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Regulatory mechanisms of muscarinic acetylcholine receptors

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REGULATORY MECHANISMS OF MUSCARINIC ACETYLCHOLINE RECEPTORS

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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To my parents, Laren and Barbara Tolbert, for their love and support, and my sisters Sara and Katie, the two best gifts my parents have given me.

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Regulatory Mechanisms of Muscarinic
Acetylcholine Receptors
Lara M. Tolbert
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ABSTRACT

Muscarinic acetylcholine receptors belong to the superfamily of G protein-coupled receptors (GPCRs). These receptors participate in a wide range of physiological functions, and are targeted by numerous pharmaceutical compounds. Thus, an understanding of the mechanisms by which the activity of these receptors is regulated is crucial in the development of novel therapeutic agents that target these receptors.

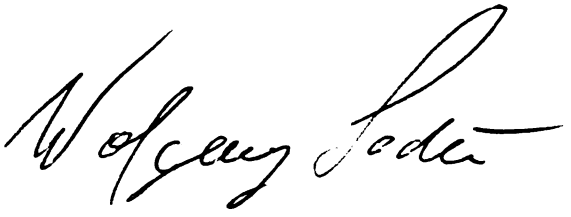
In addition to receptor activation of G proteins, a number of events have been found to occur following agonist stimulation which are thought to regulate the activity of these receptors. These events include uncoupling of the receptors from their associated G proteins, or desensitization, and trafficking of the receptors from the plasma membrane into other regions of the cell. These processes were investigated for the m1 and m3 muscarinic acetylcholine receptors.

Internalization of the m1 receptor from the cell surface into intracellular vesicles was studied by immunofluorescence confocal microscopy. Treatment with acetic acid to disrupt clathrin-mediated internalization abolished agonist-induced internalization of the m1 receptor, whereas exposure to a phorbol ester to block caveolae formation had no effect on m1 internalization. Furthermore, m1 receptors in intracellular vesicles colocalized with proteins associated with clathrin-coated vesicles, including clathrin itself, but not with caveolin. These results suggested that m1 internalization occurred via clathrin-coated vesicles.

An N-terminally epitope-tagged m1 receptor was found to internalize following treatment with an antibody against the epitope. Antibody-induced internalization similarly occurred via clathrin-coated vesicles, but in the

absence of second messenger production. Thus, second messenger stimulation and receptor internalization were demonstrated to be independent processes.

Phosphorylation on serine and threonine residues of GPCRs is thought to play a role in receptor regulation. The effect of mutation of these residues in parallel sites in the third intracellular loops of the m1 and m3 receptors was investigated. The mutation had no effect on receptor desensitization, but differentially affected receptor trafficking. The mutation completely abrogated m3 receptor internalization and down-regulation, but did not block these processes for the m1 receptor. Thus, this motif may have different regulatory roles for different receptor subtypes, and conversely, alternative motifs may be responsible for m1 and m3 receptor desensitization.



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INTRODUCTION

BACKGROUND

G protein-coupled receptors

The G protein-coupled receptor superfamily is a class of integral membrane proteins involved in transducing signals from such diverse molecules, termed agonists, as neurotransmitters, hormones, odorants, inflammatory mediators, as well as from light. Expressed in most, if not all, eukaryotic cells, G protein-coupled receptors (GPCRs) play a role in mediating a diverse array of physiological responses, including regulation of heart rate, gastrointestinal function, glandular secretion, and inflammation. Thus, it is no surprise that these receptors are targeted, either directly or indirectly, by over half of the drugs currently available (Grady *et al.*, 1997). The m1 muscarinic acetylcholine receptor, one of five muscarinic subtypes and one of several hundred GPCRs known to date, is expressed predominantly in the cerebral cortex and hippocampus areas of the brain where it is thought to function in memory. Cholinergic agonists have been found to facilitate learning and memory, and conversely cholinergic antagonists inhibit these two mental processes (Jerusalinsky *et al.*, 1997). This observation, coupled with the observed deterioration of cholinergic neurons in the hippocampus and cerebral cortex in postmortem brain tissue of Alzheimer's patients, formed the rationale behind the development of drugs that act to enhance cholinergic receptor function, such as the acetylcholinesterase inhibitor tacrine, for the treatment of memory loss associated with Alzheimer's disease (Rinton and Yamazaki, 1998).

Despite the physiological diversity of GPCR effects, these receptors appear to share significant structural homology. Hydropathy analysis suggests the existence of seven transmembrane segments, each made up of 22-

28 hydrophobic amino acids in the form of α-helices, and three intervening extracellular and intracellular loops. The receptors are situated in the membrane such that the N-terminus is located extracellularly and the C-terminus is intracellular, as depicted in Figure 1. The N terminus, extracellular loops, and transmembrane regions are thought to participate in agonist binding, while the intracellular loops interact with proteins involved in signal transduction and receptor regulation.

As their name suggests, G protein-coupled receptors transmit extracellular signals into the cytoplasm of cells by interacting with a heterotrimeric G protein. Each G protein is made up of one α, β, and γ subunit. Upon agonist binding to the extracellular and/or transmembrane regions of the receptor, the receptor is thought to undergo a conformational change, allowing for activation of its cognate G protein. Receptor activation catalyzes the release of GDP from the G protein α subunit, allowing GTP into the nucleotide binding site. The GTP-bound form is the active form of the G protein. Its dissociation from the βγ dimer allows both entities to activate downstream signaling events via enzymes and ion channels which, in turn, produce second messengers (Figure 1) (Bourne, 1997). The best understood of the second messenger pathways are stimulation of adenylyl cyclase by the α subunit of the G protein Gs and of phospholipase C by the α subunit of the G protein Gq (Figure 2). The m1 receptor couples to the latter of these two signaling pathways by preferentially interacting with Gq.

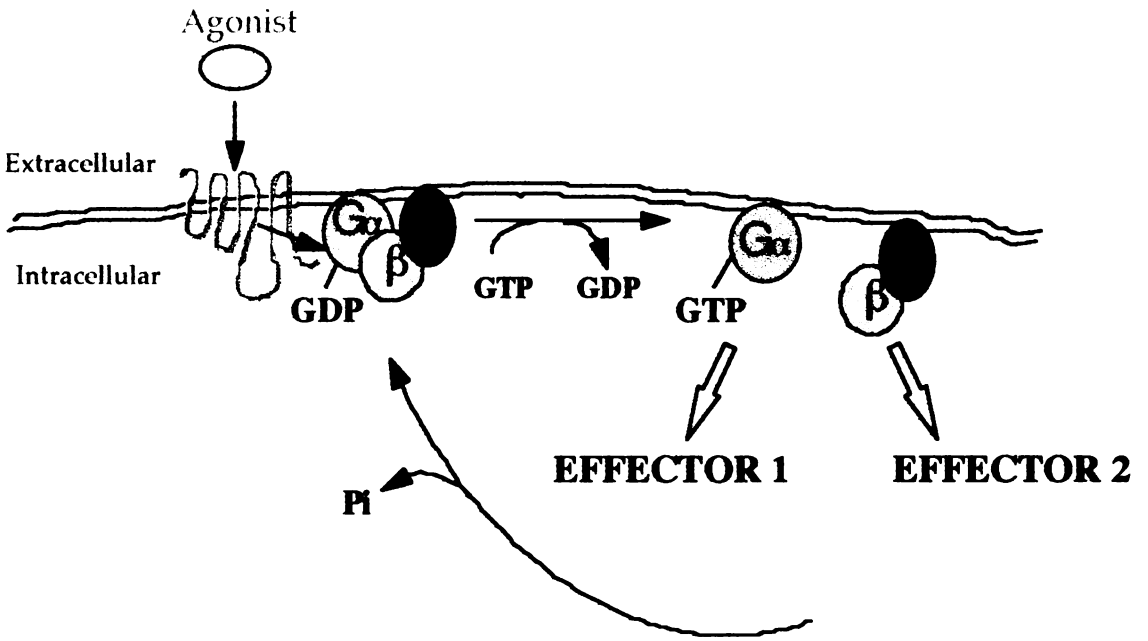


Figure 1. *G protein activation cycle.* The agonist-bound conformation of a G protein-coupled receptor promotes the dissocation of GDP from the α subunit of its associated G protein, allowing GTP into the nucleotide binding site. The GTP-activated α subunit dissociates from the βγ dimer, and both entities are then able to regulate the activity of effector proteins, such as adenylyl cyclase, phospholipase Cβ, or ion channels. The intrinsic GTPase activity of the α-subunit hydrolyzes the GTP, serving to turn off its activity by placing the α-subunit in its GDP-bound, inactive form, which reassociates with the βγ dimer.

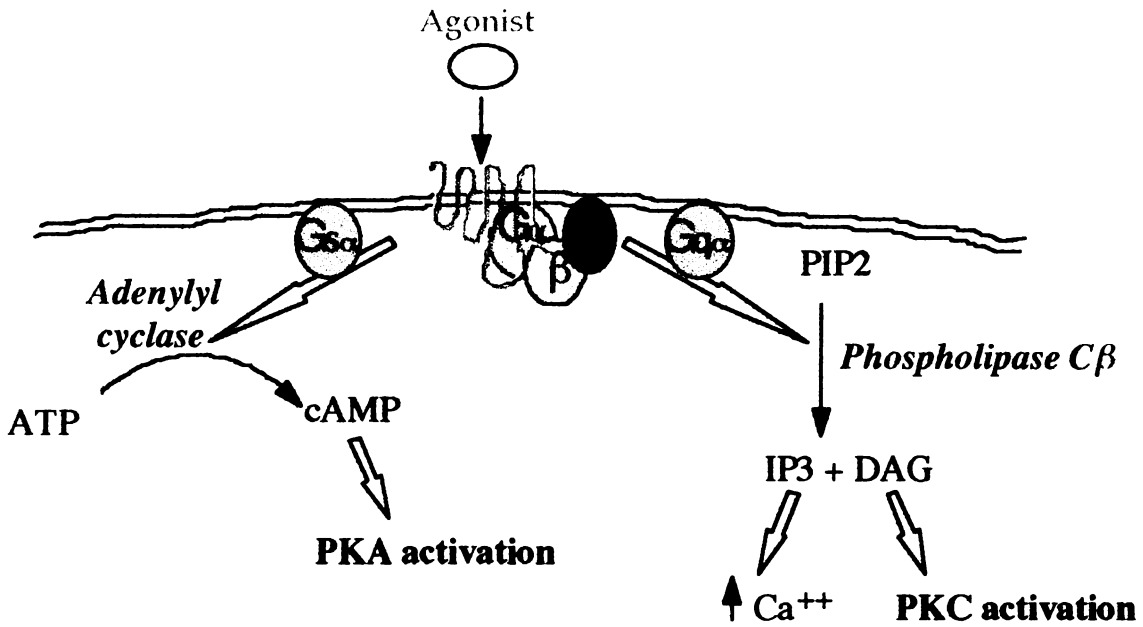


Figure 2. *Gs- and Gq-coupled second messenger pathways.* Activation of Gs-coupled receptors results in stimulation of adenylyl cyclase, leading to an increase in intracellular cyclic AMP, a second messenger that activates protein kinase A. Agonist stimulation of Gq-coupled receptors causes activation of phopholipase Cβ, which cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2), a phospholipid component of the membrane, into the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphophate (IP_3). DAG activates a Ca^{2+}-sensitive kinase, PKC, and IP3 triggers release of Ca^{2+} from intracellular stores. Activation of PKA and PKC can have many effects depending on the cell in which activation occurs, including stimulation of enzymes and alterations in gene transcription.

Regulatory mechanisms of GPCR activity

The activity of GPCRs following exposure to agonist is regulated at several levels (reviewed in Grady *et al.*, 1997). First, the duration of exposure of a receptor to agonist is tightly controlled by rapid removal of hormone and neurotransmitter agonists released from presynaptic nerve terminals. Removal of monoamine agonists (dopamine, serotonin, and norepinephrine) and amino acid neurotransmitters (GABA, glycine) occurs predominantly by uptake through transporters in presynaptic nerve terminals. Peptide agonists and the neurotransmitter acetylcholine are quickly degraded by enzymes localized within the postsynaptic cell membrane.

Second, following receptor activation of G proteins, receptors may be physically uncoupled from G proteins in a process termed homologous desensitization, which occurs within seconds of ligand binding. Desensitization may involve phosphorylation of serine and threonine residues by two families of protein kinases: G protein-coupled receptor kinases (GRKs) and the second messenger-dependent kinases protein kinase A and protein kinase C. The two kinase families differ from each other in that GRKs exclusively phosphorylate agonist-occupied receptors, whereas PKA and PKC are capable of mediating receptor phosphorylation independently of agonist activation. The desensitization process has been best characterized for the β2-adrenergic receptor. For this receptor, agonist activation is quickly followed by receptor phosphorylation on Ser/Thr residues in the cytoplasmic C tail by GRK2, also called βARK1, and in the third cytoplasmic loop by the cAMP-dependent protein kinase, PKA. Another protein, β-arrestin, binds to the βARK-phosphorylated C-terminus, and in doing so, prevents the interaction of the receptor with the G protein Gs (Hausdorff *et al.*, 1990).

A third mechanism of regulation occurs on a slightly longer time scale. Within several minutes of agonist stimulation, receptors may be transported from the cell surface into intracellular compartments in a process referred to as internalization or sequestration. The influence that this internalization event has on modulating receptor activity is not yet clear, and it appears to differ between receptors. Again, the role of internalization has been most clearly defined for the β_2-adrenergic receptor, where it has been demonstrated that internalization is required for resensitization. Following agonist exposure, arrestin binding to the phosphorylated receptor leads to translocation of the receptor into endosomes, where it is believed that the phosphates are cleaved. The dephosphorylated, resensitized receptor is then recycled back to the plasma membrane where it may undergo another cycle of agonist stimulation. While internalization appears to function similarly in the resensitization of the neurokinin NK1 receptor (Garland *et al.*, 1995), it serves to prolong desensitization for the m4 muscarinic receptor for which resensitization occurs at the plasma membrane (Bogatkewitsch *et al.*, 1996). Furthermore, internalization may contribute to desensitization for several other G protein-coupled receptors, including the secretin receptor (Holtmann *et al.*, 1996), the cholecystokinin receptor (Rao *et al.*, 1996), and the μ-opioid receptor (Pak *et al.*, 1996). Thus, the function of internalization in the regulation of GPCR activity varies among receptors.

Lastly, after several hours of agonist stimulation, a fourth means of receptor regulation, termed down-regulation, may ensue, whereby the total receptor number in a cell is decreased. Down-regulation may occur by two possible mechanisms. Internalization of the receptor, instead of being followed by recycling back to the plasma membrane, may lead to the transport of receptors from endosomes to lysosomes, where the protein itself may be

degraded. Alternatively, down-regulation can occur by reduced receptor synthesis either by reduced gene transcription or by destabilization of the corresponding mRNA transcript.

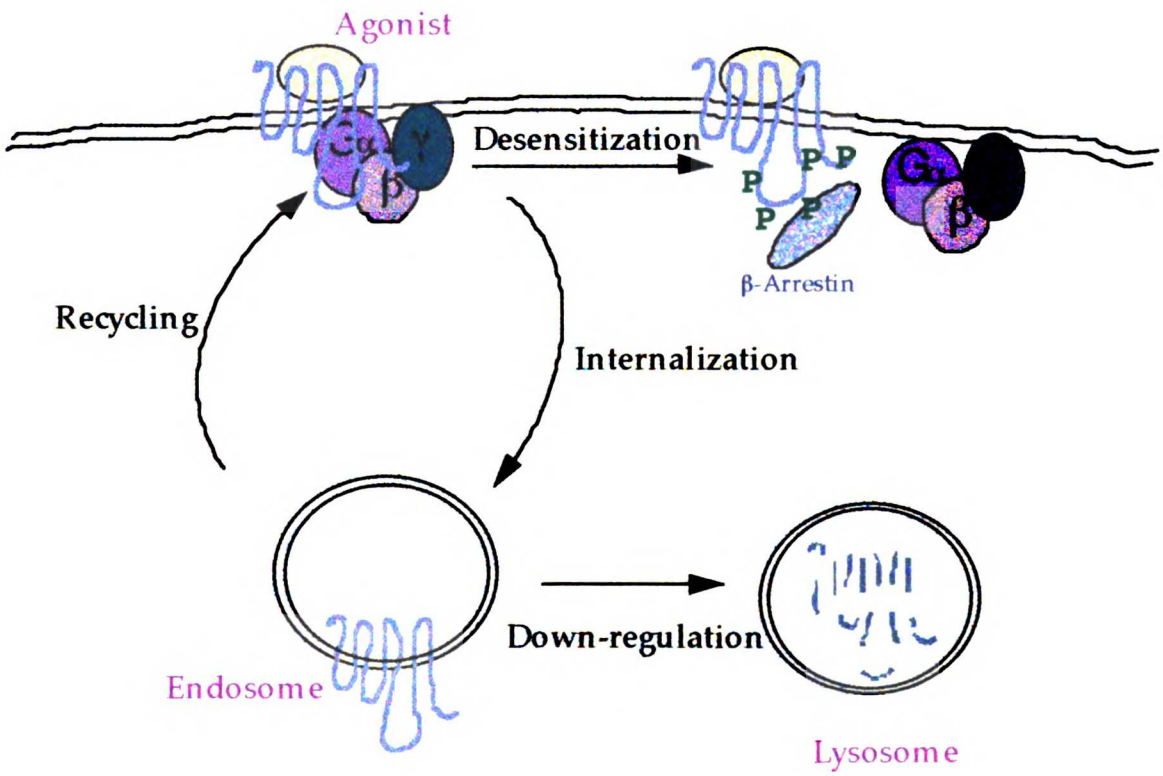


Figure 3. ***Mechanisms of GPCR regulation.*** The agonist-induced activity of G protein-coupled receptors can be regulated by a number of processes, several of which have a direct effect on the receptor itself. These include (1) an uncoupling of the receptor from its cognate G protein by receptor phosphorylation, and in some instances, arrestin binding, in a process called desensitization, (2) internalization of the receptor into an intracellular compartment inaccessible to hydrophilic ligands which may be followed by recycling of the receptor back to the plasma membrane, and (3) transport of the receptor protein into lysosomes, where the receptor is degraded.

OBJECTIVE

G protein-coupled receptors participate in many vital physiological processes and are targeted by a vast array of therapeutic agents; thus, it is essential to have an understanding of how these proteins transduce signals and the mechanisms which regulate their activity. Such knowledge will lead to development of more effective medicinal preparations which may be used at lower doses to avoid undesired side effects and could potentially bypass the complication of drug tolerance. The goal of this research project has been to clarify some of the regulatory mechanisms modulating the activity of one such receptor, the m1 muscarinic acetylcholine receptor, particularly with respect to internalization. The emphasis of project has been four-fold: (1) to determine the endocytic pathway by which the m1 receptor is internalized upon agonist stimulation in order to gain an understanding of what proteins are involved in m1 receptor internalization, (2) to investigate an agonist-independent internalization event in order to clarify the requirement for G protein activation of the second messenger pathway in m1 receptor internalization, (3) to compare the role of a potential regulatory site enriched in serine and threonine residues in the third intracellular loop in the regulation of the m1 and m3 receptors, and (4) to begin to address the contribution internalization has on regulating the signaling activities mediated by the m1 receptor.

CHAPTER 1

HUMAN m1 MUSCARINIC ACETYLCHOLINE RECEPTOR INTERNALIZES VIA CLATHRIN-COATED VESICLES

I. SUMMARY

To determine the endocytic pathway(s) responsible for muscarinic receptor internalization, we used human embryonic kidney (HEK 293) cells stably transfected with the human muscarinic subtype 1 (hm1) receptor tagged at the amino terminus with the epitope EYMPME. The subcellular location of the receptor was visualized by immunofluorescence confocal microscopy and internalization quantified with the use of binding studies. The receptor redistributed into intracellular compartments within a few minutes of agonist treatment. This process was reversible upon removal of agonist and inhibited by antagonist. Acid treatment of the cells, which disrupts internalization via clathrin-coated vesicles, inhibited carbachol-stimulated internalization. PMA, on the other hand, a phorbol ester which inhibits internalization through caveolae, had no effect on carbachol-induced endocytosis of hm1. Double labeling confocal microscopy was used to characterize the intracellular vesicles containing the hm1 receptor following agonist treatment. The hm1 receptor was colocalized with clathrin and α-adaptin, a subunit of the AP2 adaptor protein that links endocytosed proteins with clathrin in clathrin-coated vesicles. In addition, endosomes containing hm1 also contained the transferrin receptor, which constitutively internalizes via clathrin-coated vesicles. In contrast, caveolin, a major protein component of caveolae, did not colocalize with hm1 in intracellular vesicles following agonist treatment, indicating that caveolae are not involved in the agonist-induced internalization of hm1. These results indicate that agonist-induced

internalization of the hm1 receptor occurs via clathrin-coated vesicles in HEK 293 cells.

II. INTRODUCTION

Upon agonist stimulation, many cell surface receptors undergo endocytosis into compartments inaccessible to extracellular ligands. This process is known as receptor internalization. Several pathways mediating the transport of soluble material and membrane-associated proteins into the cell interior have been characterized. These include endocytosis by clathrin-coated vesicles, non-coated vesicles, and caveolae. Of the three pathways, clathrin-mediated endocytosis has been the best characterized. During internalization of tyrosine kinase receptors in a process termed receptor-mediated endocytosis, an adaptor protein, AP2, is first recruited from the cytosol to the membrane. The AP2 protein is a heterotetrameric complex consisting of α and β subunits of 100-110 kD each and two smaller subunits, $\mu 2$ and $\sigma 2$, of about 50 and 17 kD, respectively. The signal for AP2 recruitment has not yet been clearly defined. It has been proposed that interaction of the $\mu 2$ subunit of AP2 with tyrosine-containing motifs in the cytoplasmic tails of the single transmembrane domain tyrosine kinase receptors functions in AP2 recruitment; however, this interaction cannot fully account for AP2 recruitment since proteins containing these motifs are found in regions of the cell where AP2 is not localized (Robinson *et al.*, 1996; Kirchhausen *et al.*, 1997). Once AP2 is translocated to the membrane, soluble clathrin assembles around the membrane-bound AP2 complexes to form a clathrin lattice. The basic unit of the clathrin lattice is the clathrin triskelion, composed of three molecules of clathrin heavy chain (180 kD) and three molecules of clathrin light chain (33-36 kD). During assembly of clathrin at the plasma membrane, a coated

"bud" is formed, which is pinched off at the neck by the 100 kD GTPase dynamin, forming distinct clathrin-coated vesicles carrying the receptors and their bound ligands.

Clathrin-mediated endocytosis is not the sole mechanism for internalization, however, since it has been found that disruption of this pathway does not fully inhibit the uptake of soluble, or "fluid-phase," markers such as horseradish peroxidase. It has been demonstrated that following inhibition of the clathrin pathway by such treatments as K^+-depletion, hypertonicity, or expression of a temperature-sensitive mutant of dynamin, fluid-phase endocytosis is initially reduced to about 50% of the normal rate, but recovers within 30 minutes. Receptor-mediated uptake of transferrin, however, is almost completely blocked (Damke *et al.*, 1995). It has been suggested, therefore, that clathrin-independent endocytosis is a constitutive process, and clathrin serves merely to concentrate receptors for receptor-mediated endocytosis (Cupers *et al.*, 1994).

