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Site-directed mutagenesis was used to characterize structural requirements for receptor-effector coupling of the pm2 muscarinic receptor (mAchR) expressed in Chinese hamster ovary (CHO) cells. Single amino acid substitutions in the third intracellular loop of pm2 mAchR resulted in subtle changes in receptor/G-protein interactions. When alanine in amino acid position 212 of the third intracellular loop (i3) was replaced with glutamate, oxotremorine M-mediated coupling to phosphatidyl inositol (PI) hydrolysis resulted in responses similar to the partial agonist pilocarpine. This finding indicates that there are agonist specific conformations promoted by different ligands used to activate receptor coupling to effector systems. Mutation of a positively charged lysine in amino acid position 214 of i3 to alanine (K214A) resulted in a receptor that coupled to stimulation of PI metabolism via a G-protein insensitive to ADP-ribosylation by pertussis toxin (PTX). While 100 ng/ml PTX is sufficient to completely uncouple wild-type mAchR from effector systems, 1000 ng/ml failed to abolish coupling of K214A to stimulation of

PI metabolism. This finding implies that removal of the positive charge permits the muscarinic receptor to interact with G-proteins other than the PTX sensitive Gi.

Properties of receptor internalization and desensitization were dependent on receptor density and agonist used. The full agonist carbachol promoted internalization and desensitization in cells expressing both low and high receptor concentrations while the partial agonist pilocarpine was only effective in CHO cells expressing high receptor densities. When an aspartic acid in the second transmembrane domain was mutated to asparagine (D69N), coupling to effector systems was abolished for all agonists tested except oxotremorine M. Internalization of D69N occurred at rates comparable to wild-type expressed at the same receptor density, and desensitization was promoted by the full agonists but not the partial agonist pilocarpine, even if effector coupling does not occur. This suggests that effector coupling is not necessary for either internalization or desensitization of the muscarinic m2 acetylcholine receptor.

Muscarinic Receptor-Effector Coupling in Chinese Hamster Ovary Cells

by

Dylan A. Bulseco

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Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Dylan A. Bulseco, Author

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CONTRIBUTION OF AUTHORS

Chapters 2, 3 and 4 resulted in separate manuscripts, and I am the primary author on each. Chapter 2 is entitled *Single Amino Acid Substitutions in the pm2 Muscarinic Receptor Alter Receptor/G Protein Coupling Without Changing Physiological Responses* and was published in Molecular Pharmacology, **49**:132-141, 1996. Chapter 3 is entitled *Internalization of the Porcine m2 Muscarinic Receptor Does Not Require Coupling: Mutation of a Conserved Aspartic Acid in the Second Transmembrane Domain*, and has been submitted for publication to Biochemical Pharmacology. Chapter 4 is entitled *Rates of pm2 Muscarinic Receptor Internalization and Recycling is Receptor Density and Agonist Dependent*. This manuscript will be submitted for publication to the Journal of Neurochemistry.

I am the primary author on the manuscripts that resulted from Chapters 2, 3 and 4, with my advisor, Dr. Michael Schimerlik as the only co-author on chapters 2 and 4. The experiments that comprise Chapter 3 were conducted with the assistance of several co-authors. Mei Shing Pi and Mason M. Kwong are undergraduates and assisted in technical aspects of the experimentation as part of undergraduate research programs. Walter K. Vogel is a graduate student, and did some of the preliminary work on the mutant receptor D69N. The Appendix was completed with the assistance of Dr. David Broderick and Kirsten Wolthers, a graduate student in the laboratory. Their contributions provided baseline information on agonist and antagonist binding to the pm2 muscarinic receptor in the presence of high salt.

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DEDICATION

For my grandparents, who gave of themselves to their families. Their example will not be forgotten.

PREFACE

I have always been interested in signal transduction. How does a cell gather information from its environment and convert it into useful information? More importantly, once the information is gathered, how does a cell determine a meaningful response? Clearly, understanding these processes in a single cell is a daunting task.

In order to better understand regulation of these events, reductionist approaches are often employed. This may mean that processes are studied in broken cell preparations, membrane preparations or with purified components, or that it is studied in a cellular environment, intact living cells. Unfortunately, these cellular environments may introduce factors, that are not easily controlled and that reduce clarity when attempting to interpret experiments. Whatever the approach used, it is important to recognize the dependence of the experiments (and hence interpretation) on the context in which the experiment was conducted.

The research conducted for this thesis focused on two areas. The first, was an attempt to relate structural changes in the pm2 muscarinic acetylcholine receptor (mAchR) with functional effects. Although it is relatively easy to make 'structural' changes in this receptor using site-directed mutagenesis, it is not easy to evaluate the effects of these changes. Few membrane bound proteins have been purified and crystallized, making interpretation of mutagenesis studies a very difficult task. In addition, with no direct measure of agonist-promoted conformational effects, in addition to the lack of structural information, analysis relies on expression and functional evaluation of the receptor in cells

that do not normally express the mAchR. This approach has led to the second focus of this thesis research.

Results obtained on functional coupling of the mutated receptor to effector systems are complicated by several factors. These include (1) receptor density achieved in the different clonal cell lines (2) dependence on the cell lines selected to express the mAchR (3) subtle differences in the clonal lines established and (4) the expression of endogenous factors that may modulate functional expression of the mutated receptors. These factors point out the importance of considering the context in which the receptors are expressed, and necessitates careful analysis of results obtained using these expression systems.

MUSCARINIC RECEPTOR-EFFECTOR COUPLING IN CHINESE HAMSTER OVARY CELLS

Chapter 1

Introduction

Historical Overview

The concept of agonist-receptor interactions were implicit in the work of Claude Bernard and Paul Erlich in the second half of the 19th century, but it was J. N. Langley (1852-1926) of Cambridge University that coined the term "Receptor" or "receptive substance". (Limbird, 1986). Langley extended the concept that chemical signal transmission was responsible for the effects of curare and nicotine at the neuromuscular junction, and qualitatively described receptor selectivity, saturability, and mutual antagonism. In 1921, Loewi (Brown and McDonough, 1989) definitively showed that acetylcholine was transmitted chemically when fluid from the vagal endings of one stimulated frog heart was shown to have a physiological effect on a second, unstimulated heart.

In the 1920's, A. J. Clark began to quantitatively describe the qualitative observations made by his predecessors. Clark postulated that ligand-receptor interactions obey the principles of mass action, and could therefore be described mathematically.

These relationships are discussed below in greater detail within the context of the muscarinic acetylcholine receptor.

Role of Receptors

Receptors in general serve the simple purpose of ensuring that a specific signaling molecule is recognized and that its 'signal' is transduced in a relevant way (Siegel et al., 1989). Membrane receptors serve the additional purpose of transducing the signal of an extracellular ligand into an intracellular physiological response. This avoids the requirement that the ligand be capable of passing through a hydrophobic plasma membrane, and often results in an amplification of signals initiated by ligands at low concentrations. Some receptors interact with addition signal transduction machinery to effect their function. Specifically, seven-transmembrane receptors interact with G-proteins as well as effector proteins, providing three levels of interactions that can be modulated by the cellular environment (Gudermann et al., 1996).

A number of mechanisms have evolved to ensure that cellular responsiveness is maintained under conditions of constant bombardment of extracellular signals. Elevated expression of receptor ensures that cells maintain maximal sensitivity to chemical signals at low concentrations (Limbird, 1986). If these ligands are in excess, cells have evolved mechanisms of receptor desensitization and internalization to dampen the second messenger responses (Liggett, 1995). While these regulatory processes are elegant and far more complex than we have previously appreciated, information can be gained about

certain aspects of the signal transduction pathway via a reductionist approach (Cooper et al., 1986).

Site-directed mutagenesis and receptor overexpression in exogenous cellular environments have been utilized to learn more about the molecular mechanisms of receptor-mediated signal transduction. Cells are capable of expressing receptors for numerous signal transduction pathways, including tyrosine kinase/growth factor receptors and ligand gated ion channels. We have focused on a single family of membrane bound receptors, those coupled to activation of effectors via guanine nucleotide binding proteins. More specifically, one member of this family, the m2 muscarinic acetylcholine receptor, has been studied in detail using site-directed mutagenesis and expression in Chinese hamster ovary cells in order to better understand the molecular properties of signal recognition and transduction.

G-protein coupled receptors

Muscarinic receptors are in the family of G-protein coupled receptors (Figure 1.1). Members of this family have several common features. They each have seven putative transmembrane domains with the N-terminus located extracellularly and the C-terminus located in the cytosol. Each serves to transduce the signal carried by an extracellular ligand, into a cellular response via a guanine-nucleotide binding protein, or G-protein (Gudermann et al., 1996). The ligands are diverse, and can be peptides such as neurokinin, angiotensin or endothelin, or small neurotransmitters such as epinephrine,

acetylcholine and dopamine. Signal transduction is mediated via a common pathway though, through G-proteins. Figure 1.2 shows the agonist-promoted receptor activation of G-proteins, and possible effector systems that are activated.

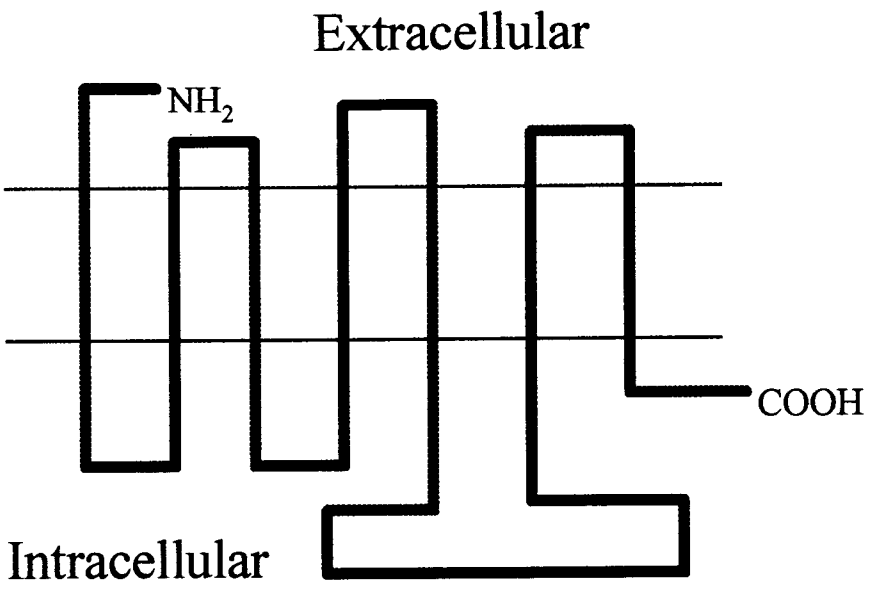

Figure 1.1: Diagram of the G-protein coupled receptor.

Common features of members of this family include seven transmembrane domains, alternating extracellular and cytosolic loops, an extracellular N-terminus and an intracellular C-terminus.

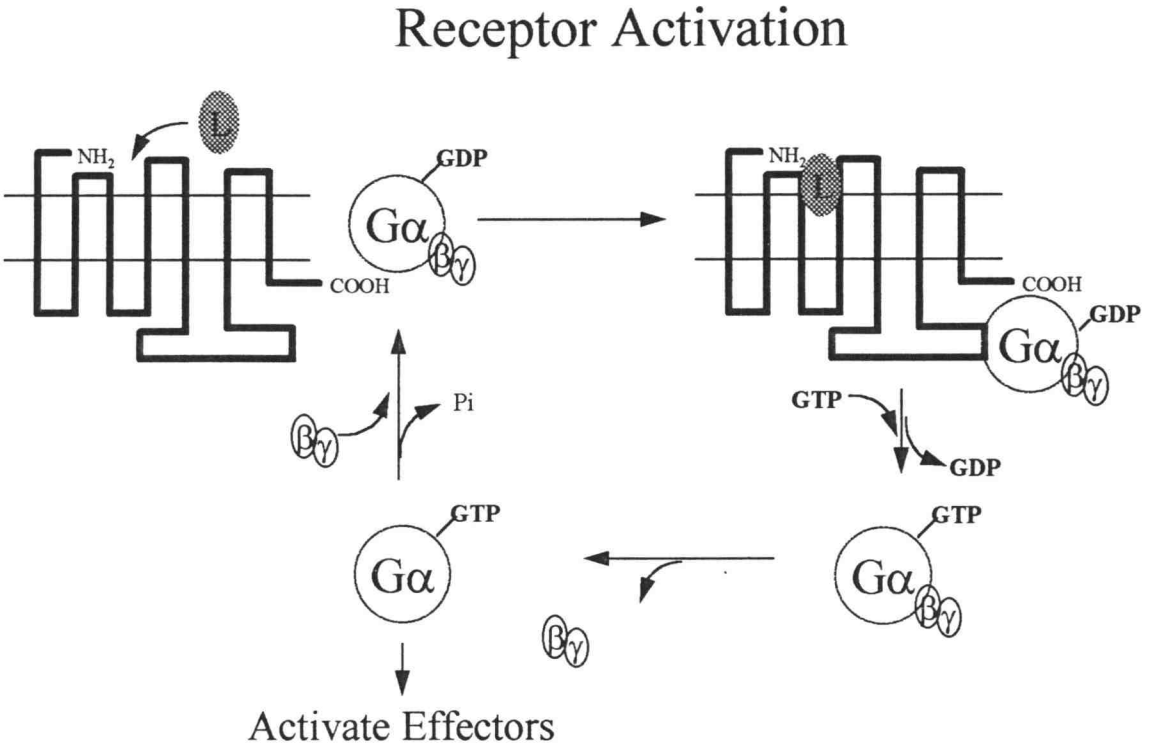

Figure 1.2: Receptor/G-protein activation cycle.

Activation of G-proteins are mediated by agonist (L) binding to the membrane-bound receptor. The activated G-proteins results in the dissociation of GDP and the binding of GTP. This exchange results in dissociation of the Gα subunit from βγ, allowing the GTP bound α subunit as well as the βγ subunit to activate effectors.

Role of G-proteins

G-proteins serve to transduce and amplify the signal carried by an extracellular ligand. After binding to the exposed surface of the receptor, the ligand presumably induces a change in receptor conformation which results in activation of a membrane delimited, heterotrimeric G-protein. It is not clear if the receptor is pre-coupled with this G-protein, or if agonist binding promotes receptor interaction with the G-protein. It is clear that agonist binding results in activation of the G-protein by a receptor-mediated change in G-protein heterotrimer conformation (Birnbaumer, 1990).

Once the activated or ligand bound receptor activates the G-protein, the heterotrimer dissociates into α and $\beta\gamma$ subunits. The change in G-protein conformation alters the affinity of the α subunit for bound GDP. The GDP dissociates, and is replaced with GTP which is at higher concentration in the cytosol. In addition, the activated Gα subunit may have a higher affinity for GTP than GDP. This activated Gα is capable of activating effector enzymes such as adenylyl cyclase and phospholipase C. Intrinsic GTPase activity of the Gα subunit results in conversion of GTP to GDP, re-association with $\beta\gamma$ subunits, and conversion of the active Gα subunit into the inactive form of the heterotrimer (Birnbaumer, 1990). The dissociated $\beta\gamma$ subunits are also capable of activating effector systems, although their importance in cellular signaling has only recently been appreciated.

Diversity of Gα subunits

The Gα subunits of the G-protein heterotrimer have many functions in signal transduction and cellular signaling. There are at least 30 identified α-subunits to date (Simon et al., 1991), characterized by sequence similarity, their effect on effector enzymes and their sensitivity to bacterial toxins. The Gαs family of Gα subunits activate adenylyl cyclase activity and are sensitive to ADP-ribosylation by cholera toxin, which functions to block GTP hydrolysis thereby activating members of this family in the absence of ligand activated receptor. Members of this family include transducin and two forms of Gαs.

The Gαi subunits mediate inhibition of adenylyl cyclase, stimulation of phosphatidyl inositol metabolism and activation of inward rectifying K+ channels. In addition, this family is sensitive to ADP-ribosylation by pertussis toxin, which results in a Gα subunit that cannot be activated by liganded receptor. This family includes three primary subtypes, Gαi1, Gαi2 and Gαi3. Other members include PTX sensitive Gαo, Gαg (gustaducin) and Gαt (transducin). In addition, transducin, which is found in retinal rods and cones, is also sensitive to ADP ribosylation by cholera toxin. Finally, Gαz, which is found primarily in brain is not sensitive to bacterial toxins.

The Gαq subunit family is insensitive to ribosylation by bacterial toxins, and mediates activation of phospholipid metabolism, including phospholipase C, D and A2, although it is unclear if activation is via a direct or an indirect mechanism. Members of this family include Gαq, Gα11, Gα14, Gα15 and Gα16. A less well characterized family of Gα subunits include Gα12 and Gα13, both of which are found ubiquitously, although

their function is unclear. Table 1.1 summarizes all of the known Gα subunits identified to date.

Since it is relatively easy to group these families of Gα subunits according to functional activation of effectors, it was always believed that G-protein specificity was conferred by the α subunits. Recent evidence indicates a larger role than previously appreciated, for the βγ subunits. In addition, the specific combination of βγ subunits appear to have large roles in determining the nature of the receptor-mediated activation of second messenger responses (Gudermann et al., 1996; Kleuss et al., 1991; Kleuss et al., 1993).

Table 1.1: Identified Gα subunits.

Family	Toxin Sensitivity	Receptors	Effectors
Gαi1,2,3	PTX	m2/m4, α2AR, D2	a
Gαo	PTX	α2AR	a
Gαz	none	m2	b
Gαg	PTX	taste receptors	c
Gαt	CTX/PTX	rhodopsin	d
Gαs	CTX	βAR, TSH, D1, NK1	e
Gαolf	CTX	olfactory receptor	f
Gαq	none	m1, α1AR	g
Gα11	none		g
Gα14	none		g
Gα15	none		g
Gα16	none		g
Gα12	none		g
Gα13	none		g

a K+ and Ca2+ channels, inhibition of adenylyl cyclase and stimulation of phospholipase C and A2
b Inhibition of adenylyl cyclase
c unknown
d Stimulation of cGMP specific phosphodiesterase
e Stimulation of adenylyl cyclase, Ca2+ and Na+ channels
f Stimulation of adenylyl cyclase
g Stimulation of phospholipase C

βγ subunits

The βγ subunits appear to have numerous direct functions as well as more subtle modulatory functions in the cell (Neer, 1994; Neer, 1995). There are at least five identified β and six identified γ subunits (Neer, 1995). Table 1.2 summarizes the β and γ subunits identified to date. The roles of these subunits have been much more difficult to characterize for a variety of reasons. First, there are no markers such as bacterial toxin sensitivity that would simplify assessing the role of the subunits. Secondly, the βγ subunits associate very tightly, and it is difficult to purify and characterize them independently of one another. In addition, since they are found ubiquitously in mammalian cells, results obtained with expression systems may be difficult to interpret since endogenous subunits are uncharacterized.

Second messenger systems

The superfamily of G-protein coupled receptors consists of a large number of receptor families, each comprised of multiple subtypes, that converge on a limited number of known second messenger effector systems. The common features of each of these families of receptors are (1) they each span the plasma membrane seven times, with an extracellular N-terminus and a cytosolic C-terminus (2) they transduce and amplify signaling by extracellular ligands and (3) their actions are mediated through guanine-nucleotide binding proteins or G-proteins.

Interestingly, there is a large redundancy in functional coupling by each of the receptor families. In other words, numerous receptors, or receptor subtypes from multiple families of GPCRs converge on coupling to the same effector systems (Gudermann et al., 1996). For example, members of the adrenergic, muscarinic, dopaminergic and serotoninergic families of GPCRs all couple to inhibition of adenylyl cyclase (Figure 1.3). Since the known G-protein α subunits are limited, the regulation of signaling pathways must be a complex cellular process, and may be dependent on simultaneous events that are competing with one another for resources (Neer, 1994; Neer, 1995). Alternatively, parsimony may dictate that cells regulate these complex signaling pathways by simply controlling expression of receptors, effectors, G-proteins or other necessary players in the signal transduction pathway. In addition, regulation may be made simpler by localizing the relevant players in the signaling pathways to delimited areas of the plasma membrane. Finally, the interactions of receptor with heterotrimeric G-proteins may be regulated to an extent by the expression and availability of specific combinations of $\beta\gamma$ subunits. Although one or all of these alternatives are likely to play a role in the regulation of signal transduction (Gudermann et al., 1996), their importance has only recently been appreciated, and are now being explored in more detail (Neer, 1994; Neer, 1995).

Before we can consider all of the possible interactions between receptor signaling and control of effector coupling, it is necessary to briefly outline the receptor families, their coupling specificity, and the G-protein heterotrimer requirements when known. Although it is impossible to provide all of the information known for GPCRs, the most

commonly studied are presented with the assumption that within these families, all or most of the known signaling pathways will be discussed.

Adrenergic receptors

There are two subfamilies of adrenergic receptors, the β and α adrenergic receptors (βAR and αAR respectively). There are at least three subtypes of β (β1, β2, and β3) and at least two subtypes of α adrenergic receptors (α1 and α2). While the βAR receptors couple to stimulation of adenylyl cyclase, the αAR couple to either stimulation or inhibition of adenylyl cyclase or stimulation of PI metabolism depending on the subtype. Members of the adrenergic receptor family interact with both Gsα and Giα G-protein subunits.

Muscarinic receptors

The muscarinic receptors can be divided into five subtypes, m1 through m5. In general, the odd numbered subtypes (m1, m3 and m5) couple to stimulation of PI metabolism through PTX insensitive G-proteins, presumably a member of the Gq family. On the other hand, m2 and m4 appears to couple through PTX-sensitive G-proteins, Gi or Go to inhibit adenylyl cyclase. In addition, when m2 is expressed in CHO cells, coupling to both an inhibition and a stimulation of adenylyl cyclase is observed (Ashkenazi et al., 1989; Ashkenazi et al., 1987). The stimulatory phase is not sensitive to PTX, although the molecular mechanism mediating this response is not clear. Further details on the porcine m2 acetylcholine receptor are provided below.

Dopamine receptors

Dopamine is a catecholamine that is synthesized via the same pathway as epinephrine and norepinephrine. Dopaminergic cells express tyrosine hydroxylase and DOPA decarboxylase but lack the additional enzymes required to convert dopamine to norepinephrine and epinephrine.

Receptors for dopamine are classified as D1 or D2, depending on whether or not coupling is to stimulation (D1) or inhibition (D2) of adenylyl cyclase. In addition, the D2 receptor subtype has been shown to be expressed in either long (D2long) or short (D2short) forms.

Serotoninergic receptors

Receptors that bind serotonin (5-HT) couple to inhibition (5-HT1, forms A, B, D, E and F) or stimulation (5-HT drosophila1) of adenylyl cyclase. In addition, serotonin receptor subtypes 5-HT2, 1C and 2F couple to stimulation of PI metabolism.

Peptide G-protein coupled receptors

There are several peptide receptors that are members of the GPCR family. These include receptors for endothelin, neurokinin, neuropeptide Y, cholecystokinin, thrombin, ACTH, and bradykinin (Birnbaumer, 1990). One common feature of the peptide receptors is a large N-terminus, which plays an important role in ligand binding (Strader et al., 1994). The peptide GPCR are capable of coupling to all effector systems discussed thus far.

Basic Structure of G-protein coupled receptors

Several domains of G-protein coupled receptors have been reported to be important in ligand binding. For peptide receptors, the large N-terminus as well several transmembrane domains play important roles. For G-protein coupled receptors that bind neurotransmitters, the second, third and seventh transmembrane domains are most important in determining ligand binding properties (Jackson, 1991; Strader et al., 1987; Strosberg, 1991).

The important domains for receptor-effector coupling is reported to include the second and third intracellular loops as well as the C-terminus. Most studies localize the major determinants to the N- and C- termini of the third intracellular loop (i3) (Wess et al., 1990; Wess et al., 1989). Large portions of the central part of this loop can be deleted without complete loss of functional coupling (Shapiro and Nathanson, 1989). In addition, replacement of an acidic amino acid residue in the second transmembrane domain results in complete loss of agonist-mediated activation of effectors. This residue is conserved across all families of G-protein coupled receptor as either an aspartic acid or glutamic acid. Conversion of this amino acid to asparagine or glutamine results in loss of coupling (Bihoreau et al., 1993; Fraser et al., 1989; Hu et al., 1992; Rose et al., 1995; Wang et al., 1991; Wang et al., 1993).

Cloning and Muscarinic receptor subtypes

Muscarinic Receptor Subtypes

Five subtypes of the muscarinic acetylcholine receptor have been cloned and characterized (Bonner et al., 1987; Kubo et al., 1986; Peralta et al., 1987). The odd numbered subtypes, m1, m3 and m5 couple to stimulation of PI metabolism while the even numbered subtypes, m2 and m4 couple to inhibition of adenylyl cyclase. In addition, the porcine m2 couples to stimulation of PI metabolism when expressed in Chinese hamster ovary cells (Ashkenazi et al., 1987).

Functional Coupling of the subtypes

The pm2 muscarinic acetylcholine receptor couples to inhibition of adenylyl cyclase via pertussis toxin (PTX) sensitive G-proteins. PTX functions to ADP ribosylate the α subunits of Gi and Go, leading to receptor/G-protein uncoupling. Treatment of cells with 100 ng/ml PTX results in complete loss of agonist-mediated activation of effector systems (Bulseco and Schimerlik, 1996). Chinese hamster ovary cells do not appear to express Go (Dr. Janet Robishaw, unpublished results), which limits the interaction of pm2 to either Gαi1,2 or 3. Others have shown that pm2 expressed in CHO cells preferentially interacts with Gαi2 and Gαi3 (Dell'Acqua et al., 1993).

When m2 AchR is expressed in CHO cells, coupling to stimulation of adenylyl cyclase is also observed. The mechanism mediating this response is not clear, but it is not

mediated by phospholipase C or Ca2+, or Gαq mediated events (Vogel et al., 1995). Others have proposed that this activation of adenylyl cyclase is due to coupling to Gαs at high agonist concentrations (Eason et al., 1992).

Structure-function studies of the muscarinic receptor

Much of the structural information obtained for G-protein coupled receptors is inferred from experiments with site-directed mutants. Conclusions can be drawn about ligand binding properties once membranes are prepared from cell homogenates, but information obtained on receptor-effector coupling properties in whole cells may be difficult to interpret without taking into consideration the cell type used for receptor expression, and the receptor densities achieved in these systems. The following discussion will focus on information relating receptor structure to three basic properties—ligand binding, receptor-effector coupling and affects on desensitization/internalization properties.

Ligand binding

The ligand binding pocket appears to be formed by a ring-like arrangement of the seven transmembrane domain (Hulme et al., 1990). Several critical amino acid residues are proposed to interact with the ligand in this conserved hydrophilic cavity (Brann et al., 1993; Wess, 1993). A critical aspartic acid residue in the third transmembrane domain has been shown to be important in ligand binding (Brann et al., 1993; Hulme et al., 1990;

Wess, 1993). Affinity labeling with antagonist and agonist mustards indicate that this residue interacts with the ammonium headgroup of muscarinic ligands. Site-directed mutagenesis of the rat m1 AchR implies that this aspartic acid residue is critical for proper ligand binding (Fraser et al., 1989). In addition, this residue has also been shown to be important for ligand binding in the β-adrenergic receptor (Strader et al., 1987).

Proline residues conserved in the fourth through seventh transmembrane domains are believed to introduce bends in the transmembrane helices (Hulme et al., 1990; Wess, 1993). This combination of straight and kinked transmembrane domains may be important for the proper agonist-induced conformational changes. One proline in transmembrane VII of m1 is believed to play an indirect role in agonist binding. Mutating this proline to alanine resulted in a receptor with 80 to 400 times lowered affinity for agonists (Wess et al., 1993). Helix wheel models proposed places this proline facing the hydrophobic lipid environment rather than the ligand binding pocket, so any affect on ligand binding may be indirect, perhaps via an affect on receptor conformation.

A series of conserved serine, threonine and tyrosine residues are located in the transmembrane domains of all muscarinic receptors. It has been proposed that the hydroxyl groups on these side chains function to interact with the acetylcholine ester moiety (Brann et al., 1993; Wess et al., 1991; Wess et al., 1992; Wess et al., 1993). Mutations of a threonine and tyrosine located in transmembrane domains V and VI respectively had the most dramatic effect on ligand binding affinities (Brann et al., 1993) but tyrosine residues of transmembrane III, VI and VII were also required for high affinity acetylcholine binding (Wess, 1993).

Two conserved cysteine residues on the extracellular loops 1 and 2 appear to form a disulfide bridge that is important in receptor stabilization (Hulme et al., 1990). Although reduction of this disulfide results in receptor with reduced affinity for both agonists and antagonists, the effect appears to be at the level of protein stability and/or conformation rather than a direct interaction with muscarinic ligands.

Receptor-effector coupling

Ligand-mediated receptor activation of G-proteins appears to require several intracellular domains of GPCRs. The third intracellular loop (i3), or "loop 5-6" connects the fifth and sixth transmembrane domains. This loop is highly variable in size, and within a receptor family, is conserved among receptor subtypes. Specifically, the N-terminal and C-terminal portions of i3 appear to be important in determining coupling properties.

The third intracellular loop of G-protein coupled receptors plays an important role in the regulation of receptor-effector coupling. Deletion analysis of i3 of the m1 AchR indicates that the middle 126 amino acids are not necessary for agonist-mediated stimulation of PI metabolism (Shapiro and Nathanson, 1989). When the entire i3 along with part of the fifth transmembrane region were switched in m1/m2 chimeric receptors and expressed in oocytes, the chimera exhibited the cellular response properties of the subtype that contributed the loop (Kubo et al., 1988).

The first 15-20 amino acid residues of the N-terminal region of i3 appear to determine G-protein specificity (Lechleiter et al., 1990; Wess et al., 1990; Wess et al., 1990; Wess et al., 1989). Wess et al (1990) expressed m2/m3 chimeric receptors in A9L

cells where the first 16 residues of m2 or 17 residues of m3 were swapped. The m3/m2-16 residue chimera did not couple to PI hydrolysis but did weakly inhibit the formation of cAMP via a PTX sensitive G-protein. The m2/m3-17 residue chimera on the other hand, coupled to PI hydrolysis by a PTX insensitive G-protein while coupling to the inhibition of adenylyl cyclase occurred through a PTX sensitive G-protein (Wess et al., 1990).

Although the N- and C- termini of i3 are rich in charged residues, the distribution of charge does not appear to be essential for efficient coupling to G-proteins. Replacement of residues conserved in the m1/m3 functional family with those conserved in the m2/m4 subtype family did not alter coupling of the human m1 AchR to PI metabolism (Arden et al., 1992). The converse experiment (replacement of m2/m4 residues with m1/m3 conserved residues) supports this finding, but further suggests slight effects on G-protein coupling with no apparent effect on cellular response (Bulseco and Schimerlik, 1996).

In addition to intracellular loops, one transmembrane mutant appears to have identified a critical amino acid residue involved in transducing a receptor conformation change upon ligand binding, into activation of G-proteins. Changing a single aspartic acid in the second transmembrane domain of GPCRs to asparagine consistently abolishes coupling of ligand activated receptor to effector systems, and presumably to activation of G-proteins. These mutations have been made in the m1 and m2 muscarinic, the β_2 adrenergic, α2 adrenergic, dopamine D2, 5-HT1 and 5-HT2A, endothelin, neurokinin-1 and angiotensin II receptors (Bihoreau et al., 1993; Brodbeck et al., 1995; Ceresa and Limbird, 1994; Fraser et al., 1989; Hu et al., 1991; Neve et al., 1991; Strader et al., 1987;

Strader et al., 1987; Wang et al., 1991). Antagonist binding is consistently unaffected. Agonist binding has been affected differently depending on the receptor system, and in some cases, specific agonists display an increase in affinity while others show a reduction. In addition, guanine nucleotide sensitivity is not consistently observed for the receptor systems in which this was studied.

Desensitization and internalization

Several domains of GPCRs appear to be important for agonist-mediated internalization and desensitization. Most of the site-directed mutagenesis has focused on regions of potential phosphorylation sites, with specific consensus sequences of specific kinases considered. Protein Kinase A, Protein Kinase C and G-protein coupled receptor kinase (GRK) appears to phosphorylate different regions of the $\alpha 2_A$ adrenergic receptor (Liggett, 1995).

The C-terminal end of GPCRs appear to be important for phosphorylation by PKC and PKA. These kinases appear to affect slower processes of receptor downregulation and desensitization. Phosphorylation by the G-protein coupled receptor kinases appear to mediate a rapid uncoupling of the receptor from effector systems.

The third intracellular loop appears to be the site of phosphorylation for the GRK family of kinases. These kinases include the β-adrenergic receptor kinases (βARK) as well as a number of G-protein receptor kinases that are numbered in order of discovery. GRK2 (or βARK1) appears to phosphorylate the m2 AchR in vivo, when expressed in COS cells (Tsuga et al., 1994).

Summary

Agonist binding to the muscarinic receptor is believed to trigger conformational changes in the transmembrane helices which are transmitted to the cytosolic loops that interact with G-proteins. While specific domains are often discussed, it is clear that changes in domains thought to be important in ligand binding (e.g. transmembrane domains) also participate in efficient coupling to effector systems. Likewise, mutations in the "G-protein coupling domain" are likely to also have effects on ligand binding while not affecting the ligand binding domain directly. These alterations may reflect modest changes in receptor conformation or alterations in agonist-induced conformation resulting in changes in receptor activation of G-proteins. Therefore, this implies that conformational changes of the receptor may involve the concerted contributions of several amino acid residues simultaneously making assignment of a specific G-protein coupling domain difficult.

This premise is supported by the finding that an aspartic acid in the second transmembrane region, conserved in almost all G-protein coupled receptors (Probst et al., 1992) is critical for the proper agonist-induced receptor conformation that is required to couple to G-proteins and effector systems. Mutating this aspartic acid in m1 AchR adrenergic and dopamine receptors (Fraser et al., 1989; Neve et al., 1991; Strader et al., 1989; Strader et al., 1987) as well as the pm2 AchR (Chapter 3) resulted in the elimination of coupling.

The porcine m2 muscarinic acetylcholine receptor

Expression in CHO cells

Clones of wild-type and mutant pm2 mAchR were stably transfected into Chinese hamster ovary cells and expression amplified by increasing the concentration of methotrexate. Overexpression in CHO cells allowed the characterization of receptor-effector coupling properties in intact cells, as well as isolation of large quantities of membranes enriched in muscarinic acetylcholine receptor.

Expression of pm2 mAchR in CHO cells resulted in several experimental problems. It is not easy to isolate clonal cell lines expressing similar receptor densities. Since properties of functional coupling are dependent on receptor density (Vogel et al., 1995; Whaley et al., 1994), effective characterization relies on our ability to normalize responses for receptor density. This required that receptor density be reduced with the slowly dissociated antagonist, *l* -QNB.

Coupling to second messenger systems

The porcine m2 muscarinic acetylcholine receptor expressed in CHO cells couples to inhibition of adenylyl cyclase and stimulation of PI metabolism via pertussis toxin sensitive G-proteins (Ashkenazi et al., 1989; Ashkenazi et al., 1987). In addition the pm2 mAchR also couples to stimulation of adenylyl cyclase at high agonist concentrations. The mechanism of adenylyl cyclase activation has not yet been determined, but does not

appear to be mediated via Gαq or Ca2+ dependent processes (Vogel et al., 1995). The pm2 mAchR also couples to other effector systems including modulation of ion channels (Hulme et al., 1990; Nathanson, 1987) and nitric oxide (NO) mediated elevation of cGMP (Bauer et al., 1994; Vila Echague et al., 1994).

Chapter 2

Single Amino Acid Substitutions in the pm2 Muscarinic Receptor Alter Receptor/G Protein Coupling Without Changing Physiological Responses

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Summary

The N-terminus of the third cytoplasmic loop of the porcine m2 muscarinic receptor plays an important role in receptor-effector coupling. While large changes in coupling properties are easily detected, subtle changes are often overlooked. Three mutant receptors were characterized after expression in Chinese hamster ovary cells, and two of these exhibited subtle changes in coupling properties. Substitution of amino acids 219-223 (KDKKE) with those conserved in the m1,3,5 receptor subtype family (ELAAL) had little effect on coupling to effector systems indicating that altering the charge distribution in this region did not affect receptor-G-protein interactions. Substitution of Ala with Glu at amino acid position 212 (A212E) or Lys with Ala in position 214 (K214A) resulted in receptors with IC_{50} for inhibition of adenylyl cyclase that resembled wild-type although maximal percent inhibition was reduced. All mutants moderately decreased coupling to PI metabolism but mutant A212E caused oxotremorine-M to become a weak partial agonist compared to carbachol suggesting that receptor conformation is agonist-dependent even for ligands normally thought of as full agonists. K214A coupled to PI metabolism through both PTX-sensitive and PTX-insensitive G-proteins. These results indicated that these mutants superficially possessed effector coupling characteristics similar to wildtype, but upon more detailed examination G-protein-receptor interactions were altered.

Introduction

mAchRs[1] mediate intracellular responses through coupling to heterotrimeric guanine nucleotide-binding proteins. They are members of a large family of G-protein coupled receptors that possess seven hydrophobic transmembrane regions connected by alternating intracellular and extracellular loops with ligand binding domains located within the transmembrane regions. Five unique subtypes of mAchRs (m1-m5) have been cloned and characterized (Bonner et al., 1987; Kubo et al., 1986; Peralta et al., 1987). The odd numbered subtypes (m1, m3 and m5) stimulate phospholipid metabolism while the even numbered subtypes (m2 and m4) preferentially couple to inhibition of adenylyl cyclase (Hulme et al., 1990). The m2 subtype also couples to the inward rectifying K^+-channel in the heart. The m2 muscarinic receptor subtype couples to both inhibition of adenylyl cyclase and stimulation of PI metabolism when expressed in CHO cells (Ashkenazi et al., 1987).

Guanine nucleotide-binding proteins that mediate the actions of mAchRs can be distinguished by their sensitivity to ADP ribosylation by PTX. The m2 mAchR couples to inhibition of adenylyl cyclase and stimulation of PI hydrolysis in CHO cells via G-proteins with differing PTX sensitivities (Ashkenazi et al., 1987). When m1 and m3 receptor subtypes are expressed in CHO cells, they couple to stimulation of PI hydrolysis either through both PTX-sensitive and insensitive G-proteins or via a G-protein with intermediate sensitivity (Ashkenazi et al., 1989). These results indicate that different mAchR subtypes are capable of evoking similar physiological responses by coupling through different G-proteins in the same cell line. Chimeras of i3 or the N-terminal

portion of i3 made with the β_1-adrenergic and m2 receptors indicate that promiscuous receptor G-protein coupling occurs with both PTX sensitive and PTX insensitive G-proteins when the wrong i2 is included (Wong and Ross, 1994).

The N-terminal and C-terminal regions of i3 have been shown to play important roles in regulation of receptor coupling to G-proteins and activation of effector systems for both the muscarinic (Lechleiter et al., 1990; Wess et al., 1990; Wess et al., 1989) (Bonner, 1992; Dixon et al., 1987) and β-adrenergic receptors . For the mAchR, the first 15-20 N-terminal residues of i3 are sufficient to determine selective coupling to G-proteins. Wess et al. (Wess et al., 1990) used chimeric m2/m3 receptors stably expressed in A9 L cells to show that the first 17 N-terminal residues of i3 from the m3 mAchR rendered the m2 subtype PTX-insensitive for coupling to PI metabolism while PTX-sensitive inhibition of adenylyl cyclase was retained. This finding suggests that the selectivity determinant for receptor-G-protein interaction lies within the first 17 N-terminal amino acid residues. This domain of i3 is proposed to have α-helical secondary structure and best correlates with the second messenger preferences exhibited by the odd and even numbered receptor subtypes in this region (Bonner, 1989; Hulme et al., 1990). Arden et al. (Arden et al., 1992) reported that substitution of three N-terminal i3 residues (the 5th, 7th and 12th) from m1 mAchRs (Glu, Lys and Glu) with residues found in the m2 subtype (Ala, Lys and Lys) did not alter the characteristics of carbachol-mediated m1 mAchR coupling to PI hydrolysis. In this mutant, the charge distribution was changed to that seen in the m2 mAchR which couples to inhibition of adenylyl cyclase by a PTX-sensitive G-protein. Blüml et al (Blüml et al., 1994a; Blüml et al., 1994b) showed that a critical Tyr at

the in the N-terminal of i3 was necessary for efficient coupling to PI hydrolysis. Carbachol was the only agonist utilized in these studies and PTX-sensitivity was not assessed.

In the experiments presented below, pm2 mAchRs with mutations in charged residues at the N-terminus of i3 were characterized using carbachol as well as other full and partial agonists (Vogel et al., 1995). The results of these experiments suggest that even single amino acid mutations in this region may subtly affect ligand binding behavior as well as the manner in which mutant receptors interact with G-proteins. Alterations in G-protein specificity can be as dramatic as selectively changing the behavior of a full agonist to a partial agonist for PI stimulation but not inhibition of cAMP formation or as subtle as altering the sensitivity of a response to uncoupling by PTX, suggesting interaction with additional G protein(s). Taken together, these results emphasize the difficulty in assigning precise "structural" domains in i3 of the pm2 mAchR without thorough receptor characterization.

Materials and Methods

Materials

[^{3}H] *l* -QNB (43 Ci/mmol), [^{3}H]NMS (79 Ci/mmol), and [^{3}H]*myo*-inositol (17.8 Ci/mmol) were purchased from Du Pont-New England Nuclear. Carbachol and atropine were purchased from Aldrich Chemical Company and *l* -QNB, NMS, oxo M, acetylcholine and pilocarpine from Research Biochemicals International. Glass fiber filters were from Schleicher and Schuell (#32) or Whatman (GF/B). Restriction enzymes and T4 DNA polymerase were from Promega, Gibco-Bethesda Research Laboratories or New

England Biolabs. The mammalian expression vector (pSVE) and the clone for the wild-type pm2 mAchR were gifts from Genentech, Inc. dhfr$^-$ CHO cells were obtained from America Type Culture Collection (Rockville, MD). Pertussis toxin was from List Biological Laboratories and Ro20-1724 from Bio Mol Research Labs.

Site Directed Mutagenesis and Expression

Site-directed mutagenesis was carried out as described by the mutagenesis procedure of BioRad. After cloning the gene for the pm2 mAchR into the Hind III-EcoRI sites of the polylinker region of m13mp18, uracil containing single stranded DNA was annealed to mutagenic primers. The primers for A212E (5'-CCTGCTCTTACTTTCCCGGGATATGTGC-3') and K214A (5'-GTCCTTCTTAATTCTAGAGGCACTGGCTCGGGA-3') were designed to introduce a single amino acid change as well as new restriction enzyme sites to facilitate screening for mutants. The mutagenic oligonucleotide primer 5'-CTCCTTAATCCTGCTCTCACTCTCCCGGGATATGTGCCA-3' was designed to replace five amino acids in position 219 through 223 (KDKKE(219-223)ELAAL). After mutant clones were identified, the mutations were confirmed by dideoxy DNA sequencing (Sanger et al., 1977).

