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Sustained morphine treatment augments prostaglandin E2-evoked Calcitonin Gene-Related Peptide release from primary sensory neurons in a PKA- dependent manner

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Abstract

Tissue damage leads to pain sensitization due to peripheral and central release of excitatory mediators such as prostaglandin E₂ (PGE₂). PGE₂ sensitizes spinal pain neurotransmitter such as calcitonin gene-related peptide (CGRP) release via activation of cyclic AMP (cAMP)/Protein kinase A (PKA) -dependent signaling mechanisms. Our previous data demonstrates that sustained morphine pretreatment sensitizes adenylyl cyclase(s) (AC) toward the direct stimulator, forskolin, in cultured primary sensory neurons (AC superactivation). In the present work we investigated the hypothesis that morphine pretreatment also sensitizes ACs toward Gs protein-coupled excitatory modulators (such as PGE₂), leading to augmented PKA-dependent CGRP release from PGE₂-stimulated primary sensory dorsal root ganglion (DRG) neurons. Our results show that sustained morphine treatment potentiated PGE₂-mediated cAMP formation and augmented PGE₂-evoked CGRP release from cultured primary sensory neurons in a PKA-dependent manner. Our data suggests that attenuation of AC-superactivation in primary sensory neurons may prevent the development of opioid-induced hyperalgesia.

Keywords

Morphine; PGE₂; PKA; CGRP release; DRG neurons; opioid-induced hyperalgesia; Gs protein signaling

1. Introduction

Tissue injury leads to the release of inflammatory mediators, such as prostaglandins at the site of injury as well as in the spinal cord (Mariott et al., 1991, Malmberg and Yaksh, 1995).

Prostaglandins are a well-established group of inflammatory mediators that produce peripheral

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and central pain sensitization *in vivo*, and cause augmented evoked excitatory neurotransmitter, such as CGRP release in the spinal cord of rodents (Andreeva and Rang, 1993) as well as *in vitro* in isolated primary sensory neurons (Vasko et al., 1994).

Functional opioid receptors are present at both the peripheral and the central termini of primary sensory neurons, and systemic morphine administration leads to pain relief through both peripheral and central mechanisms. Preventive sustained morphine treatment was found to aggravate post-operative hyperalgesia in clinical patients (Simonnet G, 2008). Recent data indicate that chronic pain (such as inflammation or nerve damage) as well as sustained systemic morphine treatment also augments prostaglandin E₂ (PGE₂) concentration in the spinal cord (Watkins et al., 2005).

PGE₂ acts via cell-surface G-protein coupled receptors (EP₁₋₄) (Shamir et al., 2004). The EP₄ and EP₂ prostaglandin receptor types are coupled to G_s proteins and activate adenylate cyclase, leading to augmented intracellular cAMP formation and activation of cAMP-dependent protein kinases such as PKA. It was shown previously that in rodents, PGE₂-induced pain sensitization is mediated by activation of G_s-protein coupled prostaglandin receptor types (Lin et al., 2006). It was also demonstrated earlier that PGE₂-mediated augmentation of capsaicin-evoked CGRP release from cultured rat primary sensory neurons is also primarily due to activation of the G_s protein coupled EP receptor types (Southhall and Vasko, 2001).

Our earlier investigations have indicated that sustained morphine pretreatment leads to augmented basal- and forskolin-stimulated cAMP formation (cAMP overshoot) in cultured neonatal rat dorsal root ganglion (DRG) neurons (Yue et al., 2008; Tumati et al., 2009). Our previous data also indicates that morphine pretreatment-mediated cAMP overshoot may play a role in the regulation of pain neurotransmitter release from primary sensory neurons, since PKA inhibitors prevented sustained morphine-mediated augmentation of basal (Yue et al., 2008) and capsaicin-evoked CGRP release (Tumati et al., 2009) from these cells.

In the present study, we investigated the hypothesis that a/ sustained morphine treatment also potentiates the efficacy of G_s protein coupled PGE₂ receptors to modulate pain neurotransmitter (CGRP) release from cultured primary sensory DRG neurons; and b/ that inhibition of PKA will attenuate morphine-mediated augmentation of PGE₂-evoked CGRP release from DRG cells.

2. Materials and methods

2.1.Primary culture of neonatal rat DRG neurons

The protocols for the use of experimental animals was in compliance with the guidelines of the NIH and has been approved by the Institutional Animal Care and Use Committee of the University of Arizona. Neonatal (1–3 day old) Sprague-Dawley rats were euthanised and DRGs were aseptically dissected from all spinal levels. The isolated tissue was digested with 0.1% collagenase (Sigma, St. Louis, MO) (3–5 min) and 0.25% trypsin (Invitrogen, Carlsbad, CA) (10 min) in Neurobasal A medium (Invitrogen, Carlsbad, CA) containing 0.5 mM L-glutamine and penicillin/streptomycin (1:100; Sigma, St. Louis, MO) (Neurobasal A/LG/PS medium) in the presence of 0.1 mg/ml DNase I (Sigma, St. Louis, MO) and 5 mM MgSO₄; and dissociated by trituration through a siliconized fire-polished pasteur pipette. After centrifugation, the cells were resuspended in Neurobasal A/LG/PS medium containing 2% B27 (Invitrogen, Carlsbad, CA); (Neurobasal A/LG/PS/B27 medium) and 250 ng/ml NGF (Sigma, St. Louis, MO). The cells were seeded onto 24 well plates to a density of $\sim 1.6 \times 10^4$ cells/well and incubated in a humidified 5% CO₂ incubator at 37°C. After 4 h incubation, anti-mitotic drugs (uridine (150 μM) and 5-fluo-deoxy-uridine (50 μM); Sigma, St. Louis, MO) were added to the medium to prevent the proliferation of non-neuronal cell types. The cells were then allowed to

differentiate for 7–9 days. The medium was changed every other day. On the day before the experiments, the cells were washed with NGF- and mitotic inhibitor-free Neurobasal A/LG/PS/B27 medium and the incubation continued in the absence of NGF/ mitotic inhibitors for an additional 24 h.

