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Effect of a calcitonin gene-related peptide antagonist ($CGRP_{8-37}$) on skin vasodilatation and oedema induced by stimulation of the rat saphenous nerve

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1 The effect of the calcitonin gene-related peptide antagonist ($CGRP_{8-37}$, 400 nmol kg^{-1}, i.v.) on the increased blood flow induced by calcitonin gene related peptide (CGRP), vasodilator prostaglandins, and topical capsaicin was measured with a laser Doppler blood flow meter in rat abdominal skin.

2 The saphenous nerve was electrically stimulated and the effect of $CGRP_{8-37}$ (400 nmol kg^{-1}, i.v.) on the increased blood flow (measured by laser Doppler flowmetry) and oedema formation (measured by the extravascular accumulation of $[^{125}I]$-albumin) was investigated in the rat hind paw.

3 $CGRP_{8-37}$ (400 nmol kg^{-1}, i.v.) had no effect on basal cutaneous blood flow at uninjected sites and sites injected with Tyrode buffer, but acted selectively to inhibit the increased blood flow induced by intradermal CGRP (10 pmol/site, $P < 0.05$), but not that induced by prostaglandin E_2 (PGE_2, 300 pmol/site) or carba-prostacyclin ($cPGI_2$, 100 pmol/site).

4 Capsaicin (0.1–33 mM), applied topically, acted in a dose-related manner to increase blood flow. $CGRP_{8-37}$ (400 nmol kg^{-1}, i.v.) almost totally inhibited blood flow induced by capsaicin (10 mM; $P < 0.05$) but did not significantly inhibit blood flow induced by a higher dose of capsaicin (33 mM).

5 The increased blood flow induced by short stimulation of the saphenous nerve (10 V, 1 ms, 2 Hz for 30 s) was inhibited by 76%, 5 min after i.v. $CGRP_{8-37}$ (400 nmol kg^{-1}, i.v., $P < 0.05$).

6 A longer (5 min) electrical stimulation of the saphenous nerve caused oedema formation, in addition to increased blood flow. The oedema formation was significantly inhibited by $CGRP_{8-37}$ (400 nmol kg^{-1}, i.v., $P < 0.05$).

7 The results suggest that the potent microvascular vasodilator neuropeptide, CGRP, is responsible for the increased blood flow observed after short stimulation of the saphenous nerve and that endogenous CGRP contributes in a pro-inflammatory manner to neurogenic oedema formation in the rat hind paw.

Keywords: Calcitonin gene-related peptide (CGRP); substance P; neurokinin-1 receptors; saphenous nerve; skin blood flow; laser Doppler flowmetry; neurogenic inflammation

Introduction

Our group has previously studied the release and activity of the 37 amino acid vasodilator sensory neuropeptide calcitonin gene-related peptide (CGRP) in the rabbit cutaneous microvasculature. The studies show that the increased blood flow induced in rabbit skin by intradermal injection of capsaicin and analogues (e.g. olvanil), can be inhibited by the co-injection of the 8–37 amino acid C-terminal peptide of CGRP ($CGRP_{8-37}$), a selective competitive antagonist of CGRP (Chiba *et al.*, 1989; Gardiner *et al.*, 1990; Han *et al.*, 1990). Early experiments suggested that $CGRP_{8-37}$ could partially inhibit capsaicin-induced vasodilatation (Hughes & Brain, 1991), whilst more recent experiments using $CGRP_{8-37}$ synthesized by improved techniques have shown that a 60% increase in blood flow in response to capsaicin (100 nmol/site) is totally inhibited by $CGRP_{8-37}$ (Hughes *et al.*, 1992). We have concluded from these results that CGRP is an important vasodilator neuropeptide released from capsaicin sensitive nerves in rabbit skin. It is generally assumed that substance P, which is often co-localised with CGRP also has an important vasoactive effect. However, substance P is an extremely weak vasodilator in rabbit skin (Brain *et al.*, 1985) and a neurokinin-1 (NK_1) receptor selective antagonist had little effect on capsaicin-induced blood flow in this species (Hughes *et al.*, 1992).

