REGULATION OF MUSCARINIC ACETYLCHOLINE RECEPTORS

by

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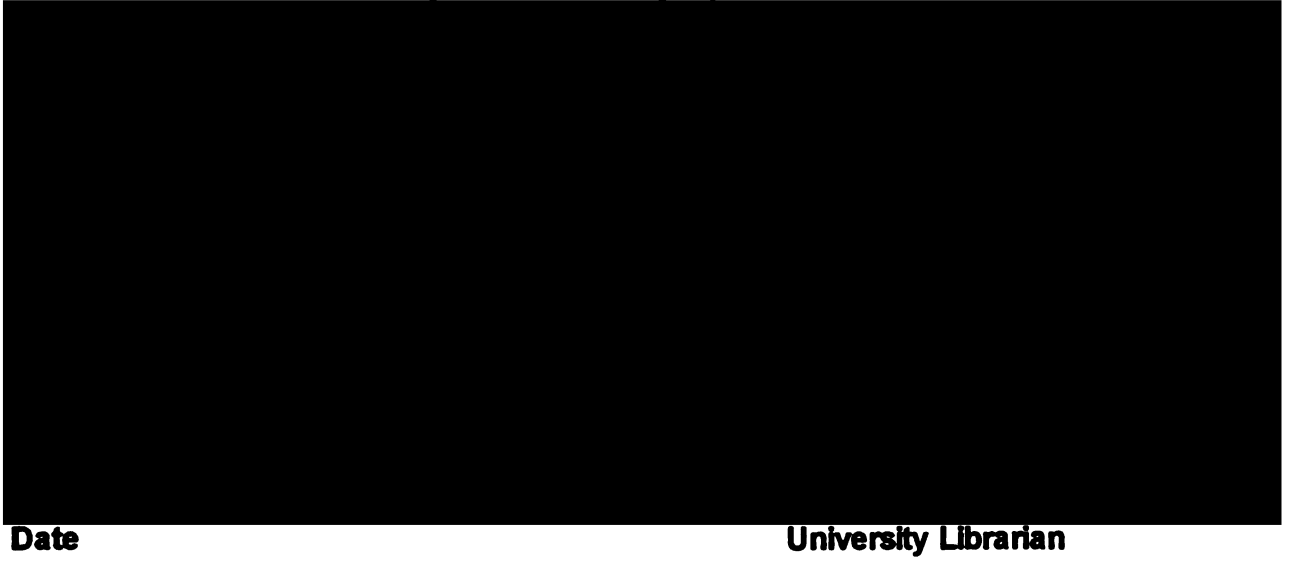

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To my parents, James and Carolyn Shockley,

for their love, support, and encouragement.

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MELINDA S. SHOCKLEY
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ABSTRACT

Mechanisms for regulation of G protein-coupled receptor activity include the agonist-induced processes of receptor internalization and down-regulation. The cellular and molecular determinants of these two events were examined using the phospholipase-C linked muscarinic acetylcholine receptor (mAChR) subtypes, m1 and m3.

Mutagenesis studies of the intracellular regions of the m1 AChR identified several regions important for receptor/G-protein interaction, internalization, and down-regulation. It was demonstrated that the second intracellular loop and the junctions of the third intracellular loop are involved in the activation of G proteins. Although several mutants (L131A, V127A/L131A) examined were defective in both internalization and G protein coupling, a stronger correlation was observed between down-regulation and G protein activation. Mutants V127A, I211A, E360A, and K362A were identified as defective in down-regulation and G protein coupling, but not internalization. These results suggested a potential structural overlap between the G proteins that couple to the m1 AChR and the factors involved in down-regulation.

The cellular pathway of internalization and the effects of blocking this pathway on down-regulation were examined for m1 and m3 AChRs expressed in Chinese hamster ovary cells. Immunofluorescence confocal microscopy allowed for the observation of internalization of both muscarinic subtypes into intracellular vesicles. Internalization was shown to occur via an endocytic pathway characterized by clathrin-coated vesicles. Down-regulation of both m1 and m3 AChRs required prior internalization of the receptor via this pathway. These data in combination with the mutagenesis studies indicated that internalization and down-regulation are sequential processes governed by distinct factors.

Further understanding of the mechanisms of internalization and down-regulation will thus require the identification of the cellular factors and the receptor domains specifically involved in the endocytosis of G protein coupled receptors via clathrin-coated pits and the subsequent sorting of these receptors to a degradative pathway.

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INTRODUCTION

I. OBJECTIVE

Signal transduction by G protein-coupled receptors (GPCRs) may be modulated by agonist-induced and agonist-independent regulatory events. The overall objective of this study was to examine the molecular mechanism(s) of two key agonist-induced regulatory processes: receptor internalization and receptor down-regulation. Two muscarinic cholinergic receptor subtypes, m1 and m3, were used as model systems. The specific aims of this research were to 1) elucidate the molecular domains of the receptor involved in sequestration/internalization 2) determine the receptor domains mediating receptor down-regulation 3) assess the contribution of both G protein activation and stimulation of second messengers to both internalization and down-regulation 4) examine the relationship between the pathway(s) of receptor internalization and down-regulation.

II. BACKGROUND

A. G Protein-Coupled Receptors

Integral membrane proteins act to transmit signals from the external environment across a cell membrane. Membrane spanning proteins are classified into three major families based on structure and mode of signal transduction. Growth factor receptors consist of a single transmembrane domain and transduce signals through the tyrosine kinase portion of their cytoplasmic tails. Ligand-gated ion channels are comprised of multiple subunits and function in the transport of charged molecules across the cell membrane. The most diverse and complex family of transmembrane receptors is that of the G protein-coupled receptor (GPCR) superfamily. GPCRs are organized into seven transmembrane domains and activate intracellular signal transduction pathways via intermediary GTP binding proteins (G proteins).

Several hundred distinct G protein-coupled receptors have been cloned and sequenced (Probst et al., 1992). The first identified G protein-coupled receptor was cloned as the gene encoding the visual opsin, bovine rhodopsin (Ovchinnikov et al., 1982). As the number of cloned GPCRs increased, the diversity of this receptor superfamily became obvious. The range of signals transmitted by GPCRs include numerous neurotransmitters, chemoattractants, hormones, cytokines, peptides, and sensory stimuli such as photons and odorants.

The characteristic seven transmembrane domain structure of GPCRs (Figure I) is similar to that of the structure of bacteriorhodopsin, a light-activated protein pump found in the purple membrane of *Halobacterium halobium*. By use of electron scattering and neutron diffraction analysis, bacteriorhodopsin was the first predicted seven transmembrane domain protein and thus served as a model for the structure of GPCRs (Engelman et al., 1980). Based on amino acid sequence and hydropathy analysis, the structure of the cloned bovine rhodopsin was predicted to be similar to that of bacteriorhodopsin with each of the seven spanning domains consisting of 20-25 amino acids (Ovchinnikov et al., 1982). This data provided a basis for the construction of a GPCR model analogous to the structure of bacteriorhodopsin (Henderson et al., 1990; Schertler et al., 1993).

While the organization of the transmembrane domains has been suggested based on hydropathy analysis, the structures of the extracellular and the intracellular portions of the receptors are unknown. A highly conserved pair of cysteines in the second and the third extracellular (e2 and e3) loops are thought to be required for the maintenance of proper receptor conformation (Lameh et al., 1990). Recently, the junctions of the third intracellular loop have been suggested to be α-helical extensions of transmembrane domains five and six (TMD V and TMD VI) (Blüml et al., 1994). Palmitoylation of

Figure I: Deduced Amino Acid Sequence of a G Protein-Coupled Receptor, the Human Muscarinic Acetylcholine m1 Receptor. Indicated are the putative seven transmembrane domains, the extracellular N-terminus, and the intracellular carboxyl-tail.

NH2
COO-

cysteine residues in the carboxyl-tail of some GPCRs is thought to function in the organization of the intracellular tail of the receptor into a potential fourth loop (Ovchinnikov et al., 1988). Recent attempts to define the structure of the intracellular and extracellular portions of a GPCR have relied upon modeling of the individual loop regions, although without much success. Our studies of GPCR function have therefore been limited due to the lack of structural information on the regions of the receptor most likely to be involved in ligand binding and G protein activation.

B. G Proteins

G proteins mediate the transmission of signal from activated G protein-coupled receptors to one or more signal transduction pathways. G proteins are heterotrimers consisting of α, β, and γ subunits (Birnbaumer et al., 1990). Binding of a ligand to a GPCR induces a conformational change that enables the receptor to bind to the heterotrimeric G protein(s). This interaction promotes the exchange of GDP for GTP on the α subunit thereby releasing activated Gα from the G$\beta\gamma$ subunits. While Gα proteins were originally believed to be the primary signaling subunits, a role for G$\beta\gamma$ subunits in transduction of signal has been defined recently (Clapham and Neer, 1993; Sternweis, 1994). Multiple G protein subunits have been cloned and sequenced, and these multiple subunits give rise to divergence in signal transduction through the interaction with different effector systems (Conklin and Bourne, 1993; Neer, 1995).

C. Signal Transduction

G protein activation of specific effector systems (enzymes or ion channels) leads to changes in the concentration of various cytosolic signaling molecules known as second messengers. Several pathways of signal transduction may be activated by the binding of an agonist to a GPCR. Pathways of signal transduction most often associated with the activation of a GPCR are stimulation/inhibition of adenylyl cyclase and the activation of

phospholipase C, although phospholipid hydrolysis by phospholipases A and D has also been shown to occur (Conklin et al., 1988; Sandmann et al., 1991). Recently, GPCRs have been proposed to be involved in mitogenic signaling as well (Simonson and Herman, 1993; Luttrell et al., 1995; van Biesen et al., 1996).

1. Adenylyl Cyclase

The first second messenger characterized was cyclic 3', 5'-monophosphate (cAMP), and the enzyme responsible for the generation of this second messenger was identified and named "adenyl cyclase" (Rall and Sutherland, 1958; Sutherland et al., 1962). Subsequently, the link between activation of a receptor and adenylyl cyclase activity was found to be a G protein (Pfeuffer, 1977; Ross and Gilman, 1977). Therefore, the signal transduction pathway consisting of receptor-G protein-adenylyl cyclase was established. Stimulation of receptor may lead not only to activation, but also inhibition, of adenylyl cyclase activity (Katada and Ui, 1982). Selectivity is determined by the $G\alpha$ subunit activated by the receptor with stimulation associated with Gs and inhibition with Gi activity.

2. Phospholipase C

Receptors which couple to $G_{q/11}$ activate phospholipase C (PLC) to alter phospholipid metabolism and Ca^{2+} concentrations within the cell (Figure II). Early observations suggested that changes in phosphatidyl inositol metabolism could be mediated by acetylcholine (Hokin and Hokin, 1953; Durell et al., 1969). Subsequently, it was shown that acetylcholine could stimulate a reduction in phosphatidylinositol-4, 5-bisphosphate (PIP_2) levels accompanied by increases in inositol trisphosphate (IP_3) and inositol monophosphate (IP). Berridge et al. (1982, 1983) demonstrated that hydrolysis of PIP_2 yields IP_3 and diacylglycerol (DAG). IP_3 binds to the IP_3 receptor on the endoplasmic reticulum to release calcium intracellularly (Streb et al., 1983), which then

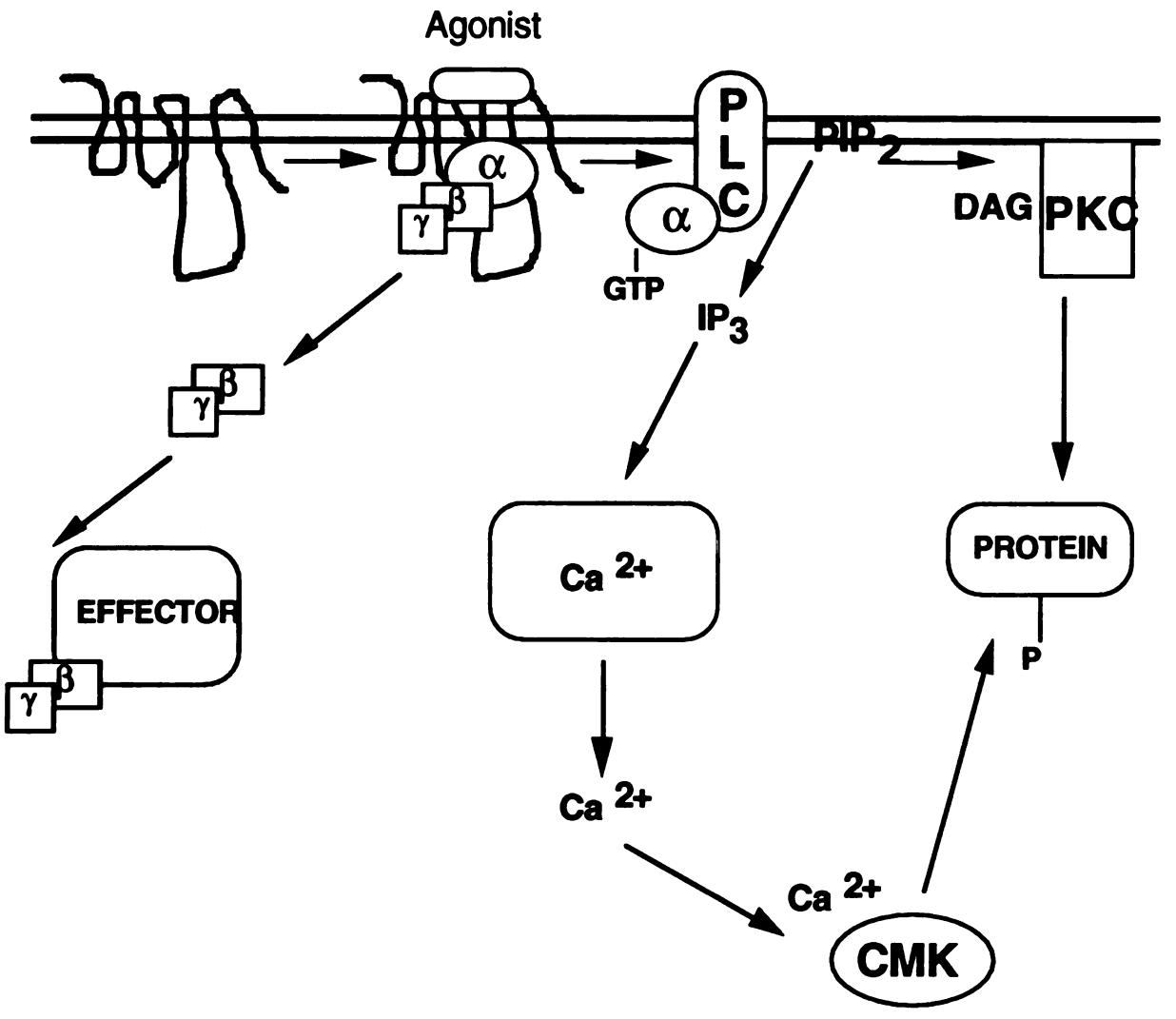


Figure II: The Phosphoinositide Hydrolysis Pathway. Agonist binding to the receptor triggers the activation of a G protein by stimulating the exchange of GDP for GTP on the α subunit. The G protein dissociates into the $G_{q/11}$ α-subunit and a βγ complex. $G_{q/11}$ stimulates the activity of phospholipase C (PLC) leading to the conversion of phosphoinositide-4, 5-bisphosphate (PIP_2) to inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to receptors on the endoplasmic reticulum to release Ca^{2+} intracellularly. The increase in cytosolic Ca^{2+} activates Ca^{2+}/calmodulin-dependent kinases (CMKs). DAG activates protein kinase C (PKC). Activated kinases lead to an increase in phosphorylated proteins within the cell. The βγ complex may also interact with effector systems to initiate cellular changes.

activates calcium-dependent kinases (Kakiuchi and Yamazaki, 1970). Diacylglycerol activates protein kinase C (Takai et al., 1979). Thus, the phosphatidylinositide hydrolysis pathway involves the following events: receptor-G protein coupling-activation of PLC-breakdown of PIP_2 to IP_3 and DAG-Ca^{2+} release-activation of calcium dependent kinases and activation of PKC by DAG.

D. Receptor Regulation

Several regulatory events modulate the activity of G protein-coupled receptors (Figure III). Agonist-induced changes in receptor functioning may result from acute or chronic stimulation of receptor. Short term regulatory events include desensitization by physical uncoupling of the receptor from G protein and sequestration/internalization of receptor to a compartment inaccessible to ligand. Long term agonist exposure usually triggers a down-regulation of the receptor pool by either an increase in the degradation of existing proteins or by a decrease in synthesis of new receptor (Klein et al., 1979; Galper and Smith, 1980; Wang et al., 1990).

1. Desensitization

The process of receptor desensitization has been most extensively analyzed for the β_2-adrenergic receptor (β_2-AR). Receptor phosphorylation appears to account for the rapid loss of responsiveness observed with the β_2-AR. Three types of kinases have been identified which phosphorylate β_2-ARs: cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and the β-adrenergic receptor-specific kinase (Hausdorff et al., 1989; Hausdorff et al., 1990). This later kinase is a member of the recently identified G protein coupled receptor kinase (GRK) family and is thought to phosphorylate only activated receptors (Palczewski and Benovic, 1991). Phosphorylation of the receptor by a GRK is thought to be followed by binding of an arrestin protein, which interferes with signal

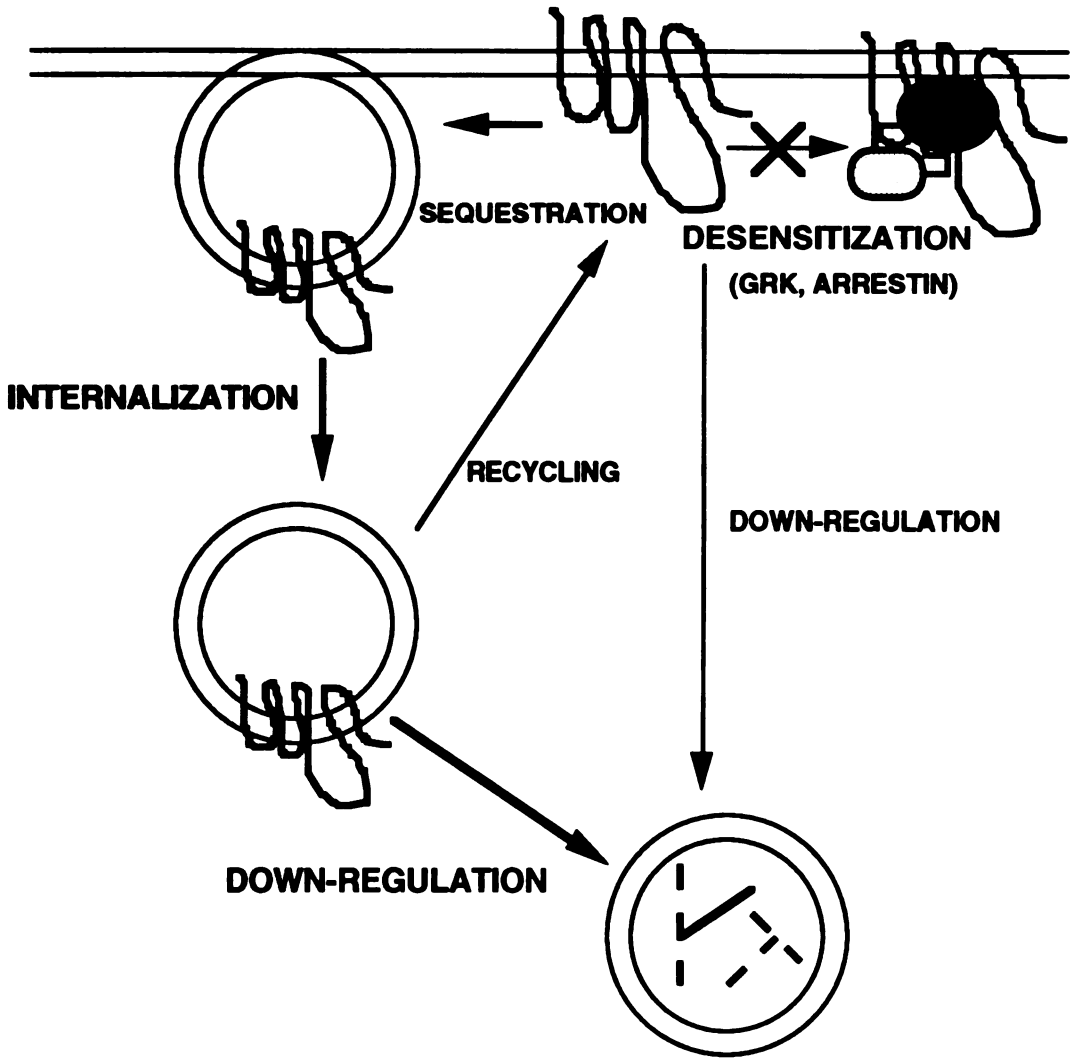


Figure III: Mechanisms of G Protein-Coupled Receptor Regulation. Agonist binding to the receptor triggers several events which modulate signaling. Rapid uncoupling of the receptor from G proteins occurs by phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs) and binding of arrestin. Rapid removal of the receptor from the plasma membrane occurs first with the sequestration of receptors at the cell surface, followed by endocytosis of the receptor into intracellular vesicles (endosomes). Internalized receptors may be transported back to the plasma membrane (recycling) or may be sorted to lysosomes for degradation (down-regulation). Down-regulation of receptors may also occur independent of internalization.

transduction by inhibiting receptor-G protein binding (Lefkowitz, 1993).

2. Sequestration/Internalization

A second regulatory mechanism governing receptor activity is sequestration/internalization. Agonist treatment induces rapid (minutes) endocytosis of the receptor away from extracellular stimuli. Initially, receptors may be sequestered at the plasma membrane prior to endocytosis. Although evidenced by ligand binding, sucrose density centrifugation and immunocytochemical studies, the process of GPCR internalization is not well understood (Galper et al., 1982; Harden et al., 1985; Raposo et al., 1987). Several endocytic pathways are known to mediate the transport of cell surface proteins, including those defined by clathrin-coated vesicles (McGraw and Maxfield, 1990), non-coated vesicles (Huet et al., 1980; Montesano et al., 1982), and caveolae (Anderson et al., 1992). Recent evidence suggest that GPCRs are internalized primarily via a pathway involving clathrin-coated vesicles (von Zastrow and Kobilka, 1992; Sloweijko et al., 1996; Tolbert and Lameh, 1996), although internalization via non-coated vesicles has been documented as well (Raposo et al., 1989). Studies with the β_2-adrenergic receptor have recently suggested that phosphorylation by GRKs and binding of arrestin may also function in the internalization process (Ferguson et al., 1995; Ferguson et al., 1996).

3. Down-regulation

Down-regulation is characterized by a decrease in total cellular receptor number following prolonged (hours) agonist exposure. Loss in receptor number is irreversible, and recovery requires de novo protein synthesis. As with the process of internalization, very little is known about the mechanism(s) of down-regulation for the GPCR family. Multiple events may contribute to the overall regulation of receptor number in a cell, including transcriptional, posttranscriptional, translational, and posttranslational changes

(Hadcock and Malbon, 1993). Down-regulation via lysosomal degradation of receptor protein is most commonly associated with prolonged agonist-stimulation, and this form of down-regulation may be mediated in part by the cellular and molecular determinants of receptor internalization (von Zastrow and Kobilka, 1992).

