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## Second Messengers Mediating the Expression of Neuroplasticity in a Model of Chronic Pain in the Rat

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### Abstract

Hyperalgesic priming is a model of the transition from acute to chronic pain, in which previous activation of cell surface receptors or direct activation of protein kinase C epsilon (PKC $\epsilon$ ) markedly prolongs mechanical hyperalgesia induced by pronociceptive cytokines. We recently demonstrated a role of peripheral protein translation, alpha-calmodulin-dependent protein kinase II ( $\alpha$ CaMKII) activation and the ryanodine receptor, in the induction of hyperalgesic priming. In the present study we tested if they also mediate the prolonged phase of PGE<sub>2</sub>-induced hyperalgesia. We found that inhibition of  $\alpha$ CaMKII and local protein translation eliminates the prolonged phase of PGE<sub>2</sub> hyperalgesia. While priming induced by receptor agonists or direct activation of PKC $\epsilon$  occurs in male but not female rats, activation of  $\alpha$ CaMKII and the ryanodine receptor also produces priming in females. As in males, the prolonged phase of PGE<sub>2</sub>-induced hyperalgesia in female rats is also PKC $\epsilon$ -,  $\alpha$ CaMKII- and protein translation-dependent. In addition, in both male and female primed rats the prolonged PGE<sub>2</sub>-induced hyperalgesia was significantly attenuated by inhibition of MEK/ERK. Based on these data we suggest that the mechanisms previously shown to be involved in the induction of the neuroplastic state of hyperalgesic priming also mediate the prolongation of hyperalgesia.

### Keywords

Second messengers; hyperalgesic priming; sensory neuron; mechanical hyperalgesia; rat

### Introduction

Hyperalgesic priming, a model of the transition from acute to chronic pain produced by a prior inflammatory insult, is expressed as a long-lasting neuroplastic state in which there is enhanced hyperalgesia induced by agents such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), adenosine and

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serotonin<sup>3,38,41</sup>. The induction of hyperalgesic priming is triggered by a transient activation of protein kinase C epsilon (PKC $\epsilon$ )<sup>3,39</sup> or molecules downstream of PKC $\epsilon$ , including alpha calmodulin-dependent protein kinase II ( $\alpha$ CaMKII) and the ryanodine receptor<sup>20</sup>, inducing translation of mRNAs in the peripheral terminal of the nociceptor<sup>19,20</sup> that are responsible for the chronic maintenance of this state<sup>6,19</sup>.

In the naïve rat, PGE<sub>2</sub>-induced hyperalgesia is of short duration (~1 h) and dependent on activation of stimulatory G-protein (Gs) and protein kinase A (PKA)<sup>1</sup>. However, in the primed state, the hyperalgesia induced by PGE<sub>2</sub> is markedly prolonged, lasting more than 24 h<sup>3</sup>, due to a novel linkage of prostaglandin receptor activation to an additional signaling pathway involving an inhibitory G-protein (Gi), phospholipase C beta 3 (PLC $\beta$ 3) and PKC $\epsilon$  during the prolonged phase of the PGE<sub>2</sub>-induced hyperalgesia<sup>3,15,22,28,32,37,41</sup>.

In this study we show that the prolonged phase of PGE<sub>2</sub>-induced hyperalgesia also involves  $\alpha$ CaMKII, local protein translation, and the MEK/ERK pathway, mechanisms not involved in signaling for PGE<sub>2</sub>-induced hyperalgesia in the naïve state<sup>2</sup>. We also show that these mechanisms contribute to the prolonged phase of PGE<sub>2</sub>-induced hyperalgesia in female rats in which hyperalgesic priming can be induced by  $\alpha$ CaMKII or ryanodine receptor activation.

## Materials and Methods

### Animals

All experiments were performed on adult male and female Sprague Dawley rats (220–400 g; Charles River Laboratories). Animals were housed, 3 per cage, under a 12-h light/dark cycle in a temperature- and humidity-controlled room in the animal care facility of the University of California, San Francisco. Food and water were available *ad libitum*. All nociceptive testing was done between 10:00 am and 5:00 pm and the experimental protocols were approved by the Institutional Animal Care and Use Committee at University of California at San Francisco and adhered to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All effort was made to minimize the number of animals used and their suffering.

### Mechanical nociceptive threshold testing

Mechanical nociceptive threshold was quantified using an Ugo Basile Analgesymeter<sup>®</sup> (Randall-Selitto paw-withdrawal test, Stoelting, Chicago, IL), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw, as previously described<sup>41,48,50</sup>. The nociceptive threshold was defined as the force in grams at which the rat withdrew its paw, and baseline paw-pressure threshold defined as the mean of the three readings taken before the test agents were injected. Each paw was treated as an independent measure and each experiment performed on a separate group of rats. Data are presented as mean change from baseline mechanical nociceptive threshold.

## Drugs

The following drugs were used in this study: prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), the ryanodine receptor activator ryanodine, and the specific inhibitor of MEK 1/2 U0126 ([1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene]<sup>13</sup>, all from Sigma-Aldrich (St. Louis, MO); alpha calcium/calmodulin-dependent protein kinase II recombinant (activated  $\alpha$ CaMKII, New England Biolabs, Ipswich, MA), the CaMKII inhibitor peptide CaM2INtide (GenScript, Piscataway, NJ), PKC $\epsilon$ V<sub>1-2</sub>, a PKC $\epsilon$  specific translocation inhibitor peptide (PKC $\epsilon$ -I)<sup>27,33</sup> (Calbiochem, La Jolla, CA), the PKC $\epsilon$  activator  $\psi$ eRACK (Biomatik, Delaware, USA), and the protein translation inhibitors cordycepin 5'-triphosphate sodium salt [Sigma-Aldrich (St. Louis, MO)] and rapamycin [EMD Chemicals (Gibbstown, NJ)]. The selection of the drug doses used in this study was based on our previous studies<sup>3,19,33,36,37,49</sup>.

