Internalization of the m2 Muscarinic Acetylcholine Receptor

ARRESTIN-INDEPENDENT AND -DEPENDENT PATHWAYS*

(Received for publication, July 2, 1997, and in revised form, July 17, 1997)

Robin Pala-Rylaarsdam,§ Vsevolod V. Gurevich, Katharine B. Lee, Judith A. Ptasienski, Jeffrey L. Benovic, and M. Marlene Hosey**

From the Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, Illinois 60611, ‡San Health Research Institute, Sun City, Arizona 85372, and the Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Recent studies have identified agonist-dependent phosphorylation as a critical event in the rapid uncoupling of the m2 muscarinic cholinergic receptors (mAChR) from G-proteins and sequestration of the receptors away from the cell surface. However, mutant m2 mAChRs were identified that were phosphorylated but unable to desensitize in adenyl cyclase assays, while they internalized like wild type (WT) mAChRs. We have tested whether these properties might stem from differences in the abilities of the WT and mutant mAChR to bind arrestins, proteins implicated in both receptor/G-protein uncoupling and internalization. We have determined that arrestin binding requires phosphorylation at a cluster of Ser/Thr residues in amino acids 307–311 in the m2 mAChR. A strong correlation was found between the ability of WT and mutant mAChRs to bind arrestins and proteins implicated in receptor internalization. We have determined that arrestins require phosphorylation of the m2 mAChR in HEK-tsA201 cells did not require arrestins and did not proceed via clathrin-mediated endocytosis. While the m2 mAChR was able to enter a clathrin- and arrestin-dependent pathway when arrestin 2 or arrestin 3 was significantly overexpressed, the preferred pathway of internalization of WT and certain mutant m2 mAChR in HEK-tsA201 cells did not involve participation of arrestins. The results suggest that the phosphorylation-mediated regulation of the m2 mAChR may involve arrestin-dependent and -independent events.

Desensitization plays an important role in turning off receptor-mediated signal transduction pathways. In the large family of G-protein coupled receptors (GPRs), two processes associated with the rapid phase of desensitization are receptor/G-protein (R/G) uncoupling and receptor internalization. Models of short term desensitization of GPRs posit that activation of GPRs leads to a rapid, agonist-dependent phosphorylation of the GPRs, which in turn allows the GPRs to bind arrestins, proteins that bind to phosphorylated GPRs and preclude further R/G interaction (for review, see Ref. 1). Recently, an additional function has been identified for non-visual arrestins, namely recruitment of receptors to clathrin-coated pits to allow for agonist-induced receptor internalization (2). The non-visual arrestins, arrestin 2 and arrestin 3 (β-arrestin and β-arrestin 2), but not visual arrestin (arrestin 1), bind both to the activated, phosphorylated β-arrestin and to clathrin, to act as adaptors to recruit the β-arrestin to the endocytic vesicles (3).

We have studied the m2 muscarinic cholinergic receptors (mAChRs) as a model of G-coupled GPRs to elucidate molecular events involved in desensitization. In recent studies we have observed that agonist-induced phosphorylation of the m2 mAChR on defined serine/threonine (Ser/Thr) residues in the third intracellular domain (i3 loop) was a key event in initiating both R/G uncoupling and receptor internalization (4). However, results with several mutant m2 mAChRs strongly suggested that distinct downstream events might be involved in R/G uncoupling and internalization (4, 5). An m2 mAChR with four Ser/Thr residues in amino acids 286–290 mutated to alanines (the “NAla4 mutant”) desensitized and internalized similarly to WT type receptor. On the other hand, an m2 mAChR with four Ser/Thr residues in amino acids 307–311 mutated to alanine (the “CAla4 mutant”) was unable to desensitize, but internalized in a manner that was not different from WT m2 mAChR (4). Furthermore, mutation of both Ser/Thr clusters (N,CAla8) abolished agonist-induced receptor phosphorylation, uncoupling from G-proteins, and reduced agonist-induced receptor internalization (4). The results of these studies suggested that: 1) phosphorylation of Ser/Thr residues in the cluster of Ser/Thr residues at aa 307–311 is critical to allow for R/G uncoupling; and 2) multiple pathways of internalization exist, as phosphorylation-dependent internalization was observed in the stable cell lines for the WT, NAla4, and CAla4 mutants, while phosphorylation-independent internalization was observed in the stable cell lines for the N,CAla8 mutant. The results highlight the observation that R/G uncoupling and internalization for the m2 mAChR are likely to occur via distinct pathways downstream of mAChR phosphorylation. We hypothesized that these mutations might have differentially impaired the ability of these receptors to bind arrestin proteins, thus affecting one or more of the regulatory events associated with m2 mAChR desensitization. In this study, we have analyzed the abilities of WT and mutant m2 mAChR to interact with arrestins in vitro and in intact HEK-tsA201 cells.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin-streptomycin, SB9001 medium, restriction en-

* This work was supported in part by National Institutes of Health Grants HL30121 (to M. M. H.), EY11500 (to V. V. G.), and GM4494 and GM47417 (to J. B. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Predoctoral fellow of the Howard Hughes Medical Institute.

** To whom correspondence should be addressed: Dept. of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, 303 E. Chicago Ave. S215, Chicago, IL 60614. Tel.: 312-503-3692; Fax: 312-503-5349; E-mail: mhosey@nwu.edu.

