Intrathecal CGRP$_{8-37}$ results in a bilateral increase in hindpaw withdrawal latency in rats with a unilateral thermal injury

O. Lofgren*, L-C. Yu*, E. Theodorsson§, P. Hansson~, T. Lundeberg*+

*Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden
†Department of Plastic and Reconstructive Surgery, Karolinska Hospital, Stockholm, Sweden
‡Department of Rehabilitation Medicine, Karolinska Hospital, Stockholm, Sweden
§Department of Clinical Chemistry, Karolinska Hospital, Stockholm, Sweden
~Neurogenic Pain Unit, Dept of Rehabilitation Medicine, Karolinska Hospital, Stockholm, Sweden

Summary The present study was performed to explore the effects of intrathecal administration of calcitonin gene-related peptide$_{8-37}$ (CGRP$_{8-37}$) on the hindpaw withdrawal latency (HWL) to pressure in rats with one thermally injured hindpaw. Furthermore, the interaction of CGRP$_{8-37}$ and naloxone was studied. Thermal injury was performed by dipping the left paw into 60°C for 20 s. This induced a significant increase in the volume of the left hindpaw (P<0.001) and significant bilateral decreases of the latency of hindpaw withdrawal response to mechanical stimulation (Left: P<0.001; right: P<0.05). Intrathecal administration of 10, 20 and 40 nmol of CGRPs$_{8-37}$, but not of 1 or 5 nmol, induced a significant bilateral increase in HWLs (P<0.001). The effect of CGRPs$_{8-37}$ was partly reversed by intrathecal injection of naloxone at a dose of 32 and 64 µg respectively.

Using radioimmunoassay, we found a significant bilateral increase in the concentration of CGRP-like immunoreactivity in the perfusate of both hindpaws 24 h after unilateral thermal injury (left: P<0.001; right: P<0.05). There was also an increase in the amount of CGRP-like immunoreactivity in the cerebrospinal fluid (P<0.001), but not in plasma.

The results indicate that CGRP plays a role in the transmission of nociceptive information in the spinal cord of thermally injured rats. Furthermore, our findings suggest that opioids can modulate CGRP-related effects in the spinal cord.

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is known to be present in widespread areas of the peripheral and central nervous system. It has been reported that CGRP may be involved in the transmission of presumed nociceptive information in the spinal cord. Biella et al. reported that CGRP has a facilitatory role on the excitation of rat spinal dorsal horn neurones induced by substance P and peripheral noxious stimuli. Furthermore, Kawamura et al., Kuraishi et al. and Satoh et al. reported that intrathecal injection of antiserum against CGRP induced prolonged reflex latencies in rats subjected to cold stress or inflammation. On the other hand, Cridland and Henry found that intrathecal injection of CGRP in the rat attenuated the excitatory effect on the tail flick reflex induced by either substance P or noxious cutaneous stimulation.

The role of the nervous system in inflammation is complex. It has been reported that experimentally induced acute or chronic inflammation results in an enhanced release of CGRP-like immunoreactivity from dorsal horn slices in vitro. Recently, it was found that unilateral intra-articular injection of pro-inflammatory substances induced a bilateral increase in substance P-, CGRP-, neurokinin A-, and neuropeptide Y-like immunoreactivity in rat synovial fluid, as well as an increase in the amount of the previously mentioned peptides in the cerebrospinal fluid.
fluid during acute monoarthritis. Inflammation has also been shown to induce an up-regulation of the expression of preprodynorphin mRNA and the mRNA of the proto-oncogene c-fos in the dorsal horn of the spinal cord.

The objectives of the present study were to further the understanding of the role of CGRP in spinal transmission of presumed nociceptive information in a 'natural' inflammation model in rats, using thermal injury, and to investigate the interaction between opioids and CGRP in this model. In addition, the effect of an acute unilateral thermal injury on CGRP-like immunoreactivity in bilateral hindpaw perfusates, cerebrospinal fluid and plasma was investigated.

