British Journal of Pharmacology (2009), 156, 826–834

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

The role of PKA and PKCε pathways in prostaglandin E_2-mediated hypernociception

D Sachs, CF Villarreal, FQ Cunha, CA Parada and SH Ferreira

Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

Background and purpose: Protein kinase (PK) A and the ε isoform of PKC (PKCε) are involved in the development of hypernociception (increased sensitivity to noxious or innocuous stimuli) in several animal models of acute and persistent inflammatory pain. The present study evaluated the contribution of PKA and PKCε to the development of prostaglandin E_2 (PGE_2)-induced mechanical hypernociception.
Experimental approach: Prostaglandin E_2-induced mechanical hypernociception was assessed by constant pressure rat paw test. The activation of PKA or PKCε was evaluated by radioactive enzymic assay in the dorsal root ganglia (DRG) of sensory neurons from the hind paws.
Key results: Hypernociception induced by PGE_2 (100 ng) by intraplantar (i.pl.) injection, was reduced by i.pl. treatment with inhibitors of PKA [A-kinase-anchoring protein St-Ht31 inhibitor peptide (AKAPI)], PKCε (PKCεI) or adenylyl cyclase. PKA activity was essential in the early phase of the induction of hypernociception, whereas PKC activity was involved in the maintenance of the later phase of hypernociception. In the DRG (L4-L5), activity of PKA increased at 30 min after injection of PGE_2 but PKC activity increased only after 180 min. Moreover, i.pl. injection of the catalytic subunit of PKA induced hypernociception which was markedly reduced by pretreatment with an inhibitor of PKCε, while the hypernociception induced by paw injection of PKCε agonist was not affected by an inhibitor of PKA (AKAPI).
Conclusions and implications: Taken together, these findings are consistent with the suggestion that PKA activates PKCε, which is a novel mechanism of interaction between these kinases during the development of PGE_2-induced mechanical hypernociception.
British Journal of Pharmacology (2009) **156**, 826–834; doi:10.1111/j.1476-5381.2008.00093.x; published online 13 February 2009

Keywords: PKA; PKC; cAMP; PGE_2 and rat mechanical hypernociception

Abbreviations: AC, adenylyl cyclase; AKAPI, A-kinase-anchoring protein St-Ht31 inhibitor peptide; i.pl., intraplantar; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKAcs, catalytic subunit of PKA; PKC, protein kinase C; PKCε, ε isoform of protein kinase C; ΨεRACK, pseudo receptor octapeptide for activated PKCε, a specific agonist of PKCε; PKCεI, $PKC\varepsilon V_{1-2}$ peptide, a selective PKCε inhibitor

Introduction

Tissue injury and inflammation are associated with increased prostanoid synthesis which sensitizes primary sensory neurons (Martin *et al.*, 1987; Schaible and Schmidt, 1988; Rueff and Dray, 1993). This sensitization of the nociceptive neurons in humans results in hyperalgesia (Ferreira, 1972) (an increased response to a stimulus which is normally painful) or allodynia (pain from stimuli that are not normally painful). However, in animal behaviour models of mechanical nociception, hyperalgesia and allodynia can be distinguished by the use of apparently different mechanical stimuli, as in the Randall-Selitto and the electronic von Frey tests. Moreover, the terms hyperalgesia and allodynia have been developed for use in clinical practice rather than for experimental work, physiology or anatomical purposes (see IASP Pain Terminology). Therefore, we have used the term hypernociception to describe the decrease of behavioural nociceptive threshold in experimental animals.

Mechanical hypernociception induced by prostaglandin E_2 (PGE_2), assessed by modification of the Randall-Selitto mechanical test, was linked with increased adenosine 3′,5′-cyclic monophosphate (cAMP) in neurons (Ferreira and Nakamura, 1979). This conclusion was later supported and extended (Taiwo *et al.*, 1989; Taiwo and Levine, 1991; Ouseph *et al.*, 1995; Aley and Levine, 1999; Cunha *et al.*, 1999). The adenylyl cyclase (AC) activator, forskolin or inhibitors of the phosphodiesterases enhanced the mechanical hypernociception induced by PGE_2 (Taiwo *et al.*, 1989; Taiwo and Levine,

Correspondence: Sérgio Henrique Ferreira, Faculdade de Medicina de Ribeirão Preto – USP, Av. Bandeirantes, 3900, Ribeirão Preto, SP, Brazil 14049-900. E-mail: shferrei@uol.com.br
Received 7 July 2008; revised 29 September 2008; accepted 6 November 2008

1991; Ouseph *et al*., 1995; Cunha *et al*., 1999; Kassuya *et al*., 2007). In primary nociceptive neurons, increased cAMP is associated with the activation of protein kinase A (PKA) *in vitro* and *in vivo* (Scott, 1991; Beebe, 1994; England *et al*., 1996; Liao *et al*., 1999; Smith *et al*., 2000; Distler *et al*., 2003; Wang *et al*., 2007). In this context, PKA participates in the inflammatory hypernociception induced by PGE_2 (Malmberg *et al*., 1997; Aley and Levine, 1999; Cunha *et al*., 1999; Kassuya *et al*., 2007).

Besides PKA, there is an extensive literature documenting a role for PKC in nociceptor activation as well as sensitization. Studies *in vitro* (Barber and Vasko, 1996; Leng *et al*., 1996; Di Castro *et al*., 2006) demonstrated that phorbol-activated PKC sensitizes primary nociceptive neurons. In this context, the hypernociception caused by inflammatory nociceptive mediators, such as adrenaline, endothelins and bradykinin, is related to the PKC pathway (Cesare *et al*. 1999; Khasar *et al*., 1999a; Souza *et al*., 2002; Cunha *et al*., 2004). In primary afferent neurons, the ε isoform of PKC (PKCε) participates in inflammatory hypernociception, as selective PKCε inhibitors reduced hypernociception induced by a variety of nociceptive stimuli (Khasar *et al*., 1999b; Dina *et al*., 2000; 2006; Hucho *et al*., 2005; Parada *et al*., 2005; Summer *et al*., 2006; Yamamoto *et al*., 2006). Furthermore, PKCε was up-regulated in dorsal root ganglia (DRG) after peripheral administration of carrageenan (Zhou *et al*., 2003).

Although the second messenger cascade associated with PGE_2-induced hypernociception involves the participation of PKA downstream from cAMP, this evidence does not exclude the participation of PKC in this pathway. Thus, the aim of the present study was to investigate whether, in mechanical hypernociception, PKC participates in the PGE_2/cAMP/PKA intracellular signalling pathway in the primary nociceptive neurons.

Methods

Animals

Animal care and handling procedures were in accordance with International Association for the Study of Pain guidelines for the use of animals in pain research and with the approval of the Ethics Committee of the School of Medicine of Ribeirão Preto (University of São Paulo). All efforts were made to minimize discomfort for the animals. Experiments were performed with 180 to 200 g male Wistar rats kept in a 12 h light–dark cycle, with controlled humidity (60–80%) and temperature (22–25°C). Food and water were available *ad libitum*. The animals were taken to the testing area at least 1 h before testing. Each experiment used five rats per group, previously found to be the minimum number of animals necessary to detect significant variations of the paw nociceptive sensitivity. All behavioural testing was performed between 9:00 AM and 4:00 PM.

