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Characterization of $CGRP_1$ receptors in the guinea pig basilar artery

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Abstract

The purpose of the present study was to characterise receptors mediating calcitonin gene-related peptide (CGRP)-induced relaxation of guinea pig basilar artery. This was done by investigating vasomotor responses in vitro and performing autoradiographic binding studies. We also intended to study the importance of an intact endothelium. Agonist studies showed that peptides of the CGRP family induced relaxation of the guinea pig basilar artery with the following order of potency: human β-CGRP = human α-CGRP ≫ adrenomedullin = [acetamidomethyl-$Cys^{2,7}$]α-human CGRP ([$Cys(ACM)^{2,7}$]CGRP) = amylin. These data are in concord with those of the autoradiographic binding studies that showed displacement of [^{125}I] human α-CGRP binding with the following order of potency: human α-CGRP = human β-CGRP ≫ adrenomedullin = human α-CGRP-(8–37) ≫ [$Cys(ACM)^{2,7}$]CGRP. In blockade experiments, the relaxant responses to human α- and human β-CGRP were competitively blocked by the $CGRP_1$ receptor antagonist human α-CGRP-(8–37), while those of adrenomedullin and amylin were blocked non-competitively. In order to examine whether amylin induced relaxation via amylin or CGRP receptors, we studied the antagonistic effect of amylin-(8–37) on the weak relaxant response to amylin and found that it was not blocked by amylin-(8–37). These findings, together with the finding that the $CGRP_2$ receptor agonist [$Cys(ACM)^{2,7}$]CGRP only induced a weak relaxation in the highest concentrations examined, suggest that the CGRP family of peptides mediate relaxation by $CGRP_1$-type receptors. Removal of the endothelium, the addition of N^G-nitro-L-arginine methyl ester (L-NAME), methylene blue or indomethacin did not affect the concentration–response curves of the CGRP analogues, neither in the presence nor in the absence of human CGRP-(8–37). The study shows the presence of a relaxant $CGRP_1$ receptor on the smooth muscle cells of guinea pig basilar artery. Various endothelial factors did not influence relaxant responses.

Keywords: Adrenomedullin; Amylin; Autoradiography; Endothelium; Intracranial blood vessel; Receptor pharmacology; Vasodilatation; Vasomotor response

1. Introduction

Calcitonin gene-related peptide (CGRP) is present in the sensory perivascular nerve fibres innervating the cerebral arteries of animals and humans. (Uddman et al., 1985; Edvinsson et al., 1989; Jansen et al., 1990; Suzuki et al., 1989). Human and rat CGRP exists in two forms, denoted as α- and β-CGRP, which are encoded by two different genes (Amara et al., 1982). The authentic α- and β-CGRP are both complete agonists for all CGRP receptor subtypes (Wimalawansa, 1996). Based on the effects of the CGRP receptor antagonist human α-CGRP-(8–37), it has been proposed that CGRP receptors are divided into $CGRP_1$ and $CGRP_2$ subtypes. In some biological systems, such as guinea pig atrium and ileum, human α-CGRP-(8–37) acts as a competitive antagonist and the receptor is classified as a $CGRP_1$ receptor. However, this fragment has a weaker antagonistic effect on the electrically stimulated rat vas deferens and CGRP-mediated hyperthermia (Dennis et al., 1990). The synthetic analogue [acetamidomethyl-$Cys^{2,7}$]α-human CGRP ([$Cys(ACM)^{2,7}$]CGRP) is, however, equipotent to authentic CGRP in this system, and a potent agonist in the rat vas deferens (Dennis et al., 1989). The receptor mediating this effect has been pharmacologically classified as a $CGRP_2$ receptor (Chakder and Rattan, 1990; Mimeault et al., 1992; Chiba et al., 1989). Other members of the calcitonin/CGRP family are adrenomedullin and amylin (Poyner, 1995). Adrenomedullin is a 52-amino acid peptide originally isolated from human phaeochromocytoma

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(Kitamura et al., 1993). Immunoreactivity for adrenomedullin has been found in cultured vascular endothelial cells and in cultured vascular smooth muscle cells (Sugo et al., 1994). The cardiovascular and cerebrovascular activities of adrenomedullin are inhibited by the CGRP receptor antagonist human α-CGRP-(8–37), which suggests that adrenomedullin in large part acts via $CGRP_1$ receptors in cardiovascular tissues (Yoshimoto et al., 1998a; Lang et al., 1997). However, it is also possible that it acts via its own receptor.

Amylin is a 37-amino acid peptide first isolated from an insulinoma (Westermark et al., 1987). Amylin has a 46% sequence homology with human α-CGRP and has previously been shown to be a dilator of some arteries (Brain et al., 1990; Baskaya et al., 1995). In addition, rat amylin was recently shown to act as a non-competitive antagonist at $CGRP_1$ receptors in rat coronary small arteries (Sheykhzade and Nyborg, 2000).

Receptors showing pharmacological profiles similar to $CGRP_1$ receptors have been cloned from rats and pigs (Njuki et al., 1993; Elshourbagy et al., 1998). When the first cloned structures appeared, they were orphans and named calcitonin receptor-like receptors due to homology to the calcitonin receptor (Fluhmann et al., 1997; Njuki et al., 1993). The pharmacology of these peptides is complicated, but recent data suggest that the CGRP and adrenomedullin receptors may be produced by the same gene that is equal to calcitonin receptor-like receptor (Aiyar et al., 1996). Specificity is determined by accessory proteins belonging to a family of receptor activity modifying proteins (RAMPs). RAMP1 coexpression with calcitonin receptor-like receptor leads to a CGRP receptor and coexpression with RAMP2 leads to an adrenomedullin receptor (McLatchie et al., 1998). In concord, an amylin receptor is formed when a calcitonin receptor is co-expressed with RAMP1 or RAMP3 (Muff et al., 1998, 1999).

