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# The peripheral pro-nociceptive state induced by repetitive inflammatory stimuli involves continuous activation of protein kinase A and protein kinase C epsilon and its $Na_V1.8$ sodium channel functional regulation in the primary sensory neuron

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ABSTRACT

In the present study, the participation of the $Na_V1.8$ sodium channel was investigated in the development of the peripheral pro-nociceptive state induced by daily intraplantar injections of $PGE_2$ in rats and its regulation in vivo by protein kinase A (PKA) and protein kinase C epsilon (PKCε) as well. In the prostaglandin $E_2$ ($PGE_2$)-induced persistent hypernociception, the $Na_V1.8$ mRNA in the dorsal root ganglia (DRG) was up-regulated. The local treatment with dipyrone abolished this persistent hypernociception but did not alter the $Na_V1.8$ mRNA level in the DRG. Daily intrathecal administrations of antisense $Na_V1.8$ decreased the $Na_V1.8$ mRNA in the DRG and reduced ongoing persistent hypernociception. Once the persistent hypernociception had been abolished by dipyrone, but not by $Na_V1.8$ antisense treatment, a small dose of $PGE_2$ restored the hypernociceptive plateau. These data show that, after a period of recurring inflammatory stimuli, an intense and prolonged nociceptive response is elicited by a minimum inflammatory stimulus and that this pro-nociceptive state depends on $Na_V1.8$ mRNA up-regulation in the DRG. In addition, during the persistent hypernociceptive state, the PKA and PKCε expression and activity in the DRG are up-regulated and the administration of the PKA and PKCε inhibitors reduce the hypernociception as well as the $Na_V1.8$ mRNA level. In the present study, we demonstrated that the functional regulation of the $Na_V1.8$ mRNA by PKA and PKCε in the primary sensory neuron is important for the development of the peripheral pro-nociceptive state induced by repetitive inflammatory stimuli and for the maintenance of the behavioral persistent hypernociception.



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## 1. Introduction

The sensitization of nociceptors, which is associated with inflammatory pain, is described as the decrease in the nociceptive threshold or shortening of the nociceptive behavior end point. This decrease in the nociceptor threshold may correspond to the clinical state known as hyperalgesia, which is better defined in animal models such as hypernociception because the pain perception in animals is not obvious. It has been described that adenosine 3′,5′-cyclic monophosphate (cAMP) plays a pivotal role in the sensitization of nociceptors induced by inflammatory stimuli [1–6]. The increase of cAMP is frequently associated with the activation of protein kinase A (PKA) in distinct cell types [7–9], including primary sensory neurons [10]. In addition, it has been demonstrated that PKA participates in the inflammatory hypernociception [3,5,6,11]. There is growing evidence that cAMP could also activate protein kinase C (PKC) to signal metabolic events in the rat pheocromocytoma cell line PC12 [12] and in B lymphocytes [13]. Among the five PKC isoforms present in dorsal root ganglion (DRG) neurons, only the ε isoform is translocated, and consequently is activated after inflammatory stimuli [14]. Furthermore, PKCε is significantly up-regulated in the DRG after peripheral administration of carrageenan [15] and contributes greatly to the development of inflammatory hypernociception and sensitization of nociceptors [16,17].

Current evidences indicate that the downstream mechanisms leading to the increase of cytosolic cAMP during inflammatory hyperalgesia involve the activation of PKA and PKC, which ultimately up-regulates the tetrodotoxin-resistant (TTX-R) sodium currents [4,6,16,18–21]. In fact, the activation of PKC and PKA appears to be a necessary step in the inflammatory stimuli-dependent up-regulation of TTX-R sodium currents and consequently, sensitization of sensory neurons induced by inflammatory mediators [21,22]. The $Na_V1.8$ is a TTX-R sodium channel present in nociceptive primary afferent neurons, A-delta and C fibers, which participate in inflammatory hyperalgesia [21–25]. The $Na_V1.8$ channels have high thresholds for activation and rapid recovery from inactivation and slow inactivation rates, all contributing to a decrease in the threshold and an increase in the number of action potentials evoked from a sensitized neuron [26]. These biophysical properties make the $Na_V1.8$ channel a candidate for maintaining the sustained repetitive firing of nociceptors. We previously demonstrated that $Na_V1.8$ channels are important for the maintenance of persistent hypernociception in an animal model of chronic inflammatory sensitization of nociceptor [27]. In the prostaglandin $E_2$ ($PGE_2$)-induced persistent hypernociception, the $Na_V1.8$ transcripts in the DRG were up-regulated. In addition, the intrathecal administration of antisense oligodeoxynucleotides (ODN) against $Na_V1.8$ decreased the mRNA encoding $Na_V1.8$ in DRG and reduced ongoing $PGE_2$-induced persistent hypernociception. A parallel restoration of the persistent hypernociception and up-regulation of $Na_V1.8$ mRNA were observed after the cessation of ODN antisense treatment. However, the molecular mechanisms by which the $Na_V1.8$ up-regulation and the ongoing hypernociception itself are maintained were not established. Therefore, the aim of this investigation was to test the hypothesis that the functional interaction between PKA, PKCε and the $Na_V1.8$ channel in primary sensory neurons is an important mechanism for the maintenance of the inflammatory persistent hypernociception. For this purpose, we used a rat model of persistent mechanical hypernociception [28] in which a state of sensitization of the nociceptors lasts for at least 30 days following the cessation of 14 successive daily intraplantar injections of $PGE_2$.

## 2. Materials and methods

### 2.1. Animals

Experiments were performed on 180–400 g male Wistar rats housed in an animal care facility of the University of São Paulo and taken to the testing area at least 1 h before testing. Food and water were available ad libitum. Animal care and handling procedures were in accordance with the International Association for the Study of Pain (IASP) guidelines for the use of animals in pain research [29] 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 the number of animals used and any discomfort. All behavioral testing was performed between 9:00 a.m. and 4:00 p.m.

