British Journal of Pharmacology (2005) 145, 646–655
www.nature.com/bjp

Increased αCGRP potency and CGRP-receptor antagonist affinity in isolated hypoxic porcine intramyocardial arteries

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1 This study describes the effects of hypoxia on relaxing responses and cAMP production induced by the known vasodilator peptides: αCGRP, amylin (AMY) and adrenomedullin (AM) on isolated pig coronary arteries *in vitro*.

2 Hypoxic incubation increased the vasorelaxant effect of αCGRP (four-fold; $P<0.05$), AMY (3.2-fold; $P<0.05$), but not significantly for AM (two-fold; NS).

3 Whereas hypoxia had no effect on arterial cAMP levels, it significantly potentiated the production of cAMP stimulated of αCGRP and AMY, but not of AM.

4 The antagonist $\alpha CGRP_{8-37}$ also exerted an increased effect in hypoxia. The Schild plot-derived pK_B values revealed an increase in the apparent affinity of the antagonist for the $CGRP_1$ receptor from 7.0 to 7.2 under control conditions *versus* 8.0 in hypoxia.

5 Removal of endothelium, peptidase inhibitors, preincubation with the adenosine A_{2A} receptor antagonist CSC (10^{-3} M), the ATP-sensitive K-channel inhibitor glibenclamide (10^{-5} M), the cyclooxygenase inhibitor indomethacin (10^{-3} M) or *NG*-monomethyl-L-arginine (10^{-4} M) had no effect on the αCGRP-induced vasorelaxation in hypoxia; neither did hypoxia influence the levels of CGRP and AM receptor mRNA.

6 We conclude that hypoxic incubation increases the relaxation and cAMP production induced by αCGRP and AMY in rings of porcine coronary arteries *in vitro*. A concomitant release of adenosine, a cyclooxygenase product, an endothelium-derived substance, activation of vascular ATP-sensitive K-channels, peptidase inhibitors or changes in CGRP and AM receptor mRNA cannot account for the changes observed in hypoxia. Moreover, $\alpha CGRP_{8-37}$ showed increased affinity at the $CGRP_1$ receptor during hypoxia, possibly due to a conformational change at the $CGRP_1$ receptor site.
British Journal of Pharmacology (2005) **145,** 646–655. doi:10.1038/sj.bjp.0706232
Published online 18 April 2005

Keywords: CGRP; adrenomedullin; amylin; calcitonin-like receptor; receptor-activity-modifying proteins; hypoxia; normoxia; hyperoxia

Abbreviations: AM, adrenomedullin; AMY, amylin; cAMP, cyclic adenosine mono-phosphate; CGRP, calcitonin gene-related peptide; CL receptor, calcitonin-like receptor; CT, calcitonin; DMSO, dimethyl sulphoxide; Iso, isoproterenol; K_{ATP} channels, ATP-sensitive K-channels; LAD, left anterior descending; L-NMMA, *NG*-monomethyl-L-arginine; NOS, nitric oxide synthase; PCR, polymerase chain reaction; RAMP, receptor-activity-modifying proteins; RT, reverse transcriptase; SNP, sodium nitroprusside

Introduction

The function of the heart depends critically on adequate oxygen supply. Imbalance between oxygen supply and the oxygen demand leads to myocardial hypoxia, one component of myocardial ischaemia, with its clinical manifestations angina pectoris/infarction or heart failure. Calcitonin gene-related peptide (CGRP), adrenomedullin (AM) and amylin (AMY) belong to the same family of vasorelaxant peptides and they probably play an important role in the regulation of coronary circulation under physiological and pathological conditions.

CGRP is widely distributed in the peripheral and central nervous systems and is released from cardiac unmyelinated sensory C-fibres (Franco-Cereceda *et al*., 1987). CGRP has been reported to gain potency in hypoxic conditions in sheep coronary arteries (Kwan *et al*., 1990) and CGRP release itself is also enhanced during hypoxia and at low pH levels in the guinea-pig myocardium (Franco-Cereceda *et al*., 1993). Furthermore, intravenously administered CGRP delays the onset of myocardial ischaemia during treadmill exercise testing in patients with chronic stable angina (Uren *et al*., 1993).

Unlike CGRP, AM is primarily produced by non-nervous tissue, especially vascular endothelium and smooth muscle cells (Sugo *et al*., 1994a, b). Direct AM-induced vasorelaxation has been observed in a number of species and in different vascular beds, including the porcine coronary circulation (Kureishi *et al*., 1995; Hasbak *et al*., 2001). Hypoxia induces AM mRNA production in human coronary artery endothelial cells (Nakayama *et al*., 1999) and higher plasma AM levels are found in patients with myocardial infarction (Kobayashi *et al*., 1996), a condition where hypoxia is one essential component.

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AMY is co-localised and co-released with insulin in the β pancreatic cells (Pittner *et al*., 1994) and is generally considered to be a glyco-regulatory peptide that inhibits the actions of insulin (Deems *et al*., 1991; Castle *et al*., 1998). But we have previously shown that AMY also induced a weak vasorelaxant effect probably *via* the $CGRP_1$ receptor in both the human and the porcine coronary circulation (Hasbak *et al*., 2001; 2003).

On a molecular level, the $CGRP_1$ receptor has been characterised and consists of the calcitonin-like receptor (CL receptor) (according to the guidelines from the International Union of Pharmacology CGRP nomenclature subcommittee (Poyner *et al*., 2002)) previously known as calcitonin receptor-like receptor (CRLR) and receptor-activity-modifying protein 1 (RAMP1) (McLatchie *et al*., 1998). RAMPs are required both for receptor trafficking and ligand binding. The association of CL receptor with either RAMP2 or RAMP3 produces AM receptors. Previously, we reported the presence of mRNA encoding the CL receptor and RAMPs in the porcine and human coronary arteries, indicating the presence of $CGRP_1$ and AM receptors (Hasbak *et al*., 2001; 2003).

In the present study, investigations were performed at various oxygen concentrations to characterise the potential influence of hypoxia on CGRP-, AM- and AMY-induced vasodilatation and on the affinity of the CGRP receptor blocker $\alpha CGRP_{8-37}$ in porcine coronary arteries. The effects were determined by functional pharmacology using the myograph technique. Moreover, in order to characterise the responses in more detail, we investigated the alterations in intracellular cAMP, mRNA levels for the $CGRP_1$ and AM receptors, the role of endothelium, ATP-sensitive K-channels (K_{ATP} channels), nitric oxide synthase (NOS), peptidase inhibitors and possible release of adenosine and vasodilator prostanoids in porcine intramyocardial arteries.

