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# $M_3$ Muscarinic Acetylcholine Receptor-Mediated Signaling Is Regulated by Distinct Mechanisms

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## ABSTRACT

We have used RNA interference previously to demonstrate that G protein-coupled receptor kinase 2 (GRK2) regulates endogenously expressed H1 histamine receptor in human embryonic kidney 293 cells. In this report, we investigate the regulation of endogenously expressed $M_3$ muscarinic acetylcholine receptor ($M_3$ mAChR). We show that knockdown of GRK2, GRK3, or GRK6, but not GRK5, significantly increased carbachol-mediated calcium mobilization. Stable expression of wild-type GRK2 or a kinase-dead mutant (GRK2-K220R) reduced calcium mobilization after receptor activation, whereas GRK2 mutants defective in $G\alpha_q$ binding (GRK2-D110A, GRK2-R106A, and GRK2-R106A/K220R) had no effect on calcium signaling, suggesting that GRK2 primarily regulates $G_q$ after $M_3$ mAChR activation. The knockdown of arrestin-2 or arrestin-3 also significantly increased carbachol-mediated calcium mobilization. Knockdown of GRK2 and the arrestins also significantly enhanced carbachol-mediated activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), whereas prolonged ERK1/2 activation was only observed with GRK2 or arrestin-3 knockdown. We also investigated the role of casein kinase-1$\alpha$ (CK1$\alpha$) and found that knockdown of CK1$\alpha$ increased calcium mobilization but not ERK activation. In summary, our data suggest that multiple proteins dynamically regulate $M_3$ mAChR-mediated calcium signaling, whereas GRK2 and arrestin-3 play the primary role in regulating ERK activation.

Activation of G protein-coupled receptors (GPCRs) by agonist occupancy leads to a conformational change in the receptor that promotes the activation of heterotrimeric G proteins, which in turn activate a variety of effectors leading to downstream signaling events (Pierce et al., 2002). Activated GPCRs are regulated by three principal mechanisms: desensitization, internalization, and down-regulation. Receptor desensitization is initiated by the phosphorylation of serine/threonine residues by GPCR kinases (GRKs), which promotes the high-affinity binding of arrestins, uncoupling the receptor from G protein and terminating signaling (Krupnick and Benovic, 1998).

There are seven members of the GRK family that are grouped into three subfamilies based on sequence and functional similarity: GRK1 and GRK7; GRK2 and GRK3; and GRK4, GRK5, and GRK6. GRK2, GRK3, GRK5, and GRK6 are expressed ubiquitously, whereas GRK1, GRK4, and GRK7 have a restricted expression pattern. Much of the research determining specific GPCR-GRK interaction has relied on techniques such as heterologous overexpression, dominant-negative constructs, and more recently RNA interference (Krupnick and Benovic, 1998; Iwata et al., 2005; Kim et al., 2005).

The nonvisual arrestins, arrestin-2 ($\beta$-arrestin1) and arrestin-3 ($\beta$-arrestin2), bind to activated, phosphorylated GPCRs, subsequently terminating G protein activation and targeting the receptors to clathrin-coated pits for internalization (Moore et al., 2007). Arrestins have also been shown to act as scaffolding proteins to promote downstream signaling events, such as activation of mitogen-activated protein kinases (Lefkowitz and Shenoy, 2005).

The muscarinic acetylcholine receptors (mAChRs) represent a subfamily of GPCRs with five subtypes, $M_1$ to $M_5$. The $M_3$ mAChR couples to $G_q$, resulting in phospholipase C-$\beta$ (PLC-$\beta$) activation, and production of inositol trisphosphate ($IP_3$) and diacylglycerol (DAG), which leads to calcium re-

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**ABBREVIATIONS:** GPCR, G protein-coupled receptor; Bis I, bisindolylmaleimide I; CK1$\alpha$, casein kinase 1-$\alpha$; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; GRK, G protein-coupled receptor kinase; $IP_3$, inositol trisphosphate; $M_3$ mAChR, muscarinic acetylcholine receptor subtype 3; PKC, protein kinase C; PLC-$\beta$, phospholipase C-$\beta$; HEK, human embryonic kidney; siRNA, small interfering RNA; AM, acetoxymethyl ester; McN-A-343, 4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; MEK, mitogen-activated protein kinase kinase.

lease from intracellular stores and protein kinase C (PKC) activation. In addition, the $M_3$ mAChR can activate extracellular signal-regulated kinase (ERK) in a calcium-independent and PKC-dependent manner (Kim et al., 1999; Wylie et al., 1999).

Upon activation, the $M_3$ mAChR is rapidly phosphorylated on serine/threonine residues within the third intracellular loop (Tobin et al., 1997) and C-terminal tail (Budd et al., 2000), although it is unclear which kinases mediate receptor phosphorylation and regulation. Wu et al. (2000) showed that GRK2 phosphorylates the $M_3$ mAChR in a G$\beta\gamma$-dependent manner and mapped the phosphorylation sites to $^{331}$SSS$^{333}$ and $^{348}$SASS$^{351}$ in the third intracellular loop. GRK3 also has the ability to phosphorylate the receptor, but receptor regulation seems to occur primarily through modulation of PLC-$\beta$ activity (Willets et al., 2001, 2002, 2003). Willets and coworkers (Willets et al., 2001, 2002, 2003) also showed that GRK6 regulates the $M_3$ mAChR by phosphorylation, whereas GRK2 and GRK5 were found to have no effect on the receptor in SH-SY5Y cells. In addition to GRK-mediated phosphorylation, casein kinase 1$\alpha$ (CK1$\alpha$) has also been shown to phosphorylate the $M_3$ mAChR in an agonist-dependent manner, although this alone was insufficient to mediate receptor desensitization (Budd et al., 2000). Finally, arrestins do not seem to be required for $M_3$ mAChR internalization (Lee et al., 1998; Mundell and Benovic, 2000) but are involved in receptor desensitization with no discernible specificity between arrestin-2 and arrestin-3 (Mundell and Benovic, 2000).

One major unanswered question regarding the physiological regulation of GPCRs is to understand which GRKs and arrestins regulate a given receptor subtype. To date, a limited number of GRKs and arrestins have been identified, whereas more than 700 mammalian GPCRs have been cloned (Gainetdinov et al., 2004). Studies over the past decade have defined the ability of individual GRKs, second messenger-dependent kinases (e.g., protein kinase A or PKC), and arrestins to regulate GPCRs in model systems. However, the mechanisms by which GRKs target endogenous GPCRs are still unknown. Using either wild-type GRK2, kinase-dead GRK2, or mutants deficient in G$\alpha_q$ binding, we showed previously that the human H1 histamine receptor was specifically regulated by GRK2, mainly through regulation of activated $G_q$ (Iwata et al., 2005). In this report, we used RNA interference to target proteins specifically involved in the agonist-dependent regulation of the endogenous $M_3$ mAChR in HEK-293 cells. We found that there was differential GRK-mediated regulation of this receptor as assessed by calcium signaling and ERK activation. In addition, knockdown of either arrestin-2 or arrestin-3 resulted in enhanced signaling from the receptor with different temporal effects. Furthermore, we show that in addition to GRKs, CK1$\alpha$ has a negative role in $M_3$ mAChR-mediated calcium mobilization. Taken together, our results show that multiple proteins mediate agonist-dependent regulation of $M_3$ mAChR signaling.

