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Noxious Thermal and Chemical Stimulation Induce Increases in ^{3}H-Phorbol 12,13-Dibutyrate Binding in Spinal Cord Dorsal Horn as Well as Persistent Pain and Hyperalgesia, Which Is Reduced by Inhibition of Protein Kinase C

Kiran Yashpal,[1,2] Graham M. Pitcher,[1] Angèle Parent,[2,4] Rémi Quirion,[2,4] and Terence J. Coderre[1,3,5]

[1]Pain Mechanisms Laboratory, Clinical Research Institute of Montreal, Departments of [2]Psychiatry and [3]Psychology, McGill University, Montreal, [4]Douglas Hospital Research Center, Verdun, and [5]Centre de recherche en sciences neurologiques et Département de médecine, Université de Montréal, Canada

We have previously suggested that protein kinase C (PKC) contributes to persistent pain in the formalin test. This study compared the effects of pharmacological inhibition of PKC with either GF 109203X or chelerythrine on persistent pain following noxious chemical stimulation with its effects on mechanical hyperalgesia, which develops in the hindpaw contralateral to an injury produced by noxious thermal stimulation. Furthermore, we have assessed changes in membrane-associated PKC in spinal cord in response to both noxious chemical and thermal stimulation. Nociceptive responses, to a hindpaw injection of 50 μl of 2.5% formalin, and flexion reflex thresholds, to mechanical stimulation (Randall-Selitto test) in the hindpaw contralateral to a thermal injury (15 sec immersion in water at 55°C), were assessed following intrathecal injection of PKC inhibitors (GF 109203X or chelerythrine). Changes in the levels of membrane-associated PKC, as assayed by quantitative autoradiography of the specific binding of ^{3}H-phorbol-12,13-dibutyrate (^{3}H-PDBu) in spinal cord sections, were assessed in rats after noxious chemical (50 μl of 5.0% formalin) and noxious thermal (90 sec immersion in water at 55°C) stimulation. Inhibitors of PKC (GF 109203X, chelerythrine), produced significant reductions of nociceptive responses to 2.5% formalin, as well as a significant reduction in the mechanical hyperalgesia in the hindpaw contralateral to a thermal injury. In addition, both noxious chemical and thermal stimulation produced significant increases in specific ^{3}H-PDBu binding in the dorsal horn of the lumbar spinal cord, likely reflecting alterations in membrane-associated PKC. The results provide both pharmacological and anatomical evidence that persistent pain produced by chemical stimulation with formalin and mechanical hyperalgesia in the hindpaw contralateral to a thermal injury are influenced by the translocation and activation of PKC in spinal cord dorsal horn neurons.

[Key words:pain, nociception, hyperalgesia, tissue injury, formalin test, protein kinase C, phorbol ester, ^{3}H-phorbol-12,13-dibutyrate binding, protein kinase C inhibitors, neuronal plasticity]

Recent evidence suggests that following peripheral tissue injury or intense noxious stimulation there are specific changes in central neuronal excitability that contribute to persistent pain and hyperalgesia (Woolf, 1991; Coderre et al., 1993). Furthermore, there is evidence that this injury or noxious stimulus-induced hyperexcitability is influenced by activity at excitatory amino acid (EAA) and substance P/neurokinin-1 (NK-1) receptors. Repetitive C-fiber afferent nerve stimulation leads to a cumulatively increasing postsynaptic depolarization or wind-up of dorsal horn neurons that is blocked by *N*-methyl-D-aspartate (NMDA) receptor antagonists (Davies and Lodge, 1987; Dickenson and Sullivan, 1987), while injury or intense afferent nerve stimulation produces a slow prolonged excitatory postsynaptic potential that is blocked by NK-1 receptor antagonists (De Koninck and Henry, 1991). Tissue injury produces an expansion of the receptive fields of neurons responding to inputs from areas adjacent to the area of injury that is blocked by NMDA antagonists (Dubner and Ruda, 1992). Injury of a rat's limb also produces a spinally mediated increase in the excitability of ipsilateral and contralateral flexion reflexes that is blocked by both NK-1 and NMDA receptor antagonists (Coderre and Melzack, 1991; Woolf and Thompson, 1991).

It has been shown that synaptic activation of NMDA and NK-1 receptors produces brief effects lasting, at most, milliseconds to seconds for NMDA receptors (Gerber et al., 1991) and seconds to minutes for NK-1 receptors (Urban and Randic, 1984; De Koninck and Henry, 1991). Thus, it is unlikely that NMDA or NK-1 receptor activation by themselves can account for the prolonged changes in neuronal excitability that follows peripheral tissue injury. It is possible, however, that these prolonged changes in excitability depend on the ability of EAA and NK-1 receptors to stimulate the production or activation of various intracellular messengers. Both EAA and NK-1 receptor activation triggers the production of various intracellular second messengers in neuronal cells. Activity at metabotropic EAA (Sladeczek et al., 1985; Sugiyama et al., 1987) or NK-1 (Watson and Downes, 1983) receptors stimulates the hydrolysis of phosphatidyinositol (PI) leading to an increased production of the intracellular messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). Activity at ionotropic EAA (NMDA) or NK-1

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Correspondence should be addressed to Terence J. Coderre, Ph.D., Director, Pain Mechanisms Laboratory, Clinical Research Institute of Montreal, Quebec, Canada, H2W 1R7.

receptors also stimulates the influx of calcium (Ca^{2+}) through NMDA receptor-operated Ca^{2+} channels (MacDermott et al., 1986) and voltage-gated Ca^{2+} channels (Womack et al., 1989), respectively. Intracellular Ca^{2+} is also increased in response to the production of IP_3, which promotes the release of intracellular Ca^{2+} from its stores within the endoplasmic reticulum (Womack et al., 1988). The increased Ca^{2+} influx and production of DAG are essential elements for the stimulation of various forms of the enzyme protein kinase C (PKC) (Hug and Sarre, 1993), and likely explain why PKC activation is triggered by both EAA (Manzoni et al., 1990) and NK-1 (Koizumi et al., 1992) receptor activity.

Activation of conventional forms of PKC by Ca^{2+} and DAG involves the translocation of the enzyme from a cytosolic location to a membrane-associated site (Hug and Sarre, 1993). Following translocation and activation, PKC phosphorylates specific substrate proteins that contribute to various cellular processes, including neurotransmitter release and transduction (Nishizuka, 1986). These changes in synaptic transmission may account for findings that PKC contributes to various forms of neuronal plasticity including long-term potentiation (Malenka et al., 1986; Hu et al., 1987), neurotoxicity (Felipo et al., 1993), and cellular growth (Housey et al., 1988). The translocation and activation of PKC has also been shown to be critical for memory processes including associative and discriminative learning (Olds et al., 1989, 1990).