The abbreviations used are: GFP, G-protein coupled receptor; R/G, receptor/G-protein; mAChR, muscarinic cholinergic receptor; WT, wild type; aa, amino acid(s); DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; HA, hemagglutinin; HEK, hamster embryonic kidney; NMS, N-methylscopolamine.
zymes, and G418 were purchased from Life Technologies, Inc. HEPESE-buffered DMEM:F12 was obtained from JRH Biosciences or Sigma. [3H]-Methylscopolamine ([3H]NMS) was purchased from NEN Life Science Products. [3H]-CGP-12177 was purchased from Amersham. Linearized baculovirus DNA and Sf9 cell transfection reagent were obtained from CLONTECH. Heidi Pitt (Northwestern University School) provided βγ subunits of transducin. Kathy Green (Northwestern University Medical School) provided COS cells. Other reagents were from Sigma or previously described sources (5).

**Cell Culture and Transfection—** COS-20 cells (from K. Green) and HEK-ta2A01 cells, a clone of human embryonic kidney (HEK) 293 cells stably expressing human virus 40 large protein (6), were grown in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin at 37 °C in a 5% CO2 environment. HEK-ta2A01 cells were transfected using the calcium phosphate precipitation method, followed by a 5–6 min shock with 30% Me2SO in DMEM. The following amounts of each expression plasmid was used for transfection of each 100-mm culture plate in the combinations described in the text: 10 μg of pCR3-human m2 WT (or mutant) (4); 5 μg of pBc12B1-bovine arrestin 1 (7), arrestin 2 (8), or arrestin 3 (9); 0.1–5 μg of pCDNA3-human dynamin (WT or mutant) (10); or 5 μg of pCDNA1-βγ-AR-FLAG (11). COS-20 cells were transfected using Life Technologies, Inc. LipofectAMINE reagent using the manufacturer's directions, with the same amounts of plasmids used for calcium phosphate transfection. Cells were assayed for total cell protein expressed on the cell surface, as measured by [3H]quinuclidinyl benzilate binding. Approximately half of expressed mACHRs were present on the cell surface, as measured by [3H]NMS binding. Expression levels of the βγ-AR were slightly higher, with 2–3 pmol of receptor/total cell protein expressed on the cell surface, as measured by [3H]-CGP-12177 binding.

**Receptor Internalization Assay—** The approach used was to measure changes in the number of receptors located on the cell surface using 2 mM amounts of the hydrophilic ligand, [3H]NMS, which cannot cross cellular membranes. On the second day after transfection, 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 subsequently for the indicated times with 1 mM carbachol (or other concentration as indicated). At the end of the incubation time, cells were rinsed five times with 5 ml of ice-cold phosphate-buffered saline, removed from the plates by pipetting with ice-cold HEPESE-buffered DMEM:F12, and subjected to radioligand binding with saturating concentrations of [3H]NMS for 2 h at 4 °C. The binding was terminated in the presence of 10 μM atropine. Protein assays were performed to control for differences in cell density on each treated plate. Data were expressed as the percent of internalization of receptors, as assessed by a loss of [3H]NMS binding as compared with levels observed in untreated cells. Experiments with the βγ-AR were performed in the same manner, using 10 μM isoproterenol to induce internalization of the receptor, which was measured as a reduction in the binding sites for 10 nM [3H]-CGP-12177.

**Sf9 Cell Culture and Infection—** Spodoptera frugiperda (Sf9) insect cells were maintained in spinner cultures of Life Technologies, Inc. SF900II medium at 27 °C. Large scale infections were performed by infecting 1 × 109 cells/ml with a multiplicity of infection of 5 (12).

**Baculovirus Construction—** Recombinant baculoviruses directed expression of the WT m2 mAChR or the Ser/Thr cluster mutant m2 mAChRs to allow for purification and reconstitution of the receptors and their subsequent use in phosphorylation and arrestin binding assays (13, 14). Previous studies established that the WT m2 mAChR is an excellent substrate for GRK2 (βARK1) and GRK3 (βARK2) in vitro as it undergoes agonist-dependent phosphorylation to a stoichiometry of 3–5 mol of phosphate (mol of P/mol of receptor (mol of R)) (13). It was important to compare the phosphorylation of the WT m2 mAChR and the mutant constructs to determine if the pattern of phosphorylation in vitro matched the results we observed in phosphorylation studies performed in vivo. Previous studies in HEK-ta2A01 cells in vitro demonstrated that the WT m2 mAChR, m2-N60Cα, and m2-C44αβ variants were all phosphorylated to similar extents (3–5 mol of P/mol of R), while the m2-N60Cαα mutant lost all agonist-induced phosphorylation (4). In the present studies, we assessed the ability of the purified WT and mutant m2 mAChRs to serve as substrates for GRK2 in vitro. Purified GRK2 phosphorylated the WT m2 mAChR to a stoichiometry of 3–5 mol of phosphate/molecule of receptor. The purified receptors were reconstituted into phospholipid vesicles containing 100% phosphatidylserine, and analyzed for [3H]quinuclidinyl benzilate binding activity. Purified, reconstituted receptors were immediately phosphorylated with GRK2 using protocols described previously (13) and subsequently stored at −80 °C until used for studies with arrestins. HEK-ta2A01 and HEK-tsA201 cells, a clone of human embryonic kidney (HEK) 293 cells stably expressing human virus 40 large protein (6), were grown in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin at 37 °C in a 5% CO2 environment. HEK-ta2A01 cells were infected with baculovirus DNA and Sf9 earized baculovirus DNA and biotinylated arrestins was monitored by immunoblotting analyses. Whole cell lysates were subjected to SDS-PAGE using 10% polyacrylamide gels. The proteins were transferred to nitrocellulose, blocked for 30 min with 5% powdered milk in Tris-buffered saline (TBS), and incubated with primary antibody overnight. HA-tagged dynamin was detected using a 1:1000 dilution of mouse anti-HA ascites. Arrestins were detected using a mouse monoclonal antibody directed against an epitope common to all arrestins. The filters were incubated with horseradish peroxidase-coupled anti-mouse IgG. Enhanced chemiluminescence was used to detect antibody staining.

