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Reduced hyperalgesia induced by nerve injury, but not by inflammation in mice lacking protein kinase Cγ isoform

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Abstract

Protein kinase C is one of protein kinases which might be involved in the nerve injury- or inflammation-induced hyperalgesia. The present study was designed to investigate the hyperalgesia with thermal paw-withdrawal test induced by sciatic nerve ligation or by intraplantar injection of a complete Freund's adjuvant solution in protein kinase Cγ knockout and its wild-type mice. Either sciatic nerve ligation or intraplantar injection of a complete Freund's adjuvant caused a marked decrease of the paw-withdrawal latency only on the ipsilateral, but not on the contralateral side of the paw in wild-type mice. This ipsilateral hyperalgesia induced by sciatic nerve ligation was significantly attenuated in protein kinase Cγ knockout mice. On the other hand, the ipsilateral hyperalgesia induced by complete Freund's adjuvant remained about the same in protein kinase Cγ knockout mice as in wild-type mice. The results indicate that protein kinase Cγ is involved in the development of the thermal hyperalgesia induced by nerve ligation, but not by complete Freund's adjuvant-induced inflammation. © 2001 Published by Elsevier Science B.V.

Keywords: Protein kinase Cγ; Hyperalgesia; Nerve ligation; Inflammation; Spinal cord; Protein kinase Cγ knockout mice

1. Introduction

Nerve injury or tissue inflammation may lead to neuropathic pain, accompanied by hypersensitivity due to alteration of sensory neurons. The mechanisms of development and maintenance of neuropathic pain are complex and not well understood. Several animal models of neuropathic pain have been used for investigating pathological changes that accompany the pain behaviors (Coderre et al., 1993). Using these models, several second messenger systems have been implicated in the development or maintenance of hyperalgesia induced by nerve injury or tissue inflammation.

Activation of protein kinase C, which triggers the sustained activation of *N*-methyl-D-aspartate receptors, has been implicated in changes in pain perception. Protein kinase C has been involved in spinal hyperexcitability and persistent pain. Activation of PKC with phorbol esters enhances thermal pain sensitivity in mice (Ohsawa and Kamei, 1999). Translocation of protein kinase C from cytosol to membrane is increased in dorsal horn neurons in a model of experimental peripheral mononeuropathy (Mao et al., 1992). Thus, activation and translocation of protein kinase C may underlie the hypersensitivity that causes hyperalgesia. However, we recently reported that the hyperalgesia induced by nerve-ligation, but not by inflammation, is attenuated by intrathecal pretreatment with protein kinase C inhibitor, calphostin C (Ohsawa et al., 2000). It is possible that mechanisms underlying hyperalgesia induced by nerve injury are different from the hyperalgesia caused by inflammation.

While wild-type mice develop a severe mechanical and thermal hyperalgesia after nerve injury, protein kinase Cγ isoform knockout mice show only a very modest expression of mechanical and thermal hyperalgesia (Malmberg et al., 1997). Mao et al. (1992) reported an increase in protein kinase Cγ isoform-like immunoreactivity in the dorsal horn of rats with nerve injury. In acute inflammation-induced pain with formalin test, protein kinase Cγ knockout mice also show attenuation of the second phase response,

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which is driven largely by tissue inflammation (Malmberg et al., 1997). In the long-term inflammatory hyperalgesia model, the persistent increase in protein kinase Cγ-like immunoreactivity is seen in the ipsilateral superficial dorsal horn of the L4 and L5 segments (Martin et al., 1999). Unfortunately, there has been no experimental comparison of hyperalgesia induced by chronic inflammation with that in the nerve-ligation in protein kinase Cγ knockout mice. Therefore, in the present study we compared nerve injury- and long-term inflammation-induced thermal hyperalgesia in protein kinase Cγ knockout and wild-type mice.

2. Materials and methods

2.1. Animals

Male mice weighing 23–27 g (The Jackson Lab., Barharbor, MA) were used. Animals were housed five per cage in a room maintained at 22 ± 0.5 °C with an alternating 12-h light–dark cycle. Food and water were available ad labium. Animals were used only once.

