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Unilateral injection of calcitonin gene-related peptide (CGRP) induces bilateral oedema formation and release of CGRP-like immunoreactivity in the rat hindpaw

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1 The contribution of calcitonin gene-related peptide (CGRP) to bilateral oedema formation in the rat hindpaw following an unilateral challenge with CGRP was investigated.

2 Rats were injected into the left hindpaw with either saline, CGRP or a CGRP antagonist ($CGRP_{8-37}$). All injections were given in a double blind fashion and in a volume of 100 μl. CGRP and $CGRP_{8-37}$ were administered in concentrations of 75, 150 or 300 pmol. Volumes of the right and left hindpaw were measured every hour for 5 h by plethysmometry.

3 Injection of CGRP 300 pmol into the left hindpaw resulted in a bilaterally increased hindpaw volume after 5 h as compared with the groups given saline. No changes were found in hindpaw volumes following the injection of either 75 or 150 pmol of CGRP or 75, 150 or 300 pmol of $CGRP_{8-37}$ as compared with saline injection.

4 To elucidate whether or not the bilateral oedema formation was related to a release of endogenous CGRP, microdialysis of the contralateral hindpaw was carried out, and concentrations of CGRP-like immunoreactivity (-LI) were determined by radioimmunoassay and high performance liquid chromatography. Injection of CGRP 300 pmol into the left hindpaw increased the release of CGRP-LI into the right hindpaw perfusate after 4 and 5 h. No changes in CGRP-LI were detected in the right hindpaw perfusate following challenge with saline or $CGRP_{8-37}$.

5 To study the contribution of the nervous system to the contralateral release of CGRP-LI, sciatic nerve ligated and intact sham-operated rats were used. Sciatic nerve ligation but not sham-operation on the non-injected side abolished the increased release of CGRP-LI following contralateral administration of CGRP 300 pmol.

6 To study the spinal cord mechanisms resulting in the bilateral oedema formation following unilateral challenge with 300 pmol of CGRP, intrathecal pretreatment with either 10 nmol bicuculline ($GABA_A$ receptor antagonist) or 10 nmol $CGRP_{8-37}$ was carried out. Bicuculline but not $CGRP_{8-37}$ abolished the bilateral oedema formation induced by CGRP 300 pmol.

7 In order to study the mechanisms by which administration of CGRP 300 pmol induces oedema, CGRP 300 pmol was administered concomitantly with either 300 pmol of $CGRP_{8-37}$ (CGRP receptor antagonist), or 3 nmol of promethazine (H_1 receptor antagonist), or 3 nmol of s(−)-propranolol ($5\text{-}HT_1$ receptor antagonist), or 3 nmol of cyproheptadine ($5\text{-}HT_2$ receptor antagonist) or 3 nmol of ICS 205–930 ($5\text{-}HT_3$ receptor antagonist). Oedema formation was measured at 1, 5, 7 and 24 h.

8 Injection of CGRP 300 pmol into the left hindpaw induced a bilateral oedema formation which was still significant at 24 h. Concomitant administration of either $CGRP_{8-37}$, ICS 205–920 or cyproheptadine blocked the oedema formation at 24 h. No effect on oedema formation was found when CGRP 300 pmol was co-administered with either promethazine or s(−)-propranolol (H_1 and $5\text{-}HT_1$ receptor antagonists, respectively).

9 The results of the present study show that both the nervous system and local inflammatory processes contribute to bilateral hindpaw oedema formation following unilateral challenge with CGRP 300 pmol. Our results indicate that endogenous release of CGRP following inflammatory response may play an important role in inducing oedema formation.

Keywords: CGRP, calcitonin gene-related peptide; $CGRP_{8-37}$ calcitonin gene-related peptide antagonist; oedema; hindpaw; rat; microdialysis; nervous system; 5-HT-receptor antagonists.

Introduction

Already in 1876 Stricker hypothesized that sensory afferent nerves might play a significant role in the inflammatory process (Stricker, 1876). In 1901 it was reported that nerve stimulation induces vasodilation (Bayliss, 1901) and in 1930 oedema response was shown to be due to antidromic stimulation of sensory nerves (Hinsey & Gasser, 1930). This so-called neurogenic inflammation is caused by the activation of Aδ- and C-fibres (Jancso *et al*., 1967; Gamse *et al*., 1980) resulting in the antidromic release of sensory neuropeptides. Among the sensory neuropeptides playing a major role in neurogenic inflammation are substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) (Lembeck & Holzer, 1979; Foreman, 1987; Kidd *et al*., 1991; Maggi, 1995). CGRP is a very potent vasodilator and the vasodilatory response seen

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after sensory stimulation has been attributed mainly to the release of CGRP (Brain *et al*., 1985; 1986, Escott *et al*., 1995). In addition to its potent vasodilatory action, CGRP is considered to be an important modulator of oedema responses. This is supported by a study showing that an injection of a CGRP antagonist ($CGRP_{8-37}$) inhibits neurogenically induced oedema formation (Escott & Brain, 1993). Many vasodilating agents potentiate the oedema formation in response to other inflammatory mediators, most likely by increasing hydrostatic pressure in small venules where the plasma extravasation occurs (cf. Raud & Lindbom, 1994). This appears to be true also for CGRP, which is a very potent vasodilator with little or no ability to cause plasma extravasation alone (Brain *et al*., 1985; 1986; 1992; Gamse & Saria, 1985; Foreman, 1987). In a series of studies, CGRP's potentiating effect in oedema formation was demonstrated, when administered concomitantly with SP, serotonin, histamine and other mediators of inflammation (Brain *et al*., 1985; Brain & Williams, 1988; 1989; Buckley *et al*., 1991a,b; Cambridge & Brain, 1992). The results of experimental studies also show that the influence of CGRP on oedema formation is dose and strain dependent (Karimian & Ferrell, 1994; Newbold & Brain, 1993).