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press, Washington, DC, U.S.A.

Tissue preparation

Porcine hearts were obtained fresh from the local abattoir (Roskilde Slagteriskole, Roskilde, Denmark) and transported to our laboratory in ice-cold physiological salt solution (154 mM NaCl, DAK, Denmark). Intramural segments of the distal part of the left anterior descending (LAD) coronary artery were isolated from the heart under a microscope. The arteries, 0.4–0.6 mm in outer diameter, were cut into ring segments, 1 mm long. Arteries were used immediately for *in vitro* pharmacology.

Vasomotor responses

Each vessel segment 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. Vessels with intact endothelium were confirmed by histological examination and substance P (10^{-6} M) evoked relaxation. Some artery segments were denuded of endothelium by inserting and rubbing the internal surface of the vessel segments with a human scalp hair (Prieto *et al*., 1991). Removal of endothelium was considered effective if substance P (10^{-6} M) no longer induced relaxation. To measure the isometric circular wall tension of the vessels, each segment was suspended between two L-shaped metal holders (0.1 mm in diameter) in a Myograph® (Model 700MO, J.P. Trading, 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), followed by an

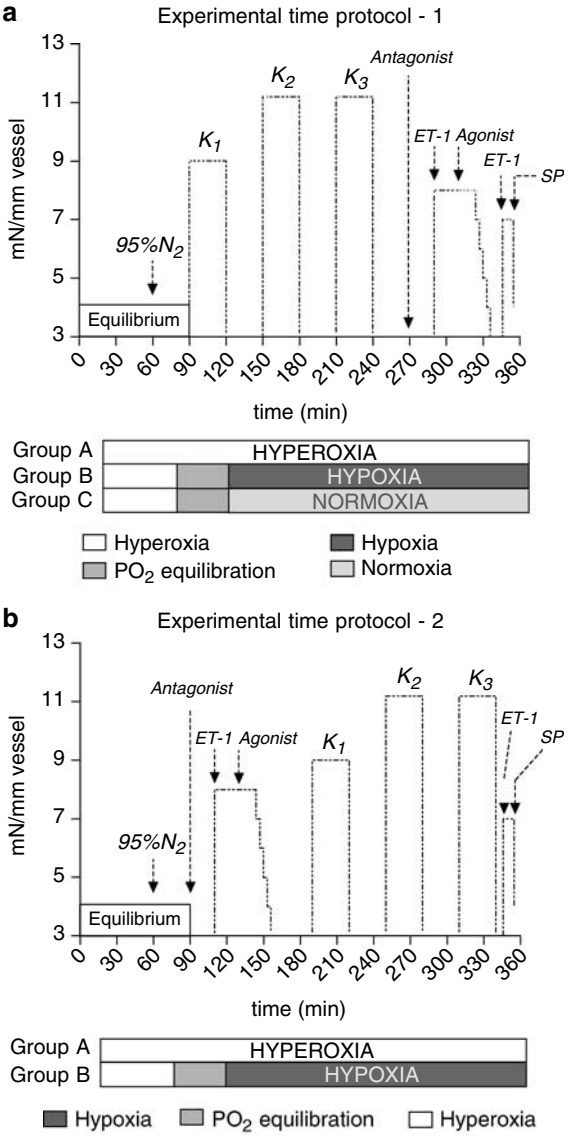

Figure 1 (a, b) Two different experimental time protocols outlined with a common 60 min initial equilibration period with 95% O_2/5% CO_2 (hyperoxia), followed by gas mixture change to 95% N_2/5% CO_2 (hypoxia) in group B and 21% O_2/5% CO_2/74% N_2 (normoxia) in group C, whereas group A continues with 95% O_2/5% CO_2 (hyperoxia). Arrows or abbreviations indicate the addition of KCl (K_{1-3}), endothelin 1 (ET-1), agonist ($\alpha CGRP$, AM or AMY), antagonist ($\alpha CGRP_{8-37}$) and substance P (SP).

equilibration period of 1 h in 95% O_2/5% CO_2. The experimental time protocol is outlined in Figure 1a.

The vessel segments were then tested in three settings: (A) hyperoxia/control was operationally defined as aeration with 95%O_2/5%CO_2, (B) hypoxia as aeration with 95% N_2/5% CO_2 ($PO_2 < 35$ mmHg) and (C) normoxia as aeration with 21% O_2/5% CO_2/74% N_2 (175 mmHg > PO_2 > 125 mmHg). Oxygen tension was measured using an oxygen electrode and oxygen meter (Microelectrodes Inc., New Hampshire, U.S.A.) immersed in the organ bath. Aliquots of the bathing medium were removed by syringe, and PCO_2 and pH were measured using a blood gas analyser (Radiometer, Denmark). Hypoxia in the bathing medium was reached in 22–27 min ($n = 10$) after changing from 95% O_2/5% CO_2 to 95% N_2/5% CO_2, whereas normoxia was reached after 11–17 min ($n = 6$) after switching gases. Thus after 30 min in (A) hyperoxia, (B) hypoxia or (C) normoxia, the vessels were exposed to a buffer solution high on KCl, obtained by supplementing the previously described buffer solution with KCl to a final concentration of 60 mM. Only vessel segments responding with a reproducible potassium-induced contraction after washout with normal buffer solution were used for further investigation. Maximally, three KCl exposures were allowed per vessel.

The αCGRP, AM and AMY were added in cumulative concentrations from 10^{-11} to 10^{-6} M every 3 min to vessel segments, which had been precontracted for 20 min with endothelin-1 (ET-1, $10^{-7.5}$ M). The contraction induced by ET-1 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 receptor antagonist $\alpha CGRP_{8-37}$, this fragment was added 20 min prior to precontraction. Thus, the vessels were exposed to hypoxia for up to 4.5 h totally in this protocol (Figure 1a).

The incubation periods were changed in an additional protocol (Figure 1b) investigating the effects of αCGRP (10^{-10}–10^{-7} M) and $\alpha CGRP_{8-37}$ (10^{-6} M). Thus, after 1 h of equilibration followed by hyperoxia/hypoxia exposure for 30 min, the vessels were exposed to $\alpha CGRP_{8-37}$/ET-1/αCGRP and subsequently by up to three KCl buffer exposures.

