A calcitonin gene-related peptide (CGRP) antagonist (CGRP₈₋₃₇) inhibits microvascular responses induced by CGRP and capsaicin in skin

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1 The effect of the calcitonin gene-related peptide (CGRP) antagonist CGRP₈₋₃₇ on responses to CGRP and other mediators was investigated in rabbit dorsal skin.

2 Blood flow changes at intradermally-injected sites were measured by a multiple site ¹³³xenon clearance technique. CGRP₈₋₃₇ had little effect on blood flow at doses up to 0.3 nmol/site, when injected alone, although a significant increase in blood flow was observed at the highest dose tested (1 nmol/site).

3 CGRP₈₋₃₇ dose-dependently inhibited the increased blood flow induced by human α CGRP and human β CGRP, but had no effect on equivalent vasodilator responses induced by vasoactive intestinal peptide (VIP) and prostaglandin E₁ (PGE₁). CGRP₈₋₃₇ showed a preferential ability to inhibit α CGRP (IC₅₀ 0.04 nmol), when compared with β CGRP (IC₅₀ $\geq$ 0.3 nmol).

4 Capsaicin, which selectively activates sensory nerves, caused a dose-dependent increase in blood flow when injected intradermally into rabbit skin. The effects of capsaicin (0.01–0.1 μ mol/site) were inhibited by CGRP₈₋₃₇ (0.3 nmol/site), with a partial but significant attenuation of blood flow induced by the highest dose of capsaicin.

5 Oedema formation, induced by intradermal histamine injection (3 nmol/site), was measured in rabbit skin by the local accumulation of intravenously-injected ¹²⁵I-labelled albumin. Vasodilator doses of CGRP, PGE₁ and capsaicin potentiated, in a synergistic manner, oedema formation induced by histamine. CGRP₈₋₃₇ totally inhibited the potentiating effect of CGRP, partially inhibited the synergistic effect of capsaicin, but did not affect PGE₁-induced responses.

6 The results suggest that capsaicin acts to release a rabbit form of CGRP in skin and that CGRP₈₋₃₇ is a useful antagonist for investigating the potential of CGRP as a neurogenic mediator of inflammation.

Keywords: CGRP; blood flow; capsaicin; oedema; skin

Introduction

Calcitonin gene-related peptide (CGRP) is a potent vasodilator neuropeptide which stimulates increased blood flow when injected intradermally into skin, as demonstrated in a number of species (Brain *et al.*, 1985; Brain & Williams, 1988). CGRP increases blood flow in a dose-dependent manner. The increased blood flow is long lasting at picomolar doses and, in studies carried out in human skin, the vasodilator activity is clearly observed as a long lasting local erythema (Brain *et al.*, 1985; 1986b). Studies in rabbit skin have revealed that CGRP, as a consequence of its vasodilator activity, potentiates oedema formation induced by a range of mediators of increased microvascular permeability including the neuropeptide substance P (with which CGRP is co-localized in sensory nerves), histamine, bradykinin and platelet activating factor (PAF) (Brain & Williams, 1985; 1989).

Two forms of human CGRP (α and β) and two forms of rat CGRP (α and β) have been determined from molecular biology studies and purification studies (Amara *et al.*, 1982; Morris *et al.*, 1984; Rosenfeld *et al.*, 1984; Steenbergh *et al.*, 1985). All structures determined to date have greater than 90% structural homology. In previous studies in rabbit skin we have demonstrated that all the forms of CGRP tested exhibit a similar potency in increasing microvascular blood flow (Brain *et al.*, 1986a); whilst amylin amide, a peptide with 46% structural homology to human α CGRP, is 100 fold less potent (Brain *et al.*, 1990). These findings strongly suggest the existence of CGRP receptors in the rabbit skin microvasculature.

