British Journal of Pharmacology (2004) 143, 1066–1073
www.nature.com/bjp

Noncompetitive antagonism of BIBN4096BS on CGRP-induced responses in human subcutaneous arteries

*,[1]Majid Sheykhzade, [2]Henrik Lind & [2]Lars Edvinsson

[1]Department of Pharmacology, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark and [2]Department of Internal Medicine, Lund University Hospital, 22185 Lund, Sweden

1 We investigated the antagonistic effect of 1-piperidinecarboxamide, *N*-[2-[[5amino-l-[[4-(4-pyridinyl)-l-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl) (BIBN4096BS) on the calcitonin gene-related peptide (CGRP)-induced responses by using isometric myograph and FURA-2 technique in human subcutaneous arteries removed in association with abdominal surgery.

2 BIBN4096BS, at the concentration of 1 pM, had no significant effect on the CGRP-induced relaxation in these vessels.

3 At the concentration of 10 pM, BIBN4096BS had a competitive antagonistic-like behaviour characterized by parallel rightward shift in the log CGRP concentration-tension curve with no depression of the E_{max}.

4 At the higher concentrations (0.1 and 1 nM), BIBN4096BS had a concentration-dependent noncompetitive antagonistic effect on the CGRP-induced responses.

5 The efficacy and potency of CGRP was significantly greater in the smaller (lumen diameter $\sim$200 μm) human subcutaneous arteries compared to the larger ones.

6 The apparent agonist equilibrium dissociation constant, K_A, for $CGRP_1$ receptors in the human subcutaneous arteries was approximately 1 nM. Analysis of the relationship between receptor occupancy and response to CGRP indicates that the receptor reserve is relatively small.

7 Using reverse transcriptase-polymerase chain reaction (RT-PCR), the presence of mRNA sequences encoding the calcitonin receptor-like receptor, receptor activity modifying protein (RAMP1, RAMP2, RAMP3) and receptor component protein were demonstrated in human subcutaneous arteries, indicating the presence of $CGRP_1$-like receptor and the necessary component for the receptor activation.

8 In conclusion, the inhibitory action of BIBN4096BS at the low concentration (10 pM) on the CGRP-tension curve (but not intracellular calcium concentration ($[Ca^{2+}]_i$) resembles what is seen with a reversible competitive antagonist. However, at the higher concentrations (0.1 and 1 nM), BIBN4096BS acts as a selective noncompetitive inhibitor at $CGRP_1$ receptors in human subcutaneous arteries.

British Journal of Pharmacology (2004) **143,** 1066–1073. doi:10.1038/sj.bjp.0705967

Keywords: Affinity; calcitonin gene-related peptide; BIBN4096BS; human subcutaneous artery

Abbreviations: BIBN4096BS, 1-piperidinecarboxamide, *N*-[2-[[5amino-l-[[4-(4-pyridinyl)-l-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl); Ca^{2+}, calcium; $[Ca^{2+}]_i$, intracellular calcium concentration; CGRP, calcitonin gene-related peptide; CRC, concentration–response curve; CRLR, calcitonin receptor-like receptor; E/E_{max}, relative vessel response to agonist; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-*bis*(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; K_A, apparent receptor agonist equilibrium dissociation constant; pK_A, receptor agonist affinity $= -\log(K_A[M])$; PSS, physiological salt solution; R/R_t, relative receptor agonist occupancy; RAMP, receptor activity modifying protein; RCP, receptor component protein; RT–PCR, reverse transcriptase–polymerase chain reaction

Introduction

Calcitonin gene-related peptide (CGRP) immunoreactive nerves have been demonstrated throughout the central and peripheral nervous system. As a neurotransmitter CGRP is found predominantly in the sensory nerve fibres innervating blood vessels located both peripherally and centrally (Uddman *et al*., 1986; Holzer, 1988). Upon stimulation, CGRP can be released from these nerve fibres both *in vitro* and *in vivo* and cause vasodilatation. For instance, this occurs both in cardiovascular system following ischaemia (Kallner, 1998), during migraine headache (Goadsby *et al*., 1990) and following subarachnoid haemorrhage (Juul *et al*., 1990).

*Author for correspondence; E-mail: mash@dfuni.dk
Advance online publication: 11 October 2004

CGRP receptors belong to the family of G-protein-coupled receptors characterized by seven transmembrane helices (Chatterjee *et al*., 1993) and are subdivided into two types, designated $CGRP_1$ and $CGRP_2$ receptors (Quirion *et al*., 1992). An important progress has been made in the field of receptor cloning and signalling by demonstrating that CRLR (calcitonin receptor-like receptor), in association with receptor activity modifying proteins (RAMPs: RAMP1, RAMP2, RAMP3) determined the receptor affinity to CGRP and adrenmedullin, while calcitonin receptor in association with RAMPs determines amylin affinity (McLatchie *et al*., 1998; Muff *et al*., 1999). Furthermore, it was shown that the RAMPs (particularly RAMP1) associate with the receptor component protein (RCP) (Luebke *et al*., 1996) to insure high-affinity coupling between the CGRP receptor and the G protein (G_S), suggesting that these protein chaperones are required for activation of signal transduction leading to the production of cAMP (Evans *et al*., 2000; Juaneda *et al*., 2000).

