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Inotropic and lusitropic effects of calcitonin gene-related peptide in the heart

Mustafa Al-Rubaiee,1 Pandu R. Gangula,2 Richard M. Millis,1 Robin K. Walker,1 Nsini A. Umoh,1 Valerie M. Cousins,1 Miara A. Jeffress,1 and Georges E. Haddad1

1Department of Physiology and Biophysics, College of Medicine, Howard University, Washington, District of Columbia; and 2Department of Physiology, Center for Women’s Health Research, Meharry Medical College, Nashville, Tennessee

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Al-Rubaiee M, Gangula PR, Millis RM, Walker RK, Umoh NA, Cousins VM, Jeffress MA, Haddad GE. Inotropic and lusitropic effects of calcitonin gene-related peptide in the heart. Am J Physiol Heart Circ Physiol 304: H1525–H1537, 2013. First published April 12, 2013; doi:10.1152/ajpheart.00874.2012.—Previous studies have demonstrated positive-inotropic effects of calcitonin gene-related peptide (CGRP), but the mechanisms remain unclear. Therefore, two experiments were performed to determine the physiological correlates of the positive-inotropic effects of CGRP. Treatments designed to antagonize the effects of physiologically active CGRP1–37 included posttreatment with CGRP8–37 and pretreatment with LY-294002 (LY, an inhibitor of phosphatidylinositol 3-kinase), 17β-estradiol (E), and progesterone (P) were also used to modulate the effects of CGRP1–37.

Experiment 1 was in vitro studies on sarcomeres and cells of isolated adult rat cardiac myocytes. CGRP1–37, alone and in combination with E and P, decreased sarcomere shortening velocities and increased shortening percentages, effects that were antagonized by CGRP8–37, but not by LY. CGRP1–37 increased resting intracellular calcium ion concentrations and Ca2+ influxes, effects that were also antagonized by both CGRP8–37 and LY. Experiment 2 was in vivo studies on left ventricular pressure-volume (PV) loops. CGRP1–37 increased end-systolic pressure, ejection fraction, and velocities of contraction and relaxation while decreasing stroke volume, cardiac output, stroke work, PV area, and compliance. After partial occlusion of the vena cava, CGRP1–37 increased the slope of the end-systolic PV relation—ship. CGRP 8–37 and LY attenuated most of the CGRP-induced effects. In human cardiomyocytes, CGRP binding to a receptor juxtapositioned on L-type Ca2+ channels is reported to increase calcium channel activation, are found to mediate the effects of CGRP on smooth muscle (24). The inotropic activity of CGRP is reported to be mediated mainly by cAMP/PKA or PKC signal transduction molecules (35, 41, 47). In human embryonic kidney-296 cells, CGRP appears to produce downstream activation of ERK by PKA and phosphatidylinositol 3-kinase (PI3K), as well as p38 MAPK activation via PKA signaling (35). These intracellular signal transduction pathways have recently been implicated in the regulation of cardiac contraction. Indeed, our laboratory has reported that ERK1/2 and PI3K inhibit K+ current and intermediate-conductance K+ current channels in normal and hypertrophied cardiomyocytes (42), which has depolarizing effects. In human cardiomyocytes, CGRP binding to a receptor juxtapositioned on L-type Ca2+ channels is reported to increase Ca2+ transport (5, 6); other researchers report decrement in the activity of transient outward current channels (41). Such increases in Ca2+ transport are known to increase the durations of cardiomyocyte action potentials and calcium transients and are important factors in cardiac hypertrophy and heart failure. Our laboratory has recently shown that the survival antiapoptotic PI3K signaling pathway is critical to cardiac mechanical function by calcium channel mechanisms, especially during cardiac hypertrophy (1). Moreover, increased plasma levels of CGRP are associated with these complications of cardiovascular disease (8, 14). Our laboratory has previously reported that estradiol and progesterone enhance CGRP-induced contractile effects in vascular smooth muscle, and we sought to consolidate these findings in the heart (10–12). The present study was, therefore, designed to determine the physiological correlates of CGRP-induced positive inotropy and to evaluate the roles of sex steroids and PI3K as mediators of CGRP signaling.

MATERIALS AND METHODS

Conformity Statement

All the procedures conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH), publication no. 85–23, revised 1996. The animal protocols were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH), publication no. 85–23, revised 1996.
have been independently approved by Howard University Institutional Animal Care and Use Committee.

**Animal Preparation**

Male Sprague-Dawley rats of 200–250 g body weight were purchased from Charles Rivers. The rats were allowed to recover and acquaint with their new environment upon arrival to the animal house of the Howard University College of Medicine for 1 wk. The animals were kept under secure, clean, and controlled room temperature (70–74°F) with a 600–1800 light cycle and were fed food and water ad libitum.

**Isolation of Cardiomyocytes**

All the reagents were purchased from Sigma Chemicals (St. Louis, MO). Double-distilled water from a MilliQ system (Millipore, MA) was used to prepare all solutions. Prepared solutions were oxygenated for 20 min before perfusion with 5% CO₂-95% O₂ and pH at 7.4 with HCl or NaOH.