2.2. Nerve injury and inflammatory pain models

The mice were anesthetized with i.p. sodium pentobarbital (60 mg/kg). A partial nerve ligation of sciatic nerve was made by tying a tight ligature with 7-0 silk suture around approximately 1/3 to 1/2 dorsal portion of the sciatic nerve, similar to the procedure described in rats by Seltzer et al. (1990) and in mice by Malmberg and Basbaum (1998). For producing an unilateral inflammation, mice were injected with 0.05 ml of complete Freund's adjuvant (*Mycobacterium tuberculosis;* Sigma, St. Louis, MO) subcutaneously in the plantar surface of the right hind paw. This dose of complete Freund's adjuvant produced significant hind paw swelling, but the animals exhibited normal behavior.

2.3. Hind paw withdrawal response induced by thermal stimulus

To measure withdrawal latency to radiant heat, mice were placed on a glass plate preheated to a constant temperature surrounded by a clear plastic chamber (model 336 Analgesia Meter; IITX/Life Science Instruments, Woodland Hills, CA) (Mansikka et al., 1999). A radiant heat stimulus was applied from underneath the glass floor with a high-intensity projector lamp bulb and the withdrawal latency was measured using an electronic timer (Hargreaves et al., 1988). The heat stimulus was focused on the plantar surface of each hind paw. The intensity of the heat stimulus was adjusted to derive an average baseline latency of paw withdrawal latency of approximately 9 s in naive mice. A 20-s cut-off was used to prevent tissue damage. Paw withdrawal latency was determined as the average of two measurements per paw. Left and right hind paws were tested alternately in no less than 2 min.

2.4. Western blotting

The Western blot analysis was performed followed by the method previously described (Narita et al., 2001). An aliquot of tissue sample was diluted with an equal volume of 2 × electrophoresis sample buffer (Protein Gel Loading Dye-2 ×; AMRESCO, Solon, OH) containing 2% sodium dodecyl sulfate and 10% glycerol with 0.2 M dithiothreitol. Proteins (5–20 μg/lane as determined by the method of Bradford, 1976) were separated by size on 4–20% sodium dodecyl sulfate-polyacrylamide gradient gel using the buffer system of Laemmli (1970) and transferred to nitrocellulose membranes in Tris–glycine buffer containing 25 mM Tris and 192 mM glycine. For immunoblot detection of protein kinase Cγ, membranes were blocked in Tris-buffered saline containing 5% nonfat dried milk (BIO-RAD Laboratories, Hercules, CA) for 1 h at room temperature with agitation. The membrane was incubated with primary antibody diluted in Tris-buffered saline (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) containing 5% nonfat dried milk overnight at 4 °C. The membrane was then washed twice for 5 min and then twice for 10 min in Triton X–Tris-buffered saline containing Tris-buffered saline and 0.05% Triton X-100 followed by 2-h incubation at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunogloblin G (Southern Biotechnology Associates, Birmingham, AL) diluted 1:10,000 in Tris-buffered saline containing 5% nonfat dried milk. After this incubation, the membranes were washed twice for 5 min and then three times for 10 min in Triton X–Tris-buffered saline. The antigen–antibody peroxidase complex was then finally detected by enhanced chemiluminescence (PIERCE, Rockford, IL) according to the manufacturer's instructions and visualized by exposure to Amersham Hyperfilm (Amersham Life Sciences, Arlington Heights, IL).

2.5. Statistical analysis

The behavioral data are presented as the mean ± S.E. at different time points after the nerve injury or adjuvant injection. The statistical significance of differences between groups was assessed with an analysis of variance (ANOVA) followed by the Newman–Keuls test; $p < 0.05$ was considered significant.

3. Results

Western blot analysis of total spinal cord proteins with a rabbit polyclonal immunogloblin G specific to protein kinase Cγ showed that the protein kinase Cγ protein was detected concentration-dependently in membrane fractions of the spinal cord of wild-type mice (Fig. 1). On the other hand, immunoreactivity of protein kinase Cγ protein was not detected in membrane fraction of spinal cord of protein

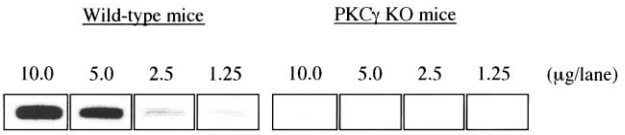

Fig. 1. Immunoblot analysis of protein levels of membrane fraction of protein kinase Cγ isoforms in the spinal cord from wild-type and protein kinase Cγ knockout mice. Proteins (1.25, 2.5, 5.0 and 10.0 μg/lane) were separated by size on 4–20% sodium dodecyl sulfate-polyacrlyamide gradient gel.

kinase Cγ knockout mice (Fig. 1), confirming the specific deletion of the protein kinase Cγ genes in protein kinase Cγ knockout mice.