In this study we have investigated the release and activity of CGRP in rat skin, where neurokinins (especially substance P) have been established as potent vasoactive mediators (Lembeck & Holzer 1979; Gamse & Saria 1985; Brain & Williams 1989). We know, from a previous study that $CGRP_{8-37}$ cannot be administered intradermally in the rat due to a pro-inflammatory activity, possibly related to basic residues in the N-terminus region (Brain *et al.*, 1992). Thus we have administered $CGRP_{8-37}$ intravenously (i.v.) which has been shown to be effective in inhibiting the hypotensive effects of i.v. CGRP in several studies (Donoso *et al.*, 1990; Gardiner *et al.*, 1990). We have examined the ability of $CGRP_{8-37}$ to influence the sensory nerve-dependent pro-inflammatory responses in the rat in response to capsaicin administration and electrical stimulation of the saphenous nerve.

Methods

Blood flow and oedema formation were measured in the skin of male Wistar rats (200–300 g), anaesthetized with sodium pentobarbitone (Sagatal, May and Baker, initial i.p. injection 50–80 mg kg^{-1}, maintained by additional 20 mg kg^{-1} h^{-1}, given i.v.). Body temperature was maintained at 36–38°C by automatic control of a heating pad. Rats were pretreated with guanethidine (20 mg kg^{-1}, s.c.) 24 h before saphenous nerve stimulation, to prevent vasoconstriction induced by concomitant stimulation of the sympathetic fibres of the saphenous nerve (Gamse & Saria, 1987).

Measurement of blood flow in abdominal skin

The animal was anaesthetized, and the abdominal skin

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shaved and depilated. Thirty minutes later $CGRP_{8-37}$ or vehicle were given i.v. and blood flow was measured at several sites with a laser Doppler flowmeter. Prostaglandins, CGRP or Tyrode were injected intradermally (50 μl) and capsaicin or vehicle (20% alcohol: 10% Tween 80: 70% saline) were applied topically (5 μl). Blood flow was measured again between 5–10 min after application of test agents. Blood flow changes were measured with a Moor dual probe laser Doppler (MBF3D) and measured as flux, as previously discussed in detail (Lawrence & Brain, 1992). The results are expressed as percentage change in blood flow compared with pre-injected sites in Figures 1 and 2. In some experiments the protocols were designed to measure responses to vasoactive agents and to saphenous nerve stimulation in the same animals.

Measurement of blood flow after saphenous nerve stimulation

After induction of anaesthesia the hind limbs of the rat were shaved and depilated with a commercial cream. The jugular vein was cannulated for i.v. administration of anaesthetic and test agents. The saphenous nerves were carefully dissected in both legs (stimulated or sham legs) tied centrally, placed on bipolar platinum electrodes and immersed in mineral oil. In order to study antidromic vasodilatation one laser Doppler probe was positioned and secured over the hind paw skin in a region innervated by the saphenous nerve, i.e. medio-dorsal side of the skin of the hind paw, as confirmed by the observation of Evans Blue dye extravasation after saphenous nerve stimulation. The other probe was positioned in a corresponding site on the sham treated hind paw to monitor basal blood flow throughout the experiment. Not earlier than 30 min after the preparation was finished, antidromic stimulation was performed using the following parameters: 10 V, 1 ms, 2 Hz for 30 s. Three stimulations were given at 20–30 min intervals. To investigate the effect of $CGRP_{8-37}$ on blood flow changes induced by saphenous nerve stimulation, $CGRP_{8-37}$ (400 nmol kg^{-1}), or vehicle (0.1% BSA in saline) were administered i.v. 5 min prior to the second stimulation. Results in Figure 3 show the flux in 3a and 3b; whilst Figure 3c shows percentage change in the vasodilator response to saphenous nerve stimulation, after i.v. treatments.

Measurement of oedema in the hind paw after saphenous nerve stimulation

The saphenous nerves were prepared as described above and left for 5 min. Then Evans Blue (25 mg kg^{-1}) and ^{125}I-labelled human serum albumin (50 kBq) were injected into the rat via the tail vein. $CGRP_{8-37}$ (400 nmol kg^{-1}) or vehicle (0.1% BSA) were administered via the jugular vein, at least 5 min prior to stimulation. Then the nerve was stimulated: 10 V, 1 ms, 2 Hz for 5 min. After stimulation, a blood sample was obtained by cardiac puncture and then the animal killed by anaesthetic overdose. The oedematous area of skin of the hind paw (as observed by Evans blue dye extravasation) was removed and weighed. An approximately equal amount of skin was also removed from the sham hind paw and weighed. Radioactivity was counted in 100 μl of plasma and hind paw skin samples. Plasma extravasation was expressed as μl of plasma in 100 mg of skin and a ratio calculated: sham versus stimulated hind paw oedema for each rat.