Stock solutions of PGE<sub>2</sub> in absolute ethanol (1  $\mu$ g/ $\mu$ l) were further diluted in 0.9% NaCl (1:50,  $C_{final}$ =0.2  $\mu$ g/ $\mu$ l) immediately before injection. The ethanol concentration of the final PGE<sub>2</sub> solution was ~2% and the injection volume 5  $\mu$ l. Stock solutions of cordycepin (10  $\mu$ g/ $\mu$ l, dissolved in a 1:1 mixture of 0.9% NaCl and absolute ethanol) or rapamycin (20  $\mu$ g/ $\mu$ l, dissolved in absolute DMSO) were further diluted in 0.9% NaCl or distilled water, respectively, immediately before injection. The ethanol or DMSO concentration in the final solutions was ~2%.

Activation of  $\alpha$ CaMKII was performed *in vitro* and a dose of 25 ng, in a volume of 2.5  $\mu$ l, of the activated  $\alpha$ CaMKII was injected intradermally on the dorsum of the rat hind paw.  $\alpha$ CaMKII was diluted in 1X NEBuffer for PK (50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 2mM DTT, 0.01% Brij 35, pH 7.5 at 25°C) supplemented with 200  $\mu$ M ATP, 1.2  $\mu$ M calmodulin and 2 mM CaCl<sub>2</sub>, and incubated for 10 min at 30°C before injection.

Drugs were administered intradermally on the dorsum of the hind paw via a beveled 30-gauge hypodermic needle attached to a Hamilton<sup>®</sup> microsyringe (Reno, NV, USA) by a short length of polyethylene (PE-10) tubing. The administration of all drugs, except PGE<sub>2</sub>, was preceded by a hypotonic shock to facilitate cell permeability to these agents (2  $\mu$ l of distilled water, separated by a bubble of air to avoid mixing in the same syringe), to get compounds into the nerve terminal<sup>7,9</sup>.

### Oligodeoxynucleotide antisense to $\alpha$ CaMKII

The oligodeoxynucleotide (ODN) antisense sequence for the  $\alpha$ -subunit of CaMKII, 5'-GGT AGC CAT CCT GGC ACT-3' (Invitrogen), was directed against a unique region of the rat mRNA sequence. The corresponding NCBI GenBank accession number and ODN position within the mRNA-sequence are NM\_012920 and 33 to 50, respectively. That this antisense can be used to downregulate the expression of  $\alpha$ CaMKII has been shown previously<sup>11</sup>. The ODN mismatch<sup>10</sup> sequence 5'-GGT AGC CAT **AAG** GGC ACT-3' corresponds to the antisense sequence with 3 bases mismatched (denoted in bold).

Before use, the ODNs were lyophilized and reconstituted in 0.9% NaCl to a concentration of 2  $\mu$ g/ $\mu$ l. During each injection, rats were briefly anesthetized with 2.5% isoflurane in 95% O<sub>2</sub>. A 30-gauge hypodermic needle was inserted into the subarachnoid space on the midline,

between the L4 and L5 vertebrae. A total of 40 µg ODN in a volume of 20 µl was slowly injected. Proper intrathecal injections were systematically confirmed by checking for a sudden flicking of the tail, a reflex that is evoked by subarachnoid space access and bolus injection<sup>35</sup>. The animals regained consciousness approximately 1 minute after the injection. The use of antisense to manipulate the expression of proteins in nociceptors, important for their role in nociceptor sensitization, is well supported by previous studies by others<sup>40,45,46,47</sup> as well as our group<sup>6,18,21,39</sup>.

### **Prolonged phase of PGE<sub>2</sub>-induced mechanical hyperalgesia**

To evaluate signaling mechanisms involved in the prolongation phase of the PGE<sub>2</sub>-induced mechanical hyperalgesia observed in our model of chronic pain, we injected one of 3 agents that induce priming,  $\psi$ εRACK, activated αCaMKII or ryanodine, intradermally on the dorsum of the rat's hind paw<sup>3,20</sup>. These agents induce mechanical hyperalgesia that resolves in 3–5 days ( $\psi$ εRACK), ~10 days (activated αCaMKII) or less than 24 h (ryanodine), and after the return of the mechanical thresholds to baseline values, the intradermal injection of PGE<sub>2</sub> at the same site produces enhanced and prolonged mechanical hyperalgesia that lasts more than 4 h and is still significant at 24 h, as opposed to the effect of intradermal injection of PGE<sub>2</sub> in control animals, that lasts ~1 h<sup>3,37,39</sup>. In this study we performed the injection of PGE<sub>2</sub> one ( $\psi$ εRACK- and ryanodine-treated rats) or two (αCaMKII-treated rats) weeks after the priming stimulus. Of note, while activation of PKCε was shown to produce hyperalgesic priming only in male rats<sup>29</sup>, the activation of αCaMKII or ryanodine receptors is able to induce priming in female as well as male rats<sup>20</sup>.

### **Statistics**

In all experiments, the dependent variable was paw-withdrawal threshold, expressed as the percentage change from baseline. The average paw withdrawal thresholds before and after (1 or 2 weeks, depending on the stimulus) the injection of the priming stimuli was 116.0 ± 1.4 g and 115.8 ± 1.2 g, respectively ( $n = 210$  paws). Of note, no statistically significant difference between paw withdrawal threshold values was observed between groups of rats subsequently injected with  $\psi$ εRACK ( $t_{11}=0.08305$ ,  $p = 0.9353$ ), activated αCaMKII ( $t_{89}=0.3258$ ,  $p = 0.7453$ ) or ryanodine ( $t_{107}=0.8813$ ,  $p = 0.3801$ ), and in these groups immediately before the injection of PGE<sub>2</sub> (paired Student's  $t$ -test). In addition, the αCaMKII antisense treatment did not induce changes in the mechanical nociceptive threshold by itself ( $p = \text{NS}$ ). To compare the hyperalgesia induced by PGE<sub>2</sub> injection in different groups, two-way repeated-measures ANOVA, followed by Bonferroni post-test, was performed. Graph Pad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) was used to plot the graphics and to perform the statistical analysis;  $P < 0.05$  was considered statistically significant. Data are presented as mean ± SEM.

