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. © 2000 Elsevier Science B.V. All rights reserved.

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 cumula-

tive 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
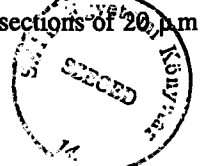
were cut in a cryostat at $-20°C$. The sections were rinsed in PBS ($25°C$) and to avoid endogenous peroxidase activity sections were pretreated by 2% H_2O_2. CGRP immunoreactivities were detected following three successive incubations separated by washings in PBS. (1) Anti-CGRP raised in rabbit (Sigma 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 cover-slipped.
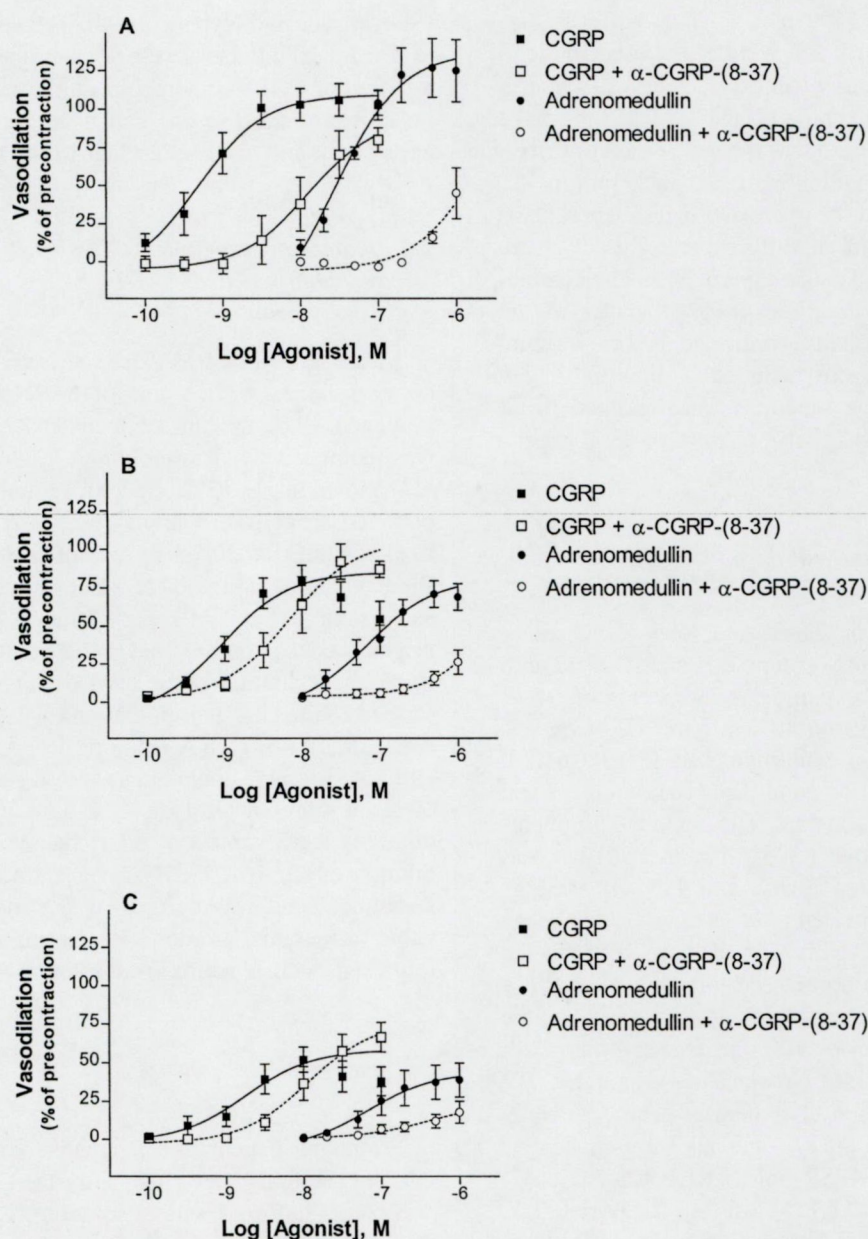

Fig. 1. Effects of CGRP and adrenomedullin in different sizes of human cerebral arteries. The dose response curves illustrate the vasodilatory effects of CGRP and adrenomedullin in the absence and in the presence of α-CGRP-(8-37). The vasodilatory responses are given in % of the prostaglandin $F_{2α}$-induced 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 = Log (DR − 1) − 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).

