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Nociceptor Subpopulations Involved in Hyperalgesic Priming

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Abstract

We have previously developed a model for the transition from acute to chronic pain, hyperalgesic priming, in which a long-lasting neuroplastic change in signaling pathways mediates a prolongation of proinflammatory cytokine-induced nociceptor sensitization and mechanical hyperalgesia, induced at the site of a previous inflammatory insult. Induction of priming is mediated by activation of protein kinase C ϵ (PKC ϵ) in the peripheral terminal of the primary afferent nociceptor. Given that hyperalgesic mediator-induced PKC ϵ translocation occurs in IB4(+)-nonpeptidergic but not in TrkA(+)-peptidergic nociceptors, we tested the hypothesis that hyperalgesic priming was restricted to the IB4(+) subpopulation of nociceptors. After recovery from NGF- and GDNF-induced hyperalgesia, a proinflammatory cytokine, prostaglandin E₂ induced, PKC ϵ -dependent, markedly prolonged hyperalgesia, two features that define the development of the primed state. Thus, hyperalgesic priming occurs in both the IB4(+)-nonpeptidergic and TrkA(+)-peptidergic subpopulations of nociceptive afferents. Of note, however, while attenuation of PKC ϵ prevented NGF-induced priming, the hyperalgesia induced by NGF is PKC ϵ -independent. We propose that separate intracellular pools of PKC ϵ , in the peripheral terminals of nociceptors, mediate nociceptor sensitization and the induction of hyperalgesic priming.

Keywords

primary afferent neuron; NGF; GDNF; hyperalgesia; PKC ϵ

In studies of the mechanisms that distinguish acute from chronic pain, we have recently demonstrated that inflammation produces a long-lasting neuroplastic change in the signaling pathway mediating proinflammatory cytokine-induced nociceptor sensitization and mechanical hyperalgesia, at a previously inflamed site (Aley et al., 2000; Reichling and Levine, 2009). The induction of this hyperalgesic priming is mediated by activation of protein kinase C ϵ (PKC ϵ) in the peripheral terminals of primary afferent nociceptors (Aley et al., 2000; Parada et al., 2003a). While PKC ϵ is found in almost all dorsal root ganglion neurons (Hundle et al., 1995; Cesare et al., 1999; Khasar et al., 1999; Numazaki et al., 2002; Summer et al., 2006; Yamamoto et al., 2006), its translocation from the cytoplasm to the plasma membrane during nociceptor sensitization, occurred in isolectin B4 (IB4)(+)-nonpeptidergic but not TrkA(+)-

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peptidergic nociceptors (Hucho et al., 2005), suggesting that some forms of chronic pain may be generated by changes in a defined subset of nociceptors. In the present study we tested the hypothesis that hyperalgesic priming occurs in the IB4(+)-nonpeptidergic, but not the TrkA (+)-peptidergic subpopulation of primary afferent nociceptors. To test our hypothesis we first evaluated if two neurotrophins, nerve growth factor (NGF) and glial cell-derived neurotrophic factor (GDNF), which have been shown previously to sensitize TrkA(+)- and IB4(+)-positive afferent nociceptor populations, respectively (Lewin and Mendell, 1993; Woolf et al., 1994; McMahon, 1996; Sammons et al., 2000; Amaya et al., 2004; Malik-Hall et al., 2005; Malin et al., 2006; Bogen et al., 2008), are also able to induce hyperalgesic priming. Since the mechanical hyperalgesia induced by NGF is independent of PKC ϵ (Malik-Hall et al., 2005), whereas priming is PKC ϵ -dependent (Aley et al., 2000; Parada et al., 2003a; Parada et al., 2005), we studied the TrkA-expressing peptidergic nociceptors, at which NGF acts to produce hyperalgesia and priming, to determine if the hyperalgesic priming induced by NGF is PKC ϵ dependent.

