Distinct Kinetic and Spatial Patterns of Protein Kinase C (PKC)- and Epidermal Growth Factor Receptor (EGFR)-dependent Activation of Extracellular Signal-regulated Kinases 1 and 2 by Human Nicotinic Acid Receptor GPR109A*\(^{[S]}\)

(\(2\) To whom correspondence should be addressed: College of Agriculture, Hainan University, Haikou, Hainan 570228, China. \(2\) Present address: College of Agriculture, Hainan University, Haikou, Hainan 570228, China. Tel.: 571-88206748; Fax: 571-88206134-8000; E-mail: znm2000@yahoo.com.)

Nicotinic acid (niacin) has been widely used as a lipid-lowering drug for several decades, and recently, orphan G protein-coupled receptor GPR109A has been identified as a receptor for niacin. Mechanistic investigations have shown that, upon niacin activation, GPR109A couples to a G\(_i\) protein and inhibits adenylyl cyclase activity, leading to inhibition of liberation of free fatty acid. However, the underlying molecular mechanisms for GPR109A signaling remain largely unknown. Using CHO-K1 cells stably expressing GPR109A and A431 cells, which are a human epidermoid cell line with high levels of endogenous expression of functional GPR109A receptors, we found that activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) by niacin was rapid, peaking at 5 min, and was significantly blocked by pertussis toxin. Furthermore, time course experiments with different kinase inhibitors demonstrated that GPR109A induced ERK1/2 activation via the matrix metalloproteinase/epidermal growth factor receptor transactivation pathway at both early and later time points (2–5 min); this pathway was distinct from the PKC pathway-mediated ERK1/2 phosphorylation that occurs at early time points (\(\leq 2\) min) in response to niacin. Overexpression of G\(\beta\)\(\gamma\) subunit scavengers \(\beta\)ARK1-CT and the Go subunit of transducin led to a significant reduction of ERK1/2 phosphorylation, suggesting a critical role for \(\beta\)\(\gamma\) subunits in GPR109A-activated ERK1/2 phosphorylation. Using arrestin-2/3-specific siRNA and an internalization-deficient GPR109A mutant, we found that arrestin-2 and arrestin-3 were not involved in GPR109A-mediated ERK1/2 activation. In conclusion, our findings demonstrate that upon binding to niacin GPR109A receptors initially activate G\(_i\), leading to dissociation of the G\(\beta\)\(\gamma\) subunit from activated G\(_i\), and subsequently induce ERK1/2 activation via two distinct pathways, one PKC-dependent pathway occurring at a peak time of \(\leq 2\) min and the other matrix metalloproteinase-dependent growth factor receptor transactivation occurring at both early and later time points (2–5 min).

Nicotinic acid (niacin), a B group vitamin, has been demonstrated to be the first pharmacologic agent that is able to clinically modify plasma lipids favorably (1, 2). Previous clinical studies have revealed that niacin has the abilities to lower levels of total plasma cholesterol, free fatty acids, and triglycerides and to strongly raise high density lipoprotein cholesterol compared with other lipid-lowering drugs (3, 4). In addition, niacin has been shown to effectively reduce the progression of atherosclerosis and mortality from coronary heart disease. However, its exact mechanism was not understood until the orphan receptor GPR109A (HM74a in human and protein up-regulated in macrophages by IFN-\(\gamma\) (PUMA-G) in mice) was identified as a receptor for niacin in 2003 (5–7). Niacin-mediated activation of GPR109A 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 (6–8). Recent studies have indicated that niacin-induced flushing was also mediated by GPR109A through the release of prostaglandins D\(_{2}\) and E\(_{2}\) (9, 10). Additionally, based on the pharmacological effects of niacin, new drugs with non-flushing, low toxicity that act via GPR109A are being developed to treat dyslipidemia and prevent cardiovascular diseases (11). Our recent studies have demonstrated that the niacin-induced internalization of GPR109A receptors is regulated by G protein-coupled receptor kinase 2 and arrestin-3 in a pertussis toxin-sensitive manner and that internalized receptors are rapidly recovered back to the cell surface (12).

Almost all GPCRs signal through the mitogen-activated protein kinase (MAPK) cascades, which are traditionally associated with growth factor receptor signaling and are involved in the control of cell proliferation and growth (13), mobility (14), differentiation (15) and apoptosis (16). It is now known that many
GPCRs\textsuperscript{3} regulate MAPK cascades via distinct G\(_i\), G\(_s\), and G\(_q\)-dependent signaling pathways, leading to activation of the ERK1/2, which function as transcriptional regulators (17–19). Previous studies demonstrated that activation of GPR109A in stably transfected CHO-K1 cells by niacin or acifran evokes phosphorylation of ERK1/2 in a pertussis toxin-sensitive way (7, 20). Although in endogenous GPR109A-expressing A431 cells, a human epidermoid carcinoma cell line, only moderate promotion of activation of ERK1/2 by niacin was observed, co-treatment with bradykinin and niacin synergistically stimulated phosphorylation of ERK1/2 (21). Interestingly, a number of GPR109A pyrazole agonists were identified to be capable of fully inhibiting lipolysis in vitro and in vivo but failed to induce both a flushing response and receptor internalization (22). Moreover, a recent study has indicated that arrestin-2/3 is involved in the activation of ERK1/2 by GPR109A (23). In contrast, we found that specific small interfering RNA (siRNA)-mediated knockdown of arrestin-3 in HEK-293 cells led to an inhibition of agonist-induced internalization but not to a blockade of ERK1/2 phosphorylation (12). However, the precise mechanism of regulation of niacin-mediated ERK1/2 activation remains largely unknown. Further elucidation of ERK1/2 activation via GPR109A will be important for the development of a new generation of lipid-lowering drugs that avoid the unwanted cutaneous flushing side effect.

