The effects of p21 $^{N\text{-}ras}$ overexpression on the proliferation and transformation of NIH3T3 cells and its role in cellular signalling.

Thesis presented for
the degree of
Doctor of Philosophy

by
Shireen-Anne Davies
Department of Biochemistry
University of Glasgow

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgements

The three years spent on this project have rushed past at an astonishing speed, not least because of the general good humour and deviant behaviour of my colleagues. I would like to extend my thanks to many people and will endeavour to include them all. In particular, I would like to thank Sue Palmer who has been a wonderful friend and supportive scientist during this writing period and also for the last two and a half years. Cheers must also go to Trevor Lakey (no longer a scientist), Brian Lavan, Greg Murphy, Simon Cook and Nigel Pyne for being friends, conspirators and perpetrators, not necessarily in any order, in Glasgow. I would also like to thank Sandra Gardner, Fiona Black, Caroline Evans, Mark Saville and Fiona Mitchell for various edibles and other assorted examples of kindness and support during the writing of this thesis and while in the lab. Finally, I would like to thank Fergus McKenzie and Ian Mullaney for help and suggestions during brief spells of G protein dabbling on my part.

My supervisors, Michael Wakelam and Miles Houslay, receive my thanks for their help, support, constructive scientific suggestions and the provision of departmental facilities during this PhD project and for the writing of this thesis. Also, thanks must go to them for annual Christmas lunches and the Dollar Glen Spree. I would like to thank Graeme Milligan for healthily cynical discussions, constructive career guidance and party attendance during the course of this PhD.

Also, I would like to extend my thanks to my family for their support, material and otherwise, without which this PhD thesis would not exist. Finally, I must thank Fraser for his unqualified support and affection during this writing period, his taxi service to and from the University at all times of day and night and his patience and tolerance during my insufferable phases.

Contents **Page**

Contents **Page**

List of figures

List of Tables

Abbreviations

All abbreviations used are recommended by the Biochemical Journal including the following:

cyclic AMP	adenosine 3', 5' cyclic-monophosphate
DAG	sn 1,2-diacylglycerol
EGF	Epidermal Growth Factor
GPI	Glycerophosphatidylinositol
GPIP	Glycerophosphatidylinositol monophosphate
$GPIP_2$	Glycerophosphatidylinositol bisphosphate
GRP	Gastrin-releasing peptide
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
PDGF	Platelet derived growth factor
PGF2α	Prostaglandin F2α
PtdIns 4,5 P_2	Phosphatidylinositol 4,5 bisphosphate
PK C	Protein kinase C
TGF	Transforming growth factor

Summary

The ras oncogene family has been implicated in the growth and development of a high percentage of human tumours. However, the biochemical role of p21 ras, the protein encoded by this oncogene, is not well defined. Therefore, the effects of p21 ras transformation of murine fibroblasts on cellular signal transduction were investigated. The study utilised a clone of NIH3T3 cells which overexpressed the p21 N-ras proto-oncogene in the presence of a glucocorticoid inducer. Overexpression of p21 N-ras in this cell line results in uncontrolled cell proliferation and transformation. The effects of transformation on growth factor mediated signal transduction pathways were studied.

Data obtained from these studies indicate that p21 N-ras overexpression may have a direct role in cell proliferation by causing the amplification of growth factor stimulated inositol phosphate production without a change in the basal levels of inositol phosphates. Bombesin stimulated inositol phosphate production in p21 N-ras transformed NIH3T3 cells occurs as a consequence of receptor mediated phosphoinositide specific phospholipase C activation. This results in the production of inositol 1,4,5 trisphosphate which acts as a calcium mobilising second messenger. This effect of p21 N-ras overexpression occurs at the post-receptor level, does not involve the guanine nucleotide binding proteins of the adenylate cyclase system and suggests that p21 N-ras may act as a coupling protein to mediate receptor activated phosphoinositide signal transduction in this cell line. Therefore, this evidence demonstrates that overexpressed p21 N-ras directly causes an amplification in growth factor stimulated inositol phosphate production which may act as an initial signal for cell proliferation.

This study also indicates that p21 N-ras overexpression results in reduced intracellular levels of cyclic AMP which are associated with rapidly proliferating cells. However, it is probable that this effect of p21 N-ras on the adenylate cyclase system is indirect and a consequence of protein kinase

C activation upon p21 $^{N\text{-}\underline{ras}}$ overexpression.

The results from this study are consistent with some reports in the literature on the function of p21 $\underline{ras}$ in cell proliferation and transformation. It appears that p21 $^{N\text{-}\underline{ras}}$ overexpression may induce rapid proliferation and consequent transformation of murine fibroblasts by the modulation of two major signal transduction pathways in this cell line.

Chapter 1: Introduction

1. Malignant transformation and the cancer cell

A cancer cell is defined by certain characteristics that make it distinct from normal cells. Some of these are the morphological transformation of the cells and their ability to proliferate rapidly, their invasion of other surrounding tissue and their non-differentiated state. Some of these characteristics are associated with cells in the early stages of embryogenesis and are only transiently expressed. The development of cancer then, may be associated with a loss of control over key events which influence normal cell proliferation.

The development of a transformed cell and therefore, cancer, is now recognised to be a multistage process involving genetic lesions and the deregulation of normal growth control pathways. (Spandidos, 1986; Ohlsson and Pfeiffer-Ohlsson, 1987)

A. Transformation as an effect of genetic changes.

The genetic changes associated with cellular transformation have been studied in detail (Marshall, 1985; Bishop,1985) and constitute a major cause of cancer development.

Mutation of certain genes can cause cellular transformation as has been shown with the Harvey-ras-1 oncogene in the T24 human bladder carcinoma cell line (Tabin et al, 1982). Point mutations within this gene sequence leading to a single amino acid substitution in the gene product can cause activation of the encoded protein resulting in cellular transformation. It has also been shown that amplification of several gene families including myc, myb and erb-B have been found in several tumour cell lines and in tumour tissue isolated from cancer patients (Alitalo et al, 1985). Other genetic changes include chromosome translocation, where it occurs in Burkitt's lymphoma and transduction events by retroviral vectors (Alitalo, 1987). The transduction event occurs when a slow transforming virus

recombines with cellular proto-oncogenes resulting in an activated transduced oncogene. At least twenty different transforming oncogenes are a result of this type of genetic rearrangment, including v-src, v-ros, v-Kirsten-ras, v-Harvey-ras and v-erb-A.

B.Oncogenes and growth regulatory pathways.

An oncogene is defined as a sequence of DNA that will cause cellular transformation in vitro and will be tumoregenic in vivo. An activated oncogene may be a result of retroviral transduction, as already described above, ie a v-onc, or by other genetic mechanisms which include DNA hypermethylation resulting in a mutated cellular oncogene, c-onc (See Marshall, 1985). Oncogenes fall into distinct groups according to the location of the protein product in the cell. Table 1 shows some oncogene families and their protein products:

Table 1 Heldin et al (1987)

Oncogene	Protein	Cellular location	Function
sis	p28 sis B chain PDGF	Extracellular	Growth factor
src	pp60 src	Inner surface plasma membrane	Tyrosine kinase
erb-B	Truncated EGF receptor	Plasma membrane	Tyrosine kinase
ros	receptor?	Plasma membrane	Growth factor receptor and tyrosine kinase
fms	CSF-1 receptor	Plasma membrane	Growth factor receptor and tyrosine kinase
ras	p21 ras	Plasma membrane	?
mil	p71/p73 doublet	Cytoplasm	Serine/threonine kinase
myc	p58 c-myc	Nucleus	DNA binding
fos	p55 c-fos	Nucleus	DNA binding
myb	p75 c-myb	Nucleus	DNA binding
jun	p39 c-jun	Nucleus	Mammalian transcription factor AP-1, binds p55 c-fos

The effect of activated oncogenes on cellular transformation is mediated by either growth factor interactions at the cell surface or by events associated with the nucleus. It is possible that the cytoplasmic oncogene products of mos and raf also have functions in cell transformation but the functions of the protein products of these oncogenes have not been identified.

The sis oncogene transduced by the Simian Sarcoma Virus (SSV), v-sis and the cellular counterpart, c-sis both encode the B chain of PDGF. It has been shown that antibodies to PDGF will reverse morphological transformation to the normal phenotype (Johnsson et al, 1985) and that suramin, which blocks PDGF binding to its receptor (Williams et al, 1984) will also abolish transformation (Betsholtz et al, 1986). The sis oncogene thus acts directly to increase the rate of cell proliferation by its direct action as a growth factor.

In contrast, the v-erb-B oncogene encodes a truncated form of the EGF receptor which lacks the EGF binding and autophosphorylation domains. The tyrosine kinase domain is intact and seems essential to cell transformation (Livneh et al, 1986). The aberrant EGF receptor synthesised as a result of v-erb B expression may be constitutively activated as a result of the lack of autophosphorylation and EGF binding sites. The oncogenic effects must therefore be mediated solely through the tyrosine kinase activity of the receptor.

It is notable that a large number of oncogene families exert their effects via tyrosine phosphorylation, however, the major substrates for these kinases remain to be identified.

The expression of nuclear oncogenes, c-myc and c-fos have been implicated in the control of DNA synthesis as they bind to DNA at specific sites (Heldin et al, 1987). These oncogenes are also co-expressed with oncogene products at the plasma membrane. For example, c-myc and the ras oncogene are co-expressed in transformed primary and secondary rat

embryo cells (Land et al, 1983; Schwab et al, 1985). This indicates that oncogene cooperativity is a necessary event for cell transformation in some systems. Also, the expression of the EJ c-Ha-ras-1 oncogene lacks complete transforming ability when injected into normal NIH3T3 fibroblasts with a limited life span but can transform fibroblasts immortalised by carcinogens (Newbold and Overell, 1983). This indicates that the expression of this oncogene is not sufficient to transform these cells and that in carcinogen transformed cells there may be other oncogenes expressed which co-transform the cells when the c-Ha-ras oncogene is expressed.

Growth factor signalling pathways can also induce oncogene expression. It has been demonstrated that PDGF, EGF and FGF can induce c-myc and c-fos expression in fibroblasts. In the case of c-fos, this is mediated through a serum-responsive element (a DNA sequence) which binds a phosphoprotein, the serum-responsive factor (SRF) which then activates c-fos expression in response to serum (Treisman, 1986).The importance of coordinated oncogene expression between oncogene products at the cell surface and the nucleus in cell proliferation and transformation has been demonstrated in several experimental systems apart from the examples cited above. The existence of oncogene products as either growth factors or growth factor receptors indicates that the growth factor regulatory pathways are crucial in the development and maintenance of tumouregenesis both in vitro and in vivo and indicates the importance of tight regulation of these pathways in normal cell growth.

C.Regulation of growth factor pathways in transformation.

1.Mitogenic and growth inhibitory pathways

The central role of oncogene product involvement in growth factor pathways has already been described and in this section the regulation of these pathways and their effects on cancer cells will be discussed. It has been shown that some cancer cells are growth factor autonomous, that is, these cells do not require exogenous serum factors for mitogenesis (See Sporn et

al, 1985). This could be explained by either increased production of a growth factor or increased production of growth factor receptors or of both these events taking place together. To be growth factor autonomous, a cell would have to secrete particular growth factors which would then bind to specific receptors on the cell surface. This endogenous production of growth factors which then bind to the producer cells is termed autocrine secretion.

At least three growth factors, TGFα, PDGF and bombesin are secreted in an autocrine manner by cancer cells. In rodent cells transformed with Kirsten-sarcoma virus (p21 Ki-ras) there is autocrine secretion of TGFα (Anzano et al ,1985) which then binds to EGF receptors on the same cell. Also, ras transformed cells and sis transformed cells express PDGF-like molecules in an autocrine manner. The autocrine PDGF secreted by SSV transformed cells is due to the expression of the sis oncogene and the protein it encodes. The fact that T24 human carcinoma cells and p21 Ki-ras transformed cells also secrete PDGF (Bowen-Pope et al, 1984) implies that there must be some oncogenic cooperativity to induce transformation via growth factor effects.

Bombesin is a tetradecapeptide isolated from frog skin and its mammalian analogue is the gastrin releasing peptide. Both these polypeptides bind to the bombesin receptor and it has been demonstrated that bombesin-like peptides can function as autocrine growth factors in human small cell lung cancer (SCLC) cells. A monoclonal antibody to the C terminus of amphibian bombesin will block the binding of bombesin to its receptor in SCLC cells and inhibit clonal growth of these cells in vitro and prevent tumouregenesis in vivo (Cuttita et al, 1985).

Autocrine secretion can also be affected by a change in the number of high affinity receptors for a growth factor. Human cancers derived from the head and neck and the A431 cell line have very high levels of the EGF receptor (Sporn et al, 1985) and may explain part of the tumouregenic capacity of these cells. This may be one way in which cancer cells can have

accelerated growth rates at a fixed concentration of growth factor(s) as compared to normal cells where the receptor number(s) is limiting.

Growth factor autonomy may be conferred also by post-receptor mechanisms along the growth factor axis. The ras family of proteins have been thought to act as coupling proteins analogous to the G proteins in the adenylate cyclase system. If this is so, then the ras protein could permanantly couple growth factor receptors to their effector systems without the need for exogenous growth factors or could permanently activate the effector systems without receptor occupation. So far, the mechanisms of growth factor pathways on transformation have been discussed in terms of positive growth factors that induce mitogenesis.There are autocrine systems that inhibit growth and these include TGFβ.

TGFβ has many effects depending on the cell type, but in some cell types it is an anti-proliferative factor. TGFβ has been shown to inhibit the proliferation of epithelial cells and functions in an inhibitory autocrine manner in the MCF-7 breast cancer cell line (Sporn et al 1987). It has been demonstrated that TGFβ inhibits the growth of NIH3T3 cells (Liboi et al, 1988) and this is associated with sustained c-fos expression. It is also known that fibroblast growth regulator (FGR) is an endogenous fibroblast product and will inhibit 3T3 cells growth in an autocrine manner (Wang et al, 1986).

The possibilitiy exists therefore, that oncogene expression in cancer cells can cause growth factor autonomy not only by increasing growth factors or their receptors by autocrine secretion, but may also suppress the responsiveness of the cell to inhibitory factors that would otherwise regulate cell growth. The mechanisms by which this latter process occurs may involve the down-regulation of the receptors for these inhibitory ligands, decreased synthesis or increased degradation of the inhibitors at the transcriptional and translational levels. There may also be a defect in the post-receptor coupling mechanisms in these growth inhibitory pathways.

2. The role of suppressor genes in transformation

Some oncogenes exert their effects on cell proliferation by modifying growth factor pathways to an extent that uncontrolled proliferation results. Studies on somatic cell hybrids between normal and transformed cells have demonstrated the existence of a family of genes that can suppress the neoplastic phenotype. Cell hybrids between tumouregenic HeLa cells and human diploid fibroblasts express the non-malignant phenotype, and from these studies, chromosomes 11 and 14 have been implicated in tumour suppression (Saxon et al, 1986).

It has also been shown that hybrids between ras-transformed murine, human and hamster fibroblasts with their non-transformed counterparts result in non-tumouregenic cells although there is no change in the levels of p21 ras in the hybrid cells as compared to the parental tumour cells. The possibility exists, then, that normal cells have suppressor genes that regulate the activity of the ras oncogene in vitro (Heldin et al ,1987).

The retinoblastoma gene (Rb gene) has been implicated in the suppression of cell proliferation as inherited deletion of the gene has led to specific tumours, retinoblastomas and osteosarcomas. The suppressive function of the Rb gene product can be removed by binding to transforming DNA virus encoded proteins (Whyte et al, 1988). The ability of the adenovirus or SV40 virus to transform cells may depend partly on the binding of the Rb gene product which abolishes its function.

The role of suppressor genes in transformation has been clearly established although the mechanisms of function are unclear. It may be that suppressor genes inhibit transformation by a direct effect on specific DNA regions, either to activate or inhibit transcription. It is not known if these events (if they occur) affect growth factor pathways in the cell, but hypothetically, controlled cell proliferation is probably restored to transformed cells by modifying responses to activating and inhibitory growth factors.

2.The Cell Cycle in relation to cell proliferation.

The cell cycle describes the distinct phases which occur during cell proliferation. These phases of the cell cycle are G_1, S, G_2 and M phase. The G_0 phase is occupied by dormant cells. S phase is the phase in which DNA synthesis takes place and M phase is where cell mitotic division occurs. The phases G_0-G_1 and G_2 are phases of the cell cycle that are not clearly defined in terms of associated events except that it is known that there are important control points in the cell cycle within these phases.

It is known however, that cells in vitro will become quiescent in the absence of growth factors when they have reached confluence at the G_0-G_1 phase. If serum growth factors are replaced in the medium of these cells, they will enter S phase *i.e.* cell proliferation occurs. Growth factor stimulation of cells in the G_0-G_1 phase is probably the most important signal for the initiation of cell growth although other agents, for example cell permeability modulators, cyclic nucleotide elevating agents, microtubule displacing agents and tumour promoters can stimulate DNA synthesis in fibroblasts (Rozengurt, 1985).

Cell cycle progression has been shown to be a pH dependent process and that an increase in intracellular pH is necessary for DNA synthesis to occur. Musgrove et al (1987) have demonstrated in human melanoma PMC 22 cells that pH values of approximately 7.5 are associated with the S, G_2 and M phases of the cell cycle but a lower pH of 7.2 is associated with G_1. When cells are quiescent, they arrest in the G_0-G_1 phase and this occurs at an extracellular pH of between 6.8-6.3 and it seems that a specific extracellular pH-dependent restriction point exists in the G_1 phase. Therefore, there is an intracellular pH variation associated with exponentially growing cells.

It is not known however, if the high intracellular pH value associated with cell progression through the cell cycle is a regulatory signal or if it is coincidental with the process of cell proliferation. Recently, Perona and Serrano (1988) have demonstrated that the expression of the gene for yeast plasma membrane H^+/ATPase in fibroblast cells causes a sustained

increase in intracellular pH. This had a proliferative and tumouregenic effect on the cells and is the first demonstration that sustained intracellular alkalinity directly influences cell proliferation. This experimental technique by-passes the requirement for growth factor stimulation of the cells and therefore, a clear demonstration of the direct effects of increased intracellular pH can be achieved.

The events that induce cells from the G_0-G_1 phase into S phase are growth factor associated and involve the increase in cytosolic Ca^{2+} and the activation of protein kinase C. These effects of growth factors will be discussed in the following section.

3.The role of second messengers in cell proliferation

A.The inositol phospholipid signalling pathway

Acetylcholine stimulated inositol phospholipid metabolism in pancreatic acinar cells was first shown by Hokin and Hokin in 1953. Since then, it has been demonstrated that inositol phospholipid metabolism is the signalling pathway activated by various mitogenic and non-mitogenic stimuli in different cell types (Berridge and Irvine, 1984). The transduction of extracellular signals at the plasma membrane occurs via this signalling pathway by the generation of two second messengers, inositol 1,4,5 trisphosphate (Ins1,4,5 P_3) and sn-1,2 diacylglycerol (DAG).

Phosphatidylinositol is phosphorylated at the 4 position on the inositol ring to yield phosphatidylinositol 4 phosphate, a distinct kinase then phosphorylates this molecule on position 5 on the inositol ring to give phosphatidylinositol 4,5 bisphosphate (PtdIns 4,5 P_2) which acts as a substrate for the receptor mechanism. The inositol phospholipids have been proposed to exist in hormone sensitive and insensitive pools in the membrane (Monaco et al, 1983)with only a small percentage of the total inositolphospholipid existing in a hormone sensitive state.

However, such a notion of pool segregation is perhaps not widely accepted now.

1. Formation of Ins 1,4,5 P3 as a second messenger

The phosphodiesterase stimulated cleavage of PtdIns 4,5 P_2 resulting in the production of Ins 1,4,5 P_3 in iris smooth muscle and hepatocytes indicated that PtdIns 4,5 P_2 was the substrate for enzymatic production of Ins1,4,5 P_3 and DAG. The phosphodiesterase involved has been called phosphoinositidase, a phospholipase C (Berridge, 1987a).

The Ins 1,4,5 P_3 produced can be removed in two ways. It can either be dephosphorylated to form Ins 1,4 P_2 (Berridge, 1987a) or can be phosphorylated by Ins1,4,5 P_3 kinase to Ins 1,3,4,5 P_4 (Irvine et al, 1986). The amount of Ins 1,4,5 P_3 in a cell on agonist stimulation thus depends upon both the rates of phosphorylation and dephosphorylation

The role of Ins1,4,5 P_3 as a calcium mobiliser was proposed from initial observations that Ins1,4,5 P_3 production either coincided with or lagged a few seconds behind calcium dependent physiological processes in liver and GH_3 cells (Berridge and Irvine, 1984). This confirmed that the generation of Ins 1,4,5 P_3 was rapid enough to qualify it both as a second messenger and also as a calcium mobiliser.

Using permeabilised pancreatic acinar cells, Streb et al (1983) demonstrated that exogenously added Ins1,4,5 P_3 caused a release of Ca^{2+} from a vesicular store thought to be the smooth endoplasmic reticulum (SER). This has since been shown to occur in many other cell systems (Berridge, 1987). The specific binding of Ins1,4,5 P_3 to the SER causes a passive efflux of Ca^{2+} from the SER into the cytoplasm.Ins 1,4,5 P_3 has been shown to bind to a specific receptor on the SER (Spat et al, 1986) and it is clear that the vicinal 4 and 5 phosphate groups on the inositol ring are essential for receptor binding.In rat liver microsomal fractions, the release of Ca^{2+} from the SER in the presence or absence of Ins1,4,5 P_3 is increased in the presence of GTP (Dawson et al, 1986). However, Gill et al (1986) have shown that GTP directly activates Ca^{2+} release from the SER of permeabilised NIE-115 cells. This mechanism of Ca^{2+} release appears to

be distinct from the Ins 1,4,5 P_3 Ca^{2+} release mechanism and may constitute an as yet undefined system for stimulated Ca^{2+} release.

The increase in intracellular Ca^{2+} (Ca^{2+}_i) has been associated with secretory, mitogenic and metabolic processes and thus it is essential for the levels of Ca^{2+}_i to be maintained at or above threshold levels for these processes to occur. It has been proposed that Ins 1,4,5 P_3 also controls the re-filling of the SER from the extracellular space. The emptying of the SER Ca^{2+} pool itself is a signal for Ca^{2+} influx and will depend on the levels of Ins 1,4,5 P_3 in the cell (Putney, 1986).

The role of Ca^{2+} in cell proliferation has been determined from observations that rapid changes in Ca^{2+}_i is a feature of many mitogenic peptides. PDGF will stimulate an intracellular Ca^{2+} increase in Swiss 3T3 cells and this may constitute an early event in cellular mitogenesis. It has been shown that an Ins 1,4,5 P_3 induced Ca^{2+} efflux from intracellular stores occurs in permeabilised T cells and Ehrlich Ascites Tumour cells (Berridge, 1987b).

The Ca^{2+}_i increase seems crucial to the activation of the c-myc and c-fos genes (Coughlin et al, 1985) and may be the first signal for the onset of mitogenesis. Also, the onset of fertilisation in Xenopus oocytes is accompanied by a large increase in Ca^{2+}_i and this can be mimicked by microinjection of Ins 1,4,5 P_3 (Busa et al, 1985).

2. The DAG/Protein kinase C pathway

When PtdIns4,5P_2 has been enzymatically cleaved it yields both Ins 1,4,5 P_3 and an additional second messenger, DAG, which also has several effects. Firstly, DAG can be acted upon by DAG lipase to generate arachidonic acid and monoglyceride. Arachidonic acid can feed into the prostaglandin and leukotriene pathways and this can have effects on DNA synthesis through autocrine secretion which can, following receptor stimulation, result in Ca^{2+}_i and cAMP increases (Macphee et al, 1984 ; Rozengurt et al, 1983).

DAG which is generated in the membrane can activate protein kinase C (PK C) which is a serine and threonine specific kinase and phosphatidylserine dependent enzyme (Takai et al, 1979). The enzyme is also Ca^{2+} dependent and this demonstrates the interaction of the two arms of the phosphoinositide signalling pathway ie. the dual effects of Ca^{2+} and DAG on PK C. The activation of PK C is accompanied by a translocation of the enzyme from the cytosol to the membrane.

PK C has been shown to be the cellular receptor for tumour promoting phorbol esters (Castagna et al, 1982) and these agents can mimick the action of PK C. The action of tumour promoters to induce tumouregenesis may thus be to modulate DNA synthesis by activating PK C. The protein substrates for PK C include the EGF receptor (Hunter et al, 1984), vinculin, lipocortin and an 80kD substrate in the cytoplasm (Berridge,1987a). These events may influence growth factor pathways and lead to mitogenesis. PK C has also been shown to activate the Na^+/H^+ exchanger resulting in increased cytoplasmic pH. This increased cytoplasmic pH is a permissive event for cell proliferation and it has been shown that growth factor stimulation of cells such as Swiss 3T3 and A431 results in increased intracellular pH. This increase in intracellular pH is also associated with cell proliferation in the PMC-22 cell line (Musgrove et al, 1987). It has recently been demonstrated that multiple forms of PK C exist (Nishizuka, 1988) and it is proposed that their activation might be differentially regulated by different growth factors. The multiple forms of PK C may have a role in other second messenger pathways depending on the specific growth factor stimulus.

3. Interaction between Ins1,4,5 P3/Ca2+ and DAG/PK C pathways and their role in transformation

The cooperative effect of both the arms of the phosphoinositide signalling system has been shown by the use of Ca^{2+} ionophores and phorbol esters to activate cellular processes normally activated by an agonist (Berridge, 1987b). The effect of PK C on the Na^+/H^+ antiporter has

already been mentioned above. The Na^+/H^+ antiporter is a transmembrane protein and is amiloride sensitive. The activation of this antiporter system causes an intracellular alkalisation leading to an increased intracellular pH (pH_i). Increased pH_i has been associated with growth factor stimulation of nearly all cell types including such diverse cells as fibroblasts and lymphocytes in rodents and in humans. Growth factor stimulation of fibroblasts leads to an increased pH_i which precedes DNA synthesis and it is now believed that this increase in pH_i is necessary for DNA synthesis and therefore, mitosis. The mechanism of action of growth factors on the Na^+/H^+ antiporter is unclear but it is possible that growth factor mediated increased PK C activity results in a conformational change at the allosteric site of the Na^+/H^+ antiporter. This may cause an apparent increase in the affinity for cytoplasmic H^+ ions, which leads to increased alkalinity of the cytoplasm (L'Allemain et al, 1984; Moolenar et al, 1983).

The role of the Na^+/H^+ antiporter in uncontrolled cell proliferation has been demonstrated in Chinese hamster embryo fibroblasts (CHEF). Transformed CHEF cells exhibit a constantly elevated pH_i as compared to the non-transformed CHEF cells. This is due to sustained activation of the Na^+/H^+ antiporter and therefore, a direct role for this antiporter system in the development of tumouregenic cells has been postulated (Ober and Pardee, 1987).

It has been shown that PK C can modulate the activity of the Na^+/H^+ antiporter. The increase in pH_i on growth factor stimulation occurs on a time scale of minutes and it is possible that this increases in pH_i is dependent upon the generation of second messengers in response to growth factors. Thus, it is possible that the phosphorylation of the Na^+/H^+ antiporter by PK C is a necessary event before increased pH_i can occur.

The interaction between Ca^{2+} and PK C can be demonstrated by their effects on the Na^+/H^+ antiporter. It has been shown that the Na^+/H^+ antiporter is not affected directly by Ca^{2+} or Ca^{2+}/calmodulin changes (Mitsuhashi and Ives, 1988; Ives and Daniel, 1987; Ober and Pardee,

1987). However, growth factor stimulation of Swiss 3T3 cells causes an initial Ca^{2+} induced acidification of the cytoplasm (Ives and Daniel, 1987) which is then followed by an increase in pH_i due to the Na^+/H^+ antiporter. It has been also shown that reduced intracellular Ca^{2+} will reduce growth factor induced activation of the Na^+/H^+ antiporter in vascular smooth muscle cells (Mitsuhashi and Ives, 1988). These data together suggest that firstly, an increase in intracellular Ca^{2+} on growth factor stimulation is a necessary event which precedes increased pH_i. This may be a reflection of the fact that PK C is a Ca^{2+} dependent enzyme and it is only in the presence of sufficient Ca^{2+} will PK C activate the Na^+/H^+ antiporter via a phosphorylation reaction.

Secondly, the initial Ca^{2+} induced acidification of the cytoplasm may be an important modulator of Na^+/H^+ antiporter activity. The possible phosphorylation of the antiporter by PK C may then sensitise it to excess H^+ ions in the cytoplasm caused by the Ca^{2+} induced acidification. These processes may together lead to increased activity of the Na^+/H^+ antiporter resulting in increased PH_i.Thus, although Ca^{2+} does not play a direct role in controlling Na^+/H^+ activity, an increase in $Ca^{2+}{}_i$ is necessary for PK C induced activation of the antiporter.

It has been demonstrated that the growth factor induction of c-fos (Grinstein et al, 1988) and c-myc (Kaibuchi et al, 1986a) expression can be achieved using a phorbol ester or Ca^{2+} ionophore.This also suggsests the cooperativity between the two second messenger systems in the coordination of growth control processes.

The numerous reports that phorbol ester pre-treatment can inhibit growth factor induced inositol phosphate production (Berridge, 1987b) have suggested that the two pathways can also be antagonistic in effect. PK C has been thought to inhibit PtdIns 4,5 P_2 hydrolysis possibly by phosphorylation of the G protein and to activate Ca^{2+} pumps which reduce Ca^{2+}i in two distinct negative feedback loops. PK C has also been shown

to phosphorylate platelet Ins 1,4,5 P_3 5' phosphomonoesterase, increasing the enzyme activity and reducing the levels of Ins 1,4,5 P_3 in the cell (Connolly et al, 1986). This would have the effect of reducing the levels of the Ca^{2+} mobilising second messenger.

The effect of PK C in inhibiting stimulated inositolphospholipid metabolism may also be at the receptor level since phorbol esters inhibit bombesin induced inositol phosphate generation but not PDGF induced inositol phosphate generation in Swiss 3T3 cells (Sturani et al, 1987). This result suggests that PK C may phosphorylate the bombesin receptor but not the PDGF receptor, or that the two agonists activate distinct G proteins or phospholipase C's in the same cell.

Cell proliferation may be partly a consequence of the activation of the phosphoinositide signalling pathway which may be one of the key events in normal cell growth. In neoplastic cell growth,the expression of oncogenes that may be part of growth factor transducing mechanisms may result in excessive production of the two second messengers. This is consistent with several findings by different workers. Firstly, it has been shown in several ras expressing cell lines (Fleishman et al, 1986, Chiarugi et al, 1986 and Wakelam et al, 1986) that there is increased inositol phosphate metabolism. Microinjection of Ha-ras into Xenopus oocytes results in increased DAG levels (Lacal et al, 1987a) and ras, src and fms transformed cells show increased levels of DAG (Wolfman et al, 1987). Other transformed cells have the capacity to grow in low Ca^{2+} medium compared to normal cells suggesting that they have high Ca^{2+}_i levels (Berridge, 1987b). Finally, Housey et al (1988) have shown that overproduction of the β1 form of PK C in Rat 6 cells leads to neoplastic growth in culture.

It may be then, that the aberrant growth and transformation caused by oncogene expression could be due in part to modulation of the phosphoinositide signalling system by the protein products of certain oncogenes. The sustained activation of the pathway may be sufficient to

induce DNA synthesis and uncontrolled growth. It is interesting that work by Wolfman et al (1987), whilst showing elevated levels of DG in ras, src and fms transformed fibroblasts also demonstrated that PK C is downregulated in the transformed cells. It is not clear what the mechanism of this downregulation might be, although Young et al, (1987), have suggested that phorbol ester induced downregulation of PK C in rat glioma cells is due to increased degradation of PK C and therefore, increased DAG production in this cell line may promote PK C degradation.

The downregulation of PK C in transformed cells seems to antagonise the effects of growth pathway activation and is an interesting puzzle to further complicate the unresolved issue of how oncogenes exert their effects on the phosphoinositide signalling pathway.

B. Modulation of second messengers by G proteins

Guanine nucleotide-binding proteins (G proteins) were first discovered to couple adenylate cyclase activity to hormone receptors (See Gilman, 1984). The adenylate cyclase enzyme, which converts ATP to cAMP is a major component in the cAMP signal transduction pathway. cAMP has many effects in the cell,among them the stimulation of cAMP-dependent protein kinases, cAMP-dependent phosphodiesterases and to regulate cell growth.

The two major classes of G proteins known to interact with the adenylate cyclase system are G_s and G_i. These G proteins are composed of an alpha, beta and gamma subunit heterotrimer. Activation of the G protein by GTP binding in exchange for GDP causes the dissociation of the complex to the alpha subunit and the beta/gamma dimer. The alpha subunit posseses sites for GTP/GDP binding and for NAD-dependent ADP-ribosylation by bacterial toxins (Gilman, 1984).

Other characterised G proteins include $G_{t\alpha 1}$ and $G_{t\alpha 2}$ or transducin which activate cGMP phosphodiesterase in retinal rods and cones respectively. The heterogeneity between the different G proteins is due to

differences in the alpha subunit sequence and there are now known differences between subtypes of each G protein subclass (Casey and Gilman, 1988).

Whereas the regulatory G proteins in the adenylate cyclase and light transducing systems have been well characterised, there has only been circumstantial evidence for the existence of G protein modulation of the phosphoinositide signalling pathway. Gomperts (1983) demonstrated that guanine nucleotide analogues could promote histamine secretion in mast cells on addition of Ca^{2+}. Also, permeabilised platelets show stimulated DAG production in the presence of GTPγS, a non-hydrolysable analogue of GTP (Haslam and Davidson, 1984). It has since been demonstrated that guanine nucleotide analogues regulate the phosphoinositide activity in the absence and presence of receptor binding ligands in a variety of cell types (Cockcroft, 1987).

The current concept is that phosphoinositide activity is regulated by a stimulatory G protein (called G_p) although the identity and the structure of this protein remains unidentified.PtdIns 4,5P_2 hydrolysis and the generation of Ca^{2+} and DAG has been shown to be modified by bacterial toxins in some cell types. The phosphoinositide signalling pathway is inhibited by pertussis toxin in mast cells, macrophages,neutrophils and lymphocytes (Cockcroft, 1987). It is also inhibited in certain fibroblasts but only with certain stimulatory growth factors and not others (Hoshijima et al, 1988, Taylor, et al, 1988 and Paris and Pouyssegur, 1986).

However, polyphosphoinositide hydrolysis is not pertussis toxin sensitive in GH_3 cells (Martin et al, 1986) and this has also been shown in pancreas, liver, heart and adipocyte cells (Cockcroft, 1987).There has been a recent report of novel GTP-binding proteins in neutrophils which are not pertussis toxin sensitive (Bokoch et al, 1988). A novel cholera toxin sensitive G protein in pituitary clonal cells which regulate phosphoinositide signalling has been proposed by Low and Hughes (1987) since pre-treatment of these cells with cholera toxin inhibits the phosphoinositide

response to agonists. However, whilst similar effects of cholera toxin have been observed in L6 skeletal myoblasts, the effects have been demonstrated to be cyclic AMP mediated and to be a consequence of reduced agonist binding (Gardner et al, submitted for publication). It is clear, therefore, that the sensitivity of the phosphoinositide system to pertussis toxin is not an indicator of the identity of G_p in the same way as toxin sensitivity can be used as an index of G_i function. The hypotheses that G_i can function as G_p, or is G_p was recently outlined (Ui, 1986) although it would be surprising if the overlap of two such distinct second messenger systems would exist at the G protein level. The heterogeneity in what appears to be G_p in terms of pertussis and cholera toxin sensitivity is apparently confusing but with the advantages of cloning and monoclonal antibody techniques the identity of G_p may be resolved.

The existence of inhibitory G proteins in the phosphoinositide signalling pathway was suggested in a report by Journot et al (1987) who showed that dopamine inhibition of angiotensin and thyrotropin-releasing hormone in anterior pituitary cells could be abolished by pertussis toxin treatment. Also, Baudry et al (1986) have shown that N-methylaspartic acid and other acidic amino acids inhibit stimulated phosphoinositide metabolism in hippocampus slices.

Therefore, experimental evidence suggests that the activity of the phosphoinositide signalling system is controlled by G proteins and this is probably the mechanism by which 'fine-tuning' of the signalling system occurs.

4. The ras oncogene

Research into the ras oncogene family has been on-going for twenty years since the genes were first identified as the transforming principle of Harvey (Ha) and Kirsten (Ki) strains of rat sarcoma virus (Barbacid, 1987). The transforming genes have been identified as mutated cellular genes and have been detected in human tumours. Since the identification of ras genes in human tumours,

the occurance of ras genes has been estimated at between 5-40% of all human tumours with the majority of these being mutated genes. Approximately 1% have been detected as being amplified normal cellular genes (proto-oncogenes) (Barbacid,1987). There is also evidence of hypermethylation of ras genes in human tumours (Feinberg and Vogelstein, 1983). The ras gene family comprises Ha-ras-1, Ha-ras-2, Ki-ras-1, Ki-ras-2 and N-ras. Ras genes have been identified in mammals, birds, insects, molluscs, plants fungi and yeasts and the existence of ras-related genes including the ral gene (Chardin et al, 1986) have now been described.The genes code for very closely related proteins of 21kD molecular weight. The proteins are 189 amino acids long (188 in the case of Ki-ras-2) and are identical to each other for the first 85 amino acid residues between Ha-ras-1, Ki-ras-2 and N-ras.The next 80 amino acids are 85% homologous between different ras genes and the rest of the molecule contains a hypervariable sequence except for the last four amino acids where there is a site for palmitic acid acylation of the molecule, essential for membrane attachment. The acylation site is present in all members of the ras protein family and it has been proposed recently that an active acylation-deacylation cycle exists and that may play a role in the signal-transduction function of the molecule (Magee et al, 1987).

The p21ras protein is bound on the inner leaflet of the plasma membrane and displays intrinsic GTPase activity at a rate similar to other G proteins (Gibbs et al, 1985). There is also some sequence homology to the alpha subunits of G proteins, including transducin (Bourne and Sullivan, 1986). The phosphorylation of p21 ras by EGF has been demonstrated but has only been shown for the Ha-ras oncogene protein (Kamata and Feramisco, 1984). The physiological significance of this growth factor induced phosphorylation is unknown.

Mutations in the ras gene leading to gene activation and cell transformation are associated with a reduction in GTPase activity. Ras gene mutations at codons 12, 59 or 61 result in transforming ras proteins and the

GTPase activity in these mutants is severely impaired (Gibbs et al, 1985). Lacal et al (1986), in a series of elegant deletion experiments using p21 Ha-ras, showed that regions 5-23 and 152-165 were absolutely required for GTP binding and cell transformation. They also proposed that these regions were essential for the normal function of the ras proto-oncogene which has been found in all tissues with high levels in rapidly proliferating tissue, embryonic tissue and terminally differentiated tissue (Barbacid, 1987).

A cytoplasmic protein, GTPase activating protein (GAP) has been shown to stimulate normal p21ras GTPase activity but not oncogenic mutant p21ras (Trahey and McCormick, 1987). The GAP protein is thought to act at region 30 -40 of the ras protein although this interaction of GAP at this 'effector region' is attenuated in mutant p21ras (Cales et al, 1988) . The oncogenicity of mutant p21ras may be due to the conformational change induced at the mutation site which prevents GAP binding, thus leading to persistent GTP binding to the ras protein.

The role of p21ras in cell proliferation, transformation and differentiation has not been resolved yet although there has been intense research in this field over the last five years. Oncogenic p21ras has been shown to induce morphological differentiation of PC12 pheochromacytoma cells (Bar-Sagi and Feramisco, 1985) and the use of anti-ras antibody reverses this differentiation. This clearly implicates p21ras in the differentiation process normally induced by nerve growth factor (Hagag et al, 1986).

It is known that the ras protein plays an important role in regulating cell entry into the S-phase of mitosis (Mulcahey et al, 1985) and that mutant p21 Ha-ras expression results in intracellular alkalinisation and initiation of S-phase (Doppler et al, 1987). Other functions ascribed to p21 ras are the control of cell endocytosis and exocytosis (Bar-Sagi et al, 1987) and the ras induced phosphorylation of mitochondrial proteins (Backer et al, 1986). Also, expression of the ras protein is necessary for the c-myc induced transformation of NIH3T3 fibroblasts (Smith et al, 1986).

The main function ascribed to the ras protein is the involvement of p21ras in growth factor pathways. The mutated versions of the ras proto-oncogene may cause transformation by activating certain growth factor pathways and by suppressing inhibitory controls. It is known that transformation or differentiation in experimental systems can be induced with mutant p21ras but it requires larger amounts of normal p21ras to achieve the same effects. The mutated ras proteins may transform cells by providing a necessary 'limiting factor' for the constitutive activation of growth factor pathways.

The RAS yeast proteins, RAS1 and RAS2 are closely related to mammalian ras proteins and have been shown to regulate adenylate cyclase activity (See Results chapter 6). In a recent report, it has been suggested that RAS proteins may be involved in glucose-induced inositolphospholipid turnover in S. Cerevisiae (Kaibuchi et al, 1986b).

It is conceivable, then given the biochemical properties of p21ras, its cellular location and its involvement in mitogenesis, transformation and differentiation that p21ras may function as a major growth regulatory factor utilising second messenger systems.

The Aims of this Project

The occurance of the ras oncogene in human tumours has already been described although the role of p21ras is still not clear in spite of the research in this direction. The importance of ras gene amplification in tumours led to the possibility of investigating the role of normal cellular p21ras in transformation.The role of p21ras in regulating second messengers as part of a signal transduction mechanism has never been resolved and it was from this starting point that this project was conceived.

This project was undertaken to study the effects of p21 N-ras overexpression on the proliferation and transformation of the NIH3T3 cell line. A number of reports have shown the involvement of the ras oncogene

with the inositolphospholipid signalling pathway (See Chapter 3) and this was studied during this project. Also, it was necessary to define the effects (if any) of p21 $^{N\text{-}\underline{ras}}$ on the cAMP signalling pathway. There has been much speculation in this area but there have been no conclusions drawn (See Chapter 6). The system used to study the effects of p21 $^{N\text{-}\underline{ras}}$ in cell proliferation and transformation was the T15 cell line, an inducible N-ras expressing clone of the NIH3T3 cell line (See Chapter 3 for details).

The purpose, therefore, of the project was to try to provide additional clues to the function of the ras oncogene and even perhaps answer the question 'What does ras do?'

Chapter 2: Materials and Methods

1.Cell Culture Methods

a. Cell Culture

T15, NIH3T3 and N 866 cells were cultured in Dulbecco's Modification of Eagle's Medium supplemented with 100 units/ml of penicillin/streptomycin, 2mM glutamine , 0.375%(w/v) sodium hydrogen carbonate (referred to as DMEM) and 10% (v/v) donor newborn calf serum. T15- cells were grown in the absence of dexamethasone while T15+ cells were grown in medium containing 2μM dexamethasone which causes the induction of p21 $^{N\text{-}ras}$ expression in these cells. Sis 771 cells were grown in DMEM/5% (v/v) calf serum. The cells were grown at 37°C in a 5% CO_2/95% air, 95% humidity atmosphere.

b. Cell passage

Confluent cells (typically 10^6 cells per 25 cm^2 flask) were passaged using a trypsin solution containing 0.1% trypsin (w/v), 0.025% EDTA (w/v) and 10 mM glucose in phosphate buffered saline. The trypsinisation process was inhibited when the cells were 'rounded up' by the addition of 2 ml of 10% serum/DMEM and the cells washed off the bottom of the flasks. An additional 2 ml of 10% serum/DMEM was added to the flask to ensure that most of the cells were removed. The cells were collected after trypsinisation, centrifuged at 800 g in a MSE Centaur for 5 minutes to pellet the cells, resuspended in DMEM/10% calf serum and plated as required.

c.Maintenance of cell lines.

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

To plate out frozen cells, each frozen vial was thawed and resuspended in

1 ml of normal medium and the cells plated out in a final volume of 4 ml medium in a 25 cm^2 flask.

d. Preparation of inositol free calf serum

Preparation of inositol-free calf serum was necessary as this calf serum was used in experiments to label cell with tritiated inositol.

1. Preparation of dialysis tubing: The dialysis tubing was boiled in 20mM EDTA for 20 minutes and washed in distilled water. The tubing was either used immediately or stored in 20% (v/v) ethanol at 4°C.

2. Dialysis of calf serum: Calf serum was aliquoted into the dialylsis tubing in either 50 or 100 ml lots and dialysed against 4 changes of 10 times its volume of dialysis medium at 4°C. The dialysis medium consisted of a 1:19 (v/v) dilution of a 20x strength Earle's Salt solution (See Materials section) in distilled, deionised water adjusted to pH 7.4 with 1 M sodium hydroxide.The dialysed calf serum was filter sterilised, aliquoted into tubes and stored at -20°C.

e. Preparation of steroid-free calf serum

The following buffer was made as required for the preparation of steroid-free calf serum: 20% (v/v) 0.1 M HEPES, pH 7.4, 0.0075% (v/v) 0.2 M EDTA, pH 7.4 and 10% (v/v) glycerol in water.100 ml of this buffer was mixed with 500 mg charcoal and 5 mg dextran for 30 minutes at room temperature.This mixture was sufficient to treat 50 ml of calf serum. The charcoal suspension was aliquoted into 2 x 50 ml lots and centrtifuged in a MSE Centaur 2 at 800 g for 10 minutes to pellet the charcoal. 50 ml of serum was added to one of the charcoal aliquots, mixed and incubated at 56°C for 15 minutes. The tube was centrifuged as before, the serum was removed and added to the second charcoal aliquot. The charcaol and serum was mixed and incubated at 56°C for 15 minutes as before and the tube centrifuged to pellet the charcoal as previously described. The serum was removed, filter sterilised and stored at -20°C.

2. Tritiated thymidine incorporation

These experiments were performed to provide a measure of cell proliferation. To ensure that cells were synchronised, it was necessary to make them quiescent prior to the [^{3}H] thymidine incorporation experiments. NIH3T3 and T15 cells were plated out in 6 cm diameter petri dishes at a density of10^6 cells per ml in 10% calf serum/ 90% DMEM with dexamethasone as necessary. The next day, the medium was changed to 1% calf serum/99% DMEM and the cells left for 5 to 7 days to reach quiescence.

At this stage, the cells were labelled with 1μCi ml^{-1} [^{3}H] thymidine per dish in 10% calf serum/90% DMEM for the appropriate times. At these times, the cells were washed twice with 5 ml of Hanks Buffered Saline. 5 ml of 5% (w/v) trichloroacetic acid were added to each plate and the plate scraped and the contents removed to a 50 ml centrifuge tube. This was repeated 3 more times and the contents of the plate pooled each time, making a total of 20 ml in all. The tubes were spun in an MSE Centaur 2 at 2000 g for 5 minutes to pellet the acid-insoluble material which contained the [^{3}H] thymidine incorporated into DNA. The supernatant was removed and 5 ml of ethanol were added to the pellet and the pellet resuspended. The tubes were centrifuged as before for 10 minutes, the supernatant removed and the pellet solubilised in 1 ml of 0.3 M sodium hydroxide. 0.5 ml of this was added to 0.1 ml of 1.5 M hydrochloric acid in a scintillation vial and the radioactivity in the sample determined by scintillation counting in 6 ml of scintillation fluid.

3.Separation of inositol phospholipids

1.Sample preparation

Two confluent 25 cm^2 flasks of T15 and NIH3T3 cells were used for each time point. The cells were labelled for 16 hours prior to the experiment with 10μCi ml^{-1} of [^{3}H] inositol in inositol- and serum-free DMEM. After the labelling period, the cells were washed extensively with Hanks buffered saline, scraped and collected by centrifugation at 800 g for 5 minutes. The collected cells were resuspended in a final volume of 1.5 ml of Hank's saline with 1% BSA (w/v) and 10mM glucose.The cells were aliquoted in 0.5 ml volumes into plastic scintillation vials (triplicate determinations were made).The cells were killed with 500μl (20% v/v) ice-cold trichloroacetic acid and samples were kept on ice at all times after this stage.

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

2.The supernatant was discarded and the pellet washed with 1 ml ice-cold water.The samples were spun as above.

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

4. 0.33 ml chloroform : methanol : hydrochloric acid (200:100:1, v/v) was added to the remaining pellet from step 3. The tubes were mixed and spun and the supernatant was added to the supernatant from the previous wash and combined.

5. 0.5 ml chloroform and 0.37 ml 0.1M hydrochloric acid were added to the combined supernatants. The samples were mixed, allowed to phase split and the upper phase discarded.

6. 0.7 mls of Folch's Synthetic Upper Phase was added to each of the samples and the tubes mixed and spun.

7. The aqueous upper layer was discarded and the samples could be stored frozen at

this stage under nitrogen .

Folch Synthetic Phases :

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

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

These were mixed and left to stand in order to separate into two phases which were stored separately at room temperature.

2.Separation of the polyphosphoinositides

a.Hydrolysis of polyphosphoinositides to glycerophosphoinositol monophosphate (GPIP) and glycerophosphoinositol bisphosphate ($GPIP_2$)

The samples from step 7 above were dried down in a Hetovac centrifuge and the following were added: 0.5 ml chloroform, 100µl methanol, 100µl 1 M sodium hydroxide in methanol. The samples were mixed and left for 15 minutes to allow deacylation to proceed. The following were then added: 0.5 ml chloroform, 0.3 ml methanol, 0.3 ml water and the samples were mixed and centrifuged in a Hettich microfuge at 16 000 g for 5 minutes.

0.5 ml of the upper phase were transfered to clean tubes and the samples neutralised by the addition of 290µl of 0.1 M Boric acid. 150µl of 3 M ammonium formate and 125µl of 0.1 M sodium tetraborate were added to each sample and mixed. Then,1.5 ml of water were 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 GPIP and $GPIP_2$ from Dowex formate columns

The samples were applied to 1ml Dowex-1-formate columns and washed with 2 x17 ml of 0.18M ammonium formate/5mM sodium tetraborate. Following this, GPIP was eluted with 15 ml of 0.35 M ammonium formate/0.1 M formic acid and $GPIP_2$ was eluted with 20 ml of 1M ammonium formate/0.1 M formic acid.

The amount of radioactivity in each sample was determined upon a known volume of each fraction by liquid scintillation counting.

4. Determination of inositol phosphate production in T15 and NIH3T3 cells

Sample preparation

T15 and NIH3T3 cells were grown in 25 cm^2 flasks and labelled with 2μCi of [^{3}H] inositol in 10% calf serum/ 90% DMEM (v/v ratios) in a total of 3 ml per flask for a period of 48 hours or in 10% inositol free calf serum and 90% inositol free DMEM for 16 hours. The different labelling protocols employed did not affect the magnitude of the inositol phosphate response to agonists of the cells but the latter increased the amount of radioactivity in the samples.

After the labelling period, the cells were washed twice with Hanks Buffered Saline containing 1% BSA and 10mM glucose (HBG). The cells were scraped and collected by centrifugation in an MSE Centaur 2 centrifuge at 800 g for 5 minutes. The pellet was washed with HBG and spun as before.The cell pellet was resuspended in fresh HBG to a final volume containing approximately 5 x10^4 cells ml^{-1} and aliquoted into plastic scintillation vials containing the appropriate ligand or water, in the case of control samples, and 10mM lithium chloride solution in a final volume of 250 μl. The samples were incubated in a shaking waterbath at 37°C for the appropriate time period and reactions terminated by the addition of 0.94 ml chloroform : methanol (1:2, v/v). The tubes were left to stand for 10 minutes after mixing.

Extraction of inositol phosphates and inositol phospholipids

0.31 ml of water and 0.31 ml of chloroform were added to each sample and mixed. The tubes were centrifuged at 2000 g in a MSE Centaur 2 for 5 minutes to separate the aqueous and organic phases.

Inositol phosphate extraction

The aqueous layer contains the inositol phosphates which were batch eluted from Dowex formate. 0.8 ml of this layer was transferred to clean tubes and 2.2 ml water were added. 0.25 ml of Dowex formate suspension was added to each tube, vortexed and left until the Dowex had settled. The supernatant

was removed and 3 ml of 60 mM ammonium formate/5 mM sodium tetraborate were added to the samples and the supernatant aspirated off when the Dowex had settled. This step was repeated once more and then the resin was washed with 3 ml of water three times.

Inositol phosphates were eluted from the resin with 0.6 ml of 1M ammonium formate/0.1M formic acid. The tubes were vortexed and the Dowex left to settle. 0.5 ml of the supernatant were removed to a scintillation vial. 0.6 ml of 1M ammonium formate/0.1 formic acid were added to the Dowex and after mixing, 0.6 ml of the supernatant were added to the scintillation vial. This procedure was repeated once more, making a total of 1.7 ml in the scintillation vials from each sample. 12 ml of Ecoscint scintillation fluid was added to each vial and the radioactivity in each sample determined by scintillation counting.

Inositol phospholipid extraction

The lower phase contains the inositol phospholipids. 0.25 ml of this phase were dried down in scintillation vials and the radioactivity in each sample detemined by scintillation counting in 3 ml of Ecoscint.

5. Separation of inositol phosphates

T15+, T15- and NIH3T3 cells were plated in 24 well plates and grown to a density of 10^5 cells per well in DMEM/10% calf serum.16 hours prior to experimentation,the medium was changed to inositol-free DMEM containing 10μCi ml^{-1} of [^{3}H] inositol to label the inositol phospholipid pool. Following this incubation period,the cells were washed twice in Hanks buffered saline and then incubated at 37° C for 5 minutes in Hanks buffered saline containing 1%(w/v) BSA,10 mM glucose (HBG) and10 mM lithium chloride. Cells were stimulated with 2.5 μm bombesin in HBG containig 10 mM lithium chloride in a final volume of 25 μl for various times and incubations quenched with 50μl (10%,v/v) ice-cold perchloric acid.The wells were scraped with the addition of 50μl water, transfered to eppendorf tubes and the samples centrifuged at 16 000 g in a Hettich microfuge at 4°C. The supernatants were removed to eppendorf tubes and neutralised with 1.5 M potassium hydroxide /60 mM HEPES in the presence of universal indicator.The samples were centrifuged as before to pellet the precipated salt and the supernatants were added to 1 ml of 5 mM sodium tetraborate/0.5mM EDTA if the samples were run on Dowex columns,or if the samples were analysed by HPLC no sodium tetraborate/EDTA was added.