Experimental Procedures

Animals

Experiments were performed on adult male Sprague Dawley rats (250-350 grams; Charles River, Hollister, CA). Animals were housed three per cage, under a 12 hr light/dark cycle, in a temperature and humidity controlled environment at the University of California at San Francisco (UCSF) animal care facility. Food and water were available *ad libitum*. All testing was done between 10:00 am and 4:00 pm. Experimental protocols, approved by the UCSF Committee on Animal Research, conformed to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Nociceptive testing

The nociceptive flexion reflex was quantified with an Ugo Basile Analgesymeter (Stoelting, Chicago, IL USA), which applies a linearly increasing mechanical force to the dorsum of a rat's hindpaw. Nociceptive threshold, defined as the force in grams at which the rat withdrew its paw, was the mean of three readings taken at 5-minute intervals. Rats were lightly restrained in cylindrical transparent acrylic restrainers designed to provide adequate comfort and ventilation, allow extension of the hind leg from the cylinder, and minimize stress. All rats were acclimatized to the testing procedures in order to reduce variability and produce a more stable baseline of the paw-withdrawal threshold measurement. Briefly, before starting each experiment, the animals were individually placed in the restrainers for an hour and on each test day for 30 minutes before starting the experiment (Dina et al., 2006). The paw pressure threshold was determined before and after administration of test agents. Each paw was treated as an independent measure and each experiment performed on a separate group of rats. The results are expressed as percentage change from baseline mechanical nociceptive threshold.

Drugs

Drugs employed in this study were prostaglandin E₂ (a direct-acting hyperalgesic agent) and NGF, from Sigma (St. Louis, MO), and GDNF from EMD Biosciences (La Jolla, CA). Drugs were applied by intradermal injection on the dorsum of the hindpaw. A stock solution of NGF (1 μ g/ μ l in 0.9% NaCl containing 0.5% BSA) was diluted in 0.9% NaCl at the time of injection (final concentration 0.2 μ g/ μ l, dose 1 μ g) (Malik-Hall et al., 2005). GDNF was similarly prepared, as described previously (Bogen et al., 2008). 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%. All drugs (except antisense and mismatch oligodeoxynucleotide) were administered intradermally in a volume of 5 μ l using a 30-gauge

hypodermic needle attached to a Hamilton syringe. The selection of the drug doses used in this study was based on dose-response curves determined during previous studies (Khasar et al., 1993; Malik-Hall et al., 2005; Bogen et al., 2008).

Antisense and mismatch oligodeoxynucleotide

Oligodeoxynucleotide antisense and mismatch to PKC ϵ mRNA were prepared as described previously (Parada et al., 2003a). The oligodeoxynucleotide antisense sequence, 5'-GCC AGC TCG ATC TTG CGC CC-3', was directed against a unique sequence of rat PKC ϵ mRNA. The corresponding GenBank (National Institute of Health, Bethesda, MD) accession number and oligodeoxynucleotide position within the cDNA sequence are XM345631 and 226-245, respectively. We have previously shown that spinal intrathecal administration of antisense oligodeoxynucleotide with this sequence decreases PKC ϵ protein in dorsal root ganglia (Parada et al., 2003b; Parada et al., 2003a). The sequence of the mismatch oligodeoxynucleotide, 5'-GCC AGC **GCG** ATC TTT CGC CC-3', corresponds to the PKC ϵ antisense sequence with 2 bases mismatched (in bold typeface).

Prior to use, lyophilized oligodeoxynucleotide was reconstituted in nuclease-free 0.9% NaCl to a concentration of 10 μ g/ μ l and stored at -20°C until use. A dose of 40 μ g of antisense or mismatch oligodeoxynucleotide was administered intrathecally in a volume of 20 μ l once daily for 3 consecutive days. Prior to each injection, rats were anesthetized with 2.5% isoflurane in oxygen. Oligodeoxynucleotide was injected using a 30-gauge hypodermic needle inserted between the fifth and sixth lumbar vertebrae, at the level of the cauda equina; intrathecal location of the injection needle was confirmed by a flicking of the rat's tail (Papir-Kricheli et al., 1987).

Statistical analysis

In all experiments, the dependent variable was change in paw withdrawal threshold as a percent of baseline paw withdrawal threshold. To test for significant differences in the effect of experimental interventions on paw withdrawal mechanical threshold over time, repeated measures ANOVAs were performed. For experiments with only one group, a significant main effect of time was followed by simple contrasts in which responses at each time point were compared to the initial time point using simple contrasts. Because these analyses required multiple comparisons, the alpha level ($p < 0.05$) was divided by the number of comparisons as a Bonferroni-type correction. For experiments with two groups, two-way repeated measures ANOVAs were performed with one between subjects factor (*i.e.*, treatment) and one within subjects factor (*i.e.*, time). The Mauchly criterion was tested 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 the main effect of treatment was significant, t -tests were performed for each time point to determine when the significant differences occurred. As with simple contrasts (above), the alpha level was divided by the number of t -tests as a Bonferroni-type correction. Data are presented in figures as mean $\pm$ standard error of the mean (S.E.M.).

