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RESEARCH PAPER

Inhibitory effect of BIBN4096BS, $CGRP_{8-37}$, a CGRP antibody and an RNA-Spiegelmer on CGRP induced vasodilatation in the perfused and non-perfused rat middle cerebral artery

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Background and purpose: A new concept for the inhibition of CGRP signalling has been developed by interaction with the CGRP molecule *per se* by using a CGRP antibody or a CGRP binding RNA-Spiegelmer (NOX-C89). We have compared these CGRP scavengers with two known receptor antagonists ($CGRP_{8-37}$ and BIBN4096BS) on CGRP-induced relaxations in the rat middle cerebral artery (MCA). Furthermore, the role of the endothelial barrier has been studied.
Experimental approach: We used the luminally perfused MCA in an arteriograph, pressurized to 85 mm Hg and myograph studies of isolated ring segments of the MCA.
Key results: In myograph studies and in the perfusion system during abluminal application, αCGRP and βCGRP induced concentration-dependent dilatation of the MCA. Given luminally neither peptide was significantly vasodilator. Adrenomedullin and amylin induced weak dilatations. In myograph experiments, relaxation induced by αCGRP was prevented by the four CGRP blockers ($CGRP_{8-37}$, BIBN4096BS, the CGRP antibody and NOX-C89.). In abluminal perfusion experiments, the relaxant response to αCGRP was prevented by these agents to a varying degree. Dilatation induced by abluminal application of αCGRP was inhibited by luminal $CGRP_{8-37}$ but not by luminal BIBN4096BS, CGRP antibody or NOX-C89.
Conclusions and Implications: α or βCGRP acted on smooth muscle cell CGRP receptors in rat MCA and were effectively prevented from reaching these receptors by the endothelial barrier. The CGRP blockers significantly inhibited αCGRP induced relaxation but were also prevented from reaching the CGRP receptors by the arterial endothelium.
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Keywords: migraine; CGRP; amylin; adrenomedullin; CGRP-antibody; $CGRP_{8-37}$; BIBN4096BS; middle cerebral artery; RNA-Spiegelmer

Abbreviations: CBF, cerebral blood flow; CGRP, calcitonin gene-related peptide; MCA, middle cerebral artery

Introduction

Trigeminal sensory C-fibres innervating the cranial vasculature contain several vasoactive peptides such as calcitonin gene-related peptide (CGRP), neurokinin A (NKA), substance P (SP) and pituitary adenylate cyclase activating peptide (PACAP) (Gulbenkian *et al*., 2001). Stimulation of the trigeminal ganglion liberates vasoactive peptides into the perivascular space. CGRP may thus interact mainly with smooth muscle cells, as all receptor elements are expressed on the smooth muscle cells (Oliver *et al*., 2002), whereas presynaptic receptors on the perivascular nerves have only been inferred indirectly (Tajti *et al*., 1999). CGRP is a potent dilator of cerebral (pial) (Edvinsson *et al*., 1987) and dural blood vessels (Williamson *et al*., 1997; Jansen-Olesen *et al*., 2003), and participates as a vasodilator but not to induce plasma extravasation in dural neurogenic inflammation (Grant *et al*., 2005). Furthermore, CGRP levels are increased during acute migraine and cluster headache attacks (Goadsby *et al*., 1990; Edvinsson and Goadsby, 1994; Goadsby and Edvinsson, 1994) and the direct infusion of CGRP induces headache and migraine attacks in migraine patients (Goadsby *et al*., 1990; Goadsby and Edvinsson, 1994; Lassen *et al*., 2002).

BIBN4096BS is a novel, well-characterized CGRP receptor antagonist that is highly selective and potent in both animal

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and human experiments (Doods *et al*., 2000; Edvinsson *et al*., 2002; Petersen *et al*., 2005). As this compound is safe for human administration and, in a proof of concept phase-II study, effective in treating acute migraine attacks (Olesen *et al*., 2004; Petersen *et al*., 2005), research has focused on the development and understanding of the site of action of CGRP antagonists for migraine treatment. Both peptide ($CGRP_{8-37}$) and non-peptide ('compound 1' and SB-273779) molecules effectively antagonize CGRP *in vivo* and *in vitro* (Aiyar *et al*., 2001; Edvinsson *et al*., 2001; Williamson *et al*., 2001). These antagonists are, for a variety of reasons, however, not suitable for human use.

Recently, a new concept for the inhibition of CGRP signalling was developed, namely a CGRP-binding RNA-Spiegelmer. This is a single-stranded mirror-image oligonucleotide that is highly resistant to nuclease degradation and is capable of tightly and specifically binding to CGRP and thus to inhibit its function (Vater *et al*., 2003). In addition, a humanized CGRP-binding monoclonal antibody has been raised in rabbits for treatment of CGRP-related disorders such as migraine.