Another clathrin-independent pathway for internalization occurs via vesicles that are slightly smaller (50-80 nm) than either clathrin-coated vesicles (100-150 nm) or the non-clathrin-coated vesicles that mediate the uptake of horseradish peroxidase (~100 nm). These smaller vesicles, and the invaginations from which they are derived, are termed caveolae. Caveolae have a distinctive flask-shaped structure with a spiral coat consisting largely of the integral membrane protein VIP21, or caveolin. The function of caveolae in the cell is currently under intense investigation. In previous years, a number of functions for caveolae have been suggested. Caveolae have been proposed to mediate the uptake of ions and small molecules in a process termed potocytosis. During potocytosis, molecules bound to GPI-linked proteins, such as the folate receptor, are concentrated as the caveolar

membrane transiently seals off, enclosing a small portion of extracellular fluid. Then, the concentrated ligands are delivered into the cytoplasm through carriers or channels (Anderson, 1993; Mukherjee *et al.,* 1997). Caveolae have also been implicated in the transcytosis of insulin and albumin (Schnitzer *et al.,* 1994). The finding that heterotrimeric G proteins as well as Src family protein tyrosine kinases are concentrated in caveolin-rich membrane fractions has led to the suggestion that caveolae play a role in mediating signal transduction (Anderson, 1993; Li *et al.,* 1995; Schnitzer *et al.,* 1995). Consistent with a role for caveolae in signaling are several reports of the association of G protein-coupled receptors, such as the endothelin subtype A receptor (Chun *et al.,* 1994), with caveolae, and the agonist-induced targeting of m2 muscarinic receptor to caveolae in cardiac myocytes (Feron *et al.,* 1997). Furthermore, the presence of an inositol-1,4,5-triphosphate-sensitive calcium channel and ATP-dependent calcium pump (Fujimoto, 1993) implicate caveolae in the regulation of calcium entry. Conclusions drawn from many of the studies investigating the function of caveolae have been called into question, however, because of two major findings. First, antibodies to GPI-anchored proteins can themselves induce redistribution of these proteins into caveolae under normal fixation conditions (Mayor *et al.,* 1994). Secondly, membrane fractions resistant to Triton X-100, previously considered to be an exclusive characteristic of caveolar membranes, persist in cells devoid of caveolae; and GPI-linked proteins partition into this phase in either situation (Mukherjee *et al.,* 1997). Thus, the true role of caveolae in cell function remains somewhat elusive.

The mechanism by which G protein-coupled receptors are internalized has not been as well characterized as that for the tyrosine kinase family of receptors. Gradually the pathways mediating the internalization of many of

the receptors in the GPCR family are being identified. In general, endocytosis involving clathrin appears to be the predominant pathway for these receptors, demonstrating that internalization by clathrin-coated vesicles is not unique to receptors with a single transmembrane domain. A number of investigators have used indirect methods involving agents found to inhibit clathrin-mediated endocytosis to show the requirement of clathrin for the internalization of several G protein-coupled receptors. Slowiejko *et al.* (1996) have thoroughly investigated the effect of inhibitors of clathrin-mediated endocytosis in the internalization of muscarinic receptors of the m3 subtype in SH-SY5Y cells. They found that under each treatment condition, including hyperosmolar sucrose, K^+ depletion, and acidification of the cytosol, that m3 receptor internalization was inhibited. Similarly, Garland *et al.* (1994) have demonstrated for the substance P (or neurokinin$_1$) receptor and Grady *et al.* (1994) for the gastrin-releasing peptide receptor, that treatment with either hyperosmolar sucrose or phenylarsine oxide results in inhibition of agonist-induced receptor internalization, suggesting a role for clathrin in the internalization of each of these receptors. Several other groups have purified clathrin-coated vesicles from bovine brain and demonstrated the presence of such receptors as A_1 adenosine receptors (Gonzalez-Calero *et al.*, 1990, 1992), D_1 and D_2 dopamine receptors (Ozaki *et al.*, 1994), and muscarinic receptors (Silva *et al.*, 1986). More direct evidence was obtained for the human chorionic gonadotropin hormone/leutinizing hormone receptor, for which electron microscopy revealed the initial clustering of receptor into clathrin-coated pits upon hormone treatment and subsequent internalization into clathrin-coated vesicles within 5 minutes after treatment (Ghinea *et al.*, 1992). Clathrin-mediated endocytosis is not a universal mechanism for GPCR internalization, however, and different pathways may mediate the internalization of a given

receptor depending on the cell line studied. For example, whereas Silva *et al.* found muscarinic receptors in clathrin-coated vesicles from bovine brain (1986), Raposo *et al.* (1987) have shown by electron microscopy that the vesicles mediating the uptake of muscarinic receptors in CCL137 human fibroblast cells are non-coated, and based on their small size (50-70 nm) are likely to be caveolae. Different pathways have also been found to mediate the internalization of β-adrenergic receptors. Using electron microscopy, Raposo *et al.* (1989) showed that, like the muscarinic receptors in CCL137 cells, β_2-adrenergic receptors in A431 carcinoma cells, underwent internalization into non-clathrin-coated vesicles, although the authors note that EGF receptors, which normally internalize by a clathrin-dependent mechanism, utilize both coated and non-coated vesicles for internalization in this cell line. Conversely, β_2-adrenergic receptors in transfected HEK 293 cells, upon agonist treatment, transverse the same endocytic pathway as the transferrin receptor, which constitutively internalizes via clathrin-coated vesicles (von Zastrow and Kobilka, 1992). Receptors can also internalize by separate pathways in the same cell line. Roettger *et al.* (1995) have demonstrated that, while the prominent pathway of internalization for the cholecystokinin receptor in CHO cells is via clathrin-coated vesicles, a population of receptors can also be found in non-coated plasma membrane invaginations and vesicles, which the authors suggest may be caveolae. Thus, it is apparent that the pathway of GPCR internalization may vary on a cell-specific and receptor-specific basis. This chapter describes the contribution that I have made into identifying the pathway involved in the agonist-induced internalization of the m1 muscarinic receptor in HEK 293 cells. Here, I have shown by confocal microscopy, both directly and indirectly, that in this cell line, the hm1 receptor internalizes via clathrin-coated vesicles.

III. EXPERIMENTAL PROCEDURES

A. Materials

Fish gelatin, poly-L-lysine, and carbachol were purchased from Sigma Chemical Co. (St. Louis, MO), and Fluoromount G was obtained from Fisher Scientific (Pittsburgh, PA). The monoclonal antibody to the epitope EYMPME (anti-EE) was purchased from Onyx Inc. (Richmond, VA). The Cy5 (indodicarbocyanine) labeling kit and Cy3 (indocarbocyanine)-conjugated goat anti-mouse IgG antibodies were obtained from Biological Detection Systems Inc. (Pittsburgh, PA). FITC-conjugated goat anti-mouse IgG antibodies were purchased from Cappell Technika (Durham, NC). Monoclonal antibody to transferrin receptor was purchased from Amersham (Arlington Heights, IL). Monoclonal and polyclonal antibodies to clathrin heavy chain and monoclonal antibody to α-adaptin were generous gifts from Dr. Frances Brodsky, UCSF. Monoclonal and polyclonal antibodies to caveolin were purchased from Transduction Laboratories (Lexington, KY). The polyclonal antibody to the C tail of the Hm1 receptor was a generous gift from Drs. Stefan Nahorsky and Andrew Tobin, University of Leceister, U.K.

B. Epitope Tag and Stable Expression of hm1 Receptor

The gene encoding the human muscarinic cholinergic subtype 1 receptor (hm1) was obtained from a human placental genomic library as previously described (Maeda *et al.*, 1990). An epitope tag with the sequence EYMPME was added to the N-terminal of the receptor using the polymerase chain reaction (Arden and Lameh, 1996). The 5' primer with sequence TGAATTCACCATGGAATACATGCCAATGGAAAACACTTCAGCCCCACCTGCTGTC was synthesized which contained the following components: an EcoRI restriction site, a 3'-nucleotide spacer, an initiating methionine, the sequence coding for the tag, and a portion of the hm1 sequence. The 3' primer with sequence

TTGGCGCCTGCTCGGTTCTCTGTCTCCCGGTA contained a BamHI restriction site. The double-digested PCR product was ligated into pSG5 and the construct was used to transfect DH5α by electroporation (BioRad Gene Pulser, Hercules, CA). The sequence of a selected clone was verified and the plasmid was co-transfected with $pRSV^{neo}$ into human embryonic kidney (HEK 293) cells by the calcium phosphate precipitation method. Clonal cell lines expressing EE-hm1 sites were selected in DMEM/H-16/F-12 with 10% fetal calf serum, 1% penicillin/streptomycin, and 400 μg/ml G418 in 5% CO_2 and maintained in this medium with 200 μg/ml G418. A clone expressing >3 X 10^5 sites/cell as determined by [^{3}H]-NMS binding was selected for further experiments.

C. Receptor Binding Assay

Cells were seeded onto 12-well dishes, allowed to attach overnight, and then treated for specified times with 1 mM carbachol or buffer in incomplete medium at 37°C. Cells were placed on ice, washed three times with ice-cold PBS, and incubated with 1.5 - 2.0 nM [^{3}H]-N-methyl scopolamine (NMS) at 12°C for 90 minutes. After labeling, cells were placed on ice, harvested with PBS, and filtered (S&S #32 glass fiber filter), followed by three rinses with ice-cold PBS. The radioactivity on the filters was determined by scintillation counting.

D. Agonist-induced Internalization of hm1 by Immunofluorescence Confocal Microscopy

HEK 293 cells expressing tagged Hm1 receptor were grown overnight on chamber slides (Nunc Inc., Napperville, IL) which had been pre-treated with poly-L-lysine. Treatment with 1 mM carbachol, 10 μM atropine, or 1 mM carbachol and 10 μM atropine were carried out at 37°C for 30 minutes. For recycling experiments, cells were treated with 1 mM carbachol for 15 minutes,

washed 3 times with phosphate-buffered saline and allowed to incubate in agonist free medium at 37°C for 1 hour prior to fixation. At the end of drug treatment, cells were washed once with PBS, fixed for 10 minutes at room temperature with 3.7% paraformaldehyde in PBS, permeabilized in PBS containing 0.25% fish gelatin, 0.04% saponin, and 0.05% NaN_3. After permeabilization, cells were labeled with anti-EE monoclonal antibody for 1 hour, washed four times with PBS, incubated with FITC (fluorescein isothiocyanate)-conjugated goat anti-mouse secondary antibody, followed by four more washes with PBS and one wash with water. Slides were mounted using Fluoromount G containing a trace amount of phenylenediamine, and stored at 4°C (Wong and Brodsky, 1992). Samples were visualized by laser scanning confocal microscopy using a krypton-argon laser coupled to a BioRad MRC-600 confocal head attached to an Optiphot II Nikon microscope with a Plan Apo 60X 1.4 NA objective lens. FITC emission was detected with a blue high sensitivity filter block.

E. Inhibition of Caveolae and Clathrin-mediated Endocytosis

Cells grown overnight on chamber slides were pretreated with either 5 mM acetic acid in HEPES (pH 5.0) for 5 minutes (Sandvig *et al.*, 1987) or 1 μM phorbol-12-myristate-13-acetate (PMA) for 30 minutes at 37°C (Smart *et al.*, 1994). Following pretreatment, 1 mM carbachol or buffer was added and cells were incubated for an additional 30 minutes at 37°C. Cells were permeabilized, stained and visualized as described above.

F. Colocalization Studies of EE-hm1 Receptor Internalization

For double labeling studies, we directly conjugated a fluorophore (Cy5) to the anti-EE mouse monoclonal antibody. Detection of the second protein of interest was carried out sequentially with primary antibody and secondary

antibody conjugated to a second fluorophore (Cy3). This allowed us to use two mouse primary antibodies to localize the proteins of interest without the possibility of interaction of the secondary antibody with both primary antibodies. The colocalization assay was carried out as follows; cells grown for one or two days on poly-L-lysine-treated chamber slides were treated for specified times with 1 mM carbachol or buffer in serum-free medium at 37°C. Cells were washed, fixed, and permeabilized as above, then incubated for one hour with monoclonal antibody to clathrin, AP2, transferrin receptor, or caveolin. After four washes with PBS, cells were incubated with Cy3-labeled goat anti-mouse secondary antibody for 30 minutes followed by four washes with PBS prior to incubation with Cy5-conjugated anti-EE mAb. After washing, cells were mounted with mounting media and visualized as above. When polyclonal antibodies to clathrin or caveolin were used, the cells were sequentially incubated with primary antibody to clathrin or caveolin, followed by Cy3-labeled secondary antibody (donkey anti-rabbit) and then with anti EE-antibody followed by Cy5 labeled goat anti-mouse secondary antibody. For the polyclonal hm1 receptor antibody, Cy3-conjugated donkey anti-rabbit secondary antibody was used to label anti-hm1 antibody, followed by primary monoclonal antibody to clathrin or α-adaptin and Cy5 conjugated goat anti-mouse secondary antibody. Cy3/Cy5 double emission was detected using a C1/C2 filter block combination (Sargent, 1994). Images of a mid-section of cells from two distinct photomultiplier tubes were collected simultaneously and then superimposed to identify areas of colocalization. When the images are merged, hm1 is arbitrarily colored red, the other proteins (clathrin, α-adaptin, transferrin receptor, or caveolin) are colored green, and areas of colocalization appear yellow.

IV. RESULTS

A. Internalization of Tagged and Untagged hm1 Receptor by Binding Assay

Cells stably transfected with the tagged or untagged hm1 receptor were treated with or without 1 mM carbachol in serum-free medium for varying times. After carbachol treatment, cells were washed and ^{3}H-NMS binding measured. The time course of receptor internalization for the tagged and untagged receptors (Fig. 1.1) were similar.

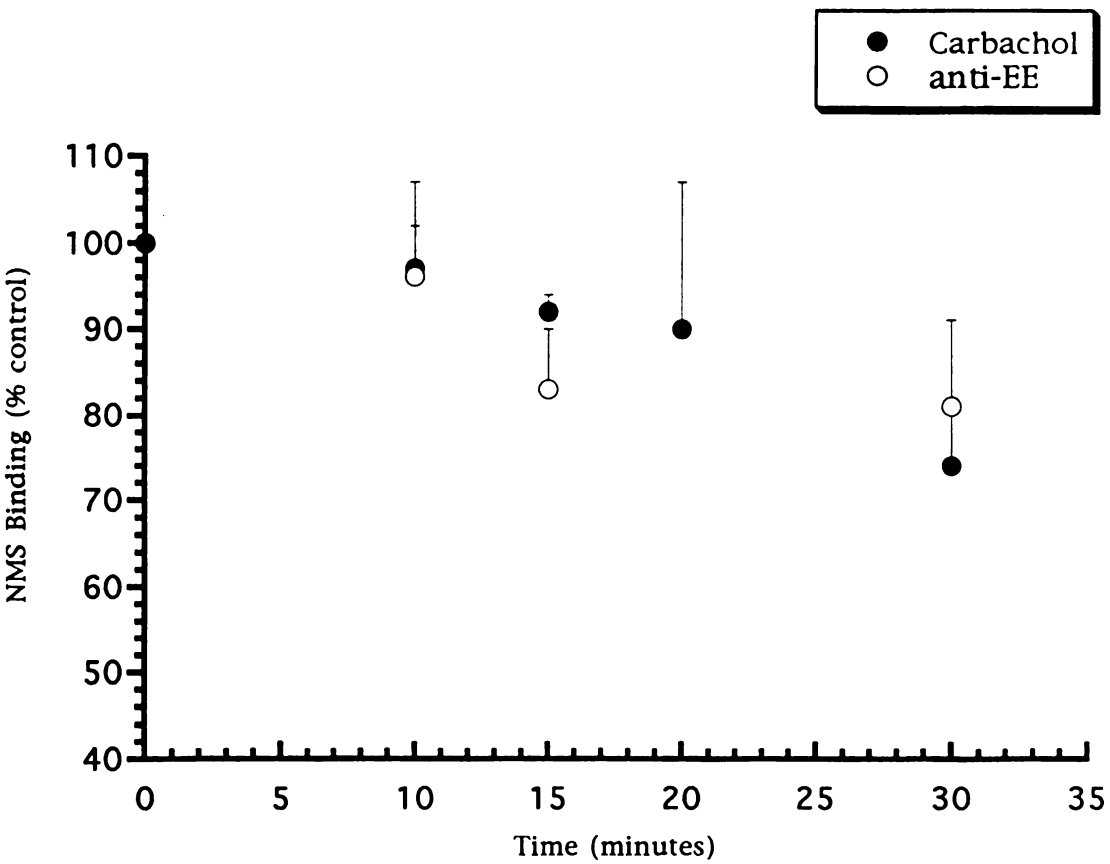


Figure 1.1. ^{3}H-NMS binding profile of EE-hm1 and untagged Hm1 receptors after carbachol treatment. Human embryonic kidney cells stably transfected with EE-tagged or untagged hm1 receptors were seeded onto 12-well cell culture dishes, allowed to attach overnight, and treated with 1 mM carbachol for times varying between 0 and 2 hours. After washing with ice-cold PBS, cell surface receptor binding was assessed using the hydrophilic ligand ^{3}H-NMS. Each point is average of quadruple points. Error bars represent standard deviation.

B. Internalization of Tagged hm1 Receptor by Confocal Microscopy

Carbachol-induced internalization of the tagged hm1 receptor was visualized by immunofluorescence confocal microscopy using the antibody to the epitope tag followed by a secondary antibody conjugated to a fluorophore (FITC, Cy3, or Cy5). In the absence of carbachol, receptors were predominantly localized at the cell surface (Fig. 1.2a), although in some cells a small degree of intracellular staining could also be observed. The intracellular staining was partly diffuse staining throughout the midsection of cells, but in some cells, vesicular intracellular staining was also observed. These vesicles in unstimulated cells could represent hm1 receptors in transit to the surface following synthesis or a population of receptors cycling continuously from the cell surface into the interior and back to the cell surface. After 30 minutes of carbachol treatment, receptors were redistributed to endosomes within the cell interior (Fig. 1.2b), and the cell surface staining of the receptor was substantially reduced. Atropine, a muscarinic antagonist, did not cause redistribution of the hm1 receptors from the cell surface (Fig. 1.2c), and blocked the internalization of hm1 induced by carbachol (Fig. 1.2d). Internalization was reversible following the removal of carbachol and incubation of the cells in agonist-free medium for 1 hour (Fig. 1.2e).

C. Effect of PMA and Acetic Acid on hm1 Receptor Internalization

To determine whether or not carbachol-stimulated internalization involves caveolae, cells were pretreated with 1 μM PMA for 30 minutes prior to a 30 minute treatment with 1 mM carbachol (Anderson *et al.*, 1992). Visualization of receptors by confocal microscopy revealed that PMA had no effect on carbachol-induced internalization (Fig. 1.3d).

To disrupt clathrin-mediated endocytosis, cells were pretreated with acetic acid as previously described (Sandvig *et al.*, 1987). Cells were pretreated

for 5 minutes with 5 mM acetic acid at 37°C prior to treatment with 1 mM carbachol for 30 minutes. Confocal images indicated that acid pretreatment completely blocked carbachol-induced internalization (Fig. 1.3b), indicating a role for clathrin-coated vesicles in hm1 internalization.

D. Double-Labeling of hm1 Receptor and Clathrin

To further investigate the potential role of clathrin in hm1 internalization, colocalization studies using antibodies to clathrin were performed. Cells were treated with carbachol for times varying between 0 and 20 minutes to determine the time course of hm1 colocalization with clathrin. Before agonist treatment, although some intracellular staining is observed, the majority of the receptors are diffusely distributed at the plasma membrane. Double-labeling studies using a monoclonal antibody against clathrin heavy chain demonstrated a high degree of colocalization between hm1 and clathrin at the cell surface prior to carbachol treatment (Fig. 1.4a). After 5-10 minutes of carbachol treatment, hm1 staining was shifted from the plasma membrane to intracellular vesicles, the majority of which were also found to contain clathrin, as indicated by the yellow color (Fig. 1.4b-d). Double-labeling of hm1 and clathrin using a polyclonal antibody to clathrin gave similar results (data not shown).

In the above study, we used directly labeled monoclonal antibody to the epitope tag on the receptor along with monoclonal antibody to clathrin. In order to rule out the possibility of any false positive signal resulting from cross-reactivity of secondary antibody with directly conjugated antibody, double-labeling studies of clathrin were also carried out using a polyclonal antibody to the C tail of the hm1 receptor. Prior to agonist treatment, hm1 was diffusely localized around the cell surface, where it colocalized with clathrin (data not shown). Following 10 minutes of carbachol treatment, cell surface

staining of hm1 was dramatically reduced, and the receptor was translocated into intracellular vesicles containing clathrin (Fig. 1.4e). The high degree of colocalization between hm1 and clathrin in intracellular vesicles is still observed (Fig 1.4e, *lower panel*) indicating that the observed colocalization is in fact real and not due to any cross-reactivity of antibodies.

We also studied the effect of treatment with the muscarinic antagonist atropine on the colocalization pattern of the hm1 receptor with clathrin. When double-labeling studies were performed in the presence of atropine alone or in combination with carbachol, little vesicular hm1 staining was observed (as seen in Fig. 1.2c-d), and hm1 receptors at the cell surface colocalized with clathrin (data not shown). Thus, the antagonist blocks carbachol-induced redistribution of the receptor into intracellular vesicles but not its association with clathrin at the plasma membrane.

E. Double-Labeling of hm1 Receptor and α-Adaptin

To confirm the role of clathrin-coated vesicles in the internalization of hm1, double-labeling studies were also carried out with α-adaptin, a subunit of the AP2 adaptor protein. α-Adaptin appeared in a punctate staining pattern throughout the interior of the cell (Fig. 1.5a, *upper panel).* The AP2 complex has been shown to be localized to vesicles in the proximity of the plasma membrane (Wong and Brodsky, 1992); thus, the rather evenly distributed punctate staining of α-adaptin indicates that the optical section chosen for visualization is located above the cell nucleus. This staining did not dramatically change after carbachol treatment (Fig. 1.5b-e, *upper panels*). Hm1 receptors were localized primarily at the cell surface in the absence of any treatment (Fig. 1.5a, *middle panel*). Again, a small degree of intracellular staining was present in some cells prior to agonist treatment; however, part of the intracellular labeling represented a hazy background staining which is

accentuated in the color photographs compared to the black and white micrographs (Fig. 1.2 and 1.3). The vesicular intracellular staining again possibly corresponds to receptors coming to the cell surface after synthesis and/or continuously cycling receptors. Following agonist treatment, staining of hm1 markedly shifted from the plasma membrane to intracellular vesicles (Fig. 1.5b-e, *middle panels*). The hm1 receptor and α-adaptin were colocalized at the cell surface prior to any agonist treatment (Fig. 1.5a, *lower panel*) as shown by the yellow color. Although some intracellular vesicles containing hm1 were present prior to agonist treatment, α-adaptin was absent from some of these vesicles, as indicated by red-colored vesicles (Fig. 1.5a, *lower panel*). After several minutes of agonist treatment, a marked relocation of the receptor from the cell surface to the cell interior occurred (Fig. 1.5b-d, *lower panels*). Many intracellular vesicles containing both the receptor and α-adaptin (yellow vesicles) appeared, and colocalization between hm1 and α-adaptin at the plasma membrane strongly decreased. To confirm these results, we carried out the experiment with the polyclonal antibody to hm1. The staining prior to agonist treatment was similar to that observed with the monoclonal antibody (data not shown). Once again, after agonist treatment, a dramatic shift of the receptor staining from the cell surface to intracellular vesicles was observed (Fig. 1.5e), and vesicles containing both receptor and α-adaptin were predominant (yellow vesicles). In all these cells (Fig. 5, *lower panels*), some of the intracellular vesicles appeared red, suggesting that a population of intracellular receptors exists that are not colocalized with these adaptor proteins. We propose that hm1 receptors not colocalized with the AP2 protein are either being transported from the Golgi to the cell surface following synthesis or recycled back to the cell surface following internalization. This recycling mechanism is important in HEK 293 cells

where no receptor downregulation occurs (Lameh *et al.,* 1992). Either transport process would account for an association with clathrin, which is involved in a number of transport processes (Pearse and Robinson, 1990; Stoorvogel *et al.,* 1996) and not the AP2 protein, which is exclusively involved in the endocytic pathway.

F. Double-Labeling of hm1 and Transferrin Receptors

In addition to clathrin and α-adaptin, we also performed double-labeling studies with anti-transferrin receptor antibody to further confirm the role of clathrin-coated vesicles in internalization of hm1. As expected, transferrin receptor was present at the cell periphery and throughout the cell interior (Fig. 1.6a-b, *upper panels*). Hm1 was present primarily at the cell surface prior to agonist treatment (Fig. 1.6A, *middle panel*) as seen by strong plasma membrane staining. After agonist treatment, this peripheral staining was replaced by predominantly intracellular, vesicular staining (Fig. 1.6b, *middle panel*). The merged images of the two receptors (*lower panel*) indicated a colocalization primarily at the cell surface prior to carbachol treatment (Fig. 1.6a), with a shift to intracellular vesicles after agonist treatment (Fig. 1.6b). Contrary to results with α-adaptin, most of these vesicles were colored yellow (i.e. stained with both antibodies), suggesting that the majority of hm1 receptors located intracellularly were localized to the same compartments as the constitutively recycling transferrin receptors. The presence of a few intracellular vesicles containing both hm1 and transferrin receptors in the absence of any carbachol treatment, supports the notion that a small population of the hm1 receptors cycle between the plasma membrane and intracellular vesicles at the resting state. However, there is a marked difference in the degree of punctate intracellular staining before and after carbachol treatment (compare Fig. 1.2 a and b).