The Hind III-EcoRI fragment containing the pm2 mAchR coding region was isolated from m13mp18 Rf DNA by agarose gel electrophoresis and subcloned into the multiple cloning region of the expression vector, pSVE (Peralta et al., 1987). Mutant pm2 mAchR-pSVE constructs were transfected into dhfr$^-$ CHO dhfr-cells by calcium

phosphate precipitation. Cells expressing recombinant receptor were grown in selective media (modified D-MEM low in glycine and thymine) with 10% dialyzed calf serum, 100 mg/ml insulin and methotrexate. Increasing concentrations of methotrexate resulted in amplification of receptor expression.

Receptor expression was assessed in whole cells on tissue culture dishes by the addition of 10 nM [^{3}H] *l*-QNB or [^{3}H]NMS in the presence or absence of 100 mM atropine or 1 mM unlabeled *l*-QNB. Cells were incubated for 1.5 hours at 37°C, then washed twice with ice cold phosphate buffered saline, pH 7.4. Cells were solubilized with 1 ml of 1% triton X-100 in PBS, then counted for tritium. Once receptors were expressed, membranes were prepared and binding of ligands assayed as described below.

Membrane Preparation and Ligand Binding

Suspension cultures were grown in MFD at 37°C in spinner culture flasks. Cells were harvested and the pellet resuspended in two volumes of homogenization buffer (250 mM sucrose, 50 mM EDTA, 25 mM imidazole buffer, pH 7.4) containing benzamidine, soybean trypsin inhibitor, and pepstatin A at final concentrations of 1 mg/ml. The cell suspension was homogenized with a polytron homogenizer, PTA 10S probe, for 2x20 sec at 4°C under argon and cell membranes were prepared by sucrose gradient centrifugation as previously described (Peterson and Schimerlik, 1984). In some cases, 5 mM sodium butyrate was added 24 hours before harvesting cells to further enhance receptor expression. Untreated and butyrate-treated cultures resulted in membrane preparations with specific activities of 20 to 300 pmol NMS sites/mg protein. All ligand binding experiments were conducted in buffer containing 10 mM HEPES, 5 mM $MgCl_2$, 1 mM

EDTA, 1 mM EGTA, pH 7.4 at 25°C by displacement of either [^{3}H] *l*-QNB or [^{3}H]NMS. Samples were filtered through glass fiber filters and washed with 3X 4 ml of ice cold 50 mM Na phosphate buffer pH 7.4. When [^{3}H]NMS was used, the glass fiber filters were treated with 0.1% (w/v) PEI before use. Nonspecific binding was determined in the presence of 10^{-4} M *l*-hyoscyamine and was less than 10% of the total radiolabel bound.

Assays for Effector Coupling

Stably transfected CHO cells expressing wild-type or mutant pm2 mAchRs were assayed in 24 well dishes for agonist-stimulated PI metabolism. Cells were plated at an initial density of 2.0 x 10^5 cells per well in Dulbecco's Modified Eagle Medium (D-MEM) with F-12 Nutrient Mixture (Gibco/Bethesda Research Laboratories), 10% calf serum (Sigma) and after addition of 0.5 ml of media containing 1 μCi/ml [^{3}H]-myo-inositol per well, incubated at 37°C for 24 hours. IP1 accumulation was measured as described by Lee et al. (Lee et al., 1990) thirty minutes after addition of ligands. To maximize fold stimulation of PI metabolism when constructing PTX dose-response curves (Figure 5), IP1 accumulation was measured fifty minutes after carbachol addition.

Assays for inhibition of forskolin-stimulated cAMP levels were conducted on CHO cells expressing wild-type or mutant receptors by a modification of the Salomon method (Salomon, 1979). Cells were plated on 35 mm tissue culture dishes and grown to a final density of 1.0 x 10^6 cells per plate. Prior to the assay for cAMP, cells were incubated with [^{3}H]-adenine (1 μCi/ml) for 2 hours in the presence of 0.5 mM Ro 20-1724 at 37°C in D-MEM + F12 Nutrient Mixture. Total tritium labeled cAMP was separated with successive columns of AG50W-X4 resin (BioRad) and Alumina (Sigma). The cAMP was eluted

from the alumina columns directly into scintillation vials with 4 ml of 0.1 M imidazole buffer, pH 7.4, and samples counted in a liquid scintillation counter. In specific experiments, PTX was added 6 to 12 hours prior to the assay to give the appropriate final concentration.

Initial experiments indicated that in CHO cells expressing wild-type pm2 mAchR over the range of 10^5 to 2 x 10^6 receptors per cell the IC_{50}, but not the maximal inhibition for cAMP formation and the maximal fold stimulation, but not the EC_{50}, for PI stimulation were dependent on the number of receptors per cell (Vogel et al., 1995). Thus it was necessary to compare the IC_{50} for adenylyl cyclase inhibition and the maximal fold stimulation of PI metabolism of mutants with wild-type cell lines containing an approximately identical number of receptors per cell. After many attempts, it was not possible to isolate an appropriate distribution of cell lines nor was it possible to reduce wild-type receptor number sufficiently with the alkylating agent benzilylcholine mustard, which was able to maximally remove about 90% of the surface sites from wild-type pm2 mAchR cell lines expressing 1-2 x 10^6 sites/cell. An alternative approach in which receptor number was reduced by addition of various amounts of the slowly dissociating antagonist *l*-QNB was utilized. After an hour, the plates were rinsed twice with media to remove any free *l*-QNB. The physiological assays were then initiated with the appropriate agonists and the average numbers of surface receptors per cell determined by addition of [^{3}H]NMS in the presence or absence of 10^{-4} M *l*-hyoscyamine. Plots of IC_{50} (for inhibition of cAMP formation) and fold maximal response (for PI stimulation) versus pm2 receptors/cell were made and the appropriate wild-type parameter determined from the

standard curves and used for comparison with the values for mutants expressing receptors at the same receptor number per cell. Parameters for mutant receptors were compared to wild-type pm2 mAchR expressed at the appropriate receptor density (Tables 2 and 3).

Data Analysis

Functional assays and agonist ligand binding data were analyzed using non-linear least squares curve-fitting in Origin (MicroCal Software, Inc.). Data from effector coupling assays were either fit to the logistic equation (1) for PI assays or equation (2) for cAMP assays.

$$Y = A + \frac{(B-A)}{1 + (C/[x])^D} \quad (1)$$

$$Y = A + \frac{(B-A)}{1 + (C/[x])^D} + \frac{(E-A)}{1 + ([x]/F)^G} \quad (2)$$

For each of these equations, [x] is the ligand concentration, A and B (or E) the minimum and maximum plateaus of the curve, C the EC_{50} for PI or adenylyl cyclase stimulation, F the IC_{50} for adenylyl cyclase inhibition and D (or G) the Hill coefficient or slope factor. The logistic equation is customarily used to evaluate dose-response curves (Barlow and Blake, 1989). Competition displacement curves were fit according to a mass action model for receptor-ligand interactions at three independent classes of sites (equation 3, below) as described in Tota et al. (Tota et al., 1987).

$$Y = \left(\frac{[L]}{K} \right) \left[\frac{(F_1)}{1 + [L]/K + [I]/K_1} + \frac{(F_2)}{1 + [L]/K + [I]/K_2} + \frac{(1 - F_1 - F_2)}{1 + [L]/K + [I]/K_3} \right]$$

In equation 3, Y is the fractional saturation of the receptor and [L] is the free concentration of antagonist used in the experiment with dissociation constant K. [I] is the total concentration (assumed to equal the free concentration since significant displacement does not occur until [I total] >> [total receptor]) of the competing agonist with dissociation constants K_1, K_2, and K_3. F_1, F_2 and F_3 are the corresponding fractions of total binding sites where $F_3 = 1 - F_1 - F_2$. Antagonist binding was analyzed using Scatchard plots (Scatchard, 1949).

Results

Expression of pm2 AchR in CHO cells

The pm2 mAchR constructs in pSVE were stably transfected into dhfr$^-$ CHO cells and amplified by growing in the presence of increasing concentrations of methotrexate. The wild-type pm2 cell line expressed 1.2 x 10^6 total receptors per cell, while A212E, K214A and KDKKE(219-223)ELAAL expressed 3.0 x 10^5, 3.8 x 10^5, and 1.0 x 10^6 receptors/cell respectively. Comparison of specific [^{3}H]NMS versus [^{3}H] *l*-QNB binding indicated that wild-type and KDKKE(219-223)ELAAL expressed 100% of their receptors on the cell surface and A212E and K214A about 70% to 80%.

Ligand binding characteristics

The ligand binding properties of pm2 mutants and wild-type receptor are summarized in Table 2.1. The affinity of [^{3}H] *l*-QNB was within a factor of two for the mutants compared to wild-type while [^{3}H]NMS binding was weaker to the mutants by

three to five fold. Thus the antagonist binding properties of the mutants for these two ligands were affected in the mutant pm2 receptors, but not very strongly.

Agonist binding data for wild-type pm2 mAchR for carbachol, acetylcholine and oxo M were consistent with fits to three independent classes of sites (K_1, K_2 and K_3) while pilocarpine data were best fit by assuming two classes of sites (i.e. $K_2 = K_3$). Agonist binding properties were affected in a selective manner by specific mutations. The mutation A212E appears to reduce the affinity of the pm2 mAchR for acetylcholine but not carbachol, and slightly increased the affinity for oxo M. The mutation K214A increased the affinity for oxo M by five to 27 fold. The mutation KDKKE(219-223)ELAAL also increased the affinity for oxo M at the low and intermediate affinity sites and for carbachol at the high affinity site by factors of eight to 10 fold. The binding properties of the partial agonist pilocarpine were essentially unaffected for the three mutants.

Table 2.1 Ligand Binding Properties of Mutant pm2 mAchRs[a]

Antagonists

	l-QNB		NMS	
	pK_d (M)	n	pK_d (M)	n
wildtype	4.68 ± 0.04	3	8.9 ± 0.1	3
A212E	4.5 ± 0.1	6	*8.1 ± 0.1	4
K214A	4.6 ± 0.1	3	*8.4 ± 0.1	3
KDKKE(219-223)ELAAL	*4.33 ± 0.04	6	*8.3 ± 0.1	2

Agonists

	pK_1 (M)	pK_2 (M)	pK_3 (M)	F_1	F_2	n
Carbachol						
wildtype	8.1±0.2	5.6±0.3	4.3±0.1	0.20±0.05	0.52±0.10	4
A212E	8.3±0.1	5.3±0.1	4.4±0.2	0.21±0.02	0.31±0.14	3
K214A	*8.42±0.02	6.0±0.1	*5.1±0.1	0.31±0.02	0.48±0.03	4
KDKKE(219-223)ELAAL	*9.0±0.2	5.7±0.2	*4.5±1	0.25±0.04	0.50±0.07	4
Acetylcholine						
wildtype	9.3±0.1	6.6±0.2	5.2±0.1	0.36±0.02	0.52±0.03	2
A212E	*8.0±0.2	*5.3±0.1	*4.4±0.2	0.25±0.09	0.54±0.25	4
K214A	8.9±0.2	6.7±0.1	5.9±0.2	0.37±0.08	0.32±0.08	2
KDKKE(219-223)ELAAL	9.5±1.1	*7.2±0.3	5.4±0.3	0.12±0.06	0.39±0.07	5
Oxotremorine-M						
wildtype	8.3±0.1	6.1±0.1	4.7±0.2	0.17±0.05	0.13±0.08	3
A212E	8.3±0.3	6.7±0.6	*5.2±0.1	0.23±0.01	0.50±0.02	4
K214A	*9.0±0.1	*6.76±0.03	*6.2±0.2	0.15±0.01	0.57±0.08	4
KDKKE(219-223)ELAAL	8.2±0.1	*7.7±0.1	*5.7±0.1	0.25±0.02	0.4±0.03	2
Pilocarpine						
wildtype	6.6±0.2	4.9±0.3	---	0.29 ± 0.11	0.71 ± 0.11	3
A212E	6.8±0.1	5.6±0.1	---	0.53 ± 0.12	0.47 ± 0.10	2
K214A	6.6±0.2	5.2±0.2	---	0.52 ± 0.05	0.48 ± 0.09	2
KDKKE(219-223)ELAAL	6.5±0.2	5.6±0.2	---	0.20 ± 0.04	0.80 ± 0.10	2

[a] Values represent the mean ± S.D. of 2-4 experiments.

* Significantly differs ($p \leq 0.05$) from pm2 wild-type mAchR.

Stimulation of PI metabolism

Data comparing the coupling of wild-type and mutant pm2 mAchRs to stimulation of PI is summarized in Figures 2.1, 2.2 and Table 2.2. Carbachol, acetylcholine and oxo M stimulated accumulation of IP1 up to 2 fold over basal levels in cells expressing wild-type pm2 mAchRs. EC_{50}s for these agonists were 1.7 μM, 0.4 μM and 0.30 μM respectively. The maximal fold stimulation values reported for wild-type pm2 mAchRs are those estimated from the standard curve at 3.0×10^5, 3.8×10^5 and 1.0×10^6 receptors per cell respectively and mutant pm2 mAchRs at their respective receptor densities.

Compared to wildtype, the mutant pm2 mAchRs stimulated PI hydrolysis with EC_{50}s that are selectively altered, depending on the ligand and mutant. Wild-type receptor, A212E, K214A and KDKKE(219-223)ELAAL display EC_{50}s of 1.7 μM, 2.6 μM, 4.2 μM and 6.6 μM respectively when carbachol is the agonist, with maximal percent stimulation of 60-72%, 66%, 85% and 98%. These findings indicate that these mutants have little or no effect on carbachol mediated coupling to PI metabolism.

Acetylcholine and oxo M appear to have different effects on the coupling of mutants to stimulation of PI metabolism than carbachol. While K214A coupled to PI metabolism with an EC_{50} approximately 10 fold higher than wild-type for acetylcholine, the EC_{50} for oxo M did not significantly differ. This is also observed for KDKKE(219-223)ELAAL but not A212E.

Although A212E shows high affinity binding to both carbachol and oxo M (Table 2.1), oxo M does not stimulate PI hydrolysis in the same manner as other ligands. Carbachol gave maximal fold stimulation of IP1 formation at levels similar to wild-type with an EC_{50} of 2.6 μM. On the other hand, both acetylcholine and oxo M behaved like partial agonists for PI stimulation (Figures 2.1, 2.2, 2.3 and 2.4), although maximal inhibition of cAMP formation was similar to carbachol (Table 2.3; Figures 2.4, 2.5 and 2.6). Maximum fold stimulation significantly differed from wild-type for both of these agonists (Table 2.2) and the EC_{50} significantly increased for acetylcholine. The response for oxo M was consistently too low to determine this parameter accurately.

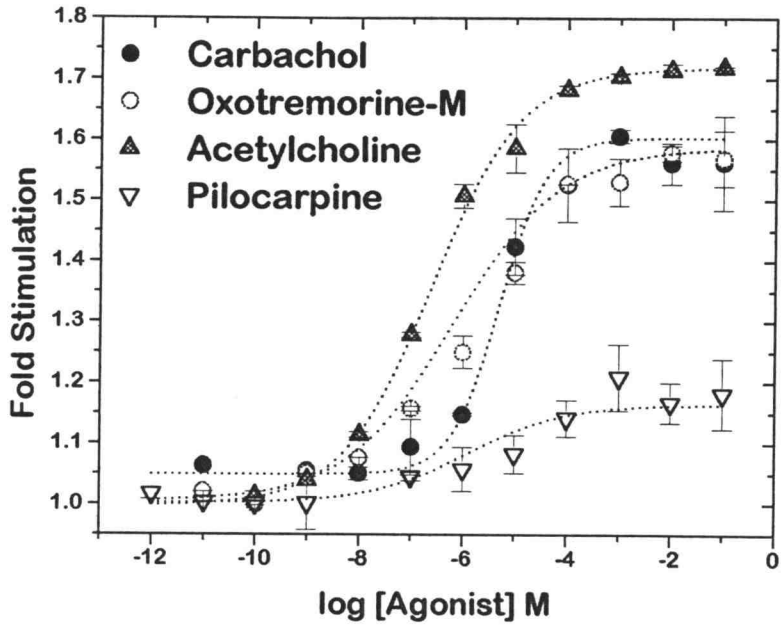

Figure 2.1: Agonist stimulation of PI hydrolysis by wild-type pm2 mAChR

Experiments were conducted as described in Experimental Procedures. Data are presented as fold stimulation of PI hydrolysis over basal levels of released inositol monophosphates. The data shown are representative of 3-6 experiments with each agonist concentration done in duplicate. Curves were derived from a least squares fit to equation 1 and average values for EC_{50} and maximal fold stimulation are summarized in Table 2.2 Wild-type pm2 mAchR, were expressing $1.2x10^6$ receptors/cell. The fitted parameters were: $EC_{50} = 4.9 \pm 1.4$ μM, 1.6 fold; $EC_{50} = 0.59 \pm 0.09$ μM, 1.6 fold; $EC_{50} = 0.24 \pm 0.02$ μM, 1.7 fold; and $EC_{50} = 1.4 \pm 1.9$ μM, 1.2 fold for carbachol, oxo M, acetylcholine and pilocarpine respectively. Slope factors used were 1.0 for carbachol, and 0.5 for oxo M, acetylcholine and pilocarpine.

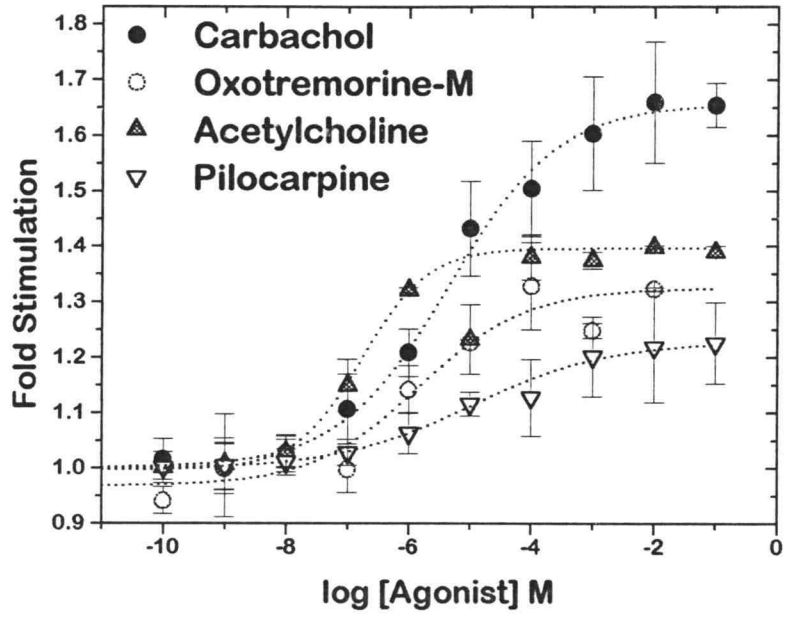

Figure 2.2: Agonist stimulation of PI hydrolysis by A212E mutant mAchR.

Experiments were conducted as described in Experimental Procedures. Data are presented as fold stimulation of PI hydrolysis over basal levels of released inositol monophosphates. The data shown are representative of 3-6 experiments with each agonist concentration done in duplicate. Curves were derived from a least squares fit to equation 1 and average values for EC_{50} and maximal fold stimulation are summarized in Table 2.2. A212E m2 mAchR mutant, $3x10^5$ receptors/cell with fitted parameters: $EC_{50} = 7.1 \pm 3.9$ μM, 1.7 fold; $EC_{50} = 2.0 \pm 0.37$ μM, 1.3 fold; $EC_{50} = 0.17 \pm 0.07$ μM, 1.4 fold; and $EC_{50} = 24.0 \pm 16.1$ μM, 1.2 fold for carbachol, oxo M, acetylcholine and pilocarpine respectively. Slope factors were 1.0 for carbachol and acetylcholine and 0.5 for oxo M and pilocarpine.

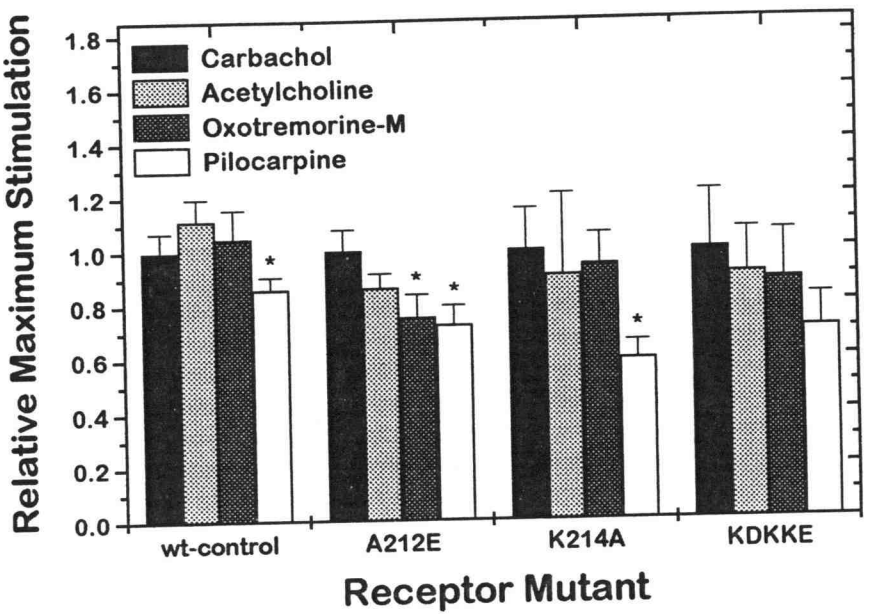

Figure 2.3: Normalized agonist-mediated maximum stimulation of PI hydrolysis for wild-type and mutant mAchRs.

Experiments were conducted as described in Experimental Procedures. Data are presented as the ratio of agonist-mediated maximal response relative to carbachol for each receptor mutant. Expression levels are $1.2x10^6$ receptors/cell for pm2 mAchR, and $3.0x10^5$, $3.8x10^5$ and $1.0x10^6$ receptors/cell for A212E, K214A and KDKKE(219-223)ELAAL respectively. The data shown are 2-10 experiments done in duplicate (± SD). Pilocarpine stimulated PI hydrolysis between 64% and 83% of carbachol stimulated levels, while oxo M and acetylcholine stimulated 90% to 110%. The one exception was oxo M for A212E which maximally stimulated PI hydrolysis at 79% of carbachol stimulated levels. Statistically significant differences ($p \leq 0.05$) are marked with *.

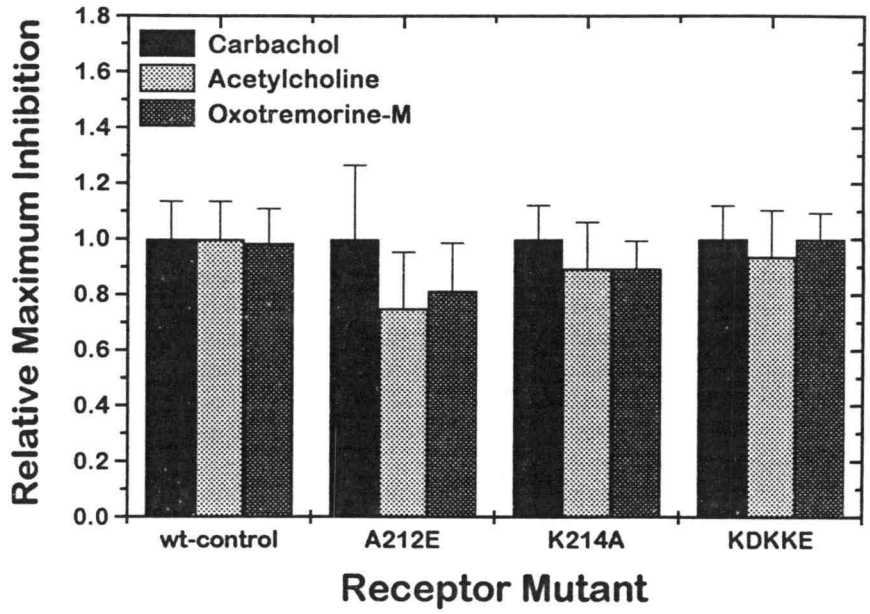

Figure 2.4: Normalized agonist-mediated maximum stimulation of PI hydrolysis and maximum inhibition of adenylyl cyclase.

Experiments were conducted as described in Experimental Procedures. Data are presented as the ratio of agonist-mediated maximal response relative to carbachol for each receptor mutant. Expression levels are $1.2x10^6$ receptors/cell for pm2 mAchR, and $3.0x10^5$, $3.8x10^5$ and $1.0x10^6$ receptors/cell for A212E, K214A and KDKKE(219-223)ELAAL respectively. The data shown are 2-9 experiments done in duplicate (± SD). All agonists inhibited cAMP formation between 75% to 100% of carbachol inhibition. Statistically significant differences ($p \leq 0.05$) are marked with *.

To compare the relative efficacy of each agonist for mutant receptors, maximal stimulation of IP1 accumulation and maximal inhibition of adenylyl cyclase were normalized to values obtained for carbachol (Figures 2.3 and 2.4). Pilocarpine stimulated PI hydrolysis between 64% and 83% of carbachol stimulated levels, while oxo M and acetylcholine stimulated 90% to 110% compared to carbachol for all receptors characterized. The one exception was oxo M for A212E which maximally stimulated PI hydrolysis at 79% of carbachol stimulated levels. While the observed maximal stimulation by acetylcholine significantly differed from wild-type for A212E (Table 2.2, Figures 2.1 and 2.2), it did not appear to differ significantly from carbachol stimulated levels for this mutant (Figure 2.3).

Table 2.2 Mutant pm2 mAchR Coupling to Stimulation of Inositol Phospholipid Metabolism

	log EC_{50} (M)	Maximal Fold Stimulation	n
Agonists			
Carbachol			
wildtype	5.8 ± 0.2	(1.60, 1.63, 1.72)[b]	[a]
A212E	5.6 ± 0.3	1.6 ± 0.1	7
K214A	*5.4 ± 0.3	1.9 ± 0.2	5
KDKKE(219-223)ELAAL	*5.3 ± 0.3	2.0 ± 0.3	7
Acetylcholine			
wildtype	6.6 ± 0.4	(1.65, 1.70, 1.82)[b]	[a]
A212E	*6.1 ± 0.1	*1.43 ± 0.04	6
K214A	*5.6 ± 0.5	1.7 ± 0.5	11
KDKKE(219-223)ELAAL	*5.4 ± 0.2	1.8 ± 0.2	4
Oxotremorine-M			
wildtype	7.1 ± 0.9	(1.50, 1.52, 1.62)[b]	[a]
A212E	[c]	*1.3 ± 0.1	6
K214A	6.5 ± 0.9	1.8 ± 0.1	7
KDKKE(219-223)ELAAL	6.3 ± 0.3	1.8 ± 0.2	5
Pilocarpine			
wildtype	4.6 ± 0.7	(1.25, 1.30, 1.58)[b]	[a]
A212E	[c]	1.2 ± 0.1	2
K214A	[c]	*1.10 ± 0.01	2
KDKKE(219-223)ELAAL	4.9 ± 0.1	1.4 ± 0.1	2

[a] Values represent the mean ± S.D. of 3-12 experiments.

[b] Wild-type % maximal stimulation at $3x10^5$, $3.8x10^5$, $1.0x10^6$ pm2 mAchR/cell.

[c] EC_{50} is not well determined due to small observed response to agonist treatment.

* Significantly differs ($p \leq 0.05$) from pm2 wild-type mAchR expressed at comparable receptor densities

Inhibition of cAMP formation

Data describing the coupling of wild-type and mutant pm2 mAchRs to the inhibition of cAMP formation in CHO cells is summarized in Figure 2.4 and Table 2.3. Reduced maximal percent inhibition was observed for all agonists for each of the mutant receptors characterized although IC_{50} values for carbachol were similar to wild-type pm2 mAchRs expressed at the same level (Table 2.3). It is unclear if this finding reflects any specific changes in receptor-G-protein interaction or if a general reduction in coupling efficiency is observed due to the slight modifications of i3. This explanation is consistent with the observation that most chimeric receptors couple to effector systems with lower efficiency than the wild-type receptor (Bonner, 1992). Unlike the results for stimulation of PI metabolism, maximal inhibition of adenylyl cyclase relative to carbachol was the same for all mutants (Figure 2.4). Increased IC_{50} values were observed for acetylcholine and oxo M for both A212E and K214A, although no significant deviation from wild-type was observed for carbachol. KDKKE(219-223)ELAAL did not exhibit any agonist-dependent increase in IC_{50}.

The data in Figures 2.5 and 2.6 showed that muscarinic agonists caused both inhibitory and stimulatory effects on cAMP levels. At high concentrations, all agonists appeared to mediate stimulation of adenylyl cyclase. The EC_{50} of stimulation for the pm2 mAchR was variable and highly dependent on receptor number with values ranging from 10 to 1000 μM.

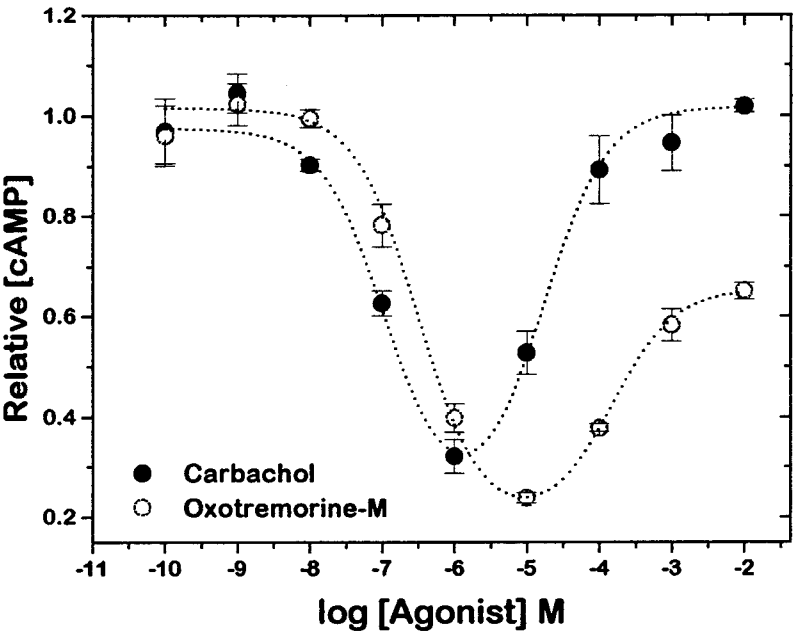

Figure 2.5: Inhibition of cAMP formation by wild-type pm2 mAChR

Experiments were conducted as described in Experimental Procedures. Data are presented as levels of cAMP generated relative to forskolin-stimulated levels. The data shown are representative of 3-4 experiments with duplicate data points. Curves were derived from a least squares fit to equation 2 and average values for IC_{50} and % maximal inhibition are summarized in Table 2.3. Wild-type pm2 mAchR expressed $1.2x10^6$ receptors/cell. The fitted parameters were: $IC_{50} = 0.11 \pm 0.03$ μM, 75% inhibition for carbachol; $IC_{50} = 0.3 \pm 0.06$ μM, 80% inhibition for oxo M.

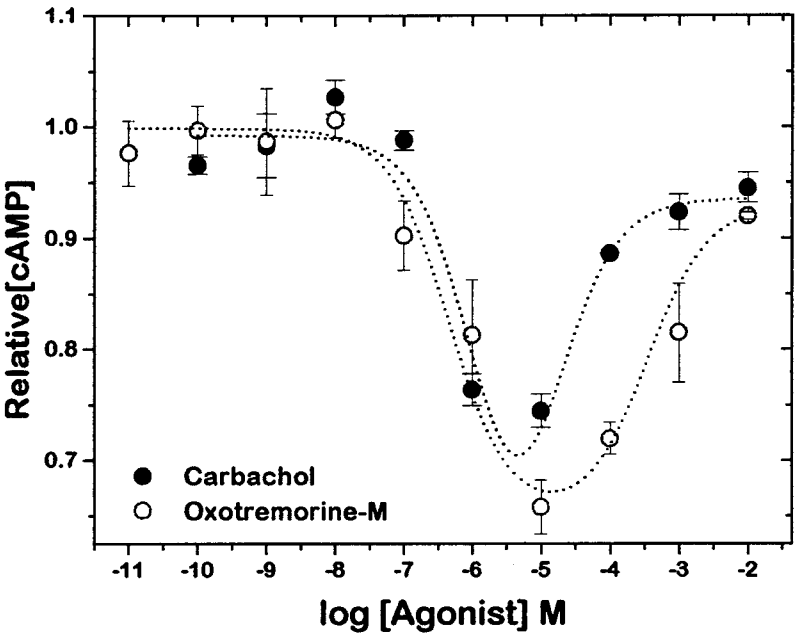

Figure 2.6: Inhibition of cAMP formation by A212E mAchR

Experiments were conducted as described in Experimental Procedures. Data are presented as levels of cAMP generated relative to forskolin stimulated levels. The data shown are representative of 3-4 experiments with duplicate data points. Curves were derived from a least squares fit to equation 2 and average values for IC_{50} and % maximal inhibition are summarized in Table 2.3. A212E pm2 mAchR mutant expressed $3x10^5$ receptors/cell, with fitted parameters: $IC_{50} = 0.7 \pm 0.3$ μM, 40% inhibition for carbachol; $IC_{50} = 0.3 \pm 0.1$ μM, 35% inhibition for oxo M. Slope factor = 1.0 for both agonists in wild-type and A212E.

Table 2.3 Mutant pm2 mAchR Coupling to Inhibition of cAMP Formation

Agonists

	log IC_{50} (M)	% Maximal Inhibition	n
Carbachol			
wildtype	(6.5, 6.6, 7.0)[b]	74 ± 7	[a]
A212E	*6.1 ± 0.3	*48 ± 9	6
K214A	6.8 ± 0.9	*47 ± 4	6
KDKKE(219-223)ELAAL	7.2 ± 0.5	*48 ± 4	9
Acetylcholine			
wildtype	(7.4, 7.5, 8.0)[b]	74 ± 7	[a]
A212E	*6.8 ± 0.3	*36 ± 7	3
K214A	*7.0 ± 0.1	*42 ± 7	4
KDKKE(219-223)ELAAL	7.6 ± 0.4	*45 ± 7	3
Oxotremorine-M			
wildtype	(6.9, 7.0, 7.4)[b]	73 ± 6	[a]
A212E	6.4 ± 0.4	*39 ± 4	3
K214A	6.7 ± 0.4	*42 ± 3	3
KDKKE(219-223)ELAAL	*7.96 ± 0.04	*48 ± 2	2

[a] Values represent the mean ± S.D. of 3-4 experiments.

[b] Wild-type EC_{50} values at 3.0×10^5, 3.8×10^5 and 1.0×10^6 pm2 mAchRs/cell.

* Significantly differs ($p \leq 0.05$) from pm2 wild-type mAchR expressed at comparable receptor densities.

PTX Sensitivity

Selectivity of G-protein coupling appeared to be affected by removal of a positive charge at the seventh N-terminal residue of i3 in the mutant K214A. Inhibition of cAMP formation was mediated by a PTX-sensitive G-protein for K214A (data not shown) while coupling to PI metabolism occurred through G-proteins with different PTX sensitivity than wild-type pm2 mAchRs (Figures 2.7, 2.8, 2.9 and 2.10). Wild-type receptor, KDKKE(219-223)ELAAL (Figure 2.9) and A212E (data not shown) coupled to PI metabolism by a G-protein that was PTX sensitive since treatment with 100 ng/ml pertussis toxin for 12 hours resulted in complete uncoupling. For K214A, treatment with 100 ng/ml PTX failed to significantly reduce the measured accumulation of IP1 (Figure 2.8) although the EC_{50} for carbachol was left-shifted by about 20 fold.

The PTX concentration dependence on maximal fold IP1 accumulation was assessed for K214A and wild-type receptor (Figure 2.10). While PTX treatment reduced the fold accumulation of IP1 for K214A there was a 100 fold right-shift in PTX sensitivity compared to wildtype. In addition, treatment with 1000 ng/ml PTX failed to completely abolish the response to carbachol. To maximize the carbachol-stimulated increase in PI hydrolysis, these experiments were allowed to proceed for 50 minutes instead of 30. While this may explain the greater fold accumulation of IP1 for both wild-type pm2 receptor and K214A, it does not account for the apparent difference in PTX sensitivity.

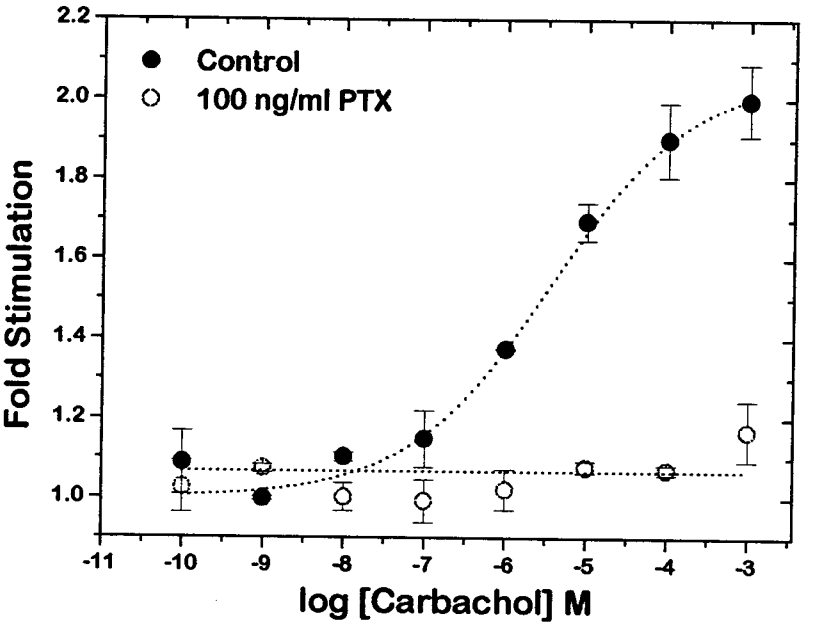

Figure 2.7: Effect of PTX on PI hydrolysis by wild-type m2 mAchR.

Cells expressing wild-type or mutant pm2 mAchRs were treated with 100 ng/ml PTX for 12 hours in the presence of 1 μCi/ml [^{3}H]*myo*-inositol prior to the assay. Data are presented as fold stimulation of PI metabolism over basal levels for each experiment. The data shown are from representative experiments. Curves through the data were derived from a least squares fit to equation 1. Wild-type pm2 mAchR (n=2) expressing 1.2×10^6 receptors/cell. Fitted parameters for the control were: $EC_{50} = 2.9 \pm 0.7$ μM, 2 fold.

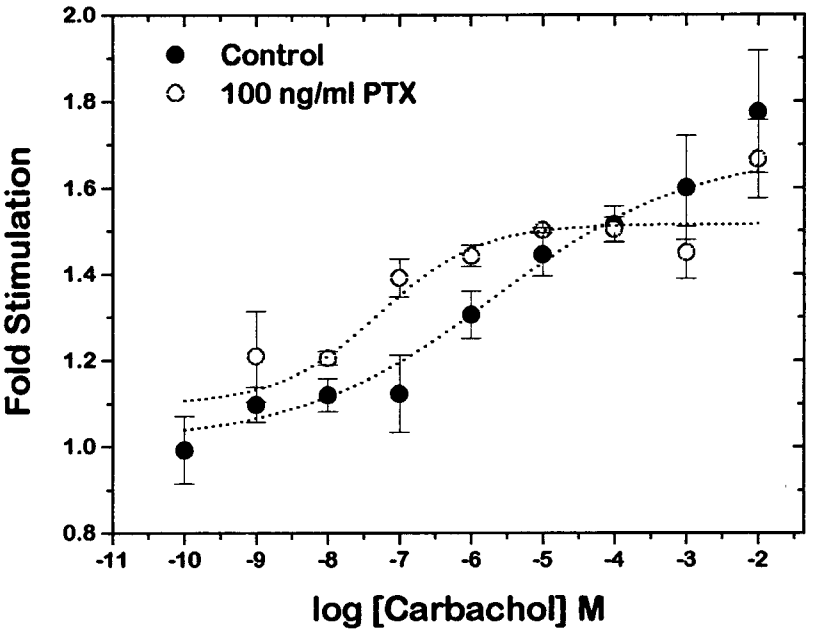

Figure 2.8: Effect of PTX on PI hydrolysis by K214A mutant mAchR.

Cells expressing wild-type or mutant pm2 mAchRs were treated with 100 ng/ml PTX for 12 hours in the presence of 1 μCi/ml [^{3}H]*myo*-inositol prior to the assay. Data are presented as fold stimulation of PI metabolism over basal levels for each experiment. The data shown are from representative experiments. Curves through the data were derived from a least squares fit to equation 1. K214A pm2 mAchR mutant (n=4) expressed 3.8×10^5 receptors/cell with fitted parameters $EC_{50} = 1.3 \pm 0.7$ μM, 1.7 fold; $EC_{50} = 0.08 \pm 0.04$ μM, 1.5 fold for control and PTX treated respectively.

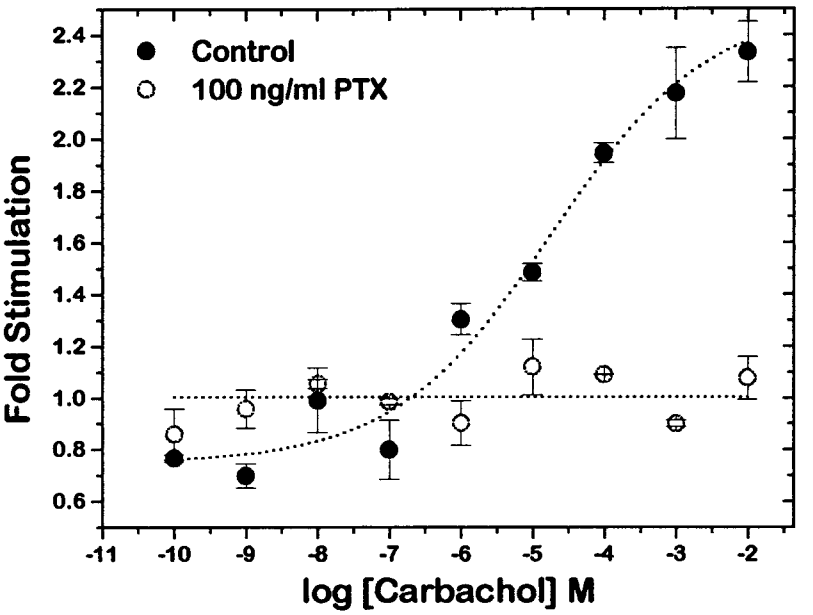

Figure 2.9: Effect of PTX on PI hydrolysis by KDKKE mutant mAchR.

