).

Mulcahey et al. (1985) demonstrated that NIH-3T3 cells induced to divide by adding serum to the culture medium are unable to enter the S phase of the cell cycle after microinjection of anti-ras antibody, demonstrating that p21^{ras} plays an important role in regulating entry into S phase. The p21^{ras} proteins are also necessary for transformation with other oncogenes: for example, NIH-3T3 cells transformed by v-src cannot enter S phase when microinjected with anti-ras Y13-259 antibodies (Smith et al., 1986).

Several lines of evidence support the idea that p21^{ras} functions in a G-protein-like manner coupling the receptors for certain growth factors to the stimulation of PIC. This proposal was raised when it was realised that PIC activation is important in control of growth by normal proliferative stimuli and by oncogenes (Berridge & Irvine, 1984). Support for an effect on PIC is the observation that transformation of Balb-3T3 fibroblasts with the EJ-Ha-ras oncogene increases the responsiveness of PIC to carbamylcholine stimulation, resulting in elevated levels of inositol phosphates compared to control cells (Chiarugi et al., 1985). Fleishman et al. (1986) showed that both NRK and NIH-3T3 cells stably transformed by three different ras genes always display

a steady state increase in the levels of DAG and inositol phosphates relative to their precursor PtdIns(4,5)P_2. Preiss et al. (1986) and Lacal et al. (1987a) have also demonstrated that there are raised levels of DAG in cells transformed by ras genes. Wakelam et al. (1986) showed that an amplification of the PIC response to growth factors can also be achieved by the over-production of normal p21$^{N\text{-}ras}$, under the control of a steroid inducible promoter. In the presence of the inducer dexamethasone, p21$^{N\text{-}ras}$ is over-expressed and the cells ($T15^+$) become transformed (McKay et al., 1986). $T15^+$ cells over-expressing p21$^{N\text{-}ras}$ showed no change in basal inositol phosphate production, but exhibited an increased responsiveness to several growth factors, particularly bombesin, compared to cells grown in the absence of the inducer ($T15^-$). This increase is observed with no accompanying change in the number or affinity of the bombesin receptors on each cell. It has now been shown that the elevated inositol phosphate response to bombesin is a consequence of increased PtdIns(4,5)P_2 hydrolysis leading to an elevated release of intracellular stored Ca^{2+} (Lloyd et al., 1989). These results are consistent with the proposal that p21^{ras} may directly or indirectly increase the coupling of certain growth factor receptors to PIC and that in normal cells the concentration of this protein is an important factor that regulates the intensity of signalling through the inositol phospholipid pathway.

Transformation of NIH-3T3 cells with activated, point-mutated N- or Ha-ras did not increase the apparent coupling of receptors to PIC, but it appeared to constitutively activate PIC resulting in an enhanced basal rate of production of total inositol phosphates (Hancock et al., 1988; Wakelam, 1988). Transformation or differentiation in experimental systems can be induced by mutant

p21^{ras}, but it requires larger amounts of normal p21^{ras} to achieve the same effects. The mutated ras proteins may transform cells by providing a necessary limiting factor for the constitutive activation of signal transduction pathways or by suppressing inhibitory control mechanisms.

The role of the ras proteins in amplifying inositol phospholipid turnover has, however, been questioned as differences in inositol phospholipid metabolism have been reported for ras transformed cells. Parries et al. (1987) showed that transformation of NIH-3T3 cells by viral Ha- or Ki-ras caused the desensitization of the PDGF-stimulated inositol phosphate response with a markedly attenuated mitogenic response while the bradykinin response was amplified. Receptor binding studies demonstrated that the number of high affinity bradykinin binding sites was increased in the v-Ha-ras transformed cells, whilst there was no change in PDGF binding. Benjamin et al. (1988) observed a similar desensitization in EJ-ras transformed NIH-3T3 cells and they suggest that EJ-ras p21 may uncouple the PDGF receptor from PIC resulting in inhibition of PDGF-stimulated activity of PIC, Ins(1,4,5)P_3 generation and Ca^{2+} mobilisation. The bombesin-stimulated inositol phosphate response in T15^{+} cells is desensitized by high density culturing (Wakelam, 1988). This desensitization can be reversed by culturing in the presence of the growth factor antagonist suramin and it has been suggested (Wakelam, 1988) that the effect is induced by the known secretion of autocrines by ras-transformed cells (Owen & Ostrowski, 1987). This effect of autocrines is probably mediated by PKC since phorbol esters can mimic the desensitization induced by high density culturing. Morris et al. (1989) have demonstrated that activation of PKC occurs within 10 min of treatment with TPA or scrape-loading

[val-12]p21^{ras} into Swiss 3T3 cells. Price et al. (1989) demonstrated that this activation of PKC by TPA or [val-12]p21^{ras} leads to a desensitization of growth factor-stimulated inositol phosphate generation. Brown et al. (1987) demonstrated that prolonged treatment of Swiss 3T3 cells with TPA, to down regulate PKC levels, leads to an increase in both basal and growth factor-stimulated levels of inositol phosphates, presumably due to a decrease in feedback regulation of phosphoinositide metabolism. The variability of effects of transfecting ras proteins into fibroblasts upon inositol phospholipid metabolism may therefore be due to desensitization mediated by PKC.

Increased DAG levels have been consistently observed in ras-transformed cells. Wolfman and Macara (1987) show that NIH-3T3 cells transformed by Ha- or Ki-ras possess elevated DAG levels without significant increases in the level of inositol phosphates. Wolfman and Macara (1987) also demonstrate that the basal phosphorylation of the PKC substrate 80kDa protein was significantly increased in all the ras transformed cells examined. However, when the ras transformed cells were incubated with TPA, phosphorylation of the 80kDa protein was reduced, suggesting that DAG elevation induces partial down-regulation of PKC activity in these cells. Lacal et al. (1987c) demonstrated the importance of PKC activity in ras induced transformation. They demonstrated that the mitogenic activity of the Ha-ras oncogene in p21^{Ha-ras}-microinjected quiescent Swiss 3T3 cells is markedly reduced under conditions in which PKC is down-regulated by chronic phorbol ester treatment. The mitogenic activity was restored when both Ha-ras and purified PKC were co-injected, demonstrating that the mitogenic response to Ha-ras requires functional PKC. Lacal et al. (1987b) have also examined the effects of microinjection of Ha-ras into Xenopus

oocytes. Increases in inositol phosphate generation were detectable within 2 min and reached a maximum after 6 min, but then appeared to be desensitized. DAG levels examined in parallel experiments demonstrated a similar rapid increase. However, the increased DAG generation was maintained for at least 20 min post-injection. Lacal et al. (1987a) demonstrated reproducible increases in DAG levels in the absence of detectable increases in inositol phosphates in Ha-ras transformed NIH-3T3 cells. These observations suggest that DAG might be generated from the metabolism of phospholipids other than PtdIns(4,5)P_2 (see Section 1.5.2).

Therefore the biochemical properties, cellular location and their involvement in mitogenesis, transformation and differentiation indicate that $p21^{ras}$ may function in a G_p-like manner regulating second messengers in signal transduction.

Research Aims

Despite extensive research, the mechanism of action of ras is still unknown. It has been proposed that $p21^{ras}$ proteins function in a G-protein-like manner mediating the effects of certain growth factors, by coupling their receptors to the stimulation of PIC. To study the role of $p21^{ras}$ in regulating second messengers as part of a signal transduction mechanism, NIH-3T3 fibroblasts, which have been transformed by constitutive expression of the c-Ha-ras-1, c-Ki-ras-2 or N-ras genes, were used. These cell lines are transformed by a several-fold over-expression of the normal ras proteins. Wild-type NIH-3T3 fibroblasts which express Ha-, Ki- or N-ras at very low levels were used as a control.

In chapter three the effect of over-expression of normal ras genes in NIH-3T3 fibroblasts upon inositol phospholipid hydrolysis was examined. The cell line H8/22, an inducible Ha-ras expressing clone of the NIH-3T3 cell line was also used to study the

effects of short term transformation by the ras proto-oncogene. NIH-3T3 cells transformed by over-expression of ras genes demonstrated a reduced $PGF_{2\alpha}$ -stimulated inositol phosphate response and this was further investigated.

This led to an investigation of the role of inositol phospholipid turnover in the stimulation of cell proliferation (Chapter four).

In Chapter five PtdCho metabolism in NIH-3T3 cells transformed by over-expression of ras genes was studied.

The aim of this study was to provide additional 'clues' as to the exact function of the ras proto-oncogene product p21 and to determine its role in lipid signalling pathways.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Materials and cell lines

Cells:

NIH-3T3, EC816, EC806, EC807, H8/22, Ki858, Ki860, N866 N872, T15	Kindly donated by C.J. Marshall, Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London SW3 6JB
AmNIH-3T3	Kindly donated by D.W. Stacey, Roche Institute of Molecular Biology, Nutley, New Jersey 07110, U.S.A.
Swiss 3T3	Kindly donated by K.D. Brown, A.F.R.C. Institute of Animal Physiology, Brabraham, Cambridge CB2 4AT, U.K.

Amersham International plc, Amersham, Buckinghamshire, England

myo[^{3}H]Inositol (specific activity = 22.8Ci mmol^{-1})

myo[^{3}H]Inositol 1-monophosphate (specific activity = 1Ci mmol^{-1})

myo[^{3}H]Inositol 1,4-bisphosphate (specific activity = 1 or 44Ci mmol^{-1})

myo[^{3}H]Inositol 1,4,5-trisphosphate (specific activity = 52Ci mmol^{-1}

myo[^{3}H]Inositol 1,3,4,5-tetrakisphosphate (specific activity = 1Ci mmol^{-1})

[methyl-^{3}H]Thymidine (specific activity = 1Ci mmol^{-1})

[methyl-^{3}H]Choline Chloride (specific activity = 76Ci mmol^{-1})

[^{3}H]Prostaglandin $F_{2\alpha}$ (specific activity = 180Ci mmol^{-1})

BDH Chemical Company, Poole, England

Ammonium formate dimethylsulphoxide, EDTA (Ethylenediamine tetra-acetic acid), Hepes (N-2-hydroxyethylpiperazine-N^1-2 ethane sulphonic acid), lithium chloride, orthophosphoric acid, potassium hydrogen phosphate, potassium dihydrogen phosphate, perchloric acid, sodium hydroxide, sodium carbonate, trichloroacetic acid and universal indicator.

Biomedical Technologies Ltd., England

Epidermal Growth Factor

Bioprocessing Ltd., Consett, England

Platelet Derived Growth Factor

Boehringer (UK) Ltd., Lewes, England

ATP and Tris

Cambridge Research Biomedicals, Cambridge, England

Bradykinin, bombesin and gastrin releasing peptide

Formachem (Research International) Plc, Strathhaven, Scotland

Boric acid, D-glucose and sodium hydrogen carbonate

FSA Laboratory Supplies, Loughborough, England

OptiPhase 'Hisafe' 3

Flow Laboratories, Rickmansworth, England

0.2 micron filters

Gibco, Paisley, Scotland

All cell culture medium including amino acids mixture (50x) and glutamine (100x)

Koch-Light Ltd., Suffolk, England

Calcium chloride, magnesium sulphate, potassium chloride and sodium potassium tartrate

Laserchrom, East Grinstead, England

Whatman Partisil 5 Wax column

May and Baker, Dagenham, England

Formic acid, hydrochloric acid and sodium tetraborate

National Diagnostics, Aylesbury, England

Ecoscint

Riedel-DettaenAg Seelze-Hannover, Germany

Magnesium chloride and sodium dihydrogen carbonate

Whatman Ltd., Maidstone, England

0.45 micron millipore filters

Pierce and Warriner, London, England

Micropor (Spectrapor 3) dialysis tubing

All other chemicals were obtained from Sigma Chemical Company, Poole, England

2.2 Cell lines and methods of cell culture

2.2.1 Cell lines

NIH-3T3 mouse fibroblasts, which have been transformed by constitutive expression of the c-Ha-*ras*-1, c-Ki-*ras*-2 or N-*ras* were used. These cell lines are transformed by a several fold over expression of the normal *ras* proteins. Wild type NIH-3T3 cells which express Ha-, Ki- and N-*ras* at very low levels were used as a control.

Swiss mouse 3T3 fibroblasts and NIH-3T3 (AmNIH-3T3) cells obtained from America (gifted by Dennis W. Stacey, Roche Institute of Molecular Biology, Nutley, New Jersey 07110) were also used. Table 2.1 lists the cell lines used.

T15 and H8/22 cell lines, which are both clones of NIH-3T3 cells were also used. The T15 cell line contains the normal human foetal N-*ras* proto-oncogene under the transcriptional control of a glucocorticoid-inducible promoter MMTV-LTR (murine mammary tumour virus long terminal repeat). The H8/22 cells contain the normal human foetal Ha-*ras* proto-oncogene under the control of the same

Table 2.1 Cell lines

Cell type	*ras*-Gene
NIH-3T3	
AmNIH-3T3	
Swiss 3T3	-
EC806	
EC807	normal Ha-*ras*
EC816	
H8/22+	
Ki858	
Ki860	normal Ki-*ras*
N866	
N872	normal N-*ras*
T15+	

glucocorticoid-inducible promoter. Using the steroid dexamethasone the expression of N-ras in the T15 cells or Ha-ras in the H8/22 cells can be switched on and off and the cellular concentration of $p21^{ras}$ can be controlled. Cells grown in the absence of the inducer dexamethasone contain negligible human $p21^{N\text{-}ras}$ ($T15^-$) or $p21^{Ha\text{-}ras}$ ($H8/22^-$) and resemble wild-type NIH-3T3 cells in morphology and growth characteristics. In cells grown in the presence of 2uM ($T15^+$) or 100nM ($H8/22^+$) dexamethasone there is a vast over production of the $p21^{ras}$ amounting to 1-2% of the plasma membrane protein and the cells exhibit the transformed phenotype.

2.2.1.1 T15 cells

Dose-response curves for the effect of dexamethasone on $p21^{N\text{-}ras}$ expression and on bombesin stimulated inositol phosphate generation in T15 cells were performed by Wakelam et al. (1986). Addition of increasing concentrations of dexamethasone to T15 cells previously grown without this inducer ($T15^-$) resulted in a concentration dependent increase in expression of the human N-ras proto-oncogene as determined by immunoprecipitable $p21^{N\text{-}ras}$ (Wakelam et al., 1986). Maximum expression of $p21^{N\text{-}ras}$ and bombesin-stimulated inositol phosphate generation was found in cells grown in the presence of 1.5uM dexamethasone for 48h. Routinely 2uM dexamethasone was used to induce $p21^{N\text{-}ras}$ expression in T15 cells.

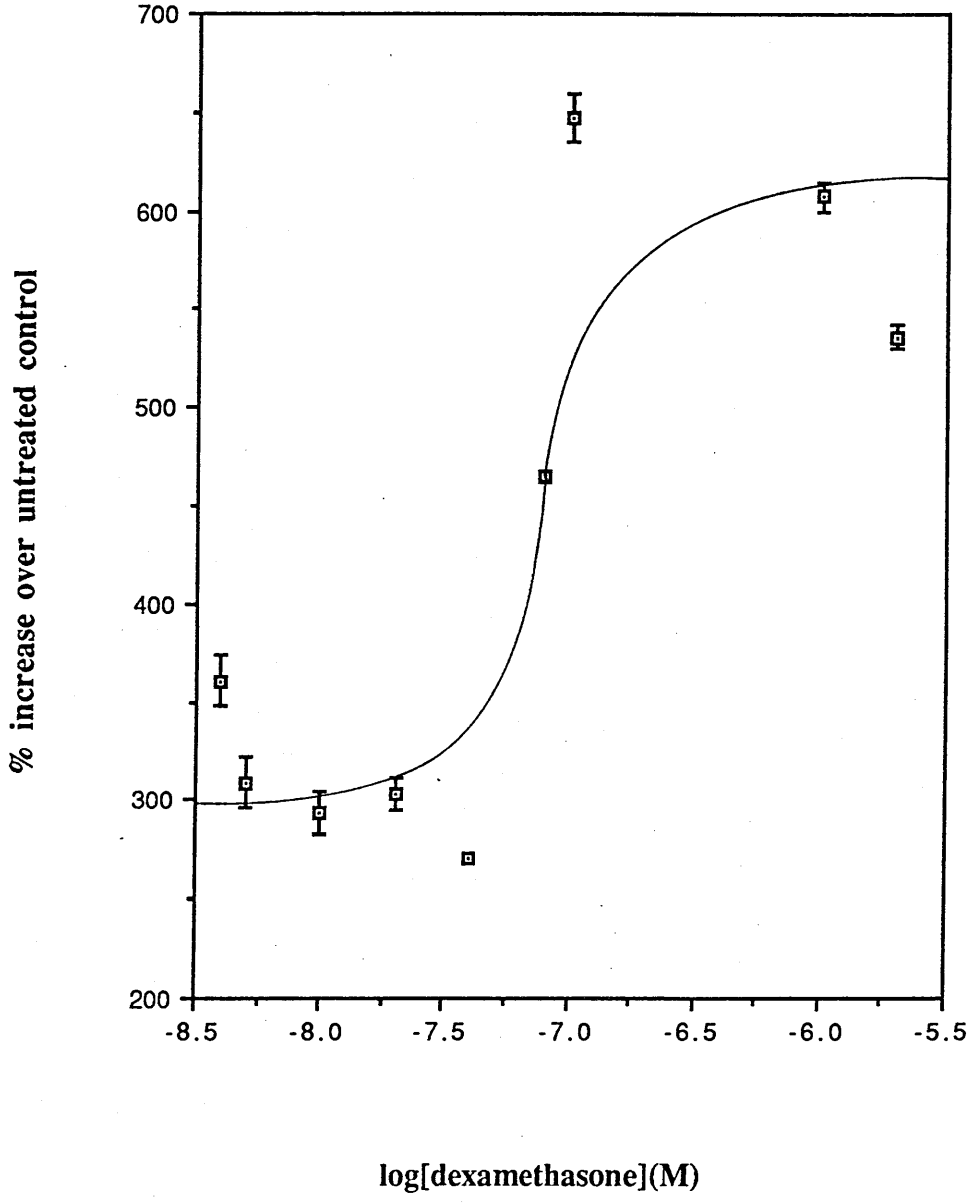
2.2.1.2 H8/22 cells

Figure 2.1 shows the dose-response curve for the effect of dexamethasone on PDGF-stimulated inositol phosphate generation in H8/22 cells. Maximum PDGF-stimulated inositol phosphate generation was found in cells grown in the presence of 100nM dexamethasone for 24h. In subsequent experiments 100nM dexamethasone was used.

Figure 2.1 Dose response curve for the effect of dexamethasone on PDGF-stimulated inositol phosphate generation in H8/22 cells

Cells were labelled with [^{3}H]inositol (1uCi ml^{-1}) in the presence of the stated concentration of dexamethasone for 24h. PDGF (1.32ug ml^{-1}) stimulated inositol phosphate generation was determined as described in Section 2.6, sample preparation, Method A and the results are means $\pm$ S.D. of one experiment where n=4.

Figure 2.1


700
600
500
400
300
200
-8.5
-8.0
-7.5
-7.0
-6.5
-6.0
-5.5
% increase over untreated control
log[dexamethasone](M)

C.J. Marshall (Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London) confirmed that in this cell line addition of 100nM dexamethasone for 24h to H8/22 cells previously grown without this inducer (H8/22) resulted in maximum expression of $p21^{Ha\text{-}\underline{ras}}$.

2.2.2 Cell Culture

All cells were grown as monolayers on plastic surfaces at 37°C in a 5% (v/v) CO_2, 95% humidified atmosphere in Dulbecco's modified Eagle's Medium (DMEM) containing donor newborn calf serum 10% (v/v) for NIH-3T3, AmNIH-3T3 and Swiss 3T3 cells and 5% (v/v) for NIH-3T3 cells transformed by overexpression of the Ha-, Ki- and N-ras genes.

2.2.3 Cell passage

The 'spent' medium from confluent flasks (approximately 10^6 cells per $25cm^2$ flask) was removed by aspiration. Trypsin solution (1-2ml) containing 0.1% trypsin (w/v), 0.025% EDTA (w/v) and 10mM glucose in phosphate buffered saline (pH7.3) was added to each flask, and left at 37°C for approximately 2-3 min. Serum-containing medium (3ml) was added and the cells collected by centrifugation at 800g in an MSE Centaur centrifuge for 5 min, resuspended in DMEM containing serum and plated as required. An Industrial D Coulter counter was used to determine the cell concentration if necessary.

2.2.4 Maintenance of cell lines

Confluent cells were passaged as above and the cells resuspended in biofreeze vials at a density of 10^6 cells ml^{-1} in freezing medium (8% DMSO (v/v), 20% calf serum (v/v) and 72% DMEM (v/v)). The vials were packed in cotton wool, placed in polystyrene boxes, frozen overnight at -80°C and then transferred to the vapour phase of a liquid nitrogen container for storage.

When frozen cells were to be recovered, a vial was removed from the liquid nitrogen container and placed into a 37^{o}C water bath for thawing. The cell suspension was aseptically transferred to 25cm^{2} flasks containing 4ml fresh serum-containing medium and placed into the incubator at 37^{o}C.

2.2.5 Preparation of inositol free calf serum

Donor newborn calf serum was dialysed to remove inositol, for use in experiments where cells were labelled with [^{3}H]inositol.

Calf serum was aliquoted into dialysis tubing in 50ml lots and dialysed against four changes of 500ml of a 1:20 (v/v) dilution of a 20x strength Earle's salt solution (see Buffer composition section) in distilled, deionised water adjusted to pH7.4 with 1M sodium hydroxide. The dialysed calf serum, was filter sterilised using a 0.2 micron filter unit and stored frozen.

2.3 [^{3}H]thymidine incorporation

Cells were plated on to 24 well plates at a density of 10^{5} cells per well. The medium was changed 24h later to one containing 0.5% (v/v) calf serum and the cells were left for three days to become quiescent. They were then washed in DMEM and incubated in fresh DMEM, containing the appropriate additions together with 1uCi ml^{-1} [^{3}H]thymidine, 0.4ng ml^{-1} EGF, 0.4ug ml^{-1} transferrin and 0.04ng ml^{-1} insulin. The medium was removed 24h later and the monolayers were washed twice with Hanks Buffered Saline. The monolayers were then washed four times with 1ml of 5% (w/v) trichloroacetic acid and the cells solubilised in 1ml of 0.3M sodium hydroxide. The solubilised cells were removed to a scintillation vial containing 0.2ml of 1.5M hydrochloric acid and the radioactivity determined by scintillation counting in 10ml of ecoscint scintillation fluid.

2.4 Growth curves

Cells were plated on to 35mm dishes at a density of 10^4 cells per dish, and the medium was changed 24h later to one containing the ligand being tested. If the cells were to be incubated serum free, the following additions were also made; 0.4ng ml^{-1} EGF, 0.4ug ml^{-1} transferrin and 0.04ng ml^{-1} insulin. At 24h intervals, cell number was determined following trypsinisation, by counting in an Industrial D Coulter counter.

2.5 Separation of inositol phospholipids

2.5.1 Sample preparation

Two confluent $25cm^2$ flasks of NIH-3T3 and T15 cells were used for each time point. The cells were labelled for 0, 2, 8, 12, 24 and 36h prior to the experiment with 5uCi ml^{-1} of [^{3}H]inositol in inositol and serum free DMEM. After the labelling period, the cells were washed twice with Hanks buffered saline, scraped and collected by centrifugation at 800g for 5 min. The cells were resuspended in 1.5ml Hank's buffered saline containing 1% (w/v) bovine serum albumin and 10mM glucose. Triplicate 0.5ml aliquots were dispensed into plastic scintillation vials. The cells were killed with 500ul (20% w/v) ice-cold trichloroacetic acid and samples kept on ice at all times after this stage.

(a) The samples were vortex-mixed and centrifuged at 2000g in an MSE Centaur 2 for 5 min. The supernatant was discarded and the pellet washed with 1ml of ice-cold 5% (w/v) trichloroacetic acid/1mM EDTA. The samples were centrifuged as above.

(b) The supernatant was discarded and the pellet washed with 1ml ice-cold distilled water. The samples were centrifuged as above.

(c) The supernatant was discarded and 1ml of chloroform:methanol:hydrochloric acid (100:100:1, v/v/v) was added. The samples were vortex-mixed and left at room temperature for 20 min. The samples were then centrifuged and the supernatant removed to clean tubes.

(d) 0.33ml chloroform:methanol:hydrochloric acid (200:100:1, v/v/v) was added to the remaining pellet from step (c). The tubes were vortex-mixed and the supernatant was added to the supernatant from the previous wash and combined.

(e) 0.5ml chloroform and 0.37ml 0.1M hydrochloric acid were added to the combined supernatants. The samples were vortex-mixed, allowed to phase split and the upper phase discarded.

(f) 0.7ml of Folch's Synthetic Upper Phase was added to each of the samples and the tubes vortex-mixed and centrifuged.

(g) The aqueous upper layer was discarded and the samples could be stored frozen at this stage under nitrogen.

Folch Synthetic Phase

The following were mixed in the ratios below (v/v/v):

chloroform:methanol:0.1M hydrochloric acid, 10:5:3

The two phases were stored separately at room temperature.

2.5.2 Separation of the polyphosphoinositides

(a) Hydrolysis of polyphosphoinositides to glycerophosphoinositol monophosphate (GroPtdInsP) and glycerophosphoinositol bisphosphate ($GroPtdInsP_2$)

The samples from step (g) above were dried down in a Hetovac centrifuge and the following were added: 0.5ml chloroform, 100ul methanol, 100ul 1M sodium hydroxide in methanol. The samples were vortex-mixed and left for 15 min to allow deacylation to proceed. The following were then added: 0.5ml chloroform, 0.3ml

methanol, 0.3ml water and the samples were vortex-mixed centrifuged in a Hettich microfuge at 16000g for 5 min.

0.5ml of the upper phase of each sample was transferred to clean tubes and the samples neutralised by the addition of 190ul of 0.1M boric acid. 150ul of 3M ammonium formate and 125ul of 0.1M sodium tetraborate were added to each sample and vortex-mixed. Then, 1.5ml of distilled water was added and the samples mixed again. This procedure reduced the ionic strength of the samples to permit binding of the samples to the ion exchange chromatographic resin (see below). The samples could be stored frozen at this stage of the assay.

(b) Elution of GroPtdInsP and GroPtdInsP$_2$ from Dowex formate columns

The samples were loaded onto 1ml Dowex-1-formate columns and washed with 2x 17ml of 0.18M ammonium formate 5mM sodium tetraborate. Following this, GroPtdInsP was eluted with 15ml of 0.35M ammonium formate/0.1M formic acid and GroPtdInsP$_2$ was eluted with 20ml of 1M ammonium formate/0.1M formic acid. The amount of radioactivity in each sample was determined upon a known volume of each fraction by liquid scintillation counting.

2.6 Determination of inositol phosphate production

The generation of inositol phosphates was determined by two methods, both of which gave identical results. This methodology permitted the determination of accumulated inositol phosphates which has been shown to be the most reliable and easily applied measure of receptor stimulated inositol lipid hydrolysis.

Sample preparation, Method A

Cells were cultured in 25cm^2 flasks and labelled by incubation in inositol free DMEM containing dialysed calf serum and 1uCi ml^{-1} [^{3}H]inositol for 24h. The monolayers were washed

twice with Hank's buffered saline (37°C) and scraped with a teflon coated spatula into the same buffer containing 10mM glucose and 1% (w/v) bovine serum albumin (HBG). The cells were collected by centrifugation in an MSE Centaur 2 centrifuge at 800g for 5 min, washed in the same buffer and resuspended in HBG containing 10mM lithium chloride. Following a 5 min incubation at 37°C, the cell suspension (containing approximately $2x10^5$ cells ml^{-1}) was dispensed into plastic insert vials containing agonist or buffer and incubated in a shaking water bath at 37°C. Incubations were terminated after the appropriate time by the addition of chloroform:methanol (1:2 v/v). The vials were vortex-mixed and left to extract for 20 min at room temperature.

Extraction of inositol phosphates

The accumulation of radiolabelled inositol phosphates was determined upon an aliquot of the upper aqueous phase by batch chromatography on Dowex 1 x 8-formate resins. 0.8ml of this phase was transferred to a clean vial and 2.2ml of distilled water were added. 0.25ml of Dowex was added to each tube, vortex-mixed and left until the Dowex had settled. The supernatant was removed and 3ml 60mM ammonium formate/5mM sodium tetraborate added to the samples and the supernatant aspirated off when the Dowex had settled. This step was repeated once more and then the Dowex was washed four times with 3ml distilled water. Inositol phosphates were eluted from the Dowex with 3x 0.6ml of 1M ammonium formate/0.1M formic acid. Radioactivity in each sample was determined by scintillation counting in 12ml of ecoscint.

The effect of storage at 4 and -18°C on total radiolabelled inositol phosphates in the upper aqueous phase was checked, and they were found to remain stable for at least 14 days.

Extraction of inositol phospholipids, Method A

The radioactivity associated with the inositol phospholipids was determined by scintillation counting of 250ul of the lower chloroform phase following air drying.

Sample preparation, Method B

Cells were cultured on 24 well plates and grown to a density of 10^5 cells per well prior to incubation in inositol free DMEM containing dialysed calf serum and 1uCi ml^{-1} [^{3}H]inositol for 24h. The monolayers were washed twice with Hank's buffered saline (37^{o}C) and then incubated in HBG containing 10mM lithium chloride for 5 min prior to the addition of agonist or buffer to give a final volume of 150ul.

Following incubation for the appropriate time, at 37^{o}C, incubations were terminated by rapid aspiration of the medium followed by the addition of 100ul of ice-cold 10% (w/v) perchloric acid. The cells were scraped, transferred to an Eppendorf tube and each well rinsed twice with 200ul of distilled water this being transferred to the same Eppendorf tube. The tubes were centrifuged for 4 min at 16000g in a Hettich microfuge at 4^{o}C and 550ul of the supernatant from each sample was transferred to a separate tube containing 130ul of 10mM EDTA (pH7). The samples were neutralised by adding 360ul of a 1:1 (v/v) mixture of Freon and tri-n-octylamine, followed by vigorous mixing of the separate phases on a vortex mixer (Sharpes and McCarl, 1982). After centrifugation for 1 min at 4000g in a Hettich microfuge the upper phase containing the radiolabelled inositol phosphates was removed for analysis as Method A.

Extraction of inositol phospholipids, Method B

Lipid extraction of the cell pellet by addition of 470ul of chloroform:methanol:hydrochloric acid (40:80:1, v/v/v). The

samples were vortex-mixed and left to extract overnight at 4°C. The phases were split by addition of 155ul chloroform and 155ul distilled water and an air dried 250ul aliquot of the lower chloroform phase was counted as Method A.

2.7 Separation of inositol phosphates

NIH-3T3 cells were plated on to 24 well plates at a cell density of $7.5x10^4$ cells per well. The medium was changed 24h later to inositol-free DMEM containing 2uCi ml^{-1} of [^{3}H]inositol, 0.4ng ml^{-1} EGF, 0.4ug ml^{-1} transferrin and 0.04ug ml^{-1} insulin. Following a 24h incubation period, the monolayers were washed twice in Hank's buffered saline and then incubated at 37°C for 5 min in HBG and 10mM lithium chloride. Cells were stimulated with 2.1uM $PGF_{2\alpha}$ in HBG containing 10mM lithium chloride in a final volume of 50ul. Following incubation for the appropriate time at 37°C incubations were terminated by the addition of 50ul ice-cold 10% (w/v) perchloric acid. The cells were scraped, transferred to an Eppendorf tube and each well rinsed three times with 75ul of distilled water, this being transferred to the same Eppendorf tube.

The tubes were centrifuged for 4 min at 16000g in a Hettich microfuge at 4°C and 275ul of the supernatant from each sample was transferred to a separate tube containing 65ul of 10mM EDTA (pH7). The samples were neutralised by adding 185ul of a 1:1 (v/v) mixture of Freon and tri-n-octylamine, followed by vigorous mixing of the separate phases on a vortex mixer. After centrifugation for 1 min at 4000g in a Hettich microfuge the upper phase containing the radiolabelled inositol phosphates was removed for analysis. If the samples were run on Dowex columns 1ml of 5mM sodium tetraborate/0.5mM EDTA was added.

The radioactivity associated with the inositol phospholipids was determined by scintillation counting of 250ul of the lower phase following air drying.

Separation of inositol phosphates from cell extracts were carried out by two methods:

2.7.1 Separation of inositol phosphates using Dowex-formate resin

Samples were applied to 1ml Dowex-formate columns and the following [^{3}H]labelled compounds eluted as follows:

i) Inositol was eluted with 12ml of distilled water

ii) Inositol and glycerophosphoinositol were eluted with 12ml of 60mM ammonium formate/5mM sodium tetraborate

iii) Inositol monophosphates were eluted with 18ml of 0.2M ammonium formate/0.1M formic acid

iv) Inositol bisphosphates were eluted with 18ml of 0.4M ammonium formate/0.1M formic acid

v) Inositol trisphosphates were eluted with 15ml of 0.8M ammonium formate/0.1M formic acid.

The amount of radioactivity in each sample was determined by liquid scintillation counting.

The elution profile of [^{3}H]-labelled inositol phosphate standards from a Dowex-formate column is shown in Figure 2.2.

2.7.2 Separation of inositol phosphates using HPLC

HPLC analysis was performed using a Whatman Partisil 5 Wax column employing a non-linear step gradient from 1-35% (v/v) 1M diammonium phosphate adjusted to pH3.7 with orthophosphoric acid (solution B). Samples were loaded onto the column and eluted at a

Figure 2.2 Resolution of [^{3}H]inositol phosphate standards by Dowex anion exchange chromatography

The figure shows the elution of tritiated inositol 1-monophosphate standards from Dowex formate columns using 18ml of 0.2M ammonium formate/0.1M formic acid applied in 1ml aliquots. Tritiated inositol 1,4-bisphosphate was eluted using 18ml of 0.4M ammonium formate/0.1M formic acid and tritiated inositol 1,4,5-trisphosphate was eluted using 15ml of 0.8M ammonium formate/0.1M formic acid applied in 1ml aliquots. Known amounts of each standard were added to acid extracts of unlabelled cells. This figure indicates that there is clear resolution of inositol 1-monophosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate. Recoveries of [^{3}H]-labelled standards were greater than 95%. Results are from a single experiment typical of three.

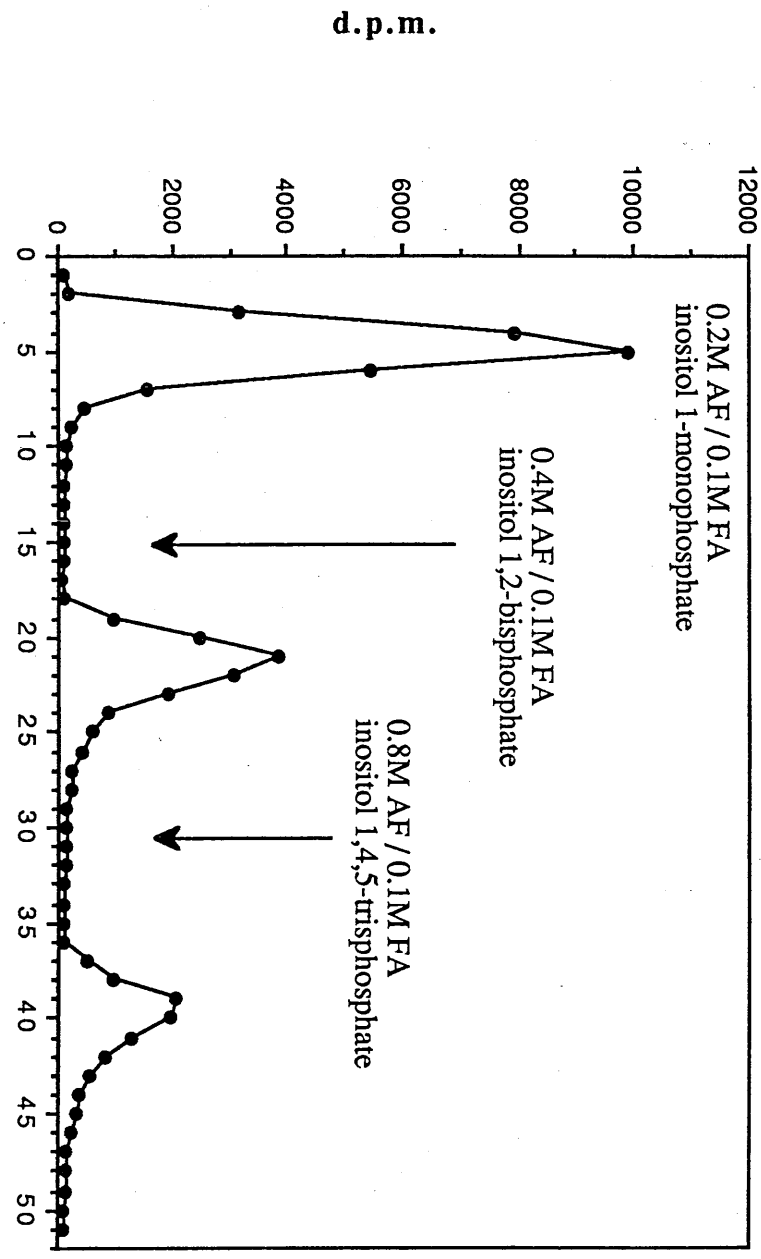

d.p.m.
12000
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
30
35
40
45
50
elution volume (ml)
0.2M AF / 0.1M FA
inositol 1-monophosphate
0.4M AF / 0.1M FA
inositol 1,2-bisphosphate
0.8M AF / 0.1M FA
inositol 1,4,5-trisphosphate

Figure 2.2

flow rate of 1ml min^{-1} as follows:-

0 - 10 min 1% B

10 - 11 min linear gradient to 8% B

11 - 21 min 8% B

21 - 22 min linear gradient to 17% B

22 - 32 min 17% B

32 - 34 min linear gradient to 35% B

After each run, the column was eluted with 35% B for an additional 3 min and then eluent was returned linearly to 1% B over 2 min. 1% B was run through the column for a further 10 min before the next sample was injected.

All column solutions were filtered through 0.45 micron Whatman filters prior to use. Fractions of 0.25ml were collected at 15 sec intervals. The radioactivity in each sample was determined by liquid scintillation counting. The identity of fractions was determined by running standard [^{3}H]-labelled samples of Ins(1)P, Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(1,3,4)P_3 (gifted by R.F. Irvine, Department of Biochemistry, AFRC Institute of Animal Physiology, Brabraham, Cambridge CB2 4AT) and Ins(1,3,4,5)P_4. The elution profile of [^{3}H]-labelled inositol phosphate standards from this column is shown in Figure 2.3. Recoveries of the inositol phosphates were greater than 95%.

2.7.3 Preparation of [^{3}H]-labelled inositol 1,3,4,5-tetrakisphosphate standard for HPLC

[^{3}H]-labelled Ins(1,3,4,5)P_4 was prepared by incubating [^{3}H]Ins(1,4,5)P_3 with the 100000g supernatant of homogenised bovine adrenal cortex. The incubation buffer consisted of 0.25M sucrose, 20mM $MgCL_2$, 10mM ATP and 50mM Tris (pH9.0). At pH9 the Ins(1,4,5)P_3 kinase retains its activity while the phosphatases are inactive, resulting in an almost 100% conversion of

Figure 2.3 Separation of [^{3}H]inositol phosphate standards by HPLC

The figure shows the separation of [^{3}H]inositol phosphate standards by HPLC using a diammonium phosphate gradient as previously described. The data was obtained from the resolution of [^{3}H]inositol phosphate standards eluted from a Partisil 5 Wax column using the gradient described and peaks of inositol 1-monophosphate (A), inositol 1,4-bisphosphate (B), inositol 1,3,4-trisphosphate (C), inositol 1,4,5-trisphosphate (D) and inositol 1,3,4,5-tetrakisphosphate (E) are indicated. The dotted line indicates the elution gradient used. Results are from a single experiment typical of three.

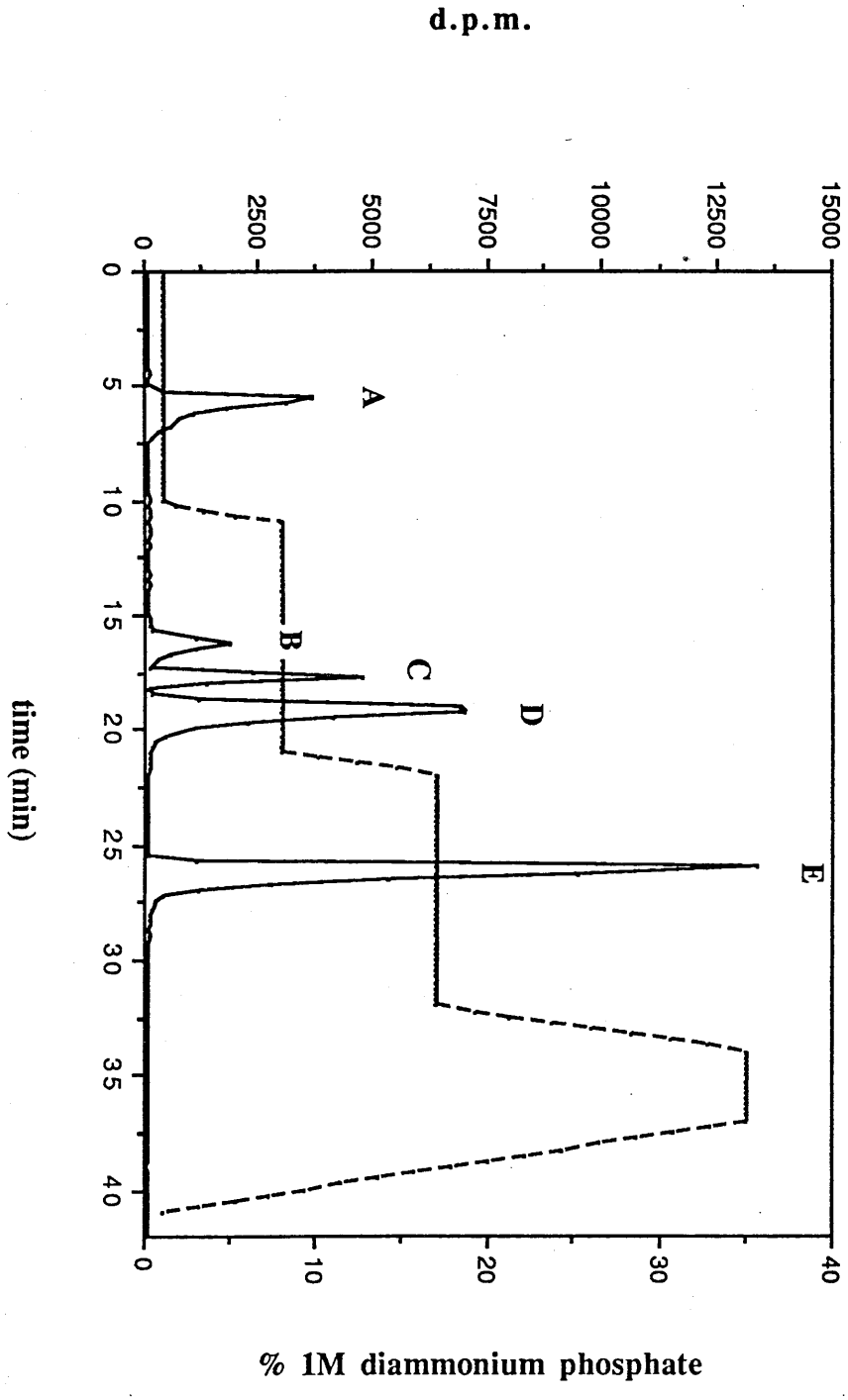

d.p.m.
0
2500
5000
7500
10000
12500
15000
A
B
C
D
E
time (min)
0
5
10
15
20
25
30
35
40
% 1M diammonium phosphate
0
10
20
30
40

Figure 2.3

$Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$. The $[^3H]Ins(1,4,5)P_3$ was stored in an ethanol:water solution, 9:1 and 100ul (100uCi) was blown to dryness under nitrogen. The $[^3H]Ins(1,4,5)P_3$ was reconstituted in 480ul of incubation buffer and incubated for 45 min at 37°C with 20ul of the 100000g supernatant of adrenal cortex. The incubation was terminated by the addition of 200ul of 10% perchloric acid, and left on ice for 10 min. The sample was centrifuged at 10000g in a Hettich microfuge for 5 min and 650ul of the supernatant was transferred to a separate Eppendorf tube. The samples were neutralised by adding 424ul of a 1:1 (v/v) mixture of Freon and tri-n-octylamine, followed by vigorous mixing of the separate phases on a vortex mixer. After centrifugation for 1 min at 4000g in a Hettich microfuge, the upper phase containing the radiolabelled inositol phosphates was removed. The resulting $Ins(1,3,4,5)P_4$ and the $Ins(1,4,5)P_3$ were separated on a Dowex anion exchange column as follows:

i) $Ins(1,4,5)P_3$ was eluted with 25ml of 0.8M ammonium formate/0.1M formic acid

ii) $Ins(1,3,4,5)P_4$ was eluted with 10ml of 1.2M ammonium formate/0.1M formic acid.

