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Characterisation of the effects of a non-peptide CGRP receptor antagonist in SK-N-MC cells and isolated human cerebral arteries

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Abstract

The cerebral circulation is innervated by calcitonin gene-related peptide (CGRP) containing fibers originating in the trigeminal ganglion. During a migraine attack, there is a release of CGRP in conjunction with the head pain, and triptan administration abolishes both the CGRP release and the pain at the same time. In the search for a novel treatment of migraine, a non-peptide CGRP antagonist has long been sought. Here, we present data on a human cell line and human and guinea-pig isolated cranial arteries for such an antagonist, Compound 1 (4-(2-*Oxo*-2,3-dihydro-benzoimidazol-1-yl)-piperidine-1-carboxylic acid [1-(3,5-dibromo-4-hydroxy-benzyl)-2-*oxo*-2-(4-phenyl-piperazin-1-yl)-ethyl]-amide). On SK-N-MC cell membranes, radiolabelled CGRP binding was displaced by both CGRP-(8–37) and Compound 1, yielding pK_i values of 8.9 and 7.8, respectively. Functional studies with SK-N-MC cells showed that CGRP-induced cAMP production was antagonised by both CGRP-(8–37) and Compound 1 with pA_2 values of 7.8 and 7.7, respectively. Isolated human and guinea pig cerebral arteries were studied with a sensitive myograph technique. CGRP induced a concentration-dependent relaxation in human cerebral arteries which was antagonized by both CGRP-(8–37) and Compound 1 in a competitive manner. In guinea pig basilar arteries, CGRP-(8–37) antagonised the CGRP-induced relaxation while Compound 1 had a weak blocking effect . The clinical studies of non-peptide CGRP antagonists are awaited with great interest.

Keywords: CGRP receptor; Cerebral vessel; Human; cAMP production; Dilatation

1. Introduction

The cerebral circulation is innervated by calcitonin-gene related peptide (CGRP)-containing nerve fibres originating in the trigeminal ganglion (Edvinsson, 1985; Uddman et al., 1985). These fibres, which release CGRP following activation by electrical stimulation (Goadsby et al., 1988) or by capsaicin (Jansen-Olesen et al., 1996), are activated in primary headaches and following subarachnoid haemorrhage (Goadsby et al., 1990; Goadsby and Edvinsson, 1994; Juul et al., 1990, 1995). Trigeminal fibres can mediate dilatation of brain vessels (Edvinsson et al., 1986) and increase cerebral blood flow (Goadsby, 1993) and they may also mediate the trigemino-vascular reflex, thereby counterbalancing cerebrovascular contraction (McCulloch et al., 1986; Edvinsson et al., 1987). In migraineurs, elevated jugular blood levels of CGRP are found during an attack and these levels are normalised on administration of sumatriptan concomitant with relief from headache pain (Edvinsson and Goadsby, 1998). CGRP is a potent vasodilator and through this action may be the key in the pathogenesis of migraine headache. A non-peptide CGRP antagonist has long been sought as an attractive and novel approach in treating migraine.

mRNAs encoding CGRP receptors and accessory proteins (RAMPs) required for CGRP receptor functionality have been detected in various cerebral and cranial arteries (Edvinsson et al., 1997; Sams and Jansen-Olesen, 1998). Pharmacological characterisation of CGRP receptors in isolated cerebral vessels has relied largely on testing a limited number of CGRP analogues and on the ant-

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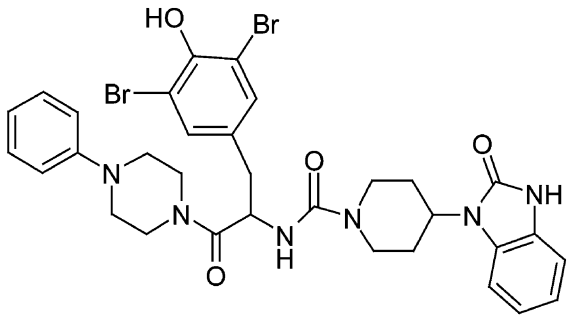

