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A Novel Nociceptor Signaling Pathway Revealed in Protein Kinase C ϵ Mutant Mice

Sachia G. Khasar,* Yu-Huei Lin,† Annick Martin,† Jahan Dadgar,† Thomas McMahon,† Dan Wang,† Bhupinder Hundle,† K. O. Aley,* William Isenberg,* Gordon McCarter,* Paul G. Green,* Clyde W. Hodge,† Jon D. Levine,* and Robert O. Messing†‡

*Department of Internal Medicine
and Oral Surgery
National Institutes of Health/University
of California, San Francisco Pain Center
†Department of Neurology
Ernest Gallo Clinic and Research Center
University of California, San Francisco
San Francisco, California 94143

Summary

There is great interest in discovering new targets for pain therapy since current methods of analgesia are often only partially successful. Although protein kinase C (PKC) enhances nociceptor function, it is not known which PKC isozymes contribute. Here, we show that epinephrine-induced mechanical and thermal hyperalgesia and acetic acid–associated hyperalgesia are markedly attenuated in PKCϵ mutant mice, but baseline nociceptive thresholds are normal. Moreover, epinephrine-, carrageenan-, and nerve growth factor– (NGF-) induced hyperalgesia in normal rats, and epinephrine-induced enhancement of tetrodotoxin-resistant Na^+ current (TTX-R I_{Na}) in cultured rat dorsal root ganglion (DRG) neurons, are inhibited by a PKCϵ-selective inhibitor peptide. Our findings indicate that PKCϵ regulates nociceptor function and suggest that PKCϵ inhibitors could prove useful in the treatment of pain.

Introduction

Recent evidence suggests that protein kinase C (PKC) regulates nociceptor function. Tumor-promoting phorbol esters, which activate most PKC isozymes (Nishizuka, 1992), sensitize nociceptors (Schepelmann et al., 1993) and enhance, in nociceptors, a current activated by a noxious thermal stimulus (Cesare and McNaughton, 1996). In addition, PKC is an important contributor to diabetic neuropathic hyperalgesia (Ahlgren and Levine, 1994), bradykinin-induced activation and sensitization of nociceptors (McGuirk and Dolphin, 1992), and epinephrine-induced hyperalgesia and nociceptor sensitization (Khasar et al., 1999). However, PKC is a family of at least ten serine-threonine kinases, and while there are functional differences between PKC isozymes (Nishizuka, 1992), it is not known which isozyme contributes to nociceptor responses.

Recent findings suggest that PKCϵ may be important in nociceptor function. PKCϵ is activated by nerve growth factor (NGF) (Ohmichi et al., 1993) and modulates several of NGF's actions, including activation of mitogen-activated protein (MAP) kinases Erk1 and Erk2 and neurite outgrowth (Hundle et al., 1995, 1997). In addition, NGF is elevated at sites of inflammation (Safieh-Garabedian et al., 1995) and produces hyperalgesia (Lewin et al., 1993). The observations that NGF exerts hyperalgesic effects and that PKCϵ regulates responses to NGF led us to postulate that PKCϵ plays a role in pain signaling.

In the present study, we tested this hypothesis by examining epinephrine- and acetic acid–associated hyperalgesia in PKCϵ mutant mice and epinephrine-, carrageenan-, and NGF-induced hyperalgesia in normal rats treated with a selective peptide inhibitor of PKCϵ. Our findings provide strong evidence that PKCϵ regulates nociceptor function.

Results

Characterization of PKCϵ Mutant Mice

We used embryonic stem (ES) cells to generate PKCϵ mutant mice by homologous recombination (Thompson et al., 1989). A recombination vector was constructed containing mouse genomic PKCϵ sequences harboring a 1.2 kb deletion. Integration of this vector was predicted to result in loss of the start codon (Figure 1A). This vector was transfected into ES cells, and nine clones containing the homologous integration were identified. Two were injected into blastocysts to produce chimeric animals. Three chimeric males mated to C57BL/6J females transmitted the mutation through the germline. Heterozygous progeny were intercrossed, and offspring were typed by Southern analysis for the PKCϵ mutation (Figure 1B).

Using Western blot analysis, we found that PKCϵ immunoreactivity could not be detected in dorsal root ganglia (DRG) (Figure 1C) or in the central nervous system (data not shown) of mutant mice. All other PKC isozymes except PKCγ could be detected in DRG from wild-type mice, and their abundance was similar in wild-type and PKCϵ mutant mice (Figure 1C). From 75 litters of heterozygous breeding pairs, 826 mice were genotyped at weaning: 193 (23%) were wild-type, 441 (54%) were heterozygotes, and 192 (23%) were homozygotes, showing an expected Mendelian ratio ($\chi^2 = 1.83$, $p = 0.40$). Food and water intake were normal in mutant mice (data not shown), and body weight measured daily over 2 weeks in 8-week-old animals was similar in PKCϵ-mutant (24.73 ± 0.9 g, $n = 8$) and wild-type (23.5 ± 0.9 g, $n = 8$) mice. Locomotor activity (Figure 1D) and performance on a 3-arm maze (data not shown) were also similar in mutant and wild-type mice, and mutants could not be distinguished from wild-type littermates in the normal cage environment. Mutant mice have survived for over 1 year, and the spontaneous mortality rate among 500 mice of each genotype maintained at least 6 weeks was similar for PKCϵ mutant (2.6%) and wild-type (1.2%) animals ($\chi^2 = 1.28$, $p = 0.26$).