2.8 Determination of water soluble choline metabolites

2.8.1 Sample preparation

Cells were plated on to 24 well plates at a density of $5x10^4$ cells per well. After 24h the medium was changed to DMEM containing 2% (v/v) donor calf serum and 1uCi ml^{-1} [methyl ^3H]choline chloride. Experiments were performed 48h later on the cells which were then confluent and quiescent. After removal of the labelling medium the cells were washed in DMEM and incubated in fresh DMEM containing 1% BSA (w/v) for 1h. The monolayers were washed with 0.5ml Hank's buffered saline (37°C) for 5 min and then

incubated in HBG for 10 min. The HBG was aspirated and the cells incubated in HBG containing agonist or buffer to give a final volume of 150ul.

Following incubation for the appropriate time at 37^{o}C, incubations were terminated by the addition of 500ul ice-cold methanol, except in those experiments in which intracellular choline levels were examined where incubation medium was aspirated immediately prior to addition of methanol. The plates were placed on ice for 10 min and the contents of each well scraped, transferred to an Eppendorf tube and each well rinsed with 150ul distilled water and 250ul of methanol this being transferred to the same Eppendorf tube. Then 375ul of chloroform was added, the samples vortex-mixed and left to extract for 1h. The phases were split by addition of 375ul chloroform and 375ul distilled water. After centrifugation for 1 min at 14000g in a Hettich microfuge the upper aqueous methanolic phase containing the radiolabelled water soluble choline containing metabolites was removed for analysis.

The radioactivity associated with the choline containing phospholipids was determined by scintillation counting of 250ul of the lower chloroform phase following air drying.

2.8.2 Extraction of choline metabolites

Choline metabolites were separated on Dowex 50H^{+} columns as described by Cook & Wakelam (1989). The methanolic aqueous phase of samples were diluted to 4ml with distilled water and applied to 1ml Dowex 50-W-H^{+} columns and the following [^{3}H]-labelled compounds eluted as follows:

i) Glycerophosphocholine was eluted with 5ml of distilled water

ii) Cholinephosphate was eluted with 15ml of distilled water

iii) Choline was eluted with 20ml of 1M hydrochloric acid.

The amount of radioactivity in each sample was determined by liquid scintillation counting of aliquots of the collected samples.

Figure 2.4 shows the elution profile obtained when [^{3}H]GroPCho, [^{14}C]ChoP and [^{3}H]Cho, in the presence of a non-radioactive aqueous methanolic phase of a cell extract, were separated on a 1ml Dowex 50-W-H^{+} column. The recovery of each metabolite and cross-contamination of the samples were calculated. These results are shown in Table 2.2 and demonstrate greater than 93% recovery of each standard. Table 2.2 also shows that there was essentially no cross-contamination of [^{3}H]Cho with [^{3}H]GroPCho or [^{14}C]ChoP. A small (6%) cross-over between [^{3}H]GroPCho and [^{14}C] ChoP was detected, but this was not of a significant magnitude to affect the results.

2.9 Prostaglandin $F_{2\alpha}$ binding studies

Confluent and quiescent cultures of cells on 24 well plates (approximately 1.25 x 10^{5} cells per well) were washed twice with DMEM and incubated for 1h in DMEM containing 1% (w/v) BSA at 37^{o}C. The monolayers were washed with Hank's buffered saline supplemented with 0.2% (w/v) BSA, 10mM Hepes and 0.01mM indomethacin (pH7.3) and incubated for 15 min at 4^{o}C in ligand-free binding medium. The binding medium consisted of Hank's buffered saline supplemented with 0.05% (w/v) BSA, 10mM Hepes, 0.01mM indomethacin, cell culture grade amino acids (1:99 (v/v) dilution of GIBCO 100x physiological amino acid mixture and 2mM bacitracin (pH7.3). This medium was then replaced with fresh binding medium containing the stated concentration of [^{3}H] $PGF_{2\alpha}$. After a 2h incubation on ice the monolayers were rapidly washed four times with ice-cold Hank's buffered saline containing 0.1% (w/v) BSA, 10mM Hepes and 0.01mM indomethacin (pH7.3). Washed cells were then solubilised in

Figure 2.4 Resolution of [^{3}H]glycerophosphocholine [^{14}C]cholinephosphate and [^{3}H]choline standards by Dowex cation exchange chromatography

The figure shows the elution of [^{3}H]GroPCho from a Dowex-H^{+} form column using 9ml of distilled water applied in 1ml aliquots. [^{14}C]ChoP was eluted using 15ml of distilled water and [^{3}H]Cho was eluted using 20ml of 1M hydrochloric acid applied in 1ml aliquots. Known amounts of each standard were added to a non-radioactive aqueous methanolic phase of a cell extract. Results are means $\pm$ S.D. of one experiment typical of 8 others. This figure shows that there is clear resolution of glycerophosphocholine, cholinephosphate and choline.

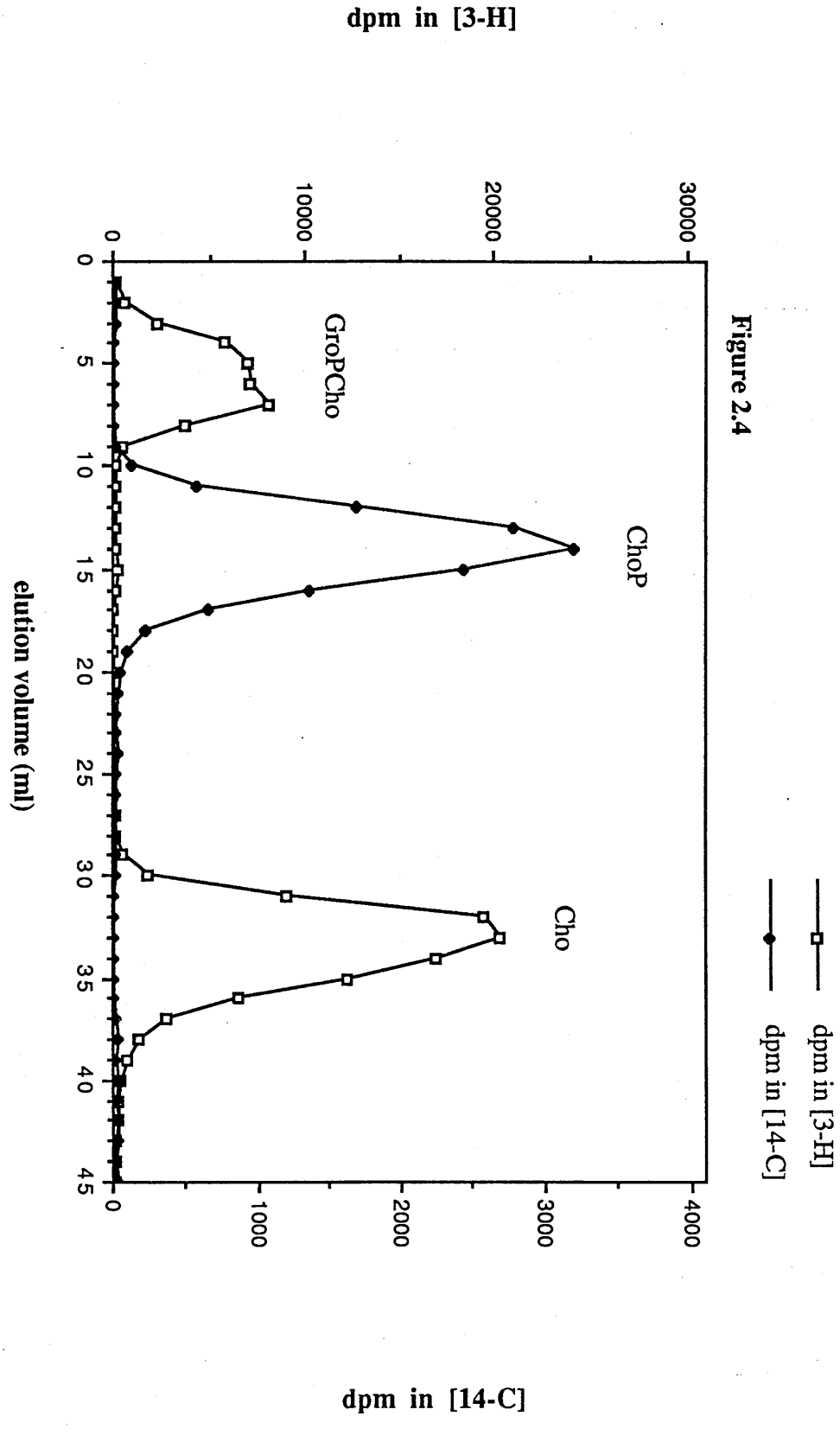

dpm in [3-H]
dpm in [14-C]
elution volume (ml)
GroPCho
ChoP
Cho
dpm in [3-H]
dpm in [14-C]
0
10000
20000
30000
0
1000
2000
3000
4000
0
5
10
15
20
25
30
35
40
45

Figure 2.4

Table 2.2 Analysis of recoveries of and cross contamination between [^{3}H]Cho labelled metabolites separated on Dowex columns

Fraction	Recovery	% Contamination with		
		Cho	ChoP	GroPCHo
Cho	96.0	-	0	0
ChoP	94.4	3.1	-	2.2
GroPCHo	93.3	0	5.9	-

Known amounts of [^{3}H]GroPCho, [^{14}C]ChoP and [^{3}H]Cho were added to a non-radioactive aqueous methanolic phase of a cell extract and separated on Dowex columns. Recoveries and cross over were determined by dual label liquid scintillation counting. The results are means calculated from 4 separate experiments where standard errors did not exceed 6%.

0.5M sodium hydroxide containing 2% (w/v) sodium carbonate and 1% (w/v) Na Dod SO_4,

Following neutralisation with 1M hydrochloric acid the total cell-associated radioactivity was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 1mM unlabelled $PGF_{2\alpha}$ and was approximately 37%. Cell number was determined on parallel wells incubated as above, except in the absence of ligand, following trypsinisation, by counting in an Industrial D Coulter counter.

2.10 Determination of choline kinase activity

Choline phosphate (ChoP) can be generated in cells either by hydrolysis of phosphatidylcholine or during its biosynthesis. The phosphorylation of choline by choline kinase is the first committed step in the biosynthetic pathway and has been shown to be stimulated in quiescent NIH-3T3 fibroblasts by serum and insulin (Warden & Friedkin, 1985). Choline kinase activity was determined in control and Ha-, Ki- and N-ras transformed NIH-3T3 cells.

2.10.1 Assay for choline kinase activity

Confluent cultures were washed three times with Hank's buffered saline (37°C), scraped and collected by centrifugation at 800g for 5 min. The cells were permeabilised by resuspension in 10mM Tris HCl (pH8.5), containing 0.05% (v/v) digitonin at 4°C. The cells were then centrifuged at 10000g in a Hettich microfuge at 4°C for 5 min and the supernatant removed to a clean Eppendorf tube. Choline kinase activity was assayed in the supernatants by a modification of the method of Macara (1989). The assay buffer contained 100mM Tris HCl (pH8.5), 10mM $MgCl_2$, 10mM Na_2 ATP, and 0.25mM [^{3}H] choline chloride (1.25uCi ml^{-1}).

2.10.2 Separation of the reaction products by ion exchange chromatography

Samples were diluted to 4ml with distilled water and applied to 1ml Dowex-hydrogen form columns, [^{3}H]-labelled compounds eluted as follows:

i) Cholinephosphate was eluted with 16ml of distilled water

ii) Choline was eluted with 20ml of 1M hydrochloric acid.

The amount of radioactivity in each sample was determined by liquid scintillation counting of aliquots of the collected samples.

2.11 Protein Determination

The method is based on that of Lowry et al. (1951). The stock solutions were:

A. 2% (w/v) sodium carbonate in 0.1M sodium hydroxide

B. 1% (w/v) copper sulphate

C. 2% (w/v) sodium potassium tartrate.

Just before use the reagents are mixed in the following v/v ratio: A:B:C, 100:1:1. Protein standards were prepared using 1mg ml^{-1} bovine serum albumin fraction V and a standard curve was constructed for a maximum of 30ug protein per sample. 2,5,10ul of the unknown samples were assayed in triplicate. 1ml of the A:B:C solution above was added to each sample, mixed and left to stand for 10 min. 100ul of Folin-Ciocalteau reagent, diluted 1:4 with water was added to each sample. The samples were mixed and left for 30 min. The absorbance of the samples were determined spectrophotometrically at 750nM in an LKB Ultrospec II spectrophotometer.

2.12 Buffer composition

2.12.1 Earle's Salt (x20)

2.33M sodium chloride

107mM potassium chloride

33.2mM magnesium sulphate

23.33mM sodium dihydrogen phosphate

110mM glucose

The pH of the buffer was adjusted to 7.3 with 1M sodium hydroxide.

2.12.2 Hank's buffered saline

1.26mM calcium chloride

0.5mM magnesium chloride

0.9mM magnesium sulphate

5.37mM potassium chloride

137mM sodium chloride

4.2mM sodium hydrogen carbonate

0.35mM sodium dihydrogen phosphate

The pH of the buffer was 7.3 when freshly made.

2.12.3 Phosphate buffered saline (PBS)

146mM sodium chloride

5.36mM potassium chloride

9.6mM disodium hydrogen phosphate

1.46mM potassium dihydrogen phosphate.

The pH of the buffer was adjusted to 7.3.

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

2.13 Preparation of Dowex 1 x 8-200 formate form

Dowex 1 x 8-200 chloride form was (obtained from Sigma Chemical Company) treated as follows to obtain Dowex formate for use in anion exchange chromatography of inositol phosphates.

A known packed volume of Dowex was washed with distilled water, left to settle and the unsettled Dowex discarded. This process was repeated twice. The Dowex was transferred to a scintered glass funnel and washed with approximately 20 volumes of 2M sodium hydroxide. The Dowex was then washed with 10x its volume of water followed by approximately 5 volumes of 1M formic acid. Finally, the Dowex was washed with 50 volumes of water until the pH of the Dowex slurry was constant at approximately pH5.5.

2.14 Preparation of Dowex 50-W-H^+

Dowex 50-W-H^+ was (obtained from Sigma Chemical Company) treated as follows to obtain Dowex for use in cation exchange chromatography of water soluble choline metabolites.

A known packed volume of Dowex was washed with distilled water, left to settle and the unsettled Dowex discarded. This process was repeated twice. The Dowex was then washed three times in 3 volumes of 1M hydrochloric acid. Finally the Dowex was washed four times in 3 volumes of distilled water or until the pH was constant at approximately pH5.5

2.15 Preparation of Dowex columns

Columns were prepared by adding 1ml of Dowex to a glass wool plugged pasteur pipette.

CHAPTER 3

DESENSITIZATION OF PROSTAGLANDIN $F_{2\alpha}$-STIMULATED INOSITOL PHOSPHATE GENERATION IN NIH-3T3 FIBROBLASTS TRANSFORMED BY OVER-EXPRESSION OF NORMAL RAS GENES

3.1 INTRODUCTION

The function of $p21^{ras}$ in cell proliferation and transformation is not known. Ras proteins bind guanine nucleotides, express an intrinsic GTPase activity and have been suggested to function as G-proteins, coupling the receptors for certain growth factors to the stimulation of PIC (Barbacid, 1987).

Evidence for this proposition came from the finding that in the T15 cell line induction of a steroid-hormone regulated ras construction, causing over-expression of the N-ras gene, resulted in the apparent amplification of the coupling of the bombesin receptor to PIC (Wakelam et al., 1986; Lloyd et al., 1989). In addition Chiarugi et al. (1986) demonstrated that transformation of Balb-3T3 fibroblasts with the EJ/T24-Ha-ras oncogene increased the responsiveness of PIC to muscarinic stimulation. An increase in the turnover of inositol phospholipids was detected by Fleishman et al. (1986) in both NRK and NIH-3T3 cells transformed by three different ras genes. In NIH-3T3 cells transformed by activated, point-mutated N- or Ha-ras genes, the agonist independent stimulation of PIC was found to be increased (Wakelam et al., 1987; Hancock et al., 1988). These activating mutations result in a reduction in the GTPase activity of the $p21^{ras}$ protein.

Other studies have failed to demonstrate consistent stimulatory effects of ras on PIC. Benjamin et al. (1987) found that NIH-3T3 cells expressing high levels of the EJ-Ha-ras oncogene show markedly reduced PDGF-stimulated PIC activity, as determined by $Ins(1.4.5)P_3$ generation suggesting that the EJ-Ha-ras uncouples the PDGF receptor from the $PtdIns(4,5)P_2$-specific phospholipase C. This work has been confirmed by Parries et al. (1987) who also found markedly reduced PDGF-stimulated inositol phosphate generation as a consequence of the expression of EJ-Ha-ras.

Since it is unclear what, if any, general effect upon inositol phospholipid metabolism occurs following transformation of cells by ras, basal and agonist-induced total inositol phosphate generation was examined in a range of transformations of NIH-3T3 cells induced by the over-expression of normal Ha-, Ki- or N-ras genes. These experiments were performed in the presence of LiCl (10mM) to prevent metabolism of the generated inositol phosphates beyond inositol monophosphate. This methodology permitted the determination of accumulated inositol phosphates and has been shown to be the most reliable and easily applicable measure of receptor stimulated inositol phospholipid hydrolysis.

3.2 RESULTS

3.2.1 Characterisation of [^{3}H]inositol labelling of lipids in NIH-3T3 and N-ras transformed NIH-3T3 cells

To determine the characteristics of [^{3}H]inositol labelling of lipids in NIH-3T3 and N-ras transformed NIH-3T3 cells and to investigate the possibility of different turnover rates of inositol phospholipids in these cells, NIH-3T3 and T15$^+$ cells were labelled with [^{3}H]inositol for increasing times. The radioactivity associated with the lipid fraction from chloroform/methanol extracted cells was determined as described in Section 2.5. After 36h the T15$^+$ cells were found to have incorporated 2.9 times the amount of total [^{3}H]inositol into their lipids compared to NIH-3T3 control cells.

Figure 3.1 illustrates the [^{3}H]inositol labelling of GroPtdInsP and GroPtdInsP$_2$ in NIH-3T3 and T15$^+$ cells. The results in Figure 3.1b show that T15$^+$ cells demonstrated significantly higher incorporation of [^{3}H]inositol into GroPtdInsP and GroPtdInsP$_2$ than control cells (Fig.3.1a). As the data has been normalised for the amount of protein assayed, the increased

Figure 3.1 [^{3}H]inositol labelling of inositol lipids in NIH-3T3 and T15$^+$ cells

The [^{3}H]inositol labelled phospholipids were deacylated to glycerophosphoinositol (GroPtdIns), glycerophosphoinositol monophosphate (GroPtdInsP) and glycerophosphoinositol bisphosphate (GroPtdInsP$_2$) which were separated on Dowex-formate columns as described in Section 2.5. The data is from one experiment and is expressed as mean dpm/mg protein $\pm$ S.D., n=3. Only results for GroPtdInsP and GroPtdInsP$_2$ are shown.

Figure 3.1a

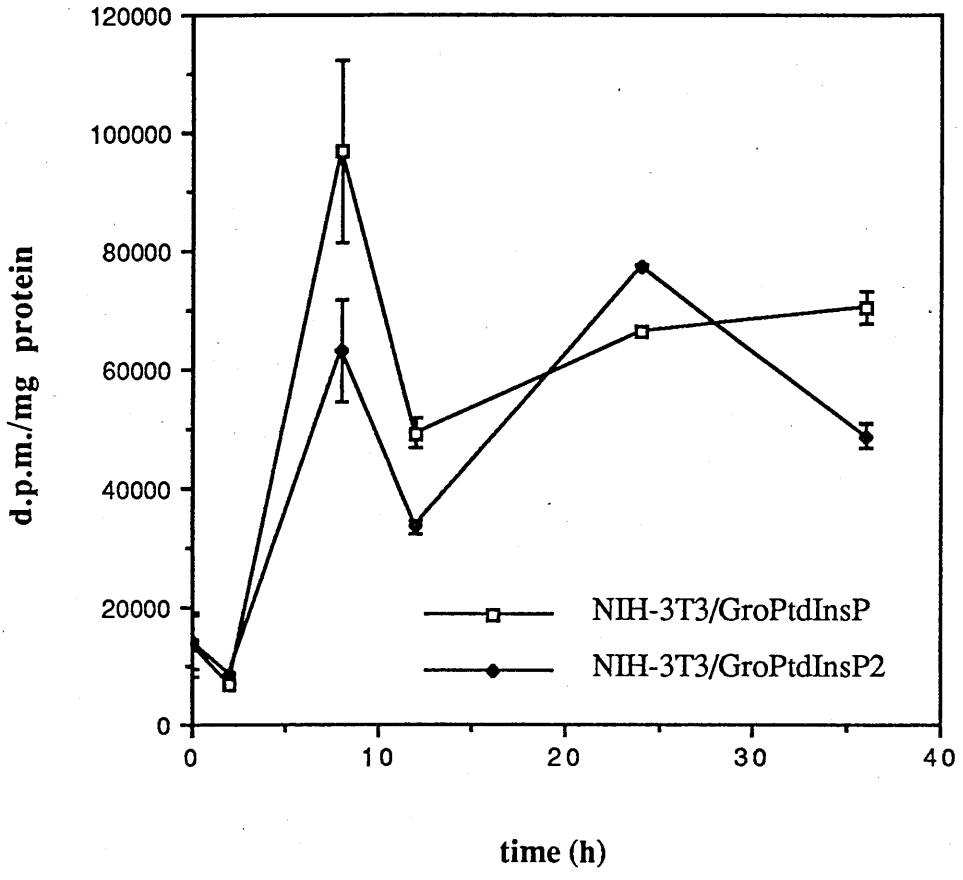
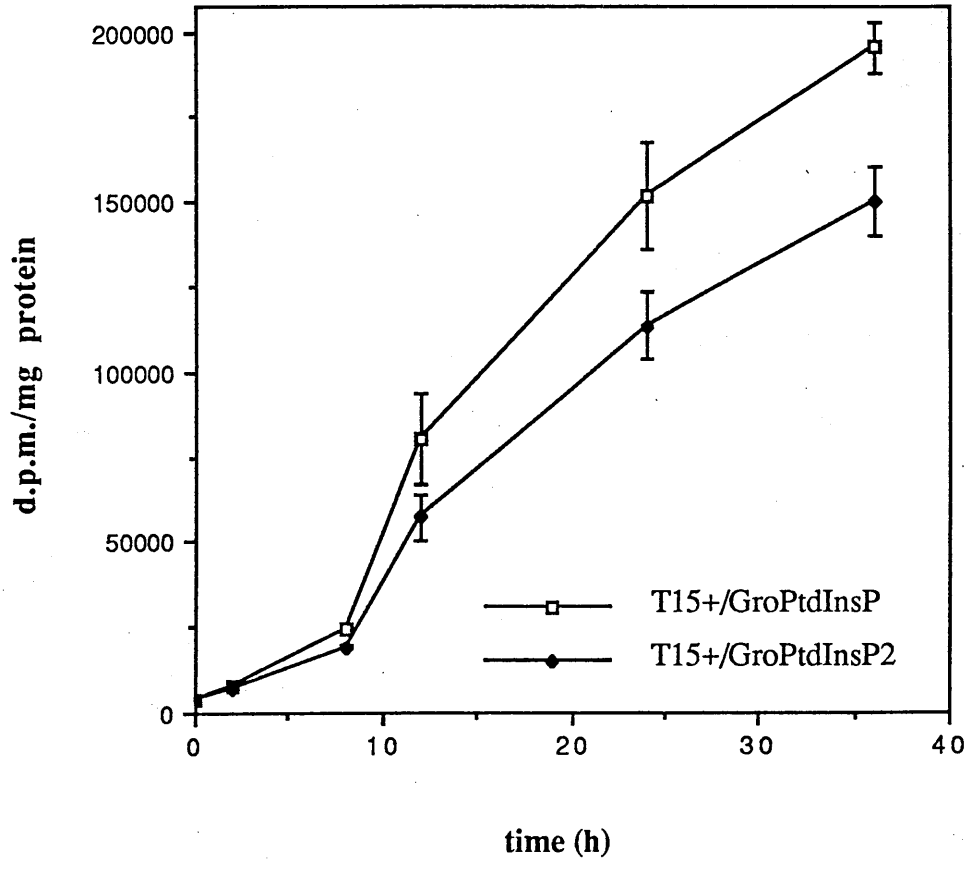


Figure 3.1b



labelling of [^{3}H]GroPtdInsP and [^{3}H]GroPtdInsP$_2$ (also [^{3}H]GroPtdIns, results not shown) were not due to differences in cell number. The peak levels of [^{3}H]GroPtdInsP and [^{3}H]GroPtdInsP$_2$ in NIH-3T3 cells appeared after 8h. There was then a decrease in [^{3}H]GroPtdInsP and [^{3}H]GroPtdInsP$_2$ levels. The incorporation of [^{3}H]inositol into GroPtdInsP and GroPtdInsP$_2$ in NIH-3T3 cells maintained a steady level after 12h, whilst the incorporation of [^{3}H]inositol into GroPtdInsP and GroPtdInsP$_2$ continued to increase in T15$^+$ cells. This increased labelling of inositol phospholipids in T15$^+$ cells as compared to NIH-3T3 cells (Fig.3.1a-1b) suggests a role for PtdIns(4,5)P$_2$ hydrolysis in cell proliferation and transformation.

3.2.2 Growth factor stimulated generation of inositol phosphates

The time course of $PGF_{2\alpha}$-stimulated inositol phosphate generation is shown in Figure 3.2. $PGF_{2\alpha}$-stimulated inositol phosphate generation increased linearly with time to a maximum at 30 min. The response then plateaued till 60 min. Unless otherwise stated, when measuring ligand stimulated total inositol phosphate generation the cells were stimulated for 30 min.

To determine if there were changes in stimulated inositol phosphate generation in cells transformed by over-expression of normal Ha-, Ki- or N-ras genes, a range of agonists were screened. Bradykinin, calf serum and PDGF stimulated significant inositol phosphate generation in all the cell lines examined. Figure 3.3 shows the effect of saturating concentrations of bradykinin, calf serum and PDGF on stimulated inositol phosphate generation in [^{3}H]inositol-labelled NIH-3T3 cells and Ha-, Ki- and N-ras transformed cells.

In cells expressing high levels of the normal Ha-, Ki- or N-ras proto-oncogene, there was a marked increase compared with the

Figure 3.2 The time course of $PGF_{2\alpha}$-stimulated inositol phosphate generation in NIH-3T3 cells

Cells were labelled with [^{3}H]inositol ($1uCiml^{-1}$) and 24h later cells were stimulated with $PGF_{2\alpha}$ for the times stated. Inositol phosphate generation was determined as described in Section 2.6 - sample preparation method A - and the results are means $\pm$ S.D. of one experiment typical of 3 others.

Figure 3.2

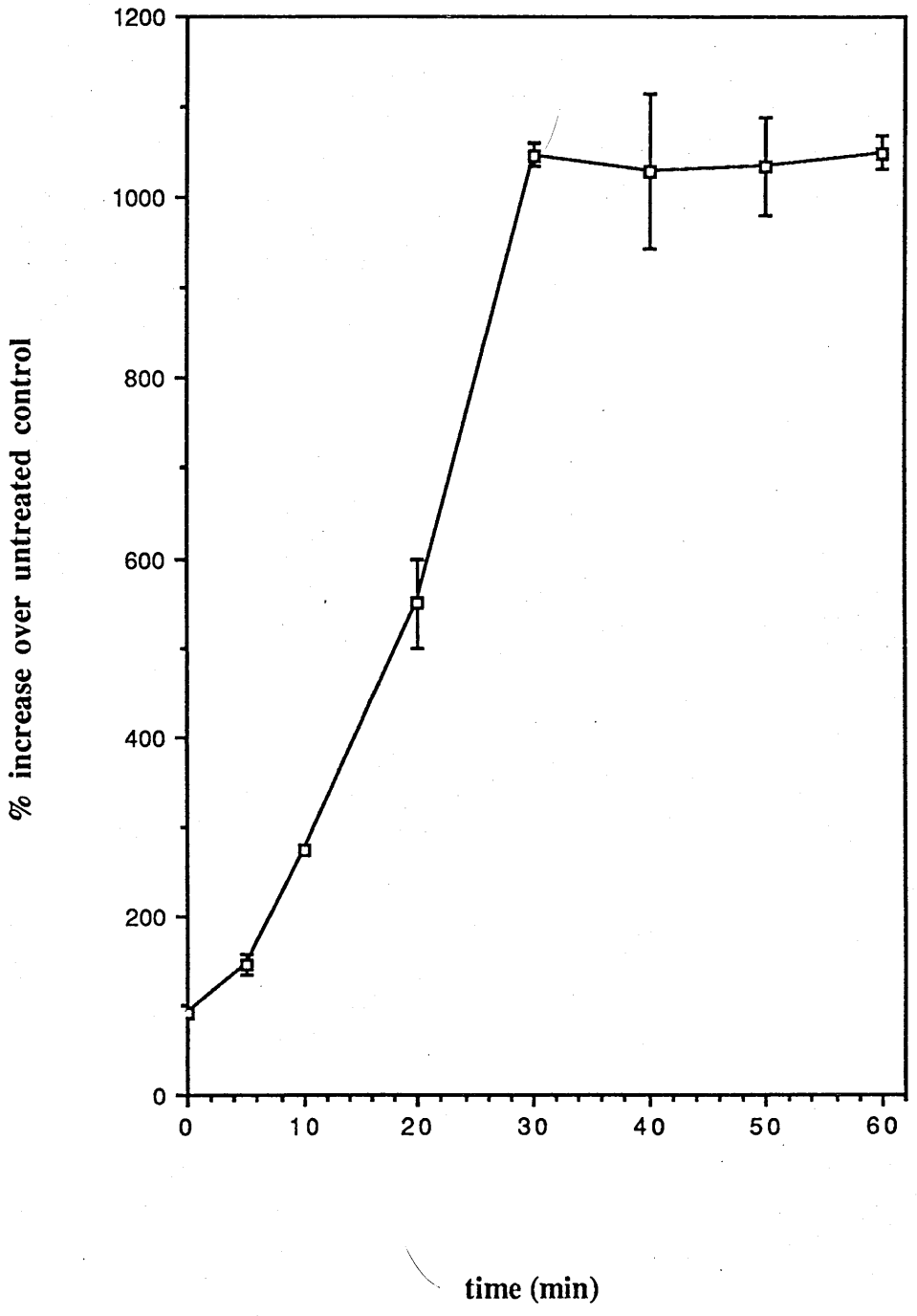
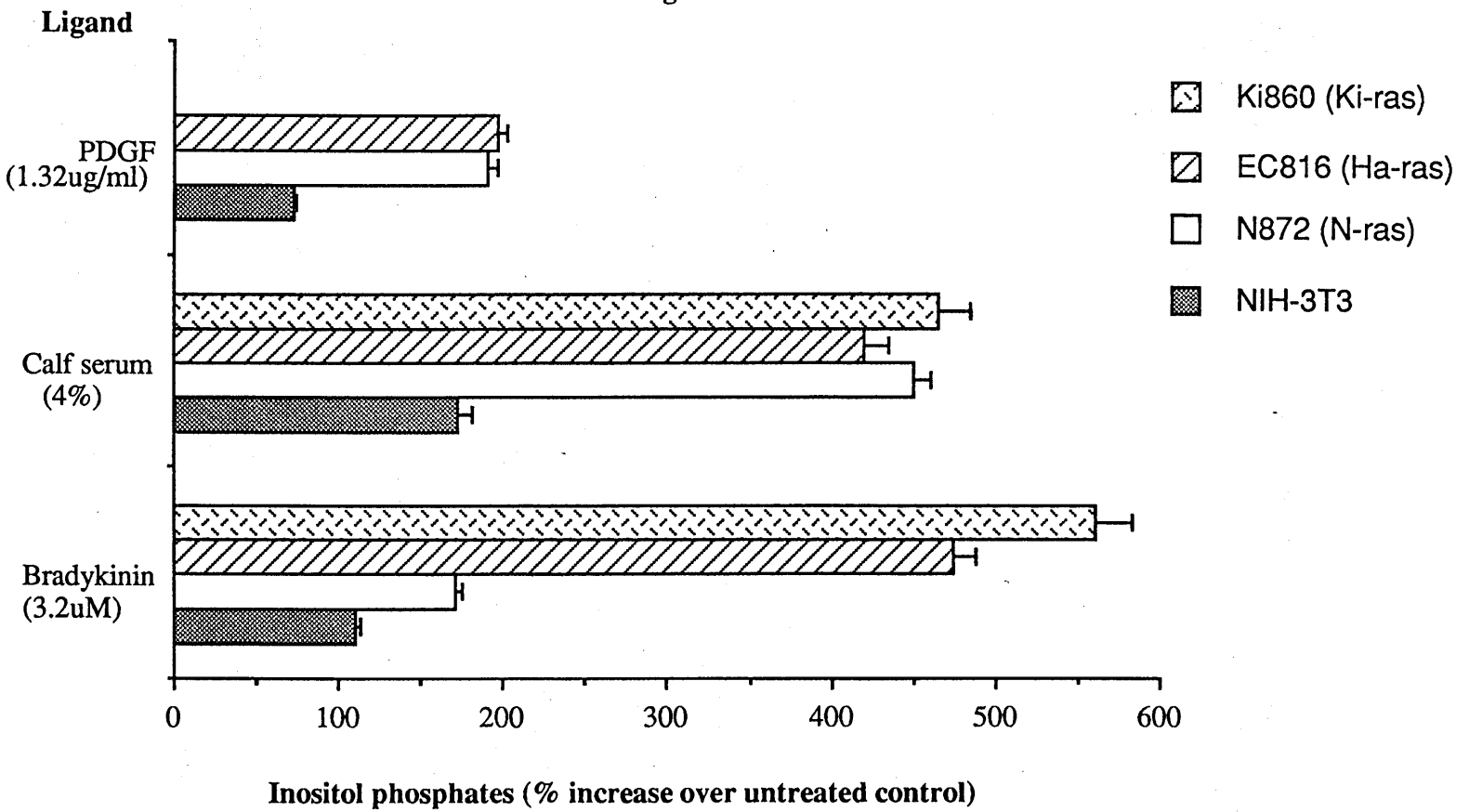

1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
% increase over untreated control
time (min)

Figure 3.3 Growth factor stimulation of inositol phosphate generation in control and Ha-, Ki- and N-ras transformed NIH-3T3 cells

Cells were labelled with [^{3}H]inositol (1uCiml^{-1}) for 24h and after a 30 min stimulation, inositol phosphate generation was determined as described in Section 2.6 - sample preparation method A. The results are means $\pm$ S.D., n=4 in each case and the data is from one experiment which gave qualitatively the same results as three others.

Figure 3.3


Ligand
PDGF
(1.32ug/ml)
Calf serum
(4%)
Bradykinin
(3.2uM)
Ki860 (Ki-ras)
EC816 (Ha-ras)
N872 (N-ras)
NIH-3T3
0
100
200
300
400
500
600
Inositol phosphates (% increase over untreated control)

NIH-3T3 cells, in inositol phosphate generation in response to stimulation by bradykinin, calf serum and PDGF.

Addition of bradykinin to Ha-, Ki- or N-ras transformed cells gave a 4.3-, 5.1- and 1.6-fold stimulation respectively, compared with the control cells. Addition of calf serum to Ha-, Ki- or N-ras transformed cells resulted in a fold increase of 2.4, 2.7 and 2.6 respectively, while addition of PDGF to Ha- or N-ras transformed cells gave 2.7- and 2.6-fold stimulation compared with control cells. Therefore stimulated inositol phosphate generation in ras transformed cells is amplified compared to NIH-3T3-control cells.

3.2.3 Basal rates of production of inositol phosphates

The higher [^{3}H]inositol labelling of inositol phospholipids in T15$^+$ cells as compared to NIH-3T3 cells (Fig.3.1) and the increased agonist stimulated inositol phosphate response (Fig.3.3) could be due to increased basal rates of turnover (i.e. in the basal activity of PIC) in the ras transformed cells. If the production of inositol phosphates is measured in the absence of agonist then a basal level of inositol phosphate generation can be determined. Table 3.1 shows there were only small differences in basal rates of production of inositol phosphates, between NIH-3T3 cells and the ras transformed NIH-3T3 cell lines examined, demonstrating that an increase in inositol phospholipid hydrolysis was only apparent during receptor activation.

3.2.4 Stimulation of inositol phosphate generation by $PGF_{2\alpha}$ in control, Ha-, Ki- or N-ras transformed cells

Stimulation of confluent NIH-3T3 cells with $PGF_{2\alpha}$ (2.1uM) induced a 12-fold increase in the generation of inositol phosphates within 30 min as compared to unstimulated controls (Table 3.2). It is demonstrated in Chapter 4 that this generation of inositol

Table 3.1 Basal rates of production of inositol phosphates

Cell Line	ras-Gene	Rate (dpm produced in inositol phosphates/min) x (100/dpm in inositol lipids at zero times)	No. of Expt.
NIH-3T3		0.047 ± 0.004	n=6
N866	normal N-ras	0.046 ± 0.006	n=1
N872		0.044 ± 0.002	n=2
T15$^-$		0.033 ± 0.007	n=6
T15$^+$		0.042 ± 0.010	n=6
EC806	normal Ha-ras	0.034 ± 0.007	n=4
EC807		0.051 ± 0.005	n=4
EC816		0.032 ± 0.004	n=5
H8/22$^-$		0.023 ± 0.002	n=1
H8/22$^+$		0.023 ± 0.002	n=1
Ki858	normal Ki-ras	0.033 ± 0.004	n=5
Ki860		0.020 ± 0.004	n=4

Cells were labelled with [^{3}H]inositol (1uCi ml^{-1}) for 24h, washed extensively and the rate of generation of inositol phosphates measured in the presence of Li^+, as described in Section 2.6 - sample preparation method A. Production was linear over the 30 min time course followed. Results are pooled from the stated number of experiments and are expressed as means ± S.D.

Table 3.2 The stimulation of inositol phosphate generation by $PGF_{2\alpha}$ in control, Ha-, Ki- and N-ras transformed NIH-3T3 cells

Cell Line	ras-Gene	Inositol phosphate generation (% increase over untreated control)
NIH-3T3		1250 ± 78
N866		263 ± 27
N872	normal N-ras	263 ± 49
T15+		380 ± 73
EC806	normal Ha-ras	355 ± 34
EC807		261 ± 34
EC816		413 ± 56
H8/22+		322 ± 22
Ki858	normal Ki-ras	181 ± 24
Ki860		219 ± 10

Cells were labelled with [^{3}H]inositol (1uCi ml^{-1}) for 24h and after a 30 min stimulation with $PGF_{2\alpha}$ (2.1uM) inositol phosphate generation was determined as described in Section 2.6. Results are expressed as means ± S.E.M., pooled from several experiments with n=6-51.

phosphates was a consequence of agonist stimulated PtdIns(4,5)P_2 breakdown. However, when NIH-3T3 cells which had been transformed by a several fold over-expression of the normal Ha-, Ki- or N-ras genes were stimulated with $PGF_{2\alpha}$ (2.1uM), only a 2-4 fold increase in inositol phosphate generation was observed (Table 3.2). There thus appears to be a ras induced desensitization to $PGF_{2\alpha}$ in the transformed cells.

The NIH-3T3 clone H8/22, in which the expression of the human Ha-ras proto-oncogene can be switched on by culturing in the presence of dexamethasone was used to demonstrate that the markedly reduced $PGF_{2\alpha}$ -stimulated inositol phospholipid turnover in ras transformed cells is directly related to the expression of $p21^{ras}$ and is not a transformation effect. Addition of increasing concentrations of dexamethasone to H8/22 cells previously grown without this inducer ($H8/22^-$) resulted in a concentration-dependent increase in expression of the human Ha-ras proto-oncogene as demonstrated by immuno-precipitable $p21^{Ha\text{-}ras}$ (C.J. Marshall, personal communication).

H8/22 cells were cultured for increasing lengths of time in the presence of dexamethasone (100nM; see Fig.2.1, Section 2.2.1.2) and basal and PDGF ($1.32ugml^{-1}$) stimulated inositol phosphate generation determined. Figure 3.4a shows that significant inositol phosphate generation occurred 8h after dexamethasone treatment in response to PDGF. A decrease in the stimulated levels of inositol phosphates was observed after 12h of dexamethasone treatment, but the levels of stimulated inositol phosphates then increased after this time with a maximum response observed after 48h of a 1608% increase over basal. These results indicated that stimulated inositol phosphate generation in $H8/22^+$ cells occurred prior to morphological changes. Figure 3.4a demonstrates that the

Figure 3.4 Time course for the effect of dexamethasone on PDGF and $PGF_{2\alpha}$ -stimulated inositol phosphate generation in H8/22 cells

Cells were labelled with [^{3}H]inositol (1uCiml^{-1}) for 48h and cultured in the presence of dexamethasone (100nM) for the times stated. PDGF (1.32ug ml^{-1}) or $PGF_{2\alpha}$ (2.1uM) stimulated inositol phosphate generation was determined as described in Section 2.6 - sample preparation method A. Results are means $\pm$ S.D. of one experiment where n=4.

Problems were encountered in repeating these experiments as it was difficult to obtain a very pure PDGF preparation. Therefore, bradykinin (3,2uM) was used as a ligand in an experiment and results demonstrated very similar trends. At time zero with no dexamethasone treatment bradykinin stimulated a significant inositol phosphate response of 41% increase over basal. The levels of stimulated inositol phosphates then increased with a maximum response at 48h after dexamethasone treatment of 224% increase over basal. In this experiment the decrease in the magnitude of the $PGF_{2\alpha}$ stimulated response with increasing time of dexamethasone treatment was demonstrated to be very similar to that shown in Figure 3.4b.

