RECEPTOR-MEDIATED CATECHOLAMINE RELEASE
FROM CHROMAFFIN CELLS: THE ROLE OF PROTEIN KINASE C

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By

Nouf AL-Rasheed BSc. (Hons).

Department of Cell Physiology and Pharmacology,
University of Leicester

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Modulation of neurotransmitter release by ionotropic nicotinic receptors and Gαq/11-coupled receptors may be essential for events such as regulation of cardiovascular function and the stress response. Previous studies indicated that the exocytotic mechanism is enhanced by protein kinase C (PKC). However, the circumstances in which PKC plays a role, the extent of regulation and the mechanisms underlying both PKC activation and its regulation of exocytosis are unclear. In this study, bovine chromaffin cells were used to explore the role of PKC, particularly the various PKC isoforms, in nicotinic receptor- and Gαq/11-coupled receptor-mediated catecholamine release.

This study reveals that bovine chromaffin cells express PKCα, -β, -ε and -δ and that they were differentially activated by nicotinic receptors and a range of Gαq/11-coupled GPCRs. Thus, nicotinic receptor stimulation recruited PKCα, -β and -ε from the cytoplasm to the plasma membrane, indicative of activation. In contrast, activation of Gαq/11-coupled receptors with histamine activated all the expressed PKC isoforms and angiotensin II only activated PKCα and -ε.

Inhibition of PKC using general or isoform-selective inhibitors potentiated catecholamine release in response to activation of Gαq/11-coupled receptors, most likely as consequence of potentiated phospholipase C-mediated signalling. However, inhibition of PKC, particularly PKCα, markedly inhibited nicotinic receptor-mediated catecholamine release. PKCα is a classical isoform of PKC, activated by Ca2+ and diacylglycerol (DAG).

The current study suggests that Ca2+ influx across the plasma membrane in response to nicotinic receptor activation is largely through the nicotinic receptors themselves. Furthermore, this Ca2+ entry activates PLC, generating both Ins(1,4,5)P3 and DAG. This activation of PLC contributes significantly to the activation of PKC. The mechanism through which PKC facilitates the release of catecholamines requires the PKC-dependent phosphorylation of myristoylated alanine-rich C protein kinase substrate (MARCKS), the subsequent disassembly of the cortical F-actin cytoskeleton and probably therefore, increased access of a reserve exocytotic vesicle pool to release sites at the plasma membrane. Moreover, this study also suggests that PLC-dependent generation of DAG recruits Munc13-1, a known vesicle priming agent. Thus, activation of Munc13-1 may also contribute to nicotinic receptor-mediated catecholamine release in a manner dependent on the activation of PLC.
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Dedicated to my husband and children.
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1: INTRODUCTION

Exocytosis from neuroendocrine cells can be triggered by different receptor types such as ionotropic nicotinic receptors and \( \text{G}_{\alpha_{q/11}} \)-coupled receptors. These different receptors may differentially regulate exocytosis by using different mechanisms: importantly, one possibility is through PKC. PKC is well documented to regulate exocytosis, although there is debate about the contribution of various PKC isoforms to the release process and the mechanisms underlying PKC activation. Work described within this thesis focuses on examining the role of PKC in catecholamine secretion in response to ionotropic nicotinic receptors and \( \text{G}_{\alpha_{q/11}} \)-coupled receptors.

1.1 NEUROENDOCRINE SECRETORY MODELS USED FOR STUDYING EXOCYTOSIS

Secretory granules and their regulated exocytosis have been most extensively studied in a number of cell types. These have served either as model systems that have certain experimental advantages or have been of particular interest due to their crucial physiological roles and alterations in disease states. A well-used cell type is the adrenal chromaffin cell and its tumour counterpart, PC12 cells, both of which have allowed biochemical and electrophysiological investigations (Gillis and Chow, 1997; Morgan and Burgoyne, 1997; Burgoyne and Morgan, 1998a). Among the wide range of secretory cells such as neurons (Bean et al., 1994; Zucker, 1996), pancreatic \( \beta \)-cells (Easom, 2000), mast cells (Lindau and Gomperts, 1991), platelets (Lemons et al., 1997) and neutrophils (Nusse and Lindau, 1988), PC12 cells and chromaffin cells were selected in this study for a variety of reasons that will be discussed later.
1.1.1 PC12 cell line

Pheochromocytomas are neuroendocrine tumours of adrenal chromaffin cells, characterized by over production of catecholamines (Parmar and Zinder, 2002). They are rare in all species except rats but occur with increased frequency in several human familial syndromes. Pheochromocytomas are inducible in rats by a variety of non-genotoxic substances that may act indirectly by stimulating chromaffin cell proliferation (Tischler, 2002).

Initially the work of this project was conducted using a PC12 cell line, which was originally isolated by Greene and Tischler (1976) from a rat pheochromocytoma. It has been extensively employed in neurobiological studies and for almost 30 years has served as a research tool. Under conventional condition these cells resemble chromaffin cells. Nevertheless, when treated with nerve growth factor (NGF) PC12 cells stop growing, enlarge and acquire a neuronal-like phenotype. For example, they develop flattened, non-spherical cell bodies and extend long, branching neurites (Greene and Tischler, 1976). Their secretory vesicles are redistributed from the somata to the neurites, and they secrete both catecholamines and acetylcholine in a Ca\(^{2+}\)-dependent manner (Harkins and Fox, 1998). Because of these properties, PC12 cells are popular model of both neurosecretory and neuronal-type cells (Janigro et al., 1989). PC12 cells express a variety of G\(\alpha_{q/11}\)-coupled receptors and ligand gated ion channels as well as T-, L-, N-, and P/Q-type Ca\(^{2+}\) channels (Shafer and Atchison, 1991). Moreover, PC12 cells synthesize and store catecholamines in secretory vesicles that have many characteristics in common with neuronal large dense-core vesicles, and release their contents by Ca\(^{2+}\)-dependent exocytosis (Shoji-Kasai et al., 2001). In addition, the ability to produce homogeneous populations of cells
from pheochromocytomas expands the usefulness of these tumours as models for neurotoxicology and for developmental and degenerative neural disorders (Tischler, 2002). An additional advantage in using PC12 cells is the possibility of selecting mutant subclones useful for the analysis of various cellular functions. In spite of these advantages, there are also disadvantages including, an apparently less stable phenotype. Thus, it is liable to change its morphology when cultured for long periods and it becomes heterogeneous after a large number of cell divisions due to spontaneously generated mutations (Shoji-Kasai et al., 2001).

PC12 cells have been widely used in studies investigating the molecular mechanisms involved in neurotransmitter release (Ritchie, 1979; Stallcup, 1979; Williams and McGee, 1982; Pozzan et al., 1986; Appell and Barefoot, 1989; Weiss and Atlas, 1991; Rhoads et al., 1993; Suh and Kim, 1994). They have also been used extensively to study signalling, particularly by GPCRs (Fasolato et al., 1988; Rhee et al., 1989; Berridge, 1993; Nardone et al., 1994; Suh et al., 1995; Koizumi et al., 2002; Kudlacek et al., 2003; Ohta et al., 2004). Despite such reports, experiments conducted on PC12 cells in this study failed to show the expression of $\mathrm{G}_{\alpha_q/11}$-coupled receptors in these cells (see Chapter 3) and attention was therefore directed to its physiological counterpart, the neuroendocrine chromaffin cells. These are relatively easy to prepare and maintain in culture, have a stable phenotype (Schwarz, 1994; Powell et al., 2000), can be stimulated to elicit robust catecholamine secretion (Burgoyne, 1991), and express a range of $\mathrm{G}_{\alpha_q/11}$-coupled receptors (O'Sullivan and Burgoyne, 1989; Plevin and Boarder, 1988; Artalejo et al., 1990; Dahmer and Perlman, 1988; Wilson, 1988; Marley, 2003; Burgoyne, 1991) and ion channels (Kilpatrick et al., 1982; Bormann and Clapham, 1985; Lara et al., 1998; Teschemacher and Seward, 2000).
1.1.2 Bovine chromaffin cells

The adrenal glands, located at the superior poles of the two kidneys, are composed of two distinct layers, the adrenal cortex and adrenal medulla. The outer adrenal cortex, which develops from the abdominal mesothelium surrounding the medulla during embryogenesis, synthesizes and secretes the adrenocortical hormones (i.e., mineralocorticoids and glucocorticoids). The adrenal medulla, which comprises the central 20% of the gland, originates from the neural crest. The adrenal medulla is a modified sympathetic ganglion, which secretes the catecholamines adrenaline and noradrenaline in response to sympathetic neural stimulation from the splanchnic nerves (Morgan and Burgoyne, 1997; Perlman and Chalfie, 1977; Eaton and Duplan, 2004).

Chromaffin cells of the adrenal medulla store their secretory products in specialized membrane-bound organelles, the chromaffin vesicles. In response to stimuli, these vesicles fuse with the plasma membrane and release their soluble contents (catecholamines, ATP, peptides) to the cell exterior by exocytosis (Rosé et al., 2002). In addition, chromaffin granules are the counterparts of LDCVs (large dense core vesicles) found in at least two compartments, the release-ready granule pool and the reserve pool comprising the vast majority of granules (Trifaró et al., 1985). Thus, these cells serve as an excellent model for studying the biosynthesis and functional role of the different peptides and proteins contained within LDVs. There is large body of evidence indicating that the co-stored neuropeptides and proteins are also co-released together with the catecholamines from cultured bovine chromaffin cells (Livett et al., 1981; Laslop and Mahata, 2002).
A number of factors have contributed to the success of the bovine chromaffin cell model. Of great importance is convenience. Large numbers of chromaffin cells can be obtained in relatively pure populations from the adrenal medulla. Other normal neuroendocrine cells have until recently not been as easily accessible, and chromaffin cells has therefore had broad appeal (Burgoyne, 1991; Tischler, 2002). Chromaffin cells of bovine source have been intensively studied by those interested in endocrine mechanisms, neuronal function and the basic cell biology of the secretory process (Burgoyne, 1984a; Knight et al., 1989). Bovine chromaffin cells provide an ideal model system for studying regulated exocytosis and in the last few decades they have been used extensively as a model system to study Ca\textsuperscript{2+}-triggered exocytosis. Secretion of catecholamines can be evoked by a wide variety of stimuli and readily quantified using a variety of approaches including electrophysiological, and biochemical analyses (Burgoyne, 1991; Holz et al., 1992; Lawrence et al., 2002) In addition, they offer the advantage of allowing the use of techniques for studying the kinetics of exocytosis in single cells with high temporal resolution. Thus, measurement of membrane capacitance and electrochemical detection of catecholamine release allows the study of exocytosis in the millisecond range (Ashery et al., 2000). For those interested in neuronal function, chromaffin cells possess the advantage that they are derived during embryogenesis from the same precursors as sympathetic neurons and possess some properties in common with neurons such as the expression of voltage-dependent Na\textsuperscript{+} and Ca\textsuperscript{2+} channels and a variety of ‘neuronal-specific’ proteins (Burgoyne, 1991). Furthermore, it has recently become possible to over-express recombinant proteins in chromaffin cells including those located at synapses, making them an ideal system to study their role in regulated exocytosis.
(Ashery et al., 1999; Duncan et al., 1999). Despite these advantages there are some important points to consider when using chromaffin cells. Foremost is the existence of species differences. Furthermore, populations of chromaffin cells are heterogenous and may have varied responsiveness to neurotrophic or mitogenic factors in vitro, and utilization of varied signal transducers in physiological processes (Suzuki and Kachi, 1996). To overcome these problems chromaffin cells should be used after only short culture periods and where possible the results confirmed with freshly isolated cells. In addition, differential plating may reduce the percentage of contaminating cells in chromaffin cell culture (Waymire et al., 1983).

1.2 NEUROTRANSMITTER RELEASE

Exocytosis is the process by which a membrane-bound vesicle fuses with the plasma membrane of a cell, resulting in the release of vesicle contents (Craig et al., 2003). Exocytosis takes place along two pathways. Firstly, constitutive exocytosis which occurs in all eukaryotic cells. In this pathway the vesicles fuse with the plasma membrane in the absence of external signals. In contrast, in regulated exocytosis, vesicles only undergo exocytosis in response to particular stimuli; for example an increase in intracellular \([\text{Ca}^{2+}]\) ([Ca\(^{2+}\)). Regulated exocytosis plays diverse roles including the release of neurotransmitters and hormones (Lin and Scheller, 2000). In neurons, synaptic vesicles that are considered the key organelles in this process, undergo a complex cycle of fusion and fission events that regulate the release process at the synapse (Betz et al., 1998). In 1950, Katz and his colleagues suggested the idea of synaptic vesicles and since then, synaptic vesicle exocytosis has been explored using a wide variety of techniques, which has led to the identification of many of the proteins that
participate not only in synaptic vesicle fusion but also in other steps of synaptic vesicle trafficking such as targeting, docking and priming. Modifications to any of these steps may modulate synaptic transmission and this may play a role in synaptic plasticity underlying events such as learning and memory (Lin and Scheller, 2000).

1.2.1 Biosynthesis of catecholamines

Chromaffin cells in the adrenal medulla are specialized for the synthesis, storage and secretion of catecholamines. These cells are innervated by preganglionic sympathetic neurons in the splanchnic nerves and activation by these nerves appears to be the most important determinant of adrenomedullary function (Perlman and Chalfie, 1977).

Chromaffin cells store catecholamines, nucleotides, opioid peptides, dopamine β-hydroxylase (DBH) and chromogranin A (CgA) in membrane-bound organelles: the chromaffin granules (Trifaro, 1977). Upon stimulation, soluble contents of the vesicles are released to the cell exterior by exocytosis. Therefore, chromaffin vesicles are considered true organelles, which in addition to their soluble contents contain enzymes involved in catecholamine synthesis (Trifaro, 2002). At least three types of chromaffin cells are present in the adrenal medulla: adrenaline (85%), noradrenaline (14-15%), and dopamine (1%) containing cells. Adrenaline, noradrenaline, and dopamine are synthesized in the chromaffin cell from tyrosine which is a common precursor (Udenfriend, 1953). Tyrosine is converted initially to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH). This enzyme is found only in catecholamine-containing cells. It is probably free in the cytosol and is considered the rate-limiting enzyme in catecholamine synthesis (Levitt et al., 1965; Ikeda et al., 1966). TH is inhibited by the end-
product of the biosynthetic pathway or by DOPA and as this provides the mechanism for regulation of the rate of synthesis (Weiner and Rabadjija, 1968). Following the production of DOPA, this is converted to dopamine by DOPA decarboxylase, a cytosolic enzyme confined to catecholamine-synthesising cells. Dopamine can be converted to noradrenaline by DBH, a membrane bound mixed-function oxidase present in chromaffin vesicles (Winkler and Westhead, 1980; Brooks and Treml, 1983; Pender and Burgoyne, 1992; Trifaró, 2002). DBH is not, however subject to rapid degradation or uptake and its concentration in the plasma can be used as index of overall sympathetic nerve activity. The subsequent formation of adrenaline from noradrenaline is catalysed by phenylethanolamine N-methyltransferase (PNMT) (Axelrod, 1962; Trifaró, 2002). This enzyme is present in the population of adrenaline-releasing chromaffin cells of the adrenal medulla but not in the smaller proportion of noradrenaline releasing cells. The adrenaline-releasing chromaffin cells, which appear only after birth, lie adjacent to the adrenal cortex and there is evidence that the production of PNMT is triggered specifically by glucocorticoid hormones produced by the cortex (Kalcheim et al., 2002).

Following synthesis of catecholamines and other neurotransmitter amines in the cytoplasm, they are taken up into storage vesicles by vesicular monoamine transporters (VMATs) to be ready for subsequent exocytotic events (Henry et al., 1994; Parmer and Zinder, 2002; Flatmark et al., 2002). VMATs act as an electrogenic antiporters (exchangers) of protons and monoamines using a proton electrochemical gradient (Johnson, 1988; Kanner and Schuldiner, 1987). Two homologous but distinct VMAT genes have been cloned from rat, bovine and human adrenal glands (Erickson and Eiden, 1993). In rat, VMAT₁ is expressed in
the adrenal gland whereas VMAT$_2$ is expressed in the brain. In contrast, bovine adrenal glands express both VMAT$_1$ and VMAT$_2$ (Krejci et al., 1993).

1.2.2 The life cycle of granules and synaptic vesicles

Neurons possess two classes of secretory vesicles, namely LDCVs and synaptic vesicles. LDCVs store neuropeptides and some small molecules such as ATP and catecholamines. Classical neurotransmitters such as acetylcholine, GABA (gamma aminobutyric acid), glutamate and glycine are stored in small synaptic vesicles (Kelly, 1993). Neuroendocrine cells such as chromaffin cells have only LDCVs and these are commonly referred to as chromaffin granules. They are known to store catecholamines, neuropeptides (neuropeptide Y and enkaphalin), nucleotides and proteins (e.g. chromogranin A and B, secretogranin II) (Huttner and Natori, 1995). In contrast, PC12 cells possess both LDCVs, which contain monoamines, and small synaptic like-microvesicles (SLMVs) thought to store acetylcholine (Weihe et al., 1996; Ninomiya et al., 1997). In addition, SLMVs are also present in other endocrine cells such as pancreatic $\beta$-cells, which store and secrete molecules such as GABA, the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) and the degradative enzyme GABA-transaminase (Reetz et al., 1991; Braun et al., 2004). Furthermore, in pancreatic $\beta$-cells, GABA and GAD are excluded from insulin-containing LDCVs (Garry et al., 1988). Although both classes of vesicles share the ability to undergo Ca$^{2+}$-triggered exocytosis, they differ in their biogenesis. Immature granules bud directly from the trans-Golgi network (TGN). In contrast, synaptic vesicles derive from the endosome and acquire specific proteins via constitutive secretion and endocytosis from the plasma membrane (Régnier-Vigouroux et al., 1991).
In neurons, synaptic vesicles are not directly bound to the actin cytoskeleton and a small proportion are tightly docked to neurotransmitter sites at the presynaptic plasma membrane ("the active zone"). The vast majority of synaptic vesicles are cross-linked by the vesicle protein synapsin I, which itself is associated with the actin cytoskeleton (Trifaró and Vitale, 1993; Burgoyn and Morgan, 1995; Calakos and Scheller, 1996).

The life cycles of synaptic vesicles and chromaffin granules are shown in Figure 1.2.2. Neuronal exocytosis occurs through a pathway consisting of multiple functionally definable stages including secretory vesicle recruitment, priming, and docking, followed by Ca\(^{2+}\)-triggered membrane fusion (Burgoyn and Morgan, 1993a; Südhof, 1995). In neurons, following the action potential, a small proportion of synaptic vesicles at the active zone (neurotransmitter release site on the presynaptic plasma membrane) undergo exocytosis within 200\(\mu\)s in response to high Ca\(^{2+}\) concentrations at the mouths of presynaptic Ca\(^{2+}\) channels where synaptic vesicles are tethered (Burgoyn and Morgan, 1995; Calakos and Scheller, 1996). Influx of Ca\(^{2+}\) through voltage-operated Ca\(^{2+}\) channels (VOCCs) is the classical mechanism of initiating transmitter release. There are multiple types of Ca\(^{2+}\) channels (L-, T-, N-, Q-, P-, and R-types). However, it is now widely accepted that N- and P/Q-type Ca\(^{2+}\) channels are the predominant species in presynaptic nerve terminals and these channels couple to proteins that form the release machinery for synaptic vesicles (Burgoyn, 1991; Artalejo et al., 1994). Mobilization of synaptic vesicles from a reserve pool of synapsin I-linked vesicles allows replenishment of those lost from the active zone as a result of rapid exocytosis. Ca\(^{2+}\) influx leads to activation of calmodulin-dependent protein kinase II (present in synaptic vesicles) which phosphorylates synapsin I leading to the
liberation of synaptic vesicles from the cytoskeletal constraint allowing them to move to and dock at the active zone (Ryan, 1999). In contrast, in chromaffin cells, the initial stage is recruitment of granules to the subplasmalemmal area, which follows disassembly of a cortical actin barrier (Cheek and Burgoyne, 1986). This is an ATP- and Ca\(^{2+}\)-dependent step, although it can also be activated by alternative Ca\(^{2+}\)-independent pathways (Burgoyne and Morgan, 1998a). The protein(s) which Ca\(^{2+}\) acts on to trigger cytoskeletal disassembly are unclear but there is evidence that scinderin, a Ca\(^{2+}\)-dependent actin-severing protein is involved in this process in chromaffin cells (Trifaro and Vitale, 1993; Roth and Burgoyne, 1995). Thus, it is proposed that Ca\(^{2+}\) acts at least two distinct stages in exocytosis: an early stage of actin rearrangement and a late stage of membrane fusion (Morgan and Burgoyne, 1997). This mobilisation step is followed by vesicle docking, which consists of the formation of protein complexes linking the presynaptic plasma membrane with the vesicle membrane. This complex set of interactions is thought to complete the docking of synaptic vesicles and primes them within the active zone (Lin and Scheller, 2000). Fusion is then triggered by influx of Ca\(^{2+}\) through presynaptic voltage-gated Ca\(^{2+}\) channels following membrane depolarization. Finally, the synaptic vesicle membrane and protein components are retrieved via endocytosis and are recycled for additional rounds of release. Clathrin-coated budding is implicated in the endocytotic process (Heuser, 1989; Palfrey and Artalejo, 1998). The binding of clathrin to the membrane is enhanced by certain adaptor proteins. Synaptotagmin serves as a receptor for the clathrin adaptor protein AP-2 (Zhang et al., 1994). The clathrin coat forms a regular lattice around the pit, which finally pinches off as a small coated vesicle. In addition, the pinching off of the vesicle depends on a
cytoplasmic GTPase, dynamin, which forms a constricting helical ring around the neck of the vesicle during endocytosis. Endocytosed vesicles may then proceed through recycling endosomes or may shed their coat of clathrin and directly re-enter the reserve or releasable pool of vesicles (Murthy and Stevens, 1998).

Exocytosis in neurons and neuroendocrine cells has now been shown to occur through two distinct modes (Burgoyne et al., 2001; Artalejo et al., 1998). In addition to the classical model of exocytosis that involves full incorporation of vesicles into the plasma membrane (‘full fusion’), followed by clathrin-mediated endocytosis, exocytosis can occur by a mechanism in which the vesicle rapidly pinches off without full integration into the plasma membrane. Retrieved vesicles are rapidly refilled with cargo or those that have not dispensed their full cargo are immediately available for further exocytosis (Valtorta et al., 2001). This so called ‘kiss-and-run’ mode of exocytosis is generally favoured by strong stimulation and high Ca\(^{2+}\) conditions and provides a mechanism for very fast vesicle recycling in response to intense signalling (Ales et al., 1999; Stevens and Williams, 2000). In the kiss-and-run mode of exocytosis, complete integration of vesicle and plasma membranes rarely occur (Sim et al., 2003). For example, in PC12 cells, secretory granules are recycled virtually intact after exocytosis (Taraska et al., 2003). In chromaffin cells, high Ca\(^{2+}\) levels are required to switch the mode to kiss-and-run (Ales et al., 1999). Recently, three forms of kiss-and-run have been described: ‘pure’, which releases only very small molecules, ‘mixed’ where the fusion pore size is greater to allow exocytosis of larger molecules, and ‘full’, where the release of all vesicle cargo is permitted (Tsuboi and Rutter, 2003; Sim et al., 2003).
Some of the most striking advances in neurobiology over the past decade have been the identification of proteins responsible for neurotransmitter release. Therefore, the next part of this section will discuss briefly these proteins that participate in vesicle targeting and docking.
Figure 1.2.2. The stages of exocytosis in neurons and neuroendocrine cells. Secretory granules or synaptic vesicles are excluded from the cell periphery by an actin cytoskeleton network. Disassembly of the actin barrier allows granules or vesicles to approach the plasma membrane and at one or more stages of exocytosis (before, during, or after), the exocytotic machinery undergoes ATP-dependent priming. In this process, granules or vesicles become attached to the plasma membrane and may be initially tethered before becoming tightly docked via the exocytotic machinery. Finally, membrane fusion is triggered by Ca$^{2+}$ in an ATP-independent stage. Adapted from Burgoyne and Morgan, 1998a and Schiavo and Stenbeck, 1997.
1.2.3 Proteins that are involved in exocytosis

The exocytotic process is regulated by a number of proteins such as those in SNARE complex. The most well known among the proteins involved in exocytosis are the SNARE (soluble n-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) proteins (Figure 1.2.3). There are two distinct categories of SNARE proteins that have been identified. SNAREs present on the vesicle (or donor) compartment are known as v-SNAREs (synaptic vesicle associated SNARE) while those on the target (or acceptor) compartment are known as t-SNAREs (target-membrane associated SNARE). The SNARE hypothesis was proposed in 1993, before most of the current knowledge became available, as the first working model to explain vesicle docking and fusion in molecular terms (Söllner et al., 1993). It was postulated that association of the v-SNARE, VAMP (vesicle-associated membrane protein, also known as synaptobrevin) with the t-SNAREs, syntaxin and SNAP-25 (synaptosomal-associated protein 25kDa) leads to the formation of a core complex needed for membrane fusion and the release of the vesicle's contents. This ‘core complex’ is very stable which is reflected in its resistance to SDS denaturation, protease digestion, clostridial neurotoxin cleavage and temperature up to ~90°C (Leenders and Sheng, 2005). In vivo its disassembly is carried out by two proteins, the ATPase, NSF (n-ethylmaleimide-sensitive fusion protein) and an adaptor protein, α-SNAP (soluble NSF attachment protein), which act as molecular chaperones to prepare the SNARE proteins for subsequent events in docking or fusion or both as they allow recycling of SNARE components (Bock and Scheller, 1997; Chen and Scheller, 2001; Leenders and Sheng, 2005). There is no doubt that the proteins underlying the SNARE hypothesis are key players in steps leading to vesicle
docking and fusion. In addition, an essential point of the SNARE hypothesis was the role of v- and t-SNARE pairing in determining the specificity of targeting (Burgoyne and Morgan, 1998a). Synaptotagmin serves importantly as a Ca\(^{2+}\) sensor through Ca\(^{2+}\)-dependent interactions with both SNAP-25 and syntaxin. Synaptotagmin possesses two C2 domains that are homologous to the Ca\(^{2+}\)-binding domain of protein kinase C (PKC). The first C2 domain (C2A) binds syntaxin in a Ca\(^{2+}\)-dependent fashion. Furthermore, synaptotagmin has been reported to have a Ca\(^{2+}\)-dependent interaction with SNAP-25 (Schiavo et al., 1997). The role of synaptotagmin in synaptic vesicle docking and fusion is most likely based on such interactions. An influx of Ca\(^{2+}\) into the cytosol drives these interactions, which subsequently cause conformational changes and allows both core complex formation and membrane fusion to progress (Jan and Stevens, 2000). Although there are 13 synaptotagmin isoforms, not all of them are able to bind Ca\(^{2+}\) (Burgoyne and Morgan, 2003). Only some (synaptotagmin I, II, III, V, VII) bind Ca\(^{2+}\) (Li et al., 1995). Currently there is little information regarding the role of synaptotagmin in chromaffin cell exocytosis, where the only isoform definitely reported is synaptotagmin I whose Ca\(^{2+}\) affinity is poorly matched to the concentrations of Ca\(^{2+}\) likely to mediate granule exocytosis (Morgan and Burgoyne, 1997).

Over the past decade, significant progress has been made in identifying other proteins involved in the modulation and regulation of vesicular release and the nature of the protein-protein interactions that underlie these events (Chen and Scheller, 2001; Li and Chin, 2003; Murthy and De Camilli, 2003). For instance, rabphilin3A, DOC2 (double C2 domain), Munc13 (mammalian uncoordinated mutant 13), and RIM (rab3 interacting molecule), all of which may also serve as
Ca\textsuperscript{2+} sensors, like synaptotagmin contain PKC-like C2 domains and bind both Ca\textsuperscript{2+} and phospholipids. Moreover, they have been identified as important factors in synaptic vesicle priming (Augustin et al., 1999b; Verhage et al., 2000; Schoch et al., 2002). DOC2 is a soluble protein and its over-expression in PC12 cells enhances Ca\textsuperscript{2+}-dependent granule exocytosis (Morgan and Burgoyne, 1997). The mechanism underlying this enhancement is unknown, although DOC2 has been shown to interact with both Munc18 (also known as nSec1) and another C2 domain containing protein, Munc13. Rabphilin3A is soluble protein containing two tandem C2 domains that mediate binding to phospholipids (especially phosphatidylinositol 4, 5-bisphosphate). Evidence for a role of this protein in granule exocytosis comes from work on chromaffin cells based on its binding in a GTP-dependent manner to Rab3a (a low molecular GTPase) (Yamaguchi et al., 1993; Chung et al., 1995). Munc13 (four isoforms: 1, 2, 3 and 4) is the mammalian homolog of C. elegans UNC-13 and contains domains homologous to both the C1 and C2 domains of PKC (Brose et al., 1995; Duncan et al., 1999). These proteins are phorbol ester/diacylglycerol binding proteins (Betz et al., 1998). The first evidence for its role in exocytosis comes from a study in neurons (Augustin et al., 1999a). In addition, over-expression of Munc13-1 in chromaffin cells increases the magnitude of both the exocytotic burst and the subsequent slower phase of release, which is consistent with a role for this protein in vesicle priming (Ashery et al., 2000; Rhee et al., 2002; Junge et al., 2004).

Other Ca\textsuperscript{2+}-binding proteins that may have a role in granule exocytosis include calmodulin, annexin II, and CAPS (Ca\textsuperscript{2+}-dependent activator protein for secretion). Calmodulin also plays a late role in certain forms of vesicle trafficking (Okabe et al., 1992). Annexins are a family of EF hand (Ca\textsuperscript{2+} binding domain)-
containing proteins are able to bind phospholipids, which may promote lipid fusion (Ali et al., 1989). CAPS is another candidate that has been proposed to mediate a late post-docking stage in the exocytotic pathway specific for dense-core vesicles (Walent et al., 1992; Richmond and Broadie, 2002).

Other components of the exocytotic machinery which are not known to associate with the SNARE complex include Munc18, cysteine string (Csp), and Rab3a. Munc18 identified initially in chromaffin cells as nSec1 (Hodel et al., 1994), is well known as a presynaptic syntaxin-binding protein that is involved in neurotransmitter release by binding tightly to syntaxin and holding it in a closed conformation, thereby preventing its assembly into a SNARE complex (Dulubova et al., 1999; Verhage et al., 2000). By an unknown mechanism, Munc18 releases syntaxin in a state that is primed for its interaction with other SNARE proteins, thus permitting the formation of SNARE complexes (Leenders and Sheng, 2005).

Csp is a secretory vesicle membrane protein, which interacts with several presynaptic proteins including syntaxin and synaptotagmin (Nie et al., 1999; Evans and Morgan, 2002). Csp is essential for a Ca\(^{2+}\)-dependent step of synaptic vesicle exocytosis, downstream of Ca\(^{2+}\) entry (Ranjan et al., 1998; Dawson-Scully et al., 2000). More recently, Csp has been shown to be expressed in chromaffin cells (Chamberlain and Burgoyne, 1996) where it is associated with chromaffin granules (Chamberlain et al., 1996) suggesting a role in exocytosis. However, it is also possible that Csp is involved in endocytotic recycling after exocytosis (Südhof, 1995) since it interacts with hsc70, a protein that is required for uncoating clathrin-coated vesicles. Rab3 (four isoforms, A-D) is localized to both synaptic vesicles and chromaffin granules. Functional studies have indicated the involvement of rab3A in exocytosis of both vesicle classes (Holz et al., 1994;
Geppert et al., 1994), although its precise molecular function remains unknown (Morgan and Burgoyne, 1997).

In conclusion, the SNARE hypothesis, which proposes that vesicle targeting is based on specific interactions between vesicular (v-SNARE) and target membrane proteins (t-SNAREs) (Kretzschmar et al., 1996), facilitates an understanding of the general mechanisms of the fusion of either synaptic vesicles or secretory granules. By combining electrophysiological and imaging techniques, it has recently been possible to study in detail the events involved in vesicle fusion, mainly on the basis of studying transmitter release from neuroendocrine cells, where transmitter is packaged in LDCVs that contain an electron-dense material (Lindau and Almers, 1995).

The identities of many of the proteins that participate in membrane fusion during exocytosis and that have key roles in the regulation of the fusion machinery are well established (Lin and Scheller, 2000) and summarized above. The exocytotic process can also be positively and negatively regulated by presynaptic receptors; either G protein-coupled receptors or ligand gated ion channels (Majewski and Iannazzo, 1998). Receptor activation in chromaffin cells results either in depolarisation and opening of voltage-dependent channels or direct activation of phospholipase C with subsequent generation of the Ca$^{2+}$-mobilising signal inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$) which is in some cases accompanied by opening of store-operated Ca$^{2+}$ channels (SOCs). The details of regulation of exocytosis by G protein-coupled receptors and ligand gated ion channels will be considered later (Section 1.3.3 and 1.4.4, respectively).
Figure 1.2.3. Major proteins expressed in chromaffin cells that are involved in regulating secretory granule exocytosis. The SNARE complex comprises three proteins: VAMP/synaptobrevin, syntaxin and SNAP-25 and interaction between these proteins leads to formation of the SNARE complex, which is essential for membrane fusion. NSF and α-SNAP act as molecular chaperones to help disassemble SNARE complexes to release SNARE proteins to engage in further membrane fusion events. Munc18 binds Syntaxin; phosphorylation of Munc18 by PKC would allow it to dissociate from syntaxin so that syntaxin could take part in SNARE complex assembly. Rab effectors are involved in the initial tethering of vesicles to target membranes. Synaptotagmin is likely to function as a component of the SNARE complex and acts as a Ca^{2+}-sensitive clamp. Voltage-gated Ca^{2+} channels (VOCCs) are also associated with syntaxin and upon cell depolarisation that triggers Ca^{2+} influx via VOCCs, the dissociation of synaptotagmin occurs allowing membrane fusion to proceed in a SNARE-dependent fashion. Csp acts to stabilise VAMP, allowing it to interact with plasma membrane-associated syntaxin and SNAP-25 to form an active SNARE complex for membrane fusion. This picture is modified from Burgoyne and Morgan (2003).
1.3 G PROTEIN-COUPLED RECEPTORS (GPCRs)

1.3.1 The superfamily of GPCRs

GPCRs constitute the largest family of cell surface molecules involved in signal transmission. There are more than 800 GPCR genes in the human genome (Fredriksson et al., 2003) and these receptors play key roles not only in many physiological processes, acting as receptors for light, odorants, hormones and neurotransmitters but also in pathological conditions including cardiovascular disease, cancer and irregularities in body weight homeostasis. In addition, these receptors constitute one of the principal targets of drugs, especially in the CNS (reviewed in Tsao and Von Zastrow, 2001; Hermans, 2003).

GPCRs can be classified into five families, namely glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin forming the GRAFS classification system, which is based on the initials of the family names. Several classification systems have been used, some systems group the receptors by how their ligand binds and other are based on structural features. The most commonly used systems uses clans (or classes) A, B, C, D, E, and F. This A-F system is designed to include all GPCRs in both vertebrates and invertebrates and clans D and E do not exist in human. Class A, B and C correspond to the rhodopsin, secretin and glutamate families respectively. The rhodopsin family constitutes the largest family and forms four main groups (α, β, γ and δ) with 13 sub-branches. The α-group of rhodopsin has five branches such as the prostaglandin receptors, amine receptors, opsin receptors, melatonin receptors, and MECA receptors (this includes melanocortin receptors, endothelial differentiation G-protein coupled receptors, cannabinoid receptors and adenosin binding receptors). The β-group includes 36 receptors such as tachykinin receptors, cholecystokinin receptors, neuropeptide Y.
receptors, endothelin-related receptors, neuromedin B receptor, vasopressin receptors, gonadotrophin-releasing hormone receptors. The $\gamma$-group of rhodopsin receptors constitutes three main branches including SOG receptors (suboesophageal ganglion receptors), MCH receptors (melanin-concentrating hormone) and the chemochine receptors. The $\delta$-group of the rhodopsin family has four main branches: the MAS-related receptors (a large family of GqPCRs called MAS-related genes (Mrgs) also known as sensory neuron-specific receptors or dorsal root receptors); glycoprotein receptors; purine receptors; and olfactory receptors. The secretin/glucagon receptor family contains the receptors for distinct hormones and peptides (e.g., calcitonin, glucagons, parathyroid hormone, secretin, vasoactive intestinal polypeptide, growth hormone-releasing hormone (GHRH), pituitary adenylate cyclase-activating polypeptide (PACAP) and diuretic hormone. The glutamate receptor family includes receptors for glutamate receptors and the calcium-sensing receptor.

1.3.2 Structural features of GPCRs

GPCRs are referred to as seven-transmembrane domain (7TM) receptors based on their highly conserved backbone structure (Muller, 2000). Thus, all members of this family are composed of a single peptide containing seven hydrophobic regions of similar length separated by hydrophilic loops of variable sizes. In other words, these receptors are characterised by the presence of seven $\alpha$-helices crossing the plasma membrane separated by intracellular and extracellular loops. The $\mathbf{NH}_2$ terminus is exposed to the extracellular environment and the $\mathbf{COOH}$ terminus is intracellular. The transmembrane domains of these receptors show most homology but there is much less homology between the N- and C-termini of different receptors. This diversity plays a role in allowing activation by
different ligands, coupling to different G-proteins and regulation by different intracellular proteins. For example, rhodopsin family receptors display short amino-terminal tails and have highly conserved amino acid residues within each transmembrane helix. The ligands for most of the rhodopsin receptors bind within a cavity between the TM regions (Baldwin, 1994) with exceptions that include the glycoprotein binding receptors where the ligand-binding domain is within the N-terminus. Receptors of the secretin family have an N-terminus between ~60 and 80 amino acids long containing a conserved cysteine bridge that is particularly important for binding of the ligand to these receptors. Receptors subtypes belonging to the metabotropic glutamate receptor family also have a relatively long N-terminus (250-580 residues). The N-terminus is thought to form two distinct lobes separated by a cavity in which glutamate binds (a “venus fly trap”) and causes the lobes to close around the ligand. However, the calcium-sensing receptor, which also belongs to family C also has a long cysteine-rich N-terminus but it is uncertain if it is involved in the binding of Ca\(^{2+}\) although it is crucial for mediating Ca\(^{2+}\) signalling. The N-terminal of the GABA receptors is long and contains the ligand-binding site but lacks the cysteine-rich domain found in other receptors of this family (Fredriksson et al., 2003).

The extracellular domains of GPCRs (including both the N-terminus and the extracellular loops) and the pocket formed by the assembly of the seven-transmembrane helices form the ligand recognition sites for many small molecules. In contrast, peptides often bind to the N-terminal but may well make connections within the TM helices. The intracellular receptor surfaces of GPCRs, including the intracellular loops and the C-terminal domain are important for G-protein recognition and activation although the precise sites vary amongst
receptors (reviewed in Ulloa-Aguirre et al., 1999; Wess, 1998; Gether, 2000; Klabunde and Hessler, 2002).

1.3.3 G protein coupled receptor (GPCR) signalling

Upon activation, GPCRs associate with distinct classes of heterotrimeric G proteins comprised of three subunits: the α subunit that has the guanine-nucleotide binding site and GTPase activity and the βγ complex (Neer, 1995; Surya et al., 1998). Up to now, at least 23 α-subunits have been identified and are classified into four families (Go/0, Go, Go/11, and Go/12). At least 6 and 12 different molecular species of β- and γ-subunits have been described, respectively (Hur and Kim, 2002; Vanderbeld and Kelly, 2000).

In the classical model of GPCR signalling, stimulation of a GPCR leads to activation of heterotrimeric G-proteins, which promote the exchange of a molecule of GDP for a molecule of GTP within the active site of the α-subunit. This is then followed by dissociation of Goα-GTP and Gβγ (Gilman, 1987). Both Goα-GTP and Gβγ can regulate ion channels indirectly via second messengers and protein kinases or directly via physical interactions between G-protein subunits and the channel protein (Wickman and Clapham, 1995). Alternatively, these subunits activate effector molecules which are enzymes that generate second messengers (Hur and Kim, 2002). Two families of ion channels have been suggested to be directly regulated by G proteins: voltage-activated Ca2+ channels which are inhibited by Gβγ (Ikeda and Dunlap, 1999) and G-protein-activated inwardly rectifying K+ channels which are activated by Gβγ (Catterall, 2000) and Go (Wickman and Clapham, 1995). The activated subunits affect their target molecules until the intrinsic GTPase activity of Go hydrolyses the GTP to inactivate the Go and cause it to re-associate with Gβγ thus completing the cycle.
Among the G\(\alpha\) protein families, this project is particularly interested in those of the G\(\alpha_{q/11}\) family and their roles in regulation of exocytosis since very little is known about the mechanisms underlying facilitation of stimulus-secretion coupling by receptors coupled to G\(\alpha_{q/11}\). Activation of G\(\alpha_{q/11}\)-coupled receptors results in generation of important second messengers including inositol 1,4,5-trisphosphate (Ins(1,4,5)P\(_3\)), which mobilizes intracellular Ca\(^{2+}\), and diacylglycerol that alone or in combination with Ca\(^{2+}\) can activate Protein kinase C (PKC) (Figure 1.3.3). The receptor-dependent generation of Ins(1,4,5)P\(_3\) and DAG is dependent on the activation of PLC\(\beta\) mediated by the G\(q/11\) class of \(\alpha\) subunits and the hydrolysis of a plasma membrane lipid, phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P\(_2\)). Ins(1,4,5)P\(_3\) activates the endoplasmic reticulum (ER) IP\(_3\) receptor (IP\(_3\)R) to release Ca\(^{2+}\) and this is often followed by activation of Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels (I\(_{\text{crac}}\)) or SOCs in the plasma membrane to promote Ca\(^{2+}\) entry and allow refilling of Ca\(^{2+}\) stores (Kiselyov \textit{et al.}, 2002). Ca\(^{2+}\) and PKC have been reported to play a crucial role in regulation of exocytosis (refer to Section 1.5.6 for details).
Figure 1.3.3. Activation of the Gαq/11-mediated signal transduction pathway. After ligand-induced receptor activation, the activated (GTP-bound) Ga- subunit of the heterotrimer dissociates from the Gβγ complex and stimulates the effector enzyme phospholipase Cβ (PLCβ) which in turn causes PtdIns(4,5)P2 hydrolysis leading to formation of the second messengers Ins(1,4,5)P3 which diffuses through the cytoplasm and releases Ca^{2+} from the endoplasmic reticulum, and DAG, which alone or in combination with Ca^{2+} activates PKC resulting in a cascade of protein phosphorylation (Ulloa-Aguirre et al., 1999). Interestingly DAG may also activate Munc-13 directly which may be involved in mediating synaptic vesicle priming and fusion (Ashery et al., 2000). This picture is modified from Ulloa-Aguirre et al. (1999).
1.3.4 Regulation of GPCR function

The functional status of GPCRs is determined predominantly by ligand binding. The exposure of GPCRs to agonists often results in a rapid attenuation of receptor responsiveness. This process is known as desensitisation, which is the consequence of a combination of different mechanisms. These mechanisms include the uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation (Lohse et al., 1990), the internalisation (endocytosis) of cell surface receptors to intracellular membranous compartments (Hermans et al., 1997; Anborgh et al., 2000) and the down-regulation of the total cellular complement of receptors due to reduced receptor mRNA and protein synthesis as well as both the lysosomal and plasma membrane degradation of pre-existing receptors (Pak et al., 1999). The time frames over which these processes occur range from seconds (phosphorylation) to minutes (endocytosis) and hours (down-regulation). Phosphorylation of GPCRs occurs rapidly and involves phosphorylation of residues located in the intracellular loops and/or the COOH-terminal tail of the stimulated receptors by second messenger-dependent activated kinases (PKA or PKC) and/or by special class of serine/threonine-specific kinases called G protein-coupled receptor kinases (GRK1-7) (Freedman and Lefkowitz, 1996; Krupnick and Benovic, 1998; Hermans, 2003; Kristiansen, 2004). Moreover, β-arrestin which belongs to a group of soluble inhibitory proteins binds to phosphorylated receptors leading to uncoupling from their respective G proteins. The binding of β-arrestin also targets some receptors to clathrin-coated pits from where they are internalised via endocytosis (Barak et al., 1997). The β-arrestin mediates this process by interacting not only with agonist-occupied, phosphorylated receptor but also with a component of the clathrin-coated pit
machinery such as the heavy chain of clathrin itself and the β2-adaptin subunit of the clathrin adaptor protein AP-2. Interaction of β-arrestin with clathrin and AP-2, as well as phosphoinositides targets the GPCRs to punctate clathrin-coated pits at the cell surface. These pits are then pinched off the cell surface by the actions of dynamin, a GTPase. Following internalisation, receptors can be recycled to the plasma membrane or targeted for degradation in lysosomes. Internalisation can also occur in a β-arrestin-independent manner by, for example, association with caveolae. Caveolae are flask-shaped membrane invaginations that are rich in caveolin proteins as well as cholesterol. However, the mechanisms by which GPCRs targeted to caveolae are not well understood (reviewed in Pierce et al., 2002). Receptor internalisation removes receptors from the cell surface whilst proteolytic degradation results in permanent loss of receptors and represents the predominant mechanism of down-regulation (Tsao and Von Zastrow, 2001). The pathway that mediates proteolytic down-regulation of GPCRs involves endocytosis followed by trafficking to lysosomes. Additional proteolytic machinery such as proteasomes or cell-associated endoproteases are also implicated in mediating down-regulation of certain GPCRs (Tsao and Von Zastrow, 2001).

