

RESEARCH PAPER

Activated human hydroxy-carboxylic acid receptor-3 signals to MAP kinase cascades via the PLC-dependent PKC and MMP-mediated EGFR pathways

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BACKGROUND AND PURPOSE

3-Hydroxy-octanoate, recently identified as a ligand for the orphan GPCR, HCA₃, is of particular interest given its ability to treat lipid disorders and atherosclerosis. Here we demonstrate the pathway of HCA₃-mediated activation of ERK1/2.

EXPERIMENTAL APPROACH

Using CHO-K1 cells stably expressing HCA₃ receptors and A431 cells, a human epidermoid cell line with high levels of endogenous expression of functional HCA₃ receptors, HCA₃-mediated activation of ERK1/2 was measured by Western blot.

KEY RESULTS

HCA₃-mediated activation of ERK1/2 was rapid, peaking at 5 min, and was *Pertussis* toxin sensitive. Our data, obtained by time course analyses in combination with different kinase inhibitors, demonstrated that on agonist stimulation, HCA₃ receptors evoked ERK1/2 activation via two distinct pathways, the PLC/PKC pathway at early time points (≤ 2 min) and the MMP/epidermal growth factor receptor (EGFR) transactivation pathway with a maximum response at 5 min. Furthermore, our present results also indicated that the $\beta\gamma$ -subunits of the G_i protein play a critical role in HCA₃-activated ERK1/2 phosphorylation, whereas β -arrestins and Src were not required for ERK1/2 activation.

CONCLUSIONS AND IMPLICATIONS

We have described the molecular mechanisms underlying the coupling of human HCA₃ receptors to the ERK1/2 MAP kinase pathway in CHO-K1 and A431 cells, which implicate the G_i protein-initiated, PLC/PKC- and platelet-derived growth factor receptor/EGFR transactivation-dependent pathways. These observations may provide new insights into the pharmacological effects and the physiological functions modulated by the HCA₃-mediated activation of ERK1/2.

Abbreviations

ADAM, a disintegrin and metalloproteinase; CRE, cAMP response element; EGFR, epidermal growth factor receptor; ET-18-OCH₃, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine; FIPI, 5-fluoro-2-indolyl des-chlorohalopemide; G_i, inhibitory G-protein; HCA, hydroxy-carboxylic acid; HDL, high-density lipoproteins; IBC293, 1-(1-methylethyl)-1H-benzotriazole-5-carboxylic acid; PDGFR, platelet-derived growth factor receptors; PTX, *Pertussis* toxin; siRNA, small interfering RNA

Introduction

The human hydroxy-carboxylic acid HCA₃ receptor, also known as GPR109B or HM74, was first cloned as an orphan GPCR during a search for novel leukocyte chemoattractant receptors (Nomura *et al.*, 1993; receptor nomenclature follows Alexander *et al.*, 2011). Amplification of HCA₃ from human spleen cDNA as a template resulted in the discovery of one close paralogue, termed HCA₂ also known as GPR109A or HM74a (Soga *et al.*, 2003). Recently, three research groups identified HCA₂ receptors as the high-affinity receptor for nicotinic acid, responsible for raising levels of high-density lipoproteins (HDL) and, thus treating lipid disorders including dyslipidaemia and atherosclerosis (Soga *et al.*, 2003; Tunaru *et al.*, 2003; Wise *et al.*, 2003). Although HCA₃ receptors share a high degree of similarity with HCA₂ receptors, displaying 96% identity to the HCA₂ receptors and with a 24-amino acid extension at its carboxyl terminus (Wise *et al.*, 2003; Tunaru *et al.*, 2005), they are not simply polymorphic variants or splice variants, as indicated by their tandem location on the human chromosome 12q24, together with GPR81 (Lee *et al.*, 2001). In addition, mRNA expression analyses have indicated that the tissue distributions of HCA₂ and HCA₃ receptors partially overlap and are partially distinct (Irukayama-Tomobe *et al.*, 2009).

Despite a high sequence homology with HCA₂ receptors, the affinity of nicotinic acid to HCA₃ receptors is quite low, that is, millimolar levels (Wise *et al.*, 2003; Li *et al.*, 2010). Furthermore, binding assay results have confirmed that the HCA₃ receptor does not appreciably bind nicotinic acid (Soga *et al.*, 2003; Tunaru *et al.*, 2003), which suggests that the HCA₃ receptor has little or no function as a nicotinic acid receptor in humans. Recently, three aromatic D-amino acids have been demonstrated to act as specific agonists to activate HCA₃ receptors (Irukayama-Tomobe *et al.*, 2009) and 3-hydroxylated β -oxidation intermediates, in particular, 3-hydroxy-octanoate has been identified as an endogenous ligand that decreases the activity of adenylate cyclase through the activation of Pertussis toxin (PTX)-sensitive G-proteins (Ahmed *et al.*, 2009). Although the effect of niacin on the antilipolytic activity is mediated via HCA₂ receptors, HCA₃ receptors have also been demonstrated to inhibit isoprenaline-induced lipolysis in primary human adipocytes (Semple *et al.*, 2006). Acifran (4,5-dihydro-5-methyl-4-oxo-5-phenyl-2-furancarboxylic acid), which possesses the same antilipolytic and triglyceride-lowering effects as nicotinic acid, activates both HCA₂ and HCA₃ receptors (Wise *et al.*, 2003). These data suggest that HCA₃ receptors are likely to be involved in modulating lipolysis and, hence, could represent an interesting target for the treatment of dyslipidemia (Skinner *et al.*, 2009).

Because the HCA₃ receptor is of great interest as a target for new antidyslipidemic drugs, in the present study, we aimed to characterize MAPK signalling pathways triggered by HCA₃ receptors using the model cell system CHO-K1, which recombinantly expresses human HCA₃ receptors, and A431 cells, a human epidermoid carcinoma cell line that endogenously expresses functional human HCA₃ receptors (Zhou *et al.*, 2007). We document here, for the first time, the molecular mechanisms underlying the coupling of human HCA₃ receptors to the ERK1/2 MAP kinase pathway in

CHO-K1 and A431 cells, which implicate the G_i protein-initiated, PLC/PKC- and platelet-derived growth factor receptor (PDGFR)/epidermal growth factor receptor (EGFR) transactivation-dependent pathways.

Methods

Molecular cloning and plasmid construction

HCA₂ and HCA₃ receptors were cloned as previously described (Li *et al.*, 2010).

Cell culture and transfection

CHO-K1 cells were grown as monolayers in 50:50 Dulbecco's modified Eagle's medium (DMEM) or Ham's F-12 medium containing 10% (v/v) FBS and glutamine (2 mM). Clonal CHO-K1 lines transfected with HCA₂, HCA₃ receptor or empty vector were grown in the media mentioned earlier, but with the addition of G418 (400 mg·L⁻¹). HEK-293 and A431 cells were grown in DMEM supplemented with 10% (v/v) FBS and glutamine (2 mM). Clonal HEK-293 lines transfected with HCA₃ were grown in the media mentioned earlier, but with the addition of G418 (800 mg·L⁻¹). Plasmid constructs were transfected or co-transfected into CHO-K1 and HEK-293 cells using Lipofectamine 2000 according to the manufacturer's instructions. All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂/95% air.

siRNA synthesis and transfection

siRNAs for β -arrestin1 and 2 were purchased as a SMARTpool from Dharmacon RNA Technologies (Lafayette, CO). The transfection protocol for β -arrestin1/2 siRNAs has been previously reported (Luo *et al.*, 2008; Li *et al.*, 2011). Forty-eight hours after transfection, cells were split for the indicated assay on the following day.

cAMP accumulation

After seeding in a 48-well plate overnight, stable CHO-K1 cells co-transfected with HCA₃ or HCA₂ receptors and pCRE-Luc were grown to 90–95% confluence, stimulated with 10 μ M forskolin alone or with 10 μ M forskolin and different concentrations of octanoic acid, the selective agonist 1-(1-methylethyl)-1H-benzotriazole-5-carboxylic acid (IBC293; Semple *et al.*, 2006) and acifran in DMEM without FBS, and incubated for 4 h (37°C). Luciferase activity was detected using a firefly luciferase kit (Promega, Madison, WI, USA). When required, cells were treated overnight with or without PTX (100 ng·mL⁻¹) in serum-free DMEM/F12 before the experiment.

Intracellular calcium measurement

Calcium mobilization was performed as described previously with slight modifications (Li *et al.*, 2010). The CHO-HCA₃ or CHO-K1 cells were harvested with Cell Stripper (Mediatech, Herndon, VA, USA), washed twice with PBS and resuspended to 5×10^6 cells·mL⁻¹ in Hank's balanced salt solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, 1 mM CaCl₂, 1 mM MgCl₂, 1 mg·mL⁻¹ glucose) containing 0.025% BSA. The cells were then loaded with 3 μ M Fura-2 acetoxymethyl ester

derivative (Fura-2/AM) (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C. Cells were washed once in Hank's solution, resuspended in Hank's, incubated at room temperature for 15 min, washed twice in Hank's solution, and then resuspended in Hank's at a concentration of 3×10^7 cells·mL⁻¹. These cells were then stimulated with 100 µM IBC293. Calcium flux was measured using excitation at 340 and 380 nm in a Tecan Infinite 200 pro series Microplate Reader (Tecan, Switzerland). When required, cells were treated overnight with or without PTX (100 ng·mL⁻¹) in serum-free DMEM/F12 before the experiment.

Western blot analysis

To analyse the knock-down of siRNA-targeted proteins and phosphorylation of ERK1/2, siRNA-transfected HEK-293 cells or agonist-stimulated cells in a six-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, Indianapolis, IN, USA) per 50 mL] on a rocker for 30 min (4°C). The lysates were centrifuged at 13 500 × g for 15 min (4°C). The supernatants were separated by electrophoresis (10% SDS-PAGE), transferred to a PVDF membrane, and immunoblotted using an anti-β-arrestin1/2 monoclonal antibody (BD Biosciences Pharmingen) or monoclonal anti-phospho-MAPK E10 antibody (Thr202/Tyr204) (Cell Signaling Technology). The membrane was then probed with HRP-labelled secondary antibodies, and chemiluminescence was detected using a HRP substrate (Cell Signaling Technology). The blots were stripped and reprobed using an anti-tubulin (1:7500) monoclonal antibody as a control for protein loading and anti-total ERK1/2 (1:2000) as a control for p-ERK1/2.

Measurement of receptor internalization by confocal imaging

HEK-293 cells stably expressing HCA₃-EGFP were transiently transfected with specific β-arrestin siRNA or a non-specific control siRNA. After transfection (72 h), cells were stimulated with 100 µM IBC293 for 40 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 LSM5 computer system. Excitation was performed at 488 nm, and fluorescence detection was performed using a 525 ± 25 nm bandpass filter. Images were collected using QED camera software and processed with Adobe Photoshop.

Measurement of receptor internalization by ELISA

HCA₃ receptors on the cell surface were quantitatively assessed by ELISA as previously described (Li *et al.*, 2010). Briefly, HEK-293 cells stably expressing Flag-HCA₃ were transiently transfected with specific β-arrestin siRNA or a non-specific control siRNA. After transfection (72 h), cells were stimulated with 100 µM IBC293 for 1 h, the medium was aspirated, and the cells were washed once with Tris-buffered saline (TBS). After fixing 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% BSA/TBS. Cells were then incubated for 1 h with a monoclonal antibody

directed against the Flag epitope (1:2000). The cells were then washed three times with TBS and incubated for 1 h with a HRP-labelled secondary antibody. Finally, the cells were washed three times, and antibody binding was visualized by adding 0.2 mL of HRP 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, Hercules, CA, USA) using Microplate Manager software.

Data analysis

All results are expressed as mean ± SEM from *n* assays. Data was analysed 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.

