BIBN4096BS is a potent competitive antagonist of the relaxant effects of α-CGRP on human temporal artery: comparison with CGRP(8-37)

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1   Release of CGRP during migraine may produce harmful dilatation of cranial arteries, thereby possibly causing pain. We have compared the antagonism by BIBN4096BS and CGRP(8-37) of the relaxant effects of α-CGRP on rings of human temporal artery.

2   α-CGRP relaxed the arteries precontracted with 9 – 24 mM KCl ($-\log EC_{50} = 9.4$) nearly as efficaciously as sodium nitroprusside (10 μM).

3   BIBN4096BS (0.1 – 100 nM) antagonized the effects of α-CGRP in surmountable manner with slopes of Schild-plots not different from unity. $-\log K_B$ values of 10.1 and 10.4 were estimated for BIBN4096BS when administered before or during the KCl-contracture respectively.

4   BIBN4096BS (1 μM) did not modify the relaxant effects of papaverine and sodium nitroprusside.

5   CGRP(8-37) (1 – 10 μM) antagonized the effects of α-CGRP in a surmountable manner with slopes of Schild-plots not different from unity. $-\log K_B$ values of 6.6 and 6.7 were estimated for CGRP(8-37) administered before or during the KCl-contracture respectively.

6   The high affinity of BIBN4096BS for CGRP receptors of human temporal artery makes it an excellent tool to explore the hypothesis of CGRP-evoked cerebral vasodilation in migraine.
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Keywords: Human temporal artery; α-CGRP-evoked relaxation; BIBN4096BS; CGRP(8-37); migraine

Abbreviations: Ach, acetylcholine; 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*)]-; α-CGRP, α-calcitonin gene-related peptide; 5-HT, 5-hydroxytryptamine; SNP, sodium nitroprusside

Introduction

The temporal artery can dilate during migraine, thereby possibly contributing to pain (Graham & Wolff, 1938; Lance, 1992). One candidate for vasodilatation of cranial and extracranial arteries is CGRP, which can be released from trigeminal nerves in man (Goadsby *et al.*, 1988) and which has been found to be elevated in the jugular blood of patients during migraine (Goadsby *et al.*, 1990). The human temporal artery appears to be surrounded by nerve fibres containing CGRP (Uddman *et al.*, 1986) and CGRP relaxes contractions induced by prostaglandin $F_{2\alpha}$ (Jansen *et al.*, 1986; Jansen-Olesen *et al.*, 1995), consistent with the existence of receptors for CGRP. In order to gain insight into the CGRP-receptor characteristics we have now studied the α-CGRP-evoked relaxation of rings from human temporal artery and compared the mode of blocking action and affinity of two blocking agents.

A non-peptide CGRP receptor antagonist, BIBN4096BS, was recently reported to possess picomolar affinity for recombinant CGRP receptors and to potently inhibit the facial vasodilatation induced by trigeminal stimulation in marmoset monkeys (Doods *et al.*, 2000). We investigated the mode of antagonism by BIBN4096BS of the relaxant effects

of α-CGRP in the human temporal artery and provide the first analysis of the mode of interaction of BIBN4096BS with human vascular CGRP receptors. For comparison we also used the peptidic fragment of human α-CGRP, CGRP(8-37), as antagonist (Chiba *et al.*, 1989) of the effects of α-CGRP. Both blockers were competitive antagonists, BIBN4096BS being 3000~5000 times more potent than CGRP(8-37). A progress report of this work has been communicated (Verheggen *et al.*, 2001).

Methods

Patients

The experiments were approved by the Ethics Committee of the University of Göttingen. Temporal arteries were obtained from 22 patients (11 males, 11 females; age range 18 – 67 years) undergoing neurosurgery. To prevent arterial damage, care was taken to avoid electrocoagulation as described (Verheggen *et al.*, 1996). Patients were operated on for the following diseases: brain tumours (meningiomas eight, metastasis three, B-cell lymphona one), aneurysms four, orbital tumours two, head injury four. Anaesthesia was induced and maintained with midazolam, flunitrazepam,

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propofol, fentanyl, alfentanyl, thiopental or ketamine. Muscle relaxation was obtained with pancuronium and/or rocuronium. Until and during surgery patients received some of the following premedication: dexamethasone, heparin, midazolam, mannitol, calcium channel antagonists, carbamazepine, antibiotics and angiotensin II receptor antagonist.

