STUDIES ON THE MECHANISM OF ACTION OF THE POTENT MITOGEN *PASTEURELLA MULTOCIDA* TOXIN

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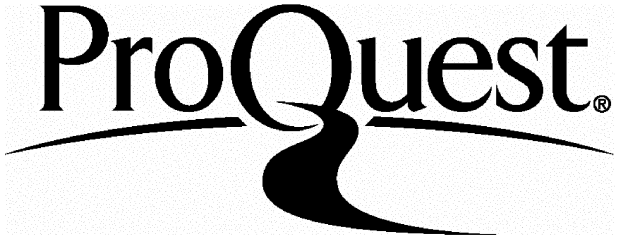

ABSTRACT

The mechanisms of action of bacterial toxins have provided novel approaches to elucidating cellular and molecular regulatory mechanisms. Recently *Pasteurella multocida* toxin (PMT) was shown to be an extremely effective mitogen for Swiss 3T3 cells and stimulates a phospholipase C-mediated increase in inositol phosphates in these cells. Hence, rPMT provided a novel tool for studying this signalling pathway. Here we analysed the effect of recombinant PMT (rPMT) on the G protein and tyrosine kinase-mediated pathways for the activation of phospholipase C. The results in this thesis demonstrate that rPMT selectively potentiates neuropeptide-mediated inositol phosphate production. Treatment of Swiss 3T3 cells with a subsaturating concentration of rPMT markedly potentiated the production inositol phosphates induced by bombesin, vasopressin and endothelin in both a time- and dose-dependent manner. Under similar conditions rPMT had no potentiating effect on the production of inositol phosphates induced by PDGF (AA and BB homodimers). rPMT treatment also markedly enhanced bombesin-induced enhancement of inositol(1,4,5)trisphosphate, the direct product of phosphatidylinositol 4,5-bisphosphate hydrolysis. In addition, rPMT pretreatment greatly reduces the Ca^{2+}-mobilising action of bombesin, consistent with Ca^{2+} mobilisation from a common intracellular pool. In contrast, treatment of cells with rPMT had no effect on the tyrosine phosphorylation of phospholipase Cγ. Depletion of protein kinase C markedly increased the accumulation of inositol phosphates induced by rPMT in a manner similar to that observed for bombesin but not PDGF.

The action of rPMT on phosphatidylinositol 4,5-bisphosphate hydrolysis also persisted in permeabilized cells. The addition of guanosine 5'-O-(β-thiodiphosphate) to permeabilized cells markedly reduced rPMT induced accumulation of inositol phosphates in a time and dose dependent manner. rPMT also increased the sensitivity of phospholipase C for free calcium. These results strongly suggest that the action of rPMT is to facilitate the coupling of G protein to phospholipase C.

Recently, the pertussis toxin-insensitive Gq subfamily of G proteins have been found to couple neuropeptide receptors (including vasopressin) to the β isoform of phospholipase C. Overexpression of the alpha subunits of this G-protein family (α_q and α_{11}) by transfection in COS-1 cells resulted in an increased production of inositol phosphates induced by rPMT. These results demonstrate that Gq may be involved in the production of inositol phosphates stimulated by PMT.

Serum and other growth factors are known to transiently induce the expression of the early protooncogenes c-fos and c-myc. The induction of these genes is subject to strong feedback inhibition. At least part of this autoregulatory mechanism could be the result of receptor desensitization. At the beginning of this work nothing was known about the ability of rPMT to stimulate gene expression. Since PMT by-passes receptor-mediated signal generation we have analysed the effect of rPMT on the induction of these early protooncogenes. The results in this thesis demonstrate that rPMT also stimulates the induction of c-fos and c-myc in Swiss 3T3 cells. The induction of these genes by rPMT occurs after a lag period of 3-4 h and levels of c-fos and in particular c-myc message can be detected for prolonged time periods. Maximal levels of c-fos induced by rPMT were approximately 50% of the maximum levels induced by bombesin. In contrast, maximum levels of c-myc induced by rPMT were slightly higher than maximum levels induced by bombesin. In PKC-down-regulated cells the levels of c-fos and c-myc mRNA induced by rPMT were severely attenuated but not abolished. Similar results are observed for bombesin-stimulation of c-fos and c-myc in PKC-depleted cells. These results demonstrate that rPMT stimulates early gene expression through both PKC-dependent and independent pathways. At least in the case of c-myc, the striking and prolonged expression of this protooncogene may be attributed to the persistent activation of early signalling pathways by rPMT.

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ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine 3': 5'-cyclic monophosphate
CSF-1	Colony stimulating factor
DAG	1,2-Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylenebis(oxyethyleneitrilo)tetracetic acid
EGF	Epidermal growth factor
FDGF	Fibroblast derived growth factor
G protein	Guanine nucleotide binding protein
GAP	GTPase activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDPβS	Guanosine 5'-O-(β-thiodiphosphate)
GRP	Gastrin releasing peptide
GTC	Guanidine isothiocyanate
GTP	Guanosine-triphosphate
GTPγS	Guanosine-5'-O-(3-thiotriphosphate)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IBMX	Isobutyl methylxanthine
IGF	Insulin-like growth factor
Inositol	*myo*-inositol
Ins(1,4,5)P_3	Inositol (1,4,5) trisphosphate
MOPS	3-N-morpholino-propanesulphonic acid
NECA	5'-N-ethyl-carboxyadenosine
OAG	1-oleoyl-2-acetyl-glycerol
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBt_2	Phorbol-12,13-dibutyrate
PDGF	Platelet derived growth factor
PKC	Protein kinase C
PGE1	Prostaglandin E1
PI3K	Phosphatidylinositol 3' kinase

PIPES	1,4-piperazinediethanesulfonic acid
PI-PLC	Phosphatidylinositol-specific Phospholipase C
PMT	*Pateurella multocida* toxin
PMSF	Phenylmethylsulphonyl flouride
PtdIns	Phosphatidylinositol
PtdIns(4)P	Phosphatidylinositol 4-monophosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
RNA	Ribonucleic acid
rPMT	recombinant *Pateurella multocida* toxin
S.E.M.	Standard error of the mean
SDS	Sodium dodecylsulfate
SH2	src homology domain 2
SH3	src homology domain 3
TCA	Trichloroacetic acid
TGF	Transforming growth factor
VGF	Vaccinia virus-induced growth factor
VIC	Vasoactive intestinal contractor
VIP	Vasoactive intestinal peptide

CHAPTER 1: INTRODUCTION

1.1. GROWTH REGULATION

Cells in a living organism exist in a dynamic equilibrium. In most tissues cell turnover is slow, with the majority of cells viable and metabolically active but in a non-proliferative state. Many cells however retain the capacity to respond to appropriate extracellular signals by reinitiating a program of cell proliferation. Typical examples are fibroblastic proliferation after tissue injury, the stimulation of lymphocytes in the generation of an immune response and regeneration of the liver after partial hepatectomy. In this manner the growth of individual cells is tightly regulated according to the requirements of the organism. The elucidation of the molecular mechanisms underlying the control of cell proliferation remains one of the fundamental problems in biology and may prove crucial for understanding the unrestrained proliferation of cancer cells.

The observation that animal cells duplicate their DNA during a discrete interval between cell divisions allowed the cell cycle to be divided into four classical phases; G_1 (growth phase), S (DNA synthetic period), G_2 and M (mitosis). G_1 is the gap between mitosis and the initiation of DNA synthesis; and G_2 is the period between S and M (reviewed in Baserga, 1976, Pardee *et al.*, 1978). It was found that, in most cases, cells respond to suboptimal growth conditions by reversibly arresting in the G_1 (or G_1/G_0) phase of the cell cycle (Pardee, 1974, Baserga, 1976). This arrest is observed in normal fibroblasts in culture (Pardee, 1974). Since the crucial control events for the regulation of growth seem to reside in G_1 (Pardee *et al.*, 1978, Pardee, 1989), elucidation of the mechanisms leading to restimulation of DNA synthesis is therefore a necessary step in the understanding of the control of cell proliferation *in vivo*.

It became evident from the first attempts to propagate animal cells *in vitro* that serum was an essential requirement for the proliferation of most cells. Cells ceased to proliferate and became quiescent in G_1/G_0 when they depleted serum of its growth promoting activity. Furthermore, addition of fresh serum caused the cells to reinitiate a program of RNA and protein synthesis, DNA synthesis and cell division (reviewed in Pardee *et al.*, 1978). Many virally and chemically transformed cells were found to have either partially or wholly lost the ability to arrest in G_1 and exhibited a decreased dependence on serum for proliferation. These findings led to the hypothesis that animal sera contained "growth factors" that controlled cellular proliferation (Wolstenholme and Knight, 1971, Temin *et al.*, 1972, Pardee and Rozengurt, 1975).

The majority of experiments presented in this thesis were performed on Swiss 3T3 cells, a murine fibroblast line established by repeated sub-culture of disaggregated embryonic cells (Todaro and Green, 1963). Cultures of this 3T3 cell line become "quiescent" in the G_1 to G_0 phase of the cell cycle when the cultures reach confluence at a saturation density determined by the serum concentration (Holley, 1975). This arrest appears therefore to be due to depletion of growth-promoting activity in the serum. Readdition of fresh serum to quiescent cultures of these cells stimulates cellular metabolism and after a variable lag period (10-15 hours) initiation of DNA synthesis.

The use of Swiss 3T3 cells in culture has a number of advantages for the study of growth control. Identical monolayer cultures of a clonal population of cells can be easily and consistently produced, allowing for reproducibility of results. In addition a chemically-defined nutrient media has been devised for these cells that allows large and reproducible increases in DNA synthesis to be monitored in the presence of growth-promoting agents. Also, since most cells are arrested in the same phase of the cell cycle (G_1/G_0), on restimulation they may be regarded as a synchronised cell population.

It should be noted, however, that established cell lines often display abnormal karyotypes and therefore cannot be directly correlated with any cell type *in vivo*. In this respect, the 3T3 cell can be considered as a general model for growth control, but results obtained from this cell line necessarily require further investigation to determine their relevance *in vivo*. However, many mechanisms of growth control identified initially in 3T3 cells have been demonstrated to be of general importance. For example, monovalent ion transport across the plasma membrane (Rozengurt and Heppel, 1975) has since been accepted as a universally important part of growth factor action (Moolenaar, 1986).

The 3T3 cell system has proved particularly useful in two major areas of growth control research:

1. The identification of agents that modulate the growth state of the cell, and their purification from various biological sources.

2. Elucidation of the mechanisms by which serum, purified growth factors and other mitogens initiate DNA synthesis. Attention has been focused on the early signalling events in mitogenesis as the initial steps in the proliferative response.

1.2. GROWTH FACTORS AND CELL PROLIFERATION

Within the last decade rapid progress has been made towards the elucidation of the mechanisms of action of the diverse extracellular factors that control the growth of cells. The availability of cell culture in nutrient media together with the purification of various polypeptide growth factors (reviewed in Rozengurt, 1980, Rozengurt and Collins, 1983, James and Bradshaw, 1984) allowed the investigation of mitogen action under chemically defined conditions (Bottenstein *et al.*, 1979, Shipley and Ham, 1981). From this work it has become clear that a variety of mitogenic factors can regulate the proliferation of normal cells. In this respect the 3T3 fibroblast has proved a particularly useful system for identification of growth promoting factors (Rozengurt, 1980, Rozengurt, 1986). It is now recognised that the proliferation of 3T3 cells may be regulated by several classes of mitogen (Table 1.2.1.). These include a).a large family of polypeptide growth factors including PDGF, EGF and Insulin-like growth factors and b) the neuropeptides, a rapidly growing family of small regulatory peptides that classically behave as local hormones or fast-acting neurotransmitters. In addition various pharmacological agents including phorbol esters and synthetic diacylglycerols can act as mitogens for these cells (Table 1.2.1.).

Both the polypeptide growth factors and the neuropeptides stimulate their growth promoting effects by binding to specific receptors located on the cell surface and activating multiple signalling pathways. At least two major signal transduction pathways initiate the cascades of molecular events stimulated by these two classes of mitogens. The polypeptide growth factors e.g. PDGF and EGF bind to receptors with intrinsic tyrosine kinase activity while the neuropeptides act through receptors coupled by guanine nucleotide binding proteins (G proteins) to effector activation. During the past few years considerable advances have been made in the identification of extracellular factors and in the elucidation of signal-transduction events that induce cell proliferation through both the tyrosine kinase and G protein-mediated pathways. In what follows is a description of the initial events induced by growth factors that stimulate cell proliferation through both the G protein and the tyrosine kinase-mediated pathways.

1.2.1. SIGNAL TRANSDUCTION THROUGH TYROSINE KINASE RECEPTORS.

Several polypeptide growth factors, including PDGF, EGF and FGF, bind to specific receptors on the plasma membrane of the target cell that possess intrinsic tyrosine kinase activity. The PDGF system has served as a prototype for studying the signalling pathways utilised by receptor tyrosine kinases. PDGF is a potent mitogen for connective tissue cells and has been implicated in a wide variety of physiological and pathological processes (Ross *et al.*,

Table 1.2.1.

Stimulation of 3T3 fibroblast DNA synthesis by extracellular factors.

Polypeptide growth factors	PDGF	Heldin and Westermark, 1984
	IGFs	Rozengurt *et al.*, 1983c, Lopez-Rivas *et al.*, 1984
	EGF	Carpenter and Cohen, 1979, Froesch *et al.*, 1979
Polypeptides released from tumours and virally infected cells.	α-TGF	Todaro *et al.*, 1981, Heldin and Westermark, 1984, Goustin *et al.*, 1986
	β-TGF	
	IGF	Marquardt *et al.*, 1980
	FDGF	Dicker *et al.*, 1981, Bowen-Pope *et al.*, 1984, Stroobant *et al.*, 1985, Ross *et al.*, 1986
	VGF	Twardzik *et al.*, 1985
Neuropeptides	Bombesin	Rozengurt and Sinnett-Smith, 1983
	Vasopressin	Rozengurt *et al.*, 1979
	Oxytocin	Dicker and Rozengurt, 1980, Rozengurt and Mendoza, 1980
	Bradykinin	Woll and Rozengurt, 1988
	VIP	Zurier *et al.*, 1988
	Endothelin	Brown and Littlewood, 1989, Takuwa *et al.*, 1989
	VIC	Fabregat and Rozengurt, 1990
Tumour promoters	Phorbol esters	Dicker and Rozengurt, 1978, Dicker and Rozengurt, 1980, Dicker and Rozengurt, 1981, Collins and Rozengurt, 1982a, Collins and Rozengurt, 1982b
Diacylglycerol	OAG	Rozengurt *et al.*, 1984
Vitamin A derivatives	Retinoic acid	Dicker and Rozengurt, 1979, Dicker and Rozengurt, 1980
Permeability modulators	Mellitin	Gelehrter and Rozengurt, 1980, Rozengurt *et al.*, 1981b

Cyclic nucleotide elevating agents.	Cholera toxin	Rozengurt *et al.*, 1981c, Rozengurt, 1982b, Rozengurt, 1982a
	Forskolin	Rozengurt *et al.*, 1981c,
	PGE_1	Rozengurt *et al.*, 1983a,
	IBMX	Rozengurt *et al.*, 1983c
	Adenosine agonists	
	cAMP derivatives.	
	Mastoparan	Gil *et al.*, 1991
Microtubule disrupting agents	Colchicine	Friedkin and Rozengurt, 1981
	Podolotoxin	Wang and Rozengurt, 1983
	Colemid	
	Vinblastine	
	Nocodazole	

1986). Structurally, PDGF is a 32 kDa dimer consisting of two homologous polypeptide chains, A and B, which are encoded by different genes. All three dimeric forms of PDGF have been found to occur naturally. PDGF A and B chains can bind differently to two distinct receptor molecules, the PDGFα and β receptors. As with other tyrosine kinase receptors, the PDGF receptors possess a large glycosylated, extracellular ligand recognition domain, a single hydrophobic transmembrane region and a cytoplasmic region that contains a highly conserved tyrosine kinase domain (Ullrich and Schlessinger, 1990 and fig 1.2.1.). The extracellular domain of the PDGF receptor is characterised by five immunoglobulin domains and the catalytic domain is interrupted by the insertion of amino sequences. The α receptor binds both PDGF A and B chains whereas the β receptor binds only the PDGF B chain (reviewed in Westermark and Heldin, 1991)

There is now considerable evidence to suggest that the binding of PDGF to its receptors results in receptor dimerization and subsequent transphosphorylation at specific tyrosine residues (reviewed in Ullrich and Schlessinger, 1990). Similar events occur for other growth factor receptors, including the EGF receptor. The ligand-stimulated autophosphorylation (or cross-phosphorylation) on tyrosine not only enhances the tyrosine kinase activity of the receptors but also creates binding sites for recruitment of specific cytosolic proteins implicated in intracellular signal transduction pathways. These include the γ1 isoform of polyphosphoinositide specific phospholipase C (PI-PLCγ_1) and phosphatidylinositol 3' kinase (PI3K), p21^{ras} GTPase-activating protein (GAP), tyrosine kinases of the src family and other cellular proteins including some that have not, as yet been identified (reviewed in Cantley *et al.*, 1991). In every case, the formation of stable complexes between receptor and cellular protein requires the tyrosine kinase activity of the receptor.

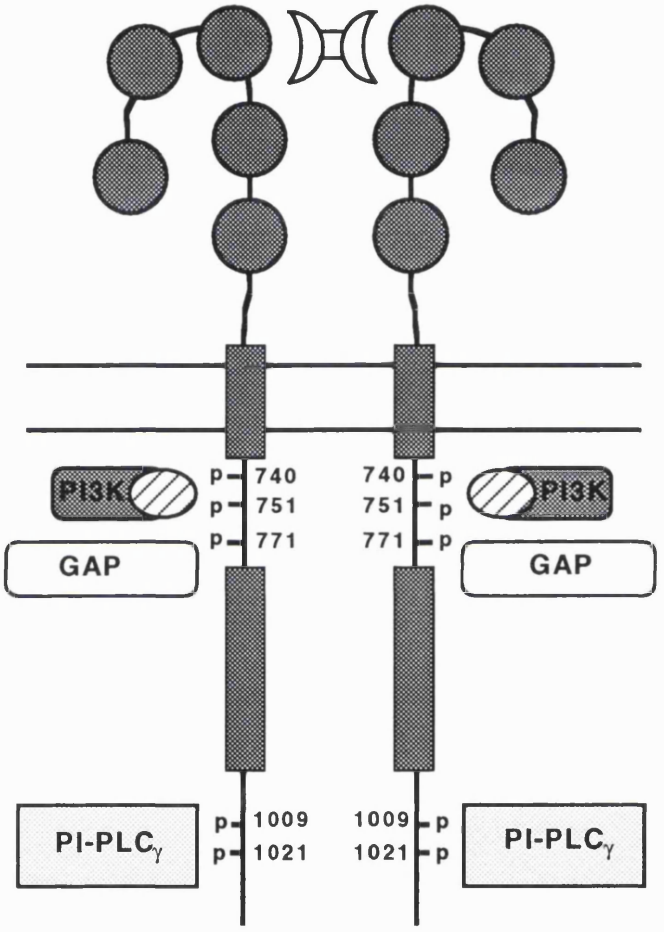
The association of cytoplasmic proteins with the activated receptors is mediated by a non-catalytic domain of ~100 amino acids, the src homology (SH)2 region (reviewed in Koch *et al.*, 1991). These domains, expressed as bacterial fusion proteins, are sufficient to form stable complexes *in vitro* with the liganded receptors (Anderson *et al.*, 1990, Mohammadi *et al.*, 1991). It has been proposed that tyrosine phosphorylation of specific residues in the polypeptide chain of the receptor acts as a switch to induce high-affinity binding of SH2-containing cytoplasmic proteins. Differences in the amino acid sequences between SH2 domains appear to result in distinct binding affinities (Koch *et al.*, 1991). Hence, SH2 domains play a crucial role in the association of cytoplasmic proteins with cellular tyrosine kinases. In addition, a number of small SH2-containing proteins have recently been identified which lack any catalytic sequences such as those responsible for phospholipase C or kinase activities. These proteins are thought to act as adaptors that bind to receptors through their SH2 domains and to downstream targets through other sequences (reviewed in Pawson and Gish, 1992).

The first evidence for ligand-dependent recruitment of enzymes to a tyrosine kinase -type receptor was the discovery that PI3K (a cytosolic enzyme in resting cells) is recruited to the PDGF receptor within less that 1 min following stimulation of fibroblasts with PDGF (Kaplan *et al.*, 1987, Whitman *et al.*, 1987). This association was shown to require the 'kinase insert' domain (a region of sequence unique to the PDGF receptor that lies in the middle of the kinase domain)(Coughlin *et al.*, 1989). A tyrosine residue in this domain undergoes autophosphorylation in response to addition of PDGF, and mutation of this residue to a phenylalanine dramatically reduces (but does not eliminate) the PI3K recruitment (Kazlauskas and Cooper, 1989). In addition, a synthetic peptide representing the conserved region of the 'kinase insert' domain blocked the interaction in vitro of the PI3K with the phosphorylated β-PDGF receptor. The autophosphorylation sites in the 'kinase insert' of the PDGF receptor required to mediate PI3K binding have been identified as Tyr740 and Tyr751 (Coughlin *et al.*, 1989, Fantl *et al.*, 1992, Kashishian *et al.*, 1992). The precise biological significance of PI3K remains unknown but it is thought that it plays a role in eliciting cell proliferation (Cantley *et al.*, 1991).

PI-PLCγ has also been shown to undergo ligand-dependent association with both EGF and PDGF receptors (Wahl *et al.*, 1988, Margolis *et al.*, 1989, Meisenhelder *et al.*, 1989, Wahl *et al.*, 1989, Morrison *et al.*, 1990). The regulation of this enzyme by growth factor receptor activation will be discussed in section 1.3.1.

The products of the *ras* family of protooncogenes are low-molecular-weight, guanine nucleotide-binding proteins that play essential roles in the control of cellular growth and differentiation (for review see Satoh *et al.*, 1992). They possess intrinsic GTPase activity, being biologically active when bound to GTP and inactive when bound to GDP. The low basal rate of GTP hydrolysis on *ras*-encoded protein ($p21^{ras}$) is stimulated by GAPs such as $p120^{GAP}$ and neurofibromin, the product of the neurofibromatosis type 1 gene. These proteins therefore inactivate $p21^{ras}$ but may also have roles as downstream effectors. The findings that $p120^{GAP}$ associates with and serves as a substrate for phosphorylation by liganded tyrosine kinase receptors suggested a link between receptors and $p21^{ras}$ mediated by GAP, but substantial evidence was lacking. It is known that phosphorylated $p120^{GAP}$ associates with two other cellular proteins, p62 and p190 (Moran *et al.*, 1991). EGF treatment stimulates GAP phosphorylation and induces complex formation between $p120^{GAP}$ and p190. Interestingly this complex has reduced GAP activity suggesting that $p120^{GAP}$ phosphorylation plays a role in the regulation of $p21^{ras}$ activity. It has been reported that proteins with SH2 domains which bind to the PDGF receptor (e.g. GRB2) may transduce the signal to ras (Lowenstein *et al.*, 1992). Indeed it has recently been demonstrated by a number of groups that GRB2 couples the EGF receptor to the Sos nucleotide exchange

Fig.1.2.1.



Schematic illustration of PDGF receptor. Shows the specificity in the interaction between different substrates and the different activated autophosphorylation sites in the activated and dimerized PDGF(β) receptor.

factor which acts as a ras activator (Buday and Downward, 1993, Gale *et al.*, 1993, Li *et al.*, 1993, Rozakis-Adcock *et al.*, 1993).

A summary of specificity in the interaction between different substrates and different activated autophosphorylation sites in the activated PDGF receptor is shown in Fig. 1.2.1.

A number of other tyrosine kinase receptor systems have been elucidated recently. Many of these receptor tyrosine kinases also associate with enzymes such as PI3K and PI-PLCγ as well as with other proteins that are known to bind to the PDGF receptor. Many of these receptors appear to have specificity for distinct targets. For example the CSF-1 receptor associates with PI3K and ras^{GAP} but appears not to associate with PI-PLCγ. Thus the activation of receptor tyrosine kinases such as the PDGF receptor results in the stimulation of multiple cellular responses that are secondary to the activation of the enzymes that bind to the receptors. Some of these signalling pathways will be discussed in section 1.3. It is likely that other proteins associate with these activated receptors that have not yet been identified. Their eventual identity may reveal even more information about the complex signalling pathways initiated by receptor tyrosine kinase activation.

1.2.2. SIGNAL TRANSDUCTION THROUGH G PROTEIN COUPLED RECEPTORS

An increasing number of hormones, small regulatory peptides or neuropeptides (e.g. bombesin, vasopressin, endothelin and bradykinin) have been shown to act as potent cellular growth factors and have been implicated in a variety of normal and abnormal biological processes including development and tumorigenesis. These growth factors transduce their mitogenic signals to the intracellular environment by specific interaction with a class of receptors coupled to G proteins. These receptors are characterised by the presence of seven hydrophobic regions thought to represent membrane-spanning domains (reviewed in Birnbaumer *et al.*, 1990). Structural homology in the putative transmembrane regions between different members of the receptor superfamily has facilitated the molecular cloning of cDNAs encoding novel receptor sequences. Indeed, the cloning and sequencing of the cDNA for several mitogenic peptide receptors including bombesin (Battey *et al.*, 1991) neuromedin-B (Corjay *et al.*, 1991), bradykinin (McEachern *et al.*, 1991), thrombin (Vu *et al.*, 1991) and vasoactive intestinal peptide (Sreedharan *et al.*, 1991) has been accomplished recently.

In addition to the extracellular domains which are responsible for ligand binding, intracellular or cytoplasmic domains of some receptors have been identified which are responsible for

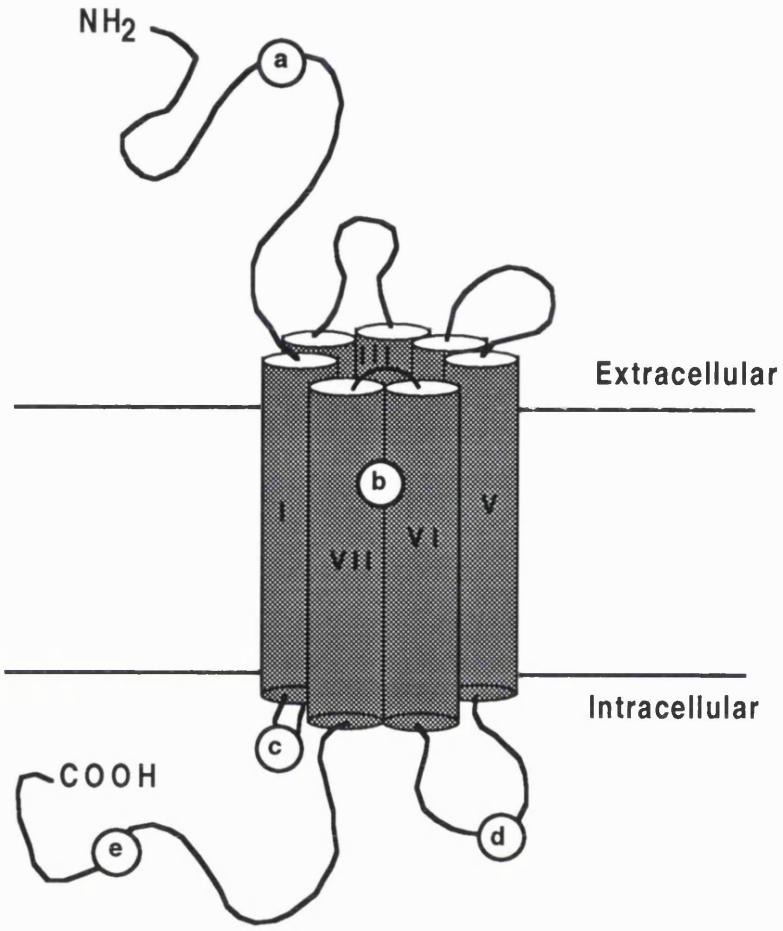
protein coupling and receptor desensitization. General structural and functional features of G protein coupled receptors are summarised in Fig.1.2.2.

The G proteins that mediate regulation of several effector molecules by coupling to hormone receptors constitute a large family of highly homologous proteins. The proteins are heterotrimers consisting of α-, β- and γ-subunits. The α subunits appear to be most diverse and have been traditionally used to define the purified heterotrimeric proteins. Thus, frequently the α-subunits can also account for the primary activity of the G proteins. A simple model for the activation G proteins is shown (Fig.1.2.3.). In the basal state, the α–subunit contains bound GDP, and association of α- and βγ-subunits is highly favoured. Stimulation of the G protein results when it binds GTP rather than GDP. Receptors interact most efficiently with the heterotrimeric form of the G protein. However, hormone binding and receptor activation decreases the affinity between the α- and βγ-subunits of the G protein and accelerates activation by increasing the rate of dissociation of GDP and potentially enhancing association of GTP. The dissociation of subunits thus generates two potential pathways [α(GTP) and free βγ-subunits] for downstream regulation. Finally, the G protein α-subunit has an intrinsic hydrolytic activity that slowly (rates < 10 min^{-1}) converts GTP to GDP and returns the G protein to its inactive form. The stimulation of G protein coupled receptors results in the activation of effector proteins (e.g. phospholipases, adenylate cyclase and ion channels), which mobilize chemical second messengers that initiate characteristic actions within the cell.

The ubiquitous hormone-stimulated adenylate cyclase system (Gilman, 1987) and the cGMP phosphodiesterase pathways (Stryer, 1986) have served as models for understanding G protein-receptor and G protein effector interactions. Reconstitution studies using purified components (activated α-subunit and effector protein) of both these systems provided fundamental evidence for the involvement of G proteins in specific signalling pathways (reviewed inGilman, 1987). It is now clear that the α-subunits of heterotrimeric G proteins regulate many other effector systems including multiple ion channels and phospholipases (Hepler and Gilman, 1992). To date cDNAs that encode at least 21 distinct G protein α-subunits have been cloned. These can be grouped into four major classes on the basis of amino acid identity i.e. those represented by Gs, Gi, Gq and G_{12}. In addition at least four distinct β- and six γ-subunits have been described. A summary of the classes of α-subunits and their effector systems is shown in Table 1.2.2.

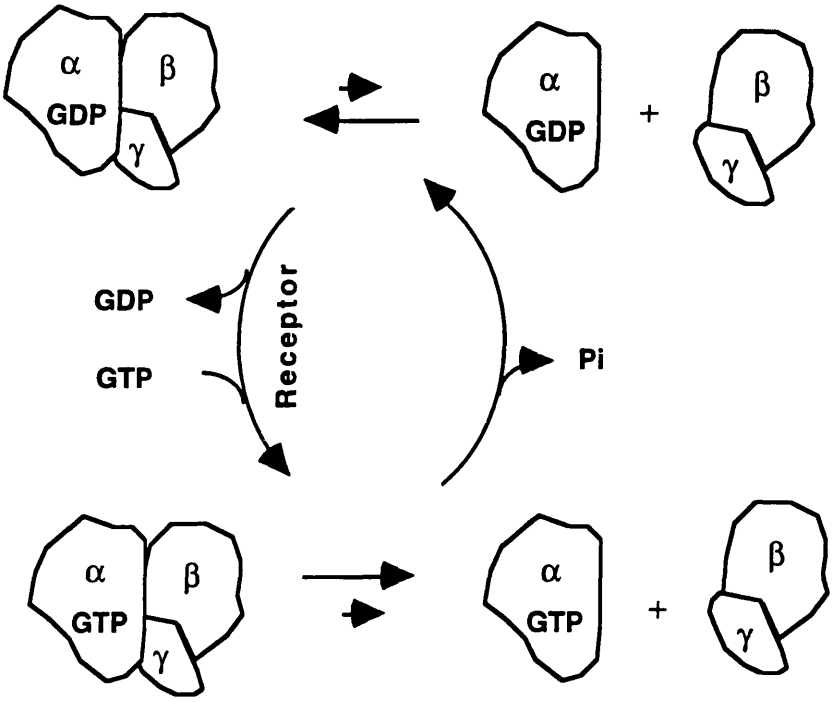
In some cases α-subunits possess specific residues that can be covalently modified by bacterial toxins. Cholera toxin catalyses the transfer of the ADP-ribose moiety of NAD to a specific arginine residue in certain α-subunits. Similarly, pertussis toxin ADP-ribosylates those α-subunits that possess a specific cysteine residue near the carboxyl terminus. Modification of α by cholera toxin constituitively activates these proteins (by inhibiting their

Fig. 1.2.2.



A schematic representation of a G protein coupled receptor. Putative membrane-spanning domains are depicted as cylinders. Letters refer to structural domains to which some general but not necessarily universal functional features have been assigned. (a) The amino terminal extracellular domain contains potential N-Linked glycosylation sites in most receptors and is the ligand binding domain for glycoprotein hormone receptors. (b) The transmembrane domains (important for ligand binding in some receptors) (c) The first and second cytoplasmic loops are implicated in coupling to G proteins (d) The third cytoplasmic loop is thought to be involved in determining specificity of coupling to different G proteins. (e) The carboxyl-terminal cytoplasmic tail is implicated in coupling to G proteins

Fig. 1.2.3.



Model for activation of G proteins. The functional state of a G protein is determined by bound nucleotide. With GDP, the G protein is inactive and subunit association is favoured. With bound GTP, the G protein is activated and the affinity between its α- and βγ-subunits is markedly reduced. Receptors stimulate G proteins by catalysing exchange of GDP for GTP.

Table 1.2.2.

Mammalian G protein α-subunits and their effector proteins

Family/α-subunit	Forms	Toxin	Effector
Gs			
α_s	4 splice variants from a single gene	Cholera	Adenylyl cyclase Ca^{2+} channel Na^+ channel
α_{olf}	1	Cholera	Adenylyl cyclase
Gi			
α_i	3 genes	Pertussis	Adenylyl cyclase K^+ channel Na^+ channel phospholipase A_2 (?)
α_t	2 genes	Cholera/Pertussis	Cyclic GMP phosphodiesterase
α_o	2 splice variants	Pertussis	K^+ channel Ca^{2+} channel
α_z		-	adenylyl cyclase (?)
Gq			
α_q		-	Phospholipase C
α_{11}		-	Phospholipase C
α_{14}		-	Phospholipase C
α_{15}		-	Phospholipase C
α_{16}		-	Phospholipase C
G_{12}			
α_{12}		-	?
α_{13}		-	?

GTPase activity), whereas modification by pertussis toxin prevents receptor-mediated activation of G proteins. Sensitivity to pertussis toxin has also provided a useful means for identifying and classifying G proteins. The involvement of a pertussis toxin-insensitive G protein in receptor-mediated activation of PI-PLC has been known for some time. Recently the Gq family (table 1.2.2.) of G proteins have been identified as regulators of this pathway. The regulation of PI-PLC by this family of G proteins will be discussed in section 1.3.1.

Since even a single G protein-receptor subtype can be coupled to multiple effectors and multiple receptor subtypes can activate a single effector (reviewed in Gilman, 1987, Pfeuffer and Helmreich, 1988, Birnbaumer *et al.*, 1990), the G protein coupled interactions form complicated networks. Furthermore, characterization of effectors of these systems has revealed that they too are specified by extensive gene families (Birnbaumer *et al.*, 1990). The continued discovery of new receptor subtypes with novel structural and functional features as well as new G proteins and effectors demonstrates that we are far from resolving the complexities of this signal transduction pathway.

1.3. EARLY SIGNALS IN MITOGENESIS

As described in the previous section the binding of polypeptide growth factors or neuropeptides to their receptors results in the activation of two major signal transduction pathways. These initial signalling events lead to the generation of an array of rapid molecular events in the membrane, cytosol and nucleus. These events, termed early signals are necessary to transduce the information required for the initiation of a proliferative response (Rozengurt, 1986). In what follows is a description of the early signals that have been implicated in growth control, with particular reference to those stimulated by the neuropeptides and PDGF in Swiss 3T3 fibroblasts.

1.3.1. PHOSPHOLIPASE C-MEDIATED HYDROLYSIS OF INOSITOL PHOSPHOLIPIDS

The phosphoinositides are a small group of membrane phospholipids which are unique in that their *myo*-inositol headgroup can be phosphorylated at multiple sites (Hawthorne, 1982). They form a minor component of most, if not all, eukaryotic membranes with the three predominant phosphoinositides being phosphatidylinositol [PtdIns] which forms the majority, phosphatidylinositol 4-monophosphate [PtdIns(4)P] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]; the numbers referring to the position of the phosphates on the inositol ring. Collectively PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 usually constitute less than 8% of total membrane phospholipids (Hawthorne, 1982) and they can readily interconvert via

a series of phosphorylation and dephosphorylation reactions (Shears, 1989) which are governed by specific kinases and phosphatases (Irvine, 1982). The metabolism of these phospholipids is shown in Fig.1.3.1.

There is now considerable evidence that stimulation of inositol phospholipid metabolism via activation of phosphoinositide-specific phospholipase C (PI-PLC) is a major signal transduction pathway for a variety of receptors in eukaryotic cells. Indeed, many extracellular signalling molecules including hormones, peptide growth factors, neurotransmitters and immunoglobulins, on binding to their cell surface receptors, elicit intracellular responses by activating PI-PLC. One of the major reactions catalysed by PI-PLC enzymes is the hydrolysis of PtdIns(4,5)P_2 to generate the two second messengers, inositol1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). Ins(1,4,5)P_3 mobilizes Ca^{2+} by binding to specific intracellular receptors that promote the opening of calcium channels in the vesicular storage sites associated with the endoplasmic reticulum (Berridge, 1987). The other product of PtdIns(4,5)P_2 hydrolysis, diacylglycerol, binds to and activates protein kinase C, resulting in the phosphorylation of a number of intracellular proteins (Nishizuka, 1986). This bifurcating pathway is one of the most commonly used transduction and transmembrane signalling mechanisms in a wide range of tissues and cells and is known to regulate a large array of cellular processes, including metabolism, secretion, contraction, neural activity, and proliferation (Rana and Hokin, 1990).

The action of PI-PLC enzymes is not solely confined to PtdIns(4,5)P_2 hydrolysis, nor are Ins(1,4,5)P_3 and DAG the only products of this reaction. These enzymes hydrolyse PtdIns and PtdIns(4)P as well as PtdIns(4,5)P_2 and an enormous number of both cyclic and non-cyclic inositol phosphates have now been found in eukaryotic cells. Details of some of the pathways linking them have been elucidated (see Fig 1.3.1.). The complexity of the inositol phosphate metabolic network has revealed that inositol phosphates other than Ins(1,4,5)P_3 might have important cellular functions. However for the purpose of this thesis only Ins(1,4,5)P_3 signalling will be discussed.

PI-PLC Isoforms

Determining the mechanism of receptor-coupled hydrolysis of PtdIns(4,5)P_2 and how the resulting products interact with their cellular targets has involved the isolation and characterization of PI-PLC, the enzyme directly responsible for second messenger production. The existence of multiple isoforms of PI-PLC was described very early on in studies of many different tissues including heart , brain, platelets, liver and kidney (Chau and Tai, 1982, Hirasawa *et al.*, 1982b, Hirasawa *et al.*, 1982a, Low and Weglicki, 1983). When

Fig. 1.3.1.

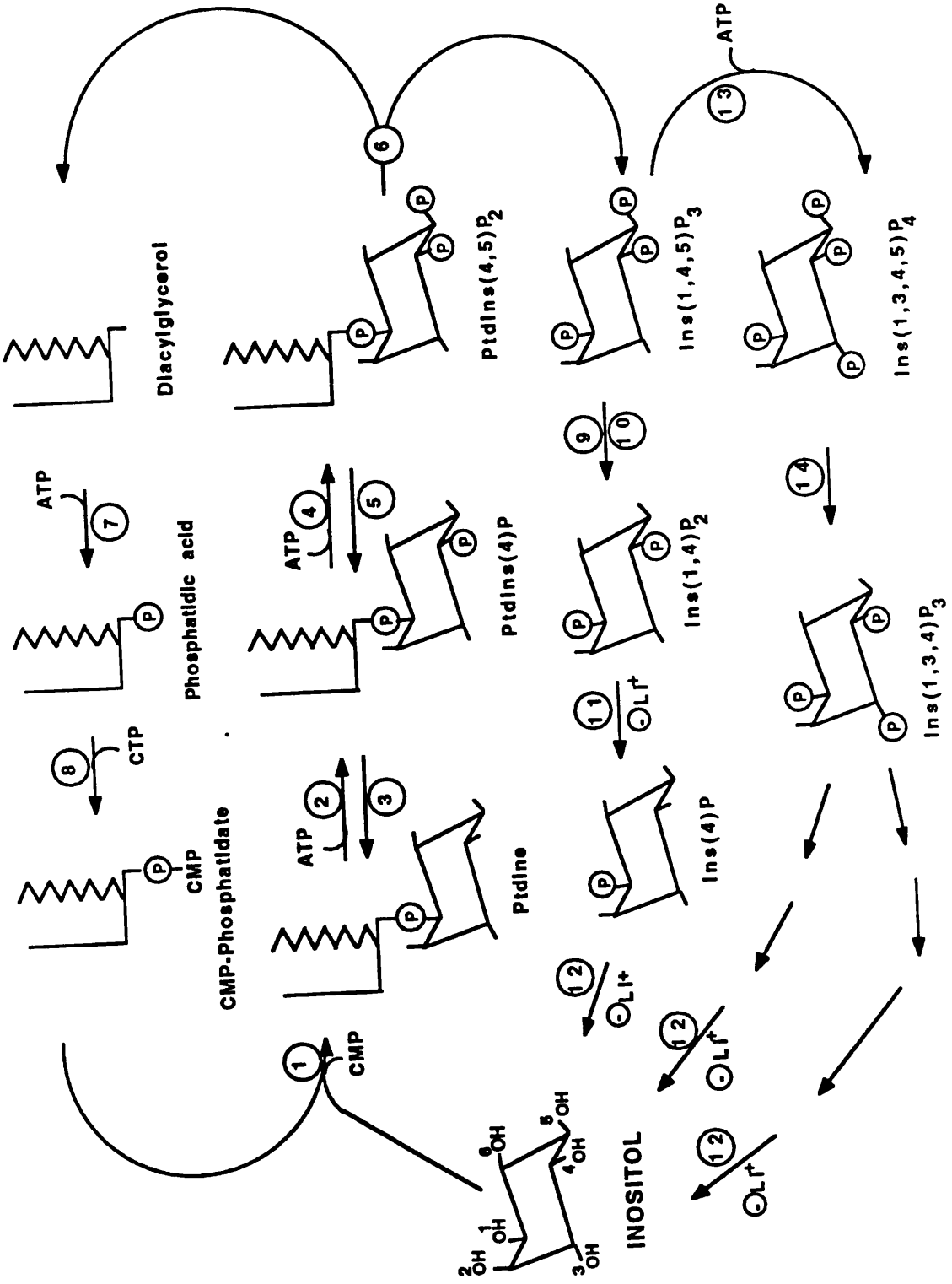

Diacylglycerol
PtdIns(4,5)P2
Ins(1,4,5)P3
Ins(1,3,4,5)P4
ATP
Phosphatidic acid
PtdIns(4)P
Ins(1,4)P2
Ins(1,3,4)P3
CTP
CMP
CMP-Phosphatidate
PtdIns
Ins(4)P
INOSITOL
Li+

Fig. 1.3.1. Diagram showing the main pathways of inositol lipid and inositol phosphate metabolism. The enzymes are 1. PtdIns synthetase (CMP-PA; inositol phosphatidyl transferase); 2. PtdIns-4-kinase (type II); 3. PtdIns(4)P phosphomonoesterase; 4. PtdIns(4)P-5-kinase; 5. PtdIns(4,5)P_2 phosphomonoesterase; 6. Phosphatidyl inositol specific phospholipase C (PI-PLC); 7. diacylglycerol kinase; 8. CMP-phosphatidic acid synthetase; 9. Ins(1,4,5)P_3/Ins(1,3,4)P_4-5-phosphatase; 10. Ins(1,4,5)P_3-5-phosphatase; 11. inositolpolyphosphate-1-phosphatase; 12. InsP phosphatase; 13. Ins(1,4,5)P_3-3-kinase. -Li^+ denotes enzymes which are inhibited by lithium.

the importance of PI-PLC in the generation of the two second messengers, Ins(1,4,5)P_3 and DAG was recognised, many groups went on to purify these enzymes. Several distinct PI-PLC isoforms have subsequently been purified from a variety of mammalian tissues (for review see Rhee *et al.*, 1989) and several forms have been molecularly cloned and sequenced (Bennett *et al.*, 1988, Katan *et al.*, 1988, Stahl *et al.*, 1988, Suh *et al.*, 1988a, Suh *et al.*, 1988b, Emori *et al.*, 1989). Comparison of their deduced amino acid sequences and immunological cross-reactivity has indicated that the PI-PLCs can be divided into three types (PLCβ, PLCγ, and PLCδ) each of which is a discrete gene product (Rhee *et al.*, 1989). Within each family, there are several subtypes e.g. PI-PLCβ_1, β_2, β_3. A classification of the currently identified members of these families is provided in Table 1.3.1

A comparison of the linear sequence of the PI-PLCβ, γ, and δ families (Fig.1.3.2) reveals that only two regions of homology, designated X and Y, are shared by the three families (Rhee *et al.*, 1989). These X and Y regions comprise of 150 and 240 amino acids respectively and are about 60% and 40% identical. Separately or jointly these regions might constitute the catalytic domain which is responsible for specific recognition of Ca^{2+} and phosphoinositides or the hydrolysis of the phosphodiester bond. Whereas PI-PLCβ and PI-PLCδ contain short sequences of 50-70 amino acids separating the X and Y regions, PI-PLCγ has a long sequence of ~400 amino acids, which contains the src homology domains, SH2 and SH3 (first identified as non-catalytic regions common to a variety of src family tyrosine kinases). These domains govern protein-protein interactions; the SH2 domain targets the molecule to tyrosine phosphorylated sequences present in other proteins (e.g. the PDGF receptor) and the SH3 domain targets it to cytoskeletal components.

Single polypeptide PI-PLCs with molecular masses of 62-68 kDa have also been purified (Rhee *et al.*, 1989). A cDNA corresponding to one of these smaller enzymes was cloned and sequenced, but the deduced amino acid sequence showed no regions corresponding to the X and Y domains. Although these smaller enzymes were named PI-PLCα (Rhee *et al.*, 1989), recent studies showed that the cDNA clone of PI-PLCα actually encodes an endoplasmic reticulum protein that carries no PI-PLC activity (Martin *et al.*, 1991) and that the 62-68 kDa enzymes may be proteolytic fragments derived from PI-PLCβ, γ, and δ types.

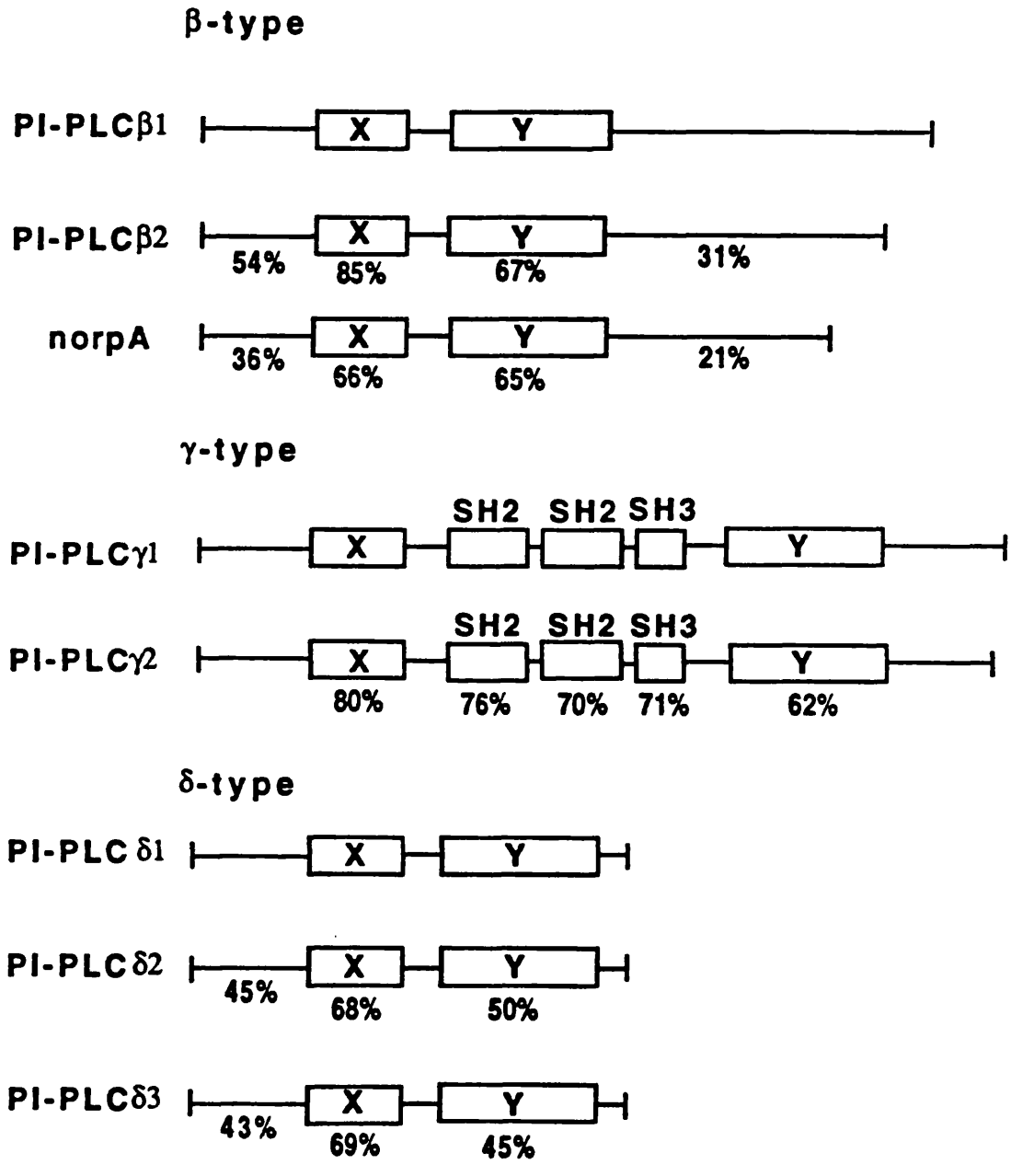
Other phospholipases that have been identified by protein purification and biochemical characterization but have not been sequenced or cloned also exist. Many of these can be classified into the known PLC-β, γ, and δ families but in addition other families can be predicted including the PI-PLCα family discussed above. Another group of 85-88 kDa PI-PLC enzymes have been found and were classified into a new family called PI-PLCε. These enzymes have not been sequenced as yet, but were classified into this new group because

Table 1.3.1.

Classification of PI-PLC isoenzymes characterised from different tissues

PLC isozyme	Size (kDa)	Source (reference)
β family		
β1	150-154	Bovine and rat brain (Ryu, *et al.*, 1986, Katan and Parker, 1987 Ryu, *et al.*, 1987a, Ryu, *et al.*, 1987b, Katan *et al.*, 1988)
β2	134	Human HL60 cDNA (Kriz *et al.*, 1990)
β3	158	human fibroblast cDNA (Kriz *et al.*, 1990, Carozzi *et al.*, 1992)
norp A	125	Drosophilia genome (Bloomquist *et al.*, 1988)
plc-21	-	Drosophilia genome (Shortridge *et al.*, 1991)
γ family		
γ1	145	Bovine and rat brain (Ryu, *et al.*, 1986, Ryu, *et al.*, 1987a, Ryu, *et al.*, 1987b, Suh *et al.*, 1988b)
γ2	146	HL60, spleen, lung (Ohta *et al.*, 1988, Emori *et al.*, 1989, Banno *et al.*, 1990, Homma *et al.*, 1990)
δ family		
δ1	85	Rat and bovine brain (Ryu, *et al.*, 1986, Ryu, *et al.*, 1987a, Ryu, *et al.*, 1987b, Homma *et al.*, 1988, Suh *et al.*, 1988a)
δ2	85	Bovine brain (Meldrum *et al.*, 1989, Meldrum *et al.*, 1991)
δ3	84	Human fibroblast cDNA (Kriz *et al.*, 1990)

Fig. 1.3.2.



Linear representation of the members of the PI-PLCβ, γ and δ families.

they showed different biochemical properties to the 85-88 kDa δ-family of PI-PLCs (for review see Cockcroft and Thomas, 1992).

Regulation of PI-PLC isoforms.

All three of the main subtypes of PI-PLC catalyse the hydrolysis of inositol-containing phospholipids: PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 of which PtdIns and PtdIns(4,5)P_2 are the preferred substrates. In addition, the catalytic activities of all three subtypes of PI-PLC are dependent on Ca^{2+}. However, the regulation of these PI-PLC isoforms is distinct. Numerous studies have shown that the PI-PLCβ family is regulated by G proteins while the PI-PLCγ family is regulated by tyrosine kinases. The regulation of PI-PLCδ remains unclear.

1. Regulation of PI-PLC by G Proteins.

The involvement of a regulatory G-protein in the signal transduction mechanism of agonists stimulating the hydrolysis of PtdIns(4,5)P_2 has been strongly implicated for some time. In 1985, several laboratories reported that guanine nucleotide binding proteins may couple certain Ca^{2+} mobilizing receptors to PI-PLC (reviewed in Harden, 1989, Martin, 1989). Studies in which labelled PtdIns(4,5)P_2 was added to membrane preparations showed that PtdIns(4,5)P_2 could be hydrolysed as a consequence of activation of membrane bound PI-PLC by either GTPγS or receptor-directed agonists. Guanine nucleotides were found to augment agonist stimulation of PI-PLC or directly activate the enzyme in many types of cell membrane, including blowfly salivary glands (Litosch *et al.*, 1985) and human neutrophils (Cockcroft and Gomberts, 1985, Gonzales and Crews, 1985). Further evidence for a role for guanine nucleotides in receptor-coupled PI-PLC activation came from studies using pertussis toxin. Pertussis toxin was originally purified from the culture supernatant of the pathogenic bacteria *Bordetella pertussis*, on the basis of its ability to interfere with α-adrenergic receptor-mediated inhibition of insulin secretion in rat pancreatic islets (Yajima *et al.*, 1978). Pertussis toxin exerts its effects by abolishing GTP-dependent and receptor-mediated inhibition of adenylate cyclase (reviewed in Ui, 1984), by ADP-ribosylation of a membrane associated guanine nucleotide binding protein, G_i (Katada and Ui, 1982a, Katada and Ui, 1982b). The α subunit of G_i contains a GTP-binding site and a site for pertussis toxin-catalysed ADP-ribosylation (Bokoch *et al.*, 1984, Katada *et al.*, 1984a, Katada *et al.*, 1984b). It is now recognised that pertussis toxin can ADP-ribosylate and inhibit the function of a family of G_i-like proteins, some of which have been purified and cloned (Table 1.2.2.), the functions for which have not been completely elucidated (reviewed in Ui, 1990). In particular, in certain cell types, including neutrophils (Okajima *et al.*, 1985) and mast cells (Nakamura and Ui, 1984), pertussis toxin blocks agonist stimulation of phosphoinositide breakdown. However, in

GH_3 cells (Martin *et al.*, 1986), astrocytoma cells (Masters *et al.*, 1985) and liver cells (Lynch *et al.*, 1986) agonist stimulation of phosphoinositide breakdown is mediated through a G protein not sensitive to pertussis toxin. Thus receptors coupling to PI-PLC may be grouped into two classes based on sensitivity to pertussis toxin (Cockcroft, 1987). Indeed, pertussis toxin sensitive and insensitive G proteins can couple receptors to phosphoinositide breakdown in the same cell (Ashkenazi *et al.*, 1989). In a similar manner, activation of PI-PLC by bombesin and vasopressin in Swiss 3T3 cells has been shown to be mediated through a pertussis toxin insensitive G protein (Zachary *et al.*, 1987b, Erusalimsky *et al.*, 1988, Fisher and Schonbrunn, 1988, Cattaneo and Vincentini, 1989, Erusalimsky and Rozengurt, 1989).