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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, $P = 0.0006$),
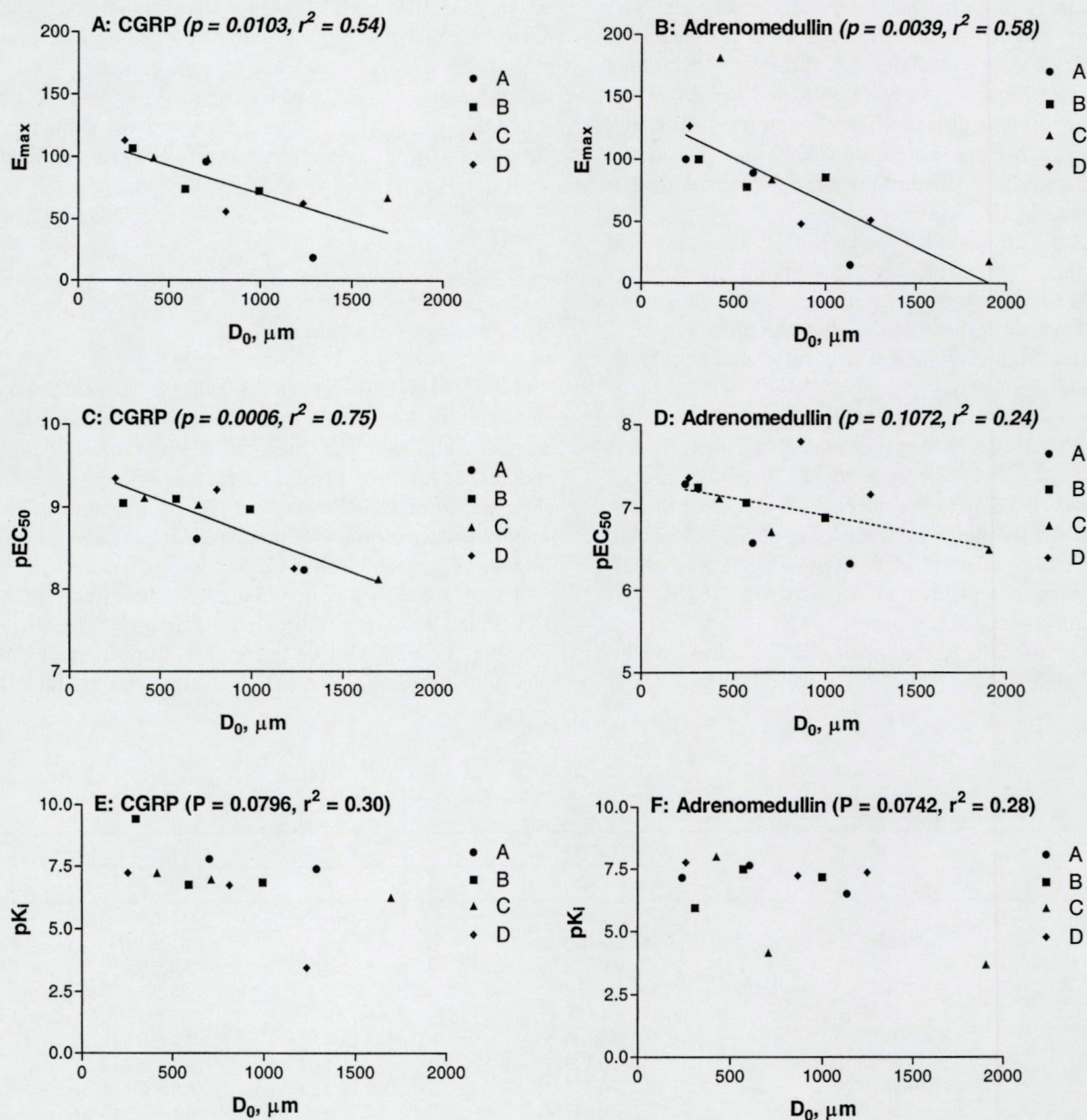

