BIBLID : ISSN 0253-9756 中国药理学报 *Acta Pharmacologica Sinica* 1993 Jul; 14 (4) : 306—311

Central expression of c-*fos* protein after peripheral noxious thermal stimulation in awake rats[1]

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ABSTRACT This study applied immunohistochemistry method to examine the pattern of c-*fos* expression in the neuraxis following peripheral noxious thermal stimulation accomplished by immersion of tail of awake rat into hot water (50℃). In unstimulated control rats, no obvious baseline expression of c-*fos* protein was found except in nucleus paraventricularis hypothalami and colliculus inferior, probably associated with restraint-induced stress and auditory stimulus, respectively. Noxious thermal stimulation resulted in the activation of c-*fos* expression, and bilateral increased nuclear immunostaining was counted in dorsal horn of lumbar and sacral segments of spinal cord (laminae I, II), nucleus raphe dorsalis, substantia grisea centralis (ventralis), nucleus paraventricularis thalami, nucleus anterior thalami, nucleus ventralis thalami, nucleus medialis thalami, nucleus reuniens, nucleus rhomboideus, nucleus habenulae lateralis, nucleus paraventricularis hypothalami, nucleus arcuatus, nucleus lateralis hypothalami, nucleus preopticus lateralis, nucleus septi lateralis, nucleus amygdala, nucleus striae terminalis, nucleus tractus diagonalis, and cortex cerebri. The results demonstrated that peripheral noxious stimulation induced central c-*fos* protein expression in a pattern of labeling nociresponsive cells.

KEY WORDS pain; proto-oncogene proteins c-*fos*; brain; spinal cord; immunohistochemistry; physical stimulation

Proto-oncogene c-*fos* belongs to a family of cellular immediate early genes. Its expression within some neurons can be induced in response to membrane depolarization resulted from a variety of stimuli[1]. Its protein product, c-*fos* protein, has been viewed as the 3rd messager which acts as a transcriptional regulator that couples extracellular signals to alternations in gene expression[2], and it presents a valuable tool as a metabolic marker for neuronal activity at cellular level, with a higher resolution and a wider application than 2-deoxyglucose autoradiography[3]. Although the usefulness of c-*fos* protein expression evoked by noxious stimulation has been proposed, the work was limited at the spinal level[4] or done on animals under anesthesia[5], which itself was among factors that affect c-*fos* protein expression. In this study, we try to undertake a quantitative study of c-*fos* expression at various central levels following peripheral noxious thermal stimulation in awake rats in order to give a more precise and comprehensive understanding of the involvement of certain nuclei relevant to nociceptive regulation under physiological condition.

Received 1992-03-09 Accepted 1993-02-17

[1] Preliminary report at the 5th National Symposium on Neuropharmacology, 1992 Mar 16—18, Shanghai

MATERIALS AND METHODS

Chemicals Polyclonal antibody against c-*fos* protein and ABC kit were purchased from Oncogene Science, USA and Vector Laboratories, USA, respectively. Paraformaldehyde and diaminobenzidine were the products from Merck and Sigma, respectively.

Noxious stimulation Experiments were done on ♀ Sprague-Dawley rats (form Shanghai Institute of Planned Parenthood Research), weighing 201±s 14 g. Rats were housed with food and water *ad lib*. Because c-*fos* protein can be evoked by various sensory stimuli, all the rats were gentlely banded with Shurtape around the body leading to a comfortable position. To avoid nonspecific c-*fos* protein elevation, study was performed under quiet surrounding at a room temperature of 20℃. In awake rats receiving peripheral

noxious thermal stimulation, the caudal 1/3 of their tails were immersed in 50℃ water for 10 s per minute, and 10 s was generally the latency of tail flick reflex. The whole stimuli lasted 1 h and then the rats were killed. In control, awake rats were restrained without immersion of their tails into hot water.

Immunohistochemistry Rats were anesthetized with sodium pentobarbital (40 $mg \cdot kg^{-1}$, ip) and perfused via aorta with 200 ml of normal saline, followed by 400 ml of freshly prepared 4% paraformaldehyde in phosphate-buffered 0.1 $mol \cdot L^{-1}$ saline (PBS, pH = 7.4). The brain and spinal cord were then removed and postfixed in the same fixative at 4℃ overnight and then cryoprotected in PBS with 30% sucrose at 4℃ until the tissue sank. Serial sections (25 μm) of the brain and spinal cord were cut transversely on a freezing microtome and collected in PBS, and every 8th slice was processed for c-*fos* protein by avidin-biotin technique.