2.2. Immunocytochemistry and image analysis

Neonatal rat DRG neurons were cultured for 7 days on poly D-lysine coated glass cover slips and then fixed with 4% paraformaldehyde. Post fixation, the cells were incubated overnight with primary antibodies against β -tubulin III (host mouse, 1:500, Sigma, St. Louis, MO) and/or CGRP (host rabbit, 1:10,000, Peninsula Laboratories Inc., San Carlos, CA) and/or glial fibrillary acidic protein (GFAP, host rabbit, 1:5,000, Sigma, St. Louis, MO) followed by washings in PBS and incubation with AlexaFluor 488-conjugated anti-goat (1:1,000, Molecular Probes), and/or Alexa Fluor 594-conjugated anti-mouse (1:1,000, Molecular Probes) secondary antibodies for 1 h. The cells were washed with PBS and sections were mounted, dried and sealed with mounting medium (Vector Laboratories, Burlingame, CA). The images were acquired with a Nikon E800 fluorescence microscope outfitted with 4 $\times$ /NA 0.2, 10 $\times$ /NA 0.45, 20 $\times$ /NA 0.75 and 40 $\times$ /NA 0.75 objectives, a filter set (excitation 540–580 nm/emission 560–620 nm) and a Hamamatsu C5810 color CCD camera and its proprietary Image Processor software (Hamamatsu Photonic System, Bridgewater, NJ, USA). Digital images were output using Adobe Photoshop 6.0 (Adobe System Inc., San Jose, CA, USA) and analyzed using MetaMorph imaging system 5.5 (Universal Imaging Corporation, West-Chester, PA, USA) on a Pentium PC.

2.3. DRG cell membrane preparation

For time course experiments, DRGs were collected from neonatal rats, cultured for 1–15 days as described earlier. After washing with Ca^{2+} , Mg^{2+} -deficient phosphate-buffered saline, the cells were harvested in the same buffer containing 0.02% EDTA. After centrifugation at 600g for 10 min, the cells were homogenized in ice-cold 10 mM Tris-HCl and 1 mM EDTA, pH 7.4 buffer. The collected cells were homogenized, centrifuged twice at 20,000 g and 4°C for 20 min, and the membrane pellet was resuspended in assay buffer (50 mM Tris, pH 7.4, containing 50 $\mu\text{g/mL}$ bacitracin, 30 μM bestatin, 10 μM captopril, 100 μM phenylmethylsulfonyl fluoride (PMSF), 1 mg/mL BSA, pH 7.4). Protein concentration was determined using Bradford assay (Bradford, 1976).

2.4. Radioligand binding

The number of PGE_2 binding sites was measured in cultured (1–15 days) DRG cell membranes preparations (20 μg) by determining [^3H] PGE_2 (1 nM) (PerkinElmer Life and Analytical Sciences, Boston, MA) specific binding, according to the procedure described by Boie et al. (1997). The reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters using a Brandel Cell Harvester (Brandel Inc., Gaithersburg, MD). The filters were rinsed five times with 4 ml of ice-cold saline containing 0.01% BSA. Filter-bound radioactivity was measured in EcoLite scintillation cocktail (MP Biomedicals, Irvine, CA) using a Beckman LS 6000SC liquid scintillation counter (Beckman Coulter, Fullerton, CA). Nonspecific binding was determined in the presence of 10 μM PGE_2 (Sigma, St. Louis, MO). Specific binding was expressed as pmol/mg protein.

2.5. Drugs

PGE_2 was purchased from Sigma, St. Louis, MO. Morphine sulfate was obtained from National Institute of Drug Abuse, Bethesda, MD. Naltrexone was purchased from Tocris, Ellisville, MO. The selective EP_2 antagonist, AH6809 (6-Isopropoxy-9-xanthone-2-carboxylic acid), was purchased from Sigma (St. Louis, MO). The selective cell permeable PKA inhibitor,

myristoylated PKI 14, 22 amide (PKI) and the selective EP₄ antagonist, L-161982 (N-[[4'-[[3-Butyl-1,5-dihydro-5-oxo-1-[2-(trifluoromethyl)phenyl]-4H-1,2,4-triazol-4-yl]methyl][1,1'-biphenyl]-2-yl]sulfonyl]-3-methyl-2-thiophenecarboxamide) were purchased from Tocris Bioscience (Ellisville, Missouri).