CGRP relaxes vascular smooth muscle cells via endothelium-dependent and endothelium-independent transduction pathways, dependent on the vascular region. In the endothelium-dependent pathway, CGRP elevates nitric oxide (NO) directly via an endothelial receptor without the involvement of adenylyl cyclase. NO then acts on smooth muscle cells by activating guanylyl cyclase and cyclic GMP production, leading to smooth muscle relaxation (Wimalawansa, 1996). In the endothelium-independent pathway, CGRP bypasses the endothelium and directly binds to CGRP receptors located on smooth muscle cells. Subsequently adenylyl cyclase is activated, which in turn produces cyclic AMP, leading to vascular relaxation (Sams et al., 1999; Jansen-Olesen et al., 1996; Edvinsson et al., 1985). Both pathways can be activated by adenylyl cyclase and the production of cyclic AMP (Gray and Marshall, 1992a; Marshall, 1992). The increased levels of cAMP may in some vascular regions activate the enzyme nitric oxide sythase (NOS), leading to increased levels of NO (Gray and Marshall, 1992a).

The present study was carried out to examine in detail the pharmacology of CGRP in the guinea pig cerebral circulation. Previous studies in the same tissue have suggested that the α- and β-forms of CGRP may act via separate subsets of receptors (Jansen, 1992). With these agents, together with [$Cys(ACM)^{2,7}$]CGRP, adrenomedullin and amylin, new tools are available for the in depth investigation of receptor subsets. In addition, we studied the long discussed question of endothelial involvement. Furthermore, for the first time we show autoradiographic binding of human α-CGRP to the guinea pig basilar artery wall and competition for these binding sites by other CGRP analogues.

2. Materials and methods

2.1. Vasomotor responses

Young male guinea pigs (250–350 g, Hvidesten, Statens Serum Institut, Denmark) of either sex were exanguinated during barbiturate anaesthesia (Pentobarbital, 500 mg/kg i.p.). The brains were removed and the basilar arteries were carefully dissected out under an operation microscope. Each vessel was cut into 2-mm-long circular segments and placed in an ice-cold buffer solution gassed with 5% CO_2 in O_2. The composition of the buffer was (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 1.5, KH_2PO_4 1.18, $MgSO_4$ 1.17, EDTA 0.027 and glucose 5.5, pH 7.4. Some experiments were performed in the absence of endothelium. These vessel segments were perfused with a buffer solution containing 0.1% Triton X-100 for 15 s (Hamel et al., 1987). Verification of the absence of endothelium was always checked by the lack of a dilator response to acetylcholine (Furchgott and Zawadzki, 1980).

In order to determine vessel tension, each segment was mounted on two parallel pins of 150 μm in diameter in a myograph (Model 610M, Danish Myo Technology, Denmark). The buffer solution was continuously aerated with 5% CO_2 in O_2 to maintain a stable pH of 7.4. The artery segments were allowed to equilibrate for approximately 30 min. The vessels were stretched to the internal circumference (L_1, equal to 90% of the circumference, L_{100}) the vessel would have if relaxed and exposed to a passive transmural pressure of 100 mm Hg (13.3 kPa). This was in order to achieve maximal active force development (Nyborg et al., 1987). Following a second 30-min equilibration period, the vessels were constricted twice by 123.7 mM KCl in a modified buffer solution in which NaCl was substituted for KCl on an equimolar basis. The contraction amounted to 5.28 ± 0.52 mN ($n = 29$) in arteries with endothelium and 2.75 ± 0.63 mN ($n = 25$) in arteries without endothelium. After another 30-min equilibration period, the vessel segments were pre-contracted with 3×10^{-6} M prostaglandin $F_{2\alpha}$. Following approximately 15 min of pre-contraction, when a stable level of tension was

Table 1
I_{max} and pIC_{50} values obtained from relaxant responses to CGRP receptor agonists in the presence and absence of endothelium

Agonist	With endothelium			Without endothelium		
	I_{max}	pIC_{50}	n	I_{max}	pIC_{50}	n
Human α-CGRP	96 ± 4	8.82 ± 0.24	8	$86 \pm 11^{n.s.}$	$8.63 \pm 0.15^{n.s}$	11
Human β-CGRP	$96 \pm 2^{n.s.}$	$9.08 \pm 0.14^{n.s.}$	8	$93 \pm 7^{n.s.}$	$9.25 \pm 0.37^{n.s.}$	8
Adrenomedullin	63 ± 9^{a}	7.36 ± 0.11^{b}	13	$84 \pm 9^{n.s.}$	$7.38 \pm 0.05^{n.s.}$	4
Amylin	36 ± 11^{a}	7.26 ± 0.12^{b}	12	$30 \pm 6^{n.s.}$	$7.10 \pm 0.17^{n.s.}$	5
Cys[$ACM^{2,7}$]CGRP	11 ± 2^{c}	7.36 ± 0.16^{b}	8	n.d.	n.d.	–

Values are given as means ± S.E.M. n = number of animals. Statistical analysis (Mann Whitney U-test) was performed comparing agonist responses in tissue with endothelium to those found in tissue without endothelium. The P values obtained are shown in the column of data without endothelium. Comparison was also made of responses found for the different agonists to the response obtained with human α-CGRP. P values are shown in the column of data with endothelium: $^{a}P < 0.05$; $^{b}P < 0.005$; $^{c}P < 0.001$.

reached, each vessel segment was challenged with 10^{-6} M acetylcholine. In order to study the relaxant effects of agonists, the cerebral arteries were pre-contracted with 3×10^{-6} M prostaglandin $F_{2\alpha}$. This resulted in a stable tension of 3.81 ± 0.44 mN ($n = 29$) in arteries with endothelium and 3.05 ± 0.67 mN ($n = 25$) in arteries without endothelium, to which the agonist was added in cumulative concentrations. The tension lasted for at least 20–30 min without a significant fall in tone. In blockade experiments, the antagonists were added to the tissue bath 15 min before the addition of preconstrictor and subsequently agonist in increasing concentrations. Out of eight tissue segments two served as controls (i.e. without antagonist), and the others were treated with antagonist in different concentrations.