‡To whom correspondence should be addressed (e-mail: romes@itsa.ucsf.edu).

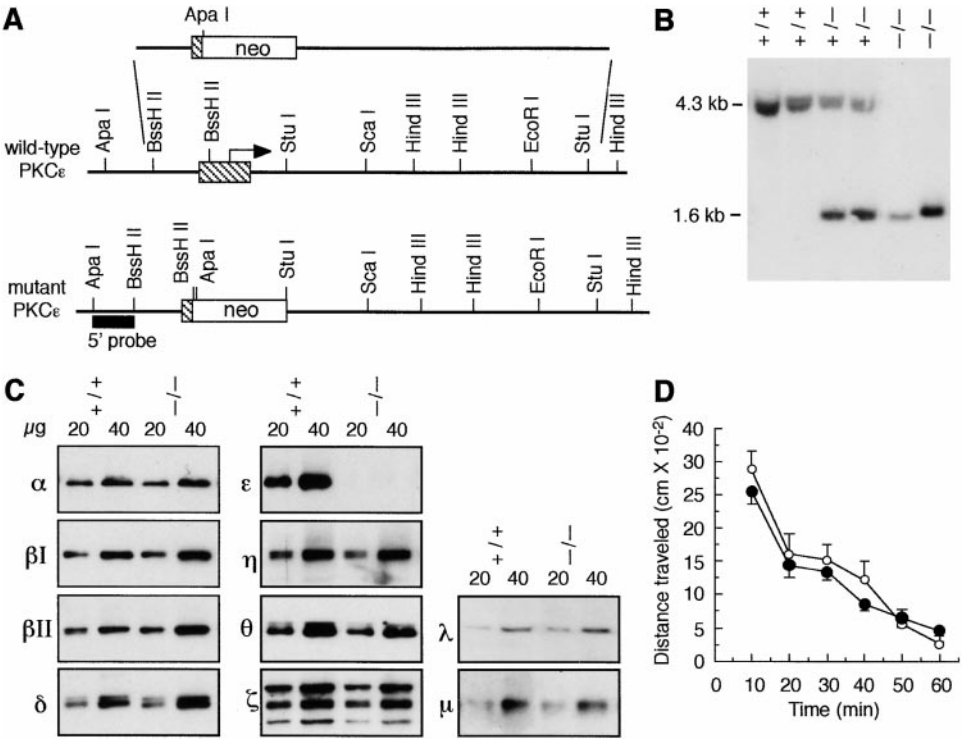

Figure 1. Gene Targeting of Mouse PKCε

(A) Schematic structures of wild-type and recombinant loci, the targeting vector, and the 5′ probe used for screening ES cell clones and mice.
(B) Southern blot of genomic DNA following digestion with ApaI and ScaI, showing fragments corresponding to wild-type (4.3 kb) and targeted (1.6 kb) alleles.
(C) Western blots of DRG tissue from wild-type (+/+) and PKCε mutant (−/−) mice, using antibodies to PKC isozymes. Three immunoreactive bands of 65, 74, and 84 kDa were detected with the anti-PKCζ antibody. The 74 kDa band corresponds to what was reported for rat brain PKCζ (Hunter et al., 1995).
(D) Spontaneous locomotor activity and habituation of wild-type (white circles) and PKCε mutant (black circles) mice over 1 hr. Data are mean ± SE values from six mice of each genotype.

PKCε Mutant Mice Show Decreased Epinephrine-Induced and Acetic Acid–Associated Hyperalgesia

To study the role of PKCε in mechanical and thermal nociceptor function, we first examined mice treated with epinephrine. Epinephrine-induced hyperalgesia and nociceptor sensitization depend on both PKC and protein kinase A (PKA) (Khasar et al., 1999). We chose to use epinephrine rather than bradykinin, which also activates PKC, because epinephrine acts directly on the primary afferent nociceptor (Khasar et al., 1999), whereas bradykinin can act indirectly (Andreev et al., 1995). We then compared epinephrine-induced responses with responses induced by prostaglandin E2 (PGE_2) since PGE_2-stimulated sensitization is mediated only by PKA (Taiwo and Levine, 1991).