The aim of the present study was to investigate if a unilateral challenge with CGRP into the hindpaw is able to induce a bilateral oedema formation. The mechanisms behind this phenomenon are also investigated.

Methods

Experimental procedure for measuring hindpaw volume

Experiments were performed on male albino Sprague Dawley rats (B&K Universal AB, Sollentuna, Sweden) weighing 200–250 g and were approved by the Karolinska Institutet local ethical committee. All rats were accustomed to the testing conditions five times daily for 4 days before the experiment was run. On the day of the experiment the right and left hindpaw volumes were first measured (basal values) and then the left hindpaw was injected with either saline, CGRP or $CGRP_{8-37}$. All injections were given in a randomized double-blind way and in a volume of 100 μl. CGRP and $CGRP_{8-37}$ were injected in concentrations of 75, 150 and 300 pmol. CGRP 300 pmol was also given concomitantly with either 300 pmol of $CGRP_{8-37}$, 3 nmol of promethazine, 3 nmol of s(−)-propranolol, 3 nmol of cyproheptadine or 3 nmol of ICS 205–930. No injection was given into the right hindpaw. Volumes of both left and right hindpaws were measured using a plethysmometer (UGO Basile, type 7150, Italy) either every hour up to 5 h, or at 1, 5, 7 and 24 h, according to the experimental protocol. Three measurements were carried out on each occasion and the average value was used for statistical analysis.

Doses of the serotonin and histamine receptor antagonists were chosen according to the data of additional experimental studies. Administration of 3 nmol of serotonin into the rat hindpaw induces oedema formation at approximately 25–30% of the basal values 30 min after injection. The oedema formation was reduced following concomitant administration of serotonin with 3 nmol of either serotonin or histamine receptor antagonists used in the present study ($5\text{-}HT_3 \leqslant 5\text{-}HT_1 \leqslant H_1 < 5\text{-}HT_2$). In order to reduce inflammatory responses, the use of these selective serotonin and histamine receptor antagonists has been previously reported by Hua & Yaksh (1993) (for $5\text{-}HT_1$ and $5\text{-}HT_3$), Eschalier *et al*. (1989) (for $5\text{-}HT_3$), Cole *et al*. (1995) (for $5\text{-}HT_2$) and Hirschelmann *et al*. (1975) (for H_1).

Experimental procedure for intrathecal injection

Intrathecal administration was performed after 4 days of behavioural training. Before experimentation 2% lidocaine was injected subcutaneously into the area to be penetrated for intrathecal injection. A stainless steel needle, with an outer diameter of 0.5 mm, was then directly inserted into the subarachnoid space between the L3–L4 vertebrae. Ten μl of solution consisting of either saline, $CGRP_{8-37}$ (10 nmol, Yu *et al*., 1996) or bicuculline (10 nmol, Rees *et al*., 1995) were infused intrathecally over 1 min. Two hours after intrathecal injection, an injection of CGRP 300 pmol was given into the left hindpaw. Hindpaw volumes were measured prior to CGRP 300 pmol injection, and hourly for 5 h following CGRP 300 pmol injection into the left hindpaw.

Experimental procedure for perfusation of the hindpaw

Characterization of CGRP-LI release. Rats were anaesthetized with an intraperitoneal injection of chloralhydrate (350 mg kg^{-1}). A microdialysis probe (CMA/20, CMA Microdialysis, AB, Stockholm, Sweden), with an external membrane diameter of 0.5 mm and 4 mm membrane length, was implanted subplantarially into the right hindpaw. The hindpaw was perfused with either saline or saline-thiorphan 10^{-6} mol L^{-1} solution. The experiment started with 1 h perfusation of the right hindpaw to determine the basal release of CGRP-LI, thereafter the right hindpaw was perfused for 2 h with capsaicin solution 10^{-6} mol L^{-1} (with or without thiorphan 10^{-6} mol L^{-1}) and after that for 1 h with saline/thiorphan solution. The flow rate was kept at 4 μl min^{-1} by the use of a CMA/100 microinjection pump (CMA/Microdialysis, Stockholm, Sweden). Collection of 60 min samples on ice was begun 1 h after probe implantation and each experiment lasted for 5 h. Collected samples were frozen immediately and kept at −70°C until analysis.

To test the recovery of CGRP during the perfusation and radioimmunoassay procedures, probes were placed into a solution containing 1000 pmol of CGRP, samples were collected every hour for 5 h and analysed in the way described for the experimental procedure. The recovery coefficient includes both the ability of the probe to exchange CGRP in the medium as well as the loss of neuropeptide during the radioimmunoassays procedure. The recovery of CGRP in the analysed samples was less than 2%, which means that we were able to analyse less than 2% of the total amount of CGRP in the hindpaw's perfusate.

Perfusation following either saline, CGRP or $CGRP_{8-37}$ injection. Anaesthetized rats were injected into the left hindpaw with either saline, CGRP (300 pmol) or $CGRP_{8-37}$ (300 pmol). The right hindpaw of the rat was perfused with thiorphan 10^{-6} mol L^{-1} as described above. Collection of 60 min samples on ice was started 1 h after probe implantation and each experiment lasted for 6 h. Following injection of either saline, CGRP or $CGRP_{8-37}$ into the left hindpaw, samples of the right hindpaw perfusate were collected every hour for 5 h.