Figure 3.4a

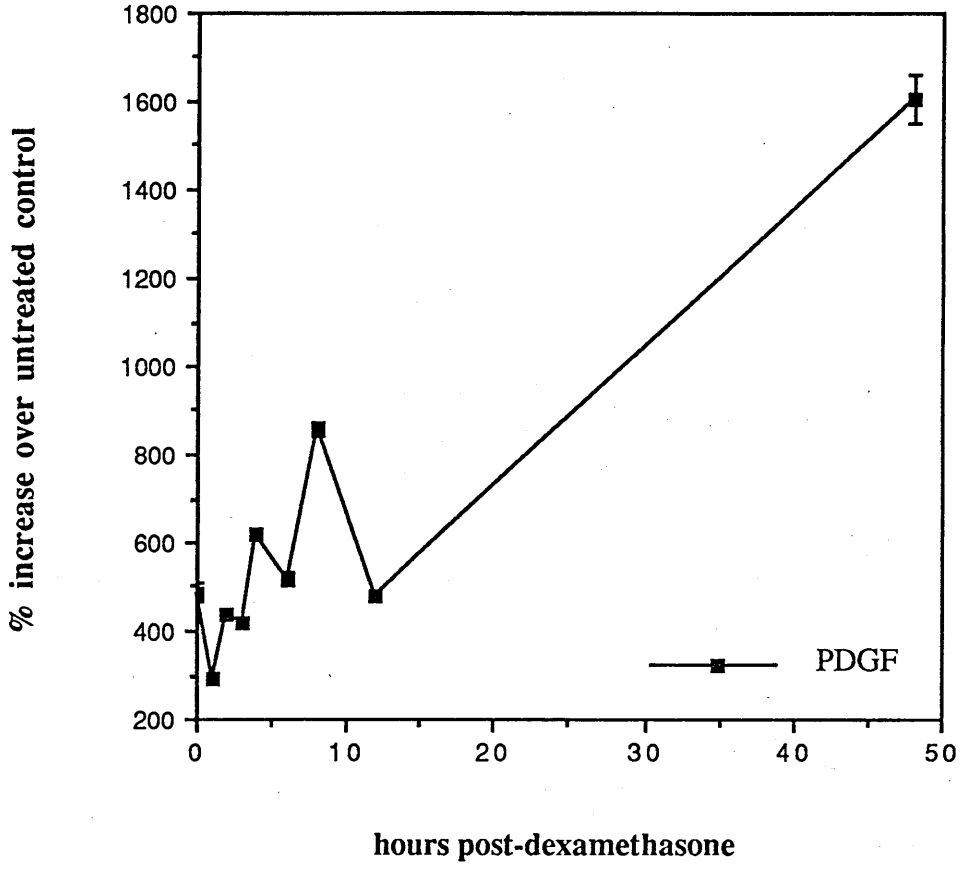
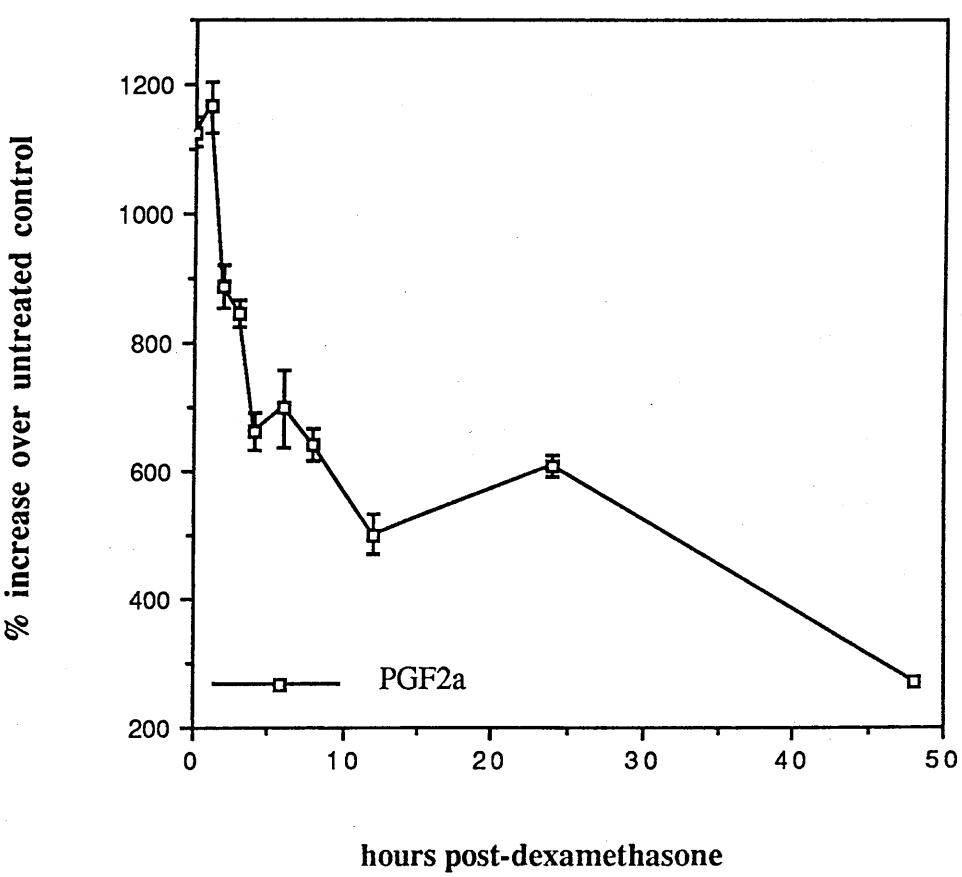


Figure 3.4b



progressive elevation of $p21^{Ha\text{-}\underline{ras}}$ concentration was paralleled by a comparable increase in the magnitude by which PDGF stimulated inositol phosphate generation, implying that increased efficiency of coupling of the PDGF receptor to inositol phosphate production is related to the concentration of $p21^{Ha\text{-}\underline{ras}}$.

However, when cells treated with dexamethasone, as above, were stimulated with $PGF_{2\alpha}$ (2.1uM), the progressive elevation of $p21^{Ha\text{-}\underline{ras}}$ concentration was paralleled by a comparable decrease in the magnitude of the inositol phosphate response (Fig.3.4b). With no dexamethasone treatment $PGF_{2\alpha}$ stimulated an inositol phosphate response of 1127% increase over basal. However, after 48h of dexamethasone treatment $PGF_{2\alpha}$ only stimulated a response of 272% increase over basal.

The reduction in the stimulation of inositol phosphate generation in <u>ras</u> transformed cells was observed at all $PGF_{2\alpha}$ concentrations tested. Figure 3.5a-5b illustrates the dose-dependence of $PGF_{2\alpha}$ -stimulated inositol phosphate generation as a percentage increase over unstimulated controls. For all the cell lines tested half-maximal stimulation occurred at a concentration of 0.18uM, while a saturating response was observed at approximately 2uM. At this concentration there was a dramatic difference in the magnitude of the $PGF_{2\alpha}$ -stimulated inositol phosphate response in the NIH-3T3 cells (12 fold) compared with the Ha-, Ki- or N-<u>ras</u> transformed cells (1.8 to 3.4 fold). A summary of the EC_{50} values obtained for the cell lines tested is shown in Table 3.3. No change in the EC_{50} value for $PGF_{2\alpha}$ as detected between the cell lines, EC_{50} = 0.18uM, indicating that over-expression of the <u>ras</u> genes in NIH-3T3 cells affected the response to $PGF_{2\alpha}$ with no associated change in receptor affinity.

Figure 3.5 Dose-dependence of $PGF_{2\alpha}$ -stimulated inositol phosphate generation in normal and ras transformed NIH-3T3 cells

Cells were labelled with [^{3}H]inositol (1uCiml^{-1}) for 24h and stimulated for 30 min with various concentrations of $PGF_{2\alpha}$ in the presence of 10mM LiCl. Inositol phosphate generation was determined as described in Section 2.6. Results are expressed as means $\pm$ S.D., pooled from several experiments.

Figure 3.5a

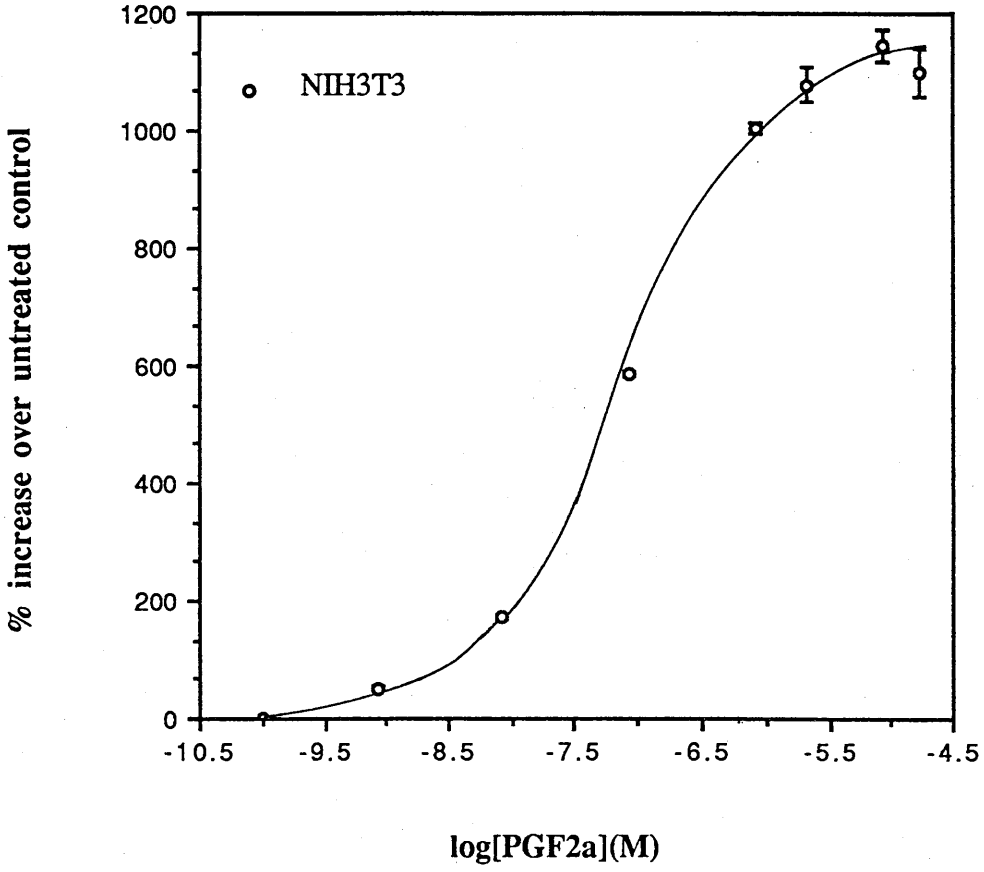


Figure 3.5b

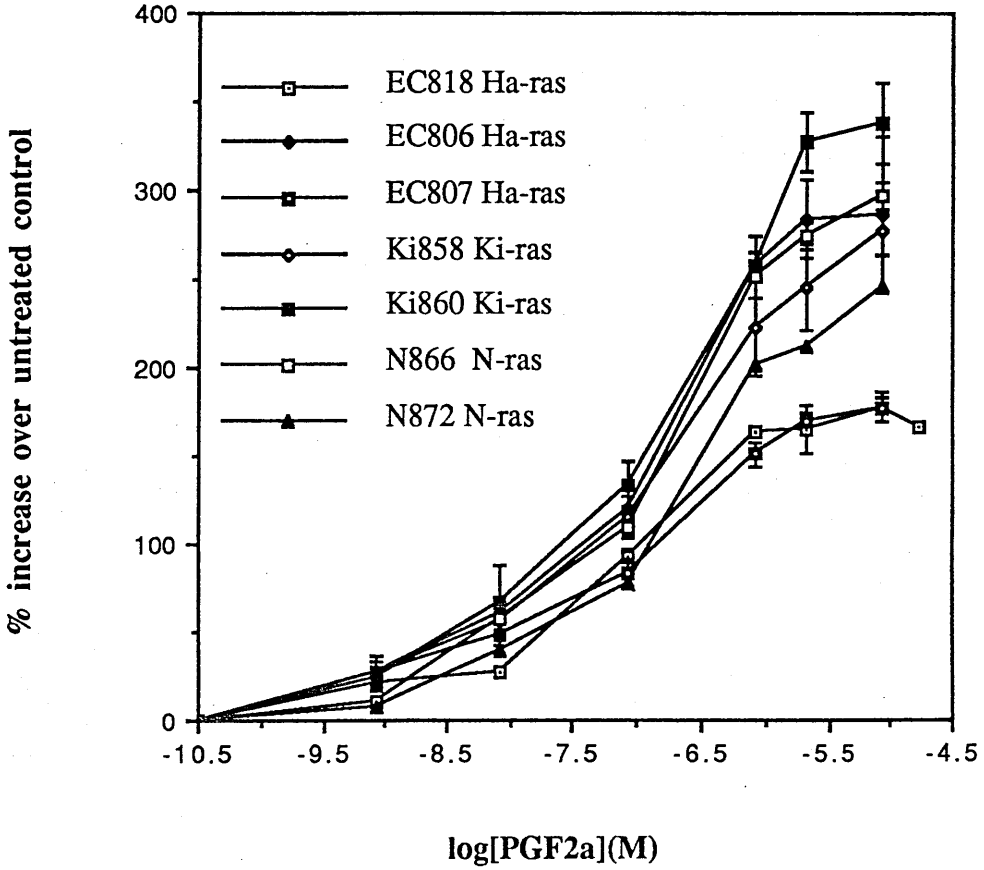


Table 3.3 Summary of EC_{50} values of $PGF_{2\alpha}$ -stimulated inositol phosphate generation

Cell line	ras-Gene	$EC_{50}(x10^{6}M)$	No. of expt.
NIH-3T3		0.178 ± 0.030	11
EC816		0.083 ± 0.001	2
EC806	Ha-*ras*	0.105 ± 0.040	2
EC807		0.083 ± 0.002	2
Ki858	Ki-*ras*	0.188 ± 0.050	4
Ki860		0.186 ± 0.020	4
N866	N-*ras*	0.187 ± 0.020	3
N872		0.175 ± 0.030	3

Cells were labelled with [^{3}H]inositol (1uCi ml^{-1}) for 24h and stimulated for 30 min with various concentrations of $PGF_{2\alpha}$ in the presence of 10mM LiCl. Inositol phosphate generation was determined as described in Section 2.6. Results are pooled from the stated number of experiments and are expressed as means ± S.D.

3.2.5 Characterisation of $PGF_{2\alpha}$ receptors on control and ras-transformed cells

Binding studies were performed in order to determine if the reduced stimulation in inositol phosphate generation in response to $PGF_{2\alpha}$ in the ras-transformed cells was due to a reduction in receptor number.

When intact, quiescent NIH-3T3 cells were incubated with [^{3}H]$PGF_{2\alpha}$ (2.5uM) at 4^{o}C, cell-associated radioactivity increased steadily reaching a maximum value at 2h (Fig.3.6). Figure 3.7 shows that the specific binding of [^{3}H]$PGF_{2\alpha}$ to intact quiescent NIH-3T3 cells as a function of radiolabelled ligand concentration was clearly saturable. Scatchard analysis of the binding data (Fig.3.7, insert) demonstrates that NIH-3T3 cells possess a single class of cell-surface receptor of Kd = 0.44 x 10^{-6}M with approximately 400,000 receptors per cell. Binding experiments performed upon the ras transformed cells demonstrated that, with the exception of T15 cells, there was no significant difference in either the number of $PGF_{2\alpha}$ receptors per cell or in their Kd values (Table 3.4). The increase in receptor number in the T15 cell line may be due to non-specific effects of the inducer dexamethasone as these cells are grown continuously in the presence of dexamethasone. Glucocorticoids can have a variety of effects upon cells. It is possible that dexamethasone may have caused an inhibition of $PGF_{2\alpha}$ synthesis and thus a consequent reduction in agonist induced down-regulation of receptor number as has been observed for mouse fibrosarcoma cells (Tashjian et al., 1975).

The H8/22 cell line did not show significantly greater $PGF_{2\alpha}$ receptor numbers. However, 20-fold less dexamethasone is used to induce expression of $p21^{Ha\text{-}ras}$ in this cell line.

Figure 3.6 Time dependence of the binding of [^{3}H]PGF$_{2\alpha}$ to intact quiescent NIH-3T3 cells

The figure shows the time dependence of the binding of [^{3}H]PGF$_{2\alpha}$ to intact quiescent NIH-3T3 cells at 4^{o}C, determined as described in Section 2.9.

Figure 3.6

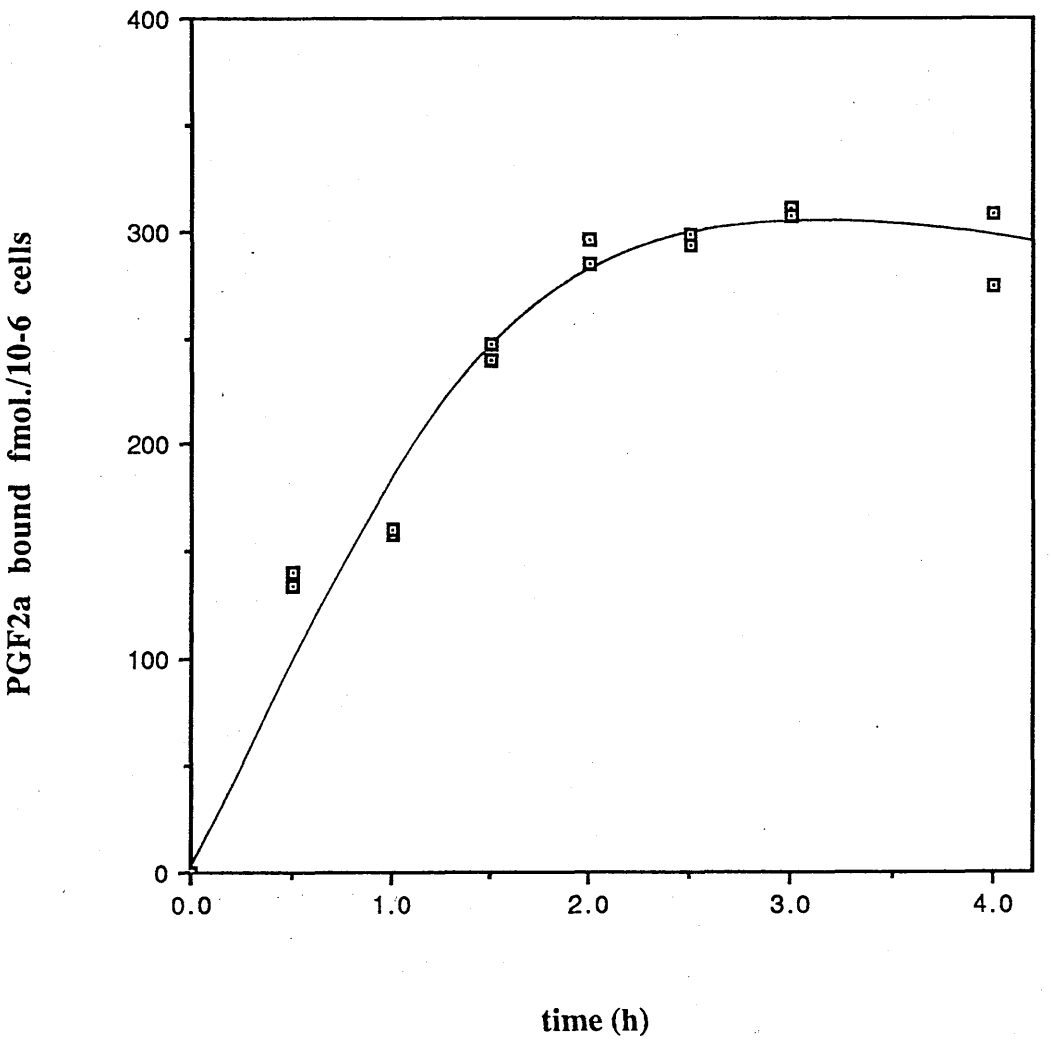

400
300
200
100
0
0.0
1.0
2.0
3.0
4.0
PGF2a bound fmol./10-6 cells
time (h)

Figure 3.7 Binding characteristics of [^{3}H]PGF$_{2\alpha}$ to NIH-3T3 cells

The figure shows the concentration dependence of the binding of [^{3}H]PGF$_{2\alpha}$ to intact quiescent NIH-3T3 cells at 4^{o}C determined as described in Section 2.9.

Inset: Scatchard plot of the same data: bound (B), radiolabelled ligand is expressed in fmol per 10^6 cells; the concentration of PGF$_{2\alpha}$ in the medium (F) is in fmol.

Figure 3.7

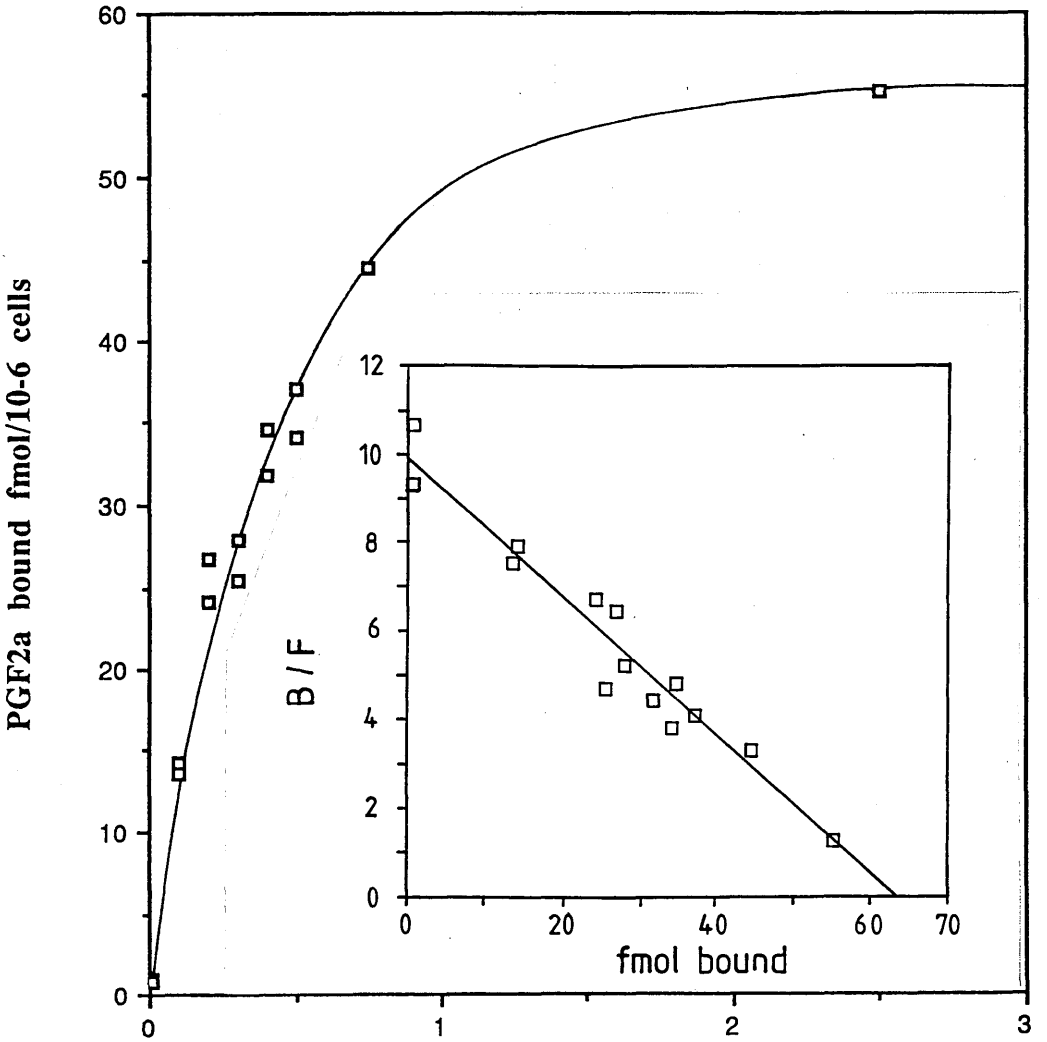

PGF2a bound fmol/10-6 cells
PGF2a (uM)
B / F
fmol bound
0
10
20
30
40
50
60
1
2
3
0
2
4
6
8
10
12
20
40
60
70

Table 3.4 $PGF_{2\alpha}$ receptors on normal and ras transformed NIH-3T3 cells

Cell line	ras-Gene	Kd($\times 10^{-6}$M)	Receptor number	No.of Expt.
NIH-3T3		0.44 ± 0.06	392000 ± 63500	12
EC806	Ha-ras	0.46 ± 0.04	268000 ± 74000	2
EC807		0.32	311000 ± 3500	1
EC816		0.50 ± 0.05	392000 ± 69000	2
H8/22+		0.21	502000 ± 69000	2
Ki858	Ki-ras	0.37 ± 0.04	456028 ± 27000	2
Ki860		0.65	400000 ± 6000	1
N866	N-ras	0.80 ± 0.72	625000 ± 201000	2
N872		0.84 ± 0.67	538000 ± 78000	2
T15+		0.44 ± 0.18	1731000 ± 67000	2

Binding studies were performed as described in Section 2.9. Results are pooled from the stated number of experiments and are expressed as means ± S.D.

Binding experiments performed upon NIH-3T3 cells cultured in the presence of dexamethasone (2uM) for 72h demonstrate that dexamethasone had no significant effect either on the number of $PGF_{2\alpha}$ receptors (426272 $\pm$ 4736, n=2) or in the Kd values of the receptors (Kd = 0.426 $\pm$ 0.05, n=2).

Thus the desensitization of $PGF_{2\alpha}$-stimulated inositol phosphate generation in ras transformed cells is not due to any reduction in receptor number or change in affinity or dissociation constant.

3.2.6 The effect of indomethacin on inositol phosphate generation

Homologous desensitization can be induced in cells as a consequence of increased autocrine secretion. In order to determine if there was increased synthesis of $PGF_{2\alpha}$ in the ras transformed cells, which could lead to an agonist-induced desensitization of the inositol phosphate response, control and ras transformed cells were cultured in the presence of the cyclo-oxygenase inhibitor indomethacin (0.1mM) for 72h. Cells were stimulated with $PGF_{2\alpha}$ and inositol phosphate generation determined. Indomethacin treatment had no effect upon the responsiveness of the cells to a subsequent stimulation with $PGF_{2\alpha}$ (Table 3.5).

The time course of $PGF_{2\alpha}$ stimulated inositol phosphate generation in NIH-3T3 cells incubated in the presence or absence of indomethancin is shown in Figure 3.8. There was a linear increase in $PGF_{2\alpha}$-stimulated inositol phosphates, a maximum stimulation of 16-18 fold of control at 30 min, with the response being desensitized at 40 min. Indomethacin did not affect the rate of $PGF_{2\alpha}$ stimulated inositol phosphate generation.

Table 3.5 $PGF_{2\alpha}$ -stimulated inositol phosphate generation in control and ras transformed NIH-3T3 cells grown in the presence or absence of indomethacin

Cell line	ras-Gene	Inositol phosphates (% increase over untreated control)	
		cells grown in absence of indomethacin	cells grown in presence of indomethacin
NIH-3T3		2169 ± 83	2165 ± 107
EC807	Ha-ras	362 ± 48	378 ± 72
Ki860	Ki-ras	244 ± 33	240 ± 25
N872	N-ras	364 ± 28	312 ± 24

Cells were cultured in the presence or absence of 0.1mM indomethacin for 72h and labelled with [^{3}H]inositol (1uCi ml^{-1}) for the last 24h. $PGF_{2\alpha}$ (2.1uM) stimulated inositol phosphate generation was determined as described in Section 2.6. Results are means ± S.D. where n=4 and are pooled from three separate experiments.

Figure 3.8 Time course for the effect of indomethacin on $PGF_{2\alpha}$-stimulated inositol phosphate generation in NIH-3T3 cells

Cells were labelled with [^{3}H]inositol (1uCi ml^{-1}) for 24h. Cells stimulated with $PGF_{2\alpha}$ (2.1uM) in the presence of indomethacin (0.1uM) were also pre-incubated for 10 min in the presence of indomethacin. Inositol phosphate generation was determined as described in Section 2.6 - sample preparation Method A. Results are means $\pm$ S.D., where n=4 of one experiment, which gave qualitatively the same results as three others.

Figure 3.8

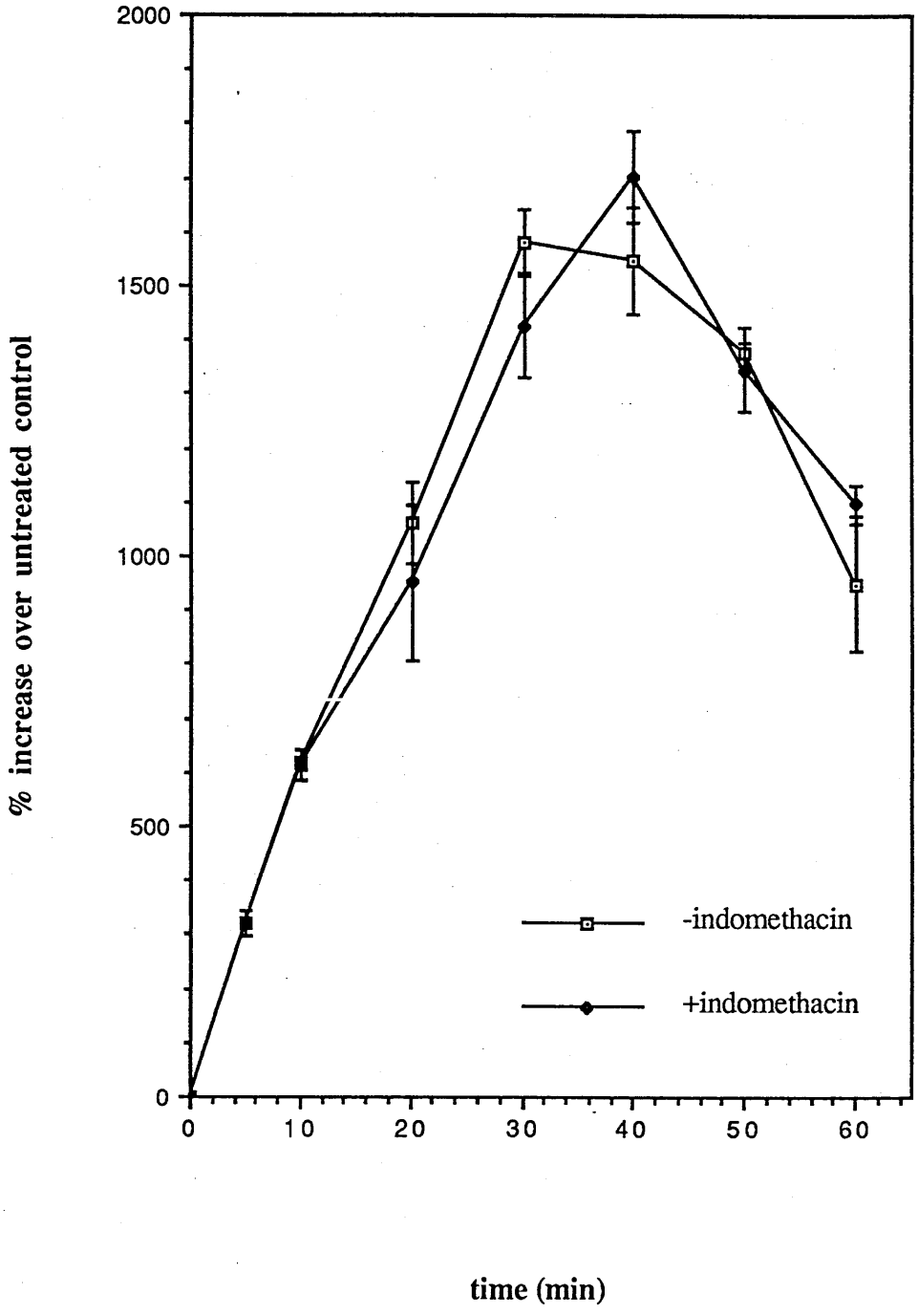

2000
1500
1000
500
0
0
10
20
30
40
50
60
% increase over untreated control
time (min)
-indomethacin
+indomethacin

Figure 3.9 illustrates growth curves of NIH-3T3 cells cultured in the presence or absence of indomethacin (0.1uM) and demonstrates that indomethacin had no effect on cell proliferation.

It is therefore unlikely that the reduced response to $PGF_{2\alpha}$ is due to the agonist itself (i.e. not homologous desensitization), but must occur via some other mechanism.

3.2.7 Effect of cell culture density upon the responsiveness of NIH-3T3 cells to $PGF_{2\alpha}$

It was noted that NIH-3T3 cells cultured to different cell densities gave differing responses to $PGF_{2\alpha}$. Table 3.6 shows that as the number of cells in a culture dish is increased and consequently the number of actively dividing cells is diminished, the magnitude of the stimulation of inositol phosphate generation by $PGF_{2\alpha}$ is increased. This increase in response is not due to the number of cells per incubation since when cells scraped from the same culture dishes were diluted in incubation medium, there was no effect upon the magnitude of the $PGF_{2\alpha}$ stimulated inositol phosphate generation (Table 3.7). Table 3.7 also demonstrates that the magnitude by which bradykinin stimulated the generation of inositol phosphates was unchanged following cell dilution. When there are 25,000 or less cells per incubation, the bradykinin and $PGF_{2\alpha}$ stimulated inositol phosphate responses were lower. However, at this low cell density the measurement of inositol phosphates is unreliable, being at the limit of accurate detection particularly in control samples.

$PGF_{2\alpha}$ receptor number was determined at different cell densities. Problems were encountered when binding experiments were performed on NIH-3T3 cells cultured at low cell density, since low levels of cell-associated radioactivity meant accurate measurements of receptor number could not be determined. However, estimates

Figure 3.9 The effect of indomethacin on the growth of NIH-3T3 cells

Cells were cultured in the presence (◆ pres. indomethacin) or absence (□ abs.indomethacin) of indomethacin (0.1uM). Cell numbers were determined by Coulter counting as described in Section 2.4. Results are means ± S.D. of 4 counts of duplicate plates and are from one experiment.

Figure 3.9

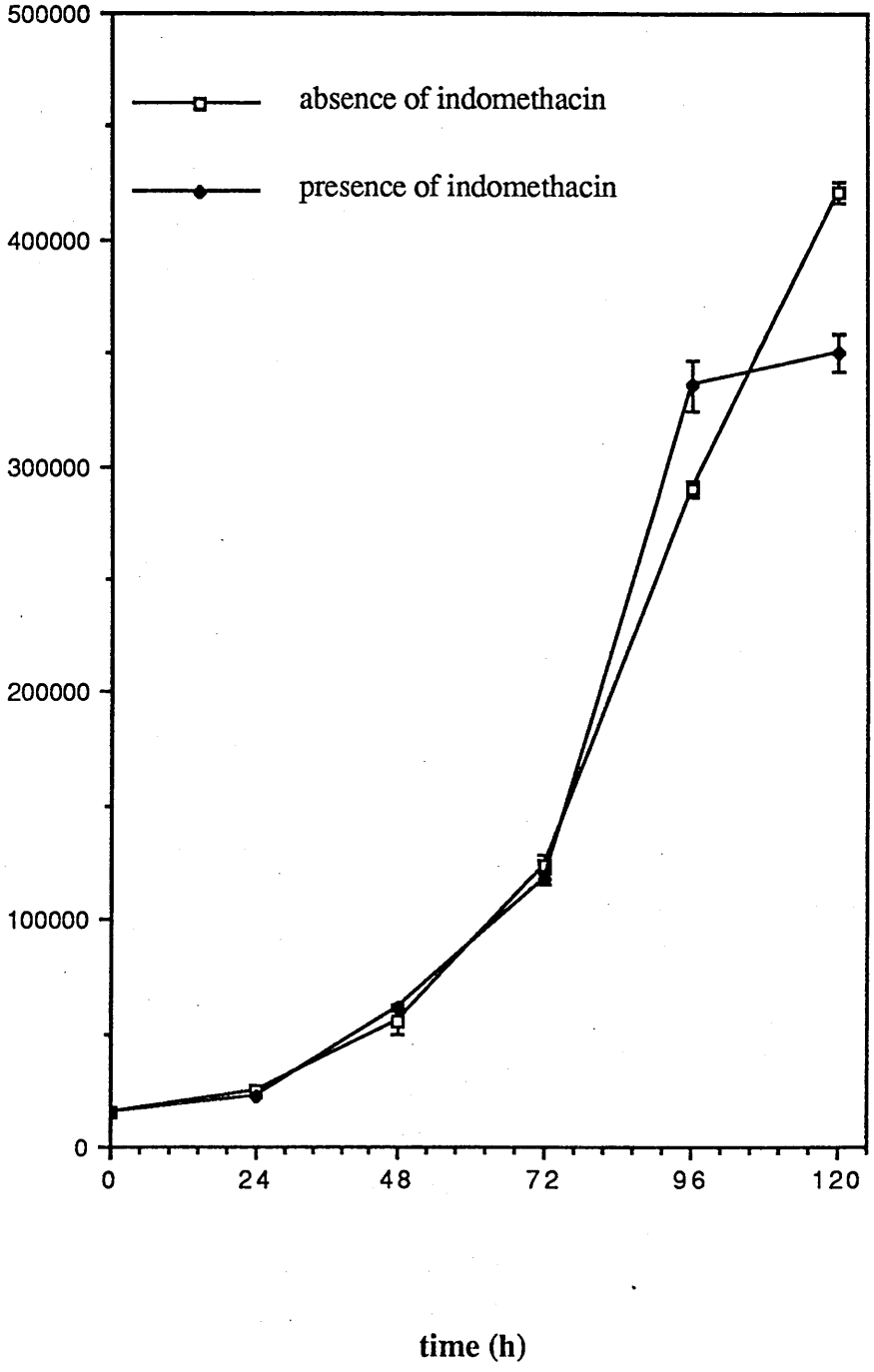

cell number
500000
400000
300000
200000
100000
0
absence of indomethacin
presence of indomethacin
0
24
48
72
96
120
time (h)

Table 3.6 Effect of cell culture density upon the responsiveness of NIH-3T3 cells to $PGF_{2\alpha}$

Cell density at plating	Dpm in control	Dpm with $PGF_{2\alpha}$	Dpm in control inositol lipids	Inositol phosphates % increase over untreated control
5×10^5	620 ± 14	11983 ± 108	21115 ± 269	1832 ± 27
2.5×10^5	625 ± 6	11185 ± 309	11923 ± 283	1690 ± 49
1×10^5	288 ± 14	2565 ± 72	7420 ± 147	790 ± 25
7.5×10^4	204 ± 19	1547 ± 17	5761 ± 91	659 ± 9
5×10^4	143 ± 5	358 ± 12	2188 ± 86	151 ± 9

NIH-3T3 cells were plated at the stated density. 16h later the medium was changed to one containing [^{3}H]inositol and 24h later $PGF_{2\alpha}$ (2.1uM) stimulated inositol phosphate generation was determined as described in Section 2.6 - sample preparation method A. Results are means ± S.D., n=4 in each case and the data are from one experiment which gave qualitatively the same results as three others.

Table 3.7 The effect of cell number dilution upon Bradykinin and $PGF_{2\alpha}$ -stimulated inositol phosphate generation in NIH-3T3 cells

Cell number per incubation	Dilution Factor	dpm in control	% increase over untreated control	
			Bradykinin	$PGF_{2\alpha}$
200,000	1	619 ± 19	356 ± 12	1775 ± 63
100,000	1.2	316 ± 17	348 ± 21	1771 ± 32
50,000	1.4	173 ± 8	351 ± 3	1805 ± 68
25,000	1.8	107 ± 8	263 ± 7	1218 ± 11
12,500	1.16	67 ± 5	189 ± 38	969 ± 70

Cells labelled with [^{3}H]inositol (1uCiml^{-1}) for 24h were scraped from four 25cm^3 flasks and diluted as described in the table. Bradykinin (3.2uM) and $PGF_{2\alpha}$ (2.1uM)-stimulated inositol phosphate generation was determined as described in Section 2.6 - sample preparation method A. Results are means ± S.D. of one experiment where n=4.

made from the results obtained indicated that there was no difference in receptor number expressed in cells cultured at different densities.

3.2.8 The effect of the presence of serum growth factors during [^{3}H]inositol labelling on subsequent growth factor stimulation of inositol phosphates

Removal of growth factor containing serum from proliferating NIH-3T3 cells, but not from their transformed derivatives, causes a cessation of proliferation. NIH-3T3 cells and a Ha-ras transformed NIH-3T3 cell line (EC807) were cultured either serum free in medium supplemented with insulin ($1ngml^{-1}$), transferrin ($10ng\ ml^{-1}$) and EGF ($10pgml^{-1}$) or in medium containing 10% calf serum. NIH-3T3 cells cultured serum free and subsequently stimulated with $PGF_{2\alpha}$ showed an inositol phosphate response, 60% greater than that observed in cells cultured in the presence of 10% calf serum (Table 3.8). However, no such change in response was observed in ras-transformed cells cultured in the absence of serum (Table 3.8).

3.2.9 The effect of TPA pre-treatment upon $PGF_{2\alpha}$-stimulated inositol phosphate generation and concentration of PKC

Desensitization of glucagon stimulated adenylate cyclase has been shown to be a PKC mediated process (Murphy et al., 1987). In order to determine if such a mechanism could be involved in the desensitization observed here, control and ras transformed NIH-3T3 cells were cultured in the presence of TPA (100nM) for 12, 24 and 48h and with the non PKC activating β-phorbol (100nM) for 24h (Table 3.9). The inhibitory effect of TPA on $PGF_{2\alpha}$-stimulated inositol phosphate generation was greater with a 12h pre-treatment compared with a 24 or 48h TPA pre-treatment for both control and ras transformed cells. A 12h TPA pre-treatment resulted in the

Table 3.8 Effect of different labelling conditions upon $PGF_{2\alpha}$-stimulated inositol phosphate generation in NIH-3T3 and EC807 (Ha-ras) cells

Cell line	Labelling conditions	dpm in control	dpm with $PGF_{2\alpha}$	Inositol phosphates % increase over untreated control
NIH-3T3	growth factor supplement	456 ± 10	25075 ± 3048	5398 ± 668
NIH-3T3	10% calf serum	543 ± 22	12653 ± 805	2230 ± 148
EC807	growth factor supplement	9218 ± 401	51433 ± 633	457 ± 8
EC807	10% calf serum	10231 ± 1385	54345 ± 1705	431 ± 17

NIH-3T3 and EC807 (cells transformed by over-expressed Ha-ras) were labelled with [^{3}H]inositol in medium containing a defined growth factor supplement (1ngml^{-1} insulin, 10ngml^{-1} transferrin and 10pgml^{-1} EGF) or medium supplemented with 10% donor calf serum (v/v). The cells were then stimulated for 30 min with $PGF_{2\alpha}$ (2.1uM) and inositol phosphate generation was determined as described in Materials and Methods. Results are means ± S.D., where n=4 and are from one experiment typical of two others.

Table 3.9 Effect of pre-treatment with TPA upon $PGF_{2\alpha}$ -stimulated inositol phosphate generation in control, Ha-ras (EC807) and N-ras (N866) transformed NIH-3T3 cells

Pretreatment	NIH-3T3		EC807		N866	
	Control dpm	Inositol phosphates (% increase over untreated control)	Control dpm	Inositol phosphates (% increase over untreated control)	control dpm	Inositol phosphates (% increase over untreated control)
None	498 ± 40	595 ± 10	2119 ± 79	299 ± 5	3462 ± 189	369 ± 10
12h TPA	472 ± 35	275 ± 13	2631 ± 150	33 ± 6	3765 ± 180	35 ± 2
24h TPA	519 ± 25	347 ± 20	2131 ± 202	82 ± 1	4055 ± 19	58 ± 2
48h TPA	523 ± 23	364 ± 90	2365 ± 304	81 ± 4	3485 ± 92	62 ± 4
24h beta phorbol	457 ± 28	609 ± 33	2033 ± 56	305 ± 4	3283 ± 86	385 ± 10

Cells were pre-treated with TPA (100nM) of β-phorbol (100nM) for the times stated. Cells were labelled with [^{3}H]inositol for 24h, prior to stimulation with $PGF_{2\alpha}$ (2.1uM). Inositol phosphate generation was determined as described in Section 2.6. Results are means ± S.D. where n=4 and are from one experiment typical of three others.

stimulation of inositol phosphate generation being reduced by 54, 89 and 90% in the NIH-3T3, EC807 (Ha-ras) and N866 (N-ras) cell lines, respectively (Table 3.9). A 24h TPA pre-treatment resulted in the stimulation of inositol phosphate generation being reduced by 42, 73 and 84%, while a 48h TPA pre-treatment resulted in a reduction of 39, 77 and 83% in the NIH-3T3, EC807 and N866 cell lines, respectively. The non PKC activating β-phorbol had no inhibitory effect on $PGF_{2\alpha}$-stimulated inositol phosphate generation.

