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Immunocytochemical study of parvalbumin, calbindin D-28k, and calretinin in the superficial dorsal horn of the rat spinal cord following unilateral hindpaw inflammation

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Abstract: The effect of noxious stimulation on the immunoreactivity of the calcium-binding proteins parvalbumin (PV), calbindin-D-28k (CB) and calretinin (CR) was investigated in the superficial dorsal horn of lumbar levels L5–L3 of the rat spinal cord. Freund's adjuvant was injected unilaterally into the hindpaw to induce inflammation. Immunohistochemical techniques were utilized to investigate changes in the calcium-binding proteins 2 h and 1, 2, 4, and 7 days after injection. At 24 h after injection, a decrease in the intensity of fluorescence of PV-immunoreactive (IR) fibers was observed in the superficial layer (substantia gelatinosa) of the ipsilateral dorsal horn (L5–L3) in most animals. Comparatively fewer animals exhibited changes in the CB- and CR-IR fibers, except at the L3 level 2 days after, and at the L4 level 7 days after the hindpaw injection. After the peak response, at 24 h in most animals, there was a decline in the number of responders at 2 days and no differences were noted at 4 days. However, at 7 days, there was again an increase in the number of animals revealing diminished fluorescence intensity in the ipsilateral substantia gelatinosa. Changes in immunoreactivity of calcium binding proteins in the interneurons of the superficial lumbar dorsal horn may reflect hyperactivity within these neurons following noxious stimulation.

Key words: Parvalbumin, Calbindin D-28k, Calretinin, Spinal cord, Immunohistochemistry

Introduction

Influx of Ca^{2+} into neuronal cells occurs following a variety of physiological and stressful stimuli [1–2]. Certain calcium-binding proteins (CBPs), such as parvalbumin (PV), calbindin D-28k (CB), and calretinin (CR) are believed to be involved in the regulation of intracellular free calcium concentrations as "buffer" proteins [3]. Each of these CBPs has a unique localization in the central nervous system, although their functions are still unknown. Variation in the concentration of free calcium in neurons is believed to play a major role in neuroexcitation. Since CBPs may help to regulate intracellular calcium, we investigated whether the expression of these proteins in the spinal cord was affected by paw stimulation. The localization of CBPs within the spinal cord has been studied, and the relationship of these proteins to pain has recently attracted increased interest [4–9]. If the presence of CBPs is related to physiological events in neurons, then immunohistochemical alterations in the staining density of these proteins could provide a useful tool to study the subpopulation of interneurons involved in the transmission of noxious stimulis. Accordingly, we investigated the immunofluorescence intensity of three CBPs (PV, CB, CR) in the superficial dorsal horn of the lumbar spinal cord following unilateral hindpaw noxious stimulation. Our focus of attention was on the L3–L5 region of the superficial dorsal horn, since it has been shown that this level was the somatotopic target for primary sensory neurons in the plantar region of the hindpaw [10–11].

Materials and methods

Forty-two male Sprague-Dawley rats (weight, 200–300 g) were assigned to two groups: the non-stimulated control group (n = 7) and the stimulated group (n = 35). Seven rats in each stimulated group were injected subcutaneously in the left hindpaw with 0.2 ml of Freund's adjuvant emulsion (Sigma, St Louis, Miss.) diluted 1:1 with saline, carried out without anesthesia. The injections were made approximately in the midportion of the hindpaw. Slight variations in the depth of the needle could not be avoided. At 2 and 24 h

