British Journal of Pharmacology (2004) 142, 1091–1098
www.nature.com/bjp

The calcitonin gene-related peptide (CGRP) receptor antagonist BIBN4096BS blocks CGRP and adrenomedullin vasoactive responses in the microvasculature

[1,2]A.D. Grant, [1,2]C.W. Tam, [1]Z. Lazar, [1]M.K. Shih & *,[1]S.D. Brain

[1]Centre for Cardiovascular Biology & Medicine, New Hunt's House, King's College London, Guy's Campus, London SE1 1UL, U.K.

1 Calcitonin gene-related peptide (CGRP) is a potent microvascular dilator neuropeptide that is considered to play an essential role in neurogenic vasodilatation and in maintaining functional integrity in peripheral tissues.

2 We have examined the effect of the nonpeptide CGRP antagonist BIBN4096BS on responses to CGRP and the structurally related peptide adrenomedullin, AM, in murine isolated aorta and mesentery preparations, and in the cutaneous microvasculature *in vivo*.

3 We show for the first time that BIBN4096BS is an effective antagonist of CGRP and AM responses in the murine mesenteric and cutaneous microvasculature, and of CGRP in the murine aorta. After local administration, BIBN4096BS selectively inhibits the potentiation of microvascular permeability in the cutaneous microvasculature by CGRP and AM, with no effect on responses induced by other microvascular vasodilators. BIBN4096BS reversed both newly developed and established vasoactive responses induced by CGRP.

4 The ability of CGRP to potentiate plasma extravasation was lost when coinjected with compound 48/80 (where mast cells would be activated to release proteases), but regained when soybean trypsin inhibitor was coinjected with compound 48/80.

5 These results demonstrate that BIBN4096BS is a selective antagonist of responses induced by CGRP and AM in the mouse microvasculature, and CGRP in the mouse aorta. The ability of BIBN4096BS to block an established CGRP microvascular vasodilatation indicates that the sustained vasodilator activity of CGRP is due to the retention of the active intact peptide and the continued involvement of the CGRP receptor.

British Journal of Pharmacology (2004) **142,** 1091–1098. doi:10.1038/sj.bjp.0705824

Keywords: CGRP; adrenomedullin; microvasculature; BIBN4096BS; mouse

Abbreviations: AM, adrenomedullin; AM_{13-52}, adrenomedullin 13–52; BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; CL, calcitonin receptor-like receptor; PGD_2, prostaglandin D_2; PGE_1, prostaglandin E_1; RAMP, receptor activity-modifying protein; SBTI, soybean trypsin inhibitor; SNP, sodium nitroprusside; VIP, vasoactive intestinal peptide

Introduction

Calcitonin gene-related peptide (CGRP) is synthesised in nociceptive sensory neurons and is commonly colocalised with other neuropeptides, especially substance P (Lundberg *et al*., 1985). It is a potent arterial vasodilator and induces a hypotensive response when injected intravenously in a number of species including man (Franco-Cereceda *et al*., 1987). However, studies with the CGRP antagonist $CGRP_{8-37}$ in rats and dogs have failed to identify any effect on basal blood pressure (Gardiner *et al*., 1990; Gardiner *et al*., 1991; Shen *et al*., 2001). Certain strains of CGRP knockout mice also have the same basal blood pressure as their wild-type counterparts (Lu *et al*., 1999). These results provide evidence that CGRP does not play an important role in regulating systemic blood pressure under basal conditions. By comparison, CGRP has a distinct profile of activity in that it is one of the most potent microvascular vasodilator substances identified, with a microvascular potency approximately 10-fold greater than that of the vasodilator prostaglandins and the structurally distinct peptide vasoactive intestinal peptide (VIP), 30–100 times that of the structurally related peptides AM and amylin and 100–1000 times greater than other established vasodilators (e.g. acetylcholine, adenosine, 5-hydroxytryptamine and substance P; Brain *et al*., 1985). Rather than playing a role in the regulation of systemic blood pressure, it is likely that CGRP regulates blood flow at the level of individual organs. The intravenous infusion of subvasodepressor doses into the conscious rat leads to specific relaxant effects in a range of tissues, such as a reduction in hindquarters vascular resistance (Gardiner *et al*., 1989). The concept of CGRP as a highly targeted vasodilator is enhanced by the observation of increased microvascular blood flow induced in the ipsilateral, but not contralateral, skin of the hind leg of the anaesthetised rat, after stimulation of CGRP-containing nerves, demonstrating

*Author for correspondence; E-mail: sue.brain@kcl.ac.uk
[2]These authors contributed equally to this study
Advance online publication: 5 July 2004

that its activity is primarily at the site of release (Escott & Brain, 1993). The potency of CGRP as a localised vasodilator has also been demonstrated by studies in which adenoviral transfer of CGRP prevents the vasospasm observed in models of subarachnoid haemorrhage in the rabbit (Toyoda *et al*., 2000) and dog (Satoh *et al*., 2002). The potent vasodilator activity of CGRP has also been identified in the cerebral circulation and led, in part, to the suggestion that it plays a role in the pathology of migraine (Goadsby *et al*., 1990). This in turn has highlighted a need to identify small nonpeptide receptor antagonists.

In addition to its potency, CGRP also differs from other vasodilator substances in that it has a long duration of action. A dose of 15 pmol injected into Caucasian human skin produces a local reddening, due to increased blood flow, which lasts for 5–6 h (Brain *et al*., 1985), and prolonged CGRP activity is also observed in rat skin (Brain & Williams, 1988). These results are in contrast to the finding that the half-life of intravascular CGRP is only 7 min (Kraenzlin *et al*., 1985), suggesting that it is rapidly metabolised in the blood. It is unclear whether this difference is due to greater CGRP stability in the skin, or an alternative mechanism, perhaps dependent on prolonged activation of the CGRP receptor or an intracellular effector mechanism.

Adrenomedullin (AM) is structurally related to CGRP and also possesses vasodilator activity, although it is 30–100 times less potent than CGRP in most systems. It is upregulated in inflamed tissues (Sugo *et al*., 1994a, b) and increased levels are found in a range of cardiovascular diseases, such as congestive heart failure (Jougasaki *et al*., 1995; Kato *et al*., 1996) and hypertension (Kitamura *et al*., 1994; Kohno *et al*., 1996). Furthermore, upregulation of AM mRNA occurs in the cutaneous microcirculation *in vivo*, in response to interleukin-1β (Chu *et al*., 2000). Interestingly, AM, or its biologically active fragment AM_{13-52} (that has similar potency) is known to act *via* the CGRP receptor in the microcirculation of several species, including hamster and rat (Hall *et al*., 1995; Chu *et al*., 2000).