Fig. 2. Correlations between artery diameter and E_{max}, pEC_{50} and pK_i-values. For each of the three artery groups from each of the four patients, the mean E_{max} and pEC_{50} values for CGRP ($n = 21$)- and adrenomedullin ($n = 20$)-induced vasodilation are plotted vs. the corresponding mean optimal 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.
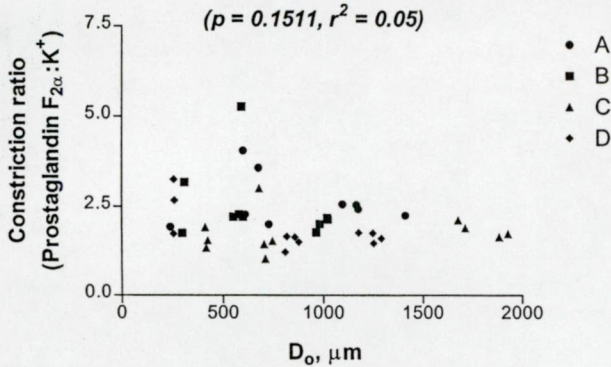
Fig. 3. Correlation between artery diameter and precontraction level. As vasoconstrictive mechanisms of prostaglandin $F_{2\alpha}$ (receptor mediated) and K^+ (depolarisation mediated) are different, the ratio of constriction induced by $3 \cdot 10^{-6}$ M prostaglandin $F_{2\alpha}$ and by 125 mM K^+ 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
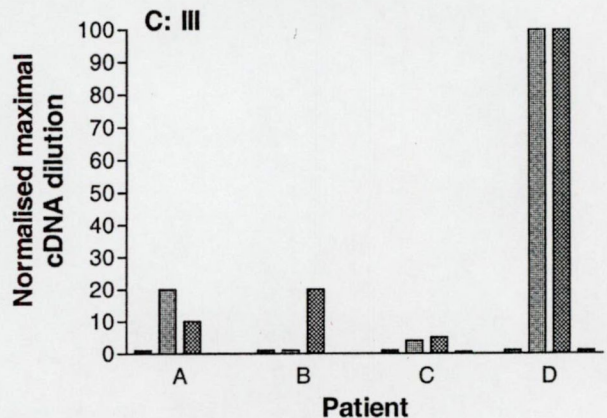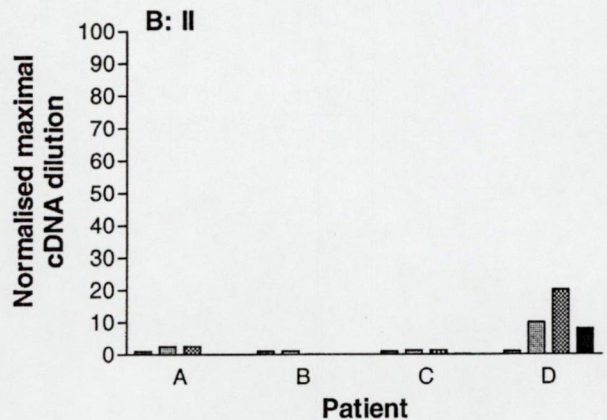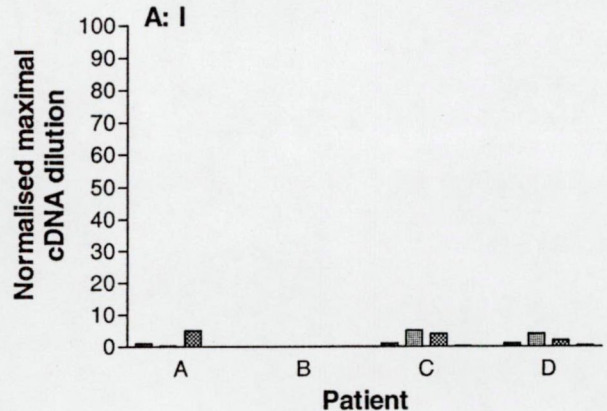
Fig. 4. Relative levels of calcitonin receptor-like receptor and RAMP mRNAs in the different sizes of arteries. Messenger RNA encoding both CGRP and adrenomedullin receptors were demonstrated in cerebral arteries of different diameters. The ratios of maximal dilution of each RAMP cDNA vs. calcitonin 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.

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

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In depth pharmacological characterization of endothelin B receptors in the rat middle cerebral artery

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Abstract

Whereas the endothelin A receptor is generally believed to mediate vasoconstriction; the endothelin B receptor seems elusive; both dilative and constrictive responses have been reported. Using the in vitro arteriograph, a method allowing compartmentalized study of vessel segments, segments of rat middle cerebral artery were cannulated with micropipettes, pressurized and luminally perfused. Vessel diameters were evaluated using a microscope equipped with an imaging system. Both intra- and extraluminal applications of endothelin-1 produced constriction. Intraluminal administration of a selective endothelin B receptor agonist sarafotoxin 6c in precontracted cerebral arteries and in the presence of the endothelin A receptor blocker FR139317 caused vasodilation in a concentration-dependent manner. Inhibition of nitric oxide synthase significantly reduced the dilation induced by sarafotoxin 6c, whereas inhibition of cyclooxygenase and endothelium-derived hyperpolarizing factor did not. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Endothelins; Endothelin receptors; In vitro pharmacology; Endothelium; Vascular smooth muscle; Middle cerebral artery; Rats

Endothelin (ET) is one of the most potent endogenous vasoconstrictors acting both peripherally and centrally. So far three isopeptides, endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3) have been identified. The amino acid sequences (21 residues) are quite unique in comparison with other mammalian peptides; only the sequence of the rare snake venom sarafotoxin has been found to be similar to that of ET [9]. The ETs can exert both constrictor and dilator effects. These opposite actions have been attributed to the existence of different endothelin receptors. The discovered receptor subtypes in mammals are the endothelin A (ET_A) and B (ET_B) receptors. Stimulation of ET_A receptors, which are located on vascular smooth muscle cells (SMC), causes vasoconstriction [2,15]. ET_B receptors, present on endothelial cells (EC), however, mediate vasodilation [4,8]. The relaxant ET_B receptor is coupled to nitric oxide (NO) production in cerebral EC [13]. This straightforward distribution of contractile and dilatory ET receptors has been confounded by the discovery

that ET_A receptors may also be present on brain capillary EC [16] as well as the fact that contractile ET_B receptors have been demonstrated in SMC [6,17].

The current standard for in vitro pharmacological characterization is to hook up a vessel segment on two prongs, one fixed and the other connected to a strain gauge. The advent of the pressurized arteriograph allows for a compartmentalized study of the vessel distinguishing between luminal (acting on EC) and abluminal (acting directly on SMC) application of drugs, an option not available to the classical strain gauge method [3,18]. Using this method we have sought to more thoroughly characterize the ET_B receptor in rat middle cerebral arteries (MCA) and to pinpoint the mediator mediating the vasodilative effect of endothelins between the endothelium and the vascular smooth muscle cells.

