G Protein-coupled Receptor Kinase 2–mediated Phosphorylation of Ezrin Is Required for G Protein-coupled Receptor–dependent Reorganization of the Actin Cytoskeleton

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G protein-coupled receptor kinase 2 (GRK2) phosphorylates and desensitizes activated G protein-coupled receptors (GPCRs). Here, we identify ezrin as a novel non-GPCR substrate of GRK2. GRK2 phosphorylates glutathione *S*-transferase (GST)-ezrin, but not an ezrin fusion protein lacking threonine 567 (T567), in vitro. These results suggest that T567, the regulatory phosphorylation site responsible for maintaining ezrin in its active conformation, represents the principle site of GRK2-mediated phosphorylation. Two lines of evidence indicate that GRK2-mediated ezrin-radixin-moesin (ERM) phosphorylation serves to link GPCR activation to cytoskeletal reorganization. First, in Hep2 cells muscarinic M1 receptor (M1MR) activation causes membrane ruffling. This ruffling response is ERM dependent and is accompanied by ERM phosphorylation. Inhibition of GRK2, but not rho kinase or protein kinase C, prevents ERM phosphorylation and membrane ruffling. Second, agonist-induced internalization of the β_2-adrenergic receptor (β_2AR) and M1MR is accompanied by ERM phosphorylation and localization of phosphorylated ERM to receptor-containing endocytic vesicles. The colocalization of internalized β_2AR and phosphorylated ERM is not dependent on Na$^+$/H$^+$ exchanger regulatory factor binding to the β_2AR. Inhibition of ezrin function impedes β_2AR internalization, further linking GPCR activation, GRK activity, and ezrin function. Overall, our results suggest that GRK2 serves not only to attenuate but also to transduce GPCR-mediated signals.

INTRODUCTION

G protein-coupled receptor (GPCR) kinase 2 (GRK2) is the most extensively characterized member of the GRKs, a family of serine/threonine kinases that specifically phosphorylate agonist-occupied GPCRs (Penela *et al.*, 2003). Typically, such a phosphorylation event leads to the binding of cytosolic β-arrestin proteins to the receptor, which cause the receptor to become uncoupled from its associated G protein and initiate the sequestration of the receptor via clathrin-mediated endocytosis. In this way, GRK-mediated phosphorylation of GPCRs such as the β_2-adrenergic receptor (β_2AR) and the M1 muscarinic receptor (M1MR) is impor-

tant in controlling their signaling activity as well as the number at the cell surface (Claing *et al.*, 2002).

Agonist-occupied GPCRs allosterically activate GRKs and can enhance their phosphorylation of nonreceptor substrates (Pitcher *et al.*, 1998a). For GRK2, nonreceptor substrates identified to date include tubulin, phosducin, ribosomal protein P2, synucleins, the inhibitory γ subunit of the type 6 retinal cyclical guanosine monophosphate (cGMP) phosphodiesterase, and the β-subunit of the epithelial Na$^+$ channel (Carman *et al.*, 1998; Haga *et al.*, 1998; Pitcher *et al.*, 1998b; Pronin *et al.*, 2000; Ruiz-Gomez *et al.*, 2000; Wan *et al.*, 2001; Freeman *et al.*, 2002; Dinudom *et al.*, 2004). These findings have led to the hypothesis that ligand binding not only instigates receptor desensitization by GRK2 but also may promote other, novel GPCR-mediated signaling pathways downstream of the activated GRK.

The actin and microtubule cytoskeletons have previously been shown to play important roles in receptor endocytosis and vesicle trafficking (Apodaca, 2001; da Costa *et al.*, 2003). Because tubulin is a component of the cellular cytoskeleton, we investigated whether other cytoskeletal proteins were substrates of GRK2. Here, we identify ezrin as a novel substrate of GRK2.

Ezrin is a member of the ezrin-radixin-moesin (ERM) family of proteins that cross-link cortical actin to the plasma membrane (Bretscher *et al.*, 2002). These proteins contain an amino-terminal four-point-one, ERM (FERM) domain that interacts with both plasma membrane-associated proteins and phosphatidylinositol 4,5-bisphosphate (PIP$_2$) as well as a carboxy-terminal actin binding region. In the cytosol, ERM

proteins adopt an inactive conformation wherein intramolecular or intermolecular interactions between the amino and carboxy termini mask the membrane and actin binding sites (Gary and Bretscher, 1995). Activation of ERM proteins, which results in the unmasking of ligand binding sites, requires PIP_2 binding to the FERM domain and subsequent phosphorylation of a carboxy-terminal threonine (T567 in ezrin) (Matsui *et al.*, 1998; Fievet *et al.*, 2004; Chambers and Bretscher, 2005). Rho kinase and protein kinase C (PKC) have been shown to mediate ERM activation via T567 phosphorylation both in vitro and in vivo (Matsui *et al.*, 1998; Simons *et al.*, 1998).

In this report, we show that ezrin is a substrate of GRK2 in vitro and in cultured mammalian cells. Furthermore, GRK2-mediated ERM phosphorylation is shown to be required for M1MR-dependent membrane ruffle formation in Hep2 cells and agonist-induced internalization of the β_2AR in human embryonic kidney 293 (HEK293) cells. Together, these results suggest a signaling function for GRK2, linking GPCR activation with GPCR-dependent reorganization of the actin cytoskeleton.

MATERIALS AND METHODS

Cell Culture

HEK293 cells stably expressing His6-tagged β_2AR (HEKβ_2 cells, a generous gift of Dr. Stefano Marullo, Institut Cochin, Paris, France) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum (FCS), 100 IU penicillin, and 100 μg/ml streptomycin (all from Sigma-Aldrich, St. Louis, MO) and 2 mg/ml Geneticin (Invitrogen, Paisley, United Kingdom) at 37°C, 5% CO_2. COS, Hep2, and HEK293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FCS, penicillin, and streptomycin at 37°C, 5% CO_2.

cDNA Constructs

The plasmids for pGEX-2T-ez$_{1-586}$, pGEX-2T-ez$_{1-533}$, pGEX-2T-ez$_{310-586}$, and pGEX-2T-ez$_{1-310}$ (1–586, 1–533, 310–586, and 1–310; Roy *et al.*, 1997) were generously provided by Dr. Christian Roy (Université Montpellier, Montpellier, France). pCB6-Ezrin-VSVG, pCB6-Ezrin-Nter-VSVG, and pCB6-Ezrin-Cter-VSVG (WT-ezrin, N-ezrin, and C-ezrin; Algrain *et al.*, 1993) were generously provided by Dr. Monique Arpin (Institut Curie, Paris, France). GRK2(45-178)-GFP and D110A-GRK2(45-178)-GFP (GRK2-RH and GRK2-mRH; Sterne-Marr *et al.*, 2003) were generously provided by Dr. Rachel Sterne-Marr (Loudenville, New York). pEGB-βARK-CT (GRK2-CT; Pierce *et al.*, 2001), pBC12BI-βARK1 and pBC12BI-βARK1-K220R (GRK2 and GRK2$_{K220R}$; Freedman *et al.*, 1995), pSV2-neo-FLAG-β_2AR and pSV2-neo-FLAG-β_2AR(L413A) (Hall *et al.*, 1998b), and pRK5-HA-M1MR and pRK5-HA-M2MR (Claing *et al.*, 2000) were generously provided by Prof. Robert Lefkowitz (Howard Hughes Medical Institute, Durham, NC). To generate a glutathione *S*-transferase (GST)-tagged ezrin construct with threonine-567 mutated to alanine (T567A), the T567A-ezrin cDNA was amplified from pCB6-Ezrin-T567A-VSVG (Dr. Monique Arpin, Institut Curie) by using PCR. The PCR primers were designed so that the resulting T567A-ezrin cDNA had a 5′ *Eco*RI site and a 3′ *Xho*I site. The cDNA was then inserted into the pGEX-5X-1 vector (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom) downstream of and in frame with the GST moiety.