Isolated arterial rings

All arteries were free of macroscopic atheroma. Arterial segments of 400 – 700 μm outer diameter, obtained during neurosurgery, were placed in oxygenated Ringer solution containing (mM): Na^+ 147, K^+ 4, Ca^{2+} 2.3, Cl^- 155.6, at room temperature, transferred immediately to the laboratory, dissected and set up in a physiological solution containing (mM): Na^+ 142, Cl^- 126, K^+ 5.84, HCO_3^- 25, Ca^{2+} 2.5, $H_2PO_4^-$ 1.175, SO_4^{2-} 1.175, glucose 5.56. The arterial segments were cleaned of adhering fat and connective tissue and cut into up to 10 rings of 3 – 4 mm in length. Each ring was mounted on an L-shaped brace in an organ bath containing 10 ml of physiological solution. The solution was gassed with 20% O_2 in 75% N_2 and 5% CO_2 at 37°C. The rings were stretched once to 10 mN and left at that length thereafter. Changes in isometric arterial tension were transformed by a mechanoelectrical transducer (TF6V5-Fleck, Mainz, Germany) and recorded continuously on a multichannel recorder (Linearcorder Mark VII WR 3310-Graphtec, Tokyo, Japan). Tissues were allowed to equilibrate for at least 2 h.

To decrease endothelial function, we used a procedure with Triton X-100, previously applied to rabbit pial arteries (Verrecchia *et al.*, 1986; Hamel *et al.*, 1987) and to human cranial arteries (Jansen-Olesen *et al.*, 1997). Physiological solution, containing 0.1% Triton X-100, was flushed for 12 s through one half of the temporal artery, followed immediately by further flushing without the detergent. This method produces oedema and vacuolization of endothelial cells (Verheggen, 2000).

Experimental protocol

To obtain a reference standard, experiments were initiated with a contraction to 3 μM 5-HT. After observing a maximal contraction to 5-HT, the functional integrity of the endothelium was assessed with the relaxation caused by 3 μM acetylcholine followed by washout of the agonists (Figure 1). To enhance arterial tone, rings were exposed to increasing concentrations of KCl (9 – 24 mM), titrated to achieve a contractile tension equivalent to approximately 30 – 45% of the maximum tension induced with 5-HT (Figures 1 and 2). After a stable contractile plateau had been ensured, a cumulative concentration-effect curve for α-CGRP was determined followed by a maximum relaxant concentration of sodium nitroprusside (10 μM). An antagonist was either administered before or during the KCl-contracture. When administrated during the KCl-contracture, the blocking agent was pre-incubated 1 h before a concentration-effect curve for α-CGRP was begun. When administered before the KCl-contracture, the blocking agent was pre-incubated 2 h. To verify the integrity of the arterial function, drugs were washed out and the experiment terminated with a contracture evoked by 100 mM KCl (Figure 1). Only a single concentration-effect curve for α-CGRP, in the absence or presence of a blocking agent, was determined on each arterial ring.

To assess whether pathways unrelated to CGRP receptors were affected, we also investigated the effects of BIBN4096BS on the relaxation elicited by SNP and papaverine. SNP was used as a tool for effects mediated *via* a cyclic GMP pathway (Böhme *et al.*, 1978). Papaverine was used as a vascular relaxant through a cyclic AMP-dependent pathway (Demesy-Waeldele & Stoclet, 1977; Kaneda *et al.*, 1998), presumably through phosphodiesterase inhibition (Chasin & Harris, 1976), and cyclic AMP-unrelated pathways (Fujioka, 1984). A single concentration-effect curve for SNP or papaverine was determined in the absence or presence of 1 μM BIBN4096BS on arterial rings obtained from the same patient. After a challenge to 5-HT followed by acetylcholine and washout, BIBN4096BS was administered before the titration curve to KCl and the curve for SNP or papaverine begun 2 h thereafter. Concentration-effect curves for the relaxant effects of SNP and papaverine were expressed as percentage decreases of the KCl-contracture.