G. Double-Labeling of hm1 Receptor and Caveolin

To confirm our preliminary conclusion that caveolae are not involved in carbachol-induced hm1 internalization, we performed double-labeling studies using a monoclonal antibody to caveolin, a major protein component of caveolae. Cells were treated in the presence or absence of 1 mM carbachol, fixed, and sequentially labeled with antibody to caveolin and hm1 receptor. Caveolin staining (Fig. 7, *upper panels*) was primarily at the cell surface with some hazy intracellular staining also present. In the absence of carbachol, some degree of colocalization was apparent at the surface of the cells (Fig. 1.7a). The shift of hm1 staining from the cell surface to intracellular compartments was dramatic, as described earlier. After carbachol treatment, almost no colocalization between hm1 and caveolin was observed (Fig. 7b-e). Most intracellular vesicles were stained only with antibody to the tagged hm1 receptor and appeared red. Intracellular regions appeared yellow only when an area of dense staining was present in the cell, suggesting that the colocalization is coincidental. Wherever the vesicles were evenly distributed (see Fig. 7c vs. 7d and 7e), no colocalization was observed (no yellow vesicles). Results from separate experiments using a polyclonal antibody to caveolin support these findings (data not shown). In addition, these negative results obtained with caveolin further confirm that the colocalization of hm1 with clathrin, α-adaptin, and transferrin receptor is not due to the cross-reactivity of the antibodies.

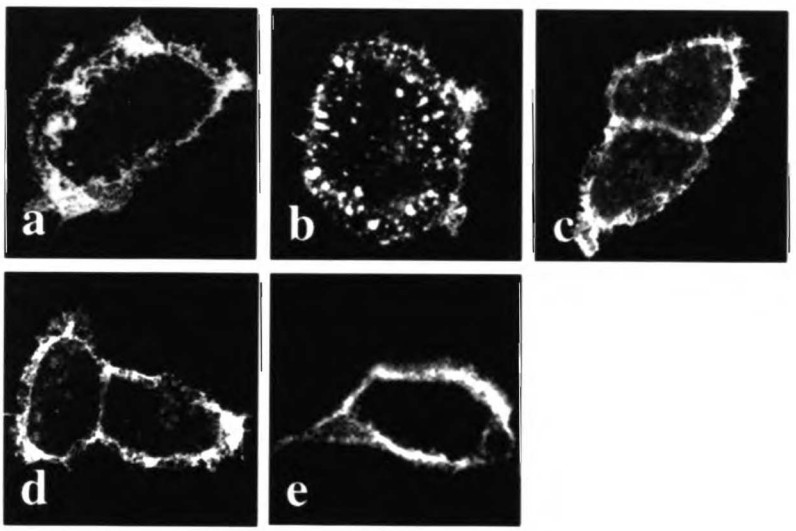


Figure 1.2. Effect of agonist and antagonist on cellular localization of hm1 receptor. HEK 293 cells expressing EE-hm1 were grown on chamber slides overnight and treated with 1 mM carbachol in the presence or absence of 10 μM atropine for 30 minutes at 37°C prior to fixation and addition of primary and secondary antibodies. FITC-conjugated goat anti-mouse secondary antibody was used in these experiments. Recycling of the receptor to the cell surface was determined after treatment of the cells with carbachol for 15 minutes, removing carbachol-containing medium, and washing the cells three times with PBS. The cells were then incubated at 37°C for 1 hour prior to fixation to allow for recycling of the receptor. (a) No treatment, (b) 1 mM carbachol , (c) 10 μM atropine, (d) 1 mM carbachol plus 10 μM atropine, (e) 1 mM carbachol followed by washing and 1 hour recycling.

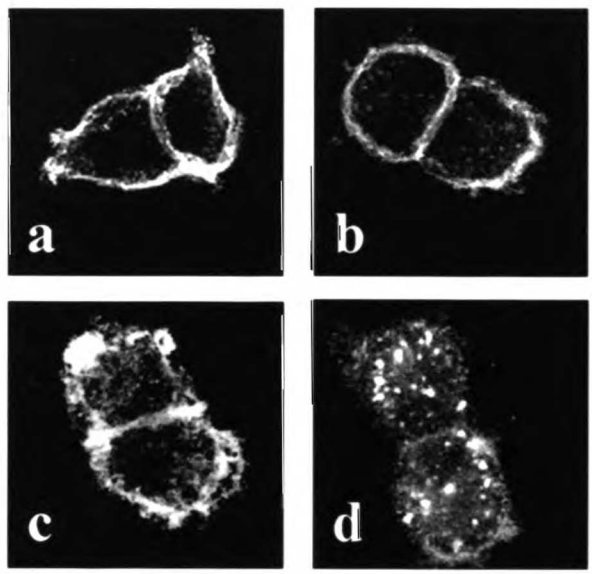


Figure 1.3. Effect of biochemical treatments on carbachol induced internalization of EE-hm1. HEK 293 cells expressing EE-hm1 were grown on chamber slides overnight and treated with either 5 mM acetic acid for 5 minutes (a,b) or 1 μM PMA for 30 minutes at 37°C (c,d) prior to addition of carbachol. Buffer (a,c) or 1 mM carbachol (b,d) was added to the incubation medium and incubation continued for 30 minutes longer. Images from a midsection of the cells are shown.

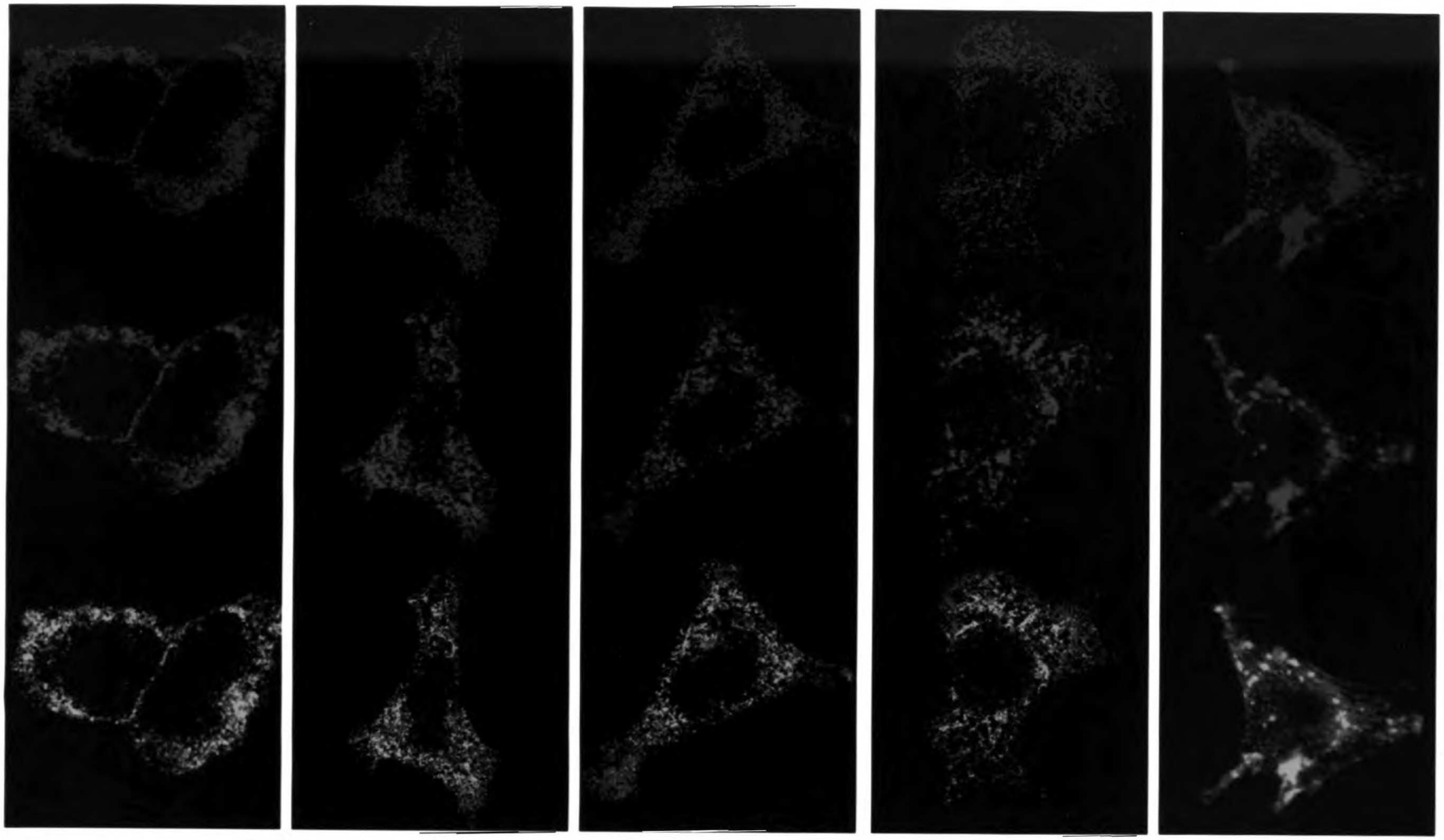


Figure 1.4. Colocalization of EE-hm1 and clathrin. HEK 293 cells expressing EE-hm1 were treated with 1 mM carbachol for varying times. After fixing and permeabilizing, cells were sequentially labeled with monoclonal antibody to clathrin followed by Cy3-labeled goat anti-mouse secondary antibody. Then hm1 was labeled with Cy5-conjugated anti-EE antibody (a-d), or polyclonal antibody to the C terminus of hm1 followed by Cy5-labeled donkey anti-rabbit secondary antibody (e). The green color indicates the localization of clathrin (*upper panel)*, red is the localization of hm1 (*middle panel*), and yellow is indicative of colocalization of the two proteins in the merged image (*lower panel*). (a) No treatment, (b) 5 minutes carbachol, (c) 10 minutes , (d) 20 minutes , (e) 10 minutes with polyclonal antibody to hm1. Images from a midsection of the cells are shown.

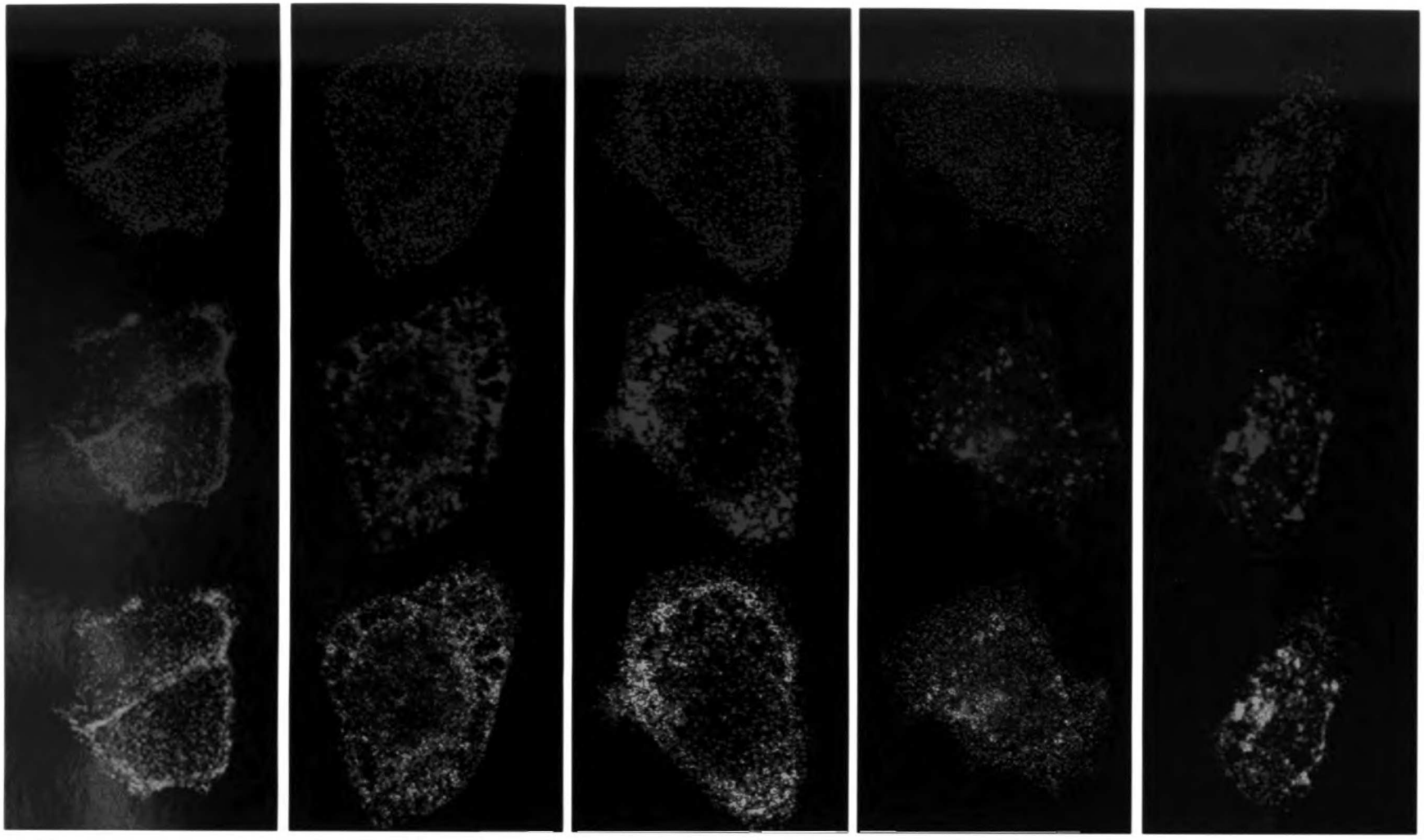


Figure 1.5. Colocalization of EE-hm1 and α-adaptin. HEK 293 cells expressing EE-hm1 were treated with carbachol for varying times. After fixing and permeabilizing, cells were sequentially labeled with monoclonal antibody to α-adaptin followed by Cy3-labeled goat anti-mouse secondary antibody. Then hm1 was labeled with Cy5-conjugated anti-EE antibody (a-d), or polyclonal antibody to the C terminus of hm1 followed by Cy5-labeled donkey anti-rabbit secondary antibody (e). The green color indicates the localization of α-adaptin (*upper panel*), red is the localization of hm1 (*middle panel*), and yellow is indicative of colocalization of the two proteins in the merged image (*lower panel*). (a) No treatment, (b) 5 minutes carbachol, (c) 10 minutes carbachol, (d) 20 minutes carbachol, (e) 10 minutes carbachol with polyclonal antibody to hm1.

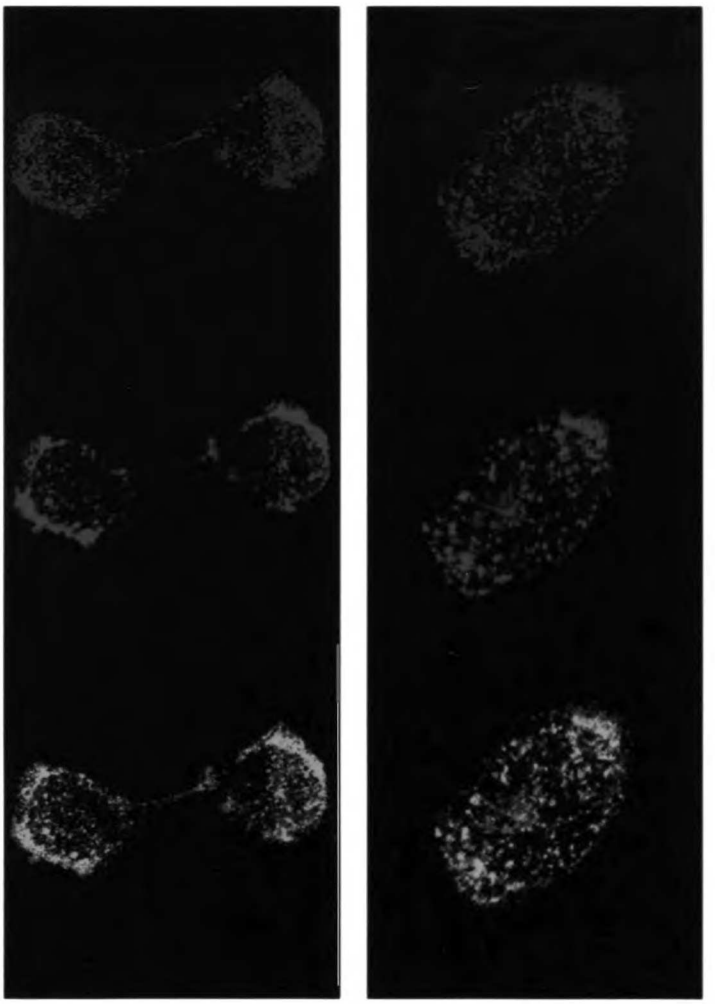


Figure 1.6. Colocalization of EE-hm1 and Transferrin Receptor. Cells were treated as described for Figures 2 and 3. The green color indicates the localization of transferrin receptor (*top panel*), red is the localization of hm1 (*middle panel*), and yellow is indicative of colocalization of the two proteins in the merged image (lower panel). (a) No carbachol treatment, (b) 10 minutes carbachol.

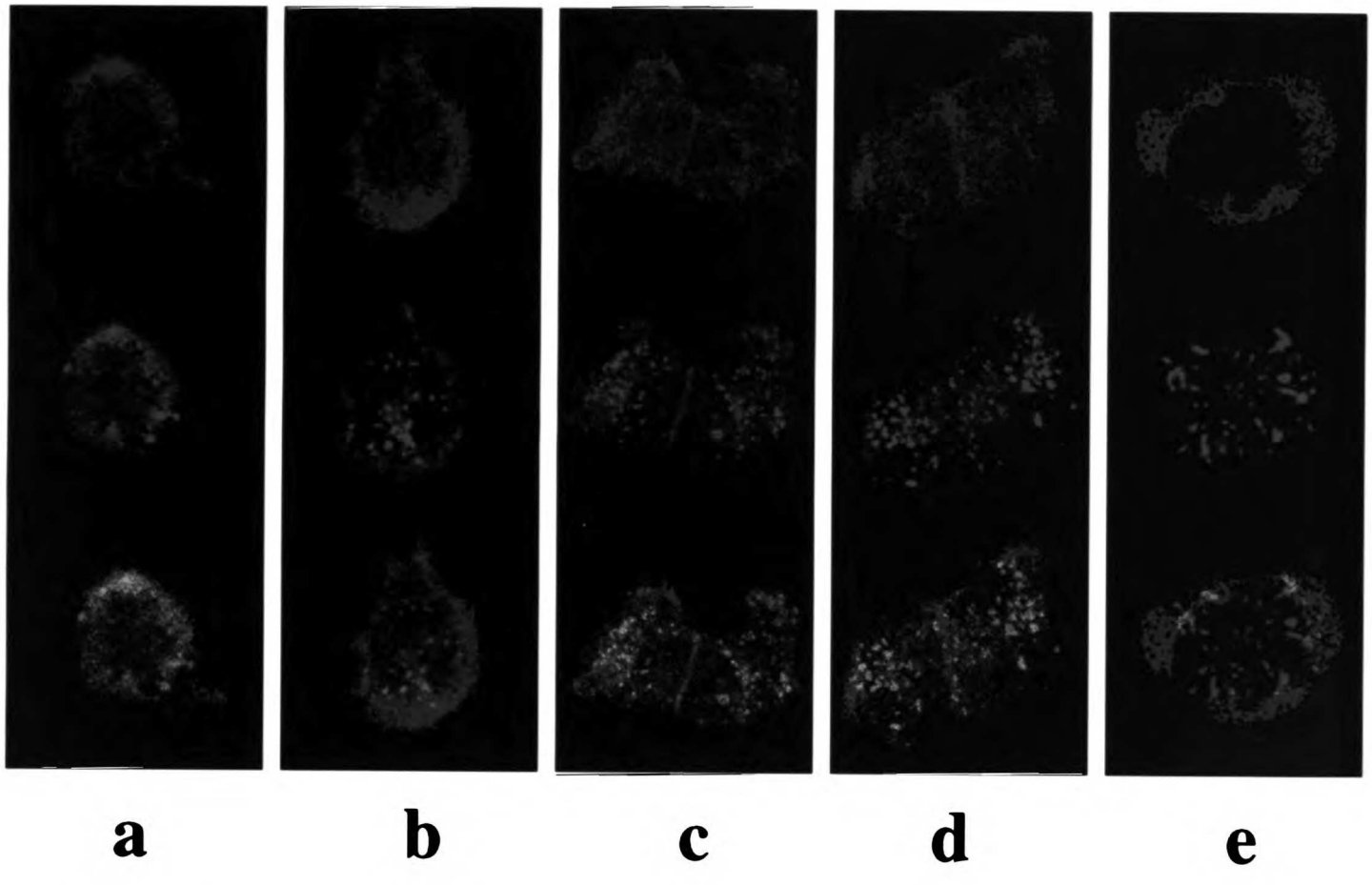


Figure 1.7. Double-Labeling of EE-hm1 and caveolin. HEK 293 cells expressing EE-hm1 were treated with carbachol for varying times. After fixing and permeabilizing, cells were sequentially labeled with monoclonal antibody to caveolin followed by Cy3-labeled goat anti-mouse secondary antibody. Then hm1 was labeled with Cy5-conjugated anti-EE antibody. The green color indicates the localization of caveolin (*upper panel*), red is the localization of hm1 (*middle panel*), and yellow is indicative of colocalization of the two proteins in the merged image (*lower panel*). (a) No treatment, (b) 5 minutes carbachol, (c) 10 minutes, (d) 20 minutes, (e) 30 minutes.

V. DISCUSSION

Receptor regulation remains a poorly understood phenomenon for the G-protein coupled receptors in general and for hm1 specifically. Agonist-induced receptor internalization, as a mechanism of receptor regulation, remains to be clarified. The first step towards a clearer understanding of the mechanism of internalization of hm1 is to define the pathway by which the receptor is transported into the interior of the cell. In this study we report the first comprehensive work describing the pathway of internalization of hm1 using immunofluorescence confocal microscopy.

We have shown that the hm1 receptor internalizes into intracellular vesicles following agonist treatment. This process is inhibited by the antagonist atropine and is reversible. Our results suggest that carbachol-stimulated internalization of hm1 receptors in HEK 293 cells occurs via clathrin-coated vesicles, consistent with an earlier study that demonstrated the presence of muscarinic receptors in coated vesicles purified from bovine brain (Silva *et al.,* 1986) and with a more recent study that showed inhibition of muscarinic receptor internalization in SH-SY5Y cells following perturbation of clathrin distribution (Slowiejko *et al.,* 1996). The fact that internalization is blocked by acetic acid, which prevents internalization by clathrin-coated vesicles, but not by PMA, which inhibits caveolae formation, provides indirect evidence that clathrin-coated vesicles, and not caveolae, are involved in hm1 internalization. Double-labeling studies of hm1 with clathrin, α-adaptin, transferrin receptor, and caveolin provide further evidence in support of this hypothesis. The colocalization after carbachol treatment between hm1 and clathrin in intracellular vesicles confirms that carbachol-induced internalization involves the association of hm1 receptors with clathrin. The apparent colocalization at the cell surface before and after

agonist treatment may be due to the association of these two proteins at the plasma membrane, i.e. the presence of hm1 in clathrin coated pits. This prelocalization in coated pits has been shown previously by electron microscopy for the hCG/LH receptor (Ghinea *et al.,* 1992). The pre-association of hm1 with clathrin in the absence of agonist may be indicative of a population of hm1 receptors recycling at the steady state level, as was demonstrated for the β_2 adrenergic receptor (von Zastrow and Kobilka, 1992). In addition, the presence of an intracellular population of hm1 at the steady state levels suggests that a small population of the receptor might be cycling in and out of the cell without any agonist treatment. A similar phenomenon has previously been observed for the β_2 adrenergic receptor (Aoki *et al.,* 1989) and the α_2-adrenergic receptor (Aoki *et al.,* 1994) using electron microscopy. Thus, it is possible that this basal internalization is a common feature of these receptors, which is generally not detected by less sensitive methods such as radioligand binding assays. The use of confocal microscopy and electron microscopy, however, allows us to focus on individual cells, enabling us to observe this phenomenon.

