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RESEARCH PAPER

Involvement of PKCα and G-protein-coupled receptor kinase 2 in agonist-selective desensitization of μ-opioid receptors in mature brain neurons

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Background and purpose: The ability of an agonist to induce desensitization of the μ-opioid receptor (MOR) depends upon the agonist used. Furthermore, previous data suggest that the intracellular mechanisms underlying desensitization may be agonist-specific. We investigated the mechanisms underlying MOR desensitization, in adult mammalian neurons, caused by morphine (a partial agonist in this system) and DAMGO (a high-efficacy agonist).
Experimental approach: MOR function was measured in locus coeruleus neurons, by using whole-cell patch-clamp electrophysiology, in rat and mouse brain slices (both wild-type and protein kinase C (PKC)α knockout mice). Specific isoforms of PKC were inhibited by using inhibitors of the receptors for activated C-kinase (RACK), and *in vivo* viral-mediated gene-transfer was used to transfect neurons with dominant negative mutants (DNMs) of specific G-protein-coupled receptor kinases (GRKs).
Key results: Morphine-induced desensitization was attenuated by using RACK inhibitors that inhibit PKCα, but not by other isoform-specific inhibitors. Further, the PKC component of morphine-induced desensitization was absent in locus coeruleus neurons from PKCα knockout mice. The PKC-enhanced morphine-induced desensitization was not affected by over-expression of a GRK2 dominant negative mutant (GRK2 DNM). In contrast, DAMGO-induced MOR desensitization was independent of PKC activity but was reduced by over-expression of the GRK2 DNM but not by that of a GRK6 DNM.
Conclusions and implications: In mature mammalian neurons, different MOR agonists can induce MOR desensitization by different mechanisms, morphine by a PKCα-mediated, heterologous mechanism and DAMGO by a GRK-mediated, homologous mechanism. These data represent functional selectivity at the level of receptor desensitization.
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Keywords: morphine; PKC; opioid; opiate; desensitization; tolerance; G-protein-coupled receptor kinase (GRK)

Abbreviations: DNM, dominant negative mutant; GFP, green fluorescent protein; GRK, G-protein-coupled receptor kinase; LC, locus coeruleus; MOR, μ-opioid receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RACK, receptors for activated C-kinase

Introduction

The μ-opioid receptor (MOR), a G-protein-coupled receptor, rapidly desensitizes in response to various opioid agonists. This desensitization can be mediated by different kinases depending upon the opioid agonist used to activate the receptor (see Bailey *et al.*, 2006; Kelly *et al.*, 2007). In human embryonic kidney (HEK)-293 cells expressing recombinant MORs desensitization induced by the high-efficacy peptide agonist DAMGO was largely mediated through a G-protein-coupled receptor kinase (GRK)-dependent mechanism whereas morphine-induced desensitization was largely mediated by protein kinase C (PKC) (Johnson *et al.*, 2006). It is now

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important to establish whether these distinct mechanisms are also operational in mature neurons. In rat brainstem neurons *in vitro*, morphine has been reported to produce significantly less receptor desensitization than other MOR agonists (Alvarez *et al*., 2002; Bailey *et al*., 2003; 2004; Blanchet *et al*., 2003; Dang and Williams 2005). However, morphine-induced MOR desensitization is significantly enhanced, if the level of PKC activity is elevated above basal by concomitant activation of a Gq-coupled receptor (Bailey *et al*., 2004). It is assumed that, in mature brain neurons, MOR desensitization by other opioid agonists such as DAMGO is GRK-mediated; however, to date there are no convincing data to substantiate this assumption.

In the present study, using both highly selective PKC isoform inhibitors and PKC knockout mice we have identified PKCα to be the isoform responsible for morphine-induced desensitization of MOR in mature brainstem locus coeruleus (LC) neurons. We have also used an adenoviral vector to over-express GRK dominant negative mutants (DNM) in mature LC neurons and have found that DAMGO-induced, but not morphine-induced, desensitization was mediated by GRK2 but not by GRK6.

Methods

Electrophysiological recordings

Brain slice preparation. All animal care and experimental procedures were in accordance with the UK Animals (Scientific Procedures) Act 1986, the European Communities Council Directive 1986 (86/609/EEC) and the University of Bristol ethical review document. Male Wistar rats (130–170 g) or C57 wild-type and PKCα knockout mice (Braz *et al*., 2004) of either sex (20–25 g; supplied by Dr A Poole, University of Bristol from animals originally developed by Dr JD Molkentin, University of Cincinnati) were killed by cervical dislocation, and horizontal brain slices (250 μm thick) containing the LC were prepared as described (Bailey *et al*., 2003).

Whole-cell patch-clamp recordings. Slices were submerged in a slice chamber (0.5 mL) mounted on the microscope stage and superfused (2.5–3 mL·min^{-1}) with artificial cerebrospinal fluid (aCSF) at 33–34°C. In those experiments in which LC neurons had been transuded with adenoviral vector to express enhanced green fluorescent protein (EGFP), transfected neurons were first identified by their fluorescence. For patch-clamp recordings, LC neurons were visualized by Nomarski optics using infrared light, and individual cell somata were cleaned by gentle flow of aCSF from a pipette. Whole-cell voltage clamp recordings (V_h = –60 mV) were made by using electrodes (3–6 MΩ) filled with (mmol·L^{-1}) 115 K-gluconate, 10 HEPES, 11 EGTA, 2 $MgCl_2$, 10 NaCl, 2 MgATP, 0.25 Na_2GTP (pH 7.3, osmolarity 270 mOsm). Recordings of whole-cell currents were filtered at 2 kHz and analysed off-line.

