British Journal of Pharmacology

DOI:10.1111/bph.13254
www.brjpharmacol.org

RESEARCH PAPER

Role for engagement of β-arrestin2 by the transactivated EGFR in agonist-specific regulation of δ receptor activation of ERK1/2

Le-Sha Zhang[1#], Yu-Jun Wang[1#], Yun-Yue Ju[1], Gui-Ying Zan[1], Chi Xu[1], Min-Hua Hong[1], Yu-Hua Wang[2], Zhi-Qiang Chi[1] and Jing-Gen Liu[1]

[1]Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica and Collaborative Innovation Center for Brain Science, Chinese Academy of Science, Shanghai, China, and [2]School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China

Correspondence
Jing-Gen Liu, PhD, Department of Neuropharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Shanghai, China.
E-mail: jgliu@mail.shcnc.ac.cn.
Yu-Hua Wang, PhD, School of Pharmacy, Nanjing University of Chinese Medicine, 138 Xian Lin Road, Nanjing, China.
E-mail: rain7943@126.com
#These authors contributed equally to this work.

Received
1 April 2015
Revised
11 June 2015
Accepted
16 July 2015

BACKGROUND AND PURPOSE

β-Arrestins function as signal transducers linking GPCRs to ERK1/2 signalling either by scaffolding members of ERK1/2s cascades or by transactivating receptor tyrosine kinases through Src-mediated release of transactivating factor. Recruitment of β-arrestins to the activated GPCRs is required for ERK1/2 activation. Our previous studies showed that δ receptors activate ERK1/2 through a β-arrestin-dependent mechanism without inducing β-arrestin binding to the δ receptors. However, the precise mechanisms involved remain to be established.

EXPERIMENTAL APPROACH

ERK1/2 activation by δ receptor ligands was assessed using HEK293 cells *in vitro* and male Sprague Dawley rats *in vivo*. Immunoprecipitation, immunoblotting, siRNA transfection, intracerebroventricular injection and immunohistochemistry were used to elucidate the underlying mechanism.

KEY RESULTS

We identified a new signalling pathway in which recruitment of β-arrestin2 to the EGFR rather than δ receptor was required for its role in δ receptor-mediated ERK1/2 activation in response to H-Tyr–Tic–Phe–Phe–OH (TIPP) or morphine stimulation. Stimulation of the δ receptor with ligands leads to the phosphorylation of PKCδ, which acts upstream of EGFR transactivation and is needed for the release of the EGFR-activating factor, whereas β-arrestin2 was found to act downstream of the EGFR transactivation. Moreover, we demonstrated that coupling of the PKCδ/EGFR/β-arrestin2 transactivation pathway to δ receptor-mediated ERK1/2 activation was ligand-specific and the Ser^{363} of δ receptors was crucial for ligand-specific implementation of this ERK1/2 activation pathway.

CONCLUSIONS AND IMPLICATIONS

The δ receptor-mediated activation of ERK1/2 is via ligand-specific transactivation of EGFR. This study adds new insights into the mechanism by which δ receptors activate ERK1/2.

Abbreviations

DPDPE, [D-Pen2, D-Pen5] enkephalin; HB-EGF, heparin-binding EGF-like growth factor; IGFR, insulin-like growth factor receptor; NG108-15, cell mouse neuroblastoma x rat glioma hybrid cell; RTK, receptor tyrosine kinase; TIPP, H-Tyr-Tic-Phe-Phe-OH

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[c]
δ receptor	ERK1
Catalytic receptors[b]	ERK2
EGFR	PKCδ
IGFR	PKCε
TrkA	MMP
	Src

LIGANDS	
AG879	GF109203X
AG1478	HB-EGF
DPDPE	Morphine
	Naltrindole

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

The δ receptor is a member of the GPCRs. Activation of δ receptors leads to inhibition of adenylyl cyclase activity and voltage-gated Ca^{2+} channels and the activation of inwardly rectifying K^+ channels through coupling to *Pertussis toxin* (PTX)-sensitive G-proteins (Law and Loh, 1999). In addition, δ receptors can also signal to other cellular effectors such as ERK1/2 (Eisinger *et al.*, 2002; Audet *et al.*, 2005; Hong *et al.*, 2009). It has been shown that ERK1/2 plays an important role in the neuronal plasticity induced by drugs of abuse and blockade of ERK1/2 activation prevents long-lasting behavioural changes, including psychomotor sensitization and conditioned place preference induced by these drugs (Girault *et al.*, 2007). Although most of their actions are mediated by G-proteins, our recent study has shown that δ receptors can activate ERK1/2 in a G-protein-independent but β-arrestin-dependent manner (Xu *et al.*, 2010). However, the precise mechanisms involved remain to be established.

β-Arrestins have been shown to function as signal transducers linking GPCRs to ERK1/2 signalling either by scaffolding members of ERK1/2 cascades (DeFea *et al.*, 2000; Luttrell *et al.*, 2001; Tohgo *et al.*, 2002) or by transactivating receptor tyrosine kinases (RTKs) through Src-mediated release of transactivating factor (Pierce *et al.*, 2001; Noma *et al.*, 2007). Given that β-arrestins are generally thought to be regulators of GPCRs, their recruitment to the activated GPCRs has been assumed to be required for linking GPCRs to ERK1/2 signalling (McDonald *et al.*, 2000; Luttrell *et al.*, 2001). However, in recent studies we found that, although the stimulation of δ receptors with opioid ligands H-Tyr-Tic-Phe-Phe-OH (TIPP) and morphine failed to recruit β-arrestin1/2 to these receptors (Hong *et al.*, 2009), they were able to activate ERK1/2 through a β-arrestin-dependent mechanism (Xu *et al.*, 2010). Additionally, previous studies have also reported that stimulation of the β_2 adrenoceptor with its ligands ICI118551 and propranolol activates ERK1/2 through a β-arrestin-dependent mechanism without promoting β-arrestin binding to the receptors (Azzi *et al.*, 2003; Wisler *et al.*, 2007). These studies suggest that recruitment of β-arrestins to the GPCRs may not be a prerequisite for their function as signal transducers linking GPCRs to ERK1/2 signalling. However, how GPCRs such as the δ receptor and β_2 adrenoceptor, which fail to recruit β-arrestins in response to specific ligand stimulation, can robustly engage a β-arrestin-dependent signalling pathway to activate ERK1/2 remains to be addressed.

GPCRs including δ receptors have also been shown to activate ERK1/2 by transactivating RTK (Prenzel *et al.*, 1999; Eisinger and Schulz, 2004; Werry *et al.*, 2005). Several previous studies have also suggested that RTKs such as the EGFR and insulin-like growth factor receptor (IGFR) can recruit β-arrestins in response to their respective growth factors (Povsic *et al.*, 2003; Girnita *et al.*, 2007), suggesting that β-arrestins are not restricted to binding to GPCRs. This raises the possibility that GPCRs, which are unable to recruit β-arrestins in response to specific ligand stimulation, might use β-arrestins as a scaffold to activate ERK1/2 through transactivated RTKs. Indeed, a recent study demonstrated that the V_2 vasopressin receptor can activate ERK1/2 through a mechanism involving the engagement of β-arrestin by a transactivated IGFR (Oligny-Longpre *et al.*, 2012). Given that δ receptors can activate ERK1/2 through the transactivation of EGFR (Schulz *et al.*, 2004; Eisinger and Ammer, 2008a) and IGFR (Eisinger and Ammer, 2011) and that IGFR and EGFR can recruit β-arrestins (Lin *et al.*, 1998; Dalle *et al.*, 2001; Zheng *et al.*, 2012), we hypothesized that δ receptors stimulated by TIPP or morphine might activate ERK1/2 in a β-arrestin-dependent manner through the transactivation of EGFRs. This study was therefore undertaken to test this hypothesis.

Methods

Mutagenesis of δ receptor

The serine codon (TCC) for amino acid 363 of the mouse δ receptor was mutated into an alanine codon (GCC) by Dr Chi Xu (Xu *et al.*, 2010).

Cell culture and transfection

The cDNA of pcDNA3.0 containing the mouse δ receptor with the HA epitope tag inserted to the N-terminus of δ receptor or the mutated δ receptor was transfected into HEK293 cells

using Transmarter transfection reagent. Cells stably expressing HA-δ receptor and S363A-HA-δ receptor were obtained by MACS cell selection kits (Miltenyi Biotec, Bergisch Gladbach, Germany) and maintained by 0.2 mg · mL^{-1} G418. The HEK293δ receptor cells transiently expressing the cDNA of pcDNA3.1 containing the β-arrestin2-GFP were transfected using Transmarter transfection reagent.

Immunoprecipitation and immunoblotting

Cells were solubilized in 1 mL of glycerol lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol v v^{-1}, 0.5% Nonidet P-40 v v^{-1}, 2 mM EDTA, 100 μM Na_3VO_4 and proteinase inhibitor cocktail) and clarified by centrifugation at 10 000 × *g* for 10 min. Immunoprecipitation was performed using the corresponding antibody with constant agitation overnight at 4°C, then 30 μL of 50% slurry of protein A/G agarose beads with constant agitation for 4 h at 4°C. Immune complexes were washed three times and boiled in Laemmli sample buffer. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membrane for immunoblotting. Chemiluminescence detection was performed by using the ECL plus Western blotting detection reagent (GE Health, Little Chalfont, UK), and immunoblots were quantified by densitometry using Quantity One (Bio-Rad, Hercules, CA, USA). For repeated immunoblotting, membranes were stripped in ReBlotPlus mild stripping solution for 20 min.

siRNA transfection

Double-stranded siRNAs targeting PKCδ were purchased from Santa Cruz Biotechnology. Chemical synthesis of double-stranded siRNAs targeting β-arrestin1 and β-arrestin2 was performed as described previously (Zhang *et al.*, 2005). A non-silencing RNA duplex was used as control. For siRNA experiments, HEK293 δ receptor cells or S363A-HEK293 δ receptor that were 20–30% confluent on 24-well plates were transfected with 20 pmol of siRNA using the lipofectamine RNAiMAX reagent. Cells were stimulated by ligands after 48 h and then harvested.

Animal treatment

Sprague Dawley male rats weighing 250–300 g were obtained from the Laboratory Animal Centre, Chinese Academy of Sciences (Shanghai, China). Rats were housed two to three per cage and maintained on a 12 h light/dark cycle with access to food and water *ad libitum*. A total of 127 rats were divided into 10 groups of at least seven rats each. All protocols were reviewed and approved by the IACUC of Shanghai Institute of Materia Medica, which received the full accreditation of AAALAC in May 2011. All studies involving animals are reported in accordance with the ARRIVE guidelines for experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Intracerebroventricular injection

Rats were anaesthetized with sodium pentobarbital (55 mg · kg^{-1}, i.p.) in combination with atropine (0.4 mg · kg^{-1}, i.p.). Depth of anaesthesia was checked by testing for lack of a pain response to gentle pressure on the hind paws. Then, the animals were placed in the stereotaxic apparatus (Narishige, Setagaya-ku, Tokyo, Japan). Guide cannulas were implanted bilaterally in the rats' brain (AP: +0.8 mm; ML: ±1.5 mm; and DV: +2.0 mm). A stainless steel blocker was inserted into each cannula to keep them patent and prevent infection. The rats were allowed to recover from surgery for 5 days. Five days later, bilateral microinfusions were made through 31 gauge injection cannulae (1.8 mm under the tip of guide cannulae) over 2 min at rate of 2.5 μL · min^{-1} and given an additional 1 min for drug diffusion. Morphine was dissolved in ACSF at a concentration of 20 nmol · 10 μL^{-1}, while [D-Pen2, D-Pen5] enkephalin (DPDPE) was dissolved in ACSF containing 10% DMSO at a concentration of 100 nmol · 10 μL^{-1}. GM6001 and AG879 were respectively diluted with ACSF to a final concentration of 10 ng · 10 μL^{-1} and 5 mM (injection volume 10 μL) , then bilaterally microinjected 1 h before agonist injection. The δ receptor antagonist naltrindole was dissolved in ACSF at a concentration of 5 μg · 10 μL^{-1} and bilaterally microinjected 15 min before agonist injection. Rats in the control group were injected with the corresponding solvent at the same time. During the microinjection, the rats were gently restrained by hand.

