Synergistic Regulation of m2 Muscarinic Acetylcholine Receptor Desensitization and Sequestration by G Protein-coupled Receptor Kinase-2 and β-Arbustin-1*

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The m2 muscarinic acetylcholine receptor (m2 mAChR) belongs to the superfamily of G protein-coupled receptors and is regulated by many processes that attenuate signaling following prolonged stimulation by agonist. We used a heterologous expression system to examine the ability of G protein-coupled receptor kinase-2 (GRK2) and β-arrestin-1 to regulate the phosphorylation state and to promote desensitization and sequestration of the m2 mAChR. Treatment of JEG-3 cells transiently expressing the m2 mAChR with a muscarinic agonist induced an ~4- or 8-fold increase in receptor phosphorylation in the absence or presence of cotransfected GRK2, respectively, compared with untreated cells transfected with receptor alone. Using the expression of a cAMP-regulated reporter gene to measure receptor function, we found that transiently transfected m2 mAChRs underwent functional desensitization following exposure to agonist. Transfected GRK2 enhanced agonist-induced functional desensitization in a manner that was synergistically enhanced by cotransfection of β-arrestin-1, which had no effect on m2 mAChR function when coexpressed in the absence of GRK2. Finally, GRK2 and β-arrestin-1 synergistically enhanced both the rate and extent of agonist-induced m2 mAChR sequestration. These results are the first to demonstrate that agonist-induced desensitization and sequestration of the m2 mAChR in the intact cell can be enhanced by the presence of GRK2 and β-arrestin-1 and that show these molecules have multiple actions on the m2 mAChR.

The family of muscarinic acetylcholine receptors (mAChRs) belongs to the superfamily of G protein-coupled receptors that couple extracellular stimuli to intracellular effector molecules through the actions of heterotrimeric G proteins (1, 2). Five subtypes of muscarinic receptors have been cloned (3–8) and classified according to their ability to couple to different signaling pathways: the m1, m3, and m5 subtypes preferentially couple to activation of phospholipase C via the Gq family of G proteins, whereas the m2 and m4 subtypes preferentially couple to inhibition of adenylyl cyclase via the Gi family of G proteins (1, 2). As with other members of the G protein-coupled receptor superfamily, the family of mAChRs is exquisitely regulated by processes that function to attenuate signaling in the presence of prolonged exposure to agonist. These processes, which differ in their time course and mechanism, have been termed desensitization, internalization (or sequestration), and down-regulation (9, 10).

Desensitization, the most rapid of these processes, is dependent upon receptor phosphorylation mediated either by second messenger kinases or by members of the family of G protein-coupled receptor kinases (GRKs) (11). GRK phosphorylation of G protein-coupled receptors, which occurs only in the presence of agonist, is thought to promote the binding of one of the members of the arrestin family of molecules to the activated receptors (12, 13). To date, four members of the arrestin family have been cloned and characterized: arrestin, cone arrestin, β-arrestin-1, and β-arrestin-2 each appear to be alternatively spliced, generating at least two polypeptides for each isoform (11). Together, the actions of GRK-mediated phosphorylation coupled with arrestin binding lead to receptor-G protein uncoupling and desensitization. The family of GRKs, which has at least six members termed GRK1 through GRK6, includes rhodopsin kinase (GRK1) and β-adrenergic receptor kinase-1 and -2 (GRK2 and GRK3, respectively) (11). The activated m2 mAChR serves as an excellent substrate in vitro for both GRK2 (14–17) and GRK3 (14) and, to a lesser extent, GRK5 (18) and GRK6 (19). Phosphorylation of the m2 mAChR by GRK2 and GRK3 promotes desensitization in vitro (14), whereas blockade of agonist-induced m2 mAChR phosphorylation in intact cells, either by transfection with kinase-inactive GRK2 or by removal of the putative phosphorylation sites by deletion mutagenesis, results in attenuation of desensitization (20).

Recent evidence suggests that phosphorylation of G protein-coupled receptors by GRKs (21, 22) and the subsequent binding of β-arrestin (23) play an additional role in receptor internalization, coupling receptors to a dynamin-dependent pathway in which receptors are targeted for endocytosis via clathrin-coated vesicles (24, 25). The role of internalization in signal attenuation is not well understood, but it has been suggested that the primary function of internalization of the β2-adrenergic receptor is to allow the dephosphorylation of receptors in preparation for their return to the plasma membrane (26, 27). Tsuga et al. (28) reported that agonist-induced sequestration of the m2 mAChR, when transiently expressed in COS-7 and BHK-21 cells, could be enhanced by coexpression of GRK2 and attenuated by coexpression of a kinase-inactive GRK2 mutant. In contrast, Pals-Rylaarsdam et al. (20) reported that coexpression of wild-type and kinase-inactive GRK2 had no effect on agonist-induced m2 mAChR sequestration in a human embry-
onic kidney cell line.