Purification of GST-Ezrin Fusion Proteins

GST-ezrin fusion proteins were expressed in BL21 *Escherichia coli* (Invitrogen) and grown in 100 ml of L-Broth containing 100 μg/ml ampicillin (Sigma-Aldrich) at 37°C, 250 rpm overnight. The cultures were diluted 1:10 in L-Broth/ampicillin and grown at 37°C, 250 rpm until $A_{550} \geq 0.5$. Isopropyl β-D-thiogalactoside (Sigma-Aldrich) was added subsequently to the cultures at a final concentration of 1 mM to induce fusion protein expression for an additional 4 h. Cultures were centrifuged at $2500 \times g$ for 10 min at 4°C, bacterial pellets were resuspended in 35 ml of cold phosphate-buffered saline (PBS) containing the protease inhibitors 40 μg/ml phenylmethanesulfonyl fluoride (Sigma-Aldrich) and 1 mM benzamidine (Sigma-Aldrich) and subject to sonication for 2×2 min. Triton X-100 was added to a final concentration of 1%, and the lysates were centrifuged at $40,000 \times g$ for 30 min at 4°C. Then, 1 ml of glutathione agarose (Sigma-Aldrich) was added to the clarified supernatants, which were incubated on a rotor mixer at 4°C for 2 h. The agarose was washed three times with cold 50 mM Tris, pH 8.0/1% Tween/1% Triton X-100 and once with cold 50 mM Tris, pH 8.0. GST-fusion proteins were eluted from the agarose with 10 mM glutathione (Sigma-Aldrich) in 50 mM Tris, pH 8.0, containing protease inhibitors, dialyzed against 50 mM Tris, pH

8.0, containing protease inhibitors, and concentrated in Centriprep YM-30 centrifugal filter devices (Millipore, Billerica, MA).

In Vitro Phosphorylation Assays

GRK2 purified from Sf9 cells expressing recombinant baculovirus encoding the GRK was a generous gift of Prof. Robert Lefkowitz. Phosphorylation reactions were carried out with purified and reconstituted GST-ezrin fusion proteins (1 μM) or GST (4 μM) and GRK2 (0.2 μM) in 20 mM Tris-HCl ,pH 7.5, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM dithiothreitol (all from Sigma-Aldrich) in the presence of 60 μM [γ-^{32}P]ATP (15,000 cpm/pmol) (Amersham Biosciences UK), for 1 h at 37°C in a total reaction volume of 25 μl. Where used, 0.5 mg/ml purified and sonicated lipids (PIP_2 and phosphatidylcholine [both from Sigma-Aldrich] in PBS) and purified G$\beta\gamma$ from bovine brain (Prof. Robert Lefkowitz) also were included. Either 10% PIP_2 and 2.125 μM G$\beta\gamma$ or 20% PIP_2 and 8.7 μM G$\beta\gamma$ were used, with essentially equivalent results. Reactions were incubated on ice for 15 min before addition of [γ-^{32}P]ATP. Termination of the reactions was by addition of SDS sample buffer (8% SDS, 5% β-mercaptoethanol, 10% glycerol, 25 mM Tris, pH 6.5, and bromphenol blue), and samples were resolved by SDS-PAGE on 10% polyacrylamide gels. Phosphorylated proteins were visualized by autoradiography.

Immunofluorescence

Hep2 and HEK293 cells were grown to 60–70% confluence before transfection by electroporation in HEBS buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM D-glucose) by using two 450-V, 125-μF pulses (Gene Electropulser II; Bio-Rad, Hercules, CA) and 1 μg of the relevant cDNA. Acetylcholine (ACh) (Sigma-Aldrich) was used at a concentration of 100 μM for 5 min (membrane ruffling assay) or 20 min (internalization assay). (−)-Isoproterenol (Iso) (Sigma-Aldrich) was used at a concentration of 50 μM for 15 or 30 min in the internalization assays. After treatment, cells were fixed in 10% trichloroacetic acid (TCA) for 15 min at 4°C (for detection of threonine-phosphorylated ezrin-radixin-moesin [pERM]) or 4% paraformaldehyde and quenched for 2×10 min in 0.27% NH_4Cl/0.37% glycine in PBS at room temperature (RT). Bovine serum albumin (2%) (BSA) (First Link UK, Birmingham, United Kingdom)/0.2% saponin (Sigma-Aldrich)/phosphate-buffered saline was subsequently used to block and permeabilize fixed cells. Primary antibody incubations were as follows: mouse anti-His6 antibody (BD Biosciences, San Jose, CA) at a 1:200 dilution; 297S rat anti-pERM antibody (a generous donation from Prof. Shigenobu Yonemura, RIKEN Center for Developmental Biology, Kobe, Japan; Matsui *et al.*, 1998; Hayashi *et al.*, 1999); rabbit anti-pERM antibody (Chemicon International, Temecula, CA) at a 1:300 dilution; rabbit anti-ezrin antibody (Upstate Biotechnology, Lake Placid, NY) at a 1:250 dilution; mouse anti-VSVG antibody (clone P5D4; Sigma-Aldrich) at a 1:2000 dilution; rat anti-hemagglutinin (HA) antibody (Roche Diagnostics, Mannheim, Germany) at a 1:100 dilution; mouse anti-GRK2 antibody (Upstate Biotechnology) at a 1:300 dilution; mouse anti-human epidermal growth factor (EGF) receptor (DakoCytomation, Ely, Cambridgeshire, United Kingdom) at a 1:250 dilution or mouse M2 anti-FLAG antibody (Sigma-Aldrich) at a 1:400 dilution were performed for 1 h at RT. Cells were subsequently washed with 2% bovine serum albumin/0.2% saponin/phosphate-buffered saline and incubated with AlexaFluor-594 or -488 donkey anti-mouse, anti-rabbit, or anti-rat IgG (Molecular Probes, Eugene, OR) at a 1:800 dilution for 1 h at RT. After washing, coverslips were mounted on slides in 90% glycerol (Sigma-Aldrich)/phosphate-buffered saline/3% *N*-propyl-gallate (Sigma-Aldrich). Where indicated, coverslips were stained with AlexaFluor-594 phalloidin (Molecular Probes) according to the manufacturer's instructions before mounting. Confocal images were taken at RT, by using Nikon Plan Apo 40× and 60× oil immersion lenses and a Bio-Rad 1024 MRC confocal with a Nikon microscope and Bio-Rad Lasersharp 2000 software to acquire the images. Images were optimized for contrast in Adobe Photoshop but no further manipulations were made.

Flow Cytometry and Fluorescence-activated Cell Sorting (FACS) Analysis

HEKβ_2 cells at ~50% confluence were transfected with either dynamin K44A or N-ezrin by using Gene Juice (Novagen, Madison, WI) according to manufacturer's instructions. Twenty-four hours posttransfection, cells were detached in PBS containing 10 mM EDTA, washed in ice-cold binding media (BM; RPMI 1640 medium without bicarbonate, containing 0.2% BSA, 10 mM HEPES and adjusted to pH 7.0) and resuspended at 5×10^6 cells/ml in cold BM containing 40 μM isoproterenol. Immediately, six aliquots of 100 μl (5×10^5 cells) were washed in cold BM and kept on ice. The remaining cells were incubated at 37°C for 30 min, after which time a further six aliquots were taken and treated as described above. The rest of the cells were cooled on ice, centrifuged (1500 rpm, 5 min at 4°C), washed three times in cold BM, resuspended in prewarmed BM containing 100 μM (−)-alprenolol (Sigma-Aldrich), and incubated for 30 min at 37°C. Six aliquots of cells were pelleted by centrifugation and washed in cold BM, as described above. All cells were labeled with mouse anti-His6 antibody (BD Biosciences) at a dilution of 1:100 in wash buffer for 1 h on ice. The cells were then washed twice in cold wash buffer (1% FCS/0.05% azide (Sigma-Aldrich)/phosphate-buffered saline) and

fixed in 1% FCS/1% paraformaldehyde (Taab Laboratories, Reading, United Kingdom)/phosphate-buffered saline overnight at 4°C. The fixative was removed by washing once in cold wash buffer, and the cells were incubated with an AlexaFluor-488–conjugated donkey anti–mouse antibody (Molecular Probes) at a dilution of 1:800 in wash buffer for 1 h on ice. Finally, the cells were washed three times in wash buffer. Cells were analyzed using a FAC-SCalibur flow cytometer (BD Biosciences).

RESULTS

Identification of Ezrin as a GRK2 Substrate

In an attempt to identify non-GPCR substrates for GRK2, HEK293 cells transfected with the β_2AR or the β_2AR and GRK2 were labeled with [^{32}P]orthophosphate and left untreated, or alternatively, treated with isoproterenol, a β-adrenergic receptor agonist. Cells were subsequently lysed and ^{32}P-labeled proteins subjected to two-dimensional gel electrophoresis and autoradiography. Proteins whose ^{32}P content increased upon expression of GRK2 in an agonist-dependent manner were excised and identified using matrix-assisted laser desorption ionization/time of flight mass spectrometry. One potential GRK2 substrate identified in this manner was the actin binding protein ezrin (our unpublished data).