After three 10-min washes in PBS, the slices were incubated in PBS with 10% normal goat serum and 0.3% Triton X-100 for 30 min at 20℃. The sections were incubated with polyclonal antibody against c-*fos* protein diluted with 1% normal goat serum and 0.8% Triton X-100 in PBS (dilution 1:4000) for 4 h at 37℃, followed by overnight incubation at 4℃. After three 10-min washes in PBS, sections were processed by commercial ABC kit. Sections were incubated in a second antibody (dilution 1:100) for 30 min at 20℃. After three 10-min washes in PBS, sections were incubated in ABC reagent for 1 h at 20℃. Following three 10-min washes in PBS, the sections were finally incubated with 0.05% diaminobenzidine and 0.02% H_2O_2 in Tris-HCl buffer 0.1 $mol \cdot L^{-1}$ (pH = 7.2) for 10—30 min at 20℃. After the reaction was completed, the sections were air-dried and cover-slipped. Immunohistochemical staining was abolished by omission of primary antibody from the protocol.

Cell counts were performed under light microscope at 100 ×. Nuclear groups were identified[6,7]. Camera lucida drawings of the immunoreactive nuclei were undertaken on representative sections. Each dot represents one labeled neuronal nucleus.

Statistics Results were expressed as $\bar{x} \pm s$, and statistical significance was calculated by t test.

RESULTS

Spinal cord Little c-*fos* protein immunoreactivity was found in control rats. Noxious thermal stimulation induced bilateral c-*fos* protein markedly in laminae I—II, moderately in laminae III—IV, and slightly in lamina X of lumbar and sacral segments of spinal cord. In contrast, no significant augment of immunoreactive neuronal nuclei was revealed in cervical and thoracic spinal cord after the stimulation (Tab 1, Fig 1).

Tab 1. Number of c-*fos* protein immunoreactive neuronal nuclei in bilateral sides of the spinal cord in rats receiving noxious thermal stimulation. $\bar{x} \pm s$, $^*P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* **control.**

Laminar regions		Control ($n=3$)	Stimulated ($n=4$)
Cervical	I—II	0.0±0.0	0.0±0.0*
	III—IV	0.0±0.0	0.0±0.0*
	V—VI	0.0±0.0	0.0±0.0*
	VII—IX	0.0±0.0	0.3±0.5*
	X	0.3±0.6	0.0±0.0*
Thoracic	I—II	0.3±0.6	0.0±0.0*
	III—IV	0.0±0.0	0.0±0.0*
	V—VI	0.0±0.0	0.0±0.0*
	VII—IX	0.3±0.6	0.3±0.5*
	X	0.0±0.0	0.0±0.0*
Lumbar	I—II	0.3±0.6	18.0±3.0***
	III—IV	0.3±0.6	2.8±1.3**
	V—VI	0.0±0.0	1.3±1.0*
	VII—IX	0.0±0.0	0.3±0.5*
	X	0.0±0.0	1.5±1.9*
Sacral	I—II	0.0±0.0	22.0±5.0***
	III—IV	0.3±0.6	2.0±1.8*
	V—VI	0.0±0.0	2.0±2.3*
	VII—IX	0.0±0.0	0.0±0.0*
	X	0.0±0.0	1.5±1.9*

Brain stem Survey was undertaken of c-*fos* expression within some nuclear groups in brain stem, namely, DR, RMG, RP, RGC, SN, CG, SC, and IC. Cluster of c-*fos* immunoreactivity was found in CG (ventralis) and DR in rats receiving noxious thermal stimulation (Tab 2, Fig 1). Low level of c-*fos* expression was generally shown in brain stem except IC. But following noxious thermal

