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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, HCA_3, is of particular interest given its ability to treat lipid disorders and atherosclerosis. Here we demonstrate the pathway of HCA_3-mediated activation of ERK1/2.

EXPERIMENTAL APPROACH
Using CHO-K1 cells stably expressing HCA_3 receptors and A431 cells, a human epidermoid cell line with high levels of endogenous expression of functional HCA_3 receptors, HCA_3-mediated activation of ERK1/2 was measured by Western blot.

KEY RESULTS
HCA_3-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, HCA_3 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 βγ-subunits of the G_i protein play a critical role in HCA_3-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 HCA_3 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 HCA_3-mediated activation of ERK1/2.

Abbreviations
ADAM, a disintegrin and metalloproteinase; CRE, cAMP response element; EGFR, epidermal growth factor receptor; ET-18-OCH_3, 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 HCA_3 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 HCA_3 from human spleen cDNA as a template resulted in the discovery of one close paralogue, termed HCA_2 also known as GPR109A or HM74a (Soga *et al.*, 2003). Recently, three research groups identified HCA_2 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 HCA_3 receptors share a high degree of similarity with HCA_2 receptors, displaying 96% identity to the HCA_2 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 HCA_2 and HCA_3 receptors partially overlap and are partially distinct (Irukayama-Tomobe *et al.*, 2009).

Despite a high sequence homology with HCA_2 receptors, the affinity of nicotinic acid to HCA_3 receptors is quite low, that is, millimolar levels (Wise *et al.*, 2003; Li *et al.*, 2010). Furthermore, binding assay results have confirmed that the HCA_3 receptor does not appreciably bind nicotinic acid (Soga *et al.*, 2003; Tunaru *et al.*, 2003), which suggests that the HCA_3 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 HCA_3 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 HCA_2 receptors, HCA_3 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 HCA_2 and HCA_3 receptors (Wise *et al.*, 2003). These data suggest that HCA_3 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 HCA_3 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 HCA_3 receptors using the model cell system CHO-K1, which recombinantly expresses human HCA_3 receptors, and A431 cells, a human epidermoid carcinoma cell line that endogenously expresses functional human HCA_3 receptors (Zhou *et al.*, 2007). We document here, for the first time, the molecular mechanisms underlying the coupling of human HCA_3 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

HCA_2 and HCA_3 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 HCA_2, HCA_3 receptor or empty vector were grown in the media mentioned earlier, but with the addition of G418 (400 $mg \cdot L^{-1}$). 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 HCA_3 were grown in the media mentioned earlier, but with the addition of G418 (800 $mg \cdot L^{-1}$). 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% CO_2/95% 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 HCA_3 or HCA_2 receptors and pCRE-Luc were grown to 90–95% confluence, stimulated with 10 μM forskolin alone or with 10 μM 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 $ng \cdot mL^{-1}$) 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-HCA_3 or CHO-K1 cells were harvested with Cell Stripper (Mediatech, Herndon, VA, USA), washed twice with PBS and resuspended to 5×10^6 $cells \cdot mL^{-1}$ in Hank's balanced salt solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 $mg \cdot mL^{-1}$ glucose) containing 0.025% BSA. The cells were then loaded with 3 μM 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 x 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 HCA_3-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

HCA_3 receptors on the cell surface were quantitatively assessed by ELISA as previously described (Li *et al.*, 2010). Briefly, HEK-293 cells stably expressing Flag-HCA_3 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 pEC_{50} 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-OCH_3), 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 (Thr^{202}/Tyr^{204}) 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 HCA_3 in CHO-K1 cells

To investigate the HCA_3-mediated activation of ERK1/2, we cloned human HCA_3 receptors (GenBank Accession No.D10923) from human genomic DNA as a template using PCR and created CHO-K1 cell lines that stably expressed human HCA_3 receptors. We first examined the functional signalling of HCA_3 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 HCA_2 and HCA_3 receptors (Mahboubi *et al.*, 2006), induced a ligand concentration-dependent inhibition of forskolin-stimulated cAMP increase with EC_{50} 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

