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RESEARCH PAPER

Does PKC activation increase the homologous desensitization of μ opioid receptors?

Seksiri Arttamangkul, William Birdsong and John T Williams

Vollum Institute, Oregon Health and Science University, Portland, OR, USA

Correspondence
John T Williams, Vollum Institute, Oregon Health Sciences University, 3181 SW Sam Jackson Park Rd, Portland, OR 97239, USA. E-mail: williamj@ohsu.edu

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BACKGROUND AND PURPOSE
This study examined the role of agents known to activate PKC on morphine-induced desensitization of μ-opioid receptors (MOP receptors) in brain slices containing locus coeruleus neurons.

EXPERIMENTAL APPROACH
Intracellular recordings were obtained from rat locus coeruleus neurons. Two measurements were used to characterize desensitization, the decline in hyperpolarization induced by application of a saturating concentration of agonist (acute desensitization) and the decrease in hyperpolarization induced by a subsaturating concentration of [Met]^{5}enkephalin (ME) following washout of the saturating concentration (sustained desensitization). Internalization of MOP receptors was studied in brain slices prepared from transgenic mice expressing Flag-MOP receptors. The subcellular distribution of activated PKC was examined using a novel fluorescent sensor of PKC in HEK293 cells.

KEY RESULTS
The phorbol esters (PMA and PDBu) and muscarine increased acute desensitization induced by a saturating concentration of morphine and ME. These effects were not sensitive to staurosporine. Staurosporine did not block the decline in hyperpolarization induced by muscarine. PDBu and muscarine did not affect sustained desensitization induced by ME nor did phorbol esters or muscarine change the trafficking of MOP receptors induced by morphine or ME. The distribution of activated PKC measured in HEK293 cells differed depending on which phorbol ester was applied.

CONCLUSIONS AND IMPLICATIONS
This study demonstrates a distinct difference in two measurements that are often used to evaluate desensitization. The measure of decline correlated well with the reduction in peak amplitudes caused by PKC activators implicating the modification of other factors rather than MOP receptors.

LINKED ARTICLES
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Abbreviations
LC, locus coeruleus; M1-A594, anti-Flag antibody M1-Alexa594; ME, [Met]^{5}enkephalin; MOP receptor, μ opioid receptor; PDBu, phorbol-12,13-dibutyrate; PIP2, phosphoinositide 4,5-bisphosphate; PMA, phorbol-12-myristate-13-acetate

Introduction

Morphine is a potent analgesic used for acute and chronic pain. The major problem with the use of morphine is the rapid development of tolerance resulting in the requirement of escalating doses to maintain the same therapeutic outcome. There are multiple mechanisms that underlie the development of tolerance but desensitization of the μ-opioid receptor (MOP receptor; see Alexander *et al.*, 2013a) is thought to be an important initial step. Understanding morphine-induced MOP receptor desensitization is therefore important for the development of more effective therapeutic treatment protocols.

Morphine differs from endogenous peptides in that it causes little desensitization and receptor internalization (reviewed Williams *et al.*, 2013). Studies *in vivo* and *in vitro* have demonstrated that tolerance after chronic morphine treatment is attenuated or reversed when PKC inhibitors are co-injected during morphine treatments or following the development of tolerance (Bailey *et al.*, 2006). The results indicate that an increase in PKC activity enhances morphine-induced tolerance (Levitt and Williams, 2012). Different assays have been used to study the role of PKC in MOP receptor desensitization (Mestek *et al.*, 1995; Fiorillo and Williams, 1996; Bailey *et al.*, 2004; 2009; Johnson *et al.*, 2006). Several studies indicate that acute desensitization caused by morphine is dependent on the activation of PKC but desensitization caused by [D-Ala(2), N-methyl-phe(4), glycol(5)] enkephalin (DAMGO), a potent synthetic-peptide agonist results from a G-protein receptor kinase (GRK) pathway (Johnson *et al.*, 2006; Bailey *et al.*, 2009).

Phosphorylation of MOP receptors by PKC at serine 363 or threonine 370 has been demonstrated (Chen *et al.*, 2012; Illing *et al.*, 2013). It is not known if phosphorylation at these sites increases acute desensitization or internalization of MOP receptors. Treatment of locus coeruleus (LC) neurons with staurosporine did not alter measures of acute desensitization by [Met]^{5}enkephalin (ME), but increased the rate of recovery from desensitization (Arttamangkul *et al.*, 2012). Likewise, staurosporine did not reverse DAMGO-induced desensitization in HEK cells (Johnson *et al.*, 2006). DAMGO-induced desensitization in LC cells was unaffected by the activation of PKC with phorbol esters; however, desensitization induced by ME and morphine was increased (Bailey *et al.*, 2004, 2009).

The present study examined the difference between ME- and morphine-induced MOP receptor desensitization in the presence of PKC activators. Measures included the acute decline in MOP receptor-induced hyperpolarization (acute desensitization), the hyperpolarization induced by an EC_{50} concentration of ME immediately following acute desensitization (sustained desensitization), MOP receptor internalization and PKC translocation. The results suggest that agents that activate PKC can reduce the acute hyperpolarization of LC neurons by mechanisms that are independent of the activity of PKC.

Methods

All animal experiments were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee of the Oregon Health and Science University (Portland, OR, USA). Animals were housed four (rats) or six (mice) to a cage in the animal care facility on a 12 h light/dark cycle. Food and water were available *ad libitum*.

