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Activation of G_i induces Mechanical Hyperalgesia Post Stress or Inflammation

Olayinka A. Dina, Sachia G. Khasar [Federal Agent], Robert W. Gear, and Jon D. Levine

Abstract

In studies of the role of primary afferent nociceptor plasticity in the transition from acute to chronic pain we recently reported that exposure to unpredictable sound stress or a prior inflammatory response induces long-term changes in the second messenger signaling pathway, in nociceptors, mediating inflammatory hyperalgesia; this change involves a switch from a G_s-cAMP-PKA to a G_i-PKC signaling pathway. To more directly study the role of G_i in mechanical hyperalgesia we evaluated the nociceptive effect of the G_i activator, mastoparan. Intradermal injection of mastoparan in the rat hind paw induces dose-dependent (0.1 ng – 1 µg) mechanical hyperalgesia. The highly selective inhibitor of G_i, Pertussis toxin, and of protein kinase C epsilon (PKCε), PKCεV₁₋₂, both markedly attenuate mastoparan-induced hyperalgesia in stressed rats but had no effect on mastoparan-induced hyperalgesia in unstressed rats. Similar effects were observed, at the site of nociceptive testing, after recovery from carrageenan-induced inflammation. These studies provide further confirmation for a switch to a G_i-activated and PKCε-dependent signaling pathway in primary mechanical hyperalgesia, induced by stress or inflammation.

Introduction

A prior localized inflammatory response (Aley et al., 2000) or a period of unpredictable sound stress (Khasar et al., 2008) produce long-term changes in the duration and second messenger signaling pathway for inflammatory mediator-induced mechanical hyperalgesia. This change involves a switch from G_s to G_i in the G-protein that mediates inflammatory mediator-induced mechanical hyperalgesia (Khasar et al., 2008). While this type of switch in second messenger signaling pathways, from G_s to G_i, has previously been reported for β₂-adrenergic receptor (β₂AR) signaling in cardiac myocytes, caused by chronic exposure to receptor agonists such as epinephrine (Lamba and Abraham, 2000), in cardiac muscle cells this involves an increased G_i-mediated *inhibition* of adenylyl cyclase, which would be expected to *attenuate* rather than enhance mechanical hyperalgesia (Levine and Taiwo, 1989; Taiwo et al., 1989). In contrast, we observed that while prior inflammation at the site of nociceptive testing (Fig. 1) (Aley et al., 2000) and repeated unpredictable sound stress (Khasar et al., 2008) also cause a switch in signaling pathways in the primary afferent nociceptor, from dependence on G_s and cAMP/protein kinase A to dependence on G_i and protein kinase Cε (PKCε), in the nociceptor this switch is associated with enhanced rather than attenuated mechanical hyperalgesia. The emergence of PKCε dependence may be important to our understanding of mechanisms of chronic pain, as this novel protein kinase C (PKC) isoform plays a critical role in the phenomenon of “hyperalgesic priming,” a model of the transition from acute to chronic pain (Aley et al., 2000).

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Having established an involvement of G_i in the switch to PKC ϵ -dependence for prostaglandin E_2 hyperalgesia, in this study we evaluated whether activation of G_i is *sufficient* to produce mechanical hyperalgesia, if such hyperalgesia requires or is enhanced by prior inflammation at the site of nociceptive testing or by prior exposure to unpredictable stress, and if G_i -induced hyperalgesia is also PKC ϵ dependent.

Methods

Animals

Male (220–450 g) Sprague–Dawley rats (Charles River, Hollister, CA) used in these experiments were housed in the Laboratory Animal Resource Center of the University of California, San Francisco, under a 12-h light/dark cycle. All experimental protocols were approved by the University of California, San Francisco, Institutional Animal Care and Use Committee, and conformed to NIH guidelines for the care and use of experimental animals. Effort was made to limit the numbers of animals used and their discomfort.