In the present study, we have compared the functional effects of the CGRP receptor antagonists (BIBN4096BS and $CGRP_{8-37}$), the CGRP antibody and the CGRP-binding RNA-Spiegelmer on the vasodilator response to αCGRP in isolated cerebral arteries. We have used two methods: (i) a sensitive myograph to study isolated ring segments of rat middle cerebral artery (MCA) (Hogestatt *et al*., 1983) and (ii) a pressure myograph to examine the effect of intraluminal and abluminal administration of agonists and antagonists (Bryan *et al*., 1996; You *et al*., 1997). The results suggest that CGRP receptors are located to the smooth muscle cells and that the blood–brain barrier prevents luminal CGRP and its blockers from reaching the arterial smooth muscle cells.

Materials and methods

Tissue preparation

The Animal Protocol Review committee at the University of Lund approved the experimental protocol (M111-04).

Male Sprague–Dawley rats (250–350 g) were anaesthetized using CO_2 and decapitated. The brain was immediately removed and placed in cold (4°C) buffer solution of the following composition (mM):NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 5.5. With the aid of a dissecting microscope, MCA segments were carefully harvested from each animal beginning at the circle of Willis and extending 5–8 mm distally.

Myograph experiments

Each MCA was cut into 1–2 mm long circular segments and placed in an ice-cold buffer solution gassed with 5% CO_2 in O_2 (Hogestatt *et al*., 1983). In order to determine vessel tension, each segment was mounted on two metal wires 40 μm in diameter in a myograph (Model 610M; Danish Myo Technology, Aarhus, Denmark). The artery segments were bathed in the buffer solution described above and were throughout the experiment continuously aerated with 95% O_2:5% CO_2 gas mixtures further stabilizing the pH at 7.4. The artery segments were allowed to equilibrate for approximately 30 min. The vessels were stretched to the internal circumference the vessels would have if relaxed and exposed to a passive transmural pressure of 75 mm Hg (10 kPa). This was carried out in order to achieve maximal active force development. Following a 30-min equilibration period, the vessels were constricted twice by 63 mM of KCl in a modified buffer solution in which NaCl was substituted for KCl on an equimolar basis. The force of these contractions amounted to 0.83 ± 0.07 mN ($n = 44$). In order to study the relaxant effect of CGRP, the MCA was pre-contracted with 3×10^{-6} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). This resulted in a stable tension of 0.87 ± 0.08 mN ($n = 44$) lasting for at least 30 min without significant fall in tone, to which agonists were added in increasing concentrations. In blockade experiments, a single concentration of a blocker (see below) was added 30 min before the addition of CGRP. Out of four segments run in parallel, two served as controls (i.e. without blocker) and the others were treated with blocker.

Pressurized arteriograph experiments

A section of the MCA (1–2 mm in length) was mounted in a pressurized arteriograph (Living Systems, Burlington, VT, USA) as described previously (Bryan *et al*., 1996; You *et al*., 1997). Micropipettes were inserted into both ends of the MCA and secured with 11-0 nylon ties. The MCA was bathed in the buffer solution (37°C) equilibrated with a gas mixture consisting of 5% CO_2 and 95% O_2, resulting in a pH of 7.4. Transmural pressure of the MCA was maintained at 85 mm Hg by raising reservoirs connected to the micropipettes to the appropriate height above the MCA. Luminal perfusion flow was adjusted to 100 $\mu l\,min^{-1}$ (range 0.1–0.09 ml min) by setting the two reservoirs at different heights. Pressure transducers on either side of the MCA provided direct measurement of perfusion pressure across the MCA.

The vessel was magnified 600-fold using a microscope coupled to a digital camera (Axis, Lund, Sweden) connected to a computer. The program Mary (Nihil KB, Lund, Sweden) saved the pictures at intervals of 1 s during the experiment as well as measuring the outer diameter of the vessels.

Following a resting period, the pressurized vessels attained a stable tone, usually considered to be due to shear stress. In the present study the initial vessel diameter was 197.3 ± 4.9 μm ($n = 67$) and after tone 135.1 ± 3.0 μm, which represents contraction by $30.8 \pm 1.0\%$ (Figure 1). Any MCA that did not develop spontaneous tone of at least 10% compared to the initial diameter within 1 h was excluded from the experiment. After another period of 30 min, the MCA segments were exposed to luminal ATP (10^{-5} M) in order to evaluate the function of the endothelium. A dilatation of at least 15% of the resting diameter was considered indicative of a functional endothelium. In the present study, the mean dilatation of the MCA segments to ATP was $99.0 \pm 6.1\%$ ($n = 67$). This was considered a positive sign for a functional endothelium and patency of the blood–brain barrier (Bryan *et al*., 1996; You *et al*., 1997). Experimental protocols were not initiated until the MCA diameter was stable over a 15-min period. The vascular response to