The involvement of EAA and NK-1 receptors in both injury-induced neuronal plasticity and in the stimulation of intracellular cascades leading to the translocation and activation of PKC, suggests that PKC may be involved in neuronal changes produced by peripheral tissue injury that contribute to persistent nociception. Earlier studies from our laboratory have shown that PKC inhibitors reduce, and PKC activators enhance, nociceptive responses after formalin-induced tissue injury (Coderre, 1992), suggesting a role for PKC in long-term changes in neuronal excitability associated with persistent nociception. If PKC plays a critical role in the development of persistent nociception, it might be expected that the types of tissue injury or noxious stimulation that produce persistent nociception would lead to increases in the translocation and activation of PKC in central nociceptive neurons. It has previously been shown that there is an increase in membrane-associated PKC in spinal cord in response to nerve constriction injury (Mao et al., 1992, 1993). In the present study, we examined whether noxious stimuli (noxious chemical and thermal stimulation), which produce both persistent pain and hyperalgesia, would produce increases in membrane-bound PKC in the dorsal horn of the spinal cord. Autoradiographic studies were conducted to assess changes in the levels of membrane-associated PKC in rat spinal cord in response to both noxious chemical (formalin) and thermal stimulation. Quantification of specific binding sites for ^{3}H-phorbol 12,13-dibutyrate (^{3}H-PDBu), a putative marker of membrane-bound PKC (Olds et al., 1989), was performed at various times after the noxious stimulation was applied. Furthermore, we extended studies assessing of the ability of inhibitors of PKC to attenuate both persistent nociception and hyperalgesia after tissue injury. Thus, we compared the effects of pharmacological inhibition of PKC with GF 109203X (Toullec et al., 1991) or chelerythrine (Herbert et al., 1990) on persistent pain following noxious chemical stimulation with formalin, with its effects on the mechanical hyperalgesia that develops in the hindpaw contralateral to a thermal injury.

Materials and Methods

These experiments were performed on male, Long–Evans hooded rats (Charles River) weighing 300–350 gm. They were maintained under controlled lighting conditions with food and water available ad libitum. The animals were divided into two groups; one group received noxious chemical stimulation, and the other group received noxious thermal stimulation. In all cases, the guidelines described in *Care and Use of Experimental Animals of the Canadian Council of Animal Care* (Vols. I, II) were strictly followed.

Physiological studies

Formalin test. For chemical nociception, rats were given a subcutaneous injection of 50 μl of 2.5% formalin into the plantar surface of one hindpaw. For nociceptive testing, animals were placed in a 30 × 30 × 30 cm Plexiglas box with a mirror below the surface at a 45° angle to allow an unobstructed view of the paws. Scoring of nociceptive behaviors began immediately after formalin injection and continued for 50 min. A nociceptive score was determined for each 5 min time block by measuring the amount of time spent in each of the following behavioral categories: 0, the injected paw is not favored; 1, the injected paw has little or no weight placed on it; 2, the injected paw is elevated and is not in contact with any surface; 3, the injected paw is licked, bitten, or shaken. A weighted average nociceptive score, ranging from 0 to 3, was calculated by multiplying the time spent in each category by the category weight, summing these products, and then dividing by the total time for each 5 min time block. All the rats were acclimatized to the test chamber before testing began. Furthermore, in addition to extended observations of the rats, rats were assessed for possible motor dysfunction by testing for placing and righting reflexes.

Assessment of mechanical hyperalgesia after noxious thermal stimulation. The noxious thermal stimulus consisted of the immersion of one hindpaw into water maintained at 55°C for 15 sec while rats were briefly anesthetized with halothane inhalation anesthesia. This exposure produces a profound ipsilateral hyperalgesia to thermal and mechanical stimuli, but only a mild tissue injury, with a reddening of the skin, little edema, and no tissue necrosis or scarring (Coderre and Melzack, 1985). We have found that, in addition to ipsilateral hyperalgesia, this injury produces a reliable contralateral hyperalgesia that is exclusively dependent on central sensitization, and is independent of peripheral sensitization in the ipsilateral hindpaw (Coderre and Melzack, 1985, 1991). This paradigm is useful then to assess the effects of neuropharmacological treatments that are aimed at reducing central sensitization. Thus, we have used these paradigm to assess the effects of spinally administered PKC inhibitors on hyperalgesia associated with central sensitization. Following the noxious thermal stimulation, mechanical hyperalgesia in the contralateral hindpaw was measured using the Randall-Selitto Apparatus. This apparatus exerts an accelerating force to a focused point on the rat's hindpaw. After baseline recordings for a flexion reflex response to the mechanical stimulus, the rat was briefly anesthetized, and one of the rat's hindpaws was immersed in the 55°C water for 15 sec. After recovery from the halothane anesthesia, mechanical flexion reflexes were measured again at 2, 5, 10, and 30 min, as well as 3, 12, and 36 hr and 3 d following the noxious thermal stimulation. Mechanical flexion reflex thresholds for baseline and post-treatment trials were based on an average of four tests after eliminating the highest and lowest scores. Scores are given as change from baseline using the standard formula for percent maximum possible effect (%MPE = [(postdrug score − baseline score)/(cutoff − baseline)] × 100) (Yaksh and Rudy, 1977).

Drug administration. Two agents known to potently and selectively inhibit PKC, GF 109203X hydrochloride (0.01–10 μg) and chelerythrine chloride (0.2–50 μg), were used (both from Calbiochem, San Diego, CA). Chelerythrine was dissolved in 10% DMSO in 0.9% saline and GF 109203X in 0.9% saline. Both agents were given intrathecally in a 30 μl volume by lumbar puncture between the L5 and L6 vertebrae, while the rats were under brief halothane anesthesia. For both formalin and secondary hyperalgesia trials, testing began 30–45 min after treatment with either vehicle, GF 109203X, or chelerythrine. Dose–responses trials were performed in the formalin test by administering three doses each of GF 109203X (0.01, 0.1, and 1.0 μg) and chelerythrine (0.2, 2.0, and 20 μg) to separate groups of rats, and were compared with vehicle control groups of 0.9% saline and 10% DMSO in 0.9% saline, respectively. A single dose of GF 109203X (10 μg) or chelerythrine (50 μg) was given as a pretreatment to rats tested for mechanical

hyperalgesia after the contralateral thermal injury. The effects of these treatments were compared with a vehicle control group in which half the rats received 10% DMSO in 0.9% saline and half received 0.9% saline. There were no significant differences between these two vehicle groups, so the two groups were combined.

Statistical analyses. For both the formalin and mechanical flexion reflex tests, statistical analyses included repeated measures ANOVA followed by post hoc comparisons to the control group using Dunnett *t* tests.