All concentration–response curves were plotted graphically. I_{max} (maximum relaxant effect obtained with an agonist), pIC_{50} (negative logarithm of the concentration of agonist that elicited half-maximum relaxation) were calculated arithmetically from each individual concentration–response curve. Values are given as means ± S.E.M. Number of experiments = n, one or two segments from each guinea pig. The antagonist potency (pA_2) was calculated as described by (Arunlakshana and Schild, 1997): $pA_2 = \log_{10}$ (conc. range − 1)/antagonist conc. The non-parametric, Mann Whitney U-test was used to determine statistical significance between two groups of data. Statistical significance was assumed when $P < 0.05$.

2.2. Autoradiography

One male guinea pig was exanguinated during barbiturate anaesthesia. The brain was removed and the basilar artery was carefully dissected out under an operation microscope. The artery was carefully frozen in Tissue Tek® on dry ice. The tissue was stored at −80°C until sectioned in a Leitz Cryostat (1720 Digital) at −20°C to 12 μm. The sections were placed on SuperFrost plus slides, dried at 0–4°C and stored at −80°C.

Slides with tissue sections were pre-incubated for 10 min at 0–4°C in 50 mM Tris (pH 7.4) containing 1% bovine serum albumin, 2 mM EDTA and 5 mM $MgCl_2$ and dried under a stream of N_2. The slides were then incubated at room temperature for 60 min in 500 μl 50 mM Tris (pH 7.4) containing 1% bovine serum albumin, 2 mM EDTA, 5 mM $MgCl_2$ and 100 pM [^{125}I] human α-CGRP with or without competing ligand. After incubation, slides were washed twice in ice-cold buffer for 5 min each. Slides were then dipped in ice-cold distilled water and dried using a stream of cold, N_2. Thereafter the slides were apposed to [^{3}H] Hyperfilm (Amersham, Denmark) in X-ray cassettes along with commercial plastic ^{14}C-autoradiographic standards (Amersham, Denmark). The exposure time of the films varied between 12 and 16 days, depending on the activity of the labelled CGRP. The latent images from the labelled tissue sections were examined using video-based image analysis (Imaging Research MCID-MI). Quantification was accomplished by comparing the density of the grey staining produced by the radiolabelled sections on the film to that produced by the commercial plastic standards. The density in sections in which no competing ligand was added was regarded as total binding (100 pM [^{125}I]human α-CGRP) and was set to 100%. Non-specific binding was obtained by incubation with 10^{-5} M hα-CGRP. In order to calculate pEC_{50} values (negative logarithm of concentration of unlabelled ligand that elicited

Table 2
Relaxant response of guinea pig basilar artery to human α-CGRP and human β-CGRP in the absence and presence of endothelium and during blockade of NOS, cGMP and prostaglandin I_2

	Human α-CGRP			Human β-CGRP		
	I_{max}	pIC_{50}	n	I_{max}	pIC_{50}	n
Control	87 ± 4	8.52 ± 0.06	9	89 ± 7	8.62 ± 0.10	7
No endothelium	95 ± 2	8.63 ± 0.15	11	92 ± 5	8.39 ± 0.05	7
L-NAME	88 ± 2	8.43 ± 0.09	6	89 ± 3	8.52 ± 0.07	11
Methylene blue	91 ± 3	8.75 ± 0.11	7	92 ± 5	8.55 ± 0.07	7
Indomethacin	97 ± 1	8.42 ± 0.08	6	94 ± 2	8.57 ± 0.09	7

Values given represent means ± S.E.M., n = number of animals. Statistical analysis (Mann Whitney U-test) showed no significant differences between control experiments and different types of blockade. There were also no differences between the responses to human α- and human β-CGRP with or without the different blockers.