Data analysis and statistics

Concentration-effect curves of α-CGRP were expressed as percentage of the relaxation produced by sodium nitroprusside. $-LogEC_{50}$ values for α-CGRP were estimated by interpolation from individual concentration-effect curves. The blocking potency of the antagonists against α-CGRP was estimated by analysis with EC_{50} concentration-ratios (CR; Arunlakshana & Schild, 1959). The error of CR was estimated by using log forms ($-logEC_{50} = pD_2$; $-logEC_{50}$ in the presence of blocking agent $= pD_{2B}$; Kaumann, 1990):

$$pD_2 - pD_{2B} \pm (s.e.m.pD_2^2 + s.e.m.pD_{2B}^2)^{1/2} \qquad (1)$$

pK_B values for the antagonists were calculated assuming slopes of one of the Schild-plot.

All data are expressed as mean ± s.e.mean. Concentration-effect curves were fitted as hyperbolae with variable slope. Unless stated otherwise, number of experiments refer to number of patients. Statistical significance was determined by Student *t*-test, ANOVA and Mann – Whitney rank test. Differences were considered significant at $P < 0.05$.

Drugs

5-Hydroxytryptamine creatine sulphate (5-HT) and Triton X-100 were purchased from Aldrich (Steinheim, Germany). Acetylcholine hydrochloride was obtained from Merck (Darmstadt, Germany). α-CGRP and CGRP(8-37) were purchased from Bachem (Heidelberg, Germany). 5-HT, SNP and papaverine hydrochloride were purchased from Sigma (Taufkirchen, Germany). BIBN4096BS was a gift of Dr Henri Doods (Boehringer Ingelheim, Biberach Germany).