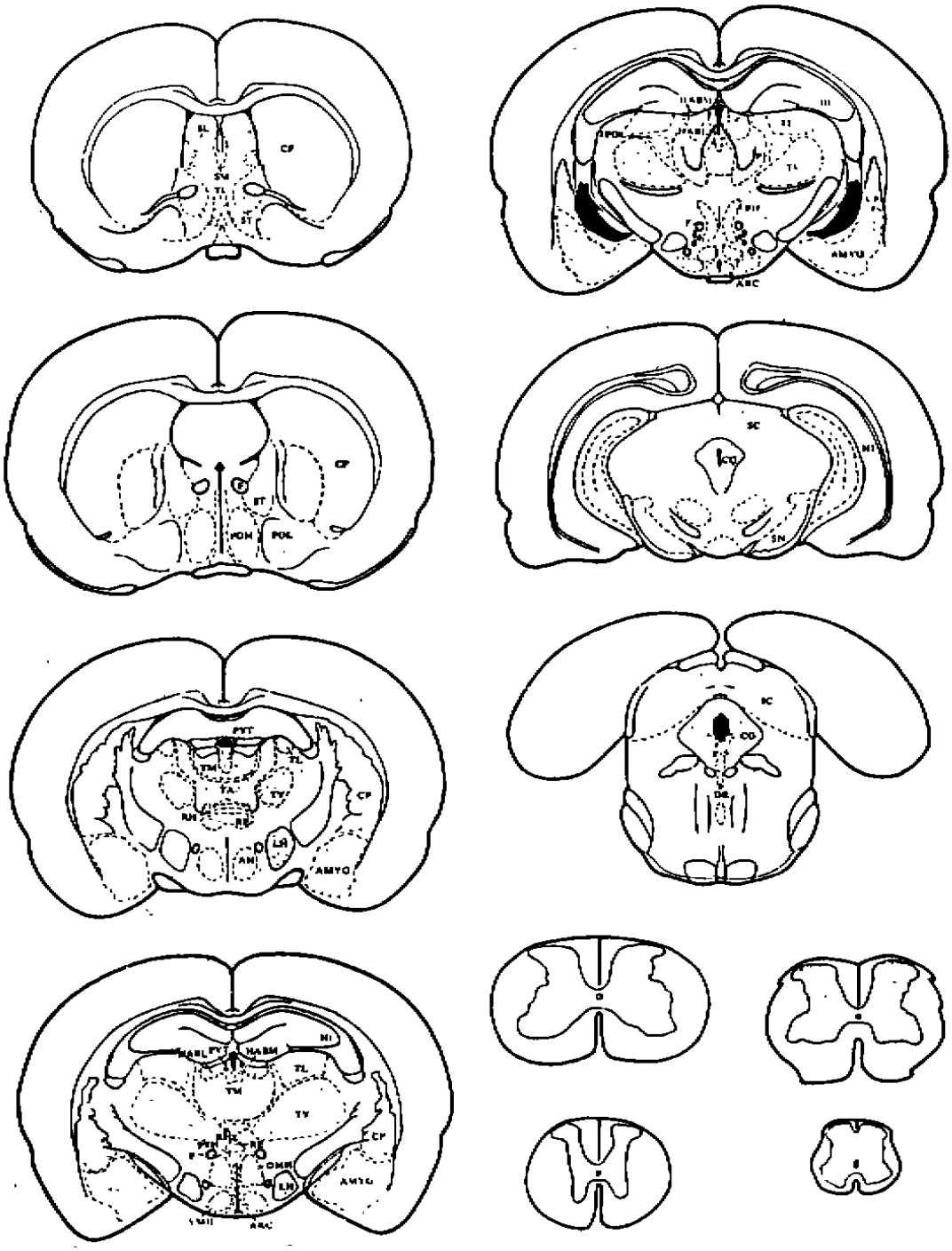

Fig 1. Camera lucida drawings of representative sections within neuraxis taken from rats receiving noxious thermal stimulation. Each dot represents one labeled neuron.