Surgical procedure for sciatic nerve ligation. To investigate the source of neuropeptide release, denervation of the right hindpaw was performed. Rats were anaesthetized with intraperitoneal chloralhydrate (350 mg kg^{-1}) and the right

sciatic nerve was cut. Control rats were sham operated. Experimentation took place 1 week to 10 days after the denervation. Both ligated and sham-operated rats received 300 pmol of CGRP into the left hindpaw. The perfusation technique used was identical with that used for intact rats.

Radioimmunoassay

Calcitonin gene-related peptide (CGRP)-LI was analysed using antiserum CGRPR8 raised against conjugated rat CGRP. HPLC-purified ^{125}I-Histidyl rat CGRP was used as radioligand, and rat CGRP as standard. The cross-reactivity of the assay to SP, neurokinin A, neurokinin B, neuropeptide K, gastrin, neurotensin, bombesin, neuropeptide Y and calcitonin was less than 0.01%. Cross-reactivity toward rat CGRP I and II was 100 and 120%, respectively. Intra- and interassay coefficients of variation were 8 and 14%, respectively. The detection limit for CGRP-LI was 7.8 fmol ml^{-1}. The average value of two tested samples (100 μl each) of the collected perfusate was used for statistical analysis.

High liquid performance chromatography

Perfusates were pooled in a total volume of 50 ml, lyophilized and redissolved in 200 μl distilled water. Rat CGRP (2 pmol in 200 μl) was used as a standard. Reverse-phase HPLC was performed using Waters Delta Pak C18 300 Å, 3.9 mm $\times$ 15 cm column, eluted with a 40 mL linear gradient of 0–40% acetonitrile. Samples were passed through Millipore GS (0.45 μm) filters before being injected onto the column. Fractions of 1 ml were collected at an elution rate of 1.0 ml min^{-1}. Each fraction was lyophilized and redissolved in 200 μl sodium phosphate buffer (pH 7.4) before analysis. The fractions were assayed for immunoreactivity in the tubes used for their collection. The detection limit for the concentrated sample was 7.8 fmol ml^{-1}.

Chemicals

Human CGRP (CGRP, Peninsula Labs Inc, Europe LIT); human $CGRP_{8-37}$ ($CGRP_{8-37}$, Peninsula Labs Inc, Europe LIT); thiorphan (Sigma Chemical Company, U.S.A.); capsaicin (Sigma Chemical Company, U.S.A.); bicuculline (Tocris, U.K.); promethazine (Sigma Chemical Company, U.S.A.); s(−)-propranolol (Sigma Chemical Company, U.S.A.); cyproheptadine (Sigma Chemical Company, U.S.A.); ICS 205–930 or 3-tropanyl-indole-3-carboxylate (Sigma Chemical Company, U.S.A.). All solutions were prepared in sterile 0.9% saline.

Statistical analysis

Statistical analysis was carried out using the SPSS software (release 6). Each experimental group included 6–8 rats.

Hindpaw volume. ANOVA test was used to test the differences between the different treatments in hindpaw volumes expressed in percentage changes from the start of the experiment (basal values). Wilcoxons rank sum test was used to test differences between the right and left hindpaw in the same animal. In rats pretreated intrathecally, the statistical analysis was performed using hindpaw values measured at 2 h after intrathecal injection, i.e. before CGRP 300 pmol injection.

CGRP-LI. Mann-Whitney U-Wilcoxon rank sum test was used to compare differences in absolute concentrations of CGRP-LI found in the right hindpaw perfusate following perfusation with either saline or thiorphan. Wilcoxons rank sum test was used to test differences within the groups following capsaicin stimulation. ANOVA test was used to test differences between saline, CGRP and $CGRP_{8-37}$ injections as well as between intact and sham-operated rats.

Results

Effects of either CGRP or $CGRP_{8-37}$ injection on the hindpaw volume as compared to saline injection

Injection of either 75 or 150 pmol of CGRP into the left hindpaw did not affect the left or right hindpaw volumes, Figure 1a and b. Injection of CGRP 300 pmol into the left hindpaw significantly enhanced the hindpaw volume of both the injected (at 4 and 5 h) and non-injected hindpaw (at 5 h), Figure 1c. No differences were found between the left and right hindpaw volumes.

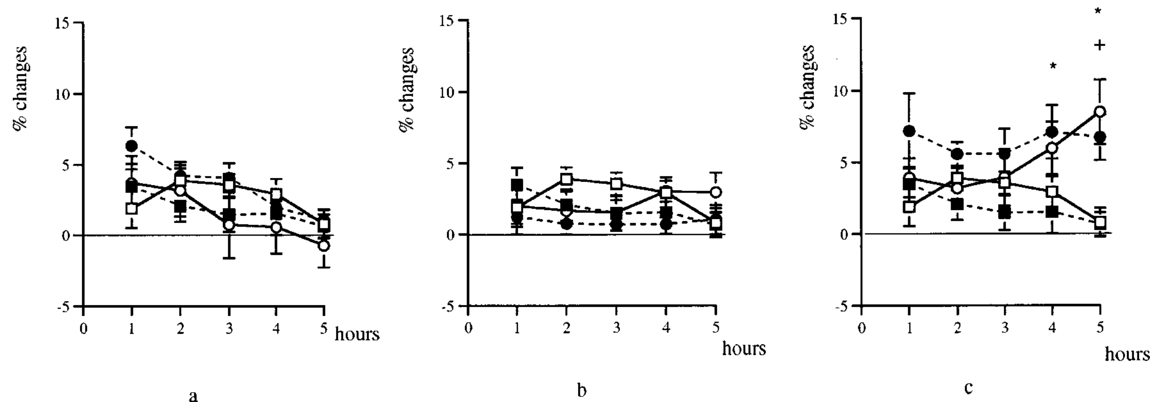

Figure 1 Changes in hindpaw volumes following different doses of a unilateral CGRP injection are presented as changes in percentage values before injection and expressed in mean ± s.e.mean (vertical axis). Horizontal axis indicates hours following injection. (a) 75 pmol, (b) 150 pmol and (c) 300 pmol. (■) represents saline injected group, injected hindpaw and (□) non-injected hindpaw; (●) represents CGRP injected group, injected hindpaw and (○) non-injected hindpaw. *Denotes significant difference in injected hindpaw volumes between the groups. + Denotes significant difference in non-injected hindpaw volumes between the groups ($P<0.05$, $n=8$).