Currently, no information exists regarding the ability of specific GRKs to phosphorylate and promote desensitization of a muscarinic receptor in an intact cell, and as noted above, the role of GRK phosphorylation in sequestration of the m2 mAChR remains controversial. Moreover, blockade of desensitization and/or sequestration by kinase-inactive GRK2 indicates that a molecule that can interact with GRK is required, but does not prove that GRK2 itself is required. Whereas both β-arrestin-1 and β-arrestin-2 are known to bind the GRK-phosphorylated m2 mAChR in vitro (29, 30), there have been no reported studies describing the effects of a β-arrestin on either desensitization or sequestration of muscarinic receptors. We have used a heterologous expression system in which activation of the m2 receptor is coupled to the expression of a sensitive, cAMP-responsive reporter gene to examine the combined role of GRK2 and β-arrestin-1 in agonist-induced desensitization of the m2 mAChR in the intact cell. We report here that GRK2 and β-arrestin-1 can synergistically promote m2 mAChR desensitization and sequestration in JEG-3 cells, providing new insight into the regulation of m2 receptor function in the intact cell.

EXPERIMENTAL PROCEDURES

Materials—[3H]quimicilidinyl benzilate (47 Ci/mmol) and [3H]methylxylscolopamine ([3H]MNS; 81–84 Ci/mmol) were purchased from Amersham Corp., and [3H]orthophosphate ([3P]) was from NEN Life Science Products. Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin, and fetal bovine serum were purchased from Life Technologies, Inc. Restriction enzymes were from New England Biolabs Inc. Forskolin was obtained from Calbiochem, and n-leuciner (potassium salt) was from Analytical Luminescence Laboratory (Ann Arbor, MI). The anti-FLAG M2 monoclonal antibody was purchased from Eastman Kodak Co. F[60] polymerase was from Stratagene, whereas other polymerase chain reaction reagents were from Perkin-Elmer Corp. Electrophoresis reagents were purchased from Bio-Rad, and Isomobil-P was from Millipore Corp. Carbamylcholine chloride (carbachol), atropine, anti-mouse IgG1-agarose, and all other reagents were purchased from Sigma. Dr. D. Capon (Genentech Inc.) kindly provided the porcine m2 mAChR (Mc7 clone) (6), and Dr. G. S. McKnight (University of Washington) provided the α16 CRE-luciferase construct (31) and the RSV-β-galactosidase construct (32). Rat Grk-2 (33) was a gift from Dr. R. R. Reed (Johns Hopkins University, Baltimore, MD). Dr. R. J. Lefkowitz (Duke University) kindly provided the GRK2 and β-arrestin-1 clones. The m2 mAChR, Grk-2, and GRK2 cDNAs were subcloned into the expression vector pcDNA3 (8), a gift from Dr. T. Bonner (National Institutes of Health, Bethesda, MD).

Transfection—JEG-3 cells, a human choriocarcinoma cell line (American Type Culture Collection, Rockville, MD), were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified 10% CO2 environment. Transfection on 24-well plates and subsequent assays of luciferase and β-galactosidase/well to correct for transfection efficiency, and 100 ng of pCDPS, G-α2, and 25 ng of the anti-FLAG M2 monoclonal antibody.

Whole Cell Phosphorylation and Immunoprecipitation of m2 mAChRs—Each 150-mm culture dish of transiently cotransfected JEG-3 cells was subcultured onto three 100-mm culture dishes 16–20 h after transfection. After 20–24 h, cells from one 100-mm plate were scraped into 0.8 ml of buffer A (20 mM KH2PO4, pH 7, 20 mM NaF, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml benzamidine, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 μg/ml pepstatin) and then used to determine receptor expression. Cells on the remaining 100-mm plates were washed twice with phosphate-free DMEM, incubated for 2–3 h in phosphate-free DMEM, and then labeled an additional 4 h at 37 °C. Cells were lysed and assayed for luciferase and β-galactosidase activity as described previously (34). Data were normalized for transfection efficiency, corrected for background, and expressed as the percent of signal seen with forskolin alone (control).