As far as the characterization of CGRP receptors is concerned, studies of CGRP receptors over the past decade were dependent on the use of the C-terminal fragment antagonist CGRP (8–37), but the recently developed small molecule selective nonpeptide CGRP receptor antagonist (BIBN4096BS, 1-piperidinecrboxamide, *N*-[2-[[5amino-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(2*H*)-quinazolinyl) has displayed much higher potency (an apparent pA_2 value in the range of 11) for human CGRP receptors expressed in SK-N-MC cells (Doods *et al*., 2000). In addition, we have recently shown that BIBN4096BS competitively antagonized the CGRP-induced relaxations in isolated human cerebral arteries (pA_2 value of 10.1) (Edvinsson *et al*., 2002). In human coronary arteries the situation is somewhat different; we observed that the vessels were less responsive to CGRP but equally sensitive to BIBN4096BS (pA_2 value of 10.4) Edvinsson *et al*., 2002). These findings are in accord with the data recently presented by Gupta *et al*. (2004). Both studies showed reduction in maximum relaxation to CGRP as the concentration of BIBN4096BS was increased. In addition, omental arteries of man (intestinal arteries) did not respond to CGRP (Edvinsson *et al*., 2002). Most strikingly, BIBN4096BS demonstrates species specificity with 100-fold lower affinity for the rat *versus* human $CGRP_1$ receptor. BIBN4096BS inhibited the vasodilation induced by the stimulation of the trigeminal ganglion in the marmoset monkey, which suggest its potential usefulness in the treatment of migraine headache (Doods *et al*., 2000; Edvinsson, 2003). With regard to discrimination between $CGRP_1$ and $CGRP_2$ receptor subtypes, the selectivity profile of BIBN4096BS is much improved compared with that of CGRP(8–37) (Wu *et al*., 2000).

The purpose of our study was to investigate the inhibitory action of BIBN4096BS on the CGRP-induced response in human subcutaneous arteries by using isometric myograph combined with FURA-2 technique. Simultaneous recording of force and intracellular calcium in these arteries will further shed light on the antagonistic profile of BIBN4096BS in peripheral vessels. Furthermore, reverse transcriptase-polymerase chain reaction (RT-PCR) technique was applied to demonstrate the presence of the necessary components for CGRP receptor expression and activation of signal transduction.

Methods

Isolated human subcutaneous arteries

The experiments were approved by the Ethics Committee of the Lund University (LU 818-01) and patient's consent. Subcutaneous arteries were surgically removed from the normal part of the abdominal wall of 25 patients (11 male and 14 female subjects; age range 46–85 years) under operation. The human subcutaneous arteries were immediately placed in 4°C cold oxygenated physiological salt solution (PSS) and cleaned of adhering fat and connective tissue and cut into rings of 1–2 mm in length for *in vitro* pharmacological experiments.

Measurement of force development

At 3–4 h after removal, the arterial ring segments were mounted on an isometric myograph (Danish Myo Technology A/S, Aarhus, Denmark) as previously described (Mulvany & Nyborg, 1980). After mounting, the arteries wee equilibrated in oxygenated (5% CO_2 in O_2) PSS at 37°C, pH 7.4, for 30 min. The vessels were then stretched to an internal circumference, L_1, equal to 90% of the circumference, L_{100}, the vessel would have if exposed to a passive transmural pressure of 100 mmHg (13.3 kPa) i n order to secure maximal active force development (Nyborg *et al*., 1987). The effective vessel lumen diameter was calculated as L_1/π.

The vessels were contracted at least six times (three times before and three times after loading with Fura-2) with the buffer solution containing 125 mM K^+ (KPSS, similar to PSS except that NaCl was exchanged for KC1 on an equimolar basis) until reproducible contractions were recorded. Vessels were accepted only if the maximal active pressure (calculated according to the Laplace relation: $\Delta P_{max} = 2 \times \Delta T_{max}/l_1$) exceeded 13.3 kPa (Mulvany & Halpern, 1977). This limit was chosen in order to secure that the measured dynamic fluorescence signals (corresponding to the $[Ca^{2+}]_i$ levels) were generated from optimal functional vessel segments.

Endothelium function

The endothelium was present in all human subcutaneous arteries. In order to assess the endothelium function in the human subcutaneous arteries, a steady-state contraction was first induced in these vessels by applying 300 nM U 46619. The vessels were then challenged with single concentration of 10 μM acetylcholine. The average acetylcholine-induced relaxation was $38 \pm 2\%$ ($n = 24$).

FURA-2 loading procedure and measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

The human subcutaneous arteries were loaded with the fluorescent $[Ca^{2+}]_i$ indicator dye (FURA-2) using exactly the same procedure as previously described (Sheykhzade & Nyborg, 2001). All experiments with FURA-2 were performed in the dark. The $[Ca^{2+}]_i$ was calculated according to the equation; $[Ca^{2+}]_i = K_d\beta[(R-R_{min})/(R_{max}-R)]$, with the assumption that the dissociation constant of FURA-2–Ca^{2+} complex, K_d, is 224 nM at 37°C (Grynkiewicz *et al*., 1985). The parameters β, R_{min} and R_{max} (all corrected for background

fluorescence signals) were determined in each vessel at the end of the experiment using exactly the same procedure as previously described (Sheykhzade & Nyborg, 2001). The mean value ($n=18$) of R_{max}, R_{min} and β were 2.66 ± 0.04, 1.12 ± 0.03 and 1.59 ± 0.10, respectively. The value in PSS and the plateau phases were designated to be 0 and 100% for both the $[Ca^{2+}]_i$ and tension, respectively.

