

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E. S. D.

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Solicitud de Patente de Invención titulada: "Antagonistas receptores de péptidos relacionados con el gen de calcitonina"

Solicitante: Bristol-Myers Squibb Company.

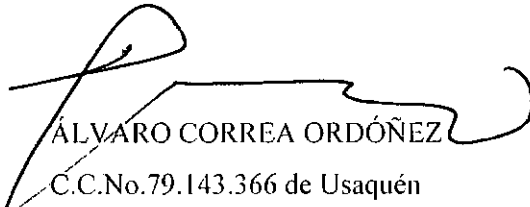
RESPUESTA AL AUTO NOTIFICADO POR FIJACIÓN EN LISTA NO. 14

DEL 22 DE ENERO DE 2010, OFICIO NO. 523 (ART. 46)

ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad en referencia, doy respuesta al auto emanado por ese Despacho dentro del término legal:

1. Atendiendo a la solicitud del Examinador, presento copia de los artículos: MALLEE JJ ET AL "Receptor Activity-modifying Protein 1 determines the species selectivity of Non-Peptide CGRP Receptor Antagonists" Journal of Biological Chemistry, American Society of Biochemical biologists, vol. 277, No. 16 y DOODS H ET AL " Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist", Journal of Pharmacology, Vol. 129, No. 3, 2000 que fueron mencionados en el reporte de búsqueda de la solicitud WO equivalente a la presente solicitud colombiana.

Atentamente,



ÁLVARO CORREA ORDÓÑEZ
C.C.No.79.143.366 de Usaquén
T.P.20.281

Anexos: Artículo MALLEE JJ ET AL "*Receptor Activity-modifying Protein 1 determines the species selectivity of non-peptide CGRP Receptor Antagonists*" Journal of Biological Chemistry, american Society of Biochemical biologists, vol. 277, No. 277, No. 16.

Artículo DOODS H ET AL "*Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist*", Journal of Pharmacology, Vol. 129, No. 3, 2000.

BOGPAM/BOGDMS-#316638-VI-P2004_121774_-_RTA_ART_46.DOC

Receptor Activity-modifying Protein 1 Determines the Species Selectivity of Non-peptide CGRP Receptor Antagonists*[§]

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The heterodimeric CGRP receptor requires co-expression of calcitonin receptor-like receptor (CRLR) and an accessory protein called receptor activity-modifying protein (RAMP) 1 (McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M. G., and Foord, S. M. (1998) *Nature* 393, 333–339). Several non-peptide CGRP receptor antagonists have been shown to exhibit marked species selectivity, with >100-fold higher affinities for the human CGRP receptor than for receptors from other species (Doods, H., Haller-mayer, G., Wu, D., Entzeroth, M., Rudolf, K., Engel, W., and Eberlein, W. (2000) *Br. J. Pharmacol.* 129, 420–423; Edvinsson, L., Sams, A., Jansen-Olesen, I., Tajti, J., Kane, S. A., Rutledge, R. Z., Koblan, K. S., Hill, R. G., and Longmore, J. (2001) *Eur. J. Pharmacol.* 415, 39–44). This observation provided an opportunity to map the determinants of receptor affinity exhibited by BIBN4096BS and the truncated analogs, Compounds 1 and 2. All three compounds exhibited higher affinity for the human receptor, human CRLR/human RAMP1, than for the rat receptor, rat CRLR/rat RAMP1. We have now demonstrated that this species selectivity was directed exclusively by RAMP1. By generating recombinant human/rat CRLR/RAMP1 receptors, we demonstrated that co-expression of human CRLR with rat RAMP1 produced rat receptor pharmacology, and vice versa. Moreover, with rat/human RAMP1 chimeras and site-directed mutants, we have identified a single amino acid at position 74 of RAMP1 that modulates the affinity of small molecule antagonists for CRLR/RAMP1. Replacement of lysine 74 in rat RAMP1 with tryptophan (the homologous amino acid in the human receptor) resulted in a ≥ 100 -fold increase in antagonist affinities, similar to the K_i values for the human receptor. These observations suggest that important determinants of small molecule antagonist affinity for the CGRP receptor reside within the extracellular region of RAMP1 and provide evidence that this receptor accessory protein may participate in antagonist binding.

CGRP is a 37-amino acid neuropeptide that is expressed in a variety of cell types in both the central and peripheral nervous systems (5). In many tissues, CGRP-containing fibers are closely associated with blood vessels (6). Among the various physiological functions reported for CGRP, the most pronounced is vasodilation. CGRP is the most powerful of the vasodilator transmitters (7), and its vasoactive effects have been demonstrated in a variety of blood vessels (8), including those in the cerebral, coronary, and mesenteric vasculature.

Mounting evidence suggests that CGRP is involved in the pathophysiology of migraine headache (9). Migraine is thought to be associated with dilation of cerebral blood vessels and activation of the trigeminovascular system (10). During the headache phase of a migraine, CGRP levels are elevated in the cranial circulation (11, 12). Successful treatment of the headache with sumatriptan resulted in normalization of CGRP levels (12), thus implicating CGRP in the pathophysiology of this disorder. Moreover, intravenous administration of CGRP to migraineurs induced a delayed migrainous headache in some patients (13). These observations suggest that inhibition of CGRP-mediated vasodilation may have therapeutic utility in the treatment of migraine.

Research in the area of CGRP has intensified in recent years due in large part to the identification of its receptor, CRLR¹ (14), and the cloning of the receptor activity-modifying proteins (RAMPs) (1) and receptor component protein (15). CGRP activity is mediated by the G_s -coupled G-protein-coupled receptor (GPCR), CRLR, which shares 55% homology with the calcitonin receptor. In an elegant study, McLatchie *et al.* (1) demonstrated that functional CGRP and adrenomedullin receptors are both derived from CRLR and that the phenotype is determined by co-expression with a particular RAMP. Co-expression of CRLR with RAMP1 results in CGRP receptor pharmacology, whereas RAMP2 or RAMP3 co-expression produces an adrenomedullin receptor. RAMPs are relatively small (148–175-amino acid) proteins containing a single predicted membrane-spanning domain, a large extracellular domain, and a short cytoplasmic domain. CRLR and RAMP1 form a 1:1 heterodimer that was recently shown to undergo internalization as a stable receptor complex in a β -arrestin- and dynamin-dependent fashion (16). The molecular function of RAMPs includes cell surface targeting (17) and may involve direct ligand binding, indirect modulation of CRLR conformation, or both (18).

In the pursuit of small molecule antagonists of the CGRP receptor, CRLR/RAMP1, Doods *et al.* (2) identified a potent and highly specific compound, BIBN4096BS (Fig. 1). BIBN4096BS

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains Tables SI–SIII and Fig. S1.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF424807.

