Positive Inotropic Effect of Calcitonin Gene-Related Peptide Mediated by Cyclic AMP in Guinea Pig Heart

Tomohisa Ishikawa, Naomichi Okamura, Akira Saito, Tomoh Masaki, and Katsutoshi Goto

The mechanism of cardiac actions of rat calcitonin gene-related peptide (CGRP) was analyzed on isolated guinea pig hearts. CGRP exerted a positive inotropic effect in a dose-dependent manner on the electrically driven left atria but not on the ventricles. Immunohistochemical studies demonstrated that CGRP-like immunoreactive nerves were distributed densely in the myocardia of the atria but only sparsely in those of the ventricles. The CGRP-induced augmentation of the contraction was accompanied by the shortening of the time to peak force and the increase in the relaxation velocity. The positive inotropic response to CGRP was significantly enhanced by isobutylmethylxanthine and was attenuated by adenosine. CGRP increased the action potential amplitude and prolonged action potential duration at the level of 50% repolarization in the left atria. In the preparations, which were partially depolarized with an increase in extracellular potassium, CGRP induced slow response action potentials. These electrophysiological results indicate that CGRP causes an increase in the slow inward Ca\(^{++}\) current. The cyclic AMP content in the left atria significantly increased following the addition of CGRP, the time course of which was nearly consistent with that of the augmentation of the contractile force. In the membrane preparation of the atria, the activity of adenylate cyclase was enhanced by CGRP in a dose-dependent manner. These effects of CGRP are qualitatively similar to those of \(\beta\)-adrenoceptor stimulation. It is concluded that the CGRP-induced response in the guinea pig atria is attributed to the activation of adenylate cyclase via stimulation of its specific receptor and the subsequent increase in the intracellular cyclic AMP level. (Circulation Research 1988;63:726–734)

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide with a 2,7-disulfide bridge and a carboxy terminal amide. The peptide was first thought to be a product resulting from an alternative processing of RNA transcripts from the calcitonin gene in the rat.\(^1,2\) Recently, CGRP was isolated from human C-cell tumors\(^3\) and porcine spinal cords.\(^4\) Immunohistochemical studies have demonstrated that CGRP-like immunoreactive (CGRP-I) fibers are widely distributed in the central nervous system and various peripheral tissues, particularly in the blood vessels and heart.\(^2,5-9\)

CGRP has been shown to possess potent actions on cardiovascular systems. Studies in various blood vessels have indicated that CGRP is one of the most potent vasodilators.\(^10-14\) It is especially noteworthy that a marked relaxing effect on the epicardial coronary arteries was shown in human subjects.\(^15\) CGRP exerts potent positive chronotropic and inotropic effects in vitro in various mammalian hearts, including the human atrium.\(^13,16-21\) Furthermore, it has been suggested that CGRP may function as a neurotransmitter of the nonadrenergic noncholinergic nerves in the guinea pig atria.\(^19-23\) CGRP is also present in the plasma of humans.\(^24\) Hence, CGRP appears to be an important biogenic product for the regulation of the cardiovascular system.

Previous investigations\(^11,14\) have suggested the intimate relation between the CGRP-induced vasodilating effect and the increased tissue content of
cyclic AMP (cAMP). In the membrane preparation of rat atria, CGRP has been shown to stimulate adenylate cyclase (AC) activity.\(^{18,23}\) Contrary to these studies, there exist some reports denying the involvement of cAMP in the actions of CGRP.\(^{26-28}\)

In the present report, therefore, the positive inotropic responses of guinea pig hearts to CGRP were precisely analyzed to elucidate the mechanisms of action of CGRP, especially in relation to the possible participation of cAMP.

**Materials and Methods**

Male albino guinea pigs (300–400 g) were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the hearts were quickly removed and immersed in ice-cold Krebs-Ringer solution of the following millimolar composition: NaCl 113, KCl 4.8, CaCl\(_2\) 2.2, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, NaHCO\(_3\) 25, and glucose 5.5.