Materials

Lipofectamine 2000 and G418 were purchased from Invitrogen (Carlsbad, CA, USA). Cell culture media and fetal bovine serum (FBS) were obtained from Hyclone (Beijing, China). The pEGFP-N1 and pCMV-Flag vectors were purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA) and Sigma (St. Louis, MO, USA) respectively. IBC293, acifran and 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) were purchased from Tocris (Ellisville, MO, USA). RIPA lysis buffer and EGTA were obtained from Beyotime (Haimen, China). PTX, Go6983, GF109203X (GFX, bisindolymaleimide), tyrphostin A9, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ET-18-OCH₃), BAPTA-AM, octanoic acid, human recombinant EGF and anti-flag M2 monoclonal antibody were purchased from Sigma. U0126, tyrphostin AG1478, GM6001, PP2 and wortmannin were from Calbiochem (La Jolla, CA, USA). Anti-phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴) and ERK1/2 antibodies and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were from Cell Signaling Technology (Danvers, MA, USA). Anti-β-arrestin1/2 monoclonal antibody was from BD Biosciences Pharmingen (San Diego, CA, USA).

Results

Functional expression of HCA₃ in CHO-K1 cells

To investigate the HCA₃-mediated activation of ERK1/2, we cloned human HCA₃ receptors (GenBank Accession No.D10923) from human genomic DNA as a template using PCR and created CHO-K1 cell lines that stably expressed human HCA₃ receptors. We first examined the functional signalling of HCA₃ receptors by assaying cAMP accumulation. As shown in Figure 1A, treatment with the endogenous ligand octanoic acid (Ahmed *et al.*, 2009), the highly selective agonist IBC293 (Semple *et al.*, 2006) and the non-selective agonist acifran, which binds to both HCA₂ and HCA₃ receptors (Mahboubi *et al.*, 2006), induced a ligand concentration-dependent inhibition of forskolin-stimulated cAMP increase with EC₅₀ values of 1.23 µM, 54 nM and 515 nM, respectively, whereas almost no inhibition of the forskolin-stimulated cAMP increase was observed in response to niacin in the range of 0.001–100 µM. The agonist-induced inhibition of

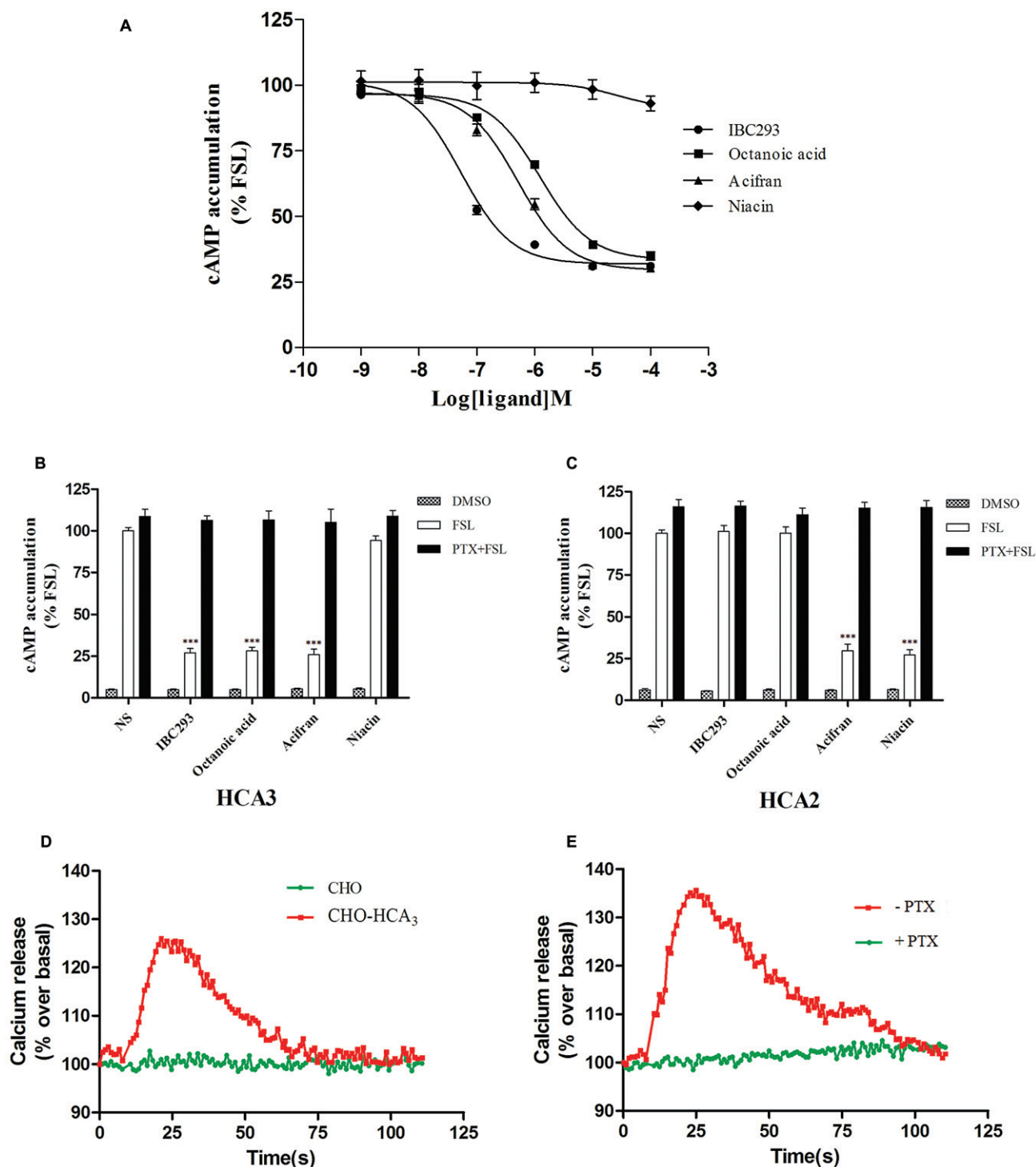


Figure 1

Characterization of HCA₃ receptors stably expressed in CHO-K1 cells. A, cAMP accumulation in CHO-K1 cells stably co-transfecting with HCA₃ and pCRE-Luc was determined in response to forskolin (FSL) and indicated ligand. Dose-dependent inhibition of forskolin-induced cAMP accumulation was measured. B and C, CHO-K1 cells stably expressing HCA₃ (B) or HCA₂ receptors (C) and pCRE-Luc were pretreated with or without 100 ng·mL⁻¹ PTX for 12 h, then stimulated with 10 µM forskolin alone or 10 µM forskolin with 100 µM octanoic acid or IBC293 or acifran or niacin in DMEM/F12 without FBS and incubated for 4 h at 37°C. Luciferase activity was detected by a firefly luciferase kit. (D) CHO-HCA₃ or CHO-K1 cells were loaded with the calcium probe Fura-2/AM followed by stimulation with 100 µM IBC293. (E) CHO-HCA₃ cells were loaded with the calcium probe Fura-2/AM followed by stimulation with 100 µM IBC293 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. Data were analysed by Student's *t*-test. ****P* < 0.001.

the forskolin-stimulated cAMP increase could be completely blocked by pretreating with 100 ng·mL⁻¹ of PTX for 12 h (Figure 1B and C). Additionally, octanoic acid (100 µM) or IBC293 (100 µM) showed no inhibitory effect on the forskolin-stimulated cAMP increase in stably HCA₂-transfected CHO cells (Figure 1C). In addition, stimulation with IBC293 (100 µM) elicited a rapid and transient increase in intracellular Ca²⁺ in CHO-K1 cells expressing HCA₃ receptors (Figure 1D) and the Ca²⁺ mobilization could be completely blocked by pretreating with 100 ng·mL⁻¹ PTX for 12 h (Figure 1E). These results suggest that HCA₃ receptors in stably transfected CHO-K1 cells are functional, and octanoic acid and IBC293 are specific ligands for HCA₃ receptors.

HCA₃ receptors activate ERK1/2 signalling via MEK 1/2 following exposure to octanoic acid, IBC293 and acifran

In CHO-HCA₃ cells, stimulation with different concentrations of agonists – octanoic acid, IBC293 and acifran – evoked ERK1/2 phosphorylation in a dose-dependent manner with EC₅₀ values of 1.52 µM, 55 nM and 470 nM, respectively (Figure 2A), whereas almost no ERK1/2 activation was observed in response to octanoic acid or IBC293 in the range of 0.1–1000 µM in CHO-HCA₂ cells (data not shown), which is consistent with the observation of intracellular cAMP accumulation with no detectable activity up to 1 mM at HCA₂ receptors (Semple *et al.*, 2006). In addition, to better characterize the HCA₃-mediated ERK1/2 signalling pathway, we also used the A431 cell line, a human epidermoid cell line with a high level of endogenous expression of functional HCA₃ receptors (Zhou *et al.*, 2007). A431 cells were cultured in serum-free DMEM media for 24 h followed by stimulation with various concentrations of IBC293 in fresh serum-free DMEM for 5 min. There was concentration-dependent activation of ERK1/2 signalling, with an EC₅₀ of 14.9 µM (Figure 2B). The HCA₃-initiated activation of ERK1/2 was time-dependent with a maximal activation at 5 min and with a subsequent reduction to baseline by 30 min in CHO-HCA₃ cells (Figure 2C). A similar result was observed during IBC293-mediated ERK1/2 activation in A431 cells (Figure 2D).

To investigate whether or not HCA₃-induced ERK1/2 phosphorylation is mediated by activation of other signalling kinases such as MEK1/2, the inhibitor U0126, a highly selective inhibitor of both MEK1 and MEK2, was used. ERK1/2 activation stimulated by octanoic acid, IBC293 and acifran were significantly inhibited by preincubation of CHO-HCA₃ cells (Figure 2E) with U0126 (1 µM). A similar result was observed for IBC293-mediated ERK1/2 activation in A431 cells (Figure 2F), which indicated that upstream MEK1/2 activation is required for HCA₃-induced ERK1/2 phosphorylation.

HCA₃ receptors initiate ERK1/2 activation via the PTX-sensitive G_i protein-dependent pathway

Previous studies have demonstrated that HCA₃ receptors act via G_i proteins to inhibit adenylyl cyclase (Semple *et al.*, 2006; Skinner *et al.*, 2007). To assess the role of the G_i protein in the regulation of HCA₃-mediated activation of

ERK1/2, CHO-HCA₃ and A431 cells were cultured in the presence or absence of 100 ng·mL⁻¹ PTX in serum-free DMEM/F-12 or DMEM, respectively, for 24 h, followed by stimulation with the indicated ligand. As illustrated in Figure 3A and C, the pretreatment of cells with PTX resulted in a nearly complete inhibition of ERK1/2 phosphorylation compared with the agonist alone in both cell lines. A similar result was observed for octanoic acid or acifran-mediated ERK1/2 activation in CHO-HCA₃ cells (Figure 3B). Together, these data demonstrate that HCA₃ receptors signal to the ERK1/2 pathway via a PTX-sensitive G_i protein-dependent mechanism.

Involvement of the PLC/PKC pathway in HCA₃-mediated ERK1/2 activation

Next, we investigated whether or not PKC plays a role in agonist-stimulated ERK1/2 phosphorylation via HCA₃ receptors. As shown earlier, in time-course studies, the HCA₃-initiated activation of ERK1/2 revealed a maximal activation at 5 min and a return to baseline by 30 min (Figure 2C and D). The CHO-HCA₃ (Figure 4A) and A431 cells (Figure 4B) were pretreated with 10 µM of GFX or 10 µM of Go6983 for 1 h, followed by the agonist IBC293 in a time course. As shown in Figure 4A and B, both treatment with GFX and Go6983 resulted in dramatic decreases (>60%) in ERK1/2 activation at an early time point (≤2 min), but very little inhibition was observed at the 5 min time point. Collectively, these data demonstrate that PKC plays a determinant role in HCA₃-mediated ERK1/2 activation at early time points (≤2 min).