## **Results**

### **Role of αCaMKII in the prolonged phase of PGE<sub>2</sub>-induced hyperalgesia in the primed animal**

We investigated if αCaMKII participates in the signaling pathway involved in the prolonged phase of PGE<sub>2</sub>-induced hyperalgesia in states of hyperalgesic priming induced by

$\psi$ eRACK, activated  $\alpha$ CaMKII or ryanodine. Male rats previously primed by activation of PKC $\epsilon$  in the hind paw, using  $\psi$ eRACK (Fig. 1A), were treated with intrathecal injection of antisense or mismatch for  $\alpha$ CaMKII mRNA for 3 consecutive days and, on the 4<sup>th</sup> day, the  $\alpha$ CaMKII inhibitor CaM2INTide or its vehicle was injected on the dorsum of the hind paw at the site of nociceptive testing. This protocol, combining intrathecal antisense treatment and pharmacological inhibition of  $\alpha$ CaMKII in the paw is based on our initial study of the role of  $\alpha$ CaMKII in hyperalgesic priming<sup>20</sup>, in which we attempted to achieve as complete inhibition of  $\alpha$ CaMKII as possible. At the same site (on the dorsum of the hind paw), 15 min later, PGE<sub>2</sub> was injected; mechanical hyperalgesia was evaluated 30 min and 4 h after PGE<sub>2</sub>. We observed that at 4 h but not 30 min the hyperalgesia induced by PGE<sub>2</sub> was significantly attenuated in the rats treated with the  $\alpha$ CaMKII antisense plus the inhibitor. In control rats treated with mismatch plus vehicle there was no significant attenuation ( $***p < 0.0001$ , when both groups are compared at the 4 h time point). Similarly, when the same protocol was applied to rats that had previously received intradermal injection of activated  $\alpha$ CaMKII (two weeks before, Fig. 1B) or ryanodine (one week before, Fig. 1C) on the dorsum of the hind paw, the magnitude of the PGE<sub>2</sub>-induced mechanical hyperalgesia was significantly decreased at 4 h but not 30 min in the  $\alpha$ CaMKII antisense plus inhibitor-treated groups, when compared to the control groups ( $***p < 0.0001$ , in both cases, when the  $\alpha$ CaMKII antisense plus the inhibitor groups are compared to the mismatch plus vehicle groups at the 4 h time point), showing that  $\alpha$ CaMKII plays a role in the prolonged phase of PGE<sub>2</sub>-induced hyperalgesia in the primed condition produced by previous activation, in the rat hind paw, of PKC $\epsilon$ ,  $\alpha$ CaMKII or the ryanodine receptor.

### **Prolonged phase of PGE<sub>2</sub>-induced hyperalgesia in male rats primed with ryanodine depends on local protein translation**

Local administration of protein translation inhibitors such as cordycepin or rapamycin at the site of nociceptive testing, on the dorsum of the rat hind paw, permanently reverses hyperalgesic priming previously produced by injection of  $\psi$ eRACK<sup>19</sup> or  $\alpha$ CaMKII<sup>20</sup> at the same site, suggesting that the maintenance of the primed state is dependent on ongoing protein translation in the peripheral terminal of the nociceptor. We tested if hyperalgesic priming induced by injection of ryanodine on the dorsum of the rat hind paw also depends on ongoing protein translation in the periphery. We observed (Fig. 2) that local injection of cordycepin or rapamycin reversed ryanodine-induced hyperalgesic priming, i.e., hyperalgesia induced by a PGE<sub>2</sub> challenge delivered 15 min after local administration of either cordycepin or rapamycin in ryanodine-primed rats is not prolonged ( $***p < 0.001$  for both cases, when compared to the vehicle group).

### **Activated $\alpha$ CaMKII- and ryanodine-induced priming: role of PKC $\epsilon$**

We tested if the prolonged phase of hyperalgesia induced by a PGE<sub>2</sub> challenge in the primed state involves a similar PKC $\epsilon$ -dependent signaling pathway to that which has been demonstrated in hyperalgesic priming induced by PKC $\epsilon$  activation<sup>3,14,16,38,39</sup>. Male and female rats previously treated with activated  $\alpha$ CaMKII (Fig. 3A) or ryanodine (Fig. 4A) on the dorsum of the hind paw, received an intradermal injection of PGE<sub>2</sub> 5 min after the administration of vehicle or PKC $\epsilon$ -I, at the same site. PKC $\epsilon$ -I, but not vehicle, significantly attenuated the prolongation of PGE<sub>2</sub> hyperalgesia, evaluated 4 h after PGE<sub>2</sub> injection (Fig.

3A, male rats:  $p = 0.0004$ ; female rats:  $p < 0.0001$ ; Fig. 4A, male rats:  $p = 0.0001$ ; female rats:  $p < 0.0015$ , when compared to the control groups).