Statistical analysis

Results are expressed as mean ± s.e.mean. Abdominal, dorsal skin and paw blood flow data were analysed by Bonferroni's modified *t* test, the standard error estimate for the analysis of variance was used to allow comparison of multiple sites. The significance of the hind paw skin oedema after saphenous nerve stimulation was tested by Student's unpaired *t* test.

Materials

The following drugs were used: prostglandin E_1 (PGE_1) from Sigma Chemical Company, U.K., human α-calcitonin gene-related peptide (CGRP) a gift from Dr U. Ney, Celltech, U.K., human $CGRP_{8-37}$ from Bachem, U.K., synthetic capsaicin-pelargonic acid vanillylamide from Fluka Chemicals Ltd., U.K., carba-prostacyclin ($cPGI_2$) from Cascade Biochem. Ltd., U.K., and guanethidine from Ciba-Geigy, U.K. CGRP, PGE_1 and PGI_2 were stored in stock solutions at − 20°C and then diluted with Tyrode solution immediately prior to use. $CGRP_{8-37}$ was prepared in 0.1% bovine serum albumin (BSA) in saline. Capsaicin was dissolved in ethanol at 100 mg ml^{-1} and diluted in a solution of alcohol:Tween 80:saline, at a ratio of 20:10:70. ^{125}I-labelled human serum albumin was obtained from Amersham International, U.K. Immac (Reckitt & Colman, U.K.) was used to remove remaining hair stubble. The composition of the Tyrode solution was a follows (mM): NaCl 136.89, KCl 2.68, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $MgCl_2$ 1.05 and glucose 5.55.

Results

The intradermal injection of CGRP, PGE_1 and carba-prostacyclin ($cPGI_2$) led to an increased blood flow as measured by laser Doppler flowmetry, in keeping with previous observations (Brain & Williams, 1989). $CGRP_{8-37}$ (400 nmol kg^{-1}, i.v.) had no significant effect on basal blood flow as shown in Figure 1. However, this figure shows that $CGRP_{8-37}$ significantly inhibited the increased blood flow induced by CGRP but not that induced by the prostaglandins. These results indicate that the administration of intravenous $CGRP_{8-37}$ can be used to investigate the release of vasoactive amounts of endogenous CGRP.

The effect of the application of topical capsaicin on rat cutaneous blood flow is shown in Figure 2. Capsaicin increased blood flow in a dose-related manner, whilst the vehicle had no effect on blood flow when tested at the highest concentration used. The graph is similar to that obtained by Lynn and co-workers (1992). $CGRP_{8-37}$ caused an almost

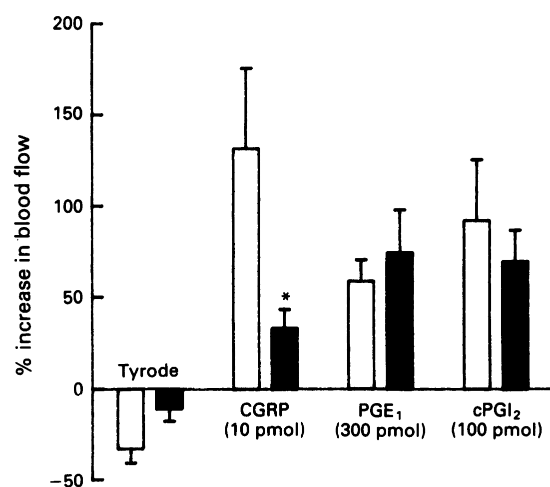

Figure 1 The effect of calcitonin gene-related peptide (CGRP) and vasodilator prostaglandins on blood flow in rat abdominal skin. Results are expressed as percentage change in local skin blood flow at each injected site compared with blood flow at the site before injection. The results from control rats (0.1% BSA, i.v.) are shown by the open columns and from rats treated with $CGRP_{8-37}$ (400 nmol kg^{-1}, i.v.) are shown by the solid columns. The results are expressed as the mean ± s.e.mean of 9 rats in each group. A significant effect of $CGRP_{8-37}$ on the vasodilator activity of CGRP is shown by $*P<0.05$.