METHODS

Animal preparation and intrathecal injection

All experiments were performed on freely moving male Sprague-Dawley rats (250–300 g; ALAB, Stockholm, Sweden). The rats were housed in cages with free access to food and water, and maintained in a room temperature of 24 ± 1°C with a 12 h light/dark cycle. All rats were accustomed to the testing conditions for at least 5 days before starting the experiment in order to get a stable response latency and to decrease the stress induced by measurements on rats. On the experimental day, rats were pre-treated with 2% lidocaine subcutaneously in order to get a stable response latency and to decrease the stress induced by measurements on rats. On the experimental day, rats were accustomed to the testing conditions for at least 5 days before starting the experiment in order to get a stable response latency and to decrease the stress induced by measurements on rats. On the experimental day, rats were pre-treated with 2% lidocaine subcutaneously in the region of intrathecal injection. A stainless steel needle with an outer diameter of 0.5 mm was inserted into the subarachnoid space between L4-L5 or L3-L4. Ten microlitres of solution (see below) were thereafter infused intrathecally during 1 min. The experimental protocol used was approved by the local animal ethical committee at Karolinska Institute.

Inflammation model

A 'natural' inflammation was produced by dipping the left paw into hot water (60°C) for 20 s under ether anaesthesia. The protocol adopted was designed to prevent the rats from excessive and unnecessary suffering. A control group was obtained by dipping the left paw into 25°C for 20 s. Four hours after thermal injury, the intrathecal injection was given and the hindpaw withdrawal tests started. The hindpaw volume was measured by a Plethysmometer (UGO Basile, type 7150, Italy).

Test of the latency to withdrawal response

The Randall Selitto Test (UGO Basile, Type 7200, Italy) was used to assess withdrawal latency to mechanical stimulation. A wedge-shaped pusher with a loading rate of 48 g/s was applied to the dorsal surface of the manually handled hindpaw and the pressure required to initiate the struggle response was assessed. The hindpaw withdrawal latency (HWL) is expressed in seconds, i.e. latency to withdrawal from start of stimulation. The measurements after thermal injury, but before intrathecal injection, were regarded as the basal HWL. The HWLs recorded during subsequent experiments were expressed as percentage change of the mean basal level for each rat (% change of basal HWL). The tests were performed before intrathecal injection as basal HWL and repeated at the times of 5, 15, 30, and 60 min after the injection as in Figure 1. In Figure 2, the tests were performed before intrathecal injection, and 5 and 15 min afterwards; next a second intrathecal injection was given and tests carried out at the times of 20, 35 and 50 min after the first injection.

Radioimmunoassay

Thirty rats were anaesthetized with chloral hydrate (0.4 g/kg) intraperitoneally, and then divided into three groups depending on the timing for examination, i.e. before (control, n=10), 4 (n=10) or 24 (n=10) h after the thermal injury of the left hindpaw. Samples of cerebrospinal fluid, plasma and the perfusate from the left and right hindpaws were obtained for radioimmunoassay. For collection of cerebrospinal fluid rats were placed in a stereotactic frame. The atlanto-occipital membrane was exposed by retracting the overlying muscles and samples of 80–150 μl of cerebrospinal fluid were obtained through a 27-gauge needle with a 1 ml syringe via a polyethylene tube. Blood (1.5–4.5 ml) was then collected by puncture of the heart with a vacutainer tube containing heparin 143 IU/ml and Trasylol 500 IU/ml. The sample of blood was centrifuged and the plasma was removed. The hindpaw perfusion was carried out through two 27-gauge needles inserted into the plantar region of the hindpaw. One millilitre of perfusate was collected. A reverse phase C18 cartridge (Sep Pak, Waters) was used for sample extraction from the perfusate or cerebrospinal fluid. All samples were rapidly cooled and stored at −80°C until analysis.

