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RESEARCH PAPER

PI3Kγ integrates cAMP and Akt signalling of the μ-opioid receptor

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BACKGROUND AND PURPOSE

The μ-opioid receptor has been characterized as the main mediator of opioid signalling in neuronal cells. Opioid-induced pain suppression was originally proposed to be mediated by μ-opioid receptor-induced inhibitory effects on cAMP, which is known to mediate inflammatory hypernociception. Recent investigations revealed PI3Kγ and Akt (PKB) as additional elements of μ-opioid receptor signalling. Hence, we investigated the interaction between pronociceptive cAMP and antinociceptive PI3K/Akt signalling pathways.

EXPERIMENTAL APPROACH

The human neuroblastoma cell line SK-N-LO and primary dorsal root ganglia (DRG) cells from mice were used to elucidate mediators of μ-opioid receptor signalling. In both cellular systems cAMP was manipulated by stimulation of adenylate cyclase and consequent effects on PI3K/Akt signalling were analysed.

KEY RESULTS

Morphine stimulated Akt phosphorylation on Ser^{473} and Thr^{308} in a dose- and time-dependent manner indicating a functional μ-opioid receptor/Akt signalling pathway in μ-SK-N-LO cells. This effect of morphine was suppressed by the μ-opioid receptor inhibitor, naloxone, Pertussis toxin, an inhibitor of G_i heterotrimeric G-proteins, and the pan PI3K inhibitor wortmannin. cAMP-elevating agents also suppressed μ-opioid receptor-dependent stimulation of PI3Kγ lipid kinase and Akt activities in SK-N-LO cells and DRG.

CONCLUSIONS AND IMPLICATIONS

The data unveil a hitherto unknown interaction of pronociceptive cAMP and antinociceptive PI3K/Akt signalling pathways in neuronal cells. PI3Kγ was identified as a mediator of the inhibitory action of cAMP on Akt in SK-N-LO cells and DRG. The data indicate that PI3Kγ has a critical role in cAMP-mediated inflammatory hypernociception and analgesic signalling via μ-opioid receptors and PI3K/Akt in neuronal cells.

Abbreviations

DRG, dorsal root ganglia; nNOS, neuronal NOS

Introduction

Opioids including morphine are well known for their analgesic effects on sensory neurons (Stein and Lang, 2009). Different receptor types have been identified as mediators of the peripheral analgesia, but the μ-opioid receptor (Alexander *et al.*, 2013) was found to be the most important for morphine signal transduction (Scherrer *et al.*, 2009). The intracellular domains of the μ-opioid receptor were shown to interact with heterotrimeric G-proteins of the G_i family. Consequently, suppression of cAMP levels has been favoured as the predominant signalling pathway for μ-opioid receptors (Law *et al.*, 2000; Endres-Becker *et al.*, 2007). According to this hypothesis the analgesic effects of opioids are mediated via inhibition of cAMP-dependent inflammatory hypernociception induced by PGs or other inflammatory mediators.

Recent investigations have revealed PI3K and Akt (also known as PKB) as additional signalling mediators of morphine and μ-opioid receptors in sensory neurons. Using pharmacological and genetic approaches Cunha *et al.* identified a signalling path of μ-opioid receptors that involves G_i, the PI3K species PI3Kγ, with subsequent stimulation of Akt and neuronal NOS (nNOS) (Cunha *et al.*, 2010). The authors suggested that nNOS-dependent production of NO mediates pain-relieving hyperpolarization of neuronal membrane potential via cGMP-dependent control of K_{ATP} potassium channel (see Alexander et al., 2013b). Additional evidence for a prominent role of PI3Kγ in opioid signal transduction has been obtained in a parallel study from our group; we revealed an essential function of PI3Kγ in the development of long-term μ-opioid receptor desensitization and tolerance in dorsal root ganglia (DRG) (König *et al.*, 2010).

PI3Kγ was originally characterized as a mediator of GPCR agonists, which have the ability to stimulate Akt activity (Stoyanov *et al.*, 1995; Stephens *et al.*, 1997; Murga *et al.*, 1998). Unexpectedly, our subsequent investigations in the heart revealed an intimate interplay between PI3Kγ and the cAMP-signalling pathway. In initial studies PI3Kγ was characterized as a stimulator of cAMP PDE activity (Patrucco *et al.*, 2004). More recent work explored the physiological relevance of cAMP/PKA-dependent suppression of PI3Kγ lipid kinase activity in the heart (Perino *et al.*, 2011). On the basis of these findings, we investigated whether an interaction between cAMP/PKA signalling and PI3Kγ/Akt signalling exists outside the heart. In the present study, we demonstrated a relationship between these major signalling pathways in peripheral sensory neurons. cAMP-dependent inhibition of μ-opioid receptor-induced stimulation of PI3Kγ lipid kinase and Akt activities could be shown in SK-N-LO cells and sensory neurons (DRG). Our data are the first to provide a mechanistic explanation for the reversible antagonism of pronociceptive signalling of inflammatory agonists via cAMP and the analgesic effects of the PI3Kγ/Akt/nNOS/NO/K_{ATP} signalling pathway.

Methods

Plasmids, antibodies and chemicals

The plasmid encoding mouse μ-opioid receptors (pcDNA3.1 HA-OPRM1 wt) has been described (Schulz *et al.*, 2004). pLKO.1 vectors encoding shRNAs targeting human PI3Kγ and plasmid pLKO.1 encoding non-targeting control shRNA were obtained from Sigma (MISSION® shRNA lentivirus-mediated transduction system, Taufkirchen, Germany). They were used in combination with lentiviral packaging plasmids pMDL. pRSV and pVSV (kindly provided by Dr. Carol Stocking, Hamburg, Germany).

Antibodies recognizing phosphorylated Akt serine 473, phosphorylated Akt threonine 308 and pan Akt were purchased from Cell Signaling Technology (Frankfurt, Germany). β-Actin monoclonal antibody was purchased from Sigma. Monoclonal mouse PI3Kγ antibody 641 was made by our own laboratory (Perino *et al.*, 2011). HRP-coupled secondary anti-mouse and anti-rabbit antibodies were obtained from KPL (Weden, Germany).

