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Non-competitive antagonism of amylin on $CGRP_1$-receptors in rat coronary small arteries

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1 We examined the interaction between rat-amylin and relaxations induced by rat-αCGRP and isoprenaline in rat isolated coronary small arteries.

2 Amylin, 0.1–100 nM, had a concentration dependent non-competitive antagonistic effect on rat-αCGRP-induced responses with an EC_{50} of approximately 1 nM. Amylin did not affect the relaxations induced by isoprenaline at a concentration of 10 nM.

3 The apparent equilibrium dissociation constant, K_A, for $CGRP_1$-receptors in the rat coronary small arteries was approximately 2 nM. Analysis of the relationship between receptor occupancy and response to rat-αCGRP indicates that the receptor reserve is small.

4 Our results show that amylin in low concentrations acts as a selective non-competitive inhibitor at $CGRP_1$-receptors in rat isolated coronary small arteries.

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Abbreviations: Ca^{2+}, calcium; CGRP, calcitonin gene-related peptide; EC_{50}[M], concentration of agonist required to give half maximal response; EDTA, ethylene diamine tetra-acetic acid; EGTA, ethylene glycolbis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid; K^+, potassium; K_A[M], apparent receptor agonist equilibrium dissociation constant; pD_2, sensitivity = $-\log(EC_{50}[M])$; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; pK_A, receptor agonist affinity = $-\log(K_A[M])$; PSS, physiological salt solution; rat-αCGRP, rat-αcalcitonin gene-related peptide; R/R_{max}, relative vessel response to agonist; R/R_t, relative receptor agonist occupancy

Introduction

Calcitonin gene-related peptide (CGRP) is released from the perivascular sensory nerve endings in the wall of flow regulating intramural coronary arteries both *in vitro* (Franco-Cereceda & Lundberg, 1985; Franco-Cereceda *et al*., 1989) and *in vivo* (Kallner, 1998) under ischaemic conditions. Activation of these sensory nerve endings has two major outcomes. First, the release of CGRP leads to a profound coronary vasodilation and increase in heart blood flow, and second CGRP causes an increase in contractile force and frequency in the atria (Kallner, 1998). This implies that CGRP has an important physiological counteracting action in the heart in emergency situations.

CGRP mediates its vasodilatation through specific receptors subdivided on the basis of in-vitro pharmacological analysis of selective peptide agonists and antagonists into $CGRP_1$ and $CGRP_2$-receptors (Poyner, 1995). Two other peptides, amylin and adrenomedullin, are relatively homologous to CGRP, which makes the pharmacological characterization of CGRP, amylin and adrenomedullin receptors difficult. This is further strengthened by molecular genetic analysis of the CGRP receptor. These studies indicate that a 7-transmembrane (7-TM) receptor, named calcitonin receptor like receptor, in association with a receptor activity modifying protein (RAMP) determines the receptor complex affinity to CGRP, amylin and adrenomedullin, respectively (McLatchie *et al*, 1998; Muff *et al*., 1998; 1999).

We have recently characterized the CGRP receptor in rat coronary small arteries to belong to the $CGRP_1$-receptor subtype (Sheykhzade & Nyborg, 1998a). In these experiments we found amylin to be a very weak agonist causing relaxations at concentration higher than 1 μM, presumably mediated *via* an interaction with $CGRP_1$-receptors (Beaumont *et al*., 1995; Vine *et al*., 1996). Because of the weak agonistic action on $CGRP_1$-receptors amylin may be expected to possess antagonistic action in the lower concentration ranges against CGRP if its affinity is high but its efficacy is low at $CGRP_1$-receptors.

We tested this hypothesis in our study by investigating the effect of rat-amylin on the rat-αCGRP concentration response relations in isolated rat coronary small arteries. Furthermore, we tested the effect of amylin on beta-adrenoceptor mediated relaxations induced by isoprenaline in arteries contracted with $PGF_{2\alpha}$.

Methods

Male Sprague Dawley rats (3 months old) were killed by cervical dislocation and the heart was rapidly removed and placed in ice-cold physiological salt solution (PSS) (composition given in Drug section below) as previously described (Nyborg *et al*., 1987).