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¹ The abbreviations used are: CRLR, calcitonin receptor-like receptor; hCRLR, human CRLR; rCRLR, rat CRLR; RAMP, receptor activity-modifying protein; hRAMP, human RAMP; rRAMP, rat RAMP; GPCR, G-protein-coupled receptor; hCGRP, human CGRP.

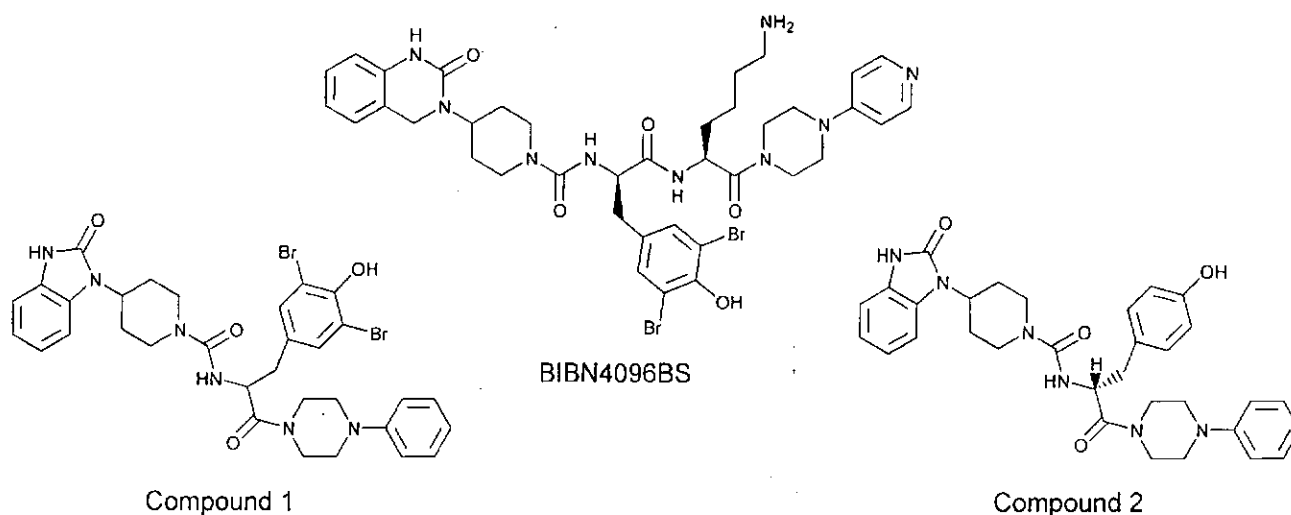


FIG. 1. Chemical structures of BIBN4096BS, Compound 1, and Compound 2.

demonstrated high affinity for the human CGRP receptor, with a K_i of 14 μM . Of particular interest was the observation that BIBN4096BS exhibited >100-fold lower affinity for CGRP receptors from rat, rabbit, dog, and guinea pig, although the affinity for the marmoset receptor was reported to be similar to that for human. These authors utilized marmoset for *in vivo* studies to evaluate the utility of BIBN4096BS as a potential anti-migraine agent. A related compound, Compound 1 (Fig. 1), was likewise shown to be a significantly more potent antagonist on human cerebral arteries than on guinea pig cerebral and porcine coronary arteries (3, 4).

To probe the species selectivity observed with the non-peptide antagonists BIBN4096BS and Compound 1, we chose to focus on the affinity differences seen for the human and rat CGRP receptors. In this report, we demonstrate that the small molecule antagonists, BIBN4096BS, Compound 1, and an analog, Compound 2, have different affinities for the human and rat receptors, and these differences are determined by the species origin of RAMP1 that is co-expressed with CRLR. Furthermore, through the use of chimeric RAMP1 proteins and site-directed mutagenesis, the exact residues responsible for the species selectivity have been identified. The results of this study may provide additional insight into the molecular basis of the CRLR-RAMP interaction and facilitate the future discovery of high affinity non-peptide antagonists.

MATERIALS AND METHODS

Marmoset RAMP1 cDNA Cloning—A partial marmoset RAMP1 cDNA was isolated from frontal brain cDNA using the PCR. The PCR primers were based upon human RAMP1 (5'-CTGCCAGGAGGCTAAC-TACG and 5'-CACCATGAAGGGGTAGAGGA). Amplification reactions consisted of 40 cycles of 45 s at 94 °C, 45 s at 58 °C, and 1 min at 72 °C and were carried out according to the manufacturer's recommended protocol for PLATINUM *Taq* PCR DNA polymerase (Invitrogen). Multiple subclones were sequenced to rule out potential errors.

Expression Constructs, Chimeras, and Mutagenesis—Human and rat cDNAs for CRLR were provided by Dr. Douglas MacNeil (Merck Research Laboratories, Rahway, NJ) and subcloned as 5' *NheI* and 3' *NotI* fragments into pcDNA3.1/Zeo(+) (Invitrogen). Human RAMP1 (hRAMP1) was provided by Dr. Bruce Daugherty (Merck Research Laboratories, Rahway, NJ) in the expression vector pcDNA3.1(+) (Invitrogen). Rat RAMP1 (rRAMP1) cloning was described previously (19); the cDNA was subcloned as a 5' *NotI* and 3' *BamHI* fragment into pcDNA3.1/Hygro(−) (Invitrogen).

Two human/rat chimeric RAMP1 cDNAs were constructed by using restriction fragments of the corresponding native cDNAs. Chimera 1 was created by replacing the nucleotides coding for the first 66 amino acids of rRAMP1 with the corresponding nucleotides of hRAMP1 by using the *BsgI* restriction site along with a *NheI* site located in the

cloning vector. Chimera 2 was created by replacing the nucleotides coding for the first 112 amino acids of rRAMP1 with the corresponding nucleotides of hRAMP1 by using the *SmaI* restriction site along with a *NheI* site located in the cloning vector.

Rat RAMP1 site-directed mutagenesis was performed by using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Lysine at position 74 of rRAMP1 was replaced with the corresponding human/marmoset amino acid tryptophan utilizing two complementary mutant oligonucleotide primers (5'-CTCACTCACTGCACCTGGCTCGTGGCAAACAAG and 5'-CTTGTGTTT-GCCACGAGCCAGGTGCAGTGAGTGAG) and the rRAMP1 expression vector construct as template. This mutation was accomplished by substituting the codon TGG corresponding to tryptophan (rK74W RAMP1). All constructs were sequenced bidirectionally with 100% coverage in each direction.

