Aspects of Phosphoinositide and Calcium Signalling in a Human Neuroblastoma Cell Line.

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by

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ASPECTS OF PHOSPHOINOSITIDE AND CALCIUM SIGNALLING IN A HUMAN NEUROBLASTOMA CELL LINE.

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In the current study, the human neuroblastoma, SH-SY5Y, was employed to investigate aspects of receptor-mediated phosphoinositide (PI) and Ca^{2+} signalling. This cell line expresses many characteristics of neuronal cells, particularly following cellular differentiation. The effects of retinoic acid (RA)-induced differentiation on phosphoinositide and Ca^{2+} signalling were examined here. Some emphasis was placed on the regulation of the signalling pathway following cellular depolarisation or protein kinase C (PKC) activation, since differentiation of neuroblastoma cells has been associated with an increased density of voltage-operated Ca^{2+} channels and alterations of the expression of PKC isozymes.

In both RA-differentiated and undifferentiated SH-SY5Y cells, depolarisation augmented receptor-mediated $[Ca^{2+}]_i$ elevation and PIC activation, providing evidence for signal integration between PIC-linked receptors and depolarising stimuli. However, differences between RA-differentiated and undifferentiated SH-SY5Y cells were minimal. Activation of PKC by phorbol ester reduced receptor-mediated PI hydrolysis in RA-differentiated and undifferentiated SH-SY5Y cells. Conversely, inhibition of PKC activity enhanced PIC activation in RA-treated cells only, suggesting PKC may play a more active role in regulation of the PI pathway in real mammalian neurones compared with neuroblastoma cells.

RA-differentiation of SH-SY5Y cells resulted in an up-regulation of muscarinic and bradykinin receptors. Stimulation of either receptor type with agonists, methacholine and bradykinin, revealed that agonist-mediated PIC activation was enhanced in the RA-treated cells. Comparisons of the concentration-effect curves for PIC activation and Ca^{2+} mobilisation from stores revealed that whilst there is a receptor reserve for agonist-mediated PIC activation in RA-differentiated cells, concomitant with up-regulation of receptor density, there is no corresponding receptor reserve for agonist-evoked Ca^{2+} mobilisation. This was despite amplification of the response, as concentration-effect curves for agonist-stimulated Ca^{2+} mobilisation lay to the left of those for PIC activation. These data suggest a complex relationship between PIC activation and Ca^{2+} mobilisation in the SH-SY5Y cell line, possibly involving a mechanism at the level of the $Ins(1,4,5)P_3$ receptor, which is known to exhibit multiple regulatory mechanisms.

The possibility that receptor agonists regulate the sensitivity of $Ins(1,4,5)P_3$ receptors, independently of PI hydrolysis, in SH-SY5Y cells was investigated. $Ins(1,4,5)P_3$-evoked $^{45}Ca^{2+}$ release was enhanced by bradykinin in a receptor-specific manner, when bradykinin alone failed to mobilise $^{45}Ca^{2+}$ in permeabilised SH-SY5Y cells. The PI 4-kinase inhibitor, wortmannin, practically abolished methacholine-mediated $Ins(1,4,5)P_3$ accumulation, but in contrast had only a small effect on methacholine-stimulated $^{45}Ca^{2+}$ mobilisation, suggesting only a very small $Ins(1,4,5)P_3$ generation is required for substantial release of stored Ca^{2+}. Depletion of agonist-sensitive $PtdIns(4,5)P_2$ pools in SH-SY5Y cells severely depressed methacholine-mediated Ca^{2+} mobilisation, and under these conditions, methacholine potentiated exogenous $Ins(1,4,5)P_3$-evoked Ca^{2+} release in a receptor-specific manner. The observed agonist-stimulated augmentation of $Ins(1,4,5)P_3$-mediated Ca^{2+} release appeared not to be mediated via activated PKC or PKG in permeabilised SH-SY5Y cells. However, the likely involvement of a G-protein in this mechanism was demonstrated by augmentation of $Ins(1,4,5)P_3$-induced $^{45}Ca^{2+}$ release by GTPγS. Thus, agonist-mediated increase in the sensitivity of $Ins(1,4,5)P_3$ receptors to $Ins(1,4,5)P_3$ may be, at least partially, responsible for the complex relationship observed between PIC activation and Ca^{2+} mobilisation.

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CONTENTS

1. INTRODUCTION

Neuronal communication in the central nervous system (CNS) involves release of biological signalling substances (or neurotransmitters) between cells. Signals released from one cell traverse the extracellular space and are recognised by an appropriate membrane-bound receptor on the receiving cell(s). The signal-receptor interaction is often transduced by a variety of second messenger cascades into information that will eventually lead to a cellular response. The interactions between neurones are largely restricted to their synapses which consist of a presynaptic terminal of one neurone and the postsynaptic site of the target neurone and information can flow from the postsynaptic to the presynaptic terminal and vice-versa. The processes by which presynaptic and postsynaptic neurones of a synapse communicate with one another are given the general term 'neurotransmission'. Disturbances in neurotransmission mechanisms are regarded as part of the basic defect in many neurological and psychiatric disorders including widespread illnesses such as schizophrenia, myasthenia gravis, Parkinson's disease and depression.

Transient changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) are a crucial element of intracellular signalling in most cell types. Ca^{2+} signalling mechanisms play a widespread role in neuronal function, with changes in $[Ca^{2+}]_i$ widely regarded to regulate neurone excitability, neurotransmitter release, metabolic reactions and gene expression. In addition, enormous interest in Ca^{2+}-dependent neuronal signalling has arisen, at least in part, from the fact that most forms of synaptic plasticity are controlled by the concentration of intracellular Ca^{2+} ions. An understanding of the mechanisms responsible for neuronal Ca^{2+} signalling and integration is expected to give critical insight into the cellular mechanisms that underlie learning and memory (see Eilers and Konnerth, 1997). The requirement for synaptic Ca^{2+} signalling during the induction of long-term potentiation is also well established (reviewed by Bliss and Collingridge, 1993). There is much, however, that remains to be elucidated regarding the initiation and the regulation of transient changes in $[Ca^{2+}]_i$ in neuronal function and the primary aim of this thesis, therefore, was to conduct an investigation into certain aspects of neuronal Ca^{2+} signalling utilising a model system.

The Ca^{2+} ions that contribute toward $[Ca^{2+}]_i$ elevations can enter the cytoplasm via a number of routes (that are not mutually exclusive) following activation of a wide variety of synaptic neurotransmitter receptors. These include Ca^{2+} entry from the extracellular space, either through Ca^{2+}-permeable channels gated directly by neurotransmitter receptors (receptor-operated Ca^{2+} channels or ROCCs), through second messenger-operated Ca^{2+} channels (SMOCCs) or through voltage-operated Ca^{2+} channels (VOCCs) that open as a consequence of neurotransmitter-induced depolarisation (Hofmann *et al.*, 1994). In addition, Ca^{2+} can also enter the cytoplasm following its release from intracellular Ca^{2+} storage organelles. In nerve cells, these Ca^{2+} stores are generally accepted to consist of specialised components of the endoplasmic reticulum (e.r.) capable of the accumulation and storage of Ca^{2+} (for reviews see Pozzan *et al.*, 1994; Verkhratsky, 1994: Simpson *et al.*, 1995). Ca^{2+} is mobilised from these stores following the activation of intracellular Ca^{2+} release channels, located on the storage organelles, which are gated by the second messenger inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), by Ca^{2+} (Furuichi *et al.*, 1994; Simpson *et al.*, 1995; Mikoshiba *et al.*, 1997) or by the putative second messenger, cyclic ADP ribose (cADPr) (Lee *et al.*, 1993; Hua *et al.*, 1994).

1.1 Ionotropic and Metabotropic Signalling Mechanisms.

Neuronal intracellular signalling mechanisms *per se*, including Ca^{2+} signalling, can essentially be segregated into two distinct mechanisms. The first are termed 'ionotropic' mechanisms, initiated following the activation of 'ionotropic' neurotransmitter receptors. Ionotropic signalling mechanisms, for example via $GABA_A$ receptors, nicotinic acetylcholine receptors and NMDA and AMPA glutamatergic receptors, generally mediate fast synaptic transmission. The second are termed 'metabotropic' mechanisms which are initiated following the activation of 'metabotropic' neurotransmitter receptors, e.g. metabotropic glutamate receptors and muscarinic acetylcholine receptors. Metabotropic signalling mechanisms tend, in general, to mediate slower synaptic responses and play a crucial role in the modulation of complex Ca^{2+} signals and other important neuronal responses, such as neurotransmitter release and synaptic plasticity (Henzi andMacDermot, 1992). Although these mechanisms are separately defined, there is great potential for considerable interaction (or cross-talk) between ionotropic

and metabotropic responses. Such interactions are discussed subsequently in more detail (sections 1.2-1.5).

1.1.2 Ionotropic Signalling Mechanisms.

Agonist stimulation of receptor-operated ion channels such as (for example) nicotinic acetylcholine receptors, P_{2x} purinoceptors and NMDA glutamate receptors cause the ion channel to open, resulting in cation influx. ROCCs belong to a distinct superfamily of proteins known as ligand-gated ion channels. Neurotransmitters are able to activate ligand-gated ion channels, which may be Ca^{2+} permeant, in which case $[Ca^{2+}]_i$ is directly and rapidly elevated. Alternatively, influx of positively charged ions (other than Ca^{2+}), such as Na^+, can stimulate cellular depolarisation which, depending on the magnitude of stimulus, can stimulate the activation of various VOCCs, through which Ca^{2+} enters the cytosol down its electrochemical gradient from the extracellular space, thus elevating $[Ca^{2+}]_i$.

There are four pharmacologically and biophysically well characterised types of VOCCs, types L, N, T and P/Q, that have been identified in different neurones (Nowcycky *et al.*, 1985; Linas *et al.*, 1989; Murakoshi and Tanabe, 1997). However, during the last few years cloning of VOCCs and the expression and characterisation of endogenous VOCCs have led to the recognition that there are a variety of other channels clearly different from these (Olivera *et al.*, 1994). It is suggested that different types of VOCCs may mediate different functions. For example, transmitter release appears to be triggered by L-, P- and N-type channels depending on the type and location of the nerve cell and on the activating transmitter (Matthies *et al.*, 1987; Lipscombe *et al.*, 1989; McMahon and Nicholls, 1991; Vaughan *et al.*, 1993). Similar to ROCCs, VOCCs also belong to a superfamily of protein receptors which have been termed 'voltage-gated ion channels'. The members of this superfamily of receptors are characterised by the presence of clusters of six transmembrane domains (Schwarz *et al.*, 1988; Kamb *et al.*, 1988; Butler *et al.*, 1989).

There is a third superfamily of receptors, which are activated by a wide variety of neurotransmitters in the CNS known as GTP-binding protein (G-protein)-coupled receptors. These receptors are characterised by the presence of seven transmembrane spanning domains and hence are often referred to as 7TM receptors (Nathans and Hogress, 1983; Frielle *et al.*,

1988; Bonner *et al.*, 1988; Fargin *et al.*, 1988). G-protein-coupled receptors have slower response characteristics compared with ROCCs and VOCCs with latency periods of 100 milliseconds or more compared with 1 to 10 milliseconds. One of the reasons for the comparatively long response time is that the 7TM receptor family are often (but not always) coupled to intracellular second messenger systems. In the nervous system G-protein coupled receptors are known as metabotropic neurotransmitter receptors.

1.1.2 Metabotropic Signalling Mechanisms.

G-proteins directly couple 7TM receptors to primary effector proteins, which can be defined as enzymes that produce intracellular second messengers, such as inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) (and other inositol phosphate molecules), diacylglycerol (DAG), adenosine 3', 5'-cyclic monophosphate (cAMP), guanosine 3', 5'-cyclic monophosphate (cGMP), nitric oxide (NO), cyclic ADP ribose (cADPr) and metabolites of arachidonic acid. Second messengers commonly activate protein kinases, enzymes capable of regulating the function of a whole range of cellular proteins by stimulating their phosphorylation (see section 1.3). However, neurotransmitters acting through G-protein-coupled receptors can also affect ion channel function, thereby integrating metabotropic and ionotropic signalling mechanisms. For instance, activation of the ionotropic NMDA receptor has been reported to both activate and potentiate certain metabotropic receptor-mediated responses (phosphoinositide hydrolysis) in neuronal cells and tissues (Nicoletti *et al.*, 1987; Baird and Nahorski, 1991; Challiss *et al.*, 1994). In addition, certain physiological agonists such as glutamate, acetylcholine and serotonin are ligands for both ionotropic and metabotropic receptors. Since individual neuronal cells express multiple receptor types and subtypes, coincident activation of metabotropic and ionotropic receptors is very likely a physiologically relevant event. It is now possible to dissect these pathways by the combination of biochemical and biophysical experimental techniques. The G-protein-coupled receptor superfamily is extremely large with at least fifty neurotransmitter receptors controlling many intracellular second messenger cascades. New members of the family, based on their structural homology, with hitherto unknown functions (termed 'orphan' receptors) are being discovered with increasing frequency, and thus the family continues to expand.

A single transmitter substance often has several closely related receptor subtypes. For instance, six α-adrenergic, three β-adrenergic and five muscarinic receptors have been cloned to date. G-proteins are also diverse and are grouped into broad families according to structural homologies of the α-subunits (although there are also several forms of the β and γ subunits). Studies on the distribution of metabotropic receptors in the CNS have revealed that almost all G-protein-coupled receptor subtypes are expressed in the brain. Indeed many subtypes of receptors are often expressed on the same neuronal cell (see Schofield *et al.*, 1990 and references therein). The great information-handling capacity of neuronal cells is likely to be attributable, at least in part, to such a diversity of metabotropic receptor subtypes in the CNS. In addition, immense specificity of the responses evoked by ligand-7TM receptor interactions in the nervous system is implied.

Despite the diversity of G-protein-coupled receptors in the nervous system, however, the number of the primary effector proteins present in the CNS are substantially less. In other words, many neurotransmitters activate diverse receptors which converge to activate (or inactivate) one particular effector protein (often) via the same G-protein family. For example, activation of muscarinic M2, α2-adrenergic, dopamine D2, serotinergic $5HT_{1a}$, adenosine A, histamine and μ and δ opioid receptors all stimulate the opening of inwardly rectifying K^+ channels in various neurones via pertussis toxin-sensitive G-proteins (North, 1989). Such a convergence of many neurotransmitter inputs onto one effector questions whether the specificity, implied by the existence of such a wide range of metabotropic receptors in the nervous system, is lost by their translation into common intracellular actions. In the endocrine system, specificity is retained via the selective expression of one appropriate 7TM receptor on the target cell. However, almost all neurones appear to express a diversity of 7TM receptors converging on common targets (Ross, 1989). Specificity seems to be compromised further by additional mechanisms. One such mechanism is the potential for 'cross-talk' between the various signal transduction mechanisms, an aspect which will be discussed in subsequent sections of this chapter.

1.2 Modulation of Ion Channel Function by G-Protein-Coupled Receptors in Neurones.

Evidence that ion channels are common effector proteins in G-protein-mediated signalling pathways has accumulated over the past fifteen years. Neurotransmitters (and hormones) control the 'tone' of the autonomic nervous system by G-protein-dependent modulation of ion channel activity. In a similar manner, vision, taste and smell are also based on G-protein-dependent mechanisms of ion channel regulation. Evidence that G-proteins are involved in receptor-mediated ion channel modulation comes from studies demonstrating that such regulation is evoked by agents, such as GTPγS and AlF_4^- that lock the G-protein in an active state, and studies demonstrating that channel regulation, in certain cases, can be disrupted by pertussis toxin or by cholera toxin (pertussis toxin: Go or Gi; cholera toxin: Gt or Gs) (reviewed by Wickham and Clapham, 1995a, 1995b). It is becoming clear, however, that ion channels can be modulated in a G-protein-dependent manner by two different mechanisms. First, it seems likely that ion channels can be modulated directly by activated G-protein, a 'membrane-delimited' mechanism. Second, ion channels can be regulated in a G-protein-dependent manner indirectly, involving diffusible second messengers.

1.2.1 Membrane delimited ion channel regulation by G-proteins.

Membrane delimited ion channel regulation essentially refers to a situation where all the elements required for channel regulation are membrane associated. Evidence for such a mechanism comes from electrophysiological studies where agonist applied to the outside, but not to the inside, of a patch of plasma membrane evoked ion channel regulation, suggesting that the regulatory pathway is confined to the membrane (Brown and Birmbaumer, 1988; Hille, 1992, 1994). However, it remains a possibility that lipid soluble channel regulators, rather than or in addition to G-protein subunits, may mediate ion channel modulation. A direct interaction between an ion channel and a G-protein remains to be directly demonstrated experimentally, which is proving a difficulty since the majority of ion channels are expressed at low densities in membranes where second messenger pathways predominate. Hence 'membrane-delimited' does not refer simply to a direct physical interaction between G-protein and ion channel. Despite the lack of absolute experimental proof for such a direct interaction

between ion channel and G-protein, neuronal Ca^{2+} channels, Na^{+} channels and K^{+} channels are all hypothesised to be modulated directly in such a manner.

In the past, hypothesised G-protein-mediated regulation of ion channel function was attributed solely to activated Gα subunits. In more recent years, however, there have been several studies which would seem to suggest that Gβγ subunits are capable of regulating effector activation (reviewed by Clapham and Neer, 1993). Thus far, a role for Gβγ in ion channel regulation has been suggested for both cloned and native (cardiac) muscarinic acetylcholine gated receptor modulation of K^{+} channels (Reuveny *et al.*, 1994; Wickman *et al.*, 1994). More recently, evidence has been presented to support a role for G-protein βγ subunits in the regulation of neuronal VOCCs (Shekter *et al.*, 1997).

1.2.2 Ion channel regulation involving diffusible second messengers.

As briefly mentioned above, activated G-proteins stimulate the regulation of a variety of effectors. The majority of these effectors act to regulate the cellular concentration of diffusible second messengers including $Ins(1,4,5)P_3$, cyclic nucleotides and Ca^{2+}. A number of ion channels are sensitive to changes in $[Ca^{2+}]_i$ and the activation of many G-protein-coupled receptors results in an increase in $[Ca^{2+}]_i$. In many neurones, Ca^{2+}-dependent K^{+} channels induce adaption of spike frequency, thus increased $[Ca^{2+}]_i$ results in lengthening of the period of time between action potential intervals during bursts of activity (Lattore *et al.*, 1989). Hence Ca^{2+}-dependent K^{+} channels are considered to be important targets for indirect modulation via G-protein-dependent mechanisms. G-proteins often regulate major classes of protein kinases via the control of cellular levels of second messengers such as diacylglycerol (protein kinase C (PKC)), cAMP (protein kinase A (PKA)), Ca^{2+} (Ca^{2+}-calmodulin-dependent protein kinase (Ca/CaM PK)) and , in a less direct manner, cGMP (protein kinase G (PKG)). Ion channel regulation by phosphorylation would appear to be a common place phenomenon in neuronal and other excitable cells (reviewed by Levitan, 1994). For example, purified L-type VOCCs have been demonstrated to be substrates for kinases, and the phosphorylated channels have increased activity in reconstituted systems (Nunoki *et al.*, 1989). Hence, by interference, the effects of protein kinase activity on ion channel function are assumed to represent important regulatory mechanisms. Ion channels are regulated by additional

mechanisms involving second messengers. For instance, receptor activation of G-proteins can often stimulate the activation of phospholipase A_2 (PLA_2), thus generating arachadonic acid (see Exton, 1994). Arachadonic acid (and various metabolites) have been demonstrated to modulate the activity of K^+ in molluscan neurones (Piomelli *et al.*, 1987). Finally, an unknown second messenger is apparently responsible for one receptor-dependent inhibitory mechanism involving N-type VOCCs in sympathetic neurones (reviewed by Hille, 1994).

Aside from G-protein-mediated regulation (via second messenger mechanism mechanisms) of the 'voltage sensor' ion channel family, electrophysiological investigations have demonstrated the influence of second messenger activated protein kinase activity on ligand gated ion channel receptor function (reviewed by Pasqualotto and Shaw, 1996). Examples of such modulation and their consequences are outlined in Table 1. Receptor phosphorylation of ligand-gated ion channels appears to be necessary for the maintenance of functional integrity of some receptors. For instance, receptor-mediated currents through ion channels gradually decline, an effect which can often be prevented by the inclusion of factors which maintain receptor phosphorylation (e.g. phosphatase inhibitors) (Stelzer *et al.*, 1988; Gynes *et al.*, 1989; Chen *et al.*, 1990; Wang *et al.*, 1993). The functional consequences of phosphorylation of a particular type of receptor appears to depend on the subtype of kinase involved (Table 1) and the subunit composition of receptors (Moss *et al.*, 1992; Krishek *et al.*, 1994). The potentiation or reduction of ligand-gated receptor-mediated currents may be accounted for by changes in properties such as channel open probability, mean open time and alterations of the rate of desensitisation (Chen and Huang, 1992; Wang *et al.*, 1991; Krisheck *et al.*, 1994).

Activated protein kinase molecules often modulate the activity of numerous other cellular proteins other than, and in addition to, ion channels in neurones. Mechanisms of activation and the functional consequences of the protein kinase species commonly found in neuronal cells are outlined in the following section.

Receptor	Kinase	Consequences
Glycine	PKA	Potentiation
	PKC	Reduction
GABAA	PKA	Potentiation
		Reduction
	PKC	Reduction
Kainate	PKA	Potentiation
NMDA	PKC	Potentiation
AMPA	PKA	Potentiation
	CaM kinaseII	Potentiation

Table 1 Modulation of ligand-gated (ionotroic) receptor-mediated currents in response to protein kinase activity.

Potentiation and reduction refer to the peak amplitude of whole cell currents (Pasqualotto and Shaw, 1996 and references therein).

1.3 Protein Kinase Signalling Pathways in Neurones.

Various protein kinase subtypes are abundantly expressed in neuronal cells and are widely regarded to mediate, following their activation, a multiplicity of cellular events via the phosphorylation of target cellular proteins. Three major protein kinase families, protein kinase A (PKA), protein kinase G (PKG) and protein kinase C (PKC) seem to have numerous functions in neurones and are activated by signal transduction cascades initiated following the interaction of ligand molecules with a whole host of receptors, many of which are members of the 7TM superfamily.

1.3.1 The protein kinase A family in neuronal signalling.

Protein kinase A can be activated following the stimulation of primary effector enzyme, adenylate cyclase (AC) or inhibition of specific phosphodiesterase (PDE) isoforms, which results in the accumulation of cAMP, which in turn stimulates the activation of PKA. There are multiple forms of these enzymes (PKA, AC and PDE) which allows cells to tailor the responsiveness of the cAMP-PKA signalling pathway to allow for its dynamic adjustment (Houslay and Milligan, 1997). The cAMP signalling pathway can influence an array of cellular processes and in neurones is believed to play a role in long-term synaptic plasticity and learning, regulation of ion channel conductance, differentiation and apoptosis (Murphy and Segal, 1997).

PKA isoforms achieve their roles in cAMP-mediated signal transduction through phosphorylation of their substrates. For example, PKA has been demonstrated to enhance NMDA receptor activity via phosphorylation in spinal cord neurones and in hippocampal microcultures (Cerne *et al.*, 1993; Raman *et al.*, 1996) which has been interpreted as providing evidence for a role of PKA in synaptic plasticity and neurotoxicity (Leonard and Hell, 1997). Adenylate cyclase is usually either stimulated or inhibited , with a resultant increase or decrease respectively of cAMP accumulation, by G-protein-coupled receptors (via G-proteins of the Gs or Gi families respectively). Hence, the above situation provides an example of cross-talk between ionotropic and metabotropic signalling pathways in neurones.

1.3.2 The cGMP-dependent protein kinase (PKG) family in neuronal signalling.

PKG achieves its roles in cGMP-mediated signal transduction events by phosphorylation of its substrates (reviewed by Butt *et al.*, 1993; Francis and Corbin, 1994). PKG is activated by the second messenger molecule cGMP in a manner analogous to that of PKA activation by cAMP. The intracellular concentration of cGMP is increased following activation of the effector enzyme guanylate cyclase (GC). There are two known pathways leading to the activation of GC. First via the activation of natriuretic peptide receptors and second via nitric oxide (NO) generation, produced following stimulation of the enzyme NO synthase (NOS).

The last few years has witnessed a great interest in how the activation of GC (with resultant activation of PKG) may control neuronal function. Such interest has resulted in the demonstration of an array of neuronal functions for the cGMP signalling pathways. These include mediation or modulation of neurotransmitter uptake and release, neuronal differentiation and gene expression, learning and memory, brain seizure activity and neurotoxicity (reviewed by Garthwaite and Boulton, 1995; Jaffery and Snyder, 1995; Wang and Robinson, 1997).

In neuronal tissues cGMP levels increase in response to depolarising stimuli. However, the mechanisms by which cGMP modulates the neuronal functions mentioned above are still not completely understood. Recent studies, however, highlight a pivotal role for PKG-mediated protein phosphorylation as the signalling pathway in many of these processes, although it is important to bear in mind that where intracellular [cGMP] is elevated, it is possible that a multiplicity of signalling pathways are activated. For instance, in a similar manner to cAMP, cGMP is known to activate (cyclic nucleotide gated) ion channels, cGMP can also regulate phosphodiesterases (PDEs) (reviewed by Conti *et al.*, 1995; Finn *et al.*, 1996).

Role of PKG in neurotransmission.

Although PKG is present at low levels in the brain, several studies have provided definite roles for PKG in certain neuronal events resulting from elevated cGMP levels. Such studies are generally based on the intracellular application of PKG or a PKG agonist or antagonist, both of which are largely without effects on PDEs or cGMP-gated ion channels. These neuronal effects of PKG include direct roles in regulation of neuronal Ca^{2+} currents (Meriney *et al.*, 1994), control of neuronal firing rates or neurotransmitter release (Akamatsu *et al.*, 1993; Pineda *et al.*, 1996), as a target in long-term potentiation (Zhuo et al., 1994; Arancio et al., 1995) and in the activation of K^+ channels (Furukawa *et al.*, 1996).

PKG substrates and action.

Much has been learned regarding the likely actions of PKG in neurones from the study of PKG substrates in non-neuronal tissues, most of which are also present in neuronal cells. These substrates (proteins) can be grouped into areas of function relating to signal

transduction: cyclic nucleotide action, protein phosphatase regulation, $[Ca^{2+}]_i$ homeostasis, ion channel regulation and G-proteins.

There are several mechanisms by which PKG substrates or PKG itself may modulate intracellular cAMP or cGMP signalling. First, cAMP may 'cross-activate' PKG (Jiang *et al.*, 1992). Second, many proteins serve as substrates for both PKA and PKG, thus providing a mechanism for convergence as well as divergence in cGMP signalling. Third, specific proteins mediating cAMP or cGMP signalling may be phosphorylated by PKG. For example, PKG autophosphorylation (Francis and Corbin, 1994). Neuronal NOS is also a substrate for protein kinases, including PKG (Dinnerman *et al.*, 1994). Phosphorylation decreases NOS activity, thus phosphorylation by PKG may serve as a feedback inhibitor of NO signalling pathways. Feedback inhibition of NOS activity by PKG in neurones may be a mechanism by which PKG could have a profound influence on neighbouring neurones.

A general functional theme for cGMP action through PKG in cells is a lowering of $[Ca^{2+}]_i$. For instance, over-expression of PKG in several cell types lowers $[Ca^{2+}]_i$ or prevents agonist-induced rises in $[Ca^{2+}]_i$ (Mery *et al.*, 1991; Ruth *et al.*, 1993). Several known mechanisms may contribute towards this phenomenon including phosphorylation by PKG of some key proteins that regulate $[Ca^{2+}]_i$. The known examples of PKG substrates that could mediate this effect are $Ins(1,4,5)P_3$ receptors, ryanodine receptors, plasma membrane associated Ca^{2+} channels and phospholamban (a regulator of e.r. Ca^{2+}-ATPase). These proteins may also be regulated by PKG through further pathways such as the effects of PKG-mediated phosphorylation of K^+ channels (discussed below). PKG may lower $[Ca^{2+}]_i$ via direct effects on Ca^{2+} channels. Opening of L-type VOCCs requires phosphorylation (Bertolino and Llinas, 1992), and major subunits of the Ca^{2+} channel are substrates for both PKA and PKG. In contrast to PKA, however, cGMP mediates channel inhibition in neurones (Nishimura *et al.*, 1992; Meriney *et al.*, 1994).This effect appears to be mediated via PKG, as demonstrated by prevention of the effect using the PKG antagonist, KT 5823 (Mery *et al.*, 1991). The target for PKG could be a Ca^{2+} channel subunit or a protein associated with it (such as G-protein (Meriney *et al.*, 1994)). Such effects in neurones would have important consequences for the generation of action potentials and on signal transduction. In addition to L-type VOCCs, N-type VOCCs in neurones are phosphorylated in vitro, although the functional consequences remain to be elucidated (Hell *et al.*, 1994). Potential modulation of N-type VOCC activity by

phosphorylation, however, may regulate release of neurotransmitters from certain nerve terminals.

Phosphorylation of intracellularly located Ca^{2+} release channels (i.e. $Ins(1,4,5)P_3$ receptors and ryanodine receptors) is very likely to contribute toward the inhibition of $[Ca^{2+}]_i$ by cGMP observed in some cell types. This aspect is discussed further elsewhere in this thesis (see section 1.5 and chapter 8). An additional mechanism located intracellularly for cGMP-evoked lowering of neuronal $[Ca^{2+}]_i$ is the phosphorylation of phospholamban by PKG. Phosphorylation of phospholamban via this pathway results in its dissociation from Ca^{2+}-ATPase with subsequent activation of the ATPase (Sarcemic *et al.*, 1989). In contrast to the effects of decreasing $[Ca^{2+}]_i$, cGMP and GCs have also been shown to stimulate the elevation of $[Ca^{2+}]_i$ in neurones, probably via several mechanisms (Leinders-Zufall *et al.*, 1995).

1.3.3 The protein kinase C family in neuronal signalling.

PKC is present in high concentrations in neuronal tissues and has been implicated in a broad spectrum of neuronal functions. The family of G-protein-coupled receptors that, following interaction with agonist, activate a signal transduction cascade which results in the activation of PKC, are the largest of the 7TM superfamily of receptors. Receptor activation stimulates the activity of the primary effector enzyme phospholipase C (PLC) (interchangeably referred to as phosphoinositidase C (PIC)) via the Gq/11 family of G-proteins. Thus activated, PIC catalyses the hydrolysis of membrane phospholipid phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) resulting in the formation of two second messenger molecules, diacylglycerol (DAG) and $Ins(1,4,5)P_3$. There are, therefore, two second messenger limbs resulting from receptor-Gq/11-PIC interactions that are separate from one another, but with potential for cross-talk between the converging pathways at various levels (see chapter 6). The function of DAG is to stimulate the activation of various isoforms of PKC, whilst the predominant action of $Ins(1,4,5)P_3$ is to release Ca^{2+} from intracellular stores (Berridge, 1993). A substantial amount of the work presented in this thesis is concerned with aspects of $Ins(1,4,5)P_3$-mediated Ca^{2+} mobilisation (in a neuronal model). The current section, however, focuses on addressing the role of PKC in neurotransmission.

Activation of various PKC isoforms in neuronal cells is frequently associated with the modulation of ion channels (Shearman *et al.*, 1989; Levitan, 1994), desensitisation of receptors (Huganir and Greengard, 1990) and enhancement of neurotransmitter release (Robinson, 1992). It is possible that, through such mechanisms, the PKC pathway modulates the efficacy of synaptic transmission, thus providing a basis for some forms of memory among other aspects of neuronal function. Upon receptor stimulation, an early but transient peak of DAG generation is often observed that quickly declines to original levels. At later phases of cellular responses, the formation of DAG has a slow onset, but is relatively sustained. This second phase of DAG generation is likely to be derived from the hydrolysis of membrane constituents such as phosphatidyl choline (PC) by another effector protein, phospholipase D (PLD) in a signal-dependent manner. Phosphatidic acid (PA) is formed by the action of PLD on PC which, in turn, is dephosphorylated to DAG (reviewed by Exton, 1990). Hence, PKC isozymes can be activated following receptor-mediated stimulation of PIC and/or PLD.

PKC isozymes.

Ten isoforms (α, βI, βII, γ, δ, ε, η, θ, ξ and τ) have been identified in mammalian tissues thus far. These isoforms exhibit subtly different enzymological properties, differential tissue expression and specific intracellular localisation. The classical PKC isozymes, the α, βI, βII and γ isoforms are activated by DAG, Ca^{2+} and phorbol esters, tumour-promoting agents that are DAG analogues and act as PKC activators in vitro (Huang *et al.*, 1986). The novel PKC isozymes, δ, ε, ηand θ isoforms do not require Ca^{2+} and exhibit their enzymatic activities in the presence of DAG or phorbol esters (Konno *et al.*, 1989). It is a distinct possibility that novel PKC isozymes are integrated directly or indirectly by a protein kinase cascade initiated by activation of growth factor receptors (Ahn *et al.*, 1990). The atypical ξ and τ PKC isoforms are not reliant on DAG, phorbol esters or Ca^{2+} for their activation (Nakanishi and Exton, 1992). So far, the members of the PKC family examined respond differently to various concentrations of Ca^{2+} and DAG and thereby produce distinct activation patterns.

The α, βI, βII, γ, δ, ε and ξ PKC isozymes have all been identified in the CNS. Immunohistochemical analysis using isoform-specific antibodies has revealed the differential

distribution of PKC isoforms in the mammalian CNS (Tanaka and Saito, 1992). PKCγ is expressed at a high concentration in the CNS, and whether it is found in other tissues is a subject of debate.

Potential roles of PKC in neuronal function.

Modulation of the sensitivity of ion channels and receptors is a major role of the PKC family (Huganir and Greengard, 1990). This function of PKC is believed to contribute toward long-term potentiation (LTP), the underlying mechanisms of which have been extensively studied in the hippocampus (Bliss and Collingridge, 1993). Briefly, LTP is initiated following activation of NMDA receptors which results in Ca^{2+} influx, triggering a synaptic response. Thereafter the response declines to a stable plateau during which another cascade of events results in enhancement of the synaptic response which can be maintained for days. Evidence is accumulating to suggest that activated PKC is required for the LTP process. For example, injection of PKC into postsynaptic cells can mimic LTP, and application of PKC inhibitors can prevent induction of LTP (Hu *et al.*, 1987). It is believed that direct phosphorylation of NMDA receptors by PKC (PKCγ is the favoured candidate) is the likely event linked to the postsynaptic component of LTP.

There is also evidence to indicate that neurotransmitter release is linked to PKC activation. Ca^{2+}-dependent neurotransmitter release evoked by depolarisation is normally potentiated by PKC activators, such as phorbol esters, and antagonised by PKC inhibitors. It's likely that the uptake and release of neurotransmitters are modulated by the PKC isoform expressed in the nerve terminals (Tanaka and Nishizuka, 1994 and references therein).

PKC itself appears to be regulated by PKG activity in neurones. For example, purified rat brain PKC (comprising α, β and γ isoforms) is phosphorylated by PKG. This PKG-evoked phosphorylation of PKC resulted in a decreased activity of PKC toward a known neuronal PKC substrate, dynamin 1 (Wang and Robinson., 1995; Gatti *et al.*, 1996). This is likely to represent a point of cross-talk between phosphoinositide and cGMP signalling pathways which could occur when both are activated simultaneously.

1.3.4 Ca^{2+}-calmodulin-dependent protein kinases in neuronal signalling.

Free cellular Ca^{2+} can bind to a small Ca^{2+}-binding protein, calmodulin. Ca^{2+}-calmodulin (CaM) can modulate the activity of a number of enzymes including CaM-dependent protein kinases (CaM kinases). Thus far, five CaM kinases have been identified, although CaM kinase II and IV have been best characterised. CaM kinase II has been extensively studied as a potential mediator of Ca^{2+}-dependent synaptic changes and is widely believed to be involved in the regulation of synaptic plasticity (Schulman, 1993). CaM kinase II is capable of autophosphorylation which switches the enzyme into a Ca^{2+}-independent form (i.e. it retains its enzymatic activity even after $[Ca^{2+}]_i$ has returned to basal levels) a property which led to the proposal that CaM kinase II may modulate long-term synaptic function. This proposal has gained support in a number of studies illustrating that stimuli that give rise to LTP also evoke significant increases in CaM kinase II activity (Molloy and Kennedy, 1991; Suzuki *et al.*, 1992; Patton *et al.*, 1993).

Similar to the PKA, PKG and PKC signalling pathways, there is potential for cross-talk between the CaM kinase signalling pathway and the transduction pathways outlined above. For example, Ca^{2+} is able to influence cAMP levels (and hence PKA activity) by the activation of CaM-dependent adenylate cyclase molecules (Ghosh and Greenberg, 1995 and references therein).

The amount of information that has been produced regarding neuronal signalling mechanisms in the last decade is vast, the current section of this thesis covering but a fraction of the information available. The aim of this section, however, was to introduce the major signal transduction pathways operating in neuronal cells, and the way in which these pathways can often diverge and then converge onto other pathways by 'cross-talk' at various levels. Specific examples of such cross-talk have been referenced, although it is important to appreciate that there are many more examples of this nature, with a large number that are likely remaining to be discovered. The majority of the work presented in this thesis, however, focuses on one particular signal transduction pathway that is activated by a wide range of neurotransmitter molecules, the phosphoinositide and Ca^{2+} signalling pathway, thus far

touched upon only lightly. The initiation, sequence of events and certain regulatory aspects of this pathway and its role in neuronal function are outlined below.

1.4 Phosphoinositide and Calcium Signalling in Neurones.

Intracellular Ca^{2+} is widely believed to represent a major mediator of development and plasticity in the nervous system, which has led to a wide-ranging interest in the array of mechanisms by which $[Ca^{2+}]_i$ is elevated and regulated in neuronal cells. The major route of Ca^{2+} entry into the neural cytoplasm is through plasma membrane associated Ca^{2+} channels. However, following exposure of many cells to a variety of stimuli that act through G-protein-coupled receptors, the second messenger $Ins(1,4,5)P_3$ is generated. $Ins(1,4,5)P_3$ is a central regulator of $[Ca^{2+}]_i$ by virtue of its action on intracellular Ca^{2+} stores, and neurones *per se* express a wide variety of 7TM receptors known to be linked to the generation of $Ins(1,4,5)P_3$. Thus, release of Ca^{2+} from internal stores is very likely to contribute towards $[Ca^{2+}]_i$ homeostasis in a neuronal environment (Henzi and MacDermot, 1992; Berridge, 1993; Kaplin *et al.*, 1994). Another mechanism by which Ca^{2+} is released from intracellular stores is by Ca^{2+}-induced Ca^{2+} release (CICR) following activation of ryanodine receptors. Both $Ins(1,4,5)P_3$ and ryanodine receptors have been extensively studied (particularly because of the key role they play in the generation of $[Ca^{2+}]_i$ oscillations). Moreover, most neuronal cells are rich in the expression of both forms of these intracellular release channels (Berridge, 1993; Ehrlich, 1995; Furuichi and Mikoshiba, 1995). There is much that remains to be uncovered, however, regarding the role of the intracellular release channels in the nervous system.

The work presented in this thesis focuses on receptor-mediated phosphoinositide (largely $Ins(1,4,5)P_3$) and Ca^{2+} signalling in a model of a neuronal cell. It is now recognised that of all the signal transduction pathways, phosphoinositide hydrolysis is linked to more plasma membrane receptor systems than any other, and can therefore be assumed to be of fundamental importance in many aspects of cell physiology. Despite this fact, however, very little is yet known of the role of this signalling pathway in neuronal function, which remains a subject of debate. One view is that $Ins(1,4,5)P_3$-mediated Ca^{2+} signalling plays an important role in a whole diversity of brain functions (see Mikoshiba and Furuichi, 1995). There is considerable evidence that the role of phosphoinositide signalling pathways in the nervous

system is the modulation of synaptic activity or efficacy, and it is anticipated that by this mechanism the pathway regulates many neuronal functions (Berridge, 1993 and references therein). The phosphoinositide signalling pathway is also believed to modulate neuronal activity and neuronal plasticity for learning and memory (Henzi and MacDermot, 1992). The difficulty in establishing the role of $Ins(1,4,5)P_3$-mediated Ca^{2+} signalling in CNS function is difficulty in interpreting data due to the complexity of nervous tissue (see section 1.5).

Ca^{2+} signalling mechanisms evoked following receptor-mediated $Ins(1,4,5)P_3$ accumulation can include Ca^{2+} release from internal stores following activation of $Ins(1,4,5)P_3$ receptors and possibly ryanodine receptors (CICR), and Ca^{2+} influx at the plasma membrane (termed 'capacitative Ca^{2+} entry') (reviewed by Berridge, 1993). These aspects and their potential roles in neuronal signalling and function are outlined below (sections 1.4.3, 1.4.4 and 1.4.5).

1.4.1 Receptor-mediated phosphoinositide hydrolysis.

Phosphoinositide synthesis and metabolism.

Major constituents of all plasma membranes are the phospholipids, of which phosphoinositides make up approximately 10%. Phosphatidylinositol (PtdIns), the most abundant of the phosphoinositides, is synthesised following the combination of CMP-phosphatidase (CMP-PA) and myo-inositol, a reaction catalysed by PtdIns synthase. The phosphorylated derivatives of PtdIns, phosphatidylinositol 4-phosphate (PtdInsP) and phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$), are synthesised from PtdIns by PI 4-kinase and PtdInsP by PI 5-kinase respectively (Downes and Mitchell, 1985). The phosphorylated forms may also be dephosphorylated by phosphomonoesterases (Berridge and Irvine, 1984), and thus the phosphoinositides are maintained in a cycle. The roles of PtdIns, PtdInsP and $PtdIns(4,5)P_2$ would appear to be as precursors for receptor-mediated generation of $Ins(1,4,5)P_3$ and DAG. $Ins(1,4,5)P_3$ itself is a short-lived second messenger and is rapidly metabolised by two enzymes, a 5-phosphatase and a 3-kinase, forming $Ins(1,4)P_2$ and $Ins(1,3,4,5)P_4$ respectively (reviewed by Cockcroft and Thomas, 1992). This dual inactivation of $Ins(1,4,5)P_3$ generates two distinct series of inositol phosphate intermediates, which are ultimately recycled into the production of an inositol phosphate pool (Figure 1).

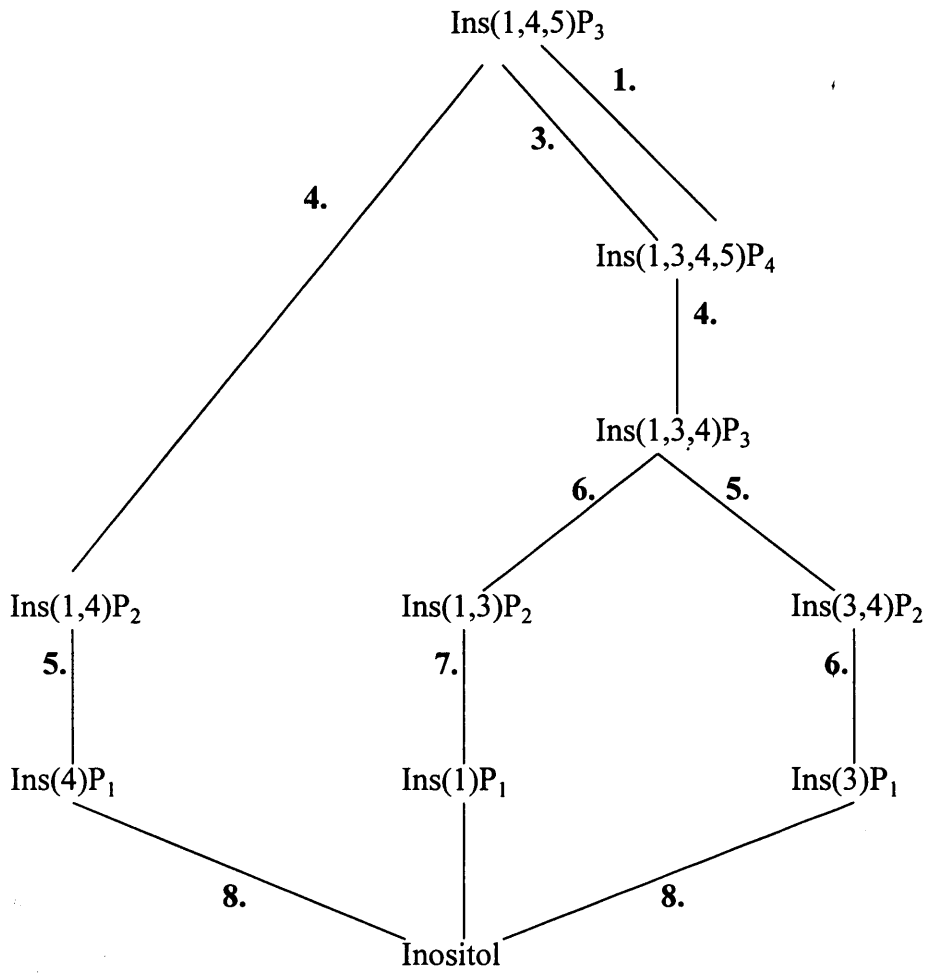


Figure 1 *Metabolism of Ins(1,4,5)P_3.*

Recycling of inositol from Ins(1,4,5)P_3. **1** = *3-kinase;* **2** = *5-kinase;* **3** = *3-phosphatase;* **4** = *5-phosphatase;* **5** = *1-phosphatase;* **6** = *4-phosphatase;* **7** = *3-phosphatase;* **8** = *monophosphatase.*

It may be of importance to note that PtdIns(4,5)P_2 is a substrate, not only for activated PIC, but also for phophoinositide 3-kinase (PI 3-kinase) activity. The action of PI 3-kinase on PtdIns(4,5)P_2 results in the generation of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), the importance of which is beginning to emerge (Stephens *et al.*, 1993; Carprnter and Cantley, 1996).

Inositol-specific PIC isoforms.

As previously stated, many neurotransmitter molecules, upon binding to their cell surface receptors, activate the primary effector enzyme, PIC, which catalyses the cleavage of PtdIns(4,5)P_2 to generate Ins(1,4,5)P_3 and DAG. The phosphosinositide signalling pathway in itself is complex, one of the factors of which is that there are multiple isoforms of PIC. The PIC isoforms have been categorised into three families, PIC β, γ and δ based on amino acid sequence homology (Lee and Rhee, 1995). The overall sequence homology is low, but two regions designated X (approximately 70 amino acids) and Y (approximately 240 amino acids) are highly conserved and thought to represent the catalytic site of the enzymes (Lee and Rhee, 1995). It is of importance to note that there are two general mechanisms by which agonists activate PIC. The first involves direct activation of PIC through G-proteins. The predominant pathway is through the Gq/11 family, although G-proteins of the Gi and Go families may also activate PIC through their βγ subunits. The second involves growth factors, cytokines and other agents that activate receptors with intrinsic tyrosine kinase activity (Lee and Rhee, 1995; Exton, 1996). The latter is likely to be of less consequence in neuronal function than the former except, perhaps, in the development of the nervous system. Differential PIC isoforms tend to be activated by the different mechanisms.

The PICβ family contains four members, PICβ1-β4. The α subunits of the Gq/11 family of G-proteins are specific activators of the β-type PIC isozymes (Taylor *et al.*, 1991; Smrcka *et al.*, 1991). The order of PIC isozymes responsiveness to activated Gq/11 is β1>β3>β4>β2 (Jhon *et al.*, 1993; Lee and Rhee, 1995). PIC β isoforms can also be activated by G-protein βγ subunits liberated from Gi or Go G-proteins. However, the rank order of the responsiveness of the isozymes to Gβγ is β3>β2>β1, which differs from that for activation by Gαq/11. PICβ4 is not affected by the βγ complex (Lee *et al.*, 1994; Jiang *et al.*, 1994). Activation of PICβ by βγ subunits occurs with 10-100 fold lower potency than activation by Gαq/11 (Park *et al.*, 1993, Blank *et al.*, 1993). It is therefore likely that activation of PICβ Gβγ requires a high concentration of βγ subunits which could only be brought about by G-proteins which exist in abundance. In the brain, the majority of G-proteins are of the Gi or Go class and it follows that activation of receptors linked to these G-proteins in neurones may well result in

the release of a high concentration of βγ subunits. Thus, receptor-mediated activation of PIC-β1 (Gαq/11), PIC-β2 (Gi/Go) and PIC-β3 (Gi/Go) under various stimuli, are all viable signalling pathways in neuronal cells.

The PIC-γ family comprises PIC-γ1 and PIC-γ2. PIC-γ1 is widely distributed in many tissues, whereas PICγ2 predominates in the spleen, B cells and lung (Fain, 1990). Activation of PICγ isoforms appears to be as a consequence of its phosphorylation by the intrinsic tyrosine kinase activity of a variety of growth factor receptors, independently of a G-protein (Hasegawa-Sasaki et al., 1988). It may be hypothesised, therefore, that activation of PIC-γ1 may play a role in neural development. Less is known about the PIC-δ isoforms (δ1 and δ2) although this form has recently been crystallised, revealing that Ca^{2+} is very likely required for its activation (Essen *et al.*, 1996).

1.4.2 Regulation of receptor-mediated phosphoinositide hydrolysis.

The mechanisms by which receptor-mediated phosphoinositide hydrolysis is regulated in a host of different cell types is, in itself, a huge area of current research. Known and potential regulatory mechanisms include acute desensitisation of receptor-mediated phosphoinositide hydrolysis, receptor internalisation and down-regulation, receptor phosphorylation by homologous and heterologous mechanisms, regulation of G-protein levels and G-protein activity, regulation of PIC by PKC and regulation of PIC by Ca^{2+}. For an extensive review of recent advances regarding regulatory mechanisms associated with receptor-mediated phosphoinositide hydrolysis the reader is referred to a recent publication by Tobin (1996). Within the scope of the current study is the regulation of receptor-mediated phosphoinositide metabolism by PKC and by Ca^{2+}. An overview of the evidence for regulation by these mechanisms is presented below.

Regulation of PIC by PKC.

Activation of PIC results, not only in the formation of Ins(1,4,5)P_3, but also in the generation of DAG which subsequently activates PKC. There is considerable evidence to suggest that PKC activation can attenuate PIC activity. For example, acute exposure of a variety of receptors expressed in different cell types to phorbol esters, which act to directly activate PKC, results in an attenuation of phosphoinositide hydrolysis following agonist stimulation (Sugiya and Putney, 1988; Boarder and Challiss, 1992; Hepler *et al.*, 1988; Oranella *et al.*, 1987; Kopp *et al.*, 1990). Furthermore, the inhibition of PKC using specific PKC inhibitors potentiates agonist-mediated PIC activation in a number of cell types (Boarder and Challiss, 1992; Challiss *et al.*, 1993). Consistent with the studies above, down-regulation of PKC with chronic exposure of cells to phorbol ester prior to agonist addition results in the potentiation of receptor-stimulated PIC activation (Brown *et al.*, 1987; Pfeilschifter *et al.*, 1989; Lin *et al.*, 1990; Lin and Chuang, 1992). This aspect of regulation is further discussed in chapter 5.

Regulation of PIC by Ca^{2+}.

In addition to its dependence on Ca^{2+}, PIC activity in some tissues may also be regulated by cytosolic Ca^{2+} concentrations. Historically this role was first suggested by observations that Ca^{2+} influx mediated by Ca^{2+} ionophores or K^+-depolarization, may initiate or enhance phosphoinositide hydrolysis (Kendall and Nahorski, 1984; Kendall and Nahorski, 1985; Baird and Nahorski, 1989; Eberhard and Holz, 1988; Del Rio *et al.*, 1994). Other studies have also revealed that receptor-mediated phosphoinositide hydrolysis in permeabilized cells can be modulated by extracellular Ca^{2+} concentrations (Taylor *et al.*, 1986; McDonough *et al.*, 1988). In addition, in a number of cell lines the sustained phase of Ins(1,4,5)P_3 generation has been shown to be reduced by the removal of extracellular Ca^{2+} (Arias-Montano *et al.*, 1994; Challiss *et al.*, 1994) suggesting at least a facilitation of PIC activity by the influx of extracellular Ca^{2+}. However, it should be noted that manipulation of the intracellular or extracellular Ca^{2+} concentration also has the potential to influence the release of neurotransmitters, particularly when neuronal tissues are employed. It is possible therefore that the observed effects were indirect through the alteration of release of other neurotransmitters (agonists) which activate PIC. However, the Ca^{2+} ionophore,

ionomycin, elevated Ca^{2+} and increased inositol phosphate accumulation in SK-N-SH cells, which have little or no capacity for neurotransmitter release (Fisher *et al.*, 1988).

The methods employed to obtain the results described above involved the measurement of total inositol phosphates, which are largely indicative of sustained PIC activation and only provide evidence for Ca^{2+}-regulation of the later phase of $Ins(1,4,5)P_3$ production. The effect of Ca^{2+} on the rapid transient phase of $Ins(1,4,5)P_3$ accumulation is less clear. Due to the large release of Ca^{2+} from intracellular stores during the initial PIC activation, it is difficult to determine how Ca^{2+} effects the peak phase of $Ins(1,4,5)P_3$ generation since it is possible that released Ca^{2+} is itself able to facilitate the activity of PIC. However, depletion of intracellular stores with thapsigargin prior to agonist challenge, thus abolishing the ability of agonists of PIC-linked receptors to elevate $[Ca^{2+}]_i$, has been shown to diminish the peak phase of $Ins(1,4,5)P_3$ generation (Wojcikiewicz *et al.*, 1994; Willars and Nahorski, 1995). This indicates that Ca^{2+} may also regulate the peak phase of $Ins(1,4,5)P_3$ production.

Recent studies have sought to examine the influence of Ca^{2+} on specific PIC isoforms. Regulation of the PIC-β isozymes by Ca^{2+} has been demonstrated in reconstituted systems, where submicromolar concentrations of Ca^{2+} have been shown to directly activate purified preparations of PIC-β isozymes (Taylor *et al.*, 1991; Smrcka *et al.*, 1991; Park *et al.*, 1993; Camps *et al.*, 1992). Ca^{2+} was also shown to enhance βγ (Park *et al.*, 1993) and $G\alpha_{q/11}$ (Smrcka *et al.*, 1991; Taylor *et al.*, 1991) stimulated PIC-β activity. The ability of Ca^{2+} to activate PIC isoforms other than PIC-β has also been investigated. PIC-δ is activated by Ca^{2+} but is not subject to regulation by G-proteins in the manner of PIC-β isoforms (Rhee and Choi, 1992) or by tyrosine phosphorylation in the manner of PIC-γ isoforms (Cockcroft and Thomas, 1992). Thus it is possible that PIC-δ isoforms are regulated primarily by Ca^{2+}, and the presence of an EF-hand motif would support this theory (Bairoch and Cox, 1990). PIC-γ isoforms are also subject to regulation by Ca^{2+}, and although PIC-β and PIC-δ hydrolyse more substrate than PIC-γ following Ca^{2+} activation, the proportional increases may be similar (Ryu *et al.*, 1987; Hepler *et al.*, 1993). This aspect of regulation is discussed further in chapter 6.

1.4.3 Ins(1,4,5)P_3-induced Ca^{2+} release.

Ins(1,4,5)P_3 receptors.

Ca^{2+} is released from intracellular stores following interaction of Ins(1,4,5)P_3 with the Ins(1,4,5)P_3 receptor/Ca^{2+} release channel complex located on the stores. There are at least three forms of the Ins(1,4,5)P_3 receptor (Ins(1,4,5)P_3R), termed Ins(1,4,5)P_3 R1-R3, of which the complete cDNA sequences have been determined (Furuichi *et al.*, 1994; Taylor and Traynor, 1995). Partial sequences, however, have also been determined for potential fourth and fifth types of Ins(1,4,5)P_3 receptor (Ross *et al.*, 1992; De Smedt *et al.*, 1994). Within this family, Ins(1,4,5)P_3R1 is the best characterised (Mikoshiba, 1993; Katayama *et al.*, 1996) and is often referred to as the 'neuronal Ins(1,4,5)P_3R' since Ins(1,4,5)P_3R1 is richly expressed and widely distributed in the mammalian brain, with highest expression in the cerebellar Purkinjie cells where it is present in the cell body, axon and dendrites (Furuichi *et al.*, 1993, 1994).

The structure of Ins(1,4,5)P_3R1, based on its amino acid sequence, has revealed that it can be structurally divided into three parts. First, the N-terminal arm which is located in the cytoplasm and possess the Ins(1,4,5)P_3 binding activity with a 'core' Ins(1,4,5)P_3 binding domain (amino acids 225-575) (Mignery and Sudhof, 1990; Yoshikawa *et al.*, 1996). Second, six putative (e.r.) membrane-spanning domains, and third a C-terminal tail. The middle section (on the N-terminal arm) of Ins(1,4,5)P_3 R1 contains modulatory sites for various agents, and possibly also acts as a transducing domain following Ins(1,4,5)P_3 binding (Furuichi and Mikoshiba, 1995 and references therein). The putative six membrane-spanning domains are believed to form the pore of the Ca^{2+} channel complex (Michikawa *et al.*, 1994). Ins(1,4,5)P_3R/Ca^{2+} channel release complexes were initially hypothesised to cluster in groups of four forming a tetramer (Maeda *et al.*, 1991), which has recently gained increasing support ((Mankawa *et al.*, 1995; Wojcikiewicz and He, 1995; Katayama *et al.*, 1996). The Ins(1,4,5)P3 receptors may either be homotetrameric or heterotetrameric assemblies of large, non-covalently bound, subunits (Hirota *et al.*, 1995). The transmembrane spanning domains are reportedly required for formation of the tetramer, and it is also a possibility that the C-terminal tail is also required (Miyawaki *et al.*, 1991).

Regulation of Ins(1,4,5)P_3-evoked Ca^{2+} release.

The function of the neuronal Ins(1,4,5)P_3R is subject to regulation by a range of other effector pathways. These include PKA, PKC, PKG, CaM kinase II and $[Ca^{2+}]_i$ (Furuichi and Mikoshiba, 1995 and references therein). The regulation of Ins(1,4,5)P_3 receptor function in neurones is very likely to affect Ca^{2+} release in response to Ins(1,4,5)P_3 generation. Thus, the coincident stimulation of receptors resulting in phosphoinositide turnover and those resulting in either cAMP accumulation, cGMP generation or Ca^{2+} influx will probably result in cross-talk between the diverse pathways.

As stated above, the middle section of Ins(1,4,5)P_3R1 contains binding sites for various modulators. Neuronal Ins(1,4,5)P_3Rs have been demonstrated to be phosphorylated by PKA and PKG at the same site (Komalavilas and Lincon, 1994). PKA has been shown to inhibit Ins(1,4,5)P_3-mediated Ca^{2+} release (Supattapone *et al.*, 1988), and therefore, by extrapolation, it may be hypothesised that the inhibition of $[Ca^{2+}]_i$ observed following elevation of cGMP in many cells (including neurones) (see section 1.3.2) may be attributable to or contributed to by PKG-phosphorylation of Ins(1,4,5)P_3 receptors. However, PKA-mediated phosphorylation of Ins(1,4,5)P_3R1 has also been reported to enhance Ins(1,4,5)P_3-evoked Ca^{2+} release in a reconstituted system (Nakade *et al.*, 1994).

Regulation of Ins(1,4,5)P_3Rs by Ca^{2+} appears to differ between the isoforms (Yameshima *et al.*, 1997). Since Ins(1,4,5)P_3R1 predominates in neurones, only the regulation of Ins(1,4,5)P_3R1 is considered in this section. Ins(1,4,5)P_3 was always presumed the endogenous agonist for this receptor since the channel only opens in the presence of Ins(1,4,5)P_3 (Ehrlich *et al.*, 1994). It has been proposed, however, that it is Ca^{2+} that actually stimulates the channel to open under physiological conditions. Evidence in support of this theory is as follows. First, the dependence of Ins(1,4,5)P_3 receptor activity on $[Ca^{2+}]$ is biphasic. Ins(1,4,5)P_3-induced Ca^{2+} release is increased with increasing $[Ca^{2+}]$ until approximately 300nM $[Ca^{2+}]$, further elevations decreasing the sensitivity of the receptor to Ins(1,4,5)P_3 (Bezprozvanny *et al.*, 1991; Taylor and Richardson, 1994). Second, Ca^{2+} oscillations can be manipulated by overexpression of e.r. Ca^{2+}-ATPase (Camacho and Lechleiter, 1993). Third, basal levels of Ins(1,4,5)P_3 in many cells are in the concentration-range sufficient to mediate Ca^{2+} release (Challiss *et al.*, 1990; Zhao *et al.*, 1990). An

alternative to this theory is that Ins(1,4,5)P_3 is the endogenous agonist, but with subtler action than proposed initially. Thus, Ca^{2+} release via opening of the Ins(1,4,5)P_3R release channel depends on the presence of both Ins(1,4,5)P_3 and Ca^{2+} (Berridge, 1997). It is generally believed that Ins(1,4,5)P_3 binds to its receptor in a co-operative manner, and there is evidence that at least three and possibly all four subunits of the Ins(1,4,5)P_3R must bind Ins(1,4,5)P_3 to initiate opening of the channel (Marchant and Taylor, 1997). It has also been suggested recently that Ca^{2+} and Ins(1,4,5)P_3 have a mutually co-operative action on the Ins(1,4,5)P_3 receptor in that increases in $[Ca^{2+}]_i$ shorten the interval between Ins(1,4,5)P_3 binding to its receptor and channel opening (Marchant and Taylor, 1997). Thus, when Ins(1,4,5)P_3 binding is complete, it is the binding of Ca^{2+} that limits the rate of channel opening, and hence provides a safeguard against spontaneous and inappropriate channel activation.

It is considered a strong possibility that the $[Ca^{2+}]$ inside the e.r. (as opposed to cytoplasmic $[Ca^{2+}]$) is also able to regulate Ins(1,4,5)P_3-mediated Ca^{2+} release. This possibility was initially proposed in order to account for the phenomenon of quantal Ca^{2+} release (QCR) (Irvine, 1990, 1991). This aspect of Ca^{2+} signalling is discussed further in chapter 5. How sequestered Ca^{2+} influences Ins(1,4,5)P_3R function remains unclear, but regardless of the mechanism, there is good evidence that a high concentration of sequestered Ca^{2+} helps to increase Ins(1,4,5)P_3R sensitivity to Ins(1,4,5)P_3 (Berridge, 1997 and references therein).

