British Journal of Pharmacology (2001) 133, 1405–1413

www.nature.com/bjp

CGRP receptors mediating CGRP-, adrenomedullin- and amylin-induced relaxation in porcine coronary arteries. Characterization with 'Compound 1' (WO98/11128), a non-peptide antagonist

*,[1,2]Philip Hasbak, [3]Anette Sams, [2]Søren Schifter, [4]Jenny Longmore & [1]Lars Edvinsson

[1]Department of Clinical Experimental Research, University Hospital of Glostrup, Glostrup, Denmark; [2]Department of Clinical Physiology and Nuclear Medicine, University Hospital of Glostrup, Glostrup, Denmark; [3]Department of Pharmacology, The Royal Danish School of Pharmacy, Copenhagen, Denmark and [4]Merck Sharp & Dohme, Neuroscience Research Centre, Eastwick Road, Harlow, Essex

1 Calcitonin gene-related peptide (CGRP), amylin and adrenomedullin (AM) belong to the same family of peptides. Accumulating evidence indicate that the calcitonin (CT) receptor, the CT receptor-like receptor (CRLR) and receptor-activity-modifying proteins (RAMPs) form the basis of all the receptors in this family of peptides.

2 Using reverse transcriptase–polymerase chain reaction the presence of mRNA sequences encoding the CRLR, RAMP1 and RAMP2 were demonstrated in porcine left anterior descending (LAD) coronary arteries, whereas porcine calcitonin (CT) receptor mRNA was not present. The partial porcine mRNA sequences shared 82–92% nucleotide identity with human sequences.

3 The human peptides αCGRP, βCGRP, AM and amylin induced relaxation with pEC_{50} values of 8.1, 8.1, 6.7 and 6.1 M respectively.

4 The antagonistic properties of a novel non-peptide CGRP antagonist 'Compound 1' (WO98/11128), $\beta CGRP_{8-37}$ and the proposed AM receptor antagonist AM_{22-52} were compared to the well-known $CGRP_1$ receptor antagonist $\alpha CGRP_{8-37}$.

5 The $\alpha CGRP_{8-37}$ and $\beta CGRP_{8-37}$ induced concentration-dependent (10^{-7}–10^{-5} M) rightward shift of both the αCGRP and βCGRP concentration-response curves. $\beta CGRP_{8-37}$ (10^{-6} M) had the same effect as $\alpha CGRP_{8-37}$ (10^{-6} M), but with less potent rightward shift of the concentration-response curves for αCGRP, AM and amylin.

6 Preincubation with 'Compound 1' (10^{-7}–10^{-5} M) and AM_{22-52} (10^{-6} M) had no significant antagonistic effect.

7 In conclusion, the building blocks forming CGRP and AM receptors were present in the porcine LAD, whereas those of the amylin receptor were not. αCGRP, βCGRP, AM and amylin mediated vasorelaxation *via* the CGRP receptors. No functional response was detected to adrenomedullin *via* the adrenomedullin receptor.

British Journal of Pharmacology (2001) **133,** 1405–1413

Keywords: CGRP; adrenomedullin; amylin; calcitonin receptor-like receptor; receptor-activity-modifying proteins; porcine coronary arteries

Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; Compound 1 (WO 98/11128), (4-(2-Oxo-2, 3-dihydro-benzoimidazol-1-yl)-piperidine-1-carboxylic acid [1-3,5-dibromo-4-hydroxy-benzyl)-2-oxo-2-(4-phenyl-piperazin-1-yl)-ethyl]-amide; CRLR, calcitonin receptor-like receptor; CT, Calcitonin; DMSO, dimethyl sulphoxide; HEK, human embryonic kidney; LAD, left anterior descending coronary artery; RAMP, receptor-activity-modifying proteins; SK-N-MC, human neuroblastoma cells

Introduction

Calcitonin gene-related peptide (CGRP), adrenomedullin (AM) and amylin are structurally related peptides with considerable homology and with different effects in the coronary circulation (Muff *et al*., 1995).

CGRP is a 37 amino acid peptide discovered in 1982 (Amara *et al*., 1982). The two isoforms of CGRP, α- and β-CGRP, have similar biological activities (Amara *et al*., 1985; Morris *et al*., 1984). CGRP is released from peripheral sensory nerves (Franco-Cereceda *et al*., 1987), and a rich supply of CGRP-immunoreactive nerve fibres has been demonstrated at the adventitial-medial border of human coronary arteries and veins (Gulbenkian *et al*., 1993; Saetrum *et al*., 1995). CGRP is a potent vasodilator causing a decrease in blood pressure and increase in heart rate when administered intravenously to healthy volunteers (Gennari & Imbimbo, 1985). CGRP potently relaxes human isolated coronary arteries (Gulbenkian *et al*., 1993), and has even

*Author for correspondence at: Department of Clinical Physiology and Nuclear Medicine, University Hospital of Copenhagen, Glostrup Hospital, Nordre Ringvej, DK-2600 Glostrup, Denmark.
E-mail: philip@post1.tele.dk

been demonstrated to cause dilation of coronary arteries at the site of atheromatous stenoses and to delay the onset of myocardial ischaemia during treadmill exercise in patients with chronic stable angina (Uren *et al*., 1993). CGRP released within the coronary circulation and in the atria is believed to be a physiological defence reaction to ongoing ischaemia (Mair *et al*., 1990; Lechleitner *et al*., 1992).

Amylin is a 37 amino acid peptide, originally found in the islet β-cells of the pancreas (Cooper *et al*., 1987). It is co-secreted from the β-cells with insulin (Kahn *et al*., 1990) in response to glucose (Kanatsuka *et al*., 1989). The expression of amylin has been demonstrated in other tissues, but never in vascular cells. Amylin is a well-known vasodilator, although its most important effect probably is to reduce the tissue-glucose response to insulin (Feuerstein *et al*., 1995). Amylin is also involved in the development of islet amyloid in the β-cells and thus in the pathogenesis of type 2 diabetes mellitus (Hoppener *et al*., 2000).

AM is a 52 amino acid peptide originally discovered in a human pheochromocytoma in 1993 (Kitamura *et al*., 1993). AM is a circulating vasodilator peptide expressed in a number of cell types (Kitamura *et al*., 1995) including endothelial cells (Sugo *et al*., 1994a) and vascular smooth muscle cells (Sugo *et al*., 1994b). The mRNA encoding AM has been detected in porcine coronary arteries (Nishimura *et al*., 1997). AM has strong hypotensive properties, and has been demonstrated to induce relaxation in different vascular beds including porcine coronary arteries (Kureishi *et al*., 1995). AM has also been shown to increase coronary blood flow in conscious sheep (Parkes, 1995). Further, AM has important antiproliferative actions on vascular cells (Kano *et al*., 1996), and recently the basal production of AM in the human coronary circulation was shown to be attenuated in subjects with coronary atherosclerosis, possibly due to the atherosclerosis-induced endothelial dysfunction and thereby decreased AM production (Hojo *et al*., 2000).

Recent investigations indicate that the calcitonin (CT) receptor and the calcitonin receptor-like receptor (CRLR) form the basis of all the receptors for the calcitonin/CGRP/amylin/AM family of peptides (Foord & Marshall, 1999). Thus, CGRP and AM bind to the same receptor, the calcitonin receptor-like receptor (CRLR), with receptor specificity being determined by receptor-activity-modifying proteins (RAMPs). Three different RAMPs have been described in human tissue, RAMP1, RAMP2 and RAMP3. Co-expression of RAMP1 and CRLR reveals a CGRP-receptor, whereas co-expression of RAMP2 or RAMP3 and CRLR form an AM receptor (Mclatchie *et al*., 1998). Similarly, RAMPs can interact with the CT-receptor gene product to induce expression of distinct amylin receptor phenotypes (Christopoulos *et al*., 1999).