2.6. Drug treatments

The stock solutions (1 mM) of PGE₂, morphine sulfate, naltrexone and PKI were prepared in double distilled water. Stock solutions of L-161982 (1 mM), AH6809 (10 mM) were prepared in cell culture grade DMSO. Aliquots of the stock solutions were stored at -20°C. The required concentrations were obtained on the day of the experiment by diluting the stock solutions in Neurobasal A/LG/PS/B27 medium. 24 hours before the experiment, the DRG neurons were gently washed twice to remove NGF and anti-mitotic drugs from the culture medium. After culturing in a NGF- and mitotic-inhibitor free medium for further 24 h, the DRG neurons were pre-incubated (37 °C, 1 h) in the absence (control) or presence of a selective PKA inhibitor, PKI (50 nM). PKI concentration was selected based on inhibitor dose-response curve (Tumati, unpublished observations). After inhibitor pretreatment, the cells were incubated with a saturating concentration of morphine (1 µM) for 24 h, in the continued presence (Mor+PKI group) or absence (Mor group) of the kinase inhibitor. The concentration of morphine was selected based on our previous studies (Yue et al., 2006). To test the involvement of opioid receptors, DRG cells were treated with the nonselective opioid receptor antagonist naltrexone (10 µM) in the presence (Mor+NTX group) or absence (NTX group) of morphine for 24 hours. Following drug pretreatment, the DRG neurons were washed twice with Neurobasal A/LG/PS/B27 medium and pre-incubated (37 °C, 1 h) in the absence (control) or presence of either a selective EP₄ (L-161982, 1 µM) or EP₂ (AH6809, 10 µM) antagonist separately, or in combination. Optimal EP receptor antagonist concentrations were selected based on previous data (Shamir et al., 2004; Funez et al., 2008). After pretreatment, the DRG neurons were incubated for 1h in the absence (Control) or presence of 100 nM PGE₂ (PGE₂, Mor+ PGE₂).

2.7. Measurement of cAMP formation

After appropriate drug treatment, opiate withdrawal was precipitated by thorough washes (3 times) with Neurobasal A medium followed by incubation in presence of naltrexone (10 µM) for 10 min. Intracellular cAMP concentrations were measured as described earlier (Rubenzik et al., 2001). Briefly, after removal of the naltrexone-containing medium the DRG neurons were incubated in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 4 mM, Sigma, St. Louis, MO) in the absence (Control) or presence of different concentrations (50 nM – 10 µM) of PGE₂ for 1h. The cells were subsequently lysed and 50 µl supernatant of the lysates were incubated with [³H]cAMP (4 nM; Perkin Elmer, Boston, MA) and protein kinase A (30 µg/ml; Sigma, St. Louis, MO) at 4°C for 2h. cAMP standards were run in parallel with each assay. After incubation, activated charcoal (26 mg/ml; NORIT, Amersfoort, The Netherlands) was added to adsorb free [³H]cAMP. Radioactivity was counted in EcoLite scintillation fluid (ICN Pharmaceuticals, Costa Mesa, CA) using Beckman LS6000SC scintillation counter.

2.8. Measurement of CGRP release

CGRP release was assayed in duplicate, using an enzyme immunoassay (EIA) kit (Cayman Chemicals, Ann Arbor, MI). Briefly, after drug treatment, the cells were gently washed twice with Phenol Red-free Neurobasal A/LG/PS medium, followed by incubation with or without PGE₂ (100 nM), in the presence or absence of EP₄ and EP₂ antagonists (separately or in combination). Forskolin (100 µM, 20 min), a direct activator of adenylyl cyclases was used as a positive control. For the CGRP release experiments we selected a PGE₂ concentration that produced ~ 50% stimulation of intracellular cAMP formation in order to ensure that the cellular

signaling pathway is not saturated. The incubation time was chosen based on time course studies (Tumati, unpublished observations). After the drug treatment, the cells were gently washed twice with Phenol Red-free Neurobasal A/LG/PS medium and incubated in 200 μ l fresh Phenol Red-free Neurobasal A medium for 10 min (37°C) in a CO₂ incubator. After incubation, the cell medium was collected, and the released CGRP content was determined using a CGRP EIA kit (Cayman Chemicals) according to the manufacturer's instructions. Standard CGRP dose-absorbance curves (range 0.1–500 pg/ml) were measured in parallel with each assay.

2.9. Data analysis

One sample t-tests were used to compare normalized mean values from each treatment group to the control group (100%). One-way ANOVA tests, followed by Newman-Keuls Multiple Comparison Tests, were performed to evaluate statistical differences between the treatment groups (*P < 0.05, **P < 0.01, and ***P < 0.001). When two groups were compared based on their raw data, unpaired t-test was used. Radioligand binding data were analyzed by nonlinear regression. Data are presented as mean $\pm$ S.E.M., unless otherwise indicated. Statistical evaluations were performed using GraphPad Prism 4.0 software.

3. Results

3.1. Immunocytochemical characterization of cultured neonatal DRG neurons

In order to assess the percentage of peptidergic neurons in our neonatal rat DRG cultures, we have performed double immunolabeling experiments using antibodies against a selective neuronal marker, β -tubulin III (red) together with an antibody against calcitonin gene-related peptide (CGRP, green) (Fig 1A). CGRP expressing neurons are represented in yellow in the Merge panel. Quantification of the immunofluorescence data indicated that approximately 60% of the total neuronal population was CGRP immunoreactive in the 7 day old primary cell culture. We also tested the culture for the presence of non-neuronal cells by co-incubating the cells with antibodies against a neuronal marker (β -tubulin III; red) and a glial (glial fibrillary acidic protein (GFAP), green) marker (Fig 1B). Quantification of the images indicates that approximately 70% of the total cells in our neonatal rat DRG cell culture were positive for β -tubulin.

3.2. The time course of PGE₂ receptor expression during differentiation of the neonatal rat DRG cell culture

Expression of the PGE₂ receptor (Fig 2) during the course of DRG cell differentiation was monitored by radioligand binding. The number of [³H] PGE₂ specific binding sites steadily increased during the first 7–9 days in culture differentiation and subsequently remained at maximal levels for at least 18 days ($B_{\max}$: 0.7 ± 0.2 pmol/mg protein). In addition, neonatal rat DRG neurons exhibited a high affinity to [³H] PGE₂ with a K_d value of 0.5 nM (n=3). We have also tested the role of sustained morphine treatment on PGE₂ receptor expression as it might be possible that sustained morphine treatment can cause an increase in PGE₂ receptors. Our data indicates that sustained morphine treatment causes no change in the $B_{\max}$ of [³H] PGE₂ binding (0.8 ± 0.1 pmol/mg of protein in both membrane preparations) (Tumati, unpublished observations). Since we have not noticed any difference in [³H] PGE₂ binding sites after sustained morphine treatment, we consider that the effect of sustained morphine treatment may not likely be associated with an increase in PGE₂ receptors.

