Oxytocin modulates the effects of galanin in carrageenan-induced hyperalgesia in rats

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In the present study we have investigated the effects of galanin and/or oxytocin on carrageenan-induced hyperalgesia, the relationship between oxytocin and galanin-containing nerve fibers in the spinal cord and the influence of galanin on oxytocin secretion. Galanin (1 μg) given intrathecally (i.t.) decreased significantly the mechanical nociceptive threshold of the carrageenan-treated hindpaw, with no significant effect on thermal nociception. The decrease in the mechanonociceptive threshold exerted by galanin was modulated by oxytocin (1 μg, i.t.) administered simultaneously. There was a close relationship between galanin- and oxytocin-immunoreactive fibers in the dorsal horn of the thoracic spinal cord, although there was no evidence for colocalization. Galanin 0.1 and 1 μg given intracerebroventricularly or intraperitoneally significantly decreased the oxytocin level in plasma 60 min after injection. Taken together, these data indicate that galanin may contribute to mechanical hyperalgesia by inhibiting the release of oxytocin from nerve terminals in the spinal cord and that oxytocin may be a potential analgesic agent.

INTRODUCTION

Galanin is a 29-amino acid peptide, originally isolated from porcine small intestine. Galanin-like immunoreactivity has been found in the superficial layers of the spinal dorsal horn, suggesting that galanin is involved in the transmission and/or modulation of nociceptive transmission at the spinal cord level. Galanin has been reported to facilitate as well as inhibit nociceptive responses to mechanical and/or thermal nociceptive stimulation. Oxytocinergic neurons project from the hypothalamic paraventricular nucleus to many regions in the brain, including areas involved in pain transmission such as the periaqueductal gray, the raphe nuclei and the superficial layers of the spinal dorsal horn. In recent articles we have shown that oxytocin administered intraperitoneally (i.p.) to rats as well as intracisternally (i.c.) to mice has an anti-nociceptive effect. A minor but significant part of this anti-nociceptive effect was independent of temperature changes and naloxone-insensitive (Lundberg et al., in preparation). Furthermore, the injection of oxytocin into the third ventricle of a patient with intractable opiate resistant cancer pain was reported to result in strong analgesia lasting 75 min. This is noteworthy because patients with neurogenic pain have been shown to be less sensitive to opiates, and galanin is suggested to play a major role in neurogenic nociception.

In the present study we wanted to investigate whether the galanin- and oxytocin-induced effects on nociception are interrelated. Three approaches were used: (1) the effect of galanin and/or oxytocin on mechanical and/or thermal carrageenan-induced hyperalgesia was studied; (2) the relationship between galanin- and oxytocin-immunoreactive fibers in the thoracic spinal cord was investigated using double-labelling immunohistochemistry; (3) the effect of galanin on oxytocin secretion was studied.

MATERIALS AND METHODS

Nociceptive tests

All experiments were carried out on freely moving male Sprague–Dawley rats (220–290 g; ALAB, Stockholm, Sweden). The rats were housed in a cage and maintained under a 12 h light/dark cycle with a room temperature of 24 ± 1°C and the rats had free access to water and food.
access to food and water. Porcine galanin, and carrageein were obtained from LabKemi, Stockholm, Sweden, and oxytocin from Ferring, Lund Sweden.

Galanin and oxytocin were dissolved in 0.9% saline. Either galanin and/or oxytocin was injected i.t. as a single dose in 0.01 ml saline through a lumbar puncture. To produce acute inflammation, carrageen (1 mg/0.1 ml saline) was injected subcutaneously into the plantar region of the right hindpaw 3 h before injection of galanin and/or oxytocin.

The effects of galanin and/or oxytocin on mechanical and thermal nociception were estimated in rats using the paw-pressure and paw heat test, respectively. The nocicceptive responses were determined with a heat analgesimeter. The heat was focused on the dorsal surface of the hindpaw and the latency of withdrawal of the paw was determined. The mechanonociceptive responses were determined using a pressure analgesimeter. The mechanical stimulation was applied to the hindpaw by a wedge-shaped pusher at a loading rate of 48 g/s and the pressure required for the struggle response was measured. Each rat was investigated with either thermal or mechanical nociceptive tests.

The statistical significance of the effects of galanin and oxytocin was assessed by analysis of variance (ANOVA), Kruskal–Wallis test of multiple comparisons or Student’s t-test. P < 0.05 was considered significant.

