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CGRP and adrenomedullin receptor populations in human cerebral arteries: in vitro pharmacological and molecular investigations in different artery sizes

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Abstract

The aim of the present study was to determine functional and molecular characteristics of receptors for calcitonin gene-related peptide (CGRP) and adrenomedullin in three different diameter groups of lenticulostriate arteries. Furthermore, the presence of perivascular neuronal sources of CGRP was evaluated in these arteries. In the functional studies, in vitro pharmacological experiments demonstrated that both CGRP and adrenomedullin induce α-CGRP-(8-37) sensitive vasodilation in artery segments of various diameters. The maximal amounts of vasodilation induced by CGRP and adrenomedullin were not different, whereas the potency of CGRP exceeded that of adrenomedullin by 2 orders of magnitude. Significant negative correlations between artery diameters and maximal responses were demonstrated for CGRP and adrenomedullin. In addition, the potency of both peptides tended to increase in decreasing artery diameter. In the molecular experiments, levels of mRNAs encoding CGRP receptors and receptor subunits were compared using reverse transcriptase polymerase chain reactions (RT-PCR). The larger the artery, the more mRNA encoding receptor activity-modifying proteins 1 and 2 (RAMP1 and RAMP2) was detected relative to the amount of mRNA encoding the calcitonin receptor-like receptor. By immunohistochemistry, perivascular CGRP containing nerve fibres were demonstrated in all the investigated artery sizes. In conclusion, both CGRP and adrenomedullin induced vasodilation via CGRP receptors in human lenticulostriate artery of various diameter. The artery responsiveness to the CGRP receptor agonists increased with smaller artery diameter, whereas the receptor-phenotype determining mRNA ratios tended to decrease. No evidence for CGRP and adrenomedullin receptor heterogeneity was present in lenticulostriate arteries of different diameters.

Keywords: Lenticulostriate artery, human; RT-PCR; CGRP (calcitonin gene-related peptide); Adrenomedullin; CRLR (calcitonin receptor-like receptor); RAMP (receptor activity-modifying protein)

1. Introduction

Calcitonin gene-related peptide (CGRP) is a potent endogenous vasodilatory agent which is released from perivascular sensory nerves in the central nervous system and in the periphery (Edvinsson et al., 1987; Hanko et al., 1985; Uddman et al., 1985, 1986). The effects of CGRP are mediated via specific CGRP receptors. Based on potencies of two CGRP derivatives, CGRP receptors have been classified into $CGRP_1$ and $CGRP_2$ receptors as reviewed by Quirion et al. (1992) and Poyner (1995). Human α-CGRP-(8-37) is a relative selective $CGRP_1$ receptor antagonist ($pA_2 \geq 7$) (Dennis et al., 1990) whereas human [$Cys(ACM)^{2,7}$]-CGRPα and human [$Cys(Et)^{2,7}$]-CGRPα are selective $CGRP_2$ receptor agonists (Dennis et al., 1989; Dumont et al., 1997). The functional $CGRP_2$ receptor has

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been demonstrated only in rat vas deferens and no molecular equivalent of this receptor has yet been identified.

Pharmacological demonstration of $CGRP_1$ receptors in different vasculature and species is numerous and the presence of mRNAs encoding human $CGRP_1$ receptors has been demonstrated in various tissues, e.g. human lung and heart (Aiyar et al., 1996), human trigeminal ganglion (Edvinsson et al., 1997; Tajti et al., 1999) and in human cranial arteries (Edvinsson et al., 1997; Sams and Jansen-Olesen, 1998).

CGRP is thought to play a significant role in certain diseases involving cerebral vasospasms. Clinical potentials for CGRP receptor agonism in the treatment of subarachnoidal haemorrhage-induced cerebral vasoconstriction have been suggested (Ahmad et al., 1996; Edvinsson et al., 1991; Juul et al., 1994; Nozaki et al., 1989; Sobey et al., 1996) and potentials for the use of CGRP receptor antagonism in the treatment of migraine have been addressed as well (Arvieu et al., 1996; Ashina et al., 2000; Doods et al., 2000; Feuerstein et al., 1995; Goadsby and Edvinsson, 1993; Goadsby et al., 1990). As CGRP receptors are widely distributed in the human organism, clinical potentials of CGRP receptor antagonism and agonism are limited by systemic side effects. However, the identification of potential (cerebro-) vascular CGRP receptor heterogeneity could challenge these limitations and could, in addition, be important for a further understanding of the role of CGRP under pathological conditions.

Adrenomedullin is a vasodilatory peptide sharing some structural similarity with CGRP (Kitamura et al., 1993; Muff et al., 1995). Immunoreactivity of adrenomedullin has been demonstrated in cultured vascular endothelial and smooth muscle cells (Sugo et al., 1994a,b). Unlike CGRP, adrenomedullin might therefore be a local acting substance not depending on an intact nervous system. Increased levels of adrenomedullin have been demonstrated during subarachnoidal haemorrhage and stroke (Kikumoto et al., 1998), and release of adrenomedullin and CGRP may therefore represent two independent pathways to counterbalance vasoconstriction.

It has elegantly been demonstrated that $CGRP_1$ and adrenomedullin receptors are derived from a common seven transmembrane domain Gs-protein coupled receptor, the calcitonin receptor-like receptor (McLatchie et al., 1998). Whether a functional CGRP or adrenomedullin receptor results from calcitonin receptor-like receptor expression depends on the coexpression of specific subunits. Calcitonin receptor-like receptor combined with the RAMP1 subunit defines a CGRP receptor and moieties of calcitonin receptor-like receptor and RAMP2 or calcitonin receptor-like receptor and RAMP3 defines adrenomedullin receptors. The calcitonin receptor-like receptor derived CGRP and adrenomedullin receptors are functionally very distinct receptors, however, CGRP and adrenomedullin show some cross-binding and cross-action at the opposite receptor (McLatchie et al., 1998). Thus, different ratios of CGRP and adrenomedullin receptors in different tissue is expected to display functional CGRP and adrenomedullin receptor heterogeneity.