Activation of MORs evoked a transmembrane K^+ current, and by performing whole-cell patch-clamp recordings, a real-time index of MOR activation could be continually recorded. In rat neurons the opioid-evoked current was continuously recorded at a holding potential of –60 mV. Opioid-evoked currents were smaller in amplitude in mouse LC neurons, and this was most marked with the partial agonist morphine. To ensure that the morphine-evoked current was sufficiently large for accurate measurement above baseline noise, the aCSF contained 10 mmol·L^{-1} K^+ (substituting KCl for NaCl) and a voltage step protocol employed. Neurons were held at –60 mV and stepped to –130 mV for 60 ms every 10 s. As the opioid-activated K^+ current is inwardly rectifying, the amplitude of the inward current was measured at –130 mV because it was greater than the outward current at –60 mV. To ensure that this voltage protocol was not in some way altering the desensitization profile we have examined in mouse LC neurons, the rate and extent of DAMGO (full agonist)-induced desensitization at –60 mV (V_h) in normal aCSF and –130 mV (using the steps protocol with raised KCL aCSF concentration) and have found no difference (data not shown).

All drugs were applied in the superfusing solution at known concentrations. Drugs and chemicals used were from Sigma (Poole, Dorset, UK), except Met-enkephalin (Bachem, St Helens, UK), Go6976 (Calbiochem, Nottingham, UK) and receptors for activated C-kinase (RACK) inhibitors of selective PKC isoforms (KAI Pharmaceuticals, South San Francisco, CA, USA).

Adenoviral vector construction. Shuttle vectors were constructed such that cDNA encoding GRK2-K220R or GRK6-L215R was placed under the control of the PRSx8 promoter, to enable selective expression in noradrenergic neurons (Hwang *et al*., 2001; Teschemacher *et al*., 2005) and upstream of an IRES followed by GFP cDNA. Recombinant viruses were subjected to three rounds of plaque purification and screened by PCR to confirm the presence of the cDNA of the GRK DNMs. Recombinant viruses were purified by $CsCl_2$ density gradient centrifugation (Graham and Prevac, 1991), dialysed into 10 mmol·L^{-1} Tris/HCl pH 8.0, 1 mmol·L^{-1} $MgCl_2$, 3% sucrose and titrated by plaque assay (1×10^{12} pfu·mL^{-1}).

Adenoviral injections. Animals were anaesthetized with an intraperitoneal injection of ketamine (75 mg·kg^{-1}) and medetomidine (500 μg·kg^{-1}) and placed in a stereotaxic head holder. The upper surface of the skull was exposed with a midline incision. Bilateral viral injections ($0.5–1 \times 10^{11}$ pfu·mL^{-1} in 0.9% saline; 1 μL per side; 0.25 μL·min^{-1}) were made at two separate sites: 1.2 mm lateral to the midline, 2.4 mm rostral to lambda, 7.1 mm from the upper surface of the skull, with the needle at an angle of 10° from the vertical. The wound was sutured, and anaesthesia was reversed with a subcutaneous injection of atipamezole (1 mg·kg^{-1}). Animals were allowed to recover for 2–3 days before further experiments.

Western blotting. The LC nuclei from one rat were microdissected and homogenized in 30 mL 50 mmol·L^{-1} Tris/HCl pH 7.5, 120 mmol·L^{-1} NaCl, 1 mmol·L^{-1} EDTA containing protease inhibitors (Complete, Roche Diagnostics Ltd., West Sussex, UK). Cell debris and nuclei were removed by centrifugation, and the supernatant was analysed by SDS-polyacrylamide gel electrophoreses. Gels were blotted onto nitrocellulose membrane and probed with rabbit polyclonal isoform-specific anti-PKC antibodies (Cell Signalling Technology Inc., Danvers, MA, USA) or with a monoclonal antibody

that recognizes an epitope within the carboxy terminus of GRK2 (gift from Professor JL Benovic, Thomas Jefferson University, Philadelphia, USA). They were then incubated with horseradish peroxidase conjugates of anti-rabbit IgG or anti-mouse IgG respectively, and bands were visualized by enhanced chemiluminescence.

Data analysis

For statistical analysis, data are presented as mean ± SEM. Two-way ANOVA and Bonferroni's post-test were used to assess significance.

Results

PKCα mediates morphine-induced MOR desensitization

In mature rat LC neurons, a receptor-saturating concentration of morphine (30 μmol·L^{-1}) applied for 7 min induced little (8 ± 3%) desensitization of the evoked current (Figure 1Ai,B). Morphine (30 μmol·L^{-1}) did, however, induce greater MOR desensitization when the PKC activity in LC neurons was enhanced by concomitant activation of M_3 muscarinic receptors in LC neurons by oxotremorine-M [oxo-M; 10 μmol·L^{-1}; Figure 1Aii, B and C; see also Bailey *et al.*, 2004 for similar results obtained with morphine plus the phorbol ester, phorbol 12-myristate 13-acetate (PMA) which is another activator of PKC]. In contrast, a receptor-saturating concentration of DAMGO (10 μmol·L^{-1}) induced marked desensitization (45 ± 5%) over the same time period (Figure 1E,F), an effect that was neither enhanced when PKC was activated by PMA (1 μmol·L^{-1}; Figure 1E) nor reduced when PKC was inhibited with Go6976 (1 μmol·L^{-1}; Figure 1F). PMA itself does not have any direct effects on these G-protein-coupled, inwardly rectifying, potassium channel (GIRK) currents themselves, as the response to noradrenaline, which activates the same population of GIRK currents as the MOR, is unaffected by PMA (North and Williams, 1985; Bailey *et al.*, 2004). In some recordings we incubated brain slices with morphine for up to 30 min, in the absence of exogenous PKC activation, and still observed minimal desensitization (data not shown), but we have observed substantial desensitization, with morphine alone, after >4 h incubations (Bailey *et al.*, 2009).

To investigate which PKC isoform is involved in morphine-induced desensitization, we have used membrane-permeable inhibitors of the RACK (Schechtman and Mochly-Rosen, 2002). Each isoform of PKC, when activated, binds to its own specific RACK, from where it can phosphorylate target proteins. Recently, small peptide inhibitors have been designed, which specifically inhibit this interaction (Chen *et al.*, 2001), and which have been rendered cell-permeable by disulphide bridge-linking to a TAT peptide. We first used a RACK inhibitor that blocks all conventional PKC isoforms (PKCα, βI, βII and γ) but does not block novel (PKCδ, ε, θ and μ) nor atypical PKC isoforms (PKCξ and λ) (Ron *et al.*, 1995).