E. Muscarinic Cholinergic Receptors

Five subtypes of muscarinic cholinergic receptors have been cloned (Kubo et al., 1986a; Kubo et al., 1986b; Bonner et al., 1987; Peralta et al., 1987a; Peralta et al., 1987b; Bonner et al., 1988) (Table I). The muscarinic receptors are structurally similar to all other GPCRs with seven transmembrane spanning domains; however, muscarinic receptors, unlike other GPCRs, have rather large third intracellular loops. A high degree of homology exists among m1, m3, and m5 subtypes with each preferentially coupling via $G_{q/11}$ to the phosphoinositide hydrolysis pathway. Likewise, subtypes m2 and m4 are homologous but couple via G_i to the inhibition of adenylyl cyclase. In addition to stimulating IP release, functions of the m1 receptor subtype include the stimulation of a chloride channel (Fukuda et al., 1987) and the inhibition of both N- and L-type Ca^{2+} currents and a M-type K^+ current (Hille, 1992). The m3 subtype also functions in the inhibition of M-type K^+ currents (Robbins et al., 1991). The m1 subtype is found predominantly in cerebral tissue while the m3 subtype is found in both the brain and in glandular tissues.

Table I: The Muscarinic Acetylcholine Receptor Family.

Subtype	m1	m2	m3	m4	m5
Primary mRNA Distribution	Brain	Heart	Smooth muscle Glands	Brain	Brain
Selective antagonist	Pirenzipine	AF-DX 116	p-fluorohexahydrosiladifenidol	-	-
Major Signaling Pathway	Stimulation IP Release	Inhibit cAMP	Stimulation IP Release	Inhibit cAMP	Stimulation IP Release
# Amino Acids (total)	460	466	590	479	531
# Amino Acids (i3 loop)	156	181	241	184	228

CHAPTER I

DOMAINS OF THE HUMAN m1 MUSCARINIC CHOLINERGIC RECEPTOR INVOLVED IN SIGNAL TRANSDUCTION AND SEQUESTRATION

I. SUMMARY

Mutational analysis of the intracellular regions of the human m1 muscarinic cholinergic receptor has led to the identification of several residues that are important for functional G protein coupling (Moro et al., 1993a). Using these previously identified mutants, we now demonstrate that alanine substitution of residues in the second intracellular loop as well as the amino- and carboxyl-termini of the third intracellular loop result in defective agonist-mediated sequestration of m1 AChR expressed in human embryonic kidney (HEK293) cells. The single point mutant L131A, which was previously shown to be highly defective in coupling, was significantly impaired with respect to sequestration. Analysis of mutant V127A, which was also deficient in coupling, revealed no defect in sequestration. However, mutation of both residues (V127A/L131A) completely abolished both processes. Additional mutations (D122N, F125A, and P130A) in the i2 loop differentially altered sequestration with similar impairments of signal transduction. The i3 loop junctions, which have been implicated as sites for G protein coupling, were shown to be involved in sequestration as two m1 mutants (W209A/I211A/Y212A and E360A/K362A/T366A) were found to be deficient in agonist-induced sequestration as well as stimulation of inositol phosphate release. These results suggest that the multi-site domain involved in signal transduction is similar to and may overlap with that involved in sequestration. Therefore, we propose that the cellular factors mediating receptor sequestration may be G proteins or factors structurally similar to G proteins.

II. INTRODUCTION

Agonist stimulation of receptors expressed at the cell surface triggers several adaptive responses which may modulate signal transduction. Characteristic of short term (minutes) agonist stimulation is a rapid sequestration of receptors, first at the cell's surface and then into the cell's interior (internalization), which may be followed by recycling or down-regulation. Different molecular requirements for receptor sequestration/internalization and down-regulation are thought to distinguish these regulatory events while little is known about the mechanism for receptor recycling (Glickman et al., 1989).

Although the molecular domains responsible for sequestration/internalization have been defined for receptors of the single transmembrane domain class (Davis et al., 1987; Rothenberger et al., 1987; Lobel et al., 1989; Chen et al., 1990; Jing et al., 1990; Thies et al., 1990), the recognition signals involved in the trafficking of the heptahelical G protein-coupled receptors (GPCRs) remain unclear. Mutational studies of several GPCRs have yielded receptors which are defective in sequestration/internalization (Strader et al., 1987; Hertel et al., 1990; Campbell et al., 1991; Hausdorff et al., 1991). Recently, Barak et al. (1994) proposed that a tyrosine residue found in the highly conserved sequence (NPXXY) in transmembrane domain VII (TMD VII) of the β_2-adrenergic receptor is required for agonist-induced sequestration/internalization. However, examination of this tyrosine residue in another GPCR, the gastrin-releasing peptide receptor, showed that it is not required for sequestration/internalization (Slice et al., 1994). Therefore, it appears that this tyrosine containing motif, which serves as a signal for the endocytosis of single transmembrane domain receptors, does not serve the same role in GPCR sequestration/internalization and suggests the presence of another domain mediating the internalization of GPCRs. Furthermore, multiple pathways for

sequestration/internalization may exist, and thus, multiple sequestration/internalization signals may exist (Raposo et al., 1987; Schvartz and Hazum, 1987).

Since G protein activation and sequestration are both dependent upon agonist stimulation, several studies have addressed the relationship between these two processes. Mutational analysis of the β_2-adrenergic receptor had initially suggested a structural overlap of receptor regions involved in G protein activation and sequestration/internalization (Cheung et al., 1989). In contrast, several m1 AChR mutants were identified with normal coupling behavior but impaired sequestration (Maeda et al., 1990; Lameh et al., 1992; Moro et al., 1993b). In addition, a m1/β_2 adrenergic receptor chimera was found to be deficient in coupling but capable of sequestration (Cheung et al., 1990). Normal sequestration/internalization was also observed for β_2-adrenergic receptors expressed in S49 cells despite functional uncoupling of these receptors from adenylyl cyclase as a result of genetic lesions in G_s (Mahan et al., 1985; Allen et al., 1989). Therefore, these results suggest the possibility that G proteins may have distinct roles in mediating signal transduction and sequestration.

In this study, a panel of m1 AChR mutants previously analyzed for contribution to G protein coupling (Moro et al., 1993a) were transiently transfected into HEK293 cells and tested for agonist-induced sequestration. Alanine substitution in three domains, i.e., both junctions of the i3 loop, and most notably, the conserved i2 loop domain, were found to affect sequestration, suggesting the hypothesis that overlapping multi-site domains mediate both signal transduction and sequestration.

III. EXPERIMENTAL PROCEDURES

A. Materials

[^{3}H]-N-methylscopolamine ([^{3}H]-NMS) (specific activity 80 Ci/mmol) and myo-[2-^{3}H]inositol (specific activity 17 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). All other reagents were of analytical grade quality.

B. Construction of Vectors Expressing m1 AChR and Mutants

The construction of m1 AChR wild-type in vector pSG5 was described previously (Lameh et al., 1992) having *Eco*RI and *Bam*HI restriction sites at the 5' and 3' ends, respectively. The point mutations were introduced using the "unique site elimination" method as was described by Moro et al. (1993a).

C. Transfection of Human Embryonic Kidney (HEK293) Cells

The cells were transfected by the calcium phosphate precipitation method (Maeda et al., 1990). Transient expression levels of m1 AChR were approximately 900 fmol/mg protein. Cells were maintained at 5% CO_2 in DME H-16/Ham's F-12 medium supplemented with 10% fetal bovine serum.

D. Phosphatidyl Inositol (PI) Hydrolysis

Cells transiently expressing m1 wild-type or mutant receptors were assayed for carbachol induced phosphoinositide hydrolysis as previously described (Maeda et al., 1990; Arden et al., 1992). Briefly, cells were incubated with [^{3}H]-myoinositol for 24 hours and were then assayed for inositol monophosphate, which accounts for most of the [^{3}H] activity in the presence of 10 mM LiCl. Results were expressed as percent of total [^{3}H] activity, and the percent values were compared between carbachol treated and

untreated cells. The coupling efficiencies of most of the mutants analyzed in this study were previously reported by Moro et al. (1993a).

E. Receptor Binding and Sequestration

Carbachol-induced sequestration of m1 receptors was determined as previously described (Maeda et al., 1990; Lameh et al., 1992). The transfected cells were replated onto 12-well cell culture dishes and allowed to attach overnight. On the day of the assay, cells were incubated in serum-free media with or without 1 mM carbachol for the indicated times. After drug treatment, the cells were washed three times with ice cold phosphate-buffered saline (PBS) to remove residual carbachol. The cells were incubated for 90 minutes at 12°C (to prevent receptor recycling) with 2 nM [^{3}H]-NMS in PBS to measure surface accessible binding sites. At the end of the incubation, the cells were filtered through glass-fiber (Schleicher and Schuell #32) filters, followed by three rapid rinses with PBS. Six independent samples were assayed for each data point unless noted otherwise. As previously reported, no measurable decrease in total receptor number was observed after two hours of carbachol treatment as determined by [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding (Lameh et al., 1992; Moro et al., 1993b).

IV. RESULTS

A. Effects of Mutation in the i2 Loop Motif DRYXXVXXPL on m1 AChR Sequestration

To test whether any residues in the i2 loop play a role in carbachol-induced sequestration, we transiently expressed mutant constructs in HEK293 cells and measured loss of surface receptor after 2 hours of carbachol treatment (Table 1.1). Mutants D122N, L131A, L131D, and the double point mutant V127A/L131A were defective in sequestration compared to wild-type m1 AChR. No effect on sequestration was observed

Table 1.1 Activation of Inositol Phosphate Release and Sequestration for Wild-type and Mutant m1 Receptors in HEK293 Cells Transiently transfected cells were incubated with 1 mM carbachol for 30 minutes to stimulate phosphoinositide hydrolysis as described previously (Moro et al., 1993a) and for two hours to induce sequestration. Cell surface receptor binding was measured with the polar tracer [^{3}H]-NMS. Overall receptor expression was determined by [^{3}H]-NMS binding prior to agonist treatment. Results from experiments yielding < 250 fmol/mg receptor protein were excluded from analysis of IP release, whereas sequestration could be measured at receptor expression levels > 150 fmol/mg protein. All data are mean ± S.D.

Mutant	Total Surface Expression (fmol/mg protein)	Stimulation of [^{3}H] Inositol Phosphate Release[a]	% Surface Receptor After Carbachol
Wild-type	920 ± 236 (19)[c]	100 ± 7	55 ± 7 (38)
D122N[b]	267 ± 45 (9)	76 ± 4 (3)	66 ± 12 (10)
F125A	500 ± 259 (14)	100 ± 11	40 ± 8 (15)
V127A	356 ± 117 (15)	48 ± 6	54 ± 7 (15)
V127A/L131A[b]	350 ± 125 (30)	8 ± 6 (4)	95 ± 15 (32)
P130A	931 ± 157 (6)	96 ± 29	62 ± 7 (6)
L131A	541 ± 189 (18)	14 ± 5	77 ± 12 (18)
L131D	676 ± 121 (11)	10 ± 12	78 ± 7 (9)
W209A/I211A/Y212A	678 ± 170 (14)	34 ± 12	72 ± 8 (14)
E360A/K362A/T366A	439 ± 121 (14)	39 ± 6	65 ± 7 (14)

[a] Stimulation of inositol phosphate release data is given for comparison and has previously been reported in Moro et al. (1993a) unless otherwise indicated.

[b] These mutants are not included with data of Moro et al. (1993a).

[c] The numbers in parenthesis correspond to number of data points.

with mutants V127A and P130A. Mutation of residue F125 resulted in significantly stronger sequestration than wild-type.

B. Sequestration of m1 AChR i3 Loop Junction Mutants

Residues proximal to transmembrane domains V and VI were mutated to test the contribution of these receptor regions to agonist-mediated sequestration (Figure 1.1). The two mutants tested (W209A/I211A/Y212A and E360A/K362A/T366A) were defective in carbachol induced sequestration (Table 1.1).

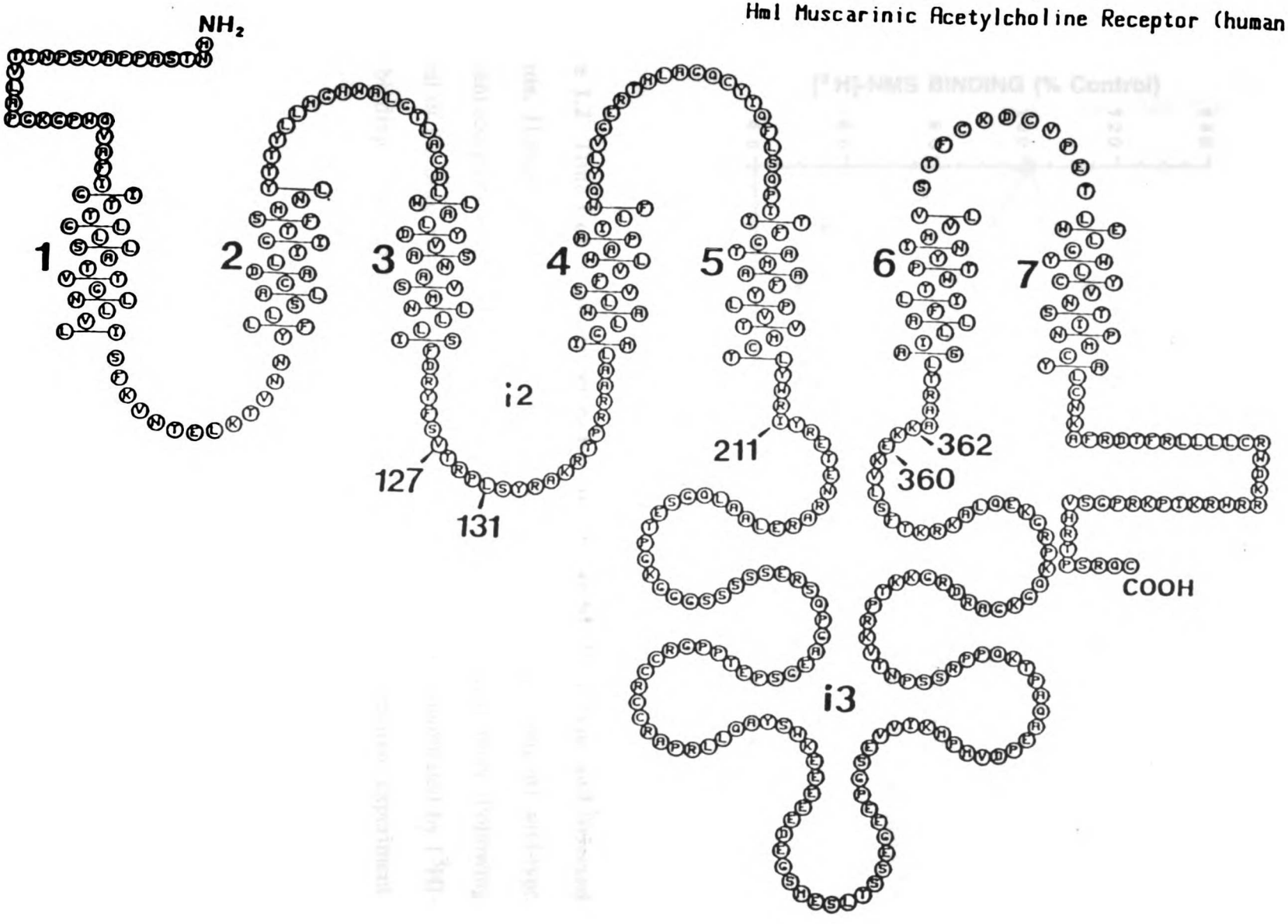
C. Time Course of Sequestration

To address whether the m1 mutants displayed altered kinetics of sequestration/internalization and recycling, we measured the time course of carbachol induced loss of [^{3}H]-NMS binding sites from the cell surface (Figure 1.2). The double mutant V127A/L131A did not measurably sequester at any time point. Each of the defective mutants tested rapidly sequestered over the initial 30 minutes, but only to an intermediate level. The low degree of sequestration, however, did not allow for accurate determination of sequestration rates. The time to reach equilibrium was comparable to wild-type for mutants L131A, W209A/I211A/Y212A, and E360A/K362A/T366A, suggesting no change in the rate of recycling for either of these mutants.

D. Comparison of Signal Transduction and Sequestration

The mutants examined in this study were initially screened for defects in stimulation of inositol phosphate release (Moro et al., 1993a), and these data are summarized in Table 1.1. As signal transduction and sequestration may be related or independent processes, the results of mutation on either process (Table 1.1) were plotted in Figure 1.3 to determine the degree of correlation. A striking correlation was observed

Figure 1.1 Amino Acid Sequence of m1 Acetylcholine Receptor. The locations of the mutations are shown. Mutations examined in this study are located in the second intracellular loop and the N- and C- junctions of the third intracellular loop.


Hm1 Muscarinic Acetylcholine Receptor (human)
NH2
1
2
3
4
5
6
7
i2
i3
127
131
211
360
362
COOH

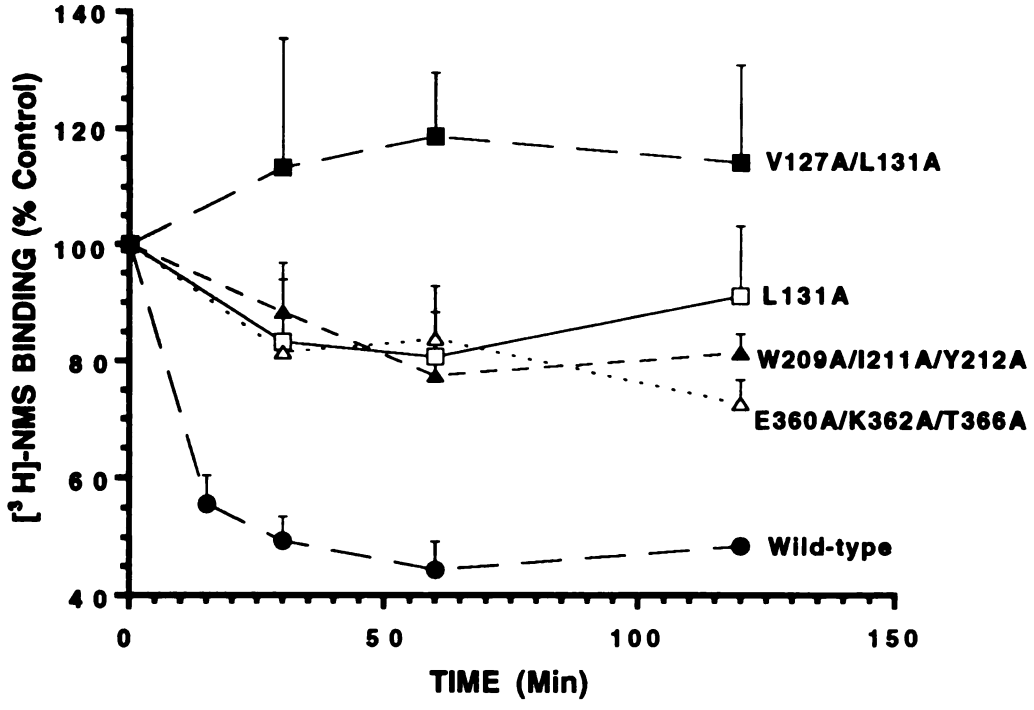


Figure 1.2 Time Course of Sequestration for m1 AChR Wild-type and Selected Mutants. Human embryonic kidney (HEK293) cells transiently expressing m1 wild-type or mutant receptors were exposed to 1 mM carbachol for the indicated times. Following removal of the drug, receptors expressed at the cell surface were quantitated by [^{3}H]-NMS binding. The results are mean ± S.D. (n =4) of one representative experiment.

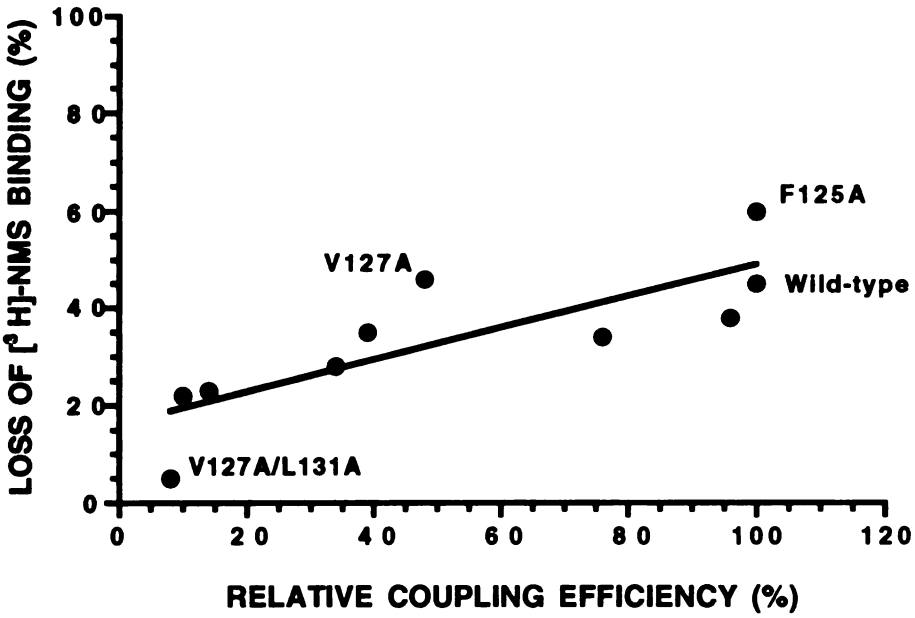


Figure 1.3 Comparison between m1 Receptor Activation of Inositol Phosphate Release and Sequestration. Coupling efficiency and loss of [^{3}H]-NMS binding after carbachol treatment for wild-type and mutant m1 receptors (data from Table 1.1) are plotted against each other. The correlation coefficient obtained by linear regression analysis is R=0.81. Mutant V127A was measurably defective in coupling, but not sequestration, while mutant V127A/L131A was completely defective in both processes. Sequestration of mutant F125A is significantly enhanced compared to wild-type.

between coupling to inositol phosphate hydrolysis and sequestration with linear regression analysis yielding a correlation coefficient R=0.81.

V. DISCUSSION

In this study, a series of alanine scanning mutants previously analyzed for G protein coupling deficiencies was tested for agonist-stimulated sequestration. Several of the mutants showed defective coupling to inositol phosphate release (mutants D122N, V127A, V127A/L131A, L131A, W209A/I211A/Y212A, and E360A/K362A/T366A) compared to wild-type m1 receptor, and we therefore proposed that these residues comprise a multi-site domain of m1 AChR which is involved in G protein coupling (Moro et al., 1993a).

To test whether G protein activation and sequestration depend on the same receptor domain, we studied the sequestration behavior of each construct in comparison to the wild-type receptor. The polar tracer [^{3}H]-NMS was used to label m1 receptor sites at the cell surface, and the carbachol induced decrease in tracer binding was used as a measure of sequestration (Maeda et al., 1990; Lameh et al., 1992). This method does not distinguish between receptors sequestered at the cell surface and receptors which have been redistributed to an intracellular compartment (internalization). Raposo et al. (1987) have demonstrated that agonist stimulation causes rapid receptor clustering at the cell surface and true internalization. In addition, Tolbert and Lameh (1996) have recently shown that m1 AChRs expressed in HEK293 cells internalize via a clathrin-mediated mechanism in response to agonist. Therefore, we surmise that the loss of surface binding observed in this study corresponds to true internalization, and we use the terms sequestration and internalization interchangeably.

The effects on sequestration observed with the mutants examined implicate domains of the i2 loop and both junctions of the i3 loop in receptor sequestration.