Radioimmunoassay of CGRP-like immunoreactivity was performed using CGRPR8° raised against conjugated rat CGRP according to Bileviciute et al.° 125I-His-tidy1 rat CGRP purified by high-performance liquid chromatography (Waters°) was used as radioligand, and rat CGRP as standard. The cross-reactivity of the assay to substance P, neurokinin A, neurokinin B, neuropeptide K, gastrin, neurotensin, bombesin, neuropeptide Y and calcitonin was less than 0.01%. Cross-reactivity toward rat α-CGRP and β-CGRP was 100% and 120%, respectively.
Effects of CGRPs following thermal injury in rats

Chemicals

Solutions for intrathecal administration were prepared with sterilized saline (0.9%), each with a volume of 10 μl: (1) 1, 5 or 10 nmol of CGRP<sub>8-37</sub> (hCGRP<sub>8-37</sub>; Peninsula Labs Inc, Europe); (2) 8, 16 or 32 or 64 μg of naloxone (naloxone hydrochloride, Sigma Chemical Company, St Louis, MO). Control groups were given 10 μl of 0.9% saline.

Statistical analysis

Data from hindpaw withdrawal tests are presented as mean ± SEM. The difference between groups was determined by one-way analysis of variance (ANOVA) or Student’s t-test (two tailed). *P<0.05, **P<0.001 and ***P<0.001 were considered as significant differences.

RESULTS

Effects of thermal injury on hindpaw volume and withdrawal latency to pressure

Before and after the thermal injury the hindpaw volume and the withdrawal latency to mechanical stimulation were assessed (Fig. 1). 4 h after thermal injury, the left hindpaw volume was significantly increased (P<0.001), but the volume of the non-injected right hindpaw showed no significant change (P = 0.83) as shown in Figure 1A. Also, there were no significant changes in volumes of the hindpaws between 1, 2, 3, and 4 h after injury (left: P = 0.59–0.74; right: P = 0.64–0.81).

Before thermal injury there was no significant difference between left and right HWLs (P = 0.56). After thermal injury the HWLs were significantly decreased bilaterally (left: P < 0.001; right: P < 0.05) compared to the HWLs before thermal injury, and the left HWL was significantly shorter than on the right side (P<0.001) as shown in Figure 1B. There was no significant change in left (P = 0.52–0.78) and right (P = 0.73–0.98) HWLs 1, 2, 3 and 4 h after the thermal injury.

Effect of intrathecal administration of CGRP<sub>8-37</sub> on the latency to withdrawal response in rats with left hindpaw inflammation

Fifty-four rats with left hindpaw inflammation, 4 h after thermal injury, were divided into four groups receiving intrathecal injections of: (1) 10 μl of 0.9% saline as a control (n = 9); (2) 1 nmol of CGRP<sub>8-37</sub> (n = 9); (3) 5 nmol of CGRP<sub>8-37</sub> (n = 9); (4) 10 nmol of CGRP<sub>8-37</sub> (n = 9); (5) 20 nmol of CGRP<sub>8-37</sub> (n = 9); and (6) 40 nmol of CGRP<sub>8-37</sub> (n = 9).

Compared to the control group, there were no significant changes in HWLs in the group receiving 1 nmol (left/left = 0.66, P = 0.49; right/right = 0.42, P = 0.58) or 5 nmol (left/left = 1.99, P = 0.31; right/right = 1.49, P = 0.33) of CGRP<sub>8-37</sub> as shown in Figure 2. The groups receiving 10, 20 or 40 nmol of CGRP<sub>8-37</sub> demonstrated significantly increased HWLs bilaterally (left/left = 15.49, 16.32, 19.61, P < 0.001; right/right = 7.01, 11.24, 14.96, P < 0.05) compared with the control group and the effect lasted for more than 60 min after CGRP<sub>8-37</sub> injection (Fig. 2).

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Fig. 2  Effects of intrathecal injection of CGRP<sub>9-37</sub> on hindpaw withdrawal latency to mechanical stimulation in rats 4 h after thermal injury of the left hindpaw. A: thermally injured left hindpaw. B: intact right hindpaw. One nmol of CGRP<sub>9-37</sub> (○; n = 9), 5 nmol of CGRP<sub>9-37</sub> (●; n = 9), 10 nmol of CGRP<sub>9-37</sub> (▲; n = 9), 20 nmol of CGRP<sub>9-37</sub> (■; n = 9), 40 nmol of CGRP<sub>9-37</sub> (▲; n = 9), 10 μl of 0.9% saline (△; n = 9) as control. HWL: hindpaw withdrawal latency. Time=0: intrathecal injection of 10 nmol of CGRP<sub>9-37</sub>.

