

Molecular mechanism for opioid dichotomy: bidirectional effect of μ -opioid receptors on P2X₃ receptor currents in rat sensory neurones

Igor Chizhnikov · Vyacheslav Kulyk · Iryna Khasabova · Sergey Khasabov · Donald Simone · Georgy Bakalkin · Dmitri Gordienko · Alexei Verkhatsky · Oleg Krishtal

Received: 25 November 2014 / Accepted: 5 January 2015 / Published online: 16 January 2015
© Springer Science+Business Media Dordrecht 2015

Abstract Here, we describe a molecular switch associated with opioid receptors-linked signalling cascades that provides a dual opioid control over P2X₃ purinoceptor in sensory neurones. Leu-enkephalin inhibited P2X₃-mediated currents with IC₅₀ ~10 nM in ~25 % of small nociceptive rat dorsal root ganglion (DRG) neurones. In contrast, in neurones pretreated with pertussis toxin leu-enkephalin produced stable and significant increase of P2X₃ currents. All effects of opioid were abolished by selective μ -opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), nonselective inhibitor naloxone, and by PLC inhibitor U73122. Thus, we discovered a dual link between purinoceptors and μ -opioid receptors: the latter exert both inhibitory (pertussis toxin-sensitive) and stimulatory (pertussis toxin-insensitive) actions on P2X₃ receptors through phospholipase C (PLC)-dependent pathways. This dual opioid control of P2X₃ receptors may provide a molecular explanation for dichotomy of opioid therapy. Pharmacological control of this newly identified facilitation/inhibition switch may open new perspectives

for the adequate medical use of opioids, the most powerful pain-killing agents known today.

Keywords Pain · Sensory neurones · Nociceptive neurones · P2X₃ receptors · Opioid receptors · Leu-enkephalin

Abbreviations

AC	adenylate cyclase
[Ca ²⁺] _i	free cytosolic Ca ²⁺ concentration
cAMP	cyclic adenosine monophosphate
DRG	dorsal root ganglion
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH ₂
DAG	diacylglycerol
Enk	leu-enkephalin
GPCRs	G protein-coupled receptors
InsP ₃	inositol 1,4,5-trisphosphate
meATP	α,β -methylene-ATP
MORs	μ -opioid receptors
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin
TRPV1	transient receptor potential vanilloid 1

I. Chizhnikov · V. Kulyk · D. Gordienko · O. Krishtal (✉)
State Key Laboratory for Molecular Biology, Bogomoletz Institute of Physiology, 4 Bogomoletz str., Kiev 01024, Ukraine
e-mail: krishtal@biph.kiev.ua

I. Khasabova · S. Khasabov · D. Simone
Department of Diagnostic and Biological Sciences, School of Dentistry, University of Minnesota, Minneapolis, MN, USA

G. Bakalkin
Department of Pharmacological Bioscience, Uppsala University, Uppsala, Sweden

A. Verkhatsky (✉)
Faculty of Life Sciences, The University of Manchester, Manchester, UK
e-mail: Alexej.Verkhatsky@manchester.ac.uk

Introduction

Neurones located in the dorsal root ganglia (DRG) are principal cells involved in transmitting information of various somatosensory modalities. Small- and medium-sized neurones localized in the DRGs initiate the sensation of pain and hence are classified as nociceptive neurones [1].

Numerous studies have indicated that homomeric P2X₃ ATP receptors expressed in the membrane of sensory neurones contribute to various types of pain behaviour, as well as pathological forms of nociception represented by hyperalgesia and allodynia [2–4].

Opioids are commonly employed for pharmacological control of pain [5], although their therapeutic usage is limited by multiple adverse effects and societal issues. It is generally acknowledged that opioids induce analgesia through their action on the central nervous system [6]. This perception, however, has been challenged with accumulation of evidence indicating that a considerable part of opioid analgesia is mediated by peripheral μ -, κ - and δ -opioid receptors [7–9]. Numerous clinical studies demonstrated that conventional and peripherally restricted opioid receptor agonists exhibit similar analgesic potency [10, 8]. The μ -opioid receptors (MORs) that belong to 7-transmembrane domain G protein-coupled receptors (GPCRs) coupled with pertussis toxin (PTX)-sensitive G_i/G_o proteins are predominantly expressed in small (nociceptive) DRG neurones [1]. Activation of MORs inhibits adenylate cyclase (AC) by the G_α subunit, whereas G_{βγ} subunits activate phospholipase C (PLC) with consequent production of second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (InsP₃) [11, 12]. Opioid-induced inhibition of AC- and cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) activity in sensory neurones reduces currents mediated by transient receptor potential vanilloid 1 (TRPV1) channels [13, 14]. Furthermore, opioids acting through MORs suppress voltage-gated Ca²⁺ channels and stimulate G protein-mediated gating of inward-rectifier K⁺ channels [15–18]. Inhibition of MORs by naloxone was also reported to remove the antinociceptive action of P2X₃ receptors antagonist A-317491 in rat models of chemogenic and inflammatory pain [19]. All these pathways represent peripheral sites of opioid analgesia, which curtails nociceptive signalling [20, 21].

In the course of chronic treatment, analgesic effects of opioids are reduced (opioid tolerance) or even reversed resulting in hyperalgesia [22–24]. At the cellular level, opioid-induced hyperalgesia has been considered as a transition from classical PTX-sensitive inhibition of AC to PTX-insensitive stimulation of AC with consequent increase in the intracellular level of cAMP [25, 26].

In the present study, we show that homomeric P2X₃ receptors are regulated by opioid signalling in a subpopulation of sensory neurones. Furthermore, we demonstrate a novel molecular mechanism for dual action of opioids on purinoceptors by presenting the data that stimulation of MORs exerts PTX-sensitive inhibitory and PTX-insensitive stimulatory effects on P2X₃ receptors.

Materials and methods

Cell culture

Lumbar dorsal root ganglia (DRG; L1 to L5) were removed following decapitation of 8-day-old Wistar rats. After isolation, ganglia were treated with trypsin (1/250–2 mg/ml) and collagenase (type IV, 1 mg/ml) at 32 °C for 35–40 min. Throughout the entire procedure, the medium (MEM) was continuously saturated with 95%O₂+5 % CO₂ gas mixture (pH 7.4). Neurones dissociated by trituration were plated on glass coverslips and incubated in MEM medium supplemented with 10 % foetal bovine serum at 37 °C. The cells were used for electrophysiological experiments after 2–3 days *in vitro*.

Electrophysiology

Whole-cell patch-clamp recordings were performed at room temperature (22±1 °C) using a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Union City, CA, USA). Signals were filtered at 2 kHz with the in-built filter, digitized at 5 kHz and stored in a laboratory computer using Digidata 1200B interface and pCLAMP 8.0 software (Molecular Devices, California, USA). All recordings were made at a holding potential of –100 mV. The extracellular solution contained (in mM) 130 NaCl, 2 CaCl₂, 5 KCl, 10 glucose and 10 HEPES (pH was adjusted to 7.4). The patch pipette solution (artificial intracellular medium) contained (in mM) 130 KCl, 10 HEPES, 10 EGTA, 0.5 GTP and 5 Mg-ATP (pH was adjusted to 7.3). The resistance of the pipettes was 5–7 MOhm.

