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Research Paper

Protein kinase C is partly involved in *c-fos* protein expression of nocuously-activated neurons but may not in concomitant modulatory action through opioid receptors at the spinal level in rats

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Abstract: The present study was aimed to examine if protein kinase C (PKC) activation is necessarily involved in both the c-fos protein expression in the nocuously-activated *c-fos* protein-like immunoreactive (Fos-LI) neurons and the concomitant opioid receptor-mediated modulation in the dorsal horn circuitry of the spinal cord. Formalin was injected into a hindpaw of rats 5 min after the rats were pretreated with intrathecal (i.t.) administration of chelerythrine (Chel) , an inhibitor of PKC, naloxone (Nal), combined administration of these two (Chel + Nal), or vehicle (n=5 in each group),respectively. By using immunocytochemical techniques, the formalin-induced Fos-LI neurons in the lumbar dorsal horn were calculated 1 h after formalin injection. The results showed that: (1) i.t. Chel significantly reduced the number of Fos-LI neurons in the dorsal horn of the spinal cord on the side ipsilateral to the formalin injection, showing a decrease by 60.3% (P<0.001) as compared to that observed in the i.t.vehicle group; (2) i.t. Nal significantly increased the number of Fos-LI neurons in the ipsilateral dorsal horn, with an increase of 46.0% (P<0.01) as compared to that in the i.t.vehicle group , the highest percentage increase being found in the deeper laminae of the dorsal horn; and (3) i.t. Chel + Nal also exhibited a significant decrease in Fos-LI neurons in the ipsilateral dorsal horn as compared to i.t. Nal group, showing a reduction of 53.2%, a value similar to that in the i.t. Chel group. These results suggest that: (1) PKC plays a role in the *c-fos* protein expression only in nearly one half of the Fos-LI neurons in the dorsal horn; and (2) PKC is possibly not involved in the concomitant modulaion on the nociception mediated by μ- (and also partly δ-) opioid receptors in the spinal cord.

Key words: immunocytochemistry; nociception; dorsal horn neurons; *c-fos* protein; protein kinase C μ-opioid receptor; formalin test; rat

蛋白激酶C部分参与伤害性刺激在脊髓背角神经元中引起的 *c-fos* 蛋白的表达而可能不参与阿片受体对脊髓痛感受的调制

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摘 要: 实验用免疫细胞化学技术观察了大鼠鞘内分别注入蛋白激酶(PKC)抑制剂Chelerythrine (Chel)、纳洛酮 (Nal)、或二者同时注入后, 由后脚掌注射福尔马林引起的脊髓腰膨大背角中 *c-fos* 蛋白样免疫活性(Fos-LI)神经元数目的改变。结果发现: (1) 鞘内注入Chel可显著降低福尔马林注射侧脊髓背角中Fos-LI神经元的数目, 同空白对照组 (鞘内注入生理盐水或10%的DMSO) 相比, 降低60.3% (P<0.001); (2)鞘内注入Nal后, 福尔马林注射侧背角中Fos-LI神经元显著增加, 同对照组相比, 增加46.0%(P<0.01), 而以背角深层增加最为明显; (3)在鞘内同时注入Chel和Nal后, 与单独注入Nal组相比, 脊髓背角中Fos-LI神经元的数目显著降低(降低53.2%), 此数值与上述单独注入Chel时引起Fos-LI神经元降低的百分率近似。结果提示: (1)PKC只参与脊髓背角中部分Fos-LI神经元中 *c-fos* 蛋白的表达; (2) PKC可能不参与背角中同时激活的μ- (以及部分δ-) 阿片受体对脊髓伤害性感受的调制。