Till now the lack of selective (preferably nonpeptide) agonists and antagonists has prevented thorough exploration of the receptor distribution and subtypes. The best receptor blockers availably for CGRP and adrenomedullin has been the peptide fragments $\alpha CGRP_{8-37}$, $\beta CGRP_{8-37}$ and AM_{22-52} (Chiba *et al*., 1989; Longmore *et al*., 1994; Eguchi *et al*., 1994). For over a decade the sensitivity to the $\alpha CGRP_{8-37}$ fragment has been used to differentiate between two proposed classes of CGRP receptors termed $CGRP_1$ and $CGRP_2$ (Dennis *et al*., 1990). Thus, $\alpha CGRP_{8-37}$ demonstrates higher affinity for the $CGRP_1$ receptor subtype ($pA_2 = 7-8$) than the $CGRP_2$ receptor ($pA_2 = 5.5-6.5$) (Juaneda *et al*., 2000). A relatively limited number of non-peptide CGRP receptor antagonists have been described. Recently a compound named BIBN4096BS was reported to have high picomolar affinity for the CGRP receptors expressed in SK-N-MC cells (Doods *et al*., 2000).

In this study the expression of mRNA encoding the porcine CRLR, CT-receptor, RAMP1–3 were investigated. Furthermore, the vasodilatatory responses of αCGRP, βCGRP, amylin, AM and the potential receptor antagonists $\alpha CGRP_{8-37}$, $\beta CGRP_{8-37}$ and AM_{22-52} were investigated in the porcine left ascending coronary artery (LAD) along with a novel non-peptide CGRP antagonist 'Compound 1' (WO 98/11128) (Edvinsson *et al*., 2001).

Methods

Vessels

Porcine hearts were obtained fresh from an abattoir (Roskilde Slagteriskole, Roskilde, Denmark) and transported to our laboratory in ice-cold physiological salt solution (154 mM NaCl, DAK, Denmark). The left anterior descending (LAD) coronary artery was isolated near the apex of the heart and fat and connecting tissue was removed under a microscope. The artery, approximately 2 mm in diameter, was cut into ring segments, 2 mm wide.

Vasomotor responses

Each vessel segment with intact endothelium was mounted in a temperature-controlled tissue bath (37°C) containing a buffer solution (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2 and glucose 5.5. The bath was continuously bubbled with a mixture of 95% O_2 and 5% CO_2, giving a pH of approximately 7.4. To measure the isometric circular wall tension of the vessels, each segment was suspended between two L-shaped metal holders (0.2 mm in diameter) in a myograph (Model 610M, Danish Myo Tecnology, Denmark). The vessels were stretched to their optimal lumen diameter in order to obtain the optimal condition for active tension development as previously described (Mulvany & Halpern, 1977). After approximately 1 h, the vessels were exposed to a buffer solution containing 60 mM KCl, obtained by substituting equimolar concentrations of NaCl for KCl in the previously described buffer solution. Only vessel segments responding with a reproducible potassium-induced contraction after washout with the normal buffer solution, was used for further investigation. Peptides were added in cumulative concentrations from 10^{-10} to 10^{-7} M or 10^{-6} M every 5 min to vessel segments, which had been precontracted for 10 min with the thromboxane A_2 agonist U46619 in a concentration of 10^{-7} M. The contraction induced by U46619 was set arbitrarily to 100% and used as an internal standard to which the relaxant response in the same vessel-segment was compared. When testing the effect of the potential receptor antagonists, 'Compound 1', $\alpha CGRP_{8-37}$, $\beta CGRP_{8-37}$ and AM_{22-52} were added 5 min prior to precontraction. Only one concentration-response experiment was allowed on each artery segment.

Drugs

Human αCGRP, βCGRP, AM, amylin and the fragments $\alpha CGRP_{8-37}$, $\beta CGRP_{8-37}$ and AM_{22-52} were all obtained from Bachem AG, Switzerland. All peptides were dissolved in distilled water. U46619 (Sigma, St. Louis, MO, U.S.A.) was dissolved in ethanol. 'Compound 1' (4-(2-Oxo-2,3-dihydro-benzoimidazol-1-yl)-piperidine-1-carboxylic acid [1-3,5-dibromo-4-hydroxy-benzyl)-2-oxo-2-(4-phenyl-piperazin-1-yl)-ethyl]-amide, Karl Thomae GmbH, WO 98/11128) (Edvinsson *et al*., 2001) was synthesized by Medicinal Chemistry, Merck Research Laboratories, U.S.A. and dissolved in DMSO (dimethyl sulphoxide). Stock solutions ($10^{-3}-10^{-4}$ M) were made and stored at −20°C. Just before the experiments the solutions were diluted in buffer containing 0.2% human serum albumin.

Molecular experiments

Primer pairs were designed to detect mRNA for porcine RAMP1 (forward: 5′-GGC AGG ACC ATC AGG AGC TA-3′ and reverse: 5′-TGC TCT GCC AGA CCA CCA GT-3′) and the porcine CT receptor (forward: 5′-AGC GCC AGT GGA ACC AAT AC-3′ and reverse: 5′-ACT TCC ATG GCG ATG ACC TC-3′). The isolation of mRNA and RT–PCR assay for CRLR, RAMP2 and RAMP3 mRNA were performed using the primers and method previously described (Sams & Jansen-Olesen, 1998). The PCR amplification products of porcine CRLR, RAMP1 and RAMP2 were purified using QIAprep spin miniprep kit protocol (QIAGEN®, QIAGEN GmbH, Germany) and sequenced using an ABI Prism™ 377 DNA Sequencer (Perkin-Elmer, Sweden). Due to the sequencing technique the sequenced product are shorter than the PCR amplification products.

Data analysis and statistics

The concentration-response curves for αCGRP, βCGRP, AM and amylin were analysed by iterative nonlinear regression analysis and the sensitivity to agonists expressed as pEC_{50}. Calculations were done using GraphPad Prism version 3.02 for Windows 95 (GraphPad Software, San Diego, CA, U.S.A., www.graphpad.com). The maximal relaxant responses of each peptide are expressed as a percentage of the contraction induced by U46619 and do not represent the E_{max} value. Results are given as mean ± s.e.mean (n), where n is the number of vessels. The effects of agonists and antagonists were examined by comparing responses before and after antagonist treatment by means of one-way analysis of variance (ANOVA) followed by Dunnett's test. Dose-ratios (DR) were calculated and used to construct full Schild plots (Arunlakshana & Schild, 1959). The pA_2-values are given with 95% confidence limits. $P<0.05$ was considered significant.

Results

Molecular experiments

The presence of mRNA encoding CRLR, RAMP1 and RAMP2 in porcine coronary arteries demonstrated by RT–PCR (Figure 1). Using the current primers, we did not succeed in finding mRNA encoding the RAMP3 molecule. Neither did we demonstrate mRNA encoding the porcine CT-receptor in the coronary arteries. Expression of the CT-receptor in the porcine kidney was used as a positive control (data not shown). The partial CRLR sequence correspond with the porcine $CGRP_1$ receptor (amino acids 252–375) sequenced by Elshourbagy *et al*. (1998) and share 98% amino acid sequence homology with the human sequence. The partial porcine sequences for RAMP1 and RAMP2 share 82–84% nucleotide sequence identity with the corresponding human sequences, whereas the amino acid sequence homology is 70–77% (Table 1).