### **$\alpha$ CaMKII and local protein translation are also involved in the PGE<sub>2</sub> signaling in female rats primed with activated $\alpha$ CaMKII or ryanodine**

When female rats previously primed with activated  $\alpha$ CaMKII (Fig. 3B) or ryanodine (Fig. 4B) on the dorsum of the hind paw and treated with intrathecal injections of antisense against  $\alpha$ CaMKII for 3 days received intradermal injection of the  $\alpha$ CaMKII inhibitor CaMINTide and, 10 min later, PGE<sub>2</sub>, both at the same site in the hind paw, a significant attenuation of the prolonged phase of hyperalgesia measured at the 4 h time point was also observed (Fig. 3B,  $p < 0.0001$ ; Fig. 4B,  $p < 0.0001$ ), compared to the primed rats treated with mismatch and CaMINTide vehicle plus PGE<sub>2</sub>. In addition, the chronic increased sensitivity to PGE<sub>2</sub>, induced by previous injection of activated  $\alpha$ CaMKII or ryanodine, on the dorsum of the hind paw of female rats, was also attenuated by the injection of the protein translation inhibitors cordycepin or rapamycin at the same site (Fig. 3C,  $p < 0.0001$ ; Fig. 4C,  $p < 0.0001$ , when compared to the control groups). These results indicate that, similarly to the hyperalgesic priming induced by activation of PKC $\epsilon$  on the dorsum of the hind paw of male rats, the prolongation of PGE<sub>2</sub> hyperalgesia induced by previous activation of  $\alpha$ CaMKII or ryanodine receptors in male and female rats, is also dependent on a switch from PKA to the addition of PKC $\epsilon$  and  $\alpha$ CaMKII signaling, and local protein synthesis.

### **Prolongation of PGE<sub>2</sub>-induced hyperalgesia in primed rats is also dependent on the MEK/ERK pathway**

It has been demonstrated that the MEK/ERK pathway plays a role in the prolongation of the PGE<sub>2</sub>-induced hyperalgesia, in rats previously primed with  $\psi$ eRACK<sup>17</sup>. Thus, since we observed similarities in the second messengers involved in the enhanced and prolonged PGE<sub>2</sub>-induced hyperalgesia in the  $\psi$ eRACK-induced priming and the priming induced by activated  $\alpha$ CaMKII or ryanodine, we investigated if MEK/ERK also has a role in the expression of the plasticity produced in the two latter models. Pretreatment with the specific inhibitor of MEK 1/2 U0126 on the dorsum of the hind paw, but not with vehicle, 10 min before the injection of PGE<sub>2</sub> in the same site that previously received intradermal injection of activated  $\alpha$ CaMKII (Fig. 5A) or ryanodine (Fig. 5B) significantly attenuated the PGE<sub>2</sub>-induced hyperalgesia at the 4<sup>th</sup> h after injection in male and female rats (Fig. 5A, male rats:  $p < 0.0001$ ; female rats:  $p = 0.0011$ ; Fig. 5B, male rats:  $p < 0.0001$ ; female rats:  $p = 0.0005$ , when compared to the vehicle groups). This result indicates that the previous treatment with activated  $\alpha$ CaMKII or ryanodine in the hind paw induced a switch in the PGE<sub>2</sub> signaling pathway, the prolongation of PGE<sub>2</sub> hyperalgesia now dependent on the MEK/ERK pathway.

## **Discussion**

Our group developed a model in rat of the transition from acute to chronic pain, referred to as hyperalgesic priming, in which a prior inflammatory stimulus induces very long-term neuroplasticity in primary afferent nociceptor function<sup>3,37,39,41</sup> that changes the signaling pathway activated by direct-acting inflammatory mediators, such as PGE<sub>2</sub>, adenosine and



serotonin<sup>3</sup>, so that the hyperalgesia they produce is markedly prolonged. One of the main signaling molecules responsible for the prolongation of PGE<sub>2</sub> hyperalgesia is PKC $\epsilon$ <sup>3,39,41</sup>.

That the primed state persists for months suggested that it is maintained by a more long-term change in the nociceptor other than simple activation of PKC $\epsilon$ , and subsequent studies showed a role of local protein translation in the priming of the peripheral nociceptor<sup>19</sup>. Furthermore, this protein translation depends on activation of 3 molecules previously shown to be involved in learning and memory (another form of long-lasting neuroplasticity<sup>20</sup>): the cytoplasmic polyadenylation element binding protein (CPEB; a molecule that regulates translation of dormant mRNAs)<sup>42,51</sup>,  $\alpha$ CaMKII<sup>8,10,12,25,26,54</sup>, and ryanodine receptors (which can activate  $\alpha$ CaMKII,<sup>43,44,53</sup>). In this study, we tested if 3 molecules that participate in the induction of the primed state, PKC $\epsilon$ ,  $\alpha$ CaMKII and the ryanodine receptor<sup>20</sup>, also play a role in the prolonged phase of hyperalgesia that is induced by a PGE<sub>2</sub> challenge in the primed state.

We previously showed that the prolonged phase of hyperalgesia induced by PGE<sub>2</sub> in the primed state in male rats is mediated by a novel addition of a delayed Gi - PLC $\beta$ 3 and PKC $\epsilon$  signaling pathway<sup>15,28,37</sup>. In this study we found that PKC $\epsilon$  also participates in the PGE<sub>2</sub>-induced hyperalgesia in male rats primed with ryanodine, confirming that the changes in the nociceptor produced by both stimuli (activated  $\alpha$ CaMKII or ryanodine) fit in the definition of hyperalgesic priming.

Another interesting feature regarding the hyperalgesic priming model is that while the activation of PKC $\epsilon$  does not induce priming in female rats<sup>29</sup>, activation of PKC $\epsilon$  does induce priming in ovariectomized female rats similarly to that observed in the male rat, an effect reversed by estrogen replacement<sup>29</sup>. These findings support the suggestion that the action of estrogen at the level of PKC $\epsilon$  was the limiting step in the cascade of events, in female rats, that lead to protein translation in the terminal of the nociceptor and, ultimately, to the development of hyperalgesic priming. In fact, we have further demonstrated that activation of  $\alpha$ CaMKII or the ryanodine receptor induces priming in female as well as male rats<sup>20</sup>. Our current experiments showed that the prolongation of PGE<sub>2</sub>-induced hyperalgesia in female rats primed with  $\alpha$ CaMKII or ryanodine is also PKC $\epsilon$ -dependent in the late phase (evaluated at the 4<sup>th</sup> h). Thus, even though PKC $\epsilon$  is not involved in the production of priming in females, it plays a role in its expression. Moreover, considering that in male rats PKC $\epsilon$  is involved in both the induction of priming and its expression, we tested if  $\alpha$ CaMKII activation, which induces priming in both male and female rats, also participates in the prolongation of PGE<sub>2</sub> hyperalgesia. Again, the inhibition of  $\alpha$ CaMKII by the combination of antisense and the  $\alpha$ CaMKII inhibitor CaMINtide significantly attenuated the prolonged PGE<sub>2</sub>-induced hyperalgesia in rats primed with  $\psi$ eRACK (male), activated  $\alpha$ CaMKII or ryanodine (male and female). Of note, once the effect of the PKC $\epsilon$ -I and the  $\alpha$ CaMKII ODN-AS/CaMINtide were washed off, the injection of PGE<sub>2</sub> again produced prolonged hyperalgesia.