In specific cell lines, it has been demonstrated that the RAMPs show competitive characteristics when interacting with calcitonin receptor-like receptor (Buhlmann et al., 1999; Muff et al., 1998). This scenario suggests partially or fully regulation of these receptors at the transcriptional level. In addition, it has been shown that levels of mRNA encoding calcitonin receptor-like receptor and RAMPs correlate with levels of adrenomedullin and CGRP binding in various rat tissues (Chakravarty et al., 2000). To our knowledge, no studies investigating function of CGRP and adrenomedullin receptors in relation to the presence of RAMP encoding mRNAs have yet been published.

In pharmacological studies performed on different vascular tissue, adrenomedullin has been shown to act partly via CGRP receptors (α-CGRP-(8-37) sensitive action) (Lang et al., 1997; Yoshimoto et al., 1998) and partly via specific adrenomedullin receptors (α-CGRP-(8-37) non-sensitive action) (Kato et al., 1995; Yoshimoto et al., 1998). No further classification of functional adrenomedullin receptors has yet been made and to our knowledge, the only convincing molecular identification of adrenomedullin receptors is the calcitonin receptor-like receptor-RAMP2 and the calcitonin receptor-like receptor-RAMP3 derived receptors.

So far, nothing is known about functional CGRP and adrenomedullin receptor populations in cerebral arteries of different origin and size, whereas diameter-dependent effects of CGRP in non-cerebral vasculature have been suggested in previous studies (Foulkes et al., 1991; Yoshimoto et al., 1998). Furthermore, mRNAs encoding both CGRP and adrenomedullin receptors have been demonstrated in human cerebral arteries of different origin (Sams and Jansen-Olesen, 1998), indicating a possible role of both receptor types in the cerebral vasculature. The aim of the present study is therefore to investigate and compare the pharmacological effects of CGRP, adrenomedullin and α-CGRP-(8-37) and in addition to compare the levels of CGRP and adrenomedullin receptor mRNAs in human lenticulostriate arteries of different sizes. Furthermore, potential sources of CGRP in the tissue of interest are to be elucidated by immunocytochemistry.

2. Materials and methods

All experiments were performed on human post-mortem tissue in accordance with the Helsinki Declaration of 1964 and the project was supported by the Human Investigation Review Board, Albert Szent-Györgyi Medical University, Szeged, Hungary (No. 1085).

Lenticulostriate branches of the human middle cerebral artery were chosen as the source of different diameter cerebral arteries.

The lenticulostriate arteries were obtained from four male patients undergoing authopsy (age: 42–89 years; cause of death: non-cerebro-vascular origins). Arteries were dissected 24–32 h post-mortem and transferred to a physiological salt solution (PSS (mM): NaCl, 118.99; KCl, 4.69; $CaCl_2$, 1.50; $MgSO_4$, 1.17; KH_2PO_4, 1.18; EDTA, 0.027, $NaHCO_3$, 0.025; glucose, 5.5). All arteries were kept at 0–4°C and were for each patient divided into three groups representing different diameter intervals: (I) Artery diameter ~300 μm; (II) Artery diameter ~700 μm; and (III) Artery diameter ~1400 μm. Each group were further divided into two parts and were either used immediately for in vitro pharmacological experiments (2–4 h after dissection) or frozen in prechilled isopentan (−70°C, 5–12 h after dissection) for subsequent molecular experiments.

Artery segments responding by >0.1 mN/mm to 125 mM K^+ were included in the pharmacological experiments and additional segments of the different artery groups were frozen for subsequent molecular experiments. The contractile function and the normalised artery diameter of the additional segments were not confirmed before freezing the segments. Patients were included in the study only when the vasoconstrictive functions were retained in all groups of arteries used for in vitro pharmacological experiments.

2.1. Functional experiments: in vitro pharmacology

From each of the four patients, a total of 12 artery segments representing artery groups I, II and III were used for the pharmacological experiments. Segments of 0.5–2 mm were mounted in PSS on 40 μm wires (I) or on 150 μm pins (II and III) in a Multimyograph (Model 610M, Danish Myotechnology) for continuous measurement and recording of the artery tension. The mounted segments were allowed to equilibrate for 30 min in PSS that was continuously aerated with 5% CO_2 and 95% O_2 at 36°C. The equilibration was repeated after each artery challenge described below. Subsequently, the distance between the sets of wires or pins was normalised to equalise $0.9 \times l_{100}$ (l_{100} is the distance between the pins or the wires when the transmural pressure equalises 100 mm Hg) and the corresponding optimal normalised circular artery diameter, D_o, was calculated as described elsewhere in detail (Mulvany and Halpern, 1977). The artery segments were challenged twice by 125 mM K^+ (KPSS (mM): KCl, 123.7; $CaCl_2$, 1.50; $MgSO_4$, 1.17; KH_2PO_4, 1.18; EDTA, 0.027, $NaHCO_3$, 0.025; glucose, 5.5) to activate and verify vasoconstrictive function. Each segment were then precontracted by prostaglandin $F_{2\alpha}$ (Dinoprost®, Upjohn), $3 \cdot 10^{-6}$ M for 30 min and was subsequently challenged with 10^{-5} M acetylcholine (Sigma) as part of the standard procedure.

Subsequently, the segments were precontracted by $3 \cdot 10^{-6}$ M Prostaglandin $F_{2\alpha}$ for 10 min prior to the cumulative addition of 10^{-10}–10^{-7} M human α-CGRP (Bachem, Switzerland) or 10^{-8}–10^{-6} M human adrenomedullin (Bachem, Switzerland) in the presence or in the absence of 10^{-6} M α-CGRP-(8-37) (Schafer-N, Copenhagen, Denmark). The antagonist were added 5 min before the preconstrictive agent was applied and each concentration of agonist were allowed to act for 4 min. Following completion of the cumulative dose response recording, the segments were washed twice with PSS before they were challenged by 125 mM K^+ for approximately 5 min (Sheykhzade and Nyborg, 1998). The vessels were allowed to rest for 30 min before the precontraction and cumulative addition of CGRP or adrenomedullin was repeated. Antagonists were added to one-half of the segments in the first experiment and to the other half in the second experiment.