Feedback regulation by high $[Ca^{2+}]_i$ onto Ins(1,4,5)P_3Rs is thought to be a mechanism for the generation of the spatiotemporal dynamics (i.e. Ca^{2+} oscillations) of $[Ca^{2+}]_i$ observed following agonist stimulation in many cell types. However, the neuronal model utilised in the current study did not exhibit spatiotemporal $[Ca^{2+}]_i$ dynamics following agonist challenge. Therefore, this aspect of Ca^{2+} signalling is not discussed here, and the reader is referred to a number of reviews that cover $[Ca^{2+}]_i$ oscillations extensively (Berridge, 1993; Ammundson and Clapham, 1993; Li *et al.*, 1995).

1.4.4 Calcium-induced calcium release.

Ryanodine receptors.

Neuronal cells also express ryanodine receptors (RyRs), the second family of intracellular Ca^{2+} release channels (Walton *et al.*, 1991; Sharp *et al.*, 1993). RyRs mediate the phenomenon of CICR following their activation by elevated $[Ca^{2+}]_i$ (reviewed by Furuichi *et al.*, 1994; Meissner, 1994). There are three major isoforms of RyRs, RyR1 (skeletal muscle), RyR2 (cardiac muscle) and RyR3 (brain) (MacPherson and Campbell, 1993). RyR2 is the most abundant isoform in the nervous system, despite the labelling of RyR3 'brain', whereas RyR3 is predominantly localised in the hippocampus, striatum and dorsal thalamus (Lai *et al.*, 1992; Furuichi *et al.*, 1994).

All three isoforms possess three functional domains, the transmembrane domain which is generally regarded to form the channel pore, a large N-terminal domain and an intermediate modulatory region. Thus, the RyR would appear to be functionally homologous to $Ins(1,4,5)P_3R1$, although mechanistically these two channel types are quite different. CICR has been demonstrated to occur in isolated sympathetic and sensory neurones (Friel and Tsien, 1992; Schmigol *et al.*, 1995) and in isolated CNS neurones (Bleakman *et al.*, 1993; Schmigol *et al.*, 1994). These above studies utilised caffeine, which directly interacts with RyRs, lowering their threshold for $[Ca^{2+}]i$ so CICR can occur at basal $[Ca^{2+}]_i$ (Sitsapesan and Williams, 1990). Hence, CICR is very likely to represent an important signalling mechanism in neuronal function. One suggested role of CICR in neuronal function is in LTP. This theory comes from observations that LTP can be blocked by a suppresser of CICR, dantrolene, or by e.r. store depletion with thapsigargin (Obenaus *et al.*, 1989; Harvey and Collingridge, 1992).

Activation of CICR.

Cellular depolarisation can activate CICR in certain neuronal preparations (Friel and Tsien, 1992; Llano *et al.*, 1994). The factors that appear to be of importance in determining the extent of CICR following depolarising stimuli are the duration and amplitude of cellular depolarisation (Kuba *et al.*, 1992; Barish, 1991) and the type of neuronal preparation selected

for experimentation (Holliday *et al.*, 1991; Kocsis *et al.*, 1994). Usually a larger amount of Ca^{2+} influx (or 'trigger Ca^{2+}') is required to initiate CICR in neurones compared with skeletal and cardiac muscle cells, which is likely to reflect differences in physiological significance of CICR in different tissues. In muscle cells, CICR is required for coupling between excitation and contraction, whereas in neurones CICR is probably designed for amplification of Ca^{2+} influx. CICR is also reported to be activated following activation of certain Ca^{2+} permeant ligand-gated ion channels. For example, CICR has been shown to be involved in $[Ca^{2+}]_i$ signalling produced by activation of NMDA receptors (Simpson *et al.*, 1993).

A second messenger molecule that has been gaining increasing attention is cyclic ADP ribose (cADPr). There is now a volume of convincing evidence to suggest that cADPr is an endogenous activator of RyRs (Galione *et al.*, 1991; Galione *et al.*, 1993; Willmott *et al.*, 1996). This action of cADPr was first reported on sea urchin oocytes and appears to play an important role in other cell types, including neurones (Hua *et al.*, 1994; Chini *et al.*, 1995). Until more recently, it was still not clear whether cADPr acts as the direct trigger and/or as a modulatory factor of RyR-mediated Ca^{2+} release. Another question is whether cADPr can activate all RyR subtypes. The activation of RyR2 seems likely, whereas the evidence for RyR1 and RyR3 is inconclusive (Sitsapesan *et al.*, 1995). cADPr is a nicotineamide adenine dinucleotide (NAD^+) metabolite, whose synthesis is stimulated by the cGMP-PKG pathway. This mechanism provides another point for cross-talk between distinct signalling pathways. Indeed, the complexity of the interactions between the Ca^{2+} and cGMP-PKG signalling pathways, mentioned at various intervals throughout this chapter, is quite large, with potentially multiple sites for interaction between the two pathways.

It may be of importance to note that Ins(1,4,5)P_3-mediated Ca^{2+} release and CICR are not necessarily two separate phenomena. For example, Ca^{2+} release mediated via the Ins(1,4,5)P_3Rs could evoke further release of Ca^{2+} through CICR via RyRs. Whether such interaction exists under physiological conditions, and the consequences of such interaction is a subject of controversy (Simpson *et al.*, 1995). Also, since the Ins(1,4,5)P_3 receptors are themselves sensitive to $[Ca^{2+}]_i$, CICR may occur at Ins(1,4,5)P_3Rs in addition to RyRs, thus providing an additional mechanism for ionotropic-metabotropic interactions.

1.4.5 Capacitative Ca^{2+} entry.

Ca^{2+} influx is regulated by the filling state of intracellular Ca^{2+} stores in many cells, i.e. when stores are depleted, Ca^{2+} entry through the plasma membrane occurs, and when stores are full Ca^{2+} entry via this pathway is inhibited. This phenomenon has been termed 'capacitative Ca^{2+} entry' and it is hypothesised that Ca^{2+} release is in some way coupled to Ca^{2+} entry (Putney, 1990). That Ca^{2+} entry of this nature is controlled by the state of filling of the Ca^{2+} pools was demonstrated using thapsigargin (depletes Ca^{2+} pools via inhibition of store Ca^{2+}-ATPase), which induces Ca^{2+} influx in a degree similar to that of PIC activation in a variety of different cell types (Thomas and Hanley, 1994). The channel through which capacitative Ca^{2+} entry occurs has been labelled the Ca^{2+} release-activated Ca^{2+} (CRAC) channel (Hoth and Penner, 1992). With an analogy to the regulation of Ins(1,4,5)P_3R1 by Ca^{2+}, capacitative Ca^{2+} entry is regulated in a biphasic manner by Ca^{2+} (Zweifach and Lewis, 1995). The CRAC channel, whilst often referred to in the literature, remains to be proven in practice. In fact, the exact route of Ca^{2+} entry is unknown, and there are theories which dispute the existence of a CRAC channel at all (Bode and Nelter, 1996). Studies in Drosophila have led to the hypothesis that a protein encoded in the 'transient receptor potential' (*Trp*) gene product, and a related, *Trp*-like (*Trpl*) protein, may function as capacitative Ca^{2+} entry channels (Hardie, 1996). Functional expression of cloned *Trp* (types 1,3,4 and 6) in expression systems results in enhancement of both agonist-stimulated Ca^{2+} entry and of Ins(1,4,5)P_3-stimulated inward currents that are, at least in part, carried by Ca^{2+} (Zhu *et al.*, 1996; Philipp *et al.*, 1996; Birnbaumer *et al.*, 1996; Zitt *et al.*, 1996). In addition, when expressed in insect Sf9 cells, *Trpl* forms a Ca^{2+} permeant non-selective cation channel activated by Ins(1,4,5)P_3 (Hu *et al.*, 1994; Dong *et al.*, 1995). Hence, convincing evidence is accumulating that, at least in a number of systems, *Trp* and *Trpl*, are likely to form plasma membrane channels associated with capacitative Ca^{2+} entry.

Capacitative Ca^{2+} entry plays an essential role in Ca^{2+} signalling, especially under conditions where Ca^{2+} stores are generating Ca^{2+} oscillations since it serves to replenish stores. The precise role of capacitative Ca^{2+} entry in neurones is unclear. However, where Ca^{2+} release mechanisms play a role in neuronal function, this mechanism of Ca^{2+} influx is likely to represent an important element of cellular machinery. In order to explain capacitative Ca^{2+} entry, a number of models have been proposed. The various models are outlined and discussed in a detailed review by Berridge (1995). The two more prominent theories are

(briefly) as follows. The first involves the release of a diffusible molecule, postulated to be released following store depletion, subsequently stimulating Ca^{2+} influx through plasma membrane Ca^{2+} channels (Ca^{2+} influx factor (CIF) model). The second is the hypothesis that intracellular Ca^{2+} release channels conformationally couple to plasma membrane Ca^{2+} channels (conformational coupling model).

The CIF model postulates that CIF is a small molecule, stored in the intracellular stores that is released following their depletion, and is proposed to modulate the hypothetical CRAC channel function by phosphorylation (Randriamampita and Tsien, 1993). Inactivation of this mechanism of Ca^{2+} entry has been suggested to be via activation of an enzymatic cascade that degrades CIF in a Ca^{2+}-dependent manner. A CIF-like factor has been isolated from T-cells and shown to regulate Ins(1,4,5)P_3-induced membrane currents in Xenopus oocytes (Thomas *et al.*, 1996). However, the CIF model will remain controversial (Bird *et al.*, 1995) until the complete purification of CIF and confirmation of its action. The conformational coupling model hypothesises that the Ca^{2+} entry channels (CRACs?) are coupled to Ins(1,4,5)P_3Rs (Berridge, 1990; Irvine, 1990). A metabolite of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, has been proposed to play a role in this mechanism (Irvine, 1990) as has store emptying (Berridge, 1990). More recently, a proposal has been outlined where Ins(1,4,5)P_3Rs communicate with the Ca^{2+} entry channels, possibly directly through protein-protein interactions causing a conformational change in the plasma membrane Ca^{2+} channels, leading to their opening and Ca^{2+} influx (Irvine, 1992; Petersen and Berridge, 1994).

1.5 Use of the SH-SY5Y Cell Line

In order to further the current understanding of the regulational of functional events which occur within the nervous system, preparations such as mammalian brain slices and synaptosomes have often been employed. Whilst providing convenient models in which to study receptor-mediated regulation of neuronal processes, such as neurotransmitter release, these preparations are less suitable for studies on the second messenger cascades initiated following receptor stimulation. This is primarily due to the heterogeneous nature of the cell populations within the tissue which will contain, in addition to neuronal cells, glial and other such non-neuronal cells. These cells are likely to have mixed receptor populations, and

therefore, activation of one receptor type may cause the release of neurotransmitter(s), thus activating different receptor types. As a result, the interpretation of functional data obtained from such preparations is extraordinarily complex. The use of neuronal cell cultures has overcome some of these difficulties and many groups have utilised primary cultures of neurones from rat cerebellum and cortex to examine regulatory aspects of second messenger signalling. However, due to the difficulties associated with the culture of such preparations (contamination etc.) (see McCarthy and DeVellis, 1980; Gallo *et al.*, 1982) many groups have moved to the use of clonal neuroblastoma cell lines.

Neuroblastoma cell lines, defined as cells derived from the neural crest (Prasad, 1975), are often used to serve as model homogeneous populations of cells expressing many of the properties of neuronal cells, and are referred to as being 'neuronal-like'. These cells are derived from neoplastic tissues and are considered to be immortal. As such, neuroblastoma cells can be cultured continuously *in vitro* and are referred to as cell lines.

1.5.1 The human neuroblastoma SH-SY5Y.

The human neuroblastoma cell line, SH-SY5Y, has been utilised as a model of a neuronal cell in which to study certain regulatory aspects of phosphoinositide signalling throughout this study. SH-SY5Y cells are descended from the parent cell line, SK-N-SH, which was established in cell culture from human metastatic neuroblastoma tissue taken from the bone marrow of a four-year-old leukaemia patient (Biedler *et al.*, 1973). Although many important studies have been undertaken using the parent cell line, one major drawback is that the SK-N-SH cell line has been shown to contain cells expressing three different phenotypes, namely: the neuroblastic N-type cells which are small, rounded and loosely adherent with numerous small neuritic processes; the epithelial/fibroblastic S-type cells with a larger, flattened, highly adherent morphology; and the intermediate I-type cells (Ciccarone *et al.*, 1989). The SH-SY5Y cell line is a thrice subcloned cell line of the N-type cells from the parent SK-N-SH cell line (Ross and Biedler, 1985).

The neuronal origin of the SH-SY5Y cell line was initially established by the presence of a high activity of dopamine-β-hydroxylase (DβH), an enzyme unique to neuronal tissue (Ross

et al., 1983). Since then SH-SY5Y cells have been shown to express many properties of mature sympathetic neurones, in particular the synthesis of noradrenaline (Pahlman *et al.*, 1990), and morphologically the cells resemble human foetal sympathetic ganglion cells (Ross *et al.*, 1983 and references therein). Thus, it is believed that these cells can act as a suitable model for sympathetic ganglia. In addition, the cell membranes of SH-SY5Y cells possess Gα subunits in proportions similar to those found in human frontal cortex, which would suggest that these cells also provide a more suitable model of central neurones than the other commonly used cell lines (Klinz *et al.*, 1987).

The PIC-catalysed hydrolysis of membrane phospholipids is one of the major routes by which intracellular messengers are generated in neuronal tissue in response to extracellular stimuli (for reviews see Nahorski *et al.*, 1986; Fisher and Agranoff, 1987). In this manner, interaction of neurotransmitters with their cell surface receptors can rapidly elevate intracellular levels of $Ins(1,4,5)P_3$ and DAG which, by virtue of their ability to release intracellularly stored Ca^{2+} and to activate PKC respectively, can alter cellular properties and function (Berridge, 1993). In recent years the SH-SY5Y cell line has been used extensively to investigate components of phosphoinositide signalling and their regulation in a neuronal-like cell (reviewed by Lambert and Nahorski, 1990; Vaughan *et al.*, 1995). These cells express muscarinic receptors, predominantly (Lambert et al., 1989) although probably not exclusively (Wall et al., 1991) of the M3 subtype. Agonist stimulation of muscarinic M3 receptors stimulates phosphoinositide hydrolysis (Caulfield, 1993) which, in these cells, seems to be via a pertussis toxin-insensitive G-protein (Lambert and Nahorski, 1990b). SH-SY5Y cells also express a bradykinin receptor (likely to be B2) linked to the activation of PIC (Willars and Nahorski, 1995). The expression of two different PIC-linked receptors in the same cell renders the SH-SY5Y cell line a useful model in which to study the receptor specificity of the phosphoinositide pathway, i.e. the possible different regulatory mechanisms following the activation of distinct receptor types G-protein-linked to the same (phosphoinositide) pathway.

In addition to the expression of receptors linked to the activation of PIC, SH-SY5Y cells also express signalling components which, whilst not directly involved in the phosphoinositide signalling pathway, may play important roles, upon activation, in the way in which the pathway is regulated. The regulation of the phosphoinositide pathway following its activation by agonist is, as previously discussed, likely to play a vital part in determining the cellular

response elicited and therefore the efficacy of an agonist-receptor interaction. For instance, the existence of VOCCs, which provide a major mechanism for Ca^{2+} entry, have been demonstrated in SH-SY5Y cells (Lambert *et al.*, 1990; Morton *et al.*, 1992; Reeve *et al.*, 1994). Since depolarisation has been reported to enhance receptor-mediated PIC activation (see section 1.4.3), depolarising stimuli may effect receptor stimulated phophoinositide hydrolysis in SH-SY5Y cells. In addition, SH-SY5Y cells express nicotinic acetylcholine receptors (Vaughan *et al.*, 1993; Purkiss *et al.*, 1995), a receptor consisting of a Na^+ channel which opens upon activation to allow Na^+ diffusion into the cell thus depolarising the plasma membrane. This in turn leads to the opening of VOCCs and the subsequent influx of Ca^{2+}. The co-expression of muscarinic acetylcholine (metabotropic) receptors and nicotinic acetylcholine (ionotropic) receptors on the cell surface of the SH-SY5Y cell line renders these cells a convenient model in which to examine potential interaction and integration of signals elicited following the activation of metabotropic and ionotropic receptors simultaneously by a single agonist. In addition to receptors linked to the activation of PIC, SH-SY5Y cells express a number of other G-protein linked receptors, whose function is to inhibit adenylate cyclase activity, including μ- and δ-opioid receptors (Yu *et al.*, 1986) and α2-adrenoceptors (Kazmi and Mishra, 1989). It has recently been demonstrated that both μ- and δ-opioid receptors, normally linked to the inhibition of adenylate cyclase (via Gi), can interact with muscarinic cholinergic systems at the level of PIC in SH-SY5Y cells (Smart *et al.*, 1995; Connor and Henderson, 1996). Interactions between, or integration of, the signal transduction pathways activated by different PIC-linked receptors and/or other plasma membrane receptors may, therefore, have cellular consequences. Such interactions may be common in an *in vivo* situation where cells are exposed to many neurotransmitters and neuromodulatory substances. The SH-SY5Y cell line therefore may be used as a model in which to study interactions between these receptors in a neuronal-like environment.

The SH-SY5Y cell line, however, has limitations as a model for the adult neurone. The major limitation is the lack of differentiation of these cells. To illustrate this fact, one must consider that mature mammalian neurones are incapable of cell division, whereas undifferentiated SH-SY5Y cells have a short dividing time. This is important since the more rapidly a cell proceeds through its cell cycle the fewer features it has in common with neurones, most likely because many of the differentiated properties of neurones are not fully articulated *in vitro* until they become post-mitotic (Temple, 1990).

Neuroblastoma cell lines in general, however, retain the capacity to differentiate into various forms of nerve cells (reviewed by Prasad, 1975). Many differentiated functions in mature neurones have been identified and measured. These include formation of neurites, an electrically excitable cell membrane, inhibition of cell division, high activities of neural enzymes associated with the synthesis or breakdown of transmitters, and increased activities of adenylate cyclase and cAMP phosphodiesterase. Undifferentiated neuroblastoma cells exhibit most of the above features, but at very low levels (Prasad, 1975). Following exposure to a variety of agents, most neuroblastoma cell lines can be induced to differentiate. That is, the neuronal functions described above become apparent or are enhanced. Since differentiated neuroblastoma cells resemble mature neurones more closely than their undifferentiated counterparts, it seems likely that the differentiated cell is a more appropriate model for studies on the functioning of the nervous system.

1.5.2 Differentiation of neuroblastoma cell lines.

Neuroblastoma cell lines have been demonstrated to undergo cellular differentiation according to the criteria listed above in response to a range of 'differentiating agents'. The current section will deal with the differentiation of neuroblastoma cells in general, the differentiation of the SH-SY5Y cell line in particular will be discussed subsequently. Following treatment with such agents, neuroblastoma cells bear a marked resemblance to mature neurones to the extent that differentiated neuroblastoma cells are now being used in neural transplants in animals to relieve the symptoms of neurological abnormalities (Prasad, 1991). Differentiating agents were first discovered in 1970 when both serum free medium (Seeds *et al.*, 1970) and 5-bromodeoxyuridine (Scubert and Jacob, 1970) were found to induce morphological differentiation (i.e. neurite extension) of murine neuroblastoma cell lines in culture. In 1971 cAMP analogues were also found to induce morphological differentiation of neuroblastoma cells (Prasad and Hsie, 1971). Since this time many agents which induce cellular differentiation have been identified. The agents more commonly used to achieve differentiation of neuroblastoma cell lines include nerve growth factor (NGF) (Reynolds and Perez-Polo 1989), dimethylsulphoxide (DMSO) (Kimhi *et al.*, 1976), phorbol ester (Heikkila

et al., 1989), ciliary neurotrophic factor (CNF) (Kalberg *et al.*, 1993) and retinoic acid (RA) (Sidell, 1982).

A number of different studies using a variety of neuroblastoma cell lines and differentiating agents have revealed that, following cellular differentiation, the expression of the components of cell signalling pathways can be altered. A widespread phenomena is a change in the expression levels of cell surface receptors. For instance, δ-opioid receptors, angiotensin II receptors and muscarinic receptors are up-regulated following differentiation of murine neuroblastoma cells, such as NG108-15 and NE1-115 cells, with RA, DMSO and NGF respectively (Beczkowska *et al.*, 1996; Seidman *et al.*, 1996; Reagan *et al.*, 1990; Vincentini *et al.*, 1985). The induction of receptors following cellular differentiation of neuroblastoma cells has also been demonstrated for glutamate, angiotensin II and dopamine receptors (Squires *et al.*, 1996; Bryson *et al.*, 1992; Farooqui, 1994). Alterations in the abundance of the Go and Gi families of G-proteins in both membrane and particulate fractions have also been associated with cellular differentiation of neuroblastoma cell lines (Brabet *et al.*, 1990; Rouot *et al.*, 1991; Li *et al.*, 1995; Ammer and Schulze, 1994. Changes in the abundance of Gq/11 following differentiation has thus far been little investigated, presumably because of its relatively low levels in cells compared with Go and Gi. In a not dissimilar manner to Go and Gi, the relative expression levels of the PKC isozymes have been reported to be altered in neuroblastoma cells following differentiation with a range of agents (Wooten *et al.*, 1992; McSwine-Kennick *et al.*, 1991; Leli *et al.*, 1993). Such alterations of signalling components following cellular differentiation could have profound effects on the signal transduction cascades following receptor activation.

1.5.3 Differentiation of the SH-SY5Y cell line.

The agents which are routinely employed for the morphological and biochemical differentiation of SH-SY5Y cells are the phorbol ester and tumour promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Pahlman *et al.*, 1981; Pahlman *et al.*, 1983), the PKC inhibitor, staurosporine (Shea and Beermann, 1991; Jalava *et al.*, 1993) and retinoic acid (Pahlman *et al.*, 1984; Yu *et al.*, 1990; Baumgartner *et al.*, 1993). SH-SY5Y cells undergo the following phenotypical changes when chronically treated with any one of the above

agents: extension of neuritic processes, inhibition of cell growth, an increased tyrosine hydroxylase activity with a concomitant increase in the concentration of noradrenaline and neurosecretory granules, an increase in the activity of neurone specific enolase and the appearance of or up-regulation of VOCCs (Pahlman *et al.*, 1984; Akerman *et al.*, 1984; Jalava *et al.*, 1992).

Differentiation of SH-SY5Y cells with TPA is also associated with a marked down-regulation of PKC activity (Heikkila *et al.*, 1989). Furthermore, treatment with the PKC inhibitor, staurosporine (Tamaoki *et al.*, 1986), also results in SH-SY5Y cell differentiation (Shea and Beermann, 1991). Hence the down-regulation or inhibition of PKC has been proposed to be correlated directly with the appearance of a differentiated phenotype (Heikkila *et al.*, 1989). TPA-mediated SH-SY5Y cell differentiation has been reported, by a number of groups, to depress muscarinic receptor-mediated signal transduction in these cells. For instance, a decrease in carbachol-stimulated phosphoinositide hydrolysis and Ca^{2+} mobilisation with a concomitant decrease in the density of muscarinic receptors has been reported following chronic TPA treatment of these cells (Cioffi and Fisher, 1990; Akerman and Heikkila, 1990). However, differentiation of SH-SY5Y cells with either TPA or staurosporine, whilst providing useful information regarding the mechanisms involved in the differentiation process may be unsuitable methods for investigations into the effects, if any, differentiation *per se* has on the phosphoinositide signalling pathway in these cells. The reasons for this are that the chronic treatment with TPA required for cellular differentiation of SH-SY5Y cells results in the down-regulation of PKC (Heikkila et al., 1989). Since the activation of PKC has been demonstrated to influence PIC activity in a number of cells (see section 1.4.3), including SH-SY5Y cells (Willars *et al.*, 1996), the effects of TPA-induced differentiation on phosphoinositide and Ca2+ signalling would be difficult to distinguish from the effects of PKC down-regulation on the pathway.

Retinoic acid, a natural derivative of vitamin A (Haussler *et al.*, 1983) is known to be a potent regulator of growth and differentiation of different types of cultured cells. Moreover, RA is thought to be a natural morphogen involved in directing pattern formation during vertebrate development (Durston *et al.*, 1989; Wagner *et al.*, 1990). The actions of RA are mediated by nuclear receptors, which belong to the steroid hormone receptors superfamily, and act as ligand-inducible transcription factors (reviewed by Guiguere, 1994). RA receptors are

expressed in neural tissues and there is increasing evidence that RA can play an important role in neural development (Cosgaya *et al.*, 1996). Since RA appears to be involved in the differentiation of neuronal cells *in vivo*, and has been widely reported to induce the differentiation of SH-SY5Y cells in culture, RA-treatment would seem an appropriate method to employ for investigations into the effects of differentiation on the phosphoinositide signalling pathway. Thus far, however, there is little information available regarding phosphoinositide signalling and its regulation in RA-treated SH-SY5Y cells. Other signalling systems on the other hand have been examined. For instance, it has been demonstrated that opiate-inhibition of adenylate cyclase activity is enhanced following RA-differentiation of SH-SY5Y cells (Yu and Sadee, 1988). Electrophysiological studies have demonstrated that RA-treatment of SH-SY5Y cells is associated with an increased excitability of cell membranes (Kuramoto *et al.*, 1981; Seward *et al.*, 1989; Toselli *et al.*, 1995). This enhanced excitability has been demonstrated to be as a result of an increased expression of high voltage-activated, ω-conotoxin-sensitive N-type Ca^{2+} channels (Reuveny and Narahashi, 1993; Seward and Henderson, 1990; Toselli *et al.*, 1991). An important effect of RA-differentiation of SH-SY5Y cells, that potentially could have a large effect on transmembrane signalling pathways in these cells, is the associated changes in the abundance of G-proteins. Undifferentiated SH-SY5Y cells express Gs, Gi, Go and Gz α-subunits, as revealed by Western blot analysis. Following RA treatment, the levels of Gi are increased whilst the levels of Gs are decreased (Ammer and Schulze, 1994). Thus, although it is clear that there are alterations of signalling components following RA-differentiation of SH-SY5Y cells, the effects on the phosphoinositide signalling pathway remain to be elucidated.

1.6 Aims.

The aims of this study were to investigate signal transduction mechanisms produced following stimulation of the muscarinic and bradykinin receptors (G-protein-linked to the activation of PIC) expressed in the SH-SY5Y cell line, and to compare these mechanisms with those produced following receptor stimulation of RA-differentiated SH-SY5Y ($SH\text{-}SY5Y_{RA6}$) cells. Differentiated neuroblastoma cells in culture resemble mature mammalian neurones more closely than their undifferentiated counterparts which are commonly utilised for investigations into the regulation of a variety of signal transduction pathways. The principal purpose of

these studies, therefore, was to compare and contrast phosphoinositide signalling, and certain regulatory aspects of this pathway, between undifferentiated and RA-differentiated SH-SY5Y cells. It was believed that these studies may yield novel information regarding how intimately the signalling mechanisms in neuroblastoma cell lines resemble those in real neuronal cells, and where the differences, if any, are located.

In the present study, differentiated SH-SY5Y cells were established in vitro using a widely utilised protocol, that is, chronic (6 days) treatment of undifferentiated SH-SY5Y cells with RA (Pahlman *et al.*, 1984, 1990). The work in this thesis does not contain a full characterisation of the parameters of differentiation in RA-treated SH-SY5Y cells. The reasons for this are that detailed studies such as these have been performed by a number of groups and therefore, such analyses would be a time-consuming repetition of previous work in this cell line. In the current study, RA-treated cells were assumed to be differentiated, according to the parameters outlined in section 1.6.2, when the cells were observed to undergo morphological differentiation (i.e. neurite extension).

The initial aim of the current study was to conduct an investigation into how RA-differentiation of SH-SY5Y cells affected agonist-stimulated phosphoinositide and Ca^{2+} signalling. To this end, both the time- and concentration-dependency of agonist-stimulated PIC activation's and $[Ca^{2+}]_i$ elevations were determined in SH-SY5Y compared with SH-$SY5Y_{RA6}$ cells. However, the major interest throughout this thesis is how the regulation of the phosphoinositide pathway is altered, if at all, following differentiation of the SH-SY5Y cell line. As previously discussed, the extent of the agonist-stimulated activation of PIC can be influenced by a range of factors, including receptor down-regulation, receptor phosphorylation, activation of PIC and the concentration of intracellular Ca^{2+}. Differentiation of neuroblastoma cell lines, including SH-SY5Y cells, has been associated with increased VOCC density (see section 1.6.3). Hence, the regulation of basal and agonist-stimulated PIC activity following cellular depolarisation was investigated in SH-SY5Y versus SH-$SY5Y_{RA6}$ cells. Cellular differentiation of neuroblastoma cell lines has also been associated with changes in the relative expression levels of the PKC isoforms, which could have cellular consequences (see section 1.6.2). Thus, PKC-mediated regulation of agonist-stimulated PIC activation was also examined in SH-SY5Y compared with SH-$SY5Y_{RA6}$ cells.

Finally, analysis of the concentration-dependency of agonist-mediated PIC activation and $[Ca^{2+}]_i$ elevation revealed a variable relationship between $Ins(1,4,5)P_3$ generation and $[Ca^{2+}]_i$ mobilisation, which depended on the agonist and on the differentiated state of the cell. A number of possible explanations for this variability are discussed in subsequent chapters. However, one potentiality was investigated in some detail, that is, the possibility that agonist can directly sensitise the $Ins(1,4,5)P_3$ receptor/ Ca^{2+} release channel complex. This possibility was examined in undifferentiated SH-SY5Y cells by examining the influence of cell surface receptor activation on the ability of exogenous $Ins(1,4,5)P_3$ to release $^{45}Ca^{2+}$ from stores within permeabilised cells.

2. GENERAL METHODS

2.1 Cell Culture and Harvesting

Undifferentiated human neuroblastoma SH-SY5Y cells, passages 15-25, were routinely grown in 175cm^2 culture flasks containing 30ml of culture medium. Culture medium consisted of Minimum Essential Medium with Earle's Salts supplemented with 50iu/ml penicillin, 50μg/ml streptomycin, 2.5μg/ml amphotericin B (fungizone), 2mM L-glutamine, 5% (v/v) foetal calf serum (FCS) and 5% (v/v) newborn calf serum (NBCS). Chinese hamster ovary (CHO)-k1 cells and CHO-k1 cells transfected with cDNA for the human muscarinic M3 receptor and the bradykinin B2 receptor (CHO BK2/M3 cells), the human muscarinic M3 receptor only (CHO-M3 cells) and the human muscarinic M1 receptor only (CHO-M1 cells) were also grown in 175cm^2 flasks containing 30ml of culture media consisting of alpha-MEM with glutamine, supplemented with 50iu/ml penilicillin, 50μg/ml streptomycin, 2.5μg/ml fungizone and 10% (v/v) NBCS. All cultures were maintained at 37°C in 5% CO_2 / 95% humidified air. Media was replaced every two days and confluent cultures were passaged weekly, in a ratio of 1:4-12, by harvesting in sterile 10mM HEPES, 154mM NaCl and 0.54mM EDTA, pH 7.4 (harvesting buffer).

2.2 Cell Differentiation

Differentiation of SH-SY5Y cells was achieved by culturing the cells, at 70% confluence, in the presence of 10μM all-trans retinoic acid (RA) for 6 days. The culture medium was identical to that used for culturing undifferentiated cells, with the exception that both FCS and NBCS were excluded from the medium and replaced with 1% (v/v) heat-inactivated FCS. Medium was replaced on day 3 and re-supplemented with fresh RA. Stock solutions of RA (10mM) were prepared in ethanol and stored at -20°C. RA was added no sooner than 12h after the transfer of harvested cells to allow sufficient time for cell attachment.

RA treatment has been used in many studies for the differentiation of neuroblastoma cell lines. The relative merits and shortcomings of this protocol are discussed in Chapter 1. The protocol employed in the current studies produced marked morphological changes consistent with differentiation (Figure 1). For clarity, throughout this study, the cells treated for 6 days with RA will be referred to either as SH-SY5Y$_{RA6}$ cells or as differentiated cells. Undifferentiated cells will be referred to simply as SH-SY5Y cells.

2.3 Cell Preparation

In addition to morphological changes consistent with cell differentiation, RA treatment of SH-SY5Y cells resulted in a cessation of cell growth. Therefore, RA treated cells (SH-SY5Y$_{RA6}$) were generally used at subconfluence (approximately 70% by area) compared to undifferentiated (SH-SY5Y) cells which were generally used, unless otherwise stated, when 100% confluence was attained.

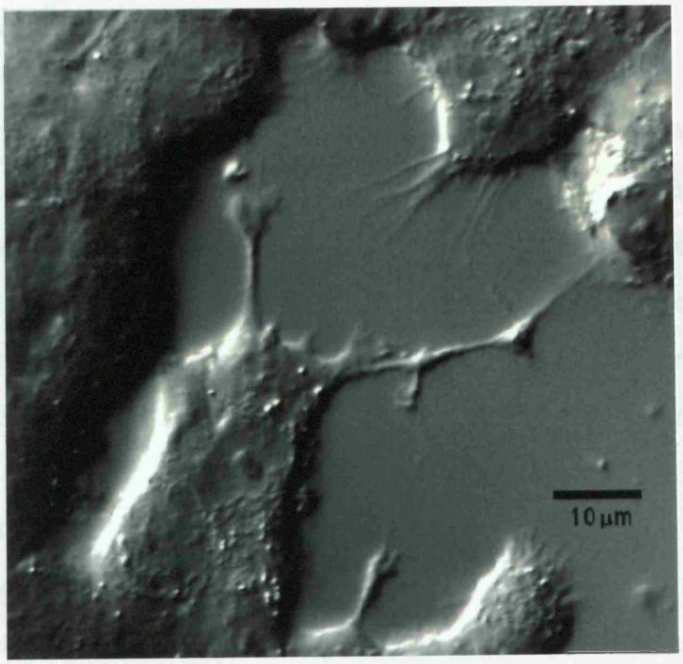
2.3.1 24 well multidishes.

Confluent cell cultures in 175cm^2 flasks were harvested and resuspended in the required amount of cell culture media. 1ml of the cell suspension was added to each well in a 24 well multidish (area of 1 well = 1.9cm^2). Multidishes were maintained at 37°C in 5% CO_2 / 95% humidified air for 24h-6 days before use, depending on the treatment.

2.3.2 Coverslips

For population intracellular Ca^{2+} concentration measurements, sterilised glass coverslips (22mm x 11mm) were placed in 8 well multidishes (1 coverslip per well). For single cell Ca^{2+} imaging, sterilised glass coverslips (20mm diameter, Chance Proper Ltd.) were placed in 35mm x 10mm culture dishes (1 coverslip per dish). Confluent cell cultures in 175cm^2 flasks were harvested and resuspended in the required amount of cell culture media and 3ml of this suspension added to each well in an 8 well multidish (to achieve 70% - 100% confluence) or culture dish (to achieve 30% - 50% confluence). Multidishes and culture dishes were maintained at 37°C in 5%CO_2 / 95% humidified air.

(A)



(B)

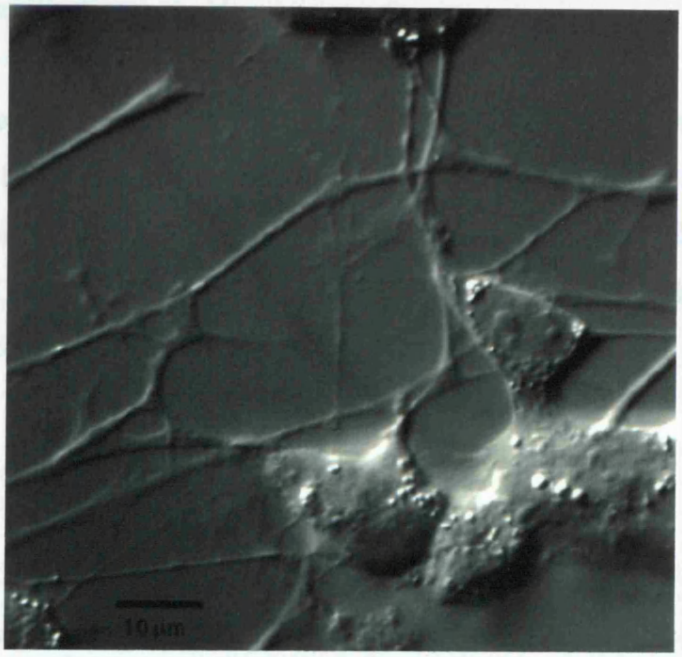


Figure 1 Photographs were taken, using differential interface contrast (Nomarski optics) with a conventional upright microscope (Zeiss Axiostep) and a water immersion lens (63 x objective), of (A) undifferentiated SH-SY5Y cells and (B) SH-SY5Y$_{RA6}$. In SH-SY5Y$_{RA6}$ cells, cell bodies are in the bottom right corner, a growth cone is apparent in the top left corner and a network of neurites is clearly visible.

2.3.3 Membrane preparation.

SH-SY5Y and SH-SY5Y$_{RA6}$ cells, grown in 175cm^2 flasks, were harvested and resuspended in ice-cold 10mM HEPES, 10mM EDTA, pH 7.4. Cells were homogenised using a polytron tissue disrupter (level 6, 4 x 5s bursts) and centrifuged at 40,000*xg* for 15min at 4°C. The resulting supernatant was discarded and the pellet resuspended in ice-cold 10mM HEPES, 0.1mM EDTA, pH 7.4 (membrane buffer). Crude membranes were re-homogenised (as above) and centrifuged at 40,000xg for a further 15min at 4°C. The resulting supernatant was discarded and the pellet resuspended in membrane buffer before a final re-homogenisation (as above). The protein concentration of the membrane suspension was determined using the Lowry method (section 2.7) and additional membrane buffer added to obtain the required protein concentration (1-2 mg/ml). Aliquots of the membrane suspension were frozen immediately by immersion in liquid nitrogen and stored at-80°C until use.

2.3.4 Cell Permeabilization.

SH-SY5Y cells were harvested and resuspended in a "cytosol-like" buffer (CLB) (see Appendix 1). Cells were centrifuged (10,000xg, 2min) and washed in 5ml ice-cold CLB per 175cm^2 flask to remove residual EDTA from the harvesting process. Cells were resuspended in 2.8ml ice-cold CLB per flask and permeabilization initiated by the addition of the detergent β-escin (final concentration of 2.5μg/ml).

2.4 Ins(1,4,5)P_3 mass assay

2.4.1 Agonist-stimulated Ins(1,4,5)P_3 generation in adherent cells.

Cells in 24 well multidishes were washed with 1ml per well of Krebs-HEPES buffer (see Appendix 2) at 37°C. Cells were incubated at 37°C prior to addition of agonist. Multidishes were maintained at 37°C, wells aspirated individually and 100μl of Krebs-HEPES buffer containing agonist at the appropriate concentration added immediately. Each data point was performed in duplicate. Reactions were terminated by the addition of 100μl of ice-cold 1M

trichloroacetic acid (TCA). For the zero time points TCA was added prior to agonist. The multidishes were maintained on ice for 15min to allow full extraction of $Ins(1,4,5)P_3$ before being prepared for the binding assay.

2.4.2 Agonist-stimulated $Ins(1,4,5)P_3$ generation in permeabilized cells.

SH-SY5Y cells were harvested, resuspended in CLB (see Appendix 1) and permeabilized with β-escin as previously described (section 2.3.4). Cell suspensions were left at room temperature for 10min followed by a 5min incubation at 37°C. At time zero, 90µl of permeabilized cell suspension was added to tubes containing 10µl of CLB or CLB containing agonist (at 10x required concentration) at 37°C. Each data point was performed in duplicate. After the required stimulation time, reactions were terminated by the addition of 100µl of ice-cold 1M TCA. For the zero time points, TCA was added prior to the cell suspension. The tubes were stored on ice for 15min, centrifuged (16,000*xg*, 1min) and returned to ice for a further 5min before being prepared for the binding assay.

2.4.3 Sample preparation.

Samples generated from experiments, in either adherent or permeabilized cell preparations, were prepared for the $Ins(1,4,5)P_3$ binding assay in an identical manner. 160µl of sample, either removed from adherent cell monolayers or from the supernatant of pelleted permeabilized cells, were transferred to microfuge tubes containing 40µl of 10mM EDTA followed by 200µl of a freshly prepared mixture of 1.1.2-trichlorotrifluoroethane and tri-n-octylamine (1:1, v/v). The resulting mixture was vortexed and left for 5min before centrifugation (16,000*xg*, 1min). After centrifugation there was a clear separation of two phases: the lower phase consisted of 1,1,2-trichlorotrifluoroethane/tri-n-octylamine plus extracted TCA; the upper aqueous phase contained inositol phosphates. 100µl of the upper aqueous phase was removed from each sample and added to tubes containing 50µl of 25mM $NaHCO_3$, which brought the pH of the extracted sample to approximately 8 (the optimal pH for $Ins(1,4,5)P_3$ binding to the $Ins(1,4,5)P_3$ receptors concentrated in the $Ins(1,4,5)P_3$ binding protein). The prepared samples were stored at -20°C prior to assay.

2.4.4 Preparation of Ins(1,4,5)P_3 binding protein.

Fresh bovine adrenal glands were de-medullated and de-capsulated on ice to obtain 60-80g of cortex. The cortex was homogenised in 8 x 50ml volumes of ice-cold 20mM $NaHCO_3$ and 1mM dithiothreitol (DTT), pH 8.0 using a Polytron tissue disrupter. The homogenate was centrifuged at 5,000*xg* for 15min at 4°C. The supernatant was removed and the pellet re-homogenised in 4 x 50ml volumes of $NaHCO_3$/DTT and re-centrifuged as above. The pooled supernatant fraction was centrifuged at 38,000*xg* for 20min at 4°C, and the 'P_2' pellet obtained was washed with $NaHCO_3$/DTT as above. The pellet was resuspended in homogenisation buffer at a protein concentration of 20mg/ml and frozen in 4ml batches at -20°C for subsequent use. This preparation has previously been described by Challiss *et al.* (1990).

2.4.5 Ins(1,4,5)P_3 binding assay.

The Ins(1,4,5)P_3 binding assay is a competition assay between unlabelled Ins(1,4,5)P_3, present in the samples or the prepared standards, and radiolabelled Ins(1,4,5)P_3. Radiolabelled and unlabelled Ins(1,4,5)P_3 compete for binding sites of the Ins(1,4,5)P_3 receptors present in the Ins(1,4,5)P_3 binding protein. The bound and the free ligand (Ins(1,4,5)P_3) can then be separated by filtration.

Assays were routinely performed in duplicate at 4°C in a total volume of 120µl as previously described by Challiss *et al.* (1990). Each assay tube contained 30µl of 100mM Tris-HCl, 4mM EDTA, pH 8.0, 30µl of D-myo-[^{3}H]-inositol 1,4,5-trisphosphate (specific activity 44 Ci/mmol, 36nCi / tube), 30µl of Ins(1,4,5)P_3 binding protein and 30µl of either neutralised sample or distilled water (dH_20) containing standard amounts of authentic, unlabelled D-myo-Ins(1,4,5)P_3 (0.036 - 12 pmol/tube, non-specific binding was determined in the presence of 10µM Ins(1,4,5)P_3). After vortexing, samples were incubated for 30min on ice before termination by filtration.

GF/B filters were predampened with ice-cold wash buffer (25mM Tris-HCl, 1mM EDTA, pH 8.0). The sample was diluted in 3ml of wash buffer and the contents of each tube filtered rapidly (Brandel Cell Harvester) to separate bound from free [^{3}H]-Ins(1,4,5)P_3. Filters were

transferred to 4ml Emulsifier Safe Scintillation Cocktail and left to extract for >12h before being counted for radioactivity by liquid scintillation spectrometry.

2.4.6 Data analysis.

A standard curve was constructed from the dpm given for the standard data using GraphPAD INPLOT (GraphPAD Software Inc., San Diego, CA) with a four parameter logistic equation. The Ins(1,4,5)P_3 concentration in the samples (in dpm) was converted to pmol/tube from the standard curve. Ins(1,4,5)P_3 mass was then converted to pmol/mg protein using protein concentrations per well deduced from protein assays (section 2.7). This value was multiplied by the dilution factor (12.5) introduced during sample preparation to give the concentration of Ins(1,4,5)P_3 generated in the experiments.

2.5 Measurement of Total [^{3}H]-Inositol Phosphates

2.5.1 Sample preparation.

Harvested cells were resuspended in culture medium containing [^{3}H]-myo-inositol (specific activity 86 Ci/mmol, 3μCi/ml). Cultures were re-seeded at 1:2 the original density in 24 well multidishes (1ml cell suspension per well). Cells were maintained at 37°C in 5% CO2 / 95% humidified air for 48h, a period previously reported to be sufficient for equilibrium to be acheived (Wojcikiewicz *et al.*, 1990; Lambert *et al.*, 1991; M. Godwyn, unpublished observations) and 100% confluence acheived. Prior to use, radiolabelled cells were washed twice in Krebs-HEPES buffer (37°C) followed by the addition of 400μl of 12.5mM LiCl in Krebs-HEPES buffer (37°C). Cells were incubated at 37°C for 15min prior to the addition of agonist. 100μl of agonist was added at the appropriate concentration (final Li^+ concentration of 10mM), and after the required stimulation time at 37°C the reaction was terminated by the addition of 500μl of ice-cold 1M TCA. Following an extraction period of 15min on ice, 800μl aliquots of sample from each well were transfered into separate polypropylene tubes and 200μl of 10mM EDTA added followed by 1ml of a freshly prepared mixture of 1,1,2-trichlorotrifluoroethane and tri-n-octylamine (1:1, v/v). After vortexing, 800μl of the upper

aqueous phase from each sample was removed and neutralised with 50µl of 250mM $NaHCO_3$. Samples were stored at 4°C prior to determination of total [^{3}H]-inositol phosphates.

2.5.2 Elution of total [^{3}H]-inositol phosphates.

Each sample was applied to a column containing 0.5ml of Dowex-1 chloride resin (8% cross-linked, 200-400 mesh). Each sample was washed with 10ml of dH_20 to remove free [^{3}H]-inositol followed by 10ml of 25mM ammonium formate to remove [^{3}H]-glycerophospho-inositol (GroPIns). Inositol phosphates, consisting of [^{3}H]-$InsP_1$, [^{3}H]-$InsP_2$, [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_4$, were then eluted with 10ml of 1M hydrochloric acid (HCl) into 20ml scintillation vials. A 5ml aliquot of the eluate from each column was counted by liquid scintillation spectrometry following addition of 15ml of Floscint Scintillation Cocktail. The columns were regenerated for further use by passing 10ml of 2M HCl through them followed by 20ml dH_20. The resin in the columns was replaced after approximately 15 uses.

2.5.3 Data analysis.

To account for differences in labelling between experiments, total [^{3}H]-inositol phosphate accumulation was expressed as % above basal accumulation.

2.6 $^{45}Ca^{2+}$ Release Assay

Cells were harvested, permeabilised and resuspended in ice-cold, cytosol-like buffer (CLB) containing 2mM ATP and 5-10µM EGTA, pH 7.2 (see section 2.3.4 and Appendix 1). The final free [Ca^{2+}] in the buffer was 90-150nM. It has previously been shown that permeabilised cell suspensions can be left on ice for a period of 1h without any effect on % $^{45}Ca^{2+}$ release induced by Ins(1,4,5)P_3 or muscarinic receptor stimulation (Burford, 1994). Intracellular Ca^{2+} stores were loaded with $^{45}Ca^{2+}$ by the addition of 0.5µCi/ml of $^{45}CaCl_2$ (1000 Ci/mmol). The suspensions were then mixed gently and left for 15min at room temperature with periodic very gentle mixing. This time period was sufficient for $^{45}Ca^{2+}$ uptake to reach equilibrium (Wojcikiewicz *et al.*, 1990; Safrany and Nahorski, 1994)).

Experimental incubations were initiated by the addition of 50µl aliquots of the permeabilised cell suspensions loaded with $^{45}Ca^{2+}$ to 50µl of drug (at 2x required concentration) or buffer in decapped, 1.5ml microfuge tubes. After a time period of 1min after the initiation of experimental incubations the tubes were microfuged (16, 000g, 2min) followed by rapid addition of 400µl of a silicone oil mixture (Dow Corning 550/556, 1:1, v/v) to each tube. The tubes were immediately re-microfuged (16, 000g, 2min) to separate cells from the supernatant. The aqueous phase and most of the silicone oil was removed carefully, without disturbing the cell pellet, by suction. The microfuge tubes containing the cell pellets were inverted for 30-60 min to remove the remaining silicone oil. 1.1ml of Emulsifier Safe Scintillation Cocktail was added to each microfuge tube which were then capped, vortex mixed, and left for 24h, allowing the radioactivity in the cell pellet to be dispersed. Radioactivity was then measured by scintillation spectrometry.

2.6.1 Data analysis.

Experiments were performed using duplicate concentrations of drug including two tubes containing buffer alone. The radioactivity in these two tubes were then taken as the total $^{45}Ca^{2+}$ content incorporated into intracellular stores. Duplicate values were averaged and compared as a % of this value to give a % of total $^{45}Ca^{2+}$ release. The amount of total $^{45}Ca^{2+}$ released by drug was expressed as a % of the total $^{45}Ca^{2+}$ released following challenge with a maximal concentration of ionomycin (5µM).

2.7 Measurement of Intracellular Ca^{2+} Concentration

2.7.1 Use of fura-2 as an indicator of intracellular Ca^{2+} concentration.

Over the past few years a series of dyes have been developed that are capable of altering their fluorescence following binding of Ca^{2+}. This has enabled their use as indicators of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Tsien, 1988). However, the $[Ca^{2+}]$ must be measured accurately without disturbing basal $[Ca^{2+}]_i$ or changes in $[Ca^{2+}]_i$ following receptor activation. In addition, Ca^{2+} indicators must be sensitive to concentration changes over a wide

range since $[Ca^{2+}]_i$ can range from 10nM to several micromolar. An indicators greatest sensitivity to Ca^{2+} occurs around the K_D value, increasing Ca^{2+} concentration beyond the K_D reduces sensitivity as the indicator reaches saturation. Therefore it is important to select an indicator with a K_D value in the same range as the $[Ca^{2+}]_i$ to be measured. Other important properties of a Ca^{2+} indicator are a rapid response to changes in Ca^{2+}, high selectivity for Ca^{2+} over other divalent cations, a high signal to noise ratio and to not alter any physiological function or influence Ca^{2+} handling by the cell.

The Ca^{2+} indicator fura-2 binds Ca^{2+} with high affinity and selectivity, has a rapid response time, a low signal to noise ratio and a K_D such that the dye saturates around 2-3μM. Binding of Ca^{2+} to fura-2 alters its fluorescence properties resulting in a shift of the fluorescence excitation spectrum such that excitation efficiency is increased at 340nm and reduced at 380nm. Peak emission occurs at 509nm. Using this dual excitation wavelength approach it is possible to calculate the properties of the dye in one of two forms (e.g. Ca^{2+} bound versus Ca^{2+} free) from the ratio of fluoresence measured at the two wavelengths. This then becomes independent of dye distribution or concentration (see Renard *et al.*, 1994). A ratio of unbound to bound forms of fura-2 can be calculated from the ratio of fluorescence intensity at 509nm using excitation wavelengths of 340 and 380nm (Tsien and Poenie, 1986). These ratios can be used to estimate $[Ca^{2+}]_i$ using ratios obtained under maximal (R_{max}) and insignificant (R_{min}) Ca^{2+} concentrations (Grynkiewicz *et al.*, 1985).

Fura-2 itself is a polycarboxylate anion that is unable to cross the lipid bilayer of cell membranes and is therefore cell impermeant. To overcome this problem cells are incubated in solutions of the esterified (acetoxymethyl (AM)) and hence hydrophobic form. The AM form can cross cell membranes freely thus loading dye into the cells. The AM group is labile to enzymatic hydrolysis by esterases present in the cytosol (Kao, 1994). Once cleaved, the indicator is returned to its original form and "trapped" within the cell. Extracellular dye is washed from the cells and thus changes in fluorescence are intracellular in origin and can be used to calculate changes in intracellular $[Ca^{2+}]_i$.

2.7.2 Measuring agonist-induced changes in intracellular Ca^{2+} concentration in cell populations.

Adherent cells on coverslips (prepared as described in section 2.3.2) were placed in 3ml Krebs-HEPES buffer (room temperature) within acrylic fluorimetry cuvettes that had been constructed to allow the coverslip to lie diagonally across the cuvette, such that alignment of the cell monolayer in the fluorimeter was at an angle to allow light from the beam in the fluorimeter into the cuvette, and to allow emitted fluorescent light out of the cuvette. The cuvette was then positioned to minimise reflectance. Following determination of autofluorescence, which involved measurement of the 340nm/380nm ratio for a period of 1min prior to addition of fura-2-AM for each experiment, a 10μl aliquot of fura-2-AM in dimethylsulphoxide (DMSO) was added (final fura-2-AM concentration 3.3μM). In order to improve the dispersion and solubilization of the dye, Pluronic F-127 (a non-ionic surfactant polyol) was pre-mixed (final concentration 0.016%) with the fura-2-AM. After 40min at room temperature, with continual agitation and protection from light (to prevent photobleaching of the dye), the cells were washed three times in fresh Krebs-HEPES buffer (37°C) to remove residual fura-2-AM. Prior to agonist stimulation the cells were incubated at 37°C for 5min to allow for de-esterification of the dye.

Cells loaded with fura-2 were placed in a Perkin-Elmer LS-5B spectrofluorimeter, aligned as described above. Changes in fluorescence prior to and following agonist stimulation were measured using excitation wavelengths of 340nm and 380nm with a 3.8s changeover time. Emission was recorded at a wavelengths of 509nm or above. Buffer was stirred continuously with a magnetic follower and the contents of the cuvette were maintained at a temperature of 37°C with a water jacket.

In situ calibration was performed in separate experiments by measuring the fluorescence ratio at a saturating concentration of Ca^{2+} (R_{max}) and in the absence of Ca^{2+} (R_{min}). An R_{max} value was defined using ionomycin (final concentration 10μM) and Ca^{2+} (final concentration 5mM). R_{min} was determined by the repeated addition of 50μl aliquots of 200mM EGTA (in 3M Tris-HCl) until the 340nm/380nm ratio remained constant. The R_{max} and R_{min} values determined in this manner were used to covert all population 340nm/380nm ratios, with the

autofluorescence values for each separate experiment subtracted, to $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985) as follows:

$$[Ca^{2+}]_i = K_D (R - R_{min} / R_{max} - R) \times sfb$$

Where K_D is the dissociation constant for fura-2 = 224nM (at 37°C) and sfb = $380_{min} / 380_{max}$.

2.7.3 Single cell Ca^{2+} imaging.

SH-SY5Y and SH-SY5Y_{RA6} cells at 30-50% confluence on glass coverslips (22mm diameter) were washed twice with Krebs-HEPES buffer. Cells were then incubated in the dark, at room temperature (22°C), for 1h with 3ml of Krebs-HEPES buffer supplemented with 2μM fura-2-AM. At the end of the loading period, the coverslips were washed twice in Krebs-HEPES buffer and incubated for a further 30min to allow for de-esterification of the dye, before being mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. Fluorescent images were obtained by alternate excitation at 340nm and 380nm (40 ms at each wavelength) using a xenon arc lamp (Osram). Emission signals were collected at wavelengths above 510nm (every 1.6s at x40 and every 400ms at x100) with an intensified charge-coupled device camera (Photonic Science). The digitized signals (after background subtraction) were collected, ratioed and analysed on the Quanticell QC 700 digital imaging system (Applied Imaging). Ratiometric values were used to estimate $[Ca^{2+}]_i$ using the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_D (R - R_{min} / R_{max} - R) \times sfb$$

Where K_D = 135nM (at 22°C) and sfb = $380_{min} / 380_{max}$.

R_{min} and R_{max} values are the minimal and maximal fluorescent ratios obtained from a sample set of either SH-SY5Y or SH-SY5Y_{RA6} cells as appropriate, using 5mM ionomycin and 6mM EGTA (for Rmin) followed by 10mM $CaCl_2$ (for Rmax). The 380min and 380max values are the fluorescent intensities after excitation at 380nm in the absence and presence of Ca^{2+} respectively.

2.8 Determination of Protein Concentration

The determination of protein concentration per well was used as an estimation of cell density. It may be possible that the total protein concentration per cell is increased following RA-differentiation of SH-SY5Y cells. In order to investigate this possibility DNA concentration versus cell counts versus protein concentration was measured for variable cell densities of both SH-SY5Y and SH-SY5Y$_{RA6}$ cells (data not shown). Unfortunately the data obtained from measurements of DNA concentration and cell counts were too variable to correlate with protein concentration (data not shown). Therefore, protein concentration, as the method accurate method attempted for estimating cell density, was utilised throughout this study.

Protein concentrations were determined using a modification of the method previously described by Lowry *et al.* (1951). Samples from 24 well multidishes were prepared by the addition of 1ml per well of 0.1M NaOH followed by a solubilisation period of >12h. Samples from permeabilised cell preparations or cell membrane preparations were prepared by the addition of 990µl of 0.1M NaOH to a 10µl aliquot of cell suspension or cell membranes. Protein standards were prepared using bovine serum albumin (BSA) diluted in 0.1M NaOH to give a range of concentrations of 0 - 1000µg of protein/ml.

To 500µl of each sample and 500µl of each standard, 1ml of a freshly prepared mixture containing 100ml of solution A (2% Na_2CO3 in 0.1M NaOH), 1ml of solution B (1% $CuSO_4$) and 1ml of solution C (2% Na^+K^+tartrate) was added. After a 10min incubation at room temperature, 200µl of Folin / Ciocalteus phenol reagent (a 1:3 dilution in dH_2O) was added to each tube. After a 20min incubation at room temperature 1ml of dH_2O was added to each tube followed by a thorough vortexing. Absorbance readings at 750nm were determined immediately. Protein concentrations were calculated for samples using the absorbance values from the protein standards.

2.9 Analysis of Data

Raw concentration-response data were analysed using the curve-fitting computer program GraphPad Prism (GraphPad Software, Inc.) by non-linear regression. Data were fitted to a four parameter logistic sigmoidal function (with equal weighting to each point) in order to obtain estimates of the maximum response and EC_{50} values. All values (unless otherwise stated) are presented as means ± Standard Error of the Means (S.E.M.) (n = number of experiments). Data obtained was analysed for statistical significance at $p< 0.05$ using unpaired, two-tailed, student's T-test, unless stated otherwise. EC_{50} values were analysed as negative $\log_{10}$ values as they are more likely to be normally distributed in this form.

3. CHARACTERISATION OF ENDOGENOUSLY EXPRESSED MUSCARINIC AND BRADYKININ RECEPTORS IN SH-SY5Y AND SH-SY5Y$_{RA6}$ CELLS.

3.1 Introduction

SH-SY5Y human neuroblastoma cells endogenously express a range of cell surface, seven-transmembrane spanning, G-protein linked receptors for neurotransmitters. So far these cells have been shown to express muscarinic receptors (Adem *et al.*, 1987; Lambert *et al.*, 1989), mu (μ) and delta (δ) opioid receptors (Kazmi and Mishra, 1987), α_2-adrenoceptors (Kazmi and Mishra, 1987), bradykinin receptors (McDonald *et al.*, 1994a) and neuropeptide Y receptors (McDonald *et al.*, 1994b). Activation of either muscarinic or bradykinin receptors in this cell line is accompanied by changes in inositol phosphates and $[Ca^{2+}]_i$ (Lambert and Nahorski, 1990; Willars and Nahorski, 1995). In view of the aims of this thesis, that is, an investigation into regulatory mechanisms underlying phosphoinositide (PI) and $[Ca^{2+}]_i$ signalling, this initial results chapter concentrates on the assessment of the levels of muscarinic and bradykinin receptors in these cells, and, to a certain extent, a characterisation of the expression of the subtype(s) of muscarinic receptors linked to the activation of PIC.

Most neurotransmitter receptors do not consist of a homogenous population, and many have been classified into subtypes using pharmacological and molecular biology techniques. Muscarinic and bradykinin receptors are of no exception. The effects of bradykinin are mediated via two main classes of receptor, B1 and B2, which have been defined pharmacologically using a variety of peptidergic agonists and antagonist (Hall, 1992). The B2 receptor subtype is linked to the activation of phosphoinositidase C (PIC), probably via the Gq/11 family of G-proteins (Derian and Moskowitz, 1986; Eggerickz *et al.*, 1992). The B1 kinin receptors are still poorly understood compared with the B2 receptors. B2 kinin receptors mediate most, if not all, the *in vivo* effects of kinins, B1 receptors becoming important in immunophysiology (when they are up-regulated). However, B1 receptors are also reportedly linked to the activation of PIC, and on the basis of the structure of the cloned receptor, may induce the recruitment of a G-protein (reviewed by Marceau, 1995). Muscarinic receptors have been classified into five genetically distinct subtypes and named M1-M5. The, M1, M3 and M5 muscarinic receptors preferentially couple to the Gq/11 family of G-proteins and, following agonist activation, stimulate PIC activation. M2 and M4

muscarinic receptors are more likely to couple to the Gi/Go G-proteins and, following agonist occupation, preferentially inhibit adenylate cyclase (Caulfield *et al.*, 1993). Clearly the complement of muscarinic receptor subtype(s) present in a cell or tissue will greatly influence the cellular response to a given agonist.