Injection of either 75, 150 or 300 pmol of $CGRP_{8-37}$ into the left hindpaw did not generally affect the left or right hindpaw volumes. Injection of $CGRP_{8-37}$ 300 pmol into the left hindpaw resulted in a slight (approximately 3% from the basal values) but significant decrease of the right hindpaw volume as compared to saline injected (at 2 h) and to the contralateral hindpaw (at 1 and 2 h), results not shown.

Characterization of CGRP-LI release into the hindpaw perfusate

The basal release of CGRP-LI into the hindpaw perfusate was approximately 20 fmol ml^{-1}, or less than 4000 fmol ml^{-1} (400 fmol/100 μl) when correcting for the recovery (less than 2%).

Perfusation with capsaicin. Effect of thiorphan. Perfusation of the hindpaw with capsaicin resulted in a slight but significantly increased release of CGRP-LI in the presence of thiorphan. The release of CGRP-LI following stimulation with capsaicin and in the absence of thiorphan (saline only) was unchanged compared to basal release, Figure 2.

High liquid chromatography. Reverse-phase HPLC analysis of pooled hindpaw perfusates showed an immunoreactive component eluting in the position of synthetic rat CGRP (Figure 3a and b). An additional component that we have not identified eluted early at the injection front (Figure 3a and b).

Effect of CGRP (300 pmol) and $CGRP_{8-37}$ (300 pmol) on the CGRP-LI release into the hindpaw perfusate

Intact rats. Injection of CGRP 300 pmol into the left hindpaw caused an increased release of CGRP-LI into the hindpaw perfusate on the contralateral side at 4 and 5 h compared to saline injection, (Figure 4a), and at 2, 3, 4 and 5 h compared to $CGRP_{8-37}$ injection (Figure 4b).

Operated rats. Sciatic nerve ligation abolished the increased release of CGRP-LI into the hindpaw perfusate on the ipsilateral side at 4 and 5 h in response to contralateral injection of CGRP 300 pmol, Figure 4c.

Effect of intrathecal pretreatment with either bicuculline or $CGRP_{8-37}$ on bilateral oedema formation induced by unilateral challenge with CGRP 300 pmol

Unilateral challenge with CGRP 300 pmol in rats intrathecally pretreated with saline, $CGRP_{8-37}$ or bicuculline did not result in bilateral oedema formation, Figure 5a and b. The ipsilateral oedema formation was inhibited by intrathecal pretreatment with bicuculline, but not by saline or $CGRP_{8-37}$, (Figure 5a,b and c). Intrathecal pretreatment with bicuculline resulted in significantly reduced hindpaw volume/oedema formation in both left (injected) and right (non-injected) hindpaws compared to those pretreated with saline, and in the left hindpaw compared to those pretreated with $CGRP_{8-37}$ (Figure 5b and c).

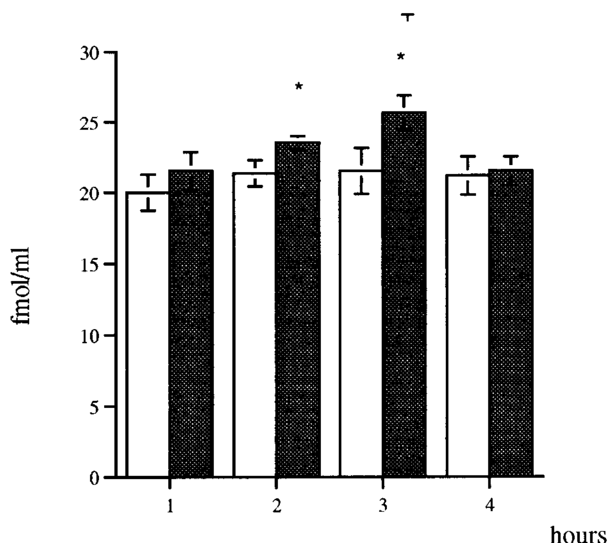

Figure 2 Concentrations of CGRP-LI in the perfusate are presented in fmol/ml as mean±s.e.mean; (■) represents group perfused with thiorphan 10^{-6} mol L^{-1} and (□) represents group perfused with saline. 1 and 4 represents basal values before and after stimulation, respectively, and 2 and 3 represents stimulation with capsaicin 10^{-6} mol/L. *Denotes significant differences between saline and thiorphan groups. + Denotes significant differences between basal values (before or after) and stimulation with capsaicin within the thiorphan group ($P<0.05$, $n=6$).