关键词: 免疫细胞化学; 伤害性感受; 背角神经元; *c-fos* 蛋白; 蛋白激酶C; m-阿片受体; 福尔马林试验; 大鼠
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Protein kinase C (PKC) is thought to be a pivotal mediator of several cellular processes, including transmembrane and intracellular signalings[1]. A great deal of effort has been devoted to clarifying the involvement of PKC in the processes of nociception at the spinal cord level using behavioral[2], electrophysiological [3], and neurochemical[4] approaches. Moreover, Dubner and Ruda[5] have reported that activation of PKC by phorbol esters can induce *c-fos* protein expression in spinal dorsal horn neurons. At the same time, it has been proposed that PKC is also involved in the signaling of μ-opioid receptors that are activated concomitantly by the peripheral nociceptive inputs[6,7]. However, we have found in a previous study that nocuously-induced activation of PKC-β subtypes occurs mainly in the superficial laminae (laminae Ⅰ ~ Ⅱ) of the dorsal horn[8]. Other authors reported that intravenous application of morphine decreased the nocuously-activated expression of *c-fos* protein with a predilective effect on neurons located in the deeper laminae (laminae Ⅴ~Ⅶ) of the dorsal horn[9]. These facts raise suspicions such as if PKC is necessarily involved in both the spinal acquisition and transmission of peripheral nociceptive inputs and the concomitant modulatory effects on them via spinal endogenous opioids. As many authors have demonstrated that the expression of *c-fos* protein in dorsal horn neurons may serve as a marker for activities of these neurons following noxious stimulation[10,11], the present study was designed to clarify these problems by using immunocytochemical techniques to examine the changes in the number of *c-fos* protein-contained dorsal horn neurons induced by subcutaneous injection of formalin in rats that were combined with pretreatment of intrathecal administration of PKC inhibitor and morphine antagonist or both, respectively.

1 METHODS

Twenty-five male Sprague-Dawley rats (150~250 g), divided randomly into 5 groups (*n*=5 for each), were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). After incising the skin over the T11~12 spinal level, a PE-10 polyethylene tube filled with saline was inserted caudally into the subarachnoid space terminating at the level of the lumbar enlargement. In the first part of the experiments, one group of rats were intrathecally (i.t.) administered with chelerythrine (Chel), an inhibitor of PKC[12], with a dosage of 20 μg in 10 μl 10% DMSO in normal saline (NS), being similar to that as used previously[13]; the other two groups were used as controls, pretreated with i.t. NS or 10% DMSO in NS, respectively. In the second part of the experiments, two groups of the rats were pretreated with either i.t. naloxone (Nal, 68 μg in 10 μl NS, a dosage as used before[14]) or combined i.t. Nal and Chel (with the same dosages as they were used alone), respectively. Five minutes after the i.t. injection, rats in all these 5 groups were injected subcutaneously with 150 μl of formalin (2.5% in saline) into the plantar surface of a hindpaw. One hour after formalin injection, all rats were deeply anesthetized and sacrificed for immunocytochemical examination of *c-fos* protein expression in the lumbar dorsal horn.

After being sacrificed, all animals were perfused via the aorta with 100 ml of normal saline followed by 1000 ml of ice-cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB, pH 7.4) and by 20% sucrose in PB (pH 7.4). The lumbar spinal cord was then removed, fixated at 4°C in the same fixative for 4~6 h, and cryoprotected overnight in 30% sucrose in 0.1 mol/L PB. Forty μm frozen sections were cut in the transverse plane on a cryostat and collected in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) for immunocytochemical analysis of *c-fos* protein by the avidin-biotin-peroxidase (ABC) method of Hsu *et al.*[15], using the polyclonal antibody against the c-fos protein (Oncogene Science INC, 1:10 000). At last, sections were stained with the peroxidase-diaminobenzidine-nickel method. Omission of the *c-fos* protein antiserum from the immunostaining protocol completely abolished the labeling. Since cross-reactivity of the antibody with the peptides or substances present in the tissue sections can not be excluded, we therefore use the term "*c-fos* protein-like immunoreactivity" (Fos-LI) as a more appropriate description of the *c-fos* protein immunostaining.