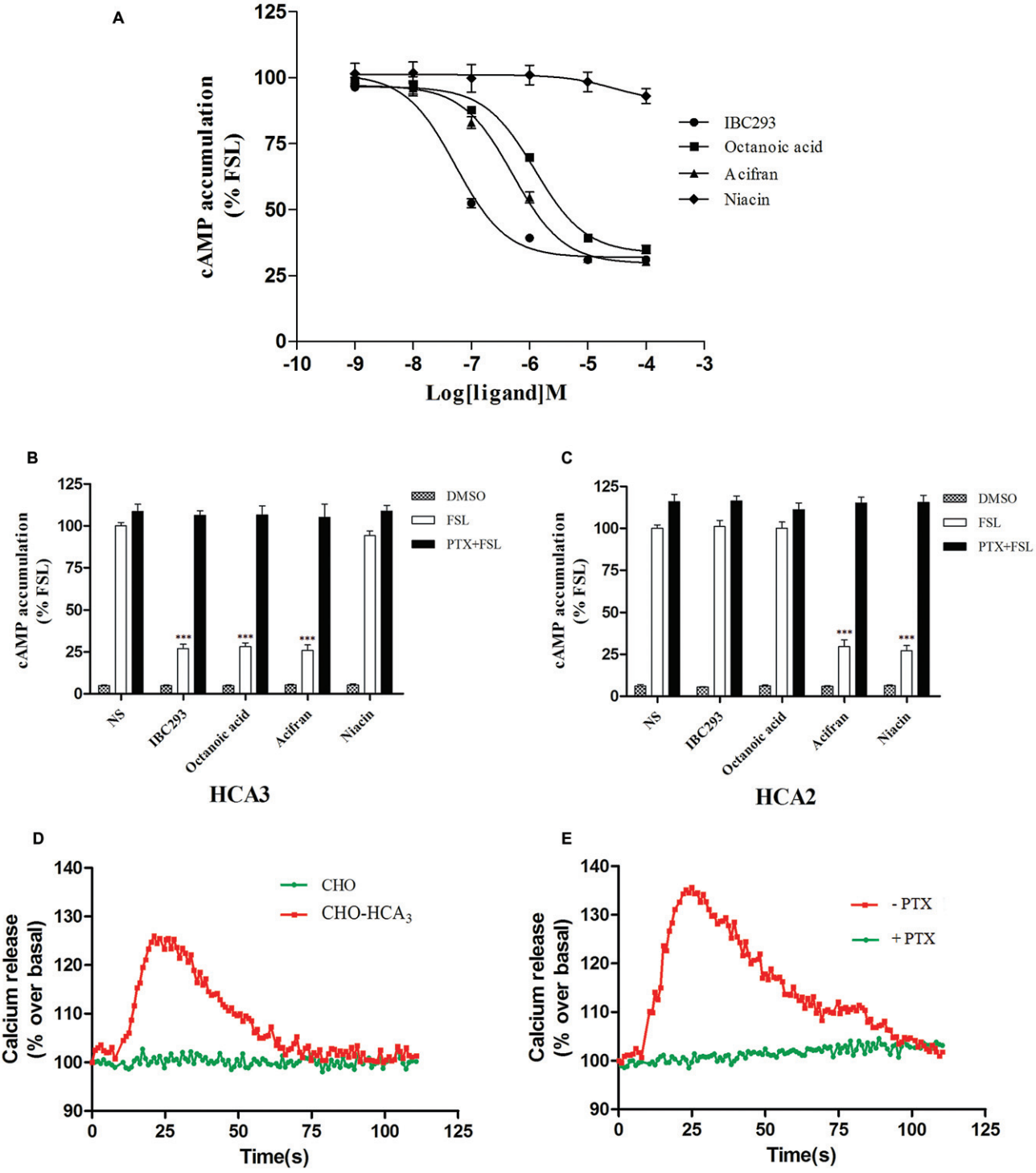


Figure 1

Characterization of HCA_3 receptors stably expressed in CHO-K1 cells. A, cAMP accumulation in CHO-K1 cells stably co-transfecting with HCA_3 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 HCA_3 (B) or HCA_2 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-HCA_3 or CHO-K1 cells were loaded with the calcium probe Fura-2/AM followed by stimulation with 100 μM IBC293. (E) CHO-HCA_3 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 HCA_2-transfected CHO cells (Figure 1C). In addition, stimulation with IBC293 (100 μM) elicited a rapid and transient increase in intracellular Ca^{2+} in CHO-K1 cells expressing HCA_3 receptors (Figure 1D) and the Ca^{2+} mobilization could be completely blocked by pretreating with 100 ng·mL^{-1} PTX for 12 h (Figure 1E). These results suggest that HCA_3 receptors in stably transfected CHO-K1 cells are functional, and octanoic acid and IBC293 are specific ligands for HCA_3 receptors.

HCA_3 receptors activate ERK1/2 signalling via MEK 1/2 following exposure to octanoic acid, IBC293 and acifran

In CHO-HCA_3 cells, stimulation with different concentrations of agonists – octanoic acid, IBC293 and acifran – evoked ERK1/2 phosphorylation in a dose-dependent manner with EC_{50} 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-HCA_2 cells (data not shown), which is consistent with the observation of intracellular cAMP accumulation with no detectable activity up to 1 mM at HCA_2 receptors (Semple *et al.*, 2006). In addition, to better characterize the HCA_3-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 HCA_3 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 EC_{50} of 14.9 μM (Figure 2B). The HCA_3-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-HCA_3 cells (Figure 2C). A similar result was observed during IBC293-mediated ERK1/2 activation in A431 cells (Figure 2D).

To investigate whether or not HCA_3-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-HCA_3 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 HCA_3-induced ERK1/2 phosphorylation.

HCA_3 receptors initiate ERK1/2 activation via the PTX-sensitive G_i protein-dependent pathway

Previous studies have demonstrated that HCA_3 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 HCA_3-mediated activation of ERK1/2, CHO-HCA_3 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-HCA_3 cells (Figure 3B). Together, these data demonstrate that HCA_3 receptors signal to the ERK1/2 pathway via a PTX-sensitive G_i protein-dependent mechanism.

Involvement of the PLC/PKC pathway in HCA_3-mediated ERK1/2 activation

Next, we investigated whether or not PKC plays a role in agonist-stimulated ERK1/2 phosphorylation via HCA_3 receptors, As shown earlier, in time-course studies, the HCA_3-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-HCA_3 (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 HCA_3-mediated ERK1/2 activation at early time points (≤2 min).

The family of PLCs classically catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG can directly activate classical types of PKC by interacting with its lipid-binding domain, and IP_3 can indirectly activate PKC by increasing intracellular Ca^{2+}, which interacts with the PKC Ca^{2+}-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-OCH_3. Pretreatment of cells with U73122 (20 μM) led to no inhibition of HCA_3-stimulated ERK1/2 activation in both CHO-HCA_3 and A431 cells (Figure 4C and D), whereas ET-18-OCH_3 significantly blocked HCA_3-mediated ERK1/2 phosphorylation in both cell lines (Figure 4C and D). Next, we examined the role of phospholipase D (PLD) in HCA_3-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-HCA_3 and A431 cells. These data suggested that PLC rather than PLD played a key role in HCA_3-mediated ERK1/2 phosphorylation.

Previous studies have shown that octanoic acid causes a rapid increase of intracellular Ca^{2+} in CHO-K1 cells expressing HCA_3 receptors (Ahmed *et al.*, 2009). Accordingly, we investigated whether or not intracellular and extracellular Ca^{2+} is involved in HCA_3-stimulated ERK1/2 phosphorylation. Pretreatment with the extracellular Ca^{2+} chelator EGTA (5 mM) or L-type Ca^{2+} channel blocker nifedipine (10 μM) significantly inhibited ERK1/2 phosphorylation in CHO-HCA_3 cells (Figure 4E). However, these two inhibitors showed no