Nociceptive test: constant pressure rat paw test

Nociception was measured in the present study by applying a constant pressure via a ring-shaped syringe piston (15 mm^2) to the dorsal surface of the hind paw. The piston pressure (20 mm Hg) was measured by a sphygmomanometer connected to the source of compressed air (Ferreira *et al*., 1978). The end point of the mechanical nociception is a typical behavioural reaction (freezing) characterized by a combination of signs: a brief apnoea, retraction of the head and forepaws and reduction in the escape movements from the position imposed by the experimental situation. Usually, the apnoea is associated with successive waves of muscular tremor. The latency of the freezing reaction is measured before (control reaction time at zero time) and at different times after intraplantar (i.pl.) administration of the tested substance in the same paws. The reduction of the latency obtained by subtracting the value of latency at a given time point from the value at zero time (31.5 ± 0.1 s, $n = 50$) quantified the intensity of mechanical hypernociception (Ferreira *et al*., 1978). In this test, local or systemic pretreatment with steroidal and non-steroidal anti-inflammatory drugs reduces the intensity of hypernociception by inflammatory stimuli (carrageenan, endotoxin, cytokines/chemokines) but does not affect hypernociception induced by directly sensitizing mediators like PGE_2 or dopamine (Nakamura and Ferreira, 1987; Cunha *et al*., 1991; 1992; Lorenzetti *et al*., 2002). Thus this test, using a constant pressure as mechanical nociceptive stimulus, allows the measurement of variations of nociceptive behaviour.

Determination of PKA and PKC activity in DRG

Rats were given a lethal overdose of sodium pentobarbital and the L4 and L5 DRG removed. These samples were frozen on dry ice and homogenized (Homogeneizador Kont). The PKA and PKC activity was measured using a commercial kit (protein kinase A assay kit, protein kinase C assay kit, Calbiochem) according to the manufacturer's instructions. Assays were only carried out using the DRG (L4-L5) ipsilateral to the hind paws receiving the i.pl. injection of PGE_2 (100 ng per paw) or N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (db-cAMP) (100 μg per paw), removed at 30 or 180 min after injection. The assay is based on the reaction of the enzyme sample with a biotinylated peptide substrate and γ^{32}P-ATP, and is expressed as pmoles of phosphate incorporated per minute.

Effect of drug treatments

To investigate the involvement of cAMP, PKA or PKCε in hypernociception induced by PGE_2 (100 ng; Ferreira and Nakamura, 1979) or db-cAMP (100 μg; Ferreira and Nakamura, 1979), the animals were pretreated with subcutaneous i.pl. injections into the same (ipsilateral) paw. Note all i.pl. treatments are shown as the dose per paw. Pretreatment with inhibitors of AC (SQ22536; 3-27 μg), of PKA [A-kinase-anchoring protein St-Ht31 inhibitor peptide (AKAPI); 0.03-0.3 μg] or of PKCε (PKCεI, 1-9 μg) was given 5 min before the i.pl. injection of PGE_2 or db-cAMP. The participation of PKA or PKCε in later phases of PGE_2 or db-cAMP-induced hypernociception was assessed by post-treatment (30 or 90 min) with AKAPI (0.3 μg) or PKCεI (9 μg). To investigate if PKA or PKCε had intrinsic hypernociceptive effects, the animals were injected with the catalytic subunit of PKA (PKAcs 0.3–9 U) or

pseudo receptor octapeptide for activated PKCε, a specific agonist of PKCε (ΨεRACK 0.3-9 μg; Dorn *et al*., 1999). To determine if PKA was able to activate PKCε, the animals were pretreated with inhibitor AKAPI (0.3 μg) or PKCε (PKCεI, 9 μg) 30 min before the PKAcs (1 U) or the specific agonist of PKCε (ΨεRACK 1 μg). In the present study, all hypernociceptive stimuli (PGE_2, db-cAMP, ΨεRACK and PKAcs) or antagonists used were given by subcutaneous injection into the hind paw.

Data analysis

Results are presented as means ± SEM for groups of five animals. Analysis of variance (one-way ANOVA) was used, followed by the Bonferroni test. The level of significance was set at $P < 0.05$. The dose–response relationships for SQ22536, AKAPI and PKCεI were analysed by non-linear regression.

Drugs and reagents

The pseudo receptor for activated PKCε octapeptide (ΨεRACK; Dorn *et al*., 1999) and $PKC\varepsilon V_{1-2}$ peptide (a selective PKCε inhibitor; Gray *et al*., 1997) were obtained from SynPep Corp (Dublin, CA, USA). SQ22536 was obtained from Biomol (Aley and Levine, 1999). The PKAcs was obtained from Calbiochem (La Jolla, CA, USA). PGE_2 and db-cAMP (Taiwo *et al*., 1989; Taiwo and Levine, 1991; Ouseph *et al*., 1995) were obtained from Sigma (St Louis, MO, USA). InCELLlect® AKAPI (Moita *et al*., 2002; Parada *et al*., 2005) was obtained from Promega Corp (Madison, WI, USA). The stock solution of PGE_2 ($1\ \mu g \cdot \mu L^{-1}$) was prepared in 10% ethanol, and additional dilutions were made in physiological saline (0.9% NaCl), yielding a final concentration of ethanol of less than 1%. All other drugs were dissolved directly in saline. Protein kinase A assay kit and protein kinase C assay kit were from Calbiochem.

Results

PKA and PKCε participate in the PGE_2-activated, cAMP second messenger cascade in primary afferent neurons

The results presented in Figure 1 show that subcutaneous i.pl. pretreatment with an inhibitor of AC (SQ22536; 3, 9 and 27 μg; ID_{50}: 4.01 μg; Figure 1A) or of PKA (AKAPI; 0.03, 0.1 and 0.3 μg; ID_{50}: 0.22 μg; Figure 1B) prevented PGE_2-induced hypernociception in a dose-dependent manner. The PKCε inhibitor (PKCεI; 1, 3 and 9 μg; ID_{50}: 2.6 μg; Figure 1C) prevented in a dose-dependent manner the hypernociception induced by PGE_2. Pretreatment with PKCεI (9 μg), or AKAPI (0.3 μg), but not with the AC inhibitor SQ22536 (27 μg), inhibited the hypernociception induced by db-cAMP (100 μg, Figure 1D). It should be stressed that SQ22536 (27 μg, Figure 1A), AKAPI (0.3 μg, Figure 1B) or PKCεI (9 μg, Figure 1C) at the highest doses used here had no nociceptive effect when given alone into normal paws.