Mutation of residue leucine 131 in the i2 loop resulted in impaired coupling and suggested the requirement for a bulky lipophilic residue in this position for G protein activation (Moro et al., 1993a). As shown in this study, mutation of this residue also impedes receptor sequestration with substitution by alanine or a polar residue such as aspartic acid leading to defective sequestration. Additionally, substitution of this residue with asparagine or methionine also impaired sequestration; although, substitution with phenylalanine, as found in the β_2-adrenergic receptor, resulted in normal functioning (Moro et al., 1994). These results suggest that a bulky lipophilic residue in the equivalent position of leucine 131 in the m1 AChR is required for receptor sequestration as well as G protein coupling. It must be noted that mutation of the equivalent position in the β_2-adrenergic receptor (phenylalanine 139) also impaired G protein activation and sequestration (Moro et al., 1994), thus , supporting a general role for a lipophilic amino acid in the i2 loop of GPCRs in both coupling and sequestration.

Additional residues in the DRYXXVXXPL motif in the i2 loop of GPCRs were also examined for their contribution to receptor sequestration. Residue 127 is highly conserved with either a valine or an isoleucine found at this position in most GPCRs (Probst et al., 1992). Mutation of valine 127 in the m1 receptor was previously shown to partially inhibit coupling to the phosphoinositide hydrolysis pathway and was also found to impair coupling to the adenylyl cyclase pathway when expressed in Chinese hamster ovary cells (see Chapter 2). Despite impaired coupling, no measurable effect on sequestration was detected. Mutation of this residue, therefore, selectively alters G protein activation without affecting sequestration (see also Chapter 2), suggesting the possibility that factors distinct from G proteins may be mediating sequestration. However, the double point mutant V127A/L131A was profoundly defective in both G protein activation and sequestration, which demonstrates that this region of the i2 loop is essential for both processes. Perhaps mutation of residue V127 contributed to the overall defects in signal transduction and sequestration observed with m1 AChR-V127A/L131A

by introducing a conformational change in the receptor, which alone is not sufficient to impede receptor sequestration but does interfere with G protein activation.

Residue Asp-122 was previously shown to play a role in m1 AChR mediated stimulation of inositol phosphate release (Fraser et al., 1989). Analysis of mutant D122N revealed that this residue is also required for sequestration, indicating the importance of a polar amino acid at this position for functional interaction with G proteins and those factors mediating sequestration.

While most mutants analyzed in this study displayed impaired abilities to couple to G protein, to sequester, or to do both, one mutation was identified that resulted in stronger sequestration behavior than wild-type. The less conserved residue phenylalanine-125 was mutated to alanine with no effect on coupling but enhanced sequestration. The reason for the enhanced sequestration is unknown but may reflect an increased affinity of the receptor for the factor mediating sequestration.

In addition to residues in the i2 loop of the m1 receptor, our results suggest that residues in the amino- and carboxyl-termini of the i3 loop play a role in the sequestration process. Previous studies have implicated these regions in the recognition and the activation of G proteins (Wess et al., 1989; Wong et al., 1990; Cotecchia et al., 1992; Kunkel and Peralta, 1993; Shapiro et al., 1993; Blüml et al., 1994; Wade et al., 1994); however, a role for these receptor regions in mediating the sequestration response is controversial (Cheung et al., 1990). We now demonstrate that the junctions of the i3 loop are important in governing the sequestration of m1 AChR. The two mutants analyzed in this study involved multi-site mutations, and one might surmise that the impairment of both G protein activation and sequestration resulted from significant conformational changes in the receptor rather than direct inhibition of protein-protein interactions. Analysis of the single amino acid mutants derived from the original triple mutants revealed that each amino acid differentially contributes to the overall ability of the m1 AChR to activate G proteins (Chapter 2 and references therein) and to influence receptor

trafficking (sequestration and down-regulation). Therefore, we propose that although important for both activation of G proteins and receptor trafficking, the i3 loop junctions of m1 AChR actually facilitate signal transduction and receptor trafficking either via the activation of distinct factors (G proteins and G protein-like proteins) or by the maintenance of distinct conformational states which are necessary for the multi-functional responses of m1 AChR.

Therefore, our results support the hypothesis that the m1 receptor binding pocket for G proteins which stimulate inositol phosphate release is similar to but not identical to the binding pocket utilized by a factor which mediates sequestration. These findings imply that a G protein and any putative sequestration protein are structurally similar but do not exclude the possibility that indirectly both sequestration and G protein activation were impaired due to conformational changes in the receptor. Several small GTP binding proteins with some structural similarity to the heterotrimeric G proteins have been shown to play a role in protein trafficking (Donaldson et al., 1991; Shpetner and Vallee, 1992; Pimplikar and Simons, 1993). In addition, a p100 protein related to both G_{α} and adaptins was implicated in receptor trafficking (Traub and Sagi-Eisenberg, 1991). Heterotrimeric G proteins have also been postulated to have a role in protein trafficking (Bomsel and Mostov, 1992; Rothman and Orci, 1992). It is possible that G proteins may mediate both signal transduction and receptor trafficking. In fact, Thompson et al. (1991) have proposed that in SK-N-SH cells the sequestration of m3 receptors, which as m1 receptors are linked to stimulation of phospholipase C activity, requires the involvement of a GTP binding protein but not activation of second messenger. Our study of the mechanism(s) of m1 receptor sequestration and down-regulation in Chinese hamster ovary cells also supports the hypothesis that activation of GTP binding proteins may be involved in receptor trafficking; however, activation of GTP binding proteins correlated with down-regulation rather than sequestration of m1 receptors (see Chapter 2). Nevertheless, these results are still consistent with the hypothesis that the factor mediating sequestration of

the m1 receptor may be structurally similar to G proteins but suggest that GTP binding to this protein may not be required for its proper functioning. In conclusion, the strategies for isolating this protein will need to focus on maintaining the native structure of the receptor as recognized by both G proteins and cellular trafficking factors.

CHAPTER TWO

DOWN-REGULATION OF HUMAN m1 MUSCARINIC ACETYLCHOLINE RECEPTORS: RELATIONSHIP TO G PROTEIN COUPLING AND INTERNALIZATION

I. SUMMARY

Human m1 muscarinic acetylcholine receptor (AChR) mutants defective in either activation of the phosphoinositide hydrolysis cascade or internalization were stably transfected into Chinese hamster ovary cells to determine receptor domains and cellular pathways relevant to down-regulation. Down-regulation of m1 AChR required prior internalization of the receptor via clathrin-mediated endocytosis, therefore any mutation affecting internalization could not be used to assess down-regulation. Four mutations, V127A in the second intracellular loop and I211A, E360A, and K362A in the junctions of the third intracellular loop, however, were identified which specifically impaired down-regulation without altering receptor internalization. These mutants were also defective in signaling via the phospholipase C and the adenylyl cyclase pathways and in G protein activation, as measured by [^{35}S]GTPγS binding. Nonetheless, a direct correlation between the level of second messenger stimulation and the extent of down-regulation was not observed. Therefore, down-regulation of m1 AChR appears not to depend on activation of signal transduction pathways but may involve a factor which interacts with receptor domains similar to those involved in G protein activation but distinct from those involved in internalization.

II. INTRODUCTION

Stimulation of receptors with agonist leads to several adaptive responses modulating receptor activity and intracellular signaling, including receptor down-regulation which occurs upon chronic agonist exposure. Mechanism(s) of receptor down-regulation include decreased gene transcription, changes in mRNA stability, and increased proteolysis of receptor protein after translocation to lysosomes (Klein et al., 1979; Habecker and Nathanson, 1992). While the mechanism(s) of down-regulation via lysosomes are well defined for single transmembrane domain receptors (Iacopetta et al., 1988; Lobel et al., 1989; Peters et al., 1990; Baenziger et al., 1991; Johnson and Kornfeld, 1992; Kurten et al., 1996), understanding of G protein-coupled receptor (GPCR) down-regulation is limited. Studying GPCR down-regulation is complicated by the fact that down-regulation is a slow response occurring over several hours, secondary to G protein signaling and rapid adaptive responses including desensitization (Hausdorff et al., 1990) and receptor sequestration/internalization. The latter distributes intact receptor from the plasma membrane to an intracellular compartment inaccessible to polar ligands (Galper et al., 1982) and may precede receptor down-regulation (von Zastrow and Kobilka, 1992). Analysis of GPCR down-regulation, therefore, must account for the regulatory events occurring short term as well as the signaling cascades activated by receptor/ligand interaction.

Agonist-dependent down-regulation of GPCRs differs even among closely related GPCR subtypes and is dependent upon the cell line expressing the receptor (Koenig and Edwardson, 1996). Proposed models of down-regulation include a sequential pathway of receptor internalization either via coated or uncoated vesicles (Raposo et al., 1987; Raposo et al., 1989; Sloweijko et al., 1996) followed by sorting to the lysosome (von Zastrow and Kobilka, 1992) in addition to a distinct pathway of receptor sorting to a lysosomal compartment without detectable net internalization of intact receptor

(Hausdorff et al., 1991). Moreover, down-regulation has been suggested to require second messenger activation (Bouvier et al., 1989), and in some instances, receptor phosphorylation by protein kinase C (PKC) has been shown to facilitate or to induce down-regulation (Liles et al., 1986). Lack of second messenger involvement in down-regulation has also been reported (Thompson et al., 1991). On the other hand, heterotrimeric G proteins are known mediators or regulators of cellular trafficking (Bomsel and Mostov, 1992; Rothman and Orci, 1992) and thus may play a role in GPCR down-regulation. Thompson et al. (1991) have postulated the involvement of a GTP-binding protein, but not the activation of the phospholipase C cascade, in the agonist-induced sequestration of mAChRs in SK-N-SH cells and have surmised a link between sequestration and down-regulation.

Molecular analysis of GPCR down-regulation using site-directed mutagenesis has also had limited success since GPCR mutations usually affect either internalization or G protein coupling in addition to down-regulation (Campbell et al., 1991; Yang et al., 1995). A few GPCR domains have been proposed to play a role specifically in agonist dependent down-regulation, including tyrosine residues in the C-terminal tails of the β_2-adrenergic receptor (β-AR) (Valiquette et al., 1990) and the m2 AChR (Goldman and Nathanson, 1994) in addition to domains in the third intracellular (i3) loop of m1 AChR (Shapiro and Nathanson, 1989; Lee and Fraser, 1993). Nevertheless, no GPCR consensus domains uniquely involved in down-regulation have been identified.

Based on the results of previous studies, several questions must therefore be addressed for each GPCR studied. Is down-regulation of a GPCR dependent upon or modulated by the generation of second messengers? Is receptor activation of a G protein a necessary component of the down-regulation process? Which internalization pathway precedes down-regulation? Finally, what are the receptor domains that play a role specifically in down-regulation?

In this study, we examined the mechanism of down-regulation for the human muscarinic acetylcholine receptor (m1 AChR). From a panel of m1 mutants which we had previously analyzed in human embryonic kidney (HEK293) cells, we selected those with a range of defects, either in carbachol-induced receptor activation of inositol phosphate (IP) release or in internalization, or in both. The mutations cover three general receptor domains, i.e., the second intracellular (i2) loop, the N- and C-junctions of the third intracellular (i3) loop, and the middle portion of the i3 loop. The i3 loop junction mutants of m1 AChR displayed widely different abilities to stimulate second messenger (inositol phosphate) production (Högger et al., 1995), but their trafficking behavior had not been studied. Additionally, mutations of the i2 loop were chosen for this study because of the variable effects on internalization characterized previously (Moro et al., 1994). The bulk of the i3 loop was examined as this region has previously been reported to serve a role in m1 AChR down-regulation (Shapiro and Nathanson, 1989) and to play a regulatory role in internalization (Moro et al., 1993b). We had chosen to examine these mutants in HEK293 cells for the coupling and the internalization studies because these cells fail to down-regulate m1 AChR during agonist exposure, thus facilitating an analysis of internalization independent of receptor down-regulation (Maeda et al., 1990). In order to study the mechanism of m1 AChR down-regulation, we expressed m1 AChR wild-type and mutants in Chinese hamster ovary (CHO) cells, which were previously shown to down-regulate muscarinic receptors as demonstrated for the m3 AChR (Yang et al., 1993). In contrast to the m3 AChR, however, agonist stimulation of m1 AChR in CHO cells resulted first in a measurable sequestration/internalization of receptor sites, followed by a more gradual down-regulation. This allowed us to determine whether the processes of internalization and down-regulation are linked or independent of each other and which receptor domains are uniquely involved in modulating the extent of internalization, down-regulation, or both. In addition, m1 AChR has been shown to couple to multiple G proteins to activate several signaling pathways in CHO cells

(Burford and Nahorski, 1996). Therefore, we also addressed the contribution of multiple G proteins and signaling pathways, including phosphoinositide hydrolysis activation by $G_q/11$ and adenylyl cyclase activation by G_s, to receptor down-regulation .

We demonstrate that internalization and down-regulation involve distinct receptor domains. We provide evidence suggesting that m1 receptor down-regulation occurs subsequent to receptor internalization via a pathway involving clathrin-coated vesicles. A strong overlap between domains involved in down-regulation and those involved in G protein activation was observed; however, stimulation of either the phospholipase C or the adenylyl cyclase pathway did not correlate well with m1 AChR down-regulation. Therefore, a factor which interacts with similar receptor domains as do G proteins may play a role in down-regulation; however, G proteins of the $G_{i/o}$, G_s, and $G_q/11$ classes appear to be unlikely candidates in modulating m1 AChR down-regulation.

III. EXPERIMENTAL PROCEDURES

A. Materials

[^{3}H]-NMS (specific activity of 85 Ci/mmol), [^{3}H]-QNB (specific activity of 41.6-47 Ci/mmol), [^{35}S]GTPγS (specific activity of 1202 Ci/mmol), myo-[2-^{3}H]inositol (specific activity of 17 Ci/mmol), and cyclic AMP[^{3}H] assay system were obtained from Amersham (Arlington Heights, IL). Carbachol was purchased from Sigma Chemical Co. (St. Louis, MO). The polyclonal antibody to the C-tail of m1 AChR was a gift from Drs. Stefan Nahorski and Andrew Tobin, University of Leceister, UK. The monoclonal antibody to the heavy chain of clathrin was a gift from Dr. Frances Brodsky, University of California, San Francisco. The Cy5 (indodicarbocyanine) goat anti-mouse and the Cy3 (indocarbocyanine) donkey anti-rabbit antibodies were obtained from Biological Detection Systems, Inc. (Pittsburgh, PA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

B. Construction of Mutants

Mutants used in this study were those reported previously (Maeda et al., 1990; Lameh et al., 1992; Moro et al., 1994; Högger et al., 1995).

C. Cell Culture and Selection of Stable Transfectants

Chinese hamster ovary (CHO) cells were transfected by the calcium phosphate precipitation method (Maeda et al., 1990) using pSG5 vector containing the wild type or mutant m1 AChR genes together with pRSV^{neo}. Stably transfected cells were selected in medium containing 400 μg/ml of the antibiotic G418 (Bethesda Research Laboratories) and tested for [^{3}H]-NMS and [^{3}H]-QNB binding. Stable transfectants were maintained at 5% CO_2 in Ham's F-12 medium supplemented with 10% fetal bovine serum and 200 μg/ml G418.

D. Receptor Binding in Intact Cells

Cells were seeded onto 12-well tissue culture dishes at $2x10^5$ cells/well. At confluency (2-3 days), cells were treated with 1 mM carbachol for varying times. At the end of agonist treatment, the cells were cooled on ice and the cell monolayer was washed 3 times with ice-cold phosphate buffered saline (PBS). The cells were incubated for 90 minutes at 12 °C with 2 nM [^{3}H]-QNB in PBS for the down-regulation assay and with 2 nM [^{3}H]-NMS in PBS to measure surface accessible binding sites. The cells were then placed on ice, the monolayers were washed 3-5 times with ice-cold PBS, and the cells were harvested with 1 ml PBS. Radioactivity was determined by scintillation counting. Non-specific binding was determined in the presence of 10 μM atropine. Percent binding values were compared between carbachol treated and untreated cells. Data for the time courses presented are the averages of quadruples in a representative experiment (repeated 2-3 times), and the error bars represent standard deviation.

E. Immunofluorescence Confocal Microscopy

CHO cells expressing m1 AChR wild-type or mutant V127A/L131A were seeded on Permanox® chamber slides (Nunc Inc., Napperville, IL) and grown to 50% confluence (~2-3 days). Cells were treated with 1 mM carbachol, 10 μM atropine, or 1 mM carbachol and 10 μM atropine for 1 hour at 37 °C. For experiments conducted under hyperosmolar conditions, cells were incubated in serum-free media containing 0.45M sucrose for 20 minutes prior to the addition of carbachol. Following drug treatment, cells were washed with PBS, fixed for 10 minutes at room temperature with 3.7% paraformaldehyde in PBS, and permeabilized in PBS containing 0.25% fish gelatin, 0.04% saponin, and 0.05% NaN_3. After permeabilization, m1 AChR was labeled by incubation of cells with anti-m1 AChR polyclonal antibody for 1 hour at room temperature. Cells were washed four times with PBS followed by incubation with Cy3-conjugated donkey anti-rabbit secondary antibody. For colocalization studies, cells were washed four times with PBS and incubated with anti-clathrin monoclonal antibody, followed by PBS wash and incubation with Cy5-conjugated goat anti-mouse antibody. Cells were then washed four times with PBS and once with H_2O. Slides were mounted using Fluoromount G (Fisher Scientific, Pittsburgh, PA) containing a trace amount of phenylenediamine and were stored at 4 °C (Tolbert and Lameh, 1996). Samples were visualized using a laser scanning confocal microscopy with a krypton-argon laser coupled with a BioRad MRC-600 confocal head attached to an Optiphot II Nikon microscope equipped with a Plan Apo 60x objective lens with 1.4 numeric aperture. Cy3/Cy5 double emission was detected with a C1/C2 filter block (Sargent, 1994). For colocalization studies, mid-sectional images from two photomultiplier tubes were collected simultaneously and superimposed to identify areas of colocalization. When the images were merged, m1 AChR is arbitrarily colored red, clathrin is green, and areas of colocalization appear yellow.

F. Phosphatidyl Inositol (PI) Hydrolysis

Cells stably expressing wild-type or mutant m1 AChR were assayed for carbachol stimulated phosphoinositide hydrolysis. Cells were plated onto 6-well culture dishes and allowed to reach ~80% confluency. Subconfluent cells were labeled with [^{3}H]-myoinositol (0.2 μM) at 37 °C overnight. Following inositol labeling, cells were incubated in serum-free media with or without carbachol in the presence of 10 mM LiCl and assayed as previously described (Maeda et al., 1990; Arden et al., 1992). Results are expressed as percent of total intracellular [^{3}H] activity in the inositol phosphates, and the percent values were compared to baseline between carbachol treated and untreated cells to determine fractional stimulation by carbachol. Data were fitted by non-linear regression to the equation $E=(E_{max} \cdot L^n)/(L^n + EC_{50}^n)$. Carbachol concentrations of 1 μM to 10 mM were used for dose-response analysis.

G. Activation of Adenylyl Cyclase

CHO cells from a confluent T-75 flask were harvested in buffer (10 mM HEPES, 0.02% EDTA, 0.9% NaCl, pH 7.4), centrifuged at 1000x g, and the pellet was resuspended in 3 ml of Krebs-HEPES buffer (NaCl, 118.6 mM; KCl, 4.7 mM; $MgSO_4 \cdot 6H_2O$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 4.2 mM; D-glucose, 11.7 mM; $CaCl_2 \cdot 2H_2O$, 1.3 mM; and HEPES (free acid), 10 mM) with pH adjusted to 7.4 with 1M NaOH. 90μL of cells in suspension were added to 10 μl of either carbachol (10 mM final concentration) or buffer in microcentrifuge tubes at 37 °C for 5 minutes. Reactions were terminated by the addition of 10μl HCl (1M). The samples were vortexed and kept on ice for 10 minutes, neutralized with 1M NaOH, and centrifuged at 16,000x g for 5 minutes. The supernatants were kept and cAMP binding assays were performed using a radioreceptor assay as described previously (Burford et al., 1995a). Data are expressed as a percentage of wild-type m1 AChR stimulation of cAMP.

H. Carbachol-Induced [^{35}S]GTPγS Binding in Cell Membranes

Crude CHO cell membranes were prepared as previously described (Burford et al., 1995b) and frozen at -70°C at a protein concentration of 5 mg/ml in 10 mM HEPES, 0.1 mM EDTA, pH 7.4. Thawed membranes were resuspended in binding buffer consisting of 10 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4 at a final protein concentration of 100 µg/ml. Incubations were conducted in a final assay volume of 1 ml for 60 minutes at 30°C in the presence of 10µM GDP and approximately 70 pM [^{35}S]GTPγS (1202 Ci/mmol) with or without 3 mM carbachol. Incubations were terminated by vacuum filtration onto Schleicher and Schuell (S&S #32) filters. Radioactivity was assessed by liquid scintillation counting. Data were expressed as carbachol-stimulated [^{35}S]GTPγS binding as a percentage of basal binding.

IV. RESULTS

A. Expression and Ligand Binding of Stable Constructs in CHO Cells

Wild-type and mutant m1 cDNAs were co-transfected with a neomycin resistance gene into Chinese hamster ovary cells lacking endogenous muscarinic receptors. Colonies resistant to G418 were isolated and screened for expression of m1 AChRs with [^{3}H]-NMS and [^{3}H]-QNB. Clonal lines with expression levels approximating 2-3 pmol/mg protein were chosen with the exception of mutants SLTSS/ALAAA and d220-314 which expressed significantly fewer receptors with multiple clonal lines tested (Table 2.1). Mutant d314-358 did not yield significant radioligand binding with [^{3}H]-NMS in intact cells and thus was excluded from further study. When appropriate, cell lines with varying receptor expression were tested to determine the effect of receptor density on

TABLE 2.1: Internalization of m1 Receptor Mutants in CHO Cells. CHO lines stably expressing wild-type and mutant m1 AChR were treated with 1 mM carbachol for 30 minutes. Cell surface receptor loss was determined with the polar tracer [^{3}H]-NMS. Total receptor expression was measured with the lipophilic tracer [^{3}H]-QNB. Data are presented as percent of cell surface binding sites prior to agonist treatment. All data are mean ± S.D.

Mutant	Total Expression (fmol/mg protein)	Cell surface receptor after carbachol treatment (% control)
Wild-type	2164 ± 709	78 ± 8 (22)[a]
V127A	3370 ± 396	81 ± 6 (4)
L131A	2563 ± 655	102 ± 7 (7)*
V127A/L131A	3359 ± 143	104 ± 5 (8)[b]*
I211A	2756 ± 377	76 ± 9 (8)[b]
E360A	3878 ± 108	79 ± 5 (11)
K362A	2436 ± 299	82 ± 9 (11)
SLTSS/ALAAA	568 ± 110	85 ± 8 (15)
d232-358	2092 ± 870	109 ± 4 (9)*
d220-314	700 ± 275	ND

[a] Number in parenthesis corresponds to number of data points.

[b] Values for 2 hr carbachol treatment. Significance given in comparison to wild-type at a 2 hour time point.

*Significantly different from wild-type m1 receptor at $p<0.01$ (Fisher PLSD).

receptor function. Comparison of binding sites detected by [^{3}H]-NMS (cell surface sites only) and [^{3}H]-QNB (total receptor sites in the cell) indicated that >90% of the total receptor pool was detected at the cell surface for the cell lines used in this study.