Arterial ring segments were isolated from the same anatomical location in the distal, intramural, part of the left coronary artery in hearts from 3-month-old male Sprague Dawley rats (Nyborg *et al*., 1987) and mounted on an isometric myograph as previously described (Mulvany & Nyborg, 1980).

The arteries were equilibrated at 37°C for 30 min in oxygenated (5% CO_2 in O_2) PSS. The vessels were then stretched to an 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 mmHg

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(13.3 kPa) (Nyborg *et al*., 1987) in order to secure maximal active force development. The effective vessel lumen diameter was calculated as L_1/π.

The vessels were repetitively contracted with 125 mM K^+-PSS (similar to PSS except that NaCl was exchanged for KCl on an equimolar basis) until reproducible contractions were recorded. The maximal contractile response of the vessels was then determined by measuring the difference in vessel wall force during contraction with activating solution (125 mM K^+-PSS to which 10 μM $PGF_{2\alpha}$ and 5-hydroxytryptamine were added) and in Ca^{2+}-free PSS (similar to PSS except that $CaCl_2$ was replaced with 0.01 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)).

The effect of rat-amylin (0.1 – 100 nM) on the rat-αCGRP concentration response characteristics was determined by constructing two cumulative concentration-response curves to CGRP (10 pM – 100 nM) in vessels contracted with 10 μM $PGF_{2\alpha}$. The first curve served as a control and the second was made in the presence of amylin. The vessels were contracted twice with 125 mM K^+-PSS between each rat-αCGRP concentration-response experiment in order to secure reproducibility of coronary artery reactivity to CGRP (Sheykhzade & Nyborg, 1998b).

In order to investigate if the effect of amylin was non-selective in rat coronary arteries, control experiments were carried out by constructing two cumulative concentration response curves to isoprenaline (1 nM – 10 μM) in vessels contracted with 10 μM $PGF_{2\alpha}$. The first curve served as a control and the second was made in the presence of 10 nM amylin.

Drugs

PSS had the following composition (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 1.5, K_2HPO_4 1.18, $MgSO_4$ 1.17, ethylene diamine tetra-acetic acid (EDTA) 0.026 and glucose 5.5, pH 7.4. Drugs used were rat-αcalcitonin gene-related peptide (rat-αCGRP), rat-amylin, (−)-isoprenaline HCl and 5-hydroxytryptamine HCl (Sigma-Aldrich, St Louis, MO, U.S.A.), $PGF_{2\alpha}$ (Dinoprost®, Upjohn, Puurs, Belgium). Rat-αCGRP and rat-amylin were dissolved in acidified distilled water and stored frozen until use. Dilutions of the stock solutions were made fresh each day.

Data analysis and statistics

Relaxations are expressed as a percentage of the $PGF_{2\alpha}$-induced tensions and $PGF_{2\alpha}$-induced tensions are expressed as a percentage of maximal contractile response of the vessels.

The concentration-response curves to rat-αCGRP were fitted to the classical 'Hill-equation': $R/R_{max} = [A]^n/([A]^n + EC_{50}[M]^n)$ using the GraphPad Prism 2.01 software. R/R_{max} is the relative vessel response to the agonist concentration, A[M]. EC_{50}[M] is concentration of agonist required to give half maximal response, and n is a fitting constant or 'Hill-coefficient'.

Because of the non-competitive antagonistic action of amylin we were able to estimate the apparent $CGRP_1$-receptor agonist affinity by applying the same mathematical method for receptor agonist affinity determination first described by Furchgott & Bursztyn (Furchgott, 1966; Furchgott & Bursztyn, 1967). Reciprocals of equieffective concentrations of CGRP in control condition (A[M]) and in the presence of 1 nM amylin (A′[M]) were determined on basis of non-linear regression analysis of the average concentration response curves data ($n = 5$). The slope of the regression line (least square method) and the y-axis intercept with 95% confidence interval was estimated in a plot of 1/A[M] vs 1/A′[M] using the GraphPad Prism 2.01 software. The estimated K_A[M] was used to estimate the relative $CGRP_1$-receptor occupancy, R/R_t, according to the equation derived by Furchgott & Byrsztyn (1967), $R/R_t = A[M]/(A[M] + K_A[M])$. The receptor reserve was calculated as $pD_2 - pK_A$.