Differential time effects of PKA and PKC inhibitors on the hypernociception induced by PGE_2 or db-cAMP

Treatment with inhibitors of PKA (AKAPI, 0.3 μg) or PKCε (PKCεI, 9 μg), either before or 30 min after the i.pl. injection of PGE_2 (100 ng) or db-cAMP (100 μg), reduced the mechanical hypernociception. However, later post-treatment (90 min after PGE_2 or db-cAMP i.pl. injection) with AKAPI was ineffective but similar treatment with PKCεI clearly reduced mechanical hypernociception. Together, these results suggest that PKA activity is essential only in the early phase of the establishment of hypernociception, whereas PKC activity is involved in the maintenance of later phases of hypernociception. In support of this suggestion, the radioactive assay for PKA activity in ipsilateral DRG (L4-L5) was increased at 30 min, but not 180 min after paw injection of PGE_2 (Figure 2B) or db-cAMP (Figure 3B). However, the PKC activity was enhanced much later, mainly at 180 min after i.pl. injection of PGE_2 (Figure 2D) or db-cAMP (Figure 3D).

PKA activates PKCε during PGE_2-induced hypernociception

We also investigated whether the hypernociception induced by a PKAcs depends on PKCε activation, and whether the hypernociceptive activity of ψεRACK depends on PKA activation. Both PKAcs (0.3, 1, 3 and 9 U; ED_{50}: 1.31 U; Figure 4A) and ψεRACK (0.3, 1, 3 and 9 μg; ED_{50}: 1.67 μg; Figure 4B) given alone induced dose-dependent mechanical hypernociception. Pretreatment (30 min before) with the inhibitor of PKCε (9 μg) significantly inhibited the hypernociception induced by PKAcs (1 U; Figure 4C). In contrast, the PKA inhibitor AKAPI (0.3 μg) given 30 min before the administration of ψεRACK (1 μg) did not change the hypernociceptive effect (Figure 4D). These results are compatible with the proposition that PKA activates PKCε during PGE_2-induced hypernociception.

Discussion

Enhanced sensitivity to nociceptive stimuli is one of the characteristics of the inflammatory response and results from an increase in the excitability of primary nociceptive neurons. There is substantial experimental evidence demonstrating that PGs, among other mediators (sympathetic amines, endothelins, 5-hydroxytryptamine (5-HT), adenosine and cytokines) sensitize the primary nociceptive neurons to innocuous mechanical (Handwerker and Neher, 1976; Pateromichelakis and Rood, 1982; Schaible and Schmidt, 1988; Mizumura *et al*., 1993; Moriyama *et al*., 2005) and chemical stimuli (Nicol and Cui, 1994; Smith *et al*., 2000). This sensitization is referred in the present study as hypernociception, thus avoiding the use of terms like hyperalgesia or allodynia, because they describe an aggregate of characteristic human pathological nociceptive symptoms (see IASP Pain Terminology).

It is now well established that inflammatory hypernociception, such as induced by PGE_2, depends on activation of signalling pathways, which require neuronal activation of PKA in neurons, downstream of the second messenger cAMP (Ferreira and Nakamura, 1979; Taiwo and Levine, 1990; 1991; 1992; Taiwo *et al*., 1992; Wang *et al*., 1996; Aley and Levine, 1999; Cunha *et al*., 1999; Kassuya *et al*., 2007). Moreover, there are a great number of studies suggesting that the PKC pathway also participates in nociceptor sensitization, induced by nociceptive mediators, such as adrenaline, endothelins and bradykinin, as well as by more general inflammatory

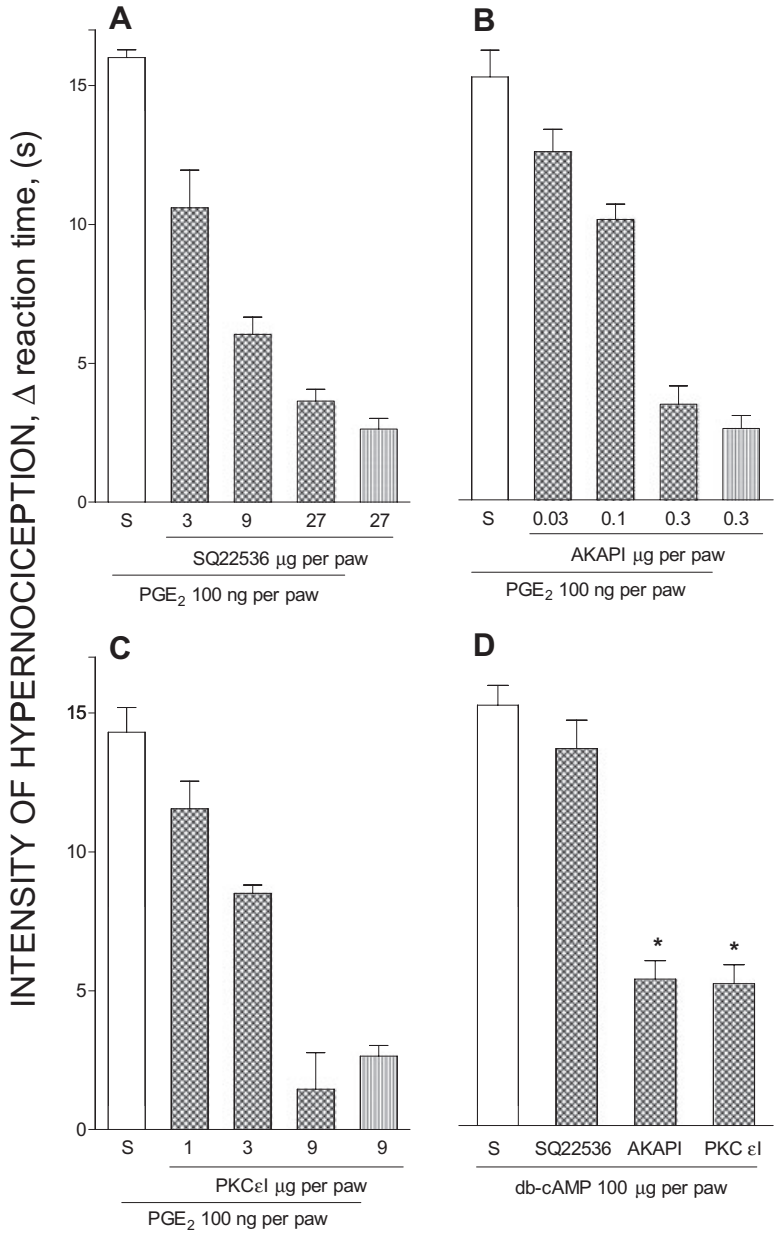

Figure 1 Effect of adenylyl cyclase, PKA or PKC inhibitors upon PGE2- or db-cAMP-evoked hypernociception. All inhibitors were given i.pl. and doses are shown as the dose per paw. In panel (A) adenylyl cyclase (SQ22536, 3, 9 or 27 μg), (B) PKA (AKAPI, 0.03, 0.1 or 0.3 μg), (C) PKCε (PKCεI, 1, 3 or 9 μg) inhibitors or saline (S, 50 μL) were injected 5 min before PGE2 (100 ng, i.pl.) injection. In (D) SQ22536 (27 μg), AKAPI (0.3 μg), PKCεI (9 μg) or saline (S, 50 μL) was injected 5 min before db-cAMP (100 μg; i.pl.) injection. The last bar of panels (A), (B) and (C) represents the effects of the inhibitors SQ22536 (27 μg), AKAPI (0.3 μg) or PKCεI (9 μg) injected alone into the paw respectively. The intensity of hypernociception was determined 3 h after i.pl. injection of PGE2 (100 ng), db-cAMP (100 μg) or saline (50 μL). The data are the means ± SEM of five animals per group. There were dose-dependent effects for SQ22536 (A; non-linear regression, R2 = 0.98), AKAPI (B; non-linear regression, R2 = 0.92) and PKCεI (C; non-linear regression, R2 = 0.96) pretreatments. *$P < 0.05$ compared with rats pretreated with saline and injected with db-cAMP (one-way ANOVA followed by Bonferroni test). AKAPI, A-kinase anchoring protein St-Ht31 inhibitor peptide; db-cAMP, N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate; i.pl., intraplantar; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKC, protein kinase C; PKCε, ε isoform of protein kinase C; PKCεI, $PKC\varepsilon V_{1-2}$ peptide, a selective PKCε inhibitor.