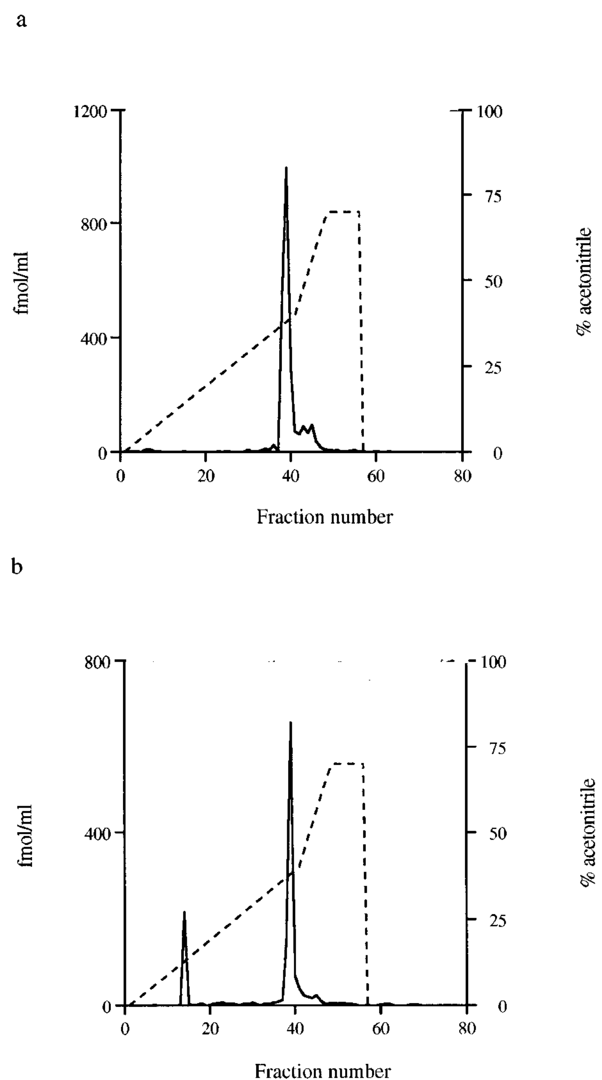

Figure 3 Reverse-phase HPLC of CGRP. (a) Synthetic rat CGRP, (b) CGRP in a pooled sample of rat hindpaw perfusates.

Effects of CGRP (300 pmol) administered either alone or concomitantly with CGRP-; histamine- or serotonin-receptor antagonists on hindpaw volumes during 24 h

Administration of CGRP 300 pmol into the left hindpaw induced bilateral oedema formation approximately at 7–9% after 24 h when compared with saline injection (Figure 6). There was a significant difference between the right and the left hindpaw in both groups at 1 h after injection, but not at 5, 7 and 24 h (significance is not indicated in Figure 6).

Injection of CGRP 300 pmol concomitantly with $CGRP_{8-37}$ 300 pmol into the left hindpaw significantly decreased both injected and non-injected hindpaw volumes at 24 h compared with the CGRP 300 pmol injection, Figure 6. In rats injected with CGRP together with $CGRP_{8-37}$, the non-injected hindpaw volume was significantly less increased at 24 h than the injected hindpaw.

Concomitant administration of H_1 and 5-HT_1 receptor antagonists had no effect on oedema formation induced by CGRP 300 pmol (Figure 7a). CGRP 300 pmol-induced oedema formation was reduced at 24 h when CGRP was co-

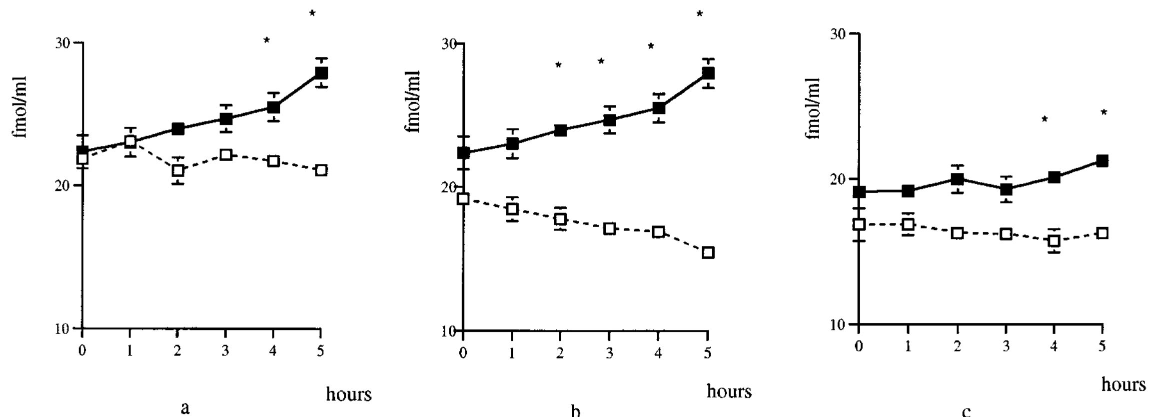

Figure 4 Comparision of CGRP-LI release into the hindpaw perfusate following different injections into the contralateral hindpaw. Concentrations of CGRP-LI in the perfusate of the non-injected hindpaw are presented in fmol/ml as mean ± s.e.mean (vertical axis). Horizontal axis indicates hours following injection. (a) Saline versus CGRP 300 pmol injection. (■) represents CGRP 300 pmol injected group and (□) represents saline injected group. *Denotes significant differences between the groups ($P<0.05$, $n=7$). (b) CGRP 300 pmol versus $CGRP_{8-37}$ 300 pmol injection. (■) represents CGRP 300 pmol injected group and (□) represents $CGRP_{8-37}$ 300 pmol injected group. *Denotes significant differences between the groups ($P<0.05$, $n=7$). (c) Sham-operated versus sciatic nerve-ligated group following CGRP 300 pmol injection. (■) Represents sham-operated group and (□) represents sciatic nerve-ligated group. *Denotes significant differences between the groups ($P<0.05$, $n=7$).