2.2. Molecular experiments: reverse transcriptase polymerase chain reactions (RT-PCR)

Messenger RNA was extracted from each of the frozen artery samples by the use of a RNeasy mini protocol (Quiagen). The amount of total RNA from each sample was estimated by determining A_{260} and A_{280} and RNA was stored at −70°C. Specific amounts of total RNA obtained from each sample were reverse transcribed in final volumes of 40 μl by use of a RT-PCR kit (Perkin Elmer). Serial dilutions of cDNAs were prepared from each sample (1, 1/10, 1/100 and 1/1000 times the original concentration) and PCR reactions of templates from each dilution were carried out as previously described (Sams and Jansen-Olesen, 1998). From the lowest concentration of cDNA resulting in a distinct band on an ethidium bromide containing agarose gel, an additional set of serial dilutions (1, 1/2, 1/4, 1/8 and 1/16 times last dilution) were subject to PCR reactions. The degree of dilution of the lowest cDNA concentration that resulted in a distinct band was defined as the maximal dilution. A value of maximal dilution was determined for each of the four mRNAs originating from each of the 12 samples.

2.3. Immunohistochemistry

From one patient, the lenticulostriate branches of the middle cerebral artery was dissected out, placed in a phosphate buffered saline solution (PSS, pH 7.4) and further dissected into the three diameter groups of interest. Immunohistochemical investigations were performed by the free-floating technique essentially as previously described in detail (Knyihar-Csillik et al., 1998). The tissue was fixed in 4% paraformaldehyde for 12 h at 4°C. The tissue was processed through graded series of glucose (10%, 20% and 30% sucrose in PBS) at 4°C and after embedding, longitudinal and transversal sections of 20 μm

were cut in a cryostat at −20°C. The sections were rinsed in PBS (25°C) and to avoid endogenous peroxidase activity sections were pretreated by 2% H_2O_2. CGRP immunoreactivities were detected following three successive incubations separated by washings in PBS. (1) Anti-CGRP raised in rabbit (Sigma RBI, diluted 1:4000, 12 h at 25°C), (2) biotinylated anti-rabbit IgG (Vector Laboratories, 1:200, 90 min at 25°C), (3) peroxidase coupled avidin (ABC, Vectastatin Elite, Vector Laboratories, 1 h at 25°C). CGRP immunoreactivity was finally visualized as a brown peroxidase product of diaminobenzidine (Polysciences) in the presence of H_2O_2 (0.01% in 1% diaminobenzidine). Sections were mounted on slides, dehydrated and coverslipped.