Mechanical hyperalgesia was evaluated by von Frey hair (VFH) stimulation. The percent withdrawal response to VFH stimulation was similar in wild-type and mutant mice (Figure 2A). However, the increase in withdrawal response following epinephrine injection was significantly reduced in mutant animals. Nevertheless, mutant mice and their wild-type littermates showed a similar increase in response produced by PGE_2. Likewise, when thermal stimulation was employed, wild-type and mutant animals demonstrated similar basal thresholds and similar responses to PGE_2, but mutant animals displayed a reduced thermal hyperalgesia compared with wild-type littermates after epinephrine administration (Figure 2B). Finally, when evaluated by the acetic acid writhing test (Bjorkman, 1995), mutant mice demonstrated a significantly lower nociceptive score than wild-type animals (Figure 2C).

A Selective Inhibitor of PKCε Decreases Epinephrine-Induced Hyperalgesia in Rats

The studies above suggest that a defect in PKCε-mediated signal transduction accounts for reduced epinephrine-induced hyperalgesia. These results were not due to a deficit in motor function since PKCε mutant mice demonstrated enhanced withdrawal responses when treated with PGE_2. However, these experiments do not exclude the possibility that this phenotype arises from the absence of PKCε during development. To address this issue, we examined nociceptive responses in adult rats treated with a selective inhibitor of PKCε, so that virtually immediate assessment of hyperalgesia following antagonism of PKCε function could be performed. In addition, we treated rats with an inhibitor of multiple PKC isozymes to evaluate possible contributions of other isozymes to nociceptor sensitization.

Upon activation, PKC isozymes translocate to specific

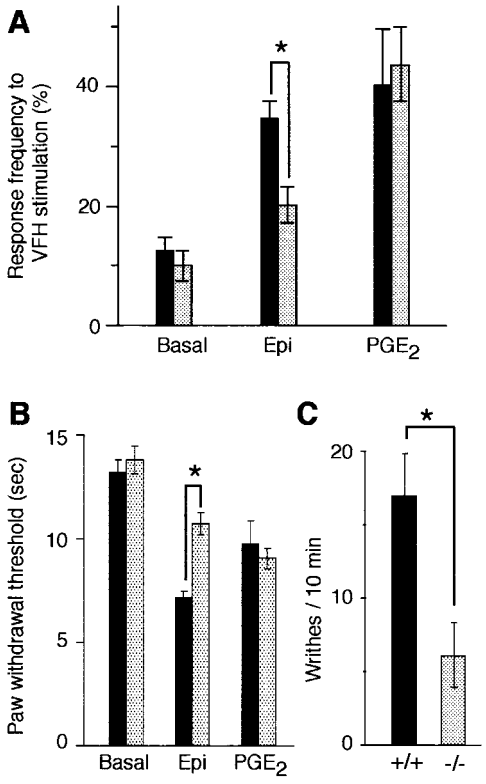

Figure 2. Mechanical and Thermal Nociception and Response to Acetic Acid in PKCε Mutant Mice

(A) Mechanical thresholds were measured in eight wild-type (black bars) and eight mutant (gray bars) mice in response to VFH (4 g) stimulation. Responses were measured before (basal) and after intraplantar injection of 100 ng epinephrine (Epi) or PGE_2. Asterisk, $p < 0.001$ (ANOVA and Scheffe F test).

(B) Thermal nociceptive thresholds in eight wild-type (black bars) and eight mutant (gray bars) mice before and after intraplantar injection of 100 ng epinephrine (Epi) or PGE_2. Asterisk, $p < 0.001$ (ANOVA and Scheffe F test).

(C) Writhing response to acetic acid administration in four wild-type (+/+) and four mutant (−/−) mice. Asterisk, $p < 0.05$ (Student's unpaired t test).

intracellular sites, where they appear to bind to anchoring proteins termed RACKs (Mochly-Rosen, 1995). The cotamer protein β′COP has recently been identified as a specific RACK for PKCε (Csukai et al., 1997). PKCε binds β′COP through its V1 region, and peptides derived from this region selectively inhibit phorbol ester–mediated translocation of PKCε in cultured cardiac myocytes (Johnson et al., 1996; Gray et al., 1997), PC12 cells (Hundle et al., 1997), and pancreatic beta cells (Yedovitzky et al., 1997). This inhibition of binding decreases phorbol ester- and norepinephrine-mediated inhibition of contraction in cardiac myocytes (Johnson et al., 1996) and inhibits protection of cardiac myocytes from hypoxia-induced cell death (Gray et al., 1997). It also reduces glucose-stimulated insulin secretion from islet cells (Yedovitzky et al., 1997) and prevents enhancement of NGF-induced neurite outgrowth and MAP kinase activation by phorbol esters in PC12 cells (Hundle et al., 1997). We used such an inhibitory peptide, εV1–2 (Johnson et al., 1996; Gray et al., 1997; Yedovitzky et al., 1997) to selectively inhibit PKCε in peripheral nerve terminals.

