Desensitization and Internalization of the m2 Muscarinic Acetylcholine Receptor Are Directed by Independent Mechanisms*

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The phenomenon of acute desensitization of G-protein-coupled receptors has been associated with several events, including receptor phosphorylation, loss of high affinity agonist binding, receptor:G-protein uncoupling, and receptor internalization. However, the biochemical events underlying these processes are not fully understood, and their contributions to the loss of signaling remain correlative. In addition, the nature of the kinases and the receptor domains which are involved in modulation of activity have only begun to be investigated. In order to directly measure the role of G-protein-coupled receptor kinases (GRKs) in the desensitization of the m2 muscarinic acetylcholine receptor (m2 mAChR), a dominant-negative allele of GRK2 was used to inhibit receptor phosphorylation by endogenous GRK activity in a human embryonic kidney cell line. The dominant-negative GRK2K220R reduced agonist-dependent phosphorylation at serine 356 and prevented acute desensitization of the receptor as measured by the ability of the m2 mAChR to attenuate adenyl cyclase activity. In contrast, the agonist-induced internalization of the m2 mAChR was unaffected by the GRK2K220R construct. Further evidence linking receptor phosphorylation to acute receptor desensitization was obtained when two deletions of the third intracellular loop were made which created m2 mAChRs that did not become phosphorylated in an agonist-dependent manner and did not desensitize. However, the mutant mAChRs retained the ability to internalize. These data provide the first direct evidence that GRK-mediated receptor phosphorylation is necessary for m2 mAChR desensitization; the likely sites of in vivo phosphorylation are in the central portion of the third intracellular loop (amino acids 282–323). These results also indicate that internalization of the m2 receptor is not a key event in desensitization and is mediated by mechanisms distinct from GRK phosphorylation of the receptor.

A logarithmic receptor phosphorylation has been demonstrated in an increasing number of studies involving members of the G-protein-coupled receptor (GPR) superfamily. These phosphorylation events have been best studied with the visual GPR, rhodopsin, and the β2-adrenergic receptor (β2-AR), where rapid phosphorylation of these receptors by a member of the G-protein-coupled receptor kinase (GRK) family underlies one or more aspects of receptor desensitization (1, 2). In these models, GRK-mediated receptor phosphorylation is believed to cause rapid desensitization by allowing a member of the arrestin family of proteins to bind the phosphorylated receptor (1, 2). Arrestin binding attenuates the ability of the receptors to modulate activity of downstream effectors by preventing further receptor/G-protein coupling.

Upon agonist activation, many GPRs are also internalized, or sequestered away from the cell surface into an altered cellular environment where they are unable to bind hydrophilic ligands. The role that internalization plays in modulation of receptor activity remains largely undefined and may well differ for different types of GPRs. For the β2-AR, increasing evidence points to receptor internalization being independent of the loss of receptor signaling, but essential for recycling of the desensitized GPRs (3–5). However, with other GPRs, internalization may well play a pivotal role in the termination of GPR signaling.

The muscarinic acetylcholine receptors (mAChR) have been the subject of many studies regarding the basis of GPR desensitization (6). There are five subtypes of mAChR (7–14) which couple to two distinct signaling pathways. The m1, m3, and m5 mAChR activate phospholipase C, while the m2 and m4 subtypes activate adenyl cyclase activity and in some cell systems weakly activate phospholipase C (12, 13, 15–18). The m2 subtype of mAChR has been the focus of several desensitization studies designed to understand the molecular basis of desensitization. In intact cells, agonists induce phosphorylation of receptors to a stoichiometry of 3–5 mol of phosphate per mol of receptor (19–21). These “in vivo” phosphorylated receptors are desensitized, as seen in their decreased ability to activate G-proteins to either bind GTP-γS or hydrolyze GTP (19), or to elicit a negative inotropic response (22). The kinase(s) responsible for agonist-dependent mAChR phosphorylation in vivo remain