Cells expressing wild-type or mutant pm2 mAchRs were treated with 100 ng/ml PTX for 12 hours in the presence of 1 μCi/ml [^{3}H]*myo*-inositol prior to the assay. Data are presented as fold stimulation of PI metabolism over basal levels for each experiment. The data shown are from representative experiments. Curves through the data were derived from a least squares fit to equation 1. KDKKE(219-223)ELAAL pm2 mAchR mutant (n=2) expressed 1.0x10^6 receptors/cell with fitted parameters for the control: EC_{50} = 15 ± 10 μM, 2.5 fold.

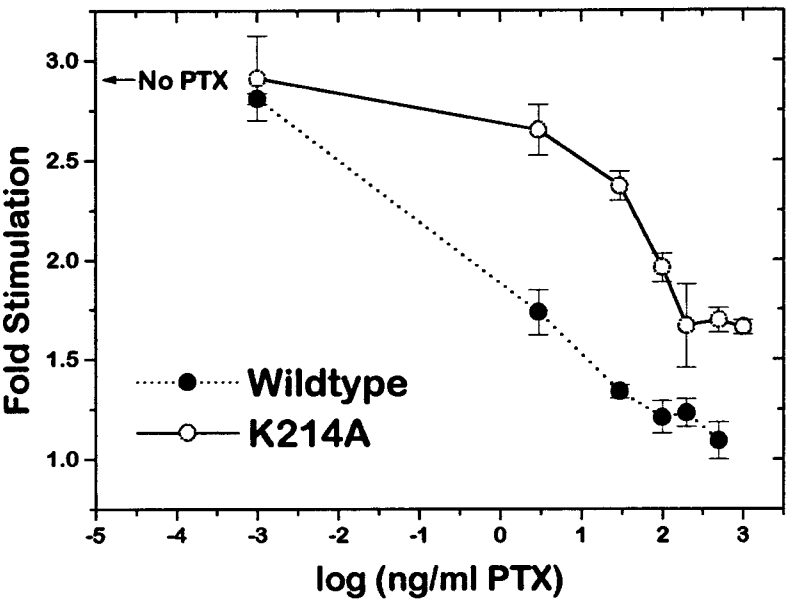

Figure 2.10: Effect of PTX concentration on IP1 accumulation by wild-type and K214A mAchR.

Cells were treated with various concentrations of PTX for 12 hours prior to conducting the PI assay and assayed for inositol monophosphate accumulation 50 minutes after addition of 1 mM carbachol. Data are presented as maximal fold increase in IP1 accumulation over basal levels for PTX-treated as well as control cells. Data shown are the means of triplicate determinations (±SD) from one representative experiment (n=2). The curves through the data have no theoretical basis.

Discussion

Specificity of coupling

The i3 loop of G-protein-coupled receptors plays an important role in regulating receptor-effector coupling. Deletion mutants of the β_2-adrenergic receptor indicated that the N- and C-terminal regions are necessary for coupling to stimulation of adenylyl cyclase (Dixon et al., 1987; Strader et al., 1987). Deletion analysis of the third intracellular loop for m1 mAchR also indicated that the middle 126 amino acids are not necessary for agonist-mediated stimulation of PI metabolism (Shapiro and Nathanson, 1989). The entire third intracellular loop and part of the fifth transmembrane region were switched in m1/m2 chimeric receptors. When expressed in oocytes, the m1/m2 chimera exhibited the physiological response characteristics of the receptor that contributed the loop (Kubo et al., 1988) also implying a role in specificity.

Other studies indicate that effector specificity is conferred by the first 15 to 20 N-terminal amino acids of i3 (Lechleiter et al., 1990; Wess et al., 1990). Wess et al. expressed m2/m3 chimeras in A9 L cells where the first 16 (of m2) or 17 (of m3) N-terminal residues were swapped between the muscarinic receptor subtypes. The m3/m2-16 residue chimera did not couple to PI hydrolysis, but did weakly inhibit formation of cAMP via a PTX-sensitive G-protein. The m2/m3-17 residue chimera on the other hand, coupled to PI metabolism through a PTX-insensitive G-protein while maintaining PTX-sensitive inhibition of cAMP formation (Wess et al., 1990). This implies that the N-terminal region of i3 plays an important role in determining coupling selectivity but may

not be the only determinant. To characterize important residues in this region, single and multiple mutations were introduced in the fifth, seventh and 12th-16th N-terminal amino acids of i3 in the porcine m2 mAchR. Our findings indicate that subtle changes in G-protein coupling characteristics and ligand binding parameters can occur with little or no change in observed physiological responses.

Effect on Ligand binding

At this time a detailed interpretation of the effects of mutations on agonist binding properties is difficult. This laboratory has analyzed agonist competition data assuming models for three classes of noninteracting sites or a two state model plus a third receptor state that does not couple to G-proteins (Vogel et al., 1995). Both approaches allow calculation of values for K_1, the agonist dissociation constant from RG, and K_2, the dissociation constant for agonist binding to the free receptor, as well as K_3, the agonist dissociation constant from a molecularly undefined receptor state which does not couple to G-proteins. The assignment of K_1 and K_2 is based on the conversion of F_1 to F_2 by guanine nucleotides (Vogel et al., 1995). However, both analyses show multiple local minima and appear to be ill-conditioned. In addition, models based on equilibrium (Chidiac and Wells, 1992) or kinetic data (Hirschberg and Schimerlik, 1994) have been proposed that evoke receptor-receptor interactions. Nevertheless, the data in Table 2.1 do indicate that even single amino acid mutations in a region of the m2 mAchR thought to be involved in coupling to G-proteins also affect the ligand binding properties of the protein.

Effect on receptor-effector coupling

The effects of the mutations on receptor-effector coupling were more straight forward. Replacing a positively charged residue with a neutral one (K214A) resulted in a receptor that coupled to effector systems through a different G-protein(s). The difference in PTX sensitivity for agonist stimulation of PI metabolism has previously been reported for receptors expressed in CHO cells (Ashkenazi et al., 1989; Ashkenazi et al., 1987). Three classes of responses were described for both transfected and endogenous receptors. Endogenous receptors for thrombin and transfected porcine m2 mAchRs coupled to PI metabolism by PTX-sensitive G-proteins while transfected m1 and m3 receptors were only partially sensitive to PTX suggesting both PTX-sensitive and insensitive G-proteins mediated the response. On the other hand, endogenous cholecystokinin receptors coupled to PI metabolism through a completely PTX-insensitive G-protein (Ashkenazi et al., 1989).

K214A appeared to couple to both inhibition of cAMP formation and stimulation of PI metabolism in a manner similar to wild-type pm2 receptor. Since inhibition of cAMP formation is mediated by a PTX-sensitive G-protein for K214A, it is clear that this mutant still efficiently couples to G_i. It has been demonstrated that wild-type m2 mAchR couples to PI metabolism through the PTX-sensitive G-proteins Gαi2 and Gαi3 in CHO cells (24). This suggests that for K214A, coupling to PI metabolism could occur through G_i as well as some other PTX-insensitive G-protein, perhaps G_q or G_{11}. G_q and G_{11} which mediate coupling to PI metabolism in a PTX-insensitive manner are expressed in our Chinese hamster ovary cell line as are Gαi2 and Gαi3, while Gαi1 and Gαo are not expressed

(Dell'Acqua et al., 1993)*. After exposure to 100 ng/ml PTX, a concentration sufficient to eliminate coupling to PI stimulation for wild-type, A212E and KDKKE(219-223)ELAAL, K214A still appeared to couple to this response with an EC_{50} for carbachol left-shifted by about 20 fold compared to the non-PTX treated control. This suggested that the major pathway for IP1 formation was through a different G-protein after PTX treatment than in non- PTX treated controls, and that this response appeared more tightly coupled in the presence of carbachol. When cells expressing K214A were treated with high concentrations of PTX, the dose-response curve was right-shifted and complete uncoupling did not occur even at high PTX concentrations (Figure 2.10). Taken together these results indicate that K214A must be coupling to PI stimulation through both PTX-sensitive and insensitive G-proteins. Whether the rightward shift of the PTX dose-response curve is due to K214A coupling to a different PTX-sensitive G-protein than wild-type receptor or to an increase in coupling through the PTX-insensitive pathway as the levels of PTX-sensitive G-protein are reduced is not yet known. When chimeric m1/m2 receptors with i3 or the N-terminal portion of i3 were reconstituted with specific G-proteins (G_i, G_o, G_z, G_q and G_s), receptor G-protein interactions occurred with both PTX sensitive and PTX-insensitive G-proteins (Wong and Ross, 1994). An alternative explanation also compatible with this result is that the K214A mutation stabilizes the receptor-G protein complex, reducing the availability of the G-protein to PTX.

* J. Robishaw, unpublished results.

Lysine is conserved at amino acid position 214 in the m2/m4 mAchR family while the m1/m3/m5 family has a conserved Glu in that position. Perhaps the positively charged side chain of Lys is important in determining the nature of G-protein coupling interactions for the m2/m4 mAchRs. Replacing this Lys with a neutral Ala may remove a constraint which allows the receptor to interact with multiple G-proteins. For the human m3 mAchR, several charged residues near the N-terminal of i3 were required for normal signal transduction (Kunkel and Peralta, 1993).

The N-terminal region of i3 is predicted to form an amphipathic α-helical structure in all G-protein coupled receptors. Studies on chimeric m2/m3 mAchRs suggest that receptors with similar function utilize regions of conserved charge and secondary structure without complete sequence identity (Lechleiter et al., 1990). While this motif may represent a structural requirement in receptor-G-protein interactions in general, the individual charged residues within this region may play a role in determining the nature of the protein-protein interaction. When the amino acid sequences of the third intracellular loop are aligned, the N-terminal regions are most conserved among the functional families (e.g. m2/m4 vs. m1/m3/m5). Replacing Lys with Glu at position 214 (K214E), which swaps residues conserved at that position between the functional families resulted in a receptor that was not properly processed. All receptor sites detected (3×10^5 sites/cell) were internalized with no expression on the cell surface (data not shown).

Replacement of a non-polar amino acid with an acidic one (A212E) resulted in ligand binding and effector coupling properties similar to wild-type receptor. The notable exceptions were the weaker binding of acetylcholine and that oxo M appears to be less

effective at stimulating PI hydrolysis than other agonists, although it bound with high affinity. Acetylcholine also appeared to be less effective at stimulating PI hydrolysis and is significantly different ($p \leq 0.05$) than wild-type pm2 (Table 2.2). When normalized to carbachol stimulated levels of IP1, this reduced efficacy is less striking and does not significantly differ from the full agonist carbachol, while oxo M does (Figure 2.3). It is not clear whether oxo M promoted a receptor conformation that was in general less efficient at coupling to G_i or if changes in $G_{i\alpha}$ subtype selectivity were altered reducing the efficiency of effector activation. Regardless of the mechanism of action, it is significant to note that this finding implies that the agonists oxo M, and to a lesser extent, acetylcholine behaved differently than carbachol.

Agonist-mediated physiological responses for A212E in CHO cells occur through PTX-sensitive G-proteins, making it difficult to ascertain whether or not subtle changes in coupling result. At pm2 mAchR numbers greater than 10^5 per cell, maximal stimulation of PI metabolism appeared to be limited by receptor expression, while maximal inhibition of cAMP formation was not. Most investigations of receptor mutants are carried out on cell lines that are overexpressing receptor. Under these conditions, inefficient coupling of receptor to G_i may go unnoticed in assays of cellular cAMP levels since the receptor is in excess. Although oxo M mediated inhibition of cAMP formation for A212E resembles wild-type receptor, it is possible that this agonist promotes a receptor conformation that is less efficient at activating the G-proteins expressed in CHO cells.

All of the mutant receptors assayed as well as wild-type pm2 mAchR exhibit both an inhibition and stimulation of cAMP formation. While the stimulatory phase is not

dependent on Ca^{2+} or activation of protein kinase-C (Baumgold, 1992), the mechanism by which this effect occurs is not known. For the α2-adrenergic receptor which also couples to both inhibition and stimulation of cAMP formation, the stimulatory phase has been reported to be mediated by a direct interaction with G_s (Eason et al., 1992). m4 mAchRs have also been shown to activate G_s directly in HEK 293 cells (Dittman et al., 1994). These observations implicate an indirect pathway in the stimulation of cAMP formation, possibly mediated by G-protein βγ subunits.

The KDKKE(219-223)ELAAL pm2 receptor mutant replaced the 12th through the 16th N-terminal residues conserved in the m2/m4 functional family with those conserved in the m1/m3/m5 family. While this change introduces alterations in the charge distribution within this domain of i3, it does not affect the predicted secondary structure of this portion of the loop. Binding of carbachol and oxo M was affected, but not effector coupling characteristics. Coupling to effector systems occurred via PTX-sensitive G-proteins as seen in wild-type receptor (Figure 2.7). Fold simulation of PI hydrolysis resembled wild-type pm2 receptor while human m1 mAchRs expressed in CHO cells elicited a 13 fold increase in IP1 accumulation (data not shown). The EC_{50} values for acetylcholine and carbachol significantly differed from wild-type while the EC_{50} for oxo M did not. Although residues 219-223 are within the region of i3 reportedly responsible for switching effector coupling characteristics between m2 and m3 mAchRs (Wess et al., 1990), this finding suggests that charge distribution in this domain of the loop does not play a large role in G-protein selectivity. This finding is supported by the converse

mutation made in the human m1 mAchR (Arden et al., 1992) where change in charge distribution played a minor role in G-protein coupling to PI metabolism.

The N-terminal domain of the third intracellular loop of the mAchR appears to play a role in G-protein coupling, but it is clearly not the only determinant. Chimeric muscarinic (Bonner, 1992; Lechleiter et al., 1990; Wess et al., 1990), and α1-adrenergic (Cotecchia et al., 1992) receptors gain the coupling selectivity of the introduced loop while characteristics of the wild-type receptor are retained to some degree. The rat m3 mAChR N-terminal domain of i3 was studied by replacing residues with corresponding amino acids found in the m2/m4 receptor family (Blüml et al., 1994b). The authors concluded that a critical Tyr residue in rat m3 was necessary for efficient coupling to PI hydrolysis, although substitution of Tyr for Ser in the corresponding position of Hm2 did not result in an enhanced PI response (Blüml et al., 1994a). Högger et al (Högger et al., 1995) studied Hm1 N- and C- terminal junctions of i3. They proposed that these domains of i3 could function as a crucial hinge region allowing exposure of specific binding pockets upon agonist binding as well as a specific recognition substrate, and cautioned against the definition of precise coupling domains in these regions of the muscarinic receptor. In addition, the G-protein selectivity of chimeric receptors (Wong and Ross, 1994) appears to depend on specific contributions by both i2 and i3, and promiscuous receptor G-protein interactions occur in the absence of the correct i2.

Since all members of the seven transmembrane receptor family interact with G-proteins, it is possible that portions of the transmembrane region (the most well conserved domains) are also important in determining G-protein recognition characteristics. A

mutation in the second transmembrane region (D69N) results in a pm2 receptor that binds agonists but does not efficiently couple to effectors. The role of intracellular loops may be to modulate coupling to G-proteins and characteristics observed for each receptor system studied may be dependent on the context in which they are expressed. Coupling selectivity and efficiency may in fact be dependent on the interaction of intracellular loops with other structural domains of the receptor as well as those of the specific G-proteins expressed in the cells being examined. Mouse m1 AChRs when expressed in Y1 adrenal carcinoma cells, CHO cells and Rat-2 fibroblasts, coupled to multiple G-proteins (as determined by PTX sensitivity) in a cell line specific manner (Shapiro et al., 1993).

Summary

In summary, we have shown that single amino acid changes in the N-terminal domain of i3 can lead to subtle changes in G-protein coupling characteristics. This finding suggests that analysis of mutants designed to assess coupling selectivity may require more detailed examination before conclusions can be drawn. Although it appears that coupling is affected by N-terminal residues of i3, the nature of these interactions may be determined by several structural domains simultaneously rather than by the i3 alone as well as the context in which the receptor is expressed. It is imperative that careful evaluation of all data pertaining to coupling characteristics be considered before conclusions be drawn on structural domains. Elucidation of explicit roles of the structural domains important in G-protein coupling by site-directed mutagenesis requires that high resolution assessment of selectivity be utilized.

Chapter 3

Internalization of the porcine m2 muscarinic receptor does not require coupling: Mutation of a conserved aspartic acid in the second transmembrane domain

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Abstract

Agonist-stimulated internalization and desensitization of effector coupling were studied in Chinese hamster ovary cells stably transfected with wild-type and mutant m2 muscarinic acetylcholine receptors. Site-directed mutagenesis was used to mutate an aspartic acid conserved in the putative second transmembrane domain of all muscarinic receptors to asparagine (D69N). Antagonist binding was unchanged by this mutation while agonist binding was slightly altered. Affinities for carbachol, acetylcholine and pilocarpine were similar to wild-type receptor, while oxo M affinity was reduced by 25 fold. Consistent with studies of other G-protein coupled receptors, the D69N mutation had a dramatic effect on receptor-effector coupling properties. Agonist-mediated inhibition of adenylyl cyclase and stimulation of PI hydrolysis was abolished for all agonists examined except oxo M. Receptor internalization and desensitization of D69N did not require coupling to second messenger systems. Agonists incapable of activating effector systems stimulated receptor internalization at rates similar to wild-type receptor. Desensitization of oxo M-stimulated receptor-effector coupling was observed for full agonists but not the partial agonist pilocarpine. Pertussis toxin, which uncouples receptor from endogenous G-proteins, failed to prevent carbachol-stimulated internalization of surface receptor sites. Cells treated with sodium nitroprusside and 8-bromo-cGMP exhibited a loss of surface receptor sites with no change in effector coupling properties while dibutyryl-cAMP and thapsigargin had no effect on receptor sites or coupling. These findings suggest that different agonists promote unique receptor conformations which can

affect G-protein coupling properties. In addition, receptor-effector coupling is not necessary for agonist-promoted internalization and that internalization and desensitization are mediated by separate mechanisms in the CHO cell heterologous expression system.

Introduction

Overview

Receptor desensitization is a common cellular response to persistent stimulation by agonists (Liggett, 1995). GPCRs are regulated by several processes in response to persistent agonist treatment including, internalization, recycling, sequestration and down-regulation It is suggested that the net effect of these processes are to uncouple the receptor from second messenger effector systems. In addition, receptor internalization and recycling may play an important role in resensitization of β_2 adrenergic receptor responses (Pippig et al., 1995; Yu et al., 1993), although the molecular mechanisms underlying these observations are still unclear.

Internalization and desensitization

Receptor internalization, down-regulation and desensitization appear to be regulated by separate mechanisms. Agonist-induced movement of receptors to localized invaginations of the plasma membrane can be separated from endocytosis for the β_2 adrenergic receptor expressed in 293 human kidney fibroblasts. Agonists may function to promote receptor movement to the appropriate cellular location where passive endocytosis can occur (von Zastrow and Kobilka, 1994). Internalized receptors are stored in endosomes and can be recycled to the plasma membrane or degraded (Koenig and Edwardson, 1994). Treatment of cells with colchicine, to prevent receptor down-regulation had no effect on rapid internalization of mAchRs in cultured neuronal cells

(Maloteaux and Hermans, 1994). Furthermore, when cells expressing dopamine D1 receptors were treated with concanavalin A or sucrose, agonist-induced receptor internalization was prevented without affecting desensitization properties (Ng et al., 1995). Human embryonic kidney cells expressing a dominant-negative allele of G-protein coupled receptor kinase 2 (GRK2) reduced agonist-dependent phosphorylation of m2 mAchR and prevented desensitization. In addition, mutations in the third intracellular loop of m2 mAchR produced receptors that were not phosphorylated in an agonist-dependent manner, yet receptor internalization was not affected (Pals Rylaarsdam et al., 1995). These results suggest that internalization, down-regulation and desensitization are regulated in an agonist-dependent manner by independent mechanisms.

Mutation of second transmembrane aspartic acid

Several aspartic acids are conserved in the second and third transmembrane domains of G-protein coupled receptors (Strader et al., 1987; Wang et al., 1991). The aspartic acid located in the putative second transmembrane of G protein-coupled receptors play an important role in mediating agonist-stimulated coupling to G-proteins (Fraser et al., 1989). Mutations analogous to aspartic acid 69 of the pm2 mAchR were made in m1 mAchR (Fraser et al., 1989; Lameh et al., 1992), in β_2 adrenergic (Strader et al., 1987), α 2 adrenergic (Ceresa and Limbird, 1994; Wang et al., 1991), serotonin (Ho et al., 1992; Wang et al., 1993), dopamine D2 (Neve et al., 1991), lutropin (Ji and Ji, 1991), angiotensin II (Bihoreau et al., 1993), neurokinin-1 (Brodbeck et al., 1995), and endothelin A receptors (Rose et al., 1995). Antagonist binding was consistently unaffected by this mutation while the effect on agonist binding varied from a reduction

(Strader et al., 1987) to an increase in agonist affinity (Fraser et al., 1989; Wang et al., 1991). Coupling to effector systems was consistently abolished by this mutation, leading to the conclusion that this acidic residue is important for agonist-mediated activation of G-proteins.

Focus of this chapter

Receptor-effector coupling does not appear to be necessary for agonist-dependent internalization and sequestration. β_2 adrenergic receptor mutants unable to stimulate effector activation undergo agonist-promoted internalization (Campbell et al., 1991; Goldman et al., 1996; Mahan et al., 1985). Contrary to these results, when the second transmembrane aspartic acid of the m1 mAchR was mutated to asparagine (D71N) and expressed in a transient transfection system, both agonist-mediated coupling and internalization were abolished (Lameh et al., 1992).

We have mutated the conserved aspartic acid to asparagine (D69N) in the second transmembrane domain of the pm2 mAchR to evaluate the role of full and partial agonists in receptor internalization and effector coupling. Coupling to inhibition of adenylyl cyclase and stimulation of PI hydrolysis is abolished by this mutation for all agonists except oxo M, which elicits maximal responses for both effector systems with about half the efficacy and a lower potency than wild-type receptor. This mutant offers a unique opportunity to evaluate the effect of agonists that promote activation of G-proteins as well as those that do not, on receptor internalization and desensitization. Internalization of D69N is observed in response to treatment with full agonists carbachol, acetylcholine and

oxo M as well as the partial agonist pilocarpine even though receptor-effector coupling is only stimulated by oxo M. Desensitization of oxo M mediated stimulation of PI hydrolysis and inhibition of forskolin-stimulated adenylyl cyclase activity was observed for all full agonists, but not the partial agonist pilocarpine. Treatment of cells expressing wild-type or D69N mAchRs with PTX does not prevent carbachol-mediated internalization of receptor. In addition, receptor internalization is not mediated by increases in Ca2+ or cAMP, although elevated cGMP may play a role. These results confirm that effector activation is not required for receptor internalization, and that desensitization and internalization are mediated by separate mechanisms. In addition, oxo M appears to promote a unique conformation in D69N pm2 mAchR capable of activating G-proteins and effector systems.

Materials and Methods

Abbreviations Used

mAchR, muscarinic acetylcholine receptor; pm2, porcine m2 subtype of the muscarinic acetylcholine receptor; CHO, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle's medium plus F12 nutrient mixture; G-protein, Guanine nucleotide-binding protein; GPCR, G-protein coupled receptors; Gpp(NH)p, guanyl imidodiphosphate; IP1, inositol monophosphate; IBMX, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PI, phosphatidylinositol; PTX, pertussis toxin; oxo M, oxotremorine M; *l*-QNB, R-(-)-quinuclidinylbenzilate; NMS, N-methyl scopolamine; and SNP, Sodium nitroprusside.

Materials

Radioactive ligands ([^{3}H] *l*-QNB, 52.3 Ci/mmol; and [^{3}H]NMS, 84 Ci/mmol) were purchased from New England Nuclear. Radioactive compounds for second messenger assays were purchased from New England Nuclear (*myo*-[^{3}H] inositol, 24.4 Ci/mmol; [^{3}H]adenine, 25.8 Ci/mmol; and [^{14}C]cAMP, 52.3 Ci/mmol) or American Radiochemical (*myo*-[^{3}H]inositol, 20 Ci/mmol; and [^{3}H]adenine, 15 Ci/mmol). Carbachol was purchased from Aldrich Chemical Company, pertussis toxin from List Biological Laboratories, forskolin, IBMX, *l*-QNB, NMS, oxo M, acetylcholine and pilocarpine were

from Research Biochemicals International, and dibutyryl-cAMP, 8-bromo-cGMP and thapsigargin from BIOMOL Research Laboratories. All other reagents were either from GIBCO-Bethesda Research Laboratories (cell culture media and supplements) or Sigma. Restriction enzymes and T4 DNA polymerase were from Promega, GIBCO-Bethesda Research Laboratories or New England Biolabs. The mammalian expression vector (pSVE) and the clone for the wild-type pm2 mAchR were gifts from Genentech, Inc. The dhfr$^-$ CHO cells were obtained from America Type Culture Collection.

Cloning and mutagenesis

Cloning and expression of mutant and wild-type mAchR-pSVE constructs are described elsewhere (Bulseco and Schimerlik, 1996; Vogel et al., 1995)*. The site-directed mutant, D69N was constructed using the oligonucleotide, 5'-GATGAGGTTAGCACAGGC-3', confirmed by dideoxy DNA sequencing (Sanger et al., 1977), and the construct stably transfected in dhfr- CHO cells by calcium phosphate precipitation. Wild-type receptor was overexpressed in CHO cells after removing 75 bases from the 5' untranslated region and replacing the weak 5' upstream Kozak sequence (CAAA) with a 'strong' Kozak sequence (CACC) (Kozak, 1987)*, to obtain receptor expression levels equivalent to those seen in the D69N mutant.

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* Bulseco, D.A. and M.I. Schimerlik, in preparation.

Receptor expression

Receptor expression was assessed in whole cells on 24-well tissue culture dishes by the addition of 10 nM [^{3}H] *l*-QNB in the presence or absence of saturating concentrations of unlabeled antagonists, NMS or *l*-QNB in DMEM. Cells were incubated for 1.5 to 2 hours, then washed twice with ice cold phosphate buffered saline (pH 7.4), solubilized with 0.5 ml 1% Triton X-100 in PBS, and counted for tritium. Surface receptor sites were determined by competition of [^{3}H] *l*-QNB with the unlabeled hydrophilic antagonist, NMS while total receptor was determined in the presence of unlabeled hydrophobic antagonist, *l*-QNB. Determination of surface receptor sites using this approach gave results within 10% of [^{3}H]NMS binding.

Receptor internalization assays were conducted after treating cells for various times in FD supplemented with 10% calf serum (Sigma). Time course of internalization was studied by incubating cells with 1 mM agonist for various times. Assays for desensitization of second messenger responses were determined after cells were incubated with 1 mM agonist for 3 to 4 hours while other treatments were for 1 (1 mM sodium nitroprusside) or 2 hours (1 μM and 1 mM dibutyryl-cAMP; 1 μM and 1 mM 8-bromo-cGMP; 100 μM thapsigargin).

Receptor ligand binding studies were performed on intact cells in 24-well dishes in DMEM. Direct binding of [^{3}H]NMS and agonist competition of [^{3}H]NMS binding for wild-type and D69N mAchR were characterized. For saturation binding isotherms, non-specific binding was determined in the presence of 100 μM *l*-hyoscyamine. Competition

binding experiments were conducted in the presence of 2.5 nM [^{3}H]NMS in the presence of unlabeled agonists (0.1 nM to 10 mM).

Assays for effector coupling and data analysis.

Assays for receptor-effector coupling are described elsewhere (Bulseco and Schimerlik, 1996). CHO cells expressing wild-type or mutant pm2 mAchRs were assayed in 24-well dishes for agonist-stimulated PI hydrolysis (Lee et al., 1990) or inhibition of forskolin-stimulated adenylyl cyclase (Salomon, 1979). Data were analyzed using nonlinear least-squares curve fitting in Origin (MicroCal Software) as described in (Bulseco and Schimerlik, 1996) for functional assays and (Vogel et al., 1995) for ligand binding experiments.

Data from effector coupling assays were fit to the logistic equation. Equation 1 was used for PI assays, with a third term added (Eq. 2) to account for a stimulatory phase observed in the cAMP assays (Bulseco and Schimerlik, 1996).

$$Y = \min + \frac{(\max - \min)}{1 + (EC50/[x])^{p}} \qquad \text{(Eq. 1)}$$

$$Y = \min + \frac{(\max 1 - \min)}{1 + ([x]/EC50i)^{p1}} + \frac{(\max 2 - \min)}{1 + (EC50s/[x])^{p2}} \qquad \text{(Eq. 2)}$$

In each of these equations [x] is the agonist concentration used; max or max1,2 and min are the maximum and minimum plateaus of the curves; and p or p1,2 the respective slope factors.

Agonist binding data were fit assuming three non-interacting classes of sites using the equation below (Vogel et al, 1995).

$$[RL] = \sum_{i=1}^{3} \frac{[Roi]([Lo]-[RL])}{Kd + (Kd/Ki)[I] + [Lo] - [RL]} \qquad \text{(Eq. 3)}$$

[Roi] is the total receptor concentration for each receptor site; [Lo], the total concentration of radiolabeled tracer used in the experiment with dissociation constant Kd; and [I], the total unlabeled competing agonist with dissociation constant Ki.

Receptor internalization kinetic experiments were fit to Equation 4. Parameters are y(t), the amount of surface receptor at time t; y0, the surface sites that remained after treatments for receptor internalization; A1, the amplitude, and *k*, the apparent rate constant for receptor internalization.

$$Y(t) = Y0 + A1\exp(-kt) \qquad \text{(Eq. 4)}$$

Results

Receptor expression levels

Wild-type and D69N mAchRs were expressed at high levels in the plasma membrane of CHO cells. Receptor densities of 2.0-4.0 x 10^6 receptors per cell were observed for both mutant and wild type, with 88-92% of the sites expressed at the cell surface. Modification of the 5' untranslated region of the wild-type receptor clone (pSVE-pm2 short cl.1) was necessary to increase receptor expression to levels observed with the D69N mutant mAchR. A wild-type clone without these 5' upstream changes maximally expressed 0.8-1.2 x 10^6 receptors per cell, although approximately 90% of these sites were localized to the plasma membrane (data not shown). All data for wild-type presented in this report refers to the high expression (pSVE-pm2 short cl.1) clone.

D69N mAchR mutant couples poorly to effectors

Coupling of D69N to inhibition of adenylyl cyclase (Figure 3.1) and stimulation of PI metabolism (Figure 3.3) is dependent on the agonist used. oxo M is the only agonist to elicit a measurable response for inhibition of adenylyl cyclase and stimulation of PI hydrolysis, although at reduced maximal responses and with lower potency. Carbachol (n=3), acetylcholine (n=3) and pilocarpine (n=1) did not elicit inhibitory responses while maximal inhibition of adenylyl cyclase was 41 ± 6 % for oxo M with an IC50 of 8.4 ± 0.5

μM (Table 3.1). This represents a 2 fold lower maximal response for oxo M and an 80 fold increase in IC50 when compared to wild-type receptor.

Table 3.1: D69N and wild-type pm2 mAchR coupling to inhibition of adenylyl cyclase and stimulation of PI hydrolysis.

Assays were conducted as described in "Materials and Methods", and analyzed as described in (Bulseco and Schimerlik, 1996). Values represent the mean ± SD of *n* experiments, each done in duplicate.

	AC Inhibition			*PI Hydrolysis*		
	IC50 (μM)	**Max Inhibition (%)**	**n**	**EC50 (μM)**	**Max Stimulation (Fold)**	**n**
Wild-type						
Carbachol	0.6 ± 0.3	61.5 ± 5.5	2	1.5 ± 0.3	1.6 ± 0.1	4
Acetylcholine	0.05 ± 0.01	61.5 ± 2.5	2	5.5 ± 2.0	1.9 ± 0.2	3
oxo M	0.10 ± 0.05	75 ± 3	2	5.1 ± 0.4	1.8 ± 0.2	2
Pilocarpine	7.9 ± 2.1	49.5 ± 3.5	2	5.6 ± 2.1	1.43 ± 0.09	3
D69N						
oxo-M	8.4 ± 0.5	41 ± 6	3	10.6 ± 1.1	1.46 ± .02	3

Data describing the coupling of wild-type pm2 mAchR to the inhibition of adenylyl cyclase and stimulation of PI hydrolysis are summarized in Figures 3.2, 3.4 and Table 3.1. Wild-type pm2 exhibits both inhibition and stimulation of adenylyl cyclase activity (Figure 3.2) for all agonists. The stimulation of adenylyl cyclase is reproducible, but with poorly characterized EC50s. A range of 15 μM to 2 mM was observed for carbachol, acetylcholine, oxo M and pilocarpine. For clarity, these parameters have been omitted from Table 3.1. Wild-type IC50 for oxo M is 0.10 ± 0.05 μM with a maximal inhibition of 75 ± 3 % at receptor densities similar to the D69N mutant.

Oxo M was the only agonist to elicit a stimulation of PI hydrolysis in CHO cells expressing the D69N mutant, with an EC50 of 10.6 ± 1.1 μM and maximum fold stimulation of 1.46 ± 0.02. These values are only slightly different from wild-type receptor; 5.1 ± 0.4 μM, 1.8 ± 0.2 maximum fold stimulation.

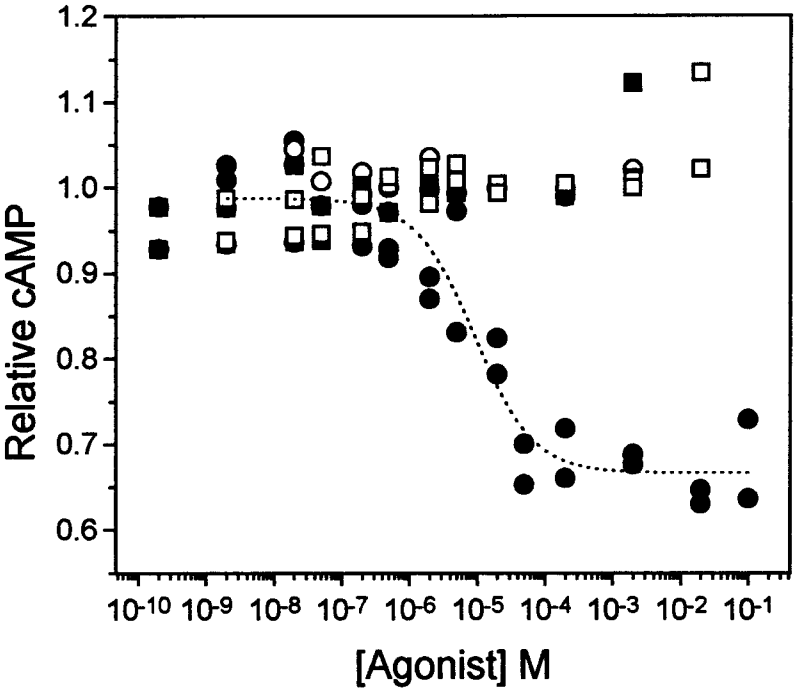

Figure 3.1: Inhibition of forskolin-stimulated cAMP formation for D69N.

Data are presented as cAMP generated relative to forskolin stimulated levels and are from representative experiments. Curves were derived from a least-squares fit as described in Bulseco and Schimerlik, (1996). Average values are summarized in Table 3.1. The fitted parameters for the D69N are IC50 = 9.0 ± 3.3 μM, 32.5 ± 1.7 % inhibition for oxo M (●), with slope fixed at 1. Data for carbachol (○), acetylcholine (■) and pilocarpine (□) were fit, but parameters are undetermined due to small measurable responses.

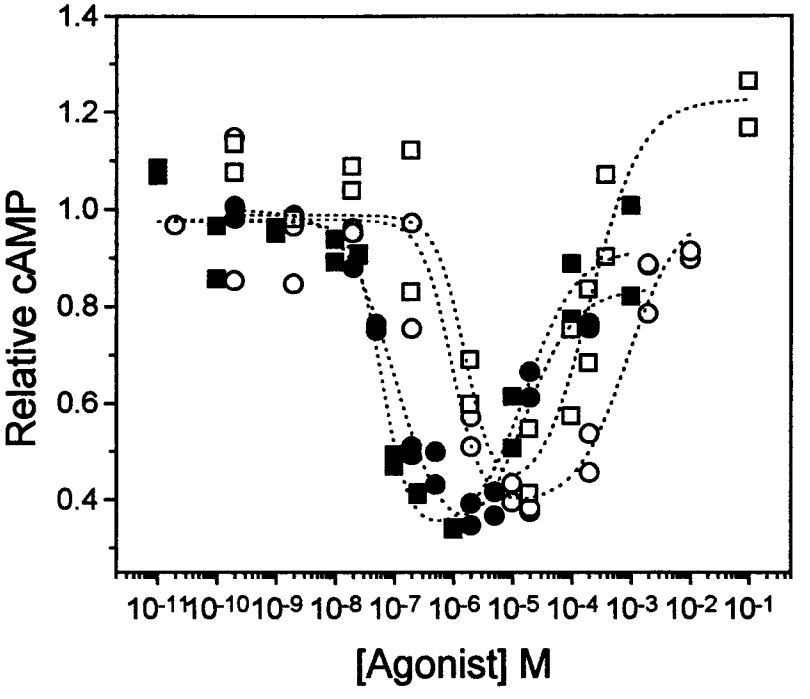

Figure 3.2: Inhibition of forskolin-stimulated cAMP formation for wild-type mAchR.

Data are presented as cAMP generated relative to forskolin stimulated levels and are from representative experiments. Curves were derived from a least-squares fit as described in Bulseco and Schimerlik, (1996). Fitted parameters for wild-type are IC50 = 0.11 ± 0.02 μM, maximal inhibition 73.2 ± 5.9 %, IC50 = 0.8 ± 0.5 μM, maximal inhibition 65.8 ± 7.8 %, IC50 = 0.06 ± 0.01 μM, maximal inhibition 64.6 ± 5.9 %, and IC50 = 1.6 ± 0.9 μM, maximal inhibition 59.8 ± 17.1 % for oxo M (●), carbachol (○), acetylcholine (■) and pilocarpine (□) respectively

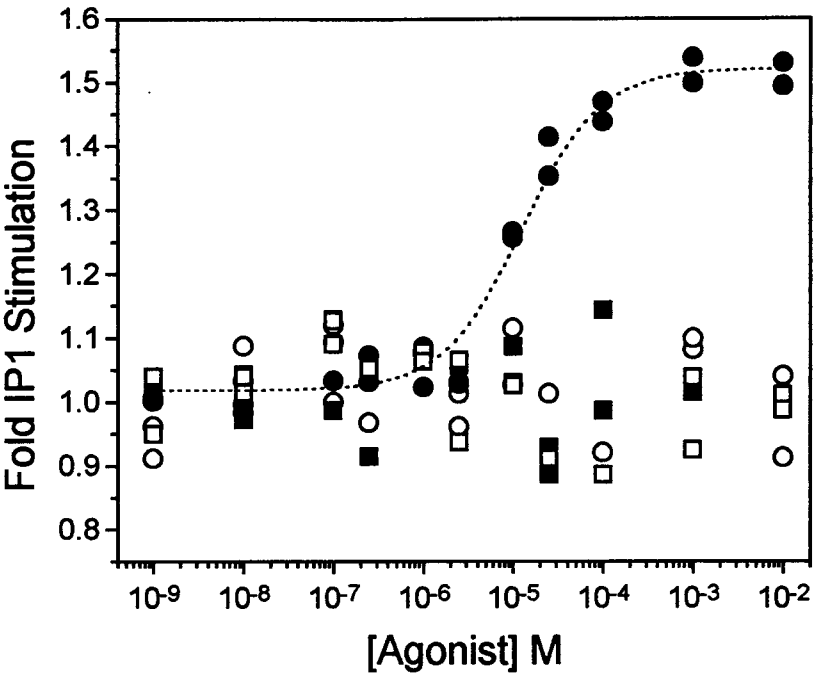

Figure 3.3: Stimulation of PI hydrolysis for D69N mutant mAchR.

The data are presented as fold stimulation of PI hydrolysis over basal levels of inositol monophosphate. Curves were derived from a least squares fit. Fitted parameters for D69N are EC50 = 12.4 ± 2.3 μM, maximal stimulation 1.50 ± 0.01 with slope factor fixed at 1 for oxo M (●). Response was too low to obtain reliable fitted values for carbachol (○), acetylcholine (■) and pilocarpine (□).

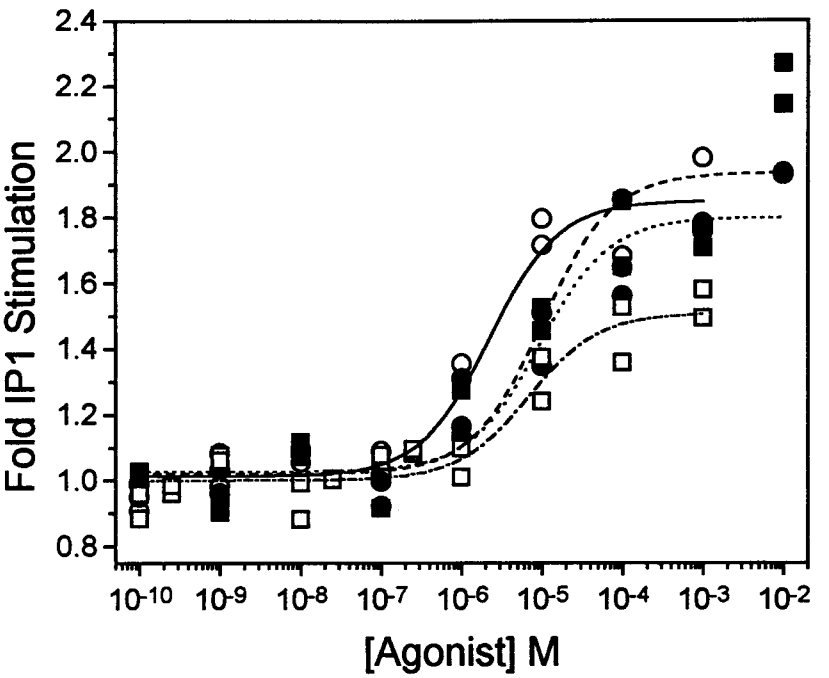

Figure 3.4: Stimulation of PI hydrolysis for wild-type mAchR.