The nature of the muscarinic receptor subtype(s) expressed in SH-SY5Y cells has been subject to controversy. Previous studies by a variety of groups have suggested the following: expression of M3 receptors alone (Lambert *et al.*, 1989), expression of a predominantly M3 population (Wall *et al.*, 1991), expression of a homogenous population of M1 receptors (Serra *et al.*, 1988; McDonald *et al.*, 1994c), expression of a mixed population of M1 and M2 receptors (Adem *et al.*, 1987) or a mixed population of M1, M2 and M3 receptors (Kukkonen *et al.*, 1992). As stated above, it is likely that both B1 and B2 kinin receptors are G-protein linked to the activation of PIC. However, the two may be distinguished since the B1 receptor has a very low affinity for bradykinin, the preferred ligands being des-Arg-BK and Lys-des-Arg-BK (see Marceau, 1995). In addition, it is likely that the B2 receptor predominates in the SH-SY5Y cell line given that B2 kinin receptors predominate in the nervous system (Wolsing and Rosenbaum, 1991). Regardless of the subtype expressed, there is a paucity of information available in the literature thus far to indicate the expression levels of bradykinin receptors in SH-SY5Y cells.

A cellular response following agonist stimulation is often not directly proportional to the number of receptors occupied by an agonist. A notable example is that occupation of only 0.25% of receptors by acetylcholine can cause a half-maximal contraction in guinea pig ileum. This is not altogether unexpected because the dose-occupancy curve lies to the right of the concentration-response curve due to amplification within the system. This phenomenon leads to the concept of "receptor reserve" (Kenakin, 1996). In systems which have a receptor reserve a difference in the receptor density can affect the sensitivity of the response to agonist, the agonist potency and/or maximal response increasing with the number of receptors present. Consequently, if a receptor reserve for PI hydrolysis exists in SH-SY5Y cells, alterations of either muscarinic or bradykinin receptor density following differentiation could result in profound alterations of PI and $[Ca^{2+}]_i$ responses.

The primary aim of this chapter was to investigate whether muscarinic and/or bradykinin receptor densities were altered following RA-differentiation of SH-SY5Y cells. The expression levels of muscarinic and bradykinin receptors were assessed by saturation binding with a tritiated muscarinic receptor antagonist, [^{3}H]-N-methyl-scopalamine ([^{3}H]-NMS) and with tritiated bradykinin ([^{3}H]-bradykinin) respectively. In view of previous claims, mentioned above, of the sole expression of M1 receptors in SH-SY5Y cells, the expression of M1 and M3 muscarinic receptors in SH-SY5Y and SH-SY5Y$_{RA6}$ cells was further investigated. Pirenzepine has been widely used as an antagonist to distinguish the M1 receptor subtype, since its affinity is 4-fold greater for M1 than M4 muscarinic receptors and over 10-fold greater for M1 receptors over other muscarinic receptors. Pirenzepine was used to displace [^{3}H]-NMS from muscarinic receptors, thus revealing the predominance of either M1 or M3 muscarinic receptors. M1 and M3 muscarinic receptors were also assessed qualitatively by Western Blot analyses using receptor-specific antisera.

3.2 Methods

Antagonist binding to muscarinic receptors expressed in SH-SY5Y and SH-SY5Y$_{RA6}$ cell membranes.

The receptor expression level (or density) of muscarinic receptors in SH-SY5Y and SH-SY5Y$_{RA6}$ cells was determined by saturation binding to cell membranes with the non-selective, radiolabelled muscarinic antagonist, [^{3}H]-N-methylscopolamine ([^{3}H]-NMS (84 Ci/mmol). Membranes were prepared (as described in General Methods) and resuspended in [^{3}H]-NMS-binding buffer (10mM HEPES, 1mM $MgCl_2$, 100mM NaCl, pH 7.4). Saturation binding experiments were conducted by incubating membranes with a range of concentrations of [^{3}H]-NMS (0.03-3.0nM) in a final assay volume of 200µl for 60min at 37°C at which point equilibrium binding was achieved (Lambert *et al.*, 1989). Each tube contained approximately 70µg of protein per incubation. Non-specific binding for each concentration of [^{3}H]-NMS was defined in the presence of 1µM atropine sulphate. Incubations were terminated by the separation of bound and free ligand by rapid vacuum filtration using ice-cold buffer (Brandel

Cell Harvester) onto pre-wetted Whatman GF/B filters. Following filtration, filters were immediately washed twice with 3ml of ice-cold buffer and transferred to separate vials into which 4ml of Emulsifier Safe Scintillation Cocktail was added. Samples were left to extract for ≥ 12h and radioactivity on the filters detected by liquid scintillation spectrometry.

Inhibition studies were performed in cell membranes using the same incubation conditions as above. Membranes were incubated with a single concentration of [^{3}H]-NMS. The concentration of [^{3}H]-NMS (0.6nM) selected was slightly higher than the calculated K_D (which was approximately 0.5nM, see Table 2.1) and increasing concentrations (10^{-4}-10^{-10}M) of pirenzepine, a muscarinic receptor antagonist which selectively binds to the M_1 and M_4 receptor subtypes (Caulfield, 1993). Incubations were terminated and radioactivity on the filters detected as described above.

Agonist binding to bradykinin receptors expressed in SH-SY5Y and SH-SY5Y$_{RA6}$ cell membranes.

The density of bradykinin receptors in SH-SY5Y and SH-SY5Y$_{RA6}$ cells was determined by modification of a previously described method (Liebmann *et al.*, 1994). Receptor expression levels were determined by saturation binding of the radiolabelled agonist [^{3}H]-bradykinin to cell membranes. Membranes were prepared (as described in General Methods) and resuspended in buffer which consisted of 25mM TES buffer, 0.1% BSA, 1mM dithiothreitol, 100μM GTP, pH 6.8 including the protease inhibitors, 1,10-phenathroline (1mM), bacitracin (100μg/ml) and captopril (10μM). GTP was included in the buffer to eliminate heterogeneity of binding sites, such that only low affinity binding sites are measured (see section 1.3.1). Saturation binding experiments were conducted by incubating membranes with a range of concentrations of [^{3}H]-bradykinin (0.02-3.0nM) in a final assay volume of 200μl for 60min at 4°C at which point equilibrium binding was achieved (Liebmann *et al.*, 1994) using approximately 70μg of protein per incubation. Non-specific binding for each concentration of [^{3}H]-bradykinin was defined in the presence of 10μM unlabelled bradykinin. Incubations were terminated by rapid vacuum filtration onto Whatman GF/B filters that had been pre-treated with 0.1% (w/v) aqueous polyethyleneimine (to suppress filter binding). Following

filtration, filters were washed immediately with 2x3ml of ice-cold 10mM TES buffer, pH 6.8. Radioactivity on the filters was detected as described above by liquid scintillation spectrometry.

Immunoblot analysis of muscarinic receptor subtypes.

Cell membranes were prepared (as described in General Methods) at a protein concentration of 1.5mg/ml and denatured by boiling 15µl of membrane sample with 15µl of sample buffer (50mM Tris, 2% SDS, 0.1% bromophenol blue, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol). Prestained marker proteins (10µl) were also denatured with an equal volume of sample buffer. Denatured samples were subjected to SDS/PAGE to separate proteins using an 8% running gel (consisting of 4.6ml H_20, 2.7ml 30% acrylamide mix, 2.5ml 1.5M Tris (pH 8.8), 0.1ml 10% SDS, 0.1ml ammonium persulphate and 6µl TEMED in a total volume of 10ml) at 200V for approximately 45min submerged in running buffer (25mM Tris-Base, 250nM glycine, 0.1% SDS). Separated proteins were transferred to nitro-cellulose (0.45µm mesh (Biorad)) pre-soaked in blotting buffer (39mM glycine, 48mM Tris-base, 0.037% SDS, 20% methanol, pH 8.3) by placing both gel and nitro-cellulose adjacent to each other, buffered by filter paper (pre-soaked in blotting buffer), in the blotting apparatus. Transfer was initiated by blotting at 0.65mA per cm^2 for ≥ 60min. Nitro-cellulose with transferred proteins was blocked overnight at 4°C with 5% (w/v) dry milk in TBST (20mM Tris-HCl, 0.5M NaCl, 0.05% Tween-20, pH 7.5). Either rabbit anti-M3 antibody (Tobin and Nahorski, 1993) (1:1000 in TBST) or rabbit anti-M1 antibody (Waugh *et al.*, 1995) (1:1000 in TBST)was then added for > 1h, followed by horseradish peroxidase-labelled anti-(rabbit IgG) antibody (1:1000 in TBST) for 1h. Incubations were at room temperature and nitro-cellulose was washed following both incubations with TBST (3x10min). Equal volumes of enhance chemiluminescence (ECL) reagents (Amersham) were mixed and immediately added to the nitro-cellulose for 2min. Excess reagents were drained and immunoreactive bands were detected by exposure to Hyperfilm-ECL.

Data analysis.

[^{3}H]-NMS saturation binding data for each experiment was analysed using a graph-fitting software package (GraphPad Software, Inc.). Data were fitted to a rectangular hyperbola according to the equation:-

$$\text{Specific bound } [^3\text{H}]\text{-NMS} = (\text{Bmax} * \text{free } [[^3\text{H}]\text{-NMS}]) / (K_D + \text{free } [[^3\text{H}]\text{-NMS}])$$

Apparent K_i values for pirenzepine inhibition of [^{3}H]-NMS binding to SH-SY5Y and SH-SY5Y$_{RA6}$ cell membranes were determined using the relevant K_D's for [^{3}H]-NMS saturation binding (see Table 2.1) and the concentration of pirenzepine to cause 50% of inhibition of [^{3}H]-NMS binding (IC_{50}) and incorporating these values into the Cheng Prussoff equation, used to calculate the affinity of a receptor for unlabelled antagonist following determination of its ability to displace a known concentration of a labelled antagonist (Cheng and Prussoff, 1973):-

$$K_i = IC_{50} / (1+(K_D / [[^3\text{H}]\text{-NMS}])$$

[[^{3}H]-NMS] is the total concentration of [^{3}H]-NMS present in each incubation (0.6nM).

It should be noted that K_i values calculated using the above equation should be equivalent to the K_D of the antagonist, in this case pirenzepine, if it were possible to measure [^{3}H]-pirenzepine binding.

3.3 Results.

Initially, it is of importance to note that data presented in this chapter, (and subsequent chapters), is expressed in terms of protein concentration, as opposed to cell numbers. Since

total protein concentration per cell may be increased following RA-differentiation of SH-SY5Y cells, protein concentration may not be an accurate representation of relative cell densites in these studies (see section 2.8). This factor is discussed in section 3.4 of the current chapter.

[^{3}H]-NMS binding to muscarinic receptors.

Specific [^{3}H]-NMS binding to SH-SY5Y and SH-SY5Y$_{RA6}$ cell membranes was saturable and concentration-dependent (Figure 1). The estimated receptor expression levels and K_D values are indicated in Table 1. The muscarinic receptor density was significantly ($p< 0.05$) greater in SH-SY5Y$_{RA6}$ cell membranes compared to SH-SY5Y cell membranes. The K_D values were not significantly different between SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

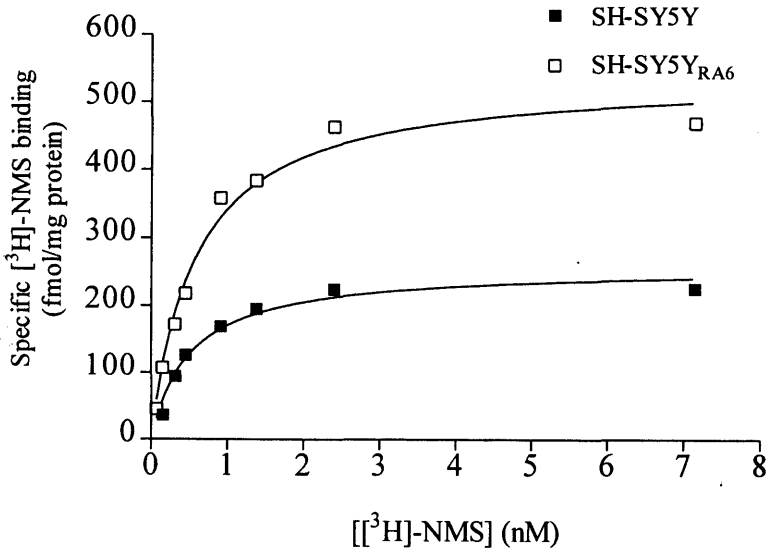


Figure 1 [^{3}H]-NMS saturation binding to membranes of SH-SY5Y and SH-SY5Y$_{RA6}$ cells. Data are representative curves from four separate experiments.

	SH-SY5Y	SH-SY5Y$_{RA6}$
B_{max} (fmol/mg protein)	272 ± 11	529 ± 22
K_D (log_{10}(M))	-9.65 ±0.28 (0.49nM)	-9.24 ± 0.03 (0.59nM)

Table 1 B_{max} represents the maximal binding capacity for [^{3}H]-NMS binding (fmol of receptor/mg protein) in SH-SY5Y and SH-SY5Y$_{RA6}$ cell membranes. K_D (log_{10} (M)) represents the dissociation constant from four separate experiments. Data are means ± S.E.M., n=4.

Pirenzepine displacement of [^{3}H]-NMS.

Pirenzepine was used over a range of concentrations to displace a single concentration of [^{3}H]-NMS (0.6nM). There was no indication of multiple affinity states and displacement curves were fitted assuming that binding occurred with a single affinity, yielding slope factors of unity. The pirenzepine displacement curves are shown in Figure 2. The affinity of pirenzepine was estimated for both SH-SY5Y and SH-SY5Y$_{RA6}$ cell membranes by the application of the Cheng Prussoff equation (see Data Analysis) The negative log K_i (pK_i) values are shown in Table 2. The pKi values were not significantly different in either cell type.

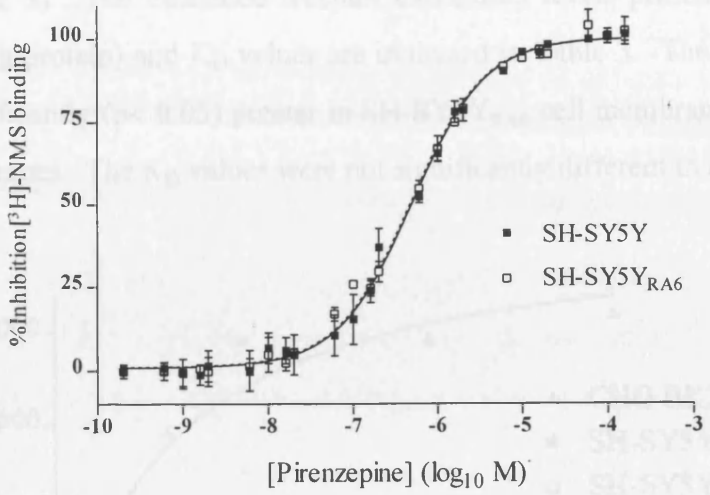


Figure 2 Pirenzepine competition binding with [^{3}H]-NMS (approximately 0.6nM) in SH-SY5Y and SH-SY5Y$_{RA6}$ cell membranes Data are means ± S.E.M., n=3.

	SH-SY5Y	SH-SY5Y$_{RA6}$
pK_i	6.56 ± 0.02	6.61 ± 0.01
Slope	0.98 ± 0.04	1.01 ± 0.02

Table 2 Pirenzepine inhibition of [3H]-NMS binding in SH-SY5Y and SH-SY5Y$_{RA6}$ cell membranes. pK_i represents the - log_{10} (M) value of the inhibition constant obtained using the Cheng-Prussof equation and the [^{3}H]-NMS KD values from Table 1. Data are means ± S.E.M., n=3.

[^{3}H]-bradykinin binding to bradykinin receptors.

The bradykinin receptor density expressed in CHO BK2/M3 (positive control), SH-SY5Y, SH-SY5Y$_{RA6}$ and untransfected CHO-k1 (negative control) cell membranes was detected by saturation binding with [^{3}H]-bradykinin. Specific [^{3}H]-bradykinin binding to CHO BK2/M3, SH-SY5Y, SH-SY5Y$_{RA6}$ and CHO-k1 cell membranes was saturable and concentration

dependent (Figure 3). The estimated receptor expression levels present in the membranes (Bmax in fmol/mg protein) and K_D values are indicated in Table 3. The bradykinin receptor density was significantly ($p< 0.05$) greater in SH-SY5Y_{RA6} cell membranes compared to SH-SY5Y cell membranes. The K_D values were not significantly different in any of the cell lines.

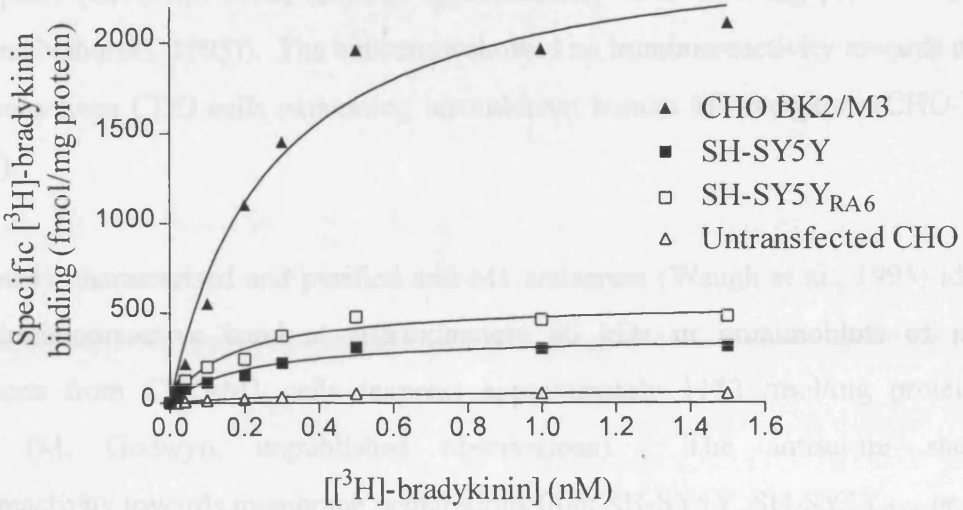


Figure 3 *[^{3}H]-bradykinin binding to SH-SY5Y, SH-SY5Y_{RA6}, CHO BK2/M3 and untransfected CHO-k1 cell membranes. Data are representative curves from four separate experiments.*

	CHO BK2/M3	SH-SY5Y	SH-SY5YRA6	CHO-k1
B_{max} (fmol/mg protein)	1772 ± 120	300 ± 21	519 ± 44	95 ± 24
K_D (log_{10} (M))	-9.57 ± 0.05 (0.27nM)	-9.70 ± 0.08 (0.20nM)	-9.74 ± 0.03 (0.18nM)	-9.44 ± 0.24 (0.36nM)

Table 3 *Bmax represents the maximal binding capacity for [^{3}H[-bradykinin binding (fmol of receptor/mg of protein) in CHO BK2/M, SH-SY5Y, SH-SY5Y_{RA6} and untransfected CHO-k1 cell membranes. K_D (log_{10} (M)) represents the dissociation constant calculated from four separate experiments. Data are means ± S.E.M., n=4.*

Immunoblot analysis of muscarinic M1 and M3 receptors.

A previously characterised and purified anti-M3 antiserum (Tobin and Nahorki, 1993) identified a diffuse immunoreactive band at approximately 90 kDa in immunoblots of membrane preparations from SH-SY5Y, SH-SY5Y$_{RA6}$ and CHO cells expressing recombinant M3 receptors (CHO-M3 cells) (express approximately 1343 fmol/mg protein M3 receptor (Tobin and Nahorski, 1993)). The antiserum showed no immunoreactivity towards membrane preparations from CHO cells expressing recombinant human M1 receptors (CHO-M1 cells) (figure 4).

A previously characterised and purified anti-M1 antiserum (Waugh et al., 1995) identified a diffuse immunoreactive band at approximately 80 kDa in immunoblots of membrane preparations from CHO-M1 cells (express approximately 1152 fmol/mg protein of M1 receptor (M. Godwyn, unpublished observations). The antiserum showed no immunoreactivity towards membrane preparations from SH-SY5Y, SH-SY5Y$_{RA6}$ or CHO-M3 cells (figure 5(a)). Purified anti-M1 antiserum was able to detect M1 muscarinic receptors expressed in CHO-M1 cells diluted such that the expression level was 9 fmol/mg protein (figure 5(b)).

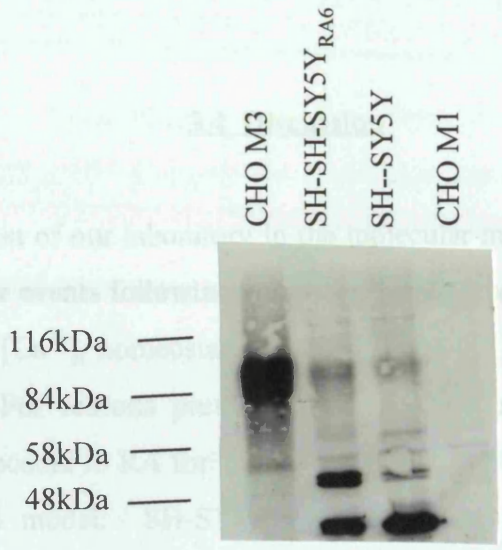


Figure 4 *Western blot using rabbit anti-M3 antibody (1:1000 dilution) to probe nitrocellulose membrane containing membrane proteins (22.5μg protein per lane) from either CHO M3, M1 cells. Position of molecular weight markers (kDa) are shown.*

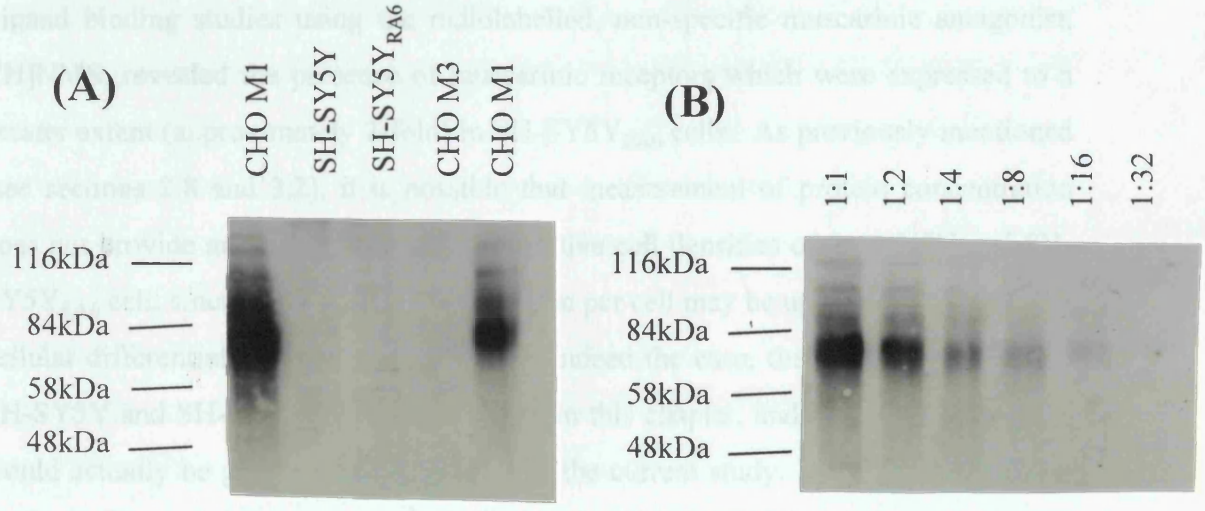


Figure 5 *Western blots using rabbit anti-M1 antibody (1:1000) dilution to probe nitrocellulose membranes containing (A) membrane proteins (22.5μg protein per lane) from either CHO M1, SH-SY5Y, SH-SY5Y$_{RA6}$ or CHO M3 cells or (B) membrane proteins from CHO M1 cells at protein concentrations of 1:1 (22.5μg protein per lane), 1:2 (11.3μg protein per lane), 1:4 (5.6μg protein per lane), 1:8 (2.8μg protein per lane), 1:16 (1.4μg protein per lane) and 1:16 (0.7μg protein per lane). Position of molecular weight markers are shown.*

3.4 Discussion

In view of the interest of our laboratory in the molecular mechanisms underlying the regulation of cellular events following the activation of receptors G-protein linked to PI metabolism and $[Ca^{2+}]_i$ homeostasis, the neuronal cell line, SH-SY5Y has been used extensively. For reasons previously discussed (Chapter 1) SH-SY5Y cells differentiated by exposure to RA for 6 days (SH-SY5Y$_{RA6}$) will potentially provide a better neuronal cell model. SH-SY5Y cells express muscarinic and bradykinin receptors, the activation of which is accompanied by PIC activation and changes in $[Ca^{2+}]_i$ (Lambert and Nahorski, 1990; Willars and Nahorski, 1995). Consequently, signal transduction following muscarinic receptor and bradykinin receptor activation in RA-treated SH-SY5Y cells was the subject of this chapter.

Ligand binding studies using the radiolabelled, non-specific muscarinic antagonist, [^{3}H]NMS, revealed the presence of muscarinic receptors which were expressed to a greater extent (approximately 2-fold) in SH-SY5Y$_{RA6}$ cells. As previously mentioned (see sections 2.8 and 3.2), it is possible that measurement of protein concentration does not provide an accurate measure of relative cell densities of SH-SY5Y and SH-SY5Y$_{RA6}$ cell, since total protein concentration per cell may be up-regulated following cellular differentiation. However, if this is indeed the case, the differences between SH-SY5Y and SH-SY5Y$_{RA6}$ cells presented in this chapter, and subsequent chapters, would actually be greater than implicated in the current study. Competition binding assays between pirenzepine and [^{3}H]-NMS yielded binding data with a slope of unity which is consistent with a single population of receptors binding a ligand in a non-co-operative manner. The binding affinity for pirenzepine was similar in SH-SY5Y and SH-SY5Y$_{RA6}$ cells (275nM and 243nM respectively). These values are similar to those obtained by other groups for pirenzepine binding to M3 receptors, in cell lines or in functional studies (Caulfield, 1993). The K_i values obtained are distinct from those which would be expected for M1 receptors, which also couple to PIC (Caulfield, 1993). In support of the binding data, Western blot analysis revealed the presence of muscarinic M3 receptors in both SH-SY5Y and SH-SY5Y$_{RA6}$ cells with a higher expression level in the differentiated cells. In contrast to previous reports (Serra *et al.*,

1988; McDonald *et al.*, 1994c) muscarinic M1 receptors were not present in either cell type to any detectable level. Collectively, these data strongly indicate that the SH-SY5Y cell line used throughout this study express a population of muscarinic M3 receptors in the undifferentiated state, which is approximately doubled following RA-induced differentiation. In agreement with Lambert *et al.* (1989) this population of receptors would appear to be homogenous. However, pharmacological characterisation of muscarinic receptor subtypes can be relatively insensitive to a small population of one subtype of receptor (less than 10%) in the presence of another receptor subtype which predominates. In effect the antagonist binding characteristics of the small population of muscarinic receptor subtypes can be masked by the antagonist binding characteristics of the predominant muscarinic receptor subtype. Therefore, in the present study, the presence of small populations of M1 receptors (less than 9 fmol/mg protein) and/or M2 receptors in SH-SY5Y cells cannot be excluded. Indeed, Wall *et al.* (1991) used muscarinic receptor subtype-selective antisera to determine subtypes of muscarinic receptor expressed in SH-SY5Y cells and found that M3 receptors predominated (74%) with lower levels of M1 (5%) and M2 (8%) receptors. However, if the population of muscarinic receptors, as revealed by [^{3}H]-NMS binding, in SH-SY5Y cells in equal to approximately 272 fmol/mg protein, then 10% of muscarinic receptors is approximately equal 27 fmol/mg protein and 5% equal to 14 fmol/mg protein. In this study, the M1 receptor population is likely to be less than 5%, if present at all, since the minimum detection of M1 receptors by Western blot analysis using an M1-specific antisera was approximately 9 fmol/mg protein (approximately equal to 3% of total receptors).

SH-SY5Y cells also express bradykinin receptors as determined by the high affinity, specific binding of the agonist [^{3}H]bradykinin. It is likely that the bradykinin receptors detected are of the B2 subtype, which are also members of the superfamily of 7TM domain G-protein coupled receptors, given that neuronal bradykinin receptors are predominantly B2 (Wolsing and Rosenbaum, 1991). In a manner similar to that of muscarinic receptors, the density of bradykinin receptors was increased approximately 2-fold following differentiation with RA. A previous study reported that in guinea pig ileum, a tissue in which bradykinin receptors have been well characterised as being of

the B2 subtype linked to phosphoinositide hydrolysis (Ransom *et al.*, 1992), [^{3}H]-bradykinin binding to purified membranes revealed the existence of two binding sites (Manning *et al.*, 1986). These binding sites consisted of a high affinity site (B_{HA}) and a low affinity site (B_{LA}) with K_D values of 10-25pM and 200-900pM respectively (Manning *et al.*, 1986; Liebmann *et al.*, 1994). However, the identification of two binding sites from the inhibition of [^{3}H]-bradykinin binding with unlabelled bradykinin is very difficult (Manning *et al.*, 1986) and quite speculative. A successful separation of B_{HA} and B_{LA} binding sites depends on good selectivity of the ligand (separation of the K_D values) and high specific radioactivity of the tracer. One group utilised ^{125}I-bradykinin as a probe for bradykinin receptors in guinea pig ileum and found that ^{125}I-bradykinin displayed enhanced affinity and selectivity for the B_{HA} site, thus confirming the existence of two separate binding sites in this tissue (Liebmann *et al.*, 1994). The same group concluded that the B_{LA} site represents the classical B2 receptor that stimulates phosphoinositide hydrolysis (Ransom *et al.*, 1992), whilst the B_{HA} site, also a B2 receptor subtype since it is recognised by classical B2 receptor antagonists, is probably G-protein linked to the inhibition of adenylate cyclase activity (Liebmann *et al.*, 1994). Binding of [^{3}H]-bradykinin to SH-SY5Y and SH-SY5Y$_{RA6}$ cell membranes in the presence of GTP yielded binding data consistent with a single receptor population with K_D values which were in good agreement with values previously found for the high affinity B2 receptor subtype linked to the metabolism of inositol phosphates (Liebmann *et al.*, 1994). Thus, it is likely that SH-SY5Y and SH-SY5Y$_{RA6}$ cells express bradykinin B2 receptors linked to phosphoinositide hydrolysis, but it is a possibility that cannot be overlooked that these cells also express B2 receptors linked to the inhibition of adenylate cyclase.

Interestingly, the untransfected CHO-k1 cells, that were used as a negative control for the expression of bradykinin receptors in the current study, appeared to express a not insignificant number of bradykinin receptors. The reason(s) for this apparent expression is unclear. It is possible that these cells express a small population of bradykinin receptors. However, challenge with bradykinin failed to evoke a $[Ca^{2+}]_i$ response in these cells (G.B. Willars, unpublished observations). Alternative explanations are that the CHO-k1 membrane preparations were somehow

contaminated with other cells expressing bradykinin receptors, or simply an error within the experiment.

Regardless of the subtypes of receptors expressed, the outcome from the present studies clearly demonstrate that differentiation of SH-SY5Y cells with RA results in the up-regulation of muscarinic (predominantly, if not exclusively, M3) and bradykinin receptors present in cell membranes. This increase could be due to increased synthesis or to decreased degradation of receptor molecules. Up-regulation of cell surface receptors following RA-induced differentiation is not restricted to muscarinic and bradykinin receptors in SH-SY5Y cells. For instance, RA-induced differentiation of SH-SY5Y cells has also been associated with an increase in VIP and mu opioid receptor populations (Preis *et al.*, 1988; Wascheck *et al.*, 1989). In addition, differentiation of neuroblastoma cell lines utilising alternative methods also results in the up-regulation of cell surface receptors. For example, differentiation of the NG108-15 or N1-E-115 neuroblastoma cell lines by serum starvation or DMSO results in an increased expression of angiotensin 2 (AT2) receptors (Seidman *et al.*, 1996; Reagan *et al.*, 1990).

The method of differentiation chosen would appear to be important in the regulation of cell surface receptor expression. For example, contrary to the findings of this study, differentiation of SH-SY5Y cells with the phorbolester TPA induces the down-regulation of muscarinic receptors (Adem *et al.*, 1987). In IMR 32 human neuroblastoma cells, differentiation with either 5-bromo-2'-deoxyuridine (BrdU) or dibutyryl cyclic adenosine 3'-5' mobophosphate (Bt_2 cAMP) had differing effects, not only on the number, but also on the functional properties of muscarinic and nicotinic receptors (Gotti *et al.*, 1987). Taken together this would suggest that the up-regulation of muscarinic and bradykinin receptors observed in the present study is as a consequence of RA-induced differentiation and not an actual requirement for differentiation. This does not however preclude the possibility that muscarinic and bradykinin receptors are not involved in other aspects of neuronal maturation. Interestingly, it has been reported that muscarinic receptors increase during the development of nervous tissue and reach their maximum in different brain regions

after different times (Kuhar *et al.*, 1980). As it would appear that the way in which the differentiating agents mentioned above modulate the number of muscarinic receptors is specific to the agent employed, this may indicate that the expression (and possibly the function) of these receptors during the maturation of the nervous system are to some extent dependent on the extracellular environment in which the neurones grow.

In conclusion, SH-SY5Y cells used in this study predominantly express the muscarinic M_3 receptor and bradykinin (probably B2) receptors. Following treatment with the differentiating agent, RA, muscarinic M3 and bradykinin receptors are up-regulated . The observed increase in cell surface receptor densities are likely to have functional consequences, which is investigated in subsequent chapters.

4. TEMPORAL ASPECTS OF AGONIST-STIMULATED PIC ACTIVATION AND CHANGES IN $[Ca^{2+}]_i$ IN SH-SY5Y AND SH-SY5Y$_{RA6}$ CELLS.

4.1 Introduction

The previous chapter described how the undifferentiated human neuroblastoma cell line, SH-SY5Y, utilised throughout this study, expresses bradykinin and muscarinic (predominantly, though possibly not exclusively M3) receptors. Following treatment with the differentiating agent, RA, bradykinin and M3 muscarinic receptors are up-regulated (chapter 3), which could result in functional consequences. Activation of muscarinic M3 receptors and bradykinin B2 receptors is known to lead predominantly to the activation of PIC (see Willars and Nahorski, 1995 and references therein). Activation of PIC results in hydrolysis of PtdIns(4,5)P_2 releasing intracellular second messengers Ins(1,4,5)P_3 and DAG. Ins(1,4,5)P_3 stimulates an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by the mobilisation of Ca^{2+} from intracellular stores, whilst DAG activates PKC resulting in phosphorylation events regulating cellular functions (Berridge, 1993). This PIC activation is thought to be mediated by G proteins of the Gq/11 family, which are insensitive to PTX-catalysed ADP-ribosylation (for review see Simon *et al.*, 1991). G-proteins of the Gq/11 family act preferentially on PICβ1 isoforms (Rhee and Choi, 1992; Smrcka *et al.*, 1991; Taylor *et al.*, 1991), the regulatory aspects of which are discussed in chapter 1.

Cellular responses following agonist challenge can be influenced by changes in receptor density. For instance, in systems which have a 'receptor reserve', an increase in the number of receptors present results in an increase of the potency, and possibly the magnitude, with which an agonist can elicit its final response. A receptor reserve denotes a spare capacity or spare receptors within the system where only a fraction of the total number of receptors are required to be activated to produce a maximal response to a full agonist (Kenakin, 1993). As a result, if there is a receptor reserve for muscarinic M3 and /or bradykinin receptors in SH-SY5Y cells, we might expect to see an increase in the magnitude of functional responses concomitant with the observable increased receptor densities following cellular differentiation. However, the complement of G-proteins, effectors and other factors which can regulate the functional response to agonist may also be affected by RA-induced differentiation. Alterations of the expression and/or the regulation of any one of these factors

could have the potential to profoundly affect functional responses following agonist challenge.

As discussed, agonist-mediated PIC activation results in an increase in $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ occurs as the result of Ca^{2+} release from intracellular stores and/or "capacitative" Ca^{2+} entry (Berridge, 1993; Putney and Bird, 1993). However, release of stored Ca^{2+} and Ca^{2+} entry will not be determined solely by agonist-induced Ins(1,4,5)P_3 accumulation. Other factors, such as the expression of Ins(1,4,5)P_3 receptor density and subtype, the size of Ca^{2+} stores and the expression of Ca^{2+} channels in the cell membrane responsible for Ca^{2+} entry will play a role in determining agonist-induced changes in $[Ca^{2+}]_i$. The way in which RA-induced differentiation of SH-SY5Y cells will effect such factors, if at all, is unknown.

The aims of the present study were to investigate how RA-induced differentiation of SH-SY5Y cells effects agonist stimulated phosphoinositide and Ca^{2+} signalling. To this end, the ability of muscarinic and bradykinin receptor agonists to activate PIC and elevate $[Ca^{2+}]_i$ in SH-SY5Y and SH-SY5Y_{RA6} was determined. Previous studies have shown that there are differences in the way in which temporal aspects of bradykinin receptor-mediated and muscarinic receptor-mediated PIC activation are regulated (Willars and Nahorski, 1995). Therefore this chapter will concentrate on the temporal aspects of phosphoinositide and Ca^{2+} signalling, to assess not only whether the magnitude of responses are altered, but also whether the time-dependency of responses are affected by RA-evoked differentiation.

The activation of PIC following stimulation with agonist was assessed in two different ways. The first was by directly measuring Ins(1,4,5)P_3 mass accumulation in stimulated cells. The disadvantage associated with using this method as an index of agonist-mediated PIC activation is that Ins(1,4,5)P_3 is metabolised by the enzymes Ins(1,4,5)P_3 5-phosphatase and Ins(1,4,5)P_3 3-kinase forming Ins(1,4)P_2 and Ins(1,3,4,5)P_4 respectively. These inositol phosphate products are sequentially dephosphorylated until monophosphates are converted to inositol by inositol monophosphatase (as reviewed by Cockcroft and Thomas, 1992). As the metabolism of Ins(1,4,5)P_3 is rapid (Fisher, 1990), the assay can only measure steady state Ins(1,4,5)P_3 levels (i.e. the net effect of both generation and metabolism), which are indicative of both PtdIns(4,5)P_2 hydrolysis and Ins(1,4,5)P_3 metabolism. Changes in the levels of Ins(1,4,5)P_3 will only provide an accurate measure of PIC activity if the rate of metabolism is

constant (Wojcikiewicz *et al.*, 1994). An alternative method used in numerous studies is the assessment of PIC activity by measuring the accumulation of total inositol phosphates in cells in the presence of lithium (Li^+). Li^+ (10mM) blocks the monophosphatase enzymes responsible for the conversion of inositol monophosphates to inositol in an uncompetitive manner. Thus, stimulation of cells in the presence of Li^+ prevents re-cycling of inositol into lipids (Nahorski *et al.*, 1991) and results in an accumulation of inositol phosphates with time (Berridge *et al.*, 1982). Measurement of total inositol phosphates in response to agonist in the presence of Li^+ provides a measure of the PIC activity involved in the breakdown of all phosphoinositides. Importantly, as metabolism is prevented by Li^+, the quantities measured reflect the rate of inositol phosphate production only. A more accurate picture of agonist-induced PIC activation will be gained by comparing PIC activation measured using both of these methods. The reasons for this are that measuring total inositol phosphate accumulation will yield a total index of PIC activity, whereas the measurement of $Ins(1,4,5)P_3$ mass accumulation is important because measuring total inositol phosphate accumulation is not necessarily a profile of second messenger generation since PtdIns and PtdInsP could be substrates for PIC whereas $PtdIns(4,5)P_2$ is the only substrate PIC cleaves that results in the formation of $Ins(1,4,5)P_3$ (Willars *et al.*, 1996).

Agonist-induced changes in $[Ca^{2+}]_i$ were determined in both cell population and single cell studies. The advantage of $[Ca^{2+}]_i$ measurement at the single cell level is that this is the only approach that can yield information about the spatial organisation of $[Ca^{2+}]_i$ changes within the cell. Furthermore, due to the asynchronous nature of most $[Ca^{2+}]_i$ responses, complex patterns may be averaged out in population measurements. This is important in RA-treated cells because any subcellular responses occurring in the neurites may be indicated. In addition, cell-to cell- variation may be highlighted in single cell studies if the cell population is heterogenous in their sensitivity to agonist. However, population measurements are more advantageous when$[Ca^{2+}]_i$ is to be correlated with measurements of metabolites, such as $Ins(1,4,5)P_3$ other and inositol phosphates, which can only be measured using a relatively large number of cells.

The results presented and discussed in this chapter are the resultant PIC activation, assessed by $Ins(1,4,5)P_3$ formation and total [^{3}H]-inositol phosphate accumulation, and changes in $[Ca^{2+}]_i$, assessed by both population and single cell $[Ca^{2+}]_i$ measurements, following

stimulation with either muscarinic or bradykinin receptors in SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

4.2 Methods

The following studies were performed in adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells cultured as described in General Methods. Ins(1,4,5)P_3 mass accumulation, total [^{3}H]inositol phosphate accumulation and single cell and population $[Ca^{2+}]_i$ elevations following stimulation with maximal doses of either methacholine (1mM) or bradykinin (10μM) were assessed as described in General Methods (sections 2.4, 2.5 and 2.7).

4.3 Results

Time-dependency of methacholine- and bradykinin-mediated Ins(1,4,5)P_3 accumulation in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

Increases in cellular Ins(1,4,5)P_3 mass in response to maximally effective concentrations of methacholine (1mM) and bradykinin (10μM) were measured over a period of 5min.

From a resting level of 11±2 pmol/mg protein, maximal concentrations of methacholine evoked a biphasic elevation of Ins(1,4,5)P_3 in SH-SY5Y cells. This consisted of a rapid (10s) peak phase (104±8 pmol/mg protein) followed by a lower but sustained plateau phase from 60-300s (77±10 pmol/mg protein, n=3). In SH-SY5Y$_{RA6}$ cells the magnitude of the peak was significantly ($p<0.01$) increased by approximately 1.5-fold compared to SH-SY5Y cells (Figure 1A). Although challenge with a maximal concentration of bradykinin (10μM) evoked a small Ins(1,4,5)P_3 response in SH-SY5Y cells (21±2 pmol/mg protein, n=3) which peaked at 10s followed by a decline to basal levels, the response was not significantly different from that elicited following addition of buffer alone (20±1 pmol/mg protein). RA treatment significantly ($p<0.01$) increased the peak Ins(1,4,5)P_3 response to bradykinin (70±6 pmol/mg

protein) (Figure 1B) which occurred at 5s, which was significantly ($p < 0.05$) different from that elicited following the addition of buffer alone (18 ± 3 pmol/mg protein, n=3).

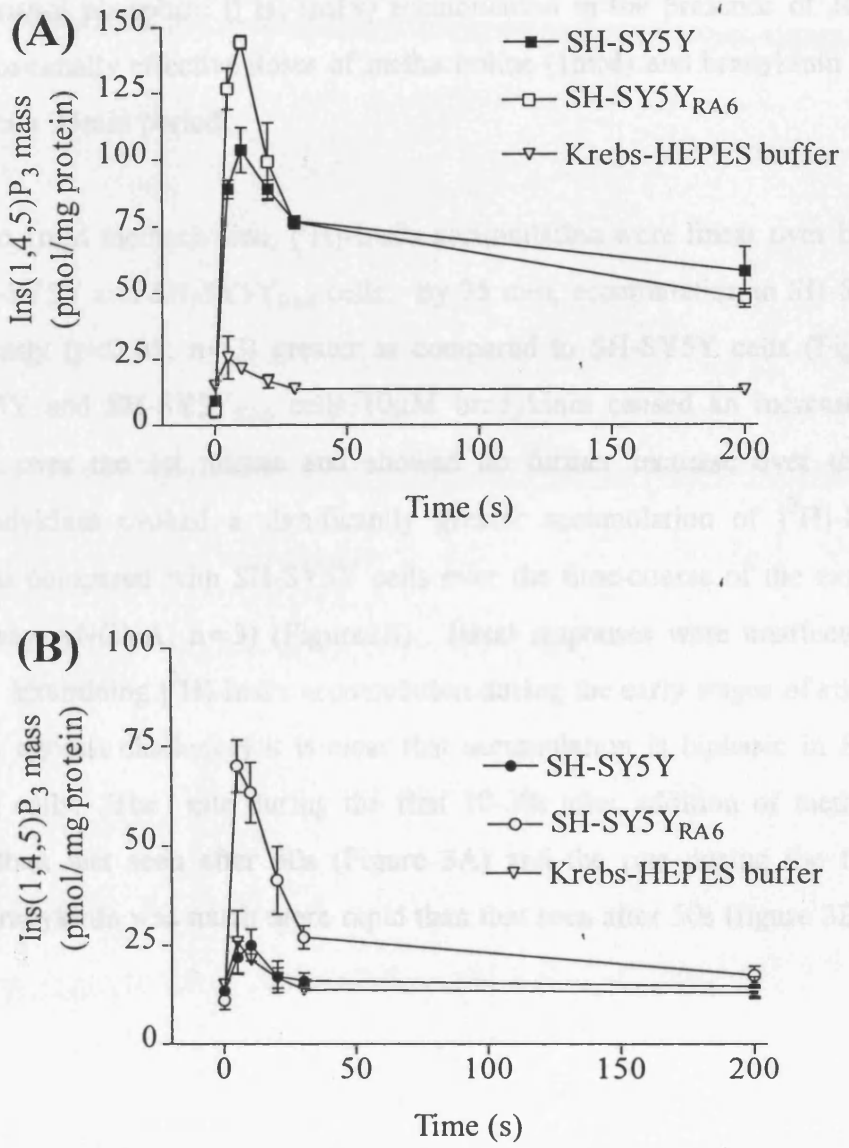


Figure 1 Time course of accumulation of Ins(1,4,5)P_3 mass in adherent SH-SY5Y and SH-SY5Y_{RA6} cells in response to a maximally effective dose of (A) methacholine (1mM) and (B) bradykinin (10μM). Data are means ± S.E.M., n=3.

Methacholine- and bradykinin-mediated accumulation of total [^{3}H]-inositol phosphates in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

Total [^{3}H]-inositol phosphate ([^{3}H]-InsPs) accumulation in the presence of 10mM LiCl in response to maximally effective doses of methacholine (1mM) and bradykinin (10μM) were measured over a 25min period.

In response to 1mM methacholine, [^{3}H]-InsPs accumulation were linear over between 2 and 25min in SH-SY5Y and SH-SY5Y$_{RA6}$ cells. By 25 min, accumulation in SH-SY5Y$_{RA6}$ cells was significantly ($p<0.05$, $n=3$) greater as compared to SH-SY5Y cells (Figure 2A). In both SH-SY5Y and SH-SY5Y$_{RA6}$ cells 10μM bradykinin caused an increased [^{3}H]-InsPs accumulation over the 1st minute and showed no further increase over the subsequent 24min. Bradykinin evoked a significantly greater accumulation of [^{3}H]-InsPs in SH-SY5Y$_{RA6}$ cells compared with SH-SY5Y cells over the time-course of the experiment ($p=0.019$, two-way ANOVA, $n=3$) (Figure2B). Basal responses were unaffected over these time periods. Examining [^{3}H]-InsPs accumulation during the early stages of stimulation (i.e. seconds after agonist challenge) it is clear that accumulation is biphasic in SH-SY5Y and SH-SY5Y$_{RA6}$ cells. The rate during the first 10-30s after addition of methacholine was more rapid than that seen after 50s (Figure 3A) and the rate during the first 10s after addition of bradykinin was much more rapid than that seen after 50s (figure 3B).

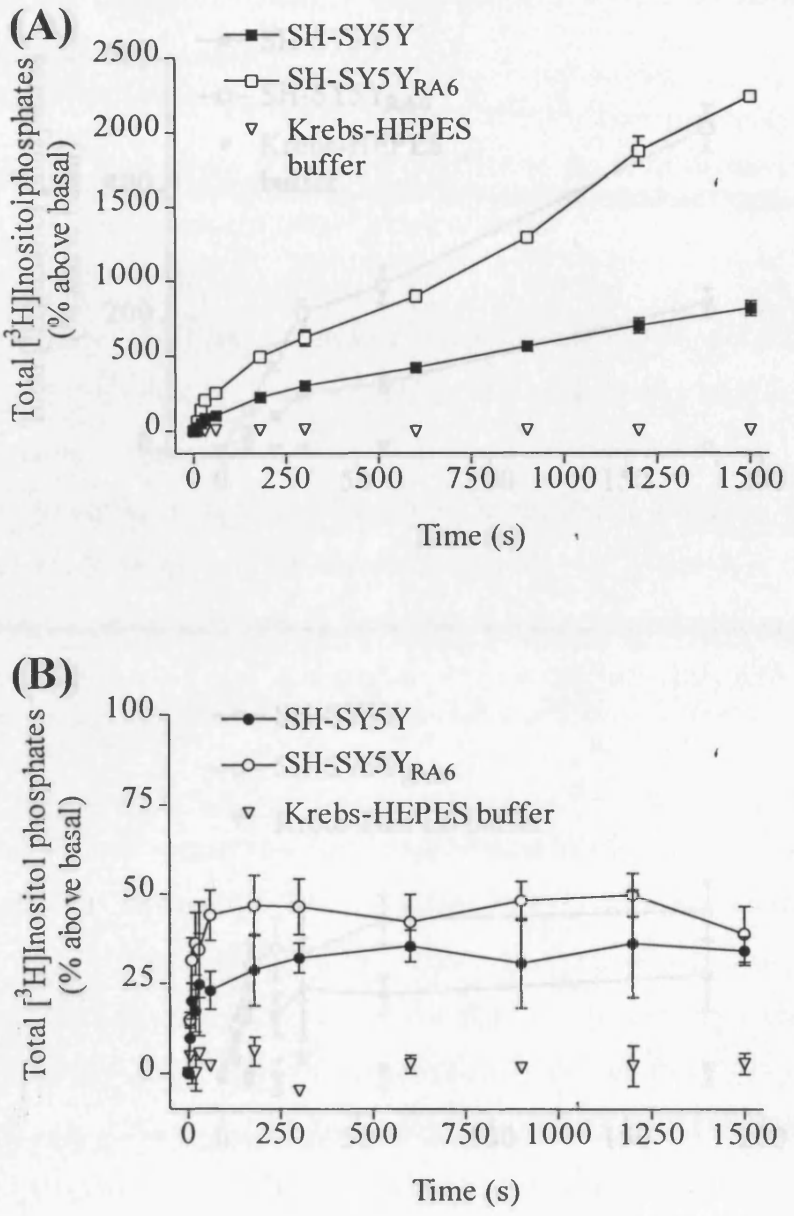


Figure 2 Time course of accumulation of [^{3}H]-inositol phosphates in adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells in response to a maximally effective dose of (A) methacholine (1mM) and (B) bradykinin (10μM). Data are percentage increase above basals and represent means ± S.E.M., n=3.

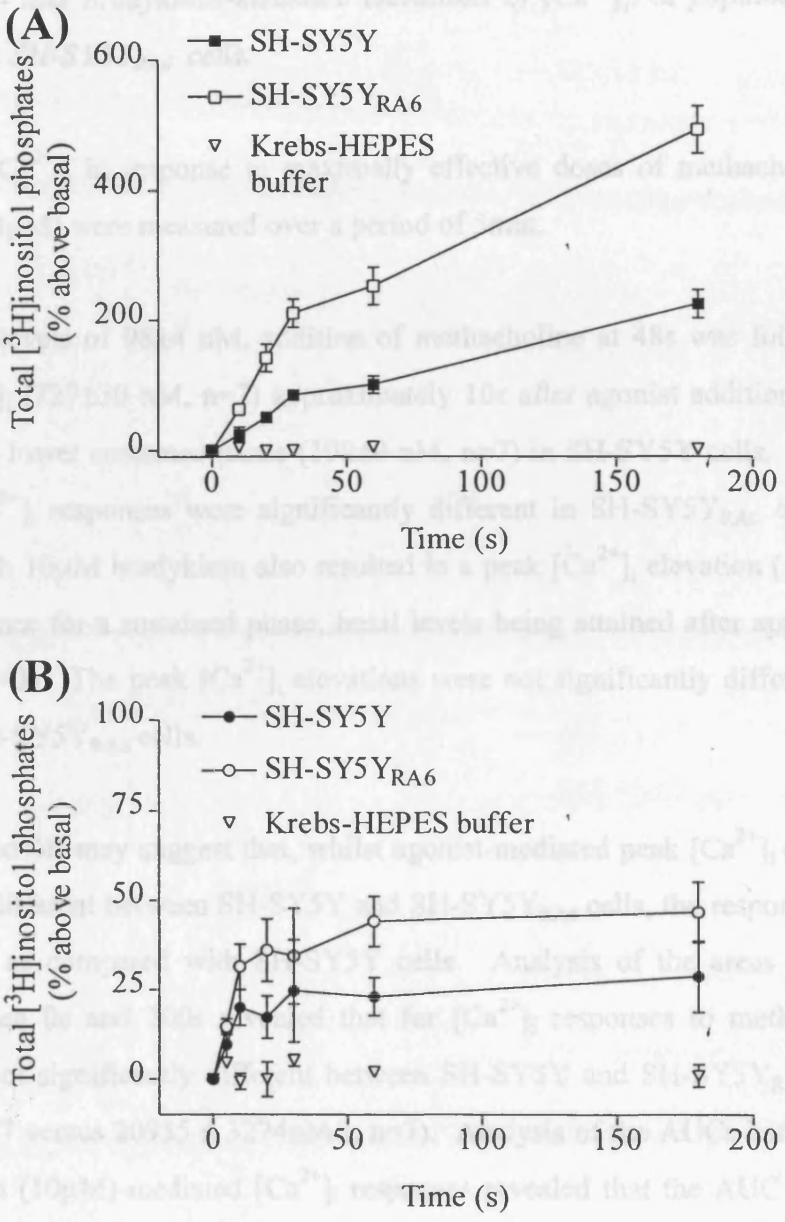


Figure 3 Biphasic kinetics of the early accumulation of [^{3}H]-inositol phosphates in adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells in response to a maximally effective dose of (A) methacholine (1mM) and (B) bradykinin (10μM). Data are percentage increases above basal values and represent means ± S.E.M., n=3.

Methacholine- and bradykinin-mediated elevations of $[Ca^{2+}]_i$ in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

Increases in $[Ca^{2+}]_i$ in response to maximally effective doses of methacholine (1mM) and bradykinin (10μM) were measured over a period of 5min.

From resting levels of 98±4 nM, addition of methacholine at 48s was followed by a rapid peak of $[Ca^{2+}]_i$ (727±30 nM, n=7) approximately 10s after agonist addition. This peak was followed by a lower sustained phase (198±9 nM, n=7) in SH-SY5Y cells. Neither peak nor sustained $[Ca^{2+}]_i$ responses were significantly different in SH-SY5Y$_{RA6}$ cells (Figure 4A). Challenge with 10μM bradykinin also resulted in a peak $[Ca^{2+}]_i$ elevation (535±41 nM, n=3) with no evidence for a sustained phase, basal levels being attained after approximately 1min (Figure 4B, n=3). The peak $[Ca^{2+}]_i$ elevations were not significantly different between SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

Figures 4A and 4B may suggest that, whilst agonist-mediated peak $[Ca^{2+}]_i$ elevations are not significantly different between SH-SY5Y and SH-SY5Y$_{RA6}$ cells, the responses are shorter in SH-SY5Y$_{RA6}$ as compared with SH-SY5Y cells. Analysis of the areas under the curves (AUC) between 0s and 200s revealed that for $[Ca^{2+}]_i$ responses to methacholine (1mM), AUCs were not significantly different between SH-SY5Y and SH-SY5Y$_{RA6}$ cells (27665 ± 5291nM/s, n=7 versus 20955 ± 3274nM/s, n=7). Analysis of the AUCs between 0s and 200s for bradykinin (10μM)-mediated $[Ca^{2+}]_i$ responses revealed that the AUC was significantly ($p< 0.05$) less in SH-SY5Y$_{RA6}$ cells as compared with SH-SY5Y cells (5911 ± 285nM/s, n=3 versus 14246 ± 1518nM/s, n=3).

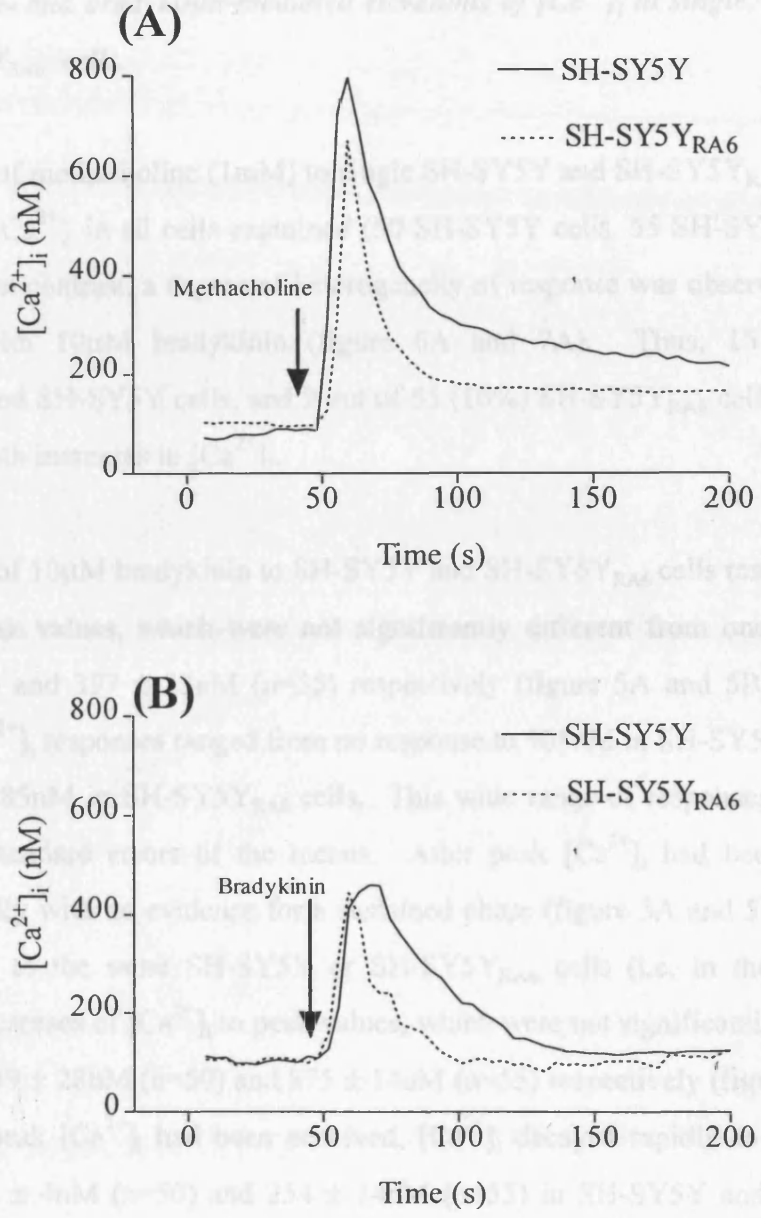


Figure 4 Time course of $[Ca^{2+}]_i$ elevations in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells in response to a maximally effective dose of (A) methacholine (1mM) and (B) bradykinin (10μM). Data are representative traces of at least three similar experiments.

Methacholine- and bradykinin-mediated elevations of $[Ca^{2+}]_i$ in single, adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

The addition of methacholine (1mM) to single SH-SY5Y and SH-SY5Y$_{RA6}$ cells resulted in a rapid rise in $[Ca^{2+}]_i$ in all cells examined (50 SH-SY5Y cells, 55 SH-SY5Y$_{RA6}$ cells) (figure 6B and7B). In contrast, a degree of heterogeneity of response was observed when cells were stimulated with 10μM bradykinin (figure 6A and 7A). Thus, 15 out of 50 (30%) undifferentiated SH-SY5Y cells, and 9 out of 55 (16%) SH-SY5Y$_{RA6}$ cells did not respond to bradykinin with increases in $[Ca^{2+}]_i$.

The addition of 10μM bradykinin to SH-SY5Y and SH-SY5Y$_{RA6}$ cells resulted in increases of $[Ca^{2+}]_i$ to peak values, which were not significantly different from one another, of 393 ± 44nM (n=50) and 397 ± 33nM (n=55) respectively (figure 5A and 5B). The bradykinin-mediated $[Ca^{2+}]_i$ responses ranged from no response to 908nM in SH-SY5Y cells and from no response to 885nM in SH-SY5Y$_{RA6}$ cells. This wide range of responses is reflected by the size of the standard errors of the means. After peak $[Ca^{2+}]_i$ had been acheived, $[Ca^{2+}]_i$ decayed rapidly with no evidence for a sustained phase (figure 5A and 5B). The addition of methacholine to the same SH-SY5Y or SH-SY5Y$_{RA6}$ cells (i.e. in the same experiment) resulted in increases of $[Ca^{2+}]_i$ to peak values, which were not significantly different from one another, of 889 ± 28nM (n=50) and 875 ± 14nM (n=55) respectively (figures 5A, 5B, 6B and 7B). After peak $[Ca^{2+}]_i$ had been acheived, $[Ca^{2+}]_i$ decayed rapidly to a sustained plateau phase of 244 ± 4nM (n=50) and 254 ± 14nM (n=55) in SH-SY5Y and SH-SY5Y$_{RA6}$ cells respectively (measurements are at 250s after the addition of methacholine) (figure 5A and 5B). Observing figure 6B compared with figure 7B, it seems that the $[Ca^{2+}]_i$ responses following challenge with methacholine were greater in SH-SY5Y compared with SH-SY5Y$_{RA6}$ cells. The reasons for this are likely to be that the cell bodies are much smaller in the SH-SY5Y$_{RA6}$ cells, and in the undifferentiated SH-SY5Y cells, the cells were clumped together. Moreover, the figures are pictures representing a small area of total cells in one of three different experiments.