Concentration of Effectors—DRG-8 cells were transiently cotransfected with 30 ng of receptor cDNA/well, 25 ng of α16 CRE-luciferase/well, 40 ng of RSV-β-galactosidase/well, 0–60 ng of pCDPS-GRK2/well, 0–60 ng of pCMV5-β-arrestin-1/well, and 100 ng of pCDPS-G-α2/well. Approximately 40–48 h after transfection, cells were pretreated without or with 0.25 ml of complete DMEM containing 15% glycerol in HEPES-buffered saline (PBS; 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4), and then triplicate wells were transfected with 0.25 ml of complete DMEM without drugs (background) or with the following concentrations of carbachol or PBS (control) for 60 min at 37 °C. In some experiments, control cells were pretreated with drug-free DMEM for either 15 or 30 min; signaling by these cells was identical to non-pretreated cells. Following pretreatment, cells were washed twice with PBS, treated with 0.25 ml of complete DMEM without drugs, and incubated an additional 4 h at 37 °C. Cells were lysed and assayed for luciferase and β-galactosidase activity as described previously (34).
The following day, immunoprecipitates were washed six to eight times with 0.5 ml of buffer C (buffer B containing 200 mM NaCl) and twice with 0.5 ml of PBS to remove nonspecifically bound proteins. The specifically adsorbed proteins were eluted from the anti-mouse IgG1-agarose by incubation in SDS-polyacrylamide gel electrophoresis sample buffer containing 8 M urea and then subjected to SDS-polyacrylamide gel electrophoresis on 9% gels containing 4 M urea as described (37), followed by electrophoretic transfer to Immobilon-P. The m2 mAChRs were analyzed by immunoblotting and autoradiography. Specifically labeled bands in the autoradiograms were analyzed by densitometry scanning on a Bio-Rad GS-670 imaging densitometer and then corrected for transfection efficiency using the amount of receptor expression levels as described under “Experimental Procedures.” Immunoprecipitated receptors were resolved by SDS-polyacrylamide gel electrophoresis using 9% gels and visualized by autoradiography. Shown is a representative example of an experiment repeated four to six times. The receptor expression levels in this experiment were as follows: m2 mAChR alone, 276 fmol/tissue culture dish; m2 mAChR + GRK2, 310; m2 mAChR + β-arrestin-1, 168; and m2 mAChR + GRK2/β-arrestin-1, 193 fmol/tissue culture dish. B, the levels of receptor phosphorylation obtained under the experimental conditions described for A were quantified and corrected for different receptor expression levels as described under “Experimental Procedures.” Data are expressed as the percent of basal phosphorylation seen in cells transfected with FLAG-m2 alone and represent the means ± S.E. of four to six independent experiments.

levels. Because the m2 mAChR exhibited agonist-dependent phosphorylation in cells transfected without added GRK2 expression vector, we determined whether agonist-dependent desensitization could be detected in these cells and whether cotransfection with GRK2 and β-arrestin-1 would lead to enhanced desensitization.

In JEG-3 cells transiently cotransfected with α168 CRE-luciferase and a construct encoding the FLAG-tagged m2 mAChR, treatment with carbachol led to a dose-dependent decrease in forskolin-stimulated luciferase expression to a maximal inhibition of 65% at 10 μM carbachol (Fig. 2A) (36). Similar results were seen in cells transfected with wild-type nontagged m2 mAChRs, indicating that addition of the FLAG epitope did not alter receptor-G protein coupling. Pretreatment of cells with 1 mM carbachol for 15 min caused a modest amount of receptor desensitization, which was demonstrated

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The m2 mAChR Synergistic Regulation by GRK2 and β-Arrestin-1

At concentrations of carbachol below 100 μM, m2 mAChR signaling was attenuated, but at concentrations of agonist of 100 μM and higher, maximal inhibition of luciferase expression was similar to that seen in the absence of pretreatment. Longer periods of pretreatment for up to 30 min did not result in additional receptor desensitization.