In the present study, we used four cellular backgrounds to characterize the mechanistic details of coupling of the human GPR109A to the ERK1/2 signaling pathway: HEK-293; CHO-K1; COS-7, which recombinantly express human GPR109A receptors; and A431 cells, a human epidermoid carcinoma cell line that endogenously expresses functional human GPR109A receptors (24). We document here, for the first time, the molecular mechanisms underlying the coupling of the human GPR109A to the ERK1/2 mitogen-activated protein kinase pathway in CHO-K1 and A431 cells and implicate the G\(_i\) protein-initiated PKC and PDGFR/EGFR transactivation-dependent pathways. Our results provide the first in-depth evidence that defines the molecular mechanism of niacin-mediated ERK1/2 activation through the human GPR109A receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lipofectamine 2000, G418, and Opti\textsuperscript{®}-MEM I reduced serum medium were purchased from Invitrogen. Cell culture media and fetal bovine serum were obtained from Hyclone (Beijing, China). The pEGFP-N1 and pCMV-FLAG vectors were purchased from Clontech and Sigma, respectively. Radioimmune precipitation assay lysis buffer was obtained from Beyotime (Haimen, China). Pertussis toxin (PTX), Go6983, GF109203X (bisindolylmaleimide), tyrphostin A9, and human recombinant EGF were purchased from Sigma. U0126, tyrphostin AG1478, GM6001, PF2, and wortmannin were from Calbiochem. Anti-phospho-ERK1/2 (Thr-202/Tyr-204) and -ERK1/2 antibodies and horseradish peroxidase-conjugated anti-rabbit IgG were from Cell Signaling Technology (Danvers, MA). Anti-GPR109A/B antibody was from Biozol (St. Louis, MO). Anti-tubulin antibody was from Beyotime. Anti-arrestin monoclonal antibody was from BD Biosciences Pharmingen.

**Cell Culture**—CHO-K1 cells were grown as monolayers in 50:50 Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 medium containing 10% (v/v) fetal bovine serum (FBS) and 2 mM glutamine. Clonal CHO-K1 lines transfected with GPR109A, GPR109B, or empty vector were grown in the above media but with the addition of G418 (400 mg/liter). COS-7 cells were transiently transfected with GPR109A, and A431 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 2 mM glutamine. HEK-293 cells stably expressing GPR109A were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 800 mg/liter G418. Plasmid constructs were transfected or co-transfected into HEK-293, CHO-K1, or COS-7 cells using Lipofectamine 2000 according to the manufacturer’s instructions. All cells were incubated at 37 °C in a humidified atmosphere with 5% CO\(_2\), 95% air.

**Molecular Cloning and Plasmid Construction**—The GPR109A cDNA and derived mutant (Δ315–328) were subcloned into pCMV-FLAG and pEGFP-N1 as reported previously (12). The truncated GPR109A mutant was obtained by overlap extension PCR. The mutant was screened for the creation of restriction enzyme sites and checked by sequencing. To generate arrestin-3-EGF, human arrestin-3 were amplified by PCR. The primers used for arrestin-3 were 5’-AAG CTT GCC ACC ATG GGG GAG AAG CTT GCC ACC ATG GGG GAG 315–328 and 5’-GTT ACC GTG CAG AAG CTT GCC ACC ATG GGG GAG AGG CGG A-3’ (forward) and 5’-GTT ACC GTG CAG AAG CTT GCC ACC ATG GGG GAG AGG CGG A-3’ (reverse). The arrestin-3 PCR product was inserted into HindIII and KpnI sites of the pEGFP-N1 vector.

**Arrestin Translocation Assay**—HEK-293 cells were transiently co-transfected with FLAG-GPR109A or FLAG-GPR109A mutant (Δ315–328) and arrestin-3-EGF, using Lipofectamine 2000 according to the manufacturer’s instructions. The following day, the cells were seeded in covered glass-bottomed 6-well plates, and after 24 h, the cells were treated with niacin for 10 min. After removal of the agonist, the cells were fixed with 3% paraformaldehyde for 15 min. Confocal images were taken on a Zeiss LSM 510 microscope with an attached Axiovert 200 microscope and an LSM5 computer system. Excitation was performed at 488 nm, and a 525 ± 25-nm bandpass filter was used for fluorescence detection. Images were collected using QED camera software and processed with Adobe Photoshop.