Immunohistochemistry

Fifteen minutes after agonist administration, rats were quickly anaesthetized with sodium pentobarbital (55 mg · kg^{-1}, i.p). Deeply anaesthetized rats were perfused with saline and then 4% cold paraformaldehyde in 0.1 mol · L^{-1} phosphate buffer. Brains were removed from the skulls and postfixed in 4% paraformaldehyde overnight at 4°C. The next day, the brains were transferred to a solution of 30% sucrose overnight until they sunk. Cryostat sections of these brains were then cut at a thickness of 30 μm on a Leica CM3050S cryostat (Leica, Wetzlar, Germany). Brain sections were stored in 10% sucrose and 0.03% sodium azide in 0.1 mol · L^{-1} phosphate buffer at 4°C for further processing. All washes were conducted in 0.1 mol · L^{-1} phosphate buffer, and sections were blocked with 10% normal goat serum for 2 h at room temperature. Sections were incubated overnight in primary antibody (1:300 dilution in 10% normal goat serum) at 4°C. Then, sections were incubated with secondary antibody (1:200 dilution in 10% normal goat serum) for 2 h at room temperature. pERK-positive sites were visualized using a SABC kit and a DAB kit. The brain sections were subsequently dehydrated in alcohol and xylene and mounted on slides under coverslips. Finally, they were imaged on an Olympus IX51 microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). To determine the level of ERK activity, the number of stained cells in CeA of the amygdala was manually counted.

Data analysis

All statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Data represent mean ± SD of at least three separate experiments. All the quantifications of phosphoproteins calculated as the ratio of total proteins. Statistical significance was determined by using Student's unpaired *t*-test when only two groups were compared. In the immunohistochemistry experiment, the data represent mean ± SEM; statistical significance was determined by one-way ANOVA followed by

Newman–Keuls comparison test. The number of animals in each group was at least seven.

Chemicals and reagents

The following antibodies were used in immunoblotting: anti-HA-tag, anti-pSer^{363}-δ receptor, anti-β-arrestin1/2, anti-pSer^{643}-PKCδ, anti-EGFR and anti-Src antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-phosphotyrosine, anti-pERK antibodies for Western blotting and anti-ERK2 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-PKCδ antibody was from Abcam (Cambridge, MA, USA). The anti-pERK antibody for immunohistochemistry was from Cell Signaling Technology. GM6001 was from Tocris Cookson, and TIPP was from Phoenix Biotech (Beijing, China). The anti-GAPDH, anti-PKCε, anti-pY416-Src antibodies, PP2, PTX and ReBlotPlus mild stripping solution (10×) were from Merck-Millipore (Bedford, MA, USA). Transmarter transfection reagent was from Abmart (Shanghai, China). LipofectamineRNAiMaxsiRNA transfection reagent was from Invitrogen (Carlsbad, CA, USA). Morphine hydrochloride was from Qinghai Pharmaceutical General Factory. All other reagents and antibodies were purchased from Sigma (St Louis, MO, USA). All the drug/receptor nomenclature confirms to BJP's Concise Guide to Pharmacology (Alexander *et al.*, 2013a,b,c).

Results

TIPP- and morphine-stimulated, but not DPDPE-stimulated, ERK1/2 activation involves the transactivation of RTKs

Our previous studies showed that the δ receptor ligands TIPP and morphine, but not DPDPE, stimulated ERK1/2 activity by using β-arrestin1/2 as a scaffold for kinases involved in the ERK1/2 cascade (Xu *et al.*, 2010), although TIPP and morphine were both unable to stimulate the recruitment of β-arrestin1/2 to the δ receptors (Hong *et al.*, 2009). Given that δ receptors can activate ERK1/2 by transactivating EGFR and IGFR (Forster *et al.*, 2007; Eisinger and Ammer, 2008a, 2011) and that EGFR and IGFR can act like GPCRs, engaging β-arrestins for downstream signalling (Lin *et al.*, 1998; Dalle *et al.*, 2001; Zheng *et al.*, 2012), we explored whether the δ receptors can employ β-arrestin1/2 as scaffolding proteins for ERK1/2 activation by transactivating EGFR in response to TIPP or morphine stimulation. To this end, we first determined whether EGFR transactivation is involved in TIPP or morphine-induced ERK1/2 activation by examining the effects of the metalloproteinase inhibitor GM6001 and EGFR inhibitor AG1478 on TIPP, morphine and DPDPE-stimulated ERK1/2 activation in HEK293 cells stably expressing δ receptors (HEK293 δ receptors). As shown in Figure 1A, stimulation of HEK293 δ receptor cells with 1 μM DPDPE, 1 μM TIPP or 10 μM morphine resulted in an enhancement of ERK1/2 phosphorylation. Pretreatment with 25 μM GM6001 or 5 μM AG1478 inhibited this TIPP- and morphine-induced but not DPDPE-induced ERK1/2 activation in HEK293 δ receptor cells, indicating that TIPP and morphine, but not DPDPE, stimulate ERK1/2 activity by transactivating EGFRs.

Mouse neuroblastoma x rat glioma hybrid cells (NG108-15 cells), which endogenously express high levels of δ receptors, have been widely used to investigate acute and chronic opioid actions. The TrkA receptor represents another member of the superfamily of RTKs that has been shown to link δ receptors to the ERK1/2 signalling pathway in NG108-15 cells (Eisinger and Ammer, 2008b). To further confirm that TIPP and morphine but not DPDPE can stimulate ERK1/2 activity by transactivating a RTK, we determined the effects of GM6001 and the TrkA inhibitor AG879 on ERK1/2 phosphorylation stimulated by TIPP, morphine and DPDPE. In line with the results obtained in the HEK293 δ receptor cells, pretreatment of cells with GM6001 (25 μM) and AG879 (10 μM) led to a decrease in the ERK1/2 phosphorylation induced by TIPP (1 μM) and morphine (10 μM) but not DPDPE (1 μM) (Figure 1B).

To exclude another target of TIPP and morphine, we also tested whether TIPP and morphine could induce ERK1/2 activation in δ receptor-null HEK293 cells, which did not express any endogenous δ receptors. The data showed that the EGFR agonist EGF, but neither TIPP nor morphine, stimulated robust ERK1/2 phosphorylation in these cells (Figure 1C).

To determine if such agonist-specific involvement of the transactivation of EGFR in δ receptor-mediated activation of ERK1/2 uncovered in cultured cells could also occur in native tissue, we investigated the effects of GM6001 and AG879 on morphine and DPDPE-stimulated ERK1/2 activation in rat brain. As shown in Figure 1D and E, i.c.v. administration of DPDPE (100 nmol · 10 μL^{-1}) and morphine (20 nmol · 10 μL^{-1}) both led to ERK1/2 activation specifically in the CeA of the amygdala, an area critically involved in the development of drug cravings (Lu *et al.*, 2005); this was revealed by the increased ERK1/2 staining detected by immunohistochemistry. Although pretreatment with the selective δ receptor antagonist naltrindole (5 μg · 10 μL^{-1}, i.c.v.) for 15 min blocked both the morphine and DPDPE-induced increase in ERK1/2 staining, pretreatment with GM6001 (10 ng · 10 μL^{-1}, i.c.v) and AG879 (5 mM · 10 μL^{-1}, i.c.v.) for 1 h significantly inhibited only the morphine but not the DPDPE-induced increase in ERK1/2 staining, confirming that the agonist-specific involvement of the transactivation of EGFR in δ receptor-mediated activation of ERK1/2 can also occur *in vivo*.

Stimulation of δ receptors with DPDPE activates Src in a PTX-sensitive manner, whereas stimulation of δ receptors by TIPP and morphine activates PKCδ in a PTX-insensitive manner

Src activation has been shown to be required for the transactivation of EGFR by GPCRs (Pierce *et al.*, 2001; Kim *et al.*, 2002; Noma *et al.*, 2007). We further determined the effects of morphine, TIPP and DPDPE on Src activation by detecting Tyr^{416} residue phosphorylation of Src. Unexpectedly, treatment of HEK293 δ receptor cells with DPDPE (1 μM), but not TIPP (1 μM) or morphine (10 μM), induced a significant Src phosphorylation at tyrosine 416, and this effect was inhibited by 10 μM PP2 (Figure 2A). Pretreatment of HEK293 δ receptor cells with PTX (100 ng · mL^{-1}, 24 h) abolished DPDPE-stimulated Src phosphorylation (Figure 2B), suggesting that phosphorylation of Src by DPDPE was Gi/o protein-dependent. To determine whether Src phosphorylation was required for DPDPE-stimulated ERK1/2 activation, we next examined the effect of PP2 on DPDPE-stimulated ERK1/2 activation. As shown in Figure 2C, pretreatment of HEK293 δ receptor cells with 20 μM PP2 for 30 min blocked DPDPE but not TIPP- or morphine-

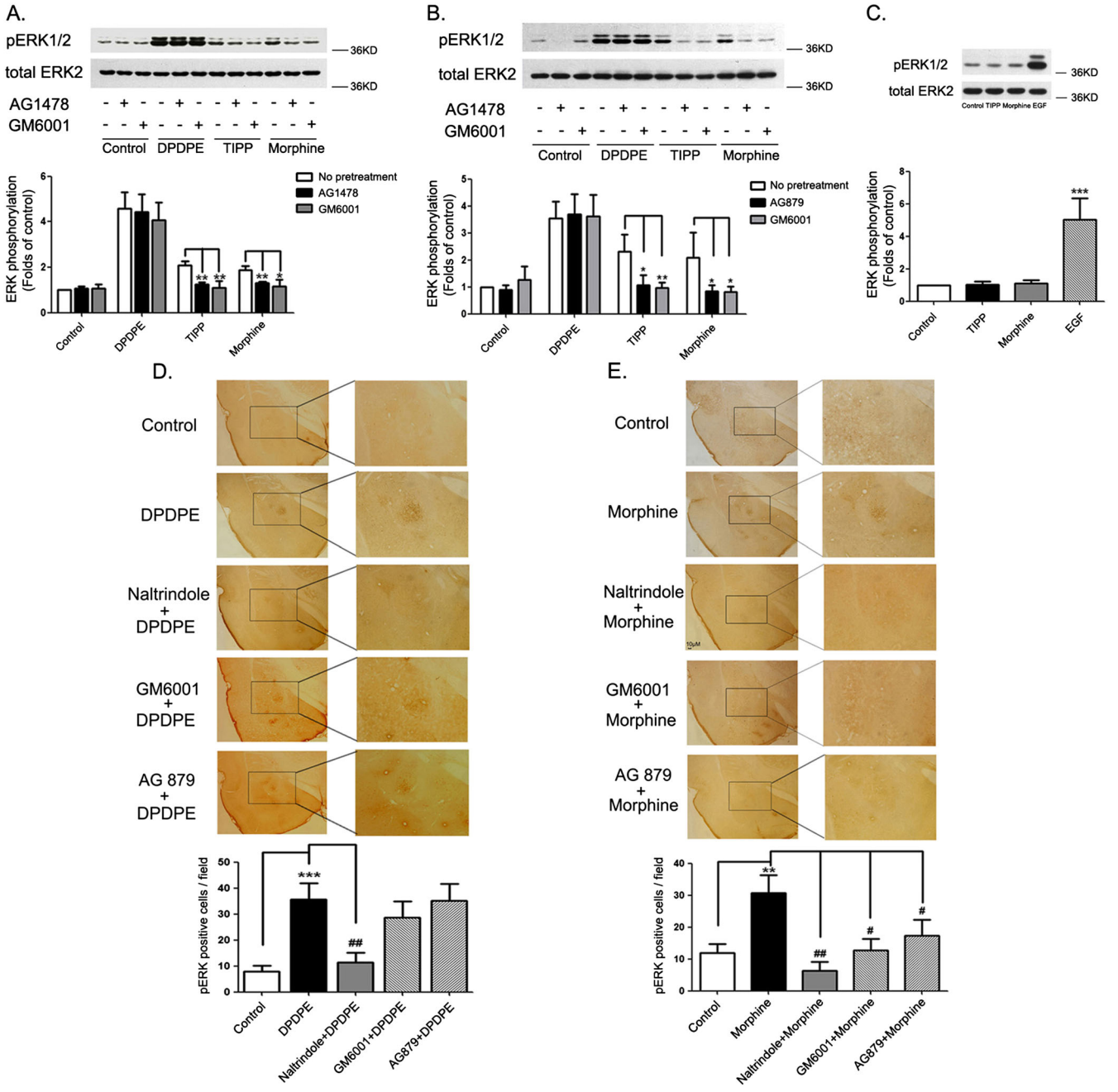

Figure 1

δ Receptor-mediated ERK1/2 activation stimulated by TIPP or morphine but not by DPDPE involves the transactivation of EGFR in cultured cells and native tissues. (A, B) Serum-starved HEK293 δ receptor cells or NG108-15 cells were pretreated or not for 30 min with the EGFR inhibitor AG1478 (5 μM), TrkA receptor inhibitor AG879 (10 μM) or metalloproteinase inhibitor GM6001 (25 μM) and then stimulated or not for 5 min with 1 μM DPDPE, 1 μM TIPP or 10 μM morphine. Phospho-ERK1/2 was detected by immunoblotting with anti-phospho-ERK1/2 antibody, and the bands were quantified by densitometry and expressed as fold of untreated control. Data represent at least three separated experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the paired ligand alone-treated groups. (C) Serum-starved HEK293 cells were stimulated with 1 μM TIPP or 10 μM morphine or EGF ($10\ ng \cdot mL^{-1}$) for 5 min. (D, E) Animals were pretreated with the δ receptor antagonist naltrindole ($5\ \mu g \cdot 10\ \mu L^{-1}$, i.c.v.) for 15 min or GM6001 ($10\ ng \cdot 10\ \mu L^{-1}$, i.c.v) for 1 h or AG879 ($5\ mM \cdot 10\ \mu L^{-1}$, i.c.v.) for 1 h and then administered DPDPE ($100\ nmol \cdot 10\ \mu L^{-1}$) or morphine ($20\ nmol \cdot 10\ \mu L^{-1}$) i.c.v. Fifteen minutes later, immunohistochemical staining of phospho-ERK1/2-positive cells in the amygdala of rats was performed as described previously. The number of animals in each group was at least seven. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the corresponding control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the corresponding ligand-untreated or ligand alone-treated control groups.