## Materials and Methods

**Materials.** HEK-293 cells were from Microbix Biosystems, Inc (Toronto, Canada), whereas carbachol was from EMD Biosciences (San Diego, CA). Pirenzepine and *p*-fluorohexahydro-sila-difenol were from Sigma-Aldrich (St. Louis, MO), and Lipofectamine 2000 and Opti-MEM were from Invitrogen (Carlsbad, CA). Phospho-specific p44/p42 polyclonal antibody was from Cell Signaling Technologies (Danvers, MA). Polyclonal ERK2, CK1$\alpha$, and GRK3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-$\beta$-arrestin monoclonal antibody was from BD Biosciences Pharmingen (San Diego, CA). Anti-GRK4–6 monoclonal antibody was from Millipore (Billerica, MA), whereas the GRK2 monoclonal antibody was produced in our laboratory; anti-$\alpha$-tubulin monoclonal antibody was from Sigma.

**Synthesis of Small Interfering RNAs.** All small interfering RNAs (siRNAs) were chemically synthesized by Dharmacon RNA Technologies (Lafayette, CO). The GRK2, GRK5, and CK1$\alpha$ siRNAs were reported previously (Liu et al., 2002; Iwata et al., 2005; Kim et al., 2005). The GRK3 siRNA sequence was 5′-GCAGAAGUCGACAAAUUUA-3′, whereas 5′-GCGCUUGGCCUACGCCUAU-3′ was used for GRK6. Arrestin-2 and -3 siRNAs were purchased as a SMARTpool. Nonspecific control siRNA VIII (5′-AAACUCUAUCUGCACGCUGAC-3′) was used as the control for all siRNA experiments.

**Cell Culture and siRNA Transfection.** HEK-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 25 mM HEPES, pH 7.2, and 0.1 mM nonessential amino acids in a 5% $CO_2$ incubator at 37°C. For transfection of GRK and casein kinase siRNAs, HEK-293 cells grown to 85 to 90% confluence in 100-mm dishes were transfected with 600 pmol of siRNA using Lipofectamine 2000 in Opti-MEM. After 6 h, cells were split 1:2, and a second transfection of 600 pmol was performed 24 h after the initial transfection. Forty-eight hours after the second transfection, cells were split for assay the following day. For arrestin SMARTpool siRNAs, cells ~70% confluent were transfected with 600 pmol of siRNA corresponding to either arrestin-2 or arrestin-3. Forty-eight hours later, cells were split for assay the following day. Control siRNA was transfected in a similar fashion as described above for each transfection condition.

**Immunoblotting.** To analyze siRNA target proteins, siRNA transfected HEK-293 cells in a six-well plate were washed twice with ice-cold phosphate-buffered saline and lysed with buffer [20 mM HEPES, pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% Triton X-100, and one tablet of Complete protease inhibitor (Roche, Indianapolis, IN) per 50 ml] at 4°C on a rocker for 30 min. The lysates were centrifuged at 4°C at 30,000 rpm in a TLA45 rotor for 30 min. The supernatants were electrophoresed on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and immunoblotted using monoclonal anti-GRK2 (1:1000), polyclonal anti-GRK3 (1:200), monoclonal anti-GRK4–6 (1:3000), monoclonal anti-$\beta$-arrestin-1 (1:1000), or polyclonal anti-CK1$\alpha$ (1:200), horseradish peroxidase-labeled secondary antibodies, and chemiluminescence. The blots were stripped and reprobed using an antitubulin (1:7500) monoclonal antibody.

**Measurement of Intracellular Calcium Mobilization.** Calcium mobilization was performed as described previously with slight modifications (Iwata et al., 2005). In brief, HEK-293 cells transfected with siRNAs were harvested with Cellstripper (Mediatech, Herndon, VA), washed twice with phosphate-buffered saline, and resuspended at $5 \times 10^6$ cells/ml in Hanks' balanced salt solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mg/ml glucose) (Invitrogen) containing 0.025% bovine serum albumin. The cells were then loaded with 3 $\mu$M Fura-2 acetoxymethyl ester derivative (Fura-2/AM; Invitrogen, Carlsbad, CA) for 30 min at 37°C. The cells were washed once in Hanks' solution, resuspended in Hanks' solution containing 0.025% bovine serum albumin, incubated at room temperature for 15 min, washed twice in Hanks' solution, and then resuspended in Hanks' at a concentration of $3 \times 10^7$ cells/ml. A typical experiment contained $1.5 \times 10^6$ cells/1.6 ml in a quartz cuvette and stimulation with different concentrations of carbachol. Calcium mobilization was measured using excitation at 340 and 380 nm and emission at 510 nm in a fluorescence spectrometer (LS55; PerkinElmer Life and Analytical Sciences, Waltham, MA). Calibration was performed using 0.1% Triton X-100 for total fluorophore release and 15 mM EGTA to chelate free calcium. When

antagonists were used, cells were preincubated with the indicated antagonist for 30 s before starting the fluorescent spectrometer and an additional 30 s before stimulation with carbachol. Intracellular calcium concentrations were calculated using a fluorescence spectrometer measurement program.

**ERK Activation Assays.** HEK-293 cells, ~90% confluent in six-well plates, were serum-starved for at least 6 h. After serum starvation, cells were stimulated with 100 $\mu$M carbachol as indicated and washed once with ice-cold phosphate-buffered saline. Lysis buffer (1% Triton X-100, 20 mM HEPES, pH 7.2, 150 mM NaCl, 10 mM EDTA, 1 $\mu$M sodium orthovanadate, 3 mM sodium pyrophosphate, 10 mM sodium fluoride, and 1 Complete protease inhibitor tablet per 50 ml) was added, and plates were stored at −80°C until harvesting. Cells were thawed and scraped into lysis buffer on ice, vortexed briefly, and debris was cleared by centrifugation at 14,000 rpm for 15 min. Equal amounts of whole-cell lysate were separated by electrophoresis on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and proteins were detected by immunoblotting. Nitrocellulose membranes were blocked for 1 h at room temperature in a 1:3 dilution of ODYSSEY blocking buffer (LI-Cor Biosciences, Lincoln, NE). A mixture of primary antibodies directed at ERK2 (monoclonal; Santa Cruz) and phospho-ERK1/2 (polyclonal; Cell Signaling Technologies) in 100% ODYSSEY blocking buffer were incubated overnight at 4°C. Nitrocellulose membranes were washed with Tris-buffered saline containing 0.1% Tween 20 over 40 min. The membranes were then incubated for 1 h at room temperature with a mixture of goat anti-rabbit Alexa Fluorophore 680 conjugated (Invitrogen) and goat anti-mouse IRDye 800 conjugated (Rockland Immunochemicals, Gilbertsville, PA) antibodies. After a 1-h incubation, the membranes were washed with Tris-buffered saline containing 0.1% Tween 20 for 60 min. Fluorescence was detected simultaneously using the ODYSSEY infrared imaging system (LI-Cor Biosciences). When antagonists were used, cells were incubated at 37°C with the indicated antagonist for 5 min before stimulation with carbachol. Fluorescence intensity of phosphorylated ERK2 was normalized to total ERK2 fluorescence, and data are represented as the fold increase over basal (± S.E.M.).

**Statistical Analysis.** Results were analyzed using a paired, two-tailed, Student's $t$ test with significance at $p \leq 0.05$.

