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Hyperalgesic Priming is Restricted to IB4-positive Nociceptors

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Abstract

We have previously described a rat model for the contribution of neuroplastic changes in nociceptors to the transition from acute to chronic pain. In this model a prior injury activates protein kinase C epsilon (PKC ϵ), inducing a chronic state characterized by marked prolongation of the hyperalgesia induced by inflammatory cytokines, prototypically prostaglandin E₂ (PGE₂), referred to as hyperalgesic priming. In this study we evaluated the population of nociceptors involved in priming, by lesioning IB4(+) nociceptors with intrathecal administration of a selective neurotoxin, IB4-saporin. To confirm that the remaining, TrkA(+)/IB4(–), nociceptors are still functional, we evaluated if nerve growth factor (NGF) induced hyperalgesia. While pretreatment with IB4-saporin eliminated the acute mechanical hyperalgesia induced by glia-derived neurotrophic factor (GDNF), NGF and ψ eRACK, a highly selective activator of PKC ϵ , induced robust hyperalgesia. After injection of NGF, GDNF or ψ eRACK, at a time at which hyperalgesia induced by PGE₂ is markedly prolonged (hyperalgesic priming) in control rats, in IB4-saporin-pretreated rats PGE₂ failed to produce this prolonged hyperalgesia. Thus, while PKC ϵ is present in most dorsal root ganglion neurons, where it can contribute to acute mechanical hyperalgesia, priming is restricted to IB4(+)-nociceptors, including those that are TrkA(+). While PKC ϵ activation can induce acute hyperalgesia in the IB4(–) population, it fails to induce priming. We suggest that hyperalgesic priming occurs only in IB4(+) nociceptors, and that in the peripheral terminals of nociceptors separate intracellular pools of PKC ϵ mediate nociceptor sensitization and the induction of hyperalgesic priming.

Keywords

Chronic pain; protein kinase C epsilon; dorsal root ganglion neurons; isolectin B4; NGF; GDNF

In studies designed to detect transition from acute to chronic pain, we demonstrated that inflammation produces a long-lasting neuroplastic change in the signaling pathway mediating inflammatory cytokine-induced nociceptor sensitization and mechanical hyperalgesia, at a previously inflamed site. We have referred to this phenomenon as hyperalgesic priming^{1,24}. The induction of priming is mediated by activation of protein kinase C epsilon (PKC ϵ) in the peripheral terminal of the nociceptor^{1,22}. While PKC ϵ is found in almost all dorsal root ganglion neurons^{5,10,17,21,25,26}, its G-protein coupled receptor activation-induced translocation from the cytoplasm to the plasma membrane is restricted to the isolectin B4-positive (IB4(+)) population of nociceptors⁹. While we have

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recently shown that both nerve growth factor (NGF) and glia-derived growth factor (GDNF) induce hyperalgesic priming⁸, roughly a third of TrkA(+) neurons are IB4(+) ^{7,15}. Since PKC ϵ is translocated to the plasma membrane in IB4(+) but not IB4(-) neurons⁹, we evaluated if NGF-induced *priming* like that induced by GDNF is mediated by its action on the TrkA(+) subpopulation of IB4(+) neurons, by lesioning the IB4(+) population of dorsal root ganglion neurons with the selective neurotoxin, IB4-saporin¹².

Experimental procedures

Animals

Experiments were performed on adult male Sprague–Dawley rats (n=27; 250–350 g; Charles River, Hollister, CA, USA). Animals were housed three per cage, under a 12 h light/dark cycle, in a temperature and humidity controlled environment at the University of California, San Francisco (UCSF) animal care facility. Food and water were available *ad libitum*. All nociceptive testing was done between 10:00 am and 4:00 pm. All experimental protocols were approved by the UCSF Committee on Animal Research and conformed to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Nociceptive testing