Chiba and colleagues (1989) demonstrated that a fragment of human α CGRP (CGRP₈₋₃₇) acted as an antagonist by dis-

placing CGRP from receptors present in rat liver plasma membranes. This study is confirmed by functional studies which demonstrated that CGRP₈₋₃₇ is an inhibitor of vascular receptors. CGRP₈₋₃₇ inhibited vasodilatation of the rat mesenteric arterial bed induced, *in vitro*, by periarterial nerve stimulation (Han *et al.*, 1990); inhibited CGRP responses of aortic tissue *in vitro* (Gray *et al.*, 1991) and inhibited the haemodynamic actions of intravenously-injected human α CGRP in the conscious rat (Gardiner *et al.*, 1990). Recent studies indicate that at least two subtypes of CGRP receptors, exist, CGRP₁ and CGRP₂ and CGRP₈₋₃₇ is suggested to be a potent antagonist of the CGRP₁ receptor (Dennis *et al.*, 1989; 1990). Maggi and co-workers (1991) have demonstrated that CGRP₈₋₃₇ competitively antagonizes human α CGRP and capsaicin-induced responses in the guinea-pig atria and rat vas deferens *in vitro*.

In this study we have investigated the effect of CGRP₈₋₃₇ on the increase in microvascular blood flow induced by CGRP and other vasodilators, including capsaicin which acts by stimulating endogenous neuropeptide release. We have also investigated the effect of CGRP₈₋₃₇ on inflammatory oedema responses which have been induced by histamine and potentiated in a synergistic manner by various vasodilators.

Methods

Blood flow and oedema formation were measured in the skin of male New Zealand White rabbits (2–2.5 kg). The rabbits were anaesthetized with sodium pentobarbitone (Sagatal, May & Baker: 30 mg kg⁻¹) given intravenous (i.v.) via the marginal ear vein. The dorsal fur of the rabbits was then removed by shaving and anaesthesia maintained by further administration as necessary. Skin blood flow (Williams, 1976) or skin oedema

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formation (Williams, 1979) was then measured in response to intradermally (i.d.)-injected test agents.

Measurement of blood flow: ^{133}Xe clearance

A site plan was marked out on the dorsal skin with six blocks of up to a maximum of nine injection sites, allocated according to a balanced site pattern. Test agents were then made up to 1 ml in saline and an aliquot (33.3 μCi) of ^{133}Xe added. The agents were then rapidly loaded into syringes and injected via 27 gauge needles i.d. into the skin sites (0.1 ml/site). The injections were given in random order in replicates of six (one into each block). The clock was started midway through injections and a clearance period of 15 min allowed. The rabbit was then killed by barbiturate overdose and the dorsal skin removed. Injection sites (16 mm diameter) were punched out and counted for radioactivity, under liquid paraffin, in a gamma-counter. Results were then calculated as % change in blood flow from control, saline-injected sites.

Measurement of oedema formation: ^{125}I accumulation

Oedema formation was measured in a separate series of experiments, as follows. A site plan was marked out on the dorsal skin with six blocks of 15 injection sites, allocated according to a balanced site pattern. A mixture of ^{125}I -labelled human serum albumin (10 μCi) and Evans Blue dye (2 ml of 2.5% w/v in saline) were administered i.v. via the marginal ear vein. Test agents were then injected i.d. in a random order and in replicates of six. After a 30 min accumulation period, a cardiac blood sample was taken (up to 10 ml) and the rabbit killed by barbiturate overdose. Injection sites were removed from the dorsal skin and counted for radioactivity in a gamma counter. Oedema formation at each skin site was then expressed as the volume of plasma accumulated, as calculated by comparing the number of counts in each skin site with that present in 1 ml of plasma.

Materials

CGRP₈₋₃₇ was obtained from Bachem (UK) Ltd, Saffron Walden, Essex. Recombinant human α and β CGRP were obtained as gifts from Dr U. Ney, Celltech, Slough, Bucks. Vasoactive intestinal peptide (VIP), histamine, PGE₁ and Evans Blue were obtained from Sigma Chemical Company, Poole, Dorset. These agents were stored in concentrated form at -20°C in either ethanol or saline containing 0.25% bovine serum albumin. Each agent was diluted in saline, as required, immediately prior to use. Capsaicin (pelargonic acid vanillylamide) was obtained from Fluka Chemicals Ltd, Glossop, Derbyshire and was stored at room temperature. Capsaicin (100 mg ml⁻¹) was dissolved in a mixture (2:1:7) of absolute alcohol: Tween 80 (Sigma): saline and diluted in saline immediately prior to use. Solvent controls were used as necessary. ^{125}I -labelled human serum albumin and ^{133}Xe were obtained from Amersham International plc, Aylesbury, Buckinghamshire and stored at 4°C .

