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Calcitonin gene-related peptide-induced suppression of atrial natriuretic peptide release through receptors for $CGRP_1$ but not for calcitonin and amylin

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Abstract

Calcitonin gene-related peptide (CGRP), a 37-amino acid neuropeptide, is found in the central nervous system as well as in the heart. CGRP shows high sequence homology with amylin, salmon calcitonin, and adrenomedullin. This study aimed to investigate the effect of CGRP on atrial hemodynamics and atrial natriuretic peptide (ANP) release by using isolated perfused beating left atria and to identify its receptor subtypes. Rat α-CGRP (0.1, 1, 10, or 100 nM) increased atrial contractility and suppressed the release of ANP in a concentration-dependent manner. However, cys-CGRP (1 μM), a $CGRP_2$ receptor agonist, slightly decreased ANP release without positive inotropism. Human α-CGRP (1 nM) showed an effect on ANP release similar to that of rat α-CGRP with potent positive inotropism. However, salmon and rat calcitonin (1 μM) caused a slight decrease or no change in ANP release. Pretreatment with a receptor antagonist for $CGRP_1$ [rat α-CGRP-(8–37)] blocked rat α-CGRP-induced suppression of ANP release and positive inotropism, whereas the antagonists for salmon or amylin did not. Therefore, we suggest that rat α-CGRP causes a suppression of ANP release with positive inotropism through the receptor for $CGRP_1$ but not that for calcitonin and amylin.

Keywords: Atrial function; CGRP (Calcitonin gene-related peptide); Calcitonin; ANP (Atrial natriuretic peptide); Amylin

1. Introduction

Cacitonin gene-related peptide (CGRP), a 37-amino acid neuropeptide (Amara et al., 1982) produced by an alternative processing of the calcitonin gene, is found in the central nervous system as well as in peripheral organs. CGRP also exists in the heart, predominantly in the left atrium of rat (Onuoha Phillips et al., 1998), in nerve fibers around coronary vessels, and in the myocardium (Lundberg et al., 1985; Onuoha Phillips et al., 1998). CGRP has potent cardiovascular effects such as vasorelaxation, increased heart rate and cardiac contractility (Fisher et al., 1983; Gennari and Fischer, 1985; Lundberg et al., 1985; Franco-Cereceda et al., 1987). Therefore, CGRP may serve as a neurotransmitter in atrial tissue.

CGRP exists in two isoforms, α- and β-CGRP (Morris et al., 1984; Amara et al., 1982), and mediates its effects via at least two functional receptor subtypes, $CGRP_1$ and $CGRP_2$ receptors (Dennis et al., 1991). CGRP shares homology with a group of peptides including amylin, salmon calcitonin, adrenomedullin, and rat calcitonin (Muff et al., 1995). This indicates that cross-reactions can occur at common receptors. A direct positive inotropic effect of CGRP mediated by both $CGRP_1$ and $CGRP_2$ receptors has been demonstrated in vitro in the isolated auricle and trabeculae of the human atrium (Du et al., 1994; Franco-Cereceda et al., 1987; Saetrum Opgaard et al., 2000), and pig myocardium (Saetrum Opgaard et al., 1999). However, Bell and McDermott (1994, 1995) have demonstrated that CGRP and amylin stimulate the contractile response in rat ventricular myocytes by interaction with $CGRP_1$ receptor. The controversy persists as to the receptor subtypes of CGRP-induced cardiac effects. It has been reported that CGRP accentuates the secretion of atrial natriuretic peptide (ANP) with increased heart rates in rat atrial strips (Yamamoto et al., 1988;

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Schiebinger and Santora, 1989). However, the receptor subtypes involved in the regulation of ANP secretion are not defined. The aim of the present study was to investigate the effects of rat α-CGRP on atrial contractility and ANP release using isolated rat atria and to identify its receptor subtypes. To assess the functional role of the receptor for rat α-CGRP, we used the selective receptor antagonists for $CGRP_1$ [CGRP-(8–37)] (Chiba et al., 1989), amylin [amylin-(8–37) and acetyl-amylin] (Deems et al., 1991), or salmon calcitonin (acetyl-[Asn^{30}, Tyr^{32}] calcitonin-(8–32), AC 187) (Beaumont et al., 1995).