**Synthesis of Small Interfering RNAs and siRNA Transfection**—All siRNAs were chemically synthesized by Dharmacon RNA Technologies (Lafayette, CO). Arrestin-2 and arrestin-3 siRNAs were purchased as a SMARTpool. The GPR109A siRNA sequences were 5’-UAUGUGAGCGCGUUGGACU-3’ and 5’-UGCUCCUUAUGUGGCUAU-3’, whereas 5’-UCAAAUAAACUAUCCAGA-3’ and 5’-CGUUCGAGUUGAGCUACUA-3’ were used for GPR109B. The transfection protocol for arrestin siRNAs in HEK-293 cells was reported previously (25). 48 h after transfection, the cells were split for

\textsuperscript{3}The abbreviations used are: GPCR, G protein-coupled receptor; CRE, cAMP response element; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; PTX, pertussis toxin; G\(_i\), inhibitory GTP-binding protein of adenyl cyclase; MMP, matrix metalloproteinase; EGFP, enhanced GFP.
the indicated assay the following day. For A431 cells transfection, we followed the double hit siRNA procedure as described previously with slight modifications (26). In brief, we seeded A431 cells at a density of 200,000 cells/6-cm dish, and after 12–16 h, the first siRNA transfection was performed using Lipofectamine 2000 (Invitrogen) and Opti-MEM I (Invitrogen). 6–8 h after the first siRNA transfection, cells were split into new 6-cm dishes. Then, on Day 2, a second siRNA transfection was performed. 24 h after the second transfection, the cells were split for the indicated assay the following day.

**cAMP Accumulation**—After seeding in a 6-well plate overnight, HEK-293 cells were transiently co-transfected with wild-type GPR109A or Δ315–328 mutant and pCRE-Luc. 24 h later, the cells were split into 48-well plates. When cells reached 90–95% confluence, the cells were stimulated with 10 mM 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).

**MAPK Assay**—Cells were plated on 6-well plates, grown to 80% confluence, rinsed with serum-free DMEM or DMEM/F-12 (2/1) and incubated overnight in serum-free medium. For PTX treatment, the cells were pretreated with 100 ng/ml PTX overnight prior to the MAPK assay. Cells were preincubated with various inhibitors for 1 h before activation with the indicated ligands. Ligand incubation was ended by washing the cells with 2 ml of ice-cold PBS followed by the addition of lysis 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)/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. The supernatants underwent electrophoresis on a 10% SDS-polyacrylamide gel, which was transferred to a PVDF membrane and immunoblotted using monoclonal anti-phospho-MAPK E10 (Thr-202/Tyr-204) (Cell Signaling Technology). Blots were probed with horseradish peroxidase-labeled secondary antibodies, and chemiluminescence was detected using HRP substrate (Cell Signaling Technology). The blots were stripped and reprobed using an antitotal ERK1/2 (1:2000) monoclonal antibody as a control for protein loading. The levels of ERK 1/2 phosphorylation were normalized to total ERK1/2, and all the immunoblots were visualized and quantified using a Bio-Rad Quantity One imaging system.

**Measurement of Receptor Internalization by ELISA**—The cell surface with the GPR109A receptor was quantitatively assessed by ELISA as described previously (27). 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% CO2 overnight and then split into 24-well plates coated with poly-1-lysine. The following day, the cells were stimulated with 100 μM niacin for 1 h. The medium was aspirated, and the cells were washed once with Tris-buffered saline (TBS). After fixation for 5 min at room temperature with 3.7% formaldehyde in TBS, the cells were washed three times with TBS and then blocked for 1 h with 1% bovine serum albumin in TBS. Cells were then incubated for 1 h with a monoclonal antibody directed against the FLAG epitope (1:1000), then the cells were washed three times with TBS and incubated for 1 h with a horseradish peroxidase-labeled secondary antibody, and finally the cells were washed three times. Antibody binding was visualized by adding 0.25 ml of horseradish peroxidase substrate (Sigma). Development was stopped by transferring 0.1 ml of the substrate to a 96-well microtiter plate containing 0.1 ml of 1% SDS. The plates were read at 405 nm in a microplate reader (Bio-Rad) using Microplate Manager software.

**Data Analysis**—All results are expressed as the mean ± S.E. of n. Data were analyzed using non-linear curve fitting (GraphPad PRISM version 5.0) to obtain pEC50 values. Statistical significance was determined using Student’s t test. Probability values less than or equal to 0.05 were considered significant.

**RESULTS**

**GPR109A Activates ERK1/2 Signaling via MEK1/2 by Niacin and Acifran**—GPR109A and GPR109B have been identified to be high and low affinity receptors for niacin, respectively. Upon stimulation by niacin, GPR109A couples to Gα protein and inhibits adenylate cyclase activity. To study niacin-mediated ERK1/2 activation, we established CHO-K1 cell lines stably expressing human GPR109A or GPR109B or empty vector. As shown in Fig. 1a, niacin induced a concentration-dependent activation of ERK1/2 with an EC50 of 49 nM in CHO-GPR109A cells, whereas almost no ERK1/2 phosphorylation was observed in response to niacin in the range of 0.1–100 μM in CHO-GPR109B cells; these findings are consistent with the observation of intracellular Ca2+ mobilization by Tunaru et al. (28). ERK1/2 phosphorylation in response to niacin was undetectable in parental CHO-K1 cells expressing empty vector (Fig. 1a), suggesting a specific activation of ERK1/2 via GPR109A by niacin. Acifran has also been found to evoke effects similar to those of niacin in preliminary clinical testing (29) and to bind to both GPR109A and GPR109B (5). We therefore determined the effects of acifran in the activation of ERK1/2 in both CHO-GPR109A and CHO-GPR109B cells. As shown in Fig. 1b, in response to acifran treatment, activation of ERK1/2 signaling was detected in a dose-dependent manner with an EC50 of 178 nM for GPR109A and an EC50 of 466 nM for GPR109B. In addition, to better characterize the GPR109A-mediated ERK1/2 signaling pathway, we also used the A431 cell line, a human epidermoid cell line with high endogenous expression levels of functional GPR109A receptors (24). A431 cells were cultured in serum-free DMEM for 24 h followed by stimulation with various concentrations of niacin in fresh serum-free DMEM for 5 min, and the concentration-dependent activation of ERK1/2 signaling was detected with an EC50 of 3.43 μM (Fig. 1c).