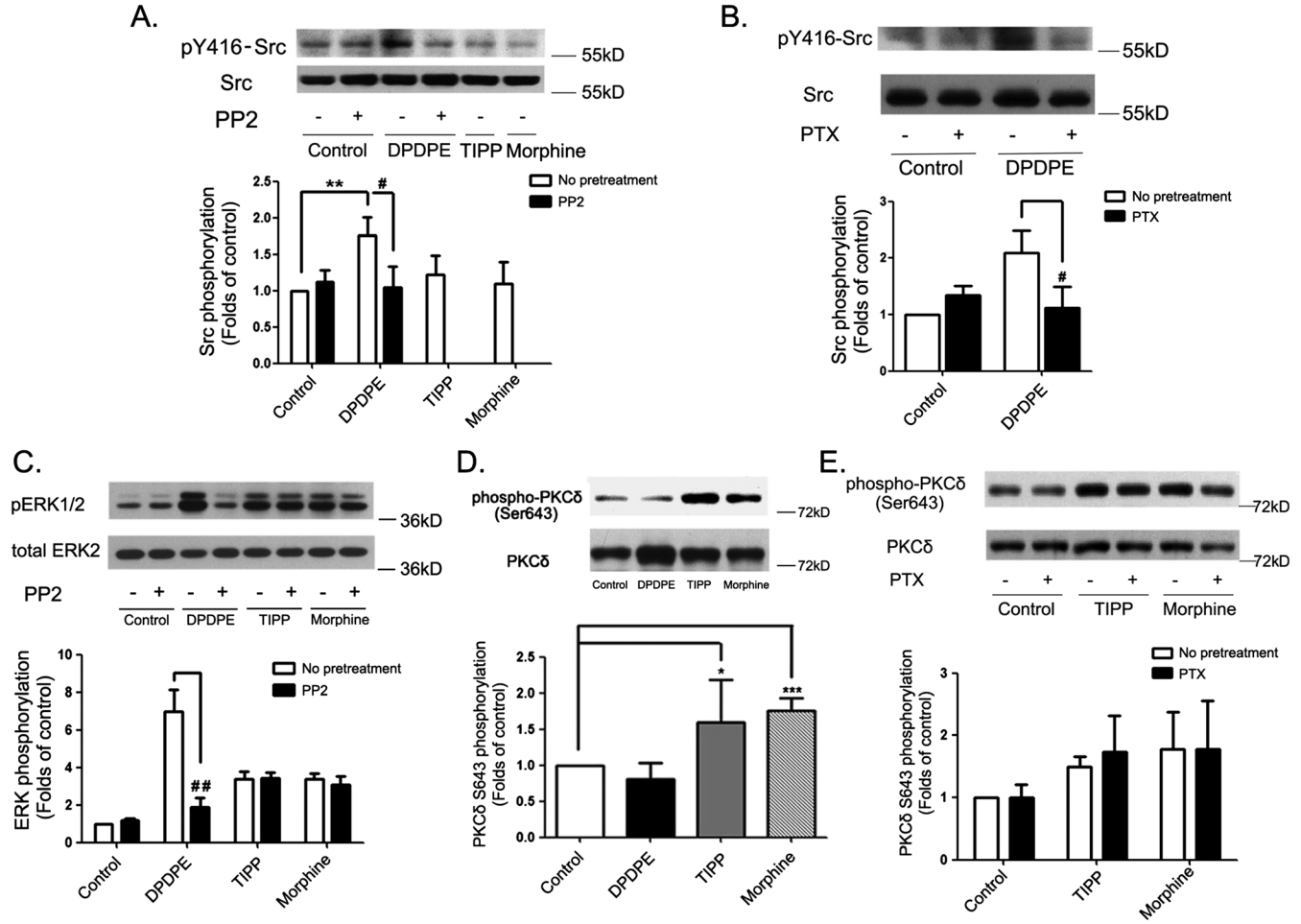

Figure 2

DPDPE, TIPP and morphine differentially regulated phosphorylation of Src and PKCδ. (A) DPDPE but not TIPP and morphine induced Src phosphorylation. (B) PTX blocked DPDPE-stimulated Src phosphorylation. (C) PP2 abolished DPDPE but not TIPP and morphine-stimulated ERK1/2 activation. Serum-starved HEK293 δ receptor cells were pretreated or not with the Src inhibitor PP2 (20 μM) for 30 min or with PTX (100 ng · mL^{-1}) for 24 h and then stimulated with 1 μM DPDPE or TIPP or 10 μM morphine for 5 min. Phosphorylation of Src or ERK1/2 was detected by immunoblotting, and the bands were quantified. (D) Morphine and TIPP but not DPDPE induced PKCδ phosphorylation. (E) PTX failed to block TIPP and morphine-stimulated PKCδ phosphorylation. Serum-starved HEK293 δ receptor cells were pretreated or not for 24 h with PTX (100 ng · mL^{-1}) and then stimulated with 1 μM TIPP or 10 μM morphine for 5 min. Data represent at least three separate experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the DPDPE alone-treated group, $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the drug-untreated control groups.

stimulated ERK1/2 activation, indicating that activation of Src was essential for DPDPE but not TIPP- or morphine-stimulated ERK1/2 activation.

These results, together with the findings mentioned previously that a metalloproteinase and RTK inhibitor fail to block DPDPE-induced ERK1/2 activation, indicate that Src activation may not be required for δ receptor-mediated ERK1/2 activation by transactivating RTKs in response to morphine and TIPP. Because PKCδ has been shown to activate metalloproteinases and induce ectodomain shedding of the proheparin-binding EGF-like growth factor (proHB-EGF) (Im *et al.*, 2007; Yang *et al.*, 2009; Kveiborg *et al.*, 2011), we next determined the effects of morphine, TIPP and DPDPE on PKCδ phosphorylation at serine 643. As shown in Figure 2D, treatment of HEK293 δ receptor cells with morphine (10 μM) or TIPP (1 μM), but not DPDPE (1 μM), induced a robust increase in PKCδ phosphorylation at serine 643, indicating that morphine and TIPP, but not DPDPE, activate PKCδ. These results suggest that PKCδ is involved in δ receptor-mediated ERK1/2 activation via transactivation of EGFR in response to TIPP and morphine stimulation. PKCδ is a downstream effector of PLC, and its activation is DAG-dependent (Olivier and Parker, 1991; Parker and Murray-Rust, 2004). Stimulation of the δ receptor results in the activation of PLC by Gβγ subunits released from activated Gi/o proteins (Murthy and Makhlouf, 1996). To determine whether PKCδ activation stimulated by morphine and TIPP was Gi/o protein-dependent, we examined the effect of PTX on morphine and TIPP-induced PKCδ activation. As shown in Figure 2E, pretreatment of HEK293 δ receptor cells with PTX (100 ng · mL^{-1}) for 24 h failed to block morphine- or TIPP-stimulated PKCδ phosphorylation, suggesting that activation of PKCδ by morphine- or TIPP-stimulated δ receptors is independent of Gi/o protein.

PKCδ activity is required for δ receptor-mediated ERK1/2 activation via transactivation of EGFR in response to TIPP or morphine but not DPDPE stimulation

Next, we investigated whether activation of PKCδ was required for the δ receptor-mediated ERK1/2 activation induced via transactivation of EGFR. We first detected δ receptor-mediated stimulation of EGFR activity. As shown in Figure 3A, treatment with morphine (10 μM) or TIPP (1 μM) but not DPDPE (1 μM) led to a marked increase in EGFR phosphorylation, and this effect was inhibited significantly by GF109203X (1 μM), a non-selective PKC inhibitor, suggestive of a role for PKCδ in TIPP- and morphine-induced EGFR phosphorylation. TIPP- and morphine-induced EGFR phosphorylation was also significantly suppressed by 25 μM GM6001 or 5 μM AG1478 (Figure 3A), indicating that metalloproteinase was involved in EGFR activation. The role of PKCδ in the transactivation of EGFR by δ receptors was further confirmed by the finding that knockdown of PKCδ abolished TIPP- and morphine-induced EGFR phosphorylation (Figure 3B).

To determine the role of PKCδ-mediated transactivation of EGFR in δ receptor-mediated ERK1/2 activation, we next examined the effect of GF109203X on TIPP or morphine-induced ERK1/2 activation. As shown in Figure 3C, pretreatment of HEK293 δ receptor cells with 1 μM GF109203X significantly attenuated 10 μM morphine- or 1 μM TIPP- but not 1 μM DPDPE-induced ERK1/2 activation. The role of PKCδ-mediated transactivation of EGFR by δ receptor was further supported by the observation that knockdown of PKCδ drastically suppressed TIPP- or morphine-induced but not DPDPE-induced ERK1/2 activation (Figure 3D–F). Because HEK293 cells also expressed PKC isoforms PKCα and PKCε (Uchiyama *et al.*, 2009), we thus also examined the effect of knockdown of PKCα and PKCε with siRNAs on TIPP- and morphine-induced