The family of PLCs classically catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). DAG can directly activate classical types of PKC by interacting with its lipid-binding domain, and IP₃ can indirectly activate PKC by increasing intracellular Ca²⁺, which interacts with the PKC Ca²⁺-binding domain (Chung *et al.*, 1997). Therefore, we tested the potential involvement of PLC in the activation of ERK1/2 using the PLC inhibitors U73122 and ET-18-OCH₃. Pretreatment of cells with U73122 (20 µM) led to no inhibition of HCA₃-stimulated ERK1/2 activation in both CHO-HCA₃ and A431 cells (Figure 4C and D), whereas ET-18-OCH₃ significantly blocked HCA₃-mediated ERK1/2 phosphorylation in both cell lines (Figure 4C and D). Next, we examined the role of phospholipase D (PLD) in HCA₃-stimulated ERK1/2 phosphorylation using the PLD inhibitor FIPI. As shown in Figure 4C and D, preincubation with FIPI also did not inhibit ERK1/2 activation in both CHO-HCA₃ and A431 cells. These data suggested that PLC rather than PLD played a key role in HCA₃-mediated ERK1/2 phosphorylation.

Previous studies have shown that octanoic acid causes a rapid increase of intracellular Ca²⁺ in CHO-K1 cells expressing HCA₃ receptors (Ahmed *et al.*, 2009). Accordingly, we investigated whether or not intracellular and extracellular Ca²⁺ is involved in HCA₃-stimulated ERK1/2 phosphorylation. Pretreatment with the extracellular Ca²⁺ chelator EGTA (5 mM) or L-type Ca²⁺ channel blocker nifedipine (10 µM) significantly inhibited ERK1/2 phosphorylation in CHO-HCA₃ cells (Figure 4E). However, these two inhibitors showed no

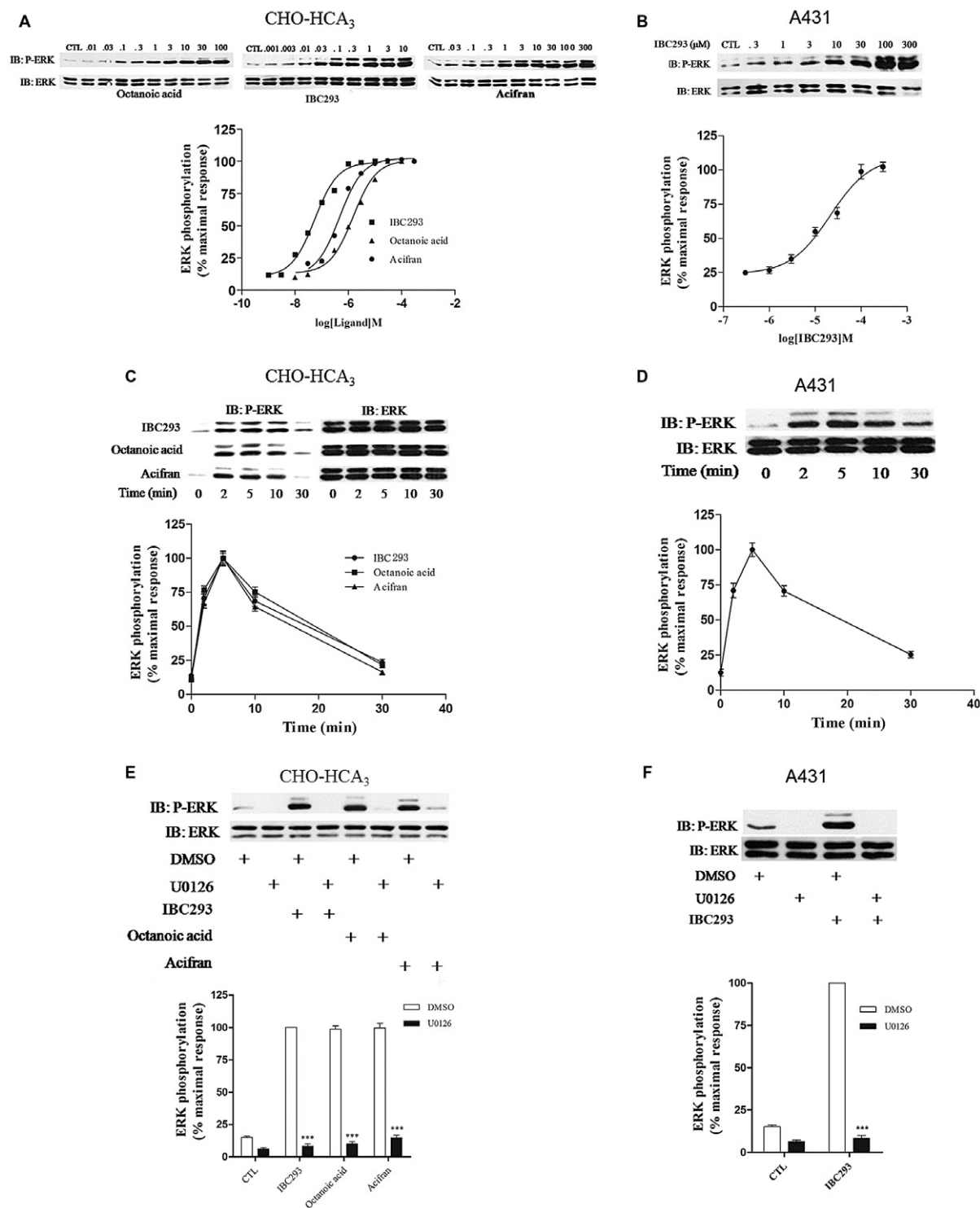


Figure 2

HCA₃ receptors activate ERK1/2 signalling via MEK1/2 pathway. (A) Serum-starved CHO-HCA₃ cells were stimulated with various concentrations of octanoic acid or IBC293 or acifran for 5 min. (B) Serum-starved A431 cells were stimulated with various concentrations of IBC293 for 5 min. (C) Serum-starved CHO-HCA₃ cells were stimulated with 1 μM IBC293 or 10 μM octanoic acid or 3 μM acifran for indicated time. (D) Serum-starved A431 cells were stimulated with 100 μM IBC293 for indicated time. (E) Serum-starved CHO-HCA₃ cells were cultured in serum-free DMEM/F12 media with or without MEK inhibitor U0126 (1 μM) for 1 h, cells were then stimulated with 1 μM IBC293 or 10 μM octanoic acid or 3 μM acifran for 5 min. (F) Serum-starved A431 cells were cultured in serum-free DMEM media with or without MEK inhibitor U0126 (1 μM) for 1 h, cells were then stimulated with 100 μM IBC293 for 5 min. The data shown are representative of at least three independent experiments. Data were analysed by Student's *t*-test. ****P* < 0.001.

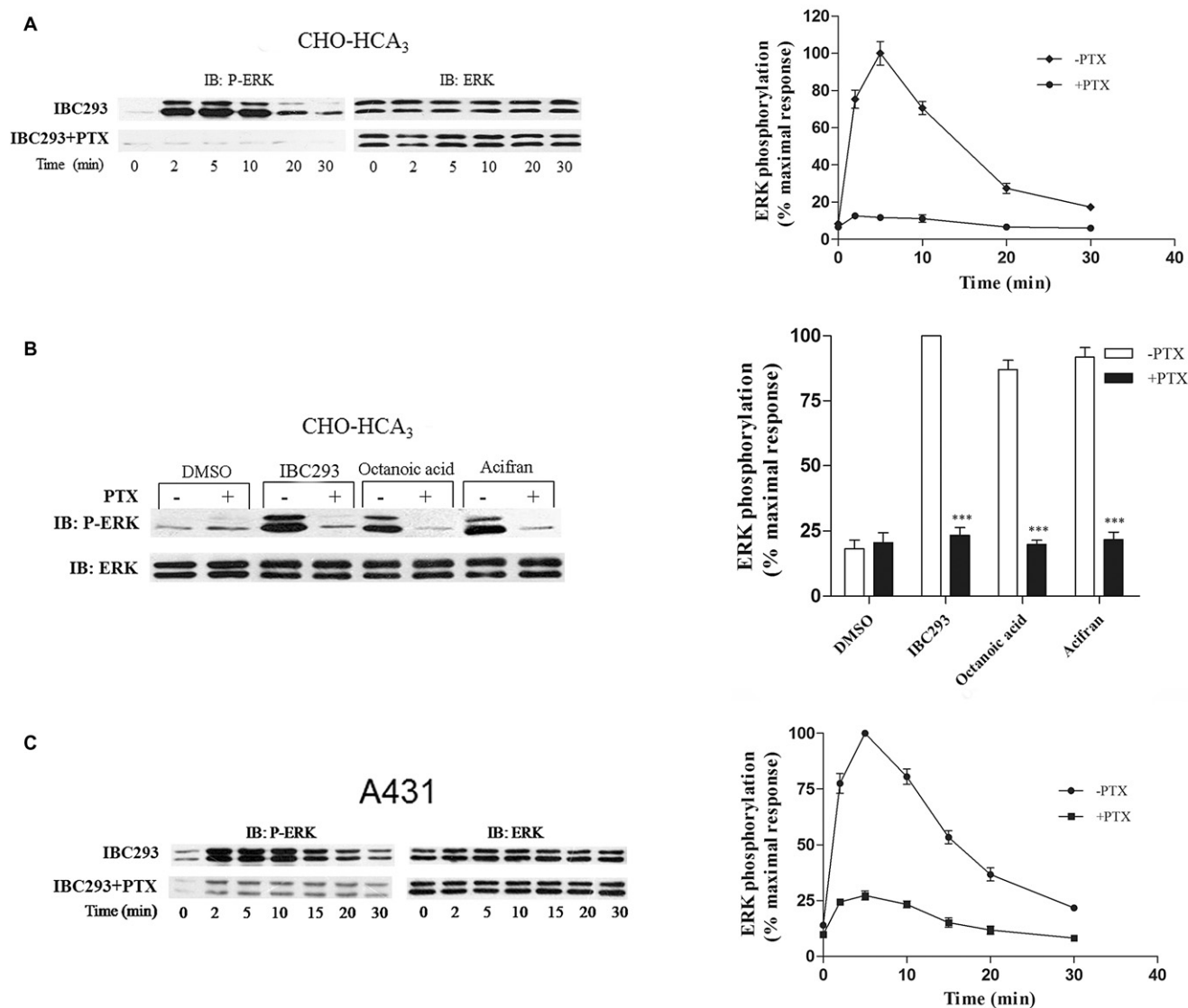
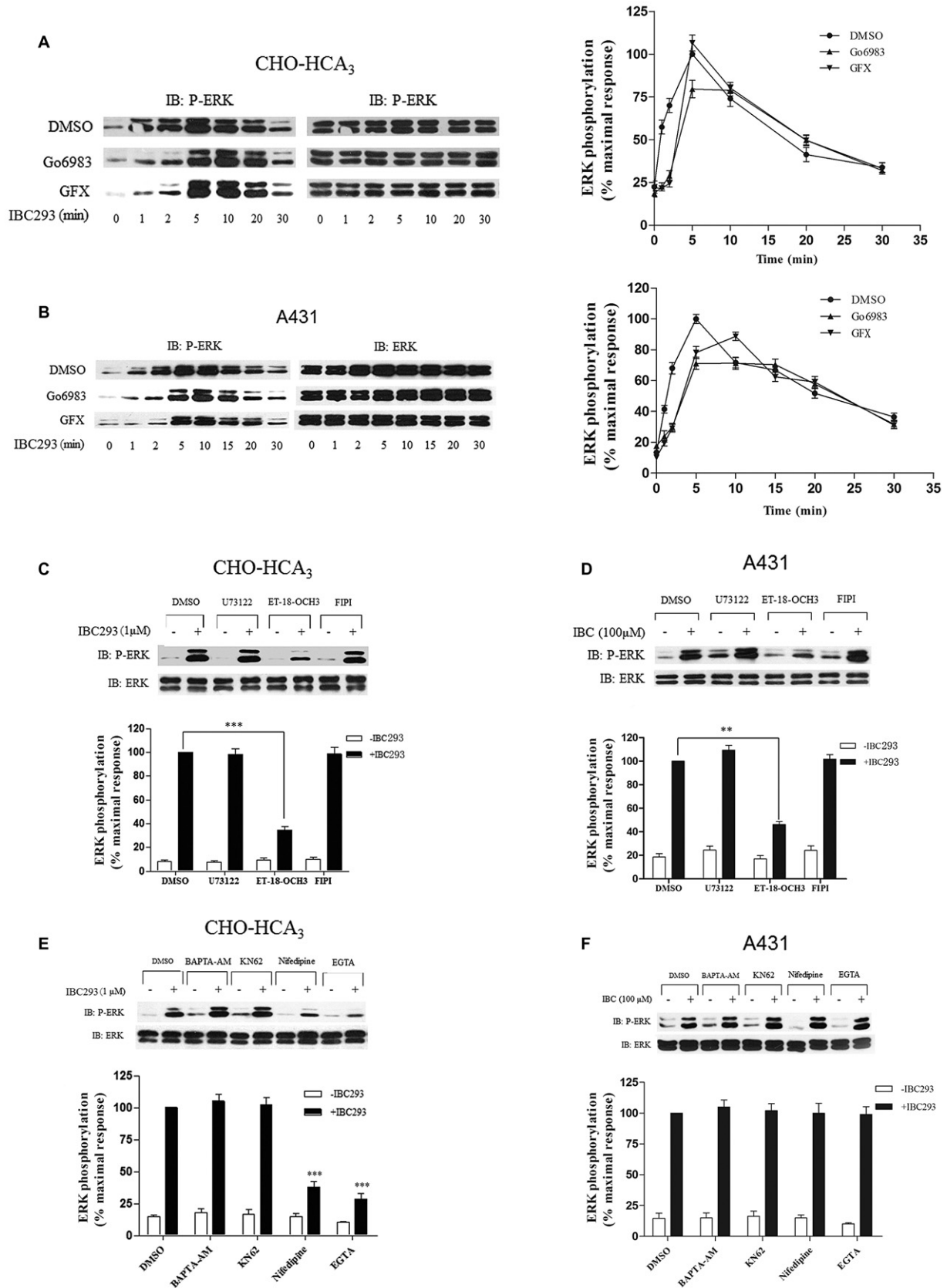