Adaptor proteins are proteins associated with clathrin-coated vesicles that mediate the interaction of receptors with the clathrin triskelion. The roles of two populations of adaptor proteins in protein trafficking have been characterized. The plasma membrane adaptor protein (AP2) associates with clathrin during endocytosis, and the Golgi adaptor protein (AP1) associates with clathrin during transport of protein from the Golgi to the cell surface (Pearse and Robinson, 1990). Double-labeling studies with α-adaptin, a subunit of AP2, again implicate a clathrin-mediated mechanism of hm1 internalization. Following carbachol treatment, hm1 receptors clearly interact with α-adaptin in intracellular vesicles. The presence of two

populations of hm1 receptors, one which colocalizes with α-adaptin and one which does not, suggests that some hm1 receptors are either being recycled back to the cell surface, transported to the cell surface following synthesis, or both, in addition to being internalized into AP2-containing intracellular compartments. Since the AP2 complex is only involved in the endocytosis of the receptors, we would not expect to observe this protein in recycling endosomes or endosomes involved in transport from the Golgi to the plasma membrane. This is in contrast to clathrin staining, since clathrin has been shown to be involved in all three processes of endocytosis, with AP2 (Pearse and Robinson, 1990); protein transport from the Golgi, with AP1 (Pearse and Robinson, 1990); and receptor recycling from endosomes to the cell surface, with an unknown adaptor protein (Stoorvogel *et al.,* 1996) that may be the newly identified AP3 complex (Simpson *et al.,* 1997; Dell'Angelica *et al.,* 1997; Dell'Angelica *et al.,* 1998). Thus, we observe a greater colocalization of hm1 with clathrin and transferrin receptor compared to α-adaptin.

Furthermore, our studies indicate that hm1 receptors are associated with transferrin receptors during the entire endocytic pathway. The continuous recycling of transferrin receptor is known to be clathrin-mediated (McGraw and Maxfield, 1990; Jing *et al.,* 1990; Goldstein *et al.,* 1985; Hopkins, 1985; McGraw *et al.,* 1991). The observation that hm1 receptors in intracellular vesicles are completely colocalized with transferrin receptors is consistent with the idea that hm1 receptors are being internalized and recycled back to the cell surface by an identical pathway as the constitutively-recycling transferrin receptors. This observation is consistent with those of other investigators who have shown colocalization of a G-protein coupled receptor with transferrin receptor (von Zastrow and Kobilka, 1992; Garland *et al.,* 1996) or transferrin (Fonseca *et al.,* 1995; Grady *et al.,* 1995).

In order to address the possibility that other mechanisms of receptor-mediated endocytosis may be involved in hm1 internalization in HEK 293 cells, we investigated the colocalization of hm1 with caveolin, a major protein in caveolae. To date, the exact role of caveolae in the cells has not been clarified. While these compartments have been shown to be involved in pinocytosis (Anderson *et al.,* 1992) they have also been implicated in cellular signaling (Lisanti *et al.,* 1994; Chang *et al.,* 1994; Chun *et al.,* 1994) Our double-labeling studies with caveolin confirm our preliminary conclusion that caveolae are not involved in agonist-stimulated internalization of hm1 receptors. There is some colocalization at the cell surface prior to agonist treatment. This colocalization might be an artifact stemming from the proximity of both proteins at the cell surface. On the other hand, the surface colocalization could indicate that the hm1 receptor is in contact with caveolae for other receptor functions such as signaling. Chun *et al.* (1994) have reported colocalization of the endothelin receptor with caveolin in the absence of agonist, indicating that this G protein-coupled receptor might in fact be in contact with caveolae to mediate signaling. The same could also be true for the hm1 receptor. More detailed experiments are necessary to investigate this phenomenon.

Receptor-mediated endocytosis could also occur via non-coated vesicles. Raposo *et al.* (1987) have reported that muscarinic receptors internalize into non-coated vesicles in CCL137 cells. The discrepancy between our results and those of Raposo *et al.* could be explained by the differences among cell lines used. Raposo *et al.* examined the distribution of endogenous m1 muscarinic receptors in CCL137 fibroblast cells by electron microscopy. They observed that after 3 hours of agonist treatment, the receptor appears in endosomes on its way to the lysosome for degradation. No down-regulation of hm1 can be

detected in HEK 293 cells (Lameh *et al.*, 1992), and here we have shown that following removal of agonist, hm1 receptors return to the cell surface. Thus, it is possible that since the fate of hm1 is different in these two cell lines, different pathways are functional in its trafficking. Another possible explanation is that the two cell lines express different proteins, possibly G proteins or other proteins involved in internalization, so that a different pathway of endocytosis of hm1 is functional in each cell line. In this study we did not address the possibility of colocalization with non-coated vesicles since we found that by blocking clathrin-mediated internalization, we completely inhibited hm1 receptor internalization. Furthermore, Roettger *et al.* (1995) have suggested that the non-coated vesicles shown to be associated with muscarinic receptors in CCL137 cells in Raposo's study may in fact be caveolae.

From the data obtained in this study, the following main conclusions can be drawn. First, hm1 receptor internalizes into intracellular vesicles after agonist treatment and this process is reversible following removal of carbachol from the medium and inhibited by the antagonist atropine. Second, the results indicate that internalization of hm1 occurs by a clathrin-mediated pathway. Examination of the internalization of other G-protein coupled receptors has yielded evidence of colocalization of these receptors with transferrin (Grady *et al.*, 1995) or the transferrin receptor (von Zastrow and Kobilka, 1992; Fonseca *et al.*, 1995; Garland *et al.*, 1996) and hence inferred the involvement of a clathrin-mediated pathway of endocytosis. Third, the results also suggest that a population of hm1 receptors may be constitutively recycling in the absence of any agonist and that treatment with agonist increases the rate of receptor internalization. Finally, our results do not exclude the interaction of the hm1 receptor with caveolae and the

CHAPTER 2

ANTIBODY TO EPITOPE TAG INDUCES INTERNALIZATION OF HUMAN m1 MUSCARINIC ACETYLCHOLINE RECEPTOR

I. SUMMARY

In this study, we looked at the effect of the antibody against the epitope EYMPME on the internalization of the human muscarinic cholinergic receptor hm1 tagged with the epitope at the N terminus. The antibody to the tag induced internalization of the hm1 receptor within minutes after exposure of HEK 293 cells transfected with the tagged receptor. This antibody-induced internalization was reversible following removal of the antibody. In contrast to hm1 internalization induced by the agonist carbachol, antibody-induced internalization was not blocked by the muscarinic antagonist atropine. The mechanism of antibody-mediated internalization did not appear to involve receptor dimerization by the antibody as Fab fragments derived from the antibody also induced internalization. The pathway of antibody-induced internalization, as with the agonist-induced process, was mediated by clathrin-coated vesicles. Antibody treatment did not result in any second messenger production nor inhibition of carbachol-stimulated second messenger production, as measured by phosphoinositide accumulation. Our data show that internalization of a G protein-coupled receptor can be triggered by interaction of the amino terminus of the receptor with an exogenous ligand and can occur independently of second messenger production. This result suggests that the receptor can exist in multiple conformations, each mediating distinct downstream events.

II. INTRODUCTION

While agonist activation of the G protein-coupled receptor signaling pathway is fairly well understood, the mechanism mediating initiation of GPCR internalization remains unclear. Induction of signaling is thought to occur when agonist occupation of the receptor induces a conformational change in the receptor, allowing for activation of G proteins. Whether or not internalization is a consequence of this conformational change allowing for interaction with proteins of the endocytic machinery, or if events downstream of G protein activation trigger internalization, has yet to be conclusively determined.

There is conflicting evidence as to the requirement for G protein activation of the second messenger system in receptor internalization. Benya *et al.* (1994), studying the effect of two point mutations in regions of the gastrin-releasing peptide (GRP) receptor required for G protein activation, found a correlation between ability of the receptor to internalize and to couple to G proteins. One of the mutants, which was completely defective in phospholipase C activation, displayed a lesser degree of internalization than the wild-type receptor and normal coupling, as measured by the effect of Gpp(NH)p on agonist affinity. The other mutant was completely defective in all three processes. Thus, the authors concluded that G protein coupling, but not activation of phospholipase C, is essential for internalization of the GRP receptor. Similarly, van Koppen *et al.* (1994) identified a deletion mutant of the m4 muscarinic receptor that was defective in internalization and in inhibition of adenylyl cyclase but retained the ability to exhibit high-affinity agonist binding. These investigators, noticing that the reduced rate of mutant receptor internalization was comparable to that of the wild-type receptor in the presence of pertussis toxin, concluded that activation of Gi proteins, but

not coupling to them, was required for m4 receptor internalization. Along similar lines, Thompson *et al.* (1991) have found that the aminosteroid U-73122, which appears to block activation of phospholipase C by Gq, disrupts agonist-induced internalization of the m3 muscarinic receptor. These authors conclude that G protein activation, but not production of phosphoinositide-derived second messengers, is required for receptor internalization.

Other studies suggest that receptor activation of second messenger pathways may be dissociated from receptor internalization. A number of studies using mutated receptors have demonstrated that, while there appears to be a correlation between residues required for coupling to G proteins and receptor down-regulation (Shockley *et al.*, 1997; Campbell *et al.*, 1990), no such relationship exists between coupling and internalization. Multiple mutant receptors have been constructed which are internalized to the same extent as wild-type receptors but are completely defective in promoting a second messenger response. These include both mutants of receptors which preferentially couple to adenylyl cyclase (Campbell *et al.*, 1990; Cheung *et al.*, 1990) and those of receptors coupled to phospholipase C (Shockley *et al.*, 1997; Conchon *et al.*, 1994; Hunyady *et al.*, 1994). Furthermore, Slowiejko *et al.* (1994) have demonstrated that internalization of the m3 muscarinic receptor occurs under conditions in which stimulation of phosphoinositide hydrolysis is blocked. These authors maintain, however, that a guanine nucleotide-binding protein may be required, based on the observation that internalization was blocked in the presence of GDPβS. Negating the requirement for activation of heterotrimeric GTP-binding proteins in GPCR internalization is the occurrence of receptor internalization in cells devoid of functional G proteins. It has been shown for the yeast α factor receptor, which belongs to the G protein-coupled receptor family, that internalization occurs both in cells in

which signal transduction is blocked by a mutation in the G protein β subunit, and in diploid cells, which do not express the pheromone-specific G protein at all (Zanolari *et al.*, 1992). Similarly, β-adrenergic receptors are fully able to undergo internalization in S49 lymphoma cells which either lack the α subunit of Gs (cyc^-) (Mahan *et al.*, 1985) or which possess a defective Gs (UNC) (Clark *et al.*, 1985). In further support of the independence of internalization from G protein activation is the observation that an antagonist of the cholecystokinin receptor induces internalization of this receptor but does not affect G protein coupling in the presence of Gpp(NH)p (Roettger *et al.*, 1996). Thus, it remains to be determined if G protein activation, of either a receptor's cognate G protein or another G protein, is required to signal receptor internalization. This chapter describes the internalization of the epitope-tagged m1 muscarinic receptor that we found to occur in response to treatment with the antibody against the epitope tag. I have characterized this internalization event with respect to site of action, pathway of endocytosis, and second messenger stimulation. These results provide further evidence that internalization can occur independently of second messenger production.

III. EXPERIMENTAL PROCEDURES

A. Materials

An antibody fragmentation kit was obtained from Pierce Co. (Rockford, IL). Cy5-conjugated goat anti-mouse IgG antibodies and Cy3 monoclonal antibody labeling kit were purchased from Amersham Corp. (Arlington Heights, IL). All other materials were identical to those used in the previous chapter.

B. Stable Expression of Epitope-Tagged hm1 Receptor

The stable cell line expressing the human muscarinic cholinergic subtype 1 (hm1) receptor with the N-terminal epitope EYMPME described in the previous chapter was used for these studies.

C. Receptor Binding Assay

Cells were seeded onto 12-well dishes, allowed to attach overnight, and then treated for specified times with 1 mM carbachol, anti-epitope antibody (1:500 or 1:1000 dilution), or buffer in incomplete medium at 37°C. Cells were placed on ice, washed three times with ice-cold PBS, and incubated with 2.0 nM [^{3}H]-N-methyl-scopolamine (NMS) at 12°C for 90 minutes. After labeling, cells were placed on ice, harvested with PBS and filtered on glass fiber filters (S&S #32), followed by three rinses with ice-cold PBS. The radioactivity on the filters was determined by scintillation counting.

D. Immunofluorescence Confocal Microscopy

Immunofluorescence assays were carried out as described in the previous chapter. Cells were treated at 37°C with 1 mM carbachol or anti-epitope antibody (1:500 or 1:1000 dilution) in serum-free medium prior to fixation. For colocalization studies, the anti-EE antibody was directly conjugated to Cy3. In these studies, clathrin was labeled first with monoclonal antibody to clathrin heavy chain followed by Cy5-conjugated goat anti-mouse secondary antibody. Then hm1 was labeled with Cy3-labeled anti-EE antibody.

E. Inhibition of Caveolae- and Clathrin-mediated Endocytosis

Cells grown on chamber slides were pretreated with either 5 mM acetic acid in HEPES (pH 5.0) for 5 minutes (Sandvig *et al.*, 1987) or 1 μM phorbol-12-

myristate-13-acetate (PMA) for 30 minutes (Smart *et al.*, 1994) at 37°C. Following pretreatment, 1 mM carbachol, 1:1000 anti-epitope antibody, or buffer was added and cells were incubated for an additional 30 minutes at 37°C.

F. Phosphatidyl Inositol (PI) Hydrolysis

PI turnover was measured as described previously (Moro et al., 1993a). Briefly, cells expressing EE-hm1 were grown in 6-well culture dishes, incubated for 24 hours with [^{3}H]-myoinositol, and then assayed for inositol monophosphate, which accounts for most of the [^{3}H] activity in the presence of 10 mM LiCl. Each assay was conducted in triplicate.

IV. RESULTS

A. Antibody-induced Internalization of hm1 Receptor Detected by Confocal Microscopy

To study receptor internalization using immunofluorescence, an epitope tag with sequence EYMPME was added by PCR to the amino terminus of the hm1 receptor (Arden and Lameh, 1996) (Fig. 2.1). This epitope is recognized by a commercially-available antibody (referred to hereafter as anti-EE) that works well for immunofluorescence studies (Arden and Lameh, 1996; Tolbert and Lameh, 1996). We showed previously that carbachol, a muscarinic agonist, induces a redistribution of hm1 receptor from the cell surface into intracellular vesicles and characterized the pathway for hm1 internalization in HEK cells (Tolbert and Lameh, 1996; see Chapter 1). Initial immunofluorescence studies designed to study redistribution of the hm1 receptor in response to carbachol revealed that treatment of cells with the anti-EE antibody alone prior to fixation also triggers the internalization process. As shown in Figure 2.2a, in the absence of any treatment, the

localization of the receptors as labeled with the anti-EE antibody after fixation was predominantly at the cell surface. When the cells were treated with a 1:1000 dilution of the anti-EE antibody in serum-free medium for 30 minutes at 37°C prior to fixation, the receptors redistributed into intracellular compartments, presumably endosomes (Fig. 2.2b). The intracellular distribution of hm1 receptor after treatment with antibody is comparable to the distribution following carbachol treatment (Fig. 2.2c). Receptor internalization following treatment with carbachol and anti-EE antibody was quantitated with binding studies using the polar tracer, [^{3}H]-N-methylscopolamine (Fig. 2.3). Antibody did not block [^{3}H]-NMS binding (data not shown). When the medium containing antibody was removed and the cells were allowed to recover in antibody-free medium, the hm1 receptor was no longer apparent in intracellular vesicles, indicating that the receptors are recycled back to the cell surface (Fig. 2.2d). Thus, antibody-induced internalization of hm1 receptors is a reversible process.

B. Observation of Antibody-Induced Internalization with an Antibody to C-terminus of hm1 Receptor

To further demonstrate the localization of hm1 receptor following anti-EE antibody treatment, we used a polyclonal antibody against a region in the carboxy terminus of the hm1 receptor to label the receptor. As shown in Figure 2.4, this antibody labels receptors at the cell surface in the absence of any treatment (Fig. 2.4a) and intracellular vesicles following treatment with 1 mM carbachol (Fig 2.4b). We have confirmed that the two antibodies recognize the same antigen by performing double-labeling studies using the anti-EE and anti-carboxy terminus antibodies simultaneously with appropriate secondary antibodies. The staining of hm1 receptors by the two antibodies completely colocalize both in the absence and in the presence of 1 mM carbachol (data not

shown). This result with antibodies against the N- and C-terminal domains indicates that the tagged-m1 receptor protein remains intact after internalization. When the carboxy-terminal antibody was used to label receptors following treatment with the anti-EE antibody, the receptors were again localized to intracellular compartments (Fig. 2.4c), demonstrating that the antibody-induced internalization of the hm1 receptor is a real effect and that the signal in intracellular vesicles is not merely due to the internalized antibody alone or antibody attached to a cleaved epitope fragment.

To confirm that the anti-EE antibody was interacting exclusively with the epitope tag to induce hm1 receptor internalization, we studied the effect of the anti-EE antibody on the untagged receptor using the antibody to the carboxy terminus of the receptor to label the receptors after treatment with the anti-EE antibody. As expected, the anti-EE antibody had no effect on the cell surface distribution of the untagged receptor (data not shown). Thus, the anti-EE antibody appears to act by binding the N-terminal epitope specifically as opposed to other regions of the receptor or other proteins. Atropine, a muscarinic antagonist which blocks both agonist-induced second messenger stimulation and internalization, had no effect on the receptor redistribution in response to the anti-EE antibody (Fig. 2.2f) in contrast to the complete inhibition of carbachol-induced internalization (Fig. 2.2g). This result supports the idea that the anti-EE antibody is acting at a region of the receptor distinct from the agonist/antagonist binding site(s).

Receptor dimerization has been shown to induce internalization of two single transmembrane receptors: the human growth hormone receptor (Saito *et al.*, 1994) and the $p185^{HER2}$ receptor (Srinivas *et al.*, 1993). To see if the anti-EE antibody was acting by dimerizing the hm1 receptors, we studied the effect of Fab fragments of the anti-EE antibody on the distribution of hm1

receptors. Fab fragments were produced using an antibody fragmentation kit containing immobilized papain. As shown in Figure 2.2e, Fab fragments from the anti-EE antibody also resulted in a redistribution of hm1 receptors into intracellular compartments. These intracellular vesicles after treatment with the Fab fragments appear to be larger in size than those after treatment with either carbachol or intact antibody. The size difference may be due to a greater extent of endosome fusion following treatment with Fab fragments, or to endosome aggregation, although the means by which the Fab fragments would mediate such events are as yet unclear. The fact that the Fab fragments also induced internalization of hm1 indicates that the anti-EE antibody is not acting by dimerizing the hm1 receptors at the plasma membrane.

C. Pathway of Antibody-Induced Internalization by Inhibition Studies and Double-Labeling Studies

Because the site on the hm1 receptor at which the anti-EE antibody acts to induce internalization differs from that of carbachol, we were interested in determining if the pathways of endocytosis following treatment with the two agents were the same. We demonstrated previously that the hm1 receptor is internalized by clathrin-coated vesicles after carbachol treatment (Tolbert and Lameh, 1996; see Chapter 1). We performed similar studies to determine if the pathway of internalization after treatment with anti-EE antibody is also clathrin-mediated. Cells were pretreated with either PMA to inhibit caveolae formation (Smart *et al.*, 1994) or acetic acid (Sandvig *et al.*, 1987) to inhibit internalization by clathrin-coated vesicles, followed by treatment with anti-EE antibody (1:1000) in serum-free medium. The confocal images show that acetic acid, but not PMA, blocks the internalization induced by anti-EE antibody (Fig. 2.5a,b) -- a result identical to that seen after carbachol treatment (Fig 2.5c,d), and indicative of a clathrin-mediated endocytic pathway.

To confirm that clathrin is in fact involved in antibody-induced hm1 receptor internalization, we performed double-labeling experiments with anti-clathrin antibody and a Cy3-conjugated anti-EE antibody. Cells were treated for 20 minutes with anti-EE antibody, then fixed and sequentially labeled with monoclonal anti-clathrin primary antibody, Cy5-conjugated goat anti-mouse secondary antibody, and Cy3-conjugated anti-EE antibody. The results show a significant degree of colocalization, represented by the yellow color in the merged image, following antibody treatment (Fig. 2.6), indicating that the vesicles into which the hm1 receptor is internalized after treatment with anti-EE antibody are indeed clathrin-coated. Thus, as for agonist-induced internalization, antibody-induced internalization occurs via clathrin-coated vesicles.

D. Measurement of Second Messenger Stimulation Following Antibody Treatment

To determine if the anti-EE antibody was capable of activating the second messenger system, the accumulation of phosphoinositides resulting from the breakdown of 1-phospho-4,5-bis-phosphate (PIP_2) in response to anti-EE treatment was measured. Following treatment with 1 mM carbachol for 30 minutes, a 5-fold stimulation above the basal level was measured, but after a 30-minute treatment with 1:100 anti-EE antibody, 10 times the concentration sufficient to induce internalization, no stimulation of PI hydrolysis was observed. Additionally, anti-EE antibody at a 1:100 dilution had no effect on carbachol-stimulated inositol monophosphate (IP_1) production (Fig. 2.7). The absence of second messenger stimulation provides further evidence in addition to that of previous investigations (Thompson *et al.*, 1991; Hunyady *et*

al., 1994; Shockley *et al.*, 1997) to support the conclusion that production of phosphoinositides is not required for internalization of Gq-linked receptors.

E. Antibody-Induced Internalization of a Mutant hm1 Receptor Defective in Agonist-induced Signaling and Internalization

To further investigate the relationship between internalization and signaling activities, we studied the effect of anti-EE antibody on the cellular localization and second messenger stimulation of a mutant receptor V127A/L131A previously characterized to be completely defective in both agonist-induced internalization and second messenger production (Moro et al., 1993a; Moro et al., 1994; Arden and Lameh, 1996). As shown in Fig. 2.8, anti-EE induces a redistribution of the mutant receptor which is similar to that of the wild-type receptor, whereas carbachol has no effect on the cell surface distribution of the mutant receptor. As for the wild-type hm1 receptor, anti-EE Fab fragments also induce internalization of the mutant receptor (Fig. 2.8d). Treatment of the mutant receptor with 1:50 dilution of the anti-EE antibody did not induce stimulation of PI hydrolysis over the basal level (data not shown). The ability of this mutant to internalize in the presence of antibody suggests that different structural or conformational motifs may be required for internalization and signaling activity.

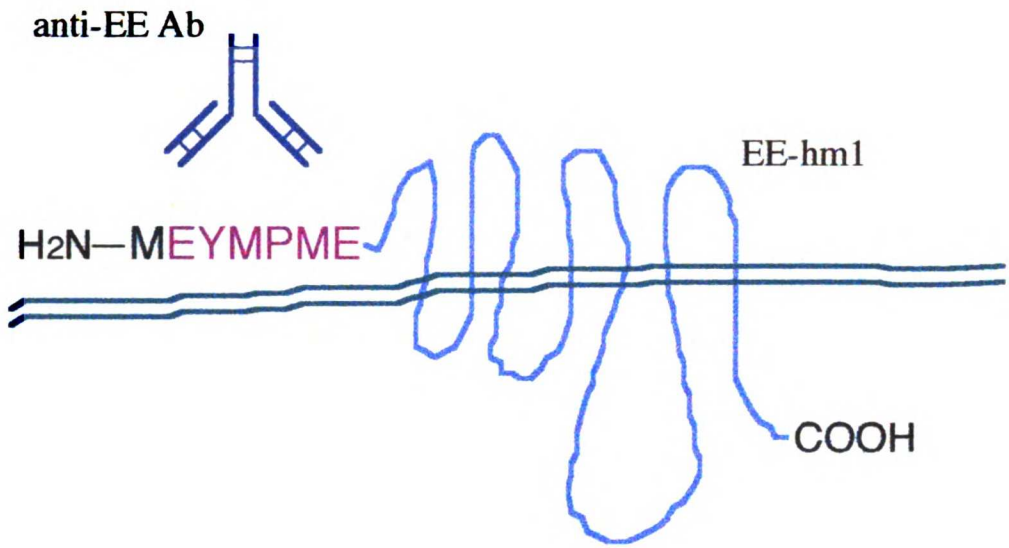


Figure 2.1. Location of the epitope tag. The epitope EYMPME was added by PCR to the N terminus of the hm1 receptor, C-terminal to the starting methionine.

