BJP British Journal of Pharmacology

DOI:10.1111/bph.12546
www.brjpharmacol.org

RESEARCH PAPER

Heterologous regulation of agonist-independent μ-opioid receptor phosphorylation by protein kinase C

Susann Illing*, Anika Mann* and Stefan Schulz

Institute of Pharmacology and Toxicology, Jena University Hospital, Friedrich Schiller University Jena, Jena, Germany

Correspondence
Stefan Schulz, Institute of Pharmacology and Toxicology, Jena University Hospital, Friedrich Schiller University Jena, Drackendorfer Straße 1, D-07747 Jena, Germany. E-mail: stefan.schulz@med.uni-jena.de

*The authors Susann Illing and Anika Mann contributed equally to this work.

Keywords
opioid receptor; MOR; PKC; phosphorylation; morphine; tolerance; analgesia

Received
7 June 2013
Revised
12 November 2013
Accepted
2 December 2013

BACKGROUND AND PURPOSE

Homologous agonist-induced phosphorylation of the μ-opioid receptor (MOR) is initiated at the carboxyl-terminal S375, followed by phosphorylation of T370, T376 and T379. In HEK293 cells, this sequential and hierarchical multi-site phosphorylation is specifically mediated by G-protein coupled receptor kinases 2 and 3. In the present study, we provide evidence for a selective and dose-dependent phosphorylation of T370 after activation of PKC by phorbol esters.

EXPERIMENTAL APPROACH

We used a combination of phospho site-specific antibodies, kinase inhibitors and siRNA knockdown screening to identify kinases that mediate agonist-independent phosphorylation of the MOR in HEK293 cells. In addition, we show with phospho site-specific antibodies were also used to study constitutive phosphorylation at S363 of MORs in mouse brain *in vivo*.

KEY RESULTS

Activation of PKC by phorbol esters or heterologous activation of substance P receptors co-expressed with MORs in the same cell induced a selective and dose-dependent phosphorylation of T370 that specifically requires the PKCα isoform. Inhibition of PKC activity did not compromise homologous agonist-driven T370 phosphorylation. In addition, S363 was constitutively phosphorylated in both HEK293 cells and mouse brain *in vivo*. Constitutive S363 phosphorylation required ongoing PKC activity. When basal PKC activity was decreased, S363 was also a substrate for homologous agonist-stimulated phosphorylation.

CONCLUSIONS AND IMPLICATIONS

Our results have disclosed novel mechanisms of heterologous regulation of MOR phosphorylation by PKC. These findings represent a useful starting point for definitive experiments elucidating the exact contribution of PKC-driven MOR phosphorylation to diminished MOR responsiveness in morphine tolerance and pathological pain.

Abbreviations

CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; DAMGO, [D-Ala^2-$MePhe^4$-Gly-ol]enkephalin; GRK, G-protein coupled receptor kinase; MOR, μ-opioid receptor; PMA, phorbol 13-myristate 12-acetate; SP, substance P

Introduction

The efficiency of μ-opioid receptor (MOR) signalling is tightly regulated and ultimately limited by the coordinated phosphorylation of serine and threonine residues in the intracellular regions of the receptor (receptor nomenclature follows Alexander *et al.*, 2013). We have recently shown that agonist-induced phosphorylation of MOR occurs at a conserved

10 residue sequence ^{370}TREHPSTANT^{379} in the receptor's carboxyl-terminal cytoplasmic tail. A range of different opioids induce receptor phosphorylation at S375, present in the middle of this sequence, but only high-efficacy opioids have the ability to drive higher order phosphorylation on flanking residues (T370, T376 and T379). S375 is the initiating residue in a hierarchical phosphorylation cascade. Such phosphorylation is carried out by G protein receptor kinases (GRK) and, for MORs, S375 can be phosphorylated by GRK2/3 as well as GRK5 isoforms, and phosphorylation of this residue is stimulated by a wide variety of opioid drugs. Phosphorylation at T370, T376 and T379 is facilitated by this priming phosphorylation, but specifically requires GRK2/3 isoforms, and phosphorylation at each of these later residues is markedly drug-selective. In fact, comparison of a variety of opioid agonists reveals an excellent correlation between phosphorylation and internalization. Analysis of point mutations of these residues indicates that multiple phosphorylations within this sequence are required for the β-arrestin-mobilizing and endocytosis-promoting activity of opioid drugs (Schulz *et al.*, 2004; Doll *et al.*, 2011; 2012; Lau *et al.*, 2011; Just *et al.*, 2013).

Although the regulation of agonist-dependent homologous MOR phosphorylation has been delineated in great detail, so far little is known about the mechanisms of agonist-independent heterologous MOR phosphorylation. Earlier studies have indicated that S363 is phosphorylated under basal conditions (El Kouhen *et al.*, 2001), and that basal phosphorylation can be blocked by inhibition of PKC activity (Johnson *et al.*, 2006). Feng *et al.* (2011) found that stimulation of PKC leads to phosphorylation of S363 in CHO cells. In contrast, using phospho site-specific antibodies, we have recently observed that S363 is constitutively phosphorylated in HEK293 cells, and that activation of PKC by phorbol esters leads to the phosphorylation of T370 in the same cellular environment (Doll *et al.*, 2011). HEK293 cells express a wide range of PKC isoforms (Atwood *et al.*, 2011), and the specific variants responsible for heterologous MOR phosphorylation have not been identified yet. Here, we address these questions and show that PKC-mediated MOR phosphorylation occurs at two different sites and that these phosphorylation events are differently regulated.

Methods

Plasmids

DNA for mouse MOR and the T370A mutant was generated via artificial gene synthesis and cloned into pcDNA3.1 by imaGenes (Berlin, Germany). In addition, the coding sequence for an amino-terminal HA-tag was added.

Antibodies

The rabbit polyclonal phospho site-specific antibodies anti-pS363 {3199}, anti-pT370 {3196}, anti-pS375 {2493}, anti-pT376 {3723}, anti-pT379 {3686} and anti-haemagglutinin (HA) {0631} antibodies have been generated and extensively characterized previously (Pfeiffer *et al.*, 2002; Plockinger *et al.*, 2008; Doll *et al.*, 2011; Just *et al.*, 2013). The phosphorylation-independent rabbit monoclonal anti-MOR antibody {UMB-3} was obtained from Epitomics (Burlingame, CA, USA) (Lupp *et al.*, 2011). The guinea pig polyclonal phospho site-specific antibody anti-pS375 {GM375-2} and the phosphorylation-independent guinea pig polyclonal anti-MOR antibody {GP6} were generated and extensively characterized previously (Grecksch *et al.*, 2006; Lupp *et al.*, 2011). The phospho site-specific antibody for the S363-phosphorylated form of the MOR{GM363-3} was 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 MOR. The anti-pS363 guinea pig polyclonal antibody {GPM363-3} has been generated and characterized in a manner identical to that previously described for the anti-pS363 rabbit polyclonal anti-MOR antibody {3199} (Doll *et al.*, 2011). The rabbit polyclonal PKCα antibody was obtained from Epitomics and the rabbit polyclonal PKC-isoform-specific anti-PKCε antibody was obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

Cell culture and transfection

HEK293 cells were obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany) and were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal calf serum. HEK293 cells were stably transfected with Lipofectamine2000 (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA). Stably transfected cells were grown in a medium supplemented with 0.1 μg·mL^{-1} puromycin (MOR) or with 0.1 μg·mL^{-1} puromycin/1 μg·mL^{-1} gentamycin (MOR-NK$_1$). HEK293 cells stably expressing MORs were characterized using radioligand-binding assays, Western blot analysis, immunocytochemistry and cAMP assays, as described previously (Koch *et al.*, 2001). Clones expressing similar amounts of receptors (~800 fmol·mg^{-1} membrane protein) were selected and used for further studies.

Analysis of opioid receptor internalization in HEK293 cells

Stably transfected cells were grown on poly-L-lysine-coated coverslips overnight. Cells were then incubated with primary antibody rabbit anti-HA antibody in serum-free medium for 2 h at 4°C. After agonist exposure, 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 with PBS. Specimens were permeabilized and then incubated with an Alexa488-conjugated goat anti-rabbit antibody (Amersham, Braunschweig, Germany). Specimens were mounted and examined using a Zeiss LSM510 META laser scanning confocal microscope (Jena, Germany).

