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Hyperalgesia induced by pituitary adenylate cyclase-activating polypeptide in the mouse spinal cord

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Abstract

The aim of the present study was to evaluate the distribution of pituitary adenylate cyclase-activating polypeptide (PACAP)-like immunoreactivity in the mouse spinal cord using an antibody against PACAP38 and to determine the behavioral profile, particularly with respect to hyperalgesia, of PACAP38 given intrathecally (i.t.) in the mouse. Immunoreactivity to PACAP38 was detected in numerous nerve fibers in the superficial layers of the dorsal horn of cervical, thoracic, lumbar and sacral segments and a few fibers extended into the deeper layers of the spinal cord. In addition, PACAP-like immunoreactivity were seen in the intermediolateral cell column of the thoracic and sacral segments. In behavioral studies, PACAP38 (0.05–0.5 μg) produced a dose-dependent decrease of the tail-flick latency when given i.t. in the mouse. At higher doses (1–10 μg), PACAP38 given i.t. elicited biting and scratching behaviors lasting 10–20 min after the injection. PACAP at high doses (1–10 μg) also produced licking at tail, paw and penis and intense grooming behaviors immediately after the i.t. injection. Similar to substance P, these behaviors produced by PACAP can be considered as pain-like syndrome. These findings suggest that PACAP may be a sensory neurotransmitter involved in nociceptive signalling in the mouse spinal cord.

Keywords: PACAP (pituitary adenylate cyclase-activating polypeptide); Dorsal horn; Intermediolateral cell column; Immunohistochemistry; Hyperalgesia; Tail-flick; Spinal cord

1. Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a newly discovered neuropeptide belonging to the secretin-, glucagon-, vasoactive intestinal polypeptide (VIP)-family of peptides. PACAP was originally isolated from the ovine hypothalamus on the basis of its ability to stimulate adenylate cyclase in rat anterior pituitary cell cultures. It has two amidated forms: PACAP38, a 38-amino-acid polypeptide (Miyata et al., 1989), and PACAP27, a truncated form of PACAP38 containing 27 residues (Miyata et al., 1990). PACAP immunoreactivity has been reported to be widely distributed in mammalian tissues, including the central nervous system (Arimura, 1992). Recently, a PACAP receptor has been purified from the bovine brain (Ohtaki et al., 1993) and its cDNA has been cloned from mouse (Inagaki et al., 1994), rat (Hashimoto et al., 1993; Hosoya et al., 1993) and human (Ogi et al., 1993). These findings provide evidence that PACAP may play an important physiological role in the central nervous system.

More recently, an abundance of PACAP-immunoreactive neural elements has been detected in the rat spinal cord and medulla (Moller et al., 1993; Dun et al., 1996b). The presence of PACAP immunoreactivity in a number of areas in the spinal cord and medulla, including the dorsal horn, lateral horn, vagal afferents, NTS and lateral reticular nuclei, suggest that PACAP may participate in a variety of sensory and autonomic functions in the rat. It has also been hypothesized that PACAP may be a transmitter in sensory C-fibers and a co-mediator of the inflammatory response and/or pain transmission that can be evoked by C-fiber stimulation (Moller et al., 1993; Zhang et al., 1993).

In the present commnuication, we describe the distribution of PACAP-like immunoreactivity in the mouse spinal cord using a PACAP38 antiserum and hyperalgesic behaviors after intrathecal (i.t.) administration of PACAP38 in the mouse. Our results indicate that PACAP may play a role in sensory nociceptive transmission.

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2. Materials and methods

2.1. Animals

Male ICR mice weighing 25–30 g (Sasco, Omaha, NE, USA) were used. Animals were housed five per cage in a room maintained at 22 ± 0.5°C with an alternating 12-h light-dark cycle. Food and water were available ad libitum. Animals were used only once. This study was conducted in concordance with the guidelines of the Ethics Committee of the International Association for the Study of Pain (Zimmermann, 1983).

2.2. I.t. administration

I.t. administration was performed following the method described by Hylden and Wilcox (1980) using a 10-μl Hamilton syringe with a 30-gauge needle. The injection volume was 5 μl.