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† The abbreviations used are: GPR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; ACh, acetylcholine; m2 mAChR, m2 muscarinic acetylcholine receptor; AR, adrenergic receptor; pARK, β-adrenergic receptor kinase; DME, Dulbecco’s modified Eagle’s medium; F-12, Ham’s F-12 medium; HRP, horseradish peroxidase; PCR, polymerase chain reaction; NMS, N-methyl scopolamine; QNB, 1-quinuclidinyl benzilate; LH(R), luteinizing hormone (receptor); PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; GTP-γS, guanosine-5′-O-(3-thiotriphosphate; HEK, human embryonic kidney.
unidentified. The m2 mAChR can be phosphorylated in vitro by members of the GRK family in a manner similar to that observed in vivo (23–26). At present, six members (GRK1–GRK6) of the family are known. Rhodopsin kinase (GRK1) was the first to be identified on the basis of its ability to phosphorylate rhodopsin in a light-dependent manner (27), while p(s)GRK1 (GRK2) was subsequently identified as a kinase that phosphorylated the β2-AR in an agonist-dependent manner (28). Some members of this family recognize the m2 mAChR in vivo in a manner that is exquisitely dependent on agonist occupancy. GRK2 and GRK3 (pGRK2 and pGRK3) are able to phosphorylate the activated m2 mAChR to a high stoichiometry (23–26). Other members of the GRK family, GRK5 and GRK6, also phosphorylate the m2 mAChR in an agonist-dependent manner in vitro, but at a low rate and extent (25, 29, 30). However, the role(s) of these kinases in regulating receptors in intact cells remains largely unexplored, in part because no selective inhibitors of these kinases are available for in vivo work. In this study, we report studies using a dominant-negative allele of GRK2 (GRK2c220R) and receptor mutants that provide new insights into events underlying receptor desensitization in intact cells.

**EXPERIMENTAL PROCEDURES**

Materials—Dulbecco’s modified Eagle’s medium (DME), fetal bovine serum, penicillin-streptomycin, restriction enzymes, and G418 were purchased from Life Technologies, Inc. HEPES-buffered DME:F-12 was purchased from DuPont. Other reagents were from Sigma or previously identified sources (19). The following individuals generously shared reagents: Michael Schimerlik, Oregon Health Sciences University. We gratefully acknowledge the contributions these people have made toward the completion of our work.

**Construction of the Deletion Mutant m2 mAChRs—**

**Transiently transfected cultures of tsA201 cells or stable cell lines at 80–90% confluence were loaded for 4 h with 0.15–0.25 μCi/ml [3H]NMS in phosphate-free DME.** The plates were then treated with drugs for 15 min at room temperature. After two washes with 5 ml of ice-cold PBS, the cells were collected by lifting the cells off the culture plate in 5 ml of buffer A (20 mM NaHPO4, 20 mM Naf, 5 mM EDTA, 5 mM EGTA, and a protease inhibitor mixture) (31) and homogenized for 10 s on ice with a Brinkman polytron OTAJS probe at setting 7. The homogenate was centrifuged at 100,000 × g for 30 min at 4°C. The resulting crude particulate fraction was then solubilized in 1 ml of buffer B (0.3 M NaCl, pH 8.0, 10 mM Tris, pH 7.4), and the resulting clear solution was centrifuged at 100,000 × g for 45 min at 4°C. The solubilized m2 mAChRs were immunoprecipitated by an overnight incubation with polyonal anti-serum made against the 13 loop of the m2 mAChR (32), which had been precipitated to protein A agarose for 2–4 h in buffer B. The immunoprecipitate was washed 8–10 times with 400 μl of buffer B to remove nonspecifically adsorbed proteins from the pellet. The proteins were eluted in the protein A-agarose mixture for 3 h at 95°C in SDS-PAGE sample buffer. The eluted proteins were separated by SDS-PAGE (37) and transferred to nitrocellulose, and the receptors were analyzed by immunoblotting and PhosphorImager analysis.