Cell Culture and DNA Transfection—293 EBNA (Invitrogen) cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose, 1 mM sodium pyruvate, and 2 mM glutamine supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin and maintained at 37 °C, 5% CO_2 , and 95% humidity. Cells were subcultured by treatment with 0.25% trypsin/0.1% EDTA in Hanks' balanced salt solution.

Twenty-four h before transfection, the cells were seeded at 2.0×10^7 cells/dish in 500- cm^2 dishes. The following day, the cells were refed with fresh growth medium 1 h before transfection. Transfections were performed by combining 60 $\mu\text{g}/\text{dish}$ DNA with 180 $\mu\text{g}/\text{dish}$ LipofectAMINE 2000 (Invitrogen). cDNAs for CRLR and RAMP1 in the mammalian expression vector pcDNA3.1 were co-transfected in equal amounts. Control transfections included pcDNA3.1 vector alone, hCRLR/pcDNA3.1, rCRLR/pcDNA3.1, and pcDNA3.1/hRAMP1. The transfection mixture was added directly to the medium, and this mixture was replaced with fresh medium 24 h later. The cells were harvested for membranes 48 h after transfection.

Membrane Preparation and Radioligand Binding Studies—Transiently transfected 293 EBNA cells were washed once with phosphate-buffered saline and harvested in harvest buffer containing 50 mM HEPES, 1 mM EDTA, and Complete protease inhibitors (Roche Molecular Biochemicals). The cell suspension was disrupted with a laboratory homogenizer and centrifuged at $48,000 \times g$ to isolate membranes. The pellets were resuspended in harvest buffer plus 250 mM sucrose. Membranes were stored at -70°C as aliquots. Membranes from rat brains stripped of the meningeal layer (Pel-Freez) were prepared in a similar manner. SK-N-MC membranes were purchased from Receptor Biology.

For binding assays, 1.5–25 μg of membranes from transiently transfected cells (dependent upon receptor expression levels), 25 μg of SK-N-MC, or 100 μg of rat brain membranes were incubated for 3 h at room temperature in binding buffer (10 mM HEPES, 5 mM MgCl_2 , and 0.2% bovine serum albumin) containing 10 pM ^{125}I -hCGRP (Amersham Biosciences) in a total volume of 1 ml. Similar results were obtained by using ^{125}I -labeled rat CGRP (Amersham Biosciences; data not shown). Incubations were terminated by filtration through GF/B 96-well filter plates that had been blocked with 0.5% polyethylenimine. Nonspecific binding was determined by using a final concentration of 300 nM

BIBN4096BS. Data were analyzed by using Sigma Plot 5.0. Saturation binding experiments were carried out in duplicate, and the dissociation constant (K_d) and the receptor density (B_{max}) were determined from the two-site binding model $y = (a * x/(b + x)) + (c * x/(d + x))$, where y = bound radioligand, x = concentration of radioligand, $a = B_{max1}$, $b = K_{d1}$, $c = B_{max2}$, and $d = K_{d2}$. K_d and B_{max} values for each membrane preparation are summarized in Supplementary Material Tables SI–SIII. K_d values typically ranged from 10 to 20 pM. Dose-response curves were plotted, and K_i values were determined for the high affinity binding site by using the equation $K_i = IC_{50}/1 + ([ligand]/K_d)$ by using Excel. Data reported in Tables I–III are expressed as the mean $\pm$ S.E. of three independent experiments. Compound 1, Compound 2, and BIBN4096BS were synthesized by the Medicinal Chemistry Department at Merck Research Laboratories.

Total binding and nonspecific binding were determined for membranes from cells transfected with cDNAs coding hCRLR/hRAMP1, hCRLR/pcDNA3.1, rCRLR/pcDNA3.1, pcDNA3.1/hRAMP1, and pcDNA3.1 alone. Membranes (25 μ g) were incubated with 10 pM [^{125} I]-hCGRP in binding buffer and incubated and processed as described above. Specific binding of [^{125} I]-hCGRP was observed only after co-transfection of CRLR with RAMP1, as expected (1) (Supplementary Material). Transfection of CRLR or RAMP1 alone resulted in little or no binding of radioligand.

Western Blotting—Membranes expressing rCRLR were treated separately with either endoglycosidase F1 or peptide-N-glycosidase F (Calbiochem) overnight at 37 °C. After the addition of protein gel loading buffer, the samples were heated at 70 °C for 10 min and then loaded onto a 4–12% gradient NuPAGE bis-Tris polyacrylamide gel (Invitrogen). After electrophoresis, the separated proteins were transferred to a 0.45 μ m nitrocellulose membrane. Rat CRLR was detected by using the WesternBreeze Immunodetection kit (Invitrogen) with affinity-purified rabbit anti-rat CRLR (Alpha Diagnostic International).

RESULTS

Small molecule antagonists of the CGRP receptor such as Compound 1 and BIBN4096BS exhibit species-selective pharmacology (2–4). For example, BIBN4096BS was reported to exhibit >100-fold higher affinity for the human CGRP receptor than for the rat receptor (2). This observation led us to examine the sequences of CRLR and RAMP1 from different species. Protein sequence alignment revealed that whereas human and rat CRLR are 91% homologous, human and rat RAMP1 share only 71% homology. This observation suggested that the pharmacological differences could be a result of the sequence dissimilarity of either protein or may result from a combined effect of differences in both CRLR and RAMP1 sequences. To determine whether the species selectivity is derived from either CRLR itself or its accessory protein, RAMP1, hybrid human/rat CGRP receptors were created by transiently transfecting cDNAs coding for human CRLR with rat RAMP1 and vice versa in 293 EBNA cells. The cells were harvested, and cell membranes were prepared for subsequent competitive ligand binding experiments. As expected, the small molecule antagonists Compound 1 and BIBN4096BS had lower affinity for rCRLR/rRAMP1 than for the transfected human CGRP receptor, hCRLR/hRAMP1 (Table I). To confirm that these transiently transfected receptors exhibited physiologically relevant pharmacology, comparisons were made to native human and rat CGRP receptors found in SK-N-MC cells (20) and rat brain, respectively. As seen in Table I, similar K_i values were obtained for both Compound 1 and BIBN4096BS on native and transiently expressed receptors. In 293 EBNA membranes expressing rCRLR/rRAMP1, [^{125} I]-hCGRP binding was inhibited by Compound 1 and BIBN4096BS with K_i values of 8670 and 2.1 nM, respectively. In contrast, co-expression of rCRLR with hRAMP1 resulted in a dramatic increase in antagonist affinities, essentially yielding human-like pharmacology with K_i values for Compound 1 and BIBN4096BS of 123 and 0.032 nM, respectively. Likewise, co-expression of hCRLR with rRAMP1 resulted in K_i values that were similar to those observed for the pure rat receptor. These results demonstrated that RAMP1

TABLE I

Summary of competitive binding experiments on membranes expressing mixed species CRLR/RAMP1 receptor complexes

hCRLR and rCRLR were transiently transfected into 293 EBNA cells along with either human or rat RAMP1. Membranes were prepared 48 h after transfection. SK-N-MC and rat brain membranes were used as controls for endogenous sources of human and rat CGRP receptors, respectively. Data represent the mean $\pm$ S.E. from three independent experiments.