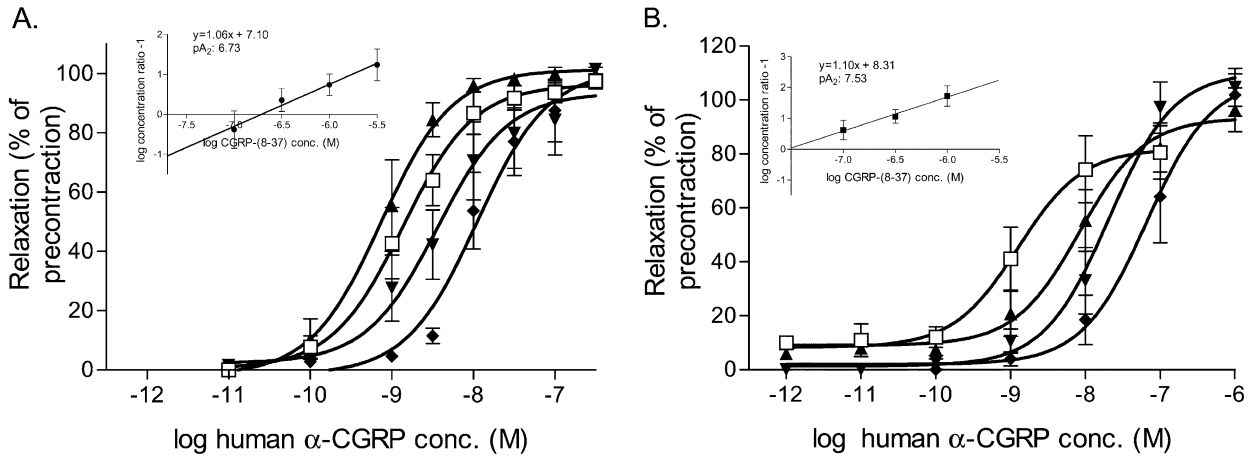

Fig. 1. Relaxation induced by cumulative concentrations of human α-CGRP in guinea pig basilar artery (A) with endothelium, (B) without endothelium. The experiments were performed without (□) and with human α-CGRP-(8–37) in different concentrations; (▲) 10^{-7} M, (▼) 3×10^{-7} M, (◆) 10^{-6} M and in some experiments (•) 3×10^{-6} M. The relaxation of each segment tested was calculated as a percentage of the pre-contraction induced by 3×10^{-6} M prostaglandin $F_{2\alpha}$, and each point represents the mean with the S.E.M. shown by vertical bars, $n = 6–13$. (Inset) Schild plot analysis yielding pA_2 values of: (A) 6.73 (slope 1.06), (B) 7.53 (slope 1.10).

half maximal displacement of [^{125}I]human α-CGRP binding), all measurements in the presence of competing ligand were calculated as % of total binding. The mean values ± S.E.M. for each concentration of competing ligand, in four identical experiments, were calculated and plotted in a graph.

2.3. Drugs

Adrenomedullin, human and rat amylin, human amylin$_{8-37}$ human α-CGRP, human β-CGRP (Bachem, Switzerland) human α-CGRP$_{8-37}$ (Schæfer-N, Denmark). [Cys(ACM)^{2,7}]-CGRP (Peninsula, USA), indomethacin, N^G-Nitro-L-arginine methyl ester (L-NAME), methylene blue (Sigma, USA) and prostaglandin $F_{2\alpha}$ (Dinoprost®, Upjohn, Sweden) were prepared by dissolving the drugs in distilled water. Indomethacin was dissolved in 70% ethanol. All drugs were further diluted in buffer solution just before the experiment. The concentrations are expressed as the final molar concentration in the tissue bath.

3. Results

3.1. Vasomotor responses

3.1.1. Agonist experiments

Human α-CGRP, human β-CGRP, adrenomedullin, amylin and [Cys(ACM^{2,7})]CGRP induced concentration-dependent relaxations of circular segments of the basilar

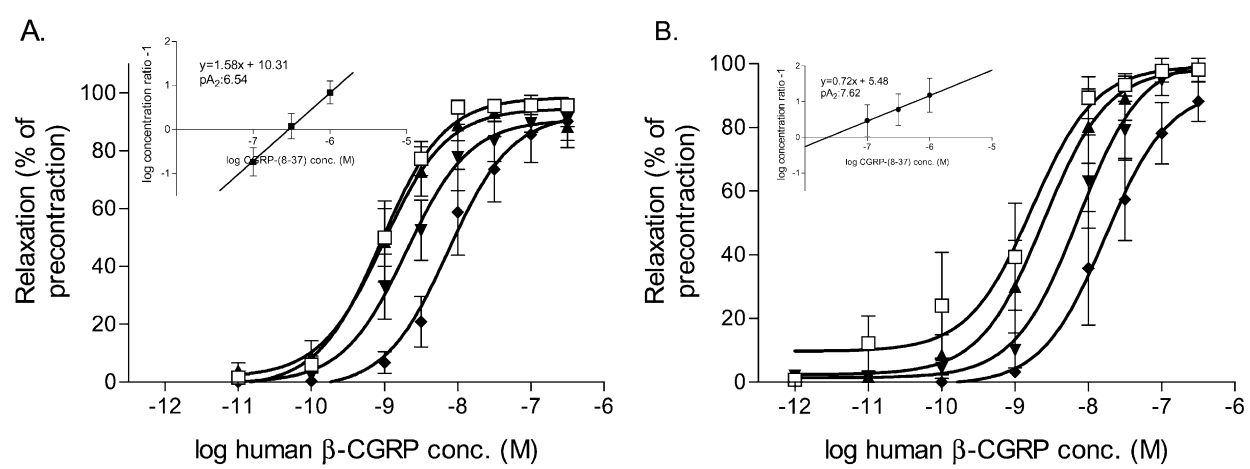

Fig. 2. Relaxation induced by cumulative concentrations of human β-CGRP in guinea pig basilar artery (A) with endothelium, (B) without endothelium. The experiments were performed without (□) and with human α-CGRP-(8–37) in different concentrations; (▲) 10^{-7} M, (▼) 3×10^{-7} M, and (◆) 10^{-6} M. The relaxation of each segment tested was calculated as a percentage of pre-contraction induced by 3×10^{-6} M prostaglandin $F_{2\alpha}$ and each point represents the mean with the S.E.M. shown by vertical bars, $n = 6–12$. (Inset) Schild plot analysis yielding pA_2 values of: (A) 6.54 (slope 1.58), (B) 7.62 (slope 0.72).