**Immunohistochemistry**

Sprague–Dawley rats (b.wt. 150–200 g) were anesthetized with sodium pentobarbital (Mebumal; 40 mg/kg, i.p.) and perfused via the ascending aorta with CaCl2-free Tyrode’s solution (37°C) followed by an ice-cold mixture of formalin-penicilic acid (4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer, pH 6.9). The thoracic spinal cord was dissected out and immediately fixed by immersion in the same fixative for 90 min. The tissue was rinsed for at least 24 h in a 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose, 0.02% bacitracin (Sigma Chemical Co., St. Louis, MO, USA) and 0.01% sodium ascorbate (Merck, Darmstadt, FRG). Sections were cut at 14 μm thickness in a cryostat (Dittes, Heidelberg, FRG) and processed for indirect immunofluorescence histochemistry. Direct comparison in the same section, a double-labelling procedure was employed. Briefly, the sections were incubated with a mixture of mouse monoclonal antibodies to oxytocin-neurophysin (final dilution 1:100; antiserum PS36) and rabbit polyclonal antiserum to rat galanin (final dilution 1:400; RAS7153, Peninsula Labs., Belmont, CA, USA) for 24 h at 4°C, rinsed in phosphate-buffered saline (PBS), and incubated for 30 min at 37°C with fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse (final dilution 1:20; Amersham Ltd., Amersham, UK) and lissamine-rhodamine (final dilution 1:10; antiserum PS36) and rabbit polyclonal antiserum to rat vasotocin (final dilution 1:400; RAST155, Peninsula Labs., Belmont, CA, USA) for 24 h at 4°C. The sections were examined in a Nikon Microphot-FX epifluorescence microscope equipped with filter combinations for FITC (450–490 nm excitation filter, 520–560 nm barrier filter) and rhodamine (546/10 nm excitation filter and 610 nm barrier filter) induced fluorescence. By switching between the filter combinations the two immunoreactivities could be compared against each other. Tri-X (Kodak, Rochester, NY, USA) black-and-white film was used for photography.

**Intracerebroventricular injection**

Male Sprague–Dawley rats (300 g), stored in air-conditioned and temperature-controlled rooms (22°C) illuminated between 07.00 and 21.00 h and fed ad libitum, were used. The animals were anaesthetized i.p. with chloral hydrate (0.5 g/kg, Apoteksbolaget, Göteborg, Sweden). For i.c.v. injection the animal was fixed in a stereotactic instrument, a hole was drilled in the right parietal bone and the needle of the syringe was inserted in the right lateral ventricle 0.90 mm posterior and 1.30 mm lateral to the Bregma. I.c.v. injections were performed using a 10 μl Hamilton syringe. Five μl peptide (or saline in control groups) was given as an injection during 10 min. After the injection, animals were released and placed on a piece of cloth. After the experiment, the brain was removed, frozen, and sectioned on a microtome, and the site of injection was confirmed.

I.c.v. as well as i.p injections were performed with 0.1 μg or 1 μg galanin dissolved in 0.9% NaCl or with saline alone. The rats were decapitated 60 min after i.c.v. or i.p. injection. Blood samples were collected into tubes containing heparin (10 IU/ml) and Trasylol (500 IU/ml). The blood samples were centrifuged and plasma was separated and stored frozen (~20°C) until analysis.

**Radioimmunoassay**

**Oxytocin.** The concentration of oxytocin in plasma and in extracts was measured with a specific radioimmunoassay as previously described by Stock and Uvnäs-Moberg, using the antibody KA19 (Milab, Malmö, Sweden). Cross-reactivity with argininevasopressin was 0.01%, with lysinevasopressin <0.01% and with argininevasotocin 0.1%. The limit of detection was 2 fmol/ml and the intra- and inter-assay coefficients of variation were 11.2 and 13%, respectively. Before plasma levels of oxytocin were determined, the hormone was separated from the plasma proteins with SEP-PAK C18 cartridges (Water Assoc. Inc., Milford, MA, USA).

The hormone levels are presented as mean values ± SD. Differences between groups were evaluated with the Kruskal–Wallis one-way analysis of variance and subsequent comparisons between two groups were made with the Mann–Whitney U-test, provided the analysis of variance first showed a significant overall effect. P < 0.05 was considered significant.