Animals were injected with sodium heparin (1,000 U/kg ip) and anesthetized with pentobarbital sodium (40 mg/kg ip), 30 min before removal of the heart. After excision, the heart was quickly transferred to a Langendorff setup for 5-min retrograde coronary perfusion through the aorta (10 ml/min at 37°C) with normal Tyrode solution containing the following (in mM): 140 NaCl, 5.4 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 20 NaHCO₃, 10 d-glucose, 0.1 EGTA, 0.4 NaH₂PO₄, 20 taurine, 10 creatine. This was followed by 20-min perfusion with nominal Ca²⁺-free Tyrode solution to which 25 mg/30 ml collagenase II (Sigma) and 3 mg/30 ml protease type XIV (Sigma) were added for the last 10 min. Then the heart was cut, minced, and filtered in a buffer solution containing the following (in mM): 114 NaCl, 4.7 KCl, 0.6 KH₂PO₄, 0.6 Na₂HPO₄, 1.2 MgSO₄·7H₂O, 12 NaHCO₃, 10 KHCO₃. Calcium was introduced gradually to the freshly isolated cardiomyocytes through five 10-min step incubations to 1.2 mM CaCl₂. Freshly isolated cardiomyocytes showing no signs of blebs or round edges were used for up to 12 h.

**Inotropic Measurements In Vitro**

The contractile properties of the cardiomyocytes were assessed at the cellular and sarcomere levels simultaneously, with the dynamic changes in intracellular calcium using the “Myocyte Calcium and Contractility Recording System” from Ionoptix (Milton, MA). A μStep light source allowing a sampling rate up to 1 kHz toggled between two excitation wavelengths of 360 and 380 μm, and a fluorescent emitted wavelength of 510 μm was filtered through a 480- to 520-μm filter and detected by a photomultiplier tube. Cells were first incubated with fura 2-AM (1 μM) for 15 min and then washed twice with Tyrode solution for 10 min before being loaded into the RC-27NE chamber from Warner Instruments (Hamden, CT) containing Tyrode solution with 1.5 mM calcium. The wired chamber was placed on an inverted IX70 Olympus microscope (Center Valley, PA) and connected to the Myopacer field stimulator (Ionoptix, MA), which was set to stimulate the cell at 1.5 × threshold for 3 ms (1 Hz). The cells were initially excited at 360 μm (0.5 s) and then switched to 380 μm for 30 s and then back to 360 μm for another 0.5 s. Emitted fluorescence of 510 μm was filtered through a 480- to 520-μm filter and captured by a photomultiplier tube connected to a Fluorescence System Interface (Ionoptic) relayed to a computer. Calcium levels were assessed by the ratio of 360/380 readings using IonWizard Software (Ionoptix), where 360-μm values were determined by extrapolation between the initial and final 360-μm acquisitions. Using Myocam-S connected to the same μStep excitation source, video images of the cells were recorded (60 Hz) simultaneously with the IonWizard software to measure the mechanical properties of sarcomeres and cells.

**Pressure-Volume Loop Acquisition**

Initially, rats were anesthetized using halothane inhalation for tracheal intubation. The endotracheal tube was then connected to a mechanical ventilator for continuous delivery of 1–2% halothane with oxygen-enriched room air. A dissection of the diaphragm exposed the apex of the heart where a 26-gauge needle was briefly inserted to introduce a conductance catheter (1.9 F from Scisense) in the left ventricle (LV) along the longitudinal axis toward the aortic valve. Once in the aorta, the catheter was retrieved back into the LV for pressure-volume (PV) measurements. The catheter was connected to the Advantage system by Scisense that acquired pressure, admittance, phase shift, and amplitude. Volume was derived in real time from admittance, phase, and amplitude data using an algorithm based on Wei’s equation to compensate for the varying wall thickness during contraction and relaxation, as well as for the distance to the LV free wall, which helps optimum positioning of the catheter tip in the middle of the chamber. The Advantage box was connected to a computer where data were analyzed, and PV loops were displayed in real time using Labscribe 2 software from iWorx (Dover, NH). Real-time data comprised heart rate, end-diastolic and end-systolic pressures (ESP) and volumes, maximum and minimum pressures, change in pressure over time, stroke volume (SV), cardiac output, ejection fraction (EF), and stroke work. Labscribe 2 was also used for offline analysis of PV area (PVA), potential energy, cardiac efficiency, maximum power, preload-recruitable stroke work (PRSW), arterial elastance (EA), τ ESP vs. volume relationship (ESPVR), and the end-diastolic pressure vs. volume relationship (EDPVR). A 26-gauge catheter connected to a low-flow syringe pump (NE-1000, New Era Pump System, Farmindale, NY) was inserted in the jugular vein for slow steady and continuous delivery of test drugs, such as CGRP₁–₃₇, CGRP₈–₃₇, and LY-294002. The syringe concentrations were adjusted to the flow rate of 0.2 μl/min to achieve a steady level of the desired blood concentration in the rat within 5 min. Blood volume was calculated using the following Lee and Blaufox (26) equation, which was originally developed for determination of blood volume in the rat based on body weight: blood volume (ml) = 0.06 × body weight (mg) + 0.77. All measurements were performed during brief suspension of the mechanical ventilation (to avoid ventilation-related changes in pressures and volumes) and at steady state, where, at the beginning of the experiment, 15 min were allowed for stabilization of the acquired hemodynamic data for controls and for steady-state drug effects. Contractility measures for ESPVR and PRSW were recorded during brief suspension of the mechanical ventilation of the hepatic vein to decrease venous return and to measure the changes in pressure within sequential PV loops at different volumes.