Western blot analysis

Cells were seeded onto poly-L-lysine-coated 60 mm dishes and grown to 80% confluence. After treatment with agonist, 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 and 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 anti-pS363 {3199}, anti-pT370 {3196}, anti-pS375 {2493}, anti-pT376 {3723} or anti-pT379 {3686} antibodies, followed by detection using an enhanced chemiluminescence detection system (Amersham). Blots were stripped and incubated again using the phosphorylation-independent anti-MOR antibody {UMB-3} (Lupp *et al.*, 2011) to ensure equal loading of the gels.

In vivo *phosphorylation studies*

All animal care and experimental; procedures were approved by the Thuringian state authorities and complied with EC 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 30 animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of N animals were used in the experiments described here. MOR knockout (–/–) mice were provided by Dr H. Loh, University of Minnesota (Minneapolis, MN, USA) and wild-type mice (MOR+/+) C57BL6/J were obtained from Charles River (Sulzfeld, Germany).

Brains from wild-type mice (male, 8–12 weeks, 25–30 g) were quickly dissected; the cerebellum was removed and the remaining brain samples were immediately frozen in liquid nitrogen. 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-MOR antibody {UMB-3} bound to protein A-agarose beads for 2 h at 4°C (Lupp *et al.*, 2011). Proteins were eluted from the beads with SDS-sample buffer for 20 min at 40°C. Samples were resolved on 7.5% SDS-polyacrylamide gels, and after electroblotting, membranes were incubated with guinea pig polyclonal anti-pS375 antibody {GM375-2} or anti-pS363 {GM363-3} antibody 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-MOR antibody {GP6} at a concentration of 0.1 μg·mL^{-1} to confirm equal loading of the gels (Lupp *et al.*, 2011).

Small interfering RNA (siRNA) silencing of gene expression

Double-stranded siRNA duplexes with 3′-dTdT overhangs were obtained from Santa Cruz Biotechnology for the following targets: PAN-PKC (5′-TGGTTCGTCTTCTGGTTGT-3′; 5′-GTGACGTGGCTGAAGTAGA-3′; 5′-AGTCAGGTAGTTGTTCGTT-3′; 5′-CCCTACACGTTTCTCTTGT-3′; 5′-TCTCTTCGTGCACAAACT-3′), PKCβ$_2$ (5′-GCTTCAGTTCTCGATTCAT-3′; 5′-GGTTCACATACCAACGAAA-3′; 5′-CCTTACCCTAAACAATGTT-3′) and PKCγ (5′-CCTACCGACCATGTTCAAT-3′; 5′-CTCTGAACTACATGGTGTA-3′; 5′-GACTGAAACCGTACACATT-3′), and from Qiagen (Hilden, Germany) for the following targets: PKCα (5′-AACCATCCGCTCCACACTAAA-3′), PKCβ$_1$ (5′-CAAGAGCTAAGTAGATGTGTA-3′), PKCε (5′-CACGGAAACACCCGTACCTTA-3′) and non-silencing RNA duplexes (5′-GCTTAGGAGCATTAGTAAA-3′; 5′-AAACTCTATCTGCACGCTGAC-3′). HEK293 cells were transfected using HiPerFect transfection reagent (Qiagen) with 150 nM for single transfection for 3 days. Silencing was quantified by immunoblotting as described (Poll *et al.*, 2010; 2011; Doll *et al.*, 2012). In all experiments shown, protein levels were reduced by ≥80%.

Data analysis

Protein bands detected on Western blots were quantified using ImageJ 1.40 g (National Institute of Health, Bethesda, MD, USA; wsr@nac.gov) or BIO-1D analysis software (Vileber, Eberhardzell, Germany). Data were analysed using GraphPad Prism 4.0 software (La Jolla, CA, USA). Statistical analysis was carried out with two-way ANOVA, followed by the Bonferroni post test. *P*-values <0.01 were considered statistically significant.

Materials

The following MOR agonists were obtained from commercial suppliers: morphine (Merck Pharma, Darmstadt, Germany), DAMGO (Sanofi-Aventis, Frankfurt, Germany) and etonitazene (Novartis, Basel, Switzerland). The NK$_1$ receptor agonist, substance P (SP) was obtained from Sigma-Aldrich (Steinheim, Germany). The PKC activator, phorbol 13-myristate 12-acetate (PMA) was obtained from Sigma-Aldrich. The following PKC inhibitors were obtained from commercial suppliers: bisindolylmaleimide II (BIM2) (Sigma-Aldrich), RO32-0432 (Sigma-Aldrich) and LY333,531 (Enzo Life Sciences, Lörrach, Germany). The GRK2 inhibitor, β-ARK1 inhibitor, and the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN62 were obtained from Calbiochem, Darmstadt, Germany; the PKA inhibitor H89 was obtained from Cell Signaling Technology (Frankfurt, Germany).

Results

We have recently developed a panel of phospho site-specific antibodies that enabled us to delineate agonist-driven MOR phosphorylation. When HEK293 cells stably expressing MOR were exposed to increasing concentrations of the PKC activator PMA for 30 min, we observed a selective and dose-dependent increase in T370 phosphorylation of MOR (Figure 1A). Unlike that seen after exposure to high-efficacy agonists, PMA exposure did not lead to an increase in phosphorylation at S375, T376 or T379. Moreover, phosphorylation at S363 was already detectable in the absence of PMA and did not dramatically increase in the presence of PMA (Figure 1A). Next, we compared the temporal dynamics of agonist-induced homologous and phorbol ester-induced heterologous T370 phosphorylation. In the presence of the high-efficacy agonist [D-Ala^2-MePhe^4-Gly-ol]enkephalin (DAMGO), T370 phosphorylation was detectable within 2 min (Figure 1B). In contrast, PMA-induced T370 phosphorylation required considerably longer time periods of up to 10 min (Figure 1B). DAMGO induces phosphorylation of a number of additional carboxyl-terminal phosphate acceptor sites, including S375, T376 and T379 (Figure 1B) (Doll *et al.*, 2012; Just *et al.*, 2013), which, in turn, facilitates a robust