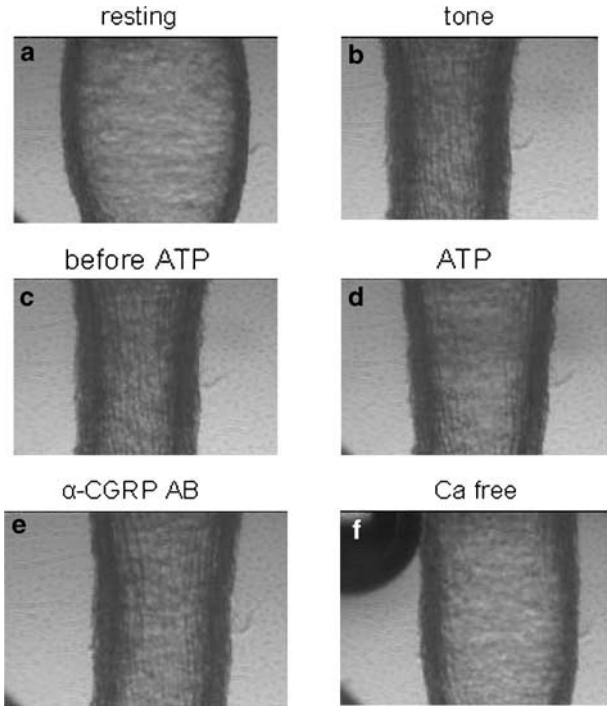

Figure 1 Illustration of the perfusion studies. (**a**) Baseline tone just after mounting of the vessel. (**b**) Following perfusion, tone was induced by the shear stress induced by perfusate flow through the lumen (0.1 ml/min). (**c**) Tone just before and (**d**) when ATP (10^{-5} M) was applied. (**e**) Tone when αCGRP (10^{-6} M) was applied abluminally and (**f**) when calcium-free buffer was perfused.

stimulation of endothelial receptors was carried out by adding αCGRP, βCGRP, adrenomedullin or amylin to the luminal perfusate (in the concentration range 10^{-12}–10^{-6} M). In addition, either CGRP peptide was added abluminally in the same concentration range to test the direct smooth muscle effect; this route of administration avoids interaction with the endothelium.

To characterize the responses to luminal and abluminal stimulation further, the two antagonists BIBN4096BS or $CGRP_{8–37}$ in doses up to 1 μM were used either alone or in the presence of increasing concentrations of αCGRP. In addition, the CGRP antibody (2.4 μg ml^{-1} when given abluminally and 12 μg ml^{-1} when given luminally) and the RNA-Spiegelmer (10^{-6} M) were used as above. Inhibitors were added either to the abluminal and the luminal perfusate 30 min before commencing application of αCGRP. At the end of each experiment, the vessel segments were exposed to a calcium-free buffer solution, which provided a measure of the maximum relaxant capability of the MCA segment (38.0±2.3 %) (Figure 1).

Data analysis

For data obtained in myograph experiments on isolated ring segments of the MCA, the amount of relaxation induced by each concentration of CGRP receptor agonist was calculated as percentage of $PGF_{2\alpha}$ precontraction. For the experiments performed on the pressurized arteriograph, the changes in measured diameters of the vessel segments are expressed as a percentage of the resting diameter. Dilatation is given as a percentage of the spontaneous tension obtained through perfusion. Values are given as mean±s.e.m. The number of experiments = n, which in the perfusion experiments equals the number of rats. E_{max} denotes the maximum response elicited by an agonist whereas pEC_{50} denotes the negative logarithm of the concentration needed to elicit half the maximum response. All concentrations expressed indicate the final concentration in the tissue baths and in the luminal or abluminal compartments of the pressurized arteriograph. Sigmoidal curve fitting was carried out using the computer program GraphPad Prism (GraphPad Software, San Diego, CA, USA). Based on the principal equation for a sigmoidal curve, the program makes iterated computations to derive a best fit based upon the actual experimental values. The non-parametric, Mann–Whitney *U*-test was used to determine statistical significance between two groups of data. Statistical significance was assumed when $P<0.05$.

Drugs

ATP, Rat αCGRP, βCGRP, adrenomedullin, amylin and $CGRP_{8–37}$ (Sigma, St Louis, MO, USA) were dissolved in distilled water. The CGRP Spiegelmer NOX-C89 (a gift from NOXXON Pharma AG, Berlin, Germany) was dissolved in the same buffer solution as used throughout the experiments. BIBN4096BS was a gift synthesized by Merck (Terlings Park, UK). BIBN4096BS was dissolved in a small volume (20 μl) of 1 N HCl, further diluted with saline (0.9%) and finally adjusted to pH 6.5–7.0 by 1 N NaOH. Stock solutions of each compound were aliquoted and frozen at –20°C. A stock solution of the humanized CGRP binding monoclonal antibody raised in rabbit was obtained from Rinat Neuroscience (San Fransisco, CA, USA). Stock solutions were stored frozen in small aliquots until use. For experiments, drugs were diluted in the buffer solution immediately before use. All chemicals were obtained from Merck (Darmstadt, Germany). Only double distilled water was used throughout the experiments.