**Immunoblot Analysis—**

**Nitrocellulose filters (0.2 μm) were blocked for 30 min to 1 h in 5% Carnation powdered milk in Tris-buffered saline.** For experiments using receptors from the stable cell lines, the rat monoclonal antibody ascites fluid (31) was diluted 1:20,000 in 5% milk/TBS. For experiments using receptors from the stable cell lines, the blots were blocked, then incubated with the 1D1 monoclonal antibody to the m2 mAChR. The amount of m2 mAChR was measured using a standard curve, the amount of receptor in purified membranes from Chinese hamster ovary cells stably transfected with the m2 mAChR (34) was assessed by ligand binding and applied to the gels. Receptor

**Intact Cell Phosphorylation and m2 mAChR Immunoprecipitation—**

**Transiently transfected current through m2 mAChR Desensitization Requires GRK-mediated Phosphorylation**

**levels from the pC3 vectors were higher in both transient and stable expression systems than the pCMV5 vector, so the epitope-tagged receptors were used for many of the experiments described in this report, as described in the text.**

**Cell Culture and Transfection—**

TsA201 cells, a clone of human embryonic kidney (HEK) 293 cells stably expressing simian virus 40 large T antigen (26), were cultured in DME supplemented with 10% calf serum, 100 units/ml penicillin, 100 units/ml streptomycin at 37°C in a 5% CO2 environment. Cells were transfected using the calcium phosphate precipitation method followed by a 5–6-min shock with 30% Me2SO in DME. The following amounts of each expression plasmid were used for transfection of each 100-mm culture plate in the combination described in the text: 10 μg of pCMV-m2 mAChR, 0.5 μg of pBCL2-GRK2, 5 μg of pB1221-GRK2c220R, and 5 μg of pCDNA1-LHR. Cells were assayed for receptor phosphorylation, cAMP production, or receptor internalization 60–72 h post-transfection. Stable cell lines were made by transfecting HEK293 cells with the pC3 vectors alone or the pCMV5 vectors with pSV-Neo. G418-resistant clones were selected and analyzed for antigenic binding to determine receptor expression levels. Stable clones were obtained for all receptors except m2A2.

Expression levels for cell lines used in this paper were measured using [3H]NQNB in ligand binding studies as follows: m2, 0.4 pmol/mg total protein; m2A1, 0.2 pmol/mg; m2KT3, 1.0 pmol/mg; m2A1KT3, 2.0 pmol/mg; m2A2KT3, 1.0 pmol/mg.

**m2 mAChR Desensitization Requires GRK-mediated Phosphorylation**

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phosphorylation was assessed by a Fuji Bas2000 PhosphorImager and 32P standards. The specific activity of ATP in the cells was determined by the method of Richardon and Hosey (19).

Receptor-mediated Modulation of CAMP Production—Cells were co-transfected with 10 μg of pcMV-m2 and 5 μg of pCDNA1-LHR encoding the human luteinizing hormone receptor (LHR) with or without 5 μg of pCDNA1-GSK (38). Each 100-mm dish of transfected cells was divided the day after transfection into a 24-well Corning culture plate that had been coated overnight with 0.012% polylysine. On the second day after transfection the cells were incubated overnight with 5% bovine serum albumin in DME, supplemented with 1 μCi/μl [3H]-ja
dene to label ATP stores. The following morning cells were assayed for their ability to the m2mAChR to mediate attenuation of basal or luteinizing hormone (LH)-induced cAMP levels. Cells were preincubated with HEPES-buffered DME:F-12 alone (control) or with medium containing 1 mM carbachol (desensitizing treatment) for 20 min at 37°C. Each 24-well plate had 12 wells as control points and 12 wells exposed to the desensitizing treatment. Following this pretreatment, cells were placed on a bed of ice and washed three times with 0.5 μl of ice-cold PBS. Triplicate wells of cells were then challenged with medium alone, 1 mM carbachol, 5 μg/ml bovine LH, or 1 mM carbachol + 5 μg/ml bovine LH for 10 min at 37°C. All challenge solutions also contained 1 mM 3-isobutyl-1-methyl-xanthine. The reactions were stopped by aspirating the medium and adding 1 ml of 5% trichloroacetic acid. Each well was spiked with 300–400 cpm of [3H]-cAMP to estimate cAMP recovery, the plates were rocked at 4°C for 1–3 h, and the cAMP was recovered by the method of Salomon (39–41). Data were normalized for recovery of cAMP and expressed as a percent of the increase over basal levels of cAMP produced by LH treatment alone. Stable cell lines were treated in the same way, except that 1 μM isoproterenol was used to activate endogenous β-adrenergic receptors to increase adenyl cyclase activity.