When this agent was applied at a concentration of 1 μmol·L^{-1} for 20 min prior to and during subsequent exposure to morphine (30 μmol·L^{-1}) in the presence of oxo-M (10 μmol·L^{-1}) it completely inhibited morphine-induced desensitization (Figure 1Aiii,B). Western blot analysis on samples of punched-out LC tissue revealed the presence of PKCα, β, γ (as well as the novel isoform PKCε) within this nucleus (Figure 1D). We then went on to use RACK inhibitors that have been reported to be selective for individual conventional PKC isoforms (Johnson *et al.*, 1996; Stebbins and Mochly-Rosen, 2001). RACK inhibitors of PKCβI, PKCβII and PKCγ, applied individually or in combination (1 μmol·L^{-1} each), failed to modify morphine-induced desensitization (Figure 1C). There is as yet no RACK inhibitor selective for PKCα but given that the RACK inhibitor that blocks all conventional PKC isoforms (α, βI, βII and γ) prevented morphine-induced desensitization but the combination of selective inhibitors of βI, βII and γ did not, we can infer that the PKC isoform involved in morphine-induced MOR desensitization is PKCα.

To confirm the involvement of PKCα in morphine-induced MOR desensitization, experiments were performed on LC neurons in brainstem slices from PKCα knockout and matched wild-type mice. Figure 2A and C show that in LC neurons from wild-type mice, as in rat LC neurons, activation of PKC with PMA (1 μmol·L^{-1} for 20 min) facilitated morphine-induced MOR desensitization whereas, in the absence of PKC activation morphine caused little MOR desensitization. In contrast, in PKCα knockout mice, morphine was unable to induce MOR desensitization either in the presence of PMA (Figure 2A,C), or in the absence of PMA (data not shown). On the other hand, DAMGO-induced desensitization was the same in LC neurons from wild-type and PKCα knockout mice (Figure 2B,C) and was unaffected by activation of PKC by PMA (Figure 2B). These data confirm that PKCα activation is required for morphine-induced, but not DAMGO-induced, MOR desensitization in LC neurons. There were no visible behavioural changes, or changes in the morphology of LC neurons between wild-type and knockout mice. No changes in initial peak current induced by morphine or DAMGO were seen, between wild-type and knockout animals (morphine wild-type: 187 ± 23 pA, morphine knockout: 182 ± 28 pA, DAMGO wild-type: 507 ± 58 pA, DAMGO knockout: 530 ± 32 pA), and there were no differences in whole-cell capacitance and holding currents.

DAMGO-induced MOR desensitization in LC neurons is mediated by GRK2 but not by GRK6

In rat LC neurons, DAMGO induced MOR desensitization even in the absence of PKC activation (Figures 1E,F and 3C,D; Bailey *et al.*, 2004). In recombinant expression systems, DAMGO-induced MOR desensitization and internalization have been shown to be GRK-mediated (Johnson *et al.*, 2006), and so we investigated the role of GRKs in DAMGO-induced desensitization in mature brain neurons. There are no small molecule, membrane-permeable, inhibitors of GRKs. Therefore to investigate the role of GRKs, we have over-expressed a DNM of GRK2 (GRK2-K220R) and a DNM of GRK6 (GRK6-L215R) in LC neurons. To do this, rats were anaesthetized and an adenoviral vector containing the noradrenergic neuron-specific promoter PRSx8 as well as the sequences of either the GRK2 DNM or GRK6 DNM and GFP (see *Methods* for details of the adenoviral vector) injected stereotactically and bilaterally into the region of the LC. Animals were allowed to

