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POSITIVE INOTROPIC EFFECTS AND RECEPTORS OF CALCITONIN GENE-RELATED PEPTIDE(CGRP) IN PORCINE VENTRICULAR MUSCLES

Miyauchi, T., **Sano, Y., **Hiroshima, O., **Yuzuriha, T., Sugishita, Y., *Ishikawa, T., *,***Saito, A. and *Goto, K.

Department of Internal Medicine, Institute of Clinical Medicine and *Department of Pharmacology, Institute of Basic Medical Sciences, University of Tsukuba, and
**Tsukuba Research Laboratories, Eisai Co. Ltd., Tsukuba, Ibaraki 305 Japan

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Summary: Calcitonin gene-related peptide (CGRP), but not capsaicin, elicited positive inotropic effects in the isolated, electrically driven false tendon of the porcine heart. Specific CGRP-binding sites were present in solubilized membrane fractions; the dissociation constant (Kd) and the maximum binding (Bmax) were 50.4 pM and 180 fmol/mg protein, respectively. SDS-PAGE analysis of CGRP-binding sites revealed the molecular mass of 70 K and 120 K. Few CGRP-like immunoreactive nerves were present in the ventricular muscle layer. These results indicate that CGRP activates specific receptor sites on the ventricular muscles and causes positive inotropic responses. CGRP receptors in ventricles are likely to be activated by circulating CGRP.

Immunohistochemical and radioimmunological studies have shown that calcitonin gene-related peptide(CGRP), a 37 amino acid peptide, is distributed not only in the nervous system (1,2) but also in plasma (3-5). CGRP elicits a positive inotropic effect, or an increase in the contractile tension, in the guinea pig atria (6,7) and is likely to function as a neurotransmitter of nonadrenergic, noncholinergic nerves (6,8,9). However there have been conflicting results of the effect of CGRP on ventricular muscles. CGRP exerted positive inotropic effects in the isolated, perfused hearts of guinea pigs (10) but not in the ventricular strips and papillary muscles of rats and guinea pigs (7,11).

Recently specific binding sites for CGRP were isolated from the porcine spinal cord (12) and human placenta (13). In the present study, pharmacological effects and binding characteristics were examined in the false tendon and ventricular muscles of porcine hearts, respectively, to assess a potential role of CGRP in controlling the cardiac function.

*** To whom correspondence should be addressed.

Abbreviations: CGRP: calcitonin gene-related peptide; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Methods and Materials

Fresh porcine hearts were obtained from a local slaughter house and ventricles of hearts were isolated in an ice-cold Krebs-Ringer's solution. The composition of the Krebs-Ringer's solution was NaCl, 113; KCl, 4.8; $CaCl_2$, 2.2; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; and glucose, 5.5 (mM).

Pharmacological studies in vitro: Since thick ventricular walls are difficult to maintain in a sufficient condition to analyze the pharmacological effects, thin false tendons (about 2 mm thick, 10 mm long) were isolated and suspended in organ baths filled with a Krebs-Ringer's solution (37 °C) constantly bubbled with 95 O_2 and 5 CO_2. The isometric contraction was measured by a transducer and recorded on a thermal pen recorder. The tissue was applied with a resting tension of 1 g and driven electrically at 1 Hz with square-wave pulses (1 msec, 2 V) generated by a stimulator. When the twitch contractions attained a steady state, human CGRP or isoproterenol was added in a cumulative manner. After washing followed by a resting period of 1 hr, capsaicin was applied.

Binding studies: Ventricles were minced and homogenized with a buffer containing 10 mM Tris-HCl (pH = 7.4), 0.5 mM phenylmethylsulfonylfluoride, 1 mM ethylenediaminetetraacetate, and 1 μg/ml each of antipain, leupeptin, pepstatin A and phosphoramidon. The homogenates were centrifuged at 1500 X g for 10 min, and the supernatant was centrifuged at 50,000 X g for 30 min. Then the pellets were resuspended in the same buffer (crude membrane fraction). The crude membrane fractions were treated with 3% digitonin, sonicated and centrifuged at 100,000 X g at 4 °C for 40 min. The supernatants were designated as solubilized fractions. The protein concentration was determined by the method of Lowry et al. (14) using bovine serum albumin as the standard.

The binding assay of CGRP was carried out according to the method described previously by Hiroshima et al. (12). In brief, the crude membrane and solubilized fractions were mixed with [^{125}I]human α-CGRP (18 to 23 pM). After incubation at 0 °C for 15 hrs, bovine γ-globulin and polyethyleneglycol 6000 were added. The free and bound radioligands were then separated by rapid filtration. The radioactivity in the filters was counted using Aloka ARC-300 γ-counter. The specific binding was defined as the difference of the amount of bound radioactivity between the absence and presence of unlabeled human α-CGRP (1.1 μM).

