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RESEARCH PAPER

Agonist-selective patterns of μ-opioid receptor phosphorylation revealed by phosphosite-specific antibodies

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BACKGROUND AND PURPOSE

Morphine activates the μ-opioid receptor without causing its rapid endocytosis. In contrast, full agonists such as [D-Ala^2-MePhe^4-Gly-ol]enkephalin (DAMGO) or etonitazene stimulate a rapid and profound internalization. However, the detailed molecular events underlying the differential regulation of receptor trafficking by distinct opioid agonists remain incompletely understood.

EXPERIMENTAL APPROACH

Here, we have generated phosphosite-specific antibodies for the carboxyl-terminal residues serine 363 (Ser363), threonine 370 (Thr370) and serine 375 (Ser375), which enabled us to selectively detect either the Ser363-, Thr370- or Ser375-phosphorylated form of the receptor.

KEY RESULTS

We showed that agonist-induced phosphorylation occurs at Thr370 and Ser375, whereas Ser363 is constitutively phosphorylated in the absence of agonist. We further demonstated that DAMGO and etonitazene stimulated the phosphorylation of both Thr370 and Ser375. In contrast, morphine promoted the phosphorylation of Ser375, but failed to stimulate Thr370 phosphorylation. In the presence of DAMGO, Ser375 phosphorylation occurred at a faster rate than phosphorylation of Thr370, indicating that Ser375 is the primary site of agonist-dependent phosphorylation. Activation of PKC by phorbol 12-myristate 13-acetate increased receptor phosphorylation only on Thr370, but not on Ser375, indicating that Thr370 can also undergo heterologous PKC-mediated phosphorylation. We also showed that μ receptor dephosphorylation can occur within minutes at or near the plasma membrane, and that agonist removal is a major prerequisite for Thr370 and Ser375 dephosphorylation.

CONCLUSIONS AND IMPLICATIONS

Together, we showed for the first time that distinct agonists stimulate site-specific patterns of phosphorylation, which are intimately related to their ability to elicit μ-opioid receptor sequestration.

LINKED ARTICLE

This article is commented on by Kelly, pp. 294–297 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2011.01387.x

Abbreviations

Con A, concanavalin A; DAMGO, [D-Ala^2-MePhe^4-Gly-ol]enkephalin; ETO, etonitazene; GRK, G protein-coupled receptor kinase; PMA, phorbol 12-myristate 13-acetate

Introduction

The opioid alkaloid morphine is among the most potent clinically used analgesic. However, the clinical utility of morphine to treat chronic pain is limited by its rapid development of tolerance and dependence (Nestler, 1996; Nestler and Aghajanian, 1997; Koob *et al.*, 1998). Morphine exerts all of its biological effects by interacting with the μ-opioid receptor (Matthes *et al.*, 1996). Like endogenous opioid peptides, morphine binds and activates the μ 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 μ-opioid receptor sequestration (Arden *et al.*, 1995; Keith *et al.*, 1996; Schulz *et al.*, 2004; Johnson *et al.*, 2006; McPherson *et al.*, 2010). To date, the molecular basis for this agonist-selective μ-opioid receptor internalization remains unknown. Numerous studies have reported the crucial role of C-terminal phosphorylation sites in regulating opioid receptor activity and trafficking. Further studies revealed that the ability of distinct opioid agonists to differentially regulate receptor endocytosis is related to their ability to promote GPCR kinase 2 (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 have suggested that phosphorylation of μ receptors occurs primarily at a cluster of three serine and threonine residues, namely serine 363 (Ser363), threonine 370 (Thr370) and serine 375 (Ser375), within the cytoplasmic tail of the receptor (El Kouhen *et al.*, 2001; Chu *et al.*, 2008). However, these three sites seem to be phosphorylated differently: Ser363 is phosphorylated only in the basal condition, Thr370 is phosphorylated both in the presence and absence of [D-Ala^2-MePhe^4-Gly-ol]enkephalin (DAMGO), and Ser375 is phosphorylated only in the presence of DAMGO (El Kouhen *et al.*, 2001). In support of these findings, it has recently been shown that Ser375 is phosphorylated by GRK2 in an agonist-dependent manner and seems to be important for the induction of opioid receptor endocytosis (Schulz *et al.*, 2004; McPherson *et al.*, 2010). So far, the kinase involved in Thr370 phosphorylation and its role in the regulation of opioid receptor trafficking is unknown. Therefore, in the present study, we have generated and extensively characterized phosphosite-specific antibodies, which allowed us to selectively detect the Ser363-, Thr370- and Ser375-phosphorylated forms of the receptor. Using these antibodies, we provide evidence for distinct agonist-selective patterns of μ-opioid receptor phosphorylation following activation by internalizing or non-internalizing agonists.

Methods

Animal care and procedures

All animal experiments were approved by the Thuringian state authorities and complied with EC regulations (86/609/EEC) for the care and reporting standards for use of laboratory animals.

Antibodies and reagents

Phosphosite-specific antibodies for the Ser363-phosphorylated form of the μ receptor were generated against the following sequence that contained a phosphorylated serine residue: EQQN(pS)ARIRQ. This sequence corresponds to amino acids 359–368 of the mouse, and 361–370 of the human μ-opioid receptor, respectively. Phosphosite-specific antibodies for the Thr370-phosphorylated form of the μ receptor were generated against the following sequence that contained a phosphorylated threonine residue: IRQN(pT)REHP. This sequence corresponds to amino acids 366–374 of the mouse, and 368–376 of the human μ-opioid receptor, respectively. Phosphosite-specific antibodies for the Ser375-phosphorylated form of the μ receptor were generated against the following sequence that contained a phosphorylated serine residue: REHP(pS)TANTV. This sequence corresponds to amino acids 371–380 of the mouse, and 373–382 of the human μ-opioid receptor respectively. The peptides were purified by HPLC and coupled to keyhole limpet haemocyanin. The conjugates were mixed 1:1 with Freund's adjuvant and injected into groups of four rabbits {3198–3201} for anti-pSer363 antibody production, {3194–3197} for anti-pT370 antibody production and {2493–2497} for anti-pS375 antibody production. The animals were injected at 4-week intervals, and serum was obtained 2 weeks after immunizations beginning with the second injection. The specificity of the antisera was initially tested using dot-blot analysis. For subsequent analysis, antibodies were affinity purified against their immunizing peptide, as well as against the non-phosphorylated peptide using the SulfoLink kit (Thermo Scientific, Rockford, IL). In addition, the anti-pSer375 antibody {catalogue number 3451} was obtained from Cell Signaling Technology (Beverly, MA). Equal loading of the gels was confirmed using the phosphorylation-independent rabbit monoclonal anti- μ-opioid receptor antibody {UMB-3}, which was extensively characterized previously (Lupp *et al.*, 2010). DAMGO was obtained from Bachem (Weil am Rhein, Germany), morphine hydrochloride was from Merck (Darmstadt, Germany) and etonitazene was from Novartis (Basel, Switzerland).