Results

GDNF and NGF induce hyperalgesic priming

In the primed state induced by local injection of carrageenan, a single injection of prostaglandin E₂, which in normal tissue induces a brief mechanical hyperalgesia that is no longer present at 4 hours, now induces prolonged hyperalgesia present 4, and even 24, hours after injection (Aley et al., 2000; Parada et al., 2003a). Following this protocol, we tested if the intradermal injection of GDNF or NGF, in place of carrageenan, induces hyperalgesic priming. The intradermal injection of GDNF (10ng/5 μ l) and NGF (1 μ g/5 μ l) on the dorsal surface of the

hindpaw induced mechanical hyperalgesia that lasted 3 weeks (Fig. 1A) and 4 days (Fig. 2A), respectively. When mechanical nociceptive threshold had returned to preneurotrophin baseline, prostaglandin E₂ (100ng/5μl) was injected into the same site on the paw and the mechanical nociceptive threshold determined 30 minutes, 4 hours and 24 hours later. In rats that had been treated with either GDNF or NGF prostaglandin E₂ produced a decrease in mechanical nociceptive threshold that lasted more than 4 hours (Figs. 1B and 2B). Thus, in contrast to our initial hypothesis, both NGF, whose receptor TrkA is selectively expressed on peptidergic neurons (Verge et al., 1992; Molliver and Snider, 1997; Bennett, 2001) and GDNF, whose receptor is selectively expressed by IB4(+)-nonpeptidergic neurons (Molliver et al., 1997; Bennett et al., 1998; Bennett, 2001) produce hyperalgesic priming.

Priming in TrkA(+)-peptidergic nociceptors is PKCε dependent

While induction of hyperalgesic priming involves activation of PKCε in the peripheral terminals of the nociceptors (Aley et al., 2000; Parada et al., 2003a), when inflammatory mediator-induced PKCε activation was assayed, *in vitro*, using translocation from the cytoplasm to the plasma membrane, PKCε was only activated in the IB4(+)-nonpeptidergic subset of nociceptors (Hucho et al., 2005). Since the acute hyperalgesia induced by NGF, which occurs in the TrkA(+)-peptidergic nociceptors, is *not* PKCε-dependent (Malik-Hall et al., 2005), we next determined if NGF-induced hyperalgesic priming is PKCε-dependent. To determine if priming produced by NGF induces a switch to PKCε signaling for prostaglandin E₂ hyperalgesia, rats were treated with oligodeoxynucleotide antisense or mismatch to PKCε mRNA, after recovery from NGF-induced hyperalgesia. When rats were treated intrathecally with antisense or mismatch oligodeoxynucleotide to PKCε for 3 days and hyperalgesia induced by prostaglandin E₂ on the fourth day, antisense but not mismatch oligodeoxynucleotide suppressed prostaglandin E₂ hyperalgesia at 4 hours, but not 30 minutes (Fig. 3A), a characteristic feature of hyperalgesic priming (Aley et al., 2000; Parada et al., 2003a). In a separate group of rats that were previously primed with NGF, antisense oligodeoxynucleotide was administered for 3 days, followed by a 3-week recovery period. In these rats, in which PKCε level has recovered from treatment with antisense (Parada et al., 2003a), prostaglandin E₂ hyperalgesia at 4 hours was no longer attenuated, demonstrating reversibility of the effect of PKCε antisense.

To demonstrate that the development of hyperalgesic priming is dependent on PKCε, we pretreated rats with oligodeoxynucleotides antisense or mismatch to PKCε starting 3 days prior to administration of NGF, and continuing for an additional 7 days, at which time NGF-induced hyperalgesia had returned to baseline. Of note, antisense did not affect the magnitude and time course of NGF hyperalgesia (Fig. 4A). Tested either 2 days (Fig. 4B) or 2 weeks (Fig. 4C) after the last administration of antisense oligodeoxynucleotide, rats failed to demonstrate prolongation of prostaglandin E₂ hyperalgesia, indicating PKCε dependence for the hyperalgesic priming induced by NGF.

