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Internalization of the Human Nicotinic Acid Receptor GPR109A Is Regulated by G_i, GRK2, and Arrestin3*[S]

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Nicotinic acid (niacin) has been widely used as a favorable lipid-lowering drug for several decades, and the orphan G protein-coupled receptor GPR109A has been identified to be a receptor for niacin. Mechanistic investigations have shown that as a G_i-coupled receptor, GPR109A inhibits adenylate cyclase activity upon niacin activation, thereby inhibiting free fatty acid liberation. However, the underlying molecular mechanisms that regulate signaling and internalization of GPR109A remain largely unknown. To further characterize GPR109A internalization, we made a construct to express GPR109A fused with enhanced green fluorescent protein (EGFP) at its carboxyl-terminal end. In stable GPR109A-EGFP-expressing HEK-293 cells, GPR109A-EGFP was mainly localized at the plasma membrane and was rapidly internalized in a dose- and time-dependent manner upon agonist stimulation. GPR109A internalization was completely blocked by hypertonic sucrose, indicating that GPR109A internalizes via the clathrin-coated pit pathway. Further investigation demonstrated that internalized GPR109A was recycled to the cell surface after the removal of agonist, and recycling of the internalized receptors was not blocked by treatment with acidotropic agents, NH_4Cl and monensin. Pertussis toxin pretreatment not only inhibited forskolin-induced cAMP accumulation and intracellular Ca^{2+} mobilization; it also significantly attenuated agonist-promoted GPR109A internalization. Moreover, RNA interference experiments showed that knockdown of GRK2 (G protein-coupled receptor kinase 2) and arrestin3 expression significantly impaired receptor internalization. Taken together, these results indicate that the agonist-induced internalization of GPR109A receptors is regulated by GRK2 and arrestin3 in a pertussis toxin-sensitive manner and that internalized receptor recycling is independent of endosomal acidification.

Nicotinic acid (niacin), a member of the water-soluble vitamins, has been used clinically to treat hyperlipidemia and coronary heart disease for over 50 years (1, 2). Previous studies revealed that niacin is capable of strongly increasing high density lipoprotein cholesterol compared with other lipid-lowering drugs (3, 4), whereas plasma concentrations of total cholesterol, free fatty acids, and triglycerides are lowered by niacin (1, 2). Niacin and its analogues also improve insulin action in short term treatments (5–8). However, the clinical use of niacin is limited due to its side effects, especially skin flushing mediated by prostaglandin release (2).

Although niacin is widely used in clinical therapy, and biochemical studies also indicated the presence of a G_i-coupled receptor in rat adipose tissue and spleen membranes (9), its exact mechanism had not been understood until the orphan receptor GPR109A was identified as a receptor for niacin in 2003 (10–12). GPR109A was identified as a high affinity receptor for niacin, whereas GPR109B, which shares 96% amino acid identity with GPR109A, is a low affinity receptor (10). Activation of GPR109A upon binding niacin functions in a G protein-coupled manner to decrease cAMP production, resulting in decreased hormone-sensitive lipase activity and reduced hydrolysis of triglycerides to free fatty acids (11–13). Recent studies have indicated that niacin-induced flushing was mediated by GPR109A through the release of prostaglandin D_2 and prostaglandin E_2 (14, 15). Additionally, based on the pharmacological effects of niacin, new drugs with non-flushing, low toxicity acting via GPR109A are in development to treat dyslipidemia and prevent cardiovascular diseases (16). However, detailed information on the signaling and internalization of GPR109A still remains unknown. Richman *et al.* (17) reported that agonists that fail to significantly activate phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2)[3] and do not cause rapid receptor internalization do not lead to a flushing response. Therefore, further elucidating the mechanism of GPR109A signaling and trafficking will be of impor-

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[3] The abbreviations used are: ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; EGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; GRK, G protein-coupled receptor kinase; CRE, cAMP-response element; PTX, pertussis toxin; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; GRK, G protein-coupled receptor kinase.

tance for the development of a new generation of lipid-lowering drugs that avoid the unwanted cutaneous flushing side effect.

Because GPR109A is of great interest as a target for new drugs with therapeutic and anti-lipolytic benefits, the present study aimed to better characterize agonist-promoted signaling and trafficking of GPR109A. We found that niacin promoted rapid GPR109A internalization in a dose- and time-dependent manner via the clathrin-coated pit pathway and that internalized receptors were recycled upon agonist removal. Further investigation demonstrated that GPR109A internalization was mediated by G_i, GRK2, and arrestin3 and that recycling was independent of endosomal acidification.

EXPERIMENTAL PROCEDURES

Materials—Cell culture medium, Lipofectamine 2000, and G418 were purchased from Invitrogen. Fetal bovine serum was obtained from Hyclone (Beijing, China). The pEGFP-N1 and pCMV-FLAG vectors were purchased from Clontech and Sigma, respectively. The membrane probe DiI and nuclear dye Hoechst 33258 and anti-α-tubulin antibody were obtained from Beyotime (Haimen, China). Pertussis toxin (PTX) and anti-FLAG M2 monoclonal antibody were purchased from Sigma. Anti-phospho-ERK1/2, anti-ERK1/2, anti-actin, and anti-β-arrestin2 were from Cell Signaling (Danvers, MA). Clathrin HC small interfering RNA (siRNA) (human) and anti-clathrin HC (TD.1) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Molecular Cloning and Plasmid Construction—GPR109A (GenBank™ accession number AY148884) and GPR109B (GenBank™ accession number D10923) were cloned by PCR using human genomic DNA as a template. The primers used for GPR109A were 5′-AAG CTT ATG AAT CGG CAC CAT CTG CAG GAT-3′ (forward) and 5′-GGT ACC TTA AGG AGA GGT TGG GCC CAG A-3′ (reverse) for pCMV-FLAG and 5′-AAG CTT GCC ACC ATG AAT CGG CAC CAT CTG CAG GAT-3′ (forward) and 5′-GGT ACC GTA GGA GAG GTT GGG CCC AGATA-3′ (reverse) for pEGFP-N1; the primers used for GPR109B were 5′-AA GCT TGC CAC CAT GAA TCG GCA CCA TCT GCA GGA T-3′ (forward) and 5′-GGT ACC GTC TCG ATG CAA CAG CCC AAC TG-3′ (reverse) for pEGFP-N1. The PCR products were inserted into the HindIII and EcoRI sites of the pCMV-FLAG vector and the HindIII and KpnI sites of the pEGFP-N1 vector. All constructs were sequenced to verify the correct sequences and orientations. To construct the firefly luciferase reporter plasmid for assaying cAMP accumulation, the human *VIP* (vasointestinal peptide) promoter (18), from nucleotide −94 to 152 with EcoRI and HindIII sites incorporated at the 5′- and 3′-end, respectively, was amplified from human genomic DNA by PCR with forward primer (5′-GAA TTC ACT TCA AGC CCT ATT CAT CCC ATG G-3′) and reverse primer (5′-AA GCT TCT CGC CCA GTC GTG CTC CCC-3′). The SV40 early mRNA polyadenylation signal was cloned from the pEGFP-N1 vector using the forward primer (5′-CTC GAG CAT AAT CAG CCA TAC CAC ATT TGT A-3′) and reverse primer (5′-GGT ACC GGA CAA ACC ACA ACT AGA ATG CA-3′); XhoI and KpnI sites were incorporated into the 5′- and 3′-ends. The cAMP-response element (CRE) was synthesized based on a previously published sequence (19, 20) and inserted into the pBluescript KS II(+) (Stratagene, La Jolla, CA) vector, followed by a minipromoter *VIP*, firefly luciferase, and SV40 poly(A) signal.

Cell Culture and Transfection—The human embryonic kidney cell line 293 (HEK-293) and COS-7 cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine (Invitrogen). GPR109A cDNA plasmid constructs were transfected into HEK-293 or COS-7 cells using Lipofectamine 2000 according to the manufacturer's instructions. Forty-eight hours after transfection, stably expressing cells were selected by the addition of 800 mg/liter G418.

Synthesis of Small Interfering RNAs and siRNA Transfection—All arrestin and GRK siRNAs were chemically synthesized by Dharmacon RNA Technologies (Lafayette, CO). The GRK2, GRK3, and GRK5 siRNAs were reported previously (21, 22). The GRK3 siRNA sequence was 5′-GCA GAA GUC GAC AAA UUU A-3′; arrestin2 and -3 siRNAs were purchased as a SMARTpool. The clathrin HC siRNA (human) was from Santa Cruz Biotechnology, Inc. The nonspecific control siRNA VIII (5′-AAA CUC UAU CUG CAC GCU GAC-3′) was used as the control for all siRNA experiments. The transfection protocol for GRK, arrestin, and CHC siRNAs was reported previously (22). Forty-eight hours after transfection, cells were split for the indicated assay the following day.

cAMP Accumulation—After seeding in a 24-well plate overnight, stable HEK-293 cells co-transfected with GPR109A and pCRE-Luc were grown to 90–95% confluence, stimulated with 10 μM forskolin alone or 10 μM forskolin with different concentrations of niacin in DMEM without fetal bovine serum, and incubated for 4 h at 37 °C. Luciferase activity was detected by a firefly luciferase kit (Promega, Madison, WI). When required, cells were treated overnight with or without PTX (50 ng/ml) in serum-free DMEM before the experiment.

Intracellular Calcium Measurement—Calcium mobilization was performed as described previously with slight modifications (21). The stably GPR109A-expressing HEK-293 cells were harvested with Cell Stripper (Mediatech, Herndon, VA), washed twice with phosphate-buffered saline (PBS), and resuspended to 5×10^6 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 $MgCl_2$, 1 mg/ml glucose) containing 0.025% bovine serum albumin. The cells were then loaded with 3 μM Fura-2 acetoxymethyl ester derivative (Fura-2/AM) (Molecular Probes, Inc., Eugene, OR) for 30 min at 37 °C. Cells were washed once in Hanks' solution, resuspended in Hanks' solution, incubated at room temperature for 15 min, washed twice in Hanks' solution, and then resuspended in Hanks' solution at a concentration of 3×10^7 cells/ml. A typical experiment contained 1.5×10^6 cells/1.6 ml in a quartz cuvette; these cells were stimulated with the indicated concentrations of niacin. Receptor specificity studies were done by measuring the calcium flux of GPR109A-expressing HEK-293 cells stimulated with 100 μM niacin as a negative control. Calcium flux was measured using excitation at 340 and 380 nm in a fluorescence spectrometer (LS55; PerkinElmer Life Sciences). Calibration was performed using 0.1% Triton X-100 for total fluorophore release and 10 mM EGTA to chelate free Ca^{2+}. Intracellular Ca^{2+} concentrations

were calculated using a fluorescence spectrometer measurement program as described previously (23).