Fig. 1. Structure of Compound 1: 4-(2-*Oxo*-2,3-dihydro-benzoimidazol-1-yl)-piperidine-1-carboxylic acid [1-(3,5-dibromo-4-hydroxy-benzyl)-2-*oxo*-2-(4-phenyl-piperazin-1-yl)-ethyl]-amide.

agonistic properties of the CGRP-(8–37) fragment. To date, a limited number of non-peptide CGRP receptor antagonists have been reported. Examples include quinine analogues (Daines et al., 1997) which have relatively weak activity; displacement of ^{125}I-CGRP from SK-N-MC membranes revealed IC_{50} values in the micromolar concentration range (Daines et al., 1997). This result contrasts with nanomolar affinity of CGRP-(8–37) (Aiyar et al., 1996). More recently, Doods et al. (2000) described BIBN4096BS (a Lys–Tyr dipeptide derivative) which had high affinity (picomolar) for CGRP receptors endogenously expressed in SK-N-MC cells and inhibited neurogenic vasodilation evoked by trigeminal stimulation in marmosets.

The aim of the present study was therefore to evaluate a second CGRP receptor antagonist (see patent number WO 98/11128, coded Compound 1: 4-(2-*Oxo*-2,3-dihydro-benzoimidazol-1-yl)-piperidine-1-carboxylic acid [1-(3,5-dibromo-4-hydroxy-benzyl)-2-*oxo*-2-(4-phenyl-piperazin1-yl)-ethyl]-amide, see Fig. 1) and use it as a research tool to characterise CGRP-mediated responses in cranial arteries. Compound 1 was characterised initially in the neuroblastoma cell line, SK-N-MC, which expresses an endogenous CGRP receptor. The effects of Compound 1 on CGRP-evoked relaxation in human and guinea-pig cranial arteries were studied next and compared with the antagonistic effects of CGRP-(8–37).

2. Materials and methods

2.1. SK-N-MC cells

2.1.1. Binding studies

The binding of ^{125}I-CGRP to receptors in SK-N-MC cell membranes was carried out as described (Semark et al., 1992) with minor modifications. Membranes (25 μg) were incubated in 250 μl of binding buffer (10 mM Hepes, pH 7.4, 5 mM $MgCl_2$ and 0.2% bovine serum albumin, BSA) containing 50 pM ^{125}I-CGRP and inhibitor. After incubation at room temperature for 90 min, the assay was terminated by filtration through GFB glass fibre filter plates (Millipore) which had been pre-wet with 0.5% polyethyleneimine. The filters were washed three times with ice-cold assay buffer, then the plates were air-dried. Scintillation fluid (50 μl) was added and the radioactivity was counted on a Topcount (Packard Instrument). Non-specific binding was determined by using a final concentration of 100 nM CGRP. Data analysis was carried out by using Prism and the K_i was determined by using the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

2.1.2. Functional studies

SK-N-MC cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C, 95% humidity, and 5% CO_2. For cAMP assays, cells were plated at 5×10^5 cells/well in 96-well poly-D-lysine coated plates (Becton-Dickinson) and cultured for ~18 h before assay.

Cells were washed with phosphate buffered saline (PBS, Sigma) then pre-incubated with 300 μM isobutylmethylxanthine in serum-free MEM for 30 min at 37°C. α-CGRP-(8–37) or Compound 1 was added and the cells were incubated for 10 min prior to the addition of CGRP. The incubation was continued for another 30 min, then the cells were washed with PBS and processed for cAMP determination according to the manufacturer's recommended protocol. Maximal stimulation over basal was defined by using 100 nM CGRP. Dose–response curves were generated by using Prism. Dose-ratios (DR) were calculated and used to construct full Schild plots (Arunlakshana and Schild, 1959).