Sections from L4~5 were analyzed under an Olympus BH2 microscope for the presence of Fos-LI neurons within the spinal cord gray matter, and the number of the neurons were counted separately from three subregions: (1) the superficial laminae (laminae Ⅰ ~ Ⅱ); (2) the deeper laminae (laminae Ⅴ~Ⅶ); and 3) other laminae of the dorsal horn. The Fos-LI neurons were identified by black labeled nuclei that were distinct from the background at magnifications of 4 ×, 10 ×, and 20 ×. Any Fos-LI neurons that were questionable under high magnification were discarded. All sections were scanned and 10 sections with the greatest number of labeled cells were selected from each animal for cell counting. The statistical significance between two groups was determined by Student's *t* test and variance analysis.

2 RESULTS

As reported previously by us[8,16] and others [10,11], formalin injection elicited a robust increase of Fos-LI neurons of the dorsal horn of the spinal cord on the side ipsilateral to the formalin injection in two control groups, while only a few Fos-LI neurons could be found on the contralateral sides; since the increases in Fos-LI neurons between two control groups showed no significant difference, data were processed and presented here together named as i.t. vehicle group. With pretreatment of i.t. Chel, as shown in Table 1 and also in Fig. 1, the number of formalin-induced Fos-LI neurons on the injected side exhibited a prominent reduction as compared to that in i.t. vehicle group; and the reduction appeared mainly in the superficial laminae I ~ II (by 63.2%) and the deeper laminae Ⅴ ~ Ⅶ (78.6%), with a total reduction of 60.3% in the formalin-injected side of the dorsal horn ($P<0.01$ in all three cases). As reported before[8,10,11,16], these two subregions of the dorsal horn were the places with high density of the nocuously-induced Fos-LI neurons.

Table 2 shows the summarized data in the second part of the experiments. It turned out that following i.t. pretreatment with naloxone, formalin injection elicited a more robust increase in the number of Fos-LI neurons in the ipsilateral dorsal horn (also see Fig. 2*A*), with an in-

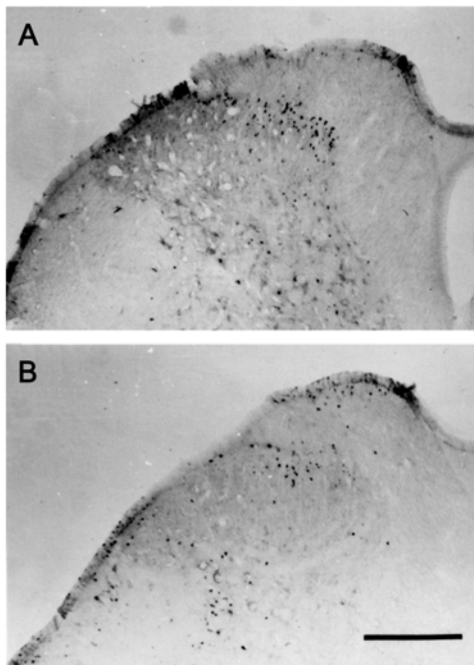

Fig. 1. Representative photomicrographs showing nociceptive Fos-LI neurons in the dorsal horn ipsilateral to the formalin injection in i.t. vehicle group (*A*) and i.t. Chel group (*B*). Bar = 100 μm.