The data are presented as fold stimulation of PI hydrolysis over basal levels of inositol monophosphate. Curves were derived from a least squares fit to a logistic equation. Fitted parameters for wild type; EC50 9.2 ± 3.8 μM, 2.2 ± 0.8 μM, 9.9 ± 5.0 μM and 6.7 ± 3.0 μM for oxo M (●, dotted line), carbachol (○, solid line), acetylcholine (■, dashed line) and pilocarpine (□, dashed-dotted line), with maximal fold stimulation of 1.75 ± 0.03, 1.83 ± 0.02, 1.90 ± 0.03, and 1.51 ± 0.02 fold respectively

Ligand binding properties of D69N are similar to wild type

Ligand binding properties of D69N were similar to wild-type pm2 AchR for all agonists except oxo M (Table 3.2). Direct binding studies of the antagonist, [^{3}H]NMS, gave dissociation constants of 0.39 ± 0.1 nM (n=10) for D69N and 0.2 ± 0.1 nM (n=6) for wild-type receptor. Agonist binding data in whole cells for D69N and wild-type pm2 mAchR for oxo M, carbachol and acetylcholine were consistent with fits to two independent classes of sites (K_H and K_L) while pilocarpine was best fit by assuming a single class of sites (K_H). The superhigh affinity state (K_{SH}) observed in membrane preparations of wild-type pm2 mAchR is not detectable in assays using intact cells, possibly due to high concentrations of endogenous guanine-nucleotides or the high expression levels achieved in these CHO cell clones. Carbachol and acetylcholine exhibited affinities similar to wild-type receptor while oxo M exhibited a 25 fold reduction in affinity. Pilocarpine bound D69N with 3 fold higher affinity than wild-type receptor, but was still best fit to a single site (Table 3.2).

Table 3.2: D69N and wild-type pm2 mAchR ligand binding properties.

Whole cell agonist competition experiments were done as described in "Materials and Methods". Data were analyzed as described in (Vogel et al., 1995) using nonlinear least-squares curve fitting. Values represent the mean ± SD of *n* experiments.

	Wild type			*D69N*		
	K_H (μM)	K_L (μM)	n	K_H (μM)	K_L (μM)	n
Carbachol[a]	1.7 ± 0.8	37.6 ± 10.3	5	2.0 ± 0.7	47.5 ± 0.3	6
Acetylcholine	0.4 ± 0.2	87.4 ± 6.7	4	0.4 ± 0.1	23.5 ± 4.0	4
oxo M	0.4 ± 0.2	43.5 ± 16.5	4	10.6 ± 3.0	254.2 ± 79.7	5
Pilocarpine	17.4 ± 9.0	[b]	3	26.6 ± 10.1	[b]	4

[a] % receptor sites in the high affinity state (K_H) is 25, 26, 33, and 100% for carbachol, acetylcholine, oxo M and pilocarpine respectively for wild-type mAchR and 31, 22, 67 and 100% for D69N.
[b] Pilocarpine is best fit to a single class of binding site.

Desensitization of oxo M-activated coupling

Desensitization of oxo M-activated coupling to inhibition of adenylyl cyclase (Figure 3.5) and stimulation of PI hydrolysis (Figure 3.7) in D69N was observed for carbachol and oxo M but not pilocarpine. Coupling properties of pm2 mAchR are dependent on receptor density (Vogel et al., 1995; Whaley et al., 1995; Whaley et al., 1994). Desensitization is therefore measured as a rightward shift in IC50 for inhibition of adenylyl cyclase activity as well as reduction in maximal inhibition, and as a reduction in maximal stimulation of PI hydrolysis with no change in EC50. Pretreating cells with acetylcholine led to inconsistent results (data not shown), presumably due to agonist degradation during the incubation period. The fitted parameters for D69N were IC50 = 3.5 ± 1.0 μM, 36.4 ± 4.1 % maximal inhibition for untreated cells, IC50 = 66.7 ± 49.0 μM, 28.2 ± 4.2 % maximal inhibition, IC50 = 71.3 ± 32.5 μM, 28.0 ± 2.1 % maximal inhibition, and IC50 = 3.0 ± 0.9 μM, 32.8 ± 2.1 % maximal inhibition for cells treated with carbachol, oxo M and pilocarpine respectively. Desensitization to PI metabolism resulted in loss of measurable response for cells treated with carbachol and oxo M, while treatment with pilocarpine resulted in a response similar to untreated cells. The fitted parameters for D69N were EC50 = 11.3 ± 3.4 μM, 1.51 ± 0.01 fold maximal stimulation, and EC50 = 4.5 ± 1.6 μM, 1.65 ± 0.02 fold maximal stimulation for control and pilocarpine treated cells respectively.

Similar results were observed for wild-type pm2 (Figures 3.6, 3.8) after treatment with various agonists. Pretreatment with both carbachol and oxo M led to desensitization of carbachol-mediated responses, but preincubation with pilocarpine did not. Fitted parameters for wild-type were IC50 = 0.38 ± 0.13 μM, maximal inhibition 72.2 ± 3.6 %, IC50 = 2.5 ± 0.2 μM, maximal inhibition 33.7 ± 4.1 %, IC50 = 3.1 ± 1.8 μM, maximal inhibition 39.9 ± 3.3 %, and IC50 = 2.7 ± 0.8 μM, maximal inhibition 53.6 ± 18.5 % for control, carbachol, oxo M and pilocarpine treated cells respectively. Carbachol and oxo M abolished measurable coupling to PI metabolism, while pilocarpine resulted in a response similar to untreated cells. Fitted parameters for wild-type are EC50 = 1.7 ± 1.2 μM, and 0.5 ± 0.2 μM, for control and pilocarpine treated cells, with maximal fold stimulation of 1.50 ± 0.04, and 1.63 ± 0.02 fold respectively. Although receptor desensitization was always accompanied by receptor internalization, a reduction in surface receptor density was not sufficient to lead to desensitization.

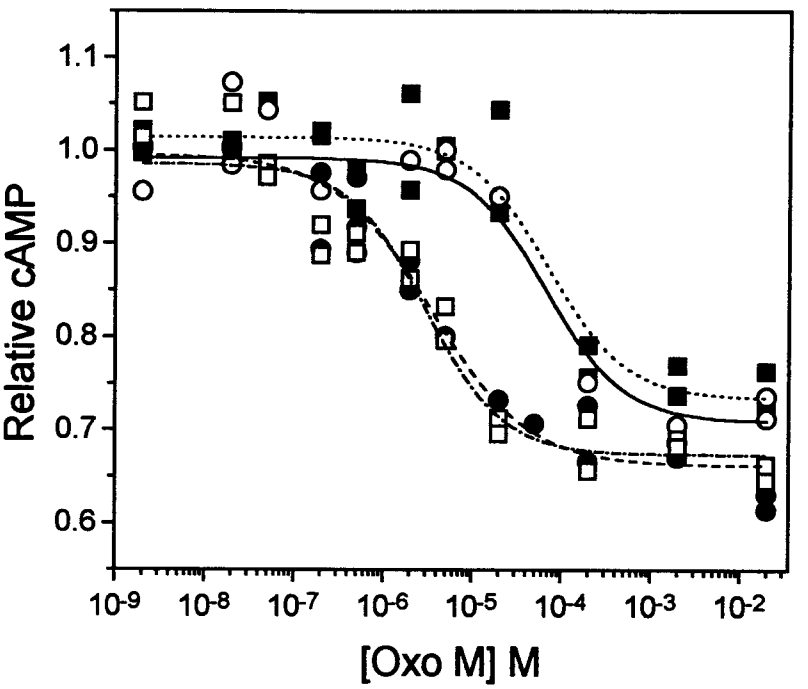

Figure 3.5: Desensitization of forskolin-stimulated inhibition of cAMP formation for D69N.

Data are presented as cAMP generated relative to forskolin-stimulated levels and are from representative experiments. Cells were pretreated with the agonists indicated (1 mM) for 4 hours, followed by second messenger assays with oxo M for D69N. Curves were derived from a least-squares fit as described in (Bulseco and Schimerlik, 1996). The fitted parameters for the D69N are IC50 = 3.5 ± 1.0 μM, 36.4 ± 4.1 % inhibition, slope 0.8 for control (●, dashed line), IC50 = 66.7 ± 49.0 μM, 28.2 ± 4.2 % inhibition for carbachol (○, solid line), IC50 = 71.3 ± 32.5 μM, 28.0 ± 2.1 % inhibition for oxo M (■, dotted line) and IC50 = 3.0 ± 0.9 μM, 32.8 ± 2.1 % inhibition for pilocarpine (□, dashed-dotted line) treated cells respectively (slope factors fixed at 1).

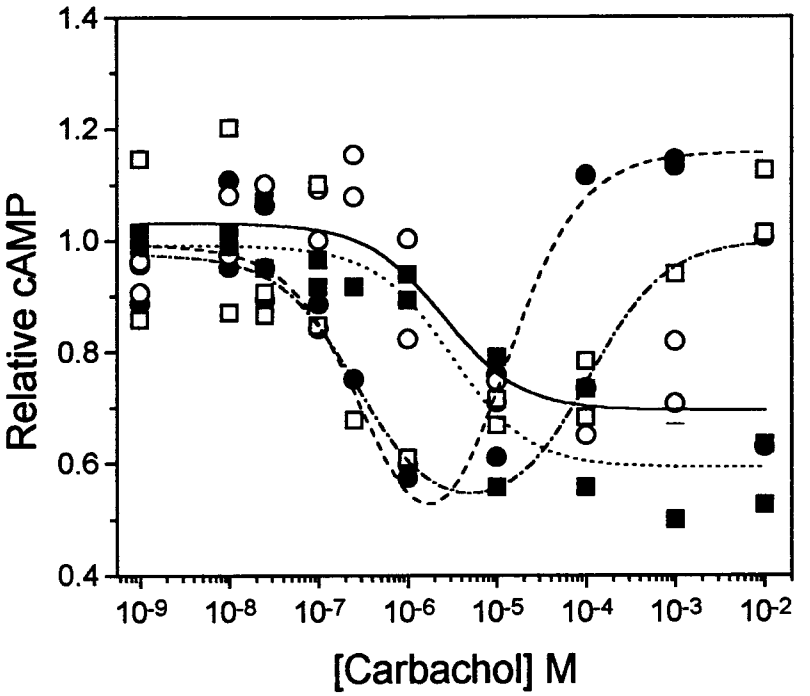

Figure 3.6: Desensitization of forskolin-stimulated inhibition of adenylyl cyclase for wild-type mAchR.

Data are presented as cAMP generated relative to forskolin-stimulated levels and are from representative experiments. Cells were pretreated with the agonists indicated (1 mM) for 4 hours, followed by second messenger assays with carbachol for wild type. Curves were derived from a least-squares fit as described in (Bulseco and Schimerlik, 1996). Fitted parameters for wild-type are IC50 = 0.38 ± 0.13 μM, maximal inhibition 72.2 ± 3.6 %, IC50 = 2.5 ± 0.2 μM, maximal inhibition 33.7 ± 4.1 %, IC50 = 3.1 ± 1.8 μM, maximal inhibition 39.9 ± 3.3 %, and IC50 = 2.7 ± 0.8 μM, maximal inhibition 53.6 ± 18.5 % for control (●, dashed line), carbachol (○, solid line), oxo M (■, dotted line) and pilocarpine (□, dashed-dotted line) treated cells respectively. Slope factors were fixed at 1.

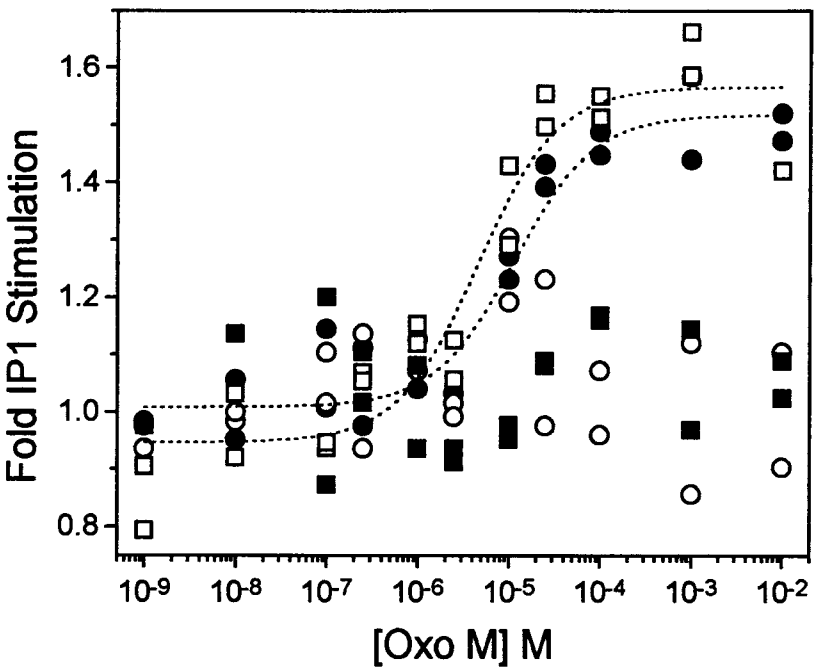

Figure 3.7: Desensitization of PI Hydrolysis for D69N mutant AchR.

The data are presented as fold stimulation of PI hydrolysis over basal levels of inositol monophosphate for control cells or after pretreatment of cells as described above. Curves were derived from a least squares fit. Fitted parameters for D69N are EC50 = 11.3 ± 3.4 μM, 1.51 ± 0.01 fold, and EC50 = 4.5 ± 1.6 μM, 1.65 ± 0.02 fold for control (●) and pilocarpine (□) treated cells. Treatment with carbachol (○) and oxo M (■) resulted in small responses and poorly determined fits.

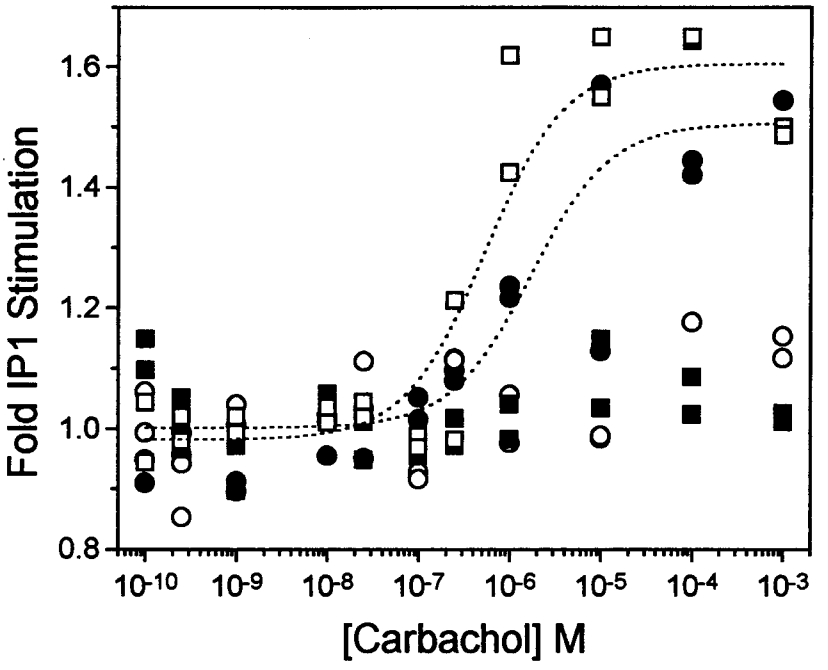

Figure 3.8: Desensitization of PI Hydrolysis by wild-type mAchR.

The data are presented as fold stimulation of PI hydrolysis over basal levels of inositol monophosphate for control cells or after pretreatment of cells as described above. Curves were derived from a least squares fit. Fitted parameters for wild-type are EC50 = 1.7 ± 1.2 μM, and 0.5 ± 0.2 μM, for control (●) and pilocarpine (□) treated cells, with maximal fold stimulation of 1.50 ± 0.04, and 1.63 ± 0.02 fold respectively. Treatment with carbachol (○) and oxo M (■) resulted in small responses and poorly determined fits.

Internalization of D69N does not require coupling

The time course of receptor internalization was similar for all agonists. The partial agonist, pilocarpine as well as full agonists carbachol, acetylcholine and oxo M, induced internalization of wild-type and D69N mAchR surface sites (Figures 3.9, 3.10). Approximately 50% of the surface sites were internalized within 30-60 minutes. Total cellular receptor was reduced maximally to 40-50%, with no further reduction after agonist treatment for up to 24 hours (data not shown). The apparent rate constants obtained for carbachol, oxo M, acetylcholine and pilocarpine were 0.04 ± 0.02, 0.05 ± 0.03, 0.06 ± 0.03 and 0.1 ± 0.08 % receptors/cell/min respectively for wild-type receptor, and 0.10 ± 0.03, 0.03 ± 0.02, 0.01 ± 0.01 and 0.11 ± 0.13 % receptors/cell/min respectively for D69N. Agonist treatment for 2 hours reduced surface receptor numbers by 40 to 70% for wild-type receptor, and by 50 to 60% for D69N. Although internalization of cell surface sites was promoted by pilocarpine, desensitization of oxo M-activated stimulation of PI hydrolysis and inhibition of forskolin-stimulated adenylyl cyclase activity was only observed after treatment with full agonists (Figures 3.5, 3.7).

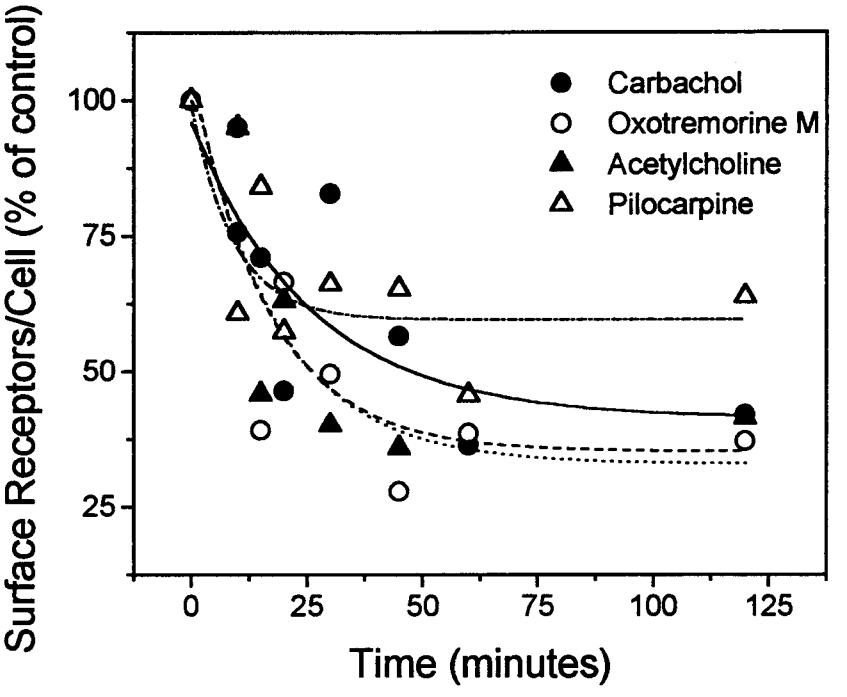

Figure 3.9: Agonist-promoted receptor internalization rates for wild-type mAchR.

CHO cells expressing wild-type pm2 mAchR were treated with 1 mM carbachol, oxo M, acetylcholine or pilocarpine in FD media supplemented with 10% calf serum for the times indicated. Fitted lines were obtained by fitting the data with equation 4, and resulted in an apparent rate estimate, *k* (in % receptor/cell/minute) an amplitude, A1, and the surface sites that remained after treatments, Y0. Rates obtained were 0.04 ± 0.02, 0.05 ± 0.03, 0.06 ± 0.03 and 0.1 ± 0.08 % receptors/cell/min for carbachol (solid line), oxo M (dotted line), acetylcholine (dashed line) and pilocarpine (dashed-dotted line) respectively, with amplitudes of 54.6 ± 17.7, 71.0 ± 18.4, 69.7 ± 18.6 and 39.1 ± 13.6. The sites that remained after agonist treatment were 41.3 ± 14.4, 32.9 ± 12.3, 35.2 ± 13.1, and 59.6 ± 6.7 % receptors/cell for carbachol, oxo M, acetylcholine and pilocarpine respectively.

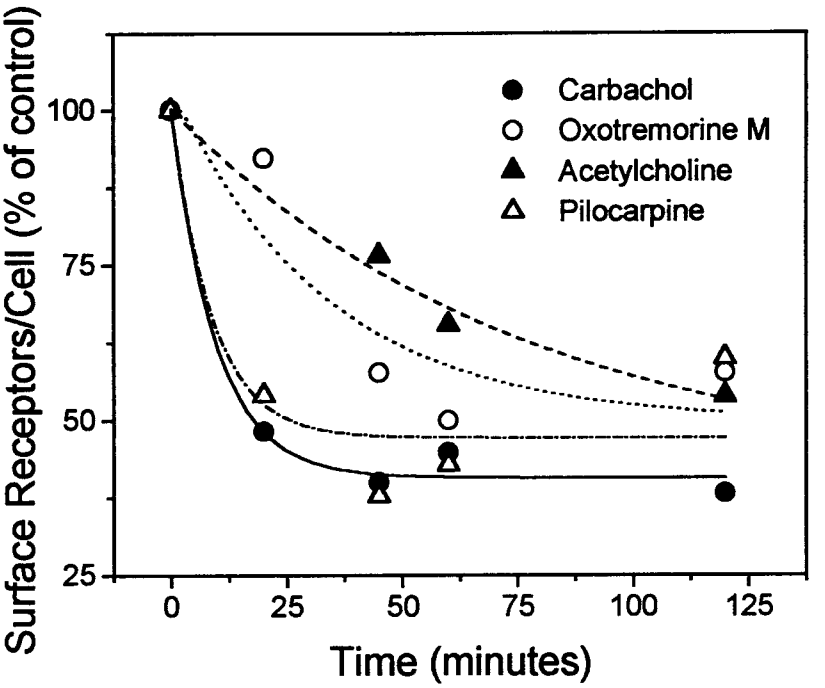

Figure 3.10: Agonist-promoted receptor internalization rates for D69N mAchR.

CHO cells expressing D69N mAchR were treated with 1 mM carbachol, oxo M, acetylcholine or pilocarpine in FD media supplemented with 10% calf serum for the times indicated. Fitted lines were obtained by fitting the data with equation 4, and resulted in an apparent rate estimate, *k* (in % receptor/cell/minute) an amplitude, A1, and the surface sites that remained after treatments, Y0. Rates obtained were 0.10 ± 0.03, 0.03 ± 0.02, 0.01 ± 0.01 and 0.11 ± 0.13 % receptors/cell/min for carbachol (solid line), oxo M (dotted line), acetylcholine (dashed line) and pilocarpine (dashed-dotted line) respectively, with amplitudes of 59.3 ± 4.0, 54.3 ± 18.7, 59.3 ± 14.2 and 52.9 ± 13.8. The sites that remained after agonist treatment were 40.7 ± 2.1, 49.8 ± 15.9, 41.0 ± 14.7, and 47.2 ± 7.2 % receptors/cell for carbachol, oxo M, acetylcholine and pilocarpine respectively.

Cells expressing wild-type or mutant receptor were treated with 100 ng/ml PTX for 12 hours prior to assays for receptor internalization. This concentration is sufficient to completely abolish receptor-effector coupling (Bulseco and Schimerlik, 1996), but did not prevent carbachol mediated internalization of surface receptor sites (Table 3.3).

cGMP induces receptor internalization while Ca2+ and cAMP do not

The effects of other second messengers were studied to determine the role of receptor-effector coupling in receptor internalization. Release of internal Ca2+ was stimulated with 1 mM thapsigargin. The cyclic nucleotide analog dibutyryl-cAMP was used at concentrations of 1μM and 1 mM. None of these treatments significantly affected cell surface pm2 mAchR expression. Thapsigargin, 1μM and 1 mM dibutyryl-cAMP resulted in 105.8 ± 14.5, 86.8 ± 3.3 and 88.9 ± 2.1% of control receptor surface sites respectively. Treating cells with 50 μM forskolin, a concentration sufficient to elevate cAMP levels in CHO cells had no effect on receptor expression levels (Table 3.3).

Sodium nitroprusside (SNP) induces internalization of m1 AchR expressed in CHO cells (Maggio et al., 1995), presumably via a nitric oxide mediated increase in cGMP levels. Treatment with 1 mM SNP, as well as the cGMP analog 8-bromo-cGMP resulted in a significant reduction of cell surface mAchR sites. Although 1 μM 8-bromo-cGMP did not significantly reduce surface receptor sites (83.6 ± 9.4%; $p = 0.27$, Student's t-test), 1 mM 8-bromo-cGMP and sodium nitroprusside did significantly ($p \leq 0.01$, Student's t-test) reduce expression of surface receptor sites to 73 ± 0.9% and 51.3 ± 0.06% respectively.

Results for D69N were similar to those obtained for wild-type pm2 mAchR for sodium nitroprusside (59.1 ± 0.5%, n=4) and 1 mM cGMP (70 ± 1.1%, n=2) treatments. Membranes prepared from CHO cells expressing wild-type pm2 AchR were treated with 1 mM SNP for 1 hour, washed, then resuspended in binding buffer followed by receptor binding assays with 10 nM antagonist [3H] *l*-QNB. This treatment had no affect on total receptor sites. Specific activities were 55.7 and 52.9 pmol/mg protein for control and SNP-treated membranes respectively, indicating that SNP requires a functioning cell to have its affect, and cannot directly reduce m2 mAchR binding sites.

Loss of surface receptor sites did not always coincide with desensitization. Figures 3.11 and 3.12 show that treatments which resulted in loss of receptor sites did not affect coupling to inhibition of adenylyl cyclase (Figure 3.11) or stimulation of PI metabolism (3.12).

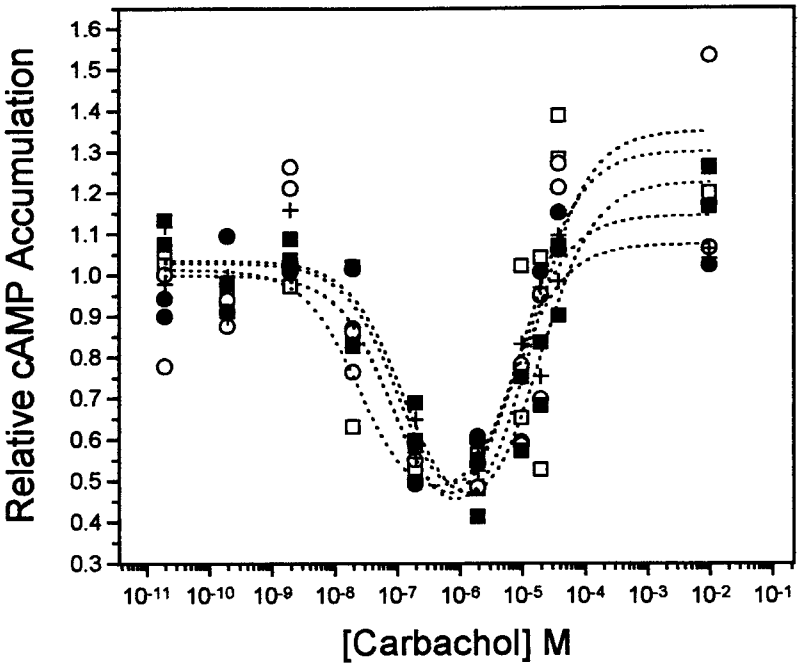

Figure 3.11: Effect of second messenger treatments on mAchR desensitization of forskolin-stimulated inhibition of adenylyl cyclase activity.

CHO cells expressing wild-type receptor were exposed to 1 mM 8-bromo-cGMP, 1 mM dibutyryl cAMP, 100 μM thapsigargin and 1 mM sodium nitroprusside and assays for accumulation of cAMP conducted as described in "Material and Methods". The lines through the data were obtained by least squares fit to equation 2. IC50s obtained for control (●), cAMP (○), cGMP (■), thapsigargin (□) and sodium nitroprusside (+) treated cells were 0.09± 0.07 μM, 0.1 ± 0.1 μM, 0.13 ± 0.07 μM, 0.02 ± 0.02 μM and 0.17 ± 0.09 μM respectively, with maximal inhibition of 68.3 ± 11.5%, 65.7 ± 11.6%, 65.9 ± 11.8%, 58.0 ± 12.0% and 70.2 ± 11.6%. EC50 for stimulation of adenylyl cyclase was 6.7 ± 4.1 μM, 14.7 ± 9.6 μM, 18.6 ± 7.3 μM, 11.7 ± 7.6 μM and 5.8 ± 3.9 μM for control, cAMP, cGMP, thapsigargin and sodium nitroprusside treated cells respectively.

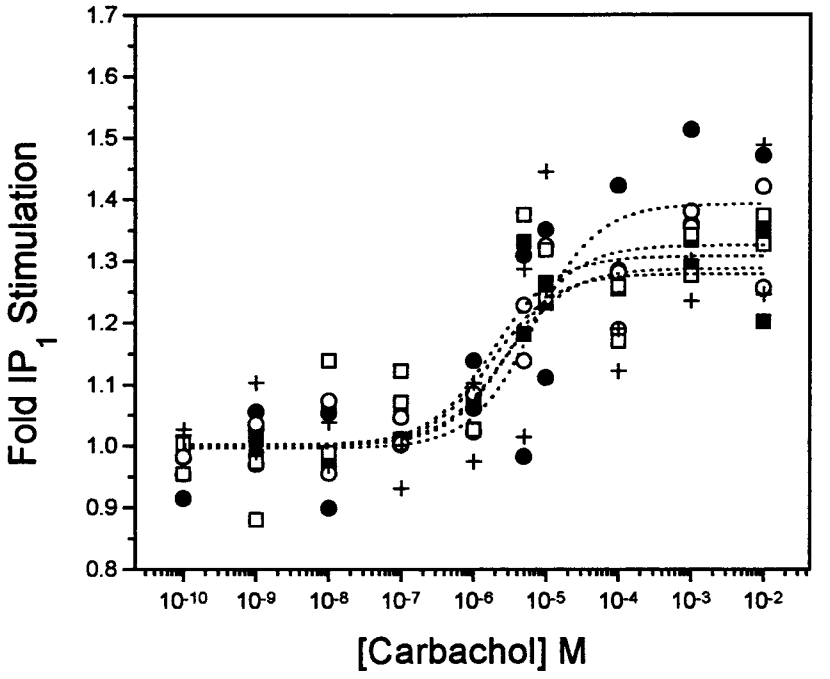

Figure 3.12: Effect of second messenger treatments on mAchR desensitization of PI hydrolysis.

CHO cells expressing wild-type receptor were exposed to 1 mM 8-bromo-cGMP, 1 mM dibutyryl cAMP, 100 μM thapsigargin and 1 mM sodium nitroprusside and PI metabolism assays conducted as described in "Material and Methods". The lines through the data were obtained by least squares fit to equation 1. EC50s obtained for control (●), cAMP (○), cGMP (■), thapsigargin (□) and sodium nitroprusside (+) treated cells were 6.7 ± 4.0 μM, 3.4 ± 1.7 μM, 1.8 ± 0.9 μM, 1.8 ± 1.4 μM and 2.8 ± 2.7 μM respectively, with maximal fold stimulation of 1.40 ± 0.06, 1.32 ± 0.04, 1.28 ± 0.04%, 1.30 ± 0.05 and 1.28 ± 0.07.

Table 3.3: Effect of other treatments on receptor internalization.

CHO cells expressing wild-type receptor were exposed to treatments as described in "Materials and Methods". Results are presented as the percent surface sites remaining (of control) after indicated treatments, and are mean ± SEM of two to seven experiments (n) each done in duplicate or triplicate.

Treatment	% Surface Binding Sites	n
Control	100	3
1 mM Carbachol	69.5 ± 1.0[b]	7
1 mM Pilocarpine	69.7 ± 5.6[b]	6
1 mM Oxotremorine M	72.3 ± 1.9[b]	2
1 μM dibutyryl-cAMP	86.8 ± 3.3	3
1 mM dibutyryl-cAMP	88.9 ± 2.1	3
1 μM 8-bromo-cGMP	83.6 ± 9.4	3
1 mM 8-bromo-cGMP	73.0 ± 0.9[b]	3
100 μM Thapsigargin	105.8 ± 14.5	3
50 μM Forskolin	97.1 ± 4.4	2
1 mM Sodium nitroprusside	51.3 ± 0.6[b]	3
100 ng/ml PTX	98.2 ± 2.1	2
100 ng/ml PTX + 1 mM carbachol	71.7 ± 1.7[b]	2

[a] Values represent the mean ± SEM of *n* determinations, each done in duplicate or triplicate.

[b] Significantly different from controls ($p \leq 0.01$, Student's t-test).

Discussion

Desensitization

Desensitization is broadly defined as the attenuation of agonist-mediated responses after prolonged agonist exposure. The mechanisms involved in receptor desensitization often include receptor phosphorylation by protein kinase A (PKA), protein kinase C (PKC) or one of a family of G-protein coupled receptor kinases (GRK) (reviewed in (Premont et al., 1995)). Rapid uncoupling of the β_2 adrenergic receptor from effectors is mediated by a member of the GRK family, βARK1 (β adrenergic receptor kinase 1 or GRK2), which rapidly phosphorylates receptors. Receptor phosphorylation is not sufficient to uncouple receptor from effector systems, and requires that β-arrestin binds the GRK2 phosphorylated receptor, before uncoupling from G-protein activation occurs (Lohse et al., 1992).

The pm2 mAchR is not rapidly uncoupled from effector systems when expressed in CHO cells. Cells must be treated for at least two hours with agonist before any appreciable change in receptor-effector coupling occurs. GRK2 can phosphorylate the m2 mAchR *in vivo*, and receptor internalization appears to depend on GRK2-mediated phosphorylation (Tsuga et al., 1994), but rapid uncoupling does not occur. Data for the β_2 adrenergic receptor is more complicated. It is clear that agonist-stimulated phosphorylation is important in β_2 adrenergic receptor sequestration and desensitization (Liggett et al., 1993), but β-arrestin is also important. Recent evidence indicates that

GRK2 phosphorylation may have a larger role than initially envisioned. Ferguson et al (1995) have recently reported that GRK2 phosphorylation facilitates β_2 adrenergic receptor sequestration and that binding of β-arrestin to GRK2-phosphorylated receptor mediates both agonist-promoted receptor internalization (Ferguson et al., 1996) and rapid uncoupling of receptor from G-proteins. pm2 mAchR expressed in CHO cells may not rapidly uncouple due to the lack of GRK or β-arrestin like proteins that would mediate rapid uncoupling of the pm2 AchR. Alternatively, these proteins may be expressed at levels too low to be effective when mAchR is overexpressed. Since coupling to inhibition of adenylyl cyclase is receptor density dependent (Vogel et al., 1995), relatively small changes in receptor-effector coupling may be masked by high receptor expression.

Internalization

The pm2 mAchR is rapidly internalized when cells are exposed to agonist. Cell surface receptor sites of both wild-type and D69N mutant mAchR are reduced to about 50% of control cells when exposed to agonist for 30 to 60 minutes. Recent evidence indicates that different mechanisms mediate internalization and desensitization (Ng et al., 1995; Pals Rylaarsdam et al., 1995). In addition, steps involved in receptor internalization may be separated into an agonist-promoted localization of receptors and an agonist-independent endocytosis event (von Zastrow and Kobilka, 1994). Internalization of receptor will "uncouple" the receptor from both the signaling ligand as well as G-proteins located in the plasma membrane. Our results indicate that desensitization as induced by full agonists is always accompanied by a reduction in receptor sites, but receptor internalization does not necessarily indicate that desensitization will be observed.

Although some of the reduction in effector coupling can be attributed to receptor internalization, our results with pilocarpine, a partial agonist, as well as cGMP and SNP treatment, emphasizes that internalization alone cannot account for receptor desensitization.

A reduced rate of desensitization for partial agonists has been reported for m1 and m3 AchR expressed in CHO cells (Hu et al., 1991). Pilocarpine, a partial agonist, affects receptor internalization and recycling properties differently than the full agonist carbachol, for pm2 mAchR expressed in CHO cells†. While both full and partial agonists resulted in surface receptor internalization, desensitization was observed only for the full agonists. The unique receptor internalization and recycling properties induced by full and partial agonists may indicate that the precise pathway taken by these internalized receptors are agonist-dependent and may impact desensitization properties.

Second messenger analogs

Other possible mechanisms of receptor internalization and desensitization were studied with the use of second messenger analogs. The permeable cyclic nucleotide analogs, dibutyryl- cAMP and 8-bromo-cGMP had different effects on cell surface expression of pm2 mAchR. 8-bromo-cGMP significantly reduced cell surface receptor expression of pm2 mAchR while dibutyryl-cAMP had no effect (Table 3.3). Coupling properties were not significantly affected by these analogs, although the slight increase in IC50 for inhibition of adenylyl cyclase and reduction in PI maximal response for 8-bromo-

† Bulseco, D.A. and M.I. Schimerlik, in preparation.

cGMP and sodium nitroprusside treatments are consistent with a reduction in surface receptor sites. These findings suggest that cAMP-dependent PKA is not involved in receptor internalization or desensitization of the pm2 mAchR expressed in CHO cells. Alternatively, the effect of phosphorylation may be masked by the high receptor expression achieved in these clonal cell lines.

cGMP may mediate receptor internalization but not desensitization. Sodium nitroprusside has been shown to promote receptor internalization of m1 AchR expressed in CHO cells (Maggio et al., 1995). Our results support these findings. Sodium nitroprusside (presumably via an increase in cGMP) and 8-bromo-cGMP reduce surface receptor sites to 51% and 73% of control cells respectively. Muscarinic receptor activation of guanylate cyclase may occur indirectly through activation of phospholipase C. Metabolism of phosphatidylinositol produces inositol monophosphates and diacylglycerol, which may subsequently activate phospholipase A2 resulting in the release of arachidonic acid or its lipoxygenase metabolites, which in turn can activate cGMP production (McKinney and Richelson, 1989). Although receptor sites are reduced, coupling to inhibition of adenylyl cyclase and stimulation of PI hydrolysis is not significantly affected by sodium nitroprusside or 8-bromo-cGMP (Figures 3.11 and 3.12). Since we did not characterize pm2 mAchR coupling to activation of guanylate cyclase in CHO cells, it is possible that this response is desensitized by these treatments while coupling to other effectors are not. These findings suggest that guanylate cyclase activation, or the action of cGMP phosphodiesterases are not involved in mediating

desensitization of pm2 mAchR to the effector systems studied, and that mechanisms mediating receptor internalization and desensitization are distinct.

Thapsigargin, which increases intracellular Ca2+ concentrations (Dell'Acqua et al., 1993) had no effect on expression of pm2 mAchR (Table 3.3) or the coupling properties of pm2 mAchR to second messenger responses (Figures 3.11 and 3.12). In addition, inclusion of EGTA-AM, a cell permeable chelater of divalent cations, did not prevent carbachol-induced loss of surface mAchR. Cells pre-treated with 100 μM EGTA-AM and untreated cells were exposed to 1 mM carbachol resulting in a reduction of cell surface receptor sites to 21.6 ± 2.3% and 37.0 ± 5.5% of control cells respectively. EGTA-AM alone reduced surface receptor sites to 68.1 ± 8.1% of untreated cells, indicating that the effects of this treatment may not be limited to chelation of internal Ca2+ or that chelation of Ca2+ results in loss of surface receptor sites. These findings suggest that Ca2+ is not directly involved in receptor internalization and desensitization. Since the pm2 mAchR couples to PI hydrolysis, it is assumed that intracellular Ca2+ concentration is elevated upon agonist stimulation, but further experiments are necessary to determine if this plays a role in receptor internalization and desensitization.

D69N Mutant

Coupling of the D69N mAchR mutant to effector systems is agonist dependent. oxo M is capable of activating both inhibition of adenylyl cyclase and stimulation of PI hydrolysis while the other agonists are not. Acetylcholine appears to elicit a slight stimulation of adenylyl cyclase, but the response is modest and difficult to characterize.

These results imply that different agonists promote different receptor conformations, which can differentially affect the interaction of receptor and G-protein. When the analogous mutation was made in the α2-adrenergic receptor (D79N), coupling to potassium channels was abolished while inhibition of adenylyl cyclase and Ca2+ currents were unaffected (Surprenant et al., 1992). This implies that agonists can promote distinct conformations of the receptor that differentially interact with G-proteins, or different G-protein heterotrimers consisting of different combinations of α and βγ subunits. Finally, we have previously shown that oxo M, and to a lesser extent acetylcholine, stimulates PI hydrolysis of the mAchR mutant A212E with reduced efficacy relative to carbachol, implying agonist specific properties of G-protein activation (Bulseco and Schimerlik, 1996).

Coupling is not necessary for internalization and desensitization

Receptor-effector coupling is not necessary to promote receptor internalization (Campbell et al., 1991; Mahan et al., 1985). When D69N was stably expressed in CHO cells, agonists that failed to elicit coupling to inhibition of adenylyl cyclase and stimulation of PI hydrolysis promoted pm2 mAchR internalization with a time course similar CHO cells expressing wild-type receptor at similar densities. This finding is supported by results obtained after treating cells with PTX. Cells were incubated with 100 ng/ml prior to experiments for receptor internalization. Although this concentration of PTX is sufficient to abolish agonist-induced effector activation (Bulseco and Schimerlik, 1996), it did not prevent carbachol stimulated internalization of surface receptor sites (Table 3.3).

Contrary to our results, Lameh et al (Lameh et al., 1992) reported that an analogous mutation in hm1 mAchR (D71N) resulted in complete loss of carbachol induced receptor internalization, although this finding may be explained by the expression system used to characterize the mutant receptor. Experiments with wild-type receptor were conducted on stably transfected human kidney cells, while mutant receptors were characterized after transient transfection of these cells (Lameh et al., 1992). Although both stable and transient transfections yielded cells expressing similar receptor levels (792 ± 347 and 676 ± 34 fmol/mg protein for wild-type and D71N respectively), the distribution of expression in the transiently transfected cell lines may play a role in determining mAchR internalization properties. While the average receptor levels are known in a transiently transfected system, the distribution of expression in these cells cannot be characterized. Our experiments conducted on D69N were in a stably transfected clonal cell line and our findings suggest that receptor density plays an important role in receptor internalization and recycling rates‡.

High affinity binding of oxo M to membranes prepared from CHO cells expressing D69N mAchR is sensitive to guanine nucleotides. When membranes were pre-incubated with 100 μM GppNHp, a small but detectable reduction in agonist high affinity binding was observed when [^{3}H]-NMS competition experiments were done with oxo M, but not with carbachol, acetylcholine or pilocarpine. Addition of GppNHp to membranes, also results in an increase in [^{3}H]-NMS binding sites, similar to that seen with wild-type pm2

‡ Bulseco, D.A. and M.I. Schimerlik, in preparation.

mAchR expressed at similar receptor levels. In addition, preliminary experiments with direct binding of [^{3}H]-oxo M also exposes a small but detectable fraction of high affinity sites that are sensitive to guanine nucleotides§. These findings indicate that the agonist bound D69N receptor/G-protein complex may be stabilized by oxo M but not the other agonists, in support of our findings that oxo M uniquely promotes coupling to inhibition of adenylyl cyclase and stimulation of PI hydrolysis. One alternative, explanation is that the D69N tightly associates with G-proteins and agonist-promoted activation and dissociation of G-protein and receptor does not occur. Since D69N is expressed at high receptor densities in CHO cells, and receptor is in large excess over available G-proteins, it is difficult to distinguish between these two possibilities.