2. Materials and methods

Male Sprague–Dawley rats weighing 300–350 g were used. The investigation was approved by the Ethic Committee in the Institute for Medical Sciences of our University.

2.1. Preparation of perfused beating rat atria

Isolated perfused beating atria were prepared by a previously described method (Cho et al., 1991; Han et al., 2003). In brief, the left atrium was dissected from the heart after killing and fixed into a Tygon cannula. The cannulated atrium was transferred into an organ chamber, immediately perfused with oxygenated HEPES buffer solution at 36.5 °C, and paced at 1.3 Hz (duration 0.3 ms, voltage 40 V). The composition of the HEPES buffer solution was as follows (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, HEPES 10, glucose, and bovine serum albumin (BSA) 0.1%. The pericardial buffer solution contained [^{3}H]-inulin to measure the translocation of extracellular fluid (ECF). Intra-atrial pressure was recorded on a Physiograph (MK-IV, Narco Bio-Systems, Houston, TX) via a pressure transducer (Statham P23Db, Oxnard, CA) and pulse pressure was calculated from the differences in systolic and diastolic intra-atrial pressures. After stabilization for 100 min, the perfusate was collected at 2-min intervals at 4 °C.

2.2. Experimental protocols

Experiments were performed with four groups. Group 1 was atrium perfused with HEPES buffer ($n=6$) throughout the experiment.

Group 2 was atrium perfused with α-CGRP or cys-CGRP. Rat α-CGRP (Bachem, Bubendorf, Switzerland, 0.1, 1, 10, 100 nM, $n=5–8$), human α-CGRP (1 nM, $n=5$), or cys-CGRP (Bachem, 1 μM, $n=6$) was introduced into the atrial lumen after a 10-min control collection period, and perfusate was collected for 50 min.

Group 3 was atrium perfused with calcitonin. Rat or salmon calcitonin (1 μM, $n=6$) in HEPES buffer was introduced into the atrial lumen after a 10-min control collection period.

Group 4 was atrium pretreated with antagonist. Receptor antagonist (all 0.3 μM) for $CGRP_1$ [CGRP-(8–37), $n=6$], salmon calcitonin (AC187, $n=7$), or amylin [amylin-(8–37), $n=6$; AC-amylin, $n=5$] in HEPES buffer was administered as a pretreatment at 40 min after the start of the perfusion. Then, rat α-CGRP (1 nM) was simultaneously infused after a 10-min control collection period. All antagonists used in this study were purchased from Bachem.

2.3. Radioimmunoassay of ANP

The concentration of immunoreactive ANP in the perfusate was measured with a specific radioimmunoassay (RIA), as described previously (Cho et al., 1989). RIA was performed in Tris–acetate buffer (0.1 M, pH 7.4) containing neomycin (0.2%), EDTA (1 mM), soybean trypsin inhibitor (50 benzoyl arginine ethyl ester units/ml), aprotinin (200 Kallikrein inhibiting unit/ml), phenylmethyl-