Until recently the identification of the pertussis toxin insensitive G protein involved in phosphoinositide hydrolysis was unknown. Several laboratories have independently made significant contributions leading to its identification. Using the polymerase chain reaction technique Strathmann and Simon obtained and sequenced cDNA clones encoding a number of previously uncharacterized α subunits of the Gq subfamily of G proteins (Strathmann and Simon, 1990). There are now known to be four distinct members ($G\alpha_q$,$G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{16}$) of the Gq subfamily (Table 1.2.2.). The amino acid sequences of $G\alpha_q$ and $G\alpha_{11}$ are 89% identical, whereas $G\alpha_{14}$ and Ga_{16} are more distantly related to $G\alpha_q$, with amino acid identities of 55-60 %. All of the Gq members lack the cysteine residue four amino acids from the carboxy terminus end that is the target for pertussis toxin-mediated ADP-ribosylation.

Pang and Sternweis purified a mixture of $G\alpha_q$ and $G\alpha_{11}$ from bovine brain with the use of an affinity matrix containing immobilised βγ-subunits (Pang and Sternweis, 1989) and subsequently it was demonstrated that these G proteins activated partially purified PI-PLC (Smrcka *et al.*, 1991). PI-PLC activation was observed only in the presence of AlF_4^-, and not with the nonhydrolyzable GTP analogue GTPγS. AlF_4^- is thought to act with the GDP-bound form of the α-subunit of a variety of heterotrimeric G proteins and stabilise them in the GTP-bound form (Bigay *et al.*, 1987) thus activating the Gα-subunit. Hence, these results imply that the purified $G\alpha_q$ and $G\alpha_{11}$ contained tightly associated GDP, which exchanges only very slowly with GTPγS. Concurrently, a mixture of $G\alpha_q$ and $G\alpha_{11}$ was purified on its ability to activate partially purified PI-PLC, from bovine liver membranes that had been incubated with GTPγS (Taylor *et al.*, 1990). When reconstituted in the presence of GTPγS with isozymes of PI-PLC, the mixture of $G\alpha_q$ and $G\alpha_{11}$ specifically activated PI-PLCβ1, but not PI-PLCγ1 and PI-PLCδ1 (Taylor *et al.*, 1990). Half maximal activation of PI-PLCβ1 required 4 μM GTPγS, again suggesting that these G proteins have a low affinity for GTP analogues. Subsequently, $G\alpha_q$ and $G\alpha_{11}$ were resolved, and both were shown to have PI-PLC stimulatory activity. In addition, a G protein isolated from turkey erythrocytes has also been shown to stimulate PI-

PLC (Waldo *et al.*, 1991). The absolute identity of the avian G protein remains to be determined, but it is immunologically related to $\alpha_{q/11}$.

Further experiments have demonstrated that members of the Gq family can interact with appropriate receptors and function as mediators in the stimulation of PI-PLC by hormones. Reconstitution of purified preparations of $G\alpha_q$ with isolated muscarinic receptors demonstrated that the M1 muscarinic acetylcholine receptors act via this protein (Berstein *et al.*, 1992). The participation of Gq in the regulation of PI-PLC by hormones was also demonstrated in native membranes by functional intervention with an antibody raised against the carboxyl terminal of $\alpha_{q/11}$. The stimulation of PtdIns(4,5)P_2 hydrolysis by a number of receptors including bradykinin, vasopressin and angiotensin-II in various membranes, was strikingly attenuated by the anti-$\alpha_{q/11}$ antibodies (Gutowski *et al.*, 1991). Using these and other techniques, a number of receptors have now been identified which activate PI-PLC via $G\alpha_q$ or $G\alpha_{11}$ (Table 1.3.2.). These results indicate that the Gq subfamily of G proteins is responsible for a broad spectrum of regulation of PI-PLC by G protein linked receptors. Interaction of these hormones with their specific receptors is thought to cause dissociation of the heterotrimeric GDP-bound Gq to yield GTP-bound $G\alpha_q$, which remains in the membrane. Membrane associated PI-PLCβ1 then binds the GTP-bound $G\alpha_q$, probably via the carboxyl-terminal regions of both proteins, which results in the activation of PI-PLCβ1.

The specificity of the interaction between different Gα subunits and PI-PLC has been further assessed by introducing cDNAs corresponding to various Gα subunits into COS-7 cells and measuring inositol phosphates formed after stimulation with AlF_4^- (Wu *et al.*, 1992b). Transfection with $G\alpha_q$ or $G\alpha_{11}$ cDNA resulted in a marked increase in inositol phosphate formation. Co-transfection of $G\alpha_q$ (or $G\alpha_{11}$) cDNA and PI-PLCβ1 cDNA resulted in even higher levels of inositol phosphate formation. The introduction of mutations (GLn-209 to Leu 209) that constitutively activate $G\alpha_q$ and $G\alpha_{11}$ resulted in persistent activation of PI-PLC and high basal levels of inositol phosphates. On the other hand, transfection with a variety of other Gα subunit cDNAs ($G\alpha_i$, $G\alpha_{oA}$, $G\alpha_{oB}$, transducin, and the constitutively activated Gln-205 to Leu mutants of $G\alpha_z$ and $G\alpha_{oA}$) did not increase inositol phosphate formation. These results are consistent with the conclusion that $G\alpha_q$ and $G\alpha_{11}$ cDNAs encode proteins that specifically activate PI-PLC.

The relative abilities of members of the Gq subfamily to activate PI-PLCβ isozymes have also been determined. $G\alpha_q$ purified from a βγ-affinity column was found to stimulate PI-PLCβ1 but not PI-PLCβ2 (Park *et al.*, 1992). More recently $G\alpha_q$ was shown to activate PI-PLCβ3 which was purified from rat brain particulate fraction (Jhon *et al.*, 1993). In addition it was found that $G\alpha_{16}$ (and less effectively, $G\alpha_q$. $G\alpha_{11}$, $G\alpha_{14}$) when expressed in COS-7 cells was most effective in reconstituting the PI-PLCβ2 (Lee *et al.*, 1992). Therefore there appears to be

Table 1.3.2.

G protein coupled receptors which have been identified to activate PI-PLC via the Gq family of G proteins.

Agonist	PLC isozyme	Reference
thromboxane A_2	?	Shenker *et al.*, 1991
Histamine	?	Gutowski *et al.*, 1991
Bradykinin	?	Gutowski *et al.*, 1991
Angiotensin II	?	Gutowski *et al.*, 1991
vasopressin	PI-PLCβ1?	Wange *et al.*, 1991
acetylcholine (M1 muscarinic receptor)	PI-PLCβ1	Berstein *et al.*, 1992
epinephrine (α1-Adrenergic receptor)	PI-PLCβ1	Wu *et al.*, 1992a
Thyrotropin-releasing hormone	PI-PLCβ1?	Aragay *et al.*, 1992
UK-14034 (α2-adrenoreceptor agonist)	?	Conklin *et al.*, 1991

specificity in the interaction of different members of the Gq subfamily with different PI-PLCβ effectors. This specificity may be important in generating tissue- or receptor-specific responses *in vivo*.

The α subunits of heterotrimeric G proteins have, for the most part, been thought of as the regulatory subunits of heterotrimeric G proteins because they bind and hydrolyse GTP and interact with receptors. The βγ-subunits have been viewed as regulatory components for α which stabilise the GDP-bound form of α. Yet the activation of G proteins yields two subunits (α and βγ) that could act on downstream targets. Recent evidence now supports the idea that free βγ can itself interact functionally with effector proteins. PI-PLC activity derived from HL60 cells was recently shown to be stimulated by preparations of βγ-subunits (Camps *et al.*, 1992b). The βγ subunits acted specifically on only one form of PI-PLC. This selectivity demonstrated that the stimulation was not simply a non-specific effect. βγ-subunits have also been shown to stimulate the activation of PI-PLCβ2 (Camps *et al.*, 1992a, Katz *et al.*, 1992, Park *et al.*, 1993) but not PI-PLCβ1. More recently, PI-PLCβ3 was shown to be stimulated by both α_q (Jhon *et al.*, 1993) and βγ-subunits (Carozzi *et al.*, 1993). These results indicate that PI-PLC isozymes may be regulated by both α and βγ-subunits. Interestingly, the ability of the βγ-subunits from pertussis toxin-sensitive G proteins (such as Go and Gi) to effect the activation of PI-PLC suggests a possible role of βγ-subunits in the pertussis toxin-sensitive stimulation of PI-PLC demonstrated by some hormones. More work is required to resolve roles of the specific subunits in the regulation of PI-PLC activity.

2. Regulation of PI-PLC-γ by tyrosine phosphorylation

The stimulation of PI-PLC by polypeptide growth factors including PDGF, appears to be independent of G proteins and to require the intrinsic tyrosine kinase activity of the receptors (see section 1.2.1). Treatment of a number of cell types with PDGF, EGF, or NGF leads to an increase in the phosphorylation of PI-PLCγ (but not PI-PLCβ1 or PI-PLCδ1) with the increased phosphorylation occurring on both serine and tyrosine residues (reviewed in Rhee and Choi, 1992). The phosphorylation of PI-PLCγ1 by the receptor tyrosine kinases of these growth factors occurs rapidly and correlates well with stimulation of PtdIns(4,5)P_2 hydrolysis. PI-PLCγ1 has been shown to undergo ligand-dependent association with both EGF and PDGF receptors (Wahl *et al.*, 1988, Margolis *et al.*, 1989, Meisenhelder *et al.*, 1989, Wahl *et al.*, 1989, Morrison *et al.*, 1990). Thus antibodies to either PI-PLCγ1 or growth factor receptors immunoprecipitates both proteins. The receptor PI-PLCγ1 association is mediated by a high affinity interaction between the SH2 domains of PI-PLCγ1 and specific tyrosine-autophosphorylated sites of the receptor (Koch *et al.*, 1991). Two autophosphorylation sites required for the interaction of PI-PLCγ1 with the PDGF receptor have recently been identified.

These sites are Tyr1009 and Tyr1021, and are found to be located in the C-terminal regions of the PDGF β-receptor (Ronnstrand *et al.*, 1992).

Association of growth factor receptors with PI-PLCγ1 precedes tyrosine phosphorylation of PI-PLCγ1 by the receptor tyrosine kinase. Both PDGF and EGF receptors appear to phosphorylate identical sites on PI-PLCγ1; Tyr-771, Tyr-783 and Tyr-1254 (Wahl *et al.*, 1990, Kim *et al.*, 1991). The role of tyrosine phosphorylation was investigated by substituting phenylalanine for Tyr at the three sites of PI-PLCγ1 and expressing the mutant enzymes in NIH3T3 cells (Kim *et al.*, 1991). Tyr-783 and to a lesser extent Tyr-1254, were shown to be essential for PDGF-stimulated inositol phosphate formation in intact cells. Like the wild-type enzyme, PI-PLCγ1 substituted for phenylalanine at Tyr-783 associated with and was phosphorylated at a serine residue in response to PDGF. These results suggest that phosphorylation of Tyr-783 is essential for PI-PLCγ1 activation and that neither the association of PI-PLCγ1 with the receptor nor its phosphorylation on serine residues is sufficient to account for PDGF-induced activation of PI-PLCγ1. In addition overexpression of PI-PLCγ1 was also shown to increase the tyrosine phosphorylation of these enzymes and inositol phosphate formation in response to PDGF (Sultzman *et al.*, 1991). These results provide direct evidence that the PDGF receptor stimulates the function of an intracellular signal-transducing protein by site-specific tyrosine phosphorylation.

Both phosphorylated and unphosphorylated PI-PLCγ1 are indistinguishable in their activities when measured *in vitro* (Kim *et al.*, 1991). However the unphosphorylated enzyme was found to be selectively inhibited in the presence of a micellar concentration of triton X-100 (Nishibe *et al.*, 1990) or in the presence of the small soluble actin-binding protein profilin, which also shows a high affinity for PtdIns(4,5)P_2 (Goldschmidt-Clermont *et al.*, 1991). Since only the phosphorylated enzyme catalyses the hydrolysis of profilin-bound PtdIns(4,5)P_2, thereby releasing profilin and altering actin polymerisation, profilin was suggested as the link between transmembrane signalling and cellular responses such as the changes in shape and increased motility induced by polypeptide growth factors (Goldschmidt-Clermont *et al.*, 1991).

The molecular events which occur after the tyrosine phosphorylation of PI-PLCγ1 are not well known. One hypothesis is the phosphorylated enzyme undergoes a conformational change that allows it to bind to the membrane cytoskeleton where its catalytic domains come into contact with the cell membrane. This hypothesis is supported by the observation that EGF and PDGF treatment of cells induces the translocation of PI-PLCγ1 from a predominantly cytosolic localisation to membrane fractions (Todderud *et al.*, 1990).

Other receptors with tyrosine kinase activity (e.g. insulin and CSF-1) fail to phosphorylate PI-PLCγ1 , indicating that all receptor tyrosine kinases do not necessarily target PI-PLCγ1 as a

substrate. In addition, it should be noted that neither EGF nor insulin cause inositol phospholipid breakdown in Swiss 3T3 cells and thus signal mitogenesis by a mechanism independent of PI-PLC in these cells.

3 . Regulation of PI-PLC by Protein kinase C

Activation of protein kinase C (PKC) (see section 1.3.3.) can also distinguish between the G protein and tyrosine kinase pathways leading to phosphoinositide hydrolysis. Phorbol esters (which directly activate PKC) inhibit inositol phosphate production induced by the neuropeptides, including bombesin and vasopressin (Brown *et al.*, 1987, Lopez-Rivas *et al.*, 1987, Brown *et al.*, 1990, Ryu *et al.*, 1990) but do not affect inositol formation in response to PDGF (Lopez-Rivas *et al.*, 1987). Accordingly, down regulation of PKC by prolonged treatment with phorbol esters (see section 1.3.3.) enhances the formation of inositol phosphates by the neuropeptides but not by PDGF (Brown *et al.*, 1990). Thus, PKC acts as a feed-back regulator. A potential site for PKC-mediated phosphorylation is PI-PLCβ (Ryu *et al.*, 1990). While phosphorylation of PI-PLCβ1 *in vitro* did not affect the activity of the enzyme, it was postulated that the modification might uncouple the phospholipase from the regulatory G protein. However evidence for this theory has not yet been demonstrated.

1.3.2. ION FLUXES

One of the earliest events to occur after the binding of most mitogens to their receptors is an increase in the fluxes of Na^+, K^+ and H^+ across the plasma membrane. (for review see Rozengurt and Mendoza, 1986). Translocation of Na^+ across the plasma membrane is mediated in part, by an amiloride-sensitive electroneutral Na^+/H^+ antiport system which is driven by the electrochemical gradient across the plasma membrane (Roos and Boron, 1981, Boron, 1983). Since the activity of the Na^+/K^+ pump in intact fibroblasts is limited by cytosolic Na^+ (Smith and Rozengurt, 1978, Mendoza *et al.*, 1980), the increase in Na^+ entry triggers a secondary stimulation of Na^+/K^+ pump activity which restores the electrochemical gradient for Na^+ by increasing intracellular K^+ concentration.

Studies to determine the mechanisms by which growth factors stimulate these monovalent ion fluxes in Swiss 3T3 cells have shown the existence of protein kinase C (PKC)-dependent and independent pathways of activation (Vara and Rozengurt, 1985, Vara *et al.*, 1985, Mendoza *et al.*, 1986b) and these will be discussed further in section 1.3.3.

In addition to stimulation of rapid monovalent ion fluxes, PDGF and other mitogens markedly stimulate Ca^{2+} efflux from radiolabelled quiescent cultures of Swiss 3T3 cells (Lopez-Rivas and Rozengurt, 1983). This effect is extremely rapid and may be observed as little as 15 seconds after addition of growth factors. Purified growth factors including PDGF, bombesin and vasopressin have also been found to stimulate efflux of Ca^{2+} from preloaded Swiss 3T3 cells (Owen and Villereal, 1983, Berridge *et al.*, 1984, Lopez-Rivas and Rozengurt, 1984, Mendoza *et al.*, 1986b). The observation that the stimulation of $^{45}Ca^{2+}$ efflux can be elicited in the absence of extracellular Ca^{2+}, suggested that these growth factors must release this cation from an intracellular store(s). Indeed, PDGF, bombesin and vasopressin cause a net decrease in cellular Ca^{2+} concentration. These results suggested that Ca^{2+} released from intracellular stores may give rise to a transient increase in cytosolic Ca^{2+} concentration before removal from the cell via a Ca^{2+}-ATPase (Moolenaar *et al.*, 1983). This was borne out when it was shown that PDGF, vasopressin and bombesin caused a rapid increase in fluorescence in quiescent Swiss 3T3 cells prelabelled with the dye quin 2 (Morris *et al.*, 1984, Mendoza *et al.*, 1986b).

The mobilization of Ca^{2+} by these mitogens is mediated by inositol $Ins(1,4,5)P_3$ which acts as a second messenger in the action of many ligands that stimulate inositol lipid turnover and Ca^{2+} efflux (Berridge and Irvine, 1984) including PDGF, bombesin and vasopressin (Berridge *et al.*, 1984, Brown *et al.*, 1984, Hasegawa-Sasaki, 1985, Heslop *et al.*, 1986, Takuwa *et al.*, 1987). Indeed $Ins(1,4,5)P_3$ releases Ca^{2+} from permeabilized Swiss 3T3 cells (Berridge *et al.*, 1984). Both vasopressin and bombesin rapidly increase $Ins(1,4,5)P_3$ simultaneously with mobilization of Ca^{2+} from intracellular stores. $Ins(1,4,5,)P_3$ is formed as a result of PI-PLC-catalysed hydrolysis of $PtdIns(4,5)P_2$ in the plasma membrane (see section 1.3.1.). The Ca^{2+}-mobilizing action of bombesin can be distinguished from that of PDGF in terms of kinetics and sensitivity to PKC-mediated feedback inhibition (Lopez-Rivas *et al*, 1987, Nanberg and Rozengurt, 1988). The increase in cytoplasmic Ca^{2+} concentration in response to PDGF occurs after a 15 second lag period and precedes the increase in $Ins(1,4,5)P_3$ formation (Lopez-Rivas *et al.*, 1987, Hasegawa-Sasaki *et al.*, 1988, Nanberg and Rozengurt, 1988). PDGF is known to stimulate inositol phosphate production in fibroblasts through the γ isoform of PI-PLC (Cuadrado and Molloy, 1990, Majerus *et al.*, 1990). The activated PDGF receptor has been shown to directly phosphorylate PI-PLCγ whereas bombesin has no effect on the tyrosine phosphorylation of this enzyme (Cuadrado and Molloy, 1990, Majerus *et al.*, 1990). The neuropeptides, including bombesin and vasopressin stimulate phosphoinositide hydrolysis via G protein coupled receptors and activate the β-isoform of PI-PLC (for review see Sternweiss and Smrcka, 1992 and section 1.3.1.). It is likely that the differences in the effects of these mitogens on Ca^{2+} mobilization reflect these fundamental differences in signal transduction. It should be noted that, neither EGF, insulin nor elevators of cAMP acutely

mobilise Ca^{2+} in Swiss 3T3 cells. These growth factors do not stimulate phosphoinositide hydrolysis in these cells.

1.3.3. ACTIVATION OF PROTEIN KINASE C

Protein kinase C (PKC) was first identified as a cyclic nucleotide-independent phosphotransferase which could be activated by proteolysis (Inoue *et al.*, 1977). This enzyme is activated by diacylglycerols and phorbol esters and comprises a family of enzymes (Nishizuka, 1988). The current family numbers seven genes (α-η). The enzymes encoded by these genes can be broadly divided into two classes; the Ca^{2+} dependent forms (α, β and γ) and those that probably do not require Ca^{2+} for activation (δ, ε, ζ, η). PKC has been implicated in the signal transduction of many short-term cellular responses, including secretion and contraction (Kikkawa and Nishizuka, 1986). More interestingly, PKC has been implicated in mediating long-term responses. Phorbol esters are not only potent tumour promoters (Hecker, 1978, Slaga *et al.*, 1982) but can also stimulate DNA synthesis and cell division in quiescent Swiss 3T3 cells in synergistic combination with insulin (Dicker and Rozengurt, 1978, Dicker and Rozengurt, 1980, Collins and Rozengurt, 1982a). The mitogenic effect is mediated by high-affinity binding sites (Collins and Rozengurt, 1982a) which were identified as PKC (Kikkawa and Nishizuka, 1986, Nishizuka, 1988). Furthermore, an important link between PKC activation and cell growth was provided by the finding that addition of the synthetic diacylglycerol 1-oleoyl-2-acetylglycerol (OAG) mimics the action of phorbol esters in stimulating re-initiation of DNA synthesis and cell division (Rozengurt *et al.*, 1984).

Another approach to testing the role of PKC in the production of biological responses is to exploit the selective removal of this enzyme caused by a prolonged pretreatment of the cells with phorbol esters. Chronic exposure to phorbol esters leads to the disappearance of measurable PKC activity in cell-free preparations (Rodriguez-Pena and Rozengurt, 1984). In parallel with this down-regulation of PKC activity, the cells become desensitized to the mitogenic effects elicited by phorbol esters or OAG (Collins and Rozengurt, 1984, Rozengurt *et al.*, 1984).

Identification of the acidic 80 kDa PKC substrate

Since activation of PKC may play a role in eliciting mitogenesis, it was of importance to test directly whether growth factors lead to activation of this enzyme in intact, quiescent cells. In 1983, Rozengurt and colleagues observed that addition of phorbol esters to quiescent

cultures of Swiss 3T3 cells causes a rapid phosphorylation of a M_r = 80,000 (80K) cellular protein (Rozengurt *et al.*, 1983b). This phosphorylation event is also induced by synthetic DAG analogues such as 1-oleoyl-2-acetylglycerol (OAG), exogenous treatment of the cells with PI-PLC from *Clostridium perfingens* (Kent, 1979, Sleight and Kent, 1983) and by PDGF (Habenicht *et al.*, 1981, Rozengurt *et al.*, 1983b). However neither insulin, EGF or elevators of cAMP induce the phosphorylation of this protein in Swiss 3T3 cells (Rozengurt *et al.*, 1983b, Vara *et al.*, 1985, Escribano and Rozengurt, 1988). Down-regulation of PKC by prolonged treatment with phorbol esters prevents the increase in phosphorylation of 80K by subsequent addition of either phorbol ester, PI-PLC or OAG (Rozengurt *et al.*, 1983b, Rozengurt *et al.*, 1984, Blackshear *et al.*, 1985). Furthermore, phosphopeptide analysis demonstrates that the same 80K phosphoprotein is generated *in vitro* by addition of partially purified PKC in the presence of Ca^{2+}, phosphatidylserine and PBt_2 (Rodriguez-Pena and Rozengurt, 1986). This prominent PKC substrate was purified to homogeneity from heat stable extracts of Swiss 3T3 cells (Brooks *et al.*, 1990). The preparation is an effective substrate of PKC and contains an unusually high proportion of acidic amino acids and of alanine. Recently, the cDNAs encoding the substrates from rat brain (Erusalimsky *et al.*, 1991), Swiss 3T3 cells (Brooks *et al.*, 1991), murine macrophage (Seykora *et al.*, 1991) and human brain (Harlan *et al.*, 1991) have been cloned. Comparison of the rodent sequences with those of the PKC substrates from human and bovine brain (termed Myristoylated Alanine-Rich C Kinase Substrate or MARCKS) revealed considerable sequence divergence, suggesting either a low level of evolutionary pressure or the existence of a gene family. These PKC substrates do not exhibit significant homology to other cellular proteins. Recently it was shown that rodent 80K and bovine and human MARCKS are not distinct members of a gene family, but represent the equivalent substrates in different species (Hergert, *et al.*, 1992). Together these results demonstrate that a rapid increase in the phosphorylation of the 80 kDa protein (now called 80K/MARCKS) specifically reflects activation of PKC in cultured Swiss 3T3 cells.

Dialysed serum and the neuropeptides bombesin and vasopressin also stimulate the phosphorylation of 80K/MARCKS in intact Swiss 3T3 cells (Rodriguez-Pena and Rozengurt, 1985, Rodriguez-Pena and Rozengurt, 1986, Zachary *et al.*, 1986). Phosphorylation of this protein is rapidly reversed upon removal of ligand, with a half-life of approximately 2 minutes (Rodriguez-Pena *et al.*, 1986). The 80K/MARCKS phosphoproteins generated in response to phorbol esters, bombesin and vasopressin were identical as judged by peptide mapping after partial proteolysis (Rodriguez-Pena and Rozengurt, 1986, Zachary *et al.*, 1986). Phosphorylation of 80K/MARCKS by bombesin and vasopressin is abolished in PKC-deficient Swiss 3T3 cells and by selective bombesin and vasopressin antagonists respectively (Rodriguez-Pena and Rozengurt, 1986, Zachary *et al.*, 1986). In addition, bombesin promotes PKC translocation as judged by its ability to induce tight association of PKC to

cellular membranes (Staddon *et al.*, 1990). These results strongly suggest that bombesin rapidly stimulates PKC activity in intact and quiescent Swiss 3T3 cells.

Significantly, down-regulation of PKC prevents not only the increase in 80K/MARCKS phosphorylation but also the stimulation of DNA synthesis induced by bombesin and vasopressin (Collins and Rozengurt, 1984, Rozengurt and Sinnett-Smith, 1987). The mitogenic response to bombesin, can be restored in the presence of high concentrations of insulin, suggesting that bombesin induces other signalling pathways not dependent on PKC activation. These findings indicate that PKC plays a central role in signal transduction by these peptides.

Phorbol esters, bombesin and vasopressin have also been shown to phosphorylate 80K/MARCKS in digitonin-permeabilized Swiss 3T3 cells (Erusalimsky *et al.*, 1988, Erusalimsky and Rozengurt, 1989). Evidence for the role of G proteins in signal transduction of specific pathways can be obtained by assessing the effects of guanine nucleotide analogues on receptor-mediated responses in permeabilized cells. Non-hydrolysable analogues of guanine nucleotides modulate 80K/MARCKS phosphorylation in response to bombesin and vasopressin but not to phorbol esters (Erusalimsky *et al.*, 1988, Erusalimsky and Rozengurt, 1989). These results suggested that both bombesin and vasopressin activate PKC through a receptor-mediated G protein linked signalling pathway. This G protein(s) was also shown to be insensitive to pertussis toxin (Zachary *et al.*, 1987a, Erusalimsky and Rozengurt, 1989).

It was recently shown that activation of PKC by phorbol esters causes a dramatic down-regulation of the expression of 80K/MARCKS mRNA and protein from Swiss 3T3 cells through a post-transcriptional mechanism (Brooks *et al.*, 1991). Stimulation of Swiss 3T3 fibroblasts with bombesin and related peptides has also been shown to cause a striking down-regulation of 80K/MARCKS mRNA (Brooks *et al.*, 1992). The down-regulation induced by bombesin could be distinguished from that induced by PBt_2 in several important aspects at both mRNA and protein levels. PBt_2 treatment results in a transient down-regulation of 80K/MARCKS mRNA with complete recovery to control levels within 48 h, correlating with the loss of PKC activity. The down-regulation of 80K/MARCKS mRNA induced by bombesin was more persistent and only partial recovery was observed after prolonged treatment. PDGF also caused a marked down-regulation of 80K/MARCKS mRNA (Brooks *et al.*, 1992). However at high concentrations of PDGF, the down-regulation of 80K/MARCKS was induced in a PKC-independent manner. PDGF is known to stimulate cAMP accumulation via an indirect pathway involving arachidonic acid metabolism (Rozengurt *et al.*, 1983c). The ability of cAMP-elevating agents such as forskolin, cholera toxin and NECA to down-regulate 80K/MARCKS mRNA (Brooks *et al.*, 1992) lead to the assumption that the action of PDGF

was through this pathway. Furthermore, down-regulation of PKC, which blocks the cellular responses to agents which act through PKC, does not prevent the induction of mitogenesis (Collins and Rozengurt, 1984) or the down regulation of 80K/MARCKS mRNA in response to these cAMP-elevating agents. Thus the ability of these agents to deplete 80K/MARCKS mRNA levels can be attributed to their ability to elevate cAMP. Interactions between the cAMP and PKC signalling pathways have previously been demonstrated. Specifically, activation of PKC by phorbol esters or bombesin has been shown to enhance the accumulation of cAMP in response to a variety of agents, possibly through a pertussis toxin-sensitive G protein (Rozengurt, *et al.*, 1987, Millar and Rozengurt, 1988). The ability of cAMP elevating agents to mediate the regulation of the expression of a prominent substrate of PKC suggest a novel mechanism of 'cross-talk' between these two major signalling pathways. More importantly, the ability of a variety of mitogens, acting through distinct signalling pathway to dramatically decrease the expression of 80K/MARCKS suggests that 80K/MARCKS may play a suppressor role in the control of cell proliferation. In addition, 80K/MARCKS was recently shown to be a calmodulin-(Graff *et al.*, 1989, McIlroy *et al.*, 1991) and actin-binding protein (Thelen *et al.*, 1991). Together these findings are important as they have implications for the coordination of cellular signalling pathways involving PKC, Ca^{2+}, and cytoskeletal organisation.

PKC may also provide an important molecular link in the sequence of events following growth factor binding to their respective receptors (Rozengurt *et al.*, 1985). In accord with this, activation of PKC, either directly with phorbol esters or indirectly by vasopressin or bombesin, leads to an increased activity of the Na^+/H^+ antiport system, which is profoundly attenuated in cells chronically exposed to phorbol esters (Mendoza *et al.*, 1980, Vara and Rozengurt, 1985, Vara *et al.*, 1985, Mendoza *et al.*, 1986b). However, the Na^+/H^+ antiport system is activated by growth factors that do not activate PKC, indicating that monovalent ion fluxes may be triggered by multiple mechanisms (Vara and Rozengurt, 1985). In addition, [^{125}I]EGF binding to specific surface receptors in Swiss 3T3 cells is markedly inhibited by bombesin and other growth factors (reviewed in Zachary and Rozengurt, 1985b). The effect is rapid in onset and results from a decrease in the apparent affinity of the EGF receptor population for EGF. Considerable evidence implicates PKC in the regulation of EGF receptor affinity by bombesin and other transmodulating agents. In particular, the inhibition of EGF binding induced by either PBt_2, diacylglycerol or bombesin is prevented by down-regulation of PKC (Sinnett-Smith and Rozengurt, 1985, Zachary and Rozengurt, 1985b, Schlessinger, 1986, Zachary, *et al.*, 1986). The EGF receptor is phosphorylated by PKC at specific sites both in vitro and in vivo (Sinnett-Smith and Rozengurt, 1985, Zachary and Rozengurt, 1985b, Zachary, *et al.*, 1986). Thus, transmodulation of the EGF receptor may result from the covalent modification of the EGF receptor catalysed by PKC, though other mechanisms are not excluded.

1.3.4. TYROSINE PHOSPHORYLATION BY G PROTEIN COUPLED RECEPTORS

The association of tyrosine kinase activity with several growth factor receptors has been discussed in detail in section 1.2.1. Classically, tyrosine phosphorylation by polypeptide growth factors and the generation of second messengers via neuropeptide receptors linked to G proteins have been regarded as separate though complementary pathways. It has recently been demonstrated that bombesin, vasopressin and endothelin stimulate a rapid increase in tyrosine phosphorylation of a group of proteins of 110-130 kDa (Zachary *et al.*, 1991) as well as minor components of 90 kDa and 70-80 kDa. Initial studies showed that these peptides do not stimulate tyrosine phosphorylation of PI-PLCγ1, PI3K or GAP, some of the known substrates for receptor tyrosine kinases (section 1.2.1).

Recently one of the substrates of neuropeptide stimulated tyrosine phosphorylation in Swiss 3T3 cells was identified as focal adhesion kinase ($p125^{FAK}$) which is itself a protein tyrosine kinase (Zachary *et al.*, 1992). The striking rapidity of neuropeptide-stimulated phosphorylation (detectable within seconds) is consistent with a pathway leading directly from neuropeptide receptors to $p125^{FAK}$. $p125^{FAK}$ has also been shown to be tyrosine phosphorylated in cells transformed by activated variants of the oncogene src ($pp60^{src}$) (Schaller *et al.*, 1992) and on activation of integrins (adhesive receptor proteins involved in cell adhesion to extracellular matrix proteins)(Guan and Shalloway, 1992). In addition to $p125^{FAK}$, tyrosine phosphorylation of other proteins such as p130 and paxillin have been identified as substrates for neuropeptide-stimulated phosphorylation (Zachary *et al.*, 1992). These proteins are also phosphorylated in src transformed cells or by the activation of integrins, and are focal adhesions associated proteins (reviewed in Zachary and Rozengurt, 1992).

These results have important implications for signal transduction and cell regulation. Most obviously they suggest that tyrosine phosphorylation of a novel type of tyrosine kinase is a point of convergence in the action of integrins oncogenes and neuropeptides and that the signal transduction pathways initiated by these diverse groups of molecules have, at least in part, similar consequences for cell function. More importantly, these results suggest the existence of an additional novel signalling pathway in the mitogenic response to these agents. The observation that inhibitors of tyrosine kinases can inhibit bombesin stimulation of DNA synthesis in Swiss 3T3 cells (Seckl and Rozengurt, 1993) supports these results.

1.3.5. CYCLIC AMP AND CELL GROWTH

The possibility that cyclic adenosine monophosphate (cAMP), the first identified second messenger for hormone action, may also modulate growth processes has been the subject of a long and controversial literature (Rozengurt, 1981). It is now recognised that a sustained increase in the cellular level of cAMP constitutes a growth promoting signal for Swiss 3T3 cells (Rozengurt *et al.*, 1981c, Rozengurt, 1982b, Rozengurt, 1982a, Rozengurt *et al.*, 1983c, Wang and Rozengurt, 1983).

A number of agents including cholera toxin, adenosine agonists including the adenosine agonist 5'-N-ethylcarboxamideadenosine (NECA), prostaglandins of the E series (PGE_1), forskolin and permeable cAMP analogues, stimulate adenylate cyclase. These agents elevate cAMP levels and stimulate DNA synthesis in Swiss 3T3 cells when added in combination with either insulin, phorbol esters, vasopressin, EGF or serum in an identical dose-dependent manner (Rozengurt *et al.*, 1981c, Rozengurt, 1982a, Rozengurt, 1982b, Rozengurt *et al.*, 1983a, Wang and Rozengurt, 1983).

Furthermore, PDGF induces a striking accumulation of cAMP in confluent and quiescent cultures of Swiss 3T3 cells in the presence of inhibitors of cAMP degradation (Rozengurt *et al.*, 1983c). PDGF releases arachidonic acid (Habenicht *et al.*, 1981), which can, in part, be metabolised by cyclooxygenase to E type prostaglandins, which in turn leave the cell and stimulate cAMP synthesis through their own receptor (Rozengurt *et al.*, 1983a). Indomethacin, a potent cyclooxygenase inhibitor, substantially inhibits both the release of E type prostaglandins, enhancement of cAMP accumulation by PDGF and to shift the dose-response for mitogenic stimulation by PDGF to the right (Rozengurt *et al.*, 1983c). These results suggest that cAMP is one of the second messengers employed by PDGF to stimulate DNA synthesis in Swiss 3T3 cells. Bombesin has also been recently shown to stimulate arachidonic acid release and consequently enhance cAMP levels in Swiss 3T3 cells (Millar and Rozengurt, 1988, Millar and Rozengurt, 1990), constituting a mitogenic signal for this neuropeptide. However, other arachidonic acid metabolites may also play a role in mitogenic signal transduction by bombesin. In contrast to PDGF and bombesin, other mitogenic agents including insulin, EGF, phorbol esters and vasopressin do not increase cellular levels of cAMP. Taken together these findings imply that cAMP represents a signalling pathway that is distinct from that utilised by agents that only activate PKC or induce tyrosine phosphorylation.

Activation of PKC and synthesis of cAMP are not, however, independent events. As discussed in section 1.3.3., the existence of interactions between these signalling systems has been demonstrated. Specifically, it has been demonstrated that, in Swiss 3T3 cells, phorbol esters and other activators of PKC, including bombesin and vasopressin, potentiate

the increase in cAMP levels caused by cAMP elevating agents such as cholera toxin or forskolin (Rozengurt *et al.*, 1987). Although the mechanism(s) underlying this effect are not known, pertussis toxin, which ADP-ribosylates and inactivates a certain class of guanine nucleotide binding protein (reviewed in Ui, 1990), abolishes the enhancing effect of PKC activation on cAMP accumulation in Swiss 3T3 cells (Rozengurt *et al.*, 1987). Since pertussis toxin does not itself promote cAMP accumulation in Swiss 3T3 cells (Rozengurt *et al.*, 1987), it is possible that a pertussis toxin substrate mediates the 'cross-talk' between the PKC and cAMP pathways.

1.3.6. EARLY PROTOONCOGENE EXPRESSION

In addition to rapid events in the membrane and cytosol, serum and growth factors rapidly and transiently induce the expression of the protooncogenes c-fos and c-myc (Reviewed in Rozengurt and Sinnett-Smith, 1988). Protooncogenes represent the cellular counterparts of transforming DNA sequences initially isolated from acutely transforming retroviruses. Thus, c-fos and c-myc are the normal cellular homologues of the transforming genes of the FBJ osteosarcoma (fos) and avian myelocytomatosis (myc) viruses, respectively. Since these cellular genes are highly conserved during evolution and because many protooncogenes are expressed in developing embryonic tissues, it is widely thought that they may play a role in the regulation of normal growth and differentiation. Indeed, expression of a transfected myc gene (Armelin *et al.*, 1984, Mougneau *et al.*, 1984, Sorrentino *et al.*, 1986) or microinjection of the c-myc protein (Kaczmarek *et al.*, 1985) into quiescent 3T3 cells stimulates entry into DNA synthesis in the presence of other growth factors (reviewed in Cole, 1986). Similarly, expression of fos antisense RNA (Nishikura and Murray, 1987) or microinjection of fos-specific antibodies (Riabowol *et al.*, 1988) blocks DNA synthesis in fibroblasts stimulated by serum.

Both c-fos and c-myc are thought to play a role in the regulation of gene expression. The demonstration that the product of the protooncogene c-jun, identified as the trans-acting factor AP-1, forms a tight complex (dimer) with fos protein is consistent with a role for c-fos in the regulation of gene transcription (reviewed in Curran, 1988). Dimerization is mediated by the leucine repeat (or leucine zipper) with two α-helices forming a coiled coil structure. In the heterodimer form both fos and jun contribute to transcriptional activation. While jun-jun homodimers are also able bind DNA, fos-fos dimers are not stable and have no DNA-binding activity (reviewed in Karin and Smeal, 1992). These dimers with DNA-binding abilities are thought to contribute to AP-1 activity and participate to varying extents in its regulation by extracellular stimuli (Karin and Smeal, 1992). The transcriptional regulation of the c-fos gene itself is complex. The c-fos promoter contains several upstream enhancer elements which

bind sequence-specific protein factors and thereby control transcription of the gene (Treisman, 1986, Curran, 1988, Prywes *et al.*, 1988, Sassone-Corsi et al., 1988a). Using in vitro assays, a number of cis-acting DNA sequences have been identified which stimulate c-fos expression in a signal-specific manner. In different cell types, the serum response element (SRE) is required for the induction of the c-fos gene by serum, EGF, PDGF or insulin (Treisman, 1986, Prywes *et al.*, 1988). Similarly, the TRE, also known as the AP-1 consensus sequence, is required for c-fos induction by phorbol esters, presumably acting through a PKC-dependent pathway, whereas the cyclic-AMP-responsive element (CRE) controls the transcription of c-fos induced by cAMP (Sassone-Corsi et al., 1988a). Transcription expression of c-fos appears to be negatively autoregulated and the phosphorylation of c-fos protein (c-Fos) in its carboxyl-region is implicated in converting c-Fos to a repressor of its own expression (Sassone-Corsi, *et al.*, 1988b, Offir, *et al.*, 1990). The target genes regulated by fos still remain to be identified.

Recent evidence also strongly suggests that the c-myc protein (c-Myc) is a transcription factor (Blackwood and Eisenman, 1991). It possesses a number of functional domains found in other proteins modulating transcription, specifically the leucine zipper characteristic of the fos-jun-CREB transcription families (Landschulz *et al.*, 1988) and the basic helix loop helix motif found in enhancer binding proteins (Murre *et al.*, 1989). Recently both a heterodimeric partner called Max (Blackwood and Eisenman, 1991) and a consensus DNA-binding sequence for Myc (Blackwell *et al.*, 1990, Prendergast and Ziff, 1991) have been identified. It has also been demonstrated that the oncogenic activity of the c-myc protein requires dimerization with Max (Amati *et al.*, 1993). Transcriptional expression of c-myc has been shown to be autoregulated by c-myc protein (c-Myc) but also requires additional trans-acting factors (Penn, *et al.*, 1990). These observations also support a role for c-myc in the regulation of cellular gene transcription. However, it is still unknown precisely which genes are regulated by myc.

As stated previously, serum and growth factors transiently induce the expression of c-fos and c-myc. c-fos mRNA is detectable as early as 10 minutes after stimulation of quiescent fibroblasts with serum or polypeptide growth factors, such as PDGF or EGF, and is maximally increased after 30 minutes (Cochran *et al.*, 1984, Greenberg and Ziff, 1984, Kruijer *et al.*, 1984, Muller *et al.*, 1984, Curran, 1988). In contrast c-myc is detectable within 30 minutes and reaches a maximum after 3 hours stimulation (Kelly *et al.*, 1983,Muller *et al.*, 1984). Bombesin also induces c-fos and c-myc expression with similar kinetics (Letterio *et al.*, 1986, Palumbo *et al.*, 1986, Bravo *et al.*, 1987a, Rozengurt and Sinnett-Smith, 1987). Since both genes encode nuclear proteins it was reasoned that their transient expression in early G_1 may play a role in the transduction of the mitogenic signal in the nucleus (Abrams *et al.*, 1982, Alitalo *et al.*, 1983, Persson and Leder, 1984, Curran, 1988).

Crucially, the increase in c-fos and c-myc mRNA levels is not prevented by inhibitors of protein synthesis (Kelly *et al.*, 1983, Greenberg *et al.*, 1986). In fact these genes are overexpressed when growth factors are added together with drugs such as cycloheximide or anisomycin (Kelly *et al.*, 1983, Greenberg *et al.*, 1986). These results indicate that the increase in c-fos and c-myc mRNA levels is not secondary to the growth response and suggests that in quiescent cells regulatory factors are present and poised to respond to environmental stimuli.

Identification of early signalling events involved in the induction of c-fos and c-myc

Since expression of c-fos and c-myc represent some of the earliest nuclear events after growth factor action, the causal relationship between early events in the membrane and cytosol and protooncogene expression, has been under intense investigation. Addition of phorbol esters to quiescent cells also causes a marked increase in c-fos and c-myc mRNA levels (Kelly *et al.*, 1983, Coughlin *et al.*, 1985, Kaibuchi *et al.*, 1986, Bravo *et al.*, 1987a, Bravo *et al.*, 1987b). The possibility that activation of PKC leads to protooncogene expression is supported by the findings that this effect is mimicked by OAG and exogenous PI-PLC in quiescent cells (Bravo *et al.*, 1987b), and that down-regulation of PKC by prolonged exposure to phorbol esters (section 1.3.3.) abolishes phorbol ester-mediated induction of these genes (Coughlin *et al.*, 1985, Kaibuchi *et al.*, 1986, Rozengurt and Sinnett-Smith, 1987). Down-regulation of PKC markedly decreases but does not abolish induction of c-fos and c-myc by bombesin (Bravo *et al.*, 1987a, McCaffrey *et al.*, 1987, Rozengurt and Sinnett-Smith, 1987). Since prolonged treatment with phorbol esters does not alter the ability of bombesin to bind to the bombesin receptor, these results imply that PKC is involved in bombesin-induced protooncogene expression. Vasopressin also induces c-fos expression in Swiss 3T3 cells by a PKC dependent pathway (Rozengurt and Sinnett-Smith, 1988). Significantly, bombesin synergistically stimulates DNA synthesis with insulin in PKC deficient Swiss 3T3 cells without affecting induction of c-fos (Mehmet et al., 1988). This implies that the large transient expression of this protooncogenes may be dissociated from mitogenic stimulation in Swiss 3T3 cells. However, it should be pointed out that c-fos is a member of an expanding family of structurally related nuclear proteins (Curran, 1988). Therefore it cannot be excluded that other fos-related proteins (e.g. fosB, fra-1) may substitute for c-fos in these situations.

The direct activation of PKC by phorbol esters at saturating concentrations induces c-fos and c-myc to only a fraction of the level induced by bombesin or PDGF alone (Rozengurt and Sinnett-Smith, 1988). Similarly, vasopressin is unable to evoke a maximal increase c-fos

mRNA levels (Rozengurt and Sinnett-Smith, 1988). This suggests that bombesin and PDGF induce additional pathways leading to c-fos expression in addition to activation of PKC. The calcium ionophore A23187 does not activate PKC, as judged by 80K/MARCKS phosphorylation, or induction of c-fos alone but greatly potentiates induction of c-fos by phorbol esters (Rozengurt and Sinnett-Smith, 1988). Since bombesin and PDGF mobilise intracellular calcium , it is plausible that an increase in intracellular calcium acts in synergy with an activation of PKC to induce c-fos. It should be noted that phorbol esters do not induce calcium mobilisation in Swiss 3T3 cells (Hesketh *et al.*, 1985, Rozengurt and Mendoza, 1985, Mendoza *et al.*, 1986a). In addition, it has recently been demonstrated that stimulation of 3T3 cells with bombesin, but not vasopressin, induces a marked release of arachidonic acid into the medium (Millar and Rozengurt, 1990). PDGF also stimulates arachidonic acid release in these cells (Mehmet *et al.*, 1990c). Arachidonic acid released by bombesin is converted into E-type prostaglandins which can enhance cAMP levels in the cell (see section 1.3.7). Recent evidence suggests that although agents that elevate intracellular cAMP do not induce c-fos expression alone (Mehmet *et al.*, 1988), they may act in synergy with either activators of PKC or agents that increase intracellular Ca^{2+} to induce the expression of c-fos (Rozengurt and Sinnett-Smith, 1988, Mehmet *et al.*, 1990b). Hence, it is likely that the induction of c-fos by PDGF and bombesin is mediated by the co-ordinated effects of PKC activation, Ca^{2+}- mobilisation and a pathway involving arachidonic acid release (Rozengurt and Sinnett-Smith, 1987, Mehmet et al., 1990b).

Furthermore, EGF stimulates c-fos expression in Swiss 3T3 cells, albeit to a lesser extent than that stimulated by bombesin. However, EGF does not stimulate PKC, mobilise intracellular Ca^{2+} or cAMP levels in these cells suggesting that additional pathways leading to c-fos expression exist. This proposal is supported by the finding that induction of c-fos by EGF is not affected by down-regulation of PKC and that EGF can synergise with activators of PKC, agents that increase intracellular Ca^{2+} or elevators of cAMP (Rozengurt and Sinnett-Smith, 1988, Mehmet *et al.*, 1990b). Recently it has been shown that AP1 activity is elevated in cells that express a variety of transforming oncogenes whose gene products act as constitutively activated intermediates in the signal transduction pathway that transmits information from receptor tyrosine kinases (e.g. EGF receptor) to the nucleus. Such oncogene products include v-src, Ha-Ras, and v-raf (Herrlich and Ponta, 1989). Binding of EGF to its receptor results in a series of events that lead to increased ras activity which appears to play a major role in the activation of downstream serine/threonine-specific protein kinases, such as Raf-1 and MAP kinase (Satoh *et al.*, 1992). The activated MAP kinase directly phosphorylates transcription factor regulators which results in the increased expression of c-fos (Treisman, 1992). Thus pathways through ras activation may be responsible for the induction of c-fos by EGF. It should be noted that PKC activation also leads to the activation these kinases.

The relationship between early signalling events and the induction of c-myc in Swiss 3T3 cells are not as well understood. However agents that elevate cAMP, activators of PKC, the calcium ionophore A23187 and EGF can individually induce c-myc expression to varying degrees in Swiss 3T3 cells (Yamashita *et al.*, 1986, McCaffrey *et al.*, 1987, Mehmet *et al.*, 1988).

Together these results suggest that protooncogenes, in particular c-fos, may be induced by multiple distinct synergistically acting early signalling pathways. The ability of serum, bombesin and PDGF to initiate multiple signalling pathways (Rozengurt, 1986) is consistent with the observation that these agents are potent inducers of c-fos and c-myc (Rozengurt and Sinnett-Smith, 1988). Thus although c-fos and c-myc expression appear not to be critical for initiation of a proliferative response, they may provide an early convergence point for synergistically acting early signals for signal transduction in the nucleus.

1.3.7. SYNERGISTIC EFFECTS AND EARLY SIGNALS IN MITOGENESIS

The results discussed in the preceding sections provide evidence for the existence of multiple signal-transduction pathways in mitogenesis. Investigation into the mechanism(s) of action of defined growth-promoting molecules lead to the discovery of striking synergistic interactions (reviewed in Rozengurt, 1986). Agents that are ineffective in stimulating DNA synthesis on their own can be as effective as serum in combination. It has emerged that these agents may be categorised into distinct groups on the basis of these synergistic interactions. It is clear that a tenable hypothesis of growth control must necessarily provide a cogent explanation for this complex pattern of synergistic effects.

As summarised in Fig. 1.3.3., PKC and cAMP represent two separate signal transduction pathways that can lead to mitogenesis. The tumour promoters phorbol esters and teleocidin, synthetic diacylglycerol OAG and the neuropeptide vasopressin and related peptides elicit a common set of early signals; namely, activation of PKC and subsequent signalling events including 80K phosphorylation and c-fos and c-myc induction, but do not alter the basal level of cAMP. These agents on their own or in combination fail to induce DNA synthesis (Dicker and Rozengurt, 1980, Collins and Rozengurt, 1982b, Rozengurt *et al.*, 1984). On the other hand agents that elevate intracellular cAMP levels, including prostaglandin E_1, cholera toxin, forskolin or the adenosine analogue, NECA, also do not stimulate DNA synthesis on their own or in combination (Rozengurt *et al.*, 1981a, Rozengurt, 1982a, Rozengurt, 1982b, Rozengurt *et al.*, 1983a). Furthermore this class of agent does not activate PKC or influx of Na^+. However, combinations of agents from these two groups such as phorbol esters and

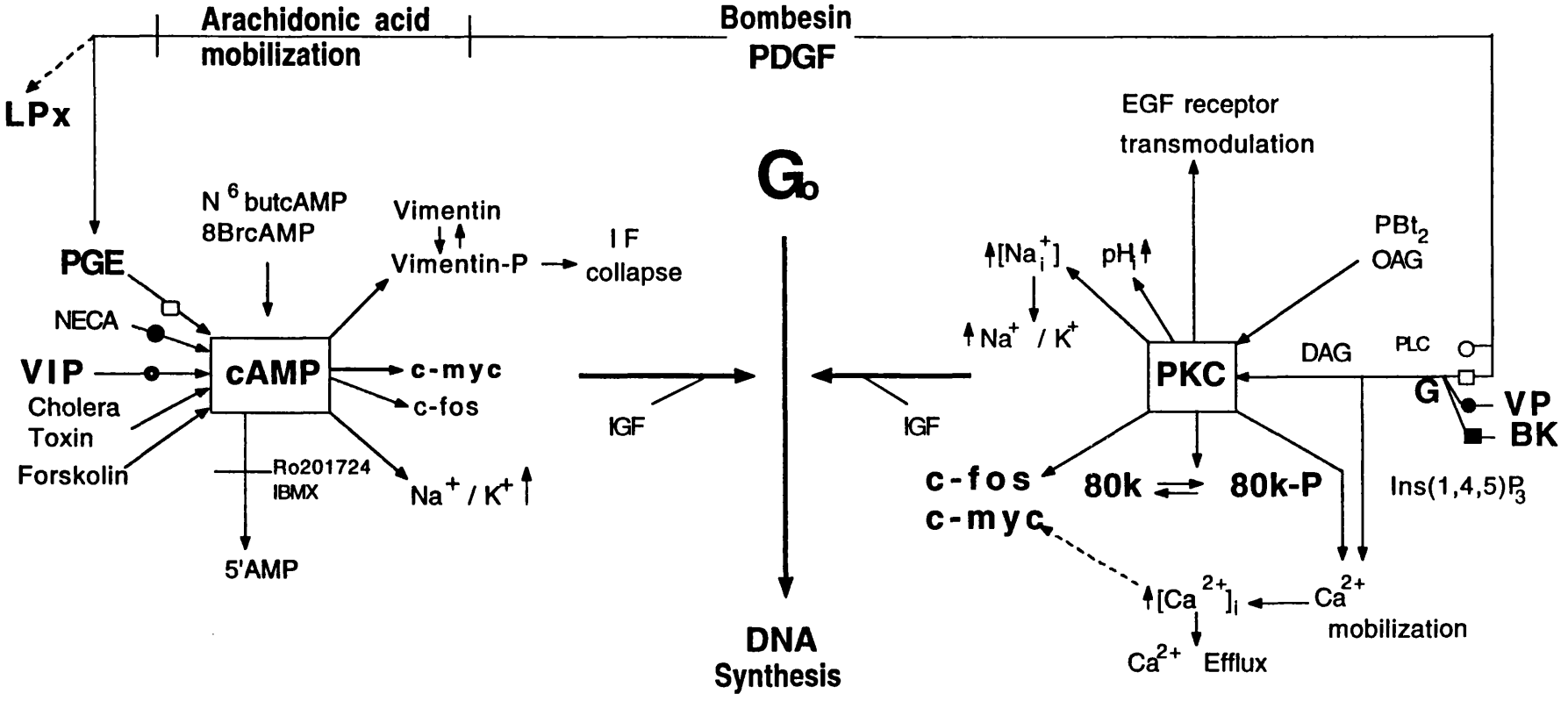

Arachidonic acid mobilization
Bombesin
PDGF
LPx
EGF receptor transmodulation
Gb
N 6 butcAMP
8BrcAMP
Vimentin
Vimentin-P
I F collapse
PGE
NECA
VIP
Cholera Toxin
Forskolin
cAMP
c-myc
c-fos
Ro201724
IBMX
Na+ / K+
5'AMP
IGF
IGF
[Na+i]
pHi
Na+ / K+
PBt2
OAG
DAG
PLC
PKC
G
VP
BK
c-fos
c-myc
80k
80k-P
Ins(1,4,5)P3
[Ca2+]i
Ca2+ mobilization
Ca2+ Efflux
DNA Synthesis

Fig. 1.3.3.

cholera toxin (Rozengurt *et al.*, 1981a) or vasopressin and butcAMP (Rozengurt, 1982a) are mitogenic for Swiss 3T3 cells. In this manner signalling mediated by an increase in intracellular cAMP, presumably via cAMP-dependent protein kinase, and by activation of PKC may result in initiation of a proliferative response (Fig. 1.3.3.). A third group of agents is characterised by insulin and EGF, the receptors for which possess tyrosine kinase activity. Neither insulin nor EGF alone reinitiate DNA synthesis on their own in Swiss 3T3 cells. Both insulin and EGF fail to activate PKC or raise cAMP levels in Swiss 3T3 cells. Nevertheless, both agents synergise with either activators of PKC or agents that elevate intracellular cAMP to stimulate DNA synthesis (Fig. 1.3.3.). Thus, synergistic interactions between various extracellular molecules seem to result from the generation of early signals that combine in a complementary manner to stimulate the necessary metabolic processes for the initiation of a proliferative response.