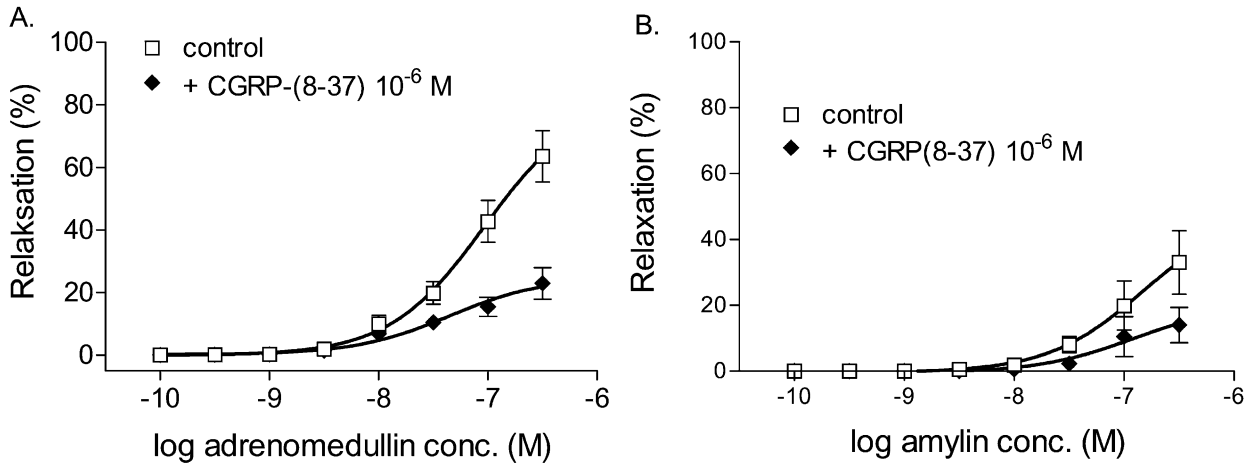

Fig. 3. Relaxation induced by cumulative concentrations of (A) adrenomedullin and (B) amylin. The experiments were performed in the absence (□) or in the presence (◆) of 10^{-6} M human α-CGRP-(8–37). Each point represents the means with the S.E.M. shown by vertical bars, $n = 6$–13.

artery pre-contracted by prostaglandin $F_{2\alpha}$ (Table 1). The mean pIC_{50} values were between 9.08 and 7.26 with the following order of potency: human β-CGRP = human α-CGRP ≫ adrenomedullin = [Cys(ACM^{2,7})]CGRP = amylin. The maximum relaxation obtained by the used concentrations was 96% for human α- and human β-CGRP, 56% for adrenomedullin, 36% for amylin and 11% for [Cys(ACM)^{2,7}]CGRP (Table 1). As [Cys(ACM^{2,7})]CGRP induced no or only a weak relaxant response, its effect was not examined in endothelium-denuded arteries or in the presence of antagonist.

Removal of the endothelium significantly changed neither the maximum contraction induced by prostaglandin $F_{2\alpha}$ nor the pIC_{50} values for any of the CGRP receptor agonists examined (Table 1).

In arteries with an intact endothelium, pre-incubation with L-NAME (3×10^{-5} M), methylene blue (10^{-5} M) or indomethacin (3×10^{-6} M) did not change the pIC_{50} values or the maximum relaxant responses to human α- or human β-CGRP (Table 2).

3.1.2. Antagonist experiments

Human α-CGRP-(8–37) (10^{-7}–10^{-6} M) significantly blocked the relaxations induced by human α- or human β-CGRP (Figs. 1A and 2A). Schild plots revealed pA_2 values of 6.73 and 6.54, respectively. Their respective slopes 1.06 and 1.58 were not significantly different from unity (Figs. 1A and 2A). The relaxations in response to adrenomedullin and amylin seemed to be non-competitively blocked by 10^{-6} M human α-CGRP-(8–37), with the maximum response being reduced while the pIC_{50} was unchanged (Fig. 3, Table 3). As the concentration–response curves obtained in the presence of antagonist were not parallel, pA_2 values could not be calculated.

Human amylin-(8–37) had no significant antagonistic effect on the relaxation induced by human amylin. The pIC_{50} value was 7.26 ± 0.12 ($n = 12$) in the absence of antagonist and 7.53 ± 0.29 ($n = 6$) in the presence of 10^{-6} M human amylin-(8–37). There was no significant difference between the observed maximum relaxation induced by amylin in the absence ($36 \pm 11\%$, $n = 12$) and the presence ($27 \pm 12\%$, $n = 6$) of human amylin-(8–37) (Fig. 4).

Table 3
Relaxant responses to CGRP receptor agonists in the presence and absence of 10^{-6} M human α-CGRP-(8–37)

Agonist	With endothelium			$+10^{-6}$ M hα-CGRP-(8–37)		
	I_{max}	pIC_{50}	n	I_{max}	pIC_{50}	n
Human α-CGRP	96 ± 4	8.82 ± 0.24	8	$98 \pm 1^{n.s.}$	7.87 ± 0.24^{b}	6
Human β-CGRP	96 ± 2	9.08 ± 0.14	8	$90 \pm 7^{n.s.}$	8.07 ± 0.22^{c}	7
Adrenomedullin	63 ± 9	7.36 ± 0.11	13	23 ± 5^{b}	$7.54 \pm 0.16^{n.s.}$	12
Amylin	36 ± 11	7.26 ± 0.12	12	10 ± 4^{a}	$7.23 \pm 0.15^{n.s.}$	7

Values are given as means ± S.E.M., n = number of animals. Statistical analysis: $^{a}P < 0.05$; $^{b}P < 0.005$; $^{c}P < 0.001$.

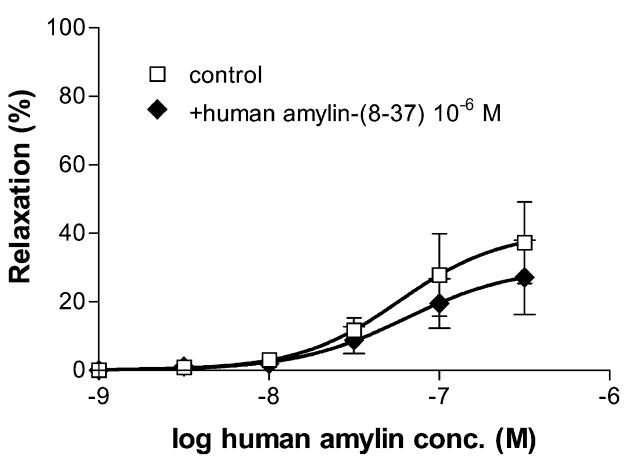

Fig. 4. Relaxation induced by cumulative concentrations of human amylin in guinea pig basilar artery. The experiments were performed without (□) and with (◆) 10^{-6} M human amylin-(8–37). The relaxation of each segment tested was calculated as a percentage of the pre-contraction induced by 3×10^{-6} M prostaglandin $F_{2\alpha}$ and each point represents the mean with the S.E.M. shown by vertical bars, $n = 6$–7.

