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NOCICEPTOR SUBPOPULATIONS INVOLVED IN HYPERALGESIC PRIMING

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Abstract—We have previously developed a model in the rat 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 Cε (PKCε) in the peripheral terminal of the primary afferent nociceptor. Given that hyperalgesic mediator-induced PKCε translocation occurs in isolectin B4 (IB4)(+)-nonpeptidergic but not in receptor tyrosine kinase (TrkA)(+)-peptidergic nociceptors, we tested the hypothesis that hyperalgesic priming was restricted to the IB4(+) subpopulation of nociceptors. After recovery from nerve growth factor (NGF)- and GDNF-induced hyperalgesia, a proinflammatory cytokine, prostaglandin E_2 (PGE_2) induced, PKCε-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 PKCε prevented NGF-induced priming, the hyperalgesia induced by NGF is PKCε independent. We propose that separate intracellular pools of PKCε, in the peripheral terminals of nociceptors, mediate nociceptor sensitization and the induction of hyperalgesic priming. **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: primary afferent neuron, NGF, GDNF, hyperalgesia, PKCε.

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 epsilon (PKCε) in the peripheral terminals of primary afferent nociceptors (Aley et al., 2000; Parada et al., 2003a). While PKCε 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 receptor tyrosine kinase (TrkA)(+)-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 PKCε (Malik-Hall et al., 2005), whereas priming is PKCε-dependent (Aley et al., 2000; Parada et al., 2003a, 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 PKCε dependent.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on adult male Sprague–Dawley rats (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 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 min intervals. Rats were lightly restrained in cylindrical transparent acrylic restrainers designed to provide adequate com-

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Abbreviations: ANOVA, analysis of variance; AS, antisense; GDNF, glial cell-derived neurotrophic factor; IB4, isolectin B4; MM, mismatch; NGF, nerve growth factor; ODN, oligodeoxynucleotide; PGE_2, prostaglandin E_2; PKCε, protein kinase C epsilon; TrkA, receptor tyrosine kinase.

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fort 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 min 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 E_2 (a direct-acting hyperalgesic agent) and NGF, from Sigma (St. Louis, MO, USA), and GDNF from EMD Biosciences (La Jolla, CA, USA). Drugs were applied by intradermal injection on the dorsum of the hindpaw. A stock solution of NGF (1 μg/μl in 0.9% NaCl containing 0.5% BSA) was diluted in 0.9% NaCl at the time of injection (final concentration 0.2 μg/μl, dose 1 μg) (Malik-Hall et al., 2005). GDNF was similarly prepared, as described previously (Bogen et al., 2008). The stock solution of prostaglandin E_2 (1 μg/μl) was prepared in absolute ethanol, and additional dilutions made with physiological saline; the final concentration of ethanol was $\leq$2%. All drugs (except antisense (AS) and mismatch (MM) oligodeoxynucleotide (ODN)) were administered intradermally in a volume of 5 μl using a 30-gauge hypodermic needle attached to a Hamilton (Reno, NV, USA) 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 ODN

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

Prior to use, lyophilized ODN was reconstituted in nuclease-free 0.9% NaCl to a concentration of 10 μg/μl and stored at −20 °C until use. A dose of 40 μg of AS or mismatch ODN was administered intrathecally in a volume of 20 μl once daily for three consecutive days. Prior to each injection, rats were anesthetized with 2.5% isoflurane in oxygen. ODN was injected using a 30-gauge hypodermic needle inserted between the fifth and sixth lumbar vertebrate, 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 (SEM).

RESULTS

GDNF and NGF induce hyperalgesic priming

In the primed state induced by local injection of carrageenan, a single injection of prostaglandin E_2, which in normal tissue induces a brief mechanical hyperalgesia that is no longer present at 4 h, now induces prolonged hyperalgesia present four, 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 (10 ng/5μ) and NGF (1 μg/5 μl) 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 pre-neurotrophin baseline, prostaglandin E_2 (100 ng/5 μl) was injected into the same site on the paw and the mechanical nociceptive threshold determined 30 min, 4 h and 24 h later. In rats that had been treated with either GDNF or NGF prostaglandin E_2 produced a decrease in mechanical nociceptive threshold that lasted more than 4 h (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 E_2 hyperalgesia, rats were treated with ODN antisense or MM to PKCε mRNA,

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