Separation of inositol phosphates from control and agonist stimulated cell extracts were carried out by two methods:

1.Separation on Dowex-formate1x 8-200 resin columns

2.HPLC using a Whatman Partisil 10 SAX column.

1.Separation of inositol phosphates using Dowex-formate resin

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

1. Inositol was eluted with 12 ml of water

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

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

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

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

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

2.Separation of inositol phosphates using HPLC

The column used was a Whatman Partisil 10 SAX and the gradient used was 0 - 1.7M ammonium formate adjusted to pH 3.7 with orthophosphoric acid (Irvine et al, 1985). The gradient was increased in a linear fashion from 0 to 43% ammonium formate solution over 19 minutes and held at this level for 2.3 minutes. The gradient was then increased to 69% ammonium formate over 7 minutes, held at this level for 6.8 minutes and then increased to 100% ammonium formate solution over 12.5 minutes. All column eluants were filtered through 0.45 micron Whatman filters before use. The 250µl sample was applied to the column at time = 0 and 0.25 ml fractions were collected. The flow rate was 1ml/min. The radioactivity in each sample was determined by liquid scintillation counting.

[^{3}H] labelled inositol phosphate standards were run on the column to ensure that resolution of the inositol phosphates could be achieved using this column and to define the mobility of the individual inositol phosphates. The elution of [^{3}H] labelled inositol phosphates from this column is shown in figure 2.1. Recoveries of the inositol phosphates were routinely between 60-85%.

Figure 2.1: Separation of [^{3}H] inositol phosphate standards by HPLC.

The figure shows the separation of inositol phosphate standards by HPLC using an ammonium formate gradient as previously described. The data was obtained from the resolution of [^{3}H] inositol phosphate standards eluted from a Partisil 10 SAX column using the gradient described and peaks of inositol 1 monophosphate, inositol 1,4 bisphosphate and inositol 1,4,5 trisphosphate are indicated. The dotted line indicates the elution gradient used.

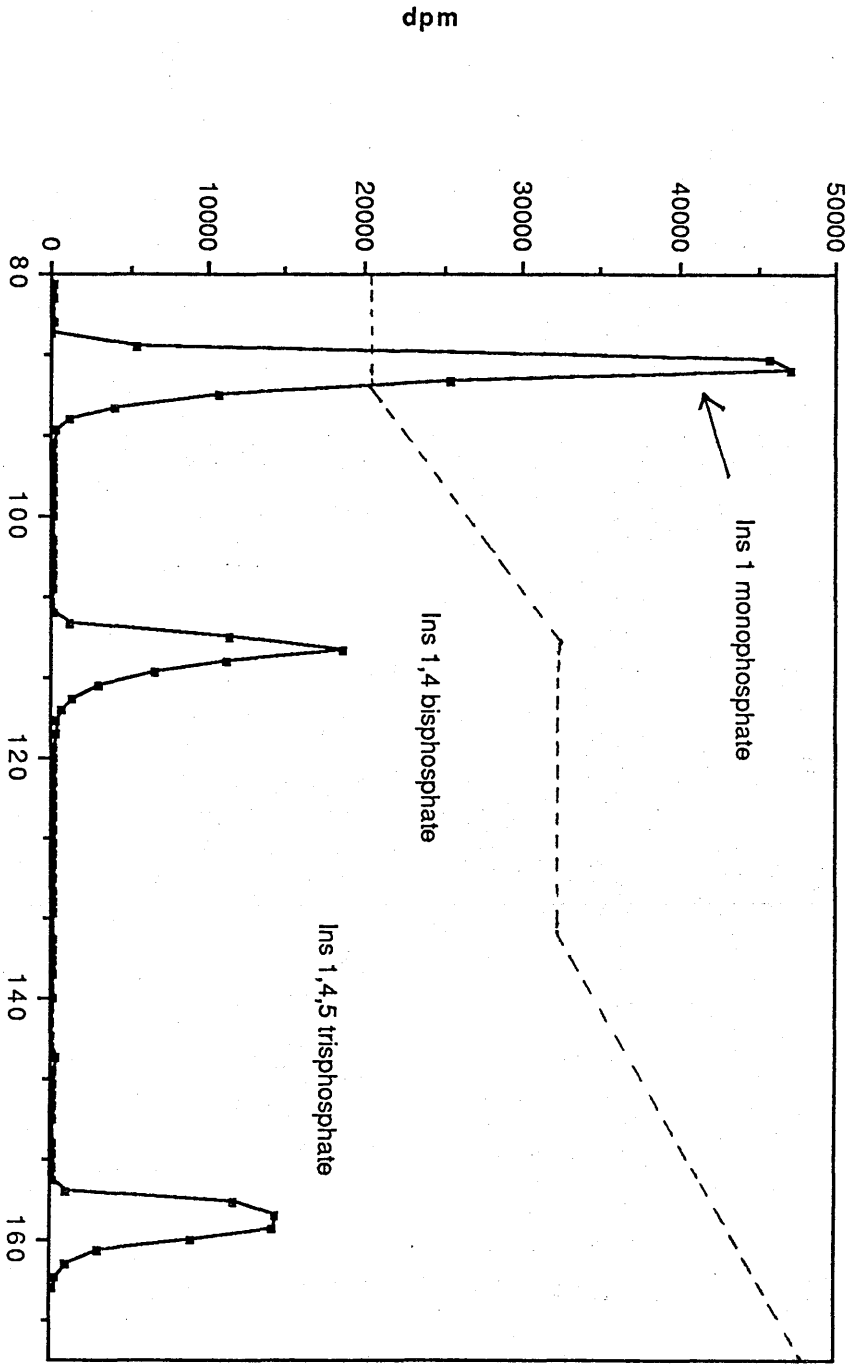

dpm
50000
40000
30000
20000
10000
0
80
100
120
140
160
fraction no.
Ins 1 monophosphate
Ins 1,4 bisphosphate
Ins 1,4,5 trisphosphate

Figure 2.1

6.Inositol 1,4,5 trisphosphate kinase assay

1.Preparation of enzyme extracts

Confluent 75 cm^2 flasks of T15+,T15-and NIH3T3 cells were washed and scraped in ice-cold PBS. The cells were collected by centrifugation at 800 g for 5 minutes. The cells were resuspended in fresh PBS and spun again.The cells were resuspended in a final volume of 1 ml (or less if a concentrated preparation was necessary) in 0.25 M sucrose/50 mM Tris/HCl buffer at pH 7.2.The cell pellet was homogenised using a teflon on glass homogeniser for 20 strokes on ice and then sonicated in a sonicating waterbath for 1 minute. The homogenate was spun at 100,000 g for 90 minutes at 4°C in a Beckman TL-100 Ultracentrifuge. The supernatant was retained and either assayed immediately or stored in 100 µl aliquots at -20°C.

2.Assay for Inositol 1, 3, 4 ,5 tetrakisphosphate formation

The high speed supernatant was incubated with the appropriate incubation buffer (see below),1- 2µCi of [^{3}H]-inositol 1,4,5 trisphosphate (Specific activity =1 or 44 Ci $mmol^{-1}$) and various concentrations of inositol 1,4,5 trisphosphate in a final volume of 150µl at 30°C for 5 minutes. The reactions were terminated by the addition of 50µl of perchloric acid (10%, v/v) and neutralised with 1.5 M potassium hydroxide/60 mM HEPES containing universal indicator. Samples were then centrifuged to remove precipitated salt as previously described.The samples were then either applied to a Partisil SAX HPLC column (as already described under 'Separation of inositol phosphates'), or, following the addition of 1 ml of 5mM sodium tetraborate/0.5 mM EDTA pH 7.2, were applied to 1 ml Dowex formate columns .The inositol phosphates were eluted from the columns as already described. In these assays, the amount of inositol monophosphate and inositol bisphosphate generated was minimal, and was the result of dephosphphorylation of the

[^{3}H] inositol 1,4,5 trisphosphate. Inositol tetrakisphosphate was eluted from columns with 15 ml 1.2 M ammonium formate/0.1 M formic acid. (See figure 2.2 for resolution of Ins 1,4,5 P_3 and Ins 1,3,4,5 P_4 standards by Dowex anion exchange chromatography.) The amount of radioactivity in each sample was determined from an aliquot of the 1.2 M ammonium formate fraction by liquid scintillation counting.

Incubation buffer 1 (based on Irvine et al, (1986))

0.25 M sucrose,20 mM magnesium chloride, 10 mM disodium ATP, 50 mM Tris adjusted to pH 9 with 1M sodium hydroxide.At this pH the activity of the IP_3 phosphatase is inhibited whilst the Ins 1,4,5 P_3 kinase remains active (See Results chapter 5) thus allowing the conversion of the Ins 1,4,5 trisphosphate substrate to Ins 1,3,4,5 tetrakisphosphate. When this buffer was used at pH 7, 2,3-diphosphoglycerate was added to the incubations at a final concentration of 2 mM to inhibit the activity of the IP_3 5'-monophosphatase (See results chapter 5).

Incubation buffer 2 (based on Harden et al, (1987))

The buffer in these experiments contained 10 mM ATP, 12 mM free Mg^{2+} and 120 nM free Ca^{2+}(based on the calcium measurements of Alison Lloyd) The buffer was: 0.02 M EDTA, 0.01 M disodium ATP, 0.376 mM calcium chloride, 22.34 mM magnesium sulphate, 115 mM potassium chloride, 5 mM potassium dihydrogen phosphate and10 mM HEPES. The buffer was used either at pH 9 or if used at pH 7, 2,3-diphosphoglyceriate was added to the incubations as described above.

Figure 2.2: Resolution of [^{3}H] inositol 1,4,5 trisphosphate and inositol 1,3,4,5 tetrakisphosphate standards by Dowex anion exchange chromatography.

Figure 2.2a indicates the elution of a tritiated inositol 1,4,5 trisphosphate standard from Dowex formate columns using 16 ml of 0.8 M ammonium formate/0.1 M formic acid followed by 16 ml 1.2 M ammonium formate/0.1 M formic acid applied in 2 ml aliquots. Figure 2.2b shows a similar experiment to determine the elution profile of tritiated inositol 1,3,4,5 tetrakisphosphate standard performed under the same conditions as experiment 2a. The figures indicate that there is clear resolution of inositol 1,4,5 trisphosphate and inositol 1,3,4,5 tetrakisphosphate using this separation procedure.

Figure 2.2a

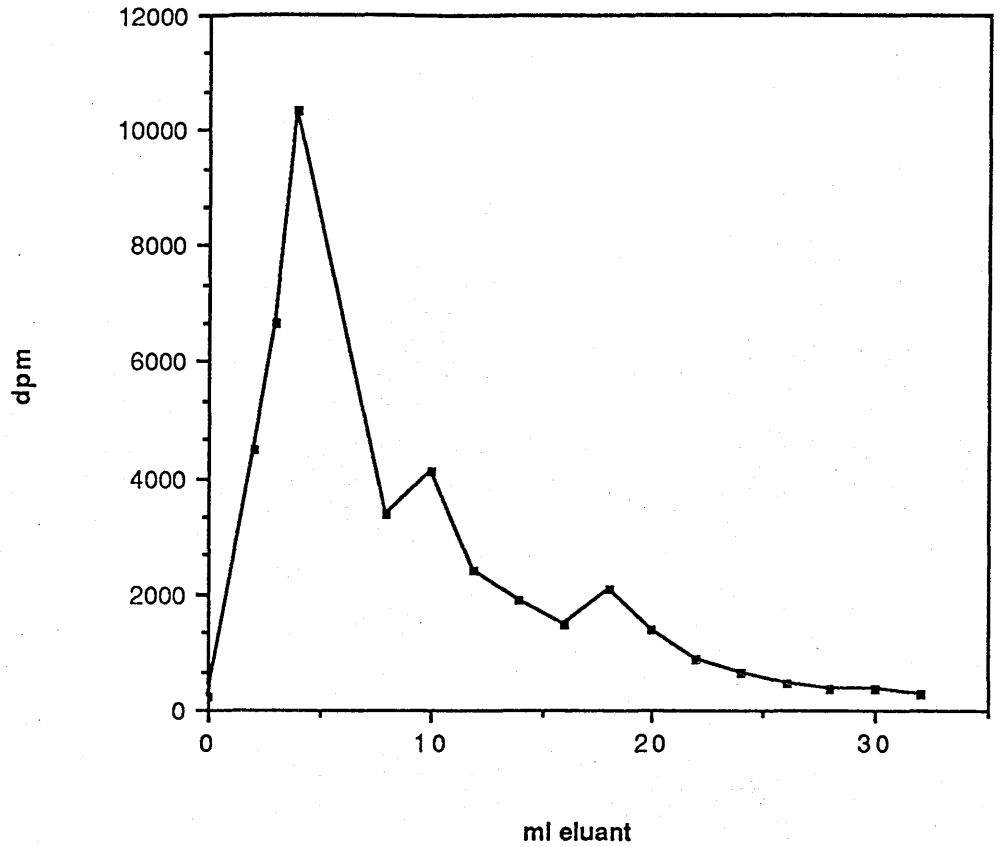
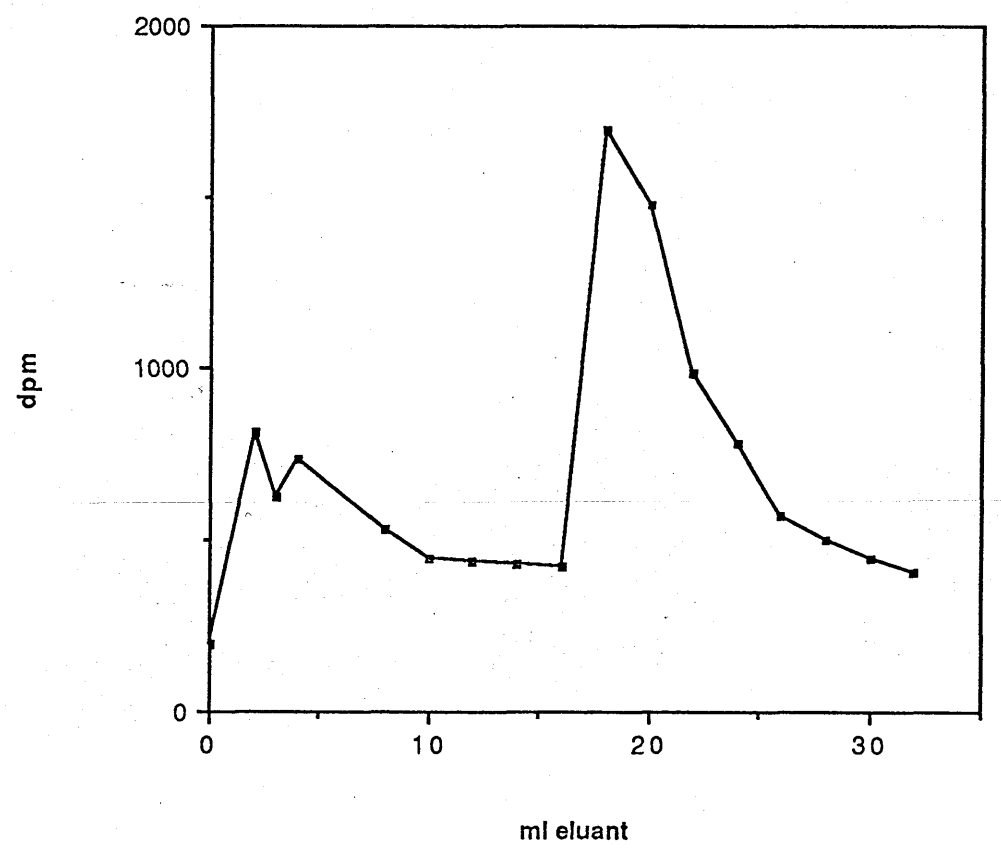


Figure 2.2b



7. Whole cell cyclic AMP determinations

a.Sample preparation

T15, N 866, NIH3T3 and sis 771 cells were grown as already described and plated in 24 well plates. T15- were cultured routinely until two days prior to experimentation, when the medium was changed to 10 % (v/v) steroid-free calf serum/90 % DMEM. This method was adopted to minimise the frequency of spontaneous transformation of T15- cells due to steroids in the growth medium.

The cells were used at a density of 10^5 per well as determined using a haemocytometer.The cells were labelled with 2μCi of [^{3}H] adenine in serum-free DMEM for 90 minutes when maximum adenine incorporation into the acid soluble fraction was obtained (Figure 2.3). After the labelling period, the cells were washed extensively with PBS at 37°C and then stimulated with the appropriate ligand in a final volume of 100-500μl of DMEM at 37°C for 10 minutes in the presence of 1mM isobutylmethylxanthine (IBMX). The reactions were terminated with 0.5 ml of 10% (w/v) trichloroacetic acid. The cylic AMP content of each well was determined immediately or the plates were stored at -20°C.

b.Preparation of Dowex and alumina columns

The method used to quantitate the amount of cyclic AMP in the samples was essentially that of Salomon et al (1974) and involves the separation of cyclic AMP from other nucleotides by Dowex and then alumina chromatography. Dowex-H+ 50x4-400 was washed in twice its packed volume with 1M hydrocholic acid and then with the same volume of water four times.The Dowex was mixed with water to a slurry (1:1, v/v ratio) and then 3 ml of this added to each column and the water left to drain out.The columns were then ready for use.After use the Dowex was washed with 2 ml of1M hydrochloric acid and left in this state. Before the next samples were applied, the columns were washed with 8 ml of water.

The alumina columns were prepared by weighing 1.2g of neutral

alumina per column and washing with 8 ml of 0.1M imidazole before use. After use, the columns were washed with 2 ml of 1M imidazole and left in this state. The columns were washed with 8 ml of 0.1M imidazole before they were used again.

c.Separation of cyclic AMP on Dowex-H+ columns.

Before samples were run on Dowex columns, the elution volumes required for the separation of cyclic AMP were determined. This was done by applying a mixture of [^{3}H] cyclic AMP and [^{32}P] ATP to the column and determining the elution profile.

Freeze-dried [^{3}H] cyclic AMP was reconstituted in either 10% trichloroacetic acid (w/v) or water and diluted so that 100µl of the solution contained approximately 10000 dpm. [^{32}P] ATP was diluted so that100µl of the solution contained approximately 2000 dpm. 100µl of each of the cyclic AMP and ATP solutions were added to 0.5 ml of 10% trichloroacetic acid and the mixture loaded onto a Dowex column. The ATP and cyclic AMP were eluted from the column with up to 3 ml of 0.1M hydrochloric acid followed by up to 10 ml of water in 0.5 ml fractions. All the fractions were collected, 3ml of Ecoscint was added to each and the radioactivity determined by liquid scintillation counting using a dual-label programme.

The elution volumes required to elute off the cylic AMP from the Dowex columns were then determined from a graph as shown in figure 2.4. Typical recovery from the Dowex columns was 80-90%.

The elution volume required to elute the cyclic AMP from alumina columns was determined as for the Dowex columns except only [^{3}H] cyclic AMP was used and the eluting buffer was 0.1M imidazole. Typical recovery from the alumina columns was 90%.

4.Determination of cyclic AMP content of the cell extracts

The supernatant from each well was added to approximately 2500 dpm of [^{14}C] cyclic AMP, the internal standard which allows for an estimation of % recovery of the nucleotide from the columns. The

percentage recovery of cyclic AMP from the Dowex and alumina columns was routinely 60-90%. When the recoveries of cyclic AMP fell below 50 %, the columns were discarded and fresh columns prepared.The supernatants were applied to prepared Dowex columns and ATP eluted with 2 ml of 0.1M hydrochloric acid. 8 ml of water was then added to the Dowex columns and this eluate allowed to run directly onto the alumina.The cyclic AMP fraction was eluted and collected with 10 ml of 0.1M imidazole.The radioactivity in this fraction was determined by counting 4 ml of this

The amount of cyclic AMP per sample was calculated from the amount of radioactivity in the sample by converting total dpm obtained (by taking into account the % recovery of cyclic AMP per sample) to pmol cyclic AMP, based on the specific activity of [^{3}H] adenine used. Figure 2.3 indicates that [^{3}H] adenine was maximal at a labelling period of 90 minutes.This experiment has since been repeated and the results obtained are similar to the experimental data shown. Also, experiments to determine the specific activity of [^{3}H] labelled intracellular ATP in all the cell types used in the experiments shown in Chapter 6 were performed (results not shown). The results indicate that there is no change in the specific activity of [^{3}H] ATP between the different cell types after the 90 minute labelling period and that the specific activity of the ATP remained constant during the experimental period.

Figure 2.3: Incorporation of tritiated adenine in T15+ cells as a function of time.

The figure shows the incorporation of tritiated adenine into the trichloroacetic acid soluble fraction of T15+ cells. The experiment was performed using T15+ cells grown in 24 well plates to a density of10^5 cells per well as previously described. The cells were labelled with [^{3}H] adenine (2μCi per ml) in serum-free DMEM at 37^{o}C and incubations were terminated at appropriate time points. The cells were washed twice with PBS at 37^{o}C and 0.5 ml 10% (w/v) trichloroacetic acid was added to each well. The wells were scraped and the contents of each well were transferred to Eppendorf tubes and the samples centrifuged for 10 minutes at 16 000 g in a Hettich microfuge at 4^{o}C. The supernatants were transferred to scintillation vials and the radioactivity in each sample determined by the addition of 3 ml of Ecoscint followed by scintillation counting.

Figure 2.3

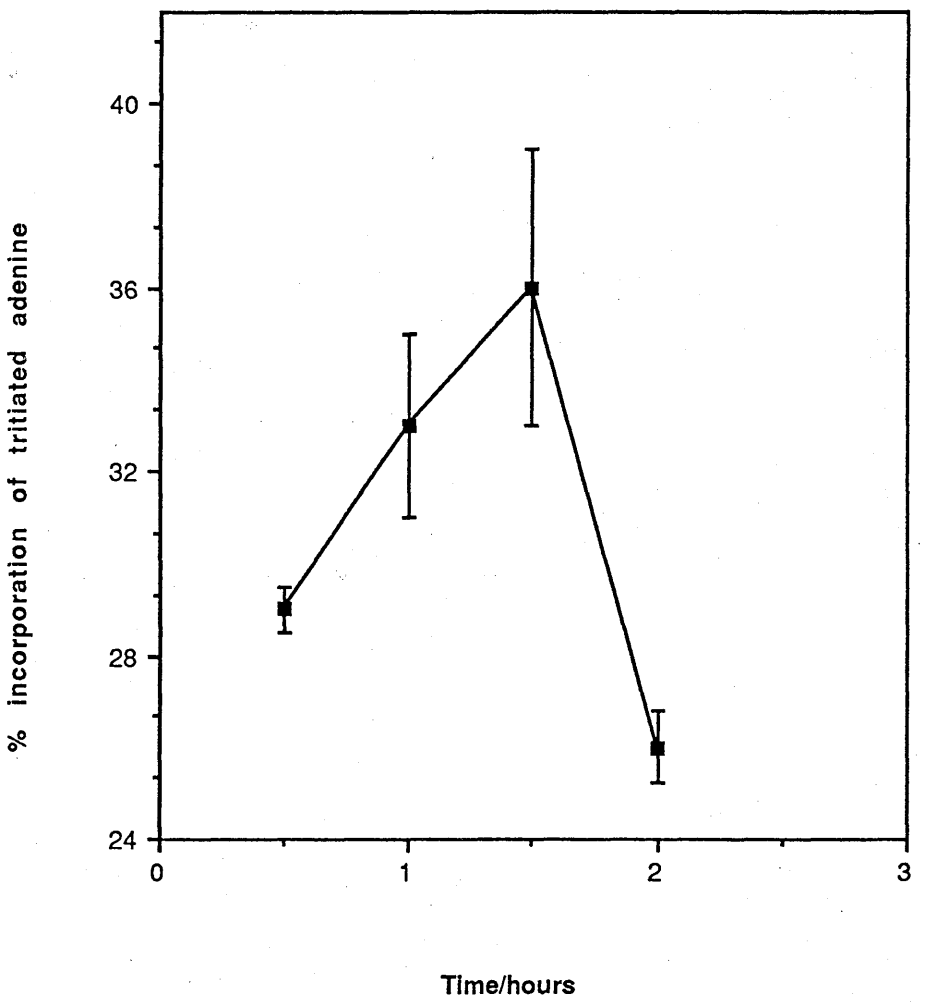
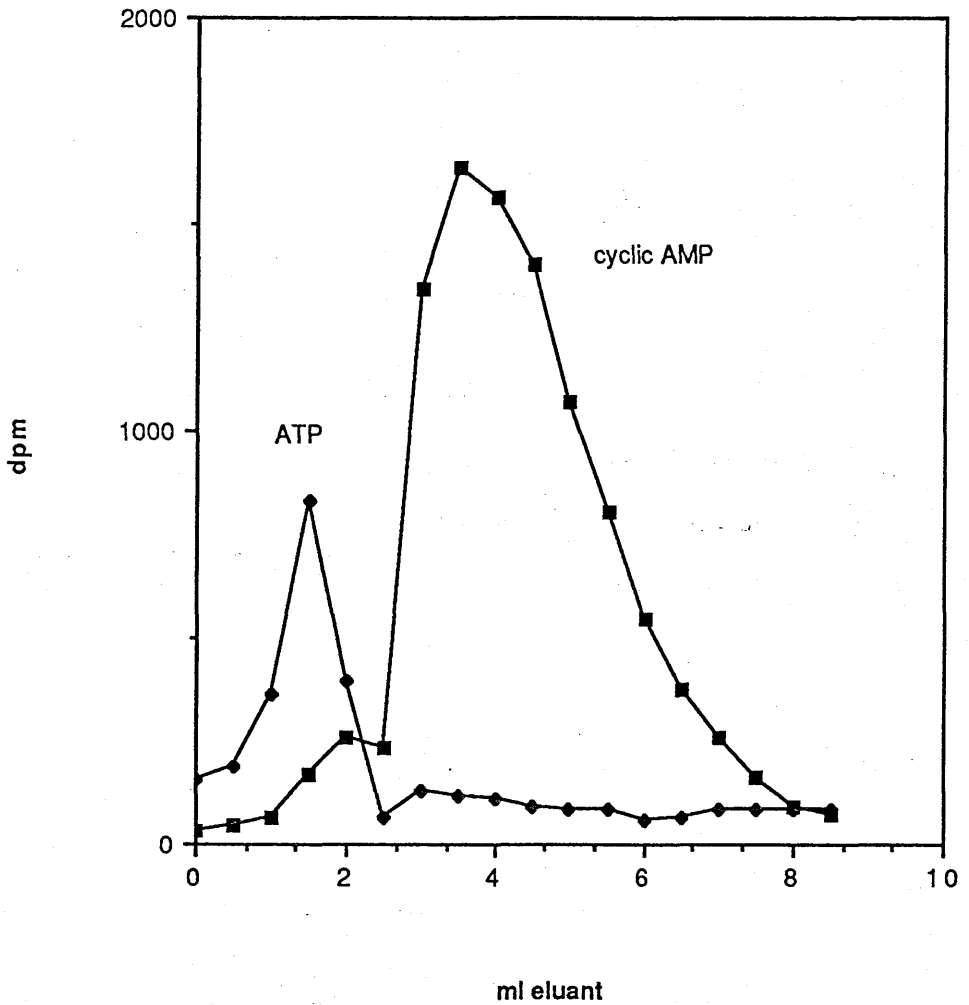

% incorporation of tritiated adenine
40
36
32
28
24
0
1
2
3
Time/hours

Figure 2.4: Elution profiles of cyclic AMP and ATP standards from Dowex columns.

The results in figure 2.4 are from an experiment to determine the elution profiles of cyclic AMP and ATP from Dowex H^+ columns. A [^{3}H] cyclic AMP standard and a [^{32}P] ATP standard were applied to the Dowex columns (as described in the text) and eluted from the Dowex resin with 6 x 0.5 ml of 0.1M hydrochloric acid follwed by 11 x 0.5 ml water. The radioactivity in each sample was determined as previously described.

Figure 2.4



8.Protein Determination

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

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

B. 1% (w/v) copper sulphate

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

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 5 and a standard curve was constructed for a maximum of 30 µg protein per sample. 10 µl of the unknown sample was assayed in duplicate.1 ml of the A:B:C solution above was added to each sample, mixed and left to stand for 10 minutes. 100µl of Folin-Ciocalteau reagent, diluted 1:4 with water was added to each sample. The samples were mixed and left for 30 minutes. The absorbance of the samples were determined spectophotometrically at 750 nM in a LKB Ultrospec 2.

9. Determination of [^{125}I] iodocyanopindolol binding in T15 cells.

The binding of [^{125} I] iodocyanopindolol to T15 cells was used as an index of β adrenergic binding since iodocyanopindolol binds to particular lipids associated with β adrenergic receptors (Levitzki, 1988) and therefore is a specific agonist for β adrenergic receptors. The following protocol was used to determine [^{125} I] iodocyanopindolol binding to T15 cells in order to demonstrate any changes that may occur in β adrenergic binding upon p21 N-ras overexpression in this cell line.

T15 cells were cultured as previously described in twenty-four well plates and 2μM dexamethasone added to the medium as required (See Chapter 4). T15- cells were maintained in DMEM/10%, v/v, steroid-free calf serum for 2-3 days prior to the experiment in order to minimise the possibility of spontaneous transformation of the cells. The cells were used at a density of 10^5 cells per well. The cells were washed twice with HBS and incubated with the following incubation medium at a volume of 1 ml per well at 4° C: 0.1% BSA, 10mM glucose, 10mM HEPES, 2 mM glutamine and cell culture grade amino acid mix (1:99 (v/v) dilution of GIBCO physiological amino acid mixture) in HBS at pH 7.2. The incubation buffer contained 10^{-9} M [^{125} I] iodocyanopindolol (approximate specific activity = 2000 Ci mmol^{-1}) and 10^{-4} M isoproterenol either in the presence or absence of 10^{-3} M pindolol to estimate non-specific binding. The cells were incubated for a period of 90 minutes as maximal binding of [^{125} I] iodocyanopindolol was obtained during this period (See figure 2.5). After the incubation period, the cells were washed with the following ice-cold buffer in 3 x 1 ml volumes : 0.1% BSA and 10mM HEPES in HBS, pH 7.2. The cells were solubilised with 0.5 ml sodium hydroxide/0.1% (v/v) triton-X-100 and radioactivity bound was determined by counting the solubilised cell samples in a LKB 1275 MINIGAMMA gamma counter.

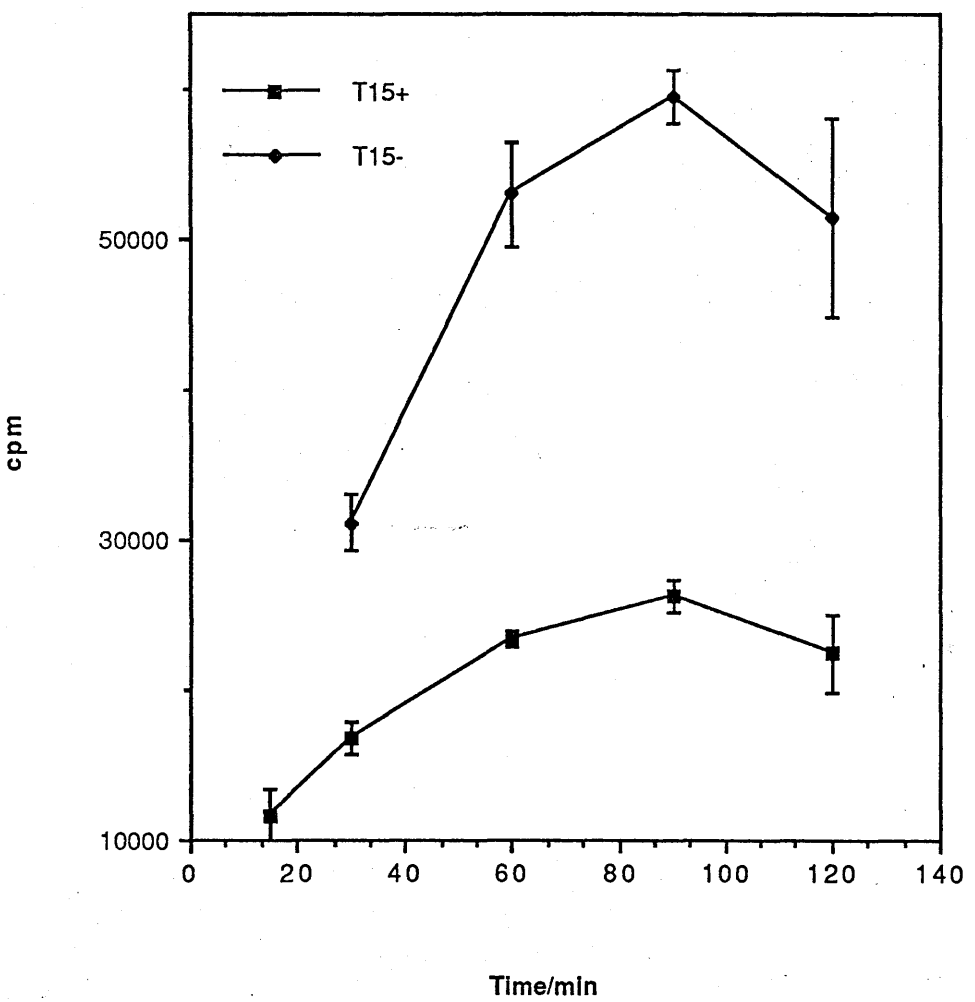
Under these conditions, the non-specific binding determined in the

presence of 10^{-3} M pindolol was estimated to be between 10-30% of the total binding. The amount of [^{125} I] iodocyanopindolol bound to T15 cells was calculated from the specific binding of [^{125} I] iodocyanopindolol in cpm which was then converted to fmol iodocyanopindolol bound using the known specific activity of the iodinated ligand.

Figure 2.5: [^{125}I] iodocyanopindolol binding in T15+ cells as a function of time.

The figure shows the results from an experiment to determine the optimum incubation time of T15 cells with iodinated iodocyanopindolol which would result in maximal binding of the ligand. The experiment was performed as described except that no isoproterenol or pindolol was present in the incubation medium *i.e.*only total iodocyanopindolol binding was estimated in this experiment. T15 cells were incubated with the incubation medium containing 1.8µl of [^{125}I] iodocyanopindolol per well and the incubations were terminated at the time points indicated in the figure. The data are the mean values of cpm [^{125}I] iodocyanopindolol bound per 10^5 cells and is ± S.D. (n=3).

Figure 2.5



10. Bacterial toxin catalysed ADP-ribosylation of T15 cell membranes.

T15 cells were grown as previously described. Membrane fractions were prepared from confluent cultures following washing of the cell monolayers with Hanks Buffered Saline at 37°C.The washed cells were homogenised with a teflon on glass homogeniser in a 10 mM Tris/ 0.1mM EDTA buffer, pH 7.5 at 4°C. The homogenate was centrifuged in a Beckman L5-50B ultracentrifuge using a Ti 50 rotor at 40 000g for 10 minutes. The pellet was resuspended in 0.5 ml of ice-cold buffer and the protein concentration determined by the Lowry method. The membrane fractions from the samples were diluted to the lowest estimated protein concentration obtained in order to apply identical amounts of protein to each lane on the gel.

20 µl of the membrane fraction from each sample containing 14.2 µg of protein was incubated with 25 µl ribosylation mix and 5 µl of either pertussis or cholera toxin (which were previously activated by incubating the toxins at 37°C for 1 hour with the addition of 50 mM dithiothreitol) in order to ribosylate the membrane samples. The final reaction volume of 50 µl was incubated at 37°C for 1 hour. The ribosylation mix contained: 0.2 M thymidine, 1.5 M potassium phosphate, 0.04 M ATP, 1 M Arginine/HCl and 1 mM NAD in water at pH 7.5. The incubations were terminated with 25 µl of 2% (w/v) sodium deoxycholate, 2.925 ml water and 1 ml 24% (w/v) trichloroacetic acid. The tubes were centrifuged in a Beckman TJ-6 centrifuge at 2000 g for 20 minutes. The supernatant was discarded and 200 µl of the following buffer was added to each sample followed by 20 µl of 1 M Tris base: 5 M urea, 0.17 M SDS. 0.38 M dithiothreotol, 50 µl of 1M Tris/ HCl per ml of buffer and a few crystals of bromophenol blue in water. The samples were then applied to a 10 % SDS polyacrylamide gel and the proteins separated by gel electrophoresis. The gel was stained to determine the positions of the protein bands and then destained before autoradiography for 3 days at -80°C. The appropriate bands on the gel as indicated by molecular weight markers were cut from the gel and the radioactivity associated with each band was determined by liquid scintillation counting.

Materials

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

Amersham International plc, Amersham, Buckinghamshire, England.

[^{3}H] adenine (Specific activity = 30 Ci mmol^{-1})

[^{3}H] cyclic AMP (Specific activity = 52 Ci mmol^{-1})

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

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

[^{3}H] Inositol 1,4 bisphosphate (Specific activity =1 Ci mmol^{-1})

[^{3}H] Inositol 1,4,5 trisphosphate (Specific activity =1 or 44 Ci mmol^{-1})

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

[^{3}H]thymidine (Specific activity = 1 Ci mmol^{-1})

[^{14}C] cyclic AMP (Specific activity = 261 mCi mmol^{-1})

[^{32}P] ATP (Specific activity = 2000 Ci mmol^{-1})

[^{125}I] iodocyanopindolol (Specific activity = 2000 Ci mmol^{-1})

BDH Chemical Company, Poole, England.

Ammoniom formate, copper sulphate, EDTA, glycerol, HEPES, 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, dithiothreotol, NAD and Tris.

Calbiochem, Cambridge, England.

Forskolin

Cambridge Research Biomedicals, Cambridge, England.

Bradykinin, bombesin, gastrin releasing peptide and 8Arginine-vasopressin.

Formachem (Research International) plc, Strathaven, Scotland.

Boric acid, D-Glucose and sodium hydrogen carbonate,

FSA Laboratary Supplies, Loughborough, England.

Sodium lauryl sulphate (SDS).

GIBCO, Paisley, Scotland.

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

Koch-Light Ltd, Suffolk, England.

Calcuim chloride, dimethyl sulphoxide (DMSO), magnesium sulphate, potassium chloride and sodium potassium tartarate.

May and Baker, Dagenham, England.

Formic acid, hydrochloric acid and sodium tetraborate.

National Diagnostics, Aylesbury, England.

Ecoscint.

Riedel-DeHaenAg Seelze-Hannover, Germany.

Magnesium chloride and sodium dihydrogen carbonate.

Whatman Limited, Maidstone, England.

Partisil 10 SAX HPLC column and 0.45 micron millipore filters.

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

Buffer Composition

Earle's Salts (x20)

2.33 M sodium chloride,

107 mM potassium chloride,

33.2 mM magnesium sulphate,

23.33mM sodium dihydrogen phosphate

110 mM glucose

The pH of the buffer was adjusted to between 7.2-7.4 with 1 M sodium hydroxide.

Hanks Buffered Saline

1.26 mM calcium chloride

0.5 mM magnesium chloride

0.9 mM magnesium sulphate

5.37 mM potassium chloride

137 mM sodium chloride

4.2 mM sodium hydrogen carbonate

0.35 mM sodium dihydrogen phosphate

The pH of the buffer should be between pH 7.2-7.4 when freshly made.

Phosphate Buffered Saline (PBS)

146 mM sodium chloride

5.36 mM potassium chloride

9.6 mM disodium hydrogen phosphate

1.46 mM potassium dihydrogen phosphate

The pH of the buffer was adjusted to between pH 7.2-7.4.

All buffers were made using distilled water and were stored at 4°C.

Preparation of Dowex 1 x8-200 formate form.

Dowex 1 x 8-200 chloride form was obtained from the Sigma Chemical Company and 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 x its volume of 2 M sodium hydroxide. The Dowex was then washed with 10 x its volume of water followed by approxomately 5 x its volume of 1 M formic acid. Finally, the Dowex was washed with 50 x its volume of water until the pH of the Dowex slurry was constant at approximately pH 5.5.

Chapter 3: Inositol phosphate production in p21 $^{N\text{-}ras}$ transformed cells.

Introduction

The function of p21 ras in cell proliferation and transformation is not known, although its role has already been discussed (see introduction). In 1985, Chiarugi et al showed that in Ha-ras transformed Balb/c3T3 murine fibroblasts, muscarinic stimulation of inositol phosphate production was increased. We utilised the T15 cell line to investigate the effects of p21 $^{N\text{-}ras}$ transformation on the inositol phospholipid signalling pathway.

The T15 cell line is a clone of NIH3T3 cells transfected with a human foetal genomic N-ras proto-oncogene. The expression of the proto-oncogene is under the control of a glucocorticoid-inducible MMTV-LTR promoter (McKay et al, 1986). The T15 cell line is derived from a clone obtained during the transfection experiments which resulted in 0% transformants in dexamethasone-free growth medium and 100% transformants when grown in dexamethasone-containing growth medium. When the cells are grown in the presence of dexamethasone (T15+) they exhibit the transformed phenotype.When T15 cells are grown in the absence of dexamethasone (T15-) they resemble wild-type NIH3T3 cells in morphology and growth characteristics. The transformation of the T15 cells is due to the overexpression of p21 $^{N\text{-}ras}$ which results in an approximately 50 fold increase in p21 $^{N\text{-}ras}$ levels compared to non-transformed NIH3T3 cells. This provides a useful experimental system for studying the effects of the ras gene product on second messenger systems.

The effects of p21 $^{N\text{-}ras}$ transformation on the phosphoinositide signalling system are shown in this chapter. This was investigated by measuring basal and agonist stimulated total inositol phosphate production in T15 cells. Total inositol phosphate measurements are a useful indication of the activation of the phosphoinositide signalling pathway and indicates if inositol mono-, bis- and tris-phosphates and their isomers are generated. The generation of total inositol phosphates is proportional to receptor mediated PtdIns 4,5 P_2

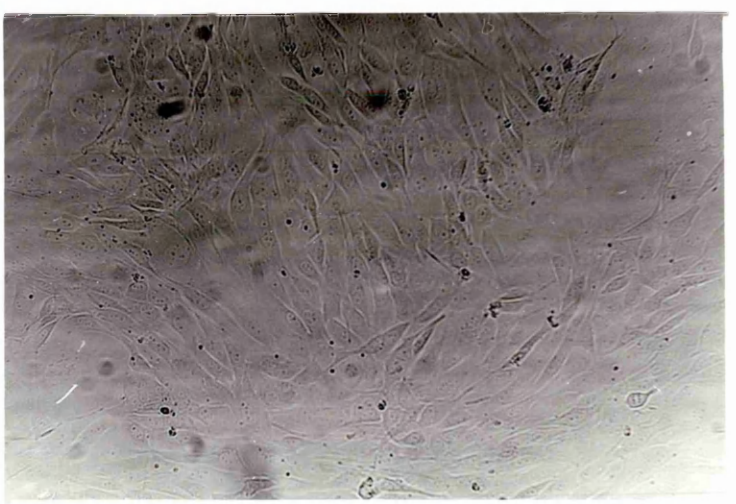
hydrolysis, although this may also be a consequence of PtdIns 4 P hydrolysis on receptor activation.

To measure total inositol phosphate accumulation on agonist stimulation, the lithium assay was employed. Lithium ions have been shown to inhibit the phosphatases which degrade inositol monophosphates to inositol (Majerus et al, 1988). If this step is blocked there will be an accumulation of inositol phosphates. Since [^{3}H] inositol labelled cells are used in these experiments, the accumulation of [^{3}H] inositol phosphates can be determined by liquid scintillation counting.In the absence of lithium, there will be very low levels of radioactivity detected in both control and stimulated inositol phosphate fractions.

Results

The photographs overleaf show confluent cultures of T15+ and T15- cells and indicate the morphological transformation associated with p21 $^{N\text{-}ras}$ overexpression in this cell line. These photogrpaphs were taken at 360 x magnification and provided by Caroline Evans.

T15+ cells



T15- cells

[^{3}H] thymidine incorporation can be used as an index of cell proliferation although it is not an absolute quantification of proliferation as is, for example, cell counting. Bearing in mind the limitations of this method, tritiated thymidine incorporation was used to determine if p21 N-ras transformed NIH3T3 cells demonstrated differences in the rate of proliferation as compared to wild type NIH3T3 or T15- cells. Rapid proliferation is one of the characteristics of transformed cells (See Introduction) and can be indicative of the activation of growth factor pathways.

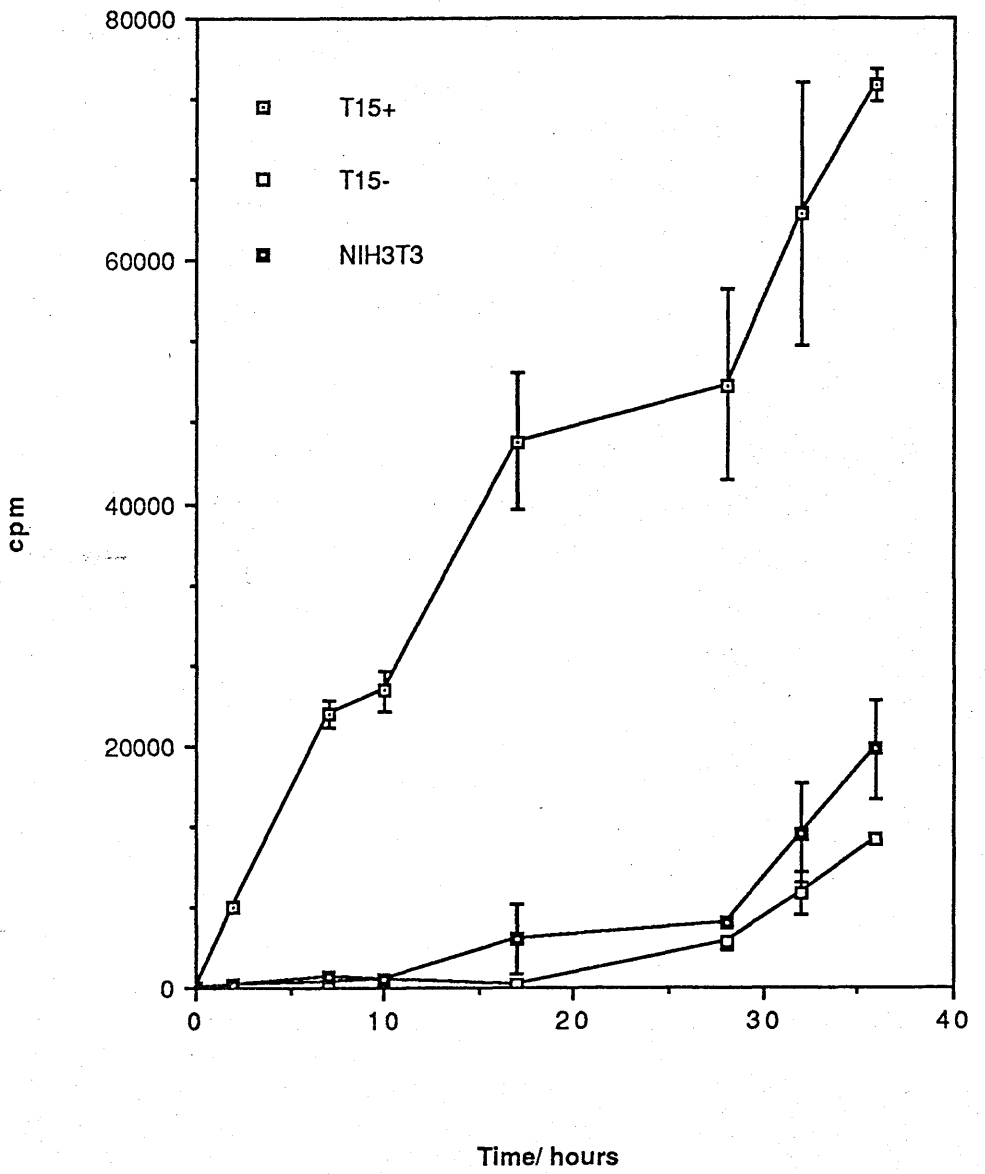
The experiments were performed on quiescent cells, that is, cells that have been growth arrested in the G_1 phase of the cell cycle (See Methods section). It was not possible to maintain T15+ cells in the G0/G1 phase since transformed cells do not contact inhibit and have lower serum growth factor requirements. This is due to the associated secretion of autocrine growth factors in transformed cells and therefore these cells can grow with very low levels of exogenous growth factors in the medium. However, the experiment utilised T15+ cells that had been treated in the same way as T15- and NIH3T3 cells *i.e.* serum deprivation. The data in figure 3.1 shows that T15+ cells incorporate thymidine into DNA more rapidly than T15- or NIH3T3 cells and this is thus an indication that T15+ cells have an increased rate of proliferation. The rates of [^{3}H] thymidine incorporation into T15- and NIH3T3 cells appear to be similar and indicates that the transfected uninduced N-ras proto-oncogene does not affect cell proliferation. The thymidine incorporation in T15- and NIH3T3 cells is negligible until after 24 hours and the increase in incorporation after this time is probably due to the entry of cells into S phase. However, in T15+ cells there does not appear to be a lag phase in the rate of [^{3}H] thymidine incorporation.This rapid incorporation of thymidine into T15+ cells without any lag phase is possibly due to the non-synchronous cell population.

Figure 3.1: Tritiated thymidine incorporation in T15 and NIH3T3 cells.

The graph in figure 3.1 shows the [^{3}H] thymidine incorporation into acid insoluble fraction of NIH3T3 and T15 cells determined as described in the Methods section. The results are cpm / dish ± S.D., n=3 and represent one experiment which is typical of two separate experiments.

The cells were incubated with [^{3}H] thymidine for the appropriate times as indicated in figure 3.1.

Figure 3.1


80000
60000
40000
20000
0
cpm
T15+
T15-
NIH3T3
0
10
20
30
40
Time/ hours

It has been demonstrated that T15+ cells have a higher proliferative rate than T15- or NIH3T3 cells. This could be due to the sustained activation of the phosphoinositide signalling system, which could be associated with cell proliferation. To investigate this possibility, experiments were performed on T15+ and NIH3T3 cells to determine if p21 $^{N\text{-}\underline{ras}}$ transformed cells exhibited a greater turnover of their inositol phospholipids than NIH3T3 cells.This would indicate that the transformed cell are turning over PtdIns 4,5 P_2 at a greater rate than non-transformed cells and this may be a transformation-related event.

It was necessary to determine the characteristics of [^{3}H] inositol labelling of lipids in T15+ and non-transformed cells to investigate the possibility of different turnover rates of inositol phospholipids in these cells. Initially, NIH3T3 and T15+ cells were labelled with tritiated inositol over a period of 48 hours. After this time, the amount of radioactivity associated with the lipid fraction from chloroform/methanol treated cells was determined (see Methods). The data obtained from these experiments showed that there was 2.7 times the amount of total [^{3}H] inositol labelled lipids in the N-ras transformed cells compared to the non-transformed cells (Results not shown).

The results in figure 3.2a show that T15+ cells have significantly higher incorporation of tritium into GPIP and $GPIP_2$ than non-transformed cells (figure 3. 2b). The increased labelling in the GPIP and $GPIP_2$ fractions in T15+ cells is not due to cell number differences as the data is normalised for the amount of protein assayed. Therefore,these higher levels of [^{3}H] GPIP and [^{3}H] $GPIP_2$ (also [^{3}H] GPI, results not shown) in the T15+ cells are probably associated with cell proliferation. The peak levels of [^{3}H] GPIP and [^{3}H] $GPIP_2$ in NIH3T3 cells appears at 8 hours and is followed by a decrease in [^{3}H] GPIP and [^{3}H] $GPIP_2$ levels.

Figure 3.2: [^{3}H] inositol labelling of inositol lipids in NIH3T3 and T15+ cells

Figure 3.2 shows the results of an experiment to label inositol phospholipids and then separate them into PI, PIP and PIP_2. The separation method involves the deacylation of these lipids into glycerophosphoinositol (GPI), glycerophosphoinositol monophosphate (GPIP) and glycerophosphoinositol bisphosphate ($GPIP_2$). The graph in figure 3.2a shows the results from experiments performed on T15+ cells and the results in figure 3.2b show the results from a similar experiment using NIH3T3 cells. Both experiments were performed under the same conditions. The data in both figures are from one experiment and are expressed as mean dpm/mg protein ± S.D., n=3. Only results for GPIP and $GPIP_2$ are shown.

The results from an experiment using [^{32}P] inorganic phosphate to label the inositol phospholipids in T15+ and NIH3T3 cells over a period of 2 hours indicated the same trends in the deacylated fractions of GPI, GPIP and $GPIP_2$. Therefore, these results also suggest that T15+ cells have a higher turnover of inositol phospholipids than NIH3T3 cells.

These experiments were not performed on T15- cells as it was thought that T15- cells, being non-transformed clones of NIH3T3 cells would exhibit similar characteristics in terms of [^{3}H] labelling of inositol phospholipids as the parental cell line. Also, during the period these experiments were performed, T15- cells were not available due to the associated problems of spontaneous transformation.