stimuli such as carrageenan and formalin (Cesare *et al.* 1999; Khasar *et al.*, 1999b; Souza *et al.*, 2002; Cunha *et al.*, 2004). Furthermore, PKCε is the isoform involved in nociceptor sensitization, as the mechanical nociception induced by carrageenan or formalin-induced nociceptive behaviour were reduced by treatment with inhibitors of PKCε (Khasar *et al.*, 1999a; Sweitzer *et al.*, 2004). Further, long-lasting hypernociception induced by PGE_2, in paws previously primed by carrageenan or TNFα, was associated with activation of neuronal PKCε (Aley *et al.*, 2000; Parada *et al.*, 2003; 2005).

In the present study, we confirmed that the treatment with cAMP or PKA inhibitors (Figure 1) prevents PGE_2-induced hypernociception, as already described (Taiwo and Levine, 1990; 1992; Aley and Levine, 1999; Parada *et al.*, 2005;

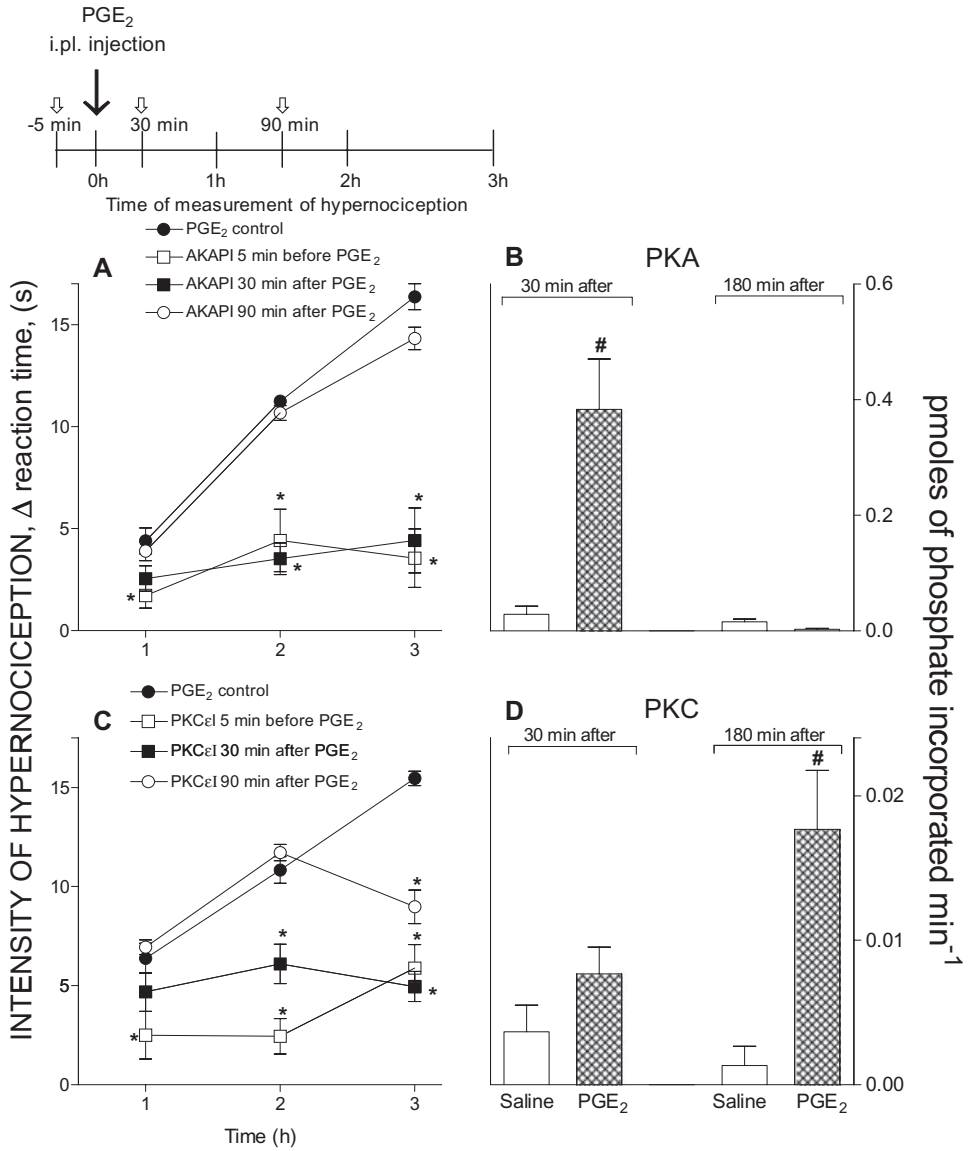

Figure 2 Time-dependence of the inhibitory effect of pre- or post-treatments with PKA (A) or PKC (C) inhibitors upon PGE_2-induced hypernociception. Note all i.pl. treatments are shown as the dose per paw. Panels (B) and (D) show the PKA and PKC activities in DRG (L4-L5) of rats injected i.pl. with PGE_2 (100 ng). (A) AKAPI (0.3 μg) or (C) PKCεI (9 μg) was administered 5 min before or 30 or 90 min after i.pl. injection of PGE_2 (100 ng). Inhibitors of PKA or PKC were given at the times indicated by the short arrows. The intensity of hypernociception was determined 1, 2 or 3 h after i.pl. injection of PGE_2. Inserted above panel (A) is a diagram showing the schedule of treatments and hypernociception determinations. The activities of PKA (B) and PKC (D) were evaluated in DRG (L4-L5) of the rats 30 or 180 min after i.pl. injection of saline (50 μL) or PGE_2 (100 ng) and expressed as pmoles of phosphate incorporated min^{-1}. The data are the means ± SEM of five animals per group in panels (A) and (C) and mean ± SEM of three animals per group in panels (B) and (D). *$P < 0.05$ compared with PGE_2-control rats. #$P < 0.05$ compared with rats injected with saline (one-way ANOVA followed by Bonferroni). AKAPI, A-kinase anchoring protein St-Ht31 inhibitor peptide; DRG, dorsal root ganglia; i.pl., intraplantar; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKC, protein kinase C; PKCε, ε isoform of protein kinase C; PKCεI, $PKC\varepsilon V_{1-2}$ peptide, a selective PKCε inhibitor.