Expression of β-arrestin-1 had very little effect on m2 receptor signaling by itself, but appeared to potentiate the effects of GRK2 expression. Cotransfection of β-arrestin-1 in the absence of GRK2 had no measurable effect on m2 mAChR signaling. Indeed, carbachol dose-response curves in the absence or presence of pretreatment for up to 30 min with 1 mM carbachol were identical to that seen with the m2 mAChR alone (Fig. 2, compare A and B). Cotransfection of β-arrestin-1 with GRK2 resulted in greater attenuation of m2 receptor signaling than was seen with GRK2 alone. In the absence of pretreatment, signaling was similar to that seen in cells transfected either with the m2 mAChR alone or with the m2 mAChR and β-arrestin-1 below 1 μM carbachol, but was attenuated above 1 μM carbachol. Pretreatment of cells cotransfected with GRK2 and β-arrestin-1 with 1 mM carbachol for 15 min had more profound effects than that of cells expressing only GRK2, resulting in a total blockade of m2 receptor signaling. Taken together, these results suggest that phosphorylation by GRK2 and subsequent binding of β-arrestin-1 are able to promote desensitization of the m2 mAChR in an agonist-dependent and synergistic fashion. This is the first report of synergistic regulation of muscarinic receptor function in the intact cell by a specific receptor kinase and β-arrestin.

**Desensitization of the m2 mAChR Is Dependent on the Presence of the Third Cytoplasmic Loop**—The third intracellular loop of the m2 mAChR has been shown to be important in agonist-induced receptor desensitization (20) as well as internalization (41) and has been reported to contain the putative GRK phosphorylation sites (16, 20, 42). To verify that the effects of GRK2 and β-arrestin-1 coexpression on m2 mAChR signaling were occurring at the level of the receptor, we deleted a portion of the third intracellular loop, encoding amino acids 227–380, from the FLAG-m2 coding sequence. This deletion mutant (termed FLAG-m2Δ3) is similar but not identical to the mutant receptor engineered by Kameyama et al. (16), which lacks amino acids 233–380, but which has expression and ligand binding properties similar to wild-type m2 mAChR. When the construct encoding the deletion mutant m2 receptor (FLAG-m2Δ3) was transiently transfected into JEG-3 cells, treatment with carbachol led to a dose-dependent inhibition of forskolin-stimulated luciferase expression that was comparable in magnitude to that seen with the full-length m2 mAChR (Fig. 3A). In contrast to full-length receptors, pretreatment for up to 30 min with 1 mM carbachol did not lead to a shift in the dose-response curve. In addition, coexpression of GRK2 and β-arrestin-1 either singly or in combination (Fig. 3B) had no effect on the ability of the FLAG-m2Δ3 receptor to signal in either the absence or presence of pretreatment with 1 mM carbachol.
carbachol for up to 30 min. In separate experiments, the FLAG-m2Δ3 receptor was found to be expressed at levels comparable to full-length receptors and, as expected from the data of Pals-Rylaarsdam et al. (20) and Nakata et al. (42), was not phosphorylated following exposure to agonist in either the absence or presence of added GRK2 expression vector. Together, these data demonstrate that the effects of GRK2 and β-arrestin-1 on m2 mAChR signaling are dependent on the presence of the third intracellular loop and suggest that they are acting at the level of the receptor and not at a site downstream in the signaling pathway.

**Titratin of GRK2 cDNA in the Presence of β-Arrestin-1 cDNA**—To further examine the specificity of the GRK2 effects on m2 mAChR signaling, JEG-3 cells were transiently cotransfected with constant amounts of FLAG-m2 and β-arrestin-1 and varying amounts of GRK2. In the absence of pretreatment, increasing amounts of GRK2 expression vector led to a dose-dependent decrease in the ability of the m2 receptor to signal, primarily at concentrations of carbachol above 1 μM (Fig. 4A). Inhibition of forskolin-stimulated luciferase expression at carbachol concentrations up to 1 μM was independent of the presence of any amount of transfected GRK2. This experiment suggests that the biphasic nature of the carbachol dose-response relationship in the presence of both GRK2 and β-arrestin-1 is largely due to rapid desensitization of the transfected receptors at the higher concentrations of carbachol and not to increased coupling to a stimulatory G protein such as Gs.