The method of [^{3}H]PDBu binding was used to estimate the amount of PKC present in control and ras transformed cells prior to and following treatment with TPA (100nM). To assess total binding, cells on 24 well plates were incubated for 15 min at 37^{o}C in the presence of 100nM [^{3}H]PDBu. Non-specific binding was determined in the presence of 10uM unlabelled PDBu and was 29 $\pm$ 10%. In control, Ha-ras (EC807) and N-ras (N866) transformed cells the amount of PDBu bound was 284 $\pm$ 75, 465 $\pm$ 3 and 451 $\pm$ 30fmol PDBu bound/mg protein, respectively (results are means $\pm$ S.D. where n=3 and are from one experiment). Following a 24h TPA pre-treatment the amount of PDBu bound in control Ha- and N-ras transformed cells was 22 $\pm$ 13, 99 $\pm$ 12 and 76 $\pm$ 4fmol PDBu bound/mg protein. Thus, following a 24h TPA pre-treatment PKC concentrations were reduced; the amount of PKC was estimated to be 7, 21 and 17% of levels in non TPA pre-treated NIH-3T3, Ha- and N-ras transformed cells, respectively. In this experiment PKC concentrations were 1.6-fold greater in the ras transformed cells compared to control cells. In two other experiments PKC concentrations were estimated to be 3-fold greater in the Ha-ras (EC807) and N-ras (N866) transformed cells compared to control cells.

The amount of PKC determined following a 24th TPA pre-treatment may be under estimated as it is difficult to remove all traces of TPA from the cells; any residual TPA may compete with [^{3}H]PDBu in the binding assay.

3.3 Discussion

Evidence indicating that p21^{ras} may be involved in a signal transduction mechanism has existed for some time. It has been suggested that p21^{ras} controls the activity of PIC, which catalyses the hydrolysis of PtdIns(4,5)P_2 (Fleishman et al., 1986; Preiss et al., 1986; Wolfman & Macara, 1987). This hypothesis is based on the fact that transformation and proliferation accompany rapid metabolism of PtdIns(4,5)P_2 (Berridge, 1984; Macara, 1985) and that a G-protein appears to control PIC activity (Cockcroft & Gomperts, 1985). Bombesin stimulation of PtdIns(4,5)P_2 hydrolysis was shown to be amplified in NIH-3T3 cells over-expressing the N-ras proto-oncogene (Wakelam et al., 1986; Wakelam, 1988). An amplification of muscarinic stimulation of PtdIns(4,5)P_2 hydrolysis in Balb/c 3T3 cells transformed by EJ-Ha-ras has also been reported (Chiarugi et al., 1986). Parries et al. (1987) demonstrated that while NIH-3T3 cells did not respond to bradykinin, NIH-3T3 cells transformed by Ha-, Ki- or N-ras expressed bradykinin stimulated PIC activity. In the same study, however, NIH-3T3 cells transformed by v-Ha-ras exhibited a loss of PDGF-stimulated PtdIns(4,5)P_2 hydrolysis. Seuwen et al. (1988) reported a desensitization of the inositol phospholipid signalling pathway in CCL39 fibroblasts transformed by activated Ha- or Ki-ras genes.

Cells transformed by ras proliferate more rapidly and demonstrate greater turnover of their inositol phospholipids than control cells (Fig.3.1). This indicates that there may be a role for increased inositol phospholipid turnover in cell proliferation and transformation. The incorporation of [^{3}H]inositol into PtdIns(4)P and PtdIns(4,5)P_2 in NIH-3T3 cells reaches a steady state level after 12h, whilst the rate of incorporation of

[^{3}H]inositol continues to increase in T15$^+$ cells (Fig.3.1). This may be due to the inhibition of mechanisms in the ras transformed cells that normally desensitize inositol phospholipid turnover in normal cells. It was considered possible that the increased inositol phospholipid turnover in the T15$^+$ cells could be due to an increase in basal turnover. However, basal rates of inositol phosphate production are not increased in ras transformed cells (Table 3.1).

In cells expressing high levels of the normal Ha-, Ki- or N-ras proto-oncogene, there is a marked increase, compared with the control cells, in inositol phosphates generated in response to stimulation by bradykinin, calf-serum and PDGF (Fig.3.3). It is possible that there could be a change in receptor number as a result of transformation by ras, but this is unlikely as Wakelam et al. (1986) demonstrated that there is no change of bombesin receptor binding characteristics as a consequence of ras transformation. However, Downward et al. (1988) demonstrated that in Rat-1 fibroblasts over-expressing normal and transforming Ha-, Ki- and N-ras genes the amplified bradykinin stimulated inositol phosphate response might be due to receptor up-regulation since bradykinin receptor numbers were found be 40-fold higher in the cells over-expressing p21^{ras} compared to control cells.

The results presented in this chapter demonstrate that in cells expressing high levels of the normal Ha-, Ki- or N-ras proto-oncogenes, the stimulation by $PGF_{2\alpha}$ of inositol phosphate generation is reduced (Table 3.2). In NIH-3T3 cells stimulation by $PGF_{2\alpha}$ results in a 12-fold increase in the level of inositol phosphates. However, in Ha-, Ki- or N-ras transformed cells $PGF_{2\alpha}$ only stimulated a 2-4-fold increase increase (Table 3.2). There thus appears to be a ras induced desensitization of $PGF_{2\alpha}$

stimulated inositol phospholipid turnover in the ras transformed cells. The NIH-3T3 clone H8/22 was used to demonstrate that the markedly reduced $PGF_{2\alpha}$ -stimulated inositol phospholipid turnover in ras transformed cells is directly related to the expression of $p21^{ras}$ and is not a transformation effect. The data in Figure 3.4 shows that $p21^{Ha\text{-}ras}$ induced amplification of PDGF-stimulated inositol phosphate generation occurred approximately 8h after dexamethasone treatment of H8/22 cells.

Results obtained by Davies (1989) in a similar experiment showed that $p21^{N\text{-}ras}$ induced amplification of bombesin-stimulated inositol phosphate generation was detected approximately 6h after dexamethasone treatment of $T15^{-}$ cells.

These data strongly suggest that the ras-induced amplification of inositol phosphate production is not associated with the transformed state, but is an early event in cell proliferation and transformation and is directly a consequence of $p21^{N\text{-}ras}$ expression. However, when H8/22- cells treated with dexamethasone, as above, were stimulated with $PGF_{2\alpha}$, the progressive elevation of $p21^{Ha\text{-}ras}$ concentration was paralleled by a comparable decrease in the magnitude of the inositol phosphate response (Fig.3.4). This ras induced desensitization of $PGF_{2\alpha}$ -stimulated inositol

phospholipid turnover is not due to any changes in receptor number, or change in EC_{50} or Kd (Table 3.3 and 3.4) and thus would appear to be due to a reduced coupling of the agonist receptor complex to PIC. It is unlikely to be due to any reduction in PIC since there is no change in the basal rate of inositol phosphate generation in cells transformed by an over-expression of normal ras genes (Table 3.1). The site of the desensitization may thus be at either the level of coupling between the receptor to G-protein, or between the G-protein and PIC. In $T15^+$ cells which over-express $p21^{N\text{-}ras}$, an amplification in both bombesin (Wakelam et al., 1986; Lloyd et al., 1989) and fluoroaluminate (Wakelam, 1989) stimulated PtdIns(4,5)P_2 hydrolysis was detected. However, $PGF_{2\alpha}$ -stimulated inositol phosphate generation is also desensitized in $T15^+$ cells (Table 3.2) with no change in affinity or dissociation constant, or a decrease in receptor number (Tables 3.3, 3.4). Therefore, at least part of the desensitization mechanism must be at the level of receptor to G-protein coupling.

In control NIH-3T3 cells desensitization of the $PGF_{2\alpha}$ -stimulated inositol phosphate response occurred at 30 min (Fig.3.2). Desensitization of agonist-stimulated PtdIns(4,5)P_2 hydrolysis is very important as it acts as an inhibitory mechanism to switch off or modulate Ins(1,4,5)P_3 production, thus effectively protecting the cell from Ca^{2+}-evoked damage during a sustained activation of Ca^{2+}-mobilising receptors.

Desensitization of an agonists second messenger response can be induced both by exposure to the agonist, that is homologously, or following exposure to an alternative agonist that is heterologously. It appears that the desensitization of the $PGF_{2\alpha}$-stimulated inositol phosphate response in the ras transformed cells is induced heterologously, since inhibition of

$PGF_{2\alpha}$ synthesis by treatment of the cells with indomethacin does not amplify the stimulation of inositol phosphate generation in response to $PGF_{2\alpha}$. In ras transformed cells there is increased secretion of a variety of autocrine growth factors (Bowen-Pope et al., 1984), e.g. PDGF, which are able to stimulate PtdIns(4,5)P_2 hydrolysis perhaps leading to the persistent activation of PKC. Huang and Ives (1989) demonstrated that exposure of vascular smooth muscle cells for 10 min to phorbol esters caused an 82% inhibition of thrombin stimulated Ins(1,4,5)P_3 generation. This effect is due to PKC activity since no inhibition was observed in PKC depleted cells. Exposure of the cells to phorbol esters for 30 min did not alter the binding affinity or maximum binding of thrombin. Other examples of phorbol ester mediated inhibition of PtdIns(4,5)P_3 hydrolysis in the absence of an effect on agonist binding include work by Brown et al. (1987) and Pfeilschifter et al. (1989). Brown et al. (1987) demonstrated that pre-treatment of Swiss 3T3 cells for 15 min with TPA caused a reduction in the bombesin-stimulated inositol phosphate response. Pre-treatment of Swiss 3T3 cells for 48h with TPA had the effect of greatly decreasing the inhibitory effect of a subsequent 15 min pre-treatment with TPA on the bombesin-stimulated inositol phosphate response (Brown et al., (1987). Pfeilschifter et al. (1989) demonstrated that pre-treatment of vascular smooth-muscle cells for 10 min with TPA decreased the angiotensin II-induced Ins(1,4,5)P_3 generation. Pre-treatment of these cells for 24h with TPA lead to an enhanced basal, as well as angiotensin II-stimulated, generation of Ins(1,4,5)P_3. Treatment of DDT_1MF-2 smooth muscle cells with bradykinin or phorbol esters lead to the desensitization of α_1-adrenergic receptor-stimulated PtdIns(4,5)P_2 hydrolysis and has been shown to be a consequence of receptor phosphorylation

(Leeb-Lundberg et al., 1985). These observations suggest that phosphorylation of receptors by PKC exerts a negative feedback control on agonist-stimulated inositol phospholipid turnover and that PKC is an important factor in limiting the production of $Ins(1,4,5)P_3$ in stimulated cells. Consistent with this possibility is the fact that the $PGF_{2\alpha}$-stimulated inositol phosphate response is reduced following exposure to TPA in both NIH-3T3 and ras transformed cells (Table 3.9). Long term TPA pre-treatment of NIH-3T3 cells lead to a reduction in the $PGF_{2\alpha}$-stimulated inositol phosphate response, whilst long term TPA pre-treatment of Swiss 3T3 cells (Brown et al., 1987) and vascular smooth muscle cells (Pfeilschifter et al., 1989) lead to an enhanced bombesin and angiotensin II-stimulated $PtdIns(4,5)P_2$ hydrolysis, respectively. The different results obtain are most likely due to the fact that PKC can be almost completely down-regulated in Swiss 3T3 cells and vascular smooth muscle cells, whilst in NIH-3T3 cells after a 24h TPA pre-treatment at least 7-21% of the PKC remains, as determined by PDBu binding. Cells transformed by Ha- or N-ras, pretreated for 24h with TPA, had greater amounts of PKC remaining compared with control cells and this is reflected in that there is greater inhibition of the $PGF_{2\alpha}$-stimulated inositol phosphate response in ras transformed cells. In non TPA treated cells the amount of PKC in Ha- and N-ras transformed cells was estimated to be at least 1.6-fold greater than control-NIH-3T3 cells and this may be a contributory factor in the desensitization of the $PGF_{2\alpha}$-stimulated inositol phosphate response in ras transformed cells. This percentage inhibition of the $PGF_{2\alpha}$-stimulated inositol phosphate response in long term TPA pre-treated control and ras transformed cells is surprising. It might have been expected that with only 7-21% of the PKC remaining that the inhibitory effect

would be less marked. Possibly the remaining PKC is adequate to induce the desensitization or perhaps only isoforms of PKC not involved in the desensitization of the inositol phospholipid signalling pathway were down-regulated. Kariya and Taki (1987) demonstrated that in rabbit aortic smooth muscle cells there exists types of PKC resistant to down-regulation after prolonged treatment with phorbol esters.

Direct demonstration that the inhibition of $PGF_{2\alpha}$ -stimulated PtdIns(4,5)P_2 hydrolysis in long term TPA treated cells and the desensitization of the $PGF_{2\alpha}$-stimulated inositol phosphate response in ras transformed cells is a result of PKC catalysed phosphorylation of the $PGF_{2\alpha}$ receptors awaits the purification of the receptor or the generation of specific antibodies.

Cells transformed by a range of ras genes have elevated levels of DAG (Preiss et al., 1986; Kamata et al., 1987: Lacal et al., 1987a, 1987b; Wolfman & Macara, 1987). Lacal et al. (1987b) have demonstrated that microinjection of $p21^{Ha\text{-}ras}$ into Xenopus oocytes increases DAG levels. Increases in DAG concentrations may lead to increases in PKC activity which has been shown to be essential for ras induced mitogenesis (Lacal et al., 1987c). The stimulation of mitogenesis in a range of normal fibroblast cell lines by growth factors has also been shown to be accompanied by an increase in DAG levels and in PKC activity (Habenicht et al., 1981; Cook et al., 1990).

If the desensitization of $PGF_{2\alpha}$ -stimulated inositol phospholipid hydrolysis is a consequence of increased PKC activity then one would expect to observe desensitization in both the transformed cells and in rapidly proliferating, non-contact inhibited NIH-3T3 cells as is indeed demonstrated in Tables 3.2 and 3.6.

Removal of growth factor containing serum from NIH-3T3 cells causes a cessation of proliferation and thus a reduction in PKC activity and consequently a reduction in the observed desensitization (Table 3.8). However, the removal of serum from the ras transformed cells does not result in resensitization. Since ras transformed cells are known to secrete a range of autocrine growth factors, including TGF_{α} and PDGF-like molecules (Owen & Ostrowski, 1987), it is not possible to remove from the culture medium factors which are able to stimulate a range of signalling pathways including PtdIns(4,5)P_2 hydrolysis. PKC activity will, therefore, continue to be stimulated in ras transformed cells even in the absence of serum, whereas in the control NIH-3T3 cells in the absence of serum, the PKC activity will be lower. It is, therefore, possible that the desensitization of $PGF_{2\alpha}$-stimulated inositol phosphate generation in both the ras transformed and in the proliferating NIH-3T3 cells is due to increased PKC activity. This suggestion is supported by the finding that when normal and ras transformed NIH-3T3 cells are cultured in the presence of the PKC activator TPA for 48h their responsiveness to $PGF_{2\alpha}$ is significantly reduced as compared to untreated control cells (Table 3.9).

The experiments presented in this chapter also suggest an explanation for the observed variability of effects of transfecting ras genes into fibroblast cells upon inositol phospholipid metabolism. Since such cells are continuously proliferating and do not exhibit 'density dependent regulation', they do not quiesce. Therefore any amplified agonist stimulated inositol phosphate response may be desensitized and not be apparent.

CHAPTER 4

ACTIVATION OF INOSITOL PHOSPHOLIPID HYDROLYSIS BY PROSTAGLANDIN $F_{2\alpha}$ WITHOUT ANY STIMULATION OF PROLIFERATION IN QUIESCENT NIH-3T3 FIBROBLASTS

4.1 INTRODUCTION

The stimulation of the proliferation of fibroblast cells in culture has been demonstrated to be achieved by a range of growth factors which are capable of activating the inositol phospholipid signal transduction pathway (Berridge, 1987b). Agonist stimulated PtdIns(4,5)P_2 hydrolysis generates two second messenger molecules, DAG and Ins(1,4,5)P_3 which activate PkC and stimulate the release of Ca^{2+} from intracellular stores respectively (Downes & Michell, 1985). A rise in intracellular free calcium concentration has been demonstrated to be one of the earliest detectable events following the stimulation of mitosis in fibroblast cells (Hesketh et al., 1985) and the activation of PkC leads to the phosphorylation of a range of cellular proteins, but in particular the Na^+/H^+ antiporter, resulting in increased cytoplasmic pH (Hesketh et al., 1985). Evidence has been obtained which suggests that Na^+-H^+ exchange is linked to DNA synthesis (L'Allemain et al., 1984; Pouyssegur et al., 1984). Alkalinisation of the cytosol appears to be obligatory for mitosis and it has been shown that growth factor stimulation of Swiss 3T3 and A431 cells results in increased intracellular pH (Schuldiner & Rozengurt, 1982). It has not yet been determined, however, whether the raised pH values associated with exponentially growing cells is a regulatory signal or if it is a consequence of cell proliferation.

$PGF_{2\alpha}$ is an agonist which has been demonstrated to be a growth factor for, and to stimulate the hydrolysis of PtdIns(4,5)P_2 in Swiss 3T3 fibroblast cells (MacPhee et al., 1984). $PGF_{2\alpha}$ stimulated inositol phosphate generation in NIH-3T3 fibroblasts and the involvement of inositol phospholipid turnover in the regulation of proliferation has been investigated.

4.2 RESULTS

4.2.1 Time-dependent $PGF_{2\alpha}$-stimulated inositol phosphate generation in NIH-3T3 cells

Although total inositol phosphate generation is a reliable measure of receptor stimulated PtdIns(4,5)P_2 hydrolysis, it is possible that it could also occur as a consequence of PtdIns hydrolysis (Majerus et al., 1988). As Ins(1,4,5)P_3 is the second messenger, it was important to obtain conclusive evidence that it is generated following stimulation with $PGF_{2\alpha}$.

Cell extracts were prepared from control and $PGF_{2\alpha}$ (2.1uM) stimulated NIH-3T3 cells in the presence of 10mM LiCl and applied to Dowex formate columns to separate the inositol phosphates. Stimulation with 2.1uM $PGF_{2\alpha}$ induced significant generation of inositol monophosphate (IP) within 10 seconds. This then increased in a linear manner and after 120 seconds it had reached 189% of control (Fig. 4.1a). Significant increases in inositol bisphosphate (IP_2) were observed by 10 seconds (approximately 48% increase). IP_2 continued to increase reaching 434% of control after 120 seconds (Fig. 4.1b). Figure 4.1c demonstrates that stimulation with $PGF_{2\alpha}$ (2.1uM) induced the generation of inositol trisphosphate (IP_3) within 5 seconds (approximately 68% increase). At 20 seconds values were approximately 377% of control. The levels declined at 30 seconds, but increased again to 440% at 60 seconds and at 120 seconds to 369% of control.

There are two isomeric forms of IP_3 known to be produced on receptor stimulation, Ins(1,4,5)P_3 and Ins(1,3,4)P_3 (Irvine et al. (1984b), which cannot be separated by Dowex anion exchange chromatography. Therefore the analysis of inositol phosphates was extended by separation upon a partisil 5 WAX HPLC column. This

Figure 4.1 Time-dependent $PGF_{2\alpha}$ -stimulated inositol phosphate generation in NIH-3T3 cells. Separation by Dowex anion exchange chromatography

[^{3}H]inositol labelled cells were stimulated with $PGF_{2\alpha}$ (2.1uM) for the times stated in the presence of 10mM LiCl. The inositol phosphates were separated on Dowex-formate 1x8-200 resin columns as described in Section 2.7a. Results are presented as the mean percentage of the radioactivity associated with the unstimulated control, n=6 in each case and the data is pooled from two separate experiments. The control d.p.m. values were: Figure 4.1a inositol monophosphate = 547 $\pm$ 1; Figure 4.1b inositol bisphosphate = 198 $\pm$ 1; Figure 4.1c inositol trisphosphate = 140 $\pm$ 6.

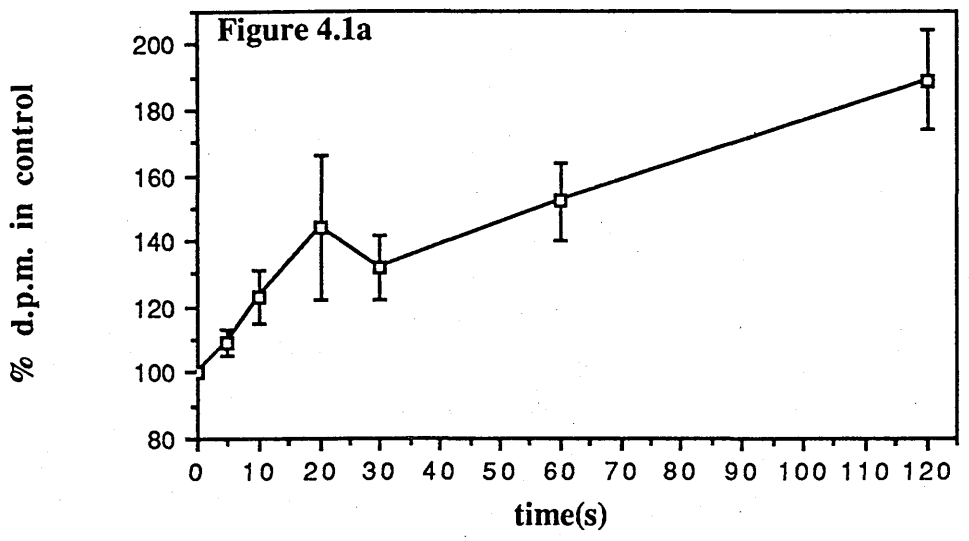


Figure 4.1a
% d.p.m. in control
200
180
160
140
120
100
80
0 10 20 30 40 50 60 70 80 90 100 110 120
time(s)

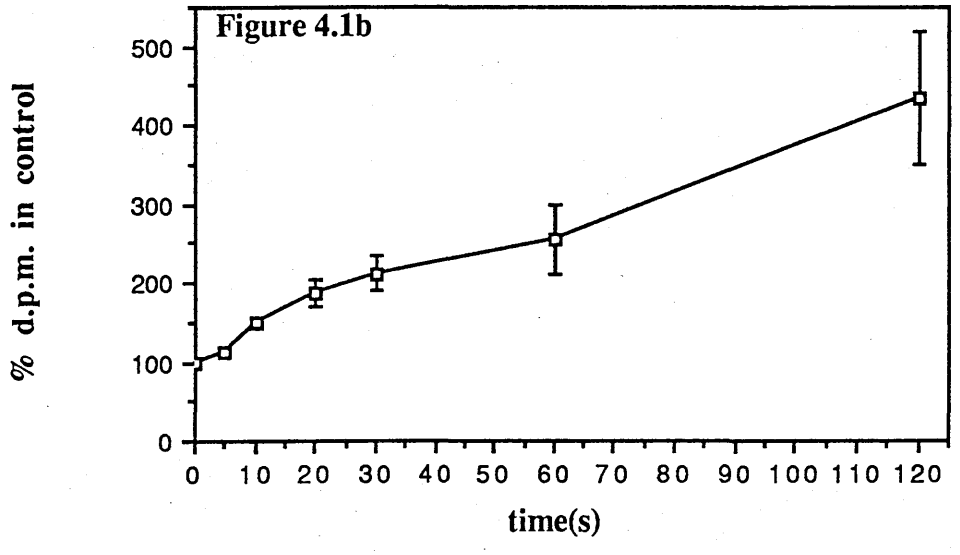


Figure 4.1b
% d.p.m. in control
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100 110 120
time(s)

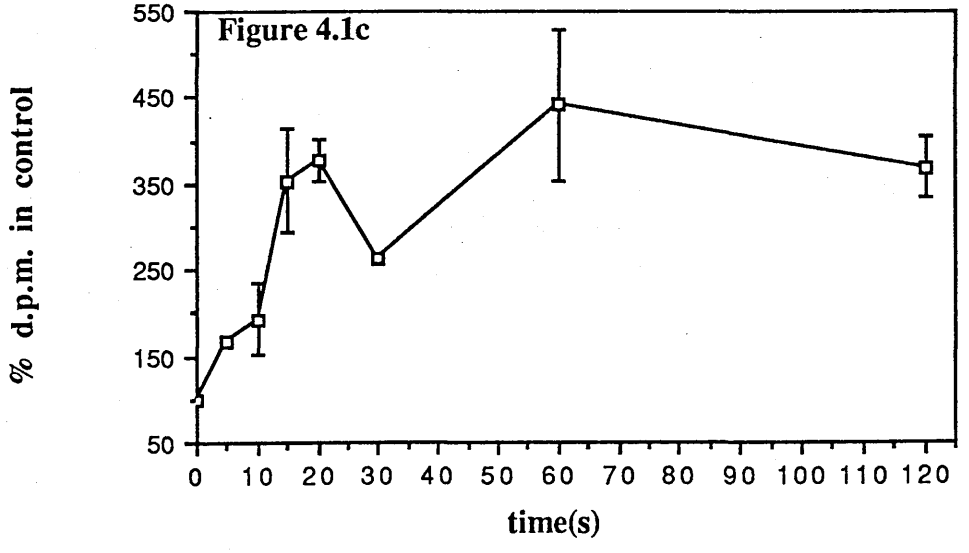


Figure 4.1c
% d.p.m. in control
550
450
350
250
150
50
0 10 20 30 40 50 60 70 80 90 100 110 120
time(s)

HPLC method also had the advantage of being able to isolate the higher inositol phosphates, for example inositol penta- or hexa-kisphosphates. Figure 4.2 shows the time-course of $PGF_{2\alpha}$-stimulated inositol phosphate generation as determined by HPLC. Stimulation with 2.1uM $PGF_{2\alpha}$ induced the generation of $Ins(1,4,5)P_3$ (Fig.4.2c) and $Ins(1,3,4,5)P_4$ (Fig.4.2e) within 5 seconds, though the magnitude of the increase in $Ins(1,3,4,5)P_4$ generation at 5 seconds (approximately 40% increase) was less than that for $Ins(1,4,5)P_3$ (approximately 150% increase). The stimulation of $Ins(1,4,5)P_3$ generation was always observed to be biphasic, as is demonstrated in Figure 4.2c. Peak accumulation of $Ins(1,4,5)P_3$, in the particular experiment demonstrated, was observed at 15 seconds when values were approximately 400% of control. The levels then declined returning to basal by 30 seconds. In some experiments the magnitude of the increase in $Ins(1,4,5)P_3$ was greater, reaching over 600% of the control value. A 10 second delay was observed before accumulation of $Ins(1,3,4)P_3$ was detected (Fig.4.2d). Accumulation of this isomer was then rapid and after 60 seconds the level was 500% of the basal; after 60 seconds the rate of generation appeared to decline. Data obtained by Dowex chromatography (Fig.4.1c) shows that after 30 seconds IP_3 levels increased and at 120 seconds were 369% of control. The analysis of IP_3 isomers by HPLC strongly suggests that the increases in IP_3 levels after 30 seconds is most likely due to increases in $Ins(1,3,4)P_3$ and not $Ins(1,4,5)P_3$. Peak accumulation of $Ins(1,3,4,5)P_4$ was detected following a 10 second stimulation but in contrast to $Ins(1,4,5)P_3$, the level appeared to remain elevated; at 120 seconds levels were 160% of control. Rapid accumulation of inositol mono- and bis-phosphates was also detected. At 120 seconds IP and IP_2 levels were 145% and 485% of the control values, respectively. Small changes were

Figure 4.2 Time-dependent $PGF_{2\alpha}$ -stimulated inositol phosphate generation in NIH-3T3 cells. Separation upon a partisil 5 WAX HPLC column

[^{3}H]inositol labelled cells were stimulated with $PGF_{2\alpha}$ (2.1uM) for the times stated in the presence of 10mM LiCl. The inositol phosphates were separated by HPLC as described in Section 2.7b. Results are presented as the mean percentage of the radioactivity associated with the unstimulated control, n=3 in each case and the data is from a single experiment typical of three. The control d.p.m. values were: Figure 4.2a inositol monophosphate = 198,976 $\pm$ 6,023; Figure 4.2b inositol bisphosphate = 7,807 $\pm$ 463; Figure 4.2c Ins(1,4,5)P_3 = 1,523 $\pm$ 97; Figure 4.2d Ins(1,3,4,)P_3 = 2,159 $\pm$ 403; Figure 4.2e Ins(1,3,4,5)P_4 = 3,409 $\pm$ 54; Figure 4.2f putative InsP_5 = 15,981 $\pm$ 1,297; Figure 4.2g putative InsP_6 = 2,711 $\pm$ 288.

Figure 4.2a

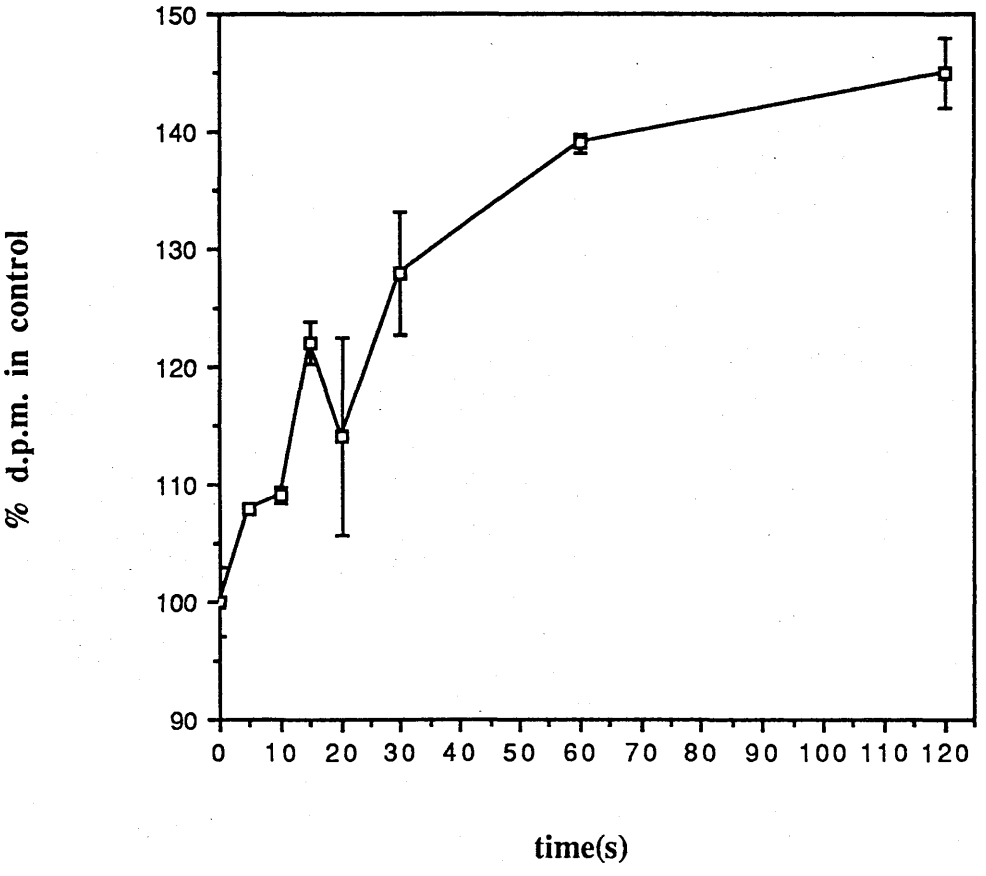


Figure 4.2b

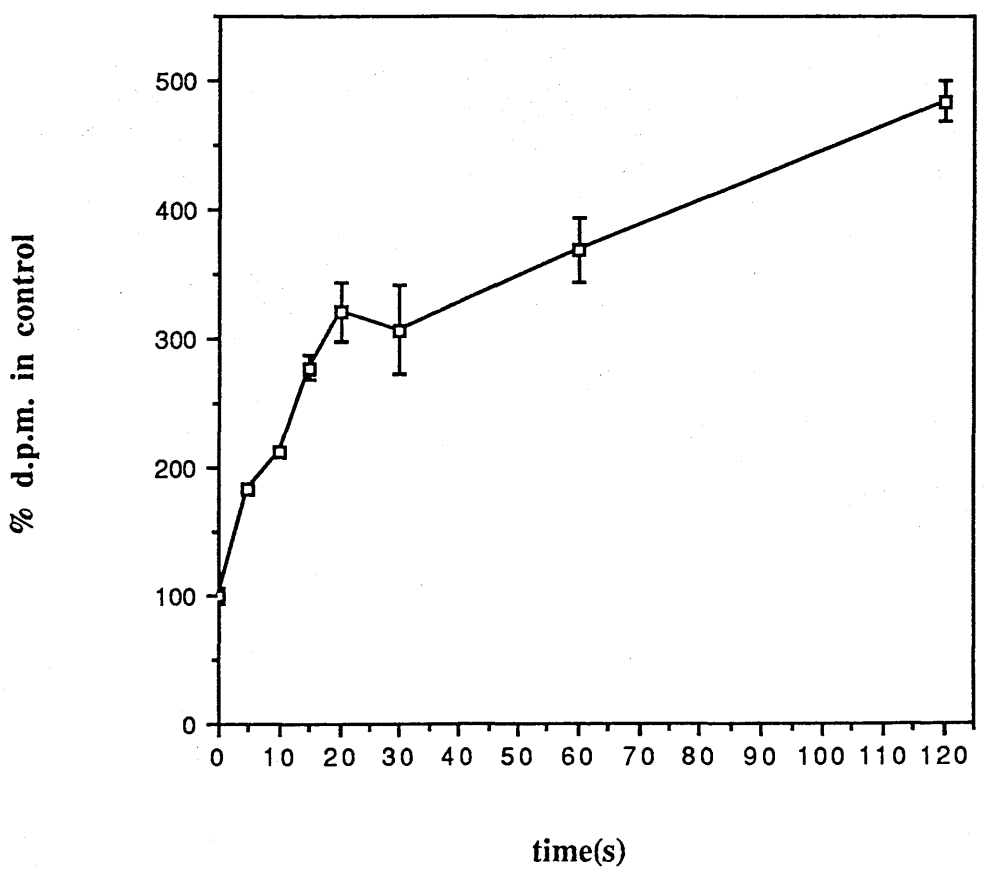


Figure 4.2c

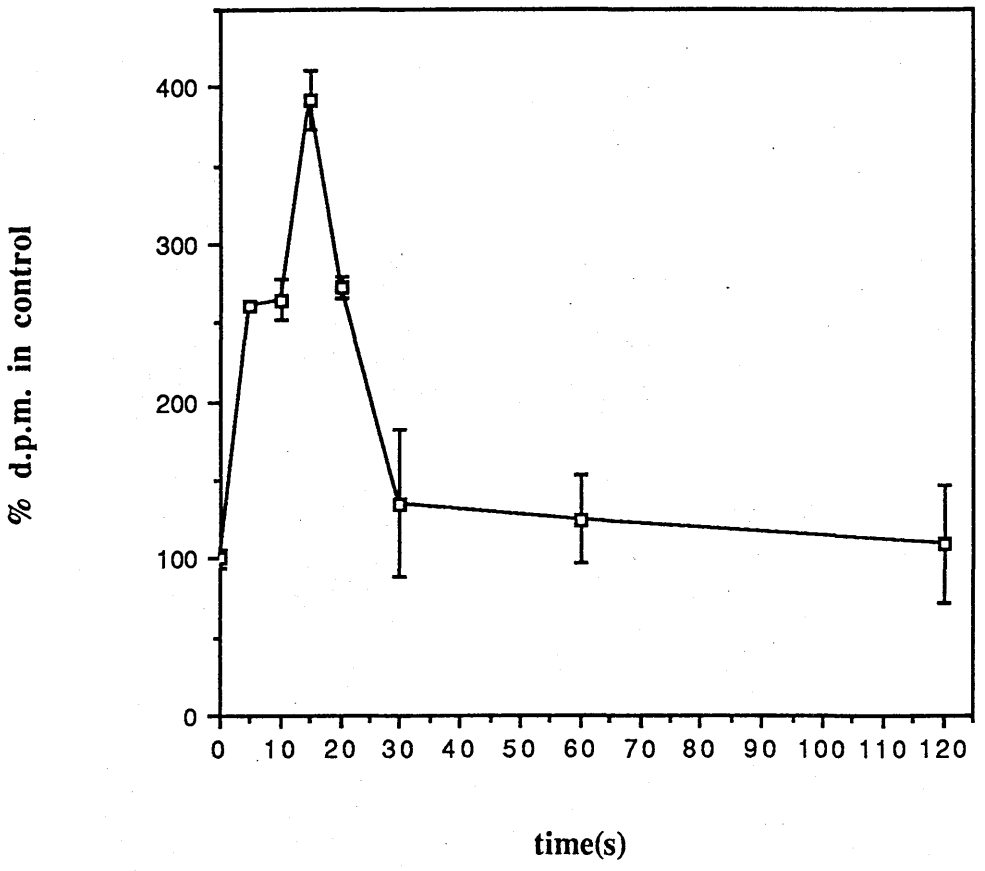


Figure 4.2d

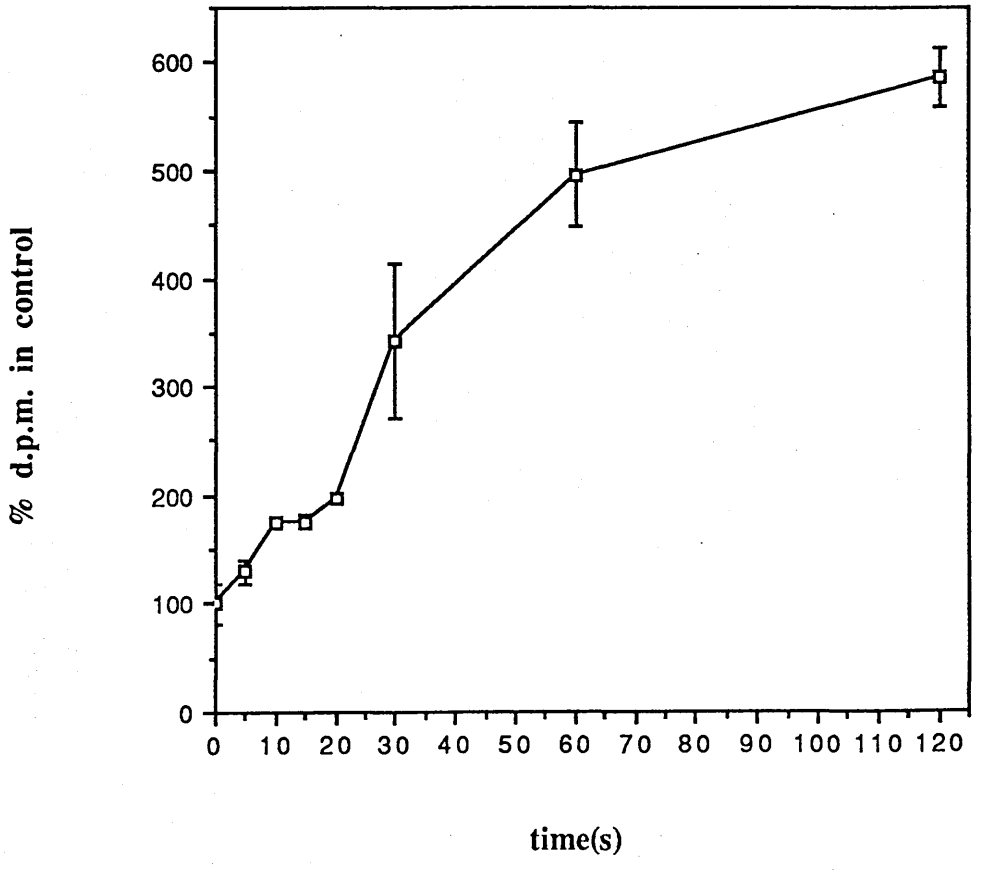


Figure 4.2e

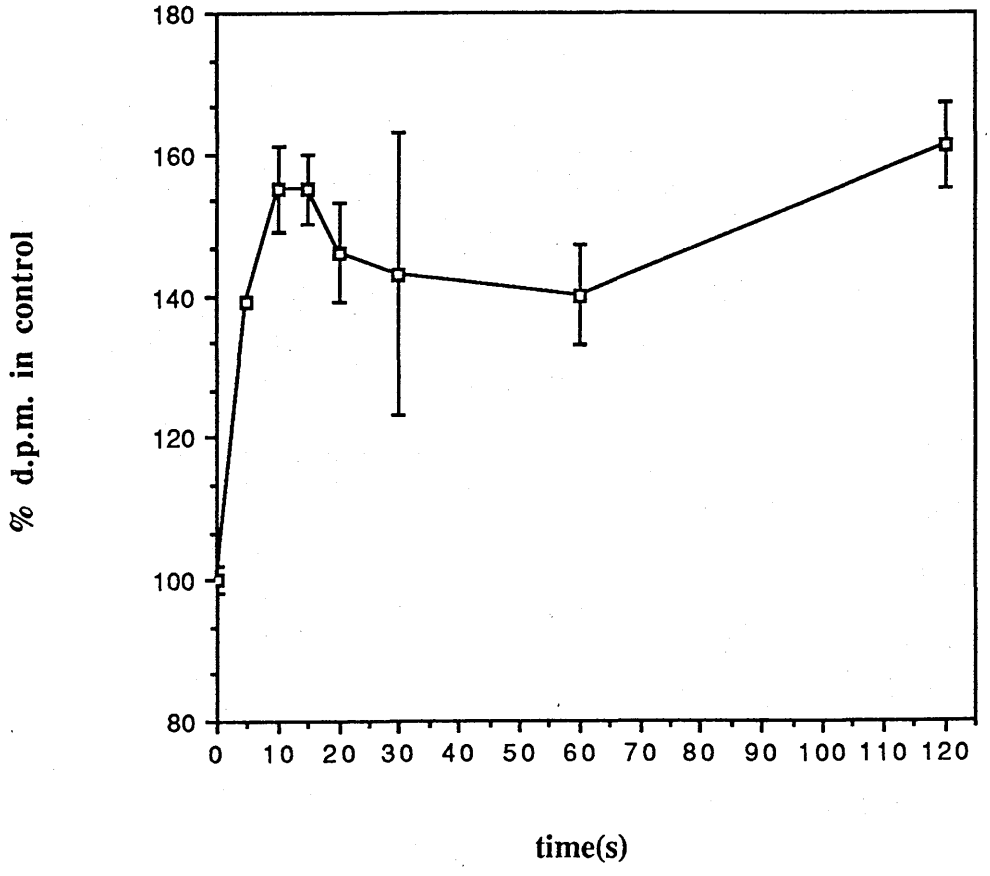

180
160
140
120
100
80
0 10 20 30 40 50 60 70 80 90 100 110 120
% d.p.m. in control
time(s)

Figure 4.2f

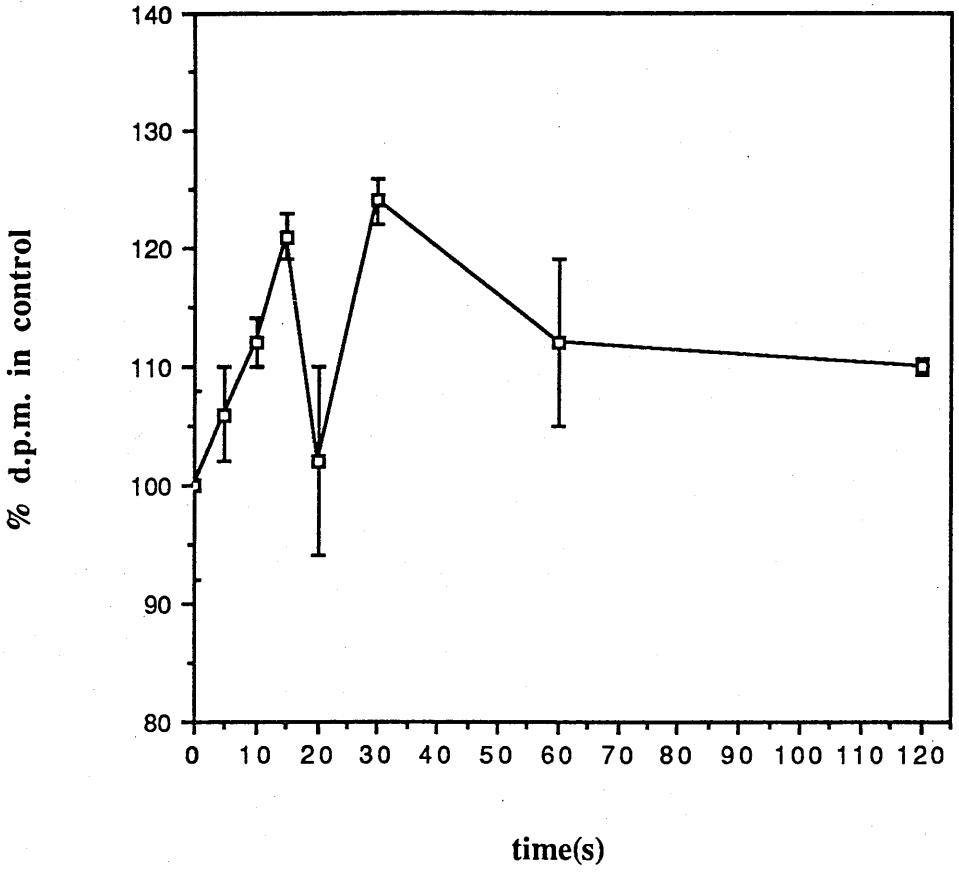
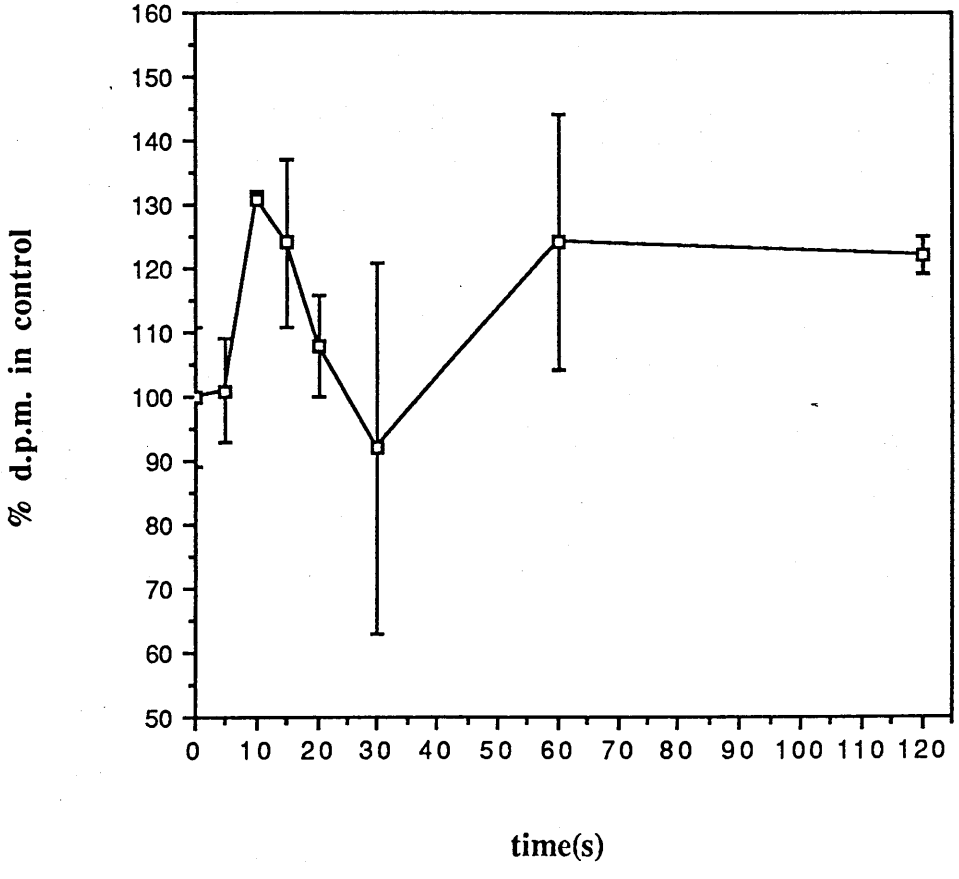


Figure 4.2g



also observed in the putative inositol penta- or hexa-kisphosphate fractions with the increases being significant after 10 seconds at about 120% and 130% of the control values respectively (Figs. 4,2f,g)

The HPLC data from IP, IP_2 and $Ins(1,4,5)P_3$ time-courses correlates with the data obtained by Dowex chromatography (see Fig.4.1) and indicates that $PGF_{2\alpha}$ stimulates $Ins(1,4,5)P_3$ production in NIH-3T3 cells as a result of PIC catalysed hydrolysis of $PtdIns(4,5)P_2$.