According to current publications, the release of vasodilatatory agents in coronary artery during hypoxia is a possibility. The interest centres on adenosine and vasodilator prostanoids. Activation of K_{ATP} channels, peptidase inhibitors and NOS are other possibilities. Thus, in separate studies adenosine (10^{-7}–10^{-3} M added every 3 min) concentration–response curves were generated using porcine arteries bubbled with 95% O_2/5% CO_2 and precontracted with ET-1 as mentioned above. In subsequent experiments the antagonistic effects of a selective A_{2A} adenosine receptor antagonist CSC (10^{-3} M), the K_{ATP} channel inhibitor glibenclamide (10^{-5} M), the cyclooxygenase inhibitor indomethacin (10^{-3} M), a mixture of peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon and thiorphan) (10^{-5} M each) and L-NMMA (10^{-4} M) were studied with αCGRP response under hyperoxia and hypoxia. All antagonists were added 20 min prior to precontraction.

To study if possible increase in sensitivity under hypoxic conditions can also be observed with isoprenaline (Iso) (10^{-11}–10^{-5} M) (a drug acting through cAMP) and sodium nitroprusside (SNP) (10^{-10}–10^{-5} M) (a NO donor acting through cGMP), concentration–response curves were generated using the above-mentioned protocol during hyperoxia and hypoxia.

Measurements of cyclic AMP

Each vessel segment was stabilised in the above-described buffer solution bubbled with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2 for more than 60 min before the experiment. αCGRP (10^{-9}–10^{-7} M), AM (10^{-8}–10^{-6} M) and AMY (10^{-8}–10^{-6} M) were added for exactly 60 s to vessels which had been precontracted for 20 min with ET-1 (10^{-8} M). Vessel segments were removed and immediately soaked in acidic ethanol (ethanol 96%; HCl, 1 M (100 : 1)) as previously described and stored at −20°C before each segment was homogenised in a glass tube by a glass pestle. Following centrifugation for 20 min at $2000 \times g$, the supernatant was isolated and evaporated under a steam of N_2 at 50°C. Using the nonacetylation protocol, the cAMP content was assayed by a RIA kit (Amersham, Denmark).

Real-time quantitative RT–PCR

Segments of porcine coronary arteries were bubbled with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2 in buffer solution for 4 h, time equivalent to the vasomotor response studies ($n = 4$ in both groups). Total RNA was isolated from coronary artery tissue with the use of TRIzol® Reagent (Introvitrogen™) according to the instructions provided by the manufacturer. First-strand cDNA was synthesised from 0.3 μg total RNA in 20 μl reaction volume using random hexamer as primers. Real-time quantitative PCR for CL receptor, RAMP1 and RAMP2 was performed based on GeneAmp® SYBR Green I assays using a GeneAmp® 5700 sequence-detection system (PE Biosystems, Applied Biosystems, Sweden), with β-actin as endogenous controls to standardise the amount of sample RNA added to a reaction. Primers for CL receptor, RAMP1, RAMP2 and β-actin were designed as follows: CL receptor forward: 5′-TGGCCAC AAATCCTGTTAGTTG-3′; reverse: 5′-CAAACACGGCCACCACAATA-3′; RAMP1 forward: 5′-CATCAGGAGCTATAAAGACCTCTCAGA-3′ and reverse 5′-CTGGTGGACTCCCAGGAAGA-3′. RAMP2 forward: WGATCMACTTTGCCAACTGCT-3′ and reverse: 5′-TGATCATGGCCAGRAGYACATC-3′ and β-actin 5′-CG GCCAGGTCATCACCAT-3′ and 5′-CCACGTCGCACTT CATGATC-3′. Wobble IUPAC-IUB symbols (Cornish-Bowden, 1985) are used in the RAMP2 primers, thus W (A or T), M (A or C), R (A or G) and Y (C or T). SYBR Green I assays were performed on all selected genes with SYBR Green PCR Master Mix (PE Biosystems). Each tube contained a total volume of 50 μl and the following: 1 × SYBR Green PCR Master Mix, 300 nM forward and reverse primers, and 6 μl of first-strand cDNA equivalent to 30 ng of total RNA. The thermal cycling parameters for PCR was 50°C for 2 min, 10 min at 95°C and 40 cycles for 15 s at 95°C, and 1 min at 60°C. All experiments were performed in duplicate.

To prove that the cDNA of β-actin, CL receptor, RAMP1 and RAMP2 were amplified with the same efficacy during real-time PCR, a standard curve were made in which the C_T values were plotted against cDNA concentration on the basis of the following equation: $C_T = [\log(1 + E)]^{-1}$ log(concentration), where C_T is the number of PCR cycles performed in one sample at a specific point of time, and E is the amplification efficiency with an optimal value of 1.

Dissociation curves ran immediately after the real-time PCR run, and possible nonspecific amplification could thus be

detected. Agarose gel electrophoretic analysis was used to verify that the amplified product corresponded to the size predicted for the amplified fragment.

Drugs

The human forms of the peptides αCGRP, $\alpha CGRP_{8-37}$, AM, AMY and ET-1 were obtained from Bachem AG, Bubendorf, Switzerland. All peptides were dissolved in distilled water. Human serum albumin (0.2%) was added to the final concentration of all reagents in the tissue bath. (1-4-Chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid (indomethacin), 1,3,7-trimethyl-8-(3-chlorostyryl)xanthine (CSC), 5-chloro-*N*-[4-(cyclohexylureido-sulphonyl)phenethyl]-2-methoxybenzamide (glibenclamide), adenosine, 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_2\alpha$ (U46619), the peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon and thiorphan), SNP dihydrate, isoprenaline hydrochloride, L-NMMA were obtained from Sigma, U.S.A.

Data analysis and statistics

The concentration–response curves for αCGRP, AM and AMY were analysed by iterative nonlinear regression analysis and the sensitivity to agonists expressed as pEC_{50} (−log of EC_{50}; concentration of the agonist that produced 50% of the maximal response), using Graphpad Prism 4.00 (GraphPad software, U.S.A.). The relaxant responses of each peptide are expressed as a percentage of the contraction induced by ET-1 ($10^{-7.5}$ M). Results are given as mean ± s.e.m. (*n*), where *n* values represent the number of hearts from which arteries were isolated. Statistical evaluation was performed by means of one-way analysis of variance (ANOVA), followed by Dunnett's test (Winer, 1971) or by unpaired Student's *t*-test.