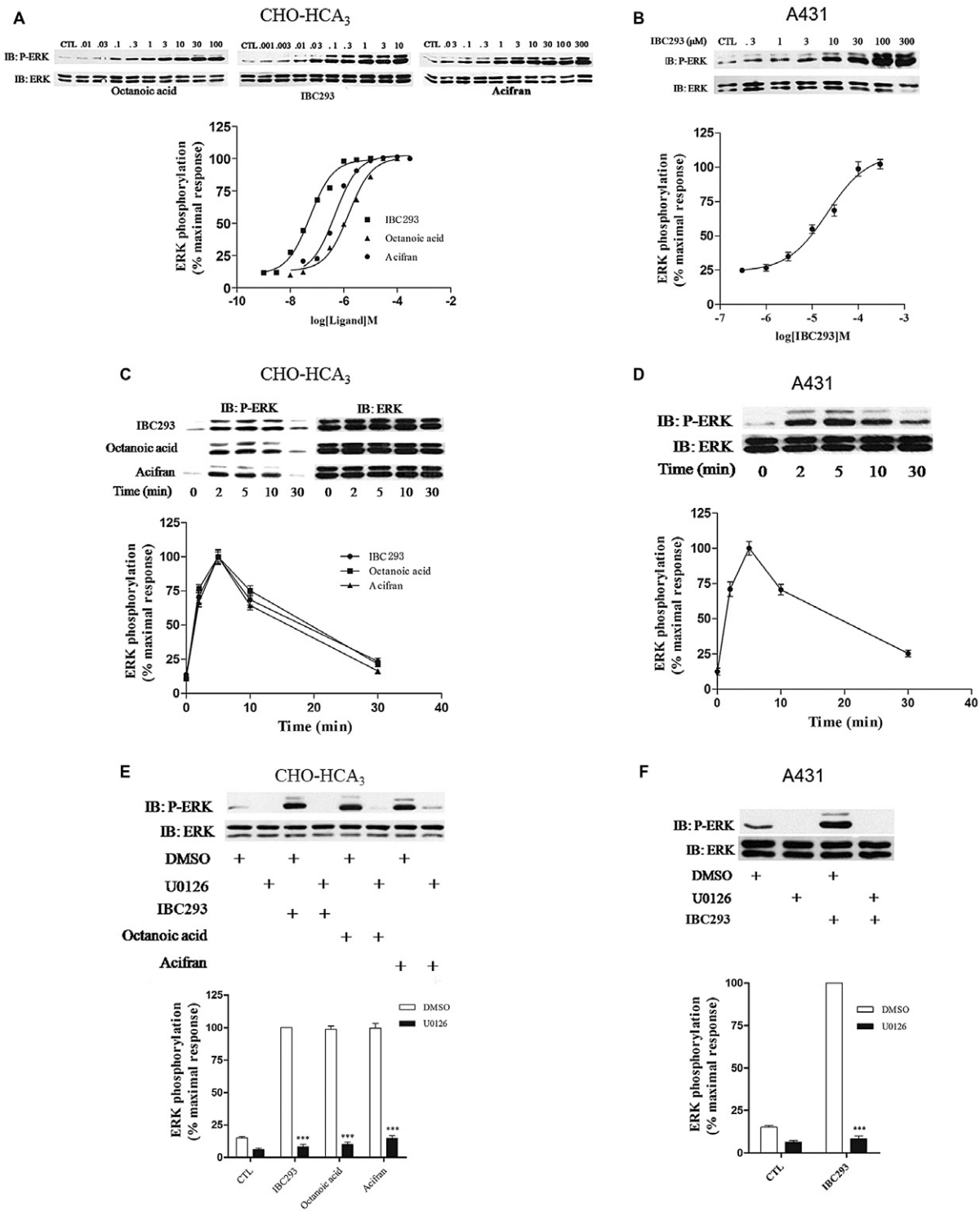


Figure 2

HCA_3 receptors activate ERK1/2 signalling via MEK1/2 pathway. (A) Serum-starved CHO-HCA_3 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-HCA_3 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-HCA_3 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. ***$P < 0.001$.