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 HA-tagged mouse μ-opioid receptor using LipofectAMINE 2000 according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). Stable transfectants were selected in the presence of 1 μg·mL^{-1} puromycine. 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. The Ser363A/Thr370A/Ser375A mutant of the mouse μ-opioid receptor was generated by gene synthesis and obtained from imaGenes (Berlin, Germany).

Western blot analysis

Cells were seeded onto poly-L-lysine-coated 60 mm dishes and grown to 80% confluence. After treatment with mor-

phine, DAMGO or etonitazene, the 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). When indicated, the cells were incubated with 600 $\mu g \cdot mL^{-1}$ concanavalin A (Con A) (Sigma, Deisenhofen, Germany) for 45 min before agonist exposure. Glycosylated proteins were partially enriched using wheat germ–lectin–agarose beads as described previously (Koch *et al.*, 2001; Schulz *et al.*, 2004). Proteins were eluted from the beads using SDS sample buffer for 20 min at 45°C. Indicated samples were dephosphorylated with lambda protein phosphatase (New England Biolabs, Frankfurt, Germany) for 2 h at 30°C, and then eluted from the beads using SDS sample buffer. 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-pSer363 {3199}, 0.1 $\mu g \cdot mL^{-1}$ anti-pThr370 {3196} or 0.1 $\mu g \cdot mL^{-1}$ anti-pSer375 {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. When indicated, drugs were washed three times with 7 mL of ice-cold buffer, either PBS) (washout), PBS supplemented with 10 μM naloxone (naloxone washout) or an acidic wash buffer (acid washout) containing 90 mM NaCl and 50 mM sodium citrate (pH 4.5). After removal of agonist, the cells were incubated in the absence of agonist at 37°C as indicated. Protein bands detected on Western blots were desensitized using ImageJ 1.40 g. Data were then analysed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA).

Immunocytochemistry

Cells were grown on poly-L-lysine-coated coverslips overnight. After the indicated drug treatment, the 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 permeablized and then incubated with anti- μ-opioid receptor antibody {UMB-3} followed by Alexa488-conjugated secondary antibodies (Amersham). The specimens were mounted and examined using a Zeiss LSM510 META (Carl Zeiss AG, Jena, Germany) laser scanning confocal microscope.

Data analysis

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

In an effort to study the spatial and temporal dynamics of agonist-driven phosphorylation of the μ-opioid receptor, we generated phosphosite-specific antibodies for the carboxyl-terminal residues Ser363, Thr370 and Ser375. These antisera were affinity purified against their immunizing peptides and initially tested in dot-blot assays using the phospho- and the corresponding non-phospho-peptides (not shown). All antibodies, which clearly detected their respective phospho-peptide and revealed only limited cross-reactivity to the corresponding non-phospho-peptide, where further characterized in Western blot assays using HEK293 cells stably expressing the μ-opioid receptor. As depicted in Figure 1A, the anti-pSer363 antibodies {3199} and {3200} detected the Ser363-phosphorylated form of the receptor in both DAMGO-treated and untreated cells. The signal of the anti-pSer363 antibody {3199} was more robust and without detectable background. As depicted in Figure 1B, the anti-pThr370 antibodies {3195} and {3196} detected the Thr370-phosphorylated form of the receptor only in DAMGO-treated, but not in morphine-treated or untreated cells. The signal of the anti-pThr370 antibody {3196} was more robust and without detectable background. As depicted in Figure 1C, the anti-pSer375 antibodies {2493} and {2494} detected the Ser375-phosphorylated form of the receptor in morphine-treated and in DAMGO-treated cells, but not in untreated cells. The signal of the anti-Ser375 antibody {2493} was comparable to that of the commercially available anti-Ser375 antibody {3451}, which has been extensively characterized previously (Schulz *et al.*, 2004). Thus, the anti-pSer363 antibody {3199}, anti-pThr370 antibody {3196} and anti-pSer375 antibody {2493} were further characterized using the Ser363A/Thr370A/Ser375A mutant of the mouse μ-opioid receptor. When HEK293 cells were transiently transfected with either the wild-type μ receptor or the Ser363A/Thr370A/Ser375A mutant, the anti-pSer363 antibody {3199} detected Ser363-phosphorylated μ receptors only in DAMGO-treated and untreated cells expressing the wild-type receptor, but not in cells expressing the Ser363A/Thr370A/Ser375A mutant (Figure 2). The anti-pThr370 antibody {3196} and the anti-pSer375 antibody {2493} detected the Thr370- and Ser375-phosphorylated μ receptors, respectively, only in DAMGO-treated cells expressing the wild-type receptor, but not in cells expressing the mutant receptor (Figure 2). To test whether the band detected in untreated cells by the anti-pSer363 antibody {3199} represents the Ser363-phosphorylated form of the μ-opioid receptor, we used lambda-phosphatase to dephosphorylate the receptors. As depicted in Supporting Information Figure S1, after phosphatase treatment, both the anti-pSer363 antibody {3199} and the anti-pSer375 antibody {2493} were no longer able to detect the Ser363- and Ser375-phosphorylated μ receptors, respectively, whereas the receptor protein was still detected using a phosphorylation state-independent antibody. These findings strongly suggest that Ser363 is constitutively phosphorylated in the absence of agonist. In contrast, Thr370 and Ser375 are sites involved in agonist-induced phosphorylation.