Results

Concentration–effect relations of CGRP, adrenomedullin and amylin

Pressurized arteriograph experiments. Abluminally applied αCGRP and βCGRP caused significant relaxations of 23±2.4% (n=12) and 11±1.1% (n=3), respectively (Figure 2). The pEC_{50} values for abluminal αCGRP and βCGRP were 9.31±0.35 and 9.02±0.43, respectively. Luminal administration of αCGRP and βCGRP showed no significant effects (1.8±0.85, and 0.0±0.0%, respectively). Slight relaxations were seen following abluminal application of amylin (E_{max}: 7.0±0.6%; pEC_{50}: 9.39±0.74) and adrenomedullin (E_{max}: 7.5±1.3%; pEC_{50}: 7.90±0.35). The response to luminally applied amylin was weak (E_{max}: 4.7±1.5%; n=3) and that of adrenomedullin did not differ from baseline (Figure 2).

Myograph experiments. The addition of increasing concentrations of αCGRP and βCGRP (10^{-10}–10^{-7} M) resulted in

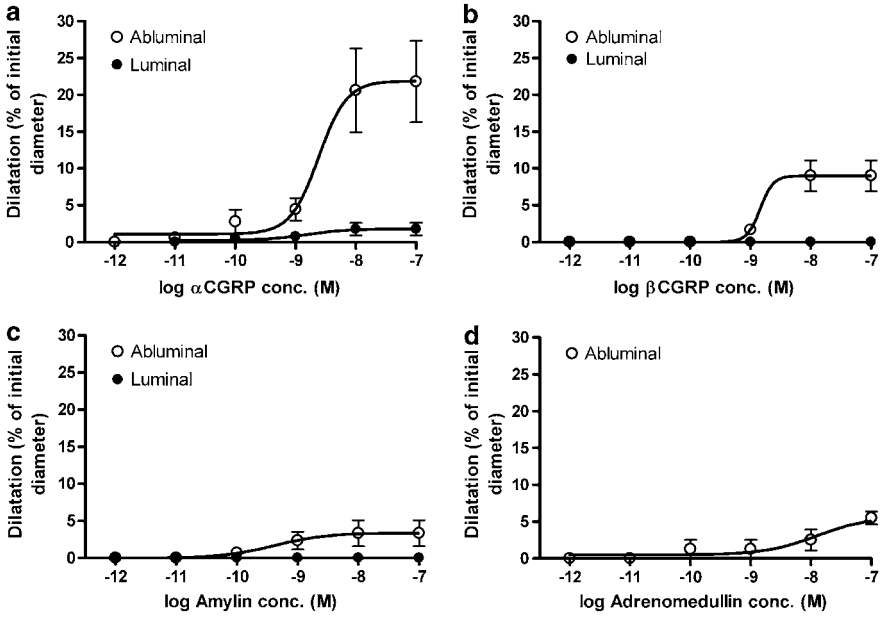

Figure 2 Concentration–dilation curves in perfused MCA for abluminally or luminally administered (**a**) αCGRP, (**b**) βCGRP, (**c**) amylin and (**d**) adrenomedullin. Values are given as means±s.e.m., $n=3$–12.

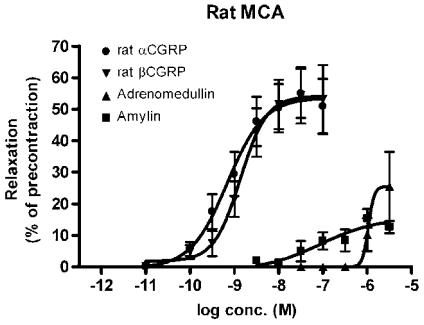

Figure 3 The concentration–effect relationship of rat αCGRP, βCGRP, adrenomedullin and amylin on rings of MCA, pre-contracted with $PGF_{2\alpha}$ (3×10^{-6} M). Values are given as means±s.e.m., $n=4$–15.

concentration-dependent relaxations of the MCA (αCGRP, E_{max}: 54±5%; pEC_{50}: 9.16±0.21) and βCGRP (E_{max}: 54±5%; pEC_{50}: 8.86±0.16) (Figure 3). The pEC_{50} values for amylin – and adrenomedullin-induced relaxations were 7.05±0.89 and 6.00±1.32, respectively. E_{max} value for amylin was 16±7% and for adrenomedullin 26±15% (Figure 3).