**Contraction Experiments**

After adhering tissues were removed, left atria, papillary muscles, and strips of right ventricles (approximately 2 mm wide and 25 mm long) were dissected from the hearts. These preparations were suspended individually by threads in organ baths containing Krebs-Ringer solution (20 ml) maintained at 37°C and aerated with a mixture of 95% O\(_2\)-5% CO\(_2\). The isometric contraction was measured with a force displacement transducer (Nihon Kohden TB-612T, Tokyo, Japan) connected to an amplifier (Nihon Kohden MEZ-8201) and displayed on an oscilloscope (Nihon Kohden VC-9). The resting force applied was 1 g for the atria and the ventricular strips and 0.5 g for the papillary muscles. The preparations were placed between a pair of platinum electrodes and electrically driven at 3.3 Hz (atria) or 1 Hz (papillary muscles, ventricular strips) with square-wave pulses of 1 msec duration and a suprathreshold intensity of about 1.5 V. Glass capillary microelectrodes filled with 2 M KCl and having resistances of 20–50 MQ were impaled in the cardiac cells. Potentials were amplified by a microelectrode amplifier (Nihon Kohden MEZ-8201) and displayed on an oscilloscope (Nihon Kohden VC-9). The preparations were allowed to equilibrate for at least 3 hours, and the cells that had resting potentials more negative than −70 mV were used for the following analyses: resting potential, action potential amplitude, maximal rate of rise, and action potential duration at 50% and 80% repolarization.

**Electrophysiological Analysis**

The isolated left atrium was pinned with the endocardial surface uppermost in a tissue bath (30 ml). The heart was perfused with Krebs-Ringer solution aerated with a mixture of 95% O\(_2\)-5% CO\(_2\) flowed over the preparation at a rate of 2–3 ml/min. The tissue bath temperature was maintained at 37°C. The preparation was stimulated electrically at 3.3 Hz with a pair of platinum electrodes by square-wave pulses of 1 msec duration and a suprathreshold intensity of about 1.5 V. Glass capillary microelectrodes filled with 2 M KCl and having resistances of 20–50 MQ were impaled in the cardiac cells. Potentials were amplified by a microelectrode amplifier (Nihon Kohden MEZ-8201) and displayed on an oscilloscope (Nihon Kohden VC-9). The preparations were allowed to equilibrate for at least 3 hours, and the cells that had resting potentials more negative than −70 mV were used for the following analyses: resting potential, action potential amplitude, maximal rate of rise, and action potential duration at 50% and 80% repolarization.

**Determination of Cyclic AMP Content**

The isolated left atrium was cut in half: one-half for the control and the other for the test. Each preparation was mounted individually in organ baths as mentioned above. After an appropriate period of incubation with (for the test) or without (for the control) 2.5 × 10^{-8} M CGRP, the tissues were taken out of the bath and immediately frozen with liquid nitrogen. The samples were homogenized in ground-glass homogenizing tubes and pestles with 1 ml of ice-cold 6% perchloric acid. Subsequently the homogenates were centrifuged at 1,500g for 10 minutes, and the supernatants were collected. This extraction procedure was repeated three times. The total supernatants were neutralized with 2N KOH and 0.5 M K-phosphate buffer (pH 7.0), and they were centrifuged at 2,000g for 20 minutes. The supernatants were processed for the analysis of cAMP content by radioimmunoassay with commercially available kits (New England Nuclear, Boston, MA).
The protein content was determined by the method of Lowry with bovine serum albumin as the standard.

### Adenylate Cyclase Assay in Membrane Preparations

The isolated heart was perfused with Krebs-Ringer solution through the coronary artery to flush any remaining blood. The membrane preparation was obtained by the method of Schumacher and coworkers. Briefly, the atria and ventricular walls were isolated and homogenized with 10 strokes of a motor-driven, ground-glass homogenizer in an ice-cold buffer A (10 mM Tris-HCl, pH 7.4, at 4°C with 2 mM dithiothreitol). After the homogenate was sedimented with successive centrifugations at 600g for 15 minutes, 400g for 10 minutes, and 200g for 10 minutes, the resulting pellet was resuspended in buffer B (buffer A plus 0.25 M sucrose) and centrifuged at 30,000g for 15 minutes. The pellet thus obtained was resuspended in buffer C (10 mM MgCl₂ and 50 mM Tris-HCl, pH 7.4, at 37°C) and resedimented at the same speed. This pellet was then resuspended in fresh buffer C and used as the membrane preparations for the enzyme assay.

Adenylate cyclase activity was determined by a modification of the method of Okamura and Sugita. The assay was initiated by the addition of the membrane preparations to prewarmed (4 minutes, 37°C) assay tubes containing a reaction mixture. The reaction mixture consisted of 40 mM Tris-HCl, 10 mM MgCl₂, 10 mM EGTA, 10 mM theophylline, 5 mM phosphoenolpyruvate, 3 μg pyruvate kinase, 1 mM ATP, 1 μM GTP, and various concentrations of CGRP. The reaction was allowed for 10 minutes at 37°C in a shaking water bath and was terminated by placement of the assay tube in a boiling water bath for 4 minutes. Denaturated protein was removed by centrifugation at 2,500g for 15 minutes. Cyclic AMP thus formed was determined by radioimmunoassay.