Morphine, naloxone and wortmannin were purchased from Sigma. *Pertussis* toxin (PTX), forskolin and H89 were purchased from Enzo Life Science (Lörrach, Germany). AS605240 was purchased from Alexis (Lausen, Switzerland). TGX221 and IC87114 were a kind gift from the Baker Heart Institute. Inhibitor A66 was purchased from Symansis (Auckland, New Zealand).

Cell culture

SK-N-LO cells were obtained from the Children's Hospital, Tübingen University, Germany. The cells were maintained in 1:1 mixture of Iscove's modified Dulbecco's medium (IMDM) : HAM's F12 (PAA Laboratories, Linz, Austria) supplemented with 10% heat-inactivated FCS (Gibco, Darmstadt, Germany) with regular splitting to avoid over confluence. The cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Creation of 6μ-SK-N-LO cells

SK-N-LO cells were transfected with plasmid pcDNA3.1 HA OPRM1 encoding mouse μ-opioid receptors and polyethylenimine (PEI) transfection reagent in the ratio of 2.5 μg of PEI per 1 μg of DNA. Then 48 h after transfection, the cells were selected using medium containing G418 (1 $mg \cdot mL^{-1}$; PAA Laboratories, Linz, Austria). The medium was replaced with fresh medium every 3 days until visible growth of cells appeared. The cells were propagated further in the media containing 1:1 mixture of IMDM: HAM's F12 supplemented with 10% heat-inactivated FCS and 1 $mg \cdot mL^{-1}$ of G418. Subsequently, the stable production of μ-opioid receptors was confirmed immunologically. These cells will be further referred to as μ-SK-N-LO cells.

Knockdown of PI3Kγ in 7μ-SK-N-LO cells by shRNA

To generate lentiviral particles, HEK293T packaging cells were maintained in DMEM (Invitrogen, Darmstadt, Germany) supplemented with 10% FCS. The cells were transiently transfected with pLKO.1 derivative plasmids in combination with packaging plasmids using PEI and lentiviral particles containing media were collected 48 h after transfection. Then 10^4 μ-SK-N-LO cells were infected three times with lentiviral particles in presence of 8 $\mu g \cdot mL^{-1}$ polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide; Sigma-Aldrich, Seelze, Deutschland). Subsequently, the transduced

cells were selected with 1 μg·mL^{-1} puromycin (Sigma-Aldrich, Seelze, Deutschland), 48 h after transduction. Sufficiently propagated cell pools (1–2 × 10^6 cells) were subjected to phenotypic characterization immediately after establishment. The corresponding control shRNA cell pools were generated and analysed in parallel.

Preparation and culture of DRG

Mice weighing 20–25 g were killed by decapitation under anaesthesia. DRGs were isolated from whole spinal cord and collected into DMEM/F12 (Gibco) medium. Subsequently, the isolated ganglia were incubated with collagenase type II (0.4 U·mL^{-1}; PAA Laboratories) for 45 min and trypsin/EDTA (PAA Laboratories) for 10 min. DRGs were washed, dissociated by mechanically triturating the ganglia using a fire-polished Pasteur pipette and suspended in medium containing DMEM/F12 supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (PAA Laboratories). Subsequently, the cells were seeded in 12-well plates in the same media. Cell cultures were maintained at 37°C in a 5% CO_2 atmosphere and experiments were performed within 24 h.

Animals

DRG were collected from adult wild-type and PI3Kγ knockout mice (C57/BL6J). Animals were housed four to six per cage and maintained in a 12 h day/light cycle in a temperature-controlled environment with food and water *ad libitum*. Twelve- to 14-week-old mice were used for the experiments. The wild-type and PI3Kγ knockout mice were derived by 10 generations of successive backcrosses of heterozygous male knockout mice from chimeric C57BL6/129Sv PI3Kγ knockout mice (Hirsch *et al.*, 2000) with female C57BL6 (Jackson Laboratories, Bar Harbor, ME, USA). Experiments were approved by the committee of the Thuringian State Government on Animal Research. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Immunohistochemistry

Sections 5 μm thick of paraformaldehyde-fixed and paraffin-embedded DRG-tissues from wild-type and PI3Kγ knockout mice were labelled with anti-PI3Kγ monoclonal antibody (641) or mouse anti-IgG2a (Sigma) as isotype negative control. Dewaxed sections were rinsed with ddH_2O and transferred to an autoclavable cuvette with 10 mM citrate buffer. Heating for 15 min of 120°C was used for antigen retrieval followed by PBS wash and blocking step with 2% serum and 0.3% Triton X-100 in PBS for 30 min at room temperature. Primary antibodies incubated over night at 4°C and were detected using biotinylated secondary antibodies (Dako, Glostrup, Denmark) and the ABC-Vectastain-KS Kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions. Visualization followed with application of the peroxidase substrate Jenchrom (MoBiTech GmbH, Göttingen, Germany). The sections were examined with a light microscope (Axioplan 2, Zeiss, Jena, Germany) coupled to a charge-coupled device video camera and an image analysing system (KS 300, Zeiss). Control experiments were carried out with omission of primary antibodies (König *et al.*, 2010).