Discussion

Hyperalgesic priming is a neuroplastic change in the primary afferent nociceptor in which proinflammatory mediators, for example prostaglandin E₂, serotonin and adenosine, now produce markedly prolonged hyperalgesia, which requires activation of a novel isoform of PKC, PKCε (Aley et al., 2000; Parada et al., 2005). Since translocation of PKCε from the cytoplasm to the plasma membrane, a marker of PKCε activation (Cesare et al., 1999; Dorn and Mochly-Rosen, 2002) in response to a hyperalgesic agent, occurred only in the IB4(+)-nonpeptidergic subset of nociceptors (Hucho et al., 2005), we tested the hypothesis that priming also occurs in the IB4(+)-nonpeptidergic but not in the TrkA(+)-peptidergic subpopulation of nociceptors, using pronociceptive neurotrophic factors that produce hyperalgesia by action at

either the IB4(+)-nonpeptidergic nociceptors, GDNF (Amaya et al., 2004; Albers et al., 2006; Bogen et al., 2008), or the TrkA(+)-peptidergic nociceptors, NGF (Woolf et al., 1994; McMahon et al., 1995; Sammons et al., 2000; Chuang et al., 2001; Malik-Hall et al., 2005; Watanabe et al., 2008). Against our initial hypothesis, however, NGF as well as GDNF produced hyperalgesic priming.

Since the mechanical hyperalgesia induced by NGF is PKC ϵ -independent (Malik-Hall et al., 2005), we determined if NGF-induced priming is PKC ϵ -dependent. Given our previous failure to detect PKC ϵ -dependent hyperalgesia in TrkA(+)-peptidergic nociceptors (Fig. 4A), our finding that NGF-induced hyperalgesic priming is PKC dependent supports the suggestion that separate pools of PKC ϵ mediate NGF hyperalgesia and the induction of priming. In support of this hypothesis, others have described separate pools of PKC ϵ , for example, in cardiac myocytes, involved in different signaling pathways, in the same cell (Costa et al., 2006). We found that NGF-induced priming, unlike NGF hyperalgesia, is PKC ϵ dependent. Compatible with the idea that the priming effect of, for example, carrageenan or the selective PKC ϵ translocation activator ψ ERACK, can be separated from its ability to produce nociceptor sensitization and mechanical hyperalgesia, we have previously shown that a low dose of carrageenan, which fails to produce mechanical hyperalgesia, still can produce hyperalgesic priming (Aley et al., 2000). The subcellular location of and different signaling pathways activated by these two PKC ϵ -dependent effects remain to be elucidated.

While PKC ϵ activation is necessary for NGF-priming, its translocation to the plasma membrane has only been observed in non-peptidergic IB4(+) fibers (Hucho et al., 2005). PKC ϵ can, however, be translocated to and activated at other subcellular sites such as the Golgi network and mitochondria (Ron et al., 1994; Lehel et al., 1995; Mochly-Rosen and Gordon, 1998; Schultz et al., 2004). Thus, we suggest that the PKC ϵ pool responsible for induction of priming in nociceptors is at a site other than the plasma membrane.

In summary, based on the observation that PKC ϵ translocation to the plasma membrane occurs in IB4(+)-nonpeptidergic but not TrkA(+)-peptidergic subpopulations of nociceptive afferents, and that NGF, which induces hyperalgesia by action on the TrkA(+)-peptidergic subpopulation of nociceptive afferents, we tested the hypothesis that hyperalgesic priming, a model of the transition from acute to chronic pain, occurs in the IB4(+)-nonpeptidergic but not TrkA(+)-peptidergic subpopulation of nociceptive afferents. The finding that NGF as well as GDNF induces hyperalgesic priming led us to evaluate whether separate pools of PKC ϵ mediate acute hyperalgesia and hyperalgesic priming. Our finding that NGF-induced priming is PKC ϵ dependent demonstrates that NGF can induce hyperalgesia that is PKC ϵ independent, while activating a pool of PKC ϵ that induces priming, without inducing hyperalgesia. The characterization of these two subpopulations of PKC ϵ is, at present, unknown.