## Results

**Pharmacological Characterization of the Muscarinic Acetylcholine Receptor Subtype Endogenously Expressed in HEK-293 Cells.** Using RNAi, we have shown previously that GRK2 regulates the endogenously expressed H1 histamine receptor in HEK-293 cells (Iwata et al., 2005). We wanted to expand this approach to determine the regulation of other endogenous GPCRs. Previous work has shown that HEK-293 cells respond to stimulation with carbachol, a nonspecific mAChR agonist, with robust $IP_3$ production and calcium mobilization that had been attributed to the $M_1$ mAChR subtype (Mundell and Benovic, 2000). However, a recent microarray analysis of commonly used cell lines suggested that the mAChR endogenously expressed in these cells is the $M_3$ receptor subtype (Hakak et al., 2003). In light of this, we sought to pharmacologically determine which mAChR subtype is actually expressed in HEK-293 cells. Cells loaded with the ratiometric calcium indicator Fura-2/AM display a robust increase in calcium mobilization in response to carbachol stimulation (Fig. 1A) with an $EC_{50}$ value of 20 $\mu$M (data not shown). Incubation with the antagonist *p*-FHHsiD, which has some selectivity for the $M_3$ mAChR ($pK_i = 7.7$) compared with the $M_1$ mAChR ($pK_i = 7.1$) (de la Vega et al., 1997), completely inhibited calcium mobilization in response to carbachol, whereas the selective $M_1$ mAChR antagonist pirenzepine only slightly inhibited calcium mobilization (Fig. 1A). This result is in line with previous reports demonstrating that pirenzepine selectively inhibits the $M_1$ mAChR ($pK_i = 8.0$) but at higher concentrations is able to inhibit the $M_3$ subtype ($pK_i = 6.7$) (de la Vega et al., 1997). In addition, there was no calcium response when the cells were

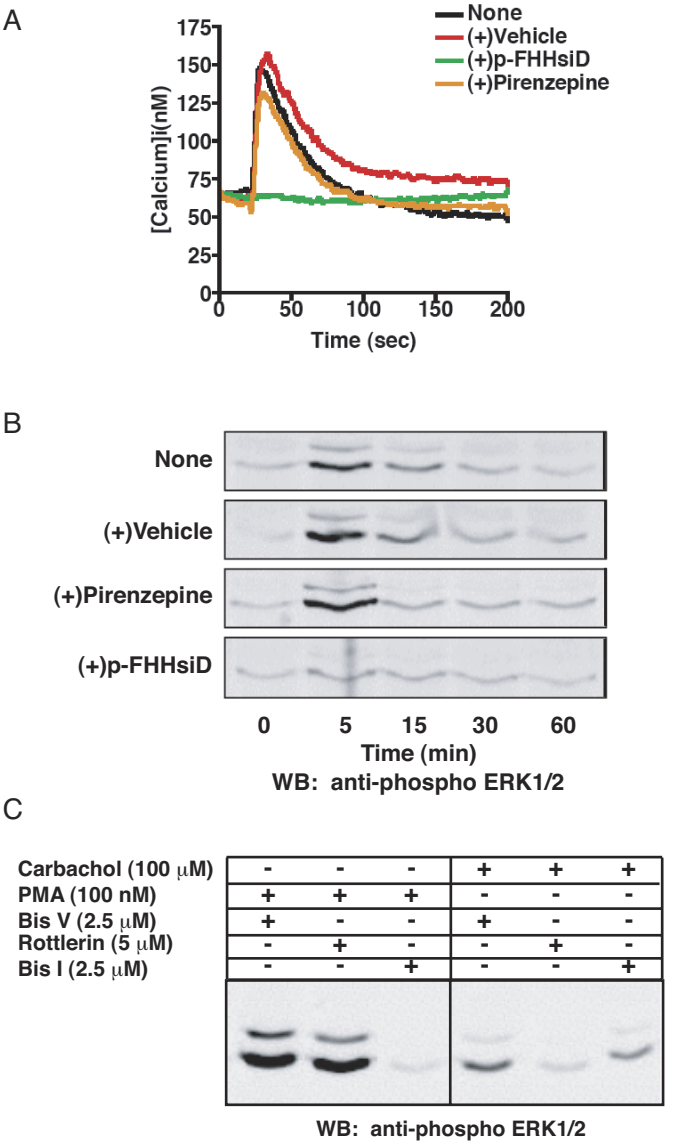


**Fig. 1.** Characterization of the muscarinic acetylcholine receptor subtype endogenously expressed in HEK-293 cells. A, HEK-293 cells loaded with the ratiometric calcium indicator Fura-2/AM were incubated with 100 nM pirenzepine (orange), 1 $\mu$M p-FHHsiD (green), vehicle (red), or not pretreated (black) and stimulated with 100 $\mu$M carbachol. Changes in calcium mobilization were assayed by monitoring the change in Fura-2/AM fluorescence. Shown is a representative tracing from three independent experiments. B, After a 6-h serum-starve, HEK-293 cells were incubated with 100 nM pirenzepine, 1 $\mu$M p-FHHsiD, vehicle, or not pretreated and stimulated with 100 $\mu$M carbachol for the indicated times. Cells from a six-well plate were harvested, and equal amounts of total cellular lysate were separated by SDS-PAGE and probed for phospho-ERK1/2 as described under *Materials and Methods*. Shown is a representative immunoblot of three independent experiments. C, cells were treated with Bis I (2.5 $\mu$M), bisindolymaleimide V (Bis V; 2.5 $\mu$M), or rottlerin (5 $\mu$M) for 30 min before stimulation with carbachol (100 $\mu$M) for 5 min or PMA (100 nM) for 15 min. pFHHsiD, *p*-fluorohexahydro-sila-difenol.

stimulated with the $M_1/M_4$ mAChR-selective agonist McN-A-343 (data not shown).

To further investigate the subtype of mAChR expressed, we also analyzed the effects of the $M_1$- and $M_3$-selective antagonists on carbachol-stimulated ERK activation. GPCRs activate ERK1/2 via a number of pathways (Werry et al., 2005), and both the $M_1$ and $M_3$ mAChRs have been shown to activate ERK1/2 in a number of cell types (Budd et al., 1999; Guo et al., 2001). Carbachol-mediated ERK1/2 activation in HEK-293 cells is dose-dependent ($EC_{50}$ ~8 $\mu$M), peaking at 5 min and returning to basal levels by 60 min (Fig. 1B, top). The addition of *p*-FHHsiD completely blocked ERK1/2 activation in response to carbachol, whereas pirenzepine had no effect (Fig. 1B). These results confirm that the primary mAChR subtype in HEK-293 cells is the $M_3$.

We also wanted to determine whether PKC was responsible for ERK activation after $M_3$ mAChR stimulation. Previous evidence suggests that the novel PKC isoforms are responsible for $M_3$ mAChR-mediated ERK activation, including PKCε in SK-N-BE2(C) cells (Kim et al., 1999) and a calcium-independent PKC in Chinese hamster ovary cells (Wylie et al., 1999). Furthermore, it has been shown recently that the $M_3$ mAChR regulates the Kir 3.1/3.2 potassium channel through activation of PKC-δ in HEK-293 cells (Brown et al., 2005). To establish whether PKC-δ is involved in $M_3$ mAChR-mediated ERK activation, we used bisindolylmaleimide I (Bis I), a general PKC inhibitor, and rottlerin, which selectively inhibits PKC-δ (Gschwendt et al., 1994). Rottlerin significantly inhibited carbachol-mediated ERK activation, whereas Bis I only partially inhibited ERK activation (Fig. 1C). The specificity of these inhibitors was confirmed by the demonstration that rottlerin had minimal effects on PMA-induced ERK activation, whereas Bis I completely inhibited PMA-promoted ERK activation (Fig. 1C). Taken together, we conclude that HEK-293 cells endogenously express the $M_3$ mAChR and that carbachol-mediated activation of the ERK1/2 cascade is dependent on PKC-δ.