Receptor Internalization Assay—The approach used was to measure changes in the number of receptors located on the cell surface using a hydrophilic ligand, [3H]NMS, which cannot cross cellular membranes. On the second day after transfection or when the stable plates were 85–90% confluent, cells from each 100-mm plate were divided equally into five 60-mm plates each, and the cells were allowed to attach overnight. The cells were incubated for 2 h in the presence of 10 μM eserine, an acetylcholinesterase inhibitor, and for 0–120 min of these 2 h in the presence of 1 mM acetylcholine (ACh). Some internalization experiments were performed with 1 mM carbachol in stable cell lines and showed no noticeable difference in response to those performed with ACh. At the end of the incubation time, cells were rinsed 5 times with 5 ml ice cold PBS, removed from the plates by pipetting with ice cold HEPES-buffered DME:F-12, and subjected to radioligand binding with saturating concentrations of [3H]-NMS for 2 h at 4°C. Nonspecific binding was defined in the presence of 100 μM atropine. Protein assays were performed to control for differences in cell density on each treated plate. Data were expressed as a percentage of the [3H]-NMS binding determined in untreated cells. In addition, whole-cell binding assays were performed with the hydrophobic ligand [3H]-QNB to confirm that total binding was unchanged during 2 h of ACh exposure.

Statistical Analysis—A Student’s t test was used to analyze the phosphorylation data. The fold increases in receptor phosphorylation relative to naive cells expressing m2mAChR alone were compared in carbachol-treated cells transfected with the m2mAChR alone versus cells cotransfected with GRK2K220R. The desensitization of adenyl cyclase response was analyzed using paired t tests, comparing the decrease in LH- or isoproterenol-induced cAMP levels produced by carbachol in naive cells versus cells pretreated with agonist.

RESULTS

Effects of Dominant-negative GRK2 on m2 mAChR Phosphorylation—In order to investigate both the identity of the kinase which phosphorylates the agonist-occupied m2mAChR and the functional effects of the phosphorylation event, a dominant-negative allele of GRK2, GRK2K220R, was coexpressed with the m2mAChR in a transient mammalian cell transfection system. This allele of GRK2 is unable to transfer phosphate from ATP to a substrate (38). GRK2K220R has been shown to compete with wild-type GRK2, preventing phosphorylation of the β2-AR in vitro, and reducing desensitization of the β2-AR in human bronchial epithelial cells (38). In the present studies, transfection of GRK2K220R with the m2mAChR in the tsA201 cells resulted in a 20–30-fold overexpression of the mutant kinase compared to endogenous GRK2 levels in the whole preparation (Fig. 1A). As the efficiency of transfection in these cells is 10–20%,2 an individual transfected cell would be estimated to overexpress GRK2K220R by 100–300-fold over endogenous GRK2 levels. Following 32P labeling, cells transfected with DNA encoding the m2mAChR with or without DNA encoding GRK2K220R were exposed to either medium alone or medium containing 1 mM carbachol for 15 min and analyzed for receptor phosphorylation. Variations in protein loading in the lanes were detected by immunoblot and analyzed by densitometry, and changes in receptor phosphorylation were normalized as a function of the amount of m2mAChR in each sample. In control cells, carbachol induced a 3-fold increase in receptor phosphorylation over basal levels (Fig. 1, C and D). The stoichiometry of phosphorylation was calculated to be 3–5 mol of phosphate per mol of receptor in the carbachol-treated cells. In cells also expressing GRK2K220R, the carbachol-induced phosphorylation of the m2mAChR was reduced by almost half (Fig. 1, C and D). This selective loss of phosphorylation caused by coexpression of GRK2K220R implicates GRK2, or another related kinase, for at least part of the agonist-dependent phosphorylation of the m2mAChR.