Effect of partial agonists

One of the primary objectives of this study was to evaluate the effect of a partial agonist on receptor internalization and desensitization for wild-type mAchR and D69N, a mutant that couples poorly to effector systems. The use of a partial agonist can provide information on internalization and desensitization properties that relates agonist efficacy, agonist affinity and receptor-effector coupling. Surprisingly, the results for internalization and desensitization were the same for both wild-type and D69N. Carbachol, oxo M, acetylcholine and pilocarpine promoted receptor internalization while desensitization was only observed with full agonists. Although pilocarpine promoted receptor internalization with the same time course for wild-type and D69N, it did not lead to desensitization.

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§ Vogel, W.K., D.J. Broderick, V.A. Mosser, G.L. Peterson, and M.I. Schimerlik, in preparation.

Although it is possible that the time course of desensitization is slower for partial agonists (Hu et al., 1991), our results are similar when cells are pretreated with pilocarpine for 3 or 12 hours. In addition, pilocarpine appears to affect receptor internalization and recycling properties in a receptor density dependent fashion different from that of carbachol**. It is likely that agonist-dependent conformational changes of the liganded receptor affects the fate of that receptor. This suggestion is supported by observations that oxo M uniquely activates effector systems for the D69N mutant, and becomes a partial agonist for an i3 mutant of the pm2 AchR (Bulseco and Schimerlik, 1996).

Summary

These results support the idea that agonist occupancy is sufficient to promote receptor internalization. Desensitization appears to be mediated by a distinct mechanism from internalization and although we observe a reduction in coupling potency and efficacy after prolonged treatment with agonists, some but not all of the observed changes can simply be attributed to a reduction in surface receptor sites. Although the partial agonist pilocarpine effectively promotes receptor internalization, only the full agonists carbachol, acetylcholine and oxo M were able to desensitize second messenger responses. Results obtained with the D69N mutant AchR support the conclusion that these responses do not require receptor-effector coupling. These findings emphasize the importance of considering both the context in which these receptors are expressed (i.e. the cell line) as well as the receptor densities achieved in these overexpression systems. Partial agonists

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** Bulseco, D.A. and M.I. Schimerlik, in preparation

may be useful tools to help distinguish the molecular mechanisms of receptor internalization and desensitization of G-protein coupled receptors.

Chapter 4

Rate of m2 muscarinic receptor internalization and desensitization is receptor density and agonist dependent

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Abstract

The porcine m2 muscarinic acetylcholine receptor (mAchR) expressed in Chinese hamster ovary cells undergoes rapid internalization and recycling upon persistent treatment with agonists. The effects of full and partial agonists were dependent on receptor densities, as were the final estimated rates of internalization ($k3$) and recycling ($k2$). When a high expression clonal cell line, pSVE short pm2 cl.1, was treated with pilocarpine or carbachol, the receptor recycling rate ($k2$) was increased two and four fold respectively, while the rate of internalization ($k3$) was increased 14 to 20 fold. Treatment of a low expression clonal cell line, pSVE pm2 cl.5, with carbachol also resulted in increased recycling (four fold) and internalization (28 fold) rates. The partial agonist, pilocarpine, had a modest affect on these rates (about 2 fold). These agonist-dependent changes in receptor recycling and internalization rates did not depend on effector-coupling. A single point mutation in the putative second transmembrane domain of the pm2 AchR, D69N resulted in a receptor that does not couple to second messenger effector systems, yet internalized and recycled at rates similar to wild-type receptor expressed at similar densities. Agonist-promoted rates of receptor degradation ($k4$) were affected by this mutant though. Carbachol did not increase the rate of receptor degradation for the mutant D69N, but increased this rate nine and 27 fold for wild-type mAchR expressed at low and high receptor densities respectively. These findings suggest that receptor internalization and recycling properties are not dependent on effector coupling, but are dependent on both receptor expression levels and the agonist used.

Furthermore, receptor degradation appears to be dependent on agonist as well as receptor-effector coupling.

Introduction

Overview

The porcine m2 mAchR activates cellular effector systems by coupling through G-proteins. In Chinese hamster ovary cells, the pm2 mAchR couples to both inhibition of adenylyl cyclase and stimulation of PI metabolism through the PTX-sensitive Gi isotypes (Ashkenazi et al., 1987). Regulation of receptor desensitization is a common cellular response to prolonged exposure to agonists (Liggett, 1995). The m2 mAchR is phosphorylated (Haga et al., 1990; Haga et al., 1993; Richardson and Hosey, 1990; Richardson and Hosey, 1992; Tsuga et al., 1994), internalized and down-regulated in response to persistent agonist treatment. It has been suggested that the net effect of these processes are to effectively uncouple the receptor from second messenger effectors. In addition, receptor internalization and recycling may play an important role in resensitization of the m2 mAchR response (Giannini and Boulay, 1995; Hein et al., 1994; Hishinuma et al., 1993; Pippig et al., 1995; Yu et al., 1993), although the mechanisms underlying these observations are still uncharacterized.

Receptor internalization and desensitization

Agonist-promoted internalization of the mAchR has been shown to occur in a variety of cell types. The rates of receptor internalization and subsequent recycling appear to be cell line and receptor subtype (Koenig and Edwardson, 1996; Koenig and Edwardson, 1994) dependent. Rapid receptor turnover appears to occur within minutes of exposure to agonist, even if the net surface receptor numbers are unchanged (Koenig and Edwardson, 1994; von Zastrow and Kobilka, 1994).

It has been proposed that receptor internalization and recycling functions to resensitize receptors following prolonged exposure to agonists (Yu et al., 1993). Phosphatases may lead to resensitization by dephosphorylating receptors that are internalized (Maloteaux and Hermans, 1994; Roettger et al., 1995). Data suggests that internalized receptors are recycled to the plasma membrane with a time course consistent with the kinetics of dephosphorylation (Giannini and Boulay, 1995). When cells expressing β2 adrenergic receptors were pre-treated with sucrose or concanavalin A, which prevents receptor internalization and sequestration, resensitization was also blocked (Yu et al., 1993).

Receptor distribution is altered after agonist treatment. β2-adrenergic receptors are rapidly redistributed to pits in the plasma membrane after exposure to agonists, from which passive receptor endocytosis can occur (von Zastrow and Kobilka, 1994). Dual pathways for agonist-promoted receptor internalization appear to exist for the cholecystokinin receptor, a G-protein coupled receptor for a peptide ligand (Roettger et al., 1995). Exposure to agonists promotes receptor internalization by both clathrin-coated

and smooth endocytotic pits. Receptors endocytosed via coated pits appear destined for degradation while those internalized via smooth endocytotic vesicles remain near the plasma membrane and appear to be rapidly dephosphorylated and recycled (Roettger et al., 1995). To date, the role of agonist efficacy on regulation of receptor trafficking has not been well characterized. Furthermore, receptor density may determine initial receptor distribution in the plasma membrane, which may affect properties of agonist-promoted receptor trafficking.

Focus of this study

In this study, we have characterized the agonist-promoted rates of receptor internalization and recycling with full (carbachol) and partial (pilocarpine) agonists at two receptor densities. At receptor densities greater than 2.0e6 receptors per cell, both carbachol and pilocarpine promoted receptor internalization similarly, while lower receptor densities (~1.0e6 receptors per cell) resulted in differences in internalization properties for the full and partial agonist. In addition, agonist-promoted internalization of a mAchR point mutant (D69N) was characterized. This mutant is expressed at high receptor densities, couples poorly to inhibition of adenylyl cyclase and stimulation of PI hydrolysis with all agonists except oxo M, yet receptor internalization is similarly promoted by carbachol, pilocarpine, oxo M and acetylcholine (Chapter 3). Our results indicate that agonist-dependent m2 mAchR internalization is receptor density dependent, and does not require receptor-effector coupling.

Materials and Methods

Abbreviations

BCM, benzilylcholine mustard; CHO, Chinese hamster ovary cells; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; D-MEM, Dulbecco's modified essential media; DNA, deoxyribonucleic acid; EDTA, ethylene diamine tetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; dhfr, dihydrofolate reductase; FD, D-MEM + F12 Nutrient Mixture; GPCR, G-protein coupled receptor; G-protein, guanine nucleotide binding protein; HEPES, 4-(2-hydroxyethyl-)-1-piperazine ethane sulfonate; IP1, inositol monophosphates; IBMX, isobutylmethylxanthine; mAb, monoclonal antibody; mAchR, muscarinic acetylcholine receptor; MFD, modified D-MEM + F12, selective media low in glycine and thymine; NMS, N-methyl scopolamine; oxo M, oxotremorine-M; PEI, polyethyline imine; pm2 mAchR, porcine m2 mAchR; PMSF, phenylmethylsulfonyl fluoride; pSVE, pSV1 derived mammalian expression vector obtained from Genentech; PTX, pertussis toxin; *l* -QNB, *l* -quinuclidinyl benzilate; RG Complex, Receptor-G-protein complex; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; SNP, Sodium nitroprusside; TCA, trichloroacetic acid; Tris, Tris (hydroxymethyl) aminomethane.

Materials

Radioactive ligands ([^{3}H] *l* -QNB, 52.3 Ci/mmol; and [^{3}H]-NMS, 84 Ci/mmol) were purchased from New England Nuclear. Radioactive compounds for second

messenger assays were purchased from New England Nuclear ([^{3}H]-myo-inositol, 24.4 Ci/mmol; [^{3}H]-adenine, 25.8 Ci/mmol; and [^{14}C]-cAMP, 52.3 Ci/mmol), Amersham ([^{3}H]-myo-inositol, 18.3 Ci/mmol) or American Radiochemical ([^{3}H]-myo-inositol, 20 Ci/mmol; and [^{3}H]-adenine, 15 Ci/mmol).

Carbachol and atropine were purchased from Aldrich Chemical Company, and forskolin, IBMX, Ro-1734, *l* -QNB, NMS, oxo M, acetylcholine and pilocarpine were from Research Biochemicals International. All other reagents were either from GIBCO-Bethesda Research Laboratories (cell culture media and supplements) or Sigma. Restriction enzymes and T4 DNA polymerase were from Promega, GIBCO-Bethesda Research Laboratories or New England Biolabs. The mammalian expression vector (pSVE) and the clone for wild-type pm2 mAchR were gifts from Genentech, Inc. The dhfr$^-$ cells were obtained from America Type Culture Collection. Glass-fiber filters were from Schleicher & Schuell (No. 32) or Whatman (GF/B).

Receptor cloning

Site-directed mutagenesis was carried out as described in Chapter 2. Briefly, uracil containing single stranded phage with the cloned pm2 mAchR was isolated from the E. coli strain, CJ236. The uracil containing phage was annealed to mutagenic primers, and second strand DNA synthesis completed. The site-directed mutant, D69N was constructed using the oligonucleotide, 5'-GATGAGGTTAGCACAGGC-3'. The mutation was confirmed by dideoxy DNA sequencing (Sanger et al., 1977). Once confirmed, the mutant mAchR was cloned into the pSVE expression vector and the construct stably transfected into dhfr$^-$ CHO cells by calcium phosphate precipitation.

Receptor expression in Chinese hamster ovary cells

The mutant pm2 mAchR constructs in pSVE were stably transfected into Chinese hamster ovary cells deficient in the gene dihydrofolate reductase (dhfr$^-$), and amplified with increasing concentrations of methotrexate. High expression wild-type pm2 mAchR was overexpressed in CHO cells after removing 75 bases from the 5' untranslated region and replacing the weak 5' upstream Kozak sequence (CAAA) with a 'strong' Kozak sequence (CACC) (Kozak, 1987)*, to obtain elevated receptor expression. This construct resulted in very high receptor expression (2.0 to 4.0 x 10^6 receptors per cell) in dhfr$^-$ CHO cells, and was necessary to achieve expression levels similar to the D69N mutant AchR. Wild-type and mutant receptor cell lines were cloned prior to characterization. The low expression clonal cell line (pSVE pm2 cl.5), the high expression clone (pSVE short pm2 cl.1) as well as the D69N (pSVE D69N cl.1/2) were characterized for internalization and desensitization properties.

Ligand binding

Receptor expression was assessed in whole cells on 24 well tissue culture dishes by the addition of 10 nM [^{3}H] *l* -QNB in the presence or absence of saturating concentrations of the unlabeled antagonists NMS or *l* -QNB in FD media. Cells were incubated for 1.5 to 2 hours, then washed twice with ice cold phosphate buffered saline (pH 7.4). Cells were solubilized with 0.5 ml 1% Triton X-100 in PBS, transferred to scintillation vials then counted for tritium. Surface receptor sites were determined by competition of [^{3}H] *l*-QNB with the unlabeled hydrophilic antagonist, NMS, while total receptor was determined in

the presence of unlabeled hydrophobic antagonist, *l* -QNB. Determination of surface receptor sites using this approach gave results within 10% of [^{3}H]-NMS binding.

Second messenger assays

Chinese hamster ovary cells expressing wild-type or mutant muscarinic receptors were assayed for coupling to second messenger effector systems. Inhibition of forskolin-stimulated adenylyl cyclase assays were conducted on either 35 mm or 24 well culture dishes. All assays for agonist-stimulated phosphatidyl inositol metabolism were conducted on 24 well dishes. Surface receptor expression as well as total receptor expressed was assessed for each second messenger assay conducted. Wild-type receptor was expressed at approximately 1.0 x 10^6 (pSVE cl.5) or 2.0 x 10^6 (pSVE short cl.1) surface sites per cell, while the D69N AchR mutant cell line expressed approximately 2.0 x 10^6 surface sites per cell.

Stably transfected CHO cells expressing wild-type or mutant pm2 were assayed for agonist-stimulated metabolism of phosphatidyl inositol as described (Bulseco and Schimerlik, 1996). Cells were plated in 24 well dishes at an initial density of 1.0 to 2.0 x 10^5 cells per well in Dulbecco's Modified Eagle Medium (FD) supplemented with 10% calf serum (Sigma). 1 μCi/ml [^{3}H]-myo-inositol was added and cells incubated at 37° C for 24 hours. Prior to the assay, cells were treated for 30 minutes with 10 mM lithium chloride in FD at 37° C. After addition of agonists, cells were incubated for an additional 30 minutes at 37° C and reactions stopped with 5% trichloroacetic acid. Extracts were diluted with distilled water, then neutralized with 1 N sodium hydroxide. Samples were applied to columns containing 0.6 ml of AG 1-X8 anion exchange resin (BioRad), washed with 10 ml

of distilled water followed by 2 ml of 5 mM sodium tetraborate, 60 mM sodium formate. Inositol monophosphates (IP1) were eluted with 2 ml of 0.2 M ammonium formate, 0.1 M formic acid.

Assays for cAMP levels were conducted on CHO cells expressing wild-type or mutant receptors plated on 35 mm or 24 well tissue culture dishes as described (Bulseco and Schimerlik, 1996). Cells were incubated with [^{3}H]-adenine (1 or 2 μCi/ml) for two to four hours at 37° C in FD supplemented with 10% calf serum. Cells were washed twice with 1 ml FD media, and assayed for inhibition of forskolin-stimulated adenylyl cyclase activity in the presence of phosphodiesterase inhibitors 0.5 mM Ro 20-1724 and 100 μM IBMX. The reaction was stopped by aspiration of the media, followed by addition of 0.3 ml 2% SDS, 1.3 mM cAMP. [^{14}C]-cAMP was added to each plate to correct for cAMP recovery, followed by 0.75 ml distilled water and 0.1 ml perchloric acid. The extract was transferred to 1.5 ml microcentrifuge tubes containing 0.1 ml potassium hydroxide at a concentration sufficient to neutralize an equivalent volume of perchloric acid (usually ~ 11.5 M). The tubes were vortexed, then centrifuged at 4° C for 10 minutes at 12,000 rpm. The supernatant from each tube was transferred to columns containing 2 ml of AG 50W-X4 Dowex (BioRad) resin. After washing with 6 ml distilled water, the samples were eluted onto columns made with 0.5 g dry Alumina resin (Sigma) with an additional 6 ml of water. The cAMP was eluted from the alumina columns directly into scintillation vials with 4 ml of 0.1 M imidazole buffer (pH 7.3), and samples counted for both [^{14}C]-cAMP and [^{3}H]-cAMP.

Assays for receptor desensitization were conducted as described above after exposing CHO cells to the appropriate treatments. All agonists were used at a concentration of 1 mM, in FD supplemented with 10% calf serum, and cells exposed for three or 12 hours.

Assays for receptor internalization and recycling

Agonist-promoted pm2 mAchR internalization and recycling rates were estimated as described (Koenig and Edwardson, 1996; Koenig and Edwardson, 1994). Receptor recycling rates were determined after blocking surface receptor binding sites with benzilylcholine mustard (BCM). Stock BCM was diluted to 10 μM in 10 mM $NaPO_4$, 1 mM EDTA, pH 7.5 and cyclized for 30 minutes at room temperature. A final concentration of 0.1 μM BCM was used to block surface receptor sites on untreated cells, cells treated with 20 μM cycloheximide for 1 hour, and cells treated with cycloheximide followed by 1 mM carbachol or pilocarpine. Cells were allowed to recover in FD media supplemented with 10% calf serum over a period of 120 minutes. At the specified times, cells were washed twice with FD media and ligand binding conducted as described above. Rate of receptor internalization was determined by following the time course of agonist-promoted loss of surface receptor sites in cells treated with carbachol or pilocarpine in FD media + 10% calf serum.

Data analysis

Data from effector coupling assays were fit to the logistic equation. Equation 1 was used for PI assays, with a third term added (Eq. 2) to account for a stimulatory phase observed in the cAMP assays (Bulseco and Schimerlik, 1996).

$$Y = \min + \frac{(\max - \min)}{1 + (EC50/[x])^{p}} \qquad \text{(Eq. 1)}$$

$$Y = \min + \frac{(\max 1 - \min)}{1 + ([x]/EC50i)^{p}} + \frac{(\max 2 - \min)}{1 + (EC50s/[x])^{p1}} \qquad \text{(Eq. 2)}$$

In each of these equations [x] is the agonist concentration used; max, max1 or max2 and min are the maximum and minimum plateaus of the curves; and p or p1 the respective slope factors.

Data from receptor internalization and recycling experiments were fit to Equation 3 (Koenig and Edwardson, 1994) where k_1 is the rate of new receptor synthesis; k_2 is the rate of receptor recycling from endosomes to the plasma membrane; k_3 the rate of receptor internalization; k_4 the rate of receptor degradation; Rs, the surface (plasma membrane) receptor sites with initial value of Rs0 at t=0; Re, the internal (endosomal) receptor sites with an initial value of Re0 at t=0.

(Eq. 3)

$$Rs = (1/(k2+k3))\left[(k1 + k2\,\mathrm{Re}0 + k2\frac{(k4-k1)}{(k2+k3)})(1-e^{-(k2+k3)t}) + Rs0(k2+k3e^{-(k2+k3)t}) - k2(k4-k1)t\right]$$

Equation 3 can be simplified by manipulation of experimental conditions.

$$Rs = \mathrm{Re}0 * (1 - e^{-k2t}) + k1t \qquad \text{(Eq. 4)}$$

In the absence of agonist, it is assumed that k_3 and k_4 are negligible. When cells are treated with BCM, all surface sites are blocked and Rs0 can be fixed at 0 resulting in Eq. 4. Treatment with cycloheximide prevents new synthesis, allowing k_1 to be fixed at 0 which eliminates the linear term ($k_1 t$) in Eq. 4. BCM experiments (n=4) done in the presence or absence of cycloheximide (in duplicate or triplicate) were pooled and the recycling rate constant (k_2), the rate of new synthesis (k_1) and the endosomal pool of mAchR (Re0) estimated by fitting the data to equation 4. These values were used in equation 3 to estimate receptor internalization rate (k_3) and rate of receptor degradation (k_4).

Agonist binding data were fit assuming three non-interacting classes of sites using the equation below (Vogel et al., 1995).

$$[RL] = \sum_{i=1}^{3} \frac{[Ro_i]([Lo]-[RL]_i)}{Kd + Kd/Ki[I]+[Lo]-[RL]_i} \qquad \text{(Eq. 5)}$$

$[Ro_i]$ is the total receptor concentration for each receptor site; [Lo], the total concentration of radiolabeled tracer used in the experiment with dissociation constant Kd; and [I], the total unlabeled competing agonist with dissociation constant Ki.

Results

Expression in Chinese hamster ovary cells

Clonal CHO cell lines expressing wild-type pm2 mAchR were selected at both low and high expression levels. The low expression cell line (pSVE pm2 cl.5) displayed between 0.8 to 1.2 x 10^6 receptors per cell, while the high expression wild-type cell line (pSVE pm2 short cl.1) expressed between 2.0 and 4.0 x 10^6 receptors per cell. In both cases, approximately 90% of the receptors expressed were present in the plasma membrane and accessible to the hydrophilic antagonist NMS. D69N mutant mAchR was also expressed at high levels in CHO cells. Between 2.0 and 2.8 x 10^6 receptors per cell were regularly expressed throughout these experiments, with at least 90% of the receptors localized to the plasma membrane.

Time course of receptor internalization

The time course of receptor internalization in response to prolonged agonist treatment is dependent on receptor density and the agonist used. Figures 4.1 and 4.2 show surface and total receptor remaining after treatment with the full agonist, carbachol, and the partial agonist, pilocarpine, for CHO cells expressing low (cl.5) and high (cl.1) pm2 mAchR levels. Carbachol significantly ($p \leq 0.01$, Student's t-test) reduced surface receptor sites to 41 ± 12 % and 37 ± 1 % of control for cells expressing both low (Figure 4.1) and high (Figure 4.2) receptor levels respectively. Pilocarpine on the other hand, significantly ($p \leq 0.01$, Student's t-test) reduced surface receptor number to 49 ± 3 % of

control only for the cl.1 CHO cell line which expresses the mAchR at high density. Cells expressing low mAchR densities were not affected by prolonged exposure to pilocarpine.

To further characterize these observations, agonist-dependent rates of receptor internalization were studied as previously described (Koenig and Edwardson, 1996; Koenig and Edwardson, 1994), in CHO cell lines expressing low or high receptor numbers. Cycloheximide did not appear to significantly affect carbachol-stimulated internalization of receptor sites for the first 120 minutes of treatment (Figure 4.3). Both surface and total receptor numbers were similar to results obtained in the absence of cycloheximide. Cycloheximide did appear to prevent the degradation of internalized receptor during longer exposure to carbachol. This resulted in greater total receptor numbers at four to six hours of carbachol treatment, than is observed in the absence of cycloheximide. Since the experiments described were conducted within 120 minutes, it is assumed that cycloheximide does not have a significant affect on the observed rates.

The time course of agonist-promoted receptor internalization was used to obtain estimates of k_3 and k_4, the rates of receptor internalization and degradation respectively. Figure 4.4 shows the effects of carbachol and pilocarpine on surface receptor number for cl.5. Carbachol treatment resulted in about 50% loss of surface receptor sites, while pilocarpine had no affect. The estimated rates of receptor recycling (k_2) for carbachol and pilocarpine treated cells were 0.054 and 0.019 % receptor/cell/minute respectively while untreated cells displayed a recycling rate of 0.012 % receptor/cell/minute (Figures 4.7 and 4.11). Carbachol treatment resulted in a 28 fold increase in rate of receptor internalization. The estimated rates of receptor internalization (k_3) were 0.0026 ± 0.0005,

0.074 ± 0.005 and 0.005 ± 0.001 % receptor/cell/minute for untreated, carbachol and pilocarpine treated cells respectively. Similarly, carbachol increased the rate of receptor degradation about 9 fold, with k_4 = 0.28 ± 0.09 % receptor/cell/minute compared to 0.031 ± 0.069 and 0.05 ± 0.01 % receptor/cell/minute for control and pilocarpine treated cells respectively (Table 4.1).

The results observed for the partial agonist, pilocarpine, were different for the CHO cell line expressing high receptor densities. Figure 4.5 shows that both carbachol and pilocarpine exposure resulted in a 50% loss of surface receptor sites. The estimated rates of receptor recycling (k_2) for carbachol and pilocarpine treated cells were 0.043 and 0.027 % receptor/cell/minute respectively while untreated cells displayed a recycling rate of 0.011 % receptor/cell/minute (Figures 4.8 and 4.12). Carbachol treatment resulted in a 22 fold increase in rate of receptor internalization while pilocarpine increased internalization rate about 16 fold. The estimated rates of receptor internalization (k_3) were 0.0027 ± 0.0005, 0.059 ± 0.011 and 0.043 ± 0.008 % receptor/cell/minute for untreated, carbachol and pilocarpine treated cells respectively. Receptor degradation was also affected by agonist treatment, with a 26 fold increase for carbachol treated cells (0.37 ± 0.24 % receptor/cell/minute) and a six fold increase for pilocarpine treated cells (0.090 ± 0.029 % receptor/cell/minute), while untreated cells displayed a degradation rate constant of 0.014 ± 0.066 % receptor/cell/minute (Table 4.1).

Agonist-promoted internalization of the pm2 mAchR is independent of receptor-effector coupling. The D69N mutant fails to couple to second messenger systems for all agonists studied except oxo M. Figure 4.6 shows that both carbachol and pilocarpine

treatments resulted in about 60% loss of surface receptor sites. The estimated rates of receptor recycling (k_2) for cells treated with carbachol and pilocarpine were 0.044 and 0.042 % receptor/cell/minute respectively while untreated cells displayed a recycling rate of 0.060 % receptor/cell/minute (Figures 4.8 and 4.12). Carbachol treatment resulted in a 15 fold increase in rate of receptor internalization while pilocarpine increased internalization rate about 14 fold. The estimated rates of receptor internalization (k_3) were 0.0072 ± 0.0011, 0.109 ± 0.011 and 0.100 ± 0.019 % receptor/cell/minute for untreated, carbachol- and pilocarpine-treated cells respectively. The results for receptor degradation were different than those seen for CHO cells expressing wild-type receptor. Carbachol had no affect on apparent rate of receptor degradation, and pilocarpine reduced the degradation rate by 80 fold. The estimated rates (k_4) were 0.081 ± 0.034, 0.090 ± 0.020 and 0.001 ± 0.003 % receptors/cell/minute for untreated, carbachol-treated and pilocarpine-treated cells respectively (Table 4.1).

Time course of receptor recycling

The rates of m2 mAchR recycling when expressed in CHO cells were affected by agonist exposure. Carbachol increased the rate of receptor recycling at low receptor densities, while both pilocarpine and carbachol increased recycling rate when receptor was expressed at high densities. In order to characterize these rates, it was necessary to eliminate receptors at the cell surface and follow the time course of receptor appearance in the absence of new protein synthesis. Cells expressing m2 mAchR were exposed to cycloheximide (20 μM, 1 hour) before agonist treatments. Benzilylcholine mustard (BCM) was then used to covalently block all surface receptor sites and the time course of

receptor recovery at the cell surface followed for 120 minutes. The treatment maximally resulted in 90% loss of surface receptor sites, although as little as 75% loss of sites were observed for some experiments. Experiments in which BCM treatment failed to reduce the surface receptor sites by at least 85% were not used in the analysis presented in this report. In each case, the residual binding after BCM treatment was subtracted prior to data analysis.

The time course of receptor appearance on the cell surface was dependent on agonist used as well as receptor density. For the low expressing cell line, the full agonist carbachol resulted in a four fold increase in recycling rate (k_2) while pilocarpine had little effect. Figures 4.7 and 4.11 show recovery of surface receptor after carbachol and pilocarpine treatments respectively. The recycling rates estimated from the analysis were 0.054 ± 0.014 % receptor/cell/minute for carbachol and 0.019 ± 0.008 % receptor/cell/minute for pilocarpine-treated cells, while cells treated with cycloheximide alone displayed a recycling rate of 0.012 ± 0.003 % receptors/cell/minute.

For the high expressing clonal cell line, carbachol and pilocarpine had similar effects on the rates of receptor recycling, increasing k_2 by four and two fold respectively. Figures 4.8 and 4.12 show recovery of surface receptor after carbachol and pilocarpine treatments. The recycling rates estimated from the analysis were 0.043 ± 0.013 % receptor/cell/minute for carbachol and 0.027 ± 0.009 % receptor/cell/minute for pilocarpine treated cells, while cells treated with cycloheximide alone displayed a recycling rate of 0.011 ± 0.004 % receptors/cell/minute.

The rate of receptor recycling for the D69N mutant AchR was unaffected by carbachol or pilocarpine treatment, although high receptor densities are expressed and both agonists promote net receptor internalization. Figures 4.9 and 4.13 show recovery of surface receptor after carbachol and pilocarpine treatments. The recycling rates estimated from this analysis were 0.044 ± 0.007, 0.043 ± 0.007 and 0.027 ± 0.011 % receptors/cell/minute for carbachol, pilocarpine and cycloheximide treated cells respectively.

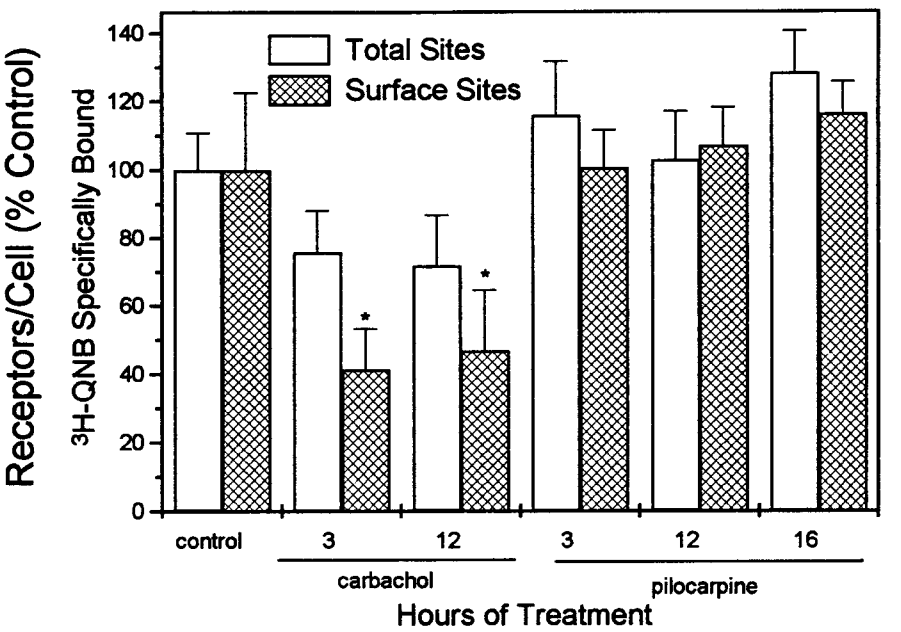

Figure 4.1: Low expression (cl.5) total and surface receptor sites remaining after agonist treatments.

Cells were treated with carbachol or pilocarpine in FD + 10% calf serum for the indicated times. [^{3}H] *l* -QNB was displaced with unlabeled *l* -QNB to determine total receptor binding sites and unlabeled NMS for determination of surface receptor sites. Data are presented as percent sites of control remaining after specified treatments (n=2 or 3, each done in triplicate). Carbachol treatments resulted in significant reduction in surface receptor sites (*, $p \leq 0.01$, Student's t-test).

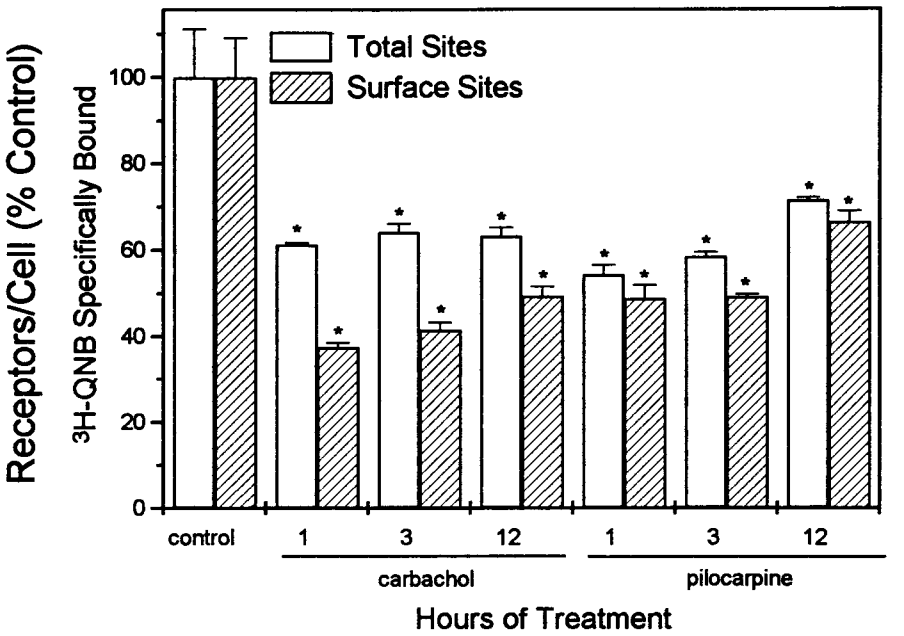

Figure 4.2: High expression (cl.1) total and surface receptor expression after agonist treatments

Cells were treated with carbachol or pilocarpine in FD + 10% calf serum for the indicated times. [^{3}H] *l* -QNB was displaced with unlabeled *l* -QNB to determine total receptor binding sites and unlabeled NMS for determination of surface receptor sites. Data are presented as percent sites of control remaining after specified treatments (n=2, each done in triplicate). Both carbachol and pilocarpine treatments resulted in differences significant from untreated cl.1 cells (*, $p \leq 0.01$, Student's t-test).

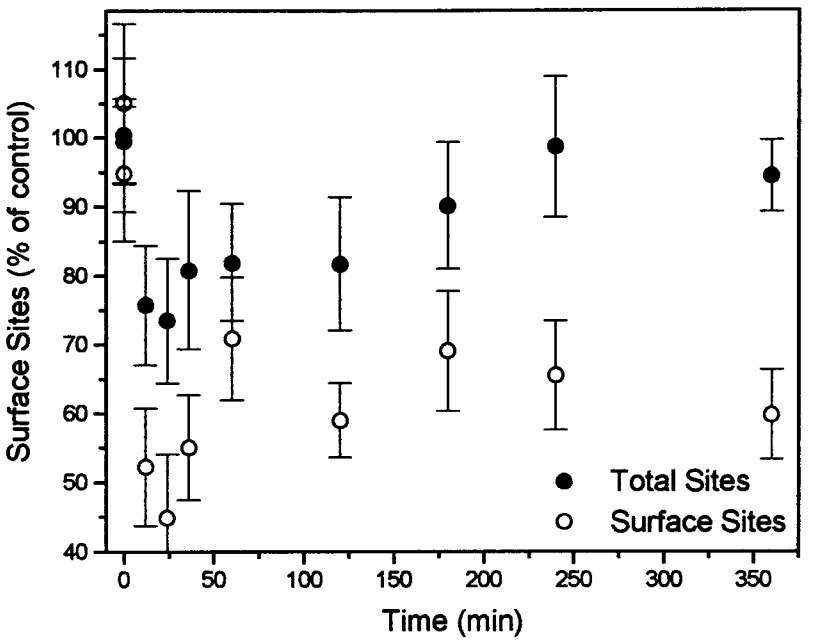

Figure 4.3: Effect of cycloheximide on cl.5 receptor down regulation.

Cycloheximide treated cells were exposed to 1 mM carbachol in FD + 10% calf serum for the times indicated. [^{3}H] *l* -QNB was displaced with unlabeled *l* -QNB to determine total receptor binding sites and unlabeled NMS for determination of surface receptor sites. Data are presented as percent sites of control remaining after carbachol treatment for the specified times (n=3, each done in triplicate).

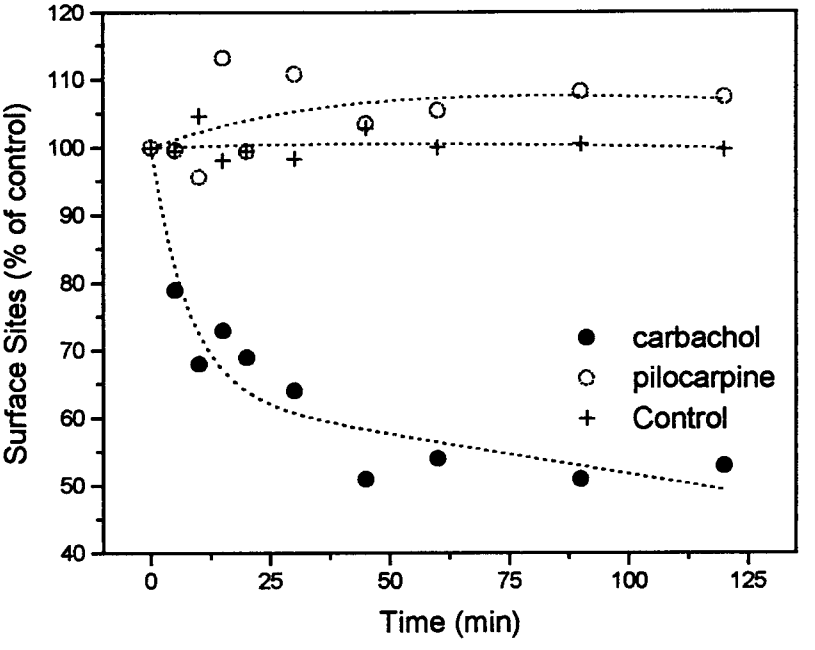

Figure 4.4: Low expression (cl.5) time course of receptor internalization after treatment with carbachol or pilocarpine.

CHO cells expressing approximately 1e6 wild-type mAchRs per cell were treated with 1 mM carbachol or pilocarpine in FD media supplemented with 10% calf serum for the times indicated. Data are reported as the mean (n=4, each done in duplicate or triplicate) of the percent surface sites of control. Error bars were omitted for clarity. Values were determined with errors of less than 10%. Fitted lines were obtained by nonlinear least squares curve fitting to equation 3. Rates are reported as % receptors/cell per minute and Re0 as % of initial surface receptor density. Values used for k_1, k_2, Re0 and Rs0 were determined in experiments utilizing BCM treatments, and fixed at the following values. k_1 was fixed at 0.00153 and Rs0 at 100 for all treatments while control, carbachol and pilocarpine treated cells were fixed at, $k_2 = 0.012$, Re0 = 23.42; $k_2 = 0.054$, Re0 = 47.15; and $k_2 = 0.019$, Re0 = 39.79 respectively. k_3 and k_4 for control, carbachol and pilocarpine treated cells were $k_3 = 0.0026 \pm 0.0005$, $k_4 = 0.031 \pm 0.069$, $k_3 = 0.074 \pm 0.005$, $k_4 = 0.28 \pm 0.09$, and $k_3 = 0.005 \pm 0.001$, $k_4 = 0.05 \pm 0.01$ respectively.

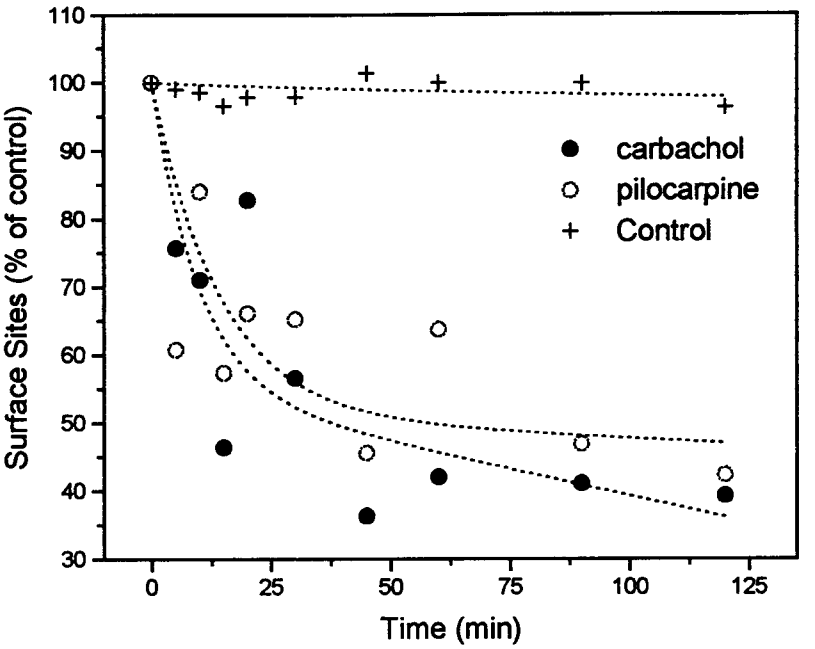

Figure 4.5: High expression (cl.1) time course of receptor internalization after treatment with carbachol or pilocarpine.

CHO cells expressing greater than 2e6 wild-type mAchRs per cell were treated with 1 mM carbachol or pilocarpine in FD media supplemented with 10% calf serum for the times indicated. Data are reported as the mean (n=4, each done in duplicate or triplicate) of the percent surface sites of control. Error bars were omitted for clarity. Values were determined with errors of less than 10%. Fitted lines were obtained by nonlinear least squares curve fitting to equation 3. Rates are reported as % receptors/cell per minute and Re0 as % of initial surface receptor density. Values used for k_1, k_2, Re0 and Rs0 were determined in experiments utilizing BCM treatments, and fixed at the following values. k_1 was fixed at 0.00285 and Rs0 at 100 for all treatments while control, carbachol and pilocarpine treated cells were fixed at, $k_2 = 0.011$, Re0 = 21.34; $k_2 = 0.043$, Re0 = 26.84; and $k_2 = 0.027$, Re0 = 29.55 respectively. k_3 and k_4 for control, carbachol and pilocarpine treated cells were $k_3 = 0.0027 \pm 0.0005$, $k_4 = 0.014 \pm 0.066$; $k_3 = 0.059 \pm 0.011$ $k_4 = 0.37 \pm 0.24$, and $k_3 = 0.043 \pm 0.008$, $k_4 = 0.090 \pm 0.029$ respectively.