### 2.2. Nociceptive test: constant pressure rat paw test

In agreement with the new IASP Pain Terminology, the terms allodynia and hyperalgesia were not used in the present study. Instead, hypernociception was used to indicate an increase in the nociception due to the sensitization of the paws to mechanical stimuli. Multiple measurements of saline-treated paws did not alter basal animal responses, which were similar to the contralateral untreated paws. Mechanical hypernociception was tested in rats as previously described [30]. In this method, a constant pressure of 20 mm Hg (measured using a sphygmomanometer) is applied (via a syringe piston moved by compressed air) to a 15-$mm^2$ area on the dorsal surface of the hindpaw and discontinued when the rat presents a typical ''freezing reaction''. This reaction is composed of brief apnea, concomitant with the retraction of the head and forepaws and the reduction in the escape movements that animals normally make to free themselves from the position imposed by the experimental situation. Usually, the apnea is associated with successive waves of muscular tremor. For each animal, the latency to the onset of the freezing reaction is measured before administration (zero time) and at different times after the administration of the hypernociceptive agents. The intensity of mechanical hypernociception is quantified as the reduction in the reaction time, calculated by subtracting the value of the second measurement from the first [30].

### 2.3. Persistent hypernociception model

The persistent hypernociception model was induced as previously described [28]. The subcutaneous intraplantar

administration of $PGE_2$ (100 ng/paw) induces acute hypernociception measured 3 h after injection [30]. The induction of persistent hypernociception was performed by injecting $PGE_2$, at the same dose, over 14 days. In order to avoid a local release of prostaglandins caused by successive injections, all animals were treated with indomethacin (2 mg/kg) by intraperitoneal route 30 min before the $PGE_2$ or saline administration. Indometacin treatment continued during the period of persistent hypernociception induction. In the control group, the rat paw was injected daily with saline (100 μl). The intensity of hypernociception was measured before and after the daily i.pl. injection, with the control reaction time being the values measured at time zero on the first experimental day. After the discontinuation of the treatment, the hypernociception was evaluated once a day for 30 days.

### 2.4. PKA and PKC activity in dorsal root ganglia

After euthanasia with an overdose of sodium pentobarbital, the L4 and L5 DRG of rats were obtained. Next, the samples were frozen in dry ice and homogenized (Homogeneizador Kont). The PKA and PKC activity were determined using a commercial kit (Protein kinase A Assay kit, Protein kinase C Assay kit, Calbiochem), according to the manufacturer's instructions on DRG (L4–L5) of rats 5 days after the $PGE_2$-persistent hypernociception induction. The assay is based on the reaction of the enzyme sample with a biotinylated peptide substrate and [$^{32}\gamma$]P-ATP, and is expressed as pmoles of phosphate incorporated per minute.

### 2.5. Real time PCR reactions

For PCR experiments, total RNA was extracted from L4/L5 DRG (three rats per group) using the Trizol reagent (GIBCO, NY, USA). Complementary DNA (cDNA) was synthesized using 3 μg of RNA through a reverse transcription reaction (Superscript II, Gibco Life Technologies, Grand Island, NY, USA). Real Time PCR quantitative mRNA analyses were performed in an ABI Prism 5700 Sequence Detection System using the SYBR Green Fluorescence Quantification System (Applied Biosystems, Warrington, UK) for quantitation of amplicons. SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 400 nM of specific primers, and 2.5 ng of cDNA were used in each reaction. The PCR conditions were 95 °C (10 min), and then 40 cycles of 94 °C (1 min), 56 °C (1 min), and 72 °C (2 min), followed by the standard denaturation curve. The sequences of primers for PKA, PKCε and beta-actin were designed using the Primer Express software (Applied Biosystems, Warrington, UK) using nucleotide sequences present in the GenBank database. Nevertheless, to normalize the real-time PCR results, amplification of the beta-actin housekeeping gene was chosen. All assays were performed in triplicate and were validated for linearity of amplification efficiency and with quantitative standard curves obtained using serial dilutions of DRG rat cDNA. To ensure the absence of amplification artifacts, endpoint PCR products were initially assessed on ethidium bromide-stained agarose gels that gave a single band of the expected size for each assay. Negative controls containing no template cDNA were run in each condition and gave no results. The reactions were quantified when the PCR product of interest was first detected (cycle threshold, Ct). Calculations for determining the relative level of gene expression were performed with the cycle threshold (Ct) method, where the mean Ct values from triplicate measurements were use to calculate expression of the target gene, with normalization to the housekeeping gene beta-actin in the samples, using the $2^{-\Delta Ct}$ formula.

### 2.6. Drugs and administration

$PKC\varepsilon V_{1-2}$ peptide (a selective PKCε inhibitor) was obtained from SynPep Corp (Dublin, CA, USA). SQ22536 (adenylate cyclase inhibitor) was obtained from Biomol International (Plymouth, PA, USA). $PGE_2$ and dipyrone were obtained from Sigma-Aldrich (St Louis, MO, USA). InCELLlect® A-kinase anchoring protein St-Ht31 inhibitor peptide (AKAPI) was obtained from Promega Corp (Madison, WI, USA). Indomethacin was obtained from Prodome (Campinas, SP, Brazil). The indomethacin was diluted in Tris HCl buffer (pH 8,0). The stock solution of $PGE_2$ (1 μg/μl) was prepared in 10% ethanol, and additional dilutions were made in physiological saline (0.9% NaCl) to yield a final ethanol concentration less than 1%. All other drugs were dissolved directly in saline. The Protein kinase A Assay kit and Protein kinase C Assay kit were obtained from Calbiochem (La Jolla, CA, USA). The drugs were administrated either locally (intraplantar, i.pl.) or centrally (intrathecal, i.t.). The i.pl. administration consisted of a 50 μl per paw injection using a 27-gauge hypodermic needle connected to a 100 μl Hamilton microsyringe. The needle was introduced subcutaneously near the third digit reaching the middle of the hindpaw plantar surface. The i.t. administration was performed in rats lightly anesthetized and with the spinal column arched. A 30-gauge needle was inserted directly into the subarachnoid space between the L4 and L5 vertebrae, and ODNs were injected in a volume of 10 μl. The $Na_V1.8$ knock-down was produced by oligodeoxynucleotide (ODN) antisense treatment. The 18-mer ODNs used in this experiment were purchased from Invitrogen (São Paulo, SP, Brazil). The ODN antisense sequence, 5-GGG GAG CTC CAT CTT CTC-3, was directed against a unique sequence of the $Na_V1.8$ [27]. The mismatch-ODN sequence, 5-GGG G**TC** TTC CA**A GC**T CTC-3, was derived from the antisense sequence by scrambling six bases (denoted by bold face). ODNs were lyophilized and reconstituted in nucleasefree water 0.9% NaCl to a concentration of 2 μg/μl. ODNs were administered intrathecal (20 μg) once daily for 4 days.