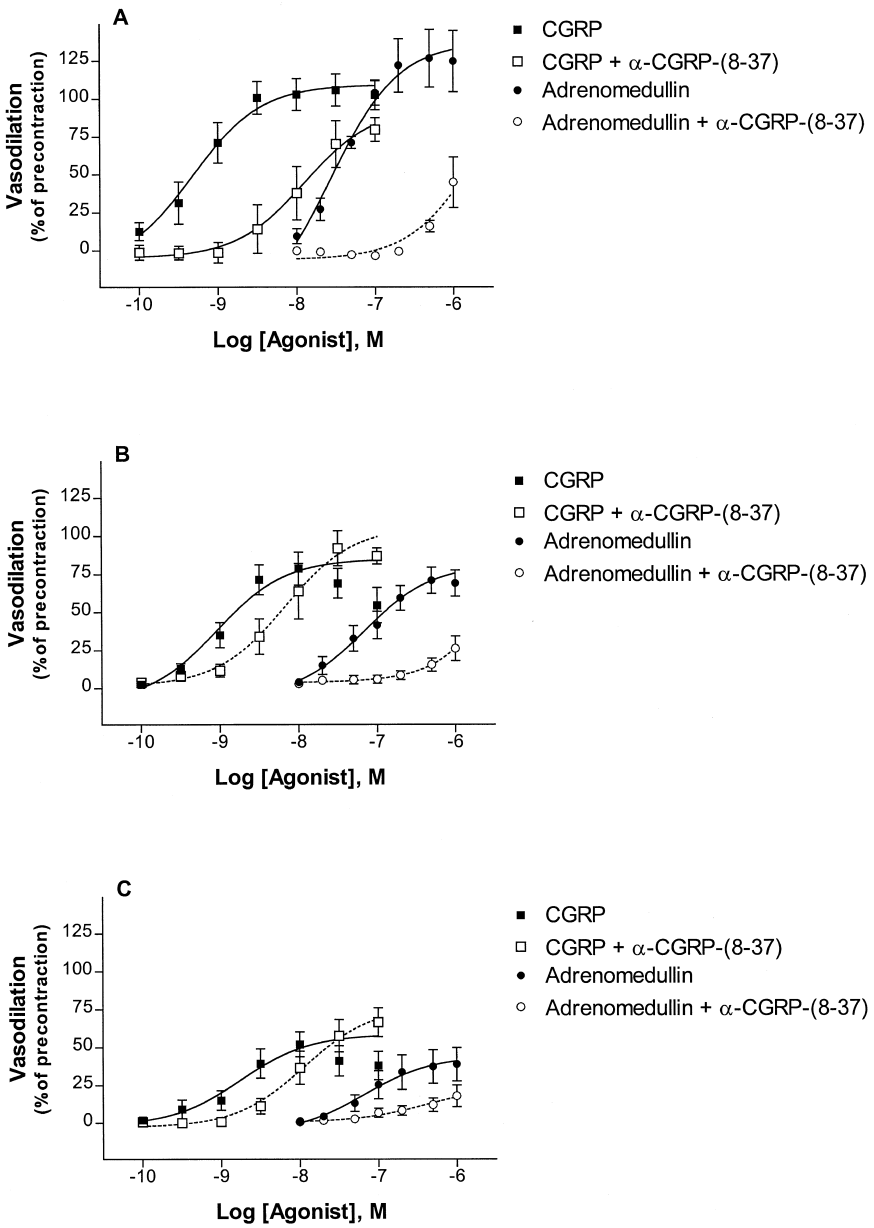

Fig. 1. Effects of CGRP and adrenomedullin in different sizes of human cerebral arteries. The dose response curves illustrate the vasodilatory effects of CGRP and adrenomedullin in the absence and in the presence of α-CGRP-(8-37). The vasodilatory responses are given in % of the prostaglandin $F_{2\alpha}$-induced preconstriction and each curve represents 6–8 segments from four patients. Each artery segment was challenged twice by cumulative concentrations of either CGRP or adrenomedullin in the presence and in the absence of α-CGRP-(8-37). The two agonist challenges of each artery segment were separated by exposure to 125 mM K^+ and for half of the segments, antagonist was present during the first dose response, the other half during the second dose response. In each of the three artery groups (A: I, 241–425 μm; B: II, 550–876 μm; C: III, 963–1727 μm), CGRP and adrenomedullin induce identical maximal amounts of vasodilation, whereas CGRP is more potent than adrenomedullin. In addition, the effects of both CGRP and adrenomedullin are inhibited by the CGRP receptor antagonist, α-CGRP-(8-37). Those findings demonstrate that both peptides act via α-CGRP-(8-37) sensitive CGRP receptors.

2.4. Calculations and statistical analysis

In the pharmacological experiments, each of the 48 artery segment obtained from the four patients were treated by either CGRP or adrenomedullin both in the presence and in the absence of α-CGRP-(8-37).

Each constriction obtained by $3 \cdot 10^{-6}$ M prostaglandin $F_{2\alpha}$ was used as a segment internal standard for calculation of CGRP- or adrenomedullin-induced vasodilation in % of the prostaglandin $F_{2\alpha}$ precontraction. From each artery segment, values of actual maximal vasodilatory response (E_{max}) were determined and the equivalent agonistic potency (pEC_{50}) were calculated by fitting the data to a sigmoidal dose response relation (GraphPad Prism). When a functional receptor desensitisation was observed at elevated agonist concentrations, these values were substituted by the actual maximal dilatory response before calculation of the estimated pEC_{50}-values.

Two values of both E_{max} and pEC_{50} were obtained from each artery segment (one representing the dose response in the presence of antagonist and one representing the dose response in the absence of antagonist). A pEC_{50} or E_{max} value from each patient was calculated as a mean of two artery segments.

From each artery segment, the antagonistic potency, pK_i of α-CGRP-(8-37) were calculated ($pK_i = \text{Log}\,(DR - 1) - \text{Log}\,10^{-6}$); DR represents EC_{50} in presence of α-CGRP-(8-37): EC_{50} in the absence of α-CGRP-(8-37); 10^{-6} M equals the molar concentration of the antagonist. The antagonistic potencies of α-CGRP-(8-37) was calculated assuming competitive antagonism on CGRP- and adrenomedullin-induced responses.

The dose ratio, $DR_{adrenomedullin:CGRP}$ (EC_{50}(adrenomedullin):EC_{50}(CGRP)) was additionally determined for each of the three artery groups from each patient and the ratio of prostaglandin $F_{2\alpha}$- and K^+-induced constriction (prostaglandin $F_{2\alpha}$:K^+) was calculated for each vessel segment.

The coincidence of non-functionality of low diameter arteries was higher than that of the larger arteries, and the experimental numbers of DR, E_{max}, pEC_{50} and pK_i were therefore lower for the small vessels.

Data manipulations: The concentrations of adrenomedullin induce only submaximal vasodilatory responses in the presence of α-CGRP-(8-37). When calculating pEC_{50} of adrenomedullin in the presence of antagonist, E_{max} was fixed to equal the E_{max} value corresponding to the same vessel segment in the absence of antagonist.

Differences in E_{max}, pEC_{50}, "prostaglandin F:K", pK_i and $DR_{adrenomedullin:CGRP}$ were evaluated for diameter dependency using linear regression analysis (GraphPad Prism).

3. Results

3.1. Functional experiments

CGRP and adrenomedullin induced concentration-dependent dilation of all the investigated human cerebral arteries (Fig. 1). The maximal amount of vasodilation induced by the two peptides were not different, however, the potency of CGRP exceeded that of adrenomedullin by approximately 2 orders of magnitude in all three groups of arteries (Fig. 1 and Table 1).

In all groups of artery diameters, the effects of both CGRP and adrenomedullin were inhibited by α-CGRP-(8-37) (Fig. 1, Table 1). Due to the order of potency of CGRP and adrenomedullin in addition to the common inhibition

Table 1
Physical and pharmacological characteristics of the investigated artery groups

		Artery group		
		I	II	III
Artery size, μm	D_o	320 ± 26 (9)	695 ± 26 (16)	1312 ± 80 (16)
	Range	241 – 425	550 – 876	963 – 1927
Prostaglandin $F_{2\alpha}$:K^+		2.1 ± 0.2 (4, 9)	2.3 ± 0.4 (4, 16)	2.0 ± 0.2 (4, 16)
E_{max}	CGRP	106 ± 4 (3, 5)	81 ± 10 (4, 8)	55 ± 12 (4, 8)
	Adrenomedullin	127 ± 19 (4, 4)	75 ± 9 (4, 8)	42 ± 17 (4, 8)
pEC_{50}	CGRP	9.2 ± 0.1 (3, 5)	9.0 ± 0.1 (4, 8)	8.4 ± 0.2 (4, 8)
	Adrenomedullin	7.3 ± 0.1 (4, 4)	7.0 ± 0.3 (4, 8)	6.7 ± 0.2 (4, 8)
pK_i	CGRP	8.0 ± 0.7 (3, 5)	7.1 ± 0.2 (4, 8)	6.9 ± 0.2 (4, 8)
	Adrenomedullin	7.2 ± 0.5 (4, 4)	7.7 ± 0.2 (4, 8)	7.1 ± 0.2 (4, 8)
Dose ratios	$DR_{adrenomedullin:CGRP}$	105 ± 26 (3)	111 ± 36 (4)	65 ± 24 (4)

The optimal normalised artery diameter (D_o) is given as mean ± S.E.M. (n) of all artery segments investigated in each group (n). In addition, the range of artery diameters in each artery group is shown.

The ratio of prostaglandin $F_{2\alpha}$-induced precontraction vs. the K^+-induced contraction are determined in order to compare the precontractive levels of segments of various diameter. The ratios are given as mean ± S.E.M. (n_p, n_e), where n_p is the number of patients examined and n_e is the number of segments examined.