Effect of CGRP inhibitors on αCGRP induced vasodilatation

Pressurized arteriograph experiments. When applied *per se* in increasing concentrations up to 1 μM none of the four CGRP blockers, BIBN4096BS, $CGRP_{8-37}$, the CGRP antibodies or NOX-C89, given luminally or abluminally had any effect on the resting tone of MCA (data not shown).

The effect of these blockers was studied only on the concentration-dependent dilatation of the pressurized MCA induced by abluminal administration of αCGRP (10^{-9}–10^{-7} M) (Figure 4). The αCGRP response was significantly inhibited by abluminally applied $CGRP_{8-37}$ (10^{-6} M) and BIBN4096BS ($P<0.05$). Luminal $CGRP_{8-37}$ also inhibited the abluminal αCGRP responses (Figure 5a), whereas luminal BIBN4096BS did not (Figures 4b and 5b).

The CGRP antibody was examined in a concentration of 2.4 μg/ml when given abluminally and 12 μg ml^{-1} when given luminally. When given abluminally, the antibody caused a significant inhibition of the αCGRP-induced relaxation only at 10^{-8} M of αCGRP (Figure 4c). There was no significant blockade of abluminal αCGRP by luminal administration of the CGRP antibody (Figure 5c).

Neither abluminal nor luminal administration of 10^{-6} M NOX-C89 showed a significant inhibition of the αCGRP induced relaxation (Figures 4d and 5d).

Myograph experiments. The responses to αCGRP were significantly attenuated by $CGRP_{8-37}$ (10^{-6} M), BIBN4096BS (10^{-7} M) and NOX-C89 (10^{-6} M) (Figure 6a, b and d) ml^{-1}. The CGRP antibody, given in a concentration of 4.8 μg, did not decrease the relaxation induced by αCGRP (data not shown). However, increasing the CGRP antibody concentration to 12 μg/ml significantly inhibited the αCGRP-induced relaxation, as shown in Figure 6c. In concentration–response experiments, NOX-C89 (10^{-6} M) and 12 μg ml^{-1} CGRP antibody produced significantly lower pEC50 values of αCGRP (10^{-10}–10^{-7} M) mediated relaxation. However, the pEC50 values were not affected by the presence of BIBN4096BS (10^{-7} M) (Table 1). In these experiments, 12 μg/ml of the CGRP antibody and BIBN4096BS (10^{-7} M), but not $CGRP_{8-37}$ (10^{-6} M) and NOX-C89 (10^{-6} M), significantly lowered maximum responses to α-CGRP (Table 1).

Discussion

The present study has demonstrated that the rat MCA contains smooth muscle cell $CGRP_1$ receptors, the CGRP

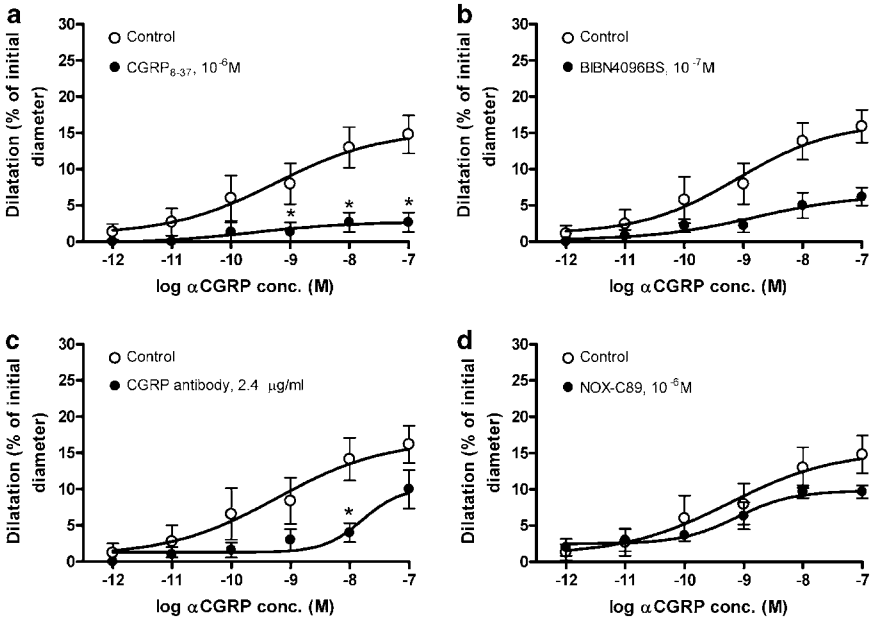

Figure 4 Relaxant effect of abluminal αCGRP in perfused MCA after abluminal application of (**a**) 10^{-6} M $CGRP_{8-37}$, (**b**) 10^{-7} M BIBN4096BS, (**c**) 2.4 $\mu g\,ml^{-1}$ CGRP antibody and (**d**) 10^{-6} M NOX-C89. Values are given as means ± s.e.m. Statistical analysis by Mann–Whitney *U*-test, $^*P<0.01$, $n=3$–7.