Vasomotor responses

The human fragments $\alpha CGRP_{8-37}$, $\beta CGRP_{8-37}$, AM_{22-52} and 'Compound 1' had no significant effect on the vasoconstriction induced by U46619 when added in doses up to 10^{-5} M. Data not shown.

Comparison of αCGRP, βCGRP, amylin and adrenomedullin

αCGRP and βCGRP induced similar concentration-dependent responses in the porcine coronary arteries (Figure 2, Table 2). The pEC_{50} values were 8.1 ± 0.1 for both peptides and the maximal relaxations 96.6 ± 1.4 and 95.8 ± 2.0% respectively. The pEC_{50} value for AM was 6.7 ± 0.1 with a maximal relaxation of 90.6 ± 2.3 and 6.1 ± 0.5% for amylin with maximal relaxation of 70.6 ± 11.5%. AM and amylin were thus significantly different from the αCGRP and

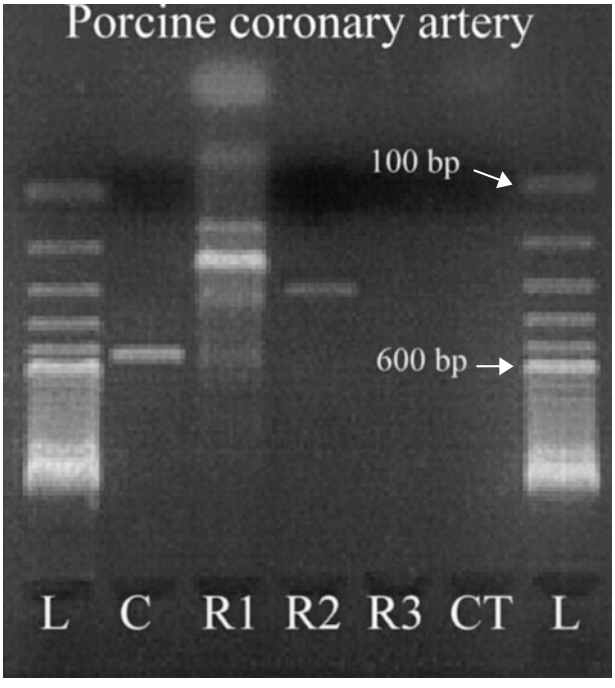

Figure 1 Demonstration of mRNA encoding the calcitonin receptor-like receptor (CRLR), RAMP1 and RAMP2 in the porcine left anterior descending coronary artery by RT–PCR. No bands are present in the lanes of RAMP3 (R3) and the calcitonin receptor (CT). L: 100 base pair ladder, C: CRLR, R1: RAMP1, R2: RAMP2, R3: RAMP3 and CT: calcitonin receptor.

Table 1 The identified partial porcine RAMP1 and RAMP2 mRNA nucleotide sequences and the NCBI Genbank accession numbers (acc. no.) are shown

(A) Porcine RAMP1

Nucleotide sequence (acc. no. AF312385), 240 base pairs:

AGGACCATCAGGAGCTATAAAGACCTCTCAGACTGCACCAGGCTCGTGGCGCAAAGGCTGGACTG
CTTCTGGCCCAACGCGGCGGTGGACAAGTTCTTCCTGGGAGTCCACCAGCAGTACTTCAGAAACT
GCCCCGTCTCCGGCAGGGCCTTGCAGGACCCGCCCAGCAGCGTCCTCTGCCCCTTCATCGTCGTC
CCCATCCTGGCGACCCTGCTCATGACCGCACTGGTGGTCTGGCAG

82% nucleotide homology to the human sequence

Amino acid sequence, 80 amino acids:

	1				
HUMAN:	RTIRSYRELA	DCTWHMAEKL	GCFWPNAEVD	RFFLAVHGRY	FRSCPISGRA
PORCINE:	RTIRSYKDLS	DCTRLVAQRL	DCFWPNAAVD	KFFLGVHQQY	FRNCPVSGRA
	51				
HUMAN:	VRDPPGSILY	PFIVVPITVT	LLVTALVVWQ		
PORCINE:	LQDPPSSVLC	PFIVVPILAT	LLMTALVVWQ		

70% amino acid homology to the human sequence

(B) Porcine RAMP2

Nucleotide sequence (acc. no. AF312383), 66 base pairs:

CAACTTTGCTGGCACGATTATAAGGATTATATGGACTCTATCAAAAAGGATTGGTGTGACTGGGCC

84% nucleotide homology to the human sequence

Amino acid sequence, 22 amino acids:

	1		
HUMAN:	QFCWNHYKDQ	MDPIEKDWCD	WA
PORCINE:	QLCWHDYKDY	MDSIKKDWCD	WA

77% amino acid homology to the human sequence

The porcine sequences correspond to (A) sequence number 213–452 in human RAMP1 (accession no. AJ001014) and (B) sequence number 264–329 in human RAMP2 (accesssion no. AJ001015). Further, the identified partial porcine RAMP1 and RAMP2 amino acod sequences are aligned. Identical amino acids in the porcine and human sequences are underlined.

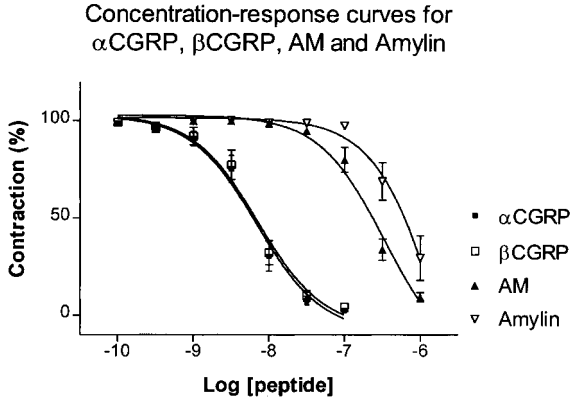

Figure 2 αCGRP, βCGRP, AM and amylin concentration-reponse relationship in porcine coronary arteries. Points represent mean values and vertical lines indicate s.e.mean. Relative responses are given as percentage fraction of the initial vessel response to U46619 (10^{-7} M) just before they were challenged with the relaxant peptides.

βCGRP responses. Further, the amylin response differed significantly from the AM response.

Effect of $\alpha CGRP_{8-37}$ and $\beta CGRP_{8-37}$

The fragments $\alpha CGRP_{8-37}$ and $\beta CGRP_{8-37}$ induced concentration-dependent (10^{-7}–10^{-5} M) rightward shift of both the αCGRP and βCGRP concentration-response curves (Figure 3a–d and Table 2). $\beta CGRP_{8-37}$ (10^{-6} M) had the same effect as $\alpha CGRP_{8-37}$ (10^{-6} M), but with less potent antagonistic properties (Figure 3c, d and Table 2). Thus, $\beta CGRP_{8-37}$ (10^{-6} M) did not antagonize the relaxant effect of βCGRP and did not affect the maximal response of αCGRP. The Schild plots revealed pA_2 values of 7.0 (6.4–8.6) and 6.9 (6.2–8.7) for $\alpha CGRP_{8-37}$ versus 6.3 (5.9–7.0) and 5.9 (5.7–6.5) for $\beta CGRP_{8-37}$ when tested with αCGRP and βCGRP respectively (Figure 4). Furthermore, preincubation with $\alpha CGRP_{8-37}$ (10^{-6} M) and $\beta CGRP_{8-37}$ (10^{-6} M) also caused a significant change in pEC_{50} values and the maximal responses for AM and amylin (Figures 5 and 6 and Table 2).