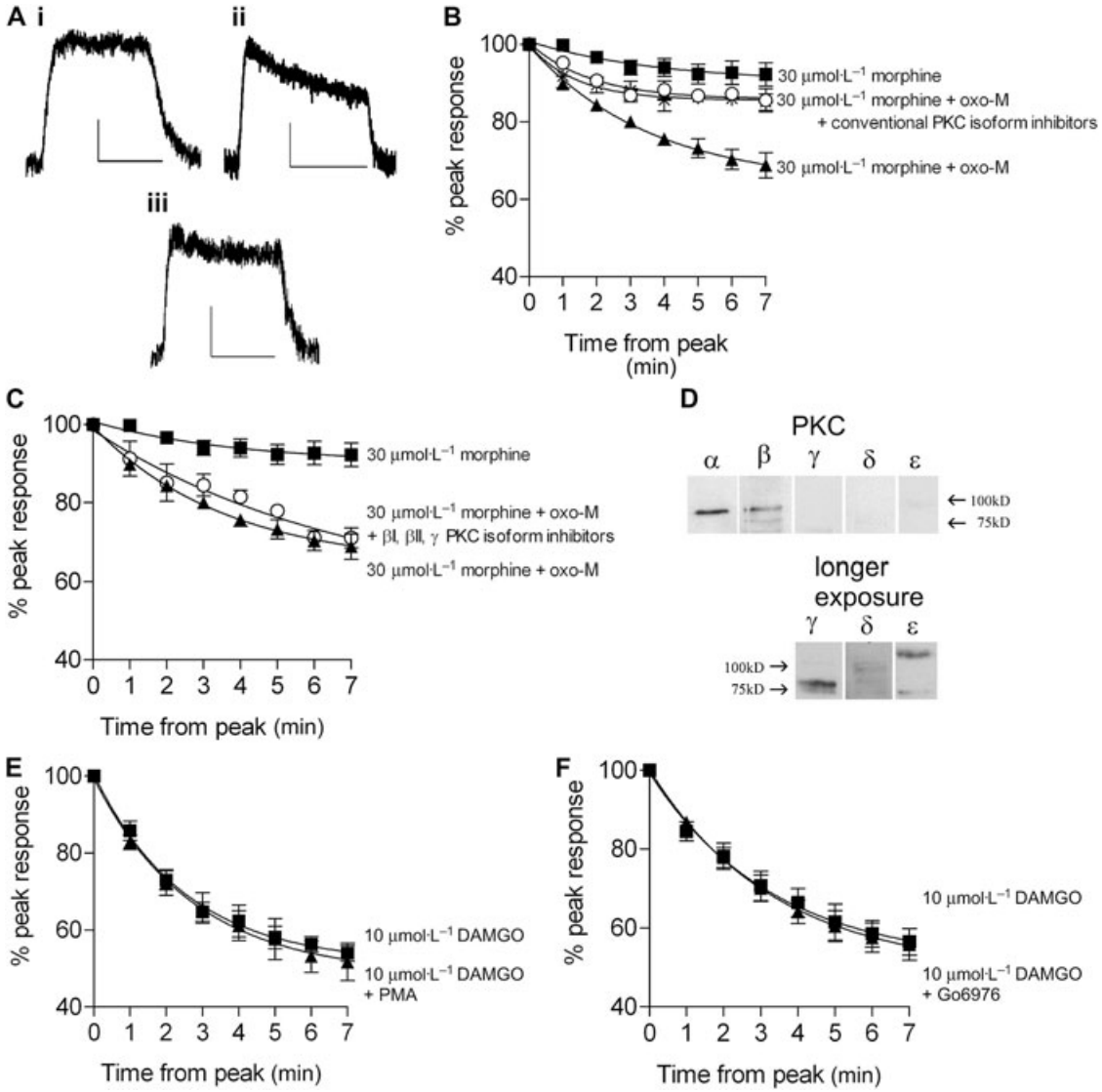


Figure 1 μ-Opioid receptor (MOR) desensitization induced by a combination of morphine and protein kinase C (PKC) activation is blocked by inhibitors of conventional PKC isoforms in rat locus coeruleus (LC) neurons. (A) Sample current recordings from rat LC neurons showing the time course of the current evoked by (i) 30 μmol·L^{-1} morphine alone, (ii) 30 μmol·L^{-1} morphine in the presence of 10 μmol·L^{-1} oxotremorine-M (oxo-M) and (iii) 30 μmol·L^{-1} morphine in the presence of 10 μmol·L^{-1} oxo-M plus 1 μmol·L^{-1} of the receptors for activated C-kinase (RACK) inhibitor of all conventional PKC isoforms (scale bars represent 50 pA and 5 min). (B) Pooled, averaged data (n = 3–13; error bars represent SEM) from experiments of the type in (A) showing the decay from the peak of the morphine-evoked current. And 30 μmol·L^{-1} morphine in the presence of 10 μmol·L^{-1} oxo-M caused significantly more MOR desensitization than 30 μmol·L^{-1} morphine alone ($P < 0.01$; two-way ANOVA and Bonferroni's post-test at the final time point only). This enhancement was markedly reduced when all of the conventional PKC isoforms (α, βI, βII, γ) were blocked (30 μmol·L^{-1} morphine + 10 μmol·L^{-1} oxo-M + 1 μmol·L^{-1} of the RACK inhibitor of all conventional PKC isoforms). $P < 0.01$ versus morphine + oxo-M. Asterisks (largely occluded by open circles) show MOR desensitization following brief application of noradrenaline (100 μmol·L^{-1}). (C) Pooled, averaged data from experiments of the type in (A) showing the decay from the peak of the morphine-evoked current. When only the βI, βII and γ conventional isoforms of PKC were inhibited, oxo-M still enhanced morphine-induced MOR desensitization (n = 3–13; error bars represent SEM; $P < 0.01$; two-way ANOVA and Bonferroni's post-test vs. morphine alone). (D) The rat LC expresses the α, β, γ and ε but not δ isoforms of PKC. Sample Western blots typical of those obtained in three separate experiments using protein from rat LC tissue. (E) Pooled, averaged data (n = 3–5; error bars represent SEM) from experiments of the type in (A) showing the decay from the peak of the DAMGO-evoked current. The desensitization induced by 10 μmol·L^{-1} DAMGO was the same in the presence and absence of 1 μmol·L^{-1} phorbol 12-myristate 13-acetate (PMA). (F) Pooled, averaged data (n = 3–4; error bars represent SEM) showing the decay from the peak of the DAMGO-evoked current. The desensitization induced by 10 μmol·L^{-1} DAMGO was the same in the presence and absence of 1 μmol·L^{-1} Go6976.

recover for 2–3 days to allow time for the GRK2 DNM, GRK6 DNM and GFP to be expressed in noradrenergic LC neurons. Animals were then killed and brainstem slices containing the LC prepared. Western blot analysis on samples of punched-out LC tissue revealed that there was a greater expression of GRK2 (using an antibody recognizing both wild-type GRK2 and GRK2 DNM) in the LC from animals injected with the GRK2 DNM- and GFP-containing virus (Figure 3B).

Virally infected neurons were identified under fluorescence microscopy by their GFP fluorescence (Figure 3A) and subsequently whole-cell patch-clamp recordings made from them. The resting membrane potential, input resistance and action