Immunoprecipitation of PI3Kγ

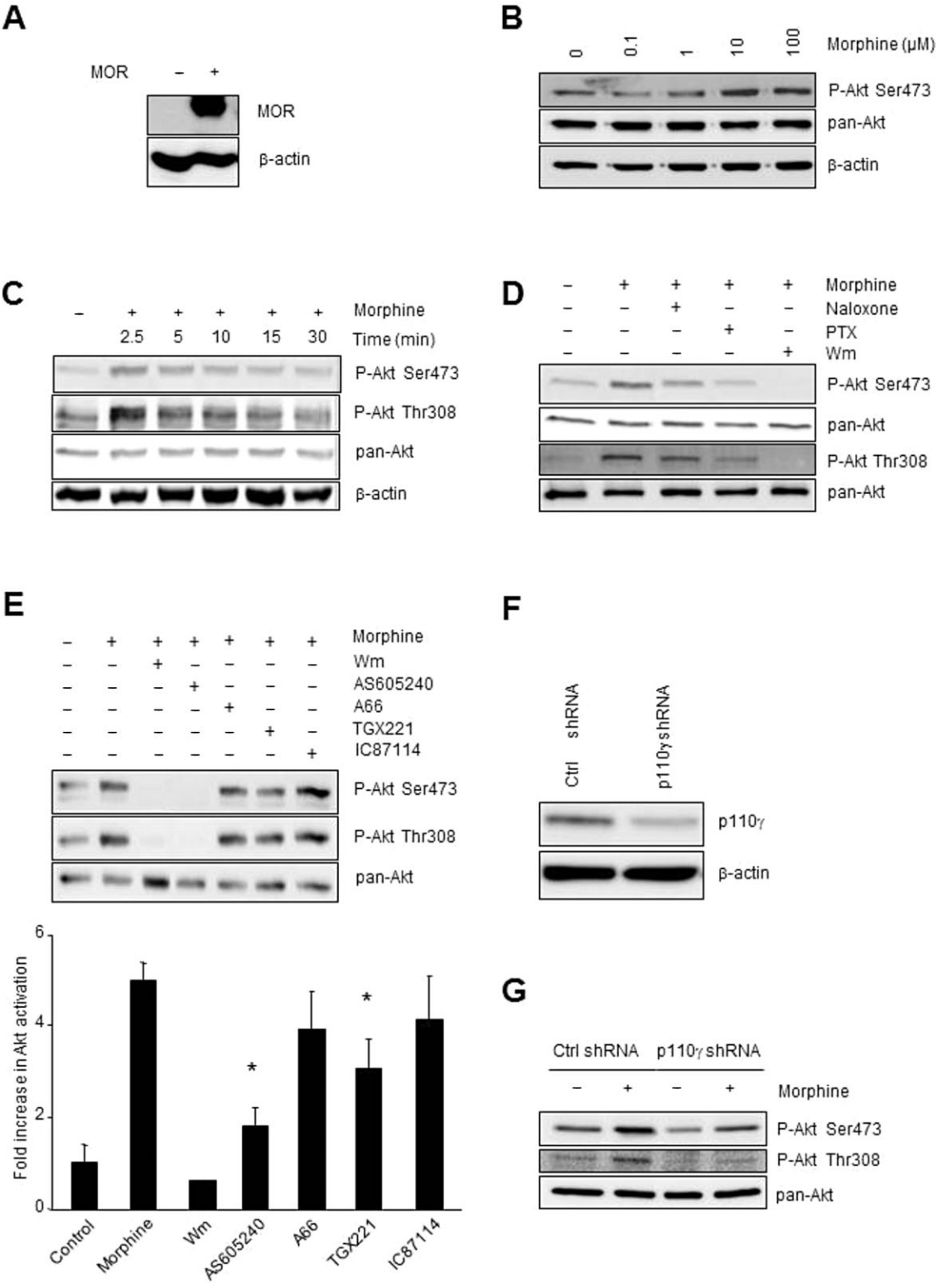
For PI3Kγ immunoprecipitation, lysis was done in 0.1% Nonidet P-40, 30 mM Tris pH 7.5, 0.15 M NaCl, 1 mM $MgCl_2$. For detection of activated signalling proteins in cell lysates, RIPA buffer containing 1% Nonidet P-40, 0.25% deoxycholate, 50 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA was used. Lysis buffers were freshly supplemented with proteinase inhibitors and phosphatase inhibitors (1 μg·mL^{-1} leupeptin, 1% aprotinin, 1 μg·mL^{-1} pepstatin A, 1 mM PMSF, 1 mM Pefabloc, 1 mM sodium orthovanadate, 1 mM glycerol phosphate), and lysis was allowed on ice for 15 min before thorough vortexing and centrifugation. PI3Kγ immunoprecipitations were performed by incubating with anti-PI3Kγ antibodies at 4°C overnight, followed by incubation with protein G-Sepharose beads. Adjusted aliquots of the cell extracts were subjected SDS-PAGE, followed by transfer to PVDF membranes (Millipore, Bedford, MA, USA), and probing with the indicated primary antibodies.

Western blot analysis

Immediately following stimulation, cells were lysed in ice cold lysis buffer containing 3.4 nM microcystin, 1 μg·mL^{-1}

