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Differential regulation of muscarinic M_1 and M_3 receptors by a putative phosphorylation domain

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Abstract

A motif consisting of several serine residues flanked N-terminally by acidic residues occurs in the third intracellular loop of both muscarinic M_1 and M_3 receptors (287SerLeuThrSerSer291 and 349SerAlaSerSer352, respectively). We examined the role of these domains in modulating agonist-induced desensitization and receptor trafficking, and for the muscarinic M_3 receptor, we assessed the contribution of phosphorylation to receptor regulation. Mutation of the above residues did not affect desensitization of phosphoinositide hydrolysis signaling for either the muscarinic M_1 or M_3 receptor and did not alter the agonist-induced phosphorylation state of the muscarinic M_3 receptor. Mutation of this domain (349SerAlaSerSer352/349AlaAlaAlaAla352) in the muscarinic M_3 receptor completely abrogated receptor internalization and subsequently, down-regulation. Mutation of the analogous domain (287SerLeuThrSerSer291/287AlaLeuAlaAlaAla291) in the muscarinic M_1 receptor had no obvious effect on internalization, but led to a more rapid down-regulation. Thus, these serine-rich regions are not required for receptor desensitization, but are differentially involved in receptor trafficking for the muscarinic M_1 and M_3 receptors.

Keywords: Receptor regulation; Internalization; Down-regulation; Desensitization; Phosphoinositide hydrolysis; Phosphorylation

1. Introduction

G protein-coupled receptors are regulated by phosphorylation at serine and threonine residues. A prominent example is the β_2-adrenoceptor, which desensitizes upon phosphorylation (Benovic et al., 1988). Receptor phosphorylation and subsequent interaction with arrestins has been shown to modulate the internalization of several G protein-coupled receptors, including muscarinic acetylcholine receptors (Pals-Rylaarsdam et al., 1995, 1997; Schlador and Nathanson, 1997; Wu et al., 1997), in addition to serving as an early step in receptor desensitization. In the case of the muscarinic M_2 receptor, two major sites in the third intracellular loop have been shown to be phosphorylated, each with different effects on receptor regulation (Pals-Rylaarsdam and Hosey, 1997). For the muscarinic M_1 and M_3 receptor subtypes, incorporation of multiple phosphates into the third intracellular loops has been demonstrated (Tobin and Nahorski, 1993; Haga et al., 1996); however, the specific sites of phosphorylation and their physiological relevance have not been addressed.

A unique motif occurs in the middle of the third intracellular loop of both the muscarinic M_1 and M_3 receptor subtypes consisting of several serine residues (287SerLeuThrSerSer291/287AlaLeuAlaAlaAla291 (SLTSS) and 349SerAlaSerSer352/349AlaAlaAlaAla352 (SASS), respectively) flanked N-terminally by acidic residues. This motif has been implicated as the target sequence for phosphorylation by the G protein-coupled receptor specific kinase family (Eason et al., 1995; Haga et al., 1996). Phosphorylation of this target sequence followed by binding of arrestins has been implicated in both

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the desensitization and the internalization of G protein-coupled receptors (Ferguson et al., 1995, 1996). Mutation of each of the serine residues and the threonine residue in these sequences to alanine were made previously, producing mutant receptors which, when expressed in human embryonic kidney (HEK293) cells, were deficient in internalization with no apparent defects in stimulating phosphoinositide hydrolysis (Moro et al., 1993). In this report, we examine the effects of these mutations on receptor regulation in detail, including receptor desensitization upon carbachol pretreatment. Furthermore, we assess the effects of these mutations on receptor trafficking using immunofluorescence confocal microscopy, which more sensitively detects internalized receptors concentrated in endocytic vesicles. To address the contribution of phosphorylation of this motif to regulatory events, we examined the phosphorylation state of the muscarinic M_3-SASS/AAAA receptor mutant before and after agonist treatment in comparison to that of the wild-type muscarinic M_3 receptor. Our results indicate that the serine-rich domain located in the third intracellular loop of the muscarinic M_3 receptor contributes to the regulation of internalization and subsequently down-regulation, possibly independently of phosphorylation, but does not affect desensitization. Examination of the analogous mutation in the muscarinic M_1 receptor similarly revealed no direct effects on receptor desensitization, but appeared to modulate cellular trafficking, influencing the net rate of receptor down-regulation. Thus, this putative phosphorylation target sequence appears to serve as a regulatory domain for cellular trafficking, but not desensitization, of the muscarinic M_1 and M_3 receptors.

2. Materials and methods

2.1. Materials

[^{3}H]*N*-methylscopolamine (specific activity 85 Ci/mmol) and [^{3}H]quinuclidinyl benzilate (specific activity 47 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Carbachol was purchased from Sigma (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 (Pittsburgh, PA). All other chemicals were purchased from Sigma.

2.2. Construction of mutants

Construction of receptor mutants M_1-SLTSS/ALAAA and M_3-SASS/AAAA was previously described by Moro et al. (1993).

2.3. Cell culture and selection of stable transfectants

Chinese hamster ovary (CHO) cells were transfected by the calcium phosphate precipitation method (Maeda et al., 1990) using pSG5 vector containing the wild type or mutant receptor 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]*N*-methylscopolamine and [^{3}H]quinuclidinyl benzilate 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.