The signalling by GPCRs is also subject to both acute and chronic regulation at sites other than the receptor, particularly by the regulation of the activity of downstream signalling molecules. These mechanisms are diverse and include, for example, the inactivation of Gα-subunits by regulators of G protein signalling (RGS proteins) which inhibit Gα by accelerating the rate of intrinsic GTPase activity of the subunits or alternatively by acting as effector antagonists
or guanine nucleotide dissociation inhibitors (Dohlman and Thorner, 1997; Hepler, 1999; Hollinger and Hepler, 2002; Hermans, 2003).

1.4 NICOTINIC ACETYLCHOLINE RECEPTORS (nAChRs)

1.4.1 nAChR subunits and subtypes

The nAChRs are pentameric ligand-gated ion channels (LGICs) and members of the superfamily of neurotransmitter receptors that includes gamma aminobutyric acid (GABA_A and GABA_C receptors), glycine and 5-hydroxytryptamine (5-HT_3) receptors (Stroud et al., 1990; Gotti et al., 1997). These receptors are known as Cys-loop receptors as all of them have a conserved sequence containing a pair of cysteines separated by 13 residues and linked by a disulfide bridge. nAChRs can be divided into two groups: muscle receptors which are found at the skeletal neuromuscular junction where they mediate neuromuscular transmission and neuronal receptors which are found throughout the peripheral and central nervous systems (Hogg and Bertrand, 2003).

To date, 17 nicotinic subunits have been cloned in vertebrates: α1, β1, γ, ε, and δ expressed in muscle tissue where they mediate transmission at the neuromuscular junction and twelve distinct “neuronal” α2-α10 and β2-β4 subunits widely expressed in the nervous system (McGehee and Role, 1995; Fucile, 2004). These subunits may constitute heteromeric or homomeric receptors. Neuronal subunits are also found in an increasing number of non-neuronal cells such as glial cells, endothelial cells, keratinocytes, macrophages, lymphocytes, ect (Sharma and Vijayaraghavan, 2002; Wang et al., 2003). In addition, although early studies indicated that human small-cell lung carcinoma (SCLC) cells, which have a ‘neuroendocrine’ phenotype, express only muscarinic cholinergic receptors (Cunningham et al., 1985), more recent studies have reported nicotinic receptor
expression (Codignola et al., 1994). Most interestingly, SCLC cells express nicotinic receptors of the neuronal type. In particular, SCLC cells express α3 and β4 nicotinic receptor subunits. In addition, SCLC cells express α7 nicotinic subunit and consistent with this both α-bungarotoxin and α-conotoxin block the secretagogue and mitogenic effects of nicotine. Moreover, other neuronal nicotinic receptor subunits have also been found in SCLC cells, such as α5 and β2 subunits (Sher et al., 2000). The distinct combinations of nAChR subunits produce receptors with different physiological properties and both α and β subunits seem to contribute to the functional diversity of the nAChRs as their molecular diversity arises from assembly of homomeric and heteromeric pentamers (Dajas-Bailader, 2002a).

Two different classes of neuronal nAChRs may be identified according to their Ca$^{2+}$ permeability: (i) neuronal nAChRs containing subunits (α7-α9) able to bind the snake venom α-bungarotoxin (α-BTX) and to form homopentameric channels (α-BTX nAChRs) exhibiting highest Ca$^{2+}$ permeability (Jagger et al., 2000; Fucile et al., 2003). (ii) heteropentameric non-α-BTX-sensitive nAChRs (non-α-BTX nAChRs) comprising at least one α (out of α2-α6) and one β (out of β2-β4) subunits which exhibit low Ca$^{2+}$ permeability (Haghighi and Cooper, 2000; Lax et al., 2002). The major brain subtype with high affinity for nicotine seems to be the α4β2 nAChR which is α-Bgt-insensitive (Flores et al., 1992). The major ganglionic subtype is α3β4 and the major subtype with high affinity for α-Bgt found in brain and ganglia is composed of homomeric arrangements of α7 (Dominguez Del Toro et al., 1994). In addition, assembling of α5 subunit either with α3β2 or α3β4 subunits yields nAChRs with high Ca$^{2+}$ permeability.
(Gerzanich et al., 1998). The α-Bgt-sensitive nAChRs, especially the α7-containing subtype, have been the target of intense investigation as they show high Ca\(^{2+}\) permeability (Vijayaraghavan et al., 1992; Séguéla et al., 1993; Rathouz et al., 1996). Pieces of evidence indicate a functional correlation between the activation of α7 nAChR and Ca\(^{2+}\)-dependent cellular processes such as neurotransmitter release, synaptic plasticity, cell growth, migration and survival (MacDermott, 1999; Belluardo et al., 2000). Some studies have suggested an association of the α7 subunit with important neuropathologies such as Alzheimer’s disease, schizophrenia and epilepsy (Leonard et al., 2001). Ca\(^{2+}\) entering the neuron through α7 channels could act as a second messenger in several cellular processes including the regulation of neurite outgrowth, neurotransmitter release and synaptic plasticity (Torrão and Britto, 2002; Fucile, 2004).

1.4.2 Structure of the nAChR

Individual nAChR subunits consist of a number of distinct functional domains (Figure 1.4.2). The large extracellular N-terminal that participates in the formation of the ligand binding domain contains putative glycosylation sites, a disulphide-linked cysteine loop between residues homologous to 128 and 142 of the α1 subunit and the interface for agonist binding (loops A, B and C in α subunits and loops D, E and F in all subunits) (Figure 1.4.2, panel B) (Corringer et al., 2000). The polypeptide chain of nAChR subunits contain four hydrophobic, putative transmembrane domains (M1-M4) (Sargent, 1993; Elgoythen et al., 1994) (Figure 1.4.2, panel A). Evidence indicates that M2 is α-helical and lines the cation pore. M3 and M4 are separated by a large variable intracellular loop which contains putative phosphorylation sites for ser/thr kinases (Hogg et al.,
2003). As mentioned above, the twelve subunits have been classified into two subfamilies of eight α and three β subunits. The α subunits have two adjacent cysteines that are homologous to those present at positions 192 and 193 of the α subunits of the muscle-type nAChR. In contrast, the β subunits (β2-β4) lack the pair of adjacent cysteines and are considered structural subunits, although it has been clearly demonstrated that both α and β subunits contribute towards the pharmacological specificity of nAChR subtypes (Luetje and Patrick, 1991). Since nAChRs result from the assembly of five subunits, they constitute an excellent prototype of allosteric proteins in which the ligand binding is distinct from the "reaction site" that is, the ionic pore. Allosteric proteins have the particularity to undergo spontaneous transitions between different conformations (Buisson and Bertrand, 1998). Upon agonist binding, nAChRs undergo an allosteric transition from the closed, resting conformation to an open state which conducts the cations Na⁺, K⁺ and Ca²⁺. In the active (open) conformation, the nAChR binds agonists with low affinity. The continued presence of agonist leads to ion channel closure and receptor desensitisation. In this condition, the nAChR is refractory to activation although it displays higher affinity for agonist binding. The rates of desensitisation and recovery differ between nAChR subtypes: for example, the α7nAChR displays very rapid desensitisation (Couturier et al., 1990a; McGehee and Role, 1995; Leonard and Bertrand, 2001). Prolonged agonist exposure may produce an inactivated state, from which recovery is very slow. The α4β2 neuronal nAChR is prone to inactivation on chronic nicotine treatment (Kuryatov et al., 2000). However, receptors composed of α3β4 or α3β4α5 display a slower time course of desensitisation than the α7 receptor (Couturier et al., 1990b; Role, 1992). Transitions between resting, open and desensitised states are reversible and
different ligands may stabilise different receptor states: agonists initially stabilise the activate (open) state whereas competitive antagonists preferentially stabilise the nAChR in a closed state, either the resting or desensitised configuration (Buisson and Bertrand, 1998).
Figure 1.4.2. Schematic representation of neuronal nicotinic acetylcholine receptor structure. Panel A: shows a structure of a neuronal nAChR subunit. Panel B: structure overview of an nAChR and agonist binding site including the model of the agonist binding site loop (modified from Leonard and Bertrand, 2001).
1.4.3 Regulation of nAChRs

Phosphorylation is an important mechanism in the regulation of ligand-gated ion channels. This regulation includes modulation of receptor expression, subcellular localization and channel properties such as desensitisation and recovery from inactivation (Hogg and Bertrand, 2003). Both serine and tyrosine residues of the nAChR can be phosphorylated (Huganir, 1991) and this regulates receptor desensitisation (Mulle et al., 1988). Investigations in cells from chick ciliary ganglia showed that α3, α4, α5, α7, β2, β3 and β4 subunits are phosphorylated by both PKA and PKC. Consensus sequences for PKA and PKC phosphorylation sites have been identified on the major intracellular loop between the M3 and M4 transmembrane segments of the rat, chick and human α7 and α4 subunits and two isoforms of the human α1 subunit. At each of these putative phosphorylation sites the phosphorylated residue is serine (Wecker et al., 2001).

The functional down-regulation of α4β2 nAChRs in permanently transfected HEK 293 cells in response to chronic nicotine exposure appears to be mediated by down-regulation of PKC activity (Eilers et al., 1997). Furthermore, recovery from down-regulation is accelerated by using inhibitors of protein phosphatases 2A and 2B suggesting that nicotine-induced down-regulation of nAChRs involves dephosphorylation at PKC phosphorylation sites. Although, phosphorylation of α7 nAChRs in vivo remain to be investigated, the intracellular domain of neuronal nAChRs is the most divergent region of the molecule. However, the phosphorylation site is highly conserved suggesting that phosphorylation may be important in modulating α7 nAChR function (Hogg and Bertrand, 2003).
1.4.4 Function of nAChR

Neuronal nAChRs are gaining credence as significant players in the nervous system, particularly as a consequence of their relationship to a number of disease states in which they are perceived as novel drug targets (Decker et al., 1998). These include Alzheimer’s and Parkinson’s diseases, schizophrenia, Tourette’s syndrome and attention deficit hyperactivity disorder. In addition, neuronal nAChRs are also targets for analgesia, anxiolysis, neuroprotection and smoking cessation (Hogg et al., 2003). A large body of evidence indicates that neuronal nAChRs are present presynaptically in the central nervous system where they facilitate neurotransmitter release, most likely through an increase in the intracellular [Ca$^{2+}$], due to a substantial influx of Ca$^{2+}$ through their channels. In addition to promoting exocytosis by opening of VOCCs through membrane depolarisation, nAChRs can also initiate exocytosis directly by virtue of their intrinsic Ca$^{2+}$ permeability. Indeed, nAChRs have a pivotal role in the regulation of neurotransmitter release as presynaptic nAChRs facilitate the Ca$^{2+}$-dependent release of many neurotransmitter, which is consistent with activation of exocytotic mechanisms (Wonnacott, 1997). In addition to provoking Ca$^{2+}$-dependent exocytosis, presynaptic nAChRs also modulate transmitter release through Ca$^{2+}$-mediated cellular mechanisms for example, PKC which may have a role in the facilitation of catecholamine release (see Section 1.5.6) and is reported to contribute to the regulation of exocytosis in adrenomedullary cells (Soliakov and Wonnacott, 2001; Dajas-Bailador and Wonnacott, 2004).

The chromaffin cell of the adrenal medulla, which receives a major cholinergic input from the splanchnic nerve to release catecholamines into the bloodstream possesses neuronal nAChRs that comprise heterologous assemblies
of α3, α5, and β4 subunits (Criado et al., 1992; Campos-Caro et al., 1997) as well as a distinct homomeric α7 receptor inhibited by α-bungarotoxin (Garcia-Guzman et al., 1994). More recently, by using a new protocol (i.e., consisting of short pulses of stimulation) and novel pharmacological tools (i.e., selective toxins to block nAChRs) it has been suggested that both α7 and α3β4 nAChR are involved in the generation of inward nAChR currents as well as in the triggering of Ca\(^{2+}\) entry and catecholamine release in response to stimulation of bovine chromaffin cells (López et al., 1998; Maneu et al., 2002).

1.5 PROTEIN KINASE C (PKC)

1.5.1 Protein Kinase C isoforms

The discovery of protein kinase C (PKC) in 1977 by Nishizuka and co-workers represented a major breakthrough in the signal transduction field (Takai et al., 1977). PKC is regarded as a key triggering step in numerous cellular processes, from the regulation of gene transcription, mitogenesis, cell proliferation, apoptosis, remodelling of the actin cytoskeleton, to the modulation of ion channels and stimulus-secretion coupling in hormone and neurotransmitter release (Newton, 1995; Toker, 1998).

PKC represents a family of at least 12 serine / threonine kinases that participate in signal transduction in response to specific hormonal, neuronal and growth factor stimuli. Differences in their structures have permitted classification of mammalian PKC isoforms into three groups (Figure 1.5.1): the first group is the conventional PKCs (cPKCs) which includes α, βI, βII, and γ isoforms. This class is considered to be Ca\(^{2+}\)- dependent and activated by the second messenger DAG in the presence of phosphatidylserine (PS). The second group is the novel PKCs (nPKCs) which comprise the δ, ε, η, μ, and θ isoforms. Members of this
group are Ca\(^{2+}\)-independent and regulated by DAG and PS (Way et al., 2000). The third group contains the atypical PKCs (aPKCs), namely \(\xi\) and \(\iota/\lambda\). PKC\(\lambda\) is a mouse homologue of the human \(\iota\) isoenzyme. These protein kinases are Ca\(^{2+}\)-insensitive and do not respond to the tumor-promoting phorbol esters, which are pharmacological agents that are extensively used to mimic the action of DAG (Mellor and Parker, 1998). Finally, the recently discovered PKC-related kinases (PRKs) represent a fourth group consisting of at least three members, PRKs 1-3. These are similar to aPKCs in being Ca\(^{2+}\)-, DAG-, and phorbol ester-insensitive (Palmer et al., 1995). The PKC isoforms differ in their structure, cofactor requirements, substrate specificity and tissue expression with specific subcellular distribution (Mochly-Rosen and Gordon, 1998). Moreover, the presence of more than one PKC isoform in a single cell type has led to the notion that each member of the PKC family plays a specific role in the processing of physiological and pathological responses to extracellular stimuli (Sena et al., 2001). Today, based on biochemical and structural differences between the isoforms, selective PKC inhibitors and activators have been developed allowing the dissection of the different signalling pathways in which the members of this family participate (Casabona, 1997). Because of the importance of PKC isoforms in major cellular functions, they are considered as potential targets for therapeutic intervention. It is widely accepted that inhibitors of PKC isoforms may be useful in treating autoimmune diseases including rheumatoid arthritis, diabetes mellitus, multiple sclerosis, Alzheimer's disease or cardiovascular diseases (Gescher, 1992; Basu, 1993; Hofmann, 1997).
Figure 1.5.1. Schematic of primary structures of PKC family members indicating the domain composition. The PKC structure can be divided into an N-terminal (N) regulatory domain and a C-terminal (C) catalytic domain. The cPKC structure comprises four conserved regions (C1-C4) and five variable regions (V1-V5). The N-terminal moiety contains the regulatory modules: the pseudosubstrate; the C1A and C1B domains (not present in the atypical PKCs) binds DAG and phorbol esters; the C2 domain for conventional PKCs binds Ca$^{2+}$. The C-terminal moiety contains ATP and substrate binding sites. The figure is a modification of a figure from Nishizuka (1992).
PKCs can be considered as ‘classical’ transducers of signal from many extracellular agonists. The initial discovery of the enzyme as a Ca$^{2+}$-activated enzyme, was immediately followed by the realization that PKC was the major target of the lipid metabolite DAG (Inoue et al., 1977; Kishimoto et al., 1980). In addition, the observation that PKC is also the major receptor for tumor-promoting phorbol esters provided a key reagent for studying the mechanism of action of this enzyme (Castagna et al., 1982).

Although, the receptor-mediated hydrolysis of inositol phospholipids was thought to be the sole mechanism leading to the activation of PKC, recent studies suggest the existence of several other routes to provide the DAG that is needed for enzyme activation (Farago and Nishizuka, 1990; Majewski and Iannazzo, 1998). For instance, phosphatidylcholine may be hydrolysed by PLD to produce DAG (Exton, 1988). In addition, both receptor-mediated, voltage-dependent and Ca$^{2+}$ influx through non-selective cation channels may cause phospholipid degradation as it may be initiated by the activation of PLC and PLD and phospholipase A2 due to Ca$^{2+}$ influx (Farago and Nishizuka, 1990).

1.5.2 Tissue distribution of PKC isoforms

PKC isoforms are widely distributed in mammalian tissues and some isoforms are localized to specific tissues. PKCα, βI, βII, δ, ε and ζ seem to be ubiquitous isoforms and are found in most or all tissues. PKCγ is largely restricted to the central nervous system (Nishizuka, 1988; Hug and Sarre, 1993). PKCη is strongly expressed in skin and lung but only slightly in brain. PKCθ is predominantly present in skeletal muscle and to a lower extent in lung, spleen, skin and brain (Osada et al., 1992). PKCμ has been found in numerous tissues and is strongly expressed in thymus and lung. Furthermore, the distribution of these
isoforms among cell types has been studied extensively for neural tissues. Such studies reveal that the conventional isoforms are present in axon terminals at the endings of the sensory nerves and in post-synaptic dendrites. In contrast, PKC$\delta$ predominates in neuronal cell bodies and PKC$\varepsilon$ is concentrated in presynaptic terminals within the central nervous system. As most tissues are made up of several different cell types, it is also a challenge to learn what isoforms are present in specific cell types (Liu and Heckman, 1998). Moreover, within a single cell, PKC isoforms exhibit differences in their distribution before and after activation (Monchly-Rosen and Gordon, 1998). For example, within non-stimulated rat cardiomyocytes PKC$\beta$II is associated with fibrillar structures and on activation translocates to both the perinuclear region and cell periphery (Disatnik et al., 1994). In these cells, PKC$\alpha$ and PKC$\zeta$ translocate from the cytosol to the perinuclear membrane while PKC$\beta$I translocates from the cytosol and perinucleus to the nucleus on activation.

Increasing evidence suggests that individual PKC isoforms can translocate to subcellular locations other than the plasma membrane including other membrane vesicles, nuclear structures, and cytoskeletal components. The subcellular location of a specific isoform may directly control the potential of that isoform to perform distinct functions because the targeting of PKCs to discrete subcellular compartments would restrict their access to potential substrates (Keenan and Kelleher, 1998). This intracellular compartmentalization could reflect the presence of intracellular PKC receptors at cytosolic and particulate level, named "receptor for activated C kinase" (RACK) that are specific for each isoform; such receptors, which bind the different PKC isoforms in the active,
"open", conformation may play an important role in their subcellular distribution and dynamics of activation (Mochly-Rosen et al., 1991a).

### 1.5.3 Structure of PKC isoforms

Members of the PKC family consist of a single polypeptide composed of an N-terminal regulatory region (approximately 20–40 kDa) and a C-terminal catalytic region (approximately 45 kDa). The regulatory region possesses the motifs that are involved in the binding of the phospholipid cofactors and Ca\(^{2+}\) and participates in protein-protein interactions that regulate PKC activity and localization. The carboxyl-terminal region is the kinase domain and includes motifs involved in ATP and substrate binding. The regulatory and catalytic domains are connected by a hinge region that is highly sensitive to proteolytic cleavage by cellular proteases (Ron and Kazanietz, 1999). Initial cloning of the first PKC group in the mid-1980s demonstrated that the polypeptide structure comprises four conserved (C1–C4) and five variable regions (V1–V5) (Coussens et al., 1986). In the NH\(_2\) terminal domain of the regulatory region, cPKCs contain two conserved sequences, C1 and C2 that are important for the regulation of the enzymatic activity. On the other hand, nPKCs and aPKCs are devoid of C2 regions. All isoforms have two conserved regions on the carboxyl terminal domain, C3 and C4 which are responsible for ATP binding and protein substrate binding respectively (Figure 1.5.1) (Azzi et al., 1992; Dekker and Parker, 1994; Hug and Sarre, 1993; Tanaka and Nishizuka, 1994).

The C1 region is present in all PKC isoforms. It is a small globular structure (approx. 8 kDa) that has a binding site for DAG (Zhang et al., 1995a; Newton, 1995). In addition, phorbol esters (non-hydrolysable analogues of the endogenous ligand) also bind to the C1 domain. Moreover, the C1 domain
specifically binds phosphatidylserine (Johnson et al., 2000). This region is also characterized by the presence of the pseudosubstrate sequence and two repeats of cysteine-rich (cys1 and cys2) zinc finger-like motifs that form a "zinc butterfly" and binds DAG and phorbol esters (Ono et al., 1989). Conventional and novel PKCs have two C1 domains but some evidence suggests that only one engages ligand in vivo. For some isoforms such as PKCδ, the C1B domain is primarily responsible for binding DAG (Szallasi et al., 1996). For others, such as PKCα, the C1A and C1B domains have equivalent roles in targeting PKC to membranes (Bogi et al., 1999). Atypical PKC and PRK isoforms are slightly different in that they have only one cysteine-rich region. As the ligand-binding pocket of the C1 domain is impaired in aPKCs and PRKs, these isoforms do not respond to either DAG or phorbol esters (Hurley et al., 1997). The function of the single zinc finger in the aPKCs is still unknown (Mellor and Parker, 1998). The beginning of the N-terminal region has a motif that resembles the consensus sequence found in the phosphorylation sites of prominent PKC substrates but possesses alanine instead of serine or threonine (House and Kemp, 1987). As a result, this motif cannot be phosphorylated and appears to be a pseudo-substrate that serves an autoregulatory function by blocking the catalytic site. This autoinhibitory domain or pseudosubstrate binds to the substrate binding site in the catalytic domain and keeps the enzyme in an inactive state in the absence of cofactors and activators (Orr et al., 1992).

Interestingly, the C1 domain is also present in several other mammalian proteins such as DAG kinase, protein kinase D (PKD), and the chimaerin family. Munc-13 also contains C1 and C2, which have been shown to interact with two synaptic vesicle proteins, namely DOC2 (double C2 domain) and syntaxin
(Mellor and Parker, 1998). Consequently, these proteins are considered other targets for DAG and phorbol esters. This lack of specificity in the stimulating action of phorbol esters raises the possibility that some of the effects attributed to PKC may in fact due to the interactions with these or other as yet not identified proteins (Kiley and Jaken, 1994).

In contrast, the C2 domain is present in conventional and novel PKCs (Nalefski and Falke, 1996; Ponting and Parker, 1996). In conventional PKCs, this 12 kDa domain serves as a membrane-targeting module that binds anionic phospholipids in a Ca\(^{2+}\)-dependent manner. The C2 domain of novel isoforms lack key residues involved in Ca\(^{2+}\) binding and as a result bind neither Ca\(^{2+}\) nor phospholipids (Newton, 2003). Like the C1 domain, the C2 domain has been found to be present in many other proteins including synaptotagmin, rabphilin-3A, phospholipases and GAPs (GTPase-activating proteins) (Ponting and Parker, 1996). Similarly, a class of proteins has been identified that interacts with the C2 domains of PKCs such as the receptors for activated PKCs (RACKs).

The C-terminal regions (C3 – V5) have been recognised in all PKC isoforms as the catalytic domain. The C3 region contains an ATP-binding sequence similar to that found in other protein kinases whereas the C4 domain, contains the sites required for the recognition of substrates (Way et al., 2000). There is evidence that the V5 domain plays a critical role in the cellular localisation. Previous studies in the human U 937 monocytic cell line have shown that PKC\(\beta\)I is localised to the microtubules while PKC\(\beta\)II is localised in part to secretory granules. The only difference between these two proteins is the V5 region (Way et al., 2000).
1.5.4 Sub-cellular targeting of PKC isoforms

Kraft et al. (1982) reported for the first time that PKC translocates from the soluble fraction of cells to the particulate fraction in response to phorbol esters. The translocation of PKC from cytoplasm to plasma membrane has become a hallmark for PKC activation (Kraft et al., 1982). The activation of PKC is triggered by a large number of extracellular signals including hormones, neurotransmitters, and growth factors that act through cell surface receptors. The activation of these receptors regulates the intracellular levels of various PKC activators including DAG, Ca\(^{2+}\), and many other lipid mediators (Feng et al., 1998). The mechanisms by which the conventional PKC isoforms become membrane-associated and thus activated have been shown to involve two sequential steps. First there is an initial Ca\(^{2+}\)- and anionic phospholipid-dependent interaction of the C2 domain with the membrane followed by binding of DAG to the C1 domains (Newton and Johnson, 1998, Medkova and Cho, 1999; Bittova et al., 2000). The interaction of DAG with the C1 domain also results in an increased specificity of both membrane-association and activation for phosphatidylserine over other anionic phospholipids (Orr and Newton, 1992; Newton and Keranen, 1994; Johnson et al., 2000). The initial interaction of the C2-domain with the membrane brings the adjacent C1 domains into close opposition with the membrane head group region. This facilitates the binding of DAG, which promotes a penetration (Newton and Johnson, 1998; Johnson et al., 2000). The combined interactions of the C1 and C2 domains with the membrane provides the free energy for structural rearrangements that lead to the dissociation of the N-terminal pseudosubstrate from the active site, which allows substrate binding. This process is further facilitated by a weak interaction of the released
pseudosubstrate with anionic head groups at the membrane surface (Mosior and McLaughlin, 1991). The regulatory domains of nPKC isoforms also contain C1 and C2 domains, but it is not known whether similar mechanisms of membrane-association and activation are involved (Slater et al., 2002). In addition to the natural activators, including DAG, the enzyme is activated with high affinity and specificity by the tumor promoting phorbol esters (Blumberg, 1991). For this reason, phorbol esters are often used in the study of the mechanisms of PKC activation, based on the assumption that they compete directly with DAG for a common binding site on the enzyme (Sharkey and Blumberg, 1985).

The isoform-specific functions may result in part from their differential subcellular localization (Mochly-Rosen and Gordon, 1998), therefore, PKC subcellular localization has been extensively studied in cultured cells using antibody staining and immunofluorescent microscopy (Mochly-Rosen, 1995). However, many signal transduction events involving PKC are rapid, transient, and difficult to follow in fixed cells. Green fluorescent protein (GFP), because of its inherent bioluminescence and stoichiometric labelling, represents a sensitive optical reporter to follow the real time localization of many proteins in live cells.

1.5.5 Regulation of PKC

PKC is regulated by three distinct mechanisms. Firstly, by phosphorylation which ‘primes’ the enzyme for catalysis (Blumberg et al., 1984). Previous studies have shown that PKC is inactive when it is synthesized but subsequent phosphorylation stabilizes the catalytically competent conformation of the kinase and causes release of the mature enzyme into the cytosol (Borner et al., 1989). Indeed, PKC is processed by a series of ordered phosphorylations that are
required for the enzyme to gain catalytic competence and the correct intracellular localization (Keranen et al., 1995; Tsutakawa et al., 1995).

The first and rate-limiting step in the processing of PKC is phosphorylation on the activation loop by phosphoinositide-dependent kinase, PDK-1, which is regarded as the upstream kinase for PKC isoforms (Chou et al., 1998; Dutil et al., 1998). PDK-1 plays a pivotal role in signalling by turning on the catalytic function of diverse members of the AGC family kinases (Toker and Newton, 2000). These kinases require activation loop phosphorylation to gain catalytic competence and this therefore functions as a switch. Although this phosphorylation is required for the maturation of PKC, once the enzyme is phosphorylated at the C-terminal sites, the activation loop phosphate is dispensable (Keranen et al., 1995). Thus, the activation loop phosphorylation is required to initiate the C-terminal processing, but once this event is completed, phosphate on the activation loop site is not necessary for activity. The importance of phosphate at the conserved segment of PKC comes from two things: it correctly positions residues for catalysis and it unmasks the entrance to the substrate binding cavity (Johnson and O’Reilly, 1996).

The second step in the processing of PKC is phosphorylation of the turn motif. Phosphorylation at the activation loop triggers the rapid phosphorylation of a motif in a pro-rich domain and this site corresponds to Ser338 in PKA which is present at the apex of a turn on the upper lobe of the kinase domain. Molecular modelling suggests that this site occupies a similar position in PKC, hence the name ‘turn motif’. Several lines of evidence suggest that this site is modified by autophosphorylation. The phosphate at the turn motif is needed for catalytic function of the enzyme, whereas dephosphorylation at this position abolishes
activity (Keranen et al., 1995). Biochemical studies indicate that phosphate at this position locks PKC in a catalytically competent, thermally stable, and phosphatase-resistant conformation (Edwards et al., 1999). Following phosphorylation of the turn motif, PKC isoforms rapidly autophosphorylate at the hydrophobic motif (Behn-Krappa and Newton, 1999). The hydrophobic site has been proposed to be modified by its own upstream kinase, PDK-2 (Alessi et al., 1997). The hydrophobic site has a second role in addition to stabilizing the structure of PKC: the sequence surrounding the hydrophobic phosphorylation motif forms a docking site for PDK-1 (Biondi et al., 2000). This site is exposed for PDK-1 binding in the unphosphorylated forms of PKC but becomes masked in the phosphorylated (and inactive) conformation. It has been suggested that newly synthesized PKC localizes to the membrane where it adopts a conformation in which the pseudosubstrate is out of the active site, thus exposing the activation loop phosphorylation site. This conformation is essential to target PKC for phosphorylation by PDK-1 (Dutil and Newton, 2000).

The second mechanism of PKC regulation is by ligand binding that promotes the membrane association of PKC and this membrane interaction provides energy to release the pseudosubstrate from the substrate-binding cavity (Kraft et al., 1982).

Initial studies on PKC focused on its requirement of cofactors for activation, and the three classical activators were identified: phosphatidylycerine, Ca$^{2+}$, and DAG. Furthermore, biochemical and biophysical studies on PKC have established the central roles of the C1 and C2 domains in driving this spatial redistribution, which leads to activation.
Regulation of PKC activity by phosphatidylserine. The activity of all isoforms of PKC is regulated by phosphatidylserine, an aminophospholipid found exclusively on the cytoplasmic leaflet of membranes which typically comprises approximately 15 mol% of the total lipid (Newton, 1993). Binding studies revealed that in the absence of DAG, PKC displays little selectivity for phosphatidylserine. The accepted model of activation of PKC by lipids is that on binding DAG (or phorbol esters) in the presence of the phospholipid cofactor, a conformational change in PKC results in the removal of the pseudosubstrate from its binding site and in the activation of enzyme. It is believed that the cysteine-rich and C2 domains are not the only regions involved in phospholipid binding. Thus the pseudosubstrate domain, once it is removed from its binding site may also contribute to membrane binding (Orr et al., 1992).

Regulation of PKC activity by diacylglycerol and phorbol esters. Phorbol esters and DAG cause a dramatic increase in the affinity of PKC for membranes. In another words it serves as a ‘molecular glue’ to recruit PKC to membranes. The nature of the ‘molecular glue’ could be a consequence of the occupancy of the ligand binding cavity in the C1 domain which results in the presentation of a contiguous hydrophobic surface. Thus, C1 domain ligands recruit PKC by altering the surface properties of the domain to favour membrane presentation (Zhang et al., 1995a). Both phorbol esters and DAG regulate PKC by the same mechanism. However, they can have very different biological effects for two reasons: firstly, membrane recruitment and activation initiated by DAG is short lived because DAG is rapidly metabolized, whereas phorbol esters are not readily metabolized and cause sustained activation of PKC. Secondly, phorbol
esters are more potent than DAG in recruiting PKC to membranes (Newton, 2001).

**Regulation of PKC activity by Ca\(^{2+}\).** Although the molecular events mediating Ca\(^{2+}\)-induced activation of cPKCs are not fully understood, there is evidence that this cation facilitates translocation by increasing the affinity of the C2 domain for anionic lipids. It is reported that increased intracellular Ca\(^{2+}\) is not essential for PKC translocation and activation especially if the C1 domain achieves sufficiently tight binding. The C2 domain does not need to be fully engaged on the membrane for activation. Therefore, phorbol ester stimulation can cause translocation of conventional PKC isoforms in the absence of changes in intracellular Ca\(^{2+}\). DAG and Ca\(^{2+}\) synergize in activating PKC but this synergism does not result from allosteric interactions between the C1 and C2 domains. Rather, each ligand independently causes the affinity of PKC for anionic lipids to increase (Hannun *et al.*, 1986). In addition, the presence of one ligand dramatically decreases the amount of the second ligand required to activate the enzyme (Oancea and Meyer, 1998).

The third mechanism of PKC regulation is by anchoring proteins: a family of proteins called RACKs (for receptors for activated C kinase) anchor the active conformation of phosphorylated PKC at specific cellular locations (Mochly-Rosen *et al.*, 1991b). PKC-RACK interaction is mediated at least in part by the C2 region in cPKCs and the C2-like region (within the V1 region) in nPKCs (Csukai *et al.*, 1997). To date, two RACKs have been identified: RACK1, the selective RACK for PKC\(\beta\)II (Ron *et al.*, 1994), and RACK2, (previously identified as \(\beta\)'COP (Stenbeck *et al.*, 1993)), the selective RACK for PKC\(\varepsilon\) (Csukai *et al.*, 1997). \(\beta\)II RACK and \(\varepsilon\) RACK bind their respective isoforms in a selective and
saturable manner (Csukai et al., 1997). In addition, both RACKs bind PKC in its active conformation. Despite the differences in the sequences of RACK1 and RACK2, the two proteins share common features. Although, RACK1 and RACK2 are not PKC substrates, both increase PKC phosphorylation of substrates (Ron et al., 1994). Other proteins named STICKs (for substrates that interact with C kinase) bind inactive, phosphorylated PKC and then release the activated kinase following their phosphorylation (Jaken, 1996).

Other mechanisms that regulate PKC activity include its proteolytic degradation. The hinge region at the border between the V3 and catalytic domains is thought to be a site sensitive to proteolytic cleavage by trypsin or by the Ca$^{2+}$-dependent neutral proteases calpain I and II (Saido et al., 1992). Prolonged exposure to phorbol esters can cause loss of certain isoforms of PKC from cells (Majewski and Iannazzo, 1998 and Liu and Heckman, 1998). For instance, after PMA (phorbol-12-myristate-13-acetate) treatment, PKC$\alpha$ is depleted through a net increase in proteolysis without a change in the rate of synthesis. Thus, PKC$\alpha$ is only rarely found to be completely down-regulated (Young et al., 1987). PKC$\beta$ is more susceptible to complete loss from many cells types (Hug and Sarre, 1993). It has been commonly observed that cPKCs are down-regulated the quickest, whilst the nPKCs are down-regulated more slowly, and aPKCs are not down-regulated (Huwiler et al., 1991; Sena et al., 1996) by prolonged exposure to phorbol esters.

1.5.6 Functions of PKC

A multiplicity of functions has been ascribed to PKC. Recurring themes are that PKC is involved in receptor desensitisation, the regulation of transcription, mediation of the immune response, regulation of cell growth and

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modulation of ion channel activity (Newton, 1995). In addition, PKC plays a crucial role in exocytosis (Terbush and Holz, 1990). One aspect of this regulation is mediated through its effects on ion channels, which was first observed in the central nervous system (CNS) where PKC may modulate noradrenaline release. In addition, there is strong evidence that the facilitatory effects of phorbol esters on action-potential-induced transmitter release are mediated by PKC (Kotsonis and Majewski, 1996; Schroeder et al., 1995). Furthermore, the effects of phorbol esters on noradrenaline release are attenuated by PKC inhibitors (Majewski et al., 1997) as well as by PKC down-regulation (Schroeder et al., 1995). In contrast, some studies revealed that phorbol esters modulate action-potential-induced transmitter release in most systems rather than induce release by themselves (Robinson, 1992). However, in some studies phorbol esters by themselves induce transmitter release in the absence of nerve stimulation such as in rat brain synaptosomes (Dekker et al., 1991), SH-SY5Y cells (Vaughan et al., 1995), PC12 cells and chromaffin cells (Burgoyne et al., 1988; Mollard et al., 1995; Teschemacher and Seward, 2000). Activation of PKC enhances Ca\(^{2+}\) channel activity and potentiates fast synaptic transmission as a result of the direct phosphorylation of the Ca\(^{2+}\) channel \(\alpha\) subunits (Maeno-Hikichi et al., 2003). The channels which are most relevant to facilitation of transmitter release are the voltage activated Ca\(^{2+}\) channel (N, L, P/Q) types in which PKC phosphorylation has been shown to increase Ca\(^{2+}\) conductance either directly or by preventing G-protein receptor mediated inhibition of the channel (Swartz, 1993; Zamponi et al., 1997). Studies on PC12 cells indicate that Ca\(^{2+}\) influx through L-type channels is the major contributory route of Ca\(^{2+}\) influx in PKC-mediated catecholamine secretion (Taylor et al., 2000). A strong coupling of secretion to L-type Ca\(^{2+}\)
channels following PKC activation might be considered surprising since this class of Ca$^{2+}$ channels has not been known to associate with the SNARE complex (El Far et al., 1995). The second channel family that may be involved are K$^+$ channels and PKC phosphorylation has been shown to inhibit K$^+$ outflow in neurons. Such inhibition results in a more persistent depolarization and enhanced Ca$^{2+}$ entry (Majewski and Iannazzo, 1998; Colby and Blaustein, 1988; Takano et al., 1995). Moreover, PKC may regulate influx of Ca$^{2+}$ through nicotinic channels (Wakade et al., 1986).

PKC may also have a key role in synaptic plasticity (Linden and Routtenberg, 1989) since treatment with phorbol ester leads to an increased recruitment of vesicles into the ready releasable pool (RRP) in hippocampal neurons (Stevens and Sullivan, 1998). Furthermore, this effect is blocked by PKC inhibition with bisindolylmaleimide. Similarly, in chromaffin cells, activation of PKC increases both the size and the rate of replenishment of the readily releasable vesicle pool (Gillis et al., 1996). This effect of phorbol ester was originally attributed to activation of PKC and although several targets for PKC phosphorylation have been identified, the link to vesicle recruitment is not clear (Nagy et al., 2002). A large number of studies on many different secretory cell types have implicated protein phosphorylation in the control of regulated exocytosis and many of these studies suggested a role for PKC (Rink et al., 1983; Shapira et al., 1987). The key proteins of the exocytotic machinery that have been identified as potential PKC substrates include, SNAP-25, VAMP, synaptotagmin I, rab3a and Munc18 (Barclay et al., 2003), although it is not clear which is responsible for the effects of PKC. There is some evidence that phosphorylation of both SNAP-25 (Shimazaki et al., 1996) and Munc18 (Fujita et al., 1996) by
PKC reduces their affinity for syntaxin. In the case of SNAP-25, this could be due to inhibition of SNARE complex formation and thereby exocytosis. In contrast, phosphorylation of Munc18 would allow it to dissociate more readily from syntaxin allowing frees syntaxin to participate in the assembly of the SNARE complex. Another substrate for PKC is the putative Ca\textsuperscript{2+} sensor synaptotagmin I. However, the physiological significance of the phosphorylation of these proteins is currently unknown (Burgoyne and Morgan, 2003). The role of these PKC substrates in catecholamine secretion from chromaffin cells has received considerable attention. However, the role of MARCKS and another DAG/phorbol ester target, Munc13, has been less well explored and the current study therefore examined their involvement in catecholamine secretion (See Chapters 5 and 6).

It is proposed that changes in the extent of release due to a switch to kiss-and-run exocytosis can be controlled by activation of PKC (Graham et al., 2000). In other words, PKC activation may speed up the release kinetics. This hypothesis is based on data derived from studies on chromaffin cells in which phorbol esters modified single granule release events such that release was initially faster and terminated more rapidly. This rapid termination of release is consistent with the activation of kiss- and -run exocytosis. Furthermore, the PKC substrate Munc18 is implicated as a potential target for the control of kiss- and -run exocytosis through PKC-mediated phosphorylation (Barclay et al., 2003). Synaptotagmin I is also a substrate for PKC (Hilfiker et al., 1999) and overexpression of this protein has been shown to modify fusion pore kinetics measured using amperometry in PC12 cells (Wang et al., 2001).