Agonists of P2X₃ receptors were applied for 500 ms by a rapid U-tube system [27] positioned at a distance between 100 and 150 μm from the cell. The time of solution exchange was approximately 20–30 ms as confirmed by liquid junction potential measurements. The time needed for subsequent wash-out of applied saline was 100–150 ms (a more distantly located second U-tube was used for this purpose). The P2X₃-receptor agonist α,β -methylene-ATP (meATP), lithium salt was dissolved in external solution immediately before the experiments. The endogenous opioid peptide neurotransmitter leu-enkephalin (Enk) and the selective μ -opioid receptor agonist endomorphin-1 were used as opioid receptor agonists. All chemicals were obtained from Sigma (Grand Island, USA).

[Ca²⁺]_i recordings

Changes in intracellular concentration of ionized calcium ([Ca²⁺]_i) in isolated DRG neurones were imaged using the high-affinity fluorescent Ca²⁺ indicator fluo-4, as previously described [28]. Briefly, the dye was loaded by 1-h incubation of the DRG neurones with 3 μM fluo-4 acetoxymethyl ester (fluo-4 AM; diluted from a stock containing 2 mM fluo-3 AM

and 0.025 % (w/v) pluronic F-127 in dimethyl sulphoxide) followed by a 1-h wash in Ringer's solution to allow time for de-esterification. The cells were imaged using a LSM 5 PASCAL laser scanning confocal microscope (Carl Zeiss, Jena, Germany). The x - y confocal images were acquired at 0.5–2 Hz using a Zeiss Plan-Apochromat 40 \times 1.3 NA oil-immersion objective. The excitation beam was produced by the 488-nm line of a 200-mW argon ion laser, and illumination intensity was attenuated to 0.5–0.8 %. Fluo-4 fluorescence was captured at wavelengths above 505 nm. To optimize signal quality, the pinhole was set to provide a confocal optical section below 2 μ m (measured with 0.2- μ m fluorescent beads). The focus was adjusted to acquire the images from the middle of the cell depth. In the figures, the intensity of fluo-4 fluorescence was normalized to the average fluorescence intensity in the images captured before agonist application (F/F_0). The temporal profiles of the agonist-induced $[Ca^{2+}]_i$ transients are illustrated by the plots showing the normalized fluo-4 fluorescence intensity (F/F_0) averaged within an entire confocal optical slice of the cell.

Data analysis

Data are presented as mean \pm S.D. (where n is a number of tested opioid sensitive cells). The IC_{50} is the concentration of an Enk that inhibited P2X₃ currents by 50 %. Commercially available software Origin 8.1 (OriginLab Corporation, USA) was used for data analysis. Statistical significance was evaluated using Student's t test. A probability value of $P < 0.05$ was considered statistically significant (when necessary, marked by asterisks on the graphs).

Results

Leu-enkephalin inhibits P2X₃ receptors

Small (15–25 μ m in diameter) DRG neurones were selected at the start of each experiment according to their responses to meATP [29]. Only neurones with fast and completely desensitizing (within 300–400 ms) ion currents characteristic for P2X₃ receptor were selected for subsequent study.

P2X₃-mediated currents were evoked by 0.5 s applications of 30 μ M meATP, which were delivered every 2–3 min. This concentration of meATP is close to saturating for the activation of P2X₃ receptors, whereas 2- to 3-min intervals between agonist applications ensure >90 % recovery of P2X₃ receptors from desensitization [30]. In all experiments, application of Enk was preceded by 2–3 applications of meATP performed to obtain a stable control current. Application of Enk resulted in two distinct outcomes: either it produced no effect (127 cells out of 171 individual neurones) or it inhibited P2X₃-

mediated currents (Fig. 1; 44 neurones or 25.8 % of the selected cells). The inhibition was accelerated when the concentration of Enk was increased (Fig. 1b), which indicates that kinetics of Enk binding to MORs rather than the rate of signal transduction is a limiting step for the inhibition of P2X₃ receptors. The inhibitory effect of Enk was dose-dependent and reversible (Fig. 1a, c). Enk in the concentration of 1 nM did not produce any change in the P2X₃ current, but subsequent application of 1 μ M Enk to the same cell resulted in complete current inhibition. After washout of Enk, the amplitude of P2X₃-mediated current fully recovered within 6–8 min (Fig. 1). Of note, application of 1 μ M Enk completely (by 0.99 ± 0.01 , $n=4$) inhibited P2X₃ receptor-mediated currents in all opioid-sensitive neurones. Similarly, selective μ -opioid receptor agonist endomorphin-1 at 100 nM almost completely inhibited P2X₃ currents (by 0.97 ± 0.02 , $n=3$, data not shown).

Slow recovery from desensitization is a unique property of P2X₃ receptors. Its rate depends on the agonist (with recovery being the slowest when ATP is used for activation [30]) and the temperature [31]; the recovery from desensitization is inhibited by purotoxin-1 from spider venom [32]. To reveal possible effects of Enk on P2X₃ receptors recovery from desensitization, the meATP application protocol was modified. Initially, the cells were stimulated every 3 min by meATP before and during application of Enk (100 nM). When Enk reached its steady-state inhibition (about 65 %), the interval between meATP applications was increased to 9 min. The amplitude of the Enk-inhibited current remained unchanged (Fig. 2a, b). Therefore, we concluded that Enk-mediated inhibition of P2X₃ receptors is not associated with modulation of the recovery from desensitization.

Prolonged application of Enk (18 min as shown on Fig. 2a and up to 20 min in other experiments) did not desensitize/internalize opioid receptors: the amplitude of inhibited P2X₃ current did not recover in the presence of Enk.

Inhibition of P2X₃ receptors is mediated by μ -opioid receptors

Naloxone is a competitive opioid antagonist, which prevents opioid-receptor interaction thus blocking opioid signalling. Naloxone alone (50 nM to 1 μ M) did not produce any changes in P2X₃ receptor-mediated currents (in cells incubated with 1 μ M naloxone for 8 min, the average amplitude of P2X₃ current was 0.99 ± 0.03 , $n=3$ of the control value). Exposure to naloxone, however, removed Enk-dependent inhibition almost completely within 6–8 min ($I/I_{\text{contr}} = 0.95 \pm 0.05$, $n=3$; Fig. 2c, d). The D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), the most selective antagonist of MOR [33] applied at 50 nM completely abolished inhibitory effect of 100 nM Enk ($I/I_{\text{contr}} = 0.97 \pm 0.07$, $n=3$, Fig. 2d). Similarly to naloxone, exposure of DRG neurones to stop alone did not change the amplitude of P2X₃ currents (the average amplitude