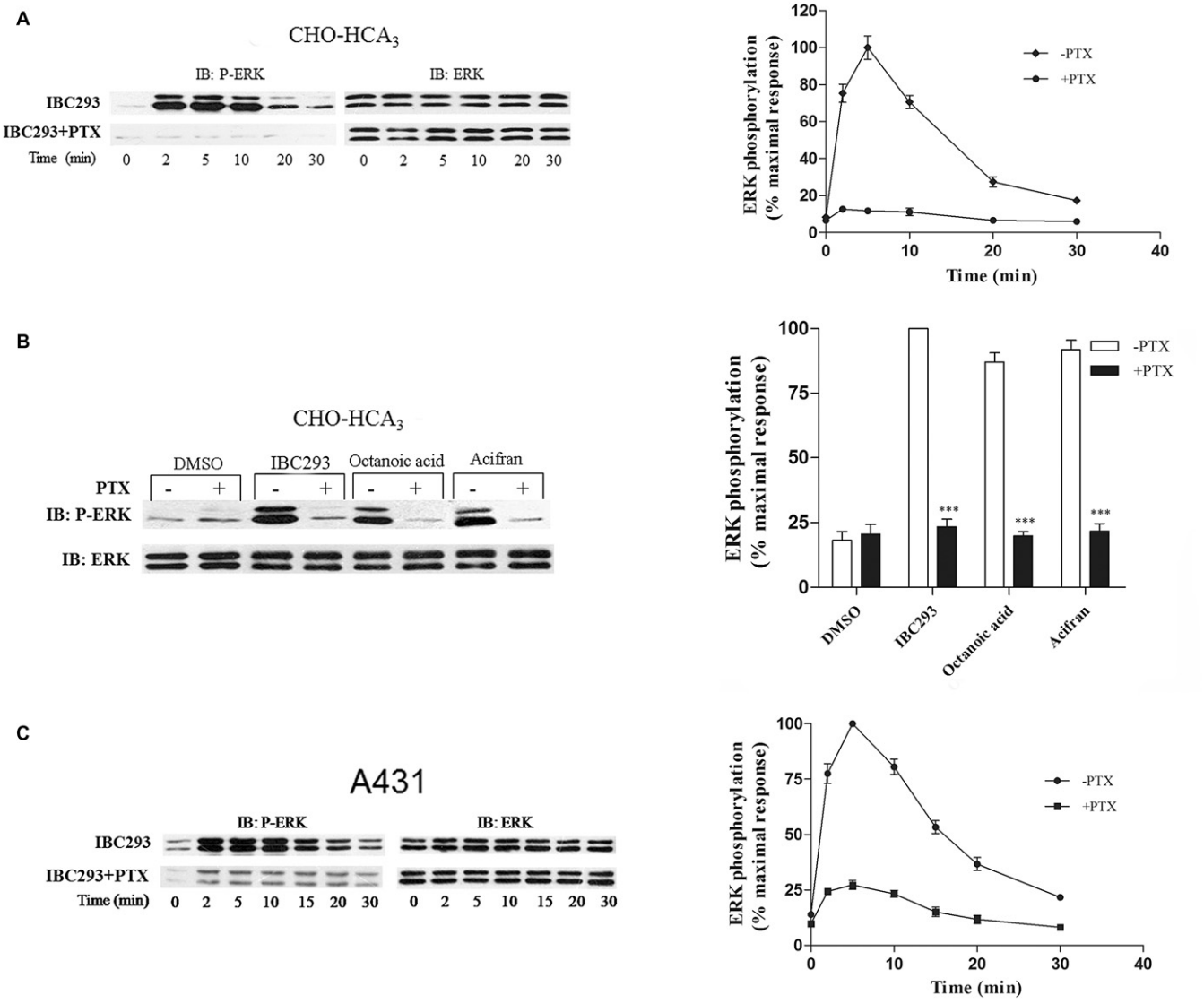
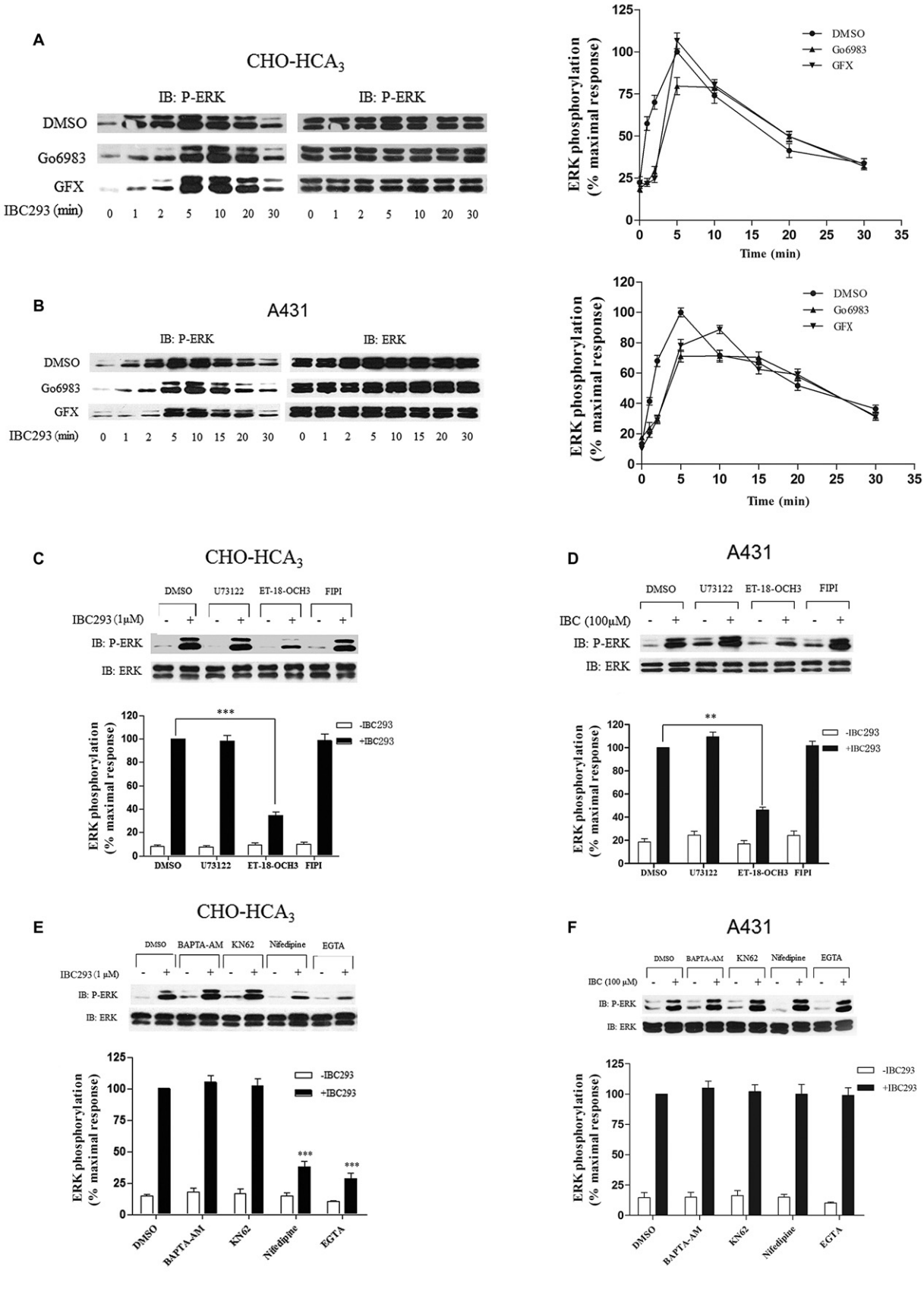


Figure 3

HCA_3 receptors initiate ERK1/2 activation via PTX-sensitive G_i protein-dependent pathway. CHO-HCA_3 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-HCA_3 or 100 μM IBC293 for A431 cells for the indicated time periods. (B) CHO-HCA_3 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 HCA_3-stimulated phosphorylation of ERK1/2. Serum-starved CHO-HCA_3 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-HCA_3 cells or 100 μM IBC293 for A431 cells for the indicated time periods. Serum-starved CHO-HCA_3 cells (C) or A431 cells (D) were pretreated with DMSO or 20 μM U73122 or 1 μM FIPI or 100 μM ET-18-OCH_3 for 1 h, and then stimulated with 1 μM IBC293 for CHO-HCA_3 cells or 100 μM IBC293 for A431 cells for 2 min. Serum-starved CHO-HCA_3 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-HCA_3 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. ***$P < 0.001$.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IB: P-ERK
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+IBC293
DMSO BAPTA-AM KN62 Nifedipine EGTA

inhibitory effect on ERK1/2 activation by HCA_3 receptors in A431 cells (Figure 4F). The intracellular Ca^{2+} chelators BAPTA-AM (100 μM) and Ca^{2+}-calmodulin kinase inhibitor KN62 (10 μM) also did not impair ERK1/2 activation by HCA_3 receptors in both CHO-HCA_3 and A431 cells (Figure 4E and F). Taken together, the results of the present study indicate that stimulation of HCA_3 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).

HCA_3-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-HCA_3 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 HCA_3-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 HCA_3 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 HCA_3 receptors evoke ERK1/2 activation via the PDGFR transactivation pathway in CHO-HCA_3 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 HCA_3-mediated ERK1/2 activation.

