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RESEARCH PAPER

Deciphering μ-opioid receptor phosphorylation and dephosphorylation in HEK293 cells

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BACKGROUND AND PURPOSE

The molecular basis of agonist-selective signalling at the μ-opioid receptor is poorly understood. We have recently shown that full agonists such as [D-Ala^2-MePhe^4-Gly-ol]enkephalin (DAMGO) stimulate the phosphorylation of a number of carboxyl-terminal phosphate acceptor sites including threonine 370 (Thr^{370}) and serine 375 (Ser^{375}), and that is followed by a robust receptor internalization. In contrast, morphine promotes a selective phosphorylation of Ser^{375} without causing rapid receptor internalization.

EXPERIMENTAL APPROACH

Here, we identify kinases and phosphatases that mediate agonist-dependent phosphorylation and dephosphorylation of the μ-opioid receptor using a combination of phosphosite-specific antibodies and siRNA knock-down screening in HEK293 cells.

KEY RESULTS

We found that DAMGO-driven phosphorylation of Thr^{370} and Ser^{375} was preferentially catalysed by G-protein-coupled receptor kinases (GRKs) 2 and 3, whereas morphine-driven Ser^{375} phosphorylation was preferentially catalysed by GRK5. On the functional level, inhibition of GRK expression resulted in enhanced μ-opioid receptor signalling and reduced receptor internalization. Analysis of GRK5-deficient mice revealed that GRK5 selectively contributes to morphine-induced Ser^{375} phosphorylation in brain tissue. We also identified protein phosphatase 1γ as a μ-opioid receptor phosphatase that catalysed Thr^{370} and Ser^{375} dephosphorylation at or near the plasma membrane within minutes after agonist removal, which in turn facilitates receptor recycling.

CONCLUSIONS AND IMPLICATIONS

Together, the morphine-activated μ-opioid receptor is a good substrate for phosphorylation by GRK5 but a poor substrate for GRK2/3. GRK5 phosphorylates μ-opioid receptors selectively on Ser^{375}, which is not sufficient to drive significant receptor internalization.

Abbreviations

DAMGO, [D-Ala^2-MePhe^4-Gly-ol]enkephalin; GRK, G-protein-coupled receptor kinase; PP, protein phosphatase

Introduction

The opioid alkaloid morphine is one of the most potent analgesics. However, the clinical utility of morphine is limited by the rapid development of tolerance and dependence (Koob *et al.*, 1998; Nestler, 1996; Nestler and Aghajanian, 1997). Morphine exerts all of its pharmacological effects by interacting with the μ-opioid receptor (Matthes *et al.*, 1996). For many opioids, the ability to induce tolerance correlates inversely with their capacity to induce μ receptor phosphorylation and internalization (Burd *et al.*, 1998; Zhang *et al.*, 1998; Arttamangkul *et al.*, 2008; Bailey *et al.*, 2009). However, morphine is a particularly poor inducer of μ-opioid receptor endocytosis, but a potent inducer of cellular

tolerance (Arden *et al.*, 1995; Keith *et al.*, 1996; Alvarez *et al.*, 2001; Schulz *et al.*, 2004; Gintzler and Chakrabarti, 2006; Johnson *et al.*, 2006; McPherson *et al.*, 2010). Conversely, full agonists such as fentanyl or etonitazene elicit robust μ receptor internalization but less tolerance (Elmer *et al.*, 1993; Gerak and France, 1996; 1997; Sala *et al.*, 1992; Walker and Young, 2001; Grecksch *et al.*, 2006; 2011; Hull *et al.*, 2009). We have recently generated phosphosite-specific antibodies for the carboxyl-terminal residues threonine 370 (Thr^{370}) and serine 375 (Ser^{375}), which enabled us to selectively detect either the Thr^{370}-phosphorylated or the Ser^{375}-phosphorylated form of the μ receptor. We have recently shown that [D-Ala^2-MePhe^4-Gly-ol]enkephalin (DAMGO) stimulated the phosphorylation of both Thr^{370} and Ser^{375} (Doll *et al.*, 2011). In contrast, morphine promoted the phosphorylation of Ser^{375} but failed to stimulate Thr^{370} phosphorylation (Doll *et al.*, 2011). As a functional consequence, DAMGO facilitates recruitment of β-arrestin1 and β-arrestin2, whereas morphine promotes recruitment of β-arrestin2 but not of β-arrestin1 (Groer *et al.*, 2011). Nevertheless, the detailed molecular events leading to site-specific phosphorylation and dephosphorylation of the MOR are still largely unknown. Here, we demonstrate using a combination of phosphosite-specific antibodies and siRNA knock-down that in HEK293 cells DAMGO-driven phosphorylation of Thr^{370} and Ser^{375} is preferentially catalysed by G-protein-coupled receptor kinases (GRKs) 2 and 3, whereas morphine-driven Ser^{375} phosphorylation is preferentially catalysed by GRK5. In addition, we identify protein phosphatase 1γ (PP1γ) as GPCR phosphatase for the μ-opioid receptor.

Methods

Reagents and antibodies

Morphine-hydrochloride was obtained from Merck (Darmstadt, Germany). DAMGO was purchased from Bachem (Weil am Rhein, Germany). Calyculin A and okadaic acid were from Sigma (Taufkirchen, Germany). The rabbit polyclonal phosphosite-specific antibodies anti-pThr^{370} {3196} and anti-pSer^{375} {2493} were generated and extensively characterized as described previously (Lupp *et al.*, 2011). The phosphorylation-independent rabbit monoclonal anti-μ-opioid receptor antibody {UMB-3} was obtained from Epitomics (Burlingame, CA, USA). Anti-GRK2, anti-GRK3, anti-GRK5, anti-GRK6 and anti-pan-PP1 antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The anti-pan-PP2 antibody was purchased from BD Biosciences (Heidelberg, Germany). Total ERK1/2 and pERK1/2 antibodies were from Cell Signaling Technologies (Frankfurt, Germany).

Cell culture and transfection

HEK293 cells were obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany). HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected with a plasmid encoding for a haemagglutinin (HA)-tagged mouse μ-opioid receptor, GRK2, GRK3 or GRK5 using Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen, Darmstadt, Germany). Stable transfectants were selected in the presence of 1 $\mu g \cdot mL^{-1}$ puromycin. HEK293 cells stably expressing mouse μ-opioid receptors were characterized using radioligand-binding assays, cAMP assays, Western blot analysis and immunocytochemistry as described previously (Koch *et al.*, 2001; Schulz *et al.*, 2004). The level of μ receptor expression was ~700 fmol mg^{-1} membrane protein.