Concentration–response curves (CRCs)

In order to investigate the effect of BIBN4096BS on the CGRP-induced responses in these vessels, two consecutive cumulative CGRP CRCS in full log increments (10 pM–100 nM) were performed on each arterial segment precontracted with 300 nM U46619. The first CGRP CRC served as the control curve (without antagonist) and the second CGRP CRC was obtained in the presence of the selective nonpeptide $CGRP_1$-receptor antagonist, BIBN4096BS. After the first CGRP control curve, the arteries were stimulated twice with KPSS and incubated with BIBN4096BS for 30 min before the second CGRP CRC was performed. This preincubation time (30 min) is the same that was used in our previous study on human cerebral arteries, where BIBN4096BS concentration-dependently induced a significant parallel-rightward shift in the log CGRP concentration–relaxation curve (Edvinsson *et al*., 2002). Time control studies showed no sign of tachyphylaxis to CGRP in these vessels as the first and the second CGRP curves were completely overlapping (data not shown). Four separate groups of arteries were used for the four different concentrations (1 pM, 10 pM, 0.1 nM and 1 nM) of the antagonist. BIBN4096BS had no effect on either the basal tone of the vessels or the U46619-induced precontration tone at the concentration range used in this study. The average internal lumen diameter of vessels in four separate groups was 377 ± 60, 324 ± 49, 333 ± 67 and $363\pm60\,\mu$M ($n=6$), respectively.

Data analysis and statistics

Relaxations are expressed as a percentage of U46619-induced tension (precontraction tone). The precontraction tone induced by 300 nM U46619 is expressed either as Nm^{-1} (newton per meter of vessel wall) or as percentage of the steady-state contractile response to KPSS. The levels of $[Ca^{2+}]_i$ are given either as percentage of the plateau levels or as absolute values (nM). The CRCs to CGRP were fitted to the classical 'Hill equation': $E/E_{max}=[A]^n/([A]^n+EC_{50}[M]^n)$ using the GraphPad Prism 3.0 software. E/E_{max} is the relative vessel response to the agonist concentration, A[M]. EC_{50}[M] is concentration of agonist required to give half-maximal response, and n is a fitting constant or 'Hill coefficient' (Kenakin, 1997). The vessel sensitivity to CGRP is given as pD_2 value, where $pD_2=-\log(EC_{50}[M])$. Results are given as mean±s.e.m. (n=number of vessels). Differences between mean values were analysed by a two-tailed Student's t-test for paired or unpaired where appropriate. Results were considered to be significant if P value is <0.05.

Estimation of the apparent antagonist affinity (pK_B)

Owing to the pseudocompetitive behaviour of BIBN4096BS at the lower concentration (10 pM), the apparent affinity of the antagonist (expressed as pK_B value) has been estimated according to the equation: $pK_B=\log(CR-1)-\log[\text{antagonist, (M)}]$ (MacKay, 1978), where CR is the agonist concentration ration between the EC_{50} (M) in the presence and absence (control condition) of the antagonist.

Estimations of the agonist equilibrium dissociation constant (K_A) and receptor reserve

Owing to the noncompetitive antagonistic action of BIBN4096BS at concentrations above 10 pM, we were able to estimate the apparent $CGRP_1$-receptor agonist affinity by applying the same mathematical method for receptor agonist affinity determination first described by Furchgott & Bursztyn (Furchgott, 1966; Furchgott & Bursztyn, 1967).

Reciprocals of equieffective concentrations of CGRP in control condition (A[M]) and in the presence of 0.1 nM BIBN4096BS (A'[M]) were determined on the basis of nonlinear regression analysis of the average CRC data ($n=6$). The slope of the regression line (least-square method) and the y-axis intercept with 95% confidence interval was estimated in a plot of $1/A$[M]) *versus* $1/A'$[M] using the GraphPad Prism 3.0 software. The estimated K_A[M] was used to estimate the relative $CGRP_1$-receptor occpancy, $R/R_t=(A[M]/A[M]/+K_A[M])$. The agonist equilibrium dissociation constant, K_A [M] for $CGRP_1$ receptor is given as pK_A value, where $pK_A=-\log(K_A[M])$.

Molecular experiments

Primer pairs were designed to detect mRNA for human CRLR (497 bp) (forward:5′-TGC TCT GTG AAG GCA TTT AC-3′ and reverse: 5′-CAG AAT TGC TTG AAC CTC TC-3′), human RAMP1 (445 bp) (forward: 5′-GAG ACG CTG TGG TGT GAC TG-3′ and reverse: 5′-TCG GCT ACT CTG GAC TCC TG-3′), human RAMP2 (283 bp) (forward: 5′-GGA CGG TGA AGA ACT ATG AG-3′ and reverse: 5′-ATC ATG GCC AGG AGT ACA TC-3′), human RAMP3 (159 bp) (forward: 5′-TGG AAG TGG TGC AAC CTG TC-3′ and reverse: 5′-TCG GCT ACT CTG GAC TCC TG-3′) and human RCP (395 bp) (forward: 5′-GTC AAG GAT GCC AAT TCT GC-3′ and reverse: 5′-TTC TTC TGC TCA GCC TCT GG-3′. The isolation of mRNA and RT-PCR assay for CRLR, RAMP1–3 and RCP were performed using the primers and the method previously described (Sams & Jansen-Olesen, 1998; Hasbak *et al*., 2003).

Drugs and solutions

PSS had the following composition (mM): NaCl 119, $NaHCO_3$ 25, KC1 4.7, $CaCl_2$ 1.5, K_2HPO_4 1.18, $MgSO_4$ 1.17, ethylene diamine tetraacetic acid (EDTA) 0.026 and glucose 5.5, pH 7.4 KPSS (125 mM K^+) was prepared by replacing NaCl with equimolar KC1. Solutions used for determination of R_{min} and R_{max} contained (in mM): 4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid (HEPES) 5, KC1 125, $MgCl_2\cdot6H_2O$ 1.17 and glucose 5.5, and then either 2 mM ethylene glycol-*bis* (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) or 5 mM $CaCl_2\cdot2H_2O$ was added, respectively. Drugs used were U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$) (Fluka Chemie AG, Switzerland), ionomycin, pluronic F127, cremophor EL, $MnCl_2$, human-αCGRP (Sigma, St Louis, MO, U.S.A.) and FURA-2AM (Molecular Probes,

Leiden, The Netherlands). BIBN4096BS was synthesised by Medical Chemistry, Merck Sharp and Dohme Research laboratories, U.K. FURA-2/AM was dissolved in loading mixture (anhydrous DMSO, pluronic F-127 and cremophor EL) just before loading. U46619 was dissolved in 50% ethanol at 10^{-2} M. Human-αCGRP and BIBN4096BS were dissolved in distilled water and stored frozen at −20°C until use. Dilutions of the stock solutions were made fresh each day.

