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Loose ligation of the rat sciatic nerve is accompanied by changes in the subcellular content of protein kinase C beta II and gamma in the spinal dorsal horn

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Abstract

This study examined whether loose ligation of the sciatic nerve was accompanied by specific changes in protein kinase C (PKC) βII and γ isozymes in the spinal dorsal horn. The isozyme staining pattern was visualized with immunocytochemistry. Their content in subcellular fractions was estimated from Western immunoblots. In control animals, PKC βII immunoreactivity extended from lamina I into lamina III, while PKC γ immunoreactivity was concentrated within laminae II and III. In ligated animals exhibiting thermal hyperalgesia, the content of both PKC βII and γ in the synaptosomal membrane fraction, but not crude cytosolic fraction, was significantly greater by an average of 40% from their respective controls. These data support suggestions that peripheral nerve injury engenders plastic changes in the dorsal horn to contribute to the development of persistent pain.

Keywords: Chronic pain; Hyperalgesia; Nociception; Sciatic ligation; Spinal plasticity

Persistent pain following nerve injury may arise from the development of central sensitization in the spinal dorsal horn. This central sensitization may be a result of plastic changes in the processing of sensory information [16].

Protein kinase C (PKC) is a family of closely related enzymes that mediate complex and varied actions [13]. In the hippocampus, the specific involvement of the PKC βII and γ isozymes is postulated to critically contribute to the full-expression of long-term potentiation (LTP), a form of activity-dependent synaptic plasticity [3].

If central sensitization is a result of plasticity, and PKC is an important intracellular mediator of such plasticity, then changes in PKC distribution may also accompany the development of persistent pain following tissue injury. The presence of both PKC βII and γ in the superficial dorsal horn has been established [1,12], and early reports by Mao and colleagues suggested an important role for PKC in the development of central plasticity following sciatic ligation [8,9]. Recent studies have provided further support for an important association between PKC and nociceptive sensory transmission [4,10], noxious thermal and chemical stimulation [14], and the sensitization of primate spinothalamic tract cells [15,18]. In addition, the lack of the PKC γ gene was reported to lead to reduced signs of neuropathic pain [7].

The specific goal of the present study was to examine whether loose ligation of the sciatic nerve was accompanied by changes in the subcellular distribution of the PKC βII and γ isozymes in the superficial dorsal horn of animals exhibiting thermal hyperalgesia as a sign of persistent pain. The translocation of these isozymes from the cytosol to the synaptic membrane is thought necessary for their activation [13], and is considered a critical step in their contribution to long-lasting synaptic plasticity [3,19]. Immunocytochemistry was employed to visualize the staining pattern of the two isozymes, and localize any change in distribution. Western immunoblots were used to obtain an estimate of potential changes in each isozyme's content in the crude cytosolic and synaptosomal membrane subcellular fractions.

Hindpaw thermal withdrawal latencies [5] were obtained from 16 adult male rats (Sprague–Dawley–Harlan). The animals were then randomly divided into two groups, and one group was subject to loose sciatic ligation [2]. Seven days after surgery, the withdrawal latencies of all animals were obtained before they were killed, and their spinal cords processed either for immunocytochemistry or Western

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immunoblots. Statistical analysis was performed with ANOVA, and significance was inferred at the $P < 0.05$ level. Experiments were conducted in accordance with guidelines accepted by the International Association for the Study of Pain [20]. The animal protocol was approved by the Animal Care Committee of the School of Veterinary Medicine at the University of Wisconsin-Madison.

Four control and four ligated animals were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and perfused with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.12 M phosphate buffer. To insure consistency, all animals were perfused on the same day, and all tissues were processed at the same time. Transverse, 50 μm thick, L5 spinal sections were incubated in: (1) rabbit antisera (Santa Cruz Biotechnology) at 1:5000 for PKC βII and 1:700 for PKC γ, (2) goat anti-rabbit IgG (1:50) and peroxidase-antiperoxidase (PAP) complex (1:80), and (3) 0.05% diaminobenzidine hydrochloride in 0.1 M phosphate-buffered saline and 0.01 hydrogen peroxide. Controls for ligand specificity included omission of primary antisera, or preabsorbtion with synthetic peptides containing the sequences against which the antibodies were raised. No staining was observed in either situation.