E_{max} values are expressed as amount of vasodilation in % of the prostaglandin $F_{2\alpha}$-induced precontraction. E_{max} and pEC_{50} values are given as mean ± S.E.M. (n_p, n_e). The mean and S.E.M. values given are calculated from means of each patient. The dose ratios are given as a mean ± S.E.M. (n_p).

by α-CGRP-(8-37) and the identical E_{max} values of the two peptides, it was demonstrated that adrenomedullin, as well as CGRP, act via CGRP receptors in the investigated tissue (Table 1).

Comparing the effects of the peptides on the different artery sizes from each patient, E_{max} values and potencies of both CGRP and adrenomedullin were consistently higher in smaller as compared to larger arteries. Performing linear regression analysis on the E_{max} values of CGRP or adrenomedullin vs. the optimal diameter of the investigated artery segment confirmed significant linear correlations (Fig. 2A and B) as the slopes of the linear regression curves were significantly different from zero ($P = 0.0380$ and $P = 0.0039$, respectively).

The pEC_{50} values of CGRP showed significant linear correlation to the artery diameter (Fig. 2C, $P = 0.0006$),

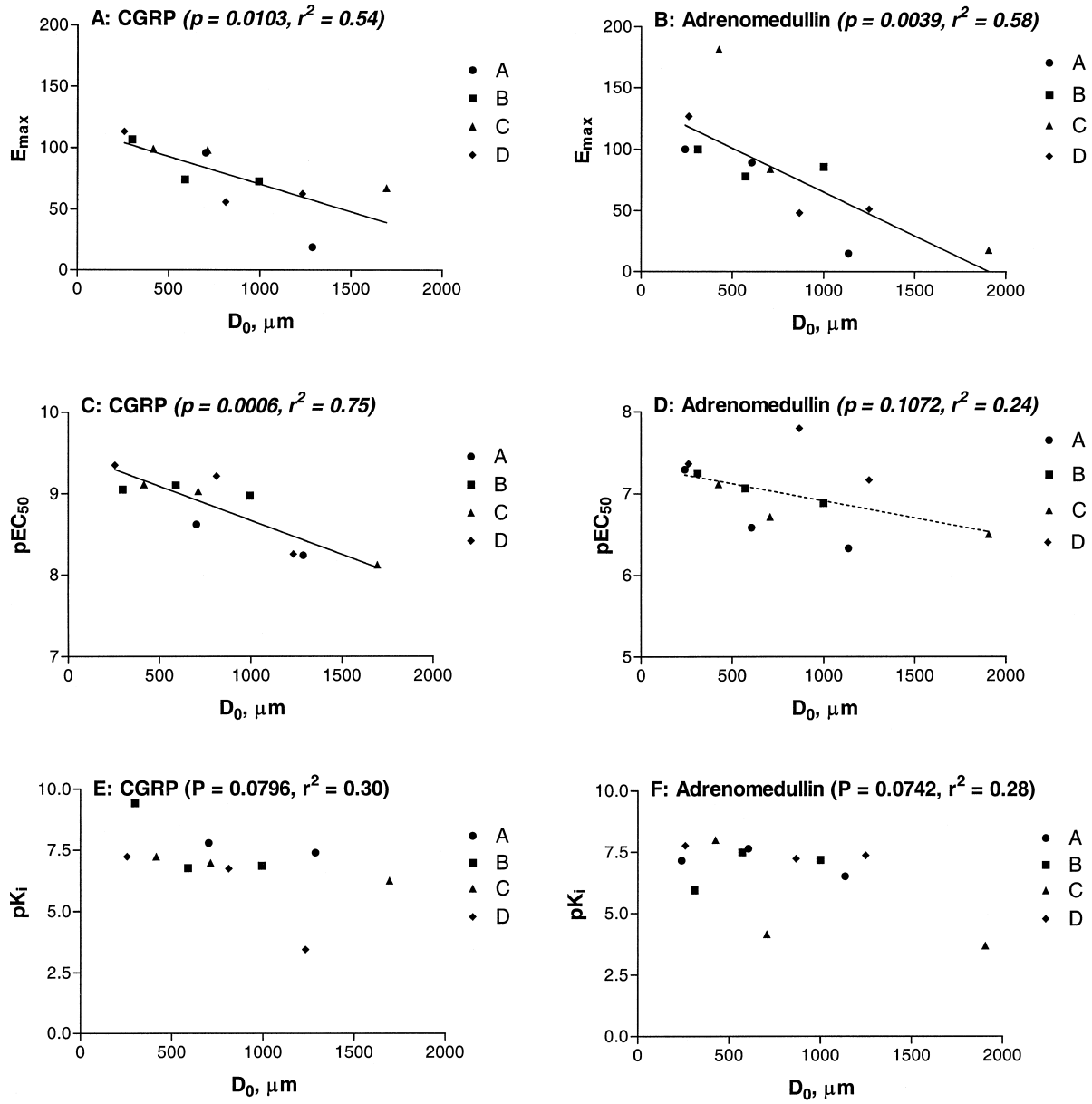

Fig. 2. Correlations between artery diameter and E_{max}, pEC_{50} and pK_i-values. For each of the three artery groups from each of the four patients, the mean E_{max} and pEC_{50} values for CGRP ($n = 21$)- and adrenomedullin ($n = 20$)-induced vasodilation are plotted vs. the corresponding mean optimal artery diameter (A, B, C and D). In addition, the antagonistic potencies of α-CGRP-(8-37) on CGRP ($n = 21$)- and adrenomedullin ($n = 20$)-induced vasodilation are plotted vs. the corresponding mean optimal artery diameter (E and F). Linear regression analysis has been performed on those mean values. Significant linear correlations for both CGRP (A)- and adrenomedullin (B)-induced E_{max} values are demonstrated as the slopes of the linear regression curves are significantly different from zero ($P = 0.0103$ and $P = 0.0039$, respectively). A significant linear correlation is seen for the potency of CGRP (C), but not that of adrenomedullin (D) ($P < 0.0006$ and $P = 0.1072$, respectively). No tendency of linear correlation between antagonistic potencies of α-CGRP-(8-37) on CGRP (E)- and adrenomedullin (F)-induced vasodilation is seen. Individual linear correlation coefficients and P-values of the linear regression analysis are shown in the right corner of the figures.