Figure 1

PI3Kγ mediates μ-opioid receptor (MOR)-dependent stimulation of Akt. (A) Expression of mouse μ-opioid receptors in SK-N-LO cells. μ-SK-N-LO cells were prepared as described in Methods, lysed and glycosylated proteins were enriched using wheat germ lectin agarose beads. Proteins were eluted from the beads separated on SDS-PAGE and immunoblotted with specific antibodies recognizing μ-opioid receptors or β-actin. (B) Effects of different concentrations of morphine on Akt phosphorylation in μ-SK-N-LO cells. After 2.5 min incubation with morphine cells were lysed, whole cell extracts were resolved by SDS-PAGE and immunoblotted with specific antibodies recognizing phosphorylated Akt Thr^{308} or Ser^{473}. Subsequently, the membranes were stripped and redeveloped with specific antibodies recognizing total Akt. (C) Time-dependent effects of 10 μM morphine on Akt phosphorylation in μ-SK-N-LO cells. (D) Involvement of μ-opioid receptors, G_i and PI3K in morphine-induced Akt activation. μ-SK-N-LO cells were pre-incubated with 10 μM naloxone (30 min) or 100 ng·mL^{-1} PTX (overnight) or 100 nM wortmannin (Wm) (30 min) before stimulation with 10 μM morphine for 2.5 min and further processed as described under (B). (E) Identification of PI3K species involved in morphine-induced activation of Akt. μ-SK-N-LO cells were pretreated with one of the PI3K isoform specific inhibitors (30 nM A66 for PI3Kα, 20 nM TGX221 for PI3Kβ, 1 μM AS605240 for PI3Kγ and 200 nM IC87114 for PI3Kδ) or 100 nM Wm as a pan-specific PI3K inhibitor and stimulated with 10 μM morphine for 2.5 min and further processed as described under (B). Statistical significance has been marked in relation to control. The data presented are representative of the results of three independent experiments. (F) Suppression of PI3Kγ catalytic subunit p110γ in μ-SK-N-LO cells. μ-SK-N-LO cells treated with control shRNA and μ-SK-N-LO cells treated with shRNA suppressing p110γ (p110γ shRNA) were resolved by 10% SDS-PAGE and immunoblotted with specific antibodies recognizing p110γ or vinculin as a control. (G) Requirement of PI3Kγ for morphine-induced activation of Akt. Control μ-SK-N-LO cells (Ctrl shRNA) and μ-SK-N-LO cells treated with shRNA suppressing p110γ (p110γ shRNA) were stimulated with 10 μM morphine for 2.5 min. After cell lysis, cell extracts were resolved by 10% SDS-PAGE and immunoblotted with specific antibodies recognizing phosphorylated Akt. As loading control, the membranes were stripped and redeveloped with β-actin antibodies.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pepstatin, 1 μg·mL^{-1} leupeptin, 100 μg·mL^{-1} pefablock-AEBSF-HCl, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM β-glycerophosphate. Protein samples were resolved by 10% SDS-PAGE and transferred on to PVDF (Immobilon™-P) membranes. The membranes were blocked with 2.5% BSA in 1X TBS with 0.01% Tween 20 or 5% dried milk powder in 1X TBS with 0.01% Tween 20 (for PI3Kγ). Subsequently, the membranes were incubated overnight at 4°C with indicated primary antibody – phosphorylated Akt serine 473 or phosphorylated Akt threonine 308 (1:1000 or 1:500 for DRG's) or PI3Kγ – and 1 h with HRP-conjugated secondary antibody (1:5000 or 1:10 000). The blots were visualized in ECL solution (Western lighting chemiluminiscence detection TM Plus ECL, PerkinElmer Life Sciences, Waltham, MA, USA) for 1 min and images were captured on Luminescent Image Analyser (LAS-4000 system, GE ImageQuant, Freiburg, Germany). For loading controls, the blots were striped and reassessed with pan Akt (1:1000) or β–actin-specific antibodies. For quantification, specific phosphorylation was calculated as the ratio of signals for phosphorylated Akt to the signal of total Akt detected. Values of unstimulated samples were set to 1.0.

Assay of PI3Kγ lipid kinase activity

Following preincubation with 10 μM forskolin or 10 μM H89 for 30 min μ-SK-N-LO cells were stimulated with 10 μM morphine for 2.5 min. After cell lysis, p110γ was immunoprecipitated using N-terminal p110γ antibody (Perino *et al.*, 2011). Corresponding samples were incubated in lipid kinase buffer (20 mM HEPES pH 7.4; 5 mM $MgCl_2$). The buffer contained phosphatidylinositol, phosphatidylserine, ATP and 5 μCi of [^{32}P]-ATP. Afterwards, samples were centrifuged for 10 min at 30°C at 100× *g*. The reaction was stopped by adding HCl. The phases were separated by adding chloroform/methanol (1:1) mixture. The organic phase was collected, dried and resuspended in chloroform/methanol (2:1) mixture. The suspension was drop wise transferred onto the TLC plates and resolved with chloroform/methanol/ammonium hydroxide/water (45:35:8.5:1.5 v v^{-1}). The dried plates were analysed by using autoradiography (Perino *et al.*, 2011).

Data analysis and statistical procedures

Data are presented as mean ± SD of at least three individual experiments. To determine statistical significance of the described results, ANOVA with Fischer's least significant difference was performed. A P value of <0.05 was considered to be statistically significant. *P < 0.05, **P < 0.01 and ***P < 0.001.

Results

PI3Kγ mediates μ-opioid receptor-dependent stimulation of Akt

In order to study involvement of PI3K in μ-opioid receptor signalling we used SK-N-LO neuroblastoma cells, which express all four species of PI3K (Spitzenberg *et al.*, 2010). SK-N-LO cells were stably transfected with a plasmid encoding μ-opioid receptors and will be referred to as μ-SK-N-LO. The expression of μ-opioid receptors in μ-SK-N-LO cells was confirmed immunologically (Figure 1A and Supporting Information Fig. S1). To characterize the signalling pattern of μ-opioid receptors, we first analysed the effects of morphine on Akt activation, which has been demonstrated to be an intracellular mediator of μ-opioid receptors. As shown in Figure 1B and Supporting Information Figure S2, Figure 1C and Supporting Information Figure S3 morphine stimulated Akt phosphorylation on Ser^{473} and Thr^{308} in a dose- and time-dependent manner indicating a functional μ-opioid receptor/Akt signalling pathway in μ-SK-N-LO cells; a 2.5 min incubation with 10 μM morphine induced maximum stimulation of Akt and this duration of incubation used for further experiments. Pre-incubation of the cells with either the μ-opioid receptor inhibitor naloxone, PTX, an inhibitor of G_i heterotrimeric G-proteins, or the pan PI3K inhibitor wortmannin decreased the stimulant effects of morphine on Akt phosphorylation (Figure 1D and Supporting Information Fig. S4). These data indicate that μ-opioid receptors, G_i heterotrimeric G-proteins and PI3K are essential signalling elements of morphine-dependent Akt stimulation.

Next, we aimed to identify the PI3K species involved in morphine-induced stimulation of Akt. μ-SK-N-LO cells have been treated with the pan PI3K-specific inhibitor wortmannin (Arcaro and Wymann, 1993) and inhibitors specifically suppressing the enzymatic activities of the PI3K species PI3Kα (A66; Jamieson *et al.*, 2011), PI3Kβ (TGX221; Chaussade *et al.*, 2007), PI3Kγ (AS605240; Camps *et al.*, 2005) and PI3Kδ (IC87114; Sadhu *et al.*, 2003). AS605240 and wortmannin produced the most significant inhibitory effects on morphine-dependent phosphorylation of Akt (Figure 1E). These pharmacological investigations suggest that PI3Kγ has a prominent role in morphine signalling to Akt. For further proof of these effects we stably transfected μ-SK-N-LO cells with PI3Kγ shRNA. As shown in Figure 1F and Supporting Information Figure S5 expression of the catalytic subunit of PI3Kγ p110γ was significantly reduced by about 80%. This given decrease in PI3Kγ expression was accompanied by a loss of morphine-dependent stimulation of Akt confirming an essential role of PI3Kγ in morphine-dependent control of Akt activity (Figure 1G and Supporting Information Fig. S6).