Electrophysiology

Male Sprague Dawley rats (150–250 g, Charles River Laboratories, Wilmington, MA, USA) were used for all electrophysiological experiments. Brain slices were prepared as described previously (Williams *et al.*, 1984). In brief, rats were anaesthetized with isoflurane and killed by thoracic percussion. The brains were removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 21.4 $NaHCO_3$, 11 glucose and 0.03 (+)MK-801. Horizontal slices were prepared (270–300 μm) using a vibratome (Leica, Nussloch, Germany) and incubated in warm (34°C) oxygenated ACSF containing (+)MK-801 (10 μM) for at least 30 min. Glass electrodes (50–60 MΩ) filled with KCl (2 M) were used for intracellular recording of membrane potential. Less than 20 pA of input current was applied to the cell to prevent cell firing. Experiments were performed at 35°C. Data were collected using Power Lab (Chart version 5.5.6, AD Instrument, Colorado Springs, CO, USA) and acquired at 200 Hz. Drugs were applied by perfusion at the rate of 1.5 mL·min^{-1}.

Flag-MOP receptor internalization

Transgenic mice in C57BL/6J background expressing Flag-tagged MOP receptors (Flag-MOP receptor) via tyrosine hydroxylase promoter were used for all internalization assays (Arttamangkul *et al.*, 2008). Data were collected from male and female mice. Mice were anaesthetized with isoflurane and killed by decapitation. Brain slices (200 μm) were prepared using a vibratome as described above. Slices were incubated in a solution of M1 antibody conjugated with Alexa 594 (M1-A594, 10 μg·mL^{-1}, 45–60 min). The labelling was visualized with an upright microscope (Olympus, Center Valley, PA, USA) equipped with a custom-built two-photon apparatus and a 60× water immersion lens (Olympus LUMFI, NA1.1, Center Valley, PA, USA). The dye was excited at 810 nm. Data were acquired and collected using Scan Image Software (Pologruto *et al.*, 2003). A z-series was collected at 1 μm intervals for 15 sections. Drugs were applied by perfusion at a rate of 1 mL·min^{-1}. All experiments were done at 35°C.

Quantification of receptor internalization

Analyses were carried out off-line with Image J (NIH) software. Details of the analysis have been previously published (Arttamangkul *et al.*, 2008). Briefly, the fluorescence in stacks of 15 images was summed. Five random regions of interest (ROIs) away from neuronal staining were selected and averaged for background fluorescence. The average background fluorescence was then subtracted from the total fluorescent intensity of the whole frame. The fluorescent intensity obtained from slices after M1-A594 incubation was considered as total fluorescent receptors (C) on the plasma membrane. Following treatment with PKC activators (10 min) and PKC activators plus opioids (15 min), the slice was lastly treated with a calcium-free ACSF containing EGTA (0.5 mM,

10 min) to remove extracellular labelling of M1-A594. The fluorescence that remained within the cells was considered to be internalized receptors (I). The % of receptors internalized was calculated by (I/C) × 100.

PKC translocation assays

The fluorescent PKC sensor (upward-DAG) DNA was obtained from Montana Molecular (http://www.montanamolecular.com). Its design and synthesis were described in detail elsewhere (Tewson *et al.*, 2012; 2013). HEK293 cells stably expressing Flag-MOP receptors (gift from Dr. Mark von Zastrow, University of California San Francisco, CA, USA) were cultured in high glucose DMEM containing 10% FBS, 0.5% HEPES, 0.8% G418. Cells were seeded on polylysine coated cover glasses (Nu-Vitro NeuVitro Corp., El Monte, CA, USA) to 70% confluence, transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and used the next day. M1-A594 (3 μg·mL^{-1}) was added to each cover glass and incubated for 15 min at 37°C in 5% CO_2 to label the Flag-MOP receptors on the plasma membrane. The cover glass was transferred to recording chamber. Drugs were diluted in ACSF and applied by perfusion at a flow rate of 2.5 mL·min^{-1}.

Imaging was carried out on an Olympus BX51WI upright microscope and Olympus LUMPlanFL N 60×/1.00 W objective. A Nipkow spinning disk confocal scanner (Solamere Technology, Salt Lake City, UT, USA) and ICCD camera (XR/MEGA 10, Stanford Photonics, Palo Alto, CA, USA) were used to acquire rapid confocal images in a single plane using Piper data acquisition software (Piper Control 2.4.51, Stanford Photonics). Upward-DAG was imaged using a 488 nm solid-state laser (CrystaLaser, Reno, NV, USA) while Alexa 594 was excited using a 561 nm solid-state laser (Cobolt Jive 25, Cobalt, Stockholm, Sweden). Rapid switching between 488 nm and 561 nm was achieved via Acusto-Optic Tunabe Filter (AOTF) (NEOS, Neos Technologies, Melbourne, FL, USA). A quadruple band-pass dichroic mirror (Di01 T403/488/568/647, Semrock, Rochester, NY, USA) and dual band-pass emission filter (FF01-523/610, Semrock) were used.

Confocal stacks from of HEK293 cells expressing both the upward-DAG (488 nm excitation) and Flag-MOP receptor (labelled with M1-A594 anti-Flag antibody, 561 nm excitation) were obtained before (control) and after 10 min in the continued presence of PKC activators (PMA 1 μM, PDBu 200 nM) or morphine (15 μM).

Image quantification was carried out using FIJI software (Schindelin *et al.*, 2012) J. and the 'Time Series Analyzer' plugin (Balaji J., Department of Neurobiology, UCLA, Los Angeles, CA, USA). Analysis was performed using Excel (Microsoft, Redmond, WA, USA) and Origin Lab software (Origin Lab Corp., Northampton, MA, USA). A single image plane was selected in which both the plasma membrane and cytosol of the cell were clearly visible and as similar as possible between control and post-drug conditions. Using the staining of M1-A594 to visualize the plasma membrane, a region of interest along the plasma membrane containing as little cytosol as possible was selected as a 'membrane' region. A second ROI containing only cytosol and avoiding the nucleus was selected as a 'cytosol' region. Five background ROIs surrounding the cell of interest were subsequently selected and the average fluorescence intensity/pixel (488 nm excitation) was measured for each region in the image frame. The average of the five background ROI intensities was subtracted from the membrane and cytosol ROIs.

Statistical analysis

Data are presented as mean and SEM. The difference was considered to be significant when the P value was less than 0.05. For PKC translocation, paired data from control and post-drug treatments were compared using paired t-tests.