We then examined the time-course of Thr370 and Ser375 phosphorylation after treatment with 10 μM morphine, 10 μM DAMGO or 0.025 μM etonitazene. Whereas Thr370 phosphorylation was detected within 2 min in DAMGO- or etonitazene-treated cells, morphine failed to stimulate a substantial phosphorylation of Thr370 even after extended exposure (Figure 3A). In contrast, all three ligands induced a clearly detectable Ser375 phosphorylation yet with a different temporal dynamics (Figure 3B). DAMGO and etonitazene stimulated a robust Ser375 phosphorylation within 2 min, which remained at high levels throughout the 30 min treatment period. The morphine-induced Ser375 phosphorylation

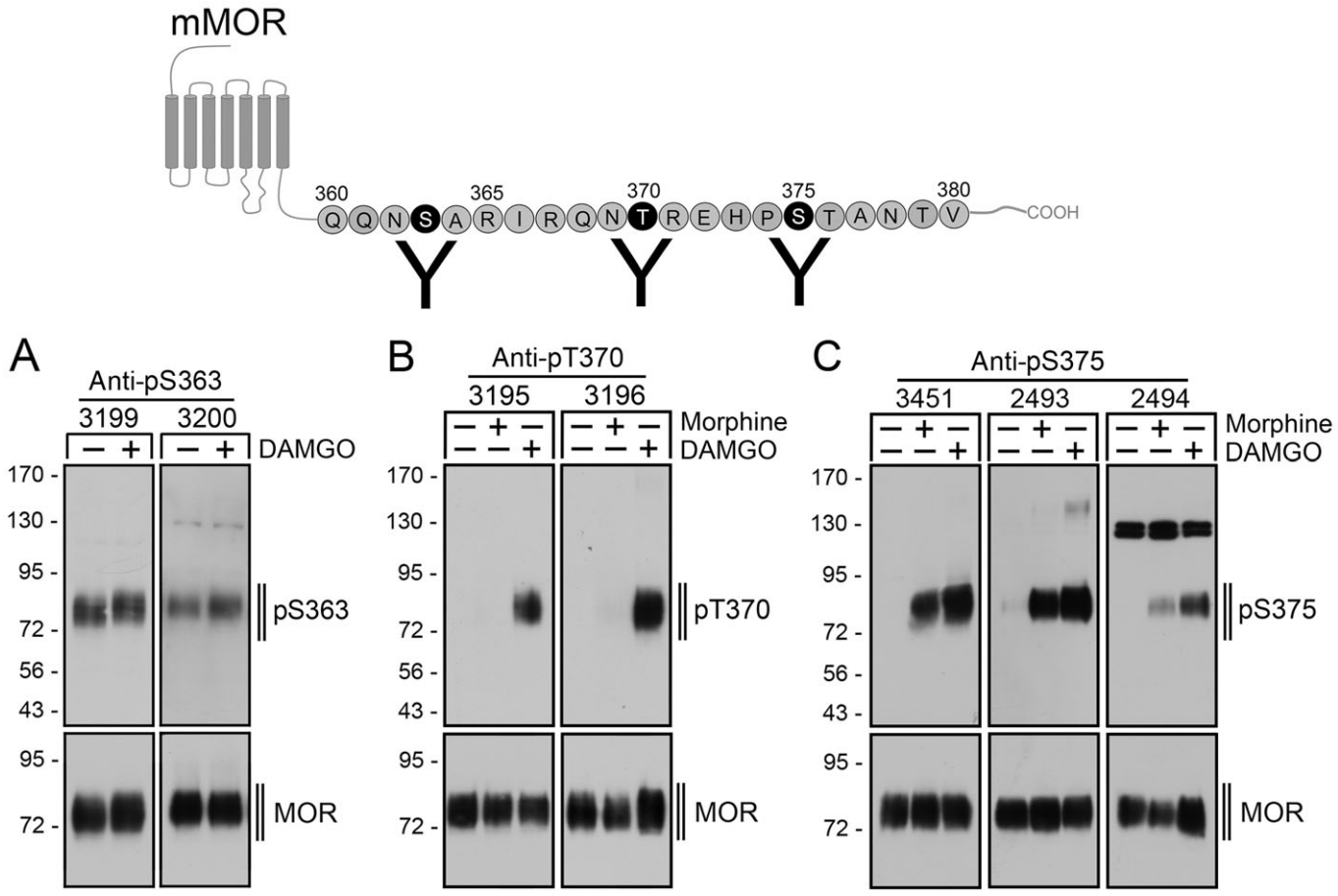

Figure 1

Characterization of phosphosite-specific antibodies for Ser363, Thr370 and Ser375. Top panel: schematic representation of the carboxyl-terminal region of the mouse μ-opioid receptor (MOR). Ser363, Thr370 and Ser375 were targeted for the generation of phosphosite-specific antibodies. (A) HEK293 cells stably expressing the μ-opioid receptor were either not exposed (–) or exposed (+) to 10 μM DAMGO for 30 min. The cells were lysed and immunoblotted with the anti-pSer363 antibodies {3199} and {3200} at a concentration of 0.1 $\mu g \cdot mL^{-1}$ (pS363, upper panel). Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody {UMB-3} at a dilution of 1:200 to confirm equal loading of the gel (MOR, lower panel). Note that Ser363 phosphorylation is detectable in both DAMGO-treated and -untreated cells. (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. The cells were lysed and immunoblotted with the anti-pThr370 antibodies {3195} and {3196} at a concentration of 0.1 $\mu g \cdot mL^{-1}$ (pT370, upper panel). Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody {UMB-3} at a dilution of 1:200 to confirm equal loading of the gel (MOR, lower panel). Note that Thr370 phosphorylation was detectable only after DAMGO, but not after morphine treatment. (C) 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. The cells were lysed and immunoblotted with the anti-pSer375 antibodies {2493} and {2494} at a concentration of 0.1 $\mu g \cdot mL^{-1}$ (pS375, upper panel). In addition, the anti-pSer375 antibody {3451} from Cell Signaling was used at a dilution of 1:1000. Blots were stripped and reprobed with the phosphorylation-independent anti- μ-opioid receptor antibody {UMB-3} at a dilution of 1:200 to confirm equal loading of the gel (MOR, lower panel). Note that the signal of the anti-Ser375 antibody {2493} was comparable to that of the commercially available anti-Ser375 antibody {3451}. Shown are representative results from one of four independent experiments per condition. The position of the molecular mass marker is indicated on the left (in kDa).

was first detected after 2 min and increased steadily throughout the 30 min treatment period. To resolve the time-courses of DAMGO-induced Thr370 and Ser375 phosphorylation in more detail, cells were exposed at room temperature and for shorter time periods. Under these conditions, maximal Ser375 phosphorylation occurred within 20 s, whereas Thr370 phosphorylation became first detectable after 40 s and reached maximal levels after 3 min, suggesting that Ser375 is the primary site of agonist-dependent phosphorylation (Figure 4A and B). Interestingly, in the Ser375A mutant, Thr370 phosphorylation was strongly reduced (Figure 4C). This finding lends further support to the hypothesis of a hierarchical μ receptor phosphorylation with Ser375 being the primary site of phosphorylation.