The CGRP family of receptors is composed of a 7-transmembrane G protein-coupled receptor, calcitonin receptor-like receptor (CL) along with a single transmembrane accessory protein, receptor activity-modifying protein (RAMP). The dominant receptor for AM is thought to be CL/RAMP2 (McLatchie *et al*., 1998), while that for CGRP is CL/RAMP1 receptor (McLatchie *et al*., 1998). However, AM can also bind to the CL/RAMP1 receptor (Kuwasako *et al*., 2003) and evidence from this and other laboratories supports the concept that in the microvasculature of the hamster and the rat, AM mediates vasodilatation *via* interaction with the CGRP receptor (Hall *et al*., 1995; Chu *et al*., 2000).

In this study, we have investigated the ability of a nonpeptide CGRP receptor antagonist (BIBN4096BS; Doods *et al*., 2000) to act selectively to block microvascular responses induced by CGRP and AM in the mouse and also used the antagonist to learn more of the sustained vasodilator activity of CGRP.

Methods

The experiments were carried out under the Animals (Scientific Procedures) Act, 1986. Normal female CD1 mice (22–27 g) were obtained from Charles River, U.K. All mice were maintained on normal diet, with free access to food and water, in a climatically controlled environment.

Measurement of vascular relaxant activity in the mouse isolated mesentery

CD1 mice were killed by exposure to a rising concentration of CO_2 or by i.p. injection of urethane (ethyl carbamate; 25% w/v solution; 2.5 mg g^{-1}) followed by cervical dislocation. The intestine was laid out, at which point the ileocolic branch of the mesentery was clamped (Berthiaume *et al*., 1997; Tam & Brain, 2004). The aorta was freed from connective tissue and at the point where the aorta branched to form the renal and mesenteric arteries, the renal artery was ligated with suture cotton (size 6/0). The aorta was cannulated and as soon as the cannula was secured, the artery and mesentery were flushed with heparinised modified Krebs' solution (composition in mM: NaCl 120, KCl, 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.5, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11). Cutting as close to the intestine as possible, the mesentery was separated from the intestine and mounted in an organ chamber. It was left to equilibrate for >60 min with continuous perfusion of modified Krebs solution containing 3.3 μM indomethacin at a flow rate of 3.5 ml min^{-1} gassed with 95% O_2/5% CO_2 and maintained at 37°C. After equilibration, the vascular tone of the tissue was raised by perfusion of 15 μM noradrenaline for approximately 30 min or until a steady state was reached, before introduction of CGRP, the biologically active fragment AM_{13-52} (referred to as AM from here on), or sodium nitroprusside (SNP). Changes in the perfusion pressure were measured by a pressure transducer (Bell and Howell, U.K.) and recorded on a MacLab (ADInstruments, U.K.).

Measurement of vascular relaxant activity in the mouse aorta

CD1 mice were killed by exposure to a rising concentration of CO_2 followed by cervical dislocation and the thoracic aortae were quickly removed and placed in Kreb's solution (composition as above) gassed with 95% O_2/5% CO_2. The aortae were dissected free of surrounding tissue and cut transversely into rings 2 mm wide. The rings were mounted on hooks in a 2 ml organ bath containing Krebs solution gassed with 95% O_2/5% CO_2 at 37°C. The rings were given a loading tension of 1 g and left to equilibrate for 60 min (with changes in bathing fluid every 10 min). Contractile and relaxant responses were measured isometrically using force displacement transducers (Lectromed, U.K.) and recorded *via* a MacLab (ADInstruments, U.K.). Aortic rings were contracted with noradrenaline (100 nM) to produce a stable contractile tone (>0.2 g tension). All rings were tested with acetylcholine (1 μM) and only tissues showing >50% relaxation (endothelium intact) were used in this study. Increasing concentrations of CGRP or AM were added cumulatively to the bath. Results are expressed as percentage relaxation of noradrenaline-induced tone.

Indirect assay of vasodilator activity by measure of ability to potentiate plasma extravasation in dorsal skin

Anaesthesia was induced using urethane, as above, and a deep, surgical level of anaesthesia was maintained throughout all

procedures, as assessed by the absence of a flinch reaction in response to pinching of the paw or the tip of the tail, or touching the eye. Direct measurements of vasodilatation by techniques such as laser Doppler flowmetry or clearance of ^{99m}Tc in skin are difficult to obtain (S. Brain, unpublished observation). Thus plasma extravasation, in response to intradermal or topical application of test agents, was measured by the extravascular accumulation of intravenously injected ^{125}I-labelled bovine serum albumin (BSA; Cao *et al*., 1999). Once anaesthetised, the dorsal skin was shaved, and a randomised injection pattern was marked out. In all cases, [^{125}I]BSA (45 kBq; Cao *et al*., 1999) was injected with saline (0.1 ml i.v. into tail vein) and then flushed through with 0.05 ml saline. After 5 min, Tyrode's solution (vehicle control; concentration in mM: NaCl 136.9, KCl 2.7, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, $MgCl_2$ 1.1 and glucose 5.6), substance P, substance P with a vasodilator or the vasodilator alone were injected intradermally into the skin in a total volume of 50 μl. The vasodilators used in this study were CGRP, AM (300 pmol site^{-1}), VIP (3 pmol site^{-1}), prostaglandin (PG) E_1 (300 pmol site^{-1}), PGD_2 (100 pmol site^{-1}) and the long-acting NO donor NOC-12 (300 nmol site^{-1}). In a further set of experiments, substance P was replaced by either histamine (3 nmol site^{-1}), or the mast cell activator compound 48/80 (500 ng site^{-1}) in the presence or absence of soybean trypsin inhibitor (3 μg site^{-1}). One site was not injected, to control for the inflammatory effects of the physical process of injection. Plasma extravasation was allowed to develop for a period of 30 min, a blood sample (0.3–0.7 ml) was then taken by cardiac puncture, and the animal killed by cervical dislocation. The blood samples were centrifuged at 6000 g for 4 min, after which plasma was taken for measurement of radioactivity in a gamma counter (Wallac, U.K.). The dorsal skin was removed and the injection sites were punched out using an 8 mm leather punch. The sites were weighed, and their radioactivity was measured. All plasma extravasation results were expressed as microlitre of plasma accumulated per gram of tissue.

Further experiments were performed to analyse effects on developing and established CGRP responses where intradermal injections were carried out at two separate time points. After shaving and marking the skin, Tyrode solution or CGRP in a total volume of 25 μl were injected intradermally. After 30 min, a second series of injections, of either Tyrode or substance P in a total volume of 25 μl was given, and plasma extravasation allowed for a further 30 min. Blood samples were then collected and radioactivity of skin sites was measured as described above.