**RESULTS**

Three hours after carrageenan, the nociceptive threshold of the carrageenan-treated hindpaw to pressure stimulation was decreased to 59% (n = 10) of the pre-carrageenan level, with no significant change in the threshold of the hindpaw contralateral to the treatment. The nociceptive latency of the carrageenan-

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<th>TABLE I</th>
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<td>Effects of intrathecal injection of 1 μg galanin on nociceptive responses of rats in paw pressure and paw heat test</td>
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<td>Values represent mean and S.E.M. Galanin significantly decreased the nociceptive threshold for pressure stimulation at 10 min (P &lt; 0.03) and this effect was reversed by oxytocin.</td>
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<th>Paw pressure threshold (g)</th>
<th>Paw withdrawal latency (s)</th>
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<tr>
<td>Before</td>
<td>10'</td>
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<tr>
<td>Galanin</td>
<td>246.8 ± 26.3</td>
</tr>
<tr>
<td>Saline</td>
<td>257.0 ± 28.4</td>
</tr>
<tr>
<td>Galanin + oxytocin</td>
<td>256.0 ± 34.0</td>
</tr>
<tr>
<td>Galanin + saline</td>
<td>219.5 ± 64.2</td>
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Fig. 1. a–d: immunofluorescence photomicrographs of sections of the thoracic spinal cord after double-labelling with mouse monoclonal antibodies to oxytocin (OXY)-neurophysin (a,c) and rabbit antiserum to galanin (GAL) (b,d). OXY-immunoreactive (IR) fibers are mainly distributed in laminae I and II, but also scattered in lamina III (a) as well as in laminae in and X, closely surrounding ependymal cells in the central canal (c). A dense plexus of GAL-IR fibers is distributed in laminae I and II (b), and some fibers extend through laminae in and X (d). Comparison of a,c with b,d reveals no colocalization of OXY- and GAL-IR fibers. However, there is a close relation of OXY- and GAL-IR fibers in laminae I, II, V and X (see open arrow in c and d. Asterisks indicate central canal. Bar = 100 μm. d, dorsal; v, ventral.
treated hindpaw to radiant heat was shortened to 51% (n = 10) of the precarrageenan level 3 h after carrageenan treatment, with no significant change in the latency of the contralateral hindpaw. An intrathecal injection of 0.9% saline did not alter the thermo- or mechanonociceptive thresholds of the carrageenan-treated paw. The nociceptive thresholds of the contralateral hindpaws were not significantly altered by saline. Motor dysfunction was not observed in any rats after intrathecal injection of saline.

The effects of i.t. injection of galanin 1 μg per rat were examined on the nociceptive threshold to hindpaw pressure stimulation (Table I). The threshold was significantly decreased by galanin 10 min after administration, but the nociceptive response of the hindpaw to radiant heat was not altered by an i.t. injection of galanin. The effects of galanin were modulated (P < 0.06 towards reversal) when oxytocin was administered simultaneously (Table I).

**Immunohistochemistry**

In single-labelled sections of the rat thoracic spinal cord, oxytocin-immunoreactive (-IR) fibers were seen mainly in laminae I and II, but also scattered in laminae III of the dorsal horn (Fig. 1a). Several oxytocin-IR fibers could be followed in laminae V and X. In lamina X, oxytocin-IR fibers were surrounding ependymal cells of the central canal (Fig. 1c). A very dense fiber plexus of galanin-IR fibers was seen in laminae I and II of the dorsal horn (Fig. 1b), and galanin-IR fiber varicosities could also be demonstrated in laminae V and X (Fig. 1d). In double-labelled sections, there was no evidence for colocalization of oxytocin- and galanin-like immunoreactivity in fibers of the dorsal horn or in laminae V or X (cf. Fig. 1a,c with 1b,d). However, a close relation between both immunoreactivities could be observed in laminae I, II, in and X (cf. Fig. 1a,c with 1b,d).

**Effects of galanin on oxytocin secretion**

Galanin 1 μg/kg administered i.c.v. as well as i.p. gave rise to a significant decrease in oxytocin levels after 60 min as is shown in Fig. 2a,b. Also 0.1 μg/kg of galanin also caused a significant decrease of oxytocin levels after i.p. injection.

**DISCUSSION**

The results of the present study show that galanin may potentiate carrageenan-induced hyperalgesia to mechanical noxious stimuli. Our results suggest that galanin present in the dorsal horn is involved in the transmission of mechanical nociceptive information.
tion of neurotransmitter release by monitoring plasma levels should be interpreted with care. Still, it is possible that galanin in the present experiments caused hyperalgesia by inhibiting the release of oxytocin in the spinal cord. It should be emphasised that the discrepancies between these and previous results on galanin may very well be related to differences in technique and/or to the different models of nociception used.

The roles of galanin and oxytocin in nociception thus need clarification and studies with this aim are presently in progress.

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