To measure the effect of increasing GRK2 expression on agonist-induced desensitization, JEG-3 cells were transfected with varying amounts of GRK2 in the presence of constant amounts of FLAG-m2 and β-arrestin-1, but then incubated in the presence or absence of carbachol for 15 or 30 min prior to...
treatment with medium containing forskolin without (control) or with 10 μM carbachol. As seen in Fig. 4A, increasing concentrations of GRK2 expression vector in the transfection mixture decreased the ability of the activated m2mAChR to signal at this concentration of carbachol in the absence of pretreatment (Fig. 4B). When cells were first pretreated for 15 min, signaling by the m2 receptor was further attenuated in a manner that correlated with increasing amounts of transfected GRK2 expression vector (Fig. 4B). Enhanced agonist-induced desensitization was apparent at all levels of GRK2 expression vector tested, and in all cases, maximal levels of desensitization were reached by 15 min. Together, these data suggest that GRK2 is able to promote desensitization of the m2mAChR in a dose-dependent manner.

Dose Dependence of Pretreatment: Effect of Altered Agonist Concentration on Desensitization—We next attempted to determine the dose dependence of agonist pretreatment in promoting desensitization of the m2mAChR in JEG-3 cells transiently transfected either alone or with various combinations of GRK2 and β-arrestin-1. Prior to stimulation with forskolin in the presence or absence of 1 μM carbachol, transfected cells were pretreated with various concentrations of carbachol or control medium for 15 min. As seen previously with this concentration of carbachol, inhibition of forskolin-stimulated luciferase expression in the absence of pretreatment was only mildly attenuated in cells transfected either with GRK2 or with GRK2 and β-arrestin-1 together (Fig. 5). The ability of the m2mAChR to inhibit forskolin-stimulated CRE-luciferase expression following pretreatment with 1 μM carbachol was almost identical to what was seen in the absence of pretreatment. Furthermore, signaling was independent of the presence of transfected GRK2 and/or β-arrestin-1, which suggests that the levels of desensitization at this concentration of carbachol are relatively mild and below the limits of our detection. Strikingly, increasing the concentration of carbachol used for pretreatment to 5 μM promoted significant (p < 0.02) levels of desensitization only in cells transfected either with GRK2 or with GRK2 and β-arrestin-1 together, reducing the amount of inhibition seen in these cells from 45 ± 6 to 23 ± 2% and from 46 ± 1 to 21 ± 5%, respectively. In contrast, signaling in cells transfected with the m2mAChR either alone or in combination with β-arrestin-1 was not significantly affected by a similar pretreatment. Of the concentrations tested for their ability to promote desensitization, only 1 mM carbachol was sufficient to cause significant attenuation of signaling in cells transfected either with FLAG-m2 alone (p < 0.02) or with FLAG-m2 and β-arrestin-1 together (p < 0.002). Taken together, these data demonstrate that the extent of m2mAChR desensitization is proportional to the concentration of agonist regardless of the presence of cotransfected GRK2, but that desensitization occurs at much lower agonist concentrations in cells cotransfected with GRK2. In addition, they suggest that desensitization of the m2mAChR, when cotransfected with GRK2, occurs primarily at concentrations of carbachol (i.e. >1 μM) at which signaling becomes attenuated in the absence of pretreatment.

Agonist-induced Sequestration: Regulation by GRK2 and β-Arrestin-1—Phosphorylation of the β₁-adrenergic receptor by GRK2, GRK3, and GRK5 (21, 22) and β-arrestin binding (23, 24) have recently been found to promote agonist-induced sequestration of this receptor. Evidence for a role of GRK phosphorylation in agonist-induced sequestration of m2mAChRs is contradictory (20, 28), whereas no data exist for the role of β-arrestin-1 in the sequestration of m2mAChRs. Because coexpression of GRK2 and β-arrestin-1 was able to regulate the m2mAChR in a functional assay, we tested the effects of these molecules on agonist-induced sequestration in transiently transfected JEG-3 cells.

Agonist-induced sequestration was measured using the membrane-impermeable muscarinic ligand [3H]NMS, which detects only cell-surface receptors. In transiently transfected JEG-3 cells, m2mAChRs underwent agonist-induced sequestration that was both time- and dose-dependent (Fig. 6, A and B, respectively) (43). Cotransfection with GRK2 led to an increase in both the rate and extent of m2mAChR sequestration over the entire time course tested (Fig. 6A). In contrast to previous experiments in which coexpression of β-arrestin-1 in the absence of GRK2 did not alter m2mAChR desensitization, coexpression of β-arrestin-1 led to an increase in the rate and extent of agonist-induced sequestration that was similar but not identical to that seen with GRK2 coexpression (Fig. 6A). Cotransfection of both β-arrestin-1 and GRK2 expression vectors led to an enhancement of the extent of m2mAChR sequestration that was slightly greater than what would be expected if the effects of each were added together (Fig. 6A). In addition, the apparent rate of m2mAChR sequestration as determined from the first-order decay curve was increased by ~130% in cells transfected with both GRK2 and β-arrestin-1 as compared with ~36 or ~45% in cells transfected with either GRK2 or β-arrestin-1, respectively (Fig. 6A, inset). This greater than additive effect implies that the two molecules are acting in concert, or synergistically, to enhance agonist-induced sequestration of the m2mAChR.