Kassuya *et al*., 2007). In addition, we found that inhibition of the PKCε prevented PGE_2 or db-cAMP-induced hypernociception. These results suggest that an increase in cAMP levels is necessary for the activation of PKCε (directly or indirectly) for the induction of PGE_2 hypernociception. Further, the activation of PKA downstream from cAMP was involved in the onset of hypernociception, mainly in the initial 30 min. After that, a continuous PKCε activity was required for the development of hypernociception induced by PGE_2. As we have shown, in contrast with PKA, PKCε inhibitors significantly reduced the hypernociception induced by PGE_2 (Figure 2A,C) or db-cAMP (Figure 3A,C), by either early (30 min after) or later (90 min after) post-treatment. Together, these results suggest that PKA activity is essential in the early phase of the establishment of hypernociception, whereas PKC activity is involved in the maintenance of later phases of hypernociception. To support this suggestion, we investigated the activities of PKA and PKCε in the cell bodies of the DRG neurons after PGE_2 and db-cAMP injection in the rat paws, assuming that they reflect biochemical events occurring at peripheral

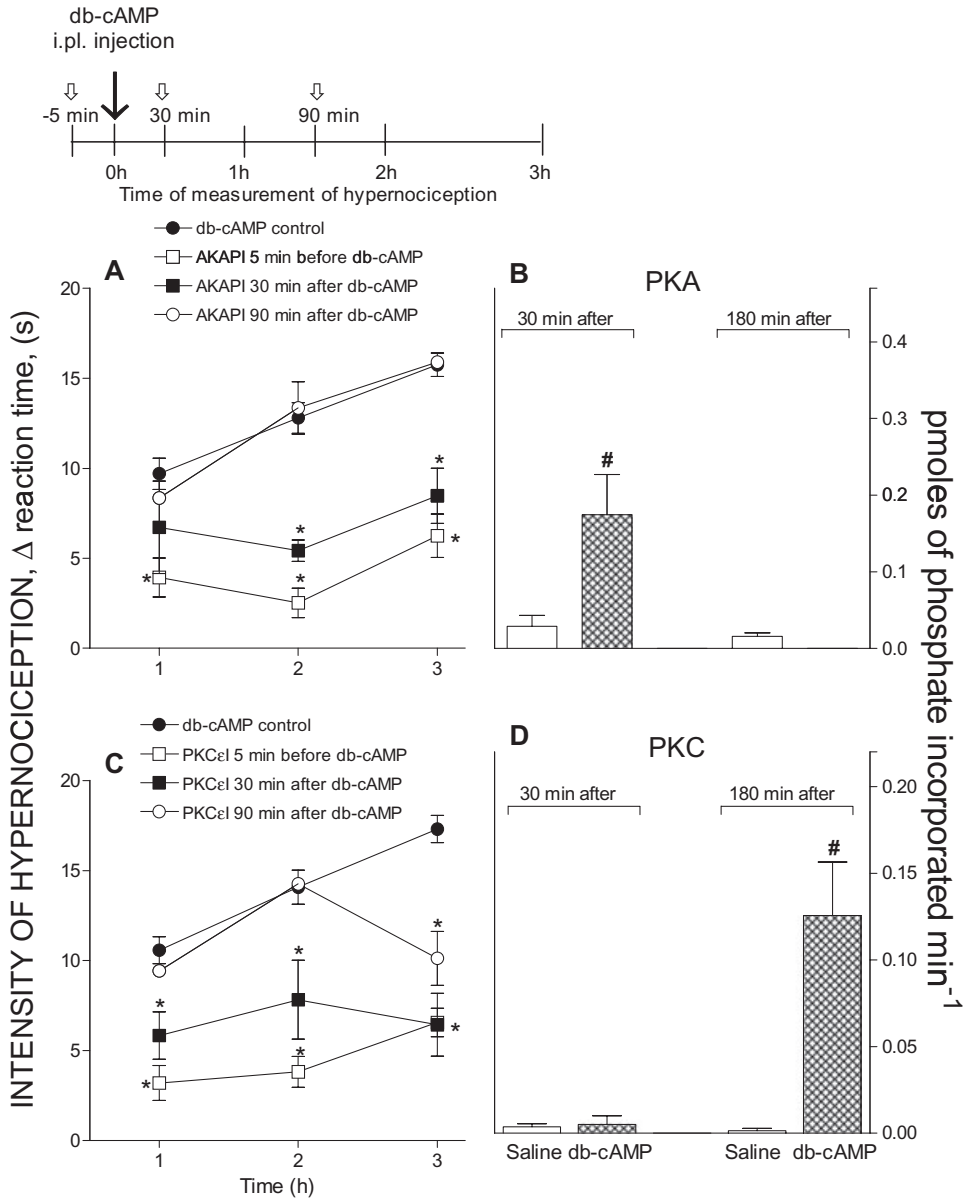

Figure 3 Time-dependence of the inhibitory effect of pre- or post-treatments with PKA (A) or PKC (C) inhibitors upon db-cAMP-induced hypernociception. All inhibitors were given i.pl. and doses are shown as the dose per paw. Panels (B) and (D) show the PKA and PKC activities in DRG (L4-L5) of rats injected i.pl. with db-cAMP (100 μg). (A) AKAPI (0.3 μg) or (C) PKCεI (9 μg) was administered 5 min before or 30 or 90 min after i.pl. injection of db-cAMP (100 μg). Inhibitors of PKA or PKC were given at the times indicated by the short arrows. The intensity of hypernociception was determined 1, 2 or 3 h after i.pl. injection of db-cAMP (100 μg). Inserted above panel (A) is a diagram showing the schedule of treatments and hypernociception determinations. The activities of PKA (B) and PKC (D) were evaluated in DRG (L4-L5) of the rats 30 or 180 min after intraplantar injection of saline (50 μL) or db-cAMP (100 μg) and expressed as pmoles of phosphate incorporated min^{-1}. The data are the means ± SEM of five animals per group in panels (A) and (C) and means ± SEM of three animals per group in panels (B) and (D). *$P < 0.05$ compared with db-cAMP-control rats treated with saline. #$P < 0.05$ compared with rats injected with saline (one-way ANOVA followed by Bonferroni). AKAPI, A-kinase anchoring protein St-Ht31 inhibitor peptide; db-cAMP, N^6,2′-O-dibutyryladenosine 3′ : 5′-cyclic monophosphate; DRG, dorsal root ganglia; i.pl., intraplantar; PKA, protein kinase A; PKC, protein kinase C; PKCε, ε isoform of protein kinase C; PKCεI, $PKC\varepsilon V_{1-2}$ peptide, a selective PKCε inhibitor.

terminations of sensory neurons. In fact, PKA activity increased in ipsilateral DRG (L4-L5) mainly in the early phase (30 min after the PGE or db-cAMP injection) and returned to basal levels 180 min after (Figures 2B and 3B). Although the kinetics of PKA activity induced by PGE_2 and db-cAMP were similar, the enzyme activity at 30 min after PGE_2 injection was higher that that induced by db-cAMP. Experiments using different doses of these mediators might explain this difference. The increase in PKC activity, however, was low when PKA levels were maximal and maximal when the PKA levels returned to the base line (Figures 2D and 3D). These results are in line with the assumption that PKC activation is downstream to the activation of PKA. Zhou *et al.* (2003) demonstrated that in paw inflammation induced by carrageenan, in which there is a significant activation of PKCε in the DRG, these kinases are synthesized and transcribed in the soma and