Effects of Dominant-negative GRK2 on m2 mAChR Desensitization—To determine the effects of GRK-mediated phosphorylation and the consequences of decreased agonist-dependent phosphorylation in cells expressing GRK2K220R, assays for receptor signaling through G-proteins were performed. Activation of the m2mAChR causes inhibition of adenyl cyclase activity by a pertussis toxin-sensitive G-protein (15, 18). For receptors that attenuate adenyl cyclase activity, changes in basal cAMP levels are difficult to measure, and the signaling of such receptors is more readily measured as the ability of the

2 X.-L. Zhao and M. M. Hosey, unpublished observations.
levels were defined as 100%. Cells were pretreated for 20 min with treatments. Basal cAMP levels were normalized to 0%; LH-induced without cDNA encoding GRK2K220R. Following a 20-min pre-co-transfected with m2 mAChR and LHR cDNAs, either with or without cDNA encoding the m2 mAChR and LHR without (A) or with GRK2K220R (B) were assessed for cAMP levels following various drug treatments. Basal cAMP levels were normalized to 0%; LH-induced levels were defined as 100%. Cells were pretreated for 20 min with medium alone (open bars) or for 20 min with 3 mM carbachol (shaded bars) to measure m2 mAChR signaling through adenylyl cyclase and the desensitization of this response. Data shown are the means ± S.E. for three independent experiments performed in triplicate. (*Significantly different from the inhibition of adenylyl cyclase activity measured in naive cells, p < 0.01.)

receptors to attenuate increases in cAMP levels caused by activation of another GPR which activates adenylyl cyclase (42). In addition, because of the low percentage of cells which became transiently transfected, signaling of an inhibitory receptor is better observed when the inhibitory receptor is co-transfected with a receptor that activates adenylyl cyclase (43), such as the LHR. In this way, LH-induced increases in cAMP are limited to the population of cells which presumably express both the inhibitory m2 mAChR and the stimulatory LHR, allowing more sensitive measurements of the m2 mAChR-mediated attenuation of adenylyl cyclase activity. Thus, cells were cotransfected with m2 mAChR and LHR cDNAs, either with or without cDNA encoding GRK2K220R. Following a 20-min pre-treatment with either medium alone, or medium containing carbachol, the cells were challenged with no drug, carbachol, LH, or carbachol + LH for 10 min. LH treatment increased cAMP levels 2–4-fold (average = 3.2 ± 0.3-fold, n = 19). These values were normalized to 100% for each experiment; basal levels were set to 0%. In control cells, carbachol significantly reduced the increase in cAMP levels caused by LH treatment by 60 ± 16%. When these cells were pretreated for 20 min with carbachol to determine if the m2 mAChR would desensitize, LH was able to stimulate cAMP production to a similar extent, but co-application of carbachol with LH caused no significant attenuation of the cAMP response (Fig. 2A). Thus, carbachol pre-treatment induced desensitization of the ability of the m2 mAChR to attenuate cyclase. This indicates that, in tsA201 cells, sufficient cellular machinery exists to cause acute desensitization of m2 mAChR.