Fig. 3  Effects of intrathecal injection of naloxone on CGRP<sub>9-37</sub> induced increase in HWLs to mechanical stimulation in thermally injured rats. A: Thermally injured left hindpaw. B: intact right hindpaw. Fifty-four thermally injured rats received an intrathecal injection of 10 nmol of CGRP<sub>9-37</sub> followed 15 min later by 8 μg (○; n = 9), 16 μg (●; n = 9), 32 μg (▲; n = 9) or 64 μg (▲; n = 9) of naloxone, or 10 μl of 0.9% saline (■; n = 9) as a control. HWL: hindpaw withdrawal latency. Another group of rats received 10 μl of 0.9% saline followed 15 min later by 32 μg of naloxone (■; n = 9). Time = 0: intrathecal injection of 10 nmol of CGRP<sub>9-37</sub>. Time = 15: intrathecal injection of either 8, 16, 32 and 64 μg of naloxone or 10 μl of 0.9% saline as control.
Effect of intrathecal administration of naloxone on the CGRP$_{8-37}$-induced increase in HWL rats with left hindpaw inflammation

Forty-five thermally injured rats received an intrathecal injection of 10 nmol of CGRP$_{8-37}$ followed 15 min later either by 8 μg ($n=9$), 16 μg ($n=9$), 32 μg ($n=9$) or 64 μg ($n=9$) of naloxone, or by 10 μl of 0.9% saline ($n=9$) as a control. The results are shown in Figure 3.

After the injection of 10 nmol CGRP$_{8-37}$ the HWL ipsilateral to the inflammation was significantly increased to mechanical stimulation in all groups. In accordance with the results mentioned above, the effect induced by CGRP$_{8-37}$ was expected to last for at least 60 min. After intrathecal injection of 32 or 64 μg of naloxone, 15 min after CGRP$_{8-37}$ administration, the increased HWLs to mechanical stimulation was reduced on the thermally injured side (left hindpaw) ($P<0.05$–$0.01$) and on the intact side (right hindpaw) ($P=0.33$–$0.49$, $P=0.27$–$0.58$). Compared to the control group, there were no significant differences in HWLs to any of the tests in the group receiving 8 μg ($n=9$, $P=0.42$; right = 3.68, $P=0.09$) or 16 μg ($n=9$, $P=0.58$; right = 1.24, $P=0.44$) of naloxone. In order to study the effect of intrathecal injection of naloxone alone, 10 rats with thermally injured left hindpaws were given an intrathecal injection of 10 μl of 0.9% saline followed 15 min later by 32 μg of naloxone. No significant change in HWL was found, as shown in Figure 3.

CGRP-like immunoreactivity in cerebrospinal fluid, plasma and the perfusate of right and left hindpaw after thermal injury

In order to determine the effects of thermal injury of the left hindpaw on the levels of endogenous CGRP, experiments were performed to measure the changes of CGRP-like immunoreactivity in cerebrospinal fluid, plasma and the perfusates of the left and right hindpaw in rats with inflammation. The results are shown in the Table.

The contents of CGRP-like immunoreactivity in the cerebrospinal fluid increased significantly 4 ($P<0.01$) and 24 h ($P<0.001$) after thermal injury, compared with pre-thermal injury data. There was no significant change in CGRP-like immunoreactivity in the plasma between the pre-thermal injury data and 4 ($P=0.66$) or 24 h ($P=0.84$) after thermal injury. Compared to pre-thermal injury data, there was a significant increase in CGRP-like immunoreactivity in the perfusate of the left hindpaw at 4 ($P<0.05$) and 24 ($P<0.001$) h after thermal injury. There was also an increased CGRP-like immunoreactivity in the perfusate of the right hindpaw at 4 ($P=0.05$) and 24 ($P<0.05$) h after the thermal injury of the left hindpaw compared to pre-thermal injury data, although the effect was less pronounced than on the left side.