2.3. Observation of aversive behaviors

A columned acrylic resin cage (diameter 21 cm, height 21 cm) was used for the observation of aversive behaviors. The mice were placed individually in the observation cage immediately after the i.t. injection. The number of scratching and biting behaviors was counted and the duration of tail-licking, paw-licking, penis-licking and grooming was measured for 20 min.

2.4. Nociceptive test

Tail-flick response was used as nociceptive test (D'Amour and Smith, 1941). For measurement of the base-line latency of the tail-flick response, mice were gently held by hand with their tail positioned in the apparatus (model TF6; EMDIE Instrument, Maidens, VA, USA) for mild radiant heat stimulation on the dorsal surface of the tail. The tail-flick latency was measured before and 20 min after the i.t. injection of PACAP38 or saline. A cut-off latency of 15 s was used to avoid tissue damage to the tail.

2.5. Immunohistochemical approach

Mice were anesthetized with a combination of ketamine hydrochloride (70 mg/kg) and xylazine (15 mg/kg) injected intraperitoneally. Animals were perfused intracardially with 0.1 M phosphate-buffered saline (PBS) followed by freshly prepared 4% paraformaldehyde in PBS (pH 7.4). Spinal cords were removed, postfixed in 4% paraformaldehyde for 2 h and left in 30% sucrose-PBS overnight. Sections of 40 μm thickness prepared by a cryostat were processed for PACAP-like immunoreactivity using the avidin biotin complex method, as described previously (Dun et al., 1996a,b).

Sections were first treated with 3% H_2O_2 to quench endogenous peroxidase, washed and blocked with 10% normal goat serum, and incubated in the primary antibody to PACAP-38 (1 : 1500–1 : 2000 dilution with 0.4% Triton X-100 in PBS; a rabbit polyclonal from Peninsula Laboratories, Belmont, CA, USA) for 48 h at 4°C with gentle agitation. After thorough rinsing, sections were incubated with biotinylated anti-rabbit immunoglobulin (1 : 150, Vector Laboratories) for 2 h. Sections were rinsed with PBS and incubated in avidin-peroxidase complex for 1 h (1 : 100, Vector Laboratories). After several rinses in Tris-buffered saline, sections were developed in diaminobenzidine-H_2O_2 solution and washed for at least 2 h with Tris-buffered saline. Section were mounted on slides with 0.25% gel alcohol, air-dried, dehydrated with absolute alcohol followed by xylene and cover-slipped with Permount.

2.6. Drug and statistical data analyses

PACAP38 was purchased from Peninsula Laboratories and dissolved in saline. The data are expressed as the mean and S.E.M. Statistical analysis of difference between groups was assessed with Student's *t*-test (comparisons of two groups for the row data), Dunnett's test (comparison with a control group) or Fisher's probability test (comparisons of two groups for the positive response rate).

3. Results

3.1. PACAP38 at lower dosages given i.t.-induced tail-flick hyperalgesia

As shown in Table 1, PACAP38 at low doses (0.05–0.5 μg), which did not caused obvious aversive behaviors, produced a decrease in tail-flick latency. The nociceptive threshold of the tail-flick response was dose-dependently decreased by i.t.-administered PACAP38. At doses of 0.1–0.5 μg, PACAP38 significantly decreased the tail-flick latency as compared to that of the i.t. saline-injected control (Table 1).

Table 1
Tail-flick hyperalgesia induced by PACAP38 injected i.t. in mice

Min after injection	Tail-flick latencies (s)	
	0 (pre)	20
Saline ($n = 10$)	7.2 ± 0.4	7.1 ± 0.3
PACAP, 0.05 μg ($n = 10$)	7.1 ± 0.4	6.7 ± 0.2
PACAP, 0.1 μg ($n = 10$)	7.5 ± 0.3	6.0 ± 0.3 [a]
PACAP, 0.5 μg ($n = 20$)	7.4 ± 0.2	5.7 ± 0.2 [b]

Groups of mice were treated i.t. with PACAP38 (0.05, 0.1 or 0.5 μg) or saline. The tail-flick latency was then measured just before (0 min) and 20 min after the i.t. injection of PACAP38 or saline.
[a] $P < 0.05$, [b] $P < 0.01$, compared with 0 min.