Western blot analysis

Cells were seeded onto poly-L-lysine-coated 60 mm dishes and grown to 80% confluence. After treatment with morphine or DAMGO, cells were lysed in detergent buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM disodium pyrophosphate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of protease and phosphatase inhibitors Complete mini and PhosSTOP (Roche Diagnostics, Mannheim, Germany). Glycosylated proteins were partially enriched using wheat germ lectin-agarose beads as described (Koch *et al.*, 2001; Schulz *et al.*, 2004). Proteins were eluted from the beads using SDS-sample buffer for 20 min at 45°C. Samples were split, resolved on 7.5% SDS-polyacrylamide gels, and after electroblotting, membranes were incubated with either 0.1 $\mu g \cdot mL^{-1}$ anti-pT370 {3196} or 0.1 $\mu g \cdot mL^{-1}$ anti-pS375 {2493} followed by detection using an enhanced chemiluminescence detection system (Amersham, Braunschweig, Germany). Blots were subsequently stripped and reprobed with anti-μ-opioid receptor antibody {UMB-3} to confirm equal loading of the gels.

Immunocytochemistry

Cells were grown on poly-L-lysine-coated coverslips overnight. Three days after siRNA transfection and the appropriate treatment with DAMGO, cells were fixed with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer (pH 6.9) for 30 min at room temperature and washed several times. Specimens were permeabilized and then incubated with anti-pSer^{375} {2493} antibody or with anti-μ-opioid receptor antibody {UMB-3} followed by an Alexa488-conjugated secondary antibody (Amersham). Specimens were mounted and examined using a Zeiss LSM510 META laser scanning confocal microscope.

Internalization assays

Cells were seeded onto 24-well plates. On the next day, cells were pre-incubated with anti-HA antibody for 2 h at 4°C. Cells were then transferred to 37°C, exposed to agonist, fixed and developed with peroxidase-conjugated secondary antibody as described previously (Lesche *et al.*, 2009; Poll *et al.*, 2010).

Small interfering RNA silencing of gene expression

Chemically synthesized double-stranded siRNA duplexes (with 3′ dTdT overhangs) were purchased from Qiagen (Hilden, Germany) for the following targets: GRK2 (5′-CCGGGAGATCTTCGACTCATA-3′ and 5′-AAGAAGTACG AGAAGCTGGAG-3′), GRK3 (5′-AAGCAAGCTGTAGAACA CGTA-3′ and 5′-GCAGAAGTCGACAAATTTA-3′), GRK5 (5′-AGCGTCATAACTAGAACTGAA-3′ and 5′-AAGCCGTGCAA

AGAACTCTTT-3′), GRK6 (5′-AACACCTTCAGGCAATACC GA-3′ and 5′-AACAGTAGGTTTGTAGTGAGC-3′) PP1α catalytic subunit (5′-AAGAGACGCTACAACATCAAA-3′), PP1β catalytic subunit (5′-TACGAGGATGTCGTCCAGGAA-3′ and 5′-GTTCGAGGCTTATGTATCA-3′), PP1γ catalytic subunit (5′-AACATCGACAGCATTATCCAA-3′ and 5′-AGAGGCAGTTGG TCACTCT-3′), PP2Aα catalytic subunit (5′-ACACCTCGTGA ATACAATTTA-3′), PP2Aβ catalytic subunit (5′-CCGACAAAT TACCCAAGTATA-3′) and a non-silencing RNA duplex (5′-GCTTAGGAGCATTAGTAAA-3′ or 5′-AAA CTC TAT CTG CAC GCT GAC-3′). HEK293 cells were transfected with 150 nM siRNA for single transfection or with 100 nM of each siRNA for double transfection using HiPerFect (Qiagen). Silencing was quantified by immunoblotting. All experiments showed protein levels reduced by ≥80%.

In vivo *phosphorylation studies*

GRK5 knockout mice ($Grk5^{tm1Rjl}$) were obtained from The Jackson Laboratory. Animals were housed in a 12 h light-dark cycle and had free access to food and water. All animal experiments were performed in accordance with the Thuringian state authorities and complied with European Commission regulations for the care and use of laboratory animals. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

After the indicated treatment, mice ($n = 40$) were killed by cervical dislocation and brains from $GRK5^{+/+}$ ($n = 20$) and $GRK5^{-/-}$ ($n = 20$) mice were quickly dissected. The cerebellum, which is devoid of μ-opioid receptors, was removed, and the remaining brain samples were immediately frozen in liquid nitrogen. In all experiments, entire brains except cerebellum were used. Samples were transferred to ice-cold detergent buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM disodium pyrophosphate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS containing protease and phosphatase inhibitors), homogenized and centrifuged at 16 000× *g* for 30 min at 4°C. The supernatant was then immunoprecipitated with the phosphorylation-independent rabbit monoclonal anti-μ-opioid receptor antibody {UMB-3} bound to protein A-agarose beads for 2 h at 4°C (Grecksch *et al.*, 2011; Lupp *et al.*, 2011). Proteins were eluted from the beads with SDS-sample buffer for 20 min at 40°C. Samples were resolved on 8% SDS-polyacrylamide gels, and after electroblotting, membranes were incubated with guinea pig polyclonal anti-pS375 antibody {GP2} at a concentration of 0.1 μg·ml^{-1} followed by detection using an enhanced chemiluminescence detection system. Blots were subsequently stripped and reprobed with phosphorylation-independent guinea pig polyclonal anti-μ-opioid receptor antibody {GP6} at a concentration of 0.1 μg·ml^{-1} to confirm equal loading of the gels (Grecksch *et al.*, 2011; Lupp *et al.*, 2011).

Data analysis

ImageJ 1.40 g software was used to densitize and quantify protein bands detected on Western blots. Data were analysed using GraphPad Prism 4.0 software. Statistical analysis was carried out with two-way ANOVA followed by the Bonferroni post-test. *P*-values of <0.05 were considered statistically significant.

Results

Site-specific phosphorylation of the μ-opioid receptor

We have recently generated phosphosite-specific antibodies that enable us to selectively detect either the Thr^{370}-phosphorylated or the Ser^{375}-phosphorylated form of the μ receptor. As depicted in Figure 1, DAMGO stimulates the phosphorylation of both Thr^{370} and Ser^{375}. In contrast, morphine promotes a selective phosphorylation of Ser^{375} without causing any substantial phosphorylation of Thr^{370} (Doll *et al.*, 2011). However, the molecular mechanisms underlying this site-specific phosphorylation of the μ-opioid receptor are far from understood.