Elevation of cAMP blocks μ-opioid receptor-dependent stimulation of Akt via PI3Kγ

Considering the major importance of cAMP in peripheral nociception (Stein and Lang, 2009), we next looked for effects of cAMP and PKA on morphine-induced activation of Akt. As shown in Figure 2A stimulation of adenylate cyclase by forskolin resulted in signification inhibition of morphine-induced stimulation of Akt indicating specific inhibitory effects of cAMP on morphine-induced stimulation of Akt. Treatment of μ-SK-N-LO cells with the PKA-specific inhibitor H89 did not significantly affect morphine-dependent stimulation of Akt suggesting that in the absence of forskolin cAMP/PKA is not involved in this effect of morphine.

We next investigated the mechanism of the inhibitory effects of cAMP on Akt activity. Following our recent study in cardiomyocytes (Perino *et al.*, 2011), we hypothesized that suppression of PI3Kγ lipid kinase activity and of Akt is induced by cAMP/PKA-dependent phosphorylation of PI3Kγ. First we looked at the effect of the cAMP-elevating agent

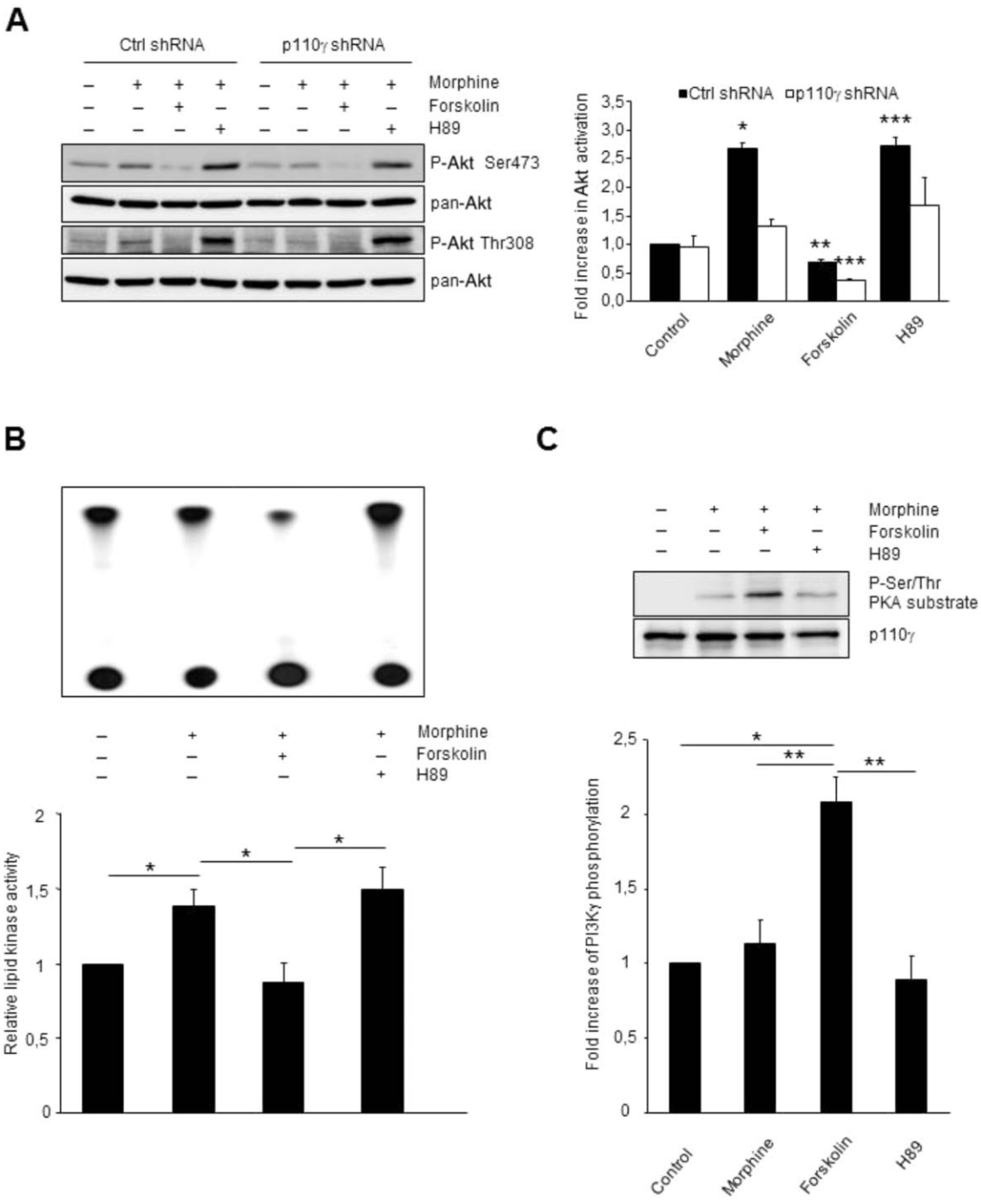

Figure 2

Elevation of cAMP blocks μ-opioid receptor-dependent stimulation of Akt via PI3Kγ. (A) cAMP-induced inhibition of μ-opioid receptor stimulation of Akt involves PI3Kγ. Control μ-SK-N-LO cells (Ctrl shRNA) and μ-SK-N-LO cells treated with shRNA suppressing the catalytic subunit of PI3Kγ were pre-incubated with 10 μM forskolin or 10 μM H89 for 30 min as indicated. Subsequently, the cells were stimulated with 10 μM morphine for 2.5 min. After cell lysis, cell extracts were resolved by 10% SDS-PAGE and immunoblotted with specific antibodies as indicated. Statistical significance has been marked in relation to wild-type (WT) – control. The data presented are representative of the results of five independent experiments. (B) cAMP-induced inhibition of PI3Kγ lipid kinase activity. μ-SK-N-LO cells were pre-incubated with 10 μM forskolin or 10 μM H89 for 30 min as indicated. Subsequently, the cells were stimulated with 10 μM morphine for 2.5 min as indicated. After cell lysis, cell extracts were immunoprecipitated using antibodies specific for the catalytic subunit of PI3Kγ. Lipid kinase activity of the precipitates was assayed after addition of [^{32}P]-ATP and phosphatidylinositol as described in Methods. The data presented are representative of the results of four independent experiments. (C) Phosphorylation of PI3Kγ catalytic subunit p110γ induced by cAMP. μOR-SK-N-LO cells were pre-incubated with 10 μM forskolin or 10 μM H89 for 30 min as indicated. Subsequently, the cells were stimulated with 10 μM morphine for 2.5 min as indicated. After cell lysis, PI3Kγ was immunoprecipitated and analysed by immunoblotting with specific antibodies detecting Ser/Thr-PKA substrates, and reprobed for PI3Kγ. The data presented are representative of the results of three independent experiments.