**Role of PKC in the reorganization of the actin cytoskeleton.** The actin cytoskeleton is a dynamic network composed of actin polymers and a large
variety of associated proteins (Geeraert et al., 2003). Most cell types possess actin filaments organized into a variety of structures including a cortical actin network found beneath the plasma membrane as in for example chromaffin cells (Burgoyne and Cheek, 1987). The idea that the cortical actin network could form a barrier to exocytosis and hence act as a site of regulation of exocytosis was first proposed by Orci and co-workers (1972). The dynamics of the cytoskeleton during exocytosis have been intensively studied in adrenal chromaffin cells (Trifarò, 1990; Trifarò and Vitale, 1993). It has been suggested that the cortical F-actin cytoskeleton is involved in regulated secretion controlling the number of secretory vesicles present at release sites in the subplasmalemmal region (Trifarò and Vitale, 1993; Vitale et al., 1995). In resting chromaffin cells, 1%-3% of the total chromaffin vesicles are either docked to the plasma membrane or within 50 nm of it (Vitale et al., 1995). This population of vesicles constitutes the release-ready vesicle pool (Neher and Zucker, 1993; Vitale et al., 1995). The rest of the chromaffin vesicles (97%-99%) form a reserve pool and remain behind a barrier of cortical F-actin (Vitale et al., 1995). The stimulation of chromaffin cells is accompanied by a focal and transient disassembly of the cortical F-actin network (Cheek and Burgoyne, 1986; Vitale et al., 1991, 1995). This allows the movement of additional secretory vesicles from the reserve pool to release sites on the plasma membrane in preparation for exocytosis (Vitale et al., 1995). Therefore, the dynamics of the cortical actin network control the size of the release-ready vesicle pool and consequently, the initial rate of exocytosis (Vitale et al., 1995).

At least two pathways control the cortical F-actin network in chromaffin cells during secretion, namely the Ca\(^{2+}\)-scinderin pathway and the PKC-MARCKS pathway (Trifarò et al., 2000; Cuchillo-Ibáñez et al., 2004). Previous
work has demonstrated that scinderin, an F-actin severing protein, has a major role in the release of chromaffin vesicles by controlling disassembly of the cortical F-actin cytoskeleton through a Ca^{2+}-dependent and PtdIns(4,5)P_2 regulated mechanism (Zhang et al., 1996a). However, several lines of research suggest that PKC can regulate catecholamine secretion without modifying scinderin activity (Rodriguez del Castillo et al., 1992; Rosé et al., 2001). For this reason, extensive studies were performed to investigate the PKC substrates that are responsible for its potentiating effect on secretion. In recent years, a substrate of PKC, MARCKS has been investigated. MARCKS has been implicated in several cellular processes such as motility, mitosis, cytoskeletal dynamics, phagocytosis, and transformation (Aderem, 1992; Allen and Aderem, 1995). MARCKS has a basic domain of 25 amino acids, located in the middle of the primary structure, which is the phosphorylation site domain (PSD) (Aderem, 1992; Blackshear, 1993). MARCKS PSD domain, in addition to containing four serines that are possible substrates for PKC, can also interact with calmodulin, phospholipids, and actin in a complex manner (Graff et al., 1989; Arbuzova et al., 1997). Phosphorylation of MARCKS in the PSD domain by PKC inhibits F-actin cross-linking (Hartwig et al., 1992) and it has also been reported that the effect of PKC on catecholamine release from chromaffin cells is mediated through MARCKS phosphorylation (Rosé et al., 2001). Other proteins believed to be involved in the regulation of the cytoskeleton that are also substrates for PKC include annexin I (Wang and Creutz, 1992), and annexin II (Johnstone et al., 1992). It is generally accepted that the pathways that trigger disassembly of the cortical F-actin could increase the size of the release-ready vesicle pool with a
consequent enhancement in the initial rate of vesicular exocytosis (Vitale et al., 1995).

Although many physiological roles for PKC have been identified, it is hard to define the physiologically relevant functions of individual PKC isoforms (Mellor and Parker, 1998). These difficulties are due to the ubiquitous and potentially redundant expression of PKC isoforms, the large number of identified PKC regulators and substrates and the presence of alternative DAG and Ca$^{2+}$ receptors that are also activated by commonly used PKC activators (Brose and Neher, 2002). Therefore, the elucidation of the precise roles of specific PKC isoforms may open a new field in which neurotransmitter release could be pharmacologically manipulated (Majewski and Iannazzo, 1998).

1.6 AIMS OF THE PROJECT

Modulation of neurotransmitter release by either ionotropic nicotinic receptors or $\text{G}_{\alpha_{q/11}}$-coupled receptors is a key feature of information processing of the mammalian nervous system and may be essential for events such as regulation of cardiovascular function and the stress response. Moreover, there is evidence that some of the modulation by either ionotropic nicotinic receptors or $\text{G}_{\alpha_{q/11}}$-coupled receptors is via PKC and changes in the exocytotic machinery. The overall aim of this project is explore the role of PKC, in particular the role of specific PKC isoforms in catecholamine secretion mediated by ionotropic nicotinic receptors and $\text{G}_{\alpha_{q/11}}$-coupled receptors and investigate the mechanisms underlying PKC activation. Previous studies, particularly using phorbol esters have indicated a facilitating role of PKC in exocytosis. However, the circumstances in which PKC plays a role, the extent of regulation and the
mechanisms underlying both PKC activation and its regulation of exocytosis are unclear.

The initial aim was to explore the use of either PC12 or bovine chromaffin cells as a model system, confirming the expression of both ionotropic nicotinic receptors and a range of Gαq/11-coupled receptors. The aim was then to investigate which PKC isoforms are expressed in these cells and to study the impact of PKC activation and particularly the role of specific PKC isoforms on catecholamine secretion evoked by either ionotropic nicotinic receptors or Gαq/11-coupled receptors. Finally, the aim was to consider the mechanism of PKC activation and the way in which PKC was able to influence exocytosis.
2: MATERIALS AND METHODS

2.1 CHEMICALS AND MATERIALS

Adrenal glands from 18- to 24-month-old cows were obtained from a local abattoir. Collagenase type 2 was supplied by Worthington Biochemical (Lakewood, NJ, USA) and DNase I was supplied by Böehringer Mannheim (Mannheim, Germany). All other cell culture reagents including PBS (phosphate buffer saline) Dulbecco’s were obtained from Gibco Life Technologies (Paisley, UK) and Sigma Aldrich (Poole, UK). Rat tail collagen used for culturing of chromaffin cells was purchased from Roche Pharmaceuticals Division (Basel, Switzerland) and Sigma (Poole, UK), respectively.

The cells were cultured in plasticware supplied by NUNC (Bibby Sterilin, Stafford, UK). Glass coverslips, both 22mm and 16mm diameter, were obtained from Chance Proper (Warley, UK) and the cuvettes used for spectrofluorimetry were obtained from Sarstedt (Leicester, UK).

Fluo-3-acetoxymethyl ester (fluo-3-AM), pluronic acid F-127, rhodamine-phalloidin, and jasplakinolide were supplied by Molecular Probes Ltd (Leiden, Holland). Adenosine triphosphate disodium salt (ATP), acetylcholine, methacholine, bradykinin, angiotensin II, atropine (sulphate salt), thapsigargin, histamine, epinephrine bitartrate, apyrase, nicotine, (+)-tubocurarine chloride hydrate, pertussis toxin (PTX), hexamethonium, cytochalasin D, BSA (bovine serum albumin), goat serum, HRP-conjugated anti-mouse and anti-rabbit (whole molecule) IgG antibodies, goat anti-mouse and anti-rabbit IgG (whole molecule) FITC (fluorescein isothiocyanate) conjugate and Bradford reagent were obtained from Sigma Aldrich (Poole, Dorset, UK). Phorbol-12-myristate-13-acetate (PMA), 4α-phorbol-12,13-didecanoate (4α-PMA) (inactive isomer), wortmannin,
myristoylated protein kinase C inhibitor, calphostin C, Ro31-8220 (1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)-maleimide, methane sulphonate), HBDDE (2,2',3,3',4,4'-hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether), and protease inhibitor cocktail set 1 containing (500μM AEBSF (4-(2-aminoethyl)-benzolsulfonylfluoride-hydrochloride), 500μM EDTA, 1μM E-64 ((2s,3s)-3-(n,{(s)-1-[n-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyloxirane-2-carboxylic acid), 1μM leupeptin, and 1μg/ml aprotinin) were supplied by Calbiochem (CN Biosciences Ltd, Nottingham, UK). LY333531 hydrochloride ((S)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9,16,21-dimetheno-1H,13H-dibenzo[e,k]pyrrolo[3,4-

h][1,4,13]oxadiazocyclohexadecene-1,3(2H)-dione hydrochloride) was obtained from A.G. Scientific, Inc. (San Diego, CA, USA). U-73122 (1-(6-((17β-3-methoxyestra-1, 3, 5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione), U-73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione), myristoylated PKCβC2-4 inhibitor (N-myristoyl-Ser-Leu-Asn-Pro-Glu-Trp-Asn-Glu-Thr), and myristoylated PKCεV1-2 inhibitor (N-myristoyl-Glu-Ala-Val-Ser-Leu-Lys-Pro-Thr) were supplied by BIOMOL Research Laboratories Inc. (Waidmannstrabe, Hamburg, Germany). Myo-[^3]H]-inositol, muscarinic antagonist 1-[N-methyl-^3]H]scopolamine methyl chloride ([^3]H]NMS, ECL plus enhanced chemiluminescent Western blotting reagent and ECL hyperfilm were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Precision plus pre-stained protein standards for Western blots and the Mini-PROTEAN II electrophoresis gel equipment were obtained from Bio-Rad Laboratories Ltd. (Hertfordshire,UK). Nitrocellulose (0.45μm pore size) for protein transfer was supplied by Schleicher and Schuell (London, UK).
Emulsifier-Safe scintillation cocktail was supplied by Packard (Pangbourne, Berks, UK). The NucleoBond Plasmid Maxi Kit was supplied by Clontech Laboratories (Palo Alto, CA, U.S.A) and transfections were carried out using either Nucleofector transfection reagent from Amaza Biosystems (Würzburg, Germany) or Lipofectamine 2000 from Invitrogen Life Technologies (Paisley, UK). Vectashield medium was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). The viral constructs containing EGFP-PKCβII and EGFP-PKCδ were generous gift of Dr. Guy Rutter (Department of Biochemistry, University of Bristol). The vectors for the Ins(1,4,5)P3 (eGFP-PHPLCδ1) and diacylglycerol (eGFP-PKCyClδ1) biosensors were provided by Professor T. Meyer (Stamford University, Stanford, CA). MARCKS-eGFP was kind gift from Dr. Hideo Mogami (Hamamatsu University, Japan). Munc13-eGFP was provided by Dr. Nils Brose (Max-Planck Institute for Experimental Medicine, Molecular Neurobiology Group, Göttingen, Germany). Bovine brain extract was a generous gift from Dr. Bazbek Davletov (Medical Research Council of Molecular Biology, Cambridge, UK). DNA encoding the human muscarinic M3 receptor that had been cloned into plasmid pcDNA3 was supplied from Invitrogen Life Technologies (Paisley, UK).

For details of all primary antibodies, please refer to Table 2.1. These commercial antibodies were purchased from Transduction Laboratories (BD Bioscience, Oxford, UK), Sigma (Poole, UK), Santa Cruz Biotechnology Inc. (California, CA, USA), and Upstate Biotechnology Inc. (Lake Placid, NY, USA).

All other standard chemical reagents of analytical grade were supplied by either Sigma Aldrich (Poole, U.K) or Fisher Scientific (Loughborough, U.K).
2.2 CELL CULTURE

2.2.1 Preparation of bovine chromaffin cells

Adult bovine chromaffin cells were prepared as described previously (Marley and Livett et al., 1987; Vitale et al., 1991; Seward and Nowycky, 1996). Adrenal glands from 18- to 24-month-old cows were obtained from a local abattoir. Excess fat was removed and glands placed in ice cold Locke's buffer (154.2mM NaCl, 2.6mM KCl, 2.2 mM K$_2$HPO$_4$, 0.85mM KH$_2$PO$_4$, 1mM glucose, 0.5mM HEPES, 1mM phenol red, pH 7.2) containing antibiotics (100μg/ml penicillin/streptomycin, 50μg/ml gentamycin). Upon arrival at the laboratory, the adrenal vein was cannulated and the gland perfused with the same buffer. The outer capsule and part of cortex were dissected from the gland taking care not to puncture the medulla. The gland was suspended in a 50ml tube and perfused at 25ml/min for 30 min at 37°C with the digestive enzymes 0.03% collagenase type 2 and 0.01% DNase I added to a Locke's buffer. Residual cortex was scraped off using a scalpel blade and the medulla minced and placed in a trypsinisation jar containing 25ml of the Locke's with added enzymes for 30 min at 37°C. This suspension was filtered through a 40μm nylon mesh and diluted with 25ml of enzyme free Locke's buffer. The cells were centrifuged at 5000g for 5 min at room temperature. A further 25ml of Locke's buffer were used to resuspend the cells. The cells were counted and the solution diluted to 2x10$^5$ cells/ml. Cells were plated on collagen-coated coverslips in 6 or 24 well plates at densities of 5x10$^5$ and 1-2x10$^5$ cells/well, respectively in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% foetal calf serum, 100μg/ml penicillin/streptomycin, 50μg/ml gentamycin, 10μM 5-fluro-deoxy-uridine (5-FDU), 10μM cytosine-β-γ-arabino-furanoside (cyto-sar) and 0.5mM glutamine.
The cells were incubated for 48 h at 37°C in 5% CO$_2$ humified atmosphere and used for 5-7 days post gland collection.

2.2.2 Cell counts

Cells were diluted with medium (1:5 dilution) then mixed with 50μl Trypan blue dye which is one of several stains recommended for use in dye exclusion procedures for viable cell. This method is based on the principle that viable cells do not take up certain dyes, whereas non-viable cells stain. Cells were counted after addition of small amount of Trypan blue-cell suspension mixture to both chambers of the hemocytometer (Superior Marienfeld, Cupertino, CA., USA), and then total cells per ml were counted by multiplying the count times dilution factor times $10^4$ (count 10 squares).

2.2.3 Collagen coating procedure

Collagen (50mg) was dissolved in 10ml of 0.1% sterile glacial acetic acid and mixed by stirring for 1 h at room temperature. Aliquots (each 200μl) were then stored at -20°C until use. A frozen aliquot (200μl) of this stock was defrosted then dissolved by adding 8ml of 60% sterile ethanol. Collagen solution was then added to cover the well or coverslip (300-500μl). Collagen coating of the well or coverslip was facilitated by incubation at room temperature until the ethanol completely evaporated and collagen coated the entire well or coverslip. Cells were then plated at the required concentration and incubated for 24 h to allow firm adherence.

2.3 WESTERN BLOTTING (IMMUNOBLOTTING)

2.3.1 Preparation of whole cell extract for immunodetection of PKC isoforms

The method was based on that previously described (Willars et al., 1999; 2001; Tovey and Willars, 2004). Multidishes (6 well) were coated with collagen
and seeded with approximately 1-2x10^6 cells. After overnight culture to allow the cells to adhere to collagen, the monolayers were washed with 1ml non-sterile PBS Dublecco's and then solubilised with 300μl solubilization buffer (10mM Tris, 10mM EDTA, 500mM NaCl, 1% (v/v) ethylphenylpolyethylene glycol (Nonidet P40), 0.1% sodium-dodecyl-sulphate (SDS), 0.5% w/v deoxycholic acid, 1mM phenylmethylsulphonyl fluoride (PMSF), 100μg/ml iodoacetamide, 100μg/ml benzamidine, and 5μl/ml protease inhibitor cocktail, pH 7.4) and left on ice for 30 min with gentle rocking. Finally, the wells were scraped gently with a fine pipette tip in order to ensure complete removal of cellular materials. The solubilised cells were then transferred to a microfuge tube and stored at -20°C until use.

2.3.2 Cell fractionation for studying the translocation of PKC isoforms

The method was based on that previously described (Willars et al., 1996; 2001). Confluent monolayers of cells in a 6 well plate were washed with 1ml of Krebs' HEPES buffer (KHB) (composition: 10mM HEPES, 4.2mM NaHCO₃, 11.7mM D-glucose, 1.2mM MgSO₄, 1.2mM KH₂PO₄, 4.7mM KCl, 118mM NaCl, and 1.3mM CaCl₂, pH 7.4). Cells were then incubated at 37°C for 5 min with 1ml KHB in the presence or absence of agonist and lysed in 200μl of ice-cold lysis buffer (20mM Tris-HCl, 5mM EGTA, 2mM EDTA, 1mM dithiothreitol (DDT), 0.5mM PMSF, 10μM benzamidine hydrochloride, 5μM iodoacetamide, pH 7.4) and left on ice for 30 min.

Separation of cytosolic and membrane fractions was performed by centrifugation (20,800g, 15 minutes, 4°C). A 100μl aliquot of supernatant, which represents the cytosolic fraction, was transferred to a 1.5ml microfuge tube and the insoluble pellet, which represents the membrane fraction, was resuspended in
200\mu l of solubilization buffer. An equal volume of 2X sample buffer (100mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% (\%\_\text{v/v}) glycerol, 200mM dithiothreitol (DDT)) was added to the cytosolic and membrane fractions. Aliquots were denatured by placing in a Grant Boekal heating block at 100°C for 5 min. Proteins within the samples (30\mu l, 30\mu g.lane\(^{-1}\) of proteins within the sample) were separated by SDS-PAGE (SDS polyacrylamide gel electrophoresis) (Bio-Rad mini-Protean II electrophoresis cell) using a 10% running gel (prepared using: 5.9ml H\(_2\)O, 5.0ml of 30% acrylamide mix, 3.8ml of 1.5M Tris (pH 8.8), 0.15ml of 10% SDS, 0.15ml of 10% ammonium persulphate and 0.006ml of TEMED) at 200 V for 45 min using a Tris and glycine-based running buffer (25mM Tris-base, 250 mM glycine, 0.1% SDS (\%\_\text{v/v})). Proteins were then transferred to nitrocellulose using a semi-dry blotter (buffer: 39mM glycine, 48mM Tris-base, 0.037% SDS (\%\_\text{v/v}), 20% methanol (\%\_\text{v/v}), pH 8.3) at 0.65mA/cm\(^2\) for 65 min. The nitrocellulose was then blocked with 5% (\%\_\text{w/v}) powdered milk in low salt TTBS (100mM NaCl, 20mM Tris- base, 0.05% Tween 20 (\%\_\text{v/v}), pH 7.5) either overnight at 4°C or for 1 h at 20°C. The nitrocellulose was then incubated with the commercially available primary antibodies against PKC isoforms at the required dilution (see Table 2.1) for 1 h at room temperature. The excess antibodies were removed by washing three times in TTBS with each wash lasting for 10 min. Subsequently, secondary antibody (HRP-linked anti-mouse IgG-peroxidase antibody) was added (1:1000) and incubated for 1 h at room temperature. The excess secondary antibody was then removed by washing three times in TTBS with each wash lasting for 10 min.

Western blots were developed with ECL plus Western blotting detection reagent for 5 min, then exposed to Amersham ECL hyperfilm for 1-10 minutes.
Immunoblots were analysed by densitometer where appropriate using Scion image analysis software (v4.02 beta, Frederick, Maryland, USA). Local background subtraction was applied to images and data either normalized to basal (nonstimulated) or calculated as percentage total cellular protein content for studying PKC translocation.

2.4 IMMUNOCYTOCHEMISTRY

2.4.1 Immunocytochemistry for studying PKC localization

Cells cultured on collagen-coated coverslips at a density of 5x10^5 cells per coverslip were washed three times with PBS. Cells were then incubated for the required period in the absence (control) or presence (stimulated) of agonists. Following the incubation, the solution was quickly removed and replaced with 4% paraformaldehyde in PBS (pH 7.4) and maintained for 30 min. After permeabilization for 10 min in PBS containing 0.2% Triton X-100, the cells were incubated for 1 h in 10% normal goat serum in PBS to block non-specific binding, then, washed three times (each 5 min) with PBS. This step was followed by overnight incubation with PKC isoform specific antibody (Transduction Laboratories) (diluted 1:100 in PBS containing 10% normal goat serum) at 4°C. Coverslips were then washed three times with PBS each for 5 min. A goat FITC-conjugated anti-mouse secondary antibody (1:512 in PBS containing 10% normal goat serum) was applied for 1 h in the dark at room temperature. Coverslips were then rinsed three times with PBS each for 5 min and mounted in Vectashield medium to reduce photobleaching.

Controls were obtained by omitting the primary antibody in the incubation. No staining of cells was observed under these conditions. Cells were observed under confocal microscopy using an excitation wavelength of 488nm.
and emitted light collected above 510nm with a narrowband pass dichroic mirror and filter and detected by a CCD camera.

### 2.4.2 Confocal microscopy for F-actin detection

Cells were plated on collagen-coated coverslips at a density of $5 \times 10^5$ cells per coverslip. Cultured cells were rinsed three times with PBS (130mM NaCl, 100mM sodium phosphate, at pH 7.4). Cells were then incubated for the required period in the absence (control) or presence (stimulated) of agonists as described in the figure legends. Following incubation, cortical F-actin integrity was determined by adaptation of the method of Lee and Trifaró (1981). Briefly, cultured cells were fixed in 4% paraformaldehyde-PBS for 30 min. Excess paraformaldehyde was quenched with 50mM NH$_4$Cl-PBS for 15 min (Li et al., 2003). The cells were permeabilized by three successive exposures of 5 min each to 50, 100, and 50% acetone and washed several times with PBS. F-actin was stained with 0.1µM rhodamine-phalloidin for 40 min in the dark at room temperature. Finally, the coverslips were rinsed several times with PBS and mounted in Vectashield.

Slides were observed with an UltraVIEW confocal microscope. Cells were excited at 568nm and emitted light collected with a broad band RGB emission filter. The effect of several treatments on the percentage of cells showing cortical F-actin disassembly was determined by examining two coverslips for each experimental condition in each experiment. One hundred single-rounded chromaffin cells were examined per coverslip. Each cell was classified as having either a "continuous" or "discontinuous" cortical rhodamine (F-actin) fluorescent ring as described previously (Vitale et al., 1991). The percentage of chromaffin cells showing cortical F-actin disassembly (discontinuous rhodamine fluorescent ring) was calculated for each experimental condition. To avoid personal bias, code
numbers were given to coverslips. The cells were examined and classified without knowing whether they were from control or treated preparations. The codes were revealed to identify the experimental conditions used, only after all results were recorded (single-blind design).

Three dimensional image analysis was performed either by using UltraVIEW confocal software where the maximum intensity of the fluorescent ring was set to 4096 arbitrary units or by using the public domain program "ImageJ" developed at the U.S. National Institutes Of Health downloaded from the internet (http://rsb.info.nih.gov/nih-image/) and the maximum intensity of fluorescent ring was set to 250 arbitrary units.

2.5 MEASUREMENT OF INTRACELLULAR ([Ca^{2+}]_{i})

2.5.1 Confocal imaging of [Ca^{2+}]_{i}

Confocal imaging of [Ca^{2+}]_{i} was performed as described previously (Werry *et al.*, 2002; Tovey and Willars, 2004). Cells were plated onto 22 mm diameter collagen coated glass coverslips and cultured for 24-48 hours. Cells were incubated in the dark for 45 min at room temperature in 2ml Krebs'/HEPES buffer containing 6μM fluo-3-acetoxymethyl ester (fluo-3-AM) and 0.044% pluronic acid F-127.

Coverslips were transferred into 2ml fresh Krebs'/HEPES buffer and left for 15 minutes at room temperature to allow for hydrolysis of the ester group. Coverslips were mounted in a chamber on the stage of an Olympus 1X70-S1F inverted microscope with a 40X oil emersion objective. The chamber was continuously perfused with Krebs'/HEPES at a rate of 5ml per minute pre-heated to 37°C with a Peltier unit. Using an UltraVIEW confocal imaging system (PerkinElmer Life Sciences, Cambridge, U.K.) cells were excited with a
krypton/argon laser at 488nm and emitted light collected at 510nm with a narrowband pass dichroic mirror and filter and detected by a CCD camera at rate of 3-4 frames per second. Agonists were perfused and the images were recorded as a single section through the cell monolayer and averaged from 5-10 cells in a field of view.

UltraVIEW LCI software was used to analyse fluorescence intensities. The images were analysed by selecting circular regions of interest in cytoplasm of all cells in a field of view and then calculating the average fluorescence intensity for this region over time. The raw data was exported as an ASCII file for further analysis and plotted against time using Origin version 6.0 (Microcal). Data are represented graphically as fold change in fluorescence intensity relative to basal levels.

2.6 BRADFORD PROTEIN ASSAY (BCA PROTEIN DETERMINATION PROCEDURE)

Protein determination procedure is an adaptation of Bradford method (Bradford, 1976). Briefly, duplicate protein standards were created by serial dilution of 1mg/ml BSA to give 0,1,2,5,10,20, and 50μg/ml. The total liquid volume in each tube was adjusted to 1ml by the addition of deionized water. The unknown samples with an approximate concentration of protein between 1-50μg/ml were prepared and the total volume also adjusted to 1ml with deionized water. Thereafter, to each tube, 2ml of Bradford Reagent was added and vortexed. Both standards and samples were incubated at room temperature for 5 min and then transferred to polystyrene cuvettes. The absorbance at 595nm was measured using a spectrophotometer (BU-65Spectrophotometer, memory PAC module).

The net absorbance versus the protein concentration of each standard was plotted and the protein concentration of the unknown sample was determined
from the standard curve plotted using GraphPad Prism (GraphPad Software, 3rd
order polynomial, San Diego, CA, USA).

2.7 DETERMINATION OF PHOSPHOLIPASE C ACTIVITY

2.7.1 Measurement of [3H]-inositol phosphate accumulation

Agonist-induced accumulation of [3H]inositol mono- and polyphosphates
(\([3H]\)-InsP\(_x\)) was determined as described previously (Willars et al., 1999). In
brief, cells were plated at equivalent density (5x10\(^5\) cells/well) in collagen-coated
24 well multidishes for 24 h before the assay. Then, cells were prelabelled with
3\(\mu\)Ci.ml\(^{-1}\) of myo-[3H]-inositol (86 Ci.mmol\(^{-1}\)) in 1ml of growth media for 48 h at
37°C to ensure equilibrium labelling (Willars and Nahorski, 1995b; Willars et al.,
1998a).

Cells were washed once with a Krebs'/HEPES buffer containing 10mM
LiCl (KHB/Li\(^+\)) to inhibit inositol monophosphatase activity, then incubated for
10 min at 37°C with 200\(\mu\)l KHB/Li\(^+\). Agonists (made up at twice the desired final
concentration in KHB/Li\(^+\)) were added in a 200\(\mu\)l volume at the appropriate time.
Reactions were stopped by the addition of 400\(\mu\)l of ice-cold 1M trichloroacetic
acid (TCA). After 15 min extraction on ice, a 400\(\mu\)l aliquot from each well was
removed and added to 100\(\mu\)l of 10mM EDTA in a 1.5ml microfuge tube. Following this, 1ml of a freshly-prepared 1:1 (v/v) mixture of tri-n-octyl-amine
and 1,1,2-trichloro-trifluoroethane was added and the tube vortexed to ensure
thorough mixing. Samples were stood for approximately 5 min to allow separation
of samples into aqueous and organic phases. A 400\(\mu\)l aliquot of the upper aqueous
phase was removed and added to 25\(\mu\)l of 250mM NaHCO\(_3\). This sample was
applied to a Dowex chloride anion exchange column (8% cross linkage; 100-200
dry mesh; AG1-X8; Sigma), which was then washed with 10ml H\(_2\)O, and then
12ml of 25mM ammonium formate. Elution of [3H]inositol phosphates ([3H]-InsP₄) from the columns was performed by washing the columns through with 10ml 1M HCl and collected into scintillation vials. A 3ml aliquot of this eluant was mixed with 15ml of Emulsifier-Safe Scintillation Cocktail (Baird et al., 1989) and radioactivity determined by scintillation counting. Dowex chloride columns were regenerated by washing with 10ml of 2M HCl followed by 10ml H₂O.

2.7.2 Single-cell measurement of Ins (1,4,5)P₃ formation using a biosensor

Chromaffin cells were seeded onto 22mm collagen coated coverslips at a density of 5x10⁵ cells per coverslips and cultured for 48 h before transient transfection with the eGFP-tagged pleckstrin homology domain of PLCδ1 (eGFP-PHPLCδ1) plasmid cDNA (1μg) (Nash et al., 2001; Tovey and Willars, 2004) using lipofectamine 2000 as described in manufacturer’s instructions (Invitrogen Life Technologies, Paisley, UK) 48 h prior to the experiment. Using an UltraVIEW confocal imaging system (PerkinElmer Life Science, Cambridge, UK) as described previously (Nash et al., 2001; Nahorski et al., 2003; Tovey and Willars, 2004), in brief, eGFP was excited with a krypton/argon laser at 488nm and emitted light collected above 510nm with a narrowband pass dichroic mirror and filter and detected by a CCD camera.

UltraVIEW LCI software was used to analyse fluorescence intensities. The images were analysed by selecting circular regions of interest in cytoplasm of responding cells and then calculating the average fluorescence intensity for this region over time. The raw data was exported as an ASCII file for further analysis in the Microsoft Excel and plotted against time using Origin version 6.0 (Microcal). Data are represented graphically as fold change in cytosolic fluorescence intensity relative to basal levels.
2.7.3 Single-cell measurement of diacylglycerol (DAG) formation using a biosensor

Chromaffin cells were transiently transfected with eGFP-tagged with C1 domain of PKCγ (eGFP-PKCγC1) (Oancea and Meyer, 1998; Tovey and Willars, 2004) using lipofectamine 2000 as described in manufacturer’s instructions (Invitrogen Life Technologies, Paisley, UK) 48 h prior to the experiment as described by. Imaging experiments were performed 48 h after transient transfection using an UltraVIEW confocal imaging system as described above (see sections 2.5.1 and 2.9.3).

2.8 CATECHOLAMINE ASSAY

The catecholamine assay was based on the oxidation of catecholamine to produce a fluorescent derivative. The method was performed as described by Von Euler and Lishajko (1961). Chromaffin cells were plated on collagen coated 24-well plates at a density of 5x10^5 cells/well. The medium was removed and cells washed three times using Krebs'/HEPES buffer. Cells were stimulated with agonist in 200-300 µl Kreb’s buffer at 37°C. Then, 130µl of the buffer over the cells was transferred into pre-chilled microfuge tubes and spun (16,000g for 1 min) at 4°C. Duplicate aliquots of 50µl were taken for assay. Each duplicate was mixed with 2ml of 1M sodium acetate buffer (pH 6) and 100µl of 0.25% potassium ferricyanide. The solution in each tube was mixed by vortexing briefly and incubated for 5 min at room temperature. The reaction was stopped by adding 1ml of a 1:10 dilution of 2% (w/v) freshly prepared ascorbic acid in 5M NaOH. The solution was thoroughly mixed and transferred to a cuvette. The absorbance was measured using a Perkin-Elmer spectrofluorimeter (model: LS-50B) at 416nm excitation and 517nm emission wavelengths.
For determination of the cellular catecholamine content, 30µl of 10% NP-40 was added to the remaining Krebs'/HEPES buffer over the cells and duplicate aliquots of 50µl were taken for assay.

The concentration of catecholamines was calculated by using the equation obtained from a standard curve generated using epinephrine. Calibration with standard epinephrine was performed by preparing various concentrations of epinephrine bitartrate (0-200µM). A duplicate (50µl) of each concentration was taken for the assay. The concentrations of epinephrine were plotted against the intensity and the data fitted with a linear function using GraphPad Prism (GraphPad Software, 3rd order polynomial, San Diego, CA, USA):

\[ Y = A + BX \]

This equation was then used to calculate the concentration of catecholamines in samples taken from the cells where A is the intercept, B is the slope, Y is the absorbance and X is the unknown concentration of catecholamine.

2.9 DETECTION OF MARCKS TRANSLOCATION AND PHOSPHORYLATION

2.9.1 Single-cell imaging of MARCKS-eGFP

Chromaffin cells were seeded onto 22mm collagen-coated coverslips at a density of 5x10^5 cells per coverslip and cultured for 48 h before transient transfection with the cDNA (1µg/well) encoding MARCKS-eGFP (Ohmori et al., 2000) using Lipofectamine 2000 as described in the manufacturer’s standard protocol 48 h prior to the experiment. The MARCKS-eGFP was then viewed using an UltraVIEW confocal imaging system (PerkinElmer Life Science, Cambridge, UK) as described previously (Nash, et al., 2001; Nahorski et al, 2003; Tovey and Willars, 2004). In brief, eGFP was excited with a krypton/argon laser
at 488nm and emitted light collected above 510nm with a narrowband pass dichroic mirror and filter and detected by a CCD camera.

UltraVIEW LCI software was used to analyse fluorescence intensities. The images were analysed by selecting circular regions of interest in the cytoplasm of cells that showed membrane localization of MARCKS-eGFP and then calculating the average fluorescence intensity for this region over time. The raw data were exported as an ASCII file for further analysis in Microsoft Excel and plotted against time using Origin version 6.0 (Microcal). Data are represented graphically as fold changes in cytosolic fluorescence intensity relative to basal levels (F/F₀).

2.9.2 Immunoblotting for studying phosphorylation of endogenous MARCKS

Cells were seeded onto collagen-coated wells in 6-well culture dishes at a density of 2x10⁶ cells per well. Cells were then incubated at 37°C for 20 min in 1ml Krebs'/HEPES buffer in the presence or absence of agonist and lysed in 300μl of ice-cold phospho-MARCKS solubilization buffer (65mM PIPES, 25mM HEPES, 8mM EGTA, 1mM EDTA, 1% Triton X-100, 1mM DDT, 40mM β-glycerolphosphate, 1mM Na₂VO₄, 10mM NaF, 1mM PMSF, pH 7.5) and left on ice for 5 min with gentle rocking. Solubilized cells were centrifuged (13000g, 5min, 4°C) to yield supernatant. The supernatants were denatured by boiling in an equal volume of sample buffer and subject to SDS/PAGE as described above (see Section 2.3.2). Proteins were transferred onto nitrocellulose membranes, blocked, and probed with phospho-MARCKS antibody overnight at 4°C. Blots were then stripped and re-probed for MARCKS for 1 h at room temperature. Immunoreactive bands of either phospho-MARCKS or MARCKS were detected by enhanced chemiluminescence (ECL) reagents and exposure to Hyperfilm-ECL.
(see Section 2.3.2 for details of immunoblotting and see Table 2.1 for details of antibodies).

2.10 DETECTION OF MUNC13-1 LOCALIZATION

2.10.1 Single-cell imaging of Munc13-1-eGFP

Chromaffin cells were seeded onto 22mm collagen-coated coverslips at a density of 5x10^5 per coverslip and cultured for 48 h before transient transfection with cDNA (1μg/well) encoding Munc13-1-eGFP (Ashery et al., 2000; Stevens et al., 2005) using Lipofectamine 2000 according to the manufacturer’s instructions. After 48 h, coverslips were mounted on the stage of a Perkin-Elmer UltraVIEW confocal microscope, maintained at 37°C with a Peltier unit and perfused with Krebs'/HEPES buffer (5ml min⁻¹) in a chamber volume of approximately 0.5ml. Visualization of changes in cytosolic fluorescence in real time was performed as described previously (Werry et al., 2002; Tovey and Willars, 2004) alone or in the presence of test agent. In brief, eGFP was excited with a krypton/argon laser at 488nm and emitted light collected above 510nm with a narrowband pass dichroic mirror and filter and detected by a CCD camera. UltraVIEW LCI software was used to analyse fluorescence intensities. The images were analysed by selecting circular regions of interest in the cytoplasm of responding cells and then calculating the average fluorescence intensity for this region over time. The raw data were exported as an ASCII file for further analysis in Microsoft Excel and plotted against time using Origin version 6.0 (Microcal). Data are represented graphically as fold change in cytosolic fluorescence intensity relative to basal levels (F/F₀).
2.10.2 Immunocytochemical localisation of Munc13-1

Chromaffin cells were seeded on 22mm collagen-coated coverslips and the subcellular localisation of Munc13-1 was determined by immunocytochemical methods using either non-transfected cells (endogenous Munc13-1) or cells transiently expressing Munc13-1-eGFP (see above). Where required, cells were challenged with test agents (37°C in Krebs' /HEPES buffer) before being fixed and processed for immunocytochemistry as described above (see Section 2.4.1 and Table 2.1).

Membrane localization was analysed by calculating the percentage of cells showing membrane localization in control and treated preparations. To avoid personal bias, code numbers were given to coverslips and cells were examined and classified in a single-blind fashion. A total of three hundred cells from three different cell cultures were examined for each experimental condition.

2.11 DATA ANALYSIS

Data from three or more identical experiments are presented as the mean ± SEM (standard error of the mean). Where statistical analysis was required, the format of the data was taken into account in the selection of an appropriate test. Two-tailed, unpaird Student’s t-test was used for direct comparison of a test value with a control, with $P<0.05$ accepted as statistical significance. For comparison of multiple data sets, one-way analysis of variance (ANOVA) was used. If $P<0.05$ using ANOVA, data was further analysed using an appropriate post-hoc tests (either Dunnet’s which is designed to restrict the number of comparisons by only comparing each group with a specified control group or Bonferroni’s test which is designed to compare only selected pairs of means). Concentration-response curves were fitted by GraphPad Prism (GraphPad Software, 3rd order polynomial, San
Diego, CA, USA) using a standard four-parameter logistic equation. EC₅₀ mean values and standard errors of the mean were generated from the mean of values generated from separate curves. EC₅₀ values are presented as log₁₀ M mean ± SEM (n).
Table 2.1: Primary antibody working concentrations

<table>
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<tr>
<th>Antibody</th>
<th>Source</th>
<th>Raised against</th>
<th>Cross-reactivity</th>
<th>Supplier</th>
<th>Working concentration (µg/mL)</th>
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<td>mouse</td>
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<td>mouse</td>
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3: CHARACTERIZATION OF RECEPTOR-MEDIATED ELEVATION OF [Ca$^{2+}$]$_i$ IN MODEL NEUROSECRETORY cells

3.1 INTRODUCTION

This chapter details preliminarily work on two neurosecretory models to select a system that expresses a range of $\text{Go}_{q11}$-coupled receptors with the aim of comparing $\text{Ca}^{2+}$ signalling evoked by different $\text{Go}_{q11}$-coupled receptor agonists with ionotropic receptors such as the nicotinic receptor. To identify the presence of $\text{Go}_{q11}$-coupled receptors, [Ca$^{2+}$]$_i$ was measured in either cell populations (PC12 cells) or single cells (chromaffin cells). Initially, the work of this project was conducted using a PC12 cell line, which was originally isolated by Green and Tischler (1976) from a rat pheochromocytoma. This clonal cell line resembles an adrenal chromaffin cell under normal conditions (see main Introduction). Following initial studies the project then focused on adrenal chromaffin cells, which are derived embryonically from the neural crest and generally regarded as modified sympathetic neurons (Burgoyne et al., 1993b; 1996; Burgoyne and Morgan, 1998b) (see main Introduction).

In the last few decades these neurosecretory models (PC12 cells and chromaffin cells) have been used extensively as model systems to study $\text{Ca}^{2+}$-triggered exocytosis (Kilpatrick et al., 1982). It has been suggested that in cell types where a rise in [Ca$^{2+}$]$_i$ is the main trigger of regulated exocytosis, $\text{Ca}^{2+}$ can be derived from entry of external $\text{Ca}^{2+}$, by $\text{Ca}^{2+}$ release from intracellular $\text{Ca}^{2+}$ stores or both. The importance of these two $\text{Ca}^{2+}$ sources varies considerably between cell types (Cheek and Burgoyne, 1985; Cheek et al., 1989; Burgoyne and Morgan, 2003). Adrenal chromaffin cells and PC12 cells have been reported to express a wide variety of $\text{Go}_{q11}$-coupled receptors, many of which elicit
exocytosis from chromaffin cells. These include the histamine $H_1$ receptor, muscarinic receptor, angiotensin AT$_{1A}$ receptor, bradykinin B$_2$ receptor, prostaglandin EP$_3$ receptor and PAC$_1$ receptors for pituitary adenylate cyclase-activating polypeptide (PACAP) (Marley, 1987; O’Sullivan and Burgoyne, 1989; Marley, 2003). Activation of these $G\alpha_{q/11}$-coupled receptors increases the activity of inositol phospholipid-specific phospholipase C and subsequently produce an elevation of $[Ca^{2+}]_i$ (Plevin and Boarder, 1988; Sasakawa et al., 1989; Bunn et al., 1990). In addition, activation of either ionotropic receptors (nicotinic receptors) or depolarisation mediated by high $K^+$ result in a rise of $[Ca^{2+}]_i$ (Burgoyne, 1991; Zhou and Neher, 1993; Harkins and Fox, 1998; Gueorguiev et al., 1999). $Ca^{2+}$ signalling can, therefore, be used as a valuable means for identification of $G\alpha_{q/11}$-coupled receptors and nicotinic receptors in these cells.

Various receptor agonists of either metabotropic or ionotropic receptors are thought to exhibit different $Ca^{2+}$ signalling profiles. Such differences might also influence their ability to trigger secretion. Furthermore, different receptors may differentially activate or use varying components of the signalling pathways (for example, different PKC isoforms), which may impact on their ability to regulate exocytosis. The aim was therefore, to select a model cell system that expresses a variety of receptors with different $Ca^{2+}$ signalling profiles.
3.2 RESULTS

Ca\textsuperscript{2+} signalling in chromaffin cells

Identification of G\textsubscript{q/11}-coupled receptors expressed in bovine chromaffin cells by studying Ca\textsuperscript{2+} signalling

In this study, single-cell confocal imaging of cells loaded with the Ca\textsuperscript{2+}-sensitive dye fluo-3 were used to identify which receptors are expressed in bovine chromaffin cells.

Stimulation of histaminergic (Figure 3.2.1), cholinergic (Figure 3.2.2), bradykinin (Figure 3.2.3) and nicotinic (Figure 3.2.4) receptors, respectively with histamine (100\mu M), acetylcholine (100\mu M), bradykinin (10\mu M) and nicotine (100\mu M) respectively in the presence of 1.3mM extracellular Ca\textsuperscript{2+} evoked peak and sustained Ca\textsuperscript{2+} signals. However, removal of extracellular Ca\textsuperscript{2+} markedly reduced the peak Ca\textsuperscript{2+} responses and abolished the sustained phases of Ca\textsuperscript{2+} signalling (Figures 3.2.1-3.2.4). There were significant differences in the peak Ca\textsuperscript{2+} responses evoked by these agonists in the presence of extracellular Ca\textsuperscript{2+} from that evoked in absence of extracellular Ca\textsuperscript{2+} (** P<0.01, unpaired Student’s t-test).