In cells that coexpressed GRK2K220R, m2 mAChR desensitization was dramatically changed. Cells pretreated with medium alone showed a similar pattern of m2 mAChR signaling as observed in control cells, in that carbachol caused a significant reduction in the LH-induced increase in cAMP to 60 ± 5% of LH levels. Note that a small but significant reduction in the magnitude of m2 mAChR-mediated cAMP attenuation in cells expressing GRK2K220R was observed, although the response was still readily measured and significantly different than the levels of cAMP observed with LH treatment alone. It is possible that GRK2K220R prevents some Gβγ-mediated attenuation of cyclase activity in these cells, as expression of all or part of this protein has been shown to prevent Gβγ effects in other systems (38, 44). Nonetheless, in contrast to cells transfected with m2 mAChR alone, cells coexpressing GRK2K220R that were pre-treated with carbachol for 20 min did not desensitize and retained their ability to attenuate the cAMP induction caused by LH treatment (78 ± 1% of LH levels, Fig. 2B). This was not significantly different from the response observed in naive cells. Taken together, the ability of GRK2K220R to ablate the phosphorylation and desensitization of the m2 mAChR data suggests that m2 mAChR phosphorylation by GRK2, or another closely related kinase, is required for desensitization of the m2 mAChR cyclase response. In addition, these data are the first direct demonstration of a requirement for agonist-dependent phosphorylation in the acute desensitization of the m2 mAChR.

Effects of Dominant-negative GRK2 on m2 mAChR Internalization—For the m2 mAChR, a role for receptor internalization in desensitization remains to be elucidated. We investigated the involvement of GRK-mediated m2 mAChR phosphorylation in receptor internalization by comparing the rate and extent of receptor internalization in cells expressing the m2 mAChR alone, or coexpressing either GRK2K220R or an overexpressed wild-type allele of GRK2. The hydrophilic antagonist [3H]NMS was used to assess surface receptor number. Changes in the amount of m2 mAChR present on the cell surface were expressed as a percentage of the levels present at zero time of agonist exposure. Exposure of cells expressing m2 mAChR to agonist led to a 40% reduction in the levels of surface receptor by 1 h of exposure and remained relatively steady through the 2-h time point (Fig. 3). Neither coexpression of GRK2K220R nor overexpression of wild-type GRK2 changed the rate or extent of receptor internalization. Parallel experiments using the hydrophobic antagonist [3H]IONB confirmed that total receptor number did not change under similar conditions (data not shown), indicating that loss of [3H]IONB binding represents internalization and not degradation of the m2 mAChR. Thus, at an agonist concentration where clear differences were observed in receptor phosphorylation and desensitization in the presence of GRK2K220R, internalization was unaffected. This suggests that m2 mAChR internalization is neither sufficient for, nor equivalent to, desensitization of the cAMP signaling pathways. The data also suggest that GRK-mediated m2 mAChR phosphorylation is not integral to the process of internalization. Alternatively, since the dominant-negative GRK2 allele only reduced
agonist-dependent phosphorylation by 50%, phosphorylation of fewer sites may required for receptor internalization than for desensitization. The remaining phosphorylation observed in the presence of GRK2/GRK3 may also be due to the action of another kinase which is involved in the internalization event.

Deletion Mutagenesis and Phosphorylation of the m2 mAChR—An important goal is the identification of the structural determinants of receptor phosphorylation, desensitization, and internalization. In order to begin to address this goal, we constructed two deletion mutants of the m2 mAChR to test whether they would be deficient in receptor phosphorylation and/or desensitization. The regions chosen for deletion mutagenesis were (S/T)-rich and relatively acidic, suggesting they might contain potential GRK phosphorylation sites (45). The receptors lacked amino acids 282–323 (m2Δ1), or 250–323 (m2Δ2), from the central region of the third intracellular (i3) loop (Fig. 4A). Previous studies have shown the agonist-dependent phosphorylation of the m2 mAChR to be predominantly on serine residues, and the only cytoplasmically localized serine residues are found in the i3 loop (21). In addition, an m2 mAChR mutant with a somewhat larger deletion in the i3 loop was previously shown to be phosphorylation-deficient in vitro assays (46).