The other four control and four ligated animals were rendered unconscious with CO_2 vapors and decapitated. The spinal cord was 'flushed' into a dish containing ice-cold homogenizing buffer, and a ~2-cm section of the L5 area was cut and divided into a dorsal and ventral half under a dissecting microscope. Homogenization, fractionation and immunoblotting procedures were performed as described previously [17]. Briefly, the dorsal half of each spinal cord was homogenized with a sonicator in 0.32 M sucrose in 20 mM HEPES buffer (pH 7.5) containing 10 mM EDTA, 2 mM EGTA, 5 mM DTT, 1 mM PMSF, 10 mg/l leupeptin, and 10 mg/l aprotinin. The homogenates were centrifuged to obtain a crude cytosolic and a solubilized membrane fraction enriched in mitochondria and synaptosomes (i.e. the P-2 fraction). The PKC βII and γ isozymes were separated by SDS-PAGE electrophoresis, and detected following incubation in: (1) rabbit polyclonal antisera (Santa Cruz Biotechnology) at 1:5000 for PKC βII and 1:300 for PKC γ, and (2) anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (1:5000). A single band of ~80 molecular weight was detected by each antibody (Fig. 2A), and the labeling of this band was blocked by preabsorption with the peptide sequences specific for each antibody.

The amount of each isozyme was estimated from scanned, chemifluorescence processed membranes (Vistra ECF Western Blotting Kit, Amersham Pharmacia Biotech) using the Molecular Dynamics Storm system and ImageQuant software. The person performing this analysis was blinded to the status of an animal (control or ligated). The average peak height of each isozyme in control animals was denoted as 100%, and the average peak height of the corresponding isozyme in the ligated animals was expressed as a percent change from this control.

All ligated animals exhibited significantly shortened ipsilateral withdrawal latencies 7 days after surgery (6.9 ± 0.4 vs. 10.4 ± 0.2 s, mean ± SEM; $F_{(1,7)} = 73.7$, $P < 0.001$). In contrast, there were no significant differences between ipsilateral baseline and day 7 latencies in control animals (10.9 ± 0.3 vs. 10.1 ± 0.6 s, $F_{(1,7)} = 1.0$, $P < 0.4$). In addition, on day 7, the latencies of ligated animals were significantly different from those of control animals ($F_{(1,14)} = 22.1$, $P < 0.001$). In pilot experiments, withdrawal latencies in sham-operated animals (exposure of the sciatic nerve without ligation) were statistically indistinguishable from controls (i.e. sham-operated animals did not develop thermal hyperalgesia).

In control animals, PKC βII immunoreactivity was diffuse and the staining expanded from lamina I into lamina III. On the other hand, PKC γ immunoreactivity was more limited and concentrated in laminae II and III (Fig. 1). Sciatic ligation appeared accompanied by increases in the staining intensity of both PKC isozymes. The increases were seen bilaterally, but were more prominent ipsilaterally (Fig. 1). This is in agreement with previous reports [8,9].

Immunoblot analysis confirmed the qualitative observations (Fig. 2). In ligated animals, the content of PKC βII in the ipsilateral synaptosomal membrane fraction was significantly greater than in controls ($38 \pm 9\%$, mean ± SEM; $F_{(1,6)} = 9.7$, $P < 0.05$). Similarly, the content of PKC γ in the ipsilateral synaptosomal membrane fraction of ligated animals was significantly greater than in the respective controls ($42 \pm 11\%$, $F_{(1,6)} = 7.3$, $P < 0.05$). On the other hand, the content of either isozyme in the ipsilateral crude cytosolic fraction in ligated animals was essentially the same as that in controls ($101 \pm 18\%$ for PKC βII, and $91 \pm 11\%$ for PKC γ).