In the absence of the sciatic nerve ligation surgery, protein kinase Cγ knockout mice exhibited the same paw withdrawal latencies to thermal stimulation as that of wild-type mice. The partial ligation of the sciatic nerve produced a marked and prolonged decrease of the paw withdrawal latency only on the ipsilateral, but not the contralateral side in wild-type mice (Fig. 2). This ipsilateral hyperalgesia developed rapidly in days 1 to 3, reached a maximum at day 7 and gradually returned to the control level in 45 to 60 days after the sciatic ligation (Fig. 2). Compared with the wild-type mice, protein kinase Cγ knockout mice had a significant decreased thermal hyperalgesia at all time points after surgery. In the wild-type mice, the latency to withdraw from heat stimulus observed at day 5 after surgery decreased to 2–3 s from a baseline response of 9 s; the latency to withdraw in the protein kinase Cγ knockout mice was 6–7 s. Thus, sciatic ligation was not completely without effect in the protein kinase Cγ knockout mice. Compared with values before nerve ligation, we recorded a modest, albeit significant decrease in thermal withdrawal latencies, but the magnitude of the change was much less than what we recorded in the wild-type mice.

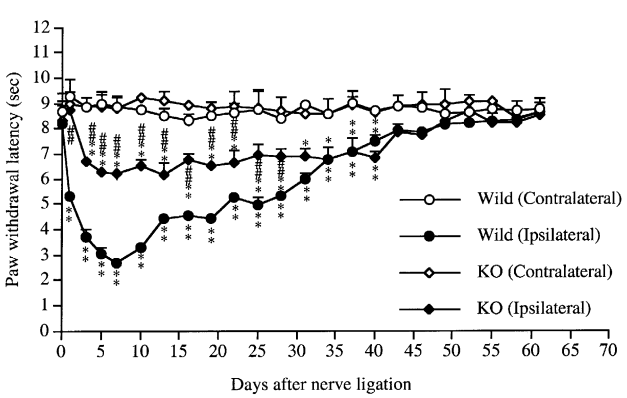

Fig. 2. Effect of sciatic nerve ligation on paw withdrawal responses to thermal stimulation in wild-type (Wild) and protein kinase Cγ knockout (KO) mice. Each point represents the mean ± S.E. to paw withdrawal latency of the injured (ipsilateral) and noninjured (contralateral) sides; $n = 4$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the contralateral side. $\#P < 0.05$, $\#\#P < 0.01$ compared to the wild-type mice.

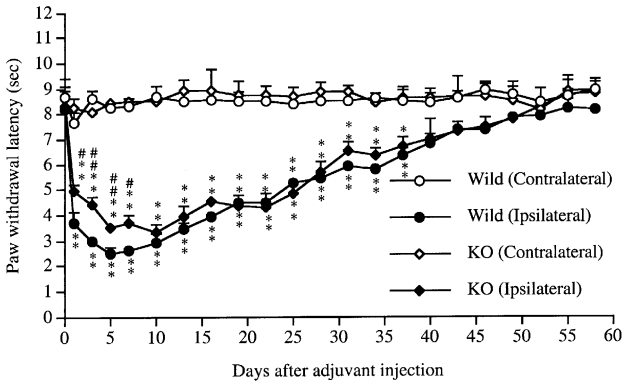

Fig. 3. Effect of intraplantar injection of complete Freund's adjuvant on paw withdrawal responses to thermal stimulation in wild-type (Wild) and protein kinase Cγ knockout (KO) mice. Each point represents the mean ± S.E. to paw withdrawal latency of the injured (ipsilateral) and noninjured (contralateral) sides; $n = 4$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the contralateral side. $\#P < 0.05$, $\#\#P < 0.01$ compared to the wild-type mice.

Unilateral intraplantar injection of a complete Freund's adjuvant solution into hind paw also caused a profound and prolong decrease of paw withdrawal latency on the ipsilateral but not on the contralateral paw side in wild-type mice. This ipsilateral hyperalgesia induced by complete Freund's adjuvant developed rapidly in day 1, reached its maximum at days 5 to 7 and gradually returned to control level in 40–60 days. Intraplantar injection of complete Freund's adjuvant in protein kinase Cγ knockout mice also produced a marked hyperalgesia similar to that of wild-type mice. However, the magnitude of the reduction of the ipsilateral paw withdrawal latencies was slightly, but significantly, less than that of wild-type mice observed at days 1 to 7. We observed no significant difference in paw withdrawal latencies during the rest of time points between protein kinase Cγ knockout mice and wild-type mice (Fig. 3).