**Experimental Design**

**Experiment 1: effects of CGRP on cardiac myocytes, in vitro.** We tested the time course of CGRP effects in vitro on sarcomeres and cells associated with dynamic changes in intracellular calcium concentrations during contractions of ventricular myocytes. Freshly isolated myocytes were stimulated at 1 Hz, and the average of 30-s recordings were analyzed in control, 5 min, 15 min, 45 min, 1 h, 2 h, and 3 h of administering 1 nM CGRP₁–₃₇. The velocity and percentage of sarcomere shortening were increased by 32.4 ± 11.1% (P < 0.05) and 46.5 ± 22.9% (P < 0.05), respectively, after 3 h of exposure to CGRP₁–₃₇ (data not shown). A similar profile was depicted for effects of CGRP₈–₃₇ on intracellular Ca²⁺ concentrations. The CGRP₁–₃₇ effects reached steady state at 3 h after exposure. Treatment with 1 nM CGRP₈–₃₇ was used to antagonize the effects of CGRP₁–₃₇. LY-294002 (1 μM) was administered to determine whether the cell survival pathway signaling molecule PI3K was involved in the effects of CGRP₁–₃₇ and of CGRP₈–₃₇. 17β-Estradiol (10 nM) and progesterone (10 nM) treatments were employed to modulate the effects of the CGRP treatments.

**Experiment 2: effects on heart function, in vivo.** Cardiac function in vivo was assessed by LV catheterization and acquisition of PV loop data. The same drugs as were used for cardiac myocyte treatments in experiment 1 were administered by bolus injection into the jugular
Compression of the vena cava was used to generate regressive PV loop data before and after the drug treatments, from which the ESPVR, EDPVR, and other inotropic properties were evaluated.

**Statistical Analysis**

All statistical analyses were performed using Prism 6.0 (Graphpad) software and verified using Microsoft Excel, which gave the same results. The paired Student’s t-test was used to compare data before and after drug treatment of the same animal group. The heteroscedastic two-sample unpaired Student’s t-test, assuming unequal variances, was used to compare the drug effects between two different animal groups. Using the null hypothesis, *P* ≤ 0.05 was significant.

**RESULTS**

**Effects of CGRP on Cardiac Myocytes In Vitro**

Mechanisms of the inotropic and lusitropic effects of CGRP on the sarcomere. Baseline resting sarcomere length did not change significantly within the test time frame. However, addition of CGRP<sub>1–37</sub> increased the baseline sarcomere length by 7.9 ± 0.2% (*P* ≤ 0.01). Figure 1 shows that CGRP<sub>1–37</sub> increased the sarcomeric velocity of contraction by 37.70 ± 7.88% (*P* ≤ 0.01) and percent shortening by 37.57 ± 7.26% (*P* ≤ 0.01). Posttreatment with CGRP<sub>8–37</sub> decreased velocity and percent shortening to less than control levels (50.67 ± 16.43% and 55.07 ± 13.40% of control, respectively; *P* < 0.05).

Figure 2 demonstrates that inhibition of PI3K by LY-294002 did not effectively antagonize the positive-inotropic effects of CGRP<sub>1–37</sub> on the sarcomere. The effects of CGRP<sub>1–37</sub> on the rate of sarcomeric relaxation were not significantly affected by CGRP<sub>8–37</sub>; however, this relaxation was blocked by inhibition of PI3K signaling using LY-294002.

Figure 3 depicts the effects of 17β-estradiol and progesterone treatments, which did not change the sarcomere length significantly in the absence of CGRP<sub>1–37</sub>. 17β-Estradiol, but not progesterone, exhibited a tendency to reduce the maximal velocity of sarcomere contraction (27.94 ± 7.28%) and percent shortening (24.36 ± 8.52%) with marginal statistical significance (*P* = 0.07). The positive-inotropic effects of CGRP<sub>1–37</sub> were enhanced in the presence of 17β-estradiol and progesterone (contraction by 47.33 ± 7.44% and percent shortening by 34.39 ± 5.48%, respectively; *P* < 0.05). Neither 17β-estradiol nor progesterone alone altered the relaxation rate constant. In the presence of both 17β-estradiol and progesterone, CGRP<sub>1–37</sub> increased the relaxation rate constant by 34.30 ± 7.21% (*P* < 0.01).