In order to demonstrate that the neuroplastic changes induced by activation of  $\alpha$ CaMKII and the ryanodine receptor fulfill all the criteria that characterizes hyperalgesic priming, i.e., PKC $\epsilon$ -dependent enhancement and prolongation of the hyperalgesia induced by PGE<sub>2</sub><sup>3,39,41</sup>



and protein translation at the terminal of the nociceptor<sup>19</sup>, we tested the effect of the protein translation inhibitors cordycepin and rapamycin, previously described to permanently reverse the hyperalgesic priming induced by  $\psi$ eRACK<sup>19</sup>, on the expression of priming produced by activated  $\alpha$ CaMKII (female rats) or ryanodine (male and female). We observed, in all cases, significant attenuation of PGE<sub>2</sub>-induced hyperalgesia at the 4<sup>th</sup> h. Based on these results, we suggest that the neuroplastic changes in the nociceptor induced by the different stimuli are similar, and probably resultant from the same process. Furthermore, treatment with the protein translation inhibitors permanently reversed the increased sensitivity to PGE<sub>2</sub> (data not shown), in line with our previous results<sup>19</sup>, suggesting that local synthesis of proteins in the terminal of the nociceptors contributes to the “memory” observed in the primed condition. However, the proteins involved in the maintenance of the hyperalgesic priming remain to be determined.

Finally, it has been described that MAPK can regulate the translation of mRNAs<sup>5,24,30,31,52</sup>, including by activation of factors that are affected by cordycepin or rapamycin, such as the mammalian target for rapamycin (mTOR)<sup>4,23,34</sup>. Since we have previously shown that these inhibitors of protein translation reversed the  $\psi$ eRACK-induced priming<sup>19</sup>, we considered the possibility that the MEK/ERK pathway could also play a role in the prolongation of PGE<sub>2</sub> hyperalgesia. In fact, we have previously shown that inhibition of this pathway attenuates the prolongation of the PGE<sub>2</sub>-induced hyperalgesia in rats primed with the PKC $\epsilon$  activator  $\psi$ eRACK<sup>17</sup>. Therefore, we evaluated if the specific inhibitor of MEK 1/2, U0126, would also attenuate the increased response to PGE<sub>2</sub> in  $\alpha$ CaMKII- and ryanodine-primed rats. As expected, the local treatment with U0126 significantly attenuated the prolongation of the PGE<sub>2</sub> hyperalgesia, showing another similarity with the priming induced by  $\psi$ eRACK. Thus, another signaling molecule that does not play a role in the PGE<sub>2</sub> effect in naïve rats<sup>2</sup> is now “recruited” by PGE<sub>2</sub> in the expression of the primed state.

In conclusion, the present study confirms that a transient activation of PKC $\epsilon$ ,  $\alpha$ CaMKII or the ryanodine receptor triggers a permanent switch in the signaling pathway activated by PGE<sub>2</sub>, in which second messengers that are not involved in its pronociceptive effect in the normal state, such as PKC $\epsilon$  and the MEK/ERK pathway, now contribute to the prolongation of PGE<sub>2</sub> hyperalgesia. Moreover, the maintenance of this neuroplasticity produced by activation of  $\alpha$ CaMKII or ryanodine receptors is dependent on local protein translation at the terminal of the sensory neuron. Such changes in the phenotype of the nociceptor, expressed as a hyperresponsiveness to inflammatory mediators, may be the underlying mechanism of clinical conditions in which, after recovery from an initiating event (such as work-related ergonomic insults, inflammation or psychological stress), the susceptibility of developing a recurrent painful condition is increased.

## Abbreviations

<b>ODN</b>	oligodeoxynucleotide
<b><math>\alpha</math>CaMKII</b>	alpha calmodulin-dependent protein kinase II
<b>PGE<sub>2</sub></b>	prostaglandin E <sub>2</sub>