Result

Effect of CGRP on the tension of U46619-precontracted arteries in control conditions

CGRP induced a concentration-dependent reduction in both the $[Ca^{2+}]_i$ and tension of human subcutaneous arteries precontracted with 300 nM U46619 (Figure 1a and b). The sensitivity of the vessels to CGRP (pD_2) was 9.34 ± 0.07 ($n = 24$), and the maximum relaxation induced by CGRP was $60 \pm 4\%$ ($n = 24$) (Figure 1a). The mean lumen diameter of vessel used was 349 ± 29 μm ($n = 24$).

Effects of CGRP on the $[Ca^{2+}]_i$ of U46619-precontracted arteries in control conditions

The maximal reduction in the $[Ca^{2+}]_i$ induced by CGRP in U46619-precontracted arteries was $57 \pm 5\%$ or 49 ± 6 nM ($n = 18$) (Figure 1b).

$[Ca^{2+}]_i$–tension relationships under resting condition and after single stimulations

The mean steady-state resting level of the $[Ca^{2+}]_i$ and tension in PSS was 37 ± 6 nM ($n = 18$) and 0.80 ± 0.07 Nm^{-1} ($n = 18$), respectively. When bathing medium was changed from normal PSS (5.9 mM) to KPSS (125 mM K^+), both the $[Ca^{2+}]_i$ and tension rapidly increased to reach (within 2 min) plateau levels of 243 ± 20 nM ($n = 18$) and 4.58 ± 0.50 Nm^{-1} ($n = 18$) respectively.

The mean steady-state level of the $[Ca^{2+}]_i$ and tension induced by 300 nM U46619 was 123 ± 10 nM ($n = 18$) and 6.92 ± 0.75 Nm^{-1} ($n = 18$), respectively.

Effect of BIBN4096BS at the low concentrations on CGRP-induced responses

At the concentration of 1 pM, BIBN4096BS was without antagonistic effect on the CGRP concentration–tension curve. The CGRP-induced maximal relaxation was 50 ± 10 *versus* $49 \pm 9\%$ ($n = 6$), and the corresponding pD_2 value was 9.21 ± 0.11 *versus* 9.22 ± 0.13, in the control condition and in the presence of 1 pM antagonist, respectively.

When the concentration of BIBN4096BS was increased to 10 pM, the antagonist induced a significant rightward shift in the log CGRP concentration–tension curve with no depression of E_{max} (Figure 1a). The CGRP-induced maximal relaxation was 60 ± 9 *versus* $59 \pm 10\%$ ($n = 6$), and the corresponding pD_2 value was 9.51 ± 0.07 *versus* 9.06 ± 0.13 (Paired *t*-test, $P < 0.0098$; $n = 6$), without and with the antagonist, respectively. Owing to the competitive behaviour of BIBN4096BS at this concentration, the apparent pK_B value for BIBN4096BS was estimated to be 11.26 ± 0.11 ($n = 6$). There was, however, a nonsignificant parallel rightward shift in the log CGRP concentration–$[Ca^{2+}]$i curve in the presence of 10 pM antagonist (Figure 1b).

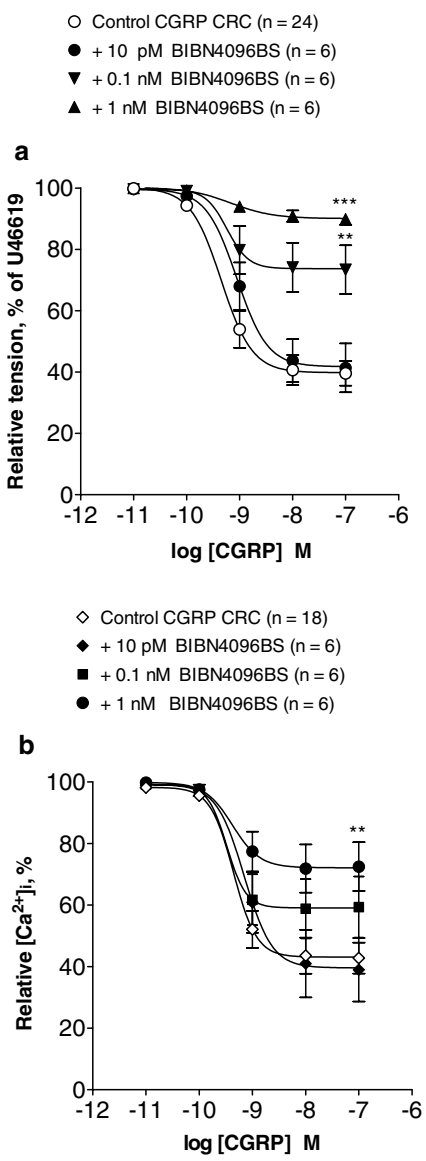

Figure 1 Effect of increasing concentrations of BIBN4096BS (10 pM, 0.1 nM, 1 nM) on the CGRP-induced reduction in the tension (a) and $[Ca^{2+}]_i$ (b) of human subcutaneous arteries. The control CGRP concentration–response curves (CRCs) are based on the pooled data. Points represent mean values and vertical bars indicate ± s.e.m. Relative tension and $[Ca^{2+}]_i$ are given as percentages of the initial steady-state levels induced by 300 nM U46619. A paired two-tailed *t*-test was used to compare the mean values between the first and second curves (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Effect of BIBN4096BS at the higher concentrations on CGRP-induced responses