**Mechanisms of the Cellular Inotropic and Lusitropic Effects of CGRP**

Figures 4 and 5 show that the cellular inotropic effects of CGRP were similar to those found at the sarcomeric level. CGRP<sub>1–37</sub> increased the baseline myocyte length by 23.31 ± 2.74% (*P* < 0.01). The baseline cell length was decreased back to the control value by LY-294002; treatment with CGRP<sub>8–37</sub> had no effect on baseline cell length. In parallel, CGRP<sub>1–37</sub> increased the velocity of cell contraction (73.76 ± 15.5%; *P* ≤ 0.05) and cell shortening (26.71 ± 6.78%; *P* ≤ 0.05). Subsequent treatment with CGRP<sub>8–37</sub> decreased the maximum velocity of cell contraction (48.85 ± 12.34%; *P* < 0.05) and the cellular shortening (42.12 ± 10.63% of control; *P* < 0.01) to
below control levels. The inotropic effect of CGRP1–37 on the latter was antagonized by LY-294002. The tendency of CGRP to increase the rate of cellular relaxation was not statistically significant (P = 0.08).

Figure 6 shows that 17β-estradiol decreased the maximal velocity of cellular contraction (30.36 ± 6.60%; P < 0.05), cellular shortening (36.87 ± 9.83%; P < 0.01), and the cellular relaxation rate constant (28.25 ± 18.63%; P < 0.05) in the presence of 17β-estradiol. In the presence of 17β-estradiol, the negative-inotropic effects of CGRP1–37 were augmented (contraction velocity by 27.43 ± 11.67% and cell shortening by 24.31 ± 7.36%, respectively; P < 0.01), and the lusitropic effects of CGRP1–37 were attenuated (39.42 ± 13.25%; P < 0.01).

Mechanism of CGRP-induced Intracellular Calcium Dynamics

Figure 7 demonstrates that treatment of freshly isolated ventricular myocytes with CGRP1–37 increased the baseline level of intracellular Ca²⁺ by 8.06 ± 2.98% (P < 0.01), the amount of Ca²⁺ transients by 49.76 ± 13.71% (P < 0.05), and the velocity of Ca²⁺ rise by 57.28 ± 13.00% (P < 0.05) during contraction. CGRP1–37 also increased the rate constant of Ca²⁺ decay during relaxation by 40.79 ± 8.51% (P < 0.05); all of these CGRP1–37-induced changes in Ca²⁺ dynamics were inhibited by CGRP8–37. Figure 8 shows that treatment of the myocytes with LY-294002 reversed the CGRP1–37 effects; the maximum velocity of calcium influx and the rise in intracellular calcium were reverted to less than control values.

Figure 9 shows that combination treatment with 17β-estradiol plus progesterone augmented the calcium response to CGRP1–37. In the presence of CGRP1–37, percent increases in Ca²⁺ influx (77.72 ± 17.79%, P < 0.05), Ca²⁺ influx velocity (85.07 ± 12.14%, P < 0.01), and the rate constant of calcium removal during relaxation (67.77 ± 10.94%, P < 0.05) were detected. Neither 17β-estradiol nor progesterone treatments changed the baseline level of Ca²⁺ or the contraction-dependent calcium dynamics in the absence of CGRP1–37.

Effects of CGRP on Heart Function In Vivo

Effects of CGRP on heart function on the heart’s PV loop. Cardiac function was assessed in vivo by LV catheterization and determination of the PV relationship from the PV loop shown in Fig. 10. CGRP1–37 increased LV ESP by 17.4% (116.0 ± 0.2 vs. 136.2 ± 1.2 mmHg; P < 0.001), velocity of LV contraction by 17% [maximum change in pressure (dPmax) 6,651 ± 26 vs. 7,767 ± 115 mmHg/s; P < 0.001] and velocity of LV relaxation by 12% [minimum change in pressure (dPmin) −6,223 ± 14 vs. −6,979 ± 101 mmHg/s; P < 0.001]. CGRP1–37 decreased the heart rate marginally (298 ± 1 vs. 288 ± 3 beats/min) and decreased the SV markedly, by 38% (170 ± 3 vs. 104 ± 1 μl; P < 0.001). This effect on SV resulted from decrements in both end-diastolic volume (EDV) (46 ± 2%; P < 0.001) and ESV (58 ± 2%; P < 0.001), which produced a 41% decrement in cardiac output (50,494 ± 741 vs. 29,930 ± 201 μl; P < 0.001). CGRP1–37 increased the EF (61.5 ± 0.6 vs. 70.1 ± 0.4%, P < 0.001) and decreased both the stroke work (34.2 ± 0.1%; P < 0.001) and the PVA (44.1 ± 0.2%, i.e.,
CGRP8–37 also increased SV (180.47 ± 2.02 ml) and decreased LV compliance measured by the quotient SV/pulse pressure from 1.60 ± 0.05 to 0.86 ± 0.02 mmHg/ml (P < 0.001). CGRP1–37 increased LV EDV by 53 ± 2% (P < 0.001 vs. CGRP1–37). EF was not decreased to control values. Posttreatment with CGRP8–37 also increased stroke work by 39.31 ± 0.05% (P < 0.001) and PVA by 22.55 ± 0.03% (P < 0.001) and decreased CGRP1–37-induced increments in the load-dependent and load-independent contractility parameters, ESPVR (Fig. 11A) and PRSW (1.40 ± 0.21 and 12.63 mmHg, respectively; P < 0.05). CGRP8–37 returned Ea and LV compliance to control values (0.64 ± 0.01 mmHg/µl and 1.96 ± 0.04 mmHg/ml, respectively; P < 0.05 for both parameters vs. CGRP1–37).