The synergistic effect was even more evident upon examination of the dose dependence of sequestration. At the lowest concentrations of agonist (i.e. 1 and 10 μM), the presence of β-arrestin-1 did not lead to enhanced sequestration in cells cotransfected with only the m2mAChR, but did promote sequestration in cells also cotransfected with GRK2 (Fig. 6B). Interestingly, cotransfection of GRK2 and β-arrestin-1 together led to a significant (p < 0.05) enhancement of sequestration at
concentrations of carbachol as low as 1 μM (Fig. 6B), a concentration at which transiently transfected m2 mAChRs failed to undergo measurable desensitization even when cotransfected with GRK2 and/or β-arrestin-1 (Fig. 5). It is unlikely that the effects of GRK2 and/or β-arrestin-1 cotransfection were due to alterations in receptor expression because basal levels of cell-surface m2 mAChR expression were fairly similar for each of the conditions tested (Fig. 6C). Taken together, these data agree with the work of Tsuga et al. (28), who suggested that GRK2 can promote agonist-induced sequestration of the m2 mAChR. In addition, we show for the first time that β-arrestin-1 is able to promote m2 mAChR sequestration in a way that appears to be synergistic with GRK2.

**DISCUSSION**

Numerous studies have demonstrated phosphorylation of the m2 mAChR by a variety of GRKs using in vitro assay systems, but it has proven difficult to determine the functional consequences of this modification within an intact cell. Pals-Rylaarsdam et al. (20) demonstrated that coexpression of kinase-inactive GRK2 in a human embryonic kidney cell line not only attenuates phosphorylation of the m2 mAChR following exposure to agonist, but dramatically inhibits the associated desensitization. The primary drawback to this type of study is that while it demonstrates that a molecule that interacts with the kinase-inactive mutant is involved in receptor desensitization, it does not prove that the GRK itself is actually involved. In addition, this type of method cannot distinguish between different functional effects potentially caused by the actions of different GRK isoforms. For example, Tiberi et al. (44) found that whereas GRK2, GRK3, and GRK5 were able to phosphorylate the dopamine D1A receptor to similar extents, the desensitization associated with GRK5 phosphorylation was significantly more profound than that seen with either GRK2 or GRK3. We describe here a system that allows reconstitution of the desensitization machinery in intact cells to test the effects of different components on the functional responsiveness of the m2 mAChR using a cAMP-regulated reporter gene. Our laboratory has previously demonstrated the utility of such an approach, showing that the m2 and m4 mAChRs differentially couple to members of the G1 and G3 class of G proteins in transiently transfected JEG-3 cells (36), as well as the effects of different mutations on the activity of G1,2 and G3,6 (45).

Initially, using whole cell phosphorylation studies, we demonstrated that the m2 mAChR is phosphorylated following exposure to agonist in JEG-3 cells in the absence of added GRK2 expression vector (Fig. 1). Coexpression of GRK2 with the m2 mAChR led to a slight enhancement of basal phosphorylation, but the predominant effect was an increase in agonist-induced phosphorylation, which indicates that the m2 mAChR serves as a substrate for GRK2 in JEG-3 cells. This is in agreement with previous studies that demonstrated that the m2 receptor is an excellent substrate for both GRK2 and GRK3 in vitro (14). Increased basal phosphorylation of other receptors has been seen in similar studies in which transient transfection has been used to overexpress GRK isoforms. For example, Menard et al. (22) reported that overexpression of GRK4 and cell-surface receptors measured in untreated cells and represent the means ± S.E. of three or four independent experiments, each performed in quadruplicate. C, receptors expressed on the surface of cells transfected with FLAG-m2 alone or in combination with GRK2, β-arrestin-1, or both GRK2 and β-arrestin-1 as indicated were measured using [3H]NMS as described under “Experimental Procedures.” Data are expressed as the percent of