	K_i	
	Compound 1	BIBN4096BS
hCRLR/hRAMP1	56.9 $\pm$ 5.3 nM	0.018 $\pm$ 0.0048 nM
rCRLR/rRAMP1	8,670 $\pm$ 570	2.1 $\pm$ 0.30
rCRLR/hRAMP1	123 $\pm$ 8.7	0.032 $\pm$ 0.0014
hCRLR/rRAMP1	6,870 $\pm$ 290	1.8 $\pm$ 0.12
SK-N-MC	46.1 $\pm$ 4.6	0.004 $\pm$ 0.0
Rat brain	8,018 $\pm$ 867	1.5 $\pm$ 0.09

TABLE II

Summary of competitive binding experiments on membranes expressing rCRLR with the RAMP1 Chimeras

hCRLR was transiently transfected into 293 EBNA cells with hRAMP1; rCRLR was similarly transfected with either rRAMP1 or a human/rat RAMP1 chimera. Membranes were prepared 48 h after transfection. Data represent the mean $\pm$ S.E. from three independent experiments. Chimera 1 was created by replacing the first 66 amino acids of rat RAMP1 with the corresponding human sequence; Chimera 2 was likewise created by replacing the first 112 amino acids of rat RAMP1 with the human sequence.

	K_i	
	Compound 1	BIBN4096BS
hCRLR/hRAMP1	32 $\pm$ 3.6 nM	0.065 $\pm$ 0.022 nM
rCRLR/rRAMP1	6,500 $\pm$ 408	1.7 $\pm$ 0.24
rCRLR/Chimera 1	4,704 $\pm$ 239	2.0 $\pm$ 0.41
rCRLR/Chimera 2	76 $\pm$ 11	0.021 $\pm$ 0.0055

determined the affinity of BIBN4096BS and Compound 1 for human and rat CGRP receptors. By contrast, the peptide antagonist CGRP_{8–37} was not species-selective, resulting in K_i values of 1.3 and 1.2 nM for the human and rat receptors, respectively (data not shown), consistent with previous reports (2, 21). The K_d of the radioligand [^{125} I]-hCGRP also was similar for both human and rat receptors, as well as for the mixed-species receptors (Supplementary Material Table SI). The species origin of CRLR in these hybrid receptors had little or no effect on the small molecule antagonist affinities.

One of the demonstrated functions of RAMPs is to ensure proper cell surface targeting of CRLR. In doing so, it was originally proposed that RAMPs also serve to influence the glycosylation state of the receptor (1). In our studies, the functional significance of glycosylation was therefore addressed because the glycosylation state of the rat CGRP receptor had not been characterized previously; furthermore, the possibility existed that co-expression of CRLR with either human or rat RAMP1 resulted in differential glycosylation of CRLR and that this effect determined the observed differences in antagonist affinities. Using an antibody to rCRLR and deglycosylation enzymes, the glycosylation state of rCRLR associated with rat or human RAMP1 was determined. The membranes from the competitive binding experiments (rCRLR/rRAMP1, rCRLR/hRAMP1, and control rCRLR/pcDNA3.1) were treated with peptide-N-glycosidase F and endoglycosidase F1. Peptide-N-glycosidase F catalyzes the hydrolysis of mature glycoproteins, whereas endoglycosidase F1 cleaves N-linked high mannose and hybrid oligosaccharides, but not complex oligosaccharides. Thus, the molecular mass of a glycosylated receptor will decrease after treatment with peptide-N-glycosidase F, and a

Supplemental Material can be found at:
<http://www.jbc.org/content/suppl/2002/04/15/277.16.14294.DC1.html>

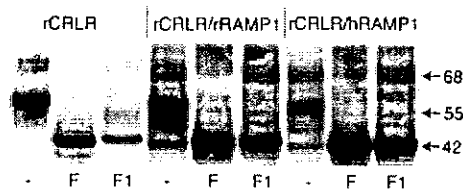


FIG. 2. Western blot analysis of rCRLR co-expressed with rRAMP1 and hRAMP1. The membranes from the competitive binding experiments, including rCRLR transfected with empty vector (pcDNA3.1), were treated with peptide-N-glycosidase F (F), endoglycosidase F1 (F1), or no enzyme. Samples were separated by SDS-PAGE, followed by Western blot analysis with anti-rat CRLR antibodies. The molecular masses (in kDa) are listed on the right.

receptor with complex glycosylation will resist endoglycosidase F1 cleavage. Co-expression of rCRLR with either human or rat RAMP1 produced species with molecular masses of 55 and 68 kDa, which were reduced to a single 42-kDa species after peptide-N-glycosidase F treatment (Fig. 2). Furthermore, the 68-kDa species represented a mature glycoprotein, as demonstrated by its resistance to endoglycosidase F1 cleavage. The negative control rCRLR alone resulted in background levels of the 55-kDa species, possibly resulting from interaction of transfected CRLR with low levels of endogenous RAMPs. The 55-kDa species likely represents a core glycosylated form of the receptor (18). These results indicated that the co-expression of either human or rat RAMP1 with rat CRLR resulted in similar levels of complex glycosylation.

RAMPs are accessory proteins predicted to contain a large extracellular amino-terminal domain and a single transmembrane-spanning domain (1). To elucidate the region of RAMP1 that is directly involved in determining the affinities of BIBN4096BS and Compound 1, human/rat RAMP1 chimeras were constructed. Chimera 1 was created by replacing the first 66 amino acids of rRAMP1 with the corresponding hRAMP1 sequence. Conversely, replacement of the first 112 amino acids of rRAMP1 with the human sequence produced Chimera 2. These constructs were then used for transient transfections in similar experiments as described above. In membranes expressing rCRLR with Chimera 1, ¹²⁵I-hCGRP binding was inhibited by Compound 1 and BIBN4096BS with *K_i* values of 4704 and 2.0 nM, respectively (Table II). These results were similar to those obtained for rCRLR/rRAMP1. By contrast, when rCRLR was co-expressed with Chimera 2, the resulting *K_i* values were similar to those obtained for hCRLR/hRAMP1. These studies demonstrated that amino acids 66–112 in the extracellular domain of RAMP1 were responsible for modulating the affinity of BIBN4096BS and Compound 1 for CRLR/RAMP1.