B. Agonist-Induced Loss of Cell Surface Wild-type and Mutant m1 AChR

The effect of carbachol on the density of m1 AChR and mutants at the cell surface was measured with the polar tracer [^{3}H]-NMS (Table 2.1). Down-regulation was negligible for m1 AChR and most of the mutants over the first 30 minutes of agonist treatment; therefore, loss of [^{3}H]-NMS binding sites was determined after 30 minutes. A significant decrease in wild-type m1 AChR binding sites occurred after 30 minutes of 1 mM carbachol treatment (*$p<0.001$, Student's t-test). Mutants V127A, I211A, E360A, K362A, and SLTSS/ALAAA did not differ significantly from the wild-type receptor, whereas mutants L131A and d232-358 displayed no detectable loss in surface binding after 30 minutes of carbachol treatment (Table 2.1). Mutant V127A/L131A was completely defective in CHO cells even after 2 hours agonist treatment.

Overall, mutational effects on m1 AChR internalization were difficult to quantitate because of the relatively small decrease in surface receptor density detectable by binding analysis. Nevertheless, mutants previously characterized as internalization deficient in HEK cells (d232-358, L131A, and V127A/L131A) (Maeda et al., 1990; Lameh et al., 1992; Moro et al., 1994) exhibited a comparable defect in CHO cells, with one exception. Mutant SLTSS/ALAAA was shown to be partially deficient in internalization when expressed in HEK293 cells (Moro et al., 1993b). When expressed in CHO cells, loss of cell surface SLTSS/ALAAA receptors was similar to that of wild-type, but down-regulation of SLTSS/ALAAA paralleled the loss in surface sites (see below) so that no net accumulation of intracellular binding sites was detectable. The three point mutants in the i3 loop junctions (I211A, E360A, K362A) had not been analyzed previously in HEK293 cells for internalization defects. Each of these mutants showed

significant loss of surface sites after 30 minutes of carbachol treatment (*$p<0.01$), similar to the wild-type. We therefore conclude that internalization of these mutants is not impaired.

C. Subcellular Distribution of m1 AChR Wild-type by Confocal Microscopy

[^{3}H]-Tracer binding studies do not distinguish between receptors sequestered at the cell surface and receptors redistributed to an intracellular vesicular compartment. To verify that loss of [^{3}H]-NMS binding sites corresponds to true internalization, we determined the subcellular distribution of m1 AChR by immunofluorescence confocal microscopy. Following carbachol treatment, m1 AChR redistributed to intracellular compartments as evidenced by the punctate staining in the presence but not the absence of carbachol (Figure 2.1A-B). This is an agonist induced redistribution as the antagonist atropine had no effect (Figure 2.1C). Accumulation of intracellular binding sites was confirmed by confocal microscopy for mutants V127A, E360A, and K362A (data not shown). The level of expression of mutant SLTSS/ALAAA was below the limit of detection for confocal microscopy. Mutants L131A and d232-358, which displayed no measurable loss in [^{3}H]-NMS binding sites after 30 minutes of carbachol (Table 2.1), accumulated in intracellular vesicles at longer time points (data not shown), suggesting that these mutants were not completely defective in internalization. No vesicular accumulation was detectable for mutant V127A/L131A (Figure 2.1E-F), which also did not exhibit a detectable loss in [^{3}H]-NMS binding following 1 mM carbachol treatment (Table 2.1). The internal background staining present in control and in carbachol treated cells may reflect an intracellular pool of receptors as previously noted with other GPCRs (Hein et al., 1994). Since this staining is consistent among treatments and the various clonal lines screened, it did not interfere with our analysis of m1 AChR internalization into vesicles. Some intracellular staining is also present, albeit at a very low intensity, in

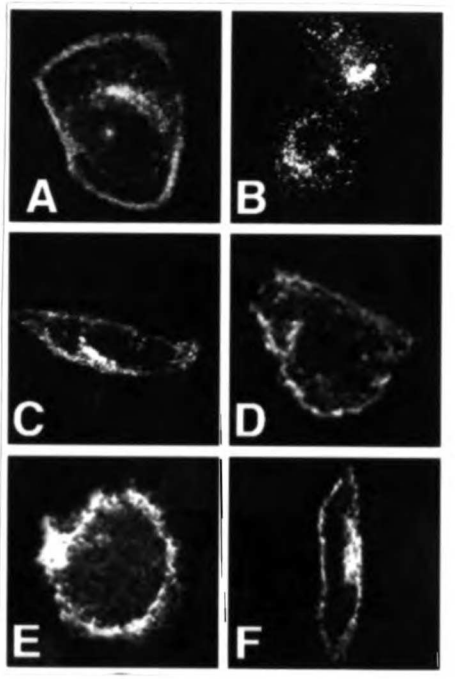


Figure 2.1: Subcellular Distribution of m1 AChR by Immunofluorescence Confocal Microscopy. Cells stably expressing m1 wild-type or mutant V127A/L131A were treated for 40 minutes with the appropriate drug, fixed, permeabilized, and visualized as described in Experimental Procedures. Panels A-D are m1 wild-type and panels E-F are mutant V127A/L131A. **(A)** No treatment. **(B)** 1 mM carbachol. **(C)** 10 μM atropine. **(D)** 1 mM carbachol, 0.45 M sucrose. **(E)** No treatment. **(F)** 1 mM carbachol.

untransfected CHO-K1, possibly representing nonspecific binding of the polyclonal anti-m1 C-tail antibody used.

D. Mechanism of m1 AChR Internalization in CHO Cells

To determine whether clathrin plays a role in the internalization of m1 AChR in CHO cells, we used a combination of biochemical and fluorescent imaging techniques. Hyperosmolar treatment with 0.45M sucrose, which blocks receptor endocytosis via clathrin coated vesicles, perturbed carbachol induced loss of [^{3}H]-NMS binding sites for wild-type m1 AChR and mutant SLTSS/ALAAA (Figure 2.2). Immunofluorescent staining of m1 AChR wild-type following 2 hours carbachol treatment under hyperosmolar conditions confirmed that the redistribution of the receptor in response to agonist was blocked (Figure 2.1D), suggesting the involvement of clathrin coated vesicles in the internalization of m1 AChR in CHO cells. Additionally, dual-labeling confocal microscopy revealed colocalization of the m1 AChR with clathrin, the major protein in clathrin coated vesicles. In the absence of carbachol, m1 AChR was primarily located at the cell surface where it did not colocalize with clathrin (Figure 2.3A). After carbachol treatment, the receptor was translocated into intracellular vesicles also containing clathrin (as depicted by the yellow color) (Figure 2.3B). These data indicate that internalization of m1 AChR in CHO cells occurs via a clathrin-dependent mechanism.

E. Down-regulation of m1 AChR Wild-type and Mutants

Loss in total receptor levels following carbachol treatment was determined for wild-type m1 AChR and mutants with the lipophilic tracer [^{3}H]-QNB (Figure 2.4), and a statistical analysis of the data is provided in Table 2.2. Less than half of the initial wild-type receptor pool remained after 12 hours of carbachol stimulation. Several mutations were identified that resulted in partial to complete impairment of m1 AChR down-regulation. Down-regulation of mutants with substitutions in the i2 loop and the i3 loop

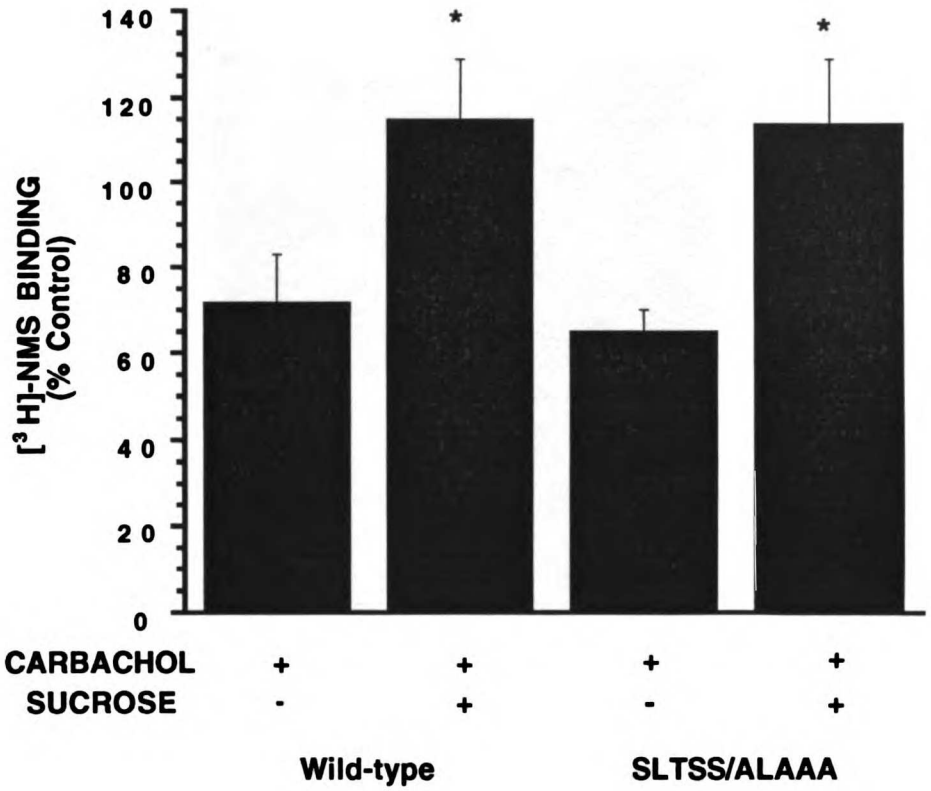


Figure 2.2: Carbachol-induced Internalization of m1 AChR Wild-type and Mutant SLTSS/ALAAA under Hyperosmolar Conditions. CHO cells stably expressing m1 wild-type or mutant SLTSS/ALAAA were treated with 1 mM carbachol for two hours in the presence or in the absence of 0.45 M sucrose. After washing with ice-cold PBS, cell surface binding was determined using the polar ligand [^{3}H]-NMS. Data are expressed as percent control binding (no carbachol). Data are presented as mean ± S.D. of 3-5 experiments performed in quadruplicate. Significance is given at *$p < 0.001$ (Fisher PLSD) in comparison to carbachol-induced internalization in the absence of sucrose.

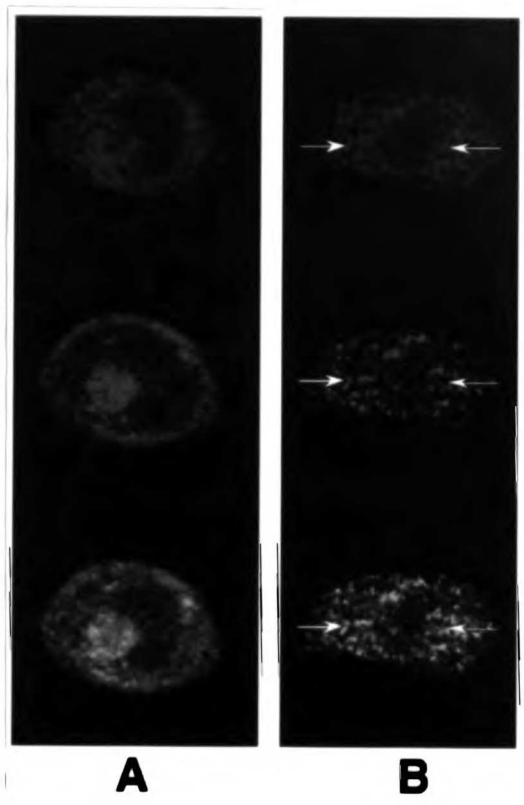


Figure 2.3: Colocalization of m1 AChR with Clathrin. Cells expressing m1 wild-type were incubated with 1 mM carbachol for 30 minutes. After fixing and permeabilizing, m1 receptors were labeled with polyclonal m1 antiserum followed by Cy3-labeled donkey anti-rabbit secondary antibody. Clathrin was then labeled sequentially with monoclonal anti-clathrin antibody followed by Cy5-conjugated goat anti-mouse antibody. The red color indicates the localization of m1 AChR, green represents the localization of clathrin, and yellow is indicative of colocalization of the receptor with clathrin in the merged image (lower panel). **(A)** No carbachol. **(B)** 1 mM carbachol. Images from the mid-section of the cells are shown. Arrows indicate representative areas of colocalization.

junctions, V127A, I211A, E360A, and K362A, was marginal with each exhibiting a decrease of only 20-25% in total binding after 12 hours (Figure 2.4A). Mutant L131A was significantly impaired with as little as 10% of the total receptor pool down-regulated after 12 hours of carbachol treatment while mutant V127A/L131A was completely defective with no measurable loss in receptor binding over 24 hours (Figure 2.4A).

Mutations in the large middle portion of the i3 loop differentially altered m1 AChR down-regulation. Deletion of the bulk of the i3 loop (d232-358) abolished down-regulation (Figure 2.4B) as previously reported with a similar m1 AChR deletion mutant (Shapiro and Nathanson, 1989). In contrast, deletion of residues 220-314 resulted in a mutant which down-regulated more rapidly than wild-type (50% decrease in total binding compared to an ~17% decrease in total wild-type receptor after 4 hours). Therefore, deletion analysis of the third intracellular loop suggested the possibility that region 315-358 may contain signals relevant to m1 AChR down-regulation; however, deletion of this region (mutant d314-358) resulted in a receptor that did not express at the surface and thus was excluded from the study.

Additionally, we examined the role of the serine/threonine rich SLTSS region located in the middle of the i3 loop in the regulation of m1 AChR down-regulation. We had previously reported that this region is important in modulating the extent of m1 AChR internalization in HEK293 cells (Moro et al., 1993b). In our present study, mutation of this region (SLTSS/ALAAA) did not affect loss of m1 receptor sites from the cell surface in CHO cells (Table 2.1). However, mutant SLTSS/ALAAA down-regulated more rapidly than wild-type over 12 hours of carbachol treatment (Figure 2.4B). It must be noted that mutant SLTSS/ALAAA expressed 4-fold less than wild-type, and we were unable to identify a stable cell line with receptor expression comparable to that of the other cell lines studied. We therefore tested down-regulation of wild-type m1 AChR at lower expression levels (1000 fmol/mg protein); however, down-regulation was

TABLE 2.2: Agonist-Induced Down-regulation of m1 Wild-type and Mutant Receptors in CHO Cells. Cells stably expressing wild-type or mutant m1 receptors at levels given in Table 2.1 were treated for indicated times with 1 mM carbachol. Receptors remaining following agonist treatment were detected with the lipophilic tracer [^{3}H]-QNB. Results are expressed as percent of the total binding sites prior to agonist treatment. All data are mean ± S.D.

Mutant	Total binding after 4 hrs carbachol (% control)	Total binding after 12 hrs carbachol (% control)
Wild-type	83 ± 4 (7)[a]	47 ± 11 (14)
V127A	86 ± 6 (8)[b]	78 ± 6 (8)*
L131A	116 ± 7 (4)*	87 ± 12 (11)*
V127A/L131A	95 ± 8 (4)*	100 ± 3 (4)*
I211A	85 ± 8 (8)[b]	78 ± 6 (8)*
E360A	92 ± 4 (4)	81 ± 3 (8)[c]*
K362A	88 ± 6 (11)[b]	71 ± 7 (12)*
SLTSS-ALAAA	70 ± 7 (8)*	26 ± 10 (9)*
d232-358	111 ± 9 (8)*	94 ± 13 (11)*
d220-314	50 ± 6 (4)*	ND[d]

[a] Numbers in parentheses correspond to the number of data points.

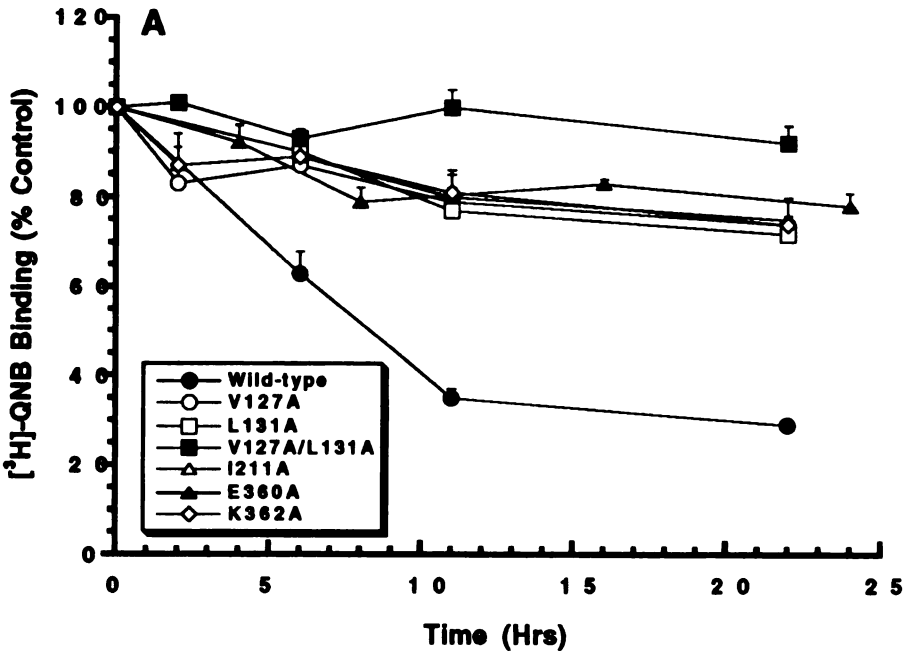
[b] Time of carbachol treatment is 6 hrs.

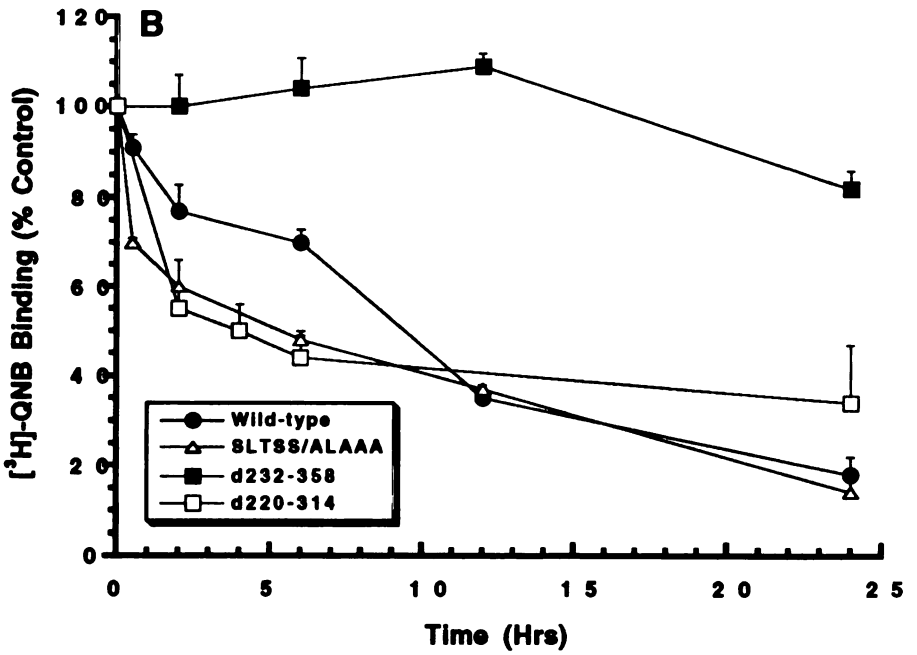
[c] Remaining QNB sites is the average of 8 hrs and 16 hrs carbachol treatment.

[d] Not determined

* Significantly different from wild-type m1 receptor at $p<0.01$ (Fisher PLSD).

Figure 2.4: Time Course of m1 AChR and Mutant Down-regulation. **(A)** Substitution mutants of the i2 and i3 loops of m1 **(B)** m1 i3 loop mutants. Cells were seeded on 12 well cell culture dishes. At confluence, cells were treated with 1mM carbachol for the indicated times. Following agonist treatment, cells were washed four times with PBS and incubated at 12 °C with 2 nM [^{3}H]-QNB for 90 minutes. Cells were then washed four times with PBS and harvested. Radioactivity was determined by liquid scintillation counting. Time course data is representative of 2-4 independent experiments performed in quadruplicate for each time point.


A
120
100
80
60
40
20
0
[3H]-QNB Binding (% Control)
0
5
10
15
20
25
Time (Hrs)
Wild-type
V127A
L131A
V127A/L131A
I211A
E360A
K362A


B
120
100
80
60
40
20
0
[3H]-QNB Binding (% Control)
0
5
10
15
20
25
Time (Hrs)
Wild-type
SLTSS/ALAAA
d232-358
d220-314

unaffected, thus arguing against the possibility that lower receptor density could account for the faster down-regulation of SLTSS/ALAAA (data not shown).

F. Effects of Hyperosmolarity on m1 AChR Down-regulation

To assess the contribution of m1 AChR internalization to the down-regulation process, the extent of agonist-induced down-regulation was monitored under conditions of hyperosmolarity which blocked m1 AChR internalization (Figures 2.1 and 2.2). Mutant SLTSS/ALAAA, which down-regulated more rapidly than wild-type following 4 hours of agonist treatment, was studied so as to minimize the exposure time of cells to hyperosmolar conditions while still allowing for detection of down-regulation. Carbachol treatment resulted in no significant loss of [^{3}H]-QNB binding sites for mutant SLTSS/ALAAA under hyperosmolar conditions (Figure 2.5).