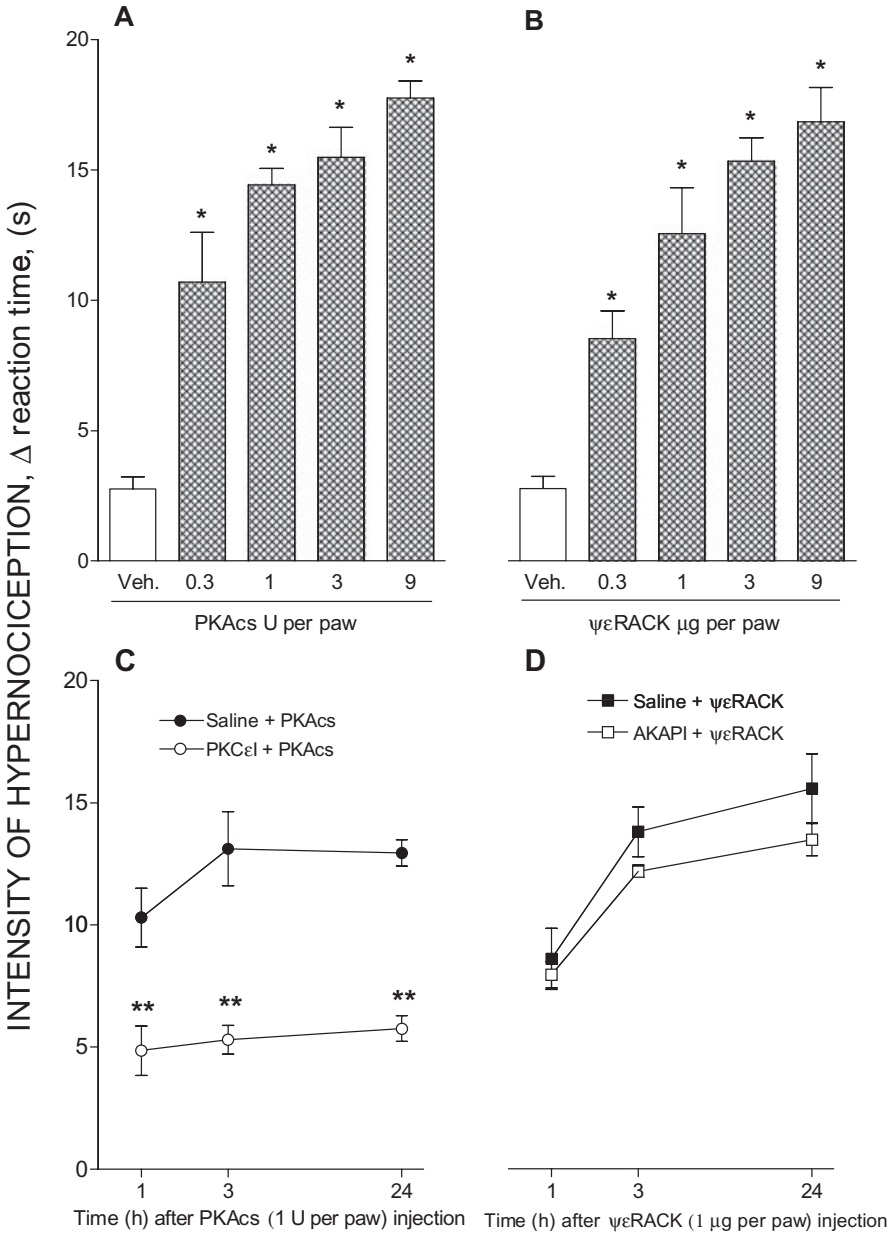

Figure 4 Crosstalk between PKA and PKCε in paw hypernociception. All i.pl. treatments are shown as the dose per paw. Panels (A) and (B): Hypernociceptive effect of the catalytic subunit of PKA (PKAcs) or a specific agonist of PKC (ΨεRACK). PKAcs (0.3, 1, 3 or 9 U) or ψεRACK (0.3, 1, 3 or 9 μg) was injected i.pl. and the intensity of hypernociception was determined 1 h after PKAcs (A) or ψεRACK (B) administration respectively. Panels (C) and (D): The effect of PKC inhibitor (PKCεI) upon PKAcs-evoked hypernociception and of PKA inhibitor (AKAP) upon ΨεRACK-evoked hypernociception. The pretreatment with PKCεI [9 μg; i.pl., panel (C)] or AKAPI [0.3 μg; i.pl., panel (D)] was performed 30 min before PKAcs (1 U; i.pl.) or ψεRACK (1 μg; i.pl.) injections respectively. The intensity of hypernociception was determined 1, 3 and 24 h after PKAcs (C) or ψεRACK (D) injection. The data are the means ± SEM of five animals per group. *$P < 0.05$ compared with control group i.pl. injected with vehicle (saline, 50 μL). **$P < 0.05$ compared with PKAcs-control rats treated with saline (50 μL). AKAPI, A-kinase anchoring protein St-Ht31 inhibitor peptide; i.pl., intraplantar; PKA, protein kinase A; PKAcs, catalytic subunit of PKA; PKCε, ε isoform of protein kinase C; ΨεRACK, pseudo receptor octapeptide for activated PKCε, a specific agonist of PKCε; PKCεI, $PKC\varepsilon V_{1-2}$ peptide, a selective PKCε inhibitor.

delivered to the nerve terminals by axonal transport. Finally, the PKCε inhibitor significantly reduced hypernociception induced by the PKAcs (Figure 4C). Nevertheless, the PKA inhibitor AKAPI did not change the hypernociceptive effect of ψεRACK (Figure 4D), thus supporting the suggestion that hypernociceptive effect induced by a PKAcs depends on PKCε activity. Hypernociception induced by PGE_2 did not cause variations in PKA and PKC activities in the paw tissues, probably because the nociceptive neurons constitute a relatively small portion of the total tissue of the paw (data not shown).

Besides, PKA and PKC and other kinases or factors such as (c-Jun N-terminalkinase (JNK), extracellular-regulated kinase, mitogen-activated protein kinases or cAMP-activated guanine exchange factor (Epac), all participate in the inflammatory response. Nevertheless, in acute mechanical hyperalgesia, PKCε and PKA (PGE_2)-mediated hyperalgesia was indepen-

dent of extracellular signal-regulated kinase (MEK) activity (Aley *et al.*, 2001). Further, JNK inhibition did not inhibit PGE_2-induced mechanical allodynia (Kassuya *et al.*, 2007). Additionally, p38 and JNK, are activated by the peripheral injection of PGE_2 and cause paw oedema in mice but no nociceptive behaviours (Claudino *et al.*, 2006). For this reason, we also did not evaluate the participation of those intracellular pathways in the inflammatory sensitization. There is an alternative mechanism by which cAMP might lead to activation of PKCε. The Epac can induce PKCε translocation via phospholipase C or D in DRG neurons (Hucho *et al.*, 2005). However, the Epac activator (CPTOMe) did not induce mechanical hypernociception in our behavioural test (data not shown).