Where only a single concentration of antagonist was used, an apparent antagonist affinity was determined according to the Gaddum equation:

$$pK_B = \log(DR - 1) - \log[B]$$

DR is the concentration ratio of the EC_{50} values in the presence and absence of the antagonist and [B] is the molar concentration of the antagonist. Where multiple concentrations of antagonists were used, pK_B was evaluated by a Schild plot with slope constrained to −1, in the case that the unconstrained slope did not differ significantly from unity (Jenkinson, 1991; Jenkinson *et al*., 1995). $P<0.05$ was considered significant.

Results

The porcine coronary arteries were tested in three settings: (A) Hyperoxia resulted in $PO_2 = 678 \pm 30$ mmHg, $PCO_2 = 33 \pm 3$ mmHg and pH = 7.4 ± 0.05, $n = 12$. (B) Hypoxia resulted in $PO_2 = 26 \pm 2$ mmHg, $PCO_2 = 34 \pm 3$ mmHg and pH = 7.4 ± 0.07, $n = 12$. (C) Normoxia resulted in $PO_2 = 147 \pm 8$ mmHg, $PCO_2 = 33 \pm 3$ mmHg and pH = 7.4 ± 0.09, $n = 8$.

Precontraction

U46619 (10^{-7}–10^{-6} M), KCl (40–60 mM) and ET ($10^{-7.5}$ M) were compared as precontracting agents at different oxygen supply. During hypoxic conditions U46619 (10^{-7}–10^{-6} M) and KCl (40–60 mM) produced lower precontraction levels compared to hyperoxia. It also appears that the contraction produced by ET-1 was slightly reduced under hypoxic conditions compared to hyperoxia and normoxia, although not significant. However, the precontraction obtained 10 min after application of ET-1 ($10^{-7.5}$ M) (8 ± 1.2 mN mm^{-1} at 95% O_2; $n = 10$ and 6.9 ± 1.1 mN mm^{-1} at 95% N_2; $n = 10$) was relatively stable and lasted for at least 1 h (6.5 ± 1.5 mN mm^{-1} at 95% O_2; $n = 10$ and 5.8 ± 1.4 mN mm^{-1} at 95% N_2; $n = 10$). To determine how much influence the level of precontraction had on the subsequent αCGRP concentration–response curves, different concentrations of ET-1 were tested during hyperoxia. Precontraction with ET-1 (10^{-7} M) induced 9.6 ± 1.7 mN mm^{-1} ($n = 8$), whereas ET-1 (10^{-8} M) induced 6.1 ± 1.6 mN mm^{-1} ($n = 10$). The subsequent αCGRP concentration–response curves generated from these experiments were almost identical with the ET-1 ($10^{-7.5}$ M)/αCGRP curves (data not shown).

αCGRP-induced vasodilatation

αCGRP induced concentration-dependent relaxation of the porcine coronary arteries. The results clearly indicate that the effect of αCGRP is enhanced under hypoxic conditions ($pEC_{50} = 9.2 \pm 0.03$, mean ± s.e.m.) compared to hyperoxic ($pEC_{50} = 8.6 \pm 0.1$) and normoxic ($pEC_{50} = 8.5 \pm 0.05$) conditions (Figure 2a–c and Table 1). At 95, 21 and 0% oxygen supply, preincubation with $\alpha CGRP_{8-37}$ induced concentration-dependent (10^{-7}–10^{-5} M) rightward shift of the αCGRP concentration–response curves (Figure 2a–c). The Schild-plot-derived pK_B values increased significantly under hypoxia compared to hyperoxia and normoxia, indicating an approximately 10-fold affinity increase for $\alpha CGRP_{8-37}$ during hypoxia (Figure 2d). The concentration–response curves obtained in the endothelium-denuded group were almost identical compared to experiments with endothelium intact vessels (Table 1). The increased potency of αCGRP and $\alpha CGRP_{8-37}$ (10^{-6} M) under hypoxia was also observed in the experiments performed using the time protocol shown in Figure 1b. Thus, under hyperoxia the pEC_{50} was 8.5 ± 0.1, mean ± s.e.m. ($n = 6$) and 7.6 ± 0.1 ($n = 8$) for αCGRP and $\alpha CGRP_{8-37}$ (10^{-6} M), respectively, whereas the pEC's$_{50}$ in the hypoxia experiments were 9.0 ± 0.1 ($n = 7$) and 7.2 ± 0.1 ($n = 6$), respectively (concentration–response curves not shown), almost identical results compared to the data obtained using the time protocol shown in Figure 1a.

AM- and AMY-induced vasodilatation

As for αCGRP hypoxia altered the pEC_{50} values for AMY, whereas the AM response was not significantly changed (Figure 3a and b). Using $\alpha CGRP_{8-37}$ in 10^{-7} and $10^{-6.5}$ M, the apparent pK_B values increased significantly during hypoxia compared with hyperoxic conditions for both peptides (Table 1).

cAMP production

In control vessels without agonist the basal cAMP concentration was 65 ± 9 fmol mg^{-1} tissue ($n = 6$), during hyperoxia *versus* 55 ± 6 fmol mg^{-1} tissue ($n = 6$) during hypoxia (NS). Thus, whereas hypoxia by itself had no significant effect on