**Regulation of $M_3$ mAChR-Mediated Calcium Mobilization in HEK-293 Cells.** We next evaluated the effect of knocking down various regulatory proteins on $M_3$ mAChR signaling. Because the phosphorylation of activated GPCRs by GRKs is often an early step in signal termination, we initially determined the effect that GRK knockdown would have on calcium mobilization after carbachol treatment. As shown in Fig. 2, A and B, we were able to selectively and specifically knock down each of the four individual GRKs expressed in HEK-293 cells. A modest increase in GRK3 expression was observed when other GRKs, in particular GRK2, were knocked down (Fig. 2B). Knockdown of GRK2, GRK3, and GRK6 led to increases of 210% ($p < 0.001$), 190% ($p < 0.001$), and 230% ($p < 0.001$), respectively, in the peak calcium transients, whereas knockdown of GRK5 had no effect on calcium mobilization (Fig. 3, A and B). This effect was also observed when methacholine was used to activate the $M_3$ mAChR (data not shown). These data suggest that multiple GRKs are involved in the desensitization of the $M_3$ mAChR.

**GRK2 Interaction with $G_q$ Is Primarily Responsible for Increased Calcium Mobilization.** The enhanced mobilization of calcium seen after silencing of GRK2 may arise from phosphorylation-dependent and/or phosphorylation-independent mechanisms (Ribas et al., 2007). Therefore, we next sought to further delineate the underlying mechanism observed for calcium mobilization when GRK2 was knocked down. Because we showed previously that GRK2 interacts with $G\alpha_q$ through the RGS homology domain of GRK2 (Carman et al., 1999), the increase in peak calcium mobilization could be a result of a loss of receptor phosphorylation, a loss of the ability of GRK2 to inhibit activated $G_q$, or both. To address this, we generated cell lines that stably express either wild-type bovine GRK2, kinase dead GRK2 (K220R), GRK2 point mutants defective in binding $G\alpha_q$ (R106A, D110A), or a GRK2 mutant that was both kinase-dead and $G_q$-deficient (R106A/K220R). Cloned cell lines expressing wild-type or mutant bovine GRK2 at levels close to endogenous GRK2 levels (1- to 5-fold overexpression) were selected for study (Fig. 3C). SDS-PAGE revealed that bovine GRK2 ran slightly slower than endogenous human GRK2 when expressed in HEK-293 cells (Fig. 3C). Stable expression of either wild-type or the kinase-dead mutant reduced carbachol-stimulated calcium mobilization by ~50% (Fig. 3D). In striking contrast, stable expression of the $G\alpha_q$ binding-deficient mutants (R106A and D110A) or the double mutant (R106A/K220R) had no effect on calcium mobilization (Fig. 3D). This suggests that GRK2 primarily regulates the activity of the $M_3$ mAChR through its ability to interact with the activated pool of $G\alpha_q$.

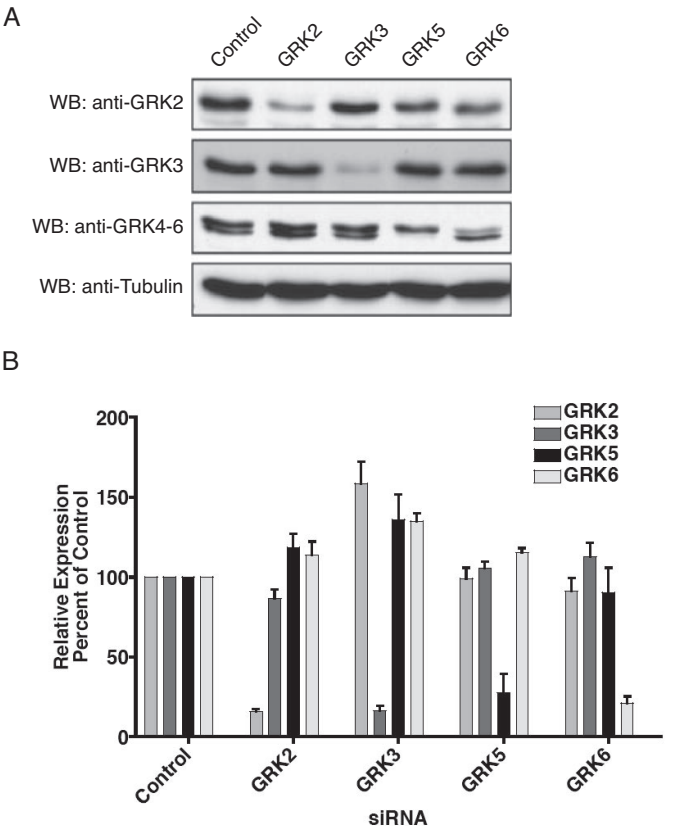


**Fig. 2.** Knockdown of endogenous GRK isoforms in HEK-293 cells. A, HEK-293 cells were transfected twice within a 24-h interval with GRK-specific or nonspecific control siRNA. Seventy-two hours after the second transfection, cells were harvested, and equal amounts of total cellular lysate were separated by 10% SDS-PAGE, transferred to nitrocellulose, and incubated with the indicated antibodies. Blots were stripped and reprobed for α-tubulin to control for loading. Shown is a representative immunoblot. B, mean relative level of GRK expression after siRNA quantified by densitometry from five separate experiments.

**The Nonvisual Arrestins Negatively Regulate $M_3$ mAChR-Promoted Calcium Mobilization.** Our data suggest that GRK-mediated phosphorylation of the $M_3$ mAChR may contribute to subsequent desensitization. Because GRK phosphorylation often promotes arrestin binding, we next determined the effect siRNA knockdown of arrestin-2 and arrestin-3 had on calcium mobilization. Pooled siRNAs targeting either arrestin-2 or arrestin-3 specifically reduced protein expression by ~90% (Fig. 4, A and B). As shown in Fig. 4, C and D, knockdown of either arrestin-2 or arrestin-3 resulted in a significant increase in the peak calcium transient upon stimulation with carbachol. The increase seen with arrestin-3 was slightly higher (74% increase) than that seen with arrestin-2 (65% increase), although silencing of arrestin-3 also led to an increase in the prolonged phase of the calcium transient (Fig. 4C), suggesting prolonged $IP_3$ production.

**Regulation of $M_3$ Muscarinic Acetylcholine Receptor-Mediated Activation of the ERK Cascade.** We next focused on understanding the roles of GRKs and arrestins in regulating the activation of ERK1/2 after $M_3$ mAChR stimulation. The kinetics of ERK1/2 activation showed a consistent peak at 5 min that returned to basal levels by 60 min (Fig. 1C). As shown in Fig. 5, A and B, knocking down GRK2 resulted in a 2.5-fold increase in the peak of ERK1/2 activation and prolonged ERK1/2 activation (Fig. 5B). Silencing of GRK5 or GRK6 also enhanced ERK1/2 activation after a 5-min stimulation, although the effects were modest and not statistically significant (1.3- and 1.5-fold increase, respectively) (Fig. 5, A and B). GRK knockdown did not change basal phospho-ERK1/2 levels (data not shown). It is interesting that in contrast to calcium mobilization, knocking down GRK3 had no effect on ERK1/2 activation (Fig. 5, A and B). Together, these data demonstrate that signaling pathways downstream of $M_3$ mAChR activation are regulated by multiple GRKs in HEK-293 cells in a separate but coordinated fashion.