GPR109A Localization and Internalization Assay—HEK-293 cells stably expressing GPR109A-EGFP were seeded in covered glass bottom 6-well plates. After 24 h, HEK-293 cells were stained with the membrane probe DiI (Beyotime) at 37 °C for 5–10 min, fixed with 2% paraformaldehyde for 15 min, and finally incubated with Hoechst 33258 (Beyotime) to stain cell nuclei for 10 min. For the internalization assay, HEK-293 cells stably expressing GPR109A-EGFP or GPR109B-EGFP were seeded in covered glass bottom 6-well plates, and cells were treated with different concentrations of niacin for 30 min or with 300 μM niacin for different incubation times at 37 °C. For the recycling assay, HEK-293 cells stably expressing GPR109A-EGFP were incubated with 100 μg/ml cycloheximide and 300 μM niacin for 30 min. After removal of the agonist, the cells were washed with PBS three times, and DMEM containing 100 μg/ml cycloheximide with or without NH_4Cl or monensin was added for different periods of time. After fixation with 2% paraformaldehyde for 15 min, cells were mounted in mounting reagent (dithiothreitol/PBS/glycerol). Confocal images were taken on a Zeiss LSM 510 microscope with an attached Axiovert 200 microscope and LSM5 computer system. Excitation was done at 488 nm, and fluorescence detection used a 525 ± 25-nm bandpass filter. Images were collected using QED camera software and processed with Adobe Photoshop.

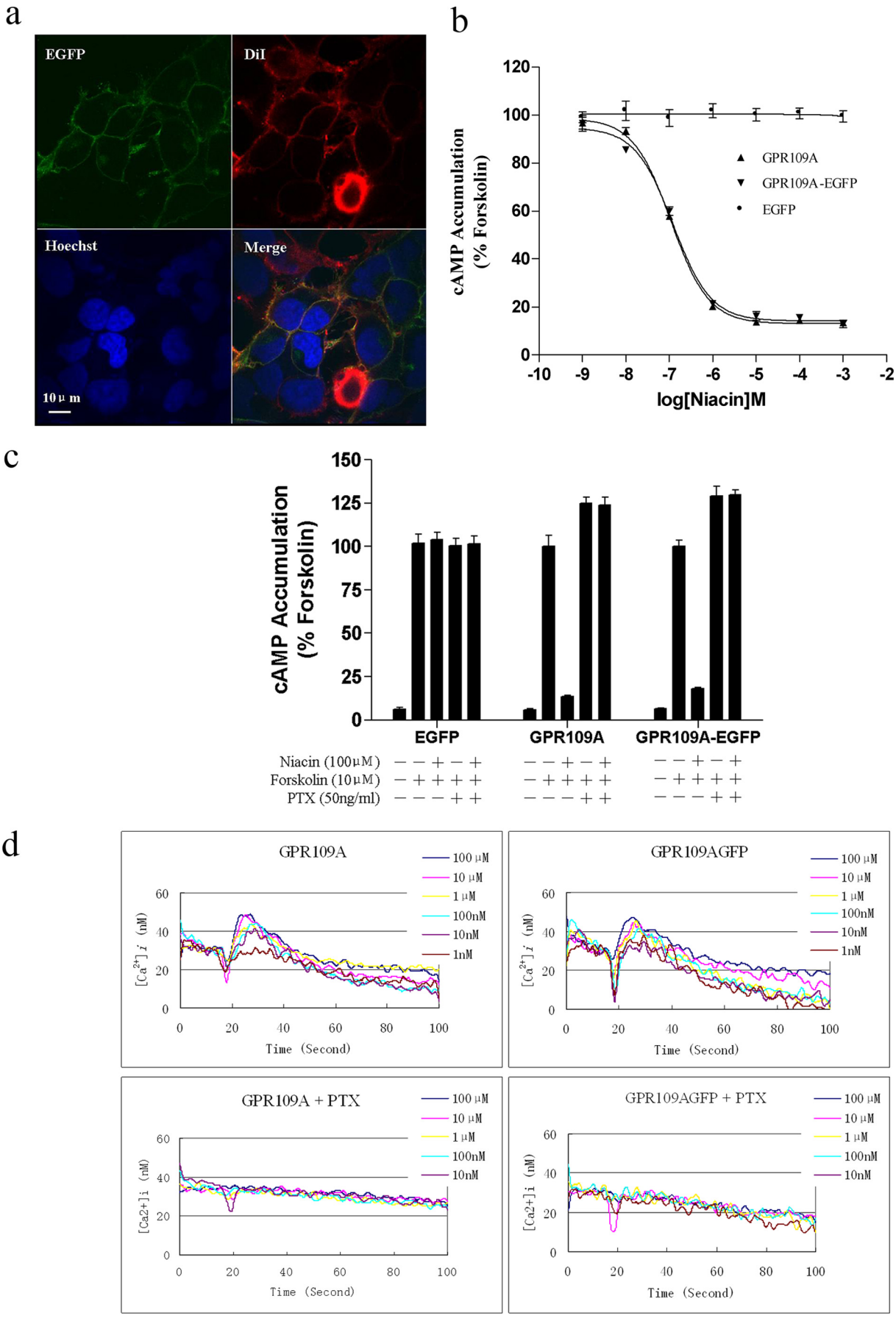
Western Blot Analysis—To analyze the knockdown of siRNA-targeted proteins and phosphorylation of ERK1/2, siRNA-transfected or agonist-stimulated HEK-293 cells in a 6-well plate were washed twice with ice-cold PBS and lysed with buffer (20 mM HEPES (pH 7.5), 10 mM EDTA, 150 mM NaCl, 1% Triton X-100, and one tablet of complete protease inhibitor (Roche Applied Science) per 50 ml) at 4 °C on a rocker for 30 min. The lysates were centrifuged at 4 °C at 12,000 rpm for 15 min. For examining arrestin3 membrane translocation, HEK-293 cells stably expressing GPR109A were incubated for 8 min with or without 100 μM niacin, and after washing twice with ice-cold PBS, cells were scraped in PBS containing complete protease inhibitor mixture. Cells were treated by brief sonication and then centrifuged at 3000 × *g* for 5 min to remove unbroken cells and nuclear fractions. Subsequently, the supernatant was centrifuged at 21,000 × *g* for 30 min to separate the membrane fraction (in the pellet) and cytosolic fraction. The proteins in the supernatant (cytosolic fraction) were precipitated with 70% ethanol, and both pellets were resuspended in PBS. Proteins in the membrane and the cytosolic fractions 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-GRK5 (1:3000), monoclonal anti-arrestin2 (1:1000), monoclonal anti-arrestin3 (1:1000), or rabbit monoclonal anti-phospho-ERK1/2 antibody (1:1000) (Cell Signaling, Danvers, MA) and next probed with horseradish peroxidase-labeled secondary antibodies, and chemiluminescence was detected using horseradish peroxidase-substrate (Cell Signaling). The blots were stripped and reprobed using an anti-tubulin (1:7500) monoclonal antibody as control for protein loading and anti-total ERK1/2 (1:2000) for phospho-ERK1/2.

Measurement of Cell Surface Receptor by ELISA—The cell surface expression of GPR109A was quantitatively assessed by ELISA as described previously (24). Briefly, after transfection with Lipofectamine 2000 according to the manufacturer's protocol, HEK-293 cells were grown in DMEM at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2 overnight and then split into 24-well dishes coated with poly-L-lysine. The following day, cells were stimulated with the indicated concentrations of niacin for the times indicated. Medium was aspirated and washed once with Tris-buffered saline. After fixing for 5 min at room temperature with 3.7% formaldehyde in Tris-buffered saline, cells were washed three times with Tris-buffered saline and then blocked for 45 min with 1% bovine serum albumin/Tris-buffered saline. Cells were then incubated for 1 h with an alkaline phosphatase-conjugated monoclonal antibody directed against the FLAG epitope diluted 1:1000. Cells were washed three times, and antibody binding was visualized by adding 0.25 ml of alkaline phosphatase substrate (Bio-Rad). Development was stopped by removing 0.1 ml of the substrate to a 96-well microtiter plate containing 0.1 ml of 0.4 M NaOH. Plates were read at 405 nm in a microplate reader (Bio-Rad) using Microplate Manager software.

RESULTS

Expression and Function of Chimeric GPR109A-EGFP Protein—In order to directly visualize the membrane localization and trafficking of GPR109A receptors, we constructed an expression vector containing GPR109A fused with EGFP at the carboxyl terminus and then transiently or stably transfected it into HEK-293 cells. Confocal microscopy revealed that the expressed GPR109A-GFP was colocalized with the membrane marker DiI with minimal intracellular accumulation in the absence of ligand in stably expressing HEK-293 cells (Fig. 1*a*), suggesting the correct localization of GPR109A-EGFP at the plasma membrane. We then examined the functional signaling of GPR109A-EGFP by assaying cAMP accumulation and intracellular Ca^{2+} flux. As shown in Fig. 1, *b* and *c*, treating HEK-293 cells that stably express GPR109A-EGFP with niacin inhibited forskolin-stimulated cAMP production comparably with cells expressing wild-type GPR109A. The agonist-induced inhibition of the forskolin-stimulated cAMP increase could be completely blocked by pretreating with 50 ng/ml PTX for 12 h (Fig. 1*c*). In addition, stimulation with niacin also elicited similar levels of intracellular Ca^{2+} mobilization in GPR109A-EGFP-expressing HEK-293 cells compared with the wild-type receptor (Fig. 1*d*). These results demonstrate that GPR109A-EGFP in stably transfected HEK-293 cells functions similarly to the wild-type receptor.