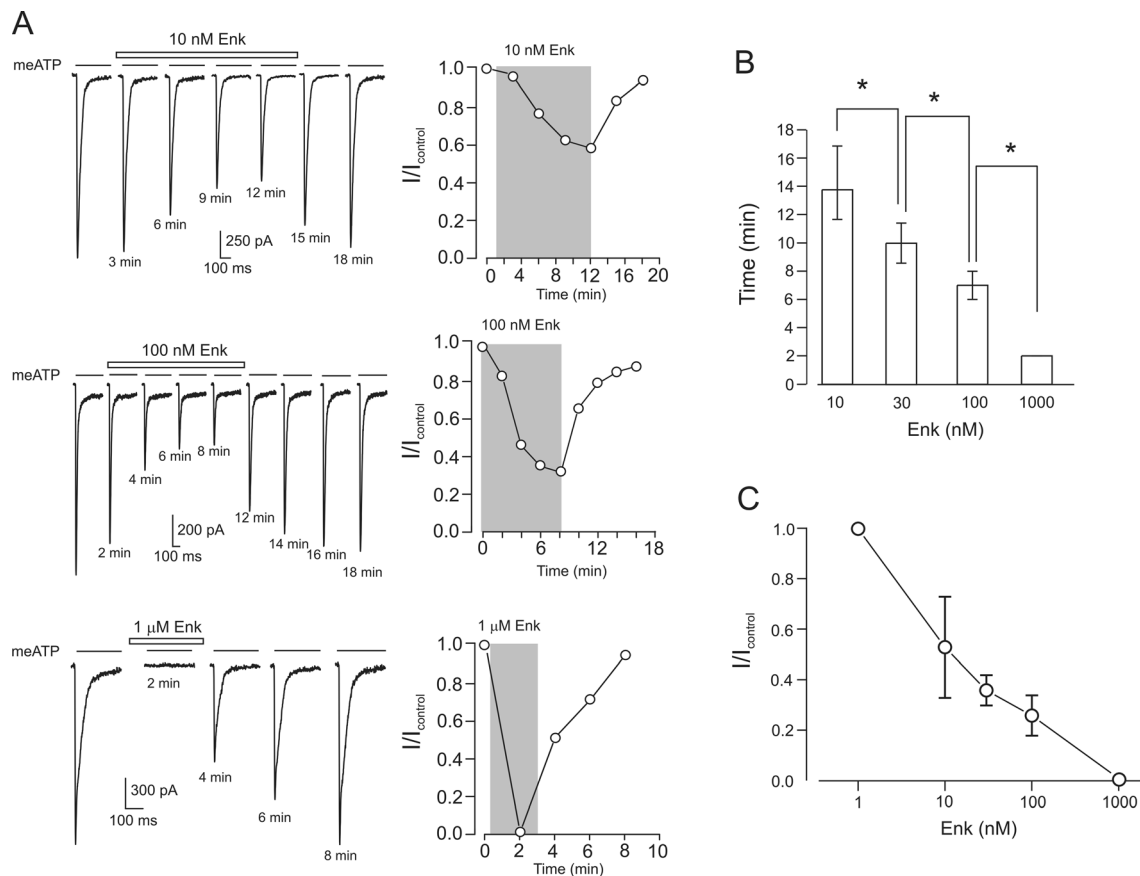


Fig. 1 Leu-enkephalin (Enk) reversibly inhibits P2X₃-mediated currents in a time- and concentration-dependent manner. **a** Current traces recorded from different neurones (left) and time course of the changes in the amplitude of the peak current (right) in control and in the presence of different concentrations of Enk. Here and below, drug application protocol is indicated on the graph, and the time under each current trace indicates the time from the start of the experiment. In graphs depicting the time course of peak current changes, the Enk application is indicated by

the grey area. **b** Time to reach steady-state level of P2X₃ current inhibition depends on the opioid concentration (indicated below bars); * $P < 0.05$, statistically significant difference ($n = 4-9$). **c** Dose-response relationship for the inhibition of P2X₃ currents by Enk. The data are presented as the ratio I/I_{control} where I_{control} is the amplitude of P2X₃ current before the drug application, and I is the current at the steady-state of inhibitory action. Each data point was obtained by averaging data from 4-6 cells

of P2X₃ current was 0.97 ± 0.02 , $n = 3$ of the control value in cells exposed to 1 μM CTOP for 8 min).

High doses of opioid antagonists transiently augment P2X₃ receptor currents

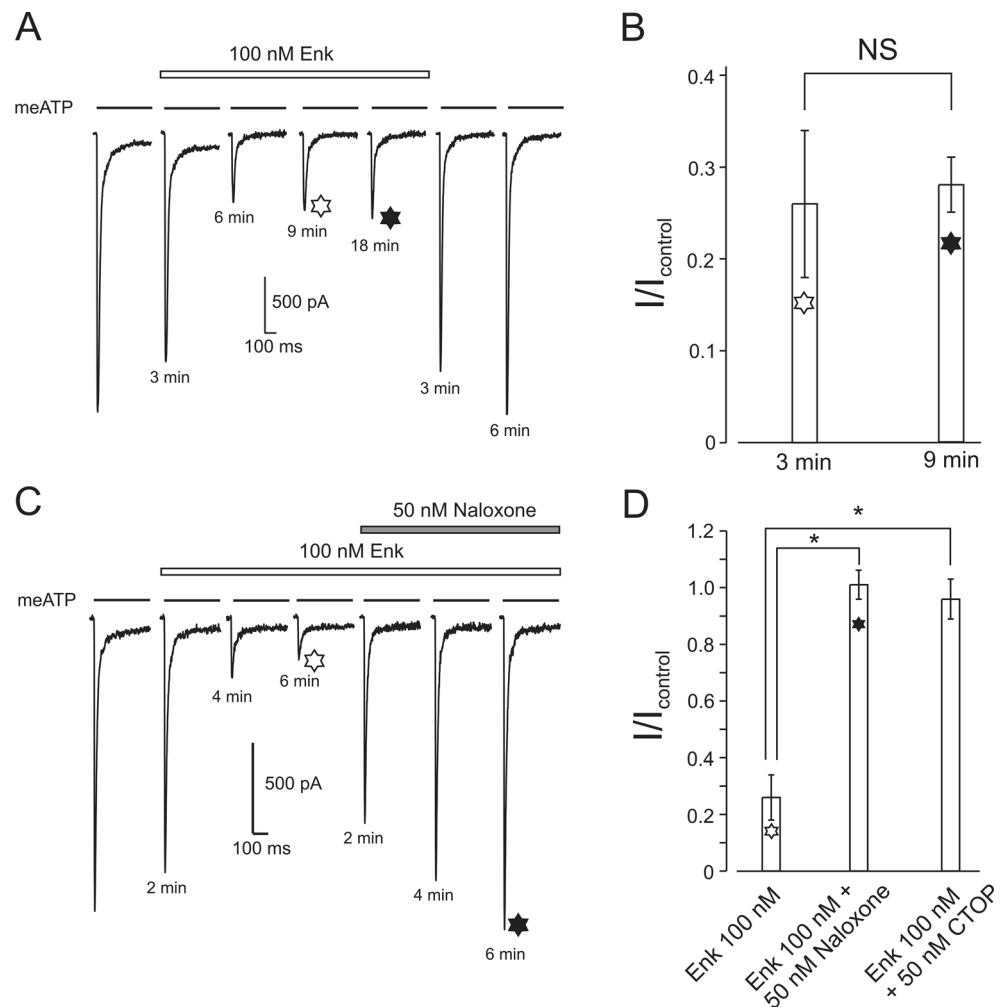
Exposure to 1 μM of naloxone not only removed MOR-mediated inhibition of P2X₃ current but also the amplitude of the P2X₃ current (in the presence of 100 nM Enk) exhibited an almost twofold and transient increase ($I/I_{\text{control}} = 2.1 \pm 0.3$, $n = 4$). A similar transient increase was obtained following application of a high dose (1 μM) of CTOP: the I/I_{control} was 2.7 ± 0.2 ($n = 4$) (Fig. 3).

This transient increase in the amplitude of P2X₃ current hinted for possible stimulatory effect of opioids (by analogy with the data on nodose neurones [34]). In DRG neurones, pre-incubated with PTX (300 ng/ml) for 17-24 h application of Enk (10 nM-1 μM) resulted exclusively in facilitating

effect on the P2X₃ current amplitude in all opioid-sensitive cells tested ($n = 11$; Fig. 4a). This facilitatory effect was completely removed by an application of naloxone ($n = 3$, Fig. 4a, lower panel).