### 2.7. Data analysis

Data are presented as means ± SEM of measurements made on 6 animals in each group and analyzed statistically by using a one-way ANOVA. When the ANOVA showed significant differences between groups, Tukey's post hoc test was used to determine the specific pairs of groups in which statistically significant differences occurred. Differences in the PKA or PKC mRNA and activity were analysed by the unpaired *t*-test. Statistical differences were considered to be significant at $P < 0.05$.

## 3. Results

### 3.1. $PGE_2$-induced persistent hypernociception

Fourteen daily i.pl. administrations of $PGE_2$ (100 ng/paw) produced hypernociceptive effects (15.2 ± 1.2 s) as described elsewhere (Fig. 1; see also [28]). Initially, the hypernociception progressed until a plateau was reached. The plateau occurred within 10 days of treatment (Fig. 1A) and lasted throughout the experimental period (30 days). There were no continuous traumatic inflammatory lesions due to the injections since the animals were pretreated with indomethacin, a typical anti-inflammatory agent. In addition, animals treated daily with saline (control) did not exhibit a significant change in their nociceptive threshold. A single intraplantar injection of dipyrone (160 μg/paw) produced a long-lasting inhibition of the $PGE_2$-induced persistent hypernociception (6.0 ± 2.3 s.) lasting the whole experimental period (Fig. 1B). A small dose of $PGE_2$ (10 ng/paw) induced a slight and short duration acute hypernociception in saline-treated rats (5.2 ± 0.4 s.). In contrast, the same dose of $PGE_2$ completely restored the hypernociceptive plateau in rats with persistent hypernociception abolished by dipyrone (Fig. 1B). The enhanced response to $PGE_2$ in these animals is suggestive of the development of a peripheral pro-nociceptive state that may be associated with primary afferent neuron plasticity. In fact, Real Time PCR (Fig. 1C) showed that the ipsilateral DRG tissues of rats with $PGE_2$-induced persistent hypernociception, treated or not with dipyrone, express more $Na_V1.8$ channel mRNA (12.1 ± 1.0 and 11.3 ± 2.2 relative mRNA transcript levels, respectively) in comparison with naïve or saline-treated control rats (6.3 ± 0.8 and 7.0 ± 0.9 relative mRNA transcript levels, respectively). In addition, when the contralateral DRG of rats with $PGE_2$-induced persistent hypernociception or control were evaluated, no difference of the $Na_V1.8$ mRNA levels was observed.

### 3.2. Effect of $Na_V1.8$ knockdown in the peripheral pro-nociceptive state induced by daily-$PGE_2$ treatment

Fig. 2 shows the effect of four daily intrathecal injections of ODNs on the nociceptive thereshold of hind paws of rats 5 days after the induction of persistent hypernociception with 14 daily injections of $PGE_2$. There was a progressive reduction in the intensity of persistent hypernociception during $Na_V1.8$ antisense treatment and a slow return to the original plateau. No effect was observed with mismatch treatment. A significant reduction of hypernociception intensity was observed 24 h after the first antisense injection (11.9 ± 1.1 s.). Differently from the dipyrone treatment (Fig. 1B), a small dose of $PGE_2$ (10 ng/paw) failed to restore the original hypernociceptive plateau (Fig. 2A) during the antisense treatment. In addition, the acute hypernociceptive response to $PGE_2$ (100 ng/paw) in antisense-treated rats with persistent hypernociception was similar to that observed in naïve rats (15.4 ± 1.3 and 15.8 ± 0.9 s, respectively; Fig. 3). As shown earlier [27], specific ODN antisense, but not mismatch treatment decreased $Na_V1.8$ transcripts. A gradual restoration of the persistent hypernociception (Fig. 2A) and $Na_V1.8$ mRNA level (Fig. 2B) was observed after the cessation of the ODN antisense treatment. When the hypernociceptive plateau was reestablished (17.4 ± 1.4 s) and $Na_V1.8$ mRNA returned to the up-regulated level (13.1 ± 0.9 relative mRNA transcript level), a small dose of $PGE_2$ (10 ng/paw) restored the persistent hypernociception blocked previously by dipyrone (160 μg/paw; Fig. 2).

### 3.3. Acute hypernociceptive effect of $PGE_2$ in $Na_V1.8$-knockdown rats

Fig. 3 represents the acute hypernociceptive response to $PGE_2$ (100 ng/paw) from rats in different conditions. The $Na_V1.8$ antisense, but not mismatch treatment, abolished the $PGE_2$-induced acute hypernociception in control rats but did not reduce the acute hypernociceptive response to $PGE_2$, at the same dose, in rats with persistent hypernociception (1.22 ± 1.1 and 15.4 ± 1.3 s, respectively).