Agonists Induce Rapid Internalization of GPR109A-EGFP—To determine whether GPR109A-EGFP stably expressed in HEK-293 cells internalized upon agonist binding, HEK-293/GPR109A-EGFP and HEK-293/GPR109B-EGFP cells were incubated with 300 μM niacin for 40 min at 37 °C and examined by confocal microscopy. As seen in Fig. 2*a*, GPR109A receptors were internalized from the cell surface into the cytoplasm with a punctate distribution upon activation by niacin, whereas the GPR109B receptors were still predominantly distributed on the cell membrane. To further investigate whether niacin can induce


a
EGFP
DiI
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Merge
10 μ m
b
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GPR109A
GPR109A-EGFP
EGFP
c
cAMP Accumulation (% Forskolin)
EGFP
GPR109A
GPR109A-EGFP
Niacin (100μM)
Forskolin (10μM)
PTX (50ng/ml)
d
GPR109A
GPR109AGFP
GPR109A + PTX
GPR109AGFP + PTX
Time (Second)
100 μM
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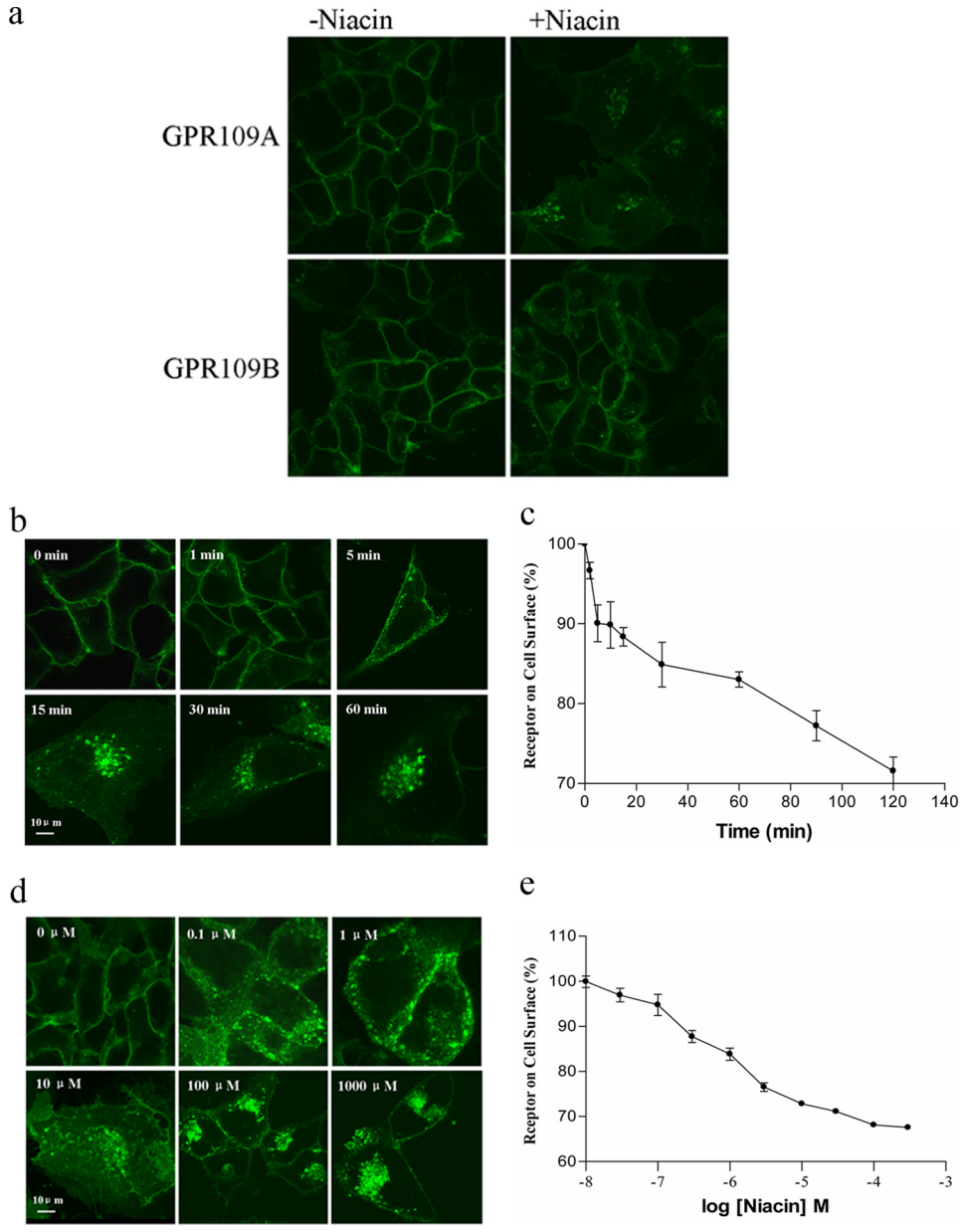


FIGURE 2. **Internalization of GPR109A-EGFP stably expressed in HEK-293 cells.** *a*, HEK-293 cells stably expressing GPR109A-EGFP or GPR109B-EGFP were stimulated with 300 μM niacin for 40 min. *b*, HEK-293 cells stably expressing GPR109A-EGFP were treated with 300 μM niacin for the indicated time periods. *d*, HEK-293 cells stably expressing GPR109A-EGFP were activated by the indicated concentrations of agonist for 40 min. *c* and *e*, ELISA measurement of GPR109A remaining on the cell surface after treatment of cells with 300 μM niacin for the indicated time period or with the indicated concentrations of agonist for 40 min. *Error bars*, S.E. for four replicates. Data were analyzed using Student's *t* test (**, $p < 0.01$; ***, $p < 0.001$). All pictures and data shown are representative of at least three independent experiments.

GPR109B receptor internalization, we treated HEK-293/GPR109B cells with different concentrations of niacin or with 100 μM 1-alkyl-benzotriazole-5-carboxylic acid, which is a specific agonist for GPR109B (25). The result demonstrated that 1 mM niacin was needed to induce weak internalization of GPR109B, whereas 100 μM 1-alkyl-benzotriazole-5-carboxylic acid induced significant endocytosis of GPR109B (see supplemental Fig. 1). These results are consistent with reports that niacin has a high affinity for GPR109A and low affinity for GPR109B (10).

To further characterize the agonist-induced internalization of GPR109A, the GPR109A-EGFP-expressing HEK-293 cells were treated with 300 μM niacin for different times. Fig. 2*b* shows the time-dependent changes in receptor internalization following agonist stimulation. Niacin evoked a rapid internalization of GPR109A-EGFP in HEK-293 cells. After 15 min of niacin exposure, internalized receptors were distributed throughout the cytoplasm, whereas they were largely clustered in the perinuclear region at 30 min. In addition, HEK293 cells expressing FLAG-GPR109A were treated with or without agonist for various times at 37 °C, and then cell surface GPR109A was quantitatively detected by ELISA. Niacin treatment induced internalization within 5 min, and the receptor continued to internalize, leading to ~30% loss of cell surface expression (Fig. 2*c*). Upon stimulation with different concentrations of niacin, a dose-dependent internalization of GPR109A-GFP was also observed (Fig. 2*d*). Quantification by cell surface ELISA showed significant loss of cell surface receptors at niacin doses of 0.1–300 μM (Fig. 2*e*).

Clathrin-coated Pits and Dynamin Are Involved in Internalization of GPR109A—To define whether GPR109A internalization involved clathrin-coated pits, GPR109A-EGFP-expressing cells were treated with hyper-

FIGURE 1. **Characterization of GPR109A-EGFP stably expressed in HEK-293 cells.** *a*, HEK-293 cells stably expressing GPR109A-EGFP were stained with a membrane plasma probe (DiI) and a nuclei probe (Hoechst). The cells stably expressing GPR109A-GFP were seeded on glass bottom 6-well plates overnight, incubated with DiI (5 μM) and Hoechst, and examined by confocal microscopy, as described under "Experimental Procedures." *b* and *c*, cAMP accumulation in HEK-293 cells stably expressing wild-type GPR109A or GPR109A-EGFP and pCRE-Luc was determined in response to forskolin and niacin. The dose-dependent inhibition of forskolin-induced cAMP accumulation in wild-type GPR109A and GPR109A-EGFP-expressing cells (*b*) and inhibition efficiency in cAMP accumulation in GPR109A-EGFP and wild-type GPR109A-expressing cells in the presence or absence of PTX (*c*) were measured. *d*, HEK-293 cells stably expressing wild-type GPR109A or GPR109A-EGFP were loaded with the calcium probe Fura-2/AM, followed by the stimulation of niacin in the presence or absence of PTX; calcium mobilization was assayed by monitoring the change in Fura-2/AM fluorescence. The data shown are representative of at least three independent experiments. *Error bars*, S.D.

tonic sucrose. Pretreatment with sucrose for 2 h had no effect on the expression or localization of GPR109A in HEK-293 cells but completely blocked niacin-induced GPR109A internalization, as analyzed by microscopy (Fig. 3*a*) and ELISA (Fig. 3*b*). To further confirm that clathrin is involved in GPR109A internalization, we used specific siRNAs targeted against clathrin heavy chain to decrease the expression of clathrin in GPR109A-expressing HEK-293 cells. The endogenous expression of clathrin was effectively and specifically knocked down by the specific siRNA treatment but was unaffected in cells treated with control siRNA (Fig. 3*c*). Silencing clathrin effectively inhibited niacin-induced GPR109A internalization, as analyzed by microscopy (Fig. 3*d*) and ELISA (Fig. 3*e*). This demonstrates that agonist-activated GPR109A is internalized via clathrin-coated pits.

In order to determine whether dynamin is involved in GPR109A internalization, GPR109A-expressing cells were transfected with the dynamin-1 dominant negative mutant K44A and analyzed 48 h after transfection. Agonist-promoted GPR109A internalization was effectively inhibited by dynamin-K44A (Fig. 3, *f* and *g*), indicating that dynamin is involved in this process.

GPR109A Colocalizes with Early and Recycling Endosomes but Not with Lysosomes—Internalized G protein-coupled receptors are generally degraded in lysosomes or recycled back to the plasma membrane via early and recycling endosomes. To investigate these two possibilities for GPR109A, GPR109A-EGFP-expressing cells were treated with niacin together with Alexa Fluor 546-labeled transferrin or LysoTracker Red DND-99. Analysis by confocal microscopy demonstrated that extensive colocalization of transferrin and GPR109A-GFP was observed (Fig. 4*a*), whereas GPR109A-GFP did not colocalize with Lysotracker Red, a marker for late endosomes and lysosomes (Fig. 4*b*), suggesting that GPR109A-GFP localizes in endosomes but not in lysosomes.

Agonist-induced GPR109A Internalization Is Src-dependent—We next investigated whether different protein kinases were involved in agonist-induced GPR109A internalization. HEK-293 cells stably expressing GPR109A-EGFP were pretreated with DMSO, protein kinase A inhibitor KT5720 (1 μM), protein kinase C inhibitor Go6983 (10 μM), EGFR-specific tyrosine kinase inhibitor AG1478 (1 μM), or Src tyrosine kinase inhibitor PP2 (20 μM) for 1 h prior to agonist stimulation. As shown in Fig. 5*a*, treatment with the protein kinase A inhibitor, protein kinase C inhibitor, and EGFR kinase inhibitor had no effect on agonist-induced GPR109A internalization, whereas treatment with the Src inhibitor effectively inhibited agonist-induced GPR109A internalization. The same result was observed by ELISA analysis (Fig. 5*b*), indicating that Src is involved in agonist-activated GPR109A internalization.

Recycling of Internalized GPR109A to the Cell Surface—In order to determine the fate of internalized GPR109A, GPR109A-EGFP-expressing cells were treated with 100 μg/ml cycloheximide and 300 μM niacin at 37 °C for 40 min, followed by the removal of residual agonist by washing and further incubation in the presence of cycloheximide for the indicated time periods. Confocal microscopy revealed that pretreatment with cycloheximide had no effect on GPR109A localization and internalization, and most of the GPR109A was internalized after treatment with 300 μM niacin for 40 min (Fig. 6*a*); however, the internalized receptors were partially recycled to the plasma membrane within 30 min after agonist removal. Further incubation resulted in full recovery of the internalized GPR109A to the cell surface as evaluated by microscopy (Fig. 6*a*) and ELISA (Fig. 6*b*). To examine the importance of endosomal acidification in the recycling of GPR109A-, GPR109A-EGFP-, or FLAG-GPR109A-expressing cells were treated with the acidotropic agents NH_4Cl and monensin. NH_4Cl showed no inhibitory effect on the recycling of GPR109A, whereas monensin had a modest effect on the recovery of internalized GPR109A to the cell surface, suggesting that endosomal acidification was not necessary to recycle internalized GPR109A receptors (Fig. 6, *c* and *d*).