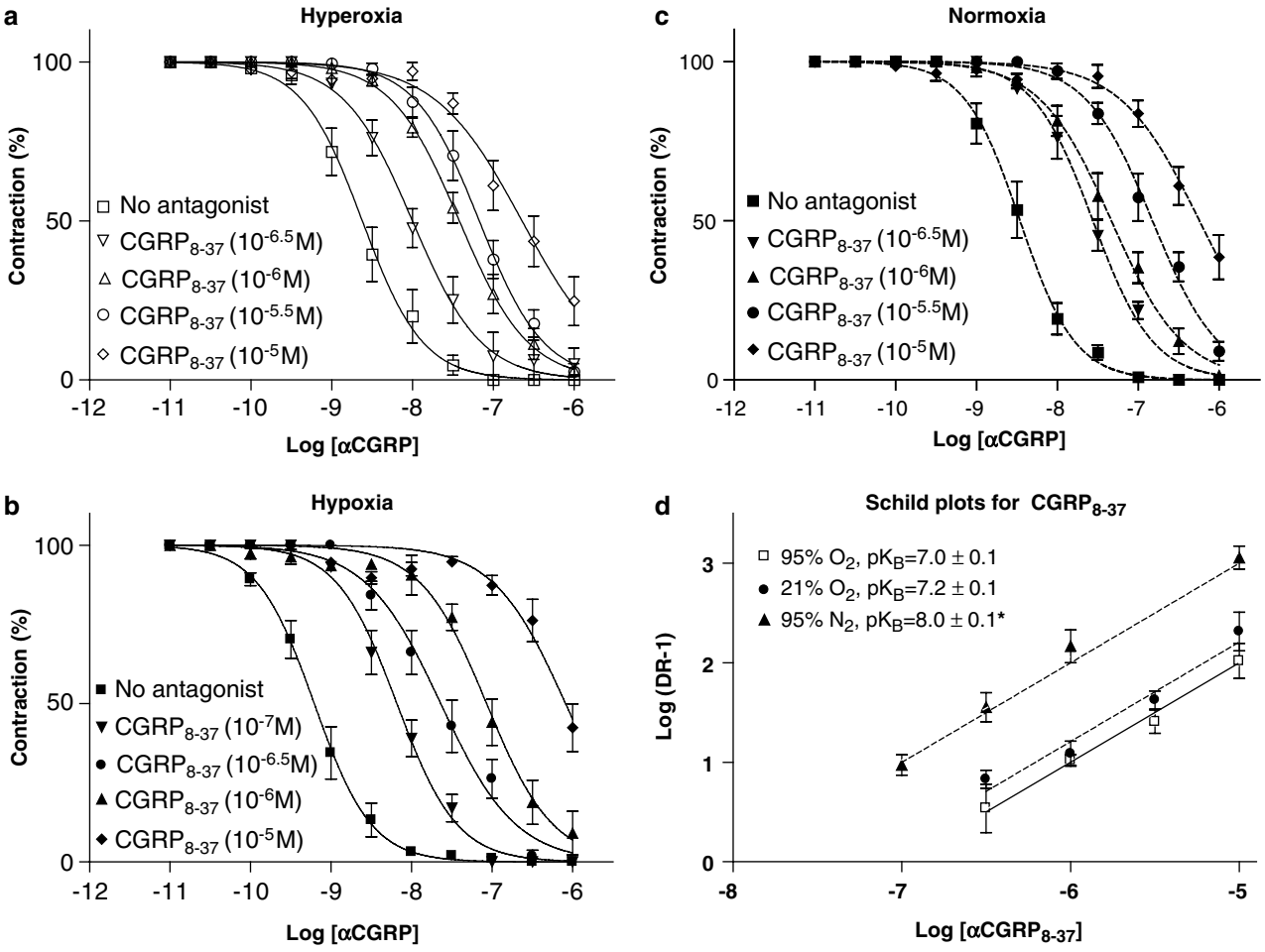

Figure 2 (a–c) αCGRP (10^{-11}–10^{-6} M) concentration–response curves under hyperoxia, hypoxia or normoxia in the presence or absence of $\alpha CGRP_{8-37}$ (10^{-7}–10^{-5} M) in vessels with endothelium. Relaxant responses are given as percentage of precontraction induced by ET-1 ($10^{-7.5}$ M). Points represent mean values and vertical lines indicate s.e.m. (d) Schild plots for $\alpha CGRP_{8-37}$ (10^{-7}–10^{-5} M) tested with αCGRP as agonist in isolated porcine LAD coronary arteries during hyperoxia, normoxia or hypoxia. Each point represents mean values and vertical lines indicate s.e.m. *Significant difference between the pK_B values obtained under hypoxia *versus* hyperoxia and normoxia.

arterial wall cAMP concentrations, these were significantly increased in presence of αCGRP. αCGRP also showed higher potency during hypoxia ($pEC_{50} = 8.6 \pm 0.1$ during hypoxia *versus* 8.2 ± 0.1 during hyperoxia (Figure 3c)). The same effects are seen for AMY (10^{-6} M) (Figure 3b), while no significant changes in cAMP concentrations or potency are demonstrated for AM (Figure 3a).

Role of adenosine, K_{ATP} channels, vasodilator prostanoids, NOS and peptidase inhibitors

Adenosine induced concentration-dependent relaxation with pEC_{50} values of 4.1 ± 0.1 ($n = 6$) mean ± s.e.m. (Figure 4). Preincubation with CSC (10^{-3} M, $n = 6$) induced the expected pronounced rightward shift of the adenosine concentration–response curve.

In subsequent studies we found no significant alteration of the αCGRP concentration–response curves under hypoxia and hyperoxia (Figure 2a and b) despite preincubation with CSC (10^{-3} M, $n = 8$), K_{ATP} channel inhibitor glibenclamide (10^{-5} M, $n = 7$), the cyclooxygenase inhibitor indomethacin (10^{-3} M, $n = 8$), L-NMMA (10^{-4} M, $n = 8$) or peptidase inhibitors ($n = 8$) (data not shown).

Iso- and SNP-induced vasodilatation

Both Iso and SNP induced concentration-dependent relaxation of the porcine coronary arteries. But, whereas the effect of Iso is enhanced under hypoxic conditions ($pEC_{50} = 7.9 \pm 0.09$; $n = 6$ during hypoxia *versus* $pEC_{50} = 7.1 \pm 0.1$; $n = 6$ during hyperoxia ($P < 0.05$)), the SNP response ($pEC_{50} = 6.2 \pm 0.12$, $n = 5$ during hypoxia *versus* $pEC_{50} = 6.4 \pm 0.13$, $n = 6$ during hyperoxia (NS)) is not (data not shown).

Real time RT–PCR

The standard curve was straight for all primers employed, yielding slopes between 2.8 and 3.2, indicating replication efficiencies close to 1 (a replication efficiency of unity would yield a slope of 3.3). All standard curves regressed with correlations between 0.98 and 0.99, indicating a uniform PCR product independent of cDNA concentrations. Electrophoresis

Table 1 pEC_{50} values of αCGRP, AM and AMY and the effect of the antagonist $\alpha CGRP_{8-37}$