The identification of amino acids 66–112 of RAMP1 as the critical region determining CGRP receptor pharmacology led us to examine the possibility that the species selectivity might be governed by specific amino acid residues. BIBN4096BS and Compound 1 were reported to have lower affinity for rat (2) and porcine (4) receptors, respectively, than for the human receptor. However, high affinity binding of BIBN4096BS was reported for both human and marmoset CGRP receptors (2). Accordingly, a partial marmoset RAMP1 cDNA was cloned, and the sequence was compared with human and other available RAMP1 sequences. Protein sequence alignment revealed numerous residues that were identical in human and marmoset but different from that found in rat, mouse, and pig (Fig. 3). However, amino acid 74 was shown to be the least-conserved residue among these species; the human and marmoset sequences contained tryptophan at this position, but a basic residue was found in the three other species. Subsequently, lysine at position 74 of rRAMP1 was replaced with the corresponding human/marmoset amino acid, tryptophan. This con-

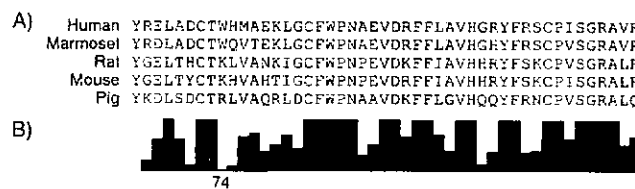


FIG. 3. A, alignment of amino acids 66–112 of RAMP1 from human, marmoset, rat, mouse, and pig. A partial marmoset RAMP1 clone was generated as described under "Materials and Methods" and deposited in GenBank™ with accession number AF424807. B, species conservation. ClustalX version 1.81 alignment quality score, demonstrating that amino acid 74 is the least-conserved residue among these species.

TABLE III
Summary of competitive binding experiments on membranes expressing rCRLR and mutant rK74W RAMP1

hCRLR was transiently transfected into 293 EBNA cells with hRAMP1; rCRLR was similarly transfected with either rRAMP1 or rK74W RAMP1. Membranes were prepared 48 h after transfection. Data represent the mean ± S.E. from three independent experiments.

	<i>K_i</i>		
	BIBN4096BS	Compound 2	Compound 1
	<i>nM</i>	<i>nM</i>	<i>nM</i>
hCRLR/hRAMP1	0.013 ± 0.0015	48 ± 4	108 ± 12
rCRLR/rRAMP1	2.9 ± 0.14	>10,000	12,033 ± 824
rCRLR/rK74W RAMP1	0.036 ± 0.0013	52 ± 7	10 ± 0.46

struct was then co-transfected with rCRLR in 293 EBNA cells. Competitive binding experiments demonstrated that human-like receptor pharmacology could be achieved by co-expression of rCRLR with rK74W RAMP1 (Table III). The affinity of BIBN4096BS increased from 2.9 nM for the rat receptor to 0.036 nM for rCRLR/rK74W RAMP1, similar to that seen for hCRLR/hRAMP1, 0.013 nM. A similar trend was observed for Compound 2. Interestingly, Compound 1 exhibited >10-fold higher affinity for the rCRLR/rK74W RAMP1 receptor than for the native human receptor, perhaps due to favorable interactions between the dibromotyrosyl moiety and the tryptophan in the RAMP1 mutant. These results suggested that the affinities of these small molecule antagonists for the CGRP receptor were heavily influenced by the nature of amino acid 74 of RAMP1.

DISCUSSION

The CGRP receptor is an atypical GPCR in that it requires heterodimerization of CRLR, a classical seven transmembrane receptor, with the accessory protein RAMP1. RAMPs confer agonist specificity to both CRLR and the calcitonin receptor. CRLR in combination with RAMP1 binds CGRP with high affinity, whereas co-expression of CRLR with RAMP2 or RAMP3 results in high affinity adrenomedullin binding (1). RAMPs also regulate the interaction of the calcitonin receptor with calcitonin and amylin, such that co-expression of calcitonin receptor with RAMP1 or RAMP3 results in amylin receptor pharmacology (22). It was recently demonstrated that the peptide agonists CGRP and adrenomedullin could be cross-linked to both CRLR and the respective RAMP, an observation that suggests that RAMPs may participate directly in the agonist binding site (18). An alternative explanation would be that RAMPs indirectly influence agonist binding sites by altering the conformation of CRLR.

Certain small molecule CGRP receptor antagonists display remarkable species selectivity, exhibiting up to 100-fold higher affinity for the human receptor than for the rat receptor. This observation is intriguing, considering the fact that the sequence homology of CRLR between these species is >90%. However, RAMP1 homology is only 71%. Using this informa-

tion, we sought to determine whether the observed differences in pharmacology between the rat and human CGRP receptors for small molecule antagonists resulted uniquely from either CRLR or RAMP1. The results of the present investigation demonstrated that the pharmacology was directed exclusively by RAMP1. Moreover, through the use of human/rat chimeric RAMP1 constructs and site-directed mutagenesis, we have determined that tryptophan 74 of RAMP1 is responsible for the high affinity binding of BIBN4096BS and Compounds 1 and 2 to the human receptor.

These observations have important implications for furthering our understanding of the heterodimeric CGRP receptor. CRLR is a member of the class B family of GPCRs that includes parathyroid hormone, corticotropin-releasing hormone, calcitonin, secretin, and glucagon receptors (23). It is well established that the large amino terminus of class B GPCRs plays a key role in agonist binding, although additional interactions with extracellular loops are still required. In the case of the CGRP receptor, RAMP1 also contains a large extracellular domain. A recent report demonstrated that CRLR and RAMP1 form a 1:1 heterodimer that is internalized as a stable receptor complex (16). Furthermore, the peptides CGRP and adrenomedullin could be cross-linked to both CRLR and RAMP1 or RAMP2, respectively (18), suggesting that either the accessory proteins are in close proximity to the agonist binding sites or the peptide agonists interact directly with both CRLR and RAMP. The present study has demonstrated that the K_d of the radioligand ^{125} I-hCGRP was unaffected by the species of RAMP1 that was co-expressed with CRLR, suggesting that any potential interaction with the RAMP likely occurs through residues that are conserved between these species. The fact that the affinity of small molecule antagonists can be affected by a single amino acid change suggests that these compounds may interact directly with RAMP1. From a mechanistic standpoint, both Compound 1 and BIBN4096BS have been shown to be competitive antagonists of the CGRP receptor (2, 3). Taken together, these observations strongly suggest that the receptor binding sites of both CGRP and these small molecule antagonists include RAMP1. The observation that the affinity of the peptide antagonist CGRP₈₋₃₇ is similar for both human and rat receptors may suggest that this antagonist does not interact with RAMP1. It is tempting, therefore, to speculate that the amino-terminal disulfide-linked ring structure of CGRP, which is lacking in the truncated antagonist CGRP₈₋₃₇, makes unique contacts with RAMP1 that trigger or facilitate signal transduction by CGRP. However, the possibility that CGRP₈₋₃₇ interacts with a conserved region of RAMP1 cannot be ruled out.