Agonist-induced GPR109A Internalization Is Pertussis Toxin-sensitive—GPR109A acts via G_i proteins to inhibit adenylyl cyclase, and pretreatment with PTX abolishes agonist-dependent G_i activation, resulting in the inhibition of forskolin-induced cAMP accumulation (Fig. 1*c*), intracellular Ca^{2+} mobilization (Fig. 1*d*), and phosphorylation of ERK1/2 (Fig. 7*a*). To investigate the involvement of the G_i protein in the internalization of GPR109A, cells were treated for 18 h at 37 °C with PTX (500 ng/ml), followed by niacin stimulation. As illustrated in Fig. 7, *b* and *c*, PTX treatment alone did not alter the expression or membrane localization of GPR109A compared with untreated cells; however, agonist-induced GPR109A internalization was significantly impaired by PTX.

Arrestins and GRKs Are Involved in Internalization of GPR109A—G protein-coupled receptor kinase (GRK)-mediated phosphorylation and arrestin binding is involved in desensitization and trafficking of many GPCRs (26, 27). To investigate the role of GRKs and arrestins in the regulation of GPR109A internalization, we used specific siRNAs to reduce the expression of GRK2, GRK3, GRK5, arrestin2, and arrestin3 in GPR109A-EGFP-expressing HEK-293 cells. The endogenous expression of GRK or arrestin was effectively and specifically knocked down by the specific siRNA treatment but was unaffected in cells treated with nonspecific or control siRNA (Fig. 8, *a* and *b*). Silencing GRK2 and arrestin3 effectively inhibited GPR109A internalization, whereas knockdown of GRK3, GRK5, or arrestin2 had no effect on the internalization of GPR109A, as analyzed by microscopy (Fig. 8*c*) or ELISA (Fig. 8*d*). We further investigated the effect of knockdown of arrestins and GRKs on activation of ERK1/2, and no difference was observed between control and knockdown cells (Fig. 8*f*).

Next we examined whether niacin-mediated stimulation promotes arrestin3 translocation to the membrane in GPR109A-expressing HEK-293 cells by Western blot analysis. As shown in Fig. 8*e*, in the absence of niacin, arrestin3 remained primarily in the cytosol, and stimulation with niacin resulted in robust recruitment of arrestin3 to the membrane. These results suggest that GRK2 mediates the phosphorylation of serine and threonine residues in GPR109A, which facilitates arrestin3 binding to the receptor, resulting in receptor internalization.

G$\beta\gamma$ Plays a Role in Regulation of GPR109A Internalization—Previous studies proposed that G$\beta\gamma$ subunits released by agonist activation of a GPCR play an important role in recruiting

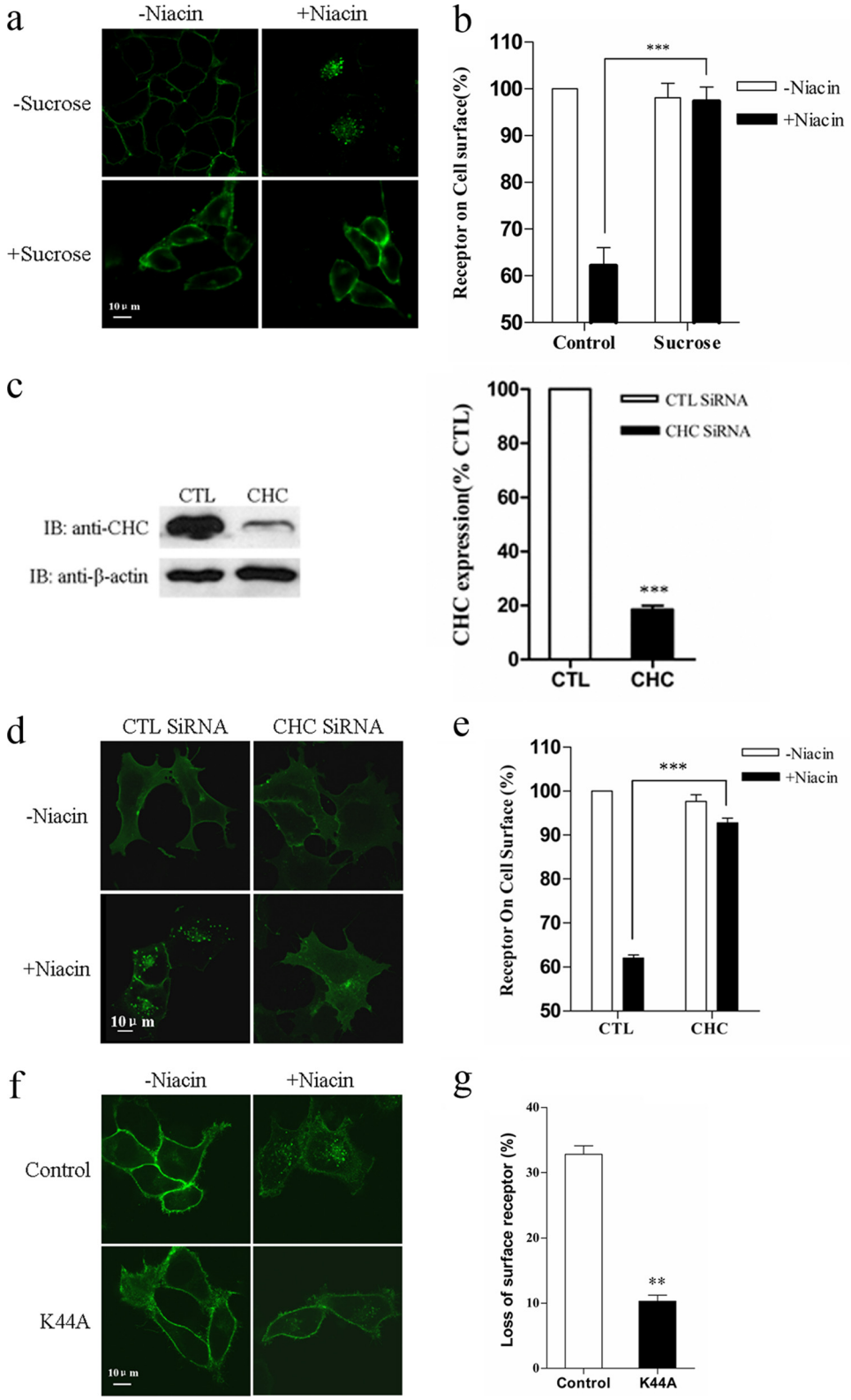


FIGURE 3. **Clathrin-coated pits and dynamin are involved in internalization of GPR109A.** *a* and *b*, HEK-293 cells stably expressing GPR109A-EGFP were treated with 450 mM sucrose for 2 h, followed by stimulation with 300 μM niacin for 40 min. *c*, HEK-293 cells stably expressing GPR109A were transfected with specific clathrin heavy chain siRNA (*CHC*) or nonspecific control siRNA (*CTL*). Seventy-two hours after 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 antibody. Blots were stripped and reprobed for β-actin to control for loading. Shown is a representative immunoblot (*IB*) from four independent experiments. *d* and *e*, 72 h after transfection with specific clathrin heavy chain siRNA or nonspecific control siRNA, cells were stimulated with 300 μM niacin for 40 min and examined with confocal microscopy (*d*) or ELISA (*e*). *f* and *g*, HEK-293 cells stably expressing GPR109A-EGFP were transfected with dynamin-K44A. Forty-eight hours after transfection, cells were treated with 300 μM niacin for 40 min and examined with confocal microscopy (*f*) or ELISA (*g*). *Error bars*, S.E. for four replicates. Data were analyzed using Student's *t* test (**, $p < 0.01$; ***, $p < 0.001$). All pictures and data shown are representative of at least three independent experiments.