Intradermal injection of εV1–2 in normal rats decreased the mechanical hyperalgesia produced by injection of epinephrine into the hindpaw (Figure 3A). There was no effect from a scrambled εV1–2 peptide (Johnson et al., 1996). The magnitude of inhibition produced by εV1–2 was similar to that produced by the PKC inhibitor bisindoylmaleimide I, which inhibits several PKC isozymes, including PKCε (Toullec et al., 1991). Administration of εV1–2 had no effect on PGE_2-induced hyperalgesia. These results strongly support the conclusion that absence of PKCε-mediated signaling in adult neurons is responsible for reduced epinephrine sensitivity in PKCε mutant mice. Furthermore, since bisindoylmaleimide I was no more effective in inhibiting epinephrine hyperalgesia than the εV1–2 peptide, it is likely that the PKCε isozyme alone is responsible for the contribution of PKC to epinephrine-induced hyperalgesia.

PKCε Inhibition Attenuates Enhancement of Tetrodotoxin-Resistant Sodium Current by Epinephrine

In DRG neurons, epinephrine enhances a tetrodotoxin-resistant sodium current (TTX-R I_{Na}) (Khasar et al., 1999) whose activity is increased by agents that act directly on primary afferent nociceptors in vivo to produce hyperalgesia (Gold et al., 1996). Bisindoylmaleimide I attenuates enhancement of TTX-R I_{Na} by epinephrine (Khasar et al., 1999), suggesting that this effect of epinephrine is partially PKC dependent. Using whole-cell patch-clamp analysis, we examined the role of PKCε in epinephrine sensitization of TTX-R I_{Na} in cultured rat DRG neurons. Epinephrine enhanced TTX-R I_{Na} by ~2-fold in neurons perfused with the inactive scrambled εV1–2 peptide (Figure 3B). Intracellular perfusion of εV1–2 peptide reduced this effect of epinephrine by approximately half. These results are consistent with our behavioral data (Figure 3A) and demonstrate a direct, PKCε-dependent action of epinephrine on TTX-R I_{Na} in primary afferent neurons.

PKCε Inhibition Reduces Carrageenan-Mediated Hyperalgesia

In addition to showing less epinephrine-induced hyperalgesia, PKCε mutant mice were less responsive than wild-type mice in the acetic acid writhing test. Since the response to acetic acid is reduced by nonsteroidal anti-inflammatory drugs (Jett et al., 1999), these results suggest that PKCε may be involved in inflammatory hyperalgesia. To investigate this possibility further, we examined rats treated with intradermal injection of the potent inflammatory agent carrageenan (Ferreira et al., 1988). Carrageenan-evoked mechanical hyperalgesia was maximal 3 hr after injection into the hindpaw (Figure 3C). Intradermal injection of εV1–2, 3.5 hr after administration of carrageenan, almost completely reversed this response. There was no effect from the scrambled εV1–2 peptide. These results indicate that PKCε contributes to inflammatory hyperalgesia.

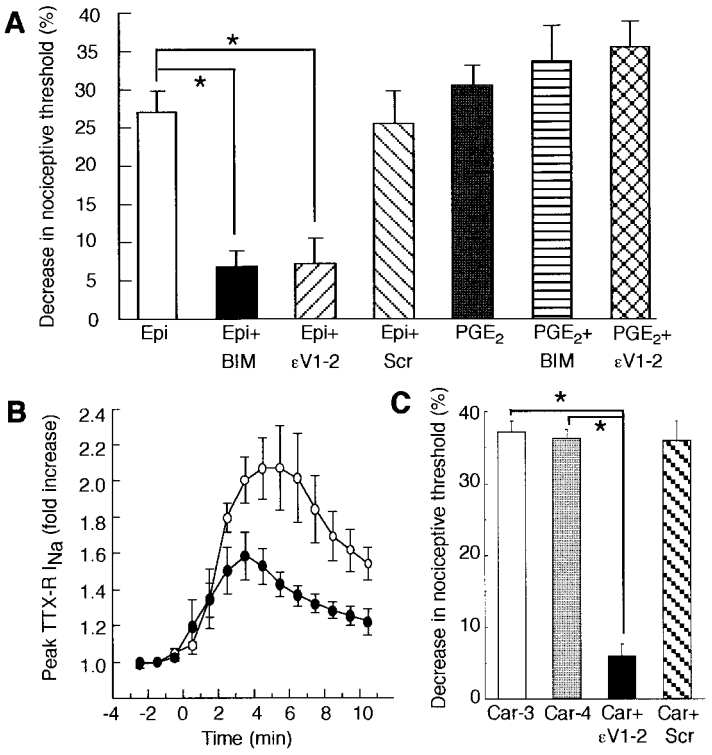

Figure 3. PKC Inhibitors Attenuate Epinephrine- and Carrageenan-Induced Mechanical Hyperalgesia and Epinephrine-Induced Enhancement of TTX-R I_{Na}

(A) Rats were treated intradermally with 100 ng epinephrine (Epi) or PGE_2 with or without local injection of either 1 μg bisindoylmaleimide I (BIM) or sterile water followed by 1 μg of εV1–2 peptide (εV1–2; EAVSLKPT) or 1 μg of scrambled εV1–2 peptide (Scr; LSETKPAV). The mean ± SE baseline threshold before drug treatment in the group receiving epinephrine was 105.3 ± 2.8 g (n = 8). Data are expressed as the percent change from baseline and are mean + SE values from eight (epinephrine) and ten (PGE_2) experiments. Asterisk, $p < 0.001$ (ANOVA and Scheffe F test).