Reagents and drugs

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). Morphine was obtained from National Institute on Drug Abuse, NIH (Rockville, MD, USA). Alexa Fluor® 594 succinimidyl ester was purchased from Invitrogen (Carlsbad, CA, USA). M1-Alexa 594 was conjugated and purified by Bio-Spin® 6 Tris Columns (Hercules, CA, USA). Dermorphin Alexa594 was synthesized and purified as previously described (Birdsong *et al.*, 2013).

Results

Desensitization induced by morphine

The peak amplitude of the hyperpolarization induced by morphine (15 μM) was 29.3 ± 0.9 mV. During a 10 min application, the hyperpolarization decreased by 5.4 ± 1.4% (n = 7, Figure 1A,B). Application of PMA (1 μM) for 10 min prior to

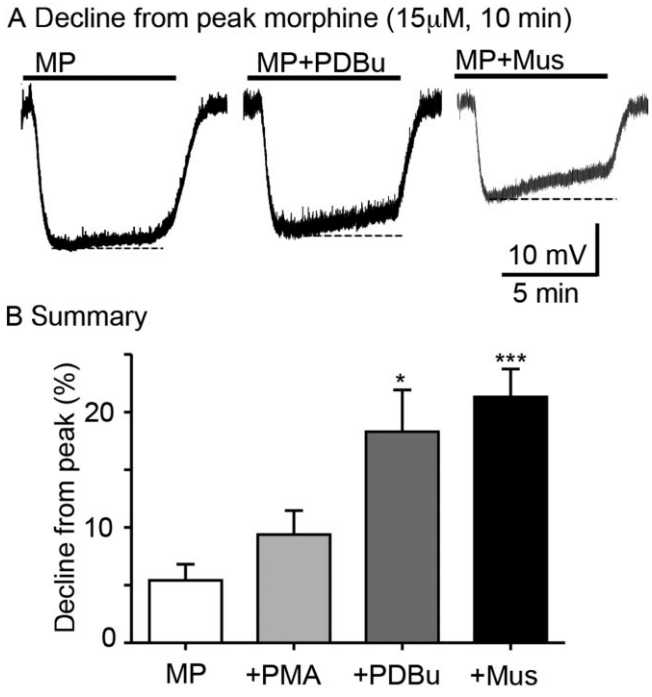

Figure 1

PKC activators increased the acute decline from peak hyperpolarization in the continuous presence of morphine (MP). (A) Intracellular recording of membrane potential during 10 min morphine application in the presence of PKC activator, control (left), PDBu (0.2 μM, middle) and muscarine (10 μM, right). (B) The decline in hyperpolarization normalized to the peak hyperpolarization (*P < 0.05; ***P < 0.001).

morphine reduced the peak amplitude of the hyperpolarization to 24.8 ± 1.6 mV and during the 10 min application, the hyperpolarization decreased by 9.4 ± 2.1% (n = 6, Figure 1B). In slices treated with PDBu (200 nM, 10 min), the peak hyperpolarization induced by morphine was 22.6 ± 1.7 mV that gradually decreased by 18.3 ± 3.6% after 10 min (n = 6, Figure 1A,B). The decline in the morphine-induced hyperpolarization in the presence of PMA was not significantly increased relative to morphine alone, whereas the decline response in the presence of PDBu was significantly larger than control (P value < 0.05, one-way ANOVA, Bonferroni's test, Figure 1B). The difference in the results obtained with PMA and PDBu were unexpected because the concentrations used for each was saturating when measured in an *in vitro* binding assay (Driedger and Blumberg, 1980). Muscarine was used to activate muscarinic acetycholine M_3-like receptors leading to the activation of PKC. Morphine, in the presence of muscarine (10 μM), induced a peak hyperpolarization of 13.6 ± 1.3 mV, which then decreased by 21.3 ± 2.4% during a 10 min application (n = 11, P value < 0.001, one-way ANOVA, Bonferroni's test, Figure 1A,B). Thus, as has been reported previously using whole-cell recordings, two treatments that are known to activate PKC increased the acute desensitization in response to morphine (Bailey *et al.*, 2004).

Reduced opioid-dependent hyperpolarization by PKC activators

One key observation shown above was that PDBu and muscarine reduced the hyperpolarization caused by morphine (15 μM). The peak hyperpolarization under control conditions was 29.3 ± 0.9 mV but was 22.6 ± 1.7 and 13.6 ± 1.3 mV following PDBu and muscarine treatment respectively. However, the absolute decline in the hyperpolarization induced by morphine was very similar in all conditions (control 1.6 ± 0.4; PMA 2.4 ± 0.6, PDBu 4.4 ± 1.0 and muscarine 2.9 ± 0.5 mV). The decrease in the peak amplitude therefore yields a large increase in acute desensitization when normalized to the peak. A strong inverse correlation between the % decline and the peak amplitude of morphine- induced hyperpolarization is shown in Figure 2D.

To confirm that the reduced hyperpolarization was the consequence of PKC activators affecting MOP receptor signalling, the experiment was done using a subsaturating concentration of ME (300 nM) before and after the presumed activation of PKC (Figure 2A,B,E). All PKC activators used in this study decreased the ME-induced hyperpolarization [Figure 2E, ME in PDBu (1 μM) 54.0 ± 7.3% of control, n = 4; ME in muscarine 47.3 ± 3.1% of control, n = 10, P < 0.01, Student's paired, two-tailed t-test]. PDBu (0.2 μM) and PMA (1 μM) also reduced the ME-induced hyperpolarization (PDBu 82.7 ± 2.3% of control, n = 8; PMA 90.3 ± 4.4% of control, n = 9, P value < 0.05, paired, two-tailed t-test). Interestingly, the hyperpolarization induced by noradrenaline acting on α_{2A}-adrenoceptors was not changed after treatment with any of the PKC activators. Both PDBu and PMA alone caused a small hyperpolarization whereas muscarine depolarized the LC neurons. In each case, the membrane potential was maintained at about –60 mV by applying current through the recording electrode. The mechanism that underlies the hyperpolarization induced by PDBu is unknown. One possibility was that PDBu activated G-protein-coupled inwardly-rectifying potassium (GIRK) channels (see Alexander *et al.*, 2013b) that partially occluded the subsequent morphine-induced hyperpolarization. This was probably not the case because the PDBu-mediated hyperpolarization did not occlude the noradrenaline-mediated current (Figure 2E), which activates the same GIRK channels. In addition, the current–voltage relationship for the PDBu-mediated outward current measured under voltage clamp was small, non-rectifying and did not reverse at the potassium equilibrium potential (data not shown).

