Desensitization of G protein-coupled receptors (GPCRs) involves the binding of members of the family of arrestins to the receptors. In the model system involving the visual GPCR rhodopsin, activation and phosphorylation of rhodopsin is thought to convert arrestin from a low to high affinity binding state. Phosphorylation of the M2 muscarinic acetylcholine receptor (mAChR) has been shown to be required for binding of arrestins 2 and 3 in vitro and for arrestin-enhanced internalization in intact cells (Pals-Rylaarsdam, R., and Hosey, M. M. (1997) J. Biol. Chem. 272, 14152–14158). For the M2 mAChR, arrestin binding requires phosphorylation at multiple serine and threonine residues at amino acids 307–311 in the third intracellular (i3) loop. Here, we have investigated the molecular basis for the requirement of receptor phosphorylation for arrestin binding. Constructs of arrestin 2 that can bind to other GPCRs in a phosphorylation-independent manner were unable to interact with a mutant M2 mAChR in which the Ser/Thr residues at 307–311 were mutated to alanines. However, although phosphorylation-deficient mutants of the M2 mAChR that lacked 50–157 amino acids from the i3 loop were unable to undergo agonist-dependent internalization when expressed alone in tsA201 cells, co-expression of arrestin 2 or 3 restored agonist-dependent internalization. Furthermore, a deletion of only 15 amino acids (amino acids 304–319) was sufficient to allow for phosphorylation-independent arrestin-receptor interaction. These results indicate that phosphorylation at residues 307–311 does not appear to be required to activate arrestin into a high affinity binding state. Instead, phosphorylation at residues 307–311 appears to facilitate the removal of an inhibitory constraint that precludes receptor-arrestin association in the absence of receptor phosphorylation.

G protein-coupled receptors (GPCRs)1 are membrane proteins that respond to a wide variety of stimuli, including sensory signals, hormones, and neurotransmitters. The ability of GPCRs to initiate signaling cascades decreases over time of exposure to agonist in a process known as desensitization (reviewed in Refs. 1 and 2). Desensitization has been most extensively studied for the visual GPCR rhodopsin (3). Within seconds following its activation, rhodopsin is phosphorylated on its C terminus in an agonist-dependent manner by rhodopsin kinase (4). This phosphorylation induces proteins termed arrestins to bind to rhodopsin and preclude the ability of rhodopsin to interact with the G protein transducin (5, 6).

Arrestins form a highly conserved family of cytosolic proteins. Arrestins 1 and 4 are found within the visual system (7, 8). Arrestins 2 and 3 are expressed ubiquitously throughout the body (9, 10) and serve multiple functions, including acting as adapters that target GPCRs to clathrin-coated pits for internalization (11). The binding of arrestins to GPCRs is thought to require both the activation and phosphorylation of the GPCRs (12). Based on evidence from both the crystal structure and mutagenesis studies on arrestin 1, a mechanism for arrestin interaction with rhodopsin has been described (12–17). A highly basic set of residues in arrestin 1, which has been shown to be responsible for recognition of phosphorylated rhodopsin, forms intramolecular charge-charge interactions that are disrupted by the phosphorylated C terminus of rhodopsin. This destabilizes the basal state of arrestin and causes arrestin to undergo a conformational change that enables binding to the activated receptor (12, 17, 18).

The events underlying the interaction of arrestins with other GPCRs have been less well studied. The M2 muscarinic acetylcholine receptor (mAChR) undergoes agonist-dependent phosphorylation within its third intracellular (i3) loop (19, 20). Phosphorylation of the M2 mAChR at multiple Ser/Thr residues in amino acids 307–311 (the C cluster) is required for desensitization and for arrestin binding both in vitro and in intact cells (20, 21), whereas phosphorylation at alternative sites (residues 286–291, the N cluster) can occur but has no apparent role in desensitization or arrestin binding (20). In the present study, we have sought to understand the role of phosphorylation of the M2 mAChR by using constructs of arrestin 2 that have been reported to interact with other GPCRs in a phosphorylation-independent manner. We have also tested whether the role of phosphorylation is to activate arrestin or...
change the conformation of the M₂ mAChR in order to allow arrestin to bind to and interact with the receptor.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium and penicillin-streptomycin were purchased from Mediatech. Fetal bovine serum was purchased from Life Technologies, Inc. Hepes-buffered Dulbecco’s modified Eagle’s medium was obtained from Sigma. N-[3H]Methylscopolamine was purchased from NEN Life Science Products. Anti-arrestin 2 antibody was a generous gift from Jeffrey Benovic (Thomas Jefferson University).

Deletion Mutagenesis—Creation of the NΔDENDEN receptor mutant was performed by deleting residues 304–319 in the vector construct of the N cluster mutant (M₂ NAla-4 mAChR pCR3 (20) using the Quick-Change site-directed mutagenesis kit (Stratagene) as described by the manufacturer’s instructions.