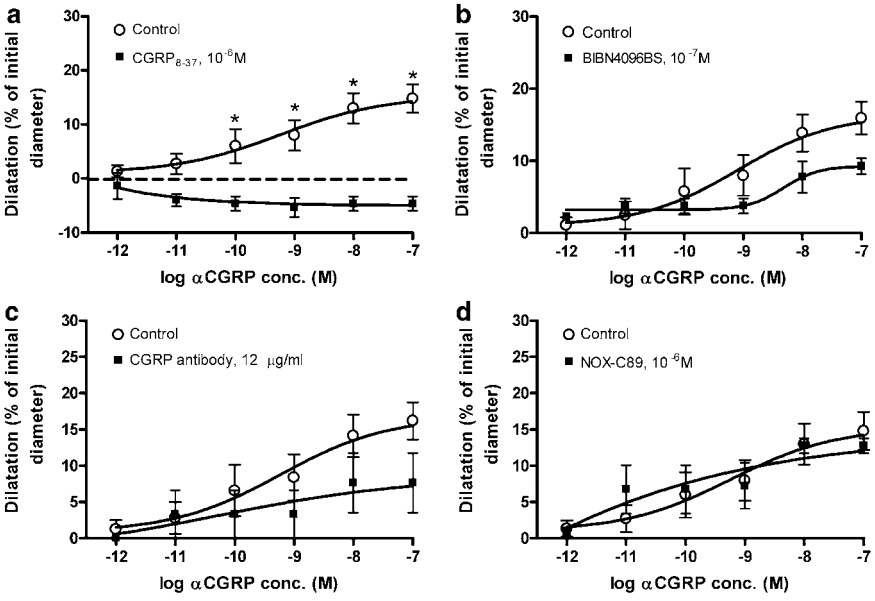

Figure 5 Relaxant effect of abluminal αCGRP in perfused MCA after luminal application of (**a**) 10^{-6} M $CGRP_{8-37}$, (**b**) 10^{-7} M BIBN4096BS, (**c**) 12 $\mu g\,ml^{-1}$ CGRP antibody and (**d**) 10^{-6} M NOX-C89. Values are given as means ± s.e.m. In (**a**), there was a significant effect of the CGRP antagonist $CGRP_{8-37}$ as indicated ($P<0.05$); the dashed line represents the baseline values. For the other antagonists (**b**, **c**), there were no statistically significant effects on dilatation induced by αCGRP (Mann–Whitney *U*-test, $^*P>0.05$, $n=3$–7).

family of peptides do not pass the endothelial barrier, the four CGRP blockers can antagonize CGRP receptor activation in myograph experiments and dilatation induced by abluminal αCGRP was inhibited by abluminal $CGRP_{8-37}$, BIBN4096BS and the CGRP antibody in the perfused MCA.

Effect of CGRP, amylin and adrenomedullin

CGRP, amylin and adrenomedullin receptors are generated by the genes *CALCR* that codes for the 7 transmembrane (TM) calcitonin receptor, whereas *CALCRL* codes for the 7 TM calcitonin receptor-like receptor. Their function and pharmacology are determined by the presence of receptor activity-modifying proteins (RAMPs) (McLatchie *et al.*, 1998). RAMPs are single TM domain proteins identified as a family of three members: RAMP1, RAMP2 and RAMP3. RAMP1, 2 and 3 in combination with *CALCR* constitute the respective amylin 1, 2 and 3 receptors (Muff *et al.*, 1999; Tilakaratne *et al.*, 2000; Zumpe *et al.*, 2000). The *CALCRL* in combination with RAMP1 represents the CGRP receptor whereas *CALCRL*