Inhibiting PKC activity with staurosporine

Staurosporine is a non-specific kinase inhibitor that potently inhibits all PKC isozymes (Rüegg and Burgess, 1989). Staurosporine (1 μM) did not reverse the reduction in ME-induced hyperpolarization induced by muscarine (Figure 2C,E, muscarine 21.3 ± 2.4%, n = 11 vs. muscarine + staurosporine 27.7 ± 3.6%, n = 6, Figure 2F). Application of PDBu caused a smaller decline in the peak hyperpolarization induced by morphine (Figure 2F) and the addition of staurosporine did not change this decline in the hyperpolarization (PDBu 18.3 ± 3.6% decrease, n = 6 vs. PDBu + staurosporine 13.3 ± 2.2% decrease, n = 6).

ME-induced desensitization

ME (30 μM) caused a peak hyperpolarization of 35.1 ± 1.0 mV that declined by 26.7 ± 4.0% (n = 8) over 10 min (Figure 3A). Neither the peak hyperpolarization (34.7 ± 1.1 mV) nor the decline from the peak (27.7 ± 2.5%, n = 5, Figure 3C) was changed after pre-incubation of slices with PMA (1 μM, n = 9). In the presence of PDBu (1 μM), the peak hyperpolarization induced by ME was smaller (28.1 ± 1.4 mV, P < 0.01, Student's two-tailed, unpaired t-test, n = 5) and the decline during a 10 min application of ME (30 μM) increased to 39.1 ± 3.3% (n = 5). In the presence of muscarine (10 μM), there was no difference in the peak hyperpolarization compared with control (34.4 ± 2.6 mV), but the acute decline increased significantly from control cells (54.5 ± 7.7% of the peak, n = 5, P < 0.01, one-way ANOVA, Bonferroni's test, Figure 3A–C).

Given the differences in the effect of PMA, PDBu and muscarine on the acute desensitization induced by ME (30 μM), a second more sensitive measure of desensitization was tested. Sustained desensitization was evaluated by the decrease in the hyperpolarization induced by a subsaturating concentration of ME immediately following the washout of the saturating concentration of ME (Connor *et al.*, 2004). The decrease in sensitivity to ME (300 nM) outlasted the washout of ME (30 μM) and recovered over a period of 30–50 min. It is unclear whether the acute and sustained desensitization are separate phenomena or two measures of the same basic observation. The hyperpolarization induced by ME (300 nM) was measured before and at several time points after the application of ME (30 μM, 10 min, Figure 3A,B). The sustained desensitization was taken as the decrease in the hyperpolarization that occurred 5 min after the washout of the high concentration of ME. Recovery from sustained desensitization was monitored for up to 45 min (Figure 3A,B,D). Sustained desensitization measured using this assay was examined in control conditions and in the presence of PMA (1 μM) or muscarine (10 μM). In control experiments, the hyperpolarization induced by ME (300 nM) decreased from