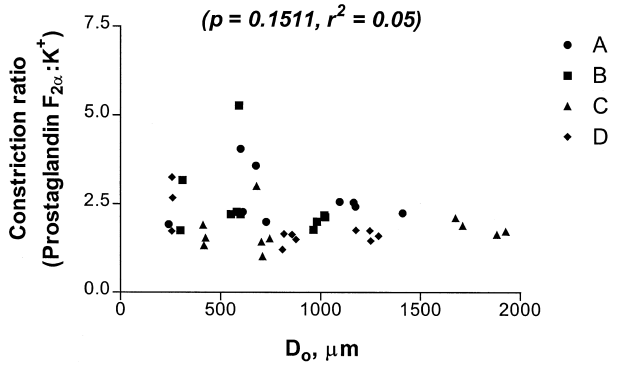

Fig. 3. Correlation between artery diameter and precontraction level. As vasoconstrictive mechanisms of prostaglandin $F_{2\alpha}$ (receptor mediated) and K^+ (depolarisation mediated) are different, the ratio of constriction induced by $3 \cdot 10^{-6}$ M prostaglandin $F_{2\alpha}$ and by 125 mM K^+ is used to evaluate whether the prostaglandin $F_{2\alpha}$-induced level of precontraction is different in different sizes of arteries. Linear regression analysis on the ratio of contraction ($n = 41$) vs. the optimal artery diameter demonstrates no correlation between precontractive level and artery size. The parameters of the linear regression analysis are shown in the right corner of the figure.

whereas the correlation between artery diameter and potency of adrenomedullin was not significant (Fig. 2D, $P = 0.1072$). However, no tendency of correlation between artery diameter and potency ratio of CGRP and adrenomedullin was observed (Table 1).

The calculated antagonistic potencies of α-CGRP-(8-37) had no tendency of diameter dependency (Fig. 2E and F). The diameter-dependent artery responsiveness to the vasodilatory peptides is not a result of a diameter-dependent precontraction, as the ratio of precontraction induced by prostaglandin $F_{2\alpha}$ vs. the contraction induced by total depolarisation (125 mM K^+) had no tendency of diameter dependence (Fig. 3) when evaluated by linear regression ($P = 0.1511$, $r^2 = 0.05$).

3.2. Molecular studies

Distinct RT-PCR bands were detected in the majority of the 12 artery segments from the four patients. However, calcitonin receptor-like receptor was not detected in artery group I from patient B and RAMP3 was not detected in group III from patient A and in groups I, II and III from patient B.

Using the described normalisation method, the mRNA ratios of calcitonin receptor-like receptor vs. RAMP1 and calcitonin receptor-like receptor vs. RAMP2 tended to increase in larger arteries as compared to smaller arteries

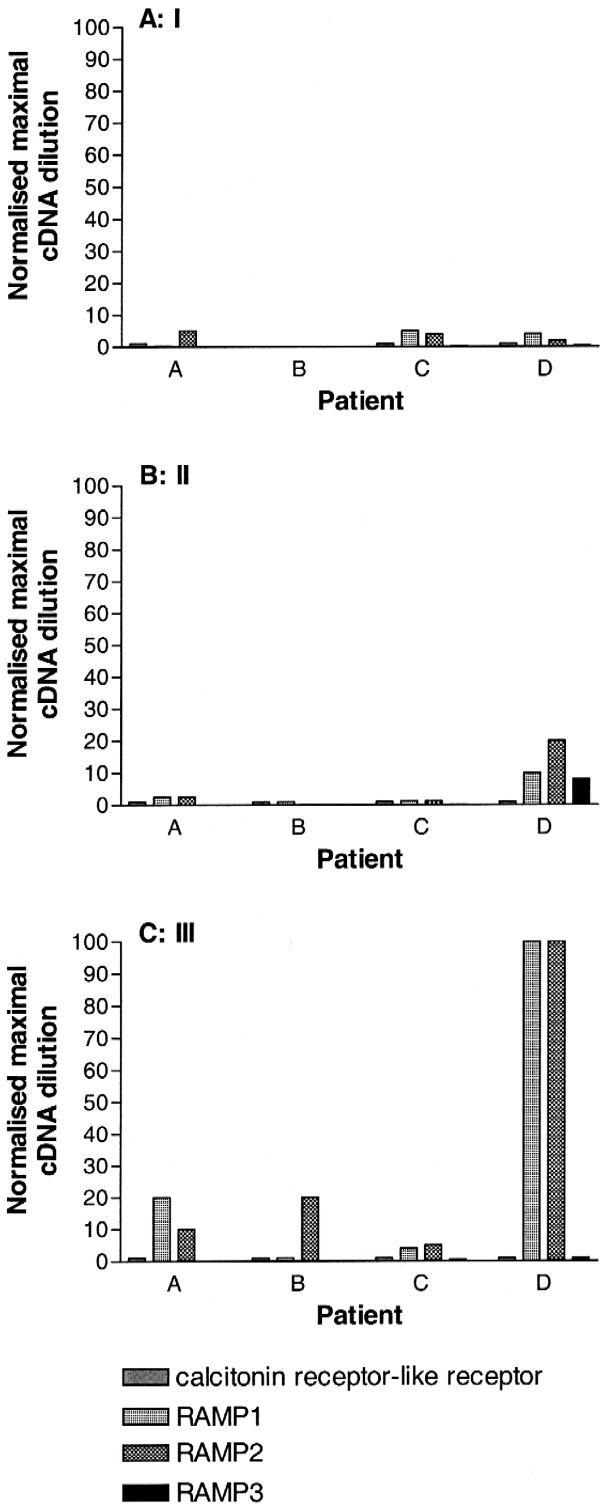

Fig. 4. Relative levels of calcitonin receptor-like receptor and RAMP mRNAs in the different sizes of arteries. Messenger RNA encoding both CGRP and adrenomedullin receptors were demonstrated in cerebral arteries of different diameters. The ratios of maximal dilution of each RAMP cDNA vs. calcitonin receptor-like receptor cDNA are calculated for each patient in each artery group. The maximal dilutions of calcitonin receptor-like receptor cDNA are fixed as unity in all three artery groups. Ratios of maximal dilution are shown as bars and symbols representing each corresponding mRNA are illustrated by legends in the figure. In three of four patients (A, B and D), consistent increases in the RAMP1-calcitonin receptor-like receptor and the RAMP2-calcitonin receptor-like receptor ratios is seen for increasing artery diameters. RAMP3 could not be detected in all samples and no consistent scenario could be demonstrated.