2.2. Isolated cranial arteries

Human intracerebral lenticulostriate arteries were removed at autopsy within 27–32 h after death. All vessels were placed in buffer solution (mM: NaCl, 119; KCl, 4.7; $CaCl_2$, 1.5; $MgSO_4$, 1.17; $NaHCO_3$, 25; KH_2PO_4, 1.18; EDTA, 0.027; glucose, 5.5, pH 7.4) aerated with 5% CO_2 in O_2 (carbogen) and transported to the laboratory for investigation. The total time between death and experiment was 40–53 h. The study was approved by the Human Investigation Review Board, Albert Szent-Györgyi Medical University, Szeged, Hungary (No. 1085).

Guinea pigs (300–550 g, Hvidesten, Statens Serum Institut, Denmark) were euthanised by pentobarbital 0.5 g/kg (i. p.) and the brains were rapidly transferred to the above buffer solution. Each basilar artery was carefully dissected out and divided into eight segments.

The arteries (human and guinea-pig) were cut into cylindrical segments of 0.4–1.6 mm in length for in vitro pharmacological experiments. Each segment was mounted on two metal prongs, one of which was connected to a force displacement-transducer and attached to a computer,

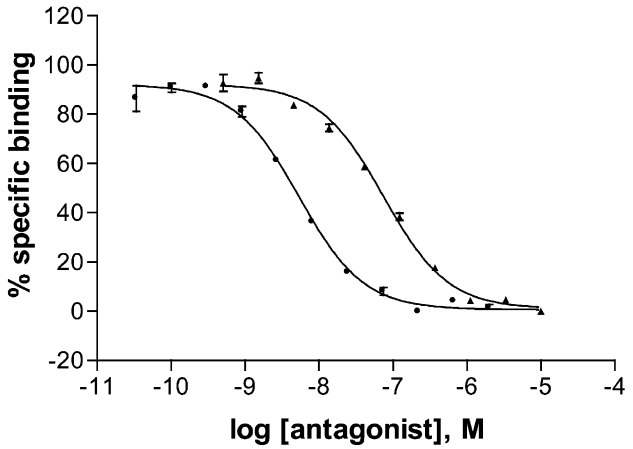

Fig. 2. Displacement of ^{125}I-CGRP binding from SK-N-MC membranes by CGRP-(8–37) (circles) and Compound 1 (triangles). Mean values ± S.E.M.

and the other to a displacement device (Mulvany and Halpern, 1977). The position of the holder could be changed by means of a movable unit allowing fine adjustments of vascular tension by varying the distance between the metal prongs. The mounted specimens were immersed in temperature-controlled tissue baths (+35°C) containing the buffer solution continuously gassed with carbogen, and the artery segments were allowed to equilibrate for approximately 30 min. The vessel tension was continuously recorded and the distance between the pins were adjusted to obtain a lumen diameter of $0.9 \times 1_{100}$. (Note, 1_{100} is an estimate of the lumen diameter of the vessel segment when a passive transmural pressure of 100 mm Hg is applied, Mulvany and Halpern, 1977.)

Following the 30-min equilibration period, the contractile capacity of each vessel segment was examined by exposure to a potassium-rich (125 mM) buffer solution which had the same composition as the standard solution except that the NaCl was exchanged for an equimolar concentration of KCl.

The vasodilatory effect of α-CGRP was examined by cumulative application of increasing concentrations of the peptide in the absence or presence of various concentrations of the antagonist (either α-CGRP-(8–37) or Compound 1). Each segment was precontracted with 3 μM prostaglandin(PG) $F_{2\alpha}$ before CGRP was added. Each segment was exposed to a single cumulative concentration–effect curve and a matched pairs protocol was used where one segment acted as control (no antagonist present) while in another six segments from the same artery, the agonist response was assessed following equilibration (10 min) with various concentrations of the antagonist.