Figure 3.2a

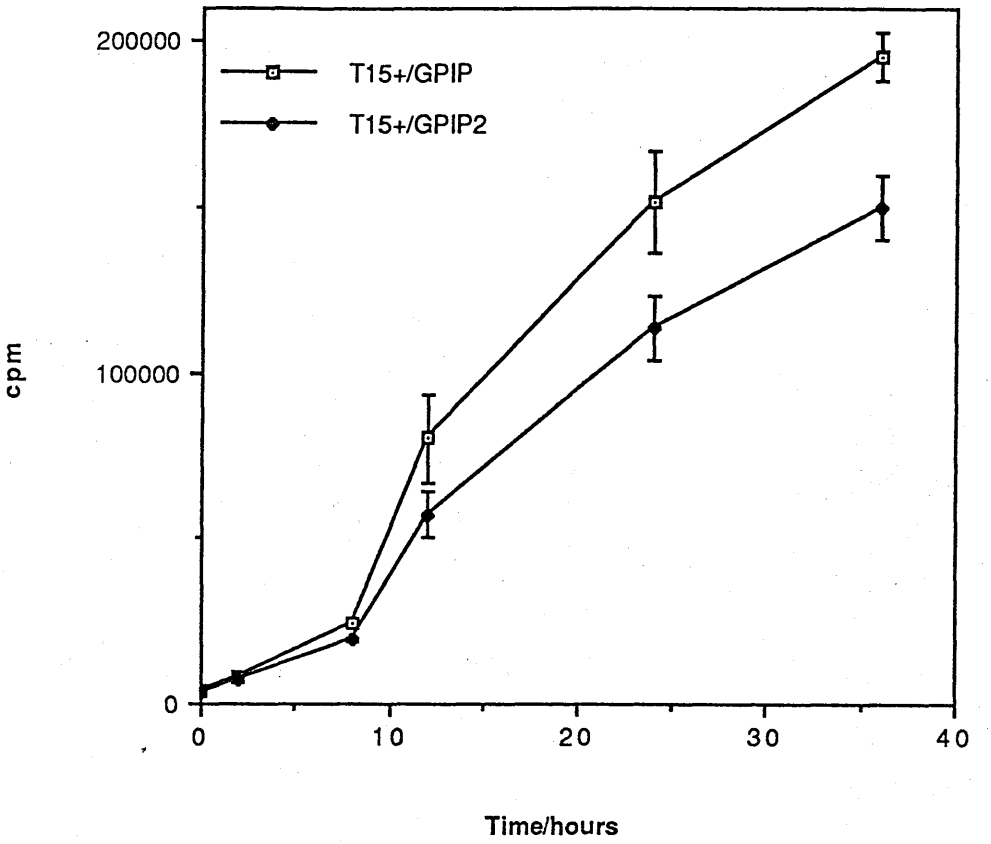
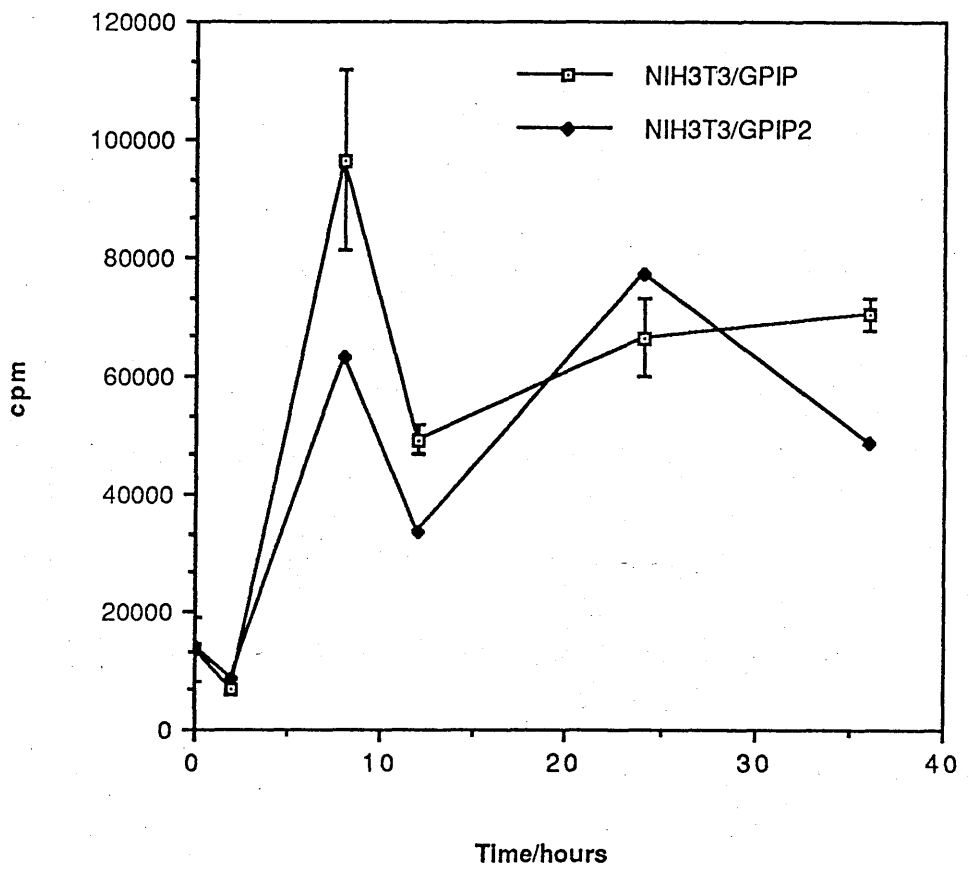


Figure 3.2b



The higher labelling([^{3}H])associated with inositol phospholipids in T15+ cells as compared to NIH3T3 cells could be due to increased basal turnover of inositol ph: ospholipids on p21 $^{N\text{-}\underline{ras}}$ transformation. Increased basal turnover has been reported in RSV transformed quail embryo cells (Diringer and Friss, 1977) and may be a consequence of oncogene activation or due to increased receptor stimulated PtdIns 4,5 P_2 hydrolysis.

If the production of inositol phosphates is measured in the absence of agonists, then a basal level of inositol phosphate production can be determined. Basal levels of inositol phosphates and inositol phospholipids were determined as in the Methods section using T15- cells as a control for T15+ cells.

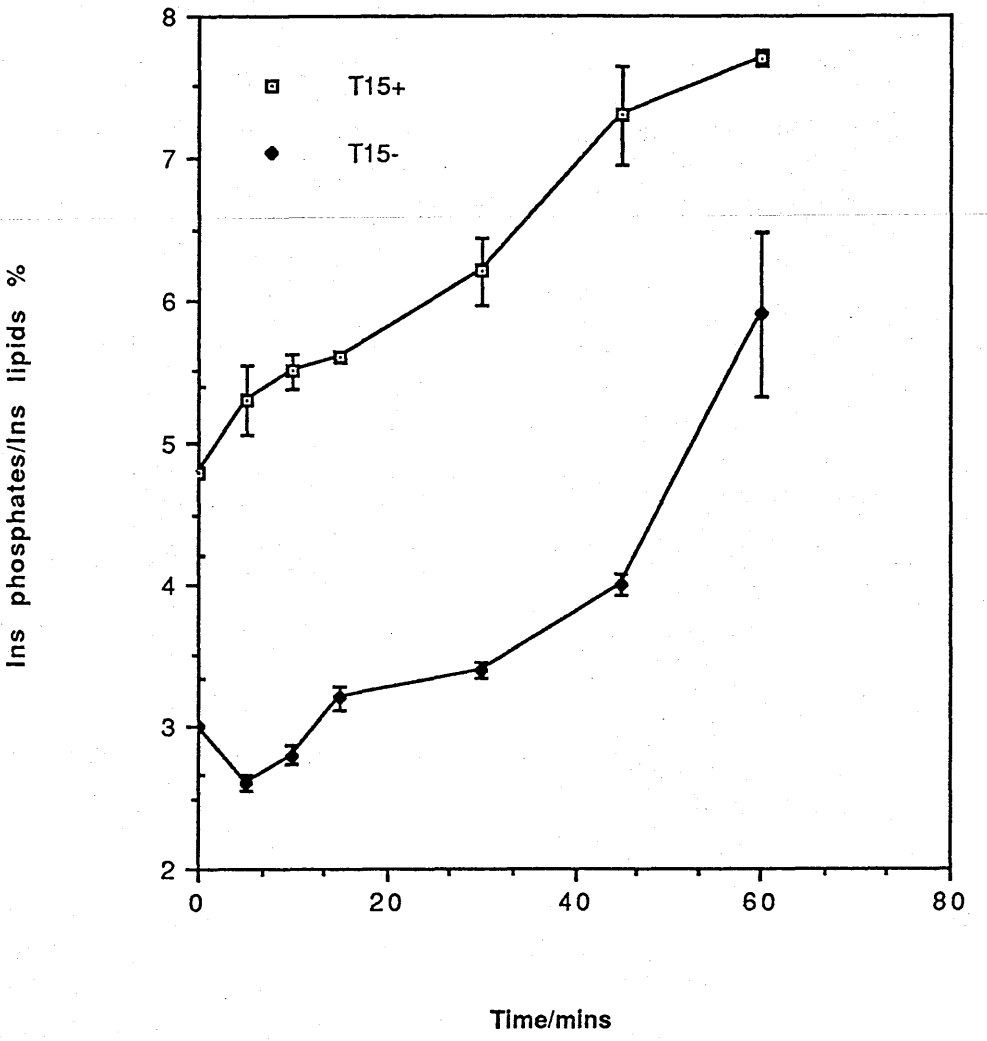
Figure 3.3 shows the results from an experiment to determine the basal rates of inositol phospholipid turnover in T15+ and T15- cells. Washed cells were incubated for the times shown in the presence of lithium ions and in the absence of any agonists and after the incubations were quenched, total inositol phosphates and phospholipids were determined.

The results show that there is no change in the basal rate of inositol phospholipid turnover between T15+ and T15- cells. The basal rate is defined as cpm phosphate / cpm lipids % / time in minutes. For both T15+ and T15- cells,this value is 0.048 min^{-1}. The figure shows that at time 0, the T15+ cells have a higher inositol phosphate/inositol phospholipid ratio than the T15- cells. This is probably due to the cell number difference between T15+ and T15- cells in the assay.

Figure 3.3: Basal rates of inositol phospholipid turnover in T15 cells.

Figure 3.3 shows the results of an experiment to determine the basal rate of inositol phosphate production in T15+ and T15- cells performed as already described. Results are means ± S.D., n=3 and values are expressed as cpm inositol phosphates/cpm inositol phospholipids (%) which are plotted against time in minutes.

Figure 3.3


T15+
T15-
Ins phosphates/Ins lipids %
Time/mins
8
7
6
5
4
3
2
0
20
40
60
80

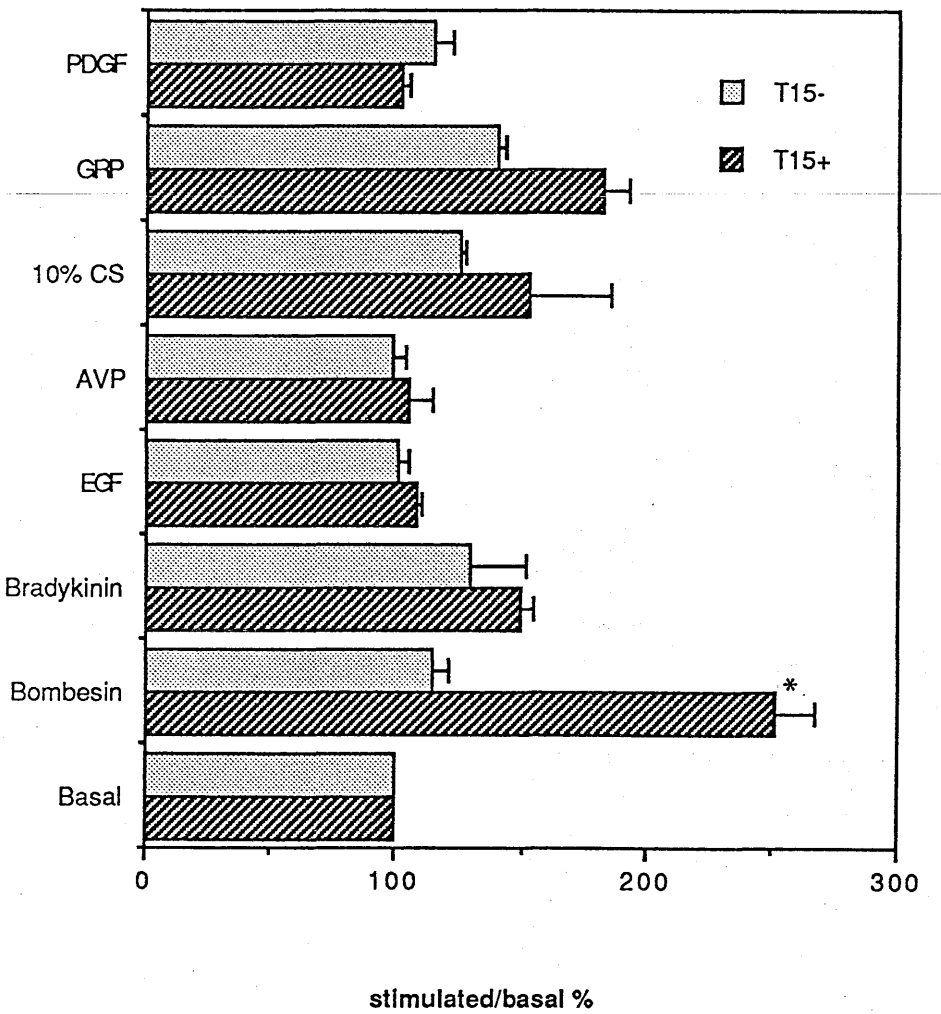
Thus, having determined that there is no change in the basal rate of inositol phospholipid turnover in T15+ and T15- cells, agonist stimulated inositol phosphate production in these cells was investigated.The production of inositol phosphates in response to agonist stimulation has been well documented (See Berridge and Irvine, 1984). A range of peptide and polypeptide agonists were screened for their ability to stimulate inositol phosphate production in T15 cells. This was to determine if there were changes in stimulated inositol phosphate production in cells transformed by the expression of p21 $^{N\text{-}ras}$. In figure 3.4, only the results from experiments using T15+ and T15- cells have been shown. Preliminary experiments with NIH3T3 cells indicated that basal and stimulated inositol phosphate production in this cell line was identical to that in the T15- cells.

The data indicates that only bradykinin, calf serum, GRP and bombesin stimulate significant inositol phosphate production in T15 cells. Bombesin stimulated an approximately 2.5-fold increase in the level of inositol phosphates in T15+ cells whilst in T15- cells bombesin stimulated a 1.3-fold increase. Also, the levels of inositol phosphates stimulated by calf serum, bradykinin and GRP in T15+ cells was approximately 1.6-, 1.5- and 2-fold respectively as compared to basal levels. In contrast, these agonists caused an increase in inositol phosphates of approximately 1.3 -fold for calf serum and bradykinin and 1.4-fold for GRP as compared to basal levels.The inositol phosphate response to agonists in T15- cells was always lower than, or the same as, T15+ cells. Therefore, stimulated inositol phosphate production in T15+ cells is amplified compared to T15- cells. Previous experiments indicated that this amplification was not a consequence of cell number differences between the two cell types *i.e.* the increase in total inositol phosphates seen in growth factor stimulated T15+ cells was not due to a greater number of T15+ cells in the assay.

Figure 3.4: Agonist stimulated total inositol phosphate production in T15 cells.

Figure 3.4 shows results from experiments to determine stimulated inositol phosphate production in response to different agonists in T15+ and T15- cells. The concentrations of the agonists were as follows:
Bombesin: 2.5μM (n=10), Bradykinin: 3.2μM (n=4), EGF: 16 nM (n=7), AVP: 0.4 μM (n=3), Calf serum: 10% (v/v) (n=3), GRP: 1.4μM (n=2) PDGF: 44 nM (n=4).
Results are expressed as stimulated value/basal value (%). These ratios are used for all experiments unless otherwise stated. Data are pooled from the number of experiments shown (n),three replicates within each and are means ± S.E.M. where n >3 and are ± S.D. where n ≤ 3. * $p \leq 0.015$
N.B. AVP used above and in the figure indicates ^{8}Arg-Vasopressin

Figure 3.4


PDGF
GRP
10% CS
AVP
EGF
Bradykinin
Bombesin
Basal
T15-
T15+
*
0
100
200
300
stimulated/basal %

Dexamethasone, which was used to induce p21 $^{N\text{-}ras}$ expression in the T15 cell line, is a glucocorticoid and can have a variety of effects upon cells, depending on the cell type. It was considered possible that dexamethasone treatment of T15 cells could have resulted in non-specific effects which could be the cause of the amplified inositol phosphate response in T15+ cells.

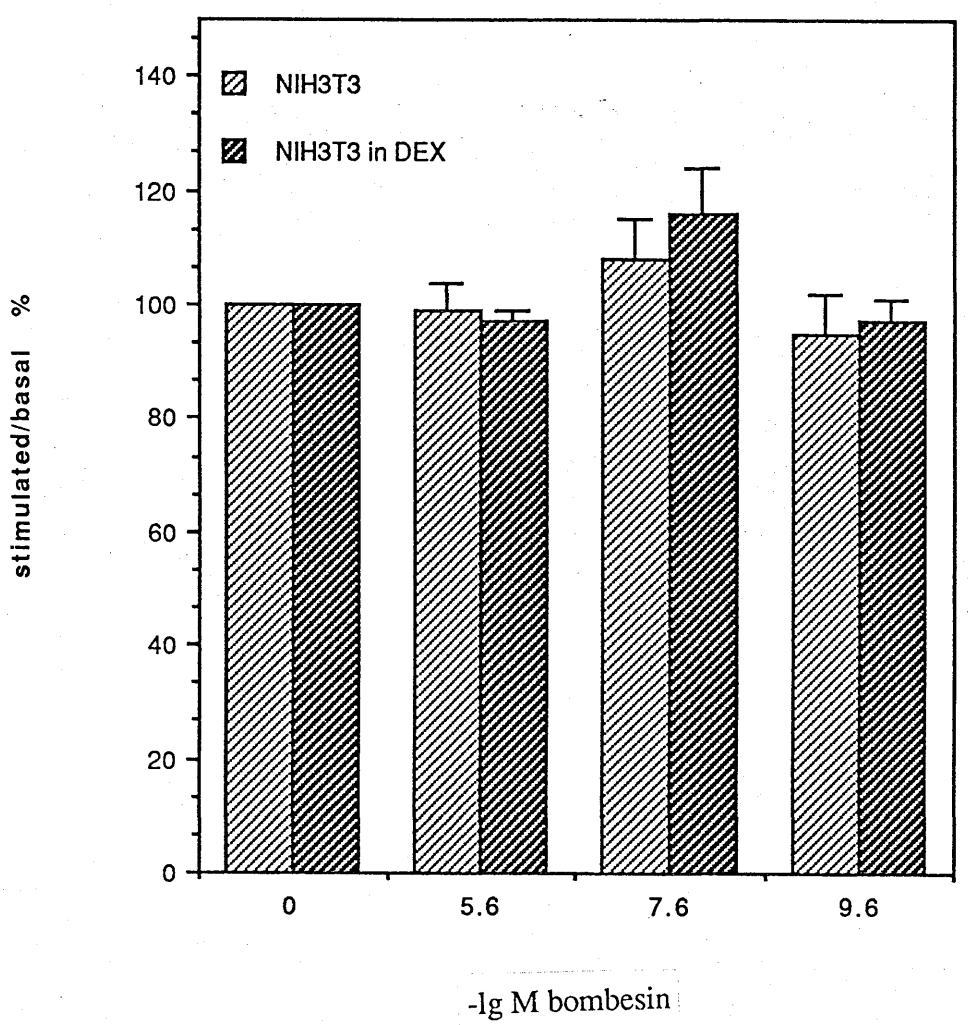
Therefore, it was necessary to determine if dexamethasone treatment of NIH3T3 cells resulted in amplified bombesin stimulated inositol phosphate production as in T15+ cells. NIH3T3 cells were treated with dexamethasone (2μM) for 72 hours and treated and untreated control cells were stimulated with 3 different concentrations of bombesin to determine stimulated inositol phosphate levels.

The graph in figure 3.5 shows that dexamethasone had no effect on stimulated inositol phosphate production at three different concentrations of bombesin. There was also no effect on the basal level of inositol phosphates in NIH3T3 cells. These results demonstrate that the amplified bombesin stimulated inositiol phosphate production in T15+ cells is not due to dexamethasone itself, but is probably associated with the overexpression of p21 $^{N\text{-}ras.}$

Figure 3.5: Basal and bombesin stimulated inositol phosphate production in dexamethasone treated NIH3T3 cells.

The results in figure 3.5 show the results from experiments to determine if dexamethasone treatment of NIH3T3 cells resulted in increased bombesin stimulated inositol phosphate levels as compared to control cells. Inositol phosphate values are expressed as % increase of basal values and are means ± S.D. and are from one experiment representative of two.

Figure 3.5


NIH3T3
NIH3T3 in DEX
stimulated/basal %
140
120
100
80
60
40
20
0
0
5.6
7.6
9.6
-lg M bombesin

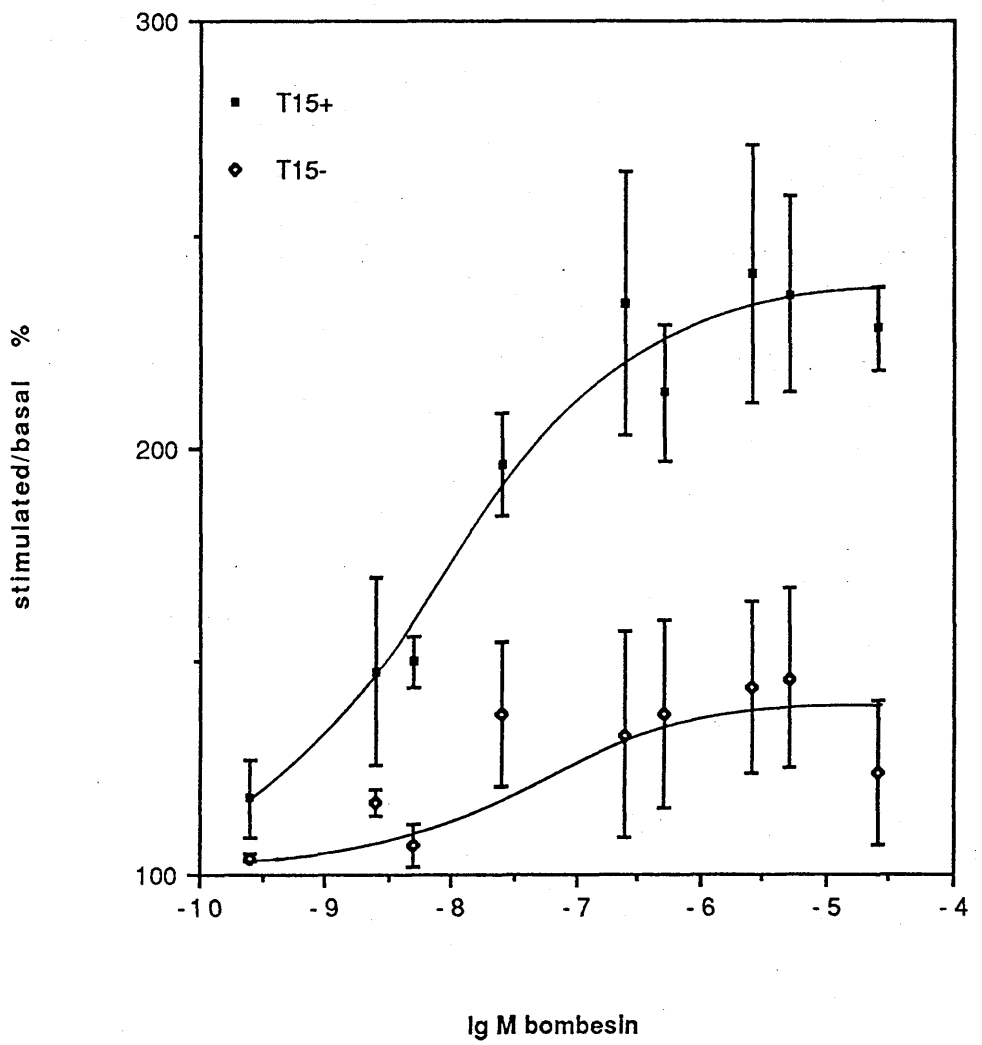
In order to investigate if there were transformation-related changes at the receptor level which caused the increase in bombesin stimlated inositol phosphate production in T15+ cells, a detailed characterisation of the bombesin response was performed. [^{125}I] bombesin binding to T15 cells demonstrated that the number of bombesin receptors in T15+ and T15- cells are the same, at approximately 4500 receptors per cell. (Wakelam et al 1986) Therefore, a dose response curve for inositol phosphate production in response to increasing concentrations of bombesin was constructed for both T15+ and T15- cells. This would indicate if there were changes in ligand binding characteristics of the bombesin receptor in T15 cells following ras induced transformation which would account for the amplified inositol phosphate response.

The results show that the bombesin stimulated inositol phosphate response in T15+ cells was amplified compared to T15- cells at all concentrations of bombesin tested. However, no change in the Ka value for the agonist was detected between the cell types, Ka=1nM bombesin. This indicates that the amplified inositol phosphate response in T15+ cells was not due to changes in the binding characteristics of the bombesin receptor. Since the number of bombesin receptors in T15+ and T15- cells was the same, (Wakelam et al, 1986) the amplification of stimulated inositol phosphate production was probably due to a post-receptor effect of p21$^{N\text{-}ras}$.

Figure 3.6: Dose response curve of bombesin stimulated inositol phosphate production in T15 cells.

The graph shows the results from experiments to determine the Ka values for bombesin stimulated inositol phosphate production in T15 cells. The data are means ± S.E.M. and are pooled from 3-5 separate experiments, where n=3 in each.

Figure 3.6



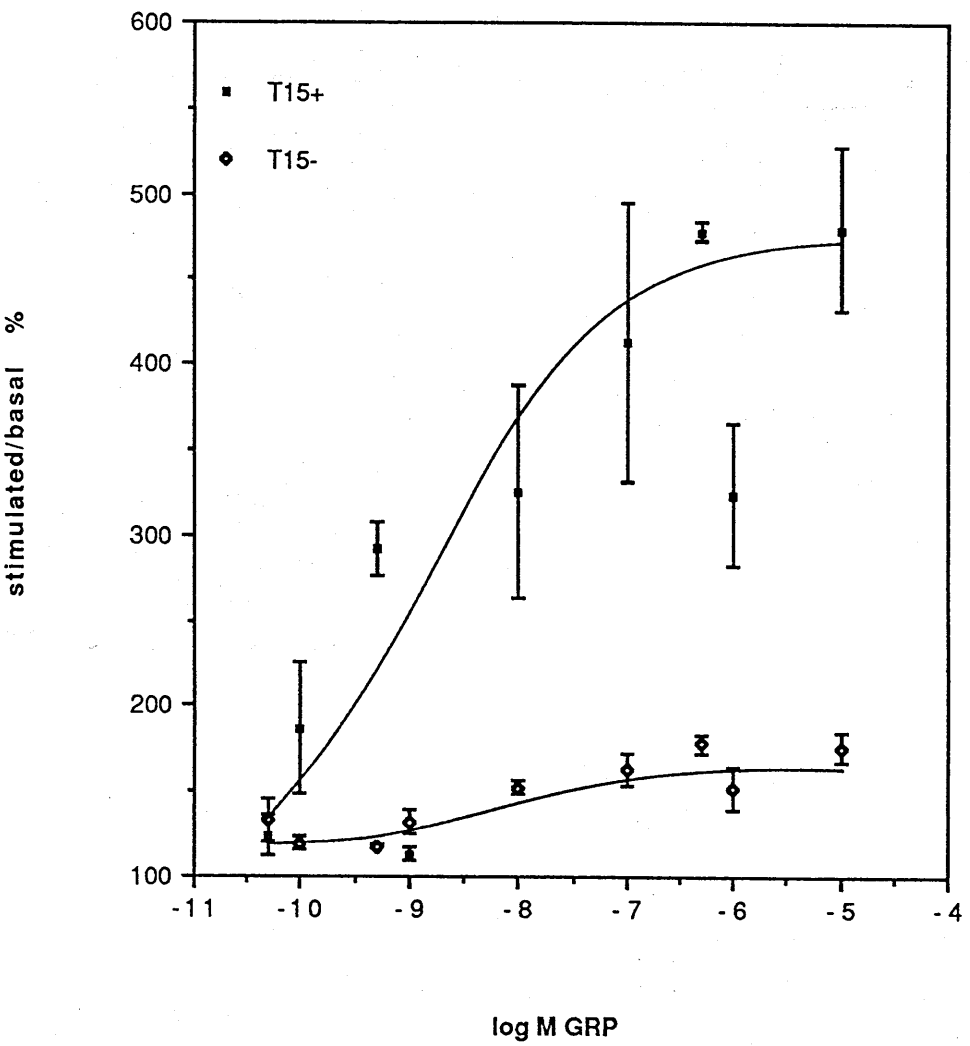
Bombesin was first isolated from frog skin and although it elicits a mitogenic response in several cell types (See Introduction) and stimulated inositol phosphate production in T15 cells, it was necessary to examine the inositol phosphate response of these cells to a mammalian equivalent of this peptide in more detail. As the data in figure 3.4 demonstrated that GRP caused an approximately 1.9-fold increase in inositol phosphate production in T15+ cells, a dose-response curve of inositol phosphate production to this peptide was constructed to determine if the characteristics of this receptor changed on p21 $^{N\text{-}\underline{ras}}$ transformtion of T15 cells.

The data in figure 3.7 shows that GRP stimulated inositol phosphate production in both T15+ and T15- cells at all concentrations tested although the response in T15+ cells was amplified. The Ka value of GRP stimulated inositol phosphate production in both cell types was estimated to be 2nM. This is in the same order of magnitude as the Ka value for bombesin and it was probable that GRP bound to the bombesin receptor in this cell line.

Figure 3.7: Dose response curve of GRP stimulated inositol phosphate production in T15 cells.

The graph shows the dose response curve of inositol phosphate production in response to GRP in T15+ and T15- cells. The experiment was carried out using the concentrations of GRP indicated in the figure. The results are means ± S.D., n=3 and are representative of two separate experiments.

Figure 3.7


600
500
400
300
200
100
T15+
T15-
stimulated/basal %
-11
-10
-9
-8
-7
-6
-5
-4
log M GRP

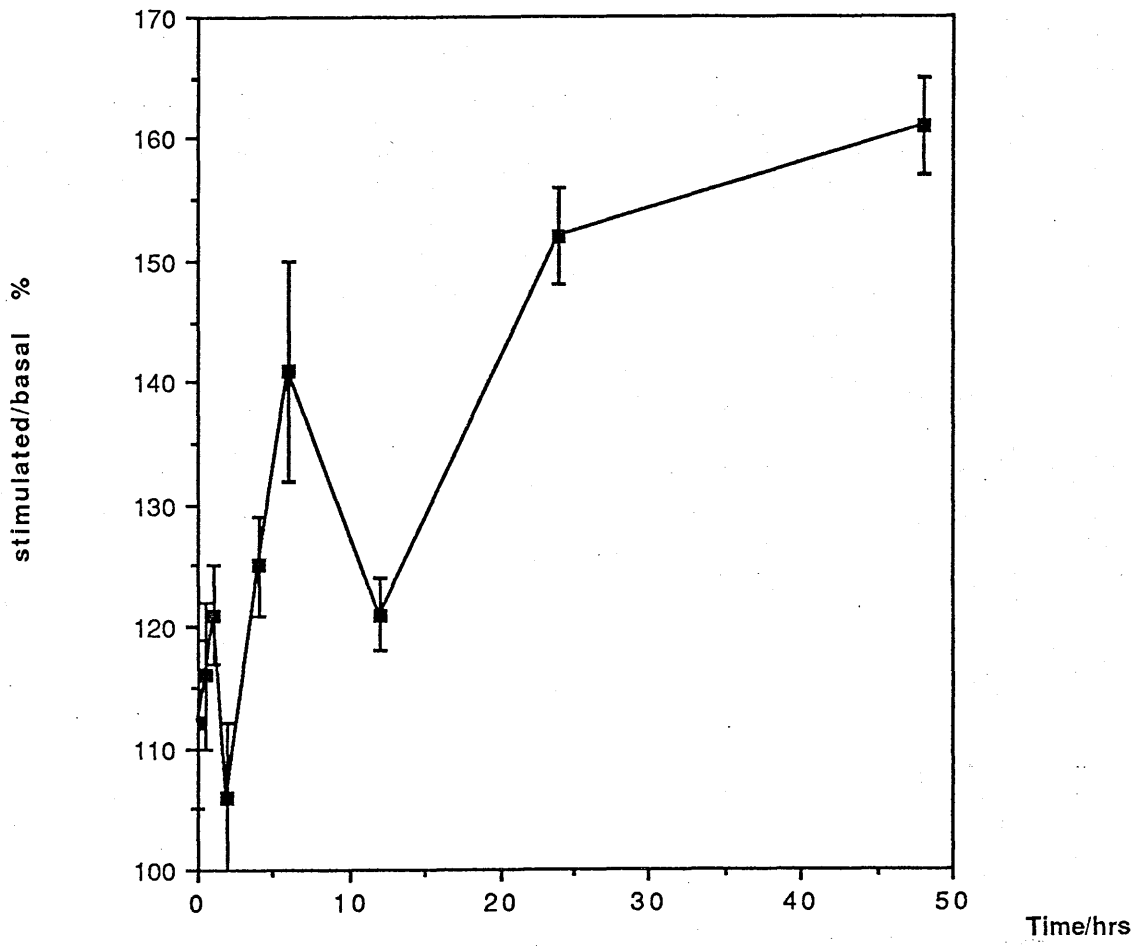
The previous results demonstrated that the inositol phosphate response to bombesin was increased in T15+ cells as compared to T15- cells. However, it was unclear if this effect was due to a ras-induced or transformation-induced effect. Therefore, it was important to demonstrate that dexamethasone-induced p21 $^{N\text{-}ras}$ expression could be correlated to stimulated inositol phosphate production in T15+ cells. The hypothesis that p21 $^{N\text{-}ras}$ was involved with stimulated inositol phosphate production could be tested in this inducible cell line and would indicate if there was a 'titratable' function of p21 $^{N\text{-}ras}$ in the phosphoinositide signalling system directly associated with cell proliferation and transformation but occuring before the onset of these processes.

A time course of dexamethasone treatment of T15- cells was performed to determine if increasing the length of time of dexamethasone exposure to T15- cells would lead to increased stimulated inositol phosphate production in response to bombesin.T15- cells were treated with 2μM dexamethasone for the times indicated and basal and bombesin (2.5μM) stimulated inositol phosphate production determined. The figure shows that significant inositol phosphate production occured six hours after dexamethasone treatment in response to bombesin. However, there was a decrease in the stimulated levels of inositol phosphates at 12 hours following the addition of dexamethasone but the levels of stimulated inositol phosphate then increase after this time point. The maximal response at 48 hours after dexamethasone treatment is 160% of the basal value and is a significant amplification in response as compared to T15- cells. These results indicated that stimulated inositol phosphate production in T15+ cells occured prior to morphological transformation and was a direct consequence of p21$^{N\text{-}ras}$ expression. These results are consistent with data obtained by C. Marshall (results not shown) which indicate that p21$^{N\text{-}ras}$ can be detected in whole cell lysates 6 hours after the addition of dexamethasone. The generation of p21 $^{N\text{-}ras}$ can thus be correlated with bombesin stimulated inositol phosphate production in this cell line.

Figure 3.8: Time course of dexamethasone induced amplification stimulated inositol phosphate production T15 cells.

The results in figure 3.8 are from experiments to determine if progressive dexamethasone treatement of T15- cells over a period of 48 hours resulted in a p21 $^{N\text{-}ras}$ induced increase in bombesin stimulated inositol phosphate production.The results are means ± S.D., n=3 and are representative of two separate experiments. The stimulated inositol phosphate levels are expressed as % stimulated inositol phosphate levels of the basal.

Figure 3.8


170
160
150
140
130
120
110
100
0
10
20
30
40
50
stimulated/basal %
Time/hrs

Reports in the literature are conflicting as to if G_p is a pertussis toxin insensitive protein or not (See Introduction). It was important to determine if the bombesin stimulated inositol phosphate production in T15 cells was bacterial toxin sensitive for two main reasons: firstly, to determine if G_p in T15 cells was sensitive to the bacterial toxins, cholera and pertussis toxins and secondly, if p21 $^{N\text{-}\underline{ras}}$ could function as, or interact with, G_i in stimulated inositol phosphate production.

Therefore, the effects of cholera and pertussis toxin treatment on bombesin stimulated production in T15+ and T15- cells were determined. T15+ and T15- cells were pre-treated with100 $ng\ ml^{-1}$ of cholera and pertussis toxin for 16 hours prior to the experiment. It is clear that using this procedure, the pertussis toxin and cholera toxin substrates in T15 cells are maximally ribosylated (See figure 3.10). Therefore, any effects of the toxins on inositol phosphate production will be due to specific effects of the toxins as a consequence of NAD ribosylation.

The data in figure 3.9 shows that pertussis toxin treatment had no effect on bombesin stimulated inositol phosphate production but cholera toxin pre-treatment of the cells resulted in a decrease of appoximately 40% of stimulated inositol phosphate production. This result indicates that bombesin stimulated inositol phosphate production in $T15^{+}$ cells was pertussis toxin insensitive but was inhibited by cholera toxin.

Figure 3.9: Effects of bacterial toxin treatment on inositol phosphate production in T15 cells.

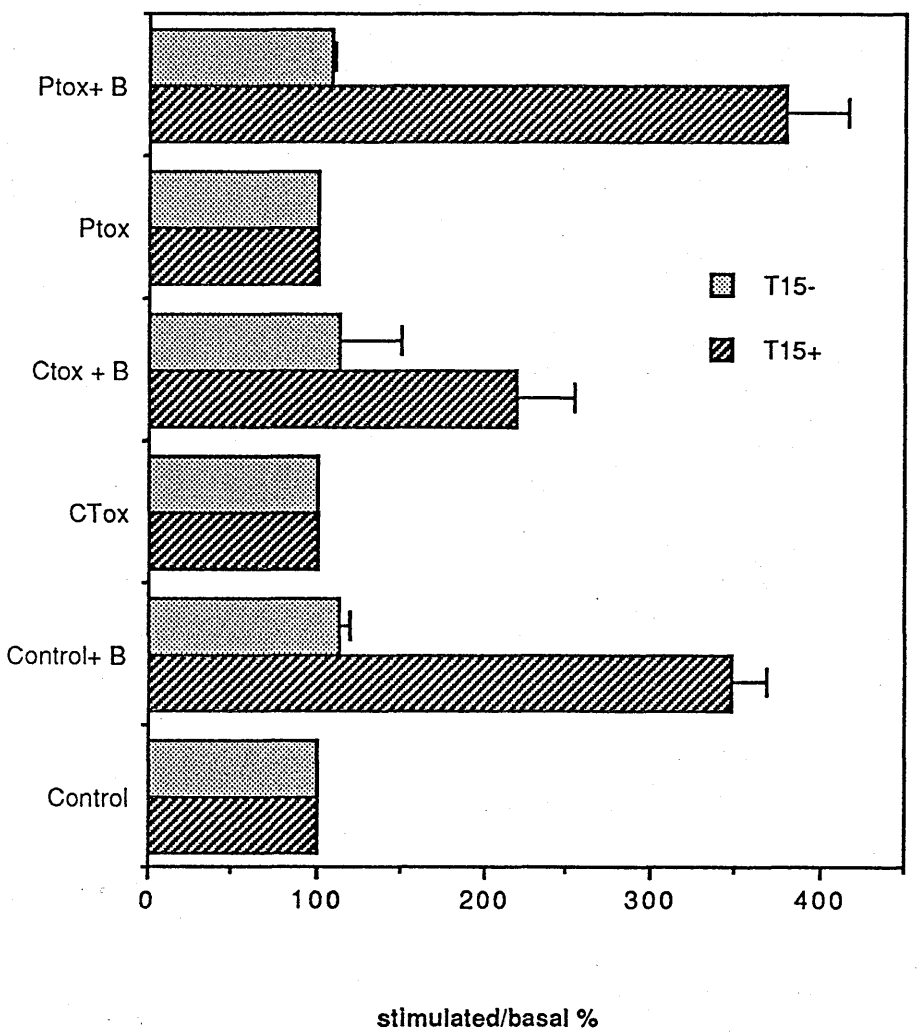
The results in figure 3.9 are from experiments to determine the effects of bacterial toxin treatment of T15 cells on basal and stimulated inositol phosphate levels. The experiments were carried out on pertussis toxin and cholera toxin treated T15 cells and basal and bombesin stimulated inositol phosphate production was measured in these cells using untreated cells as controls. The results are means ± S.D., n = 3 and are pooled from two separate experiments. The abbreviations used in the figure are:

B = bombesin

PTox = Pertussis Toxin

CTox = Cholera Toxin

Figure 3.9


Ptox+ B
Ptox
Ctox + B
CTox
Control+ B
Control
T15-
T15+
0
100
200
300
400
stimulated/basal %

As it was necessary to demonstrate that bacterial toxin treatment of T15 cells resulted in maximal ADP-ribosylation of their substrates the experiment described below was performed. T15- and T15+ cells grown as previously described to confluency were either left untreated (control cells) or treated with either cholera or pertussis toxin at a concentration of 100 $ngml^{-1}$ for 16 hours prior to the experiment. There were therefore, 6 different cell samples and these samples were treated as described in the Methods section.

If the pre-incubation of T15 cells with bacterial toxins resulted in further ribosylation of pertussis/cholera toxin pre-treated membranes by activated pertussis/cholera toxin then the conclusion must be that incubation of T15 cells with these bacterial toxins for 16 hours at a concentration of 100 $ngml^{-1}$ is not sufficient to maximally ribosylate the relevant G proteins. However, the photograph of the gel from this experiment shown in figure 3.10 clearly demonstrates that this is not the case. The lanes marked a-n are described in the figure legend.

The photograph indicates that treatment of control T15+ and T15- membranes with activated cholera and pertussis toxins resulted in significant [^{32}P] incorporation into proteins of 45 kD and 40 kD respectively. The band at 45 kD corresponds to $G_s\alpha$ whilst the band at 40 kD corresponds to $G_i\alpha$. The results indicate that there was no further cholera toxin catalysed ADP-ribosylation of cholera toxin pre-treated T15+ and T15- membranes (Lanes d and k). In contrast, there was cholera toxin catalysed ADP-ribosylation of $G_s\alpha$ in control and pertussis toxin treated T15+ and T15- cells (Lanes b,i and lanes f,m). Also, pertussis toxin ribosylation of T15+ and T15- membranes only occured in cholera toxin pre-treated cells (Lanes e and l) and in control cells (Lanes c and j) whereas there was no ADP-ribosylation of membranes from pertussis toxin pre-treated T15 cells (Lanes g and n). Therefore, pre-treatment of T15 cells with bacterial toxins for 16 hours at 100 ng ml^{-1} was sufficient to maximally ribosylate G protein substrates. The autoradiograph also indicates that there are no other cholera and pertussis toxin sensitive substrates detectable

in either T15- or T15+ cells.The bands at approx. 20 000 Mr indicate the intramolecular ADP-autoribosylated cholera toxin A chain as a result of the low amounts of sample membrane protein incubated with this bacterial toxin.

The amount of radioactivity associated with the 45 kD and 39 kD bands in each lane indicated that T15+ membranes contained approximately 6 times the amount of $G_s\alpha$ and 3 times the amount of $G_i\alpha$ compared to T15- cells. However, this result is from one experiment and was based on very low associated levels of [^{32}P]with each band and therefore cannot be accepted as a conclusive result. This data, although based on one experiment, is inconsistent with the data obtained by Milligan et al , see Discussion chapter. The reasons for this are unclear but may reflect the different cell preparations used between the experiments and may also reflect differences in cell cycle stages between these different batches of cells.

Figure 3.10 : ADP-ribosylation of T15- and T15+ membranes with bacterial toxins

The photograph shows the results from an experiment to determine the effect of pertussis and cholera toxin catalysed ADP-ribosylation of membranes prepared from bacterial toxin pre-treated T15+ and T15- cells. The lanes are as follows: (Cholera toxin and pertussis toxin have been abbreviated to CTox and PTox respectively.)

Lane a: Molecular weight markers

Lane b: Control T15+ cell membranes ADP-ribosylated with CTox.

Lane c: Control T15+ cell membranes ADP-ribosylated with PTox.

Lane d: CTox pre-treated T15+ cell membranes ADP-ribosylated with CTox.

Lane e: CTox pre-treated T15+ cell membranes ADP-ribosylated with PTox.

Lane f: PTox pre-treated T15+ cell membranes ADP-ribosylated with CTox.

Lane g: PTox pre-treated T15+ cell membranes ADP-ribosylated with PTox.

Lane h: Molecular weight markers

Lane i: Control T15- membranes ADP-ribosylated with CTox.

Lane j: Control T15- membranes ADP-ribosylated with PTox.

Lane k: CTox pre-treated T15- membranes ADP-ribosylated with CTox.

Lane l: CTox pre-treated T15- membranes ADP-ribosylated with PTox.

Lane n: PTox pre-treated T15- membranes ADP-ribosylated with CTox.

Lane m: PTox pre-treated T15- membranes ADP-ribosylated with PTox.

Figure 3.10

Discussion

The results in this chapter show that p21 $^{N\text{-}ras}$ transformed cells exhibit the growth characteristics of and the transformed phenotype associated with tumouregenic cells. The photographs of the T15 cells indicate the morphological changes associated with p21$^{N\text{-}ras}$ transformation of NIH3T3 cells. T15+ cells do not stop proliferating on contact with neighbouring cells, that is, they lose contact inhibition. T15+ cells also have elongated processes and grow in distinct clumps. This is in contrast with T15- cells which exhibit contact inhibition and therefore grow in a monolayer. T15+ cells are less adhesive than T15- cells and all these observations are consistent with the characteristics of transformed cells and reflects their ability to become invasive as tumours following injection into a mouse.

T15+ cells proliferate more rapidly and have a greater turnover of inositol phospholipids than non-transformed cells (see figures 3.1 and 3.2). This indicates that there may be a role for increased inositol phospholipid turnover in cell proliferation and transformation.In figure 3.1, the lack of a lag phase in [^{3}H] thymidine incorporation in T15+ cells may be due to the non-synchronous, non -quiescent cell population. These cells do not become quiscent when grown in 1% calf serum and DMEM unlike the non-transformed cells, which indicates the reduced growth factor dependence of transformed cells. NIH3T3 and T15- cells do not exhibit significant thymidine incorporation until after 18 hours of exposure to 10 % calf serum. This probably reflects the initiation of a new cycle of mitosis after 18 hours in these synchronous cell populations.

Figure 3.2a indicates that there is [^{3}H] inositol incorporation into inositol phospholipids in T15+ cells as compared to T15- cells (figure 3.2b) and this suggests a role for PtdIns 4,5 P_2 hydrolysis in cell proliferation and transformation. The incorporation of [^{3}H] inositol into PIP and PIP_2 in NIH3T3 cells reaches a steady level after 12 hours (figure 3.2b) although the rate of incorporation of [^{3}H] inositol continues to increase in T15+ cells (figure

3.2a). This may be due to the inhibition of mechanisms that normally desensitise inositol phospholipid turnover in normal cells in the transformed cells.This increase in inositol phospholipid turnover in T15+ cells could be due to an increased basal inositol phosphate production on p21 $^{N\text{-}ras}$ transformation. From the data shown in figure 3.3, it is clear that the basal rate of inositol phospholipid turnover is the same in T15+ and NIH3T3 cells and suggests that there is increased receptor-mediated PtdIns 4,5 P_2 hydrolysis in p21 $^{N\text{-}ras}$ transformed cells. Therefore, the increase in inositol phospholipid turnover in T15+ cells as shown in figure 3.2a cannot be explained by increased basal production of inositol phosphates but must occur by a different mechanism.

The data in figure 3.4 demonstrates that in T15+ cells there is an amplification of stimulated inositol phosphate production in response to gastrin-releasing peptide and bombesin and to a lesser extent, calf serum and bradykinin. This amplification is not due to a change in bombesin or GRP receptor number in T15+ cells (Wakelam et al, 1986) nor is it due to a change in receptor binding characteristics as a consequence of ras transformation (figures 3.6 and 3.7).The amplification of bombesin induced inositol phosphate production in T15+ cells is not due to autocrine secretion of growth factors like TGFα which have been shown to bind to the EGF receptor. This possibility has been ruled out by the fact that EGF does not stimulate significant inositol phosphate production in T15 cells (figure 3.4) and that EGF and bombesin stimulate inositol phosphate production to the same extent as bombesin alone (Wakelam et al, 1986). Therefore, EGF and by extension, TGFα cannot induce inositol phosphate production in this cell line.

The data in figure 3.4 also shows that vasopressin has no effect on inositol phosphate production in T15 cells and this indicates that this cell line may not have V_1 receptors, that is, vasopressin receptors that are linked to the phosphoinositide signalling system. PDGF did not stimulate inositol phosphate production in T15 cells although this may have been due to the relatively impure PDGF preparation used in these experiments. It has been demonstrated since

these experiments were performed that highly purified PDGF will stimulate inositol phosphate production in NIH3T3 and T15 cells although there is no amplification of the response in ras transformed cells (Black, F., Gardner, S. and Wakelam, M.J.O. unpublished data). Indeed, a desensitisation of PDGF stimulated inositol phosphate production in ras transformed cells has been reported in the literature (See Discussion chapter).

EGF, as already discussed, does not stimulate inositol phosphate production in T15 cells although it has been shown to be a potent mitogen in this cell line (Wakelam et al, 1986). The effects of EGF on mitogenesis are clearly established although the mechanism(s) by which this occurs is unclear. Besterman et al (1986) have demonstrated that inositol phospholipid hydrolysis is not associated with EGF induced mitogenesis of BALB/c3T3 fibroblasts. It has also been shown that EGF stimulated inositol phosphate production occurs via a distinct mechanism as compared to bradykinin stimulated inositol phosphate production in A431 cells (Tilly et al, 1988). EGF stimulates a small accumulation of Ins 1,4,5 P_3 after a lag period in these cells as compared to bradykinin although there is an associated EGF induced rise in cytoplasmic free calcium.Thus, it is becoming increasingly clear that the mechanism of inosit phosphate generation by EGF is distinct from that caused by other mitogenic peptides. The fact that EGF does not induce significant inositol phosphate production in the T15 cells line but does act as a mitogen is not surprising when the facts above have been considered. The mode of action of EGF may be due, in part, to the tyrosine kinase activity of the EGF receptor.

The results in figure 3.4 demonstrate that the mammalian eqivalent of bombesin, GRP, can stimulate inositol phosphate production in T15 cells to the same extent as bombesin.The binding characteristics of the receptors for these peptides appear to be similar and the Ka values for bombesin and GRP are within the same order of magnitude (see figures 3.6 and 3.7). Since the Ka values are very similar, it is probable that bombesin and GRP bind to the same receptor on T15 cells. This is clinically relevant as it has been shown that small cell lung cancer (SCLC) cells secrete bombesin and bombesin like peptides in

an autocrine fashion and that the peptides bind to the same receptor (Cuttita et al, 1985; Alexander et al, 1988). This autocrine secretion of bombesin and its related peptides is associated with the development of the tumouregenic state and indicates that bombesin and gastrin releasing peptide can have important growth factor roles in some cells.

The data in figure 3.8 shows that p21 $^{N\text{-}ras}$ induced amplification of inositol phosphate production can be detected approximately 6 hours after dexamethasone treatment of T15- cells. The production of inositol phosphates in response to bombesin decreases at 12 hours and it is possible that this effect is due to reduced synthesis of p21$^{N\text{-}ras}$ mRNA at this time point. Owen and Ostrowski (1987) have shown that dexamethasone induced expression of steroid-inducible p21 $^{Ha\text{-}ras}$ in NIH3T3 cells is reduced after 12 hours of dexamethasone treatment as indicated by the levels of mRNA for p21 $^{Ha\text{-}ras}$ in their cell system. It is possible that these effects are peculiar to the expression of the ras proto-oncogene linked to a dexamethasone-inducible promoter. It has been shown that dexamethasone induction of p21 $^{N\text{-}ras}$ is responsible for cell proliferation and transformation (McKay, et al 1986). Increased stimulated inositol phosphate production can be correlated with an increasing concentration of dexamethasone, and therefore, an increasing amount of p21 $^{N\text{-}ras}$ (Wakelam, et al 1987) .These data 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 .

The inositol phosphate response to bombesin is not pertussis toxin sensitive in T15^{+}cells although cholera toxin reduces stimulated inositol phosphate levels by approximately 40%. Low and Hughes (1987) have proposed that a novel G protein exists which is cholera toxin sensitive and inhibits inositol phosphate production in anterior pituitary cells. However, Guillon et al (1988) have shown that cholera toxin reduces the number of vasopressin receptors in WRK-1 cells, thus reducing vasopressin stimulated inositol phosphate production. Gardner et al (1989) have demonstrated that

inhibition of stimulated inositol phosphate production in L6 cells induced by cholera toxin is mediated by cAMP and is an effect upon receptor number. We have shown that cholera toxin does not alter the number of bombesin receptors in T15 cells although raising intracellular cyclic AMP levels results in an inhibition of bombesin stimulated inositol phosphate production (Milligan et al, in preparation). Therefore, the inhibition of bombesin stimulated inositol phosphate production in T15 cells could be due to a novel cholera toxin activated G protein or may be due to to an effect of cAMP in these cells. However, the data in figure 3.10 has demonstrated that there are no novel cholera toxin sensitive G protein substrates in T15 cells. Also, it has been shown that increasing cyclic AMP concentrations in T15+ cells by either forskolin treatment or via a β adrenergic agonist in the presence of a phosphodiesterase inhibitor has no effect on bombesin stimulated inositol phosphate production (Milligan et al, in preparation).