Drugs

The drugs used in these experiments were: mastoparan (Mas-7; G_i activator whose peptide sequence is: H-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Ala-Leu-Leu-NH₂; (Higashijima et al., 1990; Klinker et al., 1996; Chahdi et al., 2003) (Bachem Americas Inc., Torrance, CA); prostaglandin E_2 (direct-acting hyperalgesic proinflammatory mediator (5Z, 11 α , 13E, 15S)-11,15-Dihydroxy-9-oxoprostanoic acid; (Kessler et al., 1992; Li et al., 1995); and Pertussis toxin (PTx) (Kaslow et al., 1987; Aktories, 1997), a selective inhibitor of G_i (lyophilized powder prepared from *Bordetella pertussis*; Sigma, St Louis, MO), and PKC ϵ V₁₋₂, selective protein kinase C ϵ (PKC ϵ) (a diacylglycerol-dependent and Ca²⁺-independent PKC isozyme; (Johnson et al., 1996) translocation inhibitor peptide, (EAVSLKPT); EMD Biosciences, La Jolla, CA)). All drugs were dissolved in and diluted with 0.9% saline (Baxter Healthcare Corporation, Deerfield, IL). Because of its high viscosity, carrageenan (5 μ l of 1% solution in saline; (Dina et al., 2003) was injected using a 27-gauge hypodermic needle; all other drugs were administered using a 30-gauge hypodermic needle. In the carrageenan model of chronic inflammation, test agents were administered to test or control rats on day 5 after carrageenan, at which time mechanical nociceptive threshold had returned to pre-carrageenan baseline.

Mechanical nociceptive threshold

Mechanical nociceptive threshold was quantified using the Randall–Selitto paw pressure test (Randall and Selitto, 1957), in which a force that increases linearly over time is applied to the dorsum of the rat's hind paw (Taiwo et al., 1989; Dina et al., 2000; Dina et al., 2004), using an Algesymeter[®] (Stoelting, Chicago, IL). Rats were lightly restrained in cylindrical transparent acrylic restrainers designed to provide adequate comfort and ventilation, to allow extension of the hind leg from the cylinder, and to minimize restraint stress. All rats were acclimatized to the testing procedure, and restraint and test techniques were performed in parallel across groups. Rats were placed in individual restrainers for 1 h prior to starting each study and for 30 min prior to testing. Nociceptive threshold was defined as the force, in grams, at which the rat withdrew its paw. The baseline paw-withdrawal threshold was defined as the mean of three readings. Each paw was treated as an independent measure and each experiment performed on a separate group of rats. All behavioral testing was done between 10:00 and 16:00 hours.

Carrageenan inflammation

According to the experimental paradigm previously established in our laboratory (Aley et al., 2000), a chronic state of hyperalgesic priming was induced by intradermal injection of 5 μ l of 1% carrageenan into the dorsal hind paw. Control rats received 5 μ l of saline. Additional experimental interventions were performed 5 days after the administration of carrageenan or saline, at which time the neuroplastic changes in the primary afferent nociceptor that are responsible for prolongation and altered second messenger signaling are fully established (Aley et al., 2000).

Unpredictable sound stress

Exposure to sound stress occurred over 4 d as initially described by Singh et al. (Singh et al., 1990) and previously used in our laboratory (Strausbaugh et al., 2003; Khasar et al., 2005). Animals were placed three per cage, 25 cm from a speaker that emitted a 105 dB tone of mixed frequencies (11–19 kHz). Over a period of 30 min, rats were exposed to 5 or 10 s sound epochs each minute, at random times during the minute. After sound stress, rats were returned to the animal care facility, in their home cages. Animals were exposed to the stressor on days 1, 3, and 4. They were used for nociceptive studies 14 d after the last exposure to sound stress, at which time neuroplasticity underlying the changes in duration and second messenger signaling mediating inflammatory mediator-induced mechanical hyperalgesia were fully established (Khasar et al., 2008).

Statistics

Group data are presented as mean $\pm$ S.E.M. A two-way or three-way repeated measures ANOVA, as appropriate, was used to determine if there were significant ($p < 0.05$) differences between the groups. The within-subjects and between-subjects factors are described in the figure captions. For each repeated measures ANOVA, the Mauchly criterion was used to determine if the assumption of sphericity for the within-subjects effects was met; if the Mauchly criterion was not satisfied, Greenhouse-Geisser adjusted p -values are presented. If there was a significant interaction, groups were analyzed separately to determine the basis of the significant interaction. For between-subjects factors with more than two levels, Scheffé post hoc analyses were employed to determine the basis of the differences. Bonferroni-type corrections were applied to the alpha level as appropriate for multiple post hoc comparisons by dividing by the number of comparisons (e.g., for four comparisons, $p = 0.05/4 = 0.0125$). The level for statistical significance was set at $p < 0.05$.