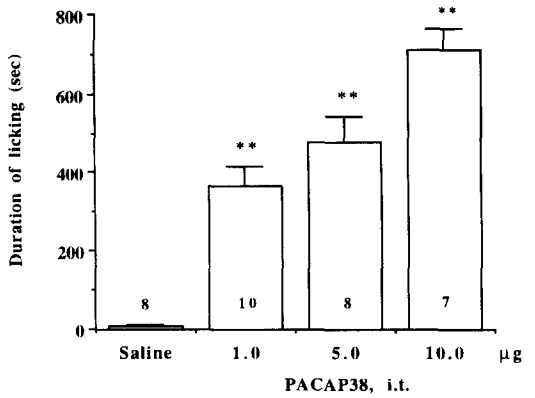

Fig. 1. The licking responses to various doses of PACAP38 injected i.t. in mice. Groups of mice were treated i.t. with PACAP38 (1, 5 or 10 μg) or saline. Immediately after the i.t. injection, the total duration of licking (tail, paw and penis) was measured for 20 min. The vertical line represents the S.E.M.; $n = 7-10$ mice for each group. ** $P < 0.01$, compared with saline pretreatment.

3.2. Aversive behaviors induced by i.t.-administered higher dosages of PACAP38

PACAP38 at high doses (1–10 μg) given i.t. produced caudally directed scratching and biting behaviors in a dose-dependent manner (Table 2). These PACAP38-induced scratching and biting behaviors started about 1–2 min after i.t. injection and lasted approximately 10–20 min. Control mice injected i.t. with saline did not produce these behaviors. The positive response rate of PACAP38-induced scratching/biting behavior was dose-dependently increased. Other behaviors, including licking at tail, paw and penis, also occurred immediately after the injection and lasted over 60 min. The total duration of licking was dose-dependently increased (Fig. 1). Severe glooming behabiors were also observed after i.t. injection of PACAP38 with a time course similar to that of licking behaviors.

3.3. Distribution of PACAP38 in the spinal cord

The distribution of PACAP-like immunoreactivity was examined in 4 mouse spinal cords and found to be similar. Numerous PACAP-like immunoreactive fibers were detected in superficial layers of the dorsal horn of cervical, thoracic, lumbar and sacral segments. In addition to the superficial layers of the dorsal horn, PACAP-like immunoreactive fibers were seen projecting into the intermediolateral cell column of the thoracic (Fig. 2) and sacral segments (data not shown). PACAP-like immunoreactive cell bodies were not observed in the spinal cord.

4. Discussion

Several recent studies indicate that PACAP may serve as a sensory transmitter in the dorsal horn. For example, a high density of specific receptors for PACAP has been observed in membranes from rat cervico-dorsal spinal cord using [I^{125}]PACAP (Cauvin et al., 1991). Immunoreactivity to PACAP was detected in numerous nerve fibers in layers I and II of the dorsal horns of all segments of the rat spinal cord as well as in a population of the dorsal root ganglion cells (Dun et al., 1996b). Similarly, it has been reported using in situ hybridization with a ^{35}S-labeled oligonucleotide probe complementary to PACAP mRNA that PACAP is synthesized in a subpopulation of rat sensory ganglion neurons (Mulder et al., 1994). The presence of PACAP-like immunoreactivity in the mouse spinal cord has yet to be documented.

In the present study, we first demonstrated the existence and distribution of PACAP-like immunoreactive fibers in the mouse spinal cord. Similar to the rat (Dun et al., 1996b) and human (Dun et al., 1996a) spinal cord, dense networks of PACAP-like immunoreactive fibers were present in the superficial layers of the dorsal horn of all segments. A few PACAP-like immunoreactive fibers were seen extending from the superficial layers of the dorsal horn to the deeper laminae. Furthermore, PACAP-like immunoreactive fibers were detected in the intermediolateral cell column of the mouse thoracic and sacral segments.