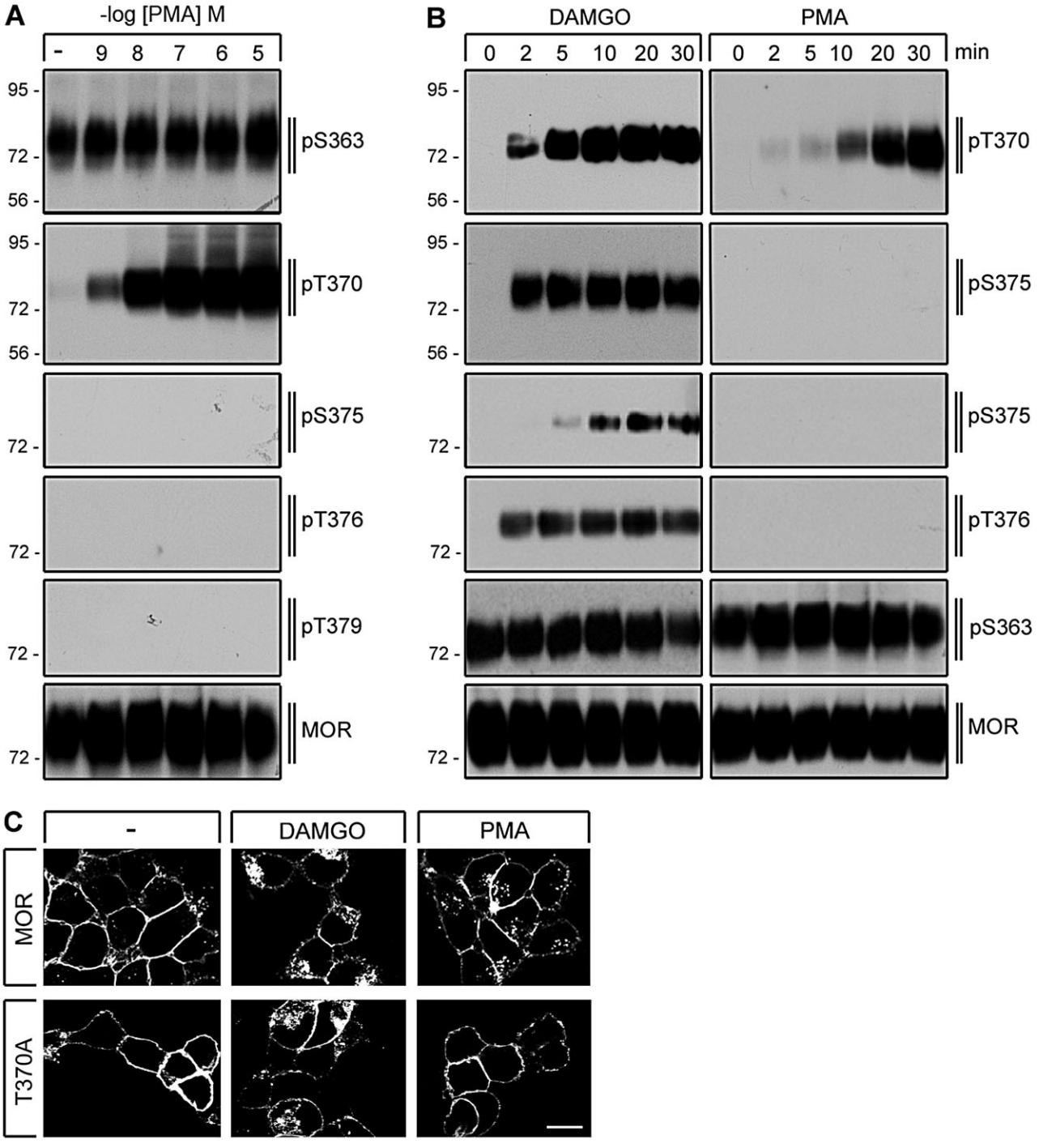

Figure 1

Activation of PKC promotes a selective MOR phosphorylation at T370. (A) HEK239 cells stably expressing MOR were either not exposed (–) or exposed to 1 nM, 10 nM, 100 nM, 1 μM or 10 μM PMA for 30 min. Cells were lysed and immunoblotted with anti-pS363, anti-pT370, anti-pS375, anti-pT376 and anti-pT379 antibodies. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gel. Note that PMA stimulates a dose-dependent phosphorylation of T370. S363 is constitutively phosphorylated. (B) Cells were stably exposed to 10 μM DAMGO or 1 μM PMA for 0, 2, 5, 10, 20 or 30 min, and then immunoblotted with anti-pS375 or anti-pT370 antibodies. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3}. Representative results from one of four independent experiments per condition are shown. The position of molecular mass markers is indicated on the left (in kDa). (C) MOR- and T370A-expressing HEK293 cells were either not exposed (–) or exposed to 10 μM DAMGO or 1 μM PMA for 30 min. Cells were then stained with anti-HA antibody, processed for immunofluorescence and examined by confocal microscopy. Note that PMA induces internalization of wild-type MOR but not of the T370A mutant. Representative images from one of three independent experiments performed in duplicate are shown. Scale bar: 20 μm.

MOR internalization (Figure 1C). Although much less pronounced, a 30 min PMA exposure also leads to a clearly detectable redistribution of MORs from the plasma membrane into the cytosol, suggesting that heterologous phosphorylation by a second-messenger kinase is able to promote MOR internalization (Figure 1C). Interestingly, the T370A mutation completely blocked the PMA-induced internalization but not DAMGO-induced internalization (Figure 1C).

We next used chemical inhibitors to determine which kinases were involved in phorbol ester-induced T370 phosphorylation. As shown in Figure 2A, the PKC inhibitors BIM2, RO32-0432 and LY333,531 completely inhibited the PMA-stimulated phosphorylation of T370, whereas the PKA inhibitor H89 and the GRK2 inhibitor βARK1 had no effect. The effects of the PKC inhibitor RO32-0432 were dose dependent, with strong inhibition of T370 phosphorylation already detectable at concentrations as low as 10 nM (Figure 2B). Under conditions when PMA-mediated T370 phosphorylation was blocked by BIM2 or RO32-0432, DAMGO still facilitated T370 phosphorylation to the same extent, suggesting that homologous phosphorylation occurred independent of PKC (Figure 2B and Supporting Information Fig. S1).

PKCs consist of three families of serine/threonine kinases that comprise (i) conventional PKCs (α, β and γ), which are activated by DAG, phosphatidylserine and calcium ions; (ii) novel PKC isoforms (δ, ε, η and θ) that are calcium ion-insensitive; and (iii) atypical PKCs (ι/λ and ζ) that require neither DAG nor calcium (Parker and Murray-Rust, 2004). All PKC inhibitors used target several PKC isoforms. Whereas BIM2 inhibits a variety of PKC isoforms, RO32-0432 and LY333,531 preferentially target conventional PKC isoforms, such as PKCα and PKCβ. Consequently, we performed a limited siRNA knockdown screen to identify the PKC isoform specifically required for PMA-driven T370 phosphorylation of MOR. As shown in Figure 3A, only inhibition of PKCα expression produced a significant reduction of T370