Vessel sensitivity, EC_{50}[M], to rat-αCGRP and the $CGRP_1$-receptor dissociation constant, K_A[M], are presented in the text as pD_2 and pK_A values, respectively, where $pD_2 = -\log(EC_{50}[M])$ and $pK_A = -\log(K_A[M])$.

Results are given as mean ± s.e.mean (n = number of vessels). Differences between mean values were analysed by Student's t-test. Results were considered to be significant if P value < 0.05.

Results

Effect of amylin on CGRP-induced relaxations

Rat-αCGRP induced a concentration dependent relaxation of rat isolated coronary small arteries with a pD_2 of 9.03 ± 0.04 ($n = 12$) in control condition (Figure 1). The mean effective lumen diameter of vessels used was 203 ± 10 μm ($n = 12$). The response induced by 10 μM $PGF_{2\alpha}$ was 71 ± 4% ($n = 12$) of the maximal contractile response of the vessels. The rat-αCGRP concentration-response curve was concentration dependently inhibited in a non-competitive fashion by rat-amylin (Figure 1). The sensitivity of the vessels to rat-αCGRP was slightly reduced, the pD_2 decreasing to 8.94 ± 0.01 ($n = 4$) and 8.76 ± 0.07 ($n = 5$) in the presence of 0.1 and 1 nM amylin, respectively. The concentration causing 50% inhibition of the

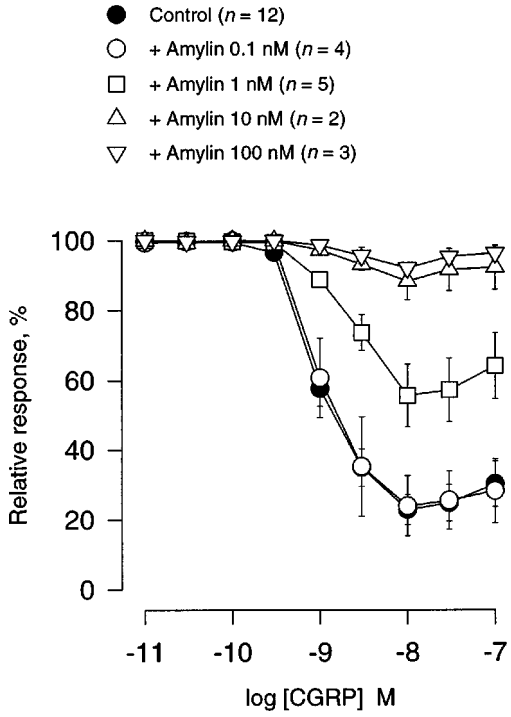

Figure 1 Effect of increasing concentrations of rat-amylin on rat-αCGRP concentration-response relations in rat isolated coronary small arteries. The responses are expressed as per cent of the initial contraction induced by 10 μM $PGF_{2\alpha}$ immediately before addition of the first concentration of rat-αCGRP. Points represent mean values and vertical bars show ± s.e.mean, where this exceeds the size of the symbol.

rat-αCGRP response was close to 1 nM. Amylin at 100 nM did not cause significant relaxation of the spontaneous basal tone in the coronary arteries.

The $CGRP_1$-receptor agonist dissociation constant, $K_A[M]$ determined in the presence of 1 nM rat-amylin (Figure 2) was 1.97 nM (1.89–2.07 nM, 95% confidence interval). The $CGRP_1$-receptor agonist affinity, $pK_A[M]$ was thus 8.70 (8.72–8.62, 95% confidence interval). The relationship between the relative $CGRP_1$-receptor agonist occupancy, R/R_t, is depicted in Figure 3. Approximately 36% of all active $CGRP_1$-receptors must be occupied for eliciting half-maximal response to rat-αCGRP. The receptor reserve calculated as $pD_2 - pK_A$ was equal to 0.26 (antilog value = 1.83) for the five vessels exposed to 1 nM amylin, their pD_2 value in control condition being equal to 8.96 ± 0.04 ($n = 5$).