Phosphorylation of G protein-coupled receptors can occur via specific GRKs or second messenger-activated kinases (e.g. PKA or PKC). We therefore treated stable HEK293 cells with forskolin or PMA, and examined Thr370 and Ser375 phosphorylation. Neither forskolin nor PMA produced a detectable phosphorylation of Ser375 (Figure 5B). Neverthe-

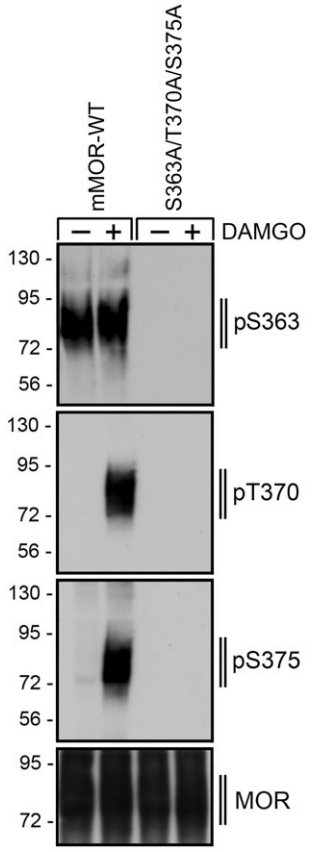

Figure 2

Characterization of phosphosite-specific antibodies using a Ser363A/Thr370A/Ser375A mutant μ receptor. HEK293 cells were transiently transfected with either the wild-type mouse μ-opioid receptor or its Ser363A/Thr370A/Ser375A mutant. Cells were either not exposed (–) or exposed (+) to 10 μM DAMGO for 30 min. The cells were lysed and immunoblotted with the anti-pSer363 antibody {3199} (pS363), anti-pThr370 antibody {3196} (pT370) or the anti-pSer375 antibody {2493} (pS375) at a concentration of 0.1 μg·mL^{-1}, or the phosphorylation-independent anti-μ-opioid receptor antibody {UMB-3} (MOR) at a dilution of 1:200 to confirm equal loading of the gel. Note that neither Ser363, Thr370 nor Ser375 phosphorylation was detectable in the Ser363A/Thr370A/Ser375A mutant. Shown are representative results from one of three independent experiments per condition. The position of the molecular mass marker is indicated on the left (in kDa).

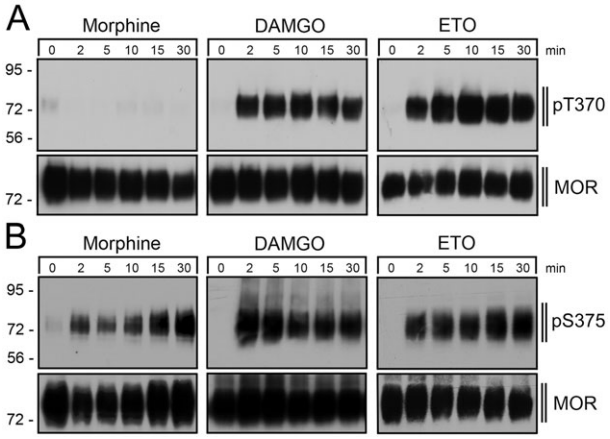

Figure 3

Time-course of agonist-induced Thr370 and Ser375 phosphorylation. (A) HEK293 cells stably expressing the μ-opioid receptor (MOR) were either exposed to 10 μM morphine, 10 μM DAMGO or 0.025 μM etonitazene (ETO) at 37°C for 0, 2, 5, 10, 15 or 30 min. Cells were lysed and immunoblotted with the anti-pThr370 antibody {3196} (pT370, upper panel). Note that Thr370 phosphorylation was detectable only after DAMGO or etonitazene, but not after morphine treatment. (B) HEK293 cells stably expressing the μ-opioid receptor were either exposed to 10 μM morphine, 10 μM DAMGO or 0.025 μM etonitazene at 37°C for 0, 2, 5, 10, 15 or 30 min. The cells were lysed and immunoblotted with the anti-pSer375 antibody {2493} (pS375, upper panel). (A and B) 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 panels). Three additional experiments gave similar results. The position of the molecular mass marker is indicated on the left (in kDa).

less, activation of PKC by PMA led to an increase in Thr370 phosphorylation, indicating that Thr370 can also undergo heterologous PKC-mediated phosphorylation (Figure 5A).

The functional recovery of desensitized μ receptors is believed to involve dephosphorylation and recycling of internalized receptors to the plasma membrane (Koch *et al.*, 2001; Schulz *et al.*, 2004; Koch and Hollt, 2008). We therefore analysed the time-course of Thr370 and Ser375 dephosphorylation upon removal of the agonist. As depicted in Figure 6, DAMGO-activated receptors were dephosphorylated within 20 min at 37°C at both Thr370 and Ser375. In contrast, etonitazene-stimulated receptors remained in a Thr370- and Ser375-phosphorylated state for at least 60 min even after extensive washout with PBS or culture medium. Given the fact that DAMGO and etonitazene are potent inducers of μ internalization, both would be expected to allow rapid receptor dephosphorylation. We then tested whether more stringent washout conditions would facilitate dephosphorylation of etonitazene-activated receptors. When etonitazene was washed out under acidic conditions (pH 4.2) or in the presence of the antagonist naloxone, a marked dephosphorylation of both Thr370 and Ser375 was detected (Figure 7A and B). Given that acidic pH is known to decrease opioid ligand binding (Pert *et al.*, 1973), we presumed that acidic pH would increase the dissociation of bound opioid ligands. Thus, under acidic conditions believed to facilitate removal of agonist (Pert *et al.*, 1973; Kohout *et al.*, 2004), a rapid μ receptor dephosphorylation was observed with all agonists tested. After exposure to DAMGO, dephosphorylation of Thr370 and Ser375 occurred within the first 10 min after the removal of the ligand (Figure 7C and D). Similar to that observed with etonitazene, the acidic washout strongly facilitated dephosphorylation of Ser375 in morphine-treated cells (Supporting Information Figure S2). Under acidic conditions, morphine-induced Ser375 phosphorylation is diminished at a comparable rate to that of the DAMGO-activated receptor (Figure 7D). Given the fact that morphine is only a weak inducer of μ receptor sequestration, these findings suggest that internalization is not an absolute requirement for receptor dephosphorylation. We therefore studied Thr370 and