Effect of antagonists

BIBN4096BS was coadministered to the perfused mesentery as a bolus dose of 300 pmol into the flow of perfusate, along with agonist. The isolated aorta was bathed in either/both BIBN4096BS (10 μM) or/and AM_{22-52} (10 μM) for 20 min prior to recordings. During measurement of potentiation of plasma extravasation by vasodilators, BIBN4096BS was coadministered as a bolus dose (300 pmol) i.d. along with the vasoactive agents. In experiments to determine the effect on developing and established CGRP responses, BIBN4096BS was either administered with the CGRP, or 30 min after the CGRP.

Materials

AM (gift from Dr Marcus Schindler, Boehringer-Ingelheim, Germany) was used for aorta and skin studies and AM_{13-52} (from Phoenix Pharmaceuticals Ltd, U.S.A.), the biologically active fragment of AM, used for mesentery studies. Human αCGRP was from Phoenix Pharmaceuticals Ltd, U.S.A. [^{125}I]BSA was purchased from ICN, U.K. BSA, urethane, substance P, VIP, SNP, NOC-12 (3-ethyl-3(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene), PGE_1, PGD_2, indomethacin, acetylcholine, histamine, compound 48/80, soybean trypsin inhibitor and noradrenaline were obtained from Sigma Chemicals, Poole Dorset, U.K. All the peptides used in this study were dissolved in a minimum volume of ultrapure water, then diluted to stock concentrations in saline containing 0.01% BSA. PGE_1 and PGD_2 were dissolved in ethanol. Urethane, noradrenaline, acetylcholine and SNP were dissolved in saline. Histamine, compound 48/80 and soybean trypsin inhibitor (SBTI) were dissolved in Tyrode solution. NOC-12 was prepared in a minimum volume of NaOH (1 M), then diluted in saline. Indomethacin was dissolved in sodium bicarbonate (5% w v^{-1}) solution. AM_{22-52} was from Bachem Ltd, U.K. AM_{22-52} was made up in 10 mM acetic acid and then diluted to a final concentration in Krebs' solution. BIBN4096BS (1-piperidinecarboxamide,*N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl)-,[R-(R*,S*)]) was kindly donated by Dr Marcus Schindler, Boehringer-Ingelheim, Germany. BIBN4096BS was dissolved in a minimum volume of HCl (1 N). The solution was made up to the required final volume with saline (at a stock concentration of 2 mg ml^{-1}), and then titrated with NaOH (1 M) to return the pH to neutral.

Analysis of results and statistical analysis

Relaxant activity in the mesenteric vasculature was measured as a change in perfusion pressure, and as the % relaxation of noradrenaline-induced tone in aortic rings. Plasma extravasation in the dorsal skin was measured as microlitre of plasma accumulated per gram of tissue. Plasma extravasation and mesenteric assays were analysed by one-way ANOVA followed by Bonferroni's modified *t* test. The isolated aorta assay data was analysed by two-way ANOVA followed by Bonferroni's modified *t* test.

Results

Initially the ability of BIBN4096BS to antagonise vasodilatation induced by exogenous CGRP was examined. The murine perfused isolated mesenteric bed *in vitro* provides a model for the direct measurement of microvascular vasodilatation. The vessels were preconstricted with noradrenaline prior to application of a vasodilator, and a decrease in perfusion pressure correlated with vasodilatation. Figure 1a shows a representative trace of the changes in perfusion pressure measured after a bolus dose of CGRP (30 pmol) alone, CGRP coadministered with BIBN4096BS (300 pmol), and then CGRP alone following washout of BIBN4096BS. These data are summarised as mean $\pm$ s.e.m. in Figure 1b. CGRP induced a significant decrease in perfusion pressure, which is abolished

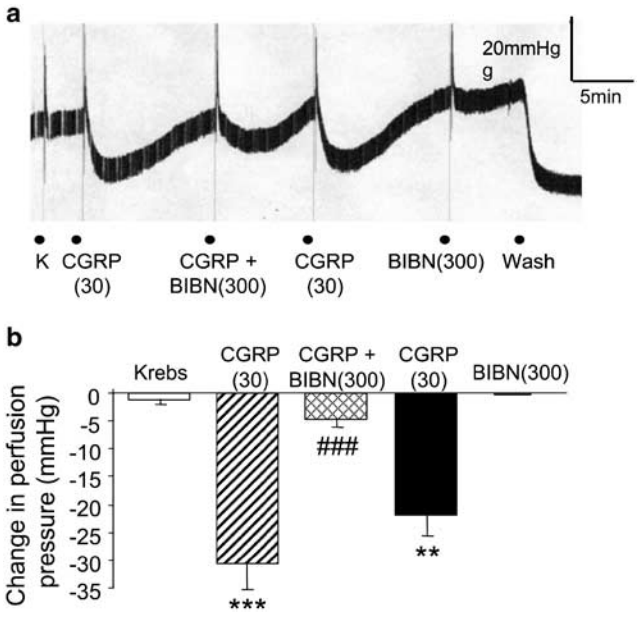

Figure 1 Effect of coadministered BIBN4096BS (BIBN; 300 pmol) on the change in perfusion pressure in the preconstricted mouse isolated mesenteric vasculature produced by a single dose of CGRP (30 pmol). (a) A typical trace illustrating the effect of CGRP administration on blood pressure after noradrenaline preconstriction. (b) Mean change in perfusion pressure produced by CGRP alone, coadministered with BIBN or after BIBN washout. Perfusion pressure, measured as an index of vasodilatation, is expressed as mmHg. Data in figure (b) is expressed as mean ± s.e.m., $n=5$. ** = $P<0.01$ and *** = $P<0.001$ compared to Krebs (K) alone. ### = $P<0.001$ compared to first CGRP dose.