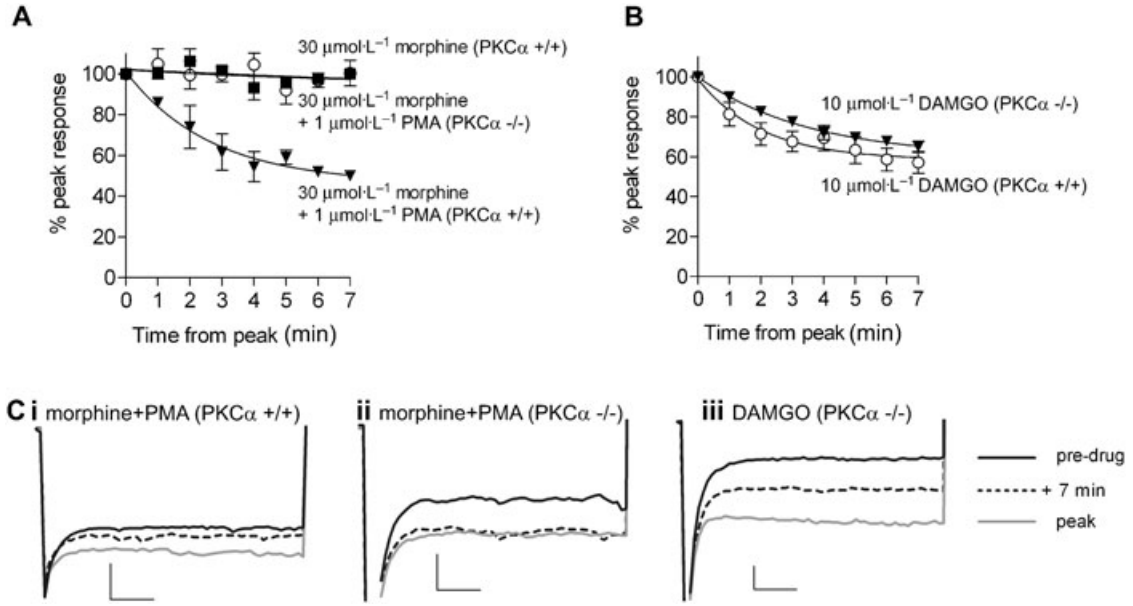


Figure 2 μ-Opioid receptor (MOR) desensitization induced by morphine or DAMGO in wild-type and protein kinase C (PKC)α knockout mice. (A) Pooled, averaged data from mouse locus coeruleus (LC) neurons (n = 3–4; error bars represent SEM). The desensitization observed in 30 μmol·L^{-1} morphine + 1 μmol·L^{-1} phorbol 12-myristate 13-acetate (PMA) in LC neurons from wild-type animals (PKCα+/+) was statistically greater than that observed with 30 μmol·L^{-1} morphine + 1 μmol·L^{-1} PMA in LC neurons from PKCα knockout (PKCα–/–) animals ($P < 0.01$; two-way ANOVA and Bonferroni's post-test at 7 min). (B) Pooled, averaged data from mouse LC neurons (n = 3–4; error bars represent SEM). Data show the decay of the 10 μmol·L^{-1} DAMGO-evoked current in LC neurons from wild-type animals (PKCα+/+) and in LC neurons from PKCα knockout animals (PKCα–/–). There was no statistically significant difference between the two curves ($P > 0.05$; ANOVA and Bonferroni's post-test). (C) Opioid-evoked currents in mouse LC neurons measured as the inward current evoked by voltage steps from –60 mV to –130 mV for 60 ms every 10 s (see *Methods* for details); scale bars represent 200 pA and 10 ms.

potential waveform of GFP-positive neurons were not significantly different from those of neurons from non-injected animals (data not shown). In GFP-positive, GRK2 DNM-expressing neurons, DAMGO (10 μmol·L^{-1}) induced a K^+ current similar in amplitude to that in control neurons (GFP-positive neurons: 134 ± 15 pA, control: 154 ± 25 pA; n = 4 and 3 respectively). The DAMGO-induced desensitization was however markedly attenuated in GFP-positive, GRK2 DNM-expressing neurons (Figure 3C,D). In contrast, in LC neurons infected with the GRK6 DNM-containing virus and exhibiting similar intensities of GFP fluorescence, the desensitization induced by DAMGO was the same as in cells not expressing the virus (Figure 3G,H).

Morphine-induced MOR desensitization did not involve GRK2

It has previously been reported that the activity of GRK2 can be enhanced by direct phosphorylation by PKC whereas GRK5 is inhibited by PKC phosphorylation (Pronin and Benovic, 1997). One possible mechanism therefore by which PKCα could enhance morphine-induced MOR desensitization would be by enhancing the activity of GRK2. In GRK2 DNM-expressing, GFP-positive neurons, morphine (30 μmol·L^{-1}) induced a K^+ current similar in amplitude to that in control neurons (GFP-positive neurons: 110 ± 14 pA, control: 107 ± 7 pA; n = 6 and 3 respectively). In the presence of PMA (1 μmol·L^{-1}), to activate PKC, morphine-induced desensitization was similar both in time course and amplitude in GRK2 DNM-expressing, GFP-positive neurons and control neurons (Figure 3E,F). Therefore PKC-mediated, morphine-induced MOR desensitization did not result from an enhancement of GRK2 activity.

Discussion

The key finding of this study is that two agonists acting on the same G–protein coupled receptor (GPCR) can induce desensitization by separate mechanisms, providing compelling evidence that different receptor agonists can induce different conformations of an endogenously expressed GPCR. To our knowledge this is the first time that the phenomenon of functional selectivity (Urban *et al.*, 2007) has been observed at the level of receptor desensitization in mature neurons. We observed that morphine-induced MOR desensitization is mediated predominantly by a heterologous, PKC-dependent mechanism whereas that induced by the peptide agonist DAMGO is homologous and mediated by GRK and not PKC. Indeed each MOR agonist may possess a different ability to recruit the various mechanisms of desensitization, as indeed we have previously reported that the desensitization induced by Met-enkephalin, likely to be GRK-mediated, can be enhanced by PKC activation (Bailey *et al.*, 2004). At present we cannot differentiate between a sequential model of desensitization where the desensitized states are interconvertible and a non-sequential, independent model where the states are not interconvertible (see Kelly *et al.*, 2007). A further complication arises from our studies of recombinant MORs expressed in HEK-293 cells where the MOR appears to be pre-phosphorylated by PKC (Johnson *et al.*, 2006).