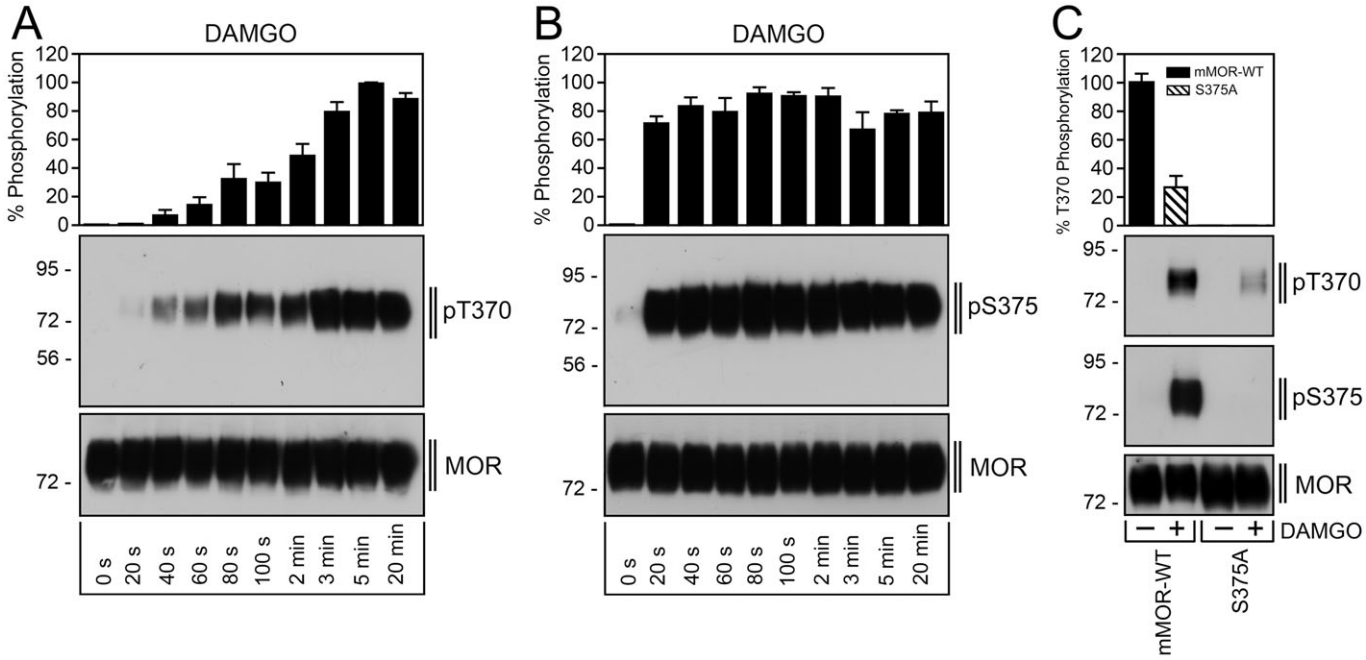

Figure 4

Time-course of DAMGO-induced Thr370 and Ser375 phosphorylation at room temperature. (A) HEK293 cells stably expressing the μ-opioid receptor (MOR) were exposed to 10 μM DAMGO at room temperature (22°C) for the indicated time periods. The cells were lysed and immunoblotted with the anti-pThr370 antibody {3196} (pT370, upper panel). (B) HEK293 cells stably expressing the μ-opioid receptor were exposed to 10 μM DAMGO at room temperature (22°C) for the indicated time periods. The cells were lysed and immunoblotted with the anti-pSer375 antibody {2493} (pS375, upper panel). (A and B) 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 panels). (C) Shows that Thr370 phosphorylation was decreased in the cells expressing the Ser375A mutant μ receptor. HEK293 cells stably expressing either the wild-type mouse μ-opioid receptor or its Ser375A mutant were used. The cells were either not exposed (–) or exposed (+) to 10 μM DAMGO for 30 min. The cells were lysed and immunoblotted with the anti-pThr370 antibody {3196} (pT370) or the anti-pSer375 antibody {2493} (pS375) at a concentration of 0.1 $\mu g \cdot mL^{-1}$, or the phosphorylation-independent anti-μ-opioid receptor antibody {UMB-3} (MOR) at a dilution of 1:200 to confirm equal loading of the gel. Results were quantified by densitometric analysis. The data were normalized to total receptor protein and expressed as % maximal phosphorylation in DAMGO-treated cells. The values represent the means ± SEM of four independent experiments. Note that Ser375 phosphorylation occurred at a faster rate than phosphorylation of Thr370. Representative blots from one of four independent experiments are shown. The position of molecular mass marker is indicated on the left (in kDa).

Ser375 dephosphorylation when μ receptor internalization was largely blocked by Con A. As depicted in Figure 8, Thr370 and Ser375 dephosphorylation occurred at a similar rate in the presence or absence of Con A, suggesting that μ-opioid receptors can be dephosphorylated at or near the plasma membrane.