The following materials were used in the in vitro experiments: human α-CGRP (Bachem or Peninsula Laboratories), human α-CGRP-(8–37), (Schafer-N, Denmark), prostaglandin F2α (Dinoprost®, Upjohn), (2-[^{125}I]iodohistidyl^{10})-hCGRP (Amersham) and isobutylmethylxanthine (Biomol). Compound 1 and α-CGRP-(8–37) used in the SK-N-MC studies were synthesised by Medicinal Chemistry (Merck Research Laboratories, USA). α-CGRP, α-CGRP-(8–37) and $PGF_{2\alpha}$ were dissolved in water and stored as aliquots at −20°C. Compound 1 was dissolved in dimethylsulphoxide (DMSO) and stored as aliquots at −20°C. SK-N-MC cells were obtained from ATCC (USA). SK-N-MC membranes were purchased from Receptor Biology (USA). ^{125}I-cAMP direct screening kits were purchased from Amersham (UK).

2.3. Analysis of data

2.3.1. Blood vessel studies

The vasodilatory response was expressed relative to the contraction evoked by prostaglandin $F_{2\alpha}$ (= 100%). For each segment, the maximum vasodilatory effect (E_{max}) was calculated. CGRP potency (expressed as pEC_{50}, i.e., negative logarithm of the molar concentration of agonist inducing a half maximum response) was determined by non-linear regression analysis (Graph Pad Prism 3.0). Data are expressed as mean values ± S.E.M. and *n* refers to the number of patients/animals from whom the vessels were collected. Statistically significant differences in pEC_{50} values were examined by Mann–Whitney *U*-test.

Dose-ratios were calculated and used to construct full Schild plots (Arunlakshana and Schild, 1959). The dissociation constants were calculated as described by Tallarida et al. (1979).

3. Results

3.1. SK-N-MC binding studies

Saturation binding studies were carried out to characterise the interaction of CGRP with the receptor in SK-N-

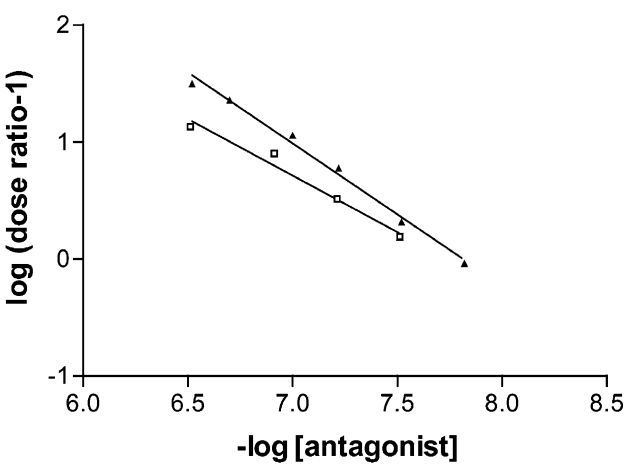

Fig. 3. cAMP effects. Schild plots showing the effects of CGRP-(8–37) (closed triangles) and Compound 1 (open squares) on CGRP evoked adenylyl cyclase stimulation in SK-N-MC cell membranes; for details see text.

MC cells. Specific binding of ^{125}I-CGRP was saturable, with a measured dissociation constant (K_D) of 15 pM and a B_{max} of 110 fmol/mg of membrane protein (data not shown). Both CGRP-(8–37) and Compound 1 displaced ^{125}I-CGRP with high affinity, yielding pK_i values of 8.9 and 7.8, respectively (Fig. 2).

3.2. Functional studies

3.2.1. SK-N-MC cells

The effect of CGRP-(8–37) and Compound 1 on CGRP-induced cAMP production in SK-N-MC cells was investigated. Both compounds caused a rightward shift in the dose–response curve for CGRP. Schild analysis of the data yielded a pA_2 value of 7.8 for CGRP-(8–37) with a slope of 1.2; a pA_2 value of 7.7 was obtained for Compound 1, with a slope of 0.96 (Fig. 3). These results demonstrated that both compounds exhibited competitive antagonism at the CGRP receptor present in SK-N-MC cells.