To determine whether indeed ezrin serves as a GRK2 substrate, GST-ezrin was expressed in, and purified from, *E. coli* and subject to in vitro phosphorylation by purified GRK2. The full-length ezrin construct (1–586) was efficiently phosphorylated by GRK2 in the presence of $G\beta\gamma$ and lipid vesicles consisting of phosphatidylcholine and PIP_2 (Figure 1, 1–586). GRK2 contains a carboxy-terminal pleckstrin homology (PH) domain that binds PIP_2 and $G\beta\gamma$ in a coordinated manner to promote membrane localization of the kinase (Pitcher *et al.*, 1995). PIP_2 additionally binds the amino terminus of ezrin; this interaction is required for phosphorylation of T567 and leads to ezrin activation (Niggli *et al.*, 1995; Fievet *et al.*, 2004). The PIP_2 and $G\beta\gamma$ dependence of GRK2-mediated ezrin phosphorylation thus presumably reflects the colocalization of both kinase and substrate to the surface of lipid vesicles and PIP_2-dependent changes in ezrin conformation.

GST-ezrin constructs with different domain deletions were used to identify the region of ezrin that contains the GRK2 phosphorylation site. A GST fusion protein encompassing residues 310–586 of ezrin was also a substrate of GRK2, localizing the GRK2 phosphorylation site to the carboxy terminus of the protein (Figure 1, top). Addition of $G\beta\gamma$- and PIP_2-containing lipid vesicles inhibited GRK2-mediated phosphorylation of this construct (Figure 1). These results are potentially consistent with the PIP_2 and $G\beta\gamma$ dependence observed for GRK2-mediated phosphorylation of full-length ezrin. GST-310-586 lacks the autoinhibitory amino-terminal PIP_2 binding domain of ezrin. Phosphorylation sites encompassed within this carboxy-terminal domain would thus be predicted to be accessible in the absence of PIP_2. Because in the presence of $G\beta\gamma$ GRK2 binds PIP_2-containing vesicles (Pitcher *et al.*, 1996), their addition may sequester GRK2 away from its non-PIP_2 binding GST-310-586 substrate. An ezrin construct lacking the carboxy terminus (1–533) was poorly phosphorylated by GRK2, both in the presence and absence of $G\beta\gamma$ and lipid (Figure 1, top).

These results indicate that GRK2 phosphorylates the carboxy terminus of ezrin, between residues 533 and 586. This region includes T567, phosphorylation of which stabilizes the activated form of ezrin (Matsui *et al.*, 1998). To assess whether GRK2-mediated ezrin phosphorylation occurs at this regulatory phosphorylation site, the ability of a mutant ezrin fusion protein, in which T567 is mutated to alanine (T567A), to serve as a GRK2 substrate was examined. GST-
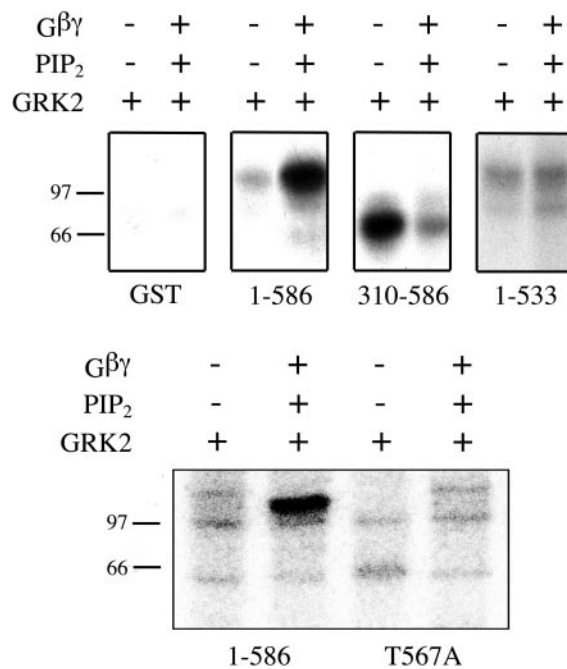

Figure 1. Ezrin is a substrate of GRK2 in vitro. GST-fusion constructs corresponding to full-length ezrin (1–586), the carboxy terminus of ezrin (310–586), a carboxy-terminal deletion (1–533), and a mutant ezrin construct lacking T567 (T567A) were overexpressed in *E. coli* and purified as detailed in *Materials and Methods*. Purified proteins (1 μM) were phosphorylated in the presence of 0.2 μM GRK2 and in the absence or presence of $G\beta\gamma$ and PIP_2. Proteins were separated on a 10% SDS-polyacrylamide gel and visualized by autoradiography. No phosphorylation of the GST moiety alone (GST) was observed. Positions of protein molecular mass standards are indicated at the left. The results shown are representative examples of at least five separate experiments.

T567A is a poor GRK2 substrate in vitro in either the presence or absence of PIP2 and $G\beta\gamma$ (Figure 1, bottom), suggesting that T567 represents the principle site of GRK2-mediated ezrin phosphorylation. Based on these observations, it is tempting to speculate that GRK2 may, like rho kinase and PKC, play a role in modulating the ability of ezrin to cross-link plasma membrane proteins to actin. Notably, the $G\beta\gamma$ dependence of GRK2-mediated phosphorylation of full-length ezrin suggests that in a cellular context this phosphorylation event may be dependent upon GPCR activation.

Ezrin and GRK2 Are Required for M1MR-mediated Membrane Ruffling

It has previously been shown that ERM proteins are involved in the biogenesis of plasma membrane structures, such as microvilli and membrane ruffles, which are formed after changes in the underlying actin cytoskeleton (Bretscher, 1989; Crepaldi *et al.*, 1997; Bonilha *et al.*, 1999; Yonemura *et al.*, 1999). We developed a model system by using Hep2 cells overexpressing HA-tagged M1MR to investigate the potential functional significance of $G\beta\gamma$-dependent GRK2-mediated ezrin phosphorylation in GPCR-mediated cytoskeletal reorganization. A 5-min agonist (ACh) treatment of Hep2 cells expressing M1MR caused rapid and transient membrane ruffles to which endogenous ezrin was localized (Figure 2A, left). Under these conditions, 87 ± 9.7% of the M1MR-transfected cells ruffled. Using an antibody (297S) that specifically recognizes pERM (Matsui *et al.*, 1998;
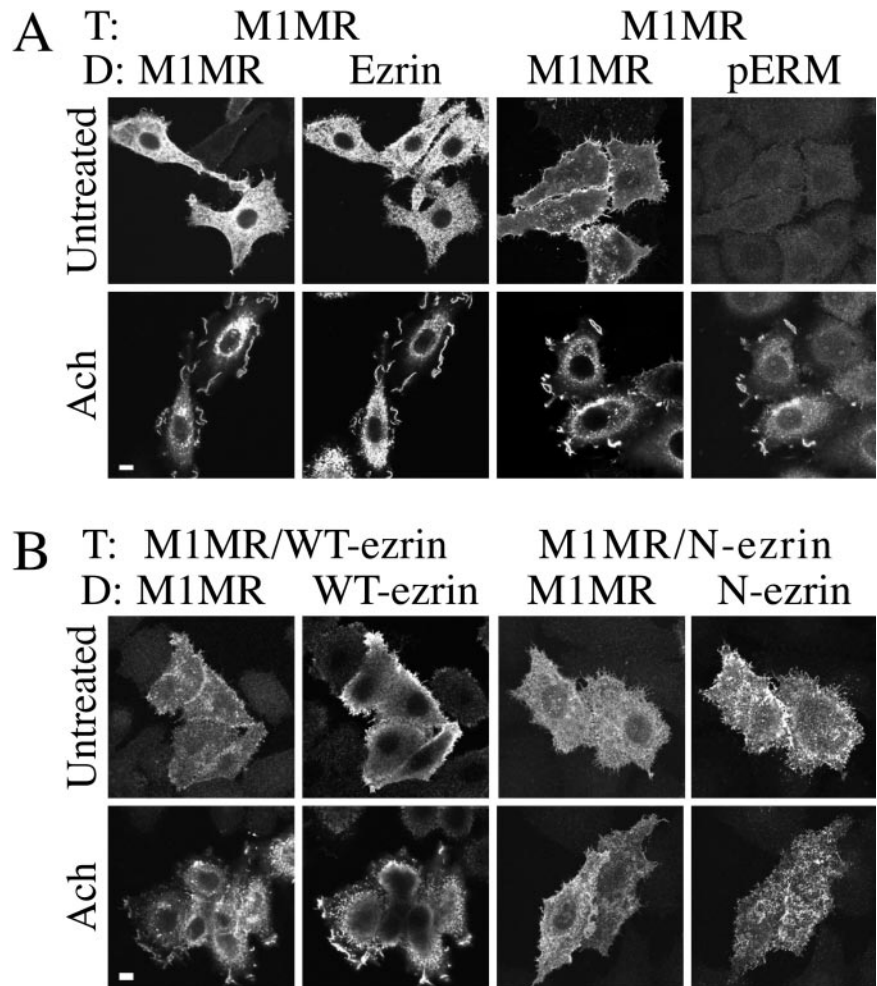
Figure 2. Ezrin is required for M1MR-mediated membrane ruffling. (A) The cellular distribution of endogenous ezrin and endogenous, activated ERM (pERM) in Hep2 cells overexpressing M1MR was visualized by immunofluorescence 48 h after transfection, as described in *Materials and Methods*. The 297S antibody was used for pERM detection. Where indicated, cells were treated with 100 μM ACh for 5 min before fixation (ACh). (B) Hep2 cells were transfected with M1MR and a wild-type ezrin construct (WT-ezrin) or M1MR and the FERM domain of ezrin (N-ezrin). Forty-eight hours post-transfection, cells were treated with agonist, 100 μM ACh, for 5 min (ACh), and the effect on membrane dynamics was visualized by immunofluorescence. T indicates cDNA constructs transfected. D indicates proteins detected. Bars, 20 μm.