In contrast to most growth promoting agents identified for Swiss 3T3 cells, PDGF and bombesin are able to stimulate DNA synthesis in the absence of other mitogens. In particular although bombesin and vasopressin elicit a striking similarity in early signalling events, vasopressin is unable to stimulate DNA synthesis in the absence of other growth-promoting agents. Since the initiation of DNA synthesis is triggered by independent signal-transduction pathways that act synergistically in mitogenic stimulation, the ability of bombesin to act as a sole mitogen could be due to activation of a signalling pathway(s) not stimulated by vasopressin.

Recently, PDGF and bombesin but not vasopressin have been shown to induce a marked biphasic release of arachidonic acid into the extracellular medium (Millar and Rozengurt, 1990, Domin and Rozengurt, 1993). The stimulation of arachidonic acid release by PDGF and bombesin is likely to contribute to mitogenesis induced by these growth factors since externally applied arachidonic acid potentiates mitogenesis induced by agents that stimulate phosphoinositide hydrolysis but not arachidonic acid release, e.g. vasopressin (Millar and Rozengurt, 1990).

Arachidonic acid released by bombesin is converted to E-type prostaglandins which act in an autocrine and paracrine manner to enhance cAMP accumulation in the cell. Since elevated cAMP levels constitute a mitogenic signal for Swiss 3T3 cells (reviewed in Rozengurt, 1991) at least one consequence of arachidonic acid release may be the modulation of intracellular cAMP levels. However other arachidonic acid metabolites may also play a role in mitogenic signal transduction by bombesin and PDGF.

In conclusion cell proliferation can be stimulated through multiple signal-transduction pathways that act in a synergistic and combinatorial fashion for transducing environmental signals from the whole organisms into the mitogenic response of a specific cell.

1.4. BACTERIAL TOXINS.

The pathological basis of the action of certain bacteria is accounted for by their ability to produce toxins that enter eukaryotic cells and subvert cellular regulatory processes. There are many known bacterial toxins and certainly more to be discovered. Among them there is diversity in host cell susceptibility, biochemical mechanisms of toxin action and, mechanisms by which the toxins enter the cells wherein their toxic biochemical actions occur. The specificity of the action of these toxins and the modification of target protein function can be exploited to make them useful as probes of cellular regulatory processes.

The intracellular activities of many of the known bacterial toxins are enzymatic. Many of these toxins catalyse the transfer of ADP-ribose from NAD^+ to specific target proteins, resulting in a protein that may be either inactive or altered in properties. Diptheria toxin and *Pseudomonas* exotoxin A block protein synthesis by ADP-ribosylating elongation factor-2(Collier, 1990, Wick and Iglewski, 1990). *Clostridium botulinum* C2 toxin ADP-ribosylates nonmuscle actin (Aktories and Just, 1990) and C3 toxin ADP-ribosylates rho, a *ras* -related GTPase (Aktories *et al.*, 1990). *P.aeruginosa* exoenzyme S preferentially ADP-ribosylates $p21^{c\text{-}H\text{-}ras}$ in vitro (Coburn *et al.*, 1989b) and has also been described to ADP-ribosylate vimentin (Coburn *et al.*, 1989a). Pertussis toxin, cholera toxin, and *Eschericia coli* heat-labile enterotoxin ADP-ribosylate the α subunits of heterotrimeric signal transducing G proteins (Moss and Vaughan, 1988, Pfeuffer and Helmreich, 1988, Ui, 1990), for example the G proteins involved in the adenylate cyclase system (see section 1.2.2.). More recently, tetanus and *botulinum* B neurotoxins have been described to be proteases which block neurotransmitter release by cleaving synaptobrevin-2, a protein that appears to play a key part in neurotransmitter release (Schiavo *et al.*, 1992).

A key to elucidating the mechanism of action of toxins is to understand how the toxins enter cells and get access to their cytoplasmic targets. In most cases that have been studied the substrates for these toxins are either located free in the cytosol or associated with the cytoplasmic side of the plasma membrane. The first step in the entry process is the binding of the toxin to a specific cell surface receptor. Many toxins are multimeric proteins, with domains which are specific for binding to these receptors. To date the biochemical nature of the receptors for most known toxins is still not clear. As eukaryotic cells are not expected to have receptors for bacterial toxins it is thought that the toxins parasitize other receptors.

Indeed, many bacterial toxins resemble glycoprotein hormones and have sequences homologous to other ligands . For example amino acid sequence homologies have been demonstrated among the A and B chains of cholera toxin, thyrotropin, lutenizing hormone, follicle-stimulating hormone and interferon. Such structural similarities also suggest similar uptake and processing mechanisms (Middlebrook and Dorland, 1984). The cell surface receptor for cholera toxin has been demonstrated to be the ganglioside G_{M1}, however another glycoprotein has also been implicated. Similarly the receptor for diptheria toxin is thought to be complex and to involve more than one molecule (reviewed in Madshus and Stenmark, 1992). The vast number of discrepancies found between the number of cell surface binding sites and the number of toxin molecules required to elicit maximal biological effect, suggests that many of the binding sites are non-productive. For example as few as 4 to 10 molecules of active cholera toxin can effect maximal stimulation of adenylate cyclase (Gill, 1975) whereas only a single molecule of activated diptheria toxin is sufficient to cause cell death. As the toxins recognise receptors which are probably reserved for other ligands or functions this might explain why many more molecules are delivered intracellularly than are required to attain a maximal biological response. In addition, if these non-productive binding sites represent different proteins then the identity of the true functional receptor is more difficult to elucidate.

One of the methods utilised by some toxins for cellular entry is the process of receptor mediated endocytosis. After binding to their receptors the toxins enter coated pits from which they are transferred to endocytic vesicles (Fitzgerald *et al.*, 1980, Morris *et al.*, 1983). These vesicles contain a proton pump which rapidly acidifies the vesicles shortly after their formation. The low pH alters the toxin structure exposing hydrophobic residues which probably help the toxin to insert into the membrane. Agents that raise intravesicular pH such as methylamine or chloroquine block the action of these toxins (reviewed in Middlebrook and Dorland, 1984). Eventually the toxin gains access to the cytosol probably as an enzymatically active fragment and acts on it s target molecule. The nature of these fragments is not very well known. The whole process can take between one and three hours before the action of the toxin on its intracellular targets is observed.

Alternative to an endocytic molecular transport mechanism, some toxins enter cells by direct traversal of the plasma membrane. Two possible methods for this kind of process are either a) by a receptor mediated process or b) by nonspecific hydrophobic diffusion. Since most toxins are rather large proteins the latter might be unlikely on grounds of energy feasibility. Bacterial toxins which are internalised directly also require activation in order to catalyse their specific reactions. Pertussis toxin for example does not require an acidic environment for its action in eukaryotic cell (reviewed in Kaslow and Burns, 1992) and does not appear to enter cells by endocytosis. A model for the activation of pertussis toxin has also been proposed

(Kaslow and Burns, 1992). It is thought that pertussis toxin becomes incorporated into the membrane where it interacts with lipophillic agents in the membrane which together with ATP cause the dissociation of the toxin to release its S1 subunit in a reduced state . In its reduced form this subunit then ADP-ribosylates G protein targets (Kaslow and Burns, 1992)). In addition, cholera toxin may enter some cells by direct penetration of the plasma membrane (Fishman, 1990), although it may enter other cells via endocytosis (Janicot *et al.*, 1991).

1.4.1. *PASTEURELLA MULTOCIDA* TOXIN

A toxin produced by some strains of *Pasteurella multocida* has been identified as the causative agent of atrophic rhinitis, a disease of growing pigs (Il'ina and Zasukhin, 1975, Rutter and Mackenzie, 1984). The disease is characterised by destruction of the nasal turbinate bones, twisting of the snout and a reduction in weight gain. The native toxin was purified in several laboratories and was found to consist of a heat labile protein with a molecular weight of between 125 and 160 kDa (Nakai *et al.*, 1984, Chanter *et al.*, 1986, Rimler and Brogden, 1986, Kamp *et al.*, 1987, Foged, 1988). Purified *Pasteurella multocida* toxin (PMT) was shown to be cytotoxic for embryonic bovine lung cells, lethal for mice and caused turbinate atrophy in gnotobiotic pigs at very low doses of toxin (Chanter *et al.*, 1986). Recently, the gene for *pasteurella multocida* toxin (PMT) was cloned and expressed in *Eschericia coli* (Petersen and Foged, 1989, Kamps *et al.*, 1990b, Lax and Chanter, 1990). The recombinant toxin (rPMT) was found to be indistinguishable from the native toxin with respect to molecular mass, antigenicity and toxicity for either experimental animals or cultured embryonic bovine lung cells (Petersen and Foged, 1989, Kamps *et al.*, 1990b, Lax and Chanter, 1990).

Interestingly, in 1990 PMT was shown to be an extremely potent mitogen for Swiss 3T3 fibroblasts (Rozengurt *et al.*, 1990b). Both PMT and rPMT were shown to be able to stimulate DNA synthesis in these cells at picomolar concentrations. The degree of rPMT-induced DNA synthesis was comparable to that elicited by 10 % bovine foetal calf serum and was observed in the complete absence of other factors. Cell proliferation is also enhanced by rPMT in these cells and in addition, the toxin is a potent mitogen for BALB/c and NIH 3T3 cells, 3T6 cells, and a tertiary mouse embryo or human fibroblasts. The mitogenic activity of rPMT is heat labile. A polyclonal antiserum to PMT is able to inhibit DNA synthesis when added early, but not late, during treatment of the Swiss 3T3 cells with rPMT. A similar time dependent action of the lysosomotrophic agent methylamine (see above) was also observed. Furthermore, transient exposure of the cells to rPMT at 37 °C, but not at 4 °C, resulted in a stimulation of DNA synthesis (Rozengurt *et al.*, 1990b). These observations suggested that

rPMT must require cellular entry and processing or activation before acting intracellularly to stimulate DNA synthesis.

As intracellularly acting bacterial toxins frequently alter key components in the signal transduction process (Middlebrook and Dorland, 1984, Pfeuffer and Helmreich, 1988) one of the first steps towards identifying the intracellular targets of PMT in cultured fibroblasts was to determine the effects of the toxin on some of the early signals leading to mitogenesis (Rozengurt, 1986). In this context, it was demonstrated (Rozengurt *et al.*, 1990b) that rPMT did not increase the intracellular concentration of cAMP in Swiss 3T3 cells. In contrast, rPMT at mitogenic concentrations, caused a striking stimulation of inositol phosphate turnover, a signal transduction mechanism leading to multiple cellular responses including cell growth (see section 1.3.1.). The ability of rPMT to elicit phosphoinositide hydrolysis also required the entry and activation of the toxin (Rozengurt *et al.*, 1990b). Thus, unlike the receptor-mediated activation of this pathway by other growth factors, rPMT enhances phosphoinositide breakdown internally. These results were extremely exciting as none of the previously described bacterial toxins that act intracellularly, were known to stimulate this transmembrane signalling system. Thus, the basis of rPMT actions are potentially useful in providing novel insights into the control of cell proliferation.

More recently, rPMT has been shown to be a potent inducer of anchorage-independent cell growth (Higgins *et al.*, 1992). The growth of many normal cells requires contact with an adhesive substratum, a requirement that is frequently abrogated in the transformed phenotype. Cells acquire the ability to grow in an anchorage-independent manner through various mechanisms. These include production of growth factors that act in an autocrine or paracrine manner, resulting in alterations in the number or structure of cellular receptors, and changes in the activity of postreceptor signalling pathways (Bishop, 1991). Rat-1 cells, a nontumorigenic cell line, exhibit anchorage-dependent growth and thus fail to form colonies when they are suspended in semi-solid medium. However on treatment of these cells with growth factors such as PDGF or EGF these cells can be induced to form colonies in semisolid medium. rPMT stimulates DNA synthesis and growth proliferation of adherent Rat-1 cells and can also stimulate inositol phosphate production and Ca^{2+} mobilization in these cells (Higgins *et al.*, 1992) . As in Swiss 3T3 cells, rPMT requires cellular entry and processing for its action. More importantly, rPMT was an extremely potent inducer of anchorage independent growth in these cells and was more effective than either EGF or PDGF (Higgins *et al.*, 1992) . Thus rPMT is an effective transforming agent for these cells and is the first bacterial toxin which has been demonstrated to induce anchorage independent cell proliferation.

In late 1990, the gene for rPMT was sequenced and was found to code for a 146 kDa protein (Buys *et al.*, 1990, Lax *et al.*, 1990, Petersen, 1990). The deduced amino acid sequence was also analysed in order to identify potentially important domains and motifs, and consequently to ascertain whether these sequences were related to the mitogenicity of rPMT (Lax *et al.*, 1990). A hydrophobicity plot indicated that rPMT might have several domains; in particular there are two hydrophobic regions which might be important in the interaction of the toxin with various cellular membranes. Comparison of the DNA and deduced amino acid sequences with the sequences on the EMBL, Genbank and Swissprot data bases, did not reveal any significant homologies with other toxins or proteins. The motif His-Glu-Trp which is common to toxins which ADP-ribosylate their substrate (Wozniak *et al.*, 1988) is found near the N terminus of the toxin. The spacing between the amino acids matched precisely the His-Glu-Trp motif of ADP-ribosylating toxins such as pertussis and cholera toxin. However, it should be noted that this motif is also found in a variety of proteins that are not thought to ADP-ribosylate any substrates. However, it should be mentioned that the available evidence indicates that rPMT does not possess ADP-ribosylating activity in the cell (discussed below). The G + C content of the *Pasteurella* genome differs markedly from the that of the toxin gene suggesting the possibility that the gene was acquired relatively recently and that there might exist a family of closely related mitogenic toxins in other species. This might be the case as recent reports have shown that the toxin gene is flanked by phage elements (Patent-Application, 1989), may be carried on a prophage (Andresen *et al.*, 1990) and that some salmonella isolates contain part of the Pasteurella toxin gene (Kamps *et al.*, 1990a). It has been suggested that other bacterial toxin genes originally had eukaryotic origins and it might also be the case that rPMT has a eukaryotic homologue involved in signal transduction.

Further investigation into the action of rPMT on signalling in Swiss 3T3 cells revealed that rPMT markedly stimulates the phosphorylation of 80K/MARCKS (see section 1.3.3), a major PKC substrate in Swiss 3T3 cells (Staddon *et al.*, 1990). The stimulation of 80K/MARCKS by rPMT was comparable to that induced by bombesin or PBt_2 except that the increase of phosphorylation by rPMT occurs after a lag period of 1-3 h compared to the relatively immediate stimulation by bombesin or PBt_2. In addition 80K/MARCKS phosphorylation by rPMT is not inhibited by cycloheximide, demonstrating that the lag period does not reflect a requirement for *de novo* protein synthesis. rPMT, like PBt_2, also stimulates the phosphorylation of 87 kDa and 33 kDa proteins in Swiss 3T3 cells. Phosphorylation of the 80K/MARCKS and 87 kDa proteins by rPMT or PBt_2 is greatly attenuated in cells depleted of PKC. In contrast, phosphorylation of the 33 kDa protein by rPMT but not by PBt_2, persists in the absence of PKC providing evidence for a PKC independent action of rPMT. These results demonstrate that rPMT, in addition to activating PKC causes a modulation of other kinases involved in regulation of the phosphorylation of the 33 kDa protein. The identity of the 33 kDa protein remains to be established. In addition, the observation that rPMT is able

to induce the transmodulation of the EGF receptor (section 1.3.3) in the absence of PKC provides further evidence for the activation of other pathways by rPMT.

The mechanisms by which rPMT stimulates PKC activity appears to be identical with that of other growth factors. rPMT, like bombesin, causes a translocation of PKC to the particulate fraction and increases the cellular content of diacylglycerol. These results together with the associated increases in inositol phosphates (Rozengurt *et al.*, 1990b) indicated that rPMT initiates events leading to the activation of PKC and that a component of these events might involve enhanced cellular PI-PLC activity.

Analysis of the inositol phosphate species present in rPMT-stimulated Swiss 3T3 cells by high performance liquid chromatography revealed that the profile of the increase in the cellular content of different inositol phosphates is very similar to that elicited by bombesin (Staddon *et al.*, 1991a). In particular rPMT causes an increase in the cellular content of $Ins(1,4,5)P_3$, a direct product of PI-PLC-mediated $PtdIns(4,5)P_2$ hydrolysis. However, unlike bombesin and GTPγS, the direct addition of rPMT to permeabilized Swiss 3T3 cells did not cause the release of inositol phosphates suggesting that PMT enters the cells by the action of endosomal/lysosomal traffic and thereby gains access to the cytosol in an activated state. The similarities in the inositol phosphate species formed in response to rPMT and bombesin suggested that rPMT modified cellular regulatory processes physiologically involved in phosphoinositide hydrolysis. These results also indicated that rPMT stimulates the PI-PLC-mediated hydrolysis of $PtdIns(4,5)P_2$ hence causing the release of $Ins(1,4,5)P_3$, Ca^{2+} mobilization as well as the increase in diacylglycerol, translocation of PKC, and phosphorylation of 80K/MARCKs observed previously.

The action of many toxins including cholera and pertussis toxin is to catalyse the ADP-ribosylation of specific cellular targets which subvert cellular regulatory activities (Middlebrook and Dorland, 1984, Pfeuffer and Helmreich, 1988). Pertussis and cholera toxin act to ADP-ribosylate the α-subunits of heterotrimeric G proteins involved in the adenylate cyclase pathway. In Swiss 3T3 cells, one of the pathways leading to the activation of PI-PLC involves signalling through a heterotrimeric G protein. Indeed bombesin and other neuropeptides act through receptors which couple to PI-PLC via a pertussis toxin-insensitive G protein (see section 1.3.1.). Recently, the α–subunits of the Gq family of pertussis toxin-insensitive G proteins were shown to be responsible for coupling of many hormone and neuropeptide receptors to the β isozyme of PI-PLC (for review see Sternweiss and Smrcka, 1992). As the deduced amino acid sequence of PMT exhibits partial homology with that of other ADP-ribosylating toxins, including cholera and pertussis (Lax *et al.*, 1990) it was plausible that rPMT might ADP-ribosylate and thereby constituitively activate the 42 kDa α-subunit of Gq. Using a novel method to detect toxin catalysed ADP-ribosylation in intact Swiss 3T3 cells, it

was found that rPMT had no ADP-ribosyltransferase activity. Under the same conditions, both cholera and pertussis toxin were shown to catalyse the ADP-ribosylation of distinct substrates in Swiss 3T3 cells. Thus, rPMT does not appear to act by ADP-ribosylation of Gq or any other substrates in Swiss 3T3 cells.

More recently, rPMT, like bombesin and PDGF was shown to cause a decrease in the ADP ribosylation of GRP78 (the 78-KDa glucose-regulated protein, also known as BiP, the immunoglobulin heavy chain-binding protein), a resident endoplasmic reticulum (ER) protein that assists in the processing of proteins destined for secretion or cell surface expression (Staddon *et al.*, 1992). GRP78/BiP has been shown to be post-translationally modified by ADP-ribosylation (Welch *et al.*, 1991). The ADP-ribosylated and unmodified forms of GRP78/BiP are functionally different as only the unmodified form has been shown to be capable of binding ER proteins (Hendershot *et al.*, 1988). Thus, the conversion of GRP78/BiP from an ADP-ribosylated state to an unmodified state might represent the conversion of GRP78/BiP from an inactive to an active state. GRP78/BiP is known as a major Ca^{2+}-binding protein of the ER (Macer and Koch, 1988). Agents that mobilize Ca^{2+} from the endoplasmic reticulum (A23187, ionomycin and thapsigargin) also caused a decrease in the ADP-ribosylation of GRP78/BiP (Staddon *et al.*, 1992) that was similar to that achieved by rPMT, bombesin, and PDGF, implicating a role for Ins(1,4,5)P_3-mediated Ca^{2+} mobilization in the action of the mitogenic agents. Thus rPMT like other potent growth factors for Swiss 3T3 cells causes the interconversion of GRP78/BiP, an early event in the stimulation of cell proliferation.

1.5. THESIS

The work in this thesis is primarily concerned with the mechanism(s) of action of rPMT on early signalling events in Swiss 3T3 cells.

A. At the beginning of the research it was known that rPMT was a potent mitogen for Swiss 3T3 cells and other established cell lines: rPMT is able to induce maximum DNA synthesis, as compared to that elicited by 10 % bovine foetal calf serum, and can do so in the complete absence of other factors (Rozengurt, *et al.*, 1990). In addition it was known that rPMT acted intracellularly to stimulate a massive and persistent production of inositol phosphates in Swiss 3T3 cells. As described in detail in section 1.3.1. there are at least two different pathways leading to inositol phosphate production in Swiss 3T3 cells. The neuropeptides stimulate inositol phosphate production by activating the β-isoform of PI-PLC via specific plasma membrane receptors coupled to a pertussis toxin-insensitive G protein. In contrast PDGF stimulates the γ-isoform of PI-PLC by acting

through receptors endowed with intrinsic tyrosine kinase activity. Since polyphosphoinositide hydrolysis and the subsequent signalling events which are activated by this pathway are known to be growth promoting signals for Swiss 3T3 cells, the following question was posed:

1. Does rPMT stimulate inositol phosphate production via either of the two known pathways in these cells and if so at what site along the pathway and what mechanisms does it employ to achieve this?

B. Cell permeabilization has provided a useful system for studying the mechanisms involved in the action of different mitogens on specific signalling pathways. In particular, permeabilized cells have been used to assess the role of G proteins in the activation of certain cellular responses. Since G proteins are known to be upstream activators of specific isozymes of phospholipase C the following question was asked.

2. Is a functional G protein required for the activation of phospholipase C by rPMT?

C. In addition to the early signalling events in the membrane and cytosol, serum and growth factors rapidly and transiently induce the expression of the cellular oncogenes c-fos and c-myc in quiescent fibroblasts (reviewed in Rozengurt and Sinnett-Smith, 1988). Since these protooncogenes encode nuclear proteins it is thought that their transient expression may play a role in the transduction of the mitogenic signal in the nucleus (Curran, 1988). Indeed, recent evidence has shown that the products of both of these genes can act as transcription factors (Curran, 1988, Rozengurt and Sinnett-Smith, 1988, Shaw, 1990, Blackwell *et al.*, 1990, Prendergast and Ziff, 1991). The induction of these genes is subject to strong feedback inhibition. In addition, receptor desensitization may play a role in the transient induction of these genes by growth factors. At the beginning of the research nothing was known about the ability of rPMT to stimulate gene expression. Hence the following question was asked.

1. Does rPMT stimulate the expression of c-fos and c-myc in Swiss 3T3 cells and if so does the persistent activation of early signalling pathways by rPMT contribute to the induction of these protooncogenes by rPMT?

CHAPTER TWO : MATERIALS AND METHODS

2.1. CELL CULTURE

2.1.1. SWISS 3T3 CELLS:

Swiss 3T3 cells (Todaro and Green, 1963) were propagated in Dulbecco's modified Eagles medium (DMEM), with 10% foetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin in a humified atmosphere of 10% CO_2 at 37 °C. Stock 90 mm Nunc dishes were maintained at subconfluent density by subculturing twice per week using a trypsin solution (0.5 mg/ml solution of trypsin in 0.2 mg/ml EDTA) at a cell density of 5 x 10^4 cells per dish. Stock cultures were replaced approximately every two months from liquid N_2 frozen cells.

For experimental purposes Swiss 3T3 cells were subcultured to 33 mm Nunc petri dishes at a density of 10^5 cells per dish or to 90 mm Nunc petri dishes at 5.5 x 10^5 cells per dish in DMEM containing 10% foetal bovine serum. After 5-7 days the cultures were confluent and quiescent (Dicker and Rozengurt, 1980), and were used between 6 and 8 days after plating.

2.1.2. COS-1 CELLS

The COS-1 cell line used in this thesis was propagated in DMEM, with 10% foetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin in a humified atmosphere of 10% CO_2 at 37 °C. Stock 90 mm Nunc dishes were maintained at subconfluent density by subculturing twice per week using a trypsin solution (0.5 mg/ml solution of trypsin in 0.2 mg/ml EDTA) at a cell density of 1 x 10^5 cells per dish. In some cases, for experimental purposes the cells were subcultured to 33 mm Nunc petri dishes at a density of 10^5 cells per dish in DMEM containing 10% foetal bovine serum. After 3-4 days the medium was switched to DMEM containing 0.5 % foetal bovine serum and the cultures used after a further 3-4 days. For transfection experiments the COS-1 cells were subcultured to 33 mm Nunc petri dishes at a density of 1-2 x 10^5 cells per dish in DMEM containing 10% foetal bovine serum and used 1-4 days after plating.

2.2. ANALYSIS OF TOTAL INOSITOL PHOSPHATES

2.2.1. FROM INTACT CELLS.

Cultures of Swiss 3T3 cells or other cell lines, in 33 mm dishes were labelled for 16-24 hours in 1 ml DMEM:Waymouth medium (1:1) containing 5-10 μCi of [2-^{3}H]inositol. Additions were made to the cells as described for each experiment and LiCl was added to a final concentration of 20 mM for the last 30 min-1hour of the incubation (Shears, 1989). Experiments were terminated by aspirating the extracellular medium and solubilising cellular inositol phosphates by replacing the medium with 1 ml of ice-cold 3% Perchloric acid. After 20 min at 4 °C the extracts were neutralised with 0.5 M KOH containing 25 mM HEPES, 5 mM EDTA, and 0.01% phenol red. Precipitated potassium perchlorate was removed by centrifugation. [^{3}H]-labelled inositol polyphosphates in the cell extracts were separated by anion-exchange over Dowex columns using Biorad AG1X8 (100-200 mesh; formate form) (Morris, *et al.*, 1988). Samples were diluted to 10 ml with water and then loaded onto 0.7 cm x 1 cm of Dowex AG1-X8 (100-200 mesh, $HCOO^-$ form) anion exchange resin in Bio-Rad (737-142) Econo-columns. The loaded columns were washed with 3 x10 ml of distilled water to remove [^{3}H]inositol and then 2 x 10 ml of 60 mM sodium formate, 5 mM borax to elute glycerophosphoinositol. Total inositol phosphates were eluted with 1 x 7 ml of 1M ammonium formate, 0.1M formic acid. The column eluates were collected and aliquots (1 ml) transferred into scintillation vials containing 10 ml of scintillation Picoflor and radioactivity determined in a Beckman β-counter.

2.2.2. FROM PERMEABILIZED CELLS

The cultures in 30 mm dishes were labelled in 1 ml of DMEM:Waymouth medium (1:1) containing 25 μCi of [2-^{3}H]inositol. Addition of toxin to the cells was as described for each experiment. The following procedure was then performed at 37 °C. The cultures were washed twice with 'K solution' (120 mM KCl, 30 mM NaCl, 2.5 mM $MgCl_2$, 1 mM K_2HPO_4, 10 mM piperazine-N,N'-bis-[2-ethanesulfonic acid] (PIPES), 2 mM EGTA, 0.5 mM $CaCl_2$, pH 7.2). The free Ca^{2+} in this solution was 64 nM. The cells were permeabilized in 1 ml of 'K solution' containing 1 mM ATP, 20 mM LiCl, either Streptolysin O or digitonin at the concentrations indicated, and the factors described for a total of 10 min. Reactions were terminated by adding 1 ml of 6% $HClO_4$ (w/v). After 30 min at 4 °C the acid extract was removed from the dish and neutralised with 1 M KOH containing 25 mM HEPES, 20 mM EDTA and 0.01% phenol red. Total inositol phosphates were analysed as described for intact cells

For the experiments involving varying concentrations of free calcium , calculations were made using a computer program based on the calculations described by Fabiato and Fabiato, 1979.

2.2.3. FROM TRANSFECTED CELLS.

One day after transfection of COS-1 cells (see below for transfection experiments) the medium was removed and the cells were washed with DMEM. Then the cells were labelled by incubation with 5-10 μCi of [2-^{3}H]inositol in 1 ml of DMEM with 10 % foetal bovine serum for 16-18 h. The cells were then washed with DMEM and the medium replaced with 1 ml of DMEM (containing 5-10 μCi of [2-^{3}H]inositol) with or without rPMT. LiCl at a final concentration of 20 mM was added to the dishes after rPMT treatment for the times indicated. Other factors were added 10 min after the addition of LiCl and reactions were terminated after a further 30 min by replacing the medium with 1 ml of 3 % perchloric acid. The samples were then neutralised and analysed for total inositol phosphates as described for intact cells..

2.3. ASSAY OF INOSITOL(1,4,5)TRISPHOSPHATE

Quiescent cultures of Swiss 3T3 cells in 90-mm Nunc dishes were washed twice with DMEM:Waymouth (1:1) and incubated for 5 h in 5 ml DMEM:Waymouth (1:1) with or without 2.5 ng/ml rPMT. The medium was then aspirated off and DMEM:Waymouth containing the required factors was added for the times indicated. Reactions were terminated on ice by replacing the medium with 500 μl of ice-cold 5% perchloric acid followed by immediate scraping with a rubber policeman. The suspension was collected, left for 20 min at 4 °C and centrifuged at 14,000 g for 5 min. The supernatant was retained and neutralised using 10 M KOH, 500 mM HEPES. The precipitated potassium perchlorate was removed by centrifugation at 14,000 g for 5 min, and 100 μl of the final supernatant was added to the assay mixture utilising [^{3}H]-Ins(1,4,5)P_3 together with a specific Ins(1,4,5)P_3-binding protein (Ins(1,4,5)P_3-receptors from bovine adrenal medulla)((Amersham, U.K.). The assay was started by the addition of the Ins(1,4,5)P_3-binding protein. Ins(1,4,5)P_3 of the cell extract was allowed to compete with the [^{3}H]-Ins(1,4,5)P_3 for the Ins(1,4,5)P_3-specific binding sites of the binding protein. After 15 min at 4 °C the reactions were centrifuged (30 min, 2,000 g) and the supernatant discarded. Pellets were resuspended in 200 μl H_2O and counted in 3 ml of Picofluor. A standard curve based on pure Ins(1,4,5)P_3 was constructed in parallel and thus the quantity of Ins(1,4,5)P_3 in the cell extracts were determined.

2.4. MEASUREMENT OF INTRACELLULAR Ca^{2+} MOBILISATION

$[Ca^{2+}]_i$ was measured with the fluorescent Ca^{2+} indicator fura 2 using a modification of the procedure of Tsien *et al.*, 1982. At the beginning of the experiment, cells on 90 mm dishes were washed twice with DMEM and incubated for 10 min in 5 ml DMEM containing 1 μM fura-2 tetraacetoxymethyl ester (fura-2/AME). The stock solution of fura-2/AME (1 mM) was dissolved in dimethyl sulphoxide. After this incubation, the dishes were washed three times with electrolyte solution which contained 120 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 25 mM glucose, 16 mM HEPES, 6 mM Tris and a mixture of amino acids at the same concentrations as those in DMEM (pH 7.2). The cells were suspended in electrolyte solution (final volume 2.0 ml) by gentle scraping and transferred to a 1 cm^2 quartz cuvette. The suspension was stirred continuously and maintained at 37 °C. Fluorescence was monitored in a Perkin-Elmer LS-5 luminescence spectrophotometer with an excitation wavelength of 336 nm and an emission wavelength of 510 nm. $[Ca^{2+}]_{cyt}$ was calculated using the formula of Tsien *et al.*, 1982.

$$[Ca^{2+}]_{cyt} \text{ in nM} = \frac{K(F - F_{min})}{(F_{max} - F)}$$

where F is the fluorescence at the unknown $[Ca^{2+}]_{cyt}$, F_{max} is the fluorescence after the trapped fluorescence is released by the addition of 0.02% Triton X-100 and F_{min} is the fluorescence remaining after the Ca^{2+} in the solution is chelated with 10 mM EGTA. The value of K was 220 for fura 2 (Mendoza *et al.*, 1986b).

2.5. MEASUREMENT OF EFFLUX OF [^{3}H]URIDINE FROM STREPTOLYSIN O PERMEABILISED CELLS.

Quiescent cultures of Swiss 3T3 cells in 30 mm dishes were washed twice with DMEM and the medium replaced with 1 ml of DMEM/ Waymouths medium (1:1) containing 1 μCi/ml of [^{3}H]uridine. Additions to the cells were as described for each experiment. After 5 h at 37 °C the cells were washed four times with DMEM and a further two times with 'K solution' (120 mM KCl, 30 mM NaCl, 2.5 mM $MgCl_2$, 1 mM K_2HPO_4, 10 mM piperazine-N,N'-bis-[2-ethanesulfonic acid] (PIPES), 2 mM EGTA, 0.5 mM $CaCl_2$, pH 7.2). The cells were permeabilized in 1 ml of 'K solution' containing 1 mM ATP and either (A) Streptolysin O or (B) digitonin at the concentrations indicated. After 10 min, 0.5 ml of the solution was removed and counted in 10 ml of scintillant in a beta counter. Total incorporated [^{3}H]uridine was

determined by washing the cells as described above and then replacing the medium with 1 ml of 5% TCA. 0.5 ml of the acid extract was then counted as before. Release of radiolabelled nucleotides was calculated as a percentage of the total.

2.6. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Slab gel electrophoresis was performed using a 5-15% acrylamide and 0.1% SDS gel (Laemlli, 1970). After electrophoresis, the gels were either used for western blot analysis or for analysis of ADP-ribosylation as described below.

In most cases, cultures in 30 mm dishes were washed twice with PBS and lysed directly into SDS/PAGE sample buffer(3% SDS, 5% glycerol, 2% b-mercaptoethanol, 10 mM Tris-HCl, pH 6.8). The cell lysates were scraped from the dishes, transferred to microfuge tubes and then heated at 100 °C for 5 min. The samples were stored at -20 °C until electrophoresis prior to which they were reheated at 100 °C for 5 min.

2.7. IMMUNOPRECIPITATION OF PI-PLCγ

Quiescent cultures of Swiss 3T3 cells in 30 mm dishes were incubated for 5 h in the absence or presence of 2.5 ng/ml rPMT. Cells were then treated with 6 nM bombesin or 25 ng/ml PDGF for 10 min. The cells were lysed with a solution containing 1% Triton, 0.25% deoxycholate, 10 mM Tris-Base, 5 mM EDTA, 50 mM NaCl, 30 mM Na pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4,1 mM phenylmethylsulfonylfluoride and 0.1% BSA. The lysates were then centrifuged and the supernatant precleared by incubation with BSA:agarose for 1 h at 4 °C. After centrifugation, the supernatant was immunoprecipitated with PI-PLCγ polyclonal antibody (1:50 dilution) for 3 h at 4 °C. The immunoprecipitate was then incubated for 1 h with protein A agarose then spun and the pellet washed four times with lysis buffer. Immunoprecipitates were analysed by one-dimensional SDS/PAGE with 8% acrylamide.

2.8. WESTERN BLOT ANALYSIS

2.8.1 BY SEMI-DRY BLOTTING (anti-Gq antibody)

Polyacrylamide SDS gels were incubated for 30 min in transfer buffer consisting of 20% methanol, 39 mM glycine, 48 mM Tris-HCl pH 9.5 and then transferred electrophoretically in transfer buffer on to nitrocellulose filters (Hybond C Extra; Amersham U.K or immobilon , millipore) using an LKB semi-dry blotting apparatus. After completion of transfer, non-specific

binding sites on the nitrocellulose sheets were blocked by incubation in PBS containing 5% dried milk powder or 5% BSA for at least 2 hours at 20 °C. After incubation overnight at 4 °C or for 2h at room temperature with an antibody raised against the C-terminal decapeptide of Gαq/11 (1:2,500 dilution) in 0.1% Tween-20 in PBS, the filters were washed three times with fresh 0.1% Tween-20 in PBS. Bound antibodies were detected by incubation with ^{125}I-protein A (0.2 μCi/ ml) in PBS containing 0.1% Tween 20 for 2 hours at 20 °C. The filters were washed several times with PBS containing 0.1% Tween 20 and then exposed to X-ray film.

2.8.2. BY WET BLOTTING (anti-Tyr antibody)

PI-PLCγ immunoprecipitates were fractionated by SDS/PAGE as described above. The gels were incubated for 30 min in transfer buffer consisting of 20% methanol, 39 mM glycine, 48 mM Tris-HCl, 0.1% SDS, pH 9.5 and then transferred electrophoretically in transfer buffer on to nitrocellulose filters (Hybond C Extra; Amersham U.K or immobilon, millipore) using a Bio-Rad wet-blotting apparatus. After completion of transfer, non-specific binding sites on the nitrocellulose sheets were blocked by incubation in PBS containing 5% dried milk powder or 5% BSA for at least 2 hours at 20 °C. After incubation overnight at 4 °C or for 2h at room temperature with a PY20 anti-Tyr(P) monoclonal antibody in 0.1% Tween-20 in PBS, the filters were washed three times with fresh 0.1% Tween-20 in PBS. Immunoreactive bands were detected by using ^{125}I-labelled rabbit anti-mouse IgG followed by autoradiography.

2.9. TRANSFECTION OF COS-1 CELLS

Transfection experiments were carried out on cultures of COS-1 cells in 30 mm dishes which were plated at 2 x 10^5 cells/dish and incubated at 37 °C for approximately 24 h or until they were 50-60 % confluent. cDNAs for α_q and α_{11} (Strathmann and Simon, 1990) which had been subcloned into a pCMV expression vector which contains the SV40 replication origin and human cytomegalovirus promoter and enhancer sequences (Foecking and Hofstetter, 1986) were used. 5 μg of the vector pCMV plasmid or cDNA corresponding to $G\alpha_q$ or $G\alpha_{11}$ were made up to 100 μl with DMEM and then mixed with 10 μl of lipofectin (Bethesda Research Laboratories) in 100 μl of DMEM. After incubating for 15 min at room temperature to allow the DNA to complex with the lipofectin agent, the mixture was made up to 1 ml with DMEM. Cells were washed twice with DMEM and the 1 ml of lipofectin/DNA mixtures were added to the dishes. After 6 h at 37 °C the medium was replaced with 1 ml of DMEM containing 10% foetal bovine serum. The level of expression of the α_q and α_{11} subunits was

determined by western blotting 2 days after transfection. Inositol phosphate production from transfected cells was as described in section 2.2.3..

2.10. DETECTION OF ADP RIBOSYLATION IN INTACT CELLS

Quiescent cultures of Swiss 3T3 cells were incubated for 16 h with 50 µCi/ml of [2-^{3}H]adenine. Additions were made directly to the medium for the required times and the cells were extracted by rapidly replacing the medium with 0.15 ml of SDS-sample buffer (section 2.6.) and fractionated by SDS-PAGE..

The gels were equilibrated in 500 ml of 7 % TCA (wt/vol) at room temperature and then placed in a 95 °C water bath for 45 min. Next, the gels were equilibrated in 25 % methanol/10 % acetic acid and then rinsed in H_2O for 2 min. After shaking gently in 1 M sodium salicylate, pH 6.0, for 20 min the gels were dried for 3 h under vacuum at 80 °C. Radioactivity was detected at -70 °C using Fuji x-ray film with exposure times of 4-14 days.

2.11. ^{125}I-GRP BINDING IN INTACT SWISS 3T3 CELLS.

For binding at 37 °C, cultures of Swiss 3T3 cells in 33 mm dishes were washed three times with DMEM at 37 °C and incubated in 1 ml of binding medium, which consisted of a 1:1 mixture of DMEM and Waymouth's medium supplemented with 1 mg/ml fatty acid and globulin free BSA, 50 mM BES, pH 7.0, and ^{125}I-GRP at the concentrations indicated. After 30 min of incubation (unless otherwise stated), cultures were rapidly washed four times with cold (4 °C) PBS supplemented with 1 mg/ml BSA. Washed cultures were extracted in 1 ml of 0.1 M NaOH containing 2% Na_2CO_3 and 1% SDS, and the total cell-associated radioactivity was determined in a Beckman γ-counter. Nonspecific binding, defined as the cell-associated radioactivity not displaced in the presence of a 500-fold excess of unlabelled GRP or bombesin, was proportional to the concentration of the labelled ligand and constituted <10% of the total at 1 nM ^{125}I-GRP. Nonspecific binding was subtracted from the total binding to obtain specific binding. All values shown are the average of triplicates that agree within 8%.

2.12. NORTHERN BLOT ANALYSIS

Quiescent cultures of Swiss 3T3 cells in 90 mm Nunc tissue culture dishes were washed twice with DMEM and incubated at 37 °C for the specified time in DMEM either in the

absence or presence of various additions as described in the individual experiments. The cultures were then washed twice with cold (4 °C) PBS and lysed in 4 M (Guanidine isothiocyanateGTC). Three dishes were pooled for each condition. Total RNA from lysates was purified by centrifugation on a caesium chloride cushion (Chirgwin *et al.*, 1979). Equal amounts of total RNA from each sample were then fractionated on a 1% agarose, 6% formaldehyde gel and transferred to nylon membranes by diffusion blotting (Thomas, 1980). Ethidium bromide staining of parallel gels showed that the amounts of ribosomal RNA analysed were approximately equal in all samples.

Hybridisation's were performed in a solution containing: 50% formamide, 5 x Denhardt's (1 x Denhardt's = 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% bovine serum albumin), 5 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M NaCitrate), 50 mM Na_2PO_4, pH 6.5, 0.1% SDS, 250 µg/ml nonhomologous single stranded DNA (from salmon sperm testis), (Wahl *et al.*, 1979) and random primed probes (specific activity 1-2 X 10^8 cpm/µg). Following hybridisation for 18 hours at 42 °C, the unbound DNA was removed by washing sequentially in 5 x SSC (20 °C), 2 x SSC (20 °C), 1 x SSC, 0.1% SDS at 37 °C and twice in 0.1 x SSC, 0.1% SDS (37 °C-55 °C). The membranes were then autoradiographed with intensifying screens at -70 °C. Following autoradiography, bound DNA probes were removed by incubating transfers at 100 °C for 20 min in 0.1 x SSC, 0.1% SDS. In this manner Northern transfers could be reprobed with various probes e.g. for c-fos (42 °C), c-myc (42 °C), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (50 °C) and mouse 18S mRNA. Radiographic bands were quantitated by scanning densitometry using a LKB scanning densitometer and normalised for differences in gel loading using the GAPDH or mouse 18 S level of mRNA as the control.

2.13. MAXI PREPARATIONS OF PLASMID DNA FOR TRANSFECTION

Plasmid containing bacteria were grown overnight at 37 °C in L-broth (ICRF standard medium) in the presence of ampicillin (100 µg/ml). Bacteria were harvested by centrifugation at 6000 r.p.m. for 10 min in a Sorvall GS3 rotor at 4 °C. The pellet was resuspended in 10 ml of buffer P1 (100 µg/ml RNAse A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0). The bacteria were lysed by the addition of 10 ml of buffer P2 (200 mM NaOH, 1% SDS) and gently mixed for 5 min at room temp. Protein was then precipitated by the addition of 10 ml of buffer P3 (2.55 M potassium acetate, pH 4.8) and the resulting mixture immediately and gently mixed. After centrifugation at 16 000 r.p.m. for 30 min in a Sorvall GSA rotor, the supernatant was removed and centrifuged again at 16 000 r.p.m. for 10 min. The supernatant was then applied to a QUIAGEN-tip 500 column which had been equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS, 15 % ethanol, pH 7,0.15 % Triton X-100) and allowed to enter

by gravity. The column was washed with 30 ml of buffer QC (1.0 M NaCl, 50 mM MOPS, 15 % ethanol, pH 7.0). The DNA was eluted with 15 ml of buffer QF (1.25 M NaCl, 50 mM MOPS, 15 % ethanol, pH 8.2). The DNA was then precipitated with 15 ml of isopropanol at room temperature, and then centrifuged at 16 000 r.p.m. for 30 min at 4 °C. After washing with 70 % ethanol, the DNA was air dried for 5 min and redissolved in a suitable volume of water. Yields of DNA from this method ranged from 200-600 μg.

2.14. PROTEIN DETERMINATION

Measurements of protein concentration were performed using a Bicinchroninic acid (BCA) Protein Assay System (Pierce Chemical Company). Aliquots of samples were mixed with a base reagent containing sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartate in 0.2 M NaOH and 0.02% Copper sulphate solution. The mixture was incubated for 30 min at 37 °C and absorbance then measured at 562 nm. BSA was used as a standard.

2.15. MATERIALS

Foetal bovine serum, Waymouth's medium and lipofectin agent were obtained from Gibco BRL (U.K.). DMEM was made up as described in the standard methods manual of the ICRF. Cell culture dishes were from Nunc, and CO_2 humidified LEEC incubators were used. Bombesin, vasopressin, EGF, phorbol 12,13-dibutyrate (PBt_2) and insulin were obtained from Sigma (U.K.). PDGF AA and BB homodimers were a gift from Dr. M. Murray (Zymogenetics, U.K.). PDGF c-sis, bFGF, [2-^{3}H]inositol (18.8 Ci/mmol; 1 Ci = 37 GBq), Ins(1,4,5)P_3 binding assay kit, ^{125}I-labelled GRP (2000 Ci/mmol; 1 Ci = 37 GBq), [^{32}P-α]dCTP, [^{3}H]uridine, [^{3}H]adenine, Hybond-N and ^{125}I-labelled rabbit anti-mouse IgG (15 μCi/μg) were all supplied by Amersham (U.K.). PY20 anti-Tyr(P) antibody was from ICN Biomedicals (U.K.). Dowex resin (AG 1-X8, 200-400 mesh) was from Bio Rad (U.K.). GTPγS and GDPβS were from Boehringer Mannheim (U.K.). Streptolysin O was from Wellcome Diagnostics (UK). c-fos DNA was a gift from Dr. D. Bentley (ICRF, London, U.K.) and c-myc DNA was a gift from Dr. G. Evan (ICRF, London, U.K.). The FM564 plasmid containing the 1.2-kb *Pst* 1 human GAPDH fragment was a gift from Drs. C. Williams and L.Lim (Institute of Neurology, London, U.K.). pCMV plasmid alone and pCMV plasmids containing the $G\alpha_q$ and $G\alpha_{11}$ DNA inserts were a gift from Dr. M. Simons (California Institute of Technology, U.S.A.). COS-1 cells were from Dr. A. Carozzi (ICRF, London, U.K.). Recombinant PMT was prepared as described previously (Lax and Chanter, 1990) and was a generous gift from Dr. A Lax (AFRC, Compton, U.K.). Samples of the rPMT used in the experiments performed in this thesis were

analysed by SDS-PAGE. These gels, stained by silver-staining, revealed a single major band migrating at the expected molecular mass (146 kDa). All other chemicals and reagents were of the highest grade available.

CHAPTER THREE: RESULTS

rPMT SELECTIVELY FACILITATES PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE HYDROLYSIS BY THE NEUROPEPTIDES BOMBESIN, VASOPRESSIN AND ENDOTHELIN.

Pasteurella multocida toxin, a potent mitogen, is the first intracellularly acting toxin that leads to the activation of PI-PLC, a major transducer of transmembrane signalling (Rozengurt *et al.*, 1990b, Staddon *et al.*, 1991a). As discussed in detail in section 1.3.1., at least two distinct signal transduction pathways lead to the activation of different PI-PLC isoforms in many cell types including Swiss 3T3 cells. The mitogenic neuropeptides bombesin, vasopressin and endothelin stimulate inositol phosphate production via specific plasma membrane receptors coupled to heterotrimeric G proteins (Heslop *et al.*, 1986, Takuwa *et al.*, 1987, Zachary *et al.*, 1987a, Nanberg and Rozengurt, 1988, Issandou and Rozengurt, 1990). Recently, the activated α subunits of the pertussis toxin-insensitive G_q subfamily have been shown to stimulate the β_1 isoform of PI-PLC (Blank *et al.*, 1991, Shenker *et al.*, 1991, Smrcka *et al.*, 1991, Taylor *et al.*, 1991, Berstein *et al.*, 1992). In contrast the γ isoform of PI-PLC is a direct target of receptors endowed with intrinsic, ligand-dependent, tyrosine kinase activity (reviewed in Cantley *et al.*, 1991). PDGF stimulates the phosphorylation of specific tyrosine residues of PI-PLCγ, thereby stimulating polyphosphoinositide hydrolysis (Kim *et al.*, 1991).

The experiments presented in this chapter were designed to determine whether rPMT utilises either of the two known pathways for the production of inositol phosphates by assessing the effect of rPMT treatment on G protein and tyrosine kinase-mediated increased production of inositol phosphates.

3.1. rPMT DISTINGUISHES BETWEEN THE NEUROPEPTIDE AND PDGF PATHWAYS FOR THE ENHANCEMENT OF INOSITOL PHOSPHATES.

In order to determine whether pretreatment of 3T3 cells with rPMT could facilitate the production of inositol phosphates by neuropeptides and PDGF, quiescent cultures of Swiss 3T3 cells labelled with [2-^{3}H]inositol were treated for 5 h with 2.5 ng/ml rPMT, a concentration that stimulated only a small increase in total inositol phosphate formation. The neuropeptides and PDGF were then added for a further 10 min and total inositol phosphates were analysed. Fig 3.1.1. shows that rPMT pretreatment caused a marked enhancement in the production of total inositol phosphates induced by bombesin, vasopressin and the mouse endothelin, VIC.

Fig. 3.1.1.

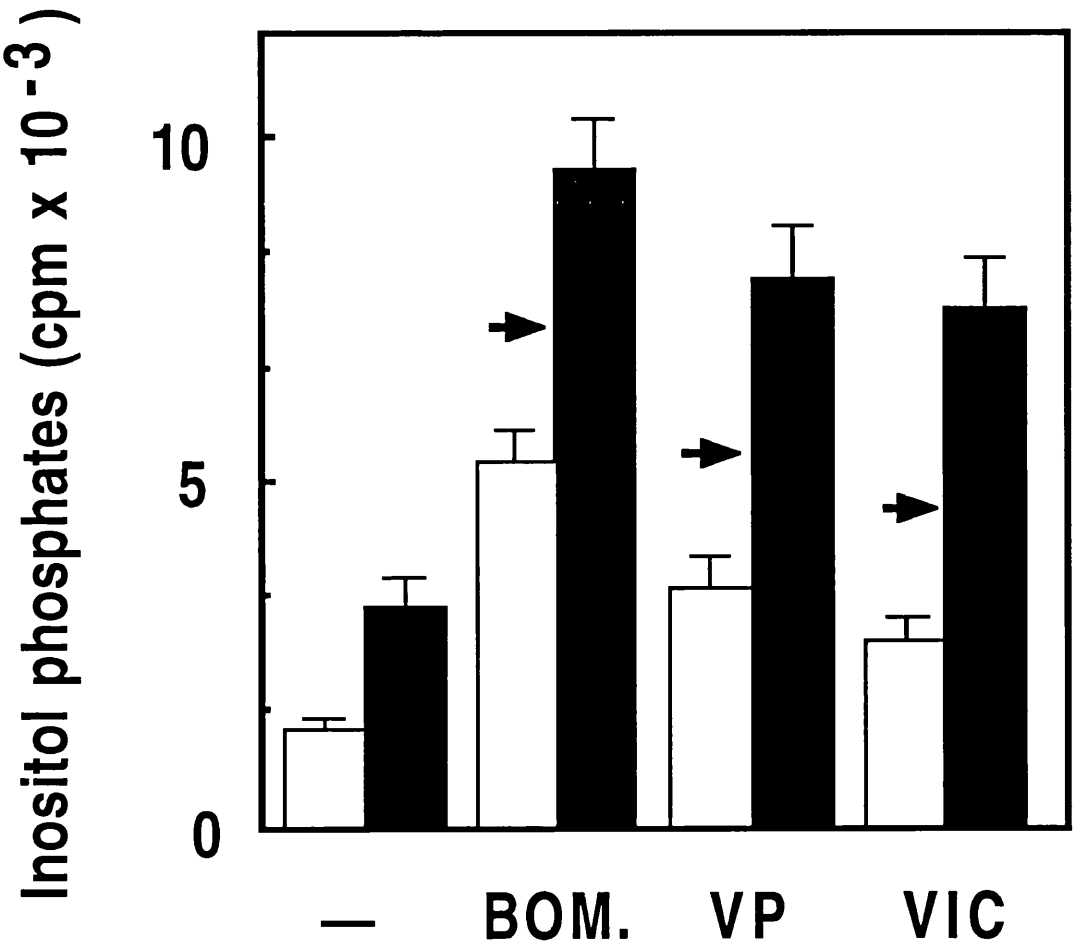


Fig. 3.1.1. Effect of rPMT pretreatment on inositol phosphate production by the neuropeptides. The cells were prelabelled with 10 μCi/ml [2-^{3}H]inositol for 16-18 h. rPMT was then added directly to the dishes to a final concentration of 2.5 ng/ml and the cultures were incubated at 37°C for 4.5 h. LiCl (20 mM) was then added and after a further 30 min the cellular inositol phosphate content was determined as described in 'Materials and Methods'. Bombesin (6 nM), vasopressin (10 nM), VIC (10 nM) were added to control (open bars) and rPMT pretreated (shaded bars) cells for 10 min prior to extraction. The increases in inositol phosphates expected from an additive effect are indicated by the arrows. The data represent means ± S.E.M. of triplicate determinations from ten individual experiments.