Cell Culture and Transfection—Human embryonic kidney cells stably expressing simian virus 40 large T antigen (HEK-tsA201 cells) were cultured as described (22). HEK-tsA201 cells were transfected using the calcium phosphate precipitation method followed by a rinse with 4 ml of culture medium and incubated with fresh culture medium until cells were utilized for assays 48–72 h posttransfection. Cells were transfected with 10 μg of receptor cDNAs and 5 μg of bovine arrestin 2 or 3 cDNA.

Receptor Internalization Assay—Changes in the number of mAChRs present on the cell surface as a function of time of agonist exposure were measured using the hydrophilic radioligand N-[3H]methylscopolamine as described previously (22). The cells used in these experiments expressed approximately 0.5–1 pmol of receptor/mg of protein at the cell surface. Data analysis was performed using GraphPad Prism software. Statistical significance was determined using Student’s t test.

Immunoblot Analysis—The expression of arrestins 2 and 3 from the various constructs used in this study was analyzed by Western blotting as described previously (22). In brief, 100 μg of protein from total cellular lysates was loaded onto SDS-acrylamide (8%) gels, transferred to nitrocellulose, and subjected to immunoblotting using antibodies raised in rabbit against arrestin 2.

RESULTS AND DISCUSSION

Effect of Phosphorylation-independent Arrestin Constructs on Internalization of M₂ mAChRs—The ability of the M₂ mAChRs to interact with arrestins is dependent on the phosphorylation of Ser/Thr residues in the sequence TVSTS in residues 307–311 (the C cluster) (21). Mutation of this sequence to AVAAA (the C cluster mutant, Fig. 1) results in a receptor that signals normally (20) and internalizes in an arrestin-independent pathway similar to that of the wild-type M₂ mAChR (20, 21) but has a greatly impaired ability to interact with arrestins 2 and 3 both in vitro and in intact cells (21). Based on the model developed for rhodopsin-visual arrestin 1 interactions (16, 17), it was possible that the inability of the C cluster mutant to interact with arrestins was due to its inability to activate arrestin 2 into a high affinity conformation that could subsequently bind to the receptors. To test this, we utilized arrestin 2 mutants that can interact with other GPCRs in a phosphorylation-independent but activation-dependent man-
bination interact with phosphorylation-deficient constructs of arrestin have been previously demonstrated to support arrestin-dependent internalization (20, 21). For the studies described below, it is important to note that it has been demonstrated previously that the overexpression of arrestins does not redirect all the receptors to the arrestin-independent pathway and that only the fractional increase in internalization that is observed upon arrestin expression represents the arrestin-dependent pathway (21).

The internalization of the M2 receptor, when transiently expressed alone in HEK-tsa201 cells, was maximal within 30 min of agonist exposure and reached an extent of 27 ± 7% (Fig. 2A). Upon co-expression of wild-type arrestin 2, the extent of internalization was enhanced approximately 3-fold after 60 min of agonist exposure (Fig. 2A), consistent with previous results (21). The phosphorylation-independent constructs arrestin 2(R169E) and arrestin 2(1–382) (23) also enhanced the extent of internalization of the M2 mAChRs to a similar extent as wild-type arrestin 2 (Fig. 2A). In marked contrast, the internalization of the C cluster mutant was not increased significantly by the overexpression of wild-type arrestin (Fig. 2B), consistent with previous results (21). Surprisingly, however, the internalization of the C cluster mutant was also not increased by the phosphorylation-independent arrestin constructs at any time point during the internalization assay (Fig. 2B). If phosphorylation of the C cluster was required to activate arrestin into a high affinity conformation, we expected the phosphorylation-independent arrestin constructs to interact with the C cluster mutant. These phosphorylation-independent constructs of arrestin have been previously demonstrated to interact with phosphorylation-deficient constructs of β2-adrenergic and δ-opioid receptors (23). As these arrestin constructs did not interact with the C cluster mutant of the M2 mAChRs, the results suggested that phosphorylation at the C cluster may be playing a previously unappreciated role.

Role of the M2 mAChR i3 Loop in Promoting Arrestin-Receptor Interaction—The i3 loops of the mAChRs play important roles in arrestin-receptor interactions, because the transfer of the i3 loop from the arrestin-sensitive M2 mAChR conferred arrestin sensitivity to the arrestin-insensitive M3 mAChR (22). We postulated that the ability of the M3/M2i3 chimera to respond to overexpressed arrestin (22) could be due to the existence of a high affinity arrestin binding site found within the M2 mAChR i3 loop. Based on the lack of interaction of the phosphorylation-independent arrestin constructs with the C cluster mutant, we further hypothesized that the i3 loop also contained an inhibitory element and that phosphorylation of the Ser/Thr residues at residues 307–311 was required to reverse the inhibition to allow arrestin binding.