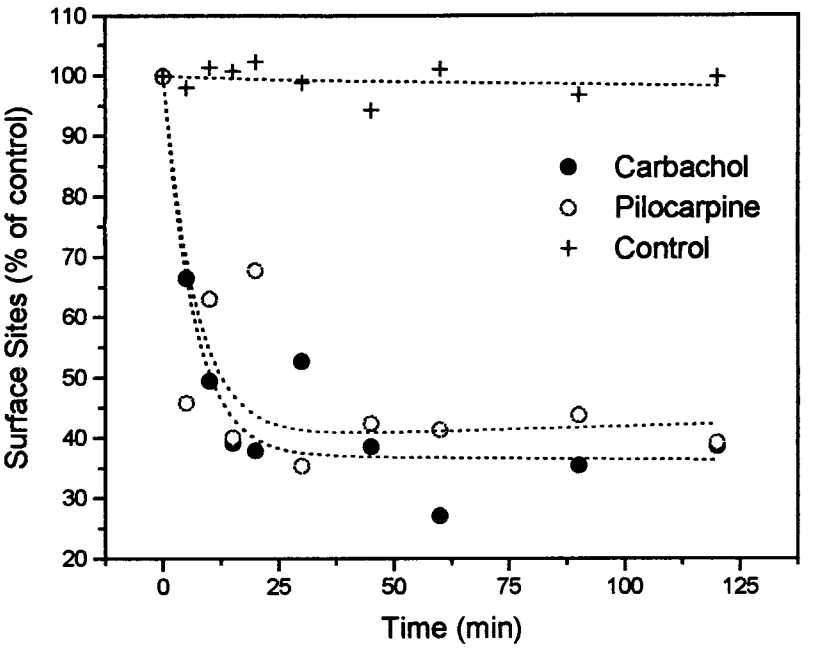

Figure 4.6: D69N mutant AchR time course of receptor internalization after treatment with carbachol or pilocarpine.

CHO cells expressing greater than 2e6 D69N mutant mAchRs per cell were treated with 1 mM carbachol or pilocarpine in FD media supplemented with 10% calf serum for the times indicated. Data are reported as the mean (n=4, each done in duplicate or triplicate) of the percent surface sites of control. Error bars were omitted for clarity. Values were determined with errors of less than 10%. Fitted lines were obtained by nonlinear least squares curve fitting to equation 3. Rates are reported as % receptors/cell per minute and Re0 as % of initial surface receptor density. Values used for k_1, k_2, Re0 and Rs0 were determined in experiments utilizing BCM treatments, and fixed at the following values. k_1 was fixed at 0.0684 and Rs0 at 100 for all treatments while control, carbachol and pilocarpine treated cells were fixed at, $k_2 = 0.0595$, Re0 = 10.37; $k_2 = 0.0435$, Re0 = 28.72; and $k_2 = 0.0424$, Re0 = 30.39 respectively. k_3 and k_4 for control, carbachol and pilocarpine treated cells were $k_3 = 0.0072 \pm 0.0011$, $k_4 = 0.081 \pm 0.034$; $k_3 = 0.109 \pm 0.011$, $k_4 = 0.090 \pm 0.020$, and $k_3 = 0.100 \pm 0.019$, $k_4 = 0.001 \pm 0.003$ respectively.

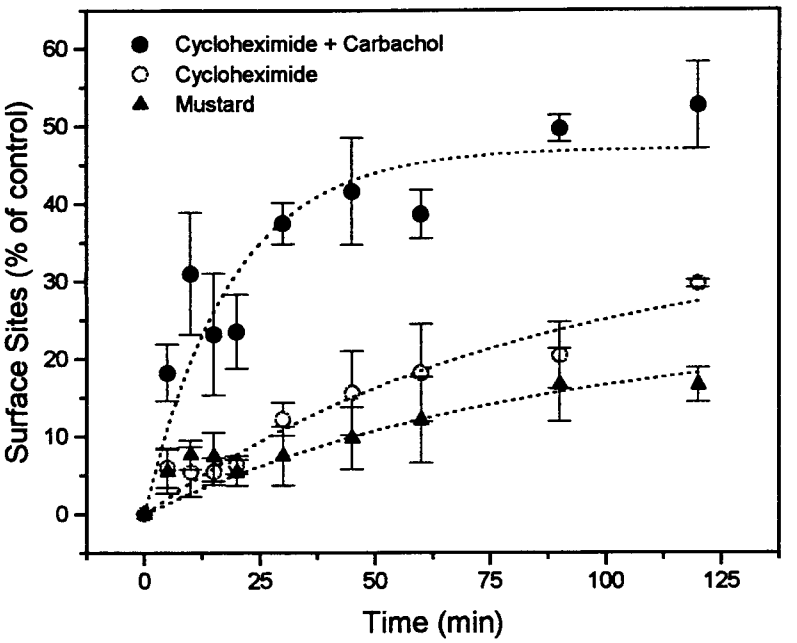

Figure 4.7: Low expression: recovery/recycling of receptor after BCM treatment.

CHO cells expressing about 1e6 wild-type mAchRs per cell were left untreated, or treated with cycloheximide alone, or 1 mM carbachol + cycloheximide in FD media supplemented with 10% calf serum for the times indicated. BCM was added to all cells and recovery of surface expression levels determined at the times specified. Data are reported as the mean ± S.E.M. (n=4 each done in duplicate or triplicate) of the percent surface sites of control. Fitted lines were obtained by nonlinear least squares curve fitting to equation 4. Rates are reported as % receptors/cell/min and Re0 as % of initial surface receptor density. Values obtained for untreated cells were $k_1 = 0.002 \pm 0.001$, k_2 fixed at 0.012, Re0 = 23.41 ± 1.94, for cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.012 \pm 0.003$, Re0 = 35.67 ± 5.30 and for carbachol + cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.054 \pm 0.014$ and Re0 = 47.15 ± 3.86.

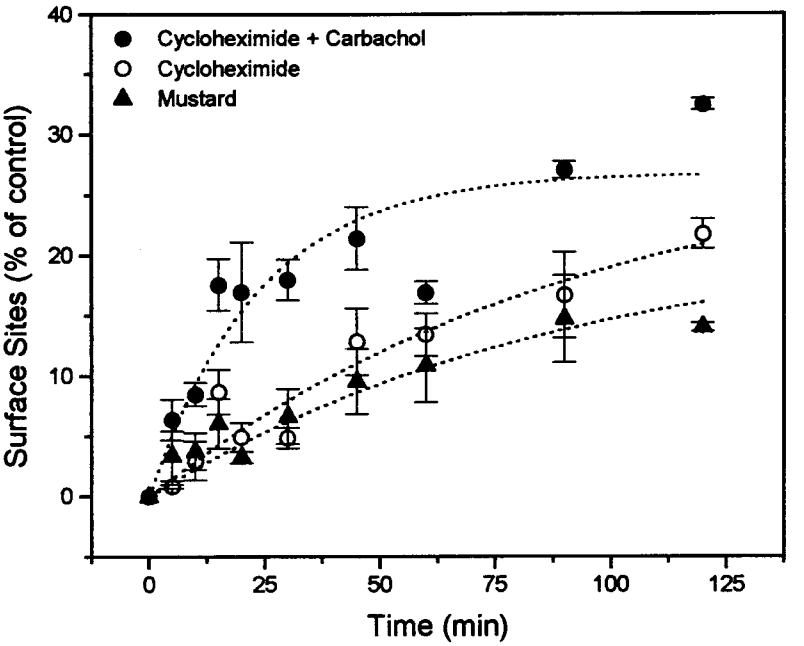

Figure 4.8: High expression: carbachol stimulated recovery/recycling of receptor after BCM treatment.

CHO cells expressing greater than 2e6 wild-type mAchRs per cell were left untreated, or treated with cycloheximide alone, or 1 mM carbachol + cycloheximide in FD media supplemented with 10% calf serum for the times indicated. BCM was added to all cells and recovery of surface expression levels was determined at the times specified. Data are reported as the mean ± S.E.M. (n=4 each done in duplicate or triplicate) of the percent surface sites of control. Fitted lines were obtained by nonlinear least squares curve fitting to equation 4. Rates are reported as % receptors/cell/min and Re0 as % of initial surface receptor density. Values obtained for untreated cells were $k_1 = 0.003 \pm 0.001$, k_2 fixed at 0.01, Re0 = 21.34 ± 1.26, for cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.011 \pm 0.004$, Re0 = 28.93 ± 6.13 and for carbachol + cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.043 \pm 0.013$ and Re0 = 26.83 ± 2.72.

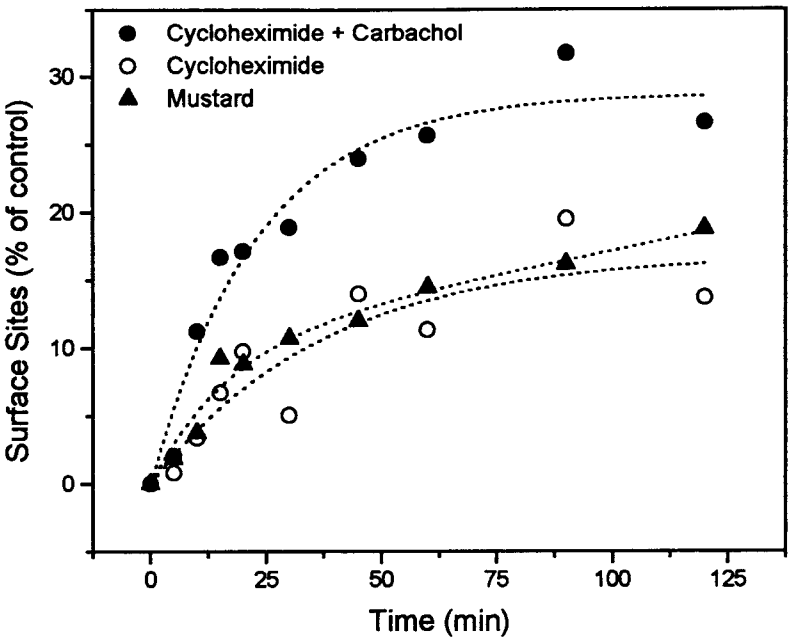

Figure 4.9: D69N: carbachol stimulated recovery/recycling of receptor after BCM treatment.

CHO cells expressing greater than 2e6 D69N mutant mAchRs per cell were left untreated, or treated with cycloheximide alone, or 1 mM carbachol + cycloheximide in FD media supplemented with 10% calf serum for the times indicated. BCM was added to all cells and recovery of surface expression levels was determined at the times specified. Data are reported as percent surface sites of control from a representative experiment. Fitted lines were obtained by nonlinear least squares curve fitting to equation 4. Rates are reported as % receptors/cell/min and Re0 as % of initial surface receptor density. Values obtained for untreated cells were $k_1 = 0.069 \pm 0.026$, k_2 fixed at 0.059, Re0 = 10.37 ± 2.5, for cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.027 \pm 0.011$, Re0 = 16.90 ± 2.69 and for carbachol + cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.0438 \pm 0.007$ and Re0 = 28.72 ± 1.60.

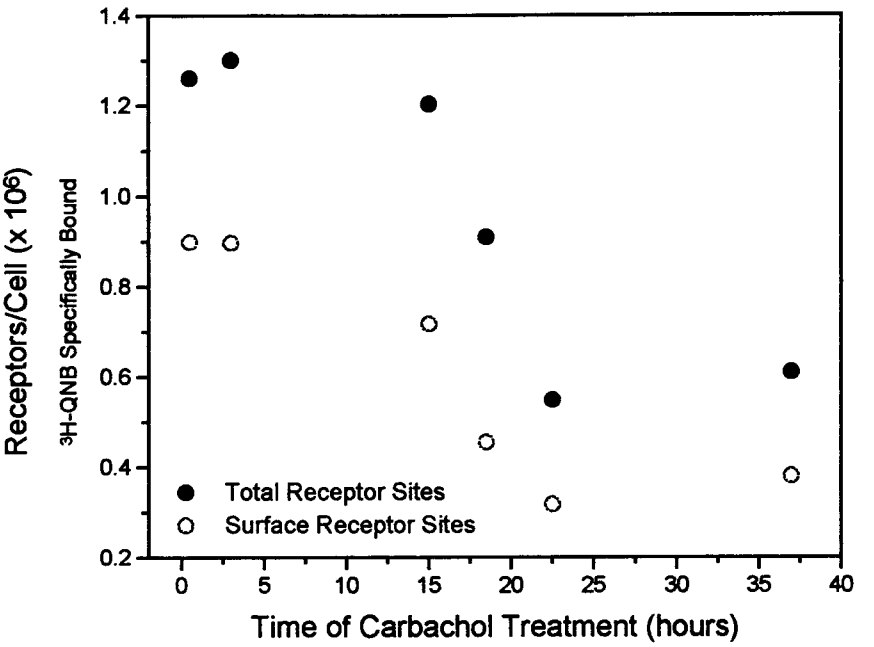

Figure 4.10: Calf serum is required for receptor internalization and down regulation.

CHO cells expressing approximately 1.2e6 wild-type mAchRs per cell were exposed to 1 mM carbachol in serum free FD media for the times indicated. [^{3}H] *l* -QNB was displaced with unlabeled *l* -QNB to determine total receptor binding sites and unlabeled NMS for determination of surface receptor sites.

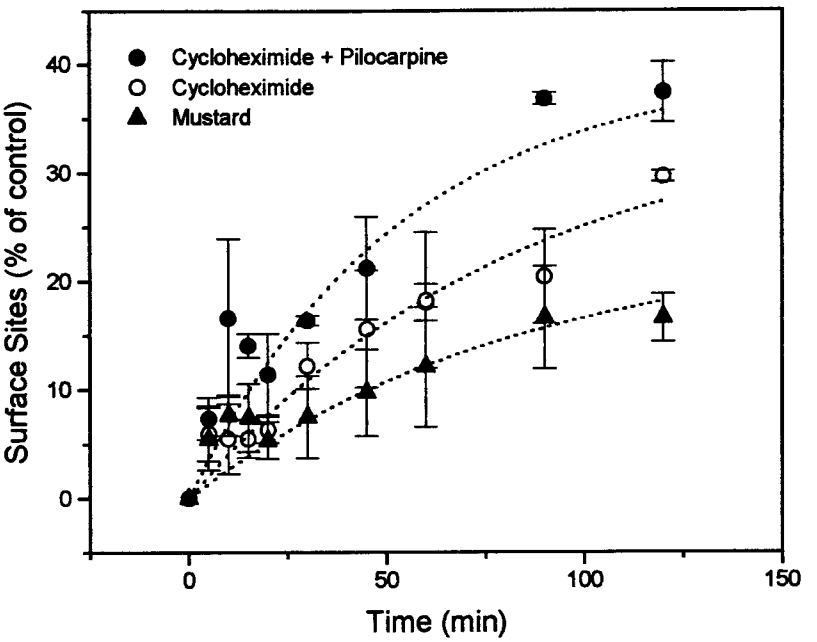

Figure 4.11: Low expression: pilocarpine stimulated recovery/recycling of receptor after BCM treatment

CHO cells expressing about 1e6 wild-type mAchRs per cell were left untreated, or treated with cycloheximide alone, or 1 mM pilocarpine + cycloheximide in FD media supplemented with 10% calf serum for the times indicated. BCM was added to all cells and recovery of surface expression levels determined at the times specified. Data are reported as the mean ± S.E.M. (n=4 each done in duplicate or triplicate) of the percent surface sites of control. Fitted lines were obtained by nonlinear least squares curve fitting to equation 4. Rates are reported as % receptors/cell/min and Re0 as % of initial surface receptor density. Values obtained for untreated cells were $k_1 = 0.002 \pm 0.001$, k_2 fixed at 0.012, Re0 = 23.41 ± 1.94, for cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.012 \pm 0.003$, Re0 = 35.67 ± 5.30 and for pilocarpine + cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.019 \pm 0.008$ and Re0 = 39.79 ± 7.45.

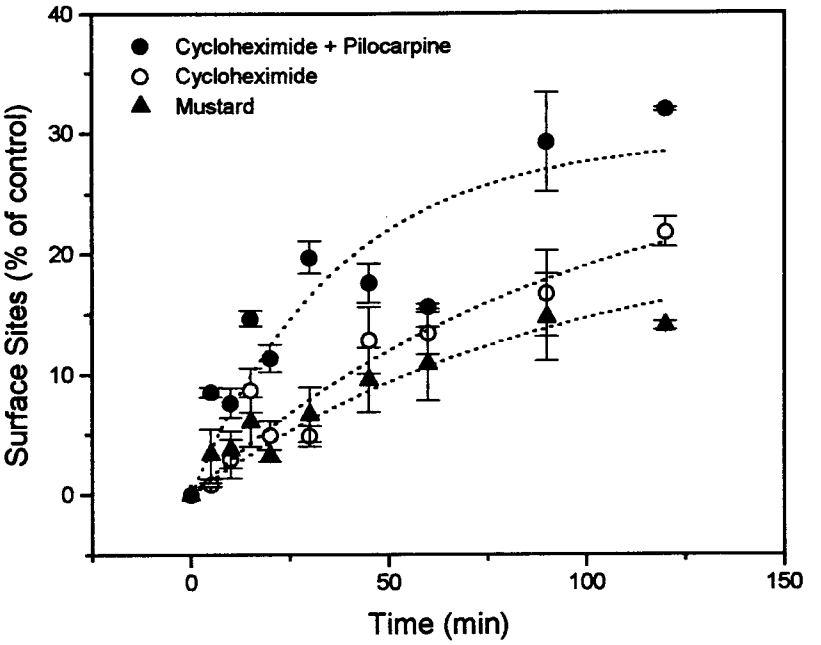

Figure 4.12: High expression: pilocarpine stimulated recovery/recycling of receptor after BCM treatment

CHO cells expressing greater than 2e6 wild-type mAchRs per cell were left untreated, or treated with cycloheximide alone, or 1 mM pilocarpine + cycloheximide in FD media supplemented with 10% calf serum for the times indicated. BCM was added to all cells and recovery of surface expression levels was determined at the times specified. Data are reported as the mean ± S.E.M. (n=4 each done in duplicate or triplicate) of the percent surface sites of control. Fitted lines were obtained by nonlinear least squares curve fitting to equation 4. Rates are reported as % receptors/cell/min and Re0 as % of initial surface receptor density. Values obtained for untreated cells were $k_1 = 0.003 \pm 0.001$, k_2 fixed at 0.01, Re0 = 21.34 ± 1.26, for cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.011 \pm 0.004$, Re0 = 28.93 ± 6.13 and for pilocarpine + cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.027 \pm 0.009$ and Re0 = 29.55 ± 4.10.

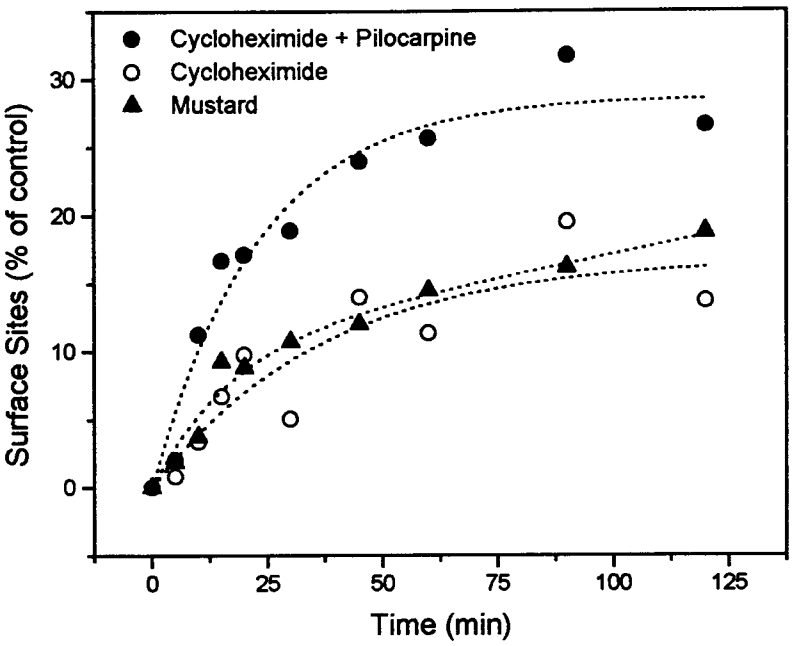

Figure 4.13: D69N: pilocarpine stimulated recovery/recycling of receptor after BCM treatment

CHO cells expressing greater than 2e6 D69N mutant mAchRs per cell were left untreated, or treated with cycloheximide alone, or 1 mM pilocarpine + cycloheximide in FD media supplemented with 10% calf serum for the times indicated. BCM was added to all cells and recovery of surface expression levels was determined at the times specified. Data are reported as percent surface sites of control from a representative experiment. Fitted lines were obtained by nonlinear least squares curve fitting to equation 4. Rates are reported as % receptors/cell/min and Re0 as % of initial surface receptor density. Values obtained for untreated cells were $k_1 = 0.069 \pm 0.026$, k_2 fixed at 0.059, Re0 = 10.37 ± 2.5, for cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.027 \pm 0.011$, Re0 = 16.90 ± 2.69 and for pilocarpine + cycloheximide treated cells, k_1 fixed at 0, $k_2 = 0.043 \pm 0.007$ and Re0 = 30.39 ± 1.63.

Agonist-promoted increase in endosomal receptor stores

Initial number of endosomal receptors may be increased by exposure to agonists. Determination of Re0, or receptor stored in endosomes at t = 0, shows that both full and partial agonists increased the estimated parameter Re0 independent of receptor density. Analysis of surface receptor recovery after cycloheximide and BCM treatment depend on recycling rate constant (k_2) and initial endosomal receptor number (Re0) after agonist treatment. Figures 4.7 and 4.11 show the time course of surface receptor recovery for CHO cells expressing wild-type pm2 mAchR at low receptor densities. Estimated values for Re0 were 23.41 ± 1.94, 47.15 ± 3.86 and 39.79 ± 7.45 % receptors for untreated, carbachol-treated and pilocarpine-treated cells respectively. For CHO cells expressing wild-type pm2 mAchR at high densities (Figures 4.8 and 4.12), these values were estimated to be 21.34 ± 1.26, 26.83 ± 2.72 and 29.55 ± 4.10 % receptors for untreated, carbachol- and pilocarpine- treated cells. Figures 4.9 and 4.13 show the time course of surface receptor recovery for D69N mutant mAchR. Re0 for this mutant was estimated to be 10.37 ± 2.5, 28.72 ± 1.60 and 30.39 ± 1.63 % receptors for untreated, carbachol- and pilocarpine-treated cells respectively.

Although Re0 is increased after treatment with agonists, cycloheximide in the absence of agonists appears to contribute to this increase. Re0 was estimated to be 35.67 ± 5.30, 28.93 ± 6.13 and 16.90 ± 2.69 % receptors for low expression wild type, high expression wild-type and D69N mutant AchR respectively. When this result is considered,

it appears that agonist treatment only affects endosomal receptor number for the low expression wild-type cell line and the CHO cell line expressing D69N mutant AchR.

Agonist-promoted receptor degradation

Prolonged exposure to agonists increased the apparent receptor degradation rate (k_4). For wild-type receptors, carbachol had a large affect on k_4, while pilocarpine's affect was modest (Table 4.1). CHO cells expressing low (cl.5) wild-type pm2 mAchR levels displayed estimated values for k_4 of 0.03, 0.28 and 0.05 % receptors/cell/minute for untreated, carbachol and pilocarpine-treated cells respectively. CHO cells expressing high receptor numbers, displayed estimated rates of 0.01, 0.38 and 0.09 % receptors/cell/minute for untreated, carbachol and pilocarpine-treated cells. Receptor-effector coupling may be necessary for agonist-promoted receptor degradation. When the D69N mutant AchR was exposed to carbachol, k_4 was unchanged, while pilocarpine treatment led to a reduction in degradation rate.

Time course of desensitization

Uncoupling of the pm2 AchR from second messenger effector systems did not follow the same time course as receptor internalization and was observed only after treatment with the full agonist carbachol. Although treatment with the partial agonist, pilocarpine reduced surface receptor numbers in CHO cells expressing greater than 2.0 x 10^6 wild-type m2 AchR, it did not lead to desensitization. In addition, cells treated with carbachol in the absence of calf serum displayed an attenuation of effector coupling with less than 10% reduction in surface receptor sites.

When CHO cells expressing wild-type mAchRs were exposed to carbachol for three or 12 hours, desensitization was observed for coupling to inhibition of adenylyl cyclase (Figures 4.14, 4.15) and stimulation of PI metabolism (Figures 4.16 and 4.17). Exposing these cells to pilocarpine for three or 12 hours did not lead to significant changes in coupling properties, although surface receptor number is reduced (Figures 4.1 and 4.2). Tables 4.3 and 4.4 summarize the results obtained for desensitization of wild-type receptor. Carbachol treatment resulted in an 18 fold and a 6 fold increase in IC50 for CHO cells expressing low and high pm2 mAchR numbers respectively, accompanied by a 35% and 12% reduction in maximal response when inhibition of adenylyl cyclase was measured. Carbachol exposure effectively eliminated measurable coupling to stimulation of PI metabolism. Pilocarpine treatment had a modest effect on IC50 for coupling to inhibition of adenylyl cyclase. CHO cells expressing low pm2 mAchR numbers displayed a two fold increase in IC50, while CHO cells expressing high receptor numbers showed roughly a two fold decrease in IC50. In each case, maximum % inhibition was unchanged when compared to control cells. Similar results were obtained for D69N when exposed to 1 mM carbachol or pilocarpine for 4 (See Figures 3.5 and 3.7) or 8 hours (n=1, data not shown) prior to second messenger assays.

Rapid receptor internalization and desensitization of CHO cells expressing pm2 mAchR requires that agonist treatment be conducted in the presence of 10% calf serum. When calf serum is omitted, the time course of carbachol promoted receptor internalization is altered (Figure 4.10), and less than 10% of the surface receptor sites are lost after 3 hours of treatment. Although very little surface receptor sites are lost, there is

a reduction in maximum percent inhibition of forskolin-stimulated inhibition of adenylyl cyclase from 71% to 57%, and a modest 4 fold increase in IC50 from 0.37 μM to 1.54 μM (Figure 4.18). EC50 for stimulation of adenylyl cyclase activity is not changed by carbachol treatment.

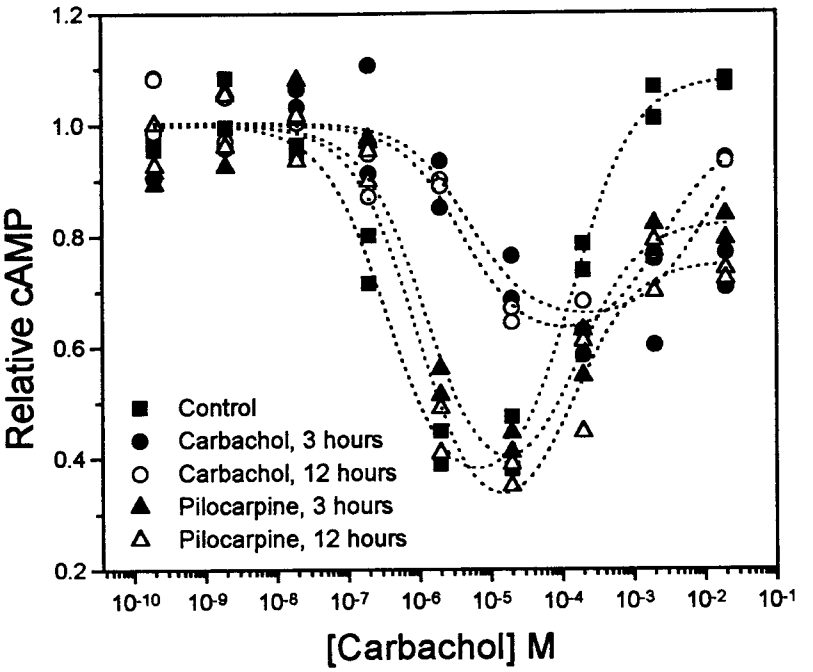

Figure 4.14: Desensitization of forskolin-stimulated inhibition of cAMP formation for low expression wild-type mAchR.

Data are presented as cAMP generated relative to forskolin-stimulated levels and are from representative experiments for pretreatment with carbachol for 3 (n=10) or 12 hours (n=2), or with pilocarpine for 3 (n=4) or 12 hours (n=2). Cells were pretreated with the agonists indicated (1 mM) for the specified times in FD + 10% calf serum, followed by second messenger assays using the agonist carbachol. Curves were derived from a least-squares fit as described in (Bulseco and Schimerlik, 1996). Average values are summarized in Table 4.3. The fitted parameters were estimated to be IC50 = 0.35 ± 0.09 μM, EC50 = 14 ± 3 μM, 68.8 ± 3.9 % inhibition; IC50 = 4.97 ± 1.52 μM, EC50 = 93.0 ± 29.3 μM, 35.7 ± 7.9 % inhibition; IC50 = 3.96 ± 1.81 μM , EC50 = 24.6 ± 15.4 μM, 39.3 ± 17.2 % inhibition; IC50 = 1.24 ± 0.56 μM, EC50 = 16.0 ± 10.0 μM, 69.4 ± 9.4 % inhibition and IC50 = 0.92 ± 0.37 μM, EC50 = 14.1 ± 8.3 μM, 75.7 ± 8.4 % inhibition for control, 3 hour carbachol treatment, 12 hour carbachol treatment, 3 hour pilocarpine treatment and 12 hour pilocarpine treatment respectively.

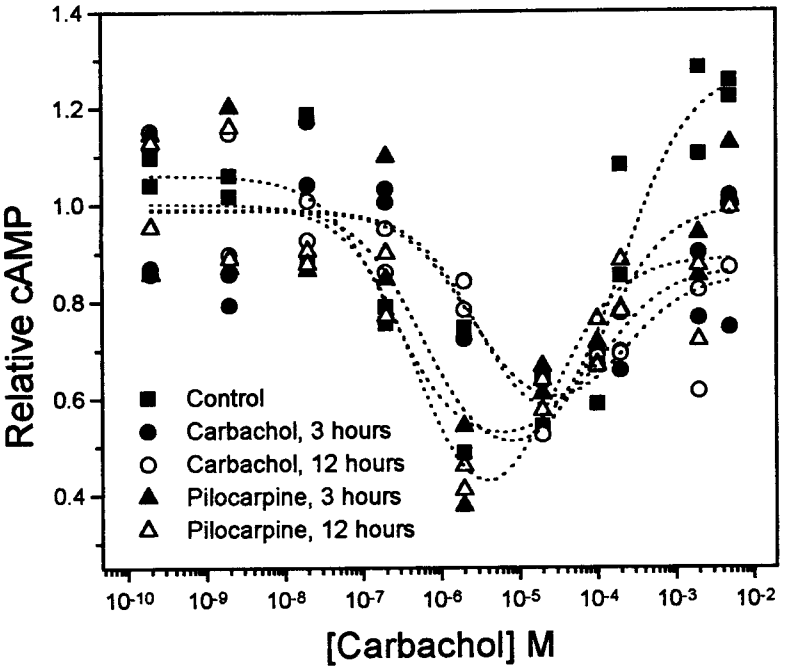

Figure 4.15: Desensitization of forskolin-stimulated inhibition of cAMP formation for high expression wild-type mAchR.

Data are presented as cAMP generated relative to forskolin-stimulated levels and are from representative experiments for pretreatment with carbachol for 3 (n=4) or 12 hours (n=2), or with pilocarpine for 3 (n=4) or 12 hours (n=2). Cells were pretreated with the agonists indicated (1 mM) for the specified times in FD + 10% calf serum, followed by second messenger assays using the agonist carbachol. Curves were derived from a least-squares fit as described in (Bulseco and Schimerlik, 1996). Average values are summarized in Table 4.3. The fitted parameters were estimated to be IC50 = 0.23 ± 0.16 μM, EC50 = 22.0 ± 11.0 μM, 54.1 ± 8.4 % inhibition; IC50 = 3.56 ± 2.42 μM, EC50 = 91.2 ± 14.4 μM, 57.6 ± 34.3 % inhibition; IC50 = 2.53 ± 1.73 μM, EC50 = 210 ± 35 μM, 46.7 ± 17.4 % inhibition; IC50 = 0.59 ± 0.26 μM, EC50 = 100.3 ± 19.6 μM, 56.9 ± 16.8 % inhibition and IC50 = 0.54 ± 0.47 μM, EC50 = 21.7 ± 12.4 μM, 76.0 ± 25.8 % inhibition for control, 3 hour carbachol treatment, 12 hour carbachol treatment, 3 hour pilocarpine treatment and 12 hour pilocarpine treatment respectively.

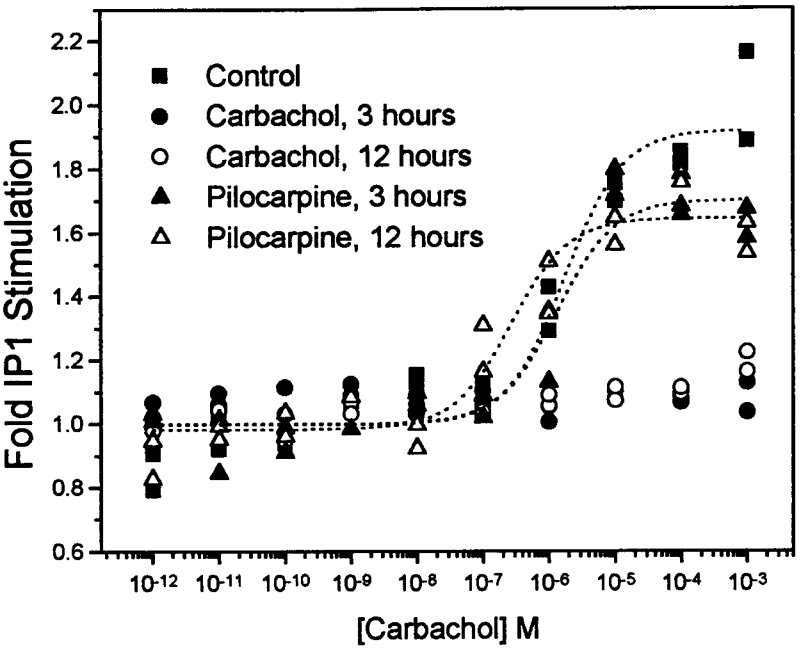

Figure 4.16: Desensitization of PI hydrolysis for low expression wild-type pm2 mAchR.

The data are presented as fold stimulation of PI hydrolysis over basal levels of inositol monophosphate for control cells and are from representative experiments for pretreatment with carbachol for 3 (n=10) or 12 hours (n=3), or with pilocarpine for 3 (n=4) or 12 hours (n=2). Cells were pretreated with the agonists indicated (1 mM) for the specified times in FD + 10% calf serum, followed by assays for PI metabolism using the agonist, carbachol. Curves were derived from a least-squares fit of equation 1. Fitted parameters were estimated to be EC50 = 1.9 ± 0.7 μM, 1.9 ± 0.3 fold stimulation; EC50 = 1.3 ± 0.5 μM, 1.7 ± 0.3 fold stimulation and EC50 = 0.3 ± 0.1 μM, 1.7 ± 0.3 fold stimulation for control, 3 hour pilocarpine and 12 hour pilocarpine treatments respectively. Carbachol treatments resulted in small responses and poorly determined fits.

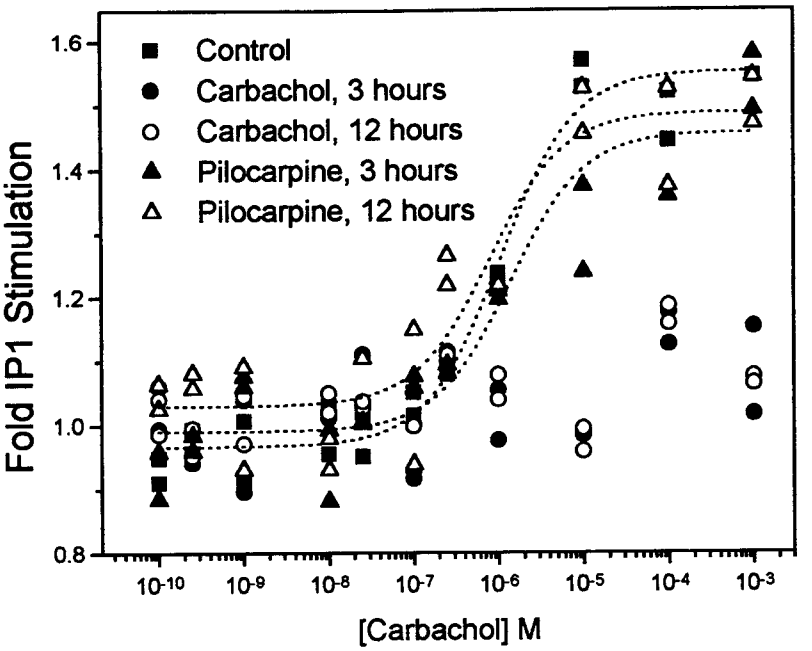

Figure 4.17: Desensitization of PI hydrolysis for high expression wild-type pm2 mAchR.

The data are presented as fold stimulation of PI hydrolysis over basal levels of inositol monophosphate for control cells and are from representative experiments for pretreatment with carbachol for 3 (n=4) or 12 hours (n=2), or with pilocarpine for 3 (n=2) or 12 hours (n=2). Cells were pretreated with the agonists indicated (1 mM) for the specified times in FD + 10% calf serum, followed by assays for PI metabolism using the agonist, carbachol. Curves were derived from a least-squares fit of equation 1. Fitted parameters were estimated to be EC50 = 1.1 ± 0.3 μM, 1.6 ± 0.1 fold stimulation; EC50 = 1.4 ± 0.6 μM, 1.5 ± 0.2 fold stimulation and EC50 = 0.8 ± 0.4 μM, 1.5 ± 0.2 fold stimulation for control, 3 hour pilocarpine and 12 hour pilocarpine treatments respectively. Carbachol treatments resulted in small responses and poorly determined fits.

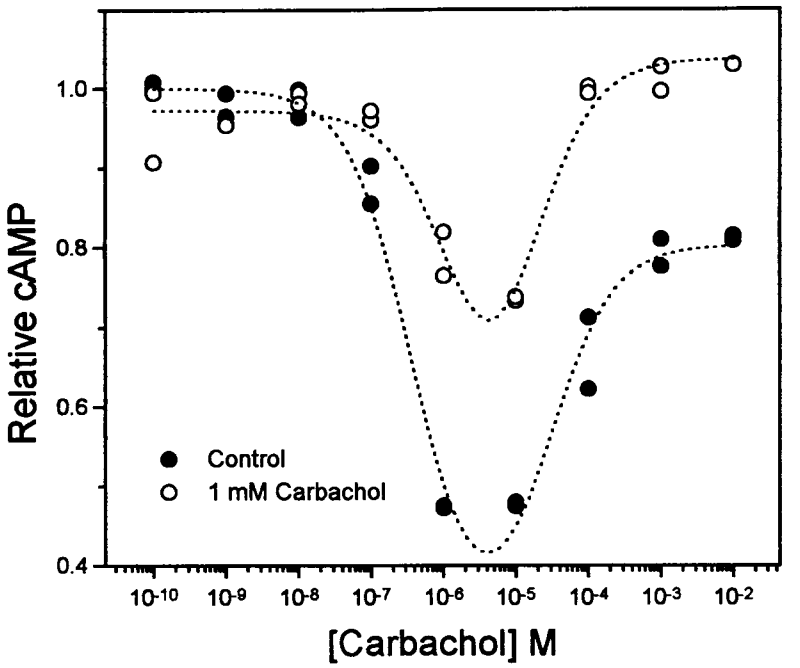

Figure 4.18: Desensitization of wild-type pm2 mAchR in the absence of calf serum.

Data are presented as cAMP generated relative to forskolin-stimulated levels and are from representative experiments. Cells were pretreated with 1 mM carbachol for 3 hours in FD media without calf serum, followed by second messenger assays using the agonist carbachol. Curves were derived from a least-squares fit as described in (Bulseco and Schimerlik, 1996). The fitted parameters were estimated to be IC50 = 0.37 ± 0.10 μM, EC50 = 30.0 ± 10.0 μM, 71.0 ± 6.8 % inhibition and IC50 = 1.5 ± 1.1 μM, EC50 = 10.2 ± 2.8 μM, 57.2 ± 5.2 % inhibition for control and carbachol treated cells respectively.

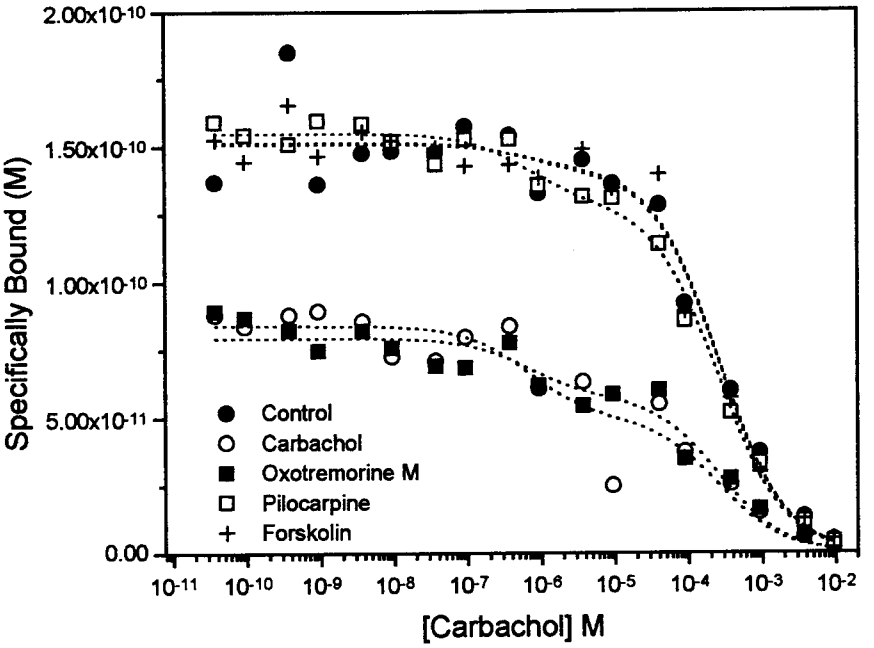

Figure 4.19: Carbachol competition binding curves after agonist treatment of cl.5.

Data are from a representative experiment presented as specifically bound [^{3}H]NMS (M) for untreated CHO cells expressing approximately 1.0 x 10^6 receptors per cell, or for cells previously treated with 1 mM carbachol (n=4), oxotremorine M (n=2), pilocarpine (n=2) or 50 μM forskolin (n=2). Curves were derived from a least-squares fit to equation 5 as described in (Vogel et al., 1995) with Lo fixed at 2.5 nM, Kd at 0.4 nM, with K1 and K2 shared across all data. The fitted parameters were estimated to be K1 = 0.09 ± 0.05 μM, K2 = 36.1 ± 4.4 μM with associated sites R1 = 12.7 ± 7.6 pM (7.2 ± 4.3 %), R2 = 164.5 ± 6.2 pM (92.8 ± 3.5 %) for untreated cells; R1 = 40.1 ± 7.2 pM (41.0 ± 7.4 %), R2 = 57.8 ± 6.3 pM (59.0 ± 6.4 %) for carbachol treated cells; R1 = 25.8 ± 7.1 pM (27.8 ± 7.7 %), R2 = 66.8 ± 5.9 pM (72.2 ± 6.3 %) for oxo M treated cells; R1 = 30.3 ± 7.6 pM (16.7 ± 4.2 %), R2 = 150.7 ± 6.5 pM (83.3 ± 3.6 %) for pilocarpine treated cells; and R1 = 10.7 ± 7.4 pM (6.0 ± 4.2 %), R2 = 165.8 ± 6.2 pM (94.0 ± 3.5 %) for forskolin treated cells.