A431 cells endogenously express GPR109A and GPR109B (24), which are high and low affinity receptors for niacin, respectively. To determine whether the ERK1/2 activation seen in response to niacin in A431 cells is likely to be mediated mainly by GPR109A, we used specific siRNAs to deplete GPR109A and GPR109B in A431. As shown in Fig. 1, d and e, the depletion of GPR109B using siRNA resulted in no alteration of niacin-induced ERK1/2 activation but did result in a significant decrease of IBC293, a GPR109B-specific agonist (30), mediated ERK1/2 activation. Vice versa, siRNA-mediated
ERK Activation Mediated by Human Nicotinic Acid Receptor

**a**

CHO-GPR109A
Empty Vector
Niacin (μM) CTL 0.01 0.03 0.1 1 3 10 100
CHO-GPR109B
Niacin (μM) CTL 0.01 0.03 0.1 1 3 10 100

**b**

CHO-GPR109A
IB: P-ERK
IB: ERK
Acifran (μM) CTL 0.01 0.03 0.1 1 3 10
CHO-GPR109B
IB: P-ERK
IB: ERK
Acifran (μM) CTL 0.01 0.03 0.1 1 3 10 30 100

**c**

A431 cell
IB: P-ERK
IB: ERK
Niacin (μM) CTL 0.01 0.03 0.1 1 3 10 30 100

**d**

siRNA CTL GPR109A GPR109B
WB: Anti-GPR109A/B GPR109B GPR109A
WB: Anti-tubulin
Receptor Expression (% CTL)
GPR109A GPR109B

**e**

siRNA CTL GPR109A GPR109B
IB: P-ERK
IB: ERK
Ligands Niacin IBC293

**f**

IB: P-ERK
IB: ERK
DMSO + + +
U0126 + + +
Niacin + +
Acifran + +

**g**

IB: P-ERK
IB: ERK
DMSO + + +
U0126 + + +
Niacin + + +
Acifran + + +
knockdown of GPR109A expression led to the significant impairment of niacin-induced ERK1/2 phosphorylation but had no inhibitory effect on IBC293-caused ERK1/2 activation in A431 cells. Taken together, these data demonstrate that niacin-induced ERK1/2 activation is mainly mediated by GPR109A, and the contribution of GPR109B to niacin-induced ERK1/2 activation in these cells is negligible.

To investigate whether or not GPR109A-induced ERK1/2 phosphorylation is mediated by MEK1/2 activation, U0126, a highly selective inhibitor of both MEK1 and MEK2, was ana-

FIGURE 1. GPR109A activates ERK1/2 signaling via MEK1/2 by niacin and acifran. CHO-K1 cells expressing either the high affinity GPR109A (closed circles) or the low affinity GPR109B niacin receptors (open circles) or control parental cells harboring neither receptor (closed squares) were cultured in serum-free DMEM/F-12 for 24 h. The next day, medium was removed, and fresh serum-free DMEM/F-12 or DMEM with or without 100 ng/ml PTX was added for 1 h, and the cells were then stimulated with 1 μM niacin or 100 μM niacin for A431 cells for the indicated time periods. c, CHO-GPR109A cells were cultured in serum-free DMEM/F-12 with or without 100 ng/ml PTX for 24 h. The next day, medium was removed, and fresh serum-free DMEM/F-12 with or without 100 ng/ml PTX was added for 1 h. The cells were then pretreated with 1 μM niacin or 3 μM acifran for 5 min. The data shown are representative of at least three independent experiments. Error bars, S.E. for three replicates. Data were analyzed using Student’s t test (***, p < 0.001). IB, immunoblot; P-ERK, phospho-ERK; NS, no stimulation.

FIGURE 2. Pertussis toxin inhibits phosphorylation of ERK1/2 induced by niacin and acifran. CHO-GPR109A cells (a) or A431 cells (b) were cultured in serum-free DMEM/F-12 or DMEM with or without 100 ng/ml PTX for 24 h. The next day, medium was removed, and fresh serum-free DMEM/F-12 or DMEM with or without 100 ng/ml PTX was added for 1 h, and the cells were then stimulated with 1 μM niacin for CHO-GPR109A cells or 100 μM niacin for A431 cells for the indicated time periods. c, CHO-GPR109A cells were cultured in serum-free DMEM/F-12 with or without 100 ng/ml PTX for 24 h. The next day, medium was removed, and fresh serum-free DMEM/F-12 with or without 100 ng/ml PTX was added for 1 h. The cells were then stimulated with 1 μM niacin or 3 μM acifran for 5 min. The data shown are representative of at least three independent experiments. Error bars, S.E. for three replicates. Data were analyzed using Student’s t test (***, p < 0.001). IB, immunoblot; P-ERK, phospho-ERK; NS, no stimulation.
Effects of PKC inhibitors on GPR109A-stimulated phosphorylation of ERK1/2.