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and 2, 4, and 7 days after the injections, the controls and stimulated group were anesthetized with sodium pentobarbital prior to intracardial perfusion of 100 ml 0.5% sodium nitrite in phosphate-buffered saline (PBS; 20 mM, pH 7.4), followed by 500 ml 10% buffered formalin (10% v/v in phosphate buffer, pH 7.0; Fisher) at 4°C. The rats were treated in conformance with the guidelines of the International Association for the Study of Pain (IASP) [12]. Following perfusion, the bony spinal column (L2–S2) was removed, exposing the dorsal part of spinal cord. The spinal cord with the bony structure was postfixed for 24 h and then transferred to 20% sucrose in PBS. After 48 h, the cord was divided into 7.5-mm blocks containing segments L2–L4 and L5–S2, and coronal sections were cut in a cryostat (20 μm) in a caudal to rostral direction. Alternate sections were placed on a set of five gelatin chrom-alum coated slides and stored at −80°C. One set of slides was stained with 1% thionine to determine the spinal cord level [13–14]. The slides were incubated in primary antiserum for 2–3 days. The antisera used were: monoclonal anti-carp muscle parvalbumin (1:20000) (Sigma), and polyclonal rabbit anti-guinea pig calretinin (1:1000) [15] in PBS containing 0.3% Triton X-100, and 1% normal goat serum. Slides prepared from animals with different survival times were incubated at the same time to avoid technical error. After incubation, the slides were washed twice with 0.2% Triton X-100 for 100 min each time, followed by 30-min incubation in fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappel) diluted 1:300 in 0.3% Triton X-100/PBS for PV and CB and incubation with goat anti-rabbit IgG (Cappel) 1:300 for CR. The slides were then washed twice (for 10 min each time) in 0.2% Triton X-100 and once in PBS (5 min). Sections were coverslipped with a glycerol-phosphate (3:1) buffer containing 0.1% *p*-phenylenediamine and examined under a fluorescence microscope.

All sections were examined for intensity of fluorescence only in the superficial layer of the dorsal horn. Particular attention was focused on detecting differences between the ipsilateral (left) side and the contralateral (right) side. Decreases in the intensity of immunohistochemical labeling in both the right and left sides were determined by making comparisons with fluorescence-positive areas (dots), using an imaging system consisting of a video camera (C2400; Hamamatsu Photonics, Shizuoka, Japan) and an image processor (Nexus Qube, Osaka, Japan). The total digital density of immunoreactivity was calculated by summing up the gray scales present in every pixel of the image. The number of animals showing asymmetries of CBP-immunoreactivity (IR) was determined at each spinal cord level (L3–L5) for each protein. Statistical analysis was performed by Student's *t*-test.

Results

After the injection of Freund's adjuvant, the left hindpaw was edematous for 1–7 days. Fluorescence microscopy in normal controls revealed a dense band of PV-IR fibers in the inner layer of lamina II (Fig. 1A). The medial portion of the band was more diffuse than the lateral portion. The fibers were varicose and only few cell bodies were noted on each 20-μm section (n = four to six cells/section). Most of the CB-IR fibers and cells were contained in lamina I and II (Fig. 1B). CR-IR fibers and cells were visible throughout lamina II in the superficial dorsal horn (Fig. 1C). Fluorescence-positive areas in the superficial dorsal horn were examined by the imaging system (Fig. 2). The symbols above the dotted line in Fig. 2 indicate decreased cases ($P < 0.001$). The mean ratio (left/right) in the control group was 1.10, and the range was 0.86–1.51. The number of animals showing a decrease in IR is shown in Table 1. Results for each of the CBPs are given below.

Parvalbumin

Injections of Freund's adjuvant into the plantar region of the left hindpaw produced changes in PV-IR that were dependent on the post-injection interval. At 2 h post-injection there was a decrease in the intensity of PV-IR at L4 on the ipsilateral (left) side in three out of seven rats (Table 1), while one rat showed a difference at L5. After 24 h, the number of animals showing a decrease increased, this being particularly notable at L4 and 5 levels. With the imaging system, the photomicrographs at 24 h demonstrated that the ratio of digital density for Fig. 3 was 2.84 [2474 dots (left)/872 dots (right)]. The range of the ratio 24 h after injection (i.e., symbols above the dotted line in Fig. 2) was 2.11–3.06. Two days after the paw injection, the number of animals

