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In-depth characterization of CGRP receptors in human intracranial arteries

Inger Jansen-Olesen[a,b,*], Linda Jørgensen[c], Ulla Engel[d], Lars Edvinsson[b,e]

[a] Department of Neurology, Glostrup Hospital, Nordre Ringvej 57 Dk-2600 Glostrup, Denmark
[b] Department of Clinical Experimental Research, Glostrup Hospital, Dk-2600 Glostrup, Denmark
[c] Department of Neurosurgery, Rigshospitalet, Dk-2100 Copenhagen Ø, Denmark
[d] Department of Anatomy and Pathology, Hillerød Hospital, Dk-3400 Hillerød, Denmark
[e] Department of Internal Medicine, University Hospital, S-22185 Lund, Sweden

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Abstract

The purpose of the present study was to characterize the effects of human (h) α- and β-calcitonin gene-related peptide (CGRP) on intracranial arteries from man and to investigate the presence of mRNA for the calcitonin receptor like receptor (CRLR) and the receptor activity modifying proteins (RAMPs) 1, 2 and 3, in cerebral and middle meningeal arteries with and without endothelium, in microvessels and in the endothelial cells isolated from the human basilar artery. Reverse transcriptase-polymerase chain reaction (RT-PCR) revealed the presence of CRLR, RAMP 1, RAMP 2 and RAMP 3 in cerebral and middle meningeal arteries with and without endothelium as well as in microvessels and in the endothelial cells. Human and rat α- and β-CGRP, amylin, adrenomedullin and [acetamidomethyl-Cys^{2,7}]human CGRP induced strong concentration-dependent relaxation of human cerebral and middle meningeal arteries. Removal of the endothelium neither changed the maximum relaxant response nor the pIC_{50} values for α- and β-CGRP as compared to the responses in arteries with an intact endothelium. Human α-CGRP-(8–37) caused a shift of hα- and hβ-CGRP-induced relaxations in cerebral and middle meningeal arteries. Calculation of pK_B values revealed that hα-CGRP-(8–37) could not significantly discriminate between relaxations induced by hα-CGRP (pK_B around 6.8) and hβ-CGRP (pK_B around 5.4). There was no significant difference in pK_B value of hα-CGRP-(8–37) on hβ-CGRP-induced relaxation of human cerebral and middle meningeal arteries with and without endothelium. In conclusion, our molecular and pharmacological data support the existence of a single type of $CGRP_1$ receptors in the human intracranial circulation.

Keywords: Calcitonin gene-related peptide; Vasomotor response; Human; Cerebral artery; Meningeal artery

1. Introduction

The 37-amino-acid peptide calcitonin gene-related peptide (CGRP) is present in sensory perivascular nerve fibres innervating cerebral arteries of animal and man (Edvinsson et al., 1989; Jansen et al., 1990; Uddman et al., 1985). In man, CGRP is released from the sensory fibres following activation by electrical stimulation of the trigeminal ganglion (Goadsby et al., 1988) or by local capsaicin treatment (Jansen-Olesen et al., 1996). Furthermore, CGRP is known to be one of the most potent endogenous vasodilators (Brain et al., 1985) that is associated with increased levels of adenylate cyclase (Edvinsson et al., 1985). CGRP exists in two isoforms, denoted as α- and β-CGRP, which differ by one or three out of 37 amino acids depending on species. β-CGRP predominates in the enteric nervous system (Mulderry et al., 1988) whereas α-CGRP is preferentially located in sensory neurones (Amara et al., 1985; Mulderry et al., 1988). Both forms of CGRP induce potent relaxations of human cerebral arteries (Jansen-Olesen et al., 1996) and are full agonists at all known CGRP receptor subtypes (Wimalawansa, 1996).

Based on potencies of two CGRP derivatives, CGRP receptors have been classified into the $CGRP_1$ and $CGRP_2$ subtypes (Poyner, 1995; Quirion et al., 1992). This is in part based on the work with α-CGRP-(8–37) as a $CGRP_1$ receptor antagonist (pA_2>7) (Dennis et al., 1990), and the synthetic analogue [acetamidomethyl-Cys^{2,7}]human CGRP ([Cys(ACM)^{2,7}]CGRP) as a $CGRP_2$ receptor agonist (Den-

* Corresponding author. Department of Neurology, Glostrup Hospital, Nordre Ringvej 57 Dk-2600 Glostrup, Denmark. Tel.: +45-43233085; fax: +45-43233985.
E-mail address: ijo@tdcadsl.dk (I. Jansen-Olesen).

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nis et al., 1989). A functional $CGRP_2$ receptor has so far only been demonstrated in rat vas deferens and there is no molecular equivalent of this receptor. Pharmacological demonstration of the $CGRP_1$ receptor in different vascular regions corresponds to the presence of mRNA encoding human $CGRP_1$ receptors, named calcitonin receptor-like receptor (CRLR) (Aiyar et al., 1996), in human trigeminal ganglia (Edvinsson et al., 1997; Tajti et al., 1999) and in human cranial arteries (Edvinsson et al., 1997; Sams and Jansen-Olesen, 1998). In addition, receptor activity modifying proteins (RAMPs) are important in defining the functional phenotype (McLatchie et al., 1998), and have been demonstrated in cerebral, middle meningeal and temporal arteries of man (Sams and Jansen-Olesen, 1998).

Elevated levels of CGRP has been detected in jugular venous blood representing the cranial drainage in migraine patients during activation of the trigeminal ganglion or during acute migraine attacks (Edvinsson and Goadsby, 1994; Goadsby et al., 1988, 1990). When these migraine attacks were treated with the $5\text{-}HT_{1B/1D}$ agonist sumatriptan, pain was relieved and the elevated CGRP levels were normalised (Goadsby and Edvinsson, 1993). The involvement of sensory nerves (and in particular CGRP) is supported by studies in experimental animals, where the antimigraine drugs sumatriptan and dihydroergotamine blocked the development of plasma extravasation and ultrastructural changes (Buzzi and Moskowitz, 1992), as well as plasma CGRP increases following electrical ganglion stimulation (Goadsby and Edvinsson, 1993). These findings suggests that there might be a role for CGRP receptor antagonists in the treatment of migraine (Edvinsson, 2001).

We have shown in three previous studies in human cerebral arteries that human (h) α-CGRP-(8–37) was able to block hα-CGRP-induced relaxations (Edvinsson et al., 2001b, 2002; Jansen-Olesen et al., 1996). In one of these studies, a weaker antagonistic effect of hα-CGRP-(8–37) on hβ-CGRP-induced relaxation was observed.

The aims of the present study were (i) to examine in detail the molecular expression (mRNA for CRLR/RAMPs) of CGRP family of receptors in human large cerebral and middle meningeal arteries, in cerebral microvessels and endothelium; (ii) to study the functional potency difference between cerebral and middle meningeal arteries for the CGRP family of peptides as examined with in vitro pharmacology; (iii) to analyse if this potency difference is related to an interaction with the endothelium.