When DRG neurones were exposed to naloxone (1 μM) for 2-4 min and subsequently naloxone solution was substituted by Enk, a substantial increase in the efficacy of the opioid inhibition of P2X₃ receptors was observed. In control settings, 1 nM Enk had no effect, whereas after pre-incubation with naloxone, the same concentration of Enk produced ~50 % inhibition of P2X₃ currents within 10-12 min (Fig. 4b, upper panel). Similarly, while 10 nM Enk inhibited P2X₃ currents by ~50 % in control conditions (cf. Fig. 1) after pre-incubation with naloxone, the same concentration of Enk led to a complete block of the P2X₃ currents within 2 min (Fig. 4b, lower panel). In summary, pre-incubation with naloxone decreases IC₅₀ for the inhibitory action of Enk from 10 to 1 nM (Fig. 4c).

Fig. 2 Inhibitory effect of Enk is not related to P2X₃ receptor desensitization and is reversed by μ -opioid receptor antagonists. **a**, **b** Increasing an interval between agonist applications from 3 min (white six-point star) to 9 min (black six-point star) does not affect the amplitude of current partially inhibited by Enk. Mean values for four experiments are presented in **b**. The time after every solution change is indicated under the current traces. *NS* indicates no significant difference ($n=4$). **c**, **d** Naloxone reverses the inhibitory action of Enk on P2X₃-mediated currents; μ -opioid specific antagonist CTOP produced the same effect. Mean values for naloxone ($n=4$) and CTOP ($n=3$) are presented in **d**; * $P<0.05$, statistically significant difference



Role of PLC and PKC in opioid-mediated regulation of P2X₃ receptors

Exposure of DRG neurones to PLC inhibitor U73122 at 1 μ M concentration for 6–8 min did not affect P2X₃ currents ($n=4$; in the presence of the drug, the average amplitude of P2X₃ current was 0.98 ± 0.04 of the control value). However, when U73122 was applied to the cells in which P2X₃ currents were completely blocked by a high dose of Enk, the current was restored to 0.87 ± 0.11 ($n=4$) of the control (Fig. 5a).

In DRG neurones pretreated with PTX for 17–24 h, both U73122 and PKC inhibitor staurosporine suppressed Enk-induced potentiation of P2X₃-mediated currents (Fig. 5b). Exposure of PTX-treated cells to Enk increased the peak amplitude of P2X₃ currents ($I/I_{\text{control}} = 1.56 \pm 0.2$, $n=7$, * $P<0.05$); this stimulation was reduced to a control level by staurosporine ($I/I_{\text{control}} = 1.04 \pm 0.1$, $n=3$, $P<0.05$) and by U73122 (0.84 ± 0.15 , $n=4$). At the same time (and similarly to PTX-untreated cells), application of U73122 and staurosporine alone for 6–8 min did not affect P2X₃ currents ($n=5$).

Recordings of $[Ca^{2+}]_i$ dynamics performed on these nociceptive neurones have shown that Enk-induced Ca^{2+} signals are associated with intracellular Ca^{2+} release from the ER stores: the Enk-induced $[Ca^{2+}]_i$ transients did not require extracellular Ca^{2+} (Fig. 6a). Treatment of these neurones with PTX for 17–24 h prior to the experiment substantially reduced but did not abolish Ca^{2+} responses stimulated by Enk (Fig. 6b), indicating that a subpopulation of PTX-insensitive G proteins link MORs to PLC; the Ca^{2+} responses were observed in all six neurones studied.

Discussion

Dual effect of opioids on P2X₃ receptors

In this study, we demonstrate that activation of MORs exerts a dual (inhibitory and stimulatory) effect on P2X₃ currents in DRG neurones. In naïve cells, the application of opioid

Fig. 3 Application of high concentrations of naloxone or CTOP removes Enk inhibition and results in transient overstimulation of the P2X₃ currents. **a** When applied in the presence of 100 nM Enk, a high dose of naloxone (1 μM) causes a transient increase in P2X₃ receptor current which subsequently returns to a control level. **b** Time course of the current changes. **c** Bar graph representing average data for similar experiments with naloxone (1 μM, *n*=4) and CTOP (1 μM, *n*=3); **P*<0.05, statistically significant difference

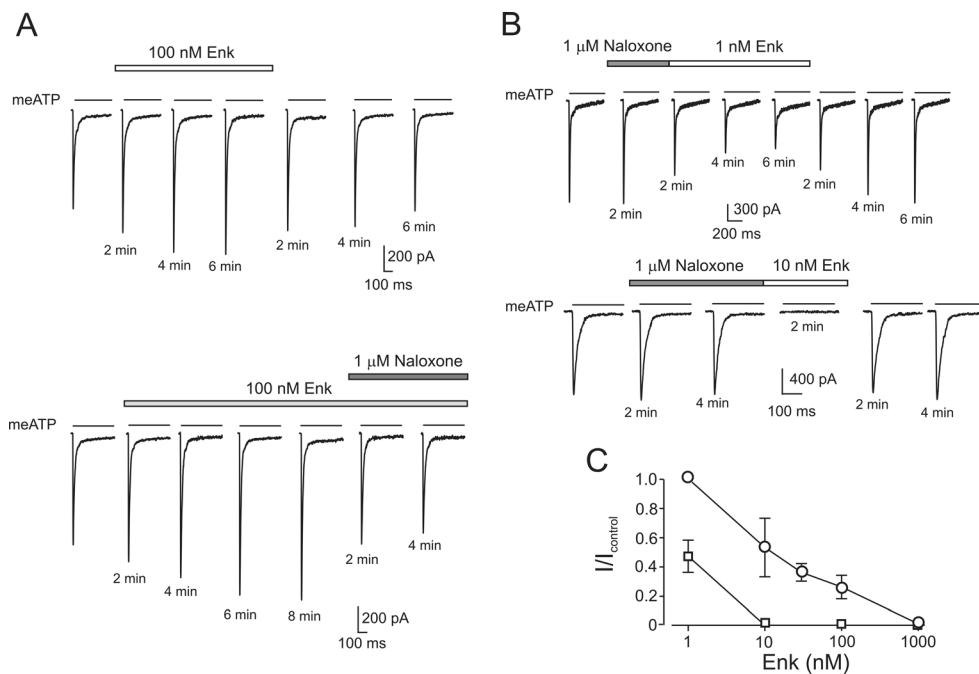
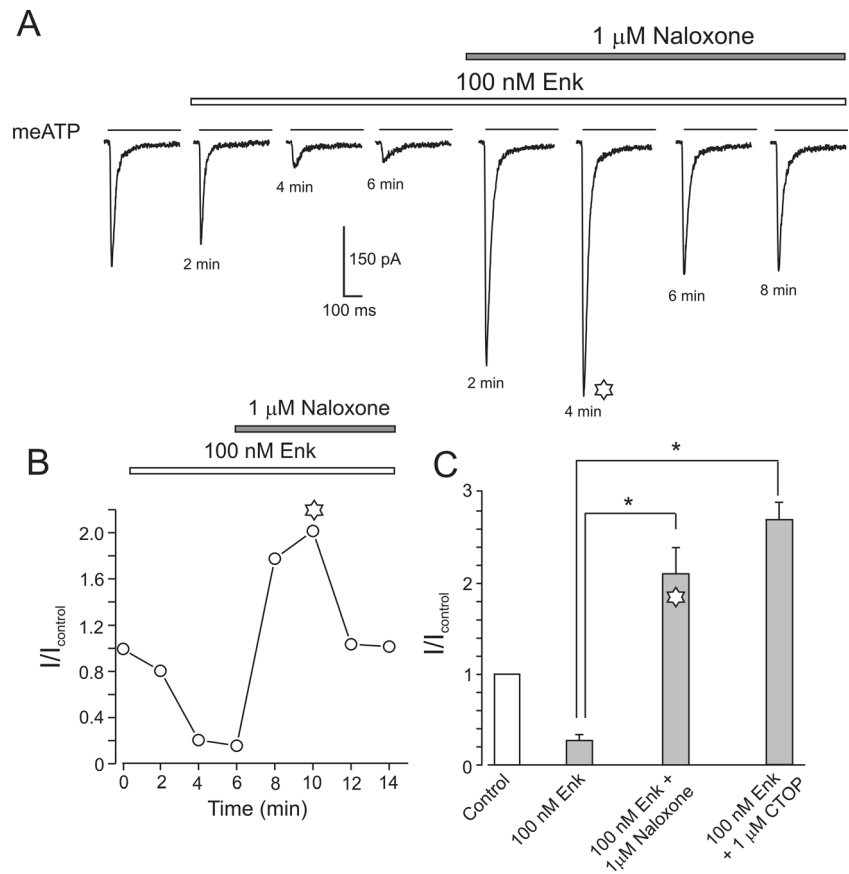