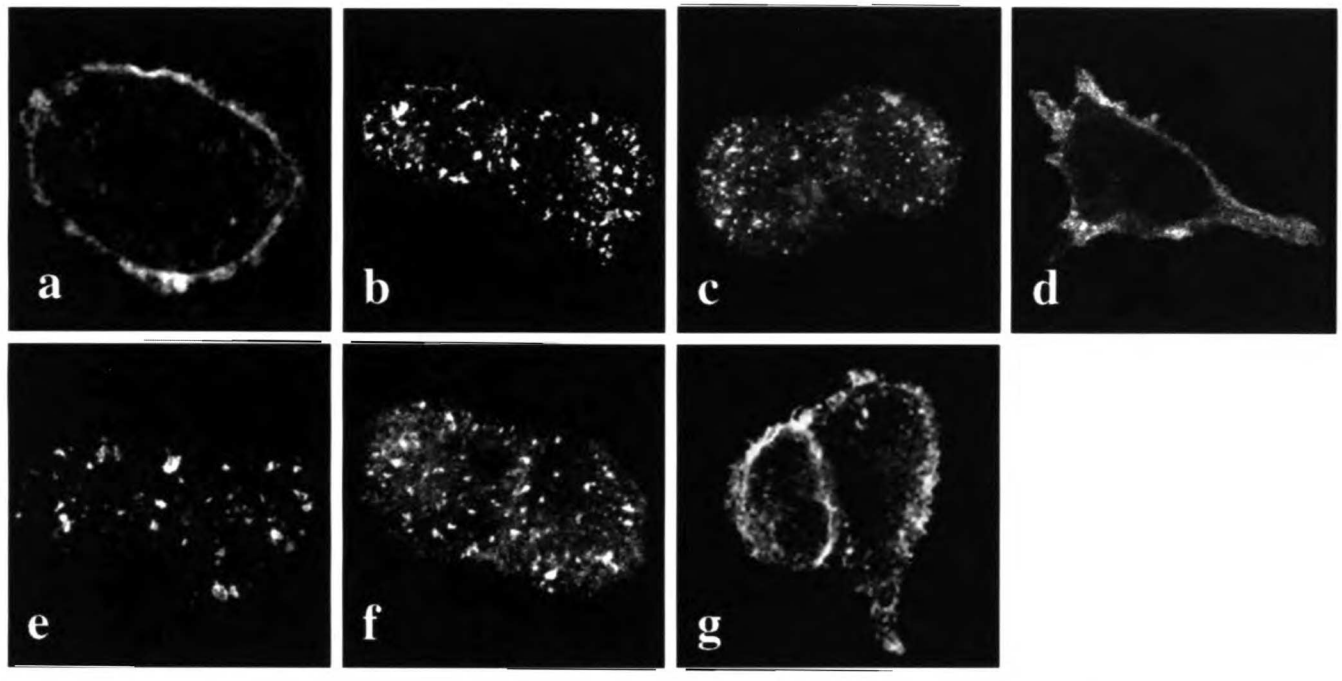


Figure 2.2. Effect of anti-EE antibody on cellular localization of hm1 receptor. Human embryonic kidney (HEK 293) cells stably transfected with EE-tagged hm1 receptor were grown on chamber slides overnight and treated for 20 minutes with indicated agent(s) at 37°C prior to fixation and addition of primary and secondary antibodies. Recycling of the receptor back to the cell surface was determined after treating the cells with 1:500 dilution of anti-EE antibody (final protein concentration approximately 20 μg/mL) for 20 minutes at 37°C, removing the medium, and washing the cells four times with PBS. The cells were then incubated in fresh medium at 37°C for one hour prior to fixation to allow for recycling of the receptor. (a) no treatment, (b) 1:500 anti-EE Ab, (c) 1 mM carbachol, (d) 1:500 anti-EE Ab followed by washing and one hour recycling, (e) 1:20 anti-EE Fab fragments (final protein concentration approximately 10 μg/mL), (f) 10 μM atropine + anti-EE Ab, (g) 10 μ M atropine + 1 mM carbachol.

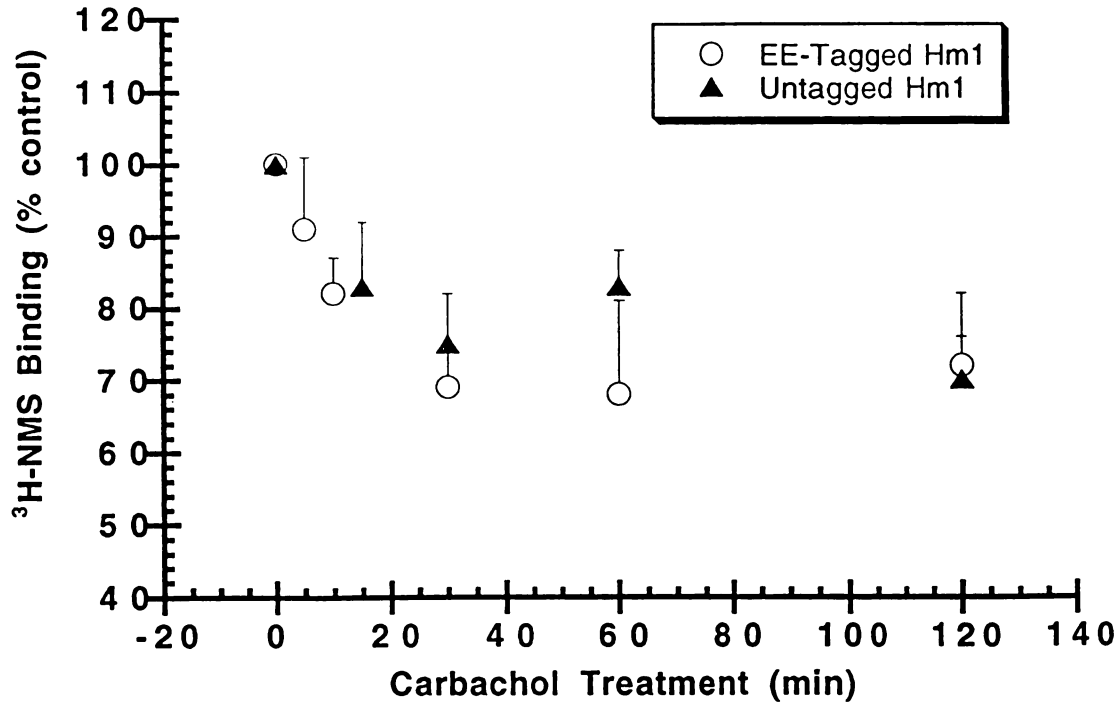


Figure 2.3. [^{3}H]-NMS binding profile of EE-hm1 receptors after treatment with 1 mM carbachol and 1:500 anti-EE Ab. HEK 293 cells expressing EE-tagged or untagged hm1 receptors were seeded onto 12-well cell culture dishes, allowed to attach overnight, and treated with 1 mM carbachol or 1:500 anti-EE Ab for varying times. After washing with ice-cold PBS, cell surface receptor binding was assessed using the hydrophilic ligand [^{3}H]-NMS. Each point is average of quadruple points. Error bars represent standard deviation. The curve is representative of three independent experiments. Open circles: treatment with anti-EE Ab. Filled circles: treatment with 1 mM carbachol.

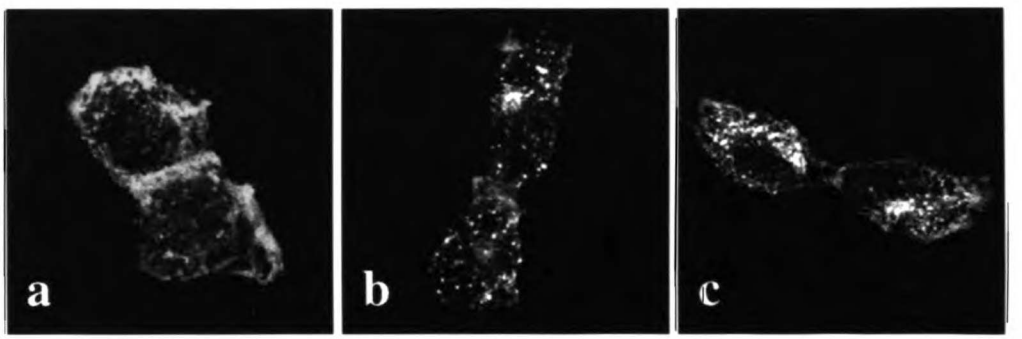


Figure 2.4. Cellular localization of hm1 receptor after treatment with carbachol and anti-EE antibody using antibody to the hm1 carboxy terminus. HEK cells expressing EE-hm1 receptor were grown on chamber slides overnight and treated for 20 minutes with the indicated agent at 37°C prior to fixation and addition of primary and secondary antibodies. The primary antibody used in this figure is directed toward a sequence at the carboxy terminus of the hm1 receptor. (a) no treatment, (b) 1 mM carbachol, (c) 1:500 anti-EE Ab.

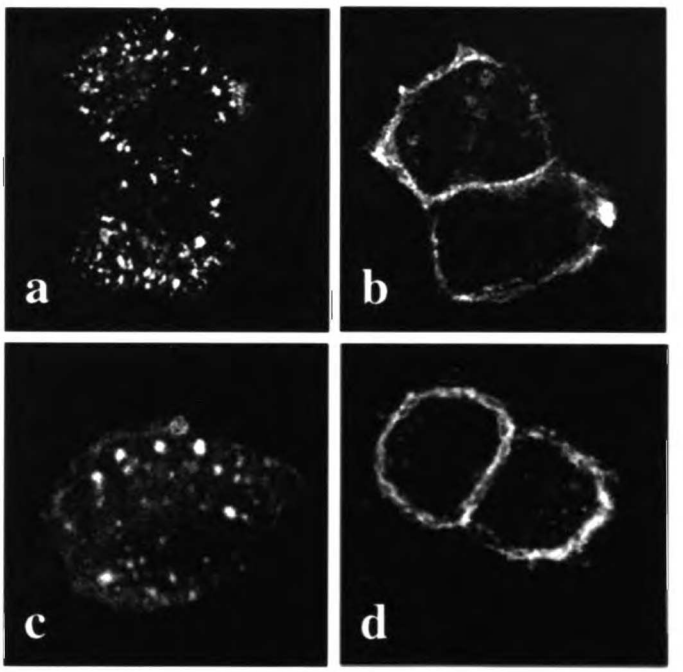


Figure 2.5. Effect of biochemical treatments on anti-EE antibody-induced internalization of EE-hm1. HEK cells expressing EE-hm1 receptor were grown on chamber slides overnight and treated at 37°C with either 5 mM acetic acid for 5 minutes (b,d) or 1 μM PMA for 30 minutes (a,c) prior to addition of 1:1000 anti-EE antibody (a,b) or 1 mM carbachol (c,d) to the medium and a further incubation for 30 minutes.

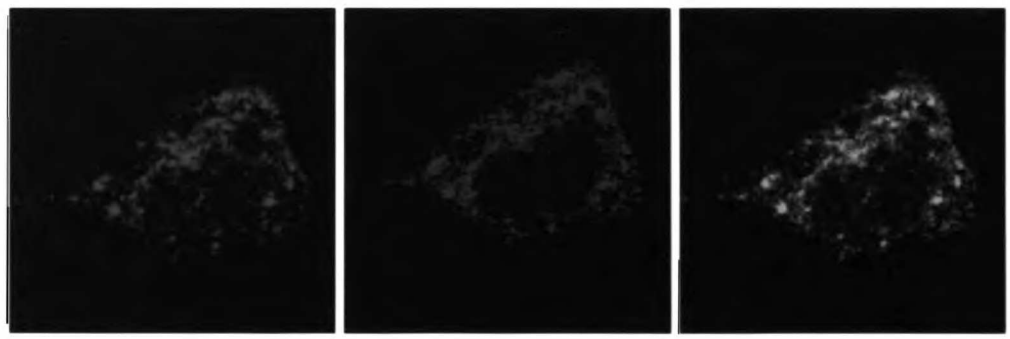


Figure 2.6. Colocalization of EE-hm1 with clathrin after treatment with anti-EE antibody. HEK cells expressing EE-hm1 were treated with 1:500 anti-EE antibody for 15 minutes at 37°C. After fixing and permeabilizing, cells were sequentially labeled with monoclonal antibody to clathrin followed by Cy3-labeled goat anti-mouse secondary antibody. Then hm1 was labeled with Cy5-labeled anti-EE antibody. The green color represents the localization of clathrin, red is the localization of hm1, and yellow is indicative of colocalization of the two proteins in the merged image.

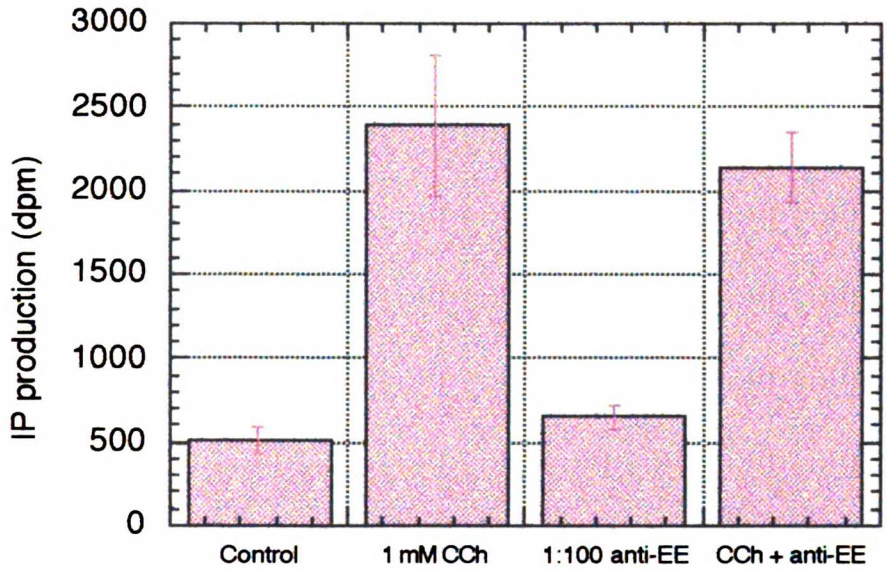


Figure 2.7. Phosphatidylinositol accumulation after carbachol and anti-EE antibody treatment. Cells expressing EE-hm1 receptor were seeded in 6-well dishes and incubated in the presence of 0.2 μM [^{3}H]*myo*-inositol overnight. Radioactive medium was replaced with fresh medium, and the cells were incubated for 15 minutes in 10 mM LiCl. Then cells were stimulated with buffer alone, 1 mM carbachol, 1:100 anti-EE antibody, or carbachol + antibody. Data is expressed as total IP eluted from the column. Assays were done in triplicate. Error bars represent standard deviation.

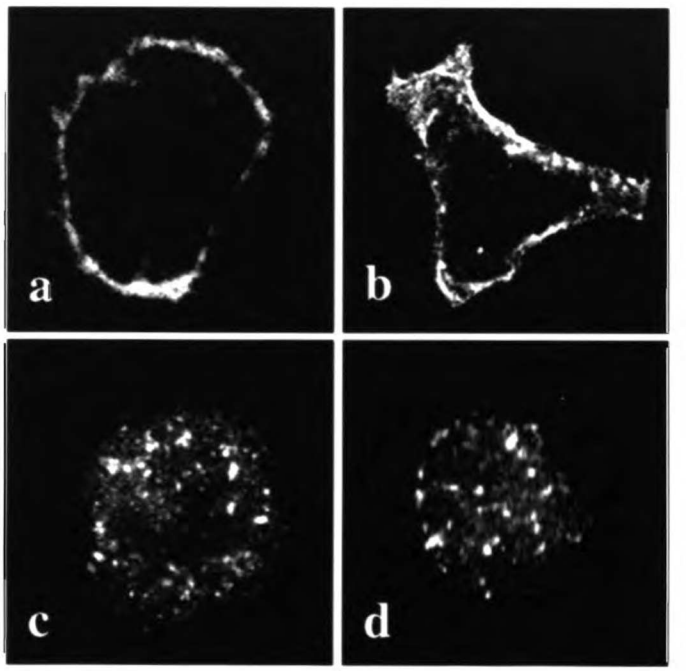


Figure 2.8. Effect of anti-EE antibody on cellular localization of hm1-V127A/L131A mutant receptor. HEK cells expressing EE-hm1-V127A/L131A mutant receptor were grown on chamber slides overnight and treated for 30 minutes with indicated agent(s) at 37°C prior to fixation and addition of primary and secondary antibodies. (a) no treatment, (b) 1 mM carbachol, (c) 1:500 anti-EE Ab, (d) 1:20 anti-EE Fab fragments.

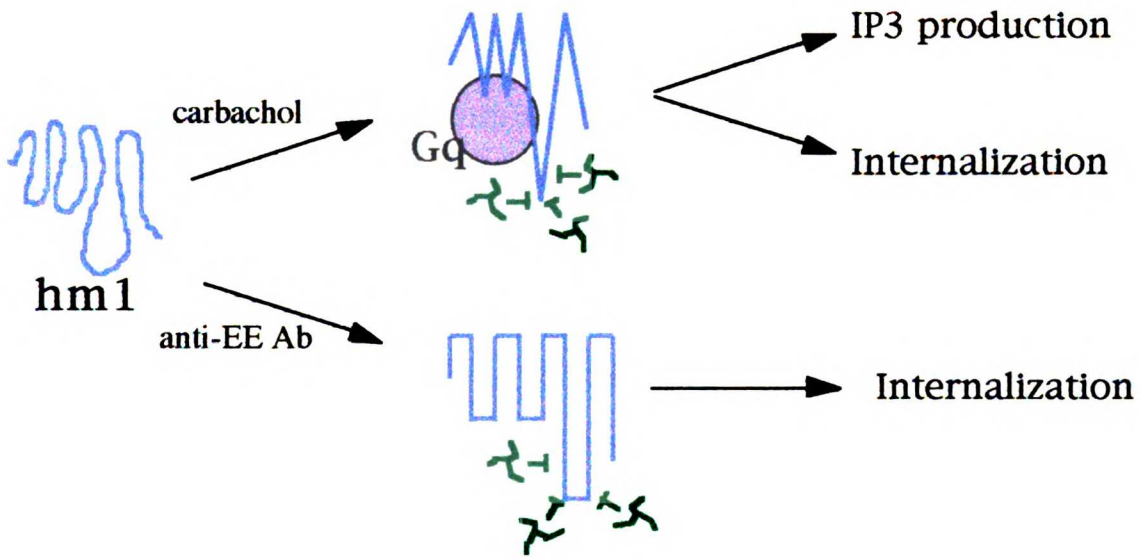


Figure 2.9. Schematic of possible conformational changes triggered by interaction of the m1 receptor with carbachol or the antibody to the epitope tag. While both the muscarinic agonist carbachol and the antibody to the N-terminal epitope cause internalization of the tagged m1 receptor, only carbachol can mediate second messenger activation. A possible explanation for the different actions of these two agents is that they induce different conformations of the receptor such that the conformation of the carbachol-bound receptor can interact both with Gq to mediate signaling and with proteins necessary for internalization. Antibody binding, however, induces a receptor conformation that only allows for interaction with the endocytic machinery, and not with Gq.

V. DISCUSSION

The observation that an agent other than a typical muscarinic agonist is capable of triggering the internalization of an epitope-tagged hm1 receptor may offer some insight into the mechanism by which G protein-coupled receptors are internalized. It is clear that the antibody interacts with the receptor at regions distinct from those sites involved in binding carbachol. First, antibody against the non-native N-terminal epitope causes internalization only of the tagged receptor and has no effect on the cell surface distribution of the untagged receptor, whereas carbachol induces internalization of both untagged and tagged receptors equally. Second, the muscarinic antagonist atropine has no effect on antibody-induced internalization, and conversely, antibody does not block [^{3}H] N-methyl-scopolamine binding. We have demonstrated that the pathway of internalization induced after antibody is the same as that resulting after carbachol treatment, which we have previously shown to occur via clathrin-coated vesicles. Thus, the primary difference between the modes of action of the two agents lies in the means by which carbachol or anti-EE antibody interacts with the receptor to induce internalization.

The differences in the way the agonist and antibody act to stimulate endocytosis may shed some light into the means by which internalization occurs for the hm1 receptor in particular, and for G protein-coupled receptors in general. We have shown that anti-EE antibody does not activate the Gq/11 second messenger system, demonstrating that second messenger production is not required for internalization to occur. A similar phenomenon has been reported for a hemagglutinin (HA)-tagged thyrotropin-releasing hormone (TRHR) receptor, a receptor which also couples to Gq (Petrou *et al.*, 1997). This receptor, also tagged at the amino terminus, behaves similarly to the EE-tagged

m1 receptor in response to treatment with antibody against the epitope tag. Like anti-EE antibody treatment of the EE-tagged m1 receptor, the monoclonal antibody against the HA epitope induces HA-TRHR internalization, but not inositol phosphate production, and does not inhibit agonist-induced IP production. Thus it is apparent that second messenger stimulation is not required for GPCR internalization, a conclusion consistent with that of previous investigations demonstrating internalization in the absence of second messenger production (Thompson *et al.*, 1991; Slowiejko *et al.*, 1994; Roettger *et al.*, 1997). An attractive explanation is that a conformational change in the receptor may expose a region of the receptor involved in interactions with proteins of clathrin-coated pits, a prime candidate being a subunit of the AP2 complex (see Fig. 2.9). The agonist carbachol may induce a conformational change that allows for interactions both with G proteins and with clathrin-coated pits. Anti-EE antibody, on the other hand, by binding to the N-terminal epitope, may cause a conformational change that allows only for recognition by a protein(s) associated with clathrin-coated vesicles. In this case, the anti-EE antibody binds to a site distinct from the agonist-binding site and induces only one event in the receptor, namely receptor internalization, without any second messenger activation. Antibody-induced internalization of the V127A/L131A double point mutant reveals that these two residues are not essential for interaction with proteins of the endocytic machinery, but rather that the mutation disrupts the ability of the agonist to interact with the receptor in such a way as to induce a conformation necessary for internalization. Thus, different structural motifs may be required for receptor internalization and coupling. A related phenomenon has been demonstrated for the μ and δ opioid receptors (Arden *et al.*, 1995; Keith *et al.*, 1996) where the peptide agonists DAMGO and DADLE, respectively,

or the alkaloid drug etorphine induce both receptor activation and internalization while morphine, another alkaloid agonist, stimulates only receptor activation and not internalization. In this case, the situation is reversed in that each of the ligands activates the receptor, but one agonist is not capable of mediating internalization. In a collaborative project with Neil Burford, I further investigated the correlation between μ receptor internalization and G protein coupling (Burford *et al.,* 1998). Here we have shown that morphine, DAMGO, and a newly identified opioid agonist, endomorphin I, activate the same set of G proteins to similar extents, but wheras DAMGO and endomorphin I cause μ receptor internalization, morphine has no effect on the distribution of the receptor. These studies indicate that the different opioid agonists may cause different conformations of the receptor, each of which cause similar signaling activity but different trafficking patterns. It is possible that more than one receptor conformation can result in the internalization of the receptor, at least one of which also mediates signal transduction. However, we have shown that for the hm1 receptor that a conformation does exist that signals internalization without G protein coupling. The possibility that G protein-coupled receptors can exist in multiple conformations has been elaborated on in separate reviews by Tucek (1997) and Bourne (1997). Tucek has proposed the existence of multiple conformational states based largely on investigations of the interaction of receptors with allosteric modulators. He cites several studies finding that association of a receptor with different agonists and antagonists can have differential effects on the affinity of the receptor for a given allosteric modulator. Different muscarinic agonists can also cause different affinities of these receptors for G proteins. Tucek has found that some allosteric modulators of muscarinic receptors have agonist-like effects that are resistant

to inhibition by antagonists. Furthermore, he notes that these allosteric compounds with agonist-like activity decrease the affinity of muscarinic receptors for acetylcholine. Thus, he concludes that the active conformation of the muscarinic receptor bound to the allosteric ligand must be different from that of the acetylcholine-bound conformation. Bourne similarly proposes that receptors can assume "plastic conformations" from the perspective of G protein activation. In support of his hypothesis, he cites studies where chimeras of two different receptors were constructed which activated G proteins that neither parent could activate. This idea would serve to explain the frequent lack of any apparent primary sequence homology among receptors that couple to the same G proteins. Thus, our observation that interaction of an antibody with the N-terminus of the tagged m1 receptor can initiate internalization but not signaling adds to the accumulating evidence in favor of a fluid model of receptor structure over the previously accepted two-state R/R* model.