Serum-starved CHO-GPR109A cells (a and b) or A431 cells (c and d) were pretreated with DMSO or 10 μM GF109203X (GFX; a and c) or 10 μM Go6983 (b and d) for 1 h, and the cell were stimulated with 1 μM niacin for CHO-GPR109A cells or 100 μM niacin for A431 cells for the indicated time periods. e, serum-starved CHO-GPR109A cells were pretreated with DMSO, 10 μM GF109203X, or 10 μM Go6983 for 1 h, and the cells were stimulated with 1 μM niacin or 3 μM acifran for 2 min. The data shown are representative of at least three independent experiments. Error bars, S.E. for three replicates. Data were analyzed using Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001). IB, immunoblot; P-ERK, phospho-ERK; NS, no stimulation.
ERK Activation Mediated by Human Nicotinic Acid Receptor

a

CHO-GPR109A

IB: P-ERK
IB: ERK

DMSO
Tyr A9 (1 μM)

b

CHO-GPR109A

IB: P-ERK
IB: ERK

DMSO
Tyr A9
Niacin
Acifran

A431 Cell

DMSO
GM6001
AG1478
Niacin (min) 0 2 5 10 30

DMSO
GM6001
AG1478

AG1478
GM6001
Niacin
EGF

COS-7-GPR109A

IB: P-ERK
IB: ERK

DMSO
AG1478
GM6001
Niacin
EGF

EGF

ERK Phosphorylation (% Maximal Response)
lyzed for its effect on the activation of ERK1/2. ERK1/2 activation stimulated by niacin was significantly inhibited by preincubation of CHO-GPR109A cells (Fig. 1f) or A431 cells (Fig. 1g) with the inhibitor U0126 (1 μM). Similar results were observed in acifran-mediated ERK1/2 activation in CHO-GPR109A cells (Fig. 1f), indicating that upstream MEK1/2 activation is required for GPR109A-induced ERK1/2 phosphorylation.

GPR109A Initiates ERK1/2 Activation via PTX-sensitive Gα Protein-dependent Pathway—GPR109A acts via Gα proteins to inhibit adenyl cyclase, and pretreatment with PTX completely abolishes agonist–dependent Gα activation, resulting in a loss of inhibition of forskolin-induced cAMP accumulation and suppression of intracellular Ca2+ mobilization (7, 9, 12). To investigate the involvement of Gα protein in the GPR109A-mediated activation of ERK1/2, cells were cultured in the presence or absence of 100 ng/ml PTX in serum-free DMEM/F-12 or DMEM, respectively, for 24 h followed by niacin stimulation. As illustrated in Fig. 2, a and b, GPR109A-initiated activation of ERK1/2 occurred in a time-dependent manner with a maximal activation at 5 min and with a subsequent reduction to base line by 30 min in both cell types after stimulation with niacin. Pretreatment of cells with PTX resulted in nearly complete inhibition of ERK1/2 phosphorylation compared with the addition of agonist alone (Fig. 2, a and b). Similar results were observed in acifran-mediated ERK1/2 activation in CHO-GPR109A cells (Fig. 2c). Together, these data demonstrate that GPR109A signals through the ERK1/2 pathway via a PTX-sensitive Gα protein-dependent mechanism.

Involvement of PKC in GPR109A-mediated ERK Activation—Previous studies have shown that niacin stimulation elicits a rapid increase of Ca2+ in GPR109A-expressing cells (9, 12, 31). Accordingly, we investigated whether or not PKC plays a role in niacin-stimulated ERK1/2 phosphorylation via GPR109A. As shown above, in time course studies, GPR109A-initiated activation of ERK1/2 revealed a maximal activation at 5 min and a return to base line by 30 min (Fig. 2, a and b). In preliminary experiments, we treated cells with two PKC inhibitors, GF109203X (bisindolymaleimide I) and Go6983, followed by stimulation with niacin for 5 min, and Western blot analysis showed little difference between the PKC inhibitor group and the control group. Next, CHO-GPR109A cells (Fig. 3, a and b) and A431 cells (Fig. 3, c and d) that had been pretreated with 10 μM GF109203X and 10 μM Go6983 for 1 h were stimulated by niacin. In the time course shown in Fig. 3, a, b, c, and d, treatment with both GF109203X and Go6983 resulted in dramatic decreases (>70%) in ERK activation at early time points (≤2 min), but only slight inhibition was observed at the 5-min time point. Acifran stimulation produced similar results in CHO-GPR109A cells (Fig. 3e). Collectively, these data demonstrate that PKC plays a dominant role in GPR109A-mediated ERK1/2 activation at early time points (≤2 min).