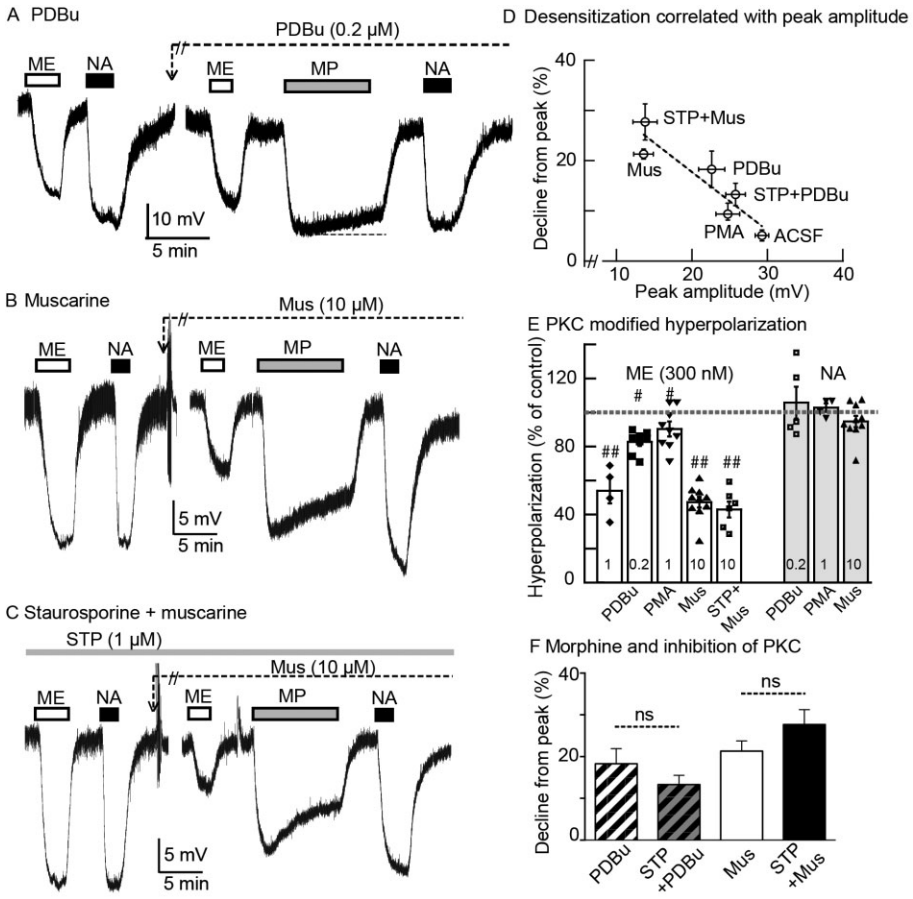

Figure 2

Representative recording in which [Met^5]-enkephalin (ME; 0.3 μM) and noradrenaline (NA; 100 μM plus 1 μM cocaine to block the NA transporter) were tested before and after applications of (A) PDBu (0.2 μM), (B) muscarine (10 μM), or (C) staurosporine (1 μM, pre-incubated for 60 min) plus muscarine (10 μM). The hatched bars indicate 10 min of cropped recording. (D) The decline in response showed an inverse linear correlation with peak amplitudes. The linear regression analysis gave a best-fit value with $R^2 = 0.8473$, slope = –1.163 ± 0.2468, y-intercept = 41.03 ± 5.54 and x-intercept = 35.28. (E) Summary showing the reduction in hyperpolarization induced by ME (300 nM) after the application of the PKC activators. Analyses using Student's paired, two-tailed *t*-test showed that the hyperpolarizations in the presence of PDBu (1 μM), PMA (1 μM), PDBu (0.2 μM) and muscarine (10 μM) were significantly different from their untreated controls (##$P < 0.01$; #$P < 0.05$). (F) Summary of the acute decline in hyperpolarization after the treatment of slices with staurosporine (1 μM, over 60 min) and then co-application with either PDBu (0.2 μM) or muscarine (10 μM). ns = not significantly different.

24.0 ± 2.1 mV before desensitization to 3.7 ± 0.8 mV 5 min after desensitization (peak desensitization = 14.6 ± 2.3% of control, n = 9). The hyperpolarization induced by ME (300 nM) recovered to 82.2 ± 7.1% (n = 4) of control after 45 min. The rate and extent of recovery in these control experiments are the same as reported previously (Harris and Williams, 1991; Osborne and Williams, 1995). In slices pre-treated with PMA (1 μM), the hyperpolarization induced by ME (300 nM) was 23.0 ± 1.3 mV and decreased to 4.0 ± 0.8 mV (peak desensitization = 18.5 ± 4.1%, n = 9). The recovery from desensitization measured at 15, 30 and 45 min was the same as in control (67.6 ± 5.5% at 45 min, n = 4, $P >$ 0.05, two-way ANOVA Bonferroni's test, Figure 3D). In the presence of muscarine (10 μM), the ME-induced desensitization remained unchanged. The hyperpolarization decreased from 13.9 ± 2.7 to 3.4 ± 0.9 mV (peak desensitization = 25.1 ± 4.3%, n = 5) and the recovery from desensitization measured at 15, 30 and 45 of the washout was the same as control (69.2 ± 8.3% at 45 min, n = 5, P value > 0.05, two-way ANOVA Bonferroni's test, Figure 3D). The results suggest that ME-induced sustained desensitization was not enhanced by agents known to activate PKC and that acute and sustained desensitization are two different phenomena.

MOP receptor trafficking

The augmentation of acute desensitization induced by PDBu, PMA and muscarine may result from an increase in MOP receptor endocytosis. This possibility was tested using a transgenic mouse that expressed Flag-MOP receptors in LC neurons. Flag-MOP receptors on LC neurons were visualized with monoclonal M1-A594 labelled antibodies in control conditions and after treatment with morphine alone or in the presence of PMA, PDBu or muscarine (Figure 4A). Morphine (15 μM, 15 min) produced a very small increase in the

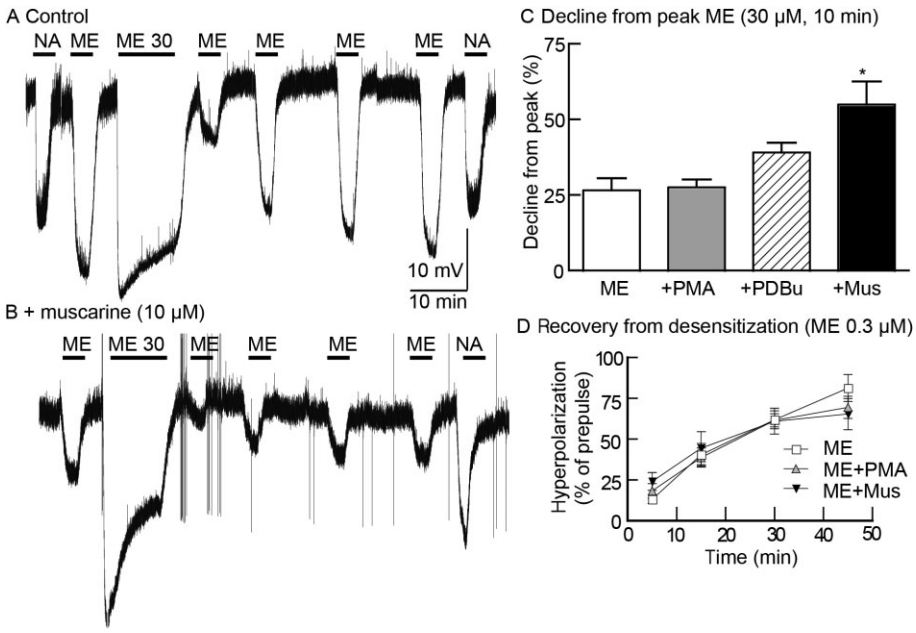

Figure 3

The effect of PKC activators on ME-induced MOP receptor desensitization. Representative recordings measuring acute desensitization, sustained desensitization, and recovery under (A) control conditions and (B) in the presence of muscarine (10 μM, pre-incubation for 10 min and in all solutions). ME (300 nM) was applied before and 5, 15, 30 and 45 min following desensitization (ME 30 μM, 10 min). (C) Summary showing the acute decline from peak hyperpolarization in the presence of PKC activators: PMA (1 μM), PDBu (1 μM) and muscarine (10 μM), *$P < 0.01$. (D) Sustained desensitization/recovery measured as the relative hyperpolarization activated by ME (300 nM) at 5, 15, 30 and 45 min post-desensitization (ME 30 μM, 10 min) normalized to the initial ME-induced hyperpolarization (300 nM, prepulse).