Effect of AM_{22-52}

Preincubation with AM_{22-52} (10^{-6} M) caused a slight but not significant rightward shift without affecting the maximal response of the concentration-response curves for AM and amylin. Neither did AM_{22-52} antagonize the response of α- and β-CGRP (Figures 5 and 6, 7a,b and Table 2).

Effect of 'Compound 1'/(WO98/11128)

The effect of αCGRP was tested after preincubation with 'Compound 1' (10^{-7}–10^{-5} M). No antagonistic effect was found (Figure 8).

Table 2 Effects of the proposed antagonists $\alpha CGRP_{8-37}$, $\beta CGRP_{8-37}$ and AM_{22-52} in the concentrations $10^{-7}-10^{-5}$ M on the pEC_{50} and the maximal relaxation

Agonist	*Antagonist* (log M)	n	*pEC_{50}*	*Maximal relaxation* (% of U46619)
αCGRP	–	**16**	**8.1±0.1**	**96.6±1.4**
	$\alpha CGRP_{8-37}$ (−7)	6	7.9±0.1	94.9±3.2
	$\alpha CGRP_{8-37}$ (−6)	10	7.3±0.2←	72.0±5.9←
	$\alpha CGRP_{8-37}$ (−5)	12	7.0±0.4←	41.7±7.3←
	$\beta CGRP_{8-37}$ (−7)	10	8.1±0.1	96.6±1.4
	$\beta CGRP_{8-37}$ (−6)	10	7.7±0.1←	90.1±2.4
	$\beta CGRP_{8-37}$ (−5)	8	7.2±0.2←	68.8±7.0←
	AM_{22-52} (−6)	10	8.1±0.1	96.9±1.4
βCGRP	–	**12**	**8.1±0.1**	**95.8±2.0**
	$\alpha CGRP_{8-37}$ (−7)	6	7.9±0.1	94.9±3.2
	$\alpha CGRP_{8-37}$ (−6)	6	7.6±0.2	87.8±5.8
	$\alpha CGRP_{8-37}$ (−5)	9	7.2±0.7←	72.2±7.1←
	$\beta CGRP_{8-37}$ (−6)	8	8.1±0.1	92.1±3.2
	$\beta CGRP_{8-37}$ (−5.5)	11	7.8±0.1←	87.2±3.9
	$\beta CGRP_{8-37}$ (−5)	10	7.5±0.1←	84.2±3.8←
	AM_{22-52} (−6)	8	8.4±0.1	90.2±4.5
AM	–	**10**	→**6.7±0.1**	**90.6±2.3**
	$\alpha CGRP_{8-37}$ (−6)	12	NE	15.7±3.4 ←
	$\beta CGRP_{8-37}$ (−6)	8	NE	48.6±11.1←
	AM_{22-52} (−6)	12	6.6±0.2	78.1±6.8
Amylin	–	**7**	→**6.1±0.5**←	→**70.6±11.5**
	$\alpha CGRP_{8-37}$ (−6)	5	NE	8.9±1.1 ←
	$\beta CGRP_{8-37}$ (−6)	5	NE	15.3±11.3←
	AM_{22-52} (−6)	5	NE	44.4±13.1

The maximal concentration of αCGRP and βCGRP tested were 10^{-7} and 10^{-6} M for AM and amylin. Arrows (→) indicate significant difference between values. NE, not estimated.

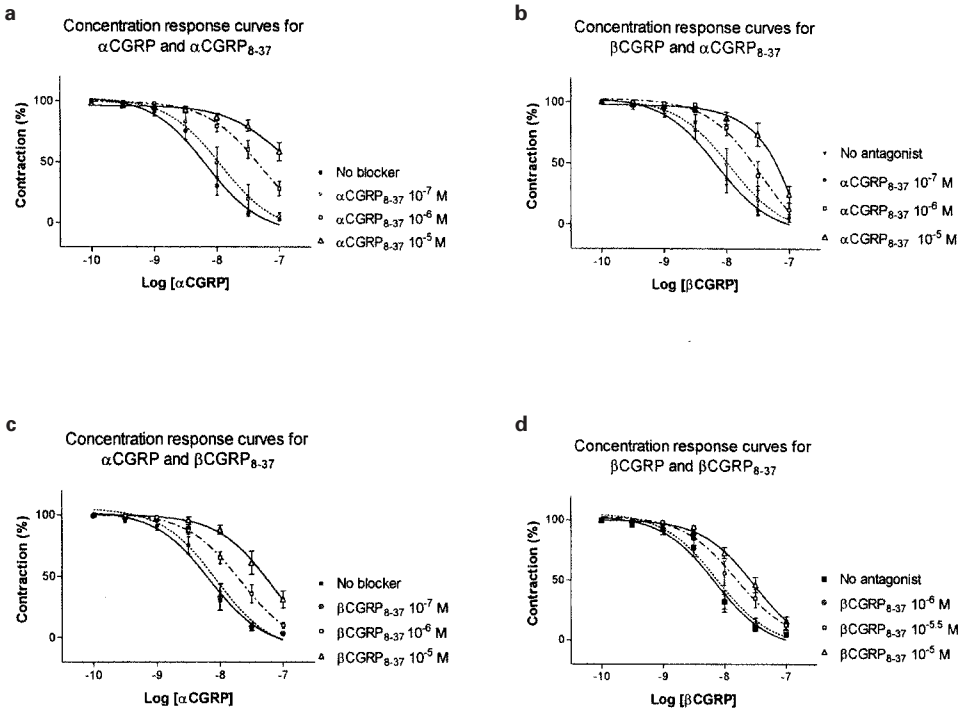

Figure 3 (a–d) Vasorelaxant effect of αCGRP ($10^{-10}-10^{-7}$ M) and βCGRP ($10^{-10}-10^{-7}$ M) in the porcine LAD contracted with U46619 (10^{-7} M). The antagonists $\alpha CGRP_{8-37}$ ($10^{-7}-10^{-5}$ M), $\beta CGRP_{8-37}$ ($10^{-7}-10^{-5}$ M) were added 15 min and U46619 10 min prior to the αCGRP/βCGRP challenge. Points represent mean values and vertical lines indicate s.e.mean. Relative responses are given as percentage fraction of the initial vessel response to U46619 (10^{-7} M) just before they were challenged with αCGRP/βCGRP.

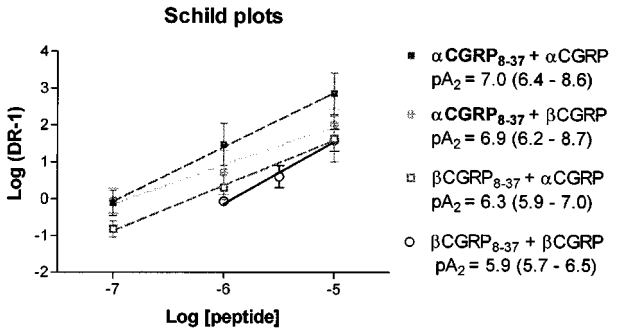

Figure 4 Schild plots for $\alpha CGRP_{8-37}$ and $\beta CGRP_{8-37}$ tested with human αCGRP and βCGRP as agonists in isolated porcine LAD. The Schild plot curve for $\alpha CGRP_{8-37}$ ($10^{-7}-10^{-5}$ M) tested with αCGRP was equal to $1.48\times$ $+10.3$ ($r=0.62$; $P=0.0011$). The pA_2 value = 7.0 (6.4–8.6). The Schild plot curve for $\alpha CGRP_{8-37}$ ($10^{-7}-10^{-5}$ M) tested with βCGRP was equal to $1.23\times$ $+7.8$ ($r=0.63$; $P=0.0007$). The pA_2 value = 6.9 (6.2–8.7). The Schild plot curve for $\beta CGRP_{8-37}$ ($10^{-7}-10^{-5}$ M) tested with αCGRP was equal to $1.05\times$ $+7.2$ ($r=0.61$; $P=0.0031$). The pA_2 value = 6.3 (5.9–7.0). The Schild plot curve for $\beta CGRP_{8-37}$ ($10^{-6}-10^{-5}$ M) tested with βCGRP was equal to $1.68\times$ $+10.0$ ($r=0.65$; $P=0.0002$). The pA_2 value = 5.9 (5.7–6.5). Each point represents mean values and vertical lines indicate s.e.mean.