Both deletion mutants bound the antagonist [3H]QNB and the agonist carbachol with affinities similar to the full-length receptor, as analyzed by Scatchard analysis and agonist–competition curves in whole cell binding studies (data not shown). All three forms of the m2 mAChR were tagged with a modified KT3 epitope. These tagged receptors also bound ligands appropriately (data not shown). Stable HEK cell lines expressing these receptors were prepared and used to measure agonist-dependent phosphorylation, desensitization, and internalization. Like the transiently expressed m2 mAChR, the stably expressed full-length receptor was phosphorylated in an agonist-dependent manner (Fig. 4, B and C). However, neither the m2Δ1 deletion mutant (Fig. 4, B and C), nor the m2Δ2 mutant (data not shown) became phosphorylated over basal levels following exposure to carbachol. Because the smaller deletion was as deficient in agonist-induced phosphorylation as the larger deletion, we conclude that the likely sites for agonist-dependant phosphorylation are located in the i3 loop within residues 282–323.

Desensitization of the Deletion Mutant m2 mAChRs—To further test the link between agonist-induced phosphorylation and desensitization of the m2 mAChR, stably expressed wild-type and mutant receptors were tested for their ability to become desensitized following exposure to agonist. This was assessed by measuring the m2 mAChR-mediated reduction in adenyl cyclase activity following stimulation of adenyl cyclase by activation of endogenous β-ARs. Treatment of cells expressing full-length m2 mAChR (0.4 pmol/mg of protein) with isoproterenol activated the β-ARs, causing an increase in cellular cAMP levels (Fig. 5A). As in the transient assays, this increase was set at 100% basal levels of cAMP were set at 0%. Coapplication of carbachol with isoproterenol reduced the cAMP levels to 35 ± 10% of that seen with isoproterenol alone (Fig. 5A). When cells expressing full-length m2 mAChR were pretreated with carbachol for 20 min, the m2 mAChR became profoundly desensitized; coapplication of carbachol and isoproterenol resulted in cAMP levels 88 ± 3% of that seen with isoproterenol alone (Fig. 5A). Thus, as in the transient expression system, m2 mAChR desensitization was observed in stable HEK cell lines.

We next tested the deletion mutants for their ability to signal and desensitize. When cells expressing m2Δ1 (0.2 pmol/mg of protein) were challenged with carbachol and isoproterenol for the cAMP assay, CAMP levels were 31 ± 8% of those seen with isoproterenol alone (Fig. 5B). Thus, this deletion mutant receptor was able to attenuate adenyl cyclase to an extent similar to wild-type receptors. However, the mutant receptor did not desensitize: in m2Δ1 cells pretreated with carbachol for 20 min the levels of cAMP were measured as 36 ± 6% (Fig. 5B) of isoproterenol levels. Thus, there was no significant change in the ability of the m2Δ1 mAChR to signal through adenyl cyclase following carbachol pretreatment. Similarly, the m2Δ2 mAChR showed no desensitization of its ability to attenuate adenyl cyclase following 20 min of carbachol pretreatment (data not shown). When the carbachol pretreatment was extended to 1 h, the m2Δ1 receptor showed only a small, statistically insignificant reduction in its ability to attenuate adenyl-
m2 mAChR Desensitization Requires GRK-mediated Phosphorylation

**Fig. 5.** Analysis of m2Δ1 coupling to adenylyl cyclase and desensitization. Stable cell lines expressing full-length m2 mAChR (A) or m2Δ1 mAChR (B and C) were pretreated with medium alone (open bars) or 1 mM carbachol (shaded bars) for 20 min (A and B) or 60 min (C), and the ability of the receptors to attenuate β2-AR-induced increases in adenylyl cyclase activity was measured. Data shown are the means ± S.E. for three independent experiments performed in triplicate (A and B) or one representative experiment performed twice (C). (*Significantly different from the inhibition of adenylyl cyclase activity measured in naive cells, p < 0.01.)

Adenylyl cyclase activity (Fig. 5C). Taken together with the results showing that these receptors did not undergo agonist-induced receptor phosphorylation, these data lend further credence to a direct role for receptor phosphorylation in desensitization, and indicate that the sites of GRK-mediated phosphorylation are most likely contained in the I3 loop.