3.3. Sustained morphine treatment augments PGE₂ mediated cAMP formation in DRG cells in a dose dependent manner

In order to test whether sustained morphine treatment affects the affinity or the efficacy of PGE₂ to stimulate cAMP formation from neonatal rat sensory neurons, we have measured PGE₂ dose - cAMP response curves in control (medium-treated) and in morphine (1 μ M, 24 h) pretreated DRG cell cultures (Fig 3). Basal cAMP formation (0 PGE₂ concentration) in cultured neonatal rat DRG neurons was 0.4 ± 0.3 pmol/50 μ l. Sustained morphine (1 μ M, 24 h) treatment augmented basal cAMP formation to 0.7 ± 0.2 pmol/50 μ l, (i.e. by 0.3 pmol/50 μ l, a 75% increase over the basal; * $P < 0.05$, unpaired t test, $n=3$). The $E_{\max}$ values for PGE₂-stimulated cAMP formation in control and morphine-pretreated cells were significantly different (0.9 ± 0.7 pmol/50 μ l in control and 1.5 ± 0.4 pmol/50 μ l in morphine treated groups respectively (* $P < 0.05$, unpaired t-test, $n=3$) while, the EC_{50} values do not show any significant difference (67 ± 19 nM in control vs. 85 ± 23 nM in morphine pretreated cells respectively) (Fig 3). The differences between PGE₂-stimulated vs. basal values in control and morphine treated cells were: 0.2 pmol/50 μ l vs 0.3 pmol/50 μ l at 100 nM PGE₂ concentration used in further experiments ($P = 0.06$, unpaired t test, $n=3$) and 0.5 pmol/50 μ l vs. 1.0 pmol/50 μ l at the $E_{\max}$ level (250 nM PGE₂) (** $P < 0.01$, unpaired t test, $n=3$).

3.4. Direct stimulation of intracellular cAMP formation leads to CGRP release from cultured neonatal rat DRG neurons

Basal CGRP release in 7 day old unstimulated DRG neurons in culture was 489 ± 18 pg/ml. Treatment of cultured neonatal rat DRG neurons with a direct activator of adenylyl cyclases, forskolin, (100 μ M, 20 min) augmented CGRP release to 1243 ± 21 pg/ml (154% over basal CGRP release *** $P < 0.001$; unpaired t-test; $n=4$) (Fig 4). This data confirms that activation of cAMP regulated intracellular signaling pathways augments CGRP release from primary sensory neurons.

3.5. Sustained morphine treatment augments PGE₂-evoked CGRP release from cultured neonatal rat DRG neurons

Basal CGRP release from control neonatal rat DRG neurons was 421 ± 41 pg/ml (100%). 24 h incubation of the cultured DRG neurons with 1 μ M morphine augmented basal CGRP release to $169 \pm 9\%$ of control (** $P < 0.01$ relative to medium-treated control, one-sample t test; $n=5$). Stimulation of the DRG neurons with 100 nM PGE₂ for 1h evoked CGRP release from the cells ($153 \pm 6\%$ of basal, * $P < 0.05$, one-sample t test; $n=5$) (Fig 5). 24 h incubation of the cells in the presence of 1 μ M morphine augmented PGE₂-evoked CGRP release to $417 \pm 13\%$ of basal, a 172% increase over PGE₂ treatment alone (*** $P < 0.001$, one way ANOVA, $n=5$). Sustained morphine-mediated augmentation of PGE₂-evoked CGRP release was completely prevented by co-incubation with the opioid antagonist, naltrexone (* $P < 0.05$ when compared with Mor+ PGE₂ group; one way ANOVA, $n=5$) demonstrating that morphine pretreatment-mediated augmentation of PGE₂-evoked CGRP release is opioid receptor-mediated. Treatment of the DRG neurons with 10 μ M naltrexone alone had no effect on basal CGRP release ($105 \pm 6\%$ of basal, $P > 0.05$ vs. control; one-sample t test, $n=5$) (Fig 5). The results (mean $\pm$ S.E.M.) are expressed as the percentage of basal CGRP release from control, medium pretreated DRG neurons.

3.6. Co-administration of selective EP₂ and EP₄ antagonists completely attenuates PGE₂-evoked CGRP release from neonatal rat DRG neurons

In order to determine the role of Gs protein-coupled PGE₂ receptor types in PGE₂-evoked CGRP release from cultured neonatal rat primary sensory neurons, we pretreated (1 h) the cells with selective EP₄ (L-161982; 1 μ M) and EP₂ (AH6809; 10 μ M) antagonists either separately or together, prior to and during PGE₂ stimulation. Pretreatment with L-161982 or AH6809

attenuated PGE₂ (100 nM, 1h)-evoked CGRP release to 108±6% and 131±8% of basal, respectively (85% and 41% reduction; ***P < 0.001 and *P < 0.05 relative to PGE₂-treated control; one way ANOVA, n=4) (Fig 6). Co-treatment of the DRG neurons with both L-161982 and AH6809 on the other hand completely attenuated PGE₂ (100 nM, 1h) evoked CGRP release (100±3% of basal; ***P < 0.001 relative to PGE₂-treated control, one way ANOVA, n=4). These data indicate that a/ both EP₄ and EP₂ receptor signaling contributes to PGE₂-mediated augmentation of CGRP release from cultured DRG neurons; and b/ that attenuation of Gs-coupled EP receptor signaling is sufficient to abolish PGE-evoked CGRP release from these cells.