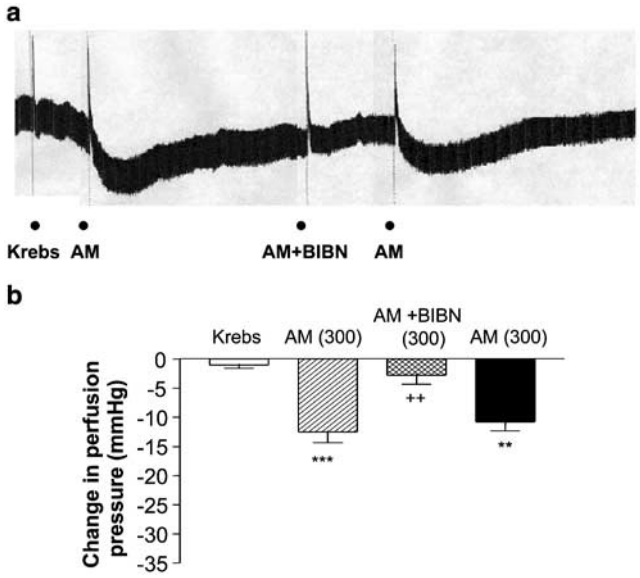

Figure 2 Effect of coadministered BIBN4096BS (BIBN; 300 pmol) on the change in perfusion pressure in the preconstricted mouse isolated mesenteric vasculature produced by a single dose of AM (300 pmol). (a) A typical trace illustrating the effect of AM administration on blood pressure after noradrenaline preconstriction. (b). Mean change in perfusion pressure produced by AM alone, coadministered with BIBN or after BIBN washout. Perfusion pressure, measured as an index of vasodilatation, is expressed as mmHg. Data in figure is expressed as mean ± s.e.m., $n=4$. ** = $P<0.01$ and *** = $P<0.001$ compared to Krebs alone. ++ = $P<0.001$ compared to first AM dose.

in the presence of BIBN4096BS. After washout of BIBN4096BS, the vasodilatation to CGRP was restored. In contrast, the NO donor compound SNP (300 nmol) produced a vasodilatation that was unaffected by treatment with BIBN4096BS (-15.5 ± 3.5 vs -13.0 ± 4.0 mmHg for SNP alone). Data for SNP + BIBN4096BS, $n=3$, are not shown. BIBN4096BS also antagonised responses induced by AM in the perfused mesentery (Figure 2). Similarly to CGRP, the response to AM was restored after washout of BIBN4096BS. This indicates that AM, in addition to CGRP, acts *via* the CGRP receptor to mediate microvascular relaxation in this tissue. However, there is evidence that AM acts *via* a CGRP-independent receptor in larger vessels of certain species, including the mouse, so the ability of BIBN4096BS to block AM responses was also investigated in the aorta (Ashton *et al*., 2000). CGRP and AM acted in a similar dose-dependent manner to induce vascular relaxation as shown in Figure 3. However, while the responses to CGRP were blocked by BIBN4096BS (Figure 3a), the responses to AM remained unaffected (Figure 3b). By comparison, the AM_{22-52} (an AM receptor antagonist) blocked the AM responses (Figure 3b). This demonstrates the ability of AM to act *via* both the AM and CGRP receptor in the mouse.

The ability of BIBN4096BS to antagonise vasodilatation induced by exogenous CGRP in murine dorsal skin microvasculature was then examined. An indirect measurement of vasodilatation was chosen, as the ability of CGRP to decrease perfusion pressure in the isolated mesentery confirms that it is a microvascular vasodilator in the mouse. Intradermal injection of substance P increases microvascular permeability, leading to an increase in plasma extravasation that can be measured by ^{125}I-albumin accumulation (Figure 4). Substance P acting *via* neurokinin NK_1 receptors is established as the principal mediator of neurogenic oedema formation (Lembeck & Holzer, 1979; Cao *et al*., 1999). Injection of CGRP alone has no effect on vascular permeability (Table 1), but coadministration of CGRP and substance P leads to a pronounced potentiation of the substance P-induced plasma extravasation, indicative of an increase in blood flow through the microvascular bed (Figure 4a). This potentiation was completely inhibited by the coinjection of BIBN4096BS (300 pmol site^{-1}), although the plasma extravasation induced by substance P still remained (Figure 4a). This pattern was mirrored with coadministration of substance P with AM (Figure 4b). The ability of other vasodilator agents to potentiate plasma extravasation after coinjection with substance P was then evaluated in dorsal skin. The susceptibility of the vasodilators to inhibition by BIBN4096BS was also examined. The structurally distinct neuropeptide VIP, the lipid prostaglandins (PGE_1 and PGD_2) and a long-acting NO-donor compound, NOC-12 (Hrabie *et al*., 1993), were also included in this study. None of the vasodilators examined produced any increase in plasma extravasation compared to Tyrode when injected alone (Table 1). However, they all significantly potentiated substance P-induced plasma extravasation. Only the potentiation by CGRP and AM was inhibited by local coinjected treatment with BIBN4096BS (Figure 4), while all the other test compounds were unaffected (Table 1).

Previous studies have demonstrated a long-lasting dilator activity of CGRP (Brain & Williams, 1989). To determine whether CGRP itself remained active in mouse skin for a

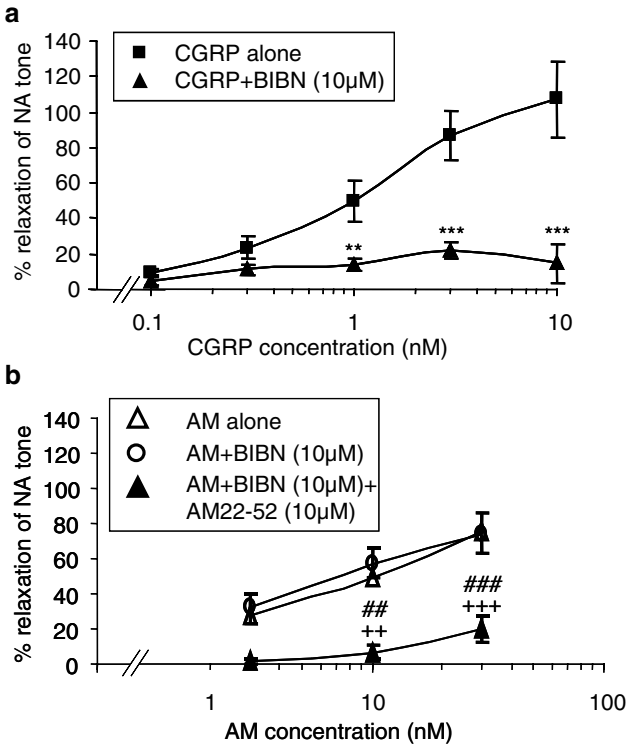

Figure 3 The effect of BIBN4096BS (BIBN; 10 μM) and AM_{22-52} (10 μM) on the relaxation induced by increasing concentrations of (a) CGRP and (b) AM in isolated rings of mouse aorta precontracted with (noradrenaline) NA. Results are expressed as % relaxation of NA-induced tone, mean$\pm$s.e.m., $n=3$–8 and 5–9, respectively. $**=P<0.01$, $***=P<0.001$ compared to CGRP alone. $++$, $\#\#=P<0.01$ and $+++$, $\#\#\#=P<0.001$ compared to AM alone and AM + BIBN, respectively.