To further test the role of the i3 loop in arrestin interactions, we used a mutant containing only a minimal amount of the i3 loop. This mutant receptor, designated M2ΔAPP, is able to couple to G proteins and signals normally,2 even though it lacks most of the i3 loop, specifically residues 223–380 (Fig. 1). This “minimal” receptor has lost all of the known phosphorylation sites within the i3 loop (19, 20). We anticipated that either 1) this receptor would be unable to interact with arrestin because it was phosphorylation-deficient and lacked most of the i3 loop, which appeared to be important for conferring arrestin sensitivity (22); or 2) if an inhibitory element exists in the i3 loop whose inhibitory influence is reversed upon phosphorylation exists in the i3 loop, then removal of the i3 loop might remove both the inhibitory element and the phosphorylation sites and thus create a receptor that can interact with overexpressed arrestin, independently of the phosphorylation state of the receptor. The M2ΔAPP receptor, like other phosphorylation-deficient receptors (20, 21), was unable to internalize when transiently expressed alone in tsa201 cells (Fig. 3A). However, upon co-expression with arrestin 2 or arrestin 3, this receptor internalized to an extent of approximately 50% (Fig. 3A), in a manner that was very similar to that seen with the WT M2.
mAChR in the presence of arrestin 2 or 3 (compare Fig. 3A with Fig. 2A). These results indicated that the i3 loop of the M2 mAChR was not absolutely necessary for arrestin interaction with the M2 mAChR and suggested that the i3 loop may contain an inhibitory element that precludes receptor-arrestin interaction in the absence of phosphorylation of the C cluster.

We also examined the ability of a minimal M3 mAChR to interact with overexpressed arrestin. Transfer of the M3i3 loop to the M2 mAChR is sufficient to inhibit M2 mAChR-arrestin interaction (22). We wondered whether the M3i3 loop was precluding the ability of the M3 mAChR to interact with overexpressed arrestins 2 or 3 (Fig. 3B) suggesting that other factors contribute to the arrestin insensitivity of this receptor (22, 25). Arrestin 3 actually appeared to inhibit the internalization of the M3i3LM receptor; however, this inhibition was not statistically significant.

To further define the portions of the i3 loop in the M2 mAChR to interact with overexpressed arrestin, transfer of the M2i3 loop to the M2 mAChR is sufficient to inhibit M2 mAChR-arrestin interaction (22). We wondered whether the M2i3 loop was precluding the ability of the M2 mAChR to interact with overexpressed arrestin. To examine this possibility, we used an M3 mAChR that also lacks its i3 loop, M3i3LM (24). However, this receptor was unable to interact with overexpressed arrestins 2 or 3 (Fig. 3B) suggesting that other factors contribute to the arrestin insensitivity of this receptor (22, 25). Arrestin 3 actually appeared to inhibit the internalization of the M3i3LM receptor; however, this inhibition was not statistically significant.

To further define the portions of the i3 loop in the M2 mAChR that contain the inhibitory element(s), we prepared and analyzed the ability of additional deletion mutants of the M2 mAChR to respond to overexpressed arrestins 2 and 3. M2Δ2 is a mutant containing a deletion of residues 250–323 of the i3 loop, whereas M2Δ1 is a slightly smaller deletion mutant with a deletion of residues 282–323 (Fig. 1) (19). These mutant receptors are both unable to undergo agonist-dependent phosphorylation (19). Like the M2ΔPP mAChR, both of these receptors were unable to internalize in tsA201 cells when transiently expressed alone (Fig. 4, A and B). However, these deletion mutants were arrestin-sensitive, as both receptors exhibited appreciable internalization when co-expressed with either arrestin 2 or 3 (Fig. 4, A and B). Thus, the results obtained with these receptors that contain smaller i3 loop deletions than the minimal mutant also suggested that the removal of an inhibitory element in the i3 loop allowed for phosphorylation-independent interaction with arrestins.

In order to characterize more specifically which residues within the i3 loop contributed to the proposed inhibitory element, we characterized a mutant in which the residues immediately surrounding the phosphorylation sites in the C cluster were deleted. This mutant, referred to as NΔDENDEN, lacks residues 304–319 of the wild-type M2 mAChR (Fig. 1), which removes the C cluster and several negative amino acid residues surrounding the C cluster (Fig. 1A). In addition, this mutant

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**Fig. 3.** Internalization of mutant M2 or M3 mAChR that contain minimal portions of the i3 loops. TsA201 cells were transfected and treated as described under “Experimental Procedures.” A, effect of arrestin 2 (arr2) or arrestin 3 (arr3) overexpression on the internalization of M2ΔPP mutant mAChR. B, effect of arrestin 2 or 3 overexpression on internalization of the M3i3LM. Data are from 5–8 experiments. *, p < 0.05 using Student’s t test.