Fig. 4 Inhibitory action of opioid on P2X₃ receptors is reversed after PTX treatment and is dramatically enhanced by pretreatment with naloxone. **a** Application of Enk and naloxone to PTX-treated neurone. (Upper panel) Effect of Enk on P2X₃-mediated currents. (Lower panel) Effect of subsequent treatment with Enk and naloxone on P2X₃ currents. **b** Current traces were recorded in the presence of naloxone and after

subsequent application of Enk (1 nM, upper panel and 10 nM, lower panel; in each panel, recordings were made from different cells). **c** Dose-response relationships in normal conditions (circles, the same plot as in Fig. 1) and after pretreatment of DRG neurone with naloxone (squares). Pairwise comparison of data points (1 and 10 nM of Enk) are significantly (*P*<0.05) different

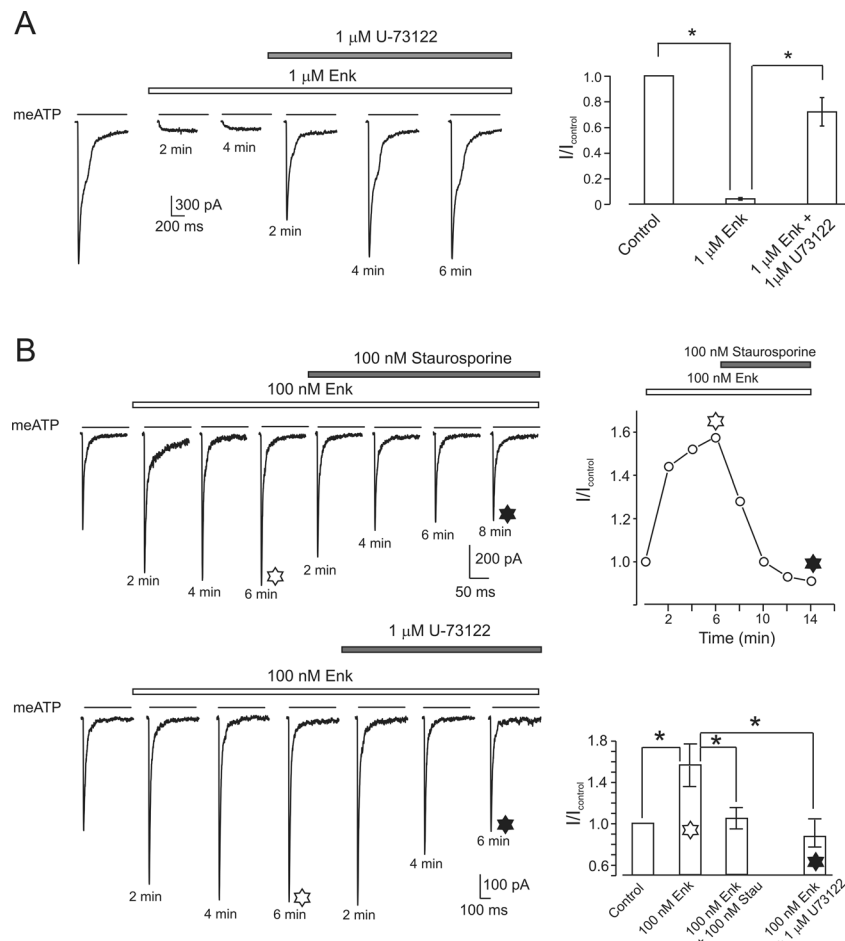


Fig. 5 PLC and PKC are involved in stimulatory and inhibitory actions of opioid on $P2X_3$ receptors. **a** *Left panel*, $P2X_3$ -mediated current is inhibited by Enk and restored by subsequent application of PLC blocker U73122. Average data from five cells are shown in the right. $*P < 0.05$, statistically significant difference. **b** *Upper panel*, representative current traces recorded after PTX treatment in the presence of opioid alone and after addition of staurosporine. The time course of the changes in the peak amplitude of $P2X_3$ -mediated current is shown on the *right*. *Lower left panel* shows $P2X_3$ -mediated currents

recorded from PTX-treated neurones in control, in the presence of Enk and after addition of PLC inhibitor U73122. *Right panel* represents a summary of the experiments, showing average values of $P2X_3$ current amplitudes measured in control and in the presence of opioids and different inhibitors. Exposure of PTX-treated cells to Enk increased the peak amplitude of $P2X_3$ currents, which action was antagonized by staurosporine and U73122. $*P < 0.05$, statistically significant difference ($n = 5$)

significantly inhibits $P2X_3$ currents (Fig. 1). In contrast, when opioids were presented to the cells pretreated with PTX, $P2X_3$ current amplitude was markedly increased (Figs. 4 and 5b). We can therefore suggest that MORs signalling directed at $P2X_3$ receptors is mediated by both PTX-sensitive and PTX-insensitive G proteins that mediate opposite effects. In normal conditions, the inhibitory effect prevails, while stimulatory is either hidden or absent. The functional expression of PTX-insensitive metabotropic pathway was also confirmed by $[Ca^{2+}]_i$ recordings: a portion of ER-originating Ca^{2+} release in response to Enk was preserved in PTX-treated neurones (Fig. 6).

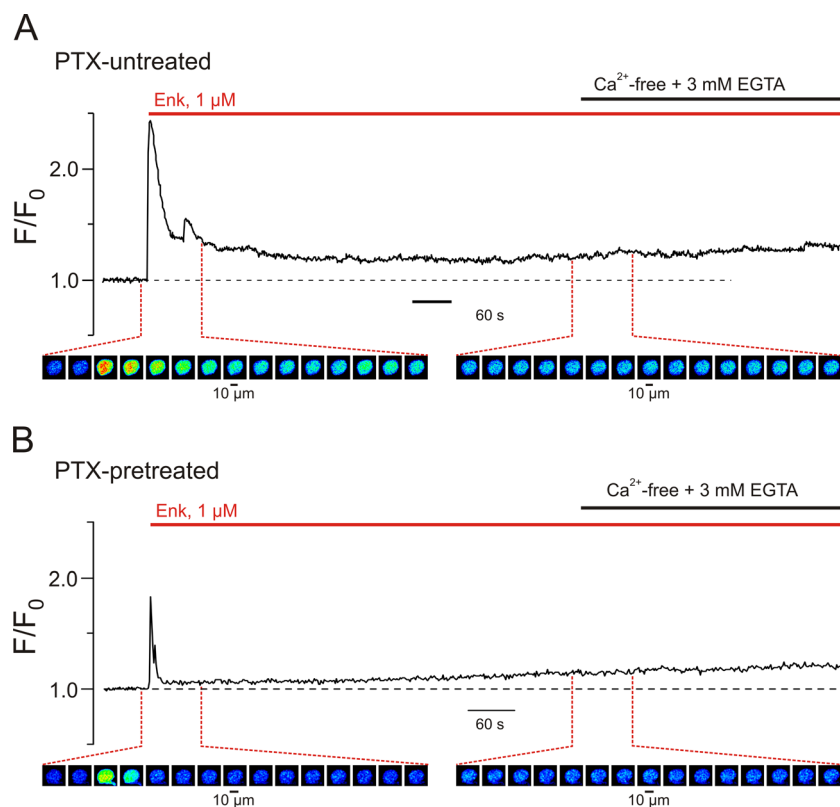
Biochemical studies indicate that MORs, like many other GPCRs, are capable of adopting several distinct active conformations coupled to different G proteins [35]. This feature of MORs-mediated signalling underlies for example dual