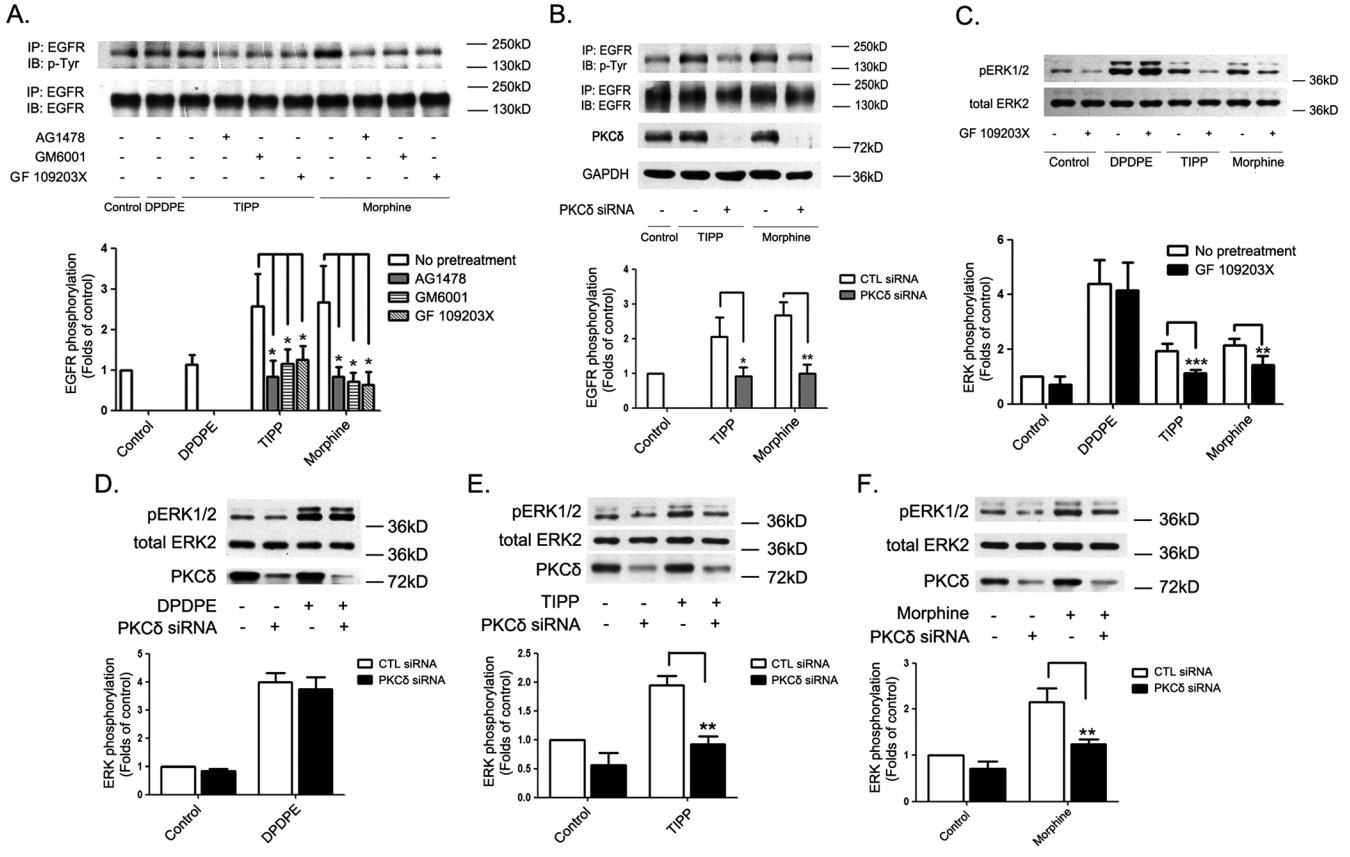

Figure 3

PKCδ activity is required for δ receptor-mediated ERK1/2 activation via transactivation of EGFR in response to TIPP or morphine but not DPDPE stimulation. (A) Serum-starved HEK293 δ receptor cells were pretreated or not for 30 min with 1 μM GF109203X or 25 μM GM6001 or 5 μM AG1478 and then stimulated or not for 5 min with 1 μM DPDPE or TIPP or 10 μM morphine. Then EGFR was immunoprecipitated, and phospho-EGFR was immunoblotted using anti-phosphotyrosine antibody. (B) HEK293 δ receptor cells transfected with siRNA targeting PKCδ or with control siRNA were treated with 1 μM TIPP or 10 μM morphine for 5 min, and phospho-Tyr-EGFR was determined. (C) GF109203X suppressed morphine or TIPP-stimulated ERK1/2 activation. Serum-starved HEK293 δ receptor cells were pretreated or not for 30 min with 1 μM GF109203X and then stimulated with 1 μM DPDPE or TIPP or 10 μM morphine for 5 min. (D–F) HEK293 δ receptor cells previously transfected with siRNA targeting PKCδ or with control siRNA were treated with 1 μM DPDPE or TIPP or 10 μM morphine for 5 min. All bands were quantified by densitometry and expressed as fold of untreated control. Data represent at least three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the paired ligand alone-treated or control siRNA-transfected groups.

ERK1/2 activation. Silencing of either PKCα or PKCε with siRNA had no significant effect on TIPP- or morphine-induced ERK1/2 activation (Figure 4), indicating that PKCα and PKCε were not involved in TIPP- and morphine-induced ERK1/2 activation. Overall, these results demonstrate that PKCδ plays a key role in δ receptor-mediated ERK1/2 activation via transactivation of EGFR.

PKCδ acts upstream of metalloproteinase-dependent transactivation of EGFR for δ receptor-mediated ERK1/2 activation in response to TIPP or morphine treatment

EGFR transactivation is thought to be initiated by cleavage of membrane-bound HB-EGF by a metalloproteinase, which leads to the release of HB-EGF and autophosphorylation of EGFR tyrosine residues (Prenzel *et al.*, 1999). Based on the observations that GM6001 and GF109203X inhibited δ receptor-mediated ERK1/2 activation, induced via transactivation of EGFR, we assumed that one or more metalloproteases function as effectors of δ receptor signalling and PKCδ may contribute to the activation of metalloproteases. To confirm this speculation, we used the transferred supernatant assay from which the involvement of PKCδ-mediated metalloprotease activity in ERK1/2 activation was detected by measuring the response in recipient cells lacking δ receptors. We first examined whether the supernatant from TIPP- or morphine-treated δ receptor-expressing cells could induce the activation of ERK1/2 in non-transfected (δ receptor null) HEK293 cells, which did not express endogenous δ receptors. As shown in Figure 5A, the addition of the supernatant from TIPP- or morphine-treated δ receptor-expressing cells to δ receptor-null cells increased ERK1/2 activity significantly above the level observed in cells treated with supernatant from non-stimulated δ receptor-expressing cells, and this effect was prevented by the addition of GM6001 to δ receptor-expressing donor cells and by the addition of AG1478 to the δ receptor-null recipient cells, suggesting that δ receptor-mediated ERK1/2 activation through transactivation of EGFR could be attributed to

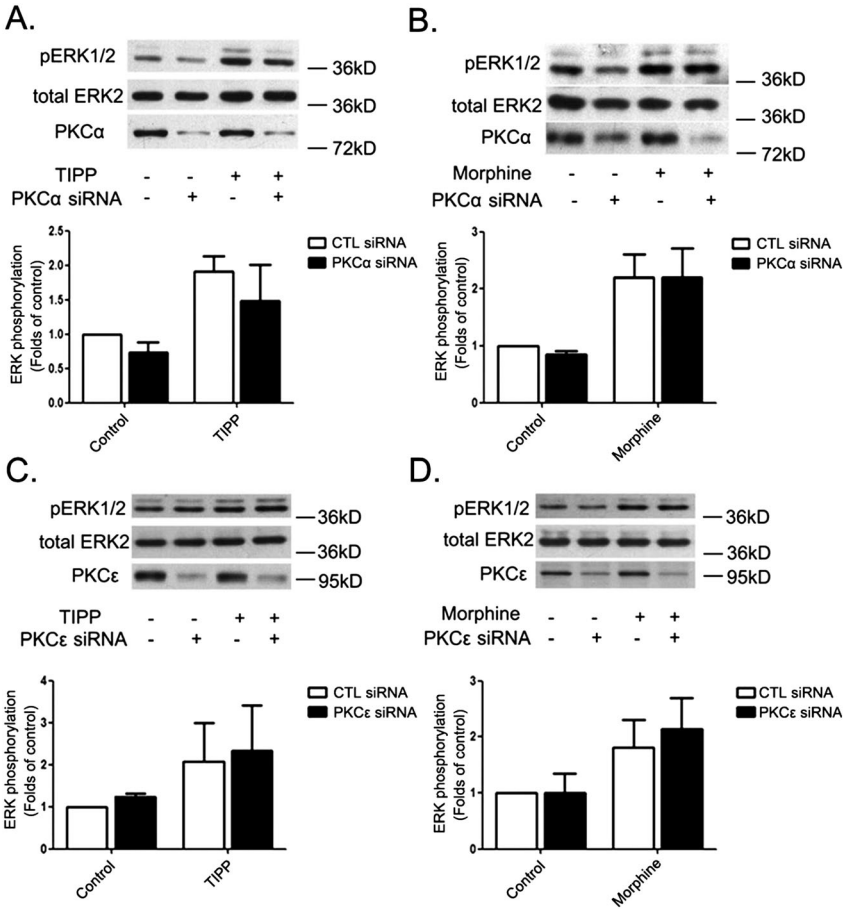

Figure 4

PKCα or PKCε is not involved in morphine or TIPP-induced activation of ERK1/2. (A, B) Silencing PKCα with siRNA had no effect on TIPP- or morphine-stimulated ERK1/2 phosphorylation. HEK293 δ receptor cells previously transfected with siRNA targeting PKCα or with control siRNA were treated with 1 μM TIPP or 10 μM morphine for 5 min. (C, D) Silencing PKCε with siRNA had no effect on TIPP- or morphine-stimulated ERK1/2 phosphorylation. HEK293 δ receptor cells previously transfected with siRNA targeting PKCε or with control siRNA were treated with 1 μM TIPP or 10 μM morphine for 5 min. Phospho-ERK1/2 was detected by immunoblotting with anti-phospho-ERK1/2 antibody, and the bands were quantified by densitometry and expressed as fold of untreated control. Data represent at least three separate experiments.

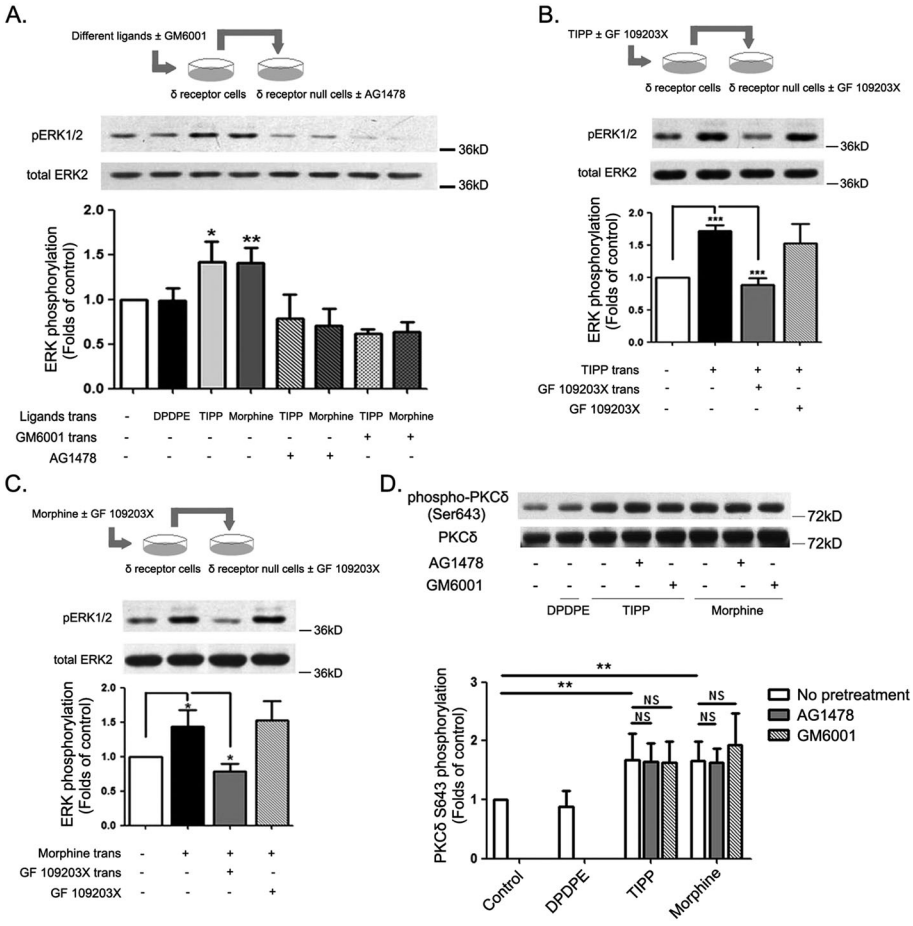

Figure 5

PKCδ acts upstream of metalloproteinase activation and is required for the release of the EGFR-activating factor. (A) Serum-starved δ receptor-null receiving cells previously treated or not for 30 min with 5 μM AG1478 were incubated for 5 min with the transferred supernatant from δ receptor-expressing donor cells previously treated or not for 30 min with 25 μM GM6001 and stimulated or not with 1 μM DPDPE, 1 μM TIPP or 10 μM morphine for 5 min. (B, C) Serum-starved δ receptor-null receiving cells previously treated or not for 30 min with 1 μM GF109203X were incubated for 5 min with the transferred supernatant from δ receptor-expressing donor cells previously treated or not for 30 min with 1 μM GF109203X and stimulated or not with 1 μM TIPP or 10 μM morphine for 5 min. Data represent at least three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the ligand-untreated or the paired ligand alone-treated groups. (E) Serum-starved HEK293δ receptor cells were pretreated or not for 30 min with 5 μM AG1478 or 25 μM GM6001 and then stimulated with 1 μM DPDPE, 1 μM TIPP or 10 μM morphine for 5 min. Data represent at least three independent experiments. $^{**}P < 0.01$ compared with the drug-untreated control groups; NS, $P > 0.05$ compared with the paired ligand alone-treated control groups.

the metalloproteinase-mediated cleavage and extracellular shedding of transactivating factors such as HB-EGF by which EGFR is activated. The requirement of δ receptor-mediated activation of metalloproteinase and shedding of transactivating factors, such as HB-EGF, for TIPP- and morphine-stimulated ERK1/2 activation was further substantiated by the observations that stimulation of δ receptor-null cells with 1 μM TIPP or 10 μM morphine failed to induce any detectable ERK1/2 phosphorylation; however, stimulation of δ receptor-null cells with EGF ($10\,ng \cdot mL^{-1}$) led to a robust ERK1/2 phosphorylation (Figure 1C). We next verified whether PKCδ was required for the metalloproteinase-dependent shedding of transactivating factors by adding GF109203X (1 μM) to δ receptor-expressing donor cells. As shown in Figure 5B and C, the addition of supernatant from TIPP or morphine-treated δ receptor-expressing donor cells that were pretreated with GF109203X, to the δ receptor-null receiving cells was unable to increase ERK1/2 activity, suggesting that PKCδ was required for the metalloproteinase-dependent shedding of transactivating factors and transactivation of EGFR.