<b>PKCε</b>	protein kinase C epsilon
<b>SEM</b>	standard error of the mean

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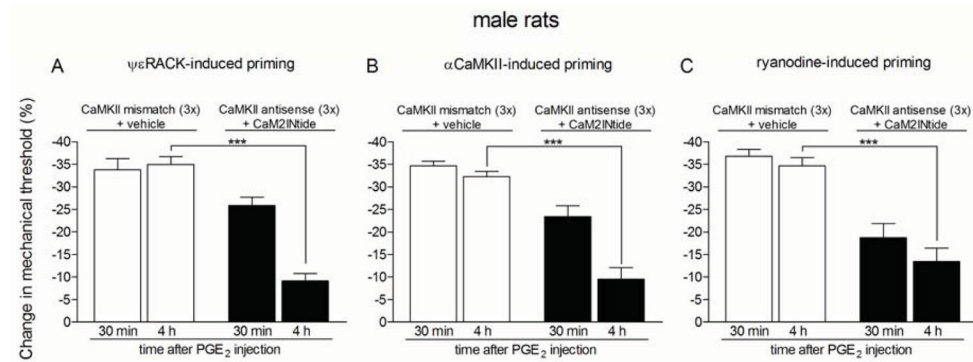
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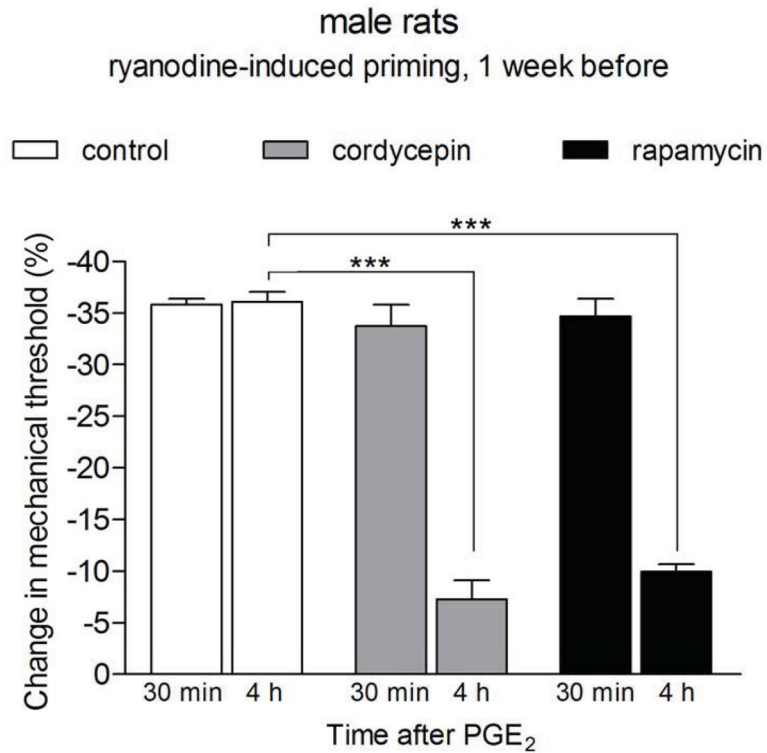
### **Perspectives**

The data provided by this study suggests that direct intervention on specific targets may help to alleviate the expression of chronic hyperalgesic conditions.



**Figure 1. The prolongation of PGE<sub>2</sub>-induced hyperalgesia in primed male rats is dependent on αCaMKII**

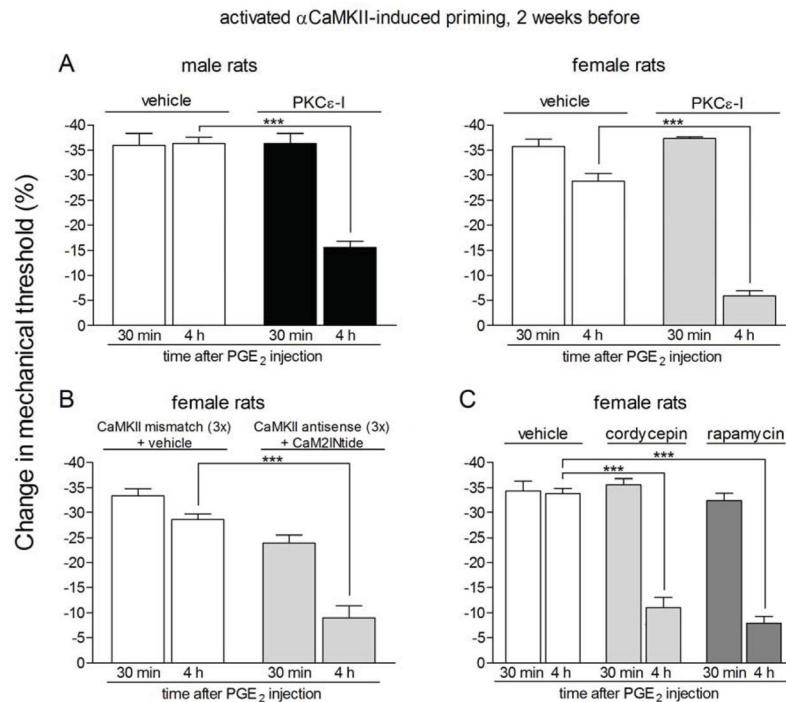
Male rats that received intradermal injection of the PKCε activator ψεRACK (1 μg, **panel A**), activated αCaMKII (25 ng, **panel B**) or the ryanodine receptor agonist (1 μg, **panel C**) on the dorsum of the hind paw one (**panels A and C**) or two (**panel B**) weeks before were treated with intrathecal injection of ODN mismatch (clear bars) or antisense (black bars) for αCaMKII for 3 consecutive days. On the 4<sup>th</sup> day, vehicle or the αCaMKII inhibitor CaM2INtide (1 μg) was administered, 15 min prior to the injection of PGE<sub>2</sub> (100 ng), at the same site. Mechanical nociceptive thresholds were evaluated 30 min and 4 h after PGE<sub>2</sub>, by the Randall-Selitto paw withdrawal test. We observed, in all cases, significant attenuation of the hyperalgesia induced by PGE<sub>2</sub> at the 4<sup>th</sup> h in the groups pretreated with the antisense/CaM2INtide (**panel A**,  $F_{1,10} = 52.07$ ,  $***p < 0.0001$ ; **panel B**,  $F_{1,10} = 68.83$ ,  $***p < 0.0001$ ; **panel C**,  $F_{1,10} = 46.14$ ,  $***p < 0.0001$ ), when compared to the mismatch/vehicle groups (two-way repeated measures ANOVA followed by Bonferroni post-test,  $n = 6$  paws per group), indicating a role of αCaMKII in the prolongation of PGE<sub>2</sub> hyperalgesia in the primed condition.