CHAPTER 3

MUTATION OF A PUTATIVE PHOSPHORYLATION DOMAIN IN THE THIRD INTRACELLULAR LOOP DIFFERENTIALLY AFFECTS THE REGULATION OF MUSCARINIC ACETYLCHOLINE m1 AND m3 RECEPTORS

I. SUMMARY

The large third intracellular (i3) loops of the muscarinic m1 and m3 receptors contain multiple serine and threonine residues representing putative phosphorylation sites that may play a role in receptor regulation. A motif consisting of several serine and threonine residues flanked N-terminally by acidic residues occurs in the center of the i3 loops of both m1 and m3 receptors ($S^{287}LTSS^{291}$ and $S^{349}ASS^{352}$, respectively). In this study, we examined the role of these receptor domains in modulating agonist-induced desensitization and receptor trafficking, and for the m3 receptor, we assessed the contribution of phosphorylation of this domain to receptor regulation. Contrary to prediction, mutation of serines and threonines in this putative phosphorylation sequence did not affect desensitization of carbachol-induced phosphoinositide hydrolysis for either the m1 or m3 receptor, and furthermore, did not alter the agonist-induced phosphorylation state of m3. However, this mutation differentially affected agonist-induced trafficking for the two receptors. Mutation of the SASS region of the m3 receptor to AAAA completely abrogated receptor internalization and subsequently, down-regulation. Mutation of the analogous domain in the m1 receptor sequence (m1-SLTSS/ALAAA), however, had no apparent effect on internalization. Thus, the serine-rich region postulated to play a role in GPCR regulation, specifically modulates m3 receptor internalization, but is not crucial to the regulation of the m1 receptor.

II. INTRODUCTION

The activity of G protein-coupled receptors is regulated by a number of processes, including desensitization, internalization, and down-regulation. Desensitization occurs rapidly following agonist stimulation (within seconds) and correlates with phosphorylation of serine and threonine residues in the third intracellular loop and/or carboxy terminus of the receptor (reviewed in Chuang *et al.*, 1996; Lefkowitz *et al.*, 1998; Krupnick and Benovic, 1998). This process has been best characterized for the β_2-adrenergic receptor, for which phosphorylation by both PKA and βARK (GRK2) contributes to uncoupling from Gs. Whereas PKA phosphorylation directly uncouples the receptor from its G protein, βARK phosphorylation increases the affinity of the receptor for a member of the arrestin family, the binding to which blocks G protein-receptor interaction. The consensus sites for βARK phosphorylation have not yet been definitively determined. It appears that βARK preferentially phosphorylates serine residues that are preceded N-terminally by acidic residues (Onorato, 1991). Fredericks *et al.* (1996) have identified three serines and one threonine within a 40-amino acid sequence in the carboxy terminus of the β_2-adrenergic receptor that are phosphorylated by βARK *in vitro*. Sites for βARK phosphorylation of the α_{2a}-adrenergic receptor are found within its third intracellular loop. Eason *et al.* (1994) have shown that phosphorylation of this receptor occurs on each of four sequential serines in the sequence EESSSS in the third intracellular loop. While similar sites exist in a number of other receptors, including receptors which have been shown to be substrates for βARK (Haga *et al.*, 1996; Richardson *et al*, 1993; DebBurman *et al.*, 1995), it remains to be determined whether or not these sites are indeed the sites phosphorylated by βARK *in vivo*.

Studies from our laboratory in previous years point to a correlation between putative sites for βARK phosphorylation and sites required for receptor internalization. Osamu Moro constructed mutants of the m1, m2, and m3 receptors with serines and threonines in analogous sites of the third intracellular loop replaced by alanine. This work demonstrated that these residues are required for m1 and m3 internalization (Moro *et al.,* 1993). For the m2 receptor, which has two such serine- and threonine-rich sites, mutation of the N-terminal site in the third intracellular loop had no effect on internalization, while replacement of serines and threonines in the C-terminal portion of the third intracellular loop partially inhibited internalization (Moro *et al.,* 1993). Pals-Rylaarsdam and Hosey (1997) further characterized the effect of these mutations on m2 receptor function. They found that the presence of either one of the clusters was sufficient for wild-type levels of phosphorylation and internalization, but simultaneous mutation of both clusters abolished phosphorylation and impaired internalization. Mutation of threonine residues in the carboxy terminus of the m3 receptor has also been found to disrupt internalization, suggesting that regulatory sites for this muscarinic subtype are located in both the third intracellular loop and carboxy terminus (Yang *et al.,* 1995). Similarly, serine- and threonine-rich sequences in the carboxy terminus have been found to be required for the internalization of the thrombin, δ-opioid, and cholecystokinin receptors (Shapiro *et al.,* 1996; Trapaidze *et al.,* 1996; Pohl, *et al.,* 1997), although in each of these cases, all serine and threonine residues were mutated rather than only those resembling putative sites for βARK phosphorylation.

The correlation between potential sites for βARK phosphorylation and sites required for internalization can be explained by recent studies demonstrating that β-arrestin, by binding to βARK-phosphorylated residues on

the C terminus of the β_2-adrenergic receptor, not only mediates homologous desensitization, but can also act as an adaptor to target these receptors to clathrin-coated vesicles (Goodman *et al.*, 1996). Consistent with this idea are observations that overexpression of β-arrestin increases the rate and extent of m2 receptor internalization in JEG-3 cells (Schlador and Nathason, 1997), and expression of a dominant-negative β-arrestin mutant blocked the internalization of β_2-adrenergic receptors (Ferguson *et al.*, 1996).

This chapter describes a collaborative project I worked on with a former graduate student in the laboratory, Melinda Shockley, characterizing the role of parallel serine- and threonine-rich sequences in the third intracellular loops of the m1 and m3 muscarinic acetylcholine receptors in regulatory events following agonist activation These sequences, ESLTSSE for the m1 receptor and ENSASS for m3, share a high degree of homology to sequences shown to be substrates for βARK (Eason *et al.*, 1995; Haga *et al.*, 1996), and thus may serve an important regulatory function in the activity of these receptors. Here we further evaluate the effect of serine/threonine to alanine mutations of the m1 and m3 receptors constructed previously (Moro *et al.*, 1993), with respect to internalization, down-regulation, desensitization, and for the m3 receptor, phosphorylation.

III. EXPERIMENTAL PROCEDURES

A. Materials

[^{3}H]-NMS (specific activity 85 Ci/mmol) and [^{3}H]-QNB (specific activity 47 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Carbachol was purchased from Sigma Chemical Co. (St. Louis, MO). 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 mutants m1-SLTSS/ALAAA and m3-SASS/AAAA was previously described by Moro *et al.* (1993).

C. Stable Expression of m1 and m3 Wild-type and Mutant Receptors

Chinese hamster ovary (CHO) cells were transfected by Melinda Shockley using the calcium phosphate precipitation method (Maeda *et al.*, 1990) with pSG5 vector containing the wild type or mutant mAChR 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 Assay

Cells were seeded onto 12-well dishes, allowed to attach overnight, and then incubated for 90 minutes at 12°C with 2 nM [^{3}H]-QNB in PBS to measure total sites or with 2 nM [^{3}H]-NMS in PBS to measure surface sites. Non-specific binding was determined in the presence of 10 μM atropine. After labeling, cells were placed on ice, harvested with PBS, and filtered (S&S #32 glass fiber filter), followed by three rinses with ice-cold PBS. The radioactivity on the filters was determined by scintillation counting. Percent binding values were compared between carbachol-treated and -untreated cells. Data for the time

courses presented are the averages of triplicate measurements from a representative experiment (repeated 2-3 times), and the error bars represent standard deviation.

E. Immunofluorescence Confocal Microscopy

To visualize m1 and m3 receptors, carbachol-treated cells were washed with PBS, fixed for 10 minutes at room temperature with 3.7% paraformaldehyde in PBS, and then simultaneously blocked and permeabilized in PBS containing 0.25% fish gelatin, 0.04% saponin, and 0.05% NaN_3. After permeabilization, receptors were labeled by incubation of cells with anti-m1 AChR polyclonal antibody or anti-m3 AChR polyclonal antibody (both from Dr. Andrew Tobin) followed by incubation with Cy3-conjugated goat anti-rabbit polyclonal antibody. For colocalization studies, cells were next 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. Slides were mounted and visualized as described in Chapter 1.

F. Desensitization of Carbachol-Induced Inositol Phosphate Release

Cells stably expressing wild-type or mutant m1 and m3 AChRs 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 for 24-48 hours. Following inositol labeling, cells were incubated in incomplete medium with or without carbachol (1 mM) for two hours. Pretreated cells were washed three times with PBS and then rechallenged with 1 mM carbachol at 37°C for the indicated time periods (0-5 minutes). Reactions were stopped by removal of carbachol-containing medium and addition of methanol to each

well. Cells were scraped and inositol phosphates recovered by methanol/chloroform extraction. Inositol (1,4,5)trisphosphate was isolated as previously described (Maeda *et al.*, 1990; Arden *et al.*, 1992). Results are expressed as the fold increase in eluted dpm over basal dpm.

G. *In vivo* Phosphorylation and Immunoprecipitation of m3 AChR Wild-type and Mutant m3-SASS/AAAA

Phosphorylation assays were carried out by Dr. Andrew Tobin at the University of Leicester. CHO cells expressing wild-type and mutant receptors were plated on 6-well culture dishes and allowed to reach ~50% confluence. The cells were washed once with phosphate-free Krebs/HEPES buffer (4.2 mM NaHCO3, 118 mM NaCl, 4.7 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM Glucose, 10 mM HEPES, pH 7.4) and incubated in 1 ml phosphate free Krebs/HEPES buffer containing 50 μCi [^{32}P] orthophosphate for 1 hour at 37°C. Agonist (1mM carbachol) was then added directly to the cells for a given time period. The reaction was stopped by washing the cells twice with 2 ml of ice-cold phosphate-free Krebs/HEPES buffer. The cells were then solubilized in 1 ml ice-cold solubilization buffer (10 mM Tris, pH 7.4; 10 mM EDTA, 500 mM NaCl, 1% Nonidet P-40, 0.5% SDS). Following 30 minutes solubilization on ice, the samples were cleared by centrifugation and the m3 receptors were immunoprecipitated as described previously (Tobin and Nahorski, 1993). Briefly, solubilized samples were incubated with the m3 muscarinic specific antiserum (Ab332) for 60 minutes. The immune complexes were isolated on protein A sepharose beads and resolved by 8% SDS-PAGE. The gels were dried and autoradiographs obtained. To ensure equal loading of protein samples, gels were stained with Coomassie blue prior to autoradiography. Data was analyzed using ImageQuant™ (Molecular Dynamics, Sunnyvale, CA).

IV. RESULTS

A. Expression and Ligand Binding Properties of Wild-type and Mutant Muscarinic Receptors

Stable Chinese hamster ovary (CHO) cell lines expressing wild-type and mutant (Fig. 3.1) receptor cDNAs were made previously in the laboratory by Melinda Shockley. B_{max} for m3 AChR wild-type expression was approximately 2.5 pmol/mg protein, and mutant m3-SASS/AAAA expressed at approximately 4.0 pmol/mg protein. Bmax for m1 AChR wild-type expression was approximately 2.0 pmol/mg protein while mutant m1-SLTSS/ALAAA expression was four-fold lower at 0.5 pmol/mg protein. None of the isolated m1-SLTSS/ALAAA clones expressed at wild-type levels. 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 m1 and m3 mAChRs. Agonist and antagonist binding affinities were unaffected by the mutations for both m1 and m3 receptors (data not shown).

mAChR1	284 S M E **S** L **T S S** E G E 295
mAChR3	345 S L E N **S** A **S S** D E E 356

Figure 3.1. Sequence alignment of the serine-rich domains in the third intracellular loops of the m1 and the m3 muscarinic receptor subtypes. Note the acidic amino acid residues flanking the serine-rich regions. Serine residues mutated to alanines are indicated in bold font.

B. Carbachol-Induced Changes in [^{3}H]-NMS Binding

Cells expressing either wild-type or mutant 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 (2nM) of [^{3}H]-NMS (Fig. 3.2). Loss of [^{3}H]-NMS binding sites was evident for wild-type m1 receptors after 15 minutes of carbachol treatment and surface sites continued to decrease for the two hours monitored with a 25% loss in binding sites observed after two hours. A similar decrease in [^{3}H]-NMS binding was observed for mutant m1-SLTSS/ALAAA. Carbachol induced a decrease in surface sites for wild-type m3 that was detectable after 15 minutes of treatment and this loss plateaued after 30 minutes. In contrast, no significant change in [^{3}H]-NMS binding was observed for mutant m3-SASS/AAAA.

C. Immunolocalization of m3 Wild-type and Mutant Receptors in CHO Cells

Prior to agonist treatment, wild-type m3 receptors resided predominantly at the cell surface (Fig. 3.3A). After carbachol treatment, receptors were localized to intracellular vesicles and surface expression of the receptor was reduced (Fig. 3.3B-D). [^{3}H]-NMS binding following agonist treatment suggested no loss in surface expression of mutant m3-SASS/AAAA (Fig. 3.2). As this mutant was expressed at relatively high levels (~4 pmol/mg protein), we wanted to confirm by confocal microscopy 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 binding studies. In the absence of agonist, m3-SASS/AAAA was expressed primarily at the cell surface (Fig. 3.3E). No agonist-induced redistribution of mutant m3-SASS/AAAA was observed over the two hour treatment period (Fig. 3.3F-H).

To characterize the vesicles containing internalized m3 receptors, 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 (Fig. 3.4A). Following agonist treatment, wild-type receptors were located in intracellular vesicles containing clathrin (Fig. 3.4B). Mutant SASS/AAAA did not colocalize with clathrin in the absence or in the presence of carbachol, consistent with a defect in internalization (data not shown).

D. Down-regulation of m1-SLTSS/ALAAA and m3-SASS/AAAA Receptors

To determine the general relevance of the serine/threonine domain in muscarinic receptor trafficking, we tested the ability of wild-type and mutant m1 and m3 receptors to undergo down-regulation. Total m1 and m3 receptor number, monitored by [^{3}H]-QNB binding, decreased following prolonged stimulation with carbachol (Fig. 3.5). Carbachol (1 mM) elicited a rapid decrease in m3 wild-type receptor number (~40% loss after 4 hours) with no additional loss observed over the 24-hour treatment period. Loss of m1 receptors was more gradual, with 75% of the receptors detectable at 4 hours and 20% of the receptor pool remaining after 24 hours of carbachol stimulation. Stimulation of CHO cells expressing m3-SASS/AAAA with carbachol resulted in no significant change in total receptor number even after 24 hours. In contrast, the extent of down-regulation of the mutant m1 receptor (m1-SLTSS/ALAAA) was not significantly different from the wild-type m1 receptor, although the m1 mutant appeared to undergo down-regulation more rapidly than the wild-type receptor (Fig. 3.5).

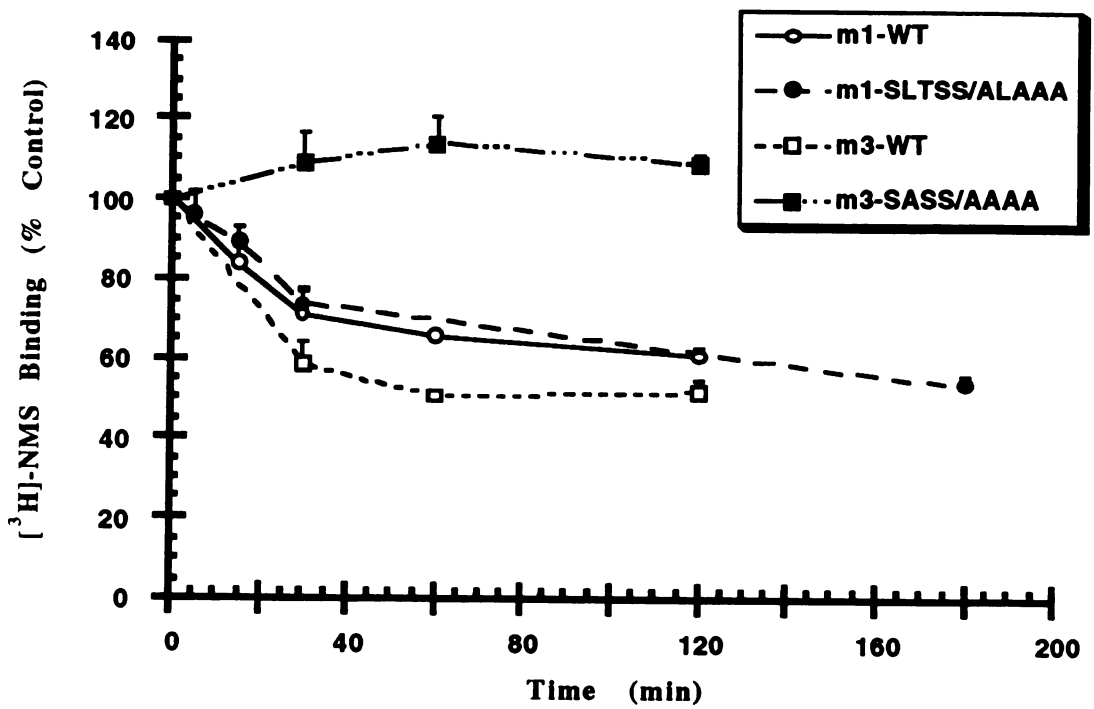


Figure 3.2. Time course of carbachol-induced internalization of m1 and m3 mutants. Confluent cells were treated with 1 mM carbachol for the indicated times. Following agonist treatment, cells were washed with PBS and incubated at 12°C with 2 nM [^{3}H]-NMS for 90 minutes. Radiolabel binding was quantified by liquid scintillation counting. Results are expressed as the percentage of surface binding sites in the absence of carbachol. Time course data are representative of three to four independent experiments performed in triplicate for each time point.

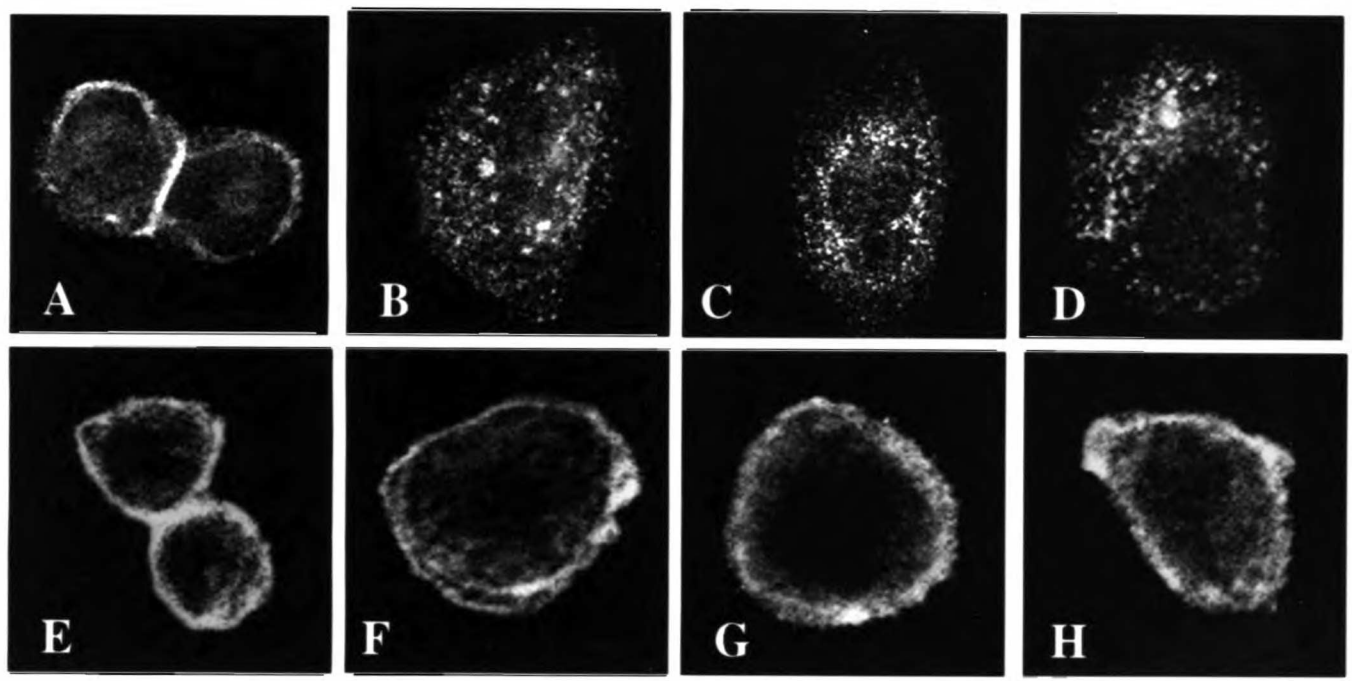


Figure 3.3. Immunolocalization of m3 wild-type and mutant receptors by confocal microscopy. CHO cells expressing either wild-type or mutant m3 receptors were treated with carbachol for the indicated times, fixed, permeabilized, and visualized as described in Experimental Procedures. **A-D:** wild-type m3 mAChR. **E-H:** mutant m3-SASS/AAAA mAChR. **A** and **E:** no carbachol treatment. **B** and **F:** 30 minutes carbachol treatment. **C** and **G:** 60 minutes carbachol treatment. **D** and **H:** 120 minutes carbachol treatment.

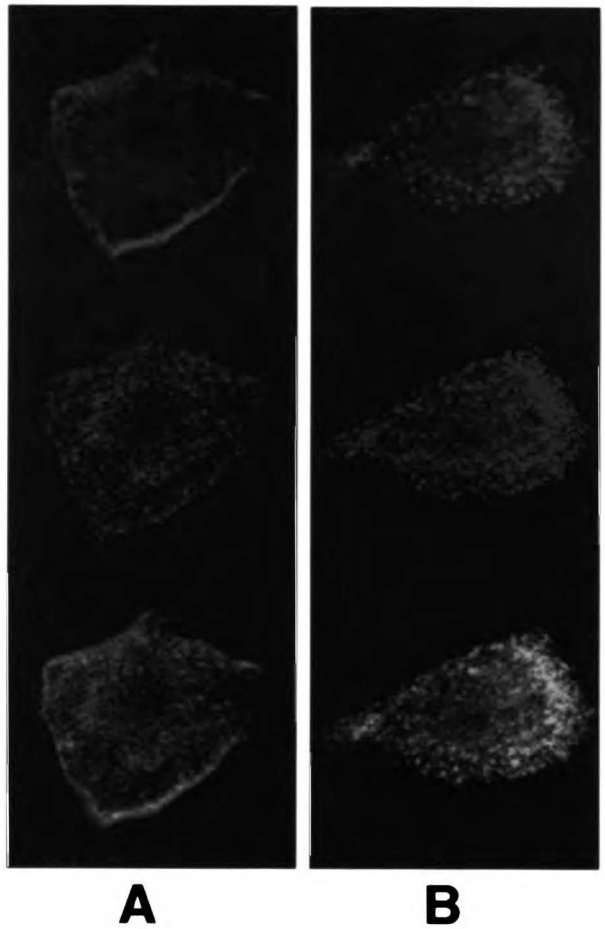


Figure 3.4. Colocalization of m3 receptors with clathrin. CHO cells stably expressing m3 wild-type receptors were treated with 1 mM carbachol for 30 minutes, fixed, permeabilized and sequentially labeled with anti-m3 receptor and anti-clathrin antibodies as outlined in Experimental Procedures. The red color indicates the localization of m3 mAChR (top panel), green corresponds to clathrin localization (middle panel), and yellow is indicative of colocalization in the merged image (lower panel). **A**: no carbachol treatment. **B**: 30 minutes carbachol treatment.