These data are in general agreement with a recent study reporting a 75–100% increase in PKC γ immunoreactivity in the ipsilateral dorsal horn following Freund's adjuvant injection in the rat hindpaw [10]. The smaller increase observed in our study probably reflects differences in the pain model used (loose ligation vs. Freund's adjuvant injection), and quantification method (isozyme content in Western immunoblots of subcellular fractions rather than staining intensity in immunohistochemical sections of the whole dorsal horn).

Sciatic ligation was not accompanied by a statistically significant increase in the number of PKC γ somata (i.e. 100 ± 9 in controls vs. 116 ± 10 in ligated animals, mean ± SEM; $F_{(1,6)} = 1.3$, $P < 0.3$). These numbers were obtained by counting all positively-stained cells (arrows, Fig. 1C,D) in three size-matched immunocytochemical sections from each control and ligated animal using a Spot 2 digital camera (Diagnostic Instruments, Sterling Heights, MI), and the Image Pro Software (v. 3, Media Cybernetics, Silver Spring, MD).

These data further support suggestions that nerve injury engenders changes in the action of PKC in the dorsal horn to perhaps contribute to persistent pain [7–10,18]. The trans-

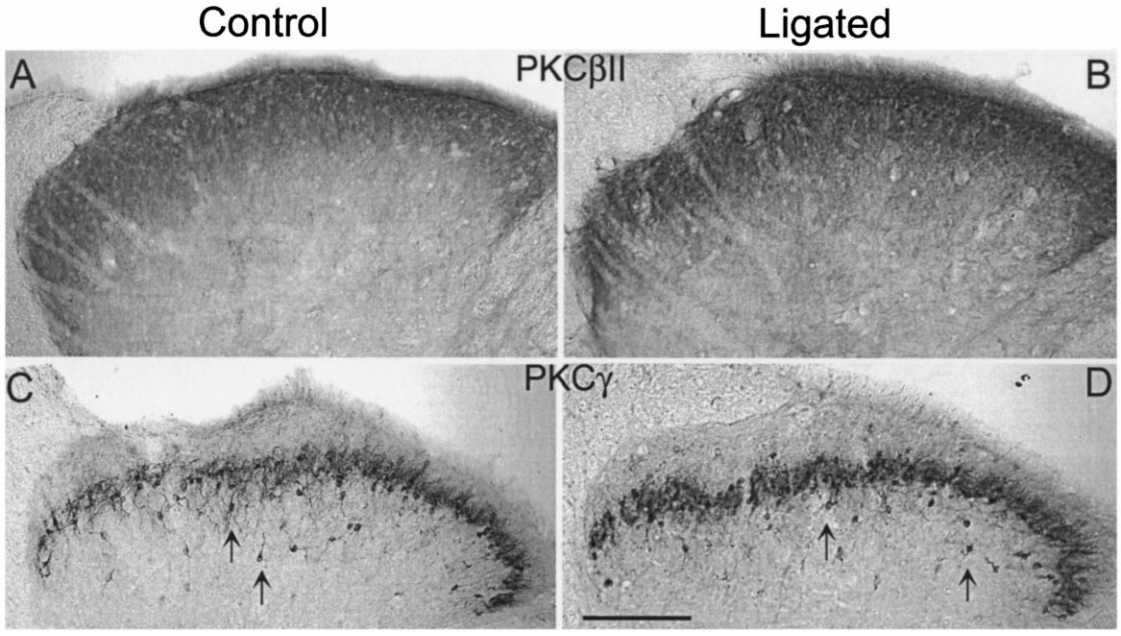

Fig. 1. Digital images illustrate the immunoreactive staining pattern of the PKC βII (A,B) and PKC γ (C,D) isozymes in the spinal dorsal horn of control (A,C) and ligated animals (B,D). Note the more extensive and diffuse staining for PKC βII (A), and the more confined and concentrated immunoreactivity for PKC γ (C). Note also the apparent increase in staining intensity in ligated animals (B,D). Arrows denote PKC γ immunoreactive cells. Dorsal is up, medial is left. Scale bar is 150 μm for all four images.