Results

α-CGRP-evoked relaxations

All arterial rings studied relaxed with acetylcholine (Figure 1), consistent with functional integrity of the endothelium
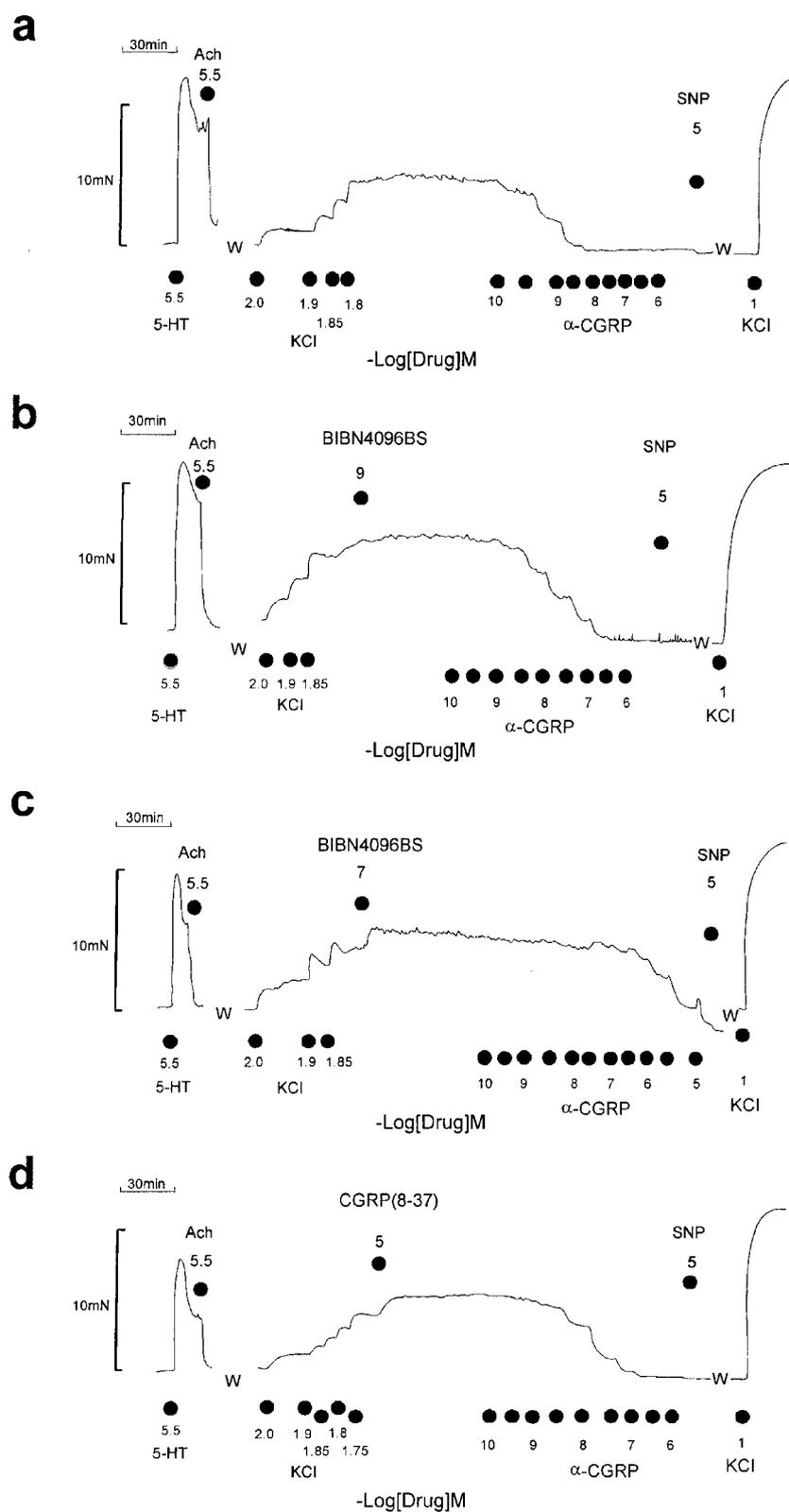
Figure 1 Antagonism of the relaxant effects of α-CGRP by BIBN4096BS and CGRP(8-37). Original tracings from the temporal artery of a 29 year old male patient with epidural haemorrhage. Each panel represents a recording from a separate ring. After a challenge with 5-HT followed by acetylcholine and washout (W), KCl was titrated to obtain a contracture equivalent to ∼30–45% of the 5-HT-evoked contraction. After a stable KCl-contracture was observed, a cumulative concentration-effect curve for α-CGRP was determined followed by SNP administration. Shown are experiments in the absence (a) and presence of BIBN4096BS (b,c) and CGRP(8-37) (d).
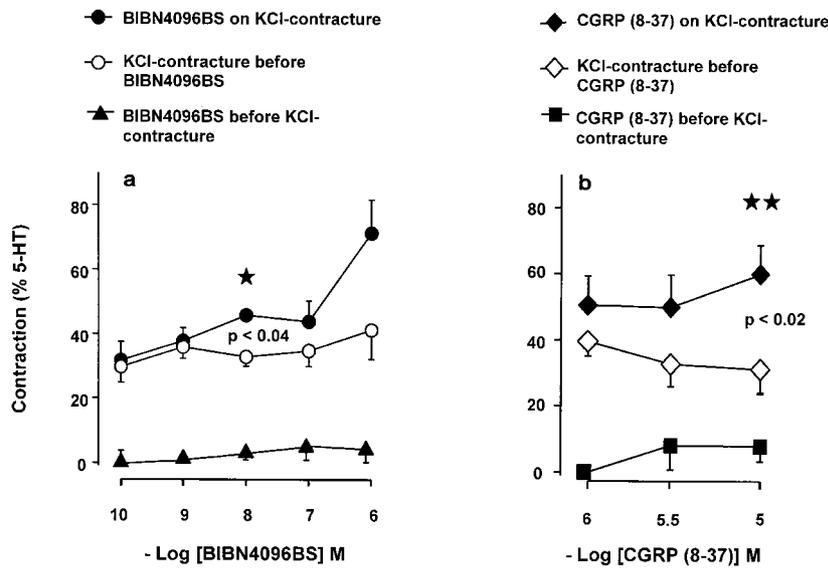
Figure 2 Marginal contractions elicited by BIBN4096BS (a) and CGRP (8-37) (b). Data from 12 patients (a) and six patients (b). Only the effects of 10 nM BIBN4096BS and 10 μM CGRP (8-37), administered during the KCl-contracture, were statistically significant.