Results from several recent studies suggest that

Table 2
Hyperalgesic responses induced by PACAP38 injected i.t. in mice

Aversive behavior	Positive animals/animals used			
	Saline 5 μl	PACAP38 1 μg	PACAP38 5 μg	PACAP38 10 μg
Scratching (> 10 times)	0/8	2/8	6/8 [a]	8/8 [a]
Self- or cage-biting (> 5 times)	0/8	4/8	6/8 [a]	8/8 [a]
Paw-licking (> 3 min)	0/8	5/8 [a]	7/8 [a]	8/8 [a]
Tail-licking (> 3 min)	0/8	6/8 [a]	8/8 [a]	8/8 [a]
Penis-licking (> 1 min)	0/8	8/8 [a]	8/8 [a]	8/8 [a]
Severe grooming (continuously > 1 min)	0/8	6/8 [a]	8/8 [a]	8/8 [a]

Groups of mice were treated i.t. with PACAP38 (1, 5 or 10 μg) or saline. Immediately after the i.t. injection, the number of scratching and biting behaviors was counted and the duration of tail-licking, paw-licking, penis-licking and grooming was measured for 20 min. The definition of 'positive animals' refers to animals that exhibited certain number of positive responses (> 5 or 10 times) or showed total duration time of responses (> 1 or 3 min).
[a] $P < 0.05$, compared with saline.