### 3.4. PKA and PKCε expression and activity in DRG during the $PGE_2$-induced persistent hypernociception

PKA and PKCε have been suggested to be involved in the primary sensory neuron sensitization. To confirm this idea, the expression and activity of PKA and PKC were evaluated in DRG from rats 5 days after the induction of persistent hypernociception by 14 daily ipl. injections of $PGE_2$ (100 ng/paw). The mechanical nociceptive threshold was evaluated to confirm the development of persistent hypernociception (data not shown). Rats were then anesthetized and the DRG (L4–L5) were removed. The expression and activity of PKA and PKCε were evaluated by Real Time PCR and radioactivity assay, respectively. The mRNA levels of PKA (1.20 ± 0.01 relative mRNA transcript level) and PKCε (1.87 ± 0.07 relative mRNA transcript level) were enhanced in ipsilateral DRG of animals with persistent hypernociception as compared with saline-treated control rats (0.33 ± 0.21 and 1.33 ± 0.02 relative mRNA transcript level, respectively. $P = 0.042$ and $P < 0.0001$; Fig. 4A). Similarly, during the persistent hypernociception the activities of PKA (0.22 ± 0.04 pmole) and PKCε (0.23 ± 0.02 pmole) were enhanced in ipsilateral DRG as compared with control rats (0.0021 ± 0.0009 and 0.005 ± 0.003 pmole, $P < 0.0001$; Fig. 4B). The contralateral DRG did not exhibit significant differences. These results suggest a role for PKA and PKCε in the persistent hypernociception induced by repeated inflammatory stimuli. To investigate this idea, behavioral studies were designed.

### 3.5. Effect of PKA and PKCε inhibition on the behavioral $PGE_2$-induced persistent hypernociception and $Na_V1.8$ expression in DRG

Fig. 5 shows the effect of PKA, PKCε or adenylate cyclase (AC) inhibition on persistent hypernociception induced by 14 daily ipl. injections of $PGE_2$ (100 ng/paw). A single i.pl. injection of the PKA inhibitor (AKAPI; PKAi; 0.3 μg/paw, panel B), PKCε inhibitor (PKCεI; PKCεi; 9 μg/paw, panel C), or its combination (PKAi 0.3 μg/paw + PKCεi 9 μg/paw; panel D), but not the AC inhibitor (SQ22536; ACi; 25 μg/paw, panel A), reduced the $PGE_2$-induced persistent hypernociception (8.5 ± 0.6; 5.1 ± 1.3; 6.1 ± 1.9 and 14.5 ± 2.1 s, respectively). The DRG of the animals used in this experiment were taken to examine the effects of