Effects of CGRP on Regressive PV Loops

Figure 10 also depicts the effects of compression of the vena cava to reduce preload and thereby generate regressive PV loops before and after the addition of CGRP1–37. CGRP1–37 increased ESPVR, a measure of LV contractility (0.98 ± 0.13 to 2.75 ± 0.29; P < 0.001), increased PRSW, a direct measure of LV contractility, which is preload and afterload independent (84.98 ± 7.51 vs. 112.93 ± 5.52 mmHg; P < 0.001); EDPRV, a measure of LV stiffness (2.16 ± 0.64 to 3.65 ± 0.75; P = 0.15), increased marginally. CGRP1–37 had no significant effect on the slope of the PVA-EDV relationship (0.022 ± 0.001 vs. 0.020 ± 0.001).

Posttreatment with CGRP8–37 reversed the effects of CGRP1–37 on the PV loop as follows: LV ESP (106.03 ± 0.69 mmHg), dPmax (6.198 ± 49 mmHg/s), and dPmin (−4.875 ± 35 mmHg/s) decreased to less than control values (P < 0.001). CGRP8–37 also increased SV (180.47 ± 2.33 µl) and cardiac output (47,778 ± 963 µl) to control values associated with increased LV EDV by 53 ± 2% (P < 0.001 vs. CGRP1–37). EF was not decreased to control values. Posttreatment with CGRP8–37 also increased stroke work by 39.31 ± 0.05% (P < 0.001) and PVA by 22.55 ± 0.03% (P < 0.001) and decreased CGRP1–37-induced increments in the load-dependent and load-independent contractility parameters, ESPVR (Fig. 11A) and PRSW (1.40 ± 0.21 and 12.63 mmHg, respectively; P < 0.05). CGRP8–37 returned Ea and LV compliance to control values (0.64 ± 0.01 mmHg/µl and 1.96 ± 0.04 mmHg/ml, respectively; P < 0.05 for both parameters vs. CGRP1–37).

P13K Dependence of CGRP-induced Inotropic and Lusitropic Effects In Vivo

To test whether the inotropic effects observed in vivo were mediated by P13K/Akt signaling, P13K was inhibited by pretreatment with LY-294002 and posttreatment with CGRP1–37. LY-294002 alone had no significant effect on the following PV loop parameters: ESP, EDP, dPmax, dPmin, SV, EF, and Ea. Pretreatment with LY-294002 increased the relaxation constant, τ Glantz, by 17.4 ± 0.6% (i.e., 21.6 ± 0.2 vs. 25.3 ± 0.4 ms; P < 0.05) and posttreatment with CGRP1–37 did not change the lusitropic effects previously observed with CGRP1–37 treatments alone. The LY-294002 pretreatment revealed a CGRP1–37-induced lusitropic effect, whereby the relaxation constant, τ Glantz, increased to 31.2 ms (i.e., 22.9 ± 1.1% vs. LY-294002 and 44.3 ± 0.9% vs. control, P < 0.001). Pretreatment with LY-294002 also blocked effects of CGRP1–37 on the load-dependent and load-independent contractility parameters, ESPVR (Fig. 11B) and PRSW, respectively, as well as the effects of CGRP1–37 on Ea.
Effects of CGRP$_{1-37}$ on the Activation Level of Akt

To confirm the relevance of Akt signaling to the effects of CGRP$_{1-37}$, it was necessary to assess the activation level of Akt in the presence of CGRP$_{1-37}$. As shown in Fig. 12, CGRP$_{1-37}$ significantly reduced the p-Akt-to-total Akt ratio (0.913 ± 0.062 vs. 0.656 ± 0.053; P < 0.025), which was negated by cotreatment with CGRP$_{8-37}$. Furthermore, inhibition of PI3K by LY-294002 prevented the CGRP$_{1-37}$-dependent decrease in the Akt activation level, which indicates that PI3K is a necessary step between the CGRP$_{1-37}$ receptor and Akt. Cotreatment with a known Akt agonist, insulin-like growth factor-I, reversed the CGRP$_{1-37}$ negative effects on the Akt activation level (1.351 ± 0.128; P < 0.033); whereas, adenoviral transfection with a dominant negative construct for Akt (Ad.myrAkt) did not produce an additive effect to CGRP$_{1-37}$ on Akt activation (0.616 ± 0.066; P < 0.024 vs. control).