Table 1. Number of animals showing a decrease in immunofluorescence activity

		2 h	24 h	2 Days	4 Days	7 Days
PV	L3	0/7	3/7	3/7	0/7	2/7
	L4	3/7	6/7	2/7	0/7	5/7
	L5	1/7	5/7	0/7	0/7	3/7
CB	L3	0/7	1/7	4/7	1/7	2/7
	L4	1/7	3/7	1/7	0/7	6/7
	L5	2/7	0/7	2/7	0/7	2/7
CR	L3	0/7	1/7	3/7	0/7	2/7
	L4	0/7	2/4	1/7	0/7	4/7
	L5	0/7	1/7	3/7	0/7	2/7

The number of animals showing a decrease in immunofluorescence intensity for each calcium-binding protein at spinal levels L3, L4, and L5 (stimulated side) was counted 2 h to 7 days after injection of Freund's solution in the ipsilateral left hindpaw.
PV, parvalbumin; CB, calbindin D-28k; CR, calretinin.

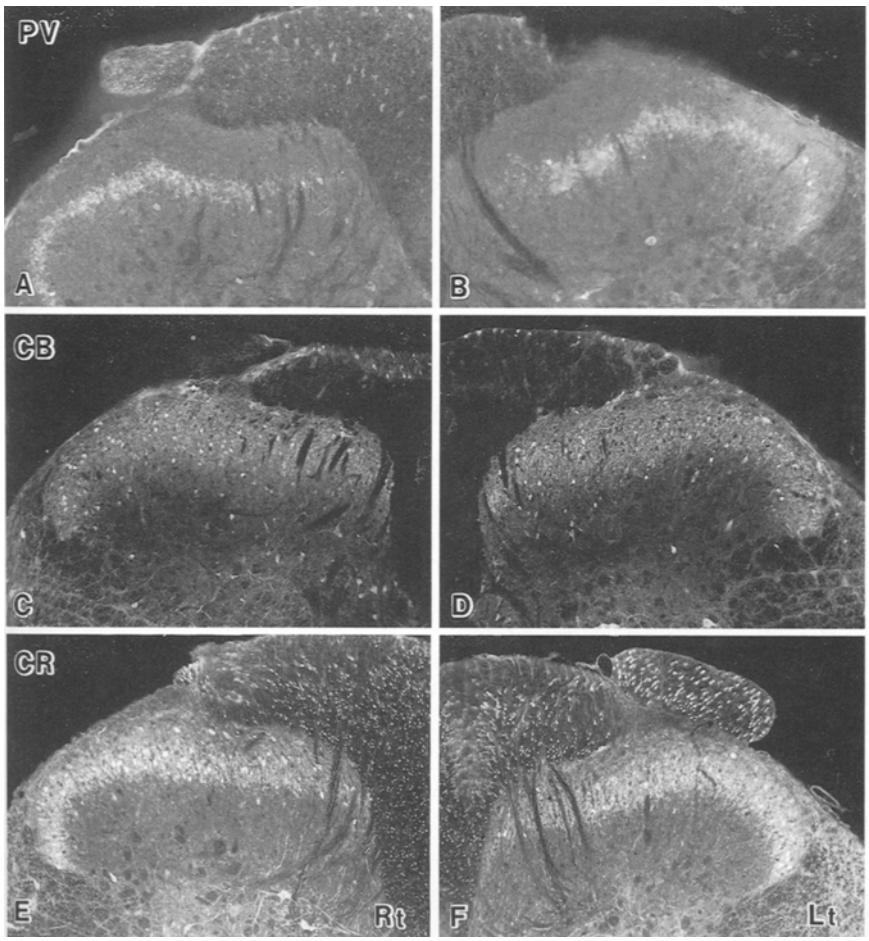

Fig. 1A–F. Distribution of immunoreactivity (*IR*) for parvalbumin (*PV*), calbindin D-28k (*CB*), and calretinin (*CR*) in controls at L4 level. Photographs on the *right* are injected side in all Figs. **A** Note the dense band of PV-IR in the lateral aspect of the superficial dorsal horn. Medial part of the band is more diffuse than the lateral portion. Few cell bodies are observed. **B** CB-IR cells and fibers are contained primarily in the superficial dorsal horn. **C** Note the dense fibers and cells with CR IR in the superficial dorsal horn, ×96