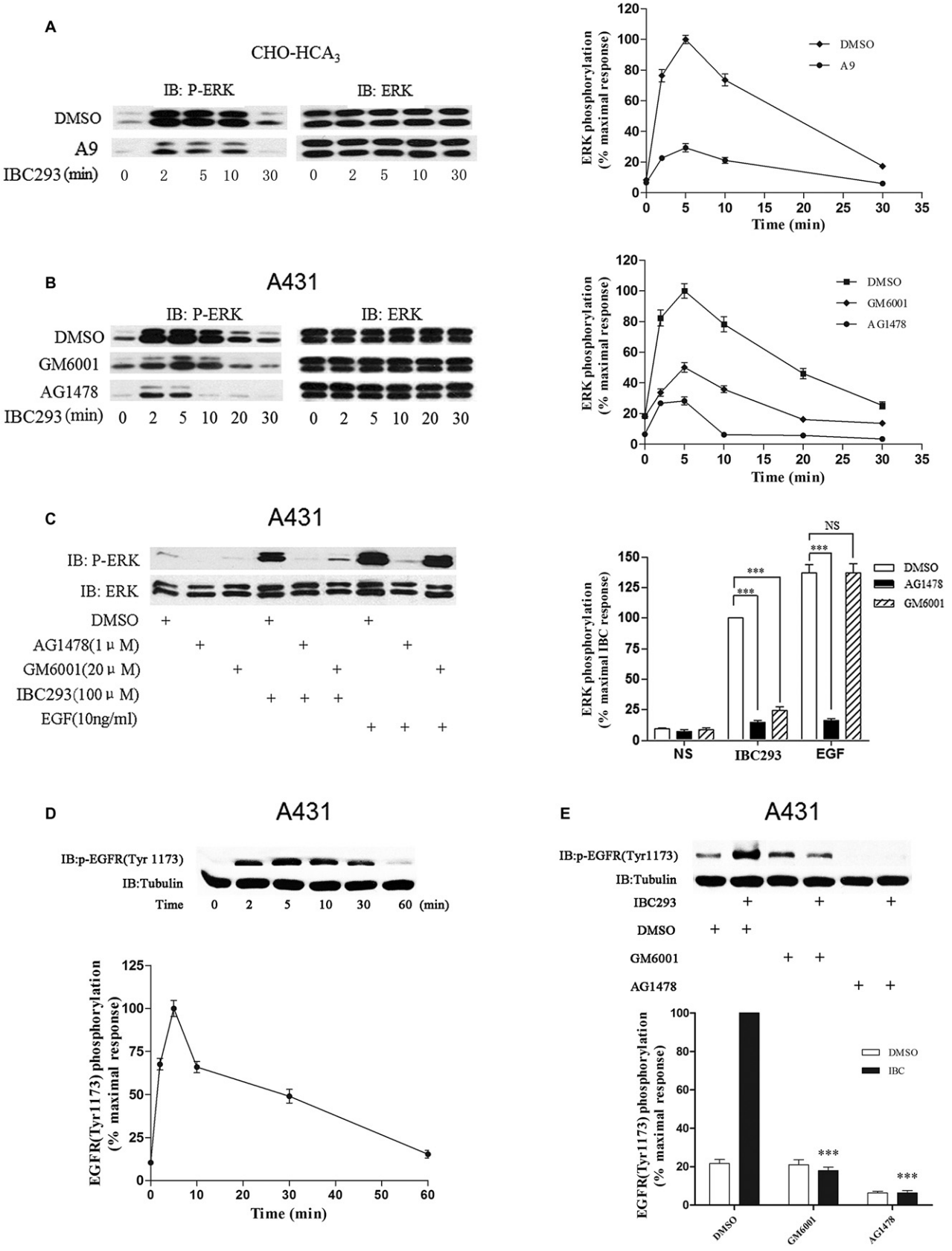
Involvement of the PI3K pathway in HCA_3-mediated ERK1/2 activation

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-Ilasaca *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-HCA_3 and A431 cells (Figure 6A and B), which suggests that PI3K plays an important role in HCA_3-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 HCA_3-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-HCA_3 and A431 cells. However, pretreatment with PP2 significantly decreased niacin-mediated ERK1/2 activation in CHO-HCA_2 cells. These results indicate that PI3K played an important role in HCA_3-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

HCA_3-induced ERK1/2 activation is dependent on growth factor receptor transactivation. (A) Serum-starved CHO-HCA_3 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 (Tyr^{1173}) antibody. Blots were stripped and reprobed 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. ***$P < 0.001$.


A
CHO-HCA3
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IB: ERK
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A9
IBC293 (min) 0 2 5 10 30 0 2 5 10 30
ERK phosphorylation (% maximal response)
Time (min)
DMSO
A9
B
A431
IB: P-ERK
IB: ERK
DMSO
GM6001
AG1478
IBC293 (min) 0 2 5 10 20 30 0 2 5 10 20 30
ERK phosphorylation (% maximal response)
Time (min)
DMSO
GM6001
AG1478
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A431
IB: P-ERK
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DMSO
AG1478(1 μ M)
GM6001(20 μ M)
IBC293(100 μ M)
EGF(10ng/ml)
ERK phosphorylation (% maximal IBC response)
NS
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DMSO
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Time 0 2 5 10 30 60 (min)
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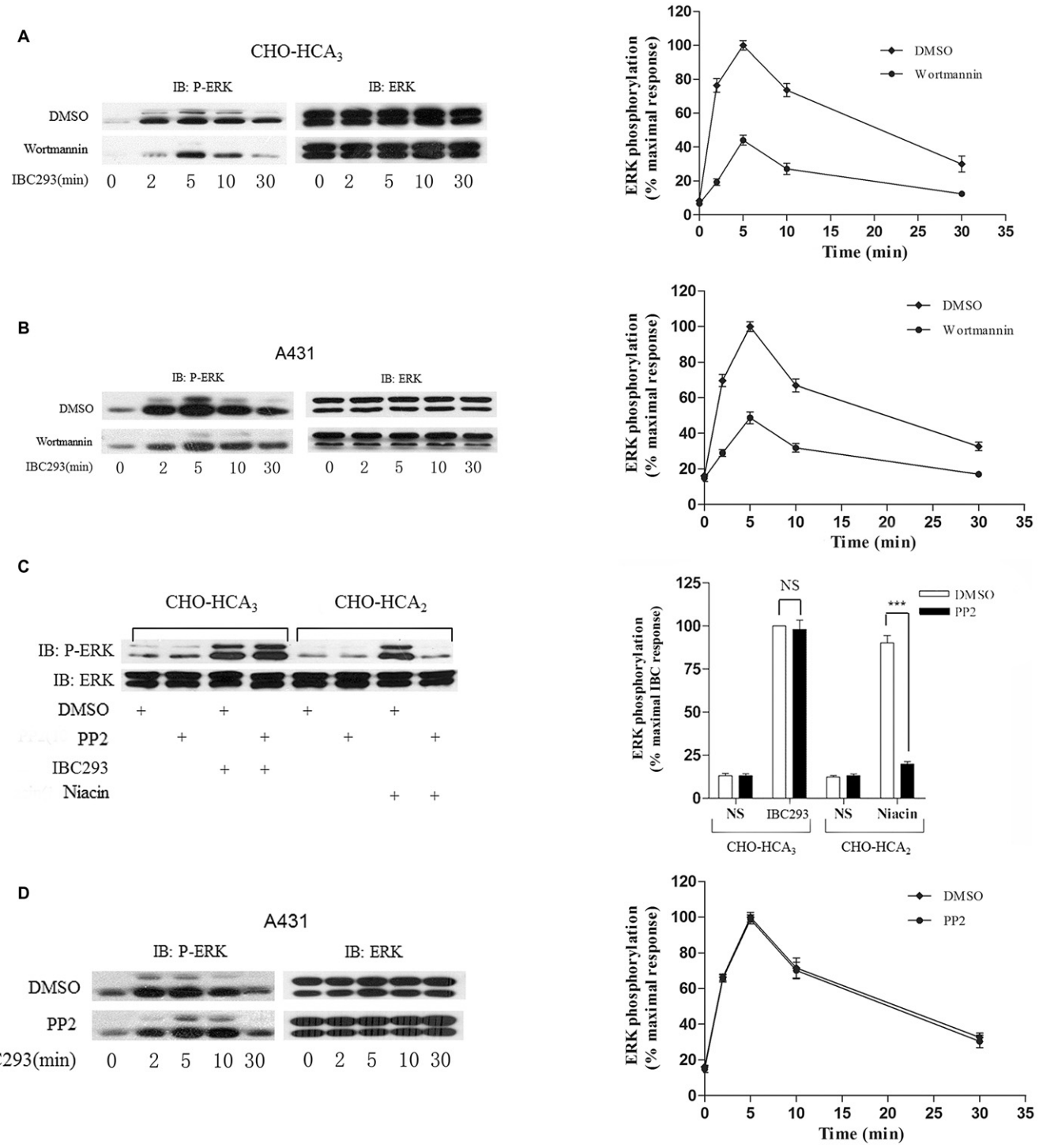