Although our results suggest an early activation of PKCδ by δ receptors upstream of the metalloproteinase-dependent EGFR transactivation, our findings do not exclude the involvement of PKCδ activity in ERK1/2 activation downstream of EGFR transactivation, because EGFRs have also been shown to regulate PKC activity (Yarden and Sliwkowski, 2001; Woods and Johnson, 2007; Chung and Ge, 2012). Thus, we next determined whether PKCδ contributed to the activation of ERK1/2 only by acting upstream of the transactivation of EGFR and not by acting downstream of EGFR. To do this, we used the transferred supernatant assay in which GF109203X (1 μM) was added to the δ receptor-null

recipient cells. Whereas the TIPP- or morphine-stimulated increase in ERK1/2 activation in the receiving cells was completely abolished by GF109203X pretreatment of the δ receptor-expressing donor cells, it was not affected by inhibition of PKCδ in the δ receptor-null receiving cells (Figure 5B and C), suggesting that, after the transactivating ligand was released in the extracellular medium, PKCδ activity was no longer required. To further confirm that PKCδ acted upstream of EGFR transactivation, we examined the effects of GM6001 and AG1478 on δ receptor-mediated activation of PKCδ. As shown in Figure 5E, pretreatment of HEK293 δ receptor cells with GM6001 (25 μM) and AG1478 (5 μM) did not alter the ability of TIPP (1 μM) or morphine (10 μM) to promote PKCδ phosphorylation at serine 643, although they completely abolished ERK1/2 activation in response to TIPP or morphine stimulation (Figure 1A), supporting the concept that PKCδ activation occurred upstream of EGFR transactivation. Taken together, these results clearly indicate that PKCδ acts only upstream of the metalloproteinase-dependent transactivation event.

β-arrestin2 is required downstream of EGFR transactivation for δ receptor-mediated ERK1/2 activation in response to TIPP or morphine stimulation

Although the data mentioned previously clearly demonstrate that ERK1/2 activation by morphine and TIPP requires the transactivation of EGFR by the δ receptors, our previous study also showed that TIPP-stimulated ERK1/2 activation is β-arrestin dependent, as the knockdown of β-arrestin expression using siRNAs abolished TIPP-stimulated ERK1/2 activation (Xu *et al.*, 2010). These results led us to assume that the engagement of β-arrestin and the transactivation of EGFR might be two correlative molecular events involved in TIPP- and morphine-stimulated ERK1/2 activation. β-arrestin may either act upstream of EGFR transactivation as a mediator of δ receptor transactivation of EGFR as it does in β_1-adrenoceptor-mediated activation of ERK1/2 (Noma *et al.*, 2007; Tilley *et al.*, 2009) or it acts downstream of EGFR transactivation as in V_2 vasopressin receptor-mediated ERK1/2 activation (Oligny-Longpre *et al.*, 2012). To investigate this, we first tested whether β-arrestins were required for TIP- or morphine-induced activation of ERK1/2. To do this, we manipulated the levels of endogenous β-arrestin1, β-arrestin2 or both using siRNAs to inhibit their expression and then determined the level of ERK1/2 activation stimulated by TIPP, morphine or DPDPE in HEK293 δ receptor cells. siRNAs, targeting β-arrestin1 or β-arrestin2 or both, selectively and effectively silenced the expression of each β-arrestin (data not shown). Consistent with our previous study (Xu *et al.*, 2010), suppression of β-arrestin1 or β-arrestin2 or both using siRNAs reduced the level of ERK1/2 activation stimulated by TIPP or morphine but not by DPDPE compared with control siRNA-transfected cells, indicating that TIPP and morphine, but not DPDPE, activated ERK1/2 in a β-arrestin-dependent manner (Figure 6A).

Compared with β-arrestin1, silencing of of β-arrestin2 had more profound effects on δ receptor-mediated ERK1/2 activation (Figure 6A); β-arrestin2 is known to have an important role in the functional adaptations in response to acute and chronic opioid agonist treatment (Bohn *et al.*, 1999, 2000; Audet *et al.*, 2012); we next focused our attention on the role of β-arrestin2 in δ receptor-mediated transactivation of EGFR. We examined whether β-arrestin2 acted upstream or downstream of EGFR transactivation using the transferred supernatant assay in which endogenous β-arrestin2 expression was interfered with respectively in donor or recipient cells. As shown in Figure 6B, β-arrestin2 knockdown in the δ receptor-expressing donor cells did not inhibit activation of ERK1/2 in the δ receptor-null recipient cells induced by supernatant transferred from the donor cells treated with 1 μM TIPP (left panel) or 10 μM morphine (right panel), whereas knockdown of the acceptor's β-arrestin2 resulted in a remarkable inhibition of ERK1/2 activation (right panel) (Figure 6C), suggesting that β-arrestin2 acted downstream of EGFR transactivation but was not required for the metalloproteinase-mediated shedding of the EGFR transactivating ligand.

The recruitment of β-arrestin to activated GPCRs is required for its function as a signal transducer linking GPCRs to ERK1/2 signalling (McDonald *et al.*, 2000; Luttrell *et al.*, 2001). Given the fact that δ receptor-null recipient cells do not express any endogenous δ receptors (Figure 1C), the role of β-arrestin2 downstream of EGFR transactivation in these cells after supernatant transfer implicates the existence of a distinct stimulating signal triggering β-arrestin engagement. Previous studies have shown that EGFR can recruit β-arrestins in response to its cognate ligand stimulation (Dalle *et al.*, 2001; Oligny-Longpre *et al.*, 2012). We thus hypothesized that EGFR could possibly conserve this signalling capacity in the context of the transactivation event by δ receptors when stimulated by TIPP or morphine. Supporting this hypothesis, co-immunoprecipitation experiments revealed that β-arrestin2 was associated with the endogenously expressed EGFR following morphine or TIPP but not DPDPE stimulation (Figure 6D).

DPDPE can activate ERK1/2 through transactivation of EGFR in HEK293 cells expressing S363A mutant δ receptor as TIPP or morphine did in cells expressing the wild-type δ receptor

The Ser^{363} residue of the δ receptor plays an important role in the regulation of receptor functions (Trapaidze *et al.*, 1996; Kouhen *et al.*, 2000; Law *et al.*, 2000). Our previous study also demonstrated that the Ser^{363} residue of the δ receptor is crucial for the ligand-specific δ receptor adoption of distinct signalling pathway to activate ERK1/2 (Xu *et al.*, 2010). To further investigate the mechanisms underlying ligand-specific engagement of β-arrestin2 for ERK1/2 activation through the transactivation of EGFR, we determined the role of the Ser^{363} residue of the δ receptor in ligand-specific engagement of β-arrestin2 for transactivation of EGFR. To this end, we tested the effects of DPDPE stimulation on PKCδ activation and EGFR phosphorylation and determined whether the silencing of PKCδ or β-arrestin2 expression with siRNA had an effect on DPDPE-stimulated ERK1/2 activation in HEK293 cells expressing the mutant δ receptor in which Ser^{363} was replaced by alanine (S363A). As shown in Figure 7A, in the cells expressing the S363 mutant δ receptors (S363A δ receptors), DPDPE (1 μM) failed to yield

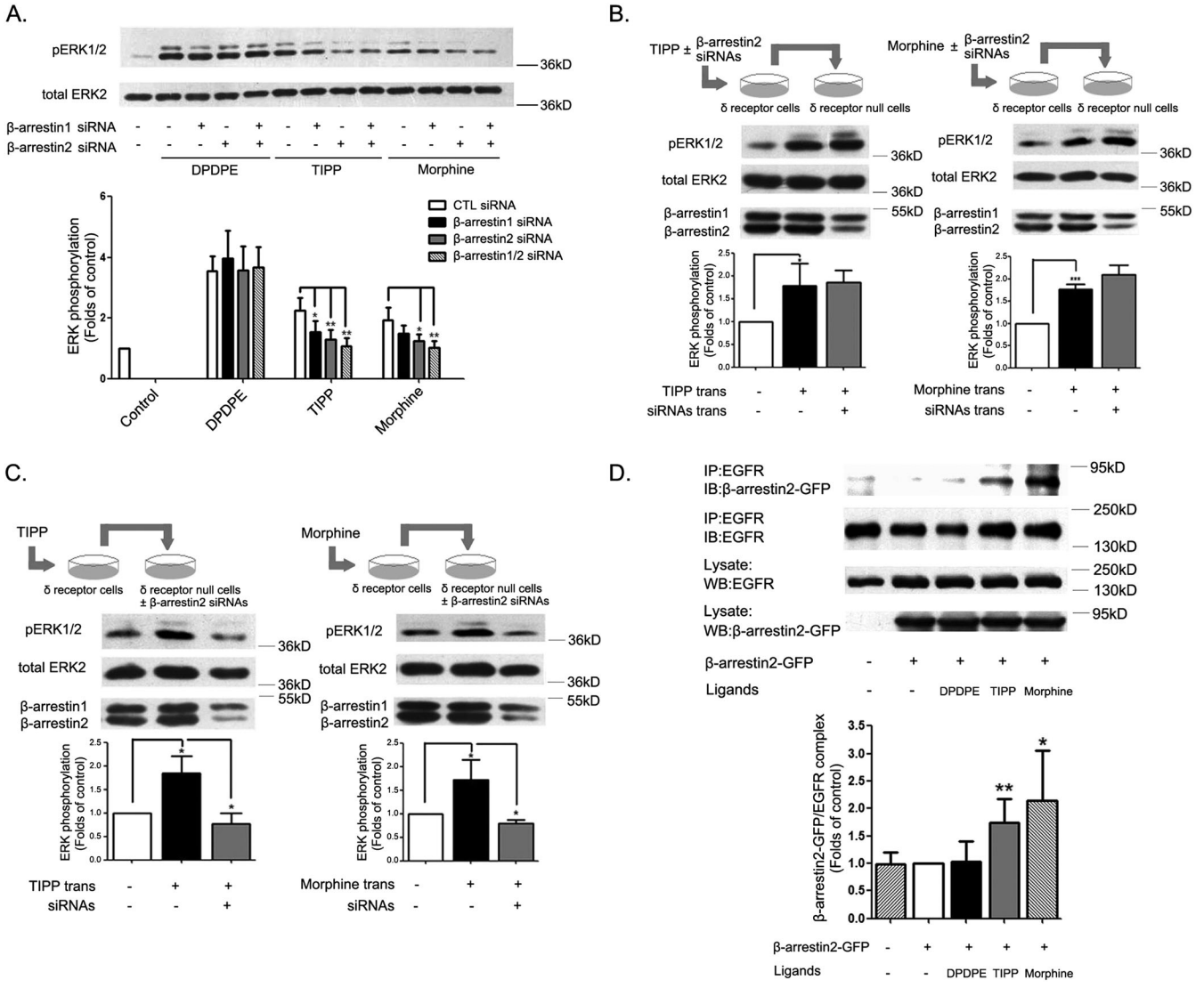

Figure 6

β-Arrestin2 is required for δ receptor-mediated ERK1/2 activation stimulated by morphine and TIPP but not by DPDPE and acts downstream of EGFR transactivation. (A) HEK293 δ receptor cells were transfected with siRNAs targeting β-arrestin1, β-arrestin2 or both, or with control siRNA, and then stimulated with 1 μM DPDPE, 1 μM TIPP or 10 μM morphine for 5 min. (B) Serum-starved δ receptor-null receiving cells were incubated for 5 min with the transferred supernatant from δ receptor-expressing donor cells previously knockeddown for either or both β-arrestins and stimulated or not with 1 μM TIPP (left panel) or 10 μM morphine (right panel) for 5 min. (C) Serum-starved δ receptor-null receiving cells previously transfected with siRNA were incubated for 5 min with the transferred supernatant from δ receptor-expressing cells stimulated or not with 1 μM TIPP (left panel) or 10 μM morphine (right panel) for 5 min. β-Arrestin2 knockdown was detected with the anti-β-arrestin1/2 antibody. (D) HEK293 δ receptor cells transiently expressing β-arrestin2-GFP or not were stimulated with 1 μM DPDPE, 1 μM TIPP or 10 μM morphine for 5 min, and then cells were subjected to immunoprecipitation. Co-immunoprecipitation of β-arrestin2 and endogenous EGFRs and transfected β-arrestin2 in cell lysates were detected. The bands were quantified by densitometry, calculated as the ratios of β-arrestin2 and EGFR and expressed as fold of untreated wild-type control. Data represent at least three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the drug-untreated and the paired control siRNA group.

phosphorylation of δ receptors at Ser^{363}, as did TIPP (1 μM) and morphine (10 μM) in cells expressing wild-type δ receptors. Additionally, in contrast to its effects in cells expressing wild-type δ receptors, DPDPE treatment failed to activate Src but activated PKCδ in a PTX-insensitive manner (Figure 7B and C). Moreover, DPDPE induced phosphorylation of EGFR and ERK1/2, which could be inhibited by 1 μM GF109203X, 25 μM GM6001 or 5 μM AG1478 (Figure 7D and E). ERK1/2 activation induced by DPDPE was also sensitive to PKCδ siRNA and β-arrestin2 siRNA (Figure 7F and G). These results indicate that stimulation of the S363A δ receptors with DPDPE uses β-arrestin2 as a signal transducer to activate ERK1/2 via PKCδ-dependent activation of metalloproteinase shedding of transactivating factors, as TIPP and morphine did in cells expressing wild-type δ receptors.