DISCUSSION

CGRP-induced Inotropy

This study is the first to show that CGRP$_{1-37}$, independent of its known vasodilatory effects, has positive-inotropic effects on the heart, partially mediated by PI3K signaling. The positive-inotropic effects of CGRP$_{1-37}$ were effectively antagonized by CGRP$_{8-37}$, also partially mediated by PI3K signaling. The effects of CGRP at the cellular level were studied in vitro and were corroborated by LV PV loop measurements studied in vivo. The positive-inotropic effects of CGRP were also found to be load independent. It is noteworthy that CGRP$_{8-37}$ decreased the effects of CGRP$_{1-37}$ below control values, at both the sarcomeric and cellular levels. These findings suggest that, in the absence of an exogenous effect, basal activity of CGRP signaling might function to maintain normal cardiac contractility. PI3K inhibition was found to have comparable effects to CGRP$_{8-37}$, which suggests that both CGRP and PI3K may activate the same intracellular signal transduction pathway. A comparable finding is reported for the role of PI3K/Akt signaling for CGRP-ergic mesenteric perivascular nerve functions in insulin-resistant rats (16).

Estrogenic Effects of CGRP-induced Inotropy

The results of this study show that 17β-estradiol decreased the heart’s sarcomeric and cellular inotropy, whereas progesterone had no effect. 17β-Estradiol is also reported to decrease cellular contractility in human and mouse male cardiomyocytes, but not in female cardiomyocytes (21). This male sex-specific negative-inotropic effect is attributed to activity of estrogen-activated myosin regulatory light chain interacting protein (21). Increased cardiac contractility in ovariectomized female rats is attributed to low activity of 17β-estradiol (30). 17β-Estradiol treatments are also reported to increase progesterone receptor activity in female but not in male cardiomyocytes (21). These findings corroborate the data in this study, demonstrating negative-inotropic effects of 17β-estradiol on sarcomeres and cardiomyocytes from male rats, without effects of progesterone. 17β-Estradiol is reported to upregulate the vasodilatory effects of CGRP$_{1-37}$ (45), with and without pro-
gesterone (10). In the present study, 17\(\beta\)-estradiol increased the positive-inotropic effects of CGRP in cardiomyocytes. This cellular effect should translate to decreased total peripheral resistance and afterload, while increasing cardiac contractility and improving cardiac function in the animal. These CGRP positive-inotropic effects on cardiomyocytes were found independent of the vascular ones. It is, therefore, likely that the CGRP-induced positive-inotropic effects that we observed in vivo did not result solely from decreased afterload, but also to an intrinsically increased cardiac contractility. As depicted in Fig. 1C, estrogen did not significantly reduce the sarcomeric relaxation rate constant. However, estrogen did enhance the positive effect of CGRP1–37, thereby prolonging the relaxation time. At the cellular level, estrogen, by itself, reduced the relaxation rate constant, but still enhanced the CGRP effect. This finding suggests that the negative effect of estrogen on the relaxation rate constant may be mediated by a separate mechanism compared with that of CGRP. Nevertheless, estrogen receptor activation may also cross-activate a CGRP-dependent signaling pathway, such as PI3K.

**CGRP-induced Cardiac Inotropy and Calcium Dynamics**

Intracellular calcium dynamics is one of the most important factors affecting cardiac contractility. In this study, we simultaneously recorded variables reflecting intracellular calcium dynamics associated with contraction-induced changes in sarcomeric and cellular lengths (Table 1). Although CGRP1–37 increased the calcium baseline, this effect was not abolished by PI3K inhibition. On the other hand, CGRP produced parallel increases in the amount and velocity of calcium release, as well as prolongation of Ca\(^{2+}\) sequestration, effects that were reversed by inhibition of PI3K. These findings are consistent with our laboratory’s previous report that the PI3K/Akt pathway does not regulate the L-type calcium channels in normal rat cardiomyocytes, but does so in hypertrophied ones (1). The results of the present study imply that the CGRP-induced positive inotropy that we observed might be mediated by at least two parallel pathways, a PI3K-dependent as well as a calcium-dependent one. Accordingly, PI3K might regulate the positive-inotropic effects of CGRP in parallel with the calcium release events; whereas CGRP-induced calcium influx might be regulated by PI3K-independent mechanisms. Although neither 17\(\beta\)-estradiol nor progesterone affected calcium handling significantly, they did increase the effects of CGRP on intracellular calcium dynamics during cardiomyocyte contraction.