Table 1
pD_2 values of α-CGRP in the absence and presence of CGRP receptor antagonists in human intracerebral lenticulostriate arteries (LBMCA) and guinea-pig basilar arteries (BA)

Antagonist (M)	Human LBMCA		Guinea pig BA	
	Compound 1	CGRP-(8–37)	Compound 1	CGRP-(8–37)
0	*9.6 ± 0.1 (3)*		*9.0 ± 0.2 (6)*	
10^{-7}	8.8 ± 0.2[a]	8.7 ± 0.3[a]	8.8 ± 0.2	8.7 ± 0.1
10^{-6}	8.1 ± 0.2[a]	7.9 ± 0.2[a]	8.7 ± 0.2	8.0 ± 0.2[a]
10^{-5}	7.6 ± 0.3[a]	6.7 ± 0.3[a]	8.4 ± 0.1[a]	7.2 ± 0.3[a]

The pD_2 values are given as mean ± S.E.M. (number of individuals = twice the number of vessel segments).

[a] pD_2 is significantly different from pD_2 control as evaluated by nonparametric *U*-test (Mann–Whitney *U*).

3.3. Human isolated cerebral and guinea pig basilar arteries.

In human isolated cerebral arteries precontracted with prostaglandin $F_{2\alpha}$, CGRP caused a concentration-depen-

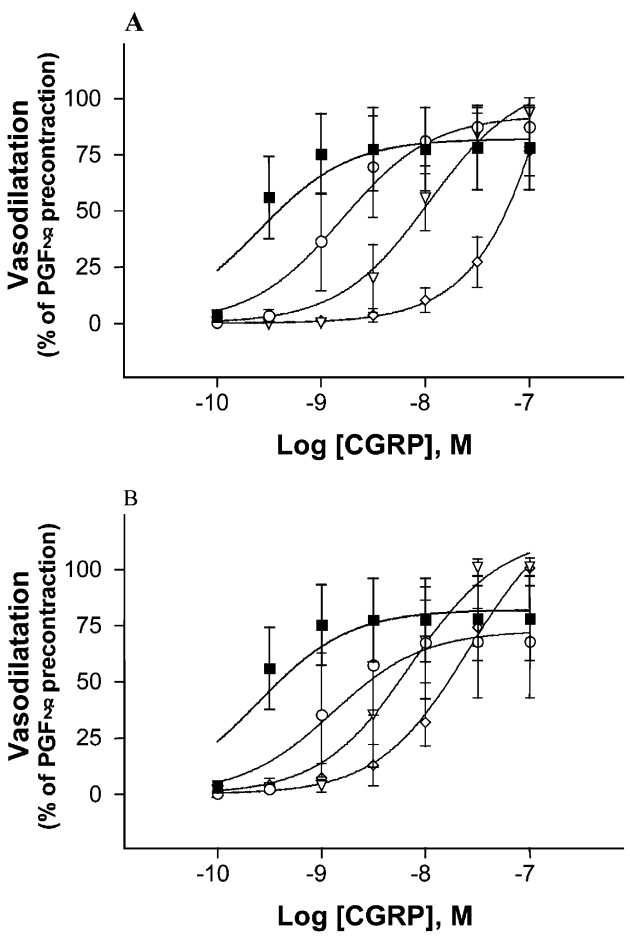

Fig. 4. Effect of αCGRP in human isolated cerebral arteries. Concentration–response curves to CGRP were obtained in the presence and absence of CGRP-(8–37) (Panel A) or Compound 1 (Panel B). Closed squares show the control relaxant curves in the absence of antagonist and open symbols show responses in the presence of increasing antagonist concentrations (circles 10^{-7} M, triangles 10^{-6} M and diamonds 10^{-5} M). Values given represent means ± S.E.M., $n = 3$.