(B) TTX-R I_{Na} was measured in cells intracellularly perfused through the patch-clamp electrode with 200 μM εV1–2 (black circles, n = 4) or 200 μM scrambled εV1–2 (white circles, n = 4) for 15 min prior to application of 1 μM epinephrine. There was a significant decrease in the effect of epinephrine in cells treated with εV1–2 peptide ($p = 0.04$ [two-way repeated measures ANOVA]).

(C) Rats were treated intradermally with 1% carrageenan (10 μl per paw) and tested for mechanical hyperalgesia 3 (Car-3, n = 12) or 4 (Car-4, n = 24) hr later. Some animals received a local injection of 1 μg εV1–2 (Car + εV1–2, n = 6) or scrambled εV1–2 (Car + Scr, n = 6) 3.5 hr after carrageenan administration and were tested 30 min later. Asterisk, $p < 0.0001$ (ANOVA and Scheffe F test).

NGF-Induced Hyperalgesia Is Not Reduced by PKCε Inhibition

In PC12 cells, NGF-induced neurite outgrowth and MAP kinase activation are enhanced by overexpression of PKCε (Hundle et al., 1995). However, downregulation (Roivainen et al., 1993) or inhibition (Hundle et al., 1997) of PKCε does not impair responses to NGF. These results indicate that PKCε modulates but is not required for NGF signaling in PC12 cells. To investigate whether PKCε is necessary for NGF-induced nociception, we examined rats treated with intradermal injection of NGF.

Rats were injected with 1 μg of either εV1–2 or scrambled εV1–2 peptide in the dorsal aspect of the hindpaw. After 15 min, all rats received an injection of 1 μg of NGF at the same site in the paw. Repeat doses of each peptide were administered 3 and 5.75 hr after NGF injection. Rats were then tested for mechanical hyperalgesia 6 hr after receiving NGF. NGF decreased the nociceptive threshold by 38.3% ± 3.6% (n = 6) in rats injected with the inactive scrambled εV1–2 peptide. In rats treated with the εV1–2 peptide, NGF was less effective, decreasing the nociceptive threshold by only 29.9% ± 3.6% (n = 6, p = 0.04, Student's t test). These results indicate that PKCε also contributes to NGF-mediated hyperalgesia.

PKCε Expression in DRG Neurons

Since peripheral injection of εV1–2 reduced epinephrine-, carrageenan-, and NGF-induced hyperalgesia, we hypothesized that PKCε in the peripheral terminals of DRG neurons mediates this effect. That PKCε is present in the peripheral processes of sensory neurons was supported by the observation of PKCε immunoreactivity in hindpaw skin from normal rats at the dermal–epidermal junction (Figure 4A), where the highest density of afferents in the skin is located (McCarthy et al., 1995; Petruska et al., 1997). Under higher magnification, PKCε immunoreactivity in this region was observed in profiles resembling nerve fibers (Figure 4A, insert). In addition, PKCε immunoreactivity was found in most small and some medium diameter DRG neurons in wild-type adult mice and rats (Figures 4C, 4D, and 4G). In wild-type mice, 75% ± 4% of all DRG neurons and 93% ± 15% of small neurons (soma area, <570 μm^2) (Scroggs et al., 1994) expressed PKCε immunoreactivity. Minimal background immunostaining was seen in DRG of PKCε mutant mice (Figure 4E). PKCε immunoreactivity was also present in the soma and process of cultured DRG neurons that express substance P (Figures 4H and 4I). Therefore, PKCε is present in most DRG neurons and in fibers in the skin, as well as in cell bodies and processes of cultured nociceptors.

Discussion

For more than a decade, PKC has been suggested to play a role in nociceptive signaling. We now demonstrate the necessary contribution of a specific isozyme of PKC to primary afferent nociceptor function. We found that PKCε is required for full expression of epinephrine-induced sensitivity to mechanical and thermal stimuli.