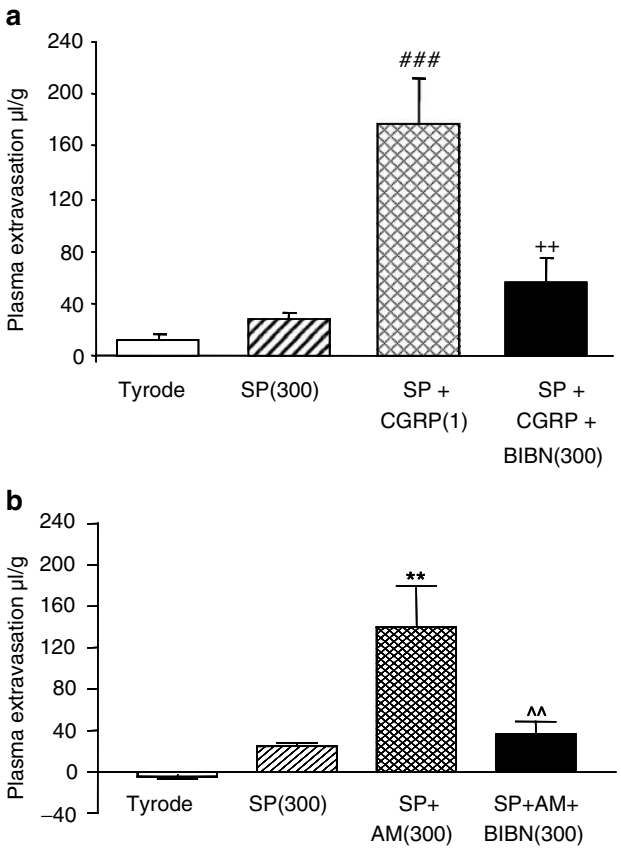

Figure 4 Effect of BIBN4096BS (BIBN; 300 pmol) on the potentiation of SP (300 pmol)-induced plasma extravasation by (a) CGRP (1 pmol) and (b) AM (300 pmol) in the dorsal skin of CD1 mice. Plasma extravasation is expressed as microlitres of plasma accumulated per gram of tissue, mean$\pm$s.e.m., $n=5$ and 9, respectively. $\#\#\#=P<0.001$ compared to SP alone (a) and $++=P<0.01$ compared to SP + CGRP. $**=P<0.01$ compared to SP alone (b) and $\wedge\wedge=P<0.01$ compared to SP + AM.

prolonged period, or whether the extended vasodilatation was due to activation of mechanisms downstream of the CGRP receptor, the dorsal skin experiments were repeated, but with CGRP administration 30 min before substance P (Figure 5). BIBN4096BS was either coadministered with CGRP (Figure 5a) or given 30 min later with substance P (Figure 5b). In both cases, CGRP administered 30 min before substance P was still active and able to potentiate the resulting plasma extravasation. Coinjection of BIBN4096BS either at 0 min with CGRP or with substance P, 30 min after CGRP, abolished this potentiation (Figure 5). These results reveal the sustained involvement of the CGRP receptor. To investigate this further, experiments were carried out to test the ability of CGRP to potentiate mast cell-dependent oedema under conditions where proteases such as tryptase would also be released. The results show that while CGRP clearly potentiates plasma extravasation induced by the mast cell amine histamine (Figure 6), it is unable to potentiate the plasma extravasation observed following mast cell activation by compound 48/80 (Figure 7a), unless the tryptase inhibitor soybean trypsin inhibitor was also present (Figure 7b).

Discussion

The present study reveals that BIBN4096BS selectively inhibits CGRP and AM-induced vasoactive responses in the murine mesenteric and cutaneous microvasculature; however, BIBN4096BS was only able to inhibit CGRP-induced responses in the mouse aorta. Evidence was also provided showing that BIBN4096BS can either be administered with CGRP to block a developing vasodilator response, or given after CGRP to block an established vasodilatation. The established vasodilatation induced by CGRP is due to a continuous interaction between CGRP and its receptor and this is supported by the finding that the potentiating ability of CGRP is lost under conditions where active proteases that degrade CGRP are present in skin.

The selective ability of BIBN4096BS to antagonise only CGRP receptor responses is demonstrated by the observation that while CGRP responses are blocked in all tissues by BIBN4096BS, AM responses were blocked in the microvascular tissues examined but not in the aorta. That is, BIBN4096BS was able to inhibit both CGRP and AM responses in the cutaneous and mesenteric microvasculature. It is unlikely that BIBN4096BS is interacting with the CL/RAMP2 (AM_1 receptor) or CL/RAMP3 (AM_2 receptor) complexes, as it has a very low affinity for these receptors (Hay *et al.*, 2003). Therefore, we suggest that BIBN4096BS is a selective antagonist at the CGRP receptor, and that AM appears to mediate its microvascular effects *via* the CGRP receptor. This confirms and strengthens our previous findings

Table 1 Summary of the abilities of vasodilator agents to potentiate plasma extravasation induced by substance P (SP; 300 pmol site^{-1}) in the dorsal skin of CD1 mice, either in the absence or presence of BIBN4096BS (BIBN; 300 pmol site^{-1})

	Vasodilator alone (μl/*g*)	*Vasodilator + SP* (μl/*g*)	*Vasodilator + SP + BIBN* (μl/*g*)
Tyrode	0.5±1.7	33.3±3.6	53.0±15.0
CGRP (1 pmol)	4.3±3.0	176.0±36.7***	55.7±19.8$^{\#\#}$
AM_{13-52} (300 pmol)	7.8±2.1	139.7±39.8**	36.7±11.7$^{\#\#}$
VIP (3 pmol)	−6.8±3.8	47.1±11.6*	63.3±13.6
PGE_1 (300 pmol)	13.1±10.4	97.2±26.8*	87.0±30.9
PGD_2 (100 pmol)	12.0±5.1	107.8±27.9*	115.6±46.4
NOC-12 (300 nmol)	16.6±3.4	118.0±21.2**	95.9±25.7

Results are expressed as microlitre of plasma accumulated per gram of tissue. * = $P<0.05$, ** = $P<0.01$ and *** = $P<0.001$ compared to substance *P* alone; $^{\#\#}$ = $P<0.01$ compared to vasodilator + SP.