**RESULTS**

**Agonist-dependent Phosphorylation of WT and Mutant m2 mAChRs—** Phosphorylation is a key event that facilitates interactions of arrestins with GPRs (1). To begin to elucidate any differences in the arrestin binding properties of the WT and the Ser/Thr cluster mutant m2 mAChRs described earlier (4), we developed recombinant baculoviruses directing expression of the WT and mutant m2 mAChRs to allow for purification and reconstitution of the receptors and their subsequent use in phosphorylation and arrestin binding assays (13, 14). Previous studies established that the WT m2 mAChR is an excellent substrate for GRK2 (βARK1) and GRK3 (βARK2) in vitro as it undergoes agonist-dependent phosphorylation to a stoichiometry of 3–5 mol of phosphate (mol of P/mol of receptor (mol of R)) (13). It was important to compare the phosphorylation of the WT m2 mAChR and the mutant constructs to determine if the pattern of phosphorylation in vitro matched the results we observed in phosphorylation studies performed in vivo. Previous studies in HEK-ta2A01 cells in vitro demonstrated that the WT m2 mAChR, m2-N60Cα, and m2-C44αβ variants were all phosphorylated to similar extents (3–5 mol of P/mol of R), while the m2-N60Cαα mutant lost all agonist-induced phosphorylation (4). In the present studies, we assessed the ability of the purified WT and mutant m2 mAChRs to serve as substrates for GRK2 in vitro. Purified GRK2 phosphorylated the WT m2 mAChR to a stoichiometry of

---

**In Vitro Phosphorylation Assay—** The ability of the m2 mAChR and the Ser/Thr cluster mutants to undergo agonist-dependent phosphorylation by GRK2 was assessed using in vitro assays. Each assay contained 20 μM Tris, pH 7.4, 5 mM MgSO4, 2 mM EDTA, 50 μM γ-[35S]ATP (specific activity 500–2000 cpm/pmol), and 0.1 μM m2 mAChR. Assays were performed in the presence of 1 mM transducin and 10 μM atropine to assess the agonist dependence of the phosphorylation. GRK2 was used at a concentration of 25 nM. Gβγ subunits from the heterotrimeric G-protein transducin were added at a concentration of 250 nM. Assays were performed at room temperature for 0–60 min, with the maximal stoichiometry of phosphorylation being observed by 30 min. Reactions were stopped by the addition of SDS-PAGE sample buffer or by freezing at −80 °C.

**Arrestin Binding Assay—** Interactions between purified m2 mAChRs and arrestins were measured using an established arrestin binding assay (14). Purified, reconstituted wild type or mutant m2 mAChRs were phosphorylated in large batches (≥50 pmol/reaction, 0.1 pmol/μl of receptor) by GRK2 as described above, but with nonradioactive ATP, or expressed arrestins in the absence of kinase as controls. Parallel small reactions with γ-[35S]ATP were performed to allow calculations of the stoichiometry of phosphorylation. Reactions were incubated for 30 min at room temperature. The receptors were divided into aliquots and stored at −80 °C until used for arrestin binding. The wild type or mutant m2 mAChRs (100 fmol) were incubated for 35 min at 30 °C with 100 fmol of radiolabeled arrestin (prepared as described previously in 14) and 0.1 μM carbachol in 50 mM Tris, pH 7.5, 50 mM potassium acetate, 0.5 mM MgCl2, and 0.2 mM dithiothreitol, in a total volume of 50 μl. Arrestin-receptor complexes (50 μl volume) were separated from free arrestin by gel filtration over 2-ml Sepharose CL-2B columns, as described previously (14).

**Immunoblot Analyses—** Expression of arrestins and HA-tagged dynamin was monitored by immunoblotting analyses. Whole cell lysates were subjected to SDS-PAGE using 10% polyacrylamide gels. The proteins were transferred to nitrocellulose, blocked for 30 min with 5% powdered milk in Tris-buffered saline (TBS), and incubated with primary antibody overnight. HA-tagged dynamins were detected using a 1:1000 dilution of mouse anti-HA ascites. Arrestins were detected using a mouse monoclonal antibody directed against an epitope common to all known arrestins, diluted 1:2000 in milk/TBS (15). After washing away unbound primary antibody with TBS, the filters were incubated for 2 h with horseradish peroxidase-coupled anti-mouse IgG. Enhanced chemiluminescence was used to detect antibody staining.
phosphorylation of the m2-N,C Ala8 receptor was significantly lower with a stoichiometry of 1.9 ± 0.3 mol of P/mol of R (Table I). The extent of the in vitro phosphorylation of the m2-N,CAla4 receptor was significantly lower with a stoichiometry of 1.9 ± 0.3 mol of P/mol of R, respectively (Table I). The results reflect the previous results obtained in studies of phosphorylation in intact cells (4), but are not identical. Thus, both receptors with only one Ser/Thr cluster mutated to alanines were phosphorylated at multiple sites by GRK2; however, the stoichiometries were somewhat reduced compared with the WT m2 mAChR. In intact cells, the phosphorylation of the WT, and N- and C-cluster mutants exhibited similar extents of phosphorylation (4). Similarly, while the in vitro phosphorylation of the double Ser/Thr cluster mutant was significantly lower than the WT receptor, this mutant was phosphorylated at two sites by GRK2 in vitro, while it exhibited no agonist-dependent phosphorylation in intact cells (4). The results suggest that the in vitro phosphorylation assays may lead to phosphorylation at additional sites that are not phosphorylated in the environment of the intact cell. Alternatively, the GRK that is suspected to phosphorylate these receptors in HEK cells (5) may recognize the receptors somewhat differently than GRK2.