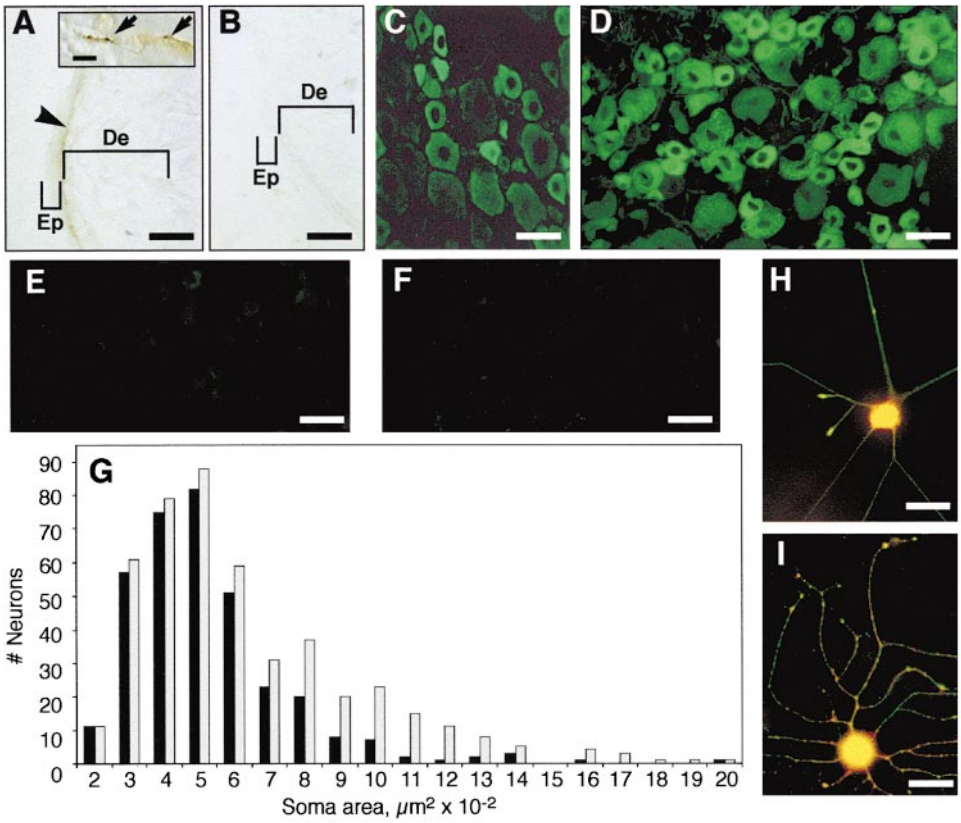

Figure 4. PKCε Immunoreactivity in Skin and DRG

Skin from normal rats was incubated with PKCε antibody in the absence (A) or presence (B) of PKCε antigen peptide. No immunoreactivity was seen when antigen was included in the incubation. PKCε-specific immunoreactivity was seen at the dermal–epidermal junction (arrowhead) and in the dermis, with characteristics of nerve fiber staining (inset, arrows). No staining was seen in the epidermis, possibly due to limited sensitivity of the primary antibody or because PKCε may be expressed in a subset of nociceptors that does not extend terminals into the epidermis. Representative sections are shown from normal rat (C), wild-type mouse (D), and PKCε mutant mouse (E) DRG cells stained with PKCε antibody. In (F), wild-type mouse DRG cells stained with secondary antibody alone. (G) shows size–frequency histogram of PKCε-expressing (black bars) and all (gray bars) neurons from seven wild-type mouse DRG. DRG neurons from rat (H) and mouse (I) were cultured for 7 hr in 25 ng/ml NGF and stained with antibody to substance P (red) and PKCε (green). Yellow color indicates colocalization of both immunoreactivities. No immunostaining was observed in cells incubated with secondary antibodies alone. Scale bar, 50 μm (A–F, H, and I) and 100 μm (inset, [A]). Abbreviations: Ep, epidermis; and De, dermis.

Because the magnitude of epinephrine-induced hyperalgesia was similar in rats treated with a nonselective PKC inhibitor and with the PKCε inhibitor peptide, PKCε may be the only PKC isozyme mediating epinephrine-induced hyperalgesia. The presence of normal PGE_2-mediated responses in PKCε mutant mice is also consistent with our recent data indicating that PGE_2 hyperalgesia and sensitization are not dependent on PKC but require PKA (Khasar et al., 1999). Epinephrine, like PGE_2, enhances TTX-R I_{Na}, which is important in nociceptor sensitization and in hyperalgesia induced by inflammation (Khasar et al., 1999). The εV1–2 peptide reduced the effect of epinephrine on TTX-R I_{Na}, indicating that TTX-R I_{Na} is a target of PKCε as well as PKA. Taken together, our findings suggest that PKCε contributes to epinephrine-induced hyperalgesia by enhancing TTX-R I_{Na} in primary afferent nociceptors.

Our findings also indicate that PKCε is required for full expression of carrageenan-induced hyperalgesia. This suggests a role for PKCε in pain due to inflammation and is consistent with evidence linking epinephrine to inflammatory hyperalgesia. For example, carrageenan-induced inflammation increases epinephrine levels (Mikhailov and Rusanova, 1993), and β-adrenergic antagonists reduce carrageenan-induced hyperalgesia (Cunha et al., 1991). Together with our data, these findings suggest that in nociceptors, PKCε participates in a β-adrenergic receptor–stimulated pathway that contributes to inflammatory hyperalgesia.