**Effects of CGRP on the PV Loop**

The in vivo PV loop measurements corroborated the in vitro measurements of this study, demonstrating that CGRP has positive-inotropic effects. These results imply that the in vivo effects of CGRP were not the consequence of its vasodilatory and afterload-reducing properties. CGRP improved the load-dependent and load-independent contractility parameters, ESPVR and PRSW, and decreased the EDV (preload). According to the force-length and force-tension relationships, decreasing the preload should decrease the SV. Indeed, decreased SVs associated with decreased cardiac outputs were found. However, the CGRP-induced interplay between the mechanically
Fig. 6. Modulation of cardiomyocyte contractile effects of CGRP1–37 by E and P at the cellular level. Bars show effects of control, 10 nM E, 10 nM P, and 1 nM CGRP1–37 with 10 nM of E and 10 nM of P (E&P&CGRP) on maximal shortening velocity of cells during contraction (A), cell shortening as a percentage of resting length (B), and cell relaxation rate constant (C). Values are means ± SE; n = 20 from 4–5 animals for each group. *Significantly different from controls at P < 0.05. #Significantly different from CGRP1–37 treatments at P < 0.05.

Fig. 7. Effects of CGRP1–37 and CGRP8–37 on cardiomyocyte calcium dynamics. Bars show effects of control, 1 nM CGRP1–37 treatments, and 1 nM CGRP8–37 posttreatments on resting intracellular calcium ion concentration ([Ca²⁺]; A), Ca²⁺ influx during cell contraction (B), [Ca²⁺] increment during cell contraction (C), and Ca²⁺ decay rate constant during cell relaxation (D). Values are means ± SE; n = 20 from 4–5 animals for each group. *Significantly different from controls at P < 0.05. #Significantly different from CGRP1–37 treatments at P < 0.05.
Fig. 8. Effects of CGRP 1–37 and LY on cardiomyocyte calcium dynamics. Bars show effects of control, 1 nM CGRP 1–37 pretreatments, and 1 μM LY posttreatments (CGRP&LY) on resting $[\text{Ca}^{2+}]_i$ (A), $\text{Ca}^{2+}$ influx during cell contraction (B), $[\text{Ca}^{2+}]_i$ increment during cell contraction (C), and $\text{Ca}^{2+}$ decay rate constant during cell relaxation (D). Values are means ± SE; $n = 20$ from 4–5 animals for each group. *Significantly different from controls at $P < 0.05$. #Significantly different from CGRP 1–37 treatments at $P < 0.05$.

Fig. 9. Modulation of cardiomyocyte calcium effects of CGRP 1–37 by E and P. Bars show effects of control, 10 nM E, 10 nM P, and 1 nM CGRP 1–37 with 10 nM of E and 10 nM of P (E&P&CGRP) on resting $[\text{Ca}^{2+}]_i$ (A), $\text{Ca}^{2+}$ influx during cell contraction (B), $[\text{Ca}^{2+}]_i$ increment during cell contraction (C), and $\text{Ca}^{2+}$ decay rate constant during cell relaxation (D). Values are means ± SE; $n = 20$ from 4–5 animals for each group. *Significantly different from controls at $P < 0.05$. #Significantly different from CGRP 1–37 treatments at $P < 0.05$. 
driven decrement in SV and the aforementioned increment in contractility was associated with increased EFs. This CGRP-induced increment in EF occurred at lower energy cost because both stroke work (which accounts for the mechanical work) and PVA (which includes the stroke work as well as the elastic potential energy accounted for by the PV reserve) were significantly reduced. The PVA is directly related to the amount of oxygen consumption by the LV per beat. Thus it appears that CGRP enabled the LV to produce greater EFs at lower energy costs. This is a significant finding in view of a report that CGRP is released from ex vivo pig hearts as a consequence of ischemia-reperfusion (33). Taken together, these findings suggest that CGRP promotes restoration of cardiac function with a small expenditure of energy. In the present study, all of the
effects of CGRP occurred with no significant change in EDPVR or in the /H9270 Glantz relaxation constant. Similar to the in vitro cellular findings, the CGRP1–37-induced increases in cardiac contractility and function were prevented by pretreatment with CGRP8–37 and by inhibition of PI3K signaling, indicative of PI3K-dependent, positive-inotropic effects for CGRP1–37. Positive-inotropic effects of CGRP1–37 antagonized by CGRP8–37 have also been reported in isolated adult rat cardiomyocytes, which were not prevented by inhibiting the production of cAMP or blocking L-type calcium channels (2). In contrast, a protein kinase A-dependent mechanism has been implicated in the positive-inotropic effects of CGRP at the cellular level (18). These positive-inotropic effects of CGRP were significantly greater than the ones we observed in the present study and also greater than others have reported (2). However, the cell contractility and intracellular calcium measurements performed in the present study are in agreement with both reports (2, 18). Moreover, the calcium measurements made in the present study are similar to previously reported CGRP-induced decrements in the calcium sequestration rate constant, attributed to increased calcium-induced calcium-release (18).