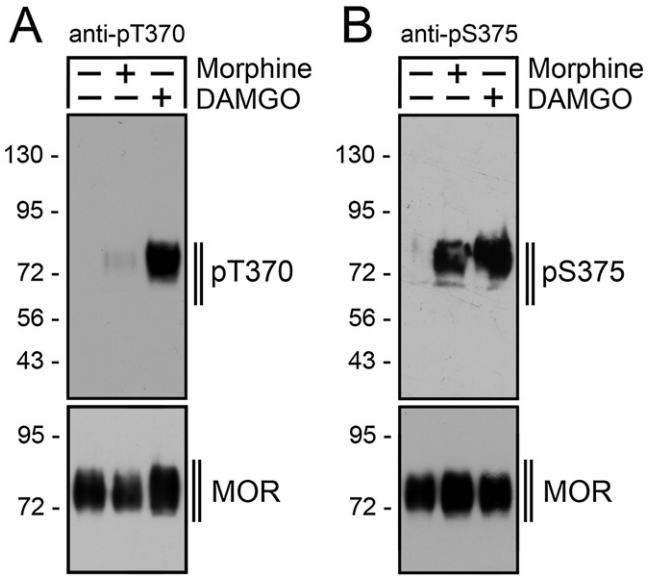

Figure 1

Site-specific phosphorylation of μ-opioid receptor. (A) HEK293 cells stably expressing the mouse μ-opioid receptor were either not exposed (–) or exposed (+) to 10 μM morphine or 10 μM DAMGO for 30 min. Cells were lysed and immunoblotted with the anti-$pThr^{370}$ (pT370, upper panel) antibody. Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody UMB-3 to confirm equal loading of the gel (MOR, lower panel). Note that Thr^{370} phosphorylation is detectable only after DAMGO but not after morphine treatment. (B) HEK293 cells stably expressing the μ-opioid receptor were either not exposed (–) or exposed (+) to 10 μM morphine or 10 μM DAMGO for 30 min. Cells were lysed and immunoblotted with an anti-$pSer^{375}$ (pS375, upper panel) antibody. Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody UMB-3 to confirm equal loading of the gel (MOR, lower panel). Shown are representative results from one of four independent experiments per condition. The position of molecular mass markers is indicated on the left (in kDa).

GRK2 and GRK3 catalyse DAMGO-induced phosphorylation of Thr^{370} and Ser^{375}

We used specific siRNA sequences to evaluate the contribution of GRK2 and GRK3 to agonist-induced μ receptor phosphorylation. Inhibition of GRK2 or GRK3 expression significantly reduced DAMGO-induced phosphorylation of both Thr^{370} and Ser^{375} (Figure 2A). Given the close relationship between GRK2 and GRK3, it is conceivable that the loss of one GRK could be compensated for by another GRK. We therefore examined the effect of inhibition of both GRK2 and GRK3 expressions. The results show that combined administration of GRK2 and GRK3 siRNAs led to a complete inhibition of Thr^{370} and Ser^{375} phosphorylation indicating that GRK2 and GRK3 function as a redundant phosphorylation system for the DAMGO-activated μ-opioid receptor (Figure 2A). In contrast, inhibition of GRK5 or GRK6 expression had no effect on DAMGO-induced μ-opioid receptor phosphorylation (Figure 2B). We have previously shown that overexpression of GRK2 can enhance morphine-induced Ser^{375} phosphorylation (Zhang *et al.*, 1998; Schulz *et al.*, 2004). We therefore examined to what extent overexpression

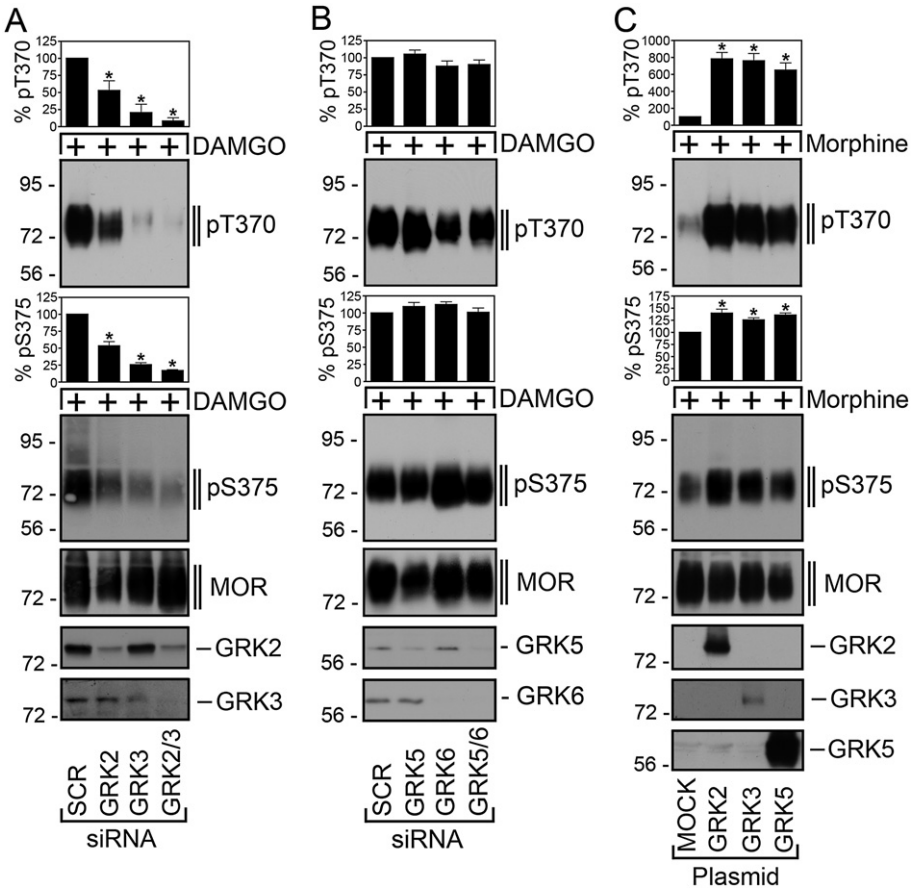

Figure 2

GRK2 and GRK3 are responsible for DAMGO-induced Thr^{370} and Ser^{375} phosphorylation. (A) HEK293 cells stably expressing the μ-opioid receptor were transfected with siRNA targeted to GRK2, GRK3, GRK2 and GRK3 or non-silencing siRNA control (SCR) for 72 h and then exposed to 10 μM DAMGO for 30 min. Cells were lysed and immunoblotted with anti-pThr^{370} (pT370, upper panel) or anti-pSer^{375} antibodies (pS375, lower panel). Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody UMB-3 to confirm equal loading of the gels (MOR). siRNA knock-down of GRK2 and GRK3 was confirmed by Western blot. Note that transfection with either GRK2 or GRK3 siRNAs resulted in a significant decrease of DAMGO-induced Thr^{370} and Ser^{375} phosphorylation. (B) HEK293 cells stably expressing the μ-opioid receptor were transfected with siRNA targeted to GRK5, GRK6, GRK5 and GRK6 or non-silencing siRNA control (SCR) for 72 h and then exposed to 10 μM DAMGO for 30 min. Cells were lysed and immunoblotted with anti-pThr^{370} (pT370, upper panel) or anti-pSer^{375} antibodies (pS375, lower panel). Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody UMB-3 to confirm equal loading of the gels (MOR). (C) HEK293 cells stably expressing the μ-opioid receptor were transfected with empty vector (MOCK), GRK2, GRK3 or GRK5 for 2 days. Cells were then treated with 10 μM morphine for 5 min. The levels of phosphorylated μ receptors were determined using anti-pThr^{370} (pT370, upper panel) and anti-pSer^{375} antibodies (pS375, lower panel). Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody UMB-3 to confirm equal loading of the gels (MOR). Overexpression of GRK2, GRK3 and GRK5 was confirmed by Western blot. Blots were quantified and expressed as percentage of maximal phosphorylation in SCR- or MOCK-transfected cells, which was set at 100%. Data correspond to the mean ± SEM from four to five independent experiments. Results were analysed by two-way ANOVA followed by the Bonferroni post-test (*, $P < 0.05$). Note that overexpression of GRK2, GRK3 and GRK5 strongly enhanced morphine-induced Thr^{370} and Ser^{375} phosphorylation. The positions of molecular mass markers are indicated on the left (in kDa).