Agonist	*Antagonist (log M)*	*Oxygen supply*	*Endothelium*	n	*pEC_{50}*	*Apparent pK_B*
αCGRP	—	**95%**	+	**12**	**8.6±0.1**	—
	$\alpha CGRP_{8-37}$ (−6.5)	95%	+	11	8.0±0.05[a]	—
	$\alpha CGRP_{8-37}$ (−6)	95%	+	10	7.6±0.03[a]	—
	$\alpha CGRP_{8-37}$ (−5.5)	95%	+	7	7.2±0.04[a]	—
	$\alpha CGRP_{8-37}$ (−5)	95%	+	9	6.6±0.06[a]	—
	—	**95%**	−	**12**	**8.6±0.07**	—
	$\alpha CGRP_{8-37}$ (−6)	95%	−	11	7.5±0.05[a]	7.1 (7.0–7.2)
	—	**21%**	+	**8**	**8.5±0.05**	—
	$\alpha CGRP_{8-37}$ (−6.5)	21%	+	7	7.6±0.06[a]	—
	$\alpha CGRP_{8-37}$ (−6)	21%	+	7	7.3±0.08[a]	—
	$\alpha CGRP_{8-37}$ (−5.5)	21%	+	6	6.8±0.07[a]	—
	$\alpha CGRP_{8-37}$ (−5)	21%	+	8	6.2±0.06[a]	—
	—	**0%**	+	**12**	**9.2±0.03[b]**	—
	$\alpha CGRP_{8-37}$ (−7)	0%	+	10	8.2±0.03[a]	—
	$\alpha CGRP_{8-37}$ (−6.5)	0%	+	7	7.6±0.05[a]	—
	$\alpha CGRP_{8-37}$ (−6)	0%	+	13	7.1±0.05[a]	—
	$\alpha CGRP_{8-37}$ (−5)	0%	+	12	6.1±0.05[a]	—
	—	**0%**	−	**11**	**9.1±0.04[b]**	—
	$\alpha CGRP_{8-37}$ (−6)	0%	−	11	7.2±0.04[a]	7.9 (7.8–8.0)[b]
AM	—	**95%**	+	**8**	**6.9±0.04**	—
	$\alpha CGRP_{8-37}$ (−6.5)	95%	+	9	6.2±0.05[a]	7.1 (7.0–7.2)
	—	**0%**	+	**8**	**7.2±0.04**	—
	$\alpha CGRP_{8-37}$ (−6.5)	0%	+	9	6.0±0.05[a]	7.6 (7.5–7.7)[b]
AMY	—	**95%**	+	**8**	**6.3±0.06**	—
	$\alpha CGRP_{8-37}$ (−7)	95%	+	8	6.0±0.06[a]	7.0 (6.7–7.2)
	—	**0%**	+	**8**	**6.8±0.02[b]**	—
	$\alpha CGRP_{8-37}$ (−7)	0%	+	8	5.9±0.11[a]	7.9 (7.6–8.1)[b]

Where only a single concentration of antagonist was used, apparent pK_B values have been calculated for $\alpha CGRP_{8-37}$. Experiments without antagonists are shown with bold characters.
[a]Significant difference between values compared with the above underlined pEC_{50} value.
[b]Significant difference between the hyperoxia/normoxia and the hypoxia groups.

verified only one product for each primer pair at the expected size (data not shown).

The β-actin was used as internal reference and relative amounts of mRNA for the CL receptor, RAMP1 and RAMP2 are presented in Figure 5. The mRNA for RAMP1 was higher than mRNA for CL receptor and RAMP2 in the porcine coronary arteries, but there was no difference between the hypoxic and hyperoxic conditions.

Discussion

This paper reports three new observations: (1) hypoxia results in increases in the potency of both αCGRP- and AMY- but not AM-induced vasodilatation, (2) increases in cAMP formation induced by αCGRP and AMY but not AM and (3) a 10-fold increase in affinity of $\alpha CGRP_{8-37}$ during hypoxic conditions.

Hypoxia strongly potentiates the vasorelaxant response of αCGRP and AMY, but does not alter the effect of AM significantly. In the early 1990s Kwan *et al*. (1990) described similar shift of the αCGRP concentration–response curve during hypoxia as compared to hyperoxia in sheep coronary arteries, but the phenomenon was not investigated further.

The increase in agonist potency during hypoxic conditions can be explained by one or more of the following possibilities alone or in combinations: (1) changes in signal transduction, (2) increase in number of receptors, (3) participation of another vasodilator agonist released from the vessel by hypoxia (e.g. adenosine), (4) peptidase inhibitors and/or (5) conformational changes in the receptor itself induced by hypoxia.

In the porcine coronary arteries, CGRP uses an NO- and endothelium-independent pathway, which correlates closely with a rise in intracellular cAMP (Kageyama *et al*., 1993; Yoshimoto *et al*., 1998; Wisskirchen *et al*., 1999). Whereas hypoxia has no effect on arterial cAMP levels in the present study, it significantly potentiates the production of cAMP stimulated by αCGRP and AMY, while AM shows no change in cAMP production. Comparing the estimated pEC_{50} values obtained from the concentration–response curves (Table 1 and Figure 3c), hypoxia increases the vasorelaxation of αCGRP four-fold and the cAMP production two-fold. Since the enhancement in the effect of CGRP is reflected by an increase in CGRP-induced accumulation of cAMP, it seems probable that the changes occur at the receptor level or anyway before cAMP production. Probably the mechanisms reside in the smooth muscle cells since endothelium denudation has no effect on the αCGRP-induced vasorelaxation.

Quantitative real-time PCR shows that acute hypoxia does not increase expression of mRNAs for CGRP or AM receptors, which is not unexpected, since others (Moller *et al*., 2002) have found that induction requires a time span of at least 7 h. However, since recruitment of receptor proteins within the applied time frame from intracellular compartments may be a possibility, Western blotting or immunofluorescence experiments could have been relevant. But so far no specific antibodies are commercially available for the porcine CL receptor or RAMPs. Interestingly, higher levels of RAMP1