G. Carbachol-Induced Inositol Phosphate Accumulation by m1 AChR and Mutants

Carbachol treatment of wild-type m1 AChR released 64% of the total inositol pool measured as inositol monophosphate (IP) over 30 minutes. The maximum level of IP release in response to carbachol was similar to the wild-type receptor for mutants E360A and d232-358 (Figure 2.6A). A decrease in potency, however, was observed for mutant E360A (EC_{50} 105 ± 14 μM) in comparison to wild-type (EC_{50} 14 ± 3 μM) with no change in carbachol binding affinity (data not shown), suggesting a possible coupling defect. This result is in contrast to the sensitizing effect of this mutation observed in HEK293 cells (Högger et al., 1995) for unknown reasons. Even though it was expressed at a lower level, mutant SLTSS/ALAAA was as effective as wild-type. Mutants V127A, V127A/L131A, I211A, and K362A stimulated lower maximal levels of IP release (Figure 2.6A). Mutant L131A, previously defined as coupling deficient when screened in HEK293 cells (Moro et al., 1993a), showed similar efficacy to that of wild-type at the high expression level; however, L131A was 3-4 fold less potent (EC_{50} 47 ± 5 μM) than

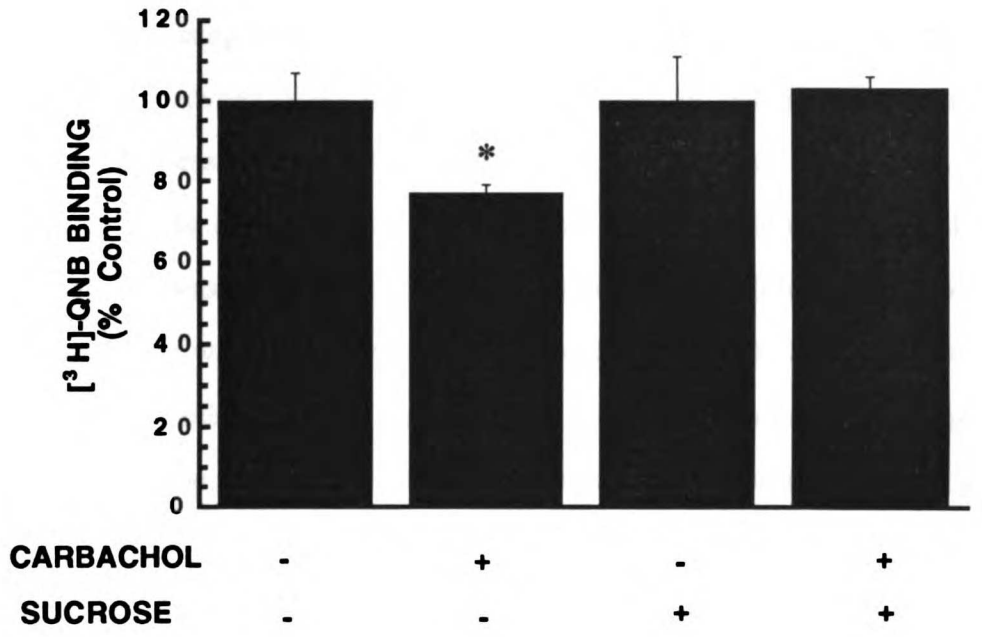


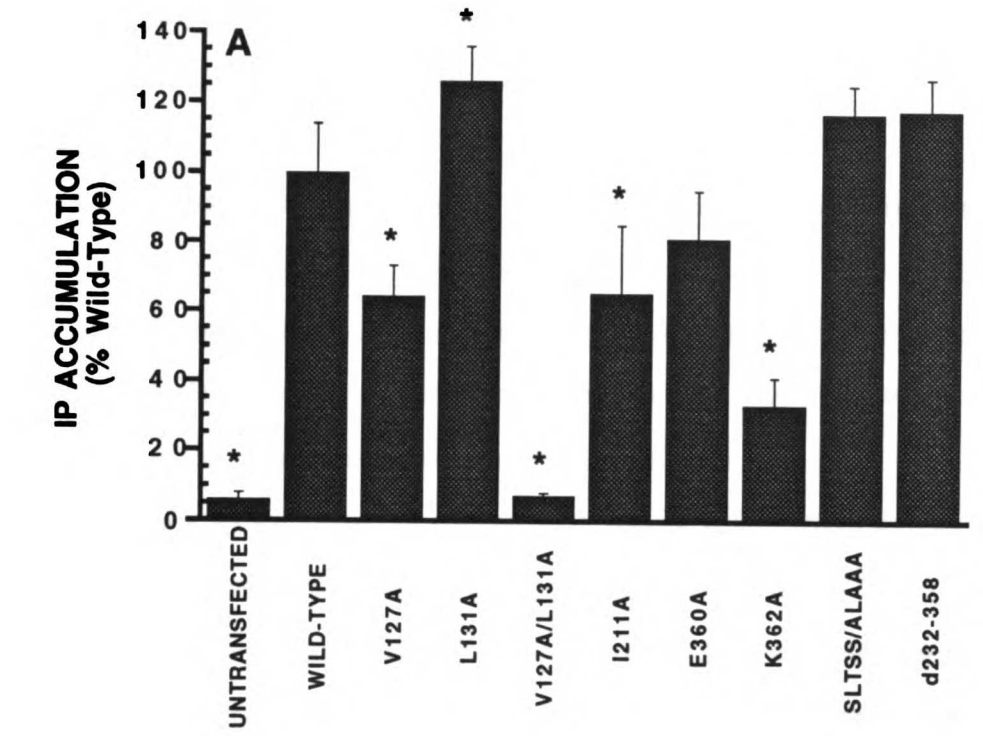
Figure 2.5: Carbachol-induced Down-regulation of m1-SLTSS/ALAAA under Hyperosmolar Conditions. Cells expressing m1-SLTSS/ALAAA were treated with 1 mM carbachol in the presence or in the absence of 0.45 M sucrose for 4 hours. Following treatment, cells were washed and incubated with 2 nM [^{3}H]-QNB as described in Experimental Procedures. Data are expressed as percent control (no carbachol) binding. Significance is given at *$p<0.001$ (Fisher PLSD) in comparison to control binding in the absence of sucrose.

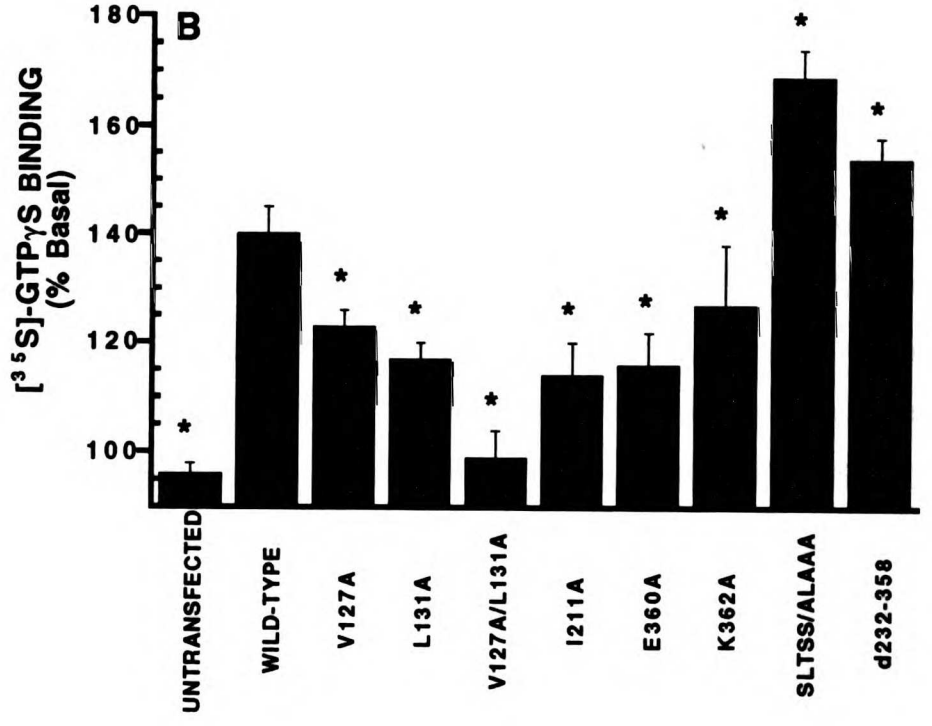
wild-type (EC_{50} 14 ± 3 μM) with no change in carbachol binding affinity (data not shown), suggesting an overall defect in coupling. Since previous reports have noted a relationship between receptor density and efficacy of second messenger coupling for receptors expressed in CHO cells (Shapiro et al., 1993), mutant L131A was tested at different levels of expression for maximal IP stimulation. Reduced maximal IP accumulation was observed at a lower expression level (680 fmol/mg protein, 13% of IP released by the wild-type receptor). The double mutant V127A/L131A was completely defective in IP release. These results are consistent with our previous studies in HEK293 cells (Moro et al., 1994) and confirm that mutants selected for impaired PI hydrolysis retain this defect when expressed in CHO cells.

H. Stimulation of Adenylyl Cyclase Activity by m1 AChR and Mutants

m1 AChR expressed in CHO cells was shown to couple to adenylyl cyclase via G_S, in addition to phospholipase C via the $G_{q/11}$ family of G proteins (Burford and Nahorski, 1996). After 5 minutes incubation with agonist, wild-type m1 AChR produced a carbachol-stimulated cAMP accumulation of 374 ± 46 pmol/mg protein (n = 4 independent experiments) from basal levels of 2-16 pmol/mg protein. No carbachol-induced cAMP accumulation was evident in untransfected CHO cells. Mutant d232-358 was able to induce wild-type levels of cAMP accumulation in response to carbachol (128 ± 18% of wild-type, n = 3 independent experiments) while activation of mutant SLTSS/ALAAA was 20 ± 3% that of wild-type (n = 3 independent experiments). Receptor expression levels influence the response measured because of an apparent lack of receptor reserve for adenylyl cyclase activation (Burford and Nahorski, 1996). Therefore, the less efficient stimulation of adenylyl cyclase by mutant SLTSS/ALAAA may have been related to its lower expression level rather than coupling changes induced by the mutation. Mutants V127A, L131A, V127A/L131A, I211A, E360A, and K362A

Figure 2.6: Carbachol-induced G-protein Activation by m1 AChR Wild-type and Mutants as Measured by (A) Inositol Monophosphate Accumulation (B) Stimulation of [^{35}S]GTPγS Binding. For IP accumulation assays, cells were treated with 1mM carbachol for 30 minutes. [^{35}S]GTPγS binding was determined with 10 mM carbachol. Data in panel (A) are presented as a percentage of m1 AChR wild-type stimulation. Data in panel (B) are presented as a percentage of basal (no carbachol) treatment for each construct tested. All data are mean ± S.D. of data points collected over 4-6 experiments. Significance of the difference between wild-type and mutant receptor is indicated by the asterisk (*) and is given at $p<0.0001$ (Fisher PLSD) for data in panel (A) and at $p<0.01$ (Fisher PLSD) for data in panel (B).


A
IP ACCUMULATION
(% Wild-Type)
140
120
100
80
60
40
20
0
UNTRANSFECTED
WILD-TYPE
V127A
L131A
V127A/L131A
I211A
E360A
K362A
SLTSS/ALAAA
d232-358


B
[35S]-GTPγS BINDING
(% Basal)
180
160
140
120
100
UNTRANSFECTED
WILD-TYPE
V127A
L131A
V127A/L131A
I211A
E360A
K362A
SLTSS/ALAAA
d232-358

produced no carbachol-induced increases in cAMP accumulation over basal levels, suggesting that they are all clearly deficient in coupling to adenylyl cyclase in CHO cells.

I. Carbachol-induced Activation of GTP Binding Proteins: Measurement of [^{35}S]-GTPγS Binding

G protein activation was measured using the non-hydrolyzable analog of GTP, [^{35}S]GTPγS, in the presence or absence of carbachol to determine which, if any, G proteins may be involved in down-regulation. Down-regulation of m1 AChR in CHO cells occurred in a PTX-insensitive manner with loss of [^{3}H]-QNB binding unaffected following 6 hours carbachol treatment in the presence of 100 ng/ml PTX (69 ± 12% of control binding in the presence of PTX compared to 78 ± 12% binding in the absence; results are averaged triplicate measurements from two independent experiments). These data suggest that G proteins of the G_i/G_o family are not required for m1 receptor down-regulation.

Carbachol-stimulated [^{35}S]GTPγS binding was therefore performed in CHO cell membranes prepared from cells that were pretreated for 16 hours in media supplemented with 100 ng/ml PTX to measure binding only to pertussis toxin-insensitive G proteins. This treatment reduced both basal and agonist stimulated [^{35}S]GTPγS binding for wild-type m1 AChR as shown previously (Burford et al., 1995b). Carbachol (3 mM) stimulated [^{35}S]GTPγS binding to 140 ± 6% of basal levels in cell membranes from CHO-m1 wild-type cells (Figure 2.6B). Basal binding of [^{35}S]GTPγS was not significantly different between each of the CHO cell clones tested (data not shown). Untransfected CHO cell membranes and membranes prepared from CHO cells expressing mutant V127A/L131A produced no increase in carbachol-stimulated [^{35}S]GTPγS binding above basal levels. Membranes prepared from CHO cells expressing mutants V127A, L131A, I211A, E360A, and K362A produced significantly lower levels of G protein activation following agonist incubation compared to membranes of wild-type m1

AChR expressing cells, despite the slightly higher expression of mutant m1 AChR in these clones as compared to wild-type m1 AChR expression (Table 2.1). Each of these m1 AChR mutants, therefore, appear to be partially deficient in coupling to PTX-insensitive G proteins compared to wild-type m1 AChR. Membranes prepared from both the SLTSS/ALAAA mutant and the i3 loop deletion mutant d232-358 produced high levels of carbachol-stimulated [^{35}S]GTPγS binding, suggesting that these mutants are fully functional in coupling to PTX-insensitive G proteins. The high level of carbachol-induced [^{35}S]GTPγS binding in the d232-358 mutant is expected considering the expression level of this mutant in comparison to wild-type m1 AChR (Table 2.1). However, the [^{35}S]GTPγS binding stimulated by mutant SLTSS/ALAAA was higher even though expression of this mutant was only 25% that of wild-type m1 AChR. This result is consistent with our finding that mutant SLTSS/ALAAA was fully active in stimulating IP release ($G_{q/11}$), which is a PTX-insensitive response.

V. DISCUSSION

In this study, we addressed the mechanisms underlying G protein-coupled receptor down-regulation with the m1 AChR as the model receptor. Down-regulation is a distinct mechanism possibly requiring cellular factors different from second messenger coupling and internalization (Thompson et al., 1991; Yang et al., 1995; Kurten et al., 1996). Since down-regulation occurs subsequent to these two events, it has been difficult to establish factors specific to the down-regulation process. The present study sheds new light on the mechanism of receptor down-regulation by parallel analysis of three key agonist-induced events: activation of G proteins, second messenger generation, and receptor internalization. The contribution of each event to the down-regulation of m1 AChR was determined by use of mutants which selectively interfere with one or all processes.

A. Cellular Pathways of Down-regulation: Relationship to Internalization

Internalization of m1 AChR was previously reported to be marginally detectable in CHO cells (Koenig and Edwardson, 1996). However, in the present study, vesicular localization of the m1 AChR following carbachol treatment by use of immunofluorescence confocal microscopy demonstrated intracellular accumulation of intact binding sites, consistent with the rapid loss of m1 AChR binding sites from the cell surface as measured by [^{3}H]-NMS binding studies.

In HEK293 cells, we have shown that m1 AChR is internalized via clathrin-coated vesicles, a pathway inhibited by either hypertonic conditions, depletion of intracellular K^+, or acidification of the cytosol (Tolbert and Lameh, 1996). Similarly, the m3 AChR, which also stimulates phosphoinositide hydrolysis, internalizes via a clathrin mediated process in SH-SY5Y neuroblastoma cells (Sloweijko et al., 1996). We show here that both internalization and down-regulation of m1 AChR are perturbed by hypertonicity and that internalized receptor colocalizes with clathrin, indicating that internalization of m1 AChR in CHO cells also occurs by a clathrin-mediated process. Moreover, since hyperosmolar treatment abolished both m1 AChR internalization and down-regulation, it appears that internalization via clathrin coated vesicles precedes down-regulation. Since m1 AChR appears to be endocytosed via identical pathways in HEK293 and CHO cells, the failure of HEK293 cells to down-regulate m1 AChR may therefore reflect a defect in the down-regulation pathway in these cells rather than differences in the internalization pathway.

To assess the effects of m1 AChR mutations interfering with internalization on the mechanism of down-regulation, we had to address the kinetics of both processes. Since internalization is relatively rapid (minutes), while down-regulation is slow (hours), we expected a net accumulation of intact wild-type receptors intracellularly, which was observable by the difference in receptor sites detectable by [^{3}H]-QNB (all receptors) and [^{3}H]-NMS (surface receptors). Any mutation that impairs internalization would be

expected to result in a reduction in the number of receptors accumulating intracellularly, and this was observed with mutant V127A/L131A, which was previously shown to be defective in internalization when expressed in HEK293 cells (Moro et al., 1994). Since down-regulation of m1 AChR is dependent on prior clathrin-mediated internalization, the failure of mutant V127A/L131A to down-regulate may result from a direct effect on internalization. Mutants L131A and d232-358, however, are only partially defective in internalization since loss of surface sites at early time points is not measurable for either mutant but vesicular accumulation of receptor at later time points (1 hour) can be detected. Furthermore, we have recently demonstrated that mutant d232-358 is capable of internalization in HEK293 cells despite no measurable loss of surface binding sites (Arden and Lameh, 1996), and mutant L131A was previously shown to be only partially defective in internalization when expressed in HEK293 cells (Moro et al., 1994). The effects of either of these two mutations on down-regulation could therefore not be assessed since we cannot rule out the possibility that the partial defects in internalization may have indirectly affected down-regulation.

In contrast, mutations could affect net internalization without altering down-regulation. Therefore, GPCR down-regulation may occur without detectable net accumulation of intracellular receptors by altering either the rate of recycling or the rate of down-regulation so that receptors that are internalized are immediately sorted to lysosomes for degradation. In the present study, the partially internalization deficient m1 AChR mutant SLTSS/ALAAA (Moro et al., 1993a) was seen to down-regulate even faster than the wild-type receptor. As a result, loss of surface binding sites of m1 AChR-SLTSS/ALAAA was comparable to wild-type, but no net accumulation of intracellular binding sites was detectable with [^{3}H]-NMS and [^{3}H]-QNB binding studies. Therefore, kinetic changes in internalization, down-regulation, and possibly recycling may account for the distinct cellular trafficking of SLTSS/ALAAA. By blocking the internalization pathway (hyperosmolar conditions), down-regulation of m1-SLTSS/ALAAA is also

suppressed, indicating that SLTSS/ALAAA endocytosis is similar to that of wild-type. The role of this serine/threonine rich region of m1 AChR in receptor sorting needs to be studied further in light of the recent findings that GRK2 and β-arrestins may play a role in receptor internalization (Ferguson et al., 1996). Haga et al. (1996) have recently reported phosphorylation of m1 AChR purified from insect Sf9 cells by GRK2 which may involve this serine/threonine rich region. Perhaps mutation of this region affects intracellular sorting of the receptor so as to favor rapid degradation in lysosomes instead of recycling back to the plasma membrane.

In this study, we have identified several mutations of m1 AChR which impair down-regulation without altering internalization. These mutations are therefore suitable for assessing direct effects on down-regulation. Mutation of the valine 127 residue in the i2 loop of m1 AChR has previously been shown to affect coupling to PI turnover, but not internalization (Moro et al., 1994). We now demonstrate a role for this residue in receptor down-regulation. Furthermore, the junctions of the i3 loop have long been implicated in G protein coupling (Arden et al., 1992; Wess, 1993); however, the contribution of these regions to the internalization process was unclear (Cheung et al., 1990; Moro et al., 1994). While all three mutants with substitutions in the i3 loop junctions, I211A, E360A, and K362A, maintained wild-type internalization behavior, these mutations similarly impaired the extent of m1 AChR down-regulation. Therefore, we have established a much closer correlation between G protein coupling and down-regulation than with internalization.

B. Role of G Protein Activation and Signaling Pathways in m1 AChR Down-regulation

Whereas internalization is thought to be independent of second messenger stimulation (Campbell et al., 1991), the role of second messenger, e.g. inositol phosphates and cAMP, in receptor down-regulation has not been clarified. Mutational analysis of the

human m1 receptor has led to the identification of several key residues important for functional G protein coupling as measured by IP accumulation. Mutants displaying the greatest defects were included with this study to determine if phosphoinositide hydrolysis is necessary for m1 down-regulation in CHO cells. We did not observe a dependency of receptor loss on extent of IP accumulation. In fact, mutant L131A, which is defective in PI hydrolysis, can stimulate levels of IP comparable to wild-type when expressed at high levels but is defective in down-regulation regardless of the level of expression. Similarly, mutant E360A stimulated IP production to wild-type levels but was defective in down-regulation when expressed at the same level as the wild-type receptor. Thus, generation of inositol phosphates is not necessary or at least not sufficient for down-regulation of m1 receptor in CHO cells.

We also examined the contribution of adenylyl cyclase activation by m1 AChR (Burford and Nahorski, 1996) to down-regulation. Phosphorylation of m1 AChR by PKA has been implicated in the heterologous regulation of this receptor (Lee and Fraser, 1993). Moreover, G_s has been identified as a factor in regulating cellular protein trafficking (Colombo et al., 1994). We observed no direct correlation between levels of cAMP accumulation and the extent of down-regulation in response to carbachol for the mutants tested (e.g., mutants V127A, E360A, and K362A did not stimulate cAMP production but did partially down-regulate). Treatment of CHO cells expressing wild-type m1 with 100μM H7, a general kinase inhibitor which blocks both PKC and PKA, had no effect on receptor down-regulation (data not shown). These data argue against the potential involvement of the adenylyl cyclase pathway in m1 AChR down-regulation and suggest that PKA and PKC are not involved. Lack of PKA involvement is consistent with a previous study demonstrating no PKA involvement in homologous regulation of m1 AChR (Lee and Fraser, 1993).

Whereas this study indicates that signaling events downstream of G protein activation are not required for m1 AChR down-regulation, considerable evidence exists

for a role of GTP binding proteins in internalization and down-regulation of GPCRs (Mahan et al., 1985; Thompson et al., 1991). To address the potential role of G proteins in the process of receptor down-regulation, we measured the ability of the m1 AChR mutants to stimulate GTP exchange in response to agonist. This measure is the most proximal coupling event that can be readily detected between receptor and G protein. Mutants defective in guanine nucleotide exchange, e.g., V127A, L131A, I211A, E360A, and K362A, were also deficient in carbachol induced down-regulation. The double point mutant, V127A/L131A, was completely defective in this functional assay as well. The point mutations in the i2 loop, V127A and L131A, both interfered with coupling and down-regulation; however, only the leucine residue appeared to play a role in internalization. Each point mutation in the i3 loop junctions, I211A, E360A, and K362A, affected G protein interaction as well as down-regulation, but not m1 AChR internalization. Therefore, a strong correlation was observed between G protein coupling and down-regulation, but not internalization.

Our mutational study indicates a potential structural overlap between G proteins that couple to m1 AChR and the factors involved in down-regulation; however, our results argue against a primary role for several G proteins, including $G_{i/o}$, $G_{q/11}$, and G_s. Pertussis toxin treatment, which interferes with $G_{i/o}$ activity, had no effect on m1 down-regulation. Previous reports have also suggested that $G_{i/o}$ does not appear to be required for down-regulation of muscarinic receptors (Thomas and Hoffman, 1986; Maloteaux and Hermans, 1994). Our data also indicate that the activity of G_s and/or $G_{q/11}$ does not correlate with the extent of m1 AChR down-regulation; therefore, direct involvement of either G_s or $G_{q/11}$ in down-regulation is unlikely. Moreover, Valiquette et al. (1993) demonstrated that normal receptor/G_s interaction is not required for down-regulation of the β_2-adrenergic receptor. In our study, full activation of either G_s or $G_{q/11}$ alone did not support functional down-regulation (d232-358 mutant), thus indicating that other factors must be involved. Nevertheless, all mutations that impaired carbachol-induced

GTP exchange also impaired down-regulation; thus, we cannot rule out the possibility that a GTP-binding protein or a factor interacting with similar receptor domains as those of the G proteins may be required for m1 AChR down-regulation.

In conclusion, we demonstrate that down-regulation of m1 AChR is under the control of factors distinct from those involved in internalization and downstream signaling events. A correlation between G protein coupling and down-regulation suggests a potential role for a factor which interacts with receptor domains similar to those involved in G protein activation. In addition, down-regulation of m1 AChR is dependent on prior internalization of the receptor via clathrin-mediated endocytosis, linking receptor internalization and down-regulation and arguing against down-regulation occurring via a separate pathway.