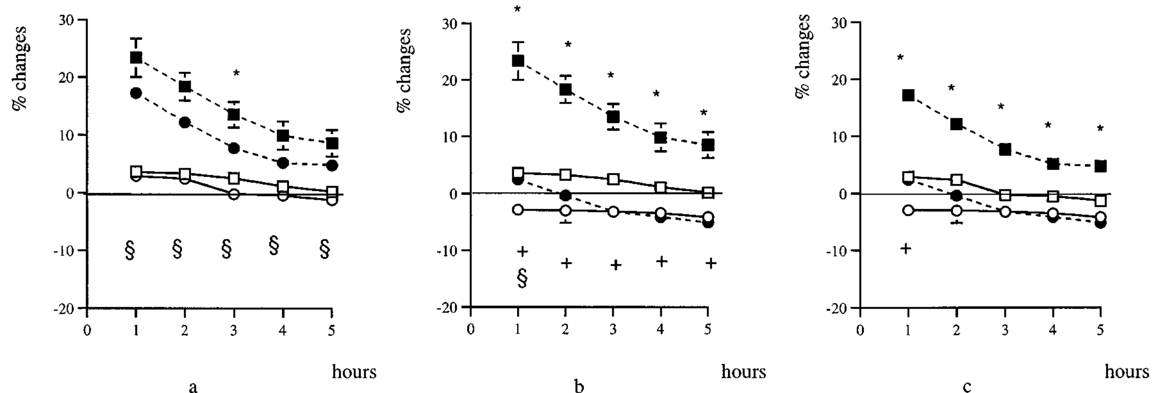

Figure 5 Comparision of different intrathecal (i.t) pretreatments given 2 h before a unilateral CGRP 300 pmol injection. Changes in hindpaw volumes are presented as changes in percentage values before hindpaw injection and expressed in mean ± s.e.mean (vertical axis). Horizontal axis indicates hours following injection. (a) Saline versus $CGRP_{8-37}$. (■) Represents group pretreated i.t. with saline, injected hindpaw and (□) non-injected hindpaw; (●) represents group pretreated i.t. with $CGRP_{8-37}$, injected hindpaw and (○) non-injected hindpaw. *Denotes significant difference in injected hindpaw volume between the groups. §Denotes significant differences between the ipsi- and contralateral side in both saline and $CGRP_{8-37}$ pretreated rats ($P<0.05$, $n=7$). (b) Saline versus bicuculline. (■) Represents group pretreated i.t. with saline, injected hindpaw and (□) non-injected hindpaw; (●) represents group pretreated i.t. with bicuculline, injected hindpaw and (○) non-injected hindpaw. *Denotes significant difference in injected hindpaw volume between the groups. + Denotes significant difference in non-injected hindpaw volume between the groups. §Denotes significant differences between the ipsi- and contralateral side in bicuculline pretreated rats ($P<0.05$, $n=7$). (c) $CGRP_{8-37}$ versus bicuculline. (■) Represents group pretreated i.t. with $CGRP_{8-37}$, injected hindpaw and (□) non-injected hindpaw; (●) represents group pretreated i.t. with bicuculline, injected hindpaw and (○) non-injected hindpaw. *Denotes significant difference in injected hindpaw volume between the groups. + Denotes significant difference in non-injected hindpaw volume between the groups ($P<0.05$, $n=8$).

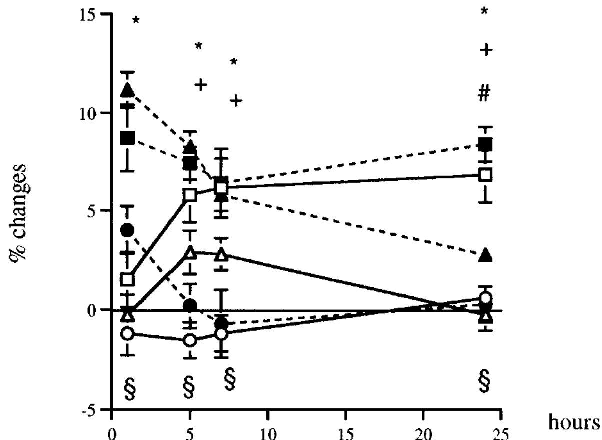

Figure 6 Changes in hindpaw volumes following a unilateral injection of 300 pmol of either CGRP or CGRP together with $CGRP_{8-37}$ are presented as changes in percentage values before injection and expressed in mean ± s.e.mean (vertical axis). Horizontal axis indicates hours following injection. (■) Represents CGRP 300 pmol injected group, injected hindpaw and (□) non-injected hindpaw; (●) represents saline injected group, injected hindpaw and (○) non-injected hindpaw; (▲) represents CGRP + CGRP 300_{8-37} injected group, injected hindpaw and (△) non-injected hindpaw. *Denotes significant difference in injected hindpaw volumes between the saline and CGRP 300 pmol groups. + denotes significant difference in non-injected hindpaw volumes between the saline and CGRP 300 pmol groups ($P<0.05$, $n=6$). #Denotes significant difference in both injected and non-injected hindpaw volumes between the CGRP and CGRP + $CGRP_{8-37}$ groups. §Denotes significant difference between the ipsi- and contralateral side of rats injected with CGRP + $CGRP_{8-37}$ ($P<0.05$, $n=6$).

administered with 5-HT_2 and 5-HT_3 receptor antagonists (Figure 7b).