**FIGURE 4.** GPR109A-induced ERK1/2 activation is dependent on growth factor receptor transactivation. Serum-starved CHO-GPR109A cells were pre-treated with DMSO or PDGF-selective receptor tyrosine kinase inhibitor tyrphostin A9 (1 μM) for 1 h and then stimulated with 1 μM niacin for the indicated time periods (a) or with 1 μM niacin or 3 μM acifran for 5 min (b). c, serum-starved A431 cells were pretreated with DMSO, EGFR-selective receptor tyrosine kinase inhibitor tyrphostin AG1478 (100 nM), or MPP inhibitor GM6001 (10 μM) for 1 h, and the cells were then stimulated with 100 μM niacin for the indicated time periods. d and e, serum-starved A431 cells (d) and COS-7-GPR109A cells (e) were pretreated with DMSO, EGFR-selective receptor tyrosine kinase inhibitor tyrphostin AG1478 (100 nM), or MPP inhibitor GM6001 (10 μM) for 1 h, and the cells were stimulated with 100 μM niacin or 10 ng/ml EGF for 5 min. The data shown are representative of at least three independent experiments. Error bars, S.E. for three replicates. Data were analyzed using Student’s t test (**, p < 0.01; ***., p < 0.001). IB, immunoblot; P-ERK, phospho-ERK; NS, no stimulation; Tyr, tyrphostin.
and Src tyrosine kinases play important roles in GPR109A-mediated ERK activation in CHO-GPR109A cell lines.

In A431 cells, pretreatment with the PI3K inhibitor wortmannin showed a result similar to that seen in CHO-GPR109A cells (Fig. 5c). Because Src activation has been shown to stimulate GPCR-mediated MMP induction and EGFR transactivation (43), we next examined whether differential activation of Src is responsible for the distinctive signaling pathways in A431 cells. Inhibition of Src by the selective Src kinase inhibitor PP2 did not attenuate niacin-induced ERK1/2 activation in A431 cells.
ERK Activation Mediated by Human Nicotinic Acid Receptor

CHO-GPR109A

| Time(min) | pCDNA3.1 | βARK-CT | Go-transducin |
|-----------|----------|---------|---------------|
| IB: P-ERK |          |         |               |
| 0         | 2        | 5       | 10            |
| 0         | 2        | 5       | 10            |
| 0         | 2        | 5       | 10            |
| IB: ERK   |          |         |               |
| 0         | 2        | 5       | 10            |
| 0         | 2        | 5       | 10            |
| 0         | 2        | 5       | 10            |

FIGURE 6. Giβγ plays central role in GPR109A-induced ERK1/2 activation. CHO-GPR109A cells were transiently transfected with the Giβγ antagonists βARK-CT or Go-transducin, and the cells were then serum-starved for 24 h and stimulated with 1 μM niacin for the indicated time periods. The data shown are representative of at least three independent experiments. Error bars, S.E. for three replicates. Data were analyzed using Student’s t test (**, p < 0.01). IB, immunoblot; P-ERK, phospho-ERK.

cells (Fig. 5d). These results show that Src kinase is not required for niacin-induced EGFR transactivation in A431 cells.

Giβγ Plays Central Role in GPR109A-induced ERK1/2 Activation—For most G protein-coupled receptors, signaling from the activated receptor to ERK1/2 involves the Giβγ subunit of heterotrimeric G proteins. Our previous study demonstrated that, upon activation of GPR109A by niacin, the membrane-bound Giβγ subunit is released from Gβγ, followed by recruitment of G protein-coupled receptor kinase 2 to catalyze the phosphorylation of the activated receptors (12). Accordingly, we sought to define the role of the Giβγ subunit in GPR109A-induced ERK1/2 phosphorylation. We transfected CHO-GPR109A cells with the β-adrenergic receptor kinase COOH domain (amino acids 495–689) (βARK1-CT) or the Ga subunit of transducin, both of which are scavengers of Giβγ subunits (40, 44, 45). Upon transfection, a significant inhibition of GPR109A-induced ERK1/2 phosphorylation was observed (Fig. 6), suggesting that the Giβγ subunit is likely to play a central role in GPR109A-induced ERK1/2 activation.

There Is No Involvement of Arrestins in GPR109A-mediated ERK1/2 Activation—Our previous data have shown that the agonist-induced internalization of GPR109A is primarily dependent on arrestin-3. Recent studies have indicated that niacin stimulation of ERK1/2 via GPR109A requires both PTX-sensitive Gβγ, protein and arrestins (7, 23). However, the result obtained from a knockdown of arrestins using siRNA showed no inhibition of ERK1/2 phosphorylation in response to niacin in HEK-293 cells stably expressing GPR109A (Fig. 7, a and b). Moreover, we constructed a GPR109A mutant with a deletion of 14 amino acids from residues 315 to 328 (∆315–328) at the C-terminal end. This mutant functions normally in stimulation of cAMP accumulation (Fig. 7c) but lacks the ability to translocate arrestins to the cell membrane (see supplemental Fig. 1) and internalize in response to niacin (Fig. 7d). The treatment of HEK-293 cells stably expressing the ∆315–328 mutant with niacin showed a sustained activation of ERK1/2 from 10 to 30 min as compared with wild-type GPR109A (Fig. 7e).