Male Sprague–Dawley rats (250–300 g) were anaesthetized using CO_2 and decapitated. The brain was immediately removed and placed in cold (4°C) buffer solution of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 5.5.

With the aid of a dissecting microscope, both MCAs were carefully harvested beginning at the circle of Willis and

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extending 5–8 mm distally. A section of the MCA (1–2 mm in length) was mounted in an arteriograph (Living System, Burlington, VT, USA) [3,18]. Micropipettes were inserted into both ends of each MCA and secured in place with nylon ties. The mounted MCA was bathed in buffer solution of the above composition (37°C) equilibrated with gas consisting of 5% CO_2 – 95% O_2. The resulting pH of the bath solution was 7.4. Luminal pressure of the MCAs was measured at either side of the vessel using pressure transducers and was maintained at 85 mmHg by raising or lowering reservoirs connected to the micropipettes. Luminal perfusion was adjusted to 100 μl/min by setting the two reservoirs at different heights. The mounted vessels were magnified *600-fold* with a microscope coupled to a video camera. The analogue output signal of the camera was converted to a digital image using a custom made digitizer, and the final image was displayed on a computer monitor. Outside diameters of the MCAs were measured directly on the computer monitor. Drugs could be delivered either to the luminal reservoir (luminal application) or to the vessel bath (abluminal application).

After mounting and pressurization, the MCAs developed spontaneous tone (approximately 25% of the initial diameter) over the equilibrating period of 1–1.5 h. Experimental protocols were not initiated until the MCA diameter was stable over a 15-min period. Any MCA that did not develop spontaneous tone was excluded. The presence of a functional endothelium was tested at the beginning of each experiment by adding adenosine triphosphate (ATP) abluminally, 10^{-5} M. A vasodilatation of 30% or more of the resting diameter was considered indicative of a functional endothelium.

ET-1 (mixed agonist), or sarafotoxin 6c (S6c, selective ET_B receptor agonist) were added cumulatively in logarithmic steps to either the abluminal bath or the luminal perfusate in the concentration range 10^{-13}–10^{-7} M. For the abluminal administration, the vascular smooth muscle was exposed to each concentration of the agonists for 5 min before next cumulative increase. The endothelium was exposed to each concentration of the receptor agonists for approximately 15 min. The steady-state change in MCA diameter was measured at each concentration. To avoid the risk of tachyphylaxis only one concentration response experiment was conducted for each MCA.

Experiments were conducted both in vessels only having developed spontaneous tone and in vessels additionally contracted by adding the thromboxane A_2 analogue U46619 abluminally (10^{-7} M).

In experiments performed with S6c as agonist, the selective ET_A receptor antagonist FR139317 was used at a concentration of $10^{-5.5}$ M to block any unwanted stimulation of the ET_A receptor, and the selective ET_B antagonist IRL 2500 was employed at a concentration of $10^{-6.5}$ M to characterize the response [1,7,10]. To elucidate the mechanisms responsible for dilatory effects of ET receptors, L-NAME (N^ω-nitro-L-arginine methyl ester, a NO synthase

inhibitor), indomethacin (a cyclooxygenase inhibitor) and charybdotoxin (a blocker of voltage sensitive and calcium activated potassium channels, by definition an inhibitor of the endothelially derived hyperpolarizing factor, EDHF) were employed at concentrations of 10^{-5}, 10^{-5}, and 10^{-8} M, respectively [12]. These drugs were added both luminally and extraluminally to the system.

Data are presented as mean ± SEM. To compensate for variation amongst vessels, measured diameters in a given experiment were indexed to either the resting diameter (contractile experiments) or the precontracted diameter (dilatory experiments). E_{max} denotes, depending on the experiment, either the maximal contractile, or maximal dilatory effect, and pEC_{50} denotes the negative logarithm to the concentration at which half the maximal effect was obtained. Data analysis was performed using GraphPad Prism from GraphPad Software Inc. Differences were statistically evaluated using Student's t-test considering P values below 0.05 significant.

The mean diameters of the MCAs after initial pressurization and after development of spontaneous tone were 266 ± 20 and 215 ± 20 μm ($n = 40$), respectively.

Both luminal and abluminal application of ET-1 produced contraction in a concentration-dependent manner (Fig. 1a). Abluminally E_{max} was 26.9 ± 0.7% and pEC_{50} was 9.6 ± 0.1; luminally the same values were 24.3 ± 1.1% and 9.8 ± 0.2, respectively. The differences were not statistically different. No dilatory effect of ET-1 was observed, not even in the presence of the ET_A antagonist FR139317.