2.4. Receptor binding in intact cells

Receptor binding studies were as previously described (Shockley et al., 1997). Cells were incubated for 90 min at 12°C with 2 nM [^{3}H]quinuclidinyl benzilate in phosphate buffered saline (PBS) to quantify total receptors and with 2 nM [^{3}H]*N*-methylscopolamine in PBS to measure surface accessible binding sites (Harden et al., 1985). Non-specific binding was determined in the presence of 10 μM atropine. Percent binding values were compared between carbachol treated and untreated cells. Data for the time courses presented are the averages of triplicate measurements from a representative experiment (repeated 2–3 times), and the error bars represent standard deviation.

2.5. Immunofluorescence confocal microscopy

Indirect immunofluorescence of muscarinic M_1 receptors was previously reported (Shockley et al., 1997). For visualization of muscarinic M_3 receptors, carbachol-treated cells were washed with PBS, fixed for 10 min 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, muscarinic M_3 receptors were labeled by incubation of cells with anti-M_3 receptor polyclonal antibody (Ab322) followed by incubation with Cy3-conjugated goat anti-rabbit polyclonal antibody. For colocalization studies, cells were washed four times with PBS and incubated with anti-clathrin monoclonal antibody, followed by PBS wash and incubation with Cy5-conjugated goat anti-mouse antibody. Slides were mounted using Fluoromount G (Fisher Scientific, Pittsburgh, PA) containing a trace amount of phenylenediamine and were stored at 4°C (Tolbert and Lameh, 1996). Samples were visualized using a laser scanning confocal microscopy with a krypton–argon laser coupled with a BioRad MRC-600 confocal head attached to an Optiphot II Nikon microscope equipped with a Plan Apo 60× objective lens with 1.4 numeric aperture.

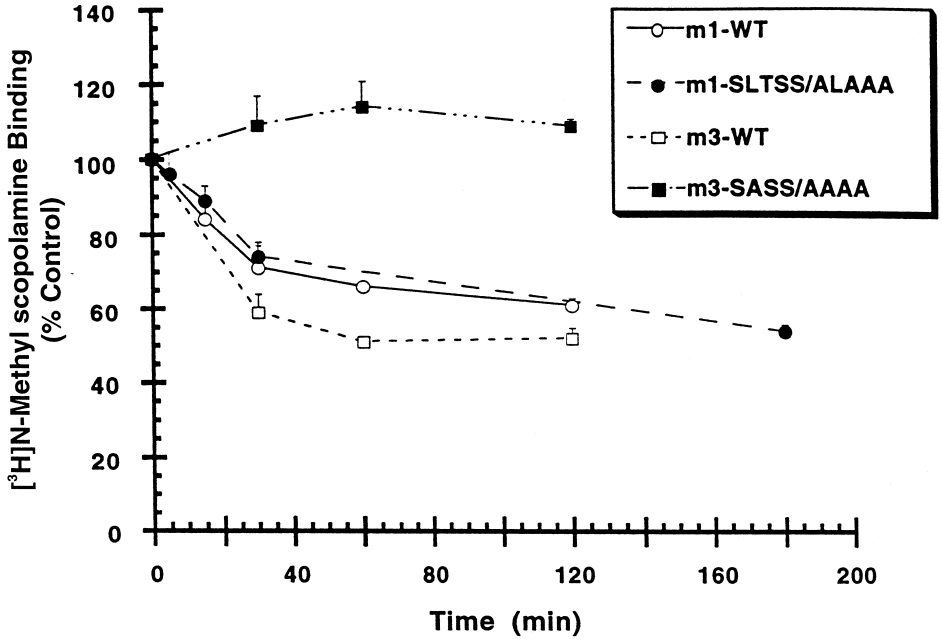

Fig. 1. Time course of carbachol-induced internalization of wild-type and mutant muscarinic M_1 and M_3 receptors. Confluent cells were treated with 1 mM carbachol for the indicated times. Following agonist treatment, cells were washed with PBS and incubated at 12°C with 2 nM [^{3}H]*N*-methylscopolamine for 90 min. Radiolabeled 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.

2.6. Desensitization of carbachol-induced inositol phosphate release

Cells stably expressing wild-type or mutant muscarinic receptors were assayed for carbachol-stimulated phosphoinositide hydrolysis. Cells were plated onto 6-well culture dishes and allowed to reach ~80% confluency. Subconfluent cells were labeled with [^{3}H]myoinositol (0.2 μM) at 37°C overnight. Following inositol labeling, cells were incubated in serum-free media with or without carbachol