location of calcium-dependent PKC isozymes from the cytosol to the synaptic membrane is thought necessary for their activation [3], and is considered critical in promoting the development of long-lasting activity-dependent synaptic plasticity [3,19]. We interpret the increases in PKC isozyme content in ligated animals similarly as a consequence of intracellular up-regulation and increased translocation (i.e. activation) elicited by peripheral injury to produce long-term modification of synaptic action in the spinal dorsal horn. Given that our immunocytochemical data revealed no significant changes in the number of visible PKC γ somata, our results suggest that, at least for this isozyme, the increased translocation occurred in cells already containing PKC γ.

In the CA1 region of the hippocampus, postsynaptic action of the PKC γ isoform may be required for the induction of *N*-methyl-D-aspartate (NMDA)-dependent LTP, while activation of PKC βII presynaptically may be necessary for LTP maintenance [3]. This is in contrast to the CA3 region of the hippocampus where translocations of PKC α and ε appear associated with the expression of the mechanistically different, non-NMDA-dependent form of LTP [19]. In the spinal dorsal horn, like in the CA1 region, LTP is NMDA-dependent [16].

Our results suggest a similar action of PKC βII and γ isozymes in the spinal dorsal horn. The diffuse staining of PKC βII suggests a predominant localization of this isozymes within neuronal terminals, and this implies a largely presynaptic action. The more confined localization of PKC γ within cell bodies and nearby processes implies, on the other hand, a largely post-synaptic role.

Our results also indicate that it is especially the neurons in laminae II and III that may be 'sensitized' by injury to primary input, and that it is the plastic changes in the neuro-

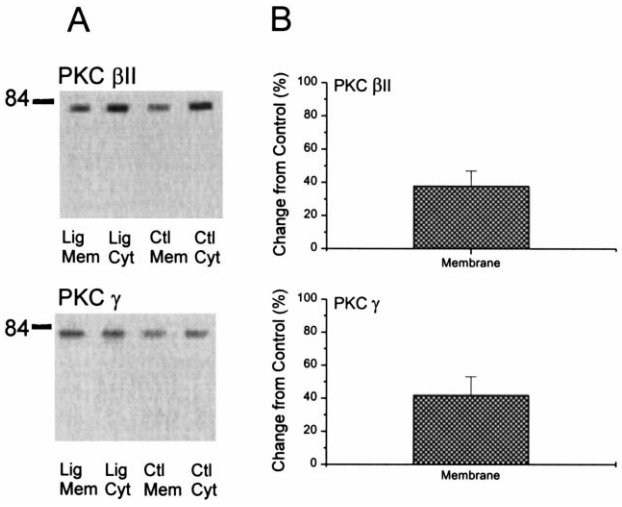

Fig. 2. (A) Examples of Western immunoblots of the PKC βII and γ isozymes in subcellular fractions from a control and a ligated animal. Note that a single band of approximately 80 MW for each PKC isozymes is shown in each ECL lumigraph. For both immunoblots, Ctl = control, Lig = ligated, Cyt = crude cytosolic fraction, Mem = synaptosomal membrane fraction. (B) Summary plots of the percent increase in the content of the PKC βII and γ isozymes in the synaptosomal membrane fraction of ligated animals. Note that the contents of both PKC βII and PKC γ are about 40% greater when compared to their respective controls. The values are averages of four animals in each group. Error bars represent the SEM.

nal circuitry of these spinal areas that may underlie the development of central sensitization in ligated animals. This is in agreement with studies in which changes in activity in Aδ and Aβ fibers [6,11] have been suggested as potential contributors to the development of persistent pain.

Overall, these results indicate that sciatic ligation is accompanied by an increase in the immunoreactive content of two PKC isozymes in the synaptosomal membrane subcellular fraction of the superficial spinal dorsal horn. The data further support the contention that plastic changes in the processing of spinal sensory information may contribute to the development of persistent pain following peripheral nerve injury.

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