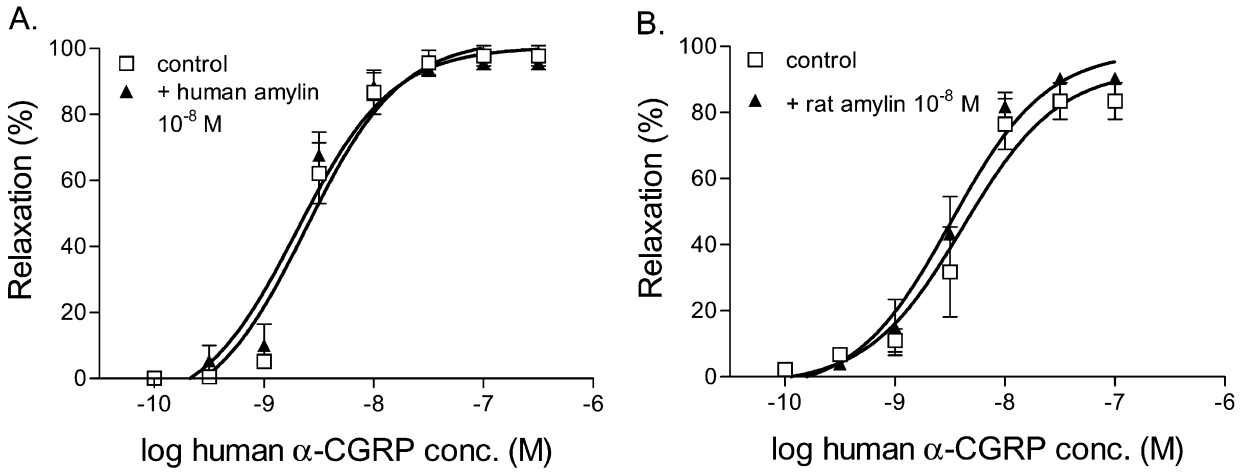

Fig. 5. Relaxation induced by cumulative concentrations of human α-CGRP in guinea pig basilar artery. The experiments were performed without (□) and with (♦), (A) 10^{-8} M human amylin or (B) 10^{-8} M rat amylin. The relaxation of each segment tested was calculated as a percentage of the pre-contraction induced by 3×10^{-6} M prostaglandin $F_{2\alpha}$ and each point represents the mean with the S.E.M. shown by vertical bars, $n = 6$–14.

Removal of the endothelium, did not significantly change the antagonistic effect of human α-CGRP-(8–37) on human α- or human β-CGRP-induced relaxations. In these arteries the Schild plot revealed pA_2 values of 7.53 and 7.82 with slopes of 1.10 and 0.72, respectively (Figs. 1B and 2B). Although human α-CGRP-(8–37) seemed to be approximately 10 times more potent in arteries without endothelium, the pA_2 values of 7.53 ± 0.41 ($n = 4$) for the human α-CGRP and 7.82 ± 0.53 ($n = 4$) for the human β-CGRP-induced relaxation in endothelium-denuded arteries were not significantly different from the pA_2 values of 6.54 ± 0.27 ($n = 4$, $P = 0.286$) and 6.87 ± 0.30 ($n = 5$, $P = 0.0571$) for the respective human α- and human β-CGRP-induced relaxation obtained in arteries with an intact endothelium.

L-NAME (3×10^{-5} M) or indomethacin (3×10^{-5} M) did not affect the antagonistic effect of human α-CGRP-(8–37) on relaxations induced by human α- or human β-CGRP. Schild plots constructed from data from blockade experiments performed in the presence of L-NAME showed pA_2 values of 7.13 ± 0.15 (slope = 0.81; $n = 4$; $P = 0.343$) and 6.71 ± 0.06 (slope = 1.06; $n = 5$; $P = 0.286$) for human α-CGRP and human β-CGRP, respectively. In the presence of indomethacin, the respective pA_2 values were 7.23 ± 0.18 (slope = 1.33; $n = 4$; $P = 0.686$) and 6.78 ± 0.06 (slope = 1.35; $n = 5$; $P = 0.400$).

A low concentration (10^{-8} M) of either human or rat amylin was given 15 min before a concentration–response curve was obtained by addition of human α-CGRP (10^{-10}–3×10^{-7} M).

There was no difference between the maximum relaxation induced by human α-CGRP in the absence ($98 \pm 3\%$, $n = 13$; $83 \pm 6\%$, $n = 4$) and the presence ($97 \pm 1\%$, $n = 13$; $91 \pm 2\%$, $n = 4$) of human or rat amylin, respectively. The pIC_{50} value was 8.51 ± 0.10 ($n = 13$) and 8.42 ± 0.12 ($n = 4$) in the absence and 8.70 ± 0.11 ($n = 13$) and 8.53 ± 0.15 ($n = 4$) in the presence of 10^{-8} M human and rat amylin, respectively (Fig. 5A and B).

3.2. Autoradiographic binding studies

[^{125}I]CGRP was used to generate autoradiograms in sections of the guinea pig basilar artery. Specific binding of [^{125}I]CGRP amounted to 75% of total binding to basilar artery sections. The respective CGRP analogues were used in the concentration range 10^{-12} to 10^{-5} M to study the displacement of [^{125}I]CGRP binding in the artery sections. In the presence of human α-CGRP or human β-CGRP (Fig. 6), specific [^{125}I]CGRP binding was totally displaced.