Activation of AT\textsubscript{1A} angiotensin receptor by angiotensin II (100nM) (Figure 3.2.5) in the presence of 1.3mM extracellular Ca\textsuperscript{2+} evoked only a slight peak Ca\textsuperscript{2+} response and no sustained plateau of Ca\textsuperscript{2+} signalling (Figure 3.2.6: Panel A). The peak Ca\textsuperscript{2+} response was slightly attenuated in the absence of extracellular Ca\textsuperscript{2+} (Figure 3.2.6: Panel B). The mean data for agonist-mediated alterations in [Ca\textsuperscript{2+]}, are shown in Figure 3.2.6.
Figure 3.2.1. **Single cell imaging of histamine-mediated Ca^{2+} signalling in chromaffin cells.** Cells were cultured on glass coverslips loaded with fluo-3-AM, excited at λ488 and imaged on the confocal microscope. Cells were challenged with 100μM histamine via perfusion after 50 seconds in the presence or absence of 1.3mM [Ca^{2+}]_e. Dotted lines indicate the presence of agonist. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Images A and B were taken at time points indicated on the graph and demonstrate the effect of agonist in the presence of extracellular Ca^{2+} while images C and D show the effect of agonist in the absence of extracellular Ca^{2+}. Data are representative of three independent experiments (three adrenal gland preparations, approximately 280 cells in total).
Figure 3.2.2. **Single cell imaging of acetylcholine-mediated Ca\(^{++}\) signalling in chromaffin cells.** Cells were cultured on glass coverslips loaded with fluo-3-AM, excited at \(\lambda\)488 and imaged on the confocal microscope. Cells were challenged with 100\(\mu\)M acetylcholine via perfusion after 50 seconds in the presence or absence of 1.3mM \([\text{Ca}^{++}]\)\(_e\). Dotted lines indicate the presence of agonist. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Images A and B were taken at time points indicated on the graph and demonstrate the effect of agonist in the presence of extracellular \(\text{Ca}^{++}\) while images C and D shown the effect of agonist in absence of extracellular \(\text{Ca}^{++}\). Data are representative of three independent experiments (three adrenal gland preparations, approximately 180 cells in total).
Figure 3.2.3. **Single cell imaging of bradykinin-mediated Ca\(^{2+}\) signalling in chromaffin cells.** Cells were cultured on glass coverslips loaded with fluo-3-AM, excited at 488nm and imaged on the confocal microscope. Cells were challenged with 10µM bradykinin via perfusion after 50 seconds in the presence or absence of 1.3mM [Ca\(^{2+}\)]\(_e\). Dotted lines indicate the presence of agonist. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Images A and B were taken at time points indicated on the graph and demonstrate the effect of agonist in the presence of extracellular Ca\(^{2+}\) while images C and D show the effect of agonist in the absence of extracellular Ca\(^{2+}\). Data are representative of three independent experiments (three adrenal gland preparations, approximately 160 cells in total).
Figure 3.2.4. **Single cell imaging of nicotine-mediated Ca^{2+} signalling in chromaffin cells.** Cells were cultured on glass coverslips loaded with fluo-3-AM, excited at λ488nm and imaged on the confocal microscope. Cells were challenged with 100μM nicotine via perfusion after 50 seconds in the presence or absence of 1.3mM [Ca^{2+}]_e. Dotted lines indicate the presence of agonist. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Images A and B were taken at time points indicated on the graph and demonstrate the effect of agonist in the presence of extracellular Ca^{2+} while images C and D show the effect of agonist in the absence of Ca^{2+}. Data are representative of three independent experiments (three adrenal gland preparations, approximately 240 cells in total).
Figure 3.2.5. **Single cell imaging of angiotensin II-mediated Ca\(^{2+}\) signalling in chromaffin cells.** Cells were cultured on glass coverslips loaded with fluo-3-Am, excited at λ488 and imaged on the confocal microscope. Cells were challenged with 100nM angiotensin II via perfusion after 50 seconds in the presence or absence of 1.3mM [Ca\(^{2+}\)]\(_e\). Dotted lines indicate the presence of agonist. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Images A and B were taken at time points indicated on the graph and demonstrated the effect of agonist in the presence of extracellular Ca\(^{2+}\) while images C and D show the effect of agonist in the absence of extracellular Ca\(^{2+}\). Data are representative of three independent experiments (three adrenal gland preparations, approximately 210 cells in total).
Figure 3.2.6. Summary data of single cell imaging of agonist-mediated Ca\textsuperscript{2+} signalling in chromaffin cells. Data are the summary data from Figures 3.2.7 to 3.2.11. Cells were cultured on coverslips, loaded with fluo-3 and imaged using the confocal microscope. A) Cells were challenged with agonists in the presence of 1.3mM [Ca\textsuperscript{2+}]\textsubscript{c}. B) Cells were challenged with agonists in the absence of extracellular Ca\textsuperscript{2+}. The hatched bars represent the average maximal change in fluorescence relative to basal levels after agonist perfusion while the black bars represent the change in fluorescence above basal 200 seconds following agonist perfusion. The dotted lines indicate the basal fluorescence. Data are mean + SEM, n=3 (* P<0.05, ** P<0.01, ***P<0.001 vs. peak value of control, as determined by paired Student’s t-test).
3.3 DISCUSSION

3.3.1 Summary of data

Initial work on the PC12 cell line indicated that the clone of PC12 cells used in this study did not express receptors coupled to Goq/11 that had previously been identified on these cells. Therefore, chromaffin cells were used as an alternative choice. Data demonstrated that bovine chromaffin cells express a wide range of receptors that elevate $[\text{Ca}^{2+}]$, most likely through activation of Goq/11 including those for: angiotensin II, histamine, acetylcholine, and bradykinin. In addition, chromaffin cells increase $[\text{Ca}^{2+}]$, in response to activation of ionotropic nicotinic receptors. The sustained phases of the Ca$^{2+}$ responses mediated by either Goq/11-coupled receptors or ionotropic nicotinic receptors were dependent on extracellular Ca$^{2+}$ whilst the peak responses to these agonists also showed some dependence on extracellular Ca$^{2+}$. Histamine and angiotensin II exhibited different Ca$^{2+}$ profiles and they were therefore selected to study their regulatory role in exocytosis (see subsequent Chapters). Specifically, histamine induced a peak and sustained plateau of $[\text{Ca}^{2+}]$ elevation. In contrast, angiotensin II evoked only a peak Ca$^{2+}$ response. As seen in Chapters 4 and 5, the subsequent focus of this work will be on comparing the effects of ionotropic receptors on catecholamine release with those of either histamine or angiotensin II Goq/11-coupled receptors.

3.3.2 Ca$^{2+}$ signalling-mediated by Goq/11-coupled receptors and ionotropic receptors in bovine chromaffin cells

The results presented in this chapter indicate that bovine adrenal chromaffin cells express a wide variety of receptors coupled to Goq/11 that mediate Ca$^{2+}$ signalling, including those for angiotensin II, histamine,
acetylcholine, and bradykinin. This is in agreement with previous findings that indicated the presence of a wide range of \( \text{Ga}_{\alpha q/11} \)-coupled receptors many of which also elicit exocytosis. This includes \( \text{AT}_1 \) angiotensin II receptors, \( \text{H}_1 \) histamine receptors, cholinergic receptors, and \( \text{B}_2 \) bradykinin receptors (Feldberg and Lewis, 1964; Wilson and Kirshner, 1977; Plevin and Boarder, 1988; Sasakawa et al., 1989; Bunn et al., 1990; Bottari et al., 1993; Herrington et al., 1995; Roberts-Thomson et al., 2000; Marley, 2003). In addition, this study highlights differences in \( \text{Ca}^{2+} \) signalling profiles evoked by these receptors. Thus, histamine receptors, cholinergic receptors and bradykinin receptors evoked a peak followed by sustained plateau \( \text{Ca}^{2+} \) response, whereas activation of angiotensin II receptors mediated only a peak \( \text{Ca}^{2+} \) signal. It has been proposed that \( \text{Ga}_{\alpha q/11} \)-protein-mediated activation of PLC with subsequent generation of the \( \text{Ca}^{2+} \)-mobilising signal Ins(1,4,5)P\(_3\) and thereby mobilization of intracellular \( \text{Ca}^{2+} \) stores contributes substantially to the peak \( \text{Ca}^{2+} \) response to \( \text{Ga}_{\alpha q/11} \)-coupled receptor agonists in chromaffin cells (Zimlichman et al., 1987; O'Sullivan and Burgoyne, 1989; Plevin and Boarder, 1988; Noble et al., 1988; Forsberg et al., 1986; Sasakawa et al., 1989; Bunn et al., 1990; Stauderman et al., 1990; Roberts-Thomson et al., 2000; Kiselyov et al., 2002). It has been known for some considerable time that chromaffin cells have an Ins(1,4,5)P\(_3\)-sensitive \( \text{Ca}^{2+} \) store (Stoehr et al., 1986) located in the endoplasmic reticulum (Bayerdorffer et al., 1984; Prentki et al., 1984; Cheek and Burgoyne, 1985; Cheek et al., 1993). It was suggested previously that localization of endoplasmic reticulum at/or near one pole of the chromaffin cells might contribute to the observed localization of \( \text{Ca}^{2+} \) signals to one pole of the cells (O'Sullivan et al., 1989). Furthermore, the Ins(1,4,5)P\(_3\)-sensitive stores in chromaffin cells were localized away from the
bulk of the secretory granules in the area between the plasma membrane and nucleus where the endoplasmic reticulum is located (Burgoyne et al., 1989; O'Sullivan et al., 1989). The sustained phase of the Ca\textsuperscript{2+} response may be due to Ins(1,4,5)P\textsubscript{3} generation, the depletion of intracellular Ca\textsuperscript{2+} stores and the subsequent activation of capacitative Ca\textsuperscript{2+} entry through store-operated Ca\textsuperscript{2+} channels (SOCs) (Putney, 1986; 1990; Putney and McKay, 1999; Barritt, 1999; Fomina and Nowycky, 1999). This study revealed the contribution of capacitative Ca\textsuperscript{2+} entry to the sustained Ca\textsuperscript{2+} responses (see Chapter 6). In addition, there is evidence that activation of voltage-operated calcium channels (VOCCs) might also be involved in Ca\textsuperscript{2+} signalling evoked by G\textsubscript{q/11}-coupled receptor agonists (Zerbes et al., 1998, Marley, 2003). Previous studies have demonstrated the presence of L, N, P and Q-type on bovine adrenal chromaffin cells (Artalejo et al., 1994; López et al., 1994a; 1994b; Lara et al., 1998).

The Ca\textsuperscript{2+} signalling data also suggests that sustained Ca\textsuperscript{2+} responses mediated either by histamine, acetylcholine or bradykinin are markedly dependent on extracellular Ca\textsuperscript{2+} influx through either VOCCs or SOCs, since elimination of extracellular Ca\textsuperscript{2+} completely abolished the sustained phase induced by these stimuli. In contrast, the peak Ca\textsuperscript{2+} responses evoked by these agonists were only partially dependent on extracellular Ca\textsuperscript{2+} influx since removal of extracellular Ca\textsuperscript{2+} partially reduced the peak Ca\textsuperscript{2+} signalling. It has been previously proposed that placing cells in low extracellular Ca\textsuperscript{2+} might itself cause some Ca\textsuperscript{2+} store depletion particularly from stores close to the membrane which contribute to Ca\textsuperscript{2+} release (Wheldon et al., 2001; Willars et al., 1995a; 1998b). The present finding is consistent with previously published work that Ca\textsuperscript{2+} responses evoked by these agonists were more transient in Ca\textsuperscript{2+} free buffer than...
in the presence of external Ca\textsuperscript{2+} (O'Sullivan et al., 1989; Stauderman and Pruss, 1990; Robinson and Burgoyne, 1991; Nassar-Gentina et al., 1997; Zerbes et al., 1998).

Data presented indicate that cholinergic receptor activation with acetylcholine in the presence of extracellular Ca\textsuperscript{2+} evoked the greatest peak Ca\textsuperscript{2+} response compared with the G\textsubscript{\alpha q/11}-coupled receptors examined in this study, but its effect was comparable to that evoked by ionotropic receptors. Since acetylcholine is the physiological secretagogue for chromaffin cells (Douglas and Rubin, 1961), similarities in Ca\textsuperscript{2+} signalling evoked by acetylcholine and nicotine might be attributed to the fact that acetylcholine activates both types of cholinergic receptors, nicotinic and muscarinic (Wilson and Kirshner, 1977). Although the biggest Ca\textsuperscript{2+} signalling might be due to the former receptor (i.e., nicotinic receptors), experiments performed in this study in the absence of extracellular Ca\textsuperscript{2+} showed some Ca\textsuperscript{2+} response mediated by acetylcholine. This suggests that some of Ca\textsuperscript{2+} response is likely to be via G\textsubscript{\alpha q/11}-coupled receptors. Another explanation for the greatest Ca\textsuperscript{2+} response evoked by acetylcholine could be due to the nature of the cholinergic receptors coupled to G\textsubscript{\alpha q/11} that triggers Ca\textsuperscript{2+} mobilization from the intracellular stores and thereby activates capacitative Ca\textsuperscript{2+} entry.

The present study demonstrated that angiotensin II exhibited a unique [Ca\textsuperscript{2+}], response profile that was different from that mediated by other G\textsubscript{\alpha q/11}-coupled receptor agonists in that it did not have a sustained plateau. Thus, Ca\textsuperscript{2+} signalling data directed the attention to select histamine and angiotensin II in order to compare their signalling pathways specifically their impact on exocytosis. Acetylcholine was excluded because it acts simultaneously on nicotinic and
muscarinic receptors present in chromaffin cells. The possible explanation for different Ca\(^{2+}\) signalling profiles mediated by histamine and angiotensin II is that angiotensin type 1A receptor rapidly desensitises (Olivares-Reyes et al., 2001). Indeed, previous studies have suggested that PKC-induced phosphorylation promotes desensitisation of the AT\(_{1A}\) receptor (Barker et al., 1995; Olivares-Reyes et al., 2001).

It is widely accepted that histamine and angiotensin II both trigger secretion of catecholamines in bovine chromaffin cells and this depends upon external Ca\(^{2+}\), with histamine being a much better secretagogue than angiotensin II (O'Sullivan et al., 1989). It has been proposed that beside the capacitative Ca\(^{2+}\) entry discussed above, the nature of the channels through which histamine and possibly angiotensin II stimulate Ca\(^{2+}\) entry and the mechanism of their opening are unknown (Robinson and Burgoyne, 1991).

Nicotinic receptor-mediated elevation of [Ca\(^{2+}\)]\(_i\) was extremely dependent on external Ca\(^{2+}\) in agreement with previously published work (Burgoyne, 1991; Nassar-Gentina et al., 1997). Activation of nAChRs is known to depolarise cells, which activates VOCCs and allows Ca\(^{2+}\) influx (Vijayaraghavan et al., 1992; Rathouz and Berg, 1994). In addition to this, nAChRs themselves can serve as Ca\(^{2+}\) entry pathways (Miledi et al., 1980; Decker and Dani, 1990; Zhou and Neher, 1993; Zhang et al., 1996b). Ca\(^{2+}\) permeability of \(\alpha 3\beta 4\) subunits of nAChRs is quite high but less than \(\alpha 7\) that exhibits high relative permeability to Ca\(^{2+}\) (Costa et al., 1994; Rathouz and Berg, 1994; McGehee and Role, 1995; Ragozzino et al., 1998). Chromaffin cells express the neuronal type of nAChR, which is represented by pentametric complexes (eight \(\alpha\) (\(\alpha 2-\alpha 9\)) and three \(\beta\) (\(\beta 2-\beta 4\)) neuronal nAChR subunits) (Sargent, 1993). Pharmacological studies indicate
the presence of multiple populations of adrenal nAChRs such as α3, α5, α7, and β4 nAChR subunits (Garcia-Guzman et al., 1995; Wenger et al., 1997). The subunit combination of nAChRs associated with the catecholamine secretion from chromaffin cells was reported to be α3β4 or α3β4α5 (Tachikawa et al., 2001). Besides Ca\(^{2+}\) influx through nAChR channels (Zhou and Neher, 1993; Harkins and Fox, 1998) and VOCCs (Kilpatrick et al., 1981; Knight and Kesteven, 1983), activation of nAChR can also induce Ca\(^{2+}\) release from ryanodine- and Ins(1,4,5)P\(_3\)-sensitive intracellular stores (Eberhard and Holz, 1987; 1988; Nakaki et al., 1988; Robinson and Burgoyne, 1991; Roberts-Thomson, 2000; Dajas-Bailador et al., 2002a). The release of Ca\(^{2+}\) from these stores and activation of capacitative Ca\(^{2+}\) entry might contribute to the sustained Ca\(^{2+}\) response triggered by nicotinic receptor activation. The sustained elevations of intracellular Ca\(^{2+}\) in response to nicotinic receptor stimulation have been observed previously in hippocampal neurones (Dajas-Bailador et al., 2000b), chick ciliary ganglia neurones (Shoop et al., 2001) and PC12 cells (Gueorguiev et al., 2000) and consistent with observations obtained from the present study on chromaffin cells. In addition, the present study shows evidence of some contribution of thapsigargin-sensitive Ca\(^{2+}\) stores to sustained Ca\(^{2+}\) response mediated by nicotinic receptor activation (see Chapter 6).

It is worthwhile to note that not all of the cells examined responded with elevated [Ca\(^{2+}\)]\(_i\) to most agonists. Indeed, the reason for this may be due to the fact that chromaffin cultures might be not entirely pure and may contain other cell types including bovine adrenal medulla endothelial cells (BAMEC) and fibroblasts (Bossu et al., 1992; Vinet and Vargas, 1999). In addition, these contaminated cells might divide in culture and can exert major distorting effects
on biochemical measurements of older cultures. Therefore, chromaffin cells should be used after only short culture periods and where possible the results confirmed with freshly isolated cells. Another explanation for failure of some chromaffin cells to respond to agonist is that the rise in $[Ca^{2+}]_i$ might be spatially restricted often to one pole of cell which might hinder $Ca^{2+}$ diffusion in cytoplasm.
4: CATECHOLAMINE RELEASE EVOKED BY ACTIVATION OF EITHER LIGAND GATED ION CHANNELS OR \( \text{Go}_{\alpha_{q/11}} \)-COUPLED RECEPTORS

4.1 INTRODUCTION

The aim of the work described in this chapter was to examine the effect of the stimulation of either nicotinic receptors or \( \text{Go}_{\alpha_{q/11}} \)-coupled receptors on catecholamine release from cultured bovine adrenal chromaffin cells.

Triggered exocytosis is the most common cellular mechanism for secretion. It consists of fusion of an intracellular vesicle with the plasma membrane and provides a highly regulated system to release a variety of substances, including neurotransmitters, as discrete packages called “quanta” (Katz, 1971). The exocytosis process is a chain of complex mechanisms that involve vesicle docking, priming, and fusion (Borges et al., 2002) (see Chapter 1). Adrenomedullary chromaffin cells are terminally differentiated secretory cells of neural crest origin, dedicated to the synthesis, storage, and release of catecholamines (Livett, 1984). Catecholamines are stored within chromaffin granules at very high concentrations (0.5-1M) (Borges et al., 2002). At least three types of chromaffin cells are present in the adrenal medulla: adrenaline (85%), noradrenaline (14-15%) and dopamine (1%) containing cells (Eaton and Dulphan, 2004). In addition, a number of different neuropeptides and proteins are co-stored and co-released with the catecholamines from the adrenal medulla (Takiyyuddin et al., 1990) including enkephalins, neuropeptide Y (NPY), dynorphin, galanin, vasoactive intestinal peptide (VIP), substance P, neurotensin, somatostatin, dopamine β-hydroxylase (DBH), chromogranins A and B (CgA, CgB), and secretogranin II (SgII) (Winkler et al., 1986; Fischer-Colbrie et al., 1995) (see Chapter 1 for details of different vesicles and differential release). In response to a
variety of stimuli, catecholamines are released and this cell model has been widely used in the study of neuroendocrine function and in investigations of the mechanisms involved in secretion.

The adrenal medulla receives cholinergic innervation from the sympathetic nervous system via the splanchnic nerve (Burgoyne, 1984a). Catecholamine secretion from chromaffin cells is evoked by acetylcholine (ACh) released from the splanchnic nerve which activates nicotinic acetylcholine receptors (nAChRs) on chromaffin cells. The influx of cations through the receptor channel causes plasma membrane depolarisation and opening of voltage-gated Ca\(^{2+}\) channels. The rapid influx of extracellular Ca\(^{2+}\) through these channels initiates a number of different processes that lead to exocytosis including docking of secretory vesicles to the plasma membrane prior to fusion and fusion of the vesicles with the plasma membrane. Following this, the emptied readily releasable pool is replenished due to breakdown of the cortical cytoskeleton and migration of secretory granules to the cell surface, and fusion of the granule membranes with the plasma membrane (Cox and Parsons, 1997; Mollard et al., 1995). In fact, both the rapid exocytotic release of catecholamines from docked vesicles (Douglas and Rubin, 1961) and the refilling of the readily releasable pool (Von Rüden and Neher, 1993) are dependent on the local rise of the \([Ca^{2+}]\). It is widely accepted that catecholamine secretion from the bovine adrenal medulla is evoked largely by nicotinic receptor activation. However, the bovine adrenal medulla also expresses a number of G protein-coupled receptors (GPCRs) that stimulate catecholamine secretion. This includes AT\(_1\) angiotensin II receptors, H\(_1\) histamine receptors, B\(_2\) bradykinin receptors, EP\(_3\) prostaglandin (PG) receptors, PAC\(_1\) receptors for pituitary adenylate cyclase-activating polypeptide (PACAP), and in some species
muscarinic cholinoceptors and P2Y purinoceptors (Marley, 2003; Marley et al., 2002; Aguilar et al., 1992). The efficacy of several of these receptors, including those for angiotensin II, bradykinin, ATP and prostaglandins is very low; however, receptors for histamine and PACAP are highly effective and comparable to powerful secretagogues such as nicotinic receptor agonists and K+ depolarization (Donald et al., 2002). Indeed, there is considerable evidence that these Gαq/11-coupled receptors play substantial physiological roles in driving adrenal catecholamine secretion in parallel to and in cooperation with nicotinic receptors. Like the secretory responses to nicotinic receptor activation and K+-evoked depolarisation, the secretion in response to Gαq/11-coupled receptor agonists depends on the influx of extracellular Ca^{2+} (Marley, 2003).

Several pieces of evidence link actin to the dynamic regulation of secretion and neurotransmission (Viviani et al., 1996). It has been proposed that the actin microfilament network localized underneath the plasma membrane of chromaffin cells (Lee and Trifaró, 1981; Trifaró et al., 1984; Cheek and Burgoyne, 1986) acts as a barrier to the movement of secretory granules, blocking their access to exocytosis sites at the plasma membrane (Trifaró et al., 1982; Burgoyne, 1991). Thus, removal of the actin barrier would allow the free movement of granules and their subsequent interaction with the plasma membrane (Lelkes et al., 1986). Prior to the disruption of the F-actin network very few secretory vesicles are found between it and the plasma membrane. This region, 0-50nm wide, is occupied by 1.2-2.4% of the total number of secretory vesicles present in chromaffin cells (Vitale et al., 1995). It has been shown that nicotinic receptor stimulation of chromaffin cells induces the disassembly of cortical filamentous actin (F-actin) (Cheek and Burgoyne, 1986 and Rodríguez Del
Castillo et al., 1992) allowing the free movement of secretory vesicles to release sites at the plasma membrane (Vitale et al., 1991). There is evidence that secretion mediated by H₁ histamine receptors is a consequence of redistribution of subplasmalemmal scinderin (an F-actin severing protein) concomitantly with cortical F-actin disassembly (Zhang et al., 1995b). Two pathways are known to be involved in the control of chromaffin cell cortical F-actin networks during secretion: the Ca²⁺-scinderin and PKC-MARCKS pathways (Cuchillo-Ibáñez et al., 2004). Several lines of research suggest the involvement of PKC in the regulation of actin cytoskeleton organization and dynamics and this may contribute to facilitating catecholamine secretion (Rosè et al., 2001). This contributory effect of PKC may be attributed to the phosphorylation of cytoskeletal proteins and the disruption of cortical F-actin near the plasma membrane. This would then allow an increase in the size of readily releasable pool of secretory granules (Gillis et al., 1996). Acute treatment of chromaffin cells with PMA disrupts the F-actin network in some cortical areas and causes a 2-3 fold increase in the number of secretory vesicles within 0-50nm of the plasma membrane (Trifaró et al., 2000). In addition, PMA does not change the basal catecholamine secretion but enhances the initial rate of secretion in response to nicotine.

In the basal (unstimulated) state, F-actin can be visualized with rhodamine-labeled phalloidin as a strong cortical fluorescent ring (Cheek and Burgoyne, 1986; Vitale et al., 1991; 1995). Upon stimulation this cortical fluorescent ring is disrupted. Since phalloidin is a probe for F-actin, the disappearance of rhodamine fluorescence indicates disassembly of actin filaments at specific subplasmalemmal areas (Vitale et al., 1991). Disassembly of cortical F-
actin in response to stimulation is thought to allow the movement of vesicles from the reserve compartment to replenish the readily releasable pool in preparation for exocytosis to occur (Vitale et al., 1991; 1995).

The aims of the experiments described in this chapter were to characterize the effect of activation of either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors on secretion of catecholamines from cultured bovine adrenal chromaffin cells. In this study KCl was used as control depolarizing agent. Catecholamine release from populations of chromaffin cells was monitored by a spectrofluorimetric assay of extracellular catecholamines and referenced (as %) to the total cellular content determined after cell lysis (see Chapter 2). The effect of activation of either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors on cytoskeletal cortical F-actin integrity was then investigated and the impact of chemical stabilization of cortical F-actin filaments on catecholamine secretion determined.
4.2 RESULTS

Time-dependent increase in catecholamine secretion-evoked by nicotinic receptors

Release (at 37°C) from monolayers of cultured (3 days) cells was monitored by a spectrofluorometric assay of extracellular catecholamines and referenced (as %) to the total cellular content determined after cell lysis as described in “Materials and Methods”. Figure 4.2.1 shows a time-dependent increase in the release of catecholamines evoked by nicotine (100µM). At the zero time, the basal release was 2.6 ± 0.8%. Catecholamine release during incubations of 30s, 1 min, 3 min, and 5 min were 4.0 ± 0.3%, 6.0 ± 0.5%, 10.3 ± 0.3%, and 17.7 ± 0.6% of the cell content, respectively. The maximal release (24.70%) was observed at 10 min stimulation with nicotine. With 10 min and 20 min treatments, the extent of release was similar (24.7% and 24.7%, respectively) release was approximately similar. Therefore, a 10 min incubation was selected as suitable time for incubation throughout the catecholamine assays.
Figure 4.2.1. Time course of nicotine-mediated secretion of catecholamines from bovine adrenal chromaffin cells. Chromaffin cells were incubated with 100μM nicotine for various periods as indicated. The amount of catecholamines released into the buffer was measured as described in “Materials and Methods” and is expressed as a percentage of total cellular content. Data show mean ± SEM from three different cell preparations. * P<0.05. ** P<0.01, *** P<0.001 by one-way ANOVA followed by post-hoc Dunnett's test vs. zero time.
Effect of the activation of either ligand gated ion channels or $G\alpha_{q/11}$-coupled receptors on secretion of catecholamines

As shown in Figure 4.2.2, either nicotine (100µM) or KCl (40mM) caused a significant release of catecholamines that was abolished by removal of 1.3mM extracellular Ca$^{2+}$ (i.e. no added Ca$^{2+}$ to Krebs'/HEPES buffer) (Figure 4.2.2). Catecholamine secretion evoked by nicotine was significant greater than that mediated by KCl (Figure 4.2.2). Activation of $G\alpha_{q/11}$-coupled receptors, particularly histamine (100µM) and angiotensin II (100nM) evoked catecholamine secretion although considerably less than that evoked by either nicotine or KCl (Figure 4.2.3)

Both nicotine and KCl evoked a concentration-dependent increase in catecholamine release, with pEC$_{50}$ values of 5.40 ± 0.75 for nicotine and 1.64 ± 0.65 for KCl (Figure 4.2.4: panel A and B). Although there was evidence of concentration-dependent increases in response to either histamine or angiotensin II, sigmoidal curves could not be fitted to the concentration-response curves making accurate determination of EC$_{50}$ values problematic (Figure 4.2.4: panel C and D) (Table 4.2.1).

To test whether either nicotine or KCl potentiate the secretion of catecholamines evoked by $G\alpha_{q/11}$-coupled receptors, chromaffin cells were treated with histamine (100µM) in combination with either nicotine (100µM) or KCl (40mM) for 10 min. Histamine had no additional impact on catecholamine secretion as it did not enhance the release evoked by either nicotine alone or KCl alone. Similarly, angiotensin II (100nM) did not influence the secretion evoked by either nicotine or KCl (Figure 4.2.3).
Figure 4.2.2. The influence of extracellular Ca\(^{2+}\) on either nicotine or K\(^+\)-evoked secretion of catecholamines from bovine adrenal chromaffin cells. Chromaffin cells were challenged with nicotine (100μM) or KCl (40mM) for 10 min at 37°C either in the presence of 1.3mM external Ca\(^{2+}\) or in Ca\(^{2+}\)-free buffer (i.e. no added Ca\(^{2+}\)). Catecholamine release was measured as described in "Materials and Methods" and is expressed as a percentage of total cellular content. (Basal release is that from unstimulated cells). Data are mean ± SEM from three different cell preparations (each in duplicate). *** P<0.001 by one-way ANOVA followed by post-hoc Bonferroni’s test. (Not all comparisons are shown on the graph).
Figure 4.2.3. Effect of $\text{G}_{\alpha_q/11}$-coupled receptors on secretion of catecholamines from bovine adrenal chromaffin cells. Chromaffin cells were challenged with either agonists of $\text{G}_{\alpha_q/11}$-coupled receptors alone (i.e. histamine (100µM) or angiotensin II (100nM)) or a combination of activators of $\text{G}_{\alpha_q/11}$-coupled receptors and ligand gated ion channels (i.e. nicotine (100µM) and KCl (40mM)) for 10 min at 37°C. Catecholamine release was measured as described in “Materials and Method” and is expressed as a percentage of total cellular content. Data show mean ± SEM from three different cell preparations. * $P<0.05$, ** $P<0.001$ vs. basal (unstimulated cells) by one-way ANOVA followed by post-hoc Dunnett’s test.
Figure 4.2.4. Concentration-dependent effects of nicotine, K\(^+\), angiotensin II and histamine on secretion of catecholamines from bovine adrenal chromaffin cells. Chromaffin cells challenged with various concentrations of nicotine (panel A, pEC\(_{50}\) 5.4 (~4.0µM)), KCl (panel B, pEC\(_{50}\) 1.64 (~23mM)), histamine (panel C, pEC\(_{50}\) 5.25 (~5µM)) and angiotensin II (panel D, pEC\(_{50}\) 8.25 (~5nM)). Full details of the pEC\(_{50}\) values are given in Table 4.2.1. Catecholamine release was measured as described in Materials and Methods and is expressed as percentage of total cellular content. Data are mean ± SEM from three different cell preparations.
**Table 4.2.1: Summary of pEC$_{50}$ values for nicotine, KCl, histamine and angiotensin II evoked secretion of catecholamines from bovine adrenal chromaffin cells.**

<table>
<thead>
<tr>
<th></th>
<th>nicotine</th>
<th>KCl</th>
<th>histamine</th>
<th>angiotensin II</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEC$_{50}$ ± SEM</td>
<td>5.40 ± 0.75</td>
<td>1.64 ± 0.65</td>
<td>5.25 ± 0.56</td>
<td>8.25 ± 0.43</td>
</tr>
<tr>
<td>Hill slope</td>
<td>4.51</td>
<td>4.19</td>
<td>0.64</td>
<td>0.39</td>
</tr>
</tbody>
</table>

- Sigmoidal curves could not be fitted to the concentration-response curves for either angiotensin II or histamine. The pEC$_{50}$ values are therefore very approximate values based on the curves.
The effect of jasplakinolide, an agent that prevents F-actin depolymerization and stabilizes the actin network (Oheim and Stuhmer, 2000) was examined in chromaffin cells. Stimulation of cells with either nicotine (100μM, 40s) or cytochalasin D (2μM, 10 min) alone caused significant disassembly of cortical F-actin, which was almost completely prevented by pretreatment of cells with jasplakinolide (10μM, 30 min) (Figures 4.2.6a and 4.2.6b).
Figure 4.2.5a. Effect of either cytochalasin D, PMA, nicotine, histamine, or angiotensin II on the cortical F-actin network. Chromaffin cells were incubated in the absence of test agents or with cytochalasin D (2 μM, 20 min), PMA (1 μM, 10 min), nicotine (100 μM, 40 s), histamine (100 μM, 10 min), and angiotensin II (100 nM, 10 min) at 37°C. Following these treatments, the cells were fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under "Materials and Methods. The arrows in the left hand corner of the upper panels point out the confocal image of the cell which were then subject to image analysis. Three-dimensional image analysis were performed using either "UltraView software" (Perkin-Elmer Life Science, Cambridge, UK) (a, b, c, d, e, and f) where the maximum intensity of the fluorescent ring was set to 4096 arbitrary units or using the public domain program "Image J" (a', b', c', d', e', and f'). Images are representative of approximately six hundred cells from three different cell preparations. Control cells show an intact cortical ring (a, a') with a uniform fluorescence intensity pattern. Cytochalasin D, PMA, nicotine, histamine, and angiotensin II treated cells show a disrupted cortical ring (b, c, d, e, and f) with irregularities such as patches of fluorescence.
Figure 4.2.5b. Effects of cytochalasin D, PMA, nicotine, histamine, and angiotensin II on the integrity of cortical F-actin. Chromaffin cells were incubated in the absence of test agents or with cytochalasin D (2μM, 20 min), PMA (1μM, 10 min), nicotine (100μM, 40s), histamine (100μM, 10 min), or angiotensin II (100nM, 10 min) at 37°C. Following these treatments, the cells were fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under “Materials and Methods”. The rhodamine cortical staining of chromaffin cells was analysed and classified as being either continuous or discontinuous (see Figure 4.2.5a) and the percentage of cells displaying cortical F-actin disassembly (disrupted cortical rhodamine staining) calculated. Six hundred cells from a total of three different cell cultures were examined. Data shown are mean ± SEM. ** P<0.01, *** P<0.001 vs. control by one-way ANOVA followed by post-hoc Dunnett’s test.
Figure 4.2.6a. Effect of a cortical F-actin stabilizer on cortical F-actin disassembly mediated by either cytochalasin D or nicotine. Chromaffin cells were incubated with or without jasplakinolide (10µM, 30 min) at 37°C before stimulation with either buffer control, cytochalasin D (2µM, 20 min) or nicotine (100µM, 40s). Following these treatments, the cells were fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under “Materials and Methods”. The arrows at the left corner of panels point out the confocal image of the cell. Three-dimensional image analysis was performed using “UltraVIEW software” (Perkin-Elmer Life Science, Cambridge, UK) where the maximum intensity of the fluorescent ring was set to 4096 arbitrary units. Images are representative of approximately six hundred cells from three different cell culture preparations. Control cells show an intact cortical ring with a uniform fluorescence intensity pattern. In the absence of jasplakinolide, either nicotine or cytochalasin D show a disrupted cortical ring with irregularities such as patches of fluorescence. However, preincubation of the cells with jasplakinolide essentially prevents the disruption of cortical F-actin by either nicotine or cytochalasin D. Mean data are shown in Figure 4.2.6b.
Figure 4.2.6b. Effect of jasplakinolide on cortical F-actin disassembly mediated by either cytochalasin D or nicotine. Chromaffin cells were incubated with either buffer control, cytochalasin D (2µM, 30 min) or nicotine (100µM, 40s) following preincubation with jasplakinolide (10µM, 30 min) at 37°C. Following these treatments, the cells were fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under “Materials and Methods”. The rhodamine cortical staining of chromaffin cells was analysed and classified as being either (see Figure 4.2.6a) continuous or discontinuous and the percentage of cells displaying cortical F-actin disassembly (disrupted cortical rhodamine staining) calculated. Six hundred cells from a total of three different cell cultures were examined. Data shown are mean ± SEM. *** P<0.001 by one-way ANOVA followed by post-hoc Bonferroni’s test. (Not all comparisons are shown on the graph).
Effect of the cortical F-actin stabilizer jasplakinolide on secretion of catecholamines evoked by either nicotinic or G<sub>αq/11</sub>-coupled receptors

Prevention of cortical F-actin disassembly by jasplakinolide (10μM, 30 min) (see Figures 4.2.6a and 4.2.5b) significantly reduced secretion of catecholamines mediated by nicotine (100μM, 10 min) (10.5 ± 0.6% vs. 21.0 ± 3.3%, *** P<0.001) (Figure 4.2.7). In contrast, catecholamine release induced either by PMA (1μM), histamine (100μM) or angiotensin II (100nM) was not affected by stabilization of cortical F-actin with jasplakinolide (Figure 4.2.7).
Figure 4.2.7. Effect of the cortical F-actin stabilizer jasplakinolide on catecholamine secretion evoked by either PMA, nicotine, histamine or angiotensin II. Chromaffin cells were incubated with or without jasplakinolide (10μM, 30 min) at 37°C before stimulation with either buffer control, cytochalasin D (2μM, 20 min) (CD), PMA (1μM, 10 min), nicotine (100μM, 10 min), histamine (100μM, 10 min) (hist), or angiotensin II (100nM, 10 min) (ATII). Catecholamine release was measured as described in “Materials and Methods” and is expressed as percentage of total cellular content. Data are mean ± SEM from three different cell preparations. ** P<0.001, *** P<0.001 by one-way ANOVA followed by post-hoc Bonferroni’s test. (Not all comparisons are shown on the graph).
4.3 DISCUSSION

4.3.1 Summary of data

The work described in this chapter demonstrates that nicotinic receptor activation caused release of catecholamines that was abolished by removal of extracellular Ca$^{2+}$ and was both time- and concentration-dependent. Activation of G$\alpha_q$-coupled receptors with either histamine or angiotensin II was able to evoke catecholamine secretion and this was also concentration-dependent. Nicotinic receptor activation caused fragmentation of the cortical F-actin ring whereas stabilization of F-actin with an F-actin stabilizer (jasplakinolide) inhibited both nicotine-mediated fragmentation and catecholamine secretion and this strongly suggests that nicotinic receptor-mediated catecholamine secretion is crucially dependent on the reserve pool to replenish the readily releasable pool with secretory granules. Although, activation of G$\alpha_q$-coupled receptors with either histamine or angiotensin II mediated disruption of the cortical F-actin filaments, catecholamine secretion evoked by either histamine or angiotensin II was not affected by stabilization of cortical F-actin. Thus, this gives an indication that G$\alpha_q$-coupled receptor-mediated catecholamine release is independent of the reserve pool.

4.3.2 Effect of K$^+$ depolarization or the activation of either ligand-gated ion channels or G$\alpha_q$-coupled receptors on secretion of catecholamines

The bovine adrenal chromaffin cell has been widely used as model to study the dynamics of exocytosis and pools of neurotransmitter-storing vesicles (Burgoyne and Morgan, 1998a; Gillis and Chow, 1997; Morgan and Burgoyne, 1997). It is generally agreed that the adrenal medulla releases catecholamines into the blood stream in response to both splanchnic nerves and humoral stimulation.
(Douglas, 1968; Alamo et al., 1991). It has been suggested that although secretion is mainly triggered by nicotinic stimulation, other receptors such as H₁ histamine receptors and AT₁ angiotensin II receptors can also promote secretion of catecholamines from adrenal medullary cells (Marley, 1987; O'Sullivan and Burgoyne, 1989). This study demonstrates that activation of either nicotinic receptors or K⁺ evoked depolarisation causes a much greater release of catecholamines than stimulation of Goq₁₁-coupled receptors and this is consistent with previous published work (Marley, 2003). The results presented in this chapter also indicate that nicotine was a more effective secretagogue than KCl. This is in agreement with previous findings (Cox and Parsons, 1997).

It is widely recognized that nicotinic receptor activation results either in membrane depolarisation with the opening of VOCCs in the plasma membrane or Ca²⁺ entry through the nicotinic receptor channel itself (ionotropic receptors) (Zhou and Neher, 1993; Harkins and Fox, 1998). Thus, the additional Ca²⁺ entry through the nicotinic receptor channel itself or the spatial relationship between nicotinic receptors and the secretory machinery could explain why nicotine was a more effective secretagogue than KCl.

This study highlights the importance of external Ca²⁺, as secretion due to either nicotinic receptor activation or K⁺-depolarization was abolished by removal of Ca²⁺. This is in agreement with many other studies demonstrating an essential role of Ca²⁺ in triggering exocytosis from chromaffin cells either in population or single cell studies (Augustine and Neher, 1992; Burgoyne, 1984b; Cheek et al., 1989; Cobbold et al., 1987; Kao and Schneider, 1986; Kim and Westhead, 1989; Stauderman and Pruss, 1989; Stauderman et al., 1990).
A rise in \([\text{Ca}^{2+}]_j\) is the main trigger of regulated exocytosis in many cell types. The \(\text{Ca}^{2+}\) can be derived from entry of external \(\text{Ca}^{2+}\), by \(\text{Ca}^{2+}\) release from intracellular stores, or both. The importance of these two \(\text{Ca}^{2+}\) sources varies considerably between cell types. This ranges from cells in which \(\text{Ca}^{2+}\) entry is the only effective stimulus for exocytosis to cells in which \(\text{Ca}^{2+}\) stores are the predominant source. In adrenal chromaffin cells the release of \(\text{Ca}^{2+}\) from intracellular stores triggers only a very low level of secretion (Cheek and Burgoyne, 1985; Cheek et al., 1989; Cheek and Thastrup; 1989; O'Sullivan et al., 1989) and this may in part be due to the capacitative (store-operated) \(\text{Ca}^{2+}\) entry that accompanies emptying of intracellular \(\text{Ca}^{2+}\) stores (Robinson et al., 1992; Fomina and Nowycky, 1999). Moreover, \(\text{Ca}^{2+}\)-dependent mechanisms triggered by nicotinic receptor stimulation include activation of PKC (Messing et al., 1989; Vainio et al., 1998; Soliakov and Wonnacott, 2001; Mahata et al., 2002), which may then influence the exocytosis process (see Chapter 5).