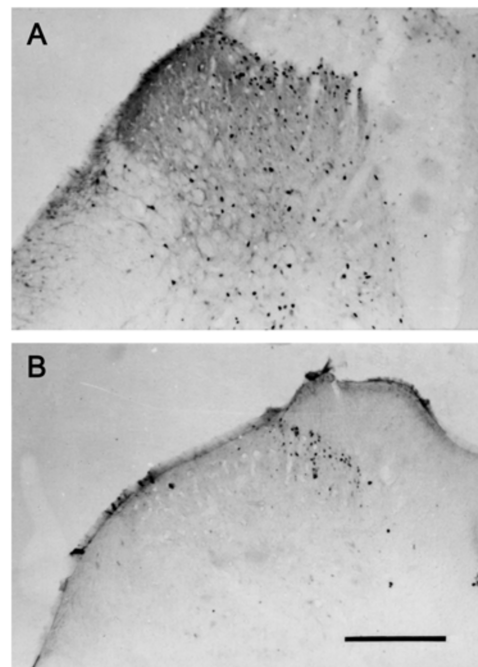

Fig. 2. Representative photomicrographs showing nociceptive Fos-LI neurons in the dorsal horn ipsilateral to the formalin injection in i. t. Nal group (*A*) and i.t. Nal + Chel group (*B*). Bar = 100 μm.

Table 1. Data summary showing the numbers and their comparisons of nociceptive Fos-LI neurons in the dorsal horn ipsilateral to the formalin injection in i.t. vehicle group (1) and i.t. Chel group (2)

Subdivisions of dorsal horn	1 Number of Fos-LI neurons in i.t. vehicle group	2 Number of Fos-LI neurons in i.t. Chel group	Percentage changes calculated by (2-1)/1×100
Laminae Ⅰ~Ⅱ	38 ± 3.0	14 ± 2.1***	−63.2%
Laminae Ⅴ~Ⅶ	14 ± 2.1	3 ± 2.3***	−78.6%
Other regions	11 ±0 .1	8 ± 2.4	−
Total	63 ± 4.8	25 ± 3.6**	−60.3%

***$P<0.001$; **$P<0.01$ as compared to that in i.t. vehicle group. Each number is mean ± SEM averaged from 50 sections of 5 rats.

Table 2. Data summary showing the numbers and their comparisons of nociceptive Fos-LI neurons in the dorsal horn ipsilateral to the formalin injection in i.t. vehicle group (1) and i.t. Nal group (3)

Subdivisions of dorsal horn	1 Number of Fos-LI neurons in i.t. vehicle group	3 Number of Fos-LI neurons in i.t. Nal group	Percentage changes calculated by (3-1)/1×100
Laminae Ⅰ~Ⅱ	38 ± 3.0	47 ± 4.7**	23.7%
Laminae Ⅴ~Ⅶ	14 ± 2.1	28 ± 2.8***	100.0%
Other regions	11 ±3 .1	17 ± 3.0	–
Total	63 ± 4.8	92 ± 5.7**	46.0%

***P<0.001; **P<0.01 as compared to that in i.t. vehicle group. Each number is mean ± SEM averaged from 50 sections of 5 rats.

Table 3. Data summary showing the numbers and their comparisons of nociceptive Fos-LI neurons in the dorsal horn ipsilateral to the formalin injection in i.t. Nal group (3) and i.t. Nal+Chel group (4)

Subdivisions of dorsal horn	3 Number of Fos-LI neurons in i.t. Nal group	4 Number of Fos-LI neurons in i.t. Nal+Chel group	Percentage changes calculated by (4-3)/3×100
Laminae Ⅰ~Ⅱ	47 ± 4.7	20 ± 2.3**	−57.4%
Laminae Ⅴ~Ⅶ	28 ± 2.8	8 ± 3.0***	−71.4%
Other regions	17 ± 3.0	15 ± 3.2	–
Total	92 ± 5.7	43 ± 6.0***	−53.2%

***P<0.001; **P<0.01 as compared to that in i.t. Nal group. Each number is mean ± SEM averaged from 50 sections of 5 rats.

crease of 46.0% as compared to that in i.t. vehicle group (P<0.01). Additionally, when the rats were pretreated with combined i.t. Nal and Chel (Table 3), the number of Fos-LI neurons also exhibited a significant reduction (also see Fig. 2*b*) as compared to that observed in group with i.t. Nal only, showing a reduction rate of –57.4% in laminae Ⅰ ~ Ⅱ, –71.4% in laminae Ⅴ ~ Ⅶ, and –53.2% in total dorsal horn (P<0.01 in all three cases), respectively. By comparing the data in Tables 1, 2 and 3, it was evident that i.t. Chel suppressed the formalin-induced Fos-LI neurons by a similar reduction rate no matter whether naloxone was present or not (–53.2% *vs* –60.3%); and also, naloxone still elicited a robust increase of Fos-LI neurons in i.t. Chel+Nal group even the number of these neurons should have been deeply suppressed by the concomitantly-administered chelerythrine, namely, the real number of Fos-LI neurons in i.t. Chel+Nal group still surpassed that in i.t. Chel group by 41.8%.