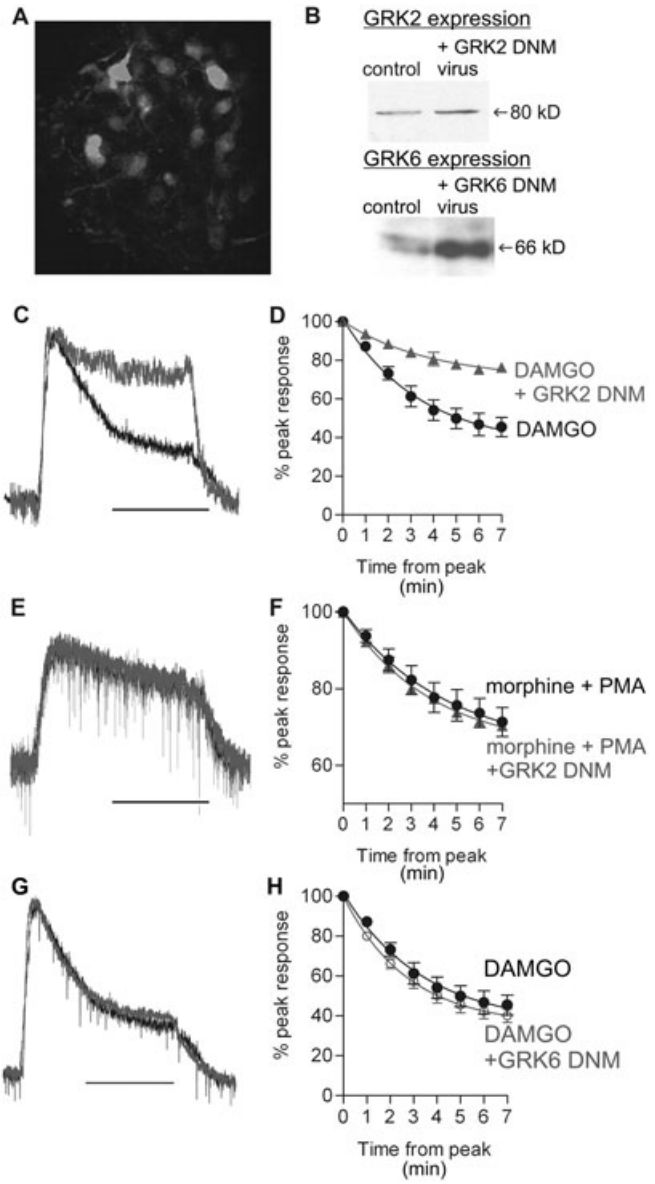
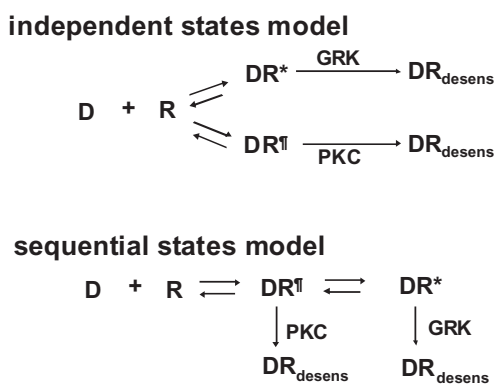


Figure 3 Inhibition of DAMGO-induced, but not morphine-induced, MOR desensitization by over-expressing a GRK2 dominant negative mutant in rat LC neurons. (A) LC neurons exhibit fluorescence, 2 days after stereotactic local injection of a PRSx8-GRK2(K220R)-IRES-eGFP-containing adenoviral vector. (B) Total GRK2 and GRK6 immunoreactivity was enhanced in rat LC tissue 2 days following injection of PRSx8-GRK2(K220R)-IRES-eGFP- or PRSx8-GRK6(L215R)-IRES-eGFP-containing adenoviral vector. Sample Western blots from three separate experiments each. (C) Sample current recordings showing DAMGO-induced desensitization. The black trace shows a recording from an LC neuron taken from a control animal and shows marked desensitization in response to DAMGO (10 μmol·L^{-1}). The grey trace shows a recording from an eGFP-positive neuron, and therefore assumed to also be expressing the GRK2 DNM. Traces are scaled to equal peak currents; scale bar represents 5 min. (D) Pooled data from experiments (n = 3–4) as in (C) showing the decay from the peak of the DAMGO-evoked current. When the GRK2 DNM was expressed, DAMGO-induced MOR desensitization was significantly attenuated compared with that observed in control neurons. Error bars represent SEM (P < 0.01; two-way ANOVA and Bonferroni's post-test at 7 min). (E) Sample current recordings showing MOR desensitization induced by morphine (30 μmol·L^{-1}) in the presence of PMA (1 μmol·L^{-1}). The black trace shows a recording from an LC neuron from a control animal; the grey trace shows a recording from a neuron expressing the GRK2 DNM. Traces are scaled to equal peak currents; scale bar represents 5 min. (F) Pooled data from experiments (n = 3–6) as in (E) showing the decay from the peak of the morphine-evoked current. Data show morphine-induced MOR desensitization in the presence of PMA (1 μmol·L^{-1}) in neurons from control animals and in neurons expressing the GRK2 DNM. Error bars represent SEM. There was no statistically significant difference between the two curves (P > 0.05; two-way ANOVA and Bonferroni's post-test at 7 min). (G) Sample current recordings showing DAMGO-induced desensitization. The black trace shows a recording from an LC neuron taken from a control animal and shows marked desensitization in response to DAMGO (10 μmol·L^{-1}). The grey trace shows a recording from an eGFP-positive neuron, and therefore assumed to also be expressing the GRK6 DNM. Traces are scaled to equal peak currents; scale bar represents 5 min. (H) Pooled data from experiments (n = 3–4) as in (G) showing the decay from the peak of the DAMGO-evoked current. When the GRK6 DNM was expressed, DAMGO-induced MOR desensitization was not significantly attenuated compared with that observed in control neurons. Error bars represent SEM (P > 0.05; two-way ANOVA and Bonferroni's post-test at 7 min). DNM, dominant negative mutant; GFP, green fluorescent protein; GRK, G-protein-coupled receptor kinase; LC, locus coeruleus; MOR, μ-opioid receptor; PMA, phorbol 12-myristate 13-acetate.



Two state model of agonist-selective MOR desensitisation where the conversion to DR* or DR¶ depends upon the specific agonist (D) that activates the receptor.

In LC neurons there is a small component of morphine-induced MOR desensitization apparent in the absence of PKC activation (Bailey *et al.*, 2004; Dang and Williams, 2005). This, coupled with the observation that in HEK-293 cells, expression of the GRK2 DNM seemed to exert a slight inhibitory effect on morphine-induced desensitization (Johnson *et al.*, 2006) could indicate that there is a component to morphine desensitization that is GRK-mediated. If this leads to arrestin binding to the MOR, it would explain the small but significant amount of receptor internalization induced by morphine in HEK-293 cells and might also explain the morphine-induced, arrestin-dependent MOR receptor internalization seen in striatal neurons (Haberstock-Debic *et al.*, 2005) although morphine-induced MOR internalization has not been observed in several types of neuron including those in LC, cortex, spinal cord and gut (Sternini *et al.*, 1996; Keith *et al.*, 1998; Trafton *et al.*, 2000; Van Bockstaele and Commons 2001). The relative amounts of PKC-mediated and

GRK-mediated morphine-induced MOR desensitization may therefore vary between cell types depending on the relative expression levels of each enzyme.