Recently, it has been demonstrated that the bombesin receptor is coupled to a pertussis toxin insensitive G protein in various cell types including Swiss 3T3 cells (Fischer and Schonbrun, 1988) and it is possible that this G protein acts as G_p in T15 cells. It is also possible that p21 $^{N\text{-}ras}$ can act as G_p or can interact with G_p as an α subunit in this cell line due to the pertussis toxin insensitivity of the bombesin stimulated inositol phosphate response in T15+ cells and may explain the post-receptor effects of p21$^{N\text{-}ras}$ on stimulated inositol phosphate production. The pertussis toxin insensitivity of this response further implies that p21$^{N\text{-}ras}$ does not amplify the effects of G_i in this cell line and that G_i is not involved in modulating stimulated inositol phosphte production.

Therefore, the results presented in this chapter indicate that the effects of p21 $^{N\text{-}ras}$ overexpression in the T15 cell line on cell proliferation and transformation may be mediated in part by the amplification of growth factor stimulated inositol phosphate production in T15+ cells.

Chapter 4: Bombesin stimulated Ins 1,4,5 P_3 production in T15 cells.

Introduction

In Chapter 3, the effects of p21 $^{N\text{-}ras}$ upon stimulated inositol phosphate production have been discussed. Although total inositol phosphate production can be an indication of PtdIns 4,5 P_2 hydrolysis as a result of phosphoinositidase activation, it could also occur as a consequence of PtdIns hydrolysis (Majerus et al, 1988). To obtain conclusive evidence as to which of these processes occured, the separation of inositol phosphates from stimulated T15 cells was performed.

Cell extracts were prepared from control and stimulated cells in the presence of 10mM lithium chloride and these extracts were applied to Dowex formate columns to separate the different inositol phosphates. This method of separation will resolve inositol mono-, bis-, tris- and tetrakisphosphates (Berridge et al, 1982) but will not separate the various isomers of these compounds. Therefore, cell extracts from basal and stimulated T15 cells were also applied to a Partisil 10 SAX column and the inositol phosphates separated by HPLC.

These methods allowed the investigation of the mechanisms of bombesin induced hydrolysis of inositol phospholipids in T15 cells. From the data obtained in figure 3.4, Chapter 3, bombesin and GRP stimulated the largest production of inositol phosphates in T15 cells compared to the other peptides tested. Since bombesin has been demonstrated to bind to the same receptor as GRP, bombesin was used as an agonist for stimulated inositol phosphate production in these cells.

Bombesin has been shown to stimulate inositol phosphate production in fibroblasts (Heslop et al, 1986) and to act as a mitogen for Swiss 3T3 (Rozengurt and Sinnet-Smith, 1983) and NIH3T3 cells (Wakelam et al, 1986). Thus, the stimulation of inositol phosphate production by bombesin can be an initial signal for cell proliferation in T15 cells if Ins1,4,5 P_3 is generated as a

second messenger.

All experiments shown in this chapter were performed using a maximal concentration of bombesin as determined from figure 3.6, Chapter 3, which was 2.5μM. This concentration of bombesin was shown to elicit maximal inositol phosphate generation in T15 cells.

Results

Figures 4.1a-1c show the separation of inositol phosphates in basal and stimulated T15- cells extracts by Dowex anion exchange chromatography. The experiment was performed as a time-course of bombesin stimulated inositol phosphate production.

Figure 4.1a shows that there was significant production of inositol monophosphate (IP) at 20 seconds of bombesin stimulation. The level of IP decreased at 30 seconds but increased up to 120 seconds. The decrease in IP levels at 30 seconds may be due to experimental error.Figure 4.1b shows that there is an increase in inositol bisphosphate (IP_2) production on bombesin stimulation of T15- cells between 20 seconds and 30 seconds. At 60 seconds there was a decrease in IP_2 production in this cell line on bombesin stimulation which remained at this level for up to 120 seconds. Although there is no significant production of inositol trisphosphate (IP_3) as shown in figure 4.1c, there are probably low, undetectable levels produced on bombesin stimulation which lead to increases in IP and IP_2 with time.Therefore, in T15- cells there was no detectable increase in IP_3 production on bombesin stimulation although there were significant increases in IP and IP_2.

Figure 4.1: Time-dependent bombesin stimulated inositol phosphate production in T15- cells. Separation by Dowex anion exchange chromatography.

The results in the figure are means ± S.D., n=3 and are from one experiment. Figure 4.1a shows inositol monophosphate accumulation in T15- cells stimulated with bombesin over a period of120 seconds.Figure 4.1b and 4.1c show inositol bisphosphate and trisphosphate respectively in bombesin stimulated T15- cell extracts over the same time period. The experimental results are representative of 4 separate experiments.

Figure 4.1a

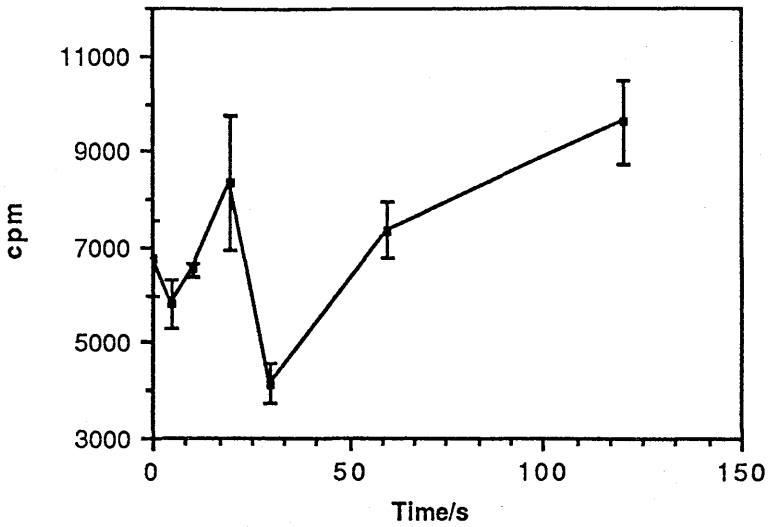


Figure 4.1b

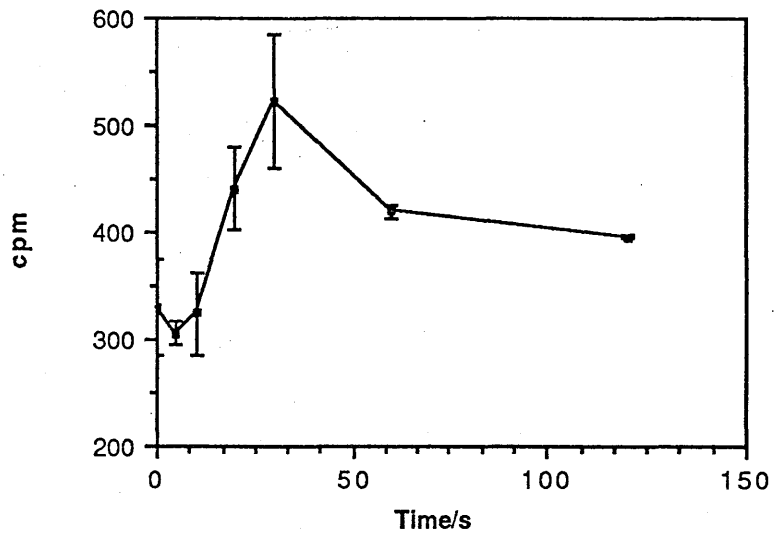
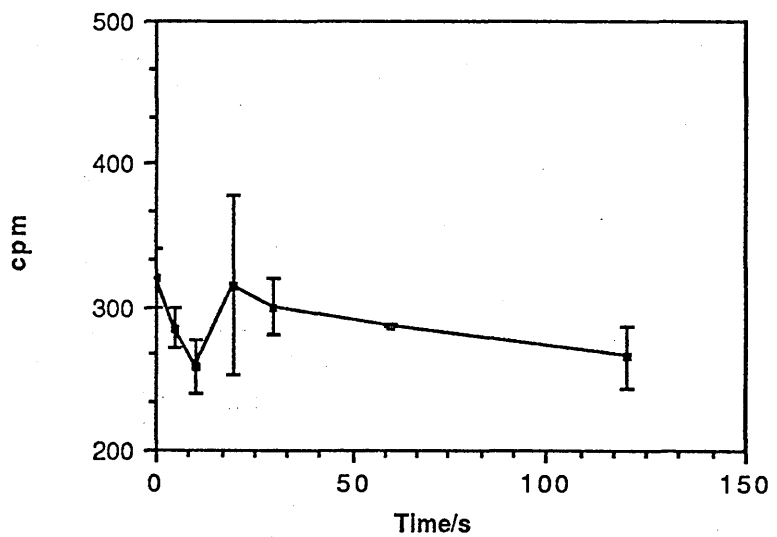


Figure 4.1c



A similar experiment to that in figure 4.1 was performed using T15+ cells to investigate the characteristics of bombesin stimulated inositol phosphate production in this cell type. Cell extracts prepared from control and bombesin stimulated T15+ cells were applied to Dowex formate columns and the results of a time-course experiment are shown in figure 4.2a-2c. Figure 4.2a shows the production of IP in bombesin stimulated T15+ cells which was first significant after 10 seconds of bombesin stimulation. The production of IP increased in a linear fashion up to 120 seconds and the stimulation of IP production was approximately 3 fold compared to basal levels.

The production of IP_2 in bombesin stimulated T15+ cells is shown in figure 4.2b and was significant at 10 seconds. The level of IP_2 then remained steady after 60 seconds, suggesting that the IP_2 was being dephosphorylated to IP at the same rate that it was being produced.

Bombesin stimulated IP_3 production in T15+ cells is shown in figure 4.2c. Peak production occured at 10 seconds after which there was a decrease to a level above the basal at 120 seconds. The production of IP_3 was rapid and transient on bombesin stimulation and is consistent with its role as a second messenger. The stimulation of IP_3 production in this experiment was 240% of the basal at 10 seconds following bombesin stimulation.

The data shows that in T15+ cells there was significant production of IP_3 in response to bombesin stimulation.The stimulation of IP_3 varied from 160-300% of the basal between experiments and was not associated with cell density i.e. the confluency of cells used in these experiments did not affect the stimulation of IP_3 production, although Wakelam (1988) has demonstrated that confluent T15+ cells exhibit a smaller bombesin stimulated inositol phosphate response than non-confluent T15+ cells. This does not appear to be the case in terms of IP_3 production based on the results of these experiments and may be due to the different labelling conditions employed.

Figure 4.2: Time-dependent bombesin stimulated inositol phosphate production in T15+ cells.Separation by Dowex anion exchange chromatography.

The results in figure 4.2 are means ± S.D., n=3 and are representative of six experiments. Figure 4.2a indicates inositol monophosphate production in bombesin stimulated T15+ cells for up to 120 seconds. Figures 4.2b and 4.2c indicate inositol bisphosphate and inositol trisphosphate production respectively in bombesin stimulated T15+ cell extracts over the same time period.

Figure 4.2a

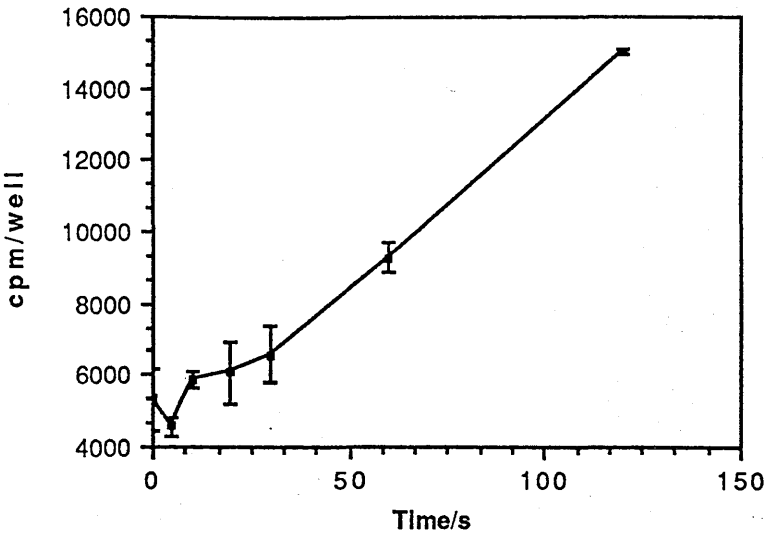


Figure 4.2b

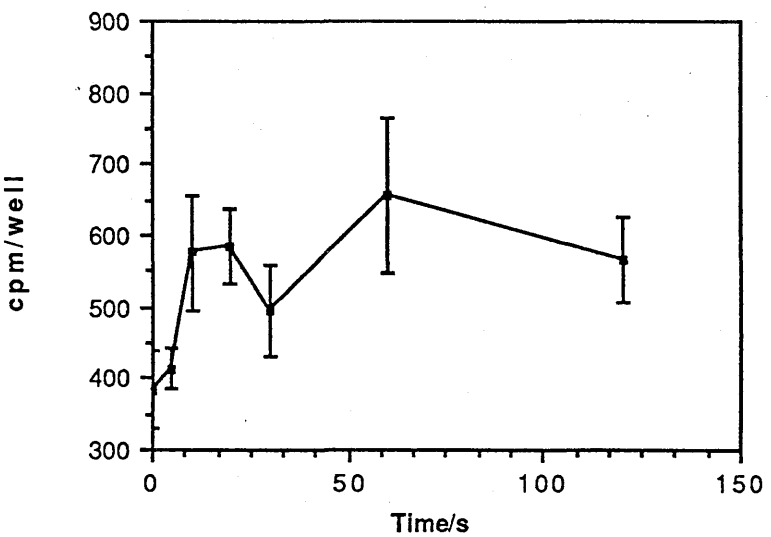
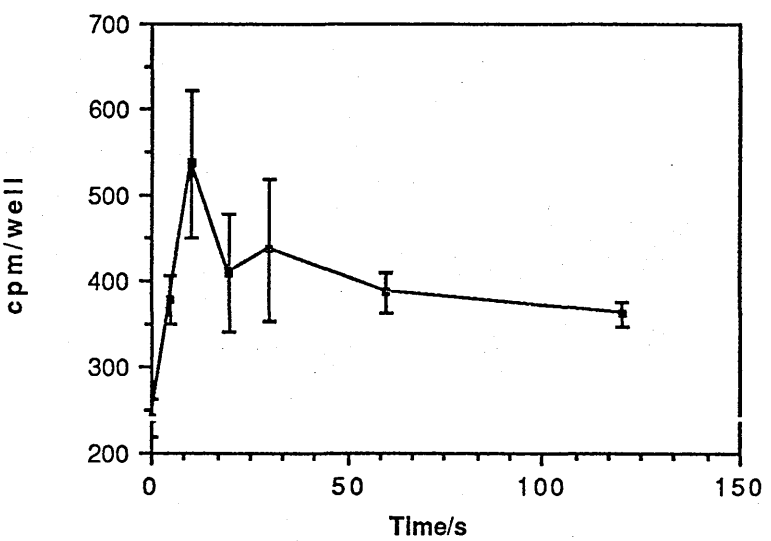


Figure 4.2c



The HPLC technique is used to separate inositol phosphates for several reasons. Firstly, it is more conveniant than Dowex anion exchange chromatography and a detailed elution profile of a given sample can be obtained more rapidly. Secondly, unlike Dowex chromatography, the use of HPLC can resolve the individual isomers of inositol phosphates . Lastly, the HPLC method can be used to isolate the higher inositol phosphates, for example, inositol pentakisphosphate.

Cell extracts prepared from basal and stimulated T15- cells were therefore analysed by HPLC to obtain additional information not provided by Dowex anion exchange chromatography. The elution profile of extracts from T15- cells stimulated for 5 seconds with bombesin is shown in figure 4.3.

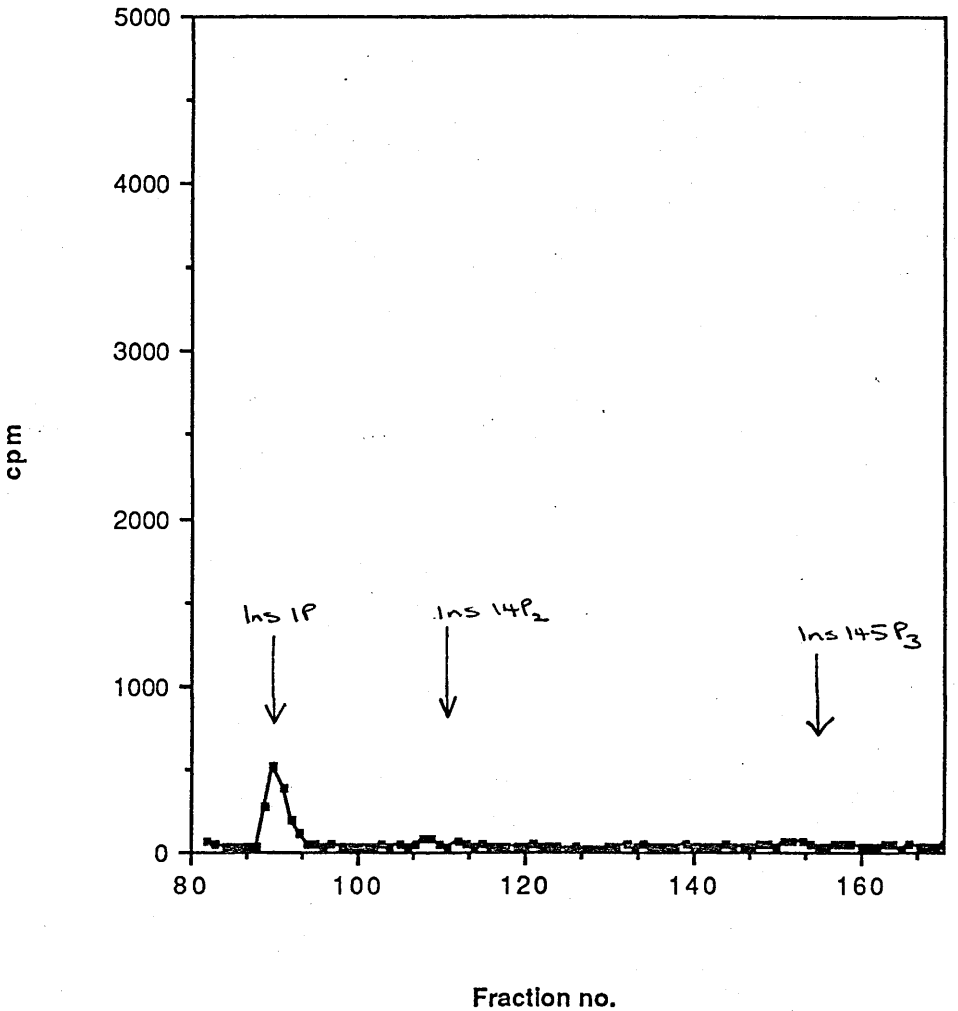
The figure illustrates that following a 5 second stimulation with bombesin, there was no significant production of IP_3 or any of its isomers, nor of IP_2 in T15- cells. There is only detectable production of IP. This IP co-elutes with an Ins 1 P standard and therefore, using our HPLC technique, the IP generated in bombesin stimulated T15- cells is probably be Ins 1 P. From the elution profile, the single peak obtained indicates that no isomers of IP are formed.

Figure 4.3: HPLC elution profile of bombesin stimulated T15- cell extract.

The results shown in figure 4.3 show the HPLC elution profile of a T15- cell extract prepared from T15- cells stimulated with bombesin for 5 seconds as described in the Methods section. The data shown in the figure is from one sample within one experiment but is typical of two. The results are drawn to a large y-axis to allow direct comparison to figure 4.4.

The experiment was not carried out using cells that were labelled with an increased amount of [^{3}H] inositol because this approach would not be cost effective.

Figure 4.3


5000
4000
3000
2000
1000
0
cpm
Ins 1P
Ins 14P2
Ins 145P3
80
100
120
140
160
Fraction no.

Time-course experiments were performed on bombesin stimulated T15+ cells and the cell extracts were analysed by HPLC both to determine the characterisitics of bombesin stimulated inositol phosphate production production over a time period of 120 seconds and to also identify the isomers of inositol phosphates generated. Figure 4.4 illustrates the elution profile obtained by HPLC of an extract of T15+ cells stimulated for 5 seconds with bombesin.

The production of Ins 1 P in T15+ cells was much greater than in T15- cells (See figure 4.3) although this is not due to cell number differences between the two cell types. There was production of IP_2 on bombesin stimulation of T15+ cells and this peak was shown to be Ins 1,4 P_2 by its elution position being identical to a tritiated Ins 1,4 P_2 standard.

There was significant production of IP_3 on bombesin stimulation of T15+ cells as already demonstrated by Dowex anion exchange chromatography. However, the use of HPLC to separate the bombesin stimulated inositol phosphates demonstrated that this IP_3 consisted solely of the Ins 1,4,5 P_3 isomer.

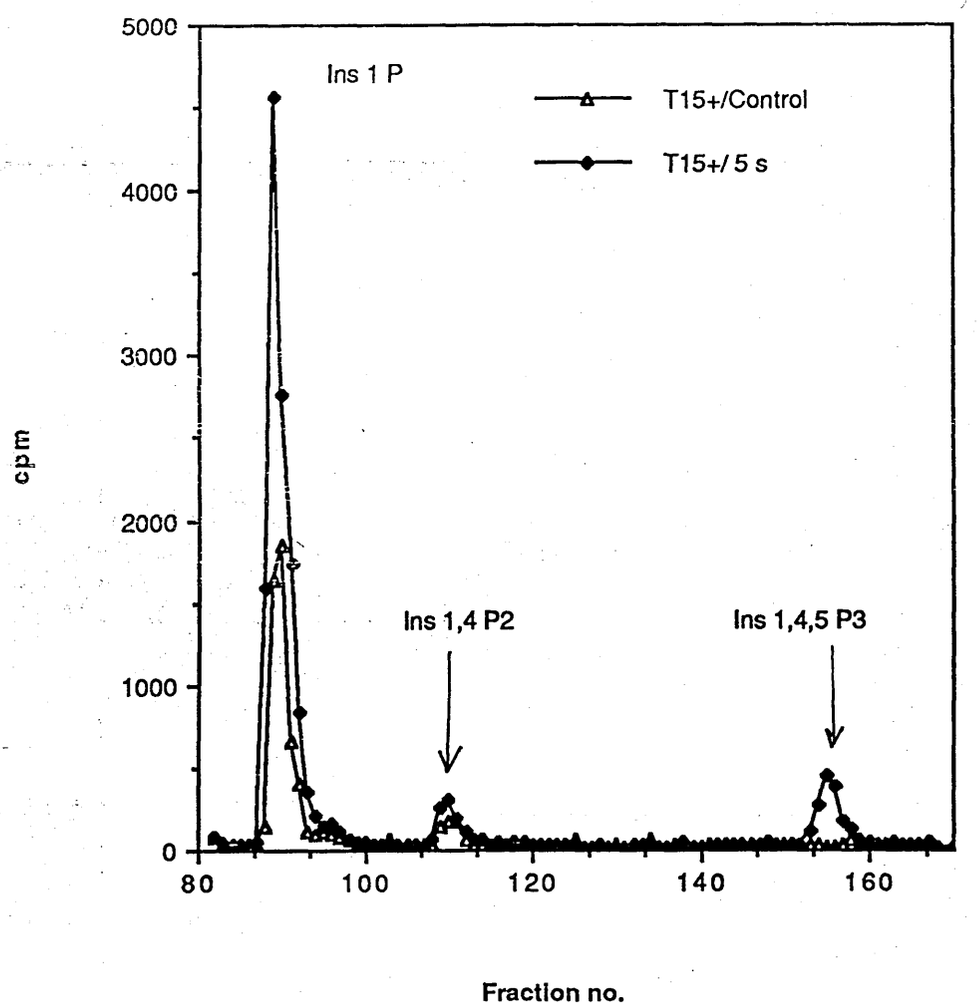
In T15+ cells, therefore, there are no other isomers of inositol phosphates produced in response to 5 second bombesin stimulation apart from Ins 1 P, Ins 1,4, P_2 and Ins 1,4,5 P_3. The Ins1P and Ins1,4 P_2 must be formed as a consequence of Ins 1,4,5 P_3 dephosphorylation.

Figure 4.4: HPLC elution profile of a bombesin stimulated T15+ cell extract.

The results in figure 4.4 are from an experiment to determine the inositol phosphates present in an extract prepared from T15+ cells stimluated for 5 seconds with bombesin. The experiment was carried out as already described and the data in the figure is from one sample within an experiment but is typical of three separate experiments.

In addition to the elution profile of a T!5+ cell sample stimulated for 5 seconds with bombesin, the elution profile of a control T!5+ sample has been included. The control elution profile indicates that there are significant levels of Ins 1 P and Ins 1,4 P_2 in this control sample although it is likely that the Ins 1 P and Ins 1,4 P_2 detected are a result of the accumulation of these inositol phosphates as a result of basal inositol phospholipid turnover in the presence of lithium chloride.

Figure 4.4


5000
4000
3000
2000
1000
0
cpm
Ins 1 P
T15+/Control
T15+/ 5 s
Ins 1,4 P2
Ins 1,4,5 P3
80
100
120
140
160
Fraction no.

The production of Ins1,4,5 P_3 in bombesin stimulated T15+ cells was determined by a time-course experiment. Bombesin was used to stimulate T15+ cells for various times up to 120 seconds and cell extracts prepared from each sample. The samples were applied to the HPLC column and from the elution profiles, the total amount of Ins1,4,5 P_3 produced in each sample was calculated by the sum of the cpm values of all the fractions within the Ins1,4,5 P_3 peak. These values were then expressed as % stimulation of the basal levels.

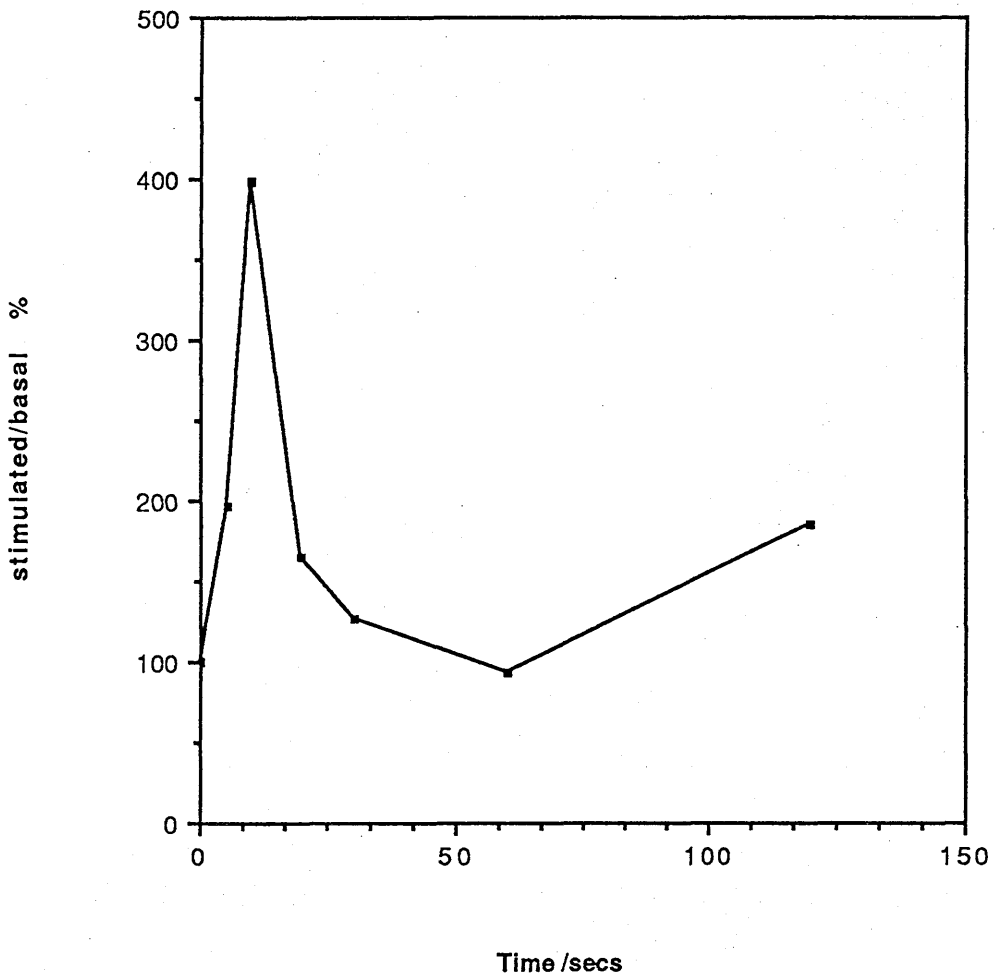
Figure 4.5 shows that the generation of Ins1,4,5 P_3 in bombesin stimulated T15+ cells was rapid and reached a peak at 10 seconds. The level of Ins1,4,5 P_3 then declined to a level above the basal by 60 seconds.

This data correlates with that obtained by Dowex chromatography (See figure 4.2c) and indicates that bombesin stimulates Ins1,4,5 P_3 production in T15+ cells as a result of phosphoinositidase hydrolysis of PtdIns 4,5 P_2.

Figure 4.5: Time-course of Ins1,4,5 P_3 production in T15+ cells.

The data in figure 4.5 is from an experiment to determine the time-course of Ins 1,4,5 P_3 production in bombesin stimulated T15+ cells.The results are obtained from HPLC analysis of bombesin stimulated T15+ cell extracts performed over a time period of 120 seconds as described. The results are expressed as % stimulated/basal levels of Ins 1,4,5 P_3 and are from one experiment but the data is typical of three separate experiments.

Figure 4.5



The data obtained in the previous experiments showed that there was no significant production of IP_3 at any time point in T15- cells (See figure 4.1c), Also, HPLC data showed that there was no significant production of Ins1,4,5 P_3 in T15- cells at 5 seconds, unlike T15+ cells.

Figure 4.6 shows part of the HPLC elution profiles of control and 10 second bombesin stimulated T15- and T15+ cells. The data shown only indicates the part of the gradient between fractions 140-170; Ins 1,4,5 P_3 elutes between fractions 150-160.

Figure 4.6a clearly shows that the peak time of Ins 1,4,5 P_3 generation in T15+ cells was at 10 seconds. There is no significant generation of Ins 1,4,5 P_3 in T15- cells (figure 4.6b). In this experiment, the T15- cells had an apparent higher basal rate than the T15+ cells and this could be due to the different HPLC columns used for the two experiments.

The control elution profiles in the Ins1,4,5 P_3 region of T15- and T15+ cells were similar to the 10 second elution profile of T15- cells (figures 4.6a and 4.6b). At no time tested was there a detectable increase in Ins 1,4,5 P_3 production in bombesin stimulated T15- cells.

Figure 4.6: HPLC elution profile of extracts prepared from 10 second bombesin stimulated T15 cells.

Figure 4.6a shows the HPLC elution profile obtained from the analysis of T15+ cell extracts prepared from control T15+ cells and T15+ cells stimulated with bombesin for 10 seconds. The region of Ins 1,4,5 P_3 elution from the Partisil SAX column is shown and the data is from one experiment which is typical of three.Figure 4.6b is the data from a similar experiment using T15- cells and shows the results from HPLC analysis of cell extracts prepared from T15- cells which were either unstimulated or stimulated with bombesin for 10 seconds. The data is from one experiment representative of two separate experiments.

Figure 4.6a

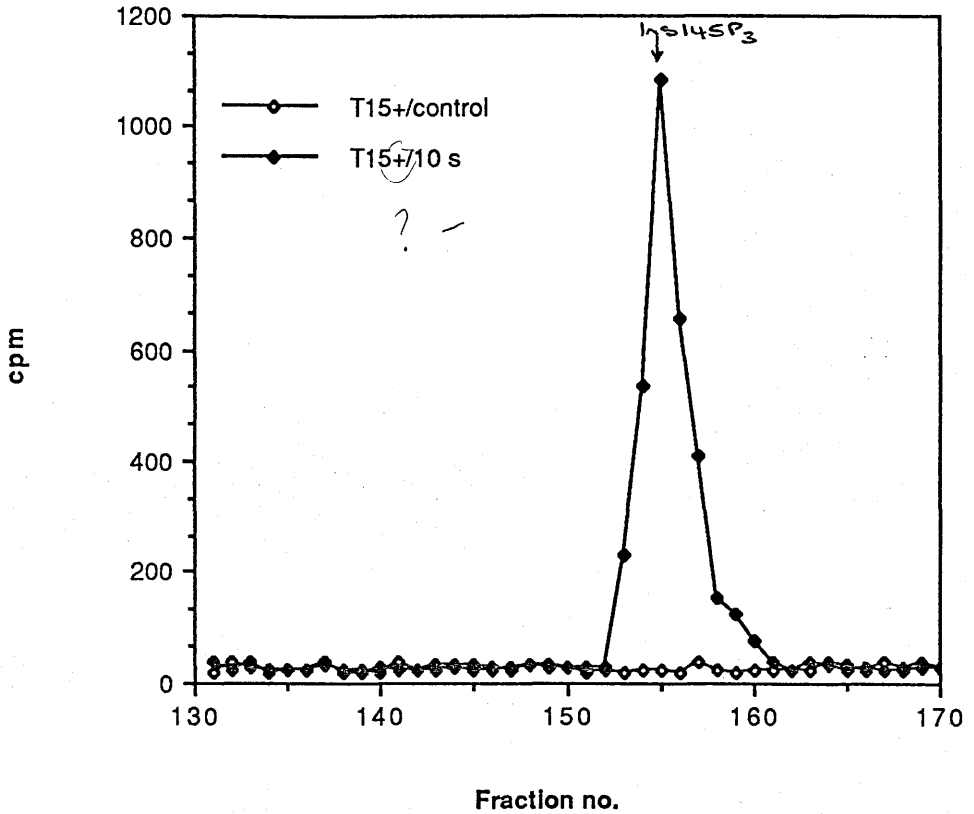
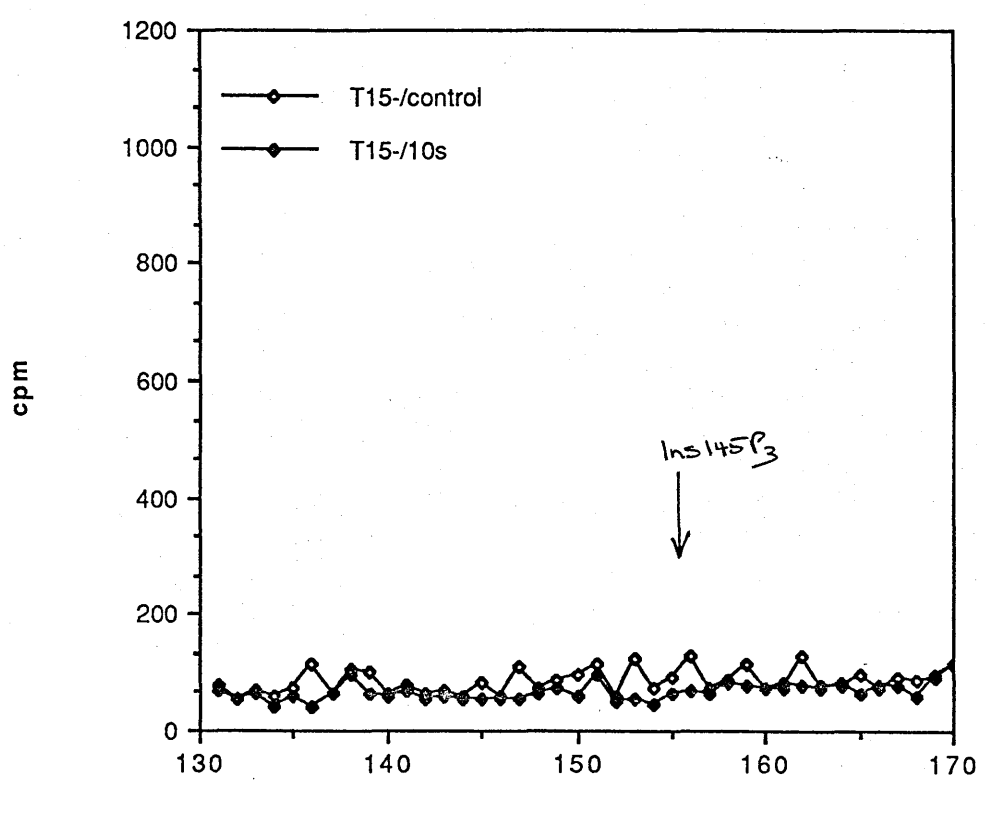


Figure 4.6b



Discussion

The data shown in this chapter illustrates that consistent results can be obtained using two different techniques to separate inositol phosphates in T15 cells. The results obtained from experiments using Dowex anion exchange chromatography to separate inositol phosphates in T15- cells (Figure 4.1) demonstrated that there was no significant production of IP_3 on bombesin stimulation. Although this result was obtained from one experiment, other experiments also failed to demonstrate an increase in IP_3 production in bombesin stimulated in T15- cells.

Since there was significant IP_2 and IP produced on bombesin stimulation of T15- cells, it must be concluded that there is IP_3 production on bombesin stimulation but in such small amounts as to be undetectable by the assay method. This would be a reasonable assumption as there is a small increase in total inositol phosphate production as compared to basal levels in bombesin stimulated T15- cells (See chapter 3) and this increase in total inositol phosphates, although consists mostly of IP is probably due to IP_3 production and its sequential dephosphorylation. However, the possibility that the IP and IP_2 generated in bombesin stimulated T15- cells as a consequence of PtdIns or PtdIns 4 P breakdown can be ruled out. This is due to the fact that there was no significant generation of IP and IP2 (figures 4.1a and 4.1b) in cell extracts prepared from bombesin stimulated T15- cells until 30 seconds following bombesin stimulation. If these inositol phosphates are generated as a consequence of PtdIns and PtdIns 4 P breakdown, then the production of IP and IP_2 would occur much more rapidly.

Analysis by HPLC data of extracts prepared from bombesin stimulated T15- cells (See figure 4.3) also showed no detectable increase in Ins 1,4,5 P_3 production. This is consistent with the data obtained from Dowex chromatography. The Ins 1 P peak in the elution profile must arise from Ins1,4,5 P_3 as a consequence of its sequential dephosphorylation and therefore, although there is no detectable Ins 1,4,5 P_3, it must be produced as a

consequence of bombesin stimulated PtdIns 4, 5 P_2 hydrolysis.

In contrast, T15+ cells exhibited a rapid, transient increase in Ins 1,4,5 P_3 production in response to bombesin stimulation. This increase reaches a peak at 10 seconds and has been demonstrated both by HPLC and Dowex chromatography (Figures 4.5 and 4.2 respectively). The generation of IP_3 as detected by Dowex chromatography in figure 4.2 when analysed by HPLC was shown to shown to consist solely of Ins 1,4,5 P_3 (figure 4.4). It is clear from figure 4.4 and figure 5.1 in chapter 5 that no Ins 1,3,4 P_3 is generated in T15+ cells following stimulation with 2.5μM bombesin. Therefore,the inositol phosphates generated in response to bombesin stimulation of T15+ cells are a consequence of Ins 1,4,5 P_3 production and that the assay for total inositol phoshates in control and stimulated T15+ cells is an accurate estimate of PtdIns 4,5 P_2 hydrolysis. Thus,the production of inositol phosphates in T15+ cells is a consequence of receptor mediated PtdIns 4,5 P_2 hydrolysis and not PtdIns hydrolysis as the generation of IP_3 preceded any significant increase in IP as shown in figure 4.2 of this chapter.

The data in figure 4.4 shows that bombesin can stimulate rapid Ins 1,4,5 P_3 production in T15+ cells which leads to an increase in Ins 1,4 P_2 and Ins 1 P as a consequence of sequential dephosphorylation of Ins 1,4,5 P_3. The generation of the Ca^{2+} mobilising second messenger, Ins1,4,5 P_3, has been shown to occur in response to bradykinin in NG115-40IL cells (Jackson et al, 1987) and A431 cells (Tilly et al, 1987) and also in Swiss 3T3 cells stimulated with PDGF (Berridge et al, 1984). It is generally accepted that the production of Ins1,4,5 P_3 in these cells in response to growth factor agonists is a consequence of PtdIns 4,5 P_2 hydrolysis by the phosphoinositidase enzyme.

The percentage stimulation of Ins1,4,5 P_3determined from Dowex chromatography in T15+cells is variable between 160-300% of the basal. The reasons for this are unclear, but this variability is not associated with cell density, although it has been demonstrated that high density culturing leads to an inhibition of bombesin stimulated inositol phosphate production in T15+ cells (Wakelam, 1988). Recently, it has been demonstrated that in T15+ cells, there is an amplification of bombesin

stimulated increase in intracellular Ca^{2+} levels compared to T15- cells.(Lloyd et al, submitted for publication). The amplified bombesin stimulated increase in intracellular Ca^{2+} levels in T15+ cells is due to the responses in individual cells and is not due to a larger number of T15+ cells in a population responding to the agonist compared to a much smaller number of T15- cells. This has been verified by the measurement of intracellular Ca^{2+} changes in single T15 cells stimulated with 2.5μM bombesin indicated by changes in Fura-2 fluorescence. Using this technique, it has been estimated that the bombesin stimulated increase in intracellular Ca^{2+} is on average 155% over basal levels in T15+ cells while in T15- cells the bombesin stimulated increase in intracellular Ca^{2+} is 39% over basal levels. However,this response is variable between cells, for example, the increase in intracellular Ca^{2+} stimulated by bombesin in T15 cells varies from 87% to 160% and it has been postulated that there are different levels of p21 $^{N\text{-}ras}$ in each cell. Therefore, the variability in Ins1,4,5 P_3 production may be a reflection of the amount of p21 $^{N\text{-}ras}$ in each cell in a particular cell population.

The basal levels of Ins 1,4,5 P_3 in T15- and T15+ cells from HPLC experiments were the same (approximately 350 cpm per well) and indicate that there is no change in the basal rate of Ins 1,4,5 P_3 production between the cell types. This supports the results obtained in figure 3.3, chapter 3 where it was demonstrated that the rate of inositol phospholipid turnover in these cells was the same.

Experiments to determine the levels of bombesin stimulated inositol phosphates in NIH3T3 cells demonstrated that the response of these cells to bombesin was the same as T15- cells. There was no detectable Ins1,4,5 P_3 production although there was Ins 1 P and Ins 1,4 P_2 produced on bombesin stimulation. Therefore, NIH3T3 and T15- cells respond in the same way to agonists linked to the phosphoinositide signalling pathway.

However, NIH3T3 cells stimulated with PGF 2α produce high levels of Ins1,4,5 P_3 and Ins1,3,4,5 P_4 and its dephosphorylated derivatives, Ins 1,3,4 P_3, Ins 3,4 P_2 and Ins 4 P which can be resolved by HPLC (Black,F.

and Wakelam, M.J.O., manuscript in preparation). T15 cells exhibit an inhibition of PGF2α induced inositol phosphate production which may be a consequence of activated protein kinase C.Activation of protein kinase C may be due to p21 $^{N\text{-}ras}$ overexpression and could result in phosphorylation of PGF2α receptors.

The results clearly show that in T15+ cells there is amplified Ins 1,4,5 P_3 production in response to bombesin as compared to T15- cells. The only isomers of IP, IP_2 and IP_3 detectable are Ins1 P, Ins 1,4 P and Ins 1,4,5 P_3 which implies that there is no Ins 1,3,4, 5 P_4 synthesised in these cells in response to bombesin stimulation, or that the levels of Ins 1,3,4,5P_4 are so low as to be undetectable. The production of Ins1,4,5 P_3 must occur as a consequence of receptor-mediated PtdIns 4,5 P_2 hydrolysis by the phosphoinoisitidase enzyme.

The amplification of bombesin stimulated inositol phosphates in T15+ cells as a result of Ins1,4,5 P_3 production as compared to T15- cells has to be due to post-receptor modifications in T15+ cells for reasons already discussed in chapter 3. It has been shown that bombesin and PDGF induced PtdIns 4,5 P_2 hydrolysis are early events in mitogenesis and that microinjection of an antibody to PtdIns 4,5 P_2 will abolish growth factor induced mitogenesis in NIH3T3 cells (Matuoka et al, 1988). Also, bombesin induces c-myc and c-fos expression in quiescent Swiss 3T3 cells (Bravo et al, 1987) and this may be due to bombesin induced production of Ins 1,4,5 P_3 with subsequent intracellular Ca^{2+} mobilisation. Thus, the production of Ins1,4,5 P_3 may be an initial signal for cell proliferation in T15+ cells and may be generated at high levels in response to different growth factors as a result of p21 $^{N\text{-}ras}$ expression. In T15- cells this process may also occur, but at a very much lower rate so that uncontrolled proliferation does not occur.

The data in this chapter shows that p21$^{N\text{-}ras}$ may have a direct role in the amplification of bombesin stimulated Ins 1,4,5 P_3 production in NIH3T3 cells. The properties of p21^{ras} as already described, make it reasonable to consider that p21 $^{N\text{-}ras}$ may act as a guanine nucleotide binding protein to stimulate the phosphoinositidase enzyme on receptor occupancy. It may be in this capacity that the overexpressed N-ras proto-oncogene can cause uncontrolled cell proliferation and transformation.

Chapter 5: Characterisation of the NIH3T3 inositol 1,4,5 trisphosphate kinase enzyme.

Introduction

In chapter 4, bombesin stimulated inositol phosphate production in T15 cells was discussed. During the course of these experiments, it was noted that there was no detectable bombesin stimulated inositol 1,3,4,5 tetrakisphosphate (Ins 1,3,4,5 P4) production in T15 and NIH3T3 cells. Ins 1,3,4,5 P4 is formed by the phosphorylation of Ins 1,4,5 P3 by inositol 1,4,5 trisphosphate 3-kinase (Ins 1,4,5 P3 kinase) an enzyme whose presence has been demonsrated in, for example, brain slices and in Xenopus oocytes (Irvine et al, 1986).

The absence of Ins 1,3,4,5 P4 generation in bombesin stimulated T15 and NIH3T3 cells could be due to several factors. It is possible that this fibroblast cell line does not synthesize the Ins 1,4,5 P3 kinase enzyme and therefore, the conversion of Ins 1,4,5 P3 to Ins 1,3,4,5 P4 cannot be catalysed. To investigate this possibility, experiments to determine Ins 1,4,5 P3 kinase activity in T15 and NIH3T3 cytosol extracts were performed. In order to measure Ins 1,4,5 P3 kinase activity in these cell types, the conversion of [3H] Ins 1,4,5 P3 to [3H] Ins 1,3,4,5 P4 in the presence of a known amount of the cytosolic preparation was measured. The amount of Ins 1,3,4,5 P4 generated in these samples was quantified by separating and collecting the [3H] labelled Ins 1,3,4,5 P4 by either HPLC or Dowex anion exchange chromatography as utilised in chapter 4.

A second possibility that may explain the undetectable levels of Ins 1,3,4,5 P4 in T15 and NIH3T3 cells is that the level of Ins 1,4,5 P3 formed following bombesin stimulation in these cells are too low to allow Ins 1,3,4,5 P4 to be detected if it is formed. Thirdly, if the phosphorylation and dephosphorylation pathways of Ins 1,4,5 P3 exist in these cell types as already discussed (See Introduction), then it may be that the dephosphorylation of Ins 1,4,5 P3 to Ins 1,4 P2 occurs at a greater rate than

the phosphorylation of Ins 1,4,5 P3 to Ins 1,3,4,5 P4. These possibilities are discussed further in this chapter based upon the results from the experiments to determine Ins 1,4,5 P3 kinase activity in T15 and NIH3T3 cells.

Results

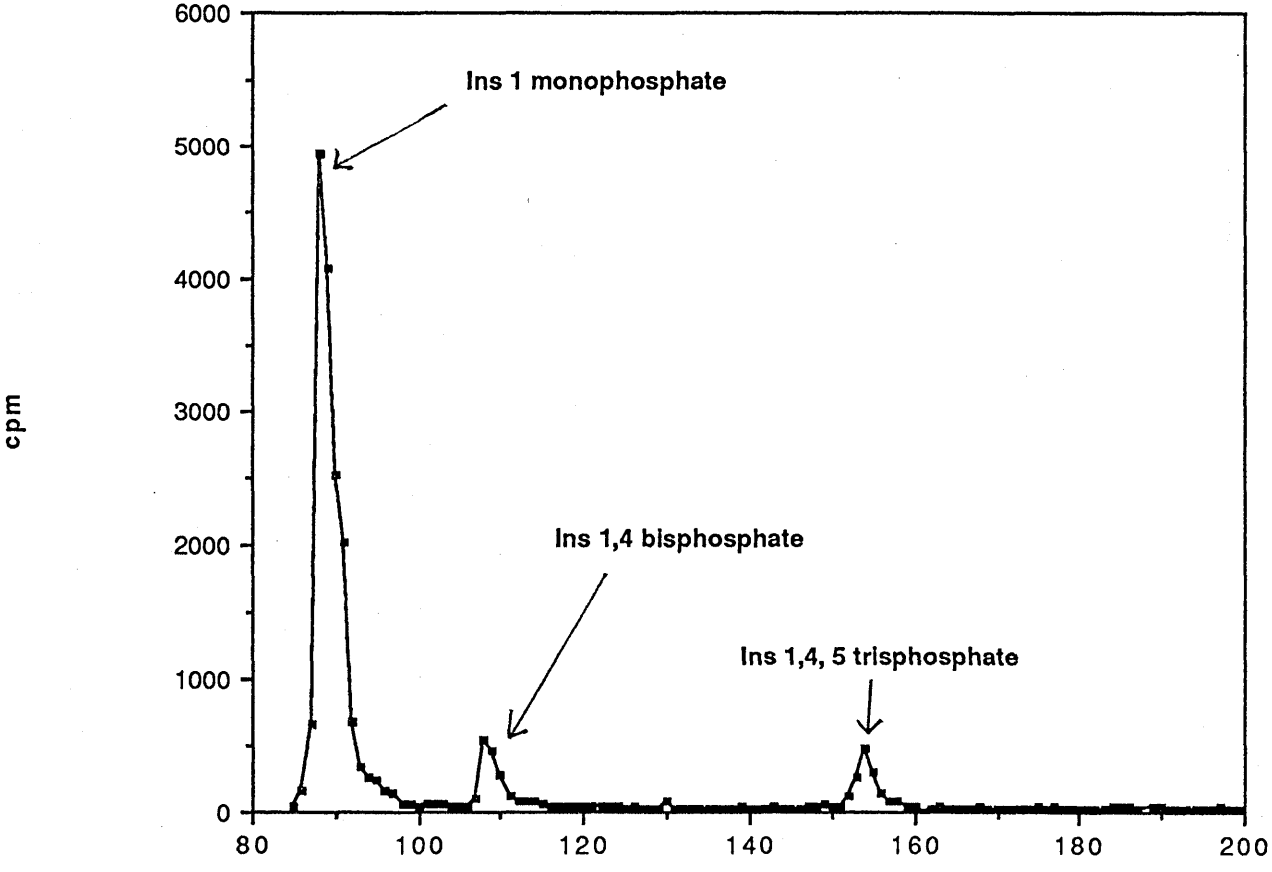
The results from the HPLC analysis of bombesin stimulated inositol phosphate production in T15 cells showed that there was no detectable Ins 1,3,4,5 P4 in this cell line and this has been discussed in Chapter 4. The results in figure 5.1 show the results from a 120 second bombesin stimulated T15+ cell extract prepared as described in Chapter 4 and analysed by HPLC to separate the different inositol phosphates.

Figure 5.1 clearly shows that at 120 second bombesin stimulation there is no detectable Ins 1,3,4,5 P_4 in T15+ cells. There are no different isomers of inositol trisphosphate, inositol bisphosphate and inositol monphosphate generated following bombesin stimulation as already discussed in Chapter 4. The figure shows that only inositol 1 monophosphate, inositol 1,4 bisphosphate and Ins 1,4,5 P3 are generated on bombesin stimulation of T15+ cells. Therefore, there are no isomers of inositol monophosphate, inositol bisphosphate and inositol trisphosphate that are products of Ins 1,3,4,5 P4 dephosphorylation for example Ins 1,3,4 P3, Ins 3,4 P2 or Ins 1,3 P2 and Ins 3 P. Therefore, it can be concluded that there is no Ins 1,3,4,5 P4 formation following bombesin stimulation of T15+ cells. Alternatively,the levels of Ins 1,3,4,5 P4 are so low as to be undetectable by this HPLC method. There was also no detectable Ins 1,3,4,5 P4 in bombesin stimulated T15- or NIH3T3 cells up to 120 seconds. Therefore, this lack of Ins 1,3,4,5 P4 is thus unlikely to be a consequence of p21 $^{N\text{-}\underline{ras}}$ transformation of NIH3T3 cells since it occurs in the wild-type and non-transformed T15 cells.

Figure 5.1: HPLC elution profile of an extract prepared from T15+ cells stimulated with bombesin for 120 seconds.

Figure 5.1 shows a HPLC elution profile of a T15+ cell extract prepared from T15+ cells stimulated with 2.5μM bombesin for 120 seconds as described in the Methods section. The data is from one sample within a single experiment but is representative of three separate experiments. The peaks are labelled to indicate the relevant inositol phosphates .

Figure 5.1


6000
5000
4000
3000
2000
1000
0
cpm
Ins 1 monophosphate
Ins 1,4 bisphosphate
Ins 1,4, 5 trisphosphate
80
100
120
140
160
180
200
Fraction no.

The absence of Ins 1,3,4,5 P_4 in bombesin stimulated T15 and NIH3T3 cells could be due to the absence of the Ins 1,4,5 P_3 kinase enzyme in this cell line. To examine this possibility, cytosolic cell extracts (high speed supernatants) were prepared from a homogenate of T15+ cells and incubated at 30°C for 5 minutes in the presence of 0.67 nmol of [^{3}H] Ins 1,4,5 P_3 (Specific activity = 1Ci mmol^{-1}) in incubation buffer A at pH 7 and at pH 9. The experimental procedure is described in detail in the Methods section. The incubations were terminated by the addition of perchloric acid and the neutralised supernatants analysed for Ins 1,3,4,5 P_4 by HPLC as already described.