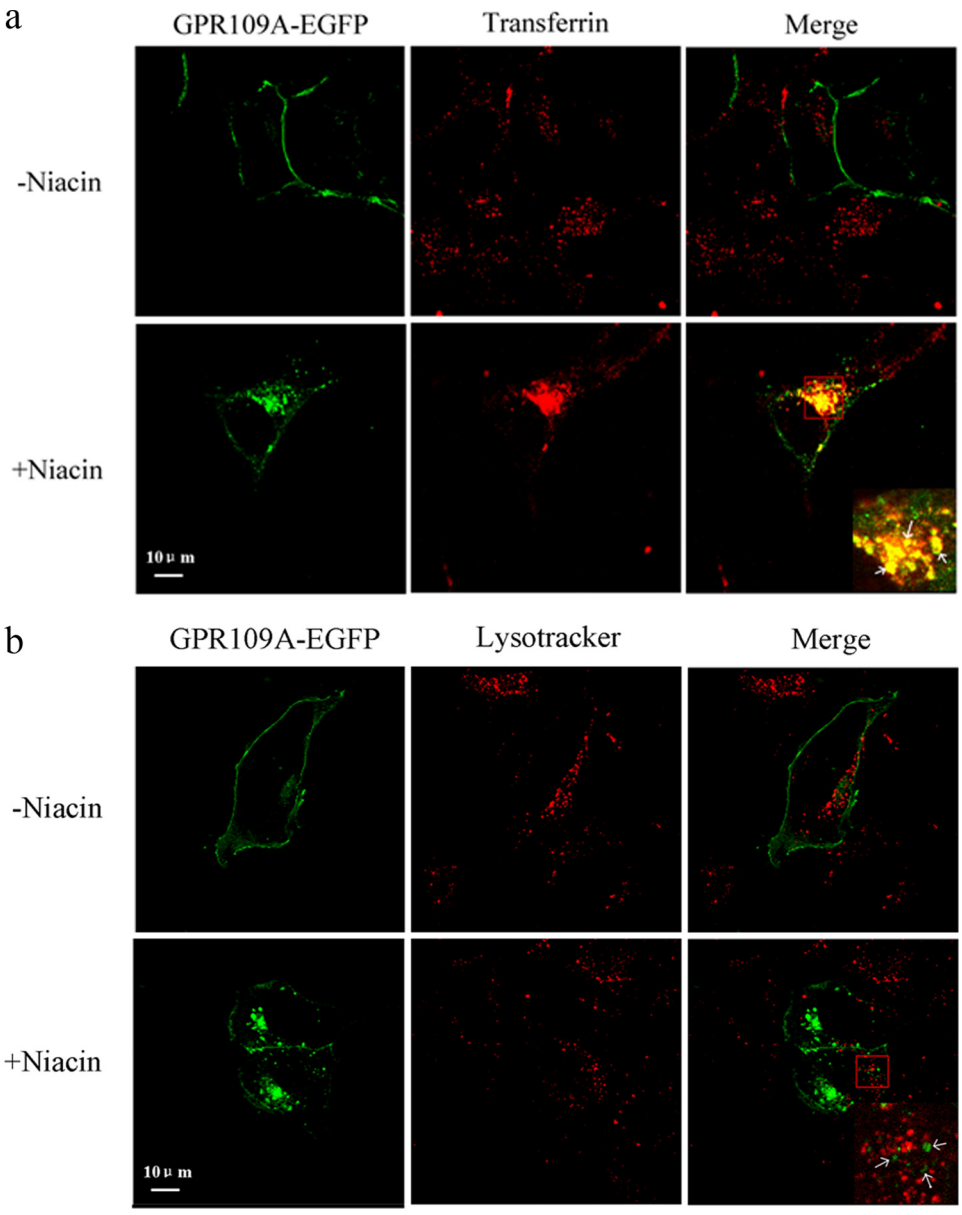


FIGURE 4. **Localization of internalized GPR109A-EGFP stably expressed in HEK-293 cells.** HEK-293 cells stably expressing GPR109A-EGFP were incubated with or without 300 μM niacin at 37 °C in the presence of either 100 μg/ml Alexa Fluor 546-labeled transferrin (*a*) or 50 nM lysotracker DND-99 (*b*) for 40 min. Cells were fixed and examined with confocal microscopy as described under "Experimental Procedures." All pictures shown are representative of at least three independent experiments.

GRK2 to the membrane to promote receptor phosphorylation (28, 29). Thus, we next investigated if the G$\beta\gamma$ subunit regulates GPR109A internalization. In preliminary experiments, we used a construct encoding the carboxyl-terminal domain (GRK2-Cter) (GRK2-Gly^{495}–Leu^{689}) of GRK2, which is widely used to inhibit G$\beta\gamma$-dependent signaling (30). However, overexpression of GRK2-Cter in HEK-293 cells showed no effects on GPR109A internalization in comparison with control. Early experiments demonstrated that catalytically inactive GRK2 blocked M_2 muscarinic acetylcholine receptor internalization in COS7 cells but had no effect on M_2 muscarinic acetylcholine receptor internalization in BHK-21 and HEK-293 cells (31, 32). Subsequently, GRK2 protein expression levels were shown to vary from cell type to cell type, with the lowest levels of GRK2 expression found in COS7 cells (33, 34). Therefore, the effectiveness of GRK2-Cter overexpression may depend on the level of GRK2 protein expressed in the particular cell line used in the experiment (35). We then used COS-7 cells to assess the effect of GRK2-Cter overexpression on GPR109A internalization. Confocal microscopy and ELISA analysis demonstrated that GRK2-Cter expression significantly inhibited agonist-promoted GPR109A-EGFP internalization (Fig. 8, *g* and *h*). These results strongly suggest that G$\beta\gamma$ subunits play an important role in the regulation of GPR109A internalization, consistent with the result of the GRK2 knockdown with specific siRNA.

DISCUSSION

Fifty years ago, niacin was introduced into the clinic as the first orally available drug to treat lipid metabolism disorders, including dyslipidemia or hyperlipoproteinemia. However, the putative mechanism of action of niacin has only recently been elucidated with the identification of GPR109A as a receptor for niacin (10–12). Recently, a number of GPR109A pyrazole agonists that failed to induce the internalization of the receptor or to activate the ERK1/2 pathway were shown to be capable of fully inhibiting lipolysis *in vitro* and *in vivo*, but they failed to elicit a flushing response (17). This suggests that there is some hope that a better elucidation of the mechanisms of GPR109A signaling and trafficking will help to develop a novel class of antidyslipidemic drugs without eliciting the flushing response. In the present study, we have characterized the trafficking of GPR109A upon stimulation by niacin. We constructed an expression vector containing EGFP fused in frame to the carboxyl terminus of GPR109A to directly visualize GPR109A expression, localization, and trafficking in cultured cells. GFP from the jellyfish *Aequoria victoria* has been widely used to monitor protein expression and localization, and its usefulness has been demonstrated for more than 30 GPCRs (16, 36–38). The potential disadvantages of a GPCR-GFP chimera are altered conformation and receptor functionality (38). Therefore, we first verified that GPR109A-EGFP functioned similarly to the wild-type receptor in membrane localization, inhibition of cAMP production, and intra-

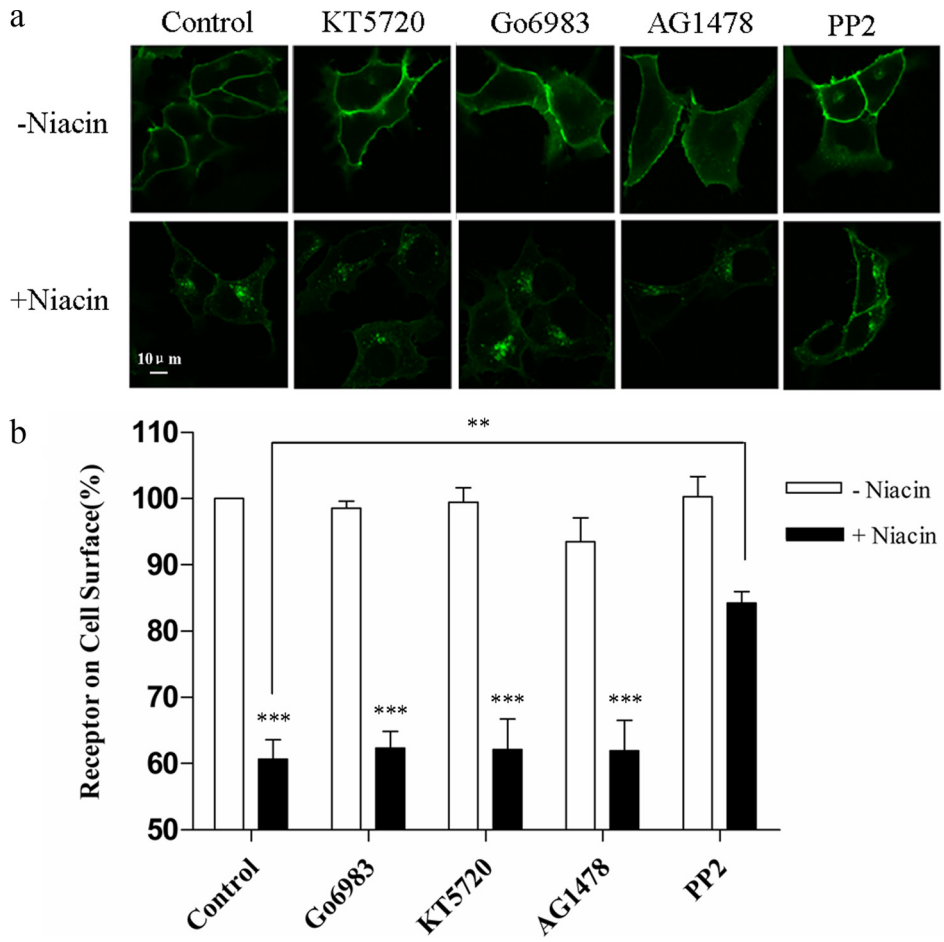
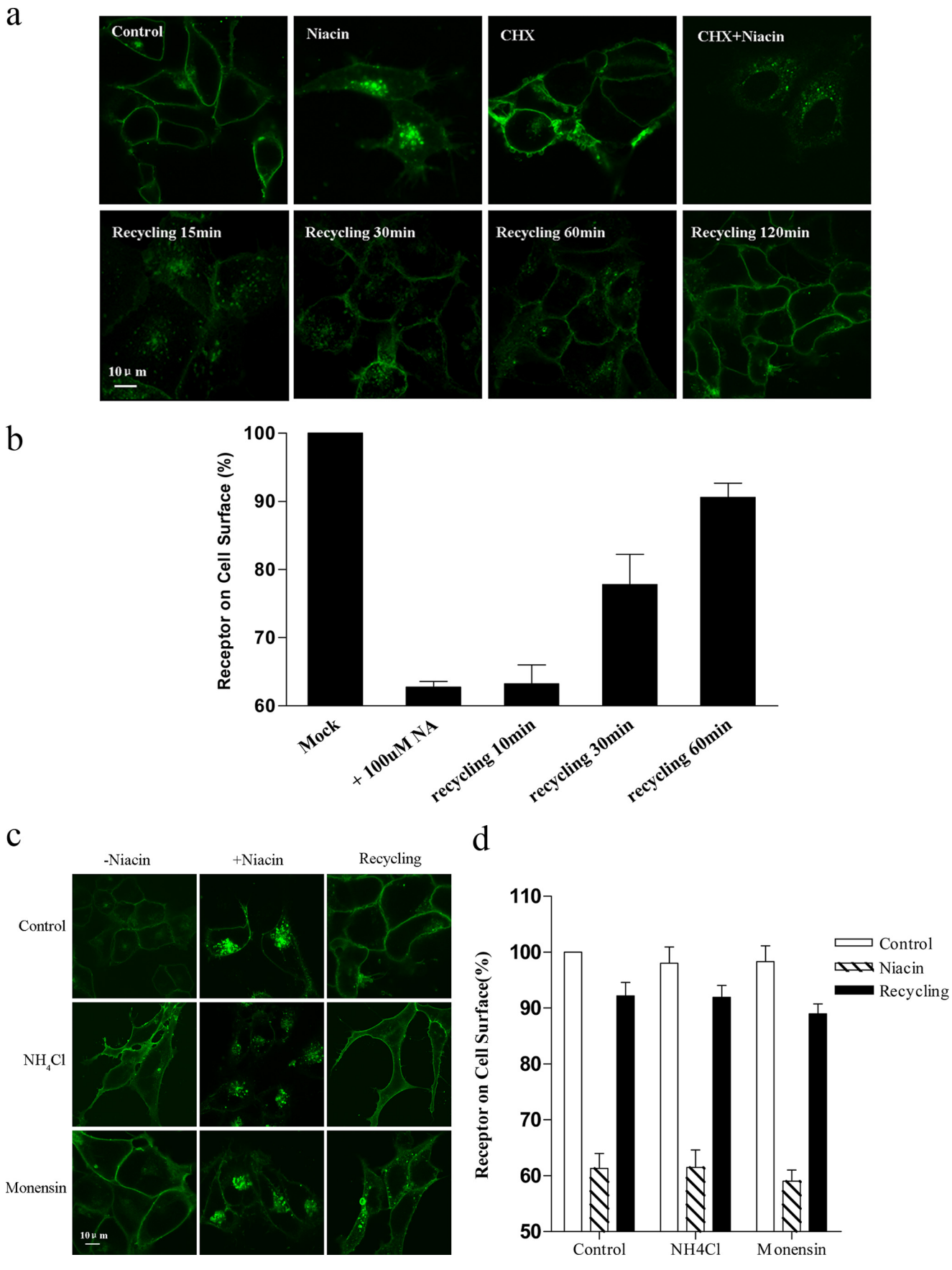