Hayashi *et al.*, 1999), it was observed that the cellular levels of pERM increase after agonist stimulation and that it is activated ERM proteins that are localized to ruffles (Figure 2A, right). These results demonstrate that M1MR activation promotes ERM phosphorylation and suggest that activated ERM may play a role in ruffle formation.

To investigate a potential functional role for ezrin in M1MR-mediated membrane ruffling, Hep2 cells were cotransfected with the M1MR and ezrin (WT-ezrin) or the amino-terminal FERM domain of ezrin (N-ezrin). Expression of the FERM domain of ezrin has been shown to inhibit ezrin function in a number of model systems (Crepaldi *et al.*, 1997; Amieva *et al.*, 1999). Expression of full-length ezrin has no effect on agonist-dependent M1MR-mediated ruffling and the expressed protein, like the endogenous, localizes to ruffles (WT-ezrin; Figure 2B). M1MR/ezrin cotransfected cells ruffled after a 5-min Ach treatment (90.9 ± 3.9%; Figure 3B). In contrast, only 31.5 ± 8% of Hep2 cells cotransfected with M1MR and the FERM domain of ezrin (N-ezrin) exhibited ruffling under identical conditions (N-ezrin; Figures 2B and 3B). These results are consistent with an obligate role for activated, phosphorylated ERM proteins in M1MR-mediated membrane ruffle formation in our system.

Because ezrin is a GRK2 substrate in vitro, we investigated a potential role for GRK2-mediated phosphorylation events in this model of cytoskeletal reorganization. Hep2 cells were cotransfected with M1MR and either the carboxy terminus of GRK2 (GRK2-CT), the catalytically inactive mutant kinase

(K220R), the amino-terminal regulator of G protein signaling homology (RH) domain of GRK2 (GRK2-RH), or a mutant RH domain of GRK2 (GRK2-mRH). Both GRK2-CT and GRK2$_{K220R}$ contain the G$\beta\gamma$/PIP$_2$ binding PH domain of GRK2, and their expression inhibits agonist-dependent translocation of endogenous GRK2 to activated GPCRs (Daaka *et al.*, 1997). The green fluorescent protein (GFP)-tagged RH domain of GRK2 binds activated Gαq and is recruited to the plasma membrane in a Gαq-dependent manner (Sterne-Marr *et al.*, 2003). The M1MR is Gαq coupled; expression of GRK2-RH would thus be anticipated to inhibit the translocation of endogenous GRK2 to the activated M1MR. Expression of GRK2-CT, GRK2$_{K220R}$, or GRK2-RH should therefore inhibit receptor-activated GRK2-mediated phosphorylation events. A mutant GFP-tagged GRK2 RH domain construct, GRK2-mRH, which fails to bind activated Gαq and so would be predicted to have no effect on the agonist-induced translocation of endogenous GRK2, was used as a control in these experiments. Cells coexpressing M1MR and GRK2-CT or M1MR and GRK2$_{K220R}$ show diminished membrane ruffling (by ~90 and 89%, respectively, compared with M1MR alone) after ACh treatment (Figure 3, A and B). Similarly, expression of the RH domain of GRK2 inhibited M1MR-dependent ruffling by ~81% (Figure 3, A and B). In marked contrast, the mutant RH domain of GRK2, GRK2-mRH, had no inhibitory effect on ruffling. These results imply that, in addition to
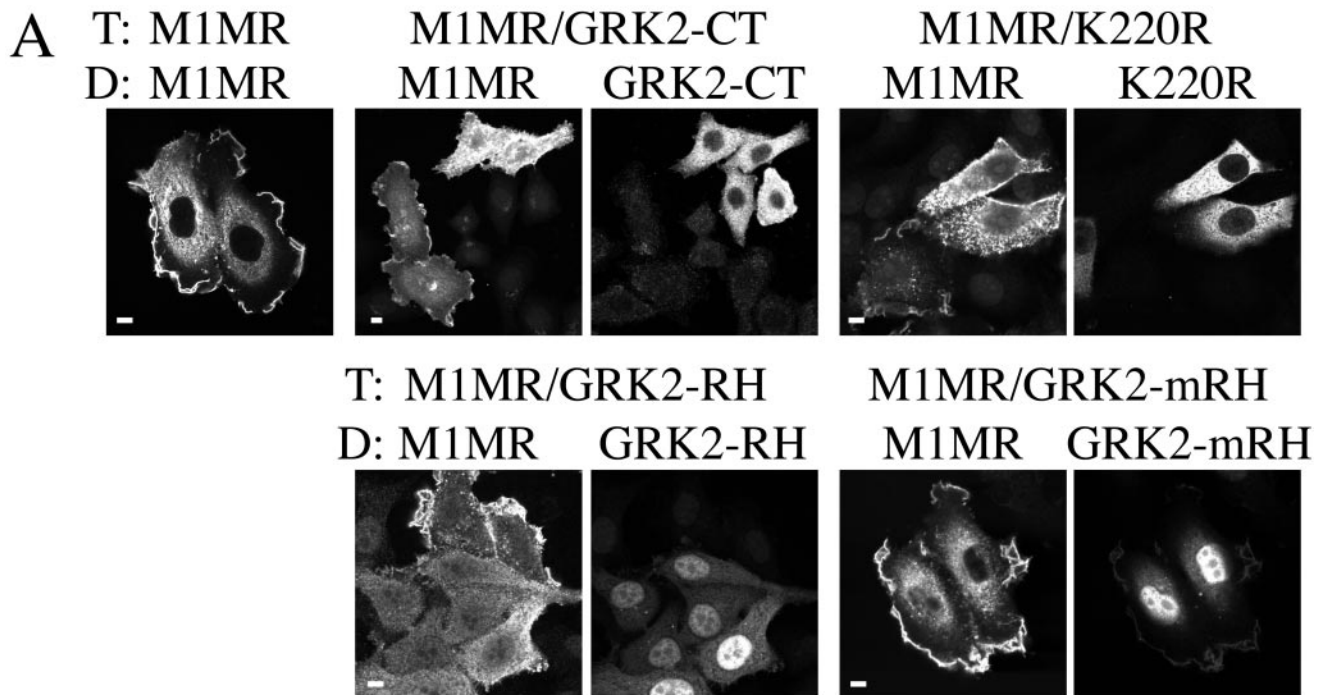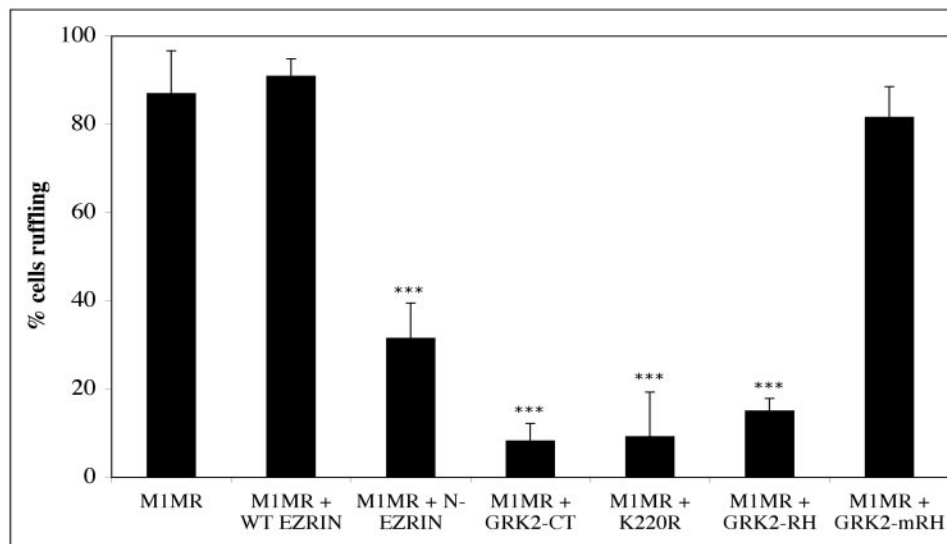
Figure 3. M1MR-mediated membrane ruffling is GRK2 dependent. (A) Hep2 cells were transfected with M1MR or cotransfected with M1MR and either the carboxy terminus of GRK2 (M1MR/GRK2-CT), catalytically inactive GRK2 (M1MR/K220R), the RH domain of GRK2 (M1MR/GRK2-RH), or a mutant RH domain (M1MR/GRK2-mRH). Forty-eight hours after transfection, the cells were treated with 100 μM ACh for 5 min (ACh), and expressed proteins were visualized by immunofluorescence. (B) The effect of different ezrin and GRK2 constructs on M1MR-mediated ruffling. Approximately 100 appropriately transfected cells were scored as ruffling or nonruffling per experiment. Data indicate the mean number of ruffling cells. Error bars represent SD of mean data collected from multiple experiments (n $\geq$ 3). ***p $\leq$ 0.001.