regardless of patients age, and with the absence of macroscopic atheroma lesions. α-CGRP was a potent relaxant ($-\log EC_{50} = 9.37 \pm 0.10$, $n = 17$ patients) and nearly as efficacious as SNP (Figures 1, 3 and 4). We inquired whether or not the endothelium participated in the relaxant effects of α-CGRP. The acetylcholine-induced relaxation of rings obtained from the Triton X-100-treated half was nearly abolished ($13.1 \pm 14.0\%$ decrease of the 5-HT-evoked contraction, five rings from three patients) compared to four rings from the untreated half ($104.5 \pm 11.5\%$ decrease of the 5-HT response). The $-\log EC_{50}$ values for α-CGRP did not differ significantly in the two groups (9.01 ± 0.30 Triton X-100 vs 8.87 ± 0.34 no Triton X-100). These results are consistent with the concept that in human temporal artery the effects of α-CGRP are mediated through receptors located at the membrane of the arterial smooth muscle and agree with a previous report (Jansen-Olesen *et al.*, 1995).

Antagonism of the effects of α-CGRP by BIBN4096BS and CGRP(8-37)

Higher concentrations of the blocking agents tended to produce small and inconsistent contractions, when administered before or during the KCl-contracture (Figures 1 and 2). Only 10 nM BIBN4096BS and 10 μM CGRP(8-37), administered during the KCl-contracture, enhanced significantly the KCl-contracture (Figure 2). The pH (7.40) of the solution remained unaltered during the contractions produced by the two blocking agents. The nature of the marginal contractions produced by BIBN4096BS and CGRP(8-37) is still unknown, but could be related to blockade of the relaxant effects of small amounts of CGRP released from the arterial nerve endings.

BIBN4096BS (0.1 – 100 nM) and CGRP(8-37) (1 – 10 μM) antagonized the effects of α-CGRP in surmountable manner, regardless of whether administered before or during (Figures 1, 3 and 4) the KCl-contracture. Although 1 μM

BIBN4096BS caused additional blockade, it was partially insurmountable (Figure 3), conceivably due to insufficient dissociation of BIBN4096BS from the receptors. Therefore data with 1 μM BIBN4096BS were not included in the Schild-plot. Schild-plots for BIBN4096BS and CGRP(8-37) had slopes not significantly different from slope one under the two conditions (Figure 5, Table 1). The blocking potency of CGRP(8-37) was not different, while for unknown reasons BIBN4096BS appeared twice as potent ($P < 0.05$, Student *t*-test between pK_B values) when administered during the KCl-contracture than before the KCl-contracture (Figure 5). pK_B values (Table 1) revealed that BIBN4096BS was a 3000 to 5000 times more potent antagonist than CGRP(8-37).

BIBN4096BS does not antagonize relaxations evoked by papaverine and SNP

BIBN4096BS (1 μM) failed to affect arterial relaxations produced by papaverine and SNP (Figure 6). The $-\log IC_{50}$ values for papaverine were 6.42 ± 0.05 and 6.48 ± 0.07 in the absence and presence of 1 μM BIBN4096BS respectively. The $-\log IC_{50}$ values for SNP were 7.77 ± 0.16 and 7.62 ± 0.16 in the absence and presence of 1 μM BIBN4096BS respectively.