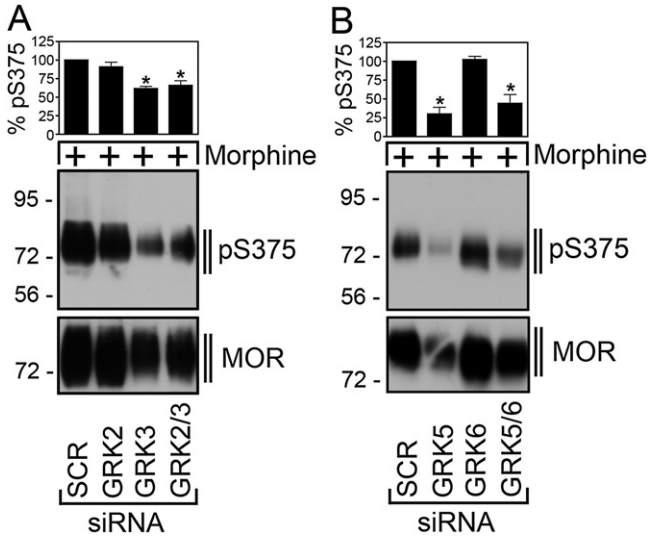

Figure 3

GRK5 is responsible for morphine-induced Ser^{375} phosphorylation. (A) HEK293 cells stably expressing the μ-opioid receptor were transfected with siRNA targeted to GRK2 and GRK3, GRK2 and GRK3 or non-silencing siRNA control (SCR) for 72 h and then exposed to 10 μM morphine for 30 min. Cells were lysed and immunoblotted with anti-pSer^{375} antibodies (pS375, lower panel). Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody UMB-3 to confirm equal loading of the gels (MOR). (B) HEK293 cells stably expressing the μ-opioid receptor were transfected with siRNA targeted to GRK5, GRK6, GRK5 and GRK6 or non-silencing siRNA control (SCR) for 72 h and then exposed to 10 μM morphine for 30 min. Cells were lysed and immunoblotted with anti-pSer^{375} antibodies (pS375, lower panel). Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody UMB-3 to confirm equal loading of the gels (MOR). Blots were quantified and expressed as percentage of maximal phosphorylation in SCR-transfected cells, which was set at 100%. Data correspond to the mean ± SEM from four independent experiments. Results were analysed by two-way ANOVA followed by the Bonferroni post-test (*, $P < 0.05$). Note that transfection with GRK5 siRNA resulted in a significant decrease of morphine-induced Ser^{375} phosphorylation. The positions of molecular mass markers are indicated on the left (in kDa).

of GRK2, GRK3 or GRK5 would alter the site-specific phosphorylation of the morphine-activated μ receptor. We found that overexpression of GRK2 or GRK3 resulted in an increase in Ser^{375} phosphorylation as well as in a clearly detectable Thr^{370} phosphorylation even after a brief exposure to morphine (Figure 2C). To our surprise, overexpression of GRK5 also resulted in robust increase in Thr^{370} and Ser^{375} phosphorylation (Figure 2C).

GRK5 catalyses morphine-induced Ser^{375} phosphorylation

Consequently, we examined the contribution of GRK5 to morphine-induced μ receptor phosphorylation. As depicted in Figure 3A, morphine-induced Ser^{375} phosphorylation was only partially inhibited (~40%) when expression of GRK2/3 was inhibited. In contrast, siRNA knock-down of GRK5 but not GKR6 strongly inhibited (~70%) morphine-induced Ser^{375} phosphorylation (Figure 3B). Combined administration of 200 nM GRK5 and 200 nM GRK6 siRNAs did not result in an inhibition of morphine-induced Ser^{375} phosphorylation over that seen with 150 nM GRK5 siRNA alone (Figure 3B). These findings suggest that the morphine-activated μ-opioid receptor is an efficient substrate for phosphorylation by GRK5.

Knock-down of GRKs leads to enhanced μ-opioid receptor signalling

Exposure of the μ-opioid receptor to both morphine and DAMGO facilitates ERK signalling. ERK activation was sensitive to pertussis toxin indicating that opioid-induced ERK signalling was largely mediated by G_i proteins (Figure 4A). We then evaluated the role of GRKs in μ-opioid receptor-dependent ERK activation. As depicted in Figures 4B and C, siRNA knock-down of GRK2/3 and GRK5/6 strongly enhanced ERK activation induced by DAMGO and morphine, respectively. These findings suggest that GRK-mediated phosphorylation of the μ-opioid receptor is involved in desensitization of its G protein signalling. We also evaluated the role of GRKs in DAMGO-induced μ-opioid receptor sequestration using a quantitative ELISA assay. In fact, GRK2/3 knock-down reduced DAMGO-induced internalization by ~50% while GRK5/6 knock-down had little effect (not shown).

GRK5 selectively contributes to morphine-induced Ser^{375} phosphorylation in vivo

GRK5$^{-/-}$ and GRK5$^{+/+}$ mice were treated with morphine (30 mg·kg^{-1} s.c.) for 30 min. When μ-opioid receptors were immunoprecipitated from brain homogenates of these mice using the rabbit monoclonal antibody UMB-3 and Ser^{375} phosphorylation detected using the guinea pig polyclonal anti-pSer^{375} antibody {GP2}, we found a strong inhibition (~50%) of morphine-induced Ser^{375} phosphorylation in GRK5$^{-/-}$ mice compared with GRK5$^{+/+}$ mice (Figure 5). In contrast, when mice were treated with etonitazene (30 μg·kg^{-1} s.c.) for 30 min, we observed a similar robust Ser^{375} phosphorylation in GRK5$^{-/-}$ and GRK5$^{+/+}$ mice (Figure 5). These findings suggest that GRK5 selectively contributes to morphine-induced Ser^{375} phosphorylation *in vivo*.

PP1γ catalyses μ-opioid receptor dephosphorylation

The phosphatase involved in μ-opioid receptor dephosphorylation has not been identified yet. Serine/threonine-specific protein phosphatases are classified into seven families PP1–PP7 (Virshup and Shenolikar, 2009). Whereas calyculin A inhibits the activity of PP1 and PP2 to a similar extent, okadaic acid blocks PP2A activity but has little effect on PP1 (Cohen, 1989; Ishihara *et al.*, 1989; Cohen *et al.*, 1990; Hardie *et al.*, 1991; Spurney, 2001). Neither calyculin A nor okadaic acid is able to reduce PP3 activity. When DAMGO was washed out, the μ receptor was completely dephosphorylated within 20 min (Figure 6). In the presence of calyculin A, Ser^{375} dephosphorylation was inhibited in a dose-dependent manner (Figure 6A). Under otherwise identical conditions, okadaic acid was not able to block Ser^{375} dephosphorylation (Figure 6B). These results strongly suggest that PP1 activity