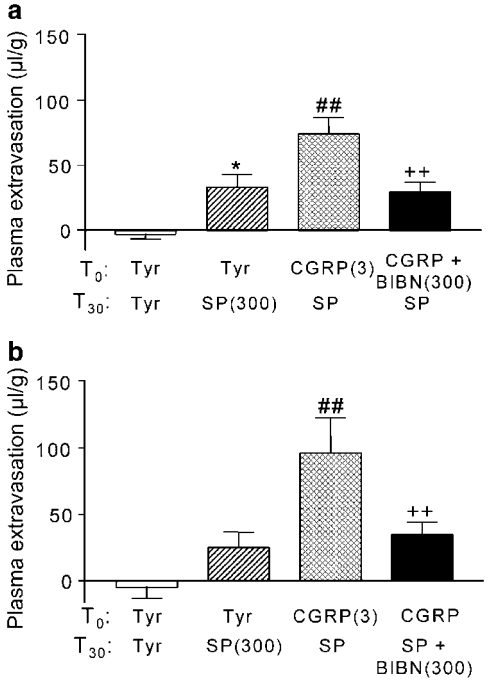

Figure 5 The effect of BIBN4096BS (BIBN; 300 pmol) on the ability of CGRP (3 pmol) administered at 0 min to potentiate plasma extravasation induced by substance P (SP; 300 pmol), administered at 30 min, in the dorsal skin of CD1 mice. (a). BIBN coinjected with CGRP at 0 min. (b). BIBN coinjected with substance P at 30 min. Results were measured over a 30 min period after substance P injection and expressed as microlitre of plasma accumulated per gram of tissue, mean±s.e.m., $n=10$ and 8, respectively. * = $P<0.05$, compared to Tyr/Tyr control site. $\#\# = P<0.01$ compared to Tyr/SP site. ++ = $P<0.01$ compared to CGRP/SP site.

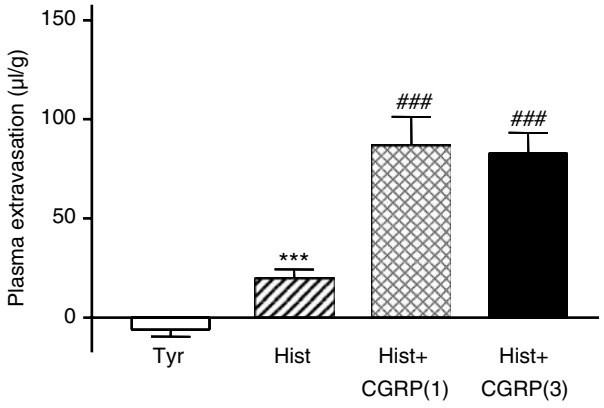

Figure 6 Effect of CGRP (1 or 3 pmol) on the plasma extravasation induced by coinjected histamine (3 nmol) in the dorsal skin of CD1 mice. Plasma extravasation is expressed as microlitre of plasma accumulated per gram of tissue, mean±s.e.m., $n=7$. *** = $P<0.001$ compared to Tyrode treatment. ### = $P<0.001$ compared to histamine alone.

that the microvascular effects of AM in the hamster and rat are mediated *via* the CGRP receptor (Hall *et al*., 1995; Chu *et al*., 2000), as BIBN4096BS is much more potent and selective than $CGRP_{8-37}$, the antagonist used in these previous studies. Overall, the available evidence indicates that BIBN4096BS is a selective antagonist at the CGRP receptor in the mouse.

These initial findings were followed by a fuller *in vivo* study of the vasoactive effect of CGRP in the mouse dorsal skin microvasculature. We used an indirect plasma extravasation assay in the mouse dorsal skin, due to the difficulty of directly measuring blood flow in this tissue, possibly due to the nature of murine skin and its movement as the mouse breathes. The ability of vasodilators to potentiate plasma extravasation induced by mediators of increased microvascular permeability was first demonstrated in the 1970s (Williams & Morley, 1973). Williams and co-workers suggested a 'two mediator hypothesis', in which microvascular vasodilators potentiate plasma extravasation as a consequence of increased blood flow. The increase in blood flow leads to a raised intravascular pressure in the postcapillary venule, the site of increased microvascular permeability and thus increased leakage. This hypothesis is supported by our finding that a range of known microvascular dilators are able to potentiate the plasma extravasation induced by substance P in the murine cutaneous microvasculature (Tam & Brain, 2004). The experiments carried out in the dorsal skin, in which coinjection of BIBN4096BS abolished the potentiation of plasma extravasation by CGRP, confirm that BIBN4096BS is a CGRP antagonist in the mouse. This activity of BIBN4096BS after local administration compares favourably with the peptide CGRP antagonist $CGRP_{8-37}$, which induces an inflammatory response after i.d. injection (S. Brain, unpublished observation). The selectivity of BIBN4096BS is demonstrated by its failure to inhibit the potentiation by a slow-release NO donor compound (Hrabie *et al*., 1993), the vasodilator prostaglandins PGE_1 and PGD_2, or VIP. The VIP data is particularly revealing as it is also a vasodilator neuropeptide that shares a second messenger system with CGRP (i.e. accumulation of cAMP). In addition, it signals *via* the VPAC-1 receptor that is suggested to interact with RAMP1, an accessory protein necessary for the formation of functional CGRP receptors (Christopoulos *et al*., 2003).

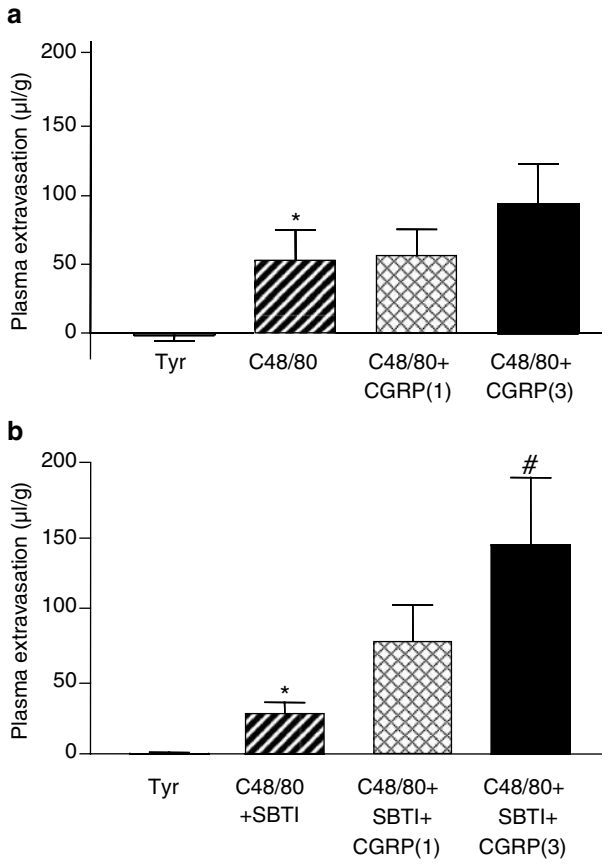

Figure 7 The effect of CGRP (1 or 3 pmol) on plasma extravasation induced by compound 48/80 (C48/80; 500 ng) in (a) the absence and (b) the presence of soybean trypsin inhibitor (SBTI; 3 μg) in the dorsal skin of CD1 mice. Results were measured over a 30 min period after compound 48/80 injection and expressed as microlitre of plasma accumulated per gram of tissue, mean $\pm$ s.e.m., $n=10$ and 9, respectively. $*=P<0.05$ compared to Tyrode site. $\#=P<0.05$ compared to compound 48/80 + SBTI (b).