FIGURE 5. **Effect of protein kinase inhibitors on agonist-induced GPR109A internalization in GPR109A-EGFP stably expressed in HEK-293 cells.** *a*, HEK-293 cells stably expressing GPR109A-EGFP were pretreated with or without protein kinase A inhibitor KT5720 (1 μM), protein kinase C inhibitor Go6983 (10 μM), EGFR inhibitor AG1478 (1 μM), and Src inhibitor PP2 (20 μM) for 1 h prior to agonist stimulation. Cells were fixed and examined with confocal microscopy as described under "Experimental Procedures." *b*, ELISA measurement of cell surface receptors in GPR109A-expressing cells treated with protein kinase inhibitors. ELISA data are expressed as a percentage of receptors detected on surface of agonist-untreated cells expressing GPR109A. *Error bars*, S.E. for four replicates. Data were analyzed using Student's *t* test (**, $p < 0.01$; ***, $p < 0.001$). All pictures and data shown are representative of at least three independent experiments.

cellular Ca^{2+} mobilization. Moreover, agonist-induced receptor internalization was also confirmed by confocal microscopy and ELISA. In the present studies with the GPR109A-GFP chimera, we demonstrated that GPR109A receptors heterologously expressed in HEK-293 cells were rapidly internalized from the cell membrane into the cytoplasm in a dose- and time-dependent manner upon agonist challenge, which could be significantly inhibited by pretreatment with PTX. The internalized receptors were recycled back to the cell surface after removing the agonist, and recycling was not disrupted by treatment with NH_4Cl or monensin. siRNA experiments revealed that GRK2 and arrestin3 play important roles in regulating the internalization of activated GPR109A.

Receptor desensitization and trafficking are the predominant mechanisms that modulate GPCR signaling. Upon activation, GPCRs are rapidly phosphorylated by GRKs or different protein kinases, followed by arrestin binding, which results in rapid receptor internalization (39, 40). GRKs are the primary mediators of agonist- dependent GPCR phosphorylation, and seven GRKs (GRK1 to -7) exist within mammals (27). GRK2-dependent phosphorylation plays a role in the internalization of the M_2 muscarinic acetylcholine receptor (31, 41), and GRK-mediated phosphorylation promotes GPCR internalization (42). In this study, we present evidence to show that knocking down GRK2 expression with specific siRNA significantly inhibited GPR109A internalization, suggesting that GRK2 plays a major role in the phosphorylation of activated GPR109A, whereas GRK3 and GRK5 knockdown had no effect on the internalization of GPR109A. This observation is consistent with the results of overexpression of GRK2-Cter (residues 495–689) in COS-7 cells, which significantly blocked GPR109A internalization. Our results also suggest that the activation of GPR109A promotes the translocation of arrestins to the plasma membrane, where the receptor preferentially binds arrestin3 rather than arrestin2. Therefore, together with the observation that treatment with hypertonic sucrose (0.45 M) inhibited GPR109A internalization, we postulate that phosphorylated GPR109A binds arrestin3 after receptor activation, is targeted to clathrin-coated pits, and is then rapidly recycled back to the cell surface from perinuclear early endosomes after internalization (43, 44). Walters *et al.* (45) reported that niacin-induced stimulation of GPR109A led to arrestin-dependent signaling to ERK1/2 activation, and this pathway mediated the adverse side effect of cutaneous flushing but not the beneficial antilipolytic effect of niacin. Our results obtained from siRNA experiments demonstrated that knocking down GRK or arrestin expression in HEK-293 cells had no effect on ERK1/2 pathway activation. The mechanism of regulation of GPR109A-stimulated ERK1/2 signaling remains under investigation.

After internalization, receptors can be recycled to the plasma membranes, targeted to the lysosome, or degraded in the proteasome (46). To further investigate the fate of internalized GPR109A, HEK-293 cells stably expressing GPR109A-EGFP were treated with 300 μM niacin for 40 min, followed by washing and further incubation to enable internalized receptors to return to the plasma membrane (Fig. 6*a*). Treatment with cycloheximide, which inhibits protein synthesis, did not affect this recovery (Fig. 6, *a* and *b*), indicating that the recycling of GPR109A is independent of *de novo* synthesis of receptors. Interestingly, pretreatment with recycling blockers, specifically the weak base NH_4Cl and the proton ionophore monensin, did


a
Control
Niacin
CHX
CHX+Niacin
Recycling 15min
Recycling 30min
Recycling 60min
Recycling 120min
10 μ m
b
Receptor on Cell Surface (%)
100
90
80
70
60
Mock
+ 100uM NA
recycling 10min
recycling 30min
recycling 60min
c
-Niacin
+Niacin
Recycling
Control
NH4Cl
Monensin
10 μ m
d
Receptor on Cell Surface(%)
110
100
90
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70
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50
Control
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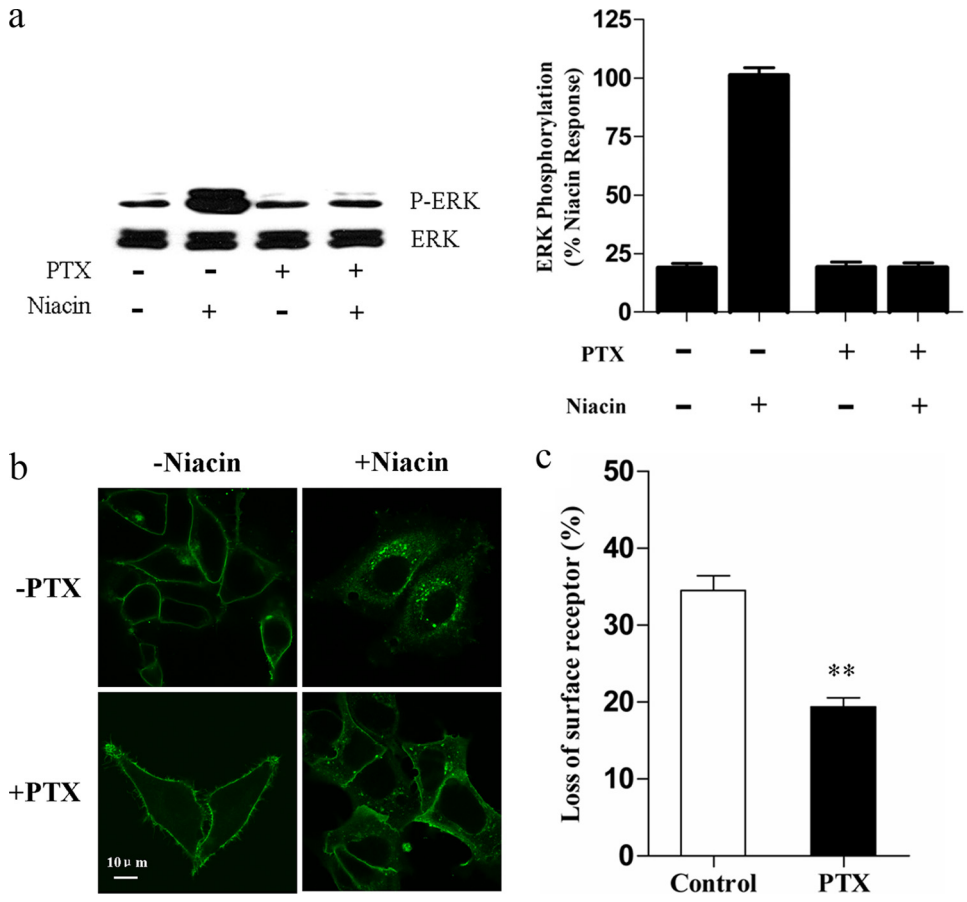

FIGURE 7. **Effects of PTX pretreatment on activation of ERK1/2 and internalization of GPR109A in HEK-293 cells.** *a*, activation of ERK1/2 in HEK293 cells pretreated with PTX (500 ng/ml) for 16 h, followed by a challenge with 300 μM niacin for 5 min was accessed by Western blot, as described under "Experimental Procedures." Signals were quantified by densitometry and expressed as a percentage of the maximal phosphorylated ERK (*P-ERK*) obtained at 5 min, and the *graphs* represent mean ± S.E. from at least four independent experiments. *b*, HEK-293 cells stably expressing GPR109A-EGFP were treated with PTX (500 ng/ml) for 16 h, followed by a challenge with 300 μM niacin for 40 min, and examined with confocal microscopy as described under "Experimental Procedures." Shown is a representative picture from five independent experiments. *c*, ELISA quantification of cell surface receptors in GPR109A-expressing cells treated with PTX. ELISA data are expressed as a percentage of receptors detected on the surface of agonist-untreated cells expressing GPR109A. *Error bars*, S.E. for four replicates. Data were analyzed using Student's *t* test (**, $p < 0.01$; ***, $p < 0.001$). All pictures and data shown are representative of at least three independent experiments.

tin2. NH_4Cl and monensin may affect receptor recycling in a number of systems (49, 53), but endosomes are likely to play a central role in controlling membrane recycling by providing an acidic but nonhydrolytic compartment in which ligands can be removed from receptors and from which membrane recycling can occur (54). Analyzing the degree of receptor association of intracellular ligand revealed that monensin prevents the dissociation of the receptor-ligand complex that normally occurs subsequent to endocytosis (53). Internalized GPR109A may rapidly recycle back to the cell surface in an NH_4Cl- and monensin-insensitive manner possibly due to the low affinity of niacin for GPR109A, resulting in dissociation from the receptor in early endosomes even under neutralizing intraluminal pH conditions. Further studies are needed, however, to elucidate the mechanism underlying the insensitivity of GPR109A recycling to NH_4Cl and monensin.