Discussion

Mutation of either S363, T370 or S375 to alanine within the carboxyl-terminal tail of the mouse μ-opioid receptor has been shown to reduce either basal or the rate and extent of DAMGO-induced μ phosphorylation and internalization (El Kouhen *et al.*, 2001; Schulz *et al.*, 2004). So far, only Ser375 has been shown directly to undergo agonist-driven phosphorylation most likely by a GRK2-dependent mechanism (Schulz *et al.*, 2004). To further explore the fundamental differences in DAMGO- and morphine-induced endocytosis, we generated phosphosite-specific antibodies, which enabled us to selectively detect either the Ser363-, Thr370- or Ser375-phopshorylated form of the μ receptor. We found Ser363 is constitutively phosphorylated and that DAMGO stimulated the phosphorylation of both Thr370 and Ser375, which is in line with previous findings (El Kouhen *et al.*, 2001). Notably, morphine promoted the phosphorylation of Ser375, but failed to stimulate Thr370 phosphorylation. In the presence of DAMGO, Ser375 phosphorylation occurred at a faster rate than phosphorylation of Thr370, indicating that Ser375 is the primary site of agonist-dependent phosphorylation. This suggestion is supported by our finding that DAMGO-induced Thr370 phosphorylation was reduced in a S375A receptor mutant. Thus, our findings show that both Thr370 and Ser375 are sites of agonist-dependent phosphorylation. In addition, we provide direct evidence for distinct patterns of μ-opioid receptor phosphorylation following activation by internalizing or non-internalizing agonists.

Earlier studies have shown that DAMGO and morphine can induce μ receptor desensitization by different mechanisms. The desensitization induced by morphine was largely GRK2-independent, but required ongoing activation of PKC, whereas for DAMGO the desensitization was driven by a

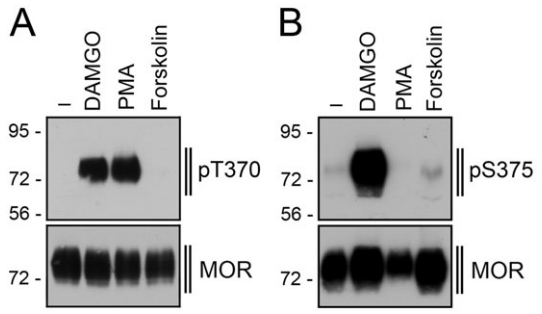

Figure 5

Thr370 and Ser375 phosphorylation by second messenger-activated kinases. (A) HEK293 cells stably expressing the μ-opioid receptor (MOR) were either exposed to 10 μM DAMGO for 30 min or to 100 nM PMA or 10 μM forskolin for 120 min, subsequently lysed and immunoblotted with the anti-pThr370 antibody {3196} (pT370, upper panel). (B) HEK293 cells stably expressing the μ-opioid receptor (MOR) were either exposed to 10 μM DAMGO for 30 min or to 100 nM PMA or 10 μM forskolin for 120 min, subsequently lysed and immunoblotted with the anti-pSer375 antibody {2493} (pS375, upper panel). (A and B) Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gel (MOR, lower panels). Note that activation of PKC by PMA increased receptor phosphorylation only on Thr370, but not on Ser375. Two additional experiments gave similar results. The position of molecular mass marker is indicated on the left (in kDa).

homologous GRK2-dependent mechanism (Johnson *et al.*, 2006; Kelly *et al.*, 2008; Bailey *et al.*, 2009; Hull *et al.*, 2009). The data presented here suggest that both Ser375 and Thr370 can be targets for homologous GRK2-dependent phosphorylation by receptor-internalizing agonists such as DAMGO or etonitazene. In contrast, the heterologous activation of PKC by PMA did not produce any detectable phosphorylation of Ser375, but led to an increase in Thr370 phosphorylation, indicating that Thr370 can also undergo heterologous PKC-mediated phosphorylation. Notably, morphine treatment alone did not induce any phosphorylation at Thr370. Therefore, these findings support the idea that morphine-driven μ receptor desensitization requires both homologous phosphorylation of Ser375 and heterologous PKC-mediated phosphorylation of Thr370 after ongoing PKC activation from another source.

We have previously shown that prolonged exposure to both the receptor-internalizing agonist DAMGO and the non-internalizing agonist morphine promoted desensitization of μ-opioid receptor signalling in transfected cells (Koch *et al.*, 1998; 2001; Wolf *et al.*, 1999). Whereas DAMGO-desensitized receptors are rapidly recycled to the plasma membrane after internalization and regain functional activity within minutes, morphine-desensitized receptors remain at the plasma membrane and largely fail to resensitize (Koch *et al.*, 2001; Schulz *et al.*, 2004). Based on these results, we proposed a model in that regulated endocytosis of the μ-opioid receptor is required for its dephosphorylation and resensitization (Koch and Hollt, 2008). The use of phosphosite-specific antibodies allowed us for the first time to directly follow agonist-induced phosphorylation and