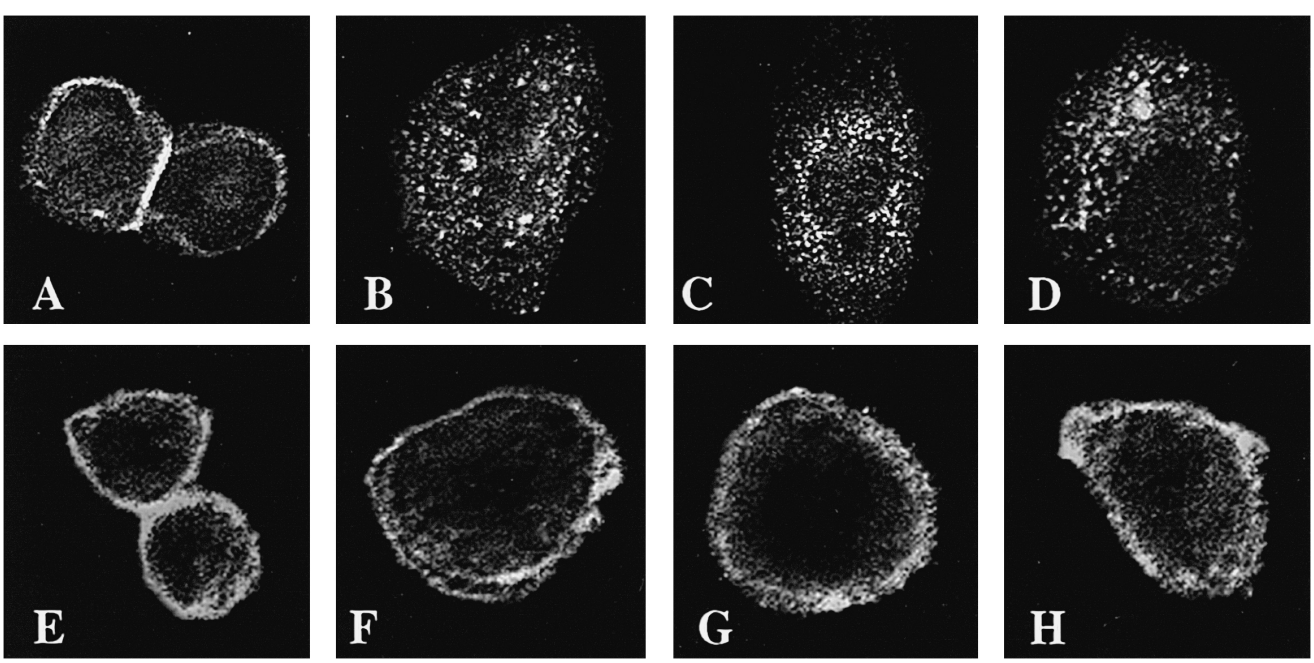

Fig. 2. Immunolocalization of wild-type and mutant muscarinic M_3 receptors by confocal microscopy. CHO cells expressing either wild-type or mutant muscarinic M_3 receptors were treated with carbachol for the indicated times, fixed, permeabilized, and visualized as described in Section 2. (A–D) Wild-type muscarinic M_3 receptor. (E–H) Mutant M_3-SASS/AAAA. (A and E) No carbachol treatment. (B and F) 30 min carbachol treatment. (C and G) 60 min carbachol treatment. (D and H) 120 min carbachol treatment. Data are representative of four independent experiments.

(1 mM) for 2 h. Following pretreatment, cells were washed four times with PBS and then challenged with 1 mM carbachol at 37°C for the indicated time periods (0–5 min). Reactions were stopped by removal of carbachol-containing media and addition of methanol to each well. Cells were scraped and inositol phosphates recovered by methanol/chloroform extraction. Inositol-1,4,5-trisphosphate (IP_3) was isolated as previously described (Maeda et al., 1990; Arden et al., 1992). Results are expressed as the fold increases in eluted dpm over basal dpm.

2.7. In vivo phosphorylation and immunoprecipitation of muscarinic M_3 wild-type and M_3-SASS/AAAA receptors

CHO cells expressing wild type and mutant receptors were plated onto 6 well culture dishes and allowed to reach ~50% confluence. The cells were washed once with phosphate-free Krebs/HEPES buffer (4.2 mM $NaHCO_3$, 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 mCi [^{32}P] orthophosphate for 1 h at 37°C. Cells were then challenged with carbachol (1 mM) for the indicated time periods (0–15 min). 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 min solubilization on ice, the samples were cleared by centrifugation and the muscarinic M_3 receptors were immunoprecipitated as described previously (Tobin and Nahorski, 1993). Briefly, solubilized samples were incubated with the muscarinic M_3 receptor specific antiserum (Ab332) for 60 min. The immune complexes were isolated on protein A sepharose beads and resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (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 were analyzed using ImageQuant™ (Molecular Dynamics, Sunnyvale, CA).

3. Results

3.1. Expression and ligand binding properties of wild-type and mutant muscarinic receptors

Wild-type and mutant receptor cDNAs were cotransfected with a neomycin resistance gene into Chinese hamster ovary cells lacking endogenous muscarinic receptors. Colonies resistant to G418 were isolated and screened for receptor expression with [^{3}H]*N*-methylscopolamine and [^{3}H]quinuclidinyl benzilate. Cell lines with the greatest expression levels were chosen for this study to facilitate the use of confocal microscopy for monitoring receptor trafficking. B_{max} for wild-type muscarinic M_3 receptor expression was approximately 2.5 pmol/mg protein, and mutant M_3-SASS/AAAA expressed at approximately 4.0 pmol/mg protein. B_{max} for wild-type muscarinic M_1 receptor expression was approximately 2.0 pmol/mg protein while mutant M_1-SLTSS/ALAAA expression was fourfold lower at 0.5 pmol/mg protein. We were unable to isolate a clonal line of M_1-SLTSS/ALAAA expressing at wild-type levels. Comparison of binding sites detected by [^{3}H]*N*-methylscopolamine (surface sites) and [^{3}H]quinuclidinyl benzilate (total sites) indicated that >90% of the total receptor pool was detected at the cell surface for both wild-type and mutant muscarinic M_1 and M_3 receptors. Agonist and antagonist binding affinities were unaffected