The nociceptive flexion reflex was quantified with an Ugo Basile Analgesymeter (Stoelting, Chicago, IL), which applies a linearly increasing mechanical force to the dorsum of a rat's hind paw. Nociceptive threshold, defined as the force in grams at which the rat withdrew its paw, was the mean of three readings taken at 5 min intervals. Rats were lightly restrained in cylindrical transparent acrylic restrainers designed to allow extension of the hind leg from the restrainer for nociceptive threshold testing. All rats were acclimatized to the testing procedures to reduce variability and produce a more stable baseline of the paw-withdrawal threshold. The mechanical paw threshold was determined before and after administration of test agents. Each paw was treated as an independent measure and each experiment performed on separate groups of rats. The results are expressed as percentage change from baseline mechanical nociceptive threshold.

Drugs

Drugs employed in this study were prostaglandin E₂ (PGE₂) (a direct-acting hyperalgesic agent) and nerve growth factor (NGF) from Sigma (St. Louis, MO, USA), glia-derived neurotrophic factor (GDNF) from EMD Biosciences (La Jolla, CA), and ψ eRACK, a selective activator of PKC ϵ ⁶ prepared by SynPep (Dublin, CA). Drugs were applied by intradermal injection on the dorsum of the hind paw. A stock solution of NGF (1 μ g/ μ l in 0.9% NaCl containing 0.5% bovine serum albumin) was diluted in 0.9% NaCl at the time of injection (dose 1 μ g)¹⁹. GDNF was similarly prepared³. ψ eRACK was dissolved in saline¹¹. The stock solution of prostaglandin E₂ (1 μ g/ μ l) was prepared in absolute ethanol and additional dilutions made with physiological saline; the final concentration of ethanol was $\leq$ 2%. IB4-saporin, which consists of isolectin B4 coupled to the neurotoxin saporin, was purchased from Advanced Targeting Systems (San Diego, CA). All drugs, except IB4-saporin, which was administered by the spinal intrathecal route, were administered intradermally in a volume of 5 μ l using a 30-gauge hypodermic needle attached to a microsyringe (Hamilton, Reno NV). The selection of the drug doses used in this study was based on dose–response curves determined during previous studies^{11,16,19}.

Intrathecal administration of IB4-saporin

IB4-saporin was diluted with saline and a dose of 3.2 μ g/20 μ L administered intrathecally 10 days prior to experiments^{3,12}. With the use of an insulin syringe, IB4-saporin was injected

into the subarachnoid space on the midline between the L4 and L5 vertebrae. For this procedure, rats were anesthetized with 2.5% isoflurane (97.5% O₂).

Statistical analysis

In all experiments, the dependent variable was change in paw withdrawal threshold represented as percentage change from baseline paw withdrawal threshold. Group data are presented as mean±standard error of the mean (SEM). Statistical comparisons were done by ANOVA or Student's t-test, as appropriate. P-values < 0.05 were considered statistically significant.

Results

Effect of IB4-saporin on hyperalgesia

Ten days after the intrathecal administration of IB4-saporin, a time at which IB4(+) terminals in the dorsal horn of the spinal cord have degenerated, GDNF (10 ng), NGF (1 µg) or ψ εRACK (1 µg), was injected intradermally, on the dorsum of the hind paw, in separate groups of rats. Pretreatment with IB4-saporin almost completely eliminated the mechanical hyperalgesia induced by GDNF. However, NGF and ψ εRACK induced robust hyperalgesia in IB4-saporin treated rats (Fig. 1A). Thus, the remaining TrkA(+) nociceptors, which are IB4(-), remain functional as NGF and PKC ϵ activation produces mechanical hyperalgesia. These findings confirm the role of PKC ϵ in nociceptor sensitization and mechanical hyperalgesia, as well as demonstrating that this function can be distinguished from its role in hyperalgesic priming. In control animals, not treated with IB4-saporin, GDNF, NGF and ψ εRACK all produced mechanical hyperalgesia (Fig. 1B).