For the analysis of the molecular weight, the solubilized fraction was incubated with [^{125}I]human α-CGRP (0.11 nM) at 0 °C either in the presence or absence of 3.0 μM unlabeled human α-CGRP. The [^{125}I]human α-CGRP-binding site complex was then separated from free ligand by gel filtration and incubated with disuccinimidyl suberate. Covalently labeled binding sites were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (15). Standard molecular markers were myosin heavy chains (Mr = 200 K), caldesmon (Mr = 155 K), α-actinin (Mr = 105 K), desmin (Mr = 55 K), α-tropomyosin (Mr = 43 K), actin (Mr = 42 K) and β-tropomyosin (Mr = 35 K). After electrophoresis, the gel was exposed to X-ray film for 2 weeks.

Immunohistochemistry: The false tendons and ventricular walls were fixed in paraformaldehyde and picric acid for 72 hrs and processed for a fluorescence immunohistochemistry according to the method described by Saito and Goto (2).

Results

In the isolated, electrically driven false tendon of the porcine ventricles, CGRP induced positive inotropic responses in a dose-dependent manner (1 nM to 300 nM)(Fig. 1). Isoproterenol, a β-adrenergic agonist, also produced dose-dependent positive inotropic responses in a similar dose range.

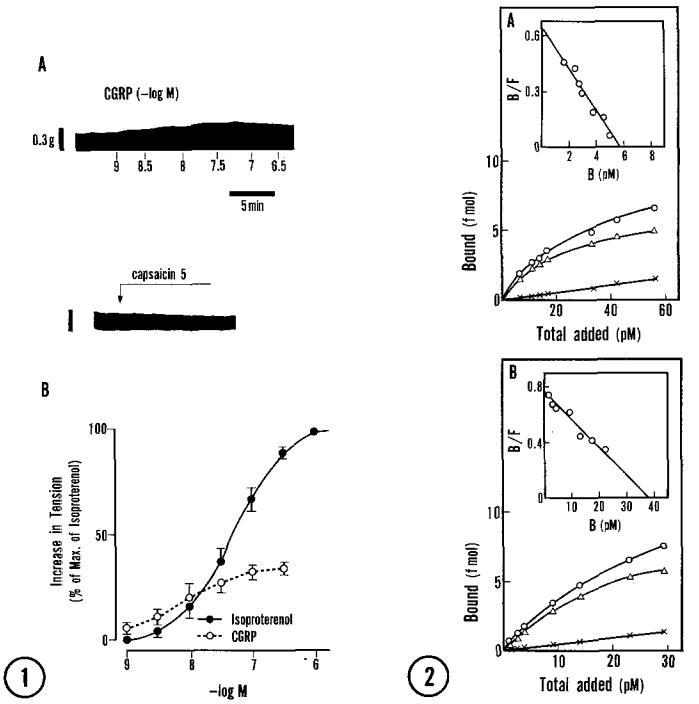
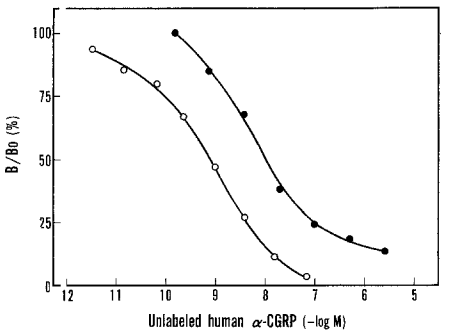


Figure 1. Typical example of the effect of CGRP (A) and dose-response curves for the positive inotropic effect of CGRP and isoproterenol (B) on the contraction of ventricular muscles. Numbers in the figure indicate the - log M. Each point and bar represents the mean and S.E. of 7 tissues. The ED50 values (with 95% confidence interval) to CGRP and isoproterenol in these preparations were 5.50 nM (1.64 - 1.88 nM) and 36.9 nM (12.7 - 107 nM), respectively. The maximum response induced by CGRP was 33.9 ± 3.2% of that by isoproterenol.

Figure 2. Total, nonspecific and specific binding of [^{125}I]CGRP and Scatchard analysis to the crude membrane (A) and solubilized (B) fractions of ventricular muscles. Each value represents the mean of four duplicate determinations.

However, the maximum response to CGRP was about 30% of that to isoproterenol. Capsaicin, which releases CGRP from intracardiac nerves (9) elicited no positive inotropic response in all the preparations examined (n = 7), but caused a slight decrease of the contractile tension in 5 out of the 7 tissues.