In the spontaneously contracted artery there was no effect of S6c neither abluminally nor luminally. However, upon precontraction with U46619 (10^{-7} M) and selective blockage of the ET_A receptor with FR139317 ($10^{-5.5}$ M) abluminally, luminal administration of S6c gave rise to a concentration-dependent dilatation with an E_{max} of 4.9 ± 0.1% and a pEC_{50} of 10.1 ± 0.1 (Fig. 1b). Abluminally administered only a negligible response was observed with an E_{max} of 0.3 ± 0.1% and a pEC_{50} of 10.2 ± 0.1. Luminal application of the selective ET_B receptor antagonist IRL 2500 ($10^{-6.5}$ M), 15 min before the application of a single dose of S6c (10^{-8} M) also administered luminally, significantly attenuated the vasodilatative effect of S6c (Fig. 2a).

The effect of pretreating the precontracted and ET_A blocked perfused MCA both abluminally and luminally with either L-NAME, indomethacin or charybdotoxin on the vasodilatative effect of a single dose of S6c administered luminally is shown in Fig. 2b. Only pretreatment with L-NAME had a significantly attenuating effect on the vasodilatative effect of S6c; 1.8 ± 2.7 versus 5.6 ± 2.5% in the non-pretreated vessel ($P < 0.05$). Pretreatment with charybdotoxin gave rise to a non-significant attenuation whereas indomethacin had no effect (Fig. 2b).

Following the advent of the arteriograph allowing discrete investigation of luminal and abluminal effects of vasoactive agents, we have investigated the MCA from
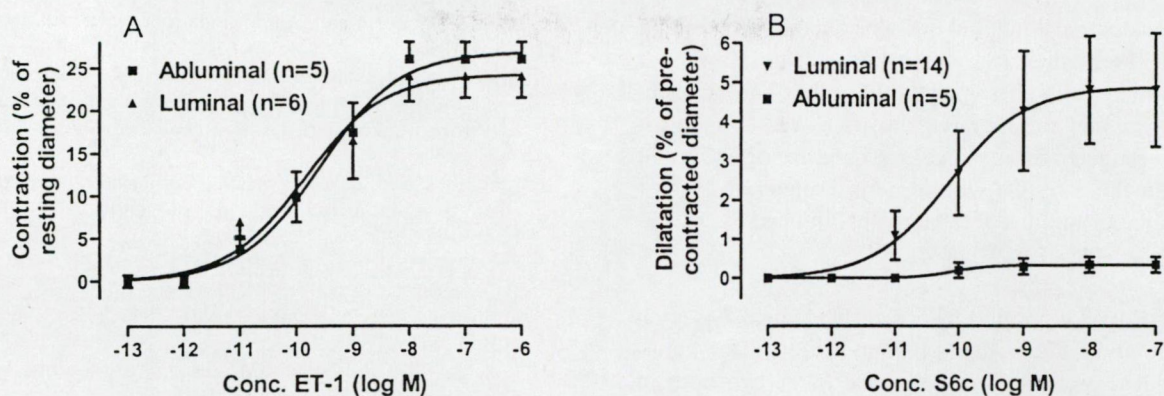
Fig. 1. (A) Vasocontraction caused by increasing concentrations (10^{-12}–10^{-7} M) of ET-1 administered luminally and abluminally on rat MCAs. (B) Vasomotor effect of increasing concentrations (10^{-12}–10^{-7} M) of S6c administered luminally and abluminally on rat MCAs precontracted with U46619 (10^{-7} M) and with selective blockage of the ET_A receptor using FR139317 ($10^{-5.5}$ M), the two latter administered abluminally. Data are given as mean ± SEM.

the rat in terms of responsiveness to ET agonists. The cerebral arteries posses the well-known blood–brain barrier (BBB) which in effect comes about through endothelial tight junctions [5,14]. In the present study, only hydrophilic substances were used thus strengthening the two-compartment approach. The effect of ET-1 was independent of administration route. Since ET-1 cannot cross the BBB passively, the clear inference must be, that contractile ET receptors are probably present both on the endothelium as well as on the SMC. In view of the dilative response of the precontracted and ET_A blocked artery to S6c, the contractile effect of luminally administered ET-1 must be through an ET_A receptor. How the endothelium mediates the contractile response is at present unknown.

The dilative effect of S6c was not very strong (E_{max} ~6%) when comparing to application of ATP (E_{max} ~30%), used for testing the presence of a functional endothelium. ATP was capable of dilating the vessel at resting tone, whereas precontraction and blockage of the ET_A receptor (despite the ET_B receptor selective nature of S6c) was required to unmask the effect of S6c.

At present three major signalling substances of endothelial origin have been defined, NO, prostaglandins (the most notable being prostacyclin (PGI_2)) and endothelium derived hyperpolarizing factor (EDHF) [11]. Through tests with an inhibitor of NO synthase, an inhibitor of cycloxygenase and a potassium channel inhibitor (the hypothesized site of action of EDHF), the results revealed that NO seems to be the principal mediator of the ET_B receptor stimulated vasodilatation, though minor a role may also be ascribed to EDHF. Cyclooxygenase products do not, however, seem to be of importance in the present set-up.