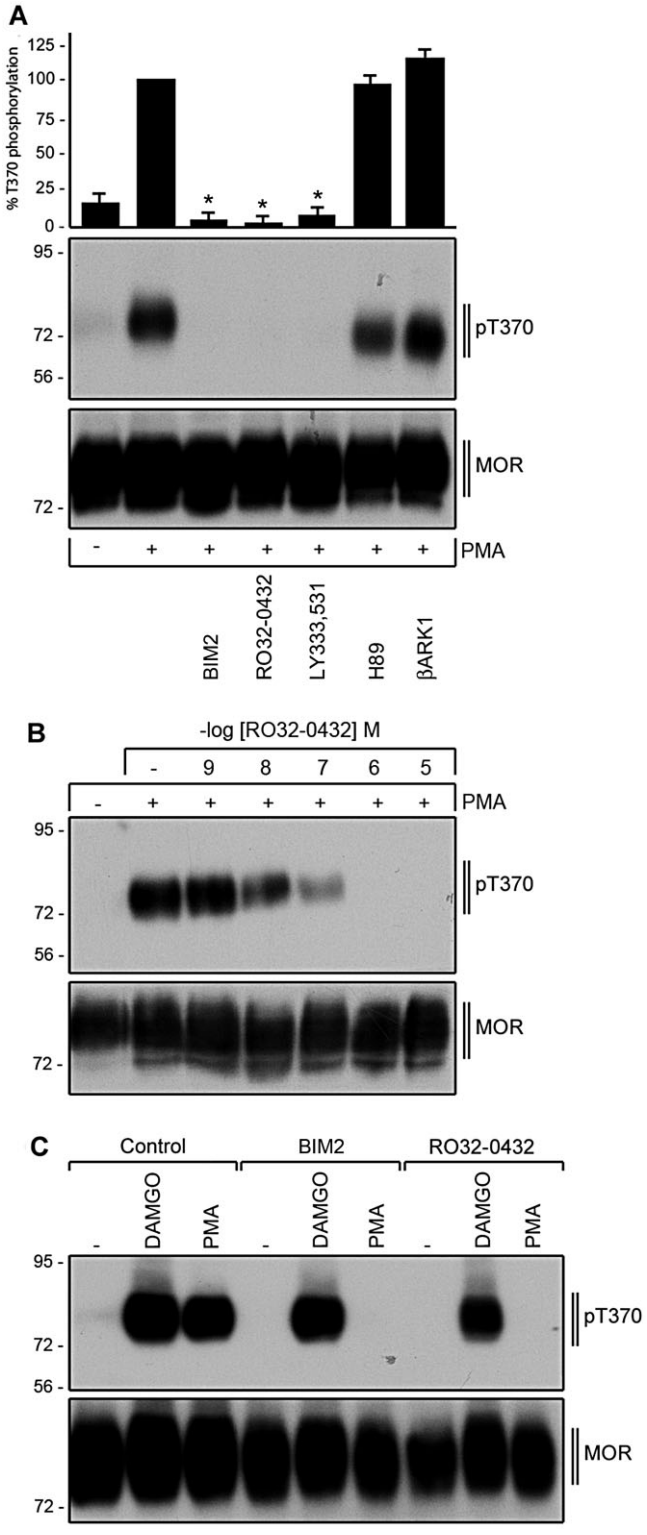

Figure 2

Heterologous T370 phosphorylation is mediated by PKC. (A) HEK239 cells stably expressing MOR were treated with 10 μM BIM2, 10 μM RO32-0432; 10 μM LY333,531; 50 μM H89 or 10 μM βARK1 inhibitor for 30 min and then either not exposed (–) or exposed to 1 μM PMA. Cells were lysed and immunoblotted with anti-pT370 antibody. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gel. T370 phosphorylation was quantified and expressed as percentage of maximal phosphorylation in PMA-treated control cells, which was set at 100% (upper panel). Data correspond to mean ± SEM from at least four independent experiments. Results were analysed by two-way ANOVA, followed by the Bonferroni post test (*P < 0.01). Note that pre-incubation with the PKC inhibitors BIM2, RO32-0432 or LY333,531 diminished T370 phosphorylation. (B) Cells were either not treated (–) or treated with RO32-0432 in concentrations ranging from 10^{-9} to 10^{-5} M for 30 min and then either not exposed (–) or exposed to 1 μM PMA for 30 min. Cells were lysed and immunoblotted with the anti-pT370 antibody. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody. (C) MOR-expressing HEK239 cells were either not treated (control) or treated with 1 μM BIM2 or 1 μM RO32-0432 for 30 min and then either not exposed (–) or exposed to 10 μM DAMGO or 1 μM PMA for 30 min. Cells were lysed and immunoblotted with the anti-pT370 antibody. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3}. Note that the PKC inhibitors BIM2 and RO32-0432 diminished PMA-induced T370 phosphorylation but did not inhibit agonist-induced homologous T370 phosphorylation. Representative results from one of four independent experiments per condition are shown. The position of molecular mass markers is indicated on the left (in kDa).

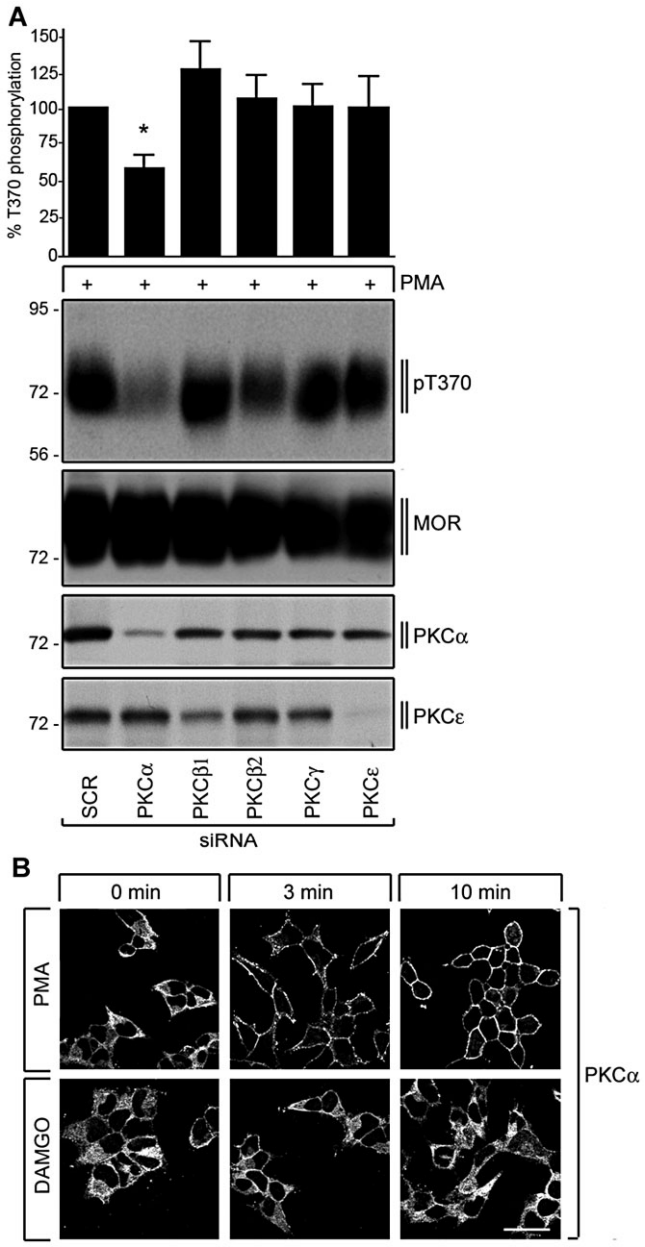

Figure 3

PMA-induced T370 phosphorylation is mediated by the PKCα isoform. (A) HEK293 cells stably expressing MOR were transfected with 150 nM siRNA targeted to PKCα PKCβ_1, PKCβ_2 PKCγ, PKCε or non-silencing siRNA (*SCR*) for 72 h and then exposed to 1 μM PMA for 30 min. Cells were lysed and immunoblotted with anti-pT370 antibody. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gels. T370 phosphorylation was quantified and expressed as percentage of maximal phosphorylation in SCR-transfected cells, which was set at 100% (upper panel). Data correspond to mean ± SEM from at least four independent experiments. Results were analysed by two-way ANOVA, followed by the Bonferroni post test (*$P < 0.01$). The positions of molecular mass markers are indicated on the left (in kDa). Note that transfection with PKCα siRNA resulted in significant inhibition of T370 phosphorylation. (B) Cells were exposed to 1 μM PMA or 10 μM DAMGO for 0, 3 or 10 min. Cells were then fixed, stained with anti-PKCα antibody, processed for immunofluorescence and examined by confocal microscopy. Note that PMA treatment leads to recruitment of PKCα to the plasma membrane. Representative images from one of three independent experiments performed in duplicate are shown. Scale bar: 20 μm.

phosphorylation to ~55% ($P < 0.001$), whereas siRNA knockdown of PKCβ_1, PKCβ_2, PKCγ or PKCε had no effect. Collectively, these results suggest that T370 phosphorylation induced by phorbol esters was predominantly mediated by the PKCα isoform. We then examined the cellular distribution of endogenous PKC-α in HEK293 cells exposed to PMA or DAMGO. In untreated cells, PKCα is distributed throughout the cytosol (Figure 3B). However, after activation by phorbol esters, PKCα was rapidly recruited to the plasma membrane outlining the cell shape (Figure 3B). In contrast, no such redistribution of PKCα was seen in DAMGO-treated cells (Figure 3B). Thus, activated PKCα exists in close proximity to MOR in PMA-treated cells and is therefore likely to contribute to T370 phosphorylation.

Next, we asked whether PKC-dependent T370 phosphorylation might also occur after heterologous activation of G_q protein-coupled receptors. When neurokinin NK_1 receptors were co-expressed with MOR and then stimulated with increasing concentrations of SP, we observed a selective and dose-dependent phosphorylation of T370 similar to that observed after direct activation of PKC with phorbol esters (Figure 4A). This SP-induced T370 phosphorylation of MOR was completely blocked by the PKC inhibitor BIM2 but not by the PKA inhibitor H89, the GRK2 inhibitor βARK1 or the CaMKII inhibitor KN62 (Figure 4B). We then used siRNA knockdown screening to identify the PKC isoform specifically required for SP-driven T370 phosphorylation of MOR. We observed that only inhibition of PKCα expression produced a significant reduction of T370 phosphorylation to ~40% ($P < 0.001$), whereas siRNA knockdown of PKCβ_1, PKCβ_2, PKCγ or PKCε had no effect (Figure 4C). We also observed a clearly detectable MOR internalization after NK_1 receptor stimulation with SP (Figure 4D). Interestingly, the SP-induced MOR internalization was completely blocked by the PKC inhibitor BIM2 (Figure 4D). These results suggested that T370 phosphorylation could occur after activation of G_q protein-coupled receptors expressed in the cells and that PKCα mediated this effect.