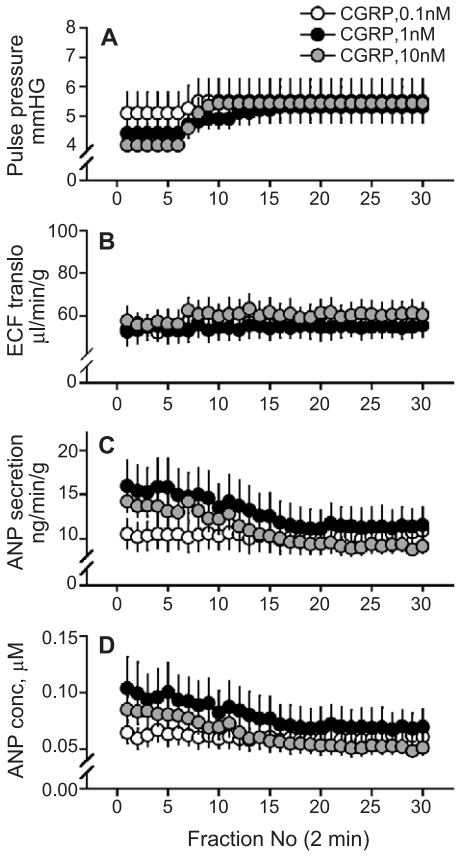

Fig. 1. Effect of rat α-CGRP on pulse pressure (A), ECF translocation (B), ANP secretion (C), and ANP concentration (D) in isolated perfused beating rat atria. After a 100-min control period, atrial perfusate was collected for 10 min at 2-min intervals as a control and then rat α-CGRP (0.1, 1, or 10 nM, $n=5–8$) was perfused into the atrial lumen. Rat α-CGRP increased pulse pressure with a slight increase in ECF translocation. The secretion of ANP in terms of ECF translocation (interstitial ANP concentration) was markedly decreased by rat α-CGRP. ECF translo, ECF translocation; ANP conc, ANP concentration; ●, ○, ●, CGRP-infused atria at doses of 0.1, 1, and 10 nM, respectively. Values are the means ± S.E.M.

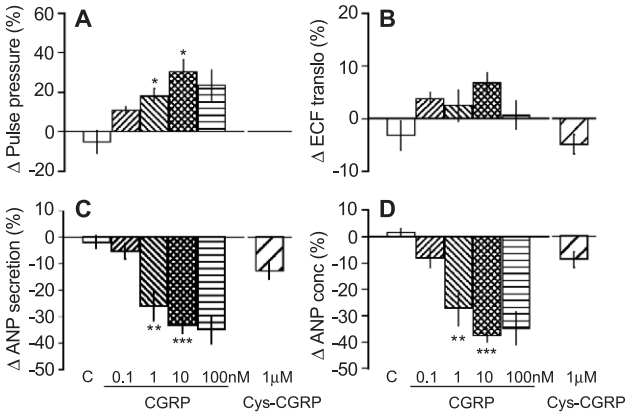

Fig. 2. Relative percent changes in pulse pressure (A), ECF translocation (B), ANP secretion (C), and ANP concentration (D) by rat α-CGRP (0.1, 1, 10, and 100 nM, $n=5-8$) and cys-CGRP (1 μM, $n=6$). Values were expressed as percent changes of the last five experimental values for exposure to CGRP, as compared to mean of the five control values. Rat α-CGRP increased pulse pressure in a concentration-dependent manner and decreased ANP secretion. Cys-CGRP decreased the ANP secretion without change in pulse pressure. The potency of rat α-CGRP was approximately 3000-fold greater than that of cys-CGRP. Legends are the same as in Fig. 1. $*P<0.05$, $**P<0.01$, $***P<0.001$ vs. the lowest dose of rat α-CGRP.

sulfonyl fluoride (0.4 mg%), sodium azide (0.02%), and bovine serum albumin (1%). Standard and samples were incubated with anti-ANP antibody and [^{125}I]ANP for 24 h at 4 °C. Bound forms were separated from the free form using a charcoal suspension or second antibody. RIA for ANP was done on the day of experiments and all samples in an experiment were analyzed in a single assay. The molar concentration of ANP release was calculated using the ANP secretion divided by ECF translocation and 3060 [molecular mass for ANP-(1–28)] and was expressed in μM (Cho et al., 1990).

2.4. Measurement of ECF translocation

We previously reported a two-step sequential mechanism of ANP secretion from the atrium (Cho et al., 1990): First, atrial release of ANP into the interstitial space occurs by means of atrial stretching, and second, the released ANP is translocated into the atrial lumen, concomitantly with ECF translocation due to contraction. The radioactivity of [^{3}H]-inulin in the atrial perfusate was measured with a liquid scintillation counter (Tris-Carb 23-TR; A Packard Bioscience, Downers Grove, IL). The amount of ECF translocated through the atrial wall was calculated from the total radioactivity in the perfusate divided by the radioactivity in the pericardial reservoir and atrial wet weight and was expressed in μl/min/g.