2. Materials and methods

2.1. Human tissue

For studies of vasomotor responses, human arteries from the cerebral cortical surface (pial artery) and dura mater (middle meningeal artery) were obtained either in conjunction with neurosurgical tumor operations or at autopsy within 6 to 24 h post-mortem. For reverse transcriptase-polymerase chain reaction (RT-PCR), major cerebral arteries (middle cerebral and basilar arteries), small cortical arteries and frontotemporal cortex were obtained at autopsy 6–24 h post mortem. Specimens were collected in accordance with Danish legislation and approved by The Danish Ethical Committee, Copenhagen (registration number KA95213m).

2.2. Reverse transcriptase-polymerase chain reaction

2.2.1. Isolation of tissue

Human intracranial arteries were cleaned from blood by perfusion with ice-cold buffer solution. The arterial segments were divided in two halves, one of which was cut open and the endothelium removed by carefully scraping it off with a scalpel blade. The arterial segments were then immediately snap frozen in liquid nitrogen and stored at − 80 °C. For isolation of endothelial cells, human basilar arteries were cleaned from blood by perfusion with ice-cold saline, cut open and the endothelial layer carefully scraped off, snap frozen in liquid nitrogen and stored at − 80 °C.

Cerebral microvessels were isolated from human frontotemporal cortex as described by Estrada et al. (1983). Briefly, 10-g human cortex was homogenized gently with a Dounce tissue grinder in ice-cold phosphate buffered saline (PBS) (0.01 mol/l, pH 7.4), and centrifuged (with the use of a Beckman GS15R swinging bucket rotor) at $2000 \times g$ for 10 min at 4 °C. The supernatant was then discarded, and the pellet was washed by resuspension in PBS and recentrifuged at $2000 \times g$ for 10 min. The supernatant was discarded, the pellet resuspended in PBS, gently layered on top of a dextran solution (15%; molecular weight, 38 400) and then centrifuged at $3500 \times g$ for 55 min. The pellet was subsequently collected, resuspended in PBS, layered over dextran, and centrifuged at $4000 \times g$ for 20 min. The final pellet was poured over a nylon mesh screen (50 μm) and washed extensively with a strong stream of ice-cold PBS. The microvessel fraction, containing small arterioles, venules and capillaries, was collected from the top of the screen and immediately snap frozen in liquid nitrogen and stored at − 80 °C. The purity of the microvessel fraction was examined in a sample by methylene blue staining and light microscopy.

2.2.2. Isolation of mRNA

Messenger RNA was isolated on poly (T)-coated magnetical particles by use of Dynalbeads mRNA direct kit (Dynal, Norway) according to the manufacturer's instructions. Isolated mRNA was not quantified.

2.2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Synthesis of first-strand cDNA and subsequent polymerase chain reaction (PCR) amplification was carried out using

the GeneAmp RNA PCR kit reagents (Perkin-Elmer, Denmark) as described by the supplier. Complementary DNA was stored at −20 °C. Reverse transcriptase negative controls were performed for each mRNA extract by substituting the reverse transcriptase enzyme in the reaction mixture with nuclease-free water.

Primer sets for the subsequent PCR amplification were designed by Primer Designer 3 software (Scientific and Educational Software, NC, USA) using nucleotide sequences purchased from NCBI Nucleotide query. Primers were designed to be specific for CRLR, RAMP1, RAMP2 and RAMP3 (Sams and Jansen-Olesen, 1998) and these do not cross-hybridize with any other known sequences. Homology with published sequences was checked using an NCBI BLAST search.

PCR reactions, initiated by the specific primer sets for CRLR, RAMP1, RAMP2 and RAMP3, were carried out with cDNA originating from human cerebral and middle meningeal arteries with and without endothelium, from cerebral microvessels isolated from the frontotemporal cortex and endothelial cells from middle cerebral and basilar arteries.

Each PCR mixture contained PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 2.5 mM $MgCl_2$, 25 mU/μl Platinum™ Taq DNA Polymerase (GibcoBRL, Invitrogen, Denmark) 0.2 mM of dATP, dTTP, dCTP and dGTP and 0.2 μM of each sense- and antisense-specific primers for the corresponding to the sequence of interest. Final volumes were 25 μl including 1 μl of cDNA solution. The reaction mixture was overlaid with mineral oil (Perkin-Elmer). PCR reactions were carried out on a RoboCycler Gradient40 (Stratagene, USA) in the following manner: an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation for 1 min at 95 °C, annealing for 90 s at 63 °C and 30 s at 72 °C. After the final cycle, the temperature was maintained at 72 °C for 7 min to allow completion of synthesis of amplified products.

2.2.4. Electrophoretic analysis

Ten microliters from each PCR amplified product was loaded on a 2% agarose gel (GibcoBRL, Invitrogen), containing 0.5 μg/ml ethidium bromide, and the size of the amplified products was verified by co-electrophoresis of a 100-base-pair nucleotide DNA ladder (GibcoBRL, Invitrogen).

The identity of the amplified sequences was identified by restriction analysis; for further details see Sams and Jansen-Olesen (1998).

2.3. Vasomotor responses

Immediately after removal, the vessel segments were placed in a buffer solution containing (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2 and glucose 11. Circular vessel segments with an inner diameter of 0.3–0.6 mm (cerebral arteries) or 0.5–1 mm (meningeal arteries) and a length of 2–4 mm were mounted in two Multi Myographs, each for parallel experiments of up to four vessels in separate tissue baths (Model 610M, JP Trading, Denmark). The vessel segments were suspended between two L-shaped metal holders (0.15 or 0.2 mm in diameter). The distance between the holders could be varied by a micropositioner coupled to one of the holders, thereby allowing adjustment of the resting tension of the segments. The other holder was connected to a transducer for registration of alterations in vascular tone. The Multi Myograph was connected to a Pentium computer with Myodaq for Windows software (Myonic Software, Denmark). The arterial segments were given a tension of 2–8 mN depending on the vessel size (usually 4 mN) and were allowed to accommodate for 1–1.5 h until the tension had stabilised at the desired level. In the tissue baths the arteries were immersed in a temperature-controlled (37 °C) buffer solution that continuously was bubbled with a mixture of 95% O_2 and 5% CO_2 giving a pH of about 7.4. The presence of intact endothelium was assessed by a good relaxant response to acetylcholine.

Some experiments were performed to examine the role of the endothelium. These vessels were treated before mounting with a 10-s perfusion with 0.1% Triton-X 100 followed by perfusion with ice-cold buffer solution for 30 s. Using this procedure the endothelium is removed without any damage to the endothelial cells (Hamel et al., 1987). Removal of the endothelium was confirmed by loss of relaxant response to 10^{-5} M acetylcholine. Vessel reactivity was tested by exposure to a buffer solution containing 125 mM KCl, obtained by an equimolar substitution of NaCl for KCl. Only vessels with a reproducible K^+-induced contraction after washout with the sodium buffer solution were used for further investigation. The K^+-induced contraction amounted to 4.79 ± 0.33 mN (n = 90, from 24 patients) and 3.03 ± 0.43 mN (n = 19 from 3 patients) in cerebral arteries, and 5.56 ± 0.58 mN (n = 62 from 25 patients) and 7.10 ± 1.47 mN (n = 17 from 6 patients) in middle meningeal arteries with and without endothelium, respectively. For the study of relaxant responses, the vessel segments were pre-contracted by the addition of prostaglandin $F_{2\alpha}$ at a concentration of 3×10^{-6} M. At the time of pre-contraction where the relaxant agent first was added to the tissue baths (the stable level of tension), the pre-contraction amounted to 5.34 ± 0.35 mN (n = 90) and 3.09 ± 0.61 mN (n = 19) in cerebral arteries, and 3.47 ± 0.34 mN (n = 62) and 4.36 ± 0.97 mN (n = 17) in middle meningeal arteries with and without endothelium, respectively.