Effect of amylin on isoprenaline-induced relaxations

Amylin at a concentration of 10 nM had no effect on isoprenaline concentration-response curves in rat isolated coronary arteries (Figure 4). The pD_2 values were 7.56 ± 0.06 vs 7.47 ± 0.06 ($n = 7$) and maximal relaxations were 72 ± 7 vs $73 \pm 8\%$ ($n = 7$), without and with amylin, respectively. The mean effective lumen diameter of vessels used was 211 ± 13 μm ($n = 7$). The responses induced by 10 μM $PGF_{2\alpha}$, without and with amylin, were 73 ± 5 vs $79 \pm 4\%$ ($n = 12$) of the maximal contractile response of the vessels, respectively.

Discussion

Amylin, or islet amyloid polypeptide, is a 37 amino acid peptide with 43% homology to CGRP (Edwards & Morley, 1992) sharing the characteristic ring structure formed by a cystine bridge between amino acid 2 and 7. This feature is also found in adrenomedullin (van-Rossum *et al.*, 1997), although this peptide is 15 amino acids longer than CGRP, but it still contains a disulphide bridge between amino acid 16 and 21. Amylin and adrenomedullin will therefore behave as weak or partial agonists at CGRP-receptors and as full agonists at their

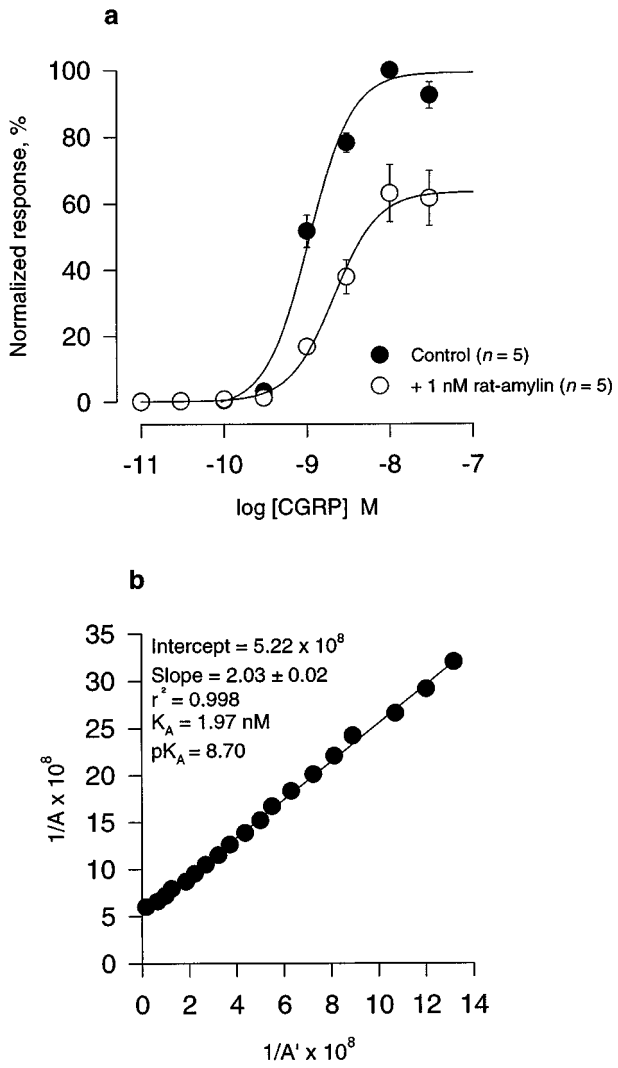

Figure 2 (a) Rat-αCGRP concentration-response relations in control condition and in presence of 1 nM rat-amylin in isolated rat coronary small arteries used for estimation of $CGRP_1$-receptor agonist affinity. Vessel responses have been normalized to the maximal response to rat-αCGRP in each vessel. (b) Regression line for the plot of reciprocals of equieffective concentrations of rat-αCGRP without (A[M]) and in the presence of 1 nM rat-amylin (A′[M]).