We have recently shown that S363 is constitutively phosphorylated in HEK293 cells. Firstly, we used an array of chemical inhibitors to identify kinases responsible for constitutive S363 phosphorylation. As shown in Figure 5 (left panel), the PKA inhibitor H89, the GRK2 inhibitor βARK1 and the CaMKII inhibitor KN62 had no effect. Although the PKC inhibitor BIM2 completely inhibited S363 phosphorylation, RO32-0432 and LY333,531 only partially inhibited S363 phosphorylation. We then performed siRNA knockdown of a variety of PKC isoforms, which did not lead to the identification of a specific PKC isoform required for constitutive S363 phosphorylation in HEK293 cells. However, when the expression of multiple PKC isoforms was inhibited using a PAN-PKC siRNA, we found a significant reduction of constitutive S363 phosphorylation to ~50% ($P < 0.001$) (Figure 5A, right panel).

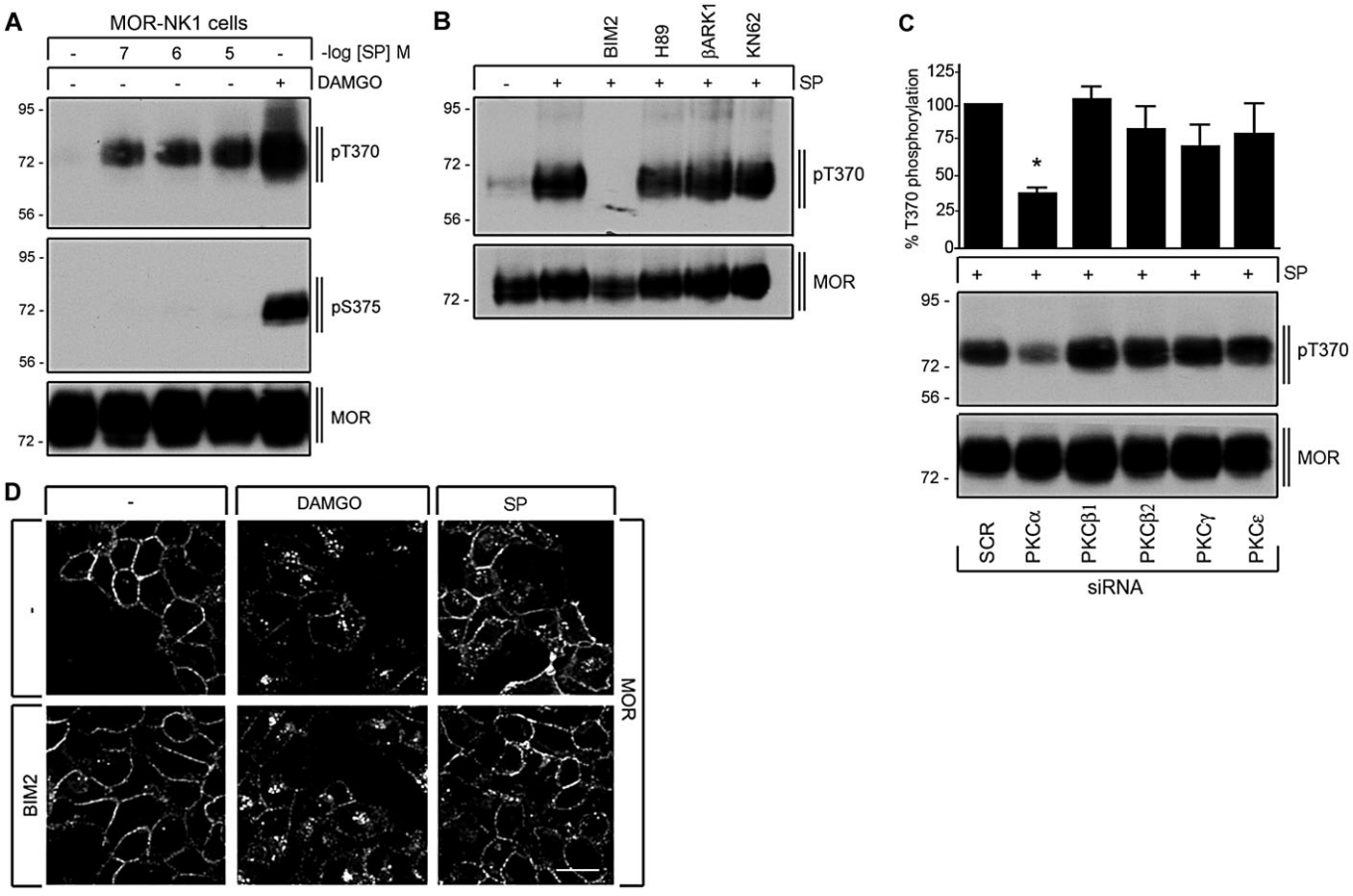

Figure 4

Substance P (SP) induces PKC-dependent T370 phosphorylation. (A) HEK293 cells stably co-expressing MOR and NK_1 receptors were either not exposed (–) or exposed to SP in concentrations ranging from 10^{-7} to 10^{-5} M or 10 μM DAMGO for 30 min. Cells were lysed and immunoblotted with anti-pT370 and anti-pS375 antibodies. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gel. Note that SP leads to selective T370 phosphorylation. (B) HEK239 cells stably co-expressing MOR and NK_1 receptors were treated with 1 μM BIM2, 10 μM H89, 1 μM βARK1 inhibitor or 1 μM KN62 for 30 min and then either not exposed (–) or exposed to 1 μM SP. Cells were lysed and immunoblotted with anti-pT370 antibody. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3}. T370 phosphorylation was quantified and expressed as percentage of maximal phosphorylation in SP-treated control cells, which was set at 100% (upper panel). Data correspond to mean ± SEM from at least four independent experiments. Results were analysed by two-way ANOVA, followed by the Bonferroni post test (*P < 0.01). Note that the PKC inhibitor BIM2 diminished heterologous pT370 phosphorylation. (C) HEK293 cells stably expressing MOR and NK_1 receptors were transfected with 150 nM siRNA targeted to PKCα $PKC\beta_1$, $PKC\beta_2$ PKCγ, PKCε or non-silencing siRNA (*SCR*) for 72 h and then exposed to 1 μM SP. Cells were lysed and immunoblotted with anti-pT370 antibody. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gels. T370 phosphorylation was quantified and expressed as percentage of maximal phosphorylation in SCR-transfected cells, which was set at 100% (upper panel). Data correspond to mean ± SEM from at least four independent experiments. Results were analysed by two-way ANOVA, followed by the Bonferroni post test (*P < 0.01). Note that transfection with PKCα siRNA resulted in significant inhibition of T370 phosphorylation. Representative results from one of four independent experiments per condition are shown. The position of molecular mass markers is indicated on the left (in kDa). (A) HEK239 cells stably expressing MOR and NK_1 receptors were either not exposed (–) or exposed to 10 μM DAMGO, 1 μM SP or /and 1 μM BIM2 for 30 min. Cells were then stained with anti-HA antibody, processed for immunofluorescence and examined by confocal microscopy. Note that BIM2 incubation inhibited internalization of wild-type MOR after SP stimulation but not after DAMGO stimulation. Representative images from one of three independent experiments performed in duplicate are shown. Scale bar: 20 μm.

These results suggested that several PKC isoforms could function as a redundant phosphorylation system, responsible for constitutive S363 phosphorylation of MOR. Under conditions when constitutive S363 phosphorylation was blocked by BIM2 or RO32-0432, DAMGO, but not morphine, facilitated a clearly detectable S363 phosphorylation, suggesting that S363 can also be a substrate for homologous PKC-independent phosphorylation (Figure 5B).