3 DISCUSSION

Several lines of earlier evidence have suggested that PKC is implicated in the cascade of events that lead to the induction of various proto-oncogenes, such as *c-fos* and *c-jun*, that encode transcription factors controlling the expression of other genes[1, 20]. As shown by the data presented above, this view is supported only partly by our present results which showed that intrathecal administration of PKC inhibitor chelerythrine reduced the number of nocuously-induced Fos-LI neurons in the spinal dorsal horn. Meaningfully, this value reminds us of the investigation we observed previously in a double staining immunocytochemical study[8], in which it was observed that only nearly one half of the nocuously-induced Fos-LI neurons in the dorsal horn co-existed with the activated PKC Ⅰ~Ⅱ subtypes. These facts coming from two kinds of experiments strongly imply that the activation of PKC or of its β-subtypes are necessary for the c-fos protein expression only in about one half of the nocuously-induced Fos-LI neurons in the spinal dorsal horn. Of course, the fact and its functional significance remain to be further clarified. It is possible that the different functional positions in the circuitry (interneuron or projection neuron[10]) and the different neurochemical characteristics (such as GABA-ergic[17]) of the nocuously-activated Fos-LI neurons might be in parallel with their different behaviors in respect of PKC activation.

On the other hand, it has also been reported that PKC modulates the responsiveness of spinal opioids receptors

that are concomitantly involved in the spinal processing of peripheral noxious inputs[6,7,18]. However, according to the present study, it seems that the antinociceptive modulatory effect exhibited concomitantly by the activation of μ- (and also partly δ-[19]) receptor-mediated signaling system might not be affected by PKC activation. If PKC had been involved in the process, the intrathecal administration of chelerythrine would not have blocked the spinal *c-fos* protein expression similarly (60.3% *vs* 53.2%) in both cases when chelerythrine was either used alone or combined with naloxone; also, naloxone should not have increased the *c-fos* protein expression similarly (46.0% *vs* 41.8%) whether naloxone was used alone or combined with chelerythrine. Although we do aware that our proposal is not decisive, it is strengthened further by following facts: by scrutinizing the data presented in Table 2, one can find that the greatest increase of Fos-LI neurons in i.t. Nal group appears in laminae Ⅴ ~ Ⅶ. This fact is consistent with an earlier report by Tolle *et al*.[9] , showing that intravenous application of morphine decreases the nocuously-induced *c-fos* protein expression with a predilective effect on neurons in deeper spinal laminae; moreover, these laminae are also the place where the chelerythrine exhibits its highest suppressive effect in the dorsal horn no matter whether chelerythrine is administered alone or combined with naloxone (78.6% *vs* 71.4%; see Tables 1 and 3). These facts implicate evidently that blockade of μ- (and also partly δ-) opioid receptors does not remarkably affect the suppressive effect of chelerythrine; and also, blockade of PKC activation does not affect the effect of naloxone. Taken together, we incline to suggest that the spinal antinociceptive effect through related opioid receptors are carried out in a way that is independent of the activation of PKC systems.

Owing to the fact that PKC family consists at least of twelve isoforms that have been implicated in a wide range of physiological functions[1,20], the functional importance of PKC in spinal nociception should be more complex than it has been proposed at the present time. Further characterization of individual isoforms of PKC and their contribution to the spinal nociceptive processing are urgently needed.

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