Figure 6

Effects of PI3K and Src on HCA_3-mediated ERK1/2 activation. Serum-starved CHO-HCA_3 cells (A) or A431 cells (B) were pretreated with DMSO or PI3K selective inhibitor wortmannin (1 μM) for 1 h, and then stimulated with 1 μM IBC293 for CHO-HCA_3 cells or 100 μM IBC293 for A431 cells for the indicated time periods. (C) Serum-starved CHO-HCA_2 or CHO-HCA_3 cells were pretreated with DMSO or the Src selective inhibitor PP2 (10 μM) for 1 h, and then stimulated with 1 μM niacin or 1 μM IBC293 respectively for 5 min. (D) Serum-starved A431 cells were pretreated with DMSO or PP2(10 μM) 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. Data were analysed by Student's *t*–test. $^{**}P < 0.01$, $^{***}P < 0.001$.

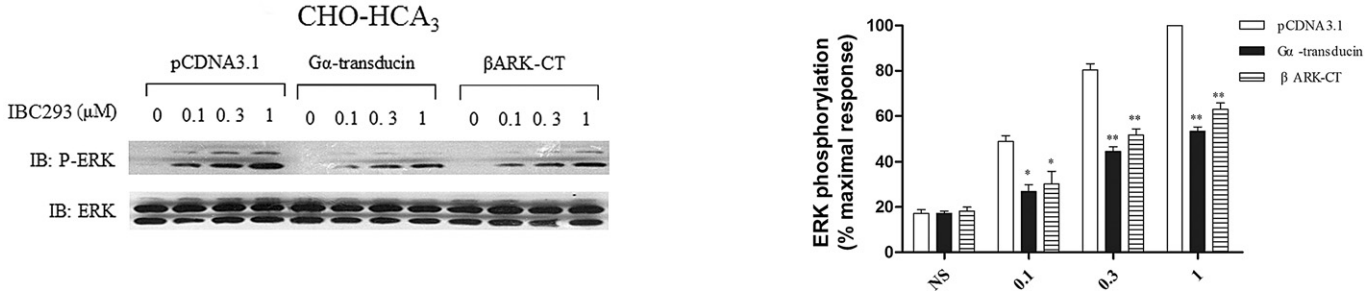

Figure 7

Effects of $G_{\beta\gamma}$ subunits on HCA_3-mediated ERK1/2 Activation. CHO-HCA_3 cells were transiently transfected with the $G_{\beta\gamma}$ scavengers βARK-CT or Gα-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 HCA_3-induced ERK1/2 activation

PLC and PI3K can be activated through a mechanism involving the $G_{\beta\gamma}$-subunits (Fields and Casey, 1997; Lopez-Ilasaca *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 HCA_3-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-HCA_3 cells with the β-adrenoceptor kinase COOH domain (495–689 aa) (βARK1-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 HCA_3-induced ERK1/2 phosphorylation was observed (Figure 7), which suggests that the $G_{\beta\gamma}$ subunit is likely to play a central role in HCA_3-induced ERK1/2 activation. Simultaneous inhibition of $G_{\beta\gamma}$ and PLC or $G_{\beta\gamma}$ and PDGFR in CHO-HCA_3 cells resulted in enhanced inhibition of HCA_3-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 HCA_3-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 HCA_3 internalization, but β-arrestins are not involved in HCA_3-mediated ERK1/2 activation

To evaluate the role of β-arrestins in the regulation of HCA_3 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 HCA_3 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 HCA_3 internalization, whereas knock-down of β-arrestin1 had no effect on the internalization of HCA_3 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 HCA_3 receptor internalization, but both β-arrestins are not required for HCA_3-mediated ERK1/2 activation.