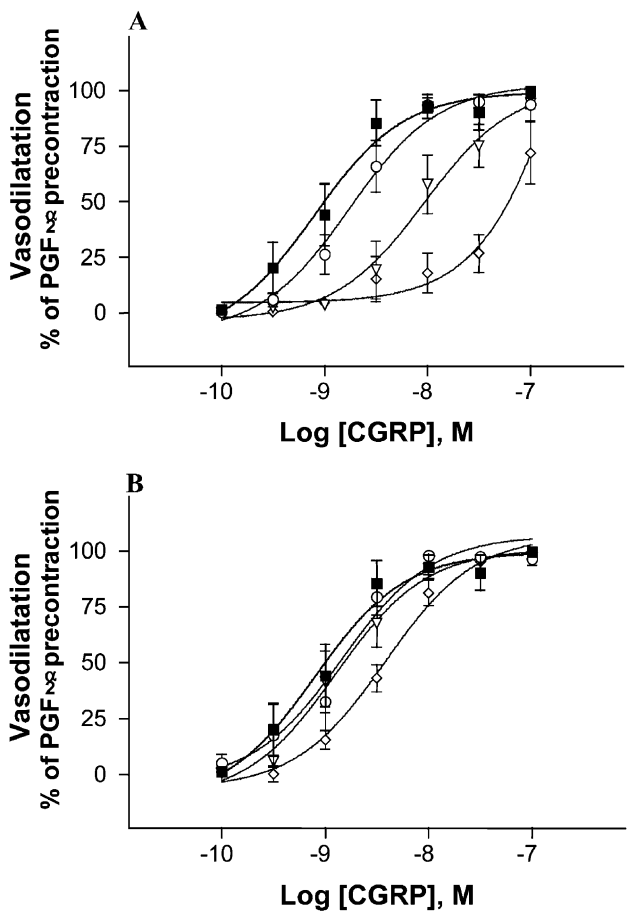

Fig. 5. Relaxant response of guinea-pig isolated basilar arteries following cumulative applications of CGRP. Concentration–response curves to CGRP were obtained in the presence and absence of CGRP-(8–37) (Panel A) or Compound 1 (Panel B). Closed squares show the control relaxant curves in the absence of antagonist and open symbols show responses in the presence of increasing antagonists concentrations (circles 10^{-7} M, triangles 10^{-6} M and diamonds 10^{-5} M). Values given represent means ± S.E.M., $n = 6$.

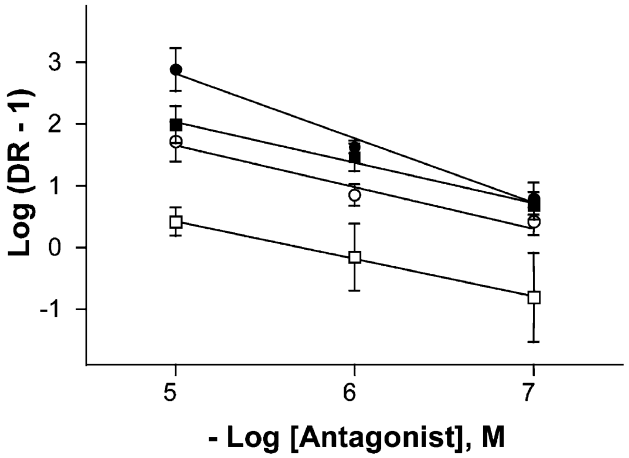

Fig. 6. Schild plot analysis of the antagonistic responses of CGRP-(8–37) (circles) and Compound 1 (squares) in human (open symbols) and guinea pig cerebral arteries (closed symbols).

dent relaxation, yielding a pEC_{50} value of 9.6 ± 0.1 and an E_{max} of 82 ± 19. CGRP-(8–37) caused concentration-dependent parallel shifts to the right of the concentration–effect curve to α-CGRP without changing the maximum relaxant response. The pEC_{50} value for CGRP was significantly reduced from 9.6 ± 0.1 in the absence of CGRP-(8–37) to 6.7 ± 0.3 in the presence of CGRP-(8–37) (Fig. 4 and Table 1). The results in guinea pig basilar artery segments were similar (Fig. 5).