In contrast to some GPCRs (Ahn et al., 2004; Lefkowitz and Shenoy, 2005), internalization is not required for $M_3$ mAChR-mediated ERK activation (Budd et al., 1999). Thus, it was not surprising that knockdown of either arrestin-2 or arrestin-3 resulted in an ~2-fold increase in ERK activation, with differential temporal effects (Fig. 5, C and D). Silencing of arrestin-2 led to enhanced ERK1/2 activation at 5 min,

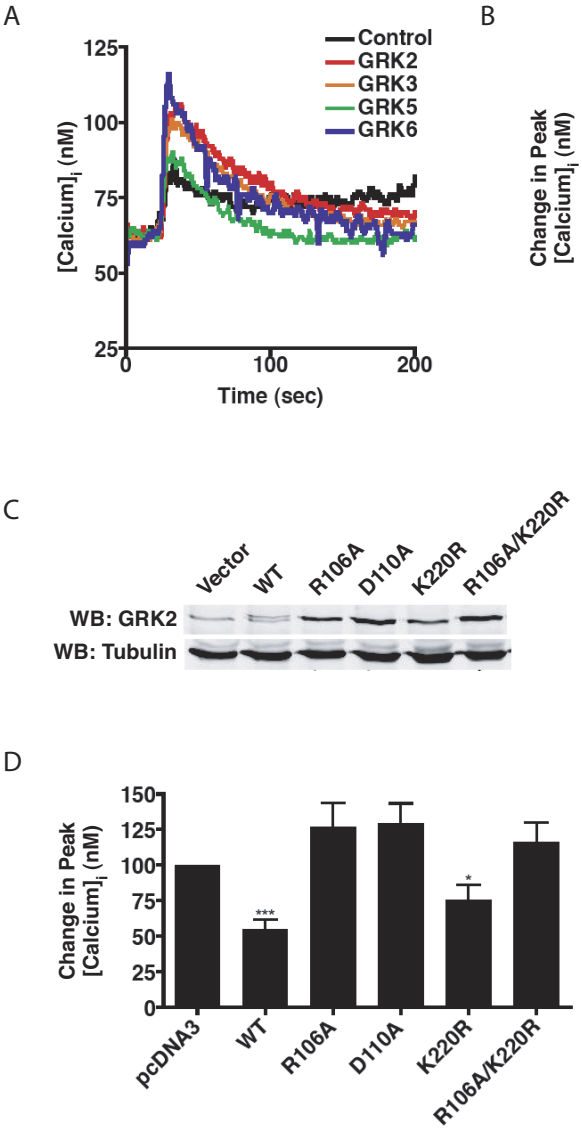


**Fig. 3.** GRK-mediated regulation of calcium mobilization after $M_3$ mAChR activation. A, effect on calcium mobilization. Seventy-two hours after the second siRNA transfection, HEK-293 cells were loaded with Fura-2/AM and stimulated with 10 $\mu$M carbachol. B, Mean (± S.E.M.) increase in the peak calcium transient after stimulation with 10 $\mu$M carbachol from five individual experiments (***, $p < 0.001$ using two-tailed $t$ test). C, representative immunoblot showing relative levels of GRK2 stably expressed in HEK-293 cells. D, calcium mobilization in HEK-293 cells stably expressing bovine GRK2. Mean (± S.E.M.) increase in peak calcium mobilization in cells expressing vector (pcDNA3), wild-type, $G_q$-binding deficient (R106A, D110A), kinase-dead (K220R), or the $G_q$-binding deficient/kinase-dead (R106A/K220R) bovine GRK2 (*, $p < 0.05$ for GRK2-K220R; ***, $p < 0.001$ for wild-type GRK2).

whereas silencing of arrestin-3 led to both enhanced and prolonged activation (Fig. 5D). These data suggest that under normal physiological conditions, either arrestin-2 or arrestin-3 is sufficient to negatively regulate acute signaling events upon $M_3$ mAChR activation, although arrestin-3 seems to play a larger role in terminating signaling in response to prolonged agonist exposure.

**Regulation of the $M_3$ Muscarinic Acetylcholine Receptor by Casein Kinase 1α.** CK1α also phosphorylates the $M_3$ receptor in an agonist-dependent manner, although it does not seem to be required for desensitization of the receptor (Tobin et al., 1997; Budd et al., 2000, 2001). CK1α has also been shown to phosphorylate the $M_1$ mAChR and rhodopsin in vitro (Tobin et al., 1997; Waugh et al., 1999). To determine whether CK1α has a role in regulating the endogenous $M_3$ mAChR, HEK-293 cells were transfected with CK1α siRNA that specifically reduced CK1α protein levels to ~40% of that seen in control cells (Fig. 6A). Knockdown of CK1α resulted in a significant increase (62%, $p < 0.01$, $n = 4$) in the peak calcium transient compared with cells treated with control siRNA (Fig. 6B). To determine whether this effect was specific to CK1α-mediated regulation of the $M_3$ mAChR and not to some other aspect of the $G_q$ signaling pathway, we also tested the ability of CK1α to regulate the histamine H1 receptor, which is regulated by GRK2 in HEK-293 cells (Iwata et al., 2005). Knockdown of CK1α had no effect on calcium mobilization upon stimulation with 100 μM histamine (data not shown), suggesting that the effect of CK1α knockdown was specific for $M_3$ mAChR signaling. It is interesting that knockdown of CK1α had no effect on carbachol-mediated activation of ERK1/2 (Fig. 6, C and D). These data demonstrate that, in addition to the GRK family, the agonist-activated $M_3$ mAChR is also regulated by CK1α.

## Discussion

GPCRs transduce extracellular stimuli into specific intracellular signals that regulate a variety of cellular functions. GPCR desensitization is typically mediated by members of the GRK family, which specifically phosphorylate the agonist-occupied receptor, promoting the subsequent high-affinity binding of arrestins. For most GPCRs, the specificity of GRKs and arrestins in cells remains poorly defined. In this report, we used an siRNA-based approach in HEK-293 cells to characterize the role of these proteins in $M_3$ mAChR signaling. We found that the $M_3$ mAChR displays a complex pattern of regulation, such that GRK2, GRK3, GRK6, arrestin-2, arrestin-3, and CK1α all participate to negatively regulate calcium signaling upon receptor activation.

It was shown previously that GRK2 can be recruited to and phosphorylate the $M_3$ mAChR at two separate serine clusters within the third intracellular loop (Wu et al., 2000). In addition to receptor phosphorylation, GRK2 is able to bind both GTP-bound $G\alpha_q$ (Carman et al., 1999) and free $G\beta\gamma$ (Pitcher et al., 1992). The crystal structure of GRK2 (Tesmer et al., 2005) suggests that it may simultaneously sequester both active $G\alpha_q$ and free $G\beta\gamma$, which, in addition to receptor phosphorylation, may increase the strength and effectiveness of GRK2-mediated receptor regulation. We and others have demonstrated that GRK2-regulated GPCRs, such as the H1 histamine (Iwata et al., 2005), $M_1$ mAChR (Willets et al., 2005), metabotropic glutamate (Dhami et al., 2005), and