Concentration–response data were obtained by cumulative addition of CGRP receptor agonists to the tissue bath. Antagonists were added to the tissue baths 20 min before the responses to CGRP receptor agonists were tested. The values for relaxation are expressed as percentage of the level of pre-contraction due to prostaglandin $F_{2\alpha}$

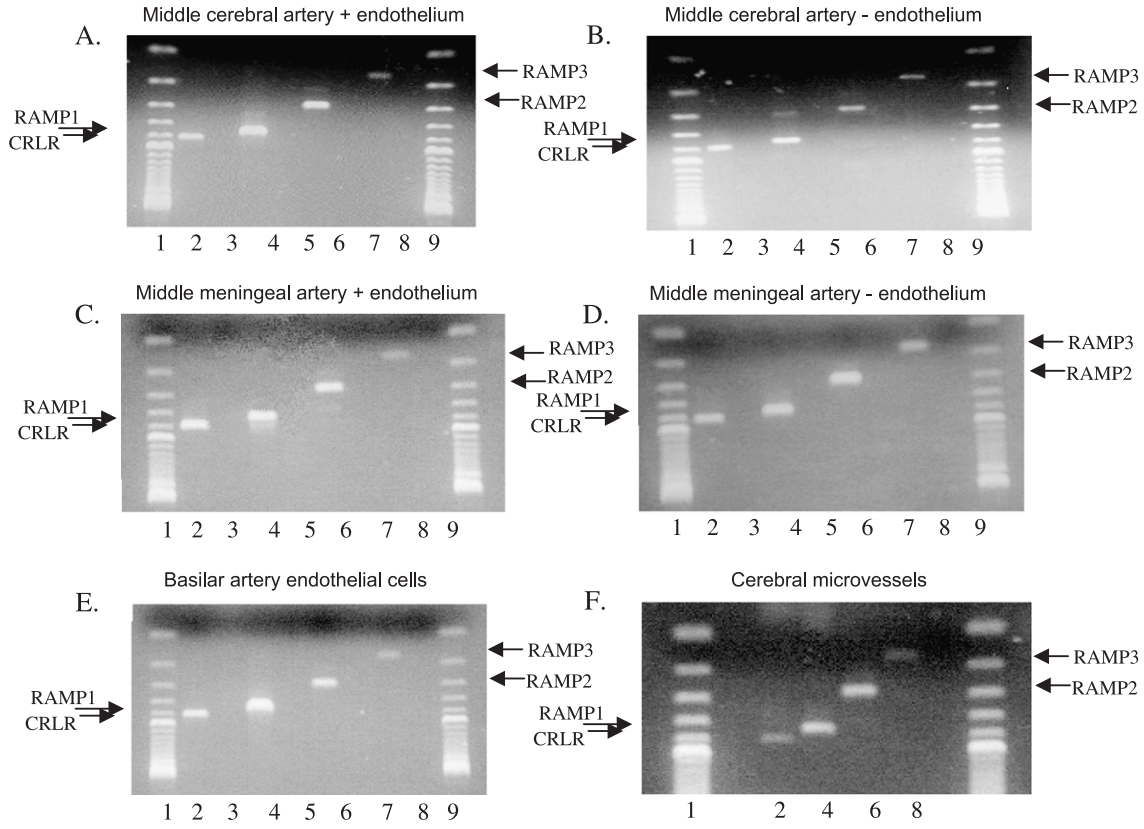

Fig. 1. Expression of CRLR and RAMP1, -2 and -3 in human middle cerebral (A and B) and middle meningeal (C and D) arteries with (A and C) and without endothelium (B and D), in human basilar artery endothelial cells (E) and in human cerebral microvessels (F) demonstrated by RT-PCR. Ladder shows 100 base-pair steps. Bands corresponding to the presence of mRNA encoding CRLR as well as RAMP1, -2 and -3 (lengths of products were 497, 445, 283 and 159 bp, respectively) are evident in all preparations. No bands are seen in the negative controls (lanes 3, 5, 7 and 9) where mRNA was not reverse-transcribed to cDNA prior to amplification (lack of RT enzyme).

(3×10^{-6} M). The responses were characterised in terms of I_{max} (maximum relaxant effect obtained with an agonist), IC_{50} (the concentration eliciting half maximum relaxant effect) and pIC_{50} (negative logarithm of IC_{50}) values. Values are given as mean ± S.E.M. Number of experiments = n, one or two segments from each patient.

Table 1
Relaxant effect of CGRP receptor agonists on human cerebral and middle meningeal arterial segments precontracted by 3×10^{-6} M prostaglandin $F_{2\alpha}$

With endothelium	Cerebral			Meningeal		
	I_{max}	pIC_{50}	n	I_{max}	pIC_{50}	n
hα-CGRP	88 ± 3	9.69 ± 0.21	28	92 ± 5, n.s.	8.76 ± 0.35, (P = 0.0364)[a]	8
hβ-CGRP	72 ± 7	9.42 ± 0.25	17	82 ± 4, n.s.	8.31 ± 0.20, (P = 0.0003)[c]	22
rα-CGRP	80 ± 7	9.64 ± 0.22	5	87 ± 5, n.s.	8.65 ± 0.18, (P = 0.0087)[b]	6
rβ-CGRP	81 ± 8	10.25 ± 0.26	5	n.d.	n.d.	
[Cys(ACM)$_{2,7}$] -CGRP	79 ± 9	7.43 ± 0.06	11	70 ± 21, n.s.	7.25 ± 0.21, n.s.	3
Amylin	82 ± 14	8.25 ± 0.73	4	n.d.	n.d.	
Adrenomedullin	72 ± 5	7.21 ± 0.06	4	n.d.	n.d.	

Data are given as means ± S.E.M.; n = number of vessel segments examined. I_{max} = maximum relaxation in % of precontraction; pIC_{50} = the negative logarithmic concentration (M) of agonist eliciting half maximum relaxation; n.d. = not done. Statistical analysis comparing the responses in cerebral to that of meningeal arteries was performed by Mann–Whitney U-test; a P value < 0.5 was regarded as significant. [a]P < 0.05, [b]P < 0.01, [c]P < 0.005.