To further confirm the role of arrestins in niacin-triggered ERK1/2 activation, we used specific siRNAs to knock down the expression of arrestin-2, arrestin-3, or both arrestin-2 and arrestin-3 in A431 cells as shown in Fig. 7, f and g. Knockdown of arrestins showed no inhibition of ERK1/2 phosphorylation in response to niacin in A431 cells. Taken together, our results indicate that arrestins play no role in GPR109A-induced ERK1/2 activation.

DISCUSSION

Niacin has been clinically demonstrated to be an antidiyslipidemic drug that is able to efficiently reduce coronary disease morbidity and mortality (1, 2) and to slow the progression of atherosclerosis (4, 46). Recent investigations have shed light on the molecular mechanism by which the human niacin receptor GPR109A leads to the inhibition of lipolysis in adipocytes but also triggers the cutaneous flushing side effect (47–49). The identification of pyrazole agonists that have the ability to fully inhibit lipolysis without causing skin flushing (50, 51) prompted a detailed mechanistic investigation into the signaling pathways downstream of GPR109A. A recent study has shown that these non-flushing agonists fail to induce receptor internalization and ERK1/2 activation (22). Moreover, niacin-activated GPR109A elicits arrestin-dependent ERK1/2 phosphorylation, resulting in skin flushing but not the beneficial antilipolytic effect of niacin (23). As a consequence, there is significant interest in understanding the precise molecular mechanisms of signaling by this receptor as it represents an important therapeutic target. However, details of the pathways linking GPR109A receptors to ERK1/2 activation remain to be further elucidated. We therefore used CHO-K1 and HEK-293 cells that were stably or transiently transfected with human GPR109A receptors and A431 cells that endogenously express functional human GPR109A receptors to characterize GPR109A-mediated ERK1/2 activation.

In the present study, the CHO-K1 cell line was selected as a cellular model system for characterizing GPR109A receptor signaling pathways because it is a commonly used cell line for investigating GPCR coupling to various signaling pathways. For better delineation of GPR109A-mediated phosphorylation of ERK1/2, we also used the A431 cell line, a human epidermoid cell line with high levels of endogenous expression of functional GPR109A receptors (24), in our current study. Although A431
ERK Activation Mediated by Human Nicotinic Acid Receptor
cells have been shown to express both GPR109A and GPR109B by quantitative real time PCR analysis (24) and there are no specific antagonists against GPR109B available to discriminate between GPR109A and GPR109B in A431 cells, previous studies have demonstrated that the amount of GPR109A mRNA is about 1.5-fold more than that of GPR109B in A431 cells, supporting the proposition that GPR109A, rather than GPR109B, mediates the major effects of niacin on lipolysis (6). Moreover, a recent study has shown that treatment of CHO-K1 cells expressing GPR109B with high concentrations of niacin (up to 1 mM) still fails to elicit Ca\(^{2+}\) mobilization (28). The present results that were derived from direct measurements of ERK1/2 phosphorylation showed that both niacin and acifran evoked ERK1/2 phosphorylation in GPR109A-expressing CHO-K1 cells with EC\(_{50}\) values of 49 and 178 nM, respectively. However, only acifran could elicit ERK1/2 activation in GPR109B-expressing CHO-K1 cells with an EC\(_{50}\) of 466 nM, and the concentration of niacin used was not enough to activate GPR109B to elicit ERK1/2 activation. In addition, in A431 cells, stimulation with different concentrations of niacin induced ERK1/2 activation with an EC\(_{50}\) of 3.43 \(\mu\)M. Together, these data suggest that the role of GPR109B in ERK1/2 activation in A431 cells that are stimulated by less than 100 \(\mu\)M niacin is likely to be negligible or nonexistent. The present study suggests that activated GPR109A signals to the ERK1/2 pathway via MMP-mediated EGFR transactivation. Additional investigations are needed to further define the molecules linking the G\(_i\) protein to the metalloproteinase cleavage of pro-heparin binding EGF.

It has been established that, as a G\(_i\)-coupled receptor, niacin-bound GPR109A inactivates adenylyl cyclase, leading to a decrease in intracellular cAMP levels. This activation is abolished by pretreatment with PTX (23). To assess whether the dominant pathway for GPR109A-mediated ERK1/2 signaling is through G protein activation, we first examined the role of the G\(_i\) protein in ERK1/2 activation. Both CHO-K1-stably expressing GPR109A and A431 cell lines showed a time-dependent activation of ERK1/2 in response to niacin, peaking at ~5 min and returning to basal levels at 30 min, but this ERK1/2 activation was completely attenuated in the presence of PTX. These results indicate that the essential involvement of a heterotrimeric G\(_i\) protein in ERK1/2 phosphorylation at an early stage is common to both CHO and A431 cells.