Fig. 3.1.2.

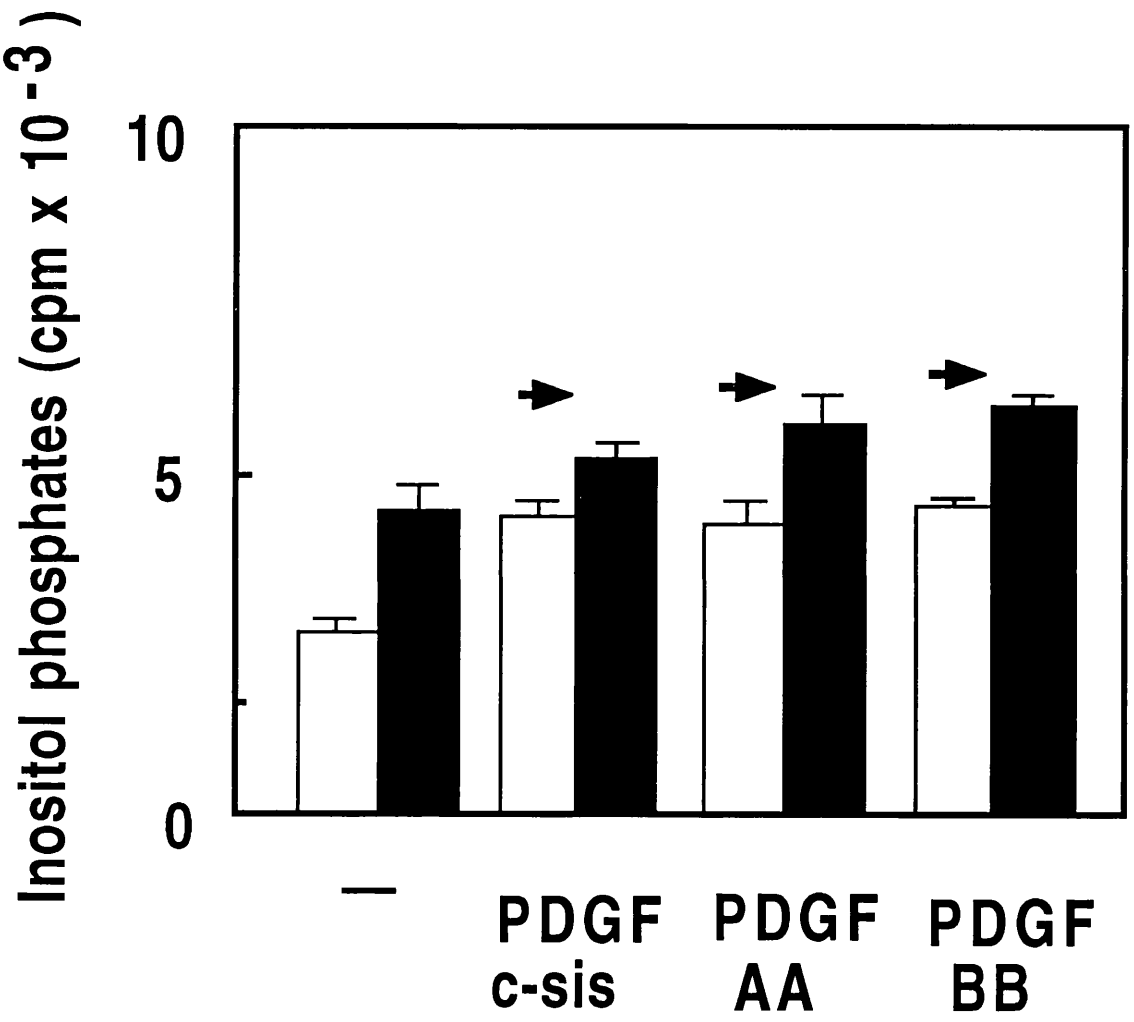


Fig. 3.1.2. Effect of rPMT pretreatment on inositol phosphate production by PDGF. Quiescent cultures of Swiss 3T3 cells were prelabelled with 10 μCi/ml [2-^{3}H]inositol for 16-18 h. rPMT was then added directly to the dishes to a final concentration of 2.5 ng/ml and the cultures were incubated at 37°C for 4.5 h. LiCl (20 mM) was then added and after a further 30 min the cellular inositol phosphate content was determined as described in 'Materials and Methods'. PDGF c-sis, AA and BB (25 ng/ml) were added to control (open bars) and rPMT pretreated (shaded bars) cells for 10 min prior to extraction. The increases in inositol phosphates expected from an additive effect are indicated by the arrows. The data represent means ± S.E.M. of triplicate determinations from four individual experiments.

Table 3.1.1.

Effects of insulin, EGF and bFGF on inositol phosphates induced by rPMT.

	Inositol phosphates (cpm)	
Growth Factor	Without rPMT	With rPMT
None	1447 ± 127	3422 ± 277
Insulin	1537 ± 15	3030 ± 126
bFGF	1620 ± 216	3915 ± 140
EGF	1402 ± 90	3090 ± 128
Bombesin	5363 ± 300	9600 ± 100

Cells were prelabelled with 10 μCi/ml of [2-^{3}H]inositol for 16 h. rPMT was then added directly to the dishes and incubated at 37 °C for 4.5 h. LiCl (20 mM) was then added and after a further 30 min the cellular inositol phosphate content was determined. bFGF (10 ng/ml), EGF (5 ng/ml), insulin (1 μg/ml) or bombesin (6nM) were added to control and rPMT pretreated cells for 10 min prior to extraction. Results are means ± S.E.M. for triplicate determinations of three individual experiments.

In all cases, the enhancement was greater than that expected from an additive effect which is indicated by the arrows in Fig. 3.1.1. In contrast, rPMT pretreatment did not potentiate the inositol phosphates produced in response to either PDGF c-sis or the homodimers PDGF AA and PDGF BB. The resulting stimulation of inositol phosphate production was additive rather than synergistic (Fig.3.1.2).

In addition to PDGF, other polypeptide growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and insulin are mitogenic for Swiss 3T3 cells (Rozengurt, 1986). These growth factors also initiate their mitogenic responses through receptors possessing intrinsic tyrosine kinase activity (Cantley *et al.*, 1991 and section 1.2). In some cell types the mitogenic effects of these growth factors occurs in the absence of phosphoinositide hydrolysis. It should be noted that bFGF has been shown to stimulate inositol phosphate production in different cell types including Swiss 3T3 cells, but these results have been controversial (Tsuda *et al.*, 1985, Kaibuchi *et al.*, 1986, Magnaldo *et al.*, 1986, Paris *et al.*, 1988, Brown *et al.*, 1989). In our laboratory, the mitogenic effect of bFGF on Swiss 3T3 fibroblasts has been demonstrated to occur without stimulation of inositol phosphates (Nanberg *et al.*, 1990). In addition, EGF has been shown to stimulate phosphoinositide hydrolysis in certain cell types through the activation of PI-PLC-γ. However, the ability of this growth factor to activate this enzyme requires that a relatively large number of EGF receptors be present (Wahl *et al.*, 1988, Margolis *et al.*, 1989). Swiss 3T3 cells express relatively low numbers of EGF receptors (~50,000 receptors per cells), which probably accounts for the inability of EGF to stimulate inositol phosphate production in these cells (Margolis *et al.*, 1989). It was therefore important to assess the effects of these growth factors on the stimulation of inositol phosphates in rPMT treated cells. In particular, if rPMT were to be acting through the PI-PLCγ pathway for the stimulation of inositol phosphates it could act intracellularly to facilitate the coupling of these growth factor receptors to PI-PLCγ. Table 3.1.1 demonstrates that, in agreement with previous results, EGF, bFGF and insulin did not stimulate inositol phosphates on their own in Swiss 3T3 cells. More importantly, addition of these growth factors to rPMT-treated cells had no effect on the production of inositol phosphates induced by rPMT. For comparison the increase in inositol phosphates observed on stimulation of these cells with bombesin, and the striking enhancement seen with bombesin in rPMT-treated cells is also shown (Table 3.1.1).

3.2. EFFECT OF LI^+ ON THE ENHANCEMENT OF INOSITOL PHOSPHATES BY rPMT.

The experiments in the previous section were carried out in the presence of LiCl. LiCl is known to inhibit some of the enzymes which hydrolyse the inositol phosphates, in particular InsP phosphatases and inositolpolyphosphate-1-phosphatase (see Fig. 1.3.1.). These

Table. 3.2.1.

Enhancement by LiCl of the production of inositol phosphates by rPMT, neuropeptides and PDGF.

	Inositol Phosphates cpm	
Addition	Control	LiCl
Control	187 ± 4	232 ± 5
rPMT	235 ± 7	573 ± 50
Bombesin	300 ± 10	653 ± 60
rPMT + Bombesin	577 ± 12	1551 ± 70
Vasopressin	209 ± 2	491 ± 24
rPMT + Vasopressin	455 ± 46	1402 ± 7
PDGF	293 ± 6	600 ± 30
rPMT + PDGF	315 ± 25	722 ± 20

Swiss 3T3 cells were labelled with 10 μCi/ml [2-^{3}H]inositol for 16-18 h. rPMT was then added directly to the dishes to a final concentration of 2.5 ng/ml and the cultures were incubated at 37 °C for 4.5 h. NaCl (Control) or LiCl (20 mM) was then added and after a further 30 min the cellular inositol phosphate content was determined as described in 'Materials and Methods'. Bombesin (6 nM), vasopressin (10 nM) as well as PDGF c-sis (25 ng/ml) were added to untreated and rPMT pretreated cells for 10 min prior to extraction. The data represent means ± S.E.M. of determinations from two individual experiments.

enzymes are important in the metabolism of $Ins(1,4,5)P_3$ the direct product of $PtdIns(4,5)P_2$ hydrolysis (Fig 1.3.1.). Thus, LiCl acts to facilitate the accumulation of inositol phosphate metabolites stimulated in response to PI-PLC activation. However, it could not be ruled out that LiCl could have other effects e.g. on the translocation and activation of rPMT. It was therefore important to assess the effect of LiCl on the production of inositol phosphates by rPMT. Table 3.2.1. demonstrates that rPMT can stimulate inositol phosphate production in 3T3 cells in the absence of LiCl. This stimulation is clearly amplified (2.4-fold) in the presence of LiCl. The inositol phosphates produced by either bombesin, vasopressin or PDGF are similarly amplified in the presence of LiCl. More importantly, the enhancement of neuropeptide-stimulated inositol phosphates in rPMT-treated cells was also apparent in the absence of LiCl while no enhancement was observed with PDGF in rPMT-treated cells.

3.3. ENHANCING EFFECT OF rPMT PRETREATMENT ON THE PRODUCTION OF INOSITOL PHOSPHATES: CONCENTRATION DEPENDENCE AND TIME-COURSE.

The substantial enhancement of total inositol phosphates by the neuropeptides but not PDGF in the presence of rPMT was used to further elucidate the pathways and mechanisms involved. The effect of pretreatment with different concentrations of rPMT on the enhancement of inositol phosphates elicited by bombesin vasopressin and PDGF is shown in Fig. 3.3.1. rPMT on its own increased the accumulation of inositol phosphates in a dose dependent manner. Addition of bombesin caused a shift in the dose response; the concentration of rPMT required to produce half maximal effect was reduced from 9 ng/ml to 3 ng/ml (Fig. 3.3.1 A). Vasopressin addition also caused a shift in the dose response of inositol phosphate production by rPMT (Fig. 3.3.1 B). In contrast, addition of PDGF to cells pretreated with different concentrations of rPMT had no effect on the half maximal concentration and the resulting dose response curve was that expected from an additive effect (Fig. 3.3.1 C).

The dose-dependent stimulation of inositol phosphates by bombesin was also enhanced by treatment of cells with rPMT for all concentrations of bombesin used. Even concentrations of bombesin which gave a maximal stimulation of inositol phosphates were greatly enhanced in cells which had been pretreated with rPMT at 2.5 ng/ml (Fig 3.3.2.).

The time course of inositol phosphate accumulation induced by either bombesin or PDGF in control and rPMT-pretreated cells is shown in Fig. 3.3.3. The rate of accumulation of inositol phosphates stimulated by bombesin in rPMT-pretreated cells is 2-fold higher than the rate induced by bombesin in control cells (Fig. 3.3.3 A). In contrast, the rate of accumulation of

Fig. 3.3.1.

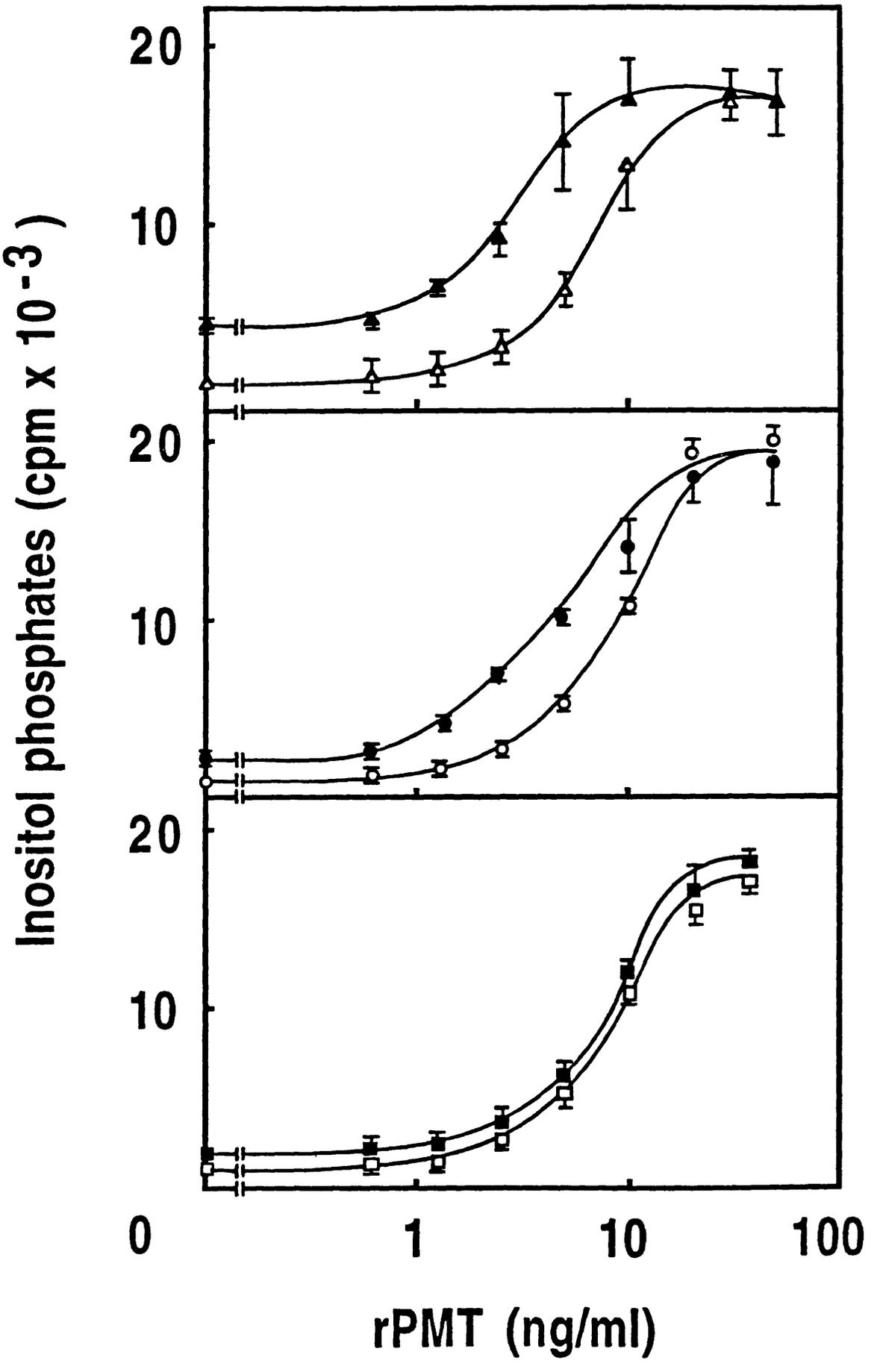

Inositol phosphates (cpm x 10-3)
20
10
20
10
20
10
0
1
10
100
rPMT (ng/ml)

Fig. 3.3.1. Effects of pretreatment with increasing concentrations of rPMT on inositol phosphate production in the absence or presence of bombesin, vasopressin and PDGF. [2-^{3}H]inositol-labelled Swiss 3T3 cells were incubated with increasing concentrations of rPMT for 4.5 h. LiCl was then added to give a final concentration of 20 mM. Total inositol phosphates were extracted after 30 min in the absence (open symbols) or presence (closed symbols) of **A.** Bombesin (6 nM) **B.** Vasopressin (10 nM) or **C.** PDGF c-sis (25 ng/ml). These factors were added directly to the cultures 10 min prior to extraction. Analysis of total inositol phosphates was as described in 'Materials and Methods'. Values shown are the mean ± S.E.M. of triplicate determinations.

Fig. 3.3.2.

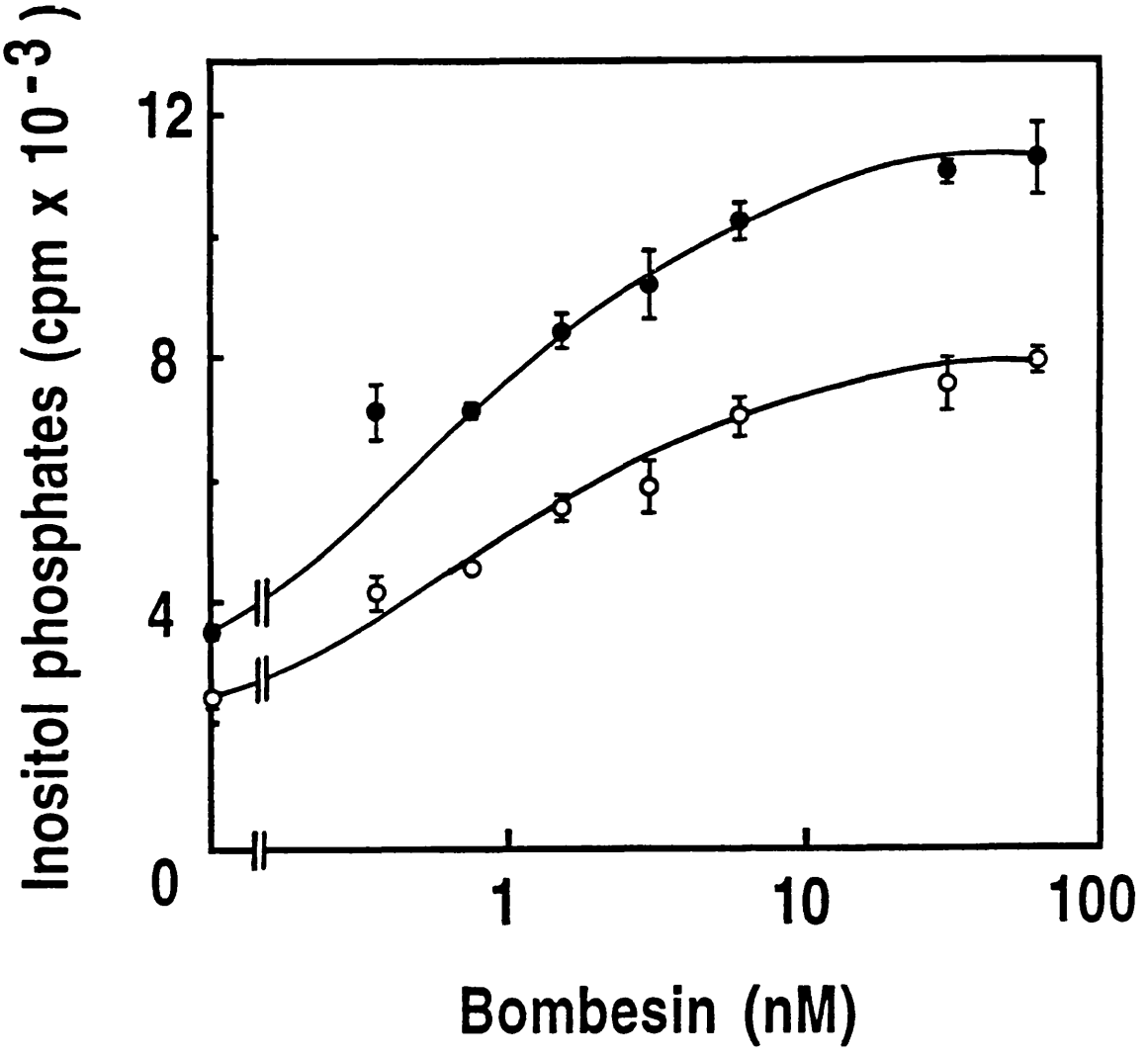


Fig. 3.3.2. The effect rPMT-treatment on the dose dependent production of inositol phosphates by bombesin. [2-^{3}H]inositol-labelled Swiss 3T3 cells were incubated with rPMT at a final concentration of 2.5 ng/ml for 4.5 h. LiCl was then added to give a final concentration of 20 mM. Total inositol phosphates were extracted after 30 min in the absence (open symbols) or presence (closed symbols) of increasing concentrations of Bombesin (6 nM) which was added directly to the cultures for 10 min prior to extraction. Analysis of total inositol phosphates was as described in 'Materials and Methods'. Values shown are the mean ± S.E.M. of triplicate determinations.

Fig. 3.3.3.

Fig. 3.3.3. The effect of rPMT pretreatment on the time course of inositol phosphate production elicited by bombesin or PDGF. The cells were labelled for 16-18 h with 10 μCi/ml [2-^{3}H]inositol in DMEM:Waymouths medium (1:1). Pretreatment with rPMT (2.5 ng/ml) was for 4.5 h when LiCl to a final concentration of 20 mM was added. **A.** Addition of 6 nM bombesin, to control (open triangles) and rPMT pretreated cells (closed triangles). **B.** Addition of PDGF (25 ng/ml) to control (open circles) and rPMT pretreated cells (closed circles). Bombesin and PDGF were added 20 min after the addition of LiCl (zero time). No additions to control (open squares) or rPMT pretreated cells (closed squares) are shown in A & B. Total inositol phosphates were extracted at the times indicated. Values shown are means ± S.E.M. of triplicate determinations and are representative of three individual experiments.

inositol phosphates stimulated by PDGF in either control or rPMT-pretreated cells was identical (Fig. 3.3.3.B).

Thus rPMT can distinguish between neuropeptide and PDGF induced inositol phosphate accumulation in Swiss 3T3 cells.

3.4. EFFECT OF rPMT TREATMENT ON THE ACCUMULATION OF INS(1,4,5)P_3 INDUCED BY BOMBESIN AND PDGF.

The preceding results have all involved the analysis of total inositol phosphates. In order to determine whether treatment with rPMT potentiates the ability of neuropeptides to stimulate the production of Ins(1,4,5)P_3, one of the products of the reaction catalysed by PI-PLC we measured the cellular content of this second messenger using a radiolabelled-ligand displacement assay. Cultures of Swiss 3T3 cells were pretreated with or without 2.5 ng/ml rPMT for 5 h and then challenged with bombesin for various times. As shown in Fig. 3.4.1., addition of bombesin to control cells caused a rapid increase in the cellular level of Ins(1,4,5)P_3 peaking 10 sec after stimulation with an 80% increase over basal. When bombesin was added to cells which have been pretreated with rPMT the accumulation of Ins(1,4,5)P_3 was dramatically increased; even 5 sec after stimulation the increase rose from 40 to 200%. These elevated levels of Ins(1,4,5)P_3 persisted for the duration of the experiment (Fig. 3.4.1.). PDGF also increased the cellular level of Ins(1,4,5)P_3 in these cells. This occurred at a slower rate , peaking 60 sec after addition with an increase of about 80% over basal levels. These kinetic results are in agreement with previous results obtained in [^{3}H]inositol-labelled Swiss 3T3 cells (Nanberg and Rozengurt, 1988). However, in contrast to the results observed for bombesin, addition of PDGF to rPMT-treated cells did not result in the potentiation of the levels of Ins(1,4,5)P_3 induced by PDGF on its own (Fig. 3.4.2.). Thus, rPMT selectively facilitates the neuropeptide-stimulated signal transduction pathway for the stimulation of inositol phosphates.

3.5. rPMT AND BOMBESIN MOBILIZE Ca^{2+} FROM A COMMON INTRACELLULAR POOL.

It is known that an increase in Ins(1,4,5)P_3 content causes a rapid mobilization of an intracellular store of Ca^{2+} (Mendoza *et al.*, 1986b, Lopez-Rivas *et al.*, 1987, Nanberg and Rozengurt, 1988). In Swiss 3T3 cells, intracellular Ca^{2+} mobilised in response to growth factors acting via Ins(1,4,5)P_3, including bombesin, is subsequently released into the extracellular medium leading to a decrease in the cellular Ca^{2+} content (Mendoza *et al.*,

Fig. 3.4.1.

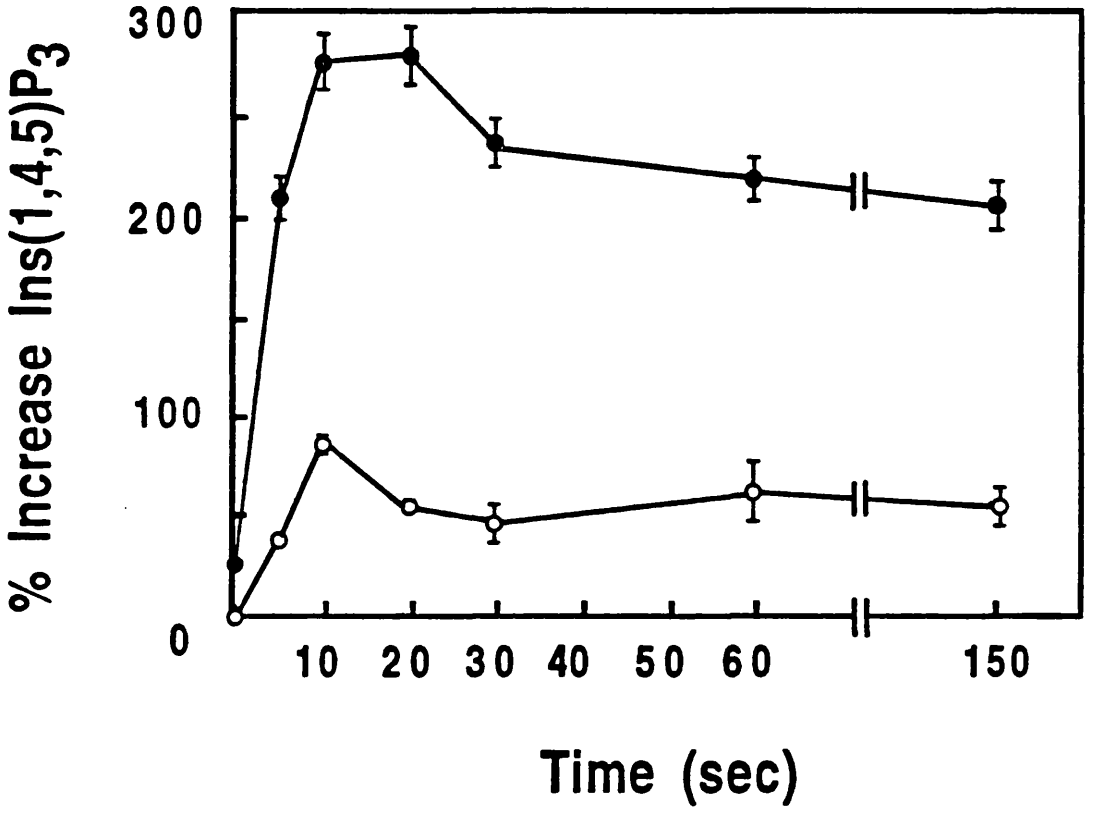


Fig. 3.4.1. Effect of bombesin on the cellular content of Ins(1,4,5)P_3 in control and rPMT treated cells. Quiescent cultures of Swiss 3T3 cells in 90 mm dishes were treated with 2.5 ng/ml rPMT for 5 h. Then, bombesin (6 nM) was added to control (open symbols) or rPMT pretreated (closed symbols), cultures for the indicated times. For each sample Ins(1,4,5)P_3 was measured as described under 'Materials and Methods' and the percentage increase in Ins(1,4,5)P_3 over basal was determined (Basal level was 11.3 ± 3 pmoles Ins(1,4,5)$P_3/10^6$ cells). Each point represents the mean ± S.E.M. of triplicate determinations taken from two individual experiments.

Fig. 3.4.2.

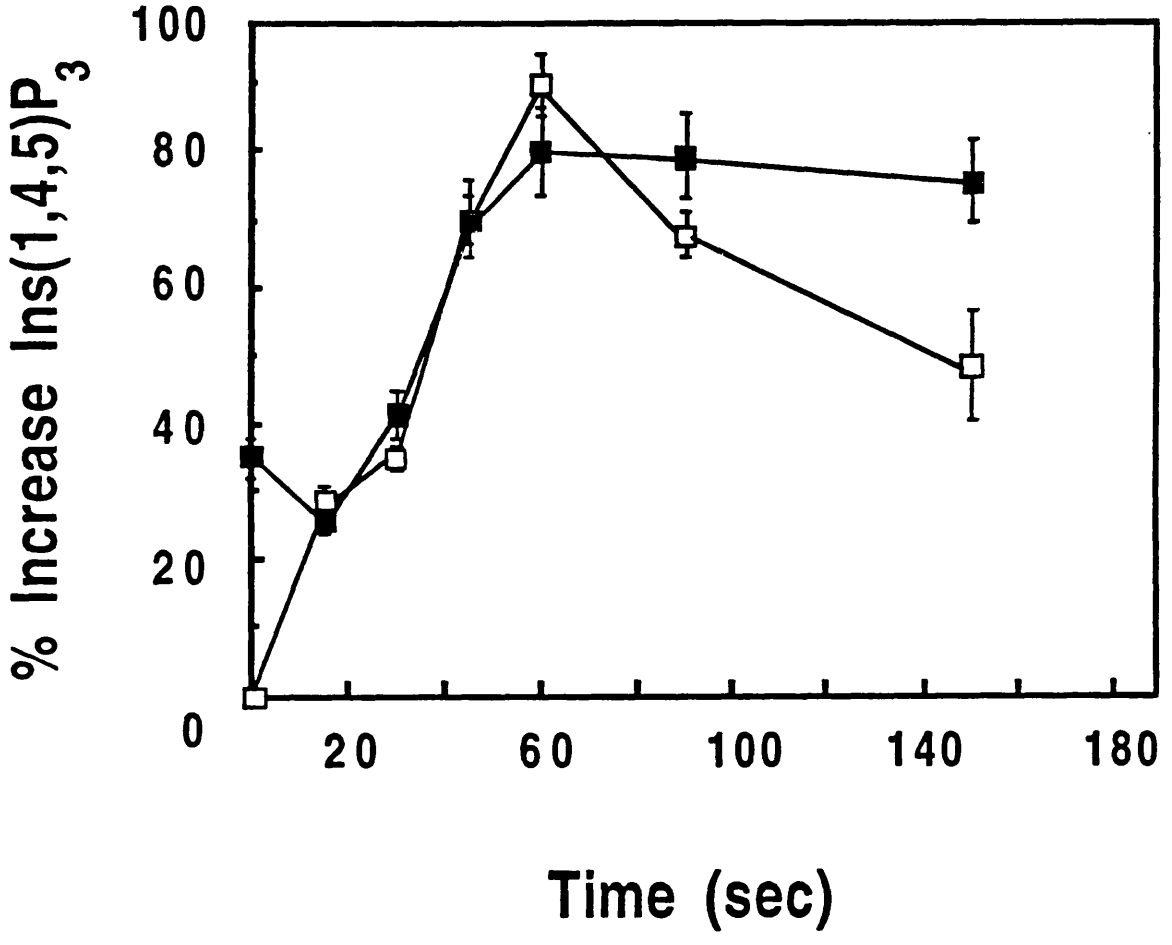


Fig. 3.4.2. rPMT pretreatment has no effect on the cellular content of Ins(1,4,5)P_3 induced by PDGF. Quiescent cultures of Swiss 3T3 cells in 90 mm dishes were treated with 2.5 ng/ml rPMT for 5 h. PDGF 25 ng/ml was then added to control (open symbols) or rPMT pretreated (closed symbols), cultures for the indicated times. For each sample Ins(1,4,5)P_3 was measured as described under 'Materials and Methods and the percentage increase in Ins(1,4,5)P_3 over basal was determined (Basal level was 13.7 ± 3 pmoles Ins(1,4,5)$P_3/10^6$ cells). Each point represents the mean ± S.E.M. of triplicate determinations taken from two individual experiments.

1986b, Lopez-Rivas *et al.*, 1987, Nanberg and Rozengurt, 1988). rPMT has been shown to mobilize intracellular Ca^{2+} in a time and dose dependent manner (Staddon *et al.*, 1991a). Our results so far demonstrate that rPMT acts to stimulate Ins(1,4,5)P_3 production through the same pathway as bombesin and therefore we might expect that these two agents mobilize Ca^{2+} from a common pool. Fig 3.5.1.A. shows that pretreatment of cells with rPMT for 5 h greatly reduced the increase in intracellular Ca^{2+} caused by bombesin. Protein kinase C activation is known to attenuate Ca^{2+} mobilization by low concentrations of bombesin (Lopez-Rivas *et al.*, 1987) and rPMT activates protein kinase C (Staddon *et al.*, 1990). However under identical conditions, it was confirmed that treatment of the cells with the protein kinase C activator PBt_2 did not decrease the Ca^{2+} mobilizing action of bombesin (Fig. 3.5.1.A). Attenuation by rPMT pretreatment was observed in the presence or absence of extracellular Ca^{2+}, indicating that the source of the Ca^{2+} mobilised by bombesin was intracellular (Fig. 3.5.1.B). Furthermore, Fig 3.5.1.C shows that the decrease in the Ca^{2+}-mobilizing action of bombesin caused by rPMT was dependent upon the time of exposure of the cells to the toxin, exhibiting a lag period which is consistent with the lag period required for rPMT to enter the cells and stimulate inositol phosphate production (Rozengurt *et al.*, 1990b). The decrease of Ca^{2+} mobilization by bombesin in rPMT-treated cells is consistent with a common pool of Ca^{2+}, presumably the Ins(1,4,5)P_3-sensitive pool.

3.6. rPMT DOES NOT AFFECT THE TYROSINE PHOSPHORYLATION OF PI-PLCγ.

In order to substantiate further the differential effects of rPMT on G protein and tyrosine kinase signal transduction we determined whether rPMT has any effect on PDGF-stimulated phosphorylation of PI-PLCγ in Swiss 3T3 cells. Cultures incubated in the presence or absence of rPMT received either bombesin or PDGF for 10 min. The cells were lysed and the extracts were immunoprecipitated with an antibody against PI-PLCγ. Western blot analysis of the immunoprecipitates with an antiphosphotyrosine antibody showed that PDGF stimulates tyrosine phosphorylation of this enzyme to the same extent in both control and rPMT-treated cells (Fig. 3.6.1.). A protein phosphotyrosine band migrating with an apparent molecular mass of 170-190 kDa which was also detected in the immunoprecipitates of PDGF-treated cells is most likely the autophosphorylated PGDF receptor which is known to become tightly associated with PI-PLCγ (Cantley *et al.*, 1991 and section 1.3.1.). In contrast, bombesin did not stimulate the phosphorylation of PI-PLCγ either in control cells or in rPMT-treated cells (Fig. 3.6.1.). Addition of 20 ng/ml rPMT, a concentration that induced a massive accumulation of inositol phosphates, did not increase tyrosine phosphorylation of PI-PLCγ (results not shown).

Fig. 3.5.1.

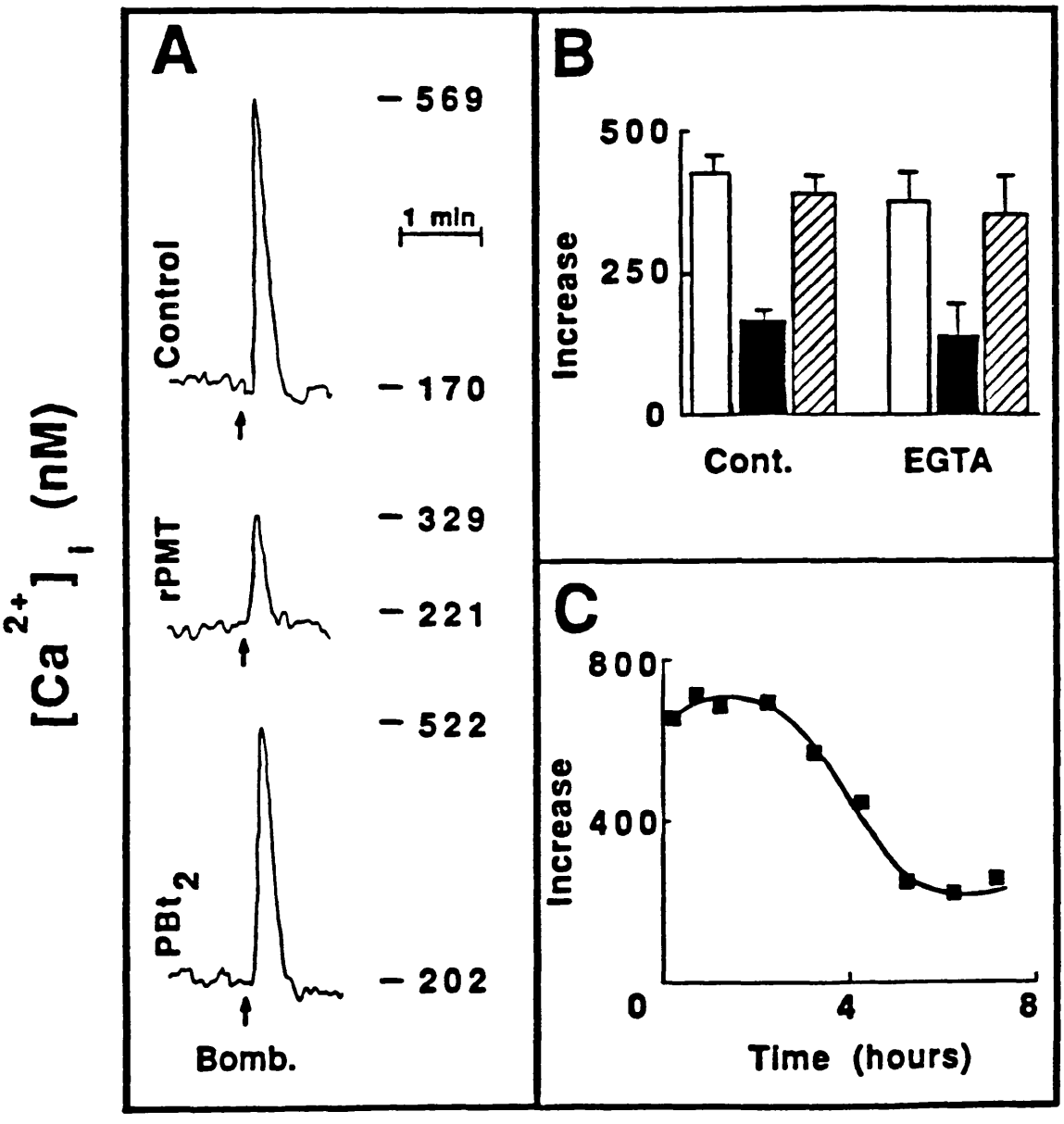

A
B
C
[Ca2+]i (nM)
Control
rPMT
PBt2
Bomb.
1 min
569
170
329
221
522
202
500
250
0
Increase
Cont.
EGTA
800
400
0
4
8
Time (hours)

Fig. 3.5.1. rPMT and Bombesin mobilize $Ca^{2+}$from a common intracellular pool. $[Ca^{2+}]_i$ was measured as described under 'Materials and Methods'. The bombesin-induced increase in $[Ca^{2+}]_i$ in control cells, cells pretreated with 20 ng/ml rPMT for 5 h or cells pretreated with 200 nM PBt_2 for 10 min is shown in **A.** Bombesin was added at a final concentration of 10 nM. For the PBt_2 pretreatment, the phorbol ester and fura-2/AM were added to the cells simultaneously. PBt_2 was then readded, at the same concentration, to the cells in the cuvette. In three independent cell preparations, the value (mean ± S.E.M.) for resting $[Ca^{2+}]_i$ in the control cells (186 ± 20 nM, 11 observations) was not statistically significantly different ($p < 0.1$, by student's *t* test) from the $[Ca^{2+}]_i$ found after the rPMT pretreatment (202 ± 30 nM, 15 observations). The values shown in **B.** summarise the data obtained from three independent experiments (mean ± S.E.M.) showing the bombesin-induced increase in $[Ca^{2+}]_i$ in control cells (open bars), or cells pretreated with rPMT (closed bars), or cells pretreated PBt_2 (striped bars). The cells were incubated as in A, in the absence of EGTA (Cont.) or in its presence (EGTA). EGTA was added 1 min prior to the addition of bombesin to give a final concentration of 2 mM. The pH of the incubation medium was not changed by the addition of EGTA. In **C.**, the cells were pretreated with 20 ng/ml rPMT for various times, and the increase in $[Ca^{2+}]_i$ induced by bombesin (10 nM) was then determined.

Fig. 3.6.1.

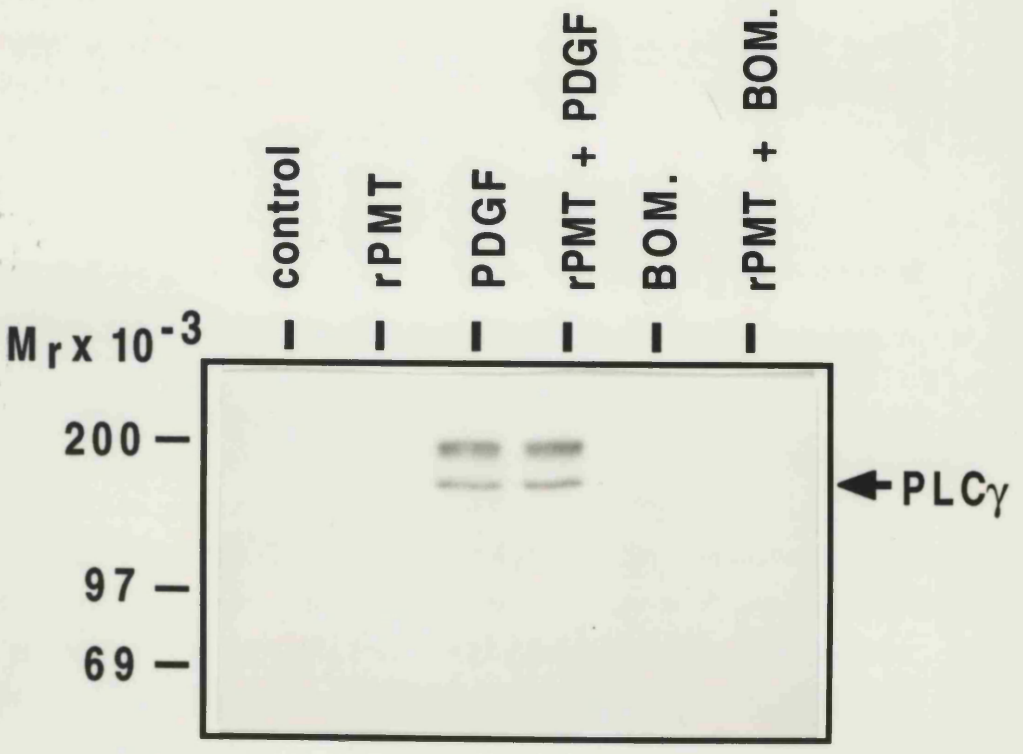

control
rPMT
PDGF
rPMT + PDGF
BOM.
rPMT + BOM.
Mr x 10-3
200
97
69
PLCγ

Fig. 3.6.1. Phosphorylation of PI-PLCγ in Swiss 3T3 cells. Quiescent Swiss 3T3 cells in 30 mm dishes were incubated with or without rPMT (2.5 ng/ml) for 5 h. Bombesin (6 nM) or PDGF (25 ng/ml) were added to control and rPMT-treated cells for 10 min. The cells were lysed and the extracts were immunoprecipitated with an anti-PLCγ polyclonal antibody; immunoprecipitates were analysed by Western blotting with PY20 anti-Tyr(P) monoclonal antibody under standard conditions and visualised as described in 'Materials and Methods'.

3.7. DOWN-REGULATION OF PKC.

Activation of PKC can also distinguish between the G protein and tyrosine kinase pathways leading to polyphosphoinositide hydrolysis. Phorbol esters inhibit inositol phosphate production induced by bombesin or vasopressin (Brown *et al.*, 1987, Lopez-Rivas *et al.*, 1987, Brown *et al.*, 1990, Ryu *et al.*, 1990) but do not affect inositol phosphate formation in response to PDGF (Lopez-Rivas *et al.*, 1987). Accordingly, down-regulation of PKC enhances the formation of inositol phosphates by neuropeptides but not by PDGF (Lopez-Rivas *et al.*, 1987). If rPMT and neuropeptides utilise the same pathway to promote activation of inositol phospholipid breakdown it could be predicted that PKC down-regulation should also potentiate the production of inositol phosphates in response to rPMT. Fig. 3.7.1. (left), shows that, in PKC-down regulated cells, rPMT, like bombesin, induced a further striking increase in inositol phosphate production. In contrast, the increase of inositol phosphates elicited by PDGF was not affected by PKC-down regulation. Fig.3.7.1. (right), shows that PKC-down regulation markedly enhanced the formation of inositol phosphates induced by various concentrations of rPMT.

3.8. THE ENHANCING EFFECT OF rPMT DOES NOT REQUIRE *DE NOVO* PROTEIN SYNTHESIS.

A possible explanation of the previous results is that the enhancing effect of rPMT could result from *de novo* synthesis of neuropeptide receptors or other signal transduction proteins. Alternatively, the toxin could induce a post-translational modification of a pre-existing constituent of the neuropeptide signal transduction pathway. We investigated the first hypothesis in a variety of ways. To test the possibility that the number or affinity of bombesin receptors may have been altered by rPMT pretreatment, Scatchard analysis of cellular binding sites was performed using ^{125}I-GRP in control cells and in cells which had been pretreated with 2.5 ng/ml rPMT for 5 h. As the results in Fig. 3.8.1. demonstrate, the affinity and total number of bombesin/GRP receptor binding sites for ^{125}I-GRP is unaffected.

We next examined the possibility that rPMT might increase the levels of the α subunits of Gq, the G protein subunits that have been demonstrated to regulate PI-PLCβ. Western blot analysis using an antibody raised against the common C-terminal decapeptide of α_q and α_{11} (Gutowski *et al.*, 1991) demonstrated the presence of a band migrating with an apparent molecular weight of 42-43 kDa. Under conditions that resulted in a marked increase in inositol phosphate production by rPMT, we did not detect any increase in the levels of these proteins (Fig. 3.8.2. inset). In addition, cycloheximide, a protein synthesis inhibitor, had no effect on inositol phosphate production induced by rPMT, bombesin or a combination of the

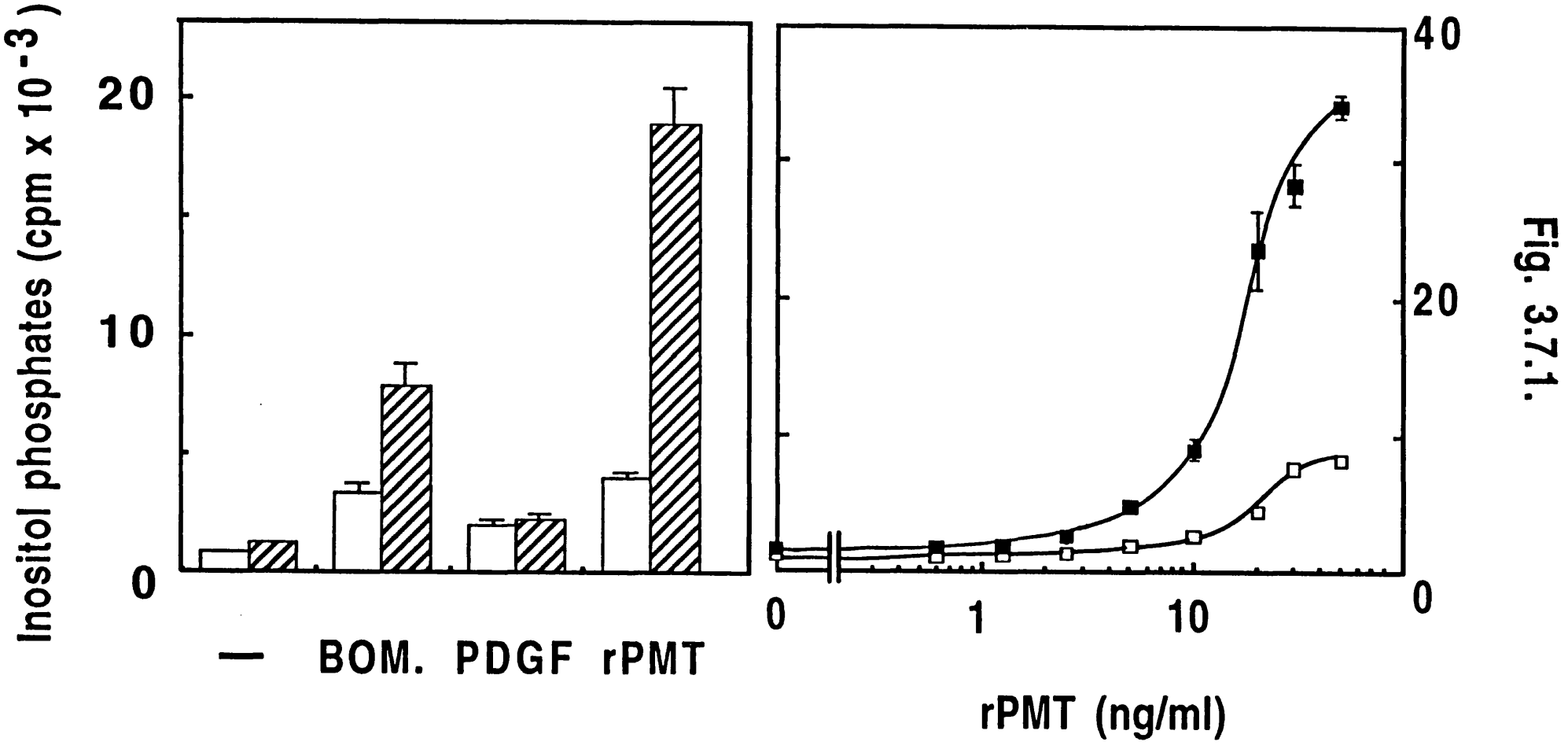

Inositol phosphates (cpm x 10-3)
20
10
0
BOM. PDGF rPMT
40
20
0
0
1
10
rPMT (ng/ml)

Fig. 3.7.1.

Fig.3.7.1. Effect of down-regulation of PKC on the enhancement of inositol phosphates by rPMT. Control cultures or those pretreated with phorbol 12,13-dibutyrate (1 mM) for 48 h were incubated with 10 μCi/ml [2-^{3}H]inositol for 16-18 h. Inositol phosphates were extracted as described previously. **Left.** Control (open bars) or PKC down-regulated (hatched bars) cells were incubated with rPMT(10 ng/ml) for 5 h or with bombesin (6 nM) and PDGF (25 ng/ml) for 10 min. In all cases LiCl was added 30 min prior to extraction at a final concentration of 20 mM. **Right.** Increasing concentrations of rPMT were added to control (open symbols) and PKC down-regulated (closed symbols) cultures. rPMT treatment was for 5 h and LiCl (final concentration 20 mM) was added 30 min prior to extraction. Values represent means ± S.E.M. of triplicate determinations.

Fig. 3.8.1.

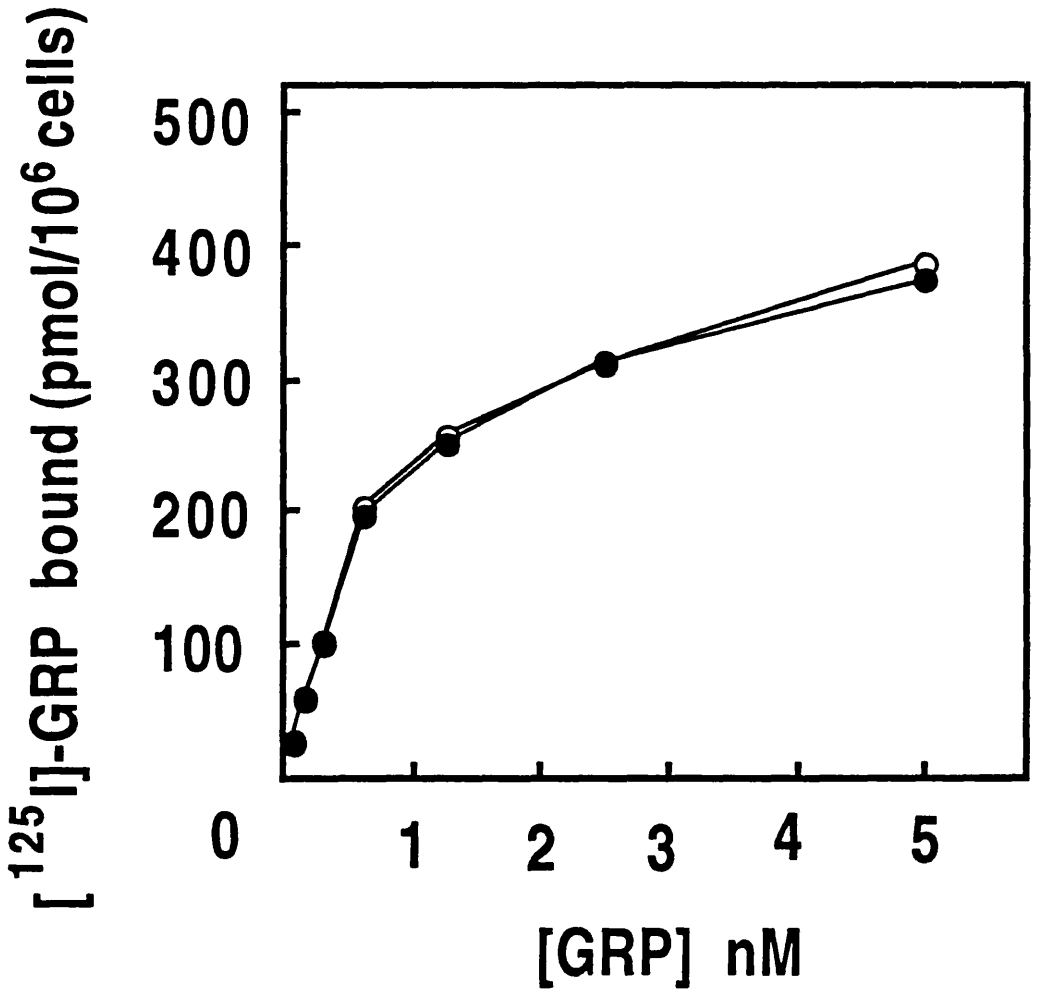

[125I]-GRP bound (pmol/10^6 cells)
500
400
300
200
100
0
1
2
3
4
5
[GRP] nM

Fig. 3.8.1. The effect of rPMT on the number and binding affinity of ^{125}I-GRP for the bombesin receptor Confluent and quiescent cultures of Swiss 3T3 cells were pretreated for 5 h in the absence (open circles) or presence (closed circles) of rPMT (2.5 ng/ml). The cells were then washed and incubated in the presence of various concentrations of ^{125}I-labelled GRP in binding medium (DMEM:Waymouth medium (1:1) containing BSA at 1 mg/ml, pH 7) for 30 min at 37 °C and 10% CO_2/90% air. After this time the cultures were washed and extracted and total cell-associated radioactivity was determined. Each point represents the mean ± S.E.M. of triplicate determinations.