The aim of the following experiments was to investigate whether PKCδ acted upstream of EGFR transactivation and/or β-arrestins acted downstream of EGFR transactivation

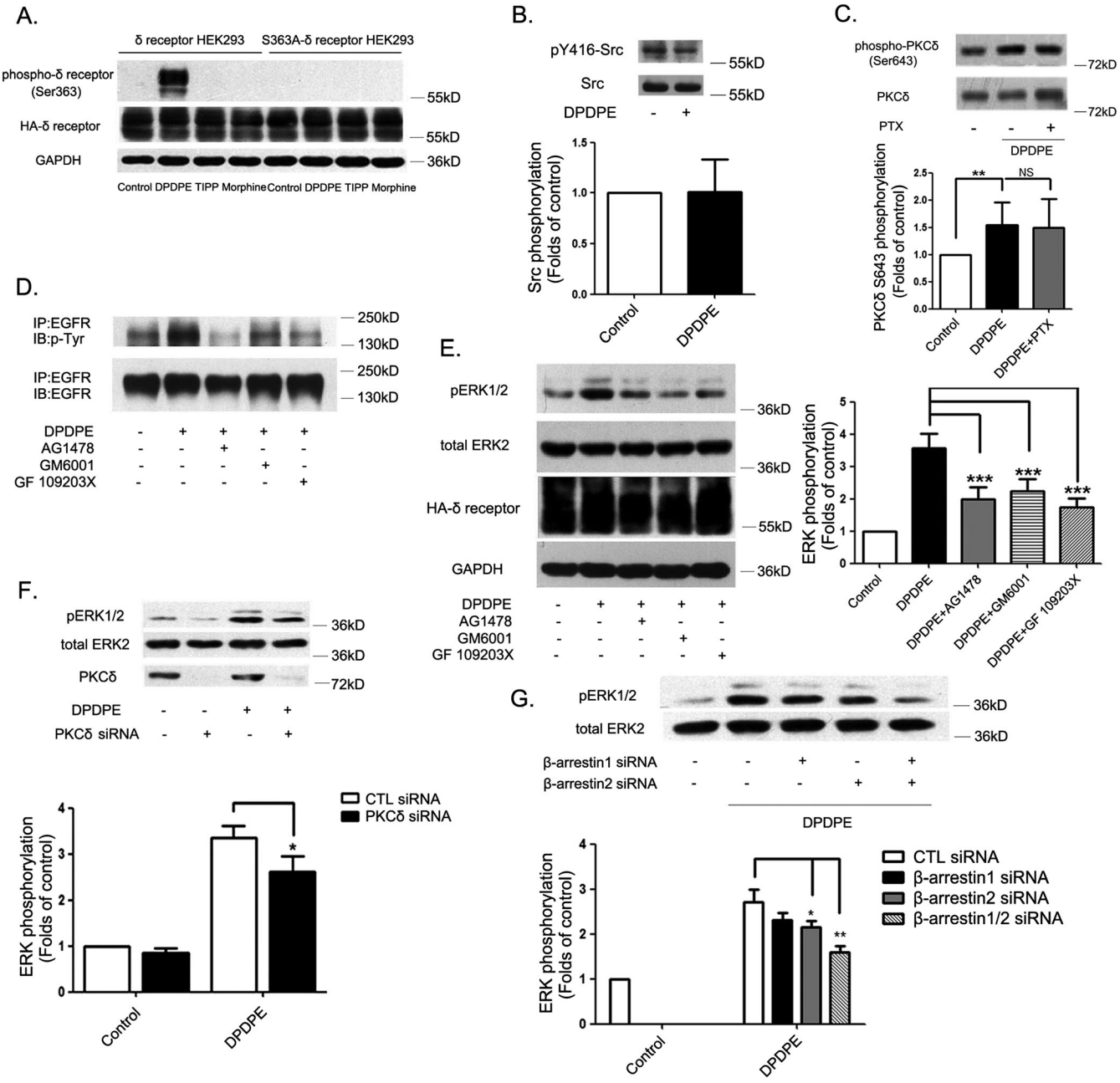

Figure 7

DPDPE can activate ERK1/2 by employing β-arrestin2 via PKCδ-mediated transactivation of EGFR in HEK293 cells expressing the S363A mutant δ receptor. (A) Cells stably expressing wild-type δ receptors or S363A δ receptors were stimulated with 1 μM DPDPE or TIPP or 10 μM morphine for 5 min. The phosphorylation of the Ser^{363} residue in the δ receptors was verified. (B, C) Cells stably expressing S363A δ receptors were pretreated or not with PTX (100 ng · mL^{-1}) for 24 h and then stimulated with 1 μM DPDPE for 5 min. The phosphorylation of Src or PKCδ was detected and expressed as fold of untreated control. (D, E) Serum-starved S363A δ receptor cells were pretreated with either 1 μM GF109203X, 25 μM GM6001 or 5 μM AG1478 for 30 min and then stimulated with 1 μM DPDPE for 5 min. (F) Cells expressing S363A δ receptors previously transfected with siRNA targeting PKCδ or control siRNA were treated with 1 μM DPDPE for 5 min. (G) Cells expressing S363A δ receptors were transfected with siRNAs targeting β-arrestin1, β-arrestin2 or with siRNA control and then stimulated with 1 μM DPDPE for 5 min. Data represent at least three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with DPDPE-untreated or the DPDPE alone-treated or the paired control siRNA groups.

in the process of DPDPE-induced ERK1/2 activation through δ receptor-mediated transactivation of EGFR in S363A δ receptor/HEK293 cells. To accomplish this, we first determined the effect of the addition of GF109203X to donor cells or δ receptor-null recipient cells on DPDPE-stimulated ERK1/2 activation using the transferred supernatant assay. As shown in Figure 8A, whereas the addition of GF109203X (1 μM) to the S363A δ receptor-expressing donor cells resulted

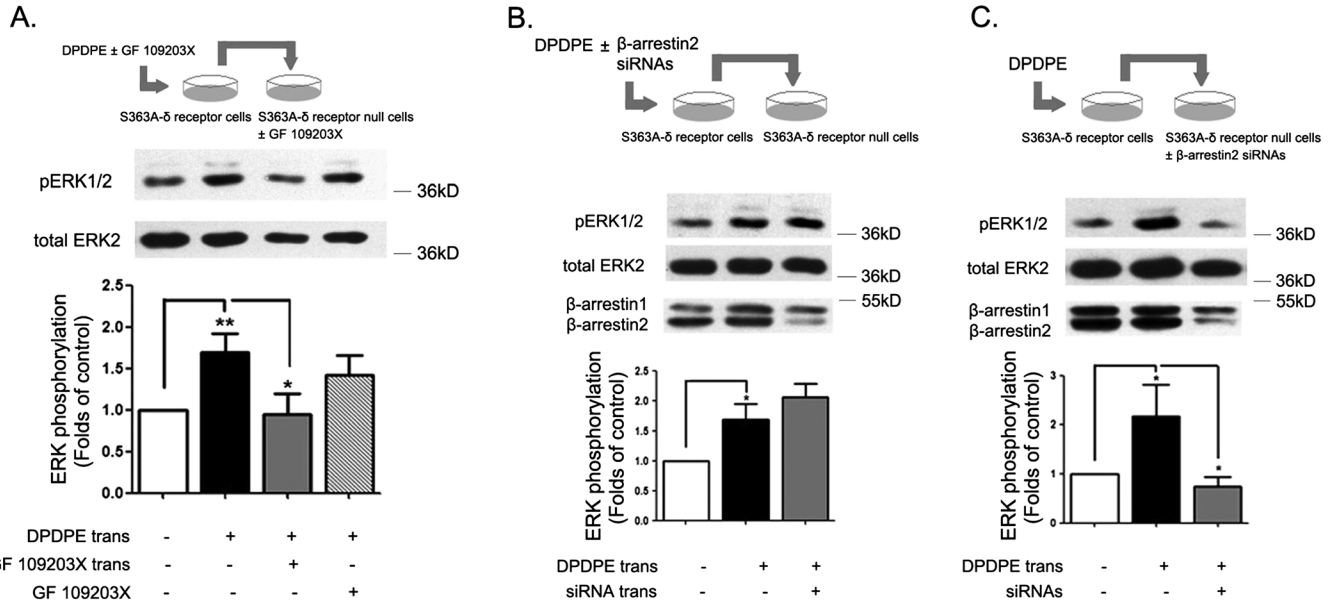

Figure 8

β-Arrestin2 is required for DPDPE-stimulated ERK1/2 activation and acts downstream of the PKCδ-mediated activation of metalloproteinase and transactivation of EGFR in HEK293 cells stably expressing the S363A δ receptor. (A) Serum-starved S363A δ receptor-null receiving cells previously treated or not for 30 min with the PKC inhibitor 1 μM GF109203X were incubated for 5 min with the transferred supernatant from S363A δ receptor-expressing donor cells previously incubated or not for 30 min with 1 μM GF109203X and stimulated or not with 1 μM DPDPE for 5 min. (B) Serum-starved S363A δ receptor-null receiving cells were incubated for 5 min with the transferred supernatant from δ receptor-expressing donor cells previously transfected with siRNA targeting β-arrestin2 or control siRNA and stimulated or not with1 μM DPDPE for 5 min. (C) Serum-starved S363A δ receptor-null receiving cells previously transfected with siRNAs targeting β-arrestin2 or control siRNA were incubated for 5 min with the transferred supernatant from S363A δ receptor-expressing donor cells previously stimulated or not with 1 μM DPDPE for 5 min. Data represent at least three separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the DPDPE-untreated or the DPDPE alone-treated or the control siRNA group.

in a drastic inhibition of ERK1/2 activation in the S363A δ receptor-null receiving cells induced by supernatant transferred from 1 μM DPDPE-treated donor cells, the presence of GF109203X in the δ receptor-null receiving cells did not inhibit the activation of ERK1/2 induced by supernatant from donor cells, indicating that PKCδ acted upstream but not downstream of EGFR transactivation. We next examined the effect of interfering with endogenous β-arrestin2 expression in donor or recipient cells on DPDPE-stimulated ERK activation. As shown in Figure 8B and C, β-arrestin2 knockdown in the S363A δ receptor-expressing donor cells treated with DPDPE did not inhibit supernatant-induced activation of ERK1/2 in the receiving cells, which differed from the effec of β-arrestin2 knockdown in the S363A δ receptor-null receiving cells induced by transferred supernatant, suggesting that β-arrestin2 acted downstream of EGFR transactivation. Taken together, these results suggest a crucial role of the Ser^{363} residue of the δ receptors in the agonist-specific engagement of β-arrestin2 for δ receptor-mediated ERK1/2 activation mediated by transactivation of EGFR (Figure 9).