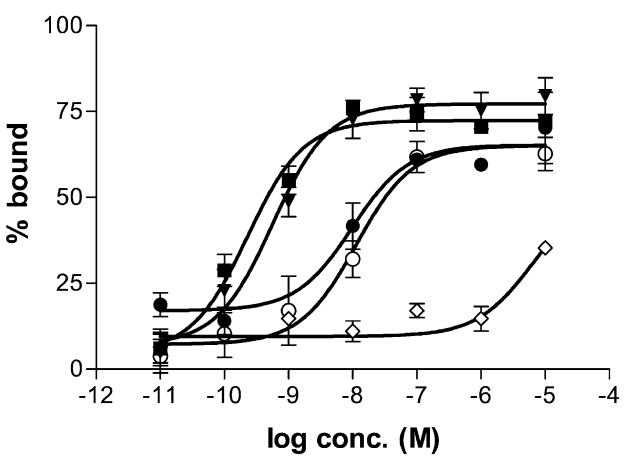

Fig. 6. Displacement of [^{125}I] human α-CGRP binding with unlabelled (■) human α-CGRP, (▼) human β-CGRP, (•) adrenomedullin (○) human α-CGRP-(8–37) or (◇) [Cys(ACM^{2,7})]CGRP in sections of guinea pig basilar artery. Each point represent means ± S.E.M., $n = 4$ individual segments from one animal.

The respective negative logarithm of the ligand concentration causing a half-maximal displacement (pIC_{50}) of [^{125}I]-CGRP binding was 9.64 ± 0.13 and 9.25 ± 0.17. In the presence of human α-CGRP-(8–37) or adrenomedullin, [^{125}I]CGRP binding amounted to 85% and 77% of the total specific binding with pIC_{50} values of 7.95 ± 0.20 and 8.01 ± 0.24, respectively (Fig. 6).

[Cys(ACM)^{2,7}]CGRP showed a much weaker displacement of [^{125}I]CGRP binding than the other ligands. Displacement only occurred at the highest concentration (10^{-5} M) given, amounting to 43% of the total specific binding.

4. Discussion

In the present study, our aim was to examine: (1) whether CGRP mediates relaxation via CGRP, adrenomedullin or amylin receptors; (2) whether the effect most likely is mediated by $CGRP_1$ or $CGRP_2$ receptors; and (3) whether endothelial factors are involved in CGRP-induced responses.

4.1. Does CGRP act via CGRP, adrenomedullin or amylin receptors in guinea pig basilar artery?

The CGRP receptor nomenclature (TiPS, 1999) and the reviews by Poyner (1995) and Wimalawansa (1996) suggest the existence of one CGRP, one adrenomedullin and one amylin receptor, with differing agonist potency. In the present study, the order of potency for vasomotor responses of guinea pig basilar artery was human αCGRP = human β-CGRP > adrenomedullin = [Cys(ACM)$_{2-7}$]-CGRP = amylin, and in binding studies it was human αCGRP = human β-CGRP > adrenomedullin = human CGRP-(8–37) ≫ [Cys(ACM)^{2,7}]CGRP. This order of potency is more consistent with that for the CGRP receptor (CGRP > adrenomedullin ≫ amylin ≫ salmon calcitonin) than for the adrenomedullin or amylin receptors (TiPS, 1999). If adrenomedullin receptors were present we would, according to the TiPS nomenclature, have expected a potency order of adrenomedullin ≫ CGRP = amylin > salmon calcitonin and if the receptors were of the amylin type, the order of potency would have been salmon calcitonin > amylin > CGRP > human calcitonin > adrenomedullin (TiPS, 1999). In the present study, we did not investigate the effects of human and salmon calcitonin. However, in a previous study performed in our laboratory with guinea pig basilar artery, we have shown that these agents only act as weak vasodilators with a maximum relaxation of about 12% of precontraction. The pIC_{50} values were 8.6 for human calcitonin and 8.1 for salmon calcitonin (Jansen, 1992). Thus, agonist experiments suggest the presence of CGRP receptors as compared to adrenomedullin or amylin receptors.

4.2. Does amylin act via CGRP or amylin receptors in guinea pig basilar artery?

In order to investigate whether the response to amylin was mediated via an amylin receptor, experiments were performed in the presence of the amylin receptor antagonist, amylin-(8–37) (Deems et al., 1991). We found that amylin-(8–37) had no significant antagonistic effect on human amylin-induced relaxations. This finding, taken together with the described poor affinity and amount of vasomotor reactivity to amylin as well as the agonist order of potency in the examined tissue, argues against a functional amylin receptor. Thus, it is most likely that amylin acts via the CGRP receptor. This finding is supported by other studies in which amylin and adrenomedullin have been described to act via $CGRP_1$ receptors (Westfall and Curfman-Falvey, 1995; Mori et al., 1997; Lang et al., 1997; Hall and Brain, 1999). In our hands, both peptides induced weaker relaxations than CGRP and they were not competitively blocked by human α-CGRP-(8–37). The CGRP receptor antagonist did, however, depress ($P < 0.05$) the maximum relaxation elicited by both peptides (Table 3). These findings could mean that adrenomedullin and amylin not only have a lower affinity for the $CGRP_1$ receptor but also a lower efficacy than human α- and human β-CGRP.

4.3. Does CGRP act via $CGRP_1$ or $CGRP_2$ receptors in guinea pig basilar artery?

The classification of CGRP receptors into $CGRP_1$ and $CGRP_2$ receptors came from work with the antagonist human α-CGRP-(8–37) and the linear agonist [Cys(ACM)^{2,7}]CGRP (Quirion et al., 1992). According to the classifications $CGRP_1$ receptors have high affinity for CGRP-(8–37) (pK_B about 7) and are not activated by [Cys(ACM)^{2,7}]CGRP, whereas $CGRP_2$ receptors have lower affinity for CGRP-(8–37) ($pK_B < 6$) but are activated by [Cys(ACM)^{2,7}]CGRP.