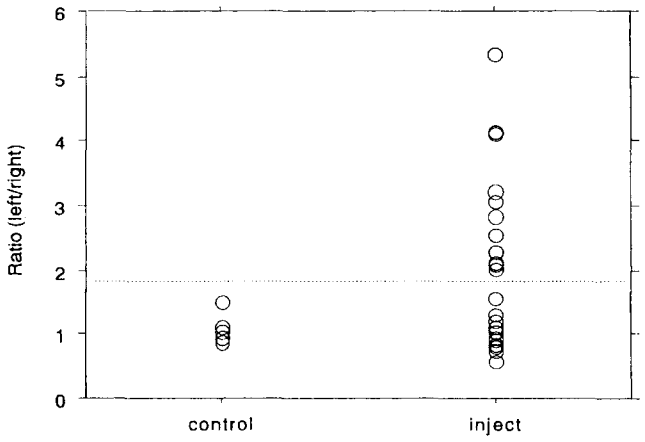

Fig. 2. Areas of positive fluorescence in the superficial dorsal horn were compared on the right and left (*injected*) sides by using an imaging system (Nexus Qube). The ratios for left/right were calculated (*circles*). *Circles above the dotted line* show the cases of decreased immunoreactivity ($P < 0.001$)

showing depletion of IR decreased. Four days after the left paw injection of Freund's adjuvant, PV-IR revealed no asymmetry, and the ratio of digital density for Fig. 4A and B was 0.76 [1108 dots.(left)/1460 dots (right)]. After 7 days, the decrease in fluorescence intensity on the injected side was marked (Fig. 5A). The ratio for PV IR for Fig. 5A and B was 5.36 [1393 dots (left)/260 dots (right)].

Calbindin D-28k

Only a few animals responded to injection, after 2 h and 24 h. Although the number of animals showing a depletion of fluorescence intensity increased, asymmetries disappeared 4 days post-injection, except in one rat at level L3. Seven days after the injection, there was a prominent decrease in fluorescence intensity (Table 1). The ratio of CB-IR for Fig. 5B was 4.14 [2647 (right)/640 (left)].

Calretinin

Immunofluorescence microscope observations revealed no visible decrease in the pattern at 2 h. Only a few animals responded after 24 h, while several rats showed ipsilateral changes, mostly at L3 and L5, after 2 days. No asymmetries were noted 4 days after injection of Freund's adjuvant. Similarly to the findings for PV and

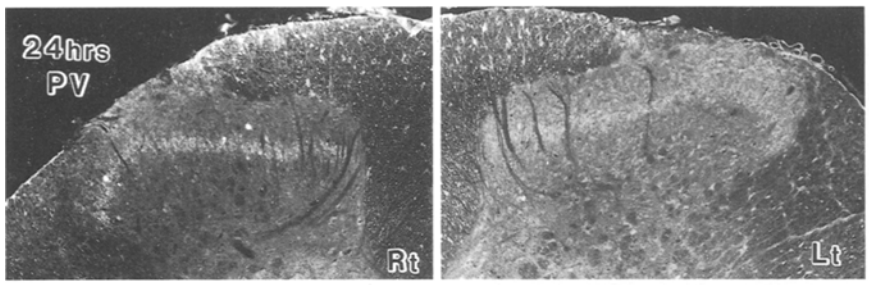

Fig. 3. PV-IR fibers and cells, 24 h after injection of Freund's adjuvant into the left (ipsilateral) hindpaw, L4 level of the spinal cord. Note decrease in both the medial and lateral portions of the left side. ×96