Discussion

The δ receptor can employ multiple mechanisms to activate ERK1/2, including G-protein dependent and independent processes. Protein tyrosine kinases play an important role in opioid receptor-mediated activation of ERK1/2. It has been reported that δ receptors activate ERK1/2 by releasing G-protein βγ subunits, leading to the recruitment of the non-RTK Src, which serves as a mediator of Ras activity and thus activates an Ras-dependent cascade (Belcheva *et al.*, 1998). The δ receptor has also been shown to activate ERK1/2 by transactivating RTKs such as the EGFR (Eisinger and Schulz, 2004; Cohen *et al.*, 2007; Forster *et al.*, 2007). Recently, we demonstrated that β-arrestins function as signal transducers linking δ receptor stimulation to ERK1/2 activation by scaffolding component kinases of the ERK cascade in a G-protein-independent manner, following stimulation of δ receptors with the specific agonist morphine or TIPP (Xu *et al.*, 2010). Moreover, we found that the recruitment of β-arrestins to the δ receptors may not be required for its scaffolding function because TIPP and morphine were both unable to promote β-arrestins bound to the activated δ receptors (Hong *et al.*, 2009), although the recruitment of β-arrestin to the activated GPCRs has been clearly shown to be necessary for its scaffolding function (Luttrell *et al.*, 1999; McDonald *et al.*, 2000; Luttrell *et al.*, 2001). The present study extends our previous findings by revealing that the recruitment of β-arrestin to the transactivated EGFR rather than the activated δ receptors is required for its role in δ receptor-mediated ERK1/2 activation and supports the notion that β-arrestins can serve as a downstream effector of crosstalk between GPCRs and RTKs (Oligny-Longpre *et al.*, 2012). In comparison with β-arrestin1, silencing β-arrestin2 produced a more pronounced inhibition

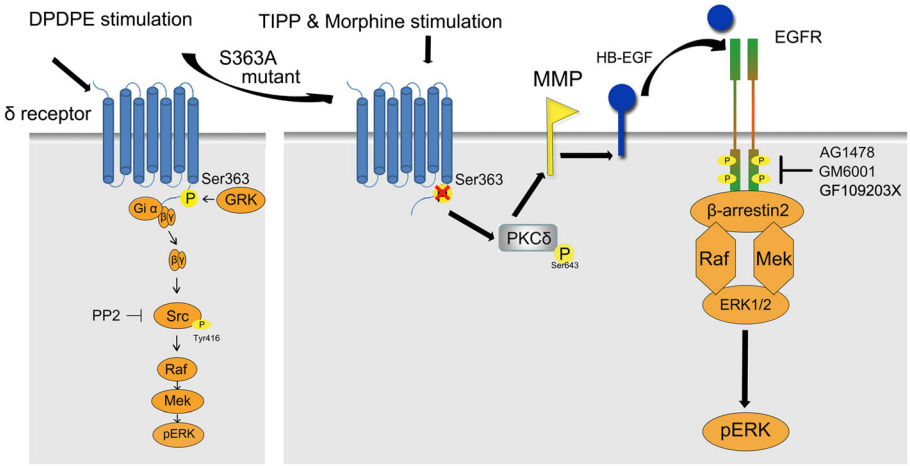

Figure 9

Schematic representation of δ receptor-mediated ERK1/2 activation via the PKCδ/EGFR/β-arrestin2 signalling pathway in response to specific agonist stimulation. DPDPE binding to δ receptors results in the dissociation of heterotrimeric G-proteins into Gα-GTP and Gβγ subunits. Gβγ subunit release leads to the recruitment and activation of Src, which causes tyrosine phosphorylation of the δ receptors, producing an SH2 site that might bind to Shc, Grb2 and additional downstream molecules to activate the Ras-dependent ERK cascade, whereas the binding of TIPP or morphine to δ receptors activates PKCδ rather than Src, which results in the activation of metalloproteinase (MMP), release of heparin-binding EGF-like factor (HB-EGF) and transactivation of EGFR. The transactivated EGFR can then activate ERK1/2 by recruiting β-arrestins as a scaffold to assemble component kinases of the ERK cascade. Mutation of the Ser^{363} residue of the δ receptor can switch DPDPE from activation of Src to activation of PKCδ and thus allows it gain the ability to appropriate the PKCδ/EGFR/β-arrestin2 signalling pathway to activate ERK1/2.

of ERK1/2 activation (Figure 6A). Although it is possible that β-arrestin1 is partially involved, our results clearly indicate that β-arrestin2 plays a critical role. We further demonstrated that β-arrestin2 acted downstream of EGFR transactivation, because β-arrestin2 knockdown in the δ receptor-expressing donor cells did not inhibit the activation of ERK1/2 in the δ receptor-null receiving cells, whereas β-arrestin2 depletion in the δ receptor-null receiving cells resulted in a drastic inhibition of the ERK1/2 activation induced by supernatant transfer from TIPP- or morphine-treated δ receptor-expressing donor cells. Moreover, an immunocomplex association of β-arrestin2 with EGFR (Figure 6D), but not with the δ receptor (Hong *et al.*, 2009), can be detected in response to stimulation of δ receptors with TIPP or morphine, indicating that the β-arrestin2 is recruited by the transactivated EGFR rather than by the activated δ receptors. Our findings suggest that β-arrestins may not be exclusively GPCR-binding adapters or scaffolds as thought previously, which is consistent with a recent study demonstrating that β-arrestins act downstream of the IGFR transactivation for the V_2 vasopressin and the platelet-activating factor receptors (Oligny-Longpre *et al.*, 2012). Additionally, the present study also suggests that the receptor complex between the δ receptor and EGFR may not be a prerequisite for EGFR-dependent and β-arrestin-dependent activation of ERK1/2 by the δ receptors, as the EGFR transactivating ligand released after stimulation of δ receptors by TIPP or morphine can activate ERK1/2 in cells devoid of δ receptors in a β-arrestin-dependent manner. This contrasts with results from several previous studies showing that a physical association between a GPCR, β-arrestins and an RTK in a multiprotein complex is involved in RTK-dependent and β-arrestin-dependent activation of ERK1/2 by a GPCR (Maudsley *et al.*, 2000; Alderton *et al.*, 2001; Tilley *et al.*, 2009).

The other novel findings of this study are that the engagement of β-arrestin2 in δ receptor-mediated ERK1/2 activation through the transactivated EGFR is agonist specific and the Ser^{363} of δ receptors is crucial for ligand-specific modification of the ERK1/2 activation pathways. DPDPE was found to activate Src, while morphine and TIPP were found to activate PKCδ. Transactivation of EGFR by GPCRs involves metalloproteinase-mediated cleavage and extracellular shedding of heparin-binding EGF (HB-EGF) (Prenzel *et al.*, 1999). Although Src has been reported to link GPCR stimulation to the activatation of metalloproteinases and transactivation of EGFR (Daub *et al.*, 1997; Pierce *et al.*, 2001; Kim *et al.*, 2002; Noma *et al.*, 2007), it fails to activate metalloproteinases and transactivate EGFR upon stimulation of δ receptors with DPDPE, as metalloproteinase and EGFR inhibitors are unable to block DPDPE-induced ERK1/2 activation. However, in contrast to previous findings that showed PKCδ induces ectodomain shedding of the proHB-EGF by activating metalloproteinases (Im *et al.*, 2007; Yang *et al.*, 2009; Kveiborg *et al.*, 2011), we found that PKCδ activated by morphine or TIPP is able to induce metalloproteinase-dependent release of HB-EGF in the extracellular medium after δ receptor stimulation, leading to the transactivation of EGFR. Although HB-EGF was not detected directly in the present study, it is clearly released in the medium of TIPP or morphine-stimulated, δ receptor-expressing cells, as small interfering RNA to PKCδ or inhibition of PKCδ activity by GF109203X inhibited TIPP- or morphine-induced EGFR phosphorylation, and the transfer of supernatant from the δ receptor-expressing donor cells led to EGFR-dependent ERK1/2 activation in HEK293 cells devoid of endogenous δ receptors. Indeed, transactivation of EGFR by δ receptors through PKCδ has been demonstrated in a previous study (Eisinger and Ammer, 2008a).

Currently, the precise mechanisms underlying the different activation of Src and PKCδ by DPDPE, TIPP and morphine are unclear. Distinct receptor configurations formed upon occupancy of receptors by these ligands might be responsible for these differences. There is accumulating evidence suggesting that the GPCRs can adopt different active configurations, which may couple to specific signalling pathways (Ghanouni *et al*., 2001; Azzi *et al*., 2003). DPDPE may stabilize a receptor configuration that couples Gi/o protein. In this case, δ receptor stimulation would result in the release of Gβγ, leading to the recruitment and activation of Src. This is supported by evidence that PTX and PP2 abolish DPDPE-induced ERK1/2 activation (Audet *et al*., 2005; Hong *et al*., 2009) and stimulation of δ receptors with DPDPE induces an association of Gβγ with Src (Xu *et al*., 2010). Whereas the configuration stabilized by TIPP and morphine may be unable to couple Gi/o protein but, instead, couples another PTX-insensitive G-protein, as PTX failed to block morphine and TIPP-induced activation of PKCδ (Figure 2E) and stimulation of δ receptors with TIPP was unable to induce an association of Gβγ with Src (Xu *et al*., 2010). The Gq family proteins may be a possible candidate for coupling to the configuration stabilized by TIPP and morphine, because δ receptor stimulation has been shown to activate Gq family proteins and yield PLC (Lee *et al*., 1998; Ho *et al*., 2001; Lo and Wong, 2004), which may result in PKCδ activation by the hydrolyzation of phosphatidylinositol-4,5-bisphosphate into IP3 and DAG.

Certain residues in the C-terminal of GPCRs may be crucial for the formation of distinct active receptor configurations in response to stimulation by specific agonists. Mutations or alterations in the state of phosphorylation of these residues may lead to changes in the receptor configurations stabilized by specific agonists, thereby resulting in coupling to distinct signalling pathways. Indeed, it has been shown that the mutation of certain residues of the β_2-adrenoceptor and angiotensin AT_{1A} receptor can shift these two receptors to activate ERK1/2 from a G-protein-dependent mechanism to a β-arrestin-dependent mechanism (Wei *et al*., 2003; Shenoy *et al*., 2006). The present study also demonstrates that the mutation of the Ser^{363} residue of the δ receptor can switch DPDPE from activation of Src to activation of PKCδ and thus allow it gain the ability to appropriate the PKCδ/EGFR/β-arrestin2 signalling pathway to activate ERK1/2. Our findings suggest that the Ser^{363} of δ receptors is crucial for ligand-specific engagement of β-arrestin2 for ERK1/2 activation through the transactivation of EGFR.

Our *in vivo* data suggested that δ receptor-mediated activation of ERK1/2 via transactivation of RTK may be physiologically relevant, because it can be observed in the central (CeA) nuclei of the amygdala in rats following morphine but not DPDPE stimulation. As found in the NG108-15 cells, the mechanism leading to the ERK1/2 activation in the CeA of the amygdala relied on the activation of a metalloproteinase and TrkA receptors, because the metalloproteinase and TrkA receptors inhibitors significantly blocked the morphine-stimulated ERK1/2 phosphorylation in the CeA of the amygdala. Our *in vitro* and *in vivo* studies also demonstrated that agonist-bound δ receptors transactivated different RTKs in different cell lines to activate ERK1/2. Specifically, EGFR was transactivated in the HEK293 cells, whereas TrkA was transactivated in the NG108-15 cells and CeA of the amygdala. These results demonstrated that δ receptors used distinct signalling partners to lead to the same outcome; this is consistent with previous findings showing that adenosine A_{2A} receptors activate downstream signalling by transactivating Trk receptors, in PC12 cells and hippocampal neurons (Wiese *et al*., 2007), but EGFR in vascular smooth muscle cells (Lin *et al*., 2012). Future work is required to establish the importance of the δ receptor-mediated activation of ERK1/2 via agonist-specific transactivation of RTK in the development of opiate drug tolerance and dependence.

Acknowledgements

This research was supported by grants 2013CB835100, 2009CB522005, 2012ZX09301001-005 and 2012BAI01B00 (to J.-G. L.) from the Ministry of Science and Technology of China, by grants 81130087 and 91232716 (to J.-G. L.) from the National Natural Science Foundation of China and by grants 13JC140680 (to J,-G. L.) from the Committee of Science and Technology of Shanghai and BK2012457 (to Y.-H. W.) from the Committee of Science and Technology of Jiangsu Province. This research was also a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Conflict of interest

The authors disclose no conflict of interest.

References

Alderton F, Rakhit S, Kong KC, Palmer T, Sambi B, Pyne S *et al.* (2001). Tethering of the platelet-derived growth factor beta receptor to G-protein-coupled receptors. A novel platform for integrative signaling by these receptor classes in mammalian cells. J Biol Chem 276: 28578–28585.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013a). The concise guide to PHARMACOLOGY 2013/14: G protein-coupled receptors. Br J Pharmacol 170: 1459–1581.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013b). The concise guide to PHARMACOLOGY 2013/14: catalytic receptors. Br J Pharmacol 170: 1676–1705.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013c). The concise guide to PHARMACOLOGY 2013/14: enzymes. Br J Pharmacol 170: 1797–1867.