Statistics

Bonferroni's modified *t* test was used to assess the significance of differences between treatments. The modified test uses the s.e. estimate for the analysis of variance to account for the fact that multiple tests were performed (**P* < 0.05 and ***P* < 0.01).

Results

The effect of increasing doses of intradermally-injected CGRP₈₋₃₇ on basal blood flow was measured by the multiple site ^{133}Xe clearance technique. Results are shown in Figure 1. At the lowest dose tested (0.03 nmol/site), the antagonist

Figure 1 The effect of CGRP₈₋₃₇ on basal blood flow in rabbit skin. The dose-related effect of CGRP₈₋₃₇ (C8-37) is shown. The response to saline is indicated by the dashed line. Results are mean values (s.e.mean indicated by vertical bars) for *n* = 4 rabbits and * indicates a significant change (*P* < 0.05) in blood flow from that measured in response to the previous dose.

caused a small, non-significant inhibition of blood flow. The two consecutive doses (0.1 and 0.3 nmol/site) had no effect but, in contrast, a significant increase in blood flow was observed following administration of the highest dose of CGRP₈₋₃₇ tested (1 nmol/site).

The effect of CGRP₈₋₃₇, at a sub-vasodilator dose (0.3 nmol/site), on the dose-dependent increases in blood flow induced by human α CGRP (0.3–3 pmol/site) and human β CGRP (0.3–3 pmol/site) is shown in Figure 2. Human α and β CGRP increased blood flow in a similar manner, and the concomitant injection of CGRP₈₋₃₇ inhibited blood flow induced by both α CGRP and β CGRP. The inhibitory effect of CGRP₈₋₃₇ was selective for the CGRP peptide, as blood flow induced by the structurally-unrelated neuropeptide, vasoactive intestinal peptide (VIP) was not affected; results were as follows: VIP (10 pmol) $58 \pm 10\%$ increase in blood flow, VIP + CGRP₈₋₃₇ $49 \pm 5\%$ increase in blood flow from control, saline-injected sites, mean $\pm$ s.e.mean, *n* = 8.

Figure 2 shows that CGRP₈₋₃₇ caused a total inhibition of the increase in blood flow induced by the highest dose of α CGRP, but only a partial inhibition of the increase in blood flow induced by the highest dose of β CGRP. We investigated this partial inhibition further by studying the ability of increasing doses of the antagonist to inhibit responses induced by a high dose of α and β CGRP respectively, as shown in Figure 3. The experiments were designed so that the effect of CGRP₈₋₃₇ on human α and β CGRP (3 pmol/site) was simultaneously tested in the same nine rabbits. CGRP₈₋₃₇ (0.03–0.3 nmol) acted dose-dependently to inhibit completely the effects of α CGRP (*IC*₅₀ = 0.04 nmol). In contrast, CGRP₈₋₃₇ inhibited blood flow induced by β -CGRP in a less potent manner (*IC*₅₀ $\geq$ 0.3 nmol).

The vasodilator response to intradermal injection of capsaicin is shown in Figure 4. CGRP₈₋₃₇ (0.3 nmol/site) caused an inhibition of capsaicin-induced blood flow at every dose in every animal tested. Figure 4 shows that, although partial, a significant inhibition of blood flow induced by capsaicin 0.1 μmol was observed. For control, Figure 4 also shows that the increased blood flow induced by the prostanoid vasodilator PGE₁ was not inhibited by CGRP₈₋₃₇.