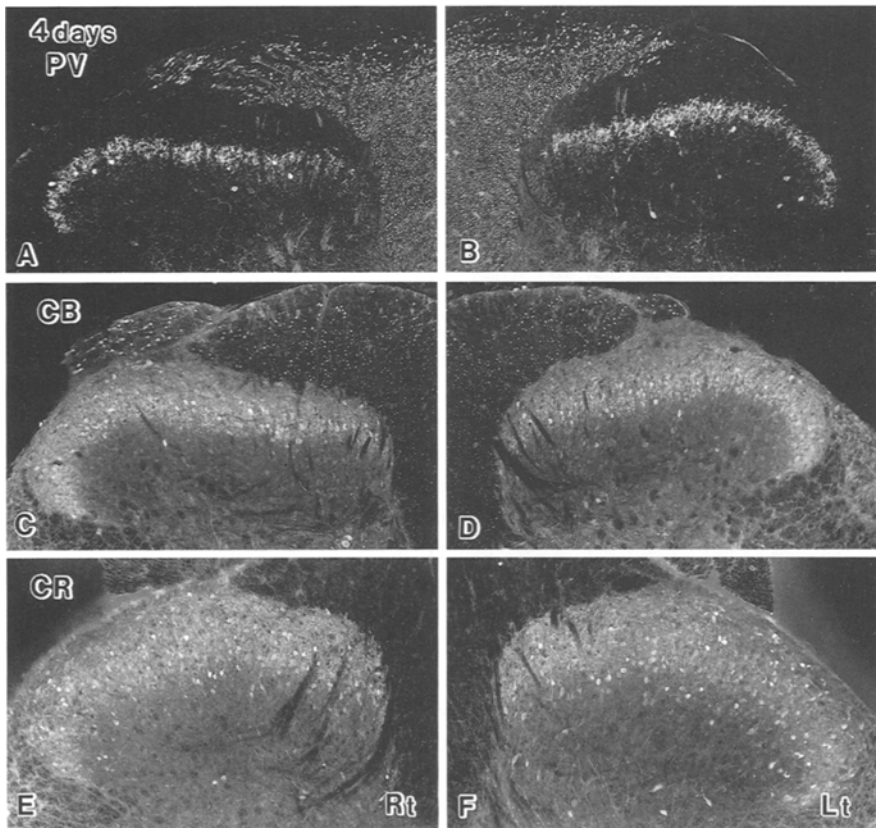

Fig. 4A–F. By 4 days after injection of Freund's solution, no change was observed for any of the three proteins

CB IR, the number of rats showing depletion of CR-IR at the injection side increased at 7 days (Table 1).

Discussion

Anatomical studies have demonstrated that fine caliber sensory fibers emanating from the skin of the rat hindpaw are distributed in superficial laminae of the dorsal horn [16–18] and that the terminal zone in the substantia gelatinosa (lamina II) has a somatotopic organization [10,11,19–24]. The central branches of the hind limb afferent fibers from intracutaneous regions of the paws were found to project in a precise manner to the medial two-thirds of the substantia gelatinosa of the spinal cord segments L3–L5. The projections from the plantar skin of the digits and dorsal foot skin were shown to be organized somatotopically in mediolateral compartments in the dorsal horn. The injection paradigm in the present study is not analogous to that of Molander and Grant [11], who injected low microliter amounts of wheat germ agglutinin-horseradish peroxidase intracutaneously. In the present study, a subcutaneous injection of 0.2 ml Freund's adjuvant resulted in edema of the entire hindpaw, including the digits. The size of the foot varied among individual rats. This variation in the inflammatory response may be due to the precise site of infusion of the Freund's solution within the paw. Following intracutaneous injections of wheat germ agglutinin-horseradish peroxidase, sensory fibers of the digit and the plantar skin projected medially within the superfical laminae of the substantia gelatinosa and the projection of the dorsal skin involved the lateral part of the substantia gelatinosa in addition to L3