In our *in vitro* studies on LC neurons, it is necessary to enhance PKC activity to observe morphine-induced MOR desensitization (this paper; Bailey *et al.*, 2004) yet *in vivo*, morphine tolerance, which is reversed by PKC inhibitors (Smith *et al.*, 2007), develops without any additional activation of PKC. This is likely due to the basal conditions that are achieved in isolated, patch-clamped neurons. Such neurons are quiescent with no ongoing calcium entry due to action potential firing; they will also not be being bombarded with synaptic inputs that would activate metabotropic Gq-coupled receptors resulting in PLC activation, raised intracellular diacylglycerol and calcium levels as well as activating ionotropic glutamate receptors to raise intracellular calcium levels. Raised intracellular calcium levels and production of diacylglycerol would result in a higher level of PKC activity.

The classical scenario for GPCR desensitization is by phosphorylation of the receptor by GRKs and subsequent arrestin binding (Pierce *et al.*, 2002). It was possible that PKC might facilitate MOR desensitization indirectly by phosphorylating and activating GRKs (Zhang *et al.*, 1996; Krasel *et al.* 2001). However we showed that that was not the case, as viral vector-mediated expression of a dominant negative form of GRK2 had no effect on PKC-facilitated morphine-induced MOR desensitization, whereas DAMGO-induced MOR desensitization was reduced. Interestingly, DAMGO-induced desensitization was not inhibited by over-expression of a GRK6 DNM even though LC neurons are likely to express GRK6 (Erdtmann-Vourliotis *et al.*, 2001) demonstrating a degree of specificity with regard to MOR–GRK interaction in mature neurons. We have previously reported that the GRK2 and GRK6 DNMs used in this study inhibit adenosine A_2 and secretin receptor desensitization respectively (Mundell *et al.*, 1997; Ghadessy *et al.*, 2003). It is unlikely that simply expressing adenovirus in LC neurons produces the selective decrease in DAMGO-induced desensitization. While the GRK2 DNM-containing virus inhibited DAMGO desensitization it was ineffective against morphine-induced desensitization and furthermore the GRK6 DNM-containing virus was ineffective at the same expression levels against both DAMGO- and morphine-induced desensitization. Therefore de facto we have shown that the virus itself does not produce the inhibition of DAMGO desensitization.

Unfortunately we have not yet been able to exclude that in the absence of GRK activity, DAMGO can desensitize the MOR by a PKC-dependent mechanism. This is because to do so requires the complete removal of GRK2 from LC neurons. The GRK2 knockout is lethal, and at present no irreversible inhibitors of GRK2 are available. Over-expression of the GRK2 DNM as used in our present study reduces, but does not abolish, GRK2 activity and thus subsequent activation of PKC is likely to result in an enhancement of endogenous GRK2 activity by direct phosphorylation of GRK2 by PKC (Pronin and Benovic, 1997) and overcome the inhibition produced by the GRK2 DNM.

It is also possible that PKC activation in some way enhances a phosphorylation-independent physical interaction between GRKs and the MOR as has been described for some G-protein-coupled receptors (Ferguson, 2007). Our attempts, however, using co-immunoprecipitation experiments on HEK-293 cells over-expressing both MOR1 and GRK2, have failed to find agonist-induced MOR/GRK binding, even though we have previously observed mGluR1a/GRK binding using this approach (Mundell *et al.*, 2004). Other workers have reported that the interaction between the MOR and GRKs is weak relative to other GPCRs (Schulz *et al.*, 2002).

Coupled with our work examining MOR desensitization in HEK-293 cells (Johnson *et al.*, 2006), we can conclude that PKC facilitates MOR desensitization by a mechanism independent of GRK and arrestin, and which operates within the neuron expressing the MOR. The likely target for PKC phosphorylation is the MOR itself, and in HEK-293 cells, MORs are phosphorylated in response to PKC activation (Johnson *et al.*, 2006; I. Rodriguez-Martin, S. Oldfield and E. Kelly, unpubl. obs.).

Protein kinase C exists as multiple isoforms (Way *et al.*, 2000), many of which are expressed in CNS neurons. In LC neurons, a single PKC isoform, PKCα, appears to be responsible for facilitating morphine-induced MOR desensitization as when PKCα was inhibited or absent, morphine caused minimal MOR desensitization, even in the presence of PKC activators. However PKCα is not the only isoform that may be involved in morphine-induced MOR desensitization and it is entirely possible that in different neuronal populations other isoforms of PKC may be involved. Indeed, *in vivo*, there is evidence from analgesia studies that morphine tolerance may involve not only PKCα but also PKCγ and PKCε (Zeitz *et al.*, 2001; Hua *et al*, 2002; Newton *et al.*, 2007; Smith *et al.*, 2007).

In conclusion, we show that in mature neurons morphine and DAMGO can induce MOR desensitization by different mechanisms, demonstrating a striking degree of kinase specificity in the desensitization of neuronal GPCRs. The desensitization induced by morphine is largely GRK2-independent but requires ongoing activation of PKCα whereas for DAMGO the desensitization is by a homologous GRK2-dependent mechanism.

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Conflict of interest

None.

References

Alvarez VA, Arttamangkul S, Dang V, Salem A, Whistler JL, von Zastrow M *et al.* (2002). μ-Opioid receptors: ligand-dependent activation of potassium conductance, desensitization, and internalization. *J Neurosci* **22**: 5769–5776.

Bailey CP, Couch D, Johnson E, Griffiths K, Kelly E, Henderson G (2003). μ-Opioid receptor desensitisation in mature rat neurons:

lack of interaction between DAMGO and morphine. *J Neurosci* **23**: 10515–10520.