Our previous study demonstrated that agonist-activated GPR109A elicits a rapid increase in intracellular Ca\(^{2+}\) in a PTX-sensitive manner (12). We next evaluated the role of PKC in the regulation of GPR109A-induced ERK1/2 phosphorylation using specific inhibitors. Our data demonstrated that the GPR109A-induced ERK1/2 activation was blocked by PKC inhibitors Go6983 and GF109203X, suggesting that the PKC pathway participates in ERK1/2 activation. An interesting and important observation arising from the current study is that both PKC inhibitors Go6983 and GF109203X exhibited significant inhibitory effects on GPR109A-mediated ERK1/2 activation at 2 min in CHO-K1 and A431 cells, whereas Go6983 was found to also slightly decrease the ERK1/2 phosphorylation at 5 min. Taken together, it seems likely that, upon GPR109A activation, G\(_\beta\)\(_\gamma\) subunits released from the activated G\(_i\) may activate phospholipase C, leading to diacylglycerol-mediated increases in PKC activity. PKC can activate Raf-1 by stimulating the formation of active RasRaf-1 complexes, leading to activation of the ERK1/2 signaling cascade (52).

There is a growing body of evidence to suggest that the transactivation of growth factor receptors is another mechanism by which GPCRs mediate ERK1/2 phosphorylation (32, 38). The role of EGFR transactivation in ERK1/2 stimulation by GPCR ligands is cell-specific. COS-7 cells express the EGF receptor (53), but CHO-K1 cells express PDGF receptors (54). Therefore, experiments were conducted to evaluate the involvement of EGFR or PDGFR in ERK1/2 activation by niacin. Our results showed that, in CHO-K1 cells, GPR109A-mediated ERK1/2 activation was potently inhibited by the PDGF receptor-selective inhibitor tyrphostin A9, the Src family tyrosine kinase inhibitor P2, and the phosphatidylinositol 3-kinase inhibitor wortmannin but was not inhibited by the EGF receptor-selective AG1478. However, in A431 cells and COS-7 cells expressing GPR109A, the EGFR receptor-selective inhibitor AG1478 and the MMP inhibitor GM6001 were found to significantly impair ERK1/2 activation by niacin. These results suggest that a transactivation of growth factor receptors participates in GPR109A-mediated ERK1/2 phosphorylation but via different pathways in different cell lines. When PTX-sensitive G\(_i\) is activated by GPCR ligands, this leads to a release of the G\(_\beta\)\(_\gamma\) subunit that, in turn, activates a matrix metalloprotease that cleaves heparin-binding EGF and leads to EGFR transactivation (36). Because GPR109A-mediated ERK1/2 activation is sensitive to PTX treatment and to overexpression of the \(\beta\)-adrenergic receptor kinase dominant negative domain or to G\(\alpha\) transducin, it is probable that GPR109A activates the ERK1/2 signaling
pathway via the βγ subunit/MMP/heparin-binding EGF/EGFR cascade. A major finding of this study is that treatment with the EGF receptor-selective inhibitor AG1478 and the MMP inhibitor GM6001 resulted in significant attenuation of ERK1/2 activation at a peak time of 5 min after stimulation by GPR109A agonists in A431 cells; this is distinct from the PKC-mediated ERK1/2 activation that occurs at early time points (≤2 min). However, additional investigations will be necessary to clarify how the βγ subunit can be linked to the activation of MMP.

Numerous distinct mechanisms exist that allow activated GPCRs to signal through the ERK1/2 cascade. A plethora of evidence has accumulated that implicates arrestins as signal transducers in the mediation of ERK1/2 activation (55). However, for the thrombin receptor PAR1, the dopamine D2 receptor, and the formyl peptide receptor, arrestins have been found to play a minor role (if any) in the activation of the ERK1/2 pathway (56–59). We next sought to define the role of arrestins in GPR109A-mediated ERK1/2 activation. We first present evidence to indicate that knocking down arrestin-2 and -3 expression levels with specific siRNAs showed no effects on GPR109A-mediated ERK1/2 phosphorylation in HEK-293 cells. This result is in disagreement with the observation that niacin treatment led to arrestin-dependent activation of ERK1/2 signaling (17). We then constructed a deletion mutant that was incapable of arrestin-3 recruitment or receptor internalization to confirm that arrestin-3 is not involved in GPR109A-mediated ERK1/2 activation. To further confirm the role of arrestins in niacin-triggered ERK1/2 activation, we used specific siRNAs to knock down either arrestin-2, arrestin-3, or both arrestin-2 and arrestin-3 in A431 cells. As shown in Fig. 7, f and g, knockdown of arrests showed no inhibition of ERK1/2 phosphorylation in response to niacin in A431 cells. Our current results suggest that arrestins are unlikely to play a major role in the GPR109A-mediated phosphorylation of ERK1/2 in stably transfected HEK-293 cells and in A431 cells that endogenously express GPR109A.

In summary, the current study provides a detailed delineation of the niacin-mediated activation of ERK1/2 in CHO cells that are stably transfected with GPR109A and in A431 cells that endogenously express GPR109A. Based on our data, we propose that, upon exposure to niacin, activated GPR109A caused dissociation of G protein from βγ subunits, causing the PKC pathway to couple to ERK1/2 phosphorylation at early time points (≤2 min) and the MMP/EGFR transactivation pathway to act at both early and later time points (2–5 min). We also present evidence that βγ subunits play a critical role in GPR109A-activated ERK1/2 phosphorylation. These observations may provide new insights into the pharmacological effects of and the physiological functions modulated by GPR109A-mediated activation of ERK1/2.

Acknowledgments—We thank Aiping Shao, Ming Ding, and Hamnin Chen for technical assistance and equipment usage.

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ERK Activation Mediated by Human Nicotinic Acid Receptor

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