3.7. Co-administration of selective EP₂ and EP₄ antagonists completely attenuates Sustained morphine-mediated augmentation of PGE₂-evoked CGRP release from neonatal rat DRG neurons

Pretreatment of the cells with either L-161982 or AH6809 also attenuated morphine pretreatment-mediated potentiation of PGE₂-evoked CGRP release (422±15% of basal) only partially - by 85% and 56%, respectively (150±4% and 241±10% of basal; ***P < 0.001 and **P < 0.01 relative to the Mor+ PGE₂ treatment group; one way ANOVA, n=4) (Fig 6). On the other hand, co-treatment of the cells with both L-161982 and AH6809 completely prevented (106±3% of basal, 98% reduction relative to the Mor + PGE₂ treatment group; ***P < 0.001; one way ANOVA, n=4) morphine pretreatment-mediated augmentation of PGE₂-evoked CGRP release. This result indicates that the effect of morphine pretreatment in the neonatal rat DRG neurons is due to compensatory adaptations in Gs protein-coupled PGE₂-receptor signaling.

3.8. Pre-treatment with a selective PKA inhibitor (myristoylated PKI-amide (PKI) attenuates sustained morphine pretreatment-mediated augmentation of PGE₂-evoked CGRP release from neonatal rat DRG neurons

In order to test the role of cAMP-dependent protein kinase (PKA) in sustained morphine-mediated augmentation of PGE₂-evoked CGRP release (162±4% of basal) in cultured primary sensory neurons, we pretreated the cells with a selective PKA inhibitor, PKI (50 nM) for 1h prior to addition of morphine (1 μM) and subsequently, co-incubated the cells with both PKI and morphine for 24h. After drug pretreatment, the cells were stimulated with PGE₂ (100 nM) in the continuous presence of PKI for 1h. The PKA inhibitor alone had no effect on basal CGRP release from DRG neurons (Fig 7). On the other hand, preincubation with PKI attenuated PGE₂-evoked CGRP release by 77% (114±5% of basal; **P < 0.01 - a relative to the PGE₂ treatment group; one way ANOVA, n=3) (Fig 7), confirming that PGE₂ causes CGRP release from DRG neurons by activating Gs protein-coupled intracellular signaling pathways. Preincubation with the PKA inhibitor also significantly attenuated sustained morphine mediated augmentation of PGE₂-evoked CGRP release by 84 % (149±6% of basal; ***P < 0.001 relative to the morphine+ PGE₂ treatment group; one way ANOVA, n=3) (Fig 7), that morphine pretreatment-mediated augmentation of PGE₂-evoked CGRP release is dependent on sustained morphine-mediated compensatory adaptations in the cAMP/PKA signaling pathway in cultured neonatal rat primary sensory neurons.

4. Discussion

Our present investigation demonstrates novel findings that a/ PGE₂ treatment evokes basal CGRP release from cultured neonatal rat DRG neurons through activation of Gs protein coupled EP receptor types (EP₂ and EP₄) and PKA; b/ morphine pretreatment augments EP₂ / EP₄ receptor activation-dependent CGRP release; and c) morphine pretreatment augments PGE₂-evoked CGRP release due to cellular compensatory adaptations in the cAMP/PKA signaling pathway.

Opioid-induced hyperalgesia is an important clinical problem (Alfonsi et al., 2000, Laoureyras et al., 2009, Richebe, 2005). Preventive opioid treatment prior to and during surgeries frequently exaggerates and extends postoperative pain. Such exaggerated pain sensitivity may also play an important role in the development of analgesic tolerance (Rivat, 2002). Indeed, numerous studies have shown that drugs that inhibit pain sensitization reduce tolerance to morphine's analgesic effects (Ossipov et al., 2005). Thus, it was found that intrathecal administration of compounds that inhibit prostaglandin synthesis (COX, cyclo-oxygenase inhibitors) attenuate morphine withdrawal hyperalgesia (Trang et al., 2002), allodynia (Lashbrook et al., 1999), and reduce antinociceptive tolerance (Powell et al., 1999, Trang et al., 1999), indicating an important role of elevated spinal prostaglandin levels in opioid treatment-mediated pain sensitization and antinociceptive tolerance. Interestingly, previous data indicate that although acute opioids exhibit increased efficacy against inflammatory hyperalgesia, inflammation highly enhances the development of tolerance to systemic morphine (Fernandez-Duenas et al., 2007). We hypothesize that morphine pretreatment sensitizes primary sensory neurons toward inflammatory mediators, leading to augmented spinal pro-nociceptive compensatory adaptations.

Prostaglandins are inflammatory lipid mediators that augment pain neurotransmitter (such as CGRP) release in the spinal cord, *in vivo* (Andreeva and Rang, 1993) and in cultured primary sensory neurons *in vitro* (Vasko et al., 1994). PGE₂ acts via cell-surface G protein coupled receptors (EP₁₋₄) that differ in their signal transduction pathways (Shamir et al., 2004). The EP₂ and EP₄ and EP₃C receptor types are coupled to Gs proteins and activate adenylate cyclases (AC) and cAMP-regulated intracellular signaling cascades. Earlier antisense oligodeoxynucleotide knockdown experiments have shown that, although primary sensory neurons express multiple EP receptor types, PGE₂-mediated augmentation of capsaicin-evoked CGRP release is chiefly due to activation of the Gs protein coupled EP receptors types (Southall and Vasko, 2001). Our current data also demonstrates that preincubation with selective Gs protein coupled EP receptor antagonists completely attenuate PGE₂-mediated CGRP release from cultured neonatal rat primary sensory neurons.