Fig. 3.8.2.

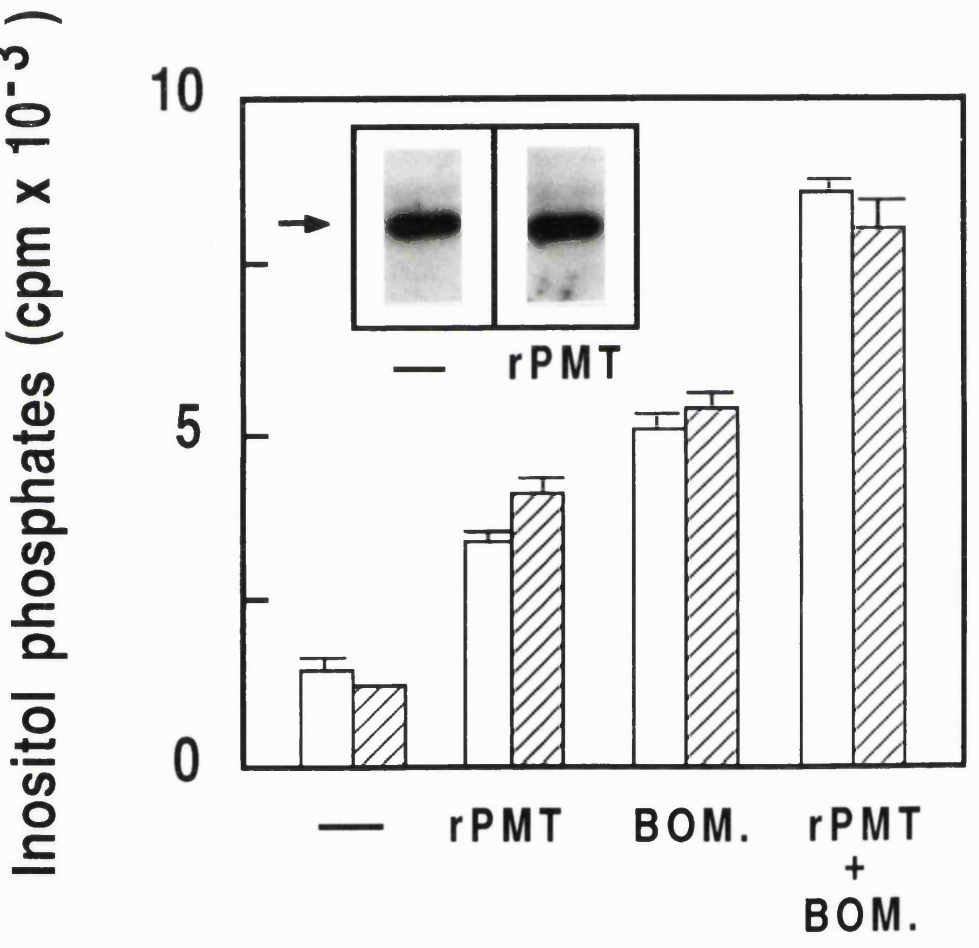


Fig. 3.8.2. rPMT-mediated enhancement of inositol phosphate production is not dependent on *de novo* protein synthesis. Confluent and quiescent cultures Swiss 3T3 cells were labelled for 16-18 h with 10 μCi/ml [2-^{3}H]inositol. The cells were incubated with rPMT in the absence (open bars) or presence (cross hatched bars) of cycloheximide (5 μg/ml) for 4.5 h. LiCl was then added and after a further 30 min total inositol phosphates were extracted as described. Stimulation with bombesin (6 nM) was for 10 min prior to extraction. Values represent the mean ± S.E.M. of triplicate determinations for two separate experiments. **Inset:** Cultures of Swiss 3T3 cells which were either untreated or pretreated with rPMT (20 ng/ml) for 5 h were lysed in SDS/PAGE sample buffer, electrophoresed in 12.5 % SDS/PAGE, and analysed by Western blotting using an antibody raised against the C-terminal decapeptide of $G\alpha_{q/11}$. The 42-kDa protein recognised specifically by the antibody is indicated by the arrow.

two (Fig. 3.8.2.). The data strongly suggest that the enhancing effect of rPMT on the production of inositol phosphates induced by neuropeptides is the result of a post-translational modification(s) rather than of increased protein synthesis.

3.9. SUMMARY AND DISCUSSION

PMT is the first intracellularly acting bacterial toxin that induces a dramatic increase in polyphosphoinositide breakdown (Rozengurt *et al.*, 1990b, Staddon *et al.*, 1991a, Higgins *et al.*, 1992) and therefore may provide a novel tool to study the effects of persistent activation of this signal transduction pathway in responsive intact cells. The mechanism of action of rPMT assumes an added importance in view of the fact that this toxin potently stimulates cellular proliferation and anchorage-independent cell growth (Rozengurt *et al.*, 1990b, Higgins *et al.*, 1992).

In many cell types including Swiss 3T3 cells polyphosphoinositide hydrolysis by PI-PLC isoforms is activated by at least two distinct mechanisms (Lopez-Rivas *et al.*, 1987, Nanberg and Rozengurt, 1988). One involves neuropeptide receptors coupled to G proteins, most likely G_q, which regulate the activity of PI-PLCβ1 (Blank *et al.*, 1991, Smrcka *et al.*, 1991, Taylor and Exton, 1991, Berstein *et al.*, 1992) and the other involves PDGF-mediated tyrosine phosphorylation of specific residues of PI-PLCγ (Kim *et al.*, 1991).

Our results indicate that rPMT selectively potentiates neuropeptide-mediated inositol phosphate production. Treatment of cells with a subsaturating concentration of the toxin markedly enhanced the accumulation of inositol phosphates induced by bombesin, vasopressin and the mouse endothelin, VIC. These peptides bind to distinct receptors (Erusalimsky *et al.*, 1988, Erusalimsky and Rozengurt, 1989, Rozengurt *et al.*, 1990a, Sinnett-Smith *et al.*, 1990) and signal through a G protein pathway, as shown previously using permeabilized cells and membrane preparations (Collins and Rozengurt, 1983, Zachary and Rozengurt, 1985a, Coffer, *et al.*, 1990, Fabregat and Rozengurt, 1990, Rozengurt, *et al.*, 1990) Cloning and sequencing of these receptors demonstrate that they belong to the superfamily of G protein-linked receptors characterised by seven putative transmembrane domains (Arai *et al.*, 1990, Sakurai *et al.*, 1990, Battey *et al.*, 1991). In sharp contrast to the effects observed with the neuropeptides, rPMT treatment did not enhance the accumulation of inositol phosphates induced by PDGF, either the BB or the AA homodimers. Furthermore, rPMT did not increase PDGF-mediated tyrosine phosphorylation of the PI-PLCγ isoform. These results clearly indicate that rPMT facilitates signal transduction through the neuropeptide-mediated pathway.

In theory, the toxin could enhance the responses initiated by bombesin, vasopressin or endothelin by increasing the efficiency of ligand binding to these receptors. However, the toxin does not change the number or affinity of the bombesin/GRP receptor for ligand and the potentiating effect occurs in the absence of *de novo* protein synthesis. Thus rPMT facilitates signal transduction at a point distal to the receptors. This conclusion is in accord with the fact that rPMT can induce a massive polyphosphoinositide breakdown when it is added in the absence of synergistic peptides but at higher concentrations than those used to amplify neuropeptide-mediated signal transduction.

A common distal point in the action of different neuropeptide receptors is the activation of G_q and PI-PLCβ (Strathmann and Simon, 1990, Blank *et al.*, 1991, Shenker *et al.*, 1991, Smrcka *et al.*, 1991, Taylor *et al.*, 1991, Wilkie *et al.*, 1991, Berstein *et al.*, 1992). It is therefore plausible that the toxin alters the properties of G_q, the coupling of this G protein to PI-PLCβ or the properties of PI-PLCβ. If this is the case, it would be expected that rPMT treatment potentiates the generation of Ins(1,4,5)P_3 one of the products of phosphatidylinositol(4,5)P_2 hydrolysis. Our data demonstrates that the increase in the intracellular level of Ins(1,4,5)P_3 induced by bombesin is dramatically enhanced in rPMT-treated cells. The amplification in the accumulation of this second messenger could be detected as early as 5 sec after bombesin addition. This kinetic result strongly suggests that rPMT treatment changes the properties of one of the elements of the signal transduction pathway immediately distal to the receptor i.e., G_q and/or PI-PLCβ.

It has been known for some time that activation of PKC can severely attenuate the polyphosphoinositide breakdown induced by a variety of neuropeptides including bombesin and vasopressin (Brown *et al.*, 1987, Lopez-Rivas *et al.*, 1987, Brown *et al.*, 1990). Although the precise molecular mechanism has not been clearly identified, activation of PKC has been shown to increase the phosphorylation at a specific serine residue of PI-PLCβ (Ryu *et al.*, 1990). PDGF-stimulated inositol phosphate accumulation that is mediated by PI-PLCγ is not inhibited by phorbol ester activation of PKC. If the facilitating effects of rPMT are mediated by the G_q/PI-PLCβ pathway, down-regulation of PKC should enhance the ability of rPMT to induce polyphosphoinositide hydrolysis. The results presented here demonstrate that down-regulation of PKC causes a striking enhancement of rPMT-induced inositol phospholipid hydrolysis and provide an independent line of evidence suggesting that rPMT acts through the G_q/PI-PLCβ pathway leading to polyphosphoinositide hydrolysis.

In conclusion, the results presented in this chapter strongly suggest that rPMT selectively enhances the production of inositol phosphates by neuropeptides.

CHAPTER FOUR: RESULTS

rPMT-INDUCED INOSITOL PHOSPHATE PRODUCTION IN PERMEABILIZED CELLS: EFFECT OF GDPβS, GTPγS AND DEPENDENCE ON Ca^{2+} CONCENTRATION.

Cell permeabilization has provided a useful approach to introduce guanine nucleotide analogues into the cytosol in order to assess the contribution of G proteins in the generation of biological responses. The neuropeptides (e.g. bombesin) stimulate phosphoinositide hydrolysis via specific plasma membrane receptors coupled to a pertussis toxin-insensitive G protein (see section 1.3.1.). The observations in chapter 3 that rPMT acts along the neuropeptide pathway for the enhancement of inositol phosphates prompted us to investigate whether a G protein was also required for its action. In addition, it is also known that Ca^{2+} can activate PI-PLC either directly or by modulating receptor mediated responses. G protein-mediated stimulation of PI-PLC activity has been shown, in some cases, to result in a decreased Ca^{2+} requirement of the enzyme as well as an increased intrinsic activity. Permeabilized cells have also been useful for studying the effects of Ca^{2+} on PI-PLC activation by allowing buffered Ca^{2+} solutions to be introduced into cells to raise or lower cytosol levels of Ca^{2+}. We have investigated the effects of the non-hydrolysable analogues GDPβS and GTPγS and also the effect of Ca^{2+} on the production of inositol phosphates induced by rPMT in permeabilized cells to determine whether a functional G protein was required for its action. Most of the experiments shown in this chapter were carried out on cells permeabilized with streptolysin O . Streptolysin O, a bacterial cytolysin, is a very effective tool for generating large pores (> 10 nm) in the plasma membrane (Buckingham and Duncan, 1983). Following addition of streptolysin O to intact cells, diffusion of molecules both out of and into the cells occurs at rates which are dependent on the size of the molecules. Hence, small molecules such as ATP or GTPγS can easily and rapidly diffuse into these permeabilized cells. Digitonin, a steroid glycoside which also acts as a permeabilization agent (Dubinsky and Cockrell, 1975, Becker *et al.*, 1980, Fiskum *et al.*, 1980), was also used to assess the effects of rPMT in permeabilized 3T3 cells.

4.1. EFFECT OF rPMT ON THE RELEASE OF THE [^{3}H]URIDINE-LABELLED NUCLEOTIDE POOL FROM STREPTOLYSIN O AND DIGITONIN PERMEABILIZED SWISS 3T3 CELLS.

Conditions required to render the plasma membrane of Swiss 3T3 cells permeable to charged solutes of low molecular weight were determined by measuring the efflux of

Fig. 4.1.1.

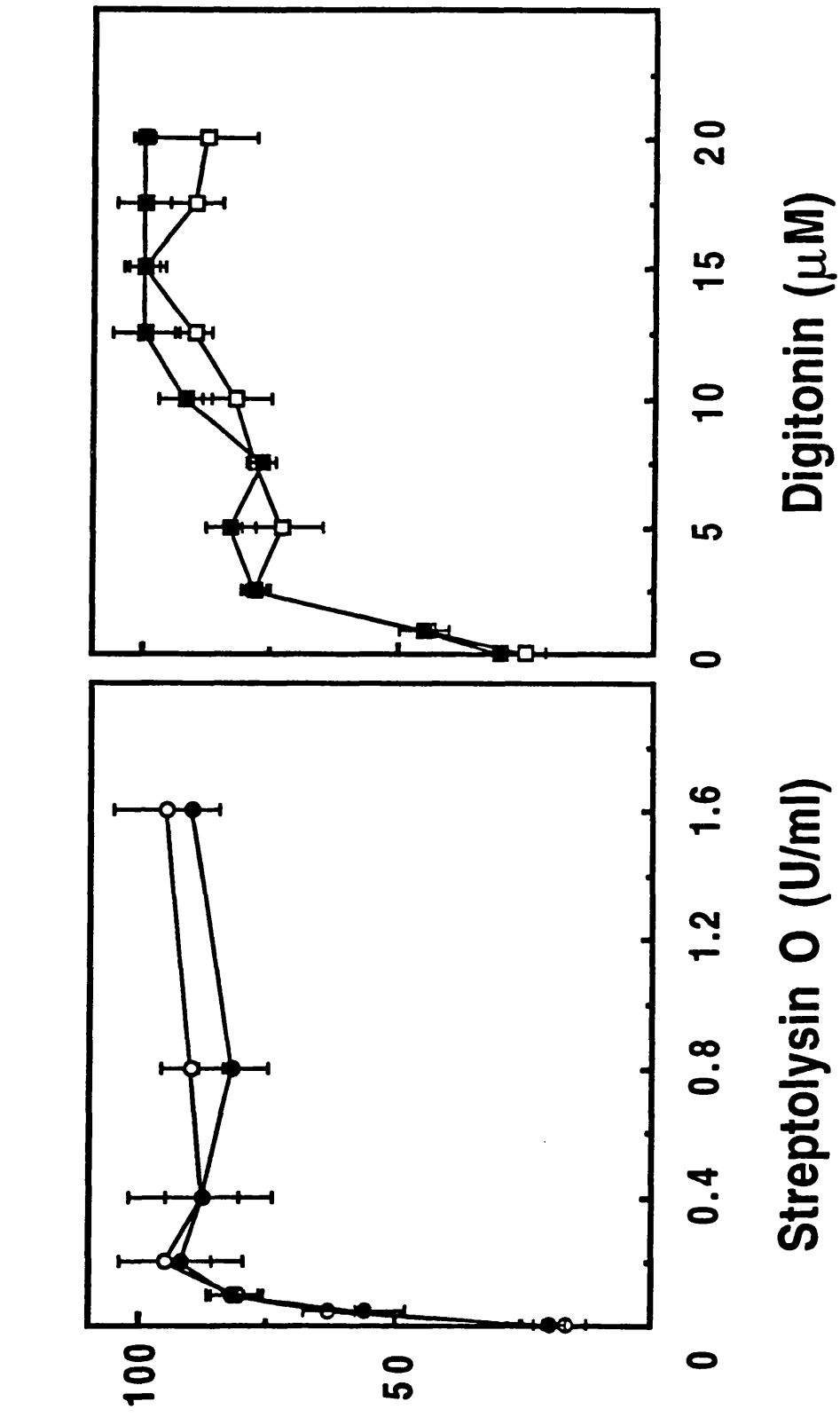

Efflux of [3H]Uridine-labeled pool
% Total incorporated label)
100
50
0
0.4
0.8
1.2
1.6
Streptolysin O (U/ml)
0
5
10
15
20
Digitonin (μM)

Fig. 4.1.1. Effect of rPMT on the release of [^{3}H]uridine in Streptolysin O and digitonin permeabilized Swiss 3T3 cells. Quiescent cultures of Swiss 3T3 cells were washed twice with DMEM and the medium replaced with 1 ml of DMEM/ Waymouths medium (1:1) containing 1 μCi/ml of [^{3}H]uridine. rPMT (20 ng/ml) was added at the same time as the [^{3}H]uridine. After 5 h at 37 °C the control (open symbols) and rPMT-treated (closed symbols) cells were washed four times with DMEM and a further two times with 'K solution'. The medium was then replaced with 1 ml of 'K solution' containing varying concentrations of either (**left**) streptolysin O or (**right**) digitonin. After 10 min, 0.5 ml of the solution was removed and counted in 10 ml of scintillant in a beta counter. Total incorporated [^{3}H]uridine was determined by washing the cells as described above and then replacing the medium with 1 ml of 5% TCA. 0.5 ml of the acid extract was then counted as before and the release of radiolabelled nucleotides by different concentrations of permeabilization agent calculated as a percentage of the total. Results are the mean ± S.E.M. for triplicate determinations.

[^{3}H]uridine-labelled nucleotides from the acid-soluble pool in quiescent monolayers. Quiescent cultures of Swiss 3T3 cells were labelled with [^{3}H]uridine for 5 h, in the absence or presence or 20 ng/ml rPMT. The cells were then washed and permeabilized using different concentrations of streptolysin O or digitonin. Permeabilization of the cells allows the release of this radiolabelled nucleotide into the medium and the amounts released were assessed by scintillation counting. Fig 4.1.1. shows that either 15 μM of digitonin or 0.4 U/ml streptolysin O released 100% of the total radiolabelled nucleotide from the cells over the time of the experiment. The percentage release of the radiolabelled nucleotide was identical in cells that were treated with rPMT. These results demonstrate that rPMT-treatment does not affect the ability of either streptolysin O or digitonin to permeabilize Swiss 3T3 cells.

4.2. THE STIMULATION OF INOSITOL PHOSPHATE PRODUCTION BY rPMT PERSISTS IN PERMEABILIZED CELLS.

We next assessed whether the production of inositol phosphates seen with rPMT in intact cells was also observed in permeabilized cells. It was shown previously that the direct addition of rPMT to saponin-permeabilized cells failed to stimulate the release of inositol phosphates (Staddon *et al.*, 1991a). This was confirmed in cells permeabilized with streptolysin O. The direct addition of rPMT at 20 ng/ml or 100 ng/ml to streptolysin O-permeabilized cells failed to stimulate the release of inositol phosphates (Table 4.2.1). Under the same conditions we have demonstrated that either bombesin or GTPγS alone stimulated inositol phosphate release (Table 4.2.1). These results are consistent with the hypothesis that rPMT must enter the cells by endocytosis and be activated before stimulating the release of inositol phosphates.

In the experiments carried out in this chapter, cells were pretreated with rPMT for 5 h to allow the toxin to enter the cells and stimulate the production of inositol phosphates prior to permeabilization. Quiescent cultures of [2-^{3}H]inositol-labelled Swiss 3T3 cells were incubated with different concentrations of rPMT for 5 h and then permeabilized with either streptolysin O or digitonin for a further 10 min. Fig 4.2.1 shows that rPMT stimulated a dose dependent increase in inositol phosphates in streptolysin O- and digitonin-permeabilized cells. In streptolysin O-permeabilized cells the concentration of rPMT which produced a half maximal response was 16 ng/ml while in digitonin permeabilized cells the half maximal response was at 20 ng/ml. In intact cells, the half maximal concentration of rPMT required for the stimulation of inositol phosphates was 9 ng/ml. The increase in the half maximal concentration of rPMT in permeabilized cells may be a result of the loss, or uncoupling of components of the phospholipase C pathway on permeabilization. In addition, a leftward shift in the dose response to rPMT observed with bombesin in intact cells was also observed in

Table. 4.2.1.

rPMT does not stimulate the release of [^{3}H]inositol phosphates from permeabilized Swiss 3T3 cells.

	Inositol phosphates (cpm)
control	451 ± 17
Bombesin	1893 ± 32
GTPγS	1656 ± 43
rPMT 20 ng/ml	448 ± 12
rPMT 100 ng/ml	462 ± 17

[2-^{3}H]inositol phosphate release from streptolysin O-permeabilized Swiss 3T3 cells was as described in 'Materials and Methods'. Permeabilization was for 10 min in the absence of other factors or in the presence of bombesin (30 nM), GTPγS (0.1 mM), rPMT (20 ng/ml) or rPMT (100 ng/ml) when inositol phosphates were extracted and analyzed as described. Values are the means ± S.E.M. of triplicate determinations.

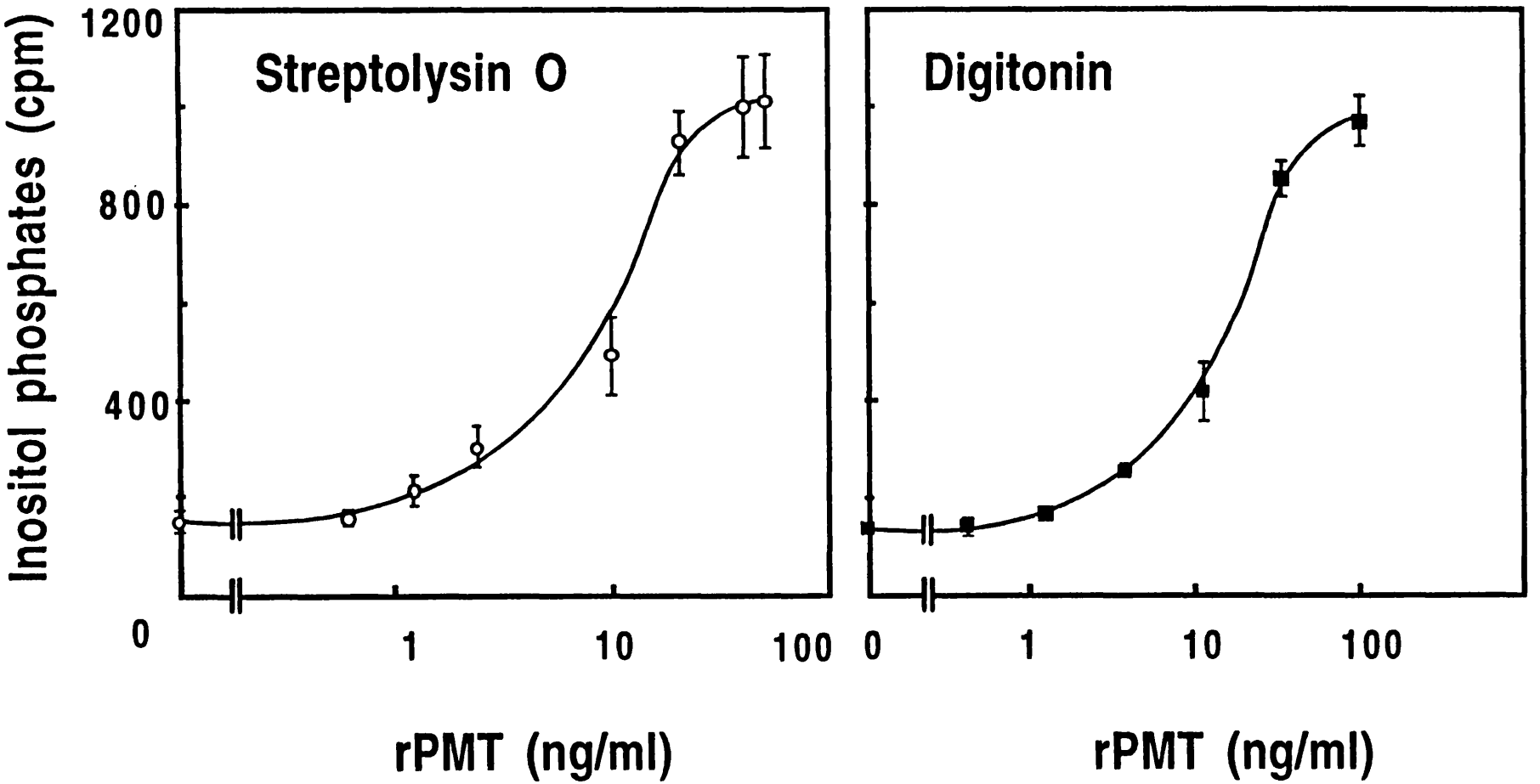

Inositol phosphates (cpm)
1200
800
400
0
Streptolysin O
1
10
100
rPMT (ng/ml)
Digitonin
0
1
10
100
rPMT (ng/ml)

Fig. 4.2.1.

Fig. 4.2.1. The stimulation of inositol phosphate production by rPMT persists in permeabilized cells. The cells were labelled for 16-18 h with 25 μCi/ml [2-^{3}H]inositol in DMEM:Waymouths (1:1). Treatment with varying concentrations of rPMT was for 5 h prior to permeabilization. Permeabilization was for 10 min. prior to extraction. **Left.** Permeabilization using streptolysin O and **Right.** Permeabilization using Digitonin. Total inositol phosphates were extracted and analysed as described. Values shown are the means ± S.E.M of triplicate determinations.

Fig. 4.2.2.

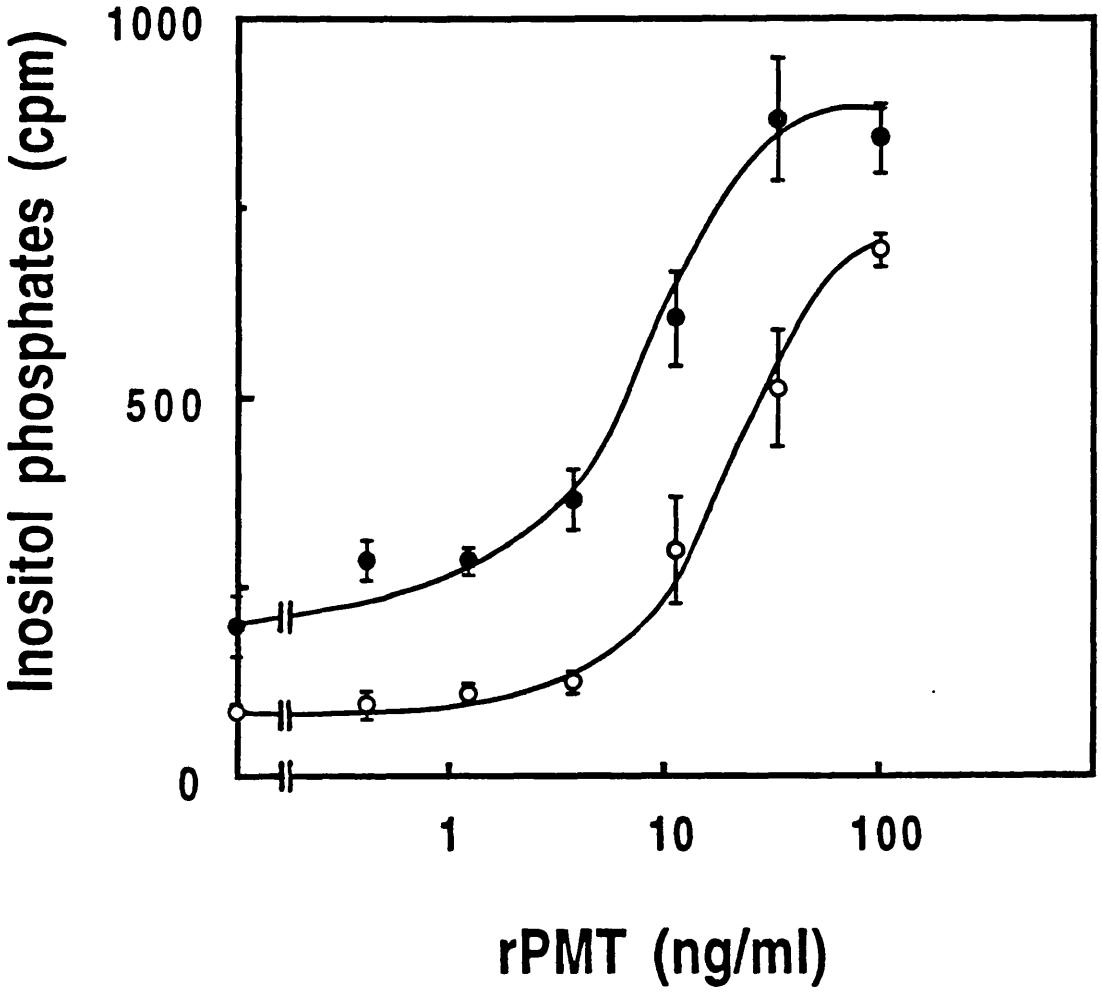


Fig. 4.2.2. The Effect of bombesin on the dose response of rPMT in permeabilized cells. Cells were labelled for 16-18 h with 25 μCi/ml [2-^{3}H]inositol in DMEM:Waymouths (1:1) when they were treated or not with varying concentrations of rPMT for 5 h prior to permeabilization. Permeabilization was for 10 min in the absence (open symbols) or presence of bombesin (6 nM). Extraction and analysis of total inositol phosphates was as described. Values represent means ± S.E.M. of triplicate determinations.

permeabilized cells (Fig. 4.2.2). Bombesin decreased the half maximal concentration of rPMT required in digitonin permeabilized cells from 22 ng/ml to approximately 9 ng/ml.

4.3. EFFECT OF GDPβS ON INOSITOL PHOSPHATES INDUCED BY rPMT.

As described in section 1.3.1., the neuropeptides are known to stimulate inositol phosphate production through receptors which are coupled to heterotrimeric G proteins. These G proteins consist of three subunits α, β, and γ. The α -subunits contain GTPase activity and are involved in the interaction with and activation of PI-PLCβ. In the basal state the α-subunits contain bound GDP and are associated with their corresponding βγ-subunits. Stimulation of the appropriate neuropeptide receptor results in the dissociation of the associated G protein into its α- and βγ-subunits and the exchange of GTP for GDP on the α-subunit. This GTP-bound α-subunit can then associate with and activate PI-PLCβ. The intrinsic GTPase activity of the α-subunit slowly converts the GTP to GDP and returns the G protein to its inactive form (see section 1.2.2. and Fig 1.2.3.). Non-hydrolysable analogues of guanine nucleotides such as GDPβS or GTPγS are therefore very useful for studying the role of G proteins in the generation of biological responses. GDPβS, a non-hydrolysable analogue of GDP, retains the α-subunit in its GDP bound or inactive state. Thus GDPβS will have the effect of inhibiting responses which require G protein activation. In contrast, GTPγS the non-hydrolysable analogue of GTP, activates the G protein and can elicit G protein mediated responses on its own. Neither of these analogues are able to penetrate the cell membrane and therefore permeabilization is necessary in order to introduce these compounds into cells.

We first examined the effects of GDPβS on the production of inositol phosphates stimulated by rPMT. Quiescent cultures of Swiss 3T3 cells labelled with [2-^{3}H]inositol were incubated with rPMT at 2.5 and 20 ng/ml for 5 h after which they were permeabilized with streptolysin O in the presence or absence of 0.5 mM GDPβS. Fig. 4.3.1. shows that the production of inositol phosphates in response to rPMT at 20 ng/ml was markedly inhibited by GDPβS (65% inhibition) and that induced by rPMT at 2.5 ng/ml was totally blocked by this G protein antagonist. Fig. 4.3.1. also shows, for comparison, that GDPβS inhibited the production of inositol phosphates elicited by either 6 nM bombesin or 1μM GTPγS whereas GTPγS at a concentration of 1mM reversed the inhibitory effect of GDPβS. The inhibition by GDPβS occurred at all concentrations of rPMT used (Fig.4.3.2.).

Fig. 4.3.1.

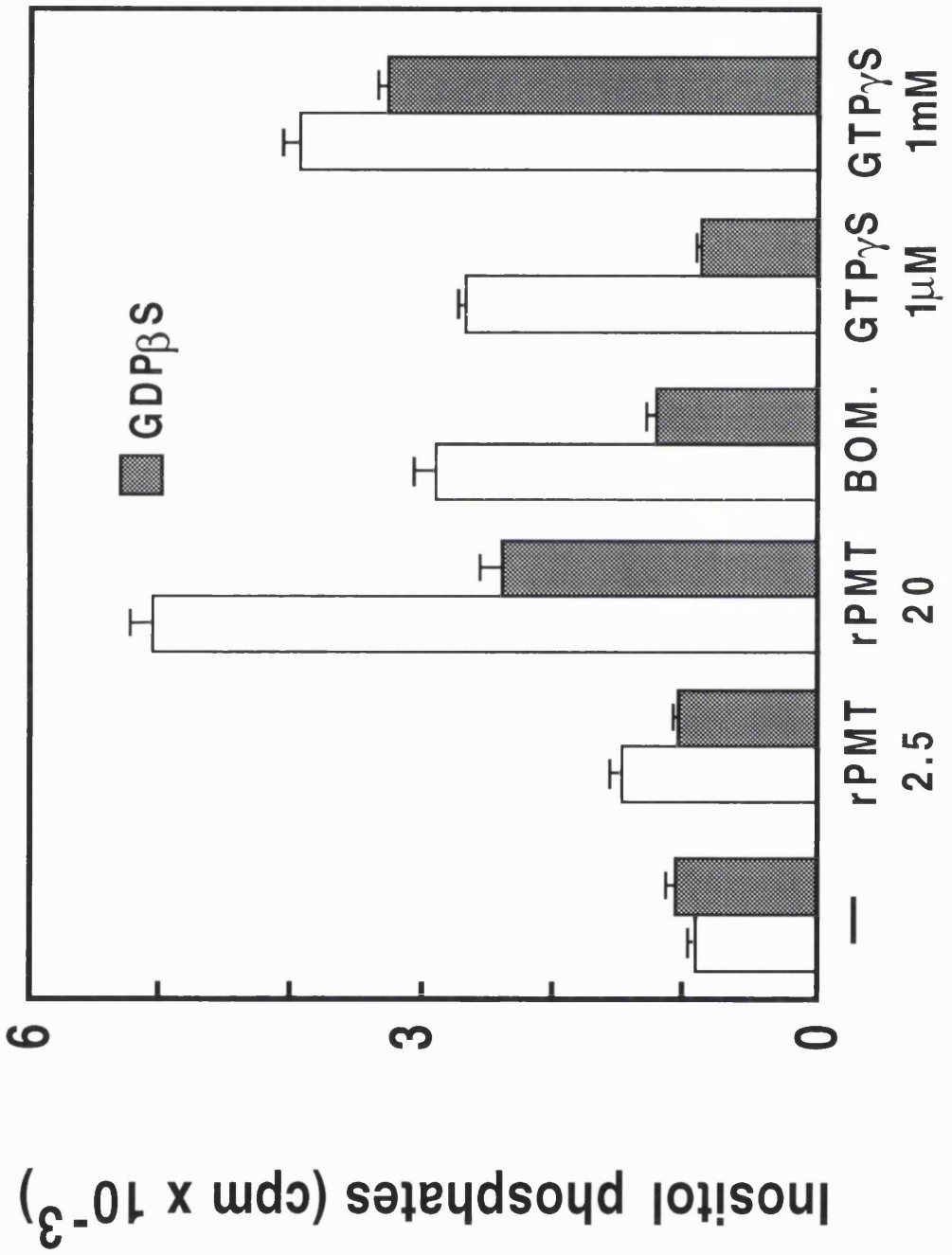

Inositol phosphates (cpm x 10^-3)
6
3
0
GDPβS
—
rPMT 2.5
rPMT 20
BOM.
GTPγS 1μM
GTPγS 1mM

Fig. 4.3.1. The effect of GDPβS on the stimulation of inositol phosphate production by rPMT in permeabilized cells. Cells were labelled with 25 μCi/ml of [2-^{3}H]inositol for 16-18 h. Permeabilization of Swiss 3T3 cells with streptolysin O was as described in 'Materials and Methods'. Treatment with rPMT was for 5 h prior to permeabilization. Permeabilization was for 10 min in the absence of other factors or in the presence of rPMT (2.5 ng/ml), rPMT (20 ng/ml), bombesin (6 nM), GTPγS (1 μM) and GTPγS (1 mM). These factors were added in the absence (open bars) or presence (shaded bars) of 0.5 mM GDPβS. Extraction and analysis of total inositol phosphates was as described. Values represent means ± S.E.M. of triplicate determinations.

Fig. 4.3.2.

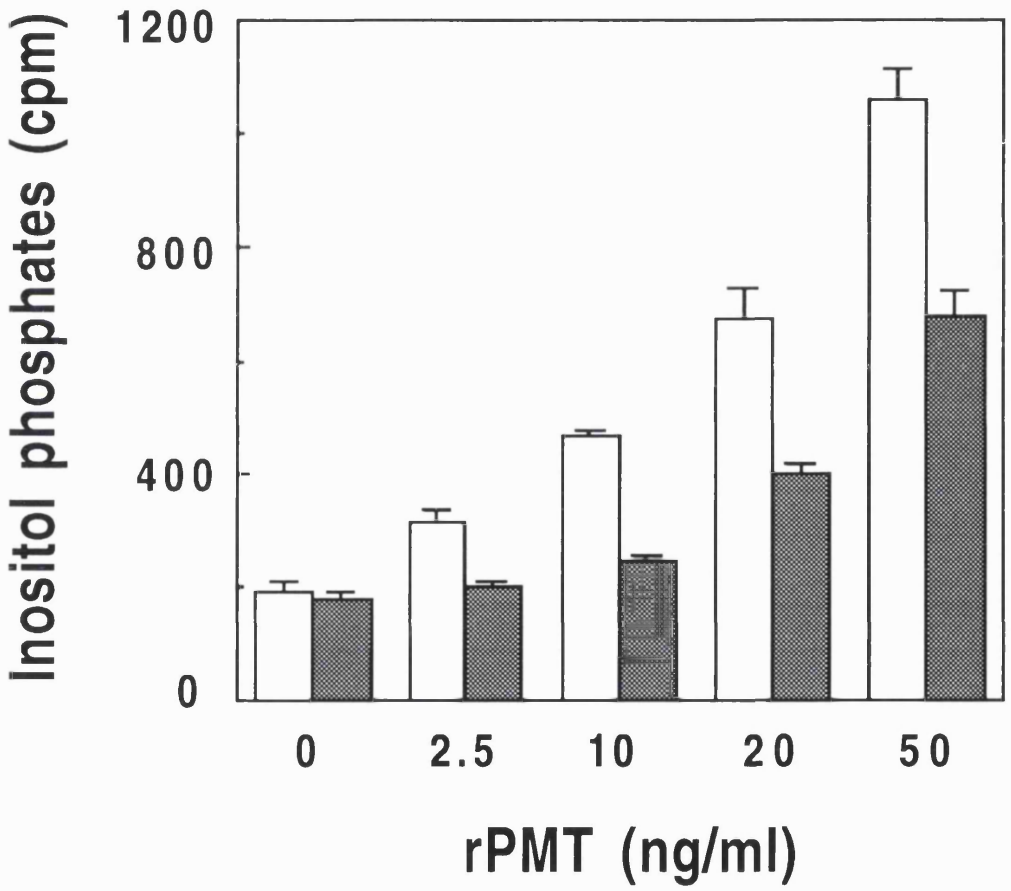


Fig. 4.3.2. Effect of GDPβS of the dose-dependent increase in inositol phosphate production induced by rPMT. [2-^{3}H]inositol-labelled Swiss 3T3 cells were permeabilized with Streptolysin O as described previously. Treatment with increasing concentrations of rPMT was for 5 h prior to permeabilization. Permeabilization was for 10 min in the absence (open symbols) or presence of (closed symbols) of 0.5 mM GDPβS. Extraction and analysis of total inositol phosphates was as described. Values shown are the means ± S.E.M. of triplicate determinations.

4.4. CONCENTRATION DEPENDENCE OF THE EFFECT OF GDPβS (EFFECT ON Ca^{2+}, rPMT).

Ca^{2+} is known to directly stimulate PI-PLC in permeabilized cells (Pachter *et al.*, 1992), isolated membranes (Taylor and Exton, 1987, Martin and Kowalchyk, 1989) and purified preparations (Smrcka *et al.*, 1991). In order to test further the specificity of the inhibitory effect of GDPβS, we determined the effect of various concentrations of this analogue on inositol phosphate production stimulated either by treatment with rPMT or by free calcium. Fig. 4.4.1. shows that GDPβS inhibited rPMT-stimulated inositol phosphate production in a concentration dependent manner. In contrast, this analogue had no effect on the free calcium-stimulated PI-PLC, indicating that its inhibitory effect was selective.

4.5. INHIBITION OF rPMT-INDUCED INOSITOL PHOSPHATES IS A RAPID CONSEQUENCE OF ADDITION OF GDPβS

Inhibition of inositol phosphate production in rPMT-treated cells was a rapid consequence of GDPβS addition to permeabilized cells. Cells which had been prelabelled with [2-^{3}H]inositol for 16 -18 h were treated or not with rPMT 20 ng/ml for 5 h. The cells were then permeabilized in the absence or presence of 0.5 mM GDPβS and the reactions terminated at various times after permeabilization. Fig 4.5.1. shows that a marked decrease in the rate of inositol phosphate formation was evident 2.5 min after the addition of GDPβS.

4.6. ATTENUATION OF GTPγS STIMULATED INOSITOL PHOSPHATES BY rPMT.

Addition of GTPγS to permeabilized cells caused a marked (220%) maximal increase in the production of inositol phosphates in a concentration-dependent manner (Fig. 4.6.1). In contrast, the stimulatory effect of this analogue was only 35% in rPMT-treated cells (Fig. 4.6.1). It is unlikely that this subadditivity between GTPγS and rPMT is due to a limiting precursor pool since an increase in the toxin concentration in the presence of GTPγS caused a further increase in the production of inositol phosphates (Table 4.6.1). The attenuation shown in Fig 4.6.1. suggests that the effects of GTPγS and those of rPMT treatment converge at the same post-receptor locus in the neuropeptide signal transduction pathway.

Fig. 4.4.1.

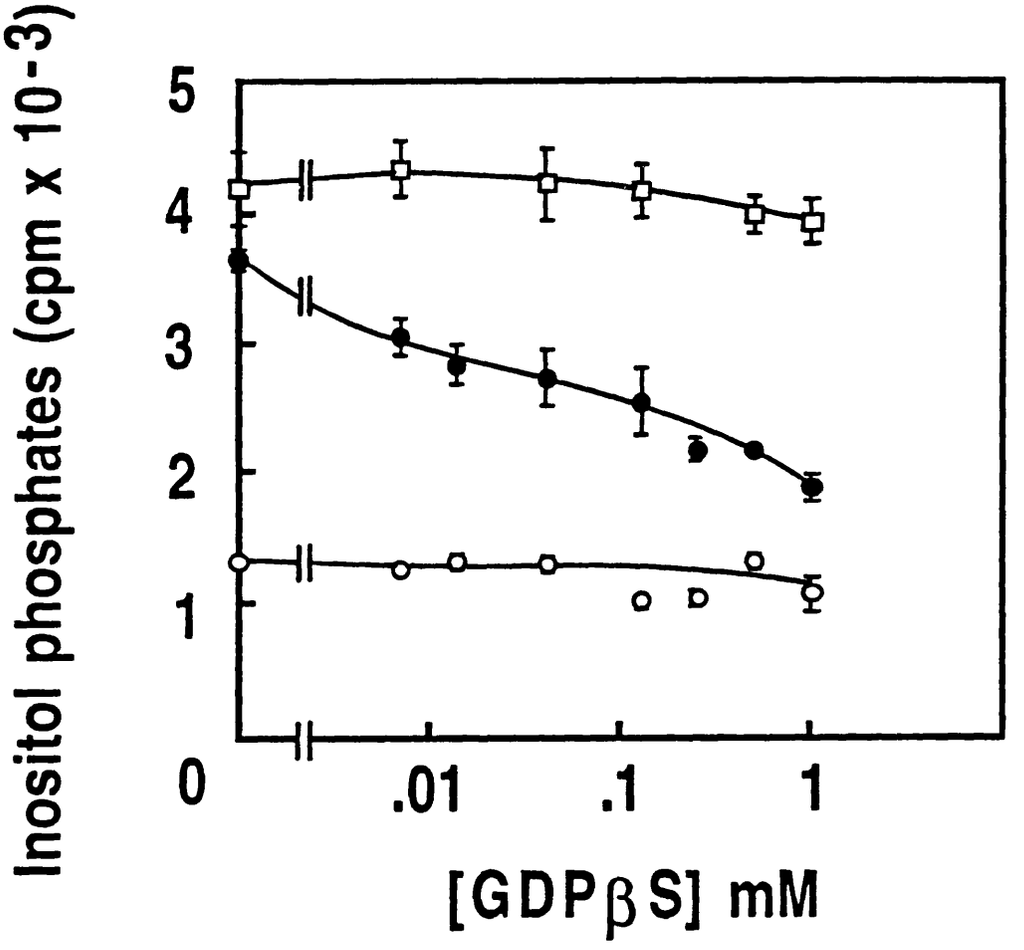


Fig. 4.4.1. Dose dependence of inhibition by GDPβS on inositol phosphates enhanced by rPMT. Quiescent cultures of Swiss 3T3 cells were labelled for 16-18 h with 25 μCi/ml [2-^{3}H]inositol in DMEM:Waymouths (1:1). Treatment with rPMT (5 ng/ml) was for 5 h prior to permeabilization. Increasing concentrations of GDPβS were added to control cells (open circles), rPMT-treated cells (closed circles), or to cells permeabilized in the presence of 10^{-5} M Ca^{2+} (open squares). Permeabilization was for 10 min. prior to extraction. Total inositol phosphates were extracted and analysed as described. Values shown are the means ± S.E.M of triplicate determinations.

Fig. 4.5.1.

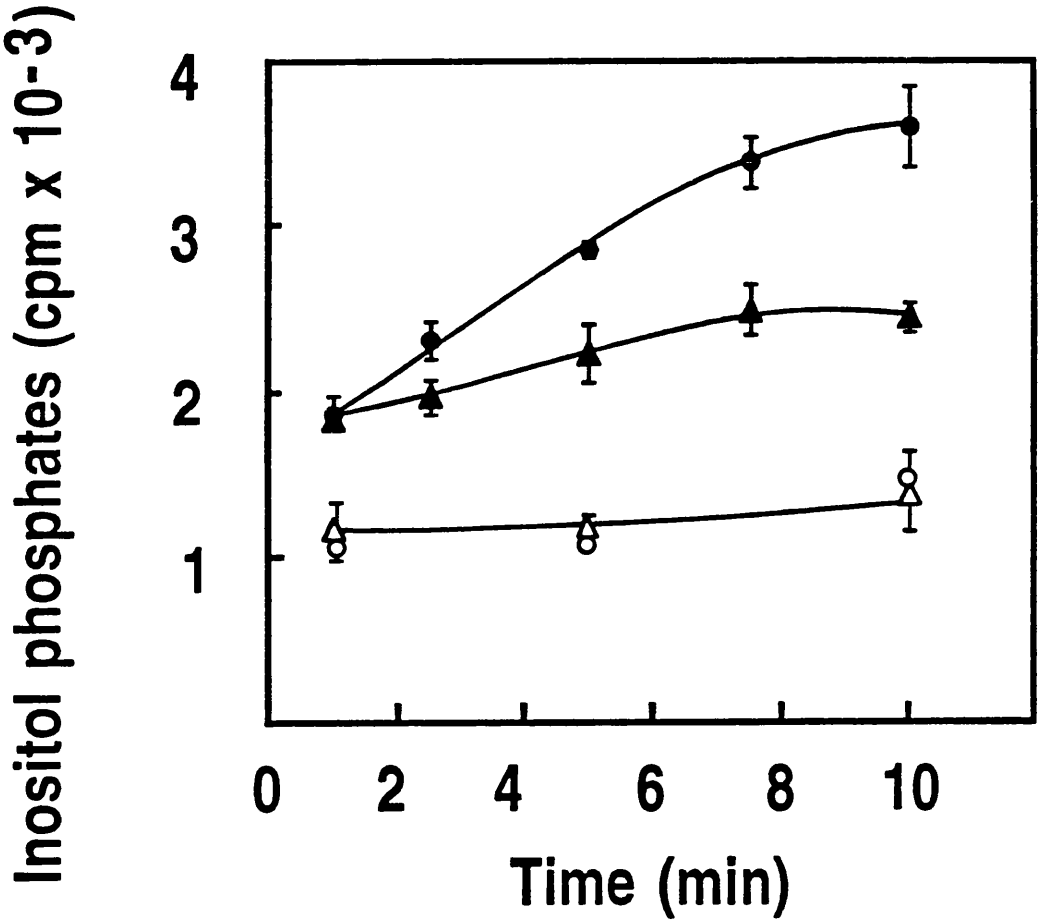


Fig. 4.5.1. Time course of inhibition by GDPβS on inositol phosphates enhanced by rPMT. The cells were labelled for 16-18 h with 25 μCi/ml [2-^{3}H]inositol in DMEM:Waymouths (1:1). Treatment with rPMT (5 ng/ml) was for 5 h prior to permeabilization. Addition of GDPβS (0.5 mM) to control (open triangles) and rPMT-treated cells (closed triangles). No additions to control (open circles) or rPMT-treated cells (closed circles) are shown. GDPβS was added at the time of permeabilization and the reactions were terminated at the times indicated. Total inositol phosphates were extracted and analysed as described. Values shown are the means ± S.E.M of triplicate determinations.

Fig. 4.6.1.

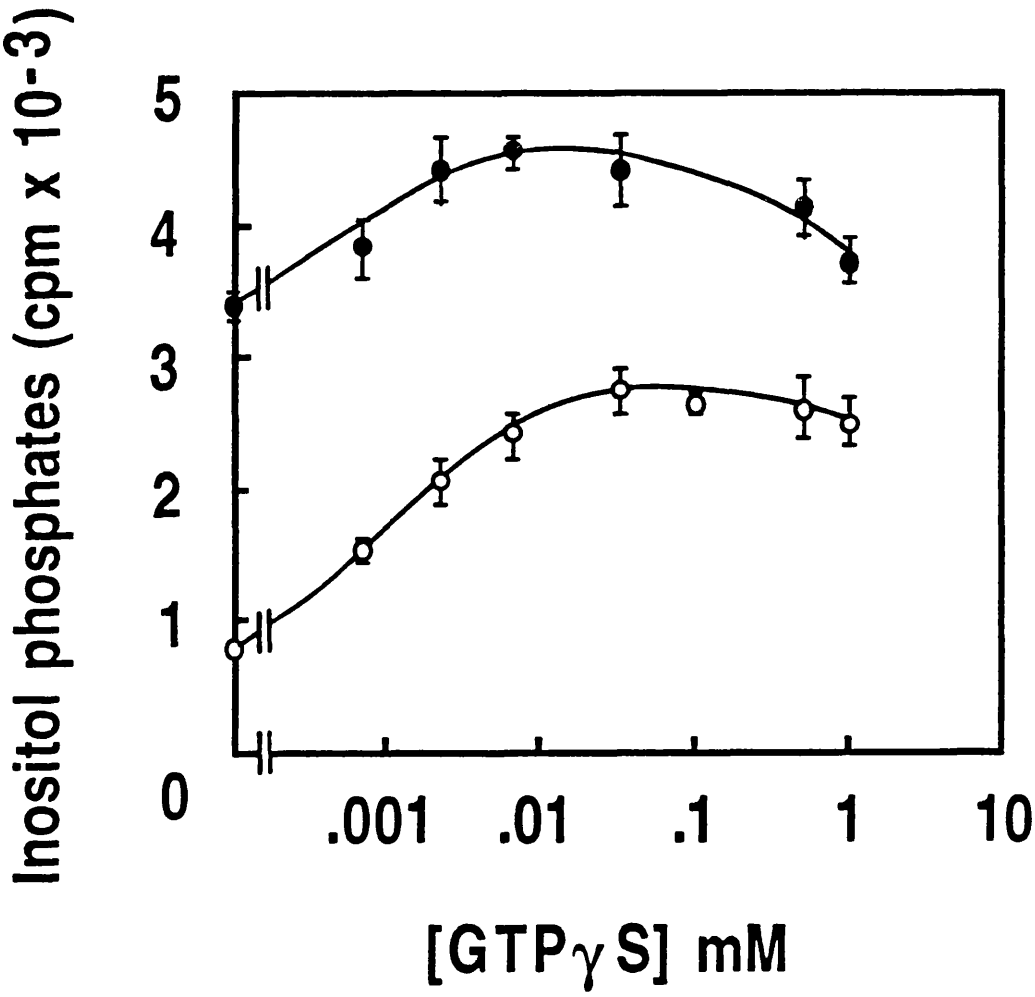


Fig. 4.6.1. The effect of rPMT-treatment on the dose-dependent increase in the production of inositol phosphates stimulated by GTPγS in permeabilized cells. [2-^{3}H]inositol phosphate release from streptolysin O-permeabilized Swiss 3T3 cells has been described in 'Materials and Methods'. Treatment with rPMT (20 ng/ml) was for 5 h prior to permeabilization. Increasing concentrations of GTPγS were added to control (open circles) or rPMT-treated cells (closed circles) at the time of permeabilization. Permeabilization was for 10 min, when inositol phosphates were extracted and analysed as described. Values are the means ± S.E.M. of triplicate determinations taken from three individual experiments.

Table 4.6.1.

Effect of GTPγS on rPMT induced inositol phosphates in permeabilized Swiss 3T3 cells.