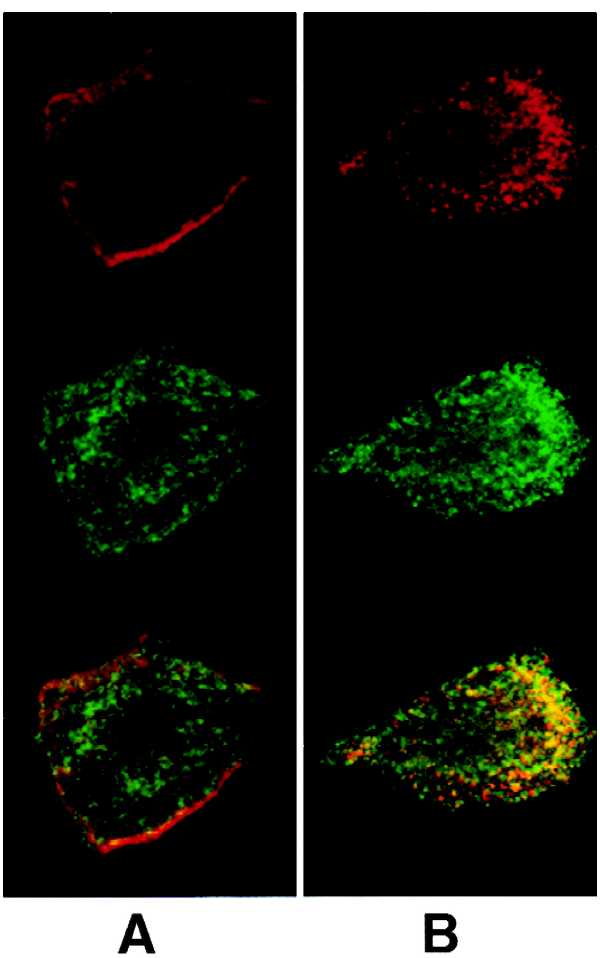

Fig. 3. Colocalization of muscarinic M_3 receptors with clathrin. CHO cells stably expressing wild-type muscarinic M_3 receptors were treated with 1 mM carbachol for 30 min, fixed, permeabilized and sequentially labeled with anti-M_3 receptor and anti-clathrin antibodies as outlined in Section 2. The red color indicates the localization of muscarinic M_3 receptors (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 min carbachol treatment. Data are representative of three independent experiments.

by the mutations for both muscarinic M_1 and M_3 receptors (data not shown).

3.2. Carbachol induced changes in cell surface expression

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 (2 nM) of [^{3}H]*N*-methylscopolamine (Fig. 1). Carbachol induced a decrease in wild-type muscarinic M_3 receptor surface sites that was detectable after 15 min of treatment and this loss plateaued after 30 min. In contrast, no significant change in [^{3}H]*N*-methylscopolamine binding was observed for mutant M_3-SASS/AAAA receptors. Loss in [^{3}H]*N*-methylscopolamine binding sites was evident for wild-type muscarinic M_1 receptors after 15 min and continued to decrease for the 2 h monitored with a 25% loss in binding sites observed at 2 h. A similar decrease in [^{3}H]*N*-methylscopolamine binding was observed for mutant M_1-SLTSS/ALAAA. Varying levels of receptor expression did not affect the extent of receptor internalization (data not shown).

3.3. Immunolocalization of wild-type and mutant muscarinic M_3 receptors in CHO cells

Prior to agonist treatment, wild-type muscarinic M_3 receptors resided predominantly at the cell surface (Fig. 2A). After carbachol treatment, receptors were localized to intracellular vesicles and surface expression of the receptor was reduced (Fig. 2B–D). Immunofluorescence imaging suggested a more dramatic decrease in surface expression of muscarinic M_3 receptors following carbachol treatment than measured by [^{3}H]*N*-methylscopolamine binding. This discrepancy results from the techniques used to image the cells. The power of the confocal microscope is that it allows for optical sectioning permitting the visualization of internal vesicles localized to the midsection of the cell without any interfering signals from the other sections like the cell surface. Furthermore, due to the high concentration of receptors clustered in vesicles, a strong signal is observed. To image under these conditions, the gain of the instrument was decreased, which resulted in a less efficient acquisition of the lower intensity signal corresponding to the surface of the cell. Because of these issues, quantitating of confocal micrographs is less reliable. Therefore, we used binding data to more accurately quantify receptor number in this study.

[^{3}H]*N*-methylscopolamine binding following agonist treatment suggested no loss in surface expression of muscarinic M_3-SASS/AAAA receptors (Fig. 1). As this mutant was expressed at relatively high levels ($\sim$ 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 undetectable by binding studies. In the absence of agonist, muscarinic