We did not address the mechanism underlying PKA/PKC crosstalk in the present study. However, there is evidence pointing to a role for PKA in the activation and translocation of PKC. In fact, activation of dopamine D_1 receptors, known to couple to $G\alpha_s$, increases PKC activity and translocation in LTK cells (Yu *et al.*, 1996). In addiction, Huang *et al.* (2001) demonstrated that PKCζ is a key downstream component of a PKA-dependent, anti-apoptotic signalling pathway activated by a G protein-coupled receptor. Recently, Yao *et al.* (2008) demonstrated that activation of PKA by Sp-cAMPS (PKA activator) was sufficient to induce activity and translocation of PKCε.

In conclusion, the present study shows that both PKA and PKCε participate in acute mechanical hypernociception downstream from PGE_2 receptor activation and suggest that PKA may activate PKCε. Thus, our results describe a novel signalling pathway, in addition to the usual cAMP/PKA pathway, in mechanical hypernociception induced by PGE_2.

Acknowledgements

This study was supported by grants from the CNPq and FAPESP, Brazil. We thank IRS Schivo, SR Rosa and FL Mestriner for excellent technical assistance.

Conflict of interest

The authors state no conflict of interest.

References

Aley KO, Levine JD (1999). Role of protein kinase A in the maintenance of inflammatory pain. *J Neurosci* **19**: 2181–2186.

Aley KO, Messing RO, Mochly-Rosen D, Levine JD (2000). Chronic hypersensitivity for inflammatory nociceptor sensitization mediated by the epsilon isozyme of protein kinase C. *J Neurosci* **20**: 4680–4685.

Aley KO, Martin A, McMahon T, Mok J, Levine JD, Messing RO (2001). Nociceptor sensitization by extracellular signal-regulated kinases. *J Neurosci* **17**: 6933–6939.

Barber LA, Vasko MR (1996). Activation of protein kinase C augments peptide release from rat sensory neurons. *J Neurochem* **67**: 72–80.

Beebe SJ (1994). The cAMP-dependent protein kinases and cAMP signal transduction. *Semin Cancer Biol* **5**: 285–294.

Cesare P, Dekker LV, Sardini A, Parker PJ, McNaughton PA (1999). Specific involvement of PKC-e in sensitization of the neuronal response to painful heat. *Neuron* **23**: 617–624.

Claudino RF, Kassuya CAL, Ferreira J, Calixto JB (2006). Pharmacological and molecular characterization of the mechanisms involved in the prostaglandin E2 (PGE2)-induced edematogenic responses in mice. *J Pharmacol Exp Ther* **318**: 611–618.

Cunha FQ, Lorenzetti BB, Poole S, Ferreira SH (1991). Interleukin-8 as a mediator of sympathetic pain. *Br J Pharmacol* **104**: 765–767.

Cunha FQ, Poole S, Lorenzetti BB, Ferreira SH (1992). The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia. *Br J Pharmacol* **107**: 660–664.

Cunha FQ, Teixeira MM, Ferreira SH (1999). Pharmacological modulation of secondary mediator systems – cyclic AMP and cyclic GMP – on inflammatory hyperalgesia. *Br J Pharmacol* **127**: 671–678.

Cunha JM, Rae GA, Ferreira SH, Cunha FQ (2004). Endothelins induce ETB receptor-mediated mechanical hypernociception in rat hindpaw: roles of cAMP and protein kinase C. *Eur J Pharmacol* **501**: 87–94.

Di Castro A, Drew LJ, Wood JN, Cesare P (2006). Modulation of sensory neuron mechanotransduction by PKC- and nerve growth factor-dependent pathways. *Proc Nat Acad Sci USA* **103**: 4699–4704.

Dina OA, Barletta J, Chen X, Mutero A, Martin A, Messing RO *et al.* (2000). Key role for the epsilon isoform of protein kinase C in painful alcoholic neuropathy in the rat. *J Neurosci* **20**: 8614–8619.

Dina OA, Messing RO, Levine JD (2006). Ethanol withdrawal induces hyperalgesia mediated by PKCepsilon. *Eur J Neurosci* **24**: 197–204.

Distler C, Rathee PK, Lips KS, Obreja O, Neuhuber W, Kress M (2003). Fast Ca2+-induced potentiation of heat-activated ionic currents requires cAMP/PKA signaling and functional AKAP anchoring. *J Neurophysiol* **89**: 2499–2505.

Dorn GW II, Souroujon MC, Liron T, Chen CH, Gray MO, Zhou HZ *et al.* (1999). Sustained in vivo cardiac protection by a rationally designed peptide that causes epsilon protein kinase C translocation. *Proc Natl Acad Sci USA* **96**: 12798–12803.

England S, Bevan S, Docherty RJ (1996). PGE_2 modulates the tetrodotoxin-resistant sodium current in neonatal rat dorsal root ganglion neurones via the cyclic AMP-protein kinase A cascade. *J Physiol* **495**: 429–440.

Ferreira SH (1972). Prostaglandins, aspirin-like drugs and analgesia. *Nat New Biol* **240** (102): 200–203.

Ferreira SH, Nakamura M (1979). I-Prostaglandin hyperalgesia, a $cAMP/Ca^{2+}$ dependent process. *Prostaglandins* **18**: 179–190.

Ferreira SH, Lorenzetti BB, Correa FM (1978). Central and peripheral antialgesic action of aspirin-like drugs. *Eur J Pharmacol* **53**: 39–48.

Gray MO, Karliner JS, Mochly-Rosen D (1997). A selective epsilon-protein kinaseC antagonist inhibits protection of cardiac myocytes from hypoxiainduced cell death. *J Biol Chem* **272**: 30945–30951.

Handwerker HO, Neher KD (1976). Characteristics of C-fibre receptors in the cat's foot responding to stepwise increase of skin temperature to noxious levels. *Pflugers Arch* **365**: 221–229.

Huang NK, Lin YW, Huang CL, Messing RO, Chern Y (2001). Activation of protein kinase A and atypical protein kinase C by A(2A) adenosine receptors antagonizes apoptosis due to serum deprivation in PC12 cells. *J Biol Chem* **276**: 13838–13846.

Hucho TB, Dina OA, Levine JD (2005). Epac mediates a cAMP-to-PKC signaling in inflammatory pain: an isolectin B4(+) neuron-specific mechanism. *J Neurosci* **25**: 6119–6126.

Kassuya CA, Ferreira J, Claudino RF, Calixto JB (2007). Intraplantar PGE2 causes nociceptive behaviour and mechanical allodynia: the role of prostanoid E receptors and protein kinases. *Br J Pharmacol* **150**: 727–737.

Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D *et al.* (1999a). A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. *Neuron* **24**: 253–260.

Khasar SG, McCarter G, Levine JD (1999b). Epinephrine produces a beta-adrenergic receptor-mediated mechanical hyperalgesia and in

vitro sensitization of rat nociceptors. *J Neurophysiol* **81**: 1104–1112.

Leng S, Mizumura K, Koda H, Kumazawa T (1996). Excitation and sensitization of the heat response induced by a phorbol ester in canine visceral polymodal receptors studied in vitro. *Neurosci Lett* **206**: 13–16.