	Inositol phosphates (cpm)	
Addition	-	GTPγS
-	924 ± 66	3960 ± 156
rPMT 20 ng/ml	5562 ± 60	6642 ± 150
rPMT 50 ng/ml	7170 ± 384	7968 ± 228

Quiescent cultures of Swiss 3T3 cells were labelled with 25μCi/ml of [2-^{3}H]inositol for 16 h. rPMT, at the concentrations indicated, was then added directly to the dishes and incubated at 37 °C for 5 h. Permeabilization with streptolysin O was as described in 'Materials and Methods'. GTPγS (0.1 mM) was added to control or rPMT-treated cells at the time of permeabilization. Permeabilization was for 10 min, when inositol phosphates were extracted and analyzed as described. Values are the means ± S.E.M. of triplicate determinations taken from three individual experiments.

4.7. DEPENDENCE ON Ca^{2+}.

It has been demonstrated that GTPγS activation of PI-PLC decreases the Ca^{2+} requirement for the activation of this enzyme (Taylor and Exton, 1987, Martin and Kowalchyk, 1989). In streptolysin O-permeabilized 3T3 cells, GTPγS causes a leftward shift of the dose-response of free calcium for the stimulation of inositol phosphate release. Since the preceding results suggested that rPMT, like GTPγS, enhances the coupling of G protein to PI-PLC, we examined the effect of toxin treatment on the Ca^{2+} requirement for the production of inositol phosphates. [2-^{3}H]inositol-labelled Swiss 3T3 cells were treated or not with 20 ng/ml rPMT for 5 h. The cells were then permeabilized using streptolysin O as described previously except the concentration of free calcium in the permeabilization medium was varied. Fig. 4.7.1. shows that permeabilized cells display an increased production of inositol phosphates in response to increasing concentrations of free calcium. In addition, treatment with rPMT also caused an increase in the sensitivity of PI-PLC for Ca^{2+}; the half maximal concentration of Ca^{2+} was reduced from 5×10^{-7} M in the control cells to 5×10^{-8} M in the rPMT-treated cells. The toxin treated cells also exhibited a marked increase in inositol phosphate production even at high concentrations of free calcium.

4.8. SUMMARY AND DISCUSSION

In the experiments shown in this chapter we found that the increased production of inositol phosphates induced by rPMT treatment is preserved after cell permeabilization with both streptolysin O and digitonin, agents which form pores in the membrane by interacting with cholesterol. This result is of added importance because it rules out the possibility that the effects of rPMT on polyphosphoinositide hydrolysis result from a change in an intermediary second messenger e.g. free calcium. Comparison of the results observed on using either streptolysin O or digitonin is important in order to demonstrate that our results are not due to non-specific effects caused by the method of permeabilization. Furthermore, we exploited this cell permeabilization procedure to determine the effects of cell-impermeable guanine nucleotide analogues on rPMT action.

Evidence for the role of G proteins in signal transduction pathways has been obtained in many systems by assessing the effects of guanine nucleotide analogues in permeabilized cells. The α-subunits of heterotrimeric G proteins bind to and hydrolyse GTP. In their inactive state these α-subunits are GDP-bound. The active or GTP-bound state of these α-subunits can be promoted by activation of G protein coupled receptors. Non-hydrolysable guanine nucleotide analogues such as GDPβS and GTPγS can therefore either inhibit or

Fig. 4.7.1.

Fig. 4.7.1. Dependence on Ca^{2+} of PI-PLC activity in control and rPMT-treated Swiss 3T3 cells permeabilized with Streptolysin O. Quiescent cultures of Swiss 3T3 cells were incubated for 18 h with 25 μCi/ml [2-^{3}H]inositol. Treatment of cells with 20 ng/ml rPMT was for 5 h prior to permeabilization. Permeabilization was as described in 'Materials and Methods' except the concentration of free calcium in the permeabilization medium was varied. The effect of various concentrations of free calcium in the control (open circles) or rPMT-treated cells (closed circles) is shown. Values are the means ± S.E.M. of triplicate determinations taken from three individual experiments.

activate respectively these α-subunits. Both GDPβS and GTPγS are membrane impermeant, thus permeabilization is necessary in order to asses the effects of these compounds. Our results show that GDPβS, inhibits the production of inositol phosphates by rPMT in permeabilized 3T3 cells. The inhibition by GDPβS was rapid and concentration-dependent. The ability to stimulate PI-PLC with free calcium in the presence of GDPβS emphasises the specificity of this inhibitory effect. This finding shows that rPMT-mediated inositol phosphate production requires a functional G protein.

The involvement of a G protein in the stimulation of inositol phosphate production by rPMT is also supported by the fact that the striking activation of PI-PLC by GTPγS is markedly attenuated by prior treatment of the cells with rPMT, suggesting that the toxin and GTPγS converge into the same signal transduction step. GTPγS reduces the Ca^{2+} requirement of PI-PLC to catalyse the cleavage of polyphosphoinositides in membrane preparations (Taylor and Exton, 1987, Martin and Kowalchyk, 1989) or in permeabilized 3T3 cells (results not shown). Similarly, rPMT treatment causes a striking decrease in the Ca^{2+} requirement for the generation of inositol phosphates in permeabilized cells. Taken together, these results imply that rPMT facilitates G protein coupling to PI-PLC.

In conclusion, the results presented in this chapter demonstrate that, using a permeabilized cell system, we have identified for the first time one of the sites of action of rPMT, namely facilitation of G protein coupling to PI-PLC.

CHAPTER FIVE: RESULTS

INVOLVEMENT OF THE NOVEL Gq FAMILY OF G PROTEINS IN THE STIMULATION OF INOSITOL PHOSPHATES BY rPMT.

In the previous two chapters it was demonstrated that rPMT selectively facilitates the neuropeptides for the production of inositol phosphates in Swiss 3T3 cells and that rPMT requires a functional G protein for its action. The neuropeptides are known to stimulate phosphoinositide hydrolysis through specific plasma membrane receptors which couple to heterotrimeric G proteins (see section 1.3.1). The α subunit proteins of the Gq subfamily of pertussis toxin-insensitive heterotrimeric G proteins have recently been shown to stimulate PI-PLCβ1 (Blank *et al.*, 1991, Shenker *et al.*, 1991, Smrcka *et al.*, 1991, Taylor *et al.*, 1991, Berstein *et al.*, 1992). The involvement of this G protein subfamily in the coupling of neuropeptide receptors, including vasopressin and bradykinin, to PI-PLC has also been demonstrated (Gutowski *et al.*, 1991, Wange *et al.*, 1991). Some of the evidence supporting the involvement of $G\alpha_q$ and $G\alpha_{11}$ in PI-PLC activation has been provided by transient transfection of cells with $G\alpha_q$ and $G\alpha_{11}$ cDNAs (Wu *et al.*, 1992a, Wu *et al.*, 1992b).

The experiments carried out in this chapter were designed to try and identify the G protein involved in the stimulation of inositol phosphate production by rPMT. By transient transfection of COS-1 cells, we overexpressed the α subunits of the $G\alpha_q$ and $G\alpha_{11}$ proteins to assess whether the Gq family of G proteins can play a role in the stimulation of PI-PLC by rPMT.

5.1. rPMT-STIMULATION OF INOSITOL PHOSPHATES IN SWISS 3T3 CELLS OCCURS THROUGH A PERTUSSIS TOXIN INSENSITIVE PATHWAY.

In some cell types, receptor-mediated hydrolysis of phosphatidylinositol,4,5-bisphosphate can be blocked by prior treatment of the cells with pertussis toxin, suggesting the involvement of pertussis toxin sensitive G proteins in the activation of PI-PLC. The neuropeptides stimulate phosphoinositide hydrolysis in Swiss 3T3 cells through a pertussis toxin-insensitive pathway and the pertussis toxin-insensitive Gq family of G proteins have been demonstrated to couple neuropeptide receptors to PI-PLC (section 1.3.1.). We examined the effects of pertussis toxin-pretreatment on the stimulation of inositol phosphates by rPMT in Swiss 3T3 cells to determine whether the stimulation of inositol phosphates by rPMT also occurs in a pertussis toxin-insensitive manner. Quiescent cultures of [2-^{3}H]inositol-labelled Swiss 3T3 cells were

Fig. 5.1.1.

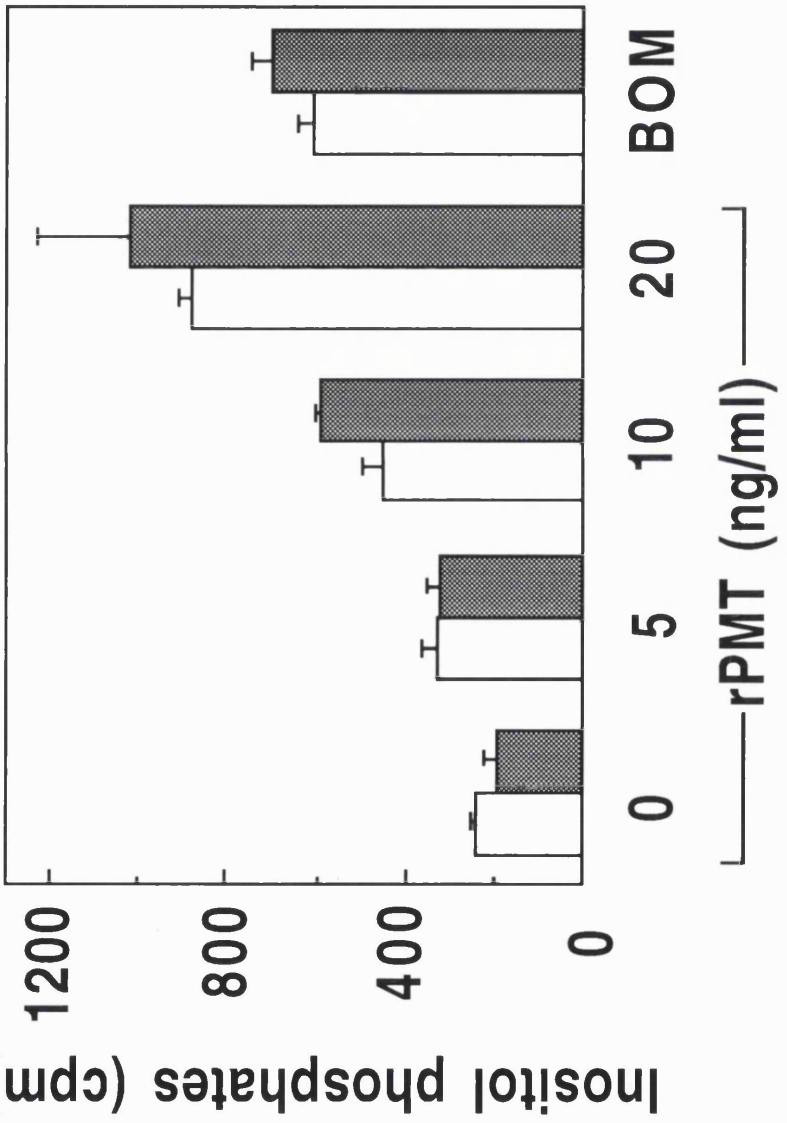


Fig. 5.1.1. Effect of pretreatment of Swiss 3T3 cells with pertussis toxin on the production of inositol phosphates stimulated by rPMT. Quiescent cultures of Swiss 3T3 cells were labelled with 10 μCi/ml [2-^{3}H]inositol for 16-18 h. Cultures were either treated (shaded bars) or not (open bars) with 20 ng/ml pertussis toxin which was added at the same time as the [2-^{3}H]inositol. Treatment with varying concentrations of rPMT was for 5 h in total and treatment with 6 nM Bombesin (BOM) was for 10 min. LiCl (20 mM) was added to all dishes 30 min prior to extraction. Total inositol phosphates were extracted and analysed as described in 'Materials and Methods'.

Fig. 5.1.2.

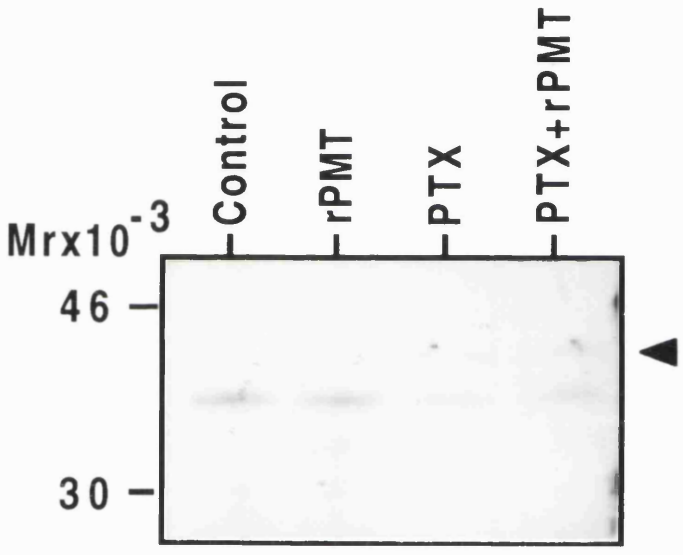


Fig. 5.1.2. Effect of rPMT on the pertussis toxin catalysed ADP-ribosylation of substrates in intact Swiss 3T3 cells. Confluent and quiescent cultures of Swiss 3T3 cells were incubated with 50 μCi/ml of [^{3}H]adenine for 16 -18 h. Treatment with pertussis toxin (100 ng/ml) was from the time of addition of label. rPMT was added to control or pertussis toxin (PTX)-treated cells for 5 h. Labelled material was analysed by SDS-PAGE and fluorography as described under 'Materials and Methods'. The fluorogram shows the effect of rPMT on the labelling of the PTX substrate in a gel treated with TCA at 95 °C, as described under 'Materials and Methods'. The arrow depicts the 40 kDa pertussis toxin substrate.

treated with pertussis toxin for 16 -18 h and then with rPMT for a further 5 h. Fig 5.1.1. demonstrates that pertussis toxin on its own does not stimulate inositol phosphate production in Swiss 3T3 cells. In addition, pretreatment of cells with pertussis toxin had no effect on the stimulation of inositol phosphates by varying concentrations of rPMT.

It was important to demonstrate that the pertussis toxin preparation used in the experiment shown in fig 5.1.1. was biologically active i.e. that it was capable of causing the ADP-ribosylation of a 40 kDa protein in intact Swiss 3T3 cells. This was demonstrated using a novel technique employing [2-^{3}H]adenine to metabolically radiolabel NAD^+, and exploits the acid stability of ADP-ribose amino-acid linkages formed by toxins, to allow clear visualisation of toxin catalysed labelling (Staddon *et al.*, 1991b). Incubation of cells with [^{3}H]adenine and without toxin treatment results in the incorporation of label into many bands. These bands are attributed to RNA labelled in the cells by [2-^{3}H]ATP and they can be removed either by acid treatment or by treatment of cells extracts with RNAse A prior to electrophoresis (Staddon *et al.*, 1991b)). Using the same technique it was also demonstrated that rPMT did not catalyse the ADP-ribosylation of any proteins in intact 3T3 cells implying that it must act by mechanisms other than ADP-ribosylation. If rPMT is acting through a pertussis toxin insensitive pathway then we might expect that rPMT should have no effect on the ADP ribosylation by pertussis toxin of its 40 kDa substrate. Fig.5.1.2. shows a fluorogram from Swiss 3T3 cells which were labelled with [2-^{3}H]adenine and assessed for toxin catalysed ADP-ribosylation after treatment with TCA at 95 °C. This fluorogram demonstrates that rPMT had no effect on the pertussis toxin-catalysed ADP-ribosylation of its 40 kDa substrate in intact 3T3 cells. The lower band observed in each lane most likely represents a residual component of the labelled acid soluble pool which was not completely removed by acid treatment. Thus, rPMT like the neuropeptides stimulates phosphoinositide hydrolysis through a pertussis toxin insensitive G protein pathway.

5.2. STIMULATION OF INOSITOL PHOSPHATES BY rPMT IN COS-1 CELLS: DOSE DEPENDENCE AND TIME COURSE.

The stimulation of inositol phosphates by rPMT has been demonstrated in Swiss 3T3 cells but has not been demonstrated in COS-1 cells. Prior to transfection experiments, we determined whether rPMT stimulated inositol phosphate production in COS-1 cells. COS-1 cells were labelled with 25 µCi/ml of [2-^{3}H]inositol as described. The effect of pretreatment of the COS-1 cells with different concentrations of rPMT for 7 h is shown in Fig. 5.2.1. rPMT increased the accumulation of inositol phosphates in a dose-dependent manner. The concentration of rPMT required to give a half maximal effect was approx. 100 ng/ml which is about ten fold greater than that required for a half maximal effect in Swiss 3T3 cells. A

Fig. 5.2.1.

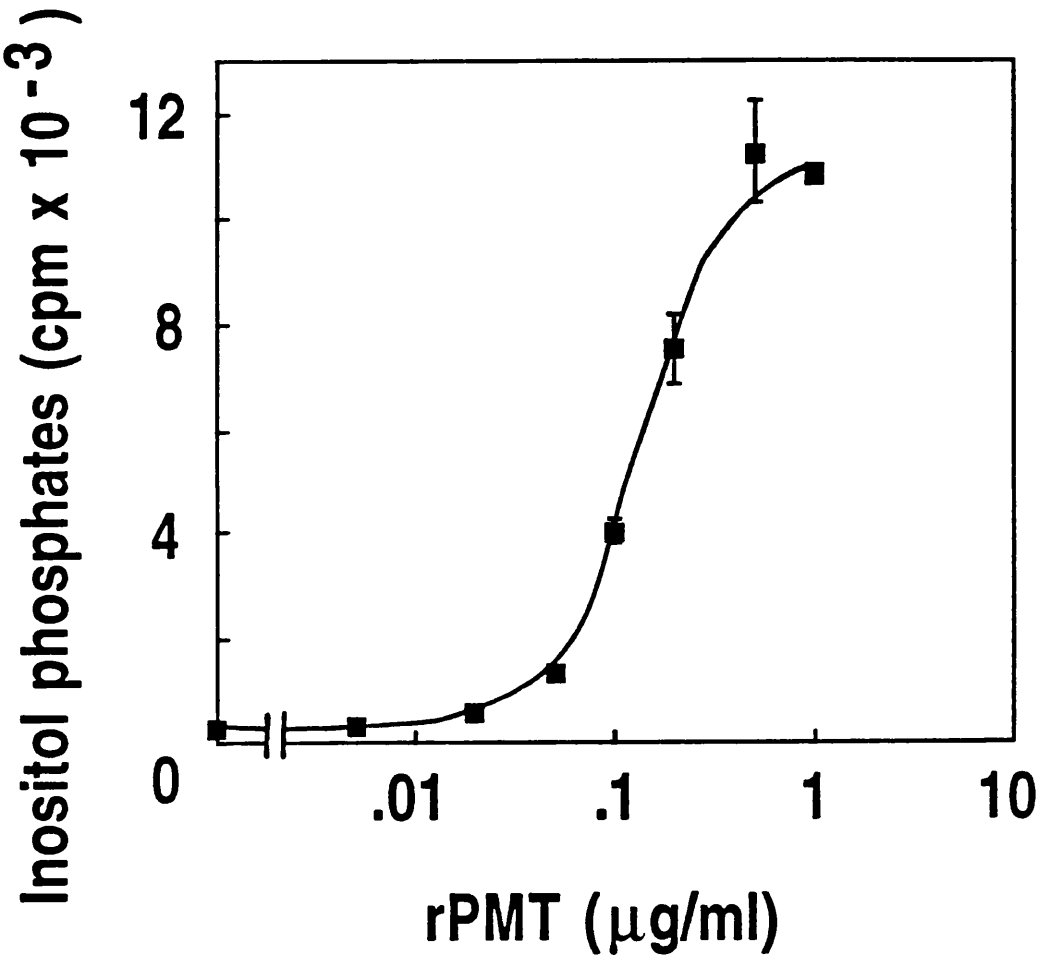


Fig. 5.2.1. Effects of pretreatment with increasing concentrations of rPMT on inositol phosphate production in COS-1 cells. COS-1 cells were labelled with 5 μCi/ml [2-^{3}H]inositol in DMEM/Waymouths medium (1:1) for 16 -18 h. The cells were then incubated with increasing concentrations of rPMT for 7 h. LiCl was then added to give a final concentration of 20 mM. Total inositol phosphates were extracted after 30 min. Analysis of total inositol phosphates was as described in 'Materials and Methods'. Values shown are the mean ± S.E.M. of triplicate determinations.

Fig. 5.2.2.

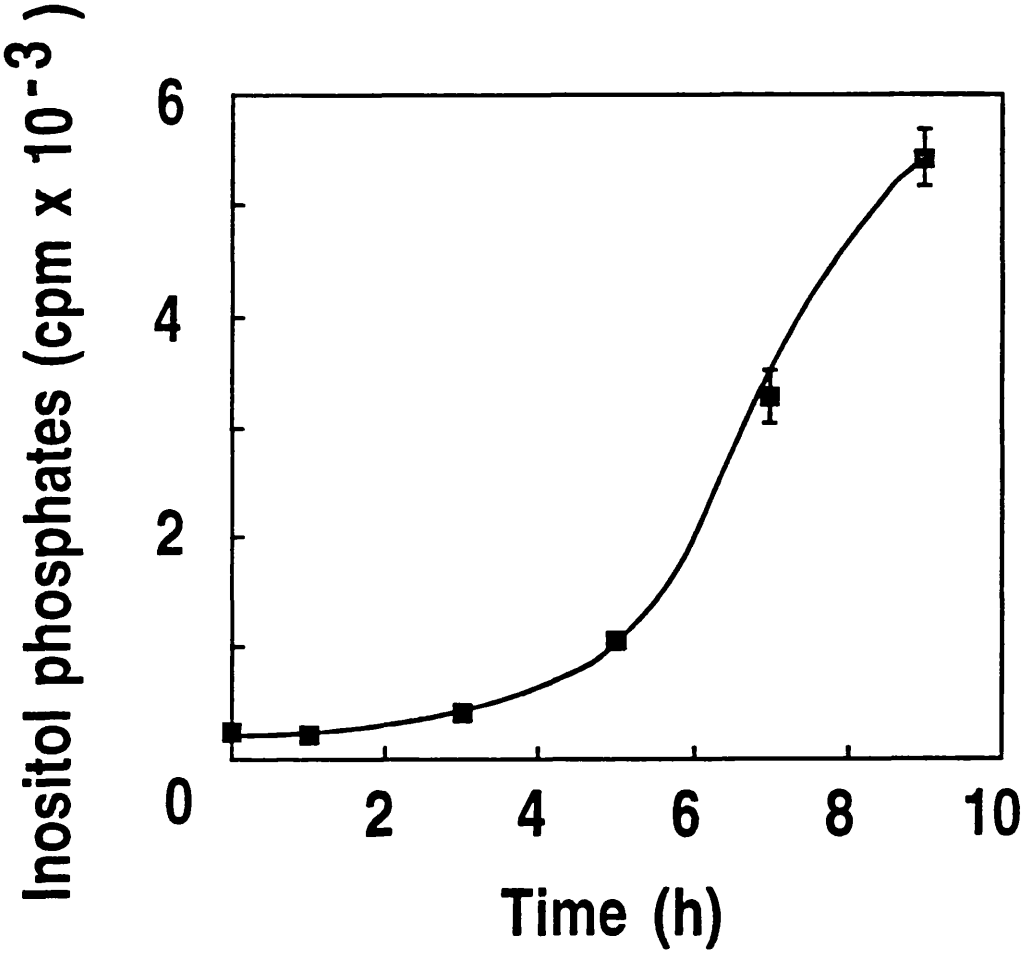


Fig. 5.2.2. The time course for the production of inositol phosphates by rPMT in COS-1 cells. The cells were labelled for 16-18 h with 5 μCi/ml [2-^{3}H]inositol in DMEM:Waymouths medium (1:1). The cells were then treated with rPMT for the times indicated. LiCl (20 mM) was added 30 min prior to extraction in each case. Total inositol phosphates were extracted and analysed as described. Values shown are means ± S.E.M. of triplicate determinations.

possible explanation for this decreased sensitivity is that COS-1 cells express fewer receptors for rPMT on their cell surface.

The time course of inositol phosphate accumulation induced by rPMT (100 ng/ml) is shown in Fig. 5.2.2.. After a lag period of about 3-4 h rPMT stimulates a massive accumulation of inositol phosphates which still persists even 9 h after addition of toxin. Thus, rPMT enters and activates PI-PLC in COS-1 cells in a time and dose-dependent manner.

5.3. METHYLAMINE BLOCKS THE STIMULATION OF INOSITOL PHOSPHATES BY rPMT IN COS-1 CELLS.

To demonstrate that the mechanism required for entry of rPMT into Swiss 3T3 cells is the same for COS-1 cells, we examined the effect of methylamine on the production of inositol phosphates by rPMT in COS-1 cells. As described previously, rPMT, like many bacterial toxins, requires entry into cells and processing for biological activity. The lysosomotrophic agent methylamine blocks the entry of rPMT into Swiss 3T3 cells and hence blocks rPMT-stimulation of inositol phosphates in a time dependent manner. Fig. 5.3.1. shows that when methylamine was added to COS-1 cells at the same time as rPMT it was able to prevent the stimulation of inositol phosphates by the toxin. However, when methylamine was added 3 h after the addition of rPMT the effect of methylamine was reduced and rPMT was still able to stimulate a nearly maximal stimulation of inositol phosphates. Thus, rPMT requires entry and processing before stimulating inositol phosphate production in COS-1 cells.

5.4. EXPRESSION OF $G\alpha_q$ AND $G\alpha_{11}$ SUBUNITS IN TRANSIENTLY TRANSFECTED COS-1 CELLS.

In order to test the function of cloned $G\alpha_q$ and $G\alpha_{11}$ subunits separately, appropriate cDNA clones which were inserted in pCMV vectors, were transiently transfected into COS-1 cells. Two days after transfection the levels of expression of the $G\alpha_q$ and $G\alpha_{11}$ proteins were assessed by western blotting using a polyclonal peptide antibody raised against a peptide representing the C terminal decapeptide common to both $G\alpha_q$ and $G\alpha_{11}$. Fig. 5.4.1. shows that in the COS-1 cells which had been transiently transfected with pCMV plasmid alone a 42 kDa band representing an unresolved mixture of $G\alpha_q$ and $G\alpha_{11}$ protein was detected. However, transient transfection with either $G\alpha_q$ or $G\alpha_{11}$ showed bands with a >50-fold increase in the reactivity with the antibody as compared to the low reactivity seen in cells which were transiently transfected with the pCMV plasmid only. The cells were treated identically to those used in subsequent experiments for the analysis of inositol phosphates.

Fig. 5.3.1.

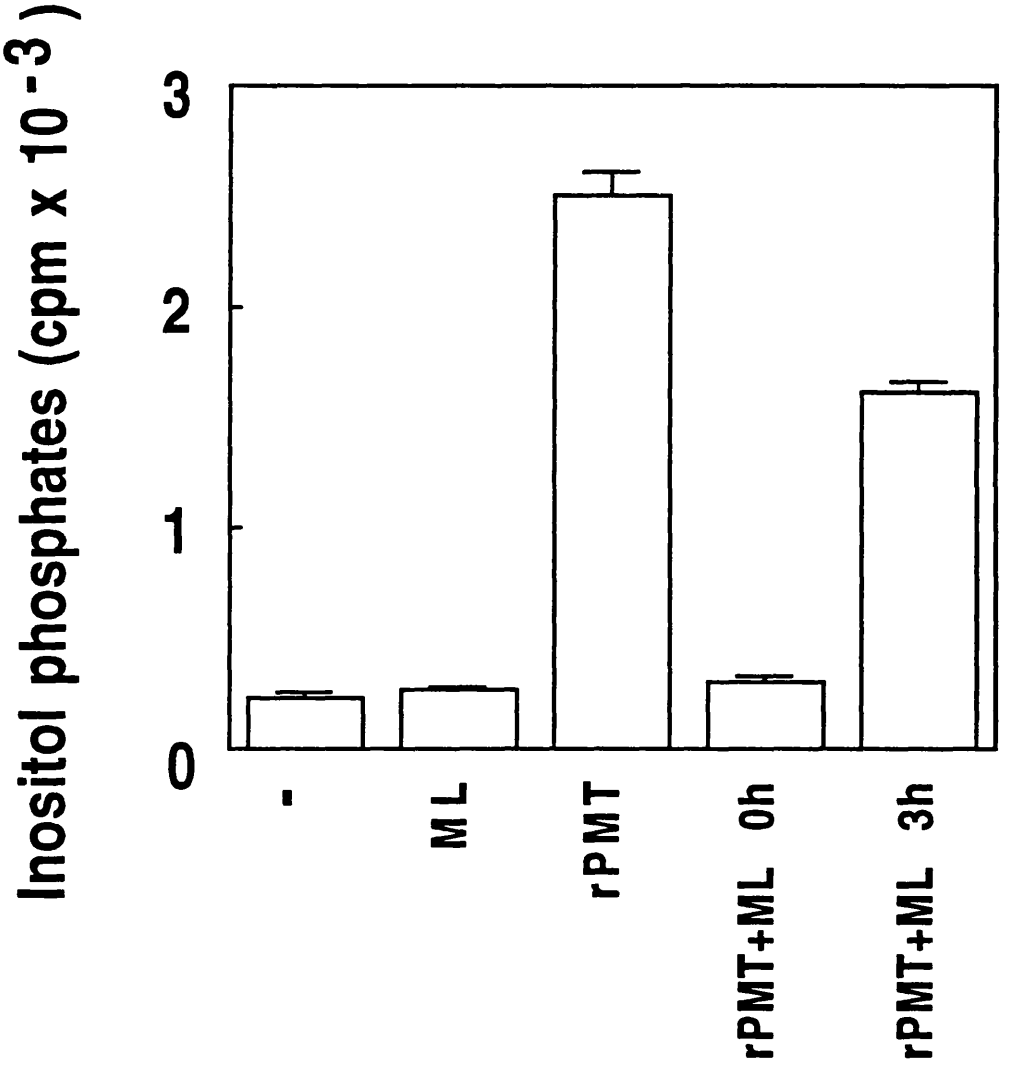

Inositol phosphates (cpm x 10 -3)
3
2
1
0
-
ML
rPMT
rPMT+ML 0h
rPMT+ML 3h

Fig. 5.3.1. The effect of methylamine on the production of inositol phosphates enhanced by rPMT in COS-1 cells. [2-^{3}H]inositol-labelled COS-1 cells were treated or not with 100 ng/ml rPMT for 7 h. Methylamine (9 mM) was added either at the same time as rPMT or 3 h after addition of rPMT. LiCl (20 mM) was added 30 min prior to the termination of the reactions. Total inositol phosphates were extracted and analysed as described. Values shown are means ± S.E.M. of triplicate determinations.

Fig. 5.4.1.

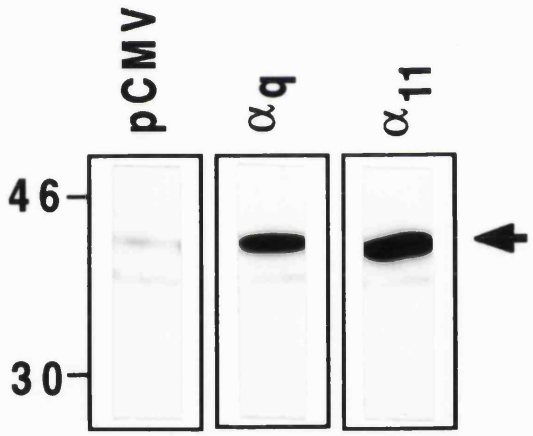


Fig. 5.4.1. Expression of $G_{\alpha q}$ and $G_{\alpha 11}$ subunits in transiently transfected COS-1 cells. Cultures of COS-1 cells were transiently transfected with either empty plasmid (pCMV) or plasmids containing cDNAs encoding $G\alpha_q$ or $G\alpha_{11}$ subunits as described in 'Materials and Methods'. The cells were maintained under the same conditions as the cells used in inositol phosphate assays for 48 h after transfection. The cells were then lysed in SDS/PAGE sample buffer, electrophoresed in 12.5 % SDS/PAGE, and analysed by Western blotting using an antibody raised against the C-terminal decapeptide of Gαq/11. The 42-kDa protein recognised specifically by the antibody is indicated by the arrow.

These results demonstrate that COS-1 cells express low levels of $G\alpha_{q/11}$ and that the transient transfection of both $G_{\alpha q}$ and $G_{\alpha 11}$ under the conditions used in our assay resulted in a massive enhancement of expression of these proteins

5.5. EFFECT OF OVEREXPRESSION OF $G\alpha_q$ AND $G\alpha_{11}$ ON THE PRODUCTION OF INOSITOL PHOSPHATES STIMULATED BY rPMT IN COS-1 CELLS.

COS-1 cells were transiently transfected with cDNAs corresponding to $G\alpha_q$ and $G\alpha_{11}$ subunits and labelled with [2-^{3}H]inositol as described. A slight increase in the steady state levels of inositol phosphates was observed in the cells transfected with $G\alpha_q$ and $G\alpha_{11}$, suggesting that the majority of the $G\alpha$ subunits may be in the plasma membrane in an inactive, *e.g.* GDP-bound state and may require activation before they could stimulate PI-PLC. Fig. 5.5.1. shows that the production of inositol phosphates stimulated by AlF_4^- was markedly increased in both $G\alpha_q$- and $G\alpha_{11}$-transfected cells. AlF_4^- is thought to act by interacting with the GDP-bound form of the α subunits of a variety of heterotrimeric G proteins and stabilise them in the GTP bound configuration (Bigay *et al.*, 1987). These results demonstrate that the $G\alpha_q$ and $G\alpha_{11}$ subunits, generated by cDNA expression, when activated can stimulate PI-PLC activity. In addition Fig. 5.5.1. shows that the production of inositol phosphates by rPMT is also markedly enhanced in both $G\alpha_q$- and $G\alpha_{11}$-transfected cells. These results demonstrate that this family of G proteins is involved in the activation of PI-PLC by rPMT.

5.6. SUMMARY AND DISCUSSION

The results in the chapters 3 and 4 demonstrate that rPMT stimulates phosphatidylinositol 4,5-bisphosphate hydrolysis in Swiss 3T3 cells through the same pathway as the neuropeptides. The action of rPMT on this pathway was shown to involve a functional G protein. The neuropeptides are known to act through receptors which couple to heterotrimeric G proteins (Heslop *et al.*, 1986, Takuwa *et al.*, 1987, Zachary *et al.*, 1987a, Nanberg and Rozengurt, 1988, Issandou and Rozengurt, 1990). Heterotrimeric G proteins consist of three subunits, α, β, and γ. The α subunits contain GTPase activity and appear to be responsible for interaction with a variety of effectors. Many α subunits have been cloned and sequenced, and they can be divided into four classes based on their sequence identity (Simon *et al.*, 1991). The sensitivity of these α subunits to pertussis toxin has also been a criteria for distinguishing between different G protein α subunits in particular those G proteins which couple receptors to phosphoinositide breakdown (Ui, 1990). Pertussis toxin ADP-ribosylates a cysteine residue four amino acids from the C-terminus of susceptible α subunit

Fig. 5.5.1.

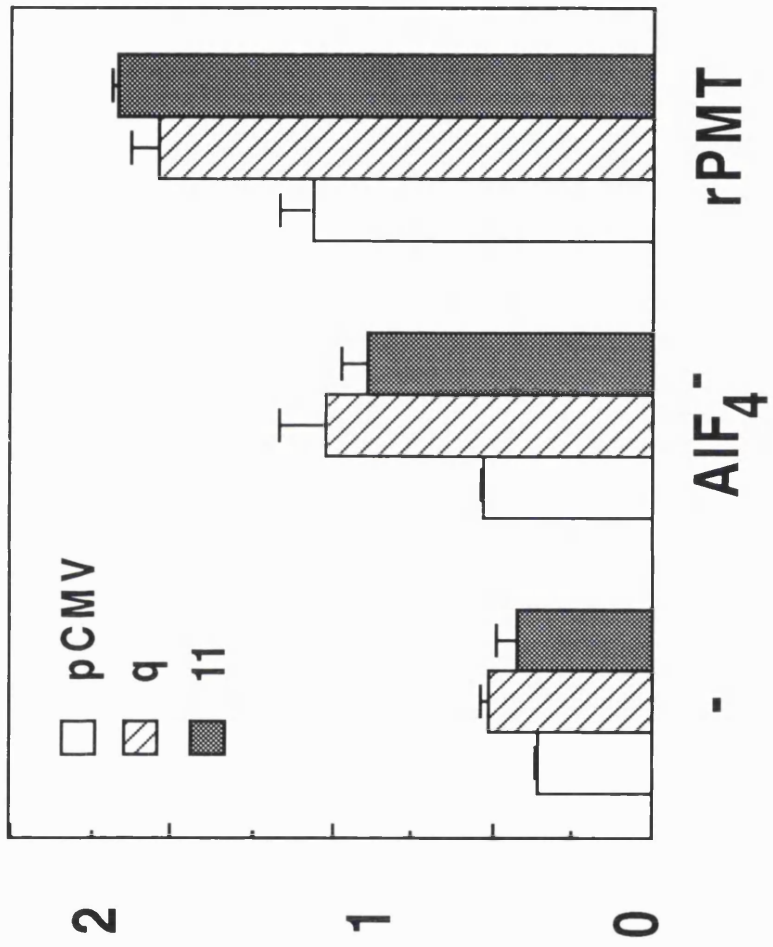


Fig. 5.5.1. Effect of overexpression of $G_{\alpha q}$ and $G_{\alpha 11}$ on the production of inositol phosphates stimulated by rPMT in COS-1 cells. COS-1 cells in 30 mm dishes were transfected as described in 'Materials and Methods'. Twenty four hours after transfection the cells were labelled with 5 μCi/ml [2-^{3}H]inositol in DMEM medium containing 10 % foetal bovine serum. After a further 16 -18 h the cells were washed twice with DMEM and the medium replaced with 5 μCi/ml [2-^{3}H]inositol in DMEM medium with or without 200 ng/ml rPMT. LiCl (20 mM) was added directly to the dishes and the reactions terminated after a further 40 min. AlF_4^- (30 μM $AlCl_3$ and 10 mM NaF) was added 10 min after the addition of LiCl. Total inositol phosphates were analysed as described previously. Values represent the means ± S.E.M. for triplicate determinations and are representative of three individual experiments.

proteins (Strathmann and Simon, 1990). Activation of phospholipase C by bombesin and vasopressin in Swiss 3T3 cells has been shown to be mediated through a pertussis toxin insensitive G protein (Zachary *et al.*, 1987a, Erusalimsky *et al.*, 1988, Fisher and Schonbrunn, 1988, Cattaneo and Vincentini, 1989, Erusalimsky and Rozengurt, 1989). The α subunits of the Gq class of G proteins have recently been demonstrated to be specific activators of the β-type PI-PLC isozymes (Blank *et al.*, 1991, Shenker *et al.*, 1991, Smrcka *et al.*, 1991, Taylor *et al.*, 1991, Berstein *et al.*, 1992). In addition, $G_{\alpha q}$ and $G_{\alpha 11}$ have recently been demonstrated to be involved in ligand mediated stimulation of PI-PLC activity by vasopressin, angiotensin, bradykinin, thromboxane A_2, thyrotropin releasing hormone and α_1-adrenergic receptors (Gutowski *et al.*, 1991, Shenker *et al.*, 1991, Wange *et al.*, 1991, Aragay *et al.*, 1992, Wu *et al.*, 1992a). Although the G protein involved in the activation of PI-PLC by bombesin has not yet been demonstrated, the most likely candidate is Gq . None of the Gq protein α subunits are substrates for ADP-ribosylation catalysed by pertussis toxin. In this chapter we have demonstrated that rPMT stimulation of inositol phosphate production, like the neuropeptides, occurs through a pertussis toxin-insensitive pathway. The results in section 5.1. demonstrate that pertussis toxin had no effect on the stimulation of inositol phosphates by rPMT nor did rPMT affect the ADP-ribosylation by pertussis toxin of its 40 kDa substrate in Swiss 3T3 cells. Clearly the cellular substrates as well as the mechanisms of action of these two toxins are distinct from one another. These results are of added importance as recent findings demonstrate that the βγ subunits of pertussis toxin-sensitive G proteins are able to activate the β isozymes of PI-PLC. Hence our results rule out the possibility that rPMT stimulation of PI-PLC is mediated through the βγ subunits of pertussis toxin sensitive G proteins.

Evidence supporting the involvement of $G\alpha_q$ and $G\alpha_{11}$ proteins in PI-PLC activation has been provided by transient transfection of cells with cDNAs corresponding to these G protein α subunits. In the present chapter we have assessed the effect of transfection of $G\alpha_q$ and $G\alpha_{11}$ on the ability of rPMT to stimulate PtdIns(4,5)P_2 hydrolysis in COS-1 cells. Overexpression of $G\alpha_q$ and $G\alpha_{11}$ proteins in COS-1 cells resulted in an increased production of inositol phosphates induced by rPMT. A similar increase in inositol phosphate production in Gαq and $G\alpha_{11}$ transfected cells was observed on stimulation of the cells with AlF_4^-, which is known to activate α subunits by stabilising them in the GTP-bound configuration. The overexpression of these α subunit proteins was assessed by western blotting using a polyclonal antibody raised against a peptide representing the C terminal decapeptide common to both α_q or α_{11} and fig 5.4.1. demonstrates that after transient transfection a >50-fold increase in the expression of both proteins was observed. The stimulation of inositol phosphates by rPMT in $G\alpha_q$- and $G\alpha_{11}$-transfected cells were similar, suggesting that the toxin can utilise either G protein subunit equally. This stimulation of inositol phosphates observed with rPMT in $G\alpha_q$ and $G\alpha_{11}$ transfected cells further substantiates the point that

rPMT stimulates the activation of PI-PLC is not mediated through βγ-subunits. If this was the case, then it might be expected that the overexpression of α-subunits would remove all free βγ-subunits resulting in an inhibition of inositol phosphates induced by rPMT rather than a stimulation.

In addition, stimulation of inositol phosphate production by rPMT in the $G\alpha_q$ and $G\alpha_{11}$ transfected cells can be compared with the results in chapter 3, where addition of the neuropeptides to rPMT-treated cells resulted in an enhancement of inositol phosphates. The activation of neuropeptide receptors by ligand binding is known to result in the dissociation of receptor-associated heterotrimeric G proteins into α- and βγ-subunits. In a similar manner to that observed in the transfected cells, these free α-subunits could then account for the enhanced stimulation of inositol phosphates observed in rPMT-treated cells.

The mechanisms of entry of rPMT and activation of PI-PLC by rPMT in COS-1 appear to be similar to that observed in Swiss 3T3 cells. Thus it is likely that $G\alpha_q$ and $G\alpha_{11}$ are also involved in the stimulation of inositol phosphates by rPMT in 3T3 cells.

CHAPTER SIX: RESULTS

INDUCTION OF EARLY ONCOGENES BY rPMT

In addition to the rapid early signalling events in the membrane and cytosol, serum and growth factors transiently induce the expression of the protooncogenes c-fos and c-myc (Kelly *et al.*, 1983, Cochran *et al.*, 1984, Greenberg and Ziff, 1984, Kruijer *et al.*, 1984, Muller *et al.*, 1984). These protooncogenes encode nuclear proteins which are thought to play a role in the transduction of the mitogenic signal in the nucleus (see section 1.3.6). The induction of these genes is subject to strong feedback inhibition (Sassone-Corsi, *et al.*, 1988b, Offir, *et al.*, 1990, Penn, *et al.*, 1990). At least part of this autoregulatory mechanism could be the result of receptor desensitization.

Pasteurella multocida toxin (PMT) is a potent mitogen for Swiss 3T3 cells in the absence of other growth-promoting agents (see section 1.4.1). Unlike other growth factors whose mitogenic effects are mediated through specific high affinity receptors, rPMT acts intracellularly and by-passes receptor-mediated signal generation. Hence, the stimulation of early signals, including inositol phosphate production, by rPMT persists for longer periods of time than the stimulation of similar pathways by growth factors. In addition, the induction of these oncogenes by growth factors has been shown to be mediated through specific signalling pathways in the cytosol (Rozengurt and Sinnett-Smith, 1988). In particular, activation of PKC by certain growth factors or mitogenic agents is known to lead to the expression of c-fos and c-myc. rPMT has been demonstrated to activate PKC in Swiss 3T3 cells (Staddon *et al.*, 1990). This makes rPMT is an attractive tool for exploring the mechanism(s) underlying early protooncogene induction.

The experiments presented in this chapter were designed to establish the effects of rPMT on the expression of c-fos and c-myc mRNA levels in quiescent Swiss 3T3 cells. In particular, to determine whether the kinetics of induction of these genes by rPMT is the same as for other growth factors and also to determine the contribution of the activation of PKC, by rPMT, on the expression of these genes.

6.1. TIME COURSE OF INDUCTION OF C-FOS AND C-MYC BY rPMT

Quiescent cultures of Swiss 3T3 cells were treated with rPMT at 20 ng/ml, a concentration which stimulates maximum DNA synthesis in this cell line. Total RNA was extracted at

various times after addition of rPMT and analysed for c-fos mRNA levels by RNA blot transfer and hybridisation to a c-fos DNA probe. The 2.2-kilobase c-fos mRNA which is at the limit of detection in quiescent cells is markedly increased by rPMT. Figure 6.1.1. shows that c-fos was first detected 3 h after rPMT addition, was maximally increased after 4 h, and decreased slowly for a further six hours until it was no longer detectable after 11 h. Densitometer tracing of the autoradiograms indicated that rPMT increased the level of c-fos mRNA in the 4 h samples by 110 ± 20-fold (n=3). The induction of c-fos mRNA by rPMT was compared to the induction of this protooncogene by bombesin. Bombesin also markedly enhances c-fos mRNA levels in Swiss 3T3 cells. In agreement with previous results (Rozengurt and Sinnett-Smith, 1988) Fig. 6.1.1. shows that the maximum level of induction of c-fos by bombesin (200 ± 28-fold) occurred 30 min after stimulation and rapidly declined to nearly basal levels in the following hour. The time course of induction of c-fos by bombesin and rPMT as assessed by scanning densitometry is also shown in Fig.6.1.1. Levels of induction are expressed as a percentage of the maximal stimulation seen with bombesin at 30 min in this experiment. Although expression of c-fos mRNA stimulated by rPMT persists for a longer time than expression stimulated by bombesin, the maximal stimulation of c-fos by rPMT is lower than that seen for bombesin.

The addition of rPMT to quiescent cultures of Swiss 3T3 cells also increased the c-myc mRNA level (Fig. 6.1.2.). The 2.4-kilobase c-myc mRNA was detectable 3-4 h after rPMT addition, and the maximal increase (89 ± 5-fold) occurred 8 h after stimulation. Levels of c-myc mRNA remained elevated at nearly maximal levels for at least another 3 hours (92% of maximal at 11h) and very slowly decreased over the subsequent hours until at 28 h, levels were back to basal (Fig. 6.1.2.). These kinetic results are again in contrast to the results observed for bombesin. Maximal stimulation of c-myc mRNA by bombesin occurred 3 h after stimulation and returned to nearly basal levels after 7h. Interestingly, maximal stimulation of c-myc by rPMT in this and other experiments (results not shown) was found to be approximately 10 % higher than maximal levels stimulated by bombesin.

These results demonstrate that rPMT, like bombesin and other growth factors, stimulates the induction of c-fos and c-myc. However the kinetics for the induction these genes by rPMT is different from that observed for other growth factors acting through receptors. The maximum stimulation of both of these oncogenes by rPMT occurs approximately 3-5 h after maximal stimulation by bombesin. In addition, the decline in the levels of expression of both c-fos and c-myc after maximal stimulation are much slower for rPMT.

Fig. 6.1.1.

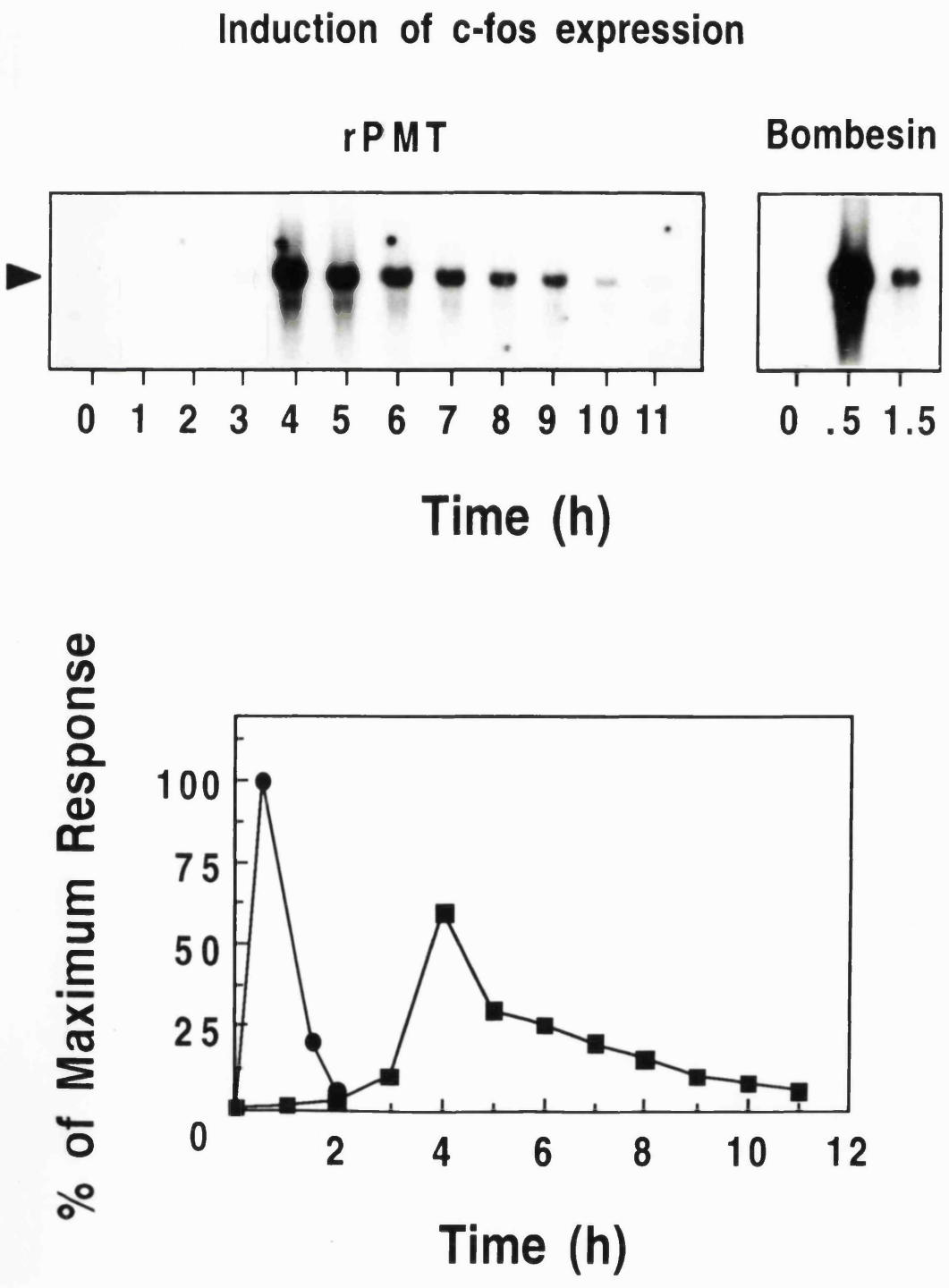

Induction of c-fos expression
rPMT
Bombesin
0 1 2 3 4 5 6 7 8 9 10 11
0 .5 1.5
Time (h)
% of Maximum Response
100
75
50
25
0
2 4 6 8 10 12
Time (h)

Fig. 6.1.1. Time course of c-fos mRNA induction by rPMT. Quiescent cultures of Swiss 3T3 cells grown in 90 mm Nunc dishes were washed twice and incubated with 5 ml of DMEM:Waymouth medium (1:1) containing either rPMT (20 ng/ml) or bombesin (6 nM) for the times indicated. Three dishes were pooled for each condition. The incubations were terminated by washing the cultures twice with ice cold PBS and total RNA was extracted and analysed for c-fos mRNA by Northern blotting as described in 'Materials and Methods'. **Top.** Autoradiogram of Northern blot of representative experiment. **Bottom**. The levels of c-fos were measured by scanning densitometry. The values are expressed as the percentage of the maximum level obtained on stimulation with either bombesin (closed circles) or rPMT (closed squares). Equal loading (10 µg RNA/lane) was confirmed by stripping the blot and reprobing with a GAPDH probe (results not shown). The level of c-fos induction was taken as a fraction over the level of GAPDH mRNA by scanning densitometry.

Fig. 6.1.2.