CHAPTER THREE

DOWN-REGULATION OF m3 RECEPTORS IN CHINESE HAMSTER OVARY CELLS IS DEPENDENT UPON PRIOR INTERNALIZATION OF THE RECEPTOR VIA A CLATHRIN-MEDIATED PATHWAY

I. SUMMARY

Agonist stimulation of G protein-coupled receptors triggers rapid (minutes) internalization of receptors to an intracellular compartment inaccessible to ligand. Internalized receptors may be sorted to lysosomes for subsequent degradation, a long term regulatory response known as down-regulation. The molecular and cellular requirements for GPCR down-regulation and the contribution of the internalization to this process are unknown. A domain in the third intracellular loops of the m1 and the m3 muscarinic receptor subtypes has been demonstrated to regulate internalization of these receptors when expressed in HEK293 cells (Moro et al., 1993b). Our study of m1 receptor internalization and down-regulation suggested that this domain does not appear to play a role in m1 receptor trafficking in Chinese hamster ovary cells (Chapter 2). We have further examined the role of this domain in the internalization and down-regulation of m3 receptors expressed in CHO cells. Mutation of the serine rich SASS domain in the i3 loop of m3 AChR appeared to inhibit not only internalization, but also down-regulation of this muscarinic receptor subtype, suggesting that down-regulation occurs sequentially downstream of the internalization event. Furthermore, internalization of m3 receptors in CHO cells occurred via clathrin-coated pits, and thus the mutation appeared to interfere specifically with clathrin-mediated endocytosis. As this region of the m3 receptor has been implicated as a target for phosphorylation by receptor specific kinases (Tobin et al., 1996), our results suggest the potential involvement of phosphorylation in the early steps of the clathrin-mediated endocytosis of m3 receptors.

II. INTRODUCTION

G protein-coupled receptors are regulated in response to agonist by several mechanisms including the redistribution of receptors to intracellular compartments inaccessible to ligand. The early phase of this redistribution involves the sequestration of receptors first at the cell surface and then in the cell's interior (internalization). Internalized receptors may then be recycled back to the cell surface or may be sorted to lysosomes for degradation (down-regulation).

Although several studies have documented the agonist triggered internalization and down-regulation of GPCRs, the cellular mechanisms and the molecular determinants involved in each process have not been well defined. Recent studies, however, have begun to elucidate the cellular pathways involved in receptor trafficking. Considerable evidence suggests that GPCRs internalize primarily via an endocytic pathway characterized by clathrin-coated vesicles (von Zastrow and Kobilka, 1992; Sloweijko et al., 1996; Tolbert and Lameh, 1996; Zhang et al., 1996), although alternative endocytic pathways have also been proposed (Raposo et al., 1989; Robinson et al., 1996). Furthermore, phosphorylation of receptors by G protein-coupled receptor specific kinases (GRKs) followed by b-arrestin binding to the phosphorylated receptor have been implicated as the early steps in the internalization of the GPCRs (Tsuga et al., 1994; Ferguson et al., 1995; Ferguson et al., 1996).

While progress has been made in the elucidation of the mechanism(s) involved in G protein-coupled receptor internalization, little is known about the mechanism(s) involved in agonist-induced receptor down-regulation. Several studies have indicated that receptor internalization and down-regulation are distinct processes (Mahan et al., 1985; Bouvier et al., 1989; Hausdorff et al., 1989). However, it has also been proposed that receptor internalization is a prerequisite for down-regulation (Thompson et al., 1991;

von Zastrow and Kobilka, 1992). Moreover, it remains controversial as to the molecular requirements governing receptor internalization and down-regulation (see Chapter 2)

Muscarinic acetylcholine receptors (mAChR) internalize and down-regulate in response to agonist treatment. Recent evidence supports internalization of the phospholipase C -linked subtypes, m1 AChR and m3 AChR, via clathrin coated vesicles (Sloweijko et al., 1996; Tolbert and Lameh, 1996). Mutational analysis of the receptor domains required for the internalization of these two muscarinic receptor subtypes has suggested a serine/threonine rich region as the putative molecular target for the internalization machinery of the cell (Moro et al., 1993b). This molecular domain has also been identified as a substrate for phosphorylation by the family of G protein-coupled receptor kinases (Haga et al., 1996). In addition, Tobin and Nahorski (1993) have demonstrated that m3 receptors expressed in CHO cells are phosphorylated in an agonist-dependent manner. Phosphorylation of the m3 receptor was predicted to occur at serine residues located within a region of the third intracellular loop containing this putative internalization domain (Tobin et al., 1996). These data therefore suggest the possible involvement of phosphorylation at sites within the putative internalization domain as an early step in the cellular trafficking of m1 and m3 receptors.

In this study, we again addressed the role of the serine rich SASS domain located within the third intracellular loop of the m3 receptor in modulating not only internalization, but down-regulation of this muscarinic subtype. We chose to re-examine the role of this domain in muscarinic receptor trafficking due to the paradoxical results obtained with the m1 receptor (see Chapter 2). Previously, the serine/threonine rich domain SLTSS was shown to regulate m1 receptor internalization as mutant m1-SLTSS/AAAA was found to be deficient in agonist-induced internalization when expressed in HEK293 cells (Moro et al., 1993b). However, this m1 receptor mutant appeared to internalize and to down-regulate in response to carbachol treatment when expressed in CHO cells; although, we were unable to determine if this mutation increased

the rate of down-regulation in such a way as to mask a deficit in internalization. Therefore, we questioned the general relevance of this domain in regulating agonist-induced muscarinic receptor trafficking.

We now investigate the role of this putative internalization domain in the trafficking of m3 receptors expressed in CHO cells. Expression of m3 wild-type and mutant SASS/AAAA receptors occurred at similarly high levels allowing for visualization of receptor distribution within the cell by confocal microscopy. Our results indicate that mutation of the SASS domain completely blocks internalization of m3 receptors in CHO cells. Furthermore, down-regulation of m3 receptors is dependent upon prior internalization of the receptor via clathrin-coated vesicles. These findings contrast those obtained with the m1 receptor, and thus it appears that the agonist-induced trafficking of m3 receptors, but not m1 receptors, is directly dependent on this domain.

III. EXPERIMENTAL PROCEDURES

A. Materials

[^{3}H]-NMS (specific activity of 85 Ci/mmol), [^{3}H]-QNB (specific activity of 41.6-47 Ci/mmol) and myo-[2-^{3}H]inositol (specific activity of 17 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Carbachol was purchased from Sigma Chemical Co.(St. Louis, MO). The polyclonal antibody to the third intracellular loop of m3 AChR was a gift from Drs. Stefan Nahorski and Andrew Tobin, University of Leceister, UK. The monoclonal antibody to the heavy chain of clathrin was a gift from Dr. Frances Brodsky, University of California, San Francisco. The Cy5 (indodicarbocyanine) goat anti-mouse and the Cy3 (indocarbocyanine) donkey anti-rabbit antibodies were obtained from Biological Detection Systems, Inc. (Pittsburgh, PA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

B. Construction of Mutants

Construction of mutant m3-SASS/AAAA was previously described by Moro et al. (1993).

C. Cell Culture and Selection of Stable Transfectants

Chinese hamster ovary (CHO) cells were transfected by the calcium phosphate precipitation method (Maeda et al., 1990) using pSG5 vector containing the wild type or mutant m3 AChR genes together with $pRSV^{neo}$. Stably transfected cells were selected in medium containing 400 μg/ml of the antibiotic G418 (Bethesda Research Laboratories) and tested for [^{3}H]-NMS and [^{3}H]-QNB binding. Stable transfectants were maintained at 5% CO_2 in Ham's F12 medium supplemented with 10% fetal bovine serum and 200 μg/ml G418.

D. Receptor Binding in Intact Cells

Cells were seeded onto 12-well tissue culture dishes at $2x10^5$ cells/well. At confluency (2-3 days), cells were treated with 1 mM carbachol for varying times. At the end of agonist treatment, the cells were cooled on ice and the cell monolayer was washed 3 times with ice-cold phosphate buffered saline (PBS). The cells were incubated for 90 minutes at 12 °C with 2 nM [^{3}H]-QNB in PBS for the down-regulation assay and with 2 nM [^{3}H]-NMS in PBS to measure surface accessible binding sites. The cells were then placed on ice, the monolayers were washed 3-5 times with ice-cold PBS, and the cells were harvested with 1 ml PBS. Radioactivity was determined by scintillation counting. Non-specific binding was determined in the presence of 10 μM atropine. Percent binding values were compared between carbachol treated and untreated cells. Data for the time courses presented are the averages of quadruples in a representative experiment (repeated 2-3 times), and the error bars represent standard deviation.

E. Immunofluorescence Confocal Microscopy

CHO cells expressing m3 wild-type or mutant SASS/AAAA were seeded on Permanox® chamber slides (Nunc Inc., Napperville, IL) and grown to 50% confluence (~2-3 days). Cells were treated with 1mM carbachol for 1 hour at 37 °C. For experiments conducted under hyperosmolar conditions, cells were incubated in serum-free media with 0.45 M sucrose for 20 minutes prior to the addition of carbachol. Following drug treatment, cells were washed with PBS, fixed for 10 minutes at room temperature with 3.7% paraformaldehyde in PBS, and permeabilized in PBS containing 0.25% fish gelatin, 0.04% saponin, and 0.05% NaN_3. After permeabilization, m3 AChR was labeled by incubation of cells with anti-m3 AChR polyclonal antibody for 1 hour at room temperature. Cells were washed four times with PBS followed by incubation with Cy3-conjugated donkey anti-rabbit secondary antibody. For colocalization studies, cells were washed four times with PBS and incubated with anti-clathrin monoclonal antibody, followed by PBS wash and incubation with Cy5-conjugated goat anti-mouse antibody. Cells were then washed four times with PBS and once with H_2O. Slides were mounted using Fluoromount G (Fisher Scientific, Pittsburgh, PA) containing a trace amount of phenylenediamine and were stored at 4 °C (Tolbert and Lameh, 1996). Samples were visualized using a laser scanning confocal microscopy with a krypton-argon laser coupled with a BioRad MRC-600 confocal head attached to an Optiphot II Nikon microscope equipped with a Plan Apo 60x objective lens with 1.4 numeric aperture. Cy3/Cy5 double emission was detected with a C1/C2 filter block (Sargent, 1994). For colocalization studies, mid-sectional images from two photomultiplier tubes were collected simultaneously and superimposed to identify areas of colocalization. When the images were merged, m3 AChR is arbitrarily colored red, clathrin is green, and areas of colocalization appear yellow.

F. Phosphatidyl Inositol (PI) Hydrolysis

Cells stably expressing wild-type or mutant m3 were assayed for carbachol stimulated phosphoinositide hydrolysis. Cells were plated onto 6-well culture dishes and allowed to reach ~80% confluency. Subconfluent cells were labeled with [^{3}H]-myoinositol (0.2 μM) at 37°C overnight. Following inositol labeling, cells were incubated in serum-free media with or without carbachol in the presence of 10 mM LiCl and assayed as previously described (Maeda et al., 1990; Arden et al., 1992). Results were expressed as percent of total intracellular [^{3}H] activity in the inositol phosphates, and the percent values were compared to baseline between carbachol treated and untreated cells to determine fractional stimulation by carbachol.

IV. RESULTS

A. Expression and Ligand Binding Properties of m3 Wild-type and Mutant Receptors in CHO Cells

Wild-type and mutant m3 cDNAs were co-transfected with a neomycin resistance gene into Chinese hamster ovary cells lacking endogenous muscarinic receptors. Colonies resistant to G418 were isolated and screened for receptor expression with [^{3}H]-NMS and [^{3}H]-QNB. Cell lines with the greatest expression levels were chosen for this study to facilitate the use of confocal microscopy for monitoring receptor trafficking. B_{max} for CHO-m3 wild-type expression was approximately 2.5 pmol/mg protein and CHO-m3-SASS/AAAA expressed at approximately 4 pmol/mg protein. Stable lines expressing significantly fewer sites were also isolated and screened for carbachol induced effects when appropriate. Comparison of binding sites detected by [^{3}H]-NMS (surface sites) and [^{3}H]-QNB (total sites) indicated that >90% of the total receptor pool was detected at the cell surface for both wild-type and mutant m3 receptor. Antagonist binding affinity for mutant m3 was similar to that of wild-type (data not shown). Agonist

binding was also unaffected as measured by carbachol displacement of [^{3}H]-NMS binding sites (IC_{50} 0.20 ± 0.03 mM for wild-type and 0.29 ± 0.05 mM for mutant SASS/AAAA).

B. Carbachol-Induced Changes in [^{3}H]-NMS Binding to Intact CHO-m3 and CHO-m3-SASS/AAAA Cells

Cells expressing either wild-type or mutant m3 receptors were treated for the indicated times with 1 mM carbachol, and the remaining surface sites were measured in the presence of a saturating concentration (2 nM) of [^{3}H]-NMS. A decrease in surface expression of m3 wild-type receptors was time-dependent with a loss in surface binding initially detected following 15 minutes of treatment (Figure 3.1). In contrast, no significant change in [^{3}H]-NMS binding was observed for mutant m3-SASS/AAAA (data not shown).

C. Subcellular Distribution of Wild-type and Mutant m3 Receptors by Confocal Microscopy

To determine whether the loss in [^{3}H]-NMS surface binding sites corresponds to true internalization, immunofluorescence confocal microscopy was used to visualize receptor distribution within individual cells. Prior to agonist treatment, wild-type m3 receptors resided predominantly at the cell surface (Figure 3.2A). After 30 minutes of carbachol treatment, receptors were localized to intracellular vesicles and surface expression of the receptor was reduced (Figure 3.2B). Vesicular accumulation of wild-type receptors occurred in a time-dependent manner (Figure 3.2C-D); however, localization of m3 receptors over time appeared to increase in the perinuclear region of the cell at the later time points as opposed to the distinct vesicular pattern seen at earlier time points (Figure 3.2B). [^{3}H]-QNB binding following two hours of carbachol

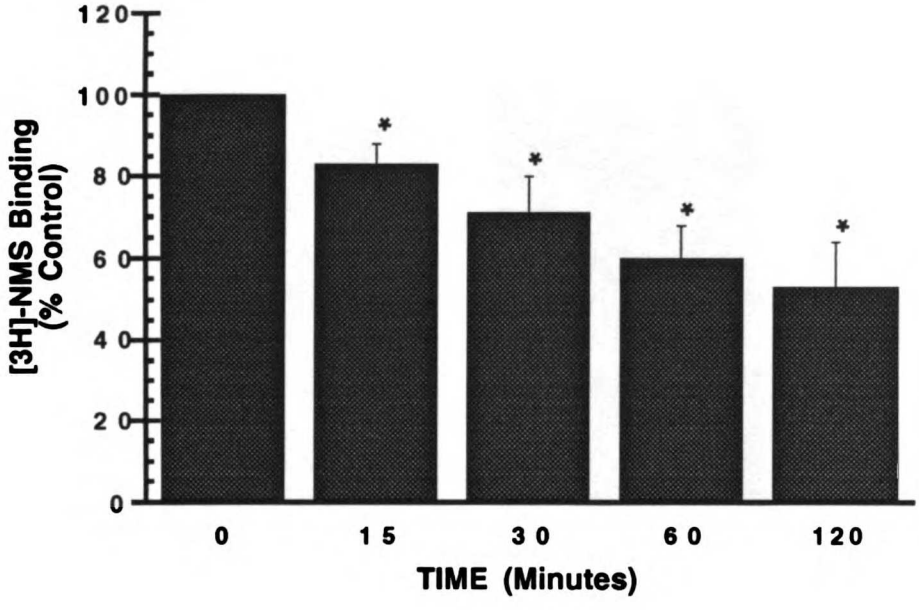


Figure 3.1 Carbachol-Induced Internalization of m3 AChR Wild-type. CHO cells stably expressing wild-type m3 receptors were treated for the indicated times with 1 mM carbachol to stimulate internalization of receptors. Following agonist treatment, cell surface binding sites were determined with a saturating concentration (2 nM) of the polar tracer [^{3}H]-NMS. Data are presented as the percent of cell surface binding sites prior to agonist treatment. Data are mean ± S.E. of three to five experiments, each performed in triplicate. Significance is given at *$p<0.01$ (Fisher PLSD and one-factor ANOVA).

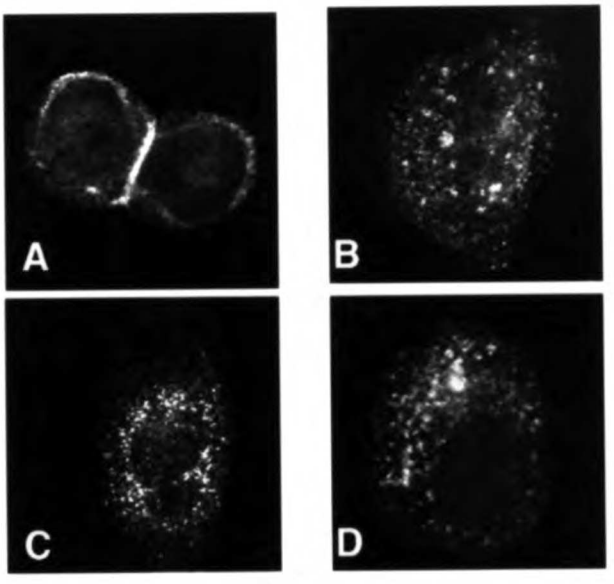


Figure 3.2 Subcellular Distribution of m3 AChR Visualized by Immunofluorescence Confocal Microscopy. Cells stably expressing m3 wild-type were treated with carbachol for the indicated times, fixed, permeabilized, and visualized as described in Experimental Procedures. **(A)** No treatment. **(B)** 30 minutes Carbachol. **(C)** 60 minutes Carbachol. **(D)** 120 minutes Carbachol. Data are representative of three or more independent experiments.

treatment indicated a loss of total cellular receptors (see Figure 3.6), and the increased perinuclear staining observed may therefore reflect a receptor population associated with late endosomes or lysosomes.

[^{3}H]-NMS binding following agonist treatment suggested no loss in surface expression of mutant m3-SASS/AAAA. As this mutant was expressed at relatively high levels (~4 pmol/mg protein), we wanted to confirm that this mutant was indeed defective in internalization and that the high expression level did not mask a small population of internalized receptors undetected by our binding studies. In the absence of agonist, m3-SASS/AAAA was expressed primarily at the cell surface (Figure 3.3A). No agonist-induced redistribution of mutant m3-SASS/AAAA was observed over the two hour treatment period (Figure 3.3B-D). Four hours of agonist treatment also had no effect on the distribution m3-SASS/AAAA (data not shown).

D. Perturbation of m3 AChR Internalization by Sucrose

To disrupt clathrin-mediated endocytosis, CHO cells were treated with agonist under hyperosmolar media conditions (0.45 M sucrose). Pretreatment with sucrose completely blocked carbachol-induced internalization of m3 wild-type receptors (Figure 3.4). No change in [^{3}H]-NMS binding was observed over two hours of carbachol treatment in the presence of sucrose (data not shown).

E. Colocalization of m3 Wild-type and Mutant Receptors with Clathrin

To further examine the potential role for clathrin in the internalization of m3 receptors in CHO cells, we used dual-label confocal microscopy to simultaneously localize m3 receptors and clathrin within the cell. In the absence of agonist, m3 receptors were localized to the cell surface and did not colocalize with clathrin (Figure 3.5A). Following agonist treatment, wild-type receptors were located in intracellular vesicles

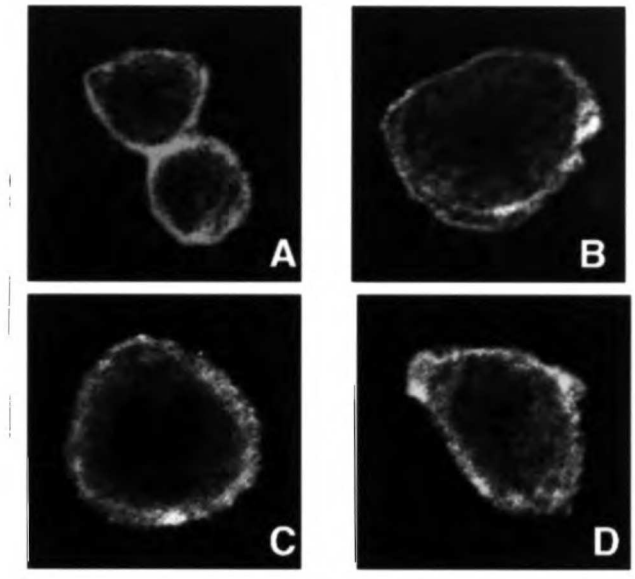


Figure 3.3 Effects of Mutation SASS/AAAA on Agonist-Induced Internalization of m3 Receptors. Cells expressing mutant m3-SASS/AAAA were treated as described in Figure 3.2. **(A)** No Carbachol. **(B)** 30 minutes Carbachol. **(C)** 60 minutes Carbachol. **(D)** 120 minutes Carbachol. Data represent the results obtained in three or more independent experiments.

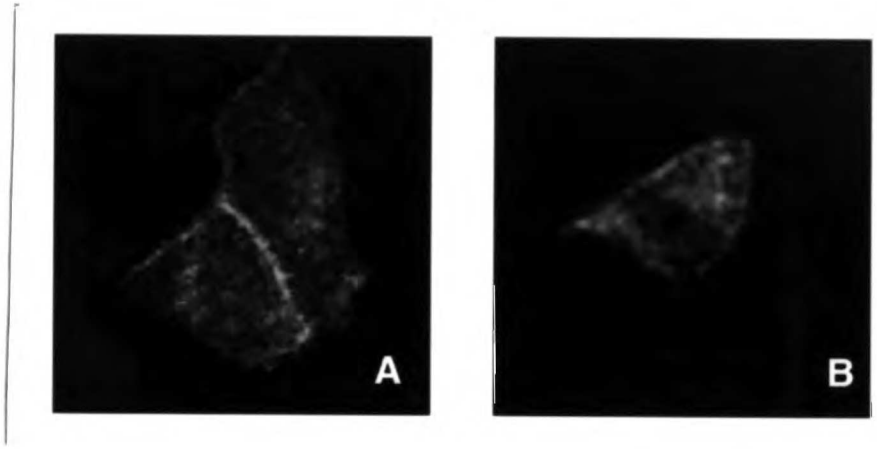


Figure 3.4 Carbachol-Induced Internalization of m3 AChR Wild-type under Hyperosmolar Conditions. CHO cells stably expressing m3 wild-type receptors were treated with 1 mM carbachol for 30 minutes in the presence of 0.45 M sucrose. After washing with PBS, receptors were visualized by confocal microscopy as described in Figure 3.2. **(A)** Control. **(B)** 1 mM Carbachol.

containing clathrin (yellow color) (Figure 3.5B). Mutant m3-SASS/AAAA, which did not internalize in response to agonist, did not colocalize with clathrin in the absence of carbachol (Figure 3.5C), although a slight colocalization was observed at the cell surface following carbachol exposure (Figure 3.5D).

F. Down-regulation of m3 Wild-type and Mutant SASS/AAAA Receptors Following Prolonged Carbachol Exposure

Total receptor number, monitored by [^{3}H]-QNB binding, was determined for wild-type and mutant m3 receptor expressing cells following prolonged stimulation by agonist (Figure 3.6). Carbachol (1 mM) elicited a rapid decrease in m3 wild-type receptor (~40% loss after 4 hours) that remained constant over time with no additional loss observed up to 24 hours. Stimulation of CHO cells expressing m3-SASS/AAAA with carbachol resulted in no significant change in total receptor number even after 24 hours.

G. Carbachol-induced Inositol Phosphate Accumulation by Wild-type and Mutant m3 AChRs

Carbachol treatment of wild-type m3 resulted in a 6.7 ± 0.4 fold increase over baseline of total inositol phosphates. Activation of phosphoinositide hydrolysis was similar to that of wild-type for mutant m3-SASS/AAAA with a 7.3 ± 0.3 fold increase observed following 30 minutes of carbachol (1mM).