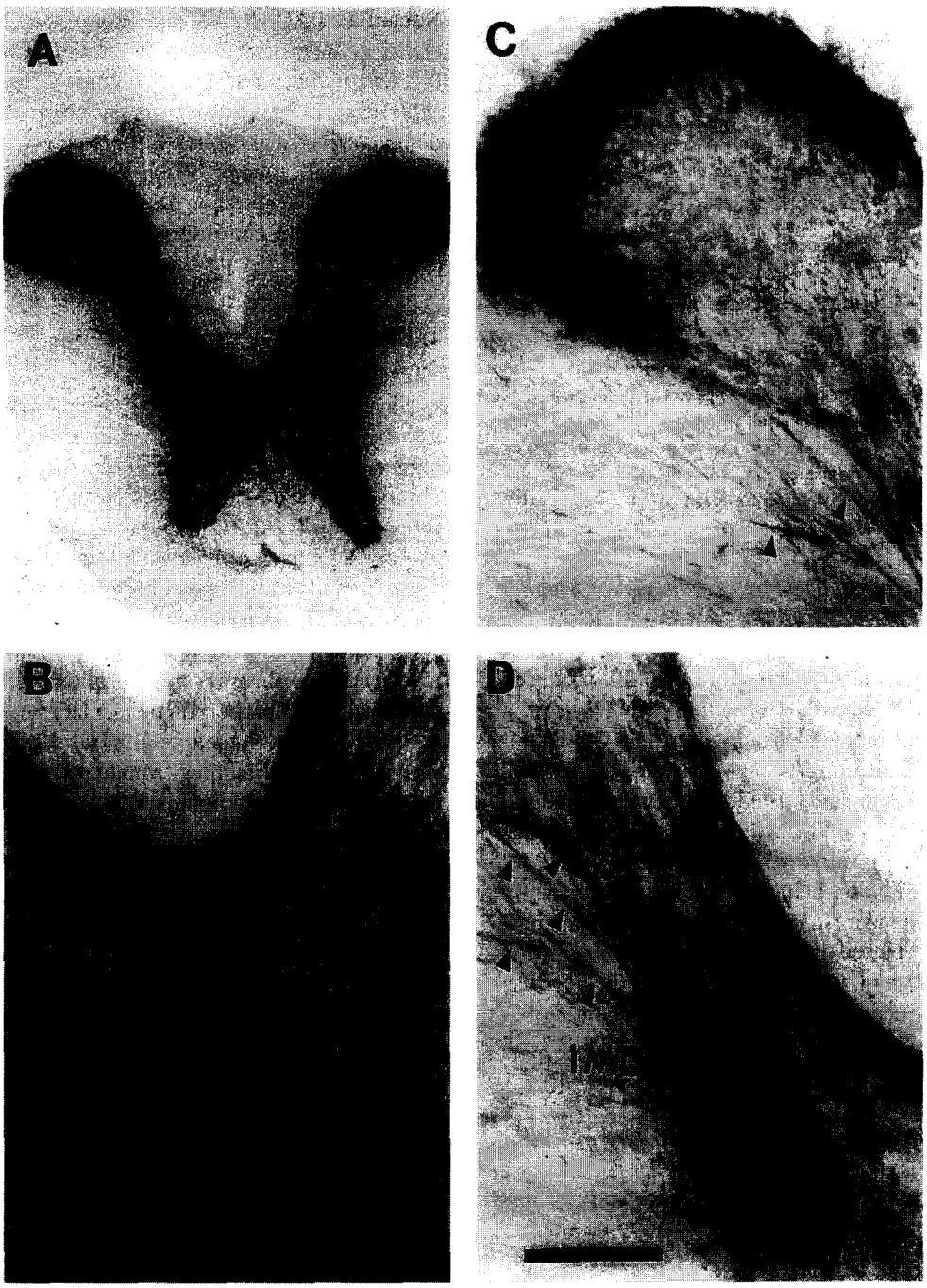

Fig. 2. Photomicrographs of a section through the mouse thoracic spinal cord labeled with PACAP-like immunoreactivity. (A) A low magnification showing 3 areas containing PACAP-like immunoreactive fibers, superficial layers of the dorsal horn, intermediolateral cell column and around the central canal. (B) Moderately labeled PACAP-like immunoreactive fibers (arrowheads) were noted above and around the central canal (CC). (C) A higher magnification of the dorsal horn from A showing numerous intensely labeled PACAP-like immunoreactive fibers in the dorsal horn and some PACAP-like immunoreactive fibers (arrowheads) projecting into the intermediolateral cell column (IML). (D) A higher magnification showing PACAP-like immunoreactive fibers projecting into the IML; PACAP-like immunoreactive somata are not seen in the IML. Calibration bar: A, 700 μm; B,C, 100 μm; D, 50 μm.

PACAP-like immunoreactive fibers in the dorsal horn may be sensory in nature. For example, PACAP-like immunoreactivity has been co-localized to some of the substance P- and calcitonin gene-related peptide (CGRP)-containing fibers in the rat dorsal horn and to a population of spinal and trigeminal ganglion cells (Moller et al., 1993). In addition, PACAP-like immunoreactive neurons were found to be sensitive to capsaicin (Moller et al., 1993; Zhang et al., 1993). As substance P has been detected by immunohistochemical techniques in primary afferent sensory ter-

minals to the spinal dorsal horn neurons (Barber et al., 1979), it is reasonable to suggest that PACAP, similar to substance P, may be one of the putative sensory neurotransmitters.

A multiplicity of endogenous systems may modulate perception and reaction to painful stimuli. For instance, i.t. injection of substance P elicits a caudally directed biting and scratching behavioral syndromes in mice (Hylden and Wilcox, 1981, 1983), indicating that the peptide may be a modulator of nociceptive signals in sensory neurons (Salt and Hill, 1983). In this study, we found that low doses of PACAP38 when given i.t. produced hyperalgesic responses against the thermal noxious stimulation in mice. At higher doses, i.t.-administered PACAP38 induced aversive behaviors, such as biting, scratching, licking and severe grooming. The hyperalgesic response induced by i.t. administration of PACAP38 was longer-lasting than that of substance P (Hylden and Wilcox, 1983). These observations provide pharmacological evidence that PACAP may play a role as a sensory neurotransmitter in nociceptive transmission in the mouse spinal cord. It is noteworthy that in the rats PACAP may suppress nociception (Zhang et al., 1993; Yamamoto and Tatsuno, 1995). The reason for these opposite observations is not clear at this time. Species difference notwithstanding, different experimental paradigms may produce varying results with respect to pain. It is also possible that the discrepancy may be due to different dosages.

The site and mechanism of hyperalgesic action of PACAP in the mouse spinal cord remain to be studied. PACAP appears to interact with two types of seven-transmembrane-domain receptors. One receptor, which is positively coupled to adenylate cyclase, also recognizes VIP, the closest family member to PACAP. A second receptor, which recognizes only PACAP, is linked to phospholipase C and adenylate cyclase (Hashimoto et al., 1993). The type of receptors interacting with PACAP to produce hyperalgesia in the mouse remains to be pharmacologically defined. The question whether PACAP interacts directly with postsynaptic receptors located on dorsal horn neurons to produce hyperalgesia or indirectly by releasing other biologically active substances in the dorsal horn remains to be addressed.

In conclusion, PACAP, which was detected in nerve fibers in the superficial layers and deeper layers of the mouse spinal cord, given i.t. at lower doses produced tail-flick hyperalgesia and elicited, at higher doses, biting and scratching behaviors. These findings suggest the possibility that PACAP plays an important role as a neurotransmitter in pain transmission.

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