**Arrestin Binding to m2 mAChRs in Vitro**—In the next series of experiments, we compared the abilities of the WT m2 and the mutant mAChRs to bind to arrestin 2 and arrestin 3 in vitro. The purified receptors were phosphorylated with GRK2 or exposed to the same phosphorylation conditions in the absence of kinase as a control for receptor phosphorylation, and subsequently used in binding assays with [3H]arrestins as described under “Experimental Procedures.” In agreement with previous studies (14, 16), the WT m2 mAChR bound arrestin 2 (Fig. 1A) and arrestin 3 (Fig. 1B) in a phosphorylation-dependent manner. Phosphorylation-dependent binding of arrestin 2 and arrestin 3 was also observed for the NAla4 mutant mAChR (Fig. 1, A and B), and the extent of binding was virtually indistinguishable from that exhibited by the WT m2 mAChR. In marked contrast, the CAla8 mutant was significantly impaired in its ability to bind both arrestins; the amount of phosphorylation-dependent arrestin binding to the CAla8 mutant was only 20–30% of that observed with either the WT or NAla4 mutant. The N,CAla4 m2 mAChR exhibited no phosphorylation-dependent binding of arrestins (Fig. 1, A and B). These results suggested that the structural requirements for phosphorylation-dependent arrestin binding include phosphorylation of the Ser/Thr residues found in aa 307–311 that are mutated in the CAla8 and N,CAla4 mutants.

**Interaction of WT and Mutant m2 mAChR with Arrestins in Intact Cells**—Of particular interest is the observation that the CAla8 mutant exhibits an internalization pattern closely resembling that of the WT m2 mAChR (4), while this mutant appears to be impaired in binding arrestins in vitro (Fig. 1). These observations raised the possibility that internalization of the m2 mAChR might occur through a phosphorylation-dependent, but arrestin-independent pathway. Interactions between the m2 mAChR and arrestins in intact cells have not yet been demonstrated. To test whether different arrestins could modulate internalization of the m2 mAChR, HEK-tsA201 cells were transiently transfected with cDNAs encoding the m2 mAChR with or without cDNAs encoding arrestin 1 (visual arrestin), arrestin 2 (β-arrestin), or arrestin 3. Coexpression of the arrestin proteins was confirmed by immunoblotting (Fig. 2A) using
Arrestin-dependent and -independent Regulation of m2 mAChR

In contrast, overexpression of arrestin 2 and/or arrestin 3 increased the levels of expression of each arrestin to approximately 50–100 pmol/mg of total cell protein in transiently transfected cells, as assessed by comparison to the immunoreactivity of a series of known amounts of purified arrestin 2. However, in a single transfected cell, the arrestins levels were even higher, as only 10–20% of the HEK cells become transfected using the standard calcium phosphate method. The agonist carbachol (1 mM) induced internalization of the m2 mAChR, as measured by loss of cell surface binding sites for the hydrophilic radioligand [3H]NMS (Fig. 2B). This internalization plateaued after approximately 1 h of agonist exposure. Coexpression of arrestin 2 or arrestin 3, but not arrestin 1, increased the rate and extent of internalization of the WT m2 mAChR (Fig. 2B). The effects of arrestin 2 and arrestin 3 were further investigated by comparing the dose-response curves for carbachol to induce m2 mAChR internalization in the presence and absence of overexpressed arrestins. Overexpression of arrestin 2 or arrestin 3 enhanced the extent of m2 mAChR internalization at 1 h at most concentrations of carbachol, while the EC50 for carbachol-induced internalization was not significantly changed (Fig. 2C).

Similar studies were performed with the Ser/Thr cluster mutant m2 mAChRs. We previously studied the rates of internalization of these mutants in stably or transiently transfected cells following exposure to high concentrations of carbachol, and found that the rate and extent of internalization of the m2-CAla4 receptor in transiently transfected cells was identical to the WT m2 mAChR, while the m2-NAla4 receptor internalized at a slightly faster rate and to a slightly greater extent than the WT m2 mAChR (4). On the other hand, the N,CAla4m2 mAChR was significantly impaired in internalization in the transiently transfected cells (4). To further analyze the properties of the mutant m2 mAChRs, we measured the concentration response curves for carbachol to induce internalization of the WT and cluster mutant m2 mAChRs following 1 h of exposure to agonist. No statistically significant shifts in the EC50 for carbachol were observed for the mutant m2 mAChRs (Table I). We next asked if overexpression of arrestin 2 or arrestin 3 could modify internalization of the mutant m2 mAChRs by incubating cells expressing the receptors ± arrestins with 1 mM carbachol for 1 h and measuring decreases in surface receptor levels. Internalization of the WT m2 mAChR following exposure to 1 mM carbachol increased from 23 ± 8% in control cells to 58 ± 6% and 65 ± 10% when arrestin 2 or arrestin 3 were overexpressed, respectively (Fig. 2B). The internalization of the NAla4-m2 mAChR after 1 h of exposure to 1 mM carbachol was modestly, but significantly, increased under similar conditions from 44 ± 2% in control cells to 55 ± 2% or 57 ± 1%, with arrestin 2 or arrestin 3 overexpression, respectively (data not shown). Interestingly, the internalization of the CAla4 and the N,CAla4 mutants was not significantly increased by overexpression of either arrestin (data not shown). These results provide new insights into the mechanisms underlying the internalization of the mAChRs in HEK cells, by suggesting that internalization of the m2 mAChR occurs via an arrestin-independent pathway.