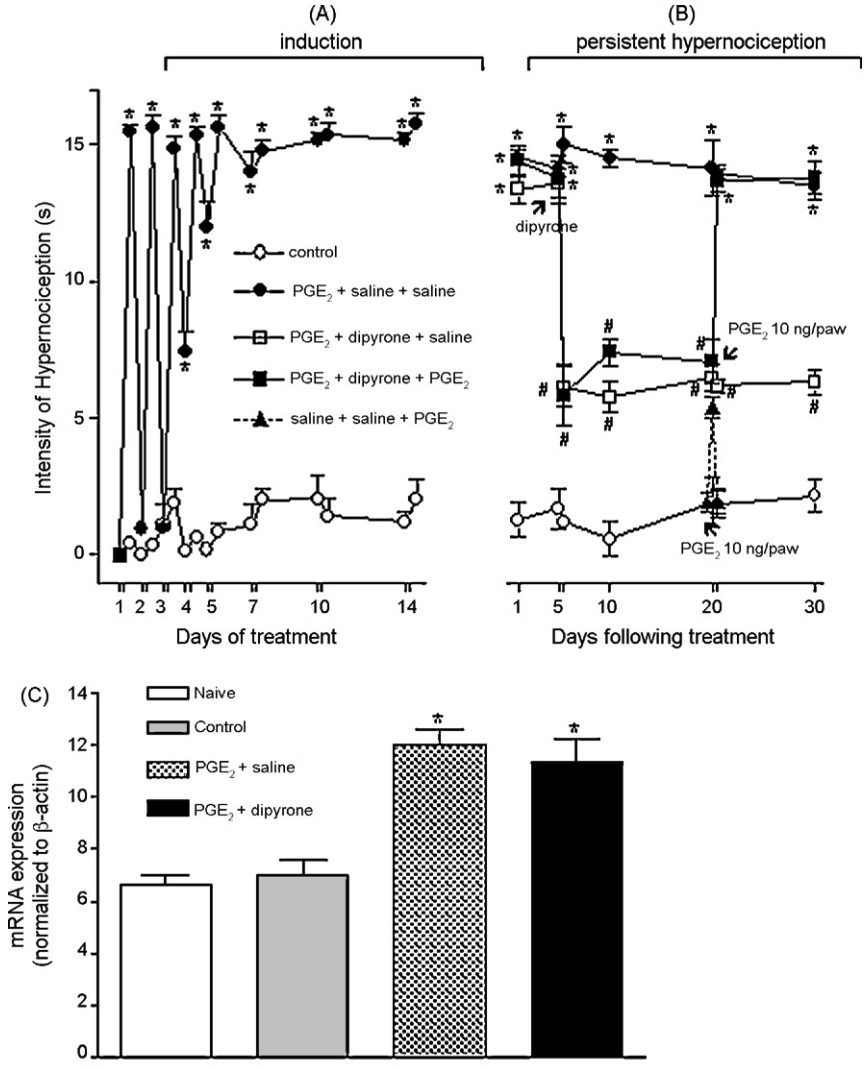


**Fig. 1 – $PGE_2$-induced persistent hypernociception and $Na_V1.8$ sodium channel expression. The intensity of hypernociception was measured before (*x* axis, first tick) and 3 h after (*x* axis, second tick) $PGE_2$ (100 ng/paw) or saline (100 μl/paw; control group) injection (induction, panel A), and once a day during the 30 days following the discontinuation of $PGE_2$ or saline injections (persistent hypernociception, panel B). The figure shows data points for only selected days. Fourteen-daily injections of $PGE_2$ (filled balls) induced a persistent hypernociception that lasted for more than 30 days, when compared with the saline (open balls) control group ($P < 0.05$). Five days after the induction, the blockade of $PGE_2$-induced persistent hypernociception was obtained by i.pl. administration of dipyrone (160 μg, filled and open squares, panel B) but not by i.pl injection of saline (100 μl, filled balls, panel B). The mechanical nociceptive threshold was measured 1 h after the i.pl. administration of dipyrone or saline (indicated by arrow). Panel B also shows the restoration of the persistent hypernociception by i.pl. administration of $PGE_2$ (10 ng) made 15 days after the dipyrone-induced blockade of persistent hypernociception (filled square). However, if $PGE_2$ at the same dose was injected in control animals, only a slight and short duration hypernociceptive effect was observed (filled triangle). Restoration of hypernociception was measured 3 h after $PGE_2$ injections. Data are expressed as mean ± SEM ($n = 6$). $^*P < 0.05$ compared with the control group; $\#P < 0.05$ compared with $PGE_2$ + saline + saline group; tested by one-way ANOVA with Tukey's post hoc. Real Time PCR data shows (panel C) the level of $Na_V1.8$ mRNA in DRG from animals with $PGE_2$–induced persistent hypernociception, 1 day after the treatment with dipyrone or saline, as well as control and naïve animals. Real Time PCR assay was performed in triplicate and with three rats per group. Beta-actin mRNA was used to normalize the relative amount of $Na_V1.8$ mRNA. Data are expressed as mean ± SEM. $^*P < 0.05$ compared with control group; tested by one-way ANOVA with Tukey's post hoc.**

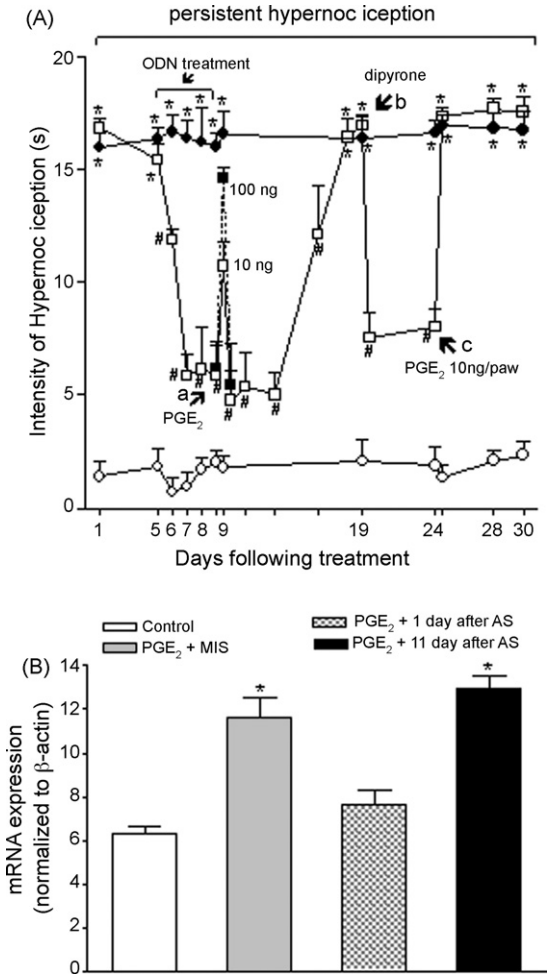


**Fig. 2 – The lack of restoration of $PGE_2$-induced persistent hypernociception in $Na_V1.8$ knock-down rats. Five days following the induction of persistent hypernociception by fourteen daily injections of $PGE_2$ (100 ng/paw), the animals received four daily intrathecal administrations of oligodeoxynucleotide (ODN, 15 μg/10 μl; panel A) antisense (open and filled square) or mismatch (filled balls). Twenty-four hours after ODN antisense treatment, the rats' paws were injected with $PGE_2$ 10 (open square) or 100 (filled square) ng/paw (arrow A). Eleven days after OND treatment, the rats' paws were injected with dipyrone (160 μg, arrow B) and, 5 days later, with $PGE_2$ 10 ng/paw (arrow C). In the control group, the rat paws were injected daily with saline (100 μl, open balls). Data are expressed as mean ± SEM ($n$ = 6). *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with mismatch group; tested by one-way ANOVA with Tukey's post hoc. Real Time PCR (panel B) shows the level of $Na_V1.8$ mRNA in DRG from rats in a state of persistent hypernociception after the ODN treatment. Real Time PCR assay was performed in triplicate and with three rats per group. The bars present groups treated with 14 daily injections of saline (control group), and 14 daily injections of $PGE_2$ + mismatch or antisense ODN treatment (1 day or 11 days after the last antisense injection). $Na_V1.8$ antisense, but not mismatch, significantly decreased the $Na_V1.8$ mRNA measured 1 day after last ODN injection. Eleven days after last ODN antisense injection, the $Na_V1.8$ mRNA was restored to its up-regulated level. Beta-actin mRNA was used to normalize the relative amount of $Na_V1.8$ mRNA. Data are expressed as mean ± SEM. *$P < 0.05$ compared with control group; tested by one-way ANOVA with Tukey's post hoc.**

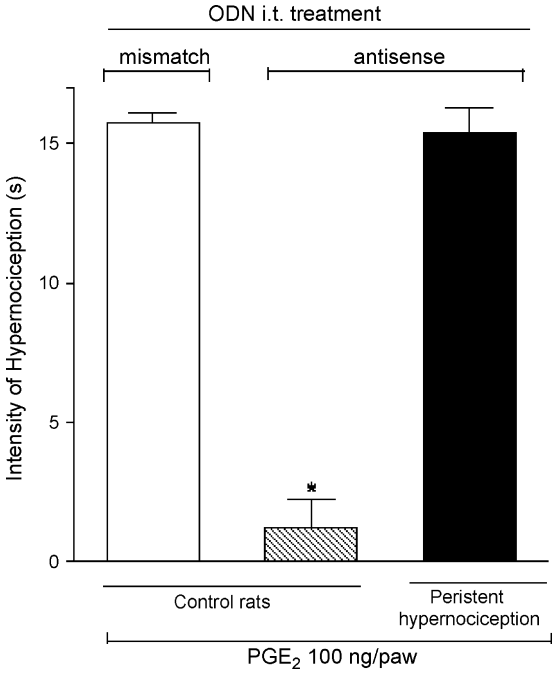


**Fig. 3 – Acute hypernociceptive response to the intraplantar injection of $PGE_2$ in $Na_V1.8$ knock-down rats with or without persistent hypernociception. Animals with persistent hypernociception induced by 14 daily i.pl. injections of $PGE_2$ (100 ng/paw) were four daily intrathecal administered with oligodeoxynucleotide (ODN, 15 μg/10 μl/i.t.) antisense (filled bar). Animals without persistent hypernociception (daily treated with saline, control rats) were four daily intrathecal administered with ODN antisense (cross-hatched bar) or mismatch (open bar). Twenty-four hours after last ODN injection, the $PGE_2$ (100 ng/paw) was injected into the rats' paws. The intensity of hypernociception was measured 3 h after the $PGE_2$ injection. Data are expressed as mean ± SEM ($n$ = 6). *$P < 0.05$ compared with remaining groups; tested by one-way ANOVA with Tukey's post hoc.**