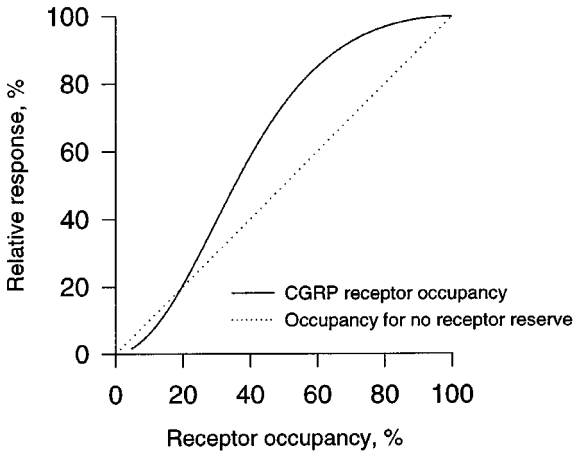

Figure 3 Relationship between relative $CGRP_1$-receptor occupancy and response to rat-αCGRP in isolated rat coronary small arteries. Dotted line indicates occupancy-response relations for an agonist with no receptor reserve.

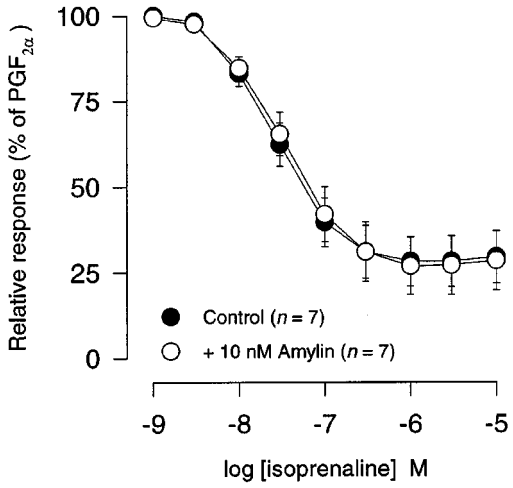

Figure 4 Isoprenaline concentration response relations in isolated rat coronary small arteries in control condition and in presence of 10 nM rat-amylin. The responses are expressed as per cent of the initial contraction induced by 10 μM $PGF_{2\alpha}$ immediately before addition of the first concentration of isoprenaline. Points represent mean values and vertical bars show ±s.e.mean, where this exceeds the size of the symbol.

own respective receptor. Both peptides are therefore used as such in order to characterize and distinguish CGRP receptors from amylin and adrenomedullin receptors *in vitro*.

We have recently characterized the CGRP receptor subtype involved in CGRP-induced relaxation of coronary small arteries precontracted with $PGF_{2\alpha}$ using amylin, adrenomedullin and the selective $CGRP_2$-receptor agonist, [Cys $(Acm)^{2,7}$]CGRP and the competitive $CGRP_1$-receptor antagonist, CGRP8-37. Our data showed that a population of CGRP1-receptor subtype mediated the CGRP-induced relaxation in these arteries (Sheykhzade & Nyborg, 1998a).

Because amylin is a weak agonist at CGRP-receptors it is most likely that its affinity to the $CGRP_1$-receptor in rat isolated coronary small arteries is low. It is therefore surprising to find that amylin acts like a non-competitive antagonist against the CGRP-induced response in rat coronary small arteries. However, a non-competitive behaviour of amylin can be explained if the affinity of amylin to the $CGRP_1$-receptor is higher and its efficacy is much lower than that of CGRP itself. The potent inhibitory action of amylin on the CGRP concentration response relations indeed indicates that amylin has a very high affinity to the CGRP receptor. The EC_{50}[M] for amylin on the CGRP-induced relaxation is close to 1 nM, which is very low and in the same affinity range as competitive receptor antagonists such as beta-adrenoceptor antagonists (Nyborg & Mikkelsen, 1985) and ketanserin (Nyborg, 1991) in this type of arteries.