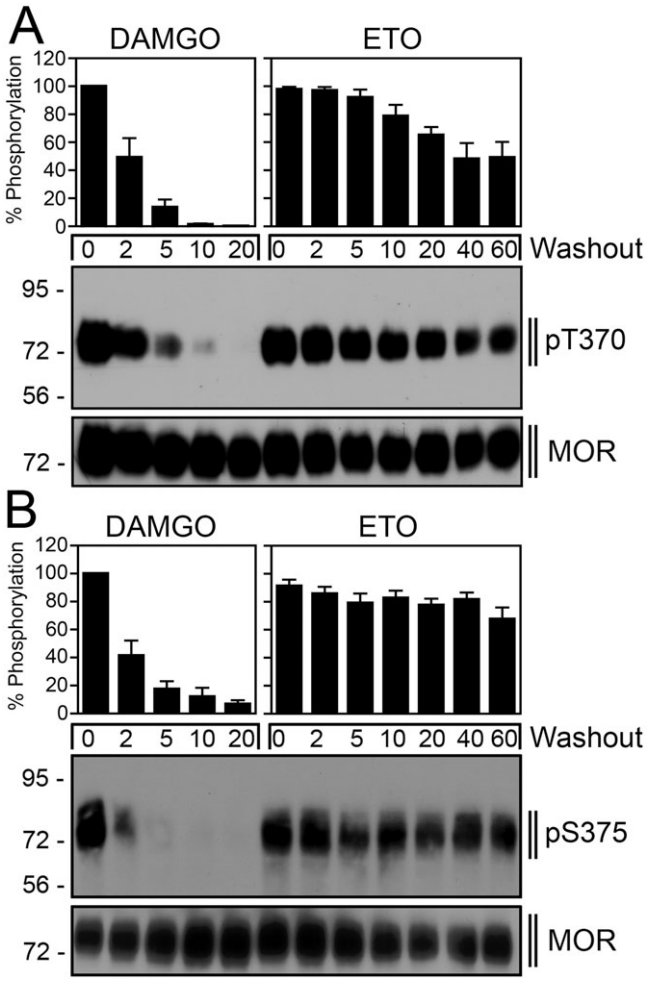

Figure 6

Time-course of Thr370 and Ser375 dephosphorylation. (A) HEK293 cells stably expressing the μ-opioid receptor (MOR) were either exposed to 10 μM DAMGO or 0.025 μM etonitazene (ETO) for 30 min; washed three times with cold PBS (pH 7.4) (washout); and incubated in the absence of agonist for 0, 2, 5, 10, 20, 40 or 60 min. The cells were lysed and immunoblotted with the anti-pThr370 antibody {3196} (pT370, upper panel). (B) HEK293 cells stably expressing the μ-opioid receptor were either exposed to 10 μM DAMGO or 0.025 μM etonitazene (ETO) for 30 min; washed three times with cold PBS (pH 7.4) (washout); and incubated in the absence of agonist for 0, 2, 5, 10, 20, 40 or 60 min. The cells were lysed and immunoblotted with the anti-pSer375 antibody {2493} (pS375, upper panel). (A and B) Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gel (MOR, lower panels). Results were quantified by densitometric analysis. The data were normalized to total receptor protein and expressed as % maximal phosphorylation in agonist-treated cells. The values represent the means ± SEM of three independent experiments. Note that in contrast to DAMGO, etonitazene-activated receptors remained in a Thr370- and Ser375-phosphorylated state for at least 60 min even after extensive washout with PBS. Blots are representative of three experiments per condition. The position of molecular mass marker is indicated on the left (in kDa).

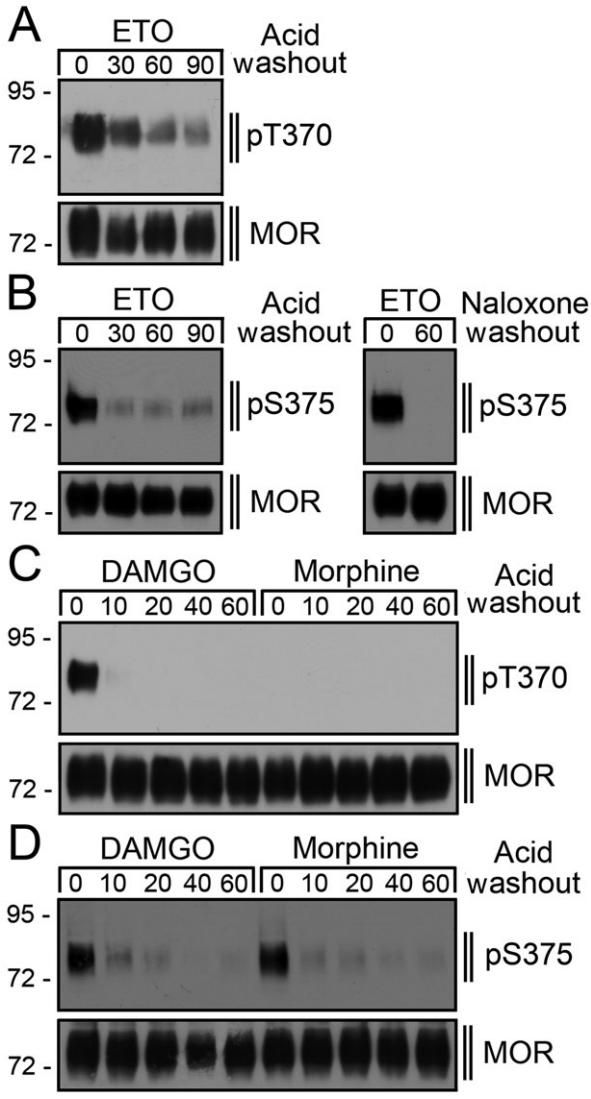

Figure 7

Time-course of Thr370 and Ser375 dephosphorylation after acidic washout. (A) HEK293 cells stably expressing the μ-opioid receptor (MOR) were exposed to 0.025 μM etonitazene (ETO) for 30 min; washed three times with cold citrate buffer (pH 4.5) (acid washout); and incubated in the absence of agonist for 0, 30, 60 or 90 min. The cells were lysed and immunoblotted with the anti-pThr370 antibody {3196} (pT370, upper panel). (B) HEK293 cells stably expressing the μ-opioid receptor were exposed to 0.025 μM etonitazene (ETO) for 30 min; washed three times with either cold citrate buffer (pH 4.5) (acid washout) or cold PBS supplemented with 10 μM naloxone (naloxone washout); and incubated in the absence of agonist for 0, 30, 60 or 90 min. The cells were lysed and immunoblotted with the anti-pSer375 antibody {2493} (pS375, upper panel). (C) HEK293 cells stably expressing the μ-opioid receptor were either exposed to 10 μM DAMGO or 10 μM morphine for 30 min; washed three times with cold citrate buffer (pH 4.5) (acid washout); and incubated in the absence of agonist for 0, 10, 20, 40 or 60 min. The cells were lysed and immunoblotted with the anti-pThr370 antibody {3196} (pT370, upper panel). (D) HEK293 cells stably expressing the μ-opioid receptor (MOR) were either exposed to 10 μM DAMGO or 10 μM morphine for 30 min; washed three times with cold citrate buffer (pH 4.5) (acid washout); and incubated in the absence of agonist for 0, 10, 20, 40 or 60 min. The cells were lysed and immunoblotted with the anti-pSer375 antibody {2493} (pS375, upper panel). (A–D) 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 panels). Note that washout under acidic conditions facilitated a rapid μ receptor dephosphorylation with all agonists tested. Representative results from one of at least three independent experiments per condition are shown. The position of molecular mass marker is indicated on the left (in kDa).