V. DISCUSSION

In this study we examined the pathways associated with the internalization and down-regulation of m3 AChRs expressed in CHO cells. Previous studies of m3 receptor trafficking in CHO cells suggested that minimal, if any, internalization of

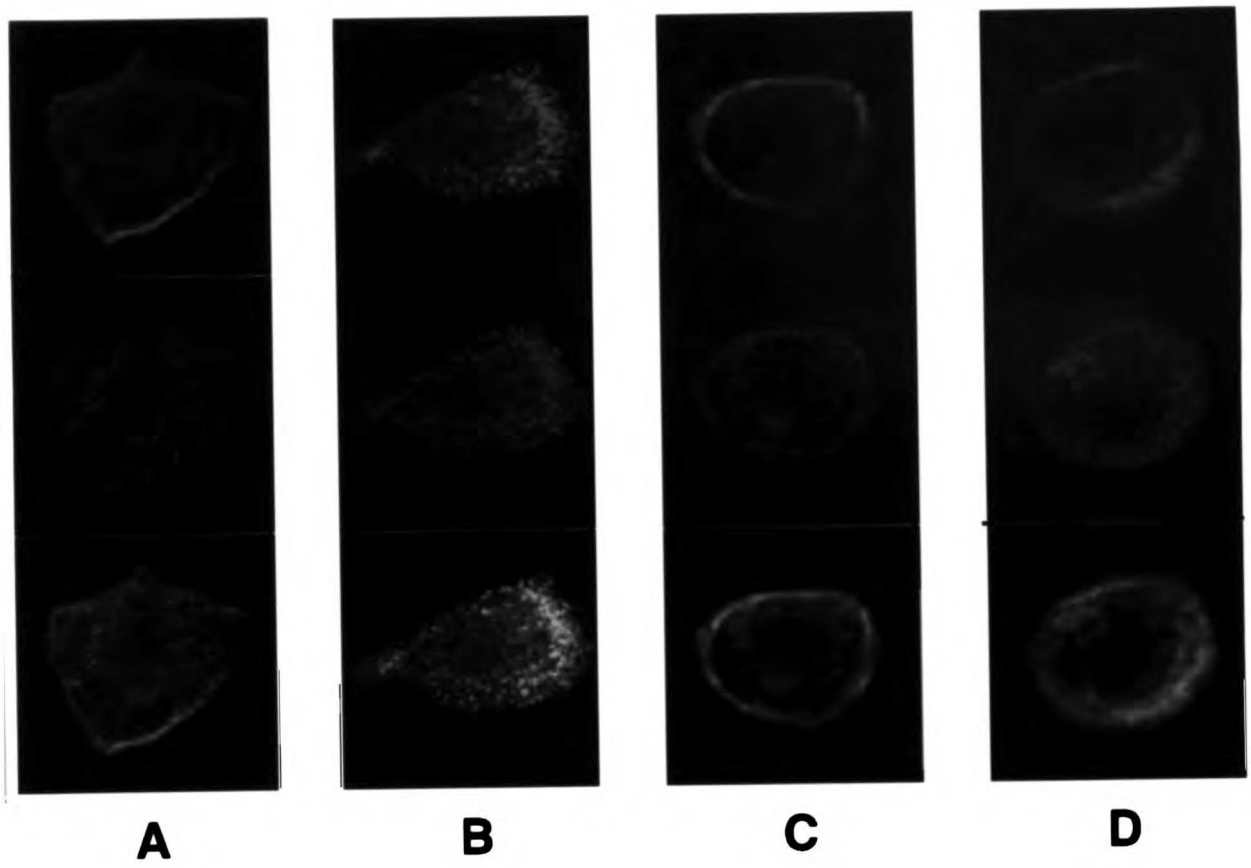


Figure 3.5 Colocalization of m3 AChR and Mutant SASS/AAAA with Clathrin. Cells expressing m3 wild-type or mutant SASS/AAAA receptors were treated for 30 minutes and 60 minutes, respectively, with 1 mM carbachol. After fixing and permeabilizing, m3 receptors were labeled with polyclonal m3 antisera followed by Cy3-labeled donkey anti-rabbit secondary antibody. Clathrin was then sequentially labeled with monoclonal anti-clathrin antibody followed by Cy5-conjugated goat anti-mouse antibody. The red color (top panel) indicates the localization of the receptor, green represents clathrin distribution, and yellow is indicative of colocalization of the receptor with clathrin in the merged image (lower panel). Panels A-B are m3 wild-type and panels C-D are mutant SASS/AAAA. **(A)** No treatment. **(B)** 1 mM Carbachol. **(C)** No treatment. **(D)** 1 mM Carbachol.

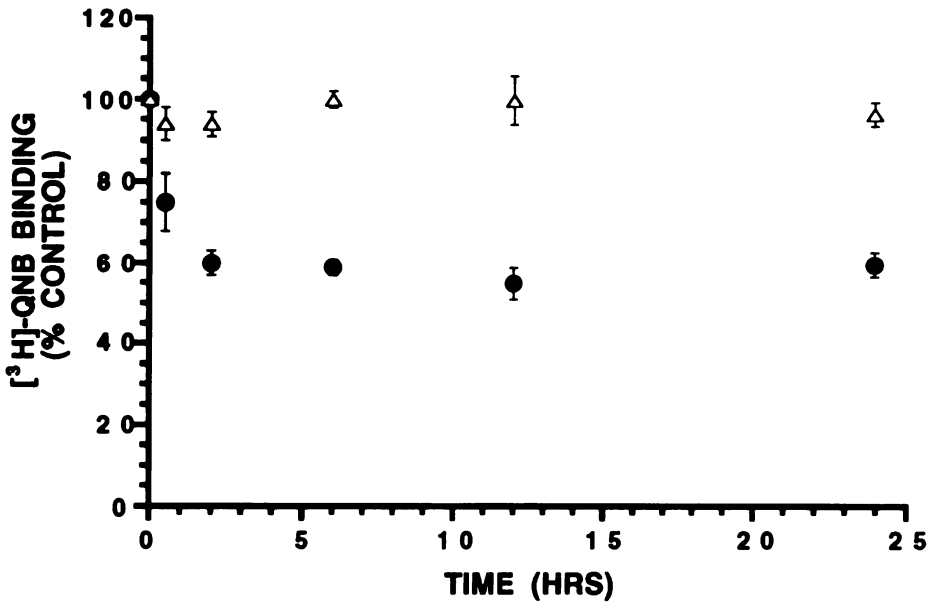


Figure 3.6 Time Course of Carbachol-Induced Down-regulation of Wild-type and Mutant m3 Receptors. Cells were treated with 1mM carbachol for the indicated times. Following agonist treatment, cells were washed and total cellular receptor was assessed by [^{3}H]-QNB binding. Time course data is representative of three independent experiments. Wild-type is indicated by filled circles (●) and mutant SASS/AAAA by open triangles (Δ).

receptor occurs with short term agonist exposure (Tobin et al., 1992; Yang et al., 1993; Koenig and Edwardson, 1996). Indeed, only after 15 minutes of carbachol treatment did we observe significant changes in surface expression of m3 receptors. To confirm that the loss in cell surface binding sites detected by [^{3}H]-NMS corresponded to true internalization, we employed immunofluorescence confocal microscopy to visualize the distribution of receptor following carbachol treatment. Consistent with binding studies, carbachol-induced a redistribution of receptors from the cell surface to intracellular vesicles. Although we were unable to quantitate the extent of internalization by confocal microscopy, the accumulation of receptor within vesicles appeared to occur with a time course similar to that observed with [^{3}H]-NMS loss. Surface staining of receptor was also substantially reduced following carbachol treatment. However, we cannot directly compare surface expression before and after treatment since optical imaging limited the visualization of the receptor to the areas of greatest intensity of signal, which corresponded to the punctate accumulations observed as vesicles rather than the cell surface after carbachol treatment. Therefore, although slow, internalization of m3 receptor does occur in CHO cells.

As we had demonstrated that m3 receptors do internalize in CHO cells, we then addressed the question as to whether m3 receptor internalization occurs via an endocytic pathway involving clathrin coated pits. The primary pathway associated with the internalization of GPCRs is that involving clathrin-coated pits (Silva et al., 1986; von Zastrow and Kobilka, 1992) Previously we had shown that m1 receptors expressed in CHO cells undergo clathrin-mediated internalization in response to carbachol treatment (see Chapter 2). Furthermore, studies have implicated clathrin-coated pits as the primary pathway of m3 internalization in SH-SY5Y cells (Sloweijko et al., 1996), although no direct relationship between clathrin and m3 receptors was shown. In this study, sucrose treatment of CHO cells expressing wild-type m3 receptors completely blocked internalization of receptor as evidenced by the lack of vesicle accumulation in response to

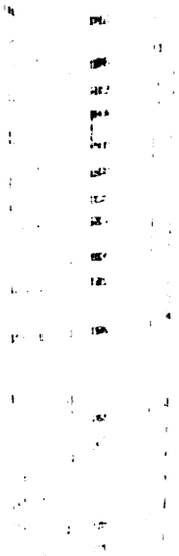
carbachol. Furthermore, m3 receptors colocalized with clathrin following carbachol treatment. Collectively, these results established a pathway involving clathrin-pits in the internalization of m3 receptors.

Mutation of the domain SASS to AAAA in the i3 loop of the m3 receptor completely abolished agonist-induced internalization as no change in [^{3}H]-NMS detectable sites and no vesicular accumulations were observable following a time course of carbachol treatment up to two hours. However, colocalization of the receptor with clathrin revealed that a slight association of receptor with clathrin was present at the cell surface following carbachol treatment. This result indicates that the mutation may not directly interfere with receptor/clathrin interactions but may instead alter some other component of the internalization machinery. Interestingly, the analogous mutation in m1 (m1-SLTSS/ALAAA) did not appear to abolish internalization of this muscarinic subtype (see Chapter 2). Therefore, it appears that this domain is not a general internalization signal for muscarinic receptors as previously predicted. Instead, this sequence may subserve the modulation of receptor trafficking by selectively interacting with specific cellular factors which are receptor-specific and may change depending on the cellular environments. In fact, intracellular trafficking of muscarinic receptors has been reported to depend upon the cell type in which a receptor is expressed (Koenig and Edwardson, 1994b; Koenig and Edwardson, 1996).

The possibility exists that the internalization defect observed with mutant m3-SASS/AAAA may have resulted from conformational changes in the receptor introduced with the mutation. Perhaps mutation of residues in the SASS sequence altered the conformation of the i3 loop such that the requisite factors for internalization no longer had access to their proper binding sites, located elsewhere in the receptor. One might postulate, however, that this conformational effect is specific to the function of internalization. First, ligand binding (affinity for carbachol) and stimulation of phosphoinositide hydrolysis by this mutant were similar to the wild-type m3 receptor.

Second, the antibody raised to the i3 loop of the m3 wild-type receptor was able to recognize the mutant construct. These data suggest that the loss of internalization was not a result of gross conformational changes, since ligand binding and signal transduction were unaffected.

Prolonged agonist stimulation results in a down-regulation of the receptor pool. If down-regulation occurs subsequent to internalization, then we would expect an internalization defective mutant to be impaired in down-regulation. However, in order to access the contribution of internalization to down-regulation, both processes need to be examined within the same cell system. Yang et al. (1993) originally identified a threonine domain in the carboxyl-terminal tail of the m3 receptor as important in modulating down-regulation only to subsequently define this region as important for sequestration as well (Yang et al., 1993; Koenig and Edwardson, 1994b; Yang et al., 1995; Koenig and Edwardson, 1996). As a consequence of the two cell lines used (CHO cells for down-regulation and HEK293 cells for internalization), Yang and colleagues were unable to derive a direct relationship between internalization and down-regulation since the defects observed may have been specific to each cell line. Likewise, we had initially identified the serine/threonine rich region as having a role in m1 and m3 receptor internalization by use of mutants expressed in HEK293 cells. However, as previously noted, mutation of this region in the m1 receptor had minimal effect on the trafficking of this receptor in CHO cells. Therefore, we addressed the contribution of the SASS region to the down-regulation of m3 cells after first noting its importance in modulating internalization within the same CHO system. Our data indicate that mutation of this region (m3-SASS/AAAA) completely impairs agonist-induced down-regulation. We may interpret these results in two ways. First, this region may have dual function with modulation of internalization over short time periods of agonist treatment and down-regulation over longer exposure times. More likely, though, this region functions in the internalization of the m3 receptor, and the effect on down-regulation is the result of the

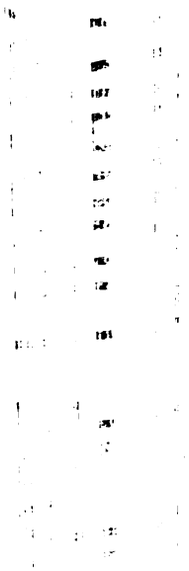


impairment of the requisite upstream internalization event. Consistent with this interpretation is the inhibition of down-regulation of m3 receptors and m1 receptors (see Chapter 2) with sucrose treatment, which biochemically blocks clathrin-mediated receptor endocytosis.

It is reasonable to postulate that internalization of m3 receptors in CHO cells may be regulated by phosphorylation of serine residues within the SASS domain. Phosphorylation of m3 receptors expressed in CHO cells has been shown to occur in an agonist-dependent manner by a kinase distinct from the previously characterized second messenger activated kinases PKC and PKA as well as the members of the GRK family (Tobin et al., 1996). Consistent with the lack of phosphorylation by either PKC and PKA, we did not observe any effects of the general kinase inhibitor H7 on the internalization of m3 wild-type receptors (data not shown), suggesting that phosphorylation by these kinases is not involved in the process of internalization. Putative sites for GRK mediated phosphorylation, however, include this highly conserved serine/threonine rich domain of the muscarinic receptors (Nakata et al., 1994; Haga et al., 1996). Indeed, the SASS domain of the m3 receptor is predicted as a putative phosphorylation site by either GRKs or similarly related kinases (Tobin et al., 1996). Considerable evidence exists for a potential role of a GRK in the internalization of G protein-coupled receptors. Tsuga et al. (1994) have shown that GRK2 or a similar kinase is involved in the internalization of m2 AChRs. Furthermore, GRK2-mediated phosphorylation has been implicated in facilitating β_2-adrenergic receptor internalization (Ferguson et al., 1995; Ferguson et al., 1996).

It remains to be seen whether phosphorylation is involved in the internalization of m3 receptors. It must be noted that despite the SASS region being a likely target for kinases, our preliminary data of the phosphorylation of mutant m3-SASS/AAAA receptors suggest that this receptor mutant is phosphorylated in a manner similar to wild-type m3 receptors (A. Tobin, personal communication). Therefore, this site may be

important in regulating clathrin-mediated endocytosis by serving as a target for the binding of a factor not requiring phosphorylated residues as recognition points. These preliminary data also call into question the general applicability of the model proposed for GPCR internalization involving the sequence: receptor phosphorylation-arrestin binding-clathrin interaction with arrestin-internalization. The role of the SASS domain in the clathrin-mediated endocytic process warrants further investigation as it may represent a site for the binding of an adaptor-like protein which serves to link the receptor to clathrin.

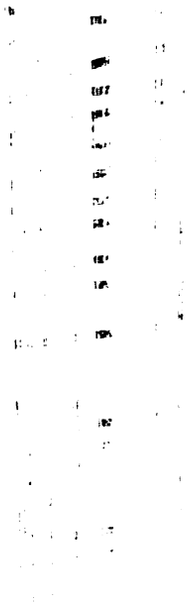


CHAPTER FOUR

BASAL ACTIVITY OF WILD-TYPE AND MUTANT HUMAN MUSCARINIC CHOLINERGIC RECEPTORS

I. SUMMARY

Stimulation of m1 AChRs by agonist leads to an increase in hydrolysis of membrane phospholipids via G protein activation of phospholipase C. Mutational analysis of the m1 receptor has led to the identification of several key residues important for G-protein coupling and subsequent phosphoinositide hydrolysis (Moro et al., 1993a; Moro et al., 1994; Högger et al., 1995). Mutation E360A in the carboxyl-terminal tail of the i3 loop was found to elevate levels of IP in the absence of agonist and to sensitize the receptor to carbachol activation. In this study, we examined the mechanism(s) for regulation of the basally active m1 receptor. As the activating mutation was initially observed in transiently transfected cells, we questioned whether the constitutive signaling could be observed in a stable cell line, which would facilitate mechanistic study of basal GPCR activity. Therefore, characterization of basal signaling by wild-type and mutant m1 receptors was performed in stably transfected HEK293 cells. While basal activity was not readily observed with m1 AChR wild-type, the mutant did display constitutive signaling when stably expressed in HEK293 cells. To address whether basal signaling by the wild-type m1 receptor could be induced, cells were pretreated with agonist. Pretreatment with carbachol did not convert the receptor to a constitutively active state. Additionally, we examined the potential involvement of receptor phosphorylation in maintaining basal signaling by initially testing whether the phosphatase inhibitor calyculin A could enhance basal IP levels generated by m1 AChR wild-type. Treatment with calyculin A did not enhance basal signaling of wild-type. Therefore, m1 AChR



wild-type does not signal in an agonist-independent manner in HEK293 cells; however, mutation of residue E360A converts the m1 receptor to a constitutively active state.

II. INTRODUCTION

G protein-coupled receptors (GPCRs) are organized into three extracellular and three intracellular loops linked via seven putative transmembrane spanning domains (TMDs). Extensive analysis of the intracellular regions of the GPCRs has led to the identification of several domains involved in G protein recognition and activation. The second and the third intracellular loops (i2 and i3 loops) have been implicated as the primary sites for G protein activation (Franke et al., 1990; Wong et al., 1990; Dalman and Neubig, 1991), although the first intracellular (i1) loop and the carboxyl-terminal tail have also been reported to be involved in coupling for several GPCRs (König et al., 1989; Liggett et al., 1991; Moro et al., 1993a).

Mutational analysis, use of synthetic peptides, and construction of receptor chimeras have suggested that the N- and the C- terminal ends of the i3 loop immediately proximal to TMD V and TMD VI are the major determinants for specificity in G protein recognition and activation (Wess et al., 1989; Cotecchia et al., 1992; Kunkel and Peralta, 1993; Shapiro et al., 1993; Blüml et al., 1994; Wade et al., 1994). While the bulk of the i3 loop region is not well conserved among the GPCR class, the junctions of the i3 loop show high sequence homology among closely related GPCR subtypes (Probst et al., 1992). Single amino acid residues within these regions have been shown to play important roles in G protein activation (Cheung et al., 1992; Moro et al., 1993a); however, a general consensus sequence for G protein coupling has yet to be identified.

To examine the role of the i3 loop junctions of m1 AChR in the activation of G proteins which couple to phosphoinositide hydrolysis, we had constructed a series of mutants with alanine substitutions in regions proposed to play a role in coupling (Moro et

al., 1993a; Moro et al., 1994). Our previous analysis of the C-terminal end of the i3 loop suggested that the consensus sequence BBXB or BBXXB (B stands for basic, X for non-basic residue) thought to play a role in G protein activation (Okamoto et al., 1990; Okamoto and Nishimoto, 1992) may not serve the same function in the case of the m1 receptor (Arden et al., 1992). Construction of the triple point mutant E360A/K362A/T366A which mutated residues within the same putative coupling site, however, resulted in a receptor mutant deficient in functional coupling to phospholipase C (Moro et al., 1993a). Analysis of the single point mutations revealed that mutation T366A alone did not alter G protein coupling while mutant K362A was highly defective in stimulation of IP release as well as overall G protein activation (see Chapter 2). Mutation of residue E360 to alanine, however, resulted in a receptor mutant displaying basal inositol phosphate release. Characterization of m1 AChR-E360A revealed an enhanced affinity to agonist, an increased potency for carbachol binding, and a strong correlation between receptor density and basal IP release, suggesting that mutation of this residue converted the m1 receptor to an agonist-independent active state (Högger et al., 1995)

In this study, we addressed the mechanism(s) by which basal signaling may be regulated. We report that atropine serves as an antagonist with negative intrinsic activity allowing for suppression of basal signaling. Additionally, mutant E360A and wild-type m1 receptors were stably expressed in HEK293 cells to facilitate mechanistic study of the constitutive state. While the mutant retained the capability to signal in an agonist-independent manner, no basal IP release was observed with wild-type m1 AChR. We tested the hypothesis that basal m1 AChR signaling is regulated by agonist conversion of the receptor to a constitutive state; however, pretreatment with agonist did not produce sustained IP release following agonist removal. Furthermore, use of the phosphatase inhibitor calyculin A to potentially enhance basal signaling of wild-type proved to be unsuccessful. We therefore propose that the wild-type receptor does not signal in an

agonist-independent manner; however, mutation of residue E360 activates the receptor such that it mimics an agonist-stimulated receptor state in the absence of agonist.

III. EXPERIMENTAL PROCEDURES

A. Materials

^{3}H] NMS (specific activity 85 Ci/mmol) and [^{3}H] myoinositol (specific activity 17.7 Ci/mmol) were obtained from Amersham Corp. All other reagents were of analytical grade.

B. Construction of Vectors Expressing m1 Wild-type and Mutant E360A Receptors

Construction of vectors were as described previously (Moro et al., 1994; Högger et al., 1995)

C. Transfection of Human Embryonic Kidney (HEK293) Cells

Cells were transfected using the calcium phosphate precipitation method (Maeda et al., 1990). Transient expression yields were 1326 ± 795 fmol/mg protein for wild-type m1 AChR and 846 ± 672 fmol/mg protein for mutant E360A. Cells expressing less than 250 fmol/mg protein were not studied further, because lower yields resulted in decreased maximal IP release for the wild type receptor (Moro et al., 1993a). Stably transfected cells were selected in medium containing 400 μg/ml of the antibiotic G418 (Bethesda Research Laboratories). Stable expression levels of wild-type and mutant m1 AChR were 2637 ± 372 fmol/mg protein and 2840 ± 241 fmol/mg protein, respectively. All cells were maintained at 5% CO_2 in DME H-16/ Ham's F-12 medium supplemented with 10% fetal bovine serum with addition of 200 μg/ml G418 for stable transfectants.

D. Receptor Binding

The assay was performed as described previously (Maeda et al., 1990). The transfected cells were replated onto 12 well cell culture dishes and allowed to attach overnight. Cells were incubated in PBS containing 2 nM [^{3}H]-NMS at 12 °C for 90 minutes. At the end of incubation, cells were harvested in reaction buffer, filtered on glass-fiber (Schleicher & Schuell #32) filters, and rapidly rinsed three times with ice-cold PBS.

E. Stimulation of Inositol Phosphate Release

Cells were plated onto six-well cell culture dishes and labeled with 0.2 μM [^{3}H] myoinositol for 24 hours. Inositol monophosphate (IP), which accounts for most of the [^{3}H]IP activity released, was measured as described previously (Lameh et al., 1992). Results were expressed as percent of total intracellular [^{3}H] activity, and percentage values were compared between carbachol treated and untreated cells. Concentrations from 0.001 to 10 mM were used for dose-response curves.

To determine the effect of atropine on basal coupling activity of m1 AChR wild-type and E360A, the assay was modified. The standard assay involved a 30 minute preincubation period with LiCl, followed by an additional incubation with LiCl in the presence of the test agent. To prevent [^{3}H]IP accumulation by basal receptor activity during the preincubation, LiCl and atropine were added simultaneously and then incubated for a total of 30 minutes. Under these conditions, atropine was capable of reversing elevated basal [^{3}H] release by E360A close to the control levels of nontransfected cells.