Discussion

One of the most striking findings of the present study was a bilateral oedema formation following unilateral challenge with CGRP 300 pmol. Increased concentrations of CGRP-LI at the time of oedema formation have been found at the present study, suggesting a contribution from endogenously released CGRP. In the present study we used thiorphan to increase the recovery of CGRP-LI found. It has been previously demonstrated that thiorphan increased release of CGRP-LI from the peripheral nerve endings in guinea-pig cerebral venous sinuses (Tramontana *et al.*, 1991), indicating that endogenous neuropeptides are involved in CGRP metabolism *in vivo* (Maggi & Giuliani, 1994). The contralateral release of CGRP-LI in the present study was blocked by nerve ligation, suggesting that the bilateral changes seen were due to neurogenic mechanisms. This is supported by the finding that intrathecal pretreatment with bicuculline prevented the ipsilateral and bilateral oedema formation. Recently, Sluka *et al.* (1995) reported that an acute peripheral inflammation results in dorsal root reflexes causing efferent activity in both myelinated an unmyelinated nerve fibres (Sluka *et al.*, 1995a,b). These reflexes could be reduced by intrathecal administration of the $GABA_A$ receptor antagonist, bicuculline (Rees *et al.*, 1995). The physiological significance of these reflexes has been speculated upon (Ochoa & Serra, 1995; Sluka *et al.*, 1995a,b). The present results clearly suggest that these reflexes contribute to oedema

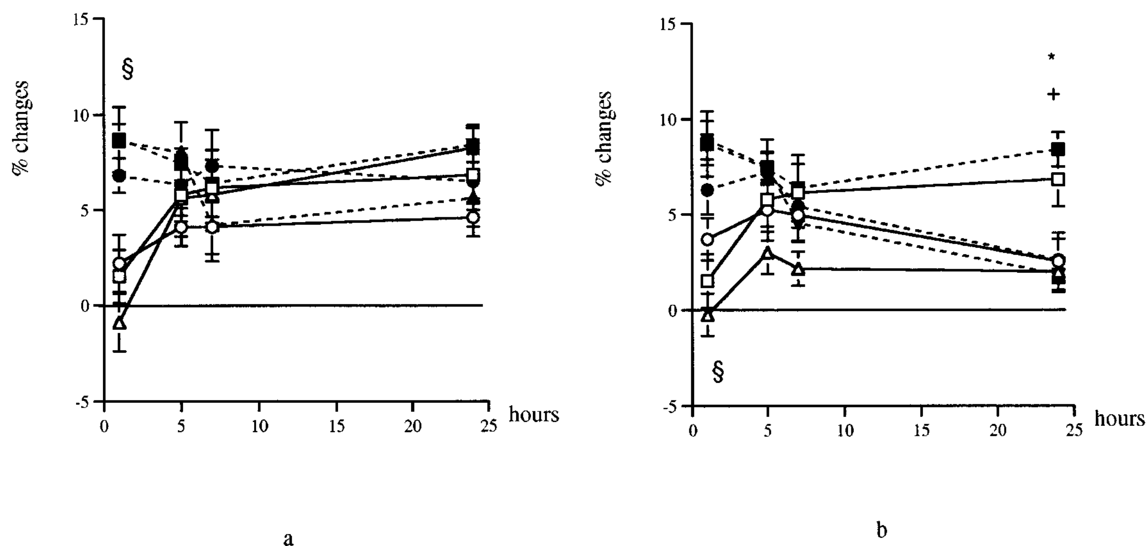

Figure 7 Changes in hindpaw volumes following a concomitant unilateral administration of CGRP 300 pmol with either H_1, or 5-HT_1 or 5-HT_2 or 5-HT_3 are presented as changes in percentage values before injection and expressed in mean ± s.e.mean (vertical axis). Horizontal axis indicates hours following injection. (a) CGRP versus CGRP + H_1 versus CGRP + 5-HT_1 (■) Represents CGRP 300 pmol injected group, injected hindpaw and (□) non-injected hindpaw; (●) represents CGRP + H_1 injected group, injected hindpaw and (○) non-injected hindpaw; (▲) represents CGRP 300 pmol + 5-HT_1 injected group, injected hindpaw and (△) non-injected hindpaw. §Denotes significant difference between the ipsi- and contralateral side in rats injected CGRP + H_1 and CGRP + 5-HT_1 ($P<0.05$, $n=6$). (b) CGRP versus CGRP + 5-HT_2 versus CGRP + 5-HT_3. (■) Represents CGRP 300 pmol injected group, injected hindpaw and (□) non-injected hindpaw; (●) represents CGRP + 5-HT_2 injected group, injected hindpaw and (○) non-injected hindpaw; (▲) represents CGRP 300 pmol + 5-HT_3 injected group, injected hindpaw and (△) non-injected hindpaw. *Denotes significant differences between CGRP versus CGRP + 5-HT_2 and CGRP versus CGRP + 5-HT_3 injected groups, injected hindpaw. + Denotes significant differences between CGRP versus CGRP + 5-HT_2 and CGRP versus CGRP + 5-HT_3 injected groups, non-injected hindpaw ($P<0.05$, $n=6$). §Denotes significant difference between the ipsi- and contralateral side in rats injected CGRP + 5-HT_3 ($P<0.05$, $n=6$).

formation. In the present study the intrathecal pretreatment with saline or $CGRP_{8-37}$ also inhibited the bilateral but not the ipsilateral oedema formation. It may be suggested that this blocking effect is due to the vehicle volume or dilution of neurogenic substances involved in the transmission at the spinal cord. However, intrathecal pretreatment with bicuculline reduced the bilateral hindpaw oedema formation, suggesting that dorsal root and spinal cord reflexes as well as central mechanisms have a tonic influence on peripheral afferent nerve activity. It has been reported that 75% of primary sensory afferents innervating the cat knee joint have a resting activity (Schaible & Schmidt, 1983) which possibly bring about the release of neuropeptides, including CGRP in the peripheral tissue. This is supported by our previous and present results showing a basal release of CGRP and other neuropeptides in knee joint synovial perfusate (Bileviciute *et al*., 1994, 1996). The functional role of this basal release could be to maintain blood circulation (Maggi, 1995) and nutrition (Jernbeck *et al*., 1990) in the innervated area.