ezrin, active GRK2 is required for the formation of membrane ruffles in Hep2 cells expressing M1MR.

GRK2 Activity Is Required for ERM Activation in Hep2 Cells

To examine the relationship between GRK2 activity and ERM phosphorylation in M1MR-mediated membrane ruf-

fling, Hep2 cells were transfected with M1MR alone, or were cotransfected with M1MR and GRK2-RH or GRK2-mRH. Transfected cells were agonist treated, fixed, and M1MR, the RH domain of GRK2, and endogenous pERM were visualized (Figure 4). In cells expressing M1MR, or coexpressing M1MR and the mutant RH domain of GRK2, pERM was observed after agonist treatment and was localized to the
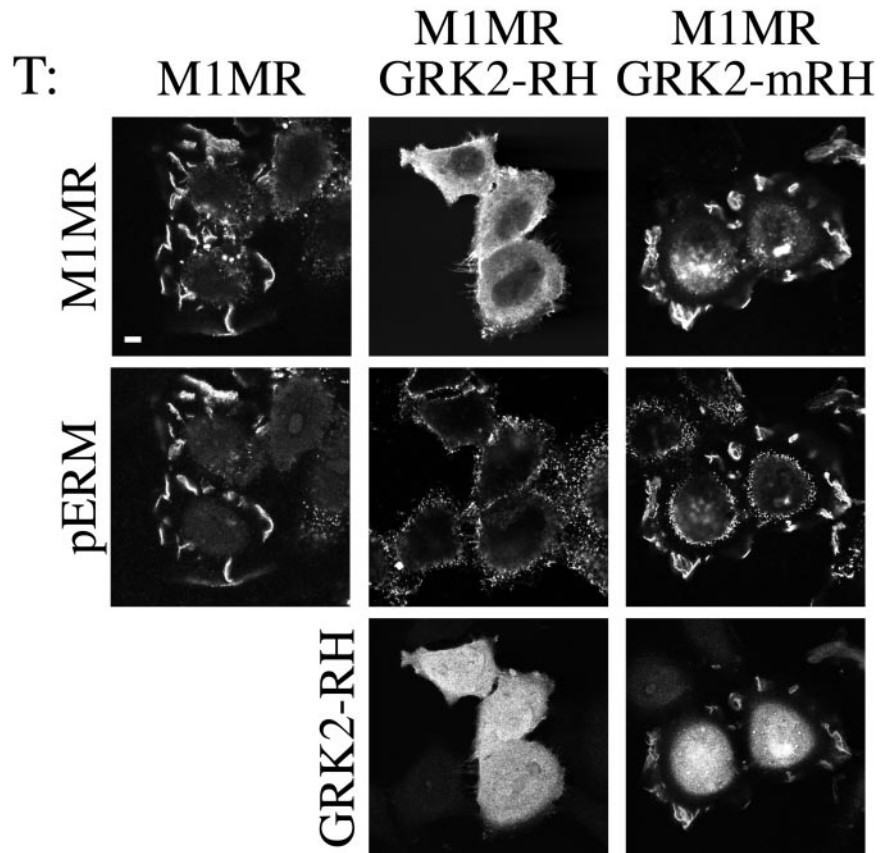
Figure 4. GRK2 is required for M1MR-mediated ERM phosphorylation. Hep2 cells were transfected with M1MR, M1MR, and GRK2-RH or M1MR and GRK2-mRH and incubated for 48 h. The cells were subsequently treated with ACh for 5 min (ACh), and M1MR, GRK2-RH, and endogenous pERM were detected by immunofluorescence. A rabbit anti-pERM antibody (Chemicon International) was used to detect pERM, as described in *Materials and Methods*. T indicates cDNA constructs transfected. Bars, 20 μm.

ensuing ruffles (Figure 4). In cells coexpressing M1MR and GRK2-RH, however, no agonist-dependent ruffling or increase in pERM staining was detected (Figure 4). The fact that endogenous GRK2 activity is required for the detection of active ERM species in the cell suggests that it is GRK2 that is responsible for the phosphorylation of ERM proteins. Furthermore, because the antibody used recognizes specifically phospho-ezrin (T567), -radixin (T564), or -moesin (T558), GRK2 is implicated as the kinase responsible for phosphorylating ERM proteins at this functionally relevant site.

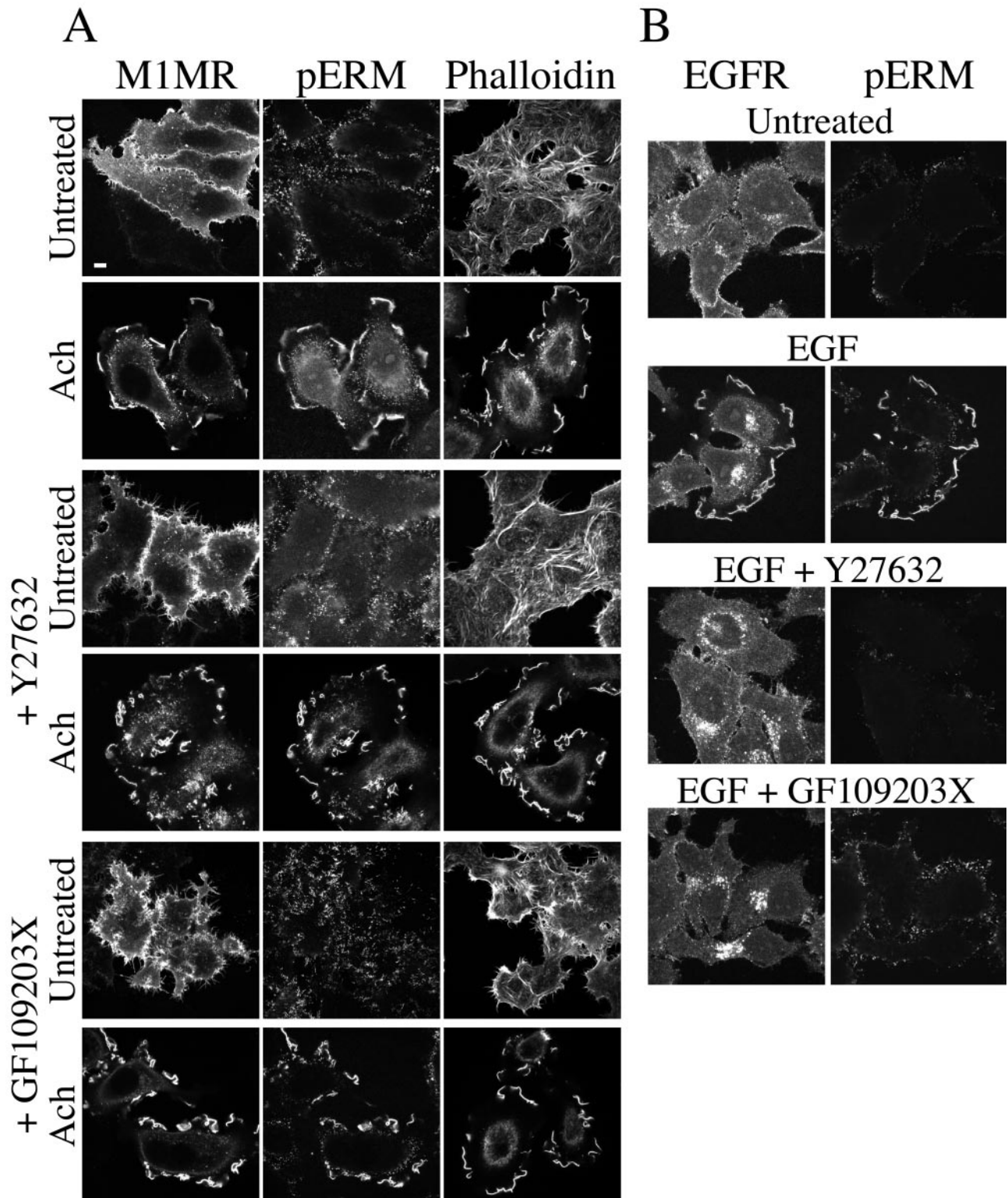### M1MR-mediated Membrane Ruffling in Hep2 Cells Is Not Rho Kinase or PKC Dependent