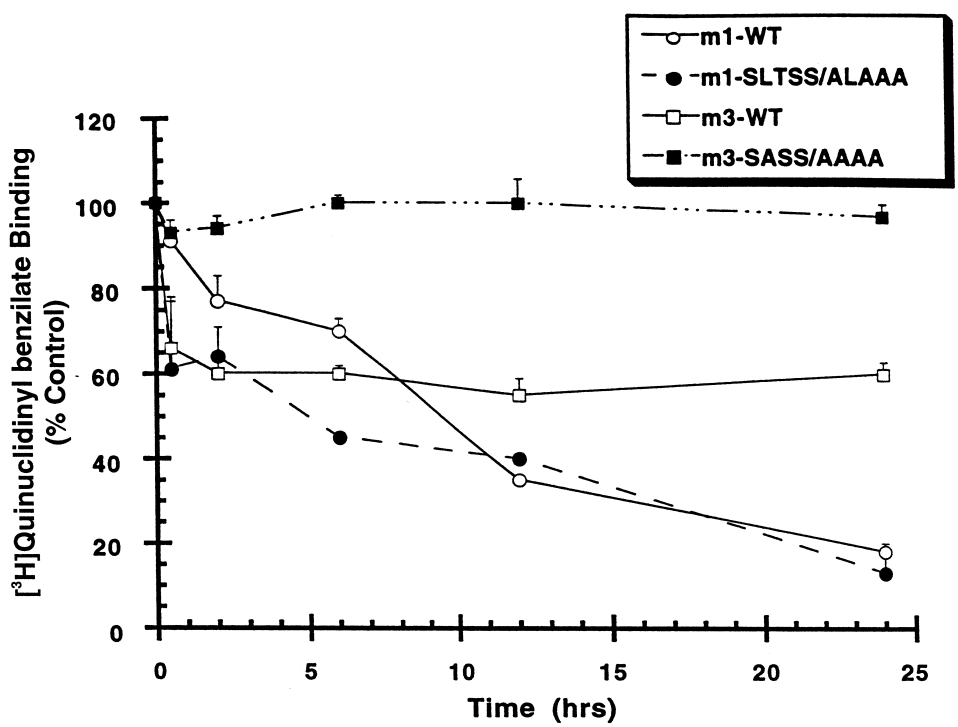

Fig. 4. Down-regulation of wild-type and mutant muscarinic M_1 and M_3 receptors. Cells expressing wild-type or mutant receptors were treated with 1 mM carbachol and the total receptor number was assessed by [^{3}H]quinuclidinyl benzilate binding. Briefly, cells were incubated with 2 nM [^{3}H]quinuclidinyl benzilate for 90 min following agonist treatment, harvested in PBS, and then radiolabeled 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.

M_3-SASS/AAAA receptors were expressed primarily at the cell surface (Fig. 2E). No agonist-induced redistribution of M_3-SASS/AAAA receptors, i.e., internal receptor-containing vesicles, was observed over the 2 h treatment period (Fig. 2F–H).

To characterize the internalized vesicles, we used dual-label confocal microscopy to simultaneously localize muscarinic M_3 receptors and clathrin within the cell. In the absence of agonist, muscarinic M_3 receptors were localized to the cell surface and did not colocalize with clathrin (Fig. 3A). Following agonist treatment, wild-type receptors were located in intracellular vesicles containing clathrin (Fig. 3B). Mutant M_3-SASS/AAAA receptors did not colocalize with clathrin in the absence or in the presence of carbachol, consistent with a defect in internalization (data not shown).

3.4. Down-regulation of muscarinic M_1-SLTSS/ALAAA and M_3-SASS/AAAA receptors

To determine the general relevance of this serine/threonine domain in muscarinic receptor trafficking, we tested the ability of wild-type and mutant muscarinic M_1 and M_3 receptors to undergo carbachol-induced down-regulation. Total muscarinic M_1 and M_3 wild-type receptor number, monitored by [^{3}H]quinuclidinyl benzilate binding, decreased following prolonged stimulation with carbachol (Fig. 4). Carbachol (1 mM) elicited a rapid decrease in wild-type muscarinic M_3 receptor number (~40% loss after 4 h) with no additional loss observed over the 24 h treatment period. Loss of muscarinic M_1 receptors was more gradual with 75% of the receptors detectable at 4 h and 20% of the receptor pool remaining after 24 h of carbachol stimulation. Carbachol stimulation of CHO cells expressing muscarinic M_3-SASS/AAAA receptors resulted in no significant change in total receptor number even after 24 h. In contrast, mutation of the muscarinic M_1 receptor (M_1-SLTSS/ALAAA) appeared to accelerate receptor down-regulation (Fig. 4). Cells expressing different levels of receptors displayed similar extent of down-regulation (data not shown).

3.5. Desensitization of the carbachol-induced IP_3 response

Peak stimulation of IP_3 by wild-type muscarinic M_3 and mutant M_3-SASS/AAAA receptors occurred within 15 s