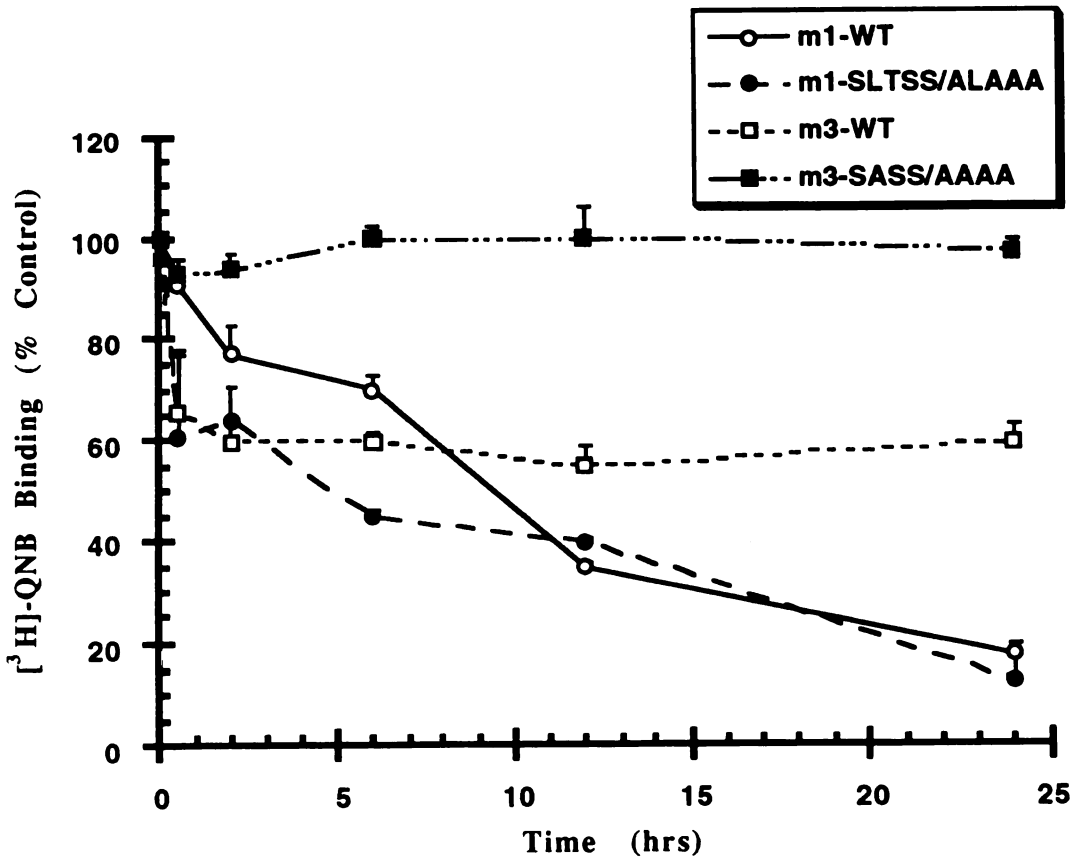


Figure 3.5. Down-regulation of wild-type and mutant m1 and m3 mAChRs. Cells expressing wild-type or mutant m1 and m3 receptors were treated with 1 mM carbachol and the total receptor number was assessed by [^{3}H]-QNB binding. Briefly, cells were incubated with 2 nM [^{3}H]-QNB for 90 minutes following agonist treatment, harvested in PBS, and then radiolabel binding was quantified by liquid scintillation counting. Data are presented as the percentage of total binding sites measured in the absence of carbachol. Time course data are representative of two to four independent experiments performed in quadruplicate.

E. Desensitization of the Carbachol-Induced Inositol (1,4,5)Trisphosphate Response

Peak stimulation of inositol (1,4,5)trisphosphate (IP_3) by m3 wild-type and mutant m3-SASS/AAAA occurred within 15 seconds and both peak responses were attenuated with carbachol pretreatment. The plateau phase of IP_3 release, or the desensitization-resistant component of m3 muscarinic receptor activity (Tobin *et al.,* 1992), was unaffected by agonist pretreatment in either case (Figures. 3.6A&B). Analysis of a time course of IP_3 release by m1 wild-type similarly revealed an initial peak stimulation within 15 seconds of 1 mM carbachol treatment which then fell to levels 4-fold above basal (Figure 3.6C). Pretreatment of m1 AChR with 1 mM carbachol for 2 hours abolished the peak response while maintaining the plateau phase. Stimulation of m1-SLTSS/ALAAA with carbachol resulted in an IP_3 peak of the same magnitude which was again absent following agonist pretreatment (Fig. 3.6D).

F. *In vivo* Phosphorylation of Wild-type and Mutant m3 Receptors in Response to Carbachol

Immunoprecipitation of m3-SASS/AAAA receptors from CHO cells revealed basal phosphorylation of this mutant receptor comparable to that seen with m3 wild-type receptors (Fig. 3.7). Phosphorylation of wild-type m3 AChR was very rapid occurring within seconds of agonist treatment, with a three-fold increase over baseline maintained for at least 15 minutes (Fig. 3.7A). A time course of m3-SASS/AAAA phosphorylation indicated that phosphorylation of the mutant was rapid and reached maximum levels after 30 seconds, as observed with the wild-type m3 receptor (Fig. 3.7B).

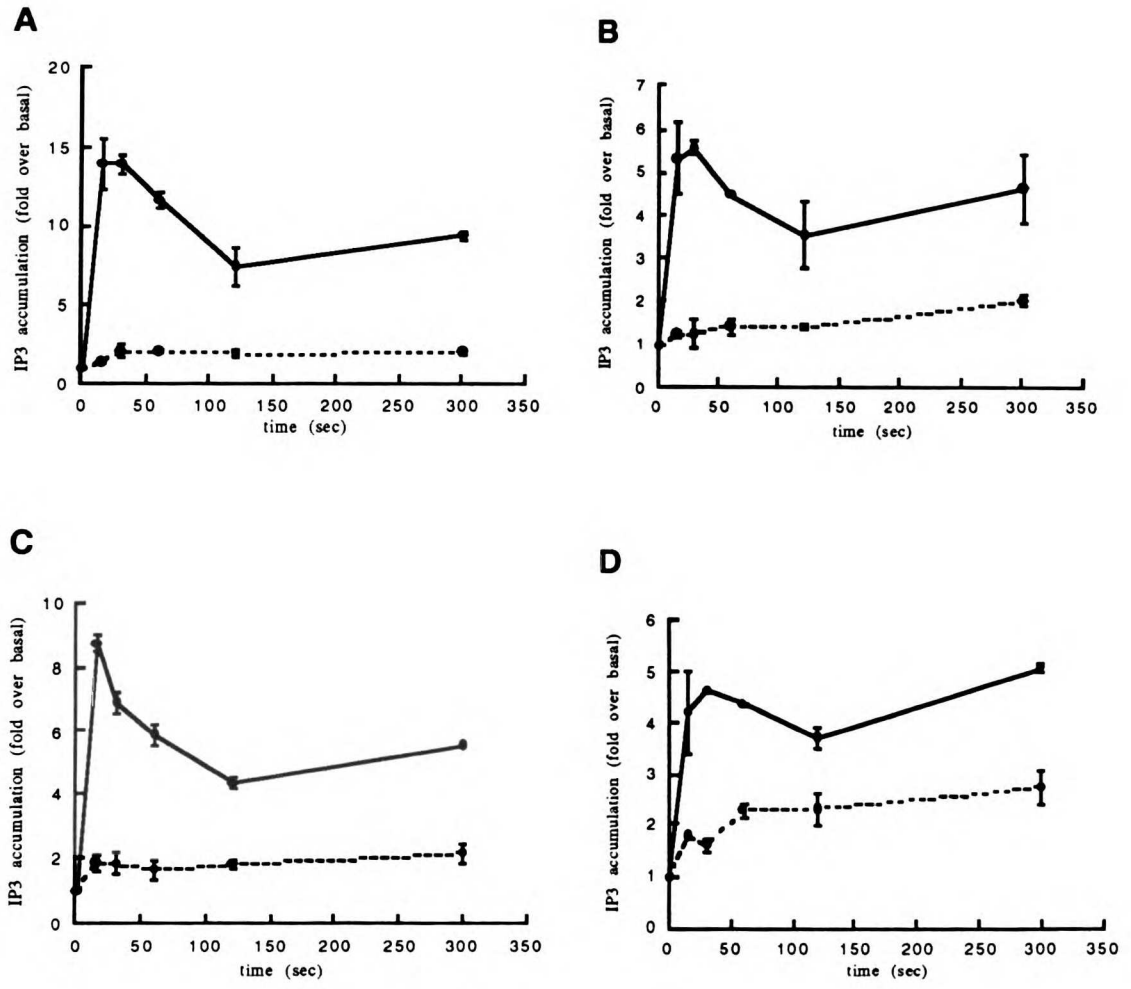


Figure 3.6. Desensitization of m1 and m3 receptor signaling. Cells stably expressing wild-type or mutant muscarinic receptors were labeled with [^{3}H]-myoinositol overnight. Following inositol labeling, cells were pretreated with carbachol (dashed line) or medium alone (solid line) for two hours, washed four times with PBS, and then challenged with 1 mM carbachol for the indicated times (0-5 minutes). Inositol (1,4,5)trisphosphates (IP_3) were isolated and quantified as described in the Experimental Procedures section. Results are expressed as the fold increase in eluted IP_3 over basal (unpretreated) levels. (A) wild-type m3 mAChR, (B) mutant m3-SASS/AAAA, (C) wild-type m1 mAChR, (D) mutant m1-SLTSS/ALAAA.

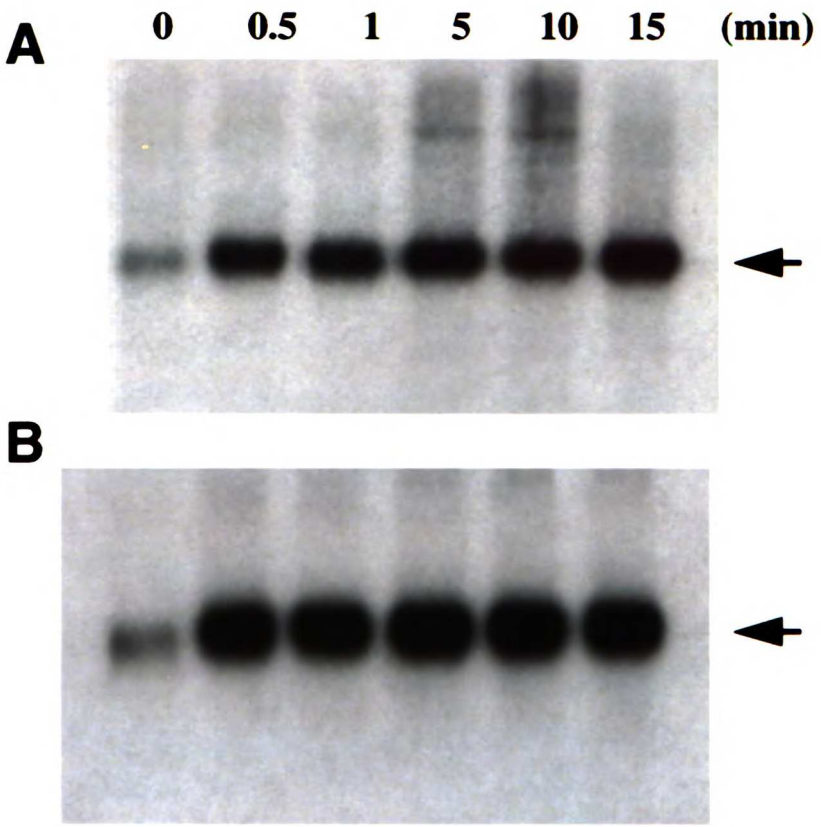


Figure 3.7. Phosphorylation of wild-type and mutant m3 mAChRs. Cells loaded with [^{32}P]orthophosphate were treated with or without 1 mM carbachol for the indicated times. The receptors were immunoprecipitated and resolved by SDS-gel electrophoresis as described in Experimental Procedures. To ensure equal loading of protein samples, the gel was stained with Coomassie blue prior to autoradiography. Arrows indicate the ~ 100 kDa band specifically corresponding to m3 receptors as previously reported (Tobin and Nahorski, 1993). (A) mutant m3-SASS/AAAA mAChR, (B) wild-type m3 mAChR.

V. DISCUSSION

This study examined the contribution of a putative phosphorylation target sequence in modulating the desensitization, internalization, and down-regulation of m1 and m3 muscarinic receptors. Previous studies identified the serine-rich domain SLTSS in the i3 loop of the m1 receptor and the homologous SASS domain in the m3 receptor as potential regulatory domains mediating muscarinic receptor internalization in human embryonic kidney (HEK293) cells (Moro *et al.*, 1993). Furthermore, both of these sequences have been predicted as sites of phosphorylation by GRKs or similarly related kinases (Tobin and Nahorski, 1993; Haga *et al.*, 1996; Tobin *et al.*, 1996). Phosphorylation by GRKs has been implicated as the initial step in both receptor desensitization and internalization (Benovic *et al.*, 1988; Ferguson *et al.*, 1995). Thus, the SLTSS and the SASS domains in m1 and m3 receptors, respectively, may serve as important sites of molecular regulation of muscarinic receptor activity.

To comprehensively examine the role of this motif in m1 and m3 receptor trafficking, we assessed the contribution of the serine-rich domains in regulating internalization in a cell line (CHO) which supports the analysis of the additional regulatory mechanism, down-regulation. It was previously reported that muscarinic receptor subtype m1 receptors expressed in CHO cells internalize via a clathrin-mediated mechanism and subsequently undergo down-regulation in response to agonist (Shockley *et al.*, 1997). Until now, studies of m3 receptor trafficking in CHO cells have suggested that minimal, if any, internalization of this muscarinic receptor subtype occurs with short term agonist exposure (Tobin *et al.*, 1992; Yang *et al.*, 1993; Yang *et al.*, 1995; Koenig and Edwardson, 1996). In this study, we were able to monitor the internal accumulation of m3 receptors in response to carbachol using

immunofluorescence confocal microscopy, thus establishing that m3 receptors are indeed internalized in CHO cells. Furthermore, m3 receptors colocalized with clathrin in intracellular vesicles following carbachol treatment, suggesting that m3 receptors, like m1 receptors, internalize via a clathrin-mediated pathway in CHO cells. Analysis of the contribution of the serine-rich regions to internalization of m3 receptors in CHO cells revealed that mutation of the SASS domain to AAAA abolished internalization with no apparent vesicular accumulation of receptor following 2 hours of carbachol stimulation and no colocalization with clathrin. However, mutation of the m1 sequence SLTSS to ALAAA had no effect on carbachol-induced m1 receptor internalization. We were unable to visualize the distribution of m1-SLTSS/ALAAA in CHO cells as the maximum expression levels obtained with this mutant were below the detection limits of the confocal microscope. Nonetheless, tracer binding studies indicated that rapid internalization of the m1 mutant did occur. Internalization of m1-SLTSS/ALAAA was blocked by hyperosmolar sucrose (data not shown), consistent with internalization via clathrin-coated vesicles. Thus, the SASS region in m3 mAChR, but not the homologous SLTSS region in m1 AChR, may serve as a molecular recognition site for factors involved in internalization. In contrast to previous analysis of m1 receptor internalization in HEK293 cells (Moro *et al.*, 1993), we observed no deficit in internalization when mutant m1-SLTSS/ALAAA was expressed in CHO cells. These recent observations may reflect cell type-specific differences in cellular trafficking pathways or may suggest a relationship between factors involved in internalization and those involved in downstream sorting events such as recycling or lysosomal targeting.

We next addressed the contribution of the serine-rich domain located in the i3 loops of both m1 and m3 receptors to receptor down-regulation.

Mutation of the SASS domain in the m3 receptor (SASS/AAAA) abolished not only internalization, but also down-regulation in CHO cells. Several interpretations of this finding exist. First, this region may have dual function with respect to modulation of internalization over short time periods of agonist treatment and down-regulation over longer exposure times. Alternatively, this region plays a role in the internalization of the m3 receptor, and the effect on down-regulation may be the result of the complete impairment of the requisite upstream internalization event. We have observed that sucrose treatment blocks m3 wild-type receptor down-regulation (data not shown) presumably by a mechanism involving the specific inhibition of clathrin-mediated internalization; therefore, the more likely hypothesis is that the SASS domain in the m3 receptor specifically directs agonist-induced trafficking via clathrin-coated vesicles.

In contrast to complete impairment of m3-SASS/AAAA internalization and down-regulation, no deficit in the extent of down-regulation was observed with mutant m1-SLTSS/ALAAA. On the contrary, mutant m1-SLTSS/ALAAA appeared to undergo down-regulation more rapidly than the wild-type m1 receptor. This result was surprising as the SLTSS/ALAAA mutation was found to impair internalization in HEK 293 cells (Moro et al., 1993) and was therefore expected to reduce down-regulation. This phenomenon was observed by Yang et al. (1995) for an m3 receptor mutant which blocked internalization in HEK 293 cells, and correspondingly, down-regulation in CHO cells. The increase in the rate of down-regulation could be accounted for by alterations in the regulation of the various trafficking pathways. For example, it is possible that the m1 mutation interferes with its ability to be transported from endosomes to the plasma membrane, so that the mutant receptors, instead of being recycled, were targeted to the lysosome for degradation. This explanation would leave

open the possibility that internalization is, in fact, defective, and the loss of receptors due to down-regulation that would normally return to the cell surface contributes to the loss of surface sites as measured by the hydrophilic ligand NMS. Resolving the molecular mechanisms governing the trafficking of wild-type and mutant m1 receptors will require additional work.

Considerable evidence exists for the potential role of a G protein-coupled receptor kinase (GRK) in the internalization of G protein-coupled receptors. The serine/threonine-rich sites of the m1 and m3 receptors examined in this study are two examples of the highly conserved domains among muscarinic receptors which are putative sites for GRK-mediated phosphorylation (Nakata *et al.*, 1994; Eason *et al.*, 1995; Haga *et al.*, 1996). Because the m3 receptor mutant SASS/AAAA displayed pronounced defects in trafficking, we were interested in investigating the possibility that phosphorylation at this site was involved in internalization. Phosphorylation of m3 receptors has previously been shown to occur in an agonist-dependent manner at a region of the third intracellular loop containing the SASS sequence (Tobin *et al.*, 1996). In this study, we found that phosphorylation of the mutant m3-SASS/AAAA was comparable to wild-type m3 AChR. This result makes it difficult to draw a correlation between internalization and phosphorylation at this site. Two possibilities are apparent. First, the SASS motif may not serve as a substrate for βARK or for other kinases, so that while this site is required for interaction of the receptor with a component of the endocytic machinery, phosphorylation at this site is not necessary. Alternatively, it remains a possibility that phosphorylation at this site *is* required for internalization, possibly via arrestin binding, but that other phosphorylation sites exist elsewhere in the receptor. In this case, wild-type levels of phosphorylation could be explained by a disinhibition of

phosphorylation at other receptor sites that were previously blocked by phosphorylation, and possibly arrestin binding, at the SASS sequence. This idea is consistent with the work of Haga *et al.* (1996) showing that m1 phosphorylation was greater when sequentially phosphorylated by βARK followed by PKC than when phosphorylated by the two kinases in the reverse order.

Previously, it was shown that m3 receptors rapidly desensitize in CHO cells independently of internalization, but in a phosphorylation-dependent manner (Tobin et al., 1992; Tobin and Nahorski, 1993). The m3 receptor has two serine-rich domains in the i3 loop which may serve as phosphorylation sites functioning in desensitization. We focused on the C-terminal SASS region of the m3 receptor since this region, and not the N-terminal serine-rich region, is located within the region of the i3 loop postulated as the target sequence for GRKs or similarly related kinases (Tobin et al., 1996). Likewise, the SLTSS region of the m1 receptor is predicted as the specific sequence phosphorylated by GRKs and thus has been predicted to play a role in m1 receptor desensitization (Haga et al., 1996). In this study, the m1-SLTSS/ALAAA and m3-SASS/AAAA mutants displayed no deficits in the ability to desensitize. It is possible that desensitization of the m3 receptor is mediated by the N-terminal serine-rich domain while the C-terminal SASS region is specific to the regulation of internalization. Desensitization of the m1 receptor may then be mediated by the phosphorylation of receptor sites other than those predicted as GRK target sequences. Further examination of other potential phosphorylation sites in the m1 and m3 receptors will be required to identify the domains specifically governing desensitization.

In summary, the predicted GRK target domains, SLTSS in the m1 receptor and SASS in the m3 receptor, are differentially involved in the

regulation of these muscarinic receptor subtypes. The SASS domain in the m3 receptor specifically modulates receptor trafficking, although a direct correlation with phosphorylation could not be determined. The SLTSS region in the m1 receptor appears to direct receptor trafficking in a cell type-dependent manner; however, the contribution of the domain SLTSS to the internalization of the m1 receptor subtype may involve more directly the regulation of the downstream targeting of the receptor for degradation. Contrary to prediction, neither the SASS domain in the m3 receptor nor the SLTSS domain in the m1 receptor is crucial for desensitization. Further studies will be needed to clarify the roles that other putative phosphorylation domains have in the regulation of muscarinic receptor activity.

BIBLIOGRAPHY

Anderson, R. G. W., Kamen, B. A., Rothberg, K. G. and Lacey, S. W. (1992) Potocytosis: Sequestration and transport of small molecules by caveolae. *Science* **255:** 410-411.

Anderson, R. G. W. (1993) Caveolae: where incoming and outgoing messengers meet. *Proc. Natl. Acad. Sci. USA* **90:** 10909-10913.

Aoki, C., Zemcik, B. A., Strader, C. D. and Pikel, V. M. (1989) Cytoplasmic loop of beta-adrenergic receptors: synaptic and intracellular localization and relation to catecholaminergic neurons in the nuclei of the solitary tracts. *Brain Res.* **493:** 331-347.

Arden, J. R. and Lameh, J. (1996) Agonist-induced internalization of human m1 muscarinic receptor mutants: Immunofluorescence confocal microscopy. *Proc. West. Pharmacol. Soc.* **39:** 51-53.

Arden, J. R., Segredo, V., Wang, Z., Lameh, J. and Sadée, W. (1995) Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged μ-opioid receptor expressed in HEK 293 cells. *J. Neurochem.* **65:** 1636-1645.

Ashworth, R., Yu, R., Nelson, E. J., Dermer, S., Gershengorn, M. C. and Hinkle, P. M. (1995) Visualization of the thyrotropin-releasing hormone receptor and its ligand during endocytosis and recycling. *Proc. Natl. Acad. Sci. USA* **92:** 512-516.

Benya, R. V., Akeson, M., Mrozinski, J., Jensen, R. T. and Battey, J. F. (1994) Internalization of the gastrin-releasing peptide receptor is mediated by both phospholipase C-dependent and -independent processes. *Mol. Pharmacol.* **46:** 495-501.

Bogatkewitsch, G. S., Lenz, W., Jakobs, K. H. and van Koppen, C. J. (1996) Receptor internalization delays m4 muscarinic acetylcholine receptor resensitization at the plasma membrane. *Mol. Pharmacol.* **50:** 424-29.

Bourne, H. R. (1997) How receptors talk to G proteins. *Curr. Op. Cell Biol.* **9:** 134-142.

Brinton, R. D. and Yamazaki, R. S. (1997) Advances and challenges in the prevention and treatment of Alzheimer's disease. *Pharm. Res.* **15:** 386-398.

Burford, N. T., Tolbert, L. M. and Sadee, W. (1998) Specific G protein activation and μ-opioid receptor internalization caused by morphine, DAMGO, and endomorphin I. *Eur. J. Pharmacol.* **342:** 123-126.