Quantitative autoradiography

Tissue processing. After noxious chemical stimulation (intraplantar injection of 50 μl of 5.0% formalin to one hindpaw), the rats (n = 3–4 per group) were decapitated at three different time points (5, 25, and 60 min). The 5.0% concentration formalin was chosen to maximize the effects in binding experiments; a lower concentration (2.5%) was used in neuropharmacological experiments since 2.5% and 5.0% formalin produce similar levels of nociceptive responding on our behavioral rating scale, and the lower concentration should avoid difficulties associated with ceiling effects that occur with the higher concentration (Coderre et al., 1993). Rats were conscious following the formalin injection, and their behaviors were observed until they were sacrificed. For noxious thermal stimulation, rats were lightly anesthetized with a mixture of sodium pentobarbital (20 mg/kg, i.p.) and chloral hydrate (120 mg/kg, i.p.) during the stimulation and until sacrifice. In the case of noxious thermal stimulation, three to four rats each were decapitated at three time points (1, 10, and 30 min) after immersion of one hindpaw in 55°C water for 90 sec. A 90 sec exposure was used to maximize the effects of the stimulus in binding experiments, which are performed in anesthetized rats; a shorter duration (15 sec) was used in the neuropharmacological experiments, since the 90 sec exposure produced substantial tissue injury, which would be unethical to administer to rats that recover from anesthesia, and are tested over a 3 d period. An additional group of four rats were sacrificed 1 min after non-noxious thermal stimulation (immersion of one hindpaw in 40°C water for 90 sec), and were otherwise treated identically to the rats given noxious thermal stimulation. Control rats included two untreated rats and two anesthetized rats that were treated identically to the noxious heat stimulated rats, except that they received no stimulus. Since there were no significant differences in ^{3}H-PDBu binding for untreated and anesthetized rats, these two groups were combined to form a single control group.

After decapitation, spinal cords were rapidly removed, dissected into specific segments, and snap frozen at −4°C in 2-methylbutane (Yashpal et al., 1990). Tissue blocks were fixed to microtome chucks and serial transverse sections (20 μm) were cut at −18°C using a cryostat microtome. Sections were then thaw mounted onto gelatin-coated microscope slides, dried overnight under a vacuum, and stored at −80°C until autoradiographic processing.

Binding assay. ^{3}H-Phorbol 12,13-dibutyrate (^{3}H-PDBu, 17 Ci/mmol, New England Nuclear, Boston, MA) binding assay was performed by preincubating sections for 1 hr at 4°C in a buffer containing 50 mM Tris-HCl (pH 7.7), 100 mM NaCl, and 1 mM $CaCl_2$, as described by Parent et al. (1993) according to Worley et al. (1986a,b). Incubations were carried out in a similar buffer containing bovine serum albumin (0.1%, Boehringer–Mannheim) and 2.5 nM ^{3}H-PDBu for 1 hr at 33°C. Nonspecific labeling was assessed by adding excess of unlabeled PDBu (1 μM, Sigma Chemicals, St. Louis, MO) to the incubation solution. At the end of the incubation period, sections were washed twice in ice-cold distilled water and immediately dried under a stream of cold air. Under these conditions, specifically bound ^{3}H-PDBu represented close to the total (>95–98%) of bound ligand. Autoradiograms were generated by opposing ^{3}H-Hyperfilm (Amersham, Chicago, IL) to slides for 7 d. Binding of ^{3}H-PDBu to sections was quantitated by microcomputer image analysis (MCID system, St. Catherines, Ontario) using tritium-labeled standards (Amersham, Chicago, IL) for film calibration (Parent et al., 1993). The regions sampled included the superficial (LI–II) and deeper (LIII–VI) laminae of the dorsal horn, lamina X, and the ventral horn.

Statistical analyses. For ^{3}H-PDBu binding studies statistical analyses included ANOVA followed by post hoc comparisons with Scheffé tests. Analyses were performed on all viable sections obtained after collecting five sections from each spinal segment of each of four controls rats, and six sections from each of the lumbar and thoracic spinal segments of three to four experimental rats in each treatment condition. However, since our preliminary experiments in this area suggested that ^{3}H-PDBu binding within the lumbar spinal cord dorsal horn is greatest at 5 min after formalin, and 10 min after noxious thermal stimulation, greater numbers of sections (10/rat) were taken at these time points. Also, since it was not expected that ^{3}H-PDBu binding levels in the cervical spinal segment would be greatly influenced by noxious stimulation of the hindpaws, cervical segments were only included at 5 and 25 min postformalin and 1 min after noxious thermal stimulation. The total numbers of sections analyzed in each spinal segment for control rats were as follows: cervical (n = 17), thoracic (n = 16), lumbar (n = 12), and sacral (n = 10). The total number of sections analyzed at each time point for stimulated rats were as follows: formalin 5 min (lumbar, n = 40; thoracic, n = 13; cervical, n = 20), formalin 25 min (lumbar, n = 21; thoracic, n = 13; cervical, n = 12), formalin 60 min (lumbar, n = 8; thoracic, n = 11), noxious thermal stimulation (NTS) 1 min (lumbar, n = 18; thoracic, n = 13; cervical, n = 10), NTS 10 min (lumbar, n = 34), NTS 30 min (lumbar, n = 14; thoracic, n = 14), non-noxious thermal stimulation 1 min (lumbar, n = 22).

Results

Physiological studies

Nociceptive scores in the formalin test for the controls and two groups of rats treated with the PKC inhibitors, GF 109203X or chelerythrine, are shown in Figure 1. The control groups, treated with saline or vehicle, exhibited the typical biphasic response after formalin injection to one hindpaw. The early phase, occurring maximally at 5 min, was followed by the intermediate phase (5–10 min) of relatively low nociceptive scores. The late nociceptive phase started at about 20 min after formalin injection and continued until the completion of testing at 50 min.

In the groups pretreated with PKC inhibitors, there was a significant reduction in the typical nociceptive responses. Nociceptive responses were dose dependently reduced by GF 109203X, in the late (10–50 min), but not the early (0–5 min) phase after formalin injection. Both the 0.1 and 1.0 μg doses of GF 109203X significantly reduced nociceptive scores throughout most of the late phase, while the 0.01 μg dose was ineffective. In the group pretreated with chelerythrine, there was also a dose-related inhibition of nociceptive responses in the formalin test. While only the highest dose of chelerythrine significantly reduced nociceptive scores in the early phase (0–5 min), both the 2.0 and the 20 μg doses significantly reduced nociceptive scores throughout the late phase (20–50 min). Even the 0.2 μg dose of chelerythrine produced a reduction in nociceptive scores; however, this effect was only significant at the 25 and 50 min time points. The PKC inhibitors did not produce any evidence of motor dysfunction since neither GF 109203X, nor chelerythrine significantly affected placing or righting reflexes.

Figure 2 illustrates the mechanical flexion reflex measured by the Randall-Selitto test in one hindpaw after the thermal injury to the contralateral hindpaw. In the control group, there was an initial brief antinociception with thresholds significantly higher than baseline up to 5 min after the injury. This initial antinociception was followed by a persistent hyperalgesia, with thresholds significantly lower than baseline between 30 min and 12 hr after the injury. Mechanical flexion reflex thresholds returned to normal levels by the 36 hr measurement and remained at this level at least up to 3 d later. In the group pretreated with GF 109203X, both the initial antinociceptive effect and the persistent hyperalgesia between 3 and 12 hr was significantly attenuated. In the group given chelerythrine, the antinociception was significantly increased, and the persistent hyperalgesia was significantly attenuated at 30 min, and 3–12 hr postinjury.