dephosphorylation at amino acids Ser375 and Thr370. Unexpectedly, we observed differences in the dephosphorylation rate of Ser375 between the receptor-internalizing agonists DAMGO and etonitazene. Like DAMGO, etonitazene is a potent inducer of μ receptor phosphorylation and internalization. However, unlike DAMGO, etonitazene-activated receptors did not undergo rapid dephosphorylation and resensitization after agonist withdrawal. Here, we show that acidic washout conditions were required to facilitate the dephosphorylation of Ser375 and Thr370, and μ receptor resensitization in etonitazene-treated cells. In contrast to the peptide agonist DAMGO, etonitazene is an extremely potent benzimidazole opiate. These differences in chemical structure may in part explain the unusual requirements for etonitazene washout. Nevertheless, a rapid μ receptor dephosphorylation was observed under acidic conditions with all agonists tested including morphine. These findings suggest that the agonist-occupied receptor is restrained in a conformation in which the dephosphorylating enzyme cannot gain access to the receptor. In fact, the present findings are not compatible with our previous hypothesis that the morphine-activated receptor cannot be dephosphorylated due to its limited ability to internalize (Schulz *et al.*, 2004; Koch and Hollt, 2008). We also showed that the μ receptors can be dephosphorylated when internalization was blocked by Con A. Thus, our present findings suggest that μ-opioid receptors can be dephosphorylated at or near the plasma membrane, a phenomenon that has also been described for other GPCRs, such as the β_2-adrenoceptor and the thyrotropin-releasing hormone receptor (Iyer *et al.*, 2006; Tran *et al.*, 2007; Gehret and Hinkle, 2010).

In summary, we showed for the first time that distinct agonists stimulate site-specific patterns of phosphorylation, which are intimately related to their ability to elicit μ receptor sequestration. Moreover, removal of an agonist from the receptor is a major prerequisite for receptor dephosphorylation. Dephosphorylation of μ-opioid receptors can also occur at the plasma membrane and is thus independent of receptor endocytosis.

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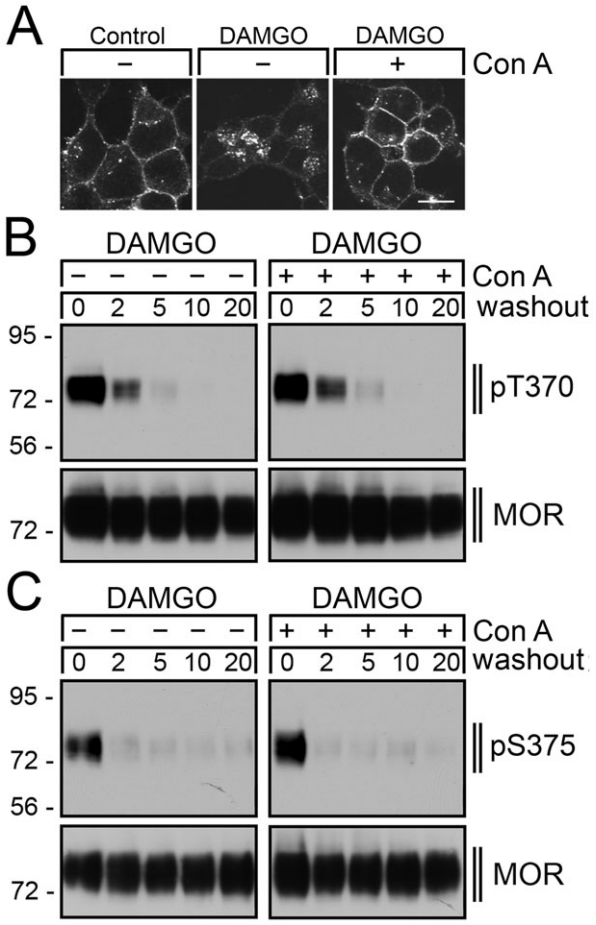

Figure 8

Time-course of T370 and S375 dephosphorylation in the presence of Con A. (A) HEK293 cells stably expressing the μ-opioid receptor (MOR) were either not exposed or exposed to 10 μM DAMGO (DAMGO) for 30 min in the presence (+) or absence (–) of 600 μg·mL^{-1} Con A. The cells were then fixed and stained with anti-MOR antibody {UMB-3}, processed for immunofluorescence and examined by confocal microscopy. Note that Con A largely prevents μ receptor internalization. (B) HEK293 cells stably expressing the μ-opioid receptor were either not exposed or exposed to 10 μM DAMGO (DAMGO) for 20 min in the presence (+) or absence (–) of Con A. The cells were washed three times with cold PBS (washout), and incubated in the absence of agonist for 0, 2, 5, 10 or 20 min. The cells were lysed and immunoblotted with the anti-pThr370 antibody {3196} (pT370). (C) HEK293 cells stably expressing the μ-opioid receptor were either not exposed or exposed to 10 μM DAMGO (DAMGO) for 20 min in the presence (+) or absence (–) of Con A. The cells were washed three times with cold PBS (washout), and incubated in the absence of agonist for 0, 2, 5, 10 or 20 min. The cells were lysed and immunoblotted with the anti-pSer375 antibody {2493} (pS375). (A and B) Blots were stripped and reprobed with the phosphorylation-independent anti-μ-opioid receptor antibody {UMB-3} to confirm equal loading of the gel (MOR). Representative results from one of three independent experiments are shown. The position of molecular mass marker is indicated on the left (in kDa). Scale bar: 20 μm.

Conflict of interest

None.

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Supporting information

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

Figure S1 Characterization of phosphosite-specific antibodies using phosphatase treatment of receptors. (A and B) HEK293 cells stably expressing the μ-opioid receptor (MOR) were either not exposed or exposed to 10 μM DAMGO (DAMGO). The cells were lysed and proteins were either dephosphorylated (+) using lambda protein phosphatase or not dephosphorylated (–). Samples were then immunoblotted with the anti-pS363 antibody {3199} (pS363) or the anti-pS375 antibody {2493} (pS375) at a concentration of 0.1 $\mu g \cdot mL^{-1}$. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gel (MOR). Shown are representative results from one of three independent experiments. The position of molecular mass marker is indicated on the left (in kDa).

Figure S2 Time-course of S375 dephosphorylation in morphine-treated cells. (A and B) HEK293 cells stably expressing the μ-opioid receptor (MOR) were either exposed to 10 μM morphine for 30 min; washed three times with either cold PBS (pH 7.4) (washout) or cold citrate buffer (pH 4.5) (acid washout); and incubated in the absence of agonist for 0, 10, 20, 40 or 60 min. Cells were lysed and immunoblotted with the anti-pS375 antibody {2493} (pS375). Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gel (MOR). Note that washout under acidic conditions facilitated S375 dephosphorylation after morphine treatment. Representative results form one of at least three independent experiments are shown. The position of molecular mass marker is indicated on the left (in kDa).

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