4.2.2 Inositol phospholipid hydrolysis and proliferation

Stimulation of NIH-3T3 cells with calf serum (4%) induced a 4-fold increase in total inositol phosphates, whilst in the same experiment a maximal dose of $PGF_{2\alpha}$ induced a 10-fold increase (Table 4.1). However, when the two stimulants were added together to the cells a 21-fold increase in inositol phosphate generation was detected (Table 4.1). This synergistic accumulation of inositol phosphates would be thought to be reflected in an equally synergistic effect upon mitosis.

Table 4.2 shows the effect of $PGF_{2\alpha}$ upon calf serum stimulated [^{3}H]thymidine incorporation into quiescent NIH-3T3 cells. [^{3}H]thymidine incorporation experiments were carried out to measure increases in the DNA content of the cells, this being a measure of mitogenesis. $PGF_{2\alpha}$, at a concentration that gave a maximal effect upon $PtdIns(4,5)P_2$ hydrolysis, had no effect upon calf serum stimulated [^{3}H]thymidine incorporation into serum-starved cells (Table 4.2). This lack of effect was observed at all calf serum concentrations tested. Since [^{3}H]thymidine incorporation into DNA is dependent upon uptake of the base into the cells it was considered possible that in these cells the prostaglandin was inhibiting thymidine uptake whilst at the same time stimulating DNA synthesis. Therefore, mitosis was also

Table 4.1 Calf serum and $PGF_{2\alpha}$ -stimulated inositol phosphate generation in NIH-3T3 cells

Stimulant	Inositol phosphate generation (% increase over untreated control)
Calf serum 4% (v/v)	426 ± 62
$PGF_{2\alpha}$ (2.1uM)	1015 ± 76
Calf serum 4% (v/v) and $PGF_{2\alpha}$ (2.1uM)	2163 ± 113

Cells were labelled with [^{3}H]inositol (1uCi ml^{-1}) for 24h and stimulated for 30 min in the presence of 10mM LiCl. Inositol phosphate generation was determined as described in Section 2.6. Results are presented as means ± S.D. pooled from two typical experiments and n=8 in each case.

Table 4.2 Effect of $PGF_{2\alpha}$ upon calf serum stimulated [^{3}H]thymidine incorporation into growth arrested NIH-3T3 cells

Additions	[^{3}H] thymidine (dpm)	Fold increase
None	11594 ± 454	
$PGF_{2\alpha}$	12337 ± 1140	1.06
0.5% calf serum	15101 ± 540	
0.5% calf serum + $PGF_{2\alpha}$	14763 ± 1459	0.98
1% calf serum	19104 ± 4588	
1% calf serum + $PGF_{2\alpha}$	18764 ± 2518	0.98
5% calf serum	18535 ± 1174	
5% calf serum + $PGF_{2\alpha}$	18879 ± 2394	1.02
10% calf serum	19136 ± 2435	
10% calf serum + $PGF_{2\alpha}$	18851 ± 1999	0.98

Quiescent NIH-3T3 cells were washed with DMEM and incubated for 24h in DMEM containing 1uCiml^{-1} [^{3}H]thymidine, 0.04ngml^{-1} insulin, 0.4ugml^{-1} transferrin, 0.4ngml^{-1} EGF and the additions stated in the table. Acid insoluble radioactivity was determined as described in Section 2.3. Results are expressed as means ± S.D., where n=4 and are from one experiment typical of three others. The $PGF_{2\alpha}$ concentration was 2.1uM, calf serum concentrations are stated on a volume basis. Fold increase refers to effect of $PGF_{2\alpha}$ upon stimulation by calf serum.

measured by determining cell number by Coulter counting, this being an absolute quantification of proliferation. Figure 4.3 illustrates growth curves for NIH-3T3 cells. Cells were plated in 35mm dishes and 24h later the medium was changed to one containing calf serum (10%) or calf serum (10%) and $PGF_{2\alpha}$(2.1uM). Additions of $PGF_{2\alpha}$(2.1uM) were made every 12h. Figure 4.3 demonstrates that $PGF_{2\alpha}$ was having no effect upon calf serum stimulated cell proliferation. The fall in cell number after 168h is probably due to cell death as cells become over confluent.

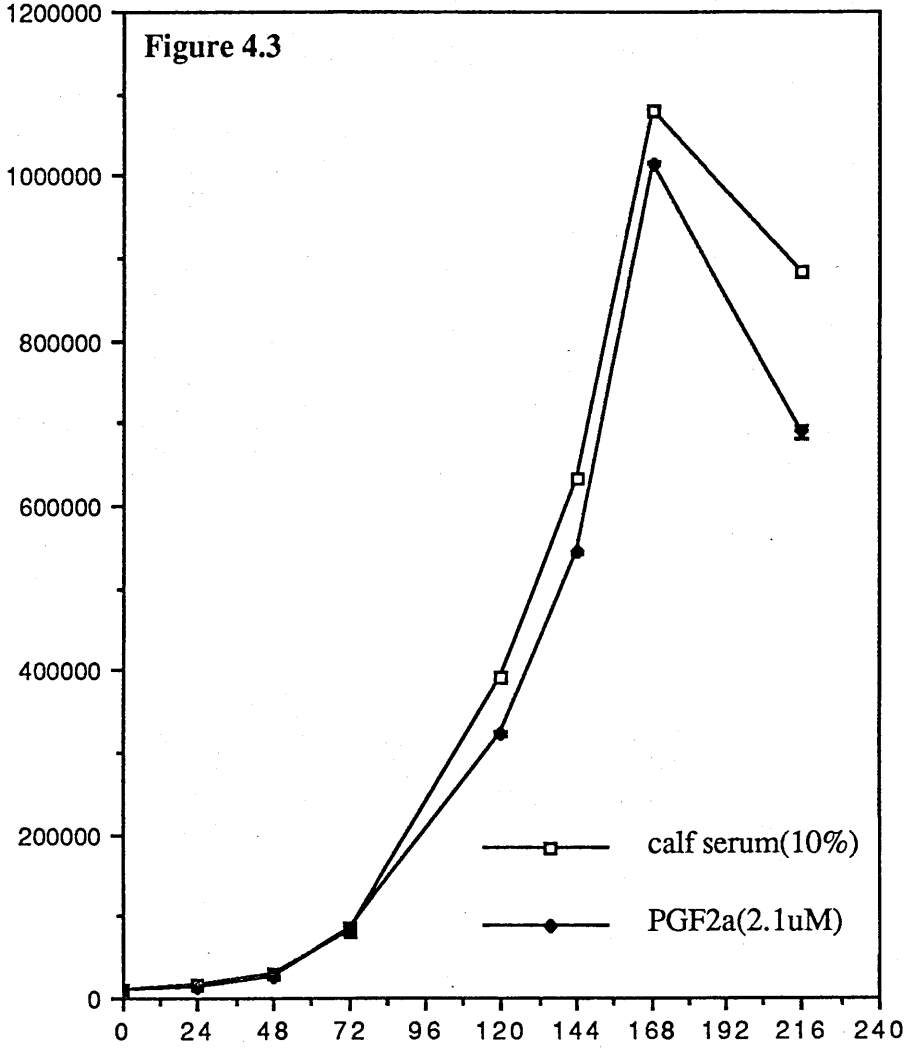
Table 4.3 shows the effect of growth factors on incorporation of [^{3}H]thymidine into growth arrested NIH-3T3 cells. In addition to having no potentiating effect upon calf serum stimulated mitosis, $PGF_{2\alpha}$ induced no DNA synthesis in quiescent NIH-3T3 cells in the presence of EGF, insulin and transferrin (Table 4.3). EGF ($0.1ugml^{-1}$), insulin ($1ngml^{-1}$), bombesin (2.5uM) and bradykinin (3.2uM) potentiated [^{3}H]thymidine incorporation by 1.83-, 2.90-, 1.09- and 2.31 fold, respectively (Table 4.3).

Contrary to the results presented in this chapter, $PGF_{2\alpha}$ has been reported to be a potent mitogen for NIH-3T3 cells (Yu et al., 1988). Consequently, NIH-3T3 cells were obtained from this group (subsequently referred to as AmNIH-3T3 cells). Table 4.4 demonstrates that in this cell line $PGF_{2\alpha}$ is indeed a potent mitogen stimulating a 2.3 fold increase in [^{3}H]thymidine incorporation. Calf serum stimulated a similar fold increase in [^{3}H]thymidine incorporation in both NIH-3T3 and AmNIH-3T3 cells 8.1 and 6.1 respectively. Figure 4.4 illustrates growth curves for AmNIH-3T3 cells cultured in the presence of calf serum (10%) or calf serum (10%) and $PGF_{2\alpha}$ (2.1uM) and demonstrates that $PGF_{2\alpha}$ potentiated calf serum stimulated cell growth to a saturation

Figure 4.3 NIH-3T3 growth curves

Cell numbers were determined by Coulter counting as described in Section 2.4. Six counts were made of duplicate plates and results are expressed as means ± S.D. from a single experiment typical of two others. □ cells grown in DMEM + 10% (v/v) calf serum, ◆ cells grown in DMEM + 10% (v/v) calf serum + 2.1uM $PGF_{2\alpha}$ (cells supplemented with $PGF_{2\alpha}$ (2.1uM) every 12h).

cell number



time (h)

Table 4.3 Effect of growth factors on incorporation of [^{3}H]thymidine into growth arrested NIH-3T3 cells

Additions	dpm[^{3}H]thymidine incorporated	Fold increase over control
Serum free	2061 ± 163	
Calf serum 10% (v/v)	12366 ± 1646	6.00
EGF (0.1ugml^{-1})	3771 ± 261	1.83
Insulin (1ngml^{-1})	5976 ± 364	2.90
Bombesin (2.5uM) + Insulin (1ngml^{-1})	5698 ± 291	2.78
Bombesin (2.5uM)	2246 ± 197	1.09
Bradykinin (3.2uM)	4760 ± 306	2.31
$PGF_{2\alpha}$ (2.1uM)	2040 ± 153	0.99

Quiescent NIH-3T3 cells were washed in DMEM and incubated for 24h in DMEM containing 10uM [^{3}H]thymidine (0.1uCimol^{-1}), 0.04ngml^{-1} insulin, 0.4ngml^{-1} transferrin, 0.4pgml^{-1} EGF and the additions stated in the table. Acid insoluble radioactivity was determined as described in Section 2.3. Results are expressed as means ± S.D. where n=4 and are from one experiment typical of two others.

Table 4.4 Stimulation of [^{3}H]thymidine incorporation in NIH-3T3 and AmNIH-3T3 cells

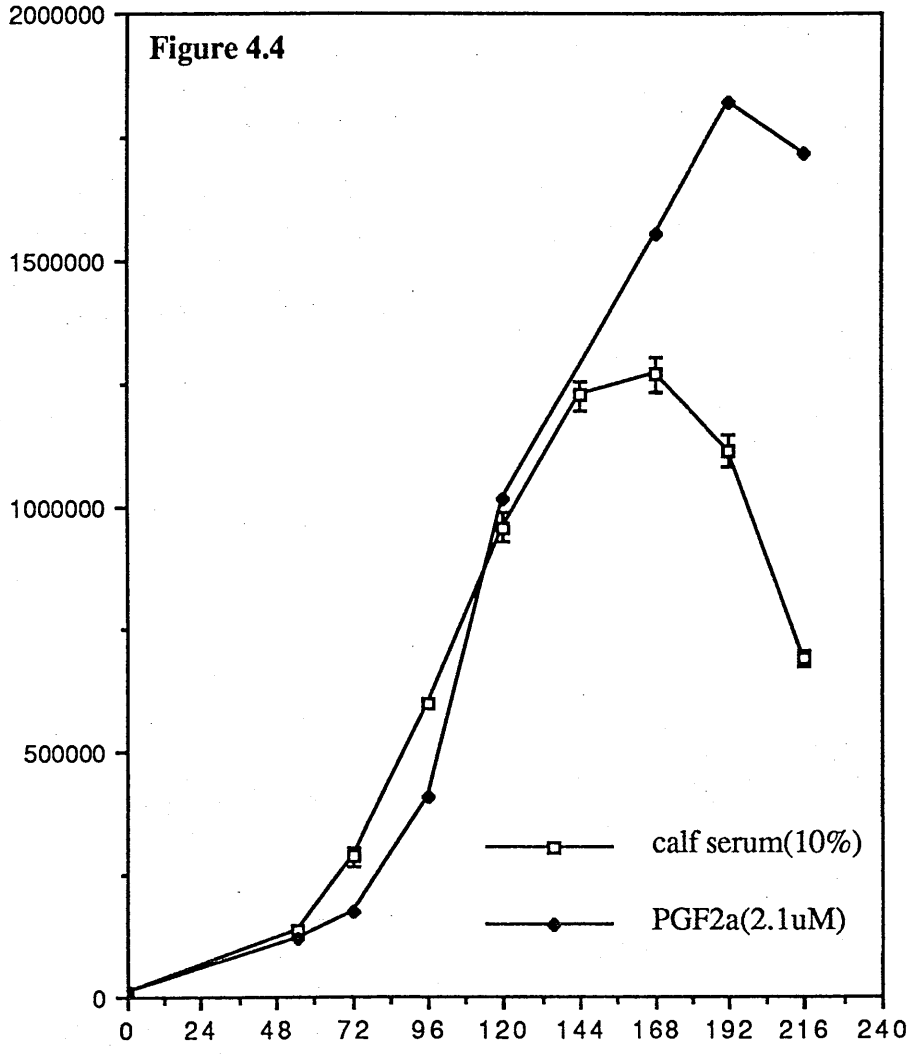
Additions	dpm[^{3}H]thymidine incorporated	Fold increase over control
NIH-3T3 cells		
serum free control	1629 ± 173	-
calf serum 10% (v/v)	13228 ± 210	8.1
$PGF_{2\alpha}$ (2.1uM)	1617 ± 103	1.0
serum free control + LiCl(10mM)	1447 ± 40	-
LiCl (10mM) + calf serum 10% (v/v)	13286 ± 324	9.1
$PGF_{2\alpha}$ (2.1uM) + LiCl(10mM)	1734 ± 174	1.2
AmNIH-3T3 cells		
serum free control	1808 ± 191	-
calf serum 10% (v/v)	11054 ± 449	6.1
$PGF_{2\alpha}$ (2.1uM)	4214 ± 210	2.3
serum free control + LiCl(10mM)	2033 ± 45	-
LiCl (10mM) + calf serum 10% (v/v)	15419 ± 414	7.6
$PGF_{2\alpha}$ (2.1mM) + LiCl(10mM)	3728 ± 183	1.8

Quiescent cells were washed in DMEM and incubated for 24h in DMEM containing 1uCiml^{-1} [^{3}H]thymidine, 0.04ngml^{-1} insulin, 0.4ugml^{-1} transferrin, 0.4ngml^{-1} EGF and the additions stated in the table. Acid insoluble radioactivity was determined as described in Section 2.3. Results are expressed as means ± S.D. where n=3-4 and are from one experiment typical of two others.

Figure 4.4 AmNIH-3T3 growth curves

Cell numbers were determined by Coulter counting as described in Section 2.4. Results are means ± S.D. of six counts of triplicate plates and are from one experiment typical of two. □ cells grown in DMEM + 10% (v/v) calf serum, ♦ cells grown in DMEM 10% (v/v) calf serum and 2.1uM $PGF_{2\alpha}$ (cells supplemented with $PGF_{2\alpha}$ (2.1uM) every 12h).

cell number



time (h)

density 160% of controls. The fall in cell number after 192h is probably due to cell death as cells become over confluent.

Table 4.4 also demonstrates that the inclusion of 10mM LiCl only had a minor effect upon $PGF_{2\alpha}$ stimulated [^{3}H]thymidine incorporation in both cell lines. Additionally, the different mitogenic effects of $PGF_{2\alpha}$ on the two cell lines were still observed when the ion was included.

MacPhee et al. (1984) have reported that the prostaglandin acts as a mitogen for Swiss 3T3 cells. Table 4.5 shows the effect of $PGF_{2\alpha}$ on incorporation of [^{3}H]thymidine into growth arrested NIH-3T3 cells and demonstrates that $PGF_{2\alpha}$ stimulates [^{3}H]thymidine incorporation in Swiss 3T3 cells in a dose dependent manner, giving a maximal 2.4-fold increase compared to a 1.3-fold increase in response to calf serum.

Figure 4.5 illustrates growth curves for Swiss 3T3 cells cultured in the presence of calf serum (10%) or calf serum (10%) and $PGF_{2\alpha}$ (2.1uM) and demonstrates that $PGF_{2\alpha}$ is a mitogen for these cells potentiating calf serum stimulated cell growth to a saturation density 140% of controls.

Since it has been demonstrated that $PGF_{2\alpha}$ stimulated DNA synthesis in Swiss 3T3 and AmNIH-3T3 cells, but not in NIH-3T3 cells a comparison of the stimulation of inositol phosphate generation in the cell types was made. In agreement with Saski (1985) the prostaglandin was found to induce a 2-3 fold increase in inositol phosphate generation in Swiss 3T3 cells, a value much lower than that found in NIH-3T3 cells. However, Table 4.6 shows that $PGF_{2\alpha}$ induced essentially equivalent increases in inositol phosphate generation in the two NIH-3T3 lines (12.5-fold). Further analysis of the $PGF_{2\alpha}$-stimulated inositol phosphate response in the AmNIH-3T3 cells revealed no major differences to that in NIH-3T3 cells (discussed further in Section 4.2.3).

Table 4.5 Stimulation of [^{3}H]thymidine incorporation in Swiss 3T3 cells

Additions	dpm[^{3}H]thymidine incorporated	Fold increase over control
serum free control	3257 ± 599	
10% calf serum	4142 ± 647	1.3
8.41×10^{-10}M $PGF_{2\alpha}$	6329 ± 1734	1.9
8.41×10^{-9}M $PGF_{2\alpha}$	5000 ± 981	1.5
8.41×10^{-8}M $PGF_{2\alpha}$	7658 ± 613	2.3
8.41×10^{-7}M $PGF_{2\alpha}$	7992 ± 185	2.4
8.41×10^{-6}M $PGF_{2\alpha}$	7095 ± 400	2.2

Quiescent Swiss 3T3 cells were washed in DMEM and incubated for 24h in DMEM containing 1uCiml^{-1} [^{3}H]thymidine, 0.04ngml^{-1} insulin, 0.4ngml^{-1} transferrin, 0.4pgml^{-1} EGF and the additions state in the table. Acid insoluble radioactivity was determined as described in Section 2.3. Results are expressed as means ± S.D. where n=4 and are from one experiment typical of two others.

Figure 4.5 Swiss 3T3 growth curves

Cell numbers were determined by Coulter counting as described in Section 2.4. Results are means ± S.D. of 4 counts of duplicate plates and are from one experiment similar to one other. □ cells grown in DMEM + 10% (v/v) calf serum, ◆ cells grown in DMEM + 10% (v/v) calf serum and 2.1uM $PGF_{2\alpha}$ (cells supplemented with $PGF_{2\alpha}$ (2.1uM) every 12h.

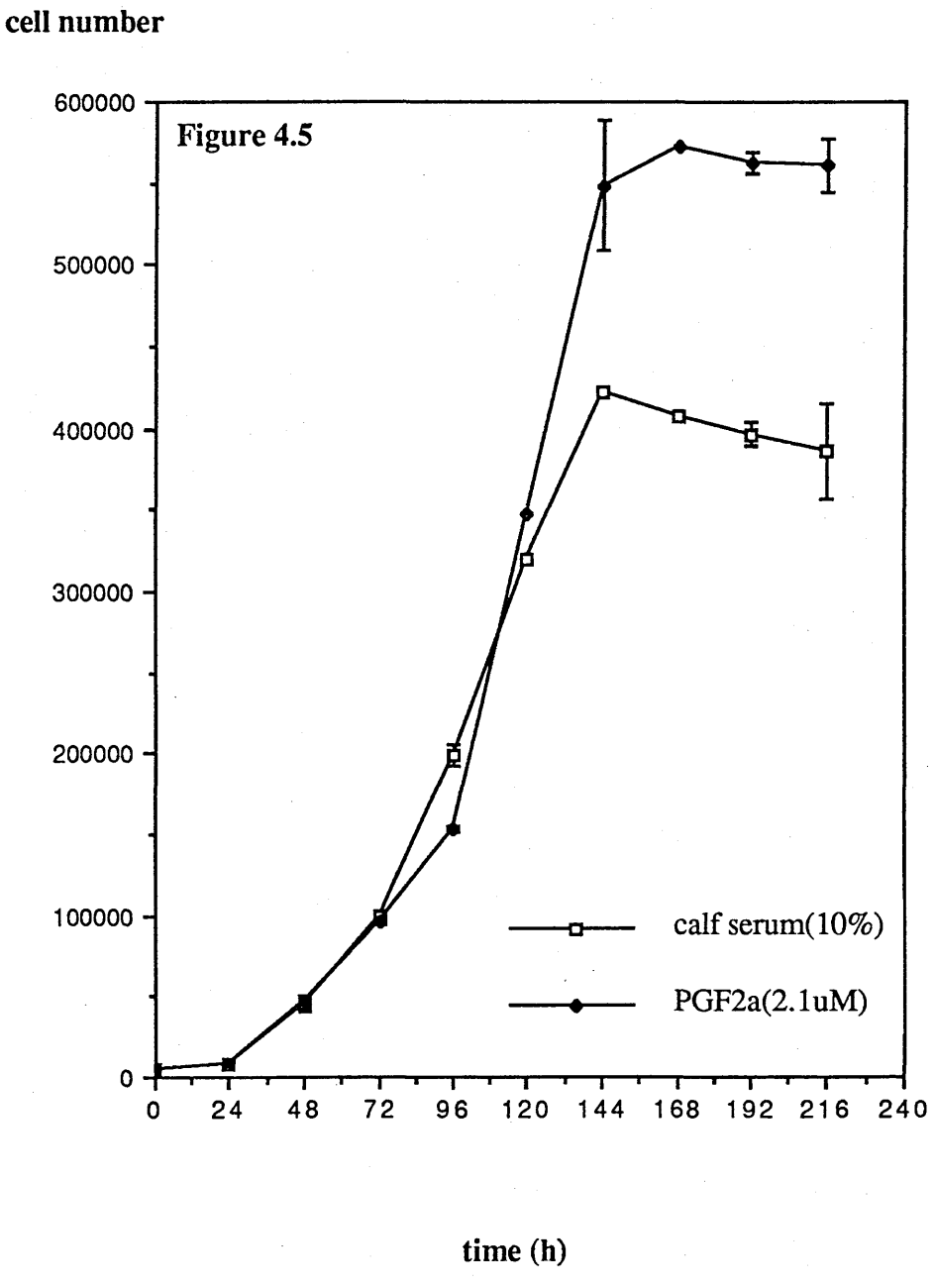

cell number
Figure 4.5
600000
500000
400000
300000
200000
100000
0
0
24
48
72
96
120
144
168
192
216
240
calf serum(10%)
PGF2a(2.1uM)
time (h)

Table 4.6 Comparison of stimulated inositol phosphate generation between NIH-3T3 and AmNIH-3T3 cells

Inositol phosphates (% increase over untreated control)

Ligand	NIH-3T3	AmNIH-3T3
Bombesin (2.5uM)	9 ± 3	193 ± 27
PDGF (1.32ugml^{-1})	43 ± 7	28 ± 10
Bradykinin (3.2uM)	104 ± 9	31 ± 3
$PGF_{2\alpha}$ (2.1uM)	1250 ± 78	1261 ± 147
Calf serum (4% v/v)	254 ± 33	259 ± 9

Cells were labelled with [^{3}H]inositol (1uCiml^{-1}) for 24h and stimulated for 30 min in the presence of 10mM LiCl, with the stated agonists. Inositol phosphate generation was determined as described in Section 2.6. Results are means ± S.E.M., pooled from three separated experiments where n=4 in each case.

Figure 4.6a (NIH-3T3) and Figure 4.6b (AmNIH-3T3) illustrate the dose-dependence of $PGF_{2\alpha}$ -stimulated inositol phosphate generation as a percentage over unstimulated controls. The EC_{50} for inositol phosphate generation for both NIH-3T3 and AmNIH-3T3 cells was essentially the same, 0.18uM and 0.11uM respectively, with a saturating response being observed at approximately 2uM.

4.2.3 Time-dependent $PGF_{2\alpha}$ stimulated inositol phosphate generation in AmNIH-3T3 cells. Separation upon a partisil 5 WAX HPLC column

Analysis of the $PGF_{2\alpha}$ -stimulated changes in individual inositol phosphates in the AmNIH-3T3 cells (Fig.4.7) demonstrated a very similar pattern to that observed in NIH-3T3 cells (Fig.4.2). Stimulation with $PGF_{2\alpha}$ (2.1uM) induced the generation of $Ins(1,4,5)P_3$ (Fig.4.7c) and $Ins(1,3,4,5)P_4$ (Fig.4.7e) within 5 seconds, though the magnitude of the increase in $Ins(1,3,4,5)P_4$ generation at 5 seconds (approximately 195% increase) was less than that for $Ins(1,4,5)P_3$ (approximately 225% increase). As in the NIH-3T3 cells stimulation of $Ins(1,4,5)P_3$ generation was observed to be biphasic in the AmNIH-3T3 cells. Peak accumulation of $Ins(1,4,5)P_3$ was observed at 20 seconds when values were approximately 375% of control. The levels then declined to 200% of basal at 120 seconds. In the NIH-3T3 cells, at 30 seconds $Ins(1,4,5)P_3$ levels had declined to basal. Peak accumulation of $Ins(1,3,4,5)P_4$ was detected following a 20 second stimulation (10 seconds later than that observed in NIH-3T3 cells) but unlike $Ins(1,4,5)P_3$, the level appeared to remain elevated and at 120 seconds was 300% of that of basal. A 10-15 second delay was observed before accumulation of $Ins(1,3,4)P_3$ was detected (Fig.4.7d), accumulation of this isomer was then rapid and after 120

Figure 4.6 Dose-dependence of $PGF_{2\alpha}$-stimulated inositol phosphate generation in NIH-3T3 and AmNIH-3T3 cells

Cells were labelled with [^{3}H]inositol (1uCi ml^{-1}) for 24h and stimulated for 30 min with various concentrations of $PGF_{2\alpha}$ in the presence of 10mM LiCl. Results are expressed as means ± S.D. Results for NIH-3T3 cells are pooled from 3 experiments, while results for AmNIH-3T3 cells are from a single experiment typical of three.

Figure 4.6a

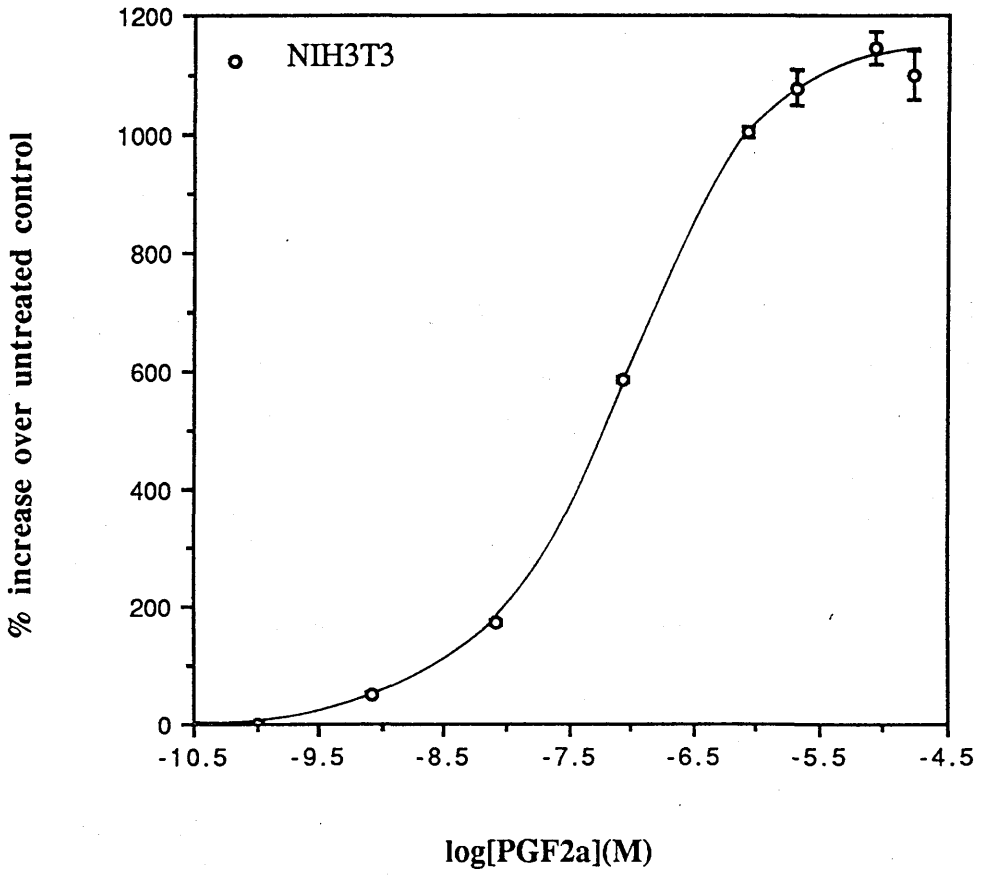


Figure 4.6b

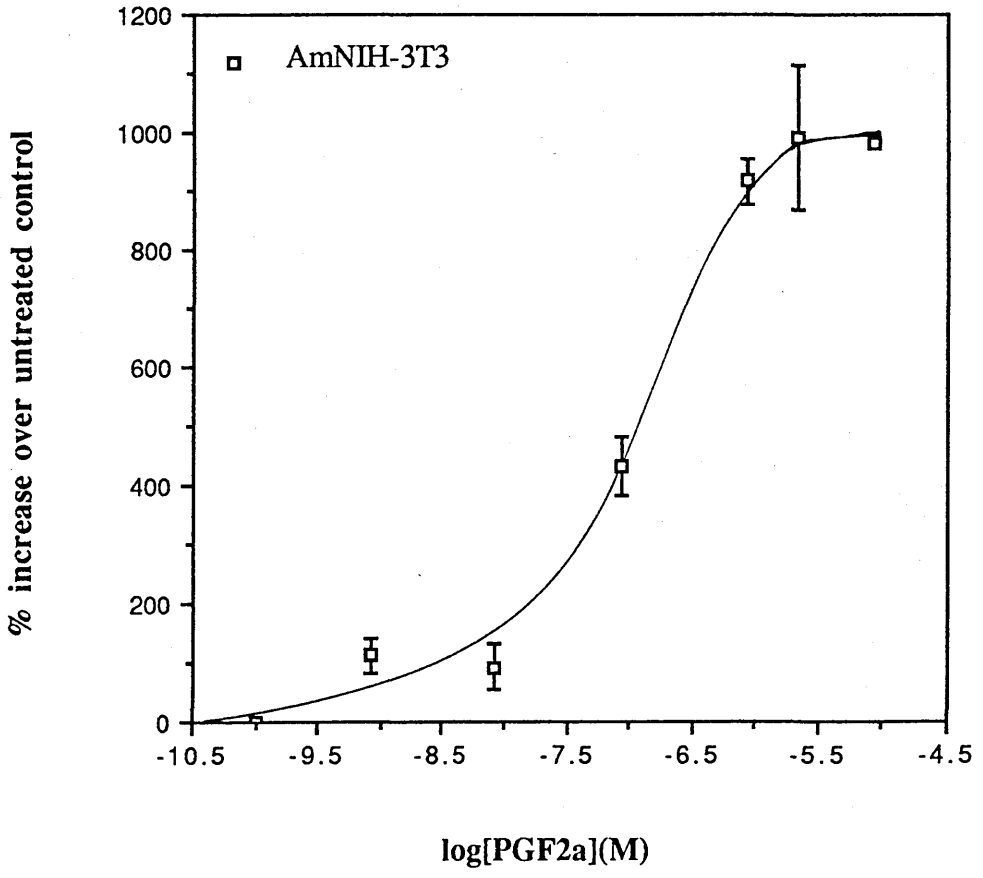


Figure 4.7 Time-dependent $PGF_{2\alpha}$-stimulated inositol phosphate generation in AmNIH-3T3 cells. Separation upon a partisil 5 WAX HPLC column

[^{3}H]inositol labelled cells were stimulated with $PGF_{2\alpha}$ (2.1um) for the times stated in the presence of 10mM LiCl. The inositol phosphates were separated by HPLC as described in Section 2.7b. Results are presented as the mean percentage of the radioactivity associated with the unstimulated control, n=3 in each case and the data is from a single experiment typical of three. The control d.p.m. values were : Figure 4.7a inositol monophosphate = 78595 $\pm$ 3089; Figure 4.7b inositol bisphosphate = 4570 $\pm$ 383; Figure 4.7c Ins (1,4,5)P_3 = 958 $\pm$ 55; Figure 4.7d Ins (1,3,4)P_3 = 1886 $\pm$ 306; Figure 4.7e Ins (1,3,4,5)P_4 = 1005 $\pm$ 143; Figure 4.7f putative Ins P_5 = 8300 $\pm$ 473; putative Ins P_6 = 1910 $\pm$ 721.