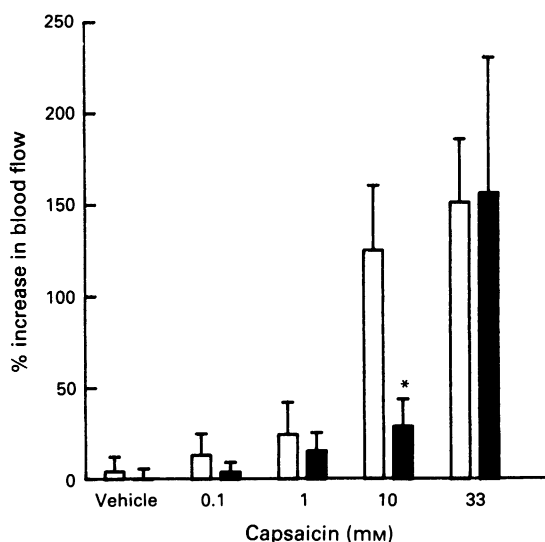

Figure 2 The effect of topical capsaicin on blood flow in rat abdominal skin. Results are expressed as percentage increase in local blood flow at each injected site compared with blood flow at the site before injection. The open columns represent control rats (0.1% BSA, i.v.) and the solid columns are animals treated with $CGRP_{8-37}$ (400 nmol kg^{-1}, i.v.). Each column is the mean ± s.e.mean of 9 rats. $CGRP_{8-37}$ significantly inhibited the vasodilator activity of capsaicin at a dose of 10 nM, as shown by $*P<0.05$.

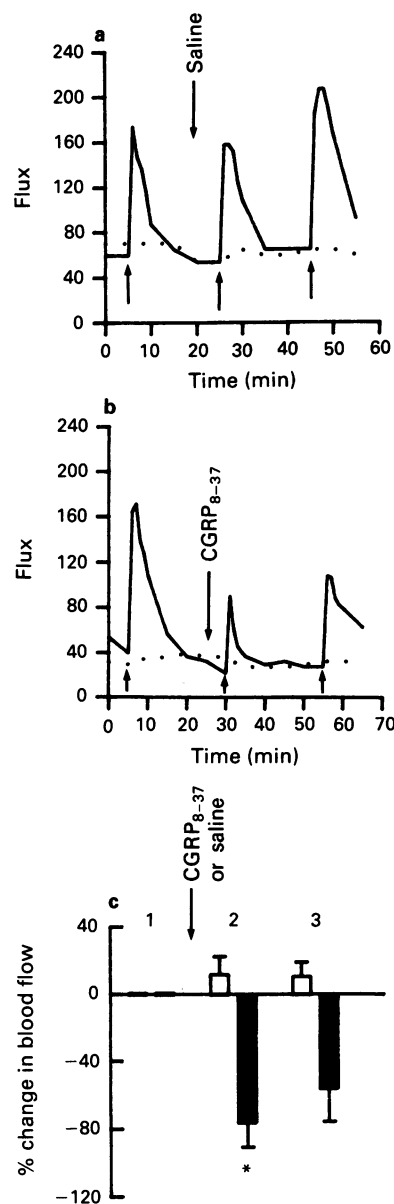

Figure 3 Effect of $CGRP_{8-37}$ on blood flow in the skin of rat hind paw after saphenous nerve stimulation (10 V, 1 ms, 2 Hz, for 30 s). (a) Effect of repeated stimulations of the saphenous nerve on blood flow in the skin of a rat hind paw. The rat received vehicle (0.1% BSA in saline, i.v.) 5 min prior to the second stimulation. (b) Effect of $CGRP_{8-37}$ (400 nmol kg^{-1}, i.v., 5 min before the second stimulation) on saphenous-induced blood flow in the rat hind paw skin. The results for (a) and (b) are expressed as Flux (arbitrary unit) and the dotted line indicates flux recorded on the contralateral paw. Stimulation of the saphenous nerve is represented by the upward arrows. (c) Collected results to show the effect of $CGRP_{8-37}$ on blood flow induced by saphenous nerve stimulation. Results are expressed as percentage change in blood flow compared to the first stimulation and are the mean ± s.e.mean of 4 rats in each group. In each group $CGRP_{8-37}$ or vehicle were administered i.v. at least 5 min prior to the second stimulation. $CGRP_{8-37}$ inhibited blood flow after the second stimulation ($*P<0.05$) and the third stimulation.

total inhibition of blood flow induced by capsaicin (10 mM). However, little inhibitory effect of a higher dose of capsaicin, 33 mM was observed.