Campbell, P. T., Hnatowich, M., O'Dowd, B. F., Caron, M. G., Lefkowtiz, R. J. and Hausdorff, W. P. (1990) Mutations of the human β_2-adrenergic receptor that impair coupling to Gs interfere with receptor down-regulation but not sequestration. *Mol. Pharmacol.* **39:** 192-198.

Chang, W.-J., Ying, Y.-S., Rothberg, K. G., Hooper, N. M., Turner, A. J., Gambiel, H. A., Gunzburg, J. D., Mumby, S. M., Gilman, A. G. and Anderson, R. G. W. (1994) Purification and characterization of smooth muscle cell caveolae. *J. Cell Biol.* **126:** 127-138.

Cheung, A. H., Dixon, R. A. F., Hill, W. S., Sigal, I. S. and Strader, C. D. 1990) Separation of the structural requirements for agonist-promoted activation and sequestration of the β-adrenergic receptor. *Mol. Pharmacol.* **37:** 775-779.

Chuang, T. T., Iacovelli, L., Sallese, M. and De Blasi, A. (1996) G protein-coupled recewptors: heterologous regulation of homologous desensitization and its implications. *Trends Pharmacol. Sci.,* **17 (11):** 416-21.

Chun, M., Liyanage, U. K., Lisanti, M. P. and Lodish, H. F. (1994) Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc. Natl. Acad. Sci USA* **91:** 11728-11732.

Clark, R. B., Friedman, J., Prashad, N. and Ruoho, A. E. (1985) Epinephrine-induced sequestration of the β-adrenergic receptor in cultured S49 WT and Cyc^- lymphoma cells. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **10:** 97-119.

Conchon, S., Monnot, C., Teutsch, B., Corvol, P. and Clauser, E. (1994) Internalization of the rat AT_{1a} and AT_{1b} receptors: pharmacological and functional requirements. *FEBS Lett.* **349:** 365-370.

Cupers, P., Veithen, A., Kiss, A., Baudhuin, P., and Courtoy, P. J. (1994) Clathrin polymerization is not required for bulk-phase endocytosis in rat fetal fibroblasts. *J. Cell Biol.* **127:** 725-735.

Damke, H., Baba, T., van der Bliek, A. M. and Schmid, S. L. (1995) Clathrin-independent pinocytosis is induced in cells overexpressing a termperature-sensitive mutant of dynamin. *J. Cell Biol.* **131:** 69-80.

DebBurman, S. K., Kunapuli, P., Benovic, J. L. and Hosey, M. M. (1995) Agonist-dependent phosphorylation of human muscarinic receptors in *Spodoptera frugiperda* insect cell membranes by G protein-coupled receptor kinases. *Mol. Pharmacol.* **47:** 224-233.

Dell'Angelica, E. C., Klumperman, J., Stoorvogel, W. and Bonifacino, J. S. (1998) Association of the AP-3 adapter complex with clathrin. *Science* **280:** 431-434.

Dell'Angelica, E. C., Ohno, H., Ooi, C. E., Rabinovich, E., Roche, K. W. and Bonifacino, J. S. (1997) AP-3: an adaptor-like protein complex with ubiquitous expression. *EMBO J.* **16 (5):** 917-928.

Eason, M. G., Moreira, S. P. and Liggett, S. B. (1995) Four consecutive serines in the third intracellular loop are the sites for β-adrenergic receptor kinase-mediated phosphorylation and desensitization of the α_{2a}-adrenergic receptor. *J. Biol. Chem.* **270 (9):** 4681-4688.

Ferguson, S. S., Downey III, W. E., Colapietro, A. M., Barak, L. S., Menard, L. and Caron, M. G. (1996) Role of β-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science* **271:** 363-366.

Fonseca, M. I., Button, D. C. and Brown, R. D. (1995) Agonist regulation of alpha 1B-adrenergic receptor subcellular distribution and function. *J. Biol. Chem.* **270:** 8902-8909.

Fredericks, Z. L., Pitcher, J. A. and Lefkowitz, R. J. (1996) Identification of the G protein-coupled receptor kinase phosphorylation sites in the human β_2-adrenergic receptor. *J. Biol. Chem.* **271 (23):** 13796-13803.

Fujimoto, T. (1993) Calcium pump of the plasma membrane is localized in caveolae. *J. Cell. Biol.* **120**, 1147-1157.

Garland, A. M., Grady, E. F., Payan, D. G., Vigna, S. R. and Bunnett, N. W. (1994) Agonist-induced internalization of the substance P (NK_1) receptor expressed in epithelial cells. *Biochem. J.* **303:** 177-186.

Garland, A. M., Grady, E. F., Lovett, M., Vigna, S. R., Frucht, M. M., Krause, J. E. and Bunnett, N. W. (1996) Mechanisms of desensitization and resensitization of G protein-coupled neurokinin1 and neurokinin2 receptors. *Mol. Pharm.* **49**, 438-446.

Ghinea, N., Vu Hai, M. T., Groyer-Picard, M. T., Houllier, A., Schoevaert, D. and Milgrom, E. (1992) Pathways of internalization of the hCG/LH receptor: Immunoelectron microscopic studies in Leydig cells and transfected L-cells. *J. Cell Biol.* **118:** 1347-58.

Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russel, D. W. and Schneider, W. J. (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Ann. Rev. Cell Biol.* **1:** 1-39.

Gonzalez-Calero, G., Martín, M., Cubero, A. and Andrés, A. (1990) Bovine brain coated vesicles contain adenosine A_1 receptors. Presence of adenylate cyclase coupled to the receptor. *J. Neurochem.* **55:** 106-113.

Gonzalez-Calero, G., Cubero, A. and Klotz, K. (1992) G-protein-coupled A_1 adenosine receptors in coated vesicles of mammalian brain: characterization by radioligand binding and photoaffinity labelling. *Cell. Signal.* **4(6):** 737-745.

Grady, E. F., Slice, L. W., Bran, W. O., Walsh, J. H. and Payan, D. G. (1995) *J. Biol. Chem.* Direct observation of endocytosis of gastrin releasing peptide and its receptor. **270:** 4603-4611.

Grady, E. F., Bohm, S. K. and Bunnett, N. W. (1997) Turning off the signal: mechanisms that attenuate signaling by G protein-coupled receptors. *Am. J. Physiol.* **273:** G586-G601.

Haga, K., Kameyama, K., Haga, T., Kikkawa, U., Shiozaki, K. and Uchyama, H. (1996) Phosphorylation of human m1 muscarinic acetylcholine receptors by G

protein-coupled receptor kinase 2 and protein kinase C. *J. Biol. Chem.* **271:** 2776-2782.

Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) Turning off the signal: desensitization of *beta*-adrenergic function. *FASEB J.* **4:** 3049.

Holtmann, M. H., Roettger, B. F., Pinon, D. I., and Miller, L. J. (1996) Role of receptor phosphorylation in desensitization and internalization of the secretin receptor. *J. Biol. Chem.* **271(38):** 23566-23571.

Hopkins, C. R. (1983a) Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. *Cell* **35:** 321-330.

Hopkins, C. R. (1985) The appearance and internalization of transferrin receptors at the margins of spreading human tumor cells. *Cell* **40:** 199-208.

Huet, C., Ash, J. F. and Singer, S. J. (1980) The antibody-induced clustering and endocytosis of HLA antigens on cultured human fibroblasts. *Cell* **21:** 429-438.

Huang, Z., Chen, Y. and Nissenson, R. A. (1995) The cytoplasmic tail of the G-protein-coupled receptor for parathyroid hormone and parathyroid hormone-related protein contains positive and negative signals for endocytosis. *J. Biol. Chem.* **270:** 151-156.

Hunyady, L., Baukal, A. J., Balla, T. and Catt, K. J. (1994) Independence of type I angiotensin II receptor endocytosis from G protein coupling and signal transduction. *J. Biol. Chem.* **269:** 24798-24804.

Jeursalinsky, D., Kornisiuk, E., and Izquierdo, I. (1997) Cholinergic neurotransmission and synaptic plasticity concerning memory processing. *Neurochem. Res.* **22(4):** 507-515.

Jing, S., Spenser, T., Miller, K., Hopkins, C. and Trowbridge, I. S. (1990) Role of the human transferrin receptor cytoplasmic domain in endocytosis: Localization of a specific signal for internalization. *J. Cell. Biol.* **110:** 283-294.

Keith, D. E., Murray, S. R., Zak, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J. and von Zastrow, M. (1996) Morphine activates opioid receptors without causing their rapid internalization. *J. Biol. Chem.* **271:** 19021-19024.

Kirchhausen, T., Bonifacino, J. S., and Riezman, H. Linking cargo to vesicle formation: receptor tail interactions with coat proteins. (1997) *Curr. Op. Cell Biol.* **9:** 488-495.

Krupnick, J. G. and Benovic, J. L. (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Ann. Rev. Pharmacol. Toxicol.* **38:** 289-319.

Lamaze, C. and Schmid, S. L. (1995) The emergence of clathrin-independent pinocytic pathways. *Curr. Op. Cell Biol.* **7:** 573-580.

Lameh, J., Philip, M., Sharma, Y. K., Moro, O., Ramachandran, J. and Sadée, W. (1992) Hm1 muscarinic cholinergic receptor internalization requires a domain in the third cytoplasmic loop. *J. Biol. Chem.* **267:** 13406-13412.

Lefkowitz, R. J., Pitcher, J., Krueger, K. and Daaka, Y. (1998) Mechanisms of β-adrenergic receptor desensitization and resensitization. *Adv. Pharmacol.* **42:** 416-420.

Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J. E., Hansen, S. H., Nishimoto, I. and Lisanti, M. P. (1995) Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. *J. Biol. Chem.* **270(26):** 15693-15701.

Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y.-H., Cook, R. F. and Sargiacomo, M. (1994) Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. *J. Cell Biol* **126:** 111-126.

Maeda, S., Lameh, J., Mallet, W., Philip, M., Ramachandran, J. and Sadée, W. (1990) Internalization of the Hm1 muscarinic cholinergic receptor involves the third cytoplasmic loop. *FEBS Lett.* **269:** 386-388.

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

Mayor, S., Rothberg, K. G. and Maxfield, F. R. (1994) Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. *Science* **24:** 1948-1951.

McGraw, T. E. and Maxfield, F. R. (1990) Human transferrin receptor internalization is partially dependent upon an aromatic amino acid on the cytoplasmic tail. *Cell Reg.* **1:** 369-377.

McGraw, T. E., Pytoeski, B., Arzt, J. and Ferrone, C. (1991) Mutagenesis of the human transferrin receptor: two cytoplasmic phenylalanines are required for efficient internalization and a second-site mutation is capable of reverting an internalization-defective phenotype. *J. Cell. Biol.* **112:** 853-861.

Montesano, R., Roth, J., Robert, A. and Orci, L. (1982) Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins. *Nature* **296:** 651-653.

Moro, O., Lameh, J., Högger, P. and Sadée, W. (1993a) Hydrophobic amino acid in the i2 loop plays a key role in receptor-G protein coupling. *J. Biol. Chem.* **268:** 22273-22278.

Moro, O., Lameh, J. and Sadée, W. (1993) Serine- and threonine-rich domain regulates internalization of muscarinic cholinergic receptors. *J. Biol. Chem.* **268 (10):** 6862-6865.

Mukherjee, S., Ghosh, R. N. and Maxfield, F. R. (1997) Endocytosis. *Phys. Rev.* **77 (3):** 759-803.

Onorato, J. J., Palczewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J. and Benovic, J. L. (1991) Role of acidic amino acids in peptide substrates of the β-

adrenergic receptor kinase and rhodopsin kinase. *Biochemistry* **30:** 5118-5125.

Ozaki, N., Moroi, K., Kadota, T., Suzuki, S. and Kadota, K. (1994) Dopamine D_1 and D_2 receptors and their signal system present in coated vesicles prepared from bovine striatal tissue. *J. Neurochem.* **62:** 582-591.

Pak, Y., Kouvelas, A., Scheideler, M. A., Rasmussen, J., O'Dowd, B. F. and George, S. R. (1996) Agonist-induced functional desensitization of the μ-opioid receptor is mediated by loss of membrane receptors rather than uncoupling from G protein. *Mol. Pharmacol.* **40:** 1214-1222.

Pals-Rylaarsdam, R. and Hosey, M. M. (1997) Two homologous phosphorylation domains differentially contribute to desensitization and internalization of the m2 muscarinic acetylcholine receptor. *J. Biol. Chem.* **272 (22):** 14152-14158.

Pearse, B. M. F. and Robinson, M. S. (1990) Clathrin, adaptors, and sorting. *Ann. Rev. Cell Biol.* **6:** 151-171.

Petrou, C., Chen, L and Tashjian, Jr., A. H. (1997) A receptor-G protein coupling-independent step in the internalization of the thyrotropin-releasing hormone receptor. *J. Biol. Chem.* **272(4):** 2326-2333.

Pippig, S., Andexinger, S. and Lohse, M. L. (1995) Sequestration and recycling of β_2-adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* **47:** 666-676.

Pohl, M., Silvente-Poirot, S., Pisegna, J. R., Tarasova, N. I. and Wank, S. A. (1997) Ligand-induced internalization of cholecystokinin receptors. *J. Biol. Chem.* **272 (29):** 18179-18184.

Rao, R. V., Roettger, B. F., Hadac, E. M., and Miller, L. J. (1997) Roles of cholecystokinin receptor phophorylation in agonist-stimulated desensitization of pancreatic acinar cells and receptor-bearing chinese hamster ovary cholecystokinin receptor cells. *Mol. Pharmacol.* **51:** 185-192.

Raposo, G., Dunia, I., Marullo, S., Andre, C., Guillet, J.-G., Strosberg, A. D., Benedetti, E. L. and Hoebeke, J. (1987) Redistribution of muscarinic acetylcholine receptors on human fibroblasts induced by regulatory ligands. *Biol. Cell* **60:** 117-123.

Raposo, G., Dunia, I., Delavier-Klutchko, C., Kaveri, S. and Strosberg, A. D. (1989) Internalization of β-adrenergic receptor in A431 cells involves non-coated vesicles. *Eur. J. Cell Biol.* **50:** 340-352.

Richardson, R. M., Kim, C., Benovic, J. L. and Hosey, M. M. (1993) Phosphorylation and desensitization of human m2 muscarinic acetylcholine receptors by two isoforms of the β-adrenergic receptor kinase. *J. Biol. Chem.* **268:** 13650-13656.

Robinson, M. S., Watts, C., and Zerial, M. (1996) Membrane dynamics in endocytosis. *Cell* **84:** 13-21.

Roettger, B. F., Rentsch, R. U., Pinon, D., Holicky, E., Hadac, E., Larkin, J. M. and Miller, L. J. (1995) Dual pathways of internalization of the cholecystokinin receptor. *J. Cell Biol.* **128:** 1029-1041.

Rothberg, K. G., Ying, Y.-S., Kolhouse, J. F. and Anderson, R. G. W. (1990a) The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. *J. Cell Biol.* **110:** 637-649.

Rothberg, K. G., Ying, Y.-S., Kamen, B. A. and Anderson, R. G. W. (1990) Cholesterol controls the clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. *J. Cell Biol.* **111:** 2931-2938.

Saito, Y., Teshima, R., Yamazaki, T., Ikebuchi, H. and Sawada, J. (1994) Ligand-induced internalization and phosphorylation-dependent degradation of growth hormone receptor in human IM-9 cells. *Mol. Cell. Endocrinol.* **106:** 67-74.

Sandvig, K., Olsnes, S., Petersen, O. W. and van Deurs, B. (1987) Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol.* **105:** 679-689.

Sargent, P. B. (1994) Double-label immunofluorescence with laser scanning confocal miscroscope using cyanine dyes. *Neuroimage* 1: 288-295.

Sargiacomo, M., Sudol, M., Tang, Z. and Lisanti, M. P. (1993) Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell Biol.* **122:** 789-807.

Schekman, R. and Orci, L. (1996) Coat proteins and vesicle budding. *Science* **271:** 1526-1533.

Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J., and Oh, P. (1995) Separation of caveolae from associated microdomains of GPI-anchored proteins. *Science* **269:** 1435-1439.

Schnitzer, J. E., Oh, P., Pinney, E. and Allard, J. (1994) Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.* **127 (5):** 1217-1232.

Schwartz, A. L. (1995) Receptor cell biology: receptor-mediated endocytosis. *Ped. Res.* **38 (6):** 835-843.

Shapiro, M. J., Trejo, J., Zeng, D. and Coughlin, S. R. (1996) Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking. *J. Biol. Chem.* **271 (51):** 32874-32880.

Shenoy-Scaria, A. M., Dietzen, D. J., Kwong, J. and Link, D. C. (1994) Cysteine3 of Src family protein tyrosine kinase determines palmitoylation and localization in caveolae. *J. Cell. Biol.* **126:** 353-363.

Shockley, M. S., Burford, N. T. and Sadée, W. (1997) Residues specifically involved in down-regulation but not internalization of the m1 muscarinic acetylcholine receptor. *J. Neurochem.* **68:** 601-609.

Silva, W. I., Andres, A., Schook, W. and Puszkin, S. (1986) Evidence for the presence of muscarinic acetylcholine receptors in bovine brain coated vesicles. *J. Biol. Chem.* **261**: 14788-14796.

Simpson, F., Peden, A. A., Christopoulou, L. and Robinson, M. S. (1997) Characterization of the Adaptor-related protein complex, AP-3. *J. Cell Biol.* **137 (4)**: 835-845.

Sloweijko, D. M., McEwen, E. l., Ernst, S. A. and Fisher, S. K. (1996) Muscarinic receptor sequestration in SH-SY-5Y neuroblastoma cells is inhibited when clathrin distribution is perturbed. *J. Neurochem.* **66**: 186-196.

Smart, E. J., Foster, D. C., Ying, Y.-S., Kamen, B. A. and Anderson, R. G. W. (1994) Protein kinase C activators inhibit receptor-mediated potocytosis by preventing internalization of caveolae. *J. Cell Biol.* **124**: 307-313.

Srinivas, U., Tagliabue, E., Campiglio, M., Menard, S. and Colnaghi, M. I. (1993) Antibody-induced activation of $p185^{HER2}$ in the human lung adenocarcinoma cell line Calu-3 requires bivalency. *Cancer Immunol. Immunother.* **36**: 397-402.

Stoorvogel, W., Oorschot, V. and Gueze, H. J. (1996) A novel class of clathrin-coated vesicles budding from endosomes. *J. Cell. Biol.* **132**: 21-33.

Thompson, A. K., Mostafapour, S. P., Denlinger, L. C., Bleasdale, J. E. and Fisher, S. K. (1991) The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J. Biol. Chem.* **266**: 23856-23862.

Tolbert, L. M. and Lameh, J. (1996) Human muscarinic cholinergic receptor Hm1 internalizes via clathrin-coated vesicles. *J. Biol. Chem.* **271**: 17335-17342.

Tolbert, L. M. and Lameh, J. (1998) Antibody to epitope tag induces internalization of human muscarinic subtype 1 receptor. *J. Neurochem.* **70**: 113-119.

Trapaidze, N., Keith, D. E., Cvejic, S., Evans, C. J. and Devi, L. A. (1996) Sequestration of the δ opioid receptor. *J. Biol. Chem.* **271 (46)**: 29279-29285.

Tucek, S. (1997) Is the R and R* dichotomy real? *Trends Pharmacol. Sci.* **18**: 414-416.

van Koppen, C. J., Sell, A., Lenz, W. and Jakobs, K. H. (1994) Deletion analysis of the m4 muscarinic acetylcholine receptor. *Eur. J. Biochem.* **222**: 525-531.

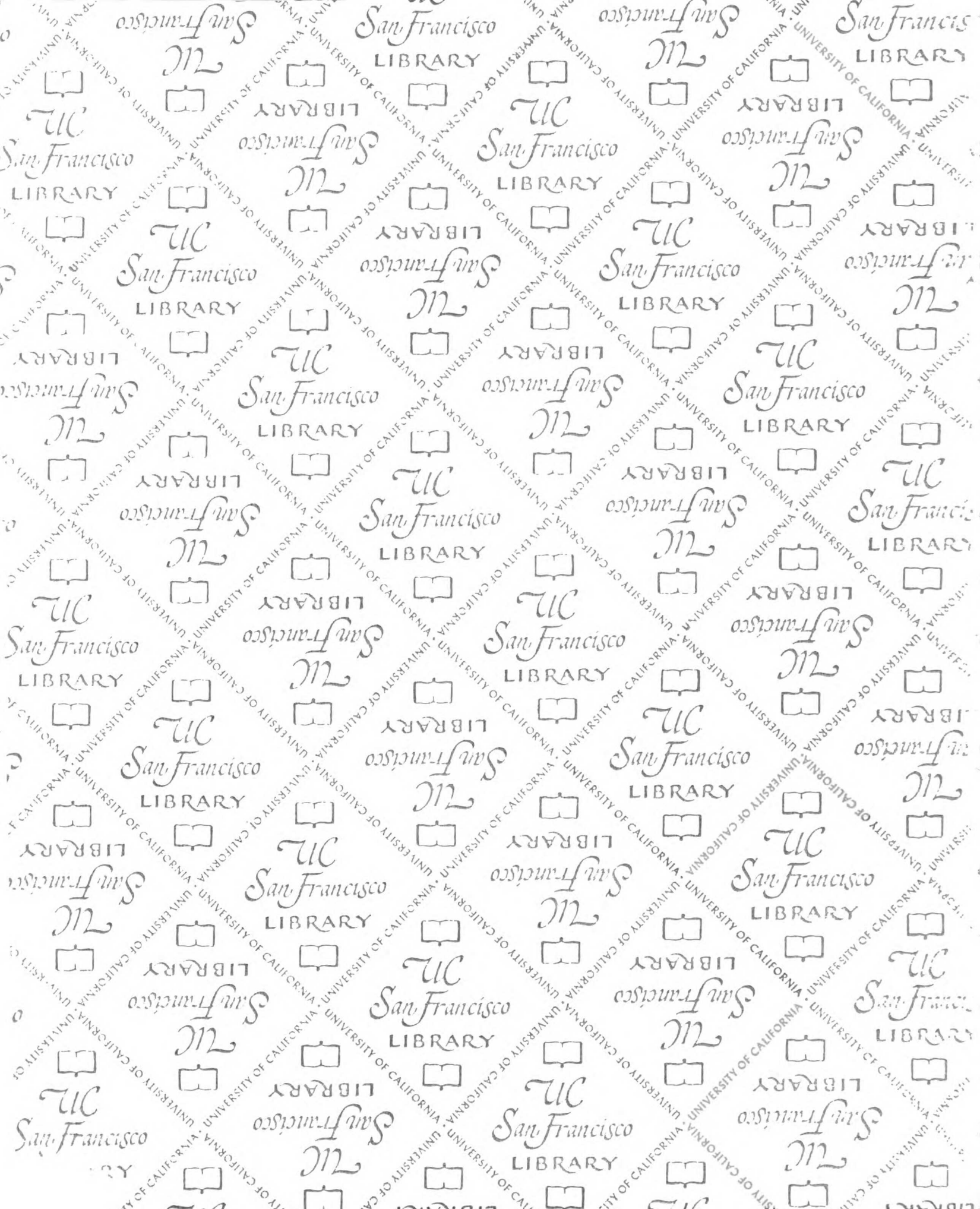
von Zastrow, M. and Kobilka, B. K. (1992) Ligand-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptor. *J. Biol. Chem.* **267**: 3530-3538.

Wong, D. and Brodsky, F. M. (1992) 100-kD Proteins of golgi- and trans-golgi network-associated coated vesicles have related but distinct membrane binding properties. *J. Cell Biol.* **117**: 1171-1179.

Yang, J., Williams, J. A., Yule, D. I. and Logsdon, C. D. (1995) Mutation of carboxyl-terminal threonine residues in human m3 muscarinic acetylcholine receptor modulates the extent of sequestration and desensitization. *Mol. Pharmacol.* **48:** 477-485.

Zanolari, B., Raths, S., Singer-Krüger, B., and Riezman, H. (1992) Yeast pheromone receptor endocytosis and hyperphosphorylation are independent of G protein-mediated signal transduction. *Cell* **71:** 755-763.

Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L. and Caron, M. G. (1996) Dynamin and β-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J. Biol. Chem.* **271:** 18302-18305.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