Figure 5.2a shows the HPLC elution profile of a T15+ cell extract incubated with [^{3}H] Ins 1,4,5 P_3 at pH 9. It clearly shows the resolution of a peak between fractions 205-220 which coelutes with a [^{3}H] Ins 1,3,4,5 P_4 standard and is therefore probably Ins 1,3,4,5 P_4. The Ins 1,3,4,5 P_4 peak has a maximal value of 900 dpm. The peak obtained between fractions 155-160 is Ins 1,4,5 P_3 and the preceeding peak at fractions 144-154 is probably Ins 1,3,4 P_3. There are detectable isomers of inositol bisphosphate and inositol monophosphate which probably include Ins 1,4 P_2, Ins 3,4 P_2 or Ins 1,3 P_2 and Ins 4 P and Ins 1 P respectively.

Therefore, it has been demonstrated that phosphorylation of Ins 1,4,5 P_3 to Ins 1,3,4,5 P_4 can be catalysed by a T15+ cell extract at pH 9. However, it has been demonstrated that the phosphomonoesterase that dephosphorylates Ins 1,4,5 P_3 is inhibited at this pH (Downes et al, 1982). It is probable that this enzyme also dephosphorylates Ins 1,3,4,5 P_4 to Ins 1,3,4 P_3 (Majerus et al, 1988) . In T15+ cells there is dephosphorylation of Ins 1,3,4,5 P_4 to Ins 1,3,4 P_3 at pH 9 and therefore, the phosphatase in these cells is not completely inhibited at pH 9.

Figure 5.2b shows the elution profile of a replicate experiment performed at pH 7. At this pH, the Ins 1,3,4,5 P_4 peak has a maximal value of 750 dpm. There are small peaks of Ins 1,4,5 P_3 and Ins 1,3,4 P_3 but the

maximal value of the of the Ins 1 monophosphate peak has increased to 1000 dpm as compared to 350 dpm at pH 9. Therefore, at this pH, the Ins 1,4,5 P3 kinase is still active, but the dephosphorylation of Ins 1,4,5 P3 and Ins 1,3,4,5 P4 to inositol 1 monophosphate is probably more rapid than the phosphorylation reactions.

A value for the activity of the Ins 1,4,5 P3 kinase was obtained by calculating the total dpm for all fractions between and including 203-220. This gave a total dpm value for Ins 1,3,4,5 P4 produced and was converted into pmols Ins 1,3,4,5 P4 produced. The value obtained for the specific activity of the Ins 1,4,5 P3 kinase in this T15+ cytosolic preparation as determined by HPLC was 2.88 pmol Ins 1,3,4,5 P4 min^{-1} mg^{-1} protein at pH 9 and 2 pmol Ins 1,3,4,5 P4 min^{-1} mg^{-1} protein at pH 7. These values were consistent with those obtained from Dowex anion exchange chromotography of replicate samples which were 2.95 ± 0.01 at pH 9 and 1.96 ± 0.3 at pH 7 (n=2, means ± S.D.).

To determine if NIH3T3 cells showed evidence of possesing Ins 1,4,5 P3 kinase activity, similar experiments as those shown in figure 5.2 were performed using cytosolic preparations. The experiments shown in Figures 5.3a and 5.3b were performed under the same experimental conditions as those in figure 5.2. Figure 5.3a shows the HPLC elution profile of a sample of NIH3T3 cytosolic extract incubated with [^{3}H] Ins 1,4,5 P3 as already described at pH 9. There is a significant peak of Ins 1,3,4,5 P4 which had a maximal value of 875 dpm. The figure also shows a large peak of Ins 1,4,5 P3 with no significant peaks of Ins 1,3,4 P3, inositol bisphosphate isomers or inositol monophosphate isomers. It is clear that firstly, there is evidence of Ins 1,4,5 P3 kinase activity in NIH3T3 cells and secondly, that the dephosphorylation of Ins 1,4,5 P3 and Ins 1,3,4,5 P4 is completely inhibited at this pH unlike in T15+ cells.

Figure 5.3b shows the HPLC elution profile of a sample of NIH3T3 cytosolic extract incubated with [^{3}H] Ins 1,4,5 P3 as above but at pH 7. At this pH, the maximal value of the Ins 1,3,4,5 P4 peak was 550 dpm as

compared to 875 dpm in figure 5.3a. There was dephosphorylation of Ins 1,3,4,5 P_4 and Ins 1,4,5 P_3 at this pH as significant peaks of Ins 1,3,4 P_3 and Ins 1,4 P_2 and Ins 3,4 P_2 or Ins 1,3 P_2 are clearly present in figure 5.3b, associated with reduced levels of Ins 1,4,5 P_3 and Ins 1,3,4,5 P_4 as compared to the data from the experiment performed at pH 9 (figure 5.3a).

The specific activity of the Ins 1,4,5 P_3 kinase assayed in this sample of NIH3T3 cytosolic extract was calculated to be 4.3 pmol Ins 1,3,4,5 P_4 $min^{-1}mg^{-1}$ protein at pH 9 and 3.04 pmol Ins 1,3,4,5 P_4 $min^{-1}mg^{-1}$ protein at pH 7 from the HPLC data. These values were confirmed from data obtained by Dowex anion exchange chromatography of the samples and were 5.6 ± 0.4 pmol Ins 1,3,4,5 P_4 $min^{-1}mg^{-1}$ protein at pH 9 and 4.7 ± 0.6 pmol Ins 1,3,4,5 P_4 min^{-1} mg^{-1} protein at pH 7 (n=2, $\pm$ S.D.)

Figure 5.2: Analysis of T15+ cytosolic extracts for Ins 1,4,5 P_3 kinase activity by HPLC.

Both figures 5.2a and 5.2b show the elution profile from HPLC analysis of T15+ cytosolic preparations incubated with 1µl of [^{3}H] Ins 1,4,5 P_3 (approximately 12500 dpm). Figure 5.2a shows the data from one sample within a triplicate set of samples incubated at pH 9. Figure 5.2b shows the data from the same experiment performed at pH 7 and represents the data from one sample. The duplicate samples within each triplicate set were applied to Dowex anion exchange columns and the Ins 1,3,4,5 P_4 quantified as already described. Recovery of standard [^{3}H] Ins 1,3,4,5 P_4 from HPLC was 62% and was 95% from Dowex anion exchange chromatography.

Figure 5.2a

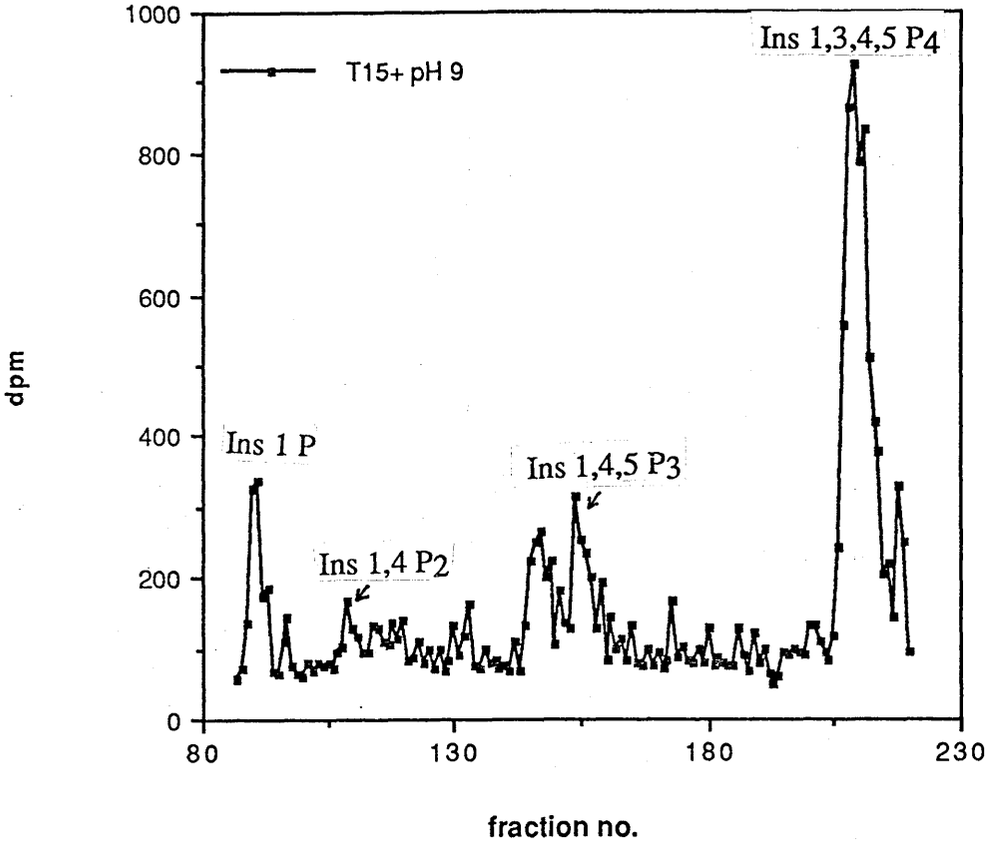


Figure 5.2b

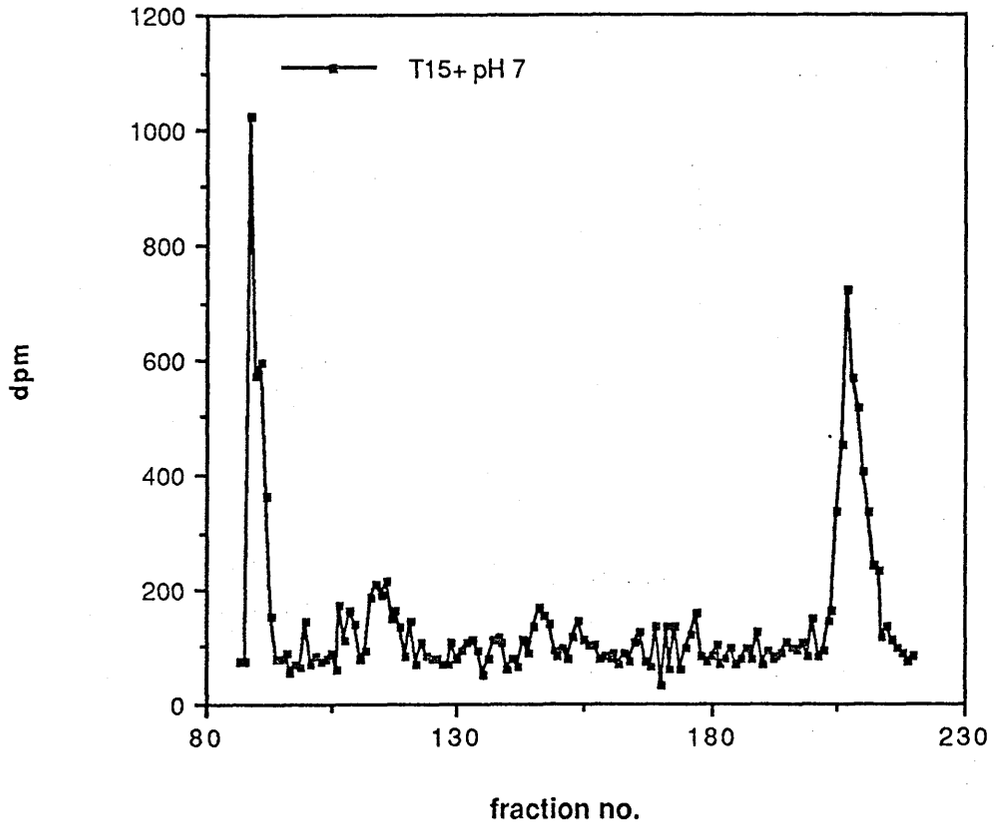


Figure 5.3: Analysis of NIH3T3 cell extracts for Ins 1,4,5 P_3 kinase activity by HPLC.

Figures 5.3a and 5.3b show the HPLC elution profiles of a NIH3T3 cytosolic preparation incubated with [^{3}H] Ins 1,4,5 P_3 as in figure 5.2 at pH 9 and pH 7 respectively. Triplicate determinations were made, one sample was analysed by HPLC and duplicate samples were analysed by Dowex anion exchange chromatography to determine if Ins 1,3,4,5 P_4 was generated as a result of Ins 1,4,5 P_3 kinase activity. The conditions of the experiment were as described in Figure 5.2.

Figure 5.3a

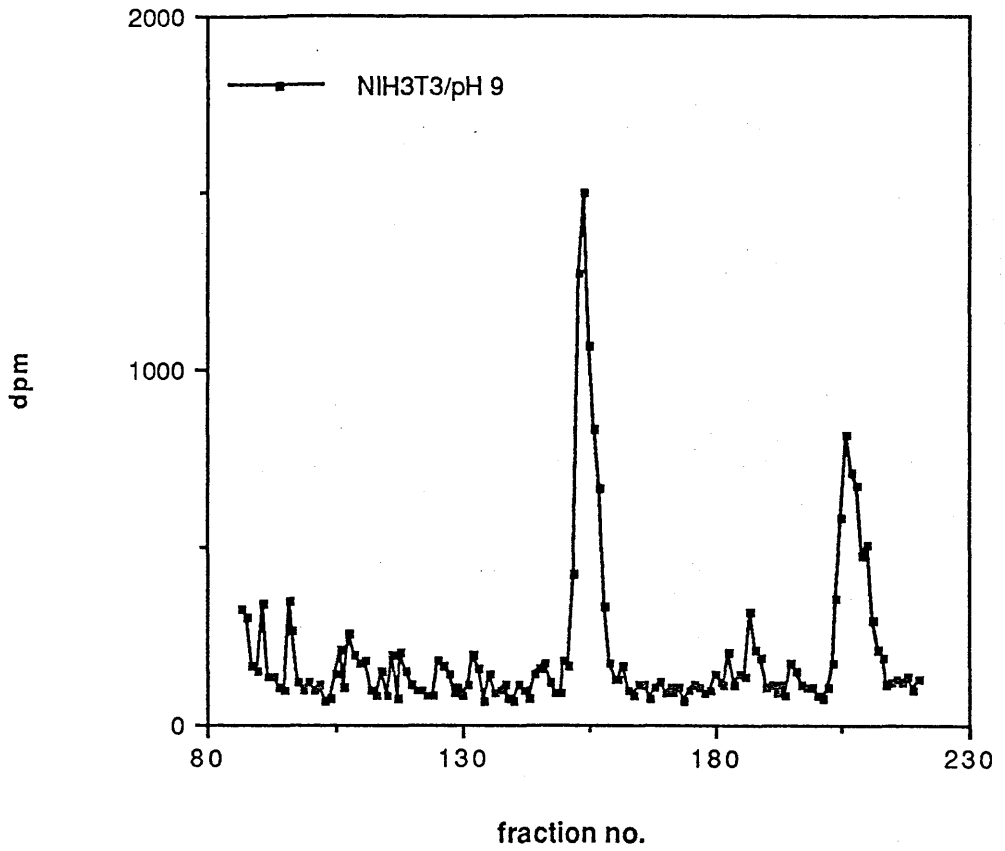
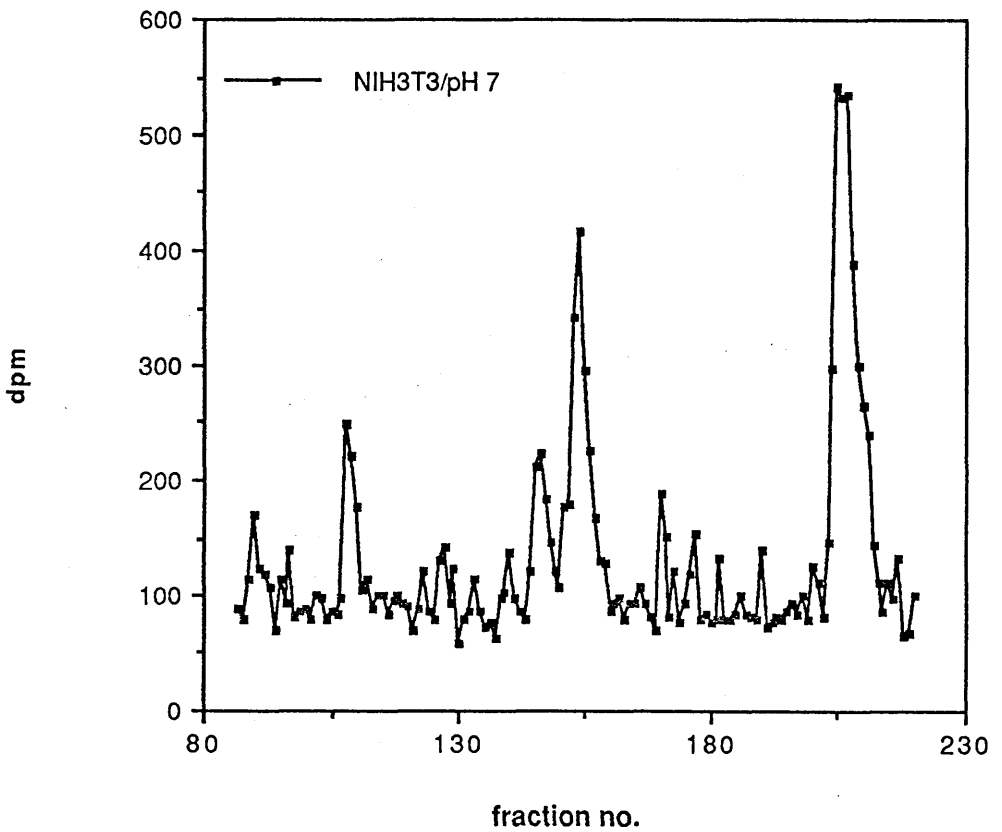


Figure 5.3b



From the preceeding results, it was clear that T15+ and NIH3T3 cells can synthesise Ins 1,3,4,5 P_4 from Ins 1,4,5 P_3 as a result of Ins 1,4,5 P_3 kinase activity. It was therefore interesting to compare these results with a rat brain cytosolic preparation under the same conditions. Rat brain high speed supernatant (a gift from Dr. S. Palmer) was incubated with [^{3}H] Ins 1,4,5 P_3 at pH 9 or pH 7 as in figures 5.2 and 3. The generation of Ins 1,3,4,5 P_4 in these samples was quantified by either HPLC or Dowex anion exchange chromatography as previously described. Figure 5.4a shows the HPLC elution profile of a sample of rat brain cytosolic extract incubated with [^{3}H] Ins 1,4,5 P_3 under appropriate conditions at pH 9. There was a large peak of Ins 1,3,4,5 P_4 with a maximal value of 1660 dpm. Dephosphorylation of Ins 1,3,4,5 P_4 at this pH occured as there was a significant peak of Ins 1,3,4 P_3 in this elution profile. There are no other peaks in this elution profile suggesting that no further dephosphorylation had taken place at pH 9.

Figure 5.4b shows that the maximal value of the Ins 1,3,4,5 P_4 peak is reduced to 1050 dpm when incubations were performed at pH 7. There is evidence of further dephosphorylation of Ins 1,3,4,5 P_4 and Ins 1,3,4 P_3 to Ins 3,4 P_2 or Ins 1,3 P_2 and Ins 1,4 P_2 and also to Ins 1 P and Ins 4 P. From this HPLC data, the specific activity of rat brain Ins 1,4,5 P_3 kinase was calculated to be 7.6 pmol Ins 1,3,4,5 P_4 min^{-1} mg^{-1} protein at pH 9 and 4.3 pmol Ins 1,3,4,5 P_4 $min^{-1}mg^{-1}$ protein at pH 7. Data obtained from Dowex anion exchange chromatography of the samples indicated that the specific activity of the rat brain Ins 1,4,5 P_3 kinase was 8.17±0.26 pmol Ins 1,3,4,5 P_4 min^{-1} mg^{-1} protein at pH 9 and 6.5±0.58 pmol Ins 1,3,4,5 P_4 min^{-1} mg^{-1} protein at pH 9. (n=2, ± SD)

Figure 5.4: Analysis of rat brain cytosolic extract for Ins 1,4,5 P_3 kinase activity by HPLC.

Figures 5.4a and 5.4b show the HPLC elution profiles of rat brain cytosolic extract incubated with [^{3}H] Ins 1,4,5 P_3 as already described (See legend Figure 5.2) at pH 9 and pH 7. The experiment was performed on triplicate samples where one sample was analysed by HPLC and duplicate samples applied to Dowex anion exchange columns to determine if Ins 1,3,4,5 P_4 was generated. The conditions of the experiment were as described for figure 5.2.

Figure 5.4a

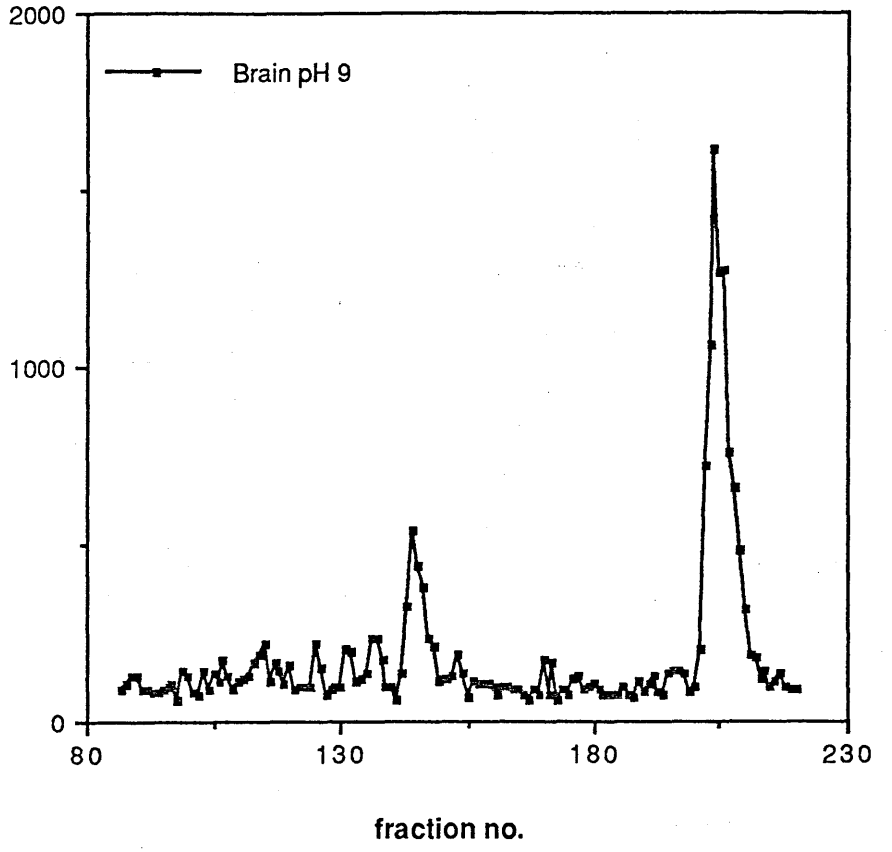
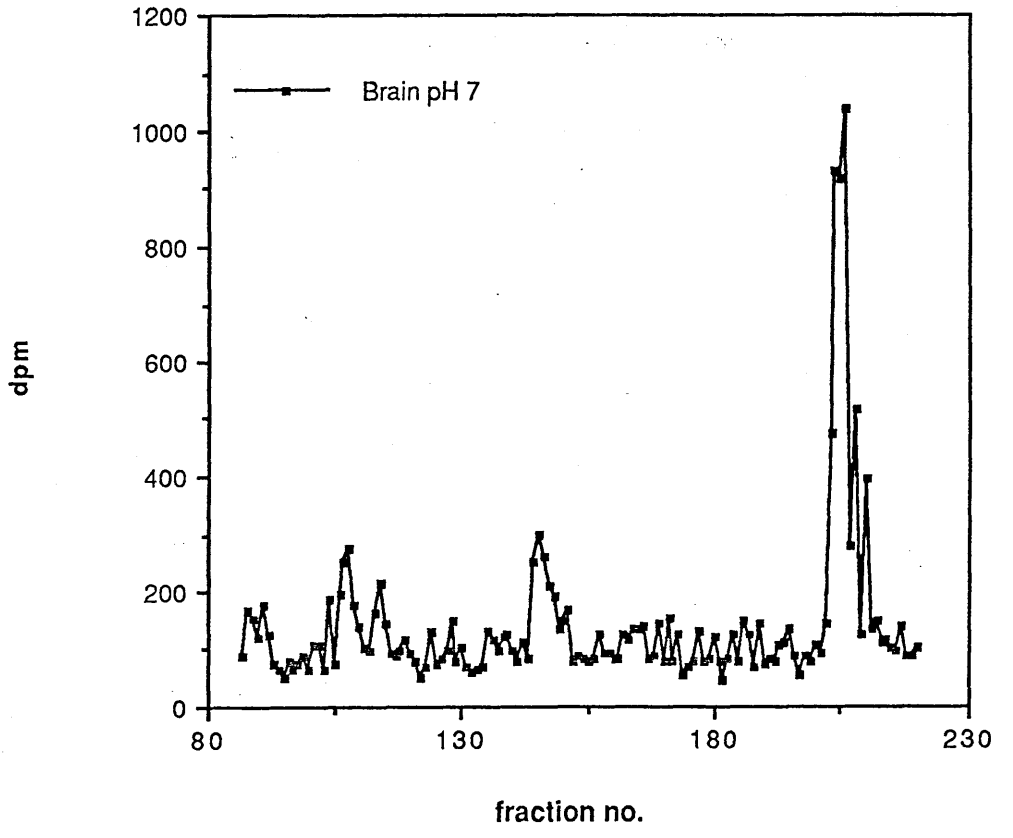


Figure 5.4b



The appropriate assay conditions for Ins 1,4,5 P_3 kinase in the NIH3T3 fibroblast cell line were determined in order to assay this enzyme routinely and to attempt to obtain consistent data between different cytosolic preparations. Therefore, Ins 1,4,5 P_3 kinase activity was assayed in T15+ and NIH3T3 cytosolic preparations at pH 9 and pH 7 in two different buffers. Buffer A is that of Irvine et al (1986) and buffer B is based on Harden et al (1987) and has a higher ionic strength than buffer A. At pH 7, it is necessary to add 2,3 diphosphoglyceric acid (2,3 DPG) to the incubations so as to prevent dephosphorylation of Ins 1,4,5 P_3 and possibly Ins 1,3,4,5 P_4 by the inhibition of the 5' monophosphatase activity. 2,3 DPG was found not to inhibit the Ins 1,4,5 P_3 kinase in a T15+ cytosolic extract at a concentration of 2mM at either pH 9 or pH 7 in buffer A *i.e.* the specific activity of Ins 1,4,5 P3 kinase in T15+ cells was unchanged in the presence of 2,3 DPG when assayed at the two pH values described.

The results in Table 2 show that the specific activity of both T15+ and NIH3T3 enzymes was inhibited at all substrate concentrations tested when incubated in buffer B at pH 7 in the presence of 2,3 DPG. The degree of inhibition between the T15+ and NIH3T3 enzymes differed. The T15+ enzyme retained some activity at pH 7 in buffer B, whilst the NIH3T3 enzyme was completely inhibited under these conditions except at 0.067μM Ins 1,4,5 P_3. In an experiment to determine if pH, different buffers and the presence of 2,3 DPG would affect Ins 1,4,5 P_3 kinase activity in cytosolic preparations from T15+ and NIH3T3 cells, it was noted that the specific activity of T15+ Ins 1,4,5 P_3 kinase was greater at pH 9 in buffer A than at pH 7 in the same buffer. Therefore, the inhibition of Ins 1,4,5 P_3 kinase specific activity in buffer B at pH 7 was not due only to the pH of the buffer, but to the composition of the buffer itself. The results in Table 2 also show that at pH 9 in buffer A, the specific activity of the T15+ enzyme was lower

than the NIH3T3 enzyme when assayed as a high speed supernatant.

On the basis of these experimental results, it was decided that most experiments to assay Ins 1,4,5 P_3 kinase activity in the NIH3T3 fibroblast cell line would be performed at pH 7 to mimick the physiological state despite the lower specific activity at this pH. Assays would be carried out in buffer A although this buffer is not a 'physiological' buffer unlike buffer B. The exceptions to these experimental conditions are the experiments described in Figure 5.8.

Table 2: Specific activity of Ins 1,4,5 P_3 kinase in T15+ and NIH3T3 cytosolic extracts under different assay conditions.

Table 2 shows the specific activity of Ins 1,4,5 P_3 kinase in T15+ and NIH3T3 cytosolic preparations in pmol Ins 1,3,4,5 P_4 min^{-1} mg^{-1} protein ± S.D. (n=3). The data is pooled from two experiments where incubations were performed in triplicate and under the conditions indicated where [S] indicates Ins 1,4,5 P_3 concentration (μM). N.D.=not determined.

	pH 7, buffer B + 2,3 DPG		pH 9,buffer A	
[S]	T15±	NIH3T3	T15±	NIH3T3
0.067	0.13±0.003	0.62±0.06	5.9±0.05	10.1±0.33
0.33	1.84±0.009	0	32.4±0.82	48±2.1
0.67	7.3±0.03	0	57.1±0.84	102±7
1.67	12.9±0.45	0	88.0±1.82	260±25
3.33	84.5±1.61	0	248±41	449±56
4.99	N.D.	N.D.	314±8	752±238
6.67	11.9±0.87	0	484±54	155±61
9.99	N.D.	N.D.	755±8	138±11
13.33	N.D.	N.D.	537±88	1291±239

Having defined the most appropriate conditions to assay the NIH3T3 fibroblast Ins 1,4,5 P3 kinase enzyme, the substrate requirements of the enzyme were investigated. This was undertaken to determine the appropriate substrate concentration at which to assay the enzyme and also to determine the kinetics of the enzyme reaction. This investigation into the characteristics of the Ins 1,4,5 P3 kinase in a cytosolic preparation was carried out mainly on the wild-type NIH3T3 cell line. Although there are some differences between the characteristics of the Ins 1,4,5 P3 kinase enzyme of T15+ and NIH3T3 cells, as seen from Table 2, it was decided to characterise the NIH3T3 enzyme initially, before any effects of transformation by p21 $^{N\text{-}\underline{ras}}$ were examined.

Initial experiments to determine the Michaelis-Menten constant (Km) for the NIH3T3 Ins 1,4,5 P3 kinase were performed using [^{3}H] Ins 1,4,5 P3 at a specific activity of 1 Ci mmol^{-1}. This had one main disadvantage *i.e.* at high substrate concentrations it was not possible to detect Ins 1,3,4,5 P4 formation above blank levels because of the low specific activity of the 3H Ins 1,4,5 P3. For example, at 20μM Ins 1,4,5 P3, there was no detectable Ins 1,3,4,5 P4 formation in samples incubated at 30^{o}C for up to 1 hour. Therefore, in the experiments shown here, 1μl of [^{3}H] Ins 1,4,5 P3 at 44 Ci mmol^{-1} was used per assay. In these experiments, samples of NIH3T3 high speed supernatants were incubated with [^{3}H] Ins 1,4,5 P3 as already described with increasing amounts of unlabelled Ins 1,4,5 P3 to give the required final concentration and the samples incubated at 30^{o}C for 5 minutes in buffer A at pH 7 in the presence of 2,3 DPG. The incubations were terminated and the neutralised supernatants were assayed for Ins 1,3,4,5 P4 formation by Dowex anion exchange chromatography.

The results in Figure 5.5a show the velocity of the reaction for Ins 1,4,5 P3 kinase in a NIH3T3 cytosolic preparation. The V_{max} calculated from this data was 23 nmol Ins 1,3,4,5 P4 min^{-1} mg^{-1} protein.

The half maximal substrate concentration determined from this plot is approximately 8.7 μM Ins 1,4,5 P_3. From this plot, the NIH3T3 Ins 1,4,5 P_3 kinase appears to display Michaelis-Menten kinetics.Figure 5.5b is the Lineweaver-Burke plot of the data in figure 5.5a. From this graph, the value of -1/Km was estimated to be -0.079 which gave a Km value of 12.7μM. These results indicated that the NIH3T3 Ins 1,4,5 P_3 kinase enzyme as assayed in a cytosolic extract prepared from the cells has a higher Km value than the enzyme from other mammalian tissues (See Discussion at the end of this chapter) and therefore, seems to have a low substrate affinity.

Figure 5.5: Substrate requirement of Ins 1,4,5 P_3 kinase specific activity from NIH3T3 high speed supernatants.

Figures 5.5a and 5.5b show the kinetics of the NIH3T3 Ins 1,4,5 P_3 kinase enzyme as determined by assaying enzyme activity of a high speed supernatant prepared from NIH3T3 cells in buffer A at pH7 in the presence of 2mM 2,3 DPG between 0.003 and 20μM Ins 1,4,5 P_3 with 1μl of [^{3}H] Ins 1,4,5 P_3 (44 Ci mmol^{-1}) per assay. The results are from one experiment performed on triplicate samples and Figure 5.5a is a plot of velocity (v) of the enzyme reaction in nmol Ins 1,3,4,5 P_4 min^{-1}mg^{-1} protein versus substrate conce ntration in μM (s). The data is representative of three separate experiments performed in triplicate and is ± S.D. (n=3). Figure 5.5b is the same data plotted as 1/v versus 1/s. The errors in this figure have been omitted for clarity.

The last two points on the Lineweaver-Burke plot have not been included on the straight line obtained as they are derived from the first two points on the V versus S plot (figure 5.5a). Both these points indicate a negligible specific activity of the Ins 1,4,5 P_3 kinase and are therefore likely to be largely erroneous when converted to the inverse values.

Figure 5.5a

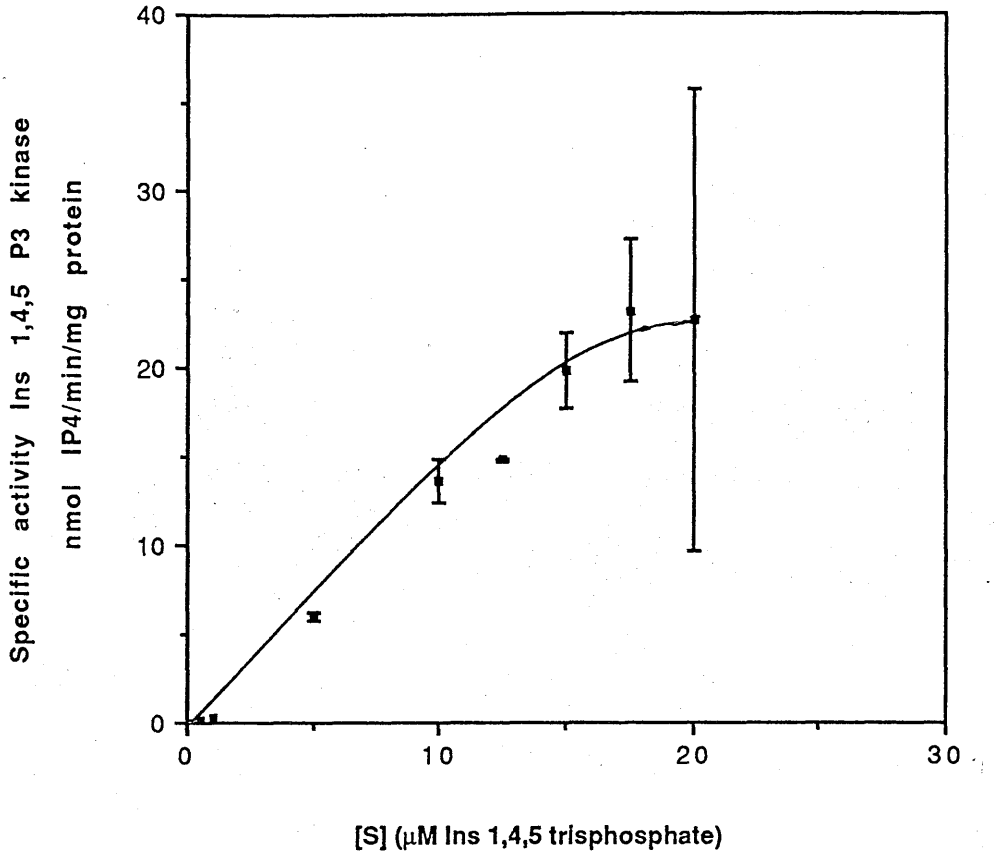
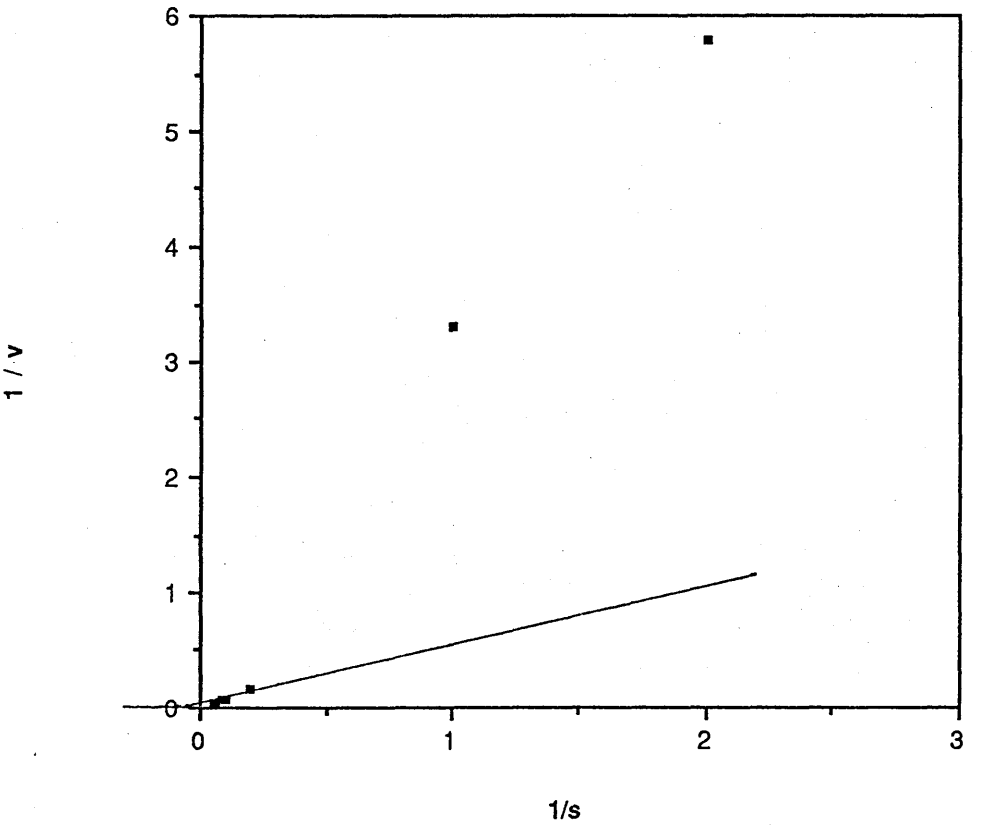


Figure 5b



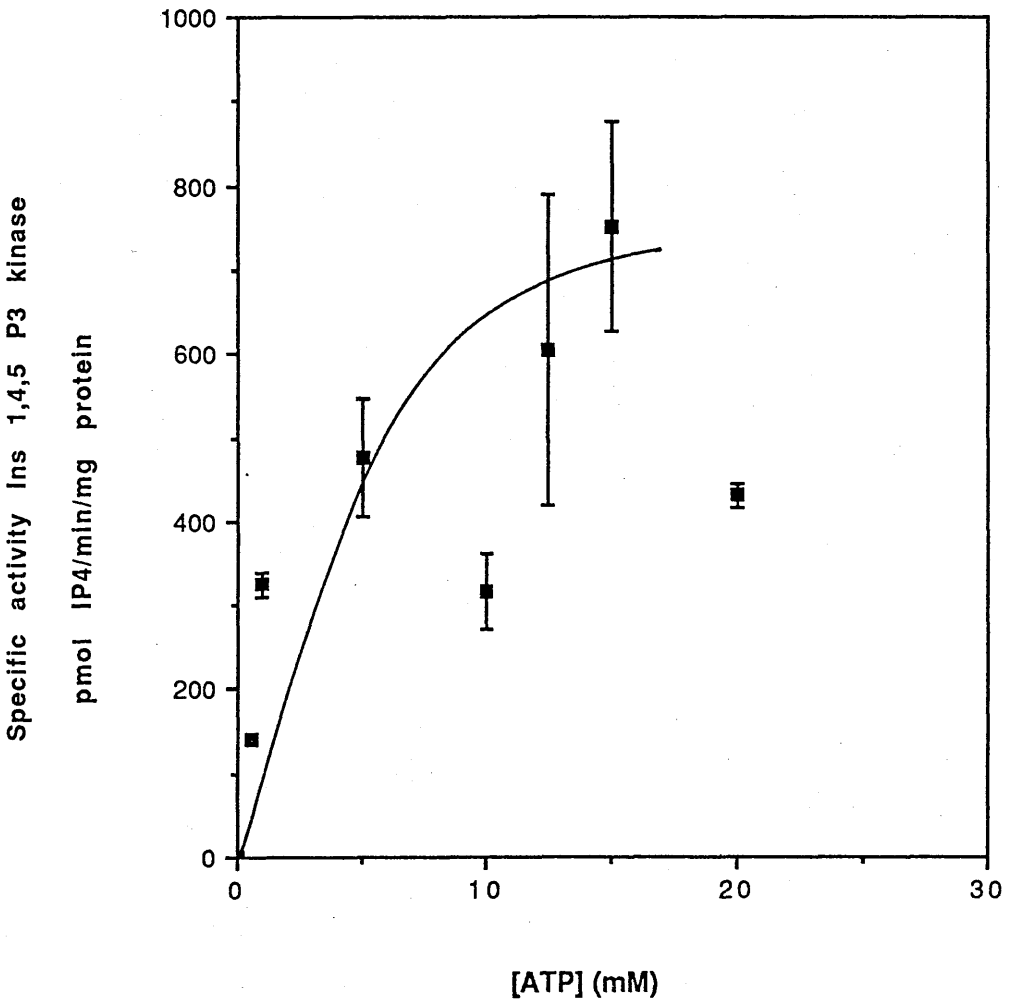
The Ins 1,4,5 P_3 concentration dependence of NIH3T3 Ins1,4,5 P_3 kinase have been demonstrated in the previous figure. Further experiments were performed to investigate the ATP requirement of the enzyme. All experiments previous to these were performed using 10 mM ATP (Irvine et al, 1986). A cytosolic preparation from NIH3T3 cells was incubated with 1µl of [^{3}H] Ins 1,4,5 P_3 (Specific activity = 44 Ci mmol^{-1}) and a final concentration of 10µM Ins 1,4,5 P_3 in ATP-free buffer A at pH 7 in the presence of 2,3, DPG with increasing concentrations of ATP at 30^{o}C for 5 minutes. The incubations were terminated as described and neutralised supernatants were analysed for Ins 1,3,4,5 P_4 content by Dowex anion exchange chromatography.

Figure 5.6 shows the results of an experiment to determine the optimum ATP concentration for NIH3T3 Ins 1,4,5 P_3 kinase activity. The results show that the specific activity for the enzyme increased up to 15 mM ATP and then decreased at 20 mM. From this experiment, the Km value for ATP was approximately 4 mM. This result suggests that an assay concentration of 10mM ATP is acceptable to measure Ins 1,4,5 P_3 kinase activity from NIH3T3 high speed supernatants routinely. Although this result is from one experiment, two separate experiments performed on different cytosolic preparations from NIH3T3 cells indicated Km values of approximately 2 mM ATP and 4 mM ATP respectively which are consistent with the result obtained from the experiment shown.

Figure 5.6: ATP concentration dependence of Ins 1,4,5 P_3 kinase activity from NIH3T3 cells.

Figure 5.6 represents the data obtained from an experiment to determine the concentration of ATP required for half-maximal Ins 1,4,5 P_3 kinase activity (Ka). The experiment was performed as described upon triplicate samples at concentrations of ATP between a range of 0- 20 mM and at 10μM Ins 1,4,5 P_3. The results are expressed as pmol Ins 1,3,4,5 P_4 $min^{-1}mg^{-1}$ protein versus ATP concentration (mM) and are means ±S.D. (n=3) and are from one experiment representative of three separate experiments.

Figure 5.6


Specific activity Ins 1,4,5 P3 kinase
pmol IP4/min/mg protein
1000
800
600
400
200
0
0
10
20
30
[ATP] (mM)

The results in Figure 5.5 demonstrate that the NIH3T3 enzyme has a high Km value for Ins 1,4,5 P_3. One of the reasons considered for this was that there may be that an endogenous inhibitor of the enzyme existing in the cytosol. One way of testing this hypotheses was to assay Ins 1,4,5 P_3 kinase activity in diluted cytosolic preparations and compare this to a non-diluted preparation. Therefore, a 'concentrated' cytosolic fraction from NIH3T3 cells was prepared by homogenising a cell pellet collected from 5 x 75 cm^2 flasks (approximately 3 x 10^7 cells in total) in 1 ml of homogenisation buffer (See Methods Section). The homogenate was centrifuged to obtain a high speed supernatant which was diluted sequentially up to 100 fold in homogenisation buffer. The supernatants were assayed for Ins 1,4,5 P_3 kinase activity at 10μM Ins 1,4,5 P_3 in buffer B at pH 7 in the presence of 2mM 2,3 DPG. Buffer B was used because the enzyme activity is inhibited in this buffer as already shown and thus if an inhibitor was present in the cytosolic preparations, the effects of dilution may enhance the enzyme activity in buffer B compared to buffer A, where no inhibition of NIH3T3 Ins 1,4,5 P_3 kinase activity was found (Refer to Table 2) .

Figure 5.7 shows the results of a dilution experiment performed as described above. The graph shows the specific activity of NIH3T3 Ins 1,4,5 P_3 kinase expressed as nmol Ins 1,3,4,5 P_4 $min^{-1}mg^{-1}$ protein plotted against fold dilution of the supernatants. There was an approximately 100 fold increase in specific activity of the enzyme when diluted 100 fold (approximately 0.5μg protein per assay). A significant increase in specific activity was first seen at a 25 fold dilution of the supernatant (approximately 12.5μg protein per assay) as compared to the undiluted preparation.

These results indicated that an increased specific activity of the Ins 1,4,5 P_3 kinase enzyme in NIH3T3 cells was associated with increased dilution of the supernatants. Therefore, there may be an endogenous inhibitor of the enzyme which is reduced in concentration upon dilution.

Figure 5.7: Specific activity of Ins 1,4,5 P_3 kinase in diluted NIH3T3 high speed supernatants.

Figure 5.7 shows the results of an experiment designed to determine if dilution of a high speed supernatant prepared from NIH3T3 cells had any effect on the specific activity of Ins 1,4,5 P_3 kinase. Diluted high speed supernatants were assayed for Ins 1,4,5 P_3 kinase activity at 10μM Ins 1,4,5 P_3 in buffer B at pH 7 in the presence of 2mM 2,3 DPG. The results are expressed in nmol Ins 1,3,4,5 P_4 $min^{-1}mg^{-1}$ protein and are means ±S.D. (n=3). Similar experiments performed at 3nM and 13nM Ins 1,4,5 P_3 gave results that showed exactly the same trend as in figure 5.7 but with lower specific activities. The protein concentration of the concentrated NIH3T3 high speed supernatant was determined to be 2.5 μg per μl. The amount of protein assayed per sample is indicated below:

Concentrated sample: 50 μg

2 fold dilution : 25 μg

4 fold dilution : 12.5 μg

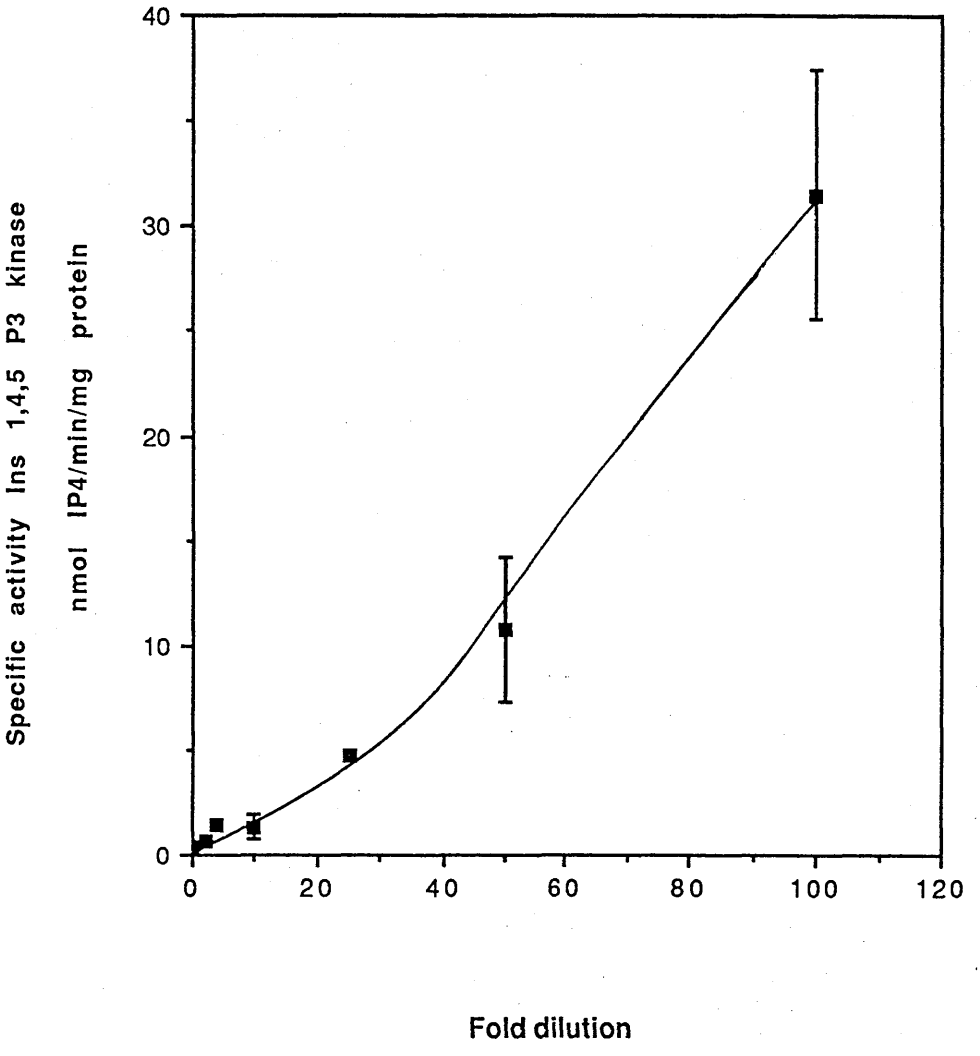
10 fold dilution : 5 μg

25 fold dilution : 2.5 μg

50 fold dilution : 1.25 μg

100 fold dilution : 0.5 μg

Figure 5.7


Specific activity Ins 1,4,5 P3 kinase
nmol IP4/min/mg protein
40
30
20
10
0
0
20
40
60
80
100
120
Fold dilution

It has already been demonstrated that the specific activities of T15+ and NIH 3T3 Ins 1,4,5 P3 kinase in high speed supernatants is reduced in buffer B compared to buffer A. Also, the results in figure 5.7 have demonstrated that there is a large increase in the specific activity of NIH3T3 Ins 1,4,5 P3 kinase following dilution of the high speed supernatant prepared from these cells. To determine if this effect was due in part to the composition of the assay buffer, the experiment as described in figure 5.7 was repeated under 2 different assay conditions.

A concentrated high speed supernatant from NIH3T3 cells was diluted up to a 100 fold in homogenisation buffer and assayed for Ins 1,4,5 P3 kinase activity as already described *i.e.* at 10μM Ins 1,4,5 P3 at pH 7 in the presence of 2,3, DPG. However, the experiment was performed in buffers A and B under the above conditions. Figure 5.8 shows the results of the experiment described above. The diluted high speed supernatant assayed in buffer B showed up to a 200 fold increase in Ins 1,4,5 P3 kinase specific activity as compared to the undiluted supernatant. In contrast, when Ins 1,4,5 P3 kinase activity was assayed in buffer A, there was only a 70 fold increase in specific activity at a 100 fold dilution. When the enzyme was assayed in buffer B, there is no significant increase in specific activity until a 10 fold dilution, but in buffer A, a significant increase in specific activity occured after a 4 fold dilution.

The data shown in this figure indicates that the increase in specific activity of NIH3T3 Ins 1,4,5 P3 kinase that occurs on sequential dilution of the high speed supernatant is greater when assayed in buffer B than in buffer A. This suggests that the composition of the assay buffer used had a role in the modultion of Ins 1,4,5 P3 kinase activity by possibly affecting an endogenous inhibitor of the enzyme present in the cytosol.

Figure 5.8: Specific activity of Ins 1,4,5 P_3 kinase from diluted NIH3T3 high speed supernatants assayed in either buffer A or B.