Because rat-amylin antagonized rat-αCGRP-induced responses non-competitively, we were able to apply the Furchgott-Bursztyn method for estimation of the agonist affinity of the $CGRP_1$-receptor (Furchgott & Bursztyn, 1967). We found a pK_A of 8.7 (K_A approximately 2 nM). [^{125}I]-CGRP is normally used to determine the binding affinity of CGRP-receptors. With this method CGRP has been estimated to have a receptor dissociation constant, K_D, between 0.07 nM in human (Luu *et al*., 1995) and 0.4 nM in bovine (Knock *et al*., 1992) coronary arteries. Our estimated K_A is thus approximately 1 decade higher than the radio-ligand binding data. However, when the agonist receptor dissociation constant is estimated with the Furchgott-Burstzyn method (Furchgott & Bursztyn, 1967) also called 'The irreversible receptor inactivation method', it is normally one or more decades higher than that obtained with radio-ligand binding technique (Oriowo *et al*., 1991). This has theoretically been ascribed to the complex interplay of intracellular second messengers and especially the available amount of intracellular G-proteins (see Kenakin, 1997). If G-protein competition is the main factor reducing the apparent receptor agonist affinity when it is determined with the Furchgott-Bursztyn method, it seems likely that our data indicates a relative unrestricted pool of second messengers involved in $CGRP_1$-receptor transduction pathway.

We determined the relative $CGRP_1$-receptor occupancy and receptor reserve in the rat coronary small arteries. Approximately 36% of all receptors must be occupied by CGRP to elicit a half-maximal response corresponding to a receptor reserve of 0.26 (antilog value = 1.83), which is the ratio between the EC_{50}[M] and K_A[M]. The $CGRP_1$-receptor reserve for rat-αCGRP is therefore relatively low in the small coronary arteries. This is in conjunction with our previous studies on the $5\text{-}HT_2$-receptor reserve using 5-hydroxytryptamine as agonist in rat coronary arteries (Nyborg, 1991), where the smaller intramural arteries had a comparable low receptor reserve for 5-hydroxytryptamine. In comparison, the alpha-adrenoceptor receptor reserve for noradrenaline, which is a full agonist, varies considerably depending on vessel type (Bevan *et al*., 1986; Oriowo *et al*., 1987), however with a tendency towards restriction of the receptor reserve in the smaller cerebral arteries (Laher & Bevan, 1985).

The agonist receptor reserve is relative and depends upon the efficacy of the agonist (Kenakin, 1997). Thus, if rat-αCGRP is not a full agonist we would also determine a low receptor reserve, but it seems unlikely that the intrinsic efficacy of rat-αCGRP should be low since it is the endogenous receptor ligand. However, the receptor density in the coronary arteries will influence the maximal response to rat-αCGRP in any case. We have recently shown that the maximal response to rat-αCGRP is inversely related to the calibre of rat coronary arteries (Sheykhzade & Nyborg, 1998b). Our present results therefore indicate that this observation can be explained by an increase in the $CGRP_1$-receptor density downstream of the coronary vasculature.

Quantitative radio-ligand binding studies on bovine coronary arteries (Knock *et al*., 1992) support the assumption of increasing CGRP-receptor density in the smaller because the binding site density was greater in distal epicardial and myocardial arteries than in proximal epicardial regions of the left anterior descending coronary artery.

Our observation of a non-competitive action of rat-amylin against rat-αCGRP-induced responses may have pathophysiological implications. CGRP released from sensory nerve endings within the coronary circulation and in the atria is believed to be a physiological defence reaction to ischaemia (Mair *et al*., 1990; Lechleitner *et al*., 1992). It may therefore be speculated that amylin plays a role for the poorer outcome in non-insulin dependent diabetes mellitus (NIDDM) patients suffering from an acute myocardial infarction (AMI), because amylin secretion and amylin plasma concentration is increased in these patients (Gagliardino *et al*., 1997) due to peripheral insulin resistance (Edwards & Morley, 1992). However, the possible role of amylin in the impairment of coronary vascular response to CGRP in NIDDM needs to be investigated in appropriate animal models and patients.

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