In the present work we tested the hypothesis that morphine pretreatment sensitizes primary sensory neurons toward the Gs protein-coupled inflammatory mediator, PGE₂, leading to augmented spinal pain neurotransmitter release. Our data indicate that indeed, 24 h pretreatment of cultured neonatal rat primary sensory neurons with morphine significantly augments PGE₂-evoked CGRP release (Fig 5) from these cells. We also show that the effect of morphine pretreatment is due to compensatory adaptation in Gs protein coupled EP receptor signaling, since sustained morphine treatment-mediated augmentation of PGE₂-evoked CGRP release was completely attenuated by co-incubation of EP₂ and EP₄ receptor antagonists (Fig 6). Interestingly, we found that the sensitization of DRG neurons after sustained morphine treatment may not be mediated at the PGE₂ receptor level.

It has been found repeatedly that sustained morphine treatment leads to a feedback regulation of intracellular cAMP formation (AC superactivation, cAMP overshoot) in cultured NG10815 cells (Sharma et al., 1977) as well as in recombinant mammalian cell lines (Avidor-Reiss et al., 1997; Malatynska et al., 1996; Rubenzik et al., 2001). Indeed, morphine pretreatment of cultured neuroblastoma/glioma cell lines augments prostaglandin-stimulated cAMP formation (Ammer and Schulz, 1993; Sharma et al., 1975). Our previous data in recombinant Chinese Hamster ovary cell lines further demonstrated the molecular mechanism of AC superactivation (cAMP overshoot) upon sustained morphine treatment and thus we had shown that sustained morphine treatment leads to the phosphorylation and sensitization of certain adenylyl cyclase isoenzymes toward stimulators in a Raf-1 dependent manner (Varga et al. 2003a,b) in these cells. Recently, we had demonstrated that morphine pretreatment augments basal and forskolin-stimulated cAMP formation (cAMP overshoot) in cultured primary sensory neurons (Tumati

et al., 2009; Yue et al., 2008). Therefore, we hypothesized that morphine pretreatment will also augment cAMP formation in response toward physiological Gs protein-coupled excitatory agents in cultured primary sensory neurons. Indeed, our present data show that sustained morphine treatment augments PGE₂-stimulated intracellular cAMP formation in cultured DRG neurons (Fig 3).

Activation of adenylyl cyclase(s) and PKA was shown to augment basal and capsaicin-evoked pain neurotransmitter (CGRP and Substance P) release from the primary sensory neurons (Southall and Vasko 2001; Vanegas and Schaible 2001). The present study confirms these findings by demonstrating that direct activation of adenylyl cyclase(s) by forskolin causes CGRP release from our *in vitro* cellular model, cultured neonatal rat primary sensory neurons (Fig 4). Finally, we show that a selective PKA inhibitor completely attenuates morphine pretreatment-mediated sensitization of PGE₂-evoked CGRP release (Fig 7), indicating that the effects of morphine are due to compensatory adaptations in intracellular cAMP/PKA-dependent pathways.

In summary, our investigation demonstrates that *in vitro* morphine pre-treatment sensitizes primary sensory neurons toward the inflammatory mediator, PGE₂, leading to an augmentation of pain neurotransmitter release in a cAMP/PKA-dependent manner. The data suggest that sustained morphine-mediated AC superactivation may sensitize the primary sensory neurons toward Gs protein-coupled inflammatory mediators, such as PGE₂, contributing to the pro-nociceptive consequences of preemptive morphine administration before/during surgeries. The finding also provides potential molecular targets for pharmacological intervention to prevent the development of such paradoxical pain sensitization.

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References

- Alfonsi P, Fletcher D, Chauvin M. Acute opioid tolerance: intraoperative remifentanyl increases postoperative pain and morphine requirement. *Anesthesiology* 2000;93:409–417. [PubMed: 10910490]
- Ammer H, Schulz R. Coupling of prostaglandin E1 receptors to the stimulatory GTP-binding protein Gs is enhanced in neuroblastoma × glioma (NG108-15) hybrid cells chronically exposed to an opioid. *Mol. Pharmacol* 1993;43:556–563. [PubMed: 8386308]
- Andreeva L, Rang HP. Effect of bradykinin and prostaglandins on the release of calcitonin gene-related peptide-like immunoreactivity from the rat spinal cord *in vitro*. *Br. J. Pharmacol* 1993;108:185–190. [PubMed: 7679028]
- Avidor-Reiss T, Nevo I, Saya D, Bayewitz M, Vogel Z. Opiate-induced adenylyl cyclase superactivation is isoenzyme-specific. *J. Biol. Chem* 1997;272:5040–5047. [PubMed: 9030567]
- Boie Y, Styocco R, Sawyer N, Slipetz DM, Ungrin MD, Neuschaffe-Rube F, Metters KM, Abramowitz M. Molecular cloning and characterization of the four rat prostaglandin E2 receptor subtypes. *Eur. J. Pharmacol* 1997;340:227–241. [PubMed: 9537820]
- Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye bindings. *Anal. Biochem* 1976;72:248–254. [PubMed: 942051]
- Cheng X, Ji Z, Tsalkova T, Mei F. Epac and PKA: a tale of two intracellular cAMP receptors. *Acta. Biochim. Biophys. Sin (Shanghai)* 2008;40:651–662. [PubMed: 18604457]
- Fernández-Dueñas V, Pol O, García-Nogales P, Hernández L, Planas E, Puig MM. Tolerance to the antinociceptive and antiexudative effects of morphine in a murine model of peripheral inflammation. *J. Pharmacol. Exp. Ther* 2007;322:360–368. [PubMed: 17468301]