Autoradiography

^{3}H-PDBu binding in the normal rat spinal cord

Figures 3*A* and 4 show specific ^{3}H-PDBu binding in four segments of the rat spinal cord. Of the four regions (i.e., superficial

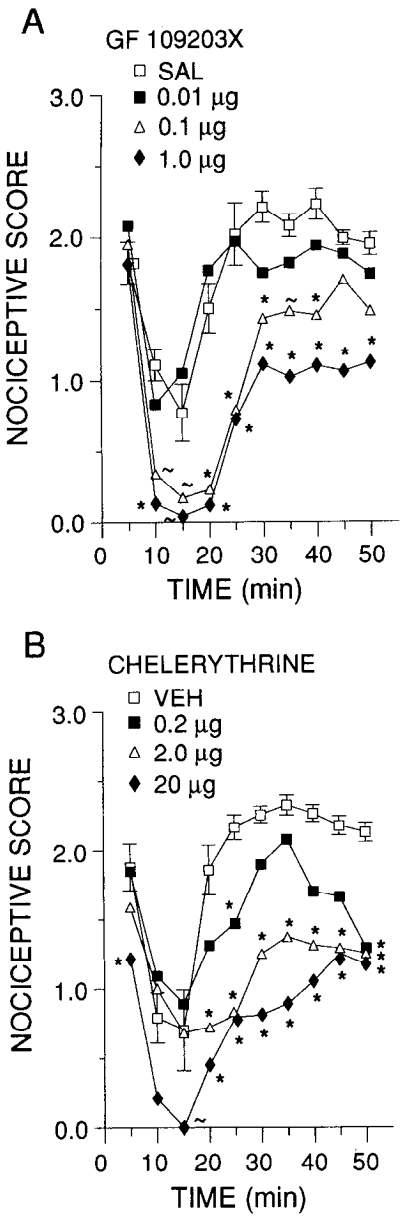

Figure 1. Nociceptive responses (± SEM) induced by injection of 50 μl of 2.5% formalin in rats pretreated with (*A*) GF 109203X (0.01–1.0 μg, n = 6,6,6) or saline (n = 7) or (*B*) chelerythrine (0.2–20 μg, n = 5,6,5) or vehicle (n = 6). Repeated measures ANOVA reveals significant main effects of dose for (*A*) [$F(3,21) = 9.4$, $p < 0.001$] and (*B*) [$F(3,18) = 14.8$, $p < 0.001$], and time for (*A*) [$F(9,189) = 38.4$, $p < 0.001$] and (*B*) [$F(9,162) = 19.3$, $p < 0.001$], as well as a significant dose × time interaction for (*A*) [$F(27,189) = 2.6$, $p < 0.001$] and (*B*) [$F(27,162) = 2.1$, $p < 0.01$. Significant differences from the vehicle control group are indicated by the following symbols (~$p < 0.05$; *$p < 0.01$, Dunnett t).

dorsal horn LI–II; deeper dorsal horn LIII–VI; lamina X; ventral horn), binding in the superficial dorsal horn laminae was the highest in all segments of the spinal cord (Fig. 4; cervical, 1351 ± 34; thoracic, 1437 ± 31; lumbar, 1413 ± 34; sacral, 1474 ± 17 fmol/mg tissue, wet weight).

In the deeper layers of dorsal horn (LIII–VI) the density of binding was moderate ranging between 410 and 630 fmol/mg tissue, wet weight (Fig. 4; cervical, 411 ± 11; thoracic, 546 ± 13; lumbar, 469 ± 13; sacral, 630 ± 15 fmol/mg tissue, wet weight).

In the lamina X region, moderate amounts of specific ^{3}H-PDBu binding were seen ranging between 345 and 830 fmol/mg tissue, wet weight; an increasing gradient of ^{3}H-PDBu binding being seen from the cervical to the sacral region (cervical 344 ± 34; thoracic 500 ± 6.6; lumbar 630 ± 44; sacral 828 ± 17 fmol/mg tissue, wet weight) (Fig. 4).

The lowest amounts of ^{3}H-PDBu binding were found in the ventral horn. Moreover, unlike the increasing rostrocaudal gradient seen in the lamina X, relatively equal amounts of ^{3}H-PDBu binding were found in the ventral horn at all levels (Fig. 4; cervical, 213 ± 11; thoracic, 283 ± 10; lumbar, 254 ± 13; sacral, 311 ± 11 fmol/mg tissue, wet weight).

Specific ^{3}H-PDBu binding in the spinal cord of rats treated with noxious chemical stimulation

Lumbar segments. The distribution of specific ^{3}H-PDBu binding in the lumbar spinal cord of the rats sacrificed at 5, 25, and 60 min after the injection of formalin is shown in Figure 3*B*. Quantification (shown in Fig. 5*A*) reveals that ^{3}H-PDBu binding increased in the superficial layers of the dorsal horn as early as 5 min after the formalin injection (1864 ± 32 vs controls, 1413 ± 34 fmol/mg of tissue, wet weight, $p < 0.01$). This increase remained significantly high, peaking at 25 min (1975 ± 41 fmol/mg tissue, wet weight, $p < 0.01$), returning toward normal values at 60 min (1566 ± 27 vs controls, 1413 ± 34 fmol/mg tissue, wet weight, $p > 0.05$) (Figs. 3*B*, 5*A*).

In laminae III–VI, significant increase in the density of binding was seen only at 25 min after the formalin injection (660 ± 27 vs control, 469 ± 13 fmol/mg tissue, wet weight, $p < 0.01$). No significant difference from the control group was observed at 5 or 60 min after the noxious chemical stimulation (527 ± 10 at 5 min; 470 ± 9.4 at 60 min vs control, 469 ± 13 fmol/mg tissue, wet weight, $p > 0.05$) (Figs. 3*B*, 5*A*).

In contrast, quantification in the lamina X and ventral horn revealed no statistical differences at any time point when compared to control values (lamina X: at 5 min, 739 ± 24; at 25 min, 783 ± 36; at 60 min, 553 ± 34, vs controls, 630 ± 44 fmol/mg tissue, wet weight; ventral horn: at 5 min, 281 ± 7.9; at 25 min, 286 ± 14; at 60 min, 231, ± 8.0, vs controls, 254 ± 13 fmol/mg tissue, wet weight) (Figs. 3*B*, 5*A*).

Thoracic and cervical segments. Although specific ^{3}H-PDBu binding appeared to be slightly elevated in laminae I–II of the dorsal horn of both thoracic and cervical spinal segments 25 min after formalin injection (e.g., thoracic, 1617 ± 44, vs controls, 1437 ± 31 fmol/mg tissue, wet weight; Figs. 3*B*, 5*B*; cervical, 1502 ± 27, vs controls 1351 ± 34; Fig. 3*B*, quantification of cervical spinal segments not shown), it did not reach statistical significance, $p > 0.05$). The only significant change at any time point after chemical stimulation was an apparent decrease in specific ^{3}H-PDBu binding at 60 min in the ventral horn of the thoracic spinal cord segment (149 ± 10 vs controls, 283 ± 10 fmol/mg tissue, wet weight, $p < 0.01$; Fig. 5*B*).