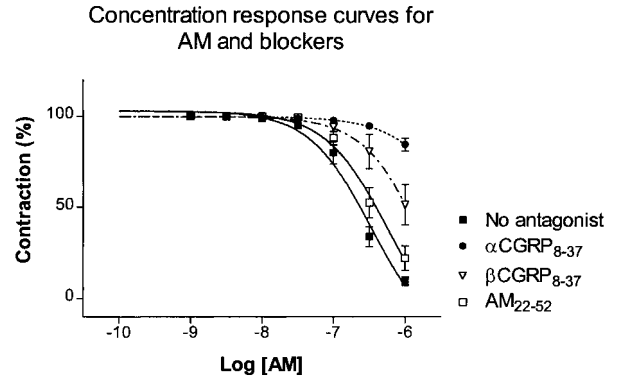

Figure 5 Vasorelaxant effect of AM in the porcine LAD contracted with U46619 (10^{-7} M). The proposed antagonists $\alpha CGRP_{8-37}$ (10^{-6} M), $\beta CGRP_{8-37}$ (10^{-6} M) and AM_{22-52} (10^{-6} M) were added 15 min and U46619 10 min prior to the αCGRP ($10^{-9}-10^{-6}$ M) challenge. Points represent mean values and vertical lines indicate s.e.mean. Relative responses are given as percentage fraction of the initial vessel response to U46619 (10^{-7} M) just before they were challenged with AM.

Discussion

RAMPs are proteins identified within the last few years. They interact and modify the phenotype of at least two family of receptors or class II group of G-protein receptors, the CGRP and CT receptor (Sexton, 1999). Three potential consequences of RAMP interaction with its associated receptors have been described: (1) intracellular transport of the receptor to the cell surface; (2) modification of the receptor glycosylation; and (3) direct and indirect modification of the ligand binding site through association with the receptor at the cell surface (Foord & Marshall, 1999). Our results

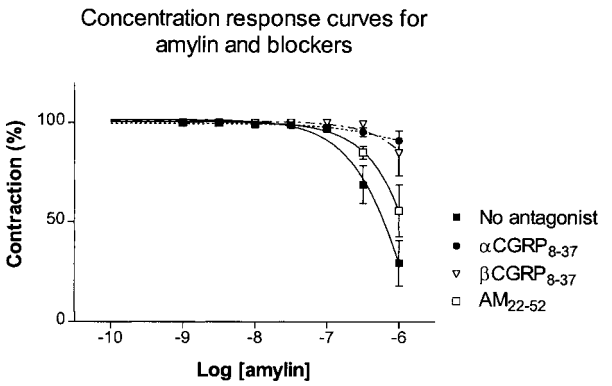

Figure 6 Vasorelaxant effect of amylin in the porcine LAD contracted with U46619 (10^{-7} M). The proposed antagonists $\alpha CGRP_{8-37}$ (10^{-6} M), $\beta CGRP_{8-37}$ (10^{-6} M) and AM_{22-52} (10^{-6} M) were added 15 min and U46619 10 min prior to the αCGRP ($10^{-9}-10^{-6}$ M) challenge. Points represent mean values and vertical lines indicate s.e.mean. Relative responses are given as percentage fraction of the initial vessel response to U46619 (10^{-7} M) just before they were challenged with amylin.

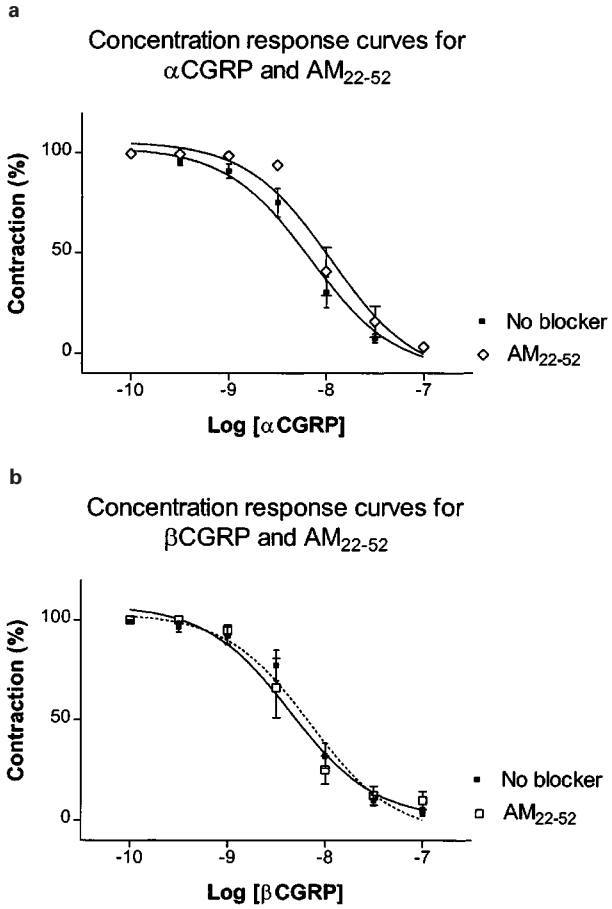

Figure 7 (a,b) Vasorelaxant effect of αCGRP ($10^{-10}-10^{-7}$ M) and βCGRP ($10^{-10}-10^{-7}$ M) in the porcine LAD contracted with U46619 (10^{-7} M). The antagonist AM_{22-52} (10^{-6} M) was added 15 min and U46619 10 min prior to the αCGRP/βCGRP challenge. Points represent mean values and vertical lines indicate s.e.mean. Relative responses are given as percentage fraction of the initial vessel response to U46619 (10^{-7} M) just before they were challenged with αCGRP/βCGRP.