Figure 4.7a

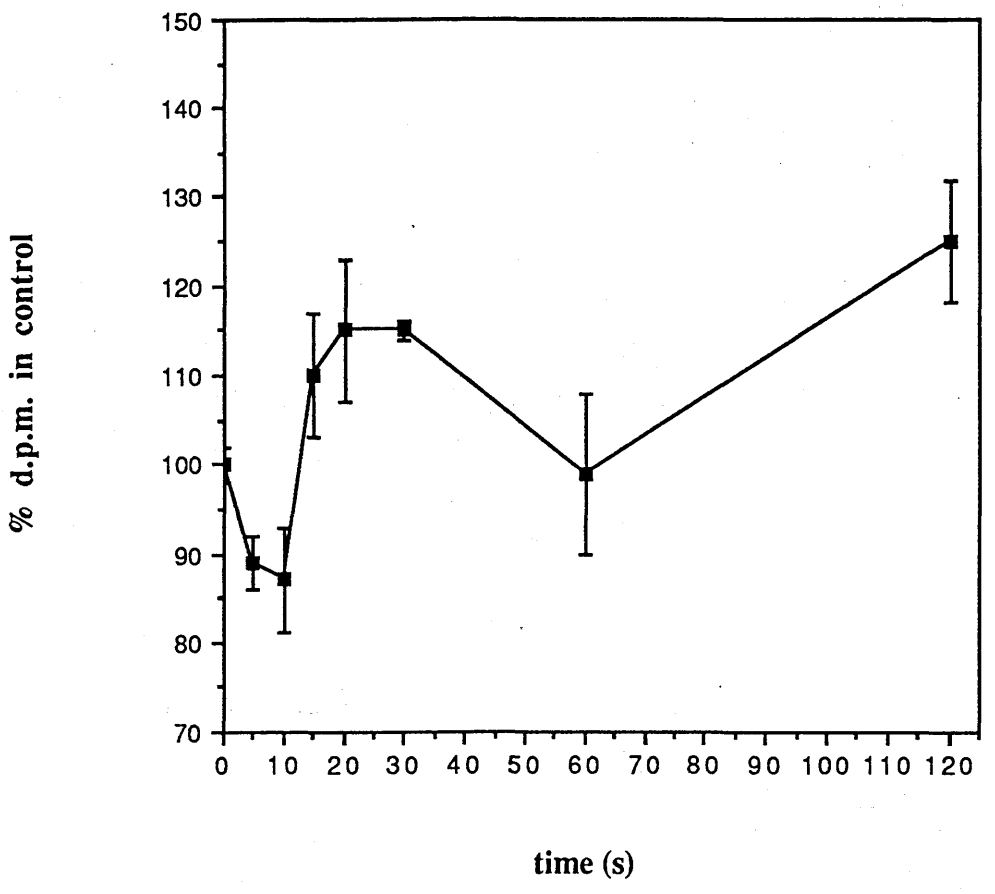


Figure 4.7b

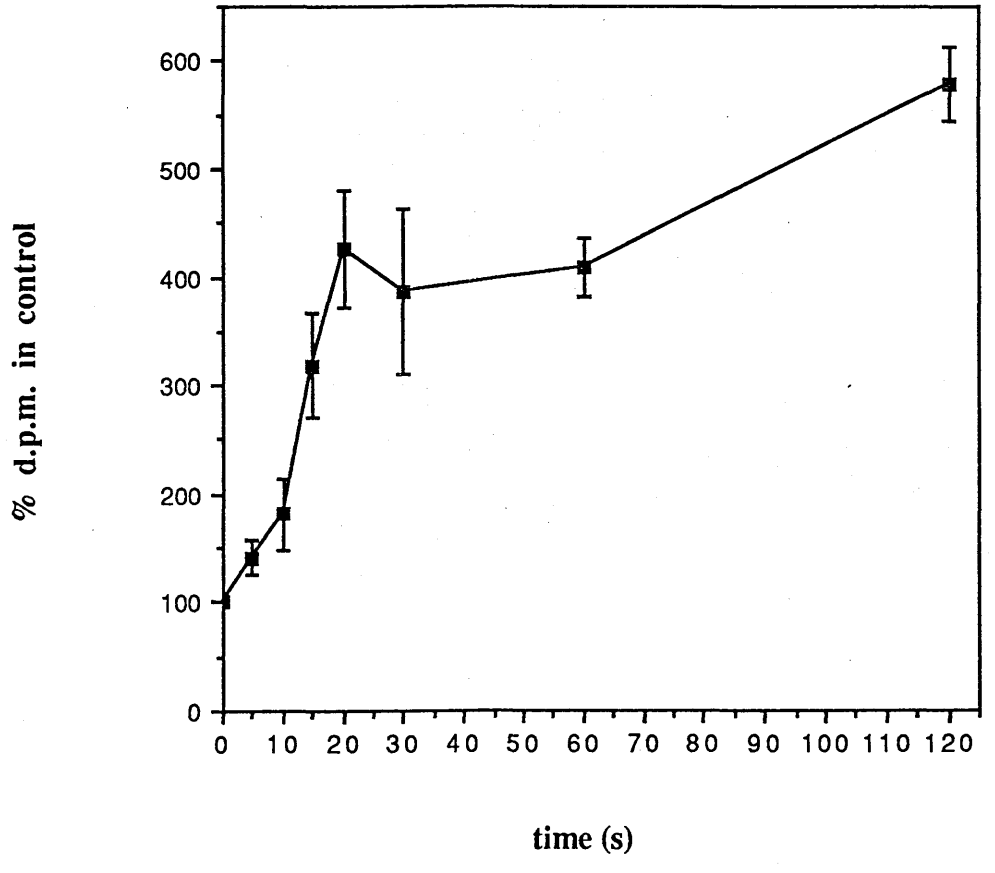


Figure 4.7c

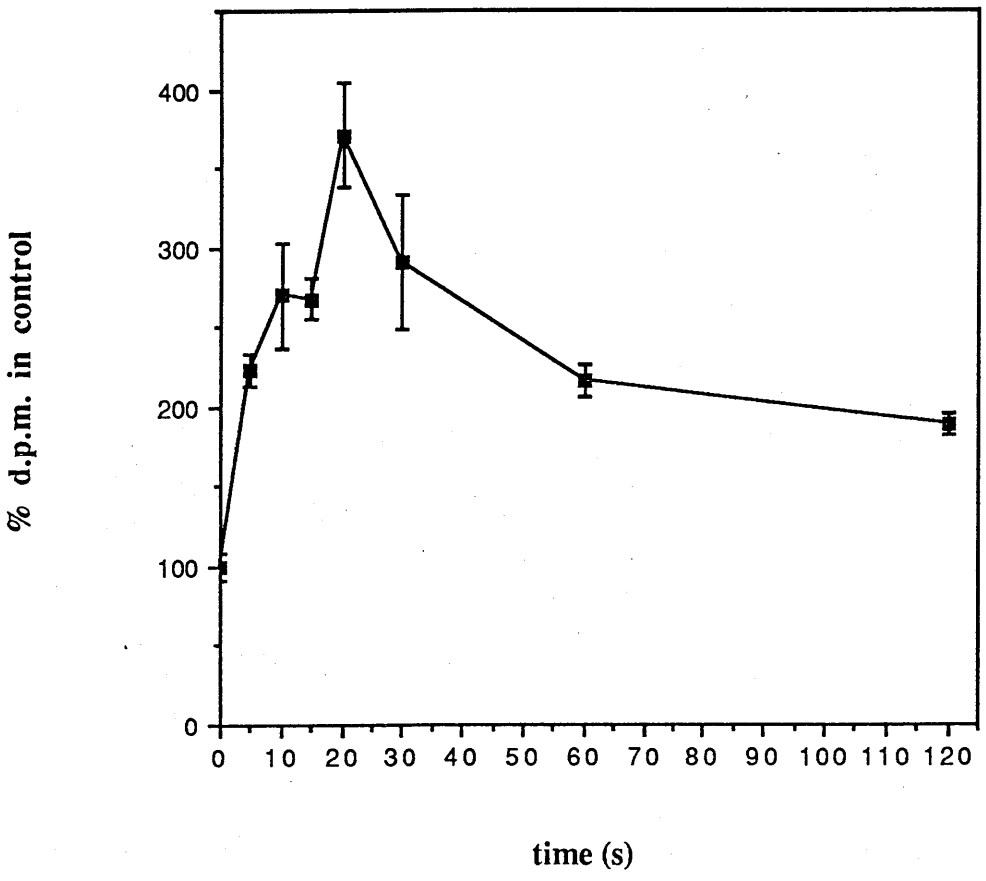


Figure 4.7d

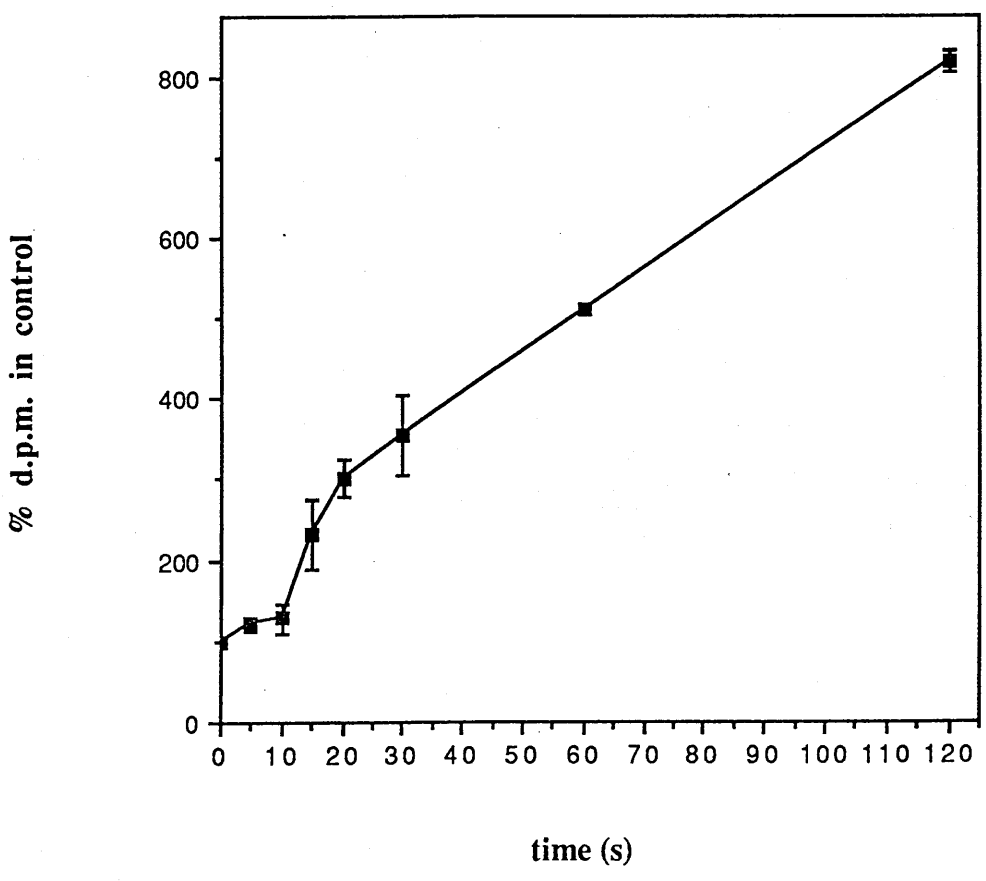


Figure 4.7e

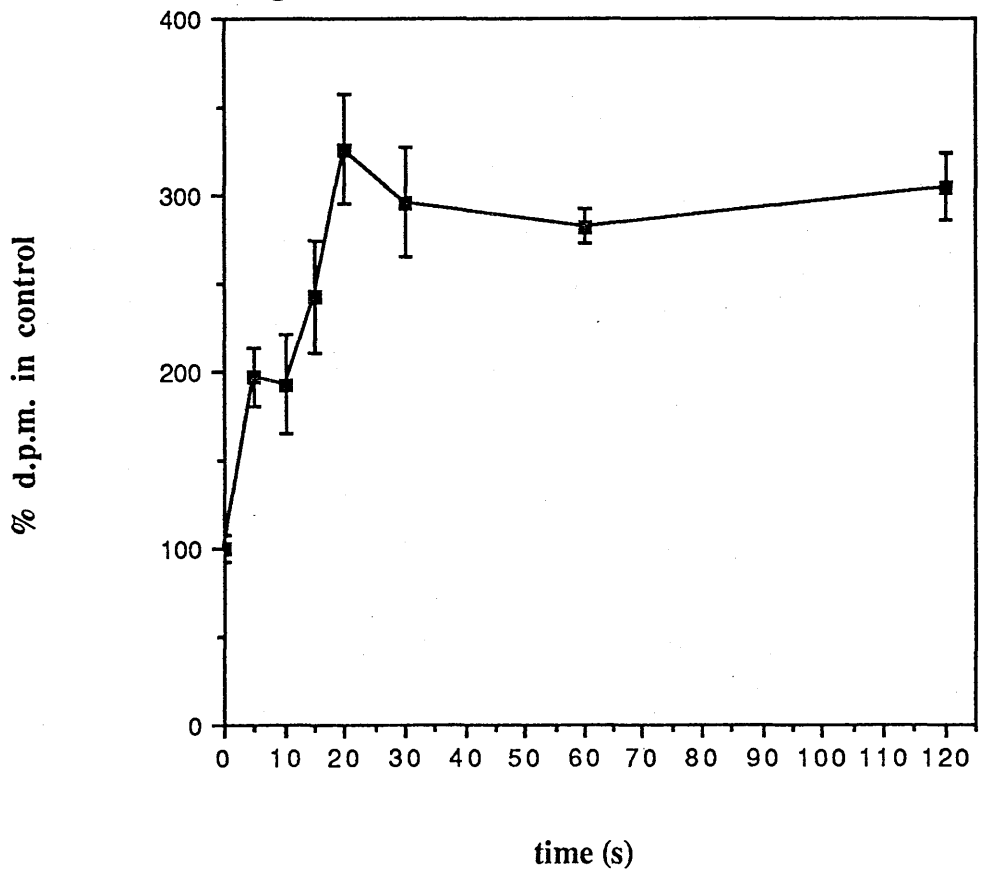

400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100 110 120
% d.p.m. in control
time (s)

Figure 4.7f

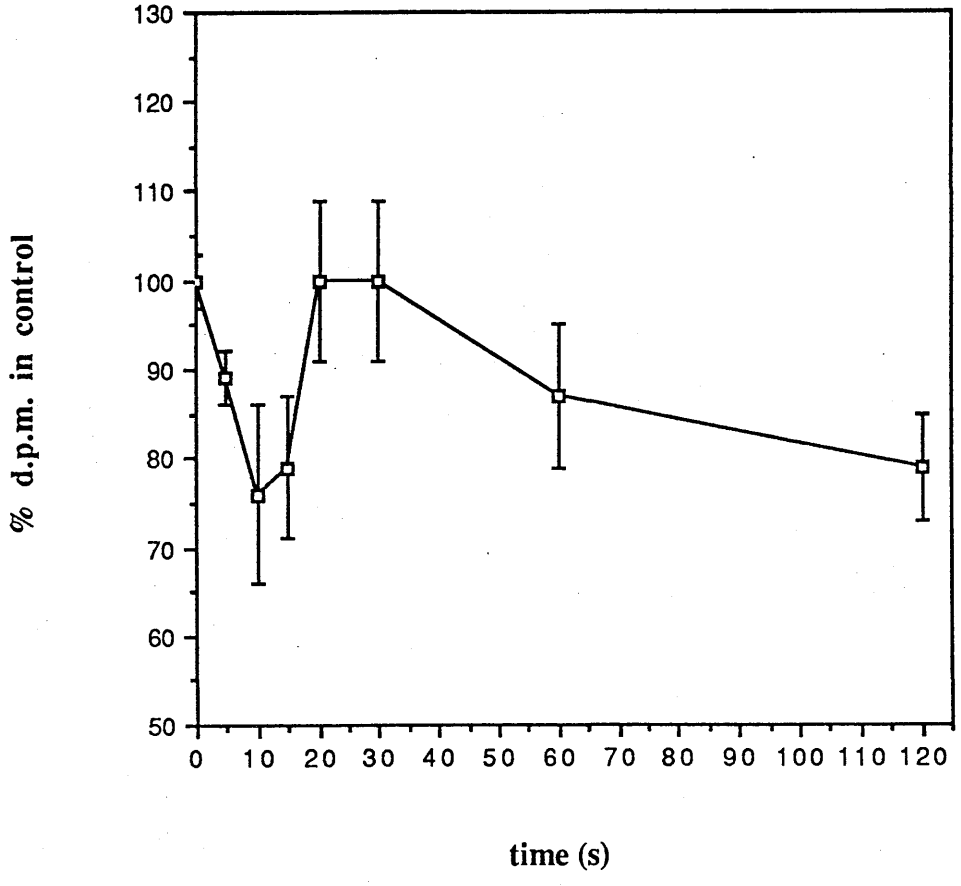
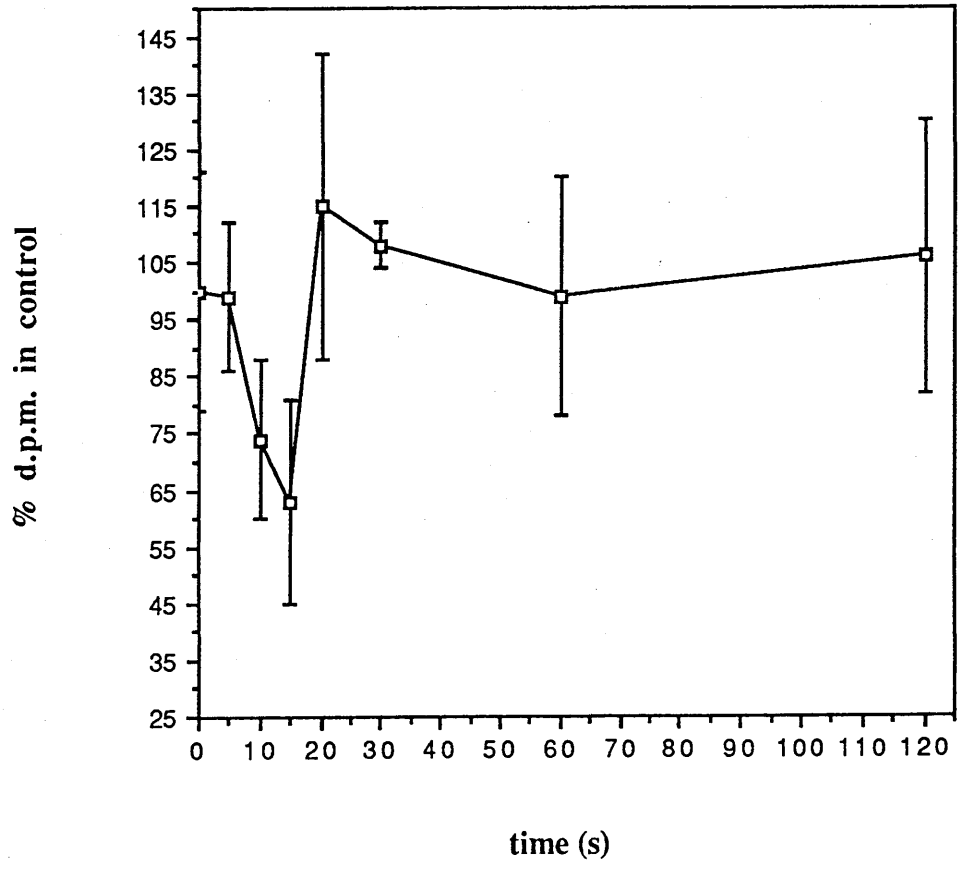


Figure 4.7g



seconds reached 800% of the basal. Rapid accumulation of inositol mono- and bis-phosphates was also detected. At 120 seconds IP and IP_2 levels were 125% and 580% of the control values, respectively (Figs.4.7a-7b). Changes were observed in the putative inositol penta- or hexa-kisphosphate fractions. Levels of penta-kisphosphate declined to 25% below levels at 10 seconds and returned to basal levels again at 20 seconds. However, levels fell to 20% below basal at 120 seconds (Fig.4.7f). Hexa-kisphosphate levels declined to 35% below basal at 15 seconds, then increased at 20 seconds where levels were 115% of the control value.

4.2.4 Characterisation of $PGF_{2\alpha}$ receptors on AmNIH-3T3 cells

In order to determine if there were differences in the binding characteristics of $[^3H]PGF_{2\alpha}$ to AmNIH-3T3 cells, binding studies were performed. Scatchard analysis of $PGF_{2\alpha}$ binding data generated a Kd of 0.56uM with approximately 500,000 receptors per cell (Fig.4.8).

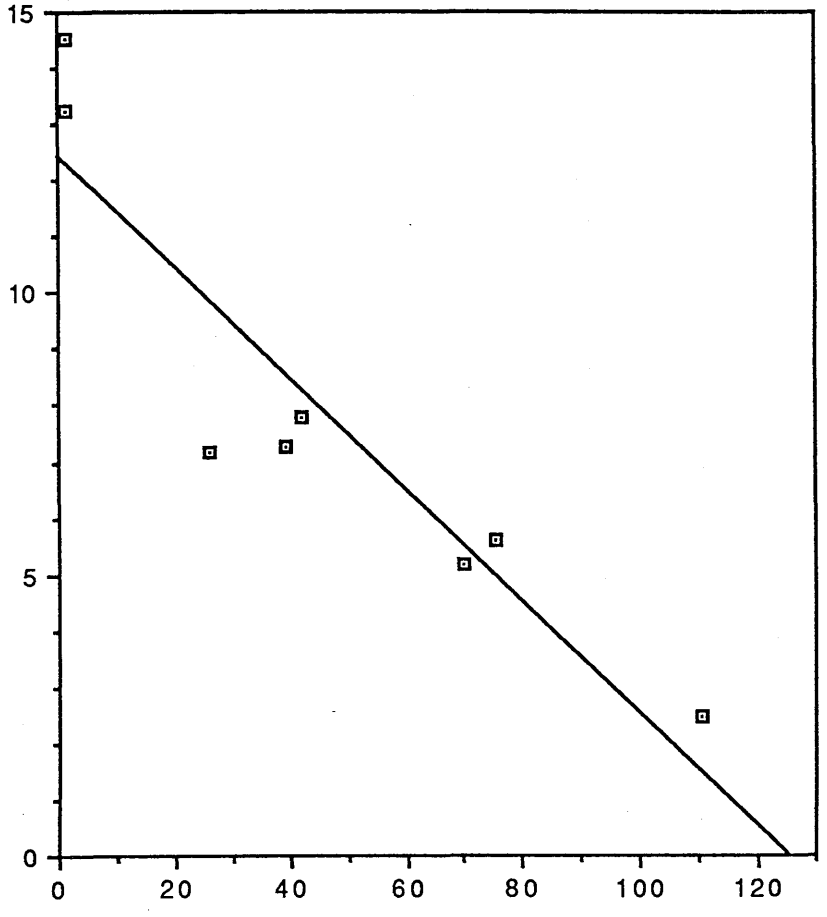
Almost identical values for the NIH-3T3 cells (Chapter 3, Section 3.2.5) have been obtained.

Whilst no differences in $PGF_{2\alpha}$-stimulated inositol phosphate generation was detected between the two cell lines, the AmNIH-3T3 cells were found to respond to a greater extent to bombesin (193% over untreated controls) compared to NIH-3T3 cells (9% over unstimulated controls). Bradykinin stimulated inositol phosphate generation to a greater extent in NIH-3T3 cells, 104% over unstimulated controls compared to 31% in AmNIH-3T3 cells (Table 4.6).

Figure 4.8 Scatchard analysis of the binding of [^{3}H]$PGF_{2\alpha}$ to AmNIH-3T3 cells

Binding was performed and assessed as described in Section 2.9. Bound $PGF_{2\alpha}$ (B) is expressed in fmol per 10^6 cells; the concentration of $PGF_{2\alpha}$ in the medium (F) is in fmol.

Figure 4.8



4.3 Discussion

$PGF_{2\alpha}$ is clearly shown in this study to stimulate the hydrolysis of PtdIns(4,5)P_2 generating Ins(1,4,5)P_3 in both the clones of NIH-3T3 cells examined (Figs. 4.1, 4.2 and 4.7). This work indicates that on stimulation with $PGF_{2\alpha}$, Ins(1,4,5)P_3 is the initial inositol phosphate generated. The other inositol phosphates found in the $PGF_{2\alpha}$ stimulated cells can be produced directly or indirectly, from Ins(1,4,5)P_3 by the actions of kinases or phosphomonoesterase activity (Hawkins et al., 1986). Their accumulation to much higher levels can be accounted for by their much slower rates of metabolism (Irvine et al., 1985). Ins(1,4,5)P_3 is metabolised in two ways: by specific removal of its 5-phosphate to give Ins(1,4)P_2 (Downes et al., 1982) or by 3-kinase action to give Ins(1,3,4,5)P_4 (Batty et al., 1985; Hawkins et al., 1986), which in turn is dephosphorylated to Ins(1,3,4)P_3 (Hawkins et al., 1986). The stimulation of Ins(1,4,5)P_3 generation was biphasic, which may imply a secondary phase of PtdIns(4,5)P_2 breakdown (Figs. 4.2 and 4.7). The significance and mechanism of this second phase of Ins(1,4,5)P_3 generation was uncertain. The initial rate of generation of Ins(1,3,4,5)P_4 was slower than that for Ins (1,4,5)P_3, but Ins(1,3,4,5)P_4 levels reached steady state much more rapidly. In contrast, Ins(1,3,4)P_3, IP_2 and IP levels rose throughout the 120 second period of stimulation with an initial short time lag of 5-10 seconds for Ins(1,3,4)P_3. Ins(1,3,4)P_3 is degraded relatively slowly, so by 120 seconds, its levels greatly exceed those of Ins(1,4,5)P_3. The delayed accumulation of Ins(1,3,4)P_3 in response to $PGF_{2\alpha}$ is consistent with this compound being a metabolite of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. Figures 4.2f-2g and 4.7f-7g show two labelled

compounds that eluted after $Ins(1,3,4,5)P_4$ on HPLC that have similar chromatographic properties to inositol penta- or hexa-kisphosphate (Heslop et al., 1985). As standards were not available, their identification is putative. Only in two experiments were fractions collected for these peaks. Therefore, insufficient data is available to determine whether these peaks are significantly influenced by $PGF_{2\alpha}$ stimulation.

The possibility that IP and IP_2 are generated in $PGF_{2\alpha}$ -stimulated cells as a consequence of PtdIns or PtdIns(4)P hydrolysis can be ruled out. This is due to the fact that the initial rate of generation of IP and IP_2 was slower than that for $Ins(1,4,5)P_3$. If these inositol phosphates were generated as a consequence of PtdIns and PtdIns(4)P hydrolysis then their production would occur much more rapidly. Thus, the inositol phosphates generated in response to $PGF_{2\alpha}$ stimulation of both clones of NIH-3T3 cells are a consequence of $Ins(1,4,5)P_3$ generation. This demonstrates that the assay for total inositol phosphates in control and stimulated cells is an accurate estimate of $PtdIns(4,5)P_2$ hydrolysis.

$Ins(1,4,5)P_3$ has been shown to release Ca^{2+} from intracellular stores in many different cells (Berridge & Irvine, 1984). Lloyd et al. (1989) have shown that stimulation of NIH-3T3 cells with a maximal $PGF_{2\alpha}$ concentration results in an increase in intracellular free Ca^{2+}. The effects of the prostaglandin upon inositol phospholipid metabolism are thus similar to those observed in response to a range of other agonists such as bradykinin and vasopressin in a variety of cell types (see Berridge, 1987a).

The stimulation of inositol phosphate generation was dose-dependent with half-maximal stimulation at 0.18uM and a saturating response observed at 2uM (Fig.4.6). This EC_{50} is

close to the Kd observed for $PGF_{2\alpha}$ receptor binding (Chapter 3, Section 3.2.5). It is also similar to that reported by MacPhee et al. (1984) for the stimulation of inositol phospholipid metabolism in Swiss 3T3 cells. In bovine corpora lutea, Powell et al. (1975) reported a single class of $PGF_{2\alpha}$ receptor of Kd 0.5-1uM, this being similar to that obtained for NIH-3T3 cells.

Whilst stimulation of NIH-3T3 cells with $PGF_{2\alpha}$ induced PtdIns(4,5)P_2 breakdown, it was without effect upon the stimulation of DNA synthesis in this clone of NIH-3T3 cells (Tables 4.2, 4.3, 4.4, Fig.4.3). This result was surprising since it has been reported that the prostaglandin is a potent growth factor for both NIH-3T3 (Yu et al., 1988) and Swiss 3T3 cells (Herschman et al., 1978; MacPhee et al., 1984). $PGF_{2\alpha}$ was unable to induce DNA synthesis even in the presence of insulin, EGF and transferrin (Tables 4.2, 4.3, 4.4); NIH-3T3 cells demonstrated an absolute requirement for low concentrations of these growth factors since they can not survive a 24h period in serum-free medium. Jimenez de Asua et al. (1985) demonstrated that insulin enhances the stimulatory effect of $PGF_{2\alpha}$ by increasing the rate of entry into S phase, whilst EGF and $PGF_{2\alpha}$ have a synergistic effect on stimulation of DNA synthesis in Swiss 3T3 cells. The results in this chapter confirm that the prostaglandin is indeed a growth factor for Swiss 3T3 cells (Table 4.5, Fig.4.5) and for the clone of NIH-3T3 cells used by Yu et al. (1988) (Table 4.4, Fig.4.4). No major differences were observed in the stimulation of inositol phosphates generation by $PGF_{2\alpha}$ between the two clones of NIH-3T3 cells (Table 4.6, Fig.4.7), nor were any differences detected in EC_{50}, Kd or $PGF_{2\alpha}$ receptor number (Figs.4.6, 4.8). It is clear, however, from the results shown in Table 4.6, that there are differences in responsiveness to other defined agonists, most

noticably to bombesin. However, this may reflect a different receptor number and deserves further study. Significantly. there is no difference in the response to calf serum (Table 4.6) which is equally effective in stimulating the proliferation of both cell types (Figs.4.3, 4.4). The stimulation of inositol phosphate accumulation by $PGF_{2\alpha}$ in Swiss 3T3 cells is less marked (3-4 fold) than in the two clones of NIH-3T3 cells, but it is clearly a growth factor for this cell type (Table 4.5).

Calf serum stimulated DNA synthesis in all three cell types (Tables, 4.2, 4.3, 4.4, 4.5, Figs. 4.3, 4.4, 4.5) and also induces inositol phosphate generation. This induction of DNA synthesis is dose-dependent, but is not potentiated in the NIH-3T3 cells by any concentration of $PGF_{2\alpha}$ (Table 4.2). This lack of potentiation is despite a synergistic effect upon inositol phosphate generation (Table 4.1). The mechanism underlying the synergistic stimulation of inositol phosphates induced by $PGF_{2\alpha}$ and calf serum is unknown. However, Paris et al. (1988) demonstrated that tyrosine kinase activating growth factors potentiate thrombin-induced PtdIns(4,5)P_2 hydrolysis in hamster fibroblasts and suggest that such growth factors enhance the coupling between G-protein and PIC, possibly through the phosphorylation of one of the two proteins. Calf serum contains tyrosine kinase activating growth factors and it is possible that such mitogens could bring about such an enhanced coupling between G-protein and PIC resulting in the synergistic inositol phosphate generation observed when NIH-3T3 cells are stimulated by both calf serum and $PGF_{2\alpha}$. NIH-3T3 cells demonstrate the same rank order for the effectiveness of calf serum, PDGF and bombesin in stimulating inositol phosphates and [^{3}H]thymidine incorporation, yet $PGF_{2\alpha}$ which stimulated a 12-fold increase in inositol phosphate generation does not stimulate [^{3}H]thymidine incorporation.

The results in this chapter, therefore, place a question upon the role of inositol phosphate metabolism in growth factor-stimulated cell proliferation. Tones et al. (1988) have demonstrated no requirement for inositol phospholipid breakdown in serum-stimulated mitogenesis in CHO-K1 cells. Taylor et al. (1988) have found that the proliferation of Swiss 3T3 cells can be inhibited by the addition of pertussis toxin with no inhibition of the stimulation of inositol phosphate generation. These studies and the experiments reported in this chapter suggest that, whilst mitogens can activate the breakdown of inositol phospholipids, it is either, not obligatory for the onset of mitosis, or in itself not a complete signal. Consequently, some additional signal must be being generated in the AmNIH-3T3 and Swiss 3T3 cells, but not in the NIH-3T3 cells, in response to $PGF_{2\alpha}$, either in early G_1 or at a later stage in the cell cycle, which is required for the onset of DNA synthesis and mitosis. The identity of this signal, is however unclear.

CHAPTER 5

THE EFFECT OF OVER-EXPRESSION OF NORMAL ras GENES IN NIH-3T3 FIBROBLASTS UPON PHOSPHATIDYLCHOLINE METABOLISM

5.1 Introduction

Many growth factors stimulate the receptor activated hydrolysis of PtdIns(4,5)P_2 generating the two second messengers Ins(1,4,5)P_3 and DAG. It has been proposed that this is the key to the mechanism whereby agonists, such as bombesin, stimulate cell proliferation (Berridge, 1987b). However, the results presented in Chapter 4 indicate that in some cell lines, breakdown of inositol phospholipids is either, not obligatory for the onset of mitosis, or in itself not a complete signal.

Mitogenesis only occurs if there is persistent activation of PKC which requires a maintained increase in cellular DAG levels. Agonist stimulated PtdIns(4,5)P_2 hydrolysis is a transient process which is rapidly desensitized (see e.g. Brown et al., 1987), whilst the cellular level of DAG has been demonstrated to remain elevated in the absence of significant amplified PtdIns(4,5)P_2 hydrolysis (Cook et al., 1990). Lacal et al. (1987a) demonstrated a reproducible increase in DAG levels in Ha-ras transformed NIH-3T3 cells, in the absence of a reproducible increase in inositol phosphates. These observations suggest that DAG might be generated from the metabolism of phospholipids other than PtdIns(4,5)P_2. In Swiss 3T3 cells it has been demonstrated that there is sustained PtdCho breakdown even when the inositol phosphate response is desensitized (Cook & Wakelam, 1989). Many agonists that stimulate inositol phospholipid metabolism have now been shown to activate the hydrolysis of PtdCho in various cell types (see Pelech & Vance, 1989).

PtdCho hydrolysis catalysed by a PtdCho specific phospholipase C was proposed (Slivka et al., 1988), since in studies demonstrating enhanced DAG production (from PtdCho), ChoP generation was also detected. However, many of these studies did not examine

the individual water soluble metabolites generated following PtdCho hydrolysis and with the demonstration that phosphatidic acid accumulates prior to DAG in hepatocytes stimulated with vasopressin (Boccking et al., 1987), the involvement of phospholipase C must be reassessed.

DAG can also arise from the sequential actions of phospholipase D (PLD) and phosphatidic acid-phosphohydrolyase. Cabot et al. (1988) demonstrated that in REF52 cells stimulation with vasopressin results in the catabolism of PtdCho to phosphatidic acid and Cho, while Cook and Wakelam (1989) have shown that in Swiss 3T3 cells stimulation with bombesin leads to the rapid generation of Cho, prior to ChoP, suggesting that agonist-stimulated PtdCho breakdown is a PLD catalysed process. It has been suggested that both phospholipase C and PLD activation can be either a direct agonist-receptor stimulated event or secondary to the activity of PKC.

The following study was carried out in order to determine whether PtdCho breakdown is a phospholipase C or PLD catalysed process and to investigate the effect of over-expression of normal Ha-, Ki- or N-ras genes in NIH-3T3 cells upon both stimulated and basal PtdCho metabolism.

5.2 Results

5.2.1 Characterisation of [^{3}H]Cho-labelling of lipids in NIH-3T3 cells

To determine the optimum labelling period it was necessary to characterise the [^{3}H]Cho-labelling of lipids in NIH-3T3 cells. NIH-3T3 cells were labelled with [^{3}H]choline chloride for increasing times. Cells were then stimulated with $PGF_{2\alpha}$ (2.1uM) or TPA (100nM; 24 and 48h time points only) and [^{3}H]Cho, [^{3}H]ChoP and [^{3}H]GroPCho were separated as described in Section

2.8 (Fig.5.1a-1c). The amount of radioactivity associated with the lipid fraction from chloroform/methanol treated cells was also determined (Fig.5.1d). Maximum incorporation of [^{3}H]Cho into Cho containing lipids occurred at 30h. Levels then dropped slightly to 72h (Fig.5.1d). Maximum levels of [^{3}H]GroPCho also occurred at 30h and levels plateaued to 72h (Fig.5.1a). No $PGF_{2\alpha}$ or TPA stimulated increases in the levels of [^{3}H]GroPCho were observed. Maximum incorporation of [^{3}H]Cho into ChoP occurred at 12h and levels decreased steadily to 72h (Fig.5.1b). No $PGF_{2\alpha}$ or TPA stimulated increases in the levels of [^{3}H]ChoP were observed.

Maximum levels of [^{3}H]Cho were observed at 30h, while maximum $PGF_{2\alpha}$ and TPA stimulated levels were observed at 24h. Both unstimulated and stimulated [^{3}H]Cho levels remained elevated for the duration of the time course. In subsequent experiments cells were labelled with [^{3}H]Cho for 30h.

5.2.2 <u>$PGF_{2\alpha}$-stimulated increases in cell associated [^{3}H]Cho-labelled metabolites in NIH-3T3 and Ha-ras transformed NIH-3T3 cells</u>

Stimulation of [^{3}H]Cho-labelled NIH-3T3 cells and Ha-<u>ras</u> (EC807) transformed NIH-3T3 cells with $PGF_{2\alpha}$ (2.1uM) caused the generation of both intracellular and total Cho (Table 5.1). In cells expressing high levels of the normal Ha-<u>ras</u> proto-oncogene, there is a significant increase, compared to control-NIH-3T3 cells, in Cho generation. No stimulated changes in the levels of [^{3}H]GroPCho and [^{3}H]ChoP were noted in either cell types. As the levels of stimulated intracellular and total [^{3}H]Cho were very similar, in subsequent experiments only total [^{3}H]Cho metabolites were analysed.

Figure 5.1 Characterisation of [^{3}H]Cho labelling of lipids in NIH-3T3 cells

Cells were labelled with [^{3}H]choline chloride ($1uCiml^{-1}$) for the times stated and stimulated with $PGF_{2\alpha}$ (2.1uM) and TPA (100nM; 24 and 48h time points only) and [^{3}H]Cho, [^{3}H]ChoP and [^{3}H]GroPCho were separated as described in Section 2.8. The results are means $\pm$ S.D. of one experiment similar to one other.

Figure 5.1a

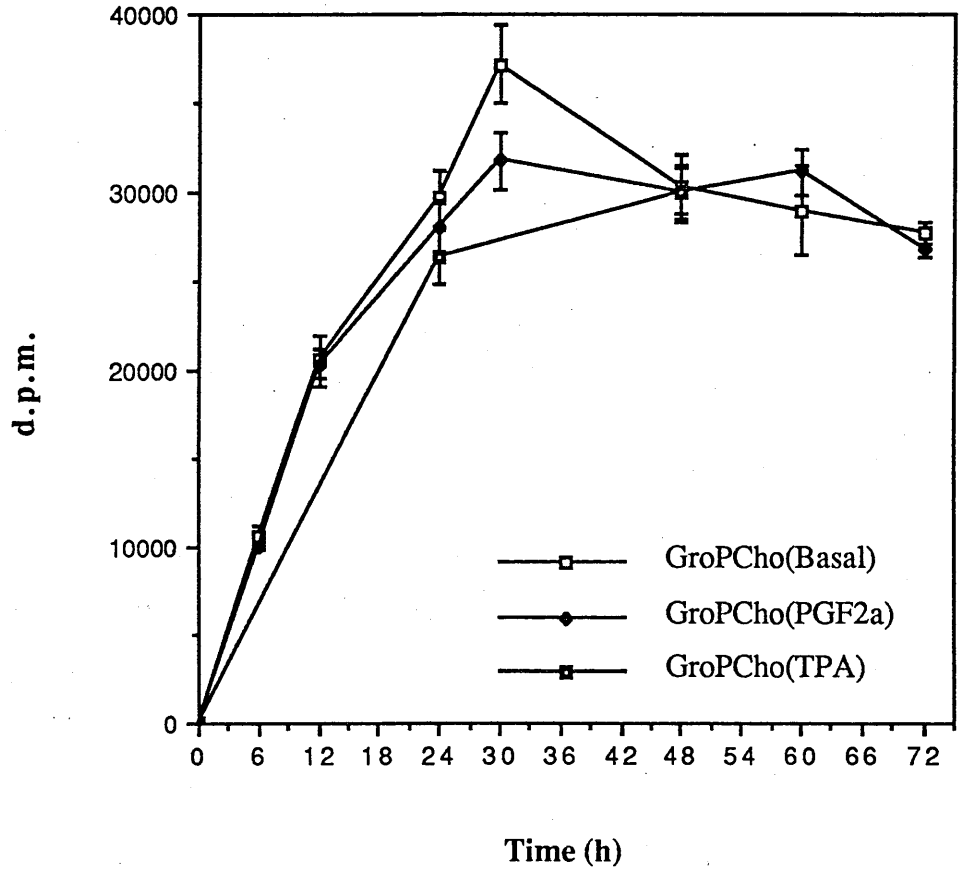


Figure 5.1b

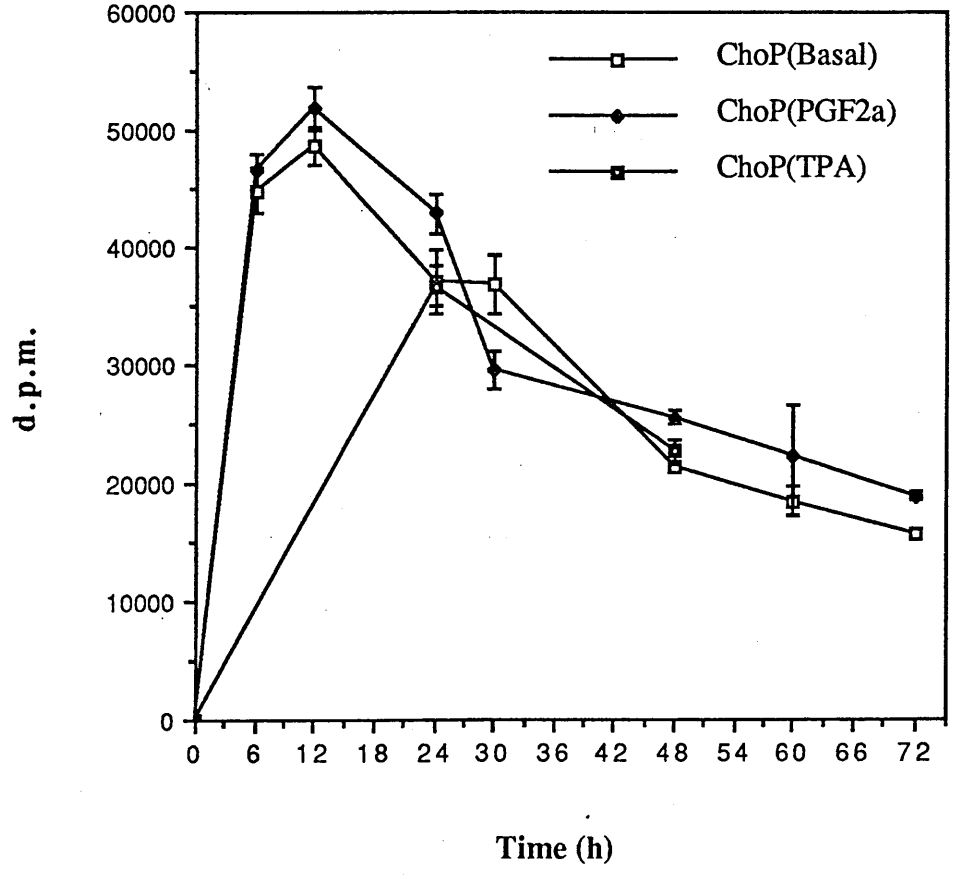


Figure 5.1c

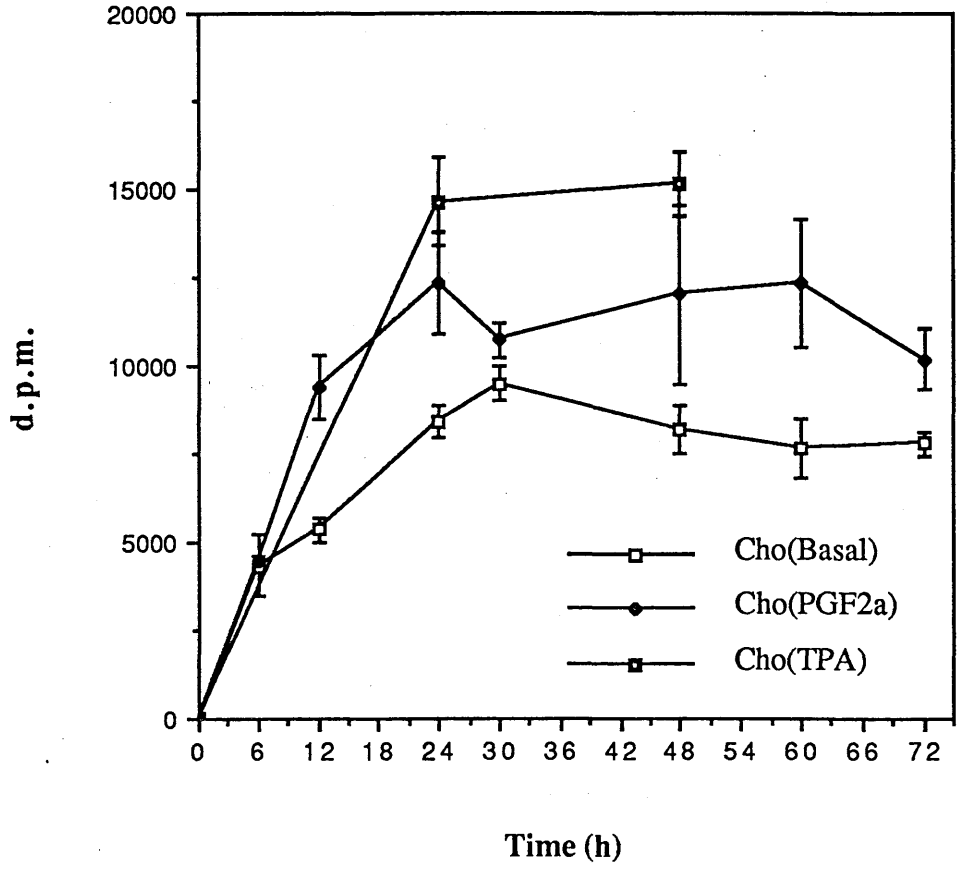


Figure 5.1d

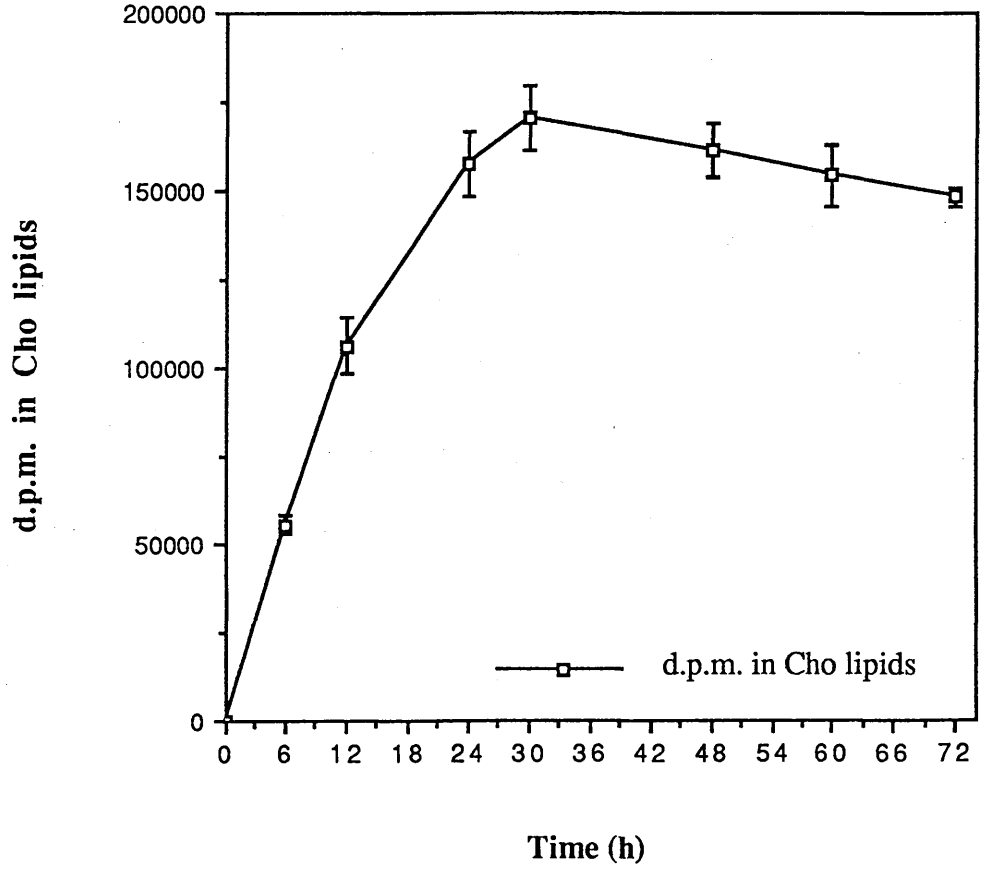


Table 5.1 $PGF_{2\alpha}$-stimulated increases in cell associated [^{3}H]Cho labelled metabolites in NIH-3T3 and Ha-ras (EC807) transformed NIH-3T3 cells

	% of untreated control					
	[^{3}H]GroPCho		[^{3}H]ChoP		[^{3}H]Cho	
	Intracellular	Total	Intracellular	Total	Intracellular	Total
NIH-3T3	93 ± 6	102 ± 6	92 ± 2	107 ± 6	132 ± 5	128 ± 6
EC807	98 ± 1	111 ± 8	98 ± 4	104 ± 5	158 ± 14	154 ± 11

Cells were labelled with [^{3}H]choline chloride ($1uCiml^{-1}$) for 30h. The monolayers were washed and stimulated with $PGF_{2\alpha}$ (2.1uM) for 20 min The medium was not removed (Total) or aspirated (Intracellular) and incubations were quenched by the addition of methanol. Aqueous methanolic extracts were prepared and the [^{3}H]Cho containing fractions isolated on Dowex columns as described in Section 2.8. Results are expressed as means ± S.D., pooled from 2 to 6 experiments.