internal fluorescence similar to previous studies (Arttamangkul *et al.*, 2008). Application of PMA, PDBu or muscarine alone did not alter the distribution of Flag-MOP receptors. Likewise, the distribution of MOP receptors on the plasma membrane before and after treatment with morphine (15 μM, 15 min) in the presence of these activators was not different from control experiments (Figure 4B). Given that acute desensitization of ME was substantially enhanced via activation of M_3-like acetylcholine receptors, ME-induced MOP receptor internalization was examined in the absence and presence of muscarine (10 μM). ME caused a significant internalization of MOP receptors and there was no difference in the internalization in slices treated with muscarine and untreated controls. Thus, activators of PKC had no significant role in MOP receptor endocytosis in LC neurons.

Subcellular distribution of PKC after PMA and PDBu

PMA and PDBu activate PKC by mimicking the actions of DAG, a lipid second messenger generated from the hydrolysis of phosphoinositide 4,5-bisphosphate (PIP2) via Gq-coupled receptor activation (Newton, 2001). Both phorbol esters bind to PKC with high affinity in nM range (Driedger and Blumberg, 1980) and are very potent and stable activators of PKC (Castagna *et al.*, 1982). PMA is more lipophilic and capable of intercalating into the lipid membrane where it activates PKC (Mosior and Newton, 1995). In contrast PDBu is more hydrophilic and thus moves more efficiently into the cytosol. The difference in results obtained with PMA and PDBU, although insensitive to staurosporine, may be dependent on an action in different subcellular compartments. To test this hypothesis, a fluorescent PKC sensor (upward-DAG) generated by combining a PKCδ fragment with circular permuted enhanced green fluorescent protein (GFP) (Tewson *et al.*, 2012; 2013) was expressed in Flag-MOP receptor expressing HEK293 cells. Phorbol ester binding was inferred from an increase in fluorescence of the sensor. Flag-MOP receptors were labelled with M1-A594 and used as a marker for the plasma membrane. Spinning disk confocal images demonstrated that both PMA and PDBu increased the total fluorescence of the upward-DAG sensor. PMA (1 μM) strongly increased the fluorescence only at the plasma membrane (Figure 5A, bottom panel, Figure 5B, membrane: 293 ± 35% of control, cytosol: 99 ± 16%, membrane: cytosol ratio: 316 ± 42%, $n = 7$), whereas PDBu (200 nM) resulted in a significant but much smaller fluorescence increase at both the plasma membrane and cytosol (Figure 5A, middle panel, membrane: 161 ± 14% of control, cytosol: 115 ± 6%, ratio 140 ± 11%, $n = 9$). Morphine (15 μM) had no effect on either cytosolic or membrane fluorescence intensity (Figure 5A top panel, membrane: 110 ± 10% of control, cytosol: 102 ± 4%, ratio: 109 ± 9%, $n = 13$). Consistent with previous work, the results indirectly imply that PMA strongly recruits and probably activates PKC at the plasma membrane while PDBu may have both cytosolic and membrane-localized actions. Thus, PMA and PDBU can act in different cellular compartments, which may account for the differential actions observed on the inhibition of opioid signalling in LC neurons.