2.5. Statistical analysis

The results are given as the means ± S.E.M. The statistical significance of the differences was assessed using repeated measure analysis of variance (ANOVA) (Fig. 1) or ANOVA (Figs. 2–4) followed by Dunnett's multiple test. The critical level of significance was set at $P<0.05$.

3. Results

3.1. Effect of rat α-CGRP on intra-atrial pressure and ANP release

After stabilization for 100 min, perfusate was collected five times every 2 min to serve as a control period and then rat α-CGRP was infused at a concentration of 0.1, 1, or 10 nM. Fig. 1 shows the effects of rat α-CGRP on pulse pressure, ECF translocation, ANP secretion, and ANP concentration with time. Rat α-CGRP caused increases in pulse pressure in a concentration-dependent manner (Fig. 1A) and a decrease in ANP secretion (Fig. 1C) without significant change in ECF translocation (Fig. 1B). The ANP released from atrial myocytes into the interstitial space is translocated into the atrial lumen, concomitantly with ECF translocation (Cho et al., 1990, 1991; Han et al., 2003). Therefore, the ANP secretion in terms of ECF translocation, which means the interstitial ANP concentration (Fig. 1D), was markedly suppressed.

Fig. 2 shows the relative percent changes in pulse pressure, ECF translocation, and ANP secretion obtained from the mean of five control values and the last five experimental values for exposure different doses of rat α-CGRP. Rat α-CGRP caused an increase in pulse pressure in

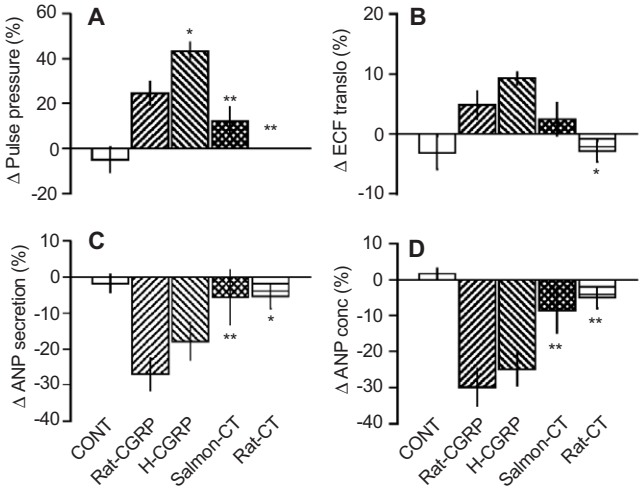

Fig. 3. Comparison of effects of rat α-CGRP (1 nM) on atrial hemodynamics and ANP secretion with human α-CGRP (1 nM), salmon calcitonin (1 μM), and rat calcitonin (1 μM). Human α-CGRP suppressed ANP release similarly to rat α-CGRP with potent positive inotropism. Calcitonin did not cause any significant changes in pulse pressure and ANP secretion. CONT, atria perfused with HEPES buffer ($n=6$); rat-CGRP, atria perfused with rat α-CGRP ($n=8$); H-CGRP, atria perfused with human α-CGRP ($n=6$); salmon-CT, atria perfused with salmon calcitonin ($n=6$); rat-CT, atria perfused with rat calcitonin ($n=6$). $*P<0.05$, $**P<0.01$, vs. rat CGRP group. Other legends are the same as in Fig. 1.

a concentration-dependent manner (Fig. 2A) and reached the peak value at 10 nM. Changes in ECF translocation by CGRP were not different from each other (Fig. 2B). The ANP secretion and ANP concentration were slightly decreased by 0.1 nM CGRP, and then markedly decreased, reaching the peak value at 10 nM CGRP (Fig. 2C and D).