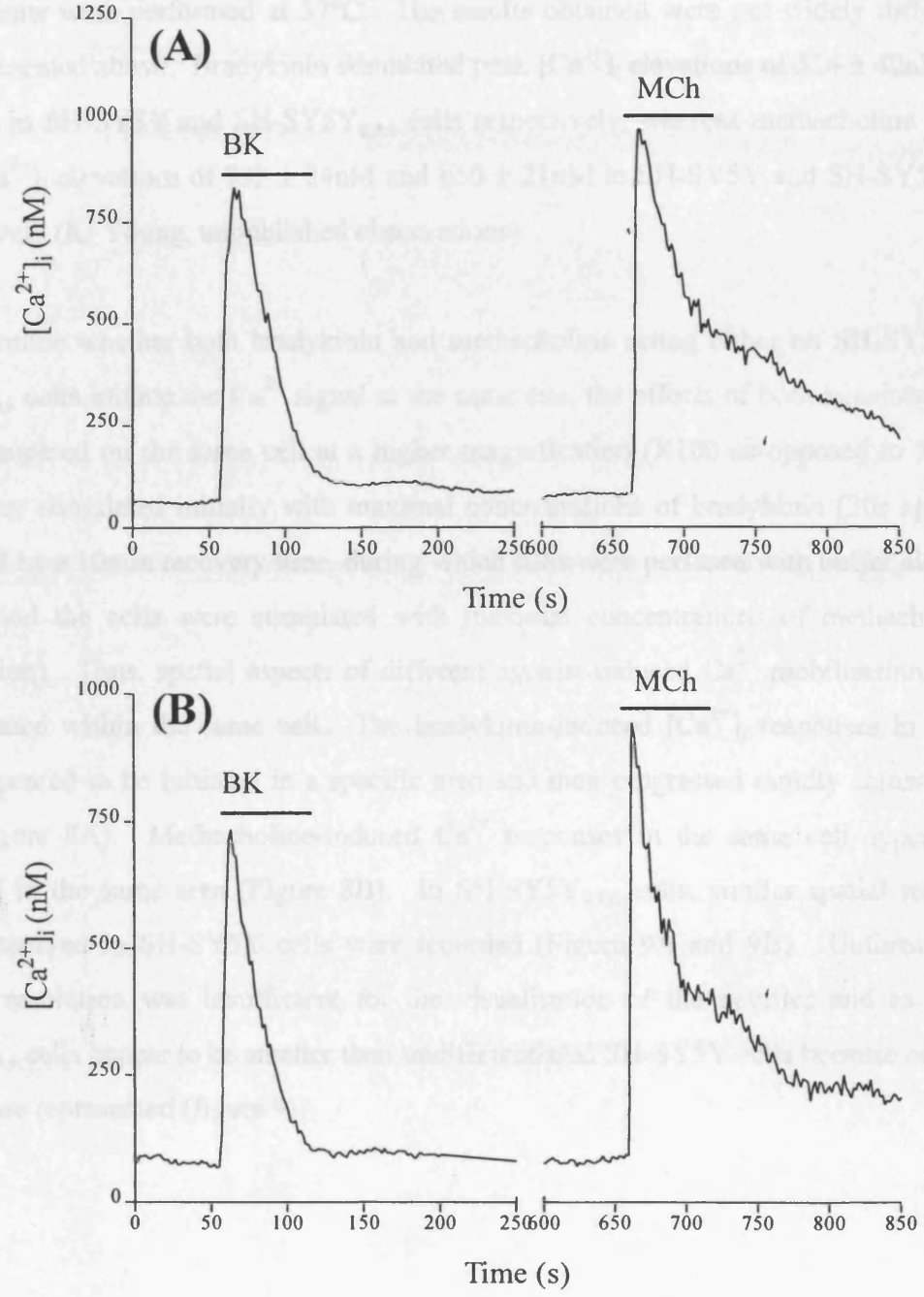


Figure 5 *Time-course of agonist-specific $[Ca^{2+}]_i$ signalling in single, adherent SH-SY5Y (A) and SH-SY5Y$_{RA6}$ (B) cells. Cells loaded with fura-2 were superfused with Krebs-HEPES buffer for 3-5min before the addition of 10μM bradykinin for 250s. Following 5min perfusion with buffer, 1mM methacholine was added for 250s. Data are representative traces of responses from a single cell of three similar experiment*

The above experiments were performed at 22°C, however, recently a similar group of experiments were performed at 37°C. The results obtained were not widely different from those presented above. Bradykinin stimulated peak $[Ca^{2+}]_i$ elevations of 514 ± 42nM and 390 ± 38nM in SH-SY5Y and SH-$SY5Y_{RA6}$ cells respectively, whereas methacholine stimulated peak $[Ca^{2+}]_i$ elevations of 732 ± 24nM and 650 ± 21nM in SH-SY5Y and SH-$SY5Y_{RA6}$ cells respectively (K. Young, unpublished observations).

To determine whether both bradykinin and methacholine acting either on SH-SY5Y or SH-$SY5Y_{RA6}$ cells initiate the Ca^{2+} signal at the same site, the effects of both agonists on $[Ca^{2+}]_i$ were compared on the same cell at a higher magnification (X100 as opposed to X40). The cells were stimulated initially with maximal concentrations of bradykinin (30s application), followed by a 10min recovery time, during which cells were perfused with buffer alone. After this period the cells were stimulated with maximal concentrations of methacholine (30s application). Thus, spatial aspects of different agonist-induced Ca^{2+} mobilisations could be investigated within the same cell. The bradykinin-induced $[Ca^{2+}]_i$ responses in SH-SY5Y cells appeared to be initiated in a specific area and then progressed rapidly across the entire cell (Figure 8A). Methacholine-induced Ca^{2+} responses in the same cell appeared to be initiated in the same area (Figure 8B). In SH-$SY5Y_{RA6}$ cells, similar spatial responses to those observed in SH-SY5Y cells were recorded (Figure 9A and 9B). Unfortunately, the camera resolution was insufficient for the visualisation of the neurites and as such, SH-$SY5Y_{RA6}$ cells appear to be smaller than undifferentiated SH-SY5Y cells because only the cell bodies are represented (figure 9).

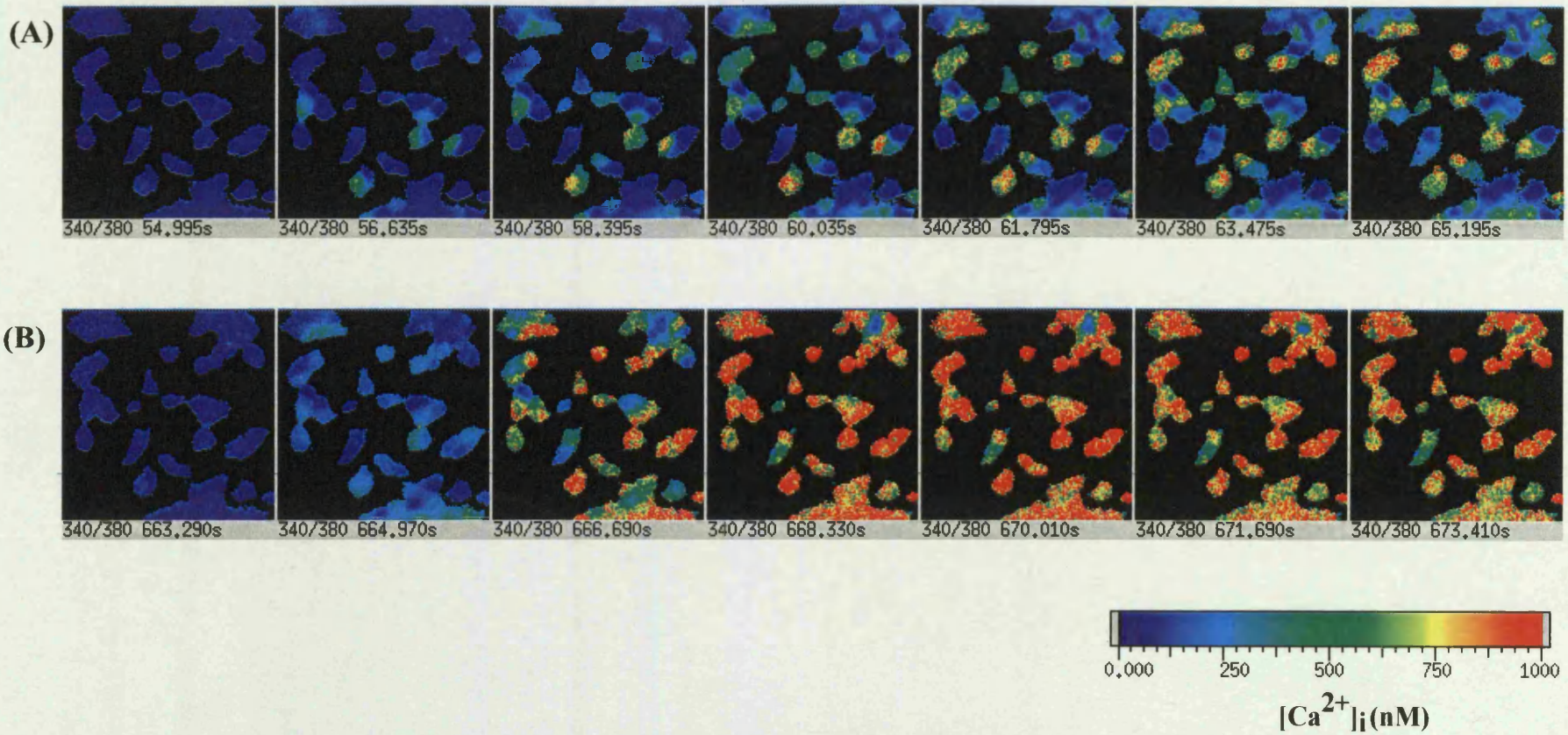


Figure 6 Changes in $[Ca^{2+}]_i$ in the same adherent SH-SY5Y cells following challenge with (A) bradykinin (10μM) and (B) methacholine (1mM).

Images of fura-2 fluorescence (magnification x40, sample rate 1.6s) were converted to approximate $[Ca^{2+}]_i$ as described in Methods (5.2). Traces are representative of 3 individual experiments.

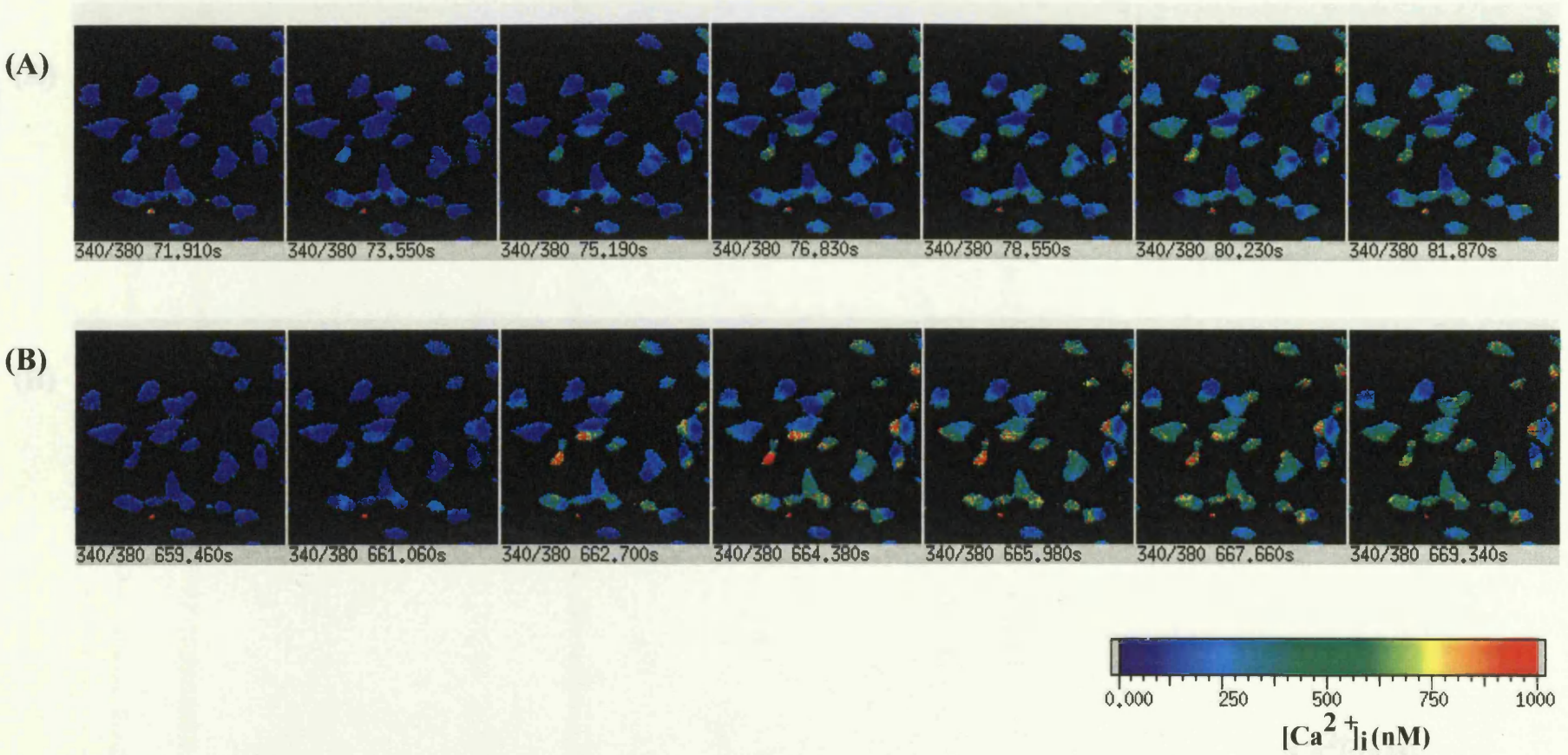


Figure 7 Changes in $[Ca^{2+}]_i$ in the same adherent SH-SY5Y$_{RA6}$ cells following challenge with (A) bradykinin (10μM) and (B) methacholine (1mM).

Images of fura-2 fluorescence (magnification x40, sample rate 1.6s) were converted to approximate $[Ca^{2+}]_i$ as described in Methods. Traces are representative of 3 individual experiments.

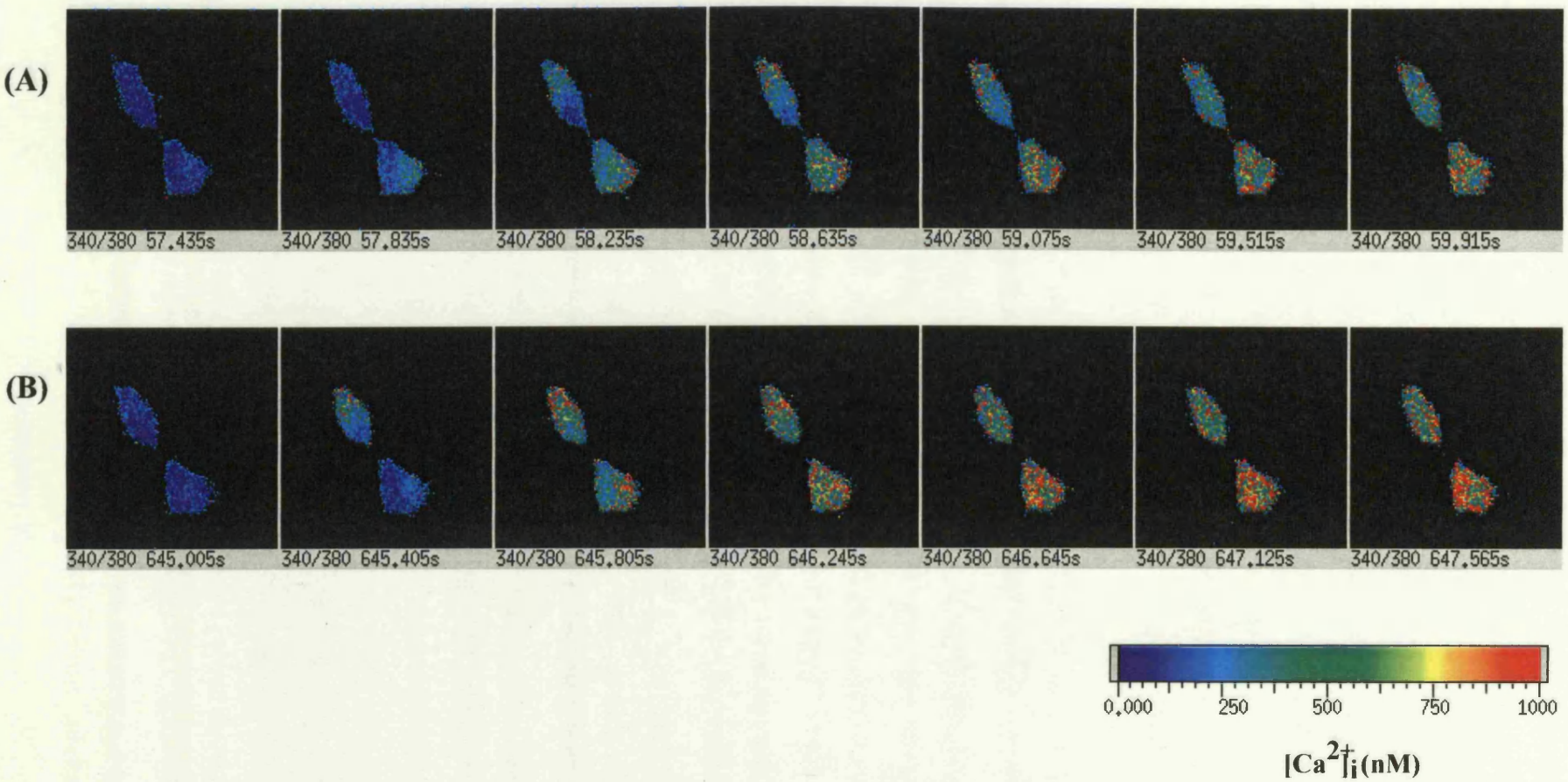


Figure 8 Changes in $[Ca^{2+}]_i$ in the same adherent SH-SY5Y cells following challenge with (A) bradykinin (10μM) and (B) methacholine (1mM).

Images of fura-2 fluorescence (magnification x100, sample time 0.4s) were converted to approximate $[Ca^{2+}]_i$ as described in Methods (5.2). Traces are representative of 3 individual experiments.

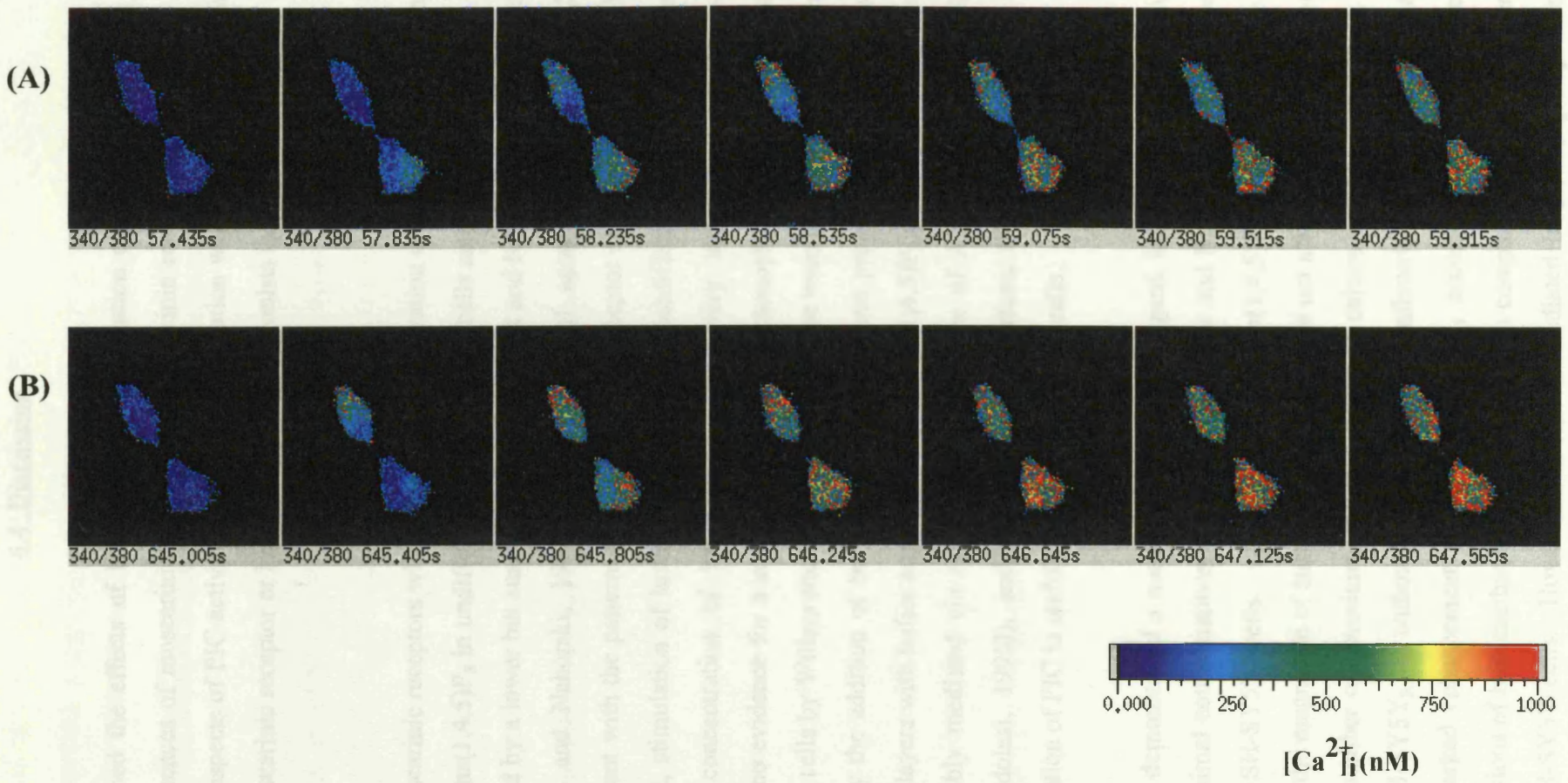


Figure 9 Changes in $[Ca^{2+}]_i$ in the same adherent SH-SY5Y$_{RA6}$ cells following challenge with (A) bradykinin (10μM) and (B) methacholine (1mM).

Images of fura-2 fluorescence (magnification x100, sample time 0.4s) were converted to approximate $[Ca^{2+}]_i$ as described in Methods (5.2). Traces are representative of 3 individual experiments.

4.4 Discussion

This study examined the effects of RA-induced differentiation of SH-SY5Y cells on the functional consequences of muscarinic receptor and bradykinin receptor activation. To this end, the temporal aspects of PIC activation and $[Ca^{2+}]_i$ elevation were investigated following challenge with muscarinic receptor or bradykinin receptor agonists in both SH-SY5Y and SH-$SY5Y_{RA6}$ cells.

Stimulation of muscarinic receptors with a maximal concentration of methacholine confirmed that elevations of $Ins(1,4,5)P_3$ in undifferentiated SH-SY5Y cells are biphasic, consisting of a rapid peak followed by a lower but sustained phase (Lambert and Nahorski, 1990; Lambert *et al.*, 1991; Willars and Nahorski, 1995). This pattern of agonist-mediated $Ins(1,4,5)P_3$ response is consistent with the pattern observed in many receptor systems (Putney and Bird, 1993). In contrast, stimulation of bradykinin receptors in undifferentiated SH-SY5Y cells, with a maximal concentration of bradykinin, elicited very small peak elevations of $Ins(1,4,5)P_3$, with no evidence for a sustained phase, i.e. a monophasic response (previously described for these cells by Willars and Nahorski (1995)), that were not significantly different to those evoked by the addition of buffer alone. It has been proposed that stimulation of adherent cell monolayers with buffer can provoke small $Ins(1,4,5)P_3$ responses due to a shear-stress effect, possibly mediated via the endogenous release of ATP (Nolert *et al.*, 1990; Grierson and Meldolesi, 1995)), and it is possible, therefore, that bradykinin does not stimulate the activation of PIC in undifferentiated SH-SY5Y cells.

SH-$SY5Y_{RA6}$ cells demonstrated a marked enhancement of peak $Ins(1,4,5)P_3$ accumulations in response to maximal concentrations of both methacholine and bradykinin compared with those elicited in SH-SY5Y cells. Basal levels of $Ins(1,4,5)P_3$, the time course of accumulations and the magnitude of sustained responses were not affected. It is worth noting that on a limited number of occasions, it was possible to culture cells for 28 days in the presence of RA (SH-$SY5Y_{RA28}$) without the cells becoming infected. SH-$SY5Y_{RA28}$ cells also demonstrated a marked enhancement of peak $Ins(1,4,5)P_3$ accumulations in response to maximal concentrations of both methacholine and bradykinin compared with those elicited in undifferentiated SH-SY5Y cells. However, the agonist-mediated $Ins(1,4,5)P_3$ responses in SH-$SY5Y_{RA28}$ cells were virtually identical to those observed in SH-$SY5Y_{RA6}$ cells. These

findings are in direct contrast with similar studies in TPA-differentiated SH-SY5Y where, following differentiation, a pronounced reduction of agonist-mediated phosphoinositide hydrolysis was observed (Cioffi and Fisher, 1990). However, it is likely that RA and TPA induce SH-SY5Y cells to differentiate along different pathways (Pahlman et al., 1984) which may account for these observations.

The transient nature of peak Ins(1,4,5)P_3 accumulations following challenge with either methacholine or bradykinin imply desensitisation of receptor-G-protein-PIC coupling. Given the biphasic nature of the response to methacholine compared with monophasic responses to bradykinin, desensitisation of the muscarinic receptor appears to be partial, whereas that of the bradykinin receptor must be complete. There are several mechanisms by which desensitisation of the response may occur. There may be a rapid depletion PtdIns(4,5)P_2 pools following agonist stimulation, and this appears to be rapid enough to account for the rapid desensitisation of the response (Fisher *et al.*, 1994). Although the desensitisation is too rapid to be accounted for by down-regulation of receptors or G-proteins (Tobin et al., 1992; Fisher et al., 1994; Mitchell et al., 1993), it is possible that the effective receptor population may be reduced by rapid mechanisms such as receptor phosphorylation, which may alter the ability of the receptor to couple to and activate G-proteins. Indeed, phosphorylation of a number of PIC-linked receptors has been reported (Kuppelberg *et al.*, 1992; Kwatra *et al.*, 1993), and phosphorylation of the muscarinic M3 receptor has been shown to occur within seconds of exposure to agonist (Tobin *et al.*, 1993) and as such, is rapid enough to account for the observed desensitisation.

The observed increase in the magnitude of peak agonist-stimulated Ins(1,4,5)P_3 accumulations in SH-SY5Y_{RA6} cells could be caused either by a potentiation of Ins(1,4,5)P_3 production or by an inhibition of Ins(1,4,5)P_3 breakdown. The possibility that the rate of Ins(1,4,5)P_3 breakdown was less rapid in SH-SY5Y_{RA6} cells, and thus responsible for the increased peak Ins(1,4,5)P_3 accumulation in response to agonist in these cells, was resolved by the analysis of the metabolism of [^{3}H]-inositol labelled inositol phosphates in the presence of Li^+, which provides a measure of the rate of PI breakdown and therefore PIC activity (Wojcikiewicz *et al.*, 1993). In the present studies, [^{3}H]-inositol phosphate accumulation in undifferentiated SH-SY5Y cells appeared to be linear over 25min. However, by measuring accumulation of [^{3}H]-inositol phosphates over the first few seconds after addition of agonist, a biphasic

accumulation of [^{3}H]-inositol phosphates was observed. These data are consistent with previous studies in both SH-SY5Y and CHO-M3 cell lines stimulated with carbachol (Wojcikiewicz *et al.*, 1993) where there was a decrease in the rate of accumulation following 20-30s of agonist exposure. Hence, in many studies where a lack of desensitisation of agonist-mediated PI hydrolysis has been reported, the first time points of inositol phosphate accumulation have often been taken several minutes after the addition of agonist, thus the early changes would have been missed (Wojcikiewicz *et al.*, 1994). It appears likely that the early change in the rate of [^{3}H]-inositol phosphate accumulation corresponds to a change in the rate of Ins(1,4,5)P_3 formation as the reduction of the rate of [^{3}H]-inositol phosphate accumulation corresponds temporally with the rapid change in Ins(1,4,5)P_3 formation. This reduction in the rate of PIC activity is likely to represent a rapid desensitisation of the PIC response (Willars and Nahorski, 1995). The biphasic [^{3}H]-inositol phosphate response following muscarinic receptor activation was greater in RA-treated SH-SY5Y cells, demonstrating an augmented muscarinic receptor-mediated PIC activation, as opposed to a decreased rate of Ins(1,4,5)P_3 metabolism.

Total [^{3}H]-inositol phosphate accumulation following challenge with bradykinin was initially rapid but small in both SH-SY5Y and SH-SY5Y$_{RA6}$ cells, the increase was, however, greater in the differentiated cells. These findings demonstrate that the lack of a sustained phase in the bradykinin-evoked Ins(1,4,5)P_3 responses was not due to enhanced metabolism of Ins(1,4,5)P_3 as there was a lack of continued accumulation of [^{3}H]-inositol phosphates. These data also suggest that the Ins(1,4,5)P_3 response elicited following challenge of undifferentiated SH-SY5Y cells with bradykinin is likely to be the result of a very small bradykinin-mediated PIC activation (as opposed to a shear-stress response) (see also Willars and Nahorski (1995)) since there is a rapid, but small, accumulation of [^{3}H]-InsPs in these cells which is absent following stimulation with buffer alone.

The results from this investigation suggest that RA-induced differentiation of SH-SY5Y cells results in an augmented agonist-induced PIC activation concomitant with up-regulation of muscarinic M3 and bradykinin receptors. The regulation of PIC activation is governed by a range of factors (see chapter 1), the expression and/or regulation of which may be altered following treatment of SH-SY5Y with RA. For instance, the expression of the Gq/11 family of G-proteins may be modified following differentiation which could result in an alteration of

the efficiency of signal transduction. Indeed, there is evidence to suggest that RA has a specific effect on the abundance of G-protein subunits in SH-SY5Y cells, which may contribute to functional changes in transmembrane signalling pathways in the differentiated cells (Ammer and Schulz, 1994). Alternatively, the regulation of the effective receptor number via kinase phosphorylation following agonist occupation may be differentially effected by RA treatment. It is possible that substrate availability is effected by the treatment of SH-SY5Y cells with RA, which would almost certainly effect the ability of agonist to generate Ins(1,4,5)P_3. PIC activity is dependent on Ca^{2+} (see chapter 1), and it therefore follows that if the way in which Ca^{2+} regulates PIC is changed following RA-induced differentiation it is likely that agonist-mediated Ins(1,4,5)P_3 production will be affected.

Stimulation of muscarinic receptors in undifferentiated SH-SY5Y cells elicited biphasic increases in $[Ca^{2+}]_i$ consisting of a rapid peak, followed by a lower, but sustained phase, as previously reported in these cells (Akerman, 1989; Lambert and Nahorski, 1990; Murphy *et al.*, 1991; Willars and Nahorski, 1995). These changes in $[Ca^{2+}]_i$ were similar in temporal pattern to the methacholine-induced, biphasic elevations of Ins(1,4,5)P_3. Similarly, stimulation of bradykinin receptors in undifferentiated SH-SY5Y cells evoked monophasic $[Ca^{2+}]_i$ elevations which were similar in temporal pattern to bradykinin-mediated Ins(1,4,5)P_3 accumulations. There is substantial evidence that the peak $[Ca^{2+}]_i$ responses are a result of Ca^{2+} release from intracellular stores by the action of Ins(1,4,5)P_3 on receptor-gated ion channels localised in specialised regions of the ER (Berridge, 1993). The sustained phase of $[Ca^{2+}]_i$ elevation following muscarinic receptor activation has previously been reported to be dependent on extracellular Ca^{2+} (Lambert and Nahorski, 1990; Murphy *et al.*, 1991) and therefore appears to be due to Ca^{2+} influx. The mechanism by which Ca^{2+} entry occurs is not fully understood and a subject of controversy. There is a great deal of evidence in favour of "capacitative" Ca^{2+} entry following store depletion (Putney, 1990; Putney and Bird, 1993; Berridge, 1995), although the method by which the stores communicate with Ca^{2+} channels in the membrane remains unknown. One suggestion is that a second messenger such as Ca^{2+} influx factor (CIF), which has been proposed to be released into the cytoplasm following store depletion (Randriamampita and Tsien, 1993; Davies and Hallett, 1995) mediates Ca^{2+} entry. However, there is also a theory that the information is transferred via the Ins(1,4,5)P_3 receptor whereby, as a result of Ca^{2+} store emptying, the Ins(1,4,5)P_3 receptor undergoes a conformational change which is then transmitted to second messenger-operated Ca^{2+} channels

(SMOCCs) in the plasma membrane which induces Ca^{2+} influx from the extracellular medium (Irvine, 1990; Berridge, 1995, 1996). For this model to work, Ins(1,4,5)P_3 receptors must be located sufficiently close to the plasma membrane in order to contact the SMOCCs.

In the current study, contrary to peak Ins(1,4,5)P_3 accumulations, peak $[Ca^{2+}]_i$ elevations in response to either methacholine or bradykinin were not effected by RA treatment. This phenomenon may represent a spare capacity within the system, i.e. generation of Ins(1,4,5)P_3 is above that required to trigger a maximal $[Ca^{2+}]_i$ response. Alternatively, agonist-mediated generation of Ca^{2+} signals in SH-SY5Y and SH-SY5Y_{RA6} cells may alter in their sensitivity to activation of PKC. Indeed, in hepatocytes the generation of Ca^{2+} signals mediated by vasopressin, angiotensin II and ATP are affected differentially by the activation of PKC (Woods *et al.*, 1987; Sanchez-Bueno *et al.*, 1990). Another explanation is that in SH-SY5Y_{RA6} cells, Ca^{2+} is actually being mobilised to a greater extent in response to agonist than in SH-SY5Y cells, but is masked by an increased rate of either sequestration into intracellular organelles or extrusion from the cell via Ca^{2+}ATPases. Indeed agonist modulation of the plasma membrane pump has previously been demonstrated in pancreatic acini (Zhang et al., 1992) and in SH-SY5Y cells (Wojcikiewicz *et al.*, 1994). Analysis of the areas under the agonist-stimulated $[Ca^{2+}]_i$ versus time curves for maximally effective concentrations of either methacholine or bradykinin revealed that the $[Ca^{2+}]_i$ response elicited by bradykinin, but not by methacholine, was shorter in SH-SY5Y_{RA6} cells. These data suggest that bradykinin may activate Ca^{2+}ATPases to a greater extent in SH-SY5Y_{RA6} cells compared with SH-SY5Y cells. However, it is unlikely that increased agonist-mediated activation of Ca^{2+}ATPases would limit the peak of $[Ca^{2+}]_i$ elevation acheived in response to agonist (Young *et al.*, 1997).

An alternative explanation for the observed phenomena is that the production of Ins(1,4,5)P_3 is compartmentalised, such that the concentration of the second messenger at the site of Ca^{2+} release is underestimated in SH-SY5Y cells. Compartmentalisation of components of the signalling pathway in this manner is an attractive theory as it would allow for the maintenance of signalling specificity following the activation of a receptor that shares its signalling pathway with other receptor types on the same cell. In support of this theory, compartmentalisation of components of the phosphoinositide signalling pathway has previously been demonstrated in pancreatic acini (Xu *et al.*, 1996a; Tortorici *et al.*, 1994). However, there is recent evidence to suggest that the regulation of Ca^{2+} mobilisation occurs

via complex processes that are little understood. For example, it has been proposed that agonists, at concentrations which are not sufficient to activate PIC, can still initiate Ca^{2+} release by increasing the affinity of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} release channel for $Ins(1,4,5)P_3$ (Xu *et al.*, 1996b).

Although distinct patterns of Ca^{2+} signalling were observed after the addition of the individual agonists, methacholine and bradykinin, to populations of SH-SY5Y cells, effects at the single cell level may have been markedly different. Similarly, although the $[Ca^{2+}]_i$ responses in cell populations following agonist challenge were unaltered following RA-induced differentiation, it is possible that differentiation may have an effect on the pattern of $[Ca^{2+}]_i$ responses in single cells. In the present study, the addition of methacholine or bradykinin to single SH-SY5Y cells resulted in the generation of Ca^{2+} signals that exhibited very similar profiles to those previously observed for both $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ elevations in cell populations. Two considerations lend support to the notion that these distinctive Ca^{2+} signals can be generated within single neuroblastoma cells. First, the SH-SY5Y cell line is clonal and has been thrice cloned from the parent SK-N-SH cell (Biedler *et al.*, 1973), thus reducing (but not eliminating) the possibility of cell heterogeneity. Second, whereas not all cells responded to bradykinin, Ca^{2+} signals were observed after the addition of methacholine in 100% of cells tested. Collectively these results indicate that distinct agonist-specific Ca^{2+} signals can be elicited within an individual SH-SY5Y cell after activation of pharmacologically distinct receptors coupled to the activation of PIC. The wide range of the magnitude of peak bradykinin-mediated $[Ca^{2+}]_i$ responses (from no response to 908nM in SH-SY5Y cells and from no response to 885nM in SH-$SY5Y_{RA6}$ cells) highlights a cell-to-cell variation in the characteristics of Ca^{2+} signals elicited by the same agonist. Similar observations have been made in other clonal cell lines (Ambler *et al.*, 1988; Prentki *et al.*, 1988; Palmer *et al.*, 1994) and although the reason for this intracellular variation is unknown, it is unlikely to be related to either the degree of fura-2 loading or to the specific stages of the cell cycle (Grynkiewicz *et al*, 1985).

The $[Ca^{2+}]_i$ responses elicited in single SH-SY5Y and SH-$SY5Y_{RA6}$ cells following challenge with methacholine were not significantly different (and in agreement with results obtained in cell populations). However, slight differences were observed for bradykinin in that 30% of SH-SY5Y cells compared with 16% of SH-$SY5Y_{RA6}$ cells did not respond to bradykinin.

Whether such differences are related to the measurable increases in $Ins(1,4,5)P_3$ is a complicated issue, mainly due to the technical procedures used to obtain these measures (i.e. cell populations versus single cells). However, studies in permeabilised hepatocytes have indicated that the $Ins(1,4,5)P_3$ concentration attained may be important in determining the percentage of cells that respond to an agonist (Renard-Rooney *et al.*, 1993). The present studies would support that conclusion because fewer SH-SY5Y cells responded to bradykinin than SH-SY5Y$_{RA6}$ cells, and SH-SY5Y$_{RA6}$ cells generate more $Ins(1,4,5)P_3$ following bradykinin challenge than SH-SY5Y cells. It may also be possible that there is a variation of bradykinin receptor density from cell-to-cell, which would influence the ability of cells to respond to bradykinin and therefore may account for the heterogeneity of the response from cell to cell.

Using single, isolated cells the spatial aspects of $[Ca^{2+}]_i$ signals generated by bradykinin and methacholine were examined to investigate the possible occurrence of localised changes in $[Ca^{2+}]_i$. The results obtained indicated that in both SH-SY5Y and SH-SY5Y$_{RA6}$ cells, the Ca^{2+} signal was initiated in a specific area and was rapidly propagated throughout the cell. This initiation site appeared not to be agonist-specific and may represent an area of relatively low cell volume, hence for a given amount of $Ins(1,4,5)P_3$, the relative local concentration in this area will be higher. These findings are in contrast to observations made by Xu *et al.* (1996) who reported that the PIC-linked agonists, carbachol, bombesin and cholesystokinin initiate $[Ca^{2+}]_i$ signals in different regions of pancreatic acinar cells, even when supramaximal concentrations of agonist were used.

In summary, following RA-induced differentiation of SH-SY5Y cells an increased peak $Ins(1,4,5)P_3$ response mediated by both methacholine and bradykinin is manifest concomitant with increased expression levels of both muscarinic and bradykinin receptors. In contrast, agonist-mediated peak $[Ca^{2+}]_i$ elevations were unaffected, either in cell populations or at the single cell level. Single cell $[Ca^{2+}]_i$ imaging revealed slight differences in bradykinin-mediated Ca^{2+} signalling between SH-SY5Y and SH-SY5Y$_{RA6}$ cells, which could possibly be explained by the differing bradykinin-mediated $Ins(1,4,5)P_3$ generations in these cells. Several potential mechanisms which could explain the observed differences have been outlined, certain aspects of which have been investigated and are presented in subsequent chapters.

5. CONCENTRATION-DEPENDENCY OF AGONIST-STIMULATED PIC ACTIVATION AND CHANGES IN $[Ca^{2+}]_i$ AND THE EFFECT OF PKC ACTIVATION OR INHIBITION ON INS(1,4,5)P_3 GENERATION IN SH-SY5Y AND SH-SY5Y_{RA6} CELLS.

5.1 Introduction

The previous chapters described how the human neuroblastoma cell line, SH-SY5Y, utilised throughout this study, expresses muscarinic M3 receptors and bradykinin (which are likely to be B2) receptors. Agonist occupation of either muscarinic or bradykinin receptors present on SH-SY5Y cells resulted in the activation of PIC and changes in $[Ca^{2+}]_i$ as revealed by Ins(1,4,5)P_3 generation, total [^{3}H]-inositol phosphate accumulation and fluorimetric $[Ca^{2+}]_i$ measurements. Following treatment with the differentiating agent, RA, PIC activation was greater following agonist challenge of both muscarinic and bradykinin receptors, concomitant with an up-regulation of muscarinic (M3) and bradykinin receptors, as determined by radioligand binding saturation analysis. In contrast, agonist-mediated $[Ca^{2+}]_i$ elevations were unaffected by RA treatment. Possible explanations for this anomaly are outlined in section 4.4.

A strong candidate as an explanation for the anomaly between agonist-stimulated PIC activation and $[Ca^{2+}]_i$ elevation in SH-SY5Y_{RA6} cells is the possibility that there is a spare capacity within the system. In other words, the generation of Ins(1,4,5)P_3 in response to a maximally effective concentration of agonist is above that required to trigger a maximal $[Ca^{2+}]_i$ response. Indeed, in many tissues and cells, dose-response curves for agonist-mediated PIC activation lie to the left of their corresponding occupancy curves, and to the right of functional, downstream responses (Kenakin, 1993). This could indicate a considerable reserve of Ins(1,4,5)P_3, or could be the result of the involvement of another mechanism. The primary aim of the current chapter was to further investigate receptor-mediated phosphoinositide hydrolysis and changes in $[Ca^{2+}]_i$ in SH-SY5Y and SH-SY5Y_{RA6} cells by examination of the agonist concentration-dependency of the responses.

The sequence of events following the activation of a PIC-coupled receptor has been well established, ie. agonist-bound receptor enhances PIC activity via a G-protein, resulting in the formation of $Ins(1,4,5)P_3$ and DAG. DAG activates PKC which, once activated, can catalyse the phosphorylation of a range of cellular proteins, some of which may play a role in the regulation of cellular events including cell growth and division (Azzi *et al.*, 1992). The activation of PKC has also been implicated in the attenuation of muscarinic receptor-mediated activation of PIC (Vincentini *et al.*, 1985; Pearce *et al.*, 1988; Willars *et al.*, 1996) and $[Ca^{2+}]_i$ elevation (Vincentini *et al.*, 1985; Murphy *et al.*, 1992; Willars *et al.*, 1996). In the above studies, activation of PKC using the tumour-promoting phorbol esters mediated the inhibition of phosphoinositide responses. One previous study from this laboratory explored the role of muscarinic receptor-mediated PKC feedback regulation on agonist-induced phosphoinositide hydrolysis and $[Ca^{2+}]_i$ elevation in the SH-SY5Y cell line (Willars *et al.*, 1996). The results revealed that there was little indication of a feedback inhibition by agonist-activated PKC in this cell line. Cellular differentiation, however, has been demonstrated to alter the expression of PKC isoforms in a number of cell lines. For instance, it has been demonstrated that NGF-differentiation of PC12 cells is associated with the up-regulation of PKC-βII (Wooten *et al.*, 1991) and PMA differentiation of HL-60 cells is associated with alterations in the relative expression levels of PKC isoforms (Lee *et al.*, 1995). It is possible, therefore, that the relative expression levels of the PKC isoforms are altered following RA-differentiation of SH-SY5Y cells, and it thus follows that the way in which PKC regulates the phosphoinositide pathway in $SH\text{-}SY5Y_{RA6}$ cells may also be altered. The secondary aim of the current chapter, therefore, was to examine the potential regulatory influence of PKC, activated by either phorbol ester or agonist occupation of muscarinic or bradykinin receptors, on PIC activation in SH-SY5Y and $SH\text{-}SY5Y_{RA6}$ cells.

5.2 Methods

The following studies were performed in adherent SH-SY5Y and $SH\text{-}SY5Y_{RA6}$ cells cultured and prepared as described in General Methods (see sections 2.1, 2.2 and 2.3). $Ins(1,4,5)P_3$ mass accumulation, total $[^3H]$-inositol phosphate accumulation and population $[Ca^{2+}]_i$ elevations following stimulation with agonist at the appropriate concentration and incubation for the appropriate length of time at 37°C were assessed as described in General Methods

(sections 2.4, 2.5 and 2.6.2 respectively). Where required the PKC inhibitor, Ro-318220 (10μM), was added 10min before and phorbol 12,13-dibutyrate (PDBu) (1μM), 5min before agonist challenge.

Data analysis.

Raw concentration-response data were analysed using the curve fitting program Graph-Pad Prism (GraphPad Software, Inc.). Data were fitted to a logistic four parameter function (with equal weighting to each point) in order to obtain estimates of the maximum response and EC_{50}. Multiple comparisons between group means were by Duncan's multiple range test at $p< 0.05$ and $p< 0.01$ following one-way ANOVA. All data are presented as means ± S.E.M.

5.3 Results

Concentration-dependence of agonist-stimulated Ins(1,4,5)P_3 generation in SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

The concentration-dependency of methacholine-, arecoline- and bradykinin-stimulated Ins(1,4,5)P_3 generation was examined at the peak phases of the biphasic responses (10s for methacholine and arecoline, 5s for bradykinin) in both SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

In SH-SY5Y and SH-SY5Y$_{RA6}$ cells, peak Ins(1,4,5)P_3 accumulation in response to methacholine was dose-dependent. Methacholine was more efficacious in SH-SY5Y$_{RA6}$ cells compared to SH-SY5Y cells ($p < 0.01$) as revealed by a decrease in the EC_{50} value and an increase in the maximal Ins(1,4,5)P_3 response to methacholine (Figure 1(A) and Table 1). The augmented muscarinic receptor-mediated Ins(1,4,5)P_3 response was further investigated using the muscarinic receptor partial agonist, arecoline. Peak Ins(1,4,5)P_3 elevations in response to arecoline were also concentration-dependent. RA treatment significantly ($p < 0.01$) increased the maximal Ins(1,4,5)P_3 response to arecoline, without altering the EC_{50} value (Figure 1(B) and Table 1).

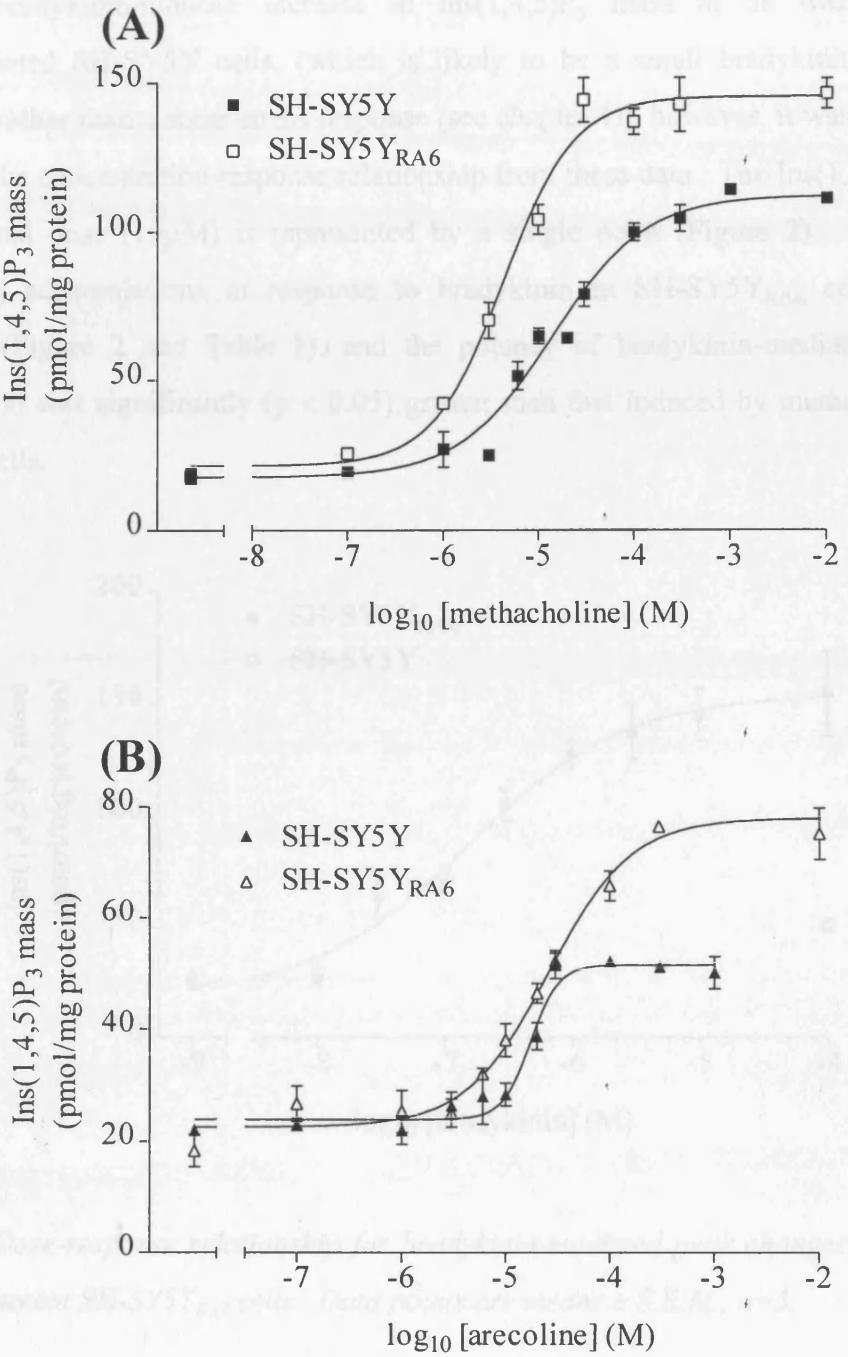


Figure 1 *Dose-response relationships for (A) methacholine- and (B) arecoline-mediated peak changes in Ins(1,4,5)P_3 mass in adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells. Data points are means ± S.E.M., n=3.*

A small bradykinin-induced increase in $Ins(1,4,5)P_3$ mass at 5s was detectable in undifferentiated SH-SY5Y cells, (which is likely to be a small bradykinin-mediated PIC activation, rather than a shear-stress response (see chapter 4)), however, it was not possible to determine the concentration-response relationship from these data. The $Ins(1,4,5)P_3$ response to a maximal dose (10μM) is represented by a single point (Figure 2). However, peak $Ins(1,4,5)P_3$ accumulations in response to bradykinin in SH-$SY5Y_{RA6}$ cells were dose-dependent (Figure 2 and Table 1), and the potency of bradykinin-mediated $Ins(1,4,5)P_3$ accumulation was significantly ($p < 0.05$) greater than that induced by methacholine in SH-$SY5Y_{RA6}$ cells.

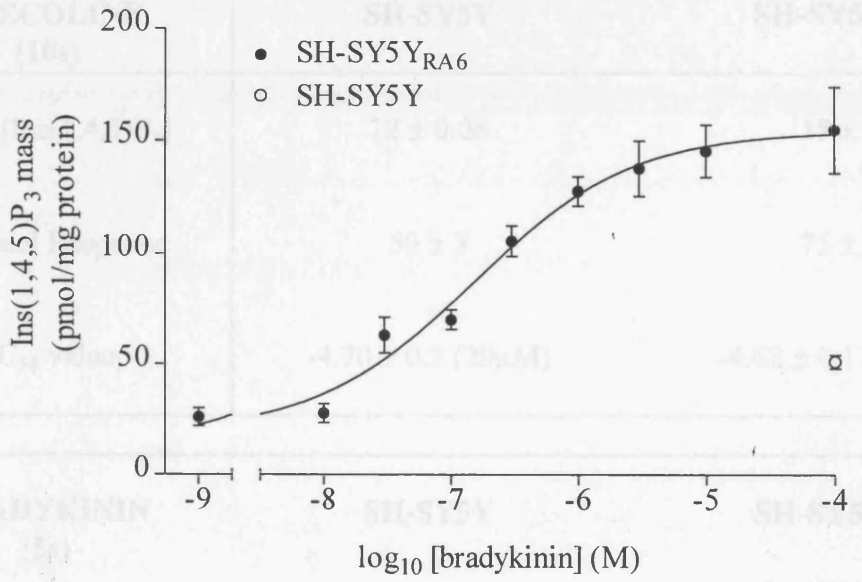


Figure 2 Dose-response relationship for bradykinin-mediated peak changes in $Ins(1,4,5)P_3$ mass in adherent SH-$SY5Y_{RA6}$ cells. Data points are means ± S.E.M., n=3.

METHACHOLINE (10s)	SH-SY5Y	SH-SY5Y$_{RA6}$
Basal [Ins(1,4,5)P_3]	18 ± 2	18 ± 2
Maximal Response	108 ± 4	144 ± 6
EC_{50} value	-4.96 ± 0.03 (11μM)	-5.36 ± 0.07 (4μM)

ARECOLINE (10s)	SH-SY5Y	SH-SY5Y$_{RA6}$
Basal [Ins(1,4,5)P_3]	22 ± 0.06	18 ± 3
Maximal Response	50 ± 3	75 ± 5
EC_{50} value	-4.70 ± 0.2 (20μM)	-4.62 ± 0.1 (24μM)

BRADYKININ (5s)	SH-SY5Y	SH-SY5Y$_{RA6}$
Basal [Ins(1,4,5)P_3]	28 ± 6	26 ± 4
Maximal Response	51 ± 3	154 ± 19
EC_{50} value	Unmeasurable	-6.54 ± 0.05 (290nM)

Table 1 *Basal and maximal responses (pmol/mg protein) and EC_{50} values (log_{10} M) for methacholine-, arecoline- and bradykinin-mediated increases in Ins(1,4,5)P_3 mass in SH-SY5Y and SH-SY5Y$_{RA6}$ cells. Values are given for peak phases of accumulation and represent means ± S.E.M. for 3 independent experiments.*

Concentration-dependence of agonist-stimulated total [^{3}H]-inositol phosphate accumulation in SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

The concentration-response relationship for total [^{3}H]-inositol phosphate accumulation following 15min exposure to methacholine or arecoline in the presence of 10mM Li^+ was investigated in SH-SY5Y and SH-SY5Y$_{RA6}$ cells. In both SH-SY5Y and SH-SY5Y$_{RA6}$ cells, challenge with methacholine produced similar maximal responses. However, the dose-response curve for undifferentiated SH-SY5Y cells lies to the right of that for SH-SY5Y$_{RA6}$ cells (Figure 3(A) and Table 2). Total [^{3}H]-inositol phosphate accumulation in response to arecoline was also dose-dependent in both SH-SY5Y and SH-SY5Y$_{RA6}$ cells. RA treatment significantly ($p< 0.05$) increased the maximal response elicited following challenge with arecoline, without significantly altering the EC_{50} value (Figure 3(B) and Table 2).

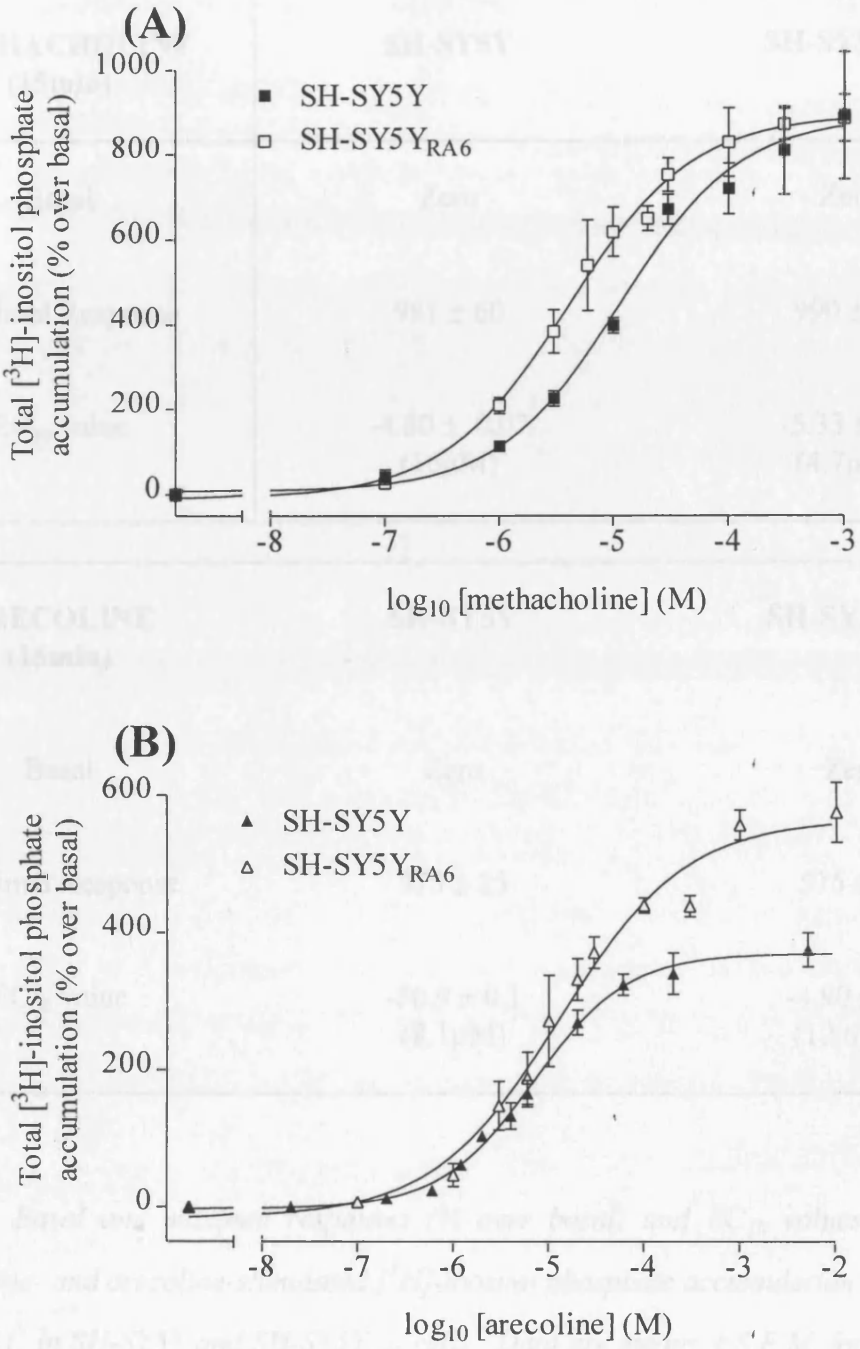


Figure 3 *Dose-response relationships for (A) methacholine- and (B) arecoline-mediated increases in total [^{3}H]-inositol phosphates in adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells. Data shown are percentage increases above basal values and represent the means ± S.E.M., n=3.*

METHACHOLINE (15min)	SH-SY5Y	SH-SY5Y$_{RA6}$
Basal	Zero	Zero
Maximal Response	981 ± 60	990 ± 43
EC_{50} value	-4.80 ± 0.07 (16μM)	-5.33 ± 0.1 (4.7μM)

ARECOLINE (15min)	SH-SY5Y	SH-SY5Y$_{RA6}$
Basal	Zero	Zero
Maximal Response	375 ± 25	575 ± 44
EC_{50} value	-50.9 ± 0.1 (8.1μM)	-4.90 ± 0.1 (12.6μM)

Table 2 *Basal and maximal responses (% over basal) and EC_{50} values (log_{10} M) for methacholine- and arecoline-stimulated [^{3}H]-inositol phosphate accumulation in the presence of 10mM Li^+ in SH-SY5Y and SH-SY5Y$_{RA6}$ cells. Data are means ± S.E.M. for 3 independent experiments.*

Concentration-dependence of agonist-stimulated $[Ca^{2+}]_i$ elevations in SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

The concentration-dependency of methacholine- and bradykinin-stimulated changes in peak intracellular Ca^{2+} concentration was examined in SH-SY5Y and SH-SY5Y$_{RA6}$ cells. In both SH-SY5Y and SH-SY5Y$_{RA6}$ cells, a concentration-dependent increase in $[Ca^{2+}]_i$ was observed in response to either methacholine or bradykinin. Maximal responses to bradykinin were significantly smaller ($p < 0.01$, 2 way ANOVA) than maximal responses to methacholine in both SH-SY5Y and SH-SY5Y$_{RA6}$ cells (compare Figure 3 and 4). The concentration-response relationships for peak $[Ca^{2+}]_i$ elevations elicited following challenge with either methacholine or bradykinin were unaltered following RA treatment in that neither the maximal responses nor the EC_{50} values were different in SH-SY5Y$_{RA6}$ cells compared with SH-SY5Y cells (Figures 4(A), 4(B) and Table 3).

In order to investigate whether the concentration-dependency of agonist-mediated peak $[Ca^{2+}]_i$ responses were unchanged (following RA-differentiation of SH-SY5Y cells) because of compensatory changes in Ca^{2+} influx versus mobilisation, the ability of agonists to mobilise Ca^{2+} from stores was investigated by performing experiments in low extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_e$). Preincubation of SH-SY5Y or SH-SY5Y$_{RA6}$ cells in low $[Ca^{2+}]_e$ buffer (Ca^{2+} excluded from Krebs-HEPES buffer) did not have a significant effect on basal $[Ca^{2+}]_i$ (Tables 3 and 4). Addition of 1mM methacholine increased peak $[Ca^{2+}]_i$ to values which were not significantly reduced compared with the responses evoked at normal $[Ca^{2+}]_i$ in either SH-SY5Y or SH-SY5Y$_{RA6}$ cells. The potency of methacholine-mediated intracellular Ca^{2+} mobilisation was also determined at low $[Ca^{2+}]_e$. The EC_{50} values for the peak phase were not significantly different from values determined at normal $[Ca^{2+}]_e$ in either SH-SY5Y or SH-SY5Y$_{RA6}$ cells (Tables 3 and 4). At low $[Ca^{2+}]_e$, addition of 10μM bradykinin increased $[Ca^{2+}]_i$ to values which were not significantly reduced compared with the responses evoked at normal $[Ca^{2+}]_e$ in either SH-SY5Y or SH-SY5Y$_{RA6}$ cells. The potency of bradykinin-mediated intracellular Ca^{2+} mobilisation was also determined at low $[Ca^{2+}]_e$. The EC_{50} values for the peak phase were not significantly different from values determined at normal $[Ca^{2+}]_e$ in either SH-SY5Y or SH-SY5Y$_{RA6}$ cells (Tables 3 and 4).