Figure 5.8 shows the results of an experiment to determine Ins 1,4,5 P_3 kinase activity in diluted samples of NIH3T3 high speed supernatant when assayed in either buffer A or buffer B at pH 7 in the presence of 2,3, DPG and with 1µl of [^{3}H] Ins 1,4,5 P_3 (Specific activity = 44 Ci mmol^{-1}) The amount of Ins 1,3,4,5 P_4 from these samples was quantified by Dowex anion exchange chromatography. The results are expressed as nmol Ins 1,3,4 5 P_4 min^{-1} mg^{-1} protein and are means ± S.D. (n=3) and are from one experiment. The amount of protein assayed per sample is indicated below:

Concentrated sample : 4.5 µg

2 fold dilution : 2.25 µg

4 fold dilution : 1.1 µg

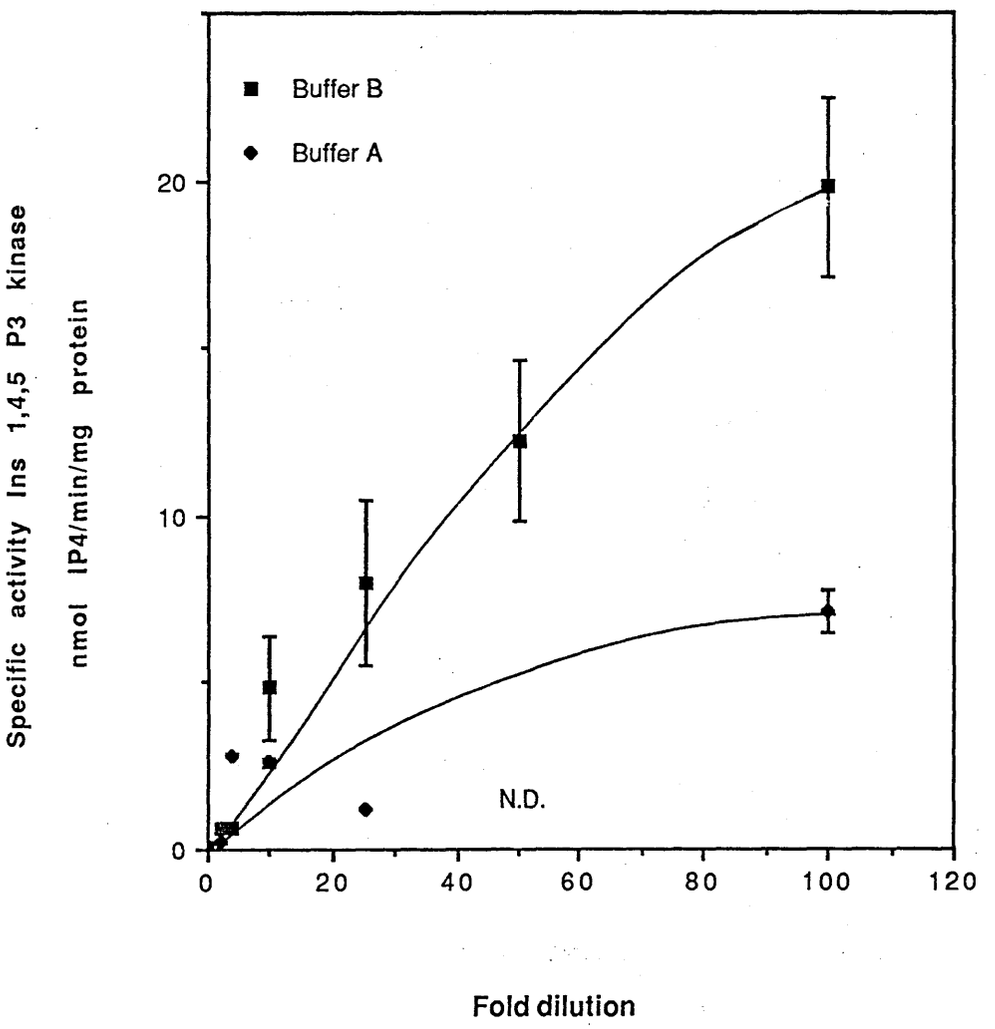
10 fold dilution : 0.45 µg

25 fold dilution : 0.18 µg

50 fold dilution : 0.09 µg

100 fold dilution : 0.045 µg

Figure 5.8


Buffer B
Buffer A
N.D.
Specific activity Ins 1,4,5 P3 kinase
nmol IP4/min/mg protein
Fold dilution
0
10
20
0
20
40
60
80
100
120

It is clear from the data shown in Figures 5.7 and 5.8 that dilution of NIH3T3 high speed supernatants resulted in higher Ins 1,4,5 P3 kinase specific activities. This increased specific activity could be due to either an increased velocity of the enzyme reaction *i.e.* increased V_{max} or a reduced Km value following dilution. To examine these possibilities, the Ins 1,4,5 P3 kinase activity in selected diluted high speed supernatants were assayed against a range of substrate concentrations as utilised in Figure 5.5.

Figures 5.9a-5.9b show the results of experiments to determine if there was a change in either the V max or Km of NIH3T3 Ins 1,4,5 P3 kinase in high speed supernatants diluted 10-, 50- and 100 fold as compared to a non-diluted supernatant. The figure shows the plot of v versus S for a non-diluted supernatant and of a 10 fold dilution of the same supernatant (figure 5.9a). The Vmax for the non-diluted sample was approximately 0.4 nmol Ins 1,3,4,5 P4 min^{-1} mg^{-1} protein although the substrate range was 0.003-15μM Ins 1,4,5 P3 as compared to 0.003-20μM Ins 1,4,5 P3 for all the diluted samples. The V max for the 10 fold diluted sample was 4 nmol Ins 1,3,4,5 P4 $min^{-1}mg^{-1}$ protein.

Figure 5.9b shows the results of similar experiments carried out on 50 fold and 100 fold diluted high speed supernatants. They clearly show that the Ins 1,4,5 P3 kinase specific activity was greatly increased compared to the 10 fold diluted and non-diluted samples of high speed supernatant. From these graphs, the Vmax value for the 50 fold diluted sample was estimated to be 14.5 nmol Ins 1,3,4,5 P4 $min^{-1}mg^{-1}$ protein and was approximately 400 nmol Ins 1, 3,4,5 P4 min^{-1} mg^{-1} protein for the 100 fold diluted sample. Thus, these results show that the activity of Ins 1,4,5 P3 kinase increased with increasing dilution of NIH3T3 high speed supernatants at all concentrations of substrate tested.

To obtain an estimate of the Km values of the Ins 1,4,5 P3 kinase in non-diluted and diluted high speed supernatants, Lineweaver-Burke plots were drawn from the data shown in Figure 5.9a and 5.9b. Figure 5.9c

shows the Lineweaver-Burke plot of the data from the non-diluted high speed supernatant. From the intercept on the x-axis, the Km value has been estimated to be 15.9μM. Figure 5.9d shows the Lineweaver-Burke plot of the data obtained for the 10 fold diluted high speed supernatant and a Km value of 15.9μM has been estimated from this graph. Figures 5.9e and 5.9f show Lineweaver-Burke plots for the 50 fold and 100 fold diluted enzymes and km values of 15.9 μM and 15 μM have been estimated have been estimated from these graphs.

Thus, these experiments demonstrate that on dilution of NIH3T3 high speed supernatants, the Km for Ins 1,4,5 P_3 of the Ins 1,4,5 P_3 kinase enzyme remains unchanged but the V_{max} increases.

Having established the possibility of an endogenous,soluble inhibitor of Ins 1,4,5 P_3 kinase in NIH3T3 fibroblasts, the following experiment was performed to determine if this inhibitor was specific for NIH3T3 Ins 1,4,5 P_3 kinase. A concentrated NIH3T3 cytosolic extract was prepared as previously described and this was mixed in a 1:1 (v/v) ratio with a high speed supernatant prepared from bovine adrenal glands by S. Palmer and F. Black. 20μl of this mixture was assayed in a final volume of 150μl for Ins 1,4,5 P_3 kinase activity at pH 7 in buffer A at 10μM Ins 1,4,5 P_3 as already described. Control incubations of adrenal high speed supernatant only, NIH3T3 extract only, adrenal extract diluted with sucrose/tris buffer (1:1,v/v ratio; See Methods for buffer composition) and NIH3T3 extract diluted with sucrose/tris buffer (1:1,v/v ratio) were also assayed for Ins 1,4,5 P_3 kinase activity under the same conditions.

The results in table 3 are from the experiment described above and show that dilution of adrenal high speed supernatant resulted in a decrease in the amount of Ins 1,3,4,5 P_4 produced as compared to the undiluted adrenal extract. However, dilution of the NIH3T3 high speed supernatant resulted in the same amount of Ins 1,3,4,5 P_4 produced as the undiluted cytosolic extract and suggested that the possible inhibition of Ins 1,4,5 P_3 kinase that occured in the NIH3T3 cell line did not occur in bovine adrenal gland. The

mixture of the adrenal and NIH3T3 high speed supernatants resulted in a smaller amount of Ins 1,3,4,5 P_4 formed compared to either diluted NIH3T3 or adrenal supernatants. Since the amount of Ins 1,3,4,5 P_4 formed in the NIH3T3/adrenal mixture was lower than either the diluted adrenal or NIH3T3 extracts which act as controls in this experiment, it is possible that there was inhibition of bovine adrenal gland Ins 1,4,5 P_3 kinase activity by the NIH3T3 cytosolic extract. This suggests that the possible inhibitor in NIH3T3 cells of Ins 1,4,5 P_3 kinase is not specific for the fibroblast enzyme.

Figure 5.9a and 5.9b: v versus [S] plot of Ins 1,4,5 P_3 kinase specific activities in undiluted and diluted NIH3T3 high speed supernatants.

These figures show the results of a v (nmol Ins 1,3,4,5 P_4 min^{-1} mg^{-1} protein) versus S plot for undiluted, 10-, 50- and 100 fold diluted NIH3T3 high speed supernatants assayed at pH 7 in the presence of 2,3, DPG in buffer B. The results are means ± S.D. (n= 3) and are from one experiment, although the experiment using a 50 fold diluted high speed supernatant was performed twice, with the same estimated Km value being derived each time.

Figures 5.9c-5.9f: Lineweaver-Burke plots of data obtained from figures 5.9a and 5.9b.

These figures are Lineweaver-Burke plots for the data shown in Figures 5.9a and 5.9b. As the data has been derived from the results in figures 5.9a and 5.9b the standard errors have been omitted for clarity.

Figure 5.9a

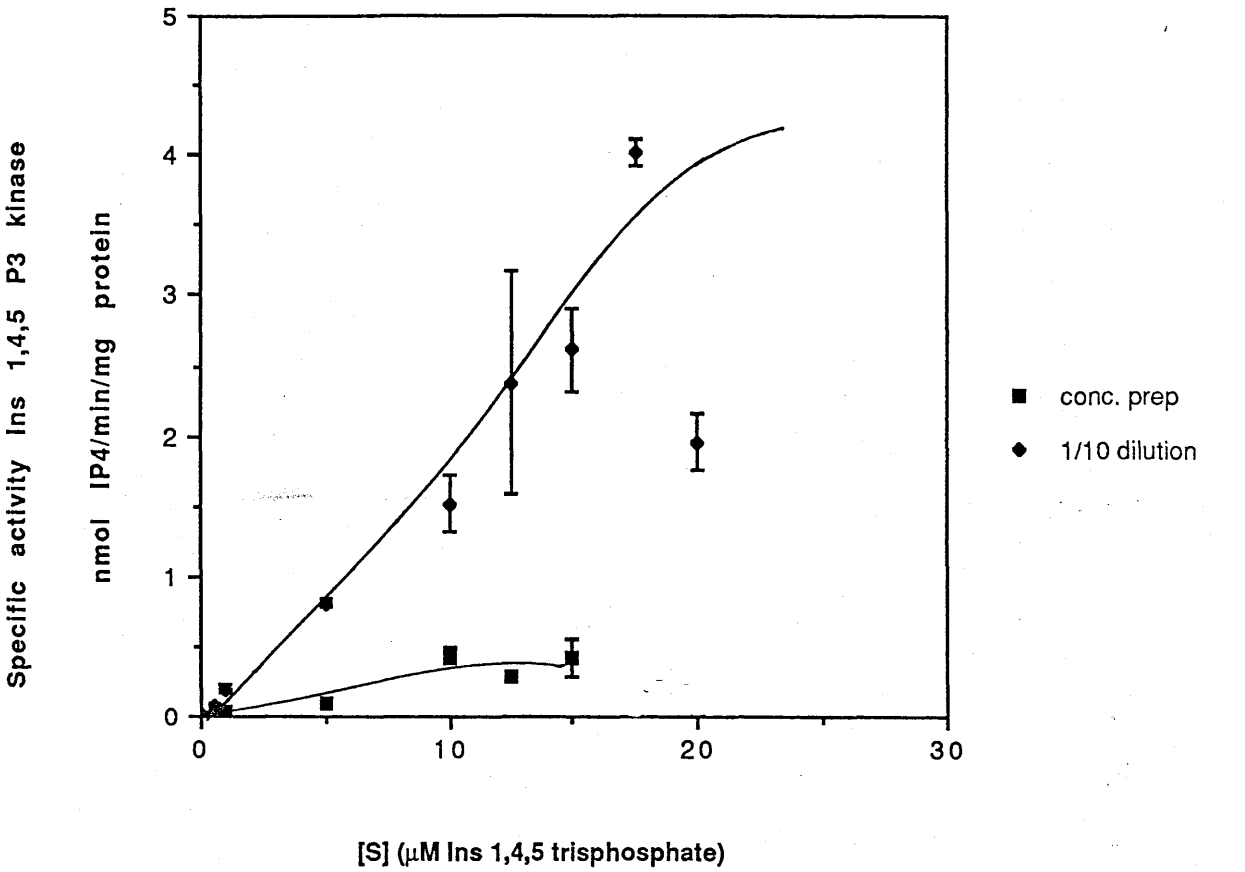


Figure 5.9b

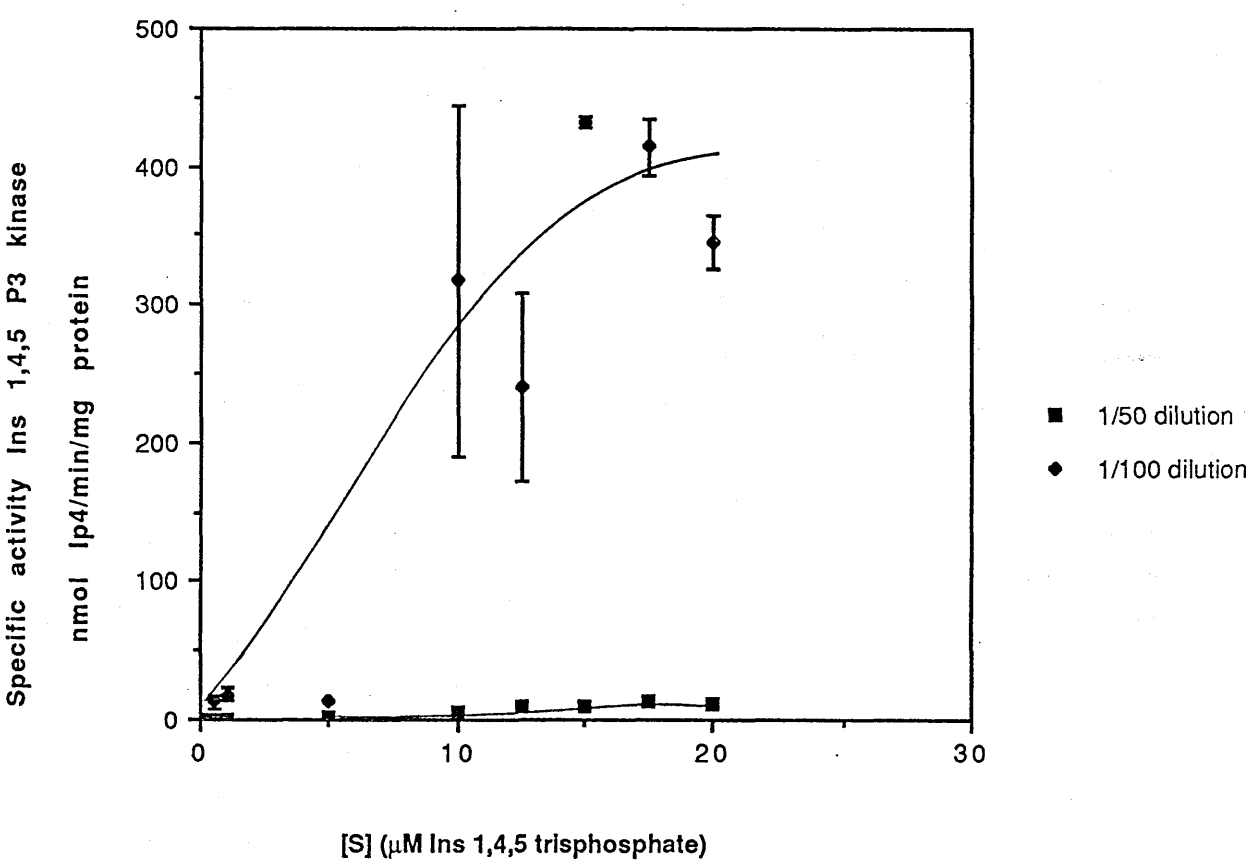


Figure 5.9c (Undiluted supernatant)

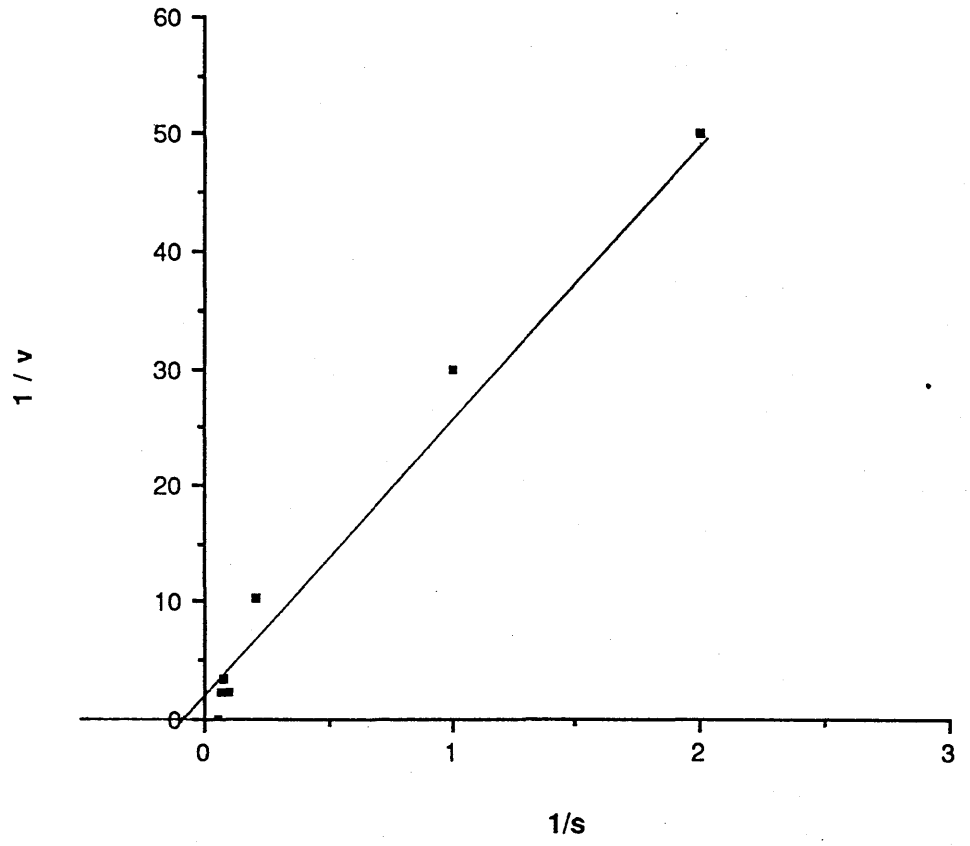


Figure 5.9d (1/10 dilution)

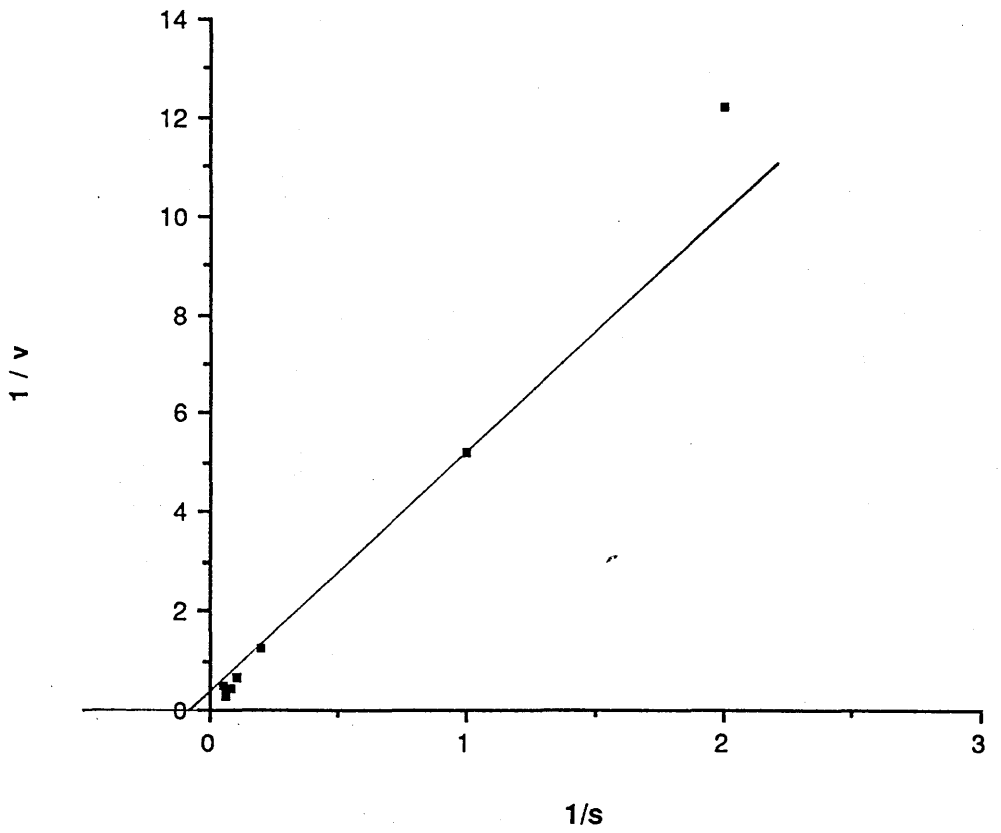


Figure 5.9e (1/50 dilution)

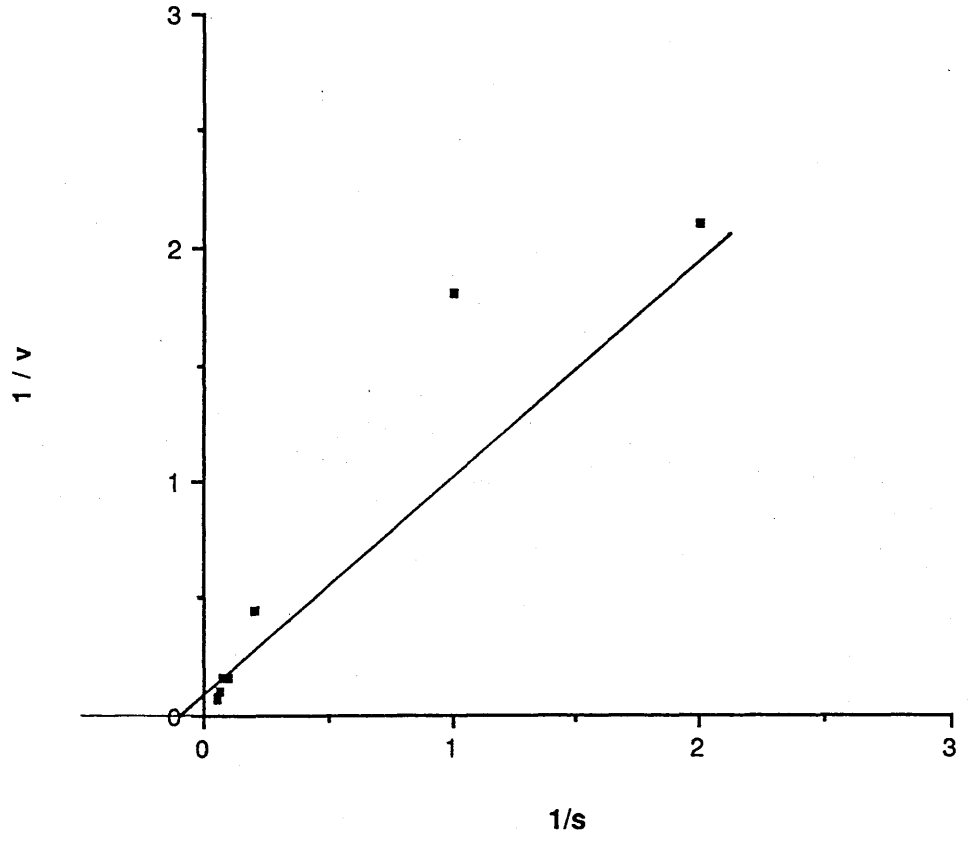
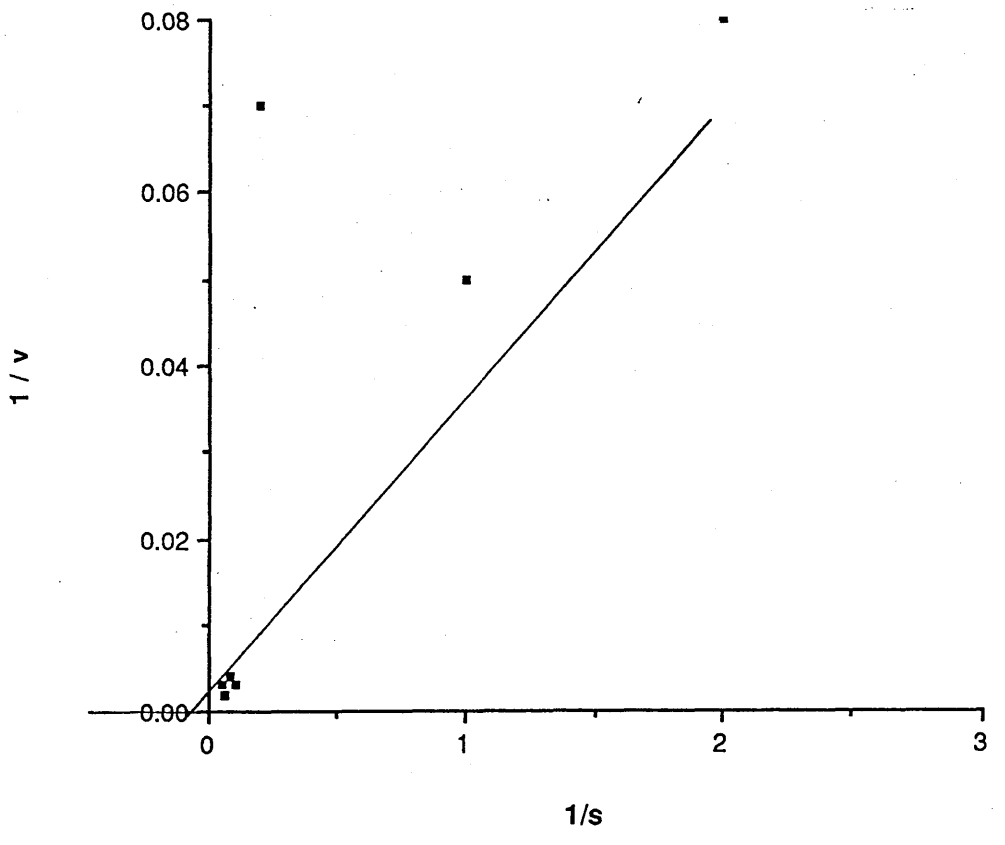


Figure 5.9f (1/100 dilution)



However,the results shown in the table are only from one experiment and the results obtained from the assays of adrenal Ins 1,4,5 P3 kinase activity indicate a value much lower than expected and was much lower than that found for concentrated NIH3T3 cytosolic extract. This could be due to the fact that the adrenal gland cytosolic extract was not a recent preparation and therefore, these experiments will have to be repeated using a more recent preparation of adrenal gland high speed supernatants. The experiments will also have to be performed over a range of dilutions of the adrenal extract with NIH3T3 extract using both buffers A and B to obtain more information on the nature of this possible non-specific inhibition of Ins 1,4,5 P3 kinase. The results in table 3 therefore, are not conclusive but are only suggestive of a possible inhibitor of Ins 1,4,5 P3 kinase that is not specific for the enzyme in this fibroblast cell line.

Table 3: Effect of NIH3T3 cytosolic extract on production of Ins 1,3,4,5 P_4 in bovine adrenal gland high speed supernatant.

The results shown in table 3 are from an experiment to determine the effect of mixing NIH3T3 concentrated high speed supernatant with high speed supernatant from bovine adrenal glands on the production of Ins 1,3,4,5 P_4. The experiment was carried out as described in the text and in the Methods section and the results are pmol Ins 1,3,4,5 P_4 produced/5 min and are means ± S.D.(n=3). The protein concentrations of the high speed supernatants were as follows:

bovine adrenal gland = 25.5 mg μl^{-1} ; NIH3T3 = 2.5 mg μl^{-1}

Incubations	pmol Ins 1,3,4, 5 P_4
Adrenal	0.62 ± 0.12
NIH3T3	0.55 ± 0.1
Adrenal/NIH3T3	0.31 ± 0.12
Adrenal/buffer	0.48 ± 0.07
NIH3T3/buffer	0.48 ± 0.12

Discussion

The results presented in this chapter are from experiments performed to investigate the possible reasons for the absence of Ins 1,3,4,5 P_4 in bombesin stimulated T15 and NIH3T3 cells. The first observation that led to these investigations was that there was no detectable Ins 1,3,4,5 P_4 in bombesin stimulated T15+ cells. In figure 5.1, the HPLC elution profile of an extract prepared from T15+ cells stimulated for 120 seconds with bombesin is shown. From this data, it is clear that there was no Ins 1,3,4,5 P_4 produced. Further, no detectable dephosphorylated derivatives of Ins 1,3,4,5 P_4 *i.e.* Ins 1,3,4 P_3 , Ins 3,4 P_2 or Ins 1,3 P_2 or Ins 3 P were detected. The reasons for this were postulated as being either that these cells lacked the Ins 1,4,5 P_3 kinase enzyme or that the levels of Ins 1,4,5 P_3 produced on bombesin stimulation were too low for Ins 1,3,4,5 P_4 formation to occur. The other possibility was that the phosphatase enzymes that dephosphorylate Ins 1,3,4,5 P_4 and Ins 1,4,5 P_3 were extremely active in these cells.

At no time point in bombesin stimulated T15 or NIH3T3 cells was there detectable Ins 1,3,4,5 P_4 despite the peak of Ins 1,4,5 P_3 reaching 1000 dpm in T15+ cells in some cases. In A431 and NG 115-40IL cells, the levels of Ins 1,3,4,5 P_4 reach approximately 50% and 25% respectively of the peak levels of Ins 1,4,5 P_3 and detectable levels of Ins 1,3,4,5 P_4 following a 15 second mitogen stimulation in these cell types (Jackson et al, 1987 and Tilly et al, 1987). If the phosphorylation and dephosphorylation pathways of Ins 1,4,5 P_3 in different cell types are similar, then from the facts above, there should be detectable Ins 1,3,4,5 P_4 production in T15+ cells on bombesin stimulation.

Although the presence of Ins 1,3,4,5 P_4 in mitogen stimulated cells is thought to occur in most cell types (See Introduction), there have been reports in the literature that demonstrate that Ins 1,3,4,5 P_4 production does not occur in all tissues. Woodcock et al (1987) have reported that there is no

Ins 1,3,4,5 P_4 formation in epinephrine perfused rat hearts and that there is no detectable Ins 1,3,4 P3. These workers only detected stimulated increases of Ins 1,4,5 P_3 and Ins 1,4 P_2 and Ins 1 P. They have suggested that there is a lack of Ins 1,4,5 P_3 kinase activity in rat heart.

Figures 5.2a and 5.2b illustrate that T15+ cells are not deficient in Ins 1,4,5 P_3 kinase activity, although from figure 5.2a it is clear that the Ins 1,4,5 P_3 5' monophosphatase enzyme is very active in this cell line and is not completely inhibited at pH 9. This contrasts with the enzyme assayed in extracts from rat liver cells (Storey et al, 1984). Figure 5.2b shows that further dephosphorylation of Ins 1,4,5 P_3 and Ins 1,3,4,5 P_4 occurs at pH 7. This suggests that the phosphorylation/dephosphorylation pathways of Ins 1,4,5 P_3 and Ins 1,3,4,5 P_4 can occur in T15+ cells.

In NIH3T3 cells, the Ins 1,4,5 P_3 5' monophosphatase is completely inhibited at pH 9 (figure 5.3a) but not at pH 7 (figure 5.3b). As in T15+ cells, the phosphorylation and dephosphorylation of Ins 1,4,5 P_3 occurs and Ins 1,3,4,5 P_4 is dephosphorylated to its derivatives. From the calculated specific activities of Ins 1,4,5 P_3 kinase in T15+ and NIH3T3 cells it is clear that T15+ cells have a lower Ins 1,4,5 P_3 kinase specific activity than NIH3T3 cells and this may be due in part to the increased Ins 1,4,5 P_3 5' monophosphatase activity in T15+ cells. However, both cell lines exhibit a lower Ins 1,4,5 P_3 kinase specific activity than the rat brain enzyme (Refer to figures 5.4a and 5.4b) and this low Ins 1,4,5 P_3 kinase specific activity may partly explain the lack of Ins 1,3,4,5 P_4 in bombesin stimulated T15 and NIH3T3 cells.

Table 2 shows that the NIH3T3 fibroblast Ins 1,4,5 P_3 kinase enzyme is inactive at pH 7 as compared to pH 9 and is less active in an ionic buffer compared to a low ionic strength, non-'physiological' buffer. Data from a similar experiment to compare the specific activities of T15 Ins 1,4,5 P_3 kinase in buffers A and B at pH 7 or pH 9 also showed that the enzyme was inhibited at pH 7 as compared to pH 9 in buffer A. This was not an effect of inhibition of Ins 1,4,5 P_3 kinase specific activity by 2,3 DPG at pH

7 as the specific activities of the enzyme assayed in the presence or absence of 2,3 DPG at pH 7 or at pH 9 were the same.This could suggest that the Ins 1,4,5 P_3 kinase enzyme has an optimum pH that is above pH 7 and is inhibited in a high ionic strength buffer. It has been shown that the optimum pH of the Ins 1,4,5 P_3 kinase from other sources is greater than pH 7, for example, the pH optimum for the enzyme from pig aortic smooth muscle is 7.7 (Yamaguchi et al, 1988) and is 8.5 for the enzyme from bovine brain (Ryu et al, 1987). The extent to which the T15+ and NIH3T3 Ins 1,4,5 P_3 kinase enzymes were inhibited differed to a marked degree. While it was possible to assay the T15 enzyme in buffer B at pH 7 in the presence of 2,3 DPG, there was no detectable NIH3T3 Ins 1,4,5 P_3 kinase activity under these conditions. For this reason, subsequent enzyme assays were performed in buffer A and at a pH that was closest to in vivo conditions *i.e.*at pH 7, although the enzyme specific activities were lower at this pH than at pH 9.

Experiments to determine the Km for Ins 1,4,5 P_3 of the Ins 1,4,5 P_3 kinase in NIH3T3 cells (figure 5.5) indicated that this enzyme has a higher Km value than the enzyme from rat brain cytosol (0.6μM, Irvine et al, 1986; 0.44μM, Morris et al, 1988), from retina (1.3μM, Tarver and Anderson, 1988), from bovine brain (0.7μM, Ryu et al, 1988) and in pig aortic smooth muscle (0.4μM, Yamaguchi et al, 1988). The Km value of the NIH3T3 enzyme as derived from experiments utilising high speed supernatants prepared from NIH3T3 cells is between 12 and 15 μM Ins 1,4,5 P_3 and is at least 10 times higher than the Km for Ins 1,4,5 P_3 kinase from other tissue and suggests that the NIH3T3 enzyme has a low affinity for Ins 1,4,5 P_3. This is supported by the fact that while bombesin stimulated NIH3T3 cells do not generate significant amounts of Ins 1,4,5 P_3 or Ins 1,3,4,5 P_4, this is not true of PGF2α stimulated NIH3T3 cells. PGF 2α stimulates a 20-30 fold increase in total inositol phosphates in NIH3T3 cells and it appears that a large increase in Ins 1,4,5 P_3 levels occurs before Ins 1,3,4,5 P_4 formation can occur in this cell type. PGF2α stimulated

NIH3T3 cells exhibit a 4 fold increase in Ins 1,4,5 P_3 production with the peak of Ins 1,4,5 P3 generation occuring at 15 seconds after agonist stimulation and an associated increase in Ins 1,3,4,5 P_4 and Ins 1,3,4 P_3 is seen in these cells (Black, F. and Wakelam, M.J.O., manuscript in preparation). Although the curve drawn in figure 5.5a indicated Michaelis-Menten kinetics for the NIH3T3 Ins 1,4,5 P_3 kinase, it is possible that this curve is sigmoidal. If this is true, it suggests another possible reason for the lack of Ins 1,3,4,5 P_3 formation at low concentrations of Ins 1,4,5 P_3 and the detection of Ins 1,3,4,5 P_4 when there are large mitogen stimulated levels of Ins 1,4,5 P_3 apart from the high Km value for Ins 1,4,5 P_3. A sigmoidal curve would also suggest that positive cooperativity was a regultory feature of this enzyme. However, until conclusive data is obtained which indicates a linear rate of enzyme reaction, the considerations above cannot be tested.

In figure 5.6, the ATP requirement of the NIH3T3 Ins 1,4,5 P_3 kinase is shown and from this data, a Km value for ATP of approximately 4 mM was derived. This value is high compared to the Km for ATP for the retinal Ins 1,4,5 P_3 kinase (1.4 mM, Tarver and Anderson, 1988),the enzyme from bovine brain (0.04 mM, Ryu et al, 1987) and the enzyme from rat brain (460 μM, Morris et al,1988). This suggests that the NIH3T3 enzyme as assyed in a high speed supernatant has a lower affinity for ATP than the enzyme from other sources. As with the substrate affinity of the enzyme, this apparently low affinity for ATP could be a factor that influencing the phosphorylation of Ins 1,4,5 P_3 to Ins 1,3,4,5 P_4 in NIH3T3 cells. Further experiments are necessary to test the possibility that the amounts of ATP in the assay were not a limiting factor for Ins 1,4,5 P_3 kinase activity as there was no ATP regenerating system present. However, on the basis of the experiments described, it is not possible to determine if these high Km values for both Ins 1,4,5 P_3 and ATP derived for NIH3T3 Ins 1,4,5 P_3 kinase were due to enzyme characteristics or to endogenous factor(s) in the cytosol which modify the affinity of the enzyme for Ins 1,4,5 P_3 and ATP.

Experiments using purified preparations of NIH3T3 Ins 1,4,5 P_3 kinase may provide a solution to these questions and may also suggest if NIH3T3 fibroblasts regulate Ins 1,4,5 P_3 kinase activity differently from other tissues, although the enzymes from different tissues seem to have different characteristics from each other (See examples cited above).

The apparent high Km value for Ins 1,4,5 P_3 of the NIH3T3 Ins 1,4,5 P_3 kinase as determined from figure 5.5 indicated that the enzyme did not have a high affinity for its substrate. Dilution of NIH3T3 high speed supernatants and the subsequent assay of the diluted high speed supernatants for Ins 1,4,5 P_3 kinase activity as shown from the results in figure 5.7 have suggested that dilution of the high speed supernatant increases the specific activity of Ins 1,4,5 P_3 kinase in the preparation. This raises the possibility of the existence of an endogenous inhibitor of the enzyme in NIH3T3 cells. Increasing specific activity was associated with decreasing amounts of protein and indicates that either the affinity of the enzyme for its substrate has increased or that the V max of the enzyme reaction had increased on dilution. As this effect is associated with reduced amounts of protein assayed for Ins 1,4,5 P_3 kinase activity it may be possibly due to the removal of an inhibitory factor(s) upon dilution of the high speed supernatant.

A further modification of NIH3T3 Ins 1,4,5 P_3 kinase specific activity occurs when the enzyme is assayed in either a physiological- type buffer (buffer B) or a non-physiological buffer (buffer A). When the dilution experiment was repeated in buffers A and B and the results compared (figure 5.8), it is clear that the increased specific activity associated with dilution of the high speed supernatant is more apparent using buffer B. In table 2, it has been shown that in this physiological type buffer there is no Ins 1,4,5 P_3 kinase activity in NIH3T3 high speed supernatants. However, dilution of these supernatants and subsequent assay of the samples in buffer B greatly enhanced the Ins 1,4,5 P_3 kinase specific activity. In buffer A, there was no inhibition of NIH3T3 Ins 1,4,5 P_3 kinase activity compared to buffer B but dilution of the high speed supernatants

and assay of these diluted supernatants in buffer A did not increase the Ins 1,4,5 P_3 kinase specific activity to the same extent as in buffer B. This suggests that the composition of the assay buffer can affect NIH3T3 Ins 1,4,5 P_3 kinase activity and that if an endogenous inhibitor is present, then its activity is modified by buffer composition, perhaps by the action of Ca^{2+} ions on the inhibitor. If the inhibitor is not active in buffer A, then detectable Ins 1,4,5 P_3 kinase activity will be observed when assayed in this system but only a small increase in Ins 1,4,5 P_3 kinase activity will be detected on dilution of high speed supernatants since the enzyme is not completely inhibited, or because only a small proportion of the total inhibitor concentration will be in an active state. However, in the physiological, high ionic strength buffer which reflects the cellular environment, the inhibitor may be in an active conformation and therefore, no activity of the Ins 1,4,5 P_3 kinase will be detectable unless the high speed supernatant is diluted. On dilution of the supernatants, therefore, a very large increase in Ins 1,4,5 P_3 kinase specific activity will be detected.Thus, the differences in the specific activities of Ins 1,4,5 P_3 kinase in undiluted and diluted NIH3T3 high speed supernatants when assayed in either buffers A or B may be a reflection of the activity of the possible inhibitor(s) in cytosolic extracts and the interaction of this inhibitor with the NIH3T3 Ins 1,4,5 P_3 kinase.The increase in specific activity of Ins 1,4,5 P_3 kinase in diluted high speed supernatants was shown to be due to an increase in V max of the enzyme reaction rather than a change in the Km of the reaction (Figures 5.9a-5.9f). This result suggests that if such an endogenous inhibitor of NIH3T3 Ins 1,4,5 P_3 kinase is present in the cells, then the inhibition is non-competitive as deduced from Lineweaver-Burke plots of the data.

The results presented in Table 3 suggest that the inhibition of NIH3T3 Ins 1,4,5 P_3 kinase may not be specific to this cell line as mixing a concentrated cytosolic extract of NIH3T3 cells with a cytosolic extract from bovine adrenal glands caused a reduction in the amount of Ins 1,3,4,5 P_4 produced as compared to either adrenal gland or NIH3T3 high speed

supernatants assayed under the same experimental conditions. More importantly, the amount of Ins 1,3,4,5 P4 generated in a sample of adrenal gland high speed supernatant 'diluted' with NIH3T3 high speed supernatant was reduced compared to Ins 1,3,4,5 P4 generated in adrenal gland and NIH3T3 high speed supernatants which were diluted in the same ratios with a sucrose/Tris buffer. This suggests that the effect of addition of NIH3T3 cytosolic extract to the adrenal gland high speed supernatant may be to inhibit the adrenal Ins 1,4,5 P3 kinase activity and therefore, shows that the 'inhibitor' of Ins 1,4,5 P3 kinase activity in NIH3T3 cells can also affect Ins 1,4,5 P3 kinase activity from other tissue.

The results in table 3 also show that dilution of NIH3T3 high speed supernatant did not affect the amount of Ins 1,3,4,5 P4 generated compared to undiluted NIH3T3 high speed supernatant whilst dilution of adrenal high speed supernatant resulted in a decrease in the amount of Ins 1,3,4,5 P4 generated compared to undiluted samples. This suggests differential regulation of Ins 1,4,5 P3 kinase activity in these two different cell types and also shows that the possible inhibition of Ins 1,4,5 P3 kinase activity in NIH3T3 cells does not occur in bovine adrenal gland.

The results presented in this chapter indicate that there may be an inhibitor of Ins 1,4,5 P3 kinase activity in the NIH3T3 cell line. The mechanism of this inhibition is at present unclear but may be non-competitive in nature. The lack of Ins 1,3,4,5 P4 production in the T15 cells may therefore be explained by the possible inhibition of Ins 1,4,5 P3 kinase activity in this cell line but from Table 1 and the data in figure 5.2, it is clear that T15+ cells exhibit a higher Ins 1,4,5 P3 5'monophosphoesterase activity than either NIH3T3 or brain cytosolic extract. The Ins 1,4,5 P3 5'monophosphoesterase activity in T15+ cells is not inhibited at pH 9 suggesting that this enzyme may be differently regulated in this transformed cell line or is present at a greater level.

The biological significance of these differences in the regulation of Ins 1,4,5 P3 and Ins 1,3,4,5 P4 dephosphorylation between T15+ and

NIH3T3 cells is not clear at present but it is possible that peptide growth factor stimulated Ins 1,3,4,5 P_4 production in T15 cells does not have a role in cell proliferation. Also, it is not known if one of the effects of p21 $^{N\text{-}\underline{ras}}$ transformation on NIH3T3 cells is to increase the rate of Ins 1,4,5 P_3 degradation or if these effects are due to the onset of transformation as a consequence of p21 $^{N\text{-}\underline{ras}}$ overexpression and are therefore not specific to the ras oncogene.Therefore, the investigation into the regulation of Ins 1,4,5 P_3 kinase activity in the NIH3T3 cell line should be carried out on the purified enzyme and studies on transformed cell lines following the study of the NIH3T3 enzyme would perhaps demonstrate the significance of these preliminary results described in this chapter. The role of the ras oncogenes and other oncogenes on this component of the phosphoinositide signalling pathway would thus possibly be more clearly defined.

Chapter 6: The effects of p21$^{N\text{-}ras}$ overexpression on cyclic AMP production in the NIH3T3 cell line.

Introduction

The results in the preceeding chapters have shown that p21 $^{N\text{-}ras}$ affects bombesin stimulated inositol phosphate production which may correlate with cell proliferation. However, the other main cellular signalling system which uses cyclic AMP as a second messenger, may also be modified by p21 ^{ras}. The adenylate cyclase signalling system is regulated by two G proteins (G_S and G_i) and the production of cyclc AMP is mediated by extracellular signals via stimulatory and inhibitory receptors coupled to the appropriate G proteins. The activity of these G proteins can be modified by ADP-ribosylation of their alpha subunits in the presence of bacterial toxins (Gilman, 1984). ADP-ribosylation catalysed by pertussis toxin will inactivate G_i while cholera toxin catalysed ADP-ribosylation will activate G_S. Therefore, the effect of both these toxins is to increase the activity of adenylate cyclase whilst acting on different substrates.

In the yeast, S. Cerevisiae, it has been shown that the RAS 2 gene product regulates adenylate cyclase activity (Broek et al, 1985). The yeast RAS proteins are closely related to the mammalian p21 ^{ras} proteins and therefore, it was possible that mammalian p21 ^{ras} was involved in the regulation of adenylate cyclase activity. The homology between p21 ^{ras} and the a subunits of GTP binding proteins, for example transducin, also suggest the possibility that p21 ^{ras} may function as a regulatory G protein for the mammalian adenylate cyclase signalling system. However, there is no evidence for this function of p21 ^{ras} in the literature although some of the reports show conflicting evidence (See Discussion at the end of this chapter).

As the previous chapters have demonstrated that p21$^{N\text{-}ras}$ overexpression is involved with increased agonist-stimulated Ins 1,4,5 P_3 production, the effects of p21$^{N\text{-}ras}$ overexpression on cyclic AMP production was investigated in the T15 cell line.The effects of p21 $^{N\text{-}ras}$ overexpression on

the adenylate cyclase system in NIH3T3 cells have not been reported in the literature. Therefore these experiments were performed to investigate the effects of p21 $^{N\text{-}\underline{ras}}$ overexpression in NIH3T3 cells on the cyclic AMP signal transduction pathway which may be associated with cell proliferation. These experiments were performed on T15- cells, T15- cells treated with dexamethasone for 24 hours prior to the experiment (T15+/24 hours) and T15- cells treated with dexamethasone for at least 2 passages resulting in complete morphological transformation (T15+ cells). This allowed the progressive increase of p21 $^{N\text{-}\underline{ras}}$ overexpression in T15 cells and the investigation of these effects on cylic AMP production. T15- cells were cultured routinely until 2 days prior to experimentation, when the medium was changed to 10 % (v/v) steroid-free serum/90 % DMEM. This was to minimise the frequency of spontaneous transformation of T15- cells due to the presence of steroids in the growth medium. Culturing of NIH3T3 cells in the steroid-free medium described above did not affect either basal or agonist stimulated intracellular cyclic AMP levels compared to cells grown in routinely used 10 % calf serum/90 % DMEM. Therefore, it can be assumed that steroid-free culture of T15- cells would not affect either basal or agonist stimulated intracellular cyclic AMP levels.

Initial attempts to characterise the effects of p21 $^{N\text{-}\underline{ras}}$ on adenylate cyclase using membrane preparations from T15 and NIH3T3 cells proved unsuccessful and therefore, cyclic AMP levels in whole cells were measured. The reasons for the lack of detectable adenylate cyclase activity in membrane preparations in T15 cells are not clear but it is possible that the procedure for the preparation of membranes from these cells resulted in the removal of necessary cytosolic factors which stimulate the activity of adenylate cyclase. It is also possible that the methods utilised for the preparation of crude membrane fractions from T15 and NIH3T3 cells affected the adenylate cyclase enzyme and the associated components in the membrane. As these problems could not be resolved, an indirect measure of adenylate cyclase activity was determined by estimating intracellular cyclic AMP content in unbroken cells in the presence of the cyclic AMP phosphodiesterase inhibitor, IBMX.

Results

Experiments using T15 cells were performed to determine cyclic AMP levels in control and agonist stimulated cells. T15- cells were grown in 2μM dexamethasone for 24 hours prior to the experiment generating T15+/24 hours cells. These cells express significant amounts of p21 $^{N\text{-}\underline{ras}}$ protein and exhibit amplified bombesin stimulated inositol phosphate production (See Chapter 3). T15+/24 hour cells differ from T15+ cells in that the latter are cultured in dexamethasone for at least two passages. The experiments were performed on these three different T15 cell types to determine if long-term p21 $^{N\text{-}\underline{ras}}$ overexpression affected cyclic AMP levels in NIH3T3 cells. These experiments demonstrate one of the advantages of this inducible cell line.

The experiments were carried out as described in the Methods section in the presence of 1mM IBMX in order to inhibit cyclic AMP phosphodiesterases. In all cell types shown in this and following experiments, the basal levels of cyclc AMP were measured in the presence of 1mM IBMX. If IBMX was not included in the assays, the levels of cyclic AMP measured in all cell types were approximately 50% of the levels measured in its presence. All concentrations of agonists were 10^{-5} M except for forskolin which was 10^{-4} M.

Figure 6.1 shows the levels of cyclic AMP in unstimulated and agonist stimulated T15 cells. The results indicate that in non-transformed cells, there was a large stimulation (approximately 7 fold) of cyclic AMP production in response to isoproterenol, adrenaline and forskolin. The isoproterenol and adrenaline stimulated levels were reduced in T15+/24 hour cells by approximately 30% and 50% respectively. However, there was no inhibition of forskolin stimulated cyclic AMP levels in T15+/24 hour cells. In contrast, in T15+ cells there was a marked reduction in isoproterenol, adrenaline and forskolin stimulated cyclic AMP levels by approximately 80%, 90% and 75% respectively. Although the stimulated levels of cyclic AMP production were inhibited when p21 $^{N\text{-}\underline{ras}}$ overexpression was induced, there was no change in the basal rate of cyclic AMP production in all three cell types, taking into

account the standard errors of the data. Therefore, the data shown in this figure suggest that overexpression of p21 $^{N\text{-}\underline{ras}}$ in T15 cells resulted in the inhibition of agonist stimulated cyclic AMP levels but did not affect the basal rate of cyclic AMP production.

From figure 6.1, it is clear that adrenaline at a concentration of 10^{-5} M acts to stimulate an increase in cyclic AMP levels in T15 cells. However, adrenaline can act via β or α_2 receptors which are stimulatory and inhibitory respectively, for adenylate cyclase. To investigate if adrenaline could act via α_2 receptors in this cell line, experiments were carried out where adrenaline was incubated with cells together with propranalol and forskolin as described in the Methods section. Propranalol, an antagonist for β receptors, will inhibit isoproterenol and adrenalin stimulation of cyclic AMP levels (See table 1). If propranalol is added with adrenaline and forskolin, then adrenaline cannot act through b receptors but must act through α_2 receptors. If adrenaline does act through α_2 receptors in this cell line, then a reduction in forskolin stimulated cyclic AMP levels in T15 cells would be observed.

The results shown in table 1 show that propranalol at 10^{-5} M effectively inhibited adrenaline stimulated cyclic AMP production in T15 cells, since propranalol and adrenaline together did not cause an increase in cyclic AMP over basal levels. When adrenaline, forskolin and propranalol are added together in T15 cells, there was no inhibition of cyclic AMP levels below the levels stimulated by forskolin alone *i.e.*under these conditions adrenaline does not act via α_2 receptors to inhibit forskolin stimulated cyclic AMP levels in T15- and T15+/24 hour cells. Therefore, these results demonstrate that adrenaline acts only through β adrenergic receptors in T15 cells and not through α_2 receptors, suggesting that the use of adrenaline as a β adrenergic agent in these experiments is valid.

Figure 6.1: Basal and stimulated cyclic AMP levels in T15-, T15+/24 hour and T15+ cells.

The figure shows the basal and agonist stimulated levels of cyclic AMP in T15-, T15+/24 hour and T15+ cells expressed as pmol cyclic AMP/10 min/10^5 cells. The experiments were carried out as described in the Methods section and incubations performed in the presence of 1mM IBMX. Concentrations of agonists were as follows: Isoproterenol=10^{-5} M, Adrenaline=10^{-5} M and forskolin=10^{-4} M. These concentrations were used in all experiments in this chapter unless otherwise stated. The results shown in this figure are pooled data from 4-6 separate experiments where n=3 in each and are means ± S.E.M.