S363 phosphorylation of MOR appears to depend on the cellular environment. Whereas S363 phosphorylation occurs constitutively in HEK293 cells, it requires activation of PKC by phorbol esters in CHO cells (Doll *et al.*, 2011; Feng *et al.*, 2011). We therefore examined S363 phosphorylation of endogenous MORs expressed in mouse brain. When crude brain homogenates from $MOR^{+/+}$ mice were immunoprecipitated with the rabbit monoclonal antibody UMB-3, the

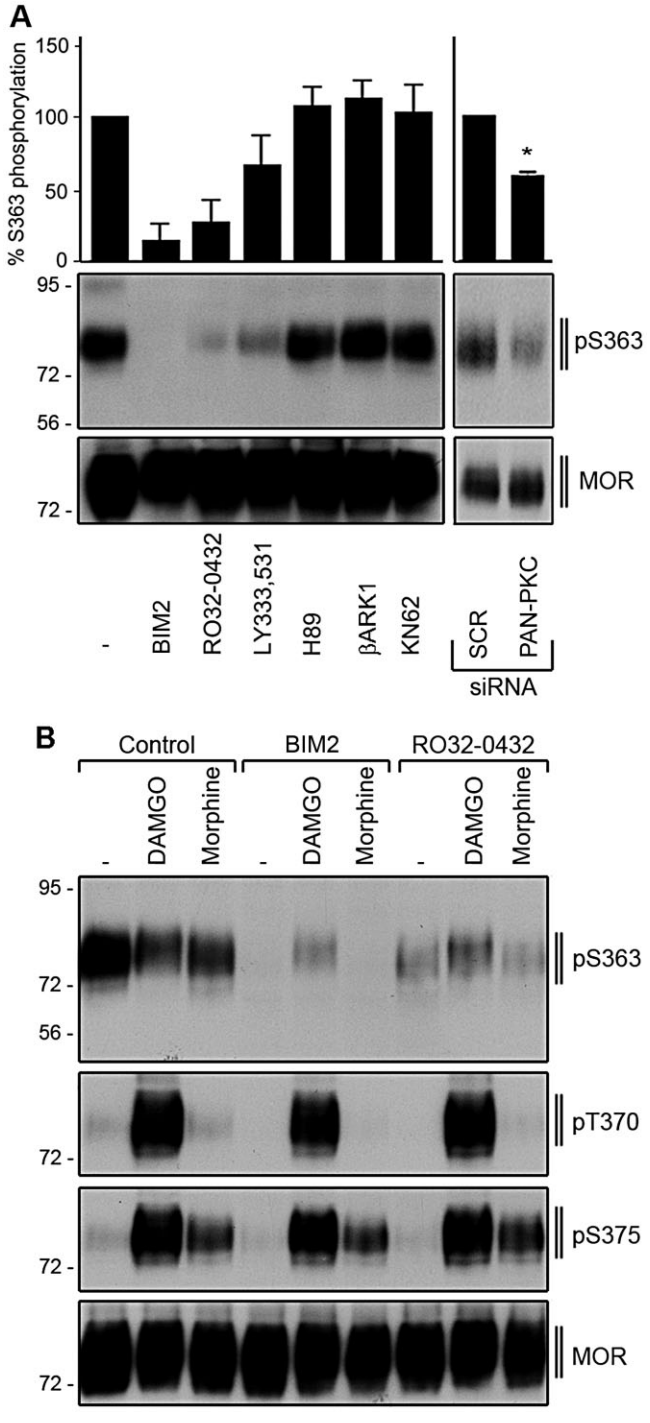

Figure 5

PKC mediates constitutive heterologous S363 phosphorylation. (A, left panel) HEK239 cells stably expressing MOR were treated with 1 μM BIM2, 1 μM Ro32-0432, 1 μM LY333,531, 10 μM H89, 1 μM βARK1 inhibitor or 1 μM KN62 for 30 min. Cells were lysed and immunoblotted with anti-pS363. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gel. Note that BIM2 completely blocked S363 phosphorylation, whereas RO32-0432 and LY333,531 partially inhibited 363 phosphorylation. (A, right panel) HEK293 cells stably expressing MOR were transfected with 150 nM siRNA targeting all PKC isoforms or non-silencing siRNA (*SCR*) for 72 h. Cells were lysed and immunoblotted with anti-pS363 antibody. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3}. S363 phosphorylation was quantified and expressed as percentage of maximal phosphorylation in control cells, which was set at 100% (upper panel). Data correspond to mean ± SEM from at least four independent experiments. Results were analysed by two-way ANOVA, followed by the Bonferroni post test (*P < 0.01). Note that transfection with PAN-PKC siRNA strongly inhibited constitutive S363 phosphorylation. (B) MOR-expressing HEK239 cells were treated with 1 μM BIM2 or 1 μM RO32-0432 for 30 min and then either not exposed (–) or exposed to 10 μM DAMGO or 10 μM morphine for 30 min. Cells were lysed and immunoblotted with the anti-pS363, anti-pT370 or anti-pS375 antibodies. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3}. Note that DAMGO treatment leads to homologous phosphorylation at S363 when ongoing PKC activity is diminished. Three independent experiments gave similar results. The positions of molecular mass markers are indicated on the left (in kDa).

guinea pig anti-pS363 antibody {GM363-3} detected a broad band migrating at M_r 70 000–80 000 in the subsequent immunoblot (Figure 6, left panel). In contrast to that observed with the anti-pS375 antibody {GM375-2}, the intensity of the pS363 band did not change markedly after treatment of mice with morphine or etonitazene (Figure 6, left panel). In contrast, no such band was detectable in brain homogenates prepared from $MOR^{-/-}$ mice under otherwise identical conditions (Figure 6, right panel). These results indicate that endogenous MORs expressed in mouse brain are constitutively phosphorylated at S363.

Discussion

In many systems, GRK-mediated phosphorylation facilitates receptor desensitization. However, morphine induces only weak phosphorylation of GRK sites on MOR (Zhang *et al.*, 1998; Schulz *et al.*, 2004; McPherson *et al.*, 2010; Doll *et al.*, 2011). This suggests that MOR desensitization by morphine may be mediated by another mechanism. There has been considerable interest in the role of PKC phosphorylation in the regulation of MOR by morphine. Johnson *et al.* (2006) demonstrated that morphine-induced desensitization was reduced by PKC inhibition in HEK293 cells. Conversely, activation of PKC enhanced the rapid desensitization induced by morphine in native locus coeruleus neurons (Bailey *et al.*, 2004; 2009). PKC appears to be involved in morphine-induced desensitization of MOR, but whether the desensitization is homologous or heterologous and whether or not the mechanism directly involves PKC phosphorylation of MOR is still uncertain (Williams *et al.*, 2013).

In the present study, we provide evidence for a selective and dose-dependent phosphorylation of T370 after activation of PKC by phorbol esters. Heterologous activation of G_q-coupled NK_1 receptors co-expressed with MOR in the same cell also leads to MOR internalization and a selective and dose-dependent phosphorylation of T370. We have previously observed a robust cross-internalization and cross-phosphorylation between NK_1 receptors and MOR, and that both receptors can exist in close proximity when co-expressed in the same cell (Pfeiffer *et al.*, 2003). The fact that SP-induced T370 phosphorylation and MOR internaliza-