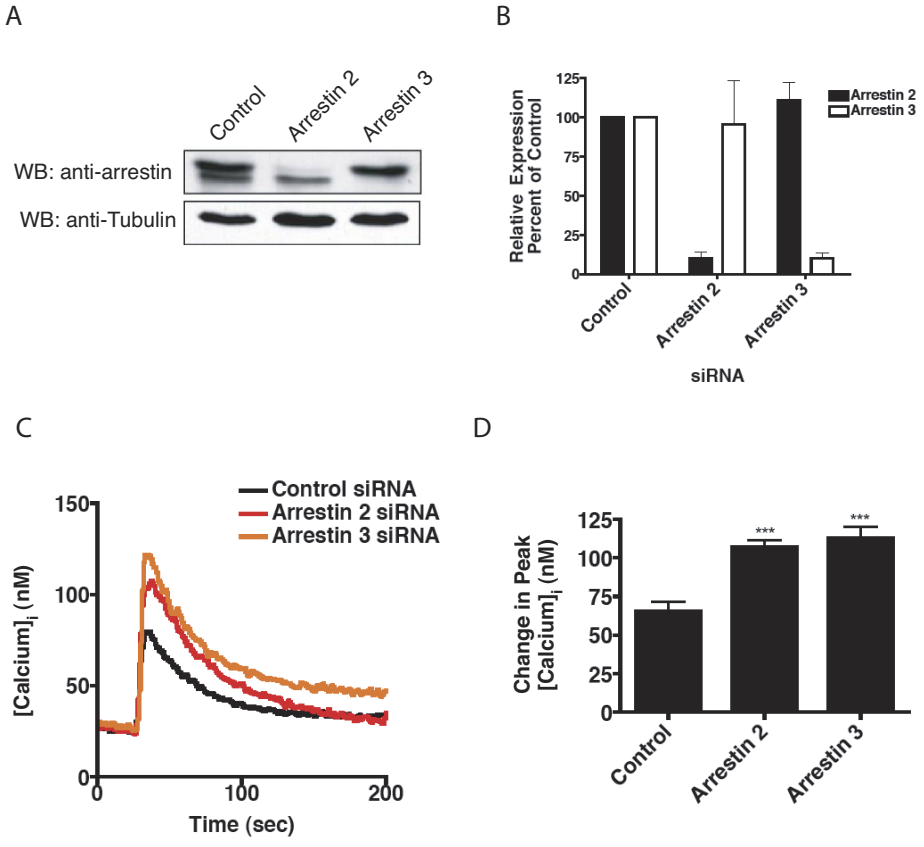


**Fig. 4.** Effect of arrestin knockdown on calcium mobilization after $M_3$ mAChR activation. A, cells were transfected with SMARTpool siRNA and harvested 72 h later. Blots were incubated with a monoclonal antibody for arrestin-2 that cross-reacts with arrestin-3. Blots were stripped and reprobed for α-tubulin to control for loading. Shown is a representative immunoblot. B, mean relative level of arrestin expression after siRNA quantified by densitometry from five separate experiments. C, effect on calcium mobilization. Cells were harvested 72 h after transfection and processed as described previously. Shown is a representative calcium trace from five independent experiments. D, mean (± S.E.M.) increase in the peak calcium transient after stimulation with 100 μM carbachol from five individual experiments (***, $p < 0.001$ using two-tailed $t$ test).

mouse cytomegalovirus GPCR M33 (Sherrill and Miller, 2006), involved the regulation of $G_q$. Studies analyzing GRK-mediated regulation of the $M_3$ mAChR in SH-SY5Y cells have shown that GRK3 and GRK6 differentially regulate the receptor, whereas GRK2 and GRK5 did not seem to be involved (Willets et al., 2001, 2002, 2003). Overexpressed GRK3 could phosphorylate the $M_3$ mAChR; however, GRK3-mediated regulation seemed to be the result of altering the activity of

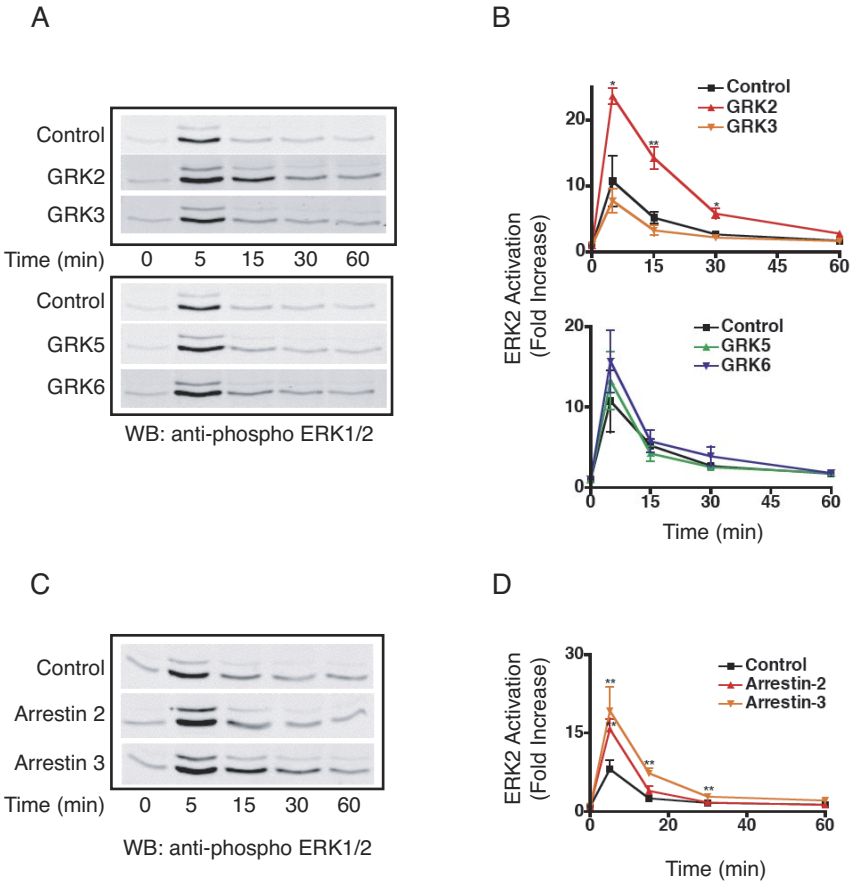


**Fig. 5.** Effect of GRK and arrestin knockdown on $M_3$ mAChR ERK activation. A, effect of GRK knockdown on ERK1/2 activation. After a 6-h serum-starve, cells were treated with 100 $\mu$M carbachol for the indicated times. Shown is a representative immunoblot from six independent experiments. B, mean fold increase in ERK2 activation. Blots were incubated simultaneously with primary antibodies specific for phospho-ERK1/2 and total ERK2 overnight. Phospho-ERK1/2 fluorescence was normalized to total ERK2 fluorescence, and data are presented as the fold increase in ERK2 activation over basal ($n = 6$, $\pm$ S.E.M.; *, $p < 0.05$; **, $p < 0.01$). C, effect of arrestin knockdown on ERK1/2 activation. After a 6-h serum-starve, cells were treated with 100 $\mu$M carbachol for the indicated times. Shown is a representative immunoblot from eight independent experiments. D, mean fold increase in ERK2 activation. Blots were incubated simultaneously with primary antibodies specific for phospho-ERK1/2 and total ERK2 overnight. Phospho-ERK1/2 fluorescence was normalized to total ERK2 fluorescence, and data are presented as the fold increase in ERK2 activation over basal ($n = 8$, $\pm$ S.E.M.; **, $p < 0.01$).