GPR109A is a $G_{i/o}$ type G protein-coupled receptor with a PTX-sensitive signaling mechanism. Pretreatment with PTX abolishes the inhibition of forskolin-induced cAMP accumulation and intracellular Ca^{2+} flux. In this study, we unexpectedly observed that PTX (500 ng/ml) pretreatment significantly abolished agonist-induced GPR109A internalization, and previous studies have shown that PTX treatment partially inhibited agonist-induced internalization of the μ-opioid receptor (55), α2-adrenoreceptor (56), and neuropeptide Y Y1 receptors (57). The G_i protein may directly or indirectly regulate GPR109A internalization. Our finding raises the question of what mechanism links G_i with receptor internalization. Normally, receptor internalization is initiated by phosphorylation of agonist-occupied receptors, mediated by the GRK family (58) or different protein kinases (39, 40). We first considered the possibility that PTX pretreatment may not affect the recycling of internalized GPR109A (Fig. 6, *c* and *d*). The role for acidification in GPCR recycling was first proposed by the finding that monensin inhibited the recovery of the internalized β-arrestin2 to the plasma membrane (47) and later was also shown for the NK1 (neurokinin 1) receptor and the CB1 (cannabinoid) receptor (48, 49). Previous investigations demonstrated that the recycling of β-arrestin2 and C5a was monensin-sensitive, whereas the recycling of β-arrestin, formyl-formyl-methionyl-leucyl-phenylalanine, and the GnRH receptor was not (50–52). Pippig *et al.* (47) reported that monensin blocked recycling but not dephosphorylation of β-arres-

FIGURE 6. **Recycling of internalized GPR109A-EGFP stably expressed in HEK-293 cells.** *a*, HEK-293 cells stably expressing GPR109A-EGFP were treated with 100 μg/ml cycloheximide and 300 μM niacin for 40 min. After thoroughly washing out the agonist, the cells were further incubated for the indicated time periods in the presence of cycloheximide. *b*, ELISA measurement of recycling of internalized GPR109A. ELISA data are expressed as a percentage of receptors detected on the surface of agonist-untreated cells expressing GPR109A. *Error bars*, S.E. for four replicates. *c*, HEK-293 cells stably expressing GPR109A-EGFP were pretreated with NH_4Cl (20 mM) or monensin (10 μM) for 2 h followed by stimulation with 300 μM niacin for 40 min. After thoroughly washing out the agonist, cells were further incubated for 2 h in the presence of cycloheximide. Cells were fixed and examined with confocal microscopy as described under "Experimental Procedures." *d*, ELISA quantification of FLAG-tagged receptors remaining on the cell surface after treatment of cells with NH_4Cl (20 mM) or monensin (10 μM). ELISA data are expressed as a percentage of receptors detected on the surface of agonist-untreated cells expressing GPR109A. *Error bars*, S.E. for four replicates. Data were analyzed using the Student's *t* test (**, $p < 0.01$; ***, $p < 0.001$). All pictures and data shown are representative of at least three independent experiments. *NA*, niacin.

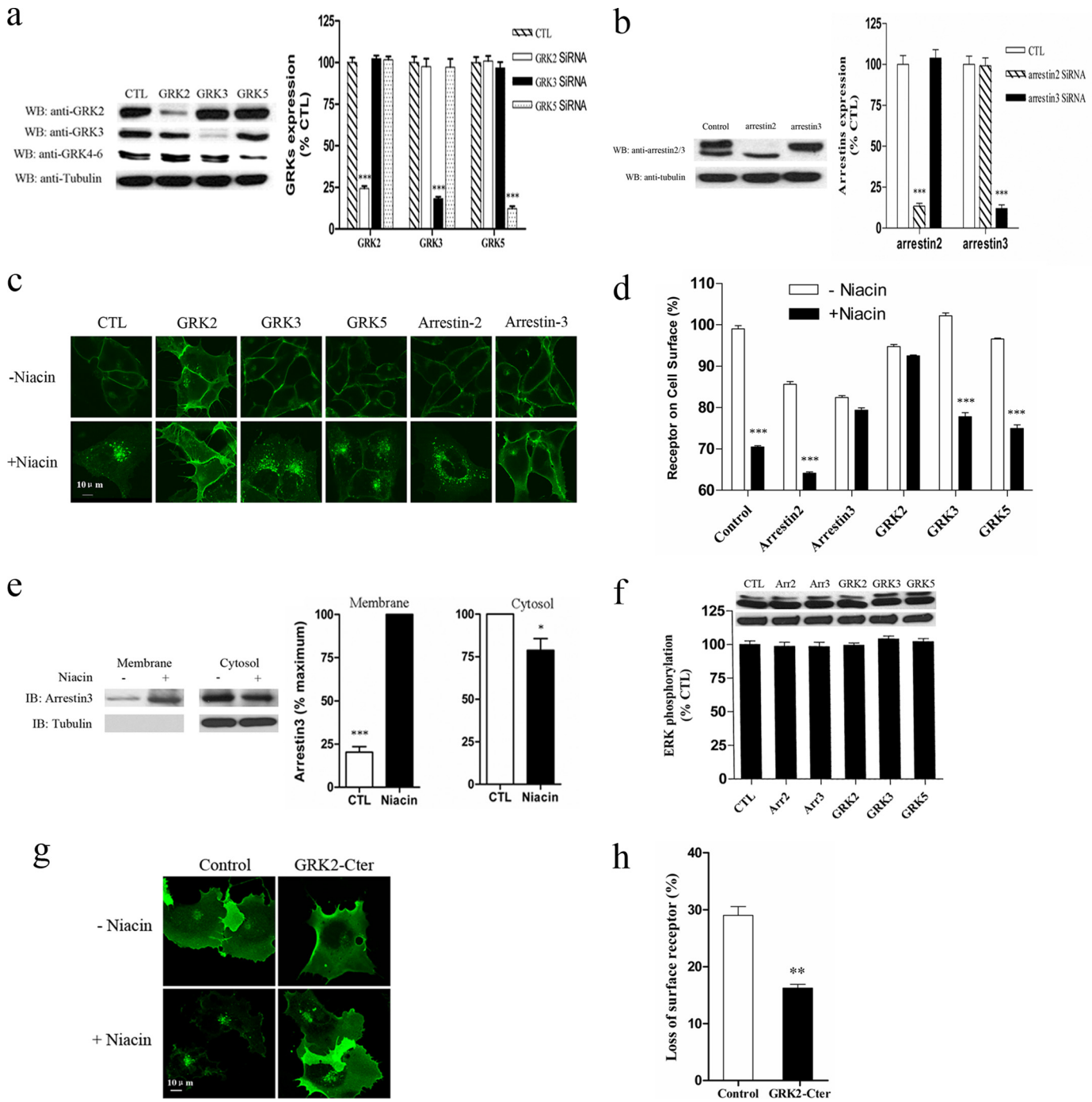

FIGURE 8. **Effect of GRK and arrestin knockdown on GPR109A internalization in HEK-293 cells.** HEK-293 cells stably expressing GPR109A-EGFP were transfected with specific GRK (*a*) or arrestin (*b*) siRNAs or nonspecific control siRNA. Seventy-two hours after transfection, cells were harvested, and 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 (*WB*) from five independent experiments. *c*, 72 h after transfection with specific GRK or arrestin siRNAs or nonspecific control siRNA, cells were stimulated with 300 μM niacin for 40 min and examined with confocal microscopy as described under "Experimental Procedures." *d*, ELISA determination of cell surface receptors in GPR109A expressing cells treated with specific GRK or arrestin siRNAs or nonspecific control siRNA. *e*, HEK-293 cells stably expressing GPR109A were incubated for 8 min with or without 100 μM niacin, and arrestin3 in the membrane and the cytosolic fractions was detected by Western blot analysis. Blots were stripped and reprobed for α-tubulin to control for loading. Shown is a representative immunoblot from four independent experiments. *f*, activation of ERK1/2 in HEK293 cells transfected with siRNA for 3 days, followed by a challenge with 300 μM niacin for 5 min, was assessed by Western blot as described under "Experimental Procedures." Signals were quantified by densitometry and expressed as a percentage of the maximal phosphorylated ERK obtained at 5 min, and the *graphs* represent mean ± S.E. from at least four independent experiments. *g*, COS-7 cells were transiently co-transfected with GPR109A-EGFP and vehicle or GRK2-Cter. Forty-eight hours after transfection, cells were stimulated with 300 μM niacin for 40 min and examined with confocal microscopy, as described under "Experimental Procedures." Shown is a representative picture from five independent experiments. *h*, ELISA analysis of cell surface receptors in GPR109A-expressing cells with overexpression of dominant negative GRK2-Cter. ELISA data are expressed as a percentage of receptors detected on surface of agonist-untreated cells expressing GPR109A. *Error bars*, S.E. for four replicates. Data were analyzed using Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Data shown are representative of at least three independent experiments.

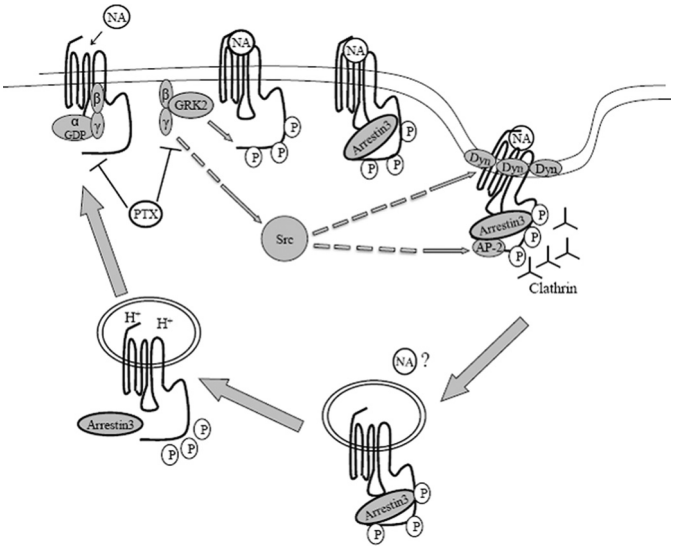

FIGURE 9. **Schematic diagram of regulation of agonist-induced GPR109A internalization and trafficking in HEK-293 cells.** Niacin (*NA*) binding to GPR109A activates the G_i family of heterotrimeric G proteins, leading to the dissociation of $G\alpha_i$ and $G\beta\gamma$. Dissociated $G\beta\gamma$ subunit recruits GRK2 to catalyze the phosphorylation of GPR109A, followed by recruitment of arrestin3 to the receptor, which results in receptor internalization. PTX treatment not only blocks activation of the G_i signaling pathway but also inhibits agonist-induced GPR109A internalization.

sibility of linking G_i to the phosphorylation of activated GPR109A via protein kinase A, but our observation that the siRNA knockdown of GRK2 completely inhibited GPR109A internalization indirectly excludes a role for protein kinase A in the regulation of GPR109A internalization. Many studies suggest that GRK2 activity also requires the binding of the $G\beta\gamma$ subunit (28, 59), and the plasma membrane translocation of these kinases is regulated by their association with the $G\beta\gamma$ subunit (29, 60). It is then more likely that PTX pretreatment abolishes GPR109A internalization by inhibiting the association of $G\beta\gamma$ subunit with GRK2. It is evident that GPR109A internalization was inhibited by the knockdown of GRK2 expression with specific siRNA in HEK-293 cells (Fig. 8, *c* and *d*) and overexpression of GRK2-Cter in COS-7 cells (Fig. 8, *g* and *h*).