Two chemically distinct vasodilators, α CGRP and PGE₁, acted in a synergistic manner to potentiate inflammatory oedema induced by histamine, as shown in Figure 5. However, only the potentiation of oedema by α CGRP was inhibited by CGRP₈₋₃₇. The effect of CGRP₈₋₃₇ on the ability of capsaicin

Figure 2 The effect of CGRP₈₋₃₇ on blood flow induced by increasing doses of human α calcitonin gene-related peptide (α CGRP) and human β CGRP. CGRP₈₋₃₇ (0.3 nmol/site⁻¹) was injected together with each dose of human α CGRP and human β CGRP: (a) shows responses to α CGRP in the absence (○) and the presence (●) of CGRP₈₋₃₇; (b) shows responses to β CGRP in the absence (□) and presence (■) of CGRP₈₋₃₇. In both graphs, the response to CGRP₈₋₃₇ alone is shown by (◆) and blood flow at control, saline-injected sites is indicated by the dashed line. Results are expressed as mean values (s.e. mean indicated by vertical bars) for $n = 4-11$ rabbits and a significant inhibitory effect of CGRP₈₋₃₇ is indicated by * ($P < 0.05$) or ** ($P < 0.01$).

to potentiate the effects of histamine is also shown. Capsaicin itself induced a small amount of oedema formation at the higher dose. The effect of CGRP₈₋₃₇ on oedema induced by histamine in the presence of capsaicin was tested. CGRP₈₋₃₇ caused a significant but partial inhibition of this response, as shown in Figure 5.

Discussion

The results show that CGRP₈₋₃₇, when injected intradermally with vasodilators, acts as a selective antagonist to inhibit CGRP responses in rabbit skin. The antagonist inhibited vasodilator responses induced by human α CGRP, β CGRP and capsaicin (which stimulates endogenous neuropeptide release) but had no effect on vasodilator responses induced by the prostanoid PGE₁ and the structurally unrelated neuropeptide vasodilator, VIP. The antagonist also inhibited the ability of CGRP and capsaicin to potentiate inflammatory oedema formation induced by histamine.

CGRP₈₋₃₇ inhibited responses to human α CGRP (IC₅₀ 0.04 nmol) in a more potent manner than β CGRP (IC₅₀ $\geq$ 0.3 nmol). In previous *in vitro* studies, CGRP₈₋₃₇ has been shown to have preferential effects on CGRP₁ receptors (Dennis *et al.*, 1989; 1990). Thus it is possible that α CGRP acts preferentially on CGRP₁ receptors in rabbit skin, whilst

Figure 3 The effect of increasing doses of CGRP₈₋₃₇ (C8-37) on blood flow induced by human α calcitonin gene-related peptide (α CGRP, 3 pmol/site) and human β CGRP (3 pmol/site): (a) shows the increased blood flow induced by human α CGRP (○) and the effect of increasing doses of CGRP₈₋₃₇ on this response (●); (b) shows the level to which blood flow is increased by β CGRP (□) and the effect of increasing doses of CGRP₈₋₃₇ on this response (■). In both graphs, the dashed line at 0% refers to control, saline-injected sites. Results are from experiments carried out in the same animals and each point is expressed as mean value (s.e. mean shown by vertical bars) for $n = 9$ rabbits. A significant inhibitory effect of CGRP₈₋₃₇ is indicated by ** ($P < 0.01$).

β CGRP causes increased blood flow by acting on more than one CGRP receptor in this tissue. This suggests that caution should be used in the interpretation of human studies where CGRP₈₋₃₇ is used. Both α and β forms of CGRP can be released and exert functional effects in man, but CGRP₈₋₃₇ might have differential inhibitory effects dependent on the form of CGRP released.