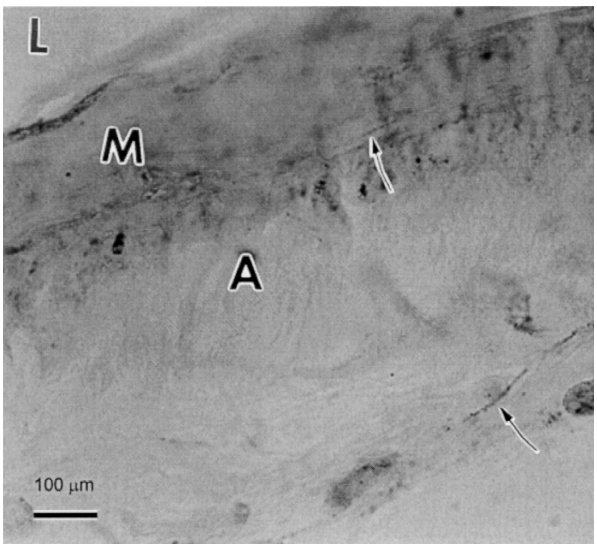

Fig. 5. CGRP immunoreactivity in human cerebral arteries. L: Lumen; M: Media and A: Adventitia. CGRP immunoreactivity was demonstrated in perivascular nerve fibres in all three sizes of lenticulostriate arteries. The arrows indicate CGRP immunoreactivity in the adventitia and media of an artery segment representing artery group I.

(Fig. 4). No consistent tendency is seen for the RAMP3-calcitonin receptor-like receptor ratio.

There was no correlation between post-mortem age and the amount of mRNA present.

3.3. Immunohistochemistry

CGRP immunoreactivity was detected in the longitudinal and transversal sections as perivascular nerve fibres in the adventitia and media of all three artery sizes (Fig. 5).

4. Discussion

The functional nature of the effects of CGRP, adrenomedullin and α-CGRP-(8-37) are similar in the three investigated artery groups: Maximal amounts vasodilation induced by CGRP and adrenomedullin are not different, CGRP is more potent than adrenomedullin and the effects of both peptides are inhibited by α-CGRP-(8-37). These characteristics are consistent with action of both peptides via CGRP receptors (McLatchie et al., 1998; Yoshimoto et al., 1998).

Despite the presence of mRNA encoding both CGRP and adrenomedullin receptors, the present study demonstrates that both CGRP and adrenomedullin induce significant concentration-dependent vasodilation only via α-CGRP-(8-37) sensitive CGRP receptors in various sizes of post-mortem human cerebral artery (Fig. 1 and Table 1). The lack of significant effects via adrenomedullin receptors could be due to significant post-mortem degradation of this receptor; however, the findings are in agreement with findings in experimental animals and tissue cultures. For example, adrenomedullin has been shown to act only via α-$CGRP_{8-37}$ sensitive CGRP receptors in human cerebromicrovascular cell cultures (Moreno et al., 1999), in porcine coronary artery (Yoshimoto et al., 1998) and in rat cerebral arteries in vivo (Lang et al., 1997) and in vitro (Mori et al., 1997). The potency of adrenomedullin in all the referred studies is lower than that of CGRP, which is in accordance with findings in the present study (Fig. 1 and Table 1). In rat aorta, however, CGRP and adrenomedullin both have pEC_{50} values of ~ 8.5, and only the vasodilation induced by CGRP is antagonised by α-$CGRP_{8-37}$ (Yoshimoto et al., 1998). Based on qualitative different effects of CGRP, adrenomedullin and α-$CGRP_{8-37}$, no CGRP and adrenomedullin receptor heterogeneity in different sizes of human lenticulostriate arteries was found in the present study.

Quantitative differences in the pharmacological profiles of CGRP, adrenomedullin and α-$CGRP_{8-37}$ were evaluated in the various artery diameters as well. The wide range of artery diameters in each of the three investigated artery groups and the narrow intervals between the groups (Table 1) strengthens the use of linear regression in the analysis of differences among various sizes of arteries. Linear regression analysis demonstrates significant negative linear correlations between artery diameter and the E_{max} values of CGRP and adrenomedullin (Fig. 2A and B). Consistency between the four specimens can be emphasised from the figures. For CGRP, this correlation has previously been demonstrated in human mesenteric vasculature (Miyauchi et al., 1996) and diameter-dependent E_{max} values of CGRP are therefore not a specific characteristic of cerebral arteries. On the other hand, it may not be the case for all arteries as no such correlation was demonstrated in the guinea pig superior mesenteric artery (Edvinsson et al., 1989).

In addition to diameter-dependent E_{max} values, a significant diameter-dependent potency of CGRP was demonstrated in this study. The correlation between CGRP potency and artery diameter was found neither in the referred studies of the human mesenteric vasculature (Miyauchi et al., 1996), nor in the guinea pig mesenteric artery (Edvinsson et al., 1989). However, a diameter-dependent potency of CGRP has previously been demonstrated in porcine coronary artery segments (Foulkes et al., 1991).

In addition to that of CGRP (Fig. 2C), the potency of adrenomedullin tended to increase in decreasing artery diameter, however, this correlation was not significant (Fig. 2D). Investigating the diameter dependency of the potency ratio of adrenomedullin and CGRP (Table 1) it is evident that CGRP is not being relatively more potent than adrenomedullin in smaller arteries as compared to larger arteries (correlation not shown), and based on potencies of CGRP and adrenomedullin, no evidence for receptor heterogeneity in lenticulostriate arteries of different diameters is present.