forskolin on lipid kinase activity of PI3Kγ. As shown in Figure 2B, forskolin inhibited lipid kinase activity of PI3Kγ in immunoprecipitates. The inhibitory effect of forskolin on PI3Kγ lipid kinase activity was accompanied by increased phosphorylation of the protein (Figure 2C). These data suggest that cAMP/PKA-induced phosphorylation and inhibition of PI3Kγ lipid kinase is the main mechanism whereby cAMP-elevating agents suppress morphine-dependent Akt stimulation.

Together, our results obtained in μ-SK-N-LO cells demonstrate that PI3Kγ is an essential mediator of morphine-dependent enhancement of Akt activity. In addition, the data suggest that PI3Kγ is the target of PKA-dependent phosphorylation, which mediates the inhibitory action of cAMP on morphine-evoked stimulation of Akt.

μ-opioid receptor- and PI3Kγ-induced stimulation of Akt and inhibitory effects of cAMP and PKA in primary DRG

In order to prove these findings in sensory neurons, we isolated DRG from mice. As shown in Figure 3A, expression of PI3Kγ could be demonstrated by immunohistochemical staining in wild-type DRG, whereas PI3Kγ-deficient cells do not show comparable staining. Next we looked for morphine-induced effects on Akt phosphorylation. As shown in Figure 3B, morphine provoked robust stimulation of Akt in wild-type DRG, whereas no significant increase in Akt phosphorylation was observed in PI3Kγ-deficient cells. Intriguingly, the stimulant effect of morphine on Akt could be suppressed by pre-incubating the DRG with forskolin. As with the μ-SK-N-LO cells, treatment with the PKA inhibitor H89 did not significantly affect morphine-dependent stimulation of Akt.

Stimulation of Akt by morphine and the inhibitory effect of forskolin on morphine-dependent stimulation of Akt was not apparent in PI3Kγ-deficient DRG. These data suggest that PI3Kγ has an important role in the analgesic effects mediated by Akt-dependent signalling and the pronociceptive action of cAMP in sensory neurons.

Discussion

The signalling activities of μ-opioid receptors are intimately associated with the molecular functional pattern of inflammatory reactions. PI3Kγ represents a paradigmatic example of this relationship. Originally, this signalling protein was characterized as a master mediator of leukocyte functions in inflammation (Hirsch *et al.*, 2000). Recent studies extended this view and revealed that PI3Kγ is an essential element of pain-relieving opioid effects in neuronal cells (Cunha *et al.*, 2010; König *et al.*, 2010). Our investigations in the cell line μ-SK-N-LO confirmed these results. Morphine-induced signal transduction of μ-opioid receptors to Akt includes G_i heterotrimeric G-proteins and PI3Kγ as essential molecular links. According to the seminal investigations of Cunha *et al.* the PI3Kγ-dependent signalling path between μ-opioid receptors and Akt may be supplemented by nNOS and NO flowing into hyperpolarized cells induced by cGMP-dependent control of K_{ATP} potassium channels (Cunha *et al.*, 2010).