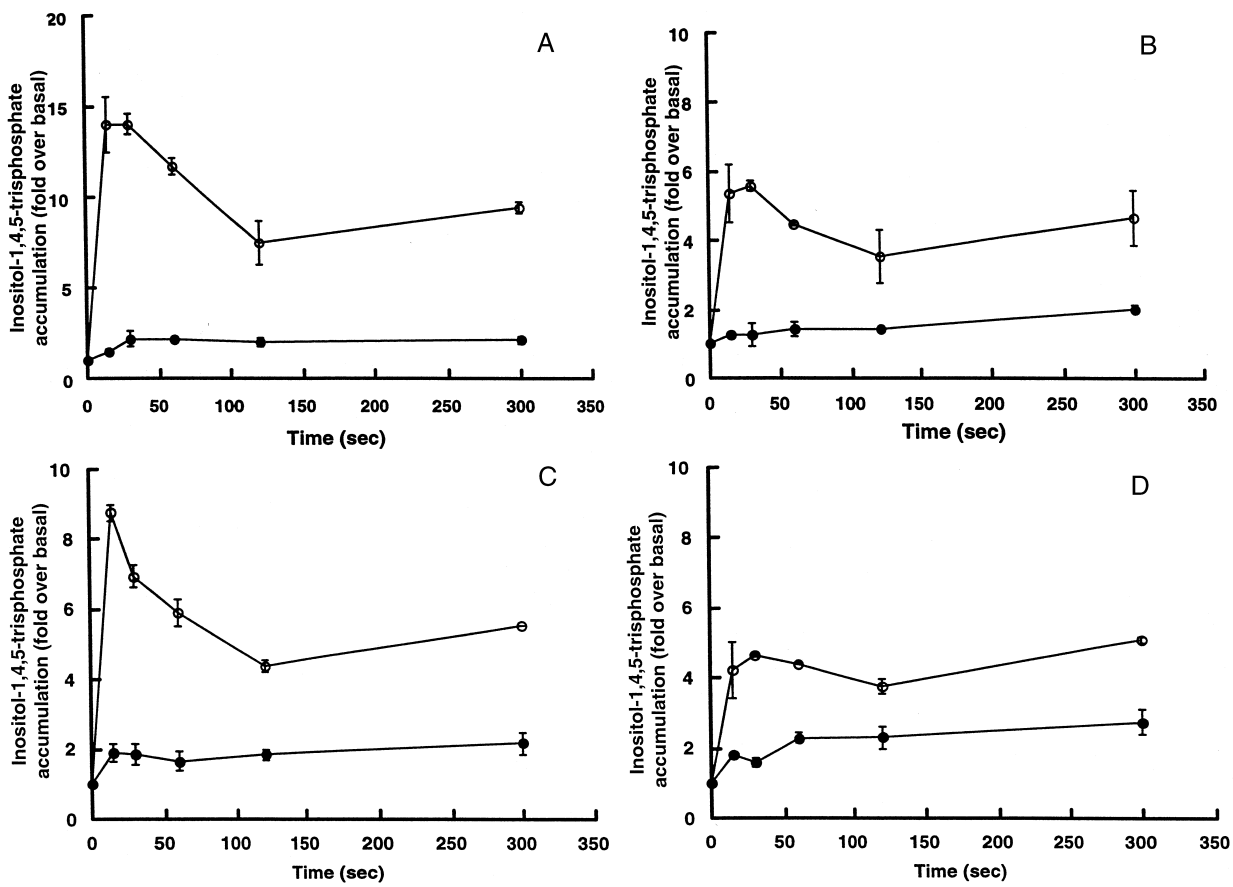

Fig. 5. Desensitization of muscarinic M_1 and M_3 receptor signaling. Cells stably expressing wild-type or mutant muscarinic receptors were labeled with [^{3}H]myoinositol overnight. Following inositol labeling, cells were pretreated with carbachol for 2 h, washed four times with PBS, and then challenged with 1 mM carbachol for the indicated times (0–5 min). IP_3 were isolated and quantified as described in Section 2. Results are expressed as the fold increase in eluted IP_3 over basal levels. (A) Wild-type muscarinic M_3 receptor. (B) Mutant M_3-SASS/AAAA. (C) Wild-type muscarinic M_1 receptor. (D) Mutant M_1-SLTSS/ALAAA. No carbachol pretreatment (open circles), pretreated with carbachol for 2 h (closed circles). Data are representative of three to six independent experiments.

and both peak responses were attenuated with carbachol pretreatment. The plateau phase of IP_3 release, or the desensitization-resistant component of muscarinic M_3 receptor activity (Tobin et al., 1992), was unaffected by agonist pretreatment in either case (Fig. 5A–B). Analysis of a time course of IP_3 release by wild-type muscarinic M_1 receptors similarly revealed an initial peak stimulation within 15 s of 1 mM carbachol treatment which then fell to levels four-fold above basal (Fig. 5C). Pretreatment of wild-type muscarinic M_1 receptors with 1 mM carbachol for 2 h abolished the peak response while maintaining the plateau phase. Stimulation of muscarinic M_1-SLTSS/ALAAA receptors with carbachol resulted in an inositol phosphate peak of the same magnitude as wild-type, which was again absent following agonist pretreatment (Fig. 5D).

3.6. In vivo phosphorylation of wild-type and mutant muscarinic M_3 receptors in response to carbachol

Immunoprecipitation of muscarinic M_3-SASS/AAAA receptors from CHO cells revealed basal phosphorylation of this mutant receptor comparable to that seen with wild-type muscarinic M_3 receptors (Fig. 6). Phosphorylation of wild-type muscarinic M_3 receptors was very rapid, occurring within seconds with the three-fold increase over baseline maintained for at least 15 min (Fig. 6A). The time course of muscarinic M_3-SASS/AAAA receptor phosphorylation indicated that phosphorylation of this mutant was also rapid and of the same magnitude as wild-type muscarinic M_3 receptors (Fig. 6B).