The rationale for the present study was in part our observation that PKCε modulates NGF responses in PC12 cells (Hundle et al., 1995, 1997). While NGF is known to induce hyperalgesia (Lewin and Mendell, 1993; Woolf et al., 1994), the mechanisms involved are not known. Therefore, we tested the hypothesis that NGF-induced hyperalgesia is mediated by PKCε. We found that inhibition of PKCε with the εV1–2 peptide decreased NGF-induced mechanical hyperalgesia in rats. Thus, these data provide the first evidence for a signal transduction pathway that underlies NGF-induced hyperalgesia.

The finding that PKCε immunoreactivity was present

in over 90% of small diameter DRG neurons in wild-type mice indicates that PKCε is expressed in nociceptors. Nevertheless, since PKCε is widely expressed in the central nervous system (Saito et al., 1993), where it could contribute to the development of hyperalgesia, our findings do not eliminate the possibility that PKCε acts by central mechanisms. However, since PGE_2-induced hyperalgesia was normal despite attenuation of epinephrine- and inflammation-induced hyperalgesia, it is unlikely that our findings reflect a change in central mechanisms in PKCε mutant mice. Furthermore, peripheral intradermal injection of εV1–2 peptide was sufficient to reduce epinephrine-induced hyperalgesia in rats.

Recent evidence suggests that another PKC isozyme, PKCγ, is also involved in inflammatory hyperalgesia. In PKCγ null mice, pain responses, tissue swelling, and plasma extravasation are attenuated during the second phase of the formalin test (Malmberg et al., 1997). Since this phase of the response to formalin appears to be driven by inflammation, these findings suggest that loss of PKCγ attenuates pain due to tissue injury. Unlike PKCε, PKCγ cannot act by modulating signal transduction in primary afferent nociceptors since it is not expressed in those neurons. However, PKCγ immunoreactivity is present in lamina II of the spinal cord dorsal horn (Malmberg et al., 1997), suggesting that it could modulate pain via spinal mechanisms. Therefore, it appears that at least two members of the PKC family modulate pain responses, but they do so at different levels of the neuraxis.

Local administration of epinephrine exacerbates symptoms in patients with neuropathic pain (Choi and Rowbotham, 1997). Therefore, our findings suggest that epinephrine-induced activation of PKCε may be clinically important. Lower scores obtained in the acetic acid writhing test in PKCε mutant mice and blockade of carrageenan-induced hyperalgesia by a specific inhibitor of PKCε in rats also suggest that PKCε contributes to inflammatory pain. Finally, since PKCε does not contribute to baseline nociceptive thresholds, and PKCε mutant mice appear normal, it may be possible to develop PKCε inhibitors that ameliorate pathologic pain without producing serious systemic side effects or interfering with normal nociceptive responses.

Experimental Procedures

Generation of PKCε Mutant Mice

PKCε was cloned from a lambda FIX II 129 mouse liver genomic library (Stratagene; 946308) with an 813 bp probe (nucleotides 470–1282) from mouse PKCε cDNA (Schaap et al., 1989). The exon containing the ATG start codon and encoding amino acids 1–116 of mouse PKCε (Schaap et al., 1989) was identified. The homologous recombination vector JD825 was constructed with the vector pNTK, obtained from Dr. R. M. Mortensen (Harvard University). A 1.0 kb BssHII fragment (beginning 60 bp 5′ from the ATG start codon) was cloned into the BamHI site of pNTK between the *neo* and TK selection markers. A 6.1 kb StuI fragment downstream of the exon was then cloned into the HindIII site of the pNTK construct, adjacent to the *neo* marker. This construct was designed to delete a 1.2 kb fragment of the *pkc*ε gene, including the exon containing the translation initiation codon. The completed construct was linearized with ClaI and transfected by electroporation using a Biorad gene pulsar at 250 V and 500 μF into 129/RF8 ES cells. Recent genotyping of RF8 cells indicates that they are isogenic with 129/SvJae lines (R. Farese, personal communication), not 129/SvTer, as originally reported (Meiner et al., 1996). Clones were selected by culture in the presence of G418 (180 μg/ml) and 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (200 nM). Genomic DNA isolated from 400 colonies was digested with ApaI and ScaI restriction enzymes, Southern blotted, and hybridized with a 0.5 kb DNA probe 5′ of the flanking region in JD825. Nine clones were identified that contained the predicted homologous integration. Two were microinjected into embryonic day 3.5 C57BL/6J blastocysts. Male chimeras with 80%–90% agouti coat color were mated with C57BL/6J females, and germline transmission was documented by Southern analysis. Breeding was accomplished using mixed pairs of 10–15 heterozygous hybrid male and female mice. Experiments were performed with wild-type and homozygous mutant littermates.