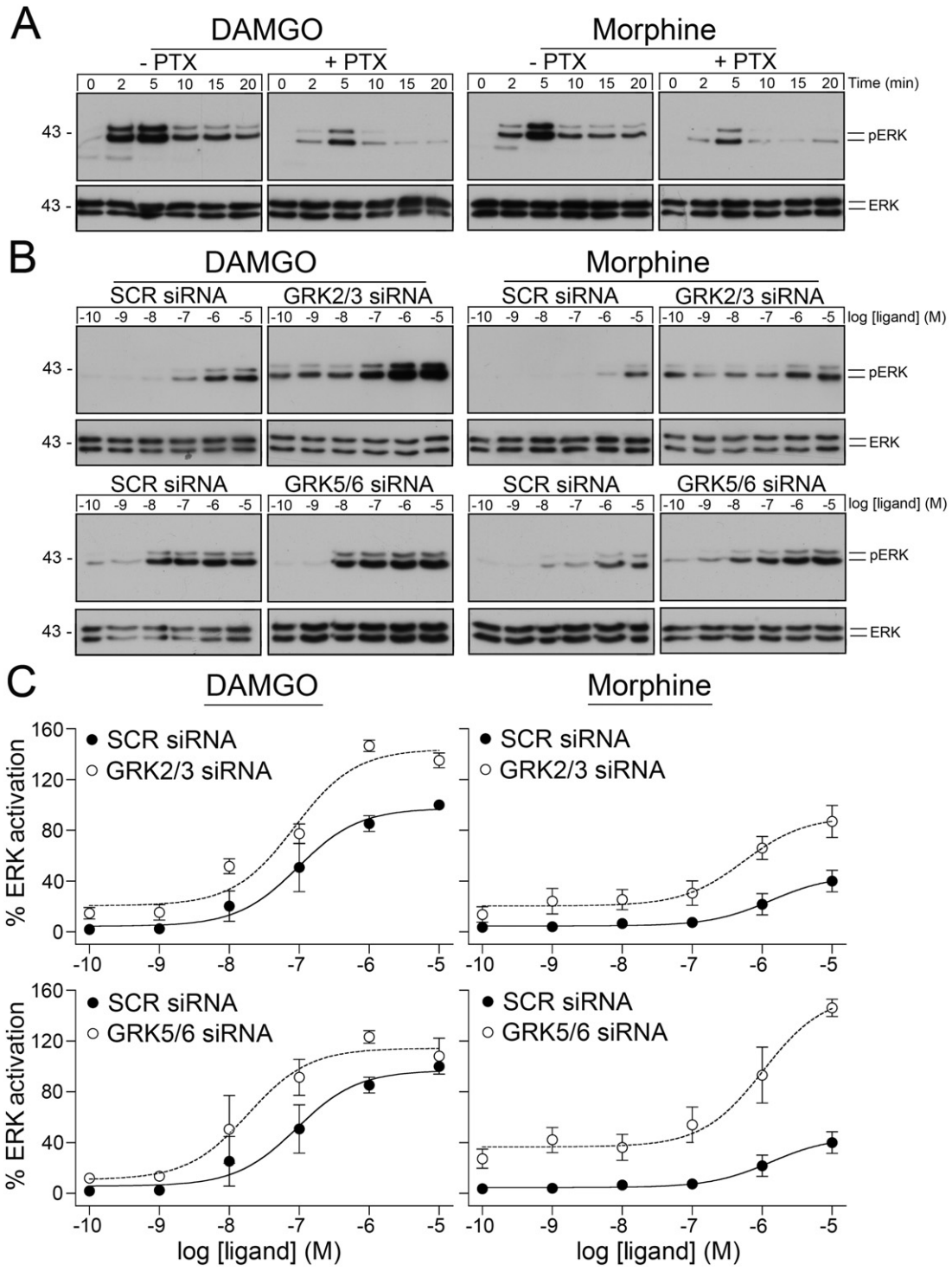

Figure 4

Inhibition of GRK expression facilitates μ-opioid receptor signalling. (A) HEK293 cells stably expressing the μ-opioid receptor were incubated with 100 ng·ml^{-1} pertussis toxin (PTX) for 16 h and then exposed to 10 μM DAMGO for 0, 2, 5, 10, 15 or 20 min (left panel) or 10 μM morphine for 0, 2, 5, 10, 15 or 20 min (right panel). The levels of phosphorylated ERK (pERK1/2) and total ERK (ERK1/2) were then determined by Western blot analysis. (B) HEK293 cells stably expressing the μ-opioid receptor were transfected with siRNA targeted to GRK2 and GRK3 (upper panel), GRK5 and GRK6 (lower panel) or non-silencing siRNA control (SCR) for 72 h and then exposed for 5 min to DAMGO (left panel) or morphine (right panel) in concentrations ranging from 10^{-10} to 10^{-5} M. The levels of phosphorylated ERK (pERK1/2) and total ERK (ERK1/2) were then determined by Western blot analysis. (C) ERK activation was quantified and expressed as percentage of maximal phosphorylation in SCR-transfected cells after stimulation with DAMGO, which was set at 100%. Data correspond to the mean ± SEM from at least five independent experiments performed in duplicate. The positions of molecular mass markers are indicated on the left (in kDa).

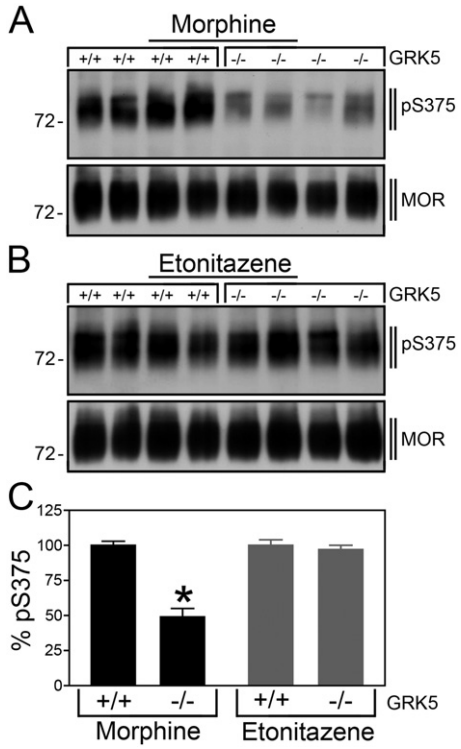

Figure 5

GRK5 selectively contributes to morphine-induced Ser^{375} phosphorylation in mouse brain. (A) GRK5$^{+/+}$ mice (n = 4) and GRK5$^{-/-}$ mice (n = 4) were treated with morphine (30 mg·kg^{-1} s.c.). (B) GRK5$^{+/+}$ mice (n = 4) and GRK5$^{-/-}$ mice (n = 4) were treated with etonitazene (30 μg·kg^{-1} s.c.). After 30 min, brains were dissected. Homogenates were prepared from entire brain after removal of the cerebellum. μ-Opioid receptors were immunoprecipitated with UMB-3 and immunoblotted with guinea pig anti-pS375 antibody {GP2} (upper panels). Blots were stripped and reprobed with the phosphorylation-independent guinea pig anti-μ-opioid receptor {GP6} to confirm equal loading of the gel (lower panels). The positions of molecular mass markers are indicated on the left (in kDa). Note that Ser^{375} phosphorylation was reduced in morphine- but not in etonitazene-treated GRK5$^{-/-}$ mice compared with GRK5$^{+/+}$ mice. (C) Data are reported as % Ser^{375} phosphorylation in GRK5$^{+/+}$ mice, which was set at 100%. Data are presented as the means ± SEM from GRK5$^{+/+}$ mice (morphine: n = 10, etonitazene: n = 10) and GRK5$^{-/-}$ mice (morphine: n = 10, etonitazene: n = 10). Differences between genotypes were analysed by two-way ANOVA followed by the Bonferroni post-test (*P < 0.05).