Figure 3

HCA₃ receptors initiate ERK1/2 activation via PTX-sensitive G_i protein-dependent pathway. CHO-HCA₃ cells (A) or A431 cells (C) were cultured in serum-free DMEM/F12 or DMEM media with or without 100 ng·mL⁻¹ PTX for 24 h. The next day, media was removed and fresh serum-free DMEM/F12 or DMEM media with or without 100 ng·mL⁻¹ PTX were added for 1 h, cells were then stimulated with 1 μM IBC293 for CHO-HCA₃ or 100 μM IBC293 for A431 cells for the indicated time periods. (B) CHO-HCA₃ cells were cultured in serum-free DMEM/F12 media with or without 100 ng·mL⁻¹ PTX for 24 h. The next day, media was removed and fresh serum-free DMEM/F12 media with or without 100 ng·mL⁻¹ PTX were added for 1 h, cells were then stimulated with 1 μM IBC293 or 10 μM octanoic acid or 3 μM acifran for 5 min. The data shown are representative of at least three independent experiments. Data were analysed by Student's *t*-test. ****P* < 0.001.

Figure 4

Effects of PKC, calcium and PLC on HCA₃-stimulated phosphorylation of ERK1/2. Serum-starved CHO-HCA₃ cells (A) or A431 cells (B) were pretreated with DMSO or 10 μM Go6983 or 10 μM GFX for 1 h, and then stimulated with 1 μM IBC293 for CHO-HCA₃ cells or 100 μM IBC293 for A431 cells for the indicated time periods. Serum-starved CHO-HCA₃ cells (C) or A431 cells (D) were pretreated with DMSO or 20 μM U73122 or 1 μM FIPI or 100 μM ET-18-OCH₃ for 1 h, and then stimulated with 1 μM IBC293 for CHO-HCA₃ cells or 100 μM IBC293 for A431 cells for 2 min. Serum-starved CHO-HCA₃ cells (E) or A431 cells (F) were cultured in serum-free DMEM/F12 or DMEM media with or without EGTA (5 mM) or nifedipine (10 μM) or BAPTA-AM(50 μM) or KN62 (1 μM) for 1 h, cells were then stimulated with 1 μM IBC293 for CHO-HCA₃ or 100 μM IBC293 for A431 cells for 2 min. The data shown are representative of at least three independent experiments. Data were analysed by Student's *t*-test. ****P* < 0.001.



inhibitory effect on ERK1/2 activation by HCA₃ receptors in A431 cells (Figure 4F). The intracellular Ca²⁺ chelators BAPTA-AM (100 µM) and Ca²⁺-calmodulin kinase inhibitor KN62 (10 µM) also did not impair ERK1/2 activation by HCA₃ receptors in both CHO-HCA₃ and A431 cells (Figure 4E and F). Taken together, the results of the present study indicate that stimulation of HCA₃ receptors by agonists leads to ERK1/2 activation at early time points (≤2 min) via the PKC pathway, consistent with the observation of angiotensin II receptor (Ahn *et al.*, 2004a) and AT_{1A} receptors (Kim *et al.*, 2009).

HCA₃-induced ERK1/2 activation is dependent on a growth factor receptor-involved transactivation mechanism

It is well known that the transactivation of a growth factor receptor participates in GPCR-mediated ERK1/2 phosphorylation (Pierce *et al.*, 2001). CHO-K1 cells are known to endogenously express PDGFR-β (Duckworth and Cantley, 1997) and lack endogenous EGFR (Shi *et al.*, 2000). Serum-starved CHO-HCA₃ cells were preincubated with the PDGFR-selective receptor tyrosine kinase inhibitor tyrphostin A9 (10 µM) for 1 h, followed by stimulating with 1 µM IBC293 for different periods of time. As shown in Figure 5A, in the A9 pretreated cells, there was a > 60% inhibition of ERK1/2 phosphorylation compared with agonist alone; however, there was no such effect in the A431 cells (data not shown), which suggests that PDGFR transactivation is involved in HCA₃-induced ERK1/2 activation in CHO-K1 cells but not in A431 cells.

To assess the role of EGFR transactivation in agonist-induced ERK1/2 activation in cells endogenously expressing HCA₃ receptors, A431 cells were utilized for further investigations. Serum-starved A431 cells were treated with AG1478, an EGFR-specific tyrosine kinase inhibitor, for 1 h before exposing them to IBC293. As shown in Figure 5B and C, AG1478 (100 nM) dramatically inhibited (>70%) IBC293-induced ERK1/2 phosphorylation. Several studies have shown that the transactivation of EGFR is sensitive to MMP inhibitors (Prenzel *et al.*, 1999; Gschwind *et al.*, 2001; Pierce *et al.*, 2001). To define the mechanism underlying the IBC293-induced transactivation of EGFR, A431 cells were treated with the MMP inhibitor GM6001 (10 µM) for 1 h before exposing them to IBC293 or EGF. GM6001 treatment led to a significant reduction (>50%) of ERK1/2 activation induced by IBC293 but not by EGF (Figure 5B and C). These results demonstrate that HCA₃ receptors evoke ERK1/2 activation via the PDGFR transactivation pathway in

CHO-HCA₃ cells and the EGFR transactivation pathway via the metalloproteinase-dependent shedding of HB-EGF in A431 cells. Simultaneous inhibition of PLC and PDGFR in CHO-HCA₃ cells and simultaneous inhibition of PLC and EGFR in A431 cells resulted in a nearly complete inhibition of ERK1/2 phosphorylation (see Supporting Information Figure S1), suggesting the involvement of PLC/PKC and MMP/EGFR or PDGFR in the HCA₃-mediated ERK1/2 activation.

Involvement of the PI3K pathway in HCA₃-mediated ERK1/2 activation

Previous studies have reported that PI3K and Src are involved in ERK1/2 activation in response to G_i-coupled receptors (Hawes *et al.*, 1996; Kranenburg *et al.*, 1997; Lopez-Illasaca *et al.*, 1997; Luttrell *et al.*, 1999; Ptasznik and Gewirtz, 2000). Pretreatment with the PI3K inhibitor wortmannin resulted in decreased IBC293-stimulated ERK1/2 phosphorylation in both CHO-HCA₃ and A431 cells (Figure 6A and B), which suggests that PI3K plays an important role in HCA₃-mediated ERK1/2 activation. Src activation has been shown to stimulate GPCR-mediated MMP induction and EGFR transactivation (Shah *et al.*, 2003). Therefore, we next examined the role of Src in HCA₃-mediated ERK1/2 activation. As shown in Figure 6C and D, Src inhibition by the selective Src kinase inhibitor PP2 did not attenuate IBC293-induced ERK1/2 activation in both CHO-HCA₃ and A431 cells. However, pretreatment with PP2 significantly decreased niacin-mediated ERK1/2 activation in CHO-HCA₂ cells. These results indicate that PI3K played an important role in HCA₃-mediated ERK1/2 activation, whereas Src kinase was not required for IBC293-induced EGFR transactivation in A431 cells.

To determine whether PKC and PI3K act upstream or downstream of the EGFR, we carried out experiments to examine the effect of PKC and PI3K inhibitors on IBC293-induced EGFR phosphorylation. As shown in Supporting Information Figure S2A, in A431 cells pretreated with specific inhibitors of PKC and PI3K, IBC293-stimulated EGFR phosphorylation was significantly reduced. Next, we examined the effect of PKC and PI3K on EGF-induced ERK1/2 activation. As shown in Supporting Information Figure S2B and C, in A431 cells pretreated with PKC and PI3K specific inhibitors, EGF stimulated ERK1/2 activation was also significantly reduced. These data indicate that PKC and PI3K are more likely to act downstream of the EGFR, but we cannot rule out the possibility that PKC and PI3K may also act upstream of the EGFR.

Figure 5

HCA₃-induced ERK1/2 activation is dependent on growth factor receptor transactivation. (A) Serum-starved CHO-HCA₃ cells were pretreated with DMSO or PDGFR selective receptor tyrosine kinase inhibitor tyrphostin A9 (10 µM) for 1 h, and then stimulated with 1 µM IBC293 for the indicated time periods. (B) Serum-starved A431 cells were pretreated with DMSO or EGFR selective receptor tyrosine kinase inhibitor tyrphostin AG1478 (1 µM) or MMP inhibitor GM6001 (20 µM) for 1 h, and then stimulated with 100 µM IBC293 for the indicated time periods. (C) Serum-starved A431 cells were pretreated with DMSO or AG1478 (1 µM) or GM6001 (20 µM) for 1 h, and then stimulated with 100 µM IBC293 or 10 ng·mL⁻¹ EGF for 5 min. (D) Serum-starved A431 cells were stimulated with 100 µM IBC293 for the indicated time periods. (E) Serum-starved A431 cells were pretreated with DMSO or AG1478 (1 µM) or GM6001 (20 µM) for 1 h, and then stimulated with 100 µM IBC293 for 5 min, cells were harvested, and equal amounts of total cellular lysate were separated by 10% SDS-PAGE, transferred to a PVDF membrane, and incubated with anti-p-EGFR (Tyr¹¹⁷³) antibody. Blots were stripped and reprobbed for tubulin to control for loading. The data shown are representative of at least three independent experiments. Data were analysed by Student's *t*-test. ****P* < 0.001.