Is the Dynamin/Clathrin Pathway Involved in m2 mAChR Internalization?—Arrestin 2 and arrestin 3 are clathrin-binding proteins that act as adaptor molecules to link GPRs to clathrin-coated endocytic vesicles (3). We hypothesized that the m2 mAChR might use a different pathway for internalization, since the CAla4 mutant was significantly impaired in its ability to bind arrestins in vitro (Fig. 1) and in vivo, yet it exhibited internalization parameters very similar to the WT m2 mAChR (Table I; see also Ref. 4). Endocytosis via clathrin-coated vesicles is a dynamin-dependent process (17), and expression of a dominant-negative mutant of human dynamin 1

![Figure 2](http://www.jbc.org/)

**FIG. 2.** Arrestin enhances ligand-induced internalization of the m2 mAChR. HEK-tsA201 cells were transiently transfected with the m2 mAChR alone, or cotransfected with arrestin 1 (visual arrestin), arrestin 2 (μ-arrestin), or arrestin 3. Untreated cells expressed 0.7–1.5 pmol of surface receptor/mg of total cell protein. Internalization of the receptor was measured 60 h post-transfection using [3H]NMS in whole cell radioligand binding assays. A, immunoblot showing levels of expression of the indicated arrestins in 350 μg of protein (whole cell lysate). Lane 1, cells transfected with m2 mAChR alone; lane 2, cotransfected arrestin 1; lane 3, cotransfected arrestin 2; lane 4, cotransfected arrestin 3. A monoclonal mouse antibody directed against a common epitope of the three arrestin isoforms was used to probe the immunoblot. B, time courses of internalization of the m2 mAChR under different cotransfection conditions following receptor exposure to 1 mM carbachol were measured using whole cell binding assays. ■, m2 mAChR alone; ○, m2 mAChR + arrestin 1; □, m2 mAChR + arrestin 2; or ▼, m2 mAChR + arrestin 3. Data were fit to a one-site exponential association function using GraphPad Prism software and represent the means ± S.E. for three independent experiments each performed in triplicate. C, dose-response relationships for arrestin 2 and arrestin 3-enhanced internalization of the m2 mAChR internalization were measured. Cells were treated with varying concentrations of carbachol for 1 h, washed, and changes in surface receptor number were measured using [3H]NMS whole cell binding assays with m2 mAChR alone (●), m2 mAChR + arrestin 2 (▼), and m2 mAChR + arrestin 3 (▼). Data were fit to one-site dose-response curves using GraphPad Prism software and represent the means ± S.E. for three independent experiments each performed in triplicate. The EC50 values for carbachol to induce m2 mAChR internalization were 4.3 ± 1.4 μM for m2 mAChR expressed alone, and 0.8 ± 0.4 μM when arrestin 2 or 1.4 ± 0.9 μM when arrestin 3 was coexpressed with the m2 mAChR.
The wild type and mutant m2 mAChRs. Cells were exposed to 1 mM alone (lane 1), or HA-dynK44A (lane 2), type HA-dynamin (lane 3), were measured using [3H]NMS whole cell binding assays following 1 h exposure to 10 μM alpranolol. Untreated cells expressed 2-3 pmol of receptor/mg of total cell protein. Changes in receptor internalization caused by arrestin or dynaminK44A overexpression were measured, and data were analyzed using Student’s t-test. Data are the means ± S.E. for three to six independent experiments. (*, significantly different from cells transfected with receptor alone, p < 0.05).

The m2 mAChR internalizes through a dynamin-independent pathway. A dominant-negative mutant of dynamin 1, dynaminK44A, was used to disrupt clathrin-mediated endocytosis in cells cotransfected with the wild type or mutant m2 mAChRs. A, overexpression of HA-tagged wild type and dynaminK44A was measured in immunoblots of 350 μg of total cellular protein from cells transfected with receptor alone (lane 1), type HA-dynamin (lane 2), or HA-dynaminK44A (lane 3). B, the ability of dynaminK44A to inhibit m2 mAChR internalization was tested for the wild type and mutant m2 mAChRs. Cells were exposed to 1 mM carbachol for 1 h, and changes in surface receptor number were measured using [3H]NMS whole cell radioligand binding assays. Carbachol for 1 h, and changes in surface receptor number were measured using [3H]NMS whole cell radioligand binding assays. All of the receptors tested continued to internalize in the presence of dynaminK44A and there was no significant difference in the amount of receptor internalization observed for the wild type or any mutant m2 mAChR with overexpression of wild type dynamin or the dominant negative mutant dynamin (Fig. 3B).

If the m2 mAChR internalizes via an arrestin-independent pathway, as suggested by the results described previously, one might not expect this receptor to internalize via clathrin-coated pits. Therefore, as a positive control in these studies, it was important to determine that overexpression of the dominant-negative dynamin was able to suppress internalization of a GPR suspected to use clathrin-mediated endocytosis. Two approaches were taken. First, we demonstrated that the overexpressed dynaminK44A protein was clearly able to reduce the enhanced internalization of the wild type m2 mAChR caused by overexpression of arrestin 2 or arrestin 3 (Fig. 3C). This latter result demonstrated that arrestin 2 or arrestin 3 overexpression shuttled the m2 mAChR to clathrin-coated vesicles for endocytosis. Importantly, because the dominant negative dynamin only inhibited that portion of internalization of the m2 mAChRs that was attributable to overexpression of arrestins, these data demonstrated that the m2 mAChR internalized predominantly by an arrestin- and dynamin-independent pathway in HEK cells.