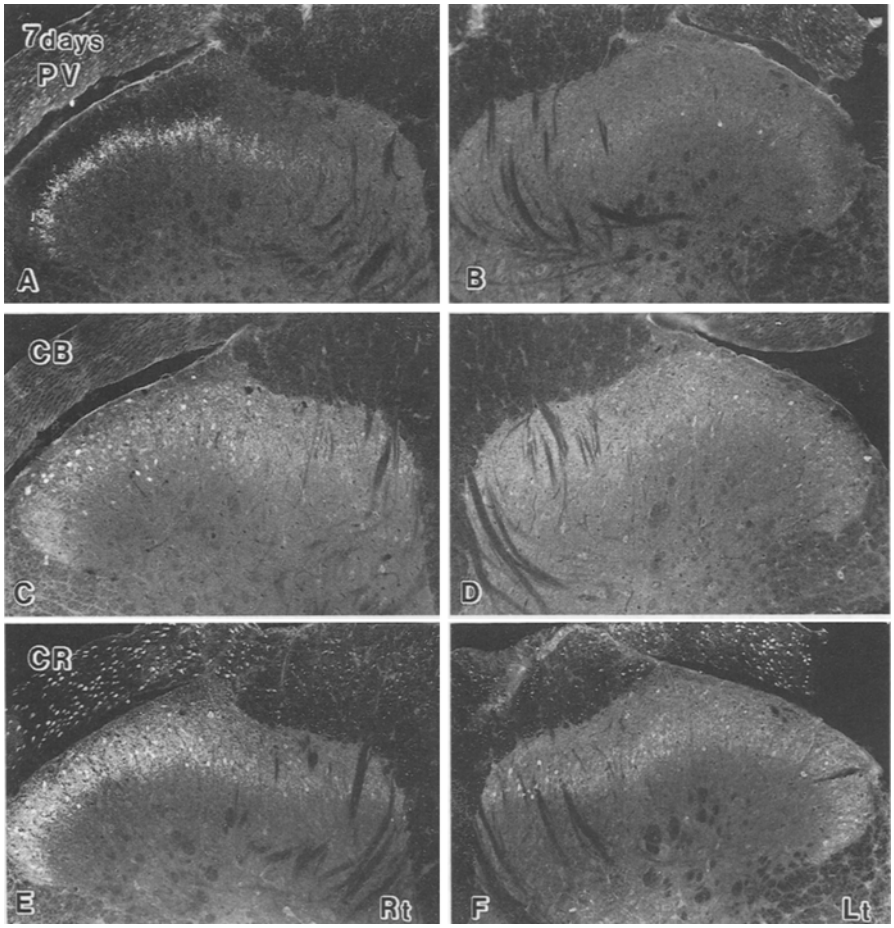

Fig. 5A–F. Superficial dorsal horn IR cells and fibers 7 days after left hindpaw injection (L4 level). **A** PV; immunofluorescence intensity is markedly decreased on the left side. **B** and **C** CB and CR; note a decrease of immunoreactivity on the left side. ×96

to L5 representation [11]. In this regard, Presley et al. [25] demonstrated a dense distribution of c-*fos* immunoreactivity at the L4–L5 level of the superficial dorsal horn (laminae I and II) of the spinal cord after injection of formalin into the rat hindpaw. We focused our analysis on areas where maximal somatotopic organization of cutaneous afferent fibers from the hind foot had been mapped in the substantia gelatinosa of the spinal cord [11]. One or 2 days after injection, the number of the animals showing a decrease in IR intensity increased. Four days after the hindpaw injection, the intensity of fluorescence of all three proteins appeared to return to control levels. However, at 7 days, most unexpectedly, there was again an increase in the number of animals that revealed a reduced amount of labeling at the L4 level. An explanation for this result is not evident. Similar studies with Freund's adjuvant injected into the hindpaw have revealed an increase in the amount of the opiate peptide, dynorphin, in spinal segments that receive sensory imput from the affected limb [26–28]. The peak dynorphin A levels were reached 3–4 days after injection of Freund's adjuvant. It is conceivable that, in 4 days, the perception of dynorphin-sensitive pain in the superficial dorsal lumbar cord is diminished, thereby resulting in a return to normal IR. Millan et al. [29] showed a rise in immunoreactive dynorphin and α-neoendorphin after 3 weeks of chronic pain, induced by intradermal injection at the tail base of *Mycobacterium butyricum*, which results in edema, inflammation, and hyperalgesia of the joints [29]. They also revealed a relative decrease in the kappa opioid receptors in the spinal cord. Dynorphin is considered to be an endogenous ligand for kappa receptors [30]. Similarly, long-term exposure to the opiate etorphin (>24 h) produced down-regulation of receptor activity in NG10815 cells [31]. In our experiment, in the rat spinal cord, a slow desensitization of opiate receptors may have been related to the renewal of pain activation 7 days after noxious stimulation, thereby resulting in the diminished intensity of fluorescence of the three calcium-binding proteins. We speculate that the initial decrease in fluorescence intensity is a reflection of diminished concentrations of PV and CB following 24 h of pain. It is possible that sensory nerve stimulation decreases the gene expression of these proteins, perhaps via Ca^{2+} influx, resulting in reduced