**Fig. 6.** Time course of agonist-induced internalization of m2Δ1 and m2Δ2 mAChRs. Stable cell lines expressing the wild-type (●), m2Δ1 (○), or m2Δ2 (■) receptors were used to analyze receptor internalization. The percent of surface receptor present after various times of exposure to agonist was measured in whole cell binding assays using the hydrophilic ligand [3H]NMS. Data shown are the means ± S.E. of three to five independent experiments performed in triplicate.

The observation that GRK-mediated receptor phosphorylation is reduced by coexpression of GRK2K220R is in agreement with several other studies. This allele of GRK2 was shown to reduce agonist-dependent phosphorylation of the β2-AR in vitro and to decrease β2-AR desensitization in vivo, while not affecting receptor internalization (38). A slightly different allele of GRK2, GRK2K220W, was shown to reduce agonist-dependent m2 mAChR phosphorylation in COS7 and BHK cells (48). This study suggested that overexpression of wild-type GRK2 could decrease the concentration of agonist required for m2 mAChR internalization. However, overexpression of the dominant-neg-
activate allele had only minimal effects on receptor internalization, and the question of receptor-effector coupling was not addressed (48). More studies will be needed to fully understand the role of receptor internalization in receptor regulation; perhaps cell-specific mechanisms play a role in the differential regulation of internalization.

The data presented here also suggest that m2 mAChR desensitization is a distinct event from receptor internalization. It has been observed that the rate of receptor desensitization is faster than the rate of receptor internalization (22, 47–49). This observation, along with data from the β2-AR system, led us to postulate that receptor internalization is indeed independent of receptor desensitization. The data presented here show that, under conditions where receptor desensitization is blocked, receptor internalization continues to occur. We conclude from this observation that receptor internalization is not a key event in eliciting a loss of signaling ability and, as discussed earlier, may occur by mechanisms distinct from GRK-mediated, agonist-dependent phosphorylation of the m2 mAChR. While much more remains to be done to elucidate the events which cause receptor internalization, it is possible that conformational changes in the receptor, analogous to those which activate G-proteins, allow the activated receptor to interact with the internalization machinery. Another tenable proposal for mechanisms of internalization might include second-messenger production for the activation of endocytosis pathways (50, 51).

Finally, the sites of phosphorylation and domains responsible for desensitization of the m2 mAChR remain to be defined. The data presented here narrow down the search for phosphorylation sites to serine and threonine residues in amino acids 282–323. However, it should be noted that such a large deletion may have disturbed secondary structural elements necessary for receptor phosphorylation, GRK activation, or arrestin binding. Thus, it is possible that the phosphorylated residues lie outside this deleted region. One concern is that receptors may activate the GRKs (46) and that the lack of phosphorylation observed here might be due to loss of a kinase activation domain rather than the loss of actual phosphorylation sites. However, a larger deletion of the I3 loop (amino acids 233–380) did not affect the ability of the m2 mAChR to increase activity of GRK2 toward a peptide substrate in vitro (46), strongly suggesting that the m2Δ11 mutant should also be able to activate GRKs. Deletion of residues 282–323 did not alter the ability of the m2 mAChR to attenuate adenyl cyclase activity but abolished desensitization of this response. This suggests that site-directed mutagenesis of individual residues in this region may well lead to fruitful discovery of the sites of receptor phosphorylation and desensitization in vivo. Data obtained from experiments using point mutants may indicate if the m2 mAChR requires full phosphorylation for receptor desensitization.

In summary, we have shown that agonist-dependent phosphorylation of the m2 mAChR in intact cells is at least partially mediated by a GRK and is absolutely required for desensitization of the G-protein-mediated attenuation of adenyl cyclase activity, while GRK activity is not needed for receptor internalization. The sites of GRK-mediated phosphorylation of the m2 mAChR are likely found in amino acids 282–323; phosphorylation in this domain appears to be required for desensitization but not for internalization, although deletion of this domain reduces the rate and extent of receptor internalization. Future studies will determine the exact residues which are phosphorylated to give a more complete picture of the structural basis of m2 mAChR desensitization.

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