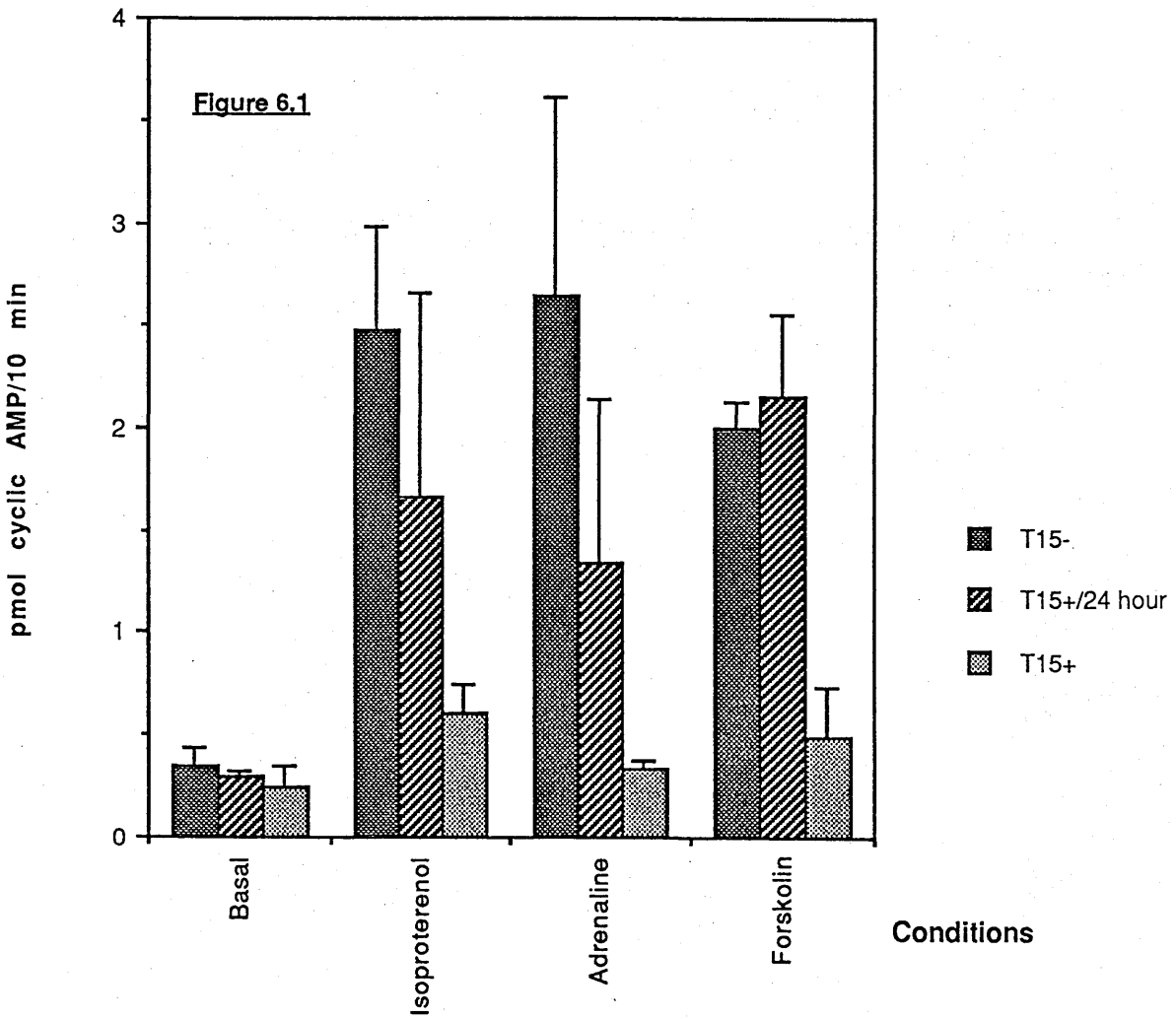


Figure 6.1
pmol cyclic AMP/10 min
4
3
2
1
0
Basal
Isoproterenol
Adrenaline
Forskolin
Conditions
T15-
T15+/24 hour
T15+

Table 1: Adrenaline acts as a β adrenergic agonist in T15 cells.

The table shows data from an experiment to determine if adrenaline acts through either β or α_2 receptors, or both, in T15 cells at a concentration of 10^{-5} M. The experiment was performed as described in the Methods section on T15-, T15+/24 hour and T15+ cells using propranalol at a concentration of 10^{-5} M and other agonists at concentrations already described. The data is from one experiment representative of 2-4 separate experiments and are expressed as mean pmol cyclic AMP/10 min/10^5 cells ± S.D., n=3 in each. N.D.= not determined.

	Cell type		
Conditions	**T15-**	**T15+/24 hour**	**T15+**
Basal	0.33±0.02	0.3±0.02	0.33±0.02
Adrenaline	3.23±0.28	1.98±0.38	0.68±0.10
Adrenaline + Propranalol	0.40±0.10	0.35±0.02	0.18±0.02
Forskolin	1.85±0.35	2.0±0.18	0.75±0.03
Forskolin+ Adrenaline+ Propranalol	1.73±0.15	1.93±0.15	N.D.

The data in the previous figures have shown that the overexpression of p21 $^{N\text{-}ras}$, induced by dexamethasone, resulted in an inhibition of agonist stimulated cyclic AMP levels. As this effect could be due to a non-specific effect of the steroid in this cell type, experiments were performed on NIH3T3 cells treated with dexamethasone to determine if such cells exhibited an inhibition of agonist stimulated cyclic AMP levels. Therefore, NIH3T3 cells were treated with 2μM dexamethasone for 24 hours prior to the experiment and control and treated cells assayed for cyclic AMP content under basal and agonist stimulated conditions as previously described.

The data shown in figure 6.2 indicates that there were no differences in the basal and agonist stimulated cyclic AMP levels in control and dexamethasone treated NIH3T3 cells. The data also shows that although the basal level of cyclic AMP in NIH3T3 cells was similar to T15- cells, the agonist and forskolin stimulated cyclic AMP levels were lower than in T15- cells. This result is surprising, as the two cell types are non-transformed cells, T15- cells being derived from NIH3T3 cells. The reasons for this variation is unclear but may represent clonal differences between the cell lines.

Figure 6.2: Basal and agonist stimulated cyclic AMP levels in control and dexamethasone treated NIH3T3 cells.

The figure shows the data from experiments to determine the cyclic AMP levels in NIH3T3 cells which were either treated or untreated with 2μM dexamethasone for 24 hours prior to the experiment. The experiment was performed under the conditions of previous experiments (See figure 6.1). All concentrations of agonists were as already described and incubations were performed in the presence of 1mM IBMX. The data is expressed as pmol cyclic AMP/10 min/10^5 cells and is pooled from two separate experiments, n=3 in each case and the results are means ± S.D.

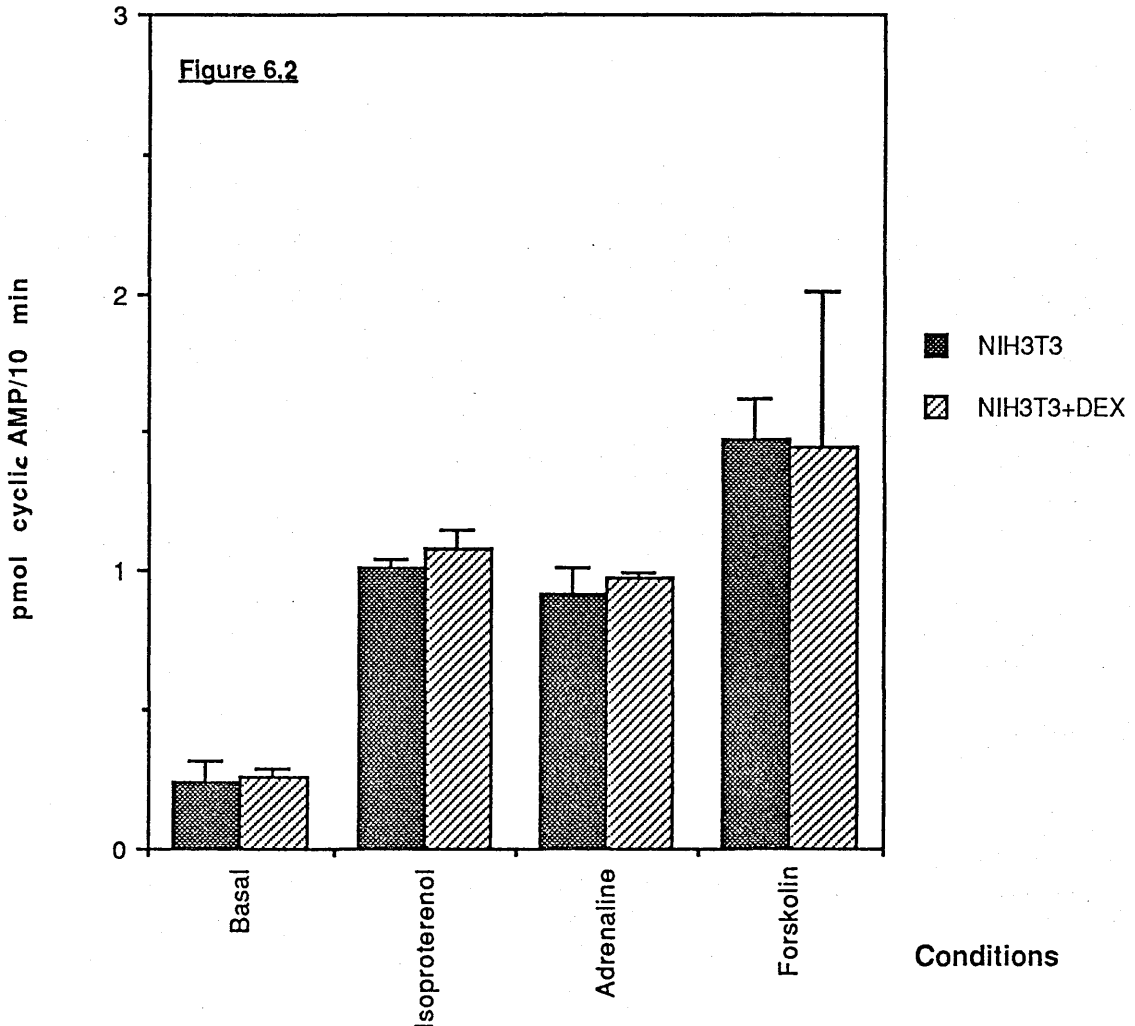


Figure 6.2
pmol cyclic AMP/10 min
3
2
1
0
Basal
Isoproterenol
Adrenaline
Forskolin
Conditions
NIH3T3
NIH3T3+DEX

The T15 cell line is transfected with a dexamethasone inducible promoter which controls p21 $^{N\text{-}\underline{ras}}$ overexpression. Transformation of the cells can thus be 'switched on' or 'switched off' by the addition of the steroid to the growth medium of the cells. As the previous results have demonstrated that p21 $^{N\text{-}\underline{ras}}$ overexpression can affect cyclic AMP generation, the constitutive overexpression of p21 $^{N\text{-}\underline{ras}}$ in NIH3T3 cells and its relevance to cyclic AMP production was examined.

The N 866 cell line was utilised to investigate if constitutive overproduction of p21 $^{N\text{-}\underline{ras}}$ would affect cyclic AMP production in NIH3T3 cells. N 866 cells are NIH3T3 cells transfected with the human foetal genomic p21$^{N\text{-}\underline{ras}}$ proto-oncogene. The difference between this cell line and T15 cells is that the N 866 cell line constitutively overproduces p21 $^{N\text{-}\underline{ras}}$, are thus permanantly 'switched on' and thus exhibit the transformed phenotype.This cell line was utilised to investigate cyclic AMP production under the same conditions as described in figure 6.1. All assays were performed in the presence of 1mM IBMX and all concentrations of agonists used were as previously described.

The data in figure 6.3 shows that both basal and agonist stimulated levels of cyclic AMP were reduced in N 866 cells compared to T15 cells. There was an approximately 33% decrease in basal levels of cyclic AMP in N 866 cells as compared to T15 cells. In N 866 cells there was no significant increase in cyclic AMP levels when the cells are incubated with isoproterenol or adrenaline. However, there was an approximately 1.6 fold increase in stimulation of cyclic AMP levels in response to forskolin stimulation. This increase, however, was small compared to the 6.7 fold increase detected in T15- cells and 2 fold increase detected in T15+ cells. Therefore, in N 866 cells there is negligible β adrenergic stimulation of cyclic AMP production. This indicates that cells that are constitutively transformed by p21 $^{N\text{-}\underline{ras}}$ have much lower cyclic AMP levels than T15 cells and indicates that the inhibition of stimulated cyclic AMP levels seen in T15+/24 hour cells and T15+ cells is

associated with p21 $^{N\text{-}\underline{ras}}$ overexpression and may affect cell proliferation.

The effects of p21 $^{N\text{-}\underline{ras}}$ overexpression on basal and stimulated cyclic AMP production as shown in the preceeding figures and Table 1 could have been mediated by the inhibitory G protein for adenylate cyclase, G_i. To determine if this was the case, T15+ and N 866 cells were treated with pertussis toxin at a concentration of 100ng ml^{-1} for 16 hours. This procedure enabled maximal ribosylation of $G_{i\alpha}$ as shown in figure 3.10 . Following pertussis toxin treatment, control and pertussis toxin treated cells were assayed for cyclic AMP content under basal and agonist stimulated conditions as already described. If G_i played a role in modulating cyclic AMP levels in these cells, then the ribosylation of $G_{i\alpha}$ and therefore, its inactivation, would result in an increase in cyclic AMP levels in the toxin treated cells as compared to control cells.

The results in figure 6.4 show that in both T15+ and N 866 cells pertussis toxin treatment did not affect either basal or agonist stimulated cyclic AMP levels. This indicates that G_i does not have a role in the inhibition of cyclic AMP production in cells that overexpress p21 $^{N\text{-}\underline{ras}}$ and that these effects are due solely to the overexpression of the p21 $^{N\text{-}\underline{ras}}$ proto-oncogene.

The data in figure 6.4 indicates that there is no stimulation of adenylate cyclase by either β adrenergic agents or forskolin in T15+ cells which are either treated or untreated with pertussis toxin. This data is not in agreement with that shown in figure 6.3, where there is a small stimulation of adenylate cyclase in T!5+ cells by the ligands above. One reason for this variability may be the different cell preparations used between the experiments. The T15+ cells used in the experiment of figure 6.4 may have been in an advanced state of transformation compared to the cells used in previous experiments and consequently respond to β adrenergic agents and forskolin in a similar manner to the N 866 cells.

Figure 6.3: Basal and agonist stimulated cyclic AMP levels in N 866 cells.

The data in figure 6.3 shows the basal, isoproterenol, adrenaline and forskolin stimulated cyclic AMP levels in N 866 cells. For comparison, cyclic AMP levels in T15- and T15+ cells under the same conditions are indicated. All concentrations of IBMX and agonists used are as already described. The data is from between 4-6 experiments for T15- and T15+ cells, n=3 within each experiment and are means ± S.E.M. but the data from N 866 cells is from two experiments, n=3 in each and are means ± S.D. The data is expressed as pmol cyclic AMP/10 min/10^5 cells.

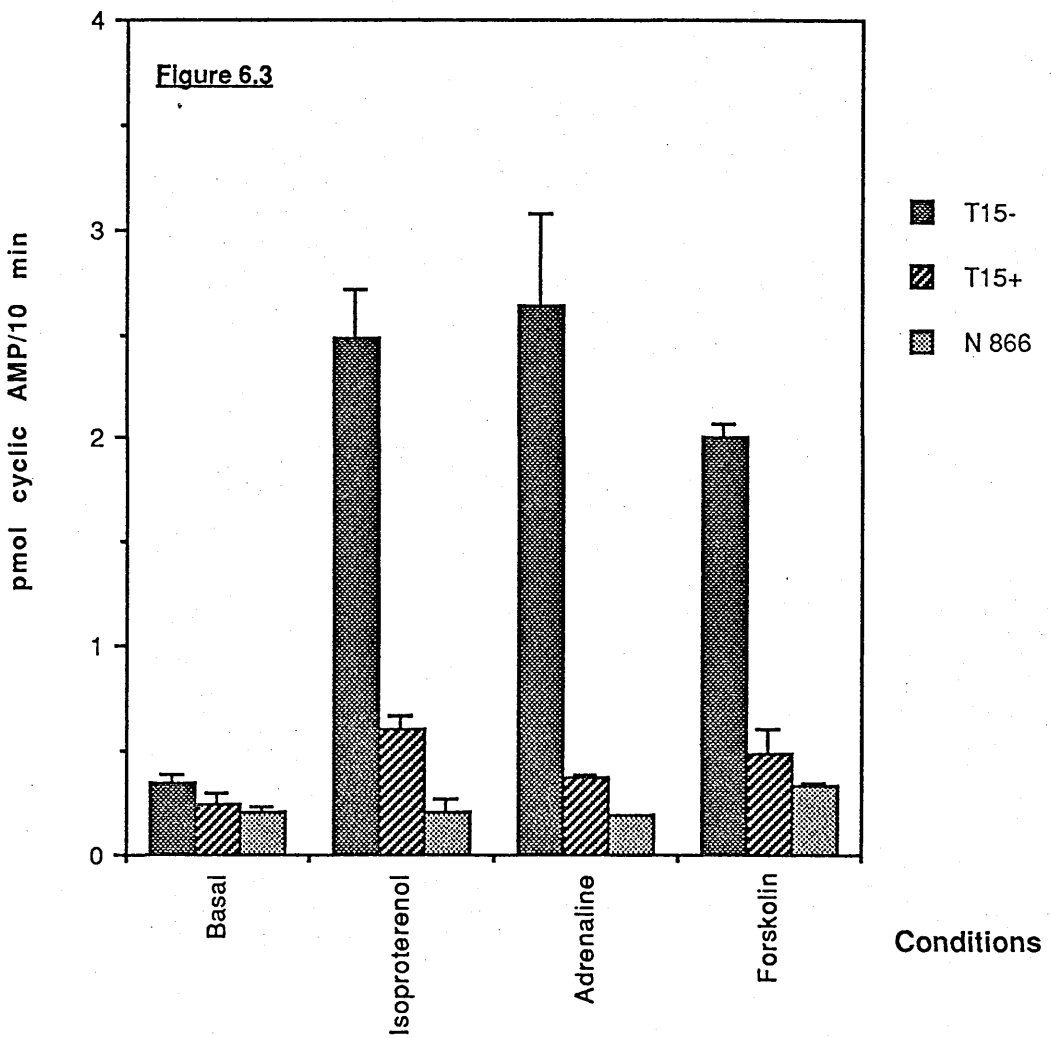


Figure 6.3
pmol cyclic AMP/10 min
4
3
2
1
0
Basal
Isoproterenol
Adrenaline
Forskolin
Conditions
T15-
T15+
N 866

Figure 6.4: The effect of pertussis toxin treatment on basal and agonist stimulated cyclic AMP levels in T15+ and N 866 cells.

The figure shows the results from experiments to determine basal and agonist stimulated cyclic AMP levels in pertussis toxin treated T15+ and N 866 cells. The cells were grown as described in the Methods section and cells were treated with 100 $ngml^{-1}$ pertussis toxin for 16 hours prior to the experiment to maximally ribosylate $G_{1\alpha}$. These cells and untreated, control cells were then assayed for cyclic AMP under basal and isoproterenol, adrenaline and forskolin stimulated conditions. All incubations were performed in the presence of 1mM IBMX and all concentrations of agonists used were as in figure 6.1. The results are expressed in pmol cyclic AMP/10min/10^5 cells and are from one experiment, n=3 (means ± S.D.) for T15+ cells and are from two pooled experiments, n=3 in each (mean ± S.D.) for N 866 cells.

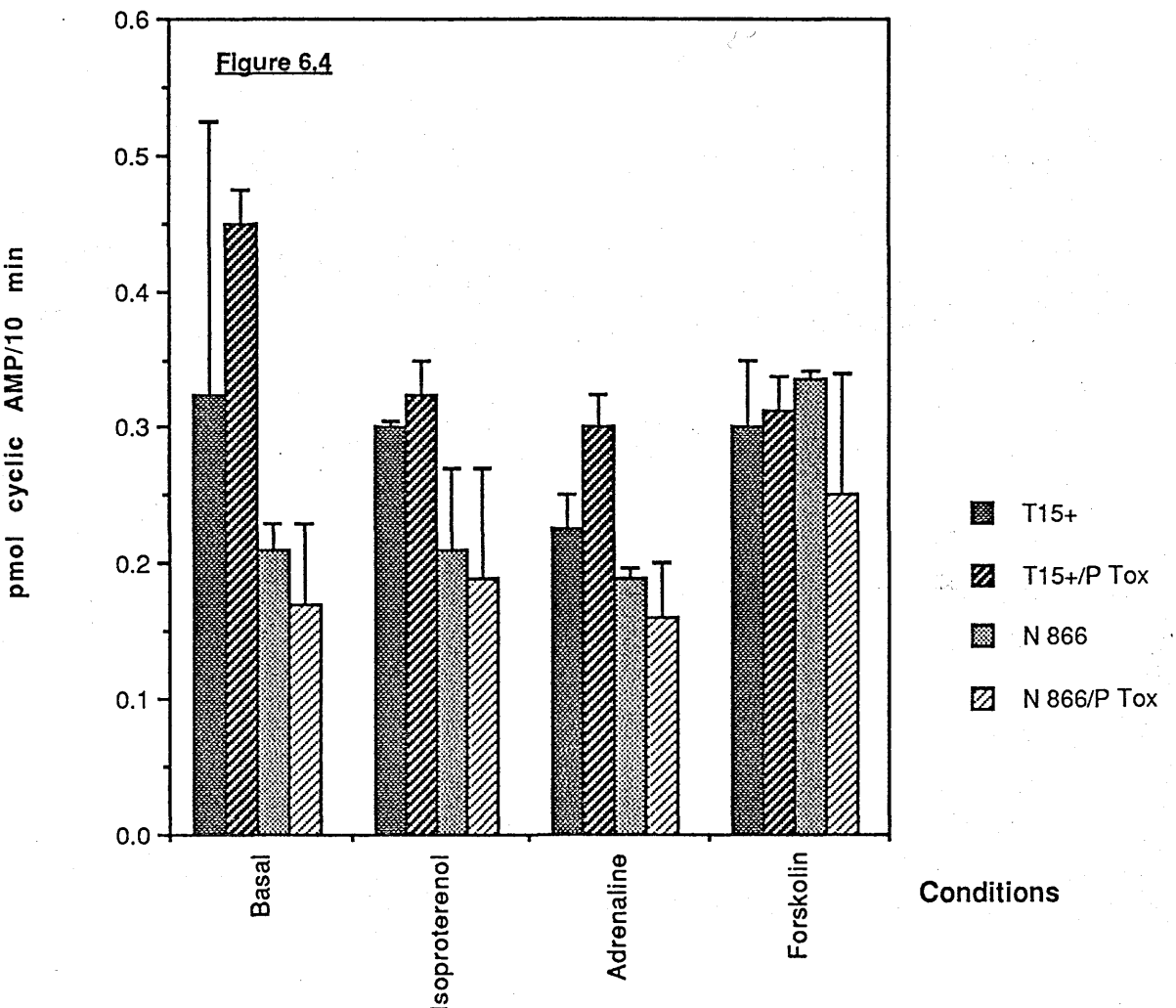


Figure 6.4
pmol cyclic AMP/10 min
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Basal
Isoproterenol
Adrenaline
Forskolin
Conditions
T15+
T15+/P Tox
N 866
N 866/P Tox

All the results in the previous figures indicate that the overexpression of the N-ras proto-oncogene can modify cyclic AMP production in the NIH3T3 cell line. In order to determine if these effects were confined to cells expressing the N-ras proto-oncogene, the cyclic AMP levels in cells expressing the c-sis oncogene were determined. The cell line used was the sis 771 cell line. The cells were grown as described in the Methods section and basal and agonist stimulated cyclic AMP levels determined as in previous experiments.

The results in figure 6.5 show that there was no change in the basal level of cyclic AMP in sis 771 cells as compared to T15- cells. The isoproterenol stimulated cyclic AMP levels in sis 771 cells were lower than in T15- cells but the adrenaline stimulated cyclic AMP levels were the same in both cell types suggesting that there was no real difference between both cell types in terms of agonist stimulated cyclic AMP levels. However, forskolin stimulated cyclic AMP levels in sis 771 cells were approximately 30% lower than in T15- cells and may suggest that c-sis expression may modify forskolin stimulation of cyclic AMP levels. Therefore, this suggests that c-sis expression does not modify the basal nor the β adrenergic agonist stimulation of cyclic AMP levels but may alter the forskolin stimulated cyclic AMP response in NIH3T3 cells.

Figure 6.5: Basal and agonist stimulated cyclic AMP levels in sis 771 cells.

The figure shows the basal and agonist stimulated levels of cyclic AMP in sis 771 cells. For comparison, corresponding values for T15- cells have been included. The data was obtained from cells grown as in the Methods section and experiments were carried out in the presence of 1mM IBMX as already described. All concentrations of agonists were used as described in figure 6.1. The data is expressed as pmol cyclic AMP/10 min/10^5 cells and is from 4-6 separate experiments, n =3 in each and are expressed as means ± S.E.M.

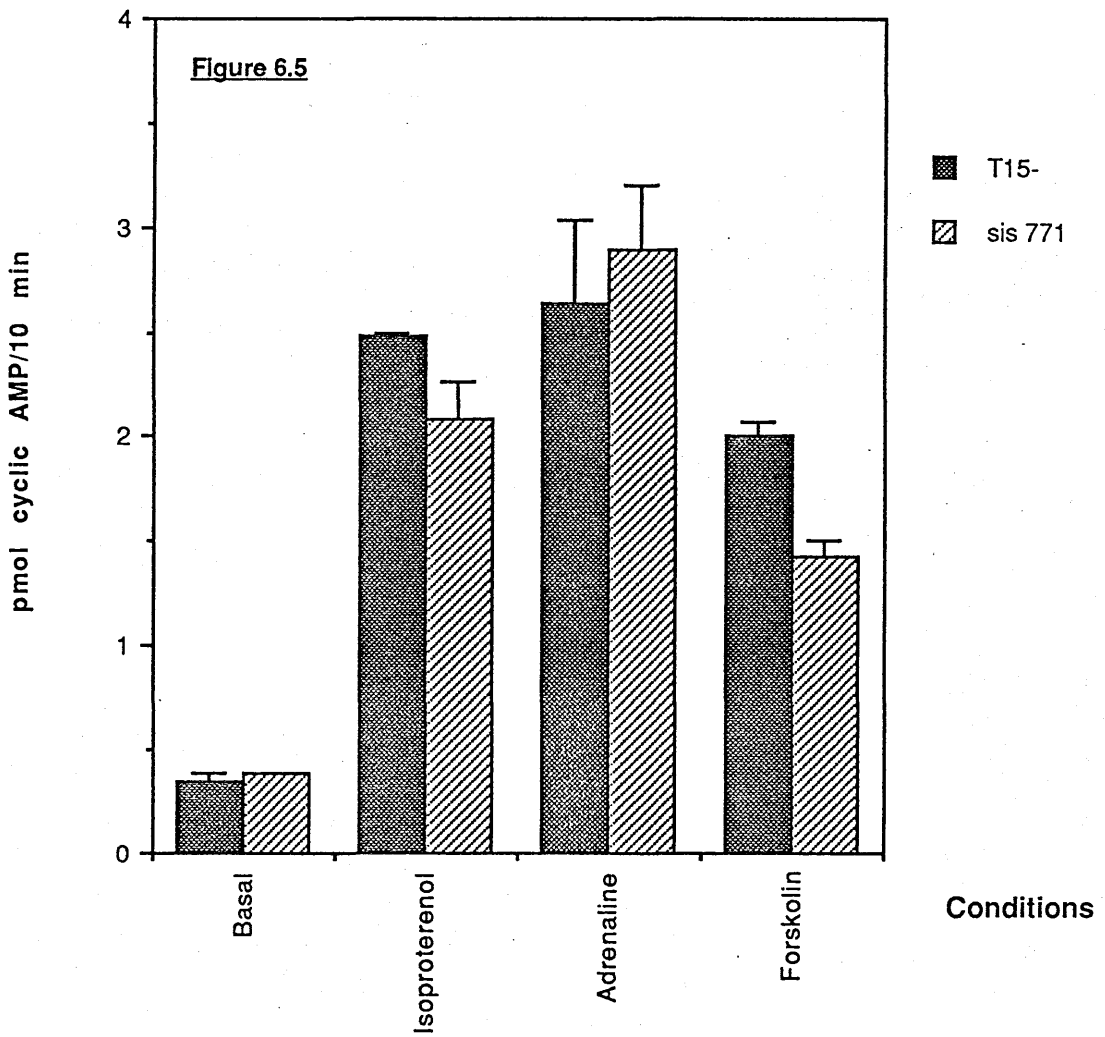


Figure 6.5
pmol cyclic AMP/10 min
4
3
2
1
0
T15-
sis 771
Basal
Isoproterenol
Adrenaline
Forskolin
Conditions

The p21 $^{N\text{-}\underline{ras}}$ induced inhibition of agonist stimulated cyclic AMP levels in NIH3T3 cells has been shown to be specific for the effects of p21$^{N\text{-}\underline{ras}}$ overexpression (See figure 6.5) and does not appear to involve G_i function (See figure 6.4). Therefore, this inhibition of stimulated cyclic AMP production may be due to a change in the β adrenergic receptor affinity in T15 cells upon p21 $^{N\text{-}\underline{ras}}$ overexpression. In order to investigate this possibility, T15-, T15+/24 hours and T15+ cells were stimulated with a range of isoproterenol concentrations and stimulated cyclic AMP levels measured to obtain a dose response curve to isoproterenol in these cell types. Only isoproterenol was used and not adrenaline as it has been demonstrated that adrenaline only acts as a ligand for β receptors in this cell line (See Table 1) and therefore, the binding characteristics of adrenaline would be essentially similar to isoproterenol .

The data in figures 6.6a - 6.6c clearly shows that the dose-response curves for isoproterenol in T15- (figure 6.6a), T15+/24 hour (figure 6.6b) and T15+ (figure 6.6c) cells were similar in shape and differ only the in magnitude of the cyclic AMP response. In all three cell types, significant amounts of cyclic AMP are generated at 10^{-5} M and the peak value of cyclic AMP generation occurs at 10^{-4} M isoproterenol. The fold stimulation of cyclic AMP production at 10^{-4} M isoproterenol compared to basal levels is approximately 4.2 in T15- cells, approximately 2.6 in T15+/24 hour cells and approximately 1.75 in T15+ cells. Basal levels of cyclic AMP for the cells were: T15-: 0.24 ± 0.04, T15+/24 hour : 0.22 ± 0.03 and T15+ : 0.22 ± 0.02. The units of measurement were: pmol cyclic AMP/10 min/10^5 cells.

The graphs also show that the Ka (concentration of isoproterenol required for half maximal stimulation of cyclic AMP levels) for all three cell types was the same at approximately 3.2 x 10^{-6} M isoproterenol.This suggests that the β receptor affiniity for isoproterenol is unchanged upon p21$^{N\text{-}\underline{ras}}$ overexpression. Therefore, the inhibition of stimulated cyclic AMP production in T15+/24 hour, T15+ and N 866 cells is probably not due to a change in

receptor properties.

The results shown in figures 6.6a - 6.6c suggest that the p21 $^{N\text{-}ras}$ induced reduction of β adrenergic stimulated cyclic AMP levels was not due to a change in receptor efficacy for β adrenergic ligands. The other mechanism by which this p21 $^{N\text{-}ras}$ induced inhibition could occur would be the alteration of the number of available binding sites for β adrenergic ligands upon p21 $^{N\text{-}ras}$ overexpression. To investigate this possibility initial attempts were made to determine the number of β adrenergic binding sites in T15-, T15+/24 hour and T15+ cells by using [^{125} I] iodocyanopindolol as a ligand for β adrenergic receptors (See Methods section) . These attempts were unsuccessful as Scatchard analysis of the data obtained from these experiments was not possible as no displacement of the radiolabelled ligand occured at any concentration of unlabelled ligand except at 10^{-3} M isoproterenol or pindolol. The reasons for this are unclear but these experimental results may suggest that these cells readily bind and internalise iodocyanopindolol and that displacement of this internalised ligand does not occur unless very high concentrations of the competing ligand are introduced. Due to these problems, the cells were labelled with [^{125} I] iodocyanopindolol in the presence of 10^{-4} M isoproterenol to obtain an estimate of the amount of [^{125} I] bound under conditions where maximal stimulation of cyclic AMP levels occured. Non-specific binding was estimated in the presence of 10^{-3} M pindolol (See Methods Section) and these values used to calculate the data shown in Table 2.

The results shown in Table 2 are from 2 experiments performed as detailed in the Methods section to determine the amount of [^{125} I] iodocyanopindolol bound to T15-, T15+/24 hour and T15+ cells. The results shown indicate that there was a reduction in the amount of [^{125} I] iodocyanopindolol bound to p21 $^{N\text{-}ras}$ transformed T15 cells. From the data in experiment 1, there was an approximately 20% decrease and 89% decrease in [^{125} I] iodocyanopindolol binding in T15+/24 hour cells and T15+ cells respectively, as compared to T15- cells. The data in experiment 2 indicates that an approximately 40% reduction in T15+/24 hour cells and an approximately

Figures 6.6a - 6.6c: Dose response curves for isoproterenol in T15-, T15+/24 hour and T15+ cells.

The data shown in figures 6.6a - 6.6c are from experiments to determine the Ka values for isoproterenol stimulated cyclic AMP production in T15-, T15+/24 hour and T15+ cells. The experiment was performed on the three cell types as already described in the presence of 1 mM IBMX and isoproterenol was used at concentrations of and between 10^{-9} M - 10^{-3} M. The data shown is from one experiment, n= 3 in each and are expressed as means ± S.D. but is representative of three separate experiments and indicates pmol cyclic AMP generated per 10 minutes per 10^5 cells. Figure 6.6a : T15- cells; Figure 6.6b : T15+/24 hour cells; Figure 6.6c : T15+ cells.

Figure 6.6a

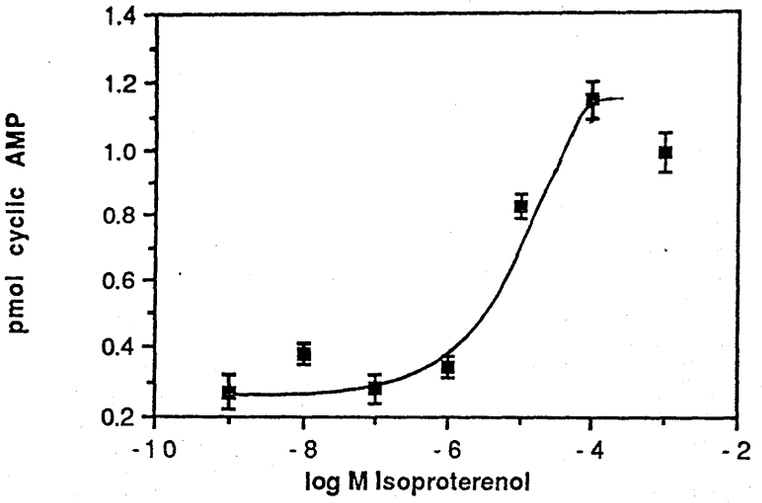


Figure 6.6b

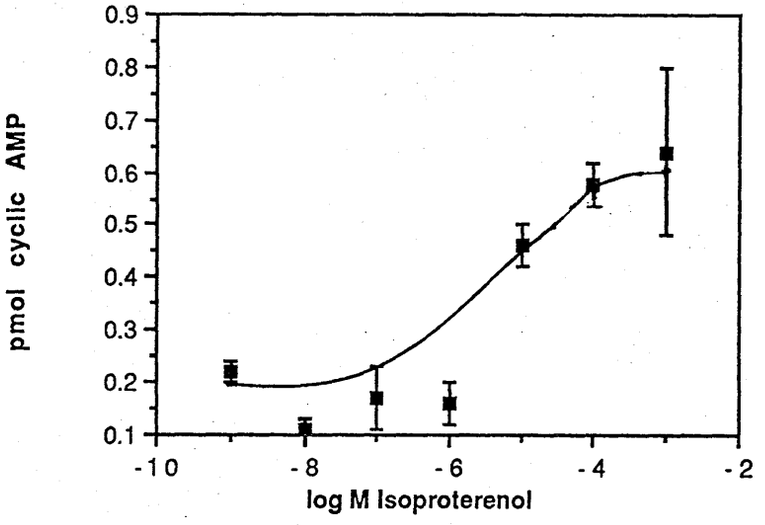
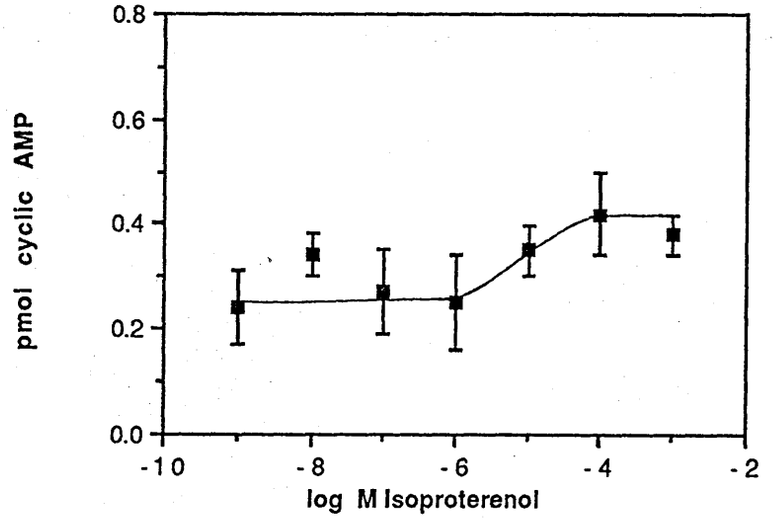


Figure 6.6c



95% reduction in T15+ cells occured in [^{125} I] iodocyanpoindolol binding as compared to T15- cells.

This suggests that p21 $^{N\text{-}ras}$ transformation results in a reduction in β adrenergic stimulated cyclic AMP levels by modifying binding of β adrenergic ligands to the cell surface receptors. It is possible that this can occur either by a downregulation of receptors on p21 $^{N\text{-}ras}$ transformtion or by a modification of the receptors which results in reduced ligand binding. However, this mechanism seems to be only a partial explanation for the reduction in agonist stimulated and basal cyclic AMP levels in NIH3T3 cells transformed with the p21 $^{N\text{-}ras}$ proto-oncogene.

Table 2: [^{125} I] iodocyanopindolol binding in T15-, T15+/24 hour and T15+ cells as an estimate of β adrenergic ligand binding in these cell types.

The data shown in the table are from experiments to determine the amount of [^{125} I] iodocyanopindolol bound to T15-, T15+/24 hour and T15+ cells. The experiments were carried out as already described and the results are expressed as mean fmol [^{125} I] iodocyanopindolol bound/10^5 cells ± S.D., n=3 in each experiment. Non-specific binding was determined in the presence of 10^{-3} M pindolol and was estimated to be approximately 10-30% of the total binding obtained.

Cell type	Experiment 1	Experiment 2
T15-	4.46 ± 0.07	6.96 ± 0.59
T15+/24 hour	3.63 ± 0.11	2.93 ± 0.7
T15+	0.5 ± 0.15	0.29± 0.01

Discussion.

The results shown in this chapter are from experiments to determine cyclic AMP levels in unbroken T15, N 866, NIH3T3 and sis 771 cells. This method was used as attempts to directly measure adenylate cyclase activity in T15 and NIH3T3 cells were unsuccesful. The reasons for this are unclear but as these could not be resolved, an indirect measure of adenylate cyclase activity was obtained by quantifying cyclic AMP levels in whole cells in the presence of IBMX.

In figure 6.1, it is shown that the overexpression of p21 $^{N\text{-}ras}$ in T15+/24 hour cells results in a reduction of agonist stimulated cyclic AMP levels compared to non-transformed cells. However, there was no change in the stimulation of cyclic AMP in response to forskolin.Prolonged expression of p21 $^{N\text{-}ras}$ in T15+ cells led to a further inhibition of β adrenergic agonist stimulated cyclic AMP levels but under these conditions, there was also a reduction in forskolin stimulated cyclic AMP levels. These results show that p21 $^{N\text{-}ras}$ overexpression may result in an inhibition of adenylate cyclase activity by either receptor- or post-receptor mediated mechanisms. The use of adrenaline as a β adrenergic agonist in T15 cells is valid as the results in Table 1 show that adrenaline acts only through β adrenergic recptors and not through α_2 receptors in these cell types. It may be that T15 cells lack α_2 receptors altogether,or that they are present in such small numbers that a significant alteration in cyclic AMP levels as a result of α_2 receptor binding by an appropriate ligand may not be detectable using this assay system.

Dexamethasone treatment of T15- cells resulted in reduced intracellular cyclic AMP levels as already shown. This effect is due to the expression of p21 $^{N\text{-}ras}$ and is not due to the effects of dexamethasone itself. This is supported by the results shown in figure 6.2 which indicate that NIH3T3 cells either treated or untreated with 2μM dexamethasone exhibit similar basal and agonist stimulated cyclic AMP levels. The data also indicates that the stimulated cyclic AMP levels in NIH3T3 cells are lower

than in T15- cells. This is an unexpected result as the two cell types are essentially similar, both are non-transformed cells and the levels of basal and stimulated inositol phosphates in these cell types are the same. However, the fold stimulation of cyclic AMP levels differ by approximately 30% although the forskolin stimulated cyclic AMP levels were the same in both cell types. The data indicates that the differences between the NIH3T3 and T15- agonist stimulated cyclic AMP levels may be due to a difference in β adrenergic receptor numbers between the two cell types although this possibility remains to be investigated.

Having demonstrated that prolonged overexpression of p21 $^{N\text{-}\underline{ras}}$ induced by dexamethasone results in a decrease in agonist stimulated cyclic AMP levels, the N 866 cell line was used to investigate the effects of constitutive p21 $^{N\text{-}\underline{ras}}$ overexpression upon intracellular cyclic AMP levels.The transformed N 866 cells exhibit an increased reduction in agonist stimulated cyclic AMP levels and also show a reduced basal level of cyclic AMP compared to chronically transformed T15+ cells (figure 6.3). This suggests that the inhibition of cyclic AMP production as a consequence of p21 $^{N\text{-}\underline{ras}}$ overexpression is not only linked to p21 $^{N\text{-}\underline{ras}}$ concentration in the cell but also to the consequences of continual p21 $^{N\text{-}\underline{ras}}$ overexpression, for example, continuous cell proliferation.

The experimental results obtained as discussed above are in agreement with published studies on the effect of p21 $^{N\text{-}\underline{ras}}$ on the adenylate cyclase signalling system. Tarpley et al (1986) have shown that p21 $^{Ha\text{-}\underline{ras}}$ expression in NIH3T3 cells led to reduced hormone stimulated adenylate cyclase activity and stimulated intracellular cyclic AMP levels. Furthermore, Chiarugi et al (1985) have demonstrated that transformation of Balb/3T3 fibroblasts with the EJ/ T24/Ha-ras oncogene causes a reduction in basal, PGE_1 and forskolin stimulated cyclic AMP levels compared to control cells. Also, C127 mouse fibroblasts infected with a temperature sensitive mutant of Kirsten Sarcoma virus (TS 371) *i.e.* the Ki-ras oncogene, when assayed for adenylate cyclase activity, exhibited reduced adenylate cyclase activity

compared to wild-type cells (Saltarelli et al, 1985). Therefore, it is clear that in some cell lines, the effect of p21 ras expression is to reduce adenylate cyclase activity with a resulting decrease in intracellular cyclic AMP levels.

Although the results discussed in this chapter have implicated the N-ras proto-oncogene in the modulation of cyclic AMP levels in mouse fibroblasts, there was the possibility that these effects were due to the expression of other oncogenes either together with, or as a result of, p21 $^{N\text{-}ras}$ overexpression. To investigate this possibility, the NIH3T3 cell line transfected with a p 5M1 c-sis cDNA containing plasmid (sis 771) was used and basal and agonist stimulated cyclic AMP levels were measured. The results in figure 6.5 show that there is no change in the basal and β adrenergic stimulated cyclic AMP levels compared to T15- cells but there is an approximately 30% decrease in the forskolin stimulated cyclic AMP level in sis 771 cells. The fact that the basal and β adrenergic agonist stimulated cyclic AMP levels are similar in sis 771 and T15- cells suggests that the expression of the c-sis proto- oncogene does not modulate adenylate cyclase activity directly. Since the basal levels of cyclic AMP are similar in T15, NIH3T3 and sis 771 cells, the inhibition of forskolin stimulated cyclic AMP levels in sis 771 cells may be due to a modification of adenylate cyclase that either inhibits forskolin binding, or inhibits forskolin stimulation of cyclic AMP production. The fact that c-sis expression does not affect basal cyclic AMP levels suggests that this modulation is specific to the regulation of adenylate cyclase activity by agonists that bind directly to this enzyme.The results in figure 6.5 suggest that the p21 $^{N\text{-}ras}$ induced inhibition of β adrenergic stimulated cyclic AMP levels is not due to c-sis oncogene expression and is a specific effect of p21 $^{N\text{-}ras}$ expression. However, the inhibition of forskolin stimulated cyclic AMP levels in T15+ cells may be a consequence of c-sis co-expression with p21 $^{N\text{-}ras.}$ It has been shown that other oncogenes that may be co-expressed with the ras oncogene do not affect cyclic AMP levels, for example, Saltarelli et al (1985) demonstrated that c-myc transformed NIH3T3 cells do not have altered cyclic AMP levels

as compared to control cells. Using src-transformed NIH3T3 cells, Tarpley et al (1986) demonstrated that stimulated cyclic AMP levels in these cells were comparable to wild-type NIH3T3 cells. These results suggest that even if oncogenes are co-expressed with p21 ras, or expressed as a consequence of p21 ras expression (See Introduction chapter) they do not have a direct role in modulating cyclic AMP levels in mouse fibroblasts. However, it is possible that c-sis proto-oncogene expression in T15+ and N 866 cells may cause the decrease in forskolin stimulated cyclic AMP levels.

The effect of p21 ras expression on intracellular cyclic AMP levels is thought to occur via G_i in some cell types. Franks et al (1987) have suggested that p21 Ki-ras interacts with G_i to inactivate the inhibitory component of the adenylate cyclase enzyme in NRK cells. However, Beckner et al (1985) have demonstrated that MDCK cell membranes ADP-ribosylated with pertussis toxin exhibited an increase in agonist stimulated adenylate cyclase activity in wild-type cells but not in p21 Ha-ras transformed cells. Therefore, they have suggested that p21 ras does not act as G_i although the cell lines transformed with p21 Ha-ras and p21 Ki-ras exhibit reduced adenylate cyclase activity. The results shown in figure 6.4 demonstrate that in T15+ and N 866 cells, pertussis toxin treatment does not increase the basal or stimulated cyclic AMP levels. Although this experiment was only performed on p21 N-ras transformed cells and not on control T15- or NIH3T3 cells, the results do suggest that p21 N-ras does not inhibit cyclic AMP production in this cell line by interacting with, or acting as, G_i. The inhibition of cyclic AMP production seen must therefore occur at a receptor level or at a post-receptor level which does not involve the inhibitory G_i proteins.

To investigate the possibility of whether the inhibition of cyclic AMP production in p21 N-ras transformed T15 cells was a consequence of a change in receptor affinity for β adrenergic ligands on p21 N-ras expression, dose-response curves to isoproterenol in T15-, T15+/24 hour and T15+ cells were performed and cyclic AMP levels measured. The results shown in

figure 6.6 indicate that there is a significant increase in cyclic AMP levels in all three cell types only between 10^{-5} M and 10^{-3} M isoproterenol. Also, in all three cell types the maximal stimulated level of cyclic AMP occurs at 10^{-4} M isoproterenol, therefore giving a Ka value of approximately 3.2 x 10^{-6} M for each cell type. Therefore, from this data it is clear that there is no change in the receptor affinity for isoproterenol on p21 $^{N\text{-}\underline{ras}}$ expression. This is also probably true for adrenaline and therefore, a change in receptor affinity is not the cause of the inhibition of cyclic AMP production in p21 $^{N\text{-}\underline{ras}}$ transformed cells.

The inhibition of β adrenergic stimulation of cyclic AMP levels in p21 $^{N\text{-}\underline{ras}}$ overexpressing T15 cells could be due to a reduction in the number of β adrenergic receptor binding sites as a consequence of p21 $^{N\text{-}\underline{ras}}$ expression. This possibility is strongly suggested by the data shown in Table 2 which indicates that T15+/24 hour and T15+ cells have reduced amounts of [^{125} I] iodocyanopindolol bound per 10^5 cells as compared to T15- cells. The reduction in the amount of iodocyanopindolol bound to T15 cells under conditions of maximal cyclic AMP stimulation *i.e.* in the presence of 10^{-4} M isoproterenol, is associated with increasing lengths of time over which p21 $^{N\text{-}\underline{ras}}$ is expressed in these cells.T15+ cells exhibit an approximately 90% reduction in [^{125} I] iodocyanopindolol binding compared to T15 cells while T15+/24 hour cells show a reduction of approximately 20% in [^{125} I] iodocyanopindolol binding as compared to T15- cells. Therefore, the reduction in stimulated cyclic AMP levels seen in p21 $^{N\text{-}\underline{ras}}$ overexpressing cells may be due to the downregulation of β adrenergic receptors in these cells. Levitzki et al (1986) have demonstrated that NIH3T3 cells which express the Ha-ras proto-oncogene and the Ha-ras oncogene have a reduced number of iodocyanopindolol binding sites and this reduction in the number of β adrenergic receptors is associated with the relative tumouregenicity of the transfected Ha-ras oncogenes.

The results discussed so far have suggested that p21 $^{N\text{-}\underline{ras}}$ overexpression causes a decrease in the number of β adrenergic receptors in

T15+/24 hour and T15+ cells leading to decreased stimulated cyclic AMP production. This modification of the cyclic AMP signalling pathway at the receptor level cannot, however, explain the inhibition of forskolin stimulated cyclic AMP levels in T15+ cells and the inhibition of both basal and agonist stimulated cyclic AMP levels in N 866 cells. As forskolin acts as a ligand for the catalytic subunit of the adenylate cyclase enzyme (Gilman, 1984) the stimulation of cyclic AMP production by forskolin is a post-receptor event. If forskolin stimulation of cyclic AMP production is reduced, then this result suggests that this modification of the adenylate cyclase signalling pathway by p21 $^{N\text{-}\underline{ras}}$ is at the enzyme level as p21 $^{N\text{-}\underline{ras}}$ has been shown not to interact with or act as G_i (Refer to figure 6.4). Therefore, it is possible that the expression of p21 $^{N\text{-}\underline{ras}}$ in NIH3T3 cells can modify the interaction between adenylate cyclase and forskolin resulting in inhibition of the forskolin stimulated cyclic AMP levels in these cells. However, the fact that the basal production of cyclic AMP is unchanged while forskolin stimulated cyclic AMP levels are reduced in T15+ cells, although both the basal and forskolin stimulated cyclic AMP levels are reduced in N 866 cells suggests that p21 $^{N\text{-}\underline{ras}}$ expression may have a limited effect on the adenylate cyclase enzyme. p21 $^{N\text{-}\underline{ras}}$ overexpression may result in the modification of adenylate cyclase only to the extent that forskolin stimulated adenylate cyclase activity is inhibited while the basal rate of adenylate cyclase activity is unchanged. Only when there is prolonged and constitutive expression of p21 $^{N\text{-}\underline{ras}}$ does this modification of the adenylate cyclase enzyme affect basal levels of cyclic AMP production. This effect of p21 $^{N\text{-}\underline{ras}}$ expression may be due to secondary effects of p21 $^{N\text{-}\underline{ras}}$ and to the possible co-expression of the c-$\underline{sis}$ proto-oncogene and is probably a cell proliferation related event. There is no plausible mechanism(s) for this effect of p21 $^{N\text{-}\underline{ras}}$ on adenylate cyclase as yet.

Therefore, the results in this chapter have shown that progressive p21 $^{N\text{-}\underline{ras}}$ overexpression can lead to an inhibition of β adrenergic agonist and forskolin stimulation of cyclic AMP production. Furthermore,

constitutive expression of p21 $^{N\text{-}\underline{ras}}$ leads to an inhibition of basal cyclic AMP production and almost complete inhibition of stimulated cyclic AMP production. These effects seem specific to the expression of p21 $^{N\text{-}\underline{ras}}$ and are not a consequence of non-specific effects of dexamethasone on T15 cells. Also, it is probable that the ras oncogene specifically exerts these effects on the cyclic AMP signalling pathway via several mechanisms although some of these effects are possibly mediated through c-sis co-expression. Firstly, it is possible that p21 $^{N\text{-}\underline{ras}}$ overexpression results in a downregulation or a modification of β adrenergic receptors such that there is an inhibition of agonist stimulated cyclic AMP levels. This occurs without a change in the receptor affinity for β adrenergic ligands but may be due to the transformation effects of p21 $^{N\text{-}\underline{ras}}$. Secondly, prolonged overexpression of p21 $^{N\text{-}\underline{ras}}$ in the T15+ cell line and the constitutive overexpression of p21 $^{N\text{-}\underline{ras}}$ in N 866 cells seems to modify the adenylate cyclase enzyme such that forskolin stimulated cyclic AMP levels and basal cyclic AMP levels are reduced. This modification does not appear to involve the stimulatory or inhibitory G proteins for the adenylate cyclase system from our data and the data of other workers.