3.2. Effect of cys-CGRP on intra-atrial pressure and ANP release

To investigate the role of $CGRP_2$ receptor subtype in atrial contractility and ANP release, cys-CGRP (1 μM), a $CGRP_2$ receptor agonist was infused after collection of perfusate during the control period. Cys-CGRP caused slight decreases in ANP secretion and concentration without significant changes in pulse pressure and ECF translocation (Fig. 2). Cys-CGRP was approximately 3000-fold less potent than rat α-CGRP.

3.3. Effects of cacitonin on intra-atrial pressure and ANP release

For the comparison of rat α-CGRP effects to those of human α-CGRP and calcitonin, human α-CGRP (I nM), salmon or rat calcitonin (1 μM) was infused after collection of perfusate during the control period. Fig. 3 shows the relative changes in pulse pressure, ECF translocation, ANP secretion, and ANP concentration by human α-CGRP and calcitonin, as compared to those in the rat CGRP-infused group. Human α-CGRP also showed suppression of ANP release similarly to rat α-CGRP and the positive inotropic effect was more prominent. Interestingly, neither salmon nor rat calcitonin caused significant changes in pulse pressure, ECF translocation, and ANP secretion (Fig. 3).

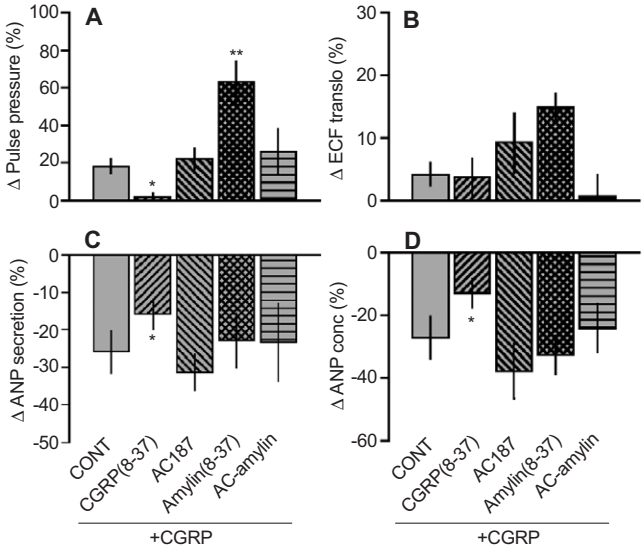

Fig. 4. Modification of rat α-CGRP-induced suppression of ANP secretion and positive inotropism by the pretreatment of various antagonists. Rat α-CGRP-(8–37) blocked the suppression of ANP release and the increased pulse pressure by rat α-CGRP (1 nM) but rat amylin-(8–37), acetyl-amylin, and AC 187 (all 0.3 μM) did not. CGRP-(8–37), rat α-calcitonin gene-related peptide-(8–37)-pretreated group ($n=6$); AC187, acetyl-[Asn^{30}, Tyr^{32}]-calcitonin(8–32)-pretreated group ($n=7$); Amylin(8–37), rat amylin-(8–37)-pretreated group ($n=6$); AC-amylin, acetyl-amylin-pretreated group ($n=6$). $^{*}P<0.05$, $^{**}P<0.01$ vs. the control atria perfused with rat CGRP without pretreatment. Other legends are the same as in Fig. 1.

3.4. Modification of rat CGRP effects on intra-atrial pressure and ANP release with receptor antagonists

To modify the rat α-CGRP-induced suppression of ANP release and positive inotropic effect, a receptor antagonist (0.3 μM) was added as a pretreatment at 40 min after the start of the perfusion of rat α-CGRP (1 nM). Fig. 4 shows the relative changes in pulse pressure, ECF translocation, ANP secretion, and ANP concentration caused by rat α-CGRP in the presence of a receptor antagonist, rat α-CGRP-(8–37), AC187, amylin-(8–37), or AC-amylin. The pretreatment with rat α-CGRP-(8–37) completely blocked the positive inotropic effect of rat α-CGRP and also attenuated the suppression of ANP release (Fig. 4A and C). However, AC 187 and AC-amylin did not cause any significant changes in rat α-CGRP-induced positive inotropism and suppression of ANP release. Amylin-(8–37) accentuated the increases in inotropic effect and ECF translocation by rat α-CGRP without affecting the suppression of ANP release (Fig. 4).