**Figure 2. Prolonged phase of PGE<sub>2</sub>-induced hyperalgesia in male rats primed with ryanodine is dependent on local protein translation**

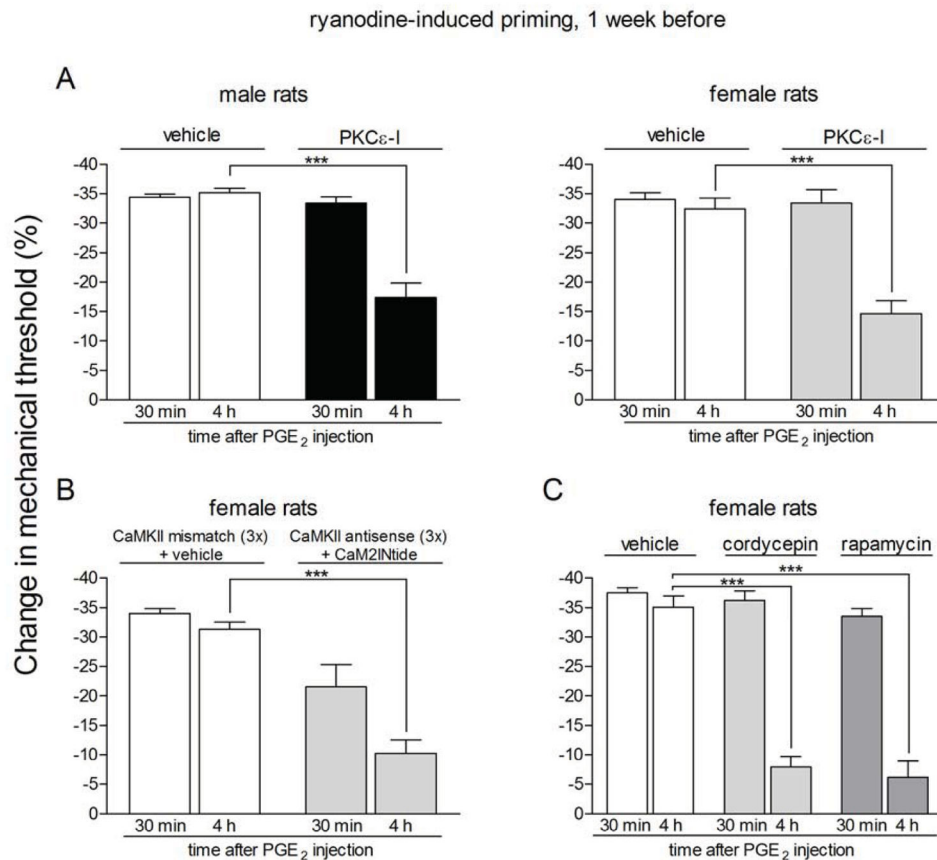
Male rats received an intradermal injection of ryanodine (1  $\mu$ g). One week later cordycepin (1  $\mu$ g), rapamycin (1  $\mu$ g), or vehicle was injected on the dorsum of the hind paw and, 15 min later, PGE<sub>2</sub> (100 ng) was injected in the same site. Mechanical nociceptive thresholds were evaluated 30 min and 4 h after PGE<sub>2</sub>, by the Randall-Selitto paw withdrawal test. In all cases, the pretreatment with the protein translation inhibitor significantly attenuated the hyperalgesia induced by PGE<sub>2</sub> at 4<sup>th</sup> h (\*\**p* < 0.001), when compared to the vehicle group ( $F_{2,15} = 85.11$ , \*\**p* < 0.0001, two-way repeated measures ANOVA followed by Bonferroni post-test, *n* = 6 paws per group).





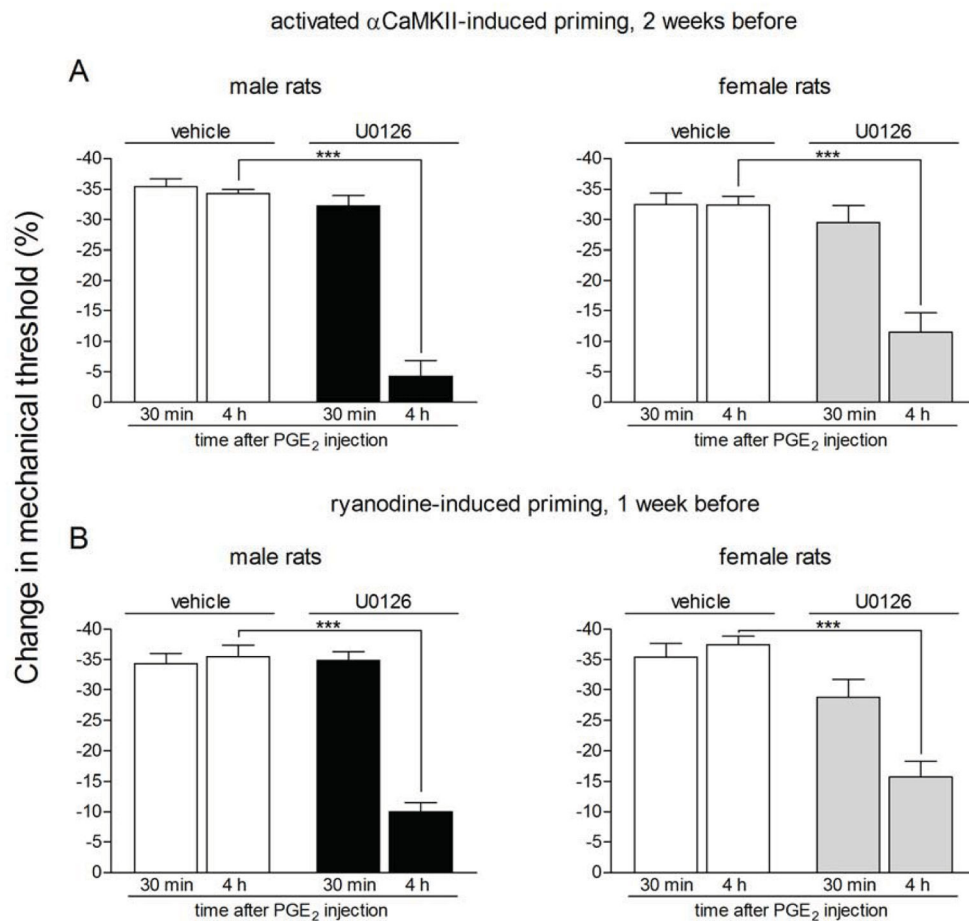
**Figure 3. Prolonged phase of PGE<sub>2</sub>-induced hyperalgesia in rats with  $\alpha$ CaMKII-induced hyperalgesic priming is dependent on PKC $\epsilon$ ,  $\alpha$ CaMKII and local protein translation**