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**Fig. 6. Dose and time dependence of agonist-induced sequestration of FLAG-tagged m2 mAChRs coexpressed with various combinations of GRK2 and/or β-arrestin-1.** A and B, JEG-3 cells were transfected with FLAG-m2 alone (squares) or in combination with β-arrestin-1 (βARR1; triangles), GRK2 (inverted triangles), or both GRK2 and β-arrestin-1 (circles) and then treated with 1 μM carbachol for the times indicated (A) or for 15 min with the concentrations of carbachol indicated (B). The inset shows the data from A fit to an exponential decay curve; the apparent first-order rate constants in cells transfected with the various constructs are as follows: m2 mAChR alone, 2.2 × 10⁻² min⁻¹; m2 mAChR + β-arrestin-1, 3.2 × 10⁻² min⁻¹; m2 mAChR + GRK2, 3.0 × 10⁻² min⁻¹; and m2 mAChR + β-arrestin-1/GRK2, 5.1 × 10⁻² min⁻¹. Cell-surface receptors were measured using the membrane-impermeable muscarinic ligand [3H]NMS as described under “Experimental Procedures.” Data are expressed as the percent of
GRK6 enhanced primarily basal phosphorylation of the β2-adrenergic receptor and that GRK5 enhanced both basal and agonist-induced phosphorylation. Interestingly, coexpression of β-arrestin-1 led to a modest reduction of agonist-induced phosphorylation in cells cotransfected with GRK2 (Fig. 1). This might be explained by recent work demonstrating that β-arrestin-1 is involved in coupling activated receptors to the internalization machinery (23–25). It has been suggested that one function of receptor internalization might be dephosphorylation of desensitized receptors in preparation for their return to the cell surface (26, 27). Indeed, a phosphatase with activity toward GRK-phosphorylated G protein-coupled receptors has been identified and found to be exclusively associated with the particulate fraction (46).

Using a heterologous expression system in which receptor activation is coupled to inhibition of CRE-luciferase expression, we demonstrated that transiently transfected m2 mAChRs become functionally desensitized when exposed to agonist (Fig. 2A). Cotransfection of GRK2 led to a modest attenuation of receptor signaling in the absence of pretreatment and, more significantly, enhanced functional desensitization following exposure to agonist (Fig. 2A). This agonist-induced desensitization in the presence of GRK2 was manifested by a shift in the dose-response curve for inhibition of forskolin-stimulated luciferase expression as well as a marked decrease in the maximal extent of inhibition. Coexpression of β-arrestin-1 led to an increase in both components of the desensitization seen in cells transfected with GRK2: signaling at high concentrations of agonist in the absence of pretreatment was decreased, and agonist-induced desensitization was enhanced (Fig. 2B). The effects of β-arrestin-1 on m2 mAChR desensitization were dependent on the presence of cotransfected GRK2 cDNA; cotransfection of β-arrestin-1 in the absence of GRK2 had no effect on m2 mAChR signaling even though transiently expressed m2 mAChRs are phosphorylated when expressed alone in JEG-3 cells (Fig. 1). The lack of effect of β-arrestin-1 (in the absence of GRK2) on m2 mAChR signaling could be explained by a number of possibilities. First, phosphorylation of the m2 mAChR by the endogenous kinase(s) might be rate-limiting and the endogenous arrestin(s) present in excess, so additional β-arrestin-1 is unable to promote additional desensitization. Alternatively, the endogenous m2 mAChR kinase activity could be distinct from GRK2, so phosphorylation by this kinase does not promote the association of transiently expressed β-arrestin-1 in these cells in a way that promotes functional desensitization. Whereas different GRKs can promote qualitatively different forms of desensitization (44), the question of whether different GRKs can promote the association of different β-arrestins or differentially regulate the functional effects of β-arrestin binding remains to be examined.

Titration of GRK2 cDNA in the presence of β-arrestin-1 caused a dose-dependent decrease in the ability of the m2 mAChR to signal in the absence of pretreatment, primarily at higher concentrations of agonist (Fig. 4A). Signaling at carbachol concentrations up to 1 μM was independent of the presence of exogenously expressed GRK2, suggesting that desensitization is negligible at these lower concentrations of carbachol. Alteration of the concentration of carbachol with which transiently transfected cells were pretreated allowed us to demonstrate that desensitization in JEG-3 cells coexpressing the m2 mAChR and either GRK2 or GRK2 in combination with β-arrestin-1 was negligible at concentrations of carbachol below 5 μM (Fig. 5). In contrast, pretreatment of these cells with 5 μM carbachol caused an ~50% decrease in signaling. This is similar to the EC50 for carbachol to induce phosphorylation of exogenously expressed m2 mAChRs in Sf9 cell membranes (11.5 μM) (17) and of the endogenous muscarinic receptors found in chick heart (20 μM) (47), which are predominately the m2 subtype (48–50). Together, these data suggest that phosphorylation of the m2 mAChR by GRK2 is responsible for the decreased ability of the receptor to signal at higher concentrations of carbachol.