Effect of IB4-saporin on priming

Intradermal injection of PGE₂ (100 ng) in naïve rats produces a robust, short-lived hyperalgesia, in which mechanical nociceptive threshold has returned to baseline by 4 hr¹. When PGE₂ is administered to rats previously treated with GDNF, NGF or ψ εRACK – when paw withdrawal threshold had returned to baseline – PGE₂ hyperalgesia is markedly prolonged, being near maximum at 4 h^{8,22}. We now show that IB4-saporin pretreatment prevents this hyperalgesic priming by GDNF (10 ng), NGF (1 µg) and ψ εRACK (1 µg), since PGE₂ (100 ng) only produced a short-lived hyperalgesia (Fig. 2A), similar to that observed in naïve rats, in IB4-saporin pretreated rats. Thus, elimination of IB4(+) neurons while leaving IB4(-) neurons intact and functional, completely eliminated hyperalgesic priming, even that induced by the selective PKC ϵ activator, ψ εRACK, which still produces mechanical hyperalgesia in the remaining IB4(-) neurons. In control animals, not treated with IB4-saporin, PGE₂ produced prolonged (>4 hr) hyperalgesia in GDNF, NGF and ψ εRACK pretreated animals (Fig. 2B).

Discussion

PKC ϵ signaling has been implicated in nociceptor sensitization, as manifest by acute mechanical hyperalgesia^{1,17} PKC ϵ has also been implicated in a neuroplastic change in nociceptor function, referred to as hyperalgesic priming¹ that has been suggested to contribute to the transition from acute to chronic pain²⁴. We have recently provided evidence that separate subcellular pools of PKC ϵ are responsible for these two functions (i.e., acute mechanical hyperalgesia and hyperalgesic priming)⁸.

In a previous study of the nociceptors involved in hyperalgesic priming, we evaluated the role of TrkA(+) versus Ret(+) neurons, by studying effects of their agonist ligands, NGF and GDNF, respectively⁸. While the mechanical hyperalgesia induced by NGF and GDNF are

PKC ϵ independent^{3,19}, both neurotrophic factors produce hyperalgesic priming⁸. Since nociceptors are commonly divided into TrkA(+) and IB4(+) ^{18,23}, we interpreted these findings to suggest that PKC ϵ -dependent hyperalgesic priming as well as acute mechanical hyperalgesia occurred in both major classes of nociceptors. And, while, in the mouse almost all Ret(+) neurons are IB4(+) ^{14,20,27}, in the rat many TrkA(+) neurons are also IB4(+) ^{7,15}. Since we have shown that G-protein coupled receptor activation of PKC ϵ causes it to translocate to the plasma membrane in IB4(+) but not in IB4(-) neurons⁹, we here tested the hypothesis that the IB4(+) subpopulation of TrkA(+) neurons, as well as the Ret(+) population of IB4(+) neurons – which together make up the IB4(+) population of neurons in the dorsal root ganglion of the rat – but not the IB4(-) population of sensory neurons, mediate hyperalgesic priming.

In our current study we observed that the destruction of IB4(+) neurons eliminated the ability of NGF as well as GDNF to induce priming (i.e., PGE₂ hyperalgesia was no longer present 4 hr). Since both NGF and GDNF induce priming in the control rat⁸, we suggest that IB4 positivity defines nociceptors that underlie hyperalgesic priming. Furthermore, while ψ εRACK, a highly selective activator of PKC ϵ ⁶, which also induces hyperalgesic priming in normal control rats¹³, did not induce priming in IB4-saporin treated animals it still produced robust mechanical hyperalgesia. This finding provides further support that there are at least two pools of PKC ϵ in primary afferent nociceptors, one mediating acute nociceptor sensitization and mechanical hyperalgesia, the other hyperalgesic priming^{1,8,9}. While the subcellular localization of these two pools of PKC ϵ remains to be established, the finding that PKC ϵ translocates to the plasma membrane in IB4(+) but not in IB4(-) neurons⁹, supports the plasma membrane as a candidate cellular site important for hyperalgesic priming.