influences on AC [36]. We may therefore assume that the dual action of MORs on $P2X_3$ receptors is similarly mediated by different MOR conformation states distinctly coupled to PTX-sensitive/insensitive G proteins. Thus, opioid-induced stimulation/inhibition of $P2X_3$ receptors is associated with distinct intracellular pathways linked to different G proteins.

Both inhibitory and stimulatory effects of MORs on $P2X_3$ receptors are mediated by the PLC

Membrane proteins and ion channels (including $P2X_3$ receptor) are regulated by a minor lipid constituent of plasma membrane, the phosphatidylinositol 4,5-bisphosphate or PIP_2 . Depletion of the membrane pool of PIP_2 leads to the inhibition of the $P2X_3$ current, which can be restored by addition of PIP_2 into the cytosol [37]; activation of MOR leads to PLC-

Fig. 6 Leu-enkephalin-induced Ca^{2+} release from intracellular stores in DRG neurones is activated via $G_{i/o}$ -independent signalling pathway. Traces of self-normalized fluo-4 fluorescence (F/F_0) reflect $[\text{Ca}^{2+}]_i$ changes induced by 1 μM Enk in DRG neurones **a** under control conditions and **b** following 24-h pretreatment with pertussis toxin (PTX). F_0 is F averaged before Enk application. Colour-coded images below the plots were captured during the periods depicted in the plots (every 5th image is shown). The initial rapid phase of Enk-induced $[\text{Ca}^{2+}]_i$ transient was substantially reduced following PTX pretreatment, whereas the sustained component of the response (that was not affected by the removal of extracellular Ca^{2+} indicating intracellular release) was attenuated to a much lesser degree



mediated hydrolyses of PIP_2 thus depleting the membrane pool of the latter. Therefore, opioid-induced PIP_2 hydrolysis is likely to be responsible for the inhibition of P2X_3 currents. The role for PLC is further corroborated by the observation that the specific blocker, U73122, completely abolished opioid-induced inhibition of P2X_3 receptors (Fig. 5a). Suppression of inhibitory component of dual opioid action on P2X_3 receptors could unmask normally hidden stimulatory component. However, in all the cells tested ($n=4$), U73122 restored the current inhibited by opioid back to the control level. This observation prompted a possibility that both opioid-induced stimulation and inhibition of P2X_3 receptors are mediated by PLC-dependent pathway. Experiments presented on Fig. 5b corroborate this conclusion: stimulatory action of opioid on P2X_3 receptors in PTX-treated cells is fully inhibited by U73122.

This, seemingly paradoxical, conclusion is in agreement with data on the regulation of P2X_3 receptors by other metabotropic pathways. For example, activation of P2Y_2 receptors inhibits P2X_3 via PTX-sensitive G_i/G_o linked to PLC, whereas bradykinin and substance P receptors coupled to PTX-insensitive G_q proteins stimulate P2X_3 currents through PLC/ PIP_2 /DAG/PKC cascade [38–40]. Assuming that similar pathways are used by MORs, one could expect that PKC is involved in the stimulatory action of MORs on P2X_3 receptors as well.

PKC is involved in the stimulatory action of MORs on P2X_3 receptors

Activation of PLC/ PIP_2 pathway results in the generation of two second messengers, InsP_3 and DAG, with the phosphokinase C (PKC) being the ubiquitous target for the latter. Regulation of P2X_3 receptors by PKC is well documented [41]. We found that inhibition of PKC by staurosporine completely abolished opioid-induced stimulation of P2X_3 currents in DRG neurones treated with PTX (Fig. 5b). The PKC can be activated either by DAG or by an increase in cytoplasmic Ca^{2+} . Since concentration of the latter was clamped in our experimental conditions, it is likely that PLC/ PIP_2 /DAG/PKC pathway is involved in the stimulation of P2X_3 receptors by MORs. That is, opioid-induced activation of PLC mediates not only inhibition but also stimulation of P2X_3 receptors.

The paradox of divergent PIP_2 signalling in the same membrane areas has been discussed in the past. It was proposed that molecular components of G protein/PLC signalling pathways and their final targets can be segregated between spatially separated microdomains [42], while distinct pools of PIP_2 in plasma membrane have been identified by electron microscopy [43]. Depletion of the PIP_2 pool associated with P2X_3 receptors leads to the inhibition of P2X_3 current [37]. We suggest that hydrolysis of another PIP_2

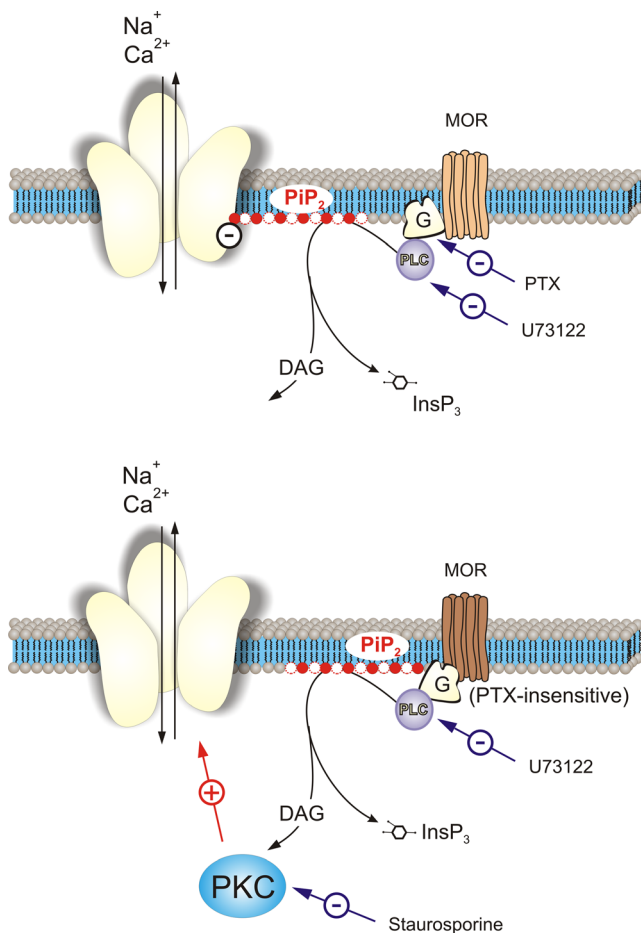


Fig. 7 Proposed bimodal signalling pathways activated by opioid and leading to inhibition and enhancement of P2X₃ receptor-mediated current in DRG neurones. Activated MORs may acquire two distinct states associated either with G proteins. Two distinct PIP₂ pools linked with P2X₃ receptor (*upper panel*) or separated from it (*lower panel*), are both hydrolyzed by PLC (that can be inhibited by U73122), which is activated either by G_{i/o} (sensitive to PTX) or by (yet unidentified) PTX-insensitive G proteins. Hydrolysis of the PIP₂ pool associated with P2X₃ receptor results in the inhibition of the latter. Hydrolysis of PIP₂ pool separated from P2X₃ receptor results in the generation of second messenger DAG, which activates PKC (that can be inhibited by staurosporine); activated PKC in turn may stimulate P2X₃ receptors. Thus, P2X₃ receptor is subjected to the two opposite regulatory influences: inhibition via G_{i/o}/PLC/PIP₂ pathway and stimulation through G_q/PLC/PIP₂/DAG/PKC pathway