Results

While, for sound stress, we have previously shown a switch to G_i - as well as development of PKC ϵ –dependence following recovery from prior inflammation we have not previously evaluated the switch to G_i dependence. In animals that had experienced prior inflammation at the site of nociceptive testing, prostaglandin E_2 (PGE $_2$) induced mechanical hyperalgesia was still unattenuated 4 hrs later (Fig. 1), a signature of hyperalgesic priming. This hyperalgesia was completely reversed by injection of Pertussis toxin (PTx; 100 ng/ μ l) at the site of nociceptive testing. In a control group of rats, recovered from prior inflammation, PTx alone had no effect on nociceptive threshold.

In the first experiments to evaluate the role of G_i in mechanical hyperalgesia, we found that the intradermal injection of mastoparan, in the hind paw, produced a dose-dependent decrease in mechanical nociceptive threshold (1 ng – 1 μ g; $n = 6$, each dose; Fig. 2). In rats that had been exposed to repeated unpredictable sound stress, with the last stress exposure 14 days prior, the dose-response curve for mastoparan-induced hyperalgesia was shifted to the left ($p < 0.001$; Fig. 3A); the peak hyperalgesia was, however, unchanged ($p = \text{NS}$). Mastoparan-induced

hyperalgesia, in both naïve and stressed rats was reversible, being no longer detectable 24 hrs after its administration (Fig. 3B).

While intradermal injection of the selective G_i inhibitor PTx (100 ng/ μ l) markedly attenuated mastoparan-induced hyperalgesia 14 days post stress ($p<0.001$; Fig. 4A), it had no effect on mastoparan-induced hyperalgesia in a control group of animals that had not been exposed to the sound stress protocol ($p=NS$; Fig. 4A). Of note, the decrease in mastoparan hyperalgesia produced by PTx in stressed rats, brought hyperalgesia to a level well below that induced by mastoparan in unstressed rats. A PKC ϵ inhibitor, PKC ϵ V₁₋₂ (1 μ g) also markedly attenuated mastoparan-induced hyperalgesia, again this was observed in stressed rats but not in unstressed rats (Fig. 4B); in fact, at the low mastoparan dose the PKC ϵ inhibitor, PKC ϵ V₁₋₂ appears to *enhance* hyperalgesia in unstressed rats.

Similar to the effect of prior stress, rats that had previously experienced a localized inflammatory response, induced by carrageenan at the site of nociceptive testing, and had recovered to pre-carrageenan baseline mechanical nociceptive threshold (Aley et al., 2000), also demonstrated mastoparan-induced hyperalgesia that was inhibited by PTx and PKC ϵ V₁₋₂, though the dose response curve for mastoparan-induced hyperalgesia was not shifted to the left (Fig. 5A,B, respectively), as it was by stress (Fig. 3A, 4A). Again, the magnitude of mastoparan-induced hyperalgesia, in the presence of PTx, was markedly less in rats that had experienced prior inflammation than the hyperalgesia induced by mastoparan (without PTx) in a control group of rats (i.e., in rats that had not experienced a prior inflammatory response, Fig. 4A).

Discussion

In the present study we evaluated the role of the 'inhibitory' heterotrimeric G- protein, G_i , in inflammatory mediator-induced mechanical hyperalgesia in the rat. We have implicated a switch in signaling, from G_s to G_i , and onset of PKC ϵ dependence for the second messenger pathway mediating prostaglandin E_2 -induced mechanical hyperalgesia, induced by prior stress (Khasar et al., 2008) or inflammation (Fig. 1; Aley et al., 2000). In the present study we evaluated whether activation of G_i was sufficient to produce PKC ϵ -mediated mechanical hyperalgesia by determining if a G_i activator, mastoparan (Higashijima et al., 1990; Klinker et al., 1996; Chahdi et al., 2003), can alone produce mechanical hyperalgesia following prior stress or inflammation, and whether this mastoparan-induced hyperalgesia is PKC ϵ dependent.