5.2.3 Analysis of growth factor stimulated generation of [^{3}H]Cho-labelled metabolites from control and Ha-ras transformed NIH-3T3 cells

To determine what effect over-expression of normal ras genes have on growth factor stimulated PtdCho breakdown, [^{3}H]Cho-labelled NIH-3T3 cells and Ha-ras (EC807) transformed NIH-3T3 cells were stimulated with saturating concentrations of bombesin, PDGF, bradykinin, calf serum and $PGF_{2\alpha}$ (Table 5.2). No stimulated changes in the levels of GroPCho or ChoP were noted in either cell type. Bombesin and PDGF did not stimulate increases in the levels of Cho, while calf serum stimulated similar increases in Cho in both cell lines (NIH-3T3=23 $\pm$ 17% above basal; EC807=34 $\pm$ 8% above basal; P=0.125). Bradykinin stimulated increases in Cho levels in Ha-ras transformed cells only (21 $\pm$ 8% above basal). $PGF_{2\alpha}$-stimulated a significant increase in Cho generation in both cell lines, but in the Ha-ras transformed cells, stimulated Cho levels are 2-fold greater (54 $\pm$ 11% above basal) compared to control cells (28 $\pm$ 6% above basal). This would suggest that in Ha-ras transformed cells there is greater $PGF_{2\alpha}$-stimulated PLD activity.

5.2.4 $PGF_{2\alpha}$-stimulated Cho generation in control and Ha-ras transformed NIH-3T3 cells

To examine the $PGF_{2\alpha}$-stimulated PLD activity in control and Ha-ras (EC807) transformed cells the dose-dependence of $PGF_{2\alpha}$-stimulated Cho generation as a percentage increase of unstimulated controls was determined (Fig.5.2). For both control and Ha-ras transformed NIH-3T3 cells half-maximal stimulation (EC_{50}) occurs at a concentration of approximately 0.17uM. A saturating response is observed at approximately 2uM; at this concentration $PGF_{2\alpha}$-stimulated Cho generation is almost 2-fold greater in the Ha-ras transformed cells (EC807= 56 $\pm$ 7% above basal)

Table 5.2 Growth factor stimulation of GroPCho, ChoP and Cho in control and Ha-ras (EC807) transformed NIH-3T3 cells

Cell Type	Ligand	% of untreated control		
		GroPCho	ChoP	Cho
NIH-3T3	Bombesin (2.5uM)	100 ± 4	106 ± 3	97 ± 7
	PDGF(1.32ugml^{-1})	97 ± 4	104 ± 7	104 ± 9
	Bradykinin (3.2uM)	103 ± 4	100 ± 8	102 ± 9
	Calf serum (4%)	99 ± 4	100 ± 8	123 ± 17
	$PGF_{2\alpha}$ (2.1uM)	101 ± 5	107 ± 5	128 ± 6
EC807	Bombesin (2.5uM)	99 ± 10	100 ± 7	110 ± 7
(Ha-ras)	PDGF (1.32ugml^{-1})	100 ± 6	101 ± 4	109 ± 9
	Bradykinin (3.2uM)	106 ± 8	100 ± 6	121 ± 8
	Calf serum (4%)	103 ± 3	100 ± 7	134 ± 8
	$PGF_{2\alpha}$ (2.1uM)	111 ± 8	104 ± 5	154 ± 11

Cells were labelled with [^{3}H]choline chloride (1uCiml^{-1}) for 30h and after a 20 min stimulation the accumulation of GroPCho, ChoP and Cho were determined as described in Section 2.8. Results are expressed as means ± S.D., pooled from 3 to 6 experiments.

Figure 5.2 Dose dependence of $PGF_{2\alpha}$ -stimulated Cho generation in control and Ha-ras (EC807) transformed NIH-3T3 cells

Cells were labelled with [^{3}H]choline chloride ($1uCiml^{-1}$) for 30h and stimulated for 30 min with various concentrations of $PGF_{2\alpha}$. [^{3}H]Cho generation was determined as described in Section 2.8. Results are expressed as means ± S.E.M., pooled from four separate experiments.

Figure 5.2a

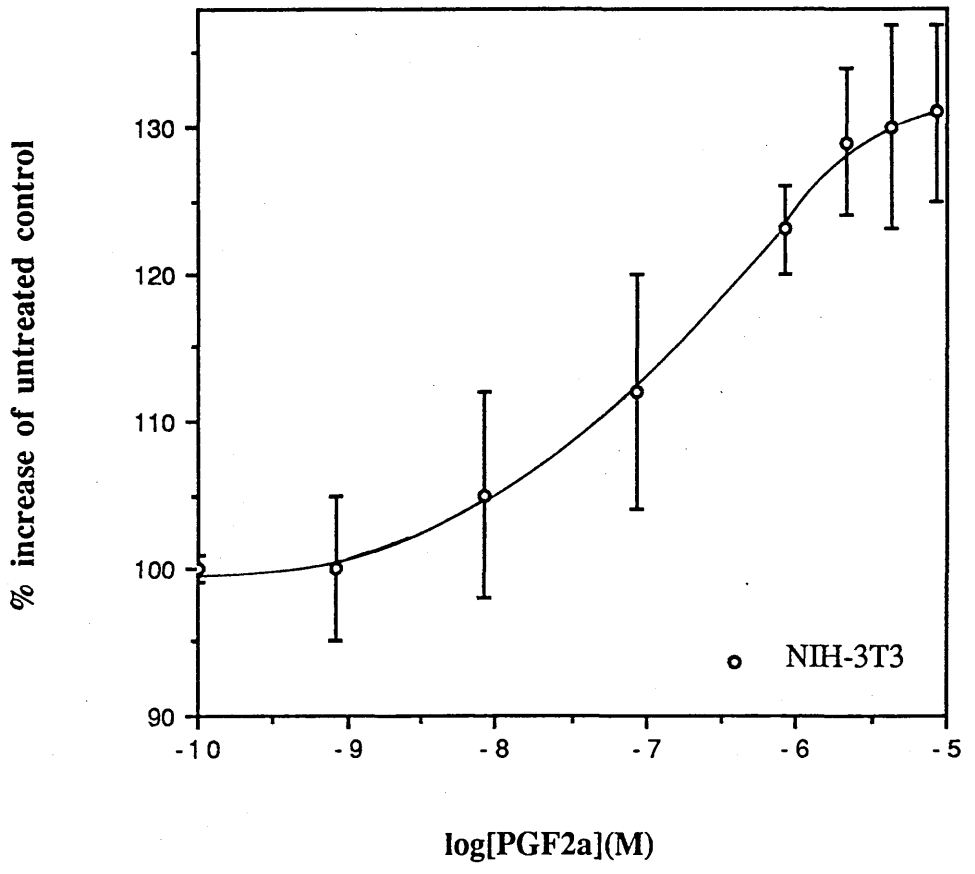
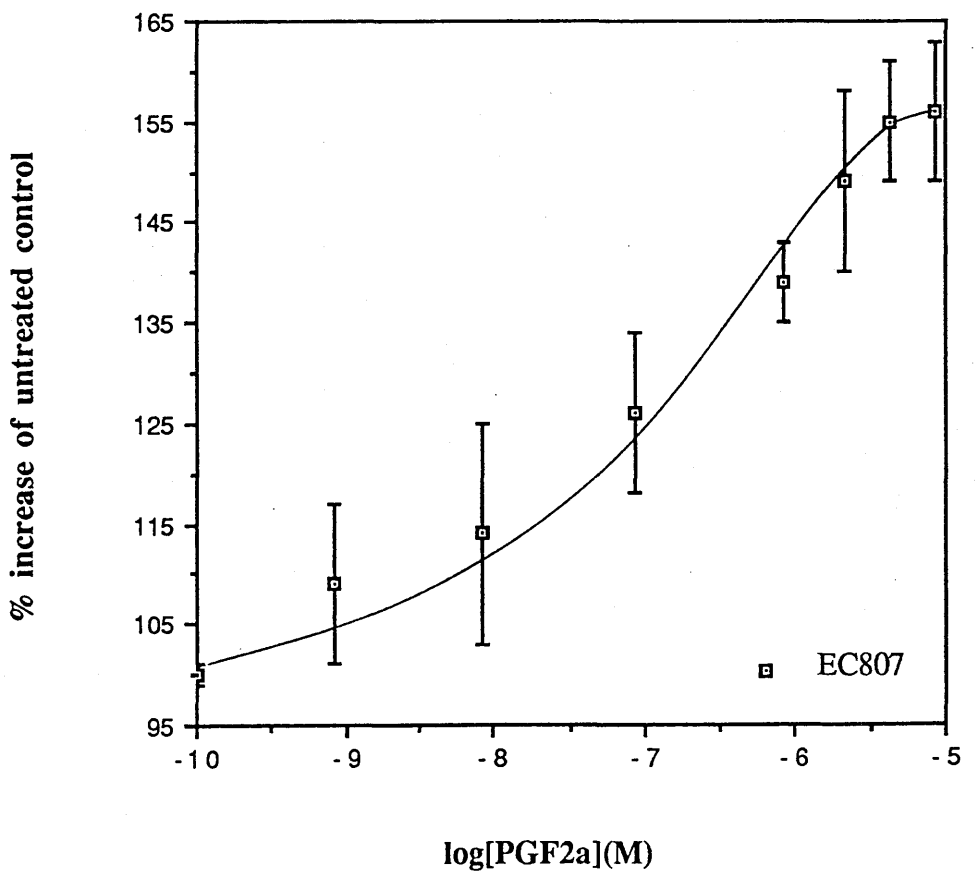


Figure 5.2b



compared to control cells (NIH-3T3= 30 ± 7% above basal). No stimulated increases in the levels of GroPCho or ChoP were observed at any of the $PGF_{2\alpha}$ concentrations tested.

The time course of $PGF_{2\alpha}$-stimulated changes in Cho levels in control and Ha-ras transformed cells is illustrated in Figure 5.3. In NIH-3T3 cells $PGF_{2\alpha}$ (2.1uM) stimulated the generation of Cho, which was significant at 1 min (20% above basal) and thereafter paralleled the basal increase in Cho resulting in levels of 28% above basal at 30 min (Fig.5.3a). In Ha-ras transformed cells, small increases in Cho generation were noted at 1 min. However, significant increases in Cho levels were only observed after 2 min (5% above basal) and increased to 55% above basal at 30 min, this being almost 2-fold greater than the $PGF_{2\alpha}$-stimulated increases observed in control cells. A similar time lag of 2 min in $PGF_{2\alpha}$-stimulated Cho generation was always observed in Ha-ras transformed cells. No significant changes in the radioactivity associated with GroPCho or ChoP were observed in response to $PGF_{2\alpha}$ (2.1uM) in either cell type.

5.2.5 TPA-stimulated Cho generation in control and Ha-ras transformed NIH-3T3 cells

In NIH-3T3 cells significant TPA (100nM)-stimulated increases in Cho generation were only observed after 5 min (7% above basal). Thereafter levels increased to 73% above basal at 1h (Fig.5.4a). A time lag of 2 min in TPA-stimulated Cho generation was always observed in control cells. In Ha-ras transformed cells a significant increase (17% above basal) in Cho generation was observed after a 1 min stimulation with TPA (100nM) and levels continued to rise for the 60 min duration of the time course to 79% above basal. This being similar to the stimulated levels in control cells. No significant change in the radioactivity

Figure 5.3 Time-course of $PGF_{2\alpha}$ -stimulated changes in Cho levels in control and Ha-ras (EC807) transformed NIH-3T3 cells

Cells were labelled with [^{3}H]choline chloride ($1uCiml^{-1}$) for 30h and after stimulation with $PGF_{2\alpha}$ (2.1uM) for the times stated, the levels of Cho were determined as described in Section 2.8. Results are expressed as means ± S.D., n=8 in each case, pooled from two separate experiments which gave qualitatively the same results as three others. Results have been normalised using d.p.m. values of untreated controls. Figure 5.3a: NIH-3T3 cells - basal rate of Cho turnover was 0.036 ± 0.009% min^{-1}; Figure 5.3b EC807 cells - basal rate of Cho turnover was 0.045 ± 0.016% min^{-1}; rate = d.p.m. Cho produced per min x 100/d.p.m. in choline containing lipids.

Figure 5.3a

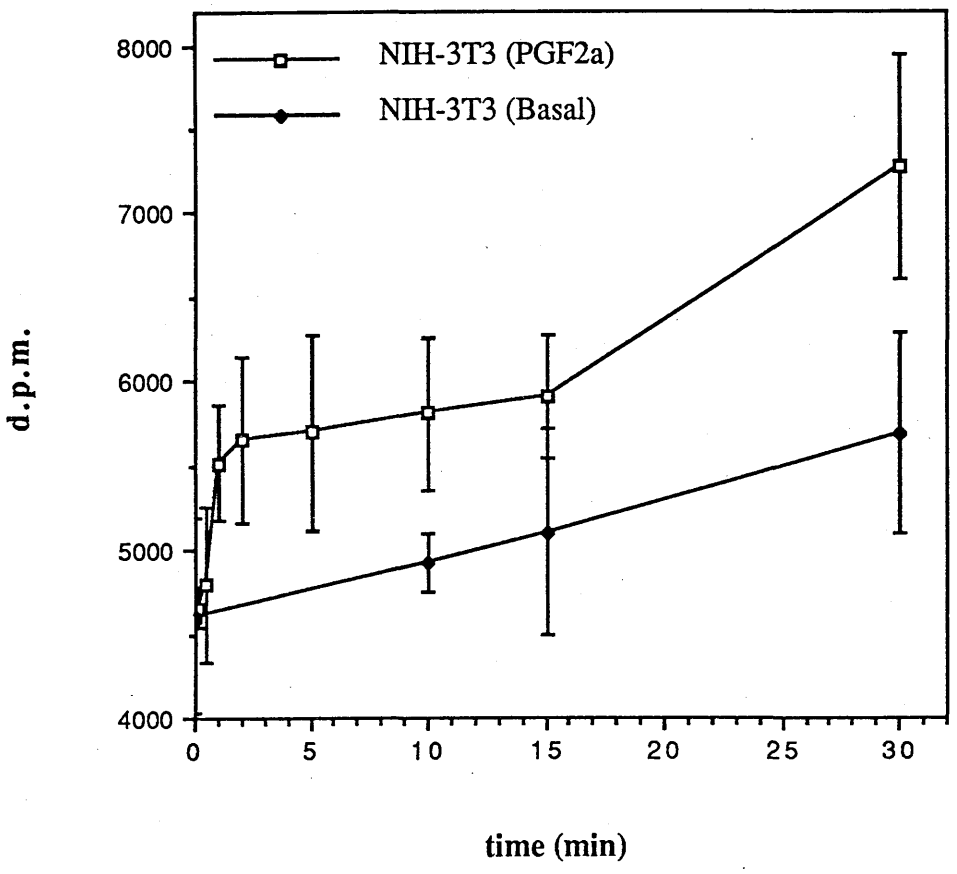


Figure 5.3b

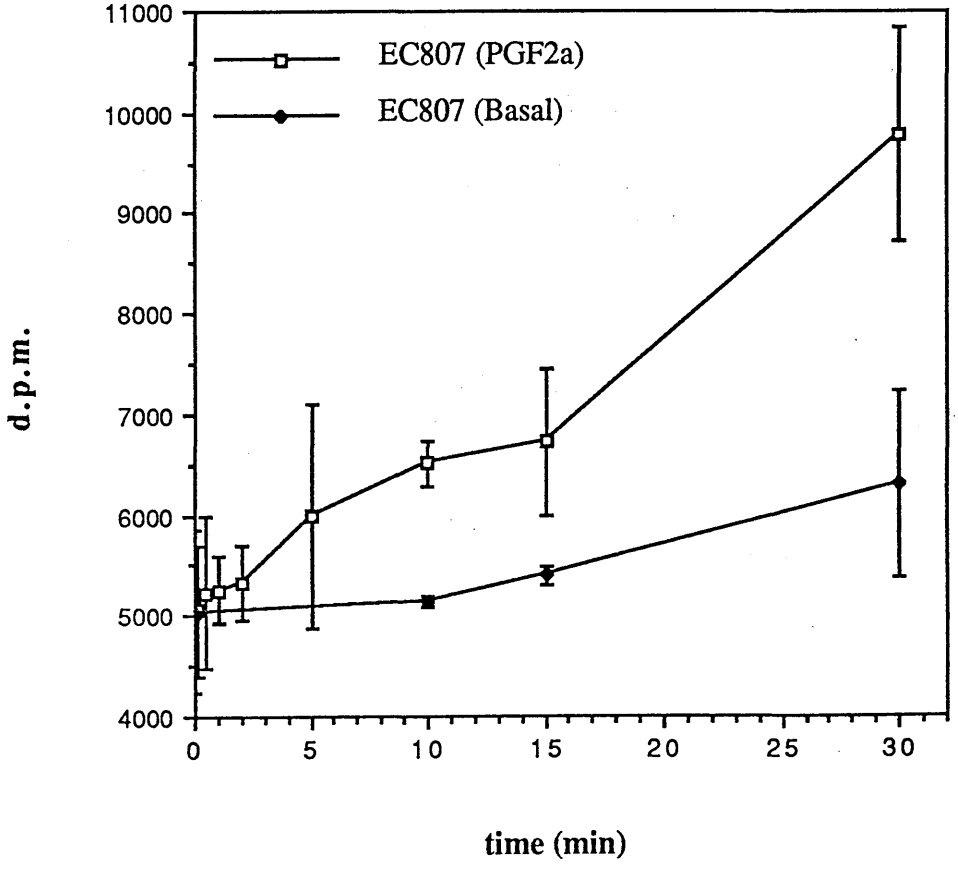


Figure 5.4 Time-course of TPA-stimulated changes in Cho levels in control and Ha-ras (EC807) transformed NIH-3T3 cells

Cells were labelled with [^{3}H]choline chloride ($1uCiml^{-1}$) for 30h and after stimulation with TPA (100nM) for the times stated, the levels of Cho were determined as described in Section 2.8. Results are expressed as means $\pm$ S.E.M., n=10 in each case, pooled from three separate experiments which gave qualitatively the same results as two others. Results have been normalised using d.p.m. values of untreated controls. Figure 5.4a: NIH-3T3 cells - basal rate of Cho turnover was 0.035 $\pm$ 0.003% min^{-1}, Figure 5.4b EC807 cells - basal rate of Cho turnover was 0.0700 $\pm$ 0.006% min^{-1}; rate = d.p.m. Cho produced per min x 100/d.p.m. in choline containing lipids.

Figure 5.4a

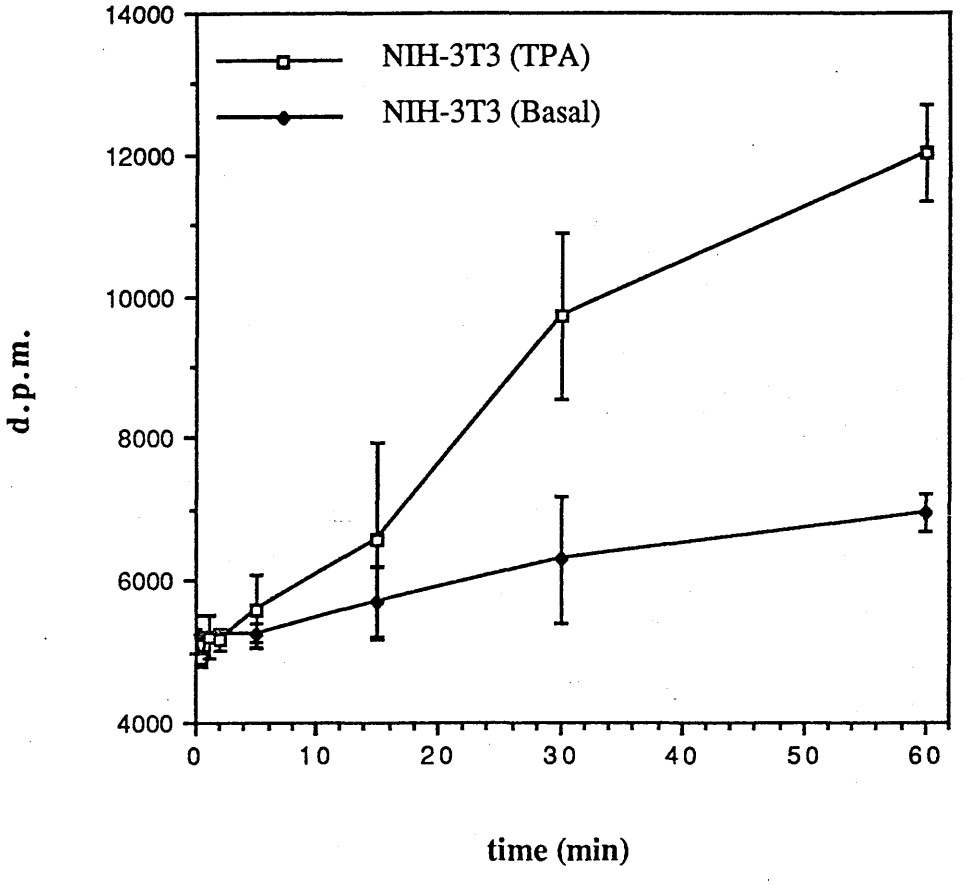
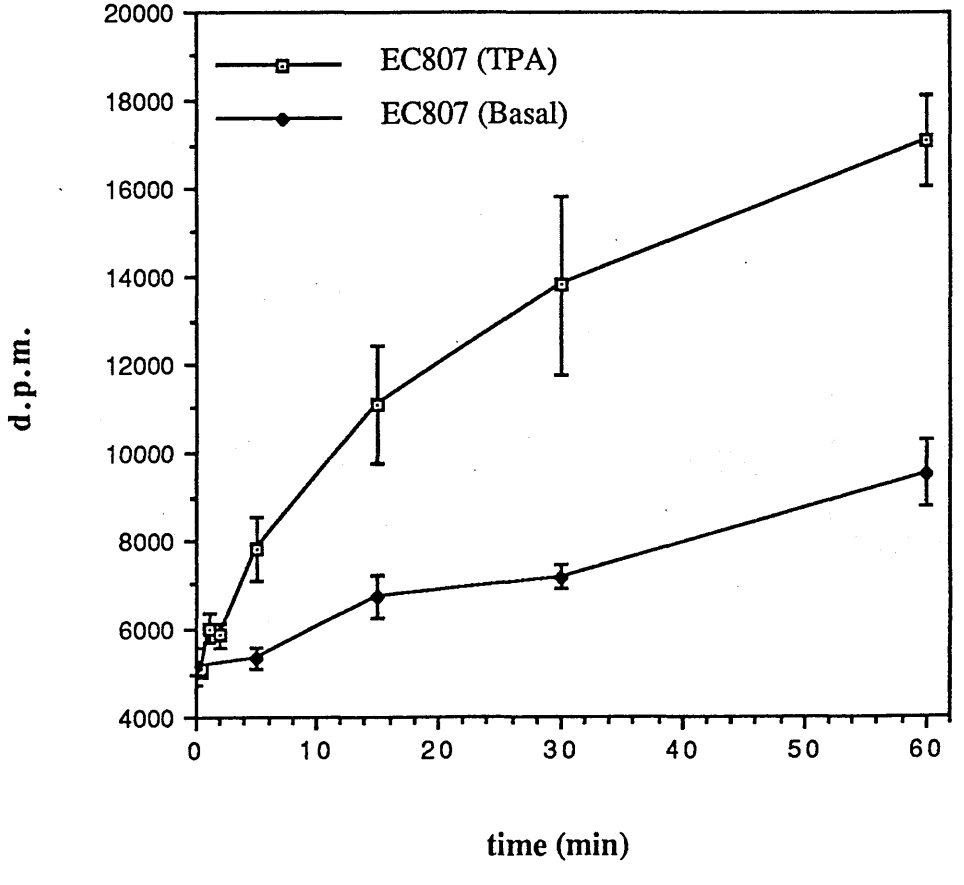


Figure 5.4b



associated with GroPCho or ChoP was observed in response to TPA in either cell type. The non C-kinase activating β-phorbol was without effect upon GroPCho, ChoP or Cho levels (results not shown).

5.2.6 Basal levels and basal rates of production of GroPCho, ChoP and Cho in control and Ha-, Ki- or N-ras transformed NIH-3T3 cells

A comparison of the basal levels of GroPCho, ChoP and Cho in control and Ha-, Ki- or N-ras transformed NIH-3T3 cells is summarised in Table 5.3. Values stated are d.p.m. in cell associated GroPCho, ChoP and Cho divided by d.p.m. in choline containing lipids. The ratio EC807:NIH-3T3 describes EC807 values divided by NIH-3T3 values and is one example of a comparison of ras transformed cell values, with control cell values.

The results show that the basal levels of GroPCho are reduced in ras transformed cells; for example, the levels of GroPCho in Ha-ras transformed cells are 41% of those in control cells. Basal levels of Cho were similar in all cell types tested. However, the basal levels of ChoP were always found to be greater in the ras transformed cells and the fold increase ranged from 1.38 to 3.25 (Table 5.3).

The basal rate of GroPCho turnover was 2.10-fold greater in Ha-ras transformed cells compared to control cells (EC807= 0.057 $\pm$ 0.03% min^{-1}; NIH-3T3= 0.027% $\pm$ 0.01% min^{-1}; [n=5-6]; rate = d.p.m. GroPCho produced per min x 100/d.p.m. in choline containing lipids). The basal rate of Cho turnover was greater in Ha-ras transformed cells (range 1.3- to 2.68-fold greater) compared to control cells (EC807= 0.053 $\pm$ 0.01% min^{-1}; NIH-3T3= 0.028 $\pm$ 0.01% min^{-1}; n=7-9), while the level of ChoP appears to be in steady state in both cell types.

Table 5.3 Basal levels of GroPCho, ChoP and Cho in control and Ha-, Ki- or N-ras transformed NIH-3T3 cells

Cell type	[(dpm in Cho metabolite/dpm in lipids)x100]			No. of
	GroPCho	ChoP	Cho	Expts.
NIH-3T3	9.42 ± 1.07	13.73 ± 1.39	5.20 ± 0.55	18
EC807(Ha-ras)	3.86 ± 0.49	28.69 ± 2.36	4.82 ± 0.72	18
Ki860(Ki-ras)	2.11	39.40	6.41	1
N872(N-ras)	1.46	28.30	6.89	1
ratio				
EC807:NIH-3T3	0.41 ± 0.05	2.09 ± 0.15	0.93 ± 0.05	

Cells were labelled with [^{3}H]choline chloride ($1 uCi ml^{-1}$) for 30h and basal levels of GroPCho, ChoP and Cho were determined as described in Section 2.8. Results are pooled from the stated number of experiments and are expressed as means ± S.E.M. Ratio describes EC807 (cells transformed by overexpressed Ha-ras) values divided by NIH-3T3 values. GroPCho ratio EC807:NIH-3T3 ranged from 0.19 to 0.75; ChoP ratio EC807:NIH-3T3 ranged from 1.38 to 3.25; Cho ratio EC807:NIH-3T3 ranged from 0.56 to 1.44.

5.2.7 Cho kinase activity in control and Ha-, Ki- or N-ras transformed NIH-3T3 cells

Lacal et al. (1987a) reported that the cellular concentration of ChoP was significantly elevated in v-Ha-ras transformed NIH-3T3 cells, 1.42- to 2.47-fold greater than control cells. This being similar to the 2.09-fold increase in ChoP levels observed in this study (Table 5.3). It was suggested that the ChoP arises from constitutive hydrolysis of PtdCho by phospholipase C, an activity that would account for the reported elevated DAG levels found in ras transformed cells (Lacal et al. 1987a). Macara (1989) however, has suggested that it is possible that the increased ChoP arises through the induction of Cho kinase activity. Therefore, experiments to determine Cho kinase activity in control and ras transformed NIH-3T3 cytosol extracts were performed. In order to measure Cho kinase activity in these cells, the conversion of [^{3}H]Cho to [^{3}H]ChoP in the presence of a known amount of the cytosolic extract was measured. The amount of [^{3}H]ChoP generated in these samples was quantified by separating the [^{3}H]-labelled ChoP by Dowex anion exchange chromatography. Phosphorylation was linear with time for at least 40 min (Fig.5.5a) and with protein concentration up to 20ug (Fig.5.5b).

Initial experiments to determine the Michaelis Menton constant (K_m) for control and Ha-ras transformed NIH-3T3 cells Cho kinase were performed using [^{3}H]Cho at a specific activity of 1Cimmol^{-1}. The results in Figure 5.6 show the velocity of the reaction for Cho kinase in a control and Ha-ras transformed NIH-3T3 cytosolic preparation. The V_{max} calculated from this data was 1.24 (NIH-3T3 cell; Fig.5.6a) and 2.83nmoles ChoP min^{-1}mg protein (EC807 cells; Fig.5.6b). The half maximal substrate concentration determined from these plots for control and Ha-ras transformed cells

Figure 5.5 Effect of time and protein concentration on Cho kinase activity in NIH-3T3 cells

Cho kinase activity was assayed as described in Section 2.10. Results are means ± S.D. of one experiment where n=3. The experiment was repeated once and gave very similar results. Figure 5.5a - effect of time on Cho kinase activity in NIH-3T3 cells; Figure 5.5b - effect of protein concentration on Cho kinase activity in NIH-3T3 cells.

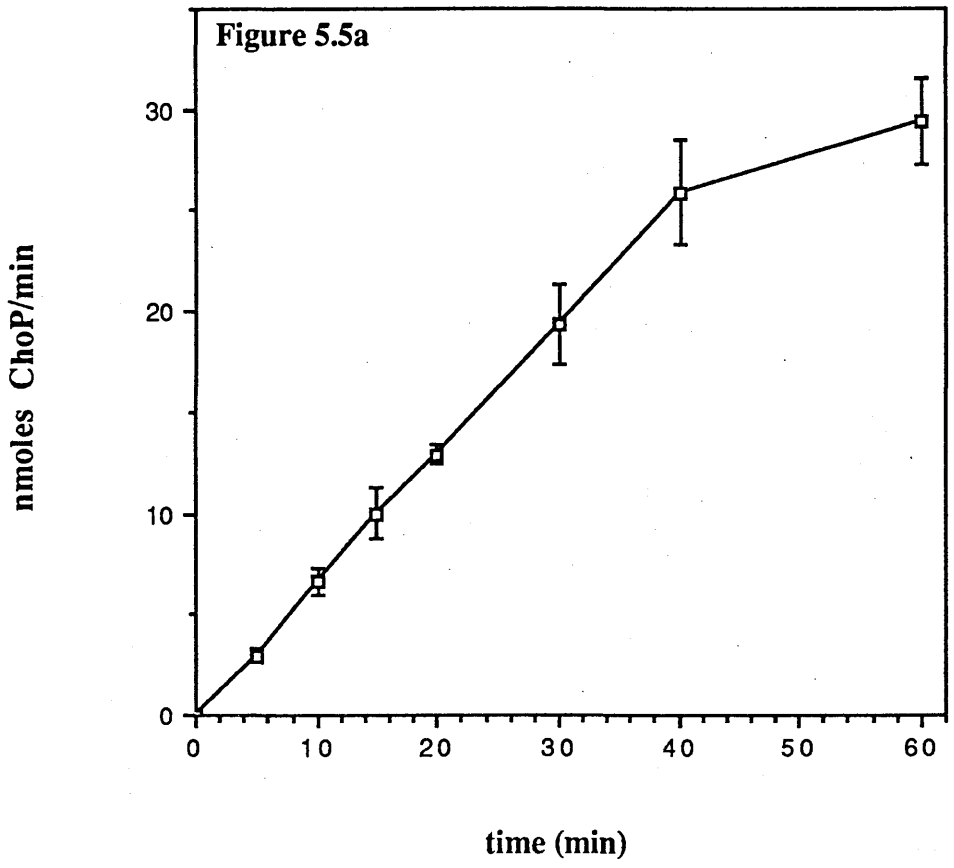


Figure 5.5a
nmoles ChoP/min
0
10
20
30
0
10
20
30
40
50
60
time (min)

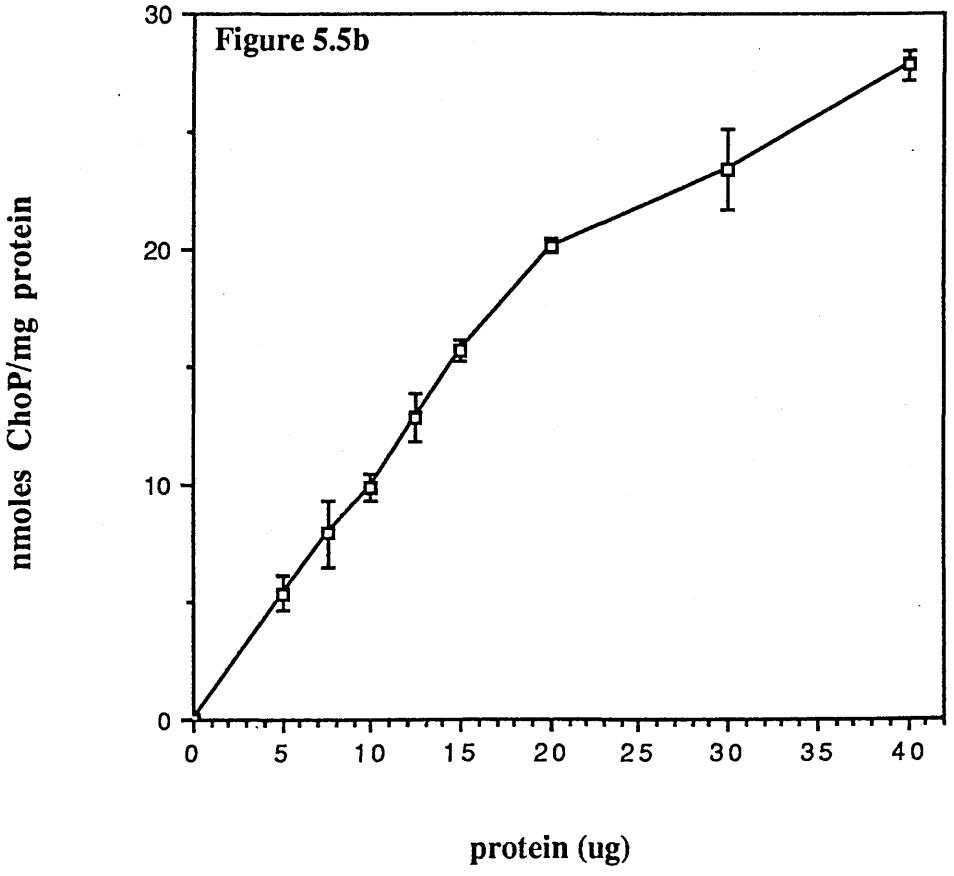


Figure 5.5b
nmoles ChoP/mg protein
0
10
20
30
0
5
10
15
20
25
30
35
40
protein (ug)

Figure 5.6 Substrate requirement of Cho kinase specific activity from control and Ha-ras transformed NIH-3T3 cytosolic preparation

The kinetics of the NIH-3T3 (Fig.5.6a and 6c) and EC807 (Fig.5.6b and 6d) Cho kinase was determined as described in Section 2.10. The results are from one experiment performed on triplicate samples and Figure 5.6a and 6b are plots of velocity (V) of the enzyme reaction in nmol ChoP $min^{-1}mg^{-1}$ protein versus substrate concentration in uM(S). Results are means $\pm$ S.D., n=3 in each case and the data are from one experiment which is representative of three others. Figure 5.6c and 6d are the same data plotted as V versus $^{V}/[S]$. Mean K_m values of four similar experiments were: NIH-3T3 cells - 18.21 $\pm$ 4.67uM; EC807 cells - 18.85 $\pm$ 1.60uM.

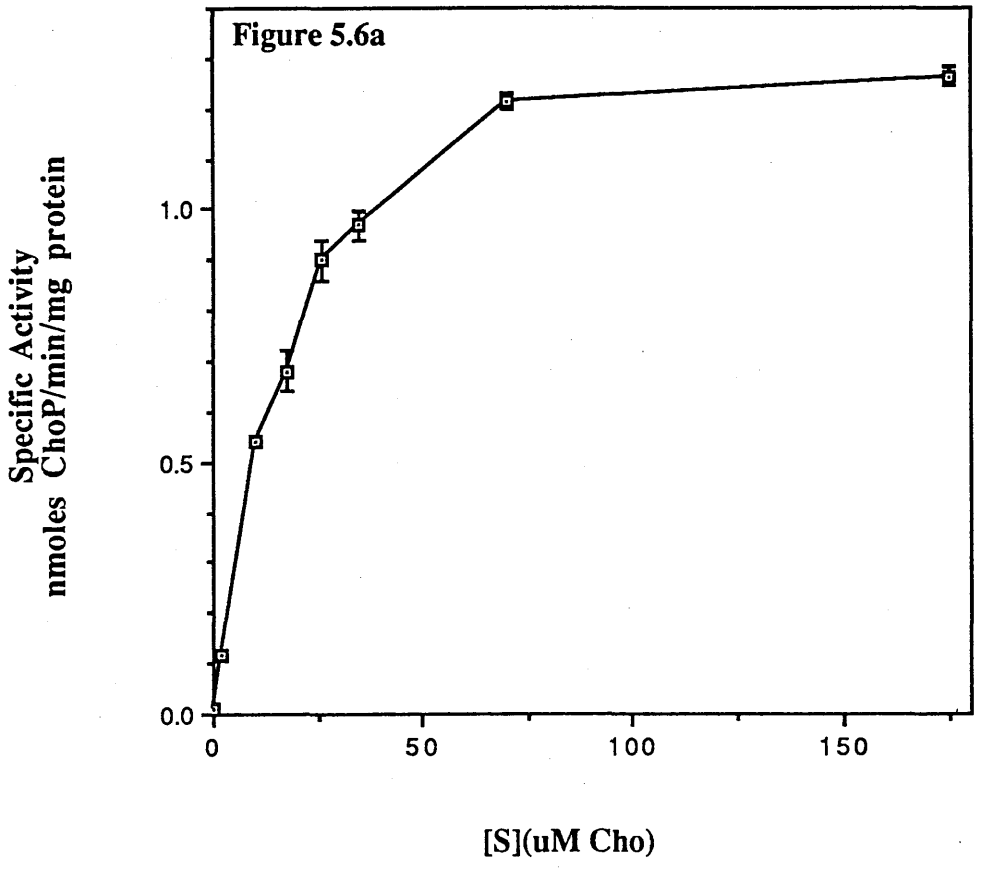


Figure 5.6a
Specific Activity
nmoles ChoP/min/mg protein
1.0
0.5
0.0
0
50
100
150
[S](uM Cho)

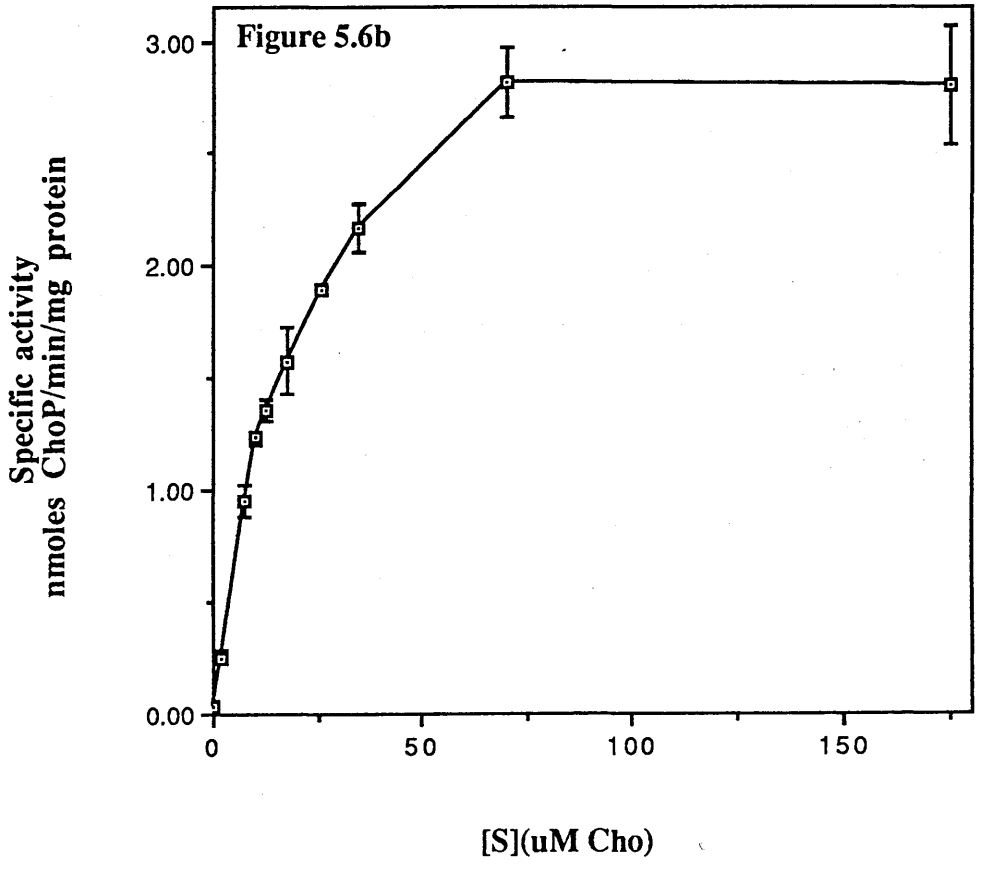


Figure 5.6b
Specific activity
nmoles ChoP/min/mg protein
3.00
2.00
1.00
0.00
0
50
100
150
[S](uM Cho)

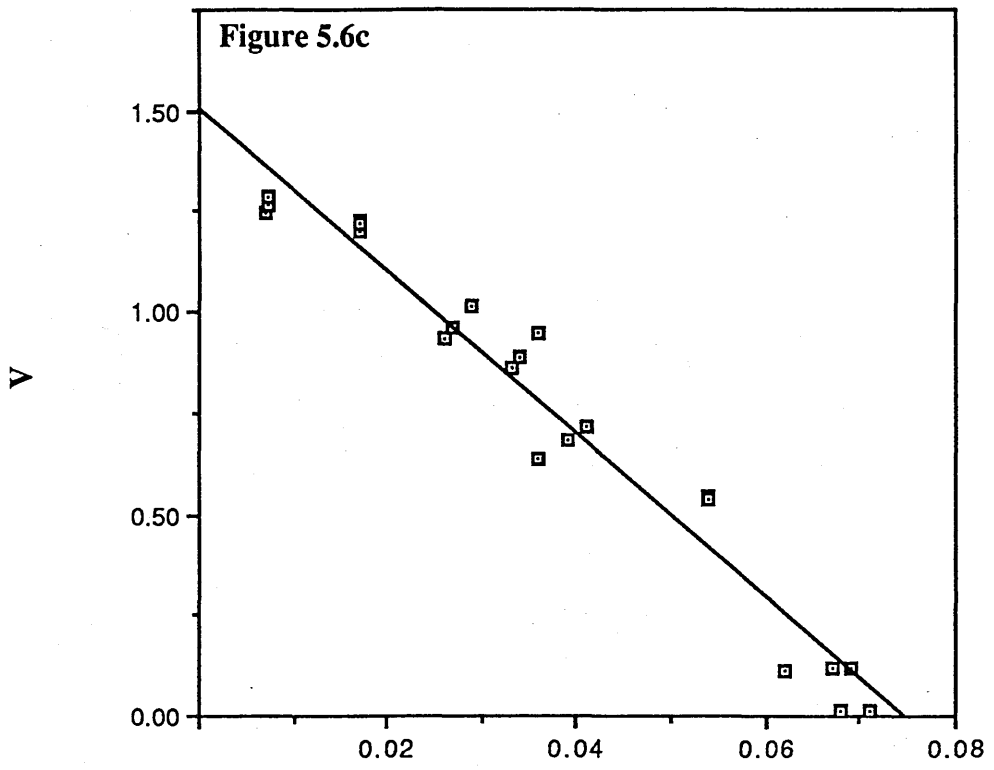


Figure 5.6c
1.50
1.00
0.50
0.00
V
0.02
0.04
0.06
0.08
V/[S]

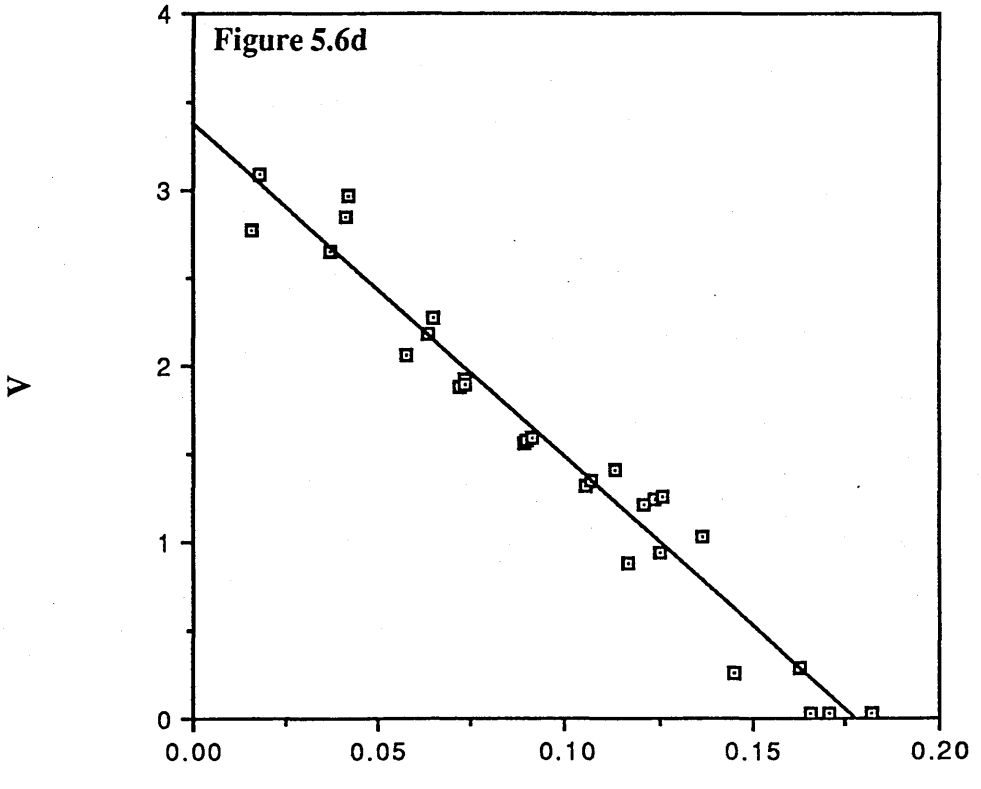


Figure 5.6d
4
3
2
1
0
V
0.00
0.05
0.10
0.15
0.20
V/[S]

was approximately 14.15 and 13.96uM Cho respectively. From these plots the control and Ha-ras transformed NIH-3T3 Cho kinase appears to display Michaelis-Menton kinetics. Figure 5.6c and 5.6d are the Eadie-Hofstee plots of the data in Figure 5.6a and 5.6b respectively. From these graphs the K_m values obtained for control and Ha-ras transformed NIH-3T3 cells were 20.15 and 19.32uM respectively; the V_{max} values obtained were 1.5 and 3.39nmoles ChoP $min^{-1}mg^{-1}$protein, respectively.