Discussion and conclusions

It is generally accepted that HCA_3 receptors, which differ from HCA_2 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 HCA_2 receptors mediates its antilipolytic and lipid-lowering effects (Zhang *et al.*, 2005), HCA_3 receptors have been shown to have very similar expression patterns to HCA_2 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 HCA_2 receptors and by PUMA-G (Benyo *et al.*, 2005), and it suggests that selective activators of HCA_3 receptors may avoid the characteristic and uncomfortable cutaneous flushing response elicited by niacin in humans (Skinner *et al.*, 2009). However, the exact role of HCA_3 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 HCA_3 receptors with physiological significance (Ahmed *et al.*, 2009; Irukayama-Tomobe *et al.*, 2009). HCA_3, together with HCA_2 receptors are of great interest as targets for the development of new antidyslipidemic drugs. Information about the signalling pathways linked to activated HCA_3 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 HCA_3 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

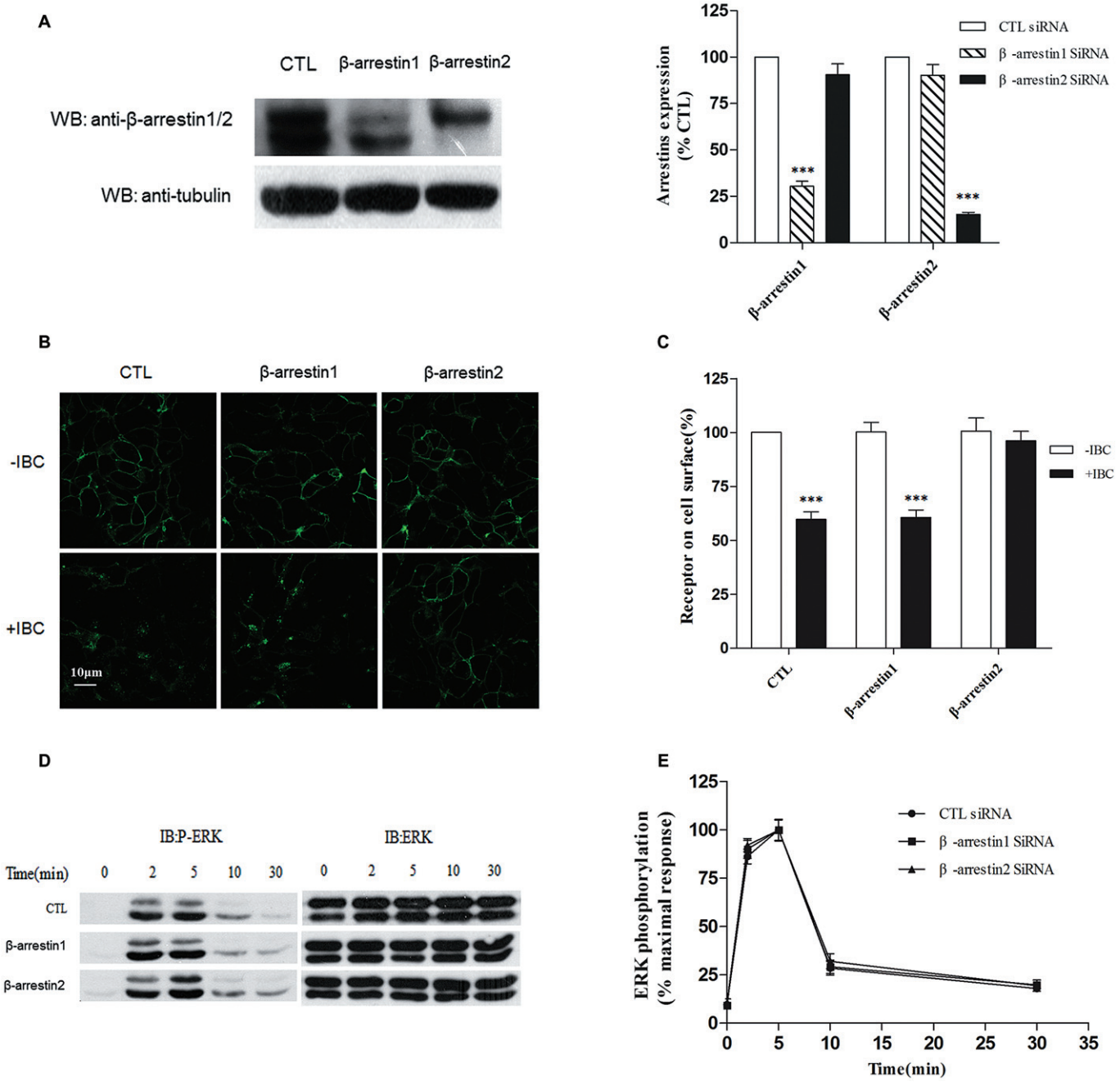

Figure 8

There is no involvement of β-arrestins in HCA_3-mediated ERK1/2 activation. (A) HEK-293 cells stably expressing HCA_3 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 HCA_3-EGFP were transfected with specific β-arrestin siRNA or a non-specific control siRNA,72 h after transfection, cells were stimulated with 100 μM IBC293 for 40 min and examined with confocal microscopy as described under 'Experimental Procedures.' (C) ELISA determination of cell surface receptors in Flag-HCA_3 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 μM 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 HCA_3-mediated ERK1/2 activation via different temporal components remains unknown. In the current study, we focused on a detailed characterization of HCA_3-mediated MAPK signalling pathways, and we demonstrated that activated HCA_3 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 HCA_3-mediated ERK1/2 activation because it is a commonly used cell line for investigating GPCR signalling pathways. For better understanding of HCA_3-induced ERK1/2 phosphorylation, A431 cells, a human epidermoid cell with high levels of endogenous expression of functional HCA_3 receptors (Zhou *et al.*, 2007) was also selected for this study. In our preliminary experiments, we found that although all three agonists of HCA_3 receptors triggered significant ERK1/2 phosphorylation in CHO-HCA_3 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 HCA_2 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 HCA_3 receptors in A431 cells.

The HCA_3 receptor is a G_i protein-coupled receptor. Upon stimulation by agonists, HCA_3 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 Ca^{2+} levels in a PTX-sensitive manner (Irukayama-Tomobe *et al.*, 2009). Thus, we explored the effect of the PLC/PKC pathway in HCA_3-mediated ERK1/2 activation. The inhibitory effect of the PKC inhibitors Go6983 and GFX suggested a critical role for PKC on HCA_3-mediated ERK1/2 activation at early time points (≤2 min). The involvement of PLC as a contributor to HCA_3-mediated ERK1/2 activation was assessed by incubating cells with two PLC inhibitors, ET-18-OCH_3 and U-73122. ET-18-OCH_3, but not U-73122, exhibited significant inhibition of ERK1/2 phosphorylation by activated HCA_3. U73122 is known to be a selective inhibitor of PLCβ (Ward *et al.*, 2003) and ET-18-OCH_3 to be a PLCγ-selective inhibitor (Souttou *et al.*, 2001; Suzuki *et al.*, 2008). ET-18-OCH_3 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-OCH_3 on HCA_3-mediated ERK1/2 activation can be accounted for suppression of both PLC and Raf in this study. Furthermore, we also demonstrated that HCA_3-induced ERK1/2 activation was abolished by the depletion of extracellular Ca^{2+} by the chelator EGTA and by nifedipine, an L-type Ca^{2+} channel blocker in CHO-HCA_3 cells, suggesting that the L-type Ca^{2+} channel may play an important part in HCA_3-mediated ERK1/2 activation in CHO-K1 cells. However, Ca^{2+} was found to play no role in HCA_3-mediated ERK1/2 activation in A431 cells. The discrepancy in the role of Ca^{2+} in HCA_3-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 AT_1 receptor-mediated activation of ERK1/2 by angiotensin II was Ca^{2+}–dependent in rat anterior pituitary cells, but Ca^{2+}–independent in hepatic C9 cells(Suarez *et al.*, 2003) (Shah and Catt, 2002). Taken together, these data suggest the involvement of both Ca^{2+}-dependent and -independent PKC isoforms in HCA_3-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 HCA_3-mediated ERK1/2 activation was potently inhibited by the PDGFR-selective tyrphostin A9 and the PI3K inhibitor wortmannin in CHO-K1 cells. However, in A431 cells, HCA_3-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 HCA_3 receptor agonists, but not by EGF, defines the intermediary action of the MMP-dependent shedding of HB-EGF in the transactivation of EGFR by HCA_3 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; Prenzel *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 HCA_3 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 HCA_3-induced EGFR transactivation in either CHO or A431 cells. However, more studies are necessary for the clarification of the exact role of PI3K in HCA_3-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 HCA_3 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 βARK-CT or Gα-transducin effectively attenuated the ERK1/2