To demonstrate that mastoparan-induced hyperalgesia is mediated by G_i after but not before stress or inflammation, we administered PTx, a highly selective G_i inhibitor (Kaslow et al., 1987; Aktories, 1997). As predicted, the hyperalgesia induced by mastoparan post-stress or -inflammation is markedly attenuated by PTx. Thus, in the setting of prior stress or inflammation, activation of G_i appears to be sufficient to induce mechanical hyperalgesia. We also found that after stress or prior inflammation, the hyperalgesia induced by mastoparan like that induced by prostaglandin E_2 became PKC ϵ dependent. Thus, the signaling pathway downstream of G_i similarly includes PKC ϵ . These findings support the suggestion that as occurs for prostaglandin E_2 -induced hyperalgesia, after stress and inflammation (Khasar et al., 2008) there is a switch to a GPCR- G_i -PKC ϵ signaling pathway.

Of note, the reduction in mastoparan-induced hyperalgesia by PTx, in rats previously exposed to stress or inflammation, reduces hyperalgesia to a level well *below* that induced by mastoparan in control rats (see Figs. 4A and 5A). This is similar to what we have previously observed for prostaglandin E_2 -induced hyperalgesia (Khasar et al., 2008). Thus this finding supports the existence of a switch in the second messenger- signaling pathway for inflammatory mediator-induced mechanical hyperalgesia, to G_i - PKC ϵ . The finding that PTx had no effect

on mastoparan-induced hyperalgesia in naïve control rats further confirms a switch from no contribution of G_i to G_i dependence, as observed for prostaglandin E_2 -induced hyperalgesia after prior stress (Khasar et al., 2008) or inflammation (Fig. 1). While Ueda and colleagues have demonstrated inhibition of nociception induced pain (for review see Ueda, 2006) this switch in G-protein coupled receptor signaling from G_s to G_i , and appearance of G_i to PKC ϵ signaling, have not been reported previously in neurons. Of note, however, such mechanisms are well established in another excitable tissue, the cardiac myocyte. Thus, in heart muscle cells, β_2 -adrenergic receptor signaling can switch from G_s to G_i , (Daaka et al., 1997; Hasseldine et al., 2003; Hill and Baker, 2003; Magocsi et al., 2007) and G_i can signal to mediators known to be upstream of PKC ϵ (Herrlich et al., 1996; Daaka et al., 1997; Steinberg, 1999; Mackay and Mochly-Rosen, 2001; Pavoine et al., 2003; Pavoine and Defer, 2005) or directly to PKC ϵ (Fraser et al., 2000; Otani et al., 2003; Paruchuri and Sjolander, 2003).

While mastoparan did produce mechanical hyperalgesia in the absence of prior stress or inflammation, this hyperalgesia was not inhibited by PTx, and therefore not G_i -mediated, nor was it antagonized by a PKC ϵ inhibitor. Of note in this regard, actions of mastoparan have been divided into PTx-sensitive, G_i -mediated, and PTx-insensitive, G_i -independent signaling pathways. Examples of G_i -independent mechanisms include: activation of MAP kinase (Miles et al., 2004), phospholipase C (Nakahata et al., 1990; Schnabel et al., 1997), phospholipase D (Mizuno et al., 1998; Chahdi et al., 2003) and two small molecular weight GTP-binding proteins *rho* and *rac* (Koch et al., 1991). Whether one of the previously described G_i -independent mechanisms of mastoparan action, or another signaling pathway, mediates the PTx-resistant hyperalgesia induced by mastoparan in the control rats not exposed to prior stress or inflammation remains to be elucidated. Alternatively, mastoparan may have activated G_i in the control rats but in the absence of prior stress or inflammation G_i is not coupled to PKC ϵ , and therefore, cannot induce hyperalgesia via this pathway.

While the present study provides evidence to support the suggestion that stress and inflammation induce a neuroplastic change in the primary afferent nociceptor, in which G_i activation can induce PKC ϵ -dependent mechanical hyperalgesia, the mechanism by which stress and inflammation induce these changes in the function of the primary afferent nociceptor, necessary to engage G_i -PKC ϵ signaling, remains to be elucidated. We have previously shown that the effect of unpredictable sound stress on primary afferent nociceptor function, needed to produce the observed neuroplastic changes in second messenger signaling, are mediated by the action of glucocorticoids and catecholamine on glucocorticoid and β_2 -adrenergic receptors, respectively, on primary afferent nociceptors (Khasar et al., 2008). That the production of hyperalgesic priming by prior stress or inflammation is mediated by changes in the primary afferent nociceptor is suggested by the fact that TNF α and IL-6, acting at their receptors on primary afferent nociceptors, each produce hyperalgesic priming (Parada et al., 2003; Dina et al., 2008).