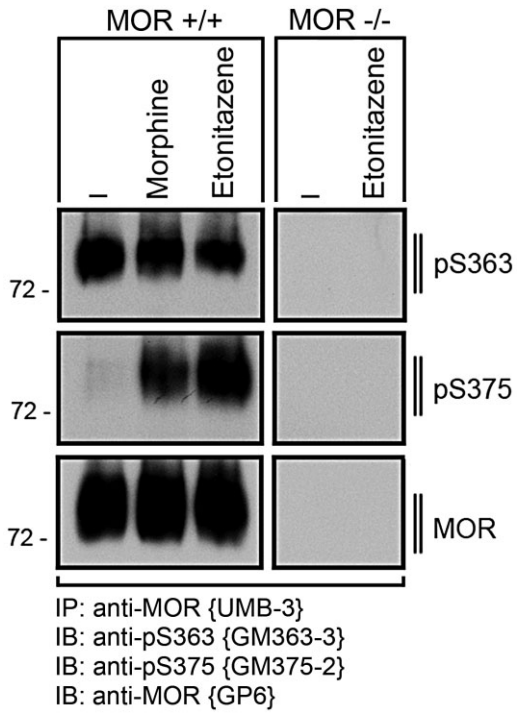

Figure 6

S363 is constitutively phosphorylated in mouse brain *in vivo*. (Right panel) Wild-type mice ($MOR^{+/+}$) mice were either not treated (–) or injected with morphine (30 mg·kg^{-1}, s.c.) or etonitazene (30 μg·kg^{-1}, s.c.). (Left panel) $MOR^{-/-}$ mice were either not treated (–) or injected 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. MORs were immunoprecipitated with phosphorylation-independent rabbit monoclonal anti-MOR antibody {UMB-3} and immunoblotted with guinea pig anti-pS363 {GM363-3} or anti-pS375 antibody {GM375-2}. Blots were stripped and reprobed with the phosphorylation-independent guinea pig anti-MOR antibody {GP6} to confirm equal loading of the gel. Representative results from one of three independent experiments performed in duplicate are shown. The positions of molecular mass markers are indicated on the left (in kDa).

tion were sensitive to PKC inhibitors suggests that crosstalk between NK1 and MOR can, in part, occur indirectly through the second-messenger kinase PKC. In contrast, inhibition of PKC activity did not compromise DAMGO-driven T370 phosphorylation. Thus, direct PKC-mediated T370 phosphorylation of MOR is unlikely to contribute to homologous desensitization. Chu *et al*. (2010) reported that morphine can promote PKCε-dependent phosphorylation of G_i proteins, which, in turn, facilitates a desensitization of MOR function in HEK293 cells. Morphine desensitization was abolished when the PKC phosphorylation sites on the G_i protein α subunit were mutated to alanines (Chu *et al*., 2010). These findings in HEK293 cells suggest that involvement of PKC activation in morphine-induced desensitization may not involve direct phosphorylation of MOR.

Recently, using mass spectrometry, Chen *et al*. (2013) showed that both S363 and T370 were phosphorylated under basal conditions. By using *in vitro* phosphorylation assays, they also observed that S363 was phosphorylated by PKC, whereas T370 was a CaMKII substrate. In the present study, we also observed that S363 undergoes basal phosphorylation and that this phosphorylation requires ongoing PKC activity. In contrast, in intact cells, agonist-dependent and agonist-independent phosphorylation of T370 were not sensitive to the CaMKII inhibitor KN62. When basal PKC activity was diminished, S363 could also be a substrate for homologous agonist-stimulated phosphorylation. However, PKC-mediated S363 phosphorylation of MOR appears to depend on the cellular environment. Whereas S363 phosphorylation occurs constitutively in HEK293 cells, it requires activation of PKC by phorbol esters in CHO cells (Doll *et al*., 2011; Feng *et al*., 2011). PKCs consists of three families of serine/threonine kinases that comprise (i) conventional PKCs (α, β and γ); (ii) novel PKC isoforms (δ, ε, η and θ); and (iii) atypical PKCs (ι/λ and ζ) (Parker and Murray-Rust, 2004). We found that phorbol ester-induced T370 phosphorylation specifically required the PKCα isoform. Constitutive S363 phosphorylation appears to require ongoing PKC activity. However, we were not able to identify a single PKC isoform required, suggesting that several PKC isoforms may function as a redundant phosphorylation system. Although the possibility that S363 phosphorylation could be a kinase reaction of killing the mouse and dumping all transmitters as a result of disrupted membrane potentials cannot be fully excluded, the present study provided strong evidence for basal S363 phosphorylation in mouse brain *in vivo*.

There is good indication that PKC plays a significant role in tolerance to the antinociceptive actions of morphine in rodents. Morphine tolerance can be reduced by co-administration of PKC inhibitors (Smith *et al*., 1999, 2003; Inoue and Ueda, 2000; Bohn *et al*., 2002; Hua *et al*., 2002). Similarly, tolerance is decreased when PKC expression is eliminated or reduced (Zeitz *et al*., 2001; Hua *et al*., 2002; Newton *et al*., 2007). These knockout/knockdown experiments as well as studies using specific inhibitors of individual PKC isoforms (Smith *et al*., 2007) suggest that PKCα, PKCγ and, to a lesser extent, PKCε are involved in tolerance to the analgesic effects of morphine. Moreover, it appears that ongoing PKC activity is necessary to maintain morphine tolerance even after it has developed. PKC inhibitors can reverse tolerance to the analgesic actions of morphine when administered 3 days after beginning the morphine treatment *in vivo* (Smith *et al*., 1999). Whereas PKC is involved in tolerance to morphine, it does not appear to be involved in tolerance to DAMGO. Nevertheless, definition of the role of direct PKC-mediated MOR phosphorylation in morphine tolerance has to await the generation of S363- and T370-phosphorylation-deficient *knock-in* mice.

This work explores the PKC-driven mechanisms of MOR phosphorylation using the currently available phospho site-specific MOR antibodies. In addition, previous studies identified phosphorylation on S356 and T357 by mass spectrometry (Lau *et al*., 2011; Chen *et al*., 2013). Therefore, it cannot be excluded that these residues could be the target for PKC-mediated phosphorylation. Interestingly, morphine does not induce phosphorylation of S363 or T370. Nevertheless, morphine-induced total MOR phosphorylation is decreased when PKC activity is inhibited by chemical

inhibitors (Johnson *et al*., 2006), suggesting that these additional sites could be involved.

In conclusion, the present results elucidate distinct modes of heterologous regulation of MOR phosphorylation by PKC. It is now possible to design definitive experiments to elucidate the exact contribution of PKC-driven S363 and T370 phosphorylation to diminished MOR responsiveness *in vivo*.

Acknowledgements

We thank Heidrun Guder, Ulrike Schiemenz and Heike Stadler for their excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft [SCH924/11-2 and SCH924/15-1].

Conflict of interest

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Authors contributions

Participated in research design: S. Schulz
Conducted experiments: S. Illing and A. Mann
Contributed new reagents: S. Schulz
Performed data analysis: S. Illing, A. Mann and S. Schulz
Wrote or contributed to the writing of the manuscript: S. Illing and S. Schulz

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al*. (2013), The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. Br J Pharmacol 170: 1459–1581

Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A (2011). Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. BMC Genomics 12: 14.

Bailey CP, Kelly E, Henderson G (2004). Protein kinase C activation enhances morphine-induced rapid desensitization of mu-opioid receptors in mature rat locus ceruleus neurons. Mol Pharmacol 66: 1592–1598.

Bailey CP, Llorente J, Gabra BH, Smith FL, Dewey WL, Kelly E *et al*. (2009). Role of protein kinase C and mu-opioid receptor (MOPr) desensitization in tolerance to morphine in rat locus coeruleus neurons. Eur J Neurosci 29: 307–318.

Bohn LM, Lefkowitz RJ, Caron MG (2002). Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin-2 knock-out mice. J Neurosci 22: 10494–10500.

Chen YJ, Oldfield S, Butcher AJ, Tobin AB, Saxena K, Gurevich VV *et al*. (2013). Identification of phosphorylation sites in the COOH-terminal tail of the mu-opioid receptor. J Neurochem 124: 189–199.

Chu J, Zheng H, Zhang Y, Loh HH, Law PY (2010). Agonist-dependent mu-opioid receptor signaling can lead to heterologous desensitization. Cell Signal 22: 684–696.

Doll C, Konietzko J, Poll F, Koch T, Hollt V, Schulz S (2011). Agonist-selective patterns of micro-opioid receptor phosphorylation revealed by phosphosite-specific antibodies. Br J Pharmacol 164: 298–307.

Doll C, Poll F, Peuker K, Loktev A, Gluck L, Schulz S (2012). Deciphering μ-opioid receptor phosphorylation and dephosphorylation in HEK293 cells. Br J Pharmacol 167: 1259–1270.

El Kouhen R, Burd AL, Erickson-Herbrandson LJ, Chang CY, Law PY, Loh HH (2001). Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid receptor internalization. J Biol Chem 276: 12774–12780.