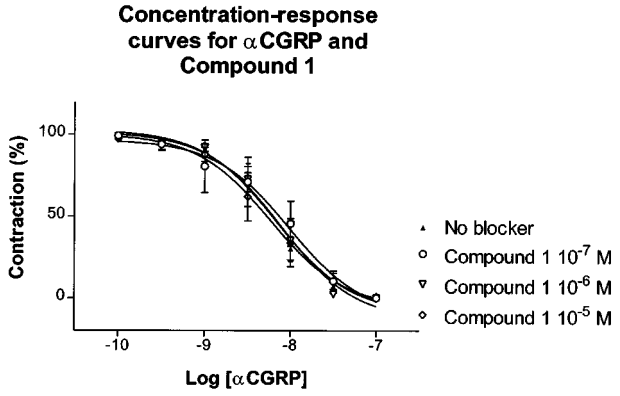

Figure 8 Vasorelaxant effect of αCGRP in the porcine LAD contracted with U46619 (10^{-7} M). The non-peptide CGRP antagonist 'Compound 1' (10^{-7}–10^{-5} M) was added 15 min and U46619 10 min prior to the αCGRP (10^{-10}–10^{-7} M) challenge. Points represent mean values and vertical lines indicate s.e.mean. Relative responses are given as percentage fraction of the initial vessel response to U46619 (10^{-7} M) just before they were challenged with αCGRP.

demonstrate the presence of mRNA sequences encoding the RAMPs and CRLR in the porcine LAD. We were not able to demonstrate RAMP3 using primers designed against the human sequence. But expression of RAMP1 and RAMP2 were demonstrated. Expression and formation of CGRP- and AM receptors are therefore possible (Mclatchie *et al*., 1998). The CT-receptor was not demonstrated. Therefore, the presence of specific amylin receptors in the porcine LAD is unlikely. To our knowledge the presence of amylin receptors have only been shown in the kidney, brain and vas deferens (Chai *et al*., 1998; Christopoulos *et al*., 1995; Tomlinson & Poyner, 1996) but never in vessels. RAMPs and CRLR have also been demonstrated in human cerebral arteries (Sams & Jansen-Olesen, 1998). The identified partial mRNA sequences demonstrate a high degree of homology with the human sequences, thereby indicating that the sequences in fact represent CRLR, RAMP1 and RAMP2 mRNA.

CGRP and AM are well known vasodilators in the coronary circulation and Yoshimoto *et al*. (1998) has previously tested these peptides in the porcine LAD. In the present study the vasorelaxant responses of αCGRP, βCGRP and AM and the blocking effect of $\alpha CGRP_{8-37}$ (10^{-6} M) on αCGRP and AM were confirmed. But in contrast to the investigations by Yoshimoto *et al*. we found significant antagonistic effect of the $\alpha CGRP_{8-37}$ (10^{-6} M) fragment on the relaxation induced by βCGRP.

The presence of CRLR and RAMP1 might be expected to give a $CGRP_1$-like receptor. However the measured pA_2 for $\alpha CGRP_{8-37}$ of 6.9–7.0 is considerably below the value of around 8 found for porcine CRLR and RAMP1 in HEK293 cell expression system (Elshourbagy *et al*., 1998). Differences between pA_2 values for $\alpha CGRP_{8-37}$ in cell lines and in real tissue are also seen with other species and one explanation could be that the receptors are better exposed in cell cultures than in real tissue with different diffusion gradient barriers. Thus, the pA_2 values of 7.0 (6.4–8.6) and 6.9 (6.2–8.7) produced by the human $\alpha CGRP_{8-37}$ fragment in the present study on the porcine coronary artery tissue is in line with previous studies where pA_2 or pKb values were 5.7–7 (Foulkes *et al*., 1991), 7.2 (Yoshimoto *et al*., 1998), 5.9–6.0 (neglecting data with high Schild plot slope) (Waugh *et al*., 1999) and 6.3–6.7 (Wisskirchen *et al*., 1999). Relatively little CGRP pharmacology has been carried out on the pig to say what pA_2 values would be expected for a $CGRP_1$ and $CGRP_2$ receptor in this species. Using the common criteria for distinguishing between the CGRP receptor subtypes the antagonist affinity for $\alpha CGRP_{8-37}$ is consistent with a $CGRP_1$ receptor, but the 95% confidence limits for the pA_2 determination covers the entire range from $CGRP_1$ to $CGRP_2$. The pA_2 values of 6.3 (5.9–7.0) and 5.9 (5.7–6.5) for $\beta CGRP_{8-37}$ indicate lower affinity at the CGRP receptor site compared to the $\alpha CGRP_{8-37}$ fragment.

The $\alpha CGRP_{8-37}$ fragment also blocked the vasodilatatory effect of amylin, indicating that amylin can act *via* the CGRP receptors. A recent study by Sheykhzade & Nyborg (2000) also shows that amylin act as a non-competitive antagonist on $CGRP_1$ receptors in rat coronary arteries.

Till now a relatively limited number of non-peptide CGRP receptor antagonists has been described. Recently a compound named BIBN4096BS was reported to have high picomolar affinity for the CGRP receptors expressed in SK-N-MC cells and was characterized as a human-selective antagonist (Doods *et al*., 2000). 'Compound 1' is a novel non-peptide CGRP antagonist and was presented as a functional CGRP receptor blocker in human SK-N-MC cells with a pK_i value of 7.8 and in human cerebral arteries with a pA_2 values of 7.7 (Edvinsson *et al*., 2001). In porcine LAD, however, 'Compound 1' demonstrated no antagonistic effect. The use of different species could explain the diverse results and perhaps 'Compound 1' has human-selective antagonistic properties as well. Similarly, in our study the $\beta CGRP_{8-37}$ fragment generally acted as $\alpha CGRP_{8-37}$, but with less potency. This is in contrast to studies in rat pulmonary artery, vas deferens and guinea-pig atria where these receptor antagonists antagonized the αCGRP responses with similar affinity (Wisskirchen *et al*., 1998; Longmore *et al*., 1994).

The human AM_{22-52} fragment has been tested as an AM receptor antagonist in many studies and in different species. Thus, in a radio-ligand binding study in porcine tissue AM_{22-52} acted as an AM receptor antagonist (Dang *et al*., 1999). AM_{22-52} has also been shown to block the vasodilatory effect of AM without affecting the response of CGRP in the vascular bed of rat and cat (Dogan *et al*., 1997; Gardiner *et al*., 1999; Saita *et al*., 1998; Takao *et al*., 1999), but in the cat hind limb vasculature the AM vasodilator response was unchanged after administration of AM_{22-52} (Champion *et al*., 1997). In the present study we only find a slight non-significant inhibition of the vasorelaxant effects of AM and amylin when preincubating with AM_{22-52}. The fragment is not a very potent AM inhibitor and its specificity has been questioned (Hinson *et al*., 2000). This is probably why different studies show different actions of AM_{22-52}. AM and amylin may still act *via* an AM receptor, but clarification of this question is not possible until a potent and specific AM receptor blocker has been identified. But CRLR has a higher affinity for RAMP1 than RAMP2 (Buhlmann *et al*., 1999), which might explain the failure to detect a functional response to adrenomedullin.

In conclusion, CGRP and AM are important in modulating blood flow in the coronary circulation. A CGRP receptor (CRLR + RAMP1) is present in the porcine coronary arteries,

but the antagonist affinity for $\alpha CGRP_{8-37}$ is neither consistent with a $CGRP_1$ nor a $CGRP_2$ receptor. The $\beta CGRP_{8-37}$ showed less potent CGRP receptor antagonism than the $\alpha CGRP_{8-37}$ fragment. The vasorelaxant effect of αCGRP, AM and amylin in the porcine coronary arteries can solely be explained by interaction with CGRP receptors. An AM receptor (CRLR + RAMP2) was also demonstrated, but no vasorelaxant effect of AM was shown *via* the AM receptor. This may be due to the lack of specificity and potency of the AM_{22-52} receptor antagonist or the fact that CRLR has a higher affinity for RAMP1 than RAMP2. No amylin receptor (CT + RAMPs) was demonstrated in the porcine LAD so the effect of amylin is probably mediated *via* the CGRP receptors. 'Compound 1', a novel non-peptide CGRP receptor antagonist, had no effect on the porcine LAD.

The study was supported through the following foundations: 'The Danish Heart Foundation', Grant No. 99-1-2-19-22679; 'The Danish Hospital Foundation for Medical Research'; 'Novo Nordisk Research Foundation'; 'The Danish Medical Association Research Fund' and 'Fonden til Lægevidenskabens fremme, A.P. Møller og Hustru Chastine Mc-Kinney Møllers Fond til almene formaal'.