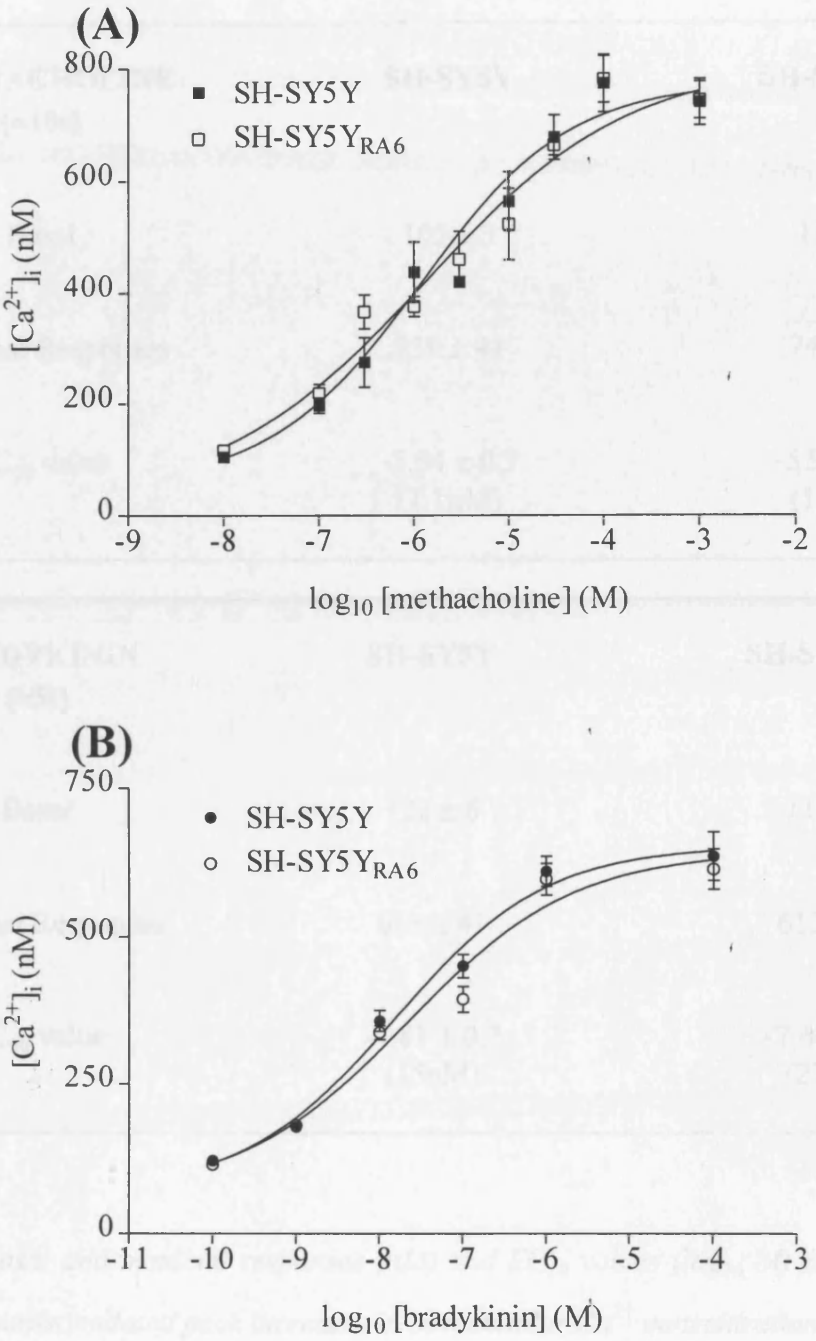


Figure 4 Dose-response relationships for (A) methacholine- and (B) bradykinin-mediated peak elevations of $[Ca^{2+}]_i$ in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells. Data points are means ± S.E.M., n=3-9.

METHACHOLINE (≈10s)	**SH-SY5Y**	**SH-SY5Y$_{RA6}$**
Basal	105 ± 3	110 ± 5
Maximal Responses	739 ± 41	742 ± 33
EC_{50} value	-5.94 ± 0.3 (1.1μM)	-5.90 ± 0.4 (1.3μM)

BRADYKININ (≈5s)	**SH-SY5Y**	**SH-SY5Y$_{RA6}$**
Basal	122 ± 6	115 ± 3
Maximal Responses	635 ± 41	613 ± 34
EC_{50} value	-7.81 ± 0.2 (15nM)	-7.66 ± 0.4 (22nM)

Table 3 Basal and maximal responses (nM) and EC_{50} values (log_{10} M) for methacholine- and bradykinin-stimulated peak increases in intracellular Ca^{2+} concentration in SH-SY5Y and SH-SY5Y$_{RA6}$ cells. Data are means ± S.E.M. for 3-7 independent experiments.

METHACHOLINE (≈10s)	SH-SY5Y	SH-SY5Y$_{RA6}$
Basal	88 ± 6	96 ± 9
Maximal Responses	675 ± 58	693 ± 46
EC_{50} value	-5.90 ± 0.5 (1.3μM)	-5.89 ± 0.3 (1.3μM)

BRADYKININ (≈5s)	SH-SY5Y	SH-SY5Y$_{RA6}$
Basal	102 ± 11	98 ± 9
Maximal Responses	589 ± 53	592 ± 47
EC_{50} value	-7.74 ± 0.4 (18nM)	-7.79 ± 0.6 (16nM)

Table 4 *Basal and maximal responses (nM) and EC_{50} values (log_{10} M) for methacholine- and bradykinin-stimulated peak increases in intracellular Ca^{2+} concentration in SH-SY5Y and SH-SY5Y$_{RA6}$ cells in a low extracellular [Ca^{2+}] buffer. Data are means ± S.E.M. for 3-7 independent experiments.*

Comparison of the relationships between peak Ins(1,4,5)P_3 accumulations and peak $[Ca^{2+}]_i$ elevations in response to methacholine and bradykinin.

The results of this study clearly show that peak Ins(1,4,5)P_3 accumulations and peak $[Ca^{2+}]_i$ elevations following agonist challenge are differentially effected following RA treatment of SH-SY5Y cells. In order to further analyse the relationship between agonist-mediated Ins(1,4,5)P_3 formation and changes in $[Ca^{2+}]_i$, we plotted peak Ins(1,4,5)P_3 accumulations versus peak $[Ca^{2+}]_i$ elevations for both methacholine and bradykinin at a range of concentrations in differentiated and undifferentiated SH-SY5Y cells. The resultant data points were fitted using a second order polynomial equation (GraphPAD Prism, GraphPAD Inc.) (Figure 5). For a given agonist-induced formation of Ins(1,4,5)P_3, the corresponding $[Ca^{2+}]_i$ elevation is different depending on the agonist and the differentiated state of the cell.

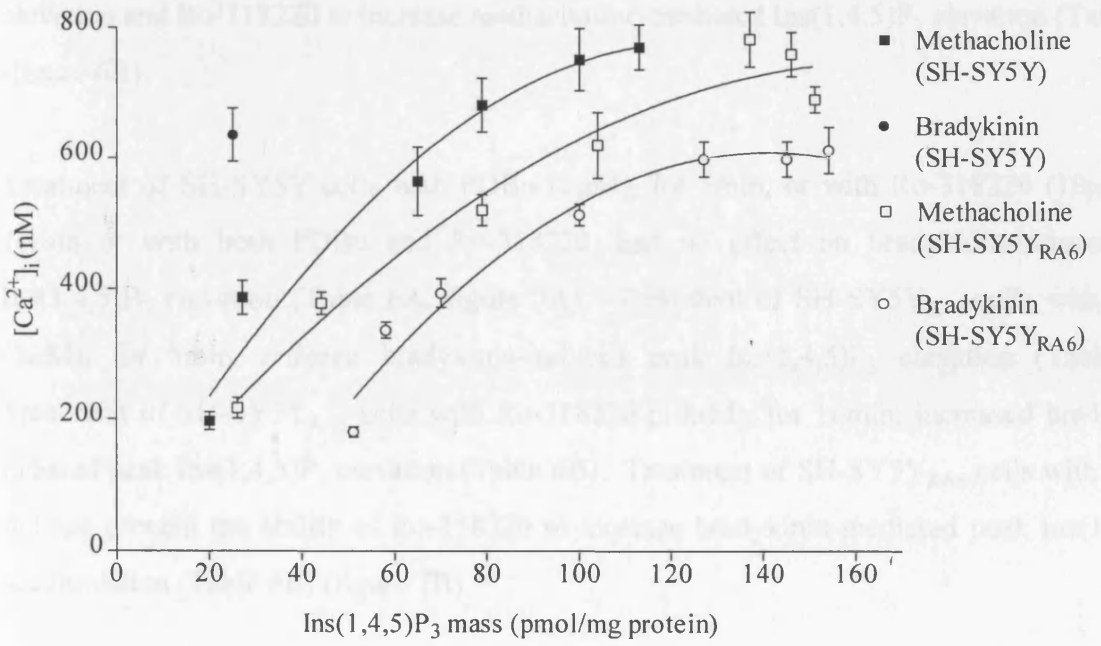


Figure 5 Relationship between peak Ins(1,4,5)P_3 mass accumulation and the equivalent $[Ca^{2+}]_i$ elevations elicited following challenge with various concentrations of either methacholine or bradykinin in adherent SH-SY5Y and SH-SY5Y_{RA6} cells. Data points were fitted with a Polynomial: second order equation and represent mean values (n=3) for peak Ins(1,4,5)P_3 mass accumulations and means ± S.E.M., n=3-9, for peak $[Ca^{2+}]_i$ elevations.

The effect of pre-treatment with PDBu and/or Ro-318220 on agonist-mediated Ins(1,4,5)P_3 accumulation in SH-SY5Y and SH-SY5Y_{RA6} cells.

Treatment of SH-SY5Y cells with the PKC activator, PDBu (1μM), for 5min, reduced methacholine-induced peak, but not sustained Ins(1,4,5)P_3 elevations (Table 5A). Treatment of SH-SY5Y cells with the PKC inhibitor, Ro-318220 (10μM), for 10min, had no effect on methacholine-induced peak or sustained Ins(1,4,5)P_3 elevations (Table 5A). However, treatment of SH-SY5Y cells with Ro-318220 prevented the ability of PDBu to reduce methacholine-mediated peak Ins(1,4,5)P_3 accumulation (Table 5A) (Figure 6A). Treatment of SH-SY5Y_{RA6} cells with PDBu (1μM), for 5min, reduced methacholine-induced peak, but not sustained, Ins(1,4,5)P_3 elevations (Table 5B). Treatment of SH-SY5Y_{RA6} cells with Ro-318220 (10μM), for 10min, increased methacholine-induced peak, but not sustained, Ins(1,4,5)P_3 elevations (Table 5B). Treatment of SH-SY5Y_{RA6} cells with Ro-318220 and PDBu prevented the ability of both PDBU to reduce methacholine-mediated peak Ins(1,4,5)P_3 elevation and Ro-318220 to increase methacholine-mediated Ins(1,4,5)P_3 elevation (Table 5B) (figure 6B).

Treatment of SH-SY5Y cells with PDBu (1μM), for 5min, or with Ro-318220 (10μM) for 10min or with both PDBu and Ro-318220, had no effect on bradykinin-induced peak Ins(1,4,5)P_3 elevation (Table 6A, figure 7A). Treatment of SH-SY5Y_{RA6} cells with PDBu (1μM), for 5min, reduced bradykinin-induced peak Ins(1,4,5)P_3 elevation (Table 6B). Treatment of SH-SY5Y_{RA6} cells with Ro-318220 (10μM), for 10min, increased bradykinin-induced peak Ins(1,4,5)P_3 elevation (Table 6B). Treatment of SH-SY5Y_{RA6} cells with PDBU did not prevent the ability of Ro-318220 to increase bradykinin-mediated peak Ins(1,4,5)P_3 accumulation (Table 6B) (figure 7B).

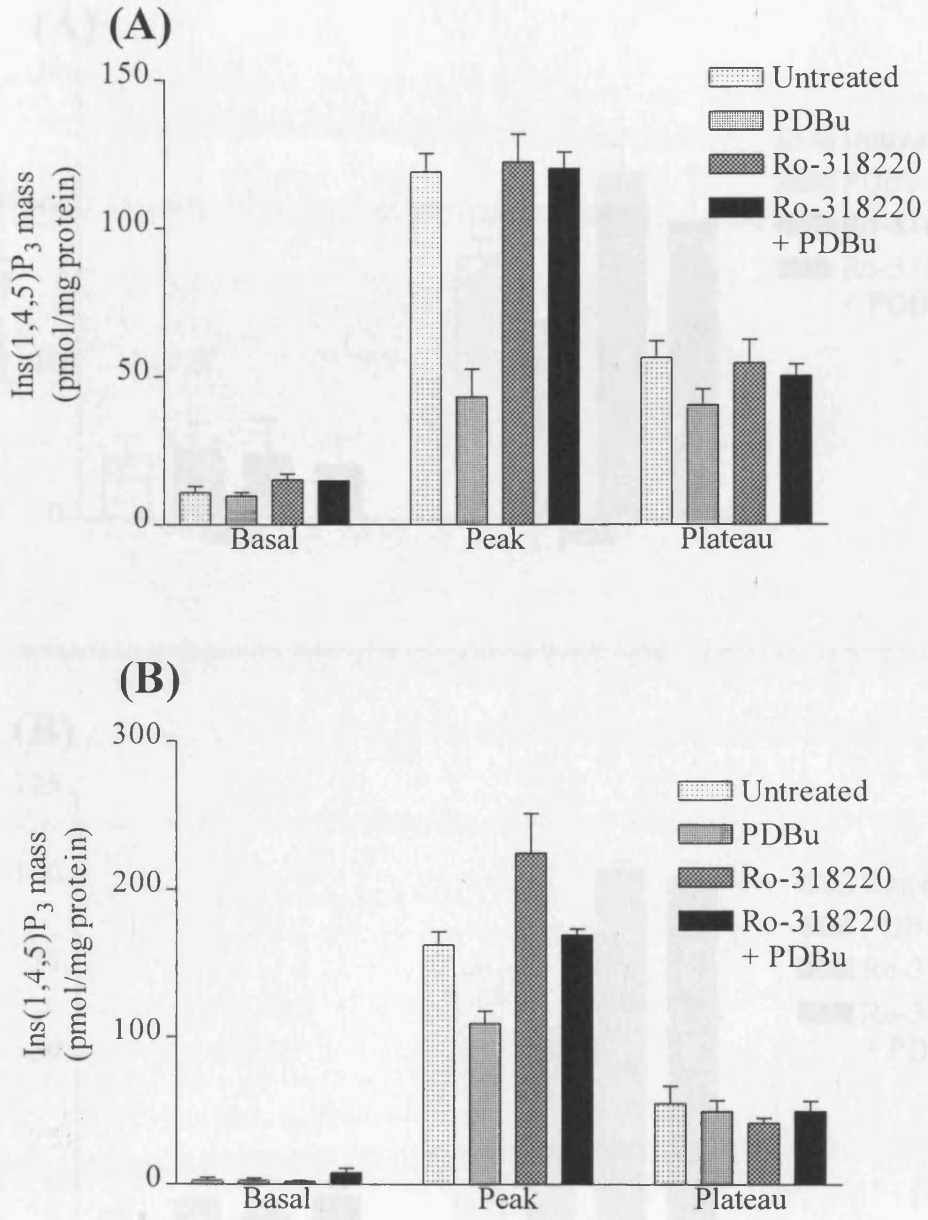


Figure 6 *Ins(1,4,5)P_3 responses to 1mM methacholine in adherent SH-SY5Y (A) and SH-SY5Y_{RA6} (B) cells treated prior to agonist challenge with 1μM PDBu for 5min, 10μM Ro-318220 for 10min, 10μM Ro-318220 for 10min plus 1μM PDBu for 5min or untreated. Data shown are means ± S.E.M., n=3-4.*

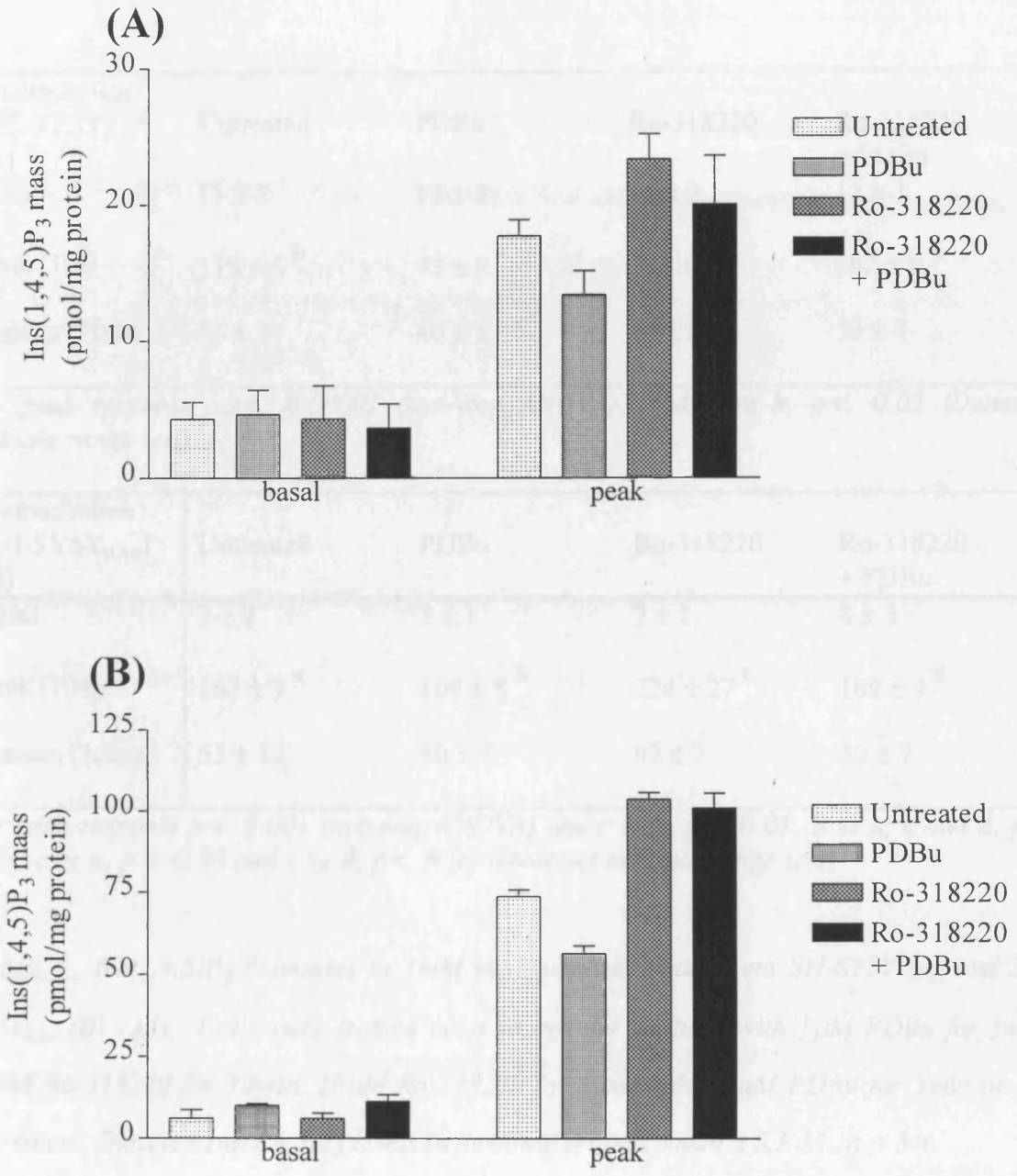


Figure 7 Ins(1,4,5)P_3 responses to 10μM bradykinin in adherent (A) SH-SY5Y and (B) SH-SY5Y_{RA6} cells treated prior to agonist challenge with 1μM PDBu for 5min, 10μM Ro-318220 for 10min, 10μM Ro-318220 for 10min plus 1μM PDBu for 5min or untreated. Data shown are means ± S.E.M., n=3.

Methacholine (SH-SY5Y) **(A)**	Untreated	PDBu	Ro-318220	Ro-318220 + PDBu
Basal	11 ± 2	12 ± 2	15 ± 2	15 ± 1
Peak (10s)	119 ± 6 **b**	43 ± 9 **a**	122 ± 9 **b**	102 ± 6 **b**
Plateau (300s)	56 ± 5	40 ± 5	54 ± 8	50 ± 4

*For peak response $p < 0.00001$ (two-way ANOVA), and **a** vs **b**, $p < 0.05$ (Duncans multiple range test).*

Methacholine (SH-SY5Y$_{RA6}$) **(B)**	Untreated	PDBu	Ro-318220	Ro-318220 + PDBu
Basal	3 ± 2	3 ± 1	2 ± 1	8 ± 3
Peak (10s)	162 ± 9 **a**	109 ± 8 **b**	224 ± 27 **c**	169 ± 4 **d**
Plateau (300s)	55 ± 12	50 ± 7	42 ± 3	50 ± 7

*For peak response $p < 0.001$ (two-way ANOVA) and **c** vs **b**, $p < 0.01$, **b** vs **a**, **c** and **d**, $p < 0.05$, **c** vs **a**, $p < 0.05$ and **c** vs **d**, $p < 0.05$ (Duncans multiple range test).*

Table 5 Ins(1,4,5)P_3 responses to 1mM methacholine in adherent SH-SY5Y (A) and SH-SY5Y$_{RA6}$ (B) cells. Cells were treated prior to agonist addition with 1μM PDBu for 5min, 10μM Ro-318220 for 10min, 10μM Ro-318220 for 10min plus 1μM PDBu for 5min or left untreated. Data are Ins(1,4,5)P_3 levels in pmol/mg protein, mean ± S.E.M., n = 3-4.

Bradykinin (SH-SY5Y) **(A)**	Untreated	PDBu	Ro-318220	Ro-318220 + PDBu
Basal	4 ± 1	5 ± 2	4 ± 2	4 ± 2
Peak (5s)	18 ± 1	13 ± 2	23 ± 2	20 ± 4

Bradykinin (SH-SY5Y_{RA6}) **(B)**	Untreated	PDBu	Ro-318220	Ro-318220 + PDBu
Basal	6 ± 1	10 ± 1	6 ± 2	11 ± 2
Peak (5s)	74 ± 2 **a**	56 ± 2 **b**	104 ± 2 **c**	101 ± 5 **d**

*For peak response $p < 0.00001$ (two-way ANOVA), and **b** vs **a**, **c** and **d**, $p < 0.05$, **c** vs **a**, $p < 0.05$ and **c** vs **d**, $p < 0.05$ (Duncans multiple range test).*

Table 6 $Ins(1,4,5)P_3$ responses to 10μM bradykinin in adherent SH-SY5Y (A) and SH-$SY5Y_{RA6}$ (B) cells. Cells were treated prior to agonist addition with 1μM PDBu for 5min, 10μM Ro-318220 for 10min, 10μM Ro-318220 for 10min plus 1μM PDBu for 5min or left untreated. Data are $Ins(1,4,5)P_3$ levels in pmol/mg protein, mean ± S.E.M., n = 3-4.

5.4 Discussion

The present studies have attempted to establish the relationship between PIC activation and changes in $[Ca^{2+}]_i$ in response to receptor occupation by agonists of either muscarinic or bradykinin receptors in undifferentiated SH-SY5Y cells as compared to SH-$SY5Y_{RA6}$ cells. These studies were performed in order to establish whether the anomaly between agonist-stimulated PIC activation and $[Ca^{2+}]_i$ elevation in RA treated SH-SY5Y cells revealed in the previous chapter was as a result of a spare capacity within the system or as a result of the involvement of another mechanism. This study also sought to examine the effects of the activation and inhibition of PKC on agonist-mediated PIC activation in both SH-SY5Y and SH-$SY5Y_{RA6}$ cells.

Analysis of the concentration-response relationships for peak Ins(1,4,5)P_3 accumulations revealed that the potency of methacholine-mediated Ins(1,4,5)P_3 accumulation, in addition to the maximal Ins(1,4,5)P_3 response elicited, was greater in SH-SY5Y_{RA6} cells than in SH-SY5Y cells. In addition, the muscarinic receptor partial agonist, arecoline, elicited a greater peak Ins(1,4,5)P_3 accumulation in the RA-treated cells, without affecting the EC_{50} value. Since muscarinic receptor density is up-regulated following RA-differentiation of SH-SY5Y cells, these results suggest that there is a receptor reserve for the peak phase of muscarinic receptor-mediated Ins(1,4,5)P_3 accumulation in SH-SY5Y_{RA6} cells. These findings are in agreement with classical receptor pharmacology where, if there is a receptor reserve for the observable response, an increased receptor number will increase the potency of the response for a given concentration of agonist (Kenakin, 1997). However, the assumption cannot be made that there is a similar receptor reserve for bradykinin-stimulated Ins(1,4,5)P_3 generation in SH-SY5Y_{RA6} cells, since submaximal concentrations of bradykinin failed to elicit a measurable Ins(1,4,5)P_3 generation in the undifferentiated SH-SY5Y cells, and it was therefore not possible to measure an EC_{50} value for the response in these cells.

An apparent receptor reserve for phosphoinositide hydrolysis has previously been proposed for muscarinic receptor-stimulated [^{3}H]-inositol phosphate accumulation in the presence of Li^+ in a number of different cell types (Ashkenazi *et al.*, 1989; Mei *et al.*, 1989; Buck and Fraser, 1990; Schwarz *et al.*, 1993; Wang and El-Fakahany, 1993). The present studies provide evidence that, in a similar manner to Ins(1,4,5)P_3 accumulation, there is a receptor reserve for methacholine stimulated [^{3}H]-inositol phosphate accumulation in SH-SY5Y_{RA6} cells. This receptor reserve is indicated by the fact that the concentration-effect curve for methacholine in SH-SY5Y_{RA6} cells lies to the left of that in SH-SY5Y cells although the magnitude of the maximal response acheived was unaffected. In addition, stimulation with arecoline elicited a greater [^{3}H]-inositol phosphate accumulation in SH-SY5Y_{RA6} cells demonstrating an enhanced muscarinic receptor-mediated phosphoinositide hydrolysis (and hence PIC activity) in SH-SY5Y_{RA6} as compared with SH-SY5Y cells. Since [^{3}H]-inositol phosphates accumulated over a 15min period are mainly representative of the sustained plateau phase of Ins(1,4,5)P_3 production, these data would suggest that there is also a receptor reserve for the sustained phase of agonist-mediated PIC activation in RA-treated cells.

Receptor theory states that agonist action at a receptor depends on two independent parameters, the ability of the agonist to bind to the receptor (affinity) and the ability of the agonist to induce a response (efficacy). However, it is difficult to determine these two parameters under the same conditions. For instance, there is evidence that the affinity of agonist for a receptor may depend not only on the receptor but also on the complement of G-protein available to interact with the receptor (MacKay, 1990; Kenakin, 1993). Thus, it is possible that the augmented agonist-mediated PIC activation observed in SH-SY5Y_{RA6} cells is due, not only to the increased expression of cell surface receptors, but also to alterations of G-protein expression. For example, if the expression levels of the $G_{q/11}$ family of G-proteins are up-regulated during RA-induced differentiation, in a manner similar to cell surface receptors, it is possible that an augmented agonist-mediated PIC activation may ensue. It is also possible that these overall changes in agonist-stimulated PIC activity may be an indication that the relative expression levels of the PIC isoforms are altered following RA-differentiation of the SH-SY5Y cell line. Such a phenomena is not entirely unlikely since the differentiation of U937 cells has been associated with the down-regulation of PIC-γ1 (Lee *et al.*, 1995). Following the binding of agonist, most PIC-coupled receptors appear to activate the PIC-β isoforms via the $G_{q/11}$ family of G-proteins (Smrcka *et al.* 1991; Taylor *et al.*, 1991). It is likely that $G_{\alpha q}$ primarily activates the PIC-β1 and PIC-β3 isoforms (Lee *et al.*, 1992; Hepler *et al.*, 1993), although activation of PIC-β4 by $G_{\alpha q}$ has also been demonstrated (Jiang *et al.*, 1994). In addition, activation of predominantly PIC-β3 and PIC-β2 by liberated βγ subunits may also occur following the activation of $G_{q/11}$ and G-proteins other than $G_{q/11}$ (Fisher, 1995). Thus, the activation profile of PIC may, to an extent, depend on the relative expression levels of the PIC isoforms which, as mentioned above, may vary between SH-SY5Y and SH-SY5Y_{RA6} cells.

Agonist-mediated elevations of $[Ca^{2+}]_i$ following occupation of cell surface receptors linked to phosphoinositide hydrolysis are widely regarded to be driven by the actions of Ins(1,4,5)P_3 on the Ins(1,4,5)P_3 receptor/Ca^{2+} release channel complex located on specialised parts of the endoplasmic reticulum (Berridge, 1993). In agreement with previous studies (Willars and Nahorski, 1995), peak $[Ca^{2+}]_i$ responses following stimulation of SH-SY5Y cells with either methacholine or bradykinin were little effected by the exclusion of extracellular Ca^{2+} which would strongly suggest that these responses are mediated by Ca^{2+} mobilisation from intracellular stores. In agreement with previous studies using cell populations (Bootman *et*

al., 1992; Willars and Nahorski, 1995), the agonist-induced peak $[Ca^{2+}]_i$ elevations in SH-SY5Y and SH-SY5Y$_{RA6}$ cell populations were dose-dependent. The exact nature of this apparent concentration-dependence is not clear. Studies using single cells have demonstrated that Ca^{2+} release often occurs in an all-or-none fashion (Shao and McCarthy, 1993; Chiavaroli *et al.*, 1994). However, there is also evidence to suggest that agonist-induced Ca^{2+} elevations in a single cell may occur in a graded fashion and are dose-dependent (Bird *et al.*, 1993) and this would appear to be the case in the SH-SY5Y cell line (Saunders *et al.*, 1996). It is suggested that dose-dependent elevations in $[Ca^{2+}]_i$ may occur by a mechanism labelled 'quantal Ca^{2+} release' (QCR) (Muallem *et al.*, 1989; Irvine, 1990), whereby sub-optimal concentrations of Ins(1,4,5)P_3 induce the rapid release of only a fraction of the total Ins(1,4,5)P_3 sensitive Ca^{2+} pool with no significant further Ca^{2+} release until additional Ins(1,4,5)P_3 is added (reviewed by Taylor and Traynor, 1995). This phenomenon of QCR allows for both rapid and graded stored Ca^{2+} mobilisation This behaviour of Ins(1,4,5)P_3 contrasts with the behaviour of other agents which mobilise Ca^{2+} from intracellular stores, such as ionomycin, sub-maximal concentrations of which ultimately produces the same net release as maximally effective concentrations (Taylor and Potter, 1990).

Several hypotheses have been proposed to explain the QCR phenomenon (reviewed by Taylor, 1992). The first, or 'digital', hypothesis envisages individual Ca^{2+} stores with different threshold sensitivities to Ins(1,4,5)P_3, where low agonist concentrations completely empty the most sensitive stores and higher concentrations empty the less sensitive stores, in other words an all-or-nothing emptying of heterogeneous Ca^{2+} stores (reviewed by Taylor and Richardson, 1991). Heterogeneous Ca^{2+} stores could result from Ins(1,4,5)P_3 receptor subtypes with different affinities for Ins(1,4,5)P_3 or different receptor densities, or a differing relationship between stores for Ins(1,4,5)P_3 receptor occupancy and channel opening. An alternative, or 'analogue', hypothesis envisages individual Ca^{2+} stores with uniform sensitivity to Ins(1,4,5)P_3, all partially emptying and then rapidly desensitising. It has been suggested that the Ins(1,4,5)P_3 receptor may switch to a conformation with a much reduced Ca^{2+} conductance (Champeil *et al.*, 1989). However this would seem unlikely since pre-incubation with Ins(1,4,5)P_3 fails to effect subsequent sensitivity of the receptor (Meyer and Stryer, 1990). Attempts have been made to define QCR at the level of the individual Ins(1,4,5)P_3 receptor. Partial depletion of the Ca^{2+} store may result in a subsequent reduction in the sensitivity of the store to Ins(1,4,5)P_3 due to a direct regulatory role of luminal Ca^{2+} on the

$Ins(1,4,5)P_3$ receptor. There is evidence that depleted Ca^{2+} stores are less sensitive to $Ins(1,4,5)P_3$ (Nunn and Taylor, 1992; Parys *et al.*, 1993), although extensive store depletion is required for these effects to become evident (Combettes *et al.*, 1996). Agonist-induced Ca^{2+} release in SH-SY5Y cells has been shown to be 'quantal' and it has recently been suggested that, in these cells, $Ins(1,4,5)P_3$ intrinsically activates and then inhibits the Ca^{2+} conductance of its receptor to produce QCR (Wilcox *et al.*, 1996).

The present study has also revealed that the potencies of both methacholine and bradykinin for the peak elevation of $[Ca^{2+}]_i$ were greater than those for the activation of PIC (as determined by measurement of agonist-mediated $Ins(1,4,5)P_3$ generation and $[^3H]$-inositol phosphate accumulation). These results are in accord with the concept that there is an amplification of the response in the phase between the production of $Ins(1,4,5)P_3$ and the release of Ca^{2+}. It is unlikely that Ca^{2+} entry contributed to the apparent amplification of the peak $[Ca^{2+}]_i$ response, since agonist stimulation in the presence of low $[Ca^{2+}]_e$ did not result in a significant decrease in the potency of methacholine- or bradykinin-stimulated peak increases in $[Ca^{2+}]_i$ in SH-SY5Y or SH-$SY5Y_{RA6}$ cells. A suggested mechanism for this amplification is that a complex relationship between $Ins(1,4,5)P_3$ receptor occupation and Ca^{2+} mobilisation exists such that Ca^{2+} itself contributes to a highly co-operative opening of the intracellular Ca^{2+} channels (Taylor and Marshall, 1992). This may be via Ca^{2+}-induced Ca^{2+} release (CICR) at the level of the $Ins(1,4,5)P_3$ and/or the ryanodine receptors (Putney and Bird, 1993) and the SH-SY5Y cell line has been demonstrated to express type-1 $Ins(1,4,5)P_3$ receptors and type-2 ryanodine receptors (Mackrill *et al.*, 1997). Provided the local $[Ca^{2+}]_i$ is sufficiently elevated, CICR may be initiated at either the $Ins(1,4,5)P_3$ or ryanodine receptors, regardless of the initial source of $[Ca^{2+}]_i$ elevation. Alternatively, a small amount of Ca^{2+} release may sensitise the $Ins(1,4,5)P_3$ receptors such that release is escalated until $[Ca^{2+}]_i$ becomes great enough to be inhibitory (Bezprozvanny *et al.*, 1991).

Despite the apparent amplification of the $[Ca^{2+}]_i$ response (illustrated by the $[Ca^{2+}]_i$ concentration-response curve lying to the left of the $Ins(1,4,5)P_3$ and $[^3H]$-inositol phosphate concentration-response curves) compared with PIC activation there appeared to be no receptor reserve for either methacholine- or bradykinin-stimulated $[Ca^{2+}]_i$ elevations. This was demonstrated by the fact that the peak $[Ca^{2+}]_i$ responses, in contrast to the PIC responses, following stimulation with either methacholine or bradykinin were not affected by RA

treatment. These results are in accord with the findings of Horie and Tsujimoto (1995) who demonstrated a suprising lack of receptor reserve for α1B-adrenoceptor-stimulated $[Ca^{2+}]_i$ elevation in CHO cells. Differences in CICR at the level of $Ins(1,4,5)P_3$ and/or ryanodine receptors are unlikely to account for this phenomenon since the expression of $Ins(1,4,5)P_3$ receptors and ryanodine receptors is reported to be unaffected by a 6 day treatment with RA of the SH-SY5Y cell line (Mackrill *et al.*, 1997). Further analysis of the relationship between the concentration of $Ins(1,4,5)P_3$ required to elicit a certain $[Ca^{2+}]_i$ elevation for either bradykinin or methacholine in both SH-SY5Y and SH-$SY5Y_{RA6}$ cells revealed that this relationship is not only different for different agonists, but also differs for the same agonist between SH-SY5Y and SH-$SY5Y_{RA6}$ cells. Analysis of the $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ data in this manner clearly demonstrates that, following agonist stimulation, the global $Ins(1,4,5)P_3$ elevation measured does not correspond with a given $[Ca^{2+}]_i$ elevation. This lack of correlation was exaggerated for bradykinin stimulated peak $Ins(1,4,5)P_3$ accumulations versus peak $[Ca^{2+}]_i$ elevations in undifferentiated SH-SY5Y cells, where very small or unmeasurable $Ins(1,4,5)P_3$ accumulations evoked relatively large Ca^{2+} mobilising responses. It is a possibility that these findings may represent an agonist-mediated, $Ins(1,4,5)P_3$-independent, mobilisation of stored Ca^{2+} in these cells. Indeed, it has recently been shown that lysophosphatidic acid (LPA) mobilises Ca^{2+} from the intracellular stores of undifferentiated SH-SY5Y cells, in the absence of $Ins(1,4,5)P_3$ receptor activation (J. Mackrill, unpublished observations). In summary, the data presented thus far suggest a more complicated relationship between agonist-induced $Ins(1,4,5)P_3$ elevation and Ca^{2+} mobilisation than has, until recently, been accepted. It is clear that the anomaly between peak $Ins(1,4,5)P_3$ generation and peak $[Ca^{2+}]_i$ elevation in SH-SY5Y and SH-$SY5Y_{RA6}$ cells is not as a result of a spare capacity within the system since there appears to be little or no receptor reserve for $[Ca^{2+}]_i$ elevation by either agonist.

The second aim of this chapter was to investigate the potential regulatory influence of PKC, activated or inhibited by phorbol ester or Ro-318220 respectively, on agonist-mediated phosphoinositide signalling. It has previously been demonstrated in rat cervical ganglion neurones that it is the activation of PKC and the subsequent phosphorylation of Ca^{2+} channels, resulting in increased channel conductance, that leads to muscarinic receptor-mediated $[Ca^{2+}]_i$ elevation, rather than $Ins(1,4,5)P_3$-mediated release of Ca^{2+} from intracellular stores (Marsh *et al.*, 1995). Since PKC seems to play a more significant role in Ca^{2+} signalling in neurones

compared with neuroblastoma cells, by the same rationale it is possible that, following cellular differentiation, the effects of PKC activation or inhibition on phosphoinositide signalling may be enhanced.

Activation of PKC with the phorbol ester, PDBu, reduced peak and sustained Ins(1,4,5)P_3 responses to methacholine to a similar extent in SH-SY5Y compared to SH-SY5Y_{RA6} cells. The greatest effect was on the peak phase of Ins(1,4,5)P_3 accumulation. This emphasises that the regulatory influences on the activation of PIC may differ during different phases of receptor activation (Willars and Nahorski, 1995), although the basis of this is unclear. In undifferentiated SH-SY5Y cells, treatment with the PKC inhibitor, Ro-318220, had no effect on methacholine-mediated Ins(1,4,5)P_3 generation. These observations would suggest that PKC activation via methacholine-induced DAG formation does not underlie the rapid desensitisation of PIC activity that occurs within seconds of agonist exposure. However, treatment with Ro-318220 enhanced methacholine-mediated peak and sustained Ins(1,4,5)P_3 responses in SH-SY5Y_{RA6} cells. Bradykinin-mediated peak Ins(1,4,5)P_3 accumulations in SH-SY5Y cells were small and any effects of PDBu or Ro-318220 were not significant. In contrast, and in agreement with results throughout this study, Ins(1,4,5)P_3 accumulation in response to bradykinin in SH-SY5Y_{RA6} cells was rapid and robust. This response, in a similar manner to those elicited by methacholine in SH-SY5Y_{RA6} cells, was attenuated by treatment with PDBu and enhanced by treatment with Ro-318220.

The current study indicates that the activation of PKC by the phorbol ester PDBu inhibits muscarinic and bradykinin receptor-mediated Ins(1,4,5)P_3 accumulation in both SH-SY5Y and SH-SY5Y_{RA6} cells. That this was a PKC-mediated effect was indicated by the reversal of the inhibition by the PKC inhibitor Ro-318220. In undifferentiated SH-SY5Y cells there was a divergence between PDBu-mediated and agonist-mediated PKC activation since Ro-318220 failed to enhance Ins(1,4,5)P_3 accumulation following challenge with either agonist. These findings are in agreement with studies in Swiss 3T3 fibroblasts, GT1-7 neuronal cells and SH-SY5Y cells (Walsh *et al.*, 1993; Zamani *et al.*, 1995; Willars *et al.*, 1996) where phorbol esters desensitised PIC-linked responses to agonists, although PKC was not involved in agonist-mediated desensitisation. In contrast, following RA-differentiation of the SH-SY5Y cell line, agonist-mediated Ins(1,4,5)P_3 generation was enhanced following treatment with Ro-318220. This enhancement is likely to be a widespread phenomenon since an effect of

agonist-mediated PKC activation on phosphoinositide signalling has been reported for a number of different receptor types (King and Rittenhouse, 1989; Challiss *et al.*, 1993; Barr and Watson, 1994; Alblas *et al.*, 1995). The ability of PKC, activated by means other than acute phorbol ester treatment, to influence PIC activity may therefore be dependent on cell type.

The reason(s) for the differences between SH-SY5Y and SH-SY5Y_{RA6} cells regarding the enhancement of agonist-induced PIC activation by Ro-318220 have not been examined in the current study. One could speculate however that, concomitant with an increased receptor number, an elevated concentration of DAG is generated following agonist challenge of SH-SY5Y_{RA6} cells in a similar manner to that observed with Ins(1,4,5)P_3. Thus PKC would, presumably, be activated to a greater extent and hence the inhibitory influence of PKC upon PIC activity would be enhanced. Under these circumstances the inhibitory effect of PKC may be great enough to be significantly reversed by Ro-318220 treatment. In theory, this hypothesis could be addressed by the down-regulation of the muscarinic and/or bradykinin receptor number in SH-SY5Y_{RA6} cells by a receptor alkylation technique using an irreversible receptor antagonist. If the SH-SY5Y_{RA6} cell surface receptor number was reduced by half in this manner, and therefore approximately equivalent to the receptor number in undifferentiated SH-SY5Y cells, a repetition of the experiments performed in this study should yield similar data for untreated SH-SY5Y and receptor alkylated SH-SY5Y_{RA6} cells. An alternative explanation for the observed differences, as discussed earlier, is that there are differences in the levels or isoforms of PKC and /or PIC expressed in SH-SY5Y and SH-SY5Y_{RA6} cells. This hypothesis could also be addressed by performing immunoblot analysis of whole cell preparations using antibodies directed against the various isoforms of both PKC and PIC.

In summary, PKC activation by phorbol ester treatment attenuated agonist-stimulated Ins(1,4,5)P_3 generation in both SH-SY5Y and SH-SY5Y_{RA6} cells. In contrast, PKC inhibition by Ro-318220 treatment enhanced agonist-mediated Ins(1,4,5)P_3 generation in SH-SY5Y_{RA6} cells only. Whether this enhancement is simply due the increased receptor number and hence enhanced agonist-mediated PKC activation, to an alteration of PKC and/or PIC isoforms or to some other factor in SH-SY5Y_{RA6} cells remains unknown. Furthermore, concomitant with an increased receptor number, PIC signalling in response to both bradykinin and muscarinic

receptor activation was greater in SH-SY5Y$_{RA6}$ compared with SH-SY5Y cells. In contrast, agonist-mediated $[Ca^{2+}]_i$ elevations were not affected by RA treatment. In conclusion, according to the concepts of classical receptor theory (Kenakin, 1993), following amplification of the response, a receptor reserve would be expected for agonist-mediated $[Ca^{2+}]_i$ responses if a receptor reserve is observed for the agonist-mediated activation of PIC. This clearly was not the case and these results cannot be accounted for by classical receptor theories. Possible mechanisms that could account for the observed anomaly include the compartmentalisation of Ins(1,4,5)P_3 or a possible agonist-mediated modulation of the Ins(1,4,5)P_3 receptor, which may be different for different agonists and/or cell types. These mechanisms have been outlined in greater detail in section 4.4., and the possible existence of a number of these mechanisms have been addressed in subsequent chapters.

6. THE EFFECT OF DEPOLARISATION-INDUCED $[Ca^{2+}]_i$ ELEVATION ON PIC ACTIVATION AND CHANGES IN $[Ca^{2+}]_i$ IN SH-SY5Y AND SH-SY5Y$_{RA6}$ CELLS.

6.1 Introduction

The phosphoinositide and Ca^{2+} signalling pathway is subject to acute regulatory features which may influence the sensitivity, temporal profile and magnitude of the responses (see Introduction). There is a large body of evidence indicating that Ca^{2+} itself may influence the activity of PIC, thus providing an important regulatory feature. It has become apparent that depolarisation-induced Ca^{2+} influx, and the resulting elevation of $[Ca^{2+}]_i$, can stimulate phosphoinositide hydrolysis, independent of agonist-mediated PIC activation, in several tissues, particularly those of a neuronal nature (Court *et al.*, 1986; Gurwitz and Sokolovsky, 1987; Audigier *et al.*, 1988). In addition, a number of studies have demonstrated that depolarisation of neuronal tissues, such as cerebral cortex, facilitates agonist-induced PIC activation in a manner which is dependent on extracellular Ca^{2+} (Kendall and Nahorski, 1984; Rooney and Nahorski, 1984; Challiss and Nahorski, 1991). However, depolarisation of the tissues used in the above experiments may have the ability to influence neurotransmitter release. Thus, it is possible that the observed effects are indirect via release of PIC-linked receptor agonists. Indeed, it has been shown previously that, in neuronal preparations from rat brain, inositol phosphate hydrolysis induced by depolarising stimuli were suppressed, at least in part, by muscarinic receptor antagonists suggesting phosphoinositide hydrolysis may be stimulated by release of endogenous acetylcholine, which acts subsequently on muscarinic receptors (Briggs *et al.*, 1985; Kendall and Nahorski, 1987; Baird and Nahorski, 1989).

Numerous studies in cell types of a more homogenous nature have provided support for a direct role for Ca^{2+} activation of PIC. Thus, under conditions where cells have little or no capacity for neurotransmitter release, cellular depolarisation results in the activation of PIC (Best, 1986; Smart *et al.*, 1995). Furthermore, in a variety of cell types, elevation of $[Ca^{2+}]_i$ using ionophores rather than depolarisation appears to result in the activation of PIC (Fisher *et al.*, 1988; McDonough *et al.*, 1988; Kolesnick and Gershengorn, 1984). However, Ca^{2+} ionophores do not stimulate PIC activation in all cell types. For example, ionomycin failed to

affect phosphoinositide responses in rat liver, adrenal glomerulosa or PC12 cells (Vincentini *et al.*, 1985; Renard *et al.*, 1987; Balla *et al.*, 1988).

Excitable cells possess high densities of voltage-operated Ca^{2+} channels (VOCCs), and extracellular Ca^{2+} is considered to be the primary source for changes in $[Ca^{2+}]_i$. As such, although neuronal cells possess a vast array of receptors linked to phosphoinositide hydrolysis, it is generally accepted that $[Ca^{2+}]_i$ changes depend predominantly on Ca^{2+} influx through VOCCs. Hence the significance of receptor-mediated PIC activation and Ca^{2+} mobilisation in excitable cells is less clear (Miller, 1987; Nahorski, 1988). Synergism between Ca^{2+}- and receptor-induced phosphoinositide metabolism would seem to be particularly significant in a neuronal context where cells may experience fluctuations in membrane potential exerted by other neuronal inputs. For instance, activation of the ionotropic NMDA receptor has been reported to both activate PIC and potentiate agonist-mediated PIC responses in neuronal cells and tissues (Nicoletti *et al.*, 1987; Baird and Nahorski, 1991; Challiss *et al.*, 1994). Ca^{2+} facilitation as opposed to activation of PIC by such mechanisms implies integration of ionotropic and metabotropic signalling. This assumes the simultaneous activation of these receptor classes on a single cell. Since individual neuronal cells express multiple receptor types and subtypes, the simultaneous activation of ionotropic and metabotropic receptors is very likely to be a physiologically relevant event. Indeed, certain physiological agonists, such as acetylcholine, glutamate and serotonin are ligands for both ionotropic and metabotropic receptors. Thus, in cells expressing both receptor classes activated by the same agonist, simultaneous receptor activation would seem to be inevitable.

The human neuroblastoma cell line, SH-SY5Y expresses metabotropic muscarinic receptors linked to phosphoinositide hydrolysis (see previous chapters). In addition, SH-SY5Y cells express nicotinic acetylcholine receptors (Vaughan *et al.*, 1993), a ligand-gated Na^+ channel, which opens upon activation to allow Na^+ diffusion into the cell, thus depolarising the plasma membrane. This could lead to the opening of VOCCs, of which both the L- and the N-type are expressed in SH-SY5Y cells (Reeve *et al.*, 1994), which results in the subsequent increase in $[Ca^{2+}]_i$. Thus, the SH-SY5Y cell line would seem an appropriate model in which to study the interaction between metabotropic and ionotropic receptors, mentioned above, following their simultaneous activation.

During neuronal differentiation, an increase of voltage-dependent Ca^{2+} currents is well supported by experimental evidence (Yaari *et al.*, 1987; Streit and Lux, 1987; Grottman *et al.*, 1988; Carbone *et al.*, 1990). Following RA-induced differentiation, SH-SY5Y cells exhibit an increased expression of VOCCs (Toselli *et al.*, 1991; Stumpo *et al.*, 1993). Since depolarisation-induced Ca^{2+} influx is reportedly enhanced in differentiated SH-SY5Y cells (see section 1.6.3), it is possible that a potential Ca^{2+} activation and/or facilitation of PIC activity following cellular depolarisation will also be enhanced in RA-treated cells. This is likely to be of importance in mammalian neurones since depolarisation and PIC activation are likely to occur simultaneously in the same cell in response to acetylcholine-mediated activation of both muscarinic and nicotinic receptors. The aims of the current chapter, therefore, were to compare the effects of challenge with the depolarising stimuli, high extracellular K^+ and nicotine, on the activation of PIC and changes in $[Ca^{2+}]_i$ in SH-SY5Y and SH-SY5Y_{RA6} cells. These studies were performed in order to establish whether depolarisation-induced Ca^{2+} influx could directly activate PIC and/or facilitate agonist-mediated PIC activation in SH-SY5Y cells, and whether these effects were enhanced following RA-induced differentiation.

6.2 Results.

The results presented in this chapter are the effects of depolarising stimuli on PIC activation (determined by the elevation of Ins(1,4,5)P_3 or by [^{3}H]-inositol phosphate accumulation) and $[Ca^{2+}]_i$ elevation in SH-SY5Y and SH-SY5Y_{RA6} cells. The depolarising stimuli utilised in the current study are nicotine (30μM), a maximal concentration shown to release [^{3}H]-noradrenaline in SH-SY5Y cells (Purkiss *et al.*, 1995), and KCl (50mM). This concentration of KCl is the approximate EC_{50} concentration required for $[Ca^{2+}]_i$ elevation in SH-SY5Y cells (Lambert *et al.*, 1991; Smart *et al.*, 1995). An EC_{50} concentration, rather than a maximal concentration of KCl was utilised as the molarity of the buffer was maintained by lowering the buffer $[Na^+]$, and there was concern that dropping the $[Na^+]$ to too great an extent may have physiological consequences, such as a disruption of the Na^+/Ca^{2+} exchange process within the cell, which may in turn lead to alterations in the Ca^{2+} gradient across the cell

membrane thus affecting Ca^{2+} influx, and/or alterations in the amount of Ca^{2+} stored within intracellular Ca^{2+} pools.

Depolarisation-mediated elevations of $[Ca^{2+}]_i$ and $Ins(1,4,5)P_3$ mass in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

From resting levels of 97 ± 3nM (n=3), addition of KCl (50mM) to undifferentiated SH-SY5Y cells was followed by a peak of $[Ca^{2+}]_i$ (305 ± 19nM, n=3) approximately 10s after addition. This peak was followed by a lower, sustained phase (170 ± 6nM). Following the addition of 50mM KCl to SH-SY5Y$_{RA6}$ cells, $[Ca^{2+}]_i$ was elevated with a peak phase of 325 ± 9nM (n=3) approximately 10s after addition, followed by a sustained phase of 161 ± 8nM (n=3) (Figure 1(A)). Neither the peak nor the sustained responses to KCl were significantly different in SH-SY5Y compared to SH-SY5Y$_{RA6}$ cells. Challenge of undifferentiated SH-SY5Y cells with nicotine (30μM) also resulted in a peak $[Ca^{2+}]_i$ elevation (209 ± 7nM) approximately 20s after addition. This peak was followed by a lower, sustained phase (133 ± 5nM, n=3). Following the addition of 30μM nicotine to SH-SY5Y$_{RA6}$ cells, $[Ca^{2+}]_i$ was elevated with a peak phase of 222 ± 8nM (n=3) approximately 10s after addition followed by a sustained phase of 137 ± 6nM (n=3) (Figure 1(B)). Neither peak nor sustained responses to nicotine were significantly different in SH-SY5Y compared with SH-SY5Y$_{RA6}$ cells.

From a resting level of 17 ± 2 pmol/mg protein (n=3) 50mM KCl and 30μM nicotine evoked very small elevations of $Ins(1,4,5)P_3$ in undifferentiated SH-SY5Y cells. Neither the responses to nicotine, nor to KCl were significantly different to those elicited following the addition of Krebs-HEPES buffer alone (Figure 2(A)). The $Ins(1,4,5)P_3$ mass responses to nicotine (30μM), KCl (50mM) or Krebs-HEPES buffer in SH-SY5Y$_{RA6}$ cells were not significantly different to those elicited in the undifferentiated cells (Figure 2(B)).

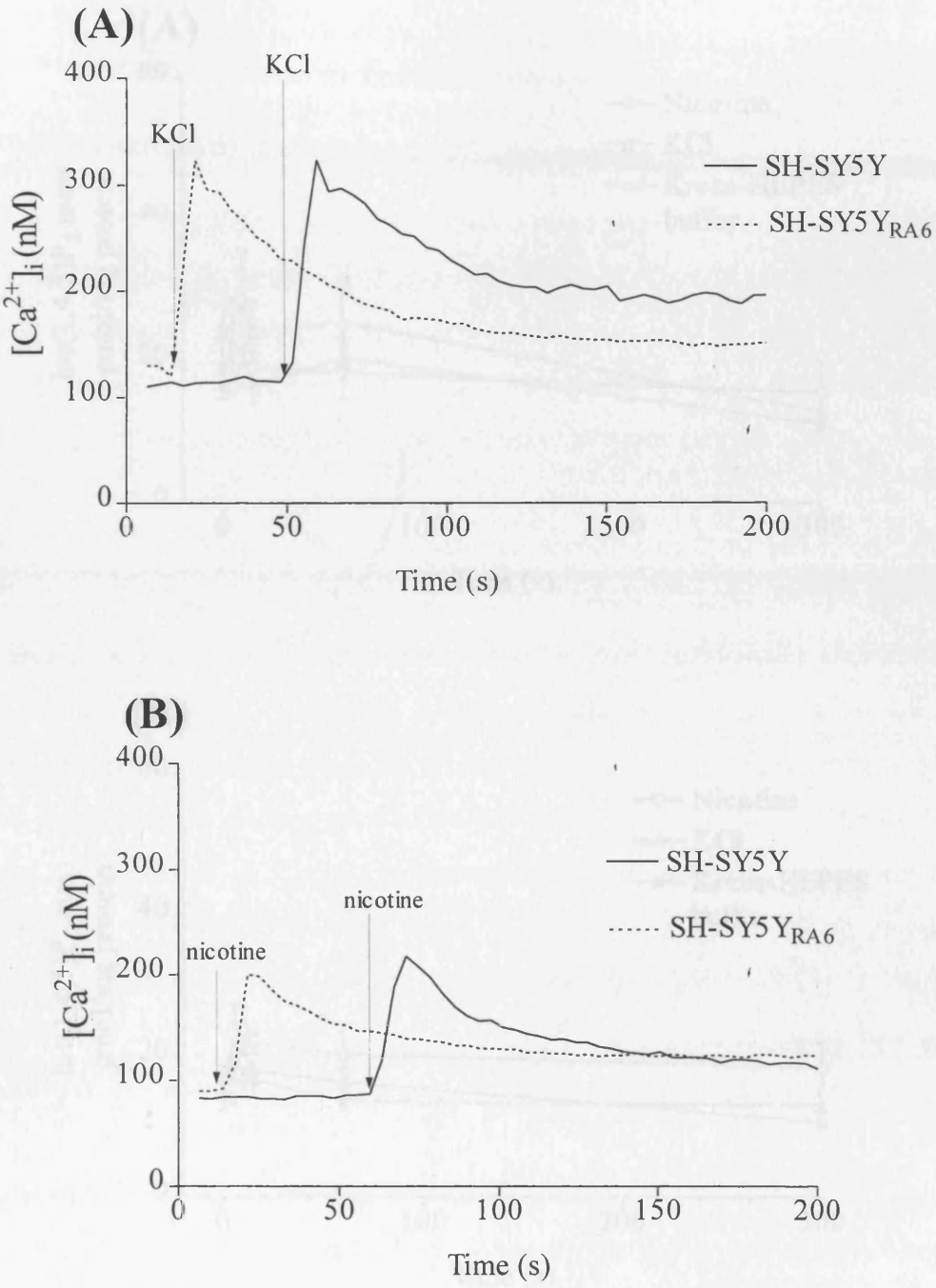


Figure 1 *Time course of $[Ca^{2+}]_i$ elevations in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells in response to (A) KCl (50mM) and (B) nicotine (30μM). Data are representative traces of at least three similar experiments.*

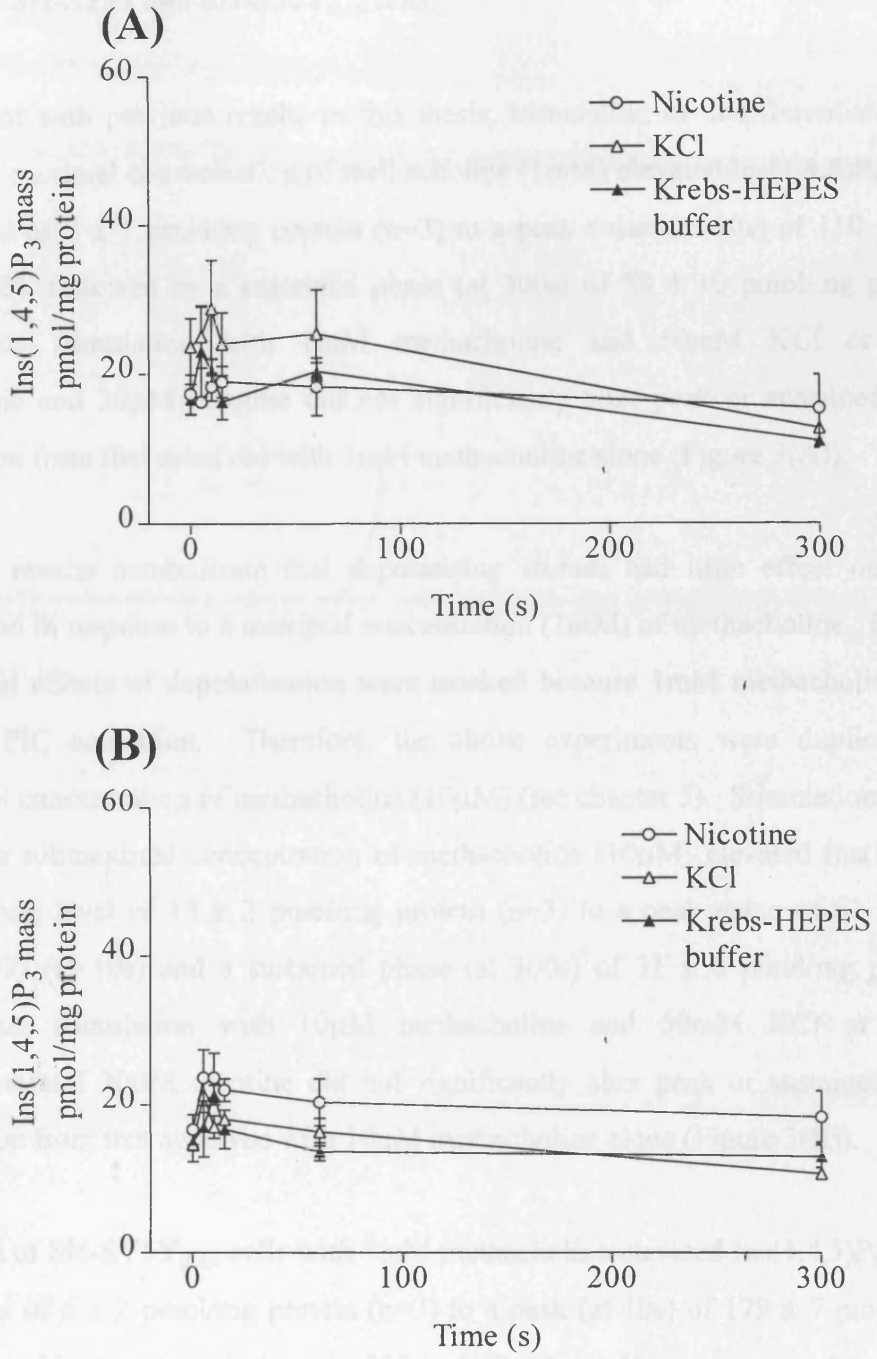


Figure 2 Time course of changes in Ins(1,4,5)P_3 mass in adherent (A) SH-SY5Y and (B) SH-SY5Y_{RA6} cells in response to KCl (50mM), nicotine (30μM) and Krebs-HEPES buffer (appropriate control). Data are means ± S.E.M., n=3.