Agonist-induced Internalization of the β2-AR in tsA201 Cells—In previous studies it has been demonstrated that the β2-AR internalize primarily via an arrestin- and clathrin-dependent pathway in HEK cells. Thus, as a second approach to demonstrate that the arrestin- and clathrin-dependent pathway was functional in the HEK-tsA201 cells, we ascertained if the internalization of the β2-AR would exhibit the expected characteristics in these cells, under conditions in which the m2 mAChR did not exhibit arrestin- or clathrin-dependent internalization. Cells were transiently transfected with cDNA encoding the β2-AR alone, or cotransfected with arrestins or dynamin. Following 1 h of exposure to 100 μM isoproterenol, 26 ± 8% of the surface β2-ARs were internalized, as measured by loss of binding sites for the hydrophilic ligand [3H]CGP-12177 (Fig. 4). The internalization of the β2-AR was enhanced by overexpression of arrestin 2 (40 ± 3% internalized) or arrestin 3 (45 ± 12% internalized) (Fig. 4). Coexpression of dom-
the m2 mAChR is not significantly internalized in this manner. The expression of a dominant-negative dynamin significantly inhibited internalization of the \( \beta_2 \)-AR (10 ± 6% internalized), while expression of wild type dynamin did not change the basal level of agonist-induced \( \beta_2 \)-AR internalization (26 ± 4% internalized). Thus, in HEK-tsa201 cells, the \( \beta_2 \)-AR seemed to prefer a dynamin-dependent internalization pathway both when the receptor was expressed alone and when it was coexpressed with arrestins. This was in marked contrast to the behavior of the m2 mAChRs in these cells. The results suggest that sufficient arrestins are expressed in the HEK-tsa201 cells to allow arrestin-dependent internalization of the \( \beta_2 \)-AR, while, under similar circumstances, the m2 mAChR utilize a phosphorylation-dependent, but arrestin-independent pathway for internalization.

**Internalization of the m2 mAChR Expressed in COS-20 Cells**—In an effort to ascertain whether the m2 mAChR heterologously expressed in another cell line would exhibit similar or different behavior as that observed in the HEK-tsaA cells, we analyzed the internalization of the m2 mAChR in COS cells. In COS cells, a very low level of m2 mAChR internalization was observed following 1 h of exposure to 1 mM carbachol (9 ± 1% internalized, Fig. 5). When arrestin 2 or arrestin 3 was overexpressed with the m2 mAChR, receptor internalization increased to 23 ± 6% and 36 ± 7% of surface receptors internalized, respectively (Fig. 5). The low basal level of internalization of the m2 mAChR in COS cells appeared to be inhibited by the dominant negative dynamin; however, the errors were large due to the difficulties in measuring the small levels of internalization that occur under these condition. The low levels of internalization in the absence of arrestin overexpression suggested that the phosphorylation-dependent, arrestin-independent pathway observed in HEK-tsa201 cells may not be available for m2 mAChR internalization in COS cells. However, overexpression of arrestins allowed the m2 mAChR to enter the arrestin- and dynamin-dependent internalization pathway. One or more components of the arrestin-independent pathway identified in tsA201 cells is likely to be missing in COS cells as the m2 mAChR is not significantly internalized in this manner.

**DISCUSSION**

Several novel findings are presented in these studies. First, in HEK-tsa201 cells, endogenous levels of arrestin 2 and arrestin 3 are detectable, but these proteins are not likely interacting with the WT or mutant m2 mAChRs to mediate internalization following agonist exposure. Coexpression of a dominant-negative mutant of dynamin, dynaminK44A, did not prevent the internalization of WT or mutant m2 mAChRs (Fig. 3B). Only upon overexpression of arrestin 2 or 3 were the WT and N-cluster mutant m2 mAChRs able to enter the arrestin- and clathrin-dependent pathway, as demonstrated by the inhibition of arrestin-enhanced endocytosis via clathrin-coated pits by dynaminK44A (Fig. 3C). As the CAla4 mutant m2 mAChR internalized in a manner equivalent to that of the WT m2 mAChR, but was severely impaired in its ability to bind arrestins (Fig. 1), this suggested that arrestin is not likely a common mediator of internalization of the m2 mAChR. Thus, it seems that internalization of the m2 mAChR in HEK-tsa201 cells proceeds by an arrestin-independent, clathrin-independent pathway. Previous results established that this pathway is greatly facilitated by phosphorylation of the m2 mAChRs at either the N or C cluster of Ser/Thr residues, since mutating both clusters (in the N,CAla4, mutant) severely impaired internalization (4). It is not clear if the internalization of the N,CAla4, mutant occurs by a phosphorylation-insensitive pathway, or if this mutant is simply a poorer substrate for the pathway facilitated by phosphorylation. Thus the preferred pathway of internalization of the m2 mAChR in the HEK-tsaA cells is a phosphorylation-facilitated but arrestin- and clathrin-independent pathway. Little is known about mechanisms that link membrane proteins to non-clathrin-coated vesicles (18). It will be important to determine if an adaptor protein for the m2 mAChR directs the receptors to non-clathrin containing vesicles for internalization. In cardiac myocytes, the m2 mAChR was recently shown to dynamically interact with caveolae following agonist stimulation (19). Perhaps this pathway will be found to be the physiologically relevant means of m2 mAChR internalization. Thus, although the clathrin-dependent endocytic pathway has been suggested as the predominant internalization pathway for other GPRs, including the \( \beta_2 \)-AR (20) and other mAChR subtypes (21, 22), it appears that the m2 mAChR can preferentially enter a clathrin-independent pathway for endocytosis.