The cDNA sequences reported in this paper have been deposited in the NCBI Genebank Database, accession numbers AF312383 and AF312385.

The authors would like to thank Betina Christensen for excellent assistance with the PCR protocols.

References

AMARA, S.G., ARRIZA, J.L., LEFF, S.E., SWANSON, L.W., EVANS, R.M. & ROSENFELD, M.G. (1985). Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. *Science,* **229,** 1094 – 1097.

AMARA, S.G., JONAS, V., ROSENFELD, M.G., ONG, E.S. & EVANS, R.M. (1982). Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature,* **298,** 240 – 244.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.,* **14,** 48 – 58.

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

CHAI, S.Y., CHRISTOPOULOS, G., COOPER, M.E. & SEXTON, P.M. (1998). Characterization of binding sites for amylin, calcitonin, and CGRP in primate kidney. *Am. J. Physiol.,* **274,** F51 – F62.

CHAMPION, H.C., SANTIAGO, J.A., MURPHY, W.A., COY, D.H. & KADOWITZ, P.J. (1997). Adrenomedullin-(22 – 52) antagonizes vasodilator responses to CGRP but not adrenomedullin in the cat. *Am. J. Physiol.,* **272,** R234 – R242.

CHIBA, T., YAMAGUCHI, A., YAMATANI, T., NAKAMURA, A., MORISHITA, T., INUI, T., FUKASE, M., NODA, T. & FUJITA, T. (1989). Calcitonin gene-related peptide receptor antagonist human CGRP-(8 – 37). *Am. J. Physiol.,* **256,** E331 – E335.

CHRISTOPOULOS, G., PAXINOS, G., HUANG, X.F., BEAUMONT, K., TOGA, A.W. & SEXTON, P.M. (1995). Comparative distribution of receptors for amylin and the related peptides calcitonin gene related peptide and calcitonin in rat and monkey brain. *Can. J. Physiol. Pharmacol.,* **73,** 1037 – 1041.

CHRISTOPOULOS, G., PERRY, K.J., MORFIS, M., TILAKARATNE, N., GAO, Y., FRASER, N.J., MAIN, M.J., FOORD, S.M. & SEXTON, P.M. (1999). Multiple amylin receptors arise from receptor activity-modifying protein interaction with the calcitonin receptor gene product. *Mol. Pharmacol.,* **56,** 235 – 242.

COOPER, G.J., WILLIS, A.C., CLARK, A., TURNER, R.C., SIM, R.B. & REID, K.B. (1987). Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 8628 – 8632.

DANG, K., DISA, J., GOUT, B. & AIYAR, N. (1999). Comparative affinities of adrenomedullin (AM) and calcitonin gene-related peptide (CGRP) for [125I] AM and [125I] CGRP specific binding sites in porcine tissues. *J. Recept. Signal. Transduct. Res.,* **19,** 803 – 817.

DENNIS, T., FOURNIER, A., CADIEUX, A., POMERLEAU, F., JOLICOEUR, F.B., ST PIERRE, S. & QUIRION, R. (1990). hCGRP8-37, a calcitonin gene-related peptide antagonist revealing calcitonin gene-related peptide receptor heterogeneity in brain and periphery. *J. Pharmacol. Exp. Ther.,* **254,** 123 – 128.

DOGAN, A., SUZUKI, Y., KOKETSU, N., OSUKA, K., SAITO, K., TAKAYASU, M., SHIBUYA, M. & YOSHIDA, J. (1997). Intravenous infusion of adrenomedullin and increase in regional cerebral blood flow and prevention of ischemic brain injury after middle cerebral artery occlusion in rats. *J. Cereb. Blood Flow Metab.,* **17,** 19 – 25.

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

EDVINSSON, L., SAMS, A., JANSEN-OLESEN, I., TAJTI, J., KANE, S.A., RUTLEDGE, R.Z., KOBLAN, K.S., HILL, R.G. & LONGMORE, J. (2001). Characterisation of the effects of a non-peptide CGRP receptor antagonist in SK-N-MC cells and isolated human cerebral arteries. *Eur. J. Pharmacol.,* **415,** 39 – 44.

EGUCHI, S., HIRATA, Y., IWASAKI, H., SATO, K., WATANABE, T.X., INUI, T., NAKAJIMA, K., SAKAKIBARA, S. & MARUMO, F. (1994). Structure-activity relationship of adrenomedullin, a novel vasodilatory peptide, in cultured rat vascular smooth muscle cells. *Endocrinology,* **135,** 2454 – 2458.

ELSHOURBAGY, N.A., ADAMOU, J.E., SWIFT, A.M., DISA, J., MAO, J., GANGULY, S., BERGSMA, D.J. & AIYAR, N. (1998). Molecular cloning and characterization of the porcine calcitonin gene-related peptide receptor. *Endocrinology,* **139,** 1678 – 1683.

FEUERSTEIN, G., WILLETTE, R. & AIYAR, N. (1995). Clinical perspectives of calcitonin gene related peptide pharmacology. *Can. J. Physiol. Pharmacol.,* **73,** 1070 – 1074.

FOORD, S.M. & MARSHALL, F.H. (1999). RAMPs: accessory proteins for seven transmembrane domain receptors. *Trends Pharmacol. Sci.,* **20,** 184 – 187.

FOULKES, R., SHAW, N., BOSE, C. & HUGHES, B. (1991). Differential vasodilator profile of calcitonin gene-related peptide in porcine large and small diameter coronary artery rings. *Eur. J. Pharmacol.,* **201,** 143 – 149.

FRANCO-CERECEDA, A., HENKE, H., LUNDBERG, J.M., PETERMANN, J.B., HOKFELT, T. & FISCHER, J.A. (1987). Calcitonin gene-related peptide (CGRP) in capsaicin-sensitive substance P-immunoreactive sensory neurons in animals and man: distribution and release by capsaicin. *Peptides,* **8,** 399 – 410.

GARDINER, S.M., MARCH, J.E., KEMP, P.A. & BENNETT, T. (1999). Influence of CGRP (8 – 37), but not adrenomedullin (22 – 52), on the haemodynamic responses to lipopolysaccharide in conscious rats. *Br. J. Pharmacol.,* **127,** 1611 – 1618.

GENNARI, C. & IMBIMBO, B. (1985). Effects of prednisone and deflazacort on vertebral bone mass. *Calcif. Tissue Int.,* **37,** 592 – 593.

GULBENKIAN, S., SAETRUM, O.O., EKMAN, R., COSTA, A.N., WHARTON, J., POLAK, J.M., MELO, J. & EDVINSSON, L. (1993). Peptidergic innervation of human epicardial coronary arteries. *Circ. Res.,* **73,** 579 – 588.

HINSON, J.P., KAPAS, S. & SMITH, D.M. (2000). Adrenomedullin, a multifunctional regulatory peptide. *Endocr. Rev.,* **21,** 138 – 167.

HOJO, Y., IKEDA, U., KATSUKI, T.A. & SHIMADA, K. (2000). Decreased adrenomedullin production in the coronary circulation of patients with coronary artery disease. *Heart,* **84,** 88.

HOPPENER, J.W., AHREN, B. & LIPS, C.J. (2000). Islet amyloid and type 2 diabetes mellitus. *N. Engl. J. Med.,* **343,** 411 – 419.