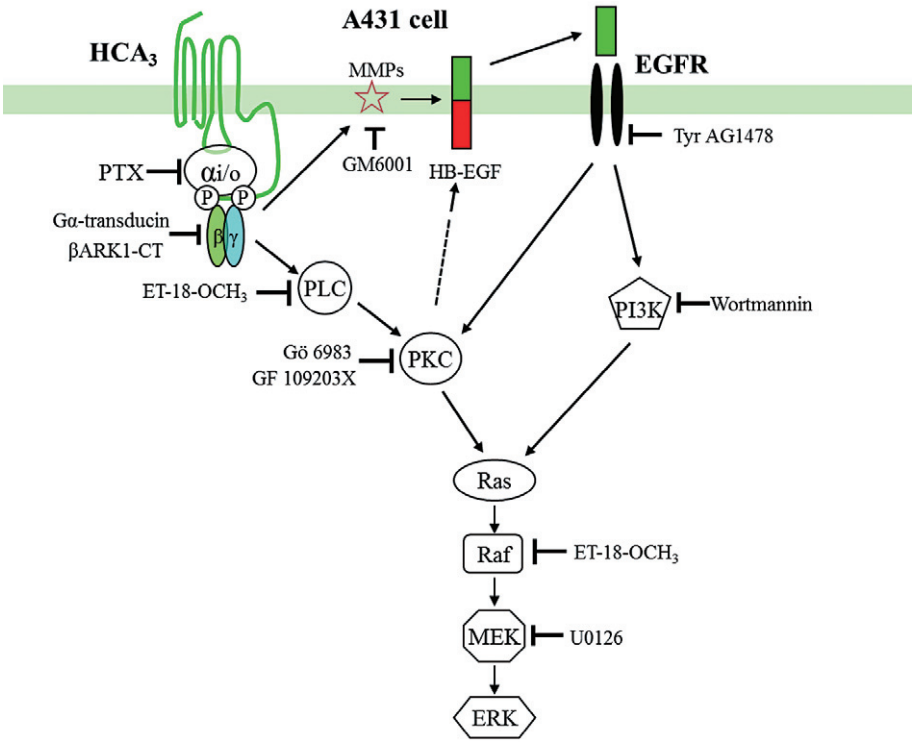

Figure 9

Schematic diagram of regulation of HCA_3-induced ERK1/2 activation in A431 cells. In response to agonists, activated HCA_3 receptors led to dissociation of G_i proteins from $G_{\beta\gamma}$-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 HCA_3 receptors (Figure 7). These results indicate that the $G_{\beta\gamma}$ subunit acts as an early signal mediating HCA_3-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 $G_{\beta\gamma}$ subunits (Crespo *et al.*, 1994; Hawes *et al.*, 1995). Previous studies have shown that G_i-type GPCRs stimulate Ca^{2+} mobilization through the binding of the $G_{\beta\gamma}$ subunits to PLC (Dorn *et al.*, 1997; Dickenson and Hill, 1998). It has also been reported that the best-understood mechanism whereby the $G_{\beta\gamma}$ 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 $G_{\beta\gamma}$ subunit protein effectors that regulate HB-EGF release remain undefined. Thus, we postulate that upon stimulation of HCA_3 receptors by agonists, activated G_i protein impairs cAMP production and its released $G_{\beta\gamma}$ 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 $G_{\beta\gamma}$ 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 HCA_2 receptors in CHO-K1 cells and A431 cells (Li *et al.*, 2011), although Src was found to play no role in HCA_3-mediated ERK1/2 activation in CHO-K1 cells stably expressing HCA_3 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), β_2-adrenoceptors (Shenoy *et al.*, 2006), vasopressin 2 (Ren *et al.*, 2005) and parathyroid hormone (Gesty-Palmer *et al.*, 2006) receptors, whereas, in the dopamine D_2 and D_3 receptors (Beom *et al.*, 2004; Quan *et al.*, 2008) and the formyl peptide receptor (Huet *et al.*, 2007; Gripentrog 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 HCA_3 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 HCA_2-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 HCA_3-mediated ERK1/2 activation in CHO cells that are stably transfected with HCA_3 receptors and in A431 cells that endogenously express HCA_3 receptors (Figure 9). In response to agonists, activated HCA_3 receptors induce the dissociation of G_i proteins from $G_{\beta\gamma}$-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 HCA_3-induced ERK1/2 activation. However, additional investigations will be necessary to further clarify the role of the ERK1/2 pathway in HCA_3-mediated lipolysis.

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Conflict of interest

The authors state no conflict of interest.

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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 HCA_3-induced ERK1/2 activation. a, Serum-starved CHO-HCA_3 cells were pretreated with DMSO or A9 or ET-18-OCH_3 or both A9 and ET-18-OCH_3 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-OCH_3 or both AG1478 and ET-18-OCH_3 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 $G_{\beta\gamma}$ and PDGFR or PLC in HCA_3-induced ERK1/2 activation. a, CHO-HCA_3 cells were transiently transfected with the $G_{\beta\gamma}$ 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-HCA_3 cells were transiently transfected with the $G_{\beta\gamma}$ scavenger βARK-CT or empty vector, the cells were serum-starved for 24 h and pretreated with DMSO or ET-18-OCH_3, 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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