A second conclusion that arises from these studies is that overexpressed arrestin 2 and arrestin 3 can act as internalization adaptor proteins for the m2 mAChR (Fig. 2, B and C). This is the first observation of an interaction between the m2 mAChR and arrestins in an intact cell. Previous reports demonstrated this function for receptors coupled to Gs (\( \alpha \)-AR) (2) and G\( \alpha_{11} \) (ATII-R) (20); our studies extend this function to the m2 mAChR, a receptor coupled to Gs, Although signaling mechanisms have been implicated in modulation of m2 mAChR internalization (23, 24), arrestin enhancement of GPR internalization does not seem to depend on which signaling pathways are activated by the GPR. However, despite the ability to demonstrate interactions between arrestin 2/3 and the m2 mAChR in a functional assay, the arrestin-mediated endocytosis was only observed when the arrestins were overexpressed, even though the pathway was available for internalization of the \( \beta_2 \)-AR. Thus, in the HEK-tsa201 cells, the primary pathway of m2 mAChR internalization appeared to be arrestin-independent. The degree of arrestin-enhanced internalization of the NAla4, mutant was reduced compared with the effects of arrestins on the wild type m2 mAChR, suggesting that although the NAla4-m2 mAChR was able to interact with arrestins, the interaction might be impaired compared with that of the wild type m2 mAChR. However, as the NAla4, mutant internalized to a greater degree in the absence of arrestin overexpression, and as the maximal extent of internalization was similar for both the WT and NAla4, receptors, an alternative explanation is that the internalization machinery may be saturated under these conditions. Importantly, the results support the suggestion that the predominant pathway of internalization of the WT, NAla4, and CAla4, m2 mAChRs in HEK cells is an arrestin-independent pathway, since the WT, NAla4, and CAla4, mutant m2 mAChR were able to internalize to similar extents in intact cells (4), although they exhibited marked differences in their abilities to bind arrestins in vitro and respond to
Thus the phosphorylation of the C Ala4 mutant, presumably in vitro, retained the ability to bind arrestins both in vitro and in intact cells, while the NAla4-m2 mAChR did not allow for arrestin-dependent enhancement of receptor internalization in intact cells, while the NAla4-m2 mAChR retained the ability to bind arrestins both in vitro and in intact cells. This difference in arrestin binding was evident even though the NAla4 mutant was phosphorylated to a similar stoichiometry as the C Ala4 mutant by GRK2 in vitro and in vitro (4). Thus the phosphorylation of the C Ala4 mutant, presumably in part on residues 286–290, poorly supported arrestin binding. Furthermore, although the N,CAla8 mutant was phosphorylated in vitro to a stoichiometry of ~2, this phosphorylation did not support any arrestin binding. Taken together, these results provided further evidence for an important regulatory role of phosphorylation of the Ser/Thr residues in the C-cluster. In previous studies we demonstrated that the WT and NAla4-m2 mAChRs desensitized in adenylyl cyclase assays, while the CAla4- and the N,CAla8-m2 mAChRs did not (4). Taken together, these results provide a strong correlation between the ability of the WT and mutant mAChRs to bind arrestins and to desensitize in adenylyl cyclase assays. These results suggest that an arrestin, or closely related protein, may recognize phosphorylation of the “C” cluster of Ser/Thr residues (aa 307–311) as a signal for receptor desensitization. While this hypothesis will require direct testing, the present experiments demonstrated that phosphorylation of Ser/Thr residues in the aa 307–311 cluster is critical to allow arrestin-receptor interaction both in intact cells in internalization assays and in vitro.

Of interest is the possibility that phosphorylation at only one or two sites may be necessary for arrestin binding, desensitization, and internalization. This is suggested by the present findings that the difference in stoichiometry of GRK2-mediated phosphorylation of the NAla4 mutant, which bound arrestins in a phosphorylation-dependent manner, and the N,CAla8 mutant, which did not bind arrestins, was 1.5–2 mol of P/mol of R (Table 1). Furthermore, we previously observed that a reduction in stoichiometry of phosphorylation of the WT m2 mAChR from ~3–5 to 1.5–2 mol of P/mol of R due to the expression of a dominant negative GRK2 caused a complete loss of desensitization (5). Finally, in previous studies, it was suggested that a stoichiometry of 2–3 was sufficient to allow for arrestin binding to the m2 mAChR (14). Future studies will be required to determine which phosphorylated amino acids are required for the processes of arrestin binding, receptor/G-protein uncoupling and internalization.

The structural determinants for internalization of the m2 mAChR seem to be quite complex. Other work has implicated regions of the seventh transmembrane domain and the intracellular carboxyl terminus as being important for internalization of the m2 mAChR (25). We and others have shown that the central portion of the third intracellular loop, and specifically clusters of Ser/Thr residues, are also important in determining the extent and rate of receptor internalization, probably in response to receptor phosphorylation (4, 26). Interestingly, the ATII-R has a similar Ser/Thr cluster in its COOH-terminal tail (27), a region that is important for internalization of that receptor (28). The ATII-R also undergoes arrestin-independent internalization in HEK cells and COS cells, and like the m2 mAChR, internalization of the ATII-R can be enhanced by overexpression of arrestin 2 (20). It may be that all four of 5 Ser/Thr residues are the preferred sites for binding of a novel adaptor protein, or a protein with an as yet unappreciated function in GPR regulation.

Our favored interpretation of the data presented in this work is that the m2 mAChR uses a phosphorylation-dependent, arrestin-independent pathway of internalization which requires either one of two Ser/Thr-rich clusters in the third intracellular loop (4). It is conceivable that this process may involve the binding of a novel adaptor to the phosphorylated m2 mAChRs to link the receptors to a dynamin- and clathrin-independent internalization pathway in tsA201 cells. The m2 mAChR is also able to utilize the arrestin-clathrin internalization pathway, but perhaps with a lower efficiency than the β2-AR, as overexpression of arrestins was required. The identification of the molecular events underlying this previously unappreciated phosphorylation-dependent, arrestin-independent pathway will be necessary to help us better understand the complex regulation of GPRs by receptor internalization. On the other hand, the structural requirements for arrestin binding to the m2 mAChR strongly correlate with the requirements identified in our previous study of m2 mAChR desensitization (4). Thus, it is conceivable that either arrestins or a similar protein mediate m2 mAChR uncoupling from G-proteins. On the other hand, if arrestins bind to the phosphorylated m2 mAChR in intact cells to mediate R/G uncoupling, why does the bound arrestin not direct the m2 mAChR to clathrin-coated pits for endocytosis? Thus, despite the strong correlation between the ability of a WT or mutant m2 mAChR to bind arrestins and to desensitize in adenylyl cyclase assays, it is conceivable that the phosphorylation-dependent uncoupling of the mAChR from G-proteins also does not involve known arrestins. Alternatively, the m2 mAChR may interact with another arrestin-like protein, which uncouples the receptor but which does not interact with clathrin. This protein might link the m2 mAChR to the clathrin-independent internalization pathway. Further studies will be necessary to directly examine the role of arrestin and other proteins in the process of R/G uncoupling. Finally, the data again draw a distinction between internalization of the m2 mAChR and receptor desensitization and support the hypothesis that these two phosphorylation-dependent events utilize different pathways.