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

KAHN, S.E., D'ALESSIO, D.A., SCHWARTZ, M.W., FUJIMOTO, W.Y., ENSINCK, J.W., TABORSKY, JR., G.J. & PORTE, JR., D. (1990). Evidence of cosecretion of islet amyloid polypeptide and insulin by beta-cells. *Diabetes,* **39,** 634 – 638.

KANATSUKA, A., MAKINO, H., OHSAWA, H., TOKUYAMA, Y., YAMAGUCHI, T., YOSHIDA, S. & ADACHI, M. (1989). Secretion of islet amyloid polypeptide in response to glucose. *FEBS Lett.,* **259,** 199 – 201.

KANO, H., KOHNO, M., YASUNARI, K., YOKOKAWA, K., HORIO, T., IKEDA, M., MINAMI, M., HANEHIRA, T., TAKEDA, T. & YOSHIKAWA, J. (1996). Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. *J. Hypertens.,* **14,** 209 – 213.

KITAMURA, K., KANGAWA, K., KAWAMOTO, M., ICHIKI, Y., NAKAMURA, S., MATSUO, H. & ETO, T. (1993). Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem. Biophys. Res. Commun.,* **192,** 553 – 560.

KITAMURA, K., KANGAWA, K., MATSUO, H. & ETO, T. (1995). Adrenomedullin. Implications for hypertension research. *Drugs,* **49,** 485 – 495.

KUREISHI, Y., KOBAYASHI, S., NISHIMURA, J., NAKANO, T. & KANAIDE, H. (1995). Adrenomedullin decreases both cytosolic Ca2+ concentration and Ca(2+)- sensitivity in pig coronary arterial smooth muscle. *Biochem. Biophys. Res. Commun.,* **212,** 572 – 579.

LECHLEITNER, P., GENSER, N., MAIR, J., DIENSTL, A., HARING, C., WIEDERMANN, C.J., PUSCHENDORF, B., SARIA, A. & DIENSTL, F. (1992). Calcitonin gene-related peptide in patients with and without early reperfusion after acute myocardial infarction. *Am. Heart J.,* **124,** 1433 – 1439.

LONGMORE, J., HOGG, J.E., HUTSON, P.H. & HILL, R.G. (1994). Effects of two truncated forms of human calcitonin-gene related peptide: implications for receptor classification. *Eur. J. Pharmacol.,* **265,** 53 – 59.

MAIR, J., LECHLEITNER, P., LANGLE, T., WIEDERMANN, C., DIENSTL, F. & SARIA, A. (1990). Plasma CGRP in acute myocardial infarction. *Lancet,* **335,** 168.

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

MORRIS, H.R., PANICO, M., ETIENNE, T., TIPPINS, J., GIRGIS, S.I. & MACINTYRE, I. (1984). Isolation and characterization of human calcitonin gene-related peptide. *Nature,* **308,** 746 – 748.

MUFF, R., BORN, W. & FISCHER, J.A. (1995). Calcitonin, calcitonin gene-related peptide, adrenomedullin and amylin: homologous peptides, separate receptors and overlapping biological actions. *Eur. J. Endocrinol.,* **133,** 17 – 20.

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

NISHIMURA, J., SEGUCHI, H., SAKIHARA, C., KUREISHI, Y., YOSHIMURA, H., KOBAYASHI, S. & KANAIDE, H. (1997). The relaxant effect of adrenomedullin on particular smooth muscles despite a general expression of its mRNA in smooth muscle, endothelial and epithelial cells. *Br. J. Pharmacol.,* **120,** 193 – 200.

PARKES, D.G. (1995). Cardiovascular actions of adrenomedullin in conscious sheep. *Am. J. Physiol.,* **268,** H2574 – H2578.

SAETRUM, O.O., GULBENKIAN, S., BERGDAHL, A., BARROSO, C.P., ANDRADE, N.C., POLAK, J.M., MELO, J.Q. & EDVINSSON, L. (1995). Innervation of human epicardial coronary veins: immunohistochemistry and vasomotility. *Cardiovasc. Res.,* **29,** 463 – 468.

SAITA, M., SHIMOKAWA, A., KUNITAKE, T., KATO, K., HANAMORI, T., KITAMURA, K., ETO, T. & KANNAN, H. (1998). Central actions of adrenomedullin on cardiovascular parameters and sympathetic outflow in conscious rats. *Am. J. Physiol.,* **274,** R979 – R984.

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

SEXTON, P.M. (1999). Recent advances in our understanding of peptide hormone receptors and RAMPs. *Curr. Opin. Drug Discov. Dev.,* **2,** 440 – 448.

SHEYKHZADE, M. & NYBORG, N.C. (2000). Non-competitive antagonism of amylin on CGRP(1)-receptors in rat coronary small arteries. *Br. J. Pharmacol.,* **130,** 386 – 390.

SUGO, S., MINAMINO, N., KANGAWA, K., MIYAMOTO, K., KITAMURA, K., SAKATA, J., ETO, T. & MATSUO, H. (1994a). Endothelial cells actively synthesize and secrete adrenomedullin [published erratum appears in Biochem Biophys Res Commun 1994 Sep 15;203(2):1363]. *Biochem. Biophys. Res. Commun.,* **201,** 1160 – 1166.

SUGO, S., MINAMINO, N., SHOJI, H., KANGAWA, K., KITAMURA, K., ETO, T. & MATSUO, H. (1994b). Production and secretion of adrenomedullin from vascular smooth muscle cells: augmented production by tumor necrosis factor-alpha. *Biochem. Biophys. Res. Commun.,* **203,** 719 – 726.

TAKAO, M., TOMITA, M., TANAHASHI, N., KOBARI, M. & FUKUUCHI, Y. (1999). Transient vasodilatory effects of adrenomedullin on cerebral parenchymal microvessels in cats. *Neurosci. Lett.,* **268,** 147 – 150.

TOMLINSON, A.E. & POYNER, D.R. (1996). Multiple receptors for calcitonin gene-related peptide and amylin on guineapig ileum and vas deferens. *Br. J. Pharmacol.,* **117,** 1362 – 1368.

UREN, N.G., SEYDOUX, C. & DAVIES, G.J. (1993). Effect of intravenous calcitonin gene related peptide on ischaemia threshold and coronary stenosis severity in humans. *Cardiovasc. Res.,* **27,** 1477 – 1481.

WAUGH, D.J., BOCKMAN, C.S., SMITH, D.D. & ABEL, P.W. (1999). Limitations in using peptide drugs to characterize calcitonin gene-related peptide receptors. *J. Pharmacol. Exp. Ther.,* **289,** 1419 – 1426.

WISSKIRCHEN, F.M., BURT, R.P. & MARSHALL, I. (1998). Pharmacological characterization of CGRP receptors mediating relaxation of the rat pulmonary artery and inhibition of twitch responses of the rat vas deferens. *Br. J. Pharmacol.,* **123,** 1673 – 1683.

WISSKIRCHEN, F.M., GRAY, D.W. & MARSHALL, I. (1999). Receptors mediating CGRP-induced relaxation in the rat isolated thoracic aorta and porcine isolated coronary artery differentiated by halpha CGRP8 - 37. *Br. J. Pharmacol.,* **128,** 283 – 292.

YOSHIMOTO, R., MITSUI-SAITO, M., OZAKI, H. & KARAKI, H. (1998). Effects of adrenomedullin and calcitonin gene-related peptide on contractions of the rat aorta and porcine coronary artery. *Br. J. Pharmacol.,* **123,** 1645 – 1654.

(Received April 1, 2001
Revised June 8, 2001
Accepted June 8, 2001)