Audet N, Charfi I, Mnie-Filali O, Amraei M, Chabot-Dore AJ, Millecamps M *et al.* (2012). Differential association of receptor-Gbetagamma complexes with beta-arrestin2 determines recycling bias and potential for tolerance of delta opioid receptor agonists. J Neurosci 32: 4827–4840.

Audet N, Paquin-Gobeil M, Landry-Paquet O, Schiller PW, Pineyro G (2005). Internalization and Src activity regulate the time course of ERK activation by delta opioid receptor ligands. J Biol Chem 280: 7808–7816.

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M *et al.* (2003). Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. Proc Natl Acad Sci U S A 100: 11406–11411.

Belcheva MM, Vogel Z, Ignatova E, Avidor-Reiss T, Zippel R, Levy R *et al.* (1998). Opioid modulation of extracellular signal-regulated protein kinase activity is Ras-dependent and involves Gbetagamma subunits. J Neurochem 70: 635–645.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG (2000). Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. Nature 408: 720–723.

Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT (1999). Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science 286: 2495–2498.

Chung CK, Ge W (2012). Epidermal growth factor differentially regulates activin subunits in the zebrafish ovarian follicle cells via diverse signaling pathways. Mol Cell Endocrinol 361: 133–142.

Cohen MV, Philipp S, Krieg T, Cui L, Kuno A, Solodushko V *et al.* (2007). Preconditioning-mimetics bradykinin and DADLE activate PI3-kinase through divergent pathways. J Mol Cell Cardiol 42: 842–851.

Dalle S, Ricketts W, Imamura T, Vollenweider P, Olefsky JM (2001). Insulin and insulin-like growth factor I receptors utilize different G protein signaling components. J Biol Chem 276: 15688–15695.

Daub H, Wallasch C, Lankenau A, Herrlich A, Ullrich A (1997). Signal characteristics of G protein-transactivated EGF receptor. EMBO J 16: 7032–7044.

DeFea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD, Bunnett NW (2000). Beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. J Cell Biol 148: 1267–1281.

Eisinger DA, Ammer H (2008a). Delta-opioid receptors activate ERK/MAP kinase via integrin-stimulated receptor tyrosine kinases. Cell Signal 20: 2324–2331.

Eisinger DA, Ammer H (2008b). Delta-opioid receptors stimulate ERK1/2 activity in NG108-15 hybrid cells by integrin-mediated transactivation of TrkA receptors. FEBS Lett 582: 3325–3329.

Eisinger DA, Ammer H (2011). Epidermal growth factor treatment switches delta-opioid receptor-stimulated extracellular signal-regulated kinases 1 and 2 signaling from an epidermal growth factor to an insulin-like growth factor-1 receptor-dependent mechanism. Mol Pharmacol 79: 326–335.

Eisinger DA, Ammer H, Schulz R (2002). Chronic morphine treatment inhibits opioid receptor desensitization and internalization. J Neurosci 22: 10192–10200.

Eisinger DA, Schulz R (2004). Extracellular signal-regulated kinase/mitogen-activated protein kinases block internalization of delta-opioid receptors. J Pharmacol Exp Ther 309: 776–785.

Forster K, Kuno A, Solenkova N, Felix SB, Krieg T (2007). The delta-opioid receptor agonist DADLE at reperfusion protects the heart through activation of pro-survival kinases via EGF receptor transactivation. American journal of physiology. Am J Physiol Heart Circ Physiol 293: H1604–1608.

Ghanouni P, Gryczynski Z, Steenhuis JJ, Lee TW, Farrens DL, Lakowicz JR *et al.* (2001). Functionally different agonists induce distinct conformations in the G protein coupling domain of the beta 2 adrenergic receptor. J Biol Chem 276: 24433–24436.

Girault JA, Valjent E, Caboche J, Herve D (2007). ERK2: a logical AND gate critical for drug-induced plasticity? Curr Opin Pharmacol 7: 77–85.

Girnita L, Shenoy SK, Sehat B, Vasilcanu R, Vasilcanu D, Girnita A *et al.* (2007). Beta-arrestin and Mdm2 mediate IGF-1 receptor-stimulated ERK activation and cell cycle progression. J Biol Chem 282: 11329–11338.

Ho MK, Yung LY, Chan JS, Chan JH, Wong CS, Wong YH (2001). Galpha(14) links a variety of G(i)- and G(s)-coupled receptors to the stimulation of phospholipase C. Br J Pharmacol 132: 1431–1440.

Hong MH, Xu C, Wang YJ, Ji JL, Tao YM, Xu XJ *et al.* (2009). Role of Src in ligand-specific regulation of delta-opioid receptor desensitization and internalization. J Neurochem 108: 102–114.

Im HJ, Muddasani P, Natarajan V, Schmid TM, Block JA, Davis F *et al.* (2007). Basic fibroblast growth factor stimulates matrix metalloproteinase-13 via the molecular cross-talk between the mitogen-activated protein kinases and protein kinase Cdelta pathways in human adult articular chondrocytes. J Biol Chem 282: 11110–11121.

Kilkenny C1, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160 (7): 1577–1579.

Kim J, Eckhart AD, Eguchi S, Koch WJ (2002). Beta-adrenergic receptor-mediated DNA synthesis in cardiac fibroblasts is dependent on transactivation of the epidermal growth factor receptor and subsequent activation of extracellular signal-regulated kinases. J Biol Chem 277: 32116–32123.

Kouhen OM, Wang G, Solberg J, Erickson LJ, Law PY, Loh HH (2000). Hierarchical phosphorylation of delta-opioid receptor regulates agonist-induced receptor desensitization and internalization. J Biol Chem 275: 36659–36664.

Kveiborg M, Instrell R, Rowlands C, Howell M, Parker PJ (2011). PKCalpha and PKCdelta regulate ADAM17-mediated ectodomain shedding of heparin binding-EGF through separate pathways. PLoS One 6: e17168.

Law PY, Kouhen OM, Solberg J, Wang W, Erickson LJ, Loh HH (2000). Deltorphin II-induced rapid desensitization of delta-opioid receptor requires both phosphorylation and internalization of the receptor. J Biol Chem 275: 32057–32065.

Law PY, Loh HH (1999). Regulation of opioid receptor activities. J Pharmacol Exp Ther 289: 607–624.

Lee JW, Joshi S, Chan JS, Wong YH (1998). Differential coupling of mu-, delta-, and kappa-opioid receptors to G alpha16-mediated stimulation of phospholipase C. J Neurochem 70: 2203–2211.

Lin CC, Lin WN, Cheng SE, Tung WH, Wang HH, Yang CM (2012). Transactivation of EGFR/PI3K/Akt involved in ATP-induced inflammatory protein expression and cell motility. J Cell Physiol 227 (4): 1628–1638.

Lin FT, Daaka Y, Lefkowitz RJ (1998). Beta-arrestins regulate mitogenic signaling and clathrin-mediated endocytosis of the insulin-like growth factor I receptor. J Biol Chem 273: 31640–31643.

Lo RK, Wong YH (2004). Signal transducer and activator of transcription 3 activation by the delta-opioid receptor via Galpha14 involves multiple intermediates. Mol Pharmacol 65: 1427–1439.

Lu L, Hope BT, Dempsey J, Liu SY, Bossert JM, Shaham Y (2005). Central amygdala ERK signaling pathway is critical to incubation of cocaine craving. Nat Neurosci 8: 212–219.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ *et al.* (1999). Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 283: 655–661.

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL *et al.* (2001). Activation and targeting of extracellular signal-regulated

kinases by beta-arrestin scaffolds. Proc Natl Acad Sci U S A 98: 2449–2454.

Maudsley S, Pierce KL, Zamah AM, Miller WE, Ahn S, Daaka Y *et al.* (2000). The beta(2)-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. J Biol Chem 275: 9572–9580.

McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT *et al.* (2000). Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. Science 290: 1574–1577.

McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160 (7): 1573–1576.

Murthy KS, Makhlouf GM (1996). Opioid mu, delta, and kappa receptor-induced activation of phospholipase C-beta 3 and inhibition of adenylyl cyclase is mediated by Gi2 and G(o) in smooth muscle. Mol Pharmacol 50: 870–877.

Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J *et al.* (2007). Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. J Clin Invest 117: 2445–2458.

Oligny-Longpre G, Corbani M, Zhou J, Hogue M, Guillon G, Bouvier M (2012). Engagement of beta-arrestin by transactivated insulin-like growth factor receptor is needed for V2 vasopressin receptor-stimulated ERK1/2 activation. Proc Natl Acad Sci U S A 109: E1028–1037.

Olivier AR, Parker PJ (1991). Expression and characterization of protein kinase C-delta. Eur J Biochem 200: 805–810.

Parker PJ, Murray-Rust J (2004). PKC at a glance. J Cell Sci 117: 131–132.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP, Davenport AP, McGrath JC, Peters JA, Southan C, Spedding M, Yu W, Harmar AJ; NC-IUPHAR. (2014) The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucl. Acids Res. 42 (Database Issue): D1098-106.

Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM, Lefkowitz RJ (2001). Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. J Biol Chem 276: 23155–23160.

Povsic TJ, Kohout TA, Lefkowitz RJ (2003). Beta-arrestin1 mediates insulin-like growth factor 1 (IGF-1) activation of phosphatidylinositol 3-kinase (PI3K) and anti-apoptosis. J Biol Chem 278: 51334–51339.

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.

Schulz R, Eisinger DA, Wehmeyer A (2004). Opioid control of MAP kinase cascade. Eur J Pharmacol 500: 487–497.

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.

Tilley DG, Kim IM, Patel PA, Violin JD, Rockman HA (2009). Beta-arrestin mediates beta1-adrenergic receptor-epidermal growth factor receptor interaction and downstream signaling. J Biol Chem 284: 20375–20386.

Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM (2002). Beta-arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. J Biol Chem 277: 9429–9436.

Trapaidze N, Keith DE, Cvejic S, Evans CJ, Devi LA (1996). Sequestration of the delta opioid receptor. Role of the C terminus in agonist-mediated internalization. J Biol Chem 271: 29279–29285.

Uchiyama K, Saito M, Sasaki M, Obara Y, Higashiyama S, Nakahata N (2009). Thromboxane A2 receptor-mediated epidermal growth factor receptor transactivation: involvement of PKC-delta and PKC-epsilon in the shedding of epidermal growth factor receptor ligands. Eur J Pharm Sci 38: 504–511.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM *et al.* (2003). Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. Proc Natl Acad Sci U S A 100: 10782–10787.

Werry TD, Sexton PM, Christopoulos A (2005). "Ins and outs" of seven-transmembrane receptor signalling to ERK. Trends Endocrinol Metab 16: 26–33.

Wiese S, Jablonka S, Holtmann B, Orel N, Rajagopal R, Chao MV *et al.* (2007). Adenosine receptor A2A-R contributes to motoneuron survival by transactivating the tyrosine kinase receptor TrkB. Proc Natl Acad Sci U S A 104 (43): 17210–17215.

Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S *et al.* (2007). A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. Proc Natl Acad Sci U S A 104: 16657–16662.

Woods DC, Johnson AL (2007). Protein kinase C activity mediates LH-induced ErbB/Erk signaling in differentiated hen granulosa cells. Reproduction 133: 733–741.

Xu C, Hong MH, Zhang LS, Hou YY, Wang YH, Wang FF *et al.* (2010). Serine 363 of the {delta}-opioid receptor is crucial for adopting distinct pathways to activate ERK1/2 in response to stimulation with different ligands. J Cell Sci 123: 4259–4270.

Yang CM, Hsieh HL, Yao CC, Hsiao LD, Tseng CP, Wu CB (2009). Protein kinase C-delta transactivates platelet-derived growth factor receptor-alpha in mechanical strain-induced collagenase 3 (matrix metalloproteinase-13) expression by osteoblast-like cells. J Biol Chem 284: 26040–26050.

Yarden Y, Sliwkowski MX (2001). Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2: 127–137.

Zhang X, Wang F, Chen X, Li J, Xiang B, Zhang YQ *et al.* (2005). Beta-arrestin1 and beta-arrestin2 are differentially required for phosphorylation-dependent and -independent internalization of delta-opioid receptors. J Neurochem 95: 169–178.

Zheng H, Shen H, Oprea I, Worrall C, Stefanescu R, Girnita A *et al.* (2012). Beta-arrestin-biased agonism as the central mechanism of action for insulin-like growth factor 1 receptor-targeting antibodies in Ewing's sarcoma. Proc Natl Acad Sci U S A 109: 20620–20625.