4. Discussion

In the present study, we demonstrated that rat α-CGRP causes suppression of ANP release with a positive inotropic effect through receptors for $CGRP_1$ but not those for calcitonin and amylin. Cys-CGRP ($CGRP_2$ receptor agonist) caused a suppression of ANP secretion, which is 3000-fold less than that with rat α-CGRP without changes in atrial contractility. The antagonistic actions of rat α-CGRP-(8–37) on CGRP effects suggest that the $CGRP_1$ receptor subtype may play a dominant role in the regulation of atrial contractility and ANP release.

CGRP exists in two forms in both rats and humans, α-CGRP and β-CGRP, with similar biological activities. The structure of CGRP is well conserved between species with 75% homology between salmon and human forms. CGRP also has a considerable conservation of structural features with the amylin family, less homology with the adrenomedullin family and the weakest homology with the salmon calcitonin. Therefore, the structural similarity of CGRP with these peptides suggests that they may act via common receptors (Poyner, 1995). Two classes of CGRP receptors $CGRP_1$ and $CGRP_2$ (Dennis et al., 1991) have been suggested, one with high affinity mediating a positive contractile response, and a second one with low affinity mediating a relaxant response (Kim, 1991). This report showed a biphasic

contractile response to CGRP. In porcine myocardium (Saetrum Opgaard et al., 1999) and human myocardial trabeculae (Saetrum Opgaard et al., 2000), functional $CGRP_1$ and $CGRP_2$ receptors may mediate a positive inotropic effect by α-CGRP. Giuliani et al. (1992) have reported that a concentration-related positive inotropic effect of human α-CGRP is mediated by α-CGRP-(8–37) in the isolated left atrium but not in urinary bladder of guinea pig. Our study showed that the positive inotropic effect of rat α-CGRP in isolated perfused rat atria was blocked by the $CGRP_1$ receptor antagonist but the $CGRP_2$ receptor agonist did not lead to any changes in atrial contractility. Human α-CGRP caused a potent inotropic effect but calcitonin did not, as compared with rat α-CGRP. Our results are consistent with those of others (Bell and McDermott, 1994). Therefore, we suggest that the $CGRP_1$ receptor may be more important in the regulation of atrial contractility.

The secretion of ANP is closely related to atrial hemodynamics, such as increased contractility and heart rate (Cho et al., 1991). The present study showed that the secretion of ANP was markedly suppressed by rat α-CGRP and human α-CGRP (1 nM), and slightly by the $CGRP_2$ receptor agonist (1 μM). Calcitonin did not cause any significant change in ANP release. Our data are not consistent with the reports showing the stimulation of ANP secretion by CGRP in rat beating atrial strips (Yamamoto et al., 1988; Schiebinger and Santora, 1989). These reports show a biphasic ANP secretory response to CGRP with increased atrial tension mediated by cAMP. The discrepancy may be partly due to a difference in atrial model used for the study. Using our model, we previously demonstrated the suppression of ANP release with increased contractility by amylin (Piao et al., in press). Ca^{2+} and cAMP act as negative regulators of ANP secretion in isolated perfused atria (Cho et al., 1994; Cui et al., 2002) but as positive regulators in atrial strips (Schiebinger and Santora, 1989; Schiebinger et al., 1994) even though the exact reason is not clear. There is no report touching on the identification of receptor subtypes involved in the regulation of ANP release by rat α-CGRP. We demonstrated that the rat α-CGRP-induced suppression of ANP release was blocked by rat α-CGRP-(8–37) but not by AC 187, amylin-(8–37), and acetyl-amylin. These results suggest that the rat α-CGRP-induced suppression of ANP release is mediated by the $CGRP_1$ receptor subtype but not by the receptor for amylin or calcitonin.

In conclusion, we suggest that the $CGRP_1$ receptor subtype may play a predominant role in the suppression of ANP release and positive inotropism by CGRP, which may be related to the development of hypertension.

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