REFERENCES

1. Liggett, S. B., and Leffkowitz, R. J. (1994) in Regulation of Signal Transduction Pathways by Desensitization and Amplification (Sibley, D. R., and Housley, M. D., eds) pp. 25–67, John Wiley & Sons, New York
2. Ferguson, S. S. G., Downey, W. E., Colapietro, A.-M., Barak, L. S., Menard, L., and Caron, M. G. (1996) Science 271, 363–366
3. Goodman, O. B., Krupeck J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 383, 447–450
4. Pals-Rylaarsdam, R., and Hosey, M. M. (1997) J. Biol. Chem. 272, 14152–14158
5. Pals-Rylaarsdam, R., Xu, Y., Witt-Enderby, P., Benovic, J. L., and Hosey, M. M. (1999) J. Biol. Chem. 274, 29004–29011
6. Margolkeese, R. F., McHendry-Èinde, B., and Horn, R. (1993) BioTechniques 15, 906–911
7. Sinohara, T., Dietzschold, B., Craft, C. M., Wistow, G., Early, J. J., Donoso, L. A., Hurwitz, J., and Tao, R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6975–6979
8. Lobse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Leffkowitz, R. J. (1996) Science 274, 1547–1549
9. Sterne-Marr, R., Gurevich, V. V., Goldsmith, P., Bodine, R. C., Sanders, C., Donoso, L. A., and Benovic, J. L. (1993) J. Biol. Chem. 268, 15460–15468
10. Danke, H., Raba, T., van der Blik, A. M., and Schmid, S. L. (1995) J. Cell Biol. 131, 69–80
11. Guan, X.-M., Koblika, T. S., and Koblika, B. K. (1992) J. Biol. Chem. 267, 21985–21990
12. Summers, D. M., and Smith, G. E. (1987) Tex. Agric. Exp. Stn. Bull. 1555, 1–56
13. Richardson, R. M., Kim, C., Benovic, J. L., and Hosey, M. M. (1993) J. Biol. Chem. 268, 13650–13656
14. Gurevich, V. V., Richardson, R. M., Kim, C. M., Hosey, M. M., and Benovic, J. L. (1993) J. Biol. Chem. 268, 16879–16882
15. Dua, H. S. (1992) Curr. Eye Res. 11, (Suppl.), 107–111
16. Gurevich, V. V., Dion, S. B., Onorato, J. J., Plasinski, J., Kim, C. M., Sterne-
Marr, R., Hosey, M. M., and Benovic, J. L. (1995) J. Biol. Chem. 270, 720–731
17. De Camilli, P., Takei, K., and McPherson, P. S. (1995) Curr. Opin. Neurobiol. 5, 559–565
18. Lamaze, C., and Schmid, S. L. (1995) Curr. Opin. Cell Biol. 7, 573–580
19. Feron, O., Smith, T. W., Michel, T., and Kelley, R. A. (1997) J. Biol. Chem. 272, 17744–17755
20. Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L., and Caron, M. G. (1997) J. Biol. Chem. 271, 18302–18305
21. Tolbert, L. M., and Lameh, J. (1996) J. Biol. Chem. 271, 17335–17342
22. Bogatkewitsch, G. S., Lenz, W., Jakobs, K. H., and VanKoppen, C. J. (1996) Mol. Pharmacol. 50, 424–429
23. Scherer, N. M., and Nathanson, N. M. (1990) Biochemistry 29, 8475–8483
24. Habecker, B. A., and Nathanson, N. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5035–5038
25. Goldman, P. S., Schlador, M. L., Shapiro, R. A., and Nathanson, N. M. (1996) J. Biol. Chem. 271, 4215–4222
26. Moro, O., Lameh, J., and Sadee, W. (1996) J. Biol. Chem. 271, 6862–6865
27. Bergsma, D. J., Ellis, C., Kumar, C., Nuthulaganti, P., Kerssen, H., Etshourbey, N., Griffin, E., Stadel, J. M., and Alyor, N. (1992) Biochem. Biophys. Res. Commun. 183, 989–995
28. Hunyady, L., Bor, M., Balla, T., and Catt, K. J. (1994) J. Biol. Chem. 269, 31378–31382

Arrestin-dependent and -independent Regulation of m2 mAChR

23689
Internalization of the m2 Muscarinic Acetylcholine Receptor:
ARRESTIN-INDEPENDENT AND -DEPENDENT PATHWAYS
Robin Pals-Rylaarsdam, Vsevolod V. Gurevich, Katharine B. Lee, Judith A. Ptasienski,
Jeffrey L. Benovic and M. Marlene Hosey

J. Biol. Chem. 1997, 272:23682-23689.
doi: 10.1074/jbc.272.38.23682

Access the most updated version of this article at http://www.jbc.org/content/272/38/23682

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 26 references, 19 of which can be accessed free at
http://www.jbc.org/content/272/38/23682.full.html#ref-list-1