Although, the ionotropic nicotinic receptors play a major role in eliciting catecholamine secretion, adrenal chromaffin cells also express \(G\alpha_{q/11}\)-coupled receptors such as histamine receptors and angiotensin II receptors (see Chapter 3) which also evoke a comparatively small release of catecholamines as shown in this chapter. This finding is supported by evidence in the literature that various GPCR agonists can trigger different amounts of release although nicotinic receptor stimulation evokes the biggest response (Ali and Burgoyne, 1990; O'Sullivan and Burgoyne, 1989; Marley, 2003). Some studies demonstrated that the efficacy of several of these agonists including angiotensin II, bradykinin, ATP and prostaglandins is very low. However, histamine and pituitary adenylate cyclase-activating polypeptide (PACAP) are highly effective and comparable to
powerful secretagogues such as nicotinic agonists and \( \text{K}^+ \)-depolarization (Burgoyne, 1991; Watanabe et al., 1992; Isobe et al., 1993). This is somewhat in contrast to the data in this study, which indicated that stimulation of either histamine receptors or angiotensin II receptors elicited a very low secretory effect compared to the powerful exocytotic effects evoked by either nicotinic receptors or \( \text{K}^+ \)-depolarization. This different amount of release triggered by different secretagogues may be attributed to the spatial organization of \( \text{Ca}^{2+} \) signals (Cheek et al., 1993) as cytosolic \( \text{Ca}^{2+} \) concentrations may not be raised in a uniform manner throughout the cytoplasm by secretagogues and the pattern of the \( \text{Ca}^{2+} \) wave may depend on the pathways through which extracellular \( \text{Ca}^{2+} \) enter the cells (Kuwashima et al., 2000). Another contributing factor to the low amount of catecholamine secretion evoked angiotensin II is likely to be that angiotensin AT\(_1\) receptors rapidly desensitise (Olivares-Reyes et al., 2001).

The secretory effects of histamine on chromaffin cells are mediated exclusively by \( \text{H}_1 \) receptors which are highly expressed in the adrenal medulla (Chang et al., 1979). In chromaffin cells as in many other cells, stimulation of \( \text{H}_1 \) receptors activates PLC via a pertussis toxin-insensitive G-protein and causes the production of \( \text{Ins}(1,4,5)\text{P}_3 \) (Noble et al., 1986; Plevin and Boarder, 1988) and the subsequent release of \( \text{Ca}^{2+} \) from intracellular stores (Stauderman and Pruss, 1990; Stauderman and Murawsky, 1991; Challiss et al., 1991). Such \( \text{Ca}^{2+} \) released from intracellular stores can cause secretion of catecholamines but this release is transient, lasting less than 1 min and is quantitatively small (Bunn and Boyd, 1992). In contrast, the substantial release of catecholamines evoked by sustained application of histamine is abolished in \( \text{Ca}^{2+} \)-free solution and reduced by 70-80% following inhibition of VOCCs (O'Farrell and Marley, 1999; Bunn and Boyd,
These studies indicate that histamine $H_1$ receptors on chromaffin cells are coupled to the activation of VOCCs, predominantly L-, N-, and P/Q-type VOCCs, leading to the influx of extracellular Ca$^{2+}$ and that this responsible for mediating the majority of the secretory effect (Wallace et al., 2002; Marley et al., 2002).

It is well established that angiotensin II type 1 receptors (AT1Rs) are coupled to a plethora of different types of G-protein (Richards et al., 1999). It has been proposed that the stimulatory effects of angiotensin II on secretion are associated with activation of PLC, a store-dependent rise in $[\text{Ca}^{2+}]_i$, and activation of PKC (Teschemacher and Seward, 2000).

Although catecholamine secretion evoked by $G\alpha_{q/11}$-coupled receptors would be expected to be concentration-dependent, sigmoidal curves could not be fitted to the concentration-response curves for either histamine or angiotensin II. The concentration-dependence of histamine-evoked catecholamine secretion has been studied by several groups but no consistent $EC_{50}$ value has been reported. The $EC_{50}$ values are approximate for angiotensin II ($EC_{50} \sim 5\text{nM}$) and consistent with other reported values (Teschemacher and Seward, 2000), whereas the determined $EC_{50}$ value for histamine ($EC_{50} \sim 5\mu\text{M}$) was slightly higher than the reported $EC_{50}$ values (100nM-1.9µM) (Houchi et al., 1997; Livett and Marley, 1986; Bunn and Boyd, 1992; Choi et al., 1995; Noble et al., 1988).

The activation of $G\alpha_{q/11}$-coupled receptors did not augment the catecholamine release mediated by either nicotine or KCl suggesting that they may share components of the secretory pathway.
4.3.3 Cortical F-actin disassembly mediated by nicotinic and \(G_{\alpha_{q/11}}\)-coupled receptor activation

The exocytotic vesicles are present in chromaffin cells in at least two compartments: the release ready vesicle pool and reserve pool (Heinemann et al., 1993; Vitale et al., 1995). The traffic of vesicles between these compartments is subject to fine regulation. Previous studies have suggested that an F-actin microfilament network plays an important role in this regulation (Vitale et al., 1991; 1995). It is evident that in chromaffin cells, F-actin forms a cortical network which excludes the large majority of secretory vesicles from plasma membrane docking (Burgoyne et al., 1982; Vitale et al., 1995). Therefore, the F-actin network acts as a barrier (negative clamp) blocking the access of secretory vesicles to exocytotic sites at the plasma membrane. In resting chromaffin cells, 1%-3% of the total chromaffin vesicles are either docked to the plasma membrane or within 50nm of it (Vitale et al., 1995). This population of vesicles constitutes the release-ready vesicle pool (Neher and Zucker, 1993; Vitale et al., 1995). The rest of the chromaffin vesicles (97%-99%) form a reserve pool and remain behind a barrier of cortical F-actin (Vitale et al., 1995). Stimulation of chromaffin cells is accompanied by disassembly of the cortical F-actin network (Cheek and Burgoyne, 1986; Vitale et al., 1995). This allows the movement of additional secretory vesicles from the reserve pool to release sites on the plasma membrane (Vitale et al., 1995). Thus, the cortical actin network controls the size of the release-ready vesicle pool and consequently, the initial rate of exocytosis (Vitale et al., 1995).

In this study confocal microscopy of rhodamine-phalloidin-stained cells was used to assess the integrity of cortical F-actin. Rhodamine-labelled phalloidin
was used as a probe for F-actin (Vitale et al., 1991) and disappearance of cortical sub-plasmalemmal rhodamine fluorescence was considered as an index of disassembly of actin filaments. As demonstrated in this study, disassembly was evoked by nicotinic receptor stimulation and this finding is consistent with a previous study showing that the number of secretory vesicles close to the membrane increased following nicotinic receptor stimulation (Burgoyne et al., 1982). Thus, disruption of cortical F-actin would result in an increase in the size of the readily releasable pool of secretory granules (Vitale et al., 1995) allowing enhanced exocytosis of catecholamines. This study highlights the importance of cortical F-actin disassembly in the secretory process mediated by nicotinic receptors since jasplakinolide stabilizes F-actin (Oheim and Stuhmer, 2000) and prevents its disruption leading to inhibition of the recruitment of secretory granules to the site of exocytosis. These data strongly suggest that nicotinic receptor-mediated catecholamine secretion is dependent on the reserve pool to replenish the readily releasable pool with secretory granules to allow exocytosis to occur and this was consistent with previous findings (Check and Burgoyne, 1986; Vitale et al., 1991; 1995; Trifaró et al., 2000). In addition, this also could be a logical explanation for the large amount of catecholamine secretion evoked by nicotinic receptor activation. This study shows that although activation of either nicotinic receptors or Goq/11-coupled receptors mediates disassembly of cortical F-actin, this is apparently only crucial for secretion mediated by nicotinic receptors, since stabilization of cortical F-actin failed to prevent secretion of catecholamines evoked by either histamine or angiotensin II. This could be attributed to the ability of Goq/11-coupled receptors to evoke release from the pre-docked vesicles in the readily releasable pool. This could also explain the low
amount of catecholamine secretion triggered by $G_{q/11}$-coupled receptors. Similarly, this study demonstrated that F-actin disassembly mediated by either cytochalasin D, an F-actin destabilizer (Brenner and Korn, 1979, Flanagan and Lin, 1980; Schliwa, 1982) and PMA failed to trigger secretion of catecholamines and this was in agreement with previous findings that cortical F-actin disassembly modifies the extent of secretion but is itself not sufficient to activate exocytosis (Burgoyne and Cheek, 1987; Vitale et al., 1995; Cuchillo-Ibáñez et al., 2004).

It has been suggested that in chromaffin cells, the cortical actin network is controlled by scinderin, a Ca$^{2+}$-dependent F-actin severing protein (Rodríguez Del Castillo et al., 1990). In addition to scinderin, PKC-MARCKS pathway may also control the cortical actin network (Trifaró et al., 2000) (see Chapter 5).

The link between nicotinic-mediated catecholamine release and that mediated by $G_{q/11}$-coupled receptors is the elevation of [Ca$^{2+}$]. Increased cytosolic Ca$^{2+}$ plays several roles including driving granule fusion with the plasma membrane through a Ca$^{2+}$ sensor possibly a member of the synaptotagmin family (Burgoyne and Morgan, 1998b). Additionally, activation of Ca$^{2+}$ entry at the plasma membrane may trigger fusion of vesicles docked close to the Ca$^{2+}$ channels or promote vesicle priming through effects on Ca$^{2+}$-binding proteins such as DOC2, rabphilin or CAPS (Benfenati et al., 1999; Elhamdani et al., 1999). Furthermore, Ca$^{2+}$ may disrupt the F-actin network located below the plasma membrane (Vitale et al., 2000; Doussau and Augustine, 2000) as Ca$^{2+}$ released from Ins(1,4,5)P$_3$-sensitive stores may act locally to produce actin disassembly through activation of Ca$^{2+}$-sensitive actin-severing proteins, leading to vesicle recruitment from the reserve pool to the release-ready vesicle pool (Zhang et al., 1995b). Moreover, generation of DAG at the plasma membrane
may increase vesicle docking and priming by activation of PKC which leads to phosphorylation of cytoskeletal proteins controlling vesicle recruitment to the release-ready vesicle pool (Vitale et al., 1995) (see Chapters 5 and 6), and/or phosphorylation of proteins that regulate SNARE complex formation (Turner et al., 1999). Additionally, DAG may also activate Munc-13 directly to regulate SNARE complex formation (Turner et al., 1999).

Since the role of PKC in neurotransmitter evoked by nicotinic receptors and Goq11-coupled receptors is still a matter of debate, the role of PKC in exocytosis mediated by either nicotinic receptors and Goq11-coupled receptors will be investigated in the next chapter.
5: THE ROLE OF PKC IN AGONIST-MEDIATED RELEASE OF CATECHOLAMINES FROM CHROMAFFIN CELLS

5.1 INTRODUCTION

Within this chapter the major aims were to determine the specific PKC isoforms present in chromaffin cells and activated in response to stimulation of the different receptors. Further, these studies were designed to explore the roles of the different isoforms in the regulation of exocytosis and determine the potential nicotinic receptor-mediated activation of MARCKS as a possible link between PKC activation, disruption of the cortical F-actin cytoskeleton and PKC-dependent enhanced catecholamine release (see Chapter 1 for details).

More than one PKC isoform is usually expressed in a single cell type, thus leading to the notion that each member of the PKC superfamily plays a specific role in the processing of physiological and pathophysiological responses to extracellular stimuli (Sena et al., 2001). PKC is generally in the cytosol in the inactive state but after cell stimulation translocates to the plasma membrane where it becomes activated in the presence of specific lipid co-factors. Translocation of PKC to the plasma membrane is considered a hallmark for activation (Mochly-Rosen et al., 1990). In addition, it is evident that specific PKC isoforms can also translocate to other subcellular locations including the membrane of other vesicles, nuclear structures and cytoskeletal components.

Translocation of PKC from the cytosolic to membrane fraction cell has been observed following activation of Gαq/11-coupled receptors that mediate PtdIns(4,5)P2 breakdown (Terbush and Holz, 1986; Willars et al., 1996; Sena et al., 1996; Teschemacher and Seward, 2000; Bartlett et al., 2005). Although, the traditional view that Gαq/11-coupled receptor activation is required for PKC
activation, there is evidence for activation of PKC by ligand-gated ion channels including nicotinic receptors (Mollard et al., 1995; Vainio et al., 1998; Soliakov and Wonnacott, 2001; Roberts-Thomson et al., 2000). There have been a number of approaches to defining precisely which PKC isoforms are activated in response to particular stimuli including the determination of cytosol to membrane translocation by Western blotting of cell fractions. Similarly, immunocytochemical studies with isoform-specific antibodies have been used to determine the subcellular localization of PKC isoforms (Goodall et al., 1997; Buchner et al., 1999). In addition, cells can be transfected to express fusion constructs of enhanced green fluorescent protein (eGFP) and PKC, which allows the dynamics of PKC translocation in response to different stimuli to be monitored in live cells in real time (Sakai et al., 1997; Shirai et al., 1998). Studies described within this chapter use this range of techniques to address the issue of which PKC isoforms are activated. (Cubitt et al., 1995; Sakai et al., 1997).

Although, a great many previous studies have used phorbol esters (Blumberg et al., 1984; Terbush and Holz, 1990; Ryves et al., 1991; Tanaka and Nishizuka, 1994; Nishizuka, 1995; Sena et al., 1996; Sakai et al., 1997; Mochly-Rosen and Gordon, 1998), this may lack specificity and result in a massive and uncontrolled stimulation of PKC that may not be physiologically relevant. The current study specifically seeks to determine receptor/agonist-dependent PKC activation.

The work described within this chapter focus on the role of PKC in nicotinic receptor- and Gαq/11-coupled receptor-mediated secretion of catecholamines in chromaffin cells. In order to pursue this there were three specific aims. Firstly, to identify the PKC isoforms expressed in bovine chromaffin cells; secondly, to identify which PKC isoforms are activated in
response to stimulation of either nicotinic receptors or $\text{G}_\alpha(q11)$-coupled receptors. Thirdly, to determine the impact of PKC activation on the integrity of cortical F-actin and finally to assess whether there was any evidence for an involvement of PKC-mediated phosphorylation of MARCKS in PKC-mediated effects on the cortical F-actin cytoskeleton.
5.2 RESULTS

Identification of PKC isoforms in bovine chromaffin cells

Immunoblotting of whole cell extracts for PKC isoform protein demonstrated that PKCα, PKCβ, PKCε and PKCζ were present (Figure 5.2.1). Antibodies to PKCγ, PKCθ, PKCδ, PKCη and PKCλ failed to locate any immunoreactive protein at the predicted molecular weight when used in immunoblots of whole chromaffin cell lysate (Figure 5.2.1, lane A).

The immunoreactivity of all PKC antibodies was verified using rat brain lysate (Figure 5.2.1, lane B) and bovine brain extract (Figure 5.2.1, lane C). Using a similar protein concentration of bovine and rat brain to that of the bovine chromaffin cell extract (30μg protein.lane⁻¹), antibodies to PKC-γ, -θ, -δ, -η and λ detected bands at the appropriate molecular weights, confirming the ability of the antibodies to detect these bovine isoforms.
<table>
<thead>
<tr>
<th>Antibody vs. PKC isoform</th>
<th>Lane</th>
<th>kDa</th>
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<tbody>
<tr>
<td>PKCα</td>
<td>A</td>
<td>82</td>
</tr>
<tr>
<td>PKCβ</td>
<td>B</td>
<td>80</td>
</tr>
<tr>
<td>PKCε</td>
<td>C</td>
<td>90</td>
</tr>
<tr>
<td>PKCι</td>
<td>A</td>
<td>74</td>
</tr>
<tr>
<td>PKCγ</td>
<td>B</td>
<td>80</td>
</tr>
<tr>
<td>PKCδ</td>
<td>C</td>
<td>79</td>
</tr>
<tr>
<td>PKCη</td>
<td>A</td>
<td>82</td>
</tr>
<tr>
<td>PKCλ</td>
<td>B</td>
<td>74</td>
</tr>
</tbody>
</table>

Figure 5.2.1. PKC isoform expression in bovine chromaffin cells. Lane A) chromaffin cell whole lysate (30μg protein.lane⁻¹) probed with a range of antibodies demonstrated the presence of PKC-α, -β, -ε and -ι isoforms. The immunoreactivity of all PKC antibodies was confirmed using rat brain lysate (30μg.lane⁻¹) (lane B) and bovine brain extract (30μg.lane⁻¹) (lane C) as positive controls. Immunoblots of chromaffin cell whole lysate are representative of five experiments (three different cell culture preparations) whereas immunoblots of either rat brain lysate or bovine brain extract are representative of three independent experiments (using the same frozen rat or bovine brain extracts).
Agonist-mediated translocation of PKC isoforms

Western blotting of cytosol and membrane fractions followed by densitometry was used to assess agonist-evoked redistribution of detectable isoforms of PKC (PKC-α, -β, -ε, and -ι, see Figure 5.2.1) in bovine chromaffin cells as an index of PKC activation (Newton, 1997; Mochly-Rosen and Gordon, 1998). To validate the translocation method, the effect of exposure to PMA (1μM) on the distribution of PKC isoforms present in chromaffin cells was examined. The basal distribution of PKC-α, -β, -ε and -ι before PMA treatment was more prominent in the cytosolic fraction (81%, 87%, 85% and 100%, respectively) compared to the membrane fraction (19%, 13%, 15% and 0%, respectively). PMA resulted in the translocation of PKC-α, -β and -ε from the cytosol to membrane fraction (Figure 5.2.2). In contrast, PKCι was located exclusively in the cytosol and showed no translocation to membrane following treatment with PMA (Figure 5.2.2).

This investigation into agonist-mediated translocation of PKC demonstrated that PKC-α, -β and -ε and -ι translocated to the membrane following histamine receptor activation with histamine (100μM). Histamine was used at 100μM as this concentration was previously shown to induce maximal Ca\textsuperscript{2+} responses in these cells (see Chapter 2). The rapid loss of PKCε from the cytosol to the membrane after 1min of histamine treatment was more profound than loss of PKCβ, PKCα and PKCι (Figure 5.2.3: panel A and B). Indeed, following 1 min of stimulation with histamine, PKCε was the only isoform to show a significant translocation (Figure 5.2.3). The pattern of loss of the PKC isoforms from the cytosol was mirrored by changes in the immunoreactivities within the membrane fraction (Figure 5.2.3: panel C and D). Stimulation with
another G\alpha_{q/11}-coupled receptor agonist, angiotensin II (100nM) (this concentration was also shown to evoke maximal Ca^{2+} responses in these cells, see Chapter 2) did not cause a significant loss of either PKC\beta or PKC\gamma from the cytosol. Furthermore, recruitment of PKC\alpha to the membrane fraction was only significant after 10 min incubation with angiotensin II. In contrast, there was a complete loss of PKC\epsilon from the cytosol at 10 min (Figure 5.2.4: panel A and B). In addition, the membrane bound PKC\epsilon dramatically increased between 2 and 10 min of stimulation (Figure 5.2.4: panel C and D).

Activation of nicotinic receptors with nicotine (100\mu M) (a concentration chosen based on its ability to evoke maximal Ca^{2+} responses in these cells, see Chapter 2) caused a translocation of PKC-\alpha, -\beta and -\epsilon to the plasma membrane. The membrane association of PKC\epsilon was greater than that of PKC\alpha and PKC\beta (Figure 5.2.5: panel C and D). In contrast, PKC\gamma showed no significant translocation from the cytosolic fraction over a 10 min stimulation period (Figure 5.2.5: panel A and B).
Figure 5.2.2. PMA-mediated translocation of PKC isoforms. A, C: immunoblots showing the effect of exposure to PMA (1μM) on PKC-α, -β, -ε, and -τ associated with either the cytosol (A) or membrane (C) fractions from cultured bovine adrenal chromaffin cells. Cells were treated for the indicated times after which membrane and cytosol fractions were prepared and immunoblotted for the PKC isoforms. The densities of the immunoblots in cytosolic (B) and membrane (D) fractions were quantified using Scion Image analysis software (v4.02 beta, Frederick, Maryland, USA). Cytosolic and membrane values were calculated as a percentage of the total content (i.e. membrane plus cytosolic). Immunoblots (A, B) representative of four experiments on different cell cultures. Mean data (C, D) are mean ± SEM, n=4. For * P<0.05, ** P<0.01, *** P<0.001 by one-way ANOVA followed by Dunnett’s test vs. zero time. The cytosolic fraction was probed with β-actin to confirm equal loading.
Figure 5.2.3. Histamine-mediated translocation of PKC isoforms. A, C: immunoblots showing the effect of exposure to histamine (100 μM) on PKC-α, -β, -ε, and -ι associated with either the cytosol (A) or membrane (C) fractions from cultured bovine adrenal chromaffin cells. Cells were treated for the indicated times after which membrane and cytosol fractions were prepared and immunoblotted for PKC isoforms. The densities of the immunoblots in cytosolic (B) and membrane (D) fractions were quantified using Scion Image analysis software (v4.02 beta, Frederick, Maryland, USA). Cytosolic and membrane values were calculated as a percentage of the total content (i.e. membrane plus cytosolic). Immunoblots (A, B) representative of four experiments on different cell cultures. Mean data (C, D) are mean ± SEM, n=4. For * P<0.05, ** P<0.01, *** P<0.001 by one-way ANOVA followed by Dunnett's test vs. zero time.
Figure 5.2.4. Angiotensin II-mediated translocation of PKC isoforms. A, C: immunoblots showing the effect of exposure to angiotensin II (100nM) on PKC-α, -β, -ε and -ι associated with either the cytosolic (A) or membrane (C) fractions from cultured bovine adrenal chromaffin cells. Cells were treated for the indicated times after which membrane and cytosol fractions were prepared and immunoblotted for PKC isoforms. The densities of the immunoblots in cytosolic (B) and membrane (D) fractions were quantified using Scion Image analysis software (v4.02 beta, Frederick, Maryland, USA). Cytosolic and membrane values were calculated as a percentage of the total content (i.e. membrane plus cytosolic). Immunoblots (A, B) representative of four experiments on different cell cultures. Mean data (C, D) are mean ± SEM, n=4. For * P<0.05, ** P<0.01, *** P<0.001 by one-way ANOVA followed by Dunnett’s test vs. zero time. Note that the distribution of PKC-β and -ι was exclusively associated with the cytosolic fraction throughout and have therefore been omitted from the graphs for clarity. The cytosolic fraction was probed with β-actin to confirm equal loading.
Figure 5.2.5. Nicotine-mediated translocation of PKC isoforms. A, C: immunoblots showing the effect of exposure to nicotine (100μM) on PKC-α, -β, -ε and -ι associated with either the cytosolic (A) or membrane (C) fractions from cultured bovine adrenal chromaffin cells. Cells were treated for the indicated times after which membrane and cytosol fractions were prepared and immunoblotted for PKC isoforms. The densities of the immunoblots in cytosolic (B) and membrane (D) fractions were quantified using Scion Image analysis software (v4.02 beta, Frederick, Maryland, USA). Cytosolic and membrane values were calculated as a percentage of the total content (i.e. membrane plus cytosolic). Immunoblots (A, B) representative of four experiments on different cell cultures. Mean data (C, D) are mean ± SEM, n=4. For * P<0.05, ** P<0.01, *** P<0.001 by one-way ANOVA followed by Dunnett’s test vs. zero time. The cytosolic and membrane fractions were probed with β-actin antibody to confirm equal loading and exclude contamination of membrane fraction with cytosol during the cell fractionation process.
Agonist-mediated subcellular redistribution of PKC isoforms

To characterize the subcellular redistribution of PKC isoforms in response to receptor stimulation and in particular to examine if there were isoform-specific differences in the subcellular localization, immunocytochemical studies were performed. The PKC isoform-specific antibodies used in this study recognized only single bands at the expected apparent mass for PKC-α, -β, -ε and ι on the immunoblots of bovine chromaffin cells. There was no evidence of cross-reactivity, therefore making them suitable probes for immunocytochemical localization of PKC isoforms in bovine chromaffin cells. To further ensure that the staining obtained by the PKC antibodies was specific, control fixed cells were also probed only with a FITC-conjugated goat anti-mouse secondary antibody. This procedure did not reveal any non-specific, background staining (Figure 5.2.6). Immunolocalization indicated that PKC-α, -ε, -β and ι were distributed throughout the cytoplasm in unstimulated cells. Treatment of cells for 10 min with either nicotine (100μM) or PMA (1μM) caused a redistribution to the plasma membrane of PKC-α, -ε, -β but not PKCι. Stimulation of Gαq/11-coupled histamine receptors with histamine (100μM) for 10 min resulted in the redistribution of all PKC isoforms (PKC-α, -ε, -β and -ι) in chromaffin cells to the plasma membrane. In contrast, activation of Gαq/11-coupled angiotensin receptors with angiotensin II (100nM) for 10 min caused the redistribution of only PKC-α and -ε to the plasma membrane (Figure 5.2.6) (PKC expression and localization are summarized in Table 5.2.1).
Figure 5.2.6. Immunolocalization of PKC-α, -ε, -β and 1 in cultured bovine adrenal chromaffin cells. Cells cultured on coverslips, were fixed and processed for immunocytochemistry as described in “Materials and Methods”. PKC-α, -ε, -β and 1 were distributed throughout the cytoplasm before stimulation with agonists. Cells treated for 10 min with PMA (1µM), nicotine (100µM), or angiotensin II (100nM) caused a redistribution of PKC-α, -ε and -β to the plasma membrane while treatment of the cells with histamine (100µM) for 10 min caused a redistribution of PKC-α, -ε, -β and 1 to the plasma membrane. Unstimulated cells were also probed only with a FITC-conjugated goat anti-mouse secondary antibody to ensure specificity of the immunochemical reactivity. Pictures are representative of four different cell culture preparations.

<table>
<thead>
<tr>
<th></th>
<th>FITC</th>
<th>unstimulated</th>
<th>PMA</th>
<th>nicotine</th>
<th>histamine</th>
<th>angiotensin II</th>
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<tbody>
<tr>
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Table 5.2.1 Summary of the expression of PKC isoforms and their translocation to the plasma membrane in response to PMA or receptor activation in bovine adrenal chromaffin cells.

<table>
<thead>
<tr>
<th>PKC isoform</th>
<th>Localisation in unstimulated cells (immunoblot)</th>
<th>Cytosol to membrane translocation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>PMA (1μM)</td>
</tr>
<tr>
<td>α</td>
<td>cytosol: 83% membrane: 17%</td>
<td>+</td>
</tr>
<tr>
<td>β</td>
<td>cytosol: 88% membrane: 12%</td>
<td>+</td>
</tr>
<tr>
<td>ε</td>
<td>cytosol: 68% membrane: 37%</td>
<td>+</td>
</tr>
<tr>
<td>γ</td>
<td>cytosol: 100% membrane: 0%</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are compiled from Figures 5.2.2, 5.2.3, 5.2.4, 5.2.5 and 5.2.6. Note that these data are compiled from both immunoblots and immunocytochemistry.

+ indicates translocation, - indicates no translocation.
Based on 10 min stimulation.
Agonist-mediated translocation of eGFP-PKCβII and eGFP-PKCδ

Whilst the immunoblotting and immunocytochemical approaches give valuable information on the translocation and activation of PKC isoforms, these techniques have limited temporal resolution. To overcome this, fusion constructs between eGFP (Tsien, 1998) and either PKCβII (Feng and Hannun, 1998) or PKCδ (Chiesa et al., 2001) were used to monitor the dynamics of membrane translocation of PKC isoforms in real time in living cells. These isoforms represent the conventional and novel classes of PKC isoforms, respectively. Even though PKCδ is not present in bovine adrenal chromaffin cells, its availability was used to enable monitoring of the activation of novel PKC isoforms. To examine whether activation of either Gaq11-coupled receptors or nicotinic receptors recruits either eGFP-PKCβII or eGFP-PKCδ to the plasma membrane, cells were transfected with either eGFP-tagged PKCβII or PKCδ. In unstimulated cells eGFP-PKCβII and eGFP-PKCδ were distributed uniformly throughout the cytoplasm and exclude from the nucleus. Application of PMA (1μM) caused a slow (50s) and permanent fluorescence movement towards the edges of the cell reflecting translocation of either eGFP-PKCβII (Figure 5.2.7) or eGFP-PKCδ (Figure 5.2.11) to the plasma membrane.

Stimulation with histamine (100μM) induced a rapid but transient translocation of either eGFP-PKCβII (Figure 5.2.8) or eGFP-PKCδ (Figure 5.2.12) from the cytosol to the plasma membrane. Similarly, angiotensin II (100nM) induced recruitment of either eGFP-PKCβII (Figure 5.2.9) or eGFP-PKCδ (Figure 5.2.13) to the plasma membrane. Following the initial translocation to the plasma membrane there was some reversal in the continued presence of
angiotensin II, although there was clearly a sustained movement (Figures 5.2.9 and 5.2.13). Nicotinic receptor stimulation with nicotine (100µM) mediated a rapid and sustained recruitment of either eGFP-PKCβII (Figure 5.2.10) or eGFP-PKCδ (Figure 5.2.14) to the plasma membrane. The mean data reflecting the reduction in cytosolic fluorescence mediated by these agonists are summarized in Table 5.2.2.
Figure 5.2.7. Single cell imaging of PMA-induced translocation of eGFP-PKCI. Chromaffin cells transiently transfected with eGFP-PKCI were perfused with 1μM PMA and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 25 cells).
Figure 5.2.8. Single cell imaging of histamine-induced translocation of eGFP-PKC\(\beta\)II. Chromaffin cells transiently transfected with eGFP-PKC\(\beta\)II were perfused with 100\(\mu\)M histamine and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations \((n=30 \text{ cells})\).
Figure 5.2.9. Single cell imaging of angiotensin II-induced translocation of eGFP-PKCβII. Chromaffin cells transiently transfected with eGFP-PKCβII were perfused with 100nM angiotensin II and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n=35 cells).
Figure 5.2.10. Single cell imaging of nicotine-induced translocation of eGFP-PKCβII. Chromaffin cells transiently transfected with eGFP-PKCβII were perfused with 100μM nicotine and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 32 cells).
Figure 5.2.11. Single cell imaging of PMA-induced translocation of eGFP-PKCδ. Chromaffin cells transiently transfected with eGFP-PKCδ were perfused with 1μM PMA and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 27 cells).
Figure 5.2.12. Single cell imaging of histamine-induced translocation of eGFP-PKCδ. Chromaffin cells transiently transfected with eGFP-PKCδ were perfused with 100μM histamine and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 25 cells).
Figure 5.2.13. Single cell imaging of angiotensin II-induced translocation of eGFP-PKCδ. Chromaffin cells transiently transfected with eGFP-PKCδ were perfused with 100nM angiotensin II and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 25 cells).
Figure 5.2.14. Single cell imaging of nicotine-induced translocation of eGFP-PKCδ. Chromaffin cells transiently transfected with eGFP-PKCδ were perfused with 100μM nicotine and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n= 35 cells).
Table 5.2.2 Summary of agonist-induced translocation of eGFP-tagged PKC constructs

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Maximal reduction of cytosolic fluorescence</th>
<th>eGFP-PKCβII</th>
<th>eGFP-PKCδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA (1μM)</td>
<td>0.45 ± 0.02** (n=25)</td>
<td>0.49 ± 0.03** (n=27)</td>
<td></td>
</tr>
<tr>
<td>Histamine (100μM)</td>
<td>0.85 ± 0.09* (n=30)</td>
<td>0.64 ± 0.05* (n=25)</td>
<td></td>
</tr>
<tr>
<td>Angiotensin II (100nM)</td>
<td>0.53 ± 0.03* (n=35)</td>
<td>0.73 ± 0.06* (n=25)</td>
<td></td>
</tr>
<tr>
<td>Nicotine (100μM)</td>
<td>0.65 ± 0.04* (n=32)</td>
<td>0.67 ± 0.04* (n=35)</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SEM. n= the number of cells examined. Data are expressed as fold change in cytosolic fluorescence relative to basal fluorescence (=1.0) and a smaller number therefore indicates a greater change in cytosolic fluorescence. * P<0.05, ** P<0.01 vs. the basal fluorescence of raw data (i.e. fluorescence units) before normalization, as determined by paired Student’s t-test.
Role of PKC in secretion of catecholamines evoked by nicotinic receptors and Gαq/11-coupled receptors

The role of PKC in either the facilitation of nicotinic receptor-mediated catecholamine release or in the regulation of Gαq/11-coupled receptor-evoked secretion of catecholamines was examined by treating cells with isoform-selective PKC inhibitors. A chemical oxidation method was used to measure the released and cellular levels of catecholamines as described (Materials and Methods).

PMA (1μM, 10 min) evoked a small secretion of catecholamines, which although not significantly different from basal release was abolished by inhibition of PKC using either Ro31-8220 (10μM, 15 min preincubation) or myristoylated pseudosubstrate peptide inhibitor (myr-ΨPKC inhibitor, 10μM, 30 min preincubation). These two non-selective PKC inhibitors were selected because of their different structures and modes of action. Thus, Ro31-8220 is a membrane-permeant bisindolylmaleimide IX derivative and acts as a highly selective, competitive inhibitor at the ATP binding site of PKC (Davis et al., 1992). In contrast, myr-ΨPKC inhibitor appears not to interfere with ATP binding and acts by interacting with the substrate binding site in the catalytic domain, thereby keeping the enzyme in the inactive state.

Preincubation of cells with Ro31-8220 significantly attenuated secretion of catecholamines evoked by either submaximal (3μM, 10 min) or maximal (100μM, 10 min) concentrations of nicotine either alone or in combination with PMA (1μM) (Figure 5.2.15). This combination of PMA and nicotine was used to examine whether PMA modulates catecholamine secretion evoked by nicotinic receptor activation. PMA also potentiated catecholamine secretion evoked by
either submaximal or maximal concentration of nicotine. Similarly, the myr-
PYPKC inhibitor markedly reduced secretion of catecholamines mediated by either
nicotine alone or in combination with PMA (Figure 5.2.15).

Stimulation of G\alpha_{q/11}-coupled receptors with either histamine (10\mu M, 10
min) or angiotensin II (10nM, 10 min) evoked a lower secretion of
catecholamines than that evoked by nicotine. This secretion was reduced in the
presence of PMA (1\mu M) (Figures 5.2.16 and 5.2.17). Inhibition of PKC with
Ro31-8220 markedly potentiated secretion of catecholamines induced by either
histamine or angiotensin II alone or in combination with PMA (Figure 5.2.16 and
Figure 5.2.17). Note that lower concentrations of either histamine (10\mu M) or
angiotensin II (10nM) were used here to avoid full saturation of receptors that
obscure any potentiation effect due to the maximal concentrations of either
histamine or angiotensin II.
Figure 5.2.15. Effect of inhibiting PKC on catecholamine secretion evoked by nicotine and PMA. Chromaffin cells were incubated with the non-selective PKC inhibitors Ro31-8220 (10μM, 15 min) or myr-ψPKC (10μM, 30 min) at 37°C before stimulation with PMA (1μM) or either submaximal (3μM) or maximal (100μM) concentrations of nicotine for 10 min. Catecholamine release was measured as described in “Materials and Methods” and is expressed as a percentage of the total content. Data are mean ± SEM from three different cell preparations. For * P<0.05, ** P<0.01, *** P<0.001 by one-way ANOVA followed by post ANOVA test (Bonferroni’s test). Note that not all comparisons are shown on the graph.
Figure 5.2.16. Effect of inhibiting PKC on catecholamine secretion evoked by histamine and PMA. Chromaffin cells were incubated with the non-selective PKC inhibitor Ro31-8220 (10μM, 15 min) at 37°C before stimulation with either PMA (1μM) or histamine (10μM) for 10 min. Catecholamine release was measured as described in “Materials and Methods” and is expressed as a percentage of the total cellular content. Data are mean ± SEM from three different cell preparations. For * P<0.05, *** P<0.001 by one-way ANOVA followed by post ANOVA test (Bonferroni’s test). Note that not all comparisons are shown on the graph.
Figure 5.2.17. Effect of inhibiting PKC on catecholamine secretion evoked by angiotensin II and PMA. Chromaffin cells were incubated with the non-selective PKC inhibitor Ro31-8220 (10µM, 15 min) at 37°C before stimulation with either PMA (1µM) or angiotensin II (10nM) for 10 min. Catecholamine release was measured as described in “Materials and Methods” and is expressed as a percentage of the total content. Data are mean ± SEM from three different cell preparations. For * P<0.05, ** P<0.01, *** P<0.001 by one-way ANOVA followed by post ANOVA test (Bonferroni’s test). Note that not all comparisons are shown on the graph.
Regulation of phospholipase C activity by PKC

Measurement of PLC activity using the accumulation of \(^3\)H-inositol phosphates (\([^3\text{H}]\)-InsP\(_x\)) under Li\(^+\)-block (Willars et al., 1998b; 1999; Tovey and Willars, 2004) was used to determine if the reason that PKC inhibition potentiated catecholamine secretion was due to a negative feedback loop in which PKC inhibits GPCR-mediated signalling. Removal of such feedback by inhibition of PKC would be expected to enhance GPCR-mediated signalling and consequently enhance catecholamine release.

In \(^3\)H-inositol-labeled cells, in which inositol monophosphatase activity had been blocked with Li\(^+\), activation of G\(\alpha_q/11\)-coupled receptors with either histamine (100\(\mu\)M) or angiotensin II (100nM) mediated a significant increase in \([^3\text{H}]\)-InsP\(_x\) accumulation. These accumulation of \([^3\text{H}]\)-InsP\(_x\) were significantly potentiated by inhibition of PKC using Ro31-8220 (Figure 5.2.18).
Figure 5.2.18. Effect of PKC inhibition on stimulation of PLC by either nicotinic receptors or G\alpha_{q/11}-coupled receptors. Cells were prelabelled with \[^3\text{H}\]-inositol for 48 h before stimulation for 30 min in the presence of Li\(^+\), with either PMA (1\(\mu\)M), nicotine (100\(\mu\)M), histamine (100\(\mu\)M) or angiotensin II (ATII, 100nM). The accumulation of \[^3\text{H}\]-InsP\(_x\) was measured as described in "Materials and Methods". Data are presented as percentage increase over basal (the basal value was 250 ± 18 dpm/well). Data are mean ± SEM from three different cell preparations (each in triplicate). For * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\) by one-way ANOVA followed by post ANOVA test (Bonferroni's test). Note that not all comparisons are shown on the graph.
Role of specific PKC isoforms in regulating the secretion of catecholamines evoked by nicotinic receptors and \( \alpha_{q11} \)-coupled receptors

A number of commercially available isoform-selective PKC inhibitors were used to probe the involvement of specific PKC isoforms in the secretion of catecholamines evoked by either nicotinic receptors or \( \alpha_{q11} \)-coupled receptors.

The selection of inhibitors was made based on the isoforms expressed and the availability of inhibitors. The first isoform-specific PKC inhibitor used was HBDDE, which is a derivative of ellagic acid that is reported to act as an isoform-selective inhibitor of PKC\( \alpha \) and \( \gamma \) with IC\textsubscript{50} values of 43 and 50\text{nM}, respectively, determined using an \textit{in vitro} assay (Kashiwada \textit{et al.}, 1994) (note that as PKC\( \gamma \) is not expressed in bovine adrenal chromaffin cells HBDDE is here considered as a PKC\( \alpha \) selective inhibitor). However, it acts in a competitive manner at the ATP-binding site in the catalytic domain and its IC\textsubscript{50} values determined in intact cells lays within the 5-10\text{pM} range (Mathur and Vallano, 2000; Rong \textit{et al.}, 2002).

Preincubation of cells with HBDDE (100\text{\mu M}, 30 min) inhibited the release of catecholamines induced by PMA (1\text{\mu M}, 10 min) (Figure 5.2.19). HBDDE also significantly reduced catecholamine release in response to both submaximal (3\text{\mu M}) and maximal (100\text{\mu M}) concentrations of nicotine (Figure 5.2.19).

In contrast, preincubation of cells with HBDDE had no significant effect on release evoked by histamine (100\text{\mu M}) (Figure 5.2.22) whereas, secretion of catecholamines induced by angiotensin II (100\text{nM}) was potentiated following pretreatment of cells with HBDDE (Figure 5.2.23).

The role of PKC\( \beta \) in regulating catecholamine release in response to activation of either nicotinic receptors or \( \alpha_{q11} \)-coupled receptors was examined by treating cells with either LY333531 (10\text{\mu M}, 30 min) or PKC\( \beta \)C2-4 (10\text{\mu M}, 30
min) before application of agonists. These two inhibitors were also selected because of their different modes of action and selectivity toward PKCβ isoforms. The bisindolylmaleimide compound LY333531 (Way et al., 2000), is a highly specific inhibitor of PKCβI and PKCβII (Ishii et al., 1996) with IC$_{50}$ values of 4.7nM and 5.9nM, respectively (Jirousek et al., 1996). These concentrations are approximately 50 fold lower than the concentration required to inhibit other isoforms (Ishii et al., 1996). This compound acts as a direct competitive inhibitor with ATP for binding to PKCβI with a K$_i$ of 2nM (Jirousek et al., 1996; Kowluru et al., 1998).

PKCβC2-4 is a nonopeptide derived from the C2 domain of PKCβ and inhibits translocation of C2-containing PKC isoforms. This peptide fragment is a highly selective inhibitor of PKCβ translocation (Ron et al., 1994).

Pre-treatment of cells with either LY333531 (10μM, 30 min) (Efendiev et al., 1999) or PKCβC2-4 (10μM, 30 min) (Yedovitzky et al., 1997; Ron et al., 1995) reduced PMA-mediated release of catecholamines (Figure 5.2.20). Similarly, release of catecholamines evoked by submaximal (3μM) or maximal (100μM) concentrations of nicotine was significantly attenuated by either LY333531 (Figure 5.2.20: panel A) or PKCβC2-4 (Figure 5.2.20: panel B).