Tab 2. Number of bilateral c-*fos* protein immunoreactive neuronal nuclei in neuronal groups above the spinal cord of rats subjected to noxious thermal stimulation. $\bar{x}\pm s$, $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

Supraspinal regions	Control ($n=3$)	Stimulated ($n=4$)
Brain Stem		
CG (dorsalis)	4.0±6.0	9.4±4.8*
(lateralis)	4.7±4.7	8.5±6.5*
(ventralis)	4.7±3.1	83±31***
DR	3.3±6.1	47±24**
IC	29±12	32±13*
RGC	0.0±0.0	0.3±0.5*
RMG	0.0±0.0	1.5±1.9*
RP	0.0±0.0	0.3±0.5*
SC	6.0±5.9	5.3±6.1*
SN	0.0±0.0	0.0±0.0
Diencephalon		
AH	1.3±1.5	5.8±6.0*
ARC	5.3±4.2	48±22**
DMH	4.3±4.0	3.0±2.6*
HABL	1.0±1.7	25±8.0***
HABM	0.3±0.6	1.5±1.9*
LH	2.0±4.0	18.0±8.9**
MM	0.3±0.6	0.3±0.5*
PF	1.0±1.7	5.7±6.9*
PH	4.4±3.4	2.8±1.3*
PVH	34±11	70±18**
PVT	4.7±3.1	48±16***
RE	1.3±1.2	23.0±8.1***
RH	2.0±2.0	16.0±4.0***
TA	4.7±4.2	34±16**
TL	0.0±0.0	0.7±1.2*
TM	5.3±4.2	49±20**
TPO	3.6±4.6	6.0±4.8*
TV	2.0±2.0	28±11**
VMH	1.7±2.1	3.0±3.5*
Telencephalon		
AMYG	0.0±0.0	37±17***
CP	0.7±0.6	1.3±1.5*
HI	0.0±0.0	0.0±0.0*
POL	4.0±2.0	68±14***
POM	3.0±1.0	7.0±7.0*
SL	3.0±5.3	85±38**
SM	3.5±2.4	4.5±4.4*
ST	1.0±1.7	36±20**
TD	1.0±1.7	34±20**

stimulation, the expression was not further enhanced.

Diencephalon In control rats, sparse distribution of c-*fos* immunostaining was in diencephalon, other than PVH where immunoreactive neurons were accumulated. A dramatic increase in number of c-*fos* immunoreactive neuronal nuclei was recorded in LH, PVH, ARC, TA, TV, TM, PVT, RE, RH, and HABL after the rats were thermal-stimulated (Fig 1), but no significant increase was shown in AH, PH, DMH, VMH, MM, TL, TPO, HABM, and PF (Tab 2).

Telecephalon In telecephalon, CP, HI, AMYG, POL, POM, SL, SM, ST, and TD were examined. Few baseline immunoreactive neuronal nuclei in these regions were found, and obvious immunoreactivity was shown in AMYG, POL, SL, ST, TD, after the rats were exposed to noxious thermal stimulation (Tab 2, Fig 1). A marked increase of immunoreactivity in cortex cerebri was revealed, but we were unable to localize the specific areas because of the scattered and variable distribution (data not shown).

DISCUSSION

Scattered distribution of c-*fos* protein immunoreactivity located laminae I—II, III—VI, and X of lumbar spinal cord has been reported[4,5]. We here found an accumulation of specific c-*fos* protein immunoreactive neuronal nuclei in laminae I and II, the region where specific nociresponsive neurons are highly concentrated[8]. The discrepancy may relate to the different sacrifice time. In addition, stimulus parameters including site, type, duration and strength, together with anaesthesic depth, c-*fos* protein segment towards which antibody is raised, should also be taken into consideration.

In agreement with the finding that c-*fos* protein was expressed in PVH after stressful stimuli[10], we found c-*fos* protein immunore-

activity in PVH in restrained but not thermal-stimulated control rats, but none in freely moving normal rats ($n=2$, unpublished data). Since it was unlikely to diminish stress factor during noxious stimulation, we hypothesized that a further elevation of c-*fos* protein expression in rats subject to noxious thermal stimulation may be, at least partially, stress-mediated. The presence of immunoreactivity in IC, an important formation in auditory system, indicated that the experiments should be undertaken in an environment excluding auditory input.

There is a general agreement that endogenous opioid peptide system is constructed as an antinociceptive mechanism which can be activated by noxious stimulation[8-14]. Based upon our finding of stained neuronal groups in spinal dorsal horn, CG, PVT, HABL, LH, ARC, POL, SL, ST, AMYG, where opioid peptides are densely invested and greatly connected with each other[14], the possible involvement of c-*fos* in activation of endogenous opioid peptide system for regulatory action following noxious thermal stimulation is strongly proposed.