In the assays involving treatment with the potent phosphatase inhibitor calyculin A, the inhibitor was present in all wash steps and during the LiCl incubation. Incubation time was decreased to 10 minutes which produced similar IP levels as detected after the 30 minute incubation period but allowed for cell survival in the presence of calyculin A.

IV. RESULTS

A. Stimulation of Inositol Phosphate Release

Stimulation of E360A with carbachol resulted in a 6-fold shift in EC_{50} value (1.2 ± 0.9 μM) compared to wild-type (7.4 ± 0.1 μM) with the E360A maximal response similar or slightly higher than wild-type (Figure 4.1). Moreover, transfection with E360A resulted in higher baseline levels of IP release (approximately 2.5-fold) compared to wild-type.

B. Reversal of Basal Inositol Phosphate Production by Atropine

To determine if the basal signaling is receptor dependent and reversible, the effects of the antagonist atropine on baseline IP levels were determined for wild-type and mutant E360A. Addition of atropine suppressed baseline IP production in a dose-dependent manner for mutant E360A with an IC_{50} of 0.55 ± 0.09 nM (Figure 4.2). The atropine dose-response curve for wild-type (2400 fmol/mg protein) indicated that 1 μM atropine induced a marginal decrease of baseline IP levels from 1.02 ± 0.04% to 0.85 ± 0.04% (*$p<0.05$, Fisher PLSD test); however, the marginal loss did not permit for determination of the IC_{50}. The variable results obtained for wild-type (ranging from no measurable decrease to marginal changes at very high expression levels) suggest that basal activity of the wild-type receptor in HEK293 cells is minimal, if present at all.

C. Effects of Carbachol Pretreatment on Basal Activity of Wild-type m1 AChR

Baseline inositol phosphate release was determined for HEK293 cells stably transfected with either m1 wild-type or mutant E360A receptor. A significant elevation in baseline IP levels was observed for E360A but could not be detected for m1 wild-type in comparison to nontransfected cells (Figure 4.3). In an attempt to enhance agonist-

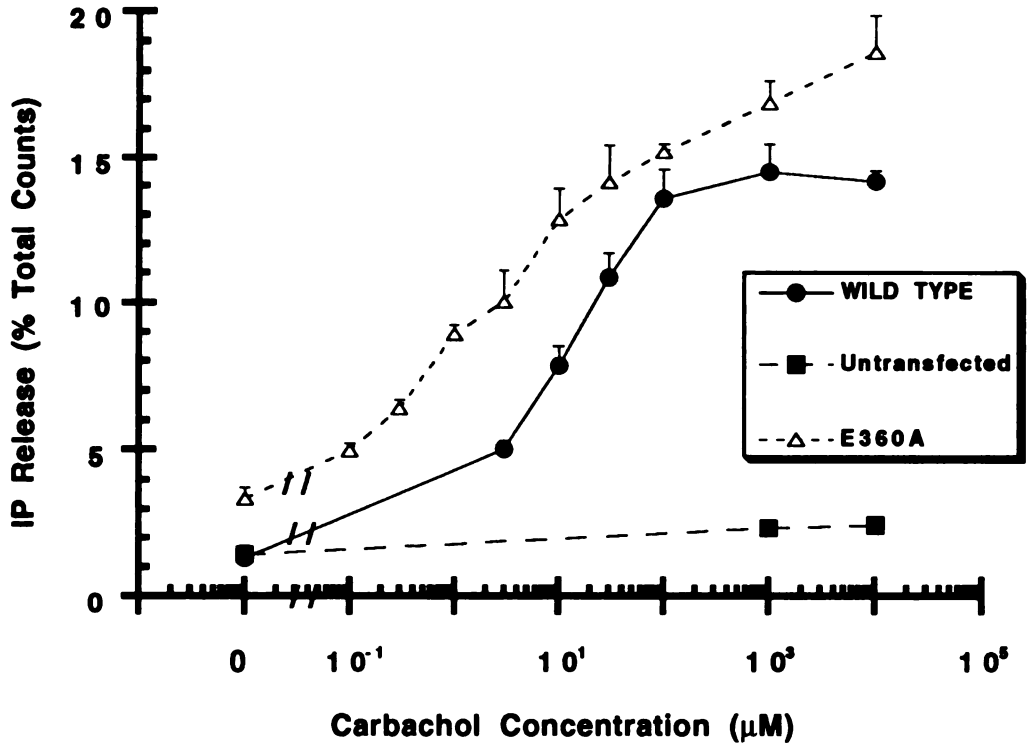


Figure 4.1 Dose-response Curves for IP Release by Wild-type m1 AChR and Mutant E360A. Concentrations from 0.1 μM to 10 mM were used for dose-response curves (n = 3-6). The dose-response curve for the wild-type m1 receptor (expression yield 1242 fmol/mg protein) is similar to that published previously (Arden et al., 1992). Expression yields were 873 and 760 fmol/mg protein for E360A in the two independent experiments averaged as shown. The carbachol EC_{50} values were 7.4 ± 1.0 μM for wild-type and 1.17 ± 0.9 μM for E360A (mean ± S.D. of three independent experiments).

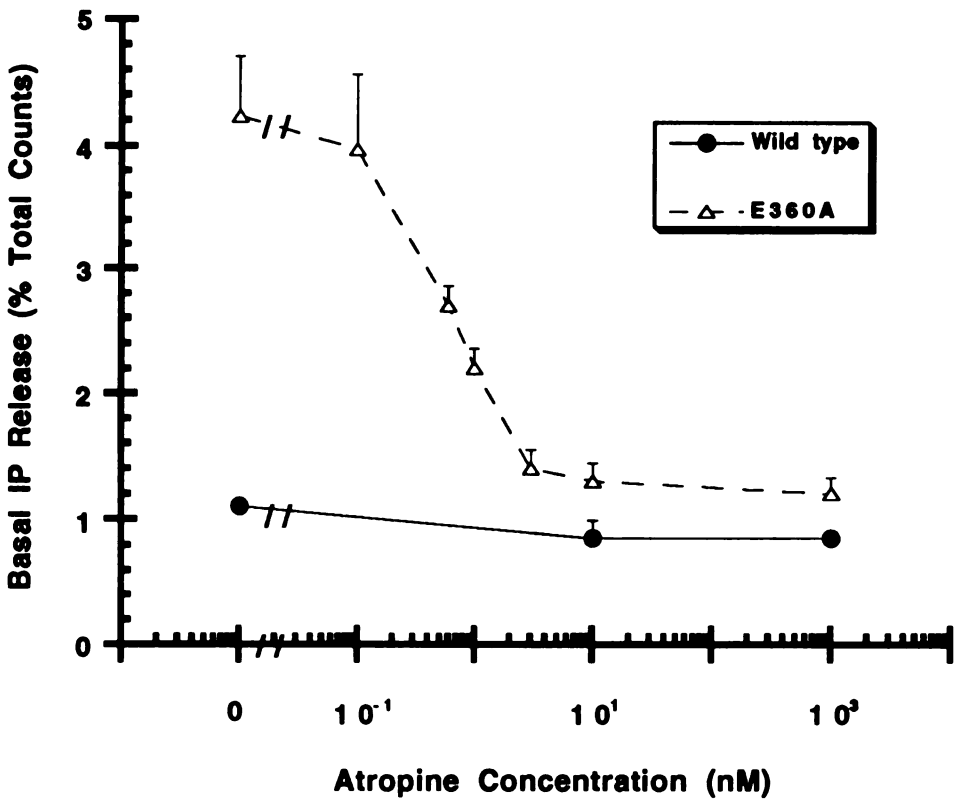


Figure 4.2: Atropine Dose-response Curve for Mutant E360A and m1 AChR Wild-type. Basal IP release in the absence of agonist was significantly higher for E360A compared to wild-type. Atropine concentrations of 0.1 nM to 1000 nM were used in the E360A dose-response curves (mean of two independent experiments, each performed in triplicate; n =6). Four other independent experiments using dose levels of 1 nM to 10 μM replicated these results. Expression of wild-type m1 AChR, measured with [^{3}H]-NMS to determine cell surface binding sites, was 2400 fmol/mg protein in the experiment shown. Expression levels for E360A were 2388 and 740 fmol/mg protein in the two averaged experiments. The calculated IC_{50} of atropine for E360A was 0.55 +/- 0.09 nM.

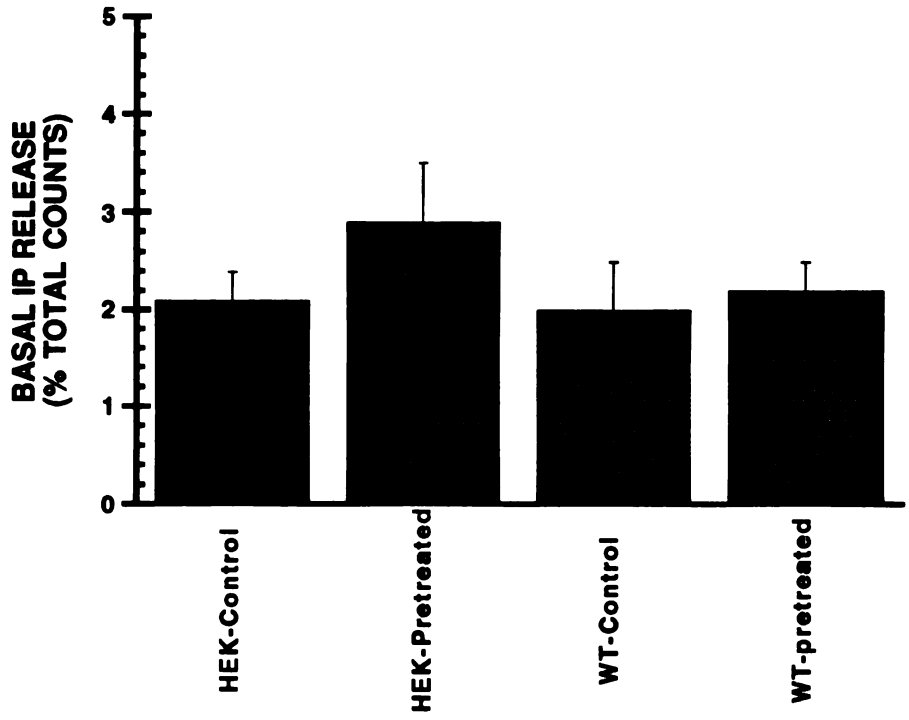


Figure 4.3 Effects of Carbachol Pretreatment on m1 AChR Wild-type Inositol Phosphate Release. Cells expressing m1 AChR wild-type were pretreated for 2 hours with 100 μM carbachol. Following agonist pretreatment, cells were washed 4x with PBS and basal inositol phosphate levels were determined as described in Experimental Procedures. Basal activity was measured in untreated cells and is presented as control basal IP release. Results are expressed as a percentage of total [^{3}H] inositol phospholipids. Data are mean ± SEM of 3-6 experiments each performed in triplicate. Data were analyzed by one-factor ANOVA.

independent signaling of wild-type m1 receptors, cells were pretreated with agonist and then were washed 4-5x prior to the assay. No change was observed in basal IP levels for wild-type following pretreatment with carbachol (100 μM, 2 hours), suggesting that basal signaling cannot be induced by prior agonist exposure.

D. Effects of Calyculin A on Basal Signaling of m1 AChR

Baseline levels of IP release for m1 wild-type were tested in the presence of calyculin A to see if a phosphatase inhibitor would enhance basal activity. Simultaneous addition of 15 nM calyculin A with LiCl resulted in no significant increase in baseline IP levels for m1 wild-type nor did it alter baseline IP profiles for nontransfected HEK293 cells or cells stably expressing m1 AChR-E360A (Figure 4.4).

V. DISCUSSION

Agonist activation of G protein-coupled receptors has been well documented for the various effector systems. Recently, several reports have suggested that some GPCRs may actively signal in an agonist-independent manner (Costa et al., 1992; Samama et al., 1993). Mutations in several GPCRs have also been shown to result in receptors which are basally active (Cotecchia et al., 1990; Lefkowitz, 1993; Parma et al., 1993; Robbins et al., 1993; Shenker et al., 1993).

Criteria for determination of basal activity for a GPCR include enhanced basal coupling as measured by second messenger stimulation/inhibition, enhanced affinity to agonist, and increased agonist potency. We have recently identified a mutation (E360A) in the C-terminus of the third intracellular loop of m1 AChR which induces constitutive activation, or agonist-independent signaling (Högger et al., 1995). In this study, we examined the regulation of the basal state by addressing the role of a receptor antagonist in modulating basal signaling. Another hallmark of constitutive receptor activity is the reversibility of basal signaling by a receptor antagonist, defined as a negative intrinsic

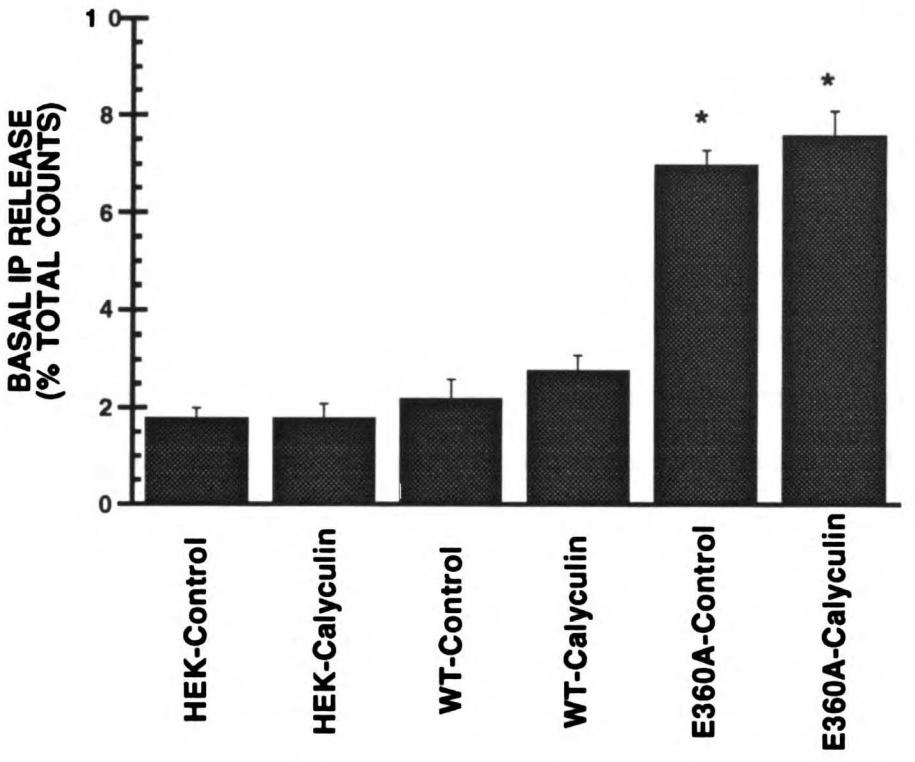


Figure 4.4 Calyculin A Effects on Basal Phosphoinositide Hydrolysis for m1 AChR Wild-type. Basal inositol monophosphate release for HEK293 cells stably expressing either wild-type or mutant m1 receptors was measured in the presence and in the absence of 15 nM calyculin for 10 minutes as described. Results are expressed as percent of total [^{3}H] inositol phospholipids. Data represent the mean ± SEM of four experiments, each performed in triplicate. For comparison, calyculin A treatment for untransfected HEK293 cells and mutant E360A are shown (duplicate experiments with triplicate measurements). Data were analyzed by one-factor ANOVA and Fisher PLSD. Significance (*$p<0.05$) is calculated in comparison to control (no calyculin) untransfected HEK293 cells.

antagonist. (Lefkowitz et al., 1993). Negative intrinsic activity has been described for 5-HT_{2C} receptor antagonists (Barker et al., 1994) and for μ-receptor antagonists (Wang et al., 1994). Examination of the effect of the muscarinic receptor antagonist atropine on the basal activity of m1 AChR-E360A revealed that this antagonist was able to decrease baseline inositol phosphate generation. This result is consistent with the high affinity of atropine at muscarinic receptors, and it indicates that the negative activity of atropine is exerted specifically at the m1 receptor in HEK293 cells. Furthermore, this result supports the hypothesis set forth by Lefkowitz (1993) suggesting that structurally a GPCR is constrained in an inactive conformation such that interaction with a G protein is prevented until an agonist binds. Since many activating mutations are found in the carboxyl-terminus of the third intracellular loop (Kjelsberg et al., 1992; Ren et al., 1993), this region must be critical for maintaining the inactive conformation of the receptor. Therefore, mutation E360A in the m1 AChR appears to relieve the receptor of its inactive conformation, thus mimicking an agonist bound state and resulting in G protein activation. Addition of an antagonist would then be expected to reverse the active conformation as was observed in this study.

These results raised the question whether basal activity was also detectable for the m1 wild-type receptor. Transient transfection of wild-type m1 AChR into HEK293 cells caused minimal, if any, changes in baseline IP levels. Furthermore, reversal of the basal state by atropine was inconsistent and was detectable with high expression only in some experiments. We therefore tested for basal activity of m1 wild-type receptors stably expressed in HEK293 cells , which consistently expressed at high levels (>2 pmol/mg protein). Again, no measurable elevation in basal IP release was detectable in cells stably expressing m1 wild-type. As the basally active state is believed to mimic the agonist-induced state of the receptor, cells were pretreated with agonist (carbachol) to examine whether basal signaling could be induced by prior agonist exposure. Agonist-dependent conversion of a GPCR to a constitutively active state, which continues signal transduction

after agonist removal, was previously reported for the μ opioid receptor (Wang et al., 1994). In this study, pretreatment of cells expressing m1 wild-type receptors with carbachol, followed by agonist removal, resulted in no significant change in basal IP release, suggesting that basal m1 receptor activity cannot be regulated by agonist.

Phosphorylation of GPCRs in response to agonist has been shown to occur by a novel class of kinases that are specific to receptors of this superfamily (Benovic et al., 1989; Haga et al., 1996). Agonist-independent phosphorylation of basally active α_2-adrenoceptors by βARK1 in HEK293 cells has been demonstrated (Ren et al., 1993). Furthermore, agonist-induced conversion of the μ opioid receptor to a constitutively active state has been shown to correlate with receptor phosphorylation (Wang et al., 1994). Since the m1 AChR has been shown to be phosphorylated *in vitro* by GRK2 (G protein-coupled receptor kinase, subtype 2)(Haga et al., 1996), phosphorylation may play a role in the regulation of baseline m1 activity. We therefore tested the possibility that inhibition of protein dephosphorylation by a phosphatase inhibitor may modify agonist-independent m1 receptor activity. Examination of the effects of the inhibitor calyculin A, which is a potent inhibitor of phosphatases PP-1 and PP-2A, revealed no significant enhancement of basal m1 receptor signaling compared to nontransfected cells. These results suggest that if phosphorylation of the m1 AChR is involved in basal signaling, it is not the primary regulatory mechanism.

In conclusion, neither agonist pretreatment nor phosphatase inhibition allowed for the identification of a regulatory mechanism possibly contributing to basal signaling by m1 wild-type receptors. Furthermore, basal signaling by m1 AChR is minimal or absent in HEK293 cells under the experimental conditions tested in this study. The m1 receptor, however, may be activated by mutation, and this suggests the possibility that m1 wild-type receptors may signal in an agonist-independent manner in other systems. Recently, Jakubik et al. (1995) reported that m1 AChRs are constitutively active in Chinese hamster ovary (CHO) cells. Our studies of m1 AChR signaling in CHO cells do not support the

hypothesis that these receptors are basally active (see Chapter 2). Nevertheless, the potential for agonist-independent signaling by muscarinic receptors exists as we (Högger et al., 1995) and others (Spaulding et al., 1995) have demonstrated. The regulatory mechanisms behind and the functional consequences of basal muscarinic receptor signaling warrant further investigation as m1 and m3 receptors have been shown to have mitogenic properties (Gutkind et al., 1991) and thus may play a role in disease states as has been documented with other GPCRs (Parma et al., 1993; Shenker et al., 1993).

SUMMARY AND CONCLUSIONS

The molecular determinants and the cellular factors involved in mediating the regulation of G protein-coupled receptors were investigated. Domains of the receptors important for G protein coupling, internalization, and down-regulation were identified by use of mutant m1 AChRs. The cellular pathways associated with m1 and m3 receptor internalization and down-regulation were also examined.

Previous studies have demonstrated that the second and the third intracellular loops of G protein-coupled receptors are the primary sites for interaction with G proteins. Using m1 receptor mutants defective in coupling via Gq/11 to phospholipase C, a relationship between sequestration and coupling was observed. Mutations which affected coupling also impaired receptor sequestration, suggesting that the cellular factors mediating sequestration may be G proteins or factors structurally similar to G proteins.

The initial coupling and sequestration studies were performed in HEK293 cells, which are amenable to transient transfection and thus provide for a rapid screening system for mutants. Down-regulation of m1 receptors was negligible in HEK293 cells. To further study the down-regulation process, receptor mutants were stably expressed in CHO cells which are capable of mediating down-regulation of GPCRs. As a new cell model was being used to study down-regulation, the processes of coupling and internalization of the m1 receptor mutants needed to be re-examined. Analysis of coupling and internalization of m1 AChRs in CHO cells revealed that these processes appear to be independent and that mutational effects differed between cell systems. A strong correlation was observed for G protein coupling and down-regulation, suggesting that perhaps G proteins or factors similar to G proteins may mediate the trafficking of receptors to a degradative pathway rather than initiating the internalization of receptors into endosomes.

Cellular trafficking of both m1 and m3 receptor was studied in CHO cells to determine the relationship between internalization and down-regulation. Both muscarinic receptor subtypes were shown to internalize via an endocytic pathway involving clathrin-coated vesicles. Down-regulation was shown to depend upon prior internalization of the receptor, although it appeared that each process was under the control of distinct molecular signals and cellular factors. These data support the hypothesis that agonist-stimulation of G protein-coupled receptors first triggers rapid internalization followed by sorting of the receptor to lysosomes. The role of a distinct sorting signal for lysosomal targeting becomes apparent as receptors may also recycle to the surface. This data further indicate that a distinct sorting signal on the receptor must be recognized by a specific cellular factor to mediate down-regulation. Since m1 and m3 receptors internalize in both HEK293 cells and CHO cells but only down-regulate in CHO cells, one might postulate that CHO cells, but not HEK293 cells, express the cellular factor required for targeting muscarinic receptors lysosomes. Further understanding of the mechanism(s) of down-regulation will require the identification of the cellular factors involved. Since a relationship between receptor down-regulation and G protein activation was observed in CHO cells, one likely candidate would be a G protein or a G protein-like factor which is not expressed in HEK293 cells.

Additionally, agonist-independent regulation of muscarinic receptors was studied. Mutant m1 AChR-E360A was shown to stimulate inositol phosphate release in the absence of carbachol when transiently expressed in HEK293 cells. Neither agonist pretreatment nor phosphatase inhibition allowed for the identification of a regulatory mechanism involved in basal signaling of muscarinic receptors. These results further support that the regulation of G protein-coupled receptors is dependent on the cellular environment in which the receptor is expressed.

These studies have provided insight into the mechanism(s) of G protein-coupled receptor regulation. Further elucidation of the mechanism(s) of receptor internalization

and down-regulation will require the identification of the cellular factors involved in each process. Modulation of receptor activity via manipulation of the internalization and down-regulation events may serve as an alternative treatment for disease states associated with gain/loss of G protein-coupled receptor function. Identification of the cellular factors involved in mediating these regulatory events may provide for potential targets for drug intervention.

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