The specific activities of Cho kinase in the cell extracts from Ha-, Ki- and N-ras transformed NIH-3T3 cells were significantly higher, 2.29, 2.02 and 2.07-fold greater respectively, than those of control cells (Table 5.4). These differences could be attributed to the fact that on reaching confluency NIH-3T3 cells quiesce whereas ras transformed cells continue in active growth. Comparison of measurements of Cho kinase activity in cultures which were 100% confluent and 50% confluent (in exponential growth) were made. Cells in logarthimic growth gave similar V_{max} and K_m values to those cells which were confluent (Table 5.5).

5.2.8 Comparison of stimulated and basal PtdCho metabolism in NIH-3T3 and AmNIH-3T3 cells

In Chapter 4 it was demonstrated that $PGF_{2\alpha}$ -induced mitosis in AmNIH-3T3 cells, but not in NIH-3T3 cells, while inducing inositol phosphate generation in both cell types. It was of interest, therefore, to determine if differences in PtdCho metabolism existed between these cell types, hence comparisons of stimulated and basal PtdCho metabolism were made. Saturating concentrations of bombesin, PDGF, bradykinin, and calf serum did not stimulate changes in the levels of GroPCho or ChoP in either cell type (Table 5.6). Saturating concentrations of $PGF_{2\alpha}$ did not stimulate changes in the levels of GroPCho in NIH-3T3 cells or ChoP

Table 5.4 Cho kinase activity in control and Ha-, Ki- and N-ras transformed NIH-3T3 cells

Cell type	Mean specific activity (nmoles/min/mg protein)	Enhancement ratio	No. of Expts.
NIH-3T3	0.861 ± 0.14		8
EC807(Ha-*ras*)	1.978 ± 0.30	2.29	8
Ki860(Ki-*ras*)	1.740 ± 0.28	2.02	2
N872(N-*ras*)	1.780 ± 0.24	2.07	2

Cho kinase was assayed as described in Section 2.10. Results are expressed as means ± S.D., pooled from the stated number of experiments. Enhancement ratio described values from cells transformed by Ha-*ras* (EC807); Ki-*ras* (Ki860); N-*ras* (N872) divided by NIH-3T3 values. The specific activity of Cho kinase from extracts of NIH-3T3 and EC807 cells ranged from 0.432 to 1.586 and 1.128 to 3.390nmoles/min/mg protein respectively.

Table 5.5 Measurements of Cho kinase activity in cultures of control and Ha-ras transformed NIH-3T3 cells which were 100% confluent and 50% confluent

Cell type	V_{max} (nmoles/min/mg protein)	K_m (uM)
NIH-3T3 100% confluent	0.814 ± 0.21	15.21 ± 2.63
NIH-3T3 50% confluent	0.973 ± 0.31	16.93 ± 3.47
EC807(Ha-ras) 100% confluent	2.324 ± 0.53	16.11 ± 3.07
EC807(Ha-ras) 50% confluent	2.462 ± 0.47	17.45 ± 1.74

Cho kinase activity was assayed as described in Section 2.10. Results are means ± S.D., pooled from 3 experiments.

Table 5.6 Growth factor stimulation of GroPCho, ChoP and Cho in NIH-3T3 and AmNIH-3T3 cells

Cell Type	Ligand	% of untreated control		
		GroPCho	ChoP	Cho
NIH-3T3	Bombesin (2.5uM)	100 ± 4	106 ± 3	97 ± 7
	PDGF(1.32ugml^{-1})	97 ± 4	104 ± 7	104 ± 9
	Bradykinin (3.2uM)	103 ± 4	100 ± 8	102 ± 9
	Calf serum (4%)	99 ± 4	100 ± 8	123 ± 17
	$PGF_{2\alpha}$ (2.1uM)	101 ± 5	107 ± 5	128 ± 6
AmNIH-3T3	Bombesin (2.5uM)	101 ± 7	108 ± 7	96 ± 17
	PDGF (1.32ugml^{-1})	94 ± 8	103 ± 20	95 ± 7
	Bradykinin (3.2uM)	95 ± 7	107 ± 11	99 ± 8
	Calf serum (4%)	100 ± 6	108 ± 8	131 ± 5
	$PGF_{2\alpha}$ (2.1uM)	119 ± 9	105 ± 13	130 ± 10

Cells were labelled with [^{3}H]choline chloride (1uCiml^{-1}) for 30h and after a 20 min stimulation the accumulation of GroPCho, ChoP and Cho were determined as described in Section 2.8. Results are expressed as means ± S.D., pooled from 4 to 6 experiments.

levels in either cell type. However, $PGF_{2\alpha}$-stimulated significant increases in GroPCho levels (approximately 19% above basal) in AmNIH-3T3 cells. Bombesin, PDGF and bradykinin did not stimulate changes in the levels of Cho, while calf serum and $PGF_{2\alpha}$-stimulated significant increases in Cho levels (approximately 30% above basal) in both NIH-3T3 and AmNIH-3T3 cell types.

A comparison of the basal levels of GroPCho, ChoP and Cho in NIH-3T3 and AmNIH-3T3 cells is summarised in Table 5.7. Values stated are d.p.m. in cell associated GroPCho, ChoP and Cho divided by d.p.m. in Cho containing lipids. The ratio AmNIH-3T3:NIH-3T3 describes AmNIH-3T3 values divided by NIH-3T3 values. No significant differences in the levels of GroPCho, ChoP or Cho were noted between the cell types (Table 5.7).

The specific activity of Cho kinase from AmNIH-3T3 cell extracts was similar (0.935 $\pm$ 0.12nmoles ChoP/min/mg protein; n=2) to that obtained from NIH-3T3 cell extracts (0.861 $\pm$ 0.14nmoles ChoP/min/mg protein; n=8).

Table 5.7 Basal levels of GroPCho, ChoP and Cho in NIH-3T3 and AmNIH-3T3 cells

Cell type	[dpm in Cho metabolite/dpm in lipids) x 100] GroPCho	ChoP	Cho
NIH-3T3	6.94 ± 1.32	7.97 ± 1.15	3.63 ± 1.28
AmNIH-3T3	7.08 ± 0.7	8.55 ± 1.27	3.94 ± 1.34
AmNIH-3T3:NIH-3T3	1.09 ± 0.18	1.07 ± 0.08	1.14 ± 0.23

Cells were labelled with [^{3}H]choline chloride ($1uCiml^{-1}$) for 30h and basal levels of GroPCho, ChoP and Cho were determined as described in Section 2.8. Results are pooled from four experiments and are expressed as means ± S.D. Ratio describes AmNIH-3T3 values divided by NIH-3T3 values.

5.3 Discussion

As $PGF_{2\alpha}$ induced PtdIns(4.5)P_2 hydrolysis in both NIH-3T3 and AmNIH-3T3 cells and was a mitogen in AmNIH-3T3 cells, but not in NIH-3T3 cells, it was of interest to determine if differences in basal and/or agonist-stimulated PtdCho metabolism existed between the two clones of NIH-3T3 cells. No differences in growth factor stimulated Cho responseswere detected between the two clones. However, in AmNIH-3T3 cells, but not in NIH-3T3 cells, $PGF_{2\alpha}$ -stimulated an increase in GroPCho levels. This may imply a difference between the cells in agonist-stimulated phospholipase A_2 activity. This was the only difference noted between the two NIH-3T3 clones as basal levels of GroPCho, ChoP and Cho were similar and deserves further study. Cho kinase activity was also very similar in both clones.

Exactly which intracellular signalling system(s) ras interacts with is not clear. Stimulation of DNA synthesis by ras requires functional PKC activity (Lacal et al., 1987c; Morris et al., 1989) and mitogenesis only occurs if there is persistent activation of PKC, thus requiring a maintained increase in cellular DAG levels. Cells transformed by ras oncogenes have elevated levels of DAG (Preiss, 1986; Lacal et al., 1987a, 1987b; Wolfman & Macara, 1987; Fleishman et al., 1986). However, in all cell types examined, agonist-stimulated PtdIns(4,5)P_2 hydrolysis is a transient process which is rapidly desensitized (e.g. Brown et al., 1987). Therefore, DAG must be generated from the metabolism of phospholipids other than PtdIns(4,5)P_2. It has been suggested that PtdCho breakdown could be a potential source of DAG and many agonists that stimulate PtdIns (4,5)P_2 hydrolysis have now been shown to also activate the hydrolysis of PtdCho in various cell types (see Pelech & Vance, 1989).

The data presented in this chapter suggests that stimulation of PKC either directly (e.g. by TPA) or indirectly, via activation of PtdIns(4,5)P_2 hydrolysis (e.g. by $PGF_{2\alpha}$ and serum), may stimulate breakdown of PtdCho by PLD. The results in Table 5.2 demonstrate that calf serum and $PGF_{2\alpha}$ induced hydrolysis of PtdCho and the formation of Cho in both control and Ha-ras transformed NIH-3T3 cells, while bradykinin induced Cho generation only in Ha-ras transformed cells. In the Ha-ras transformed cells $PGF_{2\alpha}$-stimulated Cho levels were 2-fold greater than control cells suggesting that in the transformed cells there is greater $PGF_{2\alpha}$-stimulated PLD activity (Table 5.2). No stimulated changes in the levels of GroPCho of ChoP were observed in either control or Ha-ras transformed cells, indicating that none of the growth factors tested stimulated phospholipase A_2 or PtdCho-phospholipase C activity (Table 5.2). The stimulation of Cho generation by $PGF_{2\alpha}$ was dose-dependent, with half maximal stimulation at 0.18uM and a saturating response observed at 2uM (Fig.5.2). This was observed for both the control and the Ha-ras transformed cells. This EC_{50} is close to the Kd observed for $PGF_{2\alpha}$ receptor binding (Chapter 3, Section 3.2.5) and the EC_{50} for $PGF_{2\alpha}$-stimulated PtdIns(4,5)P_2 hydrolysis (Chapter 3, Section 3.2.4).

Agonist-stimulated PLD activity in HL60 cells has been suggested by time-course experiments showing rapid formation of phosphatidic acid with slower formation of DAG (Pai et al., 1988) and by the finding of phosphatidic acid formation even in the presence of a DAG kinase inhibitor (Mahadevappa, 1988). Receptor-regulated PLD activity has also been proposed to account for PtdCho hydrolysis in vasopressin-stimulated hepatocytes (Bocckino et al., 1987) and REF-52 cells (Cabot et al., 1988).

Results obtained by Cook and Wakelam (1989) strongly suggest that in Swiss 3T3 cells bombesin stimulated PtdCho breakdown by the activation of a receptor linked PLD on the basis that Cho is elevated between 10 and 30 seconds whilst ChoP levels only rise after 2 min.

Examination of $PGF_{2\alpha}$ -stimulated PLD activity by time-course experiments demonstrated that, in NIH-3T3 cells, Cho generation was significant at 1 min while in Ha-ras transformed cells a time lag of 2 min in $PGF_{2\alpha}$ -stimulated Cho generation was observed (Fig. 5.2). Compared to bombesin-stimulated Cho generation in Swiss 3T3 cells (Cook & Wakelam, 1989), $PGF_{2\alpha}$ -stimulated Cho generation in NIH-3T3 cells is slightly delayed, as Cho generation is not significantly elevated until 0.5 to 1 min. Although $PGF_{2\alpha}$ -stimulated breakdown of PtdCho is likely to be via activation of a receptor linked PLD other possibilities cannot be ruled out. For example, the possibility exists that PLD activity is being stimulated by the increase in intracellular free calcium concentration in response to $PGF_{2\alpha}$ -stimulated $Ins(1,4,5)P_3$ generation. Lloyd et al. (1989) have shown that $PGF_{2\alpha}$ stimulates a similar Ca^{2+} response in both control ($T15^-$) and N-ras transformed NIH-3T3 cells ($T15^+$). If an increase in intracellular Ca^{2+} concentration was responsible for activating PLD activity, then it would be expected that the fold-stimulation of Cho generation would be similar in both control and Ha-ras transformed cells and this is clearly not the case. $PGF_{2\alpha}$ -stimulated increases in Cho generation are observed at 30 min indicating that the signal stimulated by $PGF_{2\alpha}$ is long lived. As $PGF_{2\alpha}$ -stimulated $PtdIns(4,5)P_2$ hydrolysis is desensitized at 30 min (Chapter 3, Fig.3.2) then the increase in intracellular free calcium concentration in response to

$PGF_{2\alpha}$-stimulated Ins(1,4,5)P_3 generation would be unlikely to account for increases in Cho generation observed at 30 min. Indeed, Taki and Kanfer (1979) have suggested that PLD is active even in the absence of Ca^{2+}. The time lag of 2 min in $PGF_{2\alpha}$-stimulated Cho generation observed in Ha-ras transformed cells is difficult to explain. It may, however, be due to the fact that the basal rate of Cho production is at least 1.3-fold greater than that observed in control cells, which may mask an early stimulated increase.

Stimulation of PtdCho hydrolysis by TPA has been described in a number of systems (see Pelech & Vance, 1989) and has been tentatively proposed to be involved in providing a second phase of increased DAG levels, through the mediation of PKC (Muir & Murray, 1987; Cook et al., 1990). In NIH-3T3 cells TPA-stimulated increases in Cho generation were only observed after 5 min (Fig.5.4). A time lag of at least 2 min in TPA-stimulated Cho generation was always observed in control cells. In Ha-ras transformed cells a significant increase in Cho generation was observed after 1 min stimulation with TPA. At 1h TPA-stimulated similar fold increases in Cho generation (1.75-fold) in both control and Ha-ras transformed cells. These results strongly suggest that PLD activity is mediated by increased PKC activity and this is supported by work by Cabot et al. (1989) who demonstrated that in REF52 cells staurosporine, a PKC inhibitor and down-regulation of PKC were dually effective in suppressing TPA-induced PLD catalysed hydrolysis of PtdCho. However, Cook and Wakelam (1989) found that whilst down-regulation of PKC in Swiss 3T3 cells caused an inhibition of both bombesin and TPA-stimulated Cho generation, staurosporine only partially inhibited the responses to both the agonist and phorbol ester in normal cells. The time-lag in

TPA-stimulated Cho generation in control cells is difficult to account for. Liscovitch et al. (1987) also demonstrated an apparent lag period of 2.5-5 min in TPA (100nM) stimulated Cho generation in NG108-15 cells. Upon TPA treatment PKC is known to re-distribute from one cell compartment to another. It is possible that the translocation of PKC from the cytoplasm to the membrane requires a period of time and this may contribute to the lag period after the addition of TPA before an increase in PtdCho metabolism is observed. The ras transformed cells are always in active growth and do not quiesce when confluent. Therefore, PKC is always activated and may be already in a membrane-bound state. Indeed in $T15^{+}$ and N866 cells, the proportion of membrane-bound PKC is 3-fold higher than in control, non-transformed cells (Davies, S.A. & Wakelam, M.J.O., unpublished data). Thus when the cells are stimulated with TPA, since PKC activity is already stimulated, TPA acts to amplify the response and changes in Cho levels are significant within 1 min.

Consequently, activation of PKC in NIH-3T3 cells may lead to both the desensitization of PtdIns(4,5)P_2 hydrolysis and to the stimulation of PtdCho breakdown, thus potentiating its own activation whilst reducing the increase in intracellular free Ca^{2+} concentration. Therefore, it is possible that the stimulation of PtdCho breakdown in response to $PGF_{2\alpha}$ is activated by the increase in PKC activity as a consequence of prior PtdIns(4,5)P_2 hydrolysis rather than being a direct receptor activated event. Stimulation of NIH-3T3 cells with maximal concentrations of either $PGF_{2\alpha}$ or TPA causes the loss of only about 5% of the label from the total PtdCho fraction. This is in agreement with the findings of Cook and Wakelam (1989) using bombesin and TPA-stimulated Swiss-3T3 cells and also Liscovitch et al. (1987) using

TPA-stimulated NG108-15 cells and is probably due to the relatively small generation of Cho as compared to the large size of the cellular PtdCho pool.

The data presented in this chapter demonstrates that in ras-transformed cells PtdCho turnover is increased both by PLD and phospholipase A_2 pathways. The results show that in ras transformed cells basal levels of GroPCho are reduced (Table 5.3), but that the rate of GroPCho production is 2.1-fold greater in Ha-ras transformed cells compared to control cells. This would imply that in ras-transformed cells there is greatly increased phospholipase A_2 activity. This may be of importance as hydrolysis of PtdCho by phospholipase A_2 can produce arachidonic acid, an immediate precursor of eicosanoids. Bar-Sagi and Feramisco (1986) have also suggested that phospholipase A_2 activity may be activated when cells are microinjected with $p21^{ras}$. However, Price et al. (1989) have demonstrated that induction of DNA synthesis by oncogenic ras was unaffected by inhibitors of prostaglandin synthesis, indicating that conversion of the released arachidonic acid to various prostaglandins is not required for stimulation of DNA synthesis by ras. No difference was found in the basal levels of Cho between normal and ras-transformed cells, but the basal rate of Cho production was 1.3 to 2.68-fold greater in the ras-transformed cells. This suggests that there is increased basal PLD activity in ras transformed cells. This increased PLD activity may result from increased PKC activity as ras-transformed cells are always in active growth. Therefore, PKC is always activated. It is also possible that the increased autocrine production of several growth factors e.g. PDGF, observed in ras-transformed cells may also amplify PKC activity and hence PLD activity. Basal levels of ChoP were always found to be

greater in the ras-transformed cells compared to control cells and the fold increase ranged from 1.38 to 3.25 (Table 5.3). The basal level of ChoP appears to be in steady state in both control and Ha-ras transformed cells. This would imply that the rate of production of ChoP equals its conversion to CDP-choline by CTP: phosphocholine cytidyltransferase.

Lacal et al. (1987a) demonstrated similar increases in ChoP levels in v-Ha-ras transformed NIH-3T3 cells. They suggested that ChoP arises from constitutive hydrolysis of PtdCho by PtdCho-phospholipase C, an activity that would account for the reported elevated DAG levels found in ras-transformed cells. Preiss et al. (1986) and Lacal et al. (1987a) have shown that levels of DAG are elevated in ras-transformed cells, but this does not seem to be derived from increased PtdIns(4,5)P_2 hydrolysis (Wolfman & Macara, 1987; Morris et al., 1989). Macara (1989) suggested that the increased levels of ChoP in the ras-transformed cells arises through the induction of Cho kinase activity. Indeed the results in Table 5.4 demonstrate that the specific activity of Cho kinase in extracts of the ras-transformed cells is at least 2-fold greater than in control cells. This fold increase being similar to the fold increase observed in ChoP levels in the ras-transformed cells compared to control cells (Table 5.3). Cho kinase activity measured in both control and Ha-ras transformed cells in exponential growth gave similar V_{max} and K_m values to cells which were confluent (Table 5.5). Therefore, differences in basal levels of ChoP and Cho kinase activity are not simply due to the increased rate of proliferation of ras-transformed cells. Transformation of NIH-3T3 cells by ras appears to induce Cho kinase activity and it is this induction, rather than increasing PtdCho-phospholipase C activity that accounts for the elevation of basal levels of ChoP in

ras-transformed cells. Indeed Price et al. (1989) demonstrate that scrape loading Val12p21^{ras} into Swiss3T3 cells elevates ChoP levels. This increase occurs 10 to 20 min after scrape loading and continues for at least 60 min. They also demonstrate that this ras-dependent increase in ChoP is abolished in PKC down-regulated cells. This suggests that Cho kinase activity is dependent upon PKC activity and as PKC is always activated in ras-transformed cells it would account for the increased ChoP levels in ras-transformed cells compared to control cells.

CHAPTER 6

DISCUSSION

6.1 Discussion

The aim of the work described in this thesis was to investigate the effects of over-expression of normal ras proteins upon lipid signalling pathways in cultured NIH-3T3 fibroblasts. The results in Chapter 3 indicate that over-expression of $p21^{ras}$ in NIH-3T3 cells is associated with increased cell proliferation and transformation (Fig.3.1). Very high levels of normal cellular $p21^{ras}$ are required to be expressed in order to induce transformation. For example McKay et al. (1986) demonstrated that in the T15 cell line a 20-50-fold over-expression of normal $p21^{N\text{-}ras}$ was required. To achieve similar effects with $p21^{V\text{-}ras}$ or a mutated cellular ras oncogene, a lower concentration of the gene product is required i.e. concentrations comparable with those in untransformed cells (see McKay et al., 1986). Feramisco et al. (1984) demonstrated that mutated $p21^{Ha\text{-}ras}$, when injected into quiescent fibroblasts, caused rapid increases in DNA synthesis and mitosis and had a profound effect on cell morphology, whereas normal cellular $p21^{Ha\text{-}ras}$ injected at the same concentration had little or no observable effect.

The biochemical properties of ras proteins, plasma membrane binding, GDP/GTP binding and GTP hydrolysis are central to their biological function. The proteins are biochemically and biologically active only when in a GTP-bound state; GDP bound forms are inactive (Trahey & McCormick, 1987). GAP stimulates the GTPase activity and thus the conversion of p21-GTP to p21-GDP. However, GAP has no effect on the GTPase activity of oncogenic $p21^{ras}$ proteins and as a result, these proteins remain in their active, GTP-bound state. This may partly explain why a lower concentration of mutant $p21^{ras}$ is required for the stimulation of cell proliferation and transformation than normal $p21^{ras}$. Mulcahey et

al. (1985) demonstrated that microinjection of neutralising anti-p21^{ras} antibodies into quiescent fibroblasts, blocked proliferation induced by the addition of growth factors. This data together with some sequence homology to other proteins strengthens the possibility that p21^{ras} can act in a G-protein-like manner in signal transduction pathways which result in cell proliferation and transformation. It is still not clear, however, which processes are being regulated and therefore with which molecules p21^{ras} interacts.

The results in Chapter 3 have suggested that p21^{ras} can function at a post-receptor level to enhance growth factor stimulated inositol phosphate generation. In Chapter 3 it was demonstrated that in cells expressing high levels of the normal Ha-, Ki- or N-ras proto-oncogene, there was a marked increase in inositol phosphate generation in response to stimulation with certain growth factors compared to the control NIH-3T3 cells. This increase is likely to be due to the enhanced production of Ins(1,4,5)P_3 and its metabolites, since Lloyd et al. (1989) demonstrated that, in the T15 cell line, the induction of the N-ras proto-oncogene caused an amplification of bombesin-stimulated Ins(1,4,5)P_3 generation and intracellular Ca^{2+} release. The effect is due specifically to the expression of P21$^{N\text{-}ras}$ and can be correlated to the amount of cellular p21$^{N\text{-}ras}$ (Wakelam et al., 1987).

Several lines of evidence support the proposal that P21^{ras} is coupled to PtdIns(4,5)P_2 hydrolysis. Fleishman et al. (1986) have shown that NIH-3T3 and NRK cells transformed by Ha-, Ki- and N-ras genes demonstrate an increase in the steady state levels of inositol phosphates and DAG compared to non-transformed cells. Lacal et al. (1987b) demonstrated that microinjection of transforming p21$^{Ha\text{-}ras}$ into Xenopus oocytes resulted in increased

generation of inositol phosphates and DAG. Hancock et al. (1988) demonstrated that NIH-3T3 cells constitutively expressing and COS-1 cells transiently expressing oncogenically activated Ha- or Ki-ras genes display a significantly enhanced basal rate of inositol phospholipid turnover. These results suggested that the ras proto-oncogenes control the activity of PIC which hydrolyses PtdIns(4,5)P_2 to form Ins(1,4,5)P_3 and DAG and the presence of an activated mutated ras oncogene would result in a constitutively activated PIC. In support of this hypothesis Chiarugi et al. (1986) demonstrated that transformation of Balb-3T3 fibroblasts with the EJ/T24-Ha-ras oncogene increased the responsiveness of PIC to muscarinic stimulation. In addition, Wakelam et al. (1986) demonstrated that, in the T15 cell line, induction of a steroid-hormone regulated ras construct, causing over-expression of the N-ras gene, resulted in an increase in the apparent coupling of the bombesin receptor to PIC. However, this effect of $p21^{ras}$ expression may be confined to certain cell lines and Xenopus oocytes as contradictory evidence has been presented.

Some workers have reported a loss of response to growth factors in cells transfected and transformed by ras. Seuwen et al. (1988) observed a desensitization of the inositol phospholipid response in CCL39 fibroblasts transformed by mutant Ha- and Ki-ras in response to serum or thrombin. However, this desensitization is apparently at the receptor level, since the response to ALF_4^- persisted. The desensitization appeared not to be specific to ras expression as a similar effect was observed in polyoma virus-transformed CCL39 cells. Parries et al. (1987) demonstrated that transformation of NIH-3T3 cells by viral Ha- or Ki-ras caused the desensitization of the PDGF-stimulated inositol phosphate response with no effect on receptor number. Benjamin et al. (1988)

observed a similar desensitization and have demonstrated that EJ-ras transformed NIH-3T3 cells have decreased PDGF-stimulated $Ins(1,4,5)P_3$ and Ca^{2+} levels compared to control cells.

In Chapter 4 differences in the agonist-stimulated mitogenic and second messenger responses between two clones of NIH-3T3 cells were observed. This may have a bearing upon the contradictory observations made on the effects of transfection of apparently the same cell type with the same ras gene upon inositol phospholipid metabolism (Wakelam et al., 1986; Seuwen et al., 1988; Downward et al., 1988). Some of the conflicting results may arise from the use of different ras proto-oncogenes and oncogenes and different growth conditions. Wakelam (1988) reported that the amplified bombesin-stimulated inositol phosphate response in N-ras transformed NIH-3T3 cells is only observed at low cell densities. These cells when cultured at high cell density, demonstrate an inhibition of the amplification; these inhibitory effects are probably caused by the increased secretion of autocrines.

The results presented in Chapter 3 further demonstrate that in cells expressing high levels of the normal Ha-,Ki- and N-ras proto-oncogene, the stimulation by $PGF_{2\alpha}$ of inositol phosphate generation is reduced. This effect is not due to any reduction in receptor number, or change in EC_{50} or Kd and thus would appear to be due to a reduced coupling of the agonist receptor complex to PIC. The desensitization of $PGF_{2\alpha}$ induced inositol phospholipid turnover is associated with proliferative state, as the desensitization is observed in both the transformed cells and in rapidly proliferating, non-contact inhibited NIH-3T3 cells (Tables 3.2 & 3.6). Since the desensitization can be mimicked by treatment of the control cells with TPA, it is possible that the increased autocrine production of growth factors in the ras-transformed cells

could lead to increased PKC activity causing phosphorylation of $PGF_{2\alpha}$ receptors. Indeed Leeb-Lundberg et al. (1985) demonstrated that bradykinin or phorbol ester treatment of DDR_1 MF-2 cells caused a desensitization of α_1-adrenergic receptor-stimulated PtdIns(4,5)P_2 hydrolysis; an effect shown to be a consequence of receptor phosphorylation. Although phosphorylation of the $PGF_{2\alpha}$ receptors appears to be the most likely explanation for the ras induced desensitization of the $PGF_{2\alpha}$-stimulated inositol phosphate response, direct demonstration of PKC catalysed phosphorylation of the $PGF_{2\alpha}$ receptor awaits the purification of the receptor or the generation of specific antibodies. PKC catalysed phosphorylation of receptors may play a crucial role in a negative feedback regulation of the Ca^{2+} mobilising pathway. Indeed in Chapter 3 it is shown that $PGF_{2\alpha}$-stimulated PtdIns(4,5)P_2 hydrolysis is desensitized following a 30 min exposure to the agonist in control-NIH-3T3 cells.

In many cell types examined agonist-stimulated PtdIns(4,5)P_2 hydrolysis has been demonstrated to be a transient process which is rapidly desensitized (e.g. Brown et al., 1987). Cook et al. (1990) demonstrated that in bombesin-stimulated Swiss 3T3 cells the levels of DAG remained elevated for at least 60 min whilst the Ins(1,4,5)P_3 concentration had returned to basal levels within 1 min. Consequently, the sustained elevation of DAG would appear to be generated from the metabolism of phospholipids other than PtdIns(4,5)P_2. It has been demonstrated that many agonists can stimulate the hydrolysis of PtdCho in addition to PtdIns(4,5)P_2 (see Pelech & Vance, 1989). The results presented in Chapter 5 demonstrate that $PGF_{2\alpha}$, a potent mitogen in some cells which has been shown to stimulate the hydrolysis of PtdIns(4,5)P_2 generating Ins(1,4,5)P_3 (Chapter 4), is also

capable of stimulating the hydrolysis of PtdCho, apparently by a PLD-catalysed mechanism. TPA has also been demonstrated to stimulate the hydrolysis of PtdCho. Therefore it is likely that PKC either directly or indirectly activates PLD. Experiments with TPA suggest that PLD activation is secondary to inositol phospholipid hydrolysis. Cook and Wakelam (1989) have demonstrated that the EC_{50} for TPA-stimulated Cho generation in Swiss 3T3 cells is very similar to that for the stimulation of purified brain C-kinase activity (Castagna et al., 1982). Also the EC_{50} for Cho generation was identical to the IC_{50} for the inhibition of bombesin stimulated inositol phosphate production in Swiss 3T3 cells (Brown et al., 1987). The ability of TPA to stimulate PtdCho hydrolysis implies that a small, transient burst in PtdIns(4,5)P_2 hydrolysis in response to an agonist might be sufficient to initiate PtdCho catabolism, resulting in the generation of lipid activators of PKC; PtdCho turnover might then provide for a sustained activation of PKC even when PtdIns(4,5)P_2 hydrolysis is desensitized. Consequently, activation of PKC in NIH-3T3 cells may lead both to the desensitization of PtdIns(4,5)P_2 hydrolysis and to the breakdown of PtdCho, thus potentiating its own activation, whilst reducing the increase in intracellular free Ca^{2+} concentration. Thus, it is possible that the stimulation of PtdCho breakdown in response to $PGF_{2\alpha}$ is activated by the increase in PKC activity as a consequence of PtdIns(4,5)P_2 hydrolysis rather than being a direct receptor activated event. This hypothesis could explain the results of Griendling et al. (1986). These authors demonstrated that in rat aortic vascular smooth muscle cells, angiotensin II caused a biphasic generation of DAG, peaking both at 15 secs and at 5 min. Only the first peak was accompanied by an accumulation of Ins(1,4,5)P_3 and a marked decrease in

PtdIns(4,5)P_2; these rapid effects were blocked by a PKC activating phorbol ester, while the sustained DAG formation remained unchanged. Thus differences in the time-course of Ins(1,4,5)P_3 and DAG generation could be explained by the above described activation of PtdIns(4,5)P_2 and PtdCho hydrolysis. Cook and Wakelam (1989) have demonstrated that down-regulation of PKC activity in Swiss 3T3 cells by chronic TPA treatment prevents bombesin-stimulated choline generation thus supporting the concept of PtdCho breakdown being secondary to prior activation of PKC.

However, it is possible that stimulated PtdCho hydrolysis is induced by the activation of a receptor linked PLD and is a parallel event to stimulated PtdIns(4,5)P_2 hydrolysis. Indeed in permeabilised hepatocytes it has been demonstrated that the stimulation of PtdIns(4,5)P_2 hydrolysis by poorly hydrolysed analogues of GTP was blocked at 'zero Ca^{2+}' (Uhing *et al.*, 1986) whereas phosphatidic acid accumulation due to PtdCho hydrolysis was not (Boccking *et al.*, 1987). These reports suggest that agonist occupancy of its receptor may lead to differential activation of PIC and PLD catalysed hydrolysis of PtdIns(4,5)P_2 and PtdCho respectively, mediated either by the same G-protein or by two different G-proteins. In support of this model Besterman *et al.* (1986) demonstrated that prior down-regulation of PKC by prolonged pretreatment of 3T3-L1 cells with phorbol esters almost totally abolished subsequent stimulation of PtdCho breakdown by phorbol esters but only partially attenuated subsequent stimulation by PDGF and serum. These observations suggest that in 3T3-L1 cells PDGF and serum act, at least partially, through a PKC independent mechanism.

These models may help explain the results obtained in the *ras* transformed fibroblasts. Over-expression of *ras* in NIH-3T3

cells causes an amplification of some agonist stimulated inositol phosphate responses and therefore concomitant increases in DAG concentration. Increased DAG levels from PtdIns(4,5)P_2 hydrolysis will cause increased PKC activity which may therefore lead to both the desensitization of PtdIns(4,5)P_2 hydrolysis and to the stimulation of PLD catalysed hydrolysis of PtdCho. Increased agonist stimulated PLD activity in ras transformed cells would then result in increased DAG levels potentiating PKC activity. Indeed, it has been suggested that elevated DAG levels may provide a continuous internal growth signal in transformed cells (Preiss et al., 1986). It may, therefore, be possible that in the ras transformed cells, $PGF_{2\alpha}$ does indeed promote an amplified inositol phosphate response compared to NIH-3T3 cells but only when in early G_1. However, the consequent enhanced DAG levels would cause increased PKC activity resulting in the very rapid desensitization of the agonist stimulated response. Enhanced PKC activity may also lead to enhanced PLD activity resulting in the 2-fold increase in $PGF_{2\alpha}$-stimulated Cho levels observed in ras transformed cells compared to control cells (Table 5.2). It may also be possible that $p21^{ras}$ functions in a G-protein-like manner coupling certain receptors to PLD which would result in increased DAG generation and thus increased PKC activity. This could conceivably lead to the desensitization of the $PGF_{2\alpha}$-stimulated inositol phosphate response in ras transformed cells.

PLD catalyses the cleavage of PtdCho to Cho and phosphatidic acid. Given that Cho is such an abundant metabolite, it is unlikely to possess any second messenger-like functions. However, the phosphatidic acid produced could have many roles. Putney et al. (1980) proposed that phosphatidic acid could function as a Ca^{2+} ionophore and may therefore be involved in growth

factor-stimulated Ca^{2+} entry into cells. It has also been demonstrated that phosphatidic acid itself can act as a growth factor stimulating DNA synthesis in A431 carcinoma cells (Moolenaar et al., 1986). Phosphatidic acid is dephosphorylated to DAG due to the ubiquitous presence of phosphatidic acid-phosphohydrolase. The importance of this enzyme has been demonstrated by Billah et al. (1989). These authors showed that incubation of fMet-Leu-Phe-stimulated human neutrophils with the phosphohydrolase inhibitor, propranolol, resulted in a decrease in the concentration of DAG and an increase in the concentration of phosphatidic acid. In rat hepatocytes, vasopressin which activates PLD (Bocckino et al., 1987) causes both translocation and activation of the phosphohydrolase (Pollard & Brindley, 1984). These events may be relevant to the elevation of DAG and activation of PKC in long term responses of cells to stimuli such as growth factors. PLD could play an important role in the physiological regulation of agonist-induced events giving rise to sustained elevated levels of DAG. These elevated levels of DAG could then activate PKC over the 15h period required for the progression of 3T3 cells through to the initiation of DNA synthesis. In addition the DAG generated from PtdCho may have a different acyl chain structure from that generated from the inositol phospholipids. Given that the fatty acid composition of DAG may influence its ability to activate PKC, coupled with the fact that different isoforms of PKC may be present, opportunities therefore exist for both the complex regulation of PKC and hence initiation of a series of diverse intracellular responses.

6.2 Activation of inositol phospholipid breakdown by $PGF_{2\alpha}$ without any stimulation of proliferation in quiescent NIH-3T3 fibroblasts

In Chapter 3 stimulated changes in total inositol phosphates were determined. It was therefore important to demonstrate that $PGF_{2\alpha}$ induced a characteristic inositol phosphate response as observed in response to other agonists in other cell types. As $Ins(1,4,5)P_3$ is the second messenger, it was important to obtain conclusive evidence that it is generated following stimulation with $PGF_{2\alpha}$. The results in Chapter 4 clearly demonstrate that in NIH-3T3 cells $PGF_{2\alpha}$ stimulates the hydrolysis of $PtdIns(4,5)P_2$ generating $Ins(1,4,5)P_3$. The second messenger is generated rapidly (within 5 seconds) and is rapidly metabolised. Lloyd *et al.* (1989) have shown that stimulation of these cells with a maximal $PGF_{2\alpha}$ concentration results in an increase in intracellular free Ca^{2+}. The effect of the prostaglandin upon inositol phospholipid metabolism are thus similar to those observed in response to bradykinin in NG115-401L cells (Jackson *et al.*, 1987) and A431 carcinoma cells (Tilly *et al.*, 1987). Whilst stimulation of NIH-3T3 cells with $PGF_{2\alpha}$ induced $PtdIns(4,5)P_2$ breakdown, it was without effect upon DNA synthesis in NIH-3T3 cells. Yet $PGF_{2\alpha}$ has been reported to be a potent growth factor for both NIH-3T3 (Yu *et al.*, 1988) and Swiss 3T3 cells (MacPhee *et al.*, 1984). The results in Chapter 4 confirm that the prostaglandin is indeed a growth factor for Swiss 3T3 cells and for the clone of NIH-3T3 cells used by Yu *et al.* (1988). No major differences were observed in the stimulation of inositol phosphates by $PGF_{2\alpha}$ between the two clones of NIH-3T3 cells, nor were there any differences detected in Ka, Kd or $PGF_{2\alpha}$ receptor number. Differences in responsiveness to other defined agonists were

detected, most noticeably to bombesin. However, this may reflect a different receptor number. Significantly, there is no difference in the response to calf serum which is equally effective in stimulating the proliferation of both cell types. NIH-3T3 cells demonstrate the same rank order for the effectiveness of calf serum, PDGF and bombesin in stimulating inositol phosphates and [^{3}H]thymidine incorporation, yet $PGF_{2\alpha}$ which stimulated a 12-fold increase in inositol phosphate generation did not stimulate [^{3}H]thymidine incorporation.

The results in Chapter 4, therefore, place a question upon the role of inositol phospholipid metabolism in growth factor stimulated cell proliferation. Taylor et al. (1988) have found that the proliferation of Swiss 3T3 cells can be inhibited by the addition of pertussis toxin. However, in this cell the toxin has no effect upon the stimulation of inositol phosphate generation. Tones et al. (1988) have found no evidence for a role of inositol phospholipid turnover in the proliferative response to serum in CHO-K1 cells. However, when these cells were stimulated with serum a 20-fold increase in PtdCho synthesis was observed. On addition of serum the concentration of phosphatidic acid increased rapidly, but at 3-4.5h after serum addition the levels of phosphatidic acid declined; at this time the cells were estimated to be in mid-G1 and the decrease in phosphatidic acid levels persisted throughout S and G2 phase. It is possible that the phosphatidic acid is hydrolysed to provide DAG for the activation of PKC. These results suggest the existence of an alternative growth factor-signalling pathway which is independent of PtdIns(4,5)P_2 hydrolysis. However, in NIH-3T3 cells $PGF_{2\alpha}$ stimulated both PtdIns(4,5)P_2 and PtdCho hydrolysis, yet is unable to induce proliferation in these cells. It would appear that the cells lack a mechanism distal to the $PGF_{2\alpha}$ receptor, related to cell proliferation.

Therefore, it appears that whilst mitogens can activate the breakdown of inositol phospholipids, it is either, not obligatory for the onset of mitosis, or in itself not a complete signal. Consequently, some additional signal must be being generated in the AmNIH-3T3 and Swiss 3T3 cells, but not in the NIH-3T3 cells, in response to $PGF_{2\alpha}$, either in early G_1 or at a later stage in the cell cycle, which is required for the onset of DNA synthesis and mitosis. The identity of this signal, however remains unclear.

6.3 Conclusion

Although much available evidence indicates a role for $p21^{ras}$ in the function of cellular growth factors and mitogens, high levels of $p21^{ras}$ have been found in non-dividing tissues. Spandidos and Dimitrov (1985) used an immunoblotting method to examine ras protein expression in a variety of normal mouse tissues and demonstrated high levels of ras proteins in heart tissue as compared to lung, liver, spleen, kidney, brain and skeletal muscle tissues from the same animal. This indicates that cellular ras expression does not always correlate well with cell proliferation. The breakdown of $PtdIns(4,5)P_2$ may be a normal control reaction in non-proliferating cells and also a stimulus to proliferation. Possibly there is a threshold of signal intensity that has to be exceeded before proliferation is triggered in competent cells. Although $PGF_{2\alpha}$ stimulated a high level of inositol phosphates in NIH-3T3 cells there is no stimulation of mitogenesis. It is concluded that these cells lack a mechanism distal to the $PGF_{2\alpha}$ receptor, related to cell proliferation.

However, there is a clear association between tumour cell growth and expression of ras oncogenes and around 10-20% of all human cancers have been shown to contain ras genes with the majority of these being mutated. Approximately 1% have been detected as

having amplified normal cellular genes. Thus it is likely that a greater understanding of what function ras gene products serve in the processes of signal transduction by growth factors will also be a major step forward in understanding the normal control mechanisms in this system and the molecular events that convert normal cells to tumours. The presence of ras oncogenes in at least 10% of human tumours certainly justifies the intense research dedicated to this gene family during the last decade.

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