Several potential mechanisms could account for the ability of Trp-74 of RAMP1 to confer high affinity binding to non-peptide antagonists of the human CGRP receptor. An indirect mechanism could involve modulation of the conformation of CRLR by residues in the extracellular domain of RAMP1. An alternative scenario could be envisioned whereby these antagonists interact, at least in part, with RAMP1 directly. Favorable hydrophobic interactions between Trp-74 and the antagonists could potentially account for the additional binding energy relative to that seen with Lys-74 of the rat receptor. Conversely, repulsive interactions with the charged lysine in rat RAMP1 could result in decreased antagonist affinity. An alternative model could involve the formation of a binding pocket that includes Trp-74 and other amino acids found within either CRLR or RAMP1. Identification of additional residues within the CRLR-RAMP1 heterodimer involved in binding of these small molecule antagonists remains to be determined. It should be pointed out that a tryptophan residue (Trp-33) was recently shown to confer high affinity binding to a small molecule antagonist of the class

B human glucagon-like peptide 1 (GLP-1) receptor (24). Trp-33 is located within the amino terminus, demonstrating further that important determinants of small molecule ligand affinity may reside within the extracellular domains of class B GPCRs.

Alignment of known RAMP1 sequences from other species revealed a lysine residue at position 74 of mouse RAMP1. A novel application of the present discovery would be generation of a humanized CGRP receptor mouse, wherein lysine 74 is replaced with tryptophan by homologous recombination. Such a knock-in mouse would have significant utility for *in vivo* pharmacological studies of CGRP receptor antagonists and would complement marmoset as a suitable animal model.

Elucidation of the role of RAMPs in modulating ligand selectivity and antagonist recognition remains a critical issue for understanding the biochemistry of this unique GPCR. The heterodimeric nature of this receptor, as well as the requirement for receptor component protein for coupling to the G-protein signaling pathway, provides a tremendous opportunity for dissecting the numerous and varied interactions in this novel receptor complex. Although defining the precise nature of the CRLR-RAMP1 heterodimer will require elucidation of its three-dimensional structure, the present investigation has provided considerable insight into the mode of interaction of small molecule antagonists with the CGRP receptor.

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SPECIAL REPORT

Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist

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Calcitonin gene-related peptide (CGRP) is one of the most potent endogenous vasodilators known. This peptide is increased during migraine attacks and has been implicated in the pathogenesis of migraine headache. Here we report on the first small molecule selective CGRP antagonist: BIBN4096BS. *In vitro*, this compound is extremely potent at primate CGRP receptors exhibiting an affinity (K_i) for human CGRP receptors of 14.4 ± 6.3 ($n=4$) pM. In an *in vivo* model, BIBN4096BS in doses between 1 and 30 $\mu\text{g kg}^{-1}$ (i.v.) inhibited the effects of CGRP, released by stimulation of the trigeminal ganglion, on facial blood flow in marmoset monkeys. It is concluded that BIBN4096BS is a potent and selective CGRP antagonist.

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Keywords: BIBN4096BS; CGRP-antagonist; migraine

Abbreviations: 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*)]-; cAMP, adenosine 3'5'-cyclic monophosphate; CGRP, calcitonin gene-related peptide; EDTA, ethylenediaminetetraacetic acid; LDF, laser doppler flow; SK-N-MC, neuroblastoma cell line of human origin; 5-HT, 5-hydroxytryptamine

Introduction CGRP, a 37 amino acid neuropeptide first identified in 1982 (Amara *et al.*, 1982) belongs to a family of peptides including calcitonin, adrenomedullin and amylin. Localization studies have shown a wide distribution of CGRP-like immunoreactive structures including receptors in the periphery and central nervous system (Sexton, 1991; Brain & Cambridge, 1996). In many tissues, CGRP containing nerves are closely associated with blood vessels. Although CGRP has a number of effects, its most pronounced action is vasodilation. It is one of the most potent endogenous vasodilators known and its vasodilatory effects have been shown in a variety of vessels, e.g. CGRP released from sensory fibres originating in the trigeminal ganglia dilates cerebral vessels (Goadsby & Edvinsson, 1993).

Migraine headache is thought to be associated with dilatation of cranial vessels and activation of the trigemino-vascular system (Moskowitz & Macfarlane, 1993; Moskowitz, 1984; Goadsby *et al.*, 1991). In man stimulation of the trigeminal ganglion results in the release of CGRP (Goadsby *et al.*, 1988). Moreover, during a migraine attack levels of CGRP are increased (Goadsby *et al.*, 1990; Gallai *et al.*, 1995). Thus, CGRP has been implicated in the pathogenesis of migraine headache. Accordingly, inhibition of the CGRP-induced vasodilatation could be expected to attenuate migraine symptoms.

So far, only C-terminal fragments, example given CGRP(8–37), have been described to act as antagonists (Chiba *et al.*, 1989; Rist *et al.*, 1998), however these compounds are not very potent and their peptidic nature limit their use, especially for *in vivo* or clinical investigations. Our goal was to identify small molecule antagonists for the CGRP receptor and to use them to assess the role of CGRP in migraine headache.

Our high throughput screening led to the identification of dipeptide-like compounds that showed weak but unequivocal

inhibition of binding to the CGRP receptor. Lead optimization led to potent CGRP antagonists, the prototype being 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*)]- (Figure 1). The present study was undertaken to examine the pharmacological profile of BIBN4096BS and to investigate its potential as an anti-migraine drug.

Methods *In-vitro experiments* Binding studies investigating CGRP-receptors from human or rat origin were performed using SK-N-MC (neuroblastoma cell line of human origin) cell membranes (approximately 0.175 Mio cells) and rat spleen homogenates (approximately 50 μg of protein per assay). ^{125}I -hCGRP was used as the radioligand. The incubation buffer contained (in mM): Tris 50, NaCl 150, MgCl_2 5 and EDTA 1, (ethylene diamine tetra-acetic acid) pH 7.4. Membrane homogenates were incubated for 180 min at room temperature with 50 pM ^{125}I -hCGRP and increasing concentrations of displacing compound. The incubation was terminated by filtration through GF/B glass fibre filters using a cell harvester. The protein-bound radioactivity was determined in a gamma counter. The nonspecific binding was defined as radioactivity bound in the presence of 1 μM CGRP. The IC_{50} values were obtained by non-linear regression analysis on the basis of a one binding site model. K_i values were calculated according to the equation $K_i = \text{IC}_{50} / (1 + L/K_d)$, according to Cheng & Prusoff (1973), where L and K_d represent the concentration and the dissociation constant of the radioligand. The K_d was 0.05 nM and 0.12 nM for the human and rat CGRP receptor, as determined by saturation binding experiments with 0.5–500 pM ^{125}I -CGRP.