was required for μ receptor dephosphorylation. We next used specific siRNA sequences to evaluate the contribution of the catalytic subunits PP1α, PP1β and PP1γ to μ receptor dephosphorylation. The results depicted in Figure 7A show that only inhibition of PP1γ expression significantly attenuated Thr^{370} and Ser^{375} dephosphorylation. In contrast, siRNA knock-down of PP1α or PP1β had no effect on μ receptor dephosphorylation (Figure 7A). Inhibition of PP1γ expression also reduced Ser^{375} dephosphorylation in morphine-treated cells (not shown). Moreover, siRNA knock-down of PP2α and PP2β also did not influence μ receptor dephosphorylation (not shown). We then evaluated the effect of PP1γ siRNA knock-down on the subcellular distribution of Ser^{375}-phosphorylated μ-opioid receptors in DAMGO-treated cells. As depicted in Figure 7B, inhibition of PP1γ expression facilitated detection of Ser^{375}-phosphorylated receptors at the plasma membrane 5 min after DAMGO exposure. These results strongly suggest that μ-opioid receptor dephosphorylation is a highly regulated process that is initiated shortly after receptor activation at or near the plasma membrane.

PP1γ facilitates μ-opioid receptor recycling

Finally, we analysed the role of dephosphorylation in receptor trafficking. As depicted in Figure 8, complete redistribution of internalized μ-opioid receptors to the plasma membrane requires a prolonged incubation (~120 min) in agonist-free medium. After PP1γ knock-down, internalized μ-opioid receptors were still detectable after 120 min in cells treated under otherwise identical conditions (Figure 8). These results suggest that PP1γ-mediated dephosphorylation facilitates μ-opioid receptor recycling.

Discussion

Like endogenous opioid peptides, morphine binds and activates the μ-opioid receptor (Arden *et al.*, 1995; Keith *et al.*, 1996; Burd *et al.*, 1998; Koch *et al.*, 2001). Unlike endogenous opioids, however, morphine does not elicit robust receptor sequestration (Arden *et al.*, 1995; Keith *et al.*, 1996; Schulz *et al.*, 2004; Johnson *et al.*, 2006; McPherson *et al.*, 2010). Up to date, the molecular basis for this agonist-selective μ receptor internalization remains unknown. Earlier studies revealed that the ability of distinct opioid agonists to differentially regulate receptor endocytosis is related to their ability to promote GRK2-dependent phosphorylation of the μ-opioid receptor (Zhang *et al.*, 1998; Ferguson, 2001; Schulz *et al.*, 2004; Kenski *et al.*, 2005). Analysis of serial truncation and site-directed mutants suggested that phosphorylation occurs primarily at Ser^{363}, Thr^{370} and Ser^{375} within the cytoplasmic tail of the receptor (El Kouhen *et al.*, 2001; Chu *et al.*, 2008). In addition, more recent studies have provided evidence for agonist-driven phosphorylation of Thr^{376} and Thr^{379} (Lau *et al.*, 2011). On the functional level, mutation of Ser^{375}, Thr^{376} or Thr^{379} to alanine resulted in a similar inhibition of μ-opioid receptor endocytosis (Lau *et al.*, 2011).

We have recently generated phosphosite-specific antibodies, which enabled us to selectively detect the Ser^{363}-, Thr^{370}- or the Ser^{375}-phosphorylated forms of the receptor (Doll *et al.*, 2011). We found that the phosphorylation of these three sites is differently regulated: DAMGO stimulated the phosphorylation of both Thr^{370} and Ser^{375} with Ser^{375} being the primary site of phosphorylation. In contrast, morphine promoted the phosphorylation of Ser^{375} but failed to stimulate Thr^{370} phosphorylation (Doll *et al.*, 2011; Grecksch *et al.*, 2011). Yet, Ser^{363} is constitutively phosphorylated in the absence of agonist (Doll *et al.*, 2011).

In the present study, we used our novel phosphosite-specific antibodies in combination with siRNA knock-down screening to identify kinases and phosphatases involved in agonist-dependent phosphorylation and dephosphorylation of the μ-opioid receptor. Our findings clearly show that the morphine-activated μ-opioid receptor acquires a conforma-