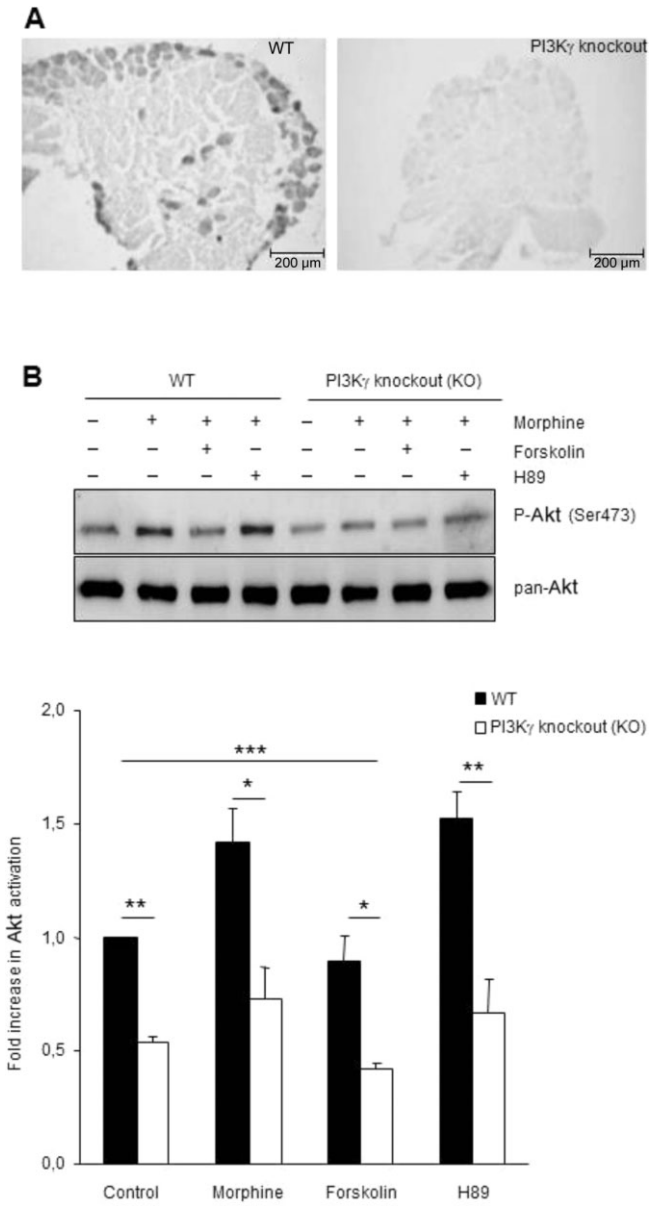

Figure 3

PI3Kγ mediates μ-opioid receptor-induced signalling in DRG. (A) Expression of PI3Kγ in DRG isolated from wild-type (WT) and PI3Kγ-deficient mice. Immunohistochemical staining of PI3Kγ in WT DRG, PI3Kγ-deficient cells do not exhibit comparable staining. Sections of paraformaldehyde-fixed and paraffin-embedded DRG from WT and PI3Kγ knockout mice were labelled with anti-PI3Kγ monoclonal antibody or mouse anti-IgG2a as described in Methods. (B) Involvement of PI3Kγ in morphine-induced signalling in DRG and inhibitory effect of cAMP. DRG neuronal cells from WT and PI3Kγ knockout mice were pre-incubated (30 min) with either 10 μM forskolin or 10 μM H89 as indicated. Subsequently, the cells were stimulated with 10 μM morphine for 2.5 min. After cell lysis, cell extracts were subjected to 10% SDS-PAGE and immunoblotted with specific antibodies recognizing phosphorylated Akt Thr^{308}. Then the membranes were stripped and redeveloped with specific antibodies recognizing total Akt. The data presented are representative of the results of five independent experiments.

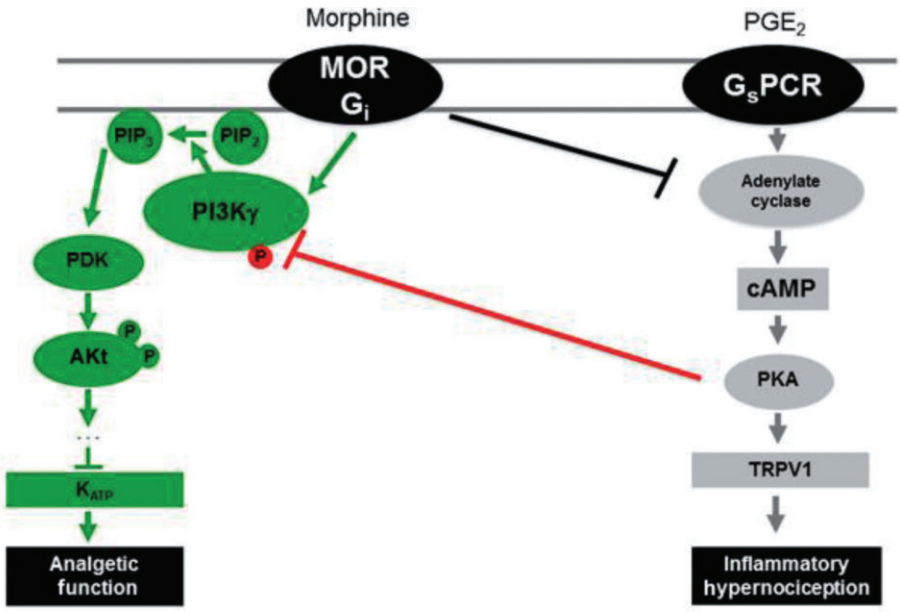

Figure 4

Summarizing overview about the signalling pathways under investigation. Classical μ-opioid receptor (MOR) signalling via suppression of cAMP by G_i heterotrimeric G-protein is illustrated in black. μ-opioid receptor-induced analgesic signalling via PI3Kγ/Akt (Cunha *et al.*, 2010) is shown in green. Antagonistic cAMP signalling induced by agonists of inflammatory hypernociception via cAMP/PKA-dependent phosphorylation of PI3Kγ and subsequent suppression of Akt activity is shown in red.

As a main focus of our study, we investigated the effects of cAMP signalling on morphine-induced Akt activation. cAMP has been characterized as a key mediator of opioid withdrawal-induced hyperalgesia (Sharma *et al.*, 1975; Spahn *et al.*, 2013). In addition, cAMP was shown to convey neuronal sensitization induced by inflammatory mediators like PGE_2. According to a series of studies the pronociceptive activities of PGE_2 are mediated by receptor-induced release of cAMP, followed by PKA-dependent phosphorylation and corresponding sensitization of transient receptor potential vanilloid 1 (TRPV1) channels and other nociceptors (Stein and Lang, 2009). In support of these findings, the inhibitory effects of morphine on TRPV1-dependent sensitization of sensory neurons were suppressed by cAMP-elevating agents (Endres-Becker *et al.*, 2007).

Our data unveil a mechanistic explanation for the inhibitory effects of cAMP and PKA on μ-opioid receptor signalling to Akt. The elevated cAMP level induced by forskolin blocked morphine-induced stimulation of Akt in μ-SK-N-LO cells. This effect was accompanied by cAMP-dependent phosphorylation of PI3Kγ and suppression of its lipid kinase activity suggesting that PKA-induced phosphorylation of PI3Kγ mediated the inhibitory effect of cAMP on opioid-induced μ-opioid receptor signalling to Akt. This assumption is supported by results from our recent study, which demonstrated an inhibitory effect of cAMP on PI3Kγ lipid kinase activity in cardiomyocytes via phosphorylation of the catalytic subunit p110γ by PKA (Perino *et al.*, 2011).

This PI3Kγ-dependent μ-opioid receptor signalling to Akt and the suppressive effects of the cAMP/PKA system on this signalling pathway observed in μ-SK-N-LO cells were fully supported by our investigations in primary sensory neurons (DRG). The proposed signalling reactions induced by morphine involved PI3Kγ as an essential mediator. In addition, the data indicate that suppression of Akt activity is mediated by cAMP/PKA-dependent phosphorylation of PI3Kγ.