At the concentrations above 10 pM (0.1 and 1 nM), BIBN4096BS concentration dependently attenuated the CGRP-induced responses in a noncompetitive fashion. The

maximal relaxation induced by CGRP was significantly (67±8 *versus* 27±8%; Paired *t*-test, $P=0.0042$; $n=6$) reduced in the presence of 0.1 nM BIBN4096BS, and there was an even further attenuation of the CGRP-induced maximal relaxation (66±7 *versus* 10±2%; Paired *t*-test, $P<0.0001$; $n=6$) when concentration of BIBN4096BS was increased to 1 nM. The CGRP-induced maximal reduction in $[Ca^{2+}]i$ was significantly (Paired *t*-test, $P=0.0089$) decreased from 59±7% or 51±5 nM ($n=6$) in the control condition to 27±8% or 25±7 nM ($n=6$) in the presence of 1 nM BIBN4096BS (Figure 1a and b). The sensitivity of the vessels to CGRP was slightly reduced, the pD_2 values decreasing from 9.39±0.06 ($n=6$) in the control condition to 9.26±0.07 ($n=6$) in the presence of 0.1 nM BIBN4096BS, and from 9.25±0.07 ($n=6$) in the control condition to 9.14±0.08 ($n=6$) in the presence of 1 nM BIBN4096BS.

Calibre dependency of CGRP-induced relaxation

Further analysis of the pooled data showed a significant correlation between the arterral lumen diameter and the magnitude of maximum relaxation induced by CGRP ($r=0.91$; $P<0.0001$; $n=24$) (Figure 2a) and the vessel sensitivity (pD_2) to CGRP (linear regression analysis: $r=0.62$; $P=0.0012$; $n=24$) (Figure 2b). The calibre of the arteries had no influence on the level of precontraction tone (% of KPSS) induced by 300 nM U46619 ($r=0.09$; $P=0.66$; $n=24$), and the level of precontraction tone in these vessels had no influence on the magnitude of maximum relaxation induced by CGRP ($r=0.03$; $P=0.90$; $n=24$).

Apparent agonist equilibrium dissociation constant and receptor reserve

CGRP concentration–response relations in control condition and in the presence of 0.1 nM BIBN4096BS in isolated human subcutaneous arteries were used for estimation of $CGRP_1$-receptor agonist affinity. Vessel responses have been normalized to the maximal response to CGRP in each vessel, the pD_2 value in control condition being equal to 9.39±0.06 ($n=6$). The $CGRP_1$-receptor agonist dissociation constant, K_A[M] determined in the presence of 0.1 nM BIBN4096BS was 1.13 nM (1.05–1.23 nM, 95% confidence interval). The $CGRP_1$-receptor agonist affinity, pK_A[M] was thus 8.95 (8.98–8.91, 95% confidence interval) (Figure 3). Analysis of the relationship between the relative $CGRP_1$-receptor agonist occupancy (R/R_t) and the relative CGRP-induced response (E/E_{max}) revealed that approximately 27% of all active $CGRP_1$ receptors must be occupied for eliciting half-maximal response (EC_{50}) to CGRP.

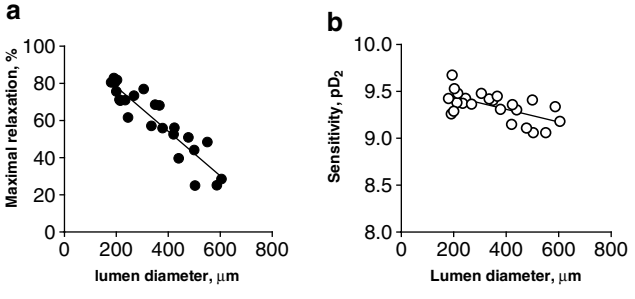

Figure 2 The relationship between the size of lumen diameter of human subcutaneous arteries and (a) the maximal CGRP-induced relaxation in these vessels (linear regression analysis: $r=0.91$; $P<0.0001$; $n=24$) and (b) the sensitivity (pD_2) of human subcutaneous arteries to CGRP (linear regression analysis: $r=0.62$; $P=0.0012$; $n=24$).

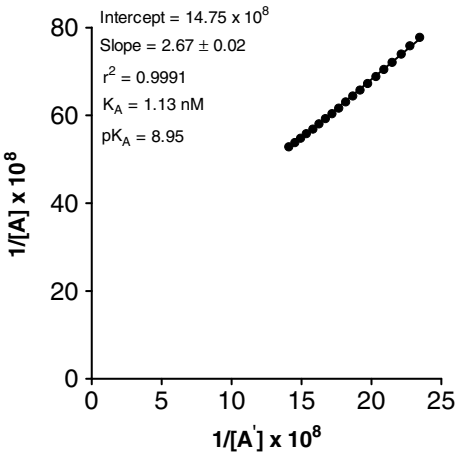

Figure 3 Regression line for the plot of reciprocals of equieffective concentrations of CGRP without (A[M] and with 0.1 nM BIBN4096BS (A'[M]).

Molecular experiments

The results from RT–PCR revealed the presence of mRNA encoding CRLR, RAMP1, RAMP2, RAMP3 and RCP in human subcuttaneous arteries ($n=4$) (Figure 4). All vessels expressed the appropriate mRNAs.