4. Discussion

In normal animals, peripheral nerve injury such as sciatic nerve ligation produces a persistent, neuropathic pain state in which pain is exaggerated and can be produced by nonpainful stimuli. We observed in the present study that partial sciatic nerve ligation surgery developed a profound hyperalgesia with paw withdrawal responses in wild-type mice. However, in mice that lack protein kinase Cγ though displayed normal response to acute pain stimuli, but they developed much less hyperalgesia after sciatic nerve ligation. Our results are consistent with results of the previous report by Malmberg et al. (1997) that thermal hyperalgesia and several neural changes evoked by partial nerve ligation found in wild-type mice are not produced in protein kinase Cγ knockout mice (Malmberg et al., 1997). Thus, protein kinase Cγ is essential for the development of partial nerve ligation-induced hyperalgesia. Previously,

it has been reported that treatment with monosialosylganglioteraglycosylceraide, which inhibits the translocation and activation of protein kinase C (Vaccarino et al., 1987), attenuates the thermal hyperalgesia in a model of peripheral nerve injury (Hayes et al., 1992). We have also found in previous study that the inhibition of protein kinase C by intrathecal pretreatment with a protein kinase C inhibitor, calphostin C, markedly attenuates the thermal hyperalgesia evoked by partial sciatic nerve ligation (Ohsawa et al., 2000). The thermal hyperalgesia induced by chronic nerve injury in rats may be related to an increase of protein kinase C bound to spinal cord membranes (Hayes et al., 1992). These findings, therefore, strongly support the view that activation of protein kinase Cγ in the spinal cord is involved in the development of thermal hyperalgesia induced by nerve-injury.

Intraplantar injection of complete Freund's adjuvant solution also produces ipsilateral hyperalgesia. However, unlike the hyperalgesia induced by partial sciatic nerve ligation, which is absent in mice lacking protein kinase Cγ, we observed that the hyperalgesia induced by complete Freund's adjuvant injection was developed to the same extent in wild-type and protein kinase Cγ knockout mice, suggesting that mechanisms of thermal hyperalgesia evoked by partial nerve ligation and complete Freund's adjuvant are different. Martin et al. (1999) found that peripheral inflammation results in somatotopically organised upregulation of protein kinase Cγ in the superficial dorsal horn. Moreover, Malmberg et al. (1997) indicates that nociceptive response induced by short-term inflammation with formalin injection is attenuated in protein kinase Cγ knockout mice compared with that in wild-type mice. On the other hand, we recently reported that intrathecal injection of protein kinase C inhibitor calphostin C attenuates the development of thermal hyperalgesia induced by partial nerve ligation, but not by long-term inflammation with complete Freund's adjuvant injection (Ohsawa et al., 2000). It is important to note that cyclic GMP-dependent protein kinase Iα in the spinal cord may be involved in the hyperalgesia induced by formalin injection (Tao et al., 2000). Thus, it appears that activation of protein kinase Cγ plays a major role in the thermal hyperalgesia induced by nerve injury, but not by long-term inflammation.

There is no difference in time courses of the recovery from the hyperalgesia induced by sciatic nerve ligation surgery and by complete Freund's adjuvant injection in wild-type and protein kinase Cγ knockout mice. The time course of development of thermal hyperalgesia induced by sciatic nerve ligation appears to correspond to the changes of substance P immunoreactivity in the spinal cord of the mouse (Malmberg and Basbaum, 1998). The recovery of substance P levels and tachykinin NK-1 receptor immunostaining after partial nerve ligation correlated to the recovery from hyperalgesia in mice (Malmberg and Basbaum, 1998). Therefore, it seems likely that protein kinase Cγ in the spinal cord is not involved in the recovery from hyperalgesia induced by nerve injury and inflammation.

In conclusion, protein kinase Cγ is involved in the development of the thermal hyperalgesia induced by nerve ligation, but not by complete Freund's adjuvant-induced inflammation. However, protein kinase Cγ is not involved in the recovery from thermal hyperalgesia induced by nerve injury and inflammation.

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