Rats primed with intradermal injection of activated  $\alpha$ CaMKII (25 ng) on the dorsum of the hind paw 2 weeks before received injection of PGE<sub>2</sub> in the presence or absence of inhibitors of the second messengers PKC $\epsilon$  (PKC $\epsilon$ -I, **panel A**) or  $\alpha$ CaMKII (ODN antisense or mismatch for  $\alpha$ CaMKII and the  $\alpha$ CaMKII inhibitor CaM2INTide, **panel B**), and the protein translation inhibitors cordycepin or rapamycin (**panel C**). Mechanical nociceptive thresholds were evaluated 30 min and 4 h after PGE<sub>2</sub>, by the Randall-Selitto paw withdrawal test. PGE<sub>2</sub> was injected 5 min (**panel A**) or 15 min (**panels B** and **C**) after the inhibitors. We observed, in all cases, significant attenuation of the hyperalgesia induced by PGE<sub>2</sub> at 4<sup>th</sup> h ( $***p < 0.001$ ) in the groups treated with the inhibitors when compared to the ODN mismatch/vehicle groups (**panel A**, male rats:  $F_{1,10} = 27.36$ ,  $p = 0.0004$ ; female rats:  $F_{1,10} = 437.96$ ,  $p < 0.0001$ ; **panel B**,  $F_{1,10} = 57.90$ ,  $p < 0.0001$ ; **panel C**,  $F_{2,15} = 26.65$ ,  $p < 0.0001$ , two-way repeated measures ANOVA followed by Bonferroni post-test,  $n = 6$  paws per group), indicating a role of PKC $\epsilon$ ,  $\alpha$ CaMKII and local protein translation in the prolongation of PGE<sub>2</sub> hyperalgesia in the  $\alpha$ CaMKII-induced hyperalgesic priming.



**Figure 4. Prolonged phase of PGE<sub>2</sub>-induced hyperalgesia in rats previously treated with ryanodine is dependent on PKC $\epsilon$ ,  $\alpha$ CaMKII and local protein translation**

Rats primed with intradermal injection of ryanodine (1  $\mu$ g) on the dorsum of the hind paw one week prior received intradermal injection of PGE<sub>2</sub> on the dorsum of the hind paw in the presence or absence of inhibitors of the second messengers PKC $\epsilon$  (PKC $\epsilon$ -I, **panel A**) or  $\alpha$ CaMKII (ODN antisense or mismatch for  $\alpha$ CaMKII and the  $\alpha$ CaMKII inhibitor CaM2INTide, **panel B**), and the protein translation inhibitors cordycepin or rapamycin (**panel C**). Mechanical nociceptive thresholds were evaluated 30 min and 4 h after PGE<sub>2</sub>, by the Randall-Selitto paw withdrawal test. PGE<sub>2</sub> was injected 5 min (**panel A**) or 15 min (**panels B and C**) after the inhibitors. We observed, in all cases, significant attenuation of the hyperalgesia induced by PGE<sub>2</sub> at the 4<sup>th</sup> h (\*\*\* $p$  < 0.001) in the groups treated with the inhibitors when compared to the vehicle groups (**panel A**, male rats:  $F_{1,10} = 36.03$ ,  $p = 0.0001$ ; female rats:  $F_{1,10} = 18.76$ ,  $p < 0.0015$ ; **panel B**,  $F_{1,10} = 56.01$ ,  $p < 0.0001$ ; **panel C**,  $F_{2,15} = 39.30$ ,  $p < 0.0001$ , two-way repeated measures ANOVA followed by Bonferroni post-test,  $n = 6$  paws per group), indicating a role of PKC $\epsilon$ ,  $\alpha$ CaMKII and local protein translation in the prolongation of PGE<sub>2</sub> hyperalgesia in the ryanodine-induced hyperalgesic priming.



**Figure 5. Prolonged phase of PGE<sub>2</sub>-induced hyperalgesia is dependent on the MEK/ERK pathway in rats previously treated with activated  $\alpha$ CaMKII or ryanodine**

Different groups of rats that were treated with intradermal injection of activated  $\alpha$ CaMKII (25 ng, **panel A**) or the ryanodine receptor agonist (1  $\mu$ g, **panel B**) on the dorsum of the hind paw two or one week prior, respectively, received PGE<sub>2</sub> (100 ng), at the same site, 5 min after the injection of vehicle or U0126 (1  $\mu$ g). Mechanical nociceptive thresholds were evaluated 30 min and 4 h after PGE<sub>2</sub>, by the Randall-Selitto paw withdrawal test. We observed, in all cases, significant attenuation of the hyperalgesia induced by PGE<sub>2</sub> at the 4<sup>th</sup> h (\*\*\*)  $p < 0.001$ ) in the groups pretreated with the U0126 (**panel A**, male rats:  $F_{1,10} = 85.58$ ,  $p < 0.0001$ ; female rats:  $F_{1,10} = 20.37$ ,  $p = 0.0011$ ; **panel B**, male rats:  $F_{1,10} = 40.34$ ,  $p < 0.0001$ ; female rats:  $F_{1,10} = 25.35$ ,  $p = 0.0005$ ), when compared to the vehicle groups (two-way repeated measures ANOVA followed by Bonferroni post-test,  $n = 6$  paws per group), indicating a role of the MEK/ERK pathway in the prolongation of PGE<sub>2</sub> hyperalgesia in the primed condition.