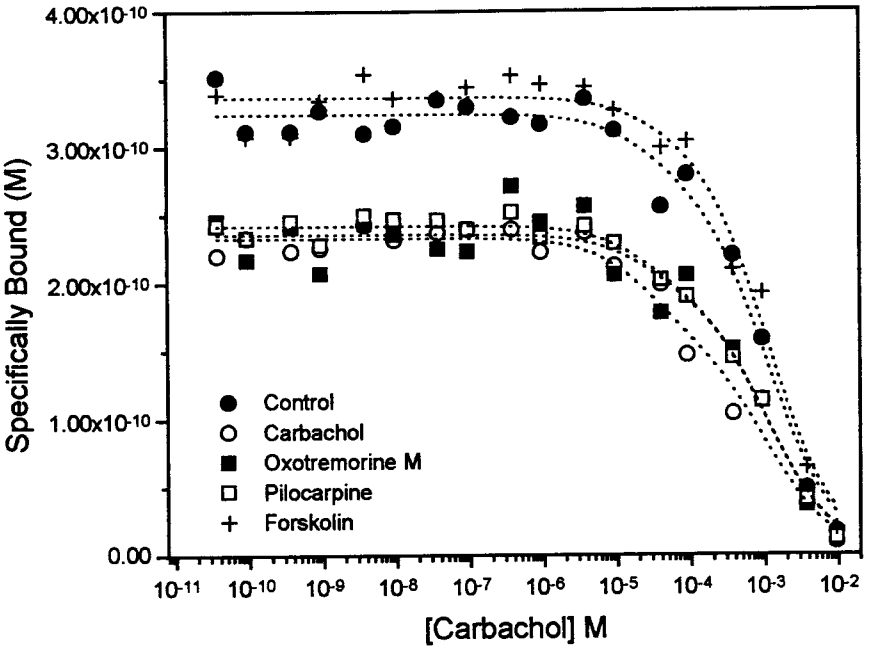

Figure 4.20: Carbachol competition binding curves after agonist treatment of cl.1.

Data are from a representative experiment (n=2) presented as specifically bound [^{3}H]NMS (M) for untreated CHO cells expressing greater than 2.0 x 10^6 receptors per cell, or for cells previously treated with 1 mM carbachol, oxotremorine M, pilocarpine or 50 μM forskolin. Curves were derived from a least-squares fit to equation 5 as described in (Vogel et al., 1995) with Lo fixed at 2.5 nM, Kd at 0.4 nM, with K1 and K2 shared across all data. The fitted parameters were estimated to be K1 = 4.1 ± 2.9 μM, K2 = 159.2 ± 25.3 μM with associated sites R1 = 57.7 ± 24.8 pM (15.1 ± 6.5 %), R2 = 323.9 ± 24.7 pM (84.9 ± 6.5 %) for untreated cells; R1 = 92.2 ± 24.3 pM (33.8 ± 8.9 %), R2 = 180.6 ± 24.4 pM (66.2 ± 9.0 %) for carbachol treated cells; R1 = 52.9 ± 21.5 pM (19.1 ± 7.8 %), R2 = 223.5 ± 20.9 pM (80.9 ± 7.6 %) for oxo M treated cells; R1 = 61.2 ± 22.2 pM (21.6 ± 7.8 %), R2 = 223.7 ± 21.9 pM (78.4 ± 7.7 %) for pilocarpine treated cells; and R1 = 33.8 ± 23.6 pM (8.5 ± 5.9 %), R2 = 363.8 ± 23.3 pM (91.5 ± 5.9 %) for forskolin treated cells.

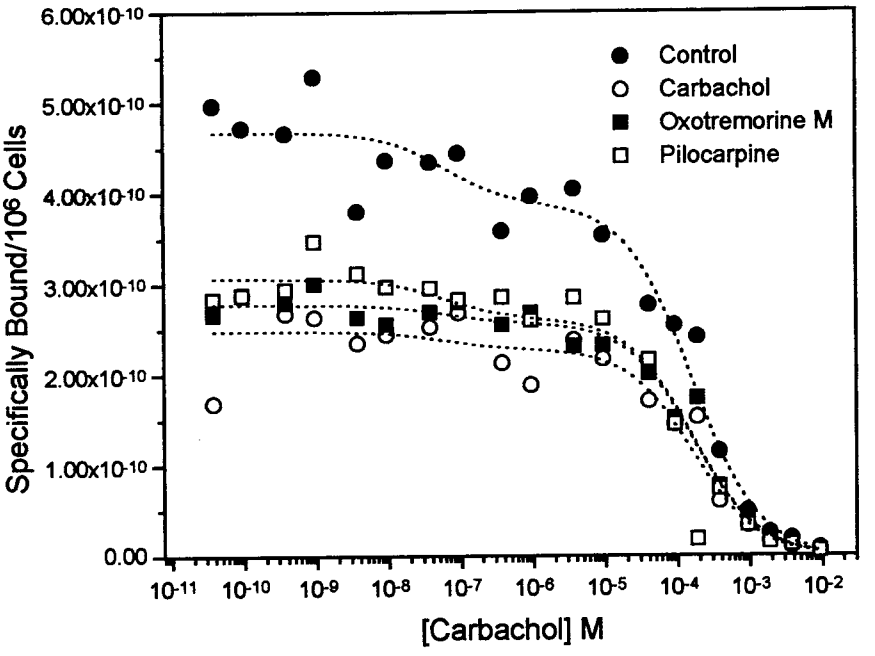

Figure 4.21: Carbachol competition binding curves after agonist treatment of D69N.

Data are from a representative experiment (n=2) presented as specifically bound [^{3}H]NMS (M) per 10^6 cells, for untreated CHO cells expressing greater than 2.0 x 10^6 D69N mutant receptors per cell, or for cells previously treated with 1 mM carbachol, oxotremorine M, or pilocarpine. Curves were derived from a least-squares fit to equation 5 as described in (Vogel et al., 1995) with Lo fixed at 2.5 nM, Kd at 0.4 nM, with K1 and K2 shared across all data. The fitted parameters were estimated to be K1 = 0.09 ± 0.01 μM, K2 = 22.8 ± 3.0 μM with associated sites R1 = 93.7 ± 22.7 pM (16.9 ± 4.1 %), R2 = 461.1 ± 19.1 pM (83.1 ± 3.4 %) for untreated cells; R1 = 24.0 ± 21.5 pM (8.2 ± 7.4 %), R2 = 267.8 ± 15.6 pM (91.8 ± 5.3 %) for carbachol treated cells; R1 = 24.7 ± 21.6 pM (7.5 ± 6.6 %), R2 = 302.8 ± 15.8 pM (92.5 ± 4.8 %) for oxo M treated cells; and R1 = 52.2 ± 21.8 pM (14.5 ± 6.0 %), R2 = 308.6 ± 16.4 pM (85.5 ± 4.5 %) for pilocarpine treated cells.

Table 4.1: Summary of internalization and recycling rate constants

Summary of the rate constants determined by nonlinear curve fitting as described in "Materials and Methods" and shown in Figures 4.4, 4.5 and 4.6. Rates are presented as % receptors/cell per minute, and Re0 in % receptors. Rates for new receptor synthesis (k_1) were determined as shown in figures 4.11, 4.12 and 4.13 and were treated as constants throughout the rest of the analysis.

Treatment	k_1	k_2	k_3	k_4	Re0
cl.5					
Control	0.002 ± 0.001	0.012 ± 0.003	0.0026 ± 0.0005	0.031 ± 0.069	23.41 ± 1.94
Carbachol	0.002 ± 0.001	0.054 ± 0.014	0.074 ± 0.005	0.276 ± 0.093	47.15 ± 3.86
Pilocarpine	0.002 ± 0.001	0.019 ± 0.008	0.005 ± 0.001	0.054 ± 0.013	39.79 ± 7.45
cl.1					
Control	0.003 ± 0.001	0.011 ± 0.003	0.003 ± 0.001	0.014 ± 0.066	21.34 ± 1.26
Carbachol	0.003 ± 0.001	0.043 ± 0.013	0.059 ± 0.011	0.375 ± 0.237	26.83 ± 2.72
Pilocarpine	0.003 ± 0.001	0.027 ± 0.009	0.043 ± 0.008	0.090 ± 0.029	29.55 ± 4.10
D69N					
Control	0.069 ± 0.026	0.0595 ± 0.013	0.007 ± 0.001	0.081 ± 0.034	10.37 ± 4.97
Carbachol	0.069 ± 0.026	0.044 ± 0.007	0.109 ± 0.011	0.090 ± 0.020	28.72 ± 1.60
Pilocarpine	0.069 ± 0.026	0.043 ± 0.007	0.100 ± 0.019	0.001 ± 0.003	30.39 ± 1.68

Table 4.2: Ratios of the rate constants are altered in response to agonist treatment

Ratios of the rate constants (k_2/k_3 and k_1/k_4) reported in Table 4.1 for CHO cells expressing about 1.0 x 10^6 receptors per cell (cl.5, low expression), greater than 2.0 x 10^6 receptors per cell (cl.1, high expression) or the D69N mutant mAchR expressing greater than 2.0 x 10^6 receptors per cell.

	Low Expression (cl.5)		High Expression (cl.1)		D69N	
	k_2/k_3	k_1/k_4	k_2/k_3	k_1/k_4	k_2/k_3	k_1/k_4
Control	4.62	0.07	3.67	0.21	8.50	0.85
Carbachol	0.73	0.01	0.73	0.01	0.40	0.77
Pilocarpine	3.80	0.04	0.63	0.03	0.43	69.00

Table 4.3: Desensitization of wild-type pm2 mAchR to inhibition of adenylyl cyclase.

Assays were conducted as described in "Materials and Methods", and analyzed as described in (Bulseco and Schimerlik, 1996). Values are the mean ± SD of *n* experiments, each done in duplicate. CHO cells expressing ~ 1.0 x 10^6 m2 AchR per cell (low expression) or greater than 2.0 x 10^6 receptors per cell (high expression) were exposed to saturating concentrations of agonists (1 mM) for either 3 or 12 hours and assays for inhibition of forskolin-stimulated adenylyl cyclase were conducted.

	Low expression			*High Expression*		
	IC50 (μM)	**Max Inhibition (%)**	**n**	**IC50 (μM)**	**Max Inhibition (%)**	**n**
Untreated[a]	0.25 ± 0.02	70.8 ± 1.8	12	0.6 ± 0.2	60.2 ± 2.6	5
(3 hours)						
Carbachol	4.7 ± 1.1	35.4 ± 2.7	10	3.9 ± 0.6	48.2 ± 4.8	4
Pilocarpine	0.61 ± 0.21	62.6 ± 3.0	4	0.3 ± 0.1	64.5 ± 2.7	4
(12 hours)						
Carbachol	4.3 ± 0.3	37.2 ± 2.2	2	3.3 ± 0.8	43.1 ± 3.6	2
Pilocarpine	0.54 ± 0.39	75 ± 3	2	0.4 ± 0.2	73.0 ± 3.0	2

Table 4.4: Desensitization of wild-type pm2 mAchR to stimulation of PI metabolism.

Assays were conducted as described in "Materials and Methods", and analyzed as described in (Bulseco and Schimerlik, 1996). Values are the mean ± SD of *n* experiments, each done in duplicate. CHO cells expressing ~ 1.0×10^6 m2 mAchR per cell (low expression) or greater than 2.0×10^6 receptors per cell (high expression) were exposed to saturating concentrations of agonists (1 mM) for either 3 or 12 hours and assays for stimulation of PI hydrolysis conducted.

	Low expression			*High Expression*		
	EC50 (μM)	**Max Stimulation (%)**	**n**	**EC50 (μM)**	**Max Stimulation (%)**	**n**
Untreated[a]	2.5 ± 0.5	1.66 ± 0.05	10	2.8 ± 0.5	1.56 ± 0.02	4
(3 hours)						
Carbachol	a	1.10 ± 0.03	10	a	1.14 ± 0.02	4
Pilocarpine	2.9 ± 0.6	1.58 ± 0.05	4	2.0 ± 0.6	1.48 ± 0.03	2
(12 hours)						
Carbachol	a	1.10 ± 0.05	2	a	1.14 ± 0.05	2
Pilocarpine	1.3 ± 0.6	1.62 ± 0.10	3	2.1 ± 1.3	1.45 ± 0.05	2

[a] Small response resulted in poorly determined fit for EC50.

Agonist binding properties after receptor internalization

Ligand binding properties of wild-type and D69N mutant AchR were difficult to characterize after long exposure to saturating concentrations of agonists. Surface receptor sites were reduced by this treatment, but agonist binding affinities of the receptor that remained was not altered in any obvious way. For this reason, competition binding experiments were analyzed by sharing parameters for high (K_H) and low (K_L) affinity binding sites for all treatments. The fractions of receptor in each affinity state were fit, and are presented as both concentration of sites and percent sites in the legends for Figures 4.19, 4.20 and 4.21.

Treatment of CHO cells expressing low and high wild-type m2 mAchR numbers resulted in a 45% and 48% loss of surface receptor sites for carbachol and oxo M treatments, and 29%, 28% and 26% reduction for carbachol, oxo M and pilocarpine treatments respectively. Treatment with 50 μM forskolin, a concentration sufficient to elevate intracellular cAMP levels, had no effect on surface receptor expression of wild-type m2 mAchR, consistent with findings presented in Chapter 3. Treatment of CHO cells expressing D69N mutant AchR resulted in 47%, 41% and 35% reduction of surface receptor numbers for carbachol, oxo M and pilocarpine respectively. Agonist treatment also appeared to alter the fraction of receptors in low and high affinity states, but not in any consistent way. This may be explained by insufficient data for the kind of analysis applied in these experiments.

Model for receptor internalization and recycling

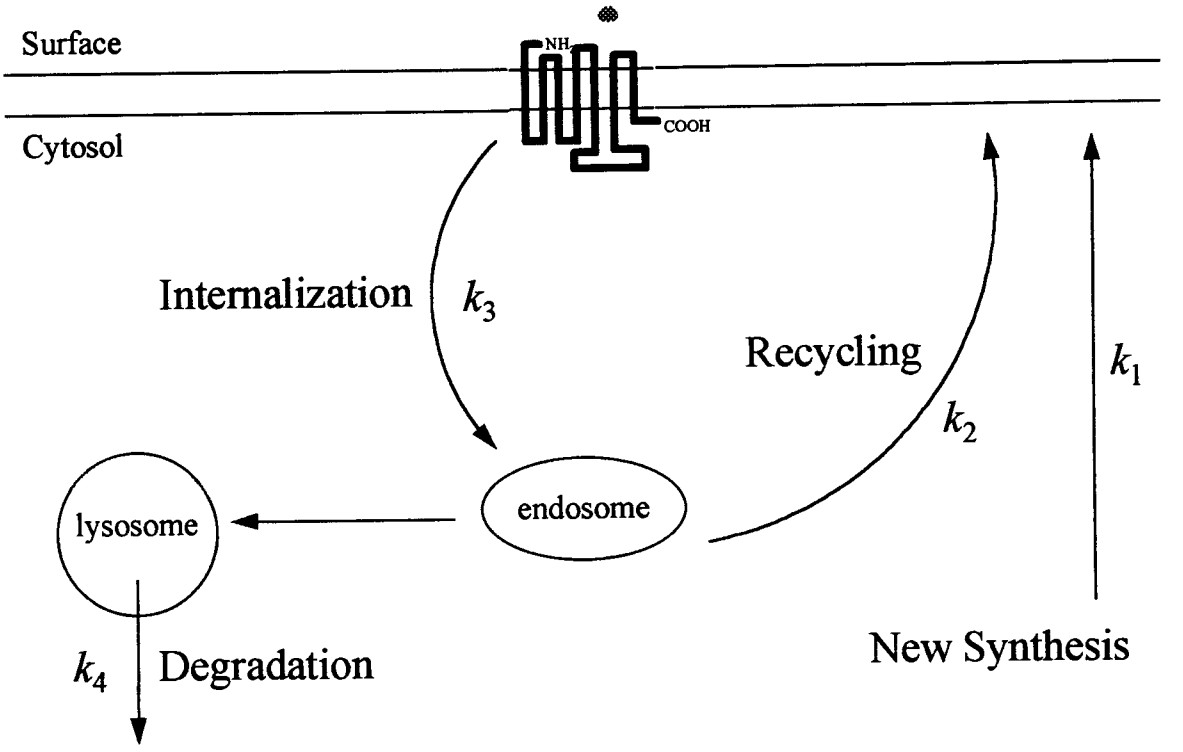

Figure 4.22: Model for receptor internalization and recycling

G-protein coupled receptors are internalized, recycled and degraded in response to persistent agonist treatment. The respective rates of internalization and recycling appear to be agonist and receptor density dependent, and are independent of effector coupling. The rate of receptor degradation also appears to be agonist-dependent, but may be independent of receptor density. In addition, effector coupling may be necessary to increase the rate of receptor degradation in response to binding of agonists.

Discussion

Role of receptor internalization and recycling

Trafficking of membrane receptors is a dynamic process. This report as well as other studies on muscarinic AchRs (Koenig and Edwardson, 1996; Koenig and Edwardson, 1994) demonstrate that receptors are being rapidly turned over even if net loss of surface receptors are not observed. When CHO cells expressing low receptor levels were exposed to pilocarpine, there was no measurable loss in surface receptor sites, although an increase in receptor trafficking was observed. Assuming initial surface receptors sites of 10^6 receptors per cell, receptor internalization was increased from 2,600 receptors/cell/minute to 5000 receptors/cell/minute after pilocarpine treatment. Using initial endosomal receptor numbers of 234,500 and 397,790 receptors/cell for untreated and pilocarpine treated cells respectively, receptor recycling was also increased from 2,809 to 7,560 receptors/cell/minute. Exposure to pilocarpine resulted in a net increase in receptor movement from 5,409 to 12,560 receptors/cell/minute, even if no measurable change in surface receptor numbers was observed.

The role of receptor internalization and recycling is not well understood, but it has been suggested that recycling is necessary for dephosphorylation and resensitization (Fonseca et al., 1995; Pippig et al., 1995; Yu et al., 1993) of G-protein coupled receptors. Although our results show agonist-dependent changes in recycling and internalization rates, it appears that desensitization may be in part due to recycled receptors that are

refractory to coupling. For wild-type receptor, the full agonist carbachol increases the rate of recycling to a greater extent than does pilocarpine at both low and high receptor expression levels. Since only the full agonist results in receptor desensitization, a period of refractoriness may be necessary to allow the recycled receptor to "reassociate" with G-proteins or other signal transduction proteins.

Pilocarpine leads to a reduction in surface receptors available for ligand binding in CHO cells with high m2 receptor densities and not in cells with low receptor numbers, but this partial agonist does not lead to desensitization. This may be explained by different fates of internalized receptor (i.e. differences in efficiency of phosphorylation or dephosphorylation) or possibly that pilocarpine leads to fewer refractory receptors in the plasma membrane than does carbachol. This is supported by the observation that stimulation of PI metabolism for wild-type m2 AchR is slightly reduced relative to control cells after treatment with pilocarpine, but is completely abolished after treatment with carbachol. If receptors that remain are refractory to stimulation after carbachol treatment but not after pilocarpine treatment, the results observed would be expected. Furthermore, carbachol treatment reduces surface receptor numbers by 50%, leaving at least 10^6 receptors per cell for the high expression cell line and 10^5 receptors for the low expression cell lines. Reduction of surface receptor to these levels with the slowly dissociating antagonist *l*-QNB resulted in a measurable coupling to PI metabolism for wild-type pm2 mAchR (Vogel et al., 1995). Clearly, the fate of receptors after antagonist treatment is different than after exposure to agonists.

Agonist-promoted internalization is dependent on receptor density

Receptor density appears to play an important role in properties of signal transduction (Vogel et al., 1995; Whaley et al., 1995; Whaley et al., 1994). In support of these findings, we are reporting that receptor density determines the internalization properties of wild-type pm2 mAchR when expressed in CHO cells. Rates of receptor internalization and recycling are similar for untreated CHO cells expressing low and high receptor numbers. For the low expression cell line, carbachol leads to an increase in both recycling and internalization rates, while pilocarpine stimulates a slight increase in rate of recycling. The rate for receptor internalization is unaffected by pilocarpine treatment at low receptor densities. Pilocarpine may promote a receptor conformation that interacts differently with available G-proteins which prevents receptor internalization. This is consistent with the finding that agonist regulates an early step in β_2 adrenergic receptor endocytosis (von Zastrow and Kobilka, 1994), specifically the redistribution of receptors to microdomains of the plasma membrane, from which endocytosis and receptor recycling can occur passively. It is not clear if pilocarpine and carbachol result in differences in plasma membrane distribution of the pm2 mAchR, but it is clear that more receptor movement, as measured by receptor internalization and recycling, occurs following treatment with carbachol. CHO cells expressing low receptor numbers internalize 74,000 receptors/minute following carbachol treatment, while pilocarpine leads to internalization of about 5,000 receptors/minute. In addition, about 25,461 receptors are recycled per minute after carbachol treatment while pilocarpine leads to recycling of 7,560 receptors/minute. These observations provide indirect support for agonist-dependent

differences in receptor redistribution, although the mechanisms regulating this receptor movement is not yet characterized.

Interestingly, when pm2 mAchR is expressed at high levels in CHO cells, both carbachol and pilocarpine result in a reduction of surface receptor. Treatment with carbachol, and to a lesser extent pilocarpine, results in increased rates of receptor internalization and recycling. Receptor movement may be modulated by other proteins, perhaps G-proteins, located in the plasma membrane of CHO cells. If receptor is in large excess over this modulatory factor, receptor movement may not be regulated, and agonist-promoted internalization may occur for both full and partial agonists. Alternatively, excess receptor may lead to a pattern of receptor distribution predisposed to agonist-mediated endocytosis, and redistribution to specific microdomains may be unnecessary. It is not clear if receptor distribution is different at low and high receptor densities, and specific experiments with epitope tagged receptors are needed to answer this question.

Both receptor distribution and the effects of specific agonists may also contribute to receptor internalization via two pathways. Studies conducted on the cholecystokinin receptor, a peptide G-protein coupled receptor, demonstrated that both coated and smooth pits are utilized during agonist-mediated receptor endocytosis (Roettger et al., 1995). Smooth vesicles appear to remain closer to the plasma membrane, and may be responsible for receptor dephosphorylation and recycling, while clathrin-coated vesicles may be responsible for receptor down-regulation and degradation. This model suggests that at least two specific microdomains exist in the plasma membranes of CHO cells. This agrees well with our observations that carbachol increases the rate of receptor degradation

to a much greater extent than does pilocarpine. For wild-type receptor, the observed rate constant is increased by 9 and 27 fold for low and high receptor expression respectively. When initial endosomal receptor is taken into account for the specific cell lines, this translates into 130,134 and 201,255 receptors degraded per minute after exposure to carbachol, while basal levels observed in untreated cells are 2,600 and 5,975 receptors/minute respectively. The pathway taken by pm2 mAchRs may be determined in part by the agonist used in the experiments, with carbachol exposure resulting in receptor internalization by coated pits (Maloteaux and Hermans, 1994) and pilocarpine predominately by non-coated pits (Roettger et al., 1995). If further experiments demonstrate that agonist-dependent trafficking does occur, this may help explain the finding that the full agonist carbachol but not the partial agonist pilocarpine results in desensitization of the pm2 AchR.

Desensitization

Assessment of desensitization is difficult since measured response is dependent on receptor density. As surface receptor number is decreased, coupling properties of the pm2 mAchR is altered as indicated by a rightward shift in IC50 for inhibition of adenylyl cyclase and a reduction in maximal response for PI hydrolysis while maximal percent inhibition of adenylyl cyclase and EC50 for PI hydrolysis remains unchanged (Vogel et al., 1995).

Exposing CHO cells expressing wild-type pm2 mAchR to carbachol results in both a rightward shift in IC50 for inhibition of AC and a reduction in maximal stimulation of PI hydrolysis. Although a reduction in total surface receptor number would lead to the same

results, this cannot explain all of the observations we have made. Exposing these cells to pilocarpine, cGMP or SNP (Chapter 3) also results in significant reduction in surface receptor sites without a measurable change in effector coupling properties. These findings imply that receptor internalization alone is not sufficient for receptor desensitization.

Desensitization is observed only after treatment with full agonists carbachol and oxo M. The D69N mutant mAchR clearly demonstrates that receptor internalization does not require receptor-effector coupling. In addition, desensitization can be promoted by full agonists that do not activate receptor-effector coupling for this mutant (Chapter 3). This implies that desensitization is also independent of effector activation, or is dependent on activation of effector systems that we have not measured. Both of these observations may also be explained by receptor internalization and recycling properties that are promoted by full and partial agonists.

Model

G-protein coupled receptors are internalized, recycled and degraded in response to persistent agonist treatment. The rate constants for each of these steps are shown in Figure 4.22. Agonist-promoted receptor internalization (k_3) and recycling (k_2) rates appear to be agonist and receptor density dependent and independent of effector coupling. The rate of receptor degradation (k_4) also appears to be agonist dependent, but may be independent of receptor density. Furthermore, effector coupling may be necessary before an increase in receptor degradation occurs in response to agonist binding. The model is discussed in greater detail below.

This model suggests that receptor internalization and recycling properties are independent of receptor desensitization. It is clear that different ligands possess different properties when bound to their receptors. While antagonists inhibit physiological responses, agonists may stimulate full or partial responses. In addition, full agonists appear to promote different receptor conformations which affect the ability of the receptor to activate effectors (Chapters 2 and 3). These examples support a model in which receptor internalization and recycling properties are dependent on agonists — different agonists may simply lead to slightly different receptor conformations that affect the fate of a liganded receptor. In addition, other factors may modulate these properties, and high receptor density may have an impact on the effectiveness of these modulators. G-proteins are down-regulated with receptors (Dell'Acqua et al., 1993; Wise et al., 1995), presumably as RG complexes, and may be limiting when receptor is in large excess. Furthermore, desensitization may be regulated by numerous events that include receptor internalization and recycling as well as receptor phosphorylation and rapid uncoupling.

The fate of internalized receptor may depend both on the bound ligand as well as coupling to effector systems. It is not clear where internalized receptors are stored, and the model proposed in Figure 4.22 combines all of these internal compartments into endosomes or lysosomes. This oversimplification may not be adequate to explain the fate of internalized receptors. For example, it is not understood how receptors stored in endosomes are targeted for degradation via lysosomes. Perhaps the more important questions are how are receptors targeted for recycling and what are the roles of specific agonists on these processes?

The specific pathways taken by receptors into the intracellular compartments may determine the fate of the receptors. Characterization of cholecystokinin receptor internalization demonstrates that the receptor can take two paths into the cell (Roettger et al., 1995). Agonist treatment promoted receptor internalization by both coated and non-coated endocytotic pits, presumably clathrin-coated and caveolin associated respectively. Furthermore, receptor phosphorylation appears to occur when the receptor is localized to the plasma membrane while dephosphorylation occurs in intracellular compartments. They have proposed that endocytosis via smooth vesicles results in receptor resensitization and recycling while clathrin mediated endocytosis results in receptor degradation and down regulation. A more complete model should therefore include internalization via two distinct pathways, with specific fates of the receptor determined by the pathway taken.

Desensitization of responses are much more difficult to assess for the pm2 mAchR expressed in CHO cells. It is not clear if loss of response occurs due to depletion of other proteins necessary for signal transduction, or an apparent loss of response is merely a passive result of reduced receptor density. The full agonist carbachol appears to promote desensitization while the partial agonist pilocarpine does not. Since both agonists will promote net receptor internalization by affecting both k_2 and k_3, desensitization must not be dependent solely on receptor movement. Cabachol treatment results in a four to five fold greater increase in receptor degradation rate (k_4) than is induced by the partial agonist pilocarpine for both low and high expression CHO cell lines. It is not clear how these agonists would promote different fates for internalized receptor. It is possible that both agonist used and receptor density affects trafficking properties of internalized receptor.

One additional possibility is that the fate of these receptors are also dependent on receptor-effector coupling. Receptor degradation rate is not increased after treatment with carbachol or pilocarpine for the D69N mutant mAchR. Since these agonists do not activate effector systems, degradation of the internalized receptor may not be stimulated, consistent with our results (Table 4.1). Desensitization cannot be dependent on degradation though, since carbachol will promote desensitization of D69N to both inhibition of adenylyl cyclase and stimulation of PI hydrolysis by the agonist oxo M (Chapter 3) even if it does not promote an increase in (k_4). This suggests that a full agonist promotes a unique series of events while receptors are being internalized and recycled, perhaps in endosomes, or that much of the desensitization observed is due to depletion of other factors required for signal transduction.

Summary

In summary, we have shown that the pm2 mAchR expressed in CHO cells undergoes rapid receptor internalization and recycling following prolonged treatment with agonists. This response is both agonist and receptor density dependent, and apparently independent of effector coupling. Studying the relative rates of agonist-promoted receptor internalization and recycling indicate that this process is regulated by mechanisms distinct from receptor desensitization. Finally, the components of this process appears to possess agonist and receptor density dependent and independent steps. Further studies should provide insight into the specific mechanisms regulating the many steps in receptor internalization and desensitization.

Chapter 5

Conclusions

m2 muscarinic receptor structure-function relationship

The primary objective of this research is to investigate the relationship between m2 muscarinic receptor structure and functional coupling to effector systems using site-directed mutagenesis. Specific amino acids in the third intracellular loop were mutated with two primary criteria—alter the charge in a particular position, or convert m2/m4 conserved amino acids to those conserved in the m1/m3/m5 family. Unfortunately, there are no available methods to follow alterations in receptor structure after mutagenesis, and data collected is limited to bioassays that are several steps removed from an agonist-promoted conformational change.

Analysis and interpretation of "structural" changes are therefore limited by our ability to assess structural changes, or the changes in agonist-promoted conformation after mutating the receptor. Studies on secondary structure have been attempted in our laboratory (Peterson et al., 1995), but information gained from circular dichroism studies fail to answer the most important questions. How are the agonist-promoted conformational changes affected by site-directed mutants? Are receptor/G-protein

interactions altered even if agonist-promoted conformational events are unchanged by the mutants? Experiments conducted using second messenger assays provide information on effector coupling efficiency and potency. Although an indirect measure of structural changes, or more precisely, a reasonably good measure of mutations that do not affect coupling properties and presumably structural properties, there are other factors that affect the observed responses in whole cells which greatly complicate analysis of these mutant receptors.

In general, the context in which the receptors are expressed appear to have a large effect on their functional properties. Since we must rely on their functional properties to draw inferences on changes in structure, it is important to characterize these effects thoroughly. Some of the more obvious factors include receptor density, co-expression of other proteins in the signal transduction pathway and expression of other as yet unidentified factors that modulate functioning of the receptor.

Finally, questions relating to organization of receptors, and receptor/G-protein effector systems in the plasma membrane may in addition play a large role in our ability to interpret functional coupling studies. Evidence indicates that certain G-proteins are localized in caveolae, non-coated pits in the plasma membranes of mammalian cells, as are specific receptor proteins (including G-protein coupled receptors) (Anderson, 1993; Chun et al., 1994; Roettger et al., 1995). Agonist-promoted diffusion of the β_2 adrenergic receptor appears to terminate at specific locations in the plasma membrane of Chinese hamster ovary cells (von Zastrow and Kobilka, 1994). Cholecystokinin receptors (a G-protein coupled receptor) appear to be protected from the effects of acid washing after

agonist treatment (Roettger et al., 1995a; Roettger et al., 1995b), presumably via insulation in membrane invaginations such as caveolae or clathrin coated pits. In addition, receptor organization in the plasma membrane may be dependent on receptor density.

Receptor-density dependence

Functional coupling of the m2 AchR to effector systems is receptor density dependent. Increasing receptor density results in greater maximal stimulation of PI metabolism for the pm2 mAchR, with no apparent change in EC50. For coupling to inhibition of forskolin-stimulated adenylyl cyclase activity, IC50 is inversely related to receptor density, while maximal percent inhibition is unchanged until very low receptor densities are achieved (Vogel et al., 1995).

This finding requires that mutant receptors be assessed after expression of wild-type receptor at comparable receptor densities, or after reduction in wild-type receptor density using the slowly dissociating antagonist, *l* -QNB. Antagonist knock-out of receptor sites is a common method used to address this, but may have inherent problems that further complicate analysis. For example, reduction of sites with *l*-QNB results in receptor-effector coupling properties that are different than reduction in sites following agonist treatment (Vogel et al., 1995). Others have normalized functional coupling after deriving mathematical relationships between receptor density, EC50, maximal response and Kd for agonist (Whaley et al., 1995; Whaley et al., 1994). The importance of considering receptor density has only recently been appreciated, and some of the

conclusions drawn by researchers regarding the functional effect of site-directed mutagenesis may be tenuous, pending more thorough investigation and analysis.

Assessment of functional coupling in transient transfection systems may lead to misleading results. Since receptor density plays a large role in coupling properties, results obtained with transiently transfected systems, where the average receptor density is known for the population of cells, but the distribution of the receptors throughout these cells is uncharacterized, may lead to incorrect conclusions. In addition, subtle effects on receptor-effector coupling may only be detectable after thorough study with multiple full and partial agonists.

Similar observations were made with respect to agonist-promoted receptor internalization and desensitization. The rates of receptor internalization and recycling appears to be dependent on initial receptor density as well as the agonist used in the experiment. In our studies, the differences between agonists were reduced with increasing receptor concentration, consistent with results on functional coupling.

Agonist dependence

The ligands used in these experiments can be classified as full and partial agonists as well as antagonists. While antagonists competitively bind receptor sites and either have no effect or an inhibitory effect, agonists function to activate coupling of the receptor to effector systems. Of these agonists, some are capable of "full" activation (full agonists) and some only partially activate the system (partial agonists)—i.e. elicit a less than maximal response at saturating concentrations of agonist.

We have found that full agonists are equal in their ability to activate coupling. While oxo M is a full agonist for wild-type receptor, it appears to have properties different from another full agonist, carbachol. For the mutant A212E, oxo M becomes a partial agonist for coupling to PI metabolism relative to wild-type receptor, while it maximally activates inhibition of adenylyl cyclase. The latter finding can be explained by the observation that there are "spare receptors" for coupling to adenylyl cyclase. The observation with the PI response is not as easily explained, and implies that oxo M is capable of promoting a receptor conformation different than carbachol.

This observation is also supported by the finding that effector coupling for the mutant D69N is only mediated by the agonist oxo M. Carbachol, acetylcholine and pilocarpine are incapable of activating the receptor/G-protein complex to promote effector activation. This implies that oxo M is uniquely capable of promoting a receptor conformation that can activate G-proteins, while the other full agonists carbachol and acetylcholine, and the partial agonist, pilocarpine cannot.

When characterizing coupling of hm1 mAchR to PI metabolism in CHO cells, the dose-response curves have very different appearances for oxo M and carbachol. Although both stimulate PI metabolism approximately 15 fold, and are considered full agonists for hm1, the slope factor for the two agonists are clearly different. Data obtained with the agonist carbachol is best fit with a slope factor of 1, while data for oxo M is often best fit with a slope factor closer to 0.5. Although the functional significance of these observations are unclear, it may be explained by cooperativity for oxo M binding and activation and lack of cooperativity for carbachol. These findings do point out that oxo M

appears to promote activation of effector systems with characteristics different from carbachol.

The same observation is made with agonist-promoted internalization of surface receptor. The agonist dependence appears to also depend on receptor density. At high receptor densities, all agonists appeared to have similar effects on internalization and recycling rates. At low receptor densities, the partial agonist pilocapine elicited a response different from the full agonist carbachol. It is not clear how these observations relate to receptor density.

High receptor density may result in different receptor organization in the plasma membrane. Increased interactions between receptors may result in dimers or multimers that have an impact on the functioning of the mAchR. The mAchR may be normally distributed or localized to particular areas of the plasma membrane, perhaps in close proximity with other proteins in the signal transduction pathway. High receptor density may result in distribution of receptors into areas of the cell membrane in which they would not normally be found. Alternatively, if another protein or factor that normally interacts with the receptor is in limited supply, removal of a constraint may also have an impact on how the receptor functions and may modulate the effects of full and partial agonists. Finally, agonist rates of dissociation and association may depend on the receptor organization in the plasma membrane. The observed responses may be a function of this organization and its effects of receptor interaction with agonists as well as G-proteins and effector systems.

One additional problem encountered when comparing different clonal cell lines is the possibility that selection of clones also resulted in selection of other properties. It is not clear if clonal lines achieve different receptor levels because metabolic rates of internalization and recycling are inherently different, or if the measured rates are different because of different receptor levels. For example, different clonal cell lines appear to possess different levels of forskolin-stimulated adenylyl cyclase. Although this potential problem is a concern, we do not think that it is very likely since baseline rates (untreated cells) of receptor internalization, recycling and degradation are very similar for the three clonal cell lines studied (Table 4.1).

Expression system dependence

Muscarinic acetylcholine receptors couple to inhibition of adenylyl cyclase or stimulation of PI metabolism, depending on the receptor subtype being characterized. The m2/m4 subtype family couples to inhibition of adenylyl cyclase activity while the m1/m3/m5 subtypes couples to stimulation of PI metabolism. When expressed in Chinese hamster ovary cells, the pm2 muscarinic acetylcholine receptor couples to both inhibition of adenylyl cyclase and stimulation of PI metabolism. Although it is unlikely that this occurs in normal tissue expressing pm2 AchR, it does illustrate that the receptor interaction with effector systems may be somewhat promiscuous. It is entirely possible that the m2 mAchR will preferentially interact with a specific subtype of Gi, but will also interact with other available G-proteins if receptor is in large excess.

Furthermore, some of the properties reported for G-protein coupled receptors are specific only for the expression system selected. Different tissues expressing endogenous muscarinic receptors, as well as different exogenous expression systems often lead to different results. This emphasizes that the context in which these receptors are expressed is also an important consideration when studying G-protein coupled receptors.

Summary

Regulation of signal transduction is a complex cellular process. Co-evolution of signaling ligands, receptors and signal transduction machinery has resulted in an elegant system of precise responses. Studying G-protein coupled receptors in an exogenous expression system places them in a cellular environment that may or may not be appropriate. In order to accurately assess the functioning of these receptors in expression systems, many things must be considered. First, the context of expression is important. Studying G-protein coupled receptors expressed in a variety of cellular environments may be necessary to gain a clear understanding of the basic mechanisms underlying function. Second, the level of receptor expression must be considered when studying the interaction of receptors with other signal transduction machinery. Since the context of receptor expression is important, overexpression may lead to artifactual interactions between the receptor being studied and signal transduction components that are part of the appropriate context. If excess receptor results in altered interactions, studying the basic properties of receptor function will be difficult. Third, full characterization requires that more than one agonist is studied. This approach will reduce the likelihood that subtle differences in receptor function will be overlooked. Finally, more refined methods of experimentation

and data analysis are required to uncover subtle interactions between components of a signal transduction system designed for precise interactions and cellular responses.

Bibliography

Anderson, R. G. (1993). Caveolae: where incoming and outgoing messengers meet. Proc Natl Acad Sci USA *90*, 10909-13.

Arden, J. R., Nagata, O., Shockley, M. S., Philip, M., Lameh, J., and Sadee, W. (1992). Mutational analysis of third cytoplasmic loop domains in G-protein coupling of the HM1 muscarinic receptor. Biochem Biophys Res Commun *188*, 1111-5.

Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J., and Capon, D. J. (1989). Functional diversity of muscarinic receptor subtypes in cellular signal transduction and growth. Trends Pharmacol. Sci. *Dec. Suppl.*, 16-22.

Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J., and Capon, D. J. (1989). Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cell. Cell *56*, 487-493.

Ashkenazi, A., Winslow, J. W., Peralta, E. G., Peterson, G. L., Schimerlik, M. I., Capon, D. J., and Ramachandran, J. (1987). An M2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. Science *238*, 672-678.

Barlow, R., and Blake, J. F. (1989). Hill coefficients and the logistic equation. Trends Pharmacol. Sci. *10*, 440-441.

Bauer, M. B., Murphy, S., and Gebhart, G. F. (1994). Muscarinic cholinergic stimulation of the nitric oxide-cyclic GMP signaling system in cultured rat sensory neurons. Neuroscience *62*, 351-9.

Baumgold, J. (1992). Muscarinic receptor-mediated stimulation of adenylyl cyclase. Trends Pharmacol Sci *13*, 339-40.

Bihoreau, C., Monnot, C., Davies, E., Teutsch, B., Bernstein, K. E., Corvol, P., and Clauser, E. (1993). Mutation of Asp74 of the rat angiotensin II receptor confers changes in antagonist affinities and abolishes G-protein coupling. Proc Natl Acad Sci USA *90*, 5133-7.

Birnbaumer, L. (1990). G Proteins in Signal Transduction. Annu. Rev. Pharmacol. Toxicol. *30*, 675-705.

Blüml, K., Mutschler, E., and Wess, J. (1994a). Functional role of a cytoplasmic aromatic amino acid in muscarinic receptor-mediated activation of phospholipase C. J Biol Chem *269*, 11537-41.

Blüml, K., Mutschler, E., and Wess, J. (1994b). Identification of an intracellular tyrosine residue critical for muscarinic receptor-mediated stimulation of phosphatidylinositol hydrolysis. J Biol Chem *269*, 402-5.

Bonner, T. I. (1992). Domains of muscarinic acetylcholine receptors that confer specificity of G protein coupling. Trends-Pharmacol-Sci *13*, 48-50.

Bonner, T. I. (1989). New subtypes of muscarinic acetylcholine receptors. Trends Pharmacol. Sci. *10*, 11-15.

Bonner, T. I., Buckley, N. J., Young, A. C., and Brann, M. R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. Science *237*, 527-532.

Brann, M. R., Klimkowski, V. J., and Ellis, J. (1993). Structure/function relationships of muscarinic acetylcholine receptors. Life Sci *52*, 405-12.

Brodbeck, R. M., Sachais, B. S., and Krause, J. E. (1995). Residue 78 in the second transmembrane domain of the neurokinin-1 receptor is important in coupling high affinity agonist binding to multiple second messenger responses. Mol-Pharmacol *47*, 1065-71.

Brown, J. H., and McDonough, P. M. (1989). Muscarinic Cholinergic Receptor Regulation of Inositol Phospholipid Metabolism and Calcium Mobilization. In The Muscarinic Receptors, J. H. Brown, ed. (Clifton, NJ: The Humana Press), pp. 259-307.

Bulseco, D. A., and Schimerlik, M. I. (1996). Single amino acid substitutions in the pm2 muscarinic receptor alter receptor/G protein coupling without changing physiological responses. Mol-Pharmacol *49*, 132-41.