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

Bailey CP, Smith FL, Kelly E, Dewey WL, Henderson G (2006). How important is protein kinase C in μ-opioid receptor desensitisation and morphine tolerance? *Trends Pharmacol Sci* **27**: 558–565.

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

Blanchet C, Sollini M, Luscher C (2003). Two distinct forms of desensitisation of G-protein coupled inwardly rectifying potassium currents evoked by alkaloid and peptide μ-opioid receptor agonists. *Mol Cell Neurosci* **24**: 517–523.

Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevitsky R *et al*. (2004). PKC-alpha regulates cardiac contractility and propensity toward heart failure. *Nat Med* **10**: 248–254.

Chen L, Wright LR, Chen CH, Oliver SF, Wender PA, Mochly-Rosen D (2001). Molecular transporters for peptides: delivery of a cardioprotective epsilonPKC agonist peptide into cells and intact ischemic heart using a transport system, R(7). *Chem Biol* **8**: 1123–1129.

Dang VC, Williams JT (2005). Morphine-induced μ-opioid receptor desensitisation. *Mol Pharmacol* **68**: 1127–1132.

Erdtmann-Vourliotis M, Mayer P, Ammon S, Riechert U, Höllt V (2001). Distribution of G-protein-coupled receptor kinase (GRK) isoforms 2, 3, 5 and 6 mRNA in the rat brain. *Mol Brain Res* **95**: 129–137.

Ferguson SSG (2007). Phosphorylation-independent attenuation of GPCR signalling. *Trends Pharmacol Sci* **28**: 173–179.

Ghadessy RS, Willets JM, Kelly E (2003). G protein-coupled receptor kinase 6 (GRK6) selectively regulates endogenous secretin receptor responsiveness in NG108-15 cells. *Br J Pharmacol* **138**: 660–670.

Graham FL, Prevac L (1991). Manipulation of adenovirus vectors. In: Murrat EJ (ed.). *Methods in Molecular Biology Volume*, Vol. 7. Human Press Inc.: Clifton, NJ, pp. 109–128.

Haberstock-Debic H, Kim KA, Yu YJ, von Zastrow M (2005). Morphine promotes rapid, arrestin-dependent endocytosis of μ-opioid receptors in striatal neurons. *J Neurosci* **25**: 7847–7857.

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.

Hwang DY, Carlezon WA, Isacson O, Kim KS (2001). A high-efficiency synthetic promoter that drives transgene expression selectively in noradrenergic neurons. *Hum Gene Ther* **12**: 1731–1740.

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

Johnson JA, Gray MO, Chen C, Mochly-Rosen D (1996). A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. *J Biol Chem* **271**: 24962–24966.

Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV *et al*. (1998). μ-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Mol Pharmacol* **53**: 377–384.

Kelly E, Bailey CP, Henderson G (2007). Agonist-selective mechanisms of GPCR desensitisation. *Br J Pharmacol* **153** (Suppl. 1): S379–S388.

Krasel C, Dammeier S, Winstel R, Brockmann J, Mischack H, Lohse MJ (2001). Phosphorylation of GRK2 by protein kinase C abolishes its inhibition by calmodulin. *J Biol Chem* **276**: 1911–1915.

Mundell SJ, Benovic JL, Kelly E (1997). A dominant negative mutant of the G protein-coupled receptor kinase 2 selectively attenuates adenosine A2 receptor desensitisation. *Mol Pharmacol* **51**: 991–998.

Mundell SJ, Pula G, McIlhinney RAJ, Roberts PJ, Kelly E (2004). Desensitization and internalization of metabotropic glutamate receptor 1a following activation of heterologous Gq/11-coupled receptors. *Biochemistry* **43**: 7541–7551.

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.

North RA, Williams JT (1985). On the potassium conductance increased by opioids in rat locus coeruleus neurones. *J Physiol* **364**: 265–280.

Pierce KL, Premont RT, Lefkowitz RJ (2002). Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* **3**: 639–650.

Pronin AN, Benovic JL (1997). Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C. *J Biol Chem* **272**: 3806–3812.

Ron D, Luo J, Mochly-Rosen D (1995). C2 Region-derived peptides inhibit translocation and function of β protein kinase C *in vivo*. *J Biol Chem* **270**: 24180–24187.

Schechtman D, Mochly-Rosen D (2002). Isozyme-specific inhibitors and activators of protein kinase C. *Methods Enzymol* **345**: 470–489.

Schulz R, Wehmeyer A, Schulz K (2002). Opioid receptor types selectively cointernalize with G protein-coupled receptor kinases 2 and 3. *J Pharmacol Exp Ther* **300**: 376–338.

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.

Stebbins EG, Mochly-Rosen D (2001). Binding specificity for RACK1 resides in the V5 region of βII protein kinase C. *J Biol Chem* **276**: 29644–29650.

Sternini C, Spann M, Anton B, Keith DJ, Bunnett NW, von Zastrow M *et al*. (1996). Agonist-selective endocytosis of μ-opioid receptor by neurons *in vivo*. *Proc Natl Acad Sci USA* **93**: 9241–9246.

Teschemacher AG, Paton JFR, Kasparov S (2005). Imaging living central neurones using viral gene transfer. *Adv Drug Deliv Rev* **57**: 79–93.

Trafton JA, Abbadie C, Marek K, Basbaum AI (2000). Postsynaptic signaling via the μ-opioid receptor: responses of dorsal horn neurons to exogenous opioids and noxious stimulation. *J Neurosci* **20**: 8578–8584.

Urban JW, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H *et al*. (2007). Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320**: 1–13.

Van Bockstaele EJ, Commons KG (2001). Internalization of μ-opioid receptors produced by etorphine in the rat locus coeruleus. *Neuroscience* **108**: 467–477.

Way KJ, Chou E, King GL (2000). Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci* **21**: 181–187.

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* **102**: 245–253.

Zhang L, Yu Y, Mackin S, Weight FF, Uhl GR, Wang JB (1996). Differential μ-opiate receptor phosphorylation and desensitisation induced by agonists and phorbol esters. *J Biol Chem* **271**: 11449–11454.