Specific ^{3}H-PDBu binding in the spinal cord of rats treated with noxious thermal stimulation

Lumbar segments. A rapid increase in specific ^{3}H-PDBu binding was seen in laminae I and II of the dorsal horn of the lumbar segment, when one of the hindpaws of the rat was immersed in hot water at 55°C for 90 sec (Figs. 3*C*, 5*A*). Binding was significantly higher 1 min after stimulation and remained significantly elevated at least up to 10 min after application of the noxious thermal stimulus (at 1 min, 1790 ± 42; at 10 min, 1763

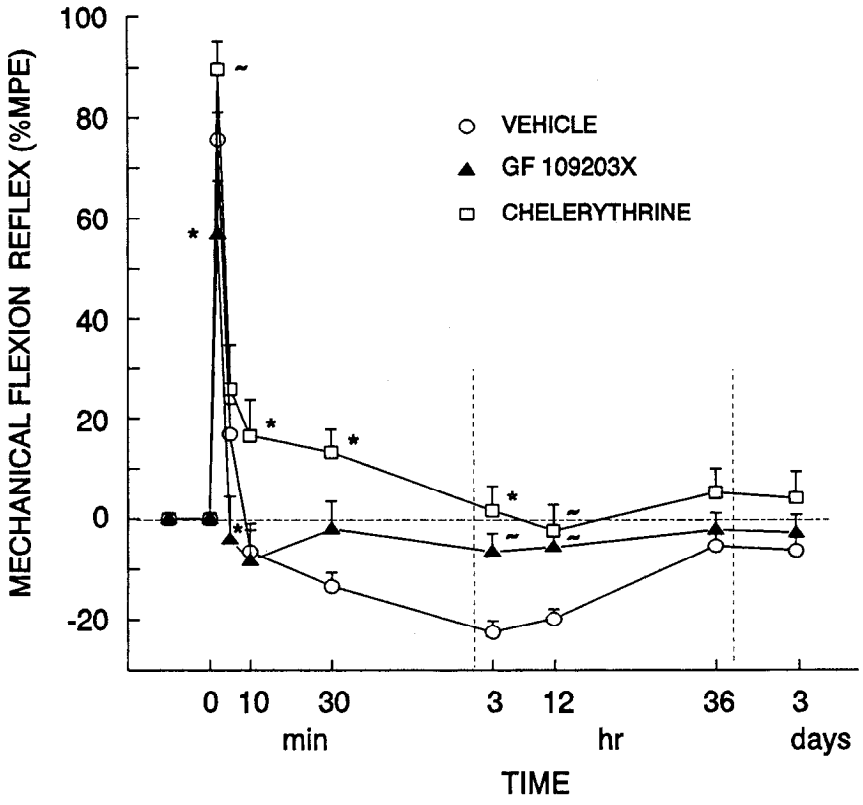

Figure 2. Percent maximum possible effect (%MPE ± SEM) for mechanical flexion reflex on Randall-Selitto apparatus after thermal injury of the contralateral hindpaw in rats pretreated with either GF 109203X (10 μg, $n = 12$), chelerythrine (50 μg, $n = 8$), or vehicle ($n = 12$). Repeated measures ANOVA reveals significant main effects for treatment group [$F(2,29) = 7.1$, $p < 0.01$ and time [$F(7,161) = 116.1$. $p < 0.001$], as well as a significant group × time interaction [$F(14,161) = 3.27$, $p < 0.001$]. Significant differences from the vehicle control group are indicated by asterisks ($\sim p < 0.05$; $^* p < 0.01$, Dunnett t).

± 22 vs controls, 1413 ± 34, or non-noxious thermal stimulation, 1530 ± 64 fmol/mg tissue, wet weight, $p < 0.05$). Specific ^{3}H-PDBu binding returned to the control levels 30 min after poststimulation (at 30 min, 1414 ± 50 vs controls, 1413 ± 34 fmol/mg tissue, wet weight). No significant change in ^{3}H-PDBu binding occurred in response to non-noxious thermal stimulation at 1 min (1530 ± 64 vs controls, 1413 ± 34 fmol/mg tissue, wet weight, $p > 0.05$) (Figs. 3*C*,5 *A*).

In the deeper laminae of the dorsal horn (LIII–VI), there was a significant increase in binding only at 10 min after the noxious thermal stimulation (589 ± 21 vs control, 469 ± 13, or non-noxious thermal stimulation, 468 ± 20 fmol/mg tissue, wet weight, $p < 0.05$). No significant difference was seen at the other time points (at 1 min, 509 ± 14; at 30 min, 500 ± 33 vs controls, 469 ± 13, or non-noxious thermal stimulation, 468 ± 20 fmol/mg tissue, wet weight, $p > 0.05$). Furthermore, ^{3}H-PDBu binding in laminae III–VI was virtually identical for controls and rats exposed to non-noxious thermal stimulation (469 ± 13 vs 468 ± 20 fmol/mg tissue, wet weight, $p > 0.05$) (Figs. 3*C*, 5*A*).

In the lamina X region, no significant change was observed at either 1, 10, or 30 min after thermal stimulation (at 1 min, 650 ± 17; at 10 min, 775 ± 23; at 30 min, 606 ± 27; vs controls, 630 ± 44 fmol/mg tissue, wet weight, $p > 0.05$) (Fig. 5*A*). However, there was a significant increase in ^{3}H-PDBu binding at 10 min after noxious thermal stimulation when compared with non-noxious thermal stimulation (775 ± 23 vs 558 ± 24 fmol/mg tissue, wet weight, $p < 0.01$). No significant changes were seen in the ventral horn at either 1 min, 10 min, or 30 min poststimulation (at 1 min, 222 ± 6.8; at 10 min, 328 ± 13; at 30 min, 207 ± 12 vs controls, 254 ± 13, or non-noxious thermal stimulation, 311 ± 11 fmol/mg tissue, wet weight, $p > 0.05$) (Figs. 3*C*, 5*A*).

Thoracic and cervical segments. Following noxious thermal stimulation, specific ^{3}H-PDBu binding in the thoracic and cervical spinal cord segments remained unaffected, with the exception of a significant decrease in the ventral horn of the thoracic segment at 1 min poststimulation (178 ± 7.0 vs controls, 283 ± 10 fmol/mg tissue, wet weight) (Fig. 5*B*).