pool separated from P2X₃ receptors induces their stimulation via the DAG/PKC pathway (Fig. 7). Involvement of PKC in the stimulatory effect of MORs on nociceptors (putative hyperalgesia) found in our *in vitro* study is in conceptual agreement with *in vivo* findings according to which opioid-induced PLC/PKC signalling contributes to hyperalgesic effects of opioids. For example, supraspinal analgesic effects of morphine are enhanced in PKC-null mice [44]. In another study, morphine applied in extremely low dose produced hyperalgesia in the hot plate test, which effect involved PLC/PKC signalling pathway [45].

Conformation-dependent interaction of MORs with antagonists separates intracellular pathways regulating P2X₃ receptors

We propose that in the subpopulation of DRG neurones, P2X₃ receptors are subjected to inhibitory and excitatory regulation mediated by diverse MOR conformation states coupled to different G proteins. In physiological conditions, an inhibition of P2X₃ receptors prevails and hence masks the effect of stimulatory arm. However, when MOR inhibitors (naloxone or CTOP) are applied at a high dose, the stimulation of P2X₃ receptors becomes transiently visible (as shown on Fig. 3). This may reflect the higher rate of interaction of antagonists with “inhibitory” MOR conformation. This phenomenon is in qualitative agreement with strong increase in efficiency of opioid inhibition after naloxone pretreatment (Fig. 4b): MOR receptors tend to bind the antagonist when being in the “inhibitory” conformation. The latter dominates when the antagonist is replaced by the agonist. Incidentally, after naloxone is washed out, MORs maintain a persistent (exceeding several minutes) “memory” for the increased opioid efficiency (Fig. 4b). A similar long-lasting MOR ‘memory’ has been described recently in other types of experiments: it was found that the affinity of MORs to an agonist is increased dramatically by prior agonist exposure [46]. These findings and results of our experiments indicate that interactions with agonists and antagonists can produce long-term changes in the conformation of MORs.

To summarize possible MOR/P2X₃ interactions (Fig. 7), we propose that MORs can acquire distinct conformational states differentially coupled to PTX-sensitive (G_{i/o}) and PTX-insensitive (most probably G_q) G proteins. The conformational state coupled to G_{i/o} proteins activates PLC, which in turn mediates hydrolysis of the PIP₂ pool associated with P2X₃ receptors. Depletion of this PIP₂ pool results in the inhibition of P2X₃ currents. In contrast, the alternative (possibly G_q-mediated) pathway hydrolyses a separate pool of PIP₂, which is not associated with P2X₃ receptors. We suggest that DAG which is synthesized during this hydrolysis may activate PKC that stimulates P2X₃ receptors. Indeed, inhibition of PKC with staurosporine eliminates stimulation of P2X₃ receptors. Naturally, we have to further suggest that DAG appears in both microdomains; however, inhibitory action of PIP₂ depletion prevails and thus overpowers the PKC-dependent stimulation. As a result, several modes of P2X₃ receptors co-exist in opioids-sensitive sensory neurones: some receptors appear in the inhibited state, some in stimulated and some can be simultaneously inhibited and stimulated. Balancing of the inhibitory and stimulatory arms defines the receptor availability and nociceptive phenotype of the neurone; when under chronic opioids treatment or in response to opioid receptor antagonist therapy, the loss of this balance may contribute to pathological nociception.

In conclusion, activation of MORs normally inhibits P2X₃ responses in DRG neurones; when treated, however, with high doses of MOR antagonists, the stimulation of P2X₃ receptors can transiently occur. Although the functional significance of the stimulatory action of opioids needs further investigation, it is possible that corresponding G protein machinery may contribute to the pro-nociceptive effects of opioids occurring in pathological conditions such as opioid-induced hyperalgesia. Another adverse effect of opioids, namely antagonist-induced opioid hypersensitivity is similarly in concordance with the phenomena observed at a single cell level. Search for pharmacological control of the mechanisms operating inhibition/excitation switch may be important for the future development of antinociceptive therapies.

Acknowledgments This work was supported by Ukrainian Fund for Fundamental Studies (grant DFFD F46.2/001) and by NIH (grant NIH 1R03TW008228-01A1).

Conflict of interest The authors declare no conflict of interest.