Previous studies have shown that ezrin can be phosphorylated at T567 by PKC and rho kinase (Matsui *et al.*, 1998; Simons *et al.*, 1998; Ng *et al.*, 2001). We examined whether these kinases were responsible for the phosphorylation of ERM proteins in our model system. Hep2 cells overexpressing M1MR were left untreated or pretreated with the rho kinase inhibitor Y27632 or the PKC inhibitor GF109203X before agonist stimulation (Figure 5A). M1MR and pERM was subsequently visualized after TCA fixation of treated cells. Neither rho kinase inhibition nor PKC inhibition prevented ruffle formation after agonist treatment and neither inhibited ERM phosphorylation or localization of pERM to the ruffles (Figure 5A, M1MR and pERM). Phalloidin staining of paraformaldehyde-fixed coverslips treated in an identical manner revealed similar agonist-dependent changes in the actin cytoskeleton in either the presence or absence of inhibitors (Figure 5A, Phalloidin). Both inhibitors, did however, significantly inhibit EGF-mediated ruffling and ERM phosphorylation (Figure 5B). Thus, inhibition of GRK2 spe-

cifically inhibits agonist-dependent M1MR-mediated ERM phosphorylation and ERM-dependent membrane ruffling in this Hep2 cell model system.
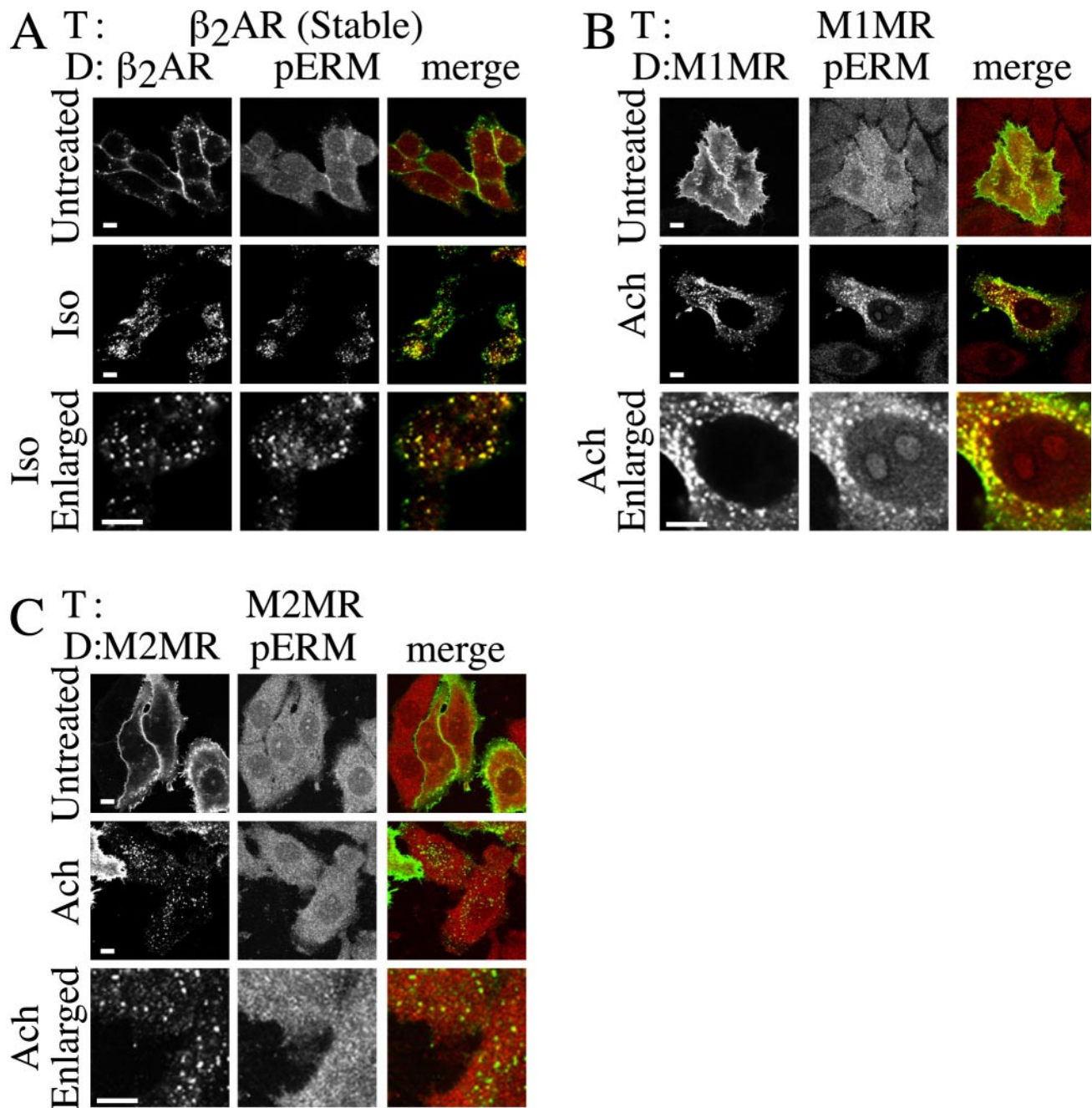
pERM Colocalizes with Internalized β₂AR and M1MR

Because we have shown that ezrin is a substrate of GRK2 with a potential role in cytoskeletal reorganization, we looked to see whether GRK2-mediated ERM phosphorylation was involved in receptor endocytosis, a process that has previously been shown to be actin dependent (da Costa *et al.*, 2003). In HEK293 cells stably expressing His$_6$-tagged β₂AR (HEKβ2), agonist (isoproterenol) treatment for 15 min induced receptor internalization (Figure 6A). This was concomitant with an increase in endogenous pERM levels, which colocalize with β₂AR at internal vesicles (Figure 6A, ISO enlarged). This phenomenon is not restricted to β₂AR or HEK293 cells. In Hep2 cells expressing M1MR, prolonged ACh treatment (20 min) resulted in receptor endocytosis, which occurred after transient membrane ruffling (Figure 6B). Agonist treatment of M1MR-transfected Hep2 cells was associated with increased endogenous pERM levels, and, in these cells also, active ERM colocalizes with internalized receptor (Figure 6B, ACh enlarged). In marked contrast, in Hep2 cells transfected with the HA-tagged M2 muscarinic receptor (M2MR), internalization but not ERM phosphorylation is detected after agonist stimulation (Figure 6C). In HEK293 cells, M2MR has been shown to internalize in a β-arrestin-, and therefore GRK-, independent manner (Pals-Rylaarsdam *et al.*, 1995; Vogler *et al.*, 1999; Claing *et al.*, 2000). These results support our contention that GRK2 is responsible for GPCR-dependent ERM phosphorylation and suggest that pERM localization to receptor-containing endocytic

Figure 5. M1MR-mediated ruffling in Hep2 is not rho kinase or PKC dependent. (A) Forty-eight hours after transfection, Hep2 cells overexpressing M1MR were treated with either the rho kinase inhibitor (Y27632, 10 μM) or the PKC inhibitor (GF109203X, 20 μM) for 1 h. Cells were then subject to agonist (100 μM ACh, 5 min) before TCA fixation (ACh). M1MR and endogenous pERM were visualized by immunofluorescence. A rabbit anti-pERM antibody (Chemicon International) was used. In parallel, a second set of identically treated coverslips were paraformaldehyde fixed and stained with phalloidin and examined for M1MR expression. The phalloidin staining from this set of coverslips is shown (Phalloidin). (B) M1MR transfected Hep2 cells were left untreated or treated with rho kinase or PKC inhibitors, as described in A. After preincubation with kinase inhibitors, cells were treated with EGF (200 ng/ml, 5 min; Sigma-Aldrich), fixed, and the EGF receptor (EGFR) and pERM were visualized by immunofluorescence as described in *Materials and Methods*. Bars, 20 μm.