In conclusion, the present experiments confirm the role of a G_i -PKC ϵ -mediated signaling pathway in primary afferent nociceptor sensitization and mechanical hyperalgesia associated with prior stress and inflammation, that may occur at or below the level of the G_i heterotrimeric G-protein in the second messenger signaling pathway. It also provides support for a change in signaling pathway, a switch that is activated by stress or prior inflammation. Thus, G_i activity is *sufficient* for reproducing, as well as necessary in the pathway mediating the neuroplastic changes in the primary afferent nociceptor, which may contribute in the transition from acute to chronic pain. Whether the mechanisms described here impacts sensitization for other stimulus modalities (e.g. heat, cold and various chemicals) remains to be elucidated.

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Figure 1.

Effect of Pertussis toxin (PTx) on prostaglandin E₂ (PGE₂)-induced mechanical hyperalgesia at a site on the dorsum of the hind paw that had experienced a prior inflammatory response. Five days after injection of carrageenan, when nociceptive threshold had returned to baseline, PGE₂ (100 ng/μl) injected at the same site produced mechanical hyperalgesia that was still maximal 4 hrs later (Aley et al., 2000). At this time injection of PTx (100 ng/μl) completely reversed PGE₂ hyperalgesia. In this and subsequent figures, data are plotted as mean ± standard error of the mean (S.E.M.). *Left three bars:* Two-way repeated measures ANOVA with one within-subjects factor (treatments with two levels, post-PGE₂ and post-PTx or saline) and one between-subjects factor (treatment group with two levels, PTx or saline) demonstrated a significant treatment × treatment group interaction ($F_{1,14}=90.834$; $p<0.001$) as well as a significant main effect of treatment group ($F_{1,14}=97.000$; $p<0.001$), indicating that PTx reverses PGE₂ hyperalgesia.

The effect of PTx alone was studied in a control group of carrageenan-treated rats. *Right two bars:* PTx administered alone and measured at 0.5 or 4 h, had no effect ($p=NS$) on mechanical nociceptive threshold in rats not previously treated with PGE₂.

Figure 2.

Dose-response relationship for the mechanical hyperalgesia induced by intradermal injection of mastoparan, in the rat hind paw.

Different groups of naïve rats (each, $n = 6$ paws) were used to determine nociceptive thresholds to each dose of mastoparan (1 ng – 1 μ g) at 0.5 h and 24 h. Two-way ANOVA showed a significant time by dose interaction ($F_{3,20}=29.751$; $p<0.001$) and a significant main effect of group ($F_{3,20}=23.977$; $p<0.001$). Because the interaction was significant, separate one-way ANOVAs (dose with four levels) were performed for each time point. The effect of dose at 0.5 h was significant ($F_{3,20}=81.476$; $p<0.001$); Scheffé post hoc analyses showed that both the 1 ng and the 10 ng doses were significantly different from all other doses, but the two highest doses (100 and 1000 ng) were not significantly different ($p=NS$) from each other. The effect at 24 h was not significant ($F_{3,20}=0.202$; $p=NS$), indicating that the effects observed at 0.5 h were dissipated by 24 hours.

Figure 3.

Mastoparan-induced hyperalgesia is enhanced by sound stress.

(A) Dose-response curves for the effect of mastoparan in naïve (non-stressed) and sound-stressed rats show a significant enhancement of mastoparan-induced hyperalgesia in sound stressed rats. The two-way ANOVA with one within-subjects factor (dose with 4 levels) and one between-subjects factor (stress treatment with two levels) showed a significant stress treatment $\times$ dose interaction ($F_{3,30}=6.364$; $p=0.004$) as well as a significant main effect of stress ($F_{1,10}=19.420$; $p=0.001$). Based on the significant interaction, a one-way between-subjects ANOVA (stress treatment with two levels) was performed for each dose to identify the doses showing significant differences. Because this method of analysis required four separate comparisons, the alpha level was set at $p<0.0125$. These analyses showed that groups that received sound stress were significantly different from the groups that did not, for each of the three lowest doses ($p=0.011$, $p<0.001$, $p<0.01$, respectively); however, the groups receiving the highest dose were not significantly different ($p=0.738$). These results suggest a leftward shift in the dose response curve such that the maximal effect of mastoparan was achieved with a dose an order of magnitude smaller in stressed animals.