Table 2
Relaxant effects of hα- and hβ-CGRP on human cerebral and middle meningeal (MM) arterial segments with and without endothelium precontracted by 3×10^{-6} M prostaglandin $F_{2\alpha}$

	With endothelium			Without endothelium		
	I_{max}	pIC_{50}	n	I_{max}	pIC_{50}	n
hβ-CGRP						
Cerebral artery	72 ± 7	9.42 ± 0.25	17	97 ± 2	9.50 ± 0.54	5
MM artery	82 ± 4	8.31 ± 0.20[c]	22	97 ± 11	8.72 ± 0.89	4
hα-CGRP						
Cerebral artery	88 ± 3	9.69 ± 0.21	28	70 ± 12	9.14 ± 0.16	7
MM artery	92 ± 5	8.76 ± 0.35[a]	8	79 ± 12	8.53 ± 0.06[b]	6

Data are given as means ± S.E.M.; n = number of vessel segments examined. I_{max} = maximum relaxation in % of precontraction; pIC_{50} = the negative logarithmic concentration (M) of agonist eliciting half maximum relaxation. Statistical analysis comparing the responses in cerebral to that of meningeal arteries was performed by Mann–Whitney U-test, a P value < 0.5 was regarded as significant. [a]P < 0.05, [b]P < 0.01, [c]P < 0.005. There was no statistical difference comparing relaxant responses in arteries with endothelium to that found in arteries without endothelium.

When analysing the effect of antagonists, the pK_B value was calculated as log[(CR − 1/B], where CR is the concentration ratio of the IC_{50} values of agonist in the presence and in the absence of a given concentration of the antagonist (B). When analysing the effect of different concentrations of antagonist on hα-CGRP relaxation, the dissociation constant (pA_2) was calculated as described by Arunlakshana and Schild (1959): pA_2 = $\log_{10}$ (conc. range − 1)/antagonist conc. Mann–Whitney U-test was used to determine statistical significance with respect to differences in I_{max}, pIC_{50} and pK_B values. Statistical significance was assumed when $P < 0.05$.

2.4. Drugs

Human and rat α-CGRP, human and rat β-CGRP (Auspep, Australia), hα-CGRP-(8–37), prostaglandin $F_{2\alpha}$ (Sigma, USA), [Cys(ACM)^{2,7}]CGRP, amylin and adrenomedullin (Peninsula, USA). A stock solution was prepared by dissolving the drugs in distilled water. All drugs were further diluted in buffer solution and added just before the experiment further diluted in buffer solution. The concentrations are expressed as the final molar concentration in the tissue bath.

3. Results

3.1. Reverse transcriptase-polymerase chain reaction

Agarose gel electrophoresis of the PCR products from human cerebral (Fig. 1A) and middle meningeal (Fig. 1C) arteries with endothelium demonstrated products of the expected size corresponding to mRNA encoding for CRLR (or the $CGRP_1$-receptor) at 497 base pairs, RAMP1 at 445 base pairs, RAMP2 at 283 base pairs and RAMP3 at 159 base pairs. In cerebral and middle meningeal arteries without endothelium, we still found bands for all four PCR products (Fig. 1B and D). Bands for CRLR, RAMP1, RAMP2 and RAMP3 were also found in basilar artery endothelial cells and cerebral microvessels (Fig. 1E and F). DNase was successfully used to eliminate any contaminating DNA since no band was detected in neg-

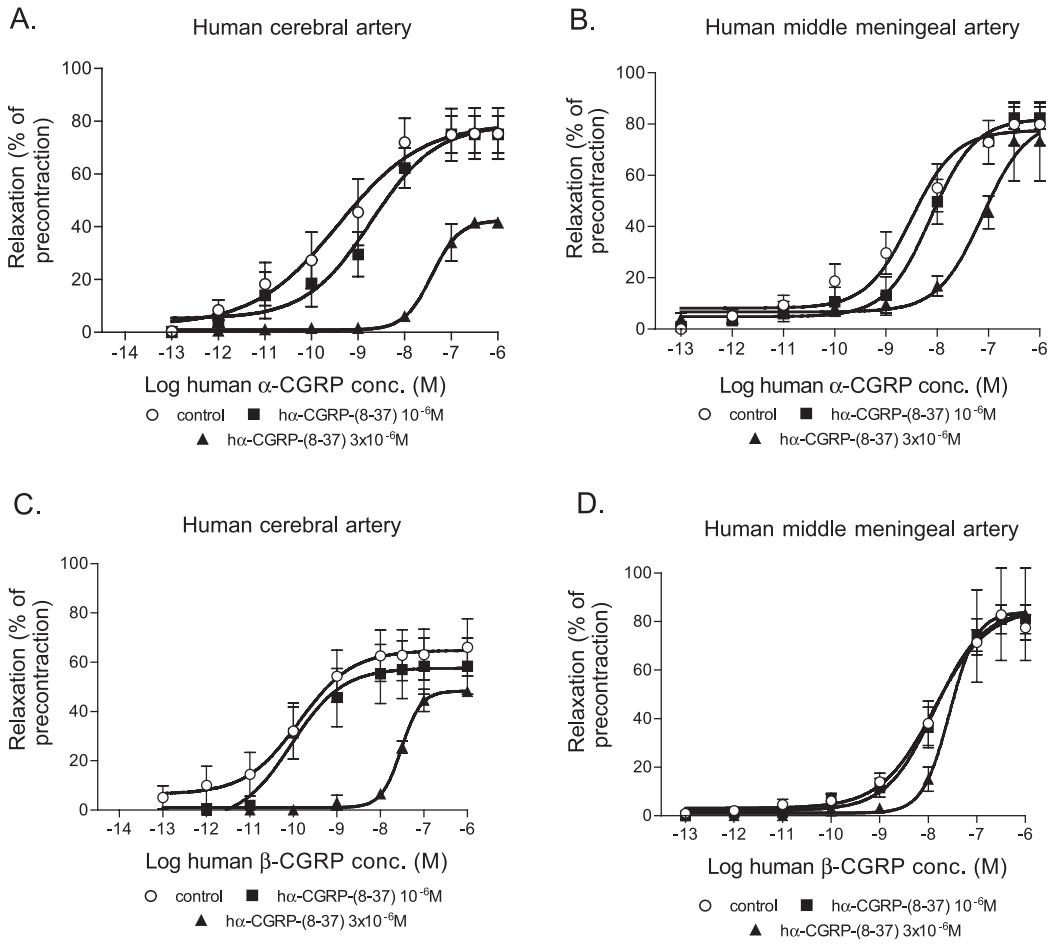

Fig. 2. Relaxant responses in human cerebral (A and C) and human middle meningeal (B and D) arteries precontracted by 3×10^{-6} M prostaglandin $F_{2\alpha}$ to the cumulative application of hα-CGRP (A and B) and hβ-CGRP (C and D) with or without hα-CGRP-(8–37) (10^{-6}–3×10^{-6} M). Values given represent mean ± S.E.M., n = 4–11.