Antagonism of BIBN4096BS was determined by measuring the formation of cyclic AMP in SK-N-MC cells. For cyclic AMP (adenosine 3'5'-cyclic monophosphate) measurements SK-N-MC cells were incubated with CGRP alone or in the

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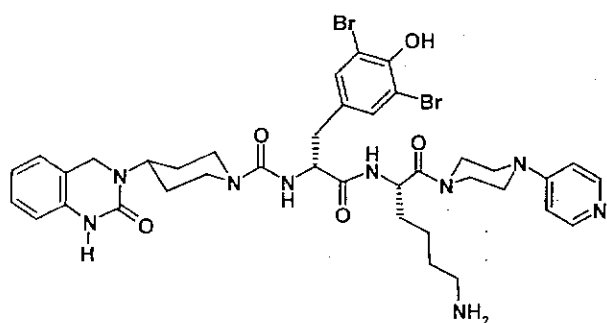


Figure 1 Structural formula of BIBN4096BS.

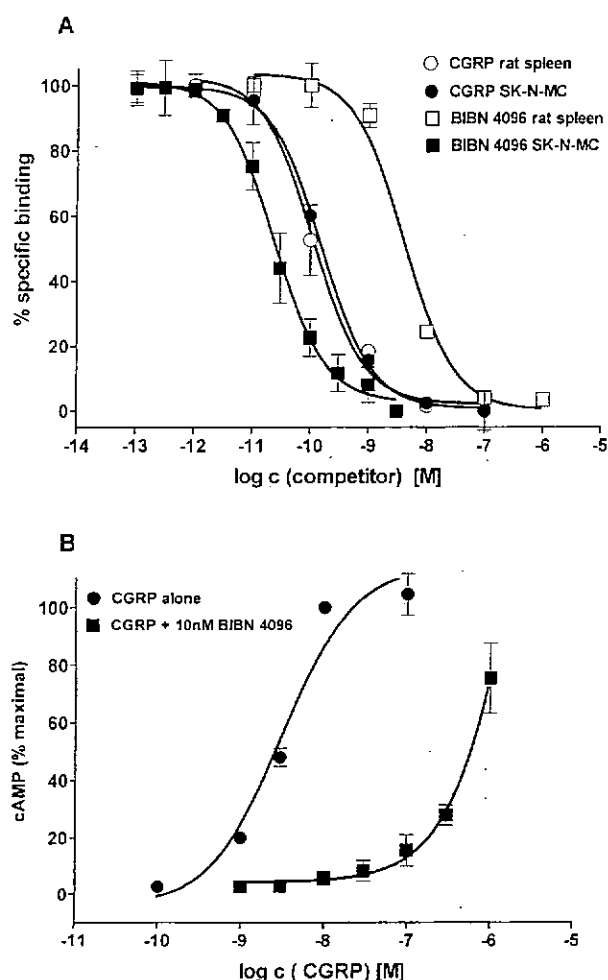


Figure 2 (A) Concentration-dependent inhibition of BIBN4096BS and CGRP of the specific binding of [¹²⁵I]-CGRP to membranes obtained from the human neuroblastoma cell line SK-N-MC or from rat spleen. (B) Stimulation of cyclic AMP formation in SK-N-MC cells by CGRP alone and in the presence of 10 nM BIBN4096BS. Data are means $\pm$ s.e.mean.

presence of 10 nM BIBN4096BS for 15 min at 37°C. Cyclic AMP was extracted with 0.1 M HCl and determined by radioimmunoassay.

An apparent pK_b value was calculated by the equation $pK_b = -\log[B] + \log(CR-1)$ (Schild, 1949), where $[B]$ is the molar concentration of the antagonist and CR is the ratio of the EC_{50} values in the presence and absence of the antagonist.

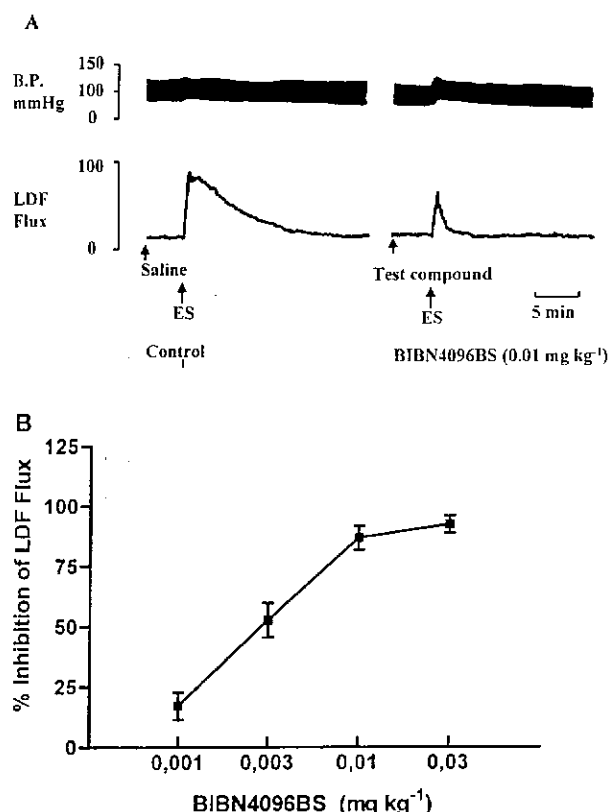


Figure 3 (A) Representative recording to illustrate the changes in blood pressure and LDF-Flux following electrical trigeminal ganglion stimulation. (B) Dose-dependent inhibition of the facial skin vasodilation induced by trigeminal ganglion stimulation in the marmoset by BIBN4096BS. Data are means $\pm$ s.e.mean.

In vivo experiments Marmosets of either sex (300–400 g) were anaesthetized with sodium pentobarbitone (induction with 30 mg kg⁻¹, i.p. and maintained with an infusion of 6 mg kg⁻¹ h⁻¹, i.m.). Body temperature was kept at 37°C by automatic control of a heating pad. The animals were placed in a stereotaxic frame and a longitudinal incision was made in the scalp. A small hole was drilled in the skull and a bipolar electrode (Rhodes SNEX 100) was lowered, using a micro-manipulator, in the trigeminal ganglion.