(B) Peak hyperalgesia to doses of mastoparan (1 ng – 1 μ g), determined after the 1 μ g dose, was not significantly different when comparing naïve and stressed rats (A and B). In both groups, hyperalgesia was no longer evident 24 h after the initial administration of mastoparan.

Figure 4.

Mastoparan-induced hyperalgesia is profoundly sensitive to PTx or PKCε inhibitor post sound stress.

(A) The selective G_i inhibitor PTx (100 ng/μl) markedly attenuated mastoparan-induced hyperalgesia 14 days post stress but not in non-stressed naïve rats. The three-way ANOVA with two between-subjects factors (stress treatment with two levels and PTx treatment with two levels) and one within-subjects factor (dose with 5 levels) demonstrated a significant 3-way interaction ($F_{4,80}=15.882$; $p<0.001$) and a significant main effect of PTx ($F_{1,20}=32.880$; $p<0.001$). Based on the significant interaction, separate two-way repeated measures ANOVAs were performed for the two stress treatment groups. The between-subjects factor was PTx treatment with two levels. These analyses revealed that, for the two groups exposed to sound stress, the group receiving PTx differed significantly from the group receiving vehicle. There was a significant dose by PTx treatment interaction ($F_{4,40}=10.116$; $p<0.001$) and a significant main effect of PTx treatment ($F_{1,10}=65.408$; $p<0.001$). There were no significant main effect or interaction between the two PTx treatment groups not exposed to sound stress, indicating that the effect of PTx depends on prior exposure to stress.

(B) Similarly, in stressed, but not in unstressed rats, mastoparan-induced hyperalgesia was markedly attenuated by a PKCε inhibitor, PKCεV₁₋₂. Three-way ANOVA with two between-subjects factors (stress treatment with two levels and PKCε inhibitor (PKCε-I) treatment with two levels, active and scrambled peptide sequences) and one within-subjects factor (dose with 5 levels) demonstrated a significant 3-way interaction ($F_{4,80}=5.858$; $p<0.001$) and a significant main effect of PKCε-I treatment ($F_{1,20}=95.435$; $p<0.001$). Based on the significant interaction, separate two-way repeated measures ANOVAs were performed for the two stress treatment groups. The between-subjects factor was PKCε-I treatment with two levels. These analyses revealed that, for the two groups exposed to sound stress, the group receiving active PKCε-I differed significantly from the group receiving scrambled PKCε-I. There was a significant dose by PKCε-I treatment interaction ($F_{4,40}=9.356$; $p<0.001$) and a significant main effect of PKCε-I treatment ($F_{1,10}=220.826$; $p<0.001$). There were no significant main effects or

interactions between the two PKC ϵ treatment groups not exposed to sound stress, indicating that the effect of PKC ϵ depends on prior exposure to stress.

Figure 5.

Mastoparan-induced hyperalgesia is profoundly sensitive to PTx or PKC ϵ inhibition following recovery from carrageenan-induced inflammation.

(A) Mastoparan hyperalgesia was inhibited by PTx (100 ng/ μ l; $p < 0.01$). Two-way ANOVA with one between-subjects factor (PTx treatment with two levels) and one within-subjects factor (dose with 5 levels) demonstrated a significant dose $\times$ PTx treatment interaction ($F_{4,40}=14.585$; $p < 0.001$) and a significant main effect of PTx treatment ($F_{1,10}=24.250$; $p < 0.001$).

(B). Mastoparan hyperalgesia was similarly inhibited by the selective PKC ϵ inhibitor peptide (1 μ g; $p < 0.001$). The two-way ANOVA with one between-subjects factor (PKC ϵ treatment with two levels) and one within-subjects factor (dose with 5 levels) demonstrated a significant main effect of PKC ϵ treatment ($F_{1,10}=96.610$; $p < 0.001$); the dose $\times$ PKC ϵ treatment interaction was not significant ($F_{4,40}=2.641$; $p < 0.073$). Both inhibitors were applied at the site of nociceptive testing that had previously experienced a localized inflammatory response induced by carrageenan.