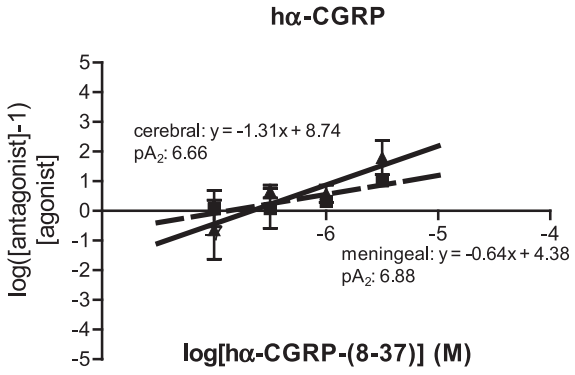

Fig. 3. Schild regression analysis for the antagonism of hα-CGRP-(8–37) to hα-CGRP-induced relaxations in human cerebral and human middle meningeal arteries. In human cerebral arteries the slope of regression line was −1.31 with a pA_2 of 6.66 and in human middle meningeal arteries the slope of regression line was −0.64 with a pA_2 value of 6.88.

ative controls where the reverse transcriptase enzyme was omitted in the first-strand cDNA reaction.

3.2. Vasomotor responses

3.2.1. Agonist experiments

3.2.1.1. With endothelium. Cumulative application of hα-CGRP, hβ-CGRP, rα-CGRP, rβ-CGRP, [Cys(ACM)^{2,7}]CGRP, amylin and adrenomedullin to the tissue baths induced concentration-dependent relaxations of circular segments of human intracranial arteries pre-contracted by prostaglandin $F_{2\alpha}$ (Table 1). Human α-, hβ- and rα-CGRP were significantly more potent in cerebral than in middle meningeal arteries ($p < 0.05$). There was no difference in relaxant responses to [Cys(ACM)^{2,7}]CGRP in the two arterial regions examined.

There were large variations in CGRP responses between arteries from different patients. In cerebral arteries the respective pIC_{50} values for hα- and hβ-CGRP were in the interval 12.19–8.20 and 10.27–8.70. In human middle meningeal arteries the respective pIC_{50} values for hα- and hβ-CGRP were between 10.46–7.75 and 11.50–7.28.

3.2.1.2. Without endothelium. Removal of the endothelium did not significantly alter the relaxant responses to hα- or hβ-CGRP in cerebral and middle meningeal arteries, neither regarding the maximum amount of relaxation nor pIC_{50} value (Table 2). Furthermore, it did not alter the difference in sensitivity to CGRP between cerebral and middle meningeal arteries, CGRP still being significantly more potent in cerebral as compared to the dural artery ($p < 0.05$).

3.2.2. Antagonist experiments

3.2.2.1. With endothelium. In cerebral and middle meningeal arteries with intact endothelium, hα-CGRP-(8–37) (10^{-7}–3×10^{-6} M) caused a rightward shift of relaxations induced by hα-CGRP (Fig. 2A and B). Construction of Schild plots revealed pA_2 values of 6.66 and 6.88 (Fig. 3). Relaxations induced by hβ-CGRP were antagonised only to a minor degree or not at all at antagonist concentrations lower than 3×10^{-6} M (Fig. 2C and D). Thus, Schild plots could not be constructed. The calculated pK_B values were for hα-CGRP-(8–37) on hβ-CGRP-induced relaxations 5.4 ± 0.8 in cerebral arteries ($n = 6$) and 5.5 ± 0.4 in middle meningeal arteries ($n = 14$) (Fig. 4). Comparing the calculated pK_B values for relaxations induced by hα-CGRP to those for hβ-CGRP, no significant difference was observed (cerebral: $P = 0.2$; meningeal $P = 0.08$; Fig. 4). Thus, no statistical evidence exists that hα-CGRP-(8–37) discriminates between relaxations induced by the two CGRP analogues in human cerebral and middle meningeal arteries.

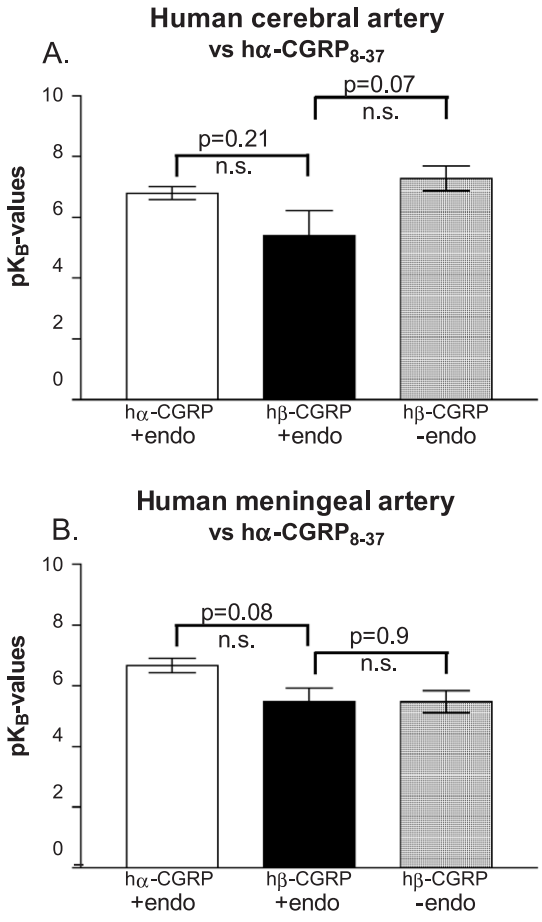

Fig. 4. Demonstration of the antagonism of hα-CGRP-(8–37) to hα- and hβ-CGRP-induced relaxations in human cerebral (A) and human middle meningeal (B) arteries. The antagonistic effect to hβ-CGRP was examined in arteries with and without endothelium. In human cerebral arteries the pK_B values were 6.81 ± 0.22 ($n = 12$) on relaxations induced by hα-CGRP and 5.41 ± 0.81 ($n = 6$) and 7.28 ± 0.40 ($n = 7$) on relaxation induced by hβ-CGRP in vessels with and without endothelium, respectively. In human middle meningeal arteries the pK_B values were 6.67 ± 0.23 ($n = 7$) on relaxations induced by hα-CGRP and 5.47 ± 0.37 ($n = 14$) and 5.47 ± 0.45 ($n = 9$) on relaxation induced by hβ-CGRP in vessels with and without endothelium, respectively.