Discussion

The results from the present study provide evidence for a contribution of PKC to the persistent nociceptive responses induced by noxious chemical or thermal stimulation of one hindpaw of the rat. The experiments provide not only pharmacological evidence in two physiological models of nociception, but also anatomical evidence using a highly specific radioligand, ^{3}H-phorbol-12,13 dibutyrate (PDBu), as an autoradiographic probe for membrane-bound PKC (Worley et al., 1986a,b; Parent et al., 1993). Both types of analyses suggest a participation of PKC in the mechanisms of persistent nociception. Thus, the pharmacological experiments indicate that intrathecal administration of each of the two inhibitors of PKC, GF 109203X (Toullec et al., 1991) and chelerythrine (Herbert et al., 1990), attenuated the persistent nociception induced by formalin injection, and blocked the remote hyperalgesia expressed in the hindpaw contralateral to a thermal injury. Activation of PKC by noxious peripheral stimulation was also apparent in the autoradiographic data, which showed an increase in specific ^{3}H-PDBu binding, or membrane-bound PKC, specifically in the dorsal horn of the lumbar segment of the spinal cord.

The presence of PKC in the central nervous system has been determined by biochemical, immunohistochemical and autoradiographic procedures in various species (Hug and Sarre, 1993). Kitano et al. (1987) and Saito et al. (1988) reported dense PKC-like immunoreactivity in the hippocampus and the substantia

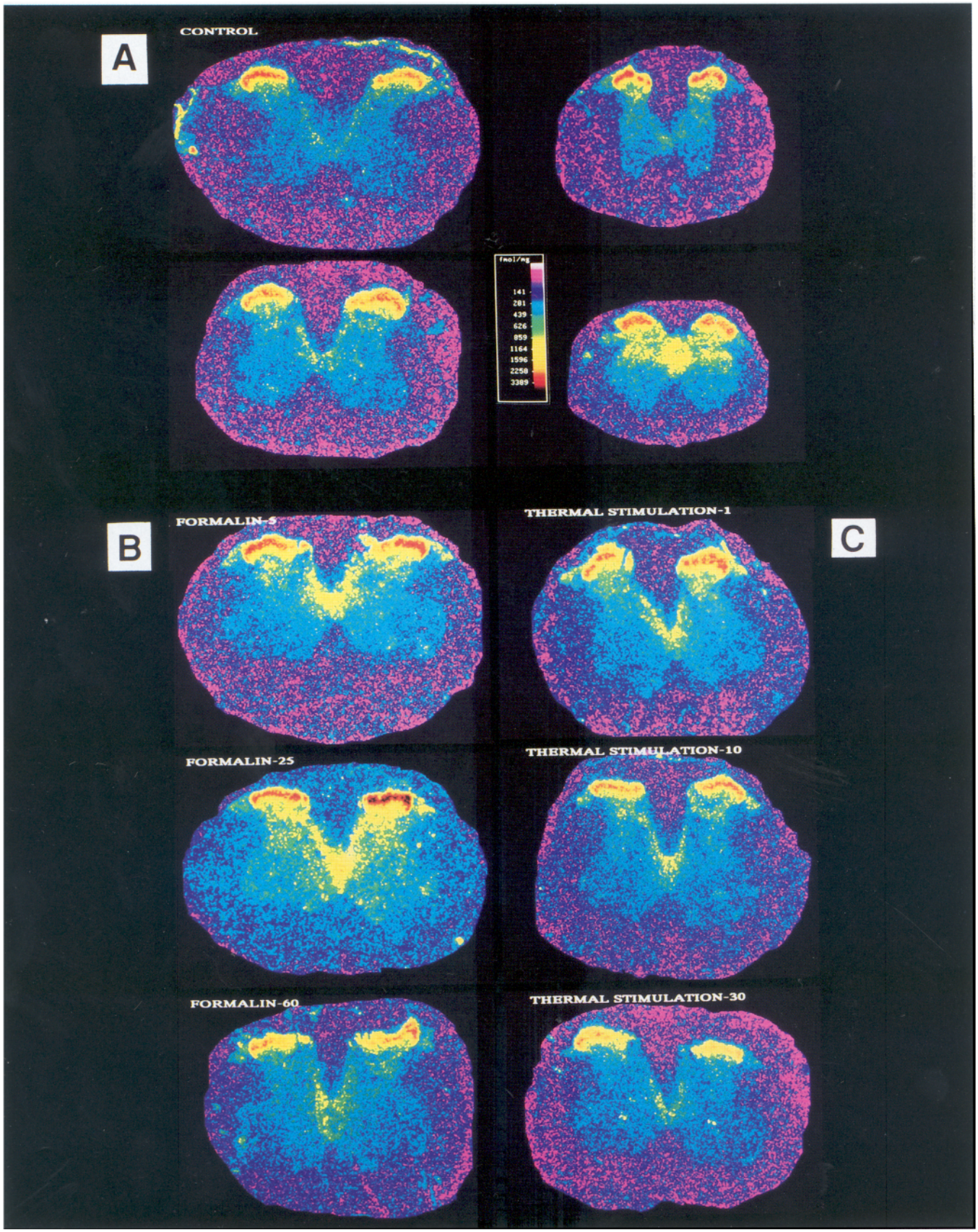

Figure 3. Photomicrographs of the autoradiographic distribution of ^{3}H-PDBu binding in the cervical (*upper left*), thoracic (*upper right*), lumbar (*lower left*), and sacral (*lower right*) spinal cord segments of the control rat (*A*), and in lumbar spinal cord segments of rats exposed to noxious (*B*) chemical (5, 25, and 60 min) or (*C*) thermal (1, 10, and 30 min) stimulation. Nonspecific binding in presence of 1.0 μM PDBu was below detectable levels.

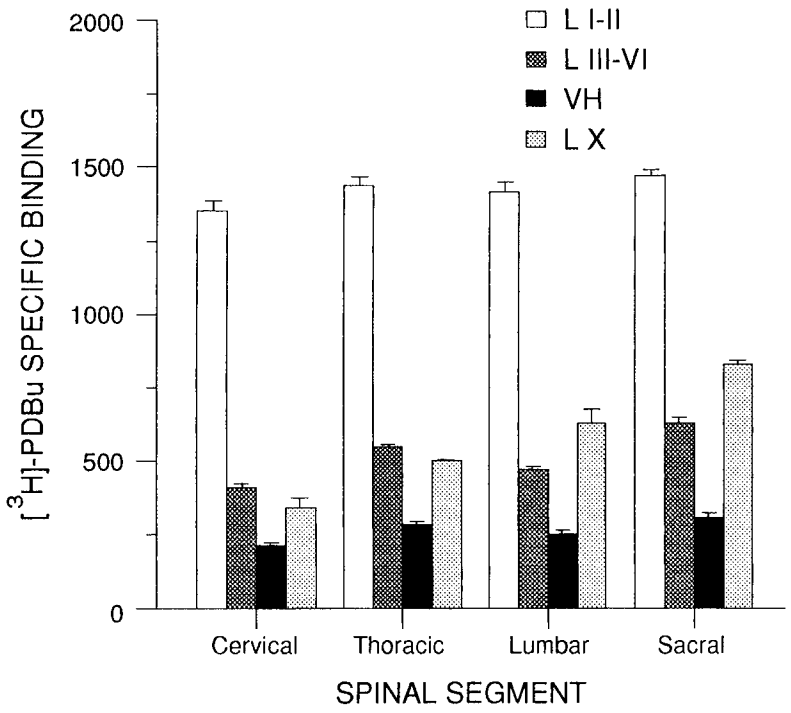

Figure 4. Quantitative autoradiographic analysis of the distribution of membrane-bound protein kinase C in the cervical, thoracic, lumbar, and sacral spinal cord of the control rat as determined by ^{3}H-PDBu binding assay. Specific ^{3}H-PDBu binding is expressed as mean ± SEM in fmol/mg of tissue, wet weight, obtained from 10–18 sections at each segmental level of spinal cords from four rats. Nonspecific binding was below detectable levels.