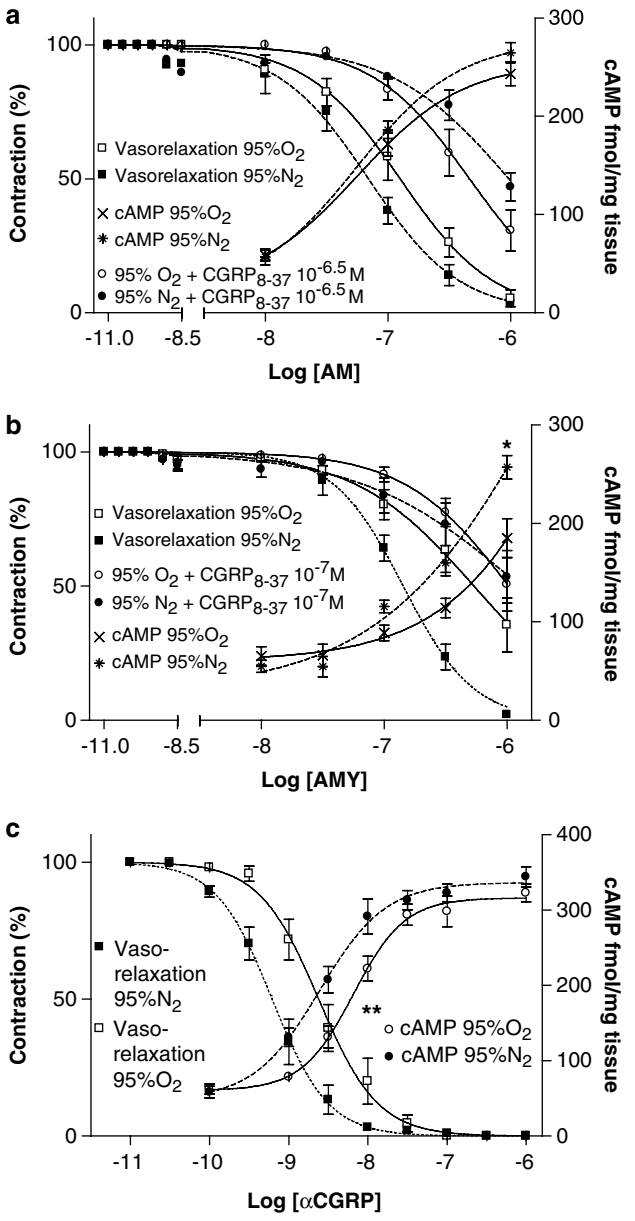

Figure 3 (a–c) cAMP production concentration–response curves during hyperoxia (95% O_2/5% CO_2) and hypoxia (95% N_2/5% CO_2) stimulated by AM (a), AMY (b) or αCGRP (c), illustrated together with the concentration–response curves for AM (a), AMY (b) or αCGRP (c) with or without $\alpha CGRP_{8-37}$. Relaxant responses are given as percentage of precontraction induced by ET-1 ($10^{-7.5}$ M). Data are mean ± s.e.m. ($n = 6$ in the cAMP groups). *Significant difference between the AMY (10^{-6} M)-induced cAMP production in the hypoxia group *versus* the hyperoxia group. **Significant difference between the αCGRP-induced cAMP production (pEC_{50} values) in the hypoxia group *versus* the hyperoxia group. All other comparisons are made by EC_{50} values and appear in Table 1.

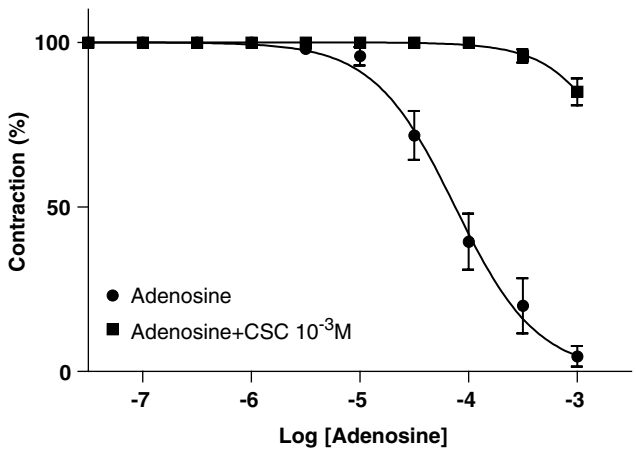

Figure 4 Adenosine concentration–response curves during hyperoxia with and without CSC (10^{-3} M). Relaxant responses are given as percentage of precontraction induced by ET-1 ($10^{-7.5}$ M). Points represent mean values and vertical lines indicate s.e.m. ($n = 6$ in each group).

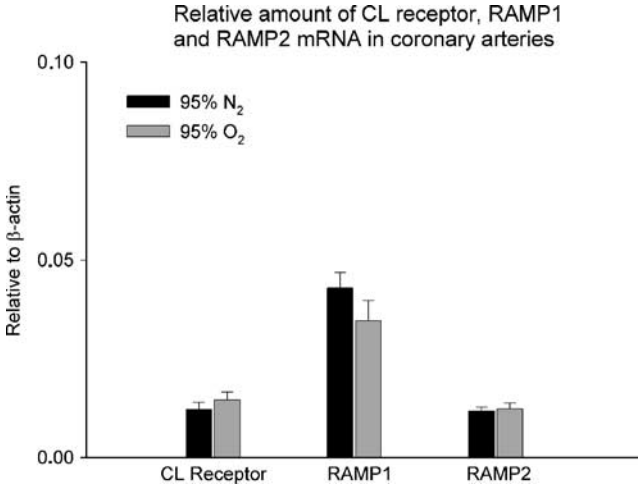

Figure 5 Amount of CL receptor, RAMP1 and RAMP2 mRNA in porcine coronary arteries in buffer solution exposed to either 95% O_2/ 5% CO_2 or 95% N_2/ 5% CO_2 for 4 h and relative to β-actin mRNA levels. Data are mean ± s.e.m. ($n = 6$ in each group).

mRNA are detected in porcine coronary artery compared to both RAMP2 and CL receptor mRNA. Knowing that RAMP1 is dominant over RAMP2 in binding the CL receptor (Buhlmann *et al*., 1999), it is likely that the formation of $CGRP_1$ receptors is more common than AM receptors in porcine coronary arteries.

It is thought that compromised ATP synthesis during hypoxia or increased myocardial workload results in the release of adenosine from myocardial cells and dilates coronary arteries, helping to match coronary blood flow with metabolic demand (Berne, 1980; Mubagwa *et al*., 1996). Adenosine release from the arteries may therefore potentially be involved in the increased potency of CGRP during hypoxia. Although four adenosine receptor subtypes (i.e. A_1, A_{2A}, A_{2B} and A_3) have been cloned from various tissues, the vasodilatation elicited by adenosine in porcine coronary arteries has been shown predominantly to be induced by the A_{2A} receptor (Lew & Kao, 1999; Hein *et al*., 2001). But, even though the commercially available A_{2A} receptor antagonist CSC (10^{-3} M) induces potent adenosine blocking, it has no effect on the increased CGRP potency during hypoxia. The endothelium, K_{ATP}-channels and vasodilator prostanoids have also been suggested as contributors to hypoxic vasodilatation in the

coronary arteries (Daut *et al*., 1990; Graser & Rubanyi, 1992; Mellemkjaer & Nielsen-Kudsk, 1994; Liu & Flavahan, 1997; Fukuda *et al*., 1999). But, neither removal of the endothelium nor preincubation with the K_{ATP} channel inhibitor glibenclamide or the cyclooxygenase inhibitor indomethacin changed the concentration–response curves for CGRP under hyperoxia/hypoxia. Neither peptidase inhibitors nor NOS seem to be involved in the mechanism of sensitisation of the vessels in hypoxia.