Effect of depolarisation on methacholine-mediated Ins(1,4,5)P_3 generation in populations of adherent SH-SY5Y and SH-SY5Y_{RA6} cells.

In agreement with previous results in this thesis, stimulation of undifferentiated SH-SY5Y cells with a maximal concentration of methacholine (1mM) elevated Ins(1,4,5)P_3 mass from a resting level of 9 ± 1 pmol/mg protein (n=3) to a peak value (at 10s) of 110 ± 9 pmol/mg protein (n=3), followed by a sustained phase (at 300s) of 58 ± 10 pmol/mg protein (n=3). Simultaneous stimulation with 1mM methacholine and 50mM KCl or with 1mM methacholine and 30μM nicotine did not significantly alter peak or sustained Ins(1,4,5)P_3 accumulation from that acheived with 1mM methacholine alone (Figure 3(A)).

The above results demonstrate that depolarising stimuli had little effect on Ins(1,4,5)P_3 accumulation in response to a maximal concentration (1mM) of methacholine. However, it is possible that effects of depolarisation were masked because 1mM methacholine maximally stimulates PIC activation. Therefore, the above experiments were duplicated using a submaximal concentration of methacholine (10μM) (see chapter 5). Stimulation of SH-SY5Y cells with a submaximal concentration of methacholine (10μM) elevated Ins(1,4,5)P_3 mass from a resting level of 13 ± 2 pmol/mg protein (n=3) to a peak value of 61 ± 9 pmol/mg protein (n=3) (at 10s) and a sustained phase (at 300s) of 31 ± 6 pmol/mg protein (n=3). Simultaneous stimulation with 10μM methacholine and 50mM KCl or with 10μM methacholine and 30μM nicotine did not significantly alter peak or sustained Ins(1,4,5)P_3 accumulation from that acheived with 10μM methacholine alone (Figure 3(B)).

Stimulation of SH-SY5Y_{RA6} cells with 1mM methacholine elevated Ins(1,4,5)P_3 mass from a resting level of 6 ± 2 pmol/mg protein (n=3) to a peak (at 10s) of 179 ± 7 pmol/mg protein (n=3) followed by a sustained phase (at 300s) of 48 ± 3 pmol/mg protein (n=3). Simultaneous stimulation with 1mM methacholine and 50mM KCl or with 1mM methacholine and 30μM nicotine did not significantly alter peak or sustained Ins(1,4,5)P_3 accumulation from that acheived with 1mM methacholine alone (Figure 4(A)). Stimulation of SH-SY5Y_{RA6} cells with 10μM methacholine (submaximal concentration) also elevated Ins(1,4,5)P_3 mass from a resting level of 12 ± 3 pmol/mg protein (n=3) to a peak value (at 10s) of 96 ± 5 pmol/mg protein (n=3) and a sustained phase (at 300s) of 37 ± 6 pmol/mg protein (n=3). Simultaneous

stimulation of SH-SY5Y$_{RA6}$ cells with 10μM methacholine and 50mM KCl or with 10μM methacholine and 30μM nicotine did not significantly alter peak or sustained Ins(1,4,5)P_3 accumulation from that acheived with methacholine (10μM) alone (Figure 4(B)). In summary, cellular depolarisation with either 50mM KCl or 30μM nicotine did not significantly alter methacholine-mediated Ins(1,4,5)P_3 accumulation (when either maximally or sub-maximally effective concentrations of methacholine were used) in SH-SY5Y or SH-SY5Y$_{RA6}$ cells.

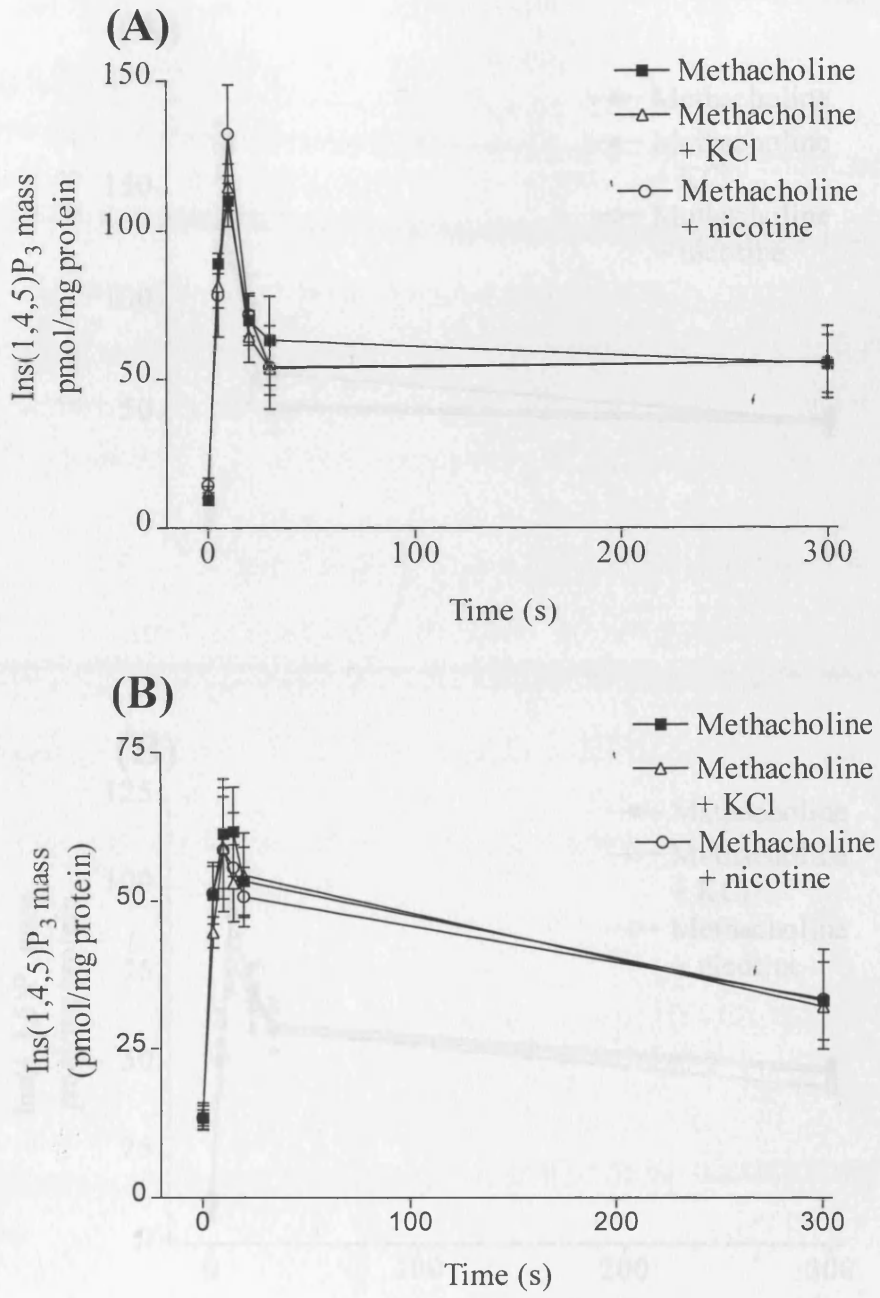


Figure 3 Time course of changes in Ins(1,4,5)P_3 mass in adherent SH-SY5Y cells in response to (A) 1mM methacholine or (B) 10μM methacholine in the absence or presence of either KCl (50mM) or nicotine (30μM). Data are means ± S.E.M., n=3.

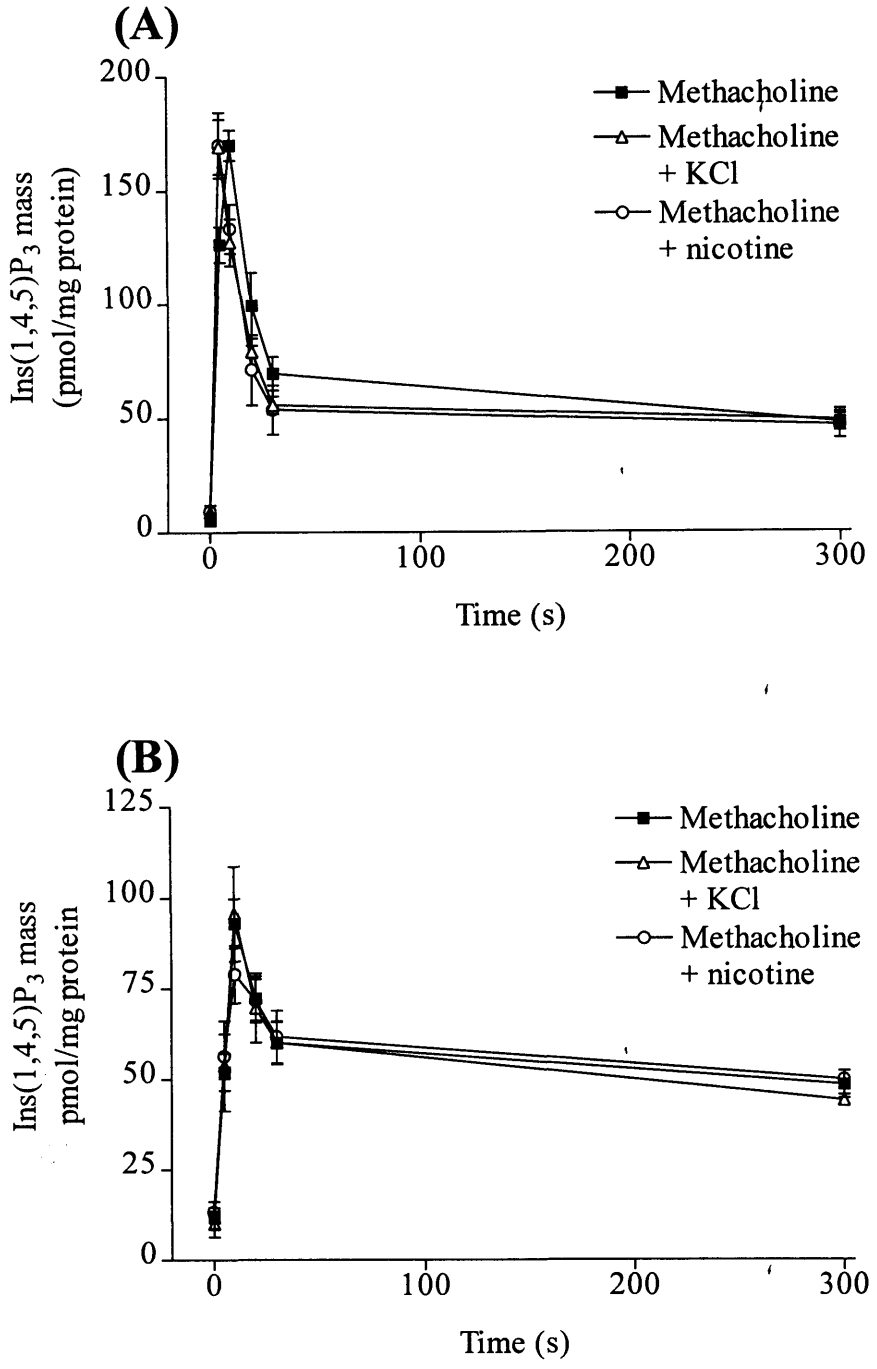


Figure 4 *Time course of changes in Ins(1,4,5)P_3 mass in adherent SH-SY5Y_{RA6} cells in response to (A) 1mM methacholine or (B) 10μM methacholine in the absence or the presence of either KCl (50mM) or nicotine (30μM). Data are means ± S.E.M., n=3.*

Effect of depolarisation on methacholine-mediated accumulation of total [^{3}H]-inositol phosphates in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

In response to 1mM methacholine, [^{3}H]-inositol phosphate accumulation was linear between 2 and 25min (as described in chapter 4) in SH-SY5Y and SH-SY5Y$_{RA6}$ cells. By 25min, accumulation in undifferentiated SH-SY5Y cells co-stimulated with 1mM methacholine and 50mM KCl was significantly ($p< 0.05$) greater as compared to SH-SY5Y cells stimulated with 1mM methacholine only (Figure 5(A)). Similarly, by 25min, [^{3}H]-inositol phosphate accumulation in SH-SY5Y$_{RA6}$ cells co-stimulated with 1mM methacholine and 50mM KCl was significantly ($p< 0.05$) greater as compared to SH-SY5Y$_{RA6}$ cells stimulated with 1mM methacholine only (Figure 5(B)). Challenge of either SH-SY5Y or SH-SY5Y$_{RA6}$ cells with 50mM KCl alone failed to significantly stimulate the accumulation of total [^{3}H]-inositol phosphates (Figures 5(A) and 5(B)).

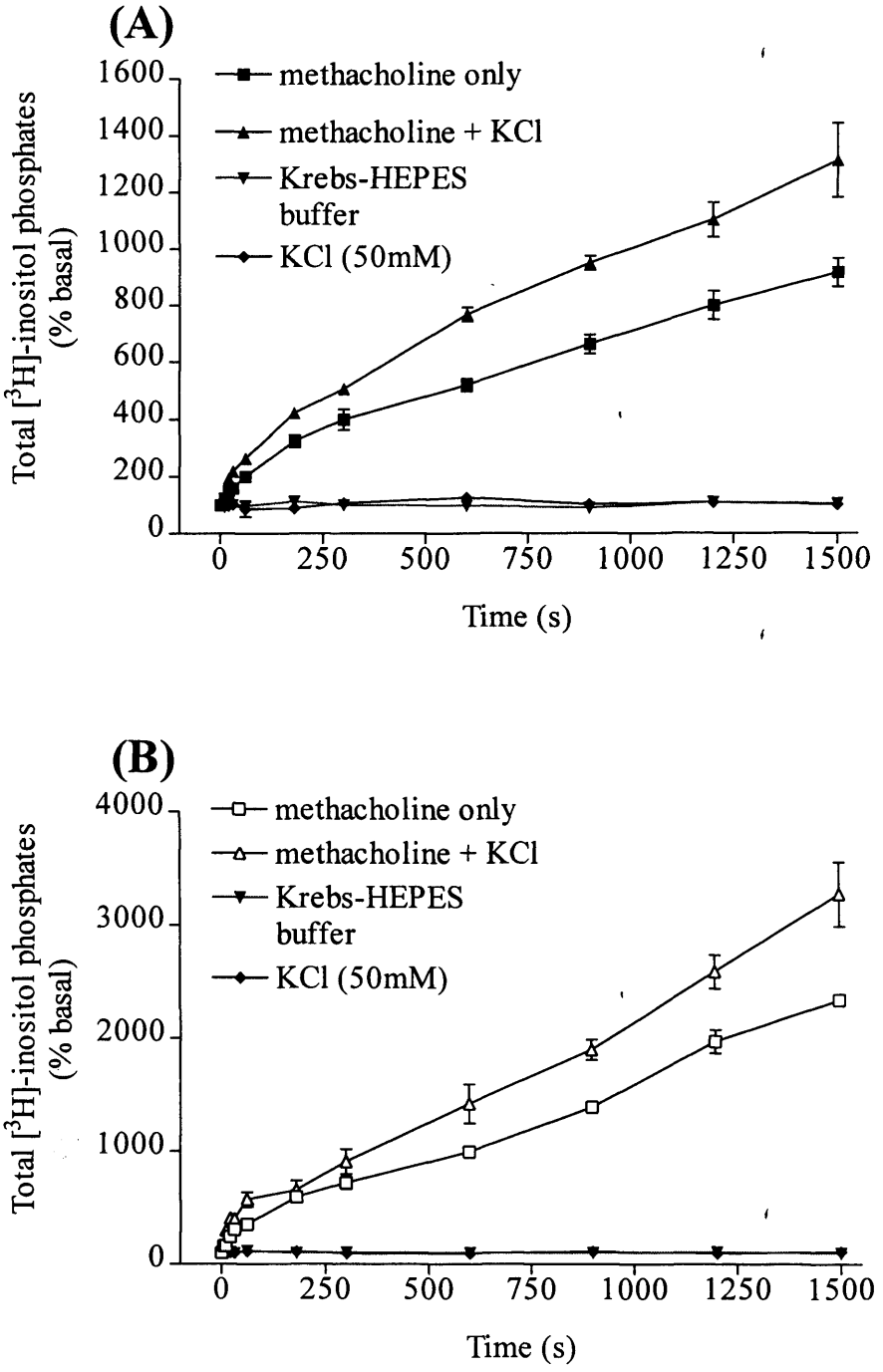


Figure 5 Time course of total [^{3}H]-inositol phosphate accumulation in adherent (A) SH-SY5Y and (B) SH-SY5Y$_{RA6}$ cells in response to methacholine (1mM) in the presence or the absence of KCl (50mM). Data are means ± S.E.M., n=3.

Effect of depolarisation on methacholine-mediated $[Ca^{2+}]_i$ elevations in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells.

In agreement with previous results in this thesis, stimulation of undifferentiated SH-SY5Y cells with a maximal concentration of methacholine (1mM) evoked a rapid rise of $[Ca^{2+}]_i$ from resting levels of 103 ± 5nM (n=7) to a peak of $[Ca^{2+}]_i$ (724 ± 33nM, n=7) approximately 10s after agonist addition. This peak was followed by a lower, sustained phase (203 ± 7nM, n=3). Neither peak nor sustained $[Ca^{2+}]_i$ elevations following challenge with 1mM methacholine were significantly altered in SH-SY5Y$_{RA6}$ cells (data not shown).

In order to investigate whether methacholine-mediated $[Ca^{2+}]_i$ responses in SH-SY5Y cells are subject to regulation by cellular depolarisation, the following experiments were designed to examine the effect of prestimulation with a depolarising stimulus on $[Ca^{2+}]_i$ responses evoked following challenge with methacholine. The aim of this set of experiments was to examine whether the cells could be 'preconditioned' by a depolarising stimulus such that subsequent stimulation of the phosphoinositide pathway resulted in $[Ca^{2+}]_i$ signals which are different from those evoked in the absence of the prior depolarisation. These experiments were duplicated in SH-SY5Y$_{RA6}$ cells to ascertain whether the effects of depolarisation on muscarinic receptor-mediated Ca^{2+} signalling are altered following RA-differentiation of these cells.

The second set of experiments were designed in order to investigate the effects of the simultaneous stimulation of SH-SY5Y cells with both a depolarising stimulus and methacholine on $[Ca^{2+}]_i$. These studies were performed in order to examine whether specific Ca^{2+} signals from different stimuli are integrated within these cells. These experiments were duplicated in SH-SY5Y$_{RA6}$ cells to investigate whether the way in which the $[Ca^{2+}]_i$ signals are integrated within the cell is altered following cellular differentiation.

Depolarisation evoked by high $[K^+]_e$

Addition of 50mM KCl to undifferentiated SH-SY5Y cells was followed by a peak of $[Ca^{2+}]_i$ (305 ± 19nM, n=3) approximately 10s after agonist addition. This peak was followed by a lower, sustained phase (187 ± 6nM, n=3). The subsequent addition of 1mM methacholine

5min later evoked a rapid peak of $[Ca^{2+}]_i$ (408 ± 47nM, n=3) approximately 10s after addition, which declined to a sustained phase of 111 ± 13nM (n=3) (Figure 6(A)), which was significantly ($p< 0.05$) reduced compared to the sustained phase evoked following stimulation with 1mM methacholine only (203 ± 6nM, n=7).

In a similar manner, addition of 50mM KCl to SH-SY5Y$_{RA6}$ cells was followed by a peak of $[Ca^{2+}]_i$ (325 ± 14nM, n=3) approximately 10s after addition, followed by a sustained phase (153 ± 8nM, n=3). The subsequent addition of 1mM methacholine 5min later evoked a rapid peak of $[Ca^{2+}]_i$ (400 ± 50nM, n=3) approximately 10s after addition, which declined to a sustained phase of 140 ±7nM (n=3) (Figure 6(A)), which was significantly ($p< 0.05$) reduced compared to the sustained phase evoked following stimulation with 1mM methacholine alone (198 ± 4nM, n=7).

The $[Ca^{2+}]_i$ responses evoked following challenge with KCl and subsequent challenge with methacholine (Figure 6(A)) were not significantly different between SH-SY5Y and SH-SY5Y$_{RA6}$ cells. However, peak $[Ca^{2+}]_i$ responses evoked in response to 1mM methacholine (subsequent to KCl) (408 ± 87nm (n=3) and 400 ± 50nM (n=3) in SH-SY5Y and SH-SY5Y$_{RA6}$ cells respectively) were significantly ($p< 0.05$) reduced compared with peak $[Ca^{2+}]_i$ elevations evoked following stimulation with 1mM methacholine only in both SH-SY5Y and SH-SY5Y$_{RA6}$ cells (724 ± 33nM (n=7) and 711 ± 28nM (n=7) respectively).

The simultaneous stimulation of undifferentiated SH-SY5Y cells with 50mM KCl and 1mM methacholine evoked an elevation of $[Ca^{2+}]_i$ which peaked approximately 10s after addition (875 ± 25nM, n=3). This peak was followed by a sustained phase of 151 ± 3nM (n=3). Simultaneous stimulation of SH-SY5Y$_{RA6}$ cells with 50mM KCl and 1mM methacholine evoked elevations of $[Ca^{2+}]_i$ which were not significantly different to those elicited in the undifferentiated SH-SY5Y cells (Figure 7(A)). The $[Ca^{2+}]_i$ responses evoked following simultaneous stimulation with 50mM KCl and 1mM methacholine were significantly ($p< 0.05$) greater than those elicited following stimulation with 1mM methacholine only in both SH-SY5Y (875 ± 25nM (n=3) versus 724 ± 33nM (n=7)) and SH-SY5Y$_{RA6}$ cells (892 ± 42 (n=3) versus 711 ± 28nM (n=7)).

Depolarisation evoked by nicotine

Addition of 30μM nicotine to undifferentiated SH-SY5Y cells was followed by a peak of $[Ca^{2+}]_i$ (242 ± 4nM, n=3) approximately 20s after addition. This peak was followed by a sustained phase (133 ± 5nM, n=3). The subsequent addition of 1mM methacholine 5min later evoked a rapid peak of $[Ca^{2+}]_i$ (967 ± 33nM, n=3) approximately 10s after addition which declined to a sustained phase of 197 ± 4nM (n=3). Addition of 30μM nicotine to SH-SY5Y_{RA6} cells was followed by a peak of $[Ca^{2+}]_i$ approximately 10s after addition which was not significantly different to that elicited in the undifferentiated cells. Subsequent addition of 1mM methacholine 5min later evoked a rapid peak of $[Ca^{2+}]_i$ (717 ± 17nM, n=3) which was significantly ($p< 0.05$) reduced compared with that elicited in SH-SY5Y cells. The rapid peak was followed by a sustained phase which was not significantly different to that evoked in SH-SY5Y cells (Figure 6(B)).

Peak $[Ca^{2+}]_i$ responses evoked following challenge with 1mM methacholine subsequent to nicotine were not significantly different to those evoked following stimulation with 1mM methacholine only in SH-SY5Y_{RA6} cells, but were significantly ($p< 0.05$) greater than peak $[Ca^{2+}]_i$ responses evoked following challenge with 1mM methacholine only in the undifferentiated SH-SY5Ycells (967 ± 33nM (n=3) versus 724 ± 33nM (n=7)).

The simultaneous stimulation of undifferentiated SH-SY5Y cells with 30μM nicotine and 1mM methacholine resulted in a rapid rise of $[Ca^{2+}]_i$ (1125 ± 63nM, n=3) which peaked approximately 10s after addition. This peak was followed by a sustained phase of 177 ± 7nM (n=3). Simultaneous stimulation of SH-SY5Y_{RA6} cells with 30μM nicotine and 1mM methacholine evoked elevations of $[Ca^{2+}]_i$ which were not significantly different to those elicited in SH-SY5Y cells (Figure 7(B)). However, the peak $[Ca^{2+}]_i$ responses evoked following simultaneous stimulation with 30μM nicotine and 1mM methacholine were significantly ($p< 0.05$) greater than those elicited following stimulation with 1mM methacholine only in both SH-SY5Y cells (1125 ± 63nM (n=3) versus 724 ± 33nM (n=7)) and SH-SY5Y_{RA6} cells (1179 ± 54 (n=3) versus 198 ± 28nM (n=7)).

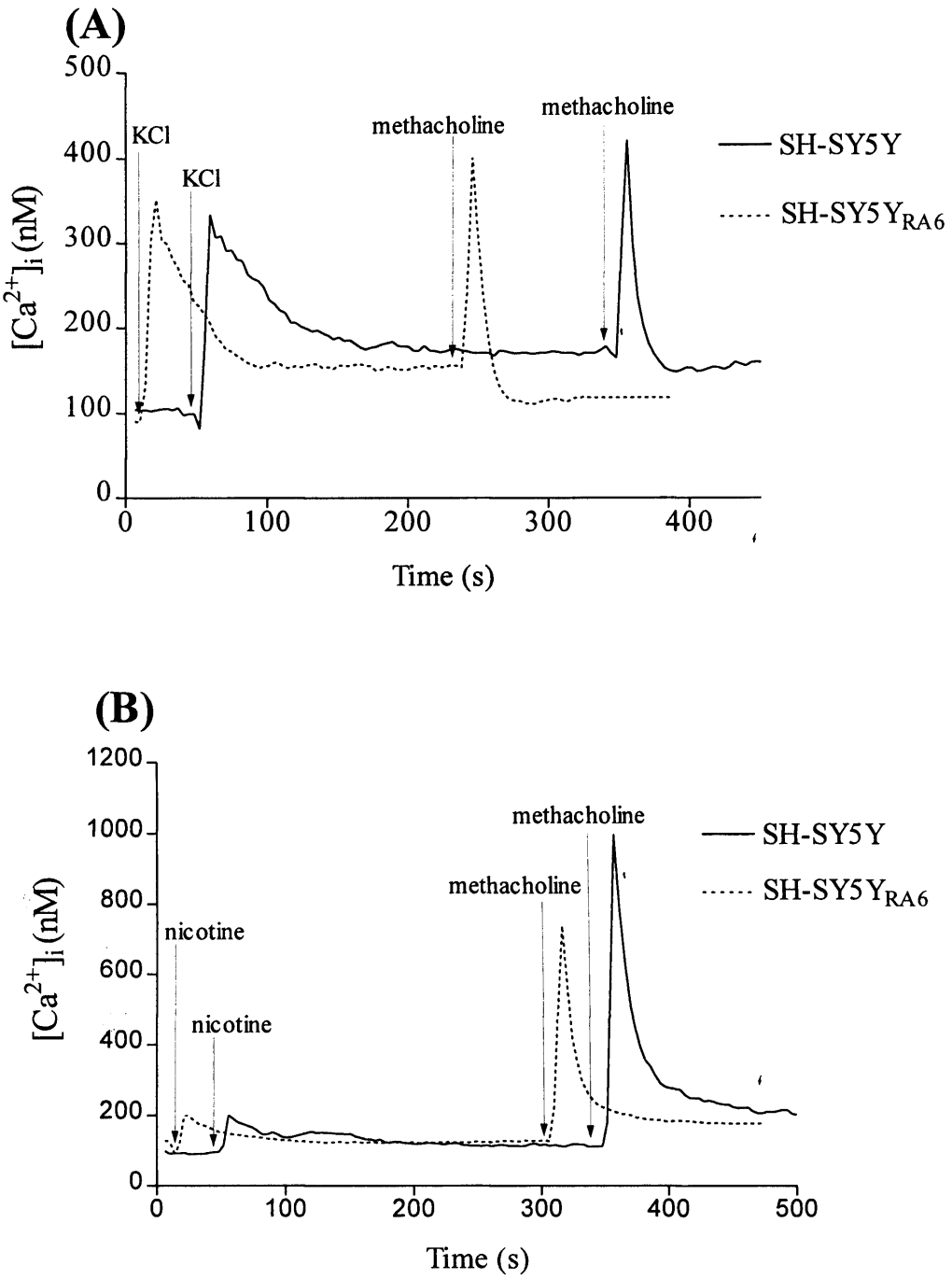


Figure 6 Time course of $[Ca^{2+}]_i$ elevations in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells in response to methacholine (1mM) following challenge with (A) KCl (50mM) and (B) nicotine (30μM). Data are representative traces of at least three similar experiments.

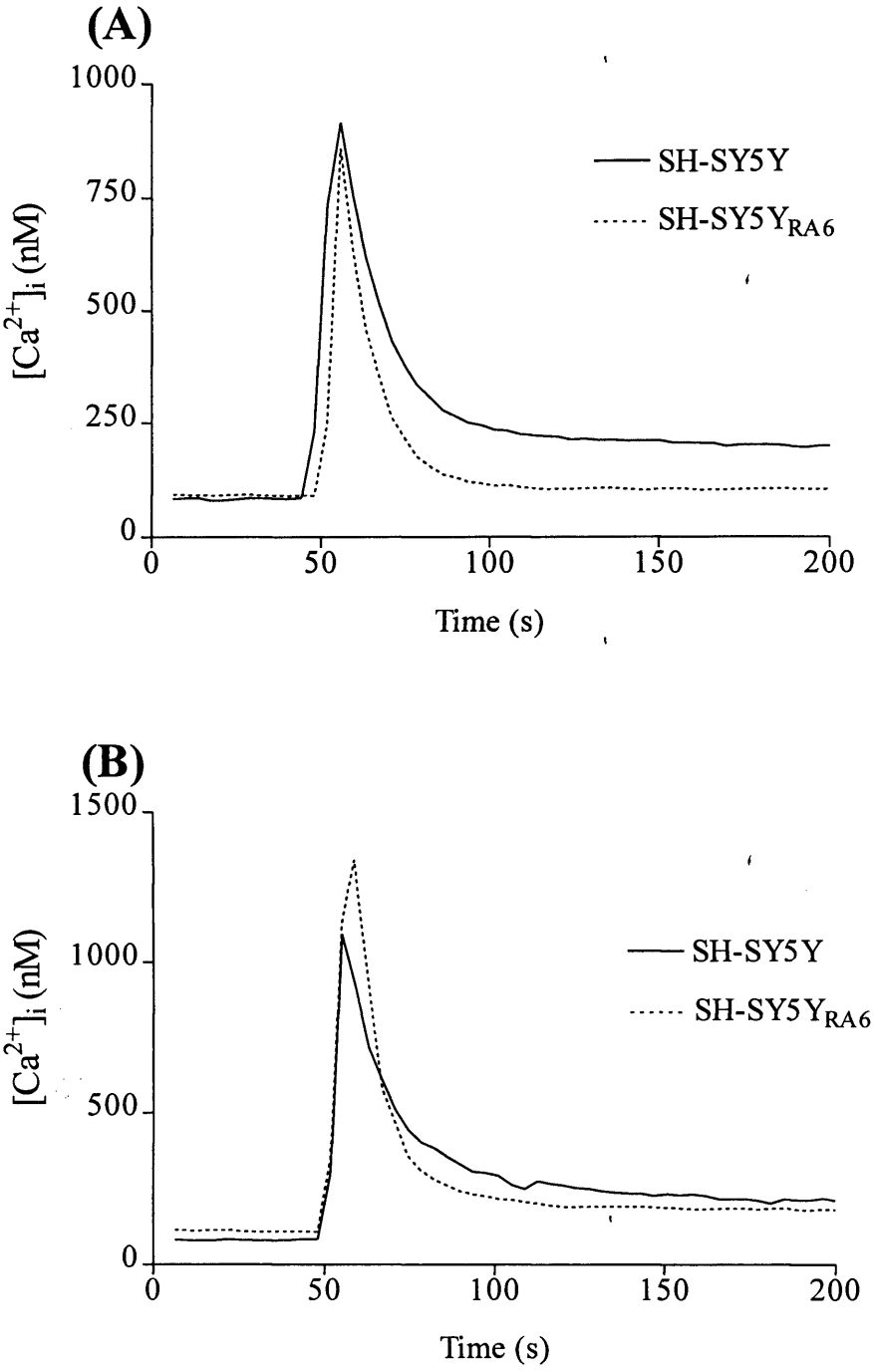


Figure 7 Time course of $[Ca^{2+}]_i$ elevations in populations of adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells following simulataneous challenge with (A) methacholine (1mM) and KCl (50mM) and (B) methacholine (1mM) and nicotine (30μM). Data are representative traces of at least three similar experiments.

6.3 Discussion.

In the present study, the ability of membrane depolarisation, using either a high extracellular K^+ concentration ($[K^+]_e$) or nicotine, to stimulate changes in $[Ca^{2+}]_i$ and to activate PIC directly in both SH-SY5Y and SH-SY5Y$_{RA6}$ cells was examined. The effects of depolarisation on agonist-stimulated PIC activation and changes in $[Ca^{2+}]_i$ in SH-SY5Y and SH-SY5Y$_{RA6}$ cells were also investigated. A specific objective of the current study was to examine whether depolarisation-induced Ca^{2+} influx, either directly via high $[K^+]_e$ or indirectly via stimulation of ionotropic nicotinic receptors, could alone activate PIC and/or facilitate muscarinic receptor-mediated PIC activation in SH-SY5Y cells. Since RA-differentiated SH-SY5Y cells have been reported to express a greater density of VOCCs than undifferentiated SH-SY5Y cells, an additional aim was to investigate whether the potential regulation of PIC by depolarisation-mediated Ca^{2+} entry was different in the SH-SY5Y$_{RA6}$ cells.

The initial finding of this study was that high $[K^+]_e$ elevated $[Ca^{2+}]_i$ in both SH-SY5Y and SH-SY5Y$_{RA6}$ cells in a manner that was clearly biphasic. The nature of the K^+-mediated $[Ca^{2+}]_i$ elevation is not clear as the nature of VOCCs expressed in SH-SY5Y cells has been controversial. The presence of both L- and N-type VOCCs on undifferentiated SH-SY5Y cells has been demonstrated by fura-2 studies using the specific blockers of N- and L-type VOCCs, ω-conotoxin and dihydropyridines respectively, and by electrophysiological studies (Lambert *et al.*, 1990; Reeve *et al.*, 1994). Conversely, in another study, undifferentiated SH-SY5Y cells have been reported not to express VOCCs at all (Connor and Henderson, 1996). The expression of VOCCs following RA-induced differentiation is also a subject of some controversy, with the expression of predominantly N-type channels (Seward and Henderson, 1990; Toselli *et al*, 1991) and the expression of both L- and N-type channels (Morton *et al.*, 1992) having been reported. Challenge with nicotine also elevated $[Ca^{2+}]_i$ in a biphasic manner in SH-SY5Y and SH-SY5Y$_{RA6}$ cells although the $[Ca^{2+}]_i$ elevations evoked by nicotine were smaller than those evoked by high $[K^+]_e$. The reasons for this were not investigated, although nicotinic acetylcholine receptors desensitise rapidly which would limit Ca^{2+} influx since, as a result of desensitisation, the depolarising pulse would only be short-lived. However, it is possible that the density of nicotinic receptors expressed in these cells is

not sufficient to elicit a large depolarising response. It is also a possibility, which was not examined in the current study, that Ca^{2+}-induced Ca^{2+} release (CICR) from ryanodine and/or $Ins(1,4,5)P_3$ sensitive Ca^{2+} stores may play a part in the $[Ca^{2+}]_i$ elevations in response to membrane depolarisation (Simpson *et al.*, 1995).

Depolarisation-induced $[Ca^{2+}]_i$ responses did not differ between SH-SY5Y and SH-$SY5Y_{RA6}$ cells. These findings were unexpected since an increased density of VOCCs during RA differentiation has been demonstrated (Toselli *et al.*, 1991), and hence depolarisation-mediated Ca^{2+} influx would be expected to be greater in SH-$SY5Y_{RA6}$ cells. According to kinetic properties, depolarisation-evoked Ca^{2+} currents are reported to be indistinguishable in RA-differentiated SH-SY5Y cells from those evokable in undifferentiated cells (Toselli *et al.*, 1991), the L- and N-like components showing time-dependent inactivation's during maintained depolarisation with time-constants of 100ms and 1000ms respectively (Reuveny and Narahashi, 1993). Hence, it is unlikely that alterations in Ca^{2+} channel kinetics are responsible for the lack of different responses in SH-SY5Y versus SH-$SY5Y_{RA6}$ cells. It is possible, however, that there is a greater expression of plasma membrane Ca^{2+}-ATPases in SH-$SY5Y_{RA6}$ cells (as discussed in chapter 4) resulting in an increased efficiency of Ca^{2+} extrusion in these cells. Thus, the inward Ca^{2+} current may be greater in SH-$SY5Y_{RA6}$ cells, but is compensated by an increased Ca^{2+}-ATPase activity, with the net result that depolarisation-mediated $[Ca^{2+}]_i$ elevations are equivalent in SH-SY5Y and SH-$SY5Y_{RA6}$ cells. However, this theory is merely speculative and electrophysiological evaluation of depolarisation-mediated inward Ca^{2+} currents would be required for its verification. Alternatively, it is possible that depolarisation-induced $[Ca^{2+}]_i$ responses are different in SH-SY5Y compared with SH-$SY5Y_{RA6}$ cells, but are sub-plasmalemmal differences (i.e. local), due to the location of Ca^{2+} channels, which would be unlikely to be registered by measuring global cytosolic $[Ca^{2+}]_i$ increases.

Elevated $[K^+]_e$ and stimulation with nicotine failed to directly stimulate $Ins(1,4,5)P_3$ mass generation above levels evoked with buffer alone in either SH-SY5Y or SH-$SY5Y_{RA6}$ cells. These findings are in contrast to a number of reports in cells and tissues, including neuronal preparations (Baird and Nahorski, 1989; Challiss and Nahorski, 1991) and undifferentiated SH-SY5Y cells (Smart *et al.*, 1995), where increasing $[K^+]_e$ evoked a phosphoinositide response *per se*. Conversely, and in agreement with the present study, a number of reports

have demonstrated that depolarisation has little or no direct effect on PIC activity (del Rio *et al.*, 1994; Chilvers *et al.*, 1994). The data in the current investigation imply that depolarisation-induced increases in $[Ca^{2+}]_i$ do not initiate independent phosphoinositide hydrolysis in either SH-SY5Y or SH-SY5Y$_{RA6}$ cells. This does not exclude the possibility that depolarisation may enhance agonist-mediated PIC activation.

Agonist-mediated depolarisation and PIC activation are likely to occur simultaneously in a physiological environment. Therefore, the effect of the simultaneous activation of muscarinic receptors and depolarisation, via elevated $[K^+]_e$ or nicotinic receptor activation, was investigated. The simultaneous application of either KCl or nicotine failed to augment Ins(1,4,5)P_3 generation in response to a maximally effective concentration of methacholine in either SH-SY5Y or SH-SY5Y$_{RA6}$ cells. One possible reason for the lack of depolarisation-mediated augmentation of muscarinic receptor-stimulated PIC activation is that methacholine (1mM) stimulated PIC to its maximal activity such that further stimulation of PIC by Ca^{2+} is not possible. Therefore, the effect of depolarisation on Ins(1,4,5)P_3 generation evoked by a submaximal concentration of methacholine (10μM) was investigated. However, neither a high $[K+]_e$, nor stimulation of nicotinic receptors, augmented Ins(1,4,5)P_3 generation in response to 10μM methacholine in either SH-SY5Y or SH-SY5Y$_{RA6}$ cells. Collectively these data would argue against a depolarisation-mediated enhancement of PIC activity in these cells. It is worth noting, however, that the rapid agonist-mediated mobilisation of Ca^{2+} from intracellular stores may, through a feed-forward activation of PIC, be sufficient to mask PIC activation by Ca^{2+} influx through VOCCs during the peak phase of Ins(1,4,5)P_3 generation (Wojcikiewicz *et al.*, 1994).

It has been previously reported that increasing $[Ca^{2+}]_i$ via Ca^{2+} entry through VOCCs may have the effect of increasing the amount of Ins(1,4,5)P_3 metabolised via 3-kinase and/or the 5-phosphatase pathway (Baird and Nahorski, 1989; Challiss and Nahorski, 1991). Hence, it is a possibility that depolarisation-mediated Ca^{2+} influx does indeed enhance PIC activity in SH-SY5Y and/or SH-SY5Y$_{RA6}$ cells, which is masked by a concomitant enhancement of Ins(1,4,5)P_3 metabolism, such that the net Ins(1,4,5)P_3 mass measured is unchanged. The simultaneous application of high $[K^+]_e$ and a maximally effective concentration of methacholine to undifferentiated SH-SY5Y cells resulted in an enhanced accumulation of $[^3H]$-inositol phosphates under Li^+ block as compared to stimulation with methacholine alone.

Similarly, in SH-SY5Y_{RA6} cells, high $[K^+]_e$ facilitated methacholine-mediated [^{3}H]-inositol phosphate accumulation to a similar extent. In both cases, basal accumulations in the presence or absence of high $[K^+]_e$ were unaffected. These findings provide evidence that K^+-depolarisation of SH-SY5Y cells facilitates methacholine-mediated PIC activation. Facilitatory effects of K^+ depolarisation and muscarinic receptor agonists have been demonstrated in several laboratories when total [^{3}H]-inositol phosphates have been assayed (Court *et al.*, 1986; Eva and Costa, 1986). However, the complication when interpreting the data from studies such as these lies with not knowing the source (i.e. PtdIns, PtdInsP and/or PtdIns(4,5)P_2) of [^{3}H]-inositol phosphates trapped with Li^+ at the inositol monophosphatase step and the possible changes in the specific radioactivity of these species under depolarising conditions. Several reports have suggested that Ca^{2+} entering the cell via VOCCs diverts the metabolism of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 by the activation of 3-kinase (Biden and Wollheim, 1986; Irvine *et al.*, 1986; Challiss and Nahorski, 1991). Thus, it may be possible that the lack of facilitation by K^+ (or nicotine) on methacholine-mediated Ins(1,4,5)P_3 generation compared with [^{3}H]-inositol phosphate accumulation in this study is the result of Ca^{2+}-mediated activation of 3-kinase. This would result in the enhanced metabolism of Ins(1,4,5)P_3. In a similar manner, it is also possible that the activity of 5-phosphatase is enhanced by depolarising stimuli in these cells, which would also result in an increased rate of Ins(1,4,5)P_3 metabolism. In the current study neither of these possibilities have been examined. The measurement of Ins(1,3,4,5)P_4 mass (see Challiss and Nahorski, 1991) following challenge with methacholine in the presence or absence of high $[K^+]_e$ would reveal whether depolarisation enhances the phosphorylation (and hence metabolism) of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 which may have consequences because of the proposed role of Ins(1,3,4,5)P_4 as a second messenger (Irvine *et al.*, 1988; Wilcox *et al.*, 1993).

Challenge of either SH-SY5Y or SH-SY5Y_{RA6} cells with methacholine following a 5min prestimulation with high $[K^+]_e$, markedly reduced the methacholine-mediated peak $[Ca^{2+}]_i$ responses, compared with those elicited by methacholine alone. The reason(s) for this effect is unclear, although a possible explanation is that the depolarisation-evoked Ca^{2+} influx, following stimulation with high $[K^+]_e$, is sufficient to evoke a Ca^{2+}-induced Ca^{2+} release (CICR) response from Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores, resulting in their partial emptying. CICR can be evoked from Ca^{2+} stimulation of Ins(1,4,5)P_3 receptors and/or

ryanodine receptors, and there is evidence that type-1 Ins(1,4,5)P_3 receptors and type-2 ryanodine receptors are co-expressed in the SH-SY5Y cell line (Mackrill *et al.*, 1997). Preliminary studies have demonstrated that SH-SY5Y cells contain a caffeine-releasable, ryanodine-sensitive Ca^{2+} pool (Hussain, 1996), suggesting that CICR via ryanodine receptors in these cells is a strong possibility. An alternative possibility is that increased $[Ca^{2+}]_i$, mediated by high $[K^+]_e$, results in the termination or reduction of Ca^{2+} influx through the CRAC channel (see Zweifach and Lewis, 1995). As a result, the refilling of the Ca^{2+} stores would be reduced such that the amount of Ca^{2+} available in the stores for mobilisation following agonist-mediated Ins(1,4,5)P_3 formation would also be reduced.

In marked contrast to the findings discussed above using high $[K^+]_e$ to increase $[Ca^{2+}]_i$, challenge with nicotine followed by methacholine resulted in methacholine-mediated peak $[Ca^{2+}]_i$ responses that were similar to those elicited by methacholine alone in SH-SY5Y_{RA6} cells, but greater than those elicited by methacholine only in the undifferentiated cells. One could hypothesise, if we assume that the theory described above is correct, that since the $[Ca^{2+}]_i$ response to nicotine is smaller than that elicited by high $[K^+]_e$, it may be insufficient to mediate a CICR response and/or inhibit capacitative Ca^{2+} entry. Since nicotinic receptors desensitise rapidly, this does not seem an unreasonable probability. It is unclear, however, why the $[Ca^{2+}]_i$ response is enhanced in SH-SY5Y but not in SH-SY5Y_{RA6} cells.

Another interesting phenomenon demonstrated in this study by both SH-SY5Y and SH-SY5Y_{RA6} cells is that 'preconditioning' of cells with the addition of high $[K^+]_e$ appears to drive down the sustained $[Ca^{2+}]_i$ plateau phase acheived following the addition of 1mM methacholine. A possible mechanism of action is through an agonist-stimulation of Ca^{2+}-ATPase in the plasma membrane, resulting in an increased ability of the cell to clear high $[Ca^{2+}]_i$ from the cytosol. In support of this hypothesis, it has previously been shown that the Ca^{2+}-ATPases can be regulated positively by agonists, probably via the activation of PKC with the subsequent phosphorylation, and hence regulation, of Ca^{2+}-ATPase (Zhang *et al.*, 1992; Wojcikiewicz *et al.*, 1994; Journeaux *et al.*, 1994; Young *et al.*, 1997).

Following the simultaneous challenge of either SH-SY5Y or SH-SY5Y_{RA6} cells with both a depolarising stimulus (either high $[K^+]_e$ or nicotine) and methacholine, peak $[Ca^{2+}]_i$ elevations elicited were enhanced compared with those evoked in response to methacholine alone. One

possible explanation for this observation is that it is simply an additive effect of Ca^{2+} entry through VOCCs and Ins(1,4,5)P_3-mediated Ca^{2+} release from intracellular stores. This is possibly the case for KCl-mediated Ca^{2+} influx although the effect is less than additive. Another explanation is that, as discussed above, a depolarising stimuli may enhance agonist-mediated Ins(1,3,4,5)P_4 generation, which may then facilitate Ins(1,4,5)P_3-mediated Ca^{2+} mobilisation. However, this theory would not explain why nicotine enhances methacholine-mediated Ca^{2+} mobilisation to a greater extent as compared with KCl, since facilitation would be expected to be greater for KCl than for nicotine if this hypothesis were correct. Ins(1,4,5)P_3-stimulated Ca^{2+} release from intracellular stores depends, not only on the cytosolic concentration of Ins(1,4,5)P_3, but also on $[Ca^{2+}]_i$ (for review see Taylor and Marshall, 1992). An alternative explanation therefore is that the ability of Ins(1,4,5)P_3 (generated by agonist) to release Ca^{2+} may be affected under depolarising conditions because depolarisation increases $[Ca^{2+}]_i$ which in turn may enhance the sensitivity of Ins(1,4,5)P_3 receptors to Ins(1,4,5)P_3. Therefore, the increase in Ins(1,4,5)P_3 generation, as well as the initial $[Ca^{2+}]_i$ rise induced by a depolarising stimulus, may explain the observed synergistic effect on $[Ca^{2+}]_i$. However, this hypothesis would also predict that facilitation of methacholine-mediated $[Ca^{2+}]_i$ elevation would be greater for high $[K^+]_e$- than for nicotine-mediated depolarisation.

In summary, neuronal cells possess a variety of signalling mechanisms, including a range of VOCCs as well as second messenger-regulated Ca^{2+} release and Ca^{2+} entry pathways. Numerous reports suggest that there is considerable interplay amongst the Ca^{2+} signalling mechanisms in neuronal cells and tissues. In the current study, direct and indirect depolarisation, by high $[K^+]_e$ and nicotine respectively, elevated $[Ca^{2+}]_i$ in a biphasic manner. Depolarisation-mediated $[Ca^{2+}]_i$ elevations did not differ between SH-SY5Y and SH-$SY5Y_{RA6}$ cells. Depolarisation by either method failed to either directly generate Ins(1,4,5)P_3 or potentiate methacholine-mediated Ins(1,4,5)P_3 generation in these cells. In contrast, high $[K^+]_e$ enhanced methacholine-stimulated [^{3}H]-inositol phosphate accumulation, without directly stimulating accumulation, to a similar extent in SH-SY5Y and SH-$SY5Y_{RA6}$ cells. Collectively these data imply a depolarisation-mediated increase in the metabolism of Ins(1,4,5)P_3. Methacholine-evoked peak $[Ca^{2+}]_i$ elevations were enhanced in the presence of depolarising stimuli, the mechanism(s) of which is unclear, but could include an

$Ins(1,3,4,5)P_4$- and/or Ca^{2+}-mediated enhancement of $Ins(1,4,5)P_3$-evoked Ca^{2+} release from intracellular stores.

7. INVESTIGATION OF A POTENTIAL MODULATION OF THE INS(1,4,5)P_3 RECEPTOR/Ca^{2+} RELEASE CHANNEL COMPLEX BY AGONIST IN SH-SY5Y CELLS.

7.1 Introduction

One of the more intriguing aspects of the work presented in previous chapters was that, whilst there was a receptor reserve for agonist-stimulated Ins(1,4,5)P_3 elevations in SH-SY5Y_{RA6} cells (concomitant with increased cell surface receptor density), there appeared to be no receptor reserve for agonist-stimulated increases in $[Ca^{2+}]_i$ in SH-SY5Y_{RA6} cells. These findings were suprising since dose-response curves for agonist-stimulated increases in $[Ca^{2+}]_i$ lay to the left of those for Ins(1,4,5)P_3 elevation in both SH-SY5Y and SH-SY5Y_{RA6} cells, indicating that there was an amplification of the response. The lack of receptor reserve for agonist-stimulated $[Ca^{2+}]_i$ elevations in SH-SY5Y_{RA6} cells was additionally suprising since it is generally believed that only small concentrations of Ins(1,4,5)P_3 are required to release Ca^{2+} from intracellular stores. Indeed, basal levels of Ins(1,4,5)P_3 in resting, intact cells are often in the micromolar range (Challiss *et al.*, 1990; Zhao *et al.*, 1990), concentrations which would be expected to fully mobilise stored Ca^{2+} from studies on microsomes and permeabilised cells. There is, however, increasing evidence to suggest that regulation of Ca^{2+} mobilisation occurs via complex processes which are not fully understood. For example, several agonists, such as bradykinin acting on 3T6 fibroblast cells (Zhao *et al.*, 1990) or parathyroid hormone acting on osteoblasts (Babich *et al.*, 1991; Pines *et al.*, 1996) cause substantial Ca^{2+} release from intracellular stores without a corresponding increase in Ins(1,4,5)P_3. In other studies agonists have been shown to activate, and antagonists to inactivate, the Ins(1,4,5)P_3 receptor/Ca^{2+} release channel, independent of PtdIns(4,5)P_2 metabolism (Zhang *et al.*, 1994; Tortorici *et al.*, 1994).

Studies in several cellular systems have demonstrated the existence of multiple mechanisms of Ins(1,4,5)P_3 receptor/Ca^{2+} release channel regulation, besides its activation by Ins(1,4,5)P_3. For example, the channel can be regulated by Ca^{2+} in a biphasic manner (Bezprozvanny *et al.*, 1993; Finch *et al.*, 1991), ATP can regulate the channel by a direct or by an indirect mechanism (Bezprozvanny *et al.*, 1993; Ferris *et al.*, 1990; Zhang *et al.*, 1993) and PKA has

been shown to either increase (Bird *et al.*, 1993; Hajnoczky *et al.*, 1993) or reduce (Cameron *et al.*, 1995) the apparent affinity of the Ins(1,4,5)P_3 receptor for Ins(1,4,5)P_3. Given such diverse mechanisms for the modulation of the Ins(1,4,5)P_3 receptor/Ca^{2+} release channel complex, a possible explanation for the above mentioned discrepancies between agonist-stimulated Ins(1,4,5)P_3 production and $[Ca^{2+}]_i$ elevation is that agonists, independent of Ins(1,4,5)P_3 production, can modulate the activity of the Ins(1,4,5)P_3 receptor/Ca^{2+} release channel complex.

The aim of the current chapter was to examine the possibility that activation of muscarinic and/or bradykinin receptors is capable of regulating the Ins(1,4,5)P_3 receptor/Ca^{2+} release channel complex, independent of Ins(1,4,5)P_3 generation in SH-SY5Y cells. This aspect was explored by examining the influence of muscarinic and bradykinin receptor activation on the ability of exogenously applied Ins(1,4,5)P_3 to release Ca^{2+} from intracellular stores, preloaded to equilibrium with $^{45}Ca^{2+}$, within a permeabilised cell. Permeabilisation of cells in suspension was performed in a cytosol-like buffer (see Appendix A) where free Ca^{2+} was buffered to the basal levels of free Ca^{2+} found in the cytosol of intact cells (approximately 100nM). In order to perform these studies, it was necessary to eliminate Ins(1,4,5)P_3 generation and $^{45}Ca^{2+}$ mobilisation in response to agonist. To this end the system was manipulated in an attempt to deplete agonist-sensitive PtdIns(4,5)P_2 pools in SH-SY5Y cells. These manipulations are described in detail in sections 7.2 and 7.3.

7.2 Methods

Ins(1,4,5)P_3- and agonist-evoked $^{45}Ca^{2+}$ release and agonist-mediated Ins(1,4,5)P_3 mass accumulations were assessed in β-escin-permeabilised cells (prepared as described in section 2.3.4) using the procedures described in sections 2.6 and 2.4 respectively.

Procedure for the depletion of agonist-sensitive PtdIns(4,5)P_2 pools.

PtdIns(4,5)P_2 pool depletion of SH-SY5Y cells was acheived using the following protocol. Cells were harvested and resuspended in Krebs-HEPES buffer (see Appendix B). Methacholine (1mM final concentration)was added to the cell suspension, vortexed and left for 1min. In order to remove the methacholine from the suspension, cells were centrifuged (10,000g, 2min) and resuspended in CLB (see Appendix A) containing wortmannin (10μM final concentration) and then washed and re-centrifuged (as described above) twice. The cell suspension in CLB containing 10μM wortmannin was left on ice for 15min, after which the cells were permeabilised with β-escin (25μg/ml). For measurement of $^{45}Ca^{2+}$ release, $^{45}CaCl_2$ was added at this point and the cells incubated at room temperature for a further 15min before initiation of experimental incubations (as described in section 2.6). For measurement of Ins(1,4,5)P_3 accumulation, cells were left following permeabilisation for 15min at room temperature before agonist stimulation (as described in section 2.4).

7.3 Results

The effect of bradykinin on exogenous Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release in permeabilised SH-SY5Y cells.

Exogenous Ins(1,4,5)P_3 dose-dependently released $^{45}Ca^{2+}$ from ionomycin-sensitive intracellular stores with a $\log_{10}$ EC_{50} value of -7.18 ± 0.03M (66nM). In the presence of bradykinin (10μM) the potency of Ins(1,4,5)P_3-evoked $^{45}Ca^{2+}$ mobilisation was significantly ($p< 0.05$) enhanced with a $\log_{10}$ EC_{50} value of -7.41 ± 0.02M (39nM). However, bradykinin alone failed to elicit a $^{45}Ca^{2+}$ mobilisation response, even at a maximal concentration of bradykinin (10μM) (figure 1).

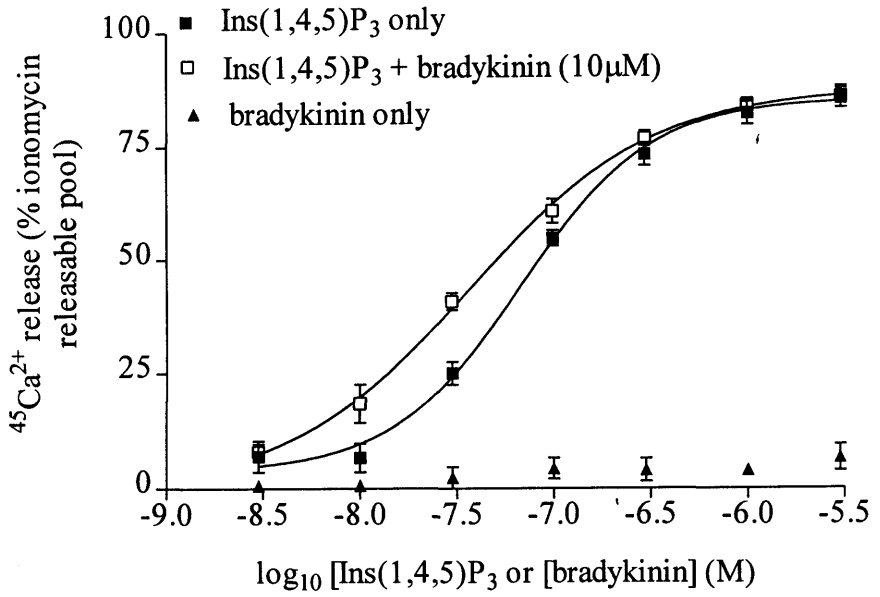


Figure 1 Dose-response relationship for Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ mobilisation in the presence or absence of 10μM bradykinin and the relationship for bradykinin alone in permeabilised SH-SY5Y cells. Data points are means ± S.E.M., n = 4.

The potentiation of Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release by bradykinin was further investigated using a single concentration of Ins(1,4,5)P_3 (30nM). Treatment of permeabilised SH-SY5Y cells with this concentration of Ins(1,4,5)P_3 alone caused a modest $^{45}Ca^{2+}$ release (19 ± 2% ionomycin release, n=4). In the presence of bradykinin (10μM) $^{45}Ca^{2+}$ release in response to 30nM Ins(1,4,5)P_3 was significantly ($p < 0.05$) potentiated (39 ± 1%, n=4) and this potentiation was reversed in the presence of the bradykinin B2-selective receptor antagonist, HOE 140 (1μM) (20 ± 0.7%, n=4) (figure 2).

In summary, these data demonstrate that bradykinin, despite being unable to evoke the release of $^{45}Ca^{2+}$ from SH-SY5Y cells, was able to increase the potency of exogenous Ins(1,4,5)P_3-evoked $^{45}Ca^{2+}$ release. This facilitation is a bradykinin receptor-dependent phenomenon as demonstrated by reversal of the potentiation in the presence of HOE 140.

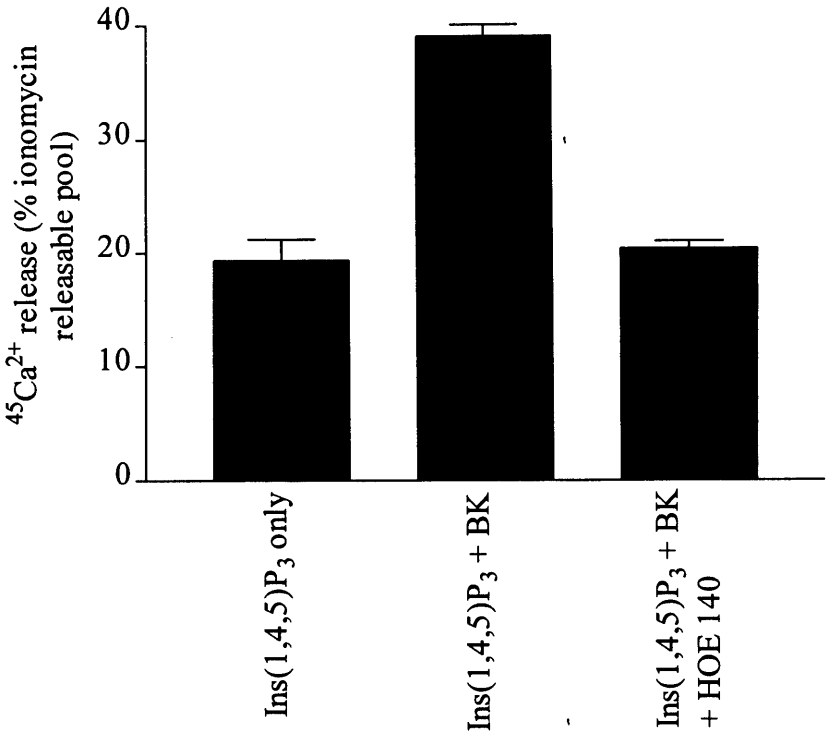


Figure 2 Effect of bradykinin (10μM) and bradykinin (10μM) + HOE 140 (1μM) $^{45}Ca^{2+}$ mobilisation in response to 30nM Ins(1,4,5)P_3 in permeabilised SH-SY5Y cells. Data are means ± S.E.M., n = 3.

The effect of wortmannin on methacholine-induced Ins(1,4,5)P_3 accumulation and $^{45}Ca^{2+}$ mobilisation in SH-SY5Y cells.

Muscarinic receptor stimulation with the full agonist, methacholine, results in a large Ins(1,4,5)P_3 accumulation which mediates a substantial and dose-dependent mobilisation of $^{45}Ca^{2+}$ in permeabilised SH-SY5Y cells (Wojcikiewicz *et al.*, 1990; Safrany and Nahorski, 1994). Thus, it is not possible to investigate a potential methacholine-mediated facilitation of exogenous Ins(1,4,5)P_3-evoked $^{45}Ca^{2+}$, as interpretation would be hindered by the accumulation of endogenous Ins(1,4,5)P_3. In order to continue the current study, a situation was required where methacholine-mediated Ins(1,4,5)P_3 elevation was reduced to such an extent that Ca^{2+} release in response to methacholine was eliminated, but coupling of the M3 receptors to G-protein was maintained.