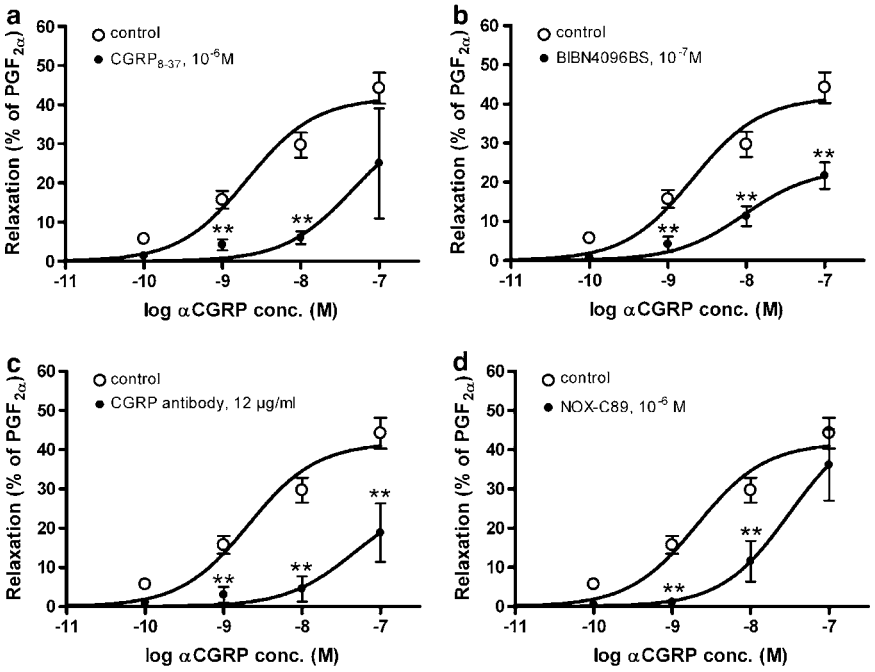

Figure 6 The effect of (**a**) 10^{-6} M $CGRP_{8-37}$, (**b**) 10^{-7} M BIBN4096BS, (**c**) 12 μg CGRP antibody and (**d**) 10^{-6} M NOX-C89 on relaxation of rings of MCA, pre-contracted with $PGF_{2\alpha}$ (3×10^{-6} M). Values are given as means ± s.e.m. Statistical analysis by Mann–Whitney *U*-test, $^{**}P<0.01$, $n=5$–7.

Table 1 Relaxant effect of αCGRP on rat middle cerebral arteries pre-contracted by 3×10^{-6} M $PGF_{2\alpha}$

Blocker tested	*pEC$_{50}$*	E$_{max}$	n
None	8.75 ± 0.16	43 ± 4	15
AB, 4.8 μg ml^{-1}	8.13 ± 0.27^{NS}	44 ± 19$^{NS.}$	6
AB, 12 μg ml^{-1}	7.65 ± 0.09**	19 ± 7*	6
NOX-C89, 10^{-6} M	7.74 ± 0.07**	36 ± 9^{NS}	7
BIBN4096BS, 10^{-7} M	8.09 ± 0.16^{NS}	14 ± 3***	6
$CGRP_{8-37}$, 10^{-6} M	8.55 ± 0.34^{NS}	25 ± 14^{NS}	5

Abbreviations: CGRP, calcitonin gene-related peptide; NS, not significant.
Data are given as means ± s.e.m.; *n* = number of vessel segments examined. E_{max} = maximum relaxation in % of pre-contraction; pEC_{50} = the negative logarithmic concentration (M) of an agonist eliciting half-maximum relaxation. Statistical analysis by Mann–Whitney *U*-test; $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.005$ vs values in the absence of blocker.
The relaxant response was studied in the absence or presence of one of four different CGRP blockers.

in combination with RAMP2 and 3 constitutes the two adrenomedullin (1 and 2) receptors (Buhlmann *et al*., 1999; Fraser *et al*., 1999; Sexton *et al*., 2001).

In the present study, the order of relaxant potency in the rat MCA was: αCGRP = βCGRP > > amylin > adrenomedullin in myograph studies. This order of potency is consistent with that of the $CGRP_1$ receptor (CGRP > amylin ⩾ adrenomedullin) and differs from the adrenomedullin (AM1: adrenomedullin > > CGRP > amylin, AM2; adrenomedullin ⩾ CGRP > amylin) or amylin receptors (amylin ⩾ CGRP > adrenomedullin) (Alexander *et al*., 2005).

This definition of the receptors in the MCA is further supported by the weak relaxant effects of adrenomedullin and amylin. Therefore, our data showed the presence of the $CGRP_1$ receptor in rat MCA. Our experiments further revealed that the $CGRP_1$ receptors are situated in the smooth muscle cells, as only abluminal administration of the peptides had effects. This is supported by data in which removal of the endothelium did not alter the CGRP-induced relaxation in brain vessels and the relaxation occurred in parallel with activation of adenylyl cyclase in the vessel walls (Jansen-Olesen *et al*., 1996; Edvinsson *et al*., 1998).