In the present study the bilateral oedema formation seen after unilateral challenge with 300 pmol of CGRP had a long latency, suggesting the involvement of local inflammatory processes including mast cell degranulation and the release of pro-inflammatory mediators such as histamine and serotonin (Richardson, 1990; Schwartz *et al*., 1991). Activation of blood platelets is another possible source of increased serotonin concentrations in the inflamed tissue (Hranilovic *et al*., 1996; Marcenac & Blache, 1985). It is known that histamine enhances oedema formation mainly through the activation of H_1 and H_2 receptors (Akoev *et al*., 1996; Schwartz *et al*., 1991), and that serotonin induces plasma extravasation through $5\text{-}HT_1$- and $5\text{-}HT_3$-receptor activation (Richardson, 1990; Taiwo & Levine, 1992). In the present study the bilateral oedema formation was blocked by $5\text{-}HT_2$ and $5\text{-}HT_3$ but not by histamine H_1 and $5\text{-}HT_1$ receptor antagonists. These findings indicate that mast cell degranulation and serotonin receptor activation are involved in CGRP induced oedema formation. It may be suggested that following the degranulation of mast cells and/or activation of blood platelets the pro-inflammatory substances are released. Thereafter, due to a close anatomical relationship between the mast cells and sensory nerves (Alving *et al*., 1991; Renda *et al*., 1992), there is an activation of nociceptive primary afferents (Akoev *et al*., 1996; Herbert & Schmidt, 1992) which triggers the release of neuropeptides, including CGRP, contributing to oedema formation (Maggi, 1995). Compared to SP, the capacity of CGRP in the activation of mast cells is less pronounced (Nilsson *et al*., 1990), most likely explaining why a dose of CGRP ten times lower (30 pmol, Newbold & Brain, 1993) than presently used did not induce hindpaw oedema formation. Moreover, Newbold & Brain (1993) used this dosage to investigate a possible anti-inflammatory effect of CGRP, also a finding reported by Raud and co-workers (Raud *et al*., 1991). The results of the present study suggest that higher doses of CGRP may have a pro-inflammatory effect. It should be stressed that challenge with saline also resulted in bilateral short-lasting oedema formation which was probably simply the reflex effects of the vehicle injection (Levine *et al*., 1985). In the present study CGRP-induced oedema lasted 24 h, possibly due to long-lasting endogenous release of CGRP-LI into the hindpaw. This finding is supported by our previous studies demonstrating long-lasting (24 h) release of neuropeptide-LI in the rat central and peripheral nervous systems following an acute challenge (Bileviciute *et al*., 1994, 1996, 1997).

The oedema formation in the present study was also blocked at 24 h by concomitant administration of CGRP with $CGRP_{8-37}$, indicating that CGRP receptors contribute to oedema formation. It is known that CGRP-receptors may be divided into $CGRP_1$ and $CGRP_2$ where the $CGRP_1$- is the receptor more sensitive to $CGRP_{8-37}$ (Poyner, 1992). Since the contralateral oedema formation was blocked by $CGRP_{8-37}$, it may be suggested that the $CGRP_1$-receptor is related to oedema formation. It has been shown that $CGRP_{8-37}$ blocks vasodilation induced by electrical nerve stimulation (Escott & Brain, 1993), supporting the role of neurogenically released CGRP in blood flow regulation. However, it is also possible that $CGRP_{8-37}$ reduces directly or indirectly the afferent input in the primary sensory neurons, resulting in decreased spinal cord and contralateral afferent activity (unpublished observations).

In the present study the concomitant administration of CGRP 300 pmol with either $CGRP_{8-37}$ or serotonin receptor antagonists reduced oedema formation only at 24 h. This may indicate that CGRP induces vasoactive effects acting through several receptors. Indeed, pretreatment of the hindpaw with dexamethasone 17 h before CGRP 300 pmol injection significantly abolished oedema formation from the start of the experiment (unpublished data), supporting an involvement of local inflammatory mechanisms in oedema formation mainly through mast cells mediator release.

It is interesting to note that following host defence responses induced by either i.p. injection of IL-1 (Bileviciute *et al*., 1994) or low doses of Freund's adjuvant (Bileviciute *et al*., 1996), an acute release of CGRP is commonly found in comparison with that of other neuropeptides (SP, NKA and NPY). The acute release of neuropeptides following an acute challenge indicates an early activation of the nervous system, possibly playing an important role in general host defence responses. Bilateral oedema formation found in the present study may be a part of the general nervous system activation following challenge. However, our unpublished studies indicate that contralateral administration of CGRP 300 pmol, in contrast to 3 nmol of 5-HT, did not cause a detectable release of SP-LI into the hindpaw perfusate. Moreover, no changes were found in hindpaw withdrawal latency to mechanical or thermal stimulation following CGRP 300 pmol injection (unpublished results). Predominant release of CGRP-LI in muscle perfusate was demonstrated during physical exercise in rats (Bucinskaite *et al*., 1998). This release of CGRP-LI under physiological non-inflammatory circumstances is suggested to contribute to muscle hyperaemia during physical exercise (Yamadama *et al*., 1997).

In conclusion, an unilateral injection of CGRP into the hindpaw of the rat induces a bilateral oedema formation, which is dependent on both neurogenic and local inflammatory mechanisms. Neuronally released CGRP-LI may be involved in mediating the contralateral oedema formation and play an important role in both non-inflammatory and inflammatory situations *in vivo*.

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