protein synthesis in 24h. A reversal of this histofluorescence response was observed by 4 days, although inflammation of the hindpaws was still observed.

The above considerations suggest that the changes in CBP-IR following pain stimulation result from changes in the concentration of CBPs in the superficial dorsal horn. However, an alternative explanation is possible. It was recently demonstrated that conformational change brought about by increasing Ca^{2+} concentration produced a decrease in the amount of monoclonal antibody bound to the calcium-binding protein troponin C [32]. That is, the decreased antibody binding to troponin C in the presence of calcium is an effect believed to be caused by a conformational change. Further, preliminary studies using purified CR and CB have revealed a similar preference of some monoclonal and polyclonal antisera for apoprotein conformations (Ca^{2+} free) [33]. These results suggest that studies revealing calcium-dependent changes in CBP-IR, or quantitative estimates of CBPs, might be affected by the CBP conformation-dependent antibody recognition characteristics of the antisera used. It is thus possible that the secondary reduction in the three CBPs observed at 7 days may reflect high levels of calcium, thereby resulting in conformational changes. Such changes could reduce the capacity of the antibody to bind to epitope(s).

Various stimuli have been reported to lead to reduced PV and CB-IR in different regions of the brain. Johansen et al. [34] reported that, 4 days after ischemia (20-min four-vessel occlusion), PV-IR disappeared from cell bodies and fibers in the CA1 portion of the hippocampus. This change was reversible within days, but was associated with a permanent loss of CB-IR in CA1 pyramidal cells. Johansen et al. [34] suggested that hippocampal PV-IR interneurons suffered from a delayed and reversible calcium accumulation in the days after ischemia, or alternatively, they suggested there could be decreased synthesis or increased destruction of PV. Nitsch et al. [35] reported less severe ischemic insult in gerbils, showing that GABAergic neurons containing PV were resistant to the effects of ischemia, although the pyramidal cells in the CA1 field had disappeared. They concluded that the presence of PV protected interneurons from the deleterious consequence of this insult. Rami et al. [36] concluded that CB is an important factor for the survival of pyramidal cells in the hippocampus after ischemia. Similarly, in seizures induced in animals by perforant path stimulation it was revealed that subpopulations of nonprincipal neurons, visualized by immunocytochemistry for PV and CB, were more resistant to excessive stimulation than CA1 pyramidal cells [37]. In addition, Sloviter [38] found a positive correlation between the presence of at least one of these CBPs in the hippocampus and relative resistance to seizure-induced neuronal damage. An investigation of the ischemic vulnerability of CR-containing non-pyramidal spiny interneurons in the hippocampus showed a selective loss of CR-IR in these neurons 12–24h after ischemia when the pyramidal cells in the CA1 region showed no sign of damage. After 2–3 days, most of the CR-IR cells had disappeared [39]. These studies indicate that there appears to be no direct relationship between CBP content and vulnerability in experimentally-induced ischemia and epilepsy. Similarly, in the present studies in the spinal cord, during the early periods (1–2 days) there was a more selective vulnerability of PV and CB-IR cells, while after 7 days, cells containing all three CBPs appeared vulnerable.

In summary, the decrease in IR of the CBPs studied, and that of PV in particular, may indicate the localization of a subpopulation of cells that respond to noxious stimulation. Alterations of IR in the interneurons of the superficial dorsal horn may signify an attempt by CBPs to buffer both the acute and the chronic influx of Ca^{2+} ions.

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