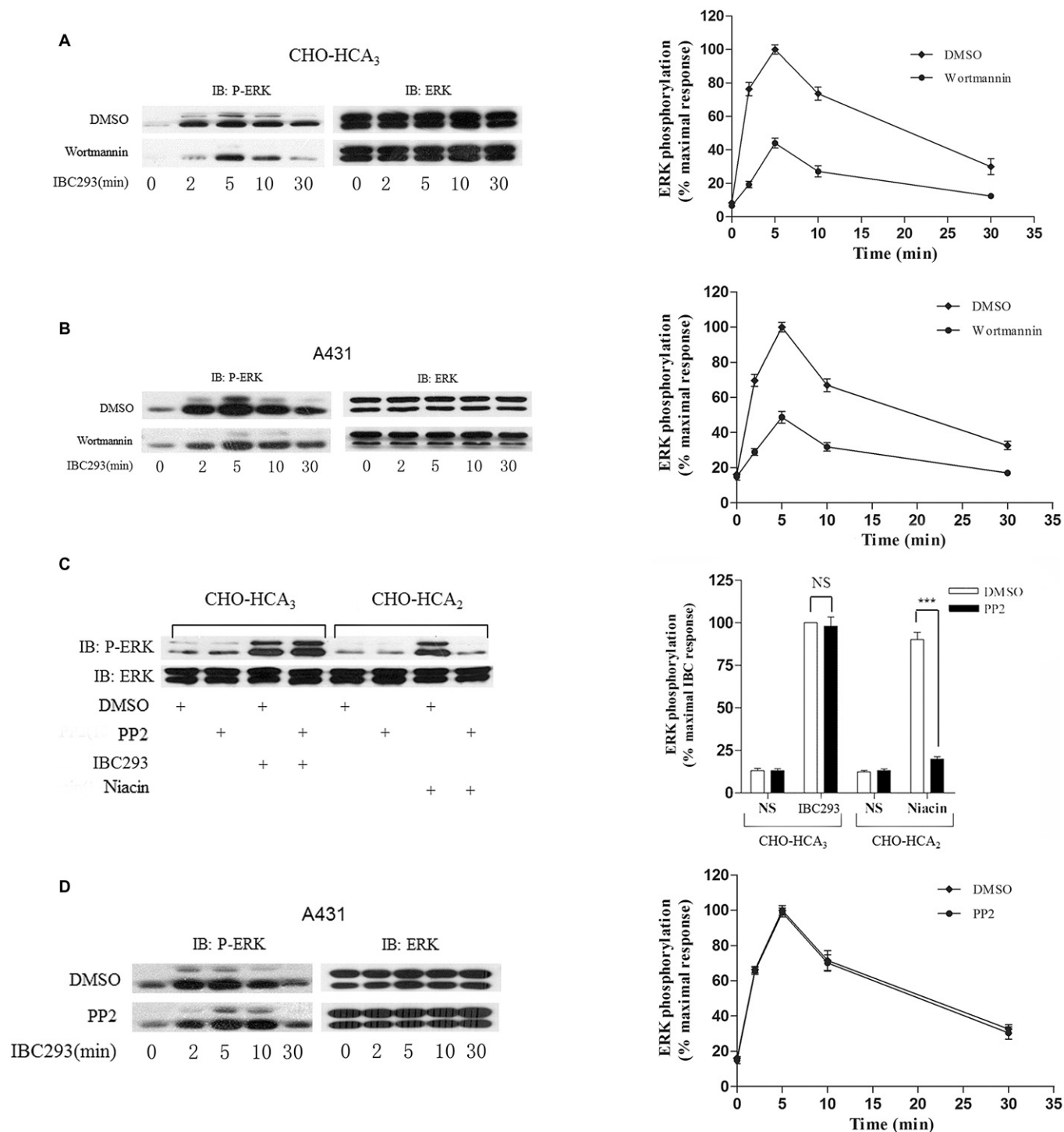


Figure 6

Effects of PI3K and Src on HCA₃-mediated ERK1/2 activation. Serum-starved CHO-HCA₃ cells (A) or A431 cells (B) were pretreated with DMSO or PI3K selective inhibitor wortmannin (1 μ M) for 1 h, and then stimulated with 1 μ M IBC293 for CHO-HCA₃ cells or 100 μ M IBC293 for A431 cells for the indicated time periods. (C) Serum-starved CHO-HCA₂ or CHO-HCA₃ cells were pretreated with DMSO or the Src selective inhibitor PP2 (10 μ M) for 1 h, and then stimulated with 1 μ M niacin or 1 μ M IBC293 respectively for 5 min. (D) Serum-starved A431 cells were pretreated with DMSO or PP2 (10 μ M) for 1 h, and then stimulated with 100 μ M IBC293 for the indicated time periods. The data shown are representative of at least three independent experiments. Data were analysed by Student's *t*-test. ***P* < 0.01, ****P* < 0.001.

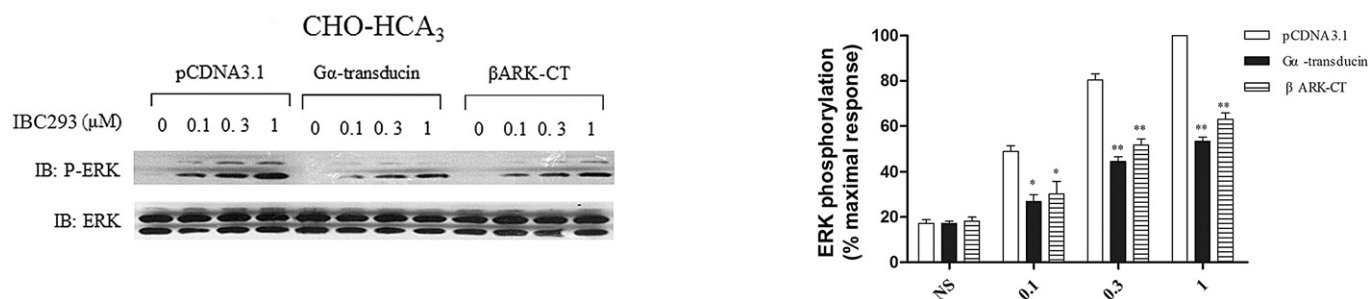


Figure 7

Effects of $G_{\beta\gamma}$ subunits on HCA₃-mediated ERK1/2 Activation. CHO-HCA₃ cells were transiently transfected with the $G_{\beta\gamma}$ scavengers β ARK-CT or G_{α} -transducin, and the cells were then serum-starved for 24 h and stimulated with various concentrations of IBC293 for 5 min. The data shown are representative of at least three independent experiments. Data were analysed by using the Student's *t*-test. **P* < 0.05, ***P* < 0.01.

$G_{\beta\gamma}$ plays a central role in HCA₃-induced ERK1/2 activation

PLC and PI3K can be activated through a mechanism involving the $G_{\beta\gamma}$ -subunits (Fields and Casey, 1997; Lopez-Illasaca *et al.*, 1997). A role for the $G_{i/o}$ -derived β - and γ -subunits was raised because overnight treatment with 100 ng·mL⁻¹ PTX eliminated HCA₃-mediated ERK1/2 activation (Figure 3A–C). To test for the involvement of the $G_{\beta\gamma}$ -subunits in ERK1/2 activation, we transfected CHO-HCA₃ cells with the β -adrenoceptor kinase COOH domain (495–689 aa) (β ARK-CT) or the G_{α} subunit of transducin, both of which are scavengers of the $G_{\beta\gamma}$ -subunits. Upon transfection, a significant inhibition of HCA₃-induced ERK1/2 phosphorylation was observed (Figure 7), which suggests that the $G_{\beta\gamma}$ subunit is likely to play a central role in HCA₃-induced ERK1/2 activation. Simultaneous inhibition of $G_{\beta\gamma}$ and PLC or $G_{\beta\gamma}$ and PDGFR in CHO-HCA₃ cells resulted in enhanced inhibition of HCA₃-mediated ERK1/2 activation, comparing with pre-treated with only one inhibitor alone (see Supporting Information Figure S3), indicating that $G_{\beta\gamma}$ subunits together with PLC and PDGFR play an important role in HCA₃-induced ERK1/2 activation in CHO-HCA₃ cells, although we could not clearly clarify the detailed mechanism of $G_{\beta\gamma}$ subunits-mediated pathways.

β -arrestin2 is involved in HCA₃ internalization, but β -arrestins are not involved in HCA₃-mediated ERK1/2 activation

To evaluate the role of β -arrestins in the regulation of HCA₃ internalization and ERK1/2 activation, we used specific siRNAs to reduce the expression of β -arrestin1 and β -arrestin2 in HEK-293 cells stably expressing HCA₃ receptors. The endogenous expression of β -arrestins was effectively and specifically knocked-down by specific siRNA treatment but was unaffected in cells treated with non-specific or control siRNAs (Figure 8A). Silencing β -arrestin2 effectively inhibited HCA₃ internalization, whereas knock-down of β -arrestin1 had no effect on the internalization of HCA₃ receptors, as analysed by microscopy (Figure 8B) or ELISA (Figure 8C). We further investigated the effect of knock-down of β -arrestins on ERK1/2 activation, and no difference was observed between control

and knock-down cells (Figure 8D). Taken together, it seems likely that β -arrestin2 is involved in HCA₃ receptor internalization, but both β -arrestins are not required for HCA₃-mediated ERK1/2 activation.

Discussion and conclusions

It is generally accepted that HCA₃ receptors, which differ from HCA₂ receptors by 16 amino acids and in an extended C-terminal end (Soga *et al.*, 2003; Wise *et al.*, 2003; Tunaru *et al.*, 2005), are the outcome of a recent gene duplication because it is only present in higher primates and absent in rodents and in most other mammals (Zellner *et al.*, 2005). Although accumulated evidence indicates that the binding of nicotinic acid to HCA₂ receptors mediates its antilipolytic and lipid-lowering effects (Zhang *et al.*, 2005), HCA₃ receptors have been shown to have very similar expression patterns to HCA₂ receptors (Soga *et al.*, 2003; Wise *et al.*, 2003; Zellner *et al.*, 2005) and to inhibit isoprenaline-induced lipolysis in primary human adipocytes (Semple *et al.*, 2006). The niacin-induced flushing has been shown to be mediated by HCA₂ receptors and by PUMA-G (Benyo *et al.*, 2005), and it suggests that selective activators of HCA₃ receptors may avoid the characteristic and uncomfortable cutaneous flushing response elicited by niacin in humans (Skinner *et al.*, 2009). However, the exact role of HCA₃ receptors in induction of the flushing side effect is currently not known. More recently, aromatic D-amino acids and the endogenous β -oxidation intermediate 3-hydroxy-octanoic acid were identified as specific agonists that activate HCA₃ receptors with physiological significance (Ahmed *et al.*, 2009; Irukayama-Tomobe *et al.*, 2009). HCA₃, together with HCA₂ receptors are of great interest as targets for the development of new antidyslipidemic drugs. Information about the signalling pathways linked to activated HCA₃ receptors and a better understanding of their functions are of major significance. Analyses of the signalling mechanisms induced by agonists of aromatic D-amino acids and endogenous 3-hydroxy-octanoic acid have demonstrated that HCA₃ receptors are coupled to PTX-sensitive G_i -proteins, which results in the inhibition of adenylyl cyclase, a transient rise of intracellular Ca^{2+} levels and the activation of ERK1/2