In summary, the present study confirms the involvement of PI3Kγ and Akt in the morphine-evoked analgesic signalling in sensory neurons. This signalling pathway originally proposed by Cunha *et al.* (2010) significantly adds to the classical understanding of μ-opioid receptor/G_i-induced inhibitory effects on cAMP-dependent nociceptor sensitization evoked by inflammatory mediators like PGE_2 (Stein and Lang, 2009). A rough overview of the 'classical' (black) and the PI3Kγ/Akt-dependent signalling reactions (green) of μ-opioid receptors is shown in Figure 4.

Our present data suggest that pronociceptive cAMP signalling interacts with the PI3Kγ/Akt pathway via PKA-dependent phosphorylation of PI3Kγ, which subsequently inhibits lipid kinase activity and correspondingly suppresses Akt activity (red in Figure 4). We hypothesize the significant relevance of these findings for the cooperative effects of opioids and inflammatory mediators *in vivo*.

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Conflict of interest

No relevant conflicts of interest to declare.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ and CGTP Collaborators (2013a). The Concise Guide to PHARMACOLOGY 2013/14: G protein-coupled receptors. Br J Pharmacol 170: 1459–1581.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ and CGTP Collaborators (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Ion channels. Br J Pharmacol 170: 1607–1651.

Arcaro A, Wymann MP (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem J 296 (Pt 2): 297–301.

Camps M, Ruckle T, Ji H, Ardissone V, Rintelen F, Shaw J *et al.* (2005). Blockade of PI3Kgamma suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. Nat Med 11: 936–943.

Chaussade C, Rewcastle GW, Kendall JD, Denny WA, Cho K, Gronning LM *et al.* (2007). Evidence for functional redundancy of class IA PI3K isoforms in insulin signalling. Biochem J 404: 449–458.

Cunha TM, Roman-Campos D, Lotufo CM, Duarte HL, Souza GR, Verri WA Jr *et al.* (2010). Morphine peripheral analgesia depends on activation of the PI3Kgamma/Akt/nNOS/NO/KATP signaling pathway. P Natl Acad Sci U S A 107: 4442–4447.

Endres-Becker J, Heppenstall PA, Mousa SA, Labuz D, Oksche A, Schafer M *et al.* (2007). Mu-opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. Mol Pharmacol 71: 12–18.

Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L *et al.* (2000). Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. Science 287: 1049–1053.

Jamieson S, Flanagan JU, Kolekar S, Buchanan C, Kendall JD, Lee WJ *et al.* (2011). A drug targeting only p110alpha can block phosphoinositide 3-kinase signalling and tumour growth in certain cell types. Biochem J 438: 53–62.

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.

König C, Gavrilova-Ruch O, von Banchet GS, Bauer R, Grun M, Hirsch E *et al.* (2010). Modulation of mu opioid receptor desensitization in peripheral sensory neurons by phosphoinositide 3-kinase gamma. Neuroscience 169: 449–454.

Law PY, Wong YH, Loh HH (2000). Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxicol 40: 389–430.

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

Murga C, Laguinge L, Wetzker R, Cuadrado A, Gutkind JS (1998). Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinasegamma. J Biol Chem 273: 19080–19085.

Patrucco E, Notte A, Barberis L, Selvetella G, Maffei A, Brancaccio M *et al.* (2004). PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. Cell 118: 375–387.

Perino A, Ghigo A, Ferrero E, Morello F, Santulli G, Baillie GS *et al.* (2011). Integrating cardiac PIP3 and cAMP signaling through a PKA anchoring function of p110gamma. Mol Cell 42: 84–95.

Sadhu C, Dick K, Tino WT, Staunton DE (2003). Selective role of PI3K delta in neutrophil inflammatory responses. Biochem Biophys Res Commun 308: 764–769.

Scherrer G, Imamachi N, Cao YQ, Contet C, Mennicken F, O'Donnell D *et al.* (2009). Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. Cell 137: 1148–1159.

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.

Sharma SK, Klee WA, Nirenberg M (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. Proc Natl Acad Sci U S A 72: 3092–3096.

Spahn V, Fischer O, Endres-Becker J, Schafer M, Stein C, Zollner C (2013). Opioid withdrawal increases transient receptor potential vanilloid 1 activity in a protein kinase A-dependent manner. Pain 154: 598–608.

Spitzenberg V, König C, Ulm S, Marone R, Ropke L, Müller JP *et al.* (2010). Targeting PI3K in neuroblastoma. J Cancer Res Clin Oncol 136: 1881–1890.

Stein C, Lang LJ (2009). Peripheral mechanisms of opioid analgesia. Curr Opin Pharmacol 9: 3–8.

Stephens LR, Eguinoa A, Erdjument-Bromage H, Lui M, Cooke F, Coadwell J *et al.* (1997). The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. Cell 89: 105–114.

Stoyanov B, Volinia S, Hanck T, Rubio I, Loubtchenkov M, Malek D *et al.* (1995). Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. Science 269: 690–693.

Supporting information

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

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Figure S1 Quantification of Figure 1A. Expression of mouse μ-opioid receptors (MOR) in μ-SK-N-LO cells. The data presented are representative of the results of three independent experiments.

Figure S2 Quantification of Figure 1B. Effects of different concentrations of morphine on Akt phosphorylation in μ-SK-N-LO cells. The data presented are representative of the results of four independent experiments.

Figure S3 Quantification of Figure 1C. Time-dependent effects of morphine on Akt phosphorylation in μ-SK-N-LO cells. The data presented are representative of the results of four independent experiments.

Figure S4 Quantification of Figure 1D. Involvement of μ-opioid receptors (MOR), G_i and PI3K in morphine-induced Akt activation. The data presented are representative of the results of three independent experiments.

Figure S5 Quantification of Figure 1F. Suppression of PI3Kγ catalytic subunit p110γ in μ-SK-N-LO cells. The data presented are representative of the results of three independent experiments.

Figure S6 Quantification of Figure 1G. Requirement of PI3Kγ for morphine-induced activation of Akt. The data presented are representative of the results of four independent experiments.