A short (30 s) electrical stimulation of the saphenous nerve caused an increased blood flow which returned to baseline levels within 5–10 min after stimulation. This response was repeatable at least three times, as shown in Figure 3a. $CGRP_{8-37}$ caused an inhibition of the peak response as shown in Figure 3b. The group data, with an inhibition of stimulated blood flow at both time points after saphenous nerve stimulation are shown in Figure 3c. No evidence of oedema formation was observed within this short stimulation period. By comparison increasing the stimulation period to 5 min led to the well established neurogenic oedema formation (shown in Figure 4) as first described by Jancso *et al.* (1967). The oedema was significantly attenuated by $CGRP_{8-37}$. The results suggest that $CGRP_{8-37}$ acted to inhibit approximately 50% of the oedema formation induced by electrical stimulation of the saphenous nerve.

Discussion

The results show that $CGRP_{8-37}$ acts in a selective manner to antagonize the actions of exogenous CGRP in increasing blood flow. $CGRP_{8-37}$ also significantly inhibited the increased blood flow induced by topical capsaicin (10 mM) and saphenous nerve stimulation, as well as oedema formation induced by saphenous nerve stimulation. $CGRP_{8-37}$ failed to inhibit the increased blood flow induced by the highest dose of capsaicin (33 mM). The reason for this is unclear; possibly a maximal response combined with the release of increased levels of CGRP resulted in an inability of $CGRP_{8-37}$ to inhibit the increased blood flow. Alternatively other vasodilator mediators might be involved in mediating the increased blood flow induced by this dose of capsaicin.

The stimulation of the saphenous nerve in rats previously treated with guanethidine, to deplete a sympathetic component, has become an established small animal model of neurogenic inflammation, since Lembeck & Holzer (1979) provided evidence that substance P acts as a neurogenic mediator of antidromic vasodilatation and neurogenic plasma extravasation. In those experiments, blood flow was measured by femoral vein outflow. In the present experiments blood flow is measured by laser Doppler flowmetry. The use of the laser Doppler blood flow meter to measure saphenous nerve-stimulated increased blood flow was first used by Gamse & Saria (1987). It became immediately apparent that an increase in blood flow could be induced by a mild and short electrical stimulation when compared with the electrical stimulation necessary for the

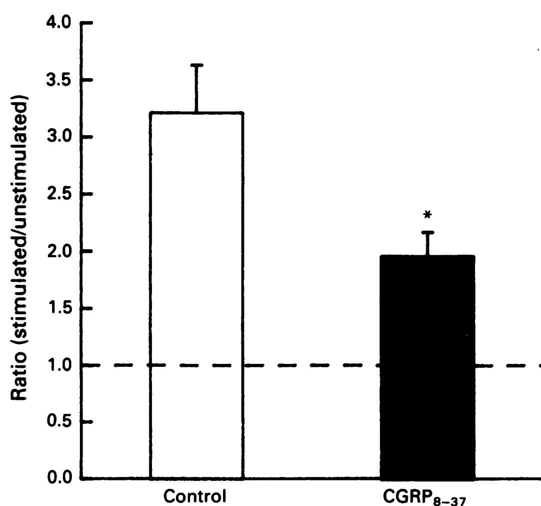

Figure 4 Effect of $CGRP_{8-37}$ (400 nmol kg^{-1}, i.v.) on oedema formation after electrical stimulation of the saphenous nerve (10 V, 1 ms, 2 Hz for 5 min). Results are expressed as the ratio of plasma extravasation in the stimulated compared to the sham-treated hind paw skin. The dashed line at 1 indicates the basal level of plasma extravasation in unstimulated paws. Results are expressed as mean ± s.e. mean of 10 rats in each group. $CGRP_{8-37}$ (solid column) significantly reduced plasma extravasation as indicated by $*P<0.05$, in comparison to the control rats (0.1% BSA, i.v., open column).

observation of oedema formation. The reason for this is not clear. It is probably related to the potency of endogenous CGRP as a vasodilator in skin. It is also possible that CGRP is released from A-delta fibres in addition to C-fibre nerves in initial saphenous nerve stimulation (Janig & Lisney, 1989). The present results are compatible with the suggestion that CGRP is the principle vasodilator released after mild and short stimulation of sensory nerves and this is in keeping with the findings of Delay-Goyet and co-workers (1992) who published a brief communication with similar findings whilst this study was in progress. They also investigated the effect of a neurokinin-1 antagonist (RP67580) and showed that this had no effect on saphenous nerve stimulation-mediated changes in blood flow.