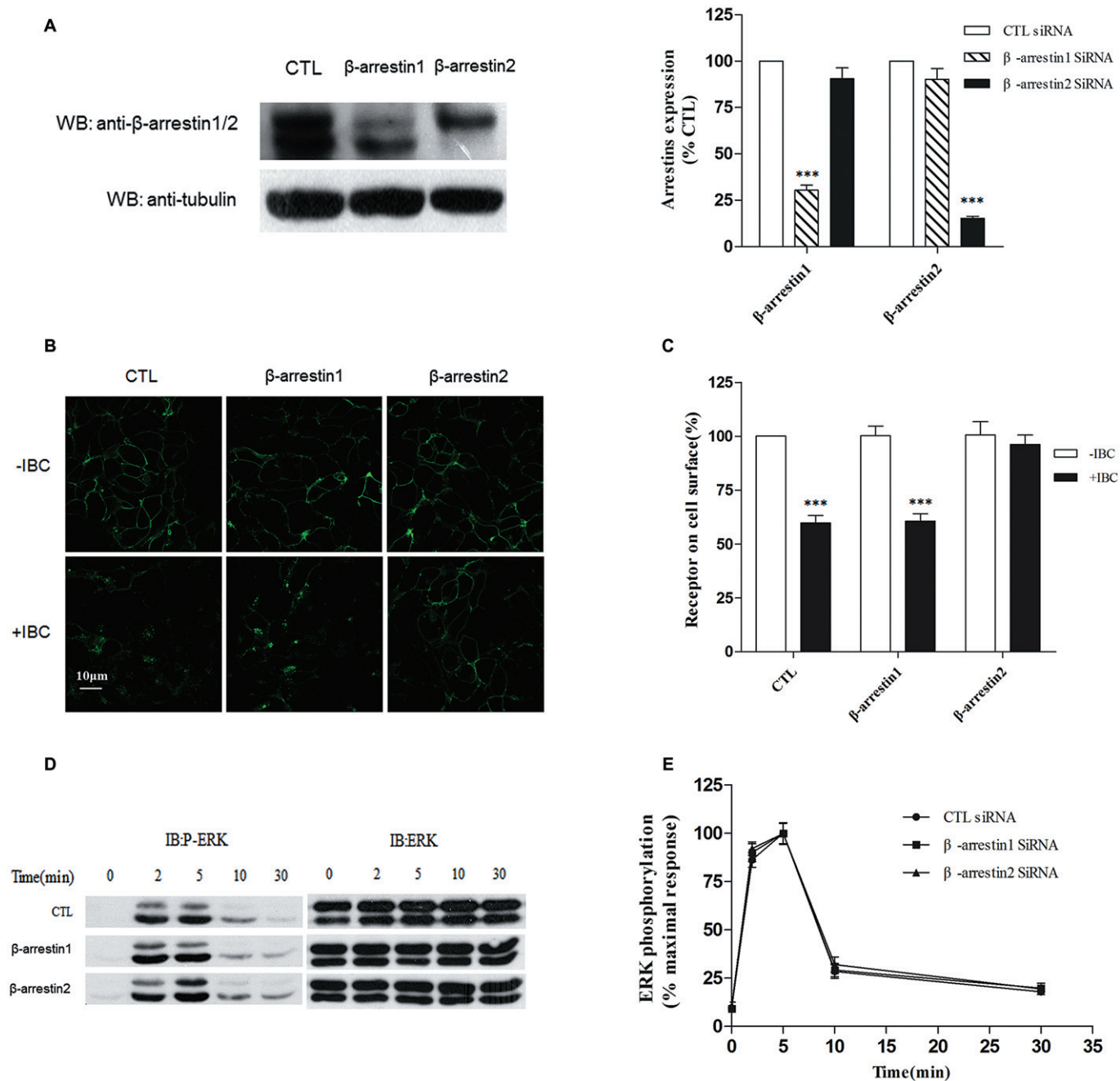


Figure 8

There is no involvement of β -arrestins in HCA₃-mediated ERK1/2 activation. (A) HEK-293 cells stably expressing HCA₃ were transfected with specific β -arrestin siRNA or a nonspecific control siRNA, 72 h after transfection, cells were harvested, and equal amounts of total cellular lysate were separated by 10% SDS-PAGE, transferred to a PVDF membrane, and incubated with anti- β -arrestin1/2 antibody. (B) HEK-293 cells stably expressing HCA₃-EGFP were transfected with specific β -arrestin siRNA or a non-specific control siRNA, 72 h after transfection, cells were stimulated with 100 μ M IBC293 for 40 min and examined with confocal microscopy as described under 'Experimental Procedures.' (C) ELISA determination of cell surface receptors in Flag-HCA₃ expressing cells treated with specific β -arrestin siRNA or nonspecific control siRNA. (D) 72 h after transfection with specific β -arrestin siRNA or non-specific control siRNA, cells were stimulated with 100 μ M IBC293 for the indicated time periods and immunoblotted using monoclonal anti-phospho-MAPK E10 (Thr202/Tyr204), and then the blots were stripped and reprobed for total ERK1/2 to control for loading. The data and pictures shown are representative of at least three independent experiments. Data were analysed by Student's *t* test ****P* < 0.01, *****P* < 0.001.

(Ahmed *et al.*, 2009; Irukayama-Tomobe *et al.*, 2009). However, the detailed mechanism of HCA₃-mediated ERK1/2 activation via different temporal components remains unknown. In the current study, we focused on a detailed characterization of HCA₃-mediated MAPK signalling pathways, and we demonstrated that activated HCA₃ receptors signal to ERK1/2 via a PLC-dependent PKC pathway and the MMP/HB-EGF-dependent EGFR transactivation pathway.

In the present study, we used the CHO-K1 cell line as a cellular model system for characterizing HCA₃-mediated ERK1/2 activation because it is a commonly used cell line for investigating GPCR signalling pathways. For better understanding of HCA₃-induced ERK1/2 phosphorylation, A431 cells, a human epidermoid cell with high levels of endogenous expression of functional HCA₃ receptors (Zhou *et al.*, 2007) was also selected for this study. In our preliminary experiments, we found that although all three agonists of HCA₃ receptors triggered significant ERK1/2 phosphorylation in CHO-HCA₃ cells, only IBC293 induced significant ERK1/2 phosphorylation in low concentrations in A431 cells, whereas both octanoic acid and acifran induced a moderate ERK1/2 phosphorylation in a very high concentration (>1 mM). This result is in good agreement with our previous observation with HCA₂ receptor (Li *et al.*, 2011); it is likely that the cell-type specificity and the cell surface expression level of receptor contributes to the different responses to agonists between two cell lines. Therefore, we chose the agonist IBC293 for further study of the ERK1/2 activation pathway induced by HCA₃ receptors in A431 cells.

The HCA₃ receptor is a G_i protein-coupled receptor. Upon stimulation by agonists, HCA₃ receptors trigger an inhibitory effect on adenylate cyclase that leads to a decrease of intracellular cAMP and, meanwhile, also elicit a transient rise of intracellular Ca²⁺ levels in a PTX-sensitive manner (Irukayama-Tomobe *et al.*, 2009). Thus, we explored the effect of the PLC/PKC pathway in HCA₃-mediated ERK1/2 activation. The inhibitory effect of the PKC inhibitors Go6983 and GFX suggested a critical role for PKC on HCA₃-mediated ERK1/2 activation at early time points (≤ 2 min). The involvement of PLC as a contributor to HCA₃-mediated ERK1/2 activation was assessed by incubating cells with two PLC inhibitors, ET-18-OCH₃ and U-73122. ET-18-OCH₃, but not U-73122, exhibited significant inhibition of ERK1/2 phosphorylation by activated HCA₃. U73122 is known to be a selective inhibitor of PLC β (Ward *et al.*, 2003) and ET-18-OCH₃ to be a PLC γ -selective inhibitor (Soultou *et al.*, 2001; Suzuki *et al.*, 2008). ET-18-OCH₃ is also a direct inhibitor of Raf (Samadder *et al.*, 2003; van der Westhuizen *et al.*, 2007). Our result with U-73122 is in agreement with observations that there are receptor-specific differences in the capacity of U-73122 to inhibit responses (Parker *et al.*, 1998; Morfis *et al.*, 2008). It is likely that the significant inhibitory effect of ET-18-OCH₃ on HCA₃-mediated ERK1/2 activation can be accounted for suppression of both PLC and Raf in this study. Furthermore, we also demonstrated that HCA₃-induced ERK1/2 activation was abolished by the depletion of extracellular Ca²⁺ by the chelator EGTA and by nifedipine, an L-type Ca²⁺ channel blocker in CHO-HCA₃ cells, suggesting that the L-type Ca²⁺ channel may play an important part in HCA₃-mediated ERK1/2 activation in CHO-K1 cells. However, Ca²⁺ was found to play no role in HCA₃-mediated ERK1/2 activa-

tion in A431 cells. The discrepancy in the role of Ca²⁺ in HCA₃-mediated ERK1/2 activation between CHO-K1 cells and A431 cells can be accounted for by cell type specificity, and our observation is in agreement with previous observation that AT₁ receptor-mediated activation of ERK1/2 by angiotensin II was Ca²⁺-dependent in rat anterior pituitary cells, but Ca²⁺-independent in hepatic C9 cells (Suarez *et al.*, 2003) (Shah and Catt, 2002). Taken together, these data suggest the involvement of both Ca²⁺-dependent and -independent PKC isoforms in HCA₃-mediated ERK1/2 activation.

The EGFR tyrosine kinase has emerged as an important transducer in signalling by GPCRs, a process termed transactivation (Schafer *et al.*, 2004a; Rozengurt, 2007). The role of EGFR transactivation in ERK1/2 stimulation by GPCR ligands is cell-specific. COS-7 cells express the EGF receptor (Shah *et al.*, 2004), but CHO-K1 cells express the PDGFR but lack endogenous EGFR (Antonelli *et al.*, 2000). Previous studies have demonstrated that proliferation of adipocytes is regulated by several growth factors, such as EGF, fibroblast growth factor and insulin-like growth factor (Smith *et al.*, 1988; Yamasaki *et al.*, 1999; Garcia and Obregon, 2002). Our results show that HCA₃-mediated ERK1/2 activation was potentially inhibited by the PDGFR-selective tyrphostin A9 and the PI3K inhibitor wortmannin in CHO-K1 cells. However, in A431 cells, HCA₃-mediated ERK1/2 activation was impaired by the EGF receptor-selective inhibitor AG1478 and the MMP inhibitor GM6001. Our finding that inhibition of metalloproteinase activity attenuated the activation of EGFR and ERK1/2 by HCA₃ receptor agonists, but not by EGF, defines the intermediary action of the MMP-dependent shedding of HB-EGF in the transactivation of EGFR by HCA₃ receptors in A431 cells. HB-EGF is synthesized in the cell as a transmembrane precursor that is proteolysed by a MMP of the zinc-dependent 'disintegrin and metalloproteinase' (ADAM) family to form a soluble growth factor that is a potent EGFR ligand (Riese *et al.*, 1998; Prenzler *et al.*, 1999). Different members of the ADAM family, including ADAM 10, ADAM 12 and ADAM 17, mediate GPCR-induced EGFR transactivation in different model systems (Schafer *et al.*, 2004b). The precise mechanism(s) by which the HCA₃ receptor stimulates ADAM activation remains to be further elucidated. Moreover, PI3Ks (Hawes *et al.*, 1996; Lopez-Ilasaca *et al.*, 1998) and Src family non-receptor tyrosine kinases (Lin *et al.*, 2008) have each been proposed as early intermediates in the pathway to induce EGF receptor transactivation. In the present study, we observed that PI3K was involved in the PDGFR- or EGFR-transactivated phosphorylation of ERK1/2, whereas the Src kinase was not required for HCA₃-induced EGFR transactivation in either CHO or A431 cells. However, more studies are necessary for the clarification of the exact role of PI3K in HCA₃-induced ERK1/2 activation.