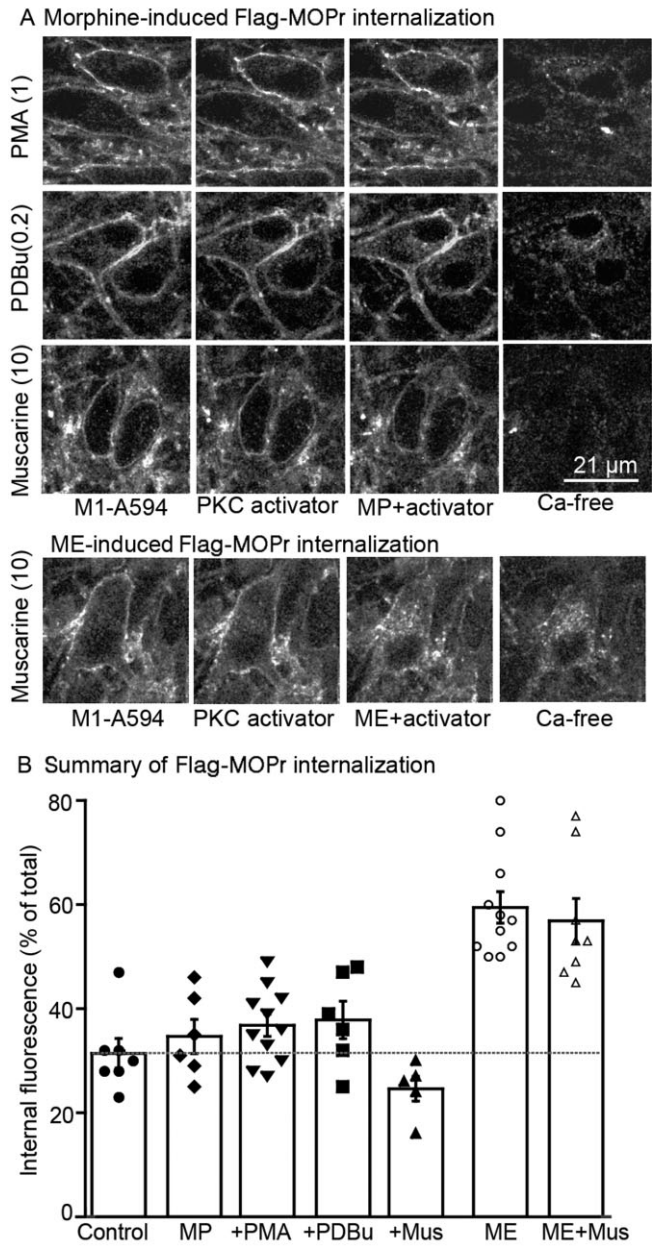

Figure 4

Opioid-induced Flag-MOP receptor (MOPr) internalization after PKC activation (A) Flag-MOP receptors in LC neurons labelled with M1-A594 (first column) in live brain slices, perfused with PKC activators 10 min (second column), then morphine (15 µM) or ME (30 µM) 10 min (third column) in the presence of PKC activators and calcium-free ACSF containing 0.5 mM EGTA 10 min (last column). (B) Summary of % receptor internalization.

In agreement with previous studies (Illing *et al.*, 2013, and personal communication with Dr. Graeme Henderson), we observed that PMA alone induced MOP receptor translocation from plasma membrane into cytoplasm of HEK293 cells (Figure 5A, bottom, right). These results could be cell-type specific because PMA did not induce MOP receptor internalization in LC neurons (Figure 4A, top panel).

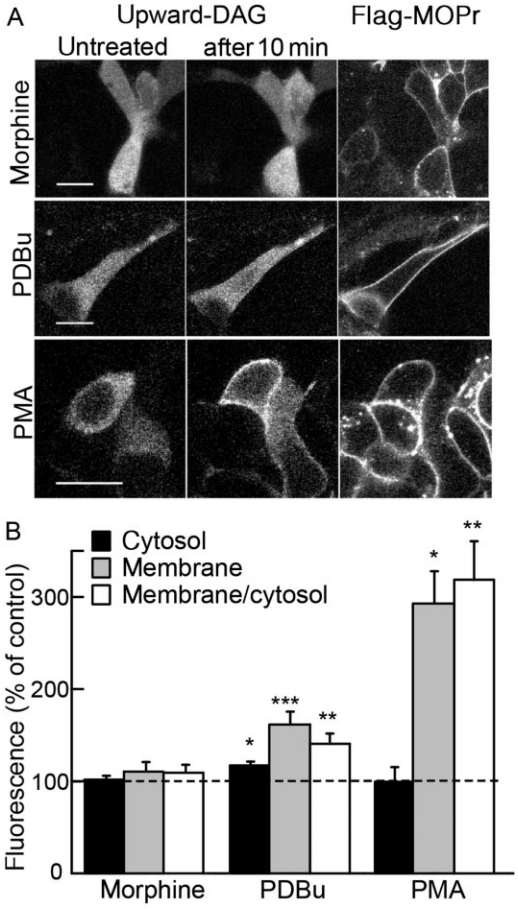

Figure 5

PMA-induced translocation of PKC to the plasma membrane. (A) A fluorescent PKC sensor (upward-DAG) was imaged in Flag-MOP receptor (MOPr) expressing HEK293 cells. Confocal images were taken before (left column) or following 10 min exposure (middle column) to either morphine (15 µM, top), PDBu (0.2 µM, middle) or PMA (1 µM, bottom). Flag-MOP receptors were labelled with M1-Alexa 594 (right column) to identify the plasma membrane and to confirm that Flag-MOP receptors were expressed in individual cells. (B) Summary of upward-DAG fluorescence intensities at the membrane and cytosol and the ratio of membrane/cytosol expressed as % of upward-DAG fluorescence measured before drug treatment. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, paired *t*-test. Scale bar = 20 µm.

Discussion

This study examined the role of reagents known to activate PKC on MOP receptor desensitization and internalization in LC neurons. The PKC activators increased the decline in the acute hyperpolarization induced by opioids (acute desensitization). However, the results from this study suggest that this measure of desensitization may differ from sustained desensitization because sustained desensitization was unaffected by muscarine and PMA. In addition, neither muscarine nor phorbol ester treatment affected the agonist-induced endocytosis of MOP receptors in LC neurons in live brain slices.

Measurement of MOP receptor desensitization

Two measures of desensitization were used in this study, the decline in the peak hyperpolarization induced by a saturating concentration of agonist (acute desensitization) and the reduction of the hyperpolarization induced by ME (300 nM) following the washout of a saturating concentration of ME (sustained desensitization). The decrease in the hyperpolarization induced by ME (300 nM) is a sensitive assay for desensitization especially in presence of receptor reserve (Christie *et al.*, 1987). As ME (300 nM) is on the steep part of the concentration- response curve, a small decrease in the sensitivity induced by desensitization results in a large decrease in the amplitude of the hyperpolarization (Connor *et al.*, 2004). This assay is, however, limited to examination of the desensitization induced by agonists such as ME that can be rapidly removed from the slice. The washout of saturating concentrations of metabolically stable agonists, such as morphine, is too slow to obtain an accurate measure of the extent of desensitization following washout.

The decline of the hyperpolarization induced by a saturating concentration of agonist can result in an underestimate in the amount of desensitization because the receptor reserve must be depleted before any decrease in the peak (Connor *et al.*, 2004). This is less of a problem with morphine because it is a partial agonist having no receptor reserve (Christie *et al.*, 1987; Osborne and Williams, 1995). Another limitation of the intracellular recording method is that changes in membrane hyperpolarization can be blunted as the membrane potential approaches the potassium equilibrium potential. However, the advantage of using intracellular recordings is that intracellular components are not 'washed out' during the experiment because of the high resistance of the recording electrode. Thus, the loss of soluble components that regulate desensitization is limited. In spite of the methodological differences between this study and previous studies (intracellular recording vs. whole-cell voltage clamp), a similar enhancement of morphine-induced decline in membrane hyperpolarization was observed following treatment with PDBu and muscarine (Bailey *et al.*, 2004 and this study). However, there were some differences. In the present study, PMA did not increase the acute decline in hyperpolarization induced by morphine. One other difference between the current and previous studies is the morphine concentration (15 μM and 30 μM, respectively). Although both concentrations are saturating, the high concentration may cause a direct inhibition of potassium conductance as shown for methadone (Rodriguez-Martin *et al.*, 2008; Matsui and Williams, 2010). The reduced potassium conductance would decrease the maximal hyperpolarization induced by morphine and overestimate the acute decline when normalized to the peak.