Using the current classification for $CGRP_1/CGRP_2$ receptors (for a review, see Juaneda *et al*., 2000) the apparent pK_B and the pK_B values obtained during hyperoxia in this study were in agreement with our previous studies (Hasbak *et al*., 2001; 2003), indicating that the vasorelaxant effect of αCGRP, AM and AMY can be explained by interaction with the $CGRP_1$ receptor. Interestingly, the $\alpha CGRP_{8-37}$ antagonist gains affinity against all peptides under hypoxia compared to hyperoxia and normoxia, indicating that this antagonist has variable affinities for the $CGRP_1$ receptor depending on the oxygen level in the experimental setting. Although $\alpha CGRP_{8-37}$ gains affinity against all peptides, it should be noticed that the apparent pK_B for $AM/\alpha CGRP_{8-37}$ is significantly lower than the pK_B values for $CGRP/\alpha CGRP_{8-37}$ and $AMY/\alpha CGRP_{8-37}$. Since $\alpha CGRP_{8-37}$ is a fragment of αCGRP, it is likely that both have the same affinity at the receptor site, and thus that αCGRP has increased affinity at the $CGRP_1$ receptor during hypoxia as well.

When co-expressed with RAMPs, the calcitonin (CT) receptor functions as an AMY receptor (Poyner *et al*., 2002). But porcine CT receptor mRNA is not present in the LAD coronary arteries (Hasbak *et al*., 2001). So considering that the AMY vasorelaxant response and the AMY-induced cAMP production are potentiated by hypoxia and blocked by the $\alpha CGRP_{8-37}$ with identical pK_B values as for $\alpha CGRP/\alpha CGRP_{8-37}$, it is very likely that AMY mediates vasorelaxation *via* the $CGRP_1$ receptor.

While hypoxia increases both αCGRP and AMY agonist potencies, in producing coronary vasodilation and cAMP generation, the effects produced by AM are not potentiated despite a substantial expression of RAMP2 (Figure 5). Hypoxia also increases the antagonist potency of $\alpha CGRP_{8-37}$ against all peptide agonists, including AM. The mechanisms underlying these changes remain unclear. In particular: αCGRP and AMY-induced effects are potentiated *via* increased production of cAMP. Apparently this is not due to increase in the expression of the receptors. On the other hand, the failure of hypoxia to increase AM-mediated effects is probably due to a different signal transduction mechanism activated by AM, as compared to αCGRP or AMY. The mechanisms *via* which AM can elicit vascular relaxation in the porcine coronary arteries are incomplete understood, but are known to involve both the CGRP and AM receptors as AM has been shown to induce vascular relaxation *via* either $CGRP_{8-37}$-sensitive or AM_{22-52}-sensitive mechanisms (Poyner *et al*., 2002). Furthermore, there is evidence that AM can act *via* an endothelium-dependent (NO-dependent) mechanism (Yoshimoto *et al*., 1998), which then relaxes the smooth muscle cells through activation of guanylate cyclase and accumulation of cGMP, which is in contrast to αCGRP and AMY.

This is indirectly supported by our Iso and SNP data. Acting through cAMP Iso shows increased sentisation during hypoxia, confirming the data of Fukuda *et al*. (1999), whereas SNP (a NO donor acting through cGMP) was unaffected by hypoxia. Perhaps the weak AM response during hypoxia can be explained by interaction with AM receptors activating NO/cGMP-dependent pathways compared to the cAMP-dependent pathway activated by the binding of CGRP/AMY to the $CGRP_1$ receptor. Taken together, these results indicate a complexity of the receptor system(s) for the CGRP superfamily of peptides in contrast to what we have previously suggested (Hasbak *et al*., 2001).

An explanation for the increase in $\alpha CGRP/\alpha CGRP_{8-37}$ potency is changes in receptor conformation during hypoxia. It is possible that hypoxia alters the $CGRP_1$ receptor-binding site, thereby increasing the affinity of αCGRP and AMY and maybe also AM for the receptor compared to binding in its normoxic state. A hypoxia-induced modification of the $CGRP_1$ receptor recognition site may be due to change in allosteric structure of the receptor, as suggested by Fritz *et al*. (1996).

In conclusion, hypoxic incubation potentiates the relaxation effect and cAMP production of CGRP and amylin in rings of porcine coronary arteries *in vitro*. This is an endothelium-independent effect, thus occurring in the smooth muscle cells. It is neither caused by the release of adenosine nor vasodilator prostanoids, and is not due to K_{ATP} channels, NOS, peptidae inhibitors or related to changes in CGRP or AM receptor mRNA. Moreover, $\alpha CGRP_{8-37}$ showed increased affinity at the $CGRP_1$ receptor during hypoxia possibly due to a conformational change at the $CGRP_1$ receptor site.

Possible physiological/pathophysiological implications

The hypoxia-induced potentiation of the CGRP and AMY vasorelaxant effect in the coronary vascular bed may be a compensatory haemodynamic mechanism to protect the hypoxic myocardium. This phenomenon may be a normal physiological response in the coronary circulation for many vasoactive substances since similar augmentation of the coronary vasorelaxant responses during hypoxia has previously been described, for example, adenosine, noradrenalin (Kwan *et al*., 1989), neuropeptide Y (Kwan *et al*., 1990), nitroglycerin (Fukuda *et al*., 1994) and Iso (Fukuda *et al*., 1999). Allosteric conformational changes of receptors resulting in increased affinity for signal molecules are possibly a general phenomenon in hypoxic environment.

The increased CGRP-receptor affinity of $\alpha CGRP_{8-37}$ is not only scientifically interesting but may also have clinical implications as well. Nonpeptide CGRP receptor antagonists are in advanced clinical development for migraine, and it is indeed important to determine if such CGRP receptor antagonists have increased affinity for the CGRP receptor during hypoxia. The implications of hypoxia-induced increased affinity of antagonists must be considered seriously as this may indicate a potential therapeutical problem for patients with ischaemic heart disease.

The study was supported by the following foundations: 'The Danish Heart Foundation', Grant No. 01-2-2-73-22943; 'The Danish Hospital Foundation for Medical Research', 'The Danish Medical Association Research Fund', 'Lundbeck Foundation' and 'Danish Medical Research Council'. We thank Kirsten Busk, Majken Gudmundsson and Helle Ludvig for excellent technical assistance.

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(Received February 8, 2005
Accepted March 14, 2005
Published online 18 April 2005)