Wortmannin, an inhibitor of myosin light-chain kinase and PtdIns 3-kinase has been reported to inhibit agonist-stimulated $Ins(1,4,5)P_3$ accumulation in cells (Nakanishi *et al.*, 1994). Wortmannin is also a potent inhibitor of PtdIns 4-kinase (Downing *et al.*, 1996), the inhibition of which results in a rapid loss of the agonist-sensitive $PtdIns(4,5)P_2$ pool in a variety of agonist-stimulated cells (Nakanishi *et al.*, 1995). In the current study wortmannin was utilised as a tool to deplete $PtdIns(4,5)P_2$ pools in SH-SY5Y cells. In order to ascertain the duration of wortmannin pre-treatment required to inhibit methacholine-mediated $Ins(1,4,5)P_3$ accumulation, SH-SY5Y cells were exposed to wortmannin (10μM) for variable time periods (0 -30min) prior to challenge with methacholine (1mM). Basal (0s), peak (10s) and sustained (5min) $Ins(1,4,5)P_3$ accumulations were then measured. The results from this study demonstrated that basal $Ins(1,4,5)P_3$ was unaffected by pre-treatment with wortmannin (10μM) for any duration of pre-treatment. However, peak and sustained methacholine-mediated $Ins(1,4,5)P_3$ accumulations were inhibited in a time-dependent manner by wortmannin pre-treatment. Maximal inhibition of peak methacholine-stimulated $Ins(1,4,5)P_3$ accumulation followed a 30min pre-treatment with 10μM wortmannin (figure 3).

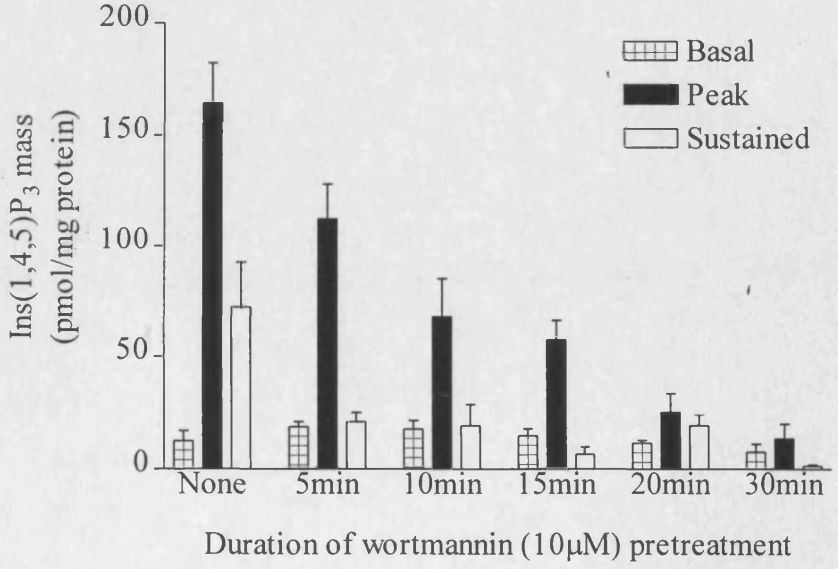


Figure 3 *The effect of wortmannin (10μM) pre-treatment (0-30min) on basal $Ins(1,4,5)P_3$ mass levels and on peak (at 10s) and sustained (at 5min) $Ins(1,4,5)P_3$ mass accumulation following challenge with 1mM methacholine in populations of adherent SH-SY5Y cells. Data are means ± S.E.M., n = 4.*

Stimulation of muscarinic receptors in permeabilised SH-SY5Y cells with a supramaximal concentration of methacholine (1mM) stimulated a time-dependent accumulation of $Ins(1,4,5)P_3$ from a basal level of 18 ± 6 pmol/mg protein (n=3) to a peak of 183 ± 11 pmol/mg protein (n=3) at 50s, which then declined to a sustained component of 98 ± 26 pmol/mg protein (n=3). Following a 30min preincubation with wortmannin (10μM), the time-dependent accumulation of $Ins(1,4,5)P_3$ in response to challenge with 1mM methacholine was markedly reduced with a peak accumulation of 45 ± 2 pmol/mg protein (n=3) occurring at 60s, with no evidence of a sustained phase (figure 4).

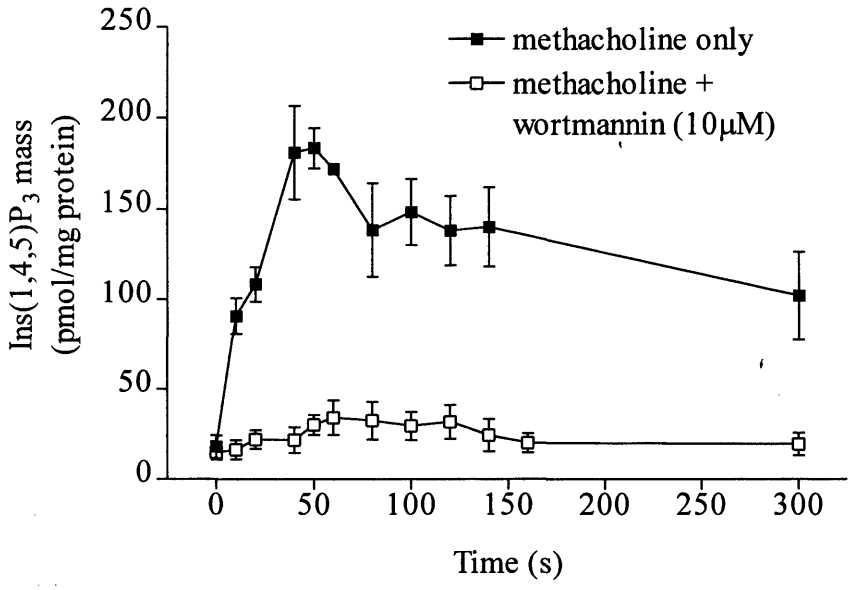


Figure 4 Time course of changes in $Ins(1,4,5)P_3$ mass following challenge with 1mM methacholine with or without 30min pre-treatment with wortmannin (10μM) in permeabilised SH-SY5Y cells. Data are means ± S.E.M., n = 3.

Methacholine alone dose-dependently mobilised $^{45}Ca^{2+}$ from ionomycin-sensitive stores with a $\log_{10}$ EC_{50} value of -5.49 ± 0.06M (3.2μM). Preincubation with wortmannin (10μM) for 30min slightly decreased the potency of methacholine-induced $^{45}Ca^{2+}$ release (approximately 2-fold) with a $\log_{10}$ EC_{50} value of -5.32 ± 0.02M (5.6μM). Surprisingly, and in contrast to the $Ins(1,4,5)P_3$ accumulations, the maximal response was only slightly reduced (13% decrease) (figure 5(A)). Exogenous $Ins(1,4,5)P_3$ alone dose-dependently released $^{45}Ca^{2+}$ from

ionomycin-sensitive stores with a $\log_{10}$ EC_{50} value of -7.21 ± 0.05M (62nM). Preincubation with wortmannin (10μM) for 30min did not significantly effect the ability of exogenous $Ins(1,4,5)P_3$ to release $^{45}Ca^{2+}$ (figure 5(B)).

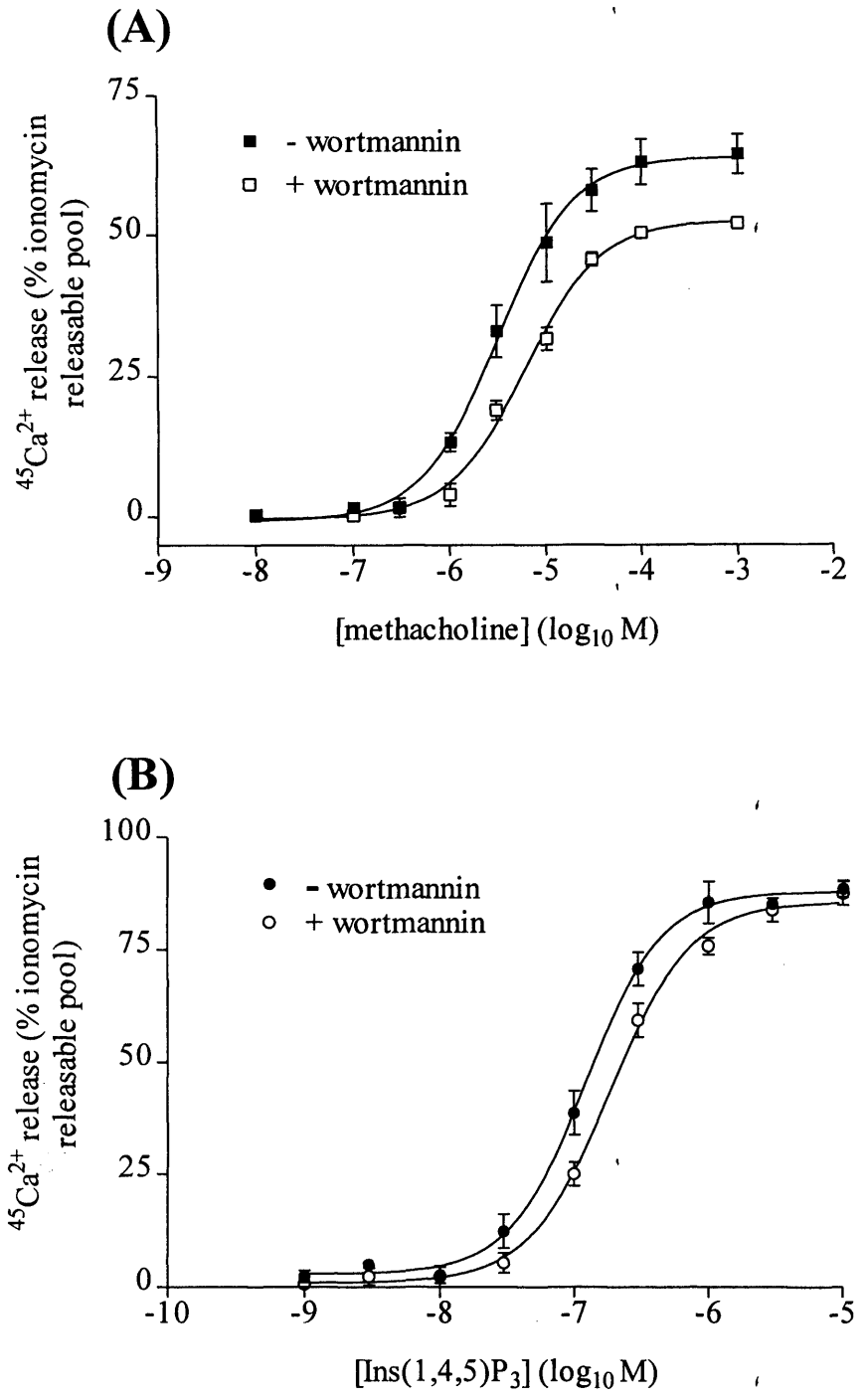


Figure 5 *Dose-response relationships for methacholine-mediated (A) and Ins(1,4,5)P_3-mediated (B) $^{45}Ca^{2+}$ mobilisation in the absence or the presence of pre-treatment with wortmannin (10μM, 30min) in permeabilised SH-SY5Y cells. Data points are means ± S.E.M., n = 3.*

The effect of heparin on methacholine-mediated $^{45}Ca^{2+}$ mobilisation and Ins(1,4,5)P_3 accumulation in permeabilised SH-SY5Y cells.

In order to establish that muscarinic receptor-mediated Ca^{2+} release was actually via the interaction of Ins(1,4,5)P_3 with its receptor/Ca^{2+} release channel, the use of heparin was employed. Heparin acts as a competitive inhibitor of the binding of Ins(1,4,5)P_3 to its receptor (Ferris *et al.*, 1989). Pre-treatment of permeabilised SH-SY5Y cells with heparin (100μg/ml) for 5min obliterated $^{45}Ca^{2+}$ mobilisation in response to methacholine (figure 6). However, heparin has also been reported to uncouple cell surface receptors from PIC (Dasso *et al.*, 1991), hence it remained possible that, in addition, heparin inhibits receptor-G-protein coupling, thus preventing generation of Ins(1,4,5)P_3. To eliminate this possibility the effect of heparin on methacholine-mediated Ins(1,4,5)P_3 accumulation was investigated. Pre-treatment of permeabilised SH-SY5Y cells with heparin (100μg/ml) for 5min significantly ($p< 0.05$) decreased time-dependent Ins(1,4,5)P_3 generation in response to 1mM methacholine with peak accumulation at 60s of 96 ± 8 pmol/mg protein (n=3) (figure 7).

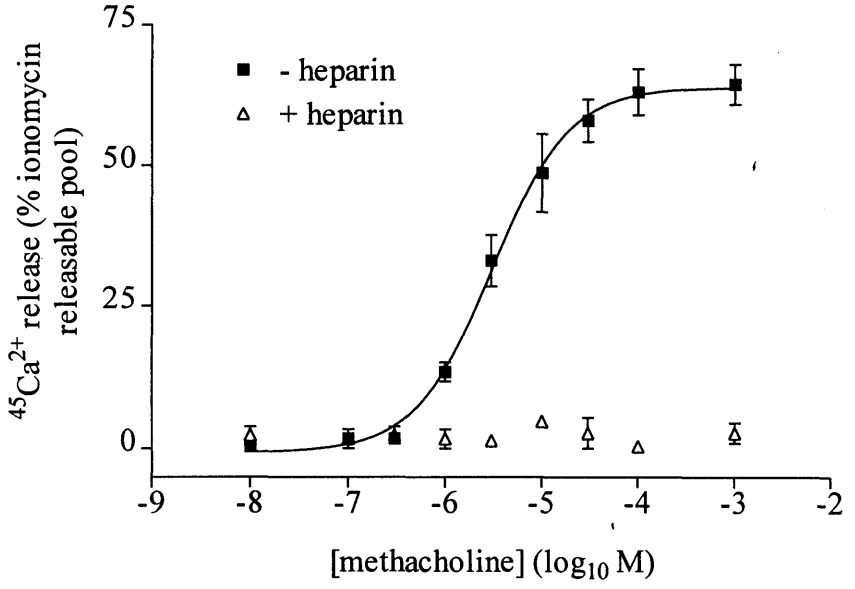


Figure 6 Dose-response-relationships for methacholine-mediated $^{45}Ca^{2+}$ mobilisation in the absence or the presence of pre-treatment with heparin (100μg/ml, 5min) in permeabilised SH-SY5Y cells. Data points are means ± S.E.M., n = 3.

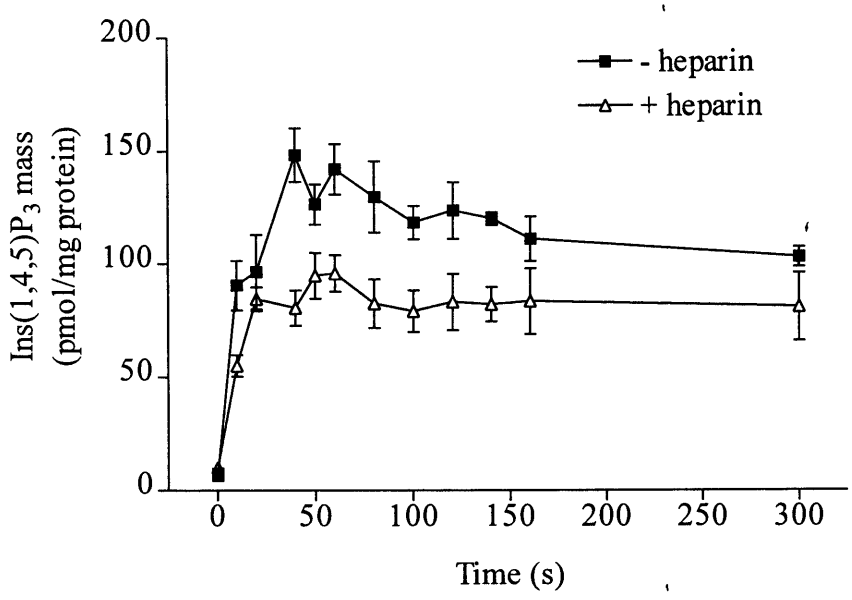


Figure 7 Time course of changes in Ins(1,4,5)P_3 mass following challenge with 1mM methacholine with or without 5min pre-treatment with heparin (100μg/ml) in permeabilised SH-SY5Y cells. Data are means ± S.E.M., n = 3.

The effect of PtdIns(4,5)P_2 pool depletion, with a methacholine and wortmannin prestimulation, on methacholine-mediated Ins(1,4,5)P_3 formation and $^{45}Ca^{2+}$ mobilisation in permeabilised SH-SY5Y cells.

It is likely that there is a residual PtdIns(4,5)P_2 pool, insensitive to wortmannin, that is available for the generation of Ins(1,4,5)P_3 following agonist-induced activation of PIC, as demonstrated by the very small methacholine-induced Ins(1,4,5)P_3 accumulation following pre-treatment with wortmannin (figure 4). This residual PtdIns(4,5)P_2 pool seems large enough for the formation of sufficient Ins(1,4,5)P_3 to initiate stored $^{45}Ca^{2+}$ release (figure 5(A)). Based on this assumption, a modified procedure was formulated in an attempt to deplete completely the agonist-sensitive PtdIns(4,5)P_2 pools in permeablised SH-SY5Y cells.

SH-SY5Y cells were exposed to methacholine (1mM) immediately prior to wortmannin pre-treatment and permeabilisation, according to the 'PtdIns(4,5)P_2 pool depletion procedure' (see section 7.2). Under these conditions, Ins(1,4,5)P_3 accumulation in response to methacholine (1mM) was maximal at 8 ± 3 pmol/mg protein (n=3) (figure 8) which was actually lower than

basal levels in the absence of prestimulation with methacholine (18 ± 2 pmol/mg protein, n=3). Similarly, under such conditions, methacholine-induced $^{45}Ca^{2+}$ release was abolished (figure 9). In parallel experiments, where cells were prestimulated with methacholine (1mM), but in the absence of wortmannin, methacholine (1mM) stimulated Ins(1,4,5)P_3 accumulation which peaked at 80s (55 ± 5 pmol/mg protein, n=3) (figure 8). Thus, pre-treatment of SH-SY5Y cells with methacholine in the absence of wortmannin resulted in an attenuation of the response, despite a 30min recovery time (compare to figure 4). Under identical conditions, methacholine-mediated $^{45}Ca^{2+}$ mobilisation was dose-dependent with a $\log_{10}$ EC_{50} value of -4.63 ± 0.02M (23μM) and maximal release of 24 ± 0.4% (n=3) (figure 9). Like the methacholine-mediated Ins(1,4,5)P_3 response under identical conditions (i.e. prestimulation with methacholine, but in the absence of wortmannin (see figure 8)) this represented an attenuated response.

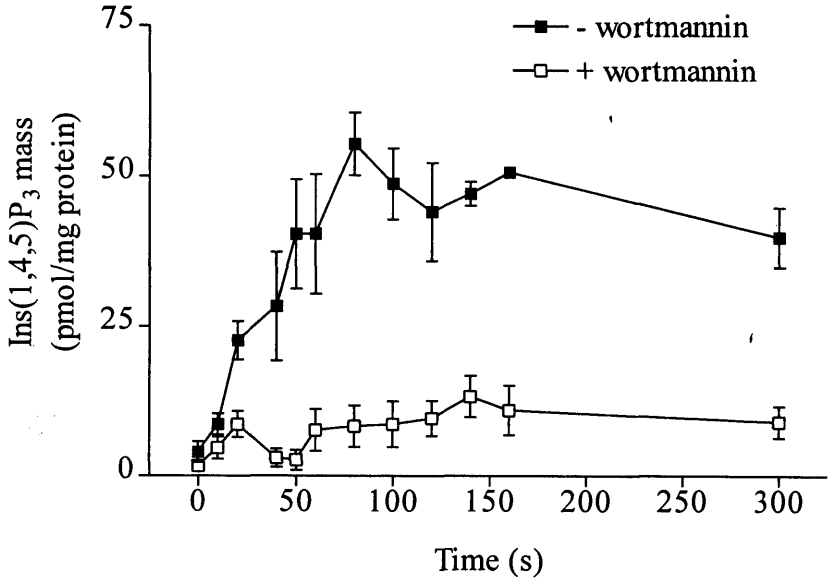


Figure 8 Time course of changes in Ins(1,4,5)P_3 mass following challenge with 1mM methacholine with or without 30min pre-treatment with wortmannin (10μM) in permeabilised SH-SY5Y cells prestimulated with methacholine (1mM, 1min), followed by a washout procedure to deplete the agonist-sensitive PIP_2 pool. Data are means ± S.E.M., n = 3.

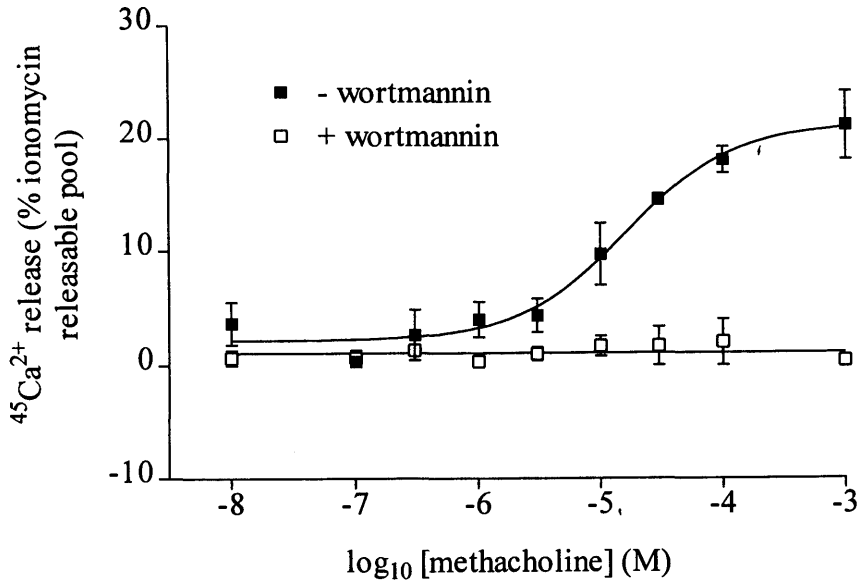


Figure 9 Dose-response relationships for methacholine-mediated $^{45}Ca^{2+}$ release with or without 30min pre-treatment with wortmannin (10μM) in permeabilised SH-SY5Y cells prestimulated with methacholine (1mM, 1min) followed by washout procedure to deplete the agonist-sensitive PtdIns(4,5)P_2 pool. Data are means ± S.E.M., n = 3.

The effect of methacholine on exogenous Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ mobilisation in PtdIns(4,5)P_2 pool depleted SH-SY5Y cells.

Under conditions of PtdIns(4,5)P_2 pool depletion, exogenous Ins(1,4,5)P_3 dose-dependently mobilised $^{45}Ca^{2+}$ from ionomycin-sensitive stores with a $\log_{10}$ EC_{50} value of -7.02 ± 0.1M (95nM) which was not significantly ($p > 0.05$) different to that in cells with intact lipid pools. However, methacholine significantly ($p < 0.05$) potentiated Ins(1,4,5)P_3 induced $^{45}Ca^{2+}$ mobilisation as demonstrated by a leftwards shift of the dose-response curve (EC_{50} value = -7.35 ± 0.01M (44nM) (figure 10). This potentiation of Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release by methacholine was further investigated using a single concentration of Ins(1,4,5)P_3 (30nM) under conditions of PtdIns(4,5)P_2 pool depletion. Challenge with Ins(1,4,5)P_3 alone stimulated a modest $^{45}Ca^{2+}$ release (23 ± 2%, n=4). In the presence of methacholine (1mM), $^{45}Ca^{2+}$ mobilisation in response to Ins(1,4,5)P_3 was potentiated (44 ± 1%, n=4) and this potentiation was reversed in the presence of the muscarinic receptor antagonist, atropine (1μM) (25 ± 4%, n=4) (figure 11).

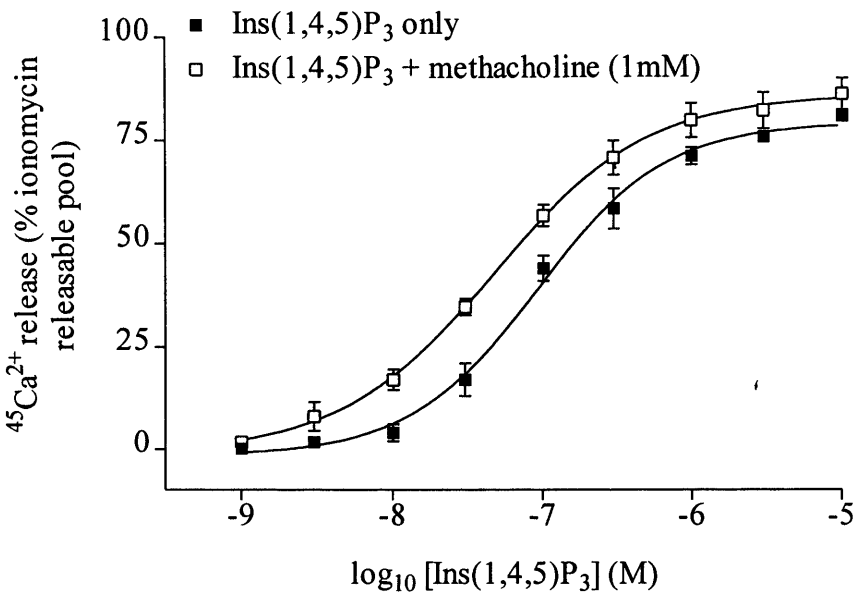


Figure 10 *Dose-response relationship for Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release in the presence or absence of 1mM methacholine in permeabilised SH-SY5Y cells prestimulated with methacholine (1mM, 1min) followed by washout procedure and 30min preincubation with wortmannin (10μM) to deplete the agonist-sensitive PtdIns(4,5)P_2 pool. Data are means ± S.E.M., n = 3.*

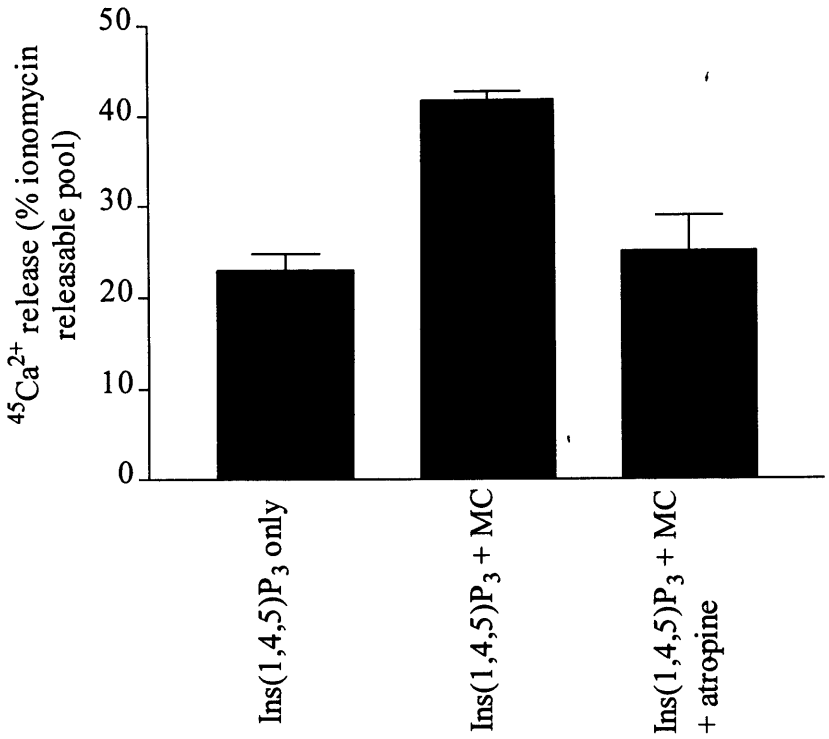


Figure 11 Dose-response relationship for Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release in the presence or absence of 1mM methacholine in permeabilised SH-SY5Y cells prestimulated with methacholine (1mM, 1min) followed by washout procedure and 30min preincubation with wortmannin (10μM) to deplete the agonist-sensitive PtdIns(4,5)P_2 pool. Data are means ± S.E.M., n = 3.

7.4 Discussion

Ins(1,4,5)P_3-induced Ca^{2+} release from intracellular stores is generally thought to mediate the Ca^{2+} mobilising action of many hormones and neurotransmitters (Berridge, 1993). The results presented in this thesis, in agreement with a previous study (Willars and Nahorski, 1995a), demonstrate that submaximal agonist-mediated Ins(1,4,5)P_3 accumulations were able to fully mobilise Ca^{2+} from intracellular stores in SH-SY5Y (and SH-SY5Y_{RA6}) cells, suggesting that agonists may stimulate the generation of Ins(1,4,5)P_3 above that required to maximally trigger the $[Ca^{2+}]_i$ response. In addition, it has been reported that in a number of different cell types agonists that activate receptors linked to the activation of PIC are capable of mobilising Ca^{2+} from intracellular stores in the apparent absence of Ins(1,4,5)P_3 accumulation (Rooney *et al.*,

1991, 1996; Xu *et al.*, 1996; Zeng *et al.*, 1996). Modulation of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} release channel complex is, therefore, a potentially important mechanism underlying discrepancies such as these. Agonist-mediated $Ins(1,4,5)P_3$ receptor modulation may also provide an explanation for why, despite an apparent amplification of the $[Ca^{2+}]_i$ response, there appeared to be no receptor reserve for agonist-stimulated $[Ca^{2+}]_i$ elevations in SH-$SY5Y_{RA6}$ cells when a receptor reserve was evident for agonist-stimulated PIC activation. Although several regulatory features of the $Ins(1,4,5)P_3$ receptor have been reported (Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991; Cameron *et al.*, 1995; Bird *et al.*, 1993), their role in agonist-dependent regulation of Ca^{2+} mobilisation has been little studied. The results presented in this chapter investigate whether stimulation of muscarinic and bradykinin receptors, in the absence of $Ins(1,4,5)P_3$ generation, is able to enhance the ability of exogenous $Ins(1,4,5)P_3$ to evoke mobilisation of stored Ca^{2+} in SH-SY5Y cells.

Stimulation of β-escin-permeabilised cells with bradykinin failed to evoke the release of $^{45}Ca^{2+}$ from intracellular stores. This lack of bradykinin-mediated Ca^{2+} release in permeabilised cells is in contrast to data presented in previous chapters where bradykinin stimulated substantial elevations of $[Ca^{2+}]_i$ both in cell populations and at the single cell level in intact, fura-2 loaded, undifferentiated SH-SY5Y cells. The observed bradykinin-stimulated $[Ca^{2+}]_i$ elevations in the intact cells were very likely to be as the result of Ca^{2+} release from intracellular stores since removal of extracellular Ca^{2+} did not effect peak $[Ca^{2+}]_i$ elevations. The reason for such a discrepancy between intact and permeabilised SH-SY5Y cells is likely to be that, since bradykinin-stimulated $Ins(1,4,5)P_3$ mass generation is very small in intact SH-SY5Y cells (see chapter 4), $Ins(1,4,5)P_3$ generated in response to bradykinin in permeabilised cell suspensions is rapidly dispersed out of the cytosol and, therefore, does not come into contact with the $Ins(1,4,5)P_3$ receptors. The $Ins(1,4,5)P_3$ thus dispersed would rapidly be diluted in the comparatively large volume of assay buffer utilised in these studies.

The differences in bradykinin-mediated Ca^{2+} mobilisation between intact and permeabilised cells may argue for compartmentalisation of $Ins(1,4,5)P_3$ following agonist challenge in intact cells. Indeed, compartmentalised generation and action of $Ins(1,4,5)P_3$ during agonist stimulation has previously been suggested in UMR-106-01 cells when it was found that agonist-induced Ca^{2+} mobilisation was less sensitive to the $Ins(1,4,5)P_3$ receptor antagonist, heparin, than mobilisation elicited by the addition of exogenous $Ins(1,4,5)P_3$ (Zhao *et al.*,

1990). It may be possible that Ins(1,4,5)P_3 is produced in specific, localised region within the cell, and does not diffuse evenly throughout the cytosol. Thus, the concentration of Ins(1,4,5)P_3 produced following stimulation of SH-SY5Y cells with bradykinin may be much greater at sites rich in Ins(1,4,5)P_3 receptors than the concentration assessed by Ins(1,4,5)P_3 mass assay, which is an average of the Ins(1,4,5)P_3 concentration in the entire cell volume and, in permeabilised cell systems, subject to media dilution. In addition, as mentioned earlier, the concentration of Ins(1,4,5)P_3 in resting, intact cells is often in the micromolar range (Challiss *et al.*, 1990; Zhao *et al.*, 1990), which would be expected to fully mobilise Ca^{2+}, but is unable to do so. Thus, resting levels of Ins(1,4,5)P_3 may also be compartmentalised and unable to access the Ins(1,4,5)P_3 receptor. At present, however, it is not possible to measure compartmentalisation of Ins(1,4,5)P_3 and, therefore, this hypothesis remains speculative.

It has previously been demonstrated that agonists and exogenous Ins(1,4,5)P_3 mobilise Ca^{2+} from the same intracellular stores (Streb *et al.*, 1983; Muallem and Beeker, 1989) by the fact that, in permeabilised cells, agonists fail to mobilise intracellularly stored Ca^{2+} when cells have previously been stimulated to release Ca^{2+} by challenge with Ins(1,4,5)P_3, and vice-versa. In the current study application of exogenous Ins(1,4,5)P_3 to permeabilised SH-SY5Y cells dose-dependently evoked $^{45}Ca^{2+}$ mobilisation. Co-stimulation of permeabilised SH-SY5Y cells with bradykinin and exogenous Ins(1,4,5)P_3 resulted in an increased potency of Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release. Collectively, the data discussed thus far demonstrate that bradykinin, despite being unable to evoke the release of $^{45}Ca^{2+}$ from intracellular stores, was able to facilitate Ins(1,4,5)P_3-evoked $^{45}Ca^{2+}$ release. This facilitation is a bradykinin B2 receptor-dependent phenomenon, as demonstrated by the reversal of the potentiation in the presence of the B2 bradykinin receptor-specific antagonist HOE 140.

Bradykinin-mediated facilitation of Ins(1,4,5)P_3-induced Ca^{2+} release, however, is relatively small. Thus, the possibility that stimulation of muscarinic M3 receptors, which are coupled to PIC with greater efficiency in SH-SY5Y cells (Willars and Nahorski, 1995a), would result in greater potentiation of Ins(1,4,5)P_3-induced Ca^{2+} release was investigated. However, muscarinic receptor stimulation with the full agonist, methacholine, results in a large accumulation of Ins(1,4,5)P_3 in permeabilised SH-SY5Y cells which mediates a substantial and dose-dependent mobilisation of $^{45}Ca^{2+}$ (Wojcikiewicz *et al.*, 1990; Safrany and Nahorski,

1994). To continue the current study, a situation was required where methacholine-mediated Ins(1,4,5)P_3 accumulation was reduced to such an extent that $^{45}Ca^{2+}$ release was eliminated, yet coupling of the muscarinic M3 receptors to G-protein was maintained. To this end, the fungal metabolite, wortmannin, was employed. Wortmannin is a potent inhibitor of the enzymes PtdIns 3-kinase and PtdIns 4-kinase, and PtdIns 4-kinase is essential for the maintenance of agonist-sensitive PtdIns(4,5)P_2 pools in most cell types (Stephens *et al.*, 1994; Nakanishi *et al.*, 1995; Downing *et al.*, 1996). Since PtdIns(4,5)P_2 is the critical plasma membrane precursor of Ins(1,4,5)P_3 (Berridge and Irvine, 1984, 1989; Nishizuka, 1988), it follows that disruption of the maintenance of PtdIns(4,5)P_2 pools by inhibition of PtdIns kinase enzymes, using wortmannin, would abolish the generation of Ins(1,4,5)P_3 stimulated by agonist. Indeed, wortmannin has been shown to deplete PtdIns(4,5)P_2 pools in SH-SY5Y cells (Willars *et al.*, 1996) and to inhibit agonist-stimulated Ins(1,4,5)P_3 production in bovine adrenal glomerulosa cells (Nakanishi *et al.*, 1994).

In the present study, preincubation of intact SH-SY5Y cells with wortmannin resulted in a time-dependent reduction of methacholine-evoked Ins(1,4,5)P_3 accumulation. This reduction was maximal following a 30min preincubation with wortmannin, at which point peak methacholine-stimulated Ins(1,4,5)P_3 generation was equivalent to basal levels of Ins(1,4,5)P_3. Stimulation of permeabilised SH-SY5Y cells with a supramaximal concentration of methacholine following a 30min preincubation with wortmannin resulted in a marked reduction, if not a complete abolition, of agonist-mediated Ins(1,4,5)P_3 accumulation. It is worth noting, however, that the time-course of methacholine-mediated Ins(1,4,5)P_3 generation in permeabilised SH-SY5Y cells is different to that in intact cells, the time taken for peak accumulation to be acheived being markedly increased. It is probable that this relates to retarded metabolism of Ins(1,4,5)P_3 due to loss of soluble enzymes, and possibly dilution of the substrate PtdIns(4,5)P_2 in permeabilised cells which may lower the amount available for cleavage by PIC (Safrany and Nahorski, 1994).

It is clear, however, that in both intact and permeabilised SH-SY5Y cells, a 30min preincubation with wortmannin depletes PtdIns(4,5)P_2 pools to an extent where agonist-stimulated Ins(1,4,5)P_3 generation is severely inhibited. In marked contrast, preincubation of SH-SY5Y cells with wortmannin for 30min only slightly reduced the potency and maximal response of methacholine-mediated $^{45}Ca^{2+}$ release. Collectively, these data demonstrate that

under conditions where $Ins(1,4,5)P_3$ responses to maximal doses of methacholine were minimised, Ca^{2+} mobilisation responses were only marginally reduced. These data provide strong evidence that only a very small agonist-mediated $Ins(1,4,5)P_3$ elevation is required to elicit a substantial release of stored Ca^{2+} in permeabilised SH-SY5Y cells. It could be argued that an initial small $Ins(1,4,5)P_3$-mediated Ca^{2+} release stimulates further release of Ca^{2+}, either directly through the interaction of Ca^{2+} with its proposed activation site on the $Ins(1,4,5)P_3$ receptor/Ca^{2+} release channel complex or indirectly through activation or inhibition of other release channel regulating enzymes (Bezprozvanny *et al.*, 1991; Zhang *et al.*, 1993). Alternatively, an initial small release of Ca^{2+} may trigger CICR from ryanodine-sensitive intracellular Ca^{2+} release channels. As discussed previously, SH-SY5Y cells express type-2 ryanodine receptors and preliminary studies may suggest that there is a caffeine-sensitive component of agonist-evoked Ca^{2+} mobilisation in this cell line (see Mackrill *et al.*, 1997). However, if such mechanisms are responsible for the observed results, one would expect that the small amount of $Ins(1,4,5)P_3$ generated in response to bradykinin in SH-SY5Y cells would also evoke substantial Ca^{2+} release, and this would appear not to be the case.

Heparin, an inhibitor of the binding of $Ins(1,4,5)P_3$ to its receptor (Ferris *et al.*, 1989), obliterated $^{45}Ca^{2+}$ mobilisation in response to methacholine, thus establishing that muscarinic receptor-mediated Ca^{2+} release is very likely to be via the interaction of $Ins(1,4,5)P_3$ with its receptor in SH-SY5Y cells. However, heparin has also been reported to uncouple cell surface receptors from PIC (Dasso *et al.*, 1991), hence it remained possible that in addition heparin inhibits receptor-G-protein coupling, thus preventing generation of $Ins(1,4,5)P_3$ (and subsequent Ca^{2+} release). This possibility was investigated by examining the effect of heparin on methacholine-mediated $Ins(1,4,5)P_3$ generation. The results obtained revealed that heparin appears to partially uncouple muscarinic receptors from PIC activation in permeabilised SH-SY5Y cells, but not to a level where Ca^{2+} release following muscarinic receptor activation would be obliterated (compare to effect of wortmannin on methacholine-stimulated $Ins(1,4,5)P_3$ accumulation and $^{45}Ca^{2+}$ release). Whether heparin partially uncouples the response at the level of G-protein activation or at the level of PIC activation is unknown.

The data discussed thus far strongly suggest that agonist-induced Ca^{2+} mobilisation in SH-SY5Y cells is mediated via activation of the $Ins(1,4,5)P_3$ receptor by its ligand, $Ins(1,4,5)P_3$. However, since there is still a very small methacholine-induced $Ins(1,4,5)P_3$ generation

following preincubation with wortmannin, it is likely that there is a residual PtdIns(4,5)P_2 pool, insensitive to wortmannin, that is available for the generation of Ins(1,4,5)P_3 following agonist-induced PIC activation. This residual PtdIns(4,5)P_2 pool seems large enough for the formation of sufficient Ins(1,4,5)P_3 to initiate stored Ca^{2+} release. This assumption led to the formation of a modified procedure referred to henceforth as the 'PtdIns(4,5)P_2 pool depletion procedure' (see section 7.2) which was designed in an attempt to deplete this hypothetical, residual PtdIns(4,5)P_2 pool. The rationale behind the design of this procedure was that an initial stimulation with a maximal concentration of methacholine would virtually deplete the agonist-sensitive PtdIns(4,5)P_2 pool which, following washout in wortmannin-containing CLB, allowed for the recovery of muscarinic receptors whilst cells were incubated in wortmannin for 30min. This subsequent incubation in wortmannin would, in theory, prevent the resynthesis of the PtdIns(4,5)P_2 pool. Under these conditions, maximal Ins(1,4,5)P_3 generation in response to a supramaximal concentration of methacholine was actually lower than basal levels in the absence of PtdIns(4,5)P_2 pool depletion. Similarly, under such conditions, methacholine-induced $^{45}Ca^{2+}$ release was abolished. These data imply that under these conditions, the small PtdIns(4,5)P_2 pool which remains following wortmannin treatment alone is eliminated. In parallel experiments where cells were pre-stimulated with methacholine, in the absence of wortmannin, muscarinic receptor-mediated Ins(1,4,5)P_3 accumulation and $^{45}Ca^{2+}$ mobilisation was reduced, despite a 30min recovery time following washout of prestimulating methacholine, which probably represents desenstisation of the responses.

Despite an apparent desensitisation of the system, PtdIns(4,5)P_2 pool depletion of permeabilised SH-SY5Y cells provided a situation where the influence of muscarinic receptor activation on the potency of exogenous Ins(1,4,5)P_3-evoked $^{45}Ca^{2+}$ release could be investigated without the interference of endogenous Ins(1,4,5)P_3 generation. Under these conditions exogenous Ins(1,4,5)P_3 alone dose-dependently mobilised $^{45}Ca^{2+}$ in a manner identical to cells with intact lipid pools. However, methacholine facilitated Ins(1,4,5)P_3-evoked $^{45}Ca^{2+}$ mobilisation, as demonstrated by a leftward shift of the dose-response curve. These data suggest that, in SH-SY5Y cells, muscarinic receptor activation results in a modulation of the Ins(1,4,5)P_3 receptor/Ca^{2+} channel release complex such that the sensitivity of the Ca^{2+} mobilising effect of Ins(1,4,5)P_3 is increased. The potentiation of Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release by methacholine is a muscarinic receptor-mediated phenomenon, as

demonstrated by the reversal of the facilitation in the presence of the muscarinic receptor antagonist, atropine.

The increased potency of $Ins(1,4,5)P_3$ to mobilise Ca^{2+} in the presence of methacholine was comparable to the increased potency of $Ins(1,4,5)P_3$-mediated Ca^{2+} mobilisation in the presence of bradykinin. As mentioned earlier, muscarinic receptors couple with greater efficiency to PIC in SH-SY5Y cells, and as a result it was expected that the potency increase evoked by methacholine would also be greater. One possible explanation is that, as muscarinic receptors appeared to be desensitised following the procedure required for complete $PtdIns(4,5)P_2$ pool depletion, muscarinic receptor-mediated responses, including potential modifications of the $Ins(1,4,5)P_3$ receptor, may be attenuated.

In summary, under conditions where agonist-mediated $Ins(1,4,5)P_3$ accumulation is insufficient to mobilise stored Ca^{2+}, activation of muscarinic or bradykinin receptors in permeabilised SH-SY5Y cells caused a facilitation of exogenous $Ins(1,4,5)P_3$-induced Ca^{2+} mobilisation. This facilitation was receptor-specific and may represent an agonist-induced modification of the $Ins(1,4,5)P_3$ receptor such that its sensitivity to $Ins(1,4,5)P_3$ is enhanced. The mechanism(s) involved in this apparent sensitisation of the $Ins(1,4,5)P_3$ receptor is, at present, unclear. Potential mechanisms are outlined and a number investigated in the subsequent chapter.

8. INVESTIGATION INTO THE MECHANISM(S) INVOLVED IN AGONIST-MEDIATED MODULATION OF THE INS(1,4,5)P_3 RECEPTOR IN SH-SY5Y CELLS.

8.1 Introduction

The data presented in the previous chapter provides evidence that in SH-SY5Y cells, agonist occupation of either muscarinic or bradykinin receptors mediates facilitation of exogenous Ins(1,4,5)P_3-evoked Ca^{2+} mobilisation under conditions where Ins(1,4,5)P_3 accumulation is not sufficient to mobilise stored Ca^{2+}. These findings may represent an agonist-induced modification of the Ins(1,4,5)P_3 receptor such that its sensitivity to Ihs(1,4,5)P_3 is enhanced. Alternatively, agonists may release a tonic inhibition of the Ins(1,4,5)P_3 receptor in SH-SY5Y cells. Whatever the mechanism, these findings have a number of potential implications for Ca^{2+} signalling in SH-SY5Y cells. First, agonist-mediated facilitation of Ins(1,4,5)P_3-induced Ca^{2+} mobilisation may provide an explanation for the amplification of agonist-stimulated responses in the phase between the production of Ins(1,4,5)P_3 and release of Ca^{2+} (see chapter 5). Second, if there is a lack of facilitation of Ins(1,4,5)P_3-induced Ca^{2+} release by agonist in SH-SY5Y_{RA6} cells this phenomenon may explain why, when agonist-mediated Ins(1,4,5)P_3 generation was greater following RA-differentiation of SH-SY5Y cells, agonist-mediated $[Ca^{2+}]_i$ responses were not affected. Finally, if different agonists vary in their ability to modulate the sensitivity of the Ins(1,4,5)P_3 receptor for Ins(1,4,5)P_3, this phenomenon may contribute towards the efficacy of agonist-mediated $[Ca^{2+}]_i$ responses, and is therefore likely to affect the efficacy of downstream responses.

Modification of the Ins(1,4,5)P_3 receptor may occur via phosphorylation or dephosphorylation of the receptor/channel complex. Indeed, the phosphorylation state of the Ins(1,4,5)P_3 receptor has been shown to regulate the sensitivity to both Ins(1,4,5)P_3 and Ca^{2+} (Joseph and Ryan, 1993). Thus, there is potential for control by a number of different protein kinases, including PKC, PKA, calcium-calmodulin-dependent protein kinase and cyclicGMP-dependent protein kinase (PKG) (Furuichi *et al.*, 1994). Recently it has been reported that infusion of purified G-protein βγ subunits into pancreatic acinar cells stimulates $[Ca^{2+}]_i$ oscillations, in the absence of Ins(1,4,5)P_3 accumulation, by increasing the affinity of the Ins(1,4,5)P_3 receptor for its ligand Ins(1,4,5)P_3 to facilitate Ca^{2+} release (Zeng *et al.*, 1996).

In view of the range of mechanisms potentially involved in agonist-mediated $Ins(1,4,5)P_3$ receptor sensitisation, the aim of this chapter was to investigate a number of possible mechanisms for the apparent agonist-stimulated facilitation of $Ins(1,4,5)P_3$-mediated Ca^{2+} release in SH-SY5Y cells.

One explanation for the results presented in the previous chapter that receptor activation may provide a mechanism where lower concentrations of $Ins(1,4,5)P_3$ are protected from metabolism. Under these circumstances, the concentration of $Ins(1,4,5)P_3$ interacting with the $Ins(1,4,5)P_3$ receptor would be greater in the presence of, than in the absence of, agonist. This eventuality was examined in the current study by investigating the ability of adenophostin, a fungal, non-metabolisable $Ins(1,4,5)P_3$ receptor agonist (Takahashi *et al.*, 1993; Wilcox *et al.*, 1995; Hartzell et al., 1997) to mobilise Ca^{2+} in the presence or the absence of methacholine. The $Ins(1,4,5)P_3$ receptor/Ca^{2+} channel release complex has previously been shown to be regulated by several kinases, including PKC (Zhang *et al.*, 1993; Cameron *et al.*, 1995). Indeed, the affinity to $Ins(1,4,5)P_3$ of the type I $Ins(1,4,5)P_3$ receptor has been shown to be regulated by the action of PKC (Cameron *et al.*, 1995). Since PKC is activated by the second messenger DAG, which is produced concomitantly to $Ins(1,4,5)P_3$ following the activation of PIC, PKC-mediated phosphorylation of the $Ins(1,4,5)P_3$ receptor would seem a prime candidate for agonist-mediated modulation of the $Ins(1,4,5)P_3$ receptor.

Nitric oxide (NO) is a short-lived, highly reactive radical that has been shown to function as a neuronal messenger involved in physiological events such as neurotransmitter release (Hanbauer, 1993), long-term potentiation (Haley *et al.*, 1992) and pathophysiological events underlying neurotoxicity (Bagetta *et al.*, 1993; Zhang *et al.*, 1994). More recently, receptor activation of the NO pathway has been demonstrated to modulate the affinity of $Ins(1,4,5)P_3$ receptors such that $Ins(1,4,5)P_3$-induced Ca^{2+} release is facilitated in guinea-pig and rat hepatocytes (Guihard *et al.*, 1996; Rooney *et al.*, 1996). The proposed mechanism of action of NO-mediated sensitisation of the $Ins(1,4,5)P_3$ receptor in these cells is that NO stimulates the generation of cyclicGMP which, in turn, stimulates the activation of PKG. Activated PKG phosphorylates $Ins(1,4,5)P_3$ receptors, thus regulating their function in these cells (Rooney *et al.*, 1996). In fact, $Ins(1,4,5)P_3$ receptors have been reported to be phosphorylated by PKG in a number of different systems (Komalavius and Lincon, 1994, 1996; Koga *et al.*, 1994), and studies using confocal laser scanning microscopy to determine the cellular

distribution of PKG suggest that the enzyme is found in the endoplasmic and sarcoplasmic reticulum where Ins(1,4,5)P_3 receptors are localised (Conwell *et al.*, 1991). Both muscarinic M3 and bradykinin B2 receptors have been reported to stimulate the NO pathway in PC-12 neuroblastoma cells (Clementi *et al.*, 1995), and hence it follows that activation of these receptor subtypes in SH-SY5Y cells might stimulate, in addition to PIC activation, the NO pathway, thus leading to phosphorylation of the Ins(1,4,5)P_3 receptor, which may facilitate Ins(1,4,5)P_3-mediated Ca^{2+} release. This potential mechanism was investigated in the current study.

There is also evidence that $[Ca^{2+}]_i$ oscillations in pancreatic acinar cells are stimulated by an agonist-mediated increase in the apparent affinity of the Ins(1,4,5)P_3 receptor/Ca^{2+} release channel complex for Ins(1,4,5)P_3 (Xu *et al.*, 1996). This effect was demonstrated to be independent of PIC activation or Ins(1,4,5)P_3 metabolism (Xu *et al.*, 1996) and is proposed to be mediated via activated G-protein βγ subunits from G-proteins other than Gq/11 (Zeng *et al.*, 1996). It is possible, therefore, that challenge of SH-SY5Y cells with agonist also stimulates, in addition to PIC activation, the release, and hence activation of, G-protein βγ subunits from G-proteins other than Gq/11. These activated βγ subunits may, in turn, modulate the Ins(1,4,5)P_3 receptor such that the Ca^{2+}-mobilising ability of Ins(1,4,5)P_3 is enhanced. This potential mechanism was also investigated in this study. The results presented in the current chapter may provide some insight into agonist-mediated modulation of Ins(1,4,5)P_3 receptors in SH-SY5Y cells. However, there are a number of other potentially relevant mechanisms, not investigated in this thesis, which are considered in the subsequent chapter.

8.2 Methods

Ins(1,4,5)P_3- and adenophostin-evoked $^{45}Ca^{2+}$ release were assessed in β-escin-permeabilised cells (prepared as described in section 2.3.4) using the procedure described in section 2.6. When the Gi and Go family of G-proteins were inhibited, cells were preincubated in 175cm^2 culture flasks with pertussis toxin (100ng/ml culture media) for 24h prior to permeabilisation. When PKC was inhibited, permeabilised cells were prestimulated with Ro 318220 (10μM) for 10min prior to initiation of experimental incubations. Sodium nitroprusside (SNP) (100μM),

8-bromo-cGMP (500μM) and GTPγS (2μM) were added 5min before agonist stimulation when required. Depletion of agonist-sensitive PtdIns(4,5)P_2 pools was acheived using the protocol described in section 7.2.

8.3 Results

The effect of methacholine on adenophostin-induced $^{45}Ca^{2+}$ release in permeabilised, PtdIns(4,5)P_2 pool depleted SH-SY5Y cells.

In order to investigate whether cell surface receptor activation may provide a mechanism where lower concentrations of Ins(1,4,5)P_3 are protected from metabolism, adenophostin-mediated $^{45}Ca^{2+}$ mobilisation was investigated. Under conditions of PtdIns(4,5)P_2 pool depletion, adenophostin dose-dependently released $^{45}Ca^{2+}$ with a $\log_{10}$ EC_{50} value of -8.04 ± 0.06M (9.1nM) in permeabilised SH-SY5Y cells. Methacholine (1mM) significantly ($p< 0.05$) potentiated adenophostin-induced $^{45}Ca^{2+}$ mobilisation as demonstrated by a leftward shift of the dose-response curve ($\log_{10}$ EC_{50} (M) value = -8.34 ± 0.1 (4.6nM) (figure 1).

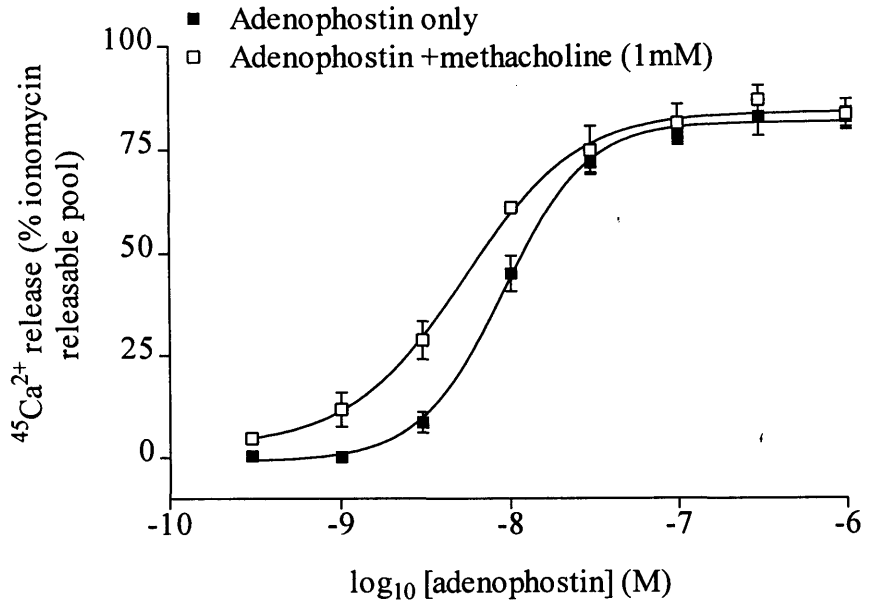


Figure 1 Dose-response relationships for adenophostin-mediated $^{45}Ca^{2+}$ release in the presence or absence of 1mM methacholine in permeabilised SH-SY5Y cells prestimulated with methacholine (1mM, 1min) followed by washout procedure and 30min incubation with wortmannin (10μM) to deplete the agonist-sensitive PtdIns(4,5)P_2 pools. Data points are means ± S.E.M., n=3.

The effect of Ro 318220 on agonist-mediated facilitation of Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release in permeabilised SH-SY5Y cells.

In order to investigate whether agonist-stimulated modulation of the Ins(1,4,5)P_3 receptor is mediated via the activation of PKC, the effect of the PKC inhibitor, Ro 318220, on the facilitation of Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release by bradykinin was further investigated using a single concentration of Ins(1,4,5)P_3 (30nM). Treatment of permeabilised cells with this concentration of Ins(1,4,5)P_3 alone caused a modest $^{45}Ca^{2+}$ release of 19 ± 2% (n=4). In the presence of bradykinin (10μM) $^{45}Ca^{2+}$ release in response to 30nM Ins(1,4,5)P_3 was significantly ($p < 0.05$) potentiated (39 ± 1%, n=4). This potentiation was not significantly reduced in the presence of Ro 318220 (30 ± 4%, n=4) (figure 2).

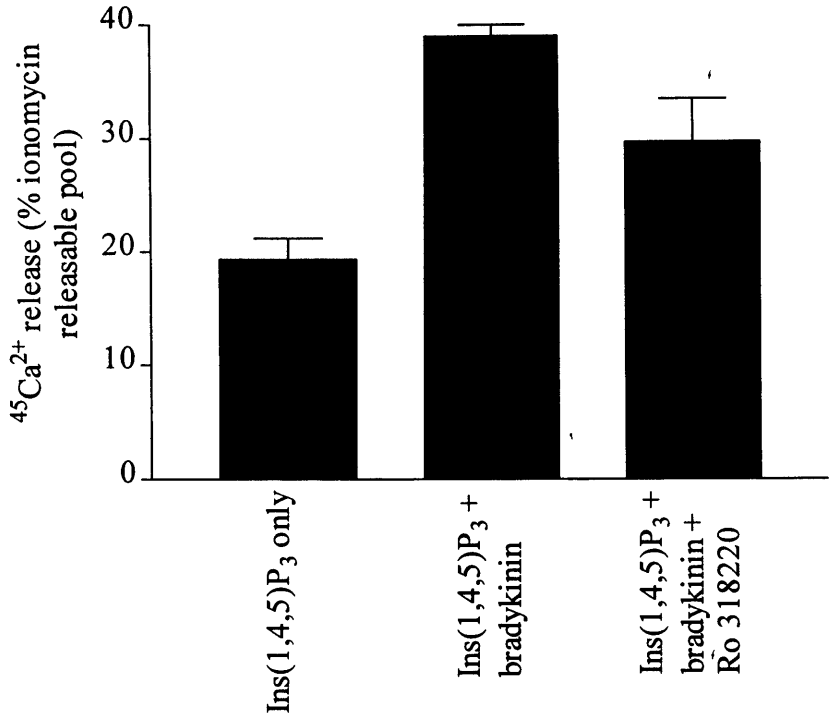


Figure 2 Effect of bradykinin (10μM) and bradykinin + Ro 318220 (10μM) on $^{45}Ca^{2+}$ mobilisation in response to 30nM Ins(1,4,5)P_3 in permeabilised SH-SY5Y cells. Data are means ± S.E.M., n=3.

In a similar set of experiments, the effect of Ro 318220 on the facilitation of Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release by methacholine, under conditions of PtdIns(4,5)P_2 pool depletion, was investigated using a single concentration of Ins(1,4,5)P_3 (30nM). Challenge with Ins(1,4,5)P_3 alone stimulated a modest $^{45}Ca^{2+}$ release of 23 ± 2% (n=4) which, in the presence of methacholine (1mM), was significantly ($p < 0.05$) potentiated (44 ± 1%, n=4). The methacholine-mediated facilitation of Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release was not significantly reduced in the presence Ro 318220 (38 ± 2%, n=4) (figure 3).

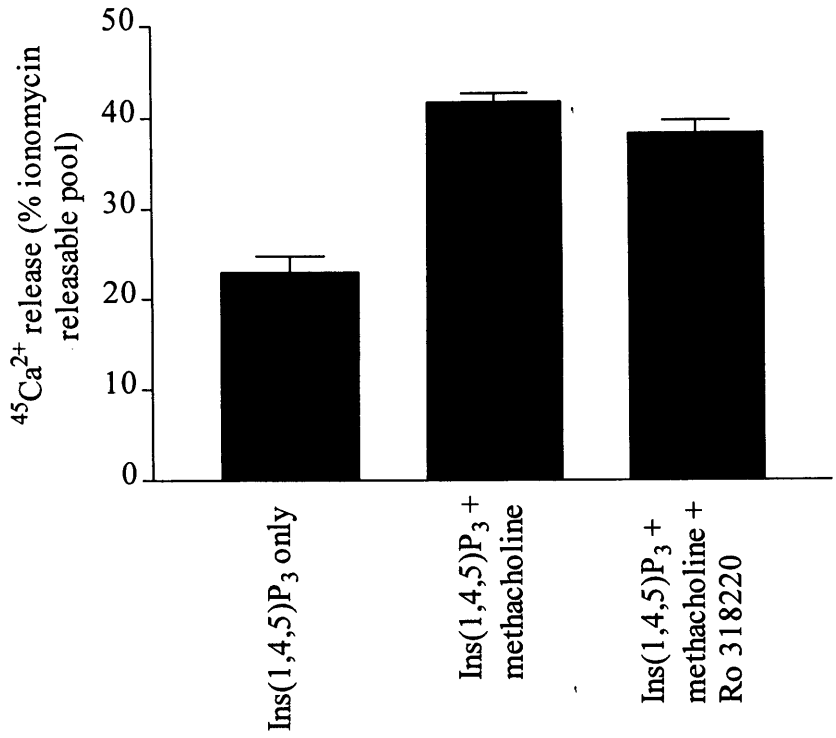


Figure 3 Effect of methacholine (1mM) and methacholine + Ro 318220 (10μM) on $^{45}Ca^{2+}$ mobilisation in response to 30nM Ins(1,4,5)P_3 in permeabilised SH-SY5Y cells prestimulated with methacholine (1mM, 1min) followed by washout procedure and 30min incubation with wortmannin (10μM) to deplete the agonist-sensitive PtdIns(4,5)P_2 pools. Data are means ± S.E.M., n=4.

The effect of PKG activation on exogenous Ins(1,4,5)P_3-evoked $^{45}Ca^{2+}$ mobilisation in permeabilised SH-SY5Y cells.

The effect of PKG activation on Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release in permeabilised SH-SY5Y cells was investigated using the non-hydrolysable cyclic GMP analogue, 8-bromo-cGMP, and the nitric oxide (NO) donor, sodium nitroprusside (SNP). Exogenous Ins(1,4,5)P_3 dose-dependently mobilised $^{45}Ca^{2+}$ from ionomycin-sensitive intracellular stores with a $\log_{10}$ EC_{50} value of -7.04 ± 0.01M (91nM). Co-stimulation of exogenous Ins(1,4,5)P_3 with maximally effective concentrations of either 8-bromo-cGMP (500μM) or SNP (100μM) did not significantly effect the potency of Ins(1,4,5)P_3-evoked $^{45}Ca^{2+}$ release ($\log_{10}$ EC_{50} (M) values = -7.12 ± 0.05 (76nM) and -7.14 ± 0.08 (72nM) respectively) (figure 4).