In human isolated cerebral arteries, Compound 1 (0.1–10 μM) caused a parallel shift to the right of the concentration–effect curve to CGRP without changing the maximum response (Fig. 4). In contrast, in guinea pig basilar artery this compound had a much lower effect when tested in the same concentration range. Only the highest concentration of Compound 1 shifted the concentration–effect curve. In human cerebral arteries, Schild plot analysis revealed pA_2 values of 7.7 and 8.1 for CGRP-(8–37) and Compound 1, respectively (the slope was not significantly different from unity) (Fig. 6). In guinea pig basilar artery (Fig. 5), the pA_2 value for CGRP-(8–37) was 7.4 and for Compound 1 it was 5.7.

4. Discussion

CGRP receptors have long been regarded as a useful target for the development of novel antimigraine therapies. However, the CGRP receptor is not a simple G-protein coupled receptor and receptor activity modifying proteins (RAMPs) are necessary for both CGRP receptor expression and function (McLatchie et al., 1998). Furthermore, association of the same G-protein coupled receptor (i.e. CRLR, calcitonin gene-related receptor-like receptor) in combination with different RAMPs can result in differences in receptor pharmacology. Previous studies have shown the expression of mRNAs encoding CRLR and RAMPs 1, 2 and 3 in human cranial arteries (Sams and Jansen-Olesen, 1998). However, the characteristics of the receptor are determined by the association of CRLR and a single RAMP protein. In addition, pharmacological characterisation has not been definitive since it has relied heavily on the use of the antagonist fragment CGRP-(8–37).

In the present study, we further characterised the receptor for CGRP in human cranial arteries by examining the effects of CGRP-(8–37) and those of a novel non-peptide antagonist. Previously, it was reported that SK-N-MC cells, which express an endogenous CGRP receptor, display similar pharmacology to a recombinant cell line expressing only CRLR and RAMP1 (McLatchie et al., 1998). We therefore used this cell line to compare the binding and antagonistic properties of CGRP-(8–37) and Compound 1. Both compounds potently displaced ^{125}I-CGRP from SK-N-MC membranes and functioned as competitive antagonists of CGRP-induced cAMP accumulation. Based on these observations, it is likely that both of these compounds act at CGRP receptors composed of CRLR and RAMP1.

In human isolated cerebral arteries, both Compound 1 and CGRP-(8–37) antagonised relaxant responses evoked by CGRP and this effect of CGRP-(8–37) is consistent with previous reports (Edvinsson et al., 1998; Jansen-Olesen et al., 1996). For both antagonists, the pA_2 values were similar to those obtained in the SK-N-MC cell line, suggesting that the receptor mediating the vasodilatory effects of CGRP is similar to the receptor in the cell line, i.e., CRLR/RAMP 1. This observation is supported by findings from RT-PCR studies showing the expression of mRNAs coding for these proteins in human cranial arteries (Sams and Jansen-Olesen, 1998).

Interestingly, in the guinea-pig cerebral artery, CGRP-(8–37) antagonised CGRP evoked relaxations with a pA_2 value similar to that obtained in the SK-N-MC cell line and in accordance with previously reported effects in other guinea-pig isolated tissues, e.g., atria (Longmore et al., 1994). However, in guinea-pig basilar artery, Compound 1 was without effect at concentrations below 10 μM. This lack of antagonistic effect of Compound 1 may reflect species differences in receptor pharmacology for this class of compounds. Indeed, a related compound BIBN4096BS has been reported to have greater than 200-fold reduced affinity for rat CGRP receptors compared to the affinity in the SK-N-MC cell line (Doods et al., 2000).

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