### Materials and Statistics

Drugs used were rat CGRP (Peninsula Laboratories, San Carlos, California), isobutylmethylxanthine, isoproterenol hydrochloride, and adenosine (Sigma Chemical, St. Louis, Missouri). Values are expressed as mean ± SEM. Comparisons were made using the one-way analysis of variance followed by Dunnett’s method, where comparisons with a common control were made, or the Student’s t test for paired (Figures 3B and 8) or unpaired (Table 1) values. The level of statistically significant difference was p<0.05.

### Results

#### Positive Inotropic Response

CGRP produced a positive inotropic response of the isolated left atria in a dose-dependent manner (Figure 1A). The maximal inotropic effect of CGRP was 71% of that of isoproterenol, and the ED₅₀ value, a half-maximal effective dose, of CGRP was 6.0×10⁻⁹ M.

As seen in the oscilloscope records (Figure 2A), CGRP shortened the time to peak force on the left atria. Figure 3A summarizes the results obtained from seven muscles, and the abbreviation of the time to peak force in a dose-dependent manner was unequivocal. Furthermore, CGRP-induced inotropic response was accompanied by an increase in the maximal contraction velocity (dF/dt) and the maximal relaxation velocity (-dF/dt). The percent increase in -dF/dt was significantly larger than that

### Table 1. Effect of CGRP (10⁻⁶ M) on Action Potential Parameters in Guinea Pig Left Atria

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CGRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential</td>
<td>76.3 ± 0.5</td>
<td>77.8 ± 0.5*</td>
</tr>
<tr>
<td>Action potential amplitude</td>
<td>103.0 ± 0.9</td>
<td>106.9 ± 0.7†</td>
</tr>
<tr>
<td>Maximal rate of rise</td>
<td>152.7 ± 5.3</td>
<td>171.1 ± 3.3†</td>
</tr>
<tr>
<td>Action potential duration at 50% repolarization</td>
<td>25.5 ± 0.6</td>
<td>27.7 ± 0.5†</td>
</tr>
<tr>
<td>Action potential duration at 80% repolarization</td>
<td>48.7 ± 0.8</td>
<td>49.7 ± 0.8</td>
</tr>
<tr>
<td>Number of cells</td>
<td>29</td>
<td>32</td>
</tr>
</tbody>
</table>

CGRP, calcitonin gene-related peptide. Values are mean ± SEM. *p<0.05, †p<0.01 from control.
in dF/dt at concentrations higher than $1.25 \times 10^{-8}$ M (Figure 3B). An increase in extracellular calcium concentration led to the augmentation of the contractile force of the left atria, the maximal response being obtained at about 7.2 mM. Figure 2B shows the actual traces of isometric contractions in the Ca$^{2+}$-rich (9 mM) medium before and after the addition of CGRP (10$^{-8}$ M). In all experiments ($n=8$), CGRP did not induce a further increase in the contractile force but shortened the duration of the contractile response in a high Ca$^{2+}$ medium, obviously by accelerating the relaxation.

The effect of isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, on the positive inotropic effect of CGRP on the left atria is shown in Figure 4. IBMX itself at a concentration of $5 \times 10^{-6}$ M produced no or only a slight increase in the contractile force. IBMX enhanced the inotropic effect of CGRP, where IBMX caused a shift of the CGRP dose-response curve to the left and an augmentation of the maximal response to CGRP. Adenosine (10$^{-3}$ M) itself exerted a transient, negative inotropic effect, with the contractile force returning to the initial level in a few minutes. CGRP was applied after a steady state in contraction was attained. As shown in Figure 4, the positive inotropic effect of CGRP was significantly attenuated by adenosine (10$^{-3}$ M).

In the papillary muscles, CGRP did not exert inotropic effects at doses up to $10^{-6}$ M, while isoproterenol produced a dose-dependent augmentation of the contractile force (Figure 1B). CGRP also produced no inotropic response in the strips of ventricular muscles ($n=6$; data not shown).

**CGRP-Like Immunoreactive Nerves**

Numerous CGRP-I nerves were found to be present within the left atrial myocardium (Figure 5A). In the ventricles, only a few CGRP-I nerves were present within the myocardium (Figure 5B), whereas bundles of CGRP-I nerves were observed in the pericardium (Figure 5C). CGRP-I nerves were frequently found within the perivascular layer of the coronary vessels (Figure 5D).