CGRP has been found to provoke migraine attacks in a human experimental model of migraine (Lassen *et al*., 2002). It is released during migraine or cluster headache attacks (Goadsby *et al*., 1990; Goadsby and Edvinsson, 1994; Juhasz *et al*., 2003); CGRP is released in parallel with increasing degree of pain during migraine attacks (Juhasz *et al*., 2005) and with enhanced intracranial vasoconstriction during subarachnoid haemorrhage (Juul *et al*., 1990). The systemic administration of CGRP can reduce cerebrovascular vasoconstriction following subarachnoid haemorrhage in man (Juul *et al*., 1994). We therefore, addressed the question of whether circulating CGRP or its family of peptides can influence the tone of the MCA. We also intended to examine the role of the endothelial barriers in brain vessels. Abluminally applied αCGRP and βCGRP concentration-dependently relaxed the MCA. However, CGRP given luminally had only minor effects on MCA diameter, which suggested that it did not pass the endothelial barrier readily. Adrenomedullin and amylin behaved in much the same way, but induced weaker responses than CGRP. This agrees with recent *in vivo* data where Petersen *et al*. (2004) observed that systemic αCGRP and βCGRP had no direct effect on cortex pial artery diameter but relaxed the middle meningeal artery, which lacks a blood–brain barrier.

The effect of CGRP on cerebral blood flow (CBF) is not clear. In most species, including rat, the peptide increases

CBF upon intravenous administration (Suzuki *et al*., 1989; Baskaya *et al*., 1995). The above-mentioned study, reported a dose-dependent increase in local CBF_{Flux} with infusion of both αCGRP and βCGRP, however; it was only significant at the highest doses given (Petersen *et al*., 2004). These results are in concert with the view that there might be some minor passage of the peptide across the endothelium of cerebral blood vessels but not as much as through the endothelium of the vessels in the dura mater, where there is no blood–brain barrier. In addition, the method for studies of CBF using laser Doppler flowmetry does not measure true tissue blood flow but rather changes in cerebral vessel tone, which could be elicited by several mechanisms (Edvinsson and Krause, 2002).

Mechanism and site of action of the antagonists

BIBN4096BS is a hydrophilic dipeptide derivative (consisting of two amino acids) and is structurally smaller than the other antagonists and, therefore, more likely to pass the blood–brain barrier, to some extent. There are, to our knowledge, no studies available that have directly measured the passage of BIBN4096BS across the blood–brain barrier. In contrast to the pial vessels, the meningeal vessels have no barrier and BIBN4096BS is likely to diffuse freely into the wall of these vessels (Knudsen *et al*., 1988; Faraci *et al*., 1989). Thus, it was observed in the closed cranial window experiments that BIBN4096BS had antagonistic effects on the middle meningeal artery following systemic administration of αCGRP and βCGRP, but only a weak effect on endogenously released CGRP in pial arteries and on CBF (Petersen *et al*., 2004). These results agree with the present data that BIBN4096BS showed an inhibitory effect when applied abluminally but none after luminal application. Thus, the blood–brain barrier seems to inhibit the passage of BIBN4096BS from the luminal to the abluminal side of the brain vessel. However, the larger hydrophilic peptide antagonist $CGRP_{8-37}$ had significant inhibitory effects on abluminal and luminal αCGRP-induced dilatation. The reason for this is unclear.

As receptors with a CGRP receptor-like pharmacological profile are situated primarily on the smooth muscle cells of cerebral arteries in man (Edvinsson *et al*., 2002; Moreno *et al*., 2002; Oliver *et al*., 2002; Petersen *et al*., 2005), systemically administered $CGRP_{8-37}$ might have to pass the blood–brain barrier to inhibit vasodilatation induced by CGRP released from sensory nerves in pial arteries and arterioles.

The hydrophilic, high molecular weight CGRP scavengers had slight antagonistic effects on relaxation to abluminal αCGRP, when given abluminally but not luminally. The CGRP-binding RNA-Spiegelmer in the concentrations used, was less effective than the CGRP antibody in the perfusion study, but not in the myograph study. However, in an *in vivo* study, we have shown that intravenous infusions of the CGRP antibodies and the CGRP-binding RNA-Spiegelmer are able to inhibit the effect of a subsequent infusion of CGRP but not of CGRP released from perivascular sensory nerves after electrical stimulation in the dura mater (Juhl *et al*., in preparation). Thus, the two novel CGRP scavengers seem to be not able to penetrate the cerebral vessel wall.

The CGRP peptide agonists were effectively prevented from reaching the $CGRP_1$ receptors in the smooth muscle cells by the endothelial barrier. The CGRP blockers significantly inhibited αCGRP-induced relaxation but were similarly prevented from reaching the receptors by the endothelial barrier. Studies of isolated human brain vessels have revealed pA_2 values for BIBN4096BS of about 0.1 nM (Edvinsson *et al*., 2002). The low penetration across the blood–brain barrier of BIBN4096BS and the antimigraine effect seen at 200 nM (Olesen *et al*., 2004) suggest that the antimigraine effect of BIBN4096BS takes place at the abluminal side of the blood–brain barrier in the MCA as BIBN4096BS passes the dural artery freely.

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Conflict of interest

The authors state no conflict of interest.

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