In contrast, LY333531 failed to influence catecholamine secretion induced by either histamine (10μM) (Figure 5.2.22) or angiotensin II (10nM) (Figure 5.2.23). Although, the peptide translocation inhibitor of PKCβ (PKCβC2-4 inhibitor) did not influence angiotensin II-mediated release it did potentiate secretion of catecholamines evoked by histamine (Figures 5.2.22 and 5.2.23).
PKCεV1-2, is a short peptide derived from the V1 region of PKCε which inhibits translocation of PKCε (Johnson et al., 1996). Inhibition of PKCε by preincubating cells with PKCεV1-2 (10μM, 30 min) (Yedovitzky et al., 1997; Mayne and Murray, 1998) reduced catecholamine secretion evoked by PMA (1μM). Furthermore, PKCεV1-2 significantly decreased secretion of catecholamines mediated by either submaximal (3μM) or maximal (100μM) concentrations of nicotine (Figure 5.2.21). In contrast, PKCεV1-2 potentiated histamine (10μM) induced catecholamine secretion (Figure 5.2.22) although it failed to affect angiotensin II (10nM) evoked secretion of catecholamines (Figure 5.2.23).
Figure 5.2.19. Effect of a PKCa/γ-selective inhibitor on catecholamine secretion evoked by nicotine or PMA. Chromaffin cells were incubated with the selective PKCa/γ inhibitor HBDDE (100μM, 30 min) at 37°C before stimulation with either PMA (1μM) or submaximal (3μM) or maximal (100μM) concentrations of nicotine for 10 min. Catecholamine release was measured as described in “Materials and Methods” and is expressed as a percentage of total cellular content. Data are mean ± SEM for three different cell preparations. For * P<0.05, *** P<0.001 by one-way ANOVA followed by post ANOVA test (Bonferroni' test). Note that not all comparisons are shown on the graph.
Figure 5.2.20. Effect of PKCβ-selective inhibitors on catecholamine secretion evoked by nicotine or PMA. Chromaffin cells were incubated with the selective PKCβ inhibitors LY333531 (10μM, 30 min, panel A) or PKCβC2-4 inhibitor (10μM, 30 min, panel B) at 37°C before stimulation with either PMA (1μM) or submaximal (3μM) or maximal (100μM) concentrations of nicotine for 10 min. Catecholamine release was measured as described in “Materials and Methods” and is expressed as a percentage of total cellular content. Data are mean + SEM for three different cell preparations. For * P<0.05, ** P<0.01, *** P<0.001 by one-way ANOVA followed by post ANOVA test (Bonferroni’s test). Note that not all comparisons are shown on the graph.
Figure 5.2.21. Effect of a PKCε-selective inhibitor on catecholamine secretion evoked by nicotine or PMA. Chromaffin cells were incubated with the selective PKCε inhibitor PKCεV1-2 (10μM, 30 min) at 37°C before stimulation with either PMA (1μM) or submaximal (3μM) or maximal (100μM) concentrations of nicotine for 10 min. Catecholamine release was measured as described in “Materials and Methods” and is expressed as a percentage of total cellular content. Data are mean ± SEM from three different cell preparations. For * P<0.05, *** P<0.001 by one-way ANOVA followed by post ANOVA test (Bonferroni’s test). Note that not all comparisons are shown on the graph.
**Figure 5.2.22. Effect of selective PKC inhibitors on catecholamine secretion**

Evoked by histamine and PMA. Chromaffin cells were incubated with HBDDE (100μM, 30 min), LY333531 (10μM, 30 min), PKCβC2-4 (10μM, 30 min), or PKCεV1-2 (10μM, 30 min) at 37°C before stimulation with either histamine (100μM) or PMA (1μM). Catecholamine release was measured as described in “Materials and Methods” and is expressed as a percentage of total cellular content. Data are mean ± SEM from three different cell preparations. For * P<0.05, ** P<0.001 by one-way ANOVA followed by post ANOVA test (Bonferroni’s test). Note that not all comparisons are shown on the graph.
Figure 5.2.23. Effect of selective PKC inhibitors on catecholamine secretion evoked by angiotensin II and PMA. Chromaffin cells were incubated with HBDDE (100μM, 30 min), LY333531 (10μM, 30 min), PKCβC2-4 (10μM, 30 min), or PKCeV1-2 (10μM, 30 min) at 37°C before stimulation with either angiotensin II (10nM) or PMA (1μM). Catecholamine release was measured as described in “Materials and Methods” and is expressed as a percentage of total content. Data are mean ± SEM from three different cell preparations. For * P<0.05 by *** P<0.001, one-way ANOVA followed by post ANOVA test (Bonferroni’s test). Note that not all comparisons are shown on the graph.
Table 5.2.3 Summary of the effects of isoform-selective PKC inhibitors on the evoked secretion of catecholamines.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>PKCα-selective inhibitor</th>
<th>PKCβ-selective inhibitors</th>
<th>PKCe-selective inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBDDE (100μM)</td>
<td>LY333531 (10μM)</td>
<td>βC2-4 (10μM)</td>
</tr>
<tr>
<td>PMA (1μM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nicotine (100μM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Histamine (10μM)</td>
<td>X</td>
<td>X</td>
<td>+</td>
</tr>
<tr>
<td>Angiotensin II (10nM)</td>
<td>++</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

- Sign shows degree of inhibition (- weak, ---- strong) (- =10-20% inhibition, -- =25-35% inhibition, --- =35-45% inhibition, and ---- =above 60% inhibition).
+ Sign shows degree of potentiation (+ weak, ++ strong) (+ = 10-20% potentiation, ++ =25-35% potentiation).
X Sign shows no effect.
The role of PKC in agonist-mediated F-actin disassembly

The role of specific PKC isoforms in mediating the disassembly of cortical F-actin in response to activation of either nicotinic receptors or Gαq/11-coupled receptors was investigated using confocal microscopy of fixed chromaffin cells stained with rhodamine-phalloidin (Vitale et al., 1991).

The confocal image for control cells (unstimulated cells) inserted in the box at the left upper corner of the image shows an intact bright fluorescent ring and weak cytosolic staining (Figure 5.2.24a). The confocal image was analysed to produce a three dimensional plot in which the image intensity is reflected in the z-axis and set to a maximum of 4096 arbitrary units (main box) (Figure 5.2.24a).

Upon stimulation of cells with cytochalasin D (2μM, 20 min), a known F-actin destabilizer, there was a breakdown (fragmentation) of the cortical fluorescent ring as seen in the confocal image (Figure 5.2.24a). This fragmented fluorescent ring was reflected in the image analysis (main box) (Figure 5.2.24a).

Stimulation of chromaffin cells with either PMA (1μM, 10 min), nicotine (100μM, 40s), histamine (100μM, 10 min), or angiotensin II (100nM, 10 min) also caused disruption of the cortical F-actin (Figure 5.2.24a). Whilst it was difficult to quantitate the integrity of the cortical F-actin ring for individual cells, cells could be scored as having either a continuous (intact) or discontinuous ring. Using a single-blind trial design and a random selection of cells, the proportion of cells with continuous and discontinuous F-actin rings were quantified for each condition (see Materials and Methods). Based upon this analysis the levels of cortical F-actin disassembly mediated by cytochalasin D > PMA > nicotine > histamine > angiotensin II (Figure 5.2.24b).
Cortical F-actin disassembly mediated by PMA was significantly reduced by either the selective PKCα inhibitor HBDDE (100μM, 30 min) or non-selective myristoylated PKC inhibitor myr-ψPKC (10μM, 30 min). Similarly, nicotine caused fragmentation of the cortical F-actin ring and this was significantly attenuated by either HBDDE or myr-ψPKC (Figure 5.2.24b). However, these inhibitors failed to prevent disruption of cortical F-actin caused by either cytochalasin D, histamine or angiotensin II (Figure 5.2.24b). HBDDE was selected in particular for this study since out of the PKC inhibitors tested it caused the greatest reduction of catecholamine secretion evoked by nicotinic receptor stimulation.
Figure 5.2.24a. Effects of PKC inhibitors on cortical F-actin disassembly mediated by cytochalasin D, PMA, nicotine, histamine, or angiotensin II. Chromaffin cells were incubated with either cytochalasin D (2µM, 30 min), PMA (1µM, 10 min), nicotine (100µM, 40s), histamine (100µM, 10 min), or angiotensin II (100nM, 10 min) following a 30 min preincubation with either vehicle (control), HBDDE (100µM) or myr-ψPKC inhibitor (10µM). Cells were then fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under “Materials and Methods”. The arrows in the left hand corner of the panels indicate the confocal image of the cell. Three-dimensional image analysis was performed using UltraView confocal software (Perkin-Elmer Life Science, Cambridge, UK) where the maximum intensity of the fluorescence was set to 4096 arbitrary units (main box). Images are representative of a total of six hundred cells for each condition from three different cell culture preparations.
Figure 5.2.24b. Effects of HBDDE and myr-ψPKC on cortical F-actin disassembly mediated by cytochalasin D, PMA, nicotine, histamine or angiotensin II. Chromaffin cells were incubated with cytochalasin D (2μM, 30 min), PMA (1μM, 10 min), nicotine (100μM, 40s), histamine (100μM, 10 min), and angiotensin II (ATII) (100nM, 10 min) following a 30 min preincubation with either vehicle (control), HBDDE (PKCα inhibitor, 100μM) or myr-ψPKC (10μM). Cells were then fixed and processed to stain actin using rhodamine-labelled phalloidin as indicated under “Materials and Methods”. The rhodamine cortical staining of chromaffin cells was analysed and classified as being either continuous or discontinuous and the percentage of cells displaying cortical F-actin disassembly (disrupted cortical rhodamine staining) was calculated. A total of six hundred cells for each condition were examined. Data shown are mean ± SEM. For *** P<0.001 for a vs. b, c, d, e and f, *** P<0.001 vs. control, one-way ANOVA followed by post ANOVA test (Bonferroni’s test). Note that not all comparisons are shown on the graph.
Nicotinic receptor-mediated phosphorylation of MARCKS and its prevention by inhibition of PKC

One of the major downstream substrates of PKC is MARCKS (Hartwig et al., 1992) and this may have particular relevance to the integrity of the cortical F-actin cytoskeleton (Hartwig et al., 1992; Trifaró et al., 2000). Indeed the phosphorylation of MARCKS has been used as a marker for activation of PKC (Bar-Am et al., 2004). Phosphorylation of endogenous MARCKS was determined by Western blotting of cell lysates with a polyclonal anti-phospho-MARCKS antibody. Phosphorylation of MARCKS was examined over a 10 min period following either PMA (1μM) or nicotine (100μM) stimulation. Treatment regimes were based on those used to stimulate catecholamine secretion (Figures 5.2.15, 5.2.19 and 5.2.20). Phospho-MARCKS was detected following these treatments as a single band with an apparent molecular mass of 86 kDa (Figure 5.2.25: panel A). The inactive phorbol ester, 4α-PMA (1μM, 10 min) did not mediate phosphorylation of MARCKS. To ensure levels of MARCKS were equivalent, lysates were also immunoblotted for total-MARCKS using a mouse monoclonal antibody specific for bovine MARCKS (Figure 5.2.25: panel A). Pre-incubation of cells with the PKC inhibitor, myr-ψPKC (10μM, 30 min) abolished the phosphorylation of MARCKS in response to challenge of cells with either PMA or nicotine (Figure 5.2.25: panel B).
Figure 5.2.25. Role of PKC in PMA- and nicotine-mediated MARCKS phosphorylation. Chromaffin cells were incubated with vehicle control (buffer) or myr-ΨPKC (10μM, 30 min) at 37°C before stimulation with either nicotine (100μM), PMA (1μM), or 4α-PMA for 20 min. Western blots of the whole cell lysate were prepared as described in “Materials and Methods”. Panel A) shows the immunoblot of phospho-MARCKS. The amount of phospho-MARCKS (p.MARCKS) was quantified using Scion Image analysis software and expressed as a percentage of control (unstimulated cells). Panel B) Cumulative data on MARCKS phosphorylation obtained from experiments carried out on four different cell culture preparations. Data are mean ± SEM. For *** P<0.001, one-way ANOVA followed by Bonferroni’s test.
Using MARCKS-eGFP to assess the activation of MARCKS in real-time

eGFP-tagged MARCKS protein was also employed to assess MARCKS activation. Under resting conditions, MARCKS localizes to the plasma membrane and upon stimulation PKC phosphorylates MARCKS, altering the charge on the molecule and causing a loss of membrane association (Arbuzova et al., 2002).

Real-time confocal imaging of cells transiently transfected with MARCKS-eGFP showed that PMA (1μM) mediated a gradual (slow) and sustained translocation of this construct from the plasma membrane to the cytosol (cytosolic fluorescence 1.45 ± 0.09 fold over basal (~18 cells). This translocation was inhibited by the PKCα-selective inhibitor HBDDE (10μM, 30 min pretreatment) (Figure 5.2.26). In contrast, nicotinic receptor activation with nicotine (100μM) caused a transient translocation of MARCKS-eGFP construct from the cell periphery to the cytosol (cytosolic fluorescence 1.79 ± 1.03 fold over basal, ~25 cells) (Figure 5.2.27). The nicotine-mediated redistribution of MARCKS-eGFP to the cytosol was completely inhibited by HBDDE (Figure 5.2.26).
Figure 5.2.26. Single cell imaging of MARCKS-eGFP. Chromaffin cells transiently transfected with MARCKS-eGFP were perfused with PMA (1µM) in the absence of HBDDE (A). Cells were preincubated with HBDDE (100µM, 30 min) at 37°C then challenged with PMA (1µM) (B). The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n=18).
Figure 5.2.27. Single cell imaging of MARCKS-eGFP. Chromaffin cells transiently transfected with MARCKS-eGFP were perfused with nicotine (100µM) in the absence of HBDDE (A). Cells were preincubated with HBDDE (100µM, 30 min) at 37°C then challenged with nicotine (100µM) (B). The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of four different cell culture preparations (n=25).
5.3 DISCUSSION

5.3.1 Summary of data

The work described within this chapter identified the PKC isoforms -α, -β, -ε and -ι representing each class of PKC isoforms within bovine adrenal chromaffin cells. Different techniques were used to assess PKC activation and each indicated that nicotinic receptor activation mediated recruitment of PKC-α, -β and -ε to the plasma membrane while it failed to mediate PKC-ι translocation. Similarly, Gαq/11-coupled receptors for either histamine or angiotensin II were able to activate PKC isoforms. Histamine caused translocation of all the expressed PKC isoforms in chromaffin cells (PKC-α, -β, -ε and -ι) to the plasma membrane whereas angiotensin II only induced redistribution of PKCα and ε to the plasma membrane.

Activation of PKC, particularly PKCα, was crucial for secretion of catecholamines mediated by nicotinic receptors. Thus, inhibition of PKC markedly reduced secretion of catecholamines evoked by nicotinic receptors. In contrast, inhibition of PKC potentiated release of catecholamines induced by Gαq/11-coupled receptor therefore PKC activation play a regulatory role in catecholamine release evoked by Gαq/11-coupled receptors due to its negative inhibitory feedback loop on Gαq/11-coupled receptor downstream PLC signalling.

In addition, investigations in this study highlight the importance of cortical F-actin disassembly in secretory process. Since stimulation of nicotinic receptor activates PKC which subsequently phosphorylates MARCKS thereby reducing its ability to cross-link F-actin filaments. The disruption of cortical F-actin would result in an increase the size of readily releasable pool of secretory granules (Vitale et al., 1995) allowing enhanced exocytosis of catecholamines.
5.3.2 PKC isoform expression in chromaffin cells

The present immunoblot analysis revealed that among the 12 PKC isoforms, bovine chromaffin cells possess only PKC-α, -β, -ε and -τ at levels detectable by immunoblotting. Interestingly, these detectable PKC isoforms in bovine chromaffin cells are representative of the three PKC subfamily classes, PKC-α and -β belong to the conventional class while PKCε represents the novel class and PKCτ represents the atypical PKC class. The presence of PKC-α, -β, -ε and -τ isoforms in chromaffin cells is in full agreement with previous studies (Pavlovic-Surjančev et al., 1993; Penberthy and Dahmer, 1994; Sena et al., 1996; 2001, Cox and Parsons, 1997; Vainio et al., 1998; Yanagita et al., 2000).

The ability of each PKC antibody to detect the PKC isoform was confirmed by the parallel study in rat brain and bovine brain extracts, which used to check antibodies. In this study all antibodies were able to detect proteins at the expected molecular weight in both tissues giving confidence that they were not expressed or expressed at very low levels in bovine adrenal chromaffin cells. It has been proposed that the absence of PKC-γ, -δ, -η, -θ and -λ may reflect either an inability of the antisera raised against the carboxyterminal peptides of rat PKC (Wetsel et al., 1992) to interact with the bovine PKC-γ, -δ, -η, -θ and -λ or a lack of expression of these isoforms in bovine chromaffin cells (Pavlović-Šurjančev et al., 1993). The present data show that each of the antibodies could detect these PKC isoforms of bovine origin supporting the view that they are not expressed or expressed at very low levels in bovine chromaffin cells.

5.3.3 PKC translocation

The translocation of PKC isoforms from cytosol to the plasma membrane is assumed to be a hallmark of PKC activation (Newton, 1997; Mochly-Rosen and
Gordon, 1998). In this study two techniques namely Western blotting and immunocytochemistry were used to study the translocation of identified PKC isoforms (PKC-α, -β, -ε and -ι) in bovine adrenal chromaffin cells.

The results clearly demonstrate that stimulation of chromaffin cells with PMA, resulted in a rapid and intense redistribution of PKC-α, -β and -ε from a predominantly cytosolic location in resting cells to membrane-associated sites. The complete loss of PKCβ from the cytosol at 10 min treatment with PMA was more profound than the loss of PKCε and PKCα. This is inconsistent with a previous study that indicated translocation of PKCε was more profound than translocation of PKCα in bovine adrenal chromaffin cells (Sena et al., 1996). Translocation of each of these isoforms in response to PMA is in agreement with other reports in bovine adrenal chromaffin cells that have showed translocation of cPKC and nPKC isoforms in response to several minutes of PMA treatment. The current study is also in agreement with these studies showing an inability of PMA to induce the redistribution of aPKC isoforms (Olivier and Parker, 1992; Ways et al., 1992; Sena et al., 1996; Sena et al., 2001; Yanagita et al., 2000).

The C1 domain constitutes the membrane-targeting regulatory domain in cPKC and nPKC and this domain is required for their interaction with PMA. However, PKCi is insensitive to PMA because the aPKC family possess only one cysteine-rich zinc finger instead of two incorporated in C1 region (Way et al., 2000).

In this study, there is clear evidence of translocation of PKC-α, -β, -ε and -ι in response to histamine receptor activation detected by both Western blotting and immunocytochemical studies. Although over a ten minutes period the degree of translocation was approximately equivalent for each isoform, the movement of
PKCε was the fastest, occurring predominantly in the first one minute of stimulation. Chromaffin cells have been shown to express histamine H₁ receptors, which are linked by Goq/11 proteins to PLC (Plevin and Boarder, 1988; Burgoyne, 1991; Challiss et al., 1991; Bunn et al., 1992) (see Chapter 3). The activation of PLC results in the generation of Ins(1,4,5)P₃ and DAG in chromaffin cells. Thus, histamine might activate PKC via elevation of intracellular Ca²⁺ and/or DAG generation resulting in the activation of DAG- and Ca²⁺-dependent isoforms of PKC (i.e., cPKC and nPKC) (Alvarez et al., 1997; Marley, 2003). The translocation of PKC isoforms to the plasma membrane mediated by histamine was inconsistent with other studies that indicate failure of histamine to induce any significant sustained activation (translocation) of any of the PKC isoforms expressed in bovine chromaffin cells (PKC-α, -ε and -ξ) (Sena et al., 1996; Donald et al., 2002), although a tendency to reduce cytosolic and increase particulate PKCε was noted in (Sena et al., 1996). Interestingly, in the present study, histamine did cause the translocation of PKCi detected by both Western blotting and immunocytochemistry. The mechanisms underlying translocation of PKCi are unclear. Indeed, it is unclear whether translocation is required for activation. However, the current data suggest that histamine, probably in a Ca²⁺- and DAG-independent manner does activate PKCi. The functional consequences of this are not clear.

Activation of angiotensin receptors by angiotensin II only recruited PKC-α and -ε to the plasma membrane with the translocation of PKCε being more prominent. However, the translocation of PKCε was slower than that following histamine receptor stimulation suggesting a different signal or strength of signal following activation of histamine and angiotensin II receptors. It has been
suggested that the angiotensin II type 1 receptor (AT₁) in chromaffin cells are coupled to a plethora of different types of G-protein (Richards et al., 1999; Teschemacher and Seward, 2000). Thus, the inhibitory effects of Gι/0 activation receptor on VOCCs and hence Ca²⁺ signalling (Takahashi et al., 1996; Powell et al., 2000) could counteract the stimulatory effect of Gαq/11-proteins on Ca²⁺ signalling. Under these circumstances, PKCα would be expected to be affected more than PKCe, since PKCα requires both Ca²⁺ and DAG for activation (Newton and Johnson, 1998) whereas PKCe is DAG-sensitive and Ca²⁺-insensitive. Indeed, the Ca²⁺ signal in response to angiotensin II was much shorter in duration than that in response to histamine. Whether this is due to differences in G-protein coupling or the rate of receptor desensitisation, this could account for the inability of angiotensin II to activate the Ca²⁺/DAG-sensitive PKCβ. Similarly, this could account for inability of angiotensin II to activate PKCi.

Nicotine caused a dramatic loss of PKCe from the cytosolic fraction that was much more marked than the loss of either PKC-α or -β. Activation of PKC-α or -β following nicotinic receptor activation could be secondary to a rise of [Ca²⁺]; (Wolf et al., 1985; Gopalakrishna et al., 1986; Akers and Routtenberg, 1987). However, in the case of Ca²⁺-independent PKCe activation could occur following Ca²⁺-triggered PLC activity and an increased formation of DAG, a necessary activator of PKCe. This will be explored further in Chapter 6. The ability of nicotine to increase membrane-bound PKCα and ε is largely in agreement with previous studies (Messing et al., 1989; Sontag et al., 1990; Rodríguez Del Castillo et al., 1992; Cox and Parsons, 1997; Vainio et al., 1998; Izrael et al., 2004).

It has been demonstrated that translocation mediated by tumor-promoting phorbol esters (in this case PMA) cause a dramatic decrease in cytosolic PKC
activity accompanied by a large increase in membrane-bound activity in many cell types (Kraft and Anderson, 1982; Nishizuka, 1995; Mochly-Rosen, 1995). This is in agreement with the present study in which PKC-α and -β translocation mediated by PMA was more prominent than translocation mediated by activation of Goq/11-coupled receptors. Interestingly the activation of Goq/11-coupled receptors increased membrane-association of PKCε more than PMA suggesting that factors other than PMA/DAG binding are crucial in the regulation of PKCε.

The problem that may emerge from using Western blotting to study the translocation of PKC isoforms in chromaffin cells is that it only allows study of changes of PKC distribution in populations of cells and not single cells. This presents a particular problem when a heterogenous population of cells like chromaffin cells are used (Tischler, 2002). Thus, chromaffin cell preparations might be contaminated by other cell types such as fibroblasts, cortical cells, and adrenal medulla endothelial cells (Bossu et al., 1992; Vinet and Vargas, 1999; Benavides et al., 2004). Furthermore, Western blotting fails to show the pattern of subcellular localization. To characterize the subcellular distribution of PKC isoforms and their potential redistribution, immunocytochemical studies were performed. Immunocytochemical studies using isoform-specific antibody confirmed the cytosolic localization of all expressed PKC isoforms in unstimulated cells and demonstrated the membrane localization of PKC-α, -β, -ε but not -τ with either PMA or nicotinic receptor activation. Histamine also caused the plasma membrane localization of all expressed PKC isoforms whereas AT1A receptor activation caused the redistribution of PKC-α and -ε to the plasma membrane.
These data are consistent with those obtained by Western blotting and demonstrate that a substantial proportion of cytosolically located PKC translocates to the plasma membrane rather than other intracellular membranes.

Whilst the immunoblotting and immunocytochemical methods give valuable information on the translocation and activation of PKC isoforms these techniques have limited temporal resolution. To overcome this, fusion constructs between enhanced green fluorescent (eGFP) (Tsien, 1998) and PKCβII (Feng and Hannun, 1998) or PKCδ (Chiesa et al., 2001) were used to monitor the dynamics of membrane translocation of PKC isoforms in real time in living cells. These eGFP-tagged constructs represent the conventional and novel classes of PKC respectively. In the present study eGFP-PKCδ was used as positive control although immunoblotting analysis indicated that PKCδ was not expressed in chromaffin cells.

As expected, PMA caused robust and sustained translocation of either eGFP-PKCβII or eGFP-PKCδ. Activation of histamine receptors caused a rapid but transient translocation of eGFP-PKCβII or eGFP-PKCδ from the cytosol to the plasma membrane. The transient nature of these translocations were inconsistent with the activation profiles that were observed using immunoblotting. It is possible that this is a reflection of high expression levels of the constructs. However, activation of either angiotensin II receptors or nicotinic receptors did cause a rapid and sustained translocation of eGFP-PKCβII and eGFP-PKCδ from the cytosol to the plasma membrane. Thus, the activation profiles of eGFP-PKCβII or eGFP-PKCδ translocation mediated by either angiotensin II or nicotine were consistent with the activation profiles observed using immunoblotting.
5.3.4 Role of PKC in secretion of catecholamines

The main function of adrenal chromaffin cells is secretion of catecholamines. As discussed elsewhere (see main introduction) PKC has been reported to regulate secretion in both chromaffin cells and other secretory cells. Given that there are structural differences in the regulatory domains of PKC isoforms expressed in chromaffin cells, these isoforms may have distinct roles in the facilitation and regulation of secretion of catecholamines from adrenal chromaffin cells (Terbush and Holz, 1986; 1990; Terbush et al., 1988; Graham et al., 2000; Taylor et al., 2000). Although, the role of PKC in secretion of catecholamines is well documented, there is debate about the underlying mechanisms (Waters and Smith, 2000). Several groups have suggested that PKC has an obligatory role in exocytosis based on the results obtained from a variety of relatively non-specific PKC inhibitors (Knight et al., 1988). In this study non-selective PKC inhibitors were initially used to confirm the role of PKC in release of catecholamines mediated either by nicotinic receptors or Gaq/coupled receptors. Isoform-selective PKC inhibitors were then used to probe the role of specific PKC isoforms. The non-selective inhibitors used were Ro31-8220 and myristoylated pseudosubstrate peptide (myr-ψPKC). The selection of these two inhibitors was based on the fact that they have different chemical structures and modes of action. Thus, Ro31-8220, a membrane-permeant bisindolylmaleimide IX derivative acts by competing with ATP for binding to PKC (Davis et al., 1992; Bit et al., 1993). Ro31-8220, which possess a straight chain alkyl side group with a cationic substituent shows little PKC isoform selectivity and inhibits most, if not all PKC isoforms known to date, including the isoforms expressed in chromaffin cells (Wilkinson et al., 1993; Shen and Glazer, 1998; Sena et al., 2001).
Furthermore, at nanomolar concentration (IC$_{50}$ = 10nM), Ro31-8220 fully inhibits PKC while still retaining specificity (Davis et al., 1992). This IC$_{50}$ value (10nM) was determined by in vitro kinase assays in which the ATP concentration is generally around 100μM. However, cellular ATP concentrations are of the order of 2mM and much higher concentrations of inhibitor are required. Ro31-8220 was used at 10μM as reported using a variety of cell types including chromaffin cells (Turner et al., 1996; Willars et al., 1999; Soliakov and Wonnacott, 2001). In contrast, myr-ψPKC is a specific, cell permeable a myristoylated peptide inhibitor and expectedly not interferes with ATP binding (Eichholtz et al., 1993). This peptide inhibitor is related to the pseudosubstrate site of PKC and competes with substrate binding site in the catalytic domain and keeps the enzyme in the inactive state (House and Kemp, 1987). This peptide is competent to inhibit all PKC isoforms as all contain a pseudosubstrate domain. Structurally, its sequence resembles a PKC phosphorylation site and it features a set of basic amino acids. However, the phosphate acceptor Ser or Thr residues are substituted by Ala (Gschwendt et al., 1991). This compound has been shown to inhibit PKC when used at 10μM in several cell types (eg. human fibroblast, jurkat cells, islets, human T lymphocytes and bovine chromaffin cells) (Ioannides et al., 1990; Eichholtz et al., 1993; Harris et al., 1996; Alexander et al., 1989; Terbush and Holz, 1990).

The current study demonstrated that inhibition of PKC using either Ro31-8220 or myr-ψPKC markedly reduced secretion of catecholamines evoked by either PMA or nicotine suggesting a crucial role for PKC in catecholamine release mediated by nicotinic receptor activation. This is consistent with previous studies in chromaffin cells in which there was a correlation between nicotinic receptor
stimulation, PKC activation and increased exocytosis (Brocklehurst et al., 1985; Wakade et al., 1986; Terbush et al., 1988; Terbush and Holz, 1990; Cox and Parsons, 1997).

PMA might be expected to enhance nicotinic receptor mediated catecholamine release when they are used concurrently based on previous studies (Wakade et al., 1986; Terbush et al., 1988; Vitale et al., 1992; Cox and Parsons, 1997). Indeed, this study demonstrated that PMA potentiated catecholamine secretion evoked by nicotinic receptor activation. To examine the influence of nicotinic receptor-mediated PKC activation on nicotinic receptor-evoked secretion, the influence of the general PKC inhibitors Ro31-8220 and myr-ΨPKC was examined. The data revealed that inhibition of PKC significantly antagonized secretion mediated by nicotine, providing strong evidence of the crucial role of PKC in facilitating secretion evoked by nicotinic receptor activation.

It has been previously demonstrated that Ca\(^{2+}\) influx due to nicotinic receptor activation induced translocation of PKC from cytosol to the membrane and that this correlates well with the secretory response (Terbush et al., 1988). In addition, it has been suggested that nicotinic receptor-mediated binding of PKC to membranes involves two components, one mediated by Ca\(^{2+}\) alone and the other by Ca\(^{2+}\) and DAG. The source of Ca\(^{2+}\) and DAG for nicotinic receptor mediated catecholamine secretion will be explored elsewhere (see Chapter 6).

Surprisingly, secretion of catecholamines evoked by the G\(\alpha_{q/11}\)-coupled receptors either histamine or angiotensin II was reduced by direct activation of PKC with PMA whilst inhibition of PKC potentiated release. G\(\alpha_{q/11}\)-coupled receptor-evoked catecholamine release from bovine adrenal chromaffin cells requires PLC activation (Plevin and Boarder, 1988; Sasakawa et al., 1989; Bunn
et al., 1990). The activation of PLC results in the hydrolysis of membrane
PtdIns(4,5)P₂. The generation of DAG and Ins(1,4,5)P₃ with subsequent Ca²⁺
release from intracellular stores evokes catecholamine secretion. In addition,
depletion of Ca²⁺ from internal stores results in store-operated Ca²⁺ entry
(capacitative Ca²⁺ entry) (Putney, 1990) and capacitative Ca²⁺ entry has been
reported to evoke secretion from both PC12 cells and chromaffin cells (Powis et
al., 1996a; Fomina and Nowycky, 1999; Taylor and Peers, 1999). The PLC-
dependent generation of DAG, and in some instances Ca²⁺ results in the activation
of PKC following Gaq/11-coupled receptor stimulation. Despite this, PKC does not
appear to potentiate catecholamine release under these conditions as inhibition of
PKC potentiates release. The reason for this is likely to be a reflection of a short
inhibitory feedback loop from PKC perhaps onto PLC (Boarder and Challis, 1992;
Bunn, 1993; Alvarez et al., 1997; Sena et al., 1996; Teschemacher and Seward,
2000; Marley, 2003). There is also evidence that PKC-dependent phosphorylation
of GPCRs may be responsible for a negative feedback loop (Willars et al., 1996;
Diviani et al., 1997; Liang et al., 1998). Thus, removal of this loop by inhibition
of PKC may potentiate receptor-mediated signalling. This negative feedback loop
from PKC onto PLC was further confirmed by measurement of PLC activity using
the accumulation of [3H]-inositol phosphates ([3H]-InsPₓ) under Li⁺-block (Willars
et al., 1998a; 1999; Tovey and Willars, 2004). This study indicated that [3H]-InsPₓ
generation mediated by Gaq/11-coupled receptors was potentiated by inhibiting
PKC using Ro31-8220. These data provide additional evidence of the negative
regulatory role of PKC on catecholamine secretion evoked by Gaq/11-coupled
receptors.
Although, Ro31-8220 and myr-ψPKC demonstrated the contribution of PKC to catecholamine secretion evoked by either nicotinic receptors or Goq11-coupled receptors, these general PKC inhibitors did not provide information on the role of individual PKC subtypes in exocytosis. Therefore, isoform-specific PKC inhibitors were used to dissect the contribution of specific PKC isoforms to nicotinic receptor-stimulated catecholamine release. It was also hoped that it may be possible to determine the isoforms responsible for the negative effects of PKC on Goq11-coupled receptor-mediating signalling, potentially enabling facilitating effects of PKC on catecholamine release to be uncovered.

The present study suggests that PKCα is the prominent isoform involved in potentiating the catecholamine release in response to nicotinic receptor activation since inhibition of this isoform using HBDDE completely abolished secretion of catecholamines evoked by nicotinic receptors. This bisindolylmaleimide compound acts in a competitive manner at the ATP-binding site in the catalytic domain (Mathur and Vallano, 2000). This compound has been used previously at the concentration used here (100μM) to selectively inhibit PKCα in chromaffin cells and other cell types (Rong et al., 2002; Markos et al., 2005). Two structurally and functionally different PKCβ inhibitors also inhibited nicotinic receptor-mediated catecholamine release. The bisindolylmaleimide compound LY333531 (Way et al., 2000) inhibits both PKCβI and PKCβII (Ishii et al., 1996) with IC₅₀ values of 4.7nM and 5.9nM, respectively (Jirousek et al., 1996). It has been to act as a direct competitive inhibitor with ATP for binding to PKCβI with a Kᵢ of 2nM (Jirousek et al., 1996; Kowluru et al., 1998). In contrast, PKCβC2-4 is derived from the C2 domain of PKC and this peptide fragment is a highly selective inhibitor of PKCβ translocation. The inhibitory effect of
PKCβC2-4 on nicotinic receptor-evoked secretion of catecholamines was slightly greater than that achieved with LY333531. In contrast, both PKCβC2-4 and LY333531 were equally effective in inhibition of PMA mediated secretion.

The PKCε inhibitor, PKCεV1-2 also had less much inhibitory effect than PKCβ inhibitors on nicotinic receptor-mediated catecholamine release. This was used at 10µM as previously reported (Ron et al., 1995; Yedovitzky et al., 1997). This peptide is derived from the V1 region of PKCε lead to inhibiting translocation of this isoforms (Johnson et al., 1996). Despite the fact that PKCε translocation to the plasma membrane mediated by nicotinic receptors was more profound than the translocation of the other expressed isoforms; PKCε contributes the least to catecholamine secretion evoked by nicotinic receptors.

From the present results it can be suggested that PKCα plays the most important role in potentiating the release of catecholamines from chromaffin cells triggered by nicotine, consistent with previously reported studies (Terbush et al., 1988; Cox and Parsons, 1997; Smith, 1999; Sena et al., 2001; Fontainhas et al., 2005). Based on the use of selective PKCβ inhibitors, this isoform would also appear to play role in catecholamine secretion mediated by nicotinic receptor activation. However, PKCα was selected as a target throughout this study since inhibition of PKCα caused the greatest attenuation of nicotinic receptor-mediated catecholamine secretion and this may require further confirmation.

In contrast, it is difficult to dissect the roles of the PKC isoforms to the regulation of catecholamine secretion evoked by Gαq/11-coupled receptors. Since the degree of potentiations caused by the wide range of isoforms-selective inhibitors were approximately comparable.
There is a range of sites of PKC action including ion channels (Wakade et al., 1986; Majewski and Iannazo, 1998; Maeno-Hikichi et al., 2003), the actin cytoskeleton (Vitale et al., 1995) and exocytotic machinery proteins that are involved in regulated exocytosis (Rink et al., 1983; Nagy et al., 2002; Barclay et al., 2003) (see Chapter 1). Although, these sites of PKC action have been extensively investigated, one PKC substrate, MARCKS and its link to F-actin and secretion has received little attention particularly in response to nicotinic receptor activation.

5.3.5 The role of PKC in agonist-mediated F-actin disassembly

It has been suggested that the actin microfilament network localized underneath the plasma membrane of chromaffin cells (Lee and Trifaró; 1981; Trifaró et al., 1984; Cheek and Burgoyne, 1986) acts as barrier (negative clamp) to the movement of secretory granules, blocking their access to exocytotic sites at the plasma membrane (Trifaró et al., 1982; Burgoyne et al., 1989; Burgoyne, 1991). In chromaffin cells, F-actin forms a cortical network that excludes the large majority of secretory vesicles from plasma membrane docking and maintains them in reserve pool. Disassembly of cortical F-actin in response to stimulation is thought to allow the movement of vesicles from this reserve compartment into the release-ready pool (Vitale et al., 1991; 1995). It is evident that stimulation of chromaffin cells produces disassembly of actin networks and removal of a barrier to secretory vesicle mobility (Trifaró et al., 1982; 1984; Cheek and Burgoyne, 1986; Burgoyne et al., 1989; Trifaró and Vitale, 1993; Zhang et al., 1995b; Trifaró et al; 2000; 2002). F-actin network disassembly has also been observed in mast cells on stimulation (Koffer et al., 1990), in depolarised synaptosomes
(Bernstein and Bamburg, 1985; Trifaro and Vitale, 1993; Walaas and Sefland, 2000) and PC12 cells (Geeraert et al., 2003).

Two pathways are known to be involved in the control of the chromaffin cell cortical F-actin network during secretion (Trifaro et al., 2000). These are the Ca$^{2+}$-scinderin and PKC-MARCKS pathways. The first pathway constitutes three Ca$^{2+}$-dependent actin-binding proteins that control actin filament length: gelsolin (Yin and Stossel, 1979), fodrin (Perrin and Aunis, 1985) and scinderin (Rodriguez Del Castillo et al., 1990). Gelsolin is an actin-servering protein expressed in many cells including chromaffin cells (Yin and Stossel, 1979; Trifaro et al., 1985; Bader et al., 1986). Fodrin, is also an actin-binding protein present in the cortical region of chromaffin cells (Perrin and Aunis, 1985). Scinderin is an 80 kDa cytosolic protein that shortens actin filaments (Rodriguez Del Castillo et al., 1990). It is evident that during cell stimulation, subplasmalemal scinderin, but not gelsolin, is redistributed in chromaffin cells and this redistribution precedes exocytosis (Vitale et al., 1991). The second pathway constitutes the PKC substrate protein, which MARCKS. MARCKS is a member of a family of PKC substrates that potentially interact with F-actin and calmodulin. MARCKS is widely distributed in the nervous system (Hartwig et al., 1992; Ouimet et al., 1990) and is also expressed in chromaffin cells (Zhang et al., 1995b; Powis et al., 1996b).

MARCKS binds actin and cross-links the actin filaments (Hartwig et al., 1992) which remain intact unless MARCKS is phosphorylated by PKC as phospho-MARCKS is unable to cross-link actin filaments (Hartwig et al., 1992). Furthermore, it has been suggested that in chromaffin cells, MARCKS mediates the effects of PKC activation by controlling the F-actin network dynamics during secretion in response to specific stimuli (Vitale et al., 1995; Trifaro et al., 2000).
The cytochemical experiments with rhodamine-labelled phalloidin (as a probe for F-actin) clearly demonstrated that the positive control, cytochalasin D depolymerises F-actin and disrupts actin filaments as previously reported for this fungal toxin (Schliwa, 1982; Flanagan and Lin, 1980; Vitale et al., 1995; Cuchillo-Ibáñez et al., 2004). The fragmentation of cortical F-actin induced by cytochalasin D was not prevented by inhibition of PKC, giving a clear indication as expected that PKC was not involved in cytochalasin D-mediated F-actin disassembly. Activation of PKC with PMA caused disassembly of the cortical F-actin network. Such PMA-induced disassembly of cortical F-actin has previously been demonstrated to facilitate the translocation to the plasma membrane (0-50 nm area) of those vesicles that are in the proximity of the plasma membrane but separated from it by the actin filament barrier (Vitale et al., 1995). The PMA result confirmed the contribution of PKC particularly α isoform to cortical F-actin disruption evoked by PMA since inhibition of PKC with selective PKCα inhibitor, HBDDE dramatically attenuated this disruption. This is consistent with previous studies that used less specific PKC inhibitors such as sphingosine, staurosporine and calphostin that blocked in a concentration dependent manner PMA-induced cortical F-actin network disassembly (Vitale et al., 1992; Rodríguez Del Castillo et al., 1992).

Previous results (see Chapter 4) demonstrated that jasplakinolide, F-actin stabilizer prevents F-actin disassembly mediated by nicotinic receptor activation and as consequence prevented release of catecholamines mediated by nicotinic receptors. This indicates that disassembly of the cortical F-actin plays a crucial role in nicotinic receptor evoked catecholamine secretion as previously suggested (Trifaró, 1984; Cheek and Burgoyne, 1986; Trifaró et al., 1989; Vitale et al.,
1991; Vitale et al., 2000; Tchakarov et al., 1998). The inability of the PKC inhibitors to fully prevent nicotinic receptor-mediated disassembly suggests that either PKC inhibition was incomplete or that a mechanism other than PKC might be involved in controlling F-actin network dynamics. This is highlighted by a previous study in chromaffin cells that revealed a Ca\(^{2+}\)-dependent scinderin mechanism to be responsible for at least 80\% of the cortical F-actin disassembly and catecholamine release in response to nicotinic receptor stimulation since PKC inhibitors only produced 20\% inhibition of F-actin disassembly and catecholamine release (Trifaró et al., 2002).

An immunoblot approach was used to study the phosphorylation of endogenous MARCKS. Data revealed that MARCKS phosphorylation increased upon stimulation with PMA and this seems to be due to PKC since this was completely abolished by inhibition of PKC. In addition, MARCKS-eGFP was used to assess the activation of MARCKS in real-time (Ohmori et al., 2000). MARCKS localizes to the plasma membrane and upon stimulation PKC phosphorylates MARCKS leading to its translocation to the cytosol (Arbuzova et al., 2002). In this study, activation of PKC by PMA treatment mediated translocation of MARCKS to the cytosol. This translocation was markedly prevented by inhibition of PKC\(\alpha\) using HBDDE suggesting a role for PKC\(\alpha\) in the activation of MARCKS and the subsequent F-actin disassembly.

Although, the effects of PMA on MARCKS phosphorylation and translocation have been studied in chromaffin cells (Vitale et al., 1992; 1995; Rosè et al., 2001; Trifaró et al., 2000; Trifaró et al., 2002), no study has previously shown a role for PKC in nicotinic receptor-mediated MARCKS phosphorylation and translocation. Clarification of the role of PKC\(\alpha\) and
MARCKS in vesicular trafficking in chromaffin cells might be achieved by studying the effect of antisense oligonucleotides or siRNAs specific for MARCKS and PKCα.

Activation of Gαq/11-coupled receptors also facilitated F-actin network disassembly supporting previously published work (Trifaró et al., 2000; 2002; Zhang et al., 1995b; Samain et al., 1999). The non-selective PKC inhibitor (myr-ψPKC) and selective PKCα inhibitor (HBDDE) failed to block F-actin filament disruption. Thus, F-actin disassembly in response to activation of Gαq/11-coupled receptors would seem to be independent of the PKC-MARCKS pathway. It is possible that Ca²⁺-scinderin pathway could therefore play a major role in controlling cortical F-actin dynamics under these conditions.