**Transmembrane Action Potential**

Figure 6 shows the traces of the action potentials before and after application of CGRP (10$^{-8}$ M) recorded from the same cell. The amplitude and duration of the plateau phase of the action potentials were increased by CGRP. Table 1 shows the effects of CGRP on the various parameters of the action potentials in left atrial preparations. CGRP (10$^{-8}$ M) prolonged the duration of action potential at 50% repolarization but not at 80% repolarization. CGRP also produced significant increases in the amplitude and maximal rate of the rise of action potentials. The resting membrane potential was slightly yet significantly hyperpolarized.

In the atria in which the fast Na$^+$ channels were voltage-inactivated by partial depolarization in elevated K$^+$ (22 mM) (Figure 7B), the addition of CGRP restored the excitability in the form of propagating slow responses with accompanying contractions (Figure 7C).

**Time Course of Changes in Force of Contraction and Intracellular cAMP Level**

The time course studies were done with CGRP 2.5$ \times 10^{-8}$ M or isoproterenol 10$^{-7}$ M. At these concentrations, CGRP or isoproterenol produced almost the maximal positive inotropic effect. As illustrated in Figure 8, CGRP caused an increase in the contractile force and an elevation of the cAMP content in the left atria. The positive inotropic response to CGRP developed slowly and required 2–3 minutes to attain a steady state. The time course of the increase in cAMP content was nearly identical with that of the augmentation of the contractile force. The cAMP content was sustained at a high level over 8 minutes. By contrast, isoproterenol caused an immediate increase in the contractile force, attaining the maximum in 40 to 60 seconds. The increase in the cAMP content also developed immediately, the extent being 55.8±17.2% ($n=8$) and 56.3±9.2% ($n=6$) at 30 seconds and 1 minute after the application of isoproterenol, respectively.

**Adenylate Cyclase Activity**

Figure 9 summarizes the changes of the AC activities induced by CGRP from four different preparations. In each preparation, two hearts were used. AC activity was present in particulate membrane fractions from atria and ventricles, and GTP (10$^{-6}$ M) augmented its basal activity. The effect of CGRP on the AC activity was examined in the
presence of GTP ($10^{-6}$ M). In the membrane from the atria, CGRP activated the AC in a dose-dependent manner. The effective dose range of CGRP for the activation of AC was almost the same as that for the positive inotropic effect. In contrast, CGRP did not stimulate the AC activity in the ventricular membrane preparations.

**Discussion**

It was clearly shown in this study that CGRP exerted a potent positive inotropic effect on the electrically driven left atria of the guinea pig. Although the maximal response to CGRP was not as great as that to isoproterenol, the ED$_{50}$ value of CGRP was comparable to that of isoproterenol. Thus, CGRP would be assumed to be one of the most potent cardiotonic substances. Our previous report$^{20}$ demonstrating that the positive inotropic effect of CGRP is not affected by $\alpha$- and $\beta$-adrenergic, histaminergic, or serotonergic antagonists suggests that CGRP acted directly on myocytes.

The present study showed that the inotropic action of CGRP was associated with an increase in the cAMP content in the guinea pig left atria. Although the elevation of the cAMP content induced by CGRP was only 1.5-fold, it was comparable to that induced by isoproterenol and it is also known that a slight increase in cAMP is enough to produce a sufficient activation of the cAMP-dependent protein kinase.$^{35,36}$ It is well established that cAMP mediates the positive inotropic action of such cardiotonic agents as $\beta$-adrenergic stimulants, and we propose that cAMP is also involved in the inotropic action of CGRP. This hypothesis is based on not only the CGRP-induced increase in the cAMP content but also characteristic features of the cardiac responses to CGRP that can be attributed to the cAMP accumulation (vide infra).

A phosphodiesterase inhibitor IBMX, which suppresses the hydrolysis of cAMP to inactive 5'-AMP, enhanced the positive inotropic response to CGRP. On the other hand, the inotropic action of CGRP was significantly suppressed by adenosine. Adenosine has been shown to attenuate the isoproterenol-induced increases in the AC activity,$^{37,38}$ the myocardial cAMP level,$^{39}$ and the slow inward $Ca^{2+}$ current$^{40}$ as well as the contractile force. The positive inotropic effects of histamine and dopamine are also suppressed by adenosine.$^{41}$ However, adenosine does not influence the inotropic response elicited by phenylephrine, a specific $\alpha$-adrenoceptor agonist,$^{42}$ which is now thought to act via increasing a
FIGURE 5. Calcitonin gene-related peptide (CGRP)-like immunoreactivities in the guinea pig hearts. A: Numerous CGRP-like immunoreactive (CGRP-I) nerves were observed within the myocardium of the left atria. B: Only a few CGRP-I nerves (arrowhead) were present within the myocardium of the left ventricles. C: Nerve bundles of CGRP-I nerves (arrows) were observed within the pericardium of the left ventricles. D: CGRP-I nerves were frequently found within the perivascular layer of the coronary vessels. V, blood vessel; M, myocardium; P, pericardium. Bar equals 100 μM.
phosphatidylinositol turnover.\textsuperscript{43} The positive inotropic response to an elevation of extracellular Ca\textsuperscript{2+} is also resistant to adenosine.\textsuperscript{41} Thus, adenosine appears to preferentially attenuate the effect of the cardiotonic substances linked to the AC-cAMP system.