Discussion

Antagonistic behaviour of CGRP in human subcutaneous arteries

The present study has for the first time shown that BIBN4096BS only at the lower concentration (10 pM) had a competitive antagonistic-like behaviour towards the CGRP-induced responses since BIBN4096BS at the relatively higher concentrations (0.1 and 1 nM) acted as a noncompetitive antagonist on the CGRP-receptors in human subcutaneous arteries. In contrast to our results, a number of studies determining the affinity of BIBN4096BS for CGRP receptors in SK-N-MC human neuroblastoma cell line ($pK_i=10.8$, Doods *et al*., 2000; $pK_i=11.4$ and $pA_2=11.2$, Edvinsson *et al*., 2002), human temporal arteries ($pK_i=10.1$–10.4, Verheggen *et al*., 2002), human cerebral arteries ($pA_2=10.1$, Edvinsson *et al*., 2002) and human coronary arteries ($pA_2=10.4$, Edvinsson *et al*., 2002) have demonstrated a competitive antagonistic action of BIBN4096BS at $CGRP_1$ receptors over a wider rang of concentrations. As far as the affinity of BIBN4096BS for the $CGRP_1$ receptor is concerned, the affinity of BIBN4096BS determined in the previous studies correlates well with the apparent antagonist affinity value estimated in our study (apparent $pK_B\sim11$) due to the competitive behaviour of BIBN4096BS at the lower

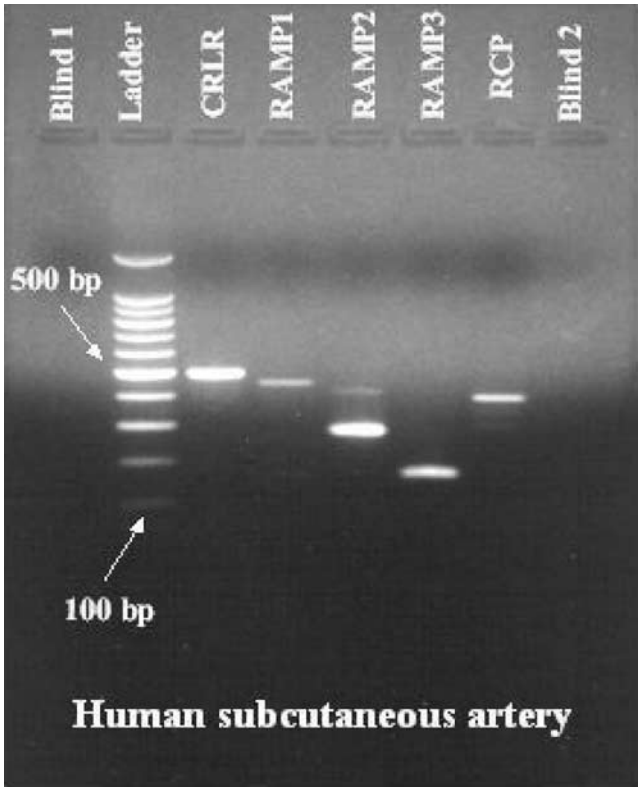

Figure 4 Demonstration of mRNA encoding the CRLR (size: 497 bp), RAMP1 (size: 445 bp), RAMP2 (size: 283 bp), RAMP3 (size: 159 bp) and RCP (size: 392 bp) in the human subcutaneous areteries by RT-PCR. Ladder: 100-base pair ladder. Blind 1 and 2 are negative controls using primers for CRLR and RAMP1, respectively, without the reverse transcriptase enzyme. Negative controls using primers for RAMP2, RAMP3 and RCP were also performed (not shown).

concentration (10 pM). However, in contrast to previous reports, it is surprising to find that BIBN4096BS at the higher concentrations (0.1 and 1 nM) acts like a noncompetitive antagonist against the CGRP-induced responses in human subcutaneous arteries. A noncompetitive behaviour of BIBN4096BS (apparent $pK_B \sim 11$) to $CGRP_1$ receptors is higher than that of CGRP ($pK_A \sim 9$), which seems to be the case in our study. Furthermore, different experimental conditions can also contribute to the discrepant findings. More generally, the extent to which the action of a competitive antagonist can be overcome by increasing the concentration of agonist is determined by the relative concentrations of two agents, by the association and dissociation rate constants for their binding, and by the duration of exposure of each (see Kenakin, 1997). The action of a competitive antagonist can therefore be surmountable under one set of experimental conditions and ionsurmountable under another.

In a previous study carried out by Verheggen *et al*. (2002) on isolated human temporal arteries, an incubation time of 2 h with BIBN4096BS was used *versus* 30 min of incubation in the present study and also in the previous studies carried out by Edvinsson *et al*. (2002) on isolated human coronary and cerebral arteries. Furthermore, Verheggen *et al*. (2002) showed that BIBN4096BS at the concentration of 1 μM caused additional blockage, which was insurmountable. The authors explained it by insufficient dissociation of BIBN4096BS from the receptors. Similar observations have been made in our previous study on human coronary arteries when relatively higher concentrations of BIBN4096BS (3 and 10 nM) were used (Edvinsson *et al*., 2002). Furthermore, recent studies carried out by Gupta *et al*. (2004) showed similar results in both proximal and distal regions of the human coronary vascular bed. The latter authors also demonstrated that the Schild plot slope was around 0.6 and hence not a perfect dissociation (Gupta *et al*., 2004). An alternative explanation for the partially surmountable antagonism by BIBN4096BS is that the antagonist, when in close enough proximity to its binding site, may form a covalent bond with it, and the antagonist–receptor complex is then converted into a tight binding slow reversible state (*irreversible competitive antagonist*). This results in insurmountable antagonism in a system with little or no receptor reserve (see Kenakin, 1997).