PKA and PKCε inhibition on the $Na_v1.8$ expression (Fig. 6). The i.pl. injection of PKAi (0.3 μg/paw) or PKCεi (9 μg/paw) did not significantly alter the $Na_v1.8$ mRNA levels 23 days after the drug administration. However, the administration of the combination of the same doses of PKA with PKCε inhibitors significantly diminished the mRNA $Na_v1.8$ levels (2.75 ± 0.15 relative mRNA transcript level). These data indicate an important role of PKA and PKCε in the regulation of the $Na_v1.8$ expression in primary sensory neurons during the $PGE_2$-induced persistent hypernociception.

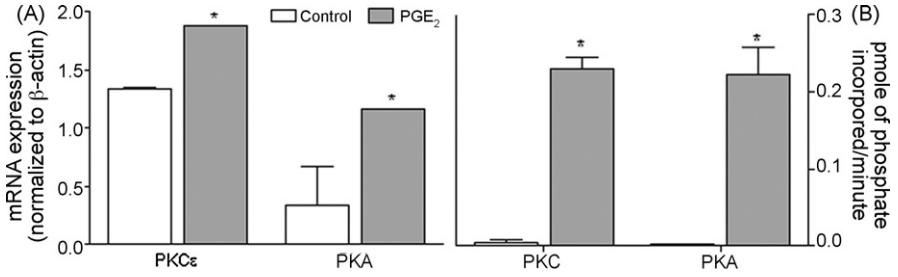


**Fig. 4 – PKA and PKCε expression and activity in DRG during $PGE_2$-induced persistent hypernociception. Five days after completion of the $PGE_2$ treatment, persistent hypernociception was confirmed using the behavioral test (data not shown in the figure). Afterward, the DRG from the animals were removed to evaluate the PKA and PKCε mRNA levels or activities. Panel A: Real Time PCR shows the level of PKCε or PKA mRNA in DRG from rats in a state of persistent hypernociception (gray bars, 14 daily i.pl. injections of $PGE_2$ 100 ng/paw) or without persistent hypernociception (open bars, 14 daily i.pl. injections of saline; control group). Panel B: Shows the PKA or PKC activity (pmole of phosphate incorporated/minute) in DRG from animals with persistent hypernociception (gray bars) or control animals (open bars). Data are expressed as mean ± SEM ($n = 6$). $^*P < 0.05$ compared with the control group; tested by Unpaired *t*-test.**

## 4. Discussion

It is generally accepted that the inflammatory hypernociception results from the sensitization of primary sensory neurons [31,32]. Experimental protocols in rats demonstrated that the metabotropic event initiated by the activation of adenylate cyclase/PKA and phospholipase C/PKC pathways results in the lowering of the nociceptor threshold [3,4,33,34], an important event associated with nociceptive sensitization. There is increasing evidence that the $Na_V1.8$ channel may play a role in the sensitization of the nociceptor, and its modulation by PKA and PKC in vitro has been described [35]. We have demonstrated here that the functional regulation of the $Na_V1.8$ mRNA by PKA and PKCε in the primary sensory neuron is important for the development of the peripheral pro-nociceptive state induced by repetitive inflammatory stimuli and for the maintenance of the behavioral persistent hypernociception.

Daily injections of $PGE_2$ caused the development of a persistent hypernociceptive state that was inhibited by a single intraplantar injection of dipyrone at doses that are known not to cause systemic effects [36]. A small dose of $PGE_2$ induces a slight and short duration of acute hypernociception in saline-treated rats. This procedure, however, fully restored the hypernociceptive state in rats in which the persistent hypernociception was inhibited by dipyrone. These data show that after the induction of the nociceptive sensitization by recurring inflammatory stimuli, an intense and prolonged nociceptive response is elicited by a minimum inflammatory stimulus. The pain hypersensitivity can be a consequence of early post-translational changes, occurring at the peripheral terminals of the primary afferent and in dorsal horn neurons, as well as later transcription-dependent changes in effector genes and again in primary sensory and dorsal horn neurons. We have previously demonstrated that the increase of $Na_V1.8$ mRNA in the DRG is essential to the maintenance of the persistent hypernociception in rats [27]. In the same way, the pro-nociceptive state described here could be associated with the $Na_V1.8$ mRNA up-regulation in primary sensory neurons. In fact, the expression and biophysical properties of $Na_V1.8$ channels can be modulated by the ongoing nociceptive input. For example, there is increased expression and redistribution of the $Na_V1.8$ channel in animal models of inflammatory [37,39] and neuropathic pain [38], injury in humans [40–42] and painful human dental pulp [43]. Similarly, local administration of inflammatory mediators increases the magnitude of the TTX-R sodium current in peripheral nerves through modulation of the voltage-dependent properties of the $Na_V1.8$ channel [22,44]. This idea is reinforced by the results shown in Figs. 1 and 2: an up-regulation of $Na_V1.8$ transcripts in DRG is observed during persistent hypernociception, confirming our previous data; after the behavioral hypernociception blocked by dipyrone, a small dose of $PGE_2$ restores the hypernociceptive plateau in rats with up-regulation of the $Na_V1.8$ mRNA; once the persistent hypernociception and $Na_V1.8$ mRNA were reduced by antisense treatment, a small dose of $PGE_2$ failed to restore the hypernociceptive plateau. In addition, the $Na_V1.8$ antisense treatment abolished the $PGE_2$–induced acute hypernociception in naïve rats but failed to reduce it in rats with persistent hypernociception (Fig. 3). These results could be explained by the $Na_V1.8$ mRNA level after the antisense treatment: in rats with persistent hypernociception and consequently, up-regulated levels of $Na_V1.8$, the reduction of the $Na_V1.8$ mRNA induced by antisense were not sufficient to affect the $PGE_2$-induced acute hypernociception.