- Funez MI, Ferrari LF, Duarte DB, Sachs D, Cunha FQ, Lorenzetti BB, Parada CA, Ferreira SH. Teletantagonism: A pharmacodynamic property of the primary nociceptive neuron. *Proc. Natl. Acad. Sci. USA* 2008;105:19038–19043. [PubMed: 18799742]
- Laboureyras E, Chateaubriand J, Richebe P, Simonnet G. Long-term pain vulnerability after surgery in rats: Prevention by nefopam, an analgesic with antihyperalgesic properties. *Anesth. Analg* 2009;109:623–631. [PubMed: 19608840]
- Lashbrook JM, Ossipov MH, Hunter JC, Raffa RB, Tallarida RJ, Porreca F. Synergistic antiallodynic effects of spinal morphine with ketorolac and selective COX1- and COX2-inhibitors in nerve-injured rats. *Pain* 1999;82:65–72. [PubMed: 10422661]
- Lin C-R, Amaya F, Barrett L, Wang H, Takada J, Samad TA, Woolf CJ. Prostaglandin E2 Receptor EP4 Contributes to Inflammatory Pain Hypersensitivity. *J. Pharmacol. Exp. Ther* 2006;319:1096–1103. [PubMed: 16966471]
- Malatynska E, Wang Y, Knapp RJ, Waite S, Calderon S, Rice K, Hraby VJ, Yamamura HI, Roeske WR. Human delta opioid receptor: functional studies on stably transfected Chinese hamster ovary cells after acute and chronic treatment with the selective nonpeptidic agonist SNC-80. *J. Pharmacol. Exp. Ther* 1996;278:1083–1089. [PubMed: 8819489]
- Malmberg AB, Yaksh TL. Cyclooxygenase inhibition and the spinal release of prostaglandin E2 and amino acids evoked by paw formalin injection: a microdialysis study in unanesthetized rats. *J. Neurosci* 1995;15:2768–2776. [PubMed: 7722627]
- Marriott D, Wilkin GP, Coote PR, Wood JN. Eicosanoid synthesis by spinal cord astrocytes is evoked by substance P; possible implications for nociception and pain. *Adv. Prostaglandin Thromboxane Leukot. Res* 1991;21B:739–741. [PubMed: 1705083]
- Ossipov MH, Lai J, King T, Vanderah TW, Porreca F. Underlying mechanisms of pronociceptive consequences of prolonged morphine exposure. *Biopolymers* 2005;80:319–324. [PubMed: 15795927]
- Powell KJ, Hosokawa A, Bell A, Sutak M, Milne B, Quirion R, Jhamandas K. Comparative effects of cyclo-oxygenase and nitric oxide synthase inhibition on the development and reversal of spinal opioid tolerance. *Br. J. Pharmacol* 1999;127:631–644. [PubMed: 10401553]
- Richebe P, Rivat C, Laulin JP, Maurette P, Simonnet G. Ketamine improves the management of exaggerated postoperative pain observed in perioperative fentanyl-treated rats. *Anesthesiology* 2005;102:421–428. [PubMed: 15681961]
- Rivat C, Laulin JP, Corcuff JB, Celerier E, Pain L, Simonnet G. Fentanyl enhancement of carrageenan-induced long-lasting hyperalgesia in rats: prevention by the *N*-methyl-D-aspartate receptor antagonist ketamine. *Anesthesiology* 2002;96:381–391. [PubMed: 11818772]
- Rubenzik M, Varga E, Stropova D, Roeske WR, Yamamura HI. Expression of alpha-transducin in Chinese hamster ovary cells stably transfected with the human delta-opioid receptor attenuates chronic opioid agonist-induced adenylyl cyclase superactivation. *Mol. Pharmacol* 2001;60:1076–1082. [PubMed: 11641436]
- Shamir D, Keila S, Weinreb M. A selective EP4 receptor antagonist abrogates the stimulation of osteoblast recruitment from bone marrow stromal cells by prostaglandin E2 *in vivo* and *in vitro*. *Bone* 2004;34:157–162. [PubMed: 14751573]
- Sharma SK, Klee WA, Nirenberg M. Opiate-dependent modulation of adenylate cyclase. *Proc. Natl. Acad. Sci. USA* 1977;74:3365–3369. [PubMed: 269396]
- Simonnet G. Preemptive antihyperalgesia to improve preemptive analgesia. *Anesthesiology* 2008;108:352–354. [PubMed: 18292670]
- Southall MD, Vasko MR. Prostaglandin receptor subtypes EP3C and EP4 mediate the prostaglandin E2-induced cAMP production and sensitization of sensory neurons. *J. Biol. Chem* 2001;276:16083–16091. [PubMed: 11278900]
- Trang T, Sutak M, Quirion R, Jhamandas K. Intrathecal co-administration of COX inhibitors also augmented the anti-allodynic efficacy of morphine in a model of neuropathic pain. *Pain* 1999;82:65–72. [PubMed: 10422661]
- Trang T, Sutak M, Quirion R, Jhamandas K. The role of spinal neuropeptides and prostaglandins in opioid physical dependence. *Br. J. Pharmacol* 2002;136:37–48. [PubMed: 11976266]