In the current study, our results demonstrate the existence of two parallel pathways by which ERK1/2 can be activated by HCA₃ receptors. One pathway involves PLC and PKC activation, which results in ERK1/2 phosphorylation at an early (2 min) time. The other is EGFR transactivation and is mediated by MMP, which leads to activation of ERK1/2 at a later time point (5 min). This activation via two pathways is abolished by pretreatment with PTX. In addition, we observed that overexpression of the G $\beta\gamma$ subunit scavenger proteins β ARK-CT or G α -transducin effectively attenuated the ERK1/2

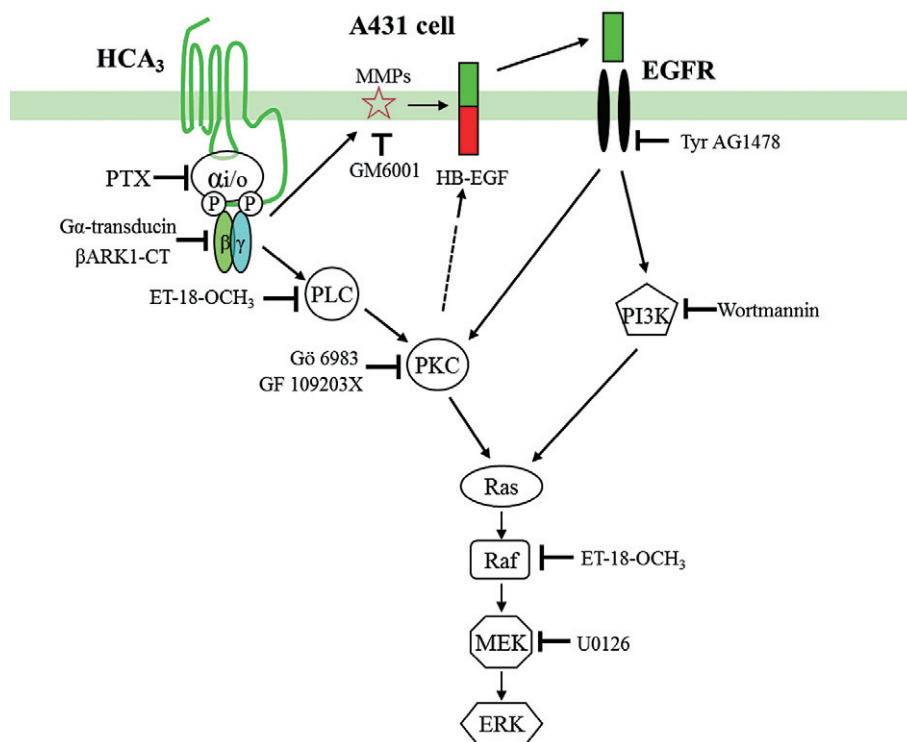


Figure 9

Schematic diagram of regulation of HCA₃-induced ERK1/2 activation in A431 cells. In response to agonists, activated HCA₃ receptors led to dissociation of G_i proteins from G_{βγ}-subunits, triggering the PKC pathway to couple to ERK1/2 phosphorylation at early time points (≤ 2 min), and the MMP/EGFR transactivation pathway with a maximum response at 5 min.

activation triggered by HCA₃ receptors (Figure 7). These results indicate that the G_{βγ} subunit acts as an early signal mediating HCA₃-induced PKC activation and EGF receptor transactivation. The major effects of G_i activation on the ERK1/2 cascade appear to be mediated via its G_{βγ} subunits (Crespo *et al.*, 1994; Hawes *et al.*, 1995). Previous studies have shown that G_i-type GPCRs stimulate Ca²⁺ mobilization through the binding of the G_{βγ} subunits to PLC (Dorn *et al.*, 1997; Dickenson and Hill, 1998). It has also been reported that the best-understood mechanism whereby the G_{βγ} subunits stimulate ERK1/2 is through the 'transactivation' of classical receptor tyrosine kinases, such as the EGF and PDGFRs (Carpenter, 2000; Gschwind *et al.*, 2001), although the G_{βγ} subunit protein effectors that regulate HB-EGF release remain undefined. Thus, we postulate that upon stimulation of HCA₃ receptors by agonists, activated G_i protein impairs cAMP production and its released G_{βγ} subunits are able to trigger the generation of DAG by directly binding to PLC, which leads to the activation of PKC, followed by a >2 min early time point peak of ERK1/2 phosphorylation. On the other hand, the free G_{βγ} subunits also cause activation of a MMP to cleave HB-EGF (Prenzel *et al.*, 1999) and lead to EGFR transactivation, which results in a >5 min late time point peak for ERK1/2 activation. These findings are consistent with our previous observation of HCA₂ receptors in CHO-K1 cells and A431 cells (Li *et al.*, 2011), although Src was found to play no role in HCA₃-mediated ERK1/2 activation in CHO-K1 cells stably expressing HCA₃ receptors.

β-Arrestins are traditionally recognized as playing a well-established role in the termination of receptor-G-protein coupling and the initiation of clathrin-dependent internalization (Luttrell and Lefkowitz, 2002). However, there is a growing body of evidence that indicates that β-arrestins function as signal transducers for many GPCRs to mediate ERK1/2 activation (Lefkowitz and Shenoy, 2005). β-Arrestins are required for later-phase activation of the ERK1/2 pathway mediated by angiotensin II AT_{1A} receptors (Ahn *et al.*, 2004b), β₂-adrenoceptors (Shenoy *et al.*, 2006), vasopressin 2 (Ren *et al.*, 2005) and parathyroid hormone (Gesty-Palmer *et al.*, 2006) receptors, whereas, in the dopamine D₂ and D₃ receptors (Beom *et al.*, 2004; Quan *et al.*, 2008) and the formyl peptide receptor (Huet *et al.*, 2007; Gripenberg and Miettinen, 2008), β-arrestins have been found to play no role or only a minor role in the activation of the ERK1/2 pathway. Our results using siRNA showed that β-arrestin2 was required for agonist-mediated internalization of HCA₃ receptors, whereas knock-down of β-arrestin2 or β-arrestin1 using siRNA had no effect on ERK1/2 activation. This result is in good agreement with our previous observation for the HCA₂-mediated activation of the ERK1/2 pathway (Li *et al.*, 2010).

Our current results have led us to propose a model for the regulation of HCA₃-mediated ERK1/2 activation in CHO cells that are stably transfected with HCA₃ receptors and in A431 cells that endogenously express HCA₃ receptors (Figure 9). In response to agonists, activated HCA₃ receptors induce the dissociation of G_i proteins from G_{βγ}-subunits, triggering the

PKC pathway to couple to ERK1/2 phosphorylation at early time points (≤ 2 min), and the MMP/EGFR transactivation pathway with a maximum response at 5 min. We present evidence that $G_{\beta\gamma}$ subunits together with PKC and EGFR/PDGFR play a central role in HCA₃-induced ERK1/2 activation. However, additional investigations will be necessary to further clarify the role of the ERK1/2 pathway in HCA₃-mediated lipolysis.

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Conflict of interest

The authors state no conflict of interest.

References

- Ahmed K, Tunaru S, Langhans CD, Hanson J, Michalski CW, Kolker S *et al.* (2009). Deorphanization of GPR109B as a receptor for the beta-oxidation intermediate 3-OH-octanoic acid and its role in the regulation of lipolysis. *J Biol Chem* 284: 21928–21933.
- Ahn S, Shenoy SK, Wei H, Lefkowitz RJ (2004a). Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* 279: 35518–35525.
- Ahn S, Wei H, Garrison TR, Lefkowitz RJ (2004b). Reciprocal regulation of angiotensin receptor-activated extracellular signal-regulated kinases by beta-arrestins 1 and 2. *J Biol Chem* 279: 7807–7811.
- Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th Edition. *Br J Pharmacol* 164 (Suppl. 1): S1–S324.
- Antonelli V, Bernasconi F, Wong YH, Vallar L (2000). Activation of B-Raf and regulation of the mitogen-activated protein kinase pathway by the G(o) alpha chain. *Mol Biol Cell* 11: 1129–1142.
- Benyo Z, Gille A, Kero J, Csiky M, Suchankova MC, Nusing RM *et al.* (2005). GPR109A (PUMA-G/HM74A) mediates nicotinic acid-induced flushing. *J Clin Invest* 115: 3634–3640.
- Beom S, Cheong D, Torres G, Caron MG, Kim KM (2004). Comparative studies of molecular mechanisms of dopamine D2 and D3 receptors for the activation of extracellular signal-regulated kinase. *J Biol Chem* 279: 28304–28314.
- Carpenter G (2000). EGF receptor transactivation mediated by the proteolytic production of EGF-like agonists. *Sci STKE* 2000: pe1.
- Chung T, Crilly KS, Anderson WH, Mukherjee JJ, Kiss Z (1997). ATP-dependent choline phosphate-induced mitogenesis in fibroblasts involves activation of pp70 S6 kinase and phosphatidylinositol 3'-kinase through an extracellular site. Synergistic mitogenic effects of choline phosphate and sphingosine 1-phosphate. *J Biol Chem* 272: 3064–3072.
- Crespo P, Xu N, Simonds WF, Gutkind JS (1994). Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature* 369: 418–420.
- Dickenson JM, Hill SJ (1998). Involvement of G-protein betagamma subunits in coupling the adenosine A1 receptor to phospholipase C in transfected CHO cells. *Eur J Pharmacol* 355: 85–93.
- Dorn GW, 2nd, Oswald KJ, McCluskey TS, Kuhel DG, Liggett SB (1997). Alpha 2A-adrenergic receptor stimulated calcium release is transduced by Gi-associated G (beta gamma)-mediated activation of phospholipase C. *Biochemistry* 36: 6415–6423.
- Duckworth BC, Cantley LC (1997). Conditional inhibition of the mitogen-activated protein kinase cascade by wortmannin. Dependence on signal strength. *J Biol Chem* 272: 27665–27670.
- Fields TA, Casey PJ (1997). Signalling functions and biochemical properties of pertussis toxin-resistant G-proteins. *Biochem J* 321: 561–571.
- Garcia B, Obregon MJ (2002). Growth factor regulation of uncoupling protein-1 mRNA expression in brown adipocytes. *Am J Physiol Cell Physiol* 282: C105–C112.
- Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Wang S *et al.* (2006). Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J Biol Chem* 281: 10856–10864.
- Gripentrog JM, Miettinen HM (2008). Formyl peptide receptor-mediated ERK1/2 activation occurs through G(i) and is not dependent on beta-arrestin1/2. *Cell Signal* 20: 424–431.
- Gschwind A, Zwick E, Prenzel N, Leserer M, Ullrich A (2001). Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene* 20: 1594–1600.
- Hawes BE, van Biesen T, Koch WJ, Luttrell LM, Lefkowitz RJ (1995). Distinct pathways of Gi- and Gq-mediated mitogen-activated protein kinase activation. *J Biol Chem* 270: 17148–17153.
- Hawes BE, Luttrell LM, van Biesen T, Lefkowitz RJ (1996). Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway. *J Biol Chem* 271: 12133–12136.
- Huet E, Boulay F, Barral S, Rabiet MJ (2007). The role of beta-arrestins in the formyl peptide receptor-like 1 internalization and signaling. *Cell Signal* 19: 1939–1948.
- Irukayama-Tomobe Y, Tanaka H, Yokomizo T, Hashidate-Yoshida T, Yanagisawa M, Sakurai T (2009). Aromatic d-amino acids act as chemoattractant factors for human leukocytes through a G protein-coupled receptor, GPR109B. *Proc Natl Acad Sci USA* 106: 3930–3934.
- Kim J, Ahn S, Rajagopal K, Lefkowitz RJ (2009). Independent beta-arrestin2 and Gq/protein kinase C pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor. *J Biol Chem* 284: 11953–11962.
- Kranenburg O, Verlaan I, Hordijk PL, Moolenaar WH (1997). Gi-mediated activation of the Ras/MAP kinase pathway involves a 100 kDa tyrosine-phosphorylated Grb2 SH3 binding protein, but not Src nor Shc. *EMBO J* 16: 3097–3105.