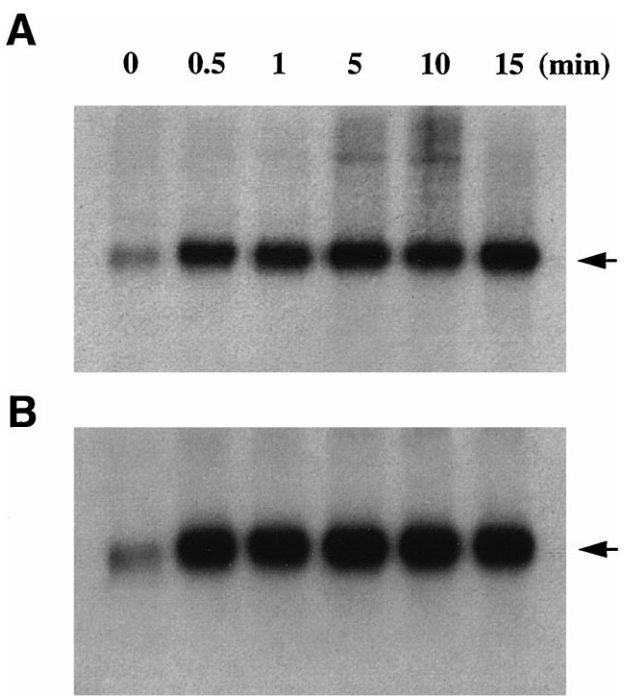

Fig. 6. Phosphorylation of wild-type and mutant muscarinic M_3 receptors. Cells loaded with [^{32}P]orthophosphate were treated with or without 1 mM carbachol for the indicated times. The receptors were immunoprecipitated and resolved by SDS–gel electrophoresis as described in Section 2. To ensure equal loading of protein samples, the gel was stained with Coomassie blue prior to autoradiography. Arrows indicate the ~100 kDa band specifically corresponding to muscarinic M_3 receptors as previously reported (Tobin and Nahorski, 1993). (A) Mutant m3-SASS/AAAA. (B) Wild-type muscarinic M_3 receptor.

4. Discussion

This study examined the role of putative phosphorylation target sequences in desensitization, internalization, and down-regulation of muscarinic M_1 and M_3 receptors. Our earlier studies had identified the serine-rich domain SLTSS in the third intracellular loop of the muscarinic M_1 receptor and the homologous SASS domain in the muscarinic M_3 receptor as potential regulatory domains. Mutations of all residues to alanine in these domains impaired internalization in transfected HEK293 cells (Moro et al., 1993). Furthermore, both of these sequences have been predicted as sites of phosphorylation by G protein-coupled receptor specific kinases or similarly related kinases (Tobin and Nahorski, 1993; Tobin et al., 1996; Haga et al., 1996). Phosphorylation by G protein-coupled receptor specific kinases 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 muscarinic M_1 and M_3 receptors, respectively, may serve as important sites of molecular regulation of muscarinic receptor activity.

To further examine the regulation of muscarinic M_1 and M_3 receptors, we revisited the contribution of the serine-rich domains in the third intracellular loop in regulating internalization in CHO cells, which display receptor down-regulation, in contrast to HEK293 cells, which do not support this process. Previously, we have reported that muscarinic M_1 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 muscarinic M_3 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, 1995; Koenig and Edwardson, 1996). In this study, we were able to monitor the internal accumulation of muscarinic M_3 receptors in response to agonist treatment using immunofluorescence confocal microscopy, thereby establishing that muscarinic M_3 receptors are indeed internalized in CHO cells. Furthermore, muscarinic M_3 receptors colocalized with clathrin in intracellular vesicles following carbachol treatment, suggesting internalization via a clathrin-mediated pathway in CHO cells. Analysis of the contribution of the serine-rich region to internalization of muscarinic M_3 receptors in CHO cells revealed that mutation of the SASS domain to AAAA

abolished internalization with no apparent vesicular accumulation of receptor following 2 h of carbachol stimulation. However, mutation of the corresponding muscarinic M_1 sequence SLTSS to ALAAA had no apparent effect on carbachol-induced loss of muscarinic M_1 receptor sites from the cell surface. We were unable to visualize the distribution of muscarinic M_1-SLTSS/ALAAA receptors in CHO cells as the maximum expression levels of this mutant in several cell lines tested were below the detection limits of the confocal microscope. Nonetheless, tracer binding studies indicated that rapid internalization of the muscarinic M_1 receptor mutant did occur and was blocked by hyperosmolar sucrose conditions (data not shown), consistent with internalization via clathrin-coated vesicles. Thus, only the SASS region in the muscarinic M_3 receptor, but not the homologous SLTSS region in the muscarinic M_1 receptor, is essential for internalization. The apparently normal loss of muscarinic M_1-SLTSS/ALAAA surface binding sites is thus in contrast to its, at least, partially deficient internalization in HEK293 cells (Moro et al., 1993). These recent observations may reflect cell type-specific differences in cellular trafficking pathways, specifically with respect to the presence of down-regulation in CHO cells, but not in HEK293 cells.

We next addressed the contribution of the serine-rich domain located in the third intracellular loops of both muscarinic M_1 and M_3 receptors in modulating receptor down-regulation. Mutation of the SASS domain in the muscarinic M_3 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 muscarinic M_3 receptor, and the absence of down-regulation could be the result of complete impairment of the requisite upstream internalization event. Since sucrose treatment blocks muscarinic M_3 receptor down-regulation (data not shown) presumably by a mechanism involving the specific inhibition of clathrin-mediated internalization, the more likely hypothesis is that the SASS domain in the muscarinic M_3 receptor is specifically involved in agonist-induced trafficking via clathrin-coated vesicles. In contrast to complete impairment of muscarinic M_3-SASS/AAAA internalization and down-regulation, no deficit in the extent of down-regulation was observed with muscarinic M_1-SLTSS/ALAAA receptors. On the contrary, muscarinic M_1-SLTSS/ALAAA receptors appeared to undergo down-regulation more rapidly than wild-type muscarinic M_1 receptors. This result was surprising since the SLTSS/ALAAA mutation was found to impair internalization in HEK293 cells (Moro et al., 1993) and thus, was expected to reduce down-regulation as was seen by Yang et al. (1995) for a muscarinic M_3 receptor mutant which blocked internalization in HEK293 cells, and correspondingly, down-regulation in CHO cells. The increase in the rate of down-regulation could be due to the SLTSS/ALAAA mutation interfering with receptor transport from endosomes to the plasma membrane, so that the mutant receptors, instead of being recycled, are targeted to the lysosome for degradation. This explanation would leave open the possibility that internalization is, in fact, deficient in this mutant. Thus, the loss of surface sites measured by the hydrophilic ligand *N*-methylscopolamine could reflect receptors that would have otherwise returned to the cell surface in the absence of mutation. Resolving the molecular mechanisms governing the trafficking of wild-type and mutant muscarinic M_1 receptors will require additional work.