Discussion

BIBN4096BS is a potent competitive antagonist

BIBN4096BS is the first non-peptide blocker that antagonizes with high potency the relaxant effects of α-CGRP on rings of human temporal artery. The surmountable antagonism of the effects of CGRP caused by 0.1 – 100 nM BIBN4096BS and linear Schild-plots with slopes not different from one suggest specific interactions with CGRP receptors. The specificity is further supported by the failure of BIBN4096BS to antagonize the relaxant effects of papaverine and SNP, at a
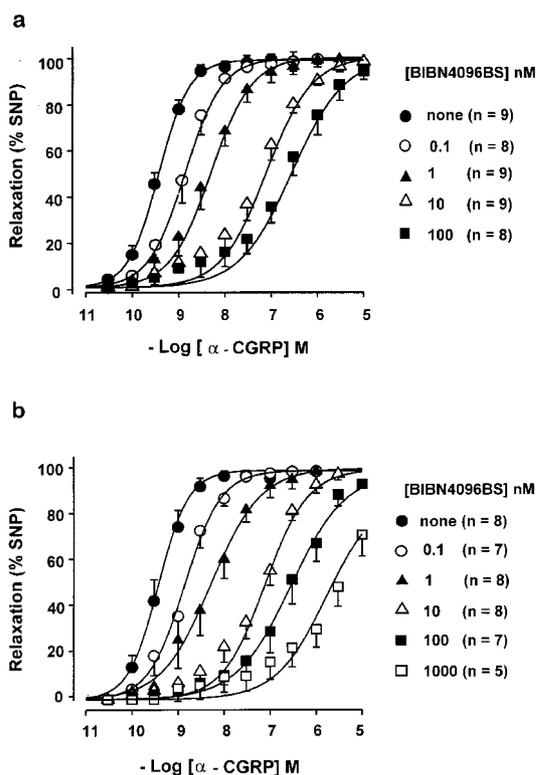
Figure 3 Antagonism of the relaxant effects of α-CGRP by BIBN4096BS, administered before (a) or during the KCl-contracture (b). Concentration-effect curves for α-CGRP are shown in the absence and presence of the indicated concentrations of BIBN4096BS. n = number of arterial rings. Data from 14 patients.
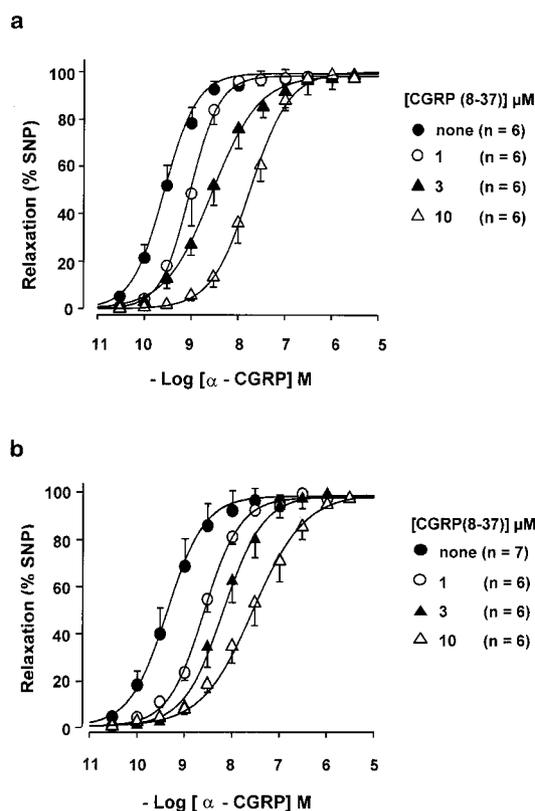

Figure 4 Antagonism of the relaxant effects of α-CGRP by CGRP(8-37) administered before (a) or during the KCl-contracture (b). Concentration-effect curves for α-CGRP are shown in the absence and presence of the indicated concentrations of CGRP (8-37). n = number of arterial rings. Data from 12 patients.

Figure 5 Schild-plots for BIBN4096BS and CGRP(8-37), administered before (solid circle) and during the KCl-contractures (open circle). For slopes and pK_B values see Table 1.

concentration (1 μM) four orders of magnitude greater than its K_B for the CGRP receptors of the human temporal artery.

Most recently, a non-peptide compound chemically related to BIBN4096BS (patent number WO 98/11128) was reported to antagonize the relaxant effects of CGRP on human lenticulostriatal arteries, obtained > 27 h after death (Edvinsson *et al.*, 2001). However, the Schild-plot of data from that report yielded a slope of 0.67 and the affinity estimate for that compound (K_B = 40 nM) was 500 to 1000 times lower than the affinity estimated for BIBN4096BS from our data of competitive inhibition of CGRP-evoked relaxation of human temporal artery.

Our affinity estimate of BIBN4096BS for the CGRP-receptors of human temporal artery (K_B = 40–90 pM) was somewhat lower than its affinity estimated from binding (K_i = 14 pM) to CGRP-receptors of the SK-N-MC human neuroblastoma cell line (Doods *et al.*, 2000). Reasons for this discrepancy are unknown but it could be that BIBN4096BS did not equilibrate completely with the CGRP-receptor population. This is unlikely though, because our affinity estimate was actually slightly lower with the longer BIBN4096BS incubation (2 h) before the KCl-contracture compared to the incubation (1 h) during the KCl-contracture.