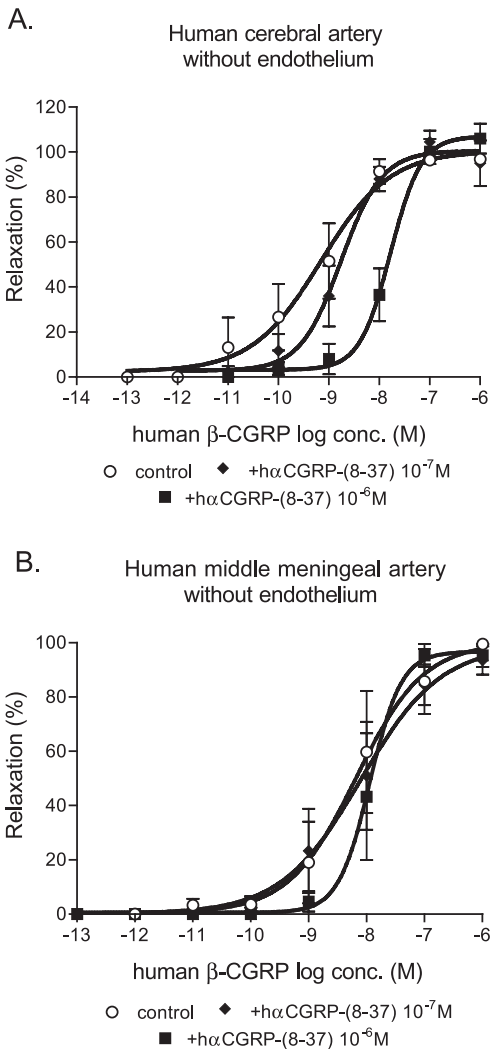

Fig. 5. Relaxant responses in human cerebral (A) and human middle meningeal (B) arteries without endothelium to the cumulative application of hβ-CGRP with or without hα-CGRP-(8–37) (10^{-7}–10^{-6} M). Prior to experiment the arteries were precontracted by 3×10^{-6} M prostaglandin $F_{2\alpha}$. Values given represent mean ± S.E.M., $n = 6$–4.

3.2.2.2. Without endothelium. In order to investigate if the endothelium has a role in the somewhat, poorer antagonistic effect seen for hα-CGRP-(8–37) of hβ-CGRP-induced relaxations, blockade experiments were performed in cerebral and middle meningeal arteries without endothelium (Fig. 5).

After removal of the endothelium, the calculated pK_B values were 7.3 ± 0.4 ($n = 7$) in cerebral and 5.5 ± 0.4 ($n = 9$) in middle meningeal arteries (Fig. 4). Statistical analysis showed no significant difference in pK_B values in cerebral ($P = 0.07$) and middle meningeal arteries ($P = 0.90$) without as compared to with endothelium (Fig. 4).

4. Discussion

In this study, we present data that demonstrate the presence of functional $CGRP_1$ receptors in human cerebral and middle meningeal arteries. We reveal that (i) the cerebral artery is approximately 10 times more sensitive to CGRP than the middle meningeal arteries. (ii) There was a slight, but nonsignificant, discrimination of hα-CGRP-(8–37) between the actions of hα- and hβ-CGRP-induced relaxation of human intracranial arteries. (iii) Removal of the endothelium did not cause a significantly higher pK_B value for hα-CGRP-(8–37) on hβ-CGRP-induced vasodilatation than that seen in arteries with an intact endothelium. Furthermore, the removal of the endothelium did not alter the sensitivity of the arteries to α- and β-CGRP. (iv) RT-PCR analysis revealed the presence of mRNA for all three RAMPs and CRLR in large cerebral and meningeal arteries, in microvessels and in isolated endothelium.

4.1. Reverse transcriptase-polymerase chain reaction (RT-PCR)

In the present study, we confirm the presence of mRNA for CRLR and the RAMPs in human cranial arteries (Sams and Jansen-Olesen, 1998) and, in addition, reveal that after removal of the endothelium, mRNA for CRLR and the RAMPs (1–3) remain. The finding of CRLR and all the RAMPs (1–3) in basilar artery endothelium and in cerebral microvessels is somewhat in contradiction to the findings of others (Moreno et al., 2002) who could only detect RAMP1 and -2 in microvascular smooth muscle cells and endothelial cells cultured from cerebral microvessels. We also detected RAMP 3. One reason for this difference might be explained for endothelial cells by differences in the size of arteries used. In regard to the finding of RAMP3 in our cerebral microvessel preparation, the possibility exists that there might have been a slight contamination of astrocytes, which in culture have been shown to contain mRNA for RAMP3. Yet another explanation could be a change in RAMP3 mRNA transcript after culture of microvascular smooth muscle cells and endothelial cells (Moreno et al., 2002). Taken together, there are molecular prerequisites for the presence of $CGRP_1$ and adrenomedullin receptors in smooth muscle cells and in endothelial cells of human intracranial arteries ranging from large arteries to cortical microvessels. The $CGRP_2$ receptor has not yet been cloned and can therefore not be studied at the molecular level.

4.2. Agonist responses

4.2.1. Relaxant responses

The CGRP analogues used acted as relaxant agents in all vessels examined. The relaxant effect to hα-CGRP has previously been described for human cerebral (Edvinsson et al., 1987, 2001b, 2002; Hanko et al., 1985; Jansen-Olesen et al., 1996; Sams et al., 2000) and middle meningeal arteries (Jansen et al., 1992). The maximum relaxant responses as well as the pIC_{50} values obtained in arteries with endothelium did not differ from that reported previously. However, we found considerable variability in po-

tency to CGRP in cerebral and middle meningeal arterial segments between different patients. The reason for this variability could be due to several factors such as genetic factors, age, sex, disease of the patients, smoking or post-mortem time. The intention of the present study has not been to investigate if eventual differences were due to any of these factors and the material is too small to draw any firm conclusions in the matter. However, the difference in sensitivity to CGRP between patients was robust and may be of importance in considering the therapeutic use of $CGRP_1$ antagonists in, e.g. migraine treatment (Edvinsson, 2001).

Relaxations induced by human and rat α- and β-CGRP were more potent in cerebral than in middle meningeal arteries. This agrees with a previous observation for α-CGRP (Jansen et al., 1992). In a clinical trial of subarachnoid haemorrhage patients, it was demonstrated that infusion of hα-CGRP could relax the constricted cerebral arteries without a dramatic drop in peripheral resistance (Juul et al., 1994). This can be explained by the fact that cerebral arteries are more sensitive to CGRP than other arteries. One reason for this might be the presence of a higher number of CGRP receptors in cerebral as compared to other vascular regions (Black and Leff, 1983); however, this observation deserves further study. Experiments with adrenomedullin and amylin were only performed in cerebral arteries. The relaxation induced by adrenomedullin was comparable to that recently shown for the human lenticulostriate artery and found to be about 100 times less potent than CGRP (Sams et al., 2000). A similar difference in potency between adrenomedullin and CGRP has been noted in the guinea pig basilar artery (Jansen-Olesen et al., 2001) and in the cat middle cerebral artery (Edvinsson et al., 2001a). Adrenomedullin has been shown in human cerebral arteries to induce a CGRP-(8–37)-sensitive vasodilatation. Taken together, these data point towards an action of adrenomedullin on $CGRP_1$-receptors (Sams et al., 2000). The effect of amylin has previously not been reported for human cerebral arteries. We found that amylin induced a pronounced relaxation, which was somewhat more potent than that of adrenomedullin. This is in contrast to that seen in the guinea pig basilar and cat middle cerebral artery where amylin only acted as a weak vasodilator (Edvinsson et al., 2001a; Jansen-Olesen et al., 2001). The ability of $[Cys(ACM)^{2,7}]$CGRP to induce relaxation was approximately 1000 times less than that of α- and β-CGRP, and there was no significant difference in potency for relaxant responses to $[Cys(ACM)^{2,7}]$CGRP in the two types of intracranial arteries. It is, therefore, not likely that human intracranial arteries are equipped with relaxant $CGRP_2$ receptors.