Receptor reserve and calibre dependency of CGRP-induced relaxation

In addition to the experimental conditions, the tissue-dependent factors such as receptor density or receptor reserve and efficiency of receptor–effector coupling can also affect the magnitude of response produced by an agonist (efficacy). In our study, approximately 27% of all receptors must be occupied by CGRP to elicit a half-maximal response (EC_{50}), indicating the presence of a relatively small $CGRP_1$-receptor reserve pool in the human subcutaneous arteries. The term receptor reserve connotes a property of a tissue, when in fact the phenomenon is dependent on both the tissue and the agonist. Therefore, the agonist receptor reserve is relative and depends upon the intrinsic efficacy of the agonist (Kenakin, 1997). However, the receptor density and the efficiency of coupling between the receptor and the stimulus–response mechanisms in the human subcutaneous arteries will influence the maximal response to CGRP in any case. In the present study, we have shown that the maximal response and the sensitivity to CGRP is inversely related to the calibre of human subcutaneous arteries, which is in concert with the previous findings in the coronary arteries of rat (Sheykhzade & Nyborg, 1998), dog (Sekiguchi *et al*., 1994) and human (McEwan *et al*., 1986), and also in human cerebral arteries (Sams *et al*., 2000). Our present results therefore indicate that this observation can be explained either by an increase in $CGRP_1$-receptor density or by increased efficiency of receptor–effector coupling downstream of the vasculature.

Presence of CGRP-receptor components

In the present study, we used RT-PCR to demonstrate the building blocks forming the functional $CGRP_1$ receptors (RAMP1 + CRLR + RCP). RAMP1 is an accessory protein that is reportedly required for intracellular trafficking and maturation of the CRLR into the CGRP1 receptor (McLatchie *et al*., 1998). Furthermore, the presence of RCP fragment ensures formation of high-affinity receptor coupled to G protein, thereby facilitating the signal transduction of G protein-coupled receptors (Evans *et al*., 2000). Thus, the presence of CRLR, RAMP1 and RCP in human subcutaneous arteries (verified by RT-PCR) might be expected to give a functional ($CGRP_1$-like receptor. However, the presence of

mRNA sequences encoding the RAMP2 and RAMP3 were also demonstrated in these vessels. An interesting feature is the dominant functional interaction between CRLR and RAMP1, which has been demonstrated by Buhlmann *et al*. (1999) in rat osteoblast-like UMR-106 cells expressing CRLR, RAMP1 and RAMP2. The association of RAMP2 with CRLR was inhibited by RAMP1, while simultaneous expression of RAMP2 did not affect the association of RAMP1 with CRLR (Buhlmann *et al*., 1999). This suggests that when all RAMPs are present, it is RAMP1 that will dictate the receptor phenotype by depressing the expression of adrenomedullin-like receptor, hence increasing likelihood of CGRP-receptor expression. Furthermore, the presence of RCP in human subcutaneous arteries indicates an effective receptor–effector coupling for $CGRP_1$ receptors, which is consistent with the observed CGRP-induced reduction in the tension and $[Ca^{2+}]_i$ of isolated human subcutaneous arteries.

In conclusion, mRNAs for the components of a functional $CGRP_1$ receptor (CRLR + RCP + RAMP1) are present in human subcutaneous arteries. The estimated apparent affinity of antagonist (BIBN4096BS) and agonist (CGRP) are consistent with the $CGRP_1$ receptor, and the observed CGRP-induced responses in these vessles (vasodilatation and reduction of $[Ca^{2+}]i$) suggest an interaction with $CGRP_1$ type receptors. Furthermore, our observation of noncompetitive action of BIBN4096BS against CGRP-induced responses in human subcutaneous arteries may have interesting clinical perspective as the antagonist has the potential to be used in the treatment of migraine and other diseases such as neurogenic inflammation and neuropathic pain, perhaps with a wider margin of safety. However, the antagonistic effects of BIBN4096BS in pathological conditions still need to be investigated.

This work is supported by the Swedish Research Council (Grant No. 5958), the Danish Research Council and the Lundbeck foundation. Professor Niels C.B. Nyborg is acknowledged for his helpful discussions.

References

BUHLMANN, N., LEUTHAUSER, K., MUFF, R., FISCHER, J.A. & BORN, W. (1999). A receptor activity modifying protein (RAMP) 2-dependent adrenomedullin receptor is a calcitonin gene-related peptide receptor when coexpreesed with human RAMP1. *Endocrinology,* **140,** 2883–2890.

CHATTERJEE, T.K., MOY, J.A., CAI, J.J., LEE, H.C. & FISHER, R.A. (1993). Solubilization and characterization of a guanine nucleotide-sensitive form of the calcitonun gene-related peptide receptor. *Mol. Pharmacol.,* **43,** 167–175.

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

EDVINSSON, L. (2003). New therapeutic target in primary headaches – blocking the CGRP receptor. *Expert Opin. Ther. Targets,* **7,** 377–383.

EDVINSSON, L., ALM, R., SHAW, D., RUTLEDGE, R.Z., KOBLAN, K.S., LONGMORE, J. & KANE, S.A. (2002). Effect of the CGRP receptor antagonist BIBN4096BS in human cerebral, coronary and omental arteries and in SK-N-MC cells. *Eur. J. Pharmacol.,* **434,** 49–53.

EVANS, B.N., ROSENBLATT, M.I., MNAYER, L.O., OLIVER, K.R. & DICKERSON, I.M. (2000). CGRP-RCP , a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. *J. Biol. Chem.,* **275,** 31438–31443.

FURCHGOTT, R.F. (1966). The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor agonist complexes. In: *Advances in Drug Research,* ed. Harper, N.J.& Simmonds, A.B. pp 21–55. London: Academic Press.

FURCHGOTT, R.F. & BURSZTYN, P. (1967). Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. *Ann. NY Acad. Sci.,* **144,** 882–898.

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

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440–3450.

GUPTA, S., MEHROTRA, S., GARRELDS, I.M., SHARMA, H.S., SAXENA, P.R. & MAASSEN VANDEN BRINK, A. (2004). Evidence for a heterogeneous CGRP receptor population in the human coronary artery. *Neuropeptides,* **38,** 122–123.