The dorsal skin assay was also used to study the long duration of action of CGRP *in vivo* and in particular whether BIBN4096BS was able to reverse an established vasodilatation. In this study, CGRP administered 30 min prior to substance P was still able to potentiate the plasma extravasation, and this potentiation could be blocked by BIBN4096BS either coadministered with CGRP or administered with the substance P, 30 min after CGRP. These data provide evidence that the prolonged duration of action of CGRP seen in human skin (Brain *et al*., 1985) is also observed in the mouse, and demonstrates the ability of BIBN4096BS to reverse an existing CGRP-mediated vasodilatation, supporting the concept of the continued involvement of the CGRP receptor, rather than a downstream effector mechanism. It has been suggested that the long duration of action of CGRP is due to its stability at extravascular sites, unless exposed to proteases such as mast cell tryptase (Brain & Williams, 1989). This hypothesis is supported by experiments presented in this manuscript. Thus, the results are in keeping with the concept that CGRP released at extravascular sites can, depending on the proteolytic environment, have an extremely long duration of action.

The precise physiological roles of AM are not fully understood, although it is believed to have developmental and vasodilator roles, and may be involved in the inflammatory response. AM plays a critical role in correct development of the vasculature, and knockout of the AM gene is a lethal phenotype (Caron & Smithies, 2001), but the identity of the receptor which mediates this response is unclear. The relative contribution of the CGRP and AM receptors to cardiovascular processes remains unclear. The demonstration that in the mouse aorta, as for other species, AM acts to induce vascular relaxation *via* a BIBN4096BS-insensitive mechanism that is blocked by the AM antagonist AM_{22-52} confirms the importance of the AM receptor in this tissue.

In conclusion, this study has established that BIBN4096BS is a selective antagonist of the CGRP receptor in the mouse and shown the ability of BIBN4096BS to block both a developing and established vasoactive response of CGRP. The importance of the CGRP receptor in mediating the microvascular responses of AM as well as CGRP is demonstrated. Furthermore, the study has revealed that the sustained duration of CGRP activity, previously observed in the skin of a range of species including humans, is due to the continuing presence within skin of active CGRP that acts *via* the CGRP receptor.

We thank the British Biological Science Research Council (BBSRC) and the British Heart Foundation (BHF) for financial support and Boehringer-Ingelheim Pharma, Germany for the gift of BIBN4096BS. ZL was supported by an Eotvos Hungarian Postdoctoral Fellowship. We also thank Jolenta Cheung, who was supported by a KCL summer studentship, for her technical help.

References

ASHTON, D., HIEBLE, P., GOUT, B. & AIYAR, N. (2000). Vasodilatory effect of AM in mouse aorta. *Pharmacology,* **61,** 101–105.

BERTHIAUME, N., HESS, F., CHEN, A., REGOLI, D. & D'ORLEANS-JUSTE, P. (1997). Pharmacology of kinins in the arterial and venous mesenteric bed of normal and B2 knockout transgenic mice. *Eur. J. Pharmacol.,* **333,** 55–61.

BRAIN, S.D. & WILLIAMS, T.J. (1988). Substance P regulates the vasodilator activity of calcitonin gene-related peptide. *Nature,* **335,** 73–75.

BRAIN, S.D. & WILLIAMS, T.J. (1989). Interactions between the tachykinins and calcitonin gene-related peptide lead to the modulation of oedema formation and blood flow in rat skin. *Br. J. Pharmacol.,* **97,** 77–82.

BRAIN, S.D., WILLIAMS, T.J., TIPPINS, J.R., MORRIS, H.R. & MACINTYRE, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. *Nature,* **313,** 54–56.

CAO, T., GERARD, N.P. & BRAIN, S.D. (1999). Use of NK(1) knockout mice to analyze substance P-induced edema formation. *Am. J. Physiol.,* **277,** R476–R481.

CARON, K.M. & SMITHIES, O. (2001). Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional AM gene. *Proc. Natl. Acad. Sci. U.S.A.,* **98,** 615–619.

CHRISTOPOULOS, A., CHRISTOPOULOS, G., MORFIS, M., UDAWELA, M., LABURTHE, M., COUVINEAU, A., KUWASAKO, K., TILAKARATNE, N. & SEXTON, P.M. (2003). Novel receptor partners and function of receptor activity-modifying proteins. *J. Biol. Chem.,* **278,** 3293–3297.

CHU, D.Q., CHOY, M., FOSTER, P., CAO, T. & BRAIN, S.D. (2000). A comparative study of the ability of calcitonin gene-related peptide and AM (13–52) to modulate microvascular but not thermal hyperalgesia responses. *Br. J. Pharmacol.,* **130,** 1589–1596.

DOODS, H., HALLERMAYER, G., WU, D., ENTZEROTH, M., RUDOLF, K., ENGEL, W. & EBERLEIN, W. (2000). Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. *Br. J. Pharmacol.,* **129,** 420–423.

ESCOTT, K.J. & BRAIN, S.D. (1993). Effect of a calcitonin gene-related peptide antagonist (CGRP8-37) on skin vasodilatation and oedema induced by stimulation of the rat saphenous nerve. *Br. J. Pharmacol.,* **110,** 772–776.

FRANCO-CERECEDA, A., GENNARI, C., NAMI, R., AGNUSDEI, D., PERNOW, J., LUNDBERG, J.M. & FISCHER, J.A. (1987). Cardiovascular effects of calcitonin gene-related peptides I and II in man. *Circ. Res.,* **60,** 393–397.

GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1989). Regional hemodynamic effects of calcitonin gene-related peptide. *Am. J. Physiol.,* **256,** R332–R338.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A., BENNETT, T., BOSE, C., FOULKES, R. & HUGHES, B. (1990). Antagonistic effect of human alpha-CGRP [8–37] on the *in vivo* regional haemodynamic actions of human alpha-CGRP. *Biochem. Biophys. Res. Commun.,* **171,** 938–943.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A., BENNETT, T., BOSE, C., FOULKES, R. & HUGHES, B. (1991). Antagonistic effect of human alpha-calcitonin gene-related peptide (8- 37) on regional hemodynamic actions of rat islet amyloid polypeptide in conscious Long–Evans rats. *Diabetes,* **40,** 948–951.

GOADSBY, P.J., EDVINSSON, L. & EKMAN, R. (1990). Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann. Neurol.,* **28,** 183–187.

HALL, J.M., SINEY, L., LIPPTON, H., HYMAN, A., KANG-CHANG, J. & BRAIN, S.D. (1995). Interaction of human AM 13-52 with calcitonin gene-related peptide receptors in the microvasculature of the rat and hamster. *Br. J. Pharmacol.,* **114,** 592–597.

HAY, D.L., HOWITT, S.G., CONNER, A.C., SCHINDLER, M., SMITH, D.M. & POYNER, D.R. (2003). CL/RAMP2 and CL/RAMP3 produce pharmacologically distinct AM receptors: a comparison of effects of AM22-52, CGRP8-37 and BIBN4096BS. *Br. J. Pharmacol.,* **140,** 477–486.

HRABIE, J.A., KLOSE, J.R., WINK, D.A. & KEEFER, L.K. (1993). New nitric oxide-releasing zwitterions derived from polyamines. *J. Org. Chem.,* **58,** 1472–1476.

JOUGASAKI, M., WEI, C.M., MCKINLEY, L.J. & BURNETT JR, J.C. (1995). Elevation of circulating and ventricular AM in human congestive heart failure. *Circulation,* **92,** 286–289.

KATO, J., KOBAYASHI, K., ETOH, T., TANAKA, M., KITAMURA, K., IMAMURA, T., KOIWAYA, Y., KANGAWA, K. & ETO, T. (1996). Plasma AM concentration in patients with heart failure. *J. Clin. Endocrinol. Metab.,* **81,** 180–183.

KITAMURA, K., ICHIKI, Y., TANAKA, M., KAWAMOTO, M., EMURA, J., SAKAKIBARA, S., KANGAWA, K., MATSUO, H. & ETO, T. (1994). Immunoreactive AM in human plasma. *FEBS Lett.,* **341,** 288–290.

KOHNO, M., HANEHIRA, T., KANO, H., HORIO, T., YOKOKAWA, K., IKEDA, M., MINAMI, M., YASUNARI, K. & YOSHIKAWA, J. (1996). Plasma AM concentrations in essential hypertension. *Hypertension,* **27,** 102–107.

KRAENZLIN, M.E., CH'NG, J.L., MULDERRY, P.K., GHATEI, M.A. & BLOOM, S.R. (1985). Infusion of a novel peptide, calcitonin gene-related peptide (CGRP) in man. Pharmacokinetics and effects on gastric acid secretion and on gastrointestinal hormones. *Regul. Pept.,* **10,** 189–197.

KUWASAKO, K., KITAMURA, K., NAGOSHI, Y., CAO, Y.N. & ETO, T. (2003). Identification of the human receptor activity-modifying protein 1 domains responsible for agonist binding specificity. *J. Biol. Chem.,* **278,** 22623–22630.

LEMBECK, F. & HOLZER, P. (1979). Substance P as neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation. *Naunyn Schmiedebergs Arch. Pharmacol.,* **310,** 175–183.

LU, J.T., SON, Y.J., LEE, J., JETTON, T.L., SHIOTA, M., MOSCOSO, L., NISWENDER, K.D., LOEWY, A.D., MAGNUSON, M.A., SANES, J.R. & EMESON, R.B. (1999). Mice lacking alpha-calcitonin gene-related peptide exhibit normal cardiovascular regulation and neuromuscular development. *Mol. Cell Neurosci.,* **14,** 99–120.

LUNDBERG, J.M., FRANCO-CERECEDA, A., HUA, X., HOKFELT, T. & FISCHER, J.A. (1985). Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. *Eur. J. Pharmacol.,* **108,** 315–319.

MCLATCHIE, L.M., FRASER, N.J., MAIN, M.J., WISE, A., BROWN, J., THOMPSON, N., SOLARI, R., LEE, M.G. & FOORD, S.M. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin- receptor-like receptor. *Nature,* **393,** 333–339.

SATOH, M., PERKINS, E., KIMURA, H., TANG, J., CHUN, Y., HEISTAD, D.D. & ZHANG, J.H. (2002). Posttreatment with adenovirus-mediated gene transfer of calcitonin gene-related peptide to reverse cerebral vasospasm in dogs. *J. Neurosurg.,* **97,** 136–142.

SHEN, Y.T., PITTMAN, T.J., BUIE, P.S., BOLDUC, D.L., KANE, S.A., KOBLAN, K.S., GOULD, R.J. & LYNCH JR, J.J. (2001). Functional role of alpha-calcitonin gene-related peptide in the regulation of the cardiovascular system. *J. Pharmacol. Exp. Ther.,* **298,** 551–558.

SUGO, S., MINAMINO, N., KANGAWA, K., MIYAMOTO, K., KITAMURA, K., SAKATA, J., ETO, T. & MATSUO, H. (1994a). Endothelial cells actively synthesize and secrete AM. *Biochem. Biophys. Res. Commun.,* **201,** 1160–1166.

SUGO, S., MINAMINO, N., SHOJI, H., KANGAWA, K., KITAMURA, K., ETO, T. & MATSUO, H. (1994b). Production and secretion of AM from vascular smooth muscle cells: augmented production by tumor necrosis factor-alpha. *Biochem. Biophys. Res. Commun.,* **203,** 719–726.

TAM, C.W. & BRAIN, S.D. (2004). The assessment of vasoactive properties of CGRP and AM in the microvasculature: a study utilising *in vivo* and *in vitro* assays in the mouse. *J. Mol. Neurosci.,* **22,** 117–124.

TOYODA, K., FARACI, F.M., WATANABE, Y., UEDA, T., ANDRESEN, J.J., CHU, Y., OTAKE, S. & HEISTAD, D.D. (2000). Gene transfer of calcitonin gene-related peptide prevents vasoconstriction after subarachnoid hemorrhage. *Circ. Res.,* **87,** 818–824.

WILLIAMS, T.J. & MORLEY, J. (1973). Prostaglandins as potentiators of increased vascular permeability in inflammation. *Nature,* **246,** 215–217.

(Received January 26, 2004
Revised March 30, 2004
Accepted April 2, 2004)