In comparison with previously published characterizations of the ET receptors in cerebral arteries, the arteriographical method of investigation has clearly demonstrated the presence of a contractile ET_A receptor on endothelial cells not hitherto appreciated. In addition, it has allowed the characterization of the ET_B receptor mediated vasodilatative response. Further advantages of the method includes both maintained intraluminal pressure as well as the possibility of maintaining shear stress through perfusion of the artery, possibilities not open to the classical in
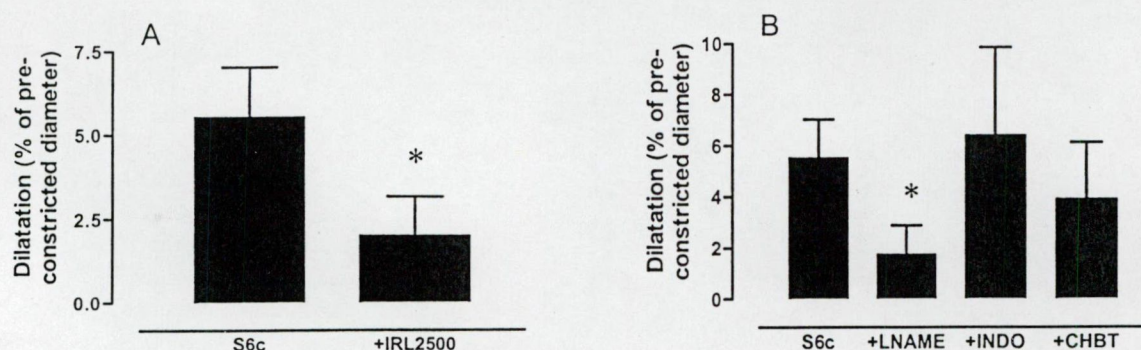

Fig. 2. (A) Vasodilative effect of a single dose of luminally administered S6c (10^{-8} M) alone on preconstricted (U46619; 10^{-7} M) rat MCAs in the presence of the ET_A receptor antagonist (FR139317; $10^{-5.5}$ M), and in the presence of ET_B receptor antagonist (IRL2500; $10^{-6.5}$ M). (B) Vasodilatation induced by luminally administered single dose (10^{-8} M) S6c in the absence ($n = 14$) and presence of L-NAME (10^{-5} M) ($n = 6$), indomethacine (10^{-5} M) ($n = 9$) and charybotoxin (10^{-8} M) ($n = 5$) on preconstricted (U46619; 10^{-7} M) rat MCAs. Values are given as mean ± SEM. *Denotes a statistically significant difference, $P > 0.05$.

vitro pharmacological method described above. Combining the two methods, the classical in vitro set-up allows for quantity (many vessel segments may be run simultaneously; with the arteriograph, only one vessel segment may be investigated at any one time) and the arteriograph complement these results with a more discrete analysis of the relative contributions of the endothelium and the vascular smooth muscle cells.

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III

ORIGINAL ARTICLE

Expression of ET_A and ET_B receptor mRNA in human cerebral arteries

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Abstract
The vascular effects of endothelins (ET) are in mammals mediated via two receptor subtypes, endothelin A (ET_A, mainly constrictive) and endothelin B (ET_B, mainly dilating) receptors. We have examined the presence of ET_A and ET_B receptor mRNA using the reverse transcription polymerase chain reaction (RT-PCR) in both normal human cerebral arteries and cerebral arteries from patients with cerebrovascular disease. Two vessel preparations were studied: macroscopic arteries and microvessels, the latter obtained through a sensitive separation method. In endothelial cells both ET_A and ET_B receptor mRNA was detected. In almost all samples from normal cerebral arteries only ET_A receptor mRNA was detected, whereas in vessel samples from patients with cerebrovascular disease as well as cerebral neoplasms, additional ET_B receptor mRNA was detected significantly more frequently. The pathophysiological significance of this difference is at present speculative, but does point to a vascular involvement of this receptor in cerebrovascular disease.

Key words: *Cerebral arteries, endothelin receptors, endothelium, human, mRNA, vascular smooth muscle cells.*

Introduction

Since the discovery of endothelin some 13 years ago this peptide has attracted profound interest in the field of cardiovascular disease, e.g. hypertension, cardiac failure and stroke.[1] Currently antagonists working at the receptors (either selectively or combined) have shown effect in various animal models of disease, and are currently considered for clinical testing or, indeed, undergoing preclinical tests. Unlike other receptor systems, where often numerous classes and subclasses of a given receptor have been identified and characterized (thus also allowing specific pharmacological targeting), only two endothelin receptors have so far been identified in mammals, the ET_A and the ET_B receptors. For this reason, any attempt at affecting the endothelin system is likely to have widespread effects.

The ET receptors are members of the rhodopsin-like seven transmembrane receptor family that couple to second messenger systems via the G_q-protein. Typically, the ET_A receptor, located on the vascular smooth muscle cells, mediates strong and long-lasting contractile effects,[2] whereas the ET_B receptor, located on the endothelium, induces vasodilatation via the

release of nitric oxide and prostaglandins.[3,4] ET_B receptors, however, have also been described as being present on the vascular smooth muscle cells of veins inducing contraction.[5] In various parts of the human circulation, mRNA for both receptors has been found expressed in the vascular smooth muscle cells.[6,7]

The present study was performed to provide information as to the presence of ET_A and ET_B receptor mRNA in conductance arteries, as well as in small arterioles and capillaries in the human cerebral circulation. In the case of conductance arteries, the vascular smooth muscle cells and the endothelial cells were studied separately.