Diameter-dependent E_{max} and pEC_{50} values of vasodilatory agents could be caused by diameter-dependent levels of precontraction obtained by $3 \cdot 10^{-6}$ M prostaglandin $F_{2\alpha}$. However, considering the constant ratio of receptor stimulated vs. depolarisation stimulated contraction, this is an unlikely explanation (Table 1, Fig. 3). Nevertheless, the artery diameter-dependent responsiveness to the two peptides may not be specific for CGRP and adrenomedullin. It may instead represent a general increased artery sensitivity to vasodilatory agents in small, as compared to large, diameter arteries.

The referred study of Foulkes et al. (1991) demonstrated a diameter-dependent antagonistic potency of α-CGRP-(8-37), which is not seen in the present study (Fig. 2E and F). Here, no significant diameter-dependent correlation could be seen for the antagonistic effect of α-CGRP-(8-37) as evaluated by linear regression of pK_i values vs. artery diameter.

The estimated antagonistic potencies (pK_i) of α-CGRP-(8-37) in the three artery groups range from 6.9 to 8.0 towards CGRP-induced vasodilation and from 7.1 to 7.7 towards adrenomedullin-induced vasodilation. According to the CGRP receptor classification, pA_2 values > 7 corresponds to action via $CGRP_1$ receptors and pA_2 values < 7 corresponds to action via $CGRP_2$ receptors. Even though the range of pK_i values found in the large diameter arteries of this study overlap the border between $CGRP_1$ and $CGRP_2$ receptors we are not likely to distinguish between the two receptor types, as there is no significant differences in the antagonistic potency in the various artery diameters. Therefore, we are unable to classify the functional CGRP receptors in the lenticulostriate arteries by use of the classical CGRP receptor classification. However, as mRNAs encoding the $CGRP_1$ receptor has been demonstrated in all investigated arteries, it is likely that the observed vasodilatory actions of CGRP and adrenomedullin are mediated via $CGRP_1$ receptors.

Based on the antagonistic potency of α-CGRP-(8-37), no CGRP and adrenomedullin receptor heterogeneity could be demonstrated in the investigated arteries.

The effects of CGRP, adrenomedullin and α-CGRP-(8-37) display no signs of CGRP and adrenomedullin receptor heterogeneity in various sizes of lenticulostriate arteries. Thus, no functional evidence for different ratios of CGRP and adrenomedullin receptors in various sizes of cerebral artery is present. Based on our findings, no artery diameter-dependent selectivity of future CGRP receptor agonistic or antagonistic drugs should therefore be expected. However, whether functional CGRP and adrenomedullin receptor heterogeneity exists in cerebral vasculature of different anatomical origin remains to be elucidated.

In the present study, each artery segment was challenged by agonist both in the absence and in the presence of an antagonist. Repeated challenges with CGRP are usually not reproducible. However, treatment with 125 mM K^+ between the CGRP challenges has been shown to reverse tachyphylaxia in rat coronary arteries (Sheykhzade and Nyborg, 1998). The same scenario has been found in guinea pig basilar arteries (unpublished). It has not been confirmed whether potassium challenge between CGRP treatments can inhibit a functional receptor desentisation in human cerebral arteries, however, comparing the present results obtained from the first and the second challenge, no bias was observed.

In the present study, presence of mRNAs encoding calcitonin receptor-like receptor, RAMP1, RAMP2, and RAMP3 was demonstrated in human lenticulostriate arteries of various diameters. Previous studies have shown that concomitant presence of RAMP1 and calcitonin receptor-like receptor in specific cell lines will result in expression of CGRP receptors, both in the absence and in the presence of RAMP2 (Buhlmann et al., 1999; Muff et al., 1998). However, when RAMP3 and calcitonin receptor-like receptor are present, adrenomedullin receptors will appear even in the presence of RAMP1 (Muff et al., 1998). Despite of the presence of mRNAs encoding RAMP1, RAMP2 and RAMP3, the present functional studies could only demonstrate functional CGRP receptors in the vascular tissue examined. When investigating the combination of cells in the tissue of this condition, no correlation to the referred previous cell line investigations are obvious. Furthermore, in addition to interacting with calcitonin receptor-like receptor, RAMP1 and RAMP2 have been shown to interact and determine the phenotype of the calcitonin receptor (Muff et al., 1999).

In the present study, the amount of cDNA dilution was calculated as relative to calcitonin receptor-like receptor for every single vessel segment. Using this normalisation parameter, amounts of RAMP1 and RAMP2 mRNA tended to increase in increasing artery size where CGRP and adrenomedullin responsiveness were decreasing. However, the reverse ratio (calcitonin receptor-like receptor vs. RAMP2 and RAMP3) increased in decreasing artery diameters. No clear tendency for RAMP3 encoding mRNA can be found and no tendency of the different RAMP mRNA ratios are obvious.

CGRP immunoreactivity was detected in perivascular nerve fibres of all the investigated arteries, indicating that the lenticulostriate arteries are likely to be regulated by CGRP in vivo. From previous studies, it is well known that various cerebral arteries are surrounded by CGRP containing nerve fibres (Edvinsson et al., 1987; Jansen et al., 1992; Uddman et al., 1985). In addition, the present study has demonstrated that certain intracerebral arteries are innervated by CGRP containing nerve fibres as well.

In conclusion, CGRP and adrenomedullin act via α-CGRP-(8-37) sensitive CGRP receptors in various sizes of human lenticulostriate arteries and artery responsiveness towards the peptides demonstrated significant negative linear correlation to artery diameter.

No heterogeneity of CGRP and adrenomedullin receptor populations in varying sizes of lenticulostriate branches of

the human middle cerebral artery was demonstrated in the present study. The pharmacological evidence for CGRP receptors in the investigated tissue is supported by the presence of mRNA encoding calcitonin receptor-like receptor and RAMP1. A possible function of the present mRNA encoding RAMP2 and RAMP3 cannot be interpreted from this study and no correlation between mRNA levels and receptor function can be suggested. The investigated arteries are likely to be regulated by CGRP in vivo, since CGRP containing nerve fibres surrounds the arteries.

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