### DISCUSSION

In the present study, thermal injury of the left hindpaw of the rat resulted in a unilateral increase in left paw volume, paralleled by an increased content of CGRP-like immunoreactivity in left and right paw perfusate. The latter finding gains indirect support from previous studies demonstrating increased biosynthesis of CGRP in the dorsal root ganglia, and increased release of CGRP-like immunoreactivity in the spinal cord in response to experimentally induced inflammation. As shown in Figure 1, the oedema formation in response to thermal injury was only seen in the thermally injured hindpaw, whereas there was a bilateral decrease in latency to hindpaw withdrawal responses induced by mechanical stimulation that was more pronounced in the thermally injured paw. Interestingly, there was a bilateral increase in CGRP-like immunoreactivity in both paw perfusates and in the cerebrospinal fluid. Taken together these findings indicate that there is a bilateral spinal hyperexcitability in neurons participating in executing the withdrawal reflex, and that this may be related to increased release of CGRP.

Mayer et al. have suggested that primary sensory neurons play a dual role in the response to injury, where the central terminals transmit information set up by the nociceptive event to the central nervous system, and the peripheral terminals mediate a local inflammatory response via the axon reflex. Several neuropeptides such as CGRP and SP, as well as other compounds, participate in this response. That CGRP has a role in transmission of presumed noxious information is supported by the findings that intrathecal administration of 10, 20 and 40 nmol of a CGRP antagonist, produced an increase in HWLs to pressure in rats with unilateral hindpaw inflammation.
inflammation. In the dorsal horn of the spinal cord it has been demonstrated that CGRP acts at both pre- and postsynaptic sites. Recently, it was demonstrated that experimentally induced inflammation enhanced the release of CGRP-like immunoreactivity in the spinal dorsal horn.

The increased amount of endogenous CGRP may exert a postsynaptic and/or presynaptic action, the latter affecting the release of substance P from primary afferents. One feasible effect of CGRP on HWLs in rats with inflammation, is in line with an increased release of endogenous CGRP in acute inflammation following thermal injury.

In the present study, the increase in HWL obtained after intrathecal administration of CGRP, in rats with left hindpaw inflammation due to thermal injury, was partly reversed by intrathecal injection of the opioid receptor antagonist naloxone, indicating that endogenous opioid peptides may be involved in the control of CGRP release and/or the pre- postsynaptic actions of CGRP. Our results are supported by the finding that opioid peptides may exert a presynaptic inhibitory effect on primary nociceptive afferents in the dorsal horn of the spinal cord, including CGRP-containing primary afferent fibres. Collin et al. recently demonstrated that endogenous opioid peptides, acting at both mu and kappa receptors, exert a tonic inhibitory control on the release of CGRP-like material in the spinal cord.

In the present study, it is shown that naloxone can block the antinociceptive effect of CGRP. It may be possible that the nociceptive input might be affected by administration of naloxone alone, but the results obtained suggest that this is unlikely. We observed no significant change of HWL to mechanical stimulation after intrathecal administration of 10 μl of 0.9% saline followed 15 min later by 32 or 64 μg of naloxone. This result is in accordance with the notion that the opioid antagonist naloxone clearly has little effect in normal animal or human subjects, as demonstrated in a number of studies. It is known that CGRP in the dorsal horn of the spinal cord originates from small diameter primary afferent fibers. In addition, spinal neurons that exhibit dynorphin-like immunoreactivity are located in laminae I–VII of the rat spinal cord. Histological studies have demonstrated a monosynaptic connection between dynorphin-containing neurons and CGRP containing axon terminals in a rat model of peripheral inflammation. The above data suggest that spinal opioid neurons may directly influence small diameter primary afferents that are likely to be involved in nociceptive transmission. In summary, the outcome of the present study shows that unilateral thermal injury of the left hindpaw results in a marked increase in the paw volume, leaving the right hindpaw volume unaffected. On the other hand, there was a bilateral decrease of HWLs and a bilateral increased release of CGRP-like immunoreactivity in hindpaw perfusates. Intrathecal administration of CGRP induced a significant bilateral increase in HWL to mechanical stimulation and these effects were partially reversed by naloxone. Overall, the results indicate that CGRP plays a role in the transmission of presumed nociceptive information in the spinal cord of rats with a ‘natural’, non-chemically induced, inflammation, and that its release and/or effects are subjected to opioid modulation.

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REFERENCES

Effects of CGRs following thermal injury in rats