**Fig. 4.** Effect of arrestins 2 and 3 on the internalization of M2 mAChR mutants that contain smaller deletions within their i3 loops. Experiments were performed as in Figs. 2 and 3. A, internalization of an M2 mAChR missing residues 250–323 (M2Δ2) in the absence and presence of overexpressed arrestin 2 (arr2) and arrestin 3 (arr3). B, internalization of an M2 mAChR missing residues 282–323 (M2Δ1) in the absence and presence of overexpressed arrestins 2 and 3. C, internalization of an N cluster mutant M2 mAChR missing residues 304–319 (NΔDENDEN) in the absence and presence of overexpressed arrestins 2 and 3. Data are from 4–11 experiments. *, p < 0.05 using Student’s t test.
has its Ser/Thr residues at the N cluster (residues 286–291) mutated to alanines to remove any effect of phosphorylation (20). We chose to make the DENDEN mutant in the context of the N cluster mutant so that the receptor would be phosphorylation-deficient and not internalize via the arrestin-independent pathway. We anticipated that this would allow for a “cleaner” interpretation of any effects of arrestins. We postulated that the NΔDENDEN mutant would only be able to respond to arrestin if the deletion of residues 304–319 removed the inhibitory element. The NΔDENDEN mutant, when expressed alone, did not undergo agonist-dependent internalization (Fig. 4C), which was similar to other phosphorylation-deficient receptor mutants. However, the NΔDENDEN deletion mutant showed extensive and rapid internalization when co-expressed with arrestin 2 and 3 (Fig. 4C). These results indicated that residues 304–319 contained an inhibitory element that precludes arrestin-receptor interaction in the absence of phosphorylation at residues 307–311. When the Ser/Thr residues in the C cluster are intact and phosphorylated, we hypothesize that this leads to a functional neutralization of the inhibitory constraints. Future studies will resolve how the Ser/Thr residues and/or the surrounding amino acids contribute to the inhibition of receptor-arrestin interaction.

The present results provide novel insights into the interaction of GPCRs and arrestins. In the well studied model of arrestin 1 interaction with visual rhodopsin, it has been proposed that arrestin 1 exists in an inactive state and that receptor activation and the subsequent phosphorylation of the C terminus of rhodopsin is required to disrupt this closed state (16, 17). This leads to a reorientation of the arrestin molecule that can now bind to phosphorylated and activated rhodopsin (26–29). Interestingly, studies have shown that C-terminally truncated rhodopsin mutants that lack phosphorylation sites can also bind to arrestin, but this requires prior “activation” of arrestin (30). Thus, if arrestin has been released from its inactive state by the binding of a phosphorylated synthetic peptide of the C terminus of rhodopsin (30) or by an appropriate mutation of arrestin that renders it phosphorylation-independent (16), binding to activated rhodopsin can occur. However, in the absence of permutations that cause activation of arrestin 1, nonphosphorylated but activated mutants of rhodopsin are unable to bind to arrestin 1 (16, 30).

Our data suggest that very different mechanisms govern the interaction of the M2 mAChRs with nonvisual arrestins 2 and 3. First, we found that two mutant arrestins that are able to convert arrestins from an inactive to active state.

Although phosphorylation of specific residues was necessary for arrestins 2 and 3 to interact with the wild-type M2 mAChR, we report here the surprising finding that the i3 loop of the M2 mAChR was not necessary for the receptor-arrestin interaction. This result was unexpected given previous results that indicated that the M3i3 loop was sufficient to confer arrestin sensitivity to the arrestin-insensitive M2 mAChR (22). This latter finding indicated that there are sufficient arrestin interaction domains located within the M3i3 loop to promote arrestin binding to the M2 mAChR. However, the present results with mutants that lack most of the i3 loop suggest that the M2 mAChRs must also contain binding sites within other intracellular loops that can support binding to overexpressed arrestin (Fig. 2). These results also suggested that the M3i3 loop inhibits the ability of these other M2 mAChR binding sites from interacting with overexpressed arrestin because transfer of the M3i3 loop to the M2 mAChR results in an arrestin-insensitive receptor (22). Our results, taken together with others, indicate that there are likely to be multiple molecular events that regulate the interactions of GPCRs and arrestins (31, 32). Future experiments will be performed to investigate the specific amino acids that contribute to the inhibitory element that impedes arrestin-M2 mAChR interaction in the absence of phosphorylation and the domains of the M2 mAChR that are able to interact with arrestin 2 in an intact cell.

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Arrestin Binding to the M₂ Muscarinic Acetylcholine Receptor Is Precluded by an Inhibitory Element in the Third Intracellular Loop of the Receptor
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