Figure 6. pERM proteins colocalize with internalized β_2AR and M1MR. (A) HEK293 cells stably expressing β_2AR were treated with 50 μM Iso for 20 min. β_2AR and endogenous pERM were detected using immunofluorescence. (B and C) Hep2 cells were transfected with M1MR (B) and M2MR (C) and incubated for 48 h. The cells were then subject to 100 μM ACh for 20 min (ACh), and receptor and endogenous pERM were visualized by immunofluorescence. 297S, a rat anti-pERM antibody, was used for visualization of endogenous pERM. T indicates cDNA constructs transfected. D indicates proteins detected. Bars, 20 μm.

vesicles may occur specifically with receptors that internalize via a GRK- and β-arrestin–dependent pathway.

Ezrin Is Required for β_2AR Endocytosis

Because pERM colocalize with internalized β_2AR, we sought to examine whether activated ezrin played a role in regulating the agonist-dependent trafficking of this receptor. Mock-transfected HEKβ2 cells were subject to 30 min of agonist treatment followed immediately by 30 min of antagonist (alprenolol)

treatment. Samples were removed before treatment (untreated), after agonist treatment (ISO [30 min]), and after agonist removal and subsequent antagonist treatment (alprenolol, ALP [30 min]). Plasma membrane-located β_2AR was labeled with an anti-His$_6$ monoclonal antibody and a fluorescently labeled secondary antibody and quantified by flow cytometry analysis. Results were compared with those of HEKβ2 cells expressing a dominant negative dynamin construct (dynamin K44A) or the dominant negative N-ezrin construct.
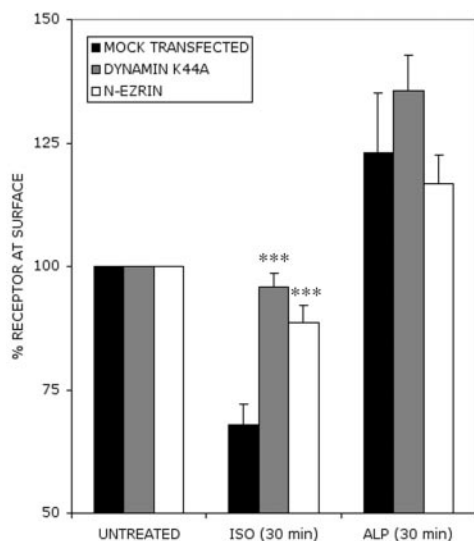
Figure 7. Ezrin is required for β_2AR endocytosis. Fluorescence flow cytometry was used to estimate internalization of β_2AR in HEK293 cells stably expressing the His6-tagged receptor (HEKβ2 cells). Cells were incubated with 40 μM isoproterenol for 30 min, washed, and then incubated with 100 μM alprenolol for 30 min to stop further endocytosis. Receptors present in the plasma membrane before treatment (untreated), after isoproterenol treatment (Iso [30 min]), and after alprenolol treatment (Alp [30 min]) were labeled with an anti-His$_6$ mAb. After fixation, the primary antibody was detected with a fluorophore-conjugated secondary antibody and quantified by flow cytometry, as described in *Materials and Methods*. This was repeated for HEKβ2 cells overexpressing a dominant negative dynamin construct (dynamin K44A) or the FERM domain of ezrin (N-ezrin). Data indicate the mean surface receptor fluorescence of cells. Error bars represent SD of mean fluorescence data collected from multiple samples of a representative experiment (n = 4). ***p $\leq$ 0.001.

After agonist treatment of mock-transfected cells, levels of β_2AR at the plasma membrane decreased by $\sim$32% (Figure 7, ISO [30 min]). Removal of agonist and treatment with antagonist resulted in the delivery of both internalized and newly synthesized receptor to the plasma membrane (Figure 7, ALP [30 min]). In cells transfected with dynamin K44A, which is known to inhibit endocytosis (van der Bliek *et al.*, 1993), <5% of total β_2AR at the cell surface was internalized. Similarly, expression of N-ezrin inhibited β_2AR internalization such that only 10% of cell surface receptor was endocytosed after isoproterenol treatment (Figure 7). Neither dynamin K44A nor N-ezrin affected delivery of β_2AR to the plasma membrane after removal of agonist. Thus, it seems that not only does phosphorylated, active ezrin colocalize with internalized β_2AR but it is also required for agonist-induced β_2AR endocytosis.

Colocalization of pERM with β_2AR Does Not Require NHERF

Previous studies have shown that the Na^+/H^+ exchanger regulatory factor (NHERF) can bind, in an agonist-dependent manner, to the carboxy terminus of the β_2AR via its PSD-95, Discs-large, ZO-1 (PDZ) domain (Hall *et al.*, 1998b) and to the FERM domain of ERM proteins (Reczek *et al.*, 1997; Reczek and Bretscher, 1998). We investigated whether NHERF was responsible for the localization of pERM with internalized β_2AR. HEK293 cells were transiently transfected with either wild-type β_2AR or a mutant β_2AR that
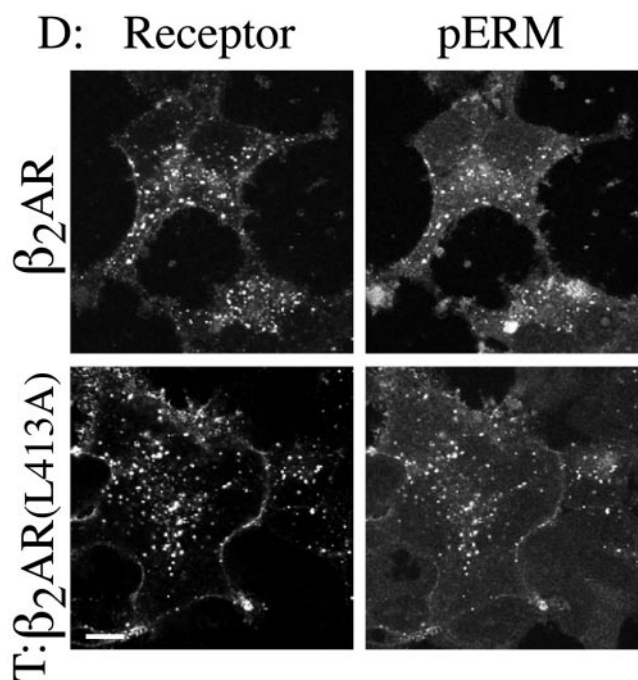
Figure 8. Colocalization of pERM and β_2AR at endocytic vesicles does not require NHERF. HEK293 cells were transfected with either the wild-type β_2AR or a mutant β_2AR that cannot bind NHERF (β_2AR$_{L413A}$). Cells were subject to 15 min of isoproterenol treatment, fixed, and permeabilized. Receptor and endogenous pERM were visualized by immunofluorescence. A rat anti-pERM antibody (297S) was used for pERM staining. T indicates cDNA constructs transfected. D indicates proteins detected. Bars, 20 μm.

cannot bind NHERF (β_2AR$_{L413A}$). Cells were subject to 15 min of isoproterenol treatment, fixed, permeabilized, and stained for receptor and endogenous pERM. As in Figure 6A, activation of transiently expressed β_2AR resulted in receptor internalization and colocalization of pERM to the internalized vesicles (Figure 8). Similarly pERM colocalized with internalized β_2AR$_{L413A}$, indicating that NHERF is not required for this interaction. Together, our results suggest that activated ezrin, but not NHERF, is required for β_2AR internalization. The colocalization of β_2AR and pERM at internalized vesicles may represent an interaction between these proteins mediated by a protein other than NHERF, a direct β_2AR/pERM interaction or alternatively a β_2AR-independent association of pERM with endocytic vesicles.

DISCUSSION

We have identified ezrin as a novel substrate of GRK2 and as a downstream effector of GPCR signaling. Our data increase the list of known nonreceptor GRK2 substrates, which already includes tubulin, phosducin, ribosomal protein P2, synucleins, the inhibitory γ subunit of the type 6 retinal cGMP phosphodiesterase, and the β-subunit of the epithelial Na^+ channel (Carman *et al.*, 1998; Haga *et al.*, 1998; Pitcher *et al.*, 1998b; Pronin *et al.*, 2000; Ruiz-Gomez *et al.*, 2000; Wan *et al.*, 2001; Freeman *et al.*, 2002; Dinudom *et al.*, 2004). These findings also corroborate the hypothesis that GRK2 may have additional functions within the cell, besides phosphorylation of agonist-occupied GPCRs.