Data in this chapter demonstrated that it is difficult to dissect which PKC isoforms are involved in the regulation of catecholamine secretion evoked by Gαq/11-coupled receptors and this aspect was not pursued further and the focus was diverted to the role of PKC in catecholamine secretion evoked by nicotinic receptors as shown in the next chapter (Chapter 6).
6: MECHANISMS UNDERLYING PKC ACTIVATION MEDIATED BY NICOTINIC RECEPTOR STIMULATION

6.1 INTRODUCTION

Catecholamine secretion from bovine adrenal chromaffin cells is physiologically stimulated by nicotinic receptor-mediated influx of extracellular Ca
\(^{2+}\) and a subsequent rise in the [Ca
\(^{2+}\)] \(_i\) (Wilson and Kirshner, 1977; Kao and Schneider, 1986). Although there is strong evidence as seen in this (see Chapter 5) and previous studies (Brocklehurst et al., 1985; Vainio et al., 1998; Soliakov and Wonnacott, 2001; Mahata et al., 2002) indicating that nicotinic receptor-mediated activation of PKC enhances secretion of catecholamines, the mechanism(s) by which PKC is activated are not fully understood. Thus, work described within this chapter sought to investigate how nicotinic receptor activation results in the activation of PKC specifically. PKC-\(\alpha\), -\(\beta\) and -\(\epsilon\) (see Chapter 5) that are either activated by DAG or Ca
\(^{2+}\) and DAG in combination. Earlier experiments (Chapter 3) demonstrated elevation of [Ca
\(^{2+}\)] \(_i\), in response to nicotinic receptor activation. Many isoforms of PLC are Ca
\(^{2+}\)-sensitive and it is possible therefore that PLC is activated in response to nicotinic receptor activation. Enhanced PLC activity would generate DAG (and potentially additional Ca
\(^{2+}\) from intracellular stores via Ins(1,4,5)P
\(_3\)) and this DAG, either alone or in combination with Ca
\(^{2+}\), could activate PKC. Indeed, under equilibrium conditions, biological membranes contain very little DAG (Hodgkin et al., 1998) and DAG generation may, therefore, be required to support PKC activation. Previous data have suggested that Ca
\(^{2+}\) influx following nicotinic- or high K
\(^{+}\)-mediated depolarisation in chromaffin cells can increase activity of PLC (Eberhard and Holz, 1988; Nakaki et al., 1988; Sasakawa et al., 1989; Roberts-Thomson et al., 2000).
Furthermore, previous studies have shown that activity of the key signal transduction enzyme PLC is enhanced by secretory stimuli in chromaffin cells (Bunn et al., 1990; Roberts-Thomson et al., 2000; Sasakawa et al., 1989). Furthermore, the PLC substrate, PtdIns(4,5)P2 is essential for secretion (Hay et al., 1995). These observations suggest that PLC activity could play an important role in the exocytotic response (O’Connel et al., 2003).

Advances in imaging techniques and the advent of GFP-tagged proteins with high selectivity for intracellular messengers make it possible to image the cellular level of Ins(1,4,5)P3 in a real time in single cells (Stauffer et al., 1998; Hirose et al., 1999). The pleckstrin homology domain of PLCδ1 (PHPLCδ1) binds specifically and with high affinity to PtdIns(4,5)P2 (Kavran et al., 1998) and a fusion construct of PHPLCδ1 and GFP (eGFP-PHPLCδ1) enriches over the plasma membrane. Therefore, agonist-stimulated Ins(1,4,5)P3 production is predominantly responsible for translocation of eGFP-PHPLCδ1 with little contribution of PtdIns(4,5)P2 depletion as result of PLC activity (Nash et al., 2001; Nahorski et al., 2003; Bartlett et al., 2005). Similarly, DAG levels can be monitored in single cells using the DAG binding domain of PKCy (eGFP-Cl2PKCy) (Oancea and Meyer, 1998; Mogami et al., 2003), which translocates to the plasma membrane in response to agonist-mediated DAG generation. In addition to these fluorescent biosensors, PLC activity can also be assessed by measurement of total [3H]-inositol phosphates ([3H]-InsPs) in Li+-blocked cells (Willars and Nahorski, 1995b).

The PLC family comprises 11 isoforms classified into four groups (four PLCβ, two PLCγ, four PLCδ and one PLCε) (Rhee and Bae, 1997; Rhee, 2001). Recently, a novel sperm-specific PLC, PLCζ has been identified (Saunders et al., 2018).
Activation of PLCβ isozymes is initiated by ligand binding to G protein-coupled receptor. Apart from Gq/11 type α subunits coupled to these receptors, Gβγ subunits can also activate PLCβ. PLCγ activation involves phosphorylation by growth-factor-activated receptor protein tyrosine kinases. Activation of PLCδ isozymes is triggered by binding of Ca2+ to EF-hand and C2 domains of PLCδ. Ca2+ activates PLCδ 50-100 fold more than β and γ isoforms. Thus, PLCδ would be expected to be responsible for activity observed following an initial elevation of [Ca2+]i. PLCε is widely expressed in human tissues and most abundantly in heart. PLCε is activated by Ras and receptors for thrombin and lysophosphatidic acid that couple to Gα12 (Rhee, 2001). Chromaffin cells express β, γ and δ isoforms of PLC (Choi et al., 1993) but whether PLCε is expressed in chromaffin cells is not yet known.

Apart from the PI-PLC pathway, DAG can also be produced by two sequential reactions in which phosphatidylcholine is broken down by phosphatidylcholine-specific phospholipase D (PC-PLD) to yield phosphatidic acid, which is then converted to DAG by phosphatidic acid phosphohydrolase (Nishizuka, 1992; Hodgkin et al., 1998; Wakelam, 1998). Indeed, PKC can also be activated by DAG generated PC-PLD pathways. Furthermore, PKC can phosphorylate and activate PLD and this may be a process that ensures continued production of DAG for sustained PKC activation (see reviews Newton, 1997; 2001). Metabolism of DAG either by DAG lipase or DAG kinase terminates its action (Hodgkin et al., 1998).

DAG is able to bind to the C1 domains of a large number of proteins with diverse function (see Chapter 1). The most prominent DAG targets belong to the PKC family of serine/threonine kinases. Binding of DAG often is synergy with
Ca\textsuperscript{2+} leads to membrane translocation and activation of PKC isoforms (Nishizuka, 1992; Newton, 1995; 1997; 2001) (see Chapter 1 for details of role of Ca\textsuperscript{2+} and DAG in PKC activation and recruitment). The modulation of cellular processes by DAG and by the functionally analogous phorbol esters has been attributed primarily to activation of PKCs (Brose and Rosenmund, 2002). However, it has been shown that DAG, as well as phorbol esters can bind to an alternative non-kinase targets including Munc13, PKD kinases, chimaerin, RacGTPase-activating proteins (GAPs) and RasGRP (Ras guanyl nucleotide-releasing protein) (Yang and Kazanietz, 2003). In this study, attention was focused on Munc13 since there is large body of evidence indicating that activation of Munc13 facilitates neurotransmitter release (Betz et al., 1998; Ron and Kazanietz, 1999; Kazanietz, 2002).

Munc13 proteins constitute a family of four mammalian homologues of *Caenorhabditis elegans* Unc13 including Munc13-1, Munc13-2, and Munc13-3 (Brose et al., 1995) and recently identified novel Munc13-like protein (Munc13-4) (Koch et al., 2000). Munc13 is specifically expressed in neuronal cells and Munc13-1 is localized at the presynaptic plasma membrane (Brose et al., 1995; Augustin, 1999b; Betz et al., 1998) whereas Munc13-4 is predominantly expressed in lung (Koch et al., 2000). Chromaffin cells express low levels of Munc13-1 and Munc13-3 while Munc13-2 is not detectable (Ashery et al., 2000). Munc13 isoforms are very proteins (195-225kDa) (Betz et al., 1997) with phorbol ester and DAG binding C1 domain and two C2 domains that are Ca\textsuperscript{2+}/phospholipid binding domains (Brose et al., 1995; Augustin et al., 1999a; 1999b). All Munc13 isoforms bind phorbol esters and DAG with high affinity and in common with PKCs translocate to the plasma membrane (Betz et al., 1998;
Ashery et al., 2000). In *C. elegans*, Unc13 is essential for coordinated movement (Brenner, 1974). It has been shown that Unc13 and Munc13 are important for synaptic vesicle priming (Augustin et al., 1999b; 2001; Richmond et al., 1999; Aravamudan et al., 1999; Varoqueaux et al., 2002). Unc13 and Munc13s act by unfolding and activating the SNARE protein syntaxin and thereby promoting SNARE complex formation (Betz et al., 1997; Brose et al., 2000; Richmond et al., 2001). In addition, it has been demonstrated that Munc13-1 interacts with the synaptic core complex by binding directly to syntaxin. Therefore, Munc13-1 has a central role in synaptic vesicle priming (Betz et al., 1997).

The work described in this chapter aimed to determine whether the activation of PKC in response to nicotinic receptor activation (which was previously shown to enhance catecholamine release (see Chapter 5)) was mediated by DAG generated by activation of PLC.

In addition, this work sought to determine whether Munc13 was activated by nicotinic receptor stimulation and whether this was also dependent on PLC activation.
6.2 RESULTS

Single cell imaging of phospholipase C activity using fluorescent biosensors

Transient transfection of chromaffin cells with eGFP-PH<sub>PLC</sub><sup>B1</sup> resulted in accumulation of fluorescence over the plasma membrane representing the association of the fusion protein with PtdIns(4,5)P<sub>2</sub>. Challenge of cells with either nicotine (100μM), histamine (100μM) or angiotensin II (100nM) resulted in loss of membrane association and enrichment of cytosolic fluorescence in chromaffin cells (approximately 40-60% of transfected cells showed translocation of eGFP-PH<sub>PLC</sub><sup>B1</sup> in response to each agonist) (Figures 6.2.1, 6.2.2 and 6.2.3). Plotting the change in cytosolic fluorescence relative to basal level against time during prolonged agonist challenge indicated that both nicotine and angiotensin II caused a transient increase in cytosolic fluorescence (Figure 6.2.3 and Figure 6.2.2). In contrast, challenge of cells with histamine induced a bigger peak than that mediated by nicotine and angiotensin II followed by a lower sustained phase. These changes in fluorescence returned to basal levels after perfusion with Krebs'/HEPES buffer to remove the agonist (Figure 6.2.1).

The potential impact of nicotinic receptors or G<sub>α</sub><sub>q11</sub>-coupled receptors on the other limb of the signalling pathway resulting from PLC-mediated hydrolysis of plasma membrane bound PtdIns(4,5)P<sub>2</sub> was assessed by determining DAG production using the eGFP-PKC<sub>γ</sub>Cl<sub>2</sub> biosensor.

In cells transiently transfected with eGFP-PKC<sub>γ</sub>Cl<sub>2</sub>, challenge with either angiotensin II (100nM) or histamine (100μM) resulted in rapid and robust loss of cytosolic fluorescence from the cytosol (Figure 6.2.4 and Figure 6.2.5) (mean data are shown in Figure 6.2.7: panel B). Nicotine (100μM) caused a rapid (40s) and transient reduction in cytosolic fluorescence and a corresponding increase at the
plasma membrane (Figure 6.2.6). The mean data for agonist-mediated changes in
cytosolic fluorescence in cells expressing either eGFP-PH_{PLC\delta1} or eGFP-PKC\gammaCl2
are summarized in Figure 6.2.7: panels A and B.
Figure 6.2.1. Single cell imaging of histamine-induced changes in the distribution of eGFP-PH\textsubscript{PLC8I} in cultured bovine adrenal chromaffin cells. Cells transiently transfected with eGFP-PH\textsubscript{PLC8I} were perfused with histamine (100\muM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 30 cells in total (~60% of examined cells responded)).
Figure 6.2.2. Single cell imaging of angiotensin II-induced changes in the distribution of eGFP-PH$_{PLC\delta1}$ in cultured bovine adrenal chromaffin cells. Cells transiently transfected with eGFP-PH$_{PLC\delta1}$ were perfused with angiotensin II (100nM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 20 cells in total (~ 40% of examined cells responded)).
Figure 6.2.3. Single cell imaging of nicotine-induced changes in the distribution of eGFP-PH$_{PLC\delta}$ in cultured bovine adrenal chromaffin cells. Chromaffin cells transiently transfected with eGFP-PH$_{PLC\delta}$ were perfused with nicotine (100μM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 24 cells in total (~ 40% of examined cells responded)).
Figure 6.2.4. Single cell imaging of angiotensin II-induced changes in the distribution of eGFP-PKC\(\gamma\)Cl\(_2\) in cultured bovine adrenal chromaffin cells. Cells transiently transfected with eGFP-PKC\(\gamma\)Cl\(_2\) were perfused with angiotensin II (100nM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 18 cells in total (~42% of examined cells responded)).
Figure 6.2.5. Single cell imaging of histamine-induced changes in the distribution of eGFP-PKCγCl2 in cultured bovine adrenal chromaffin cells. Cells transiently transfected with eGFP-PKCγCl2 were perfused with histamine (100μM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 29 cells in total (~37% of examined cells responded)).
Figure 6.2.6. Single cell imaging of nicotine-induced changes in the distribution of eGFP-PKCγCl₂ in cultured bovine adrenal chromaffin cells. Chromaffin cells transiently transfected with eGFP-PKCγCl₂ were perfused with nicotine (100μM) and confocal images were collected at a rate of 3-4 frames per second. Images A and B were taken at the points indicated on the graph. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. Data are representative of four independent experiments (four adrenal gland preparations, approximately 24 cells in total (~33% of examined cells responded)).
Figure 6.2.7. Summary of changes in cytosolic fluorescence in response to agonist stimulation in cultured bovine adrenal chromaffin cells. Cells transiently transfected with either eGFP-PH$_{PLC\delta}$ or eGFP-PK$_{CYCl_2}$ were perfused with agonists and confocal images were collected at a rate of 3-4 frames per second. Panel A: shows the mean maximal peak change in fluorescence for different agonists. Panel B: shows the mean maximal reduction in cytosolic fluorescence following agonist addition. A region of interest was selected in the cytosol and the change in fluorescence was expressed graphically as the fold change in fluorescence relative to the basal level. The dotted lines indicate the basal fluorescence. Data are mean + SEM from three different cell preparations.
Temporal profiles of total inositol phosphate generation evoked by nicotinic receptor activation

In [3H] inositol-labeled cells, in which inositol monophosphatase activity had been blocked with Li⁺, nicotine (100μM) caused a time-dependent accumulation of [3H]-InsPₓ (Figure 6.2.8). The greatest accumulation of [3H]-InsPₓ evoked by nicotine was achieved at 30 min (203.90 ± 28.20% of basal levels). Similarly, either histamine (100μM) or angiotensin II (100nM) (used here as positive controls for PLC activation) were able to mediate time-related and robust [3H]-InsPₓ accumulations (Figure 6.2.8).
Figure 6.2.8. Temporal profiles of $[^3H]$-InsP$_x$ generation evoked by activation of either nicotinic receptors or $\mathrm{G}_{\alpha_{q/11}}$-coupled receptors. Chromaffin cells were prelabelled with $[^3H]$-inositol for 48h before stimulation in the presence of 10mM Li$^+$ with either nicotine (100$\mu$M), histamine (100$\mu$M) or angiotensin II (100nM). The accumulation of $[^3H]$-InsP$_x$ was measured as described in “Materials and Methods”. Data are presented as percentage increase over basal. Data are mean ± SEM from three different cell preparations (each in triplicate). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. basal by one-way ANOVA followed by post-hoc Dunnett’s test.
Effect of wortmannin on nicotine-mediated total $[^3]H$-InsP$_x$ accumulation

Although sterol-like wortmannin is well known for its ability to block phosphatidylinositol 3-kinase (PtdIns 3-kinase) (Okada et al., 1994), at higher concentrations ($\mu$M) is also effective at blocking some isoforms of phosphatidylinositol 4-kinase (PtdIns 4-kinase) (Nakanishi et al., 1995; Downing et al., 1996; Willars et al., 1998b), a crucial enzyme in maintaining the supply of the PLC$\beta$ substrate, PtdIns(4,5)P$_2$ by the phosphorylation of PtdIns. Thus, wortmannin was used to potentially inhibit PtdIns(4,5)P$_2$ generation and to examine the impact of this on nicotinic receptor mediated PLC activation and catecholamine release.

Cells prelabeled with $[^3]H$-inositol were preincubated for 20 min under Li$^+$ block with or without wortmannin (either 1$\mu$M or 10$\mu$M). Following this incubation, cells were stimulated for 30 min with either nicotine (100$\mu$M) or angiotensin II (100nM, used as a positive control for PLC activation). The $[^3]H$-InsP$_x$ generation evoked by either nicotine or angiotensin II was not affected by preincubation of cells with 1$\mu$M wortmannin (Figure 6.2.9). However, $[^3]H$-InsP$_x$ accumulation mediated by either nicotine or angiotensin II were markedly reduced by pre-treatment of cells with 10$\mu$M wortmannin (Figure 6.2.9). In this study, the need to use 10$\mu$M wortmannin to achieve inhibition of $[^3]H$-InsP$_x$ generation was consistent with Willars et al. (1998b). In addition, the inability of 1$\mu$M wortmannin to influence PLC activity shows that the effect was not via inhibition of PtdIns 3-kinase activity as this is inhibited at lower concentrations than PtdIns 4-kinase (Nakanishi et al., 1995).
Figure 6.2.9. Effect of wortmannin on nicotinic receptor and Goqi-coupled receptor mediated stimulation of PLC. Cells were prelabelled with [3H]-inositol for 48h before treatment with wortmannin (1μM or 10μM) for 20 min at 37°C in the presence of 10mM Li+. Cells were then stimulated with either nicotine (100μM) or angiotensin II (100nM) for a further 30 min. The accumulation of [3H]-InsP₃ was measured as described in "Materials and Methods". Data are presented as percentage increase over basal (the basal values were 240 ± 16 dpm/well). Data are mean + SEM from three different cell preparations (each in triplicate). * P<0.05, *** P<0.001 vs. control by one-way ANOVA followed by post-hoc Dunnett’s test.
Effect of wortmannin on catecholamine secretion evoked by nicotinic receptors activation

Wortmannin inhibits the generation of PtdIns(4,5)P2 thereby inhibiting the activity of PLC. If either PtdIns(4,5)P2 or PLC activity are required for catecholamine release, wortmannin would therefore be expected to inhibit nicotinic receptor-mediated release.

Cells were pre-incubated with or without wortmannin (10μM) for 20 min and then stimulated with nicotine (100μM) for a further 10 min. Catecholamine secretion was measured as described in “Materials and Methods”. Inhibition of PtdIns 4-kinase by wortmannin had no effect on basal release while it markedly attenuated secretion of catecholamines evoked by nicotine (Figure 6.2.10).

Wortmannin also dramatically reduced the release of catecholamines mediated by either histamine (100μM) or angiotensin II (100nM) used as positive controls for the inhibition of PLC activity (Figure 6.2.10).
Figure 6.2.10. Effect of wortmannin on catecholamine secretion evoked by either nicotinic receptors or \( \text{G}_{\alpha_{q11}} \)-coupled receptors. Chromaffin cells were incubated with wortmannin for 20 min at 37°C before stimulation with either nicotine (100µM), histamine (100µM) or angiotensin II (100nM). Catecholamine release over 10 min stimulation with agonist was measured as described in “Materials and Method” and is expressed as percentage of total cellular content. Data are mean + SEM from three different cell culture preparations. ** \( P<0.01 \), *** \( P<0.001 \) vs. appropriate control by unpaired Student’s \( t \)-test.
Inhibition of PLC with U73122 reduces nicotine-mediated accumulation of total $[^3\text{H}]$-InsP$_x$ and catecholamine secretion

To assess the involvement of PLC in nicotinic receptor-mediated catecholamine secretion, the selective PLC inhibitor U-73122 or its inactive isomer U-73343 (Zhang et al., 1995b) were used. Initially, the effect of PLC inhibition on total $[^3\text{H}]$-InsP$_x$ generation evoked by nicotine was examined and the impact of this inhibition on secretion of catecholamines was examined subsequently.

Pretreatment of cells with the PLC inhibitor U-73122 (either 1μM or 10μM, 30 min) significantly inhibited nicotine-mediated accumulation of $[^3\text{H}]$-InsP$_x$ (Figure 6.2.11) and catecholamine secretion (Figure 6.2.12).

As a positive control, U-73122 (either 1μM or 10μM, 30 min) was also shown to inhibit histamine-mediated $[^3\text{H}]$-InsP$_x$ accumulation (Figure 6.2.11) and release of catecholamines (Figure 6.2.12). In contrast, U-73343 (10μM), the inactive analogue of U-73122 did not influence either nicotine or histamine-evoked $[^3\text{H}]$-InsP$_x$ accumulation and secretion of catecholamines (Figure 6.2.11 and Figure 6.2.12).
Figure 6.2.11. Effect of the PLC inhibitor U73122 or its negative control U73343 on nicotinic receptor and Go_q11-coupled receptor mediated stimulation of PLC. Chromaffin cells were prelabelled with [3H]-inositol for 48h before treatment with either U73122 (1 or 10μM, 30 min) or U73343 (10μM, 30 min) at 37°C in the presence of 10mM Li+ then stimulated with either nicotine (100μM) or histamine (100μM). The accumulation of [3H]-InsP_x was measured as described in “Materials and Methods”. Data are presented as percentage increase over basal (the basal values are 242 ± 17 dpm/well). Data are mean ± SEM from three different cell preparations (each in triplicate). ** P<0.01, *** P<0.001 vs. control by one-way ANOVA followed by post-hoc Dunnett’s test.
Figure 6.2.12. Effect of the PLC inhibitor U73122 or its negative control U73343 on catecholamine secretion evoked by nicotinic receptor and Goq/11-coupled receptor activation. Chromaffin cells were incubated with either U73122 (1μM or 10μM, 30 min) or U73343 (10μM, 30 min) at 37°C before stimulation with either nicotine (100μM) or histamine (100μM). Catecholamines released over 10 min stimulation with agonist were measured as described in “Materials and Methods” and are expressed as percentage of total cellular content. Data are mean ± SEM from three different cell preparations. * P<0.05, *** P<0.001 vs. control by one-way ANOVA followed by post-hoc Dunnett’s test.
The dependence on extracellular Ca\(^{2+}\) of PLC stimulation mediated by nicotinic receptors

Ca\(^{2+}\)-free buffer (i.e. no added Ca\(^{2+}\) to Krebs’/HEPES buffer) was used to test whether nicotinic receptor triggered \(\text{[^{3}H]}\)-InsP\(_x\) generation (as an index of PLC activation) was dependent upon influx of extracellular Ca\(^{2+}\).

Removal of extracellular Ca\(^{2+}\) essentially abolished nicotinic receptor-mediated accumulation of \(\text{[^{3}H]}\)-InsP\(_x\) (Figure 6.2.13). In contrast, removal of extracellular Ca\(^{2+}\) did not abolish but did attenuate \(\text{[^{3}H]}\)-InsP\(_x\) accumulation in response to either histamine or angiotensin II, particularly at later time points (i.e. 15 and 30 min) (Figure 6.2.14).
Figure 6.2.13. Time course of nicotinic receptor-mediated accumulation of total inositol phosphates in the presence or absence of extracellular Ca$^{2+}$. Cells were prelabelled with $[^{3}H]$-inositol for 48h before stimulation with nicotine (100μM) in the presence of 1.3mM [Ca$^{2+}$]_e or its absence (i.e., no added Ca$^{2+}$). The accumulation of $[^{3}H]$-InsP_x was measured as described in “Materials and Methods”. Data are presented as percentage increase over basal. Dotted line indicates the basal $[^{3}H]$-InsP_x (223 ± 15 dpm/well). Data are mean + SEM from three different cell preparations (each in triplicate). ***P<0.001, vs. control in the presence of extracellular Ca$^{2+}$ by unpaired Student’s t-test.
Figure 6.2.14. Time course of $\mathrm{Ga}_{q/11}$-coupled receptor-mediated accumulation of total inositol phosphates in the presence or absence of extracellular $\mathrm{Ca}^{2+}$. Cells were prelabelled with $[\mathrm{H}]$-inositol for 48h before stimulation with either histamine (100$\mu$M) (panel A) or angiotensin II (100nM) (panel B) in the presence of 1.3mM $[\mathrm{Ca}^{2+}]_e$ or its absence (i.e. no added $\mathrm{Ca}^{2+}$). The accumulation of $[\mathrm{H}]$-InsP$_x$ was measured as described in “Materials and Methods”. Data are presented as percentage increase over basal. Dotted lines indicate the basal $[\mathrm{H}]$-InsP$_x$ (239 ± 13 dpm/well). Data are mean ± SEM from three different cell preparations (each in triplicate). * $P<0.05$, ** $P<0.01$ vs. control in the presence of extracellular $\mathrm{Ca}^{2+}$ by unpaired Student’s t-test.
Effect of cholinergic receptor antagonists on nicotine and acetylcholine mediated activation of PLC

Since acetylcholine is the physiological secretagogue for chromaffin cells (Douglas and Rubin, 1961) and is able to activate both types of cholinergic receptors (nicotinic and muscarinic) (Wilson and Kirshner, 1977), it was used here along with selective nicotinic and muscarinic receptor antagonists to further explore the role of these receptors in the activation of PLC.

As seen in Figure 6.2.15 both nicotine (100μM) and acetylcholine (100μM) elicited [³H]-InsP₅ accumulation although that evoked by acetylcholine was significantly greater. Preincubation of cells with atropine (10μM), a selective muscarinic receptor (mAChR) antagonist significantly reduced acetylcholine-induced total [³H]-InsP₅ generation but had no effect on nicotine-evoked [³H]-InsP₅ production.

In contrast, preincubation with either d-tubocurarine (100μM) or hexamethonium (1mM), selective nicotinic receptor (nAChR) blockers, essentially abolished nicotine-mediated [³H]-InsP₅ accumulation and partially inhibited the response to acetylcholine.
Figure 6.2.15. Effect of cholinergic receptor antagonists on nicotine- and acetylcholine-mediated stimulation of PLC. Cells were prelabelled with $[^{3}H]$-inositol for 48 h before treatment with either vehicle control, atropine (10 μM), d-tubocurarine (100 μM), or hexamethonium (1 mM) for 10 min at 37°C in the presence of 10 mM Li+. Cells were then stimulated with either nicotine (100 μM) or acetylcholine (100 μM). The accumulation of $[^{3}H]$-InsP$_{x}$ was measured as described in “Materials and Methods”. Data are presented as percentage increase over basal (210 ± 11 dpm/well). Data are mean ± SEM from three different cell culture preparations (each in triplicate). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control by one-way ANOVA followed by post-hoc Dunnett’s test.
Effect of apyrase on total $[^{3}\text{H}]-\text{InsP}_x$ accumulation mediated by nicotinic receptors

Chromaffin cells of the adrenal medulla store catecholamines, nucleotides and peptides in membrane-bound organelles: the chromaffin vesicles (Trifaró, 1977; Winkler and Westhead, 1980). Upon stimulation chromaffin vesicles release their content to the cell exterior by exocytosis. Thus, ATP is co-released with catecholamines and could act via P$_2$Y purinoceptors that are coupled to PLC. Indeed, chromaffin cells express P$_2$Y receptors (Currie and Fox, 1996) and thus ATP could activate PLC following nicotinic receptor stimulation.

To test the contribution of ATP to nicotinic receptor-mediated PLC activation, the effect of apyrase (ATP diphosphohydrolase, which converts nucleotide polyphosphates to nucleoside monophosphates (Curdova et al., 1982; Tim et al., 2002) was examined as the ATP hydrolysis products adenosine and AMP do not increase $[\text{Ca}^{2+}]_i$ or stimulate inositol lipid metabolism (Gosink and Forsberg, 1993).

Preincubation of cells with apyrase (10 u.ml$^{-1}$, grade III, 10 min) under Li$^+$-block of inositol monophosphatase did not elicit any significant inhibition of total $[^{3}\text{H}]-\text{InsP}_x$ accumulation triggered by nicotinic receptor stimulation. However, apyrase significantly reduced total inositol $[^{3}\text{H}]-\text{InsP}_x$ generation evoked by UTP (300$\mu$M), a P$_2$Y$_2$ selective agonist that was used here as a positive control (Figure 6.2.16).
Figure 6.2.16. Effect of apyrase on nicotine- and UTP-mediated stimulation of PLC. Cells were prelabelled with $[^3$H]-inositol for 48h before treatment with apyrase (10 u.ml$^{-1}$) for 10 min at 37°C in the presence of 10mM Li$^+$ then stimulated with either nicotine (100μM) or UTP (300μM) for a further 30 min. The accumulation of $[^3$H]-InsP$_x$ was measured as described in “Materials and Methods”. Data are presented as percentage increase over basal (211 ± 12 dpm/well). Data are mean ± SEM from three different cell preparations (each in triplicate). *** P<0.001 vs. control, one-way ANOVA followed by post-hoc Dunnett’s test.
**Effect of PTX on nicotinic receptor-mediated PLC activation**

Opioids are also co-released with catecholamines and nucleotides from chromaffin granules (Livett et al., 1981). Although, opioids activate $\text{G}_\alpha_i$-coupled receptors, those can in some circumstances activate PLC (Charles et al., 2003; Werry et al., 2003). However, such activation is sensitive to inhibition of $\text{G}_\alpha_i$ with pertussis toxin PTX.

Chromaffin cells express opioid receptors (Bunn et al., 1988; Margioris et al., 1995). To investigate whether nicotinic receptor-mediated PLC activation is a consequence of released opioids acting on $\text{G}_\alpha_i$-coupled $\mu$- and $\delta$-opioid receptors the effect of PTX on total $[\text{H}]$-InsP$_x$ generation mediated by nicotinic receptors was studied.

Treatment of cells with PTX (100 ng.ml$^{-1}$, 24h) had no significant effect on either basal or nicotine-mediated $[\text{H}]$-InsP$_x$ accumulation. In contrast, PTX significantly attenuated total $[\text{H}]$-InsP$_x$ production mediated by angiotensin II (100nM), demonstrating that at least part of this response was mediated through $\text{G}_\alpha_{i/o}$ (Figure 6.2.17).
Figure 6.2.17. Effect of pertussis toxin on nicotine- and angiotensin II-mediated stimulation of PLC. Cells were prelabelled with \([^{3}H]\)-inositol for 24h before treatment with pertussis toxin (PTX) (100 ng.ml\(^{-1}\)) for a further 24h at 37°C. Cells were then stimulated with either nicotine (100μM) or angiotensin II (100nM) for 30 min at 37°C in the presence of 10mM Li\(^+\). The accumulation of \([^{3}H]\)-InsP\(_3\) was measured as described in “Materials and Methods”. Data are presented as percentage increase over basal. Data are mean ± SEM from three different cell preparations (each in triplicate). * P<0.05 vs. control, one-way ANOVA followed by post-hoc Dunnett’s test.
Effect of stabilization of cortical F-actin on $[^3H]$-InsP$_x$ accumulation mediated by nicotinic receptors

Although efforts were made to assess the possible contribution of released compounds to nicotine-mediated PLC activation, the experiments do not cover all possible receptor types. As an alternative, jasplakinolide was used. This blocks catecholamine release and is likely therefore to the stimulated release of any other potentially receptor active compounds. The effects of jasplakinolide on nicotinic receptor-mediated PLC activation were therefore investigated.

Pretreatment of cells with jasplakinolide (10μM, 30 min) had no significant effect on total $[^3H]$-InsP$_x$ generation induced by either nicotine (100μM) or angiotensin II (100nM) (Figure 6.2.18).
Figure 6.2.18. Effect of jasplakinolide, a cortical F-actin stabilizer, on nicotinic receptor and \( \text{G} \alpha_{q/11} \)-coupled receptor mediated stimulation of PLC. Cells were prelabelled with \( ^{[3]}\text{H} \)-inositol for 48h before incubation with or without jasplakinolide (10\( \mu \text{M} \)) for 30 min at 37\( ^{\circ} \text{C} \) in the presence of 10mM Li\(^+\) and then stimulated with either nicotine (100\( \mu \text{M} \)) or angiotensin II (100nM) for a further 30 min. The accumulation of \( ^{[3]}\text{H} \)-InsP\(_x\) was measured as described in “Materials and Methods”. Data are presented as percentage increase over basal (224 \( \pm \) 16 dpm/well). Data are mean \( \pm \) SEM from three different cell preparations (each in triplicate). * \( P<0.05 \), ** \( P<0.01 \), one-way ANOVA followed by post-hoc Bonferroni’s test (not all comparisons are shown).
**Effect of U73122 and PMA on nicotine-mediated catecholamine secretion**

This experiment aimed to investigate the role of PLC activation in nicotinic receptor-evoked secretion of catecholamines.

The results demonstrated that catecholamine release evoked by nicotinic receptor is derived by PLC not other components. To verify this, the effect of combination of either nicotine (100μM) and U73122 (10μM) or nicotine (100μM), PMA (1μM) and U73122 (10μM) on secretion were tested. Data indicated that the effect of nicotine on secretion markedly reduced in the presence of U73122 (17.68 ± 0.17% vs. 24.22 ± 0.81% of control) (** P<0.01 vs. control, as determined by ANOVA followed by Bonferroni’s test).

PMA was added to this combination to substitute for inhibited PLC but it was not able to induce any significant changes in catecholamine secretion from that induced by combination of nicotine and U73122 (17.78 ± 0.35% (data of nicotine plus U73122 plus PMA) vs. 17.68 ± 0.17% (data of nicotine plus U73122) (Figure 6.2.19). These data are difficult to interpreted for the following reasons. The inhibition of nicotine-mediated release by U73122 suggests PLC activation is important, possibly for the generation of DAG and the subsequent activation of PKC (or other DAG targets). Any such lack of DAG generation should be overcome for by PMA. The inability of PMA to overcome the inhibition by U73122 suggests either that this compound is mediating its effects via a mechanism independent of PLC or that another aspect of PLC activity (apart from DAG generation) is important. This could include PLC-dependent Ca$^{2+}$ signalling. Indeed the nicotinic receptor-evoked Ca$^{2+}$ response was reduced by both U73122 and thapsigargin, suggesting that a PLC-dependent rise of [Ca$^{2+}$], is an important component for release.
Figure 6.2.19. The effect of U73122 and PMA on nicotine-mediated catecholamine secretion. Chromaffin cells were incubated with U73122 (10μM) for 30 min at 37°C before stimulation with both PMA (1μM) and nicotine (100μM). Catecholamine released over a 10 min stimulation with agonist was measured as described in “Materials and Methods” and is expressed as percentage of total cellular content. Data are mean ± SEM from three different cell preparations. * P<0.05, ** P<0.01, one-way ANOVA followed by Bonferroni’s test. b, c, d, e and f are significantly different from a at *** P<0.001. (Not all comparisons are shown on the graph).
Effect of thapsigargin on nicotine-mediated Ca\textsuperscript{2+} signalling

The influx of Ca\textsuperscript{2+} into chromaffin cells appeared to play a major role in regulating PLC activity in response to nicotinic receptor activation (6.2.13). However, it was unclear if nicotine evoked the release of intracellular Ca\textsuperscript{2+} and whether this was involved in promoting secretion. Thus, thapsigargin has been shown to selectively prevent sequestration of Ca\textsuperscript{2+} into the endoplasmic reticulum by inhibiting the Ca\textsuperscript{2+}-ATPase pumps of the endoplasmic reticulum (Thastrup et al., 1990; Lytton et al., 1991). Confocal imaging of Ca\textsuperscript{2+} signals and fluo-3-loaded cells were used to monitor the changes in [Ca\textsuperscript{2+}]\textsubscript{i}.

Challenge of cells with nicotine (100\muM) revealed a robust (3.0 fold over basal), rapid (30s) peak followed by a lower (1.81 fold over basal) sustained phase of elevated [Ca\textsuperscript{2+}]\textsubscript{i} (Figure 6.2.20: panel A). Treatment with thapsigargin slightly but significantly reduced peak (2.10 vs. 3.0 of control fold over basal) and plateau (1.36 vs. 1.81 of control, fold over basal) Ca\textsuperscript{2+} response mediated by nicotine (6.2.20: panel B).

Histamine (100\muM), used as a positive control for a response depending upon intracellular Ca\textsuperscript{2+} stores, induced a peak (2.12 fold over basal) and sustained plateau phase (1.50 fold over basal) of Ca\textsuperscript{2+} signalling (Figure 6.2.21: panel A). However, Ca\textsuperscript{2+} signalling evoked by histamine was less robust compared to nicotine. Treatment with thapsigargin abolished both peak and sustained phase Ca\textsuperscript{2+} responses to histamine (6.2.21: panel B).

The mean data of the Ca\textsuperscript{2+} responses are summarized in Figure 6.2.20: panel C.
Figure 6.2.20. Effect of thapsigargin on nicotine-mediated Ca\(^{2+}\) signalling in bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at \(\lambda488\) nm and imaged on the confocal microscope. Panel A: cells were challenged with nicotine (100\(\mu\)M) after 30s. Panel B: cells were pretreated with thapsigargin (2\(\mu\)M, 10 min) at 37\(^\circ\)C then challenged with nicotine (100\(\mu\)M). Bar indicates the presence of nicotine. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three independent experiments (three adrenal gland preparations, approximately 180 cells in total). Panel C shows the summary data of three independent cell culture preparations. Data are mean + SEM of three independent cell culture preparations. * \(P<0.05\) vs. appropriate control (peak or plateau) by unpaired Student’s \(t\)-test.
A. Control

B. Thapsigargin pretreated

C. Summary data

Figure 6.2.21. Effect of thapsigargin on histamine-mediated Ca\(^{2+}\) signalling in bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at \(\lambda_{488}\) nm and imaged on the confocal microscope. Panel A: cells were challenged with histamine (100\(\mu\)M) after 30s. Panel B: cells were pretreated with thapsigargin (2\(\mu\)M, 10 min) at 37\(^{\circ}\)C then challenged with histamine (100\(\mu\)M). Bar indicates the presence of histamine. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three independent experiments (three adrenal gland preparations, approximately 180 cells in total). Panel C shows the summary data of three independent cell culture preparations. Data are mean + SEM of three independent cell culture preparations. * \(P<0.05\), ** \(P<0.01\) vs. appropriate control (peak or plateau) by unpaired Student’s \(t\)-test.
Effect of U73122 on nicotine-mediated Ca\textsuperscript{2+} signalling

Confocal imaging of Ca\textsuperscript{2+} signalling in fluo-3 loaded cells and U73122 were used to determine if the activation of PLC contributed to the Ca\textsuperscript{2+} signalling mediated by nicotinic receptors.

Pre-treatment of cells for 30 min with U73122 (10\mu M, 30 min) reduced both the peak (Figure 6.2.22: panel A) and sustained phase (1.05 ± 0.08% vs. 1.81 ± 0.15%) (Figure 6.2.22: panel C) of [Ca\textsuperscript{2+}]\textsubscript{i} elevation mediated by nicotine (100\mu M). U73343 (10\mu M, 30 min) used as negative control for U73122 had no effect on Ca\textsuperscript{2+} signalling evoked by nicotine (Figure 6.2.22: panel B). Mean data are summarized in Figure 6.2.22: panel D.

Preincubation of cells with U73122 abolished the Ca\textsuperscript{2+} response mediated by histamine (100\mu M), which was used here as a positive control for PLC activation (Figure 6.2.23: panel A and C). However, U73343 failed to antagonize histamine-mediated Ca\textsuperscript{2+} signalling (Figure 6.2.23: panel B). Mean data are summarized in Figure 6.2.23: panel D.
Figure 6.2.22. Effect of U73122 on nicotine-mediated Ca$^{2+}$ signalling in bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at λ488 nm and imaged on the confocal microscope. Panel A: cells were challenged with nicotine (100μM) after 30s. Cells were pretreated with either U-73343 (10μM, panel B) or U-73122 (10μM, panel C) for 30 min at 37°C then challenged with nicotine (100μM, panels B and C). Bars indicate the presence of nicotine. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three independent experiments (three adrenal gland preparations, approximately 150 cells in total for each condition). Panel D shows the summary data of three independent cell culture preparations. Data are mean ± SEM and ** $P < 0.01$ vs. appropriate control (peak or plateau) by ANOVA followed by post hoc Dunnett’s test.
Figure 6.2.23. **Effect of U73122 on histamine-mediated Ca\(^{2+}\) signalling in cultured bovine adrenal chromaffin cells.** Cells were cultured on glass coverslips, loaded with fluo-3, excited at \(\lambda 488\) nm and imaged on the confocal microscope. Panel A: cells were challenged with histamine (100\(\mu\)M) after 30s. Cells were pretreated with either U73343 (10\(\mu\)M, panel B) or U73122 (10\(\mu\)M, panel C) for 30 min at 37°C then challenged with histamine (100\(\mu\)M, panel B and C). Bars indicate the presence of histamine. The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three independent experiments (three adrenal gland preparations, approximately 150 cells in total for each condition). Panel D shows the summary data of three independent cell culture preparations. Data are mean + SEM and * \(P < 0.05\), ** \(P < 0.01\) by one-way ANOVA followed by post-hoc Dunnett’s test.
Effect of Cd\textsuperscript{2+} on nicotine and KCl mediated Ca\textsuperscript{2+} signalling

The data described previously demonstrated that PLC activation in response to nicotine was Ca\textsuperscript{2+}-dependent (see Figure 6.2.13). As Ca\textsuperscript{2+} entry in response to nicotinic receptor activation could be via either VOCCs or the nicotinic receptor channels themselves (See Chapter 3: Section 3.3.4), these studies sought to distinguish between the contribution of VOCCs and nicotinic receptor channels to Ca\textsuperscript{2+} signalling using confocal imaging of [Ca\textsuperscript{2+}], in fluo-3 loaded cells.