Although our experiment localized several neuronal populations with marked elevation of c-*fos* protein expression underlying noxious thermal stimulation, we failed to demonstrate significant c-*fos* protein enhancement in RMG, CP, which have been considered to be important sites for pain modulation. There is extensive evidence showing that c-*fos* protein is not expressed, no matter of the stimuli, in certain brain regions like SN, and the possibility of lacking the intrinsic biochemical system for c-*fos* protein expression is thus suggested[15]. Therefore, absence of c-*fos* increased should not simplely lead to the conclusion that the structure is not activated by the stimuli.

ACKNOWLEDGMENTS Thanks to Mr LU Shi-Duo for his kind assistance in camera lucida drawing preparation.

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ABBREVIATION AH = nucleus anterior hypothalami; AMYG = nucleus amygdaloidens; ARC = nucleus arcuatus; CG = substantia grisea centralis; CP = nucleus caudatus putamen; DMH = nucleus dosomedialis hypothalami; DR = nucleus raphe dorsalis; HABL = nucleus habenulae lateralis; HABM = nucleus habenulae medialis; HI = formatio hippocampi; IC = colliculus inferior; LH = nucleus lateralis hypothalami; MM = nucleus corporis mamillaris medialis; PF = nucleus parafascicularis; PH = nucleus posterior hypothalami; POL = nucleus preopticus lateralis; POM = nucleus preopticus medialis; PVH = nucleus paraventricularis hypothalami; PVT = nucleus paraventricularis thalami; RE = nucleus reuniens; RGC = nucleus reticularis gigantocellular; RH = nucleus rhomboideus; RMG = nucleus raphe magnus; RP = nucleus raphe pontis; SC = colliculus superior; SL = nucleus septi lateralis; SM = nucleus septi medialis; SN = substantia nigra; ST = nucleus striae terminalis; TA = nucleus anterior thalami; TD = nucleus tractus diagonalis; TL = nucleus lateralis thalami; TM = nucleus medialis thalami; TPO = nucleus posterior thalami; TV = nucleus ventralis thalami; VMH = nucleus ventromedialis hypothalami.

(6)

外周伤害性热刺激诱发清醒大鼠中枢 c-*fos* 蛋白表达

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摘要 实验采用免疫组织化学法观察外周伤害性热刺激所致的中枢 c-*fos* 蛋白的表达状况. 将清醒大鼠尾浸入50℃水浴中引起在脊髓腰、骶段背角 I、II 层，中缝背核，中央灰质腹侧部，丘脑，下丘脑和大脑皮层等脑区 c-*fos* 蛋白表达显著升高，表明伤害性热刺激在清醒大鼠上诱发的 c-*fos* 蛋白可显示痛相关神经元.

关键词 痛；原癌基因蛋白 c-*fos*；脑；脊髓；免疫组织化学法；物理刺激

BIBLID : ISSN 0253-9756 中国药理学报 *Acta Pharmacologica Sinica* 1993 Jul; **14** (4) : 311—314

Effects of 3,6-dimethamidodibenzopyriodonium citrate on slow inward calcium current in isolated guinea pig ventricular cells

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ABSTRACT The effects of 3,6-dimethamidodibenzopyriodonium citrate (I-65) on action potentials and slow inward calcium current (I_{si}) were examined on isolated guinea pig ventricular myocardial cells. I-65 (30—100 μmol·L^{-1}) depressed the action potential duration at 20% repolarization (APD_{20}) and, under voltage-clamp conditions, reduced the amplitude of I_{si} without changing the I-V relations. I-65 also showed use-dependent effects on I_{si}. These suggest that I-65 may block I_{si} by acting on the inactivated state of Ca channels.

KEY WORDS iodonium compounds; I-65; action potentials; myocardium; calcium channel blockers; membrane potentials

Received 1992-07-31 Accepted 1993-03-05

3,6-Dimethamidodibenzopyriodonium citrate (I-65) has been shown to cause a concentration-dependent depression of contraction and a plateau of action potential in guinea pig papillary muscles, suggesting that I-65 may block the calcium channels[1]. But the precise mode of action of this drug under voltage-clamp conditions has not been studied. This experiment examined the effects of I-65 on the slow inward calcium current (I_{si}) in isolated guinea pig ventricular myocardial cells.

MATERIALS AND METHODS

Experiments were performed on single cardiac ventricular cells isolated from guinea pig. The guinea