Materials and methods

With the approval of the local ethical committees (Szeged, Hungary and Lund, Sweden), cortical arteries and brain tissue samples were obtained from patients undergoing neurosurgical operation for brain tumours or angiomas ($n = 6$). In addition, cortical arteries, as well as larger arteries from the circle of Willis were obtained at autopsy done within 12 h from the time of death of patients who had died

from either cerebral (n = 8) or non-cerebral causes (n = 8). The mean age of the population was 48.8 years (range 9–88) and the ratio of male to female was 1:2.

Large vessel preparation

The arteries obtained were carefully dissected free of connective tissue leaving the vessel with intact intima, media and adventitia. Arteries with macroscopical signs of atherosclerosis were avoided. To separate the endothelium, the vessels were cut open longitudinally and the internal surface artery was scraped carefully with a scalpel. After preparation the material was snap frozen in liquid nitrogen and subsequently stored at -80°C.

Micro-vessel preparation

Cortical tissue samples without pial membranes were placed in ice-cold phosphate-buffered saline (PBS) solution (NaCl 145 mM, $Na_2PO_4.H_2O$ 10 mM, pH 7.4). One gram of tissue was weighed and gently homogenized in ice-cold PBS through slow up-and-down strokes in a glass-mortar. The homogenate was spun down at 1.500 g for 10 min, and the pellet subsequently washed and resuspended in PBS-buffer followed by another spin down. The tissue was then resuspended in a 15% dextran (MW 40,000) solution and spun down at 3.500 g for 45 min. The resultant pellet, containing the micro-vessel fraction, was filtered through a nylon-mesh (pore size 150 μm) and, after wash in a forceful flush of cold PBS, filtered again, though this time using a nylon mesh with a pore diameter of 50 μm, and subsequently collected in a PBS-filled test tube and spun down at 10,000 g for 10 min. The content of micro-vessels was verified light microscopically.[8,9]

Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA extraction. Blood vessel preparations (either endothelium denuded vessel or endothelium proper) were homogenized in 1 ml TriZol® reagent and left for 5 min at room temperature. Chloroform (200 μl in 1000 μl TriZol®) was added and spun down at 12,000 g for 30 min (at +4°C). The water phase was transferred to new tubes. Isopropanol (500 μl/1000 μ TriZol®) was added and mixed. The solution was left for at least 1 h at -20°C, then centrifugated for 30 min at 15,000 g (at +4°C). The resultant pellet was washed in 300 μl 75% ethanol and spun down at 900 g for 5 min (at +4°C). The pellet (containing RNA) was then redissolved in 20 μl DEPC-treated water and, unless processed immediately, stored at -80°C. Total RNA content was quantified spectrophotometrically.

The reverse transcription of RNA to cDNA was carried out using the GeneAmp RNA PCR kit (Perkin Elmer, USA) on a DNA Thermal Cycler (Perkin Elmer). cDNA was synthesized from 1 μg total RNA in 20 μl reaction volume using random hexamers as primers. Transcription was completed in one cycle; 25°C for 10 min, then 42°C for 15 min and, finally, 99°C for 5 min and subsequently cooled to 5°C.

The PCR assay was performed using the following primers: ET_A receptor forward: '5 TGGCCTTTT-GATCACAATGACTTT-3' (bases 436–459), reverse: '5-TTTGATGTGGCATTGAGCATAC AGGTT-3' (bases 737–711); ET_B receptor forward: '5-ACTGGCCATTTGGAGCTGAGATGT-3' (bases 497–521), reverse: '5-CTGCATGCCACTT TTCTTTCTCAA-3' (bases 924–901). For each primer pair a 9-μl portion from the resultant cDNA was amplified by PCR in a final volume of 50 μl. The PCR was carried out with the following amplification profile: After an initial 5 min at 95°C, the reaction mixture was taken through 30 cycles, each cycle consisting of 1 min at 95°C, 1 min at 57°C and 30 s at 72°C; the final extension period was carried on for another 7 min. A blank (water) was included in all experiments.

Electrophoretic analysis of the PCR products was carried out by placing 15-μl aliquots on a 1.5% agarose-gel stained with ethidiumbromide (1 μg/ml) in 0.5 × TBE buffer (1× TBE buffer contains 89 mM TRIS-borate, 2 mM EDTA, pH 8.0) and photographed in a UV-box. The expected size of the PCR products were 302 basepairs for ET_A receptor mRNA and 428 basepairs for ET_B receptor mRNA. For further details including validation see Möller et al.[10] A competitive RT-PCR method for analysis of ET_A and ET_B receptor mRNA revealed that the ET_A mRNA signal was stable both during organ culture[11] and in disease preparations.[12]

Results

The results of RT-PCR performed on the macro- and micro-vessel preparations are presented in Table I. ET_A receptor mRNA was present in all but one preparation, the exception being one of the micro-vessel preparations (example of PCR result is displayed in Fig. 1). In six out of eight large-vessel preparations from patients suffering from cerebral disease mRNA for the ET_B receptor was detected, whereas it was only detected in one out of eight large-vessel preparations from patients with non-cerebral disease ($p < 0.05$ using Fisher's exact test). In the micro-vessel preparations ET_B receptor mRNA was detected in two patients. The result of RT-PCR performed on endothelial cells is shown in Fig. 2, mRNA for both the ET_A and the ET_B receptor was detected.