The sarcolemmal action of CGRP was characterized by an increase in the amplitude of the action potential plateau. The plateau phase is thought to be produced by the slow inward current, which is largely carried by Ca\textsuperscript{2+}. To examine the slow inward current exclusively, the fast Na\textsuperscript{+} channel was inactivated by means of partial depolarization by elevating external K\textsuperscript{+} concentration.\textsuperscript{29} Under this condition, although the tissue showed no responses to electrical stimulation, CGRP did induce slow response action potentials qualitatively indistinguishable from those induced by isoproterenol.\textsuperscript{44} The increase in the slow inward current can be brought about by an increase in the tissue content of cAMP.

Like β-adrenergic stimulants,\textsuperscript{45-47} CGRP induced a shortening of the time to peak force and an acceleration of the relaxation velocity in the isometric contraction of the left atria. Furthermore, in a maximal increased twitch response caused by an increase of the extracellular Ca\textsuperscript{2+} concentration, CGRP shortened the duration of contraction. These results suggest that CGRP enhances the relaxation process of isometric contraction. This may be due to an accelerated Ca\textsuperscript{2+} uptake into the sarcoplasmic reticulum or by a decrease in the Ca\textsuperscript{2+}-sensitivity of the cardiac contractile protein.\textsuperscript{48} Each of the two phenomena is believed to be mediated through a phosphorylation of phospholamban, a 22,000 dalton protein in the sarcoplasmic reticulum membranes, and troponin I, one component of the regulatory protein complex of cardiac muscle, respectively. Cyclic AMP-dependent protein kinase is thought to be responsible for the phosphorylation of both proteins.\textsuperscript{48,49}

The formation of cAMP from ATP is catalyzed by AC, which is a membrane-bound enzyme. In the membrane preparations of atria, CGRP stimulated the AC activity. Goltzman and Mitchell\textsuperscript{26} showed that CGRP receptors in the central nervous system are not coupled to AC and that CGRP can stimulate the AC activity only by interacting with calcitonin receptors. In the rat atria, however, CGRP, but not...
salmon calcitonin, elicits both the activation of AC and the positive inotropic response.\(^{18,25}\) In the rat atria, furthermore, specific binding sites for CGRP have been demonstrated.\(^{18}\) It is thus reasonable to speculate that CGRP activated AC through the stimulation of its specific receptors and subsequently produced an accumulation of cAMP in the atrial muscle.

Unlike isoproterenol, CGRP induced neither positive inotropic responses nor activation of AC in the ventricular muscles. In agreement with the previous reports,\(^{2,7,20,21}\) numerous CGRP-I nerves were observed within the myocardium of the left atria. In contrast, only a few CGRP-I nerves were present within the left ventricular myocardium. In ventricles, however, there existed several bundles of CGRP-I nerves within the pericardium and CGRP-I nerve fibers within the perivascular layer of the coronary vessels. It is of particular interest that CGRP is shown to be a potent coronary vasodilator in rats, rabbits,\(^{12}\) and pigs.\(^{14}\) We therefore speculate that CGRP may function as a cardiotonic neurotransmitter of nonadrenergic noncholinergic nerves in the atrial rather than in the ventricular myocytes and that CGRP-I nerves in the ventricle are most probably concerned with a regulation of the coronary vascular tone.

In human subjects, it was recently demonstrated that CGRP exerted positive chronotropic and inotropic actions concomitant with vasodilating effects.\(^{17,50}\) Relatively dense distribution of CGRP-I nerves has been shown in the atrial musculature and around the coronary arteries of various mammals.\(^{6,7,14,19,21,25}\) Inasmuch as CGRP is released by capsaicin,\(^{16,22}\) it is supposed that CGRP is stored in C-fiber afferents and released from their terminal region. Therefore, CGRP is assumed to be released locally by some sort of reflex (e.g., during cardiac ischemia) and to play an important role in the regulation of cardiac as well as vascular functions.

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T Ishikawa, N Okamura, A Saito, T Masaki and K Goto

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