Thus our findings indicate that CGRP mediates the increase in cutaneous blood flow observed with saphenous nerve stimulation. Results of previous studies in our group show that CGRP, does not act by itself to increase microvascular permeability, but as a consequence of its vasodilator activity potentiates oedema formation induced by mediators of increased microvascular permeability such as substance P and neurokinin A, in species that include the rat (Brain & Williams, 1985; 1989). We therefore investigated whether $CGRP_{8-37}$ could affect oedema formation induced by saphenous nerve stimulation. The results show an approximately 50% inhibition. Similar experimental protocols have been used to investigate the effect of recently developed non-peptide NK_1 receptor antagonists on oedema formation induced by saphenous nerve stimulation. CP96345 (Lembeck *et al.*, 1992; Xu *et al.*, 1992) and RP67580 (Garret *et al.*, 1991) almost completely abolished oedema formation, whilst a NK_2 receptor antagonist, Men10207, had no inhibitory effect (Xu *et al.*, 1992). These results are in keeping with the previous suggestion that the NK_1 receptor mediates increased microvascular permeability (Andrews *et al.*, 1989). The partial inhibitory activity of $CGRP_{8-37}$, on oedema formation induced by saphenous nerve stimulation, indicates that endogenously-released CGRP is involved in neurogenic oedema formation. An increase in blood flow is observed and this is concomitant with oedema formation (Shepheard *et al.*, 1992). It is unlikely that substance P acts directly via NK_1 receptors to increase blood flow as the selective NK_1 receptor agonist, GR73632, does not increase blood flow in rat skin, although it has potent oedema inducing effects which are potentiated by CGRP (Birch *et al.*, 1992; Richards *et al.*, 1993). Further it is known that the selective NK_1 receptor antagonist RP67580 does not affect the increased blood flow induced by stimulation of the saphenous nerve at levels sufficient to induce oedema formation (Shepheard *et al.*, 1992). Thus a vasodilator component is likely to be involved in the resulting oedema formation. There are several possibilities as to the identity of this vasodilator. Firstly the increased blood flow could be totally induced by CGRP at concentrations that were not possible to antagonize in the present experiments when oedema formation occurs in response to a 5 min electrical stimulation period. It should be remembered that $CGRP_{8-37}$ is a peptide antagonist which is poorly characterized whilst CP96345 and RP67580 are non-peptide structures, developed on the basis of their affinity and selectivity as NK_1 receptor antagonists. Alternatively it is possible that substance P is released from the saphenous nerve in sufficient quantities to stimulate mast cell degranulation and thus the release of the vasoactive amines histamine and 5-hydroxytryptamine (5-HT). This has been suggested by previous studies where evidence for the involvement of histamine and 5-HT has been obtained (Lembeck & Holzer, 1979; Morton & Chahl, 1980). However this would appear unlikely as the amines do not participate in the oedema response which, as discussed above, is completely inhibited by NK_1 receptor antagonists. It is also possible that another distinct, perhaps novel vasodilator, in addition to CGRP, is released from sensory nerves upon saphenous nerve stimulation. This is the most exciting possibility but we have no evidence to date that this is so.

We consider that the involvement of CGRP in neurogenic oedema formation is of interest. It has recently been demonstrated that the NK_1 receptor antagonist, RP67580, inhibits oedema formation in the rat dura induced by trigeminal nerve stimulation (Shepheard *et al.*, 1993); a neurogenic model that is considered to be relevant to migraine in man. Also increased CGRP levels have been measured in venous blood samples taken from patients with migraine (Goadsby *et al.*, 1990). As CGRP is colocalized with substance P it could be released in the rat dura in vasodilator amounts. It is not known whether the endogenous release of CGRP is pro-inflammatory in terms of vasodilatation or oedema formation in the rat dura; but our results obtained in this study would suggest that such a possibility may exist. However, it should also be pointed out that CGRP, at vasodilator doses, is anti-inflammatory in the hamster cheek pouch causing a clear inhibition of mediator induced oedema formation (Raud *et al.*, 1991). Our studies suggest that this anti-inflammatory mechanism does not operate in rat skin or paw (Newbold & Brain, 1993); but the collected findings highlight the possibility that CGRP could well have multiple effects on the inflammatory response.

In conclusion we have shown that $CGRP_{8-37}$ inhibits vasodilatation induced by topical capsaicin and saphenous nerve stimulation in the rat and thus provide evidence that endogenous CGRP is the major vasodilator mediator in these models of neurogenic inflammation. $CGRP_{8-37}$ also significantly inhibited neurogenic oedema formation induced by stimulation of the saphenous nerve. We consider this is good evidence to suggest that CGRP potentiates oedema formation when co-released from sensory nerves with substance P.

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