Based on our results, we propose a model for the regulation of GPR109A internalization (Fig. 9). Upon activation of GPR109A by niacin, the membrane-bound $G\beta\gamma$ subunit is released from G_i, followed by recruitment of GRK2 to catalyze the phosphorylation of the activated receptors. GRK2-mediated phosphorylation promotes the binding of arrestin3, targeting receptors to clathrin-coated pits and causing their internalization. Both pretreatment with PTX and overexpression of GRK2-Cter disrupt GPR109A internalization, most likely by blocking the phosphorylation of activated receptors. In conclusion, GPR109A receptors undergo rapid internalization via clathrin-coated pits in HEK-293 cells following niacin exposure. The process of GPR109A internalization is regulated by GRK2 and arrestin3 and can be abolished by pretreatment with PTX. Internalized GPR109A is rapidly recovered back to the cell surface. Although this recovery is not affected by treatment with NH_4Cl or monensin, the exact mechanism underlying this insensitivity remains unknown.

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REFERENCES

1. Knopp, R. H. (1999) *N. Engl. J. Med.* **341,** 498–511
2. Carlson, L. A. (2005) *J. Intern. Med.* **258,** 94–114
3. Shepherd, J., Packard, C. J., Patsch, J. R., Gotto, A. M., Jr., and Taunton, O. D. (1979) *J. Clin. Invest.* **63,** 858–867
4. Blum, C. B., Levy, R. I., Eisenberg, S., Hall, M., 3rd, Goebel, R. H., and Berman, M. (1977) *J. Clin. Invest.* **60,** 795–807
5. Bajaj, M., Suraamornkul, S., Kashyap, S., Cusi, K., Mandarino, L., and DeFronzo, R. A. (2004) *J. Clin. Endocrinol. Metab.* **89,** 4649–4655
6. Vaag, A., Skött, P., Damsbo, P., Gall, M. A., Richter, E. A., and Beck-Nielsen, H. (1991) *J. Clin. Invest.* **88,** 1282–1290
7. Boden, G., Chen, X., and Iqbal, N. (1998) *Diabetes* **47,** 1609–1612
8. Santomauro, A. T., Boden, G., Silva, M. E., Rocha, D. M., Santos, R. F., Ursich, M. J., Strassmann, P. G., and Wajchenberg, B. L. (1999) *Diabetes* **48,** 1836–1841
9. Lorenzen, A., Stannek, C., Lang, H., Andrianov, V., Kalvinsh, I., and Schwabe, U. (2001) *Mol. Pharmacol.* **59,** 349–357
10. Wise, A., Foord, S. M., Fraser, N. J., Barnes, A. A., Elshourbagy, N., Eilert, M., Ignar, D. M., Murdock, P. R., Steplewski, K., Green, A., Brown, A. J., Dowell, S. J., Szekeres, P. G., Hassall, D. G., Marshall, F. H., Wilson, S., and Pike, N. B. (2003) *J. Biol. Chem.* **278,** 9869–9874
11. Soga, T., Kamohara, M., Takasaki, J., Matsumoto, S., Saito, T., Ohishi, T., Hiyama, H., Matsuo, A., Matsushime, H., and Furuichi, K. (2003) *Biochem. Biophys. Res. Commun.* **303,** 364–369
12. Tunaru, S., Kero, J., Schaub, A., Wufka, C., Blaukat, A., Pfeffer, K., and Offermanns, S. (2003) *Nat. Med.* **9,** 352–355
13. Zhang, Y., Schmidt, R. J., Foxworthy, P., Emkey, R., Oler, J. K., Large, T. H., Wang, H., Su, E. W., Mosior, M. K., Eacho, P. I., and Cao, G. (2005) *Biochem. Biophys. Res. Commun.* **334,** 729–732
14. Benyó, Z., Gille, A., Kero, J., Csiky, M., Suchánková, M. C., Nüsing, R. M., Moers, A., Pfeffer, K., and Offermanns, S. (2005) *J. Clin. Invest.* **115,** 3634–3640
15. Meyers, C. D., Liu, P., Kamanna, V. S., and Kashyap, M. L. (2007) *Atherosclerosis* **192,** 253–258
16. Kamanna, V. S., and Kashyap, M. L. (2007) *Am. J. Cardiol.* **100,** S53–S61
17. Richman, J. G., Kanemitsu-Parks, M., Gaidarov, I., Cameron, J. S., Griffin, P., Zheng, H., Guerra, N. C., Cham, L., Maciejewski-Lenoir, D., Behan, D. P., Boatman, D., Chen, R., Skinner, P., Ornelas, P., Waters, M. G., Wright, S. D., Semple, G., and Connolly, D. T. (2007) *J. Biol. Chem.* **282,** 18028–18036
18. Tsukada, T., Fink, J. S., Mandel, G., and Goodman, R. H. (1987) *J. Biol. Chem.* **262,** 8743–8747
19. Ray, A., Sassone-Corsi, P., and Sehgal, P. B. (1989) *Mol. Cell Biol.* **9,** 5537–5547
20. Fink, J. S., Verhave, M., Kasper, S., Tsukada, T., Mandel, G., and Goodman, R. H. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85,** 6662–6666
21. Iwata, K., Luo, J., Penn, R. B., and Benovic, J. L. (2005) *J. Biol. Chem.* **280,** 2197–2204
22. Luo, J., Busillo, J. M., and Benovic, J. L. (2008) *Mol. Pharmacol.* **74,** 338–347
23. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450
24. Orsini, M. J., Parent, J. L., Mundell, S. J., Marchese, A., and Benovic, J. L. (1999) *J. Biol. Chem.* **274,** 31076–31086
25. Semple, G., Skinner, P. J., Cherrier, M. C., Webb, P. J., Sage, C. R., Tamura, S. Y., Chen, R., Richman, J. G., and Connolly, D. T. (2006) *J. Med. Chem.* **49,** 1227–1230
26. Ferguson, S. S., Ménard, L., Barak, L. S., Koch, W. J., Colapietro, A. M., and Caron, M. G. (1995) *J. Biol. Chem.* **270,** 24782–24789
27. Moore, C. A., Milano, S. K., and Benovic, J. L. (2007) *Annu. Rev. Physiol.* **69,** 451–482
28. Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C.,

Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) *Science* **257,** 1264–1267
29. Carman, C. V., Barak, L. S., Chen, C., Liu-Chen, L. Y., Onorato, J. J., Kennedy, S. P., Caron, M. G., and Benovic, J. L. (2000) *J. Biol. Chem.* **275,** 10443–10452
30. Koch, W. J., Inglese, J., Stone, W. C., and Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268,** 8256–8260
31. Tsuga, H., Kameyama, K., Haga, T., Kurose, H., and Nagao, T. (1994) *J. Biol. Chem.* **269,** 32522–32527
32. Pals-Rylaarsdam, R., Xu, Y., Witt-Enderby, P., Benovic, J. L., and Hosey, M. M. (1995) *J. Biol. Chem.* **270,** 29004–29011
33. Aramori, I., Ferguson, S. S., Bieniasz, P. D., Zhang, J., Cullen, B., and Cullen, M. G. (1997) *EMBO J.* **16,** 4606–4616
34. Ménard, L., Ferguson, S. S., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G., and Barak, L. S. (1997) *Mol. Pharmacol.* **51,** 800–808
35. Barlic, J., Khandaker, M. H., Mahon, E., Andrews, J., DeVries, M. E., Mitchell, G. B., Rahimpour, R., Tan, C. M., Ferguson, S. S., and Kelvin, D. J. (1999) *J. Biol. Chem.* **274,** 16287–16294
36. Lippincott-Schwartz, J., and Smith, C. L. (1997) *Curr. Opin. Neurobiol.* **7,** 631–639
37. Daly, C. J., and McGrath, J. C. (2003) *Pharmacol. Ther.* **100,** 101–118
38. Kallal, L., and Benovic, J. L. (2000) *Trends Pharmacol. Sci.* **21,** 175–180
39. Wolfe, B. L., and Trejo, J. (2007) *Traffic* **8,** 462–470
40. Reiter, E., and Lefkowitz, R. J. (2006) *Trends Endocrinol. Metab.* **17,** 159–165
41. Moro, O., Lameh, J., and Sadée, W. (1993) *J. Biol. Chem.* **268,** 6862–6865
42. Ferguson, S. S. (2001) *Pharmacol. Rev.* **53,** 1–24
43. Cao, T. T., Mays, R. W., and von Zastrow, M. (1998) *J. Biol. Chem.* **273,** 24592–24602
44. Mundell, S. J., Luo, J., Benovic, J. L., Conley, P. B., and Poole, A. W. (2006) *Traffic* **7,** 1420–1431
45. Walters, R. W., Shukla, A. K., Kovacs, J. J., Violin, J. D., DeWire, S. M., Lam, C. M., Chen, J. R., Muehlbauer, M. J., Whalen, E. J., and Lefkowitz, R. J. (2009) *J. Clin. Invest.* **119,** 1312–1321
46. Hicke, L. (1999) *Trends Cell Biol.* **9,** 107–112
47. Pippig, S., Andexinger, S., and Lohse, M. J. (1995) *Mol. Pharmacol.* **47,** 666–676
48. Grady, E. F., Garland, A. M., Gamp, P. D., Lovett, M., Payan, D. G., and Bunnett, N. W. (1995) *Mol. Biol. Cell* **6,** 509–524
49. Hsieh, C., Brown, S., Derleth, C., and Mackie, K. (1999) *J. Neurochem.* **73,** 493–501
50. Liang, W., Curran, P. K., Hoang, Q., Moreland, R. T., and Fishman, P. H. (2004) *J. Cell Sci.* **117,** 723–734
51. Van Epps, D. E., Simpson, S. J., and Chenoweth, D. E. (1992) *J. Leukocyte Biol.* **51,** 393–399
52. Schvartz, I., and Hazum, E. (1987) *J. Biol. Chem.* **262,** 17046–17050
53. Harford, J., Wolkoff, A. W., Ashwell, G., and Klausner, R. D. (1983) *J. Cell Biol.* **96,** 1824–1828
54. Mellman, I., Plutner, H., and Ukkonen, P. (1984) *J. Cell Biol.* **98,** 1163–1169
55. Chakrabarti, S., Yang, W., Law, P. Y., and Loh, H. H. (1997) *Mol. Pharmacol.* **52,** 105–113
56. Olli-Lähdesmäki, T., Tiger, M., Vainio, M., Scheinin, M., and Kallio, J. (2004) *Biochem. Biophys. Res. Commun.* **321,** 226–233
57. Pheng, L. H., Dumont, Y., Fournier, A., Chabot, J. G., Beaudet, A., and Quirion, R. (2003) *Br. J. Pharmacol.* **139,** 695–704
58. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) *FASEB J.* **4,** 2881–2889
59. Haga, K., and Haga, T. (1992) *J. Biol. Chem.* **267,** 2222–2227
60. Boekhoff, I., Inglese, J., Schleicher, S., Koch, W. J., Lefkowitz, R. J., and Breer, H. (1994) *J. Biol. Chem.* **269,** 37–40