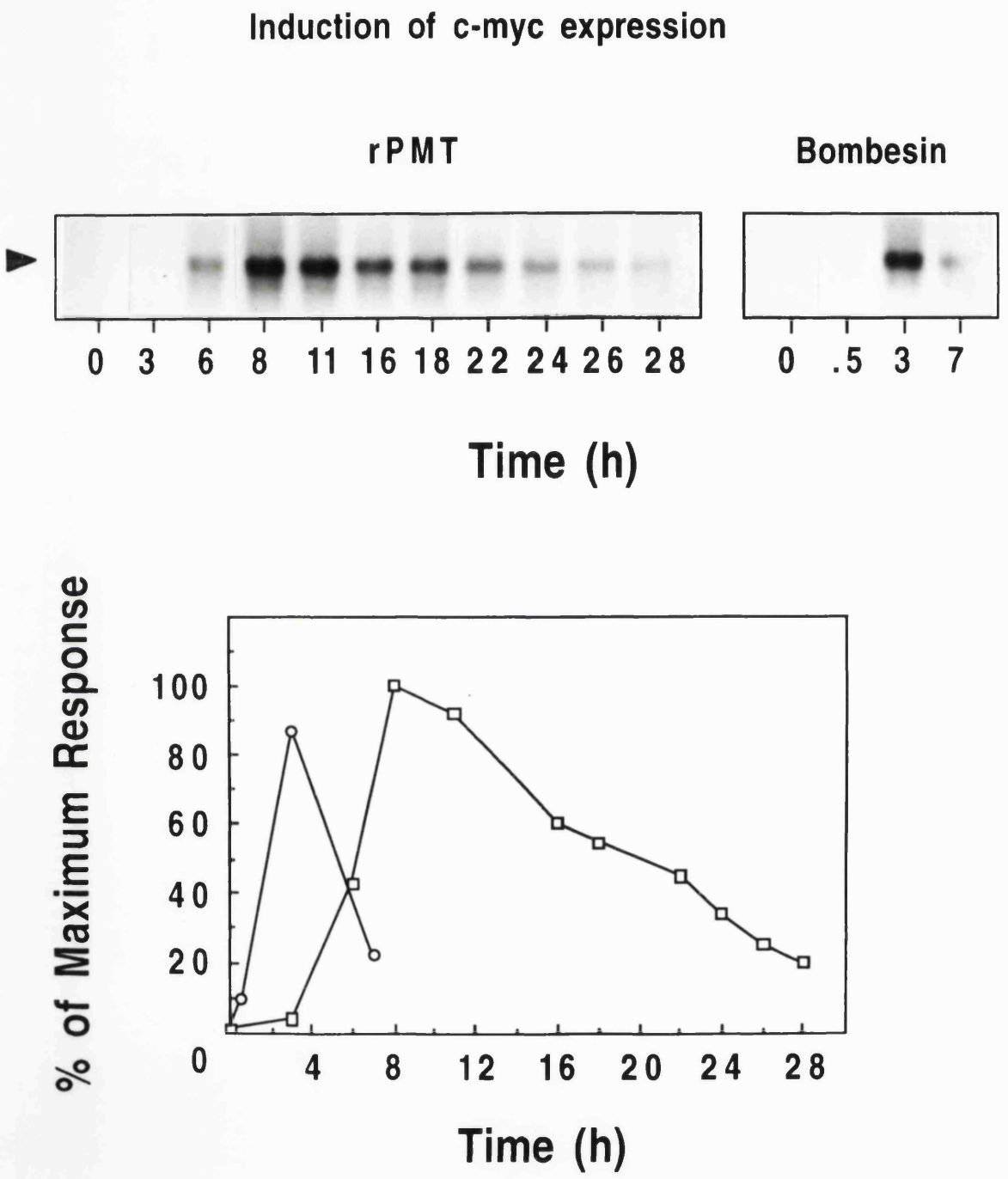

Induction of c-myc expression
rPMT
Bombesin
0 3 6 8 11 16 18 22 24 26 28
0 .5 3 7
Time (h)
% of Maximum Response
100
80
60
40
20
0
4 8 12 16 20 24 28
Time (h)

Fig. 6.1.2. Autoradiograph of the time course of c-myc mRNA induction by rPMT. Quiescent cultures of Swiss 3T3 cells grown in 90 mm Nunc dishes were washed twice and incubated with 5 ml of DMEM:Waymouth medium (1:1) containing either rPMT (20 ng/ml) or bombesin (6 nM) for the times indicated. The incubations were terminated by washing the cultures twice with ice cold PBS and dissolving in 4 M GTC. Three dishes were pooled for each condition and preparation of total RNA, Northern blotting analysis for c-myc mRNA and autoradiography were as described in 'Materials and Methods'. **Top.** Autoradiogram of Northern blot of representative experiment **Bottom.** The levels of c-myc were measured by scanning densitometry. The values are expressed as the percentage of the maximum level obtained on stimulation with either bombesin (open circles) or rPMT (open squares). Equal loading (10 µg RNA/lane) was confirmed by stripping the blot and reprobing with a mouse 18S probe (results not shown). The level of c-myc induction was taken as a fraction over the level of mouse 18S mRNA by scanning densitometry.

6.2. rPMT REQUIRES CELLULAR ENTRY AND PROCESSING IN ORDER TO STIMULATE C-FOS EXPRESSION IN SWISS 3T3 CELLS.

The induction of the expression of c-fos and c-myc mRNAs by rPMT occurs at times which are about 3-5 h later than the stimulation of these genes by bombesin. A similar time lag is observed for the stimulation by rPMT of other early signalling events in Swiss 3T3 cells, including inositol phosphate production. Thus, while bombesin stimulates the production of inositol phosphates within minutes, the production of inositol phosphates by rPMT occurs 2-3 h after addition of rPMT, depending upon the concentration used (Rozengurt *et al.*, 1990b, Staddon *et al.*, 1991a). This lag period in the action of rPMT most likely reflects the time required for its cellular entry and possible processing and activation. Many other toxins have a similar requirement for entry and processing prior to stimulating their biological responses (Neville and Hudson, 1986, Olsnes and Sandvig, 1988). To test whether this was also the case for the stimulation of c-fos expression by rPMT, we looked at the effect of either methylamine, a membrane permeant weak base which increases endosomal and lysosomal pH (Middlebrook and Dorland, 1984) or PMT-antiserum, on the ability of rPMT to induce the expression of c-fos. Fig.6.2.1. shows that addition of methylamine at the same time as rPMT (0 h) was able to block the maximal induction of c-fos by rPMT at 4 h. However, when methylamine was added 3 h after the addition of rPMT, no effect on the maximal induction of c-fos mRNA was observed. Similarly, early but not late addition of PMT-antiserum was able to block c-fos mRNA induction. In contrast, the maximal expression of c-fos by bombesin at 0.5 h was not affected by either methylamine or the PMT-antiserum demonstrating that these agents specifically inhibit responses stimulated by the toxin. These results demonstrate that rPMT requires cellular entry and processing in order to stimulate early gene expression.

6.3. STIMULATION OF C-FOS AND C-MYC EXPRESSION BY rPMT DOES NOT REQUIRE PROTEIN SYNTHESIS.

The increases in c-fos and c-myc mRNA levels induced by growth factors, including bombesin, are not prevented by inhibitors of protein synthesis (Kelly *et al.*, 1983, Greenberg and Ziff, 1984). In fact, these genes are overexpressed when growth factors are added together with drugs such as cycloheximide or anisomycin (Kelly *et al.*, 1983, Greenberg and Ziff, 1984). These findings indicated that the increase in c-fos and c-myc mRNA levels by these growth factors is not secondary to the growth response. Indeed, it would seem that the cellular machinery leading to the induction of these genes is inactive in quiescent cells but poised to respond to specific signals generated by these growth factors. In order to assess whether the increase in c-fos and c-myc mRNAs induced by rPMT also occurs in the absence of *de novo* protein synthesis, we examined the effects of cycloheximide and anisomycin on

Fig. 6.2.1.

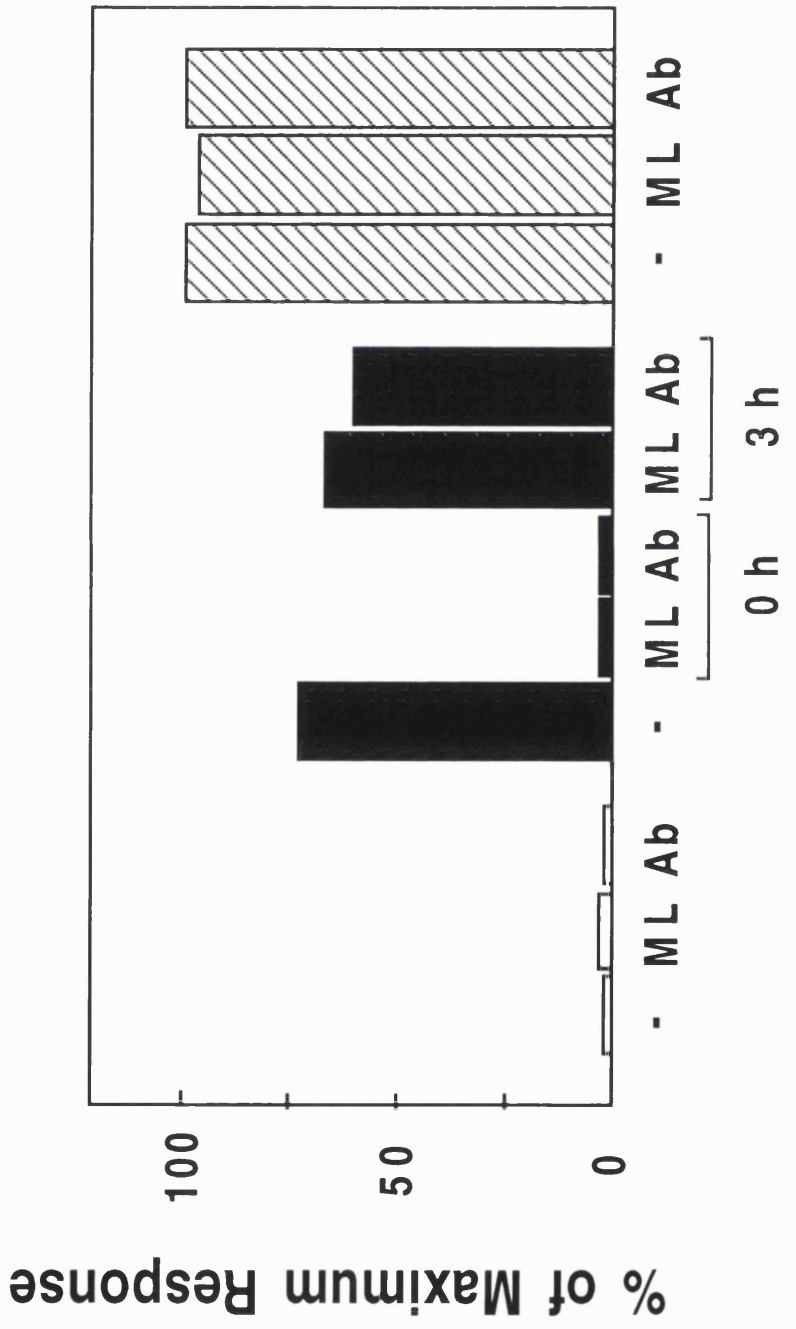


Fig. 6.2.1. Selective inhibition of the rPMT-induced expression of c-fos mRNA by methylamine and PMT antiserum. Cultures of Swiss 3T3 cells in 90 mm dishes were washed twice with DMEM and then incubated in 5 ml DMEM:Waymouth containing either rPMT (20 ng/ml) for 4 h (closed bars) or bombesin (6 nM) for 30 min (striped bars). Methylamine (ML, 9 mM pH 7.4) or PMT antiserum (Ab, 5 µl/ml) were added either at the same time as toxin (0 h) or three hours after addition of toxin (3 h). ML or Ab were added to cells stimulated with bombesin at the same time as the addition of bombesin. At the end of the incubations, three dishes were pooled for each condition and total RNA was extracted and Northern blot analysis performed using a c-fos DNA probe. Levels of c-fos mRNA were measured by scanning densitometry. The values are expressed as the percentage of the maximum levels obtained by bombesin in this experiment. Equal loading (10 µg/lane) was confirmed by reprobing the blot with a GAPDH DNA probe. The level of c-fos induction was taken as a fraction over the level of GAPDH mRNA by scanning densitometry.

Fig. 6.3.1.

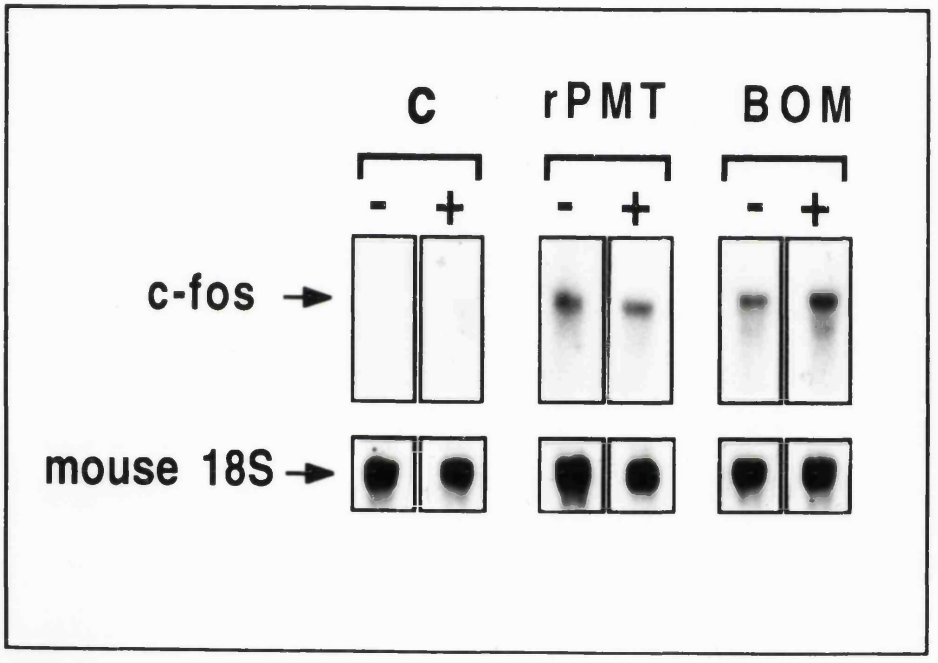


Fig. 6.3.1. Effect of cycloheximide on the induction of c-fos mRNA by rPMT. Quiescent cultures of Swiss 3T3 cells grown in 90 mm Nunc dishes were washed twice and incubated with 5ml of DMEM:Waymouth medium (1:1) containing either rPMT (20 ng/ml) for 4 h or bombesin (6 nM) for 30 min. Incubations were in the absence (-) or presence (+) of cycloheximide (0.5 µg/ml). The incubations were terminated by washing the cultures twice with ice cold PBS and dissolving in 4 M GTC. Preparation of total RNA, Northern blotting analysis for c-fos mRNA and autoradiography were as described in 'Materials and Methods'. The blot was stripped and reprobed with a mouse 18S DNA probe to confirm equal loading.

Fig. 6.3.2.

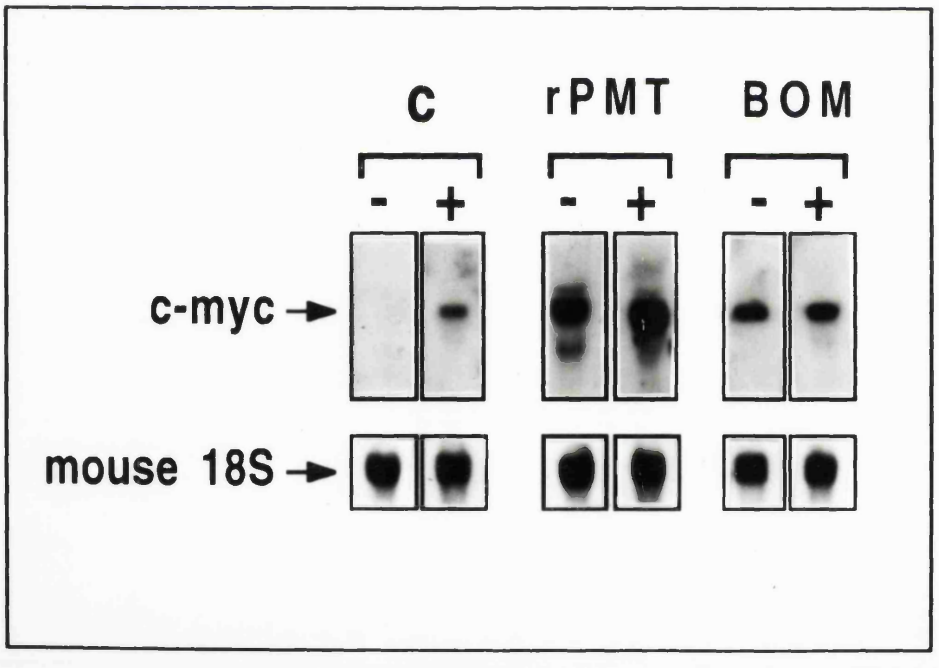


Fig. 6.3.2. Effect of anisomycin on the induction of c-myc mRNA by rPMT. Quiescent cultures of Swiss 3T3 cells grown in 90 mm Nunc dishes were washed twice and incubated with 5ml of DMEM:Waymouth medium (1:1) containing either rPMT (20 ng/ml) for 6 and 8 h or bombesin (6 nM) for 30 min. Incubations were in the absence (-) or presence (+) of anisomycin (50 μM). The incubations were terminated by washing the cultures twice with ice cold PBS and dissolving in 4 M GTC. Preparation of total RNA, Northern blotting analysis for c-myc mRNA and autoradiography were as described in Materials and Methods. The blot was stripped and reprobed with a mouse 18S DNA probe to confirm equal loading.

the induction of c-fos and c-myc mRNAs respectively, by rPMT. Fig 6.3.1. shows that the expression of c-fos mRNA levels stimulated by rPMT at 4h is not inhibited by treatment with cycloheximide. Fig. 6.3.1 also demonstrates that cycloheximide did not inhibit c-fos mRNA expression induced by bombesin. Similarly, anisomycin had no inhibitory effect on the levels of c-myc expression after treatment of cells with rPMT for 8 h or bombesin for 3 h (Fig. 6.3.2.). These results demonstrate that rPMT, like bombesin and other growth factors, induces c-fos and c-myc expression without the requirement for protein synthesis. These results are of added importance as they rule out the possibility that the delay in the expression of c-fos and c-myc mRNAs induced by rPMT might reflect a period of protein synthesis in addition to the time required for entry and activation.

6.4. THE ROLE OF PKC IN MEDIATING THE INDUCTION OF C-FOS AND C-MYC mRNA BY rPMT IN SWISS 3T3 CELLS

Prolonged exposure (>24 h) of Swiss 3T3 cells to a high concentration of phorbol 12,13-dibutyrate (PBt_2) leads to a progressive decline in the specific activity of PKC (Rodriguez-Pena and Rozengurt, 1984, Ballester and Rosen, 1985, Rozengurt *et al.*, 1985, Kaibuchi *et al.*, 1986) and virtual disappearance of immunoreactive PKC (Issandou and Rozengurt, 1989, Olivier and Parker, 1992). Cells treated in this manner are no longer responsive to a subsequent addition of phorbol esters (Collins and Rozengurt, 1984) or other mitogens acting through PKC dependent pathways (Rozengurt *et al.*, 1983b, Rodriguez-Pena and Rozengurt, 1986, Rozengurt and Sinnett-Smith, 1987, Erusalimsky *et al.*, 1988, Erusalimsky and Rozengurt, 1989), but can proliferate in response to growth factors which do not utilise PKC (Collins and Rozengurt, 1984, Vara and Rozengurt, 1985, Rozengurt and Sinnett-Smith, 1987). rPMT has previously been shown to increase the phosphorylation of an acidic 80-kDa cellular protein (80K/MARCKS see section 1.3.3.), which reflects the activation of PKC in intact and quiescent Swiss 3T3 cells (Staddon *et al.*, 1990). If the action of rPMT on c-fos and c-myc mRNA levels is mediated through activation of PKC, long term exposure to PBt_2 should block the increase in expression of these cellular oncogenes by a subsequent challenge with rPMT. To test this possibility, quiescent cultures of 3T3 cells were treated with PBt_2 at a saturating concentration (800 nM). After 40 h, control and treated cultures were washed and transferred to medium containing either rPMT, bombesin or PBt_2. As shown in Fig.6.4.1. PBt_2 stimulates c-fos expression on its own in control cells by directly activating PKC. Prolonged exposure of 3T3 cells to PBt_2 completely abolished the increase in c-fos mRNA induced by a subsequent addition of PBt_2. In contrast, down-regulation of PKC markedly decreased but did not abolish the induction of c-fos mRNA by bombesin. These results are in agreement with previous findings that the induction of this gene by bombesin occurs through both PKC-dependent and -independent pathways. Interestingly, the induction

Fig. 6.4.1.

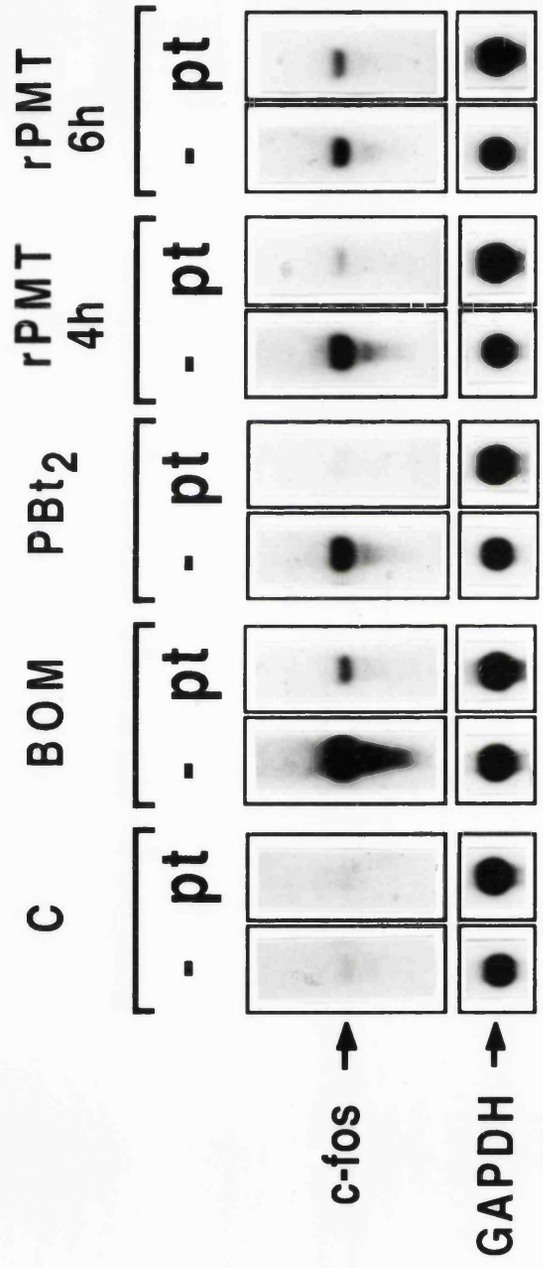


Fig. 6.4.1. Effect of treatment of 3T3 cells with phorbol dibutyrate (PBt_2) on the induction of c-fos promoted by subsequent addition of rPMT, PBt_2, or bombesin. Quiescent cultures of Swiss 3T3 cells in 90 mm Nunc dishes were incubated for 40 h in their own conditioned medium in the absence (-) or presence (pt) of PBt_2 (800 nM). At the end of the incubation period all cultures were washed with DMEM and then incubated in 5 ml DMEM:Waymouth medium (1:1) containing rPMT (20 ng/ml), bombesin (6 nM) or PBt_2 (200 nM). Incubations with bombesin and PBt_2 were for 30 min, while incubation with rPMT was for 4 h and 6h. PBt_2 (800 nM) was included in the incubations with rPMT to ensure the continued down-regulation of PKC during these long time periods. The incubations were terminated by washing the cultures twice with ice cold PBS and dissolving in 4 M GTC. Preparation of total RNA, Northern blotting analysis for c-fos mRNA and autoradiography were as described in 'Materials and Methods'. The blot was stripped and reprobed with a mouse 18S DNA probe to confirm equal loading.

Fig. 6.4.2.

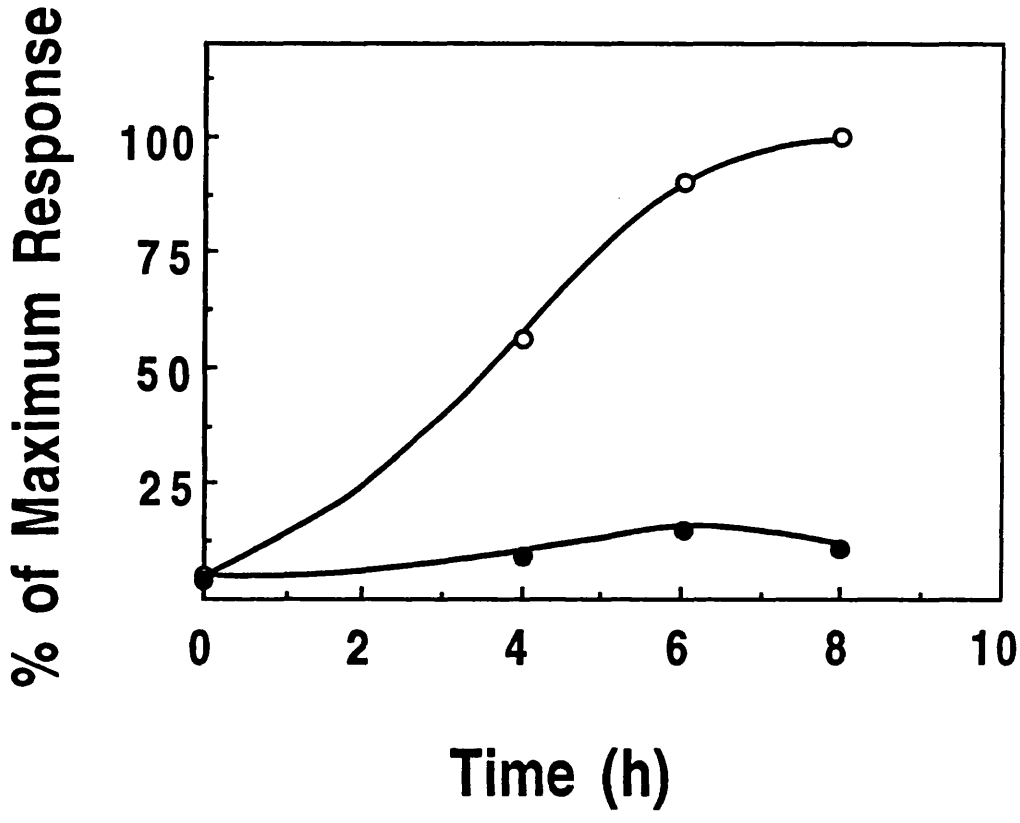


Fig. 6.4.2. Effect of treatment of 3T3 cells with phorbol dibutyrate (PBt_2) on the induction of c-myc promoted by subsequent addition of rPMT, PBt_2, or bombesin. Quiescent cultures of Swiss 3T3 cells in 90 mm Nunc dishes were incubated for 40 h in their own conditioned medium in the absence (open circles) or presence (closed) of PBt_2 (800 nM). At the end of the incubation period all cultures were washed with DMEM and then incubated in 5 ml DMEM:Waymouth medium (1:1) containing rPMT (20 ng/ml). Stimulation with rPMT was for the times indicated. In the PBt_2-treated cells PBt_2 (800 nM) was included in the incubations with rPMT to ensure the continued down-regulation of PKC during these long time periods. The incubations were terminated by washing the cultures twice with ice cold PBS and dissolving in 4 M GTC. Preparation of total RNA, Northern blotting analysis for c-myc mRNA were as described in 'Materials and Methods'. Levels of c-myc were measured by scanning densitometry and the values are expressed as the percentage of the maximum levels obtained by rPMT. The blot was stripped and reprobed with a mouse 18S DNA probe to confirm equal loading. The level of c-myc induction was taken as a fraction over the level of mouse 18S by scanning densitometry.

of c-fos mRNA by rPMT is also markedly reduced but not abolished in PBt_2 pretreated cells. A residual induction which represents about 5% of the maximum rPMT response (as assessed by scanning densitometry) is still observed in pretreated cells. These results suggest that rPMT, like bombesin can stimulate the induction of c-fos mRNA by pathways other than PKC activation.

The ability of rPMT to increase the levels of c-myc mRNA in PBt_2 treated cells was also assessed. In accordance with the previous findings for c-fos induction by rPMT, the levels of c-myc message induced by rPMT were greatly reduced but not abolished in PKC depleted cells (Fig. 6.4.2.). Thus, rPMT stimulates the induction of c-fos and c-myc through both PKC-dependent and independent pathways.

6.5. SUMMARY AND DISCUSSION.

The metabolic responses which are generated after binding of growth factors to their specific receptors often diminish over time despite the continued presence of a stimulus. This phenomenon is known as desensitization. Several mechanisms appear to regulate the responsiveness of receptors for their ligands and these mechanisms have been particularly well characterised for some receptor types in particular the β-adrenergic receptors (for review see Hausdorff *et al.*, 1990). There are rapid events involving receptor phosphorylation and slower events that involve increased receptor degradation and decreased receptor synthesis. Additional effects may involve direct modification of transduction machinery downstream of the receptor including uncoupling of receptors from transducing G proteins. Together these events blunt the effects of a given agonist and effectively stop signal transduction.

Transcription of the c-fos protooncogene is induced by stimulation of quiescent cells with serum and several growth factors, including bombesin, that act through specific high affinity receptors (Kruijer *et al.*, 1984, Muller *et al.*, 1984). The induction is rapid, transient, and can occur in the absence of new protein synthesis suggesting that post-translational modifications are involved in its transcriptional regulation (Cochran *et al.*, 1984, Greenberg *et al.*, 1986). Phosphorylation of transcription factor regulators by protein kinases, including MAP kinase, is thought to play a role in the transcriptional activation of c-fos (Treisman, 1992). In addition, expression of c-fos is negatively autoregulated by the phosphorylation of the c-fos protein (c-Fos) in its carboxyl-region (Sassone-Corsi, *et al.*, 1988b, Offir, *et al.*, 1990). The half life of c-fos mRNA is only 9 min and a sequence at the 3' end of the c-fos mRNA molecule is thought to be involved in its low stability (Shaw and Kamen, 1986).

c-myc mRNA is also transiently expressed on stimulating quiescent cells with growth factors . Levels of c-myc appear 30 min after mitogenic stimulation and reach maximum levels after 3 h (Reviewed in Rozengurt and Sinnett-Smith, 1988). C-myc expression is also known to be negatively autoregulated by complex mechanisms involving controls at the level of transcript initiation, elongation and stability (Penn *et al.*, 1990).

In addition to the autoregulatory control mechanisms which exist for the expression of these genes, part of the regulation of expression of these genes might be controlled by receptor desensitization. Since rPMT by passes receptor mediated events it was an ideal tool to study the effects of receptor desensitization on the induction of these two protooncogenes.

The results presented in this chapter demonstrate that the addition of rPMT to quiescent cultures of Swiss 3T3 cells causes a dramatic and prolonged increase in c-fos and c-myc mRNA levels without a requirement for new protein synthesis. However, the kinetics for the induction of both of these genes by rPMT were different to the kinetics for induction stimulated by bombesin. Maximal expression of both c-fos and c-myc by rPMT occurred at times which were 3-5 h later than corresponding maximal expression induced by bombesin: For c-fos mRNA levels, maximal expression was at 30 min and 4 h for bombesin and rPMT respectively (Fig.6.1.1.). Similarly, c-myc expression reached maximum levels at 3 h for bombesin and 8 h for rPMT (Fig. 6.1.2.). The delay in the induction of these protooncogenes by rPMT is in agreement with induction of other mitogenic signals by rPMT. The ability of the lysosomotrophic agent methylamine, and PMT antiserum to selectively block the induction of c-fos by rPMT suggests that rPMT requires cellular entry and activation in order to stimulate the expression of c-fos.

Once the levels of expression of c-fos or c-myc stimulated by bombesin reach maximum at 30 min and 3 h respectively, the levels of mRNA decline rapidly. c-fos mRNA have returned to nearly basal levels approximately 1.5 h after stimulation, while c-myc mRNA levels return to basal levels approximately 7-8 h after stimulation. In contrast, after maximal stimulation of c-fos and c-myc by rPMT, at 4 h and 8 h respectively, the levels of mRNA decline at a much slower rate than that observed for bombesin. Levels of c-fos induced by rPMT return to basal levels 11 h after stimulation. More notably, levels of c-myc remain at nearly maximum for 3-4 h (from 8 h to 11 h after stimulation) and do not return to basal until approximately 28 h after stimulation. These extended time courses of induction of these genes by rPMT may be a result of the persistent stimulation of early signals by rPMT which are not regulated by desensitization at receptor level. Interestingly, although the levels of expression remain elevated for longer, they do eventually return to basal levels. These results suggest that the strong autoregulatory mechanism which exist to regulate the expression of these genes are also working in rPMT-treated cells.

It should be pointed out that the maximum level of expression of c-fos mRNA by bombesin was larger than maximal levels stimulated by rPMT. Hence, a possible explanation for the extended time course of induction of c-fos by rPMT might be that entry of rPMT into these cells occurs in an asynchronous manner. The majority of cells in these cultures might stimulate maximum expression of c-fos at 4 h, a smaller population will stimulate maximal expression at longer times. Alternatively, bombesin might stimulate the induction of c-fos mRNA through signal transduction pathways that are not stimulated by rPMT. In contrast, maximal stimulation of c-myc by rPMT is slightly larger than maximal stimulation of this gene by bombesin. In addition, levels of c-myc mRNA remain at nearly maximal levels for at least 3h when they slowly return to basal levels. These results suggest that the persistent activation of early signalling pathways by rPMT may play a role in the prolonged expression of c-myc. These observations require further investigation.

PKC activation has been implicated in the sequence of events linking receptor occupancy and protooncogene induction (reviewed in Rozengurt and Sinnett-Smith, 1988). However, additional pathways involving calcium ion fluxes as well as a pathway dependent on arachidonic acid release have also been demonstrated (Rozengurt and Sinnett-Smith, 1987, Rozengurt and Sinnett-Smith, 1988, Mehmet *et al.*, 1990a). A large part of the stimulation of c-fos by bombesin is known to be mediated through a PKC-dependent pathway. We therefore assessed the role of PKC in the modulation of c-fos and c-myc mRNA levels induced by rPMT by exploiting the selective down-regulation of PKC achieved by long-term treatment with phorbol esters. We have shown that the induction of c-fos and c-myc mRNA by rPMT is greatly attenuated but not abolished in PKC down-regulated cells. These results suggest that the stimulation of these oncogenes by rPMT is mediated at least in part, by PKC activation. The PKC-independent pathway for the stimulation of these oncogenes by rPMT might involve Ca^{2+} ion fluxes and/or arachidonic acid release as rPMT is known to stimulate Ca^{2+} mobilization (Staddon *et al.*, 1991a) and arachidonic acid release (E. Rozengurt, unpublished results). Further experiments are required to determine the contribution of these signalling events to the stimulation of protooncogene expression by rPMT.

In conclusion, the evidence presented in this chapter indicates that rPMT can stimulate a prolonged expression of the early oncogenes c-fos and c-myc. At least part of the induction of these genes by rPMT is mediated through a PKC-dependent pathway.

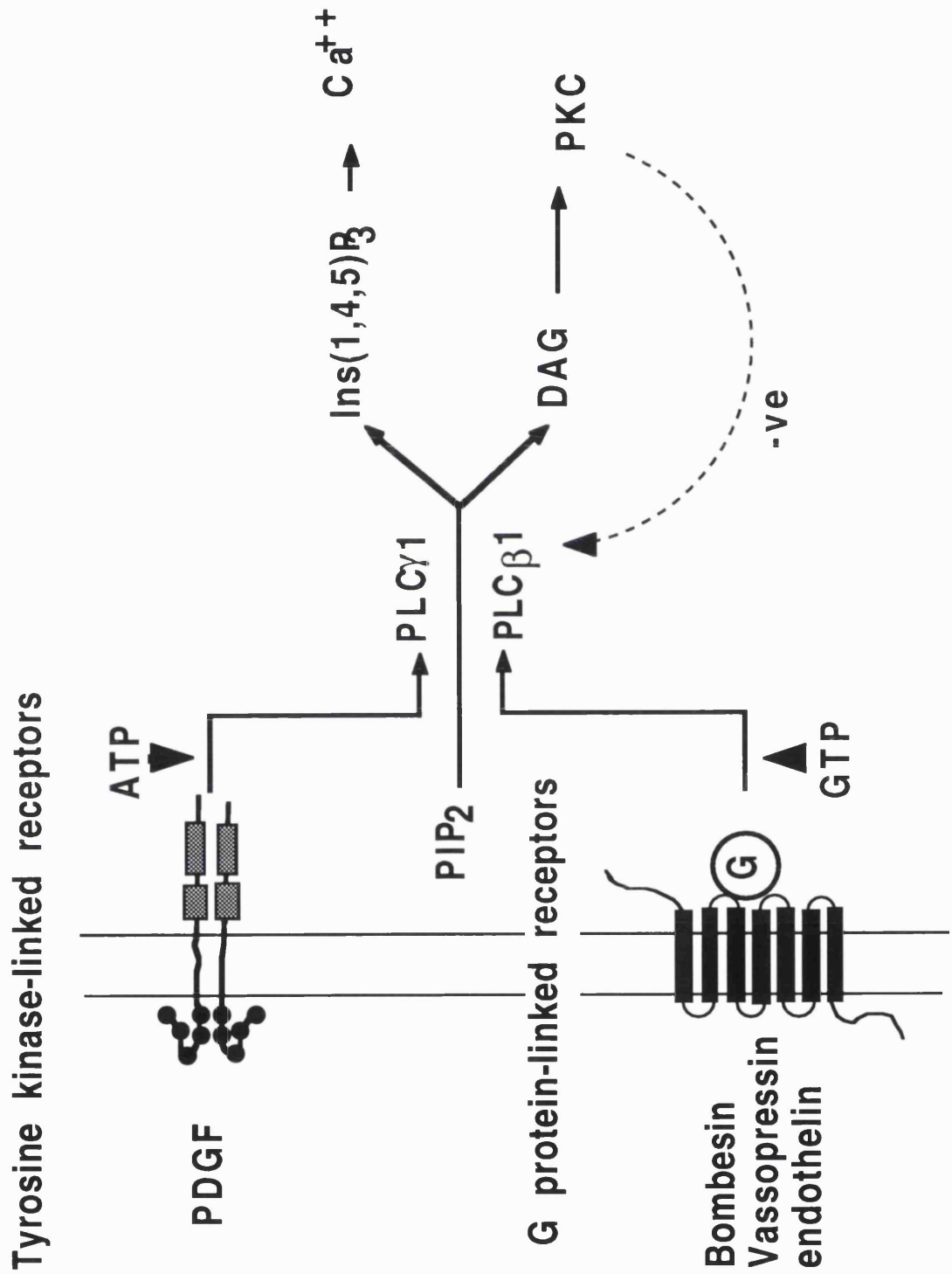
CHAPTER SEVEN: SUMMARY AND FUTURE PERSPECTIVES

7.1. rPMT STIMULATES PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE HYDROLYSIS THROUGH THE SAME SIGNALLING PATHWAY AS THE NEUROPEPTIDES; INVOLVEMENT OF THE Gq FAMILY OF G PROTEINS

The elucidation of the mechanisms of action of bacterial toxins has provided novel insights into the control of cellular regulatory processes including signal transduction and cell proliferation (Middlebrook and Dorland, 1984, Moss and Vaughan, 1988, Bourne *et al.*, 1990). Recently, *Pasteurella multocida* toxin (PMT) was found to be an extremely potent mitogen for murine Swiss 3T3 cells, other fibroblast cell lines and early passage cultures (Rozengurt *et al.*, 1990b, Higgins *et al.*, 1992). The toxin enters the cells and acts intracellularly to initiate and sustain DNA synthesis in the absence of serum or other growth factors. At the beginning of this thesis it was known that prior to the stimulation of DNA synthesis, rPMT stimulated a number of early signalling events in these cells (Rozengurt *et al.*, 1990b, Staddon *et al.*, 1991a). In particular, rPMT was found to induce a dramatic increase in polyphosphoinositide breakdown (Rozengurt *et al.*, 1990b, Staddon *et al.*, 1991a) and to mobilize Ca^{2+} from an intracellular pool (Staddon *et al.*, 1991a). Analysis of the inositol phosphate species generated in response to rPMT suggested that the toxin activated a cellular PI-PLC. In accord with this interpretation, rPMT also increased the cellular content of diacylglycerol, caused the translocation of PKC, and stimulated the phosphorylation of 80K/MARCKS (Staddon *et al.*, 1990) a major and specific substrate of PKC in cultured fibroblasts (Rozengurt *et al.*, 1983b, Blackshear *et al.*, 1985, Rodriguez-Pena and Rozengurt, 1986, Brooks *et al.*, 1991). In contrast, rPMT did not increase the cellular content of cyclic AMP (Rozengurt *et al.*, 1990b). As rPMT is the first intracellularly acting bacterial toxin that leads to the activation of PI-PLC, a major transducer of transmembrane signalling, this made rPMT an attractive and novel tool to study the effects of persistent activation of this signal transduction pathway in responsive intact cells.

In Swiss 3T3 cells, at least two distinct signal transduction pathways lead to phosphoinositide breakdown (Lopez-Rivas *et al.*, 1987, Nanberg and Rozengurt, 1988). The neuropeptides act through receptors coupled to G proteins, most likely G_q, which regulate the activity of the β-isoform of PI-PLC (Blank *et al.*, 1991, Smrcka *et al.*, 1991, Taylor and Exton, 1991, Berstein *et al.*, 1992) while PDGF acts through receptors possessing intrinsic tyrosine kinase activity and phosphorylates specific residues of PI-PLCγ (Kim *et al.*, 1991). A schematic diagram of these two pathways is shown in fig 7.1.1. A primary objective of this thesis was to determine whether rPMT stimulated inositol phosphate production through either of the known pathways that lead to the activation of PI-PLC isoforms in Swiss 3T3 cells.

Fig. 7.1.1.


Tyrosine kinase-linked receptors
PDGF
ATP
PLCγ1
PLCβ1
PIP2
Ins(1,4,5)P3
Ca++
DAG
PKC
-ve
G protein-linked receptors
G
GTP
Bombesin
Vassopressin
endothelin

Initially, using intact cells, we investigated the effect of rPMT treatment on the G protein and tyrosine kinase-mediated increased production of inositol phosphates. The results in chapter three demonstrate that rPMT potentiates inositol phosphate production induced by the neuropeptides bombesin, vasopressin and the mouse endothelin, VIC in a time and dose dependent manner. These results provided the first evidence that rPMT was stimulating PtdIns(4,5)P_2 hydrolysis through the same pathway as the neuropeptides.

The inability of rPMT to increase PDGF-mediated tyrosine phosphorylation of the PI-PLCγ isoform or to enhance inositol phosphate production induced by PDGF demonstrated that rPMT does not stimulate inositol phosphate production through this pathway.

The observations that rPMT enhanced neuropeptide stimulation of inositol phosphates presented us with a number of different possibilities for the site and mechanism of action of rPMT. In particular, rPMT could be acting to directly induce a post-translational modification of one of the components of this signal transduction pathway or alternatively could stimulate the de novo synthesis of new components of this pathway. Dissection of the neuropeptide pathway leading to the activation of PI-PLC reveals three major components that are potential targets for the action of rPMT i.e. receptor(s), G protein(s) or PI-PLC itself (see fig.7.1.1.).

Further investigation revealed that the action of rPMT appears to be the result of a post-translational modification(s) of one of the components of this signal transduction pathway rather than of increased protein synthesis. Cycloheximide, a protein synthesis inhibitor, had no effect on the stimulation of inositol phosphates induced by rPMT or on the enhancement of inositol phosphates observed on the addition of bombesin to rPMT-treated cells . In accord with these results, rPMT-treated cells do not express higher levels of the $G\alpha_{q/11}$, the G protein α-subunits thought to be involved in this pathway, nor do they express higher numbers of bombesin/GRP receptors.

It was also demonstrated that rPMT had no effect on the number or binding affinity of bombesin/GRP receptor for ligand suggesting that rPMT might be acting to facilitate this signal transduction pathway at a point distal to the receptors. The observation that the increase in the intracellular levels of Ins(1,4,5)P_3 induced by bombesin is dramatically and rapidly (within seconds) increased in rPMT-treated cells suggests that rPMT must be acting to change the properties of one of the elements of the signal transduction pathway immediately distal to the receptor, i.e.. the G protein or PI-PLC.

It has been known for some time that activation of PKC can severely attenuate inositol phosphate production induced by the neuropeptides (Brown *et al.*, 1987, Lopez-Rivas *et al.*,

1987, Brown *et al.*, 1990) but not PDGF. Although the exact mechanism is not known it is thought that it involves the phosphorylation of PI-PLCβ by PKC on specific serine residues (Ryu *et al.*, 1990). Accordingly, down-regulation of PKC by prolonged treatment of cells with phorbol esters, enhances the formation of inositol phosphates by neuropeptides but not PDGF (Brown *et al.*, 1990). Importantly, the stimulation of inositol phosphates induced by rPMT is greatly enhanced in PKC depleted cells demonstrating that PKC also acts as a potent negative regulator for the activation of PI-PLC by rPMT. These results provide an independent line of evidence that rPMT, like the neuropeptides, acts through the G protein/PI-PLCβ pathway leading to polyphosphoinositide hydrolysis.

The G protein involved in the activation of PI-PLCβ by the neuropeptides acts immediately upstream of PI-PLCβ. Thus assessing whether a G protein is also required for the stimulation inositol phosphates by rPMT might give an indication of the site of action of rPMT along this pathway. In particular, if rPMT was to stimulate this pathway upstream of the G protein or was to directly activate PI-PLC without the requirement for a G protein then it might be expected that rPMT would act in a G protein independent manner.

Hence, using a permeabilized cell system we assessed whether rPMT required a functional G protein in order to stimulate inositol phosphate production in Swiss 3T3 cells. Permeabilized cells have provided a useful approach for assessing the contribution of G proteins in the generation of biological responses. Permeabilization agents such as streptolysin O allow large pores (> 10 nm in diameter) to form in the plasma membrane of cells while maintaining their intracellular architecture and their ability to respond to appropriate effectors. Following permeabilization, diffusion of molecules both out of and into the cells occurs at rates which are governed by the size of the molecule. Indeed, large cytosolic proteins such as lactate dehydrogenase (142 kDa) or PI-PLC (145-155 kDa) have been shown to diffuse out of these permeabilized cells much more slowly than smaller proteins such as phosphoglycerate kinase (40 kDa) (reviewed in Cockcroft and Thomas, 1992). In addition small molecules such as ATP and membrane-impermeant guanine nucleotide analogues can rapidly (< 30 s after permeabilization).diffuse into permeabilized cells. Non-hydrolysable analogues of guanine nucleotides such as GDPβS or GTPγS, which act as G protein antagonists or agonists respectively, can therefore be used to determine whether a functional G protein is required for a specific response. The ability of rPMT to continue to stimulate inositol phosphate production in Swiss 3T3 cells after permeabilization allowed us to use this system to examine the effects of these G protein antagonists and agonists on rPMT activation of PI-PLC. In addition, it showed that rPMT could not change the concentration of a diffusible factor or ion that regulates PI-PLC, for example Ca^{2+}. The results in chapter four demonstrate that GDPβS, the non-hydrolysable analogue of GDP, selectively inhibits the production of inositol phosphates by rPMT in permeabilized 3T3 cells. The inhibition by GDPβS was rapid and concentration-

dependent suggesting that a functional G protein is essential for the activation of PI-PLC by rPMT. In addition, it was shown that the striking activation of PI-PLC by GTPγS is markedly attenuated by prior treatment of the cells with rPMT, suggesting that the toxin and GTPγS converge into the same signal transduction step. The conclusion from these studies is that rPMT facilitates G protein coupling to PI-PLC.

Following from these conclusions, we went on to try and identify the G protein involved in the action of rPMT on this pathway. As described in detail in section 1.3.1. the α subunits of the Gq class of G proteins have recently been demonstrated to be specific activators of the β-type PI-PLC isozymes (Blank *et al.*, 1991, Shenker *et al.*, 1991, Smrcka *et al.*, 1991, Taylor *et al.*, 1991, Berstein *et al.*, 1992). In addition, $G_{\alpha q}$ and $G_{\alpha 11}$ have recently been demonstrated to be involved in ligand mediated stimulation of PI-PLC activity by vasopressin, angiotensin, bradykinin, thromboxane A_2, thyrotropin releasing hormone and α_1-adrenergic receptors (Gutowski *et al.*, 1991, Shenker *et al.*, 1991, Wange *et al.*, 1991, Aragay *et al.*, 1992, Wu *et al.*, 1992a). Although the G protein involved in the activation of PI-PLC by bombesin has not yet been demonstrated, the most likely candidate is Gq. As rPMT stimulates the inositol phosphate production through the same pathway as the neuropeptides and requires a functional G protein, we therefore assessed whether the Gq family of G proteins were involved in the action of rPMT on this pathway.

Evidence supporting the involvement of $G\alpha_q$ and $G\alpha_{11}$ proteins in PI-PLC activation has been provided by transient transfection of cells with cDNAs corresponding to these G protein α subunits. The results in chapter 5 demonstrate that overexpression of $G\alpha_q$ and $G\alpha_{11}$ proteins in COS-1 cells resulted in an increased production of inositol phosphates induced by rPMT. A similar increase in inositol phosphate production in Gαq and $G\alpha_{11}$ transfected cells was observed on stimulation of the cells with AlF_4^-, which is known to activate α subunits by stabilising them in the GTP-bound configuration. From these results it was concluded that rPMT can utilise these G protein α-subunits to stimulate phosphoinositide hydrolysis. The stimulation of inositol phosphates by rPMT in $G\alpha_q$- and $G\alpha_{11}$-transfected cells were similar, suggesting that the toxin can utilise either G protein subunit equally. These results are consistent with the results observed in chapter three where neuropeptide-induced inositol phosphates were enhanced in rPMT-treated cells. The activation of neuropeptide receptors by ligand binding is known to result in the dissociation of receptor-associated heterotrimeric G proteins into α- and βγ-subunits. In a similar manner to that observed in the transfected cells, these free α-subunits could then be available to enhance the stimulation of inositol phosphates by rPMT.

In chapter 5 it was also demonstrated that pertussis toxin did not affect the ability of rPMT to stimulate inositol phosphate production. Thus, if other G proteins are involved in rPMT

stimulation of inositol phosphate production then they too must be pertussis toxin insensitive. In addition, rPMT had no effect on the ADP-ribosylation by pertussis toxin of its 40 kDa substrate in Swiss 3T3 cells suggesting that the cellular substrates as well as the mechanisms of action of these two toxins appear to be distinct from one another. These results are of added importance as recent findings demonstrate that the βγ–subunits of pertussis toxin-sensitive G proteins are able to activate the β isozymes of PI-PLC (Camps *et al.*, 1992a, Katz *et al.*, 1992, Carozzi *et al.*, 1993, Park *et al.*, 1993). Hence our results rule out the possibility that rPMT stimulation of PI-PLC is mediated through the βγ-subunits of pertussis toxin-sensitive G proteins. The enhanced inositol phosphate production induced by rPMT in cells transfected with these α-subunits further substantiates these conclusions. If rPMT-action was mediated by βγ-subunits then it might be expected that the overexpression of α-subunits would remove all free βγ-subunits resulting in an inhibition of inositol phosphates induced by rPMT rather than a stimulation.

In summary, the results in this thesis have demonstrated that rPMT stimulates phosphoinositide breakdown in Swiss 3T3 cells through the same pathway as the neuropeptides. We have shown that rPMT facilitates the coupling of G protein to PI-PLC and that the Gq family of G proteins are involved in this stimulation. These results are extremely exciting as no other intracellularly acting bacterial toxin has been described which can utilise this novel family of G proteins.

However, the precise mechanism that rPMT employs to deregulate this signal transduction pathway is still not known. In theory, rPMT could modify either the G protein or PI-PLC in a manner that facilitates their coupling and consequent activation of this signalling pathway. Early studies with cholera toxin were crucial to elucidate the role of Gs in the stimulatory pathway of adenylyl cyclase. Cholera toxin-catalysed ADP-ribosylation of a specific residue in α_s is sufficient to reduce the rate of GTPase activity of this subunit thereby prolonging the active (GTP-bound) state of α_s (Moss and Vaughan, 1988, Bourne *et al.*, 1990). This results in cAMP accumulation at high toxin concentrations. By analogy with cholera toxin, it might be tempting to postulate that rPMT causes ADP-ribosylation of Gq, leading to selective facilitation of neuropeptide-mediated signal transduction. However, the use of a novel procedure to detect toxin-catalysed ADP-ribosylation failed to demonstrate that rPMT stimulates this reaction in intact cells (Staddon *et al.*, 1991b). Hence the molecular basis of rPMT action on this pathway may represent a novel mechanism of toxin-induced deregulation of signal transduction.

In addition, the results presented in this thesis demonstrate that rPMT can utilise the α-subunits of Gq to stimulate PtdIns(4,5)P_2 hydrolysis. However, it cannot be ruled out that other pertussis toxin-insensitive G proteins may be involved. As these $G\alpha_q$–subunits are

ubiquitously expressed in all tissues, no cell lines exist which are deficient in this G protein. Such a cell line would be ideal for providing evidence that rPMT acts solely through this family of G proteins. As the number of identified G protein subunits continues to grow, it could be possible that other as yet unidentified pertussis toxin-insensitive G protein α-subunits are involved in the action of rPMT on this signal transduction pathway.

Similarly, the identity of the PI-PLC isozyme stimulated by rPMT has not been determined. PI-PLCβ isozymes have for the most part been thought to be involved in the stimulation of inositol phosphates through G protein pathways. However, there are indications that other non-β PI-PLC isozymes interact with G proteins although their identities need to be determined (for review see Cockcroft and Thomas, 1992). Further analysis of the role of PI-PLCβ in rPMT-mediated inositol phosphate production will be greatly facilitated by the increasing availability of molecular probes to this class of enzyme.

Clearly the elucidating of the molecular mechanisms as well as unravelling the precise nature of the components involved in the action of rPMT promises to be a fruitful area for future research. In the same way that cholera and pertussis toxins have provided useful probes for signal transduction pathways involving particular G proteins, it may be that rPMT will be an important marker for the G proteins involved in signal transduction through PI-PLC.

In addition, at least two other areas for future research might be useful in providing important information about the mechanism of action of rPMT. a) Identification of the membrane receptor utilised by rPMT to enter the cells. A crucial development to aid in this research would be the successful radiolabelling of the toxin. b) Generation of mutants of rPMT that could identify residues of rPMT that are needed for cellular entry and biological activity.

7.2. rPMT STIMULATES THE INDUCTION OF THE EARLY ONCOGENES C-FOS AND C-MYC

In addition to the early signalling events in the membrane and cytosol, serum and growth factors rapidly and transiently induce the expression of the cellular oncogenes c-fos and c-myc in quiescent fibroblasts (reviewed in Rozengurt and Sinnett-Smith, 1988). Since these protooncogenes encode nuclear proteins it is plausible that their transient expression may play a role in the transduction of the mitogenic signal in the nucleus (Curran, 1988). The demonstration that the product of the protooncogene c-jun, identified as the transacting factor AP-1, forms a tight complex with fos protein is consistent with a role for c-fos in the regulation of gene expression (Curran, 1988, Rozengurt and Sinnett-Smith, 1988, Shaw, 1990). Recent evidence also strongly suggests that the c-myc protein (myc) is a transcription factor

(Blackwood and Eisenman, 1991). Both a heterodimeric partner (Blackwood and Eisenman, 1991) and a consensus DNA-binding sequence for myc (Blackwell *et al.*, 1990, Prendergast and Ziff, 1991) have been identified.

There has been considerable interest in elucidating the signal transduction pathways involved in linking receptor occupancy and protooncogene induction. There is increasing evidence implicating PKC activation in the sequence of events involved in c-fos and c-myc induction (reviewed in Rozengurt and Sinnett-Smith, 1988). However, additional pathways involving calcium ion fluxes as well as a pathway dependent on arachidonic acid release have also been demonstrated (Rozengurt and Sinnett-Smith, 1987, Rozengurt and Sinnett-Smith, 1988, Mehmet *et al.*, 1990a). Regulation of the expression of both c-fos and c-myc involve complex autoregulatory mechanisms which for the most part account for the transient expression of these genes by growth factors (Sassone-Corsi, *et al.*, 1988b, Offir, *et al.*, 1990, Penn, *et al.*, 1990). The ability of mitogens acting through cell surface receptors to stimulate early signalling pathways often diminishes over time until the cells are no longer responsive to further addition of mitogen. This phenomenon is known as receptor desensitization. Thus, in addition to the autoregulatory mechanisms it is likely that receptor desensitization also plays a role in the transient expression of these protooncogenes. Thus, two separate regulatory mechanims lead to the feedback inhibition of protooncogene induction. An agent that induces the second messengers involved, without desensitization, could serve to dissect the contribution of each mechanism.