We have previously shown that the response to hα-CGRP was not altered after removal of the endothelium in human (Jansen-Olesen et al., 1996) or feline cerebral arteries (Edvinsson et al., 1985). In the present study we have demonstrated that also the relaxation induced by hβ-CGRP was unaffected by removal of the endothelium. In addition, the difference in sensitivity to hβ-CGRP between cerebral and middle meningeal arteries persisted after removal of the endothelium. Thus, from agonist studies it seems that the two CGRP analogues (α- and β-CGRP) induce identical responses in the two intracranial arteries examined.

4.3. Antagonist responses

4.3.1. The effect of hα-CGRP-(8–37) on hα-CGRP-induced relaxation

It is known that hα-CGRP-(8–37) is a weaker antagonist of hβ- than of hα-CGRP-induced relaxations of human cerebral arteries (Jansen-Olesen et al., 1996). In the present study, we used three different concentrations of the antagonist; thus, it should be possible to construct Schild plots for calculation of antagonist affinity. Human α-CGRP-(8–37) acted as a competitive antagonist on hα-CGRP- and hβ-CGRP-induced relaxations of cerebral and middle meningeal arteries. From Fig. 2A and C, 3×10^{-6} M of hα-CGRP-(8–37) seems to induce a noncompetitive antagonistic effect. However, this is not true as control segments from the same patients have a maximum relaxant effect of $46 \pm 9\%$ (hα-CGRP) and $46 \pm 13\%$ (hβ-CGRP). The respective pA_2 values (6.66 and 6.88) for hα-CGRP are very close to that previously reported for the guinea pig basilar artery (6.73) (Jansen-Olesen et al., 2001) and are in concert with those seen in rat and porcine coronary preparations (between 6.3 and 6.7). In human cell line SK-N-MC, it has recently been demonstrated that CGRP-induced cAMP production is antagonised by hα-CGRP-(8–37) with a pA_2 value of 7.8 (Edvinsson et al., 2001a). In the same study, the pA_2 values for the non-peptide antagonist BIBN4096BS ([*R*-(*R**,*S**)]-*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(2*H*)-quinazolinyl)-1,1-piperidinecarboxamide) on hα-CGRP-induced cAMP production in SK-N-MC cells was approximately 10 times less than the pA_2 values obtained for BIBN4096BS in hα-CGRP-induced relaxation of human cerebral arteries (Edvinsson et al., 2002). Thus, the difference in pA_2 values for hα-CGRP-(8–37) on CGRP-induced cAMP production in SK-N-MC cells (7.8) and for hα-CGRP-(8–37) on hα-CGRP-induced relaxation of human cerebral arteries (6.7) are within the same magnitude. According to the CGRP receptor classification, pA_2 values <7 suggest an action via $CGRP_1$ receptors, while pA_2 values >7 correspond to an action via $CGRP_2$ receptors. This would indicate the presence of $CGRP_2$ receptors in human cerebral and middle meningeal arteries. However, taken together with our agonist data and the finding of mRNAs encoding the $CGRP_1$ receptor, it is most likely that the observed vasodilatation of CGRP is mediated via $CGRP_1$ receptors.

4.3.2. The effect of hα-CGRP-(8–37) on hβ-CGRP-induced relaxation

At the lower concentrations (3×10^{-7} and 10^{-6} M) of hα-CGRP-(8–37), we observed no or only weak blockade.

Thus, hα-CGRP-(8–37) seems to be a somewhat weaker antagonist of hβ-CGRP than of hα-CGRP-induced relaxation in human cerebral and middle meningeal arteries. However, no significant difference could be obtained. This might be explained by the fact that hα-CGRP-(8–37) (10^{-7}–10^{-6} M) did not cause any blockade of hβ-CGRP-induced relaxation of arterial segments from about 50% of the patients and pK_B values could not be calculated in these cases. The lack of significance is in agreement with our previous findings in guinea pig basilar artery (Jansen-Olesen et al., 2001) where hα-CGRP-(8–37) was not able to discriminate between relaxations induced by hα- and hβ-CGRP. In contrast, hα-CGRP-(8–37) has been shown to be a 10-fold more potent inhibitor of hα-CGRP-induced cAMP production in astrocytes than that induced by hβ-CGRP. At first sight, hα-CGRP-(8–37) seems to show the same discrepancy between hα-CGRP and hβ-CGRP in human cerebral and middle meningeal arteries, but the differences in our studies were not significant.

4.3.3. Human β-CGRP induced relaxation in arteries without endothelium

In order to examine whether the somewhat poorer antagonistic effect of hα-CGRP-(8–37) on relaxations induced by hβ-CGRP was due to binding to endothelial receptors with a subsequent activation of endothelial factors, the antagonistic effect of the $CGRP_1$ antagonist was studied in arteries without endothelium. Even if the pK_B value increased from 5.4 to 7.5 in cerebral arteries after endothelium removal, no significant differences in pK_B values were found in either of the intracranial arteries. This is possibly due to inter-patient variability as cerebral artery segments from each patient were too small to divide into two groups (with and without endothelium), as we did for the middle meningeal arteries. Similar results have previously been obtained in the guinea pig basilar artery with a 10-fold, but nonsignificant, discrepancy of hα-CGRP-(8–37) between hα- and hβ-CGRP-induced relaxations (Jansen-Olesen et al., 2001).

It may be speculated to be due to the relation between our findings of mRNA for all components of the CGRP receptors in endothelial cells and the fact that there was no functional difference in our in vitro studies that the $CGRP_1$ receptor might not be transcribed until a pathological situation occurs. Another possibility is that the endothelial CGRP receptors are transcribed in order to react on changes in CGRP levels in the blood. However, the endothelial receptors might be less sensitive than the smooth muscle cell receptors, which results in a masked endothelium-dependent response.

4.4. Conclusion

We have shown that there is a molecular basis for the existence of CGRP and adrenomedullin receptors in human cerebral and middle meningeal arteries. As pointed out by McLatchie et al. (1998), the presence of RAMP1 + CRLR results in $CGRP_1$ receptors and this predominates over the RAMP2/3 + CRLR to form adrenomedullin receptors. The receptor mRNA is located in the vascular smooth muscle cells as well as in the endothelial cells, both in large arteries and in cortical microvessels. Functional studies have shown that the human cerebral artery is about 10 times more sensitive to CGRP than the middle meningeal artery. In antagonist studies, there was no significant discrepancy of hα-CGRP-(8–37) to relaxations induced by hα- or hβ-CGRP. Furthermore, removal of the endothelium did not significantly change the sensitivity of hα-CGRP-(8–37) as an antagonist of hβ-CGRP-induced relaxations.

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References

Aiyar, N., Rand, K., Elshourbagy, N.A., Zeng, Z., Adamou, J.E., Bergsma, D.J., Li, Y., 1996. A cDNA encoding the calcitonin gene-related peptide type 1 receptor. J. Biol. Chem. 271, 11325–11329.

Amara, S.G., Arriza, J.L., Leff, S.E., Swanson, L.W., Evans, R.M., Rosenfeld, M.G., 1985. Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science 229, 1094–1097.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. 14, 48–59.