Western Analysis, Immunohistochemistry, and Immunofluorescence

PKC isozymes were detected in freshly isolated DRG by Western analysis (Hundle et al., 1997) with polyclonal anti-PKC antibodies (0.5 μg/ml) from Santa Cruz Biotechnology (Santa Cruz, CA). Some DRG were dissected and cultured as described (Khasar et al., 1999) and then enriched for neurons and plated onto poly-DL-ornithine and laminin-coated 8-well chamber slides (Nunc, Naperville, IL) in medium containing 25 ng/ml NGF (Lindsay, 1988). After 7 hr in culture, cells were rinsed once in ice-cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.5 mM $MgCl_2$, and 0.9 mM $CaCl_2$ [pH 7.2]) and fixed in 2% paraformaldehyde in PBS for 15 min at 4°C. Cells were incubated first in blocking buffer (PBS, 1% BSA, and 0.05% Triton X-100) for 1 hr at 25°C and then in the same buffer containing rabbit anti-PKCε antibody (0.33 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) and guinea pig anti-substance P antibody (1:400 dilution; Accurate Chemical, Westbury, NY) overnight at 4°C. Cultures were then incubated at 25°C for 1 hr in buffer containing FITC-conjugated goat anti-rabbit immunoglobulin G (IgG) and Texas Red–conjugated goat anti-guinea pig IgG (Vector Laboratories, Burlingame, CA), both at a 1:500 dilution. Wells were rinsed three times in PBS, coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA), and immunofluorescence was observed with a Leitz DMRD microscope (Leica, Wetzlar, Germany).

For tissue sections, anesthetized mice and rats were transcardially perfused with PBS, and then 4% paraformaldehyde in PBS. Skin and DRGs were removed and placed in 4% paraformaldehyde for 3 hr, and then in PBS containing 20% sucrose for 24 hr and 40% sucrose for 24 hr. Sections (5–10 μm) were cut with a refrigerated microtome (Leica; SM2000R) and stored at −20°C. DRG sections were treated with methanol:acetone (1:1) for 10 min at −20°C, incubated at 25°C in PBS, 1% normal goat serum, and 0.05% Tween 20 for 1 hr, and then incubated in the same buffer containing anti-PKCε (0.33 μg/ml) for 1–2 hr. Sections were visualized with FITC-conjugated goat anti-rabbit IgG. The size of cell bodies was calculated by tracing profiles containing nuclei from digitized photographs of DRG sections with the program NIH Image 1.61. Cells were scored as expressing PKCε immunoreactivity if the intensity of immunostaining exceeded background levels observed in DRG specimens from PKCε mutant mice. Skin sections were pretreated with 3% H_2O_2 before incubation with 5% normal goat serum and 0.05% Tween 20 in Superblock (Pierce, Rockford, IL). Specimens were then incubated with anti-PKCε antibody (0.2 μg/ml) overnight in PBS, 1% normal goat serum, and 0.05% Tween 20, and immunoreactivity was detected, using the ABC kit (Vector) with diaminobenzamine followed by enhancement with 0.02% osmium tetroxide.

Behavioral Tests

Animal care and handling procedures were in accordance with University of California, San Francisco and National Institutes of Health guidelines. All behavioral testing was done between 10 am and 4 pm. Sprague-Dawley rats (200–250 g) were obtained from Bantin-Kingman, Fremont, CA. Peptides were administered to rats by intradermal injection into the dorsal aspect of the hindpaw following injection of sterile water, which was used to provide hypotonic shock and to allow entry of the peptides into cells (Keeney and Linn, 1990; Lepers et al., 1990; Schulz, 1990).

For mechanical nociceptive testing in rats, the nociceptive flexion reflex was quantified with an Ugo Basile analgesymeter (Stoelting, Chicago, IL) (Aley and Levine, 1997a, 1997b). Baseline mechanical thresholds were determined before drug treatment and then in three trials performed 15, 20, and 25 min after administration of a hyperalgesic agent. The paw withdrawal threshold was calculated as the mean of these three readings.

Mechanical nociceptive thresholds in mice were measured as the withdrawal response frequency to five applications of a 4 g VFH (Stoelting, Chicago), before and after intraplantar injection of the test agent (Kinnman and Levine, 1995). Thermal thresholds in mice were determined by the Hargreave test (Aley et al., 1996), and chemical nociception in mice was determined by the acetic acid writhing test (Ward and Takemori, 1983).

Spontaneous locomotor activity in mice was measured in 17 × 17 inch Plexiglas chambers located in sound-attenuating cubicles and equipped with two sets of 16 pulse-modulated infrared photobeams to record X–Y ambulatory movements at a 100 ms resolution (Med Associates, Lafayette, IN). Mice were handled and weighed daily for 1 week prior to activity testing. On the test day, mice were weighed and placed immediately in the activity chambers. Horizontal distance traveled (cm) was recorded for 1 hr.

Cell Culture and Electrophysiology

Primary cultures of adult rat lumbar DRGs were prepared, and TTX-R I_{Na} was examined by whole-cell voltage-clamp recording as described previously (Khasar et al., 1999). The εV1–2 and scrambled εV1–2 peptides were intracellularly perfused from the electrode for 15 min prior to bath application of 1 μM epinephrine. Throughout each experiment, the amplitude of the peak inward current was normalized to the average current measured during the 3 min prior to application of epinephrine. The time course of changes in the mean normalized current ± SE was plotted with data binned into 1 min intervals.

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