- Lee DK, Nguyen T, Lynch KR, Cheng R, Vanti WB, Arkhitko O *et al.* (2001). Discovery and mapping of ten novel G protein-coupled receptor genes. *Gene* 275: 83–91.
- Lefkowitz RJ, Shenoy SK (2005). Transduction of receptor signals by beta-arrestins. *Science* 308: 512–517.
- Li G, Shi Y, Huang H, Zhang Y, Wu K, Luo J *et al.* (2010). Internalization of the human nicotinic acid receptor GPR109A is regulated by G(i), GRK2, and arrestin3. *J Biol Chem* 285: 22605–22618.
- Li G, Deng X, Wu C, Zhou Q, Chen L, Shi Y *et al.* (2011). 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. *J Biol Chem* 286: 31199–31212.
- Lin AL, Zhu B, Zhang W, Dang H, Zhang BX, Katz MS *et al.* (2008). Distinct pathways of ERK activation by the muscarinic agonists pilocarpine and carbachol in a human salivary cell line. *Am J Physiol Cell Physiol* 294: C1454–C1464.
- Lopez-Illasaca M, Crespo P, Pellici PG, Gutkind JS, Wetzker R (1997). Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. *Science* 275: 394–397.
- Lopez-Illasaca M, Gutkind JS, Wetzker R (1998). Phosphoinositide 3-kinase gamma is a mediator of Gbetagamma-dependent Jun kinase activation. *J Biol Chem* 273: 2505–2508.
- Luo J, Busillo JM, Benovic JL (2008). M3 muscarinic acetylcholine receptor-mediated signaling is regulated by distinct mechanisms. *Mol Pharmacol* 74: 338–347.
- Luttrell LM, Lefkowitz RJ (2002). The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* 115: 455–465.
- Luttrell LM, Daaka Y, Lefkowitz RJ (1999). Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* 11: 177–183.
- Mahboubi K, Witman-Jones T, Adamus JE, Letsinger JT, Whitehouse D, Moorman AR *et al.* (2006). Triglyceride modulation by acifran analogs: activity towards the niacin high and low affinity G protein-coupled receptors HM74A and HM74. *Biochem Biophys Res Commun* 340: 482–490.
- Morfis M, Tilakaratne N, Furness SG, Christopoulos G, Werry TD, Christopoulos A *et al.* (2008). Receptor activity-modifying proteins differentially modulate the G protein-coupling efficiency of amylin receptors. *Endocrinology* 149: 5423–5431.
- Nomura H, Nielsen BW, Matsushima K (1993). Molecular cloning of cDNAs encoding a LD78 receptor and putative leukocyte chemotactic peptide receptors. *Int Immunol* 5: 1239–1249.
- Parker SL, Parker MS, Sweatman T, Crowley WR (1998). Characterization of G protein and phospholipase C-coupled agonist binding to the Y1 neuropeptide Y receptor in rat brain: sensitivity to G protein activators and inhibitors and to inhibitors of phospholipase C. *J Pharmacol Exp Ther* 286: 382–391.
- Pierce KL, Luttrell LM, Lefkowitz RJ (2001). New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene* 20: 1532–1539.
- Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C *et al.* (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 402: 884–888.
- Ptasznik A, Gewirtz AM (2000). Crosstalk between G protein-coupled receptors and tyrosine kinase signaling: Src take centre stage. *Arch Immunol Ther Exp (Warsz)* 48: 27–30.
- Quan W, Kim JH, Albert PR, Choi H, Kim KM (2008). Roles of G protein and beta-arrestin in dopamine D2 receptor-mediated ERK activation. *Biochem Biophys Res Commun* 377: 705–709.
- Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ (2005). Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci USA* 102: 1448–1453.
- Riese DJ, 2nd, Komurasaki T, Plowman GD, Stern DF (1998). Activation of ErbB4 by the bifunctional epidermal growth factor family hormone epiregulin is regulated by ErbB2. *J Biol Chem* 273: 11288–11294.
- Rozengurt E (2007). Mitogenic signaling pathways induced by G protein-coupled receptors. *J Cell Physiol* 213: 589–602.
- Samadder P, Richards C, Bittman R, Bhullar RP, Arthur G (2003). The antitumor ether lipid 1-Q-octadecyl-2-O-methyl-rac-glycerophosphocholine (ET-18-OCH3) inhibits the association between Ras and Raf-1. *Anticancer Res* 23: 2291–2295.
- Schafer B, Gschwind A, Ullrich A (2004a). Multiple G-protein-coupled receptor signals converge on the epidermal growth factor receptor to promote migration and invasion. *Oncogene* 23: 991–999.
- Schafer B, Marg B, Gschwind A, Ullrich A (2004b). Distinct ADAM metalloproteinases regulate G protein-coupled receptor-induced cell proliferation and survival. *J Biol Chem* 279: 47929–47938.
- Semple G, Skinner PJ, Cherrier MC, Webb PJ, Sage CR, Tamura SY *et al.* (2006). 1-Alkyl-benzotriazole-5-carboxylic acids are highly selective agonists of the human orphan G-protein-coupled receptor GPR109b. *J Med Chem* 49: 1227–1230.
- Shah BH, Catt KJ (2002). Calcium-independent activation of extracellularly regulated kinases 1 and 2 by angiotensin II in hepatic C9 cells: roles of protein kinase Cdelta, Src/proline-rich tyrosine kinase 2, and epidermal growth receptor trans-activation. *Mol Pharmacol* 61: 343–351.
- Shah BH, Farshori MP, Jambusaria A, Catt KJ (2003). Roles of Src and epidermal growth factor receptor transactivation in transient and sustained ERK1/2 responses to gonadotropin-releasing hormone receptor activation. *J Biol Chem* 278: 19118–19126.
- Shah BH, Yesilkaya A, Olivares-Reyes JA, Chen HD, Hunyady L, Catt KJ (2004). Differential pathways of angiotensin II-induced extracellularly regulated kinase 1/2 phosphorylation in specific cell types: role of heparin-binding epidermal growth factor. *Mol Endocrinol* 18: 2035–2048.
- Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S *et al.* (2006). Beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem* 281: 1261–1273.
- Shi W, Fan H, Shum L, Derynck R (2000). The tetraspanin CD9 associates with transmembrane TGF-alpha and regulates TGF-alpha-induced EGF receptor activation and cell proliferation. *J Cell Biol* 148: 591–602.
- Skinner PJ, Cherrier MC, Webb PJ, Sage CR, Dang HT, Pride CC *et al.* (2007). 3-Nitro-4-amino benzoic acids and 6-amino nicotinic acids are highly selective agonists of GPR109b. *Bioorg Med Chem Lett* 17: 6619–6622.
- Skinner PJ, Webb PJ, Sage CR, Dang TH, Pride CC, Chen R *et al.* (2009). 5-N,N-Disubstituted 5-aminopyrazole-3-carboxylic acids are highly potent agonists of GPR109b. *Bioorg Med Chem Lett* 19: 4207–4209.

Smith PJ, Wise LS, Berkowitz R, Wan C, Rubin CS (1988). Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *J Biol Chem* 263: 9402–9408.

Soga T, Kamohara M, Takasaki J, Matsumoto S, Saito T, Ohishi T *et al.* (2003). Molecular identification of nicotinic acid receptor. *Biochem Biophys Res Commun* 303: 364–369.

Souttou B, Carvalho NB, Raulais D, Vigny M (2001). Activation of anaplastic lymphoma kinase receptor tyrosine kinase induces neuronal differentiation through the mitogen-activated protein kinase pathway. *J Biol Chem* 276: 9526–9531.

Suarez C, Diaz-Torga G, Gonzalez-Iglesias A, Vela J, Mladovan A, Baldi A *et al.* (2003). Angiotensin II phosphorylation of extracellular signal-regulated kinases in rat anterior pituitary cells. *Am J Physiol Endocrinol Metab* 285: E645–E653.

Suzuki T, Seth A, Rao R (2008). Role of phospholipase Cgamma-induced activation of protein kinase Cepsilon (PKCepsilon) and PKCbeta1 in epidermal growth factor-mediated protection of tight junctions from acetaldehyde in Caco-2 cell monolayers. *J Biol Chem* 283: 3574–3583.

Tunaru S, Kero J, Schaub A, Wufka C, Blaukat A, Pfeffer K *et al.* (2003). PUMA-G and HM74 are receptors for nicotinic acid and mediate its anti-lipolytic effect. *Nat Med* 9: 352–355.

Tunaru S, Lattig J, Kero J, Krause G, Offermanns S (2005). Characterization of determinants of ligand binding to the nicotinic acid receptor GPR109A (HM74A/PUMA-G). *Mol Pharmacol* 68: 1271–1280.

Ward PD, Ouyang H, Thakker DR (2003). Role of phospholipase C-beta in the modulation of epithelial tight junction permeability. *J Pharmacol Exp Ther* 304: 689–698.

van der Westhuizen ET, Werry TD, Sexton PM, Summers RJ (2007). The relaxin family peptide receptor 3 activates extracellular signal-regulated kinase 1/2 through a protein kinase C-dependent mechanism. *Mol Pharmacol* 71: 1618–1629.

Wise A, Foord SM, Fraser NJ, Barnes AA, Elshourbagy N, Eilert M *et al.* (2003). Molecular identification of high and low affinity receptors for nicotinic acid. *J Biol Chem* 278: 9869–9874.

Yamasaki M, Emoto H, Konishi M, Mikami T, Ohuchi H, Nakao K *et al.* (1999). FGF-10 is a growth factor for preadipocytes in white adipose tissue. *Biochem Biophys Res Commun* 258: 109–112.

Zellner C, Pullinger CR, Aouizerat BE, Frost PH, Kwok PY, Malloy MJ *et al.* (2005). Variations in human HM74 (GPR109B) and HM74A (GPR109A) niacin receptors. *Hum Mutat* 25: 18–21.

Zhang Y, Schmidt RJ, Foxworthy P, Emkey R, Oler JK, Large TH *et al.* (2005). Niacin mediates lipolysis in adipose tissue through its G-protein coupled receptor HM74A. *Biochem Biophys Res Commun* 334: 729–732.

Zhou L, Tang Y, Cryan EV, Demarest KT (2007). Human epidermoid A431 cells express functional nicotinic acid receptor HM74a. *Mol Cell Biochem* 294: 243–248.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effect of simultaneous inhibition of PLC and PDGFR or EGFR on HCA₃-induced ERK1/2 activation. a, Serum-starved CHO-HCA₃ cells were pretreated with DMSO or A9 or ET-18-OCH₃ or both A9 and ET-18-OCH₃ for 1 h, and then stimulated with 1 µM IBC293 for the indicated time periods. b, Serum-starved A431 cells were pretreated with DMSO or AG1478 or ET-18-OCH₃ or both AG1478 and ET-18-OCH₃ for 1 h, and then stimulated with 100 µM IBC293 for the indicated time periods. The data shown are representative of at least three independent experiments.

Figure S2 Effect of PKC and PI3K on IBC293-induced EGFR phosphorylation and EGF-induced ERK1/2 activation. a, Serum-starved A431 cells were pretreated with DMSO or PKC inhibitor Go6983 (10 µM) or PI3K inhibitor wortmannin (1 µM) for 1 h, and then stimulated with 100 µM IBC293 for 5 min. b, Serum-starved A431 cells were pretreated with DMSO or PKC inhibitor Go6983 for 1 h, and then stimulated with 10 ng/ml EGF for 2 min. c, Serum-starved A431 cells were pretreated with DMSO or PI3K inhibitor wortmannin for 1 h, and then stimulated with 10 ng/ml EGF for 5 min. The data shown are representative of at least three independent experiments. Data were analyzed by using the Student's t test (** p < 0.01, *** p < 0.001).

Figure S3 Effect of simultaneous inhibition of G_{βγ} and PDGFR or PLC in HCA₃-induced ERK1/2 activation. a, CHO-HCA₃ cells were transiently transfected with the G_{βγ} scavengers βARK-CT or empty vector, the cells were serum-starved for 24 h and pretreated with DMSO or A9, and then stimulated with 1 µM IBC293 for the indicated time periods. b, CHO-HCA₃ cells were transiently transfected with the G_{βγ} scavenger βARK-CT or empty vector, the cells were serum-starved for 24 h and pretreated with DMSO or ET-18-OCH₃, and then stimulated with 1 µM IBC293 for the indicated time periods. The data shown are representative of at least three independent experiments.

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