Campbell, P. T., Hnatowich, M., O' Dowd, B., Caron, M. G., Lefkowitz, R. J., and Hausdorff, W. P. (1991). Mutations of the human beta 2-adrenergic receptor that impair coupling to Gs interfere with receptor down-regulation but not sequestration. Mol Pharmacol *39*, 192-8.

Ceresa, B. P., and Limbird, L. E. (1994). Mutation of an aspartate residue highly conserved among G-protein-coupled receptors results in nonreciprocal disruption of alpha 2-adrenergic receptor-G-protein interactions. A negative charge at amino acid residue 79 forecasts alpha 2A-adrenergic receptor sensitivity to allosteric modulation by monovalent cations and fully effective receptor/G-protein coupling. J Biol Chem *269*, 29557-64.

Chidiac, P., and Wells, J. W. (1992). Effects of adenyl nucleotides and carbachol on cooperative interactions among G proteins. Biochemistry *31*, 10908-21.

Chun, M., Liyanage, U. K., Lisanti, M. P., and Lodish, H. F. (1994). Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. Proc Natl Acad Sci USA *91*, 11728-32.

Cooper, J. R., Bloom, F. E., and Roth, R. H. (1986). The Biochemical Basis of Neuropharmacology, Fifth Edition (New York: Oxford University Press).

Cotecchia, S., Ostrowski, J., Kjelsberg, M. A., Caron, M. G., and Lefkowitz, R. J. (1992). Discrete amino acid sequences of the alpha 1-adrenergic receptor determine the selectivity of coupling to phosphatidylinositol hydrolysis. J Biol Chem *267*, 1633-9.

Dell'Acqua, M. L., Carroll, R. C., and Peralta, E. G. (1993). Transfected m2 muscarinic acetylcholine receptors couple to G alpha i2 and G alpha i3 in Chinese hamster ovary cells. Activation and desensitization of the phospholipase C signaling pathway. J Biol Chem *268*, 5676-85.

Dittman, A. H., Weber, J. P., Hinds, T. R., Choi, E. J., Migeon, J. C., Nathanson, N. M., and Storm, D. R. (1994). A novel mechanism for coupling of m4 muscarinic acetylcholine receptors to calmodulin-sensitive adenylyl cyclases: crossover from G protein-coupled inhibition to stimulation. Biochemistry *33*, 943-51.

Dixon, R., Sigal, I., Rands, E., Register, R., Candelore, M., Blake, A., and Strader, C. (1987). Ligand binding to the β-adrenergic receptor involves its rhodopsin-like core. Nature *326*, 73-77.

Eason, M. G., Kurose, H., Holt, B. D., Raymond, J. R., and Liggett, S. B. (1992). Simultaneous coupling of alpha 2-adrenergic receptors to two G-proteins with opposing effects. Subtype-selective coupling of alpha 2C10, alpha 2C4, and alpha 2C2 adrenergic receptors to Gi and Gs. J Biol Chem *267*, 15795-801.

Ferguson, S. S., Downey, W. E., Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996). Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science *271*, 363-6.

Ferguson, S. S., Menard, L., Barak, L. S., Koch, W. J., Colapietro, A. M., and Caron, M. G. (1995). Role of phosphorylation in agonist-promoted beta 2-adrenergic receptor sequestration. Rescue of a sequestration-defective mutant receptor by beta ARK1. J-Biol-Chem *270*, 24782-9.

Fonseca, M. I., Button, D. C., and Brown, R. D. (1995). Agonist regulation of alpha 1B-adrenergic receptor subcellular distribution and function. J Biol Chem *270*, 8902-9.

Fraser, C. M., Wang, C. D., Robinson, D. A., Gocayne, J. D., and Venter, J. C. (1989). Site-directed mutagenesis of m_1 muscarinic acetylcholine receptors: Conserved aspartic acids play important roles in receptor function. Mol. Pharmacol. *36*, 840-847.

Giannini, E., and Boulay, F. (1995). Phosphorylation, dephosphorylation, and recycling of the C5a receptor in differentiated HL60 cells. J-Immunol *154*, 4055-64.

Goldman, P. S., Schlador, M. L., Shapiro, R. A., and Nathanson, N. M. (1996). Identification of a Region Required for Subtype-specific Agonist-induced Sequestration of the m2 Muscarinic Acetylcholine Receptor. J Biol Chem *271*, 4215-4222.

Gudermann, T., Kalkbrenner, F., and Schultz, G. (1996). Diversity and Selectivity of Receptor-G protein Interaction. Ann. Rev. Pharmacol. Toxicol. *36*, 429-459.

Haga, K., Haga, T., and Ichiyama, A. (1990). Phosphorylation by protein kinase C of the muscarinic acetylcholine receptor. J Neurochem *54*, 1639-44.

Haga, T., Haga, K., Kameyama, K., and Nakata, H. (1993). Phosphorylation of muscarinic receptors: regulation by G proteins. Life Sci *52*, 421-8.

Hein, L., Ishii, K., Coughlin, S. R., and Kobilka, B. K. (1994). Intracellular targeting and trafficking of thrombin receptors. A novel mechanism for resensitization of a G protein-coupled receptor. J Biol Chem *269*, 27719-26.

Hirschberg, B. T., and Schimerlik, M. I. (1994). A kinetic model for oxotremorine M binding to recombinant porcine m2 muscarinic receptor expressed in Chinese hamster ovary cells. J Biol Chem *269*, 26127-26135.

Hishinuma, S., Matsumoto, Y., Uchida, M. K., and Kurokawa, M. (1993). Novel regulation of muscarinic receptors and their coupling with G proteins in smooth muscle: transient resensitization during desensitizing process. Br J Pharmacol *109*, 330-5.

Ho, B. Y., Karschin, A., Branchek, T., Davidson, N., and Lester, H. A. (1992). The role of conserved aspartate and serine residues in ligand binding and in function of the 5-HT1A receptor: a site-directed mutation study. FEBS Lett *312*, 259-62.

Högger, P., Shockley, M. S., Lameh, J., and Sadee, W. (1995). Activating and inactivating mutations in N- and C-terminal i3 loop junctions of muscarinic acetylcholine Hm1 receptors. J Biol Chem *270*, 7405-10.

Hu, J., Wang, S. Z., and el-Fakahany, E. E. (1991). Effects of agonist efficacy on desensitization of phosphoinositide hydrolysis mediated by m1 and m3 muscarinic receptors expressed in Chinese hamster ovary cells. J Pharmacol Exp Ther *257*, 938-45.

Hu, J., Wang, S. Z., and El-Fakahany, E. E. (1992). Role of conserved aspartate residue 71 in down-regulation of M1 muscarinic receptors. Pharmacol. Commun. *1*, 219-225.

Hulme, E. C., Birdsall, N. J. M., and Buckley, N. J. (1990). Muscarinic receptor subtypes. Annu. Rev. Pharmacol. Toxicol. *30*, 633-673.

Jackson, T. (1991). Structure and function of G protein coupled receptors. Pharmacol Ther *50*, 425-42.

Ji, I., and Ji, T. H. (1991). Asp383 in the second transmembrane domain of the lutropin receptor is important for high affinity hormone binding and cAMP production. J Biol Chem *266*, 14953-7.

Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G., and Wittig, B. (1991). Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. Nature *353*, 43-8.

Kleuss, C., Scherubl, H., Hescheler, J., Schultz, G., and Wittig, B. (1993). Selectivity in signal transduction determined by gamma subunits of heterotrimeric G proteins. Science *259*, 832-4.

Koenig, J. A., and Edwardson, J. M. (1996). Intracellular Trafficking of the Muscarinic Acetylcholine Receptor: Importance of Subtype and Cell Type. Mol. Pharmacol. *49*, 351-359.

Koenig, J. A., and Edwardson, J. M. (1994). Kinetic analysis of the trafficking of muscarinic acetylcholine receptors between the plasma membrane and intracellular compartments. J Biol Chem *269*, 17174-82.

Kozak, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res *15*, 8125-8148.

Kubo, T., Bujo, H., Akiba, I., Nakai, J., Mishina, M., and Numa, S. (1988). Location of a region of the muscarinic acetylcholine receptor involved in selective effector coupling. FEBS Lett *241*, 119-125.

Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., Mishina, M., Haga, T., Haga, K., Ichiyama, A., Kanagawa, K., Kohima, M., Matsuo, H., Hirose, T., and Numa, S. (1986). Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. Nature *323*, 411-416.

Kunkel, M. T., and Peralta, E. G. (1993). Charged amino acids required for signal transduction by the m3 muscarinic acetylcholine receptor. Embo J *12*, 3809-15.

Lameh, J., Philip, M., Sharma, Y. K., Moro, O., Ramachandran, J., and Sadee, W. (1992). Hm1 muscarinic cholinergic receptor internalization requires a domain in the third cytoplasmic loop. J Biol Chem *267*, 13406-12.

Lechleiter, J., Hellmiss, R., Duerson, K., Ennulat, D., David, N., Clapham, D., and Peralta, E. (1990). Distinct sequence elements control the specificity of G protein activation by muscarinic acetylcholine receptor subtypes. Embo J *9*, 4381-90.

Lee, W., Nicklaus, K. J., Manning, D. R., and Wolfe, B. B. (1990). Ontogeny of cortical muscarinic receptor subtypes and muscarinic receptor-mediated responses from rat. J Pharmacol Exp Ther *252*, 482-490.

Lee, W., Nicklaus, K. J., Manning, D. R., and Wolfe, B. B. (1990). Ontogeny of cortical muscarinic receptor subtypes and muscarinic receptor-mediated responses in rat. J Pharmacol Exp Ther *252*, 482-90.

Liggett, S. (1995). Functional Properties of Human β2-Adrenergic Receptor Polymorphisms. NIPS *10*, 265-273.

Liggett, S. B., Freedman, N. J., Schwinn, D. A., and Lefkowitz, R. J. (1993). Structural basis for receptor subtype-specific regulation revealed by a chimeric beta 3/beta 2-adrenergic receptor. Proc Natl Acad Sci USA *90*, 3665-9.

Limbird, L. E. (1986). Cell surface receptors: A short course on theory and methods (Norwell, MA: Martinus Nijhoff Publishing).

Lohse, M. J., Andexinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure, J. P., Caron, M. G., and Lefkowitz, R. J. (1992). Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. J Biol Chem *267*, 8558-64.

Maggio, R., Barbier, P., Toso, A., Barletta, D., and Corsini, G. U. (1995). Sodium nitroprusside induces internalization of muscarinic receptors stably expressed in Chinese hamster ovary cell lines. J Neurochem *65*, 943-6.

Mahan, L. C., Koachman, A. M., and Insel, P. A. (1985). Genetic analysis of β-adrenergic receptor internalization and down-regulation. Proc. Natl. Acad. Sci. USA. *82*, 129-133.

Maloteaux, J. M., and Hermans, E. (1994). Agonist-induced muscarinic cholinergic receptor internalization, recycling and degradation in cultured neuronal cells. Cellular mechanisms and role in desensitization. Biochem Pharmacol *47*, 77-88.

McKinney, M., and Richelson, E. (1989). Muscarinic Receptor Regulation of Cyclic GMP and Eicosanoid Production. In The Muscarinic Receptors, J. H. Brown, ed. (Clifton, NJ: The Humana Press), pp. 309-339.

Nathanson, N. M. (1987). Molecular Properties of the Muscarinic Acetylcholine Receptor. Ann. Rev. Neurosci. *10*, 195-236.

Neer, E. J. (1994). G Proteins: Critical control points for transmembrane signals. Protein Science *3*, 3-14.

Neer, E. J. (1995). Heterotrimeric G Proteins: Organizers of Transmembrane Signals. Cell *80*, 249-257.

Neve, K. A., Cox, B. A., Henningsen, R. A., Spanoyannis, A., and Neve, R. (1991). Pivotal role for aspartate 80 in the regulation of dopamine D2 receptor affinity for drugs and inhibition of adenylyl cyclase. Mol. Pharmacol. *39*, 733-739.

Ng, G. Y., Trogadis, J., Stevens, J., Bouvier, M., O'Dowd, B. F., and George, S. R. (1995). Agonist-induced desensitization of dopamine D1 receptor-stimulated adenylyl cyclase activity is temporally and biochemically separated from D1 receptor internalization. Proc Natl Acad Sci USA *92*, 10157-61.

Pals Rylaarsdam, R., Xu, Y., Witt Enderby, P., Benovic, J. L., and Hosey, M. M. (1995). Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms. J Biol Chem *270*, 29004-11.

Peralta, E. G., Winslow, J. W., Smith, D. H., Ashkenazi, A., Ramachandran, J., Schimerlik, M. I., and Capon, D. J. (1987). Primary structure and biochemical properties of an M2 muscarinic receptor. Science *236*, 600-605.

Peterson, G. L., and Schimerlik, M. I. (1984). Large scale preparation and characterization of membrane-bound and detergent solubilized muscarinic receptor from pig atria. Prep. Biochem. *14*, 33-74.

Peterson, G. L., Toumadje, A., Johnson, W. C., Jr., and Schimerlik, M. I. (1995). Purification of recombinant porcine m2 muscarinic acetylcholine receptor from Chinese hamster ovary cells. Circular dichroism spectra and ligand binding properties. J Biol Chem *270*, 17808.

Pippig, S., Andexinger, S., and Lohse, M. J. (1995). Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. Mol Pharmacol *47*, 666-76.

Premont, R. T., Inglese, J., and Lefkowitz, R. J. (1995). Protein kinases that phosphorylate activated G protein-coupled receptors. FASEB J *9*, 175-82.

Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Sealfon, S. C. (1992). Sequence alignment of the G-protein coupled receptor superfamily. Dna Cell Biol *11*, 1-20.

Remmers, A. E., Posner, R., and Neubig, R. R. (1994). Fluorescent guanine nucleotide analogs and G protein activation. J Biol Chem *269*, 13771-8.

Richardson, R. M., and Hosey, M. M. (1990). Agonist-independent phosphorylation of purified cardiac muscarinic cholinergic receptors by protein kinase C. Biochemistry *29*, 8555-61.

Richardson, R. M., and Hosey, M. M. (1992). Agonist-induced phosphorylation and desensitization of human m2 muscarinic cholinergic receptors in Sf9 insect cells. J Biol Chem *267*, 22249-55.

Roettger, B. F., Rentsch, R. U., Hadac, E. M., Hellen, E. H., Burghardt, T. P., and Miller, L. J. (1995a). Insulation of a G protein-coupled receptor on the plasmalemmal surface of the pancreatic acinar cell. J Cell Biol *130*, 579-90.

Roettger, B. F., Rentsch, R. U., Pinon, D., Holicky, E., Hadac, E., Larkin, J. M., and Miller, L. J. (1995b). Dual pathways of internalization of the cholecystokinin receptor. J Cell Biol *128*, 1029-41.

Rose, P. M., Krystek, S. R., Jr., Patel, P. S., Liu, E. C., Lynch, J. S., Lach, D. A., Fisher, S. M., and Webb, M. L. (1995). Aspartate mutation distinguishes ETA but not ETB receptor subtype-selective ligand binding while abolishing phospholipase C activation in both receptors. FEBS-Lett *361*, 243-9.

Salomon, Y. (1979). Adenylate cyclase assay. Adv. Cyclic Nucleotide Res. *10*, 35-55.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA *74*.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. *51*, 660-672.

Shapiro, R. A., and Nathanson, N. M. (1989). Deletion analysis of the mouse m1 muscarinic acetylcholine receptor: effects on phosphoinositide metabolism and down-regulation. Biochemistry *28*, 8946-8950.

Shapiro, R. A., Palmer, D., and Cislo, T. (1993). A deletion mutation in the third cytoplasmic loop of the mouse m1 muscarinic acetylcholine receptor unmasks cryptic G-protein binding sites. J Biol Chem *268*, 21734-8.

Siegel, G. J., Agranoff, B. W., Albers, R. W., and Molinoff, P. B. (1989). Basic Neurochemistry: Molecular, cellular and medical aspects (New York: Raven Press), pp. 984.

Simon, M. I., Strathmann, M. P., and Gautam, N. (1991). Diversity of G proteins in signal transduction. Science *252*, 802-8.

Strader, C., Dixon, R., Cheung, A., Candelore, M., Blake, A., and Sigal, I. (1987). Mutations that uncouple the β-adrenergic receptor from Gs and increase agonist affinity. J Biol Chem *262*, 16439-16443.

Strader, C., Sigal, I., and Dixon, R. (1989). Structural basis of β-adrenergic receptor function. FASEB J *3*, 1925-1832.

Strader, C., Sigal, I., Register, R., Candelore, M., Rands, E., and Dixon, R. (1987). Identification of residues required for ligand binding to the β-adrenergic receptor. Proc Natl Acad Sci USA *84*, 4384-4388.

Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. (1994). Structure and function of G protein-coupled receptors. Annu Rev Biochem *63*, 101-32.

Strosberg, A. D. (1991). Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins. Eur J Biochem *196*, 1-10.

Surprenant, A., Horstman, D. A., Akbarali, H., and Limbird, L. E. (1992). A point mutation of the alpha 2-adrenoceptor that blocks coupling to potassium but not calcium currents. Science *257*, 977-80.

Tota, M. R., Kahler, K. R., and Schimerlik, M. I. (1987). Reconstitution of the purified porcine atrial muscarinic acetylcholine receptor with purified porcine atrial inhibitory guanine nucleotide binding protein. Biochemistry *26*, 8175-8182.

Tsuga, H., Kameyama, K., Haga, T., Kurose, H., and Nagao, T. (1994). Sequestration of muscarinic acetylcholine receptor m2 subtypes. Facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2. J Biol Chem *269*, 32522-7.

Vila Echague, A., Genaro, A. M., and Sterin Borda, L. (1994). Negative inotropic effect of carbachol on rat atria mediated by nitric oxide. Acta Physiol Pharmacol Ther Latinoam *44*, 100-7.

Vogel, W. K., Mosser, V. A., Bulseco, D. A., and Schimerlik, M. I. (1995). Porcine m2 muscarinic acetylcholine receptor-effector coupling in Chinese hamster ovary cells. J Biol Chem *270*, 15485.

von Zastrow, M., and Kobilka, B. K. (1994). Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization. J Biol Chem *269*, 18448-52.

Wang, C. D., Buck, M. A., and Fraser, C. M. (1991). Site-directed mutagenesis of alpha 2A-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation by agonists. Mol Pharmacol *40*, 168-79.

Wang, C. D., Gallaher, T. K., and Shih, J. C. (1993). Site-directed mutagenesis of the serotonin 5-hydroxytrypamine2 receptor: identification of amino acids necessary for ligand binding and receptor activation. Mol Pharmacol *43*, 931-40.

Wess, J. (1993). Molecular basis of muscarinic acetylcholine receptor function. Trends Pharmacol Sci *14*, 308-13.

Wess, J., Bonner, T. I., and Brann, M. R. (1990). Chimeric m2/m3 muscarinic receptors: role of carboxyl terminal receptor domains in selectivity of ligand binding and coupling to phosphoinositide hydrolysis. Mol Pharmacol *38*, 872-7.

Wess, J., Bonner, T. I., Dorje, F., and Brann, M. R. (1990). Delineation of muscarinic receptor domains conferring selectivity of coupling to guanine nucleotide-binding proteins and second messengers. Mol Pharmacol *38*, 517-23.

Wess, J., Brann, M. R., and Bonner, T. I. (1989). Identification of a small intracellular region of the muscarinic m3 receptor as a determinant of selective coupling to PI turnover. FEBS Lett *258*, 133-136.

Wess, J., Gdula, D., and Brann, M. R. (1991). Site-directed mutagenesis of the m3 muscarinic receptor: identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding. Embo J *10*, 3729-34.

Wess, J., Maggio, R., Palmer, J. R., and Vogel, Z. (1992). Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation. A study with m3 muscarinic receptor point mutants. J Biol Chem *267*, 19313-9.

Wess, J., Nanavati, S., Vogel, Z., and Maggio, R. (1993). Functional role of proline and tryptophan residues highly conserved among G protein-coupled receptors studied by mutational analysis of the m3 muscarinic receptor. Embo J *12*, 331-8.

Whaley, B. S., Yuan, N., Barber, R., and Clark, R. B. (1995). β-Adrenergic Regulation of Adenylylcyclase: Effect of Receptor Number. Pharmacology Communications *6*, 203-210.

Whaley, B. S., Yuan, N., Birnbaumer, L., Clark, R. B., and Barber, R. (1994). Differential expression of the beta-adrenergic receptor modifies agonist stimulation of adenylyl cyclase: a quantitative evaluation. Mol Pharmacol *45*, 481-9.

Wise, A., Lee, T. W., MacEwan, D. J., and Milligan, G. (1995). Degradation of G11 alpha/Gq alpha is accelerated by agonist occupancy of alpha 1A/D, alpha 1B, and alpha 1C adrenergic receptors. J Biol Chem *270*, 17196-203.

Wong, S. K., and Ross, E. M. (1994). Chimeric muscarinic cholinergic: beta-adrenergic receptors that are functionally promiscuous among G proteins. J Biol Chem *269*, 18968-76.

Yu, S. S., Lefkowitz, R. J., and Hausdorff, W. P. (1993). Beta-adrenergic receptor sequestration. A potential mechanism of receptor resensitization. J Biol Chem *268*, 337-41.

Appendix

Receptor activation of G-proteins

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Introduction

Model of receptor activation of G-proteins

Muscarinic agonists mediate receptor-effector coupling through G-proteins. The effector system that specific muscarinic receptor subtypes couples to is determined by G-protein interactions. While receptors of the m1/m3/m5 family interact with PTX insensitive G-proteins, the m2 AchRs appear to primarily interact with PTX sensitive Gi alpha subunits. This simplified view of receptor-G-protein specificity is clearly inadequate, and many other factors must be considered when studying G-protein activation (Gudermann et al., 1996).

The guanine nucleotide binding proteins are heterotrimers comprised of α, β and γ subunits. Agonist binding to a G-protein coupled receptor is thought to promote the exchange of bound GDP for GTP. The binding of GTP leads to an uncoupling of the receptor and the heterotrimeric G-protein, as well as dissociation of the heterotrimer into α and $\beta\gamma$ subunits. Intrinsic GTPase activity converts GTP bound to the α subunit to GDP, which then reassociates with the $\beta\gamma$ subunit (Simon et al., 1991).

Principle steps in the activation process

Activation of G-proteins requires that agonist binding leads to the appropriate receptor conformation. This conformational change is the activation signal that leads to changes in state of the heterotrimeric G-protein. In order to better characterize the steps following agonist binding, each of these principle steps in the activation process must be

well characterized. This can be accomplished by characterizing the exchange of GDP and GTP using a variety of experimental approaches as well as agonist-promoted GTPase activity.

Materials and Methods

Materials

Radioactive ligands ([^{3}H] *l*-QNB, 52.3 Ci/mmol; [^{3}H]NMS, 84 Ci/mmol; γ^{32}P-GTP; 30 Ci/mmol; ^{35}GTPγS, 1156 Ci/mmol) were purchased from New England Nuclear. N-methyl-3'-O-anthranoyl (MANT) GTP analog was synthesized as described (Remmers et al., 1994). Unlabeled agonist and antagonists were from Research Biochemicals International. Cell culture reagents were from GIBCO-Bethesda Research Laboratories or Sigma.

Membrane preparation

Cells were grown as described (Bulseco and Schimerlik, 1996), harvested and the pellet resuspended in two volumes of homogenization buffer (250 mM sucrose, 50 mM EDTA, 25 mM imidazole buffer, pH 7.4) containing benzamidine, soybean trypsin inhibitor, and pepstatin A at final concentrations of 1 mg/ml. The cell suspension was homogenized with a polytron homogenizer for 2x30 sec at 4°C and cell membranes were prepared by sucrose gradient centrifugation as previously described (Peterson and Schimerlik, 1984). In some cases, 5 mM sodium butyrate was added 24 hours before harvesting cells to further enhance receptor expression. Untreated and butyrate-treated

cultures resulted in membrane preparations with specific activities of 20 to 300 pmol NMS sites/mg protein.

Ligand binding

All ligand binding experiments were conducted in buffer containing 100 mM NaCl, 10 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4 at 25°C by displacement of either [^{3}H] *l* -QNB or [^{3}H]NMS. Samples were filtered through glass fiber filters and washed with 3X 4 ml of ice cold 50 mM Na phosphate buffer pH 7.4. When [^{3}H]NMS was used, the glass fiber filters were treated with 0.1% (w/v) PEI before use. Nonspecific binding was determined in the presence of 10^{-4} M *l* -hyoscyamine and was less than 10% of the total radiolabel bound.

Assays for GTPase activity

GTPase activity was examined in membranes prepared from both fresh and frozen CHO cells expressing wild-type mAchR at low (pSVE pm2 cl.5) and high (pSVE pm2 short cl.1) expression levels as well as KDKKE(219-223)ELAAL and A212E mutant AchRs. Assays were conducted in 100 mM NaCl, 10 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM ATP, pH 7.4 at 30°C. Approximately 5 µg of total plasma membrane protein were used per data point. Reactions were initiated by addition of membrane to tubes containing buffer, saturating concentrations of agonist and 30 nM γ^{32}P-GTP (S.A. 30 Ci/mmol). Reactions were stopped by addition of ice cold 0.75 ml slurry of activated charcoal. The reaction tubes were stored on ice until ready for

centrifugation. Tubes were centrifuged at top speed in an Eppendorf Microcentrifuge, and a 500 µl aliquot removed for Cerenkov counting.

Kinetics of GTPγS binding

Agonist-promoted ^{35}GTPγS binding was examined in CHO cell membranes prepared from cells expressing wild-type (pSVE pm2 cl.5 and pKNEO pm2 cl.1) pm2 AchR. Assays were done in 100 mM NaCl, 50 nM GDP, 100 nM DTT, 10 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4 at 25°C. Membranes were equilibrated with saturating concentrations of carbachol, then assays initiated with the addition of 50 nM ^{35}GTPγS (23.7 Ci/mmol). Aliquots were removed at specific time points and filtered through glass fiber filters.

Kinetics of agonist-promoted MANT-GTP binding

Kinetics of agonist-promoted MANT-GTP binding was studied in CHO cell membranes prepared from cells expressing wild-type receptors (pSVE pm2 cl.5 and pKNEO pm2 cl.1). Assays were conducted in 100 mM NaCl, 10 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4 at 25°C using a Dionex Stopped Flow apparatus. Assays were initiated by combining membrane suspension in binding buffer with MANT-GTP at a final concentration of 0.1 mM.

Data analysis

GTPγS and MANT-GTP association kinetics were analyzed using least squares nonlinear curve fitting in Origin (MicroCal Software, Inc.) to Equation 1. Y is the

association signal being measured, with y0 the background signal. A1 is the signal amplitude; τ, the relaxation time; and x, time.

$$y = y0 + A1*(1 - \exp(-x/\tau)) \quad \text{(Eq. 1)}$$

GTPase experiments were analyzed by determining the linear initial rate in the presence and absence of the agonist, carbachol.

Results and Discussion

Ligand binding

Ligand binding properties were examined in normal binding buffer (10 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4) supplemented with 100 mM NaCl, at 25°C. Independent experiments determined that 100 mM NaCl was required to be able to measure agonist-promoted G-protein activation in CHO cell membranes. Table A.1 summarizes the dissociation constants for agonists and antagonists of the pm2 AchR. Antagonist binding affinities were very similar to wild-type receptor membranes characterized in normal binding buffer. Agonist binding affinities were similar for carbachol, oxotremorine M and pilocarpine, but not acetylcholine. All of the agonist affinities were within 2 fold of wild-type receptor membranes characterized in normal binding buffer. Acetylcholine on the other hand displayed between 10 and 100 fold higher affinity for K_1 and K_2, and about 5 fold for K_3.

Table A.1: Dissociation constants for ligands in high salt buffer.

Membranes were prepared from CHO cells expressing wild-type pm2 mAchR. Data are presented as the mean ± S.D. of n determinations. Carbachol, acetylcholine and oxotremorine M were best fit to three classes of binding sites while pilocarpine was best fit to two.

	K_1 (nM)	K_2 (nM)	K_3 (μM)	n
Carbachol	0.39 ± 0.15	71.0 ± 17	7.6 ± 2.8	2
Acetylcholine	1.8 ± 1.6	137.6 ± 80.6	4.0 ± 1.3	4
Oxotremorine M	2.2 ± 1.2	65.3 ± 23.3	2.8 ± 0.1	3
Pilocarpine		2107 ± 1438	6.1 ± 0.4	3

	K_d (nM)	n
NMS	0.38 ± 0.09	2
l-QNB	0.017 ± 0.003	3
l-Hyoscyamine	1.6 ± 0.3	2
Pirenzipine	652 ± 28	2

GTPase

Agonist-promoted GTPase activity was studied in membranes prepared from CHO cells expressing between 5 pmol/mg and 35 pmol/mg m2 AchR. Figure A.1 shows carbachol promoted increase in GTPase activity. The initial rates were determined to be 12.7 ± 27.0 for untreated membranes, and 300.0 ± 41.7 for membranes pre-incubated with carbachol. Figure A.2 shows the effect of different agonists on the production of ^{32}P from $^{32}P\gamma GTP$. The reaction velocity is assumed to be linear over the time course of the experiment. Acetylcholine, carbachol and oxo M stimulated GTPase activity, while the hydrophilic antagonist NMS did not. Both of these results are in support of agonist-mediated activation of G-proteins, and may be used to study the effects of mutant AchR on agonist-stimulated GTPase activity.

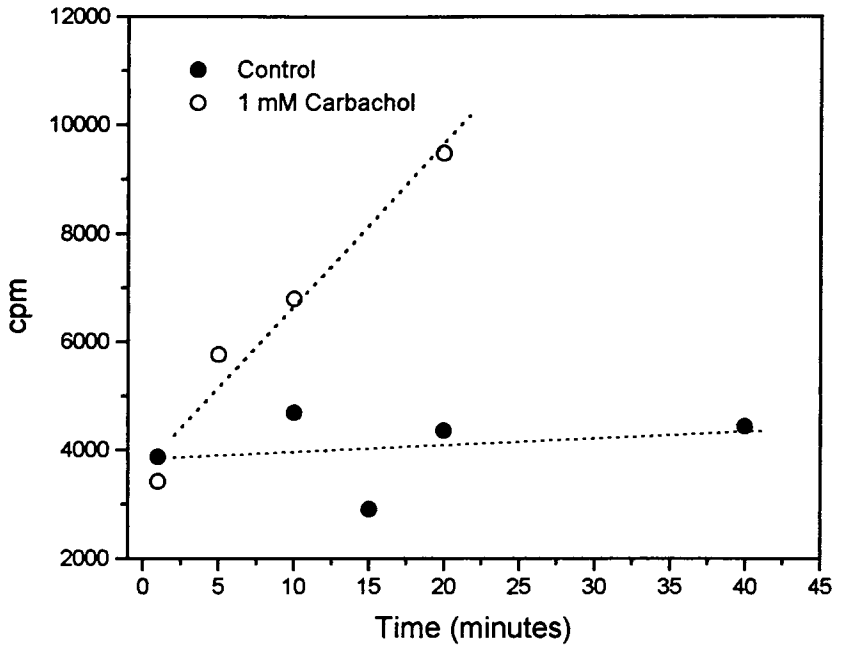

Figure A.1: Agonist-promoted GTPase activity for cl.5 wild-type pm2 AchR.

GTPase experiments were conducted as described in "Materials and Methods". Membranes were equilibrated with 1 mM carbachol prior to addition of ^{32}PγGTP to initiate reaction. The rates were estimated to be 12.7 ± 27.0 and 299.9 ± 41.7 for control and carbachol-treated cells.

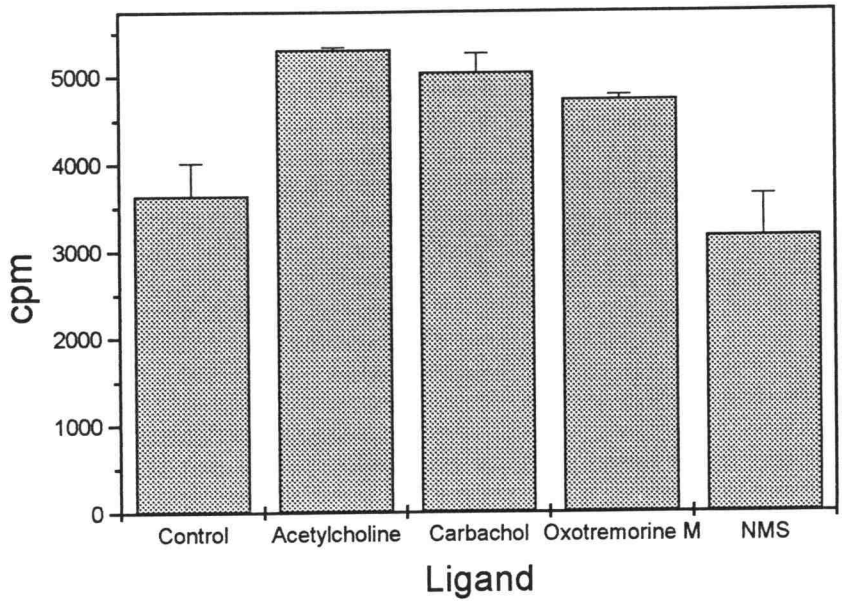

Figure A.2: Agonist-stimulated GTPase activity of cl.5 wild-type pm2 AchR.

GTPase activation was studied as described in "Materials and Methods". Data is presented as the mean ± S.D. from a single experiment done in duplicate. Membranes were equilibrated with agonists or the antagonist NMS prior to initiating the experiment with $^{32}P\gamma GTP$. The reaction was allowed to proceed for 30 minutes. The reaction velocity is assumed to be linear over this period of time.

GTPγS

Agonist-promoted association of GTP was studied using the non-hydrolyzable analog, ^{35}GTPγS. Association kinetics were rapid, and difficult to characterize. Figure A.3 shows carbachol stimulated association of GTPγS in membranes with about 25 pmol/mg m2 mAchR. The relaxation times obtained by nonlinear curve fitting to equation 1 were 36.9 ± 13.1 and 10.0 ± 1.6 sec^{-1} for control and carbachol-stimulated membranes respectively. The half time for this reaction was reduced from about 26 to 7 seconds by the agonist carbachol. Figure A.4 shows carbachol-stimulated association of GTPγS to membranes with a specific activity of about 7 pmol/mg m2 AchR. The observed relaxation times were 214 ± 37 and 44 ± 27 sec^{-1} for control and carbachol stimulated membranes respectively. It is not clear if the amplitudes observed in these experiments have any significance. Carbachol stimulation did not alter amplitude by more than about 2 fold.

The rapid association of GTPγS is not unexpected, since agonist-mediated activation of G-proteins are expected to occur rapidly. In order to follow the time course of these rapid events, we attempted to study MANT-GTP association to endogenous G-proteins in CHO cell membranes using stopped-flow rapid kinetics fluorescence spectroscopy.

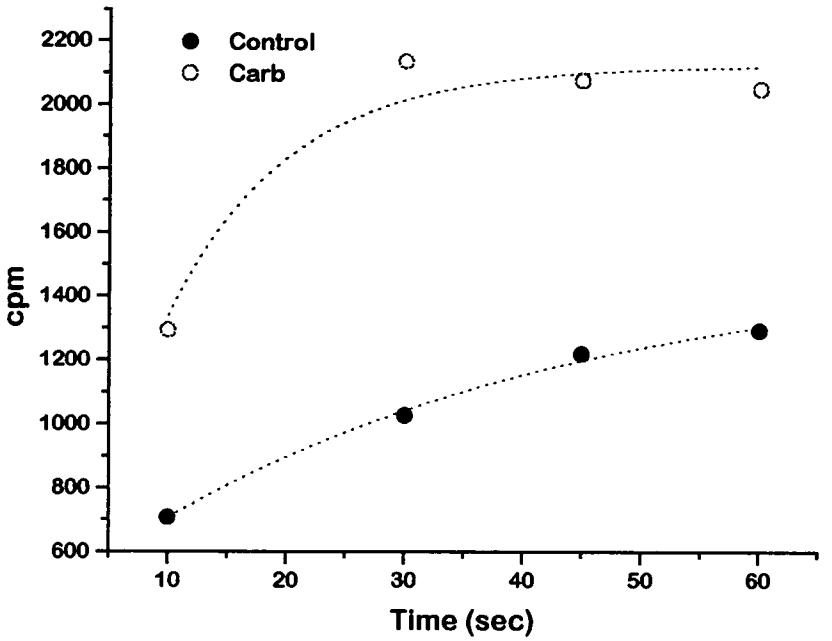

Figure A.3: ^{35}GTPγS binding to cl.5 wild-type pm2 mAchR membranes

GTPγS binding was conducted as described in "Materials and Methods". Membranes were equilibrated with carbachol before addition of ^{35}GTPγS to initiate the reaction. The amplitudes were estimated to be 1054 ± 110 and 2120 ± 73 for control and carbachol treated cells, with relaxation times of 36.9 ± 13.1 and 10.0 ± 1.6 sec^{-1} respectively.

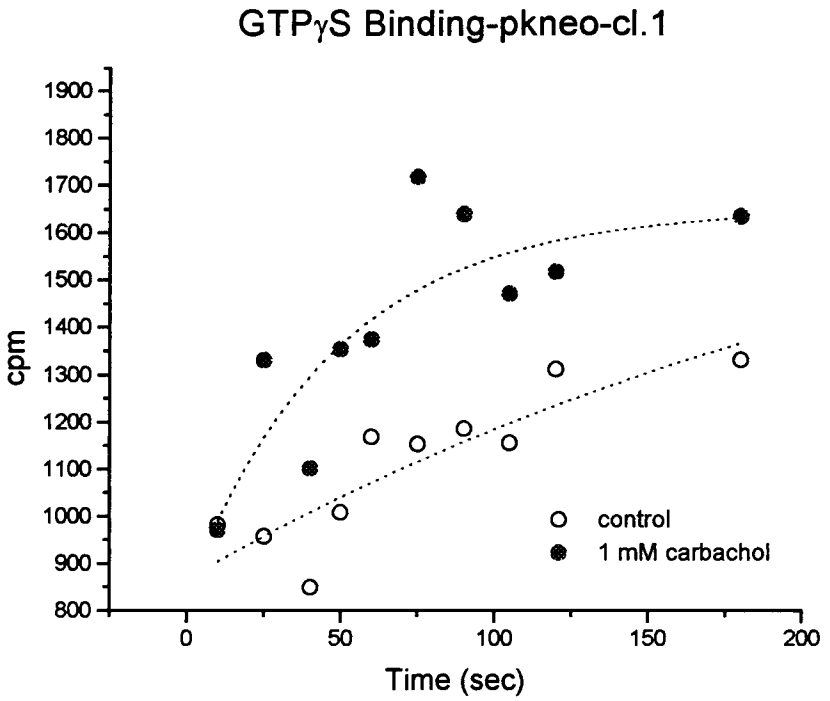

Figure A.4: ^{35}GTPγS binding to cl.1 wild-type pkneo pm2 mAchR membranes

GTPγS binding was conducted as described in "Materials and Methods". Membranes were equilibrated with carbachol before addition of ^{35}GTPγS to initiate the reaction. The amplitudes were estimated to be 867 ± 94 and 817 ± 205 for control and carbachol treated cells, with reciprocal relaxation times of 214 ± 37 and 44 ± 27 sec^{-1} respectively.

MANT-GTP binding

The binding of the fluorescently labeled analog of GTP, MANT-GTP was followed using a stopped flow rapid kinetic fluorometer. These experiments were technically difficult, and large quantities of membranes were required to see small signal changes. Other problems associated with turbidity of the suspension used, as well as non-homogenous distribution of membranes in this suspension led to many experiments with uninterpretable results. Figure A.5 shows one such experiment. Parameter estimation resulted in amplitudes of 0.036 ± 0.001 and 0.101 ± 0.001 for control and carbachol treated cells, with relaxation times of 34.3 ± 2.0 and 81.7 ± 2.5 msec respectively. This particular result was unexpected, and is not consistent with other results described in this appendix. The half time for association was actually increased by exposure to carbachol, from 24 to 56 msec, while amplitude was increased. It is likely that the data collected during these experiments will not yield any interpretable results.

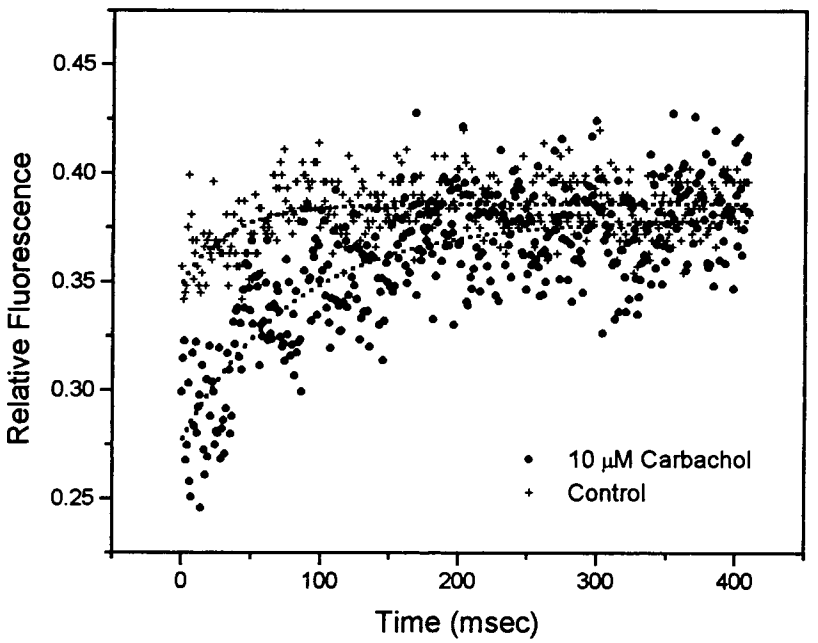

Figure A.5: MANT-GTP association to cl.1 pkneo wild-type pm2 mAchR membranes.

MANT-GTP binding was studied as described in "Materials and Methods". Data is from a single experiment, and is the average of 3 shots (12,288 data points) on the Dionex Stopped Flow apparatus. All data points were used in the data analysis, but only 409 points used for plotting purposes. The amplitudes were estimated to be 0.036 ± 0.001 and 0.101 ± 0.001 for control and carbachol treated cells, with relaxation times of 34.3 ± 2.0 and 81.7 ± 2.5 msec respectively.

Summary

The ability to measure agonist-promoted interactions between receptor and G-proteins will provide useful tools to assess the effects of receptor mutations. Questions regarding the affinity of liganded receptor for G-proteins, the specificity of these interactions and the efficiency with which agonist-bound receptors can activate G-proteins remains to be answered. Although the approaches described above allows one to begin to address these questions, they are inadequate. More refined methods for experimentation as well as data analysis are needed before these questions will be satisfactorily answered.