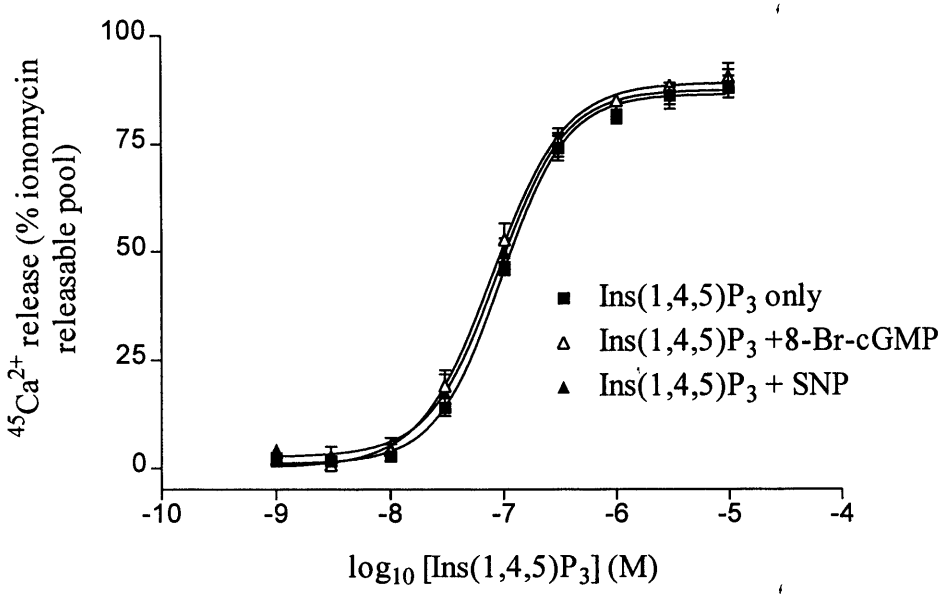


Figure 4 Dose-response relationships for Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release in the presence or absence of either 8-Br-cGMP (500μM) or sodium nitroprusside (SNP) (100μM) in permeabilised SH-SY5Y cells. Data points are means ± S.E.M., n=3.

The effect of a low concentration of GTPγS on exogenous Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ mobilisation in permeabilised SH-SY5Y cells.

In order to investigate the possible involvement of βγ subunits in agonist-mediated modulation of the Ins(1,4,5)P_3 receptor in SH-SY5Y cells, a low concentration of GTPγS, which is not sufficient to stimulate Ins(1,4,5)P_3 generation, on Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release was investigated. Exogenous Ins(1,4,5)P_3 alone released $^{45}Ca^{2+}$ dose-dependently with a $\log_{10}$ EC_{50} value of -7.10 ± 0.03M (79nM). In the presence of a low concentration of GTPγS (2μM), Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release was significantly ($p< 0.05$) enhanced ($\log_{10}$ EC_{50} (M) = -7.41 ±0.05 (39nM), as demonstrated by a leftward shift (approximately two-fold) of the dose-response curve. GTPγS alone (2μM) did not stimulate $^{45}Ca^{2+}$ release in permeabilised SH-SY5Y cells (figure 5).

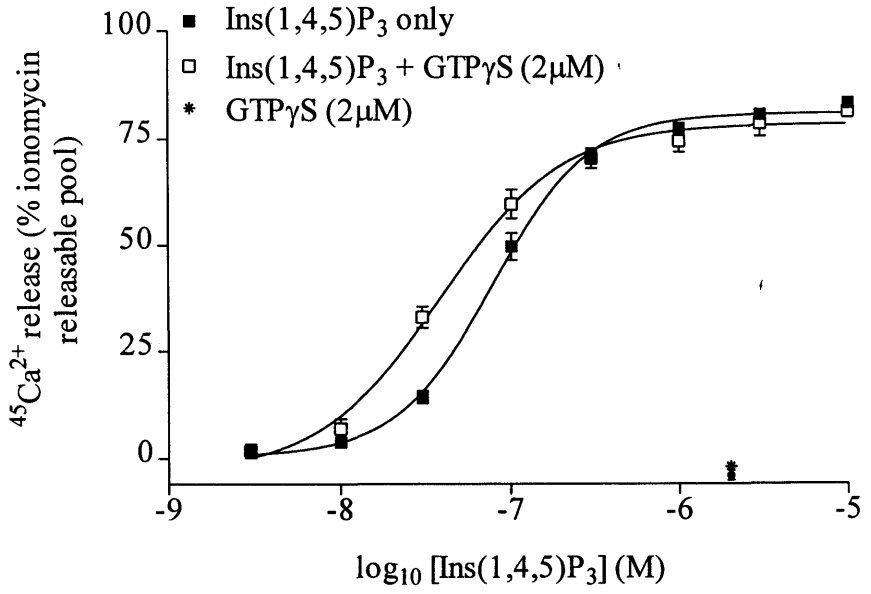


Figure 5 Dose-response relationships for Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release in the presence or absence of GTPγS (2μM) in permeabilised SH-SY5Y cells. The single data point (*) indicates the ability of GTPγS (2μM) to mobilise $^{45}Ca^{2+}$ in permeabilised SH-SY5Y cells. All data points are means ± S.E.M., n=3.

The effect of preincubation with pertussis toxin on Ins(1,4,5)P_3-induced, and agonist-mediated facilitation of Ins(1,4,5)P_3-induced, $^{45}Ca^{2+}$ release in permeabilised SH-SY5Y cells.

The possibility that activation of G-proteins of the Gi/Go family are involved in agonist-mediated modulation of the Ins(1,4,5)P_3 receptor in SH-SY5Y cells was examined by inhibition of the G-proteins Gi and Go using pertussis toxin (PTx). The effect of PTx pretreatment (100ng/ml, 24h) on Ins(1,4,5)P_3-induced, and bradykinin-mediated facilitation of Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release was investigated using a single concentration of Ins(1,4,5)P_3 (15nM). Stimulation of SH-SY5Y cells with this concentration of Ins(1,4,5)P_3 alone caused a modest $^{45}Ca^{2+}$ release (10 ± 2%, n=4). In the presence of bradykinin (10μM), $^{45}Ca^{2+}$ release induced by 15nM exogenous Ins(1,4,5)P_3 was significantly ($p< 0.05$) enhanced (20 ± 4%, n=4). Following preincubation with PTx (100ng/ml, 24h) $^{45}Ca^{2+}$ release in response to 15nM Ins(1,4,5)P_3 was also significantly ($p< 0.05$) enhanced. In the presence of

bradykinin (10μM), $^{45}Ca^{2+}$ release stimulated by 15nM Ins(1,4,5)P_3 was significantly ($p< 0.05$) greater in cells preincubated with PTx (100ng/ml, 24h) than cells without PTx treatment (31 ± 2% (n=4) versus 20 ± 1% (n=4)) (figure 6).

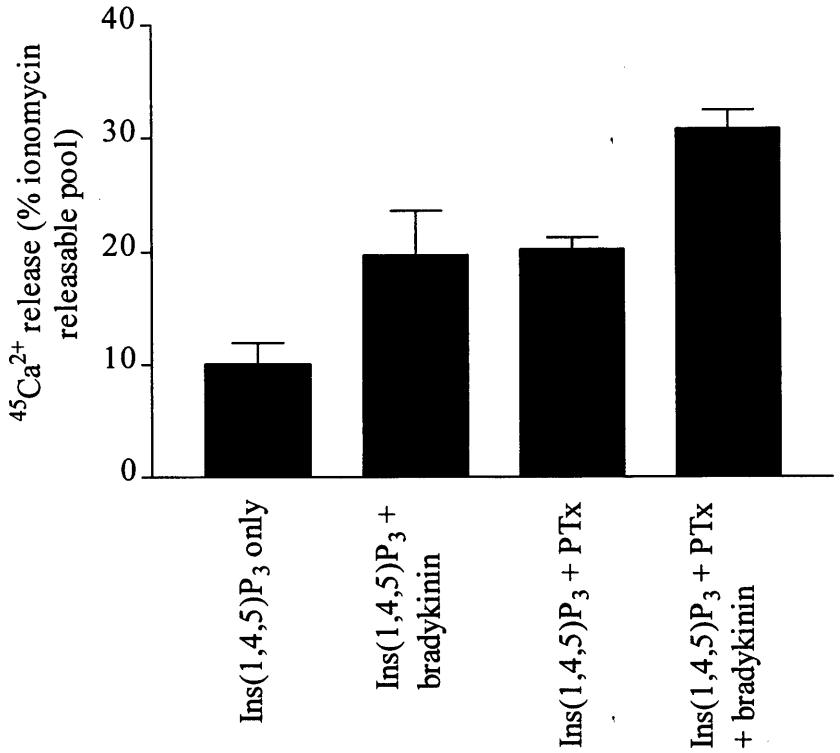


Figure 6 Effect of bradykinin (10μM) and bradykinin (10μM) + pertussis toxin (PTx) (100ng/ml) on $^{45}Ca^{2+}$ mobilisation in response to 30nM Ins(1,4,5)P_3 in permeabilised SH-SY5Y cells. Data are means ± S.E.M., n=4.

In a similar group of experiments, the effect of PTx-pretreatment on Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release and the methacholine-mediated facilitation of Ins(1,4,5)P_3-stimulated $^{45}Ca^{2+}$ release, using a single concentration of Ins(1,4,5)P_3 (15nM), was investigated under conditions of PtdIns(4,5)P_2 pool depletion. Treatment of permeabilised, PtdIns(4,5)P_2 pool depleted, SH-SY5Y cells with 15nM Ins(1,4,5)P_3 alone stimulated a small $^{45}Ca^{2+}$ release from intracellular stores of 9 ± 2% (n=4). In the presence of methacholine (1mM), $^{45}Ca^{2+}$ mobilisation in response to 15nM Ins(1,4,5)P_3 was significantly ($p< 0.05$) enhanced (26 ± 5%, n=4). Following a preincubation with PTx (100ng/ml, 24h) $^{45}Ca^{2+}$ release in response to 15nM Ins(1,4,5)P_3 was also significantly ($p< 0.05$) enhanced (20± 5%, n=4). In the presence

of methacholine (1mM), $^{45}Ca^{2+}$ release induced by 15nM Ins(1,4,5)P_3 was not significantly different in cells preincubated with PTx (100ng/ml, 24h) than in those without PTx treatment (34 ± 7% (n=4) and 26 ± 5% (n=4) respectively) (figure 7).

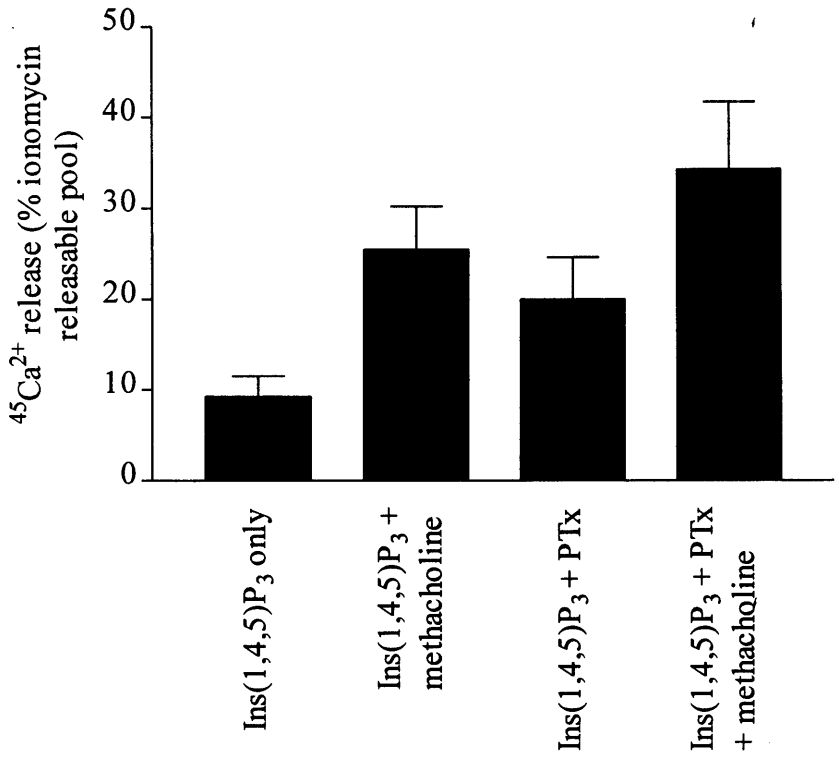


Figure 7 Effect of methacholine (1mM) and methacholine (1mM) + pertussis toxin (PTx) (100ng/ml) on $^{45}Ca^{2+}$ mobilisation in response to 30nM Ins(1,4,5)P_3 in permeabilised SH-SY5Y cells prestimulated with methacholine (1mM, 1min) followed by washout procedure and 30min incubation with wortmannin (10μM) to deplete the agonist-sensitive PtdIns(4,5)P_2 pools. Data are means ± S.E.M., n=4.

8.4 Discussion

The present studies have attempted to establish possible mechanism(s) for the apparent agonist-mediated facilitation of exogenous Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release in permeabilised SH-SY5Y cells. A number of potential mechanisms were investigated that have previously been reported to increase the sensitivity of the Ins(1,4,5)P_3 receptor/Ca^{2+}

release channel complex in a variety of model systems. These include agonist-mediated protection of lower concentrations of Ins(1,4,5)P_3 from metabolism, modulation of the Ins(1,4,5)P_3 receptor via PKC activation, stimulation of the nitric oxide (NO) pathway with the resultant activation of PKG which may modulate the Ins(1,4,5)P_3 receptor and the involvement of G-proteins, possibly other than Gq/11, in agonist-mediated facilitation of Ins(1,4,5)P_3-evoked Ca^{2+} release.

Adenophostin is a structurally unrelated compound to Ins(1,4,5)P_3. However, it has recently been reported that it is approximately 100-fold more potent than Ins(1,4,5)P_3 at releasing Ca^{2+} from intracellular stores (Takahashi *et al.*, 1994). This was evident from results presented in this chapter as adenophostin released $^{45}Ca^{2+}$ from internal stores in permeabilised SH-SY5Y cells with greater potency than Ins(1,4,5)P_3, as revealed by a lower EC_{50} value. Adenophostin also differs from Ins(1,4,5)P_3 in that it is not metabolised within the cytosol by the enzymes Ins(1,4,5)P_3 3-kinase and Ins(1,4,5)P_3 5-phosphatase that render the Ca^{2+} mobilising action of Ins(1,4,5)P_3 transient (Takahashi *et al.*, 1994; Wilcox *et al.*, 1995). Adenophostin was utilised in the current study to investigate whether the observed muscarinic receptor-mediated facilitation of Ins(1,4,5)P_3-induced Ca^{2+} release in SH-SY5Y cells was attributable to protection of lower concentrations of Ins(1,4,5)P_3 from metabolism by agonist. The data presented in this chapter show that, under conditions of PtdIns(4,5)P_2 pool depletion, muscarinic receptor activation facilitated adenophostin-evoked $^{45}Ca^{2+}$ mobilisation, as revealed by a leftward shift of the dose-response curve. Thus, it seems very unlikely that protection of Ins(1,4,5)P_3-metabolism by agonist is the mechanism for the potentiation of Ins(1,4,5)P_3-stimulated Ca^{2+} mobilisation in these cells.

Following the activation of PIC, the second messenger, DAG, concomitant to Ins(1,4,5)P_3, is generated. DAG activates the classical and novel families of PKCs which, in turn, can regulate a range of physiological events via the phosphorylation of certain cellular proteins (Berridge, 1993). As mentioned earlier in this chapter, PKC has been shown to regulate the affinity of the type-1 Ins(1,4,5)P_3 receptor for Ins(1,4,5)P_3 (Cameron *et al.*, 1995). Thus, a potential mechanism for agonist-mediated augmentation of Ins(1,4,5)P_3-induced Ca^{2+} release is modulation of the Ca^{2+} release channel via phosphorylation of the Ins(1,4,5)P_3 receptor, or the initiation of a cascade resulting in the phosphorylation or dephosphorylation of the Ins(1,4,5)P_3 receptor, by activated PKC. However, the PKC inhibitor, Ro 318220, failed to

reverse the potentiation of $Ins(1,4,5)P_3$-stimulated $^{45}Ca^{2+}$ release induced by either bradykinin or methacholine. These findings suggest that the involvement of PKC in the agonist-mediated modulation of the $Ins(1,4,5)P_3$ receptor in the SH-SY5Y cell line observed in the current study is unlikely. It is important to note, nevertheless, that under conditions of $PtdIns(4,5)P_2$ pool depletion it is unlikely that PKC is activated since DAG is generated by the action of PIC on $PtdIns(4,5)P_2$. Thus, under normal conditions (i.e. no $PtdIns(4,5)P_2$ pool depletion), PKC may modulate the $Ins(1,4,5)P_3$ receptor although this is clearly not the mechanism responsible for the facilitation observed in the current study. Hence, alternative mechanisms were investigated.

Previous studies have demonstrated that stimulation of the nitric oxide (NO) pathway potentiates noradrenaline-induced Ca^{2+} mobilisation in guinea pig hepatocytes (Guihard *et al.*, 1996) and stimulates Ca^{2+} oscillations, in the absence of $Ins(1,4,5)P_3$ accumulation, in rat hepatocytes (Rooney *et al.*, 1996). In the present study the spontaneous release of NO was mimicked through treatment with the NO donor, sodium nitroprusside (SNP). NO activates soluble guanyl cyclase with the resultant generation of cGMP (Waldman and Murad, 1987). cGMP activates protein kinase G (PKG), which has been suggested to modulate the $Ins(1,4,5)P_3$ receptor/Ca^{2+} release channel complex (Guihard *et al.*, 1996; Rooney *et al.*, 1996), presumably by phosphorylation. cGMP generation was mimicked in the current study by treatment with the non-metabolisable, cGMP analogue, 8-Br-cGMP. Activated PKG has been shown to phosphorylate two sites on the type-1 $Ins(1,4,5)P_3$ receptor, at serine 1755 and 1589 (Rooney *et al.*, 1996). The SH-SY5Y cell line has been shown to express the type-1 (and the type-2) $Ins(1,4,5)P_3$ receptor (Wojcikiewicz and Nahorski, 1991; Wojcikiewicz *et al.*, 1992; Wojcikiewicz and Oberdorf, 1996; Mackrill *et al.*, 1997) and, additionally, muscarinic M3 and bradykinin B2 receptors have previously been shown to activate NO synthase following their stimulation in PC-12 cells (Clementi *et al.*, 1995). For these reasons it seemed likely that the observed agonist-mediated facilitation of $Ins(1,4,5)P_3$-induced Ca^{2+} release was via activation of NO synthase, and subsequently PKG-mediated $Ins(1,4,5)P_3$ receptor phosphorylation. However, neither SNP nor 8-Br-cGMP had any effect on exogenous $Ins(1,4,5)P_3$-mediated $^{45}Ca^{2+}$ release, which would argue against a role for the NO pathway in the facilitation of Ca^{2+} release in the SH-SY5Y cell line.

Previous studies on platelets and vascular smooth muscle cells, however, have revealed that NO exerts an inhibitory action on agonist-evoked Ca^{2+} release from intracellular stores (Karaki *et al.*, 1988; Nguyen *et al.*, 1991; Geiger *et al.*, 1992; McDaniel *et al.*, 1992). This effect has been demonstrated to be mediated by a cGMP-dependent inhibition of PtdIns(4,5)P_2 hydrolysis (Fuji *et al.*, 1986; Hirata *et al.*, 1990), which was suggested to result from an inhibition of the interaction between activated G-proteins and PIC (Nguyen *et al.*, 1991; Clementi *et al.*, 1995). Thus, under conditions where agonist-evoked Ins(1,4,5)P_3 is not inhibited, agonist may in fact activate the NO pathway, hence modulating Ca^{2+} release from stores such that it is decreased via reduced Ins(1,4,5)P_3 generation as is, in fact, the case for muscarinic M3- and bradykinin B2-stimulated Ca^{2+} release in PC-12 cells (Clementi *et al.*, 1995). This potentiality has not been investigated in the current study.

It has recently been demonstrated that in pancreatic acinar cells, agonists and antagonists modulate the apparent affinity of the Ins(1,4,5)P_3 receptor for Ins(1,4,5)P_3 in a manner such that during agonist stimulation Ca^{2+} release is facilitated and during antagonist inhibition Ca^{2+} release is impaired (Xu *et al.*, 1996). This same research revealed that agonists and antagonists appear to modulate the affinity of the Ins(1,4,5)P_3 receptor by a mechanism dependent on activation of G-proteins, since agonist-stimulated effects were mimicked by preincubation with GTPγS. In the current study, the involvement of G-protein activation in the facilitation of Ins(1,4,5)P_3-evoked Ca^{2+} mobilisation by agonist was investigated in permeabilised SH-SY5Y cells by examining the effect of a low concentration of GTPγS on exogenous Ins(1,4,5)P_3-stimulated $^{45}Ca^{2+}$ release. The results suggested that minimal activation of G-proteins, which was insufficient to mobilise Ca^{2+} alone, is sufficient to potentiate Ins(1,4,5)P_3-evoked Ca^{2+} release. It is, however, unclear from these studies whether the G-protein(s) involved in such facilitation are of the small molecular weight variety (e.g. Rho, Ras) or of the larger, heterotrimeric family (e.g. Gq/11, Go, Gi, Gs). The small molecular weight G-proteins have been implicated in regulation of cytoskeletal functions (Aullo *et al.*, 1993; Takai *et al.*, 1995), inhibition of receptor and G-protein-mediated phospholipase D stimulation (Chong *et al.*, 1994; Schmidt *et al.*, 1996) and in the regulation of PtdIns(4,5)P_2 synthesis and, therefore, regulation of cellular signalling by PIC (Zhang *et al.*, 1996). Thus far, however, the superfamily of small molecular weight G-proteins have not been implicated in modification or modulation of the Ins(1,4,5)P_3 receptor/Ca^{2+} release channel complex. Xu *et al.* (1996) suggested that Ins(1,4,5)P_3 receptor

affinity was modulated by heterotrimeric rather than small G-protein activation since the effects of GTPγS in their study could be mimicked by low concentrations of AlF_3. If the G-protein-mediated facilitation of Ins(1,4,5)P_3-evoked Ca^{2+} mobilisation observed in the current study is via a similar mechanism to G-protein-mediated modulation of the Ins(1,4,5)P_3 receptor observed by Xu *et al.* (1996), heterotrimeric rather than small G-proteins are likely to be involved.

It has recently been shown that, in pancreatic acinii, G-protein βγ subunits transduce $[Ca^{2+}]_i$ oscillations, whereas G-protein αq/11 subunits transduce large, sustained $[Ca^{2+}]_i$ responses during agonist stimulation. It was proposed that βγ subunits transduce oscillatios by increasing the affinity of the Ins(1,4,5)P_3 receptor for Ins(1,4,5)P_3 (Zeng *et al.*, 1996). It is possible that agonist-activated βγ subunits modulate the Ins(1,4,5)P_3 receptor in SH-SY5Y cells, and are responsible for the observed agonist-induced increase in the sensitivity of the Ca^{2+} store(s) to the Ca^{2+}-mobilising effect of Ins(1,4,5)P_3. Whether these hypothetical βγ subunits are released from Gq/11 or from other G-protein families such as Gi/Go remained to be elucidated.

A number of studies using the SH-SY5Y cell line have demonstrated that receptors linked to the activation of the Gi/Go family of G-proteins can mobilise Ca^{2+}, only when cells are 'primed' with agonists that activate Gq/11. For instance, activation of neuropeptide Y (NPY) Y_2 receptors, somatostatin (sst) sst_2 receptors and δ-opioid receptors activates G-proteins of the Gi/Go family and inhibit adenylate cyclase. Activation of these receptors induces Ca^{2+} mobilisation from internal stores, only when cells are prestimulated with the muscarinic receptor agonist, carbachol. These Ca^{2+} mobilisations were shown to be pertussis toxin (PTx)-sensitive, atropine-sensitive and not affected by the concentration of carbachol used for 'priming' (Connor and Henderson, 1996; Connor *et al.*, 1996, 1997). To explain these findings it was suggested that this effect was mediated via βγ stimulation of PIC that requires prior activation by Gαq/11 (Connor *et al.*, 1997). A similar phenomenon (i.e. Gi/Go coupling to Ca^{2+} mobilisation following priming with Gq/11-coupled receptor activation) has been observed in the NG108-15 cell line (Okajima and Kondo, 1992; Okajima *et al.*, 1993), which may suggest that this is a widespread effect. The relevance of these findings to the current study is as follows. Stimulation of SH-SY5Y cells with agonist coupled to the activation of Gq/11 may also activate a certain amount of Go/Gi through receptor promiscuity (Eason *et*

al., 1992; Yamada *et al.*, 1993; Neubig, 1994). For instance, activation of both muscarinic M1 and M3 receptor expressed in CHO cells, known to be linked to the activation of G-proteins of the Gq/11 family, also stimulate Gs such that adenylate cyclase is activated, as revealed by carbachol-mediated cyclic AMP accumulation following stimulation of these receptors (Burford and Nahorski 1996). Thus, βγ subunits liberated, and hence activated, from Gi/Go (which are expressed in a far greater quantity in SH-SY5Y than Gq/11 (Akam, 1997, unpublished observations)), may interact with the $Ins(1,4,5)P_3$ receptor, which following bradykinin or muscarinic receptor activation can mobilise Ca^{2+}, even in the absence of $Ins(1,4,5)P_3$ generation.

In order to establish whether activation of Gi/Go, via promiscuous coupling of bradykinin B2 and/or muscarinic M3 receptors, was involved in agonist-mediated sensitisation of the $Ins(1,4,5)P_3$ receptor for $Ins(1,4,5)P_3$ in SH-SY5Y cells, Gi and Go were selectively inhibited using PTx. Members of the Gi and Go G-protein families contain sites susceptible to modification by PTx, which catalyses the transfer of an adenosine diphosphate (ADP)-ribose moiety from nicotinamide adenine dinucleotide (NAD^+) to the α subunit. This results in the uncoupling of modified G-protein from the receptor (Simon *et al.*, 1991). In the current study, inhibition of Gi and Go activity using PTx yielded unexpected results when $Ins(1,4,5)P_3$-evoked $^{45}Ca^{2+}$ release and agonist-mediated potentiation of $Ins(1,4,5)P_3$-evoked Ca^{2+} release was examined in permeabilised SH-SY5Y cells. The data produced revealed that inhibition of Gi and Go actually potentiated $Ins(1,4,5)P_3$-evoked $^{45}Ca^{2+}$ release, in the absence of agonist-stimulation. This potentiation is likely to be via a different mechanism from that observed in the presence of either methacholine or bradykinin in these cells, as potentiation induced by PTx plus either agonist appeared to be additive. These findings seem to suggest that neither bradykinin- nor methacholine-mediated facilitation of $Ins(1,4,5)P_3$-stimulated Ca^{2+} release are via activation of the Gi/Go family of G-proteins. In addition, these data may suggest another form of modulation of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} release channel complex. Since PTx pretreatment enhances the ability of $Ins(1,4,5)P_3$ alone to mobilise Ca^{2+}, one may speculate that, under resting conditions, a tonic inhibition is exerted on the $Ins(1,4,5)P_3$ receptor, mediated via Gi and/or Go, which is released following their inhibition. The data, however, cannot rule out the possibility that βγ subunits modulate the $Ins(1,4,5)P_3$ receptor in a positive manner.

In summary, the data presented in this chapter have demonstrated that the agonist-mediated facilitation of $Ins(1,4,5)P_3$-evoked Ca^{2+} release observed in the previous chapter is insensitive to inhibition of PKC, not mimicked by activators of PKG, but mimicked by low concentrations of GTPγS. It is suggested that this phenomenon represents an agonist-induced modification of the $Ins(1,4,5)P_3$ receptor via a G-protein-dependent mechanism. This mechanism requires further study. The results presented in this chapter also suggest that $Ins(1,4,5)P_3$ receptors in SH-SY5Y cells are modulated by a mechanism dependent on Gi and/or Go G-proteins. It is suggested that, under resting conditions, a tonic inhibition is exerted via a Gi/Go-dependent mechanism on the Ca^{2+}-mobilising ability of the $Ins(1,4,5)P_3$ receptor/Ca^{2+} release channel complex in these cells. A number of other potential mechanisms for agonist-induced facilitation of $Ins(1,4,5)P_3$-evoked Ca^{2+} release, which have not been examined in the current study, including cADP-ribose generation and activation of sphingosine kinase, are discussed in the subsequent, and final, chapter of this thesis.

9. SUMMARY AND CONCLUDING DISCUSSION.

The initial aim of this thesis was to compare and contrast agonist-mediated phosphoinostitide and Ca^{2+} signalling in the undifferentiated human neuroblastoma cell line, SH-SY5Y, with RA-differentiated SH-SY5Y cells. These studies were conducted in order to proceed towards an investigation into acute regulatory aspects of the phosphoinositide signalling pathway in SH-SY5Y cells, and to investigate whether such regulation is markedly different following the cellular differentiation of these cells. Analysis of the mechanisms responsible for regulation of signalling systems, such as the phosphoinositide pathway, is important since regulation will determine the extent of the end-point, physiological response to agonist and, therefore, will play a part in determining the efficacy of that agonist. Regulation of signalling pathways in the nervous system is particularly complex. Hence, to unravel the complexities of signal integration at synaptic junctions, it is necessary to have simplified models in which to study regulation at the molecular level.

Human neuroblastoma cell lines are routinely used as a simple model in which to study regulatory aspects of phosphoinositide signalling in a neuronal-like environment. Since differentiated neuroblastoma cells resemble mature neurones more closely than their undifferentiated counterparts, the RA-differentiated SH-SY5Y cell was considered a more appropriate model for studies on the functioning of the nervous system. In the current study, particular emphasis was placed on the regulation of the phosphoinositide signalling pathway following cellular depolarisation or the activation of PKC. The reasons for conducting such investigations are outlined in previous chapters, and discussed in brief below.

Differentiation of the SH-SY5Y cell line has been associated with an increased density of VOCCs, and since cellular depolarisation has been reported on numerous occasions to acutely regulate phosphoinositide signalling in neuronal tissue, it was of interest to investigate whether an increased VOCC density would profoundly affect depolarisation-mediated regulation of this pathway. It is conceivable that this aspect of regulation is of great importance in mammalian neurones since depolarisation and PIC activation doubtless occur simultaneously in the same cell e.g. acetylcholine activation of nicotinic and muscarinic receptors or glutamate activation of NMDA and metabotropic glutamate receptors. PKC-mediated regulation of the activity of PIC was also of interest because PKC has been reported

on many occasions to regulate the extent of PIC activity (see chapter 5). Differentiation of PC-12 and HL-60 cells in culture has been associated with alterations in the relative expression levels of PKC isoforms (Wooten et al., 1991; Lee et al., 1995). Hence it is a possibility that the relative expression levels of PKC isoforms are also altered following the cellular differentiation of SH-SY5Y cells which would be likely to affect the way in which PKC regulates the phosphoinositide signalling pathway.

The initial observation of this study was that following RA-differentiation of SH-SY5Y cells, as assessed by neurite extension (see figure 2.1) and cessation of cell division, muscarinic receptor, and bradykinin receptor, cell densities were up-regulated (approximately two-fold in each case) as detected by [^{3}H]-NMS and [^{3}H]-bradykinin binding respectively. The subtype of muscarinic receptor linked to phosphoinositide hydrolysis expressed in SH-SY5Y (and SH-$SY5Y_{RA6}$) cells, which has been subject to controversy (Lambert *et al.*, 1989; Wall *et al.*, 1991; McDonald *et al.*, 1994c; Adem *et al.*, 1987; Kukkonen *et al.*, 1992) was found to be M3 as determined by pirenzepine inhibition of [^{3}H]-NMS binding and Western blot analyses using muscarinic M1 and M3 specific antisera. It was considered very likely that bradykinin receptors expressed were of the B2 subtype given that neuronal bradykinin receptors are predominantly B2 (Wolsing and Rosenbaum, 1991). Alterations of cell surface receptor expression following cellular differentiation, although not previously reported for muscarinic or bradykinin receptors in SH-SY5Y cells, would appear to be a widespread phenomenon in which the method of differentiation is important in determining the alterations observed.

Stimulation of either muscarinic or bradykinin receptors with full agonists, methacholine and bradykinin respectively, revealed that agonist-mediated PIC activation, as determined by peak Ins(1,4,5)P_3 mass generation and [^{3}H]-inositol phosphate accumulation, was enhanced in SH-$SY5Y_{RA6}$ compared with SH-SY5Y cells. The enhanced agonist-mediated PIC activation observed was expected to be as the result of an increased cell surface receptor number in SH-$SY5Y_{RA6}$ cells. In contrast, however, agonist-mediated peak $[Ca^{2+}]_i$ responses, which represent Ca^{2+} release from intracellular stores, were not affected by RA-treatment when measured either in cell populations or at the single cell level. It was initially assumed that the difference regarding agonist-evoked PIC activation and peak $[Ca^{2+}]_i$ elevation represented a spare capacity within the system, i.e. the generation of Ins(1,4,5)P_3 was above that required to trigger a maximal $[Ca^{2+}]_i$ response in SH-$SY5Y_{RA6}$ cells. However, alternative mechanisms

such as compartmentalisation of signalling components, differential sensitivity to PKC and increased sequestration of Ca^{2+} in SH-SY5Y_{RA6} cells were also suggested (see section 4.4).

Once the effects of RA-differentiation on temporal aspects of agonist-stimulated phosphoinositide and Ca^{2+} signalling had been ascertained, investigations into regulatory aspects of this pathway in SH-SY5Y versus SH-SY5Y_{RA6} cells could proceed. PKC-regulation of agonist-mediated phosphoinositide hydrolysis, as determined by Ins(1,4,5)P_3 mass formation, was investigated initially. The data obtained revealed that activation of PKC by phorbol ester reduced agonist-mediated phosphoinositide hydrolysis to a similar extent in both SH-SY5Y and SH-SY5Y_{RA6} cells. However, inhibition of PKC activity, using Ro 318220, had no effect on agonist-mediated PIC activation in SH-SY5Y cells yet in contrast, enhanced agonist-mediated PIC activation in SH-SY5Y_{RA6} cells. Thus, it appears that inhibition of agonist-stimulated PKC activation (using Ro 318220) enhances agonist-stimulated Ins(1,4,5)P_3 generation in SH-SY5Y_{RA6} cells only suggesting that activated PKC plays a role in the regulation of phosphoinositide hydrolysis in these cells but not in the undifferentiated neuroblastoma. This hypothesis may be extended to suggest that PKC plays a more active role in the regulation of the phosphoinositide signalling pathway in adult neurones. Indeed, it has been demonstrated that in mature rat cervical ganglion neurones, it is the activation of PKC that leads to muscarinic receptor-mediated $[Ca^{2+}]_i$ elevation, rather than Ca^{2+} release from intracellular stores (Marsh *et al.*, 1995). Hence, it may be suggested that PKC does, in fact, play a more significant role in phosphoinositide and Ca^{2+} signalling in neurones compared with neuroblastoma cell lines. Possible reasons for the enhancement of agonist-induced PIC activation by Ro 318220 in SH-SY5Y_{RA6} cells are speculated upon in section 5.4. The most straightforward explanation is that stimulation of SH-SY5Y_{RA6} cells with agonist results in an increased generation of DAG, concomitant with increased receptor density, thus activating PKC to a greater extent and hence increasing it inhibitory action on PIC. Under these circumstances the inhibitory effect of PKC may be great enough to be reversed in the presence of Ro 318220. Alternatively, differing expression levels of PKC and/or PIC isoforms in SH-SY5Y versus SH-SY5Y_{RA6} cells could account for the observed differences. Experimental procedures to address these possibilities are outlined in section 5.4.

There is a substantial body of evidence to indicate that Ca^{2+} is able to either activate and/or facilitate PIC in a range of preparations both neuronal (Kendall and Nahorski, 1984, 1985,

1987; Baird and Nahorski, 1986, 1990; Chandler and Crews, 1990) and non-neuronal (Biden *et al.*, 1987; Eberhard and Holtz, 1987, 1991; Kelly *et al.*, 1994) preparations. This form of regulation provides a mechanism for integration of coincident signals, within the phosphoinositide signalling pathway, between other PIC-linked receptors and also with receptors mediating changes in $[Ca^{2+}]_i$ by other means. This form of regulation may be of particular importance in the nervous system since Ca^{2+} entry through VOCCs is considered to be the primary source for changes in $[Ca^{2+}]_i$ in excitable cells. This is despite the fact that neuronal cells possess a vast amount of receptors linked to phosphoinositide hydrolysis. Thus, it is a possibility that, in excitable cells, the extent of $[Ca^{2+}]_i$ elevation via Ca^{2+} entry through VOCCs is regulated via the activity of PIC-linked receptors. Indeed, G-protein-coupled receptors are known to regulate various ion channels (reviewed by Hille, 1994, section 1.2). It is also possible that differences in the ability of Ca^{2+} entry through VOCCs to regulate PIC activity has the potential to underlie signal differentiation, within a single cell type, following stimulation with neurotransmitters activating different PIC-linked receptors. Interactions and integration of signals from PIC-linked receptors and from ion channels, such as VOCCs, in a neuronal environment has been difficult to study, mainly because of the complexity of the tissue used as a model system. RA-differentiated SH-SY5Y cells were expected to provide a useful model in which to study interactions of this nature since the cells are homogenous and expression levels of VOCCs are up-regulated in these cells.

Depolarisation-induced Ca^{2+} influxes, mediated either by high $[K^+]_e$ or by nicotine, were equivalent in SH-SY5Y and SH-SY5Y$_{RA6}$ cells, despite the reported increased density of VOCCs in SH-SY5Y$_{RA6}$ cells. Depolarisation augmented agonist-stimulated activation of PIC, as demonstrated by [^{3}H]-inositol phosphate accumulations but not Ins(1,4,5)P_3 generations, which suggests a depolarisation-mediated increase in the metabolism of Ins(1,4,5)P_3. These findings highlight the relevance of measuring both total inositol phosphate accumulation and Ins(1,4,5)P_3 generation as an index of PIC activation, since by measuring only one of these parameters, important effects may be missed. Muscarinic receptor-mediated peak $[Ca^{2+}]_i$ elevations were also enhanced when cells were depolarised simultaneously. The mechanism for such an enhancement is unclear, but could be via Ins(1,3,4,5)P_4 and/or Ca^{2+}-mediated enhancement of Ins(1,4,5)P_3-evoked Ca^{2+} release, or via CICR by Ca^{2+} stimulation of ryanodine receptors. An interesting phenomenon was a reduction of methacholine-mediated peak and sustained $[Ca^{2+}]_i$ responses when cells were

'conditioned' by direct depolarisation with high $[K^+]_e$. Possible mechanisms of action are hypothesised upon in section 6.3, including a reduction of Ca^{2+} influx through the CRAC channel resulting in a decreased quantity of stored Ca^{2+} available for $Ins(1,4,5)P_3$-evoked Ca^{2+} mobilisation, and increased sequestration into stores via agonist-stimulation of Ca^{2+} ATPase, probably via a PKC-mediated pathway.

Collectively, the data summarised above provide evidence that there is indeed signal integration between PIC-linked receptors and depolarising stimuli in the SH-SY5Y cell line. The observed integration appears to differ for different methods of depolarisation. Thus, the SH-SY5Y cell line would seem an appropriate model in which to study signal integration of this nature. Interestingly, the way in which the different $[Ca^{2+}]_i$ signals were integrated were only minimally different between SH-SY5Y and SH-$SY5Y_{RA6}$ cells, which may suggest that the density of N-type VOCCs expressed is not important in determination of interactions and integration of these Ca^{2+} signals in SH-SY5Y cells. A possible reason for this is that only a relatively small Ca^{2+} influx may be required to evoke the observed effects.

A potentially novel regulatory mechanism of phosphoinositide and Ca^{2+} signalling in SH-SY5Y cells was uncovered by a comparison of the concentration-response relationships for both methacholine- and bradykinin-mediated PIC activation and the down-stream $[Ca^{2+}]_i$ elevations in SH-SY5Y and SH-$SY5Y_{RA6}$ cells. The data presented provide evidence that there is a receptor reserve for methacholine-stimulated [^{3}H]-inositol phosphate accumulation and peak $Ins(1,4,5)P_3$ mass generation in SH-$SY5Y_{RA6}$ cells. These findings are in agreement with classical receptor pharmacology where, if there is a receptor reserve for the observable response, an increased receptor number will increase the potency of the response for a given concentration of agonist. Submaximal concentrations of bradykinin, however, failed to evoke generation of $Ins(1,4,5)P_3$ in undifferentiated SH-SY5Y cells. Hence, the potency of this response was unmeasurable and it therefore could not be assumed that there was a receptor reserve for bradykinin-mediated $Ins(1,4,5)P_3$ generation in the RA-differentiated cells. Nevertheless, the bradykinin-mediated $Ins(1,4,5)P_3$ response was clearly greatly increased concomitant with an increased bradykinin receptor density in SH-$SY5Y_{RA6}$ cells. Dose-response curves for both methacholine- and bradykinin-stimulated peak $[Ca^{2+}]_i$ increases lay to the left of the corresponding dose-response curves for peak $Ins(1,4,5)P_3$ generation in both SH-SY5Y and SH-$SY5Y_{RA6}$ cells. These observations suggest that there is an amplification

of the response in the phase between the generation of $Ins(1,4,5)P_3$ and the release of Ca^{2+}. However, despite the apparent amplification of the $[Ca^{2+}]_i$ response, there appeared to be no receptor reserve for either methacholine- or bradykinin-stimulated peak $[Ca^{2+}]_i$ elevations in SH-SY5Y$_{RA6}$ cells as the dose-response curves overlapped those generated in the undifferentiated cells. A similar lack of receptor reserve for α1B-adrenoceptor-induced increases in $[Ca^{2+}]_i$ has been observed (Horie and Tsujimoto, 1995). The size of the peak $[Ca^{2+}]_i$ responses generated either by bradykinin or methacholine were not significantly affected by low $[Ca^{2+}]_e$ which demonstrates that it is unlikely that Ca^{2+} entry is involved in these rapid increases in $[Ca^{2+}]_i$. These findings were in agreement with a previous study using the SH-SY5Y cell line (Willars and Nahorski, 1995) who found that, despite a numerical reduction of methacholine-stimulated peak $[Ca^{2+}]_i$ responses in the absence of $[Ca^{2+}]_e$, the differences were not statistically significant. Thus, peak $[Ca^{2+}]_i$ elevations in both SH-SY5Y and SH-SY5Y$_{RA6}$ cells are likely to be solely composed of stored Ca^{2+} release.

In summary, comparisons of concentration-response data for peak $Ins(1,4,5)P_3$ generation and Ca^{2+} mobilisation from intracellular stores in SH-SY5Y versus SH-SY5Y$_{RA6}$ cells revealed that, whilst there is a receptor reserve for agonist-mediated PIC activation concomitant with up-regulation of receptor density in SH-SY5Y$_{RA6}$ cells, there is no corresponding receptor reserve for agonist-evoked Ca^{2+} mobilisation, despite amplification of the response. Further analysis of the relationship between the concentration of $Ins(1,4,5)P_3$ required to elicit a certain $[Ca^{2+}]_i$ elevation revealed that this relationship is complex, that is, different for different agonists in the same cell (e.g. SH-SY5Y) and different for the same agonist in different cells (i.e. SH-SY5Y versus SH-SY5Y$_{RA6}$). Analysis of the concentration-response data in this manner raised the question of whether there is a hitherto unknown mechanism(s) of agonist-mediated regulation of Ca^{2+} mobilisation in the SH-SY5Y cell line. Since it is becoming increasingly evident that regulation of Ca^{2+} mobilisation from internal stores occurs via complex processes that are not fully understood, this possibility seemed likely and intriguing enough to investigate.

Having thus established that the relationship between $Ins(1,4,5)P_3$ accumulation and Ca^{2+} mobilisation is particularly complex in SH-SY5Y cells, and indeed is becoming increasingly recognised as complex in a variety of cells, potential mechanisms for this complexity were sought. It seemed likely that if such a mechanism were operating, it would be at the level of

the $Ins(1,4,5)P_3$ receptor/Ca^{2+} release channel complex since the major species of the $Ins(1,4,5)P_3$-sensitive release channels (type-1), which are expressed in the SH-SY5Y cells line (Mackrill *et al.*, 1997), display multiple regulatory mechanisms (Mikoshiba, 1997). One hypothetical mechanism, which was investigated further, was that receptor agonists may regulate the sensitivity of the $Ins(1,4,5)P_3$-activated Ca^{2+} release channels, independently of phosphoinositide hydrolysis and changes in $[Ca^{2+}]_i$, in SH-SY5Y cells. If such a mechanism operates in these cells, it may offer an explanation for the apparent amplification between the PIC and the Ca^{2+} response. If separate agonists differentially modulate the sensitivity of $Ins(1,4,5)P_3$-activated Ca^{2+} release channels in SH-SY5Y cells, this could also provide an explanation for why the relationship between $Ins(1,4,5)P_3$ generation and Ca^{2+} mobilisation is different for different agonists within the same cell. In order to investigate the existence of this proposed mechanism, the sensitivity of the internal, $Ins(1,4,5)P_3$-activated Ca^{2+} releasing machinery to exogenous $Ins(1,4,5)P_3$, in the presence or absence of either methacholine or bradykinin under conditions where $Ins(1,4,5)P_3$ generation is negligible, was investigated. Thus, the ability of agonist to modulate the Ca^{2+}-mobilising activity of $Ins(1,4,5)P_3$ receptors expressed in SH-SY5Y cells, without the complication of agonist-stimulated endogenous $Ins(1,4,5)P_3$-induced Ca^{2+} release, could be examined.

Stimulation of SH-SY5Y cells with bradykinin enhanced exogenous $Ins(1,4,5)P_3$-evoked Ca^{2+} release, when stimulation with bradykinin alone failed to mobilise Ca^{2+}, even at supramaximal concentrations. This enhancement, or facilitation, was bradykinin B2 receptor specific as it was reversed by the bradykinin B2 receptor-specific antagonist, HOE 140. Methacholine alone, however, dose-dependently mobilised Ca^{2+} from stores. Treatment of cells with the PI-4-kinase inhibitor, wortmannin, practically abolished muscarinic receptor-mediated $Ins(1,4,5)P_3$ accumulation. In contrast, wortmannin pre-treatment only slightly reduced the potency and maximal response of methacholine-mediated Ca^{2+} mobilisation. These findings strongly suggest that, in SH-SY5Y cells, only a very small $Ins(1,4,5)P_3$ generation is required for substantial mobilisation of stored Ca^{2+}. The possibility that CICR, for $Ins(1,4,5)P_3$ receptors or ryanodine receptors, was responsible for the observed effect was considered unlikely since, if this was the case, the small bradykinin-mediated $Ins(1,4,5)P_3$ generation in SH-SY5Y cells would also be expected to evoke substantial Ca^{2+} mobilisation, and this is not the case. The possibility that methacholine-mediated Ca^{2+} release in SH-SY5Y cells was via a mechanism other than interaction of $Ins(1,4,5)P_3$ with its receptor was also considered

unlikely since the Ins(1,4,5)P_3 receptor antagonist, heparin, completely abolished muscarinic receptor-mediated Ca^{2+} mobilisation.

It was found that depletion of the agonist-sensitive PtdIns(4,5)P_2 pools in SH-SY5Y cells by a combined pre-treatment with methacholine and wortmannin (Willars *et al.*, 1996) severely depressed agonist-mediated Ins(1,4,5)P_3 accumulation and Ca^{2+} mobilisation. Under these conditions methacholine potentiated exogenous Ins(1,4,5)P_3-evoked Ca^{2+} release, an effect which was muscarinic receptor-specific. Since potentiation of Ins(1,4,5)P_3-evoked Ca^{2+} mobilisation following either muscarinic or bradykinin receptor occupation occurred in the absence of endogenous Ins(1,4,5)P_3-mediated effects, it was concluded that muscarinic and bradykinin receptor activation induces a modification of the Ins(1,4,5)P_3 receptor such that its sensitivity to Ins(1,4,5)P_3 is enhanced.

Potential mechanisms for the observed agonist-stimulated augmentation of Ins(1,4,5)P_3-mediated Ca^{2+} release in SH-SY5Y cells were investigated, including activation of PKC and PKG, the possibility that agonist protects lower concentrations of Ins(1,4,5)P_3 from metabolism and the involvement of G-proteins, more specifically the involvement of the Gi/Go family of G-proteins. The data from the current study suggested that this facilitation was independent of agonist-stimulated activation of either PKC or PKG. The possibility that Ins(1,4,5)P_3 was being protected from metabolism was also ruled out following studies using the non-metabolisable Ins(1,4,5)P_3 receptor agonist, adenophostin. However the data suggest the involvement of a G-protein in agonist-mediated enhanced Ins(1,4,5)P_3-evoked Ca^{2+} mobilisation, as revealed by the augmentation of agonist-stimulated Ca^{2+} release by low concentrations of GTPγS. Inhibition of the G-proteins Gi and Go with pertussis toxin did not reverse potentiation of Ins(1,4,5)P_3-mediated Ca^{2+} release by either bradykinin or methacholine, suggesting activation of these G-proteins are not involved in the observed phenomenon. However, pre-treatment with pertussis toxin facilitated the ability of Ins(1,4,5)P_3 alone to release Ca^{2+}, and further enhanced agonist-mediated potentiation of Ins(1,4,5)P_3-induced Ca^{2+} release. Thus, it was proposed that active Gi and/or Go modulate the Ins(1,4,5)P_3 receptor/Ca^{2+} release channel complex by the exertion of a tonic inhibition on the complex in SH-SY5Y cells. This effect appears to be exerted under resting conditions. It is unlikely that the agonist-mediated facilitation of Ins(1,4,5)P_3-evoked Ca^{2+} mobilisation observed in this study was via inhibition of Gi/Go-mediated tonic inhibition of the

$Ins(1,4,5)P_3$ receptor since the facilitatory effects of pertussis toxin and agonist on $Ins(1,4,5)P_3$-induced Ca^{2+} mobilisation appeared to be additive.

Thus, the involvement of a G-protein in agonist-mediated enhanced $Ins(1,4,5)P_3$-evoked Ca^{2+} mobilisation is suggested. However, the G-protein(s) involved and the mechanism responsible remain to be elucidated. An attractive mechanism of action, recently proposed by Zeng *et al.* (1996) to be responsible for $[Ca^{2+}]_i$ oscillations in pancreatic acinii, is that liberated, and hence active, βγ subunits somehow modify the $Ins(1,4,5)P_3$ receptor such that its Ca^{2+} mobilising machinery is sensitised to $Ins(1,4,5)P_3$. This possibility could be investigated in permeabilised SH-SY5Y cells by investigating whether, in the presence of purified βγ subunits, the ability of exogenous $Ins(1,4,5)P_3$ to release Ca^{2+} was enhanced. This theory could be further studied by examining the effect of the Gβγ-binding βARK fragment (Koch *et al.*, 1993, 1994) and/or the Gβγ scavenger $G\alpha_{i1G203A}$, a mutant Gα subunit which avidly binds Gβγ (Lee *et al.*, 1992; Slepak *et al.*, 1993), on agonist-mediated facilitation of $Ins(1,4,5)P_3$-evoked Ca^{2+} mobilisation. If the facilitation was reversed by these agents, a role for βγ subunits in the modulation of the $Ins(1,4,5)P_3$ receptor would seem very likely.

Although in the present study, inhibition of Gi/Go appeared to facilitate $Ins(1,4,5)P_3$-evoked Ca^{2+} release, this effect may have been mediated through Gα subunits. The possibility that agonist-mediated potentiation of $Ins(1,4,5)P_3$-evoked Ca^{2+} release is via βγ subunits liberated from Gi and/or Go cannot be excluded. To investigate the involvement of the activation (as opposed to pertussis toxin-mediated inhibition) of the Gi/Go proteins, the effect of stimulation of δ-opioid and/or neuropeptide Y and/or somatostatin receptors on $Ins(1,4,5)P_3$-evoked Ca^{2+} release in permeabilised SH-SY5Y cells could be examined. If stimulation of these receptors enhanced $Ins(1,4,5)P_3$-evoked Ca^{2+} release, a role for the activation of the Gi/Go family of G-proteins in the facilitation by agonist observed in the current study would seem conceivable. Moreover, βγ subunits released from Gi/Go would appear the likely candidates for such an effect, since data presented in this thesis suggest that active Gi/oα subunits actually inhibit $Ins(1,4,5)P_3$-mediated Ca^{2+} mobilisation.

It has previously been mentioned in this thesis, however, that a role for the small molecular weight G-proteins, such as Rho and Ras, cannot be dismissed when investigating the observed occurrence of enhanced Ca^{2+} release evoked by $Ins(1,4,5)P_3$. One way in which this

potentiality could be investigated is by the employment of *Clostridium difficile* toxin B, which gycosylates and thereby inactivates small G-proteins of the Rho family (Just *et al.*, 1994, 1995). If this toxin reversed agonist-mediated facilitation of Ins(1,4,5)P_3-evoked Ca^{2+} mobilisation in permeabilised SH-SY5Y cells, this phenomenon could be suggested to be mediated via the small molecular weight G-protein Rho.

It is not impossible that mechanisms, other than those mediated by G-proteins, contribute towards agonist-enhancement of Ins(1,4,5)P_3-stimulated Ca^{2+} release. One such mechanism is generation of the putative second messenger, cADP ribose, by agonist, independent of PIC activation. cADP ribose is known to be an activator of CICR by ryanodine-sensitive Ca^{2+} channels in several cell types (Galione *et al.*, 1991; Walseth *et al.*, 1991; Hua *et al.*, 1994) and cADP ribose has been demonstrated to trigger Ca^{2+} release in rat neurones (Currie *et al.*, 1991; Koshiyama *et al.*, 1991) and neurosecretory PC-12 cells (Clementi *et al.*, 1996). Since the SH-SY5Y cell line is reported to express type-2 ryanodine receptors (Mackrill *et al.*, 1997) it is a possibility that cADP ribose-mediated CICR is the mechanism responsible for the agonist-mediated potentiation of Ins(1,4,5)P_3-induced Ca^{2+} release in the current study. It would be possible to investigate the role of cADP ribose by examining the effect of the cADP ribose antagonist, 8-amino-cADP ribose, on agonist-mediated potentiation of Ins(1,4,5)P_3-evoked Ca^{2+} release in permeabilised SH-SY5Y cells. An alternative mechanism is an agonist-mediated activation of sphingosine kinase (SK) which produces sphingosine-1-phosphate (S-1-P), an alternative second messenger that has been demonstrated to mobilise Ca^{2+} in an Ins(1,4,5)P_3-independent manner in a variety of cell lines (Ghosh *et al.*, 1994; Mattie *et al.*, 1994; Kindman *et al.*, 1994; Choi *et al.*, 1996). Whether the activation of SK by agonist, with the resultant production of S-1-P, contributes towards the mechanism responsible for agonist-mediated facilitation of Ins(1,4,5)P_3-induced Ca^{2+} release in the current study could initially be addressed by examining the effect of exogenously applied sphingosine on exogenous Ins(1,4,5)P_3-induced Ca^{2+} mobilisation in permeabilised SH-SY5Y cells.

Although the current study provides evidence for an agonist-mediated facilitation of Ca^{2+} mobilisation, independent of Ins(1,4,5)P_3 generation in undifferentiated SH-SY5Y cells, it is unclear whether this occurrence accounts for the comparatively reduced ability of endogenous Ins(1,4,5)P_3 generation to mobilise Ca^{2+} in RA-differentiated SH-SY5Y cells. To examine

this aspect it would first be necessary to investigate whether a similar agonist-mediated facilitation of exogenous Ins(1,4,5)P_3 is evident in permeabilised SH-SY5Y_{RA6} cells. If such a facilitation were not apparent in these cells, it may suggest that the aforementioned phenomenon is not important in a more neuronal-like cell. Clearly this aspect requires a further, detailed investigation, which may highlight important mechanistic differences, regarding the phosphoinositide and Ca^{2+} signalling pathway, between the neuroblastoma SH-SY5Y and cells of a more neuronal nature.

Appendix 1: Preparation of cytosol-like buffer (CLB).

CLB was freshly prepared prior to experimentation due to the relative instability of adenosine 5'-trisphosphate (ATP) in neutral solutions. As a precaution, CLB was not used 3hr after preparation. The compounds, with their appropriate concentrations, comprising CLB are listed below.

Compound	Concentration in CLB
Potassium chloride (KCl)	120mM
Potassium dihydrogen orthophosphate (KH_2PO_4)	2mM
Sodium succinate hexahydrate ($(H_2COONa)_2.6H_20$)	5mM
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	2.4mM
HEPES free acid	20mM
Na_2ATP (grade 1, 99% pure)	2mM

CLB was always made up in plastic labware in order to avoid Ca^{2+} contamination.

The pH of the solution was adjusted to pH 7.2 with 20% (w/v) KOH. Subsequently, between 5 and 45 µl of a 10mM EGTA stock solution (made up in dH2)) was added to the CLB in order to buffer the Ca^{2+} concentration to between 90 and 150nM. The $[Ca^{2+}]$ in CLB was assessed to be between 90 and 150nM for each experiment using the following protocol.

Calculating $[Ca^{2+}]$ in CLB:

2ml of CLB buffered with 10mM EGTA was placed in a fluorimeter cuvette, to which 2µl of fura-2 free acid (200µM stock) was added. The fluorescent intensity (F) of the CLB was determined at an excitation wavelength of 340nM with emission measured at 480nM. F_{max} values were calculated following the subsequent addition of 5µl of a 200mM $CaCl_2$ solution and F_{min} values determined following the subsequent addition of 50µl EGTA (200mM, made up in 300mM Tris buffer). EGTA serves to sequester all free Ca^{2+}. The free $[Ca^{2+}]$ in CLB

was calculated by application of the values obtained for F, F_{min} and F_{max} to the following equation:

$$\text{free } [Ca^{2+}] \text{ in CLB (nM)} = F - F_{min} / F_{max} - F) \times K_D$$

Where K_D (at room temperature) = 739nM.

K_D is the equilibrium dissociation constant for the binding of Ca^{2+} to fura-2 free acid.

Appendix 2: Preparation of Krebs-HEPES Buffer.

The compounds and their appropriate concentrations comprising Krebs-HEPES buffer are listed below.

Compound	Concentration
Sodium chloride (NaCl)	119mM (6.74g/L)
Potassium chloride (KCl)	4.7mM (0.35g/L)
Magnesium sulphate hexahydrate ($MgSO_4.6H_2O$)	1.2mM (0.29g/L)
Potassium dihydrogen orthophosphate (KH_2PO_4)	1.2mM (0.16g/L)
Sodium hydrogen carbonate ($NaHCO_3$)	4.2mM (0.35g/L)
D-Glucose ($C_6H_{12}O_6$)	11.7mM (2.10g/L)
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	1.3mM (0.19g/L)
HEPES free acid	10mM (2.38g/L)

The solution was warmed to 37°C (in a water bath), and pH was corrected, at this temperature, to 7.2 using 1M NaOH.

Appendix 3: Material Suppliers.

Aldrich Chemical Company, Gillingham, Dorset, England.

Dimethylsulphoxide (DMSO)

Amersham International PLC, Aylesbury, Bucks, England.

[^{3}H]-bradykinin

$^{45}CaCl_2$

D-myo-[^{3}H]-inositol 1,4,5-trisphosphate ([^{3}H]-Ins(1,4,5)P_3)

[^{3}H]-myo-inositol

[^{3}H]-N-methyl scopolamine chloride ([^{3}H]-NMS)

Enhanced Chemiluminescence Reagents (ECL)

B.D.H. Limited, Poole, Dorset, England.

Ethylenediaminetetra-acetic acid (EDTA)

Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)

Calbiochem. Novabiochem. Ltd., Nottingham, England.

Fura-2-AM

Fura2-free acid

Ionomycin

Phorbol 12,13-dibutyrate (PDBu)

Ro-318220

Fisons Scientific Equipment, Loughborough, Leics, England.

Culpric sulphate ($CuSO_4.5H_2O$)

D-Glucose

Hydrochloric acid (Hcl)

Methanol

Potassium chloride (KCl)

Potassium sodium tartrate ($KNaC_4H_4O_6.4H_2O$)

Sodium chloride (NaCl)

Sodium carbonate (Na_2CO_3)

Sodium hydrogen carbonate ($NaHCO_3$)

Sodium hydroxide (NaOH)

Sodium succinate hexahydrate ($(NaH_2COONa)_2.6H_2O$)

Trichloroacetic acid (TCA)

GIBCO.BRL, Paisley, Scotland.

Growth media and supplements

Tissue culture flasks

Sigma Chemical Company Limited, Poole, Dorset, England.

Adenosine 5'-triphosphate (ATP)

Arecoline

Atropine sulphate

Bacitracin

Bordetella pertussis toxin (Ptx)

β-escin

Bovine serum albumin (BSA)

Bradykinin

8-bromo-cyclic guanosine monophosphate (8-Br-cGMP)

Captopril

Dithiothreitol (DTT)

Ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetraceticacid (EGTA)

Folin/ciocalteus phenol reagent

Guanosine 5'-triphosphate (GTP)

GTPγS

HEPES free acid

Horseraddish peroxidase-labelled anti9-rabbit IgG) antibody

Methacholine

1,10-phenathroline

Pirenzepine dihydrochloride

Polyethyleneimine

Retinoic acid (RA)

Sodium nitroprusside (SNP)

TES buffer

1,1,2-trichlorotrifluoroethane

Tris-HCl buffer

Tris-base buffer

Tween-20

Tri-n-octylamine

Dr. Andrew Tobin, Leicester University, England.

M1 antisera

M3 antisera

University of Rhode Island Foundation Chemistry Group, U.S.A.

D-myo-inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$)

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