Persistent pain states are known to be associated with changes in $Na_V1.8$ channels [40,41,45–47]. In line with this idea, the present study demonstrates that the up-regulated level of $Na_V1.8$ mRNA in the primary sensory neuron observed during the persistent hypernociception is, at least partially, responsible for the hypersensitivity to nociceptive stimuli. However, the biochemical mechanisms through which the $Na_V1.8$ up-regulation and the ongoing hypernociception itself are maintained are not yet established.

Biochemical, peptide mapping, and mutational analysis of sodium channel have shown that the cytoplasmic loop possesses several sites for PKA and PKC phosphorylation [48–50]. In addition, PKA activation by hyperalgesic agents results in an increase in the amplitude of peak TTX-R currents

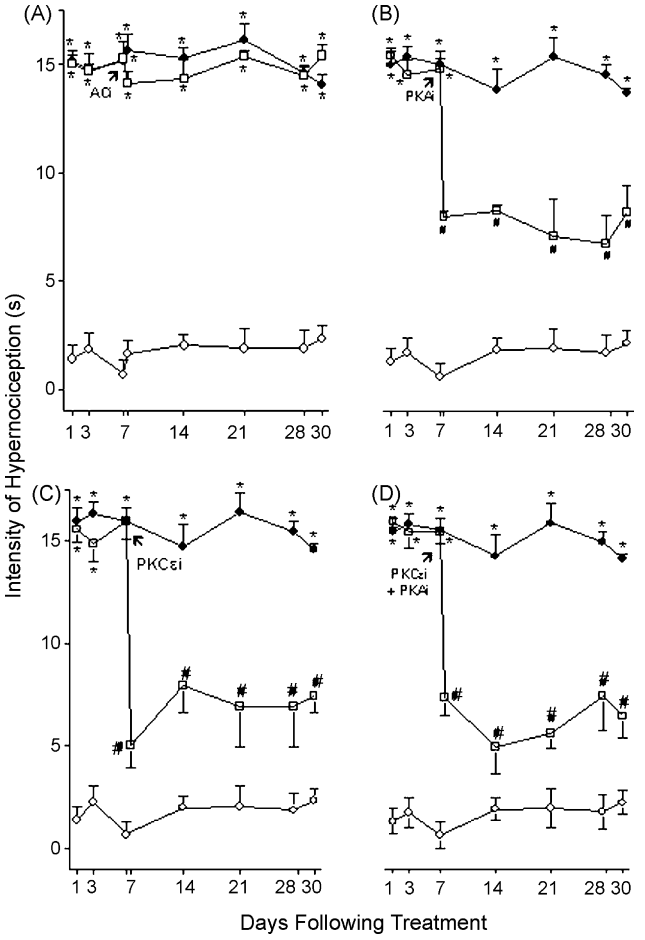


**Fig. 5 – Effect of PKA and PKCε inhibition on the behavioral $PGE_2$-induced persistent hypernociception. Seven days after the $PGE_2$ daily treatment finish, we performed an i.pl. only injection of AC inhibitor (SQ22536, ACi; 25 μg/paw, panel A), PKA inhibitor (AKAPI, PKAi; 0.3 μg/paw, panel B), PKCε inhibitor (PKCεI, PKCεi; 9 μg/paw, panel C) or a combination of PKA and PKCε inhibitors (PKAi 0.3 μg/paw + PKCεi 9 μg/paw; panel D). The intensity of hypernociception was measured after 1 h and daily during the 23 days following the injection of the inhibitors. The figure shows data points for only selected days. In the control group, rat paws were injected daily with saline (100 μl, open balls). Data are expressed as mean ± SEM ($n = 6$). *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with the remaining groups; tested by one-way ANOVA with Tukey's post hoc.**

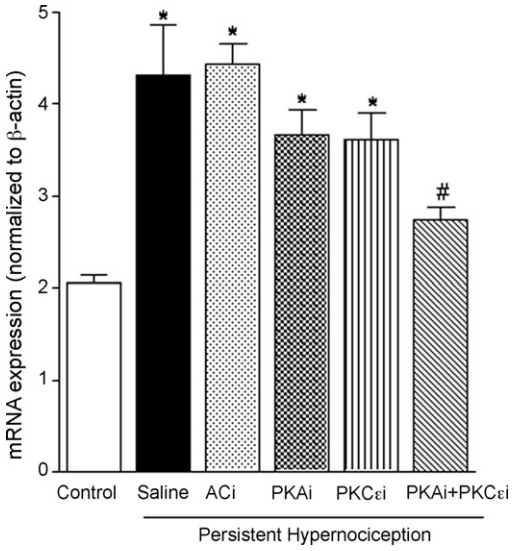


**Fig. 6 – Effect of PKA and PKCε inhibition on the $Na_v1.8$ expression in DRG. Seven days after the $PGE_2$ daily treatment finish, we performed i.pl. only injection of saline, PKA inhibitor (AKAPI, PKAi; 0.3 μg/paw), PKCε inhibitor (PKCεI, PKCεi; 9 μg/paw) or the combination of PKA and PKCε inhibitors (PKAi 0.3 μg/paw + PKCεi 9 μg/paw). Twenty-three days later, the DRGs of the animals were removed to evaluate the $Na_v1.8$ mRNA levels. In the control group, rat paws were injected daily with saline (100 μl). Data are expressed as mean ± SEM ($n = 6$). *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with the saline and ACi groups; tested by one-way ANOVA with Tukey's post hoc.**

in sensory neurons [22,51]. Similar effects are observed with cloned $Na_V1.8$ channels expressed in COS-7 cells, when they are stimulated by PKA activators [52]. Functional regulation of sodium channels by PKC also has been shown. In sensory neurons, PKC activators cause a dose-dependent increase in the amplitude of TTX-R sodium currents, and inhibitors of PKC reduce the $PGE_2$-induced modulation of TTX-R currents [21]. These in vitro studies suggest a functional regulation of TTX-R sodium channels by PKA and PKC, however, the biological relevance of this regulation still needs to be demonstrated in vivo. The next experimental series was conducted to test the hypothesis that the functional interaction between PKA, PKCε and $Na_V1.8$ channel in the primary sensory neuron is important to the maintenance of the behavioral persistent hypernociception.