Feng B, Li Z, Wang JB (2011). Protein kinase C-mediated phosphorylation of the mu-opioid receptor and its effects on receptor signaling. Mol Pharmacol 79: 768–775.

Grecksch G, Bartzsch K, Widera A, Becker A, Hollt V, Koch T (2006). Development of tolerance and sensitization to different opioid agonists in rats. Psychopharmacology (Berl) 186: 177–184.

Hua XY, Moore A, Malkmus S, Murray SF, Dean N, Yaksh TL *et al*. (2002). Inhibition of spinal protein kinase Calpha expression by an antisense oligonucleotide attenuates morphine infusion-induced tolerance. Neuroscience 113: 99–107.

Inoue M, Ueda H (2000). Protein kinase C-mediated acute tolerance to peripheral mu-opioid analgesia in the bradykinin-nociception test in mice. J Pharmacol Exp Ther 293: 662–669.

Johnson EA, Oldfield S, Braksator E, Gonzalez-Cuello A, Couch D, Hall KJ *et al*. (2006). Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. Mol Pharmacol 70: 676–685.

Just S, Illing S, Trester-Zedlitz M, Lau EK, Kotowski SJ, Miess E *et al*. (2013). Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. Mol Pharmacol 83: 633–639.

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

Koch T, Schulz S, Pfeiffer M, Klutzny M, Schroder H, Kahl E *et al*. (2001). C-terminal splice variants of the mouse mu-opioid receptor differ in morphine-induced internalization and receptor resensitization. J Biol Chem 276: 31408–31414.

Lau EK, Trester-Zedlitz M, Trinidad JC, Kotowski SJ, Krutchinsky AN, Burlingame AL *et al*. (2011). Quantitative encoding of the effect of a partial agonist on individual opioid receptors by multisite phosphorylation and threshold detection. Sci Signal 4: ra52.

Lupp A, Richter N, Doll C, Nagel F, Schulz S (2011). UMB-3, a novel rabbit monoclonal antibody, for assessing μ-opioid receptor expression in mouse, rat and human formalin-fixed and paraffin-embedded tissues. Regul Pept 167: 9–13.

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

McPherson J, Rivero G, Baptist M, Llorente J, Al-Sabah S, Krasel C *et al*. (2010). μ-Opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. Mol Pharmacol 78: 756–766.

Newton PM, Kim JA, McGeehan AJ, Paredes JP, Chu K, Wallace MJ *et al*. (2007). Increased response to morphine in mice lacking protein kinase C epsilon. Genes Brain Behav 6: 329–338.

Parker PJ, Murray-Rust J (2004). PKC at a glance. J Cell Sci 117 (Pt 2): 131–132.

Pfeiffer M, Koch T, Schroder H, Laugsch M, Hollt V, Schulz S (2002). Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization. J Biol Chem 277: 19762–19772.

Pfeiffer M, Kirscht S, Stumm R, Koch T, Wu D, Laugsch M *et al.* (2003). Heterodimerization of substance P and mu-opioid receptors regulates receptor trafficking and resensitization. J Biol Chem 278: 51630–51637.

Plockinger U, Albrecht S, Mawrin C, Saeger W, Buchfelder M, Petersenn S *et al.* (2008). Selective loss of somatostatin receptor 2 in octreotide-resistant growth hormone-secreting adenomas. J Clin Endocrinol Metab 93: 1203–1210.

Poll F, Lehmann D, Illing S, Ginj M, Jacobs S, Lupp A *et al.* (2010). Pasireotide and octreotide stimulate distinct patterns of sst2A somatostatin receptor phosphorylation. Mol Endocrinol 24: 436–446.

Poll F, Doll C, Schulz S (2011). Rapid dephosphorylation of G protein-coupled receptors by protein phosphatase 1β is required for termination of β-arrestin-dependent signaling. J Biol Chem 286: 32931–32936.

Schulz S, Mayer D, Pfeiffer M, Stumm R, Koch T, Hollt V (2004). Morphine induces terminal micro-opioid receptor desensitization by sustained phosphorylation of serine-375. EMBO J 23: 3282–3289.

Smith FL, Lohmann AB, Dewey WL (1999). Involvement of phospholipid signal transduction pathways in morphine tolerance in mice. Br J Pharmacol 128: 220–226.

Smith FL, Javed RR, Elzey MJ, Dewey WL (2003). The expression of a high level of morphine antinociceptive tolerance in mice involves both PKC and PKA. Brain Res 985: 78–88.

Smith FL, Gabra BH, Smith PA, Redwood MC, Dewey WL (2007). Determination of the role of conventional, novel and atypical PKC isoforms in the expression of morphine tolerance in mice. Pain 127: 129–139.

Williams JT, Ingram SL, Henderson G, Chavkin C, von Zastrow M, Schulz S *et al.* (2013). Regulation of μ-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. Pharmacol Rev 65: 223–254.

Zeitz KP, Malmberg AB, Gilbert H, Basbaum AI (2001). Reduced development of tolerance to the analgesic effects of morphine and clonidine in PKC gamma mutant mice. Pain 94: 245–253.

Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY *et al.* (1998). Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. Proc Natl Acad Sci U S A 95: 7157–7162.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12546

Figure S1 Homologous T370 phosphorylation is not mediated by PKC. HEK239 cells stably expressing MOR were either not pre-incubated or pre-incubated to 1 μM BIM2 and then either not exposed (–) or exposed to 1 μM DAMGO or 1 μM PMA. Cells were lysed and immunoblotted with anti-pT370 antibody. Blots were stripped and reprobed with the phosphorylation-independent anti-MOR antibody {UMB-3} to confirm equal loading of the gel. The position of molecular mass markers is indicated on the left (in kDa). Shown are representative results from one of four independent experiments per condition. T370 phosphorylation was quantified and expressed as percentage of maximal PMA-induced phosphorylation in control cells, which was set at 100% (upper panel). Data correspond to mean ± SEM from at least four independent experiments. Results were analysed by two-way ANOVA followed by the Bonferroni post test (*$P < 0.01$). Note that pre-incubation with the PKC inhibitor BIM2 diminished only the heterologous T370 phosphorylation by PMA.

Figure S2 Verification of PKCβ1/2 and PKCγ knockdown. HEK293 cells stably expressing MOR were transfected with 150 nM siRNA targeted to PKCβ1, PKCβ2 and PKCγ or non-silencing siRNA (SCR) for 72 h and total RNA was extracted from cells with the peqGOLD TOTAL RNA KIT (12-6634-01, PEQLAB GmbH, Erlangen, Germany). The positions of DNA ladder (Gene Ruler 100bp, Thermo Scientific, Waltham, MA, USA) are indicated on the left (in bp). Note that transfection with siRNA targeted to PKCβ1, PKCβ2 and PKCγ led to a decreased mRNA level of PKCβ1, PKCβ2 and PKCγ. So we assume that there is also a decreased protein level of these PKC isoforms. The reverse transcription reaction was performed using a superscript first-strand synthesis system (Invitrogen Life Technologies, Inc., Carlsbad, CA). The newly synthesized cDNA was amplified by PCR. The reaction mixture contained 1.3 μg of cDNA template, 1.5 mM MgCl2, 2.5 units of Taq polymerase and 0.5 μM primer; β-actin primer was used as an internal control. The relevant primers were as follows: PKCγ primers: 5′- GCTATCGGCCTCTTCT TCCT-3′ and 5′-AGACTTCCCATAGGGCTGGT - 3′, product size: 213; PKCβ primers: 5′-TGAAGGGGAGGATGAAGATG-3′ and 5′- TAAGGGGGCTGGATC TCTTT-3′, product size: 228 bp (Eurofins MWG Operon, Ebersberg, Germany); β-actin primers (sc-156106-PR, product size: 596 bp, Santa Cruz Biotechnology, Heidelberg, Germany). An initial incubation of 58°C for 2 min was followed by denaturing at 95°C for 10 s and then 30 cycles at 95°C for 15 s and 60°C for 1 min. All samples were tested in triplicate. Target gene expression was compared with that of the housekeeping gene, β-actin.