Downregulation of β adrenergic receptors or the modification of ligand binding to these receptors may be a consequence of receptor phoshorylation. Bouvier et al (1987) have shown that β adrenergic receptors are substrates for both protein kinase C and cyclic AMP dependent protein kinase. Therefore, one of the consequences of p21 $^{N\text{-}\underline{ras}}$ overexpression may be the activation of protein kinase C which results in phosphorylation of the β adrenergic receptors in T15+ and N 866 cells. As the basal levels of cyclic AMP are similar in T15-, T15+/24 hour and T15+ cells, it is unlikely that phosphorylation of β adrenergic receptors occurs as a consequence of cyclic AMP dependent protein kinase A activation upon p21 $^{N\text{-}\underline{ras}}$ overexpression. Therefore, the inhibition of β adrenergic stimulated cyclic AMP levels in T15+/24 hour, T15+ and N 866 cells may be due to the phosphorylation and possible downregulation of β adrenergic receptors as a

result of protein kinase C activation.

The effects of prolonged p21 $^{N\text{-}ras}$ overexpression on forskolin stimulated cyclic AMP levels may be due to either phosphorylation of the adenylate cyclase enzyme itself or the inhibition of forskolin binding to the catalytic subunit of adenylate cyclase. However, Yoshimasa et al (1987) have demonstrated that protein kinase C catalysed phosphorylation of adenylate cyclase results in a potentiation of isoproterenol, GTP and sodium fluoride stimulation of adenylate cyclase purified from frog erythrocytes. Unless different cell or tissue specific forms of adenylate cyclase exist, the phosphorylation of adenylate cyclase in T15+ and N 866 cells is therefore unlikely. Thus, the inhibition of forskolin stimulated cyclic AMP levels in T15+ and N 866 cells may be due to a yet unknown modification of adenylate cyclase resulting in an inhibition of forskolin binding or may be due to a decrease in adenylate cyclase synthesis at the transcriptional level. Although these effects are detected in T15+ and N 866 cells, the inhibition of forskolin stimulated cyclic AMP levels also occurs in sis 771 cells although to a lesser extent. Therefore, in T15+ and N 866 cells, these effects may in part be due to the co-expression of the c-sis oncogene. The implications and consequences of these modifications of the cyclic AMP signalling pathway as a result of p21 $^{N\text{-}ras}$ induced transformation of NIH3T3 cells will be discussed in the following final chapter.

Chapter 7: General Discussion

The results described in this thesis are from experiments to investigate the role of of p21 $^{N\text{-}ras}$ in the proliferation and transformtion of NIH3T3 cells. The results in chapter 3 indicate clearly that p21 $^{N\text{-}ras}$ overexpression in T15 cells is associated with increased cell proliferation and transformation. This effect of p21 ras expression has been shown to be associated with almost all cell types investigated (Barbacid, 1987). Interestingly, this result of p21 ras expression only occurs when there are very high levels of normal, cellular p21 ras expressed. For example, in T15+ cells , the cellular concentration of p21 $^{N\text{-}ras}$ is approximately 50 times that of normal NIH3T3 cells. To achieve similar effects with p21 $^{v\text{-}ras}$ or a mutant cellular ras oncogene, a lower concentration of the gene product is required than the normal proto-oncogene (See McKay et al, 1986 ; Trahey and McCormick, 1987). Also, mutant ras proteins microinjected into fibroblasts cause cellular changes which persist for a much longer length of time than those induced by normal ras proteins (Bar-Sagi and Feramisco, 1986). In addition, mutant Ha-ras when microinjected into quiscent cells causes very rapid effects whereas normal cellular Ha-ras is without effect when injected at the same concentration (Feramisco et al, 1984).

Modulation of p21^{ras} transforming ability by GAP associated GTPase activity.

Cell proliferation and transformation associated with the expression of p21 ras is associated with the guanine nucleotide binding properties of the ras protein, determined by the association of p21 ras with the GAP protein. Mutant ras protein GTPase activities are not regulated by GAP (See Introduction) resulting in a p21 ras molecule which is permanantly in a GTP bound and thus biologically active state.

However, if mutant p21 ras proteins have a reduced ability to bind GTP, there is a loss of transforming ability in most cases (Clanton et al, 1987). Furthermore, the construction of a mutant Ha-ras oncogene with an

asparagine residue substituted for serine at position 17 results in p21 Ha-ras with a preferential affinity for GDP. Expression of this oncogene in NIH3T3 cells results in an inhibition of cell proliferation which can be reversed by the coexpression of an activated ras oncogene (Feig and Cooper, 1988). Therefore, these observations suggest that the biological function of p21 ras relies on the relative binding of GDP/GTP as a consequence of GAP association. Furthermore, the relative amount of GTP or GDP bound will determine the transforming capacity of these ras oncogenes. It appears that normal p21 ras proteins exist in a preferentially GDP bound state whilst transforming mutant ras proteins are in a GTP bound state (Trahey and McCormick, 1987). This this may partly explain why a lower concentration of mutant p21 ras is required for the stimulation of cell proliferation and transformtion than normal p21 ras *i.e.* the relative amount of GDP/GTP bound will be one of the factors that determine the transforming potential of any one p21 ras protein. Therefore, GTP binding can thus be considered to be the 'limiting factor' and may affect the concentration of different ras proteins required for transformtion.

This data strengthens the possibility that p21 ras can act as a G protein in signal transduction pathways which results in cell proliferation and transformtion. The identity(ies) of these pathway(s) is a contentious issue. However, The RAS proteins in yeast have been shown to stimulate adenylate cyclase activity (Broek et al, 1985) and there has been a report that RAS encoded proteins are invoved in glucose-stimulated inositol phospholipid turnover in S. Cerevisiae (Kaibuchi et al, 1986). In yeast therefore, the RAS proteins seem to be involved in the stimulation of 2 major second messenger pathways.

p21 N-ras involvement in the inositol phospholipid signal transduction system.

The results presented in this thesis have suggested that p21 N-ras can act at a post-receptor level to enhance growth factor stimulated inositol

phospholipid turnover. In chapter 3, it was demonstrated that p21$^{N\text{-}ras}$ overexpression resulted in increased bombesin stimulated total inositol phosphate production in T15+ cells. Furthermore, the results in chapter 4 demonstrated that this increase was due to the enhanced production of Ins 1,4,5 P_3 and its dephosphorylated derivatives as a result of phosphoinositide-specific phospholipase C activation (Lloyd et al, submitted for publication). The other second messenger produced as a consequence of phospholipase C activation is DAG, which induces the activation of protein kinase C. This effect is due specifically to the expression of p21 $^{N\text{-}ras}$ and can be correlated with the amount of cellular p21 $^{N\text{-}ras}$ (Wakelam et al, 1987). Although these results clearly demonstrate that transformation of NIH3T3 cells by p21 $^{N\text{-}ras}$ may be due in part to enhancement of agonist stimulated phosphoinositide specific phospholipase C and the generation of Ins 1,4,5 P_3 with an associated increase in intracellular Ca^{2+}. However, the proposed role of p21 $^{N\text{-}ras}$ as a coupling protein between growth factor receptors at the cell surface and phosphoinositide specific phospholipase C must be questioned.

Firstly, although bombesin and GRP stimulated the largest increase in inositol phosphate production in T15+ cells, p21 $^{N\text{-}ras}$ overexpression also resulted in increased bradykinin and calf serum induced inositol phosphate production. It has been demonstrated that Ha- and Ki-ras transformation of NIH3T3 cells results in an increased number of bradykinin receptors (Parries et al, 1987). Also, a recent study of Ha-, Ki- and N-ras transformation of NIH3T3 cells has demonstrated that enhanced bradykinin stimulated inositol phosphate production in these transformed cells is associated with an increased number of bradykinin receptors (Downward et al, 1988). It is possible that an increase in the number of bradykinin receptors also occurs in T15+ cells but this hypotheses has yet to be tested. If the amplification of bradykinin and calf serum stimulated inositol phosphate production in T15+ cells is not due to an increase in receptor number, then the role of p21 $^{N\text{-}ras}$ as a specific coupling protein between the bombesin receptor and the phosphoinositide specific phospholipase C remains in doubt.

Secondly, in chapter 3, it was clearly shown that detectable bombesin stimulated inositol phosphate production occured at 6 hours after dexamethasone treatment. In addition, the time course of dexamethasone induced enhanced bombesin stimulated inositol phosphate production was closely correlated to the time course of dexamethasone induced p21 N-ras expression quantitated by immunoprecipitation of p21 N-ras with an anti-ras antibody (Marshall, C. unpublished observation). Although the immunoprecipitation of p21 N-ras was performed on whole cell lysates from T15+ cells, the p21 N-ras protein detected was the acylated form. It has been shown that p21 ras needs to be inserted into the membrane to have any biological function. However, McKay et al (1986) have shown that it takes twenty hours after dexamethasone treatment of T15- cells for p21 N-ras to be inserted into the membrane. If this figure is accurate, then the increase in bombesin stimulated inositol phosphate production seen after 6 hours of dexamethasone treatment cannot be due to the action of membrane bound p21 N-ras . This suggests firstly, that there is an obvious discrepancy between the two sets of results described and secondly, that p21 N-ras may exert these effects on the phosphoinositide signalling pathway from the cytoplasm. However, Lacal et al (1987a) microinjected non-acylated p21 ras protein into Xenopus oocytes, resulting in a very rapid increase in DAG levels and in the generation of inositol phosphates. This suggests that either p21 ras can be acylated and inserted into the membrane very rapidly, or can cause these effects from within the cytoplasm. If the latter were the case, then this is inconsistent with the hypotheses that p21 ras can function as a conventional G protein in signal transduction.

The involvement of other oncogenes in stimulated inositol phosphate turnover cannot be ignored. Lacal et al (1987b) have shown that v-sis transformed NIH3T3 cells exhibit elevated basal levels of inositol phosphates as has been demonstrated in pLHT-511 cells which overexpress p21 N-ras (Wakelam et al, 1987; Hancock et al, 1988). Owen and Owstroski (1987)

have shown that dexanethasone induction of Ha-ras transfected into NIH3T3 cells also induces c-sis expression. This implies that coexpression of other oncogenes with the ras proto-oncogene is a possibility in our cell system and may be modulating the bombesin response. However, overnight treatment of T15+ cells with suramin does not affect the bombesin stimulated inositol phosphate response (Wakelam, M.J.O., unpublished observation). Therefore, this suggests that PDGF-like molecules may not have a role in bombesin stimulated inositol phosphate production in the T15 cell line.

Smith et al (1986) have demonstrated that growth factor-like oncogenes require c-ras expression in NIH3T3 cells whereas cytoplasmic oncogenes do not, in order to transform cells. This suggests that there is a role for p21 ras in growth factor stimulated cell proliferation and transformation in rodent cells but having discussed the results above, this role may not be as direct as previously thought. It is probable that the ras oncogene plays a crucial role in mediating cell proliferation and transformation in conjunction with other coexpressed oncogenes and that this effect of ras oncogene expression is an early and necessary step in this process.

The role of the ras oncogene family in the amplification of the phosphoinositide signalling pathway has been investigated by other workers. Fleischman et al (1986) have shown that transformation of NIH3T3 and NRK cells with N-, Ha- and Ki-ras genes results in elevated basal levels of inositol phosphates and DAG compared to non-transformed cells. Hancock et al (1988) have demonstrated that mutant Ha- and N-ras transformed NIH3T3 and COS-1 cells exhibit an enhanced basal rate of inositol phospholipid turnover. By site directed mutagenesis of these ras genes, these workers have shown that there is a correlation between increased basal rates of inositol phospholipid turnover and transforming ability of the ras genes. Also, it has been shown that microinjection of p21 Ha-ras into Xenopus oocytes results in a stimulation of inositol phosphates and DAG production (Lacal et al, 1987a). Chiarugi et al (1986) demonstrated that transfection of EJ/T24/ Ha-ras oncogene into Balb c/3T3 cells caused an amplification of muscarinic

stimulation of phosphoinositide hydrolysis and an increase in intracellular Ca^{2+} levels in NIH3T3 cells. Kamata and Kung (1988) have shown that normal Ha-ras transformation of NRK cells causes an increase in both GTPγS and PDGF stimulated inositol trisphosphate production in membranes prepared from these cells as compared to normal NRK cell membranes. However, this effect of p21 ras expression may be confined to certain fibroblast cell lines and Xenopus oocytes as other workers have presented evidence contrary to the reports described above.

Seuwen et al (1988a) have demonstrated that mutant Ha- and Ki-ras transformed CCL39 cells do not exhibit increased phospholipase C activity but rather, have reduced levels of stimulated inositol phosphates compared to non-transformed cells. However, this desensitisation is apparently at the receptor level and was also observed in polyoma virus transformed CCL39 cells *i.e.* these effects were not specific to the ras oncogenes. Rat-1 fibroblasts transformed with the Ha- and Ki-ras oncogenes exhibited a decrease in PDGF stimulated inositol phosphate production compared to non-transformed cells with no effect on receptor number (Parries et al, 1987). Benjamin et al (1988) have also demonstrated that EJ-ras oncogene transformed NIH3T3 cells have decreased PDGF stimulated Ins 1,4,5 P_3 and Ca^{2+} levels compared to control cells. Finally, Alonso et al (1988) have shown that Ha- and Ki- ras transformtion of rat-1 and NIH3T3 cells results in uncoupling of receptor-mediated growth factor stimulation of phosphoinositide specific phospholipase C activity. These workers suggest that these alterations in cellular signalling are not confined to ras transformed cells but occur in src, met, trk, mos and raf transformed cells. However, the use of lithium chloride to measure the inositol phosphate content in these transformed cells remained unclear. Also, comparisons between different inositol phosphate levels in the different cell lines were based on inositol monophosphate only and may not be an accurate reflection of inositol phosphate levels in these cell lines.

Inhibition of PGF2α stimulated inositol phosphate production has

been observed in ras transformed cells when compared to control NIH3T3 cells. This may be due to receptor phosphorylation as a consequence of p21 ras expression in these cell types (Black, F.and Wakelam, M.J.O., submitted for publication). The inhibition of PDGF stimulated inositol phosphate production described above could also be a consequence of PDGF receptor phosphorylation in ras transformed cells as a consequence of DAG production and subsequent protein kinase C activity. However, it has been demonstrated that PDGF binding with subsequent stimulation of the PDGF receptor tyrosine kinase and stimulated phosphatidylinositol hydrolysis are not sufficient for mitogenesis to occur (Escobedo and Williams, 1988). Benjamin et al (1988) have shown that ras transformed cells which have decreased PDGF stimulated inositol phosphate levels still respond mitogenically to PDGF. This could imply that the stimulation of inositol phosphate production is an initial signal in the mitogenic cascade and it is possible that this response is downregulated in rapidly proliferating cells once this cascade is activated. This may explain why the inhibition of PDGF stimulated inositol phosphate production occurs in the constitutive ras transformed cell types cited in the referances above.

There is a growing body of evidence that ras transformation results in elevated basal and/or stimulated levels of DAG which would suggest that ras transformation results in the activation of protein kinase C. Hagag et al (1987) have demonstrated a rise in intracellular pH when p21 ras is microinjected into NIH3T3 cells. This is due to the activation of the Na^+/H^+ antiporter by protein kinase C and is an indication that p21 ras expression results in increased protein kinase C activity. Furthermore, Ha-ras transformed NIH3T3 cells have been shown to exhibit elevated levels of DAG but this occurs without a corresponding increase in inositol phosphates (Lacal et al, 1987b) . This suggests that other phospholipids are hydrolysed to generate diacylglycerol. It is possible that this process takes place in ras transformed cells *i.e.*the initial production of inositol phosphates in response to growth factors is downregulated and the breakdown of another

phospholipid is activated. This would result in persistent high levels of DAG with associated increased protein kinase C activity which precedes the onset of cell proliferation.

Therefore, the effects of the ras oncogene on the inositol phospholipid signal transduction system vary depending on the cell type and the particular ras (proto)-oncogene employed. It has been demonstrated, for example, that different clones of NIH3T3 cells respond differently to the same growth factors (Black, F. and Wakelam, M.J.O., manuscript in preparation) and this could be a reflection of the relative 'age' of the particular clone used or the relative spontaneous transformed state of the cells. Furthermore, murine fibroblasts spontaneously transform in culture at a high frequency. This spontaneous transformation is often associated with amplification of cellular genes. For example, the met oncogene is amplified 4-8 fold and overexpressed 20 fold in spontaneously transformed NIH3T3 cells (Cooper et al, 1986). Therefore, this is a possible occurance in and an additional difference between apparently similar cell lines utilised in studies to determine the effects of p21 ras transformation on cellular signalling.

The varying effects of the ras oncogene on signal transduction in different cell types may be due to the different ras proteins which may couple to different growth factor receptors, different species of G_p and of phospholipase C activated by p21 ras and the fact that there are cellular sources of DAG other than inositol phospholipid breakdown. Differing species of G_p and/or phospholipase C in different cell types is suggested by the different pertussis toxin sensitivities of the growth factor stimulated inositol phosphate response. The data presented in this thesis clearly shows that pertussis toxin had no effect on inositol phosphate production in T15 cells. Similarly, in GH3 cells, pancreatic acinar cells, liver cells and adipocytes, the inositol phosphate response is not pertussis toxin sensitive. However, in other cell types, for example CCL39 fibroblasts, mast cells and neutrophils, stimulated inositol phosphate production is inhibited by pertussi toxin (Cockcroft, 1987). If pertussis toxin sensitivity is used as a criterion

for G_p activity, then there must be at least two different G_p species and possibly two separate phospholipase Cs in different cell lines which have distinct pertussis toxin sensitivities. The use of pertussis toxin in experiments to determine p21 ras function will only determine if p21 ras can act as or interact with, G_i, to mediate its effects. The data in this thesis suggests that p21 N-ras cannot act as G_i but the possibility remains that p21 N-ras can interact with or act as G_p in NIH3T3 fibroblasts. In addition, bombesin has been shown to couple to a pertussis toxin insensitive G protein in four different cell types including Swiss 3T3 fibroblasts and GH_4C_1 pituitary cells (Fischer and Schonbrun, 1988).

Therefore, the role of p21 N-ras in amplified mitogen stimulated inositol phosphate production in T15+ cells is at present not clearly defined. It is certain that p21 N-ras acts at a post-receptor level and that the modulation of this second messenger pathway in this cell line does not involve G_i. Also, it has been demonstrated that dexamethasone induction of p21 N-ras expression in T15 cells does not result in changes in the levels of G_i, G_o or G_s (Milligan et al, manuscript in preparation). This indicates that bombesin stimulated inositol phosphate production is not mediated through these G proteins in this cell line.It has been demonstrated that a non-hydrolysable analogue of GTP, GTPγS, can stimulate inositol phosphate production in a broken T15+ cell preparation. The bombesin stimulated inositol phosphate production in this cell-free system was partly abolished with an anti-ras antibody (Wakelam, unpublished observation). However, these experimental results are difficult to reproduce and therefore remain inconclusive. Alternatively, experiments using a temperature sensitive mutant of p21 N-ras transfected into NIH3T3 cells could be used to provide more conclusive data to investigate if p21 N-ras does act as a G protein in phosphoinositide signal transduction. Such an experimental system would reduce the possibility of the involvement of co-expressed oncogenes or oncogenes expressed as a consequence of p21 N-ras expression in signal transduction pathways as de novo synthesis of p21 ras does not occur (Durkin, J.P., personal communication). Finally, a

conclusive experimental approach would be the reconstitution of the bombesin receptor, phosphoinositide specific phospholipase C, Ptd Ins 4,5 P_2 and p21 N-ras in an artificial membrane preparation . The question would be : will bombesin stimulated inositol 1,4,5 trisphosphate production occur in this reconstituted cell system in the presence of membrane bound p21 N-ras ?

The effects of p21 N-ras overexpression on the cyclic AMP signal transduction pathway.

In addition to its effects on the inositol phospholipid signalling pathway, p21 N-ras overexpression in T15 cells results in an associated decrease in cyclic AMP levels. As discussed in chapter 6, these results are consistent with the data of other workers using different transfected ras oncogenes to investigate the effects of ras transformtion on adenylate cyclase activity and cyclic AMP levels in the cell. However, there are a few exceptions to the general 'rule' that p21 ras expression results in a decrease in intracellular cyclic AMP levels. Franks et al (1987) have demonstrated that a temperature sensitive oncogenic viral Ki-ras in NRK cells causes an increase in cyclic AMP levels in response to cholera toxin treatment. The authors have suggested that this effect is due to the inactivation of the G_i component of the adenylate cyclase system, possibly by the action of stimulated protein kinase C on G_i. Also, it has been demonstrated that v-Ha-ras and v-Ki-ras infected rat thyroid epithelial cells exhibit increased adenylate cyclase activity and an associated increase in intracellular cyclic AMP content (Spina et al, 1987). It was suggested that the activity of the adenylate cyclase enzyme or G_s itself could be modified by the presence of p21 ras in the membrane.

It is possible that increased protein kinase C activity as a consequence of p21 ras expression results in an increase in adenylate cyclase activity. It has been demonstrated that the α subunit of certain G proteins can be phosphorylated by protein kinase C, for example, transducin (Zick et al,1986) and G_i (Katada et al, 1985). Also, the phosphorylation of adenylate cyclase by protein kinase C results in an increase in agonist stimulated activity

(Yoshimasa et al, 1987). However, this does not explain why there is an increase in cyclic AMP levels in some cell lines that express viral Ha- and Ki-ras but a decrease in other cell types studied transfected with different ras oncogenes (See Discussion chapter 6). These differences could be a result of cell type variation.

It is possible, however, that in all these cell lines transfected with ras oncogenes, protein kinase C phosphorylation of in vivo substrates as a consequence of p21 ras expression affects intracellular cyclic AMP levels. It has been shown that protein kinase C can phosphorylate β adrenergic receptors in vitro (Sibley et al, 1987). Such a modification of β adrenergic receptors by protein kinase C in T15+ cells may result in an inhibition of ligand binding and therefore of reduced stimulated cyclic AMP levels. Alternatively, protein kinase C catalysed phosphorylation may lead to an increase in receptor internalisation and thus a down-regulation of cell surface receptor number. Thus, the effect of p21 $^{N\text{-}ras}$ overexpression on the adenylate cyclase system may be via the activation of protein kinase C by DAG and is therefore an indirect effect of ras expression and is a transformation-associated phenomenon .

Production of cyclic AMP in ras transformed cells may be further downregulated by modification of either the amount of adenylate cyclase synthesised in the cells, or of the catalytic subunit of adenylate cyclase by unknown mechanisms. This may explain the inhibition of forskolin stimulated cyclic AMP production in T15+ and N 866 cells (see chapter 6). However, this effect may be mediated by the sis-oncogene as a similar observation was made in sis-771 cells. It should be noted that c-sis expression may lead to an increase in protein kinase C activity as PDGF has been shown to stimulate the activity of this kinase in some cell types (Berridge, 1987a). The data suggests therefore, that the c-sis oncogene may play a cooperative role in the process of p21 $^{N\text{-}ras}$ transformation resulting in an inhibition of cyclic AMP production via several mechanisms.

What are the main effects of decreased intracellular levels of cyclic AMP upon ras induced transformation? Smets and Van Rooy (1987) have shown that high intracellular cyclic AMP levels are associated with cells in the G_0-G_1 transition but inhibit cell progression through the G_1-G_S phase. Therefore, this suggests that proliferating cells do not require high cyclic AMP levels and that perhaps these increased levels of cyclic AMP will inhibit cell growth. Indeed, Katsaros et al (1987) have demonstrated that site-selective cyclic AMP analogues which bind to type 1 or type 2 cyclic AMP dependent protein kinases inhibit the proliferation of human cancer cell lines, including a ras expressing cell line. Inhibition of cell growth was associated with a change in cell morphology, a decrease in p21 ras levels and an increase in the type 2 cyclic AMP dependent protein kinase. Furthermore, Tagliaferri et al (1988) have shown that growth inhibition and a reversal of transformation of v-Ha-ras transformed NIH3T3 cells occured as a consequence of the addition of cyclic AMP analogues to the growth medium of these cells. Results from this laboratory have shown that the addition of 1mM dibutyryl cyclic AMP to the growth medium of p21 N-ras transformed T15 cells inhibits the growth of these cells (Davies et al, in preparation).This suggests that the concentration of cyclic AMP in the cell is an important factor in the process of cell proliferation and transformation. It is probable that one effect of p21 ras expression is to decrease adenylate cyclase activity by reducing growth factor stimulation of this signalling pathway, via protein kinase C phosphorylation of the receptor and also by a further modification of the adenylate cyclase system at a post-recptor level.

Conclusions and future prospects.

It appears therefore, that the modulation of two major signal transduction systems is necessary for cell proliferation to occur as a consequence of p21 ras expression. As protein kinase C is dependent upon DAG and Ca^{2+} for its activation, it is possible that an early effect of p21 ras

transformation is to increase levels of DAG. Protein kinase C mediated phosphorylation of the Na^+/H^+ antiporter and of various components of the adenylate cyclase system results in the entry of cells into the S phase and cell proliferation occurs. p21 ras could initially cause the amplified breakdown of PtdIns 4,5 P_2 to release DAG and Ins 1,4,5 P3 thus raising the intracellular concentration of Ca^{2+}. However, since protein kinase C desensitisation of PtdIns 4,5 P_2 breakdown has been observed in many cell types (Berridge, 1987b), this initial event may be downregulated. DAG production could be maintained at levels sufficient for cell proliferation to occur by hydrolysis of other phospholipids, for example, from phosphatidylcholine, the increased turnover of which has been observed in some ras transformed cells (Lacal et al, 1987b). This may explain why in some constitutive ras expressing cell types, there is no detectable increase in Ins 1,4,5 P_3 as a consequence of growth factor stimulation. For example, the N 866 cell line exhibits very low levels of cyclic AMP compared to T15+ cells (Chapter 6) but there is no detectable bombesin stimulated inositol phosphate production (Lloyd et al, submitted for publication). It may be that for yet unknown reasons, the downregulation of inositol phosphate production that occurs in other ras transformed cell lines does not occur in the T15 cell line.

The stimulation of DAG production and the inhibition of cyclic AMP production seems to be an important permissive event for cell growth and subsequent cell transformtion. Seuwen et al (1988b) have shown that serotonin stimulates DNA synthesis in CCL39 fibroblasts by activating phospholipase C and inhibiting adenylate cyclase. These events are pertussis toxin sensitive and the authors have suggested that G_i is involved in this process. Furthermore, Chiarugi et al (1985) have shown that Balbc/3T3 fibroblasts transformed with transfected EJ/T24 ras oncogene have lower stimulated cyclic AMP levels compared to control cells but exhibit increased muscarinic induced inositol phosphate production. Similarly, data presented in this thesis also show that p21 ras transformation of T15 cells resulted in increased growth factor stimulated inositol phosphate production and an

associated decrease in cyclic AMP levels. This dual effect of p21 ras on both the major signal transduction pathways suggests that p21 ras does act to modulate second messenger production in its capacity as a membrane bound oncogene, but the precise mechanisms are still undefined.

In any type of experiment that utilises a cell line which expresses a particular oncogene in an inducible or constitutive manner, the expression of other, possibly cooperative, oncogenes cannot be ruled out. As cancer development does occur as a muti-stage process, it is probable that the expression of a particular oncogene will have a 'knock-on' or relay effect on other oncogenes in different cellular locations. Whilst the process of transformation can be ascribed to the expression of a particular oncogene, the biochemical events invoved are a combination of different second messenger pathways and of different (proto)-oncogene actions. It is possible to inhibit cancer cell growth by the administration of antagonists to various growth factor receptors (Heldin et al, 1987). However, it is clear that this approach is not always successful. It may be that once an initial growth signal is transmitted from the cell surface, the presence of activated (proto)-oncogenes is sufficient to enable the cell to proliferate and become transformed under appropriate conditions. Thus, the inhibition of growth factor binding may not always be an effective anti-proliferative factor. Therefore, until there are experimental systems to assess the role of a particular oncogene on various biochemical pathways without the possible 'interference' of other (proto)-oncogenes, investigations into the bichemical role of (proto)-oncogenes in normal cell growth and in transformed cells will be inconclusive. This especially applies to investigations into ras (proto)-oncogene function as this gene product plays an important role in maintaining cell growth under normal conditions and, under different circumstances, can cause malignant tumour growth. A diagrammatic summary of possible events associated with p21 ^{N}-ras overexpression in T15+ cells is shown overleaf .

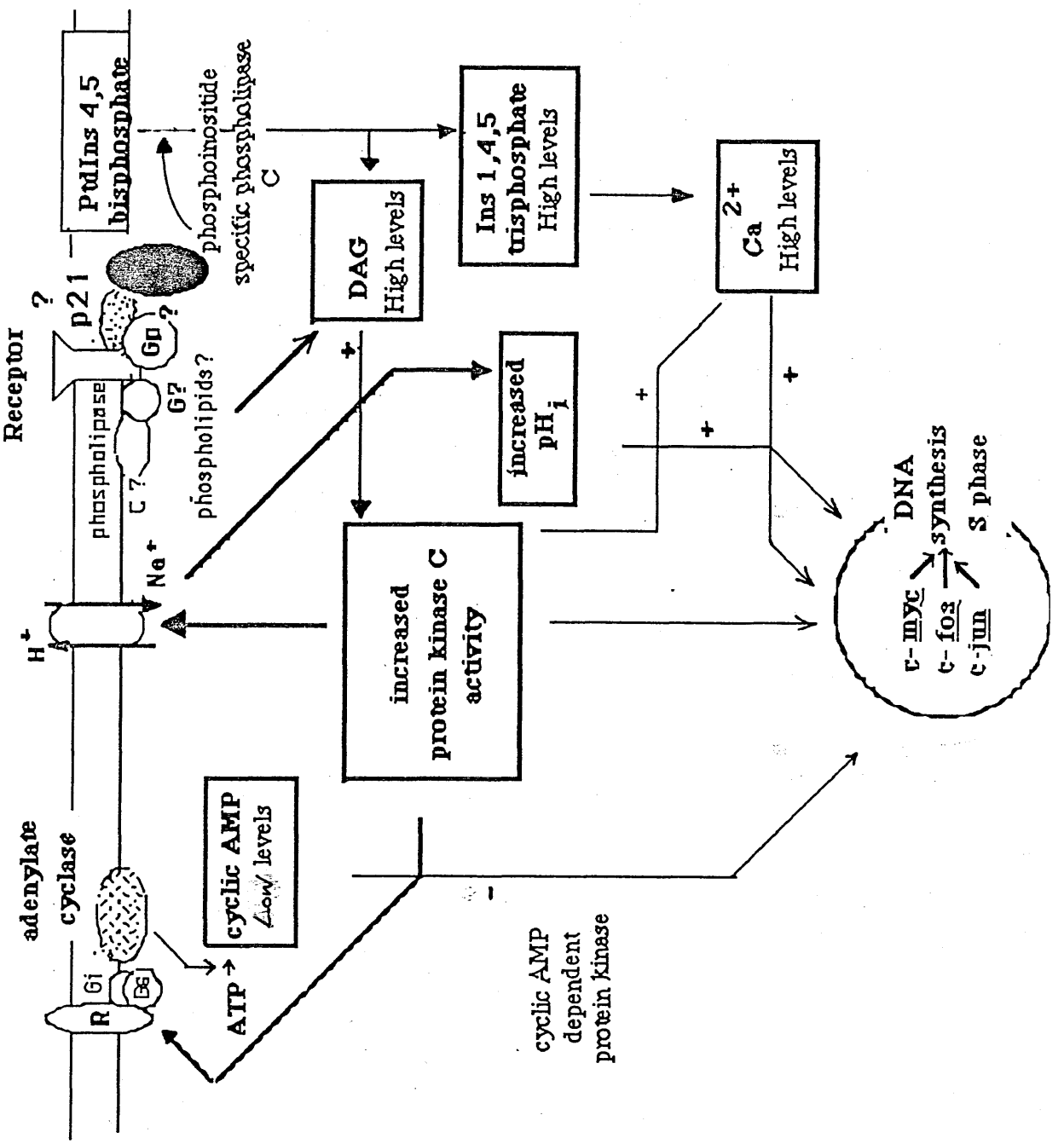
In conclusion, the role of the ras oncogene in cell proliferation and transformation is still undefined although there is growing evidence to suggest

Diagrammatic summary of possible intracellular events on p21 N-ras overexpression.

The diagram on page 211 illustrates the intracellular events that may occur on p21 N-ras overexpression in T15 cells.It has been shown that p21 N-ras overexpression in the T15 cell line directly results in an amplification of receptor mediated Ins 1,4,5 P_3 production which then acts as a second messenger to release intracellular stores of Ca^{2+}. It remains a possibility that p21 N-ras may interact with or act as Gp this cell line (See Discussion). One consequence of increased intracellular Ca^{2+} as a result of receptor mediated Ins 1,4,5 P3 production will be increased nuclear oncogene expression, for example, c-myc and c-fos, which are involved in the initiation of S phase.

Another consequence of increased receptor mediated PtdIns 4,5 P_2 hydrolysis in T15+ cells is the increased production of DAG. Increased levels of DAG in the cell will result in activation of PK C. The activation of PK C can result in increased nuclear oncogene expression resulting in the onset of DNA synthesis. Also, PK C induced phosphorylation of intracellular substrates include the phosphorylation of the Na+/H+ antiporter resulting in intracellular alkalinisation (See Introduction). An increase in intracellular pH as a result of mitogenic stimulation of T15+ cells will result in the initiation of DNA synthesis, which can only occur at this permissive intracellular pH. The production of DAG in mitogen stimulated T15+ cells may also be a consequence of phospholipase C or phospholipase D catalysed hydrolysis of other phospholipids distinct from PtdIns 4,5 P_2. Phosphatidylcholine or phosphatidylethanolamine may be the substrates for phospholipase C or D action in T15+ cells as a result of mitogen stimulation.

Finally, it has been demonstratedthat p21 N-ras overexpression in the T15 cell line results in a decrease in intracellular cyclic AMP levels as a consequence of β adrenergic receptor downregulation and a possible direct inhibition of adenylate cyclase enzyme activity. β adrenergic receptor downregulation may occur as a result of PK C phosphorylation, thus reducing intracellular cyclic AMP levels. It has been shown in ras transformed cells that reduced intracellular cyclic AMP levels are associated with the entry of cells into S phase. Also, decreased intracellular cyclic AMP levels result in the inhibition of cyclic AMP dependent protein kinase which, at high levels of enzyme activity, can inhibit cell proliferation. Therefore, the overexpression of p21 N-ras in NIH3T3 cells can result in increased cellular proliferation due to the activation and inhibition of at least two second messenger systems.


Receptor
?
p21
?
Gp
?
G?
C ?
phospholipase
phospholipids?
phosphoinositide
specific phosphoiipase
C
PdIns 4,5
bisphosphate
DAG
High levels
Ins 1,4,5
trisphosphate
High levels
Ca 2+
High levels
increased
pH i
increased
protein kinase C
activity
H+
Na+
adenylate
cyclase
R
Gi
Bg
ATP →
cyclic AMP
Low levels
cyclic AMP
dependent
protein kinase
DNA
synthesis
S phase
c- myc
c- fos
c- jun
+
+
+
+
-

that p21 ras may interact with major signal transduction pathways in a still controversial manner. It appears that the ras oncogene, like many other oncogenes, causes tumour cell growth by utilising signalling pathways already active in a normally proliferating cell. 'Subversion' of these pathways, by mechanisms that may include the constitutive activation of particular components of these signal transduction cascades, can result in uncontrolled proliferation followed by cell transformation . Furthermore, it is probable that ras oncogene expression causes the expression of other cooperative oncogenes. The 'cure' for cancer, then, cannot be a single drug aimed at a single cellular target as from clinical, molecular biological and biochemical data, the process of cell proliferation and transformation is a multistep, cooperative process which still largely eludes us.

References

Alexander, R.W., Upp, J.R., Poston, G.J., Gupta, V., Townsend, C.M. Jr.. and Thompson, J.C. (1988) Canc. Res. 48, 1439-1441

Alitalo, K. (1985) Trends Biochem. Sci. 10, 194-197

Alitalo, K., Koskinen, P., Makela, T.P., Saksela, K., Sistonea, L.and Wingvist, R. (1987) Biochim. Biophys. Acta 907, 1-32

Alonso, T., Morgan, R.O., Marvizon, J.C., Zargl, H. and Santos, E. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 4271-4275

Anzano, M.A, Roberts, A.B., De Larco, J.E., Wakefield, L.M., Assoian, R.K., Roche, N.S.,Smith, J.M., Lazarus, J.E. and Sporn, M.B. (1985) Mol. Cell Biol. 5, 242-247

Backer, J.M. and Weinstein, I.B (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6357-6361

Barbacid, M. (1987) Ann. Rev. Biochem. 56, 779-827

Bar-Sagi, D. and Feramisco, J. (1985) Cell 42, 841-848

Bar-Sagi, D. and Feramisco, J.R. (1986) Science 233, 1061-1068

Bar-Sagi, D., Fernandez, A. and Feramisco, J. (1987) Biosci. Rep. 7, 427-434

Baudry, M., Evans, J and Lynch, G. (1986) Nature 319, 329-331

Beckner, S.K., Hattori, S. and Shih, T.Y. (1985) Nature 317, 71-72

Benjamin, C.W., Connor, J.A., Tarpley, W.G. and Gorman, R.R. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 4345-4349

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

Berridge, M.J., Heslop, J.P., Irvine, R.F. and Brown, K.D. (1984) Biochem. J. 222, 195-201

Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315-321

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

Berridge, M.J. (1987b) Biochim. Biophys. Acta 907, 33-45

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

Betsholtz, C., Johnsson, A., Heldin, C.-H. and Westermark, B. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6440-6444

Bishop, J.M. (1985) Cell 48, 23-28

Black, F. and Wakelam, M.J.O. (manuscript sumitted for publication)

Bokoch, G.M. and Parkos, C.A. (1988) FEBS. Lett. 227, 66-70

Bourne, H. and Sullivan, K.A. (1986) Canc. Sur. 5, 257-274

Bouvier, M., Fredrik Leeb-Lundberg, L.M., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. (1987) J. Biol. Chem. 262, 3106-3113

Bowen-Pope, D.F., Vogel, A. and Ross, R. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 2396-2400

Bravo, R., Macdonald-Bravo, H., Muller, R., Hubsch, D and Almendral, J.M. (1987) Exp. Cell Res. 170, 103-115

Broek, D., Samiy, N., Fasano, O., Fujiyama, A., Tamanoi, F., Northup, J. and Wigler, M. (1985) Cell 41, 763-769

Busa, W.B., Ferguson, J.E., Joseph, S.K., Williamson, J.R. and Nuccitelli, R. (1985) J. Cell Biol. 101, 677-782

Cales, C., Hancock, J.F., Marshall, C.J. and Hall, A. (1988) Nature 332, 548-541

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

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

Chardin, P. and Tavitian, A. (1986) EMBO J. 5, 2203-2208

Chiarugi, V., Porciatti, F., Pasquali, F., and Brunni, P. (1985) Biochem. Biophys. Res. Commun. 132, 900-907

Chiarugi, V.P., Pasquali, F., Vannucchi, S. and Ruggiero, M. (1986) Biochem. Biophys. Res. Commun. 141, 591-599

Clanton, D.J., Lu, Y., Blair, D.G. and Shih, T.Y. (1987) Mol. Cell Biol. 7, 3092-3097

Cockcroft, S. (1987) Trends Biochem. Sci. 12, 75-78

Connolly, T.M., Laving, W.J. Jr. and Majerus, P.W. (1986) Cell 46, 951-958

Cooper, C.S., Tempest, P.R., Beckman, M.P., Heldin, C.-H. and Brookes, P. (1986) EMBO J. 5, 2623-2628

Coughlin, S.R., Lee, W.M.F., Williams, P.W., Giels, G.M. and Williams, L.T. (1985) Cell 43, 243-251

Cuttita, F., Carney, D.N., Mulshine, J., Moody, T.W., Fedorko, J., Fischler, A. and Minna, J.D. (1985) Nature 316, 823-826

Davies, S.A., Wakelam, M.J.O. and Houslay, M.D. (Oncogene, submitted for publication)

Dawson, A.P., Comerford, J.G. and Fulton, D.V. (1986) Biochem. J. 234, 311-315

Diringer, H. and Friis, R.R. (1977) Canc. Res. 37, 2979-2984

Doppler, W., Jaggi, R. and Groner, B. (1987) Gene 54, 147-153

Downes, C.P., Mussat, M.C. and Michell, R.H. (1982) Biochem. J. 203, 169-177

Downward, J., De Guzenburg, J., Riehl, R. and Weinberg, R.A. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 5774-5778

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

Feig, L.A. and Cooper, G.M. (1988) Mol. Cell Biol. 8, 3235-3243

Feinberg, A.P. and Vogelstein, B. (1983) Biochem. Biophys. Res. Commun. 111, 47-54

Feramisco, J.R., Gross, M., Kamata, T., Rosengurg, M. and Sweet, R.W. (1984) Cell 38, 109-117

Fischer, J.B. and Schonbrun, A. (1988) J. Biol. Chem. 263, 2808-2816

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

Franks, D.J., Whitfield, J.F. and Durkin, J.P (1987) Biochem. Biophys. Res. Commun. 147, 596-601

Galtwitz, D., Donath, C., Sander, C. (1983) Nature 306, 704-707

Gardner, S., Milligan, G., Rice, J.E. and Wakelam, M.J.O. (Biochem. J., in press)

Gibbs, J.B., Sigal, I.S. and Scolnick, E.M. (1985) Trends. Biochem. Sci. 10, 350-353

Gill, D.L., Ueda, T., Chueh, S.H. and Noel, M.W. (1986) Nature 320, 461-464

Gilman, A. (1984) Cell 36, 577-579

Gomperts, B. (1983) Nature 306, 64-66

Grinstein, S., Smith, J.D., Onizuka, R., Cheung, R.K., Gelfand, E.W. and Benedict, S. (1988) J. BIol. Chem. 263, 8658-8665

Guillon G., Gallo-Payet, N., Balestre, M-N. and Lombard, C. (1988) Biochem. J. 253, 765-775

Hagag, N., Halegoua, S. and Viola, M. (1986) Nature 319, 680-682

Hagag, N., Lacal, J.C., Graber, M., Aaronson, S. and Viola, M.V. (1987) Molec. Cell Biol. 7, 1984-1988

Hancock, J.F., Marshall, C.J., McKay, I.A., Gardner, S., Houslay, M.D., Hall, A. and Wakelam, M.J.O. (1988) Oncogene 3, 187-193

Harden, T.K., Stephens, L., Hawkins, P.T. and Downes, C.P. (1987) J. Biol. Chem. 262, 9057-9061

Haslam, R. and Davidson, M (1984) J. Rec. Res. 4, 605-629

Heldin, C.H., Betsholtz, C., Welsh-Claesson, L and Westermark, B. (1987) Biochim. Biophys. Acta 907, 219-244

Heslop, J.P., Blakeley, D.M., Brown, K.D., Irvine, R.F. and Berridge, M.J. (1986) Cell 47, 703-709

Hokin , M.R. and Hokin, L.E. (1953) J. Biol. Chem. 203, 967-977

Hoshijima, M., Ueda, T., Hamamori, Y., Ohmori, T. and Takai, Y. (1988) Biochem. Biophys. Res. Commun. 152, 285-293

Housey, G.M., Johnson, M.D., Hsia, W.L.W., O' Brian C.A., Murphy J.P., Kirschmeier, P. and Weinstein, I.B. (1988) Cell 52, 343-354

Hunter, T., Ling, N. and Cooper, J.A. (1984) Nature 311, 480-483

Irvine, R.F., Anggard, E.E., Letcher, A.J. and Downes, C.P. (1985) Biochem. J. 229, 505-511

Irvine, R.F., Letcher, A.J., Heslop, J.P. and Berridge, M.J. (1986) Nature 320 631-634

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

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

Johnsson, A., Betsholtz, C., Heldin, C.-H. and Westermark, B. (1985) Nature 317, 438-440

Journot, L., Homburegr, V., Pantaloni, C., Priam, M., Bockaert, J. and Enjalbert, A. (1987) J. Biol. Chem. 262, 15106-15110

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

Kaibuchi, K., Miyajima, A., Arai, K.-I. and Matsumoto, K. (1986b) Proc. Natl. Acad. Sci. U.S.A. 83, 8172-8176

Kamata, T. and Feramisco, J.R. (1984) Nature 310, 147-150

Kamata, T. and Kung, H.-F. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 5799-5803

Katada, T., Gilman, A.G., Watanabe, Y., Bauer, S. and Jakobs, K.H. (1985) Eur. J. Biochem. 151, 431-437

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

Lacal, J.C., De La Pena, P., Moscat, J., Garcia-Barreno, P., Anderson, P.S. and Aaronson, S. (1987a) Science 238, 533-536

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

L'Allemain, G., Paris, S. and Pouyssegur, J. (1984) J. Biol. Chem. 259, 5809-5815

Land, H., Parada, L.F. and Weinberg, R.A. (1983) Nature 304, 596-602

Levitzki, A., Rudick, J., Pastan, I., Vass, W.C. and Lowy, D.R. (1986) FEBS. Lett. 197, 134-138

Levitzki, A. (1988) Trends Biochem. Sci. 13, 298-301

Liboi, E., Di Francesco, P., Gallinari, P., Testa, U., Rossi, G.B. and Peschle, C. (1988) Biochem. Biophys. Res. Commun. 151, 298-305

Livneh, E., Prywes, R., Kashles, O., Reiss, N., Sasson, I., Mory, Y., Ullrich, A. and Schlessinger, J. (1986) J. Biol. Chem. 261, 12490-12497

Lloyd, A.C., Davies, S.A., Crossley, I., Whittaker, M.J., Houslay, M.D., Hall, A., Marshall, C.J. and Wakelam, M.J.O. (Biochem J., in press)

Low, W.W.Y. and Hughes, J. (1987) FEBS. Lett. 220, 327-331

Lacal, J.C., Anderson, P.S. and Aaronson, S.A. (1986) EMBO J. 5, 679-687

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

Macphee, C.H., Drummond, A.H., Otto, A.M. and Jiminez De Asua, L. (1984) J. Cell Physiol. 119, 35-40

Magee, A.I., Gutierrez, L., McKay, I.A., Marshall, C.J. and Hall, A. (1987) EMBO J. 6, 3353-3357

Majerus, P.W., Connolly, T.M., Bansal, V.S., Inhorn, R.C., Ross, T.S. and Lips, D.L. (1988) J. Biol. Chem. 263, 3051-3054

Marshall, C.J. (1985) ' Human Oncogenes' in RNA Tumour Viruses; Molecular Biology of Tumour Viruses, 2nd edition. Eds: Weiss, R., Teich, N., Varmus, H. and Coffin, J. pp 487-558

Martin, T.F.J., Lucas, D.O., Bajjalieh, S.M. and Kowalchyk, J.A. (1986) J. Biol. Chem. 261, 2918-2927

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

McKay, I. A., Marshall, C.J., Cales, C. and Hall, A. (1986) EMBO J. 5, 2617-2621

Milligan, G., Davies, S.A., Houslay, M.D. and Wakelam, M.J.O.(manuscript in preparation)

Mitsuhashi, T. and Ives, H.E. (1988) J. Biol. Chem. 263, 8790-8795

Monaco, M.E. and Woods, D. (1983) J. Biol. Chem. 258, 15125-15129

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

Morris, A.J., Murray, K.J., England, P.J., Downes, C.P. and Michell, R.H. (1988) Biochem J. 251, 157-163

Mulcahy, L.S., Smith M.R. and Stacey, D.W. (1985) Nature 313, 241-243

Musgrove, E., Seaman, M. and Hedley, D. (1987) Exptl. Cell Res. 172, 65-75

Newbold, R.F. and Overell, R.W. (1983) Nature 304, 648-651

Nishizuka Y. (1988) Nature 334, 661-665

Ober, A.S.and Pardee, A.B. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 2766-270

Ohlsson, R.I. and Pfeiffer-Ohlsson, S.B. (1987) Exptl. Cell Res. 173, 1-16

Owen, R.D. and Ostrowski, M.C. (1987) Mol. Cell Biol. 7, 2512-2520

Paris, S. and Pouyssegur, J. (1986) EMBO J. 5, 55-60

Parker, P.J., Stabel, S. and Waterfield, M.D. (1984) EMBO J. 3, 953-959

Parries, G., Hoebel, R. and Racker, E. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 2648-2652

Perona, R. and Serrano, R. (1988) Nature 334, 438-440

Putney, J.W. Jr. (1986) Cell Calcium 7, 10-12

Rozengurt, E., Stroobant, P., Waterfield, M. D., Deuel, T.F. and Keehan, M. (1983) Cell 34, 265-272

Rozengurt, E. and Sinnet-Smith, J. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 2936-2940

Rozengurt, E. (1985) in Molecular Mechanisms of Transmembrane Signalling Eds: Cohen, P. and Houslay, M.D.H., pp429-452

Ryu, S.H., Lee, S.Y., Lee, K.-Y. and Rhee, S.G. (1987) FASEB J. 1, 388-393

Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541-548

Saltarelli, D., Fischer, S. and Gacon, G. (1985) Biochem. Biophys. Res. Commun. 127, 318-325

Saxon, P.J., Srivatsan, E.S., and Stanbridge, E.J. (1986) EMBO J. 5, 3461-3466

Schwab, M., Varmus, H.E. and Bishop, J.M. (1985) Nature 316, 160-162

Seuwen, K., Lagarde, A. and Pouyssegur, J. (1988a) EMBO J. 7, 161-168

Seuwen, K., Magnaldo, I. and Pouyssegur, J. (1988b) Nature 335, 254-256

Sibley, D.R., Benovic, J., Caron, M.G. and Lefkowitz, R.J. (1987) Cell 48, 913-922

Smith, M.R., De Gudicibus, S.J. and Stacey, D.W. (1986) Nature 320, 540-543

Smets, L.A. and Van Rooy, H. (1987) J. Cell Phys. 133, 395-399

Spat, A., Bradford, P.G., McKinney, J.S., Rubin, R.P and Putney, J.W. (1986) Nature 319, 514-516

Spandidos, A. (1986) Biosci. Rep. 6, 691-708

Spina, A., Di Donato, A., Colella, G., Illiano, G., Berlingieri, M.T., Fusco, A. and Grieco, M. (1987) Biochem. Biophys. Res. Commun. 142, 527-535

Sporn, M.B. and Roberts, A.B. (1985) Nature 313, 745-747

Sporn, M.B., Roberts, A. B., Wakefield, L.M. and de Crombrugghe, B. (1987) J. Cell Biol. 105, 1039-1045

Storey, D.J., Shears, S.B., Kirk, C.J. and Michell, R.H. (1984) Nature 312, 374-376

Streb, H., Irvine, R.F., Berridge, M.J. and Shulz, I. (1983) Nature 306, 67-69

Sturani, E., Vicentini, L.M., Zippel, R., Toschi, L., Pandiella-Alonso, A., Comoglio, P.M. and Meldolesi, J. (1986) Biochem. Biophys. Res.

Commun. 137, 343-350

Tabin, C.J., Bradley, S., Bargman, C., Weinberg, R., Papageorge, A., Scolnick, E., Dhar, R., Lowr, D. and Chang, E. (1982) Nature 300, 143-149

Tagliaferri, P., Katsaros, D., Clair, T., Neckers, L., Robins, R.K. and Cho-Chung, T.S. (1988) J. Biol. Chem. 263, 409-416

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692-3695

Tarpley, W.G., Hopkins, N.K. and Gorman, R.R. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 3703-3707

Tarver, A.P. and Anderson, R.E. (1988) Biochem J. 250, 891-895

Taylor, C.W., Blakeley, D.M., Corps, A.N., Berridge, M.J. and Brown, K.D. (1988) Biochem J. 249, 917-920

Tilley, B.C., van Paridon, P.A., Verlaan, I., Wirtz, K.W.A., de Laat, S.W. and Moolenar, W.H. (1987) Biochem J. 244, 129-135

Tilley, B.C., Van Paridon, P.A., Verlaan, I., De Laat, S.W. and Moolenar, W.H. (1988) Biochem J. 252, 857-863

Trahey, M. and McCormick, F. (1987) Science 238, 542-545

Tresiman, R. (1986) Cell 46, 567-574

Ui, M. (1986) in Phosphoinositides and Receptor Mechanisms pp163-195 Receptor Biochemistry and Methodology Series Volume 7. Ed: Putney, J.R. Jr., New York: Alan R. Liss, Inc.

Wakelam, M.J.O., Davies, S.A., Houslay, M.D., McKay, I., Marshall, C.J. and Hall, A. (1986) Nature 323, 173-176

Wakelam, M.J.O., Houslay, M.D., Davies, S.A., Marshall, C.J. and Hall, A. (1987) Biochem. Soc. Trans. 15, 45-47

Wakelam, M.J.O. (1988) FEBS. Lett. 228, 182-186

Wang, J.L. and Hou, Y.M. (1986) Trends Biochem. Sci. 11, 24-26

Whyte, P., Buchkovich, K.J., Horowitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A. and Harlow, E. (1988) Nature 334, 124-129

Williams, L.T., Tremble, P.M., Lavin, M.F. and Sunday, M.E. (1984) J. Biol. Chem. 259, 5287-5294

Wolfman, A., Wingrove, T.G., Blackshear, P.J. and Macara, I.G. (1987) J. Biol. Chem. 262, 16546-16552

Woodcock, E.A., Smith, A.I., Wallace, C.A. and Schmauk White, L.B. (1987) Biochem Biophys. Res. Comm. 148, 68-77

Yamaguchi, K., Hirata, M. and Kuriyama, H. (1988) Biochem J. 251, 129-134

Yoshimasa, T., Sibley, D.R., Bouvier, M., Lefkowitz, R.J. and Caron, M.G. (1987) Nature 327, 67-70

Young, S., Parker, P.J., Ullrich, A., Stabel, S. (1987) Biochem J. 244, 775-779

Zick, Y., Sagi-Eisenberg, R., Pines, M., Gierschik, P. and Spiegel, A.M. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 9294-9297