We observed that not only the expression (Fig. 4A), but also the activity (Fig. 4B) of PKA and PKCε were enhanced in the DRG from rats with persistent hypernociception when compared with saline-treated control rats. Other studies using in vivo models have reported increased PKA and PKC activity and expression in primary sensory neurons during hyperalgesia [15,53–56]. The involvement of PKA and PKCε in the nociceptive sensitization has been suggested [2,5,6,16–18,57]. In line with this idea, we described here that the adenylate cyclase inhibitor did not reduce the installed persistent hypernociception, while both the PKA and PKCε inhibitors reduced the persistent hypernociception during the entire experimental period (Fig. 5). In addition to the behavioral hypernociception, PKA and PKCε inhibitors, but not AC inhibitor, reduced the up-regulated level of the $Na_V1.8$ mRNA in the DRG from these rats (Fig. 6).

Our results indicate that, despite of the recognized cAMP role in the acute hypernociception induction, once established, inflammatory persistent hypernociception is no longer maintained by adenylate cyclase activity with subsequent cAMP synthesis, but rather, is dependent on ongoing PKA and

PKCε activity. If cAMP levels remain elevated, cells undergo changes in their PKA signaling system; some cells alter the rate of degradation of PKA subunits, while others change the stability of the messages encoding subunits [58–60]. Therefore, after neuron exposure to hypernociceptive mediators, such as $PGE_2$, PKA activity becomes persistently elevated at basal cAMP levels [61]. On the other hand, hypernociceptive mediators activate G-protein-coupled receptors, resulting in a calcium influx from ion channels, as well as calcium release from intracellular stores. Intracellular calcium increase will lead to a direct activation of PKC and calcium/calmodulin-dependent kinase that activates adenylate cyclase causing PKA activation [62]. Taken together, these results suggest that the persistent hypernociception induced by repetitive exposure to $PGE_2$, may result from both the PKA persistent activity induced by prolonged exposure to cAMP and the resulting persistent activity of the PKC due to intracellular calcium increase. In addition, the functional modulation of the $Na_V1.8$ channel by PKA and PKCε in the primary sensory neuron can contribute to the hypernociception maintenance. In fact, the phosphorylation of TTX-R sodium channels by PKA and PKC in DRG neurons results in an increase in the TTX-R current amplitude in these cells [21,22,44], which would be expected to increase excitability of the neuron by decreasing the threshold and increasing action potential electrogenesis, thus producing an increased nociceptive response. Because the $Na_V1.8$ channel is mainly expressed in nociceptive neurons [26,63] and contributes a majority of the sodium current underlying the depolarizing phase of the action potential [47], and because the generation of action potentials is the final common pathway for neural output, $Na_V1.8$ channels are ideally positioned to serve as an important point of regulation of neuronal signal transduction.

In summary, the present data led us to postulate that the up-regulated level of $Na_V1.8$ mRNA in the primary sensory neuron is, at least in part, responsible by the delayed peripheral pro-nociceptive state induced by repetitive inflammatory stimuli. The data presented here also indicate that the continuous activity of PKCε and PKA and the $Na_V1.8$ channel functional modulation in the primary sensory neuron is crucial to the maintenance of the inflammatory persistent hypernociception (Fig. 7). The relationship between our findings and clinical pain disorders is not yet clear, but it is very probable that alterations in function and expression of these channels are intimately linked to neuronal hyperexcitability present in many types of chronic pain. Therefore, the discrete localization of the $Na_V1.8$ channel in the nociceptive neurons may provide an opportunity for the development of drugs targeted at these channels to achieve efficacious chronic pain relief with an acceptable safety profile.

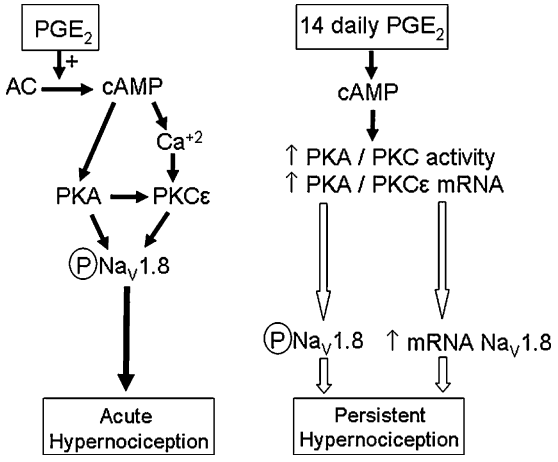


**Fig. 7 – Schematic diagram illustrating the postulated intracellular mechanisms in the primary sensory neuron involved with acute and persistent hypernociception. $PGE_2$ induces acute hypernociception by adenylate cyclase/cAMP/PKA/$Na_V1.8$ and cAMP/$Ca^{+2}$/PKC/$Na_V1.8$ second messenger cascades activation. The daily treatment with $PGE_2$ induces persistent hypernociception resulting from the PKA and PKC continuous activity induced by prolonged exposure to cAMP and its constant $Na_V1.8$ channel functional modulation. Enhanced PKA, PKCε and $Na_V1.8$ mRNA levels contribute to the ongoing hypernociception maintenance. Transient and maintained conditions are represented as black and white arrows, respectively.**

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