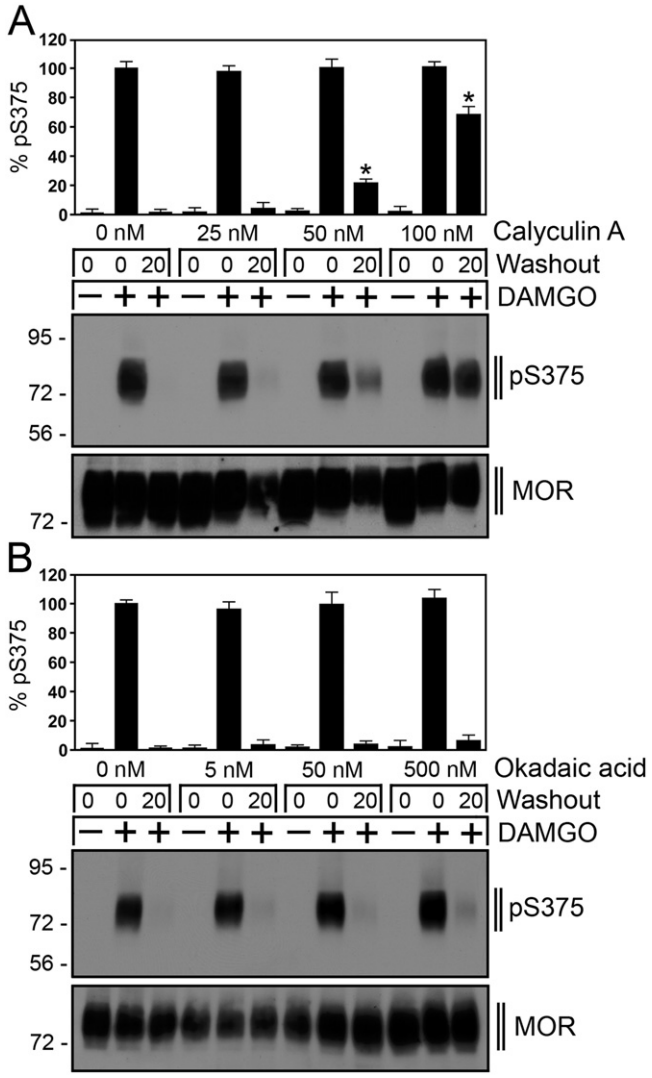

Figure 6

Inhibition of μ-opioid receptor dephosphorylation by calyculin A. (A) HEK293 cells stably expressing the μ-opioid receptor were incubated with 0, 25, 50 or 100 nM calyculin A for 5 min and then either not exposed (–) or exposed (+) to 10 μM DAMGO for 30 min. Cells were washed three times with cold citrate buffer (pH 4.5) (Washout), and then incubated in the absence of agonist for 0 or 20 min in the presence of the above indicated concentrations of calyculin A at 37°C. Cells were lysed and immunoblotted with the anti-pSer^{375} antibody (pS375). Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody UMB-3 to confirm equal loading of the gels (MOR). (B) HEK293 cells stably expressing the μ-opioid receptor were incubated with 0, 5, 50 or 500 nM okadaic acid for 5 min and then either not exposed (–) or exposed (+) to 10 μM DAMGO for 30 min. Cells were washed three times with cold citrate buffer (pH 4.5) (Washout), and then incubated in the absence of agonist for 0 or 20 min in the presence of the above indicated concentrations of calyculin A at 37°C. Cells were lysed and immunoblotted with the anti-pSer^{375} antibody (pS375). Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody UMB-3 to confirm equal loading of the gels (MOR). Shown are representative results from one of three independent experiments per condition. Blots were quantified and expressed as percentage of maximal phosphorylation in untreated cells, which was set at 100%. Data correspond to the mean ± SEM from four to five independent experiments. Results were analysed by two-way ANOVA followed by the Bonferroni post-test (* $P < 0.05$). Note that calyculin A but not okadaic acid inhibited μ-opioid receptor dephosphorylation in a dose-dependent manner. The positions of molecular mass markers are indicated on the left (in kDa).

tion that is a good substrate for phosphorylation by GRK5 but a poor substrate for phosphorylation by GRK2/3. GRK5 phosphorylates μ receptors selectively on Ser^{375}, which is not sufficient to facilitate receptor sequestration. Conversely, the DAMGO-activated μ-opioid receptor acquires a conformation that is a good substrate for phosphorylation by GRK2/3 but a poor substrate for phosphorylation by GRK5. GRK2/3 phosphorylate μ receptors at a number of carboxyl-terminal phosphate acceptor sites including Thr^{370} and Ser^{375} that in turn facilitates a robust receptor endocytosis. Thus, similar to what has been reported for the CCR7 receptor GRKs are selectively activated by distinct conformational states of the μ-opioid receptor (Zidar *et al.*, 2009). This mechanism is likely to be involved in agonist-selective μ receptor signalling. In fact, we show that inhibition of GRK expression facilitates μ-opioid receptor signalling while reducing receptor internalization. Conversely, overexpression of GRKs greatly enhances receptor phosphorylation, β-arrestin recruitment and internalization (Raehal *et al.*, 2011). Collectively, these findings indicate that the mechanisms of μ-opioid receptor regulation also depend on the subcellular complement of GRKs providing a plausible explanation for the observation that morphine can induce receptor sequestration in some cells, for example, striatal neurons but not in most other cell types (Haberstock-Debic *et al.*, 2005).

Morphine is unique in that it is a poor inducer of μ-opioid receptor internalization, but a potent inducer of cellular tolerance (Arden *et al.*, 1995; Keith *et al.*, 1996; Alvarez *et al.*, 2001; Schulz *et al.*, 2004; Gintzler and Chakrabarti, 2006; Johnson *et al.*, 2006; McPherson *et al.*, 2010). *In vitro*, prolonged exposure to both DAMGO and morphine promotes desensitization of μ-opioid receptor signalling in transfected cell lines (Chu *et al.*, 2008; 2010). Whereas DAMGO-desensitized receptors regain functional activity within minutes, morphine-desensitized receptors fail to resensitize (Koch *et al.*, 1998; 2001; Dang and Williams, 2004; Koch and Hollt, 2008). *In vivo*, morphine does not promote significant μ receptor down-regulation even under conditions that induce profound cellular tolerance (Stafford *et al.*, 2001; Gomes *et al.*, 2002; Patel *et al.*, 2002; Pawar *et al.*, 2007; Grecksch *et al.*, 2011). When administered chronically at equieffective analgesic doses, etonitazene or methadone, which are potent inducers of μ receptor internalization, produce less tolerance than morphine (Sala *et al.*, 1992; Elmer *et al.*, 1993; Gerak and France, 1996; 1997; Walker and Young, 2001; Grecksch *et al.*, 2006; Hull *et al.*, 2009). We found that GRK5 selectively contributes to morphine-induced Ser^{375} phosphorylation in mouse brain. Thus, we propose that activation of the GRK5 pathway may contribute to morphine analgesia and tolerance *in vivo*.

The mechanisms of agonist-dependent phosphorylation have been studied extensively for many GPCRs (Pitcher *et al.*,