At the beginning of this thesis, nothing was known about the ability of rPMT to stimulate gene expression. As the expression of c-fos and c-myc are thought to play a role in mitogenic signalling in the nucleus it seemed plausible that rPMT, a potent mitogen, should stimulate the expression of these genes. In addition since rPMT by passes receptor mediated activation to persistently stimulate early signalling pathways, it provided a useful tool for studying the effects of the persistent activation of early signalling events on the induction of c-fos and c-myc.

The results in chapter six demonstrate that rPMT stimulates c-fos and c-myc expression in Swiss 3T3 cells although with different kinetics to the induction of these protooncogenes by bombesin. Initially there is a delay of about 3-4 hours before either c-fos or c-myc message are detected in rPMT treated cells and the maximal stimulation of these genes by rPMT occurs approximately 3-5 h after the times at which bombesin and other growth factors maximally stimulate these genes. The ability of methylamine (a lysosomotrophic agent) and PMT-antiserum to block the induction of c-fos by rPMT in a time dependent manner demonstrated that this lag period most likely represents the time required for rPMT to enter

the cells and be activated and is consistent with the lag seen for the stimulation of other early signals by rPMT.

More interestingly, in contrast to the transient expression of c-fos and c-myc induced by bombesin, the levels of both c-fos and c-myc mRNA induced by rPMT remain elevated for extended periods of time. Maximal levels of c-fos message induced by bombesin were expressed 30 min after stimulation and then declined rapidly to nearly basal levels 1.5-2 h after stimulation. In contrast, maximal induction of c-fos by rPMT at 4 h, declined much more slowly and only returned to basal levels about 10-11 h after stimulation with rPMT. Similarly, while maximal stimulation of c-myc by bombesin at 3 h declined to basal levels approximately 7-8 h after stimulation, maximal levels of this protooncogene stimulated by rPMT at 8 h declined much more slowly and only returned to basal after 28 h. As the levels of c-fos and c-myc mRNA induced by rPMT do eventually decline to basal levels these results demonstrated that the autoregulatory mechanisms which exist to regulate the expression of these protooncogenes are still functional.

A possible explanation for the prolonged expression of c-fos and c-myc induced by rPMT might be that the persistent stimulation of mitogenic signalling pathways by rPMT plays a role in the extended expression of these protooncogenes. Alternatively the entry and activation of rPMT in the cell cultures might be occurring in an asynchronous manner and consequently not all the cells would be maximally expressing c-fos or c-myc mRNA at the same times. It would appear that, at least in the case of c-fos expression induced by rPMT, the asynchronous entry of rPMT into the cell population plays a role in the prolonged expression of this protooncogene. The results in chapter six demonstrate that, although levels of expression of c-fos induced by rPMT are detected for longer periods of time than those induced by bombesin, the maximum level of c-fos induced by rPMT is only approximately 50 % of the maximal levels induced by bombesin. These results suggest that the entry of rPMT and consequential stimulation of c-fos expression occurs in an asynchronous manner. However, it could be argued that the induction of c-fos mRNA by bombesin occurs through a signal transduction pathway(s) that is not stimulated by rPMT. Bombesin is known to induce c-fos by the co-ordinated effects of PKC activation, Ca^{2+} mobilisation and an additional pathway dependent on arachidonic acid release. The results in chapter six demonstrate that the induction of c-fos by bombesin is severely attenuated in PKC-down regulated cells suggesting that a large part of the stimulation of c-fos by bombesin is mediated through a PKC-dependent pathway. It was also shown that the induction of c-fos by rPMT in PKC-depleted cells is severely attenuated but not abolished suggesting that rPMT also stimulates c-fos through both PKC-dependent and independent pathways. rPMT, like bombesin is known to stimulate PKC activation (Staddon *et al.*, 1991a), Ca^{2+} mobilisation (Staddon *et al.*, 1991a) and arachidonic acid release (E. Rozengurt, unpublished results) in Swiss 3T3

cells. It should also be noted that down-regulation of PKC attenuates but does not abolish the mitogenic action of rPMT in these cells (E. Rozengurt and T. Higgins, unpublished work), suggesting that these other pathways may play an important role in the mitogenic action of rPMT. Since rPMT and bombesin appear to stimulate similar signalling pathways it might be expected that both of these mitogens should stimulate c-fos expression to the same degree. Thus, these results suggest that the lower maximal levels of expression of c-fos mRNA and the prolonged expression of this protooncogene induced by rPMT may indeed be explained by the asynchronous entry of the toxin into these cells. However it cannot be ruled out that the persistent activation of early signalling events by rPMT plays some part in the induction of c-fos by rPMT. In addition, the contribution of Ca^{2+} mobilisation and arachidonic acid release to the stimulation of c-fos expression by rPMT is not known. Thus, these results require further investigation

In contrast to the results observed for c-fos expression, chapter six demonstrates that the maximal levels of c-myc expression stimulated by rPMT are similar to, if not slightly higher than, the maximum levels induced by bombesin. In addition, maximum levels of expression of this protooncogene are observed for 2-3 h before they very slowly decline to basal levels. It is likely that rPMT is more effective than bombesin in inducing c-myc. Any asynchrony would tend to reduce the response, so the measurements of c-myc expression by rPMT are an underestimation of the true induction. It was also demonstrated that rPMT, like bombesin, can stimulate c-myc through both PKC-dependent and independent pathways. The identification of these pathways and their role in the mitogenic action of rPMT require further investigation. As rPMT has such a striking effect on the expression of c-myc, this toxin might provide a novel tool for the further investigation of the regulation of this protooncogene as well as for elucidating the downstream targets which are activated by the expression of this protooncogene. In addition, the prolonged expression of c-myc may explain the potent mitogenic effects of rPMT. Recently, c-myc has been implicated in the induction of apoptosis. Although the toxin, under our experimental conditions, always induces cell proliferation rather than cell death it is conceivable that 'in vivo', rPMT-induction of c-myc could be related to an apoptotic response of target cells. Whether or not induction of apoptosis plays a role in any of the 'toxic' effects of rPMT is at present unknown.

In conclusion, further analysis of the mechanisms by which rPMT stimulates the expression of these protooncogenes as well as investigating the effects of rPMT on the expression of other protooncogenes promises to be a fruitful area for future research.

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Pasteurella multocida Toxin Selectively Facilitates Phosphatidylinositol 4,5-Bisphosphate Hydrolysis by Bombesin, Vasopressin, and Endothelin

REQUIREMENT FOR A FUNCTIONAL G PROTEIN*

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Treatment of Swiss 3T3 cells with a subsaturating concentration of recombinant *Pasteurella multocida* toxin (rPMT) markedly potentiated the production of inositol phosphates induced by bombesin, vasopressin, and endothelin but not by platelet-derived growth factor (PDGF) (AA and BB homodimers). Similarly, the neuropeptides but not PDGF caused a shift in the dose-dependent increase in inositol phosphates induced by rPMT. The rate of accumulation of inositol phosphates induced by bombesin was increased 2-fold by rPMT treatment while that of PDGF was unaffected. rPMT treatment also enhanced bombesin-induced inositol(1,4,5)trisphosphate, the direct product of phosphatidylinositol 4,5-bisphosphate hydrolysis. In contrast, treatment of cells with rPMT had no effect on the tyrosine phosphorylation of phospholipase Cγ. Depletion of protein kinase C increased rPMT-induced inositol phosphates in a manner similar to that observed for bombesin but not PDGF. Thus, rPMT selectively potentiates neuropeptide-mediated inositol phosphate production. The action of rPMT on phosphatidylinositol 4,5-bisphosphate hydrolysis persisted in streptolysin *O*-permeabilized cells. Addition of guanosine 5′-*O*-(β-thiodiphosphate) to permeabilized cells markedly reduced rPMT-induced inositol phosphates in a time- and dose-dependent manner. rPMT also increased the sensitivity of phospholipase C for free calcium. Our results strongly suggest that the action of rPMT facilitates the coupling of G protein to phospholipase C.

The elucidation of the mechanism of action of bacterial toxins has provided novel insights into the control of cellular regulatory processes (1-3). Recently *Pasteurella multocida* toxin (PMT),[1] has been found to be an extremely potent mitogen for murine Swiss 3T3 cells and other fibroblast cell lines and early passage cultures (4, 5). The toxin is a monomeric 146-kDa protein and has been purified (6–9), cloned (10–12), sequenced (13–15), and expressed in *Escherichia coli*. The deduced amino acid sequence of PMT revealed no significant homologies with other toxins or proteins (14, 15). Both native and rPMT are mitogenic at picomolar concentrations. The toxin enters the cells and acts intracellularly to initiate and sustain DNA synthesis (4). Recently, rPMT has been demonstrated to induce a striking stimulation of anchorage-independent growth in Rat-1 cells (5).

Prior to the stimulation of DNA synthesis, rPMT stimulates the formation of inositol phosphates (4, 5, 16) and mobilizes Ca^{2+} from an intracellular pool (16). Analysis of the inositol phosphate species generated in response to rPMT strongly suggests that the toxin activates a cellular PI-PLC (16). In accord with this interpretation, rPMT also increases the cellular content of diacylglycerol, causes the translocation of PKC, and stimulates the phosphorylation of 80K/MARCKS (5, 17), a major and specific substrate of PKC in cultured fibroblasts (18–21). In contrast, rPMT does not increase the cellular content of cyclic AMP (4). Thus, rPMT is the first intracellularly acting toxin that leads to the activation of PI-PLC, a major transducer of transmembrane signaling.

Signal-dependent breakdown of phosphatidylinositol 4,5-bisphosphate by PI-PLC to produce the intracellular second messengers, diacylglycerol and Ins(1,4,5)P_3 is recognized as an important element in the transmission of information across the plasma membrane (for reviews, see Refs. 22–24). At least two distinct signal transduction pathways lead to the activation of different PI-PLC isoforms in many cell types including Swiss 3T3 cells. The mitogenic neuropeptides bombesin, vasopressin, and endothelin (25, 26) stimulate inositol phosphate production via specific plasma membrane receptors coupled to a pertussis toxin-insensitive G protein (27–31). Recently, activated α subunits of the pertussis toxin-insensitive G_q subfamily (32, 33) have been shown to stimulate the β_1 isoform of PI-PLC (34–38). In contrast, the γ isoform of PI-PLC is a direct target of receptors endowed with intrinsic, ligand-dependent, tyrosine kinase activity (for review, see Ref. 39). PDGF stimulates the phosphorylation of specific tyrosine residues of PI-PLCγ, thereby stimulating polyphosphoinositide hydrolysis (40).

The experiments presented here were designed to determine the effect of rPMT treatment on G protein and tyrosine kinase-mediated increased production of inositol phosphates. We report that rPMT can distinguish between neuropeptide- and PDGF-induced inositol phosphate signals in 3T3 cells: the toxin selectively enhances signal transduction initiated

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[1] The abbreviations used are: PMT, *Pasteurella multocida* toxin; rPMT, recombinant *P. multocida* toxin; PKC, protein kinase C; PI-PLC, phosphatidyl inositol-specific phospholipase C; G protein, guanine nucleotide-binding regulatory proteins; G_s and G_q, designations of classes of heterotrimeric proteins; α_q and $\alpha_{q/11}$, designations of the α subunit corresponding to a subtype of heterotrimer; GTPγS, guanosine 5′-*O*-(γ-thiotriphosphate); GDPβS, guanosine 5′-*O*-(β-thiodiphosphate); PDGF, platelet-derived growth factor; inositol, *myo*-inositol; Ins(1,4,5)P_3, inositol (1,4,5)trisphosphate; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; GRP, gastrin-releasing peptide; PIPES, 1,4-piperazinediethanesulfonic acid.

by the neuropeptides bombesin, vasopressin, and endothelin. These effects can be attributed, at least in part, to toxin-mediated facilitation of the coupling of G protein to PI-PLC.

EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of Swiss 3T3 cells (41) were propagated as described previously (42). For experimental purposes, cells were subcultured in either 33-mm Nunc dishes containing 10^4 cells or 90-mm Nunc dishes containing 5.5×10^4 cells with DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cultures were incubated in a humidified atmosphere of 10% CO_2, 90% air at 37 °C for 6–8 days before use in the experiments. After this time the cultures were confluent and arrested in the G_1/G_0 phase of the cell cycle.

Analysis of Total Inositol Phosphates—Cultures of Swiss 3T3 cells in 33-mm dishes were labeled for 16–18 h in 1 ml of DMEM/Waymouth medium (1:1) containing 10 μCi of [2-^{3}H]inositol. Additions were made to the cells as described for each experiment and LiCl was added to a final concentration of 20 mM for the last 30 min of the incubation (43). Inositol phosphates were extracted by replacing the medium with 1 ml of ice-cold 3% $HClO_4$. After 20 min at 4 °C the extract was neutralized with 0.5 M KOH containing 25 mM HEPES, 5 mM EDTA, and 0.01% phenol red. Precipitated $KClO_4$ was removed by centrifugation. Analysis of total inositol phosphates was by anion-exchange column chromatography (44). Samples were diluted to 10 ml with water and then loaded onto 1 ml of Dowex AG1-X8 (100–200 mesh, $HCOO^-$ form) in Bio-Rad Econo-columns. After washes with 3 × 10 ml of H_2O and 2 × 10 ml of 60 mM NH_4COOH, 5 mM $Na_2B_4O_7$ the inositol phosphates were eluted with 7 ml of 1 M NH_4COOH, 0.1 M HCOOH. An aliquot (1 ml) of eluate was counted in 10 ml of Picofluor.

Inositol Phosphate Release from Permeabilized Cells—The cultures were labeled in 1 ml of medium as above but containing 25 μCi of [2-^{3}H]inositol. Addition of toxin to the cells was as described for each experiment. The following procedure was then performed at 37 °C. The cultures were washed twice with K solution (120 mM KCl, 30 mM NaCl, 2.5 mM $MgCl_2$, 1 mM K_2HPO_4, 10 mM PIPES, 2 mM EGTA, 0.5 mM $CaCl_2$, pH 7.2). The cells were permeabilized in 1 ml of K solution containing 1 mM ATP, 20 mM LiCl, streptolysin O at 0.4 unit/ml and the factors described for a total of 10 min. The reaction was stopped by adding 1 ml of 6% $HClO_4$ (w/v). After 30 min at 4 °C the acid extract was removed from the dish and neutralized with 1 M KOH containing 25 mM HEPES, 20 mM EDTA, and 0.01% phenol red. For the experiments presented in Fig. 11, the calculations of free calcium concentration were as described (45). Total inositol phosphates were analyzed as described above.

Assay of Ins(1,4,5)P_3—Quiescent cultures of Swiss 3T3 cells in 90-mm Nunc dishes were washed twice with DMEM/Waymouth (1:1) and incubated for 5 h in 5 ml DMEM/Waymouth (1:1) with or without 2.5 ng/ml rPMT. The medium was then aspirated off and DMEM/Waymouth containing the required factors was added for the times indicated. The medium was then aspirated off and 5 ml of DMEM/Waymouth containing the required factors was added for the times indicated. Reactions were terminated on ice by replacing the medium with 500 μl of ice-cold 5% $KClO_4$ followed by immediate scraping with a rubber policeman. The suspension was collected, left for 20 min at 4 °C, and centrifuged at 14,000 × *g* for 5 min. The supernatant was retained and neutralized using 10 M KOH, 500 mM HEPES. The precipitated $KClO_4$ was removed by centrifugation as above, and 100 μl of the final supernatant was added to the Ins(1,4,5)P_3 assay mixture utilizing [^{3}H]-Ins(1,4,5)P_3 together with a specific Ins(1,4,5)P_3-binding protein. The assay was started by the addition of the Ins(1,4,5)P_3-binding protein. After 15 min at 4 °C the reactions were centrifuged (30 min, 2000 × *g*) and the supernatant discarded. Pellets were resuspended in 200 μl of H_2O and counted in 8 ml of Picofluor.

Generation of Antisera to $G\alpha_{q/11}$—The $G\alpha_q$- and $G\alpha_{11}$-specific polyclonal antibody was raised against the peptide QLNLKEYNLV which corresponds to the amino acid sequences from positions 350–359 in both $G\alpha_q$ and $G\alpha_{11}$. This peptide was conjugated to keyhole limpet hemocyanin using glutaraldehyde, and antisera were prepared according to the following schedule: two rabbits were immunized at multiple sites subcutaneously with 0.2 mg of the conjugated peptide emulsified in complete Freunds adjuvant and boosted 4 times at 2-week intervals with 0.2 mg of conjugated peptide in incomplete Freund's adjuvant. Antisera were collected 10 days after the second and last boost. Sera were assayed for antipeptide activity by an enzyme-linked immunoabsorbant assay using peroxidase-linked anti-rabbit Ig, 2,2′-azino-di-[3-ethlbenzthiazoline sulfonate], and $G\alpha_{q/11}$ peptide. The antiserum that gave the highest titer was used for immunoblotting. The signal obtained in Western blots was a 42-kDa band corresponding to an unresolved mixture of $G\alpha_q$ and $G\alpha_{11}$ which could be blocked using the peptide used to raise the antisera.

Immunoprecipitations—Quiescent cultures of Swiss 3T3 cells in 90-mm dishes were incubated for 5 h in the absence or presence of 2.5 ng/ml rPMT. Cells were then treated with 6 nM bombesin or 25 ng/ml PDGF for 10 min. The cells were lysed with a solution containing 1% Triton, 0.25% deoxycholate, 10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 0.1% BSA. The lysates were then centrifuged and the supernatant precleared by incubation with BSA-agarose for 1 h at 4 °C. After centrifugation, the supernatant was immunoprecipitated with PLCγ polyclonal antibody (1:50 dilution) for 3 h at 4 °C. The immunoprecipitate was then incubated for 1 h with protein A-agarose then spun and the pellet washed four times with lysis buffer. Immunoprecipitates were analyzed by one-dimensional SDS-PAGE with 8% acrylamide.

Western Blot Analysis—PLCγ immunoprecipitates of unlabeled cells were fractionated by SDS-PAGE as described above, then transferred to nitrocellulose membranes according to the manufacturer's instructions (Millipore), and immunoblotted using the PY20 anti-Tyr(P) monoclonal antibody as described (46). Immunoreactive bands were detected by using ^{125}I-labeled rabbit anti-mouse IgG followed by autoradiography.

In other experiments cells were lysed directly into SDS-PAGE sample buffer. The samples were boiled and fractionated by SDS-PAGE with 12.5% acrylamide. The proteins were transferred to nitrocellulose membranes and immunoblotted using an antibody raised against the C-terminal decapeptide of $G\alpha_q$/11 (1:2, 500 dilution). Immunoreactive bands were detected using ^{125}I-labeled protein A followed by autoradiography.

Measurement of ^{125}I-Labeled GRP Binding—Binding of ^{125}I-labeled GRP to cultures of intact Swiss 3T3 cells was performed essentially as described (47). Briefly, the cultures were washed and incubated in the presence of various concentrations of ^{125}I-labeled GRP in binding medium (DMEM/Waymouth medium (1:1) containing BSA at 1 mg/ml, pH 7) for 30 min at 37 °C. After this time the cultures were washed and extracted into 0.1 M NaOH, 2% (w/v) Na_2CO_3, 1% (w/v) SDS and total cell-associated radioactivity was determined.

Materials—Fetal bovine serum was obtained from GIBCO. Bombesin, vasopressin, epidermal growth factor, phorbol 12,13-dibutyrate, and insulin were from Sigma. PDGF AA and BB homodimers were a gift from Mark Murray of Zymogenetics. PDGF c-sis, basic fibroblast growth factor, [2-^{3}H]inositol (18.8 Ci/mmol, 1 Ci = 37 GBq), Ins(1,4,5)P_3 binding assay kit, ^{125}I-labeled GRP (2000 Ci/mmol, 1 Ci = 37 GBq), and ^{125}I-labeled rabbit anti-mouse IgG (15 μCi/μg) were all supplied by Amersham Corp. PY20 anti-Tyr(P) antibody was from ICN Biomedicals. Dowex resin (AG 1-X8, 200–400 mesh) was from Bio-Rad. GTPγS and GDPβS were from Boehringer Mannheim. Streptolysin O was from Wellcome Diagnostics (Dartford, Kent, United Kingdom). Recombinant PMT was prepared as described previously (11). All other reagents were of the highest grade commercially available.

RESULTS

rPMT Distinguishes between the Neuropeptide and PDGF Pathways for the Enhancement of Inositol Phosphates—Initially, we determined whether pretreatment of 3T3 cells with rPMT could facilitate the production of inositol phosphates by neuropeptides and PDGF. Quiescent cultures of Swiss 3T3 cells labeled with [2-^{3}H]inositol were treated for 5 h with 2.5 ng/ml rPMT, a concentration that stimulated only a small increase in total inositol phosphate formation. The neuropeptides and PDGF were then added for a further 10 min and total inositol phosphates were analyzed. Fig. 1 shows that rPMT pretreatment caused a marked enhancement in the production of total inositol phosphates induced by bombesin, vasopressin, and the mouse endothelin, VIC. In all cases, the enhancement was greater than that expected from an additive effect which is indicated by the *arrows* in Fig. 1. In contrast, rPMT pretreatment did not potentiate the inositol phos-

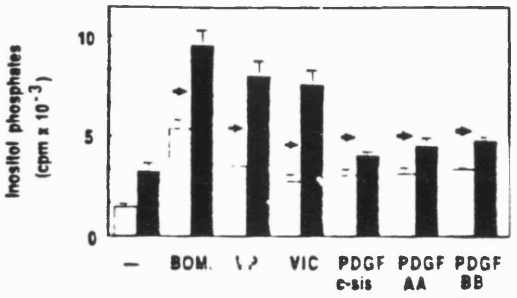

FIG. 1. **Effect of rPMT pretreatment on the inositol phosphate production by neuropeptides and PDGF.** The cells were prelabeled with 10 μCi/ml [2-^{3}H]inositol for 16–18 h. rPMT was then added directly to the dishes to a final concentration of 2.5 ng/ml, and the cultures were incubated at 37 °C for 4.5 h. LiCl (20 mM) was then added and after a further 30 min the cellular inositol phosphate content was determined as described under "Experimental Procedures." Bombesin (6 nM), vasopressin (10 nM), VIC (10 nM), as well as PDGF c-sis AA and BB (25 ng/ml), were added to control (*open bars*) and rPMT-pretreated (*shaded bars*) cells for 10 min prior to extraction. The increases in inositol phosphates expected from an additive effect are indicated by the *arrows*. The data represent means ± S.E. of determinations from four to 10 individual experiments.

TABLE I

Effects of insulin, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF), on inositol phosphates induced by rPMT

Cells were prelabeled with 10 μCi/ml of [2-^{3}H]inositol for 16 h. rPMT was then added directly to the dishes and incubated at 37 °C for 4.5 h. LiCl (20 mM) was then added, and after a further 30 min the cellular inositol phosphate content was determined. bFGF (10 ng/ml), EGF (5 ng/ml), insulin (1 μg/ml) or bombesin (6 nM) were added to control and rPMT-pretreated cells for 10 min prior to extraction. Results are means ± S.E. for triplicate determinations of three individual experiments.

Growth factor	Inositol phosphates	
	Without rPMT	With rPMT
	cpm	
None	1447 ± 127	3422 ± 277
Insulin	1537 ± 15	3030 ± 126
bFGF	1620 ± 216	3915 ± 140
EGF	1402 ± 90	3090 ± 128
Bombesin	5363 ± 300	9600 ± 100

phates produced in response to either PDGF c-sis or the homodimers PDGF AA and PDGF BB. The resulting induction of inositol phosphates was additive rather than synergistic (Fig. 1). Other growth factors that do not stimulate the production of inositol phosphates in Swiss 3T3 cells, such as insulin, epidermal growth factor, and basic fibroblast growth factor, did not enhance the formation of inositol phosphates in rPMT-pretreated cells (Table I).

Enhancing Effect of rPMT Pretreatment on the Production of Inositol Phosphates: Concentration Dependence and Time Course—The effect of pretreatment with different concentrations of rPMT on the enhancement of inositol phosphates elicited by bombesin vasopressin and PDGF is shown in Fig. 2. rPMT increased the accumulation of inositol phosphates in a dose-dependent manner. Addition of bombesin caused a shift in the dose response; the concentration of rPMT required to produce half-maximal effect was reduced from 9 ng/ml to 3 ng/ml (Fig. 2*A*). Vasopressin addition also caused a shift in the dose response of inositol phosphate production by rPMT (Fig. 2*B*). In contrast, addition of PDGF to cells

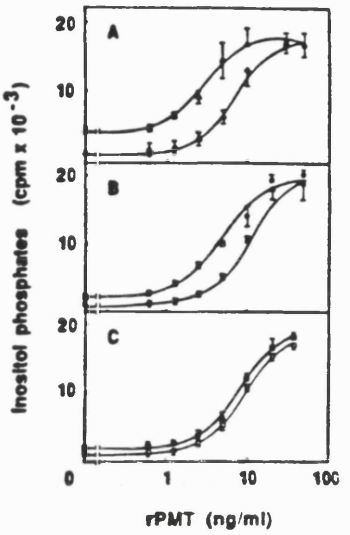

FIG. 2. **Effects of pretreatment with increasing concentrations of rPMT on inositol phosphate production in the absence or presence of bombesin, vasopressin, and PDGF.** [2-^{3}H]Inositol-labeled Swiss 3T3 cells were incubated with increasing concentrations of rPMT for 4.5 h. LiCl was then added to give a final concentration of 20 mM. Total inositol phosphates were extracted after 30 min in the absence (*open symbols*) or presence (*closed symbols*) of bombesin (6 nM) (*A*), vasopressin (10 nM) (*B*), or PDGF c-sis (25 ng/ml) (*C*). These factors were added directly to the cultures 10 min prior to extraction. Analysis of total inositol phosphates was as described under "Experimental Procedures." Values shown are the mean ± S.E. of triplicate determinations.

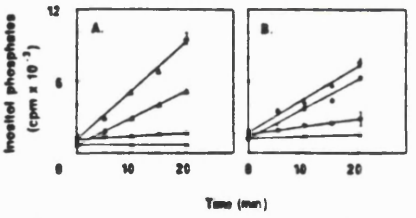

FIG. 3. **The effect of rPMT pretreatment on the time course of inositol phosphate production elicited by bombesin or PDGF.** The cells were labeled for 16–18 h with 10 μCi/ml [2-^{3}H] inositol in DMEM/Waymouths medium (1:1). Pretreatment with rPMT (2.5 ng/ml) was for 4.5 h when LiCl to a final concentration of 20 mM was added. *A*, addition of 6 nM bombesin to control (*open triangles*) and rPMT-pretreated cells (*closed triangles*). *B*, addition of PDGF (25 ng/ml) to control (*open circles*) and rPMT-pretreated cells (*closed circles*). Bombesin and PDGF were added 20 min after the addition of LiCl (zero time). No additions to control (*open squares*) or rPMT-pretreated cells (*closed squares*) are shown in *A* and *B*. Total inositol phosphates were extracted at the times indicated. Values shown are means ± S.E. of triplicate determinations and are representative of three individual experiments.

pretreated with different concentrations of rPMT had no effect on the half-maximal concentration, and the resulting dose response curve was that expected from an additive effect (Fig. 2*C*).

The time course of inositol phosphate accumulation induced by either bombesin or PDGF in control and rPMT-pretreated cells is shown in Fig. 3. The rate of accumulation of inositol phosphates stimulated by bombesin in rPMT-

pretreated cells is 2-fold higher than the rate induced by bombesin in control cells (Fig. 3*A*). In contrast, the rate of accumulation of inositol phosphates stimulated by PDGF in either control or rPMT-pretreated cells was identical (Fig. 3*B*). Thus, rPMT can distinguish between neuropeptide- and PDGF-induced inositol phosphate accumulation in Swiss 3T3 cells.

Effect of rPMT Treatment on the Accumulation of Ins(1,4,5)P_3 Induced by Bombesin and PDGF—The results shown in Figs. 1–3 prompted us to determine whether treatment with rPMT potentiates the ability of neuropeptides to stimulate the production of Ins(1,4,5)P_3, one of the products of the reaction catalyzed by PI-PLC. Cultures of Swiss 3T3 cells were pretreated with or without 2.5 ng/ml rPMT for 5 h and then challenged with bombesin for various times. As shown in Fig. 4, addition of bombesin to control cells caused a rapid increase in the cellular content of Ins(1,4,5)P_3 peaking 10 s after stimulation with an 80% increase over basal. When bombesin was added to cells which have been pretreated with rPMT the accumulation of Ins(1,4,5)P_3 was dramatically increased; even 5 s after stimulation the increase rose from 40 to 200%. These elevated levels of Ins(1,4,5)P_3 persisted for the duration of the experiment (Fig. 4). In contrast, PDGF increased Ins(1,4,5)P_3 at a slower rate, peaking 60 s after addition with an increase of about 80% over basal levels which was not potentiated in rPMT-treated cells (results not shown). Thus, rPMT selectively facilitates the neuropeptide-stimulated signal transduction pathway.

rPMT Does Not Affect the Tyrosine Phosphorylation of PI-PLCγ—In order to substantiate further the differential effects of rPMT on G protein and tyrosine kinase signal transduction, we determined whether rPMT has any effect on PDGF-stimulated phosphorylation of PI-PLCγ in Swiss 3T3 cells. Cultures incubated in the presence or absence of rPMT received either bombesin or PDGF for 10 min. The cells were lysed, and the extracts were immunoprecipitated with an antibody against PI-PLCγ. Western blot analysis of the immunoprecipitates with an antiphosphotyrosine antibody showed that PDGF stimulates tyrosine phosphorylation of this enzyme to the same extent in both control and rPMT-treated cells (Fig. 5). A protein phosphotyrosine band migrating with an apparent molecular mass of 170–190 kDa which was also detected in the immunoprecipitates of PDGF-treated cells is most likely the autophosphorylated PDGF receptor which is known to become tightly associated with PI-PLCγ (39). In contrast, bombesin did not stimulate the phosphorylation of PI-PLCγ either in control cells or in rPMT- treated cells (Fig. 5). Addition of 20 ng/ml rPMT, a concentration that induced a massive accumulation of inositol phosphates, did not increase tyrosine phosphorylation of PI-PLCγ (results not shown).

Down-regulation of PKC—Activation of PKC can also distinguish between the G protein and tyrosine kinase pathways leading to polyphosphoinositide hydrolysis. Phorbol esters inhibit inositol phosphate production induced by bombesin or vasopressin (48–51) but do not affect inositol phosphate formation in response to PDGF (48). Accordingly, down-regulation of PKC enhances the formation of inositol phosphates by neuropeptides but not by PDGF (50). If rPMT and neuropeptides utilize the same pathway to promote activation of inositol phospholipid breakdown, it could be predicted that PKC down-regulation should also potentiate the production of inositol phosphates in response to rPMT. Fig. 6 (*left*) shows that, in PKC down-regulated cells, rPMT, like bombesin, induced a further increase in inositol phosphate production. In contrast, the increase of inositol phosphates elicited by PDGF was not affected by PKC down-regulation. Fig. 6 (*right*), shows that PKC down-regulation markedly enhanced the formation of inositol phosphates induced by various concentrations of rPMT.

The Enhancing Effect of rPMT Does Not Require de Novo Protein Synthesis—A possible explanation of the previous results is that the enhancing effect of rPMT could result from *de novo* synthesis of neuropeptide receptors or other signal transduction proteins. Alternatively, the toxin could induce a post-translational modification of a pre-existing constituent of the neuropeptide signal transduction pathway. Various lines of evidence favor the later hypothesis: (*a*) binding analysis using ^{125}I-GRP revealed that rPMT treatment had no effect on the number or apparent affinity of the bombesin/GRP receptor (Fig. 7, *left*). (*b*) Scanning densitometry of Western blots using an antibody raised against the common

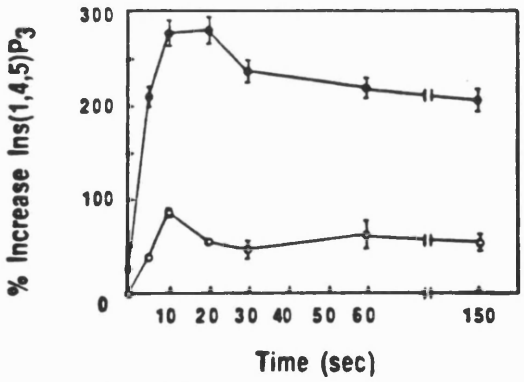

FIG. 4. **Effect of bombesin on the cellular content of Ins(1,4,5)P_3 in control and rPMT-treated cells.** Quiescent cultures of Swiss 3T3 cells in 90-mm dishes were treated with 2.5 ng/ml rPMT for 5 h. Then, bombesin (6 nM) was added to control (*open symbols*) or rPMT-pretreated (*closed symbols*) cultures for the indicated times. For each sample Ins(1,4,5)P_3 was measured as described under "Experimental Procedures," and the percentage increase in Ins(1,4,5)P_3 over basal was determined (basal level was 11.3 ± 3 pmol of Ins(1,4,5)P_3/10^6 cells). Each point represents the mean ± S.E. of triplicate determinations taken from two individual experiments.

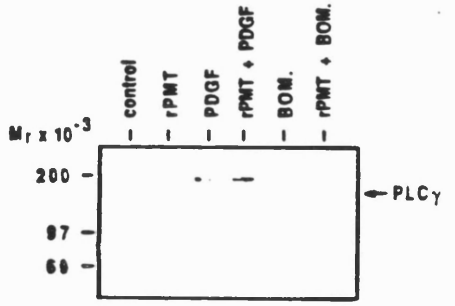

FIG. 5. **Phosphorylation of PI-PLCγ in Swiss 3T3 cells.** Quiescent Swiss 3T3 cells in 30-mm dishes were incubated with or without rPMT (2.5 ng/ml) for 5 h. Bombesin (6 nM) or PDGF (25 ng/ml) were added to control and rPMT-treated cells for 10 min. The cells were lysed, and the extracts were immunoprecipitated with an anti-PLCγ polyclonal antibody; immunoprecipitates were analyzed by Western blotting with PY20 anti-Tyr(P) monoclonal antibody under standard conditions and visualized as described under "Experimental Procedures."

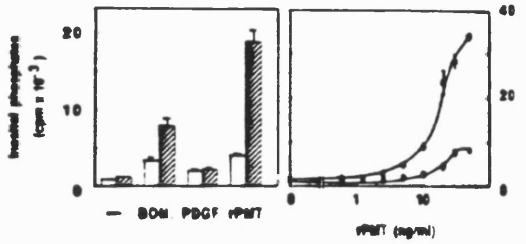

FIG. 6. Effect of down-regulation of PKC on the enhancement of inositol phosphates by rPMT. Control cultures or those pretreated with phorbol 12,13-dibutyrate (1 μM) for 48 h were incubated with 10 μCi/ml [2-^{3}H]inositol for 16–18 h. Inositol phosphates were extracted as described previously. *Left*, control (*open bars*) or PKC down-regulated (*hatched bars*) cells were incubated with rPMT (10 ng/ml) for 5 h or with bombesin (6 nM) and PDGF (25 ng/ml) for 10 min. In all cases LiCl was added 30 min prior to extraction at a final concentration of 20 mM. *Right*, increasing concentrations of rPMT were added to control (*open symbols*) and PKC down-regulated (*closed symbols*) cultures. rPMT treatment was for 5 h and LiCl (final concentration 20 mM) was added 30 min prior to extraction. Values represent means ± S.E. of triplicate determinations.

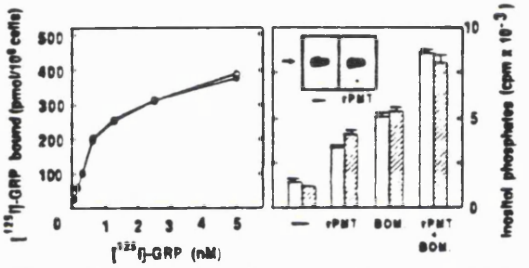

FIG. 7. rPMT-mediated enhancement of inositol phosphate production is not dependent on *de novo* protein synthesis. *Left*, binding analysis. Confluent and quiescent cultures of Swiss 3T3 cells were pretreated for 5 h in the absence (*open circles*) or presence (*closed circles*) of rPMT (2.5 ng/ml). The cells were then washed and incubated in the presence of various concentrations of ^{125}I-labeled GRP in binding medium (DMEM/Waymouth medium (1:1) containing BSA at 1 mg/ml, pH 7) for 30 min at 37 °C and 10% CO_2, 90% air. After this time the cultures were washed and extracted and total cell-associated radioactivity was determined. Each point represents the mean ± S.E. of triplicate determinations. *Right*, confluent and quiescent cultures Swiss 3T3 cells were labeled for 16–18 h with 10 μCi/ml [2-^{3}H]inositol. The cells were incubated with rPMT in the absence (*open bars*) or presence (*cross-hatched bars*) of cycloheximide (5 μg/ml) for 4.5 h. LiCl was then added, and after a further 30 min total inositol phosphates were extracted as described. Stimulation with bombesin (6 nM) was for 10 min prior to extraction. Values represent the mean ± S.E. of triplicate determinations for two separate experiments. *Inset*, cultures of Swiss 3T3 cells which were either untreated or pretreated with rPMT (20 ng/ml) for 5 h were lysed in SDS-PAGE sample buffer, electrophoresed in 12.5% SDS-PAGE, and analyzed by Western blotting using an antibody raised against the C-terminal decapeptide of $G\alpha_{q/11}$. The 42-kDa protein recognized specifically by the antibody is indicated by the *arrow*.

C-terminal decapeptide of α_q and α_{11} (52), the G protein subunits that regulate PI-PLCβ, did not detect any increase in the levels of these proteins after cultures had been treated with rPMT (Fig. 7, *inset*). (c) Cycloheximide, a protein synthesis inhibitor, had no effect on inositol phosphate production induced by rPMT, bombesin, or a combination of the two (Fig. 7, *right*). The data strongly suggest that the enhancing effect of rPMT on the production of inositol phosphates induced by neuropeptides is the result of a post-translational modification(s) rather than of increased protein synthesis.

Inositol Phosphate Production in Permeabilized Cells: Effect of GDPβS, GTPγS, and Dependence on Ca^{2+} Concentration—Next we attempted to identify the step(s) modified by rPMT within the neuropeptide-stimulated signal transduction pathway. Cell permeabilization has provided a useful approach to introduce guanine nucleotide analogues into the cytosol to assess the contribution of G proteins in the generation of biological responses. We investigated the effects of the non-hydolyzable analogues GDPβS and GTPγS on the production of inositol phosphates induced by rPMT in permeabilized cells to determine whether a functional G protein was required for its action.

Cells labeled with [2-^{3}H]inositol were incubated with rPMT at 2.5 and 20 ng/ml for 5 h after which they were permeabilized with streptolysin O in the presence or absence of 0.5 mM GDPβS. Fig. 8 shows that the increase in inositol phosphate formation induced by rPMT persisted in the permeabilized cells. The salient feature shown in Fig. 8 is that the production of inositol phosphates in response to rPMT at 20 ng/ml was markedly inhibited by GDPβS (65% inhibition) and that induced by rPMT at 2.5 ng/ml was totally blocked by this G protein antagonist. Fig. 8 also shows, for comparison, that GDPβS inhibited the production of inositol phosphates elicited by either 6 nM bombesin or 1 μM GTPγS whereas GTPγS at a concentration of 1 mM reversed the inhibitory effect of GDPβS.

Ca^{2+} is known to directly stimulate PI-PLC in permeabilized cells (53), isolated membranes (54, 55), and purified preparations (37). In order to test further the specificity of the inhibitory effect of GDPβS, we determined the effect of various concentrations of this analogue on inositol phosphate production stimulated either by treatment with rPMT or by free calcium. Fig. 9 (*left*) shows that GDPβS inhibited rPMT-stimulated inositol phosphate production in a concentration-dependent manner. In contrast, this analogue had no effect

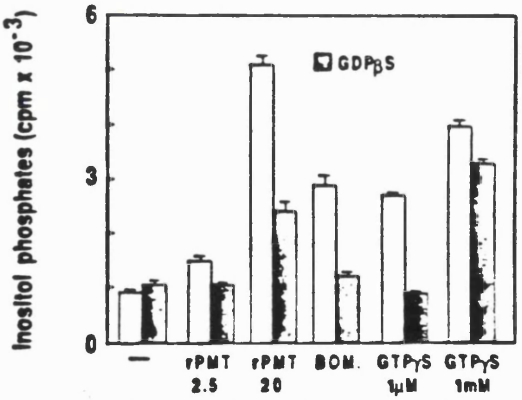

FIG. 8. The effect of GDPβS on the stimulation of inositol phosphate production by rPMT in permeabilized cells. [2-^{3}H] Inositol phosphate release from streptolysin O-permeabilized Swiss 3T3 cells has been described under "Experimental Procedures." Treatment with rPMT was for 5 h prior to permeabilization. Permeabilization was for 10 min in the absence of other factors or in the presence of rPMT (2.5 ng/ml), rPMT (20 ng/ml), bombesin (6 nM), GTPγS (1 μM), and GTPγS (1 mM). These factors were added in the absence (*open bars*) or presence (*shaded bars*) of 0.5 mM GDPβS. Extraction and analysis of total inositol phosphates was as described. Values represent means ± S.E. of triplicate determinations.

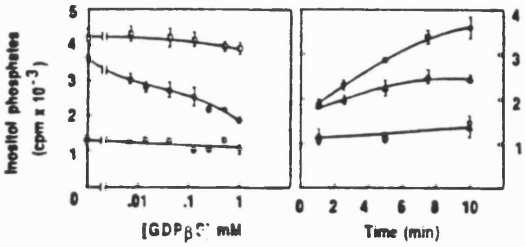

FIG. 9. **Dose dependence and time course of inhibition by GDPβS on inositol phosphates enhanced by rPMT.** The cells were labeled for 16–18 h with 25 μCi/ml [2-^{3}H]inositol in DMEM/Waymouths (1:1). Treatment with rPMT (5 ng/ml) was for 5 h prior to permeabilization. *Left*, increasing concentrations of GDPβS were added to control cells (*open circles*), rPMT-treated cells (*closed circles*), or to cells permeabilized in the presence of 10^{-5} M Ca^{2+} (*open squares*). Permeabilization was for 10 min prior to extraction. *Right*, addition of GDPβS (0.5 mM) to control (*open triangles*) and rPMT-treated cells (*closed triangles*). No additions to control (*open circles*) or rPMT-treated cells (*closed circles*) are shown. GDPβS was added at the time of permeabilization, and the reactions were terminated at the times indicated. Total inositol phosphates were extracted and analyzed as described. Values shown are the means ± S.E. of triplicate determinations.

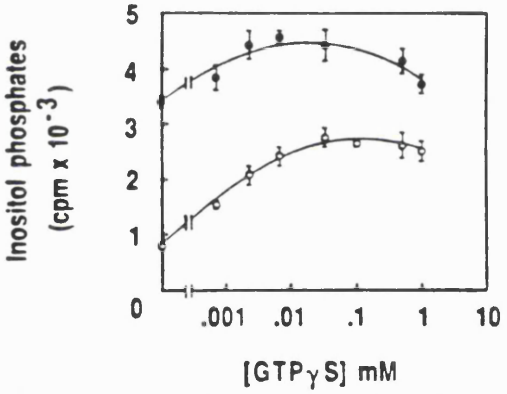

FIG. 10. **The effect of rPMT treatment on the dose-dependent increase in the production of inositol phosphates stimulated by GTPγS in permeabilized cells.** [2-^{3}H]Inositol phosphate release from streptolysin O-permeabilized Swiss 3T3 cells has been described under "Experimental Procedures." Treatment with rPMT (20 ng/ml) was for 5 h prior to permeabilization. Increasing concentrations of GTPγS were added to control (*open circles*) or rPMT-treated cells (*closed circles*) at the time of permeabilization. Permeabilization was for 10 min, when inositol phosphates were extracted and analyzed as described. Values are the means ± S.E. of triplicate determinations taken from three individual experiments.

on the free calcium-stimulated PI-PLC, indicating that its inhibitory effect was selective.

Inhibition of inositol phosphate production in rPMT-treated cells was a rapid consequence of GDPβS addition to permeabilized cells; a decrease in the rate of inositol phosphate formation was evident 2.5 min after the addition of GDPβS (Fig. 9, *right*).

Addition of GTPγS to permeabilized cells caused a marked (220%) increase in the production of inositol phosphates in a concentration-dependent manner (Fig. 10). In contrast, the stimulatory effect of this analogue was only 35% in rPMT-treated cells (Fig. 10). It is unlikely that this subadditivity between GTPγS and rPMT is due to a limiting precursor pool since an increase in the toxin concentration in the presence of GTPγS caused a further increase in the production of inositol phosphates (Table II). The attenuation shown in Fig. 10 suggests that the effects of GTPγS and those of rPMT treatment converge at the same post-receptor locus in the neuropeptide signal transduction pathway.

It has been demonstrated that GTPγS activation of PI-PLC decreases the Ca^{2+} requirement for the activation of this enzyme (54, 55). In streptolysin O-permeabilized 3T3 cells, GTPγS caused a striking leftward shift of the dose-response of free calcium for the stimulation of inositol phosphate release (results not shown). Since the preceding results suggested that rPMT, like GTPγS, enhances the coupling of G protein to PI-PLC, we examined the effect of toxin treatment on the Ca^{2+} requirement for the production of inositol phosphates. Fig. 11 shows that permeabilized cells display an increased production of inositol phosphates in response to

TABLE II

Effects of GTPγS on rPMT-induced inositol phosphates in permeabilized Swiss 3T3 cells

Quiescent cultures of Swiss 3T3 cells were labeled with 25 μCi/ml of [2-^{3}H]inositol for 16 h. rPMT, at the concentrations indicated, was then added directly to the dishes and incubated at 37 °C for 5 h. Permeabilization with streptolysin O was as described under "Experimental Procedures." GTPγS (0.1 mM) was added to control or rPMT-treated cells at the time of permeabilization. Permeabilization was for 10 min, when inositol phosphates were extracted and analyzed as described. Values are the means ± S.E. of triplicate determinations taken from three individual experiments.

Addition	Inositol phosphates	
		GTPγS
	cpm	
	924 ± 66	3960 ± 156
rPMT 20 ng/ml	5562 ± 60	6642 ± 150
rPMT 50 ng/ml	7170 ± 384	7968 ± 228

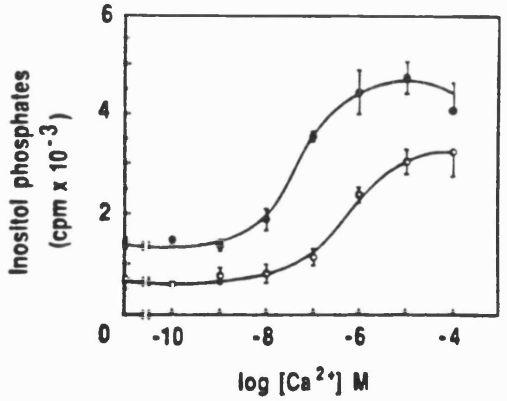

FIG. 11. **Dependence on Ca^{2+} of PI-PLC activity in control and rPMT-treated Swiss 3T3 cells permeabilized with streptolysin O.** Quiescent cultures of Swiss 3T3 cells were incubated for 18 h with 25 μCi/ml [2-^{3}H]inositol. Treatment of cells with 20 ng/ml rPMT was for 5 h prior to permeabilization. Permeabilization was as described under "Experimental Procedures" except the concentration of free calcium in the permeabilization medium was varied. The effect of various concentrations of free calcium in the control (*open circles*) or rPMT-treated cells (*closed circles*) is shown. Values are the means ± S.E. of triplicate determinations taken from three individual experiments.

increasing concentrations of free calcium. Treatment with rPMT increased the sensitivity of PI-PLC for Ca^{2+}; the EC_{50} of Ca^{2+} was reduced from 5×10^{-7} M in the control cells to 5×10^{-8} M in the rPMT-treated cells. The toxin-treated cells also exhibited a marked increase in inositol phosphate production even at high concentrations of free calcium.

DISCUSSION

PMT is the first intracellularly acting bacterial toxin that induces a dramatic increase in polyphosphoinositide breakdown (4, 5, 16) and therefore may provide a novel tool to study the effects of persistent activation of this signal transduction pathway in responsive intact cells. The mechanism of action of rPMT assumes an added importance in view of the fact that this toxin potently stimulates cellular proliferation and anchorage-independent cell growth (4, 5).

In many cell types including Swiss 3T3 cells, polyphosphoinositide hydrolysis by PI-PLC isoforms is activated by at least two distinct mechanisms (30, 48). One involves neuropeptide receptors coupled to G proteins, most likely G_q, which regulate the activity of PI-PLCβ1 (35–38), and the other involves PDGF-mediated tyrosine phosphorylation of specific residues of PI-PLCγ (40).

Our results indicate that rPMT selectively potentiates neuropeptide-mediated inositol phosphate production. Treatment of cells with a subsaturating concentration of the toxin markedly enhanced the accumulation of inositol phosphates induced by bombesin, vasopressin, and the mouse endothelin, VIC. These peptides bind to distinct receptors (56–59) and signal through a G protein pathway, as shown previously using permeabilized cells and membrane preparations (47, 60, 61). Cloning and sequencing of these receptors demonstrate that they belong to the superfamily of G protein-linked receptors characterized by seven putative transmembrane domains (62–64). In sharp contrast, rPMT treatment did not enhance the accumulation of inositol phosphates induced by PDGF, either the BB or the AA homodimers. Furthermore, rPMT did not increase PDGF-mediated tyrosine phosphorylation of the PLCγ isoform. These results clearly indicate that rPMT facilitates signal transduction through the neuropetide-mediated pathway.

In theory, the toxin could enhance the responses initiated by bombesin, vasopressin, or endothelin by increasing the efficiency of ligand binding to these receptors. However, the toxin does not change the number or affinity of the bombesin/GRP receptor for ligand, and the potentiating effect occurs in the absence of *de novo* protein synthesis. Thus, rPMT facilitates signal transduction at a point distal to the receptors. This conclusion is in accord with the fact that rPMT can induce a massive polyphosphoinositide breakdown when it is added in the absence of synergistic peptides but at higher concentrations than those used to amplify neuropeptide-mediated signal transduction.

A common distal point in the action of different neuropeptide receptors is the activation of G_q and PI-PLCβ (32–38). It is therefore plausible that the toxin alters the properties of G_q, the coupling of this G protein to PI-PLCβ, or the properties of PI-PLCβ. If this is the case, it would be expected that rPMT treatment potentiates the generation of Ins(1,4,5)P_3 one of the products of phosphatidylinositol(4,5)P_2 hydrolysis. Our data demonstrate that the increase in the intracellular level of Ins(1,4,5)P_3 induced by bombesin is dramatically enhanced in rPMT-treated cells. The amplification in the accumulation of this second messenger could be detected as early as 5 s after bombesin addition. This kinetic result strongly suggests that rPMT treatment changes the properties of one of the elements of the signal transduction pathway immediately distal to the receptor, *i.e.* G_q and/or PLCβ.

It has been known for some time that activation of PKC can severely attenuate the polyphosphoinositide breakdown induced by a variety of neuropeptides including bombesin and vasopressin (48–50). Although the precise molecular mechanism has not been clearly identified, activation of PKC has been shown to increase the phosphorylation at a specific serine residue of PI-PLCβ (51). PDGF-stimulated inositol phosphate accumulation that is mediated by PI-PLCγ is not inhibited by phorbol ester activation of PKC. If the facilitating effects of rPMT are mediated by the G_q/PI-PLCβ pathway, down-regulation of PKC should enhance the ability of rPMT to induce polyphosphoinositide hydrolysis. The results presented here demonstrate that down-regulation of PKC causes a striking enhancement of rPMT-induced inositol phospholipid hydrolysis and provide an independent line of evidence suggesting that rPMT acts through the G_q/PI-PLCβ pathway leading to polyphosphoinositide hydrolysis.

Evidence for the role of G proteins in signal transduction pathways has been obtained in many systems by assessing the effects of guanine nucleotide analogues in permeabilized cells. In the present study we found that the increased production of inositol phosphates induced by rPMT treatment is preserved after cell permeabilization with streptolysin O, an agent that causes the formation of large pores in the cell membrane by interacting with cholesterol (65). This result is important because it rules out the possibility that the effects of rPMT on polyphosphoinositide hydrolysis result from a change in an intermediary second messenger, *e.g.* free calcium. Furthermore, we exploited this cell permeabilization procedure to determine the effects of cell-impermeable guanine nucleotide analogues on rPMT action. Our results show that GDPβS, which is known to prevent G protein activation, inhibits the production of inositol phosphates in rPMT-treated cells. The inhibition by GDPβS was rapid and concentration-dependent. The ability to stimulate PI-PLC with free calcium in the presence of GDPβS emphasizes the specificity of this inhibitory effect. This finding shows that rPMT-mediated inositol phosphate production requires a functional G protein.

The involvement of a G protein in the stimulation of inositol phosphate production by rPMT is also supported by the fact that the striking activation of PI-PLC by GTPγS is attenuated by prior treatment of the cells with rPMT, suggesting that the toxin and GTPγS converge into the same signal transduction step. GTPγS reduces the Ca^{2+} requirement of PI-PLC to catalyze the cleavage of polyphosphoinositides in membrane preparations (54, 55) or in permeabilized 3T3 cells (results not shown). Similarly, rPMT treatment decreased the Ca^{2+} requirement for the generation of inositol phosphates in permeabilized cells. Taken together, these results imply that rPMT facilitates G protein coupling to PI-PLC.

Early studies with cholera toxin were crucial to elucidate the role of G_s in the stimulatory pathway of adenylate cyclase. Cholera toxin-catalyzed ADP-ribosylation of a specific residue in α_s is sufficient to reduce the rate of GTPase activity of this subunit thereby prolonging the active (GTP-bound) state of α_s (2, 3). This results in cAMP accumulation at high toxin concentration and in facilitation of ligand-dependent adenylate cyclase activation at lower toxin concentrations. By analogy with cholera toxin, it is tempting to postulate that rPMT causes ADP-ribosylation of G_q, leading to selective facilitation of neuropeptide-mediated signal transduction.

However, the use of a novel procedure to detect toxin-catalyzed ADP-ribosylation failed to demonstrate that rPMT stimulates this reaction in intact cells (66). Thus, it is likely that the molecular basis of rPMT action may represent a novel mechanism of toxin-induced deregulation of signal transduction.

In conclusion, the results presented here demonstrate that rPMT, the first intracellularly acting bacterial toxin that leads to PI-PLC stimulation, selectively potentiates neuropeptide-mediated signal transduction. Using permeabilized cells, we identified, for the first time, one of the sites of action of rPMT, namely facilitation of G protein coupling to PI-PLC.

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