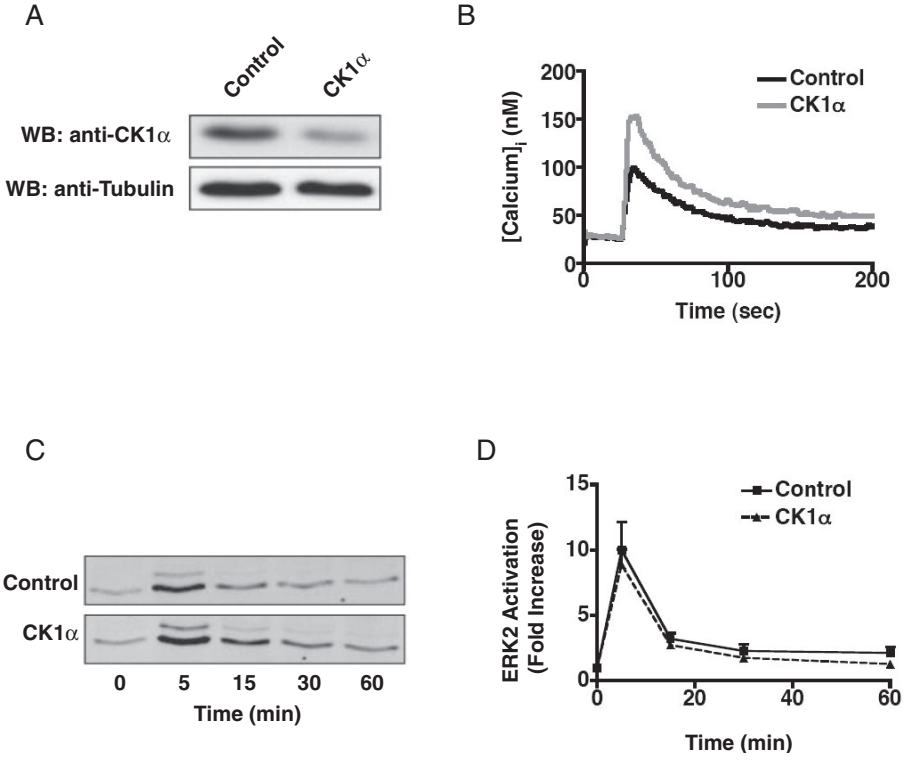


**Fig. 6.** Effect of CK1$\alpha$ Knockdown on $M_3$ mAChR Signaling. A, 72 h after the second siRNA transfection, cells were harvested, and equal amounts of total cellular lysate were separated by SDS-PAGE and immunoblotted for CK1$\alpha$ using a specific antibody. Blots were stripped and reprobed for $\alpha$-tubulin to control for loading. Shown is a representative immunoblot. B, effect on calcium mobilization. Seventy-two hours after the second siRNA transfection, cells were loaded with Fura-2/AM and stimulated with 100 $\mu$M carbachol. Shown is a representative tracing from four independent experiments (control, 103 $\pm$ 10 nM; CK1$\alpha$ siRNA, 163 $\pm$ 15 nM, $p < 0.01$). C, effect on ERK1/2 activation. After a 6-h serum-starve, cells were stimulated with 100 $\mu$M carbachol for the indicated times. Shown is a representative immunoblot from eight independent experiments. D, mean activation of ERK2. Blots were incubated simultaneously with primary antibodies specific for phospho-ERK1/2 and total ERK2 overnight. Phospho-ERK1/2 fluorescence was normalized to total ERK2 fluorescence, and data are presented as the fold increase over basal ($n = 8$, $\pm$ S.E.M.).

PLC-$\beta$ and not via receptor phosphorylation (Willets et al., 2001, 2002, 2003). In contrast, overexpressed GRK6 could phosphorylate the $M_3$ mAChR, leading to a decrease in signaling. This effect was reversed upon expression of a kinase-dead GRK6 (Willets et al., 2003).

Using siRNA coupled with stable expression of low levels of various GRK2 mutants, we found that the enhanced calcium mobilization observed upon GRK2 knockdown is primarily due to a loss in regulation of activated $G_q$ after $M_3$ mAChR stimulation (Fig. 3). Furthermore, we showed that loss of GRK2 leads to enhanced and prolonged activation of the ERK1/2 cascade (Fig. 5). The observed effects of GRK2 knockdown are 2-fold: the enhanced calcium mobilization seems to be primarily due to the loss of inhibition of activated $G_q$, whereas the enhanced and prolonged activation of ERK1/2 probably reflects enhanced DAG production/PKC-$\delta$ activation and a relief of inhibition of mitogen-activated protein kinase kinase 1 (MEK1) (Jiménez-Sainz et al., 2006). However, we cannot completely rule out the possibility that GRK2 also mediates receptor phosphorylation because endogenous $M_3$ mAChR levels are too low to evaluate phosphorylation (Tovey and Willars, 2004).

We have also found that GRK3 and GRK6 negatively regulate calcium mobilization after $M_3$ mAChR stimulation. Although knockdown of either kinase led to significant increases in calcium mobilization (Fig. 3, A and B), silencing of GRK3 had no effect on activation of ERK1/2, whereas loss of GRK6 had only a minor effect (Fig. 5, A and B). The possibility exists that there is overlap between these kinases and that regulation might involve a competition for receptor binding, as has been suggested for the angiotensin receptor (Kim et al., 2005). These previous studies suggested that GRK2 and GRK3 negatively regulate whereas GRK5 and GRK6 positively regulate ERK1/2 activation and that differences in the phosphorylation pattern mediated by GRK2/3 or GRK5/6 could alternatively promote the binding of arrestin-2 or arrestin-3, respectively (Kim et al., 2005). However, our results suggest that the $M_3$ mAChR is not subject to this type of overlapping regulation. Furthermore, the GRKs do not play a positive role in $M_3$ mAChR signaling. There is a growing number of nonreceptor substrates that have been identified for the GRKs (Ribas et al., 2007), and in line with previous findings, GRK3 could be primarily regulating PLC-$\beta$ activity via binding to $G\beta\gamma$ or $G\alpha_q$ (Willets et al., 2001). This might allow for a very rapid and robust production of $IP_3$ and subsequent calcium release that is not evident at later time points because other kinases (e.g., GRK6) may phosphorylate the receptor resulting in desensitization. In addition, mechanisms regulating downstream signaling events (e.g., $IP_3$ hydrolysis, calcium reuptake, etc.) also shape both calcium mobilization and ERK1/2 activation responses after carbachol stimulation. Because we have identified three GRKs that are involved in $M_3$ mAChR regulation, multiple proteins may need to be knocked down simultaneously to produce more prolonged signaling.

We reported previously that an ~50% reduction in arrestin levels using antisense strategies had no effect on calcium mobilization in HEK-293 cells (Mundell and Benovic, 2000). In the present study, we were able to reduce protein levels by ~90% and show that the loss of either arrestin-2 or arrestin-3 enhanced the peak calcium transient seen upon activation of the $M_3$ mAChR (Fig. 4, C and D). Taking into consideration previous reports demonstrating that the $M_3$ mAChR internalizes in an arrestin-independent manner (Lee et al., 1998), our results suggest that arrestins primarily mediate desensitization of the $M_3$ mAChR after agonist activation. Consistent with this and with previous reports (Budd et al., 1999), knockdown of either arrestin-2 or arrestin-3 also enhanced ERK1/2 activation (Fig. 5, C and D). This is in contrast to the emerging paradigm that has been proposed for a number of other GPCRs, in which arrestins promote G protein-independent signaling pathways (Lefkowitz and Shenoy, 2005) or even have opposing effects to one another, as has been shown for the angiotensin II receptor (Ahn et al., 2004). In light of the fact that HEK-293 cells express similar levels of endogenous arrestin-2 and arrestin 3 (J.L.B., unpublished results), our data suggest an inherent specificity for the $M_3$ mAChR by arrestin-3 because both calcium mobilization and ERK activation were enhanced and prolonged with arrestin-3 knockdown. This also suggests that the PLC-$\beta$/PKC arm of signaling is responsible for ERK activation, consistent with previous reports (Budd et al., 1999; Kim et al., 1999; Wylie et al., 1999). It is interesting that arrestins can also terminate muscarinic receptor signaling by recruiting diacylglycerol kinases and enhancing the degradation of the second-messenger DAG, thereby coordinately terminating GPCR/G protein interaction and promoting second-messenger degradation (Nelson et al., 2007). Taken together, the prolonged ERK activation observed after GRK2 and arrestin-3 knockdown can be attributed to enhanced $G_q$ activity, sustained DAG production, and subsequent PKC-$\delta$ activation (Fig. 7).