gelatinosa in the dorsal horn of the spinal cord in the rat. Other immunohistochemical studies have described localization of isoenzymes I, II, and III of PKC using monoclonal antibodies against each isoenzyme (Brandt et al., 1987; Nagashima et al., 1991). There are some controversies as to which specific isoenzymes are present in specific brain regions [e.g., conflicting evidence that there is either a predominance of type II (Nagashima et al., 1991) or type III (Brandt et al., 1987) isoenzymes in the dorsal horn of the spinal cord]. Nevertheless, the results indicate that PKC is clearly present in the superficial layers of the dorsal horn. Furthermore, Worley et al. (1986a,b) have characterized the distribution of membrane-bound PKC using a radioligand, ^{3}H-phorbol 12,13 dibutyrate, in the rat brain. Among the regions with the highest levels of specific ^{3}H-PDBu binding in the CNS are the superficial layers of dorsal horn of the spinal cord, while binding in the ventral horn is among the lowest. The results here confirm and extend these findings by showing the presence of high amounts of specific ^{3}H-PDBu binding in laminae I and II, intermediate levels in laminae III–VI and X, and very low binding in the ventral horn (Figs. 3*A*, 4).

Our anatomical studies use specific ^{3}H-PDBu binding as a technique to selectively measure membrane-bound PKC (Olds et al., 1989). Although there is evidence that phorbol esters may also bind to neuronal proteins such as chimaerin (Ahmed et al., 1990), there is a good correspondence between phorbol ester binding sites and PKC immunoreactivity in the brain (Saito et al., 1988). Furthermore, this method has been successfully used to show specific changes in the distribution of membrane-bound PKC in hippocampus associated with a classically conditioned response (Olds et al., 1989) or discriminative learning trials (Olds et al., 1990), as well as following neuronal injury by ischemia (Onodera et al., 1989), lesions to the entorhinal cortex (Parent et al., 1993), and in Alzheimer's diseased brains (Horsburgh et al., 1991). Recently, Mao et al. (1992, 1993) have demonstrated an increase in specific ^{3}H-PDBu binding in the dorsal horn of the spinal cord in rats subjected to nerve constriction injury. Furthermore, the increase in membrane-bound PKC, as well as nociceptive behaviors and thermal hyperalgesia induced by the nerve injury, were all attenuated by gangliosides (Hayes et al., 1992; Mao et al., 1993), which inhibit the translocation and activation of PKC (Vaccarino et al., 1987).

In the present study, both noxious chemical and noxious thermal stimulation led to significant increases in specific ^{3}H-PDBu binding in the superficial and deeper layers of the dorsal horn of the lumbar spinal cord. Neither noxious chemical nor noxious thermal stimulation produced significant increases in ^{3}H-PDBu binding in either lamina X or ventral horn regions, or in spinal segments other than the lumbar segment (with the exception of a slight, significant increase over the non-noxious thermal stimulation group at 10 min after noxious thermal stimulation in lamina X of the lumbar spinal cord). The peak response to the noxious chemical stimulus was at 25 min poststimulation, while the peak response to the noxious thermal stimulus was at 1 min poststimulation. Importantly, the two types of stimulation that produced increases in membrane-bound PKC were also capable of producing persistent nociception and hyperalgesia. Furthermore, in the case of noxious chemical stimulation, the peak increase in membrane-associated PKC corresponded with the maximal nociceptive responses to the formalin stimulus.

Although not specifically analyzed, both chemical and thermal stimulation produced bilateral increases in ^{3}H-PDBu binding in the dorsal horn (see Fig. 3*B,C*), suggesting that the stimulation of one hindpaw results in significant increases in membrane-bound PKC in the contralateral dorsal horn laminae. The increases in specific ^{3}H-PDBu binding in superficial dorsal horn are consistent with the key role of this region in the processing of spinal nociceptive information (Price, 1988; Willis, 1985). The contralateral changes in ^{3}H-PDBu binding seen after chemical stimulation are also consistent with the intense contralateral distribution of the marker of metabolic activity 2-deoxy-glucose after formalin injury (Aloisi et al., 1993). The translocation and activation of PKC in neurons contralateral to the spinal cord