Also for unknown reasons, CGRP(8-37) has been reported to bind with considerably higher affinity for CGRP receptors of the SK-N-MC cell line (K_i = 3.6 nM, Doods *et al.*, 2000; K_i = 1.3 nM, Edvinsson *et al.*, 2001) than estimated from its affinity for CGRP receptors of the human temporal artery (K_B = 200–280 nM – this work), human middle meningeal
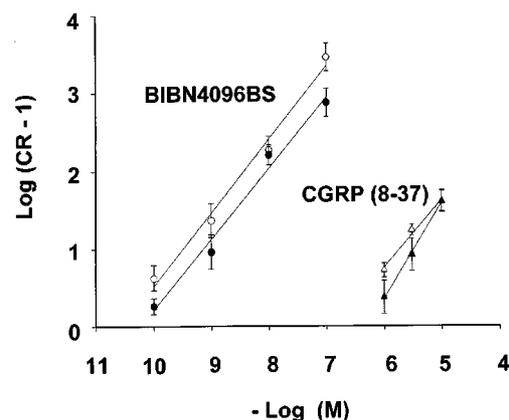
artery (K_B = 150 nM, Edvinsson *et al.*, 1998) and larger branches of lenticulostriate arteries obtained 24 h postmortem ($K_B \sim$ 300 nM, Sams *et al.*, 2000). Unlike these discrepancies, Edvinsson *et al.* (2001) found that the affinity of CGRP(8-37) as antagonist of the relaxant effects of CGRP on lenticulostriatal arteries (K_B = 15 nM) agreed with its affinity as antagonist of CGRP-evoked increases in cyclic AMP in SK-N-MC cells (K_B = 16 nM). However, it is not clear why Edvinsson *et al.* (2001) estimated a 12 times higher

Table 1 Schild-plot analysis of BIBN4096BS and CGRP (8-37)

Antagonist	Blocker administered before the KCl-contracture			Blocker administered during the KCl-contracture		
	n	Slope	pK_B	n	Slope	pK_B
BIBN4096BS	9	0.91 ± 0.08	10.10 ± 0.09	7	0.95 ± 0.07	10.42 ± 0.09
CGRP(8-37)	6	1.24 ± 0.21	6.55 ± 0.14	8	0.90 ± 0.10	6.69 ± 0.07
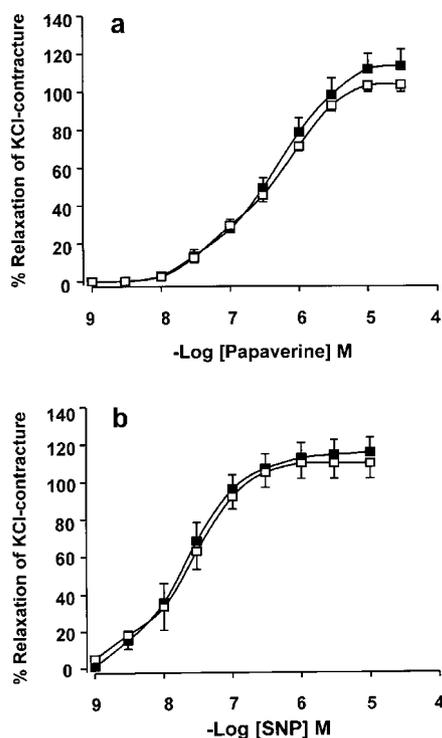
Figure 6 Lack of effects of BIBN4096BS (1 μM) on relaxations caused by papaverine (a) and SNP (b). Shown are curves obtained in the absence (solid square) and presence of BIBN4096BS (open square). Data from three patients with 5–6 arterial rings per group.

affinity of CGRP(8-37) from binding assays than from cyclic AMP assays. Futhermore, it also unclear why Edvinsson *et al*. (2001) reported a $K_B = 15$ nM for CGRP(8-37) on lenticulostriatal arteries while they estimated a 20 times lower affinity ($K_B \sim 300$ nM) on large branches ($550-1727$ μm diameter) of these arteries (Sams *et al*., 2000). One important difference that could also account for differences between the blocking potencies of CGRP(8-37) in arteries, is that the temporal arteries of our study were freshly obtained from patients undergoing surgery, whereas the lenticulostriatal arteries of Edvinsson *et al*. (2001) were obtained $27-32$ h after death.