Acknowledgments

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List of abbreviations

PKC	protein kinase C
PKC ϵ	protein kinase C epsilon isoform
IB4	isolectin B4
TrkA	receptor tyrosine kinase
NGF	nerve growth factor

GDNF	glial cell-derived neurotrophic factor
PGE ₂	prostaglandin E ₂
ODN	oligodeoxynucleotide
AS	antisense
MM	mismatch

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Figure 1. Hyperalgesic priming induced by intradermal injection of GDNF

(A) Intradermal injection of GDNF (10ng/5μl) into the rat hind paw induces prolonged mechanical hyperalgesia in the rat (one-way repeated measures ANOVA, $F(7,77)=41.674$, $p<0.001$). Three weeks later the mechanical threshold returned to the pre-GDNF administration baseline. Testing for the presence of hyperalgesic priming was performed on the 23rd day. N=12 paws;

(B) Test for hyperalgesic priming in GDNF-treated rats: intradermal injection of prostaglandin E₂ (PGE₂, 100ng/5μl) on the 23rd day after GDNF administration. GDNF-primed rats (black bars) showed decrease in mechanical nociceptive threshold that lasted at least 24 hours after

PGE₂ injection ($p < 0.01$ between GDNF+PGE₂ and naïve+PGE₂ groups at 4 and 24 hours after PGE₂ injection). White bars show the effect of PGE₂ injection in naïve animals. N=6 paws.

Figure 2. Hyperalgesic priming induced by intradermal injection of NGF

(A) Intradermal injection of NGF (1 μ g/5 μ l) into the rat hind paw induces prolonged mechanical hyperalgesia, which lasted 4 days, in the rat (one-way repeated measures ANOVA, $F(4,44) = 57.308$, $p < 0.01$). On the 6th day mechanical threshold was not different from the pre NGF administration baseline. Testing for the presence of hyperalgesic priming was performed on the 7th day. N=12 paws;

(B) Test for hyperalgesic priming in NGF-treated rats: intradermal injection of PGE₂ (100ng/5 μ l, intradermal) on the 7th day after NGF administration. Rats pretreated with NGF (black bars) show decrease in the mechanical nociceptive threshold, after PGE₂ injection, which lasted at least 24 hours ($p < 0.01$ between NGF+PGE₂ and naïve+PGE₂ groups 4 and 24 hours after PGE₂ injection). Mechanical hyperalgesia was no longer present 4 hours after injection of PGE₂ in the control rats not treated with NGF (grey bars). N=6 paws.

Figure 3. NGF-induced hyperalgesic priming is dependent on PKCε

(A) After return to baseline mechanical threshold NGF-treated rats received spinal intrathecal oligodeoxynucleotide (ODN) antisense (AS) or mismatch (MM) for PKCε mRNA for 3 consecutive days. After this period, the animals were tested for priming with intradermal injection of PGE₂ (100ng/5μl/paw). Four hours later, PGE₂-induced mechanical hyperalgesia was not present in the animals treated with AS (grey bars), while the MM group still demonstrated a decrease in the mechanical nociceptive threshold (black bars). N=6 paws ($p<0.001$ between antisense and mismatch groups at 240 minutes after PGE₂ injection); (B) Test for hyperalgesic priming in NGF-treated rats three weeks after treatment with PKCε AS or MM: both groups show hyperalgesia that lasts at least 4 hours after intradermal injection of PGE₂. N=6 paws (no difference observed between antisense and mismatch groups).

Figure 4. NGF-induced hyperalgesic priming is prevented by knockdown of PKCε

(A) Treatment with oligodeoxynucleotide (ODN) antisense (AS) or mismatch (MM) for PKCε mRNA starting 3 days before injection of NGF and continuing until the recovery of the mechanical threshold baseline (7th day after NGF). Mechanical nociceptive threshold in AS- or MM-treated rats was evaluated day 1, 3, 4 and 7 post-NGF administration. N=6 paws (no difference observed between antisense and mismatch groups);

(B) Test for priming with intradermal injection of PGE₂ (100ng/5μl/paw) was performed on the 9th day after NGF injection. In the fourth hour after PGE₂, mechanical hyperalgesia is observed in the mismatch group (black bars), but not in the rats treated with antisense (grey bars). N=6 paws ($p < 0.001$ between antisense and mismatch groups at 240 minutes after PGE₂ injection);

(C) NGF-treated rats that were pretreated with AS or MM were also tested for priming 2 weeks after the return to pre-NGF mechanical threshold baseline. Animals treated with AS still did not show hyperalgesic priming (grey bars). N=6 paws ($p < 0.001$ between antisense and mismatch groups at 240 minutes after PGE₂ injection).