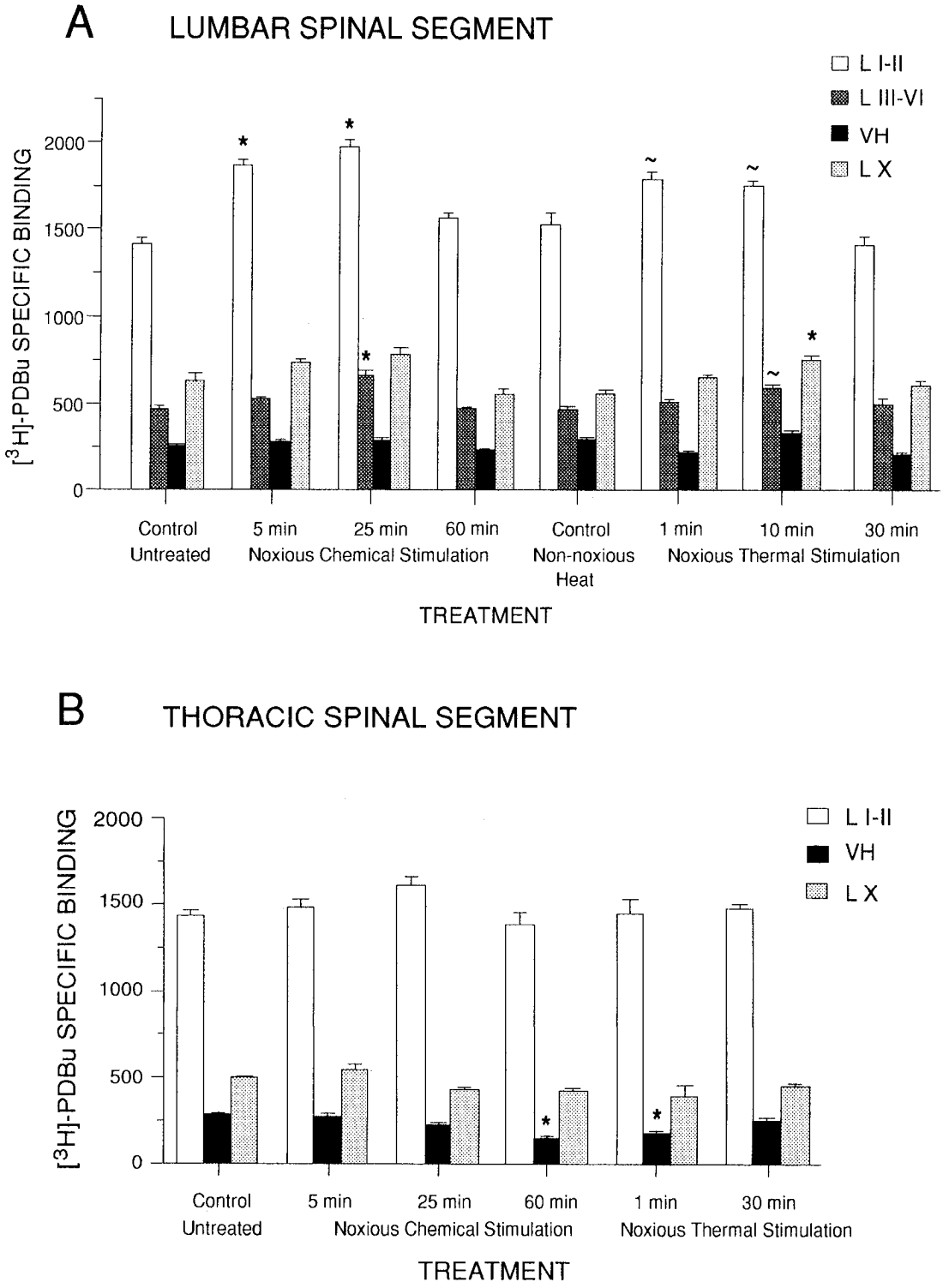

Figure 5. Quantitative autoradiographic analysis of the distribution of ^{3}H-PDBu binding in the lumbar (*A*) and thoracic (*B*) spinal cord of control rats and experimental rats at specific times after noxious chemical (5, 25, and 60 min) or thermal (1, 10, and 30 min) stimulation. Each histogram represents specific ^{3}H-PDBu binding expressed as mean ± SEM in fmol/mg of tissue, wet weight. Nonspecific binding was below detectable levels. For the lumbar spinal segments (*A*), ANOVA revealed a significant main effect of treatment group in the dorsal horn laminae I–II [$F(7,157) = 27.8$, $p < 0.001$], dorsal horn laminae III–VI [$F(7,64) = 12.3$, $p < 0.001$], lamina X [$F(7,73) = 9.2$, $p < 0.001$], and ventral horn [$F(7,142) = 11.9$, $p < 0.001$] regions. For the thoracic spinal segments (*B*), ANOVA revealed a significant main effect of treatment group in the lamina X [$F(5,32) = 4.6$, $p < 0.01$] and ventral horn [$F(5,61) = 14.5$, $p < 0.001$], but not the superficial dorsal horn [$F(5,74) = 2.2$, $p > 0.05$] regions. Significant differences from the control group are indicated by asterisks ($\sim p < 0.05$; $*p < 0.01$, Scheffé).

segment at which nociceptive inputs arrive may thus play a significant role in the development of remote secondary hyperalgesia and referred pain. The contralateral changes in ^{3}H-PDBu binding after chemical stimulation is also consistent with observations that formalin injury of one hindpaw results in nociceptive behaviors (licking and biting) directed not only at the injured hindpaw, but also frequently directed at the contralateral hindpaw (Aloisi et al., 1993; Coderre, unpublished data). The present results suggest that the contralateral activation of membrane-bound PKC after thermal injury may also be critical to the development of the hyperalgesia that develops in the hindpaw contralateral to a thermal injury (Coderre and Melzack, 1991).

Earlier studies from our laboratory have demonstrated an enhancement of nociceptive scores in the formalin test by intrathecal pretreatment with phorbol esters and activators of PKC, as well as a reduction in formalin-induced nociceptive behaviors by intrathecal pretreatment with a relatively nonspecific protein kinase inhibitor, H-7 (Coderre, 1992). Experiments presented here indicate that administration of GF 109203X and chelerythrine, much more selective inhibitors of PKC (Herbert et al., 1990; Toullec et al., 1991), reduced the nociceptive scores in late phase of the formalin test. Although very effective at inhibiting nociceptive responses in the late phase of the formalin test, the early phase of the formalin test was not as sensitive to the effects of PKC inhibitors; only the highest dose of chelerythrine significantly reduced nociceptive responses in the early phase. Furthermore, the greater potency of GF 109203X over that of chelerythrine in producing antinociception in the late phase of the formalin test is consistent with its lower IC_{50} value for inhibition of PKC (0.01 vs 0.66 μM, respectively). As for thermal injury, although the two agents produced unexplained opposite effects on the initial antinociceptive response to injury, both agents significantly blocked the persistent hyperalgesia that developed in the contralateral hindpaw and persisted for hours after the initial injury. Importantly, the presence of the PKC inhibitors at the time of injury blocked the expression of remote secondary hyperalgesia between 30 min and 12 hr postinjury. It is expected that the activation of PKC is more critically involved in mediating the sensitization of neurons leading to prolonged nociception, than in neuronal responses to brief noxious stimulation. This point is supported by the fact that the PKC inhibitors are not analgesic themselves in non-injurious measures of mechanical and thermal reflexes (Coderre and Yashpal, 1994).

The present data clearly implicate a contribution of PKC to the development of persistent nociception after chemical or thermal injury. However, the specific mechanisms by which PKC activation produces neuronal changes that contribute to persistent nociception is unknown. Alterations in neuronal excitability may result from a direct effect of PKC on ionic conductances by phosphorylation of specific ion channels (Swope et al., 1992). Stimulation of PKC has been found to enhance Ca^{2+} currents (DeRiemer et al., 1985) and to reduce both Ca^{2+}-dependent K^+ currents (Alkon et al., 1986) and Cl^- currents (Madison et al., 1986). In particular, Chen and Huang (1992) have demonstrated that PKC increases NMDA-activated currents in isolated trigeminal cells by increasing the probability of channel openings and by reducing the voltage-dependent Mg^{2+} block of NMDA receptor channels. Alternatively, the activation of presynaptic PKC has been found to enhance the release of neurotransmitters, including glutamate, from nerve terminals (Barrie et al., 1991). Recently, it has been shown that activators of PKC enhance the basal and evoked release of glutamate and aspartate in spinal cord slices, as well as depolarizing the responses of dorsal horn neurons to exogenous glutamate and NMDA (Gerber et al., 1989). Finally, PKC is implicated in the cascade of events that lead to the induction of various proto-oncogenes such as c-*fos* and c-*jun*, which code transcription factors controlling the expression of new genes (Naranjo et al., 1991). However, in addition to PKC, it is likely that other intracellular messengers, such as nitric oxide and arachidonic acid, play important roles in persistent nociception following noxious stimulation (Malmberg and Yaksh, 1992; Meller et al., 1992; Coderre and Yashpal, 1994).

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