In this report, the ability of GRK2 to phosphorylate ezrin was initially discovered in cells and subsequently confirmed

in vitro. Notably, GRK2-mediated phosphorylation of full-length ezrin is PIP_2 and $G\beta\gamma$-dependent, consistent with previous findings that show PIP_2-mediated changes in ezrin conformation are required for phosphorylation at T567 (Nakamura et al., 1999; Fievet et al., 2004). The PIP_2 and $G\beta\gamma$ dependence of GRK2-mediated phosphorylation additionally suggests that this phosphorylation event might occur in an agonist- and GPCR-dependent manner.

The GRK2 phosphorylation site within ezrin was localized to a carboxy-terminal region encompassing T567, and indeed, a mutant ezrin construct in which T567 is mutated to alanine fails to serve as a GRK2 substrate. The phosphorylation of T567 has been shown to be important for maintaining ezrin in its active conformation such that the plasma membrane and actin binding domains are exposed (Pearson et al., 2000). Our results suggest that in the presence of PIP2 and $G\beta\gamma$ GRK2-mediated ezrin phosphorylation may facilitate ezrin activation.

Stimulation of Hep2 cells overexpressing M1MR and of HEK293 cells stably expressing β_2AR leads to an increase in endogenous ERM specifically phosphorylated at T567 (ezrin), T564 (radixin), or T558 (moesin). In the Hep2 cell system, brief exposure to agonist (5 min) causes rapid and transient membrane ruffling that requires both active ezrin and GRK2. Cytoskeletal reorganization and the association of ezrin with plasma membrane and actin filaments have previously been found to be regulated by the Rho-family of small GTPases (Okamoto et al., 1999; Nakamura et al., 2000; Aspenstrom et al., 2004) and to involve phosphorylation of ezrin by either rho kinase or PKC, depending on the cellular context (Matsui et al., 1998; Simons et al., 1998). In this study, we have shown that in Hep2 cells expressing M1MR, ACh treatment leads to membrane ruffling and ERM activation even in the presence of the rho kinase inhibitor Y27632 or the PKC inhibitor GF109203X. That GRK2, but not rho kinase or PKC, inhibition ablates the M1MR-mediated ruffling response suggests that GRK2 phosphorylation facilitates ERM activation and is, at least in part, responsible for the GPCR- and ERM-dependent membrane ruffling observed in Hep2 cells.

Other GPCRS such as CCR2 (Jones et al., 2003), the sphingosine-1-phosphate receptors 1 ($S1P_1$) and 2 ($S1P_2$) (Sugimoto et al., 2003), the proteinase-activated receptor type 2 (PAR-2) (Ge et al., 2003), and CXCR4 (D'Apuzzo et al., 1997) induce membrane ruffling and cell migration on agonist activation. Although little is known about the interaction of $S1P_1$, $S1P_2$, and PAR-2 with GRKs, CCR2 and CXCR4 have been shown to undergo GRK2-dependent endocytosis after ligand binding (Aragay et al., 1998; Orsini et al., 2000). Furthermore, neutrophils and splenocytes derived from GRK6-deficient mice have altered chemotactic responses (Fong et al., 2002; Kavelaars et al., 2003; Vroon et al., 2004), indicating that GRK6 may be involved in regulating the cytoskeletal reorganization required for cell migration. In conjunction with our results, these data suggest that GRKs may have a general role in regulation of the actin cytoskeleton in processes such as cell motility. It would be interesting to investigate whether ezrin, or other ERM proteins, serve as substrates for other GRK family members, which would indicate a common pathway for actin filament modulation downstream of activated GPCRs.

Notably, not all GPCRs induce agonist-dependent ruffling when overexpressed in Hep2 cells. Indeed, the β_2AR, which would be anticipated to recruit GRK2 in an agonist-dependent manner, fails to induce a ruffling phenotype. Agonist occupancy of the β_2AR does, however, promote ezrin phosphorylation in HEK293 cells (Figure 8). These results suggest that GRK2-mediated ezrin phosphorylation, although required, is not sufficient to induce the cytoskeletal changes needed for ruffling. The additional factors required for GPCR-dependent membrane ruffling in Hep2 cells are currently a subject of investigation.

Many studies have demonstrated that the actin cytoskeleton has a role in clathrin-mediated endocytosis. Because GRK2 activity initiates GPCR desensitization and down-modulation and can also phosphorylate ezrin, a membrane-actin cross-linker, we investigated whether ezrin plays a functional role in GPCR internalization. In HEK293 cells stably expressing β_2AR and Hep2 cells expressing M1MR, prolonged agonist stimulation causes receptor endocytosis paralleled by the colocalization of pERM with receptor at internalized vesicles. Flow cytometry analysis to quantify surface β_2AR after isoproterenol treatment demonstrates that expression of the dominant negative N-ezrin construct inhibits receptor down-modulation to a similar extent as dynamin K44A expression. Dynamin is essential for clathrin-mediated endocytosis: dynamin K44A has a dominant negative effect that disrupts this process (van der Bliek et al., 1993).

Although our data suggest that ezrin may also have an important role in receptor internalization, this role may be limited to GPCRs that internalize in a GRK2- and β-arrestin–dependent manner. M2MR endocytosis is not correlated with ERM activation, and pERM is not associated with internalized M2 receptor. In HEK293 cells, M2MR has been shown to undergo endocytosis in a β-arrestin–independent manner and does not seem to internalize via typical clathrin- or caveolin-dependent pathways in HeLa or HEK293 (Pals-Rylaarsdam et al., 1995; Vogler et al., 1999; Claing et al., 2000; Roseberry and Hosey, 2001; Delaney et al., 2002). In contrast, both β_2AR and M1MR are sequestered via β-arrestin-, and therefore GRK-, dependent pathway in these cells (Claing et al., 2000). These results suggest that activated ezrin may play an obligate role in the internalization of a subset of GPCRs.

Much is known about GPCR internalization and the different mechanisms involved. Rapid sequestration of receptors such as β_2AR is characterized by GRK phosphorylation leading to β-arrestin binding and endocytosis via clathrin-coated pits; other GPCRs may internalize via caveolae or in a GRK-independent manner (von Zastrow, 2003; Chini and Parenti, 2004). Our results suggest that a requirement for activated ERM may be an additional distinguishing feature of these endocytic pathways and may correlate with GRK dependence.

Non-G-protein–coupled receptors also may use an ezrin- and clathrin-dependent endocytosis pathway. The association of the LDL receptor with ezrin has been shown to be necessary for its clathrin-mediated endocytosis (Smith et al., 2004). pERM proteins colocalize with low-density lipoprotein (LDL) particles at the plasma membrane, and although active ERM proteins do not associate with internalized LDL, GFP-N-ezrin significantly inhibited LDL uptake (Smith et al., 2004).

Further investigation is required to establish how ezrin associates with GPCRs after agonist occupation. The ERM-binding protein NHERF is known to bind to the carboxy termini of certain receptors, including the β_2AR, and has been shown to be involved in sorting β_2AR between the recycling endosome and lysosome after endocytosis (Cao et al., 1999). NHERF also has been shown to regulate the trafficking of the human κ opioid receptor and the parathyroid hormone receptor (Li et al., 2002; Sneddon et al., 2003). Our data indicate that NHERF is not required to link ERM to a receptor after stimulation because pERM colocalize with

both a mutant β_2AR that is unable to bind NHERF and to M1MR, which does not contain the carboxy terminal motif recognized by NHERF (Hall *et al.*, 1998a). It is possible that, like the integral membrane glycoprotein podocalyxin, β_2AR may be able to bind ERM both directly and via NHERF (Schmieder *et al.*, 2004). A recent study showed that Arf6 colocalizes with ezrin at the plasma membrane (Macia *et al.*, 2004). Arf6 is thought to regulate vesicle formation in endocytosis and interacts with β-arrestin, suggesting that β-arrestin may be responsible for the recruitment of Arf6 (Claing *et al.*, 2001), and hence possibly ezrin, to agonist-occupied GPCRs. It remains to be determined whether the requirement for activated ERM proteins in β_2AR internalization reflects a direct, an indirect, or is independent of an association between these two proteins.

In conclusion, we present evidence to suggest that a nonreceptor substrate of GRK2 has an effector function after GPCR activation. We show that ezrin is a novel substrate of GRK2, that ERM proteins are activated after receptor stimulation, and that GRK2-mediated ERM phosphorylation is required for GPCR-mediated cytoskeletal rearrangements as well as internalization of a subset of receptors. Recently β-arrestins, key regulators of GPCR signaling, have been shown to have additional roles as scaffold and adaptor proteins in pathways downstream of GPCRs (Ahn *et al.*, 2003). Similarly, it is becoming clear that GRK2, with its growing list of nonreceptor substrates, has a more diverse role within the cell than previously appreciated.

ACKNOWLEDGMENTS

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