CK1$\alpha$ has a variety of functions within the cell (Knippschild et al., 2005) and has been shown recently to regulate heterologously expressed $M_3$ mAChR in HEK-293 and COS7 cells (Tobin et al., 1997; Budd et al., 2000). Likewise, we demonstrate that CK1$\alpha$ knockdown results in enhanced calcium mobilization upon $M_3$ receptor activation, suggesting that CK1$\alpha$ is also involved in desensitization of endogenous $M_3$ mAChR in HEK-293 cells. Knockdown of CK1$\alpha$ had no effect on calcium mobilization upon H1 histamine receptor activation, demonstrating that this effect was specific to the $M_3$ mAChR. Previous studies have also shown that expression of a peptide corresponding to the CK1$\alpha$ binding region or overexpression of a mutated receptor lacking a portion of the third intracellular loop led to a decrease in ERK1/2 activation upon receptor stimulation, suggesting that CK1$\alpha$-mediated phosphorylation was necessary for ERK activation (Budd et al., 2001). Although we show that knockdown of CK1$\alpha$ has no effect on ERK1/2 activation (Fig. 6, C and D), indicating that CK1$\alpha$ only plays a partial role in the regulation of $M_3$ mAChR similar to GRK3 and GRK6, this may be due to the fact that we only achieved ~60% knockdown of CK1$\alpha$. The third intracellular loop of the $M_3$ mAChR contains 12 putative CK1$\alpha$ phosphorylation motifs (Tobin, 2002), two of which overlap with the proposed GRK2 phosphorylation sites (Wu et al., 2000). Thus, under physiological conditions, there could be competition between these kinases for receptor binding and phosphorylation.

In this study, we demonstrate that multiple proteins coordinately regulate the activity of the endogenous $M_3$ mAChR in HEK-293 cells (Fig. 7). Knockdown of GRK2, GRK3, GRK6, and CK1$\alpha$, but not GRK5, enhanced receptor calcium signaling, suggesting that multiple kinases regulate down-

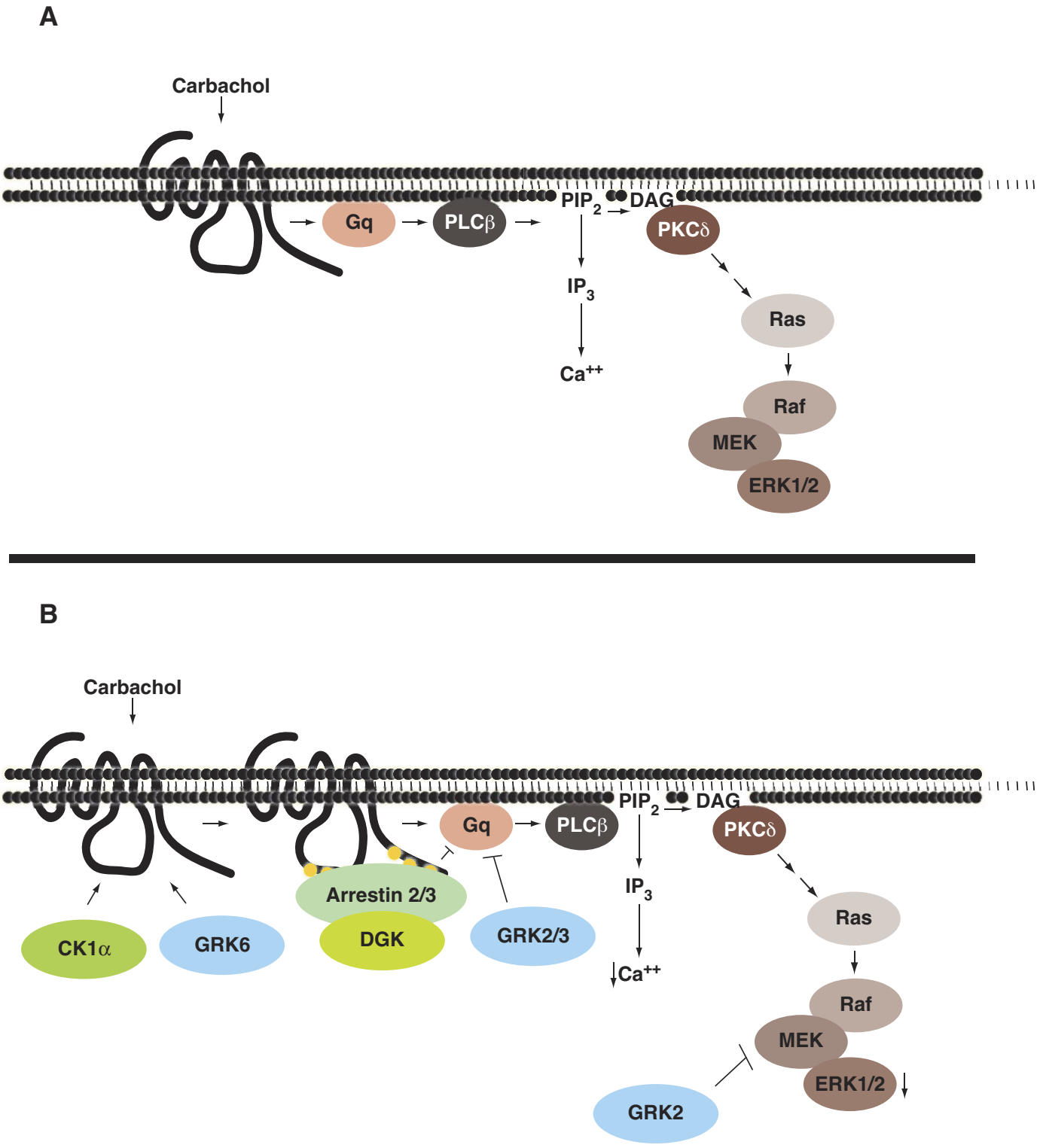


**Fig. 7.** Regulation of the endogenous $M_3$ mAChR in HEK-293 cells. A, carbachol binding to the $M_3$ mAChR results in activation of the $G_q$ family of heterotrimeric G proteins, leading to the dissociation of $G\alpha_q$ and $G\beta\gamma$. Activated $G\alpha_q$ activates PLC-$\beta$, resulting in the hydrolysis of phosphatidylinositol bisphosphate ($PIP_2$) to form the second messengers $IP_3$ and DAG. $IP_3$ interacts with the $IP_3$ receptor located at the endoplasmic reticulum, resulting in a robust but transient increase in cytosolic calcium. The formation of DAG recruits and activates the novel PKC isoform PKC-$\delta$. Once activated, PKC-$\delta$ leads to the activation of a Ras-Raf-MEK-ERK1/2 cascade. B, phosphorylation of the $M_3$ mAChR by GRK6 and possibly CK1$\alpha$ recruits arrestin-2 and arrestin-3 to the receptor, preventing further G protein activation and terminating signaling. In addition, arrestins are able to recruit diacylglycerol kinases (DGK) to the membrane and terminate the PKC-dependent arm of the signaling cascade. GRK2 and GRK3, through a conserved RGS domain, are able to interact with and sequester free $G\alpha_q$ and prevent activation of PLC-$\beta$. This results in the inhibition of both calcium mobilization and activation of the ERK1/2 cascade. GRK2 is also able to regulate the activation of the ERK1/2 cascade by interacting with and negatively regulating the activity of MEK1.

stream signaling after $M_3$ mAChR activation. An effect of GRK2 on calcium flux could be observed with both wild-type and a kinase-dead mutant but not with $G\alpha_q$-binding defective mutants, demonstrating that GRK2 primarily regulates activated $G_q$. It is interesting that only silencing of GRK2 led to both an enhanced and prolonged ERK activation. Consistent with our findings that GRK2 primarily regulated $G_q$ activity, this is probably a result of enhanced activation of the $G_q$/PLC-$\beta$/PKC-$\delta$ signaling pathway (Fig. 7). Finally, both arrestin-2 and arrestin-3 are involved in negatively regulating the $M_3$ mAChR as knockdown of either protein enhanced calcium mobilization and ERK activation. Overall, our data suggest that multiple proteins dynamically regulate $M_3$ mAChR-mediated signal transduction.

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