Shown in Figure 2 are the saturation curves of [^{125}I]human α-CGRP to the crude membrane and solubilized fractions of porcine ventricles. Scatchard analysis revealed the presence of a single, saturable binding component with an equilibrium dissociation constant (Kd) of 8.7 pM and a density (Bmax) of 35 fmol/mg protein in the crude membrane fractions and 50.4 pM and 180 fmol/mg in the solubilized fractions, respectively (n = 4). [^{125}I]human-CGRP-binding was inhibited by unlabeled human α-CGRP; IC50 values in the crude membrane and solubilized fractions were 0.85 nM and 22.0 nM, respectively (Fig. 3). Human β- and rat α-CGRP, but not angiotensin II (0.48 μM) nor human calcitonin (0.15



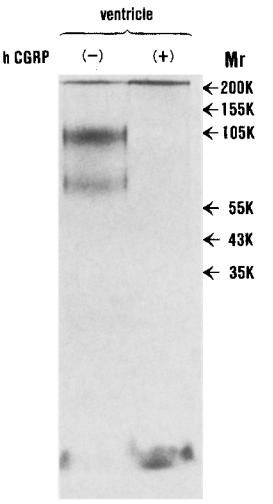


Figure 3. Inhibition of [^{125}I]CGRP-binding to the crude membrane (open circle) and solubilized (closed circle) fractions of the ventricular muscles by unlabeled human α-CGRP. Each point represents the mean of duplicate determinations.

Figure 4. Determination of the molecular size of CGRP-binding sites by cross-linking of [^{125}I]CGRP followed by SDS-PAGE. The numbers represent the molecular weight of marker proteins.

μM), inhibited the binding of [^{125}I]CGRP to the solubilized fractions (data not shown).

SDS-PAGE analysis of the CGRP-binding sites revealed the presence of intense radioactive bands at 70 K and 120 K (Fig. 4). The labeling of these bands were specific to CGRP since it was prevented by unlabeled CGRP.

Few CGRP-like immunoreactive fibers were observed in the ventricular muscle layers. In contrast, beaded varicose-type fibers were found to be present around coronary blood vessels as shown previously (16).

Discussion

CGRP and isoproterenol produced positive inotropic responses in a similar dose range, although the maximum response by CGRP was smaller than that by isoproterenol. Previous studies indicated that CGRP did not induce positive inotropic effects in ventricular strips and papillary muscles of rats (7). In guinea pigs, CGRP augmented the contraction of ventricles in isolated, perfused hearts (10) but not in the isolated, electrically driven papillary muscles and ventricular strips (11). In perfused preparations, CGRP may change the contractile force by affecting the perfusion pressure and/or heart rate. Thus it is not certain what extent of the positive inotropic response was actually induced by infused CGRP. The CGRP-induced positive

inotropic responses in the isolated, electrically driven ventricular muscles were unequivocally demonstrated in the present study.

Recently specific binding sites for CGRP with Kd and Bmax of 1.87 nM and 1.25 pmol/mg protein were isolated from the human placenta (13). The binding of CGRP to ventricular membranes was specific to CGRP since it was inhibited by CGRPs but not by angiotensin II nor calcitonin. However, the Kd and Bmax values in the crude membrane and solubilized fractions of ventricles were fairly smaller than those in the human placenta. This indicates that the CGRP-binding sites in ventricles are distinct from and have higher affinity than those in the placenta.

SDS-PAGE analysis of the CGRP-binding sites gave intense radioactive bands at 70 K and 120 K. Recently it was shown that CGRP activates the adenylate cyclase in coronary arteries (16) and atrial muscles (7,11). It is well documented that receptors coupled to adenylate-cyclase systems are usually associated with G-proteins (17). If the CGRP receptor is coupled with G-protein (Mr = ca 40 K), it is possible that the components with a high and low molecular weight are a receptor-G-protein complex and CGRP receptor, respectively. Alternatively it is also possible that the component with high molecular weight (120 K) represents another single molecule. Admittedly the exact molecular characteristics of CGRP-binding sites in the ventricles remain to be determined.

Stimulation of nonadrenergic, noncholinergic nerves elicits positive chronotropic and inotropic effects in isolated atria of guinea pigs (18,19). It has also been shown that CGRP is a transmitter in these nonadrenergic, noncholinergic nerves (6,8). CGRP-like immunoreactive fibers were scarcely present in the muscle layer of ventricles, in agreement with the previous observation in the left ventricular walls (16). Capsaicin acts on CGRP-containing nerves in vitro and releases CGRP in vasa deferentia (20), coronary arteries (21) and hearts (9,22). Like CGRP, capsaicin causes positive inotropic responses in atria of guinea pigs (9,10). Therefore, the lack of any positive effect of capsaicin on ventricular muscles but not of CGRP, indicates a poor supply of CGRP-containing nerves in the porcine ventricular muscles, although CGRP receptors are present.

Radioimmunological studies have shown the presence of CGRP-like material in the plasma in humans and rats (3-5). The plasma levels of CGRP rise in patients with medullary thyroid carcinoma (3) and in pregnant humans (4). It has also been demonstrated that calcium, pentagastrin or capsaicin elevates the levels of circulating CGRP (4,5). Intravenous infusion of CGRP induces positive inotropic responses in humans (23). Coronary perfusion of CGRP also causes positive inotropic responses in isolated hearts of guinea pigs (10).

These findings suggest that ventricular CGRP-receptors are activated by circulating CGRP.

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