Black, J.W., Leff, P., 1983. Operational models of pharmacological agonism. Proceedings of the Royal Society of London. vol. B220, pp. 141–162.

Brain, S.D., Williams, T.J., Tippins, J.R., Morris, H.R., MacIntyre, I., 1985. Calcitonin gene-related peptide is a potent vasodilator. Nature 313, 54–56.

Buzzi, M.G., Moskowitz, M.A., 1992. The trigemino-vascular system and migraine. Pathol. Biol. (Paris) 40, 313–317.

Dennis, T., Fournier, A., St-Pierre, S., Quirion, R., 1989. Structure-activity profile of calcitonin gene-related peptide in peripheral and brain tissues. Evidence for receptor multiplicity. J. Pharmacol. Exp. Ther. 251, 718–725.

Dennis, T., Fournier, A., Cadieux, A., Pomerleau, F., Jolicoeur, F.B., St-Pierre, S., Quirion, R., 1990. hCGRP-(8–37), a calcitonin gene-related peptide antagonist revealing calcitonin gene-related peptide receptor heterogeneity in brain and periphery. J. Pharmacol. Exp. Ther. 254, 123–128.

Edvinsson, L., 2001. Calcitonin gene-related peptide (CGRP) and the pathophysiology of headache: therapeutic implications. CNS Drugs 15, 745–753.

Edvinsson, L., Goadsby, P.J., 1994. Neuropeptides in migraine and cluster headache. Cephalalgia 14, 320–327.

Edvinsson, L., Fredholm, B.B., Hamel, E., Jansen, I., Verrecchia, C., 1985. Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an

endothelium-derived relaxing factor in the cat. Neurosci. Lett. 58, 213–217.

Edvinsson, L., Ekman, R., Jansen, I., Ottosson, A., Uddman, R., 1987. Peptide-containing nerve fibers in human cerebral arteries: immunocytochemistry, radioimmunoassay, and in vitro pharmacology. Ann. Neurol. 21, 431–437.

Edvinsson, L., Hara, H., Uddman, R., 1989. Retrograde tracing of nerve fibers to the rat middle cerebral artery with true blue: colocalization with different peptides. J. Cereb. Blood Flow Metab. 9, 212–218.

Edvinsson, L., Cantera, L., Jansen-Olesen, I., Uddman, R., 1997. Expression of calcitonin gene-related peptide1 receptor mRNA in human trigeminal ganglia and cerebral arteries. Neurosci. Lett. 229, 209–211.

Edvinsson, L., Goadsby, P.J., Uddman, R., 2001a. Amylin: localization, effects on cerebral arteries and on local cerebral blood flow in the cat. Sci. World J. 1, 168–180.

Edvinsson, L., Sams, A., Jansen-Olesen, I., Tajti, J., Kane, S.A., Rutledge, R.Z., Koblan, K.S., Hill, R.G., Longmore, J., 2001b. Characterisation of the effects of a non-peptide CGRP receptor antagonist in SK-N-MC cells and isolated human cerebral arteries. Eur. J. Pharmacol. 415, 39–44.

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.

Estrada, C., Hamel, E., Krause, D.N., 1983. Biochemical evidence for cholinergic innervation of intracerebral blood vessels. Brain Res. 266, 261–270.

Goadsby, P.J., Edvinsson, L., 1993. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Ann. Neurol. 33, 48–56.

Goadsby, P.J., Edvinsson, L., Ekman, R., 1988. Release of vasoactive peptides in the extracerebral circulation of humans and the cat during activation of the trigeminovascular system. Ann. Neurol. 23, 193–196.

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.

Hamel, E., Assumel-Lurdin, C., Edvinsson, L., Fage, D., MacKenzie, E.T., 1987. Neuronal versus endothelial origin of vasoactive acetylcholine in pial vessels. Brain Res. 420, 391–396.

Hanko, J., Hardebo, J.E., Kahrstrom, J., Owman, C., Sundler, F., 1985. Calcitonin gene-related peptide is present in mammalian cerebrovascular nerve fibres and dilates pial and peripheral arteries. Neurosci. Lett. 57, 91–95.

Jansen, I., Alafaci, C., Uddman, R., Edvinsson, L., 1990. Evidence that calcitonin gene-related peptide contributes to the capsaicin–induced relaxation of guinea pig cerebral arteries. Regul. Pept. 31, 167–178.

Jansen, I., Uddman, R., Ekman, R., Olesen, J., Ottosson, A., Edvinsson, L., 1992. Distribution and effects of neuropeptide Y, vasoactive intestinal peptide, substance P, and calcitonin gene-related peptide in human middle meningeal arteries: comparison with cerebral and temporal arteries. Peptides 13, 527–536.

Jansen-Olesen, I., Mortensen, A., Edvinsson, L., 1996. Calcitonin gene-related peptide is released from capsaicin-sensitive nerve fibres and induces vasodilatation of human cerebral arteries concomitant with activation of adenylyl cyclase. Cephalalgia 16, 310–316.

Jansen-Olesen, I., Kaarill, L., Edvinsson, L., 2001. Characterization of CGRP(1) receptors in the guinea pig basilar artery. Eur. J. Pharmacol. 414, 249–258.

Juul, R., Aakhus, S., Bjornstad, K., Gisvold, S.E., Brubakk, A.O., Edvinsson, L., 1994. Calcitonin gene-related peptide (human alpha-CGRP) counteracts vasoconstriction in human subarachnoid haemorrhage. Neurosci. Lett. 170, 67–70.

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.

Moreno, M.J., Terron, J.A., Stanimirovic, D.B., Doods, H., Hamel, E., 2002. Characterization of calcitonin gene-related peptide (CGRP) receptors and their receptor-activity-modifying proteins (RAMPs) in human brain microvascular and astroglial cells in culture. Neuropharmacology 42, 270–280.

Mulderry, P.K., Ghatei, M.A., Spokes, R.A., Jones, P.M., Pierson, A.M., Hamid, Q.A., Kanse, S., Amara, S.G., Burrin, J.M., Legon, S., 1988. Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. Neuroscience 25, 195–205.

Poyner, D., 1995. Pharmacology of receptors for calcitonin gene-related peptide and amylin. Trends Pharmacol. Sci. 16, 424–428.

Quirion, R., Van-Rossum, D., Dumont, Y., St-Pierre, S., Fournier, A., 1992. Characterization of CGRP1 and CGRP2 receptor subtypes. Ann. N.Y. 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., Vecsei, 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.

Tajti, J., Uddman, R., Moller, S., Sundler, F., Edvinsson, L., 1999. Messenger molecules and receptor mRNA in the human trigeminal ganglion. J. Auton. Nerv. Syst. 76, 176–183.

Uddman, R., Edvinsson, L., Ekman, R., Kingman, T., McCulloch, J., 1985. Innervation of the feline cerebral vasculature by nerve fibers containing calcitonin gene-related peptide: trigeminal origin and co-existence with substance P. Neurosci. Lett. 62, 131–136.

Wimalawansa, S.J., 1996. Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. Endocr. Rev. 17, 533–585.