CGRP₈₋₃₇ has previously been shown to cause vasoconstriction when infused alone in the rat mesenteric bed *in vitro* (Han *et al.*, 1990) and in the rat hindquarters *in vivo* (Gardiner *et al.*, 1990). Results from our experiments show that at a low concentration CGRP₈₋₃₇ caused a small non-significant decrease in blood flow, possibly as a consequence of inhibiting the actions of basally-released endogenous CGRP. However, at a higher concentration CGRP₈₋₃₇ exhibited significant vasodilator activity, suggesting some agonist activity. This result is in keeping with CGRP₈₋₃₇ acting as a partial agonist at the CGRP₁ receptor. Alternatively, Chiba and colleagues (1989) have demonstrated that CGRP₈₋₃₇ is a potent agonist at calcitonin receptors; thus perhaps CGRP₈₋₃₇ could be acting via calcitonin receptors, at higher concentrations, to increase blood flow in rabbit skin. However, we consider this unlikely as human calcitonin does not exhibit vasodilator activity in rabbit skin at similar doses (Brain *et al.*, 1985).

Figure 4 The effect of CGRP₈₋₃₇ on capsaicin and prostaglandin E₁ (PGE₁)-induced blood flow. The dose-related effect of capsaicin is shown in the absence (○) and in the presence (●) of CGRP₈₋₃₇ (C8-37, 0.3 nmol/site). The response to PGE₁ (10 pmol, open column) remains unaffected by CGRP₈₋₃₇ (filled column). Blood flow at control, saline-injected skin sites is indicated by the dashed line and the effect of CGRP₈₋₃₇ alone (0.3 nmol) is shown by (◆). Results are the mean values (s.e.mean shown by vertical bars) for $n = 4-10$ rabbits and the significant inhibitory effect of CGRP₈₋₃₇ is shown as * ($P < 0.05$).

Our first evidence for the release of a rabbit form of CGRP from capsaicin-sensitive nerves in skin comes from studies where an antibody to human α CGRP has been used to prevent the actions of endogenous CGRP. The Fab fraction of the CGRP antibody caused a significant but partial inhibition of capsaicin-induced blood flow when administered intradermally in rabbit skin (Buckley *et al.*, 1991c). The results in this paper, using the CGRP antagonist, add further support to the suggestion that capsaicin releases vasodilator quantities of CGRP in rabbit skin. Blood flow induced by low doses of capsaicin was inhibited, with higher doses inhibited partially but significantly, by CGRP₈₋₃₇. The partial inhibition of high dose capsaicin-induced blood flow by both antibody and antagonist is of interest and it is possible that sensory nerves release other vasodilators, in addition to CGRP. Alternatively, it is possible that insufficient antibody/antagonist were present for the total inhibition/antagonism of perivascular CGRP released in close proximity to vascular receptors.

The results in this study provide evidence that a CGRP antagonist can inhibit the vasodilatation which is induced in skin by endogenously-released CGRP and inflammatory oedema which occurs as a result of the synergistic action of CGRP and mediators of increased microvascular permeability (in this case histamine). In other studies, we have suggested that CGRP, as a consequence of its vasodilator activity, can

Figure 5 The effect of CGRP₈₋₃₇ (C8-37) on oedema formation which is induced by histamine (Hist, 3 nmol/site) and potentiated by calcitonin gene-related peptide (CGRP, 3 pmol/site), prostaglandin E₁ (PGE₁, 10 pmol/site) and capsaicin (Cap, 0.3 and 1 μmol/site) is shown. The response to saline is shown by the dashed line. The open columns represent responses to individual agents injected alone and the synergistic responses induced by histamine in the presence of each vasodilator, as labelled. The filled columns represent the effect of CGRP₈₋₃₇ (0.3 nmol/site) on each synergistic response and the effect of CGRP₈₋₃₇ when injected alone. Results are expressed as mean values (s.e.mean shown by vertical bars) for $n = 5$ rabbits. The significant inhibitory effect of CGRP₈₋₃₇ is shown as * ($P < 0.05$) or ** ($P < 0.01$).

potentiate neutrophil accumulation and the pro-inflammatory actions of the cytokine interleukin-1 (Buckley *et al.*, 1991a,b).

In conclusion, we would like to suggest that, if CGRP is released as a neurogenic component of inflammation, an antagonist of CGRP could act in a novel manner as an anti-inflammatory agent to limit inflammatory erythema, oedema formation and cell accumulation. Future studies will be designed to test this hypothesis.

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