The pA_2 values for human α-CGRP-(8–37) in the guinea pig basilar artery preparations were 6.73 and 6.54 for human α- and human β-CGRP, respectively. These are consistent with previous studies performed with rat and porcine coronary artery preparations (pA_2 between 6.3 and 6.7 (Wisskirchen et al., 1999; Saha et al., 1998), rat intramural coronary artery (pA_2 6.9 (Sheykhzade and Nyborg, 1998a,b)) and guinea pig left atrium (pA_2 6.9 (Dennis et al., 1990)). This suggests that the receptor in the guinea pig basilar artery might be a $CGRP_1$ receptor.

In tissues such as the rat atria, which are considered to possess $CGRP_1$ receptors, [Cys(ACM)^{2,7}]CGRP is more than 100-fold less potent than CGRP, while in tissue such as the rat vas deferens, which is regarded to contain the $CGRP_2$ receptor subtype, [Cys(ACM)^{2,7}]CGRP is only 50-fold less potent than CGRP (Dennis et al., 1990). In the guinea pig basilar artery, no or only a weak relaxant

response to [Cys(ACM)^{2,7}]CGRP was observed in concentrations up to 10^{-6} M. This finding was supported by the autoradiographic binding data in which displacement of [^{125}I]CGRP binding was not seen until [Cys(ACM)^{2,7}]-CGRP was given in a concentration of 10^{-5} M. Thus, human α- and human β-CGRP were, in our preparation, 100 if not 1000 times more potent than [Cys(ACM)^{2,7}]-CGRP. Taken together, these findings clearly support the presence of a $CGRP_1$ receptor.

4.4. Does amylin act as an antagonist of CGRP-induced relaxations in guinea pig basilar artery?

Amylin was recently suggested to be a non-competitive antagonist of CGRP-induced relaxations in rat coronary arteries (Sheykhzade and Nyborg, 2000). In order to investigate whether amylin had a similar antagonistic effect in brain arteries, 10^{-8} M of either rat or human amylin was given before the addition of human α-CGRP in increasing concentrations. This concentration was previously shown to have a maximal antagonistic effect on rat α-CGRP-induced relaxation in rat coronary arteries (Sheykhzade and Nyborg, 2000). We did, however, not find any antagonistic effect of either human or rat amylin on human α-CGRP-induced relaxations in guinea pig basilar arteries. This difference might be explained by either species or tissue differences.

4.5. Role of endothelium

In rings of rat thoracic aorta, rat α-CGRP acts via an endothelium-dependent mechanism (Brain et al., 1985; Kubota et al., 1985; Grace et al., 1987; Gray and Marshall, 1992a,b), while in cerebral arteries from cat (Edvinsson et al., 1985) and humans (Jansen-Olesen et al., 1996) CGRP acts via an endothelium-independent mechanism. Binding to an endothelial receptor is generally thought to trigger the synthesis of NO from L-arginine. However, controversy exists on whether the receptor is coupled to NO production and subsequent activation of guanylyl cyclase or not. On one hand, Grace et al. (1987) found no parallel increase in cyclic GMP levels in response to rat α-CGRP in the rat thoracic aorta. This finding is supported by the study of Prieto et al. (1991), who observed in rat coronary arteries that methylene blue could not block relaxations induced by rat α-CGRP. On the other hand, Gray and Marshall (1992a) observed increased levels of cyclic GMP in response to rat α-CGRP, and this increase was blocked by the NOS inhibitor L-NMMA in the rat thoracic aorta. The situation in the guinea pig basilar artery seems more clear-cut than that seen in the rat aorta. Removal of the endothelium did not change the relaxation in response to either of the agonists tested (see Table 1). Furthermore, in the present study, pre-treatment with neither the NOS inhibitor L-NAME nor the guanylyl cyclase inhibitor methylene blue influenced the CGRP-induced relaxations. These findings are consistent with those of other studies, where a poor effect on cyclic GMP formation and an inability of the guanylyl cyclase inhibitor to block relaxations induced by rα-CGRP were found (Grace et al., 1987; Greenberg et al., 1987; Prieto, 1991; Baskaya et al., 1995; Yoshimoto et al., 1998). In our study, the agonist responses were blocked by CGRP-(8–37) in a competitive manner, resulting in Schild slopes that did not differ from unity. The dissociation constants for CGRP-(8–37) for human α- and human β-CGRP-induced relaxations were also similar. After removal of the endothelium or treatment with indomethacin, there seemed to be an increase in pA_2 values, but the findings were not statistically significant. The experiments in the present study were performed in eight parallel experiments in order to avoid tachyphylaxis. However, within different artery segments, large variations in the response to CGRP are generally observed (unpublished observations), which could explain the differences in pA_2 values found. This is supported by other unpublished results from our group, obtained with the same segment, in which responses to CGRP were separated by two potassium-induced contractions in order to avoid tachyphylaxis (Sheykhzade and Nyborg, 1998b). In this study, we found identical relaxant responses in arteries with or without endothelium, after application of a single concentration of human α- or human β-CGRP (10^{-8} M) in the absence or in the presence of CGRP-(8–37). It is therefore most likely that the differences found in this study were due to the large variation between different segments of the guinea pig basilar artery. Thus, there is no evidence for an endothelial CGRP receptor in guinea pig basilar arteries.

In conclusion, we have shown that CGRP, adrenomedullin and amylin most probably act via the $CGRP_1$ receptor. We have also demonstrated that the CGRP-mediated relaxation of the guinea pig basilar artery is not dependent on an intact endothelium. Finally, we have shown that rat and human amylin, in our preparation, do not act as antagonists of the relaxation induced by hα-CGRP in the guinea pig basilar artery.

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