Liao X, Gunstream JD, Lewin MR, Ambron RT, Walters ET (1999). Activation of protein kinase A contributes to the expression but not the induction of long-term hyperexcitability caused by axotomy of Aplysia sensory neurons. *J Neurosci* **19**: 1247–1256.

Lorenzetti BB, Veiga FH, Canetti CA, Poole S, Cunha FQ, Ferreira SH (2002). Cytokine-induced neutrophil chemoattractant 1 (CINC-1) mediates the sympathetic component of inflammatory mechanical hypersensitivitiy in rats. *Eur Cytokine Netw* **13**: 456–461.

Malmberg AB, Brandon EP, Idzerda RL, Liu H, McKnight GS, Basbaum AI (1997). Diminished inflammation and nociceptive pain with preservation of neuropathic pain in mice with a targeted mutation of the type I regulatory subunit of cAMP-dependent protein kinase. *J Neurosci* **17**: 7462–7470.

Martin HA, Basbaum AI, Kwiat GC, Goetzl EJ, Levine JD (1987). Leukotriene and prostaglandin sensitization of cutaneous highthreshold C-mechanonociceptors in the rat. *Neuroscience* **22**: 651–659.

Mizumura K, Minagawa M, Tsujii Y, Kumazawa T (1993). Prostaglandin E_2-induced sensitization of the heat response of canine visceral polymodal receptors in vitro. *Neurosci Lett* **161**: 117–119.

Moita MA, Lamprecht R, Nader K, LeDoux JE (2002). A-kinase anchoring proteins in amygdala are involved in auditory fear memory. *Nat Neurosci* **5**: 837–838.

Moriyama T, Higashi T, Togashi K, Lida T, Segi E, Sugimoto Y *et al*. (2005). Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Mol Pain* **1**: 1–3.

Nakamura M, Ferreira SH (1987). A peripheral sympathetic component in inflammatory hyperalgesia. *Eur J Pharmacol* **135**: 145–153.

Nicol GD, Cui M (1994). Enhancement by prostaglandin E_2 of bradykinin activation of embryonic rat sensory neurones. *J Physiol* **480**: 485–492.

Ouseph AK, Khasar SG, Levine JD (1995). Multiple second messenger systems act sequentially to mediate rolipram-induced prolongation of prostaglandin E_2-induced mechanical hyperalgesia in the rat. *Neuroscience* **64**: 769–776.

Parada CA, Yeh JJ, Joseph EK, Levine JD (2003). Tumor necrosis factor receptor type-1 in sensory neurons contributes to induction of chronic enhancement of inflammatory hyperalgesia in rat. *Eur J Neurosci* **17**: 1847–1852.

Parada CA, Reichling DB, Levine JD (2005). Chronic hyperalgesic priming in the rat involves a novel interaction between cAMP and PKCepsilon second messenger pathways. *Pain* **113**: 185–190.

Pateromichelakis S, Rood JP (1982). Prostaglandin E1-induced sensitization of A delta moderate pressure mechanoreceptors. *Brain Res* **232**: 89–96.

Rueff A, Dray A (1993). Sensitization of peripheral afferent fibers in the *in vitro* neonatal rat spinal cord-tail by bradykinin and prostaglandins. *Neuroscience* **54**: 527–535.

Schaible HG, Schmidt RF (1988). Excitation and sensitization of fine articular afferents from cat's knee joint by prostaglandin E_2. *J Physiol* **403**: 91–104.

Scott JD (1991). Cyclic nucleotide-dependent protein kinases. *Pharmacol Ther* **50**: 123–145.

Smith JA, Davis CL, Burgess GM (2000). Prostaglandin E2-induced sensitization of bradykinin-evoked responses in rat dorsal root ganglion neurons is mediated by cAMP-dependent protein kinase A. *Eur J Neurosci* **12**: 3250–3258.

Souza AL, Moreira FA, Almeida KR, Bertollo CM, Costa KA, Coelho MM (2002). In vivo evidence for a role of protein kinase C in peripheral nociceptive processing. *Br J Pharmacol* **135**: 239–247.

Summer GJ, Puntillo KA, Miaskowski C, Dina OA, Green PG, Levine JD (2006). TrkA and PKC-epsilon in thermal burn-induced mechanical hyperalgesia in the rat. *J Pain* **12**: 884–891.

Sweitzer SM, Wong SM, Peters MC, Mochly-Rosen D, Yeomans DC, Kendig JJ (2004). Protein kinase C epsilon and gamma: involvement in formalin-induced nociception in neonatal rats. *J Pharmacol Exp Ther* **309**: 616–625.

Taiwo YO, Levine JD (1990). Direct cutaneous hyperalgesia induced by adenosine. *Neuroscience* **38**: 757–762.

Taiwo YO, Levine JD (1991). Further confirmation of the role of adenyl cyclase and of cAMP-dependent protein kinase in primary afferent hyperalgesia. *Neuroscience* **44**: 131–135.

Taiwo YO, Levine JD (1992). Serotonin is a directly-acting hyperalgesic agent in the rat. *Neuroscience* **48**: 485–490.

Taiwo YO, Bjerknes LK, Goetzl EJ, Levine JD (1989). Mediation of primary afferent peripheral hyperalgesia by the cAMP second messenger system. *Neuroscience* **32**: 577–580.

Taiwo YO, Heller PH, Levine JD (1992). Mediation of serotonin hyperalgesia by the cAMP second messenger system. *Neuroscience* **48**: 479–483.

Wang C, Li GW, Huang LY (2007). Prostaglandin E2 potentiation of P2X3 receptor mediated currents in dorsal root ganglion neurons. *Mol Pain* **3**: 22.

Wang JF, Khasar SG, Ahlgren SC, Levine JD (1996). Sensitization of C-fibres by prostaglandin E2 in the rat is inhibited by guanosine 5′-O-(2-thiodiphosphate), 2′,5′-dideoxyadenosine and Walsh inhibitor peptide. *Neuroscience* **71**: 259–263.

Yamamoto H, Kawamata T, Ninomiya T, Omote K, Namiki A (2006). Endothelin-1 enhances capsaicin-evoked intracellular Ca2+ response via activation of endothelin a receptor in a protein kinase Cepsilon-dependent manner in dorsal root ganglion neurons. *Neuroscience* **137**: 949–960.

Yao L, Fan P, Jiang Z, Gordon A, Mochly-Rosen D, Diamond I (2008). Dopamine and ethanol cause translocation of εPKC associated with εRACK: cross-talk between cAMP-Dependent Protein Kinase A and Protein Kinase C Signaling Pathways. *Mol Pharmacol* **73**: 1105–1112.

Yu PY, Eisner GM, Yamaguchi I, Mouradian MM, Felder RA, Jose PA (1996). Dopamine D1A receptor regulation of phospholipase C isoform. *J Biol Chem* **271**: 19503–19508.

Zhou Y, Li GD, Zhao ZQ (2003). State-dependent phosphorylation of epsilon-isozyme of protein kinase C in adult rat dorsal root ganglia after inflammation and nerve injury. *J Neurochem* **85**: 571–580.