Cd\textsuperscript{2+} (100\muM) rapidly and reversibly blocks all voltage-activated Ca\textsuperscript{2+} channels but at this concentration does not interfere with activation of nicotinic receptors (Gray et al., 1996). Pre-treatment of cells with Cd\textsuperscript{2+} abolished Ca\textsuperscript{2+} signalling evoked by KCl (40mM) demonstrating the ability of Cd\textsuperscript{2+} at this concentration to block VOCCs (Figure 6.2.24). In contrast, Cd\textsuperscript{2+} failed to inhibit nicotine-mediated Ca\textsuperscript{2+} response (Figure 6.2.25). Mean data are summarized in Figure 6.2.26.
Figure 6.2.24. Effect of Cd$^{2+}$ on KCl-mediated Ca$^{2+}$ signalling in cultured bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at $\lambda 488$ nm and imaged on the confocal microscope. Cells were challenged with KCl (40mM) in the absence or presence of Cd$^{2+}$ (100µM, 5 min pre-treatment). The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three different cell culture preparations. Mean data are shown in Figure 6.2.26.
Figure 6.2.25. Effect of Cd$^{2+}$ on nicotine-mediated Ca$^{2+}$ signalling in cultured bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at $\lambda$488 nm and imaged on the confocal microscope. Cells were challenged with nicotine (100µM) in the absence or presence of Cd$^{2+}$ (100µM, 5 min pre-treatment). The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. Data are representative of three different cell culture preparations. Mean data are shown in Figure 6.2.26.
Figure 6.2.26. Summary of the effect of Cd$^{2+}$ on nicotine- or KCl-mediated Ca$^{2+}$ signalling in cultured bovine adrenal chromaffin cells. Cells were cultured on glass coverslips, loaded with fluo-3, excited at λ488 nm and imaged on the confocal microscope. Cells were challenged with either nicotine (100μM) or KCl (40mM) in the absence or presence of Cd$^{2+}$ (100μM, 5 min pre-treatment). The change in fluorescence of all cells in the field of view was averaged and analysed by purpose written software and is represented graphically as the fold increase in fluorescence relative to initial basal levels. The peak values are analysed and data are mean ± SEM from three different cell culture preparations (number of cells for each condition is shown in parentheses). * $P<0.05$ vs. control by unpaired Student’s $t$-test.
Effect of U73122 on nicotine-mediated subcellular re-distribution of PKCα

Previous work described in this thesis demonstrated that PKCα played a major role in nicotinic receptor-evoked secretion of catecholamines (see Chapter 5). Thus, the aim here was to determine if nicotinic receptor-mediated activation of PKCα was dependent upon the activation of PLC. Western blotting of cytosol and membrane fractions followed by densitometry was used to study the effect of PLC inhibition on nicotinic receptor-mediated PKCα translocation as an index of its activation. As described earlier (see Chapter 5), to validate the translocation method, the effect of exposure to PMA (1μM, 10 min) on the subcellular distribution of PKCα was examined. PMA mediated a significant loss of PKCα from the cytosolic fraction and this was accompanied by a marked increase in the membrane fraction (Figure 6.2.27). Pretreatment of cells with either U73122 (10μM) or its inactive analogue U73343 (10μM) failed to inhibit recruitment of PKCα to the plasma membrane in response to PMA (Figure 6.2.27).

Nicotine (100μM) mediated a dramatic translocation of PKCα from the cytosolic fraction to the membrane fraction. Pre-treatment of cells with U73122 markedly inhibited translocation of PKCα-mediated by nicotine (100μM, 10 min) from the cytosolic fraction to the membrane (Figure 6.2.27). However, U73343 was unable to prevent recruitment of PKCα to the plasma membrane triggered by nicotine (Figure 6.2.27: panel B and C).

Both cytosolic and membrane fractions were probed with β-actin antibody to confirm both an equal loading and that the membrane fraction was not contaminated with cytosol during the cell fractionation process.
Figure 6.2.27. Effect of U73122 on nicotine-mediated subcellular redistribution of PKCα in cultured bovine adrenal chromaffin cells. Cells were incubated with either U73122 (10μM) or U73343 (10μM) for 30 min before stimulation with either PMA (1μM) or nicotine (100μM) for 10 min at 37°C. The cytosol and membrane fractions were prepared and immunoblots performed as described in “Materials and Methods”. The density of immunoblots in the cytosol and membrane fractions were quantified using Scion Image analysis software. Cytosolic and membrane readings were calculated as a percentage of the total (membrane plus cytosol). ** P<0.01, *** P<0.001 by one-way ANOVA followed by Dunnett’s test vs. the appropriate (cytosol or membrane) control. Data are mean + SEM from three experiments performed on different cell culture preparations.
Effect of PLC inhibition on nicotine-mediated phosphorylation of MARCKS

The phosphorylation of MARCKS has been used as a marker for PKC activation (Bar-Am et al., 2004). Earlier work described in this thesis demonstrated that phosphorylation of MARCKS in response to nicotinic receptor activation was PKC dependent (see Chapter 5). Here, further experiments were performed to determine whether PKC-dependent MARCKS phosphorylation in response to nicotinic receptor activation is PLC dependent. This question was addressed by using Western blotting of phospho-MARCKS.

Treatment of cells with PMA (1μM, 10 min) induced phosphorylation of MARCKS. This phosphorylation was not inhibited by preincubation of cells with either U73122 (10μM, 30 min) or U73343 (10μM, 30 min) (Figure 6.2.28: panels A and B).

Activation of nicotinic receptors with nicotine (100μM, 10 min) triggered phosphorylation of MARCKS and this was abolished by U73122. However, the negative control of U73122, U73343, failed to inhibit nicotine-mediated MARCKS phosphorylation (Figure 6.2.28: panels A and B).

These data suggest that phosphorylation of MARCKS-mediated by nicotinic receptors is dependent on PLC and its downstream signalling cascade.
A.

Panel A shows the immunoblot of phospho-MARCKS (p-MARCKS). The amount of phospho-MARCKS was quantified using Scion Image analysis software and expressed as a percentage of control. Panel B shows cumulative data on MARCKS phosphorylation obtained from experiments carried out on four different cell culture preparations. Data are mean ± SEM and * P<0.05, ** P<0.01 by one-way ANOVA followed by Bonferroni’s test (not all comparisons are shown on the graph).

Figure 6.2.28. Effect of PLC inhibition on nicotine-mediated phosphorylation of MARCKS in chromaffin cells. Chromaffin cells were incubated with either U73122 (10μM) or U73343 (10μM) for 30 min at 37°C before stimulation with either nicotine (100μM) or PMA (1μM) for a further 20 min 37°C. Immunoblots of the whole cell lysate were prepared as described in “Materials and Methods”. Panel A shows the immunoblot of phospho-MARCKS (p-MARCKS). The amount of phospho-MARCKS was quantified using Scion Image analysis software and expressed as a percentage of control. Panel B shows cumulative data on MARCKS phosphorylation obtained from experiments carried out on four different cell culture preparations. Data are mean ± SEM and * P<0.05, ** P<0.01 by one-way ANOVA followed by Bonferroni’s test (not all comparisons are shown on the graph).
Nicotine-mediated redistribution of MARCKS-eGFP: effects of the PLC inhibitor U73122

As described previously, the immunoblotting approach was used earlier in this chapter to demonstrate the impact of the PLC inhibitor on nicotinic receptor-mediated MARCKS phosphorylation but this technique has a limited temporal and spatial resolution. Therefore, another alternative approach, MARCKS-eGFP and confocal imaging were used to examine influence of PLC inhibitor on nicotinic receptor-mediated MARCKS activation in real time at single cell.

Real-time confocal imaging of cells transiently transfected with MARCKS-eGFP demonstrated that nicotine (100μM) caused a transient translocation of MARCKS-eGFP from the plasma membrane to the cytosol (cytosolic fluorescence 1.79 ± 0.09 fold over basal) (Figure 6.2.29: panel A).

Preincubation of cells with U73122 (10μM, 30 min) markedly inhibited nicotinic receptor-mediated redistribution of MARCKS-eGFP to the cytosol (Figure 6.2.29: panel C). However, U73343 (10μM, 30 min) did not influence the ability of nicotine to cause a translocation of MARCKS-eGFP from the plasma membrane to the cytosol (cytosolic fluorescence 1.45 ± 0.07 fold over basal (~20 cells) thereby it completely failed to inhibit MARCKS-eGFP redistribution (Figure 6.2.29: panel B).
Figure 6.2.29. Single cell imaging of nicotine-mediated redistribution of MARCKS-eGFP: effects of the PLC inhibitor U73122. Cells that had been transiently transfected with MARCKS-eGFP were perfused with nicotine (100μM) in the absence of the PLC inhibitor (panel A). Cells were incubated with either U73122 (10μM; panel C) or U73343 (10μM; panel B) for 30 min at 37°C then challenged with nicotine. The change in cytosolic fluorescence was expressed graphically as the fold increase in fluorescence relative to basal levels. Data are representative of three different cell culture preparations (n=25 cells in total).
Effect of PLC inhibition on cortical F-actin disassembly mediated by nicotinic receptor activation

Earlier experiments showed that PKC is involved in nicotinic receptor mediated cortical F-actin disassembly (see Chapter 5). To investigate whether the disruption of cortical F-actin mediated by nicotinic receptor activation is PLC dependent, the effect of inhibition of PLC on nicotinic receptor mediated cortical F-actin disassembly was studied. As previously described (see Materials and Methods), confocal microscopy was used to examine fixed chromaffin cells stained with rhodamine-phalloidin (as a probe for F-actin) (Vitale et al., 1991).

Chromaffin cell cortical F-actin disruption mediated by cytochalasin D (2μM, 20 min), an F-actin destabilizer, was not inhibited by either U73122 (10μM, 30 min) or its negative control U73343 (10μM, 30 min) based on the proportion of cells showing disruption of the cortical F-actin ring (Figure 6.2.30 and Figure 6.2.31). In contrast, the fragmentation of the cortical F-actin ring caused by nicotine (100μM, 40s) was significantly attenuated by U73122 whereas, its inactive analogue U73343 failed to inhibit this cortical F-actin disassembly (Figure 6.2.30 and Figure 6.2.31). These data suggest that PLC plays a role in mediating F-actin disassembly evoked by nicotinic receptor activation.
Figure 6.2.30. Effect of PLC inhibition on cortical F-actin disassembly mediated by either cytochalasin D or nicotine. Chromaffin cells were incubated with either cytochalasin D (2µM, 30 min) or nicotine (100µM, 40s) following preincubation with either the PLC inhibitor U73122 (10µM, 30 min) or its negative control U73343 (10µM, 30 min). Following these treatments, the cells were fixed and processed for rhodamine-labelled phalloidin staining as indicated under “Materials and Methods”. The arrows at the left corner of panels point out the confocal image of the cell. Pseudo-three-dimensional image analysis were performed using “UltraView confocal software” where the maximum intensity of the fluorescent ring was set to 4096 arbitrary units. Images are representative of six hundred cells from three different cell culture preparations. Although these images are chosen as representative, in some instance, for example, effect of nicotine on cortical F-actin in presence of U73122, the proportion of cells showing F-actin disassembly in response to nicotine is still approximately 50% (see Figure 6.2.31). The images have been chosen, however, to reflect the nature of changes following the inhibitor rather than the degree of inhibition.
Figure 6.2.31. Effect of PLC inhibition on cortical F-actin disassembly mediated by either cytochalasin D or nicotine. Chromaffin cells were incubated with either cytochalasin D (2μM, 30 min) or nicotine (100μM, 40s) following preincubation with either the PLC inhibitor U73122 (10μM, 30 min) or its negative control U73343 (10μM, 30 min). Following these treatments, the cells were fixed and processed for rhodamine-labelled phalloidin staining as indicated under “Materials and Methods”. The rhodamine cortical staining of chromaffin cells was analysed and classified as being continuous or discontinuous and the percentage of cells displaying cortical F-actin disassembly (disrupted cortical rhodamine staining) in control and treated preparations was calculated in a single-blind fashion. Six hundred cells for each condition from a total of three different cell cultures were examined. Data shown are mean ± SEM. For * P<0.05. In addition b and c are significantly different from a at P<0.001. All comparisons were by one-way ANOVA followed by post hoc Bonferroni’s test. (Not all comparisons are shown on the graph).
Nicotinic receptor-induced redistribution of transiently expressed Munc13-1-eGFP in chromaffin cells

Interestingly, previously published data demonstrated that part of the effect of phorbol esters on synaptic transmission could not be inhibited by PKC inhibitors (O’Dell et al., 1991) suggesting that there may be additional phorbol ester receptors at a synapse such as Munc13 isoforms (Brose et al., 1995). This study aimed to identify whether exocytosis mediated by nicotinic receptor activation may be regulated not only by PKC but also Munc13. This was explored initially by examining the effect of nicotinic receptor activation on the subcellular distribution of recombinant Munc13-1. Under resting (basal, non-stimulated) conditions, Munc13 is located predominantly in the cytosol. However, following activation it rapidly translocates to the plasma membrane (Betz et al., 1998; Ashery et al., 1999; 2000). In this study, cells were transfected with Munc13-1-eGFP and its potential subcellular translocation determined by confocal microscopy before and during agonist application.

Chromaffin cells transiently transfected with cDNA encoding Munc13-1-eGFP showed a uniform distribution of cytoplasmic fluorescence with nuclear sparing (Figure 6.2.32). PMA (1μM, 10 min) induced a gradual and sustained redistribution of Munc13-1-eGFP to the plasma membrane (cytosolic fluorescence 0.55 ± 0.07 fold of basal (15 cells)). In contrast, application of nicotine (100μM) failed to cause redistribution of Munc13-1-eGFP to the plasma membrane (Figure 6.2.33: panel A). These data suggest that either nicotinic receptor stimulation does not activate Munc13 or alternatively that the methodology is insufficiently sensitive, due, for example to a relatively low level of DAG formation.
In order to address this latter point, an attempt was made to enhance the level of DAG generation in response to nicotinic receptor activation by using a mixture of inhibitors of DAG lipase and kinase. This combination of RHC80267 (30μM, 5 min), an inhibitor of DAG lipase and R59949 ((1μM, 10 min), an inhibitor of DAG kinase, did not affect the inability of nicotine to induce redistribution of Munc13-1-eGFP to the plasma membrane (Figure 6.2.33: panel B).

Histamine (100μM) used as a positive control for PLC activation and downstream signalling pathways (here specifically DAG generation) also failed to induce a redistribution of Munc13-1-eGFP to the plasma membrane (Figure 6.2.34: panel A). Preincubation of cells with a mixture of DAG lipase and kinase inhibitors did not alter the cytoplasmic distribution of fluorescence intensity or its lack of re-distribution in response to histamine (Figure 6.2.34: panel B). Taken together, these data suggest that either agonist-mediated DAG generation is insufficient to induce the translocation of Munc13 or that recombinant Munc13 is insufficiently sensitive, perhaps due to the overexpression of high levels.
Figure 6.2.32. Single-cell imaging of PMA-induced translocation of transiently expressed Munc13-1-eGFP in chromaffin cells. Cells transiently transfected with Munc13-1-eGFP were challenged with PMA (1μM) after 20s. The change in cytosolic fluorescence was expressed graphically as the fold change in fluorescence relative to basal levels. Data are representative of three different cell culture preparations (n=15-20 cells).
Figure 6.2.33. Single-cell imaging of Munc13-1-eGFP during challenge of cells with nicotine. Chromaffin cells transiently transfected with Munc13-1-eGFP were challenged with nicotine (100μM) after 20s in the absence (A) or presence (B) of R59949 (1μM, 10 min preincubation) (DAG kinase inhibitor) and RHC-80267 (30μM, 5 min preincubation) (DAG lipase inhibitor). The change in cytosolic fluorescence was expressed graphically as the fold change relative to basal level. Data are representative of three different cell culture preparations (n=25-35 cells).
Figure 6.2.34. Single-cell imaging of Munc13-1-eGFP during challenge of cells with histamine. Chromaffin cells transiently transfected with Munc13-1-eGFP were challenged with histamine (100μM) after 20s in the absence (A) or presence (B) of R59949 (1μM, 10 min preincubation) (DAG kinase inhibitor) and RHC-80267 (30μM, 5 min preincubation) (DAG lipase inhibitor). The change in cytosolic fluorescence was expressed graphically as the fold change relative to basal level. Data are representative of three different cell culture preparations (n=15-20 cells).
Immunocytochemical determination of the subcellular localization of endogenous Munc13-1 in chromaffin cells: the effect of PLC inhibition on nicotine-mediated redistribution of endogenous Munc13-1

To characterize the subcellular localization of endogenous Munc13-1 in response to nicotinic receptor activation, immunocytochemical studies were performed with a specific Munc13-1 antibody (Duncan et al., 1999; Sheu et al., 2003; Groffen et al., 2004). To exclude non-specific staining, fixed cells were probed only with a FITC-conjugated goat anti-mouse secondary antibody. In control (unstimulated) cells, exogenous Munc13-1 showed a diffuse cytoplasmic distribution of fluorescence with sparing of nucleus (Figure 6.2.35).

In PMA treated cells (1μM, 10 min) fluorescence was associated with the plasma membrane. However, 4α-PMA (1μM, 10 min) failed to mediate a redistribution of the fluorescence from the cytoplasm to the plasma membrane (Figure 6.2.35). When the cells were stimulated with nicotine (100μM), the fluorescence was associated with the plasma membrane (Figure 6.2.35). These data strongly suggest that the inability of nicotine to mediate re-distribution of Munc13-1-eGFP (Figure 6.2.33) is a consequence of its high expression level. Thus, the effect of PLC inhibition on nicotine-mediated re-distribution of endogenous Munc13-1 was studied using immunofluorescence labelling and confocal microscopy.

Preincubation of cells with either U73122 (10μM, 30 min) or its negative control U73343 (10μM, 30 min) did not alter the cytoplasmic distribution of endogenous Munc13-1 of unstimulated cells (Figure 6.2.36 and Figure 6.2.37). Similarly, neither U73122 nor U73343 (Figure 6.2.36 and Figure 6.2.37) influenced the inability of 4α-PMA to alter the distribution of Munc13-1.
Furthermore, neither U73122 nor U73343 were able to inhibit membrane localization of endogenous Munc13-1 mediated by PMA (Figure 6.2.36 and Figure 6.2.37). In contrast, nicotine (100µM, 10 min) caused a redistribution of endogenous Munc13-1 that was significantly attenuated by pretreating cells with U73122. In contrast, the inactive analogue, U73343, failed to prevent recruitment of endogenous Munc13-1 to the plasma membrane in response to nicotine (Figure 6.2.36 and Figure 6.2.37).
Figure 6.2.35. Immunolocalization of Munc13-1 in cultured bovine adrenal chromaffin cells. Cells were fixed and processed for immunocytochemistry as described in “Materials and Methods” using an antibody against Munc13-1. In unstimulated cells, fluorescence showed a diffuse cytoplasmic distribution with nuclear sparing. PMA (1μM, 10 min) caused a redistribution of fluorescence to the cell periphery whereas 4α-PMA (1μM, 10 min) did not. Nicotine (100μM, 10 min) also caused a redistribution of cytosolic fluorescence to the cell periphery. Pictures are representative of three different cell culture preparations (approximately 300 cells were examined in total for each condition).
Figure 6.2.36. Effect of PLC inhibitors on nicotine-mediated Munc13-1 redistribution to the plasma membrane in cultured bovine adrenal chromaffin cells. Cells cultured on coverslips were fixed and processed for immunocytochemistry as described in “Materials and Methods” using an antibody against Munc13-1. In control, unstimulated cells Munc13-1 showed a diffuse cytoplasmic distribution with nuclear sparing. Cells showed membrane localization of Munc13-1 following pre-treatment with either PMA (1μM) or nicotine (100μM) for 10 min. However, 4α-PMA (1μM) did not influence the subcellular distribution of Munc13-1. U73343 (10μM, 30 min preincubation at 37°C) had no effect on the ability of either PMA or nicotine to cause redistribution. U73122 (10μM, 30 min preincubation at 37°C) attenuated the abilities of nicotine but not PMA to cause redistribution. Pictures are representative of three different cell culture preparations (approximately 300 cells were examined in total for each condition). Summary data are shown in Figure 6.2.37.
Figure 6.2.37. Effect of the PLC inhibitor U73122 on nicotine-mediated Munc13-1 re-distribution to the plasma membrane in cultured bovine adrenal chromaffin cells. Cells were incubated with either 4α-PMA (1μM), PMA (1μM) or nicotine (100μM) for 10 min at 37°C following preincubation with either vehicle control (Kreb’s/HEPES buffer), U73122 (10μM) or U73343 (10μM) for 30 min at 37°C. Following these treatments, the cells were fixed and processed for immunocytochemistry as described in “Materials and Methods”. Membrane localization was analysed by calculating the percentage of cells showing membrane localization in control and treated preparations. To avoid personal bias, code numbers were given to coverslips then cells were examined and classified without knowing whether they were from control or treated preparations (single-blind design). A total of three hundred cells from three different cell cultures were examined for each experimental condition. Data shown are mean ± SEM and ** P<0.01, *** P<0.001 by one-way ANOVA followed by post-hoc Bonferroni’s test. (Not all comparisons are shown on the graph).
6.3 DISCUSSION

6.3.1 Summary of data

The aim of this chapter was to investigate the mechanisms underlying PKC activation mediated by nicotinic receptor stimulation, particularly to explore whether PLC is involved. Various approaches were used to confirm the capability of nicotinic receptor stimulation to mediate PLC activation. Firstly, the subcellular re-distribution of transiently expressed eGFP-PH_{PLC\delta1} and eGFP-PKCy_{Cl2} demonstrated nicotinic receptor-mediated generation of Ins(1,4,5)P_3 and DAG generation respectively in real time at the single cell level. Nicotinic receptor activation also resulted in the accumulation of total inositol phosphates as an index of total PLC activation. Finally, pharmacological inhibition of PLC reduced nicotinic receptor-mediated inositol phosphate generation and PKC activation showing that PKC activation required the PLC signalling pathway. Further, studies revealed that extracellular Ca^{2+} was required for PLC activation as removal of extracellular Ca^{2+} inhibited [^{3}H]-InsP_x accumulation evoked by nicotine. Ca^{2+} signalling data also suggested that PLC activation is partially driven by Ca^{2+} released from intracellular stores. In addition, the PLC inhibitor U73122 attenuated Ca^{2+} signalling evoked by nicotinic receptors. Data also indicated that nicotinic receptor activation itself was responsible for PLC activation and not the activation of PLC-coupled receptors following the nicotinic receptor-mediated release of agonists from the chromaffin cells. Moreover, Ca^{2+} entry through nicotinic receptor channels constitutes the main source for entry in nicotinic receptor mediated Ca^{2+} signalling as block of VOCC by Cd^{2+} did not interfere with nicotine-mediated Ca^{2+} signalling.
Nicotinic-mediated PLC and PKC activation is likely to be linked to catecholamine secretion by MARCKS activation and subsequent F-actin disassembly, which allows access of secretory vesicles to their release sites at the plasma membrane. In addition to the activation of PKC, the current studies suggest that this DAG (and possibly Ca\(^{2+}\)) may also contribute to the activation of Munc13 which is also involved in facilitating exocytosis.

**6.3.2 Mechanisms underlying PKC activation**

Data in the previous chapter confirmed that nicotinic receptor activation resulted in the activation of PKC-\(\alpha\), -\(\beta\) and -\(\epsilon\) but not \(\tau\) in bovine chromaffin cells. Within Chapter 5 data suggest that these PKC isoforms, particularly PKC\(\alpha\) and possibly also PKC\(\beta\) contribute to catecholamine secretion evoked by nicotinic receptors. Activation of these PKC isoforms, which represent both conventional (PKC-\(\alpha\) and -\(\beta\)) and novel (PKC\(\epsilon\)) PKC classes, requires DAG alone (novel) or in combination with Ca\(^{2+}\) (conventional). However, given that DAG is required for activation of members of both of these PKC subfamilies, the activation mechanism was unclear. Thus, under basal, or unstimulated conditions biological membrane contains very little DAG (Hodgkin *et al.*, 1998) and it might be expected that production of DAG is required for PKC activation. One possibility is that DAG generation occurs following nicotinic receptor-mediated activation of PLC. Indeed, previous studies in chromaffin cells indicated that Ca\(^{2+}\) influx following nicotinic- or high K\(^+\)-mediated depolarisation could increase the activity of PLC (Eberhard and Holz, 1988; 1989; Sasakawa *et al.*, 1989). Activated PLC hydrolyses membrane PtdIns(4,5)P\(_2\), generating both Ins(1,4,5)P\(_3\) and DAG. The increased membrane DAG and intracellular Ca\(^{2+}\) could then be the driving force for the activation of PKC. The capability of nicotinic receptor stimulation to
activate PLC was addressed in this study and examined using various approaches. Firstly, measurement of the accumulation of $[^3\text{H}]-\text{InsP}_x$ against a Li$^+$-block of inositol monophosphatase activity reflects the total PLC activity (Wojcikiewicz et al., 1993; Willars et al., 1998b) and the current study demonstrated that nicotinic receptor activation caused accumulation of $[^3\text{H}]-\text{InsP}_x$ although the extent of $[^3\text{H}]-\text{InsP}_x$ generation was lower than that triggered by Go$_q$/11-coupled receptors. Secondly, the generation of Ins(1,4,5)P$_3$ and DAG in response to nicotinic receptor activation was shown using biosensors. Interestingly, not all cells expressing eGFP-PKC$_{\gamma}\text{Cl}_2$ or eGFP-PH$_{\text{PLC}\delta_1}$ responded to either nicotine, histamine or angiotensin II. This might be a reflection of the need for robust responses to mediate the translocation of the biosensors and that the cultures represent a heterogeneous collection of cells. The activation of PLC mediated by nicotinic receptor stimulation was further confirmed by using the PLC inhibitor U73122 which exerted inhibitory effects on $[^3\text{H}]-\text{InsP}_x$ generation evoked by nicotinic receptor stimulation as well as by histamine, which was used as a positive control. Wortmannin also blocked the nicotinic receptor-mediated accumulation of $[^3\text{H}]-\text{InsP}_x$ suggesting that this accumulation does indeed occur as a consequence of the hydrolysis of PtdIns(4,5)P$_2$.

6.3.3 Mechanisms underlying PLC activation mediated by nicotinic receptor stimulation

Since this study strongly suggests the involvement of PLC in nicotinic receptor-mediated cellular responses, particularly exocytosis, the crucial question that has to be addressed is how do nicotinic receptors activate PLC? The most likely mechanism for activation of PLC by nicotinic receptors is the influx of Ca$^{2+}$ through VOCC or nicotinic receptors themselves. It is, however, possible that
release of GPCR agonists following nicotinic receptor activation could be responsible for the activation of PLC. However, this is unlikely as apyrase, PTX and stabilization of cortical F-actin with jasplakinolide all failed to inhibit nicotinic receptor-mediated $[^{3}\text{H}]-\text{InsP}_x$ accumulation.

The possible sources of Ca$^{2+}$ entry triggered by nicotine are through VOCCs (Vijayaraghavan et al., 1992; Dajas-Bailador and Wonnacott, 2004) or nAChRs, which can themselves, serve as a Ca$^{2+}$ entry pathway (Decker and Dani, 1990; Zhang et al., 1996b, Harkin and Fox, 1998). The current study reveals that activation of PLC triggered by nicotinic receptors is essentially dependent on extracellular Ca$^{2+}$. In addition, this study suggests that influx of Ca$^{2+}$ via nicotinic receptors themselves contributes mostly to nicotinic receptor-mediated responses as determined by the ability of Cd$^{2+}$ to block Ca$^{2+}$ responses to KCl but not nicotine. Furthermore, the ability of thapsigargin, an inhibitor of the sarcoplasmic or endoplasmic reticulum ATPase (Thastrup et al., 1990) to attenuate Ca$^{2+}$ signalling mediated by nicotinic receptor activation suggests a contribution of intracellular Ca$^{2+}$ stores to nicotinic receptor-mediated elevation of [Ca$^{2+}$]. This Ca$^{2+}$ might therefore participate in PKC activation as suggested in previous studies (Burgoyne, 1991; Mollard et al., 1995; Dajas-Bailador and Wonnacott, 2004). An involvement of intracellular stores was also supported using U73122, which inhibited nicotinic receptor-mediated elevation of [Ca$^{2+}$].

6.3.4 A crucial role for PLC-dependent PKC activation in nicotinic receptor-mediated catecholamine secretion

Elevation of [Ca$^{2+}$], as described previously within this chapter could contribute directly to the activation of PKC, particularly the $\alpha$ and $\beta$ isoforms. However, a rise in [Ca$^{2+}$], due to Ca$^{2+}$ influx with the subsequent generation of
DAG could contribute to or be an instrumental in the activation of DAG-sensitive PKC isoforms. Thus, the role of nicotinic receptor-mediated PLC activation in the activation of PKC was examined by determining the impact of the PLC inhibitor, U73122, on the activation of PKCα. Western blotting of PKCα in cytosolic and membrane fractions to determine its subcellular localisation as an index of activation showed that U73122 inhibited activation of PKCα in response to nicotinic receptor activation suggesting that PLC and its downstream messengers are crucial to PKCα activation.

As described previously (Chapter 5), activation of PKC, particularly the α isoform in response to nicotinic receptor stimulation may trigger catecholamine secretion by phosphorylation of MARCKS, a major PKC substrate in chromaffin cells (Powis et al., 1996b). Thus, phosphorylated MARCKS loses its ability to cross-link actin filaments (Aderem, 1992; Hartwig et al., 1992) and this would be expected to result in the disorganization of the cortical actin cytoskeleton and eventually migration of secretory vesicles to exocytotic sites at the plasma membrane in preparation for exocytosis (Vitale et al., 1995). Thus, the current study suggests that activation of PKCα, mediated by nicotinic receptors, and all of its subsequent events including MARCKS phosphorylation, F-actin disassembly and catecholamine secretion are crucially dependent on PLC activation.

6.3.5 The role of targets of DAG other than PKC in nicotinic receptor-mediated catecholamine release

Although DAG may contribute significantly to PKC activation and subsequent catecholamine secretion, additional DAG targets include Munc13 (Kazanietz, 2002), which is well known to facilitate vesicle priming (Brose et al., 1995). Thus, Munc13 may act in parallel with PKC to enhance transmitter release
This study has tried to address whether nicotinic receptor stimulation mediates Munc13 activation and whether this activation is dependent on PLC activation. In this study, nicotinic receptor activation was unable to mediate redistribution of over-expressed Munc13-1-eGFP in real time at the single cell level and all attempts to elevate the level of endogenous DAG in chromaffin cells by using DAG kinase and DAG lipase inhibitors failed to cause Munc13-1-eGFP redistribution in response to nicotinic receptor activation. This might be a consequence of a massive expression of Munc13-1-eGFP whereby the DAG generated may be insufficient to cause significant translocation. This possibility was supported by the observation that nicotine could cause the redistribution of endogenous Munc13-1 to the plasma membrane. Thus, this study suggests that Munc13-1 might also contribute to catecholamine secretion evoked by nicotinic receptor activation. This is the first demonstration that Munc13-1 activation is downstream of nicotinic receptor activation. Furthermore, the data suggest that PLC-mediated generation of DAG is responsible for the activation of Munc13-1. A number of previous studies have highlighted the potential role of presynaptic Ca²⁺ influx in the activation of PLC with the subsequent generation of DAG and activation Munc13-1 to boost its priming activity (Rhee et al., 2002; Rosenmund et al., 2002).

Munc13-1 is thought to facilitate vesicle priming by localizing to the presynaptic plasma membrane with syntaxin and SNAP-25 (Betz et al., 1997). Priming of vesicles represents a crucial step in Ca²⁺-dependent exocytosis since only primed vesicles are fusion competent and can respond to an increase [Ca²⁺] by fusing with the plasma membrane. The priming process involves the assembly of syntaxin 1, SNAP-25 and synaptobrevin 2 into the SNARE-complex. The rate-
limiting factor in the SNARE-complex assembly reaction is likely to be the presence of syntaxin 1, which exists in two different conformations. In the closed conformation, the N-terminal H domain folds onto the C-terminal H3 domain, rendering this helical domain inaccessible for SNARE-complex formation (Fernandez et al., 1998; Dulubova et al., 1999). In the open conformation, the N-terminal H domain is not folded back onto the C-terminal H3 domain, leaving the C-terminal H3 domain free to interact with SNAP-25 and synaptobrevin 2 and to form the four bundle of the SNARE complex. Munc18, a cytoplasmic protein can bind syntaxin 1 in the closed conformation and may thereby inhibit SNARE complex formation (Dulubova et al., 1999). Munc13-1 binds the to N-terminus of syntaxin 1 (Betz et al., 1997) and Munc13-1 competes with Munc18 for syntaxin binding (Sassa et al., 1999). This direct interaction of Munc13-1 with the autoinhibitory domain of syntaxin 1 is required for Munc13-1 priming activity. In addition, the presence of three C2 regulatory domains suggests that Munc13-1 might also act by binding Ca\textsuperscript{2+} or phospholipids thereby acting as an exocytotic Ca\textsuperscript{2+} sensor (Aravamudan et al., 1999). Furthermore, DOC2a directly interacts with Munc13-1 in a DAG-dependent manner and this interaction plays an important role in Ca\textsuperscript{2+}-dependent exocytosis (Orita et al., 1997; Groffen et al., 2004). Moreover, its interaction with the cytoskeleton through spectrin (Sakaguchi et al., 1998) might also contribute to Munc13-1 action by rearranging the submembrane cytoskeleton.

In summary, the current data suggest that nicotinic receptor activation facilitates catecholamine secretion through activation of two parallel signalling pathways, PKC and Munc13, which are downstream of nicotinic-receptor stimulation of PLC.

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Chapter 7: Summary and concluding discussion

There is substantial evidence in the literature that PKC facilitates exocytosis. Work described within this thesis initially aimed to explore the role of PKC in exocytosis triggered by either nicotinic receptors or $G\alpha_{q/11}$-coupled receptors, and to determine whether different PKC isoforms are involved in the regulation mediated by different receptors. Initially PC12 cells were chosen as a model neurosecretory cell type that is reported to express a range of $G\alpha_{q/11}$-coupled receptors. However, preliminary work indicated that the clone of PC12 cells used in this study, although expressing, for example, ionotropic P2X receptors, did not express the range of $G\alpha_{q/11}$-coupled receptors previously reported. As a result, attention was directed to chromaffin cells, a well-recognised model neurosecretory cell that has also been reported to express a number of different GPCRs and ionotropic receptors. This study demonstrated the expression of receptors for histamine, angiotensin II, acetylcholine and bradykinin that were able to generate Ins(1,4,5)P$_3$, DAG and elevate $[Ca^{2+}]_i$, implying coupling to $G\alpha_{q/11}$. Although, activation of histamine receptors, acetylcholine receptors or bradykinin receptors mediated peak and plateau $Ca^{2+}$ responses, angiotensin II only evoked a peak $Ca^{2+}$ signal. In general, peak $Ca^{2+}$ responses as a result of the activation of $G\alpha_{q/11}$-coupled receptors arise from Ins(1,4,5)P$_3$-mediated release from intracellular $Ca^{2+}$ stores whilst the sustained plateau phase is a consequence of $Ca^{2+}$ entry across the plasma membrane. Such entry across the plasma membrane can result from depletion of intracellular $Ca^{2+}$ stores that activate capacitative $Ca^{2+}$ entry. The data presented here support this model in accord with previous reports (Plevin and Boarder, 1988; Sasakawa et al, 1989; Bunn et al., 1989).
1990; Cheek et al., 1993; Roberts-Thomson et al., 2000; Marley, 2003). In addition, chromaffin cells express ionotropic nicotinic receptors. These receptors evoked much greater peak and plateau Ca\(^{2+}\) responses than those evoked by G\(\alpha_{q/11}\)-coupled receptors. The general scheme for Ca\(^{2+}\) signalling in response to activation of nicotinic receptors would be that activation of nicotinic receptors mediate depolarisation, which subsequently activates VOCCs allowing robust Ca\(^{2+}\) entry (Rathouz and Berg, 1994; Dajas-Bailador et al., 2002). In addition, some types of nicotinic receptors are Ca\(^{2+}\) permeable; namely those consisting of \(\alpha 7\) subunits and some consisting of \(\alpha 3\beta 4\) subunits (Costa et al., 1994; Rathouz and Berg, 1994; Ragozzino et al., 1998).

Although, in many cell types a rise in intracellular Ca\(^{2+}\) is a key stimulus of regulated exocytosis, PKC also regulates secretion in both chromaffin cells and other secretory cells (Terbush et al., 1988; Terbush and Holz, 1990; Bittner and Holz, 1993; Graham et al., 2000; Taylor et al., 2000). This study reveals that bovine chromaffin cells express PKC-\(\alpha\), -\(\beta\), -\(\epsilon\) and -\(\iota\). Interestingly, these PKC isoforms are representative of the three PKC subfamilies (\(\alpha\) and \(\beta\), conventional; \(\epsilon\) novel; \(\iota\), atypical). Activation of either nicotinic receptors or G\(\alpha_{q/11}\)-coupled receptors recruited different PKC isoforms. Thus, nicotinic receptor stimulation activated PKC-\(\alpha\), -\(\beta\) and -\(\epsilon\), whilst activation of G\(\alpha_{q/11}\)-coupled receptors with histamine activated all the expressed PKC and angiotensin II only recruited PKC-\(\alpha\) and -\(\epsilon\). Several groups have suggested that PKC has an obligatory role in exocytosis based on studies using a variety of non-selective PKC inhibitors (Knight et al., 1988). In experiments described within this thesis, non-selective PKC inhibitors were also used initially to confirm the role of PKC in catecholamine release mediated either by nicotinic receptors or G\(\alpha_{q/11}\)-coupled...
receptors. These experiments indicated a pivotal role for PKC in catecholamine release mediated by nicotinic receptors. However, in contrast, inhibition of PKC potentiated release of catecholamines induced by \( \Gamma \alpha_{q/11} \)-coupled receptors. The reasons for these contrasting results are most likely due to an inhibitory role of PKC on \( \Gamma \alpha_{q/11} \)-coupled receptor activation and signalling (Lefkowitz, 1993; Willars and Nahorski, 1995b; Ferguson et al., 1996) which is removed by PKC inhibition. This finding is consistent with previous studies; particularly on chromaffin cells, which showed potentiation of \( \Gamma \alpha_{q/11} \)-coupled receptor-mediated signalling by PKC inhibitors (Boarder and Challiss, 1992). Although attempts were made using subtype-selective PKC inhibitors to determine the particular PKC isoforms involved in the regulation of signalling and release, this was not possible and the focus in this thesis was directed to exploring the role of PKC in nicotinic receptor-mediated catecholamine secretion.

Inhibition of PKC markedly inhibited nicotinic receptor-mediated catecholamine release. Many previous studies have used either down-regulation of PKC isoforms or non-selective PKC inhibitors to determine the role of PKC in catecholamine secretion mediated by nicotinic receptor activation (Vainio et al., 1998; Soliakov and Wonnacott, 2001; Mahata et al., 2002). Here this was explored further and the PKC isoforms involved in the facilitation of catecholamine secretion evoked by nicotinic receptors were determined using a range of isoform-selective inhibitors. The present study suggests that PKC\( \alpha \) is the predominant isoform involved.

Although, the role of PKC in the secretion of catecholamines is well documented, there is considerable debate about the underlying mechanisms by which PKC enhances exocytosis and the mechanisms underlying PKC activation...
in response to nicotinic receptor activation (Waters and Smith, 2000). These issues have been addressed in this study which determined that nicotinic receptors activate PKC, particularly PKCα and that activation of PKCα mediated phosphorylation of MARCKS, a PKC substrate, resulting in an inability to cross-link actin filaments and stabilize the actin network (Hartwig et al., 1992) thereby promoting cortical F-actin disassembly. Although, the effect of PMA on MARCKS phosphorylation and translocation has been extensively studied in chromaffin cells (Vitale et al., 1991; 1995; Rosè et al., 2001; Trifaró, 1989; Trifaró et al., 2002), no study has previously shown a role for PKC in nicotinic receptor-mediated MARCKS activation. Disruption of the cortical F-actin should precede the release process as removal of the cortical F-actin barrier will allow access of secretory vesicles to exocytotic sites at the plasma membrane (Trifaró et al., 1989; Vitale et al., 1991; 2000). Stabilization of cortical F-actin by jasplakinolide markedly inhibited nicotine-mediated catecholamine release highlighting the importance of the cortical F-actin disassembly in the release process. Thus, the data in this thesis emphasize the strong link between nicotinic receptor activation, the PKC-MARCKS pathway and F-actin disassembly followed by substantial increases in catecholamine secretion (Figure 7.1).

Although, it is possible that activation of PKC is triggered by elevation of 
$[\text{Ca}^{2+}]$, following activation of nicotinic receptors, evidence provided by this study suggests that the mechanism of PKC activation is rather more complicated. Although Ca$^{2+}$ can activate PKC (Vainio et al., 1998; Soliakov and Wonnacott, 2001; Mahata et al., 2002), the classical isoforms of PKC (including, therefore, PKCα and PKCβ) are activated by Ca$^{2+}$ in combination with DAG, whilst the novel isoforms (represented in this study by PKCe) are activated by DAG alone.
This suggests that Ca\(^{2+}\) alone may not be responsible for PKC activation and that it must act in combination with DAG. DAG is present in unstimulated cells at very low concentrations (Hodgkin et al., 1998) suggesting that the generation of DAG could occur and contribute to the activation of PKC. Experiments described in this thesis provide evidence that the source of DAG is from an increase in PLC activity in response to nicotinic receptor activation. Furthermore, the present study suggests that nicotinic receptors mediate entry of extracellular Ca\(^{2+}\) directly through the receptors themselves and that this activates PLC to promote the hydrolysis of PtdIns(4,5)P\(_2\) resulting in the generation of both Ins(1,4,5)P\(_3\) and DAG. The current study suggests a key role for PLC-dependent PKC activation mediated by nicotinic receptor stimulation in the cortical F-actin network disassembly by MARCKS. As a consequence of these events, the secretion of catecholamines evoked by nicotinic receptors is extremely dependent on PLC-dependent PKC activation.

This study also suggests that generation of DAG as a consequence of nicotinic receptor-mediated PLC activation could regulate exocytosis through activation of Munc13, which facilitates vesicle priming (Brose et al., 1995; Betz et al., 1998). Indeed, this study strongly suggests that Munc13-1 might contribute to catecholamine secretion evoked by nicotinic receptors in parallel with PKC. The present study proposes that the source of DAG for the activation of Munc13-1 in response to nicotinic receptor stimulation is dependent on the Ca\(^{2+}\)-mediated activation of PLC. A model by which PKC facilitates nicotinic receptor-mediated catecholamine release is presented in Figure 7.1. Although much attention has been directed to the release of catecholamines from chromaffin cells, peptide transmitter release remains less studied (Fulop et al., 2005). Catecholamines and
neuropeptides are copackaged in the same granule (Winkler and Westhead, 1980) thus; it is assumed that both types of transmitter are released by a single exocytotic mechanism and this emphasises the importance of future studies in defining the mechanisms underlying peptide release mediated by nicotinic receptor activation.
Figure 7.1. Potential mechanisms involved in the secretion of catecholamines mediated by nicotinic receptor activation. Activation of nAChRs by agonist binding causes influx of both Na⁺ and Ca²⁺. The resulting depolarisation triggers influx of Ca²⁺ through VOCCs although data from the present study suggests that a substantial proportion of the Ca²⁺ entry is via the nicotinic receptor itself. Increased [Ca²⁺]ᵢ may activate PKC directly. The current study also suggests that Ca²⁺ entry promotes activation of PLC with the subsequent generation of DAG, which then contributes significantly to the activation of PKC, particularly PKCa. PKC then phosphorylates MARCKS leading to disruption of the cortical F-actin network and recruitment of secretory vesicles from the reserve pool to the readily releasable pool in preparation for exocytosis. The elevation of [Ca²⁺]ᵢ and generation of DAG may also be responsible for the activation of Munc13, which will also directly facilitate agonist-mediated exocytosis.
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