Considerable evidence exists for the role of G protein-coupled receptor specific kinases in the internalization of G protein-coupled receptors. The serine/threonine-rich sites of the muscarinic M_1 and M_3 receptors examined in this study are two examples of conserved domains among muscarinic receptors which are putative sites for G protein-coupled receptor specific kinase-mediated phosphorylation (Nakata et al., 1994; Eason et al., 1995; Haga et al., 1996). Because the muscarinic M_3 receptor mutant SASS/AAAA displayed pronounced defects in trafficking, we were interested in investigating the possibility that phosphorylation at this site was involved in receptor internalization and down-regulation. Agonist-induced phosphorylation of muscarinic M_3 receptors by a kinase distinct from protein kinase C, protein kinase A, and G protein-coupled receptor specific kinases has been shown to occur at a region of the third intracellular loop containing the SASS sequence (Tobin et al., 1996). In this study, we found that phosphorylation of muscarinic M_3-SASS/AAAA receptors was comparable to wild-type muscarinic M_3 receptors. 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 G protein-coupled receptor specific kinases or 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 is possible that while phosphorylation at this site is required for internalization, possibly via arrestin binding, alternative phosphorylation sites exist elsewhere in the receptor. In this case, wild-type levels of phosphorylation could be explained by phosphorylation at alternative receptor sites that are less relevant in the wild-type muscarinic M_3 receptor. This idea is consistent with the work of Haga et al. (1996) showing that phosphorylation of the muscarinic M_1 receptor was greater when sequentially phosphorylated by β-adrenoceptor kinase followed by protein kinase C than when phosphorylated by the two kinases in the reverse order.

Previously, it was shown that muscarinic M_3 receptors rapidly desensitize in CHO cells, independent of internal-

ization, but in a phosphorylation-dependent manner (Tobin and Nahorski, 1993; Tobin et al., 1992). The muscarinic M_3 receptor has two serine-rich domains in the third intracellular loop, which may serve as phosphorylation sites functioning in desensitization. We focused on the C-terminally located SASS region of the muscarinic M_3 receptor since this region, and not the N-terminal serine-rich region, is located within the region of the loop postulated as the target sequence for G protein-coupled receptor specific kinases or similarly related kinases (Tobin et al., 1996). Likewise, the SLTSS region of the muscarinic M_1 receptor is predicted as the specific sequence phosphorylated by G protein-coupled receptor specific kinases and thus has been predicted to play a role in muscarinic M_1 receptor desensitization (Haga et al., 1996). In this study, the muscarinic M_1-SLTSS/ALAAA and M_3-SASS/AAAA receptor mutants displayed no deficits in the ability to desensitize. We cannot rule out the possibility that the rapid peak in IP_3 release is blunted after carbachol pretreatment because of other factors, such as depletion of inositol-4,5-bisphosphate (see Tobin et al., 1996 for a discussion). It is also possible that desensitization of the muscarinic M_3 receptor is mediated by the N-terminal serine-rich domain while the C-terminal SASS region is specific to regulation of internalization. Desensitization of the muscarinic M_1 receptor may then be mediated by the phosphorylation of receptor sites other than those predicted as target sequences for G protein-coupled receptor specific kinase. Further examination of the function of other potential phosphorylation sites in both muscarinic M_1 and M_3 receptors will be required to identify the domains specifically governing receptor desensitization.

In summary, the predicted kinase target domains, SLTSS in the muscarinic M_1 receptor and SASS in the muscarinic M_3 receptor, are differentially involved in the regulation of these muscarinic receptor subtypes. The SASS domain in the muscarinic M_3 receptor is required for receptor trafficking, but a direct correlation with phosphorylation could not be determined. The SLTSS region in the muscarinic M_1 receptor appears to modulate receptor trafficking, possibly at multiple sites along the trafficking pathway. Whereas trafficking of the muscarinic M_3-SASS/AAAA mutant cannot be observed, for the equivalent muscarinic M_1-SLTSS/ALAAA mutant, we find substantial receptor loss from the cell surface and accelerated down-regulation, possibly by reduced receptor recycling to the cell surface. This muscarinic M_1 receptor mutant is of interest as it is a singular example of selective acceleration of down-regulation. However, the molecular mechanisms of down-regulation remain unresolved. Contrary to prediction, neither the SASS domain in the muscarinic M_3 receptor nor the SLTSS domain in the muscarinic M_1 receptor is crucial for desensitization of carbachol-induced phosphoinositide signaling. Further studies will be needed to clarify the roles that other putative phosphorylation domains have in the regulation of muscarinic receptor activity.

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