TABLE I. Distribution of ET_A and ET_B receptor mRNA in the cerebral micro- and macro-vessels obtained from different patients.

Patient	Diagnosis	ET_A	ET_B
Micro-vessels			
1	Glioblastoma multiforme	+	+
2	Internal carotid aneurysm†	+	0
3	Pontocerebellar tumour	+	0
4	Arterivenous malformation	+	0
5	Aneurysm of the anterior communicating artery†	0	(+)
Macro-vessels			
1	Glioblastoma multiforme	+	+
2	Internal carotid aneurysm†	+	+
3	Pontocerebellar tumour	+	+
4	Arterivenous malformation	+	(+)
5	Aneurysm of the anterior communicating artery†	+	0
6	Cavernoma	+	+
7	Aneurysm of the anterior communicating artery†	+	+
8	Stroke†	+	0
9	Bronchiolitis following femoral fracture†	+	0
10	Ischaemic heart failure†	+	0
11	Ischaemic heart failure†	+	0
12	Ischaemic heart failure†	+	(+)
13	Ischaemic heart failure†	+	0
14	Ischaemic heart failure†	+	0

+ indicates clear presence of electrophoretic band, (+) indicates a weak band, 0 no observable reaction. †Indicates that samples were obtained postmortem.
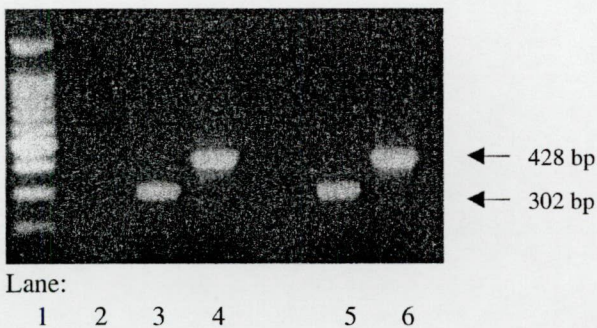

Lane:
1   2   3   4   5   6

FIG. 1. Example (patient no. 1) of agarose gel showing the RT-PCR products of both the micro- (lanes 3 and 4) and macrovessel (lanes 5 and 6) preparations. Lane 1 is a 100-base pair ladder. The products for the endothelin ET_A (302 base pairs) and ET_B (428 base pairs) were observed in both preparations. Negative control was RNA free water (lane 2).
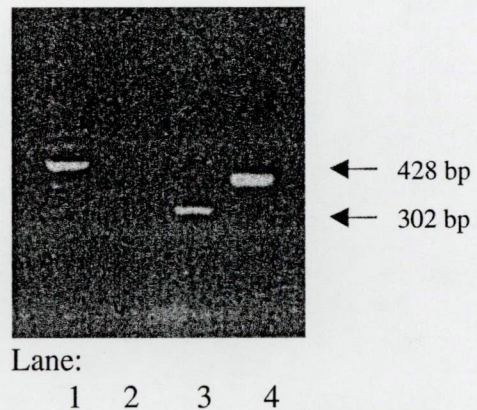

Lane:
1   2   3   4

FIG. 2. Agarose gel showing the RT-PCR products of the endothelial preparation from the macrovessel preparations. The molecular weight marker (lane 1) is a 100-base pair ladder. Negative control with RNA free water showed no reaction (lane 2). The mRNA for ET_A (lane 3) and ET_B (lane 4) receptors are shown (302 base pairs and 428 base pairs, respectively).

Discussion

The results confirm a predominant presence of ET_A receptor mRNA in larger cerebral arteries as has also been observed in peripheral arteries.[10] ET_B receptor mRNA was detected in approximately 50% of the larger arteries with a clear predominance of the arteries obtained from patients with cerebral disease. In cerebral micro-vessels ET_A receptor mRNA also dominates, although here also ET_B receptor mRNA was found in two out of five preparations. All micro-vessel preparations were obtained from patients with cerebral disease. In endothelial preparations both ET_A and ET_B receptor mRNA was detected.

In general the presence of mRNA for a given receptor may be taken as evidence of synthesis of that receptor, although no conclusion may be drawn as to the functional status of the receptor. The receptor may be fully functional and inserted into the cell membrane, but it could also be present in a modified non-functional form.[13–15]

All the patients included in this study received medication primarily in the form of cardiovascular drugs, anti-epileptic drugs and/or steroid drugs, the latter to alleviate tumour-induced intracranial hypertension. It is, of course, a possibility that the appearance of the mRNA for the ET_B receptor was caused by drugs administered. Indeed, that steroids are able to alter the endothelin system in the