The implications of our findings are, in part, dependent on the central circuits to which IB4(+) and IB4(-) neurons provide input. Of note in this regard, it has been suggested that IB4(+) and IB4(-) nociceptors input to different pain pathways in the central nervous system. Thus, IB4(+) neurons terminate in lamina II in the dorsal horn of the spinal cord, while IB4(-) neurons terminate in lamina I and IIo². Recently, Braz and colleagues have provided evidence that these two populations of nociceptors in the mouse signal to different circuits in the central nervous system, with IB4(+) neurons providing input to amygdala, hypothalamus, bed nucleus of the stria terminalis and globus pallidus while TrkA(+) neurons providing input to thalamus, parabrachial nuclei and hypothalamus⁴. Implications of the function of these two ‘circuits’ for pain syndromes remain to be established.

While the functional differences between IB4(+) and IB4(-) nociceptors in pain syndromes is poorly understood, we recently showed that elimination of IB4(+) nociceptors with IB4-saporin prevented the acute painful peripheral neuropathy induced by the cancer chemotherapeutic agent, oxaliplatin¹² suggest a potential clinical importance of this population of primary afferent nociceptors.

In summary, we have evaluated the population of primary afferent nociceptors that mediates hyperalgesic priming (i.e., persistent hyperalgesia induced by proinflammatory cytokines), a PKC ϵ -dependent mechanism in the primary afferent nociceptor that has been suggested to contribute to the transition from acute to chronic pain. We found that activation of PKC ϵ produces priming in IB4(+) (Ret(+) and TrkA(+)) but not in IB4(-) (TrkA(+)) nociceptors. Furthermore, we found that when IB4(+) nociceptors are destroyed by the IB4-binding toxin, IB4-saporin, activation of PKC ϵ in the remaining, TrkA(+)/IB4(-), nociceptors still produces hyperalgesia, but no longer produces hyperalgesic priming. Although TrkA has been considered to define a functional population of nociceptors⁴ our data suggest that IB4 positivity, or negativity, better defines a role in the development of chronic pain.

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Abbreviations

GDNF	glia-derived neurotrophic factor
IB4(+)	isolectin B4-positive
NGF	nerve growth factor
PGE₂	prostaglandin E ₂
PKCε	protein kinase C epsilon
SEM	standard error of the mean

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Figure 1. Changes in mechanical hyperalgesia in IB4-saporin pretreated rats

A. Changes in mechanical hyperalgesia in IB4-saporin pretreated rats Intradermal injection of glia-derived neurotrophic factor (GDNF) in rats that were pretreated with the neurotoxin isolectin B4 (IB4)-saporin failed to produce mechanical hyperalgesia (n=6), while in groups of rats similarly pretreated with IB4-saporin, nerve growth factor (NGF) and ψεRACK produced robust hyperalgesia (both n=6; p<0.001).

B. Hyperalgesia induced by GDNF, NGF and ψεRACK Intradermal administration of GDNF (10 ng), NGF (1 μg) and ψεRACK (1 μg) produced mechanical hyperalgesia in naïve rats. All drugs were injected in a volume of 5 μl and the paw withdrawal thresholds measured 30 min after each injection (n=6/group, p<0.001).

Figure 2. Lack of hyperalgesic priming in IB4-saporin pretreated rats

A. Lack of hyperalgesic priming in IB4-saporin pretreated rats In rats pre-treated with IB4-saporin, 10 days prior, NGF and $\psi\epsilon$ RACK, as well as GDNF, prostaglandin E₂ (PGE₂) administered in separate groups of rats, failed to induce hyperalgesic priming.

B. GDNF, NGF and $\psi\epsilon$ RACK-induced hyperalgesic priming Intradermal injection of PGE₂ produced priming (prolonged duration of PGE₂ hyperalgesia) in rats that were previously treated with GDNF, NGF or $\psi\epsilon$ RACK (n=6/group, p<0.001).