References

- Wang HB, Zhao B, Zhong YQ, Li KC, Li ZY, Wang Q, Lu YJ, Zhang ZN, He SQ, Zheng HC, Wu SX, Hokfelt TG, Bao L, Zhang X (2010) Coexpression of δ - and μ -opioid receptors in nociceptive sensory neurons. *Proc Natl Acad Sci U S A* 107:13117–13122
- Burnstock G (1996) A unifying purinergic hypothesis for the initiation of pain. *Lancet* 347:1604–1605
- Burnstock G (2013) Purinergic mechanisms and pain—an update. *Eur J Pharmacol* 716:24–40
- Prado FC, Araldi D, Vieira AS, Oliveira-Fusaro MC, Tambeli CH, Parada CA (2013) Neuronal P2X₃ receptor activation is essential to the hyperalgesia induced by prostaglandins and sympathomimetic amines released during inflammation. *Neuropharmacology* 67:252–258
- Atkinson TJ, Schatman ME, Fudin J (2014) The damage done by the war on opioids: the pendulum has swung too far. *J Pain Res* 7:265–268
- Sehgal N, Smith HS, Manchikanti L (2011) Peripherally acting opioids and clinical implications for pain control. *Pain Physician* 14:249–258
- Reichert JA, Daughters RS, Rivard R, Simone DA (2001) Peripheral and preemptive opioid antinociception in a mouse visceral pain model. *Pain* 89:221–227
- Shannon HE, Lutz EA (2002) Comparison of the peripheral and central effects of the opioid agonists loperamide and morphine in the formalin test in rats. *Neuropharmacology* 42:253–261
- Stein C, Schafer M, Machelska H (2003) Attacking pain at its source: new perspectives on opioids. *Nat Med* 9:1003–1008
- Hanna MH, Elliott KM, Fung M (2005) Randomized, double-blind study of the analgesic efficacy of morphine-6-glucuronide versus morphine sulfate for postoperative pain in major surgery. *Anesthesiology* 102:815–821
- Dhawan BN, Cesselin F, Raghurir R, Reisine T, Bradley PB, Portoghese PS, Hamon M (1996) International Union of Pharmacology. XII. Classification of opioid receptors. *Pharmacol Rev* 48:567–592
- Murthy KS, Makhlof GM (1996) Opioid μ , δ , and κ receptor-induced activation of phospholipase C- β 3 and inhibition of adenylyl cyclase is mediated by G_{i2} and G_o in smooth muscle. *Mol Pharmacol* 50:870–877
- Endres-Becker J, Heppenstall PA, Mousa SA, Labuz D, Oksche A, Schafer M, Stein C, Zollner C (2007) μ -opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. *Mol Pharmacol* 71:12–18
- Vetter I, Wyse BD, Monteith GR, Roberts-Thomson SJ, Cabot PJ (2006) The μ opioid agonist morphine modulates potentiation of capsaicin-evoked TRPV1 responses through a cyclic AMP-dependent protein kinase A pathway. *Mol Pain* 2:22
- Marker CL, Lujan R, Loh HH, Wickman K (2005) Spinal G-protein-gated potassium channels contribute in a dose-dependent manner to the analgesic effect of μ - and δ - but not κ -opioids. *J Neurosci* 25:3551–3559
- Ocana M, Cendan CM, Cobos EJ, Entrena JM, Baeyens JM (2004) Potassium channels and pain: present realities and future opportunities. *Eur J Pharmacol* 500:203–219
- Schroeder JE, McCleskey EW (1993) Inhibition of Ca²⁺ currents by a μ -opioid in a defined subset of rat sensory neurons. *J Neurosci* 13:867–873
- Wu ZZ, Chen SR, Pan HL (2004) Differential sensitivity of N- and P/Q-type Ca²⁺ channel currents to a μ opioid in isolectin B4-positive and -negative dorsal root ganglion neurons. *J Pharmacol Exp Ther* 311:939–947
- McGarraughty S, Honore P, Wismer CT, Mikusa J, Zhu CZ, McDonald HA, Bianchi B, Faltynek CR, Jarvis MF (2005) Endogenous opioid mechanisms partially mediate P2X₃/P2X_{2/3}-related antinociception in rat models of inflammatory and chemogenic pain but not neuropathic pain. *Br J Pharmacol* 146:180–188
- Stein C (2013) Opioids, sensory systems and chronic pain. *Eur J Pharmacol* 716(1–3):179–187
- Stein C, Lang LJ (2009) Peripheral mechanisms of opioid analgesia. *Curr Opin Pharmacol* 9:3–8
- Angst MS, Clark JD (2006) Opioid-induced hyperalgesia: a qualitative systematic review. *Anesthesiology* 104:570–587
- DuPen A, Shen D, Ersek M (2007) Mechanisms of opioid-induced tolerance and hyperalgesia. *Pain Manag Nurs* 8:113–121
- Lee M, Silverman SM, Hansen H, Patel VB, Manchikanti L (2011) A comprehensive review of opioid-induced hyperalgesia. *Pain Physician* 14:145–161
- Chakrabarti S, Gintzler AR (2007) Phosphorylation of Galphas influences its association with the micro-opioid receptor and is modulated by long-term morphine exposure. *Mol Pharmacol* 72:753–760
- Chakrabarti S, Regec A, Gintzler AR (2005) Biochemical demonstration of μ -opioid receptor association with G α lpha: enhancement following morphine exposure. *Brain Res Mol Brain Res* 135:217–224
- Pankratov Y, Lalo UV, Dashkin AN, Krishtal A (2001) Heterogeneity of the functional expression of P2X₃ and P2X_{2/3} receptors in the primary nociceptive neurons of rat. *Neurochem Res* 26:993–1000
- Shapovalov G, Gkika D, Devilliers M, Kondratskiy A, Gordienko D, Busslerolles J, Bokhobza A, Eschaliere A, Skryma R, Prevrarskaya N (2013) Opiates modulate thermosensation by internalizing cold receptor TRPM8. *Cell Rep* 4:504–515
- North RA (2004) P2X₃ receptors and peripheral pain mechanisms. *J Physiol Lond* 554:301–308
- Pratt EB, Brink TS, Bergson P, Voigt MM, Cook SP (2005) Use-dependent inhibition of P2X₃ receptors by nanomolar agonist. *J Neurosci* 25:7359–7365
- Khmyz V, Maximyuk O, Teslenko V, Verkhratsky A, Krishtal O (2008) P2X₃ receptor gating near normal body temperature. *Pflugers Arch* 456:339–347

32. Grishin EV, Savchenko GA, Vassilevski AA, Korolkova YV, Boychuk YA, Viatchenko-Karpinski VY, Nadezhdin KD, Arseniev AS, Pluzhnikov KA, Kulyk VB, Voitenko NV, Krishtal OO (2010) Novel peptide from spider venom inhibits P2X₃ receptors and inflammatory pain. *Ann Neurol* 67:680–683
33. Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell GI, Reisine T (1994) Pharmacological characterization of the cloned κ -, δ -, and μ -opioid receptors. *Mol Pharmacol* 45:330–334
34. Chizhnikov I, Yudin Y, Mamenko N, Prudnikov I, Tamarova Z, Krishtal O (2005) Opioids inhibit purinergic nociceptors in the sensory neurons and fibres of rat via a G protein-dependent mechanism. *Neuropharmacology* 48:639–647
35. Sanchez-Blazquez P, Gomez-Serranillos P, Garzon J (2001) Agonists determine the pattern of G-protein activation in μ -opioid receptor-mediated supraspinal analgesia. *Brain Res Bull* 54:229–235
36. Szucs M, Boda K, Gintzler AR (2004) Dual effects of DAMGO [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin and CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂) on adenylyl cyclase activity: implications for μ -opioid receptor Gs coupling. *J Pharmacol Exp Ther* 310:256–262
37. Mo G, Bernier LP, Zhao Q, Chabot-Dore AJ, Ase AR, Logothetis D, Cao CQ, Seguela P (2009) Subtype-specific regulation of P2X₃ and P2X_{2/3} receptors by phosphoinositides in peripheral nociceptors. *Mol Pain* 5:47
38. Mo G, Peleshok JC, Cao CQ, Ribeiro-da-Silva A, Seguela P (2013) Control of P2X₃ channel function by metabotropic P2Y₂ utp receptors in primary sensory neurons. *Mol Pharmacol* 83:640–647
39. Park CK, Bae JH, Kim HY, Jo HJ, Kim YH, Jung SJ, Kim JS, Oh SB (2010) Substance P sensitizes P2X₃ in nociceptive trigeminal neurons. *J Dent Res* 89:1154–1159
40. Paukert M, Osteroth R, Geisler HS, Brandle U, Glowatzki E, Ruppertsberg JP, Grunder S (2001) Inflammatory mediators potentiate ATP-gated channels through the P2X₃ subunit. *J Biol Chem* 276:21077–21082
41. Brown DA, Yule DI (2007) Protein kinase C regulation of P2X₃ receptors is unlikely to involve direct receptor phosphorylation. *Biochim Biophys Acta* 1773:166–175
42. Delmas P, Crest M, Brown DA (2004) Functional organization of PLC signaling microdomains in neurons. *Trends Neurosci* 27:41–47
43. Fujita A, Cheng J, Tauchi-Sato K, Takenawa T, Fujimoto T (2009) A distinct pool of phosphatidylinositol 4,5-bisphosphate in caveolae revealed by a nanoscale labeling technique. *Proc Natl Acad Sci U S A* 106:9256–9261
44. Newton PM, Kim JA, McGeehan AJ, Paredes JP, Chu K, Wallace MJ, Roberts AJ, Hodge CW, Messing RO (2007) Increased response to morphine in mice lacking protein kinase C epsilon. *Genes Brain Behav* 6:329–338
45. Galeotti N, Stefano GB, Guarna M, Bianchi E, Ghelardini C (2006) Signaling pathway of morphine induced acute thermal hyperalgesia in mice. *Pain* 123:294–305
46. Birdsong WT, Arttamangkul S, Clark MJ, Cheng K, Rice KC, Traynor JR, Williams JT (2013) Increased agonist affinity at the μ -opioid receptor induced by prolonged agonist exposure. *J Neurosci* 33:4118–4127