PKC activators and desensitization

Do phorbol esters increase acute desensitization induced by morphine by a PKC-dependent process? Staurosporine did not significantly inhibit the PDBu-dependent increase in acute desensitization thus raising the question whether PKC-dependent activity alone can explain the increase in desensitization. Phorbol esters can bind to and activate other C1-domain-containing proteins including chimaerins (a family of Rac GTPase activating proteins), PKD, RASGRPs (exchange factors for Ras/Rap1), Munc 13 isoforms (scaffolding proteins involved in exocytosis), and DAG kinase γ (Brose and Rosenmund, 2002; Kazanietz, 2002). Both PMA and PDBu effectively increased synaptic activity and hyperpolarized LC neurons (Bailey *et al.*, 2004 and this study) suggesting that the concentrations used were effective.

PMA has a myristate and acetate and is more lipophilic and capable of intercalating into lipid membranes (Mosior and Newton, 1995). PDBu has two butyrate side chains and is more water soluble. Application of PDBu induced a patchy distribution of PKCδ-GFP in cytoplasm of CHO-K1 cells (Wang *et al.*, 2000). In the present study using HEK293 cells, PDBu was not as efficacious in activating a fluorescent PKC sensor at the plasma membrane. PMA at the same concentration was not effective in causing an increase in morphine- or ME-induced acute desensitization in LC neurons but produced a significant increase in fluorescent of the PKC sensor at the plasma membrane of HEK293 cells. The increase in plasma membrane fluorescence was similar to the previous studies using PKCδ-GFP, PKCγ-GFP and PKCε-GFP (Shirai *et al.*, 1998; Wang *et al.*, 1999). The more pronounced effect of PDBu on opioid-induced decline therefore implies an action on a cytosolic PKC-independent effector.

PKC activation has been hypothesized to account for morphine- but not DAMGO-induced desensitization of MOP receptors (Kelly *et al.*, 2008). The present study showed that PDBu did not cause an increase in acute desensitization induced by ME. Additionally, PMA (1 μM) did not affect either acute or sustained desensitization induced by ME. Hence, ME-induced desensitization was not sensitive to phorbol esters.

Muscarine is an agonist of Gq-coupled M_3-like receptors. Upon activation, DAG is generated from the hydrolysis of PIP2 by PLC. Structural studies demonstrate that PIP2 is a required cofactor for activating GIRK channels (Whorton and MacKinnon, 2011; 2013). It is unclear whether PIP2 depletion mediated the reduction in GIRK activity and was the mechanism that accounts for the decrease in hyperpolarization induced by opioids. Given that noradrenaline-induced activation of α_{2A}-adrenoceptors that couple to the same GIRK channel was not changed by oxotremorine-M or muscarine (Bailey *et al.*, 2004; the present study Figure 2B,E), PIP2 depletion may not account for the marked decline in the opioid-induced hyperpolarization. However, receptor-specific depletion of PIP2 has been shown to reduce the activity of GIRK (Cho *et al.*, 2005b). The low lateral mobility of PIP2 and the location of the receptor-GIRK complex may account for this specific effect (Cho *et al.*, 2005a; Cui *et al.*, 2010).

PMA, PDBu and muscarine each decreased the peak opioid-induced hyperpolarization by unknown mechanisms. It is probably not a result of PKC activation because the hyperpolarization was not increased by staurosporine (see Figure 2B,C,E). The decrease in the hyperpolarization induced by the PKC activators correlated with an increase in the normalized acute desensitization. Similar results were obtained when the amplitude of the opioid-induced current was decreased with an irreversible antagonist, β-chlornaltrexamine. That too increased the apparent degree of acute desensitization (Virk *et al.*, 2009). This implies that under conditions where coupling of the receptor to effectors

(i.e. GIRK) is compromised, an increase in acute desensitization will be observed.

PKC and MOP receptor trafficking

Biochemical studies report an increase in MOP receptor phosphorylation that is time- and concentration-dependent after PMA treatment (Zhang *et al*., 1996). Using site-directed mutations and mass spectrometry, S363 was identified as a PKC phosphorylation site on MOP receptors (Feng *et al*., 2011). It has also been reported that S363 was basally phosphorylated and that T370 was phosphorylated by PMA or a peptide agonist DAMGO (Doll *et al*., 2011; Lau *et al*., 2011; Illing *et al*., 2013). Given that HEK293 cells have been reported to have high PKC activity (Johnson *et al*., 2006), it is possible that this tonic activity phosphorylates S363 but not T370. The phosphorylation state of S363 LC neurons is not known. Based on the present results, however, any PKC-dependent phosphorylation of MOP receptors is not sufficient to facilitate morphine-dependent endocytosis or sustained desensitization in LC neurons.

Conclusions

The present study examined the regulation of MOP receptors by agents known to activate PKC. The membrane hyperpolarization and MOP receptor internalization induced by morphine and ME was measured in LC slices and the activation of a novel fluorescent PKC sensor induced by PMA and PDBu was examined in HEK293 cells. The results suggest that PKC activators increase the decline in hyperpolarization induced by morphine and ME in a manner that may involve unknown mechanisms rather than a direct PKC-dependent action on MOP receptors.

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Author contributions

S. A. and W.B. performed the experiments and wrote the manuscript. J.T.W. contributed to the writing of the manuscript.

Conflict of interest

There are no competing interests.

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