The relaxant potency of α-CGRP on the human temporal artery (EC$_{50} \sim 400$ pM) was also lower than its binding affinity on the neuroblastoma cells ($K_i = 32$ pM, Doods *et al*., 2000; 15 pM Edvinsson *et al*., 2001). It may be argued that the relaxant potency of α-CGRP found by us for the temporal artery was relatively low due to our experimental conditions of partially depolarized membranes produced by small KCl elevations. However, the relaxant potency α-CGRP against KCl-precontracted arterial rings agrees with

that found for human temporal artery preparations precontracted with the prostaglandin F$_{2\alpha}$ (EC$_{50} \sim 400$ pM, Jansen-Olesen *et al*., 1995). Nanomolar concentrations of CGRP hyperpolarize the membrane of arterial smooth muscle cells (Nelson *et al*., 1990) through activation of ATP-dependent K$^+$ current (Quayle *et al*., 1994) and this mechanism contributes to vascular relaxation. Large-conductance, Ca^{2+}-activated K$^+$ (BK$_{Ca}$) channels hyperpolarize the membrane of smooth muscle cells, when activated through phosphorylation by cyclic AMP-dependent protein kinase (PKA) (Schubert & Nelson, 2001). CGRP appears to produce PKA-mediated BK$_{Ca}$ channel activation in porcine coronary arterial cells (Miyoshi & Nakaya, 1995), thereby contributing to relaxation, and we suggest that this pathway may also play a role in human temporal artery. The lower arterial relaxant potency than neuroblastoma binding affinity of CGRP could be related to the partial depolarization of the vascular cells under our conditions.

Both PKA and cyclic GMP-dependent protein kinase (GPK) phosphorylate BK$_{Ca}$ channels, thereby causing sensitization to Ca^{2+}-induced activation, hyperpolarization and relaxation of vascular smooth cells (Schubert & Nelson, 2001). It is likely that papaverine and SNP relax the temporal artery at least in part through activation of PKA and GPK respectively, *via* BK$_{Ca}$ activation. Because BIBN4096BS (1 μM) failed to modify the relaxations produced by both papaverine and SNP, the antagonist does not interfere with the PKA and PKG pathways. Furthermore, the partially insurmountable antagonism of the CGRP effects by 1 μM BIBN4096BS is therefore unlikely to be due to non-specific depression of the CGRP effects, but possibly related to the slow dissociation of BIBN4096BS from the CGRP receptors.

The lower blocking potency of the two antagonists and lower agonist potency of α-CGRP on the temporal artery compared to the corresponding binding affinities in neuroblastoma cells, could also be due to differences in CGRP receptors of the two systems or different regulation by endogenous modulators. Receptor activity modifying proteins, RAMPs, are modulators that determine the pharmacology of CGRP and other peptides (McLatchie *et al*., 1998). In addition, an accessory protein, CGRP-receptor component protein (RCP), has been found to facilitate coupling of the receptor to the G$_s$-protein/cyclic AMP pathway (Evans *et al*., 2000). We speculate that RAMP modulation of the receptor that interacts with α-CGRP and/or RCP modulation of its coupling could be different in human temporal artery and neuroblastoma cells.

Possible therapeutic relevance

Up to one third of migraine sufferers undergo dilatation of extracranial arteries (Lance, 1992), such as the temporal artery. Our results, showing that BIBN4096BS is a potent antagonist of the relaxant effects of α-CGRP in the temporal artery suggest that CGRP can be involved in migraine. CGRP, released from the sensory nerves of the temporal artery, would interact with CGRP receptors, enhance cyclic AMP levels in the smooth muscle (Jansen-Olesen *et al*., 1996) thereby inducing vasodilatation that in turn activates trigeminal fibres and migraine pain (Uddman *et al*., 1986). Blockade of the CGRP receptors by BIBN4096BS would be expected to prevent the harmful effects of CGRP. Clinical trials will be required to test this hypothesis.

We conclude that BIBN4096BS is the first potent non-peptide and competitive antagonist of CGRP-evoked relaxation of a human extracranial artery. The ability of BIBN4096BS to block CGRP-evoked vasodilatation in a human extracranial artery might lead to therapeutic benefits.

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