- Tumati S, Yamamura HI, Vanderah TW, Roeske WR, Varga EV. Sustained morphine treatment augments capsaicin evoked CGRP release in a PKA and Raf-1 dependent manner. *J. Pharmacol. Exp. Ther* 2009;330:810–817. [PubMed: 19491327]
- Vanegas H, Schaible H-G. Prostaglandins and cyclooxygenases in the spinal cord. *Prog. Neurobiol* 2001;64:327–363. [PubMed: 11275357]
- Vasko MR, Campbell WB, Waite KJ. Prostaglandin E2 enhances bradykinin-stimulated release of neuropeptides from rat sensory neurons in culture. *J. Neurosci* 1994;14:4987–4997. [PubMed: 7519258]
- Yue X, Tumati S, Navratilova E, Stropova D, St John PA, Roeske WR, Vanderah TW, Yamamura HI, Varga EV. Sustained morphine treatment augments basal CGRP release from cultured primary sensory neurons in a Raf-1 dependent manner. *Eur. J. Pharmacol* 2008;584:272–277. [PubMed: 18328477]
- Yue X, Varga EV, Stropova D, Vanderah TW, Yamamura HI, Roeske WR. Chronic morphine-mediated adenylyl cyclase superactivation is attenuated by the Raf-1 inhibitor, GW5074. *Eur. J. Pharmacol* 2006;540:57–59. [PubMed: 16750187]

(A)

(B)

Anti- β -tubulin III (red) and
Anti- GFAP (green)

Fig. 1. Immunohistochemical characterization of the primary neonatal rat DRG neuronal culture
(A) Immunofluorescence labeling of cultured neonatal rat DRG neurons for a/ a neuronal marker, β -tubulin III (red); b/ the peptide pain neurotransmitter, CGRP (green) and c. merged image to identify peptidergic DRG neurons (yellow). Scale bar for the inset represents 100 μ m.
(B) Immunofluorescent co-labeling of cultured neonatal rat DRG cells for a neuronal (β -tubulin III; red) and a glial (GFAP; green) marker. Scale bars represent 250 μ m.

Fig. 2. The time-course of PGE₂ receptor expression in differentiating neonatal rat DRG cultures After 1–16 days in culture, neonatal rat DRG neurons were incubated with [³H] PGE₂ (1 nM, 1 h). Non-specific binding was measured in presence of 10 μM unlabeled PGE₂. [³H] PGE₂ specific binding (fmol/mg of protein) was calculated at each of the time point based on three independent experiments. The number of specific binding sites steadily increased during the first 7–9 days in culture and subsequently remained at a maximal level (0.7 ± 0.2 pmol/mg protein).

Fig. 3. Sustained morphine treatment augments PGE₂-stimulated cAMP formation in cultured neonatal rat DRG neurons

Cultured DRG neurons were incubated without (■) or with (▲) morphine (1 µM) for 24h. After 24h preincubation, the cells were washed and incubated with 10 µM naltrexone for 10 min. After morphine withdrawal, the cells were stimulated with PGE₂ (50 nM – 1 µM) for 1h and cAMP concentration was measured in the cell lysates as described in the Methods section. The dose response curves show the mean ± S.E.M values from 3 individual experiments, performed in triplicates.

Fig. 4. Direct activation of adenylyl cyclase(s) evokes CGRP release from cultured neonatal rat DRG neurons

DRG neurons were incubated in the absence (Control) or presence of a direct adenylyl cyclase activator, Forskolin (100 μ M) for 20 min. CGRP concentration (pg/ml) in the supernatant was measured using an Enzyme Immunoassay kit (Cayman Chemicals). The data are shown as mean $\pm$ S.E.M. from 4 individual experiments, performed in duplicates.

Fig. 5. Sustained morphine treatment augments PGE₂-evoked CGRP release from cultured neonatal rat DRG neurons

Cultured DRG neurons were incubated in culture medium in the absence (control, dotted line) or presence (Mor) of saturating concentration of morphine (1 μ M) for 24 h. In order to test the involvement of opioid receptors, DRG cells were also co-incubated with morphine and a non-selective opioid receptor antagonist, naltrexone (NTX, 10 μ M). After 24h drug pretreatment, the cells were thoroughly washed and stimulated with PGE₂ (100 nM, 1h). The results (mean $\pm$ S.E.M) are expressed as percentage of basal CGRP release from medium-treated DRG neurons (n=5).

Fig. 6. Morphine pre-treatment mediated augmentation of *PGE*₂- evoked CGRP release from DRG neurons is EP₂ and EP₄ dependent

Cultured neonatal rat DRG neurons were preincubated in the absence (Basal, dotted line) or presence (Mor) of morphine (1 μ M) for 24 h. After pre-incubation, the cells were washed and incubated (1h) in the absence or presence of a selective EP₄- (L-161982; 1 μ M) and/or EP₂ (AH6809; 10 μ M) receptor antagonist, followed by 1 h co-incubation with PGE₂ (100 nM) PGE₂. The results (mean $\pm$ S.E.M) are expressed as percentage of basal CGRP release from control, medium-treated DRG neurons (n=4).

Fig. 7. Preincubation with a selective PKA inhibitor (PKI) attenuates PGE₂- evoked CGRP release in both control and morphine pre-treated neonatal rat DRG neurons

DRG cells were preincubated in the absence (PGE₂, Mor+ PGE₂) or presence (PKI+PGE₂, Mor+PKI+ PGE₂) of the selective PKA inhibitor, PKI (50 nM) for 1h. After inhibitor pretreatment the incubation continued in the absence (PGE₂, PKI+ PGE₂) or presence (Mor+ PGE₂, Mor+PKI+ PGE₂) of morphine (1 μ M) for a further 24 h, followed by PGE₂ (100 nM, 1 h) stimulation. Basal CGRP release (dotted line) was measured in a group of medium-treated DRG neurons. The results (mean $\pm$ S.E.M, n=3) are expressed as the percentage of basal CGRP release.