HASBAK, P., OGAARD, O.S., ESKESEN, K., SCHIFTER, S., ARENDRUP, H., LONGMORE, J. & EDVINSSON, L. (2003). Investigation of CGRP receptors and peptide pharamacology in human coronary arteries. Characterization with a nonpeptide antagonist. *J. Pharmacol. Exp. Ther.,* **304,** 326–333.

HOLZER, P. (1988). Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, CGRP and other neuropeptides. *Neuroscience,* **24,** 739–768.

JUANEDA, C., DUMONT, Y. & QUIRION, R. (2000). The molecular pharmacology of CGRP and related peptide receptor subtypes. *Trends Pharmacol. Sci.,* **21,** 432–438.

JUUL, R., EDVINSSON, L., GISVOLD, S.E., EKMAN, R., BRUBAKK, A.O. & FREDRIKSEN, T.A. (1990). Calcitonin gene-related peptide-LI in subarachnoid haemorrhage in man. Signs of activation of the trigemino-cerebrovascular system? *Br. J. Neurosurg.,* **4,** 171–179.

KALLNER, G. (1998). Release and effects of calcittonin gene-related peptide in myocardial ischaemia. *Scand. Cardivasc. J. Suppl.,* **49,** 1–35.

KENAKIN, T. (1997). *Pharmacological Analysis of Drug-Receptor Interaction*. New York: Lippincott Williams & Wilkins Publishers.

LUEBKE, A.E., DAHL, G.P., ROOS, B.A. & DICKERSON, I.M. (1996). Identification of a protein that confers calcitonin gene-related peptide responsiveness to oocytes by using a cystic fibrosis transmembrane conductance regulator assay. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 3455–3460.

MACKAY, D. (1978). How should values of pA_2 and affinity constants for pharmacological competitive antagonists be estimated? *J. Pharm. Pharmacol.,* **30,** 312–313.

MCEWAN, J., LARKIN, S., DAVIES, G., CHIERCHIA, S., BROWN, M., STEVENSON, J., MACINTYRE, I. & MASERI, A. (1986). Calcitonin gene-related peptide: a potent dilator of human epicardial coronary arteries. *Circulation,* **74,** 1243–1247.

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

MUFF, R., BUHLMANN, N., FISCHER, J.A. & BORN, W. (1999). An amylin receptor is revealed following co-transfection of a calcitonin receptor with receptor activity modifying proteins-1 or -3. *Endocrinology,* **140,** 2924–2927.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.,* **41,** 19–26.

MULVANY, M.J. & NYBORG, N.C.B. (1980). An increased calcium sensitivity of mesenteric resistance vessels in spontaneously hypertensive rats. *Br. J. Pharmacol.,* **71,** 585–596.

NYBORG, N.C.B., BAANDRUP, U., MIKKELSEN, E.O. & MULVANY, M.J. (1987). Active, passive and myogenic characteristics of isolated rat intramural coronary resistance arteries. *Pflüges Arch.,* **410,** 664–670.

QUIRION, R., VAN ROSSUM, D., DUMONT, Y., ST-PIERRE, S. & FOURNIER, A. (1992). Characterization of CGRP1 and CGRP2 receptor subtypes. *Ann. NY Acad. Sci.,* **657,** 88–105.

SAMS, A. & JANSEN-OLESEN, I. (1998). Expression of calcitonin receptor-like receptor and receptor-activity-modifying proteins in human cranial arteries. *Neurosci. Lett.,* **258,** 41–44.

SAMS, A., KNYIHAR-CSILLIK, E., ENGBERG, J., SZOK, D., TAJTI, J., BODI, I., EDVINSSON, L., VECSEL, L. & JANSEN-OLESEN, I. (2000). CGRP and adrenomedullin receptor populations in human cerebral arteries: *in vitro* pharmacological and molecular investigations in different artery sizes. *Eur. J. Pharmacol.,* **408,** 183–193.

SEKIGUCHI, N., KANATSUKA, H., SATO, K., WANG, Y., AKAI, K., KOMARU, T. & TAKISHIMA, T. (1994). Effects of calcitonin gene-related peptide on coronary microvessels and its role in acute myocardial ischemia. *Circulation,* **89,** 366–374.

SHEYKHZADE, M. & NYBORG, N.C.B. (1998). Caliber dependent calcitonin gene-related peptide-induced relaxation in rat coronary arteries: effect of K^+ on the tachyphylaxis. *Eur. J.Pharmacol.,* **351,** 53–59.

SHEYKHZADE, M. & NYBORG, N.C. (2001). Mechanism of CGRP-induced relaxation in rat intramural coronary arteries. *Br. J. Pharmacol.,* **132,** 1235–1246.

UDDMAN, R., EDVINSSON, L., JANSEN, I., STIERNHOLM, P., JENSEN, K., OLESEN, J & SUNDLER, F. (1986). Peptide-containing nerve fibres in human extracranial tissue: a morphological basis for neuropeptide involvement in extracranial pain? *Pain,* **27,** 391–399.

VERHEGGEN, R., BUMANN, K. & KAUMANN, A.J. (2002). BIBN4096BS is a potent competitive antagonist of the relaxant effects of alpha-CGRP on human temporal artery: comparison with CGRP(8–37). *Br. J. Pharmacol.,* **136,** 120–126.

WU, D., EBERLEIN, W., RUDOLF, K., ENGEL, W., HALLERMAYER, G. & DOODS, H. (2000). Characterisation of calcitonin gene-related peptide receptors in rat atrium and vas deferens: evidence for a [Cys(Et)(2,7)]hCGRP-preferring receptor. *Eur. J. Pharmacol.,* **400,** 313–319.

(Received July 2, 2004
Accepted July 5, 2004)