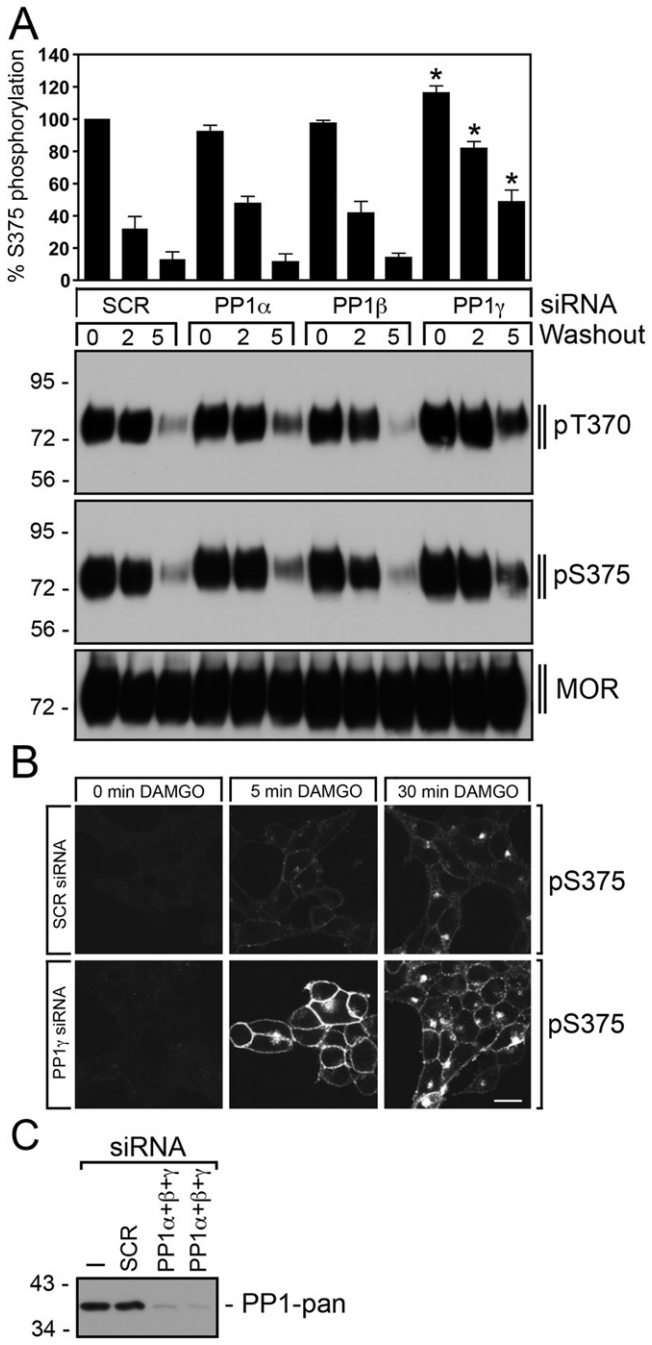

Figure 7

PP1γ is responsible for rapid μ-opioid receptor dephosphorylation. (A) HEK293 cells stably expressing the μ-opioid receptor were transfected with 150 nM siRNA targeted to PP1α, PP1β, PP1γ or non-silencing siRNA control (SCR) for 72 h and then exposed to 10 μM DAMGO for 30 min. Cells were washed three times with cold citrate buffer (pH 4.5) (Washout), and then incubated in the absence of agonist for 0, 2 or 5 min at 37°C. Cells were lysed and immunoblotted with anti-pThr^{370} (pT370) or anti-pSer^{375} antibodies (pS375). Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody UMB-3 to confirm equal loading of the gels (MOR). Ser^{375} phosphorylation was quantified and expressed as percentage of maximal phosphorylation in SCR-transfected cells, which was set at 100%. Data correspond to the mean ± SEM from at least four independent experiments. Results were analysed by two-way ANOVA followed by the Bonferroni post-test (* $P < 0.05$). Note that transfection with PP1γ siRNA resulted in a significant inhibition of μ-opioid receptor dephosphorylation. The positions of molecular mass markers are indicated on the left (in kDa). (B) HEK293 cells stably expressing the μ-opioid receptor were cultured on poly-L-lysin-coated coverslips and transfected with 300 nM siRNA targeted to PP1γ or non-silencing siRNA control (SCR) for 72 h. Cells were exposed to 10 μM DAMGO for 0, 5 or 30 min. Cells were then fixed, and stained with anti-pSer^{375} antibody (pS375), processed for immunofluorescence, and examined by confocal microscopy. Note that transfection with PP1γ siRNA facilitated detection of Ser^{375}-phosphorylated μ-opioid receptors at the plasma membrane shortly after DAMGO exposure. Shown are representative images from one of three independent experiments performed in duplicate. Scale bar, 20 μm. (C) siRNA knock-down of PP1α, PP1β and PP1γ was confirmed by Western blot using a pan-PP1 antibody detecting all PP1 isoforms. The positions of molecular mass markers are indicated on the left (in kDa).

1998; Reiter and Lefkowitz, 2006; Ribas *et al.*, 2007). In contrast, little is known about the mechanisms of receptor dephosphorylation. In *Drosophila melanogaster*, a phosphatase required for rhodopsin dephosphorylation has been identified (Steele *et al.*, 1992). The catalytic domain of this phosphatase exhibits homology to mammalian PP1, PP2A and PP2B. Here, we identified PP1γ as the μ-opioid receptor phosphatase that catalyses Thr^{370} and Ser^{375} dephosphorylation within minutes after agonist removal. Our results strongly suggest that μ-opioid receptor dephosphorylation is a highly regulated process that is initiated shortly after receptor activation at or near the plasma membrane. After our initial observation of PP1β as GPCR phosphatase for sst$_2$ somatostatin receptor (Poll *et al.*, 2011), PP1γ is only the second GPCR phosphatase to be identified. It remains unclear, however, which mechanisms regulate phosphatase specificity. It is possible that either carboxyl-terminal phosphorylation motifs, specific sequences within the intracellular loops or the β-arrestin trafficking patterns may contribute to phosphatase selection. Nevertheless, we found that PP1γ-mediated dephosphorylation facilitates μ-opioid receptor recycling.

In conclusion, the morphine-activated μ-opioid receptor is an efficient substrate for phosphorylation by GRK5 but a poor substrate for GRK2/3. GRK5 phosphorylates μ receptors selectively on Ser^{375}, which is not sufficient to drive receptor sequestration. Although this selective Ser^{375} phosphorylation promotes β-arrestin2 mobilization in morphine-treated cells, the resulting β-arrestin2-μ-opioid receptor complex may not be stable enough to facilitate efficient transfer of the receptor to the endocytic compartment. Conversely, the DAMGO-activated μ-opioid receptor acquires a conformation that is an efficient substrate for phosphorylation by GRK2/3 but a poor substrate for phosphorylation by GRK5. GRK2/3 phosphorylate μ receptors on Thr^{370} and Ser^{375} that in turn facilitates a rapid receptor endocytosis. Thus, this mechanism is likely to contribute to agonist-selective signalling at the μ-opioid receptor.

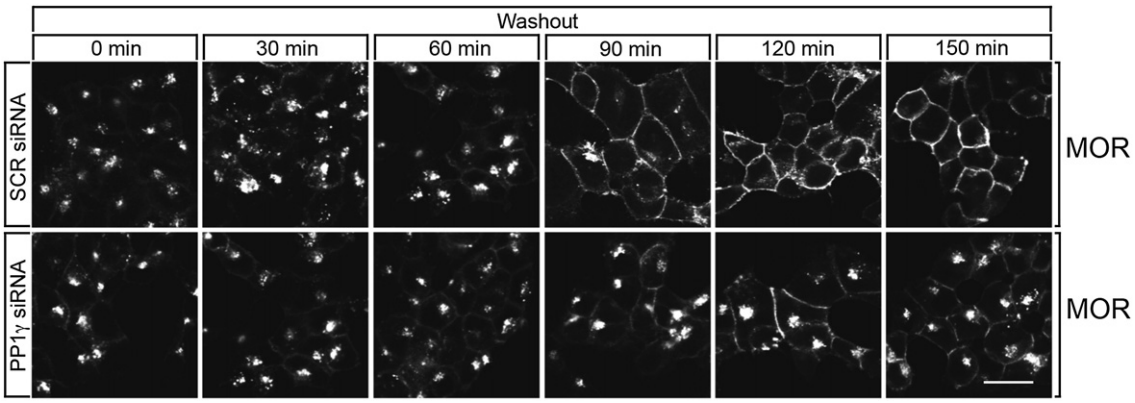

Figure 8

PP1γ facilitates μ-opioid receptor recycling. HEK293 cells stably expressing the μ-opioid receptor were cultured on poly-L-lysin-coated coverslips and transfected with 300 nM siRNA targeted to PP1γ or non-silencing siRNA control (SCR) for 72 h. Cells were exposed to 10 μM DAMGO for 30 min. Cells were washed and incubated for either 0, 30, 60, 90, 120 or 150 min in agonist-free medium at 37°C (Washout). Cells were then fixed, and stained with anti-μ-opioid receptor antibody, processed for immunofluorescence, and examined by confocal microscopy. Note that transfection with PP1γ siRNA inhibited recycling of internalized μ-opioid receptors. Shown are representative images from one of three independent experiments performed in duplicate. Scale bar, 20 μm.

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Conflict of interest

None. The authors have nothing to disclose.

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