We used a radiographic approach to identify bony landmarks as guide in placing probes in the ganglion. An X-ray was taken using a Trophy CCX digital X-Ray apparatus and the Pars petrosa was identified as guide for placing the electrodes. The positions of the electrodes in the trigeminal ganglion were checked visually at the end of each experiment following removal of the brain. The trigeminal ganglion was stimulated at 10 Hz, 2 mA, 2 ms for 30 s using a Hugo Sachs Elektronik stimulator. Neuromuscular blockade, 15 min before electrical stimulation, was achieved using pancuronium bromide (initial 1 mg kg⁻¹, i.v., followed by 0.5 mg kg⁻¹ every hour).

Microvascular blood flow changes in the facial skin were measured by Laser Doppler flowmetry with a PeriFlux laser doppler system (wave length 780 nm; Time Constant 3 s) as flux in arbitrary units and expressed as area under the flux-curve (mm²) according to Escott *et al.*, 1995.

Animals were subjected to two or three periods of electrical stimulations, separated by a 30 min interval. The first stimulation was a control for the subsequent stimulations. Test compounds were administered intravenously 5 min prior to the second or third stimulation.

Results BIBN4096BS completely inhibited the specific binding of ^{125}I -CGRP to SK-N-MC cells and displayed an affinity (K_i) of 14.4 ± 6.3 pM ($n=4$) for the human CGRP receptor (Figure 2A). The endogenous ligand CGRP itself and the peptidic antagonist CGRP(8-37) displayed affinities (K_i) of 31.7 ± 1.6 pM ($n=15$) and 3.6 ± 0.7 nM ($n=4$), respectively. Employing the same cell line it was shown that BIBN4096BS is a pure antagonist. In the presence of antagonist the concentration-response curve of CGRP to increase cyclic AMP levels was shifted to the right in a parallel fashion, indicating competitive antagonism (Figure 2B). The pK_b value calculated at an antagonist concentration of 10 nM was 11.0 ± 0.3 ($n=3$). In addition, concentrations of BIBN4096BS up to 100 μM did not show any agonistic effects. The affinity (K_i) for CGRP binding sites in rat spleen was 3.4 ± 0.5 nM ($n=3$) and 96 ± 4 pM ($n=9$) for BIBN4096BS and CGRP, respectively. Accordingly, BIBN4096BS possessed a 236 fold higher affinity for human (SK-N-MC cells) than for rat (spleen) receptors.

Electrical stimulation of the trigeminal ganglion in marmosets caused an ipsilateral increase in facial skin blood flow. Following stimulation there was a rapid increase in flow which returned to base line values after approximately 10 min. This response was obtained three times in the same animal with no significant difference between the first and third stimulation. The area under the curve for the increase in flux observed was used to determine the effects of BIBN4096BS and amounted to 112 ± 11 mm². Administration of BIBN4096BS significantly and dose-dependently inhibited the response evoked by trigeminal ganglion stimulation (Figure 3). A 50% inhibition was achieved with a dose of approximately $3 \mu\text{g kg}^{-1}$ ($n=4$) applied intravenously. An almost complete blockade was observed with a dose of $30 \mu\text{g kg}^{-1}$ (i.v.). BIBN4096BS in doses up to 1 mg kg^{-1} (i.v.) had no intrinsic cardiovascular effects.

Discussion In order to determine the affinity of BIBN4096BS for human CGRP receptors we decided to use SK-N-MC cells since the receptor expressed in this cell line is identical to the cloned human CGRP receptor (Aiyar *et al.*, 1996). The affinity of BIBN4096BS for the human CGRP receptor is even higher than that of the endogenous ligand CGRP itself and 150 fold higher compared to the peptidic antagonist CGRP(8-37). Moreover, BIBN4096BS proved to be a competitive antagonist without any intrinsic agonistic activity. BIBN4096BS had no significant affinity for a set of 75 different receptor or enzyme system ($\text{IC}_{50} > 1000$ nM) including calcitonin, amylin or adrenomedullin receptors (for complete list of assays, see Rudolf *et al.*, 1997). Therefore, it can be concluded that BIBN4096BS is a selective CGRP antagonist with high affinity. We also demonstrated that BIBN4096BS possesses a remark-

able species selectivity, exhibiting an approximately 200 fold higher affinity for primate CGRP receptors compared to rat CGRP receptors. The same was observed for other non-primate species, such as dog, guinea-pig and rabbit. Having established that BIBN4096BS is a potent and selective CGRP antagonist we addressed the question whether BIBN4096BS has the potential to be an anti-migraine drug. Measuring facial blood flow following antidromic stimulation of the trigeminal ganglion has been suggested as a model to evaluate drugs that target the trigemino-vascular system during migraine headache (Escott *et al.*, 1995). Antidromic stimulation will result in the release of neuropeptides such as CGRP. Concomitantly, an increase in blood flow will be observed in blood vessel innervated by sensory nerves. As the facial skin is innervated by the trigeminal nerve, we measured the blood flow in this area. Because BIBN4096BS has a much higher affinity for primate CGRP receptors compared to rat CGRP receptors, we used marmosets. BIBN4096BS completely inhibited the neurogenic vasodilation, demonstrating that CGRP plays a major role in the vasodilation observed following stimulation of the trigeminal nerve in the marmoset. This is in agreement with experiments performed in the rat by Escott *et al.* (1995). Although CGRP itself is a strong vasodilator, i.v. administration of the antagonist had no effect on basal blood pressure and heart rate. This might indicate that CGRP plays a minor role in basal cardiovascular homeostasis under normal conditions, and that CGRP is only involved in (human) pathophysiology. Moreover, it is of interest to mention that in our model anti-migraine drugs like sumatriptan and zolmitriptan, both 5-HT_{1B/D} agonists, inhibited the increase in facial blood flow due to trigeminal stimulation not completely, but only approximately seventy per cent (data not shown). Accordingly, it could be hypothesized that blockade of postsynaptically located vascular CGRP receptors is a more powerful way to attenuate CGRP mediated effects than inhibiting the release of the peptide by stimulating presynaptic 5-HT-receptors.

In conclusion, our results demonstrate that BIBN4096BS is a high affinity and selective CGRP antagonist. Moreover, BIBN4096BS is a very potent inhibitor of neurogenic vasodilation. Since several lines of evidence indicate that CGRP might be a key factor in the initiation of migraine headache, we expect that CGRP antagonists will be effective anti-migraine drugs. Much remains to be investigated to understand the origin of migraine and to validate the concept that CGRP is a major player in its pathophysiology. The availability of a potent and well tolerated CGRP antagonist is a prerequisite to examine whether CGRP antagonism is a new therapeutic concept. BIBN4096BS is such a tool which is presently under clinical investigation for the acute treatment of migraine headache.

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