Despite having no direct effect on agonist-induced desensitization of the m2 mAChR, expression of β-arrestin-1 in JEG-3 cells in the absence of exogenous GRK2 expression enhanced both the rate and extent of agonist-induced m2 mAChR sequestration (Fig. 6A). These effects were qualitatively similar to those seen with GRK2 expression, although the enhancement of sequestration by β-arrestin-1 at the earlier time points was slightly less than that seen with GRK2. The enhancement of sequestration seen with GRK2 and β-arrestin-1 coexpression was slightly greater than if the effects of each were simply added together, implying that the two molecules are acting synergistically. This synergism between GRK2 and β-arrestin-1 is similar to that seen with receptor desensitization (Fig. 2) and demonstrates for the first time that agonist-induced internalization of the m2 mAChR can be regulated by β-arrestin-1 and GRK2 together.

Regulation of m2 mAChR sequestration by GRK2 has been reported previously (28). It was found that overexpression of GRK2 in COS-7 and BHK-21 cells enhanced sequestration of the m2 mAChR, whereas overexpression of a kinase-inactive GRK2 allele attenuated sequestration. In contrast, Pals-Rylaarsdam et al. (20) performed similar experiments in a human embryonic kidney cell line and found that coexpression of either GRK2 or kinase-inactive GRK2 had no effect on transiently expressed m2 mAChR internalization. The data presented in this report demonstrate that GRK2, in addition to β-arrestin-1, can indeed promote m2 mAChR internalization when expressed in JEG-3 cells. These data do not, however, prove that GRK2 and β-arrestin-1 are absolutely required for sequestration to occur. Indeed, at least two sequestration pathways appear to exist in HEK-293 cells, one that is dependent on β-arrestin and dynamin and one that is independent of these molecules (24), and it is likely that multiple sequestration pathways exist in other cell types as well. In HEK-293 cells, sequestration of the angiotensin II type 1A receptor was found to be unaffected by a dominant-negative allele of dynamin unless recruited to this pathway by overexpression of β-arrestin-1. One explanation for the results of Pals-Rylaarsdam et al. (20) is that the dynamin-independent pathway may be the primary pathway by which m2 mAChRs undergo agonist-induced sequestration in human embryonic kidney cells and that this pathway may be insensitive to the effects of added GRK2 or kinase-inactive GRK2.

In summary, we have demonstrated that the m2 mAChR can be regulated synergistically by coexpression of GRK2 and β-arrestin-1 in JEG-3 cells. In addition to being the predominant muscarinic receptor in the heart, the m2 mAChR is found in a variety of peripheral tissues and throughout the brain (51). In neuronal tissues, the m2 mAChR is found associated with cholinergic as well as non-cholinergic neurons, which suggests that it is found presynaptically and may be found postsynaptically as well (52, 53). This is similar to the distribution of both GRK2 and GRK3, which are found throughout the brain associated with both postsynaptic densities and axon terminals (54). In addition, β-arrestin-1 is found to be highly expressed in the brain (13), although its ultrastructural localization has not been examined. Together, these observations suggest that both GRK2 and β-arrestin-1 may colocalize with the m2 mAChR in the brain, making them ideally situated to regulate m2 receptor function. We have shown here that GRK2 coexpression in
the intact cell enhanced phosphorylation of the m2 mACHr in
an agonist-dependent manner, resulting in increased desensiti-
zation and sequestration. β-Arrestin-1 coexpression had rel-
atively mild effects on m2 mAChR function by itself, but sig-
nificantly enhanced the effects of GRK2, suggesting that the
two molecules can act synergistically and showing that β-ar-
restin-1 can regulate the function of a muscarinic receptor in
the intact cell. These results provide new and useful informa-
tion that helps to explain the intricate mechanisms utilized by
the cell to regulate m2 mAChR signaling.

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