Interestingly, CGRP8–37 decreased LVESP, dP min, and dPmax, as well as Vmax and percent shortening (sarcomere and cellular) below control levels. This indicates that CGRP1–37 is most likely to cross talk or interact with other active contrac-

tility-related signaling pathways or mechanical elements at basal levels. PI3K inhibition was effective in reversing the CGRP1–37 effects on the cellular and whole heart levels, but not effectively on the sarcomere. The CGRP-induced sarcomere effects on the relaxation rate constant were reversed by PI3K inhibition, similar to what we have found at the cellular and whole heart levels. However, the sarcomeric shortening was not PI3K dependent. The sarcomere being the basic elemental unit of contractility, it is not unexpected that differences between sarcomere and cell or whole heart preparations may exist due to other confounding factors found at higher architectural levels that can intensify and amplify parameters found relevant at the cellular and whole organ levels. Our group has also demonstrated and correlated the effects of CGRP on NO release by endothelial cells with its strong vasodilatory effects. Because the heart has an endothelial lining, it is expected that some CGRP-induced NO release may occur at the whole heart level, but not with the cardiomyocytes or sarcomere preparations. In view of the similarity we found in the inotropic effects of CGRP on cardiomyocytes and the whole heart, it is reasonable to assume that cardiac NO release does not significantly modulate the CGRP effects on the heart.

**CGRP-induced Effects on Compliance and SV**

One interesting aspect of the CGRP-induced positive inotropy described herein is its occurrence in conjunction with decrements in LV SV and compliance. The increase in elastance indicates the need for a greater ESP to produce the same SV and thus reflects an increased rather than a decreased afterload. This is consistent with the reduced compliance. Similarly, a previous report showed an increase in contractility with reduction in elastance (22). CGRP1–37 increases the relaxation rate constant at the sarcomere and cellular level, which indicates that the relaxation is prolonged. During such prolonged relaxation, calcium is, no doubt, accumulating in sar-

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**Table 1. Effects of CGRP1–37 on baseline sarcomere and cellular lengths as well as intracellular Ca2+**

<table>
<thead>
<tr>
<th>Sarcomere Length, μm</th>
<th>Cell Length, μm</th>
<th>Intracellular Ca2+ (340/380)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting basal level</td>
<td>1.64 ± 0.02</td>
<td>94.47 ± 1.91</td>
</tr>
<tr>
<td>CGRP1–37</td>
<td>1.77 ± 0.02*</td>
<td>116.50 ± 3.20*</td>
</tr>
</tbody>
</table>

Values are means ± SE. CGRP, calcitonin gene-related peptide. *P < 0.01.
comeres, thereby creating a state of prolonged contractility and contraction, which translates to greater LV stiffness and less compliance in the whole heart. There are several lines of evidence for involvement of CGRP in mechanisms relevant to this observation of heart stiffening. Myocardial fibrosis associated with cardiomyocyte remodeling and changes in ventricular compliance are complications of many common and uncommon heart failures, from collagen/elastin gene mutation syndromes (32, 34) to myocardial ischemia and infarctions (37). CGRP is reported to participate in isoprenaline-induced cardiomyocyte remodeling (27), cardiac fibroblast proliferation (20), and platelet aggregation (31). CGRP treatment is also reported to increase the expression of collagen mRNA and collagen production (48). CGRP appears to be involved in neurogenic inflammation (19), as well as in myocardial ischemia and preconditioning (28), wherein interferences with cardiac endothelial cell functions are likely to occur. Accordingly, treatment with the proinflammatory cytokine TNF-α is reported to increase CGRP gene promoter activity associated with stimulation of transcription factor NF-κB. Jun NH2-terminal kinase, and p38 mitogen-activated protein MAPKs (7).

As previously mentioned, increased production of cAMP and activation of cAMP/PKA appear to play roles in some of the effects of CGRP (20, 31). Negative inotropy, mediated via phospholamban, a cAMP/PKA-sensitive protein, and activation of sarcoplasmic reticulum Ca2+-ATPase, have been associated with CGRP treatments (46). CGRP treatments are also shown to promote the synthesis and release of NO by endothelial cells (15). Intermedi, a vasoactive peptide derived from CGRP, is shown to have negative-inotropic effects in isolated LV papillary muscles in association with activation of NO/cGMP signaling and thin myofilament desensitization by increased phosphorylation of the troponin C protein (36). In the present study, CGRP treatments produced decrements in ventricular compliance and SV in the absence of negative inotropy. The decrements in compliance and SV described in this laboratory animal model should be useful for elucidating important contributions to heart failure in humans.

Inotropic Effects of CGRP and Akt

Our data show that CGRP1–37 reduces the activation of Akt via PI3K. This was antagonized by CGRP4–37 and reversed by insulin-like growth factor-I. Thus it seems that CGRP1–37 downregulation of PI3K and its downstream effector Akt enhances intracellular calcium availability, as depicted by the enhanced intracellular calcium levels and calcium transients, as well as faster velocity in intracellular calcium rise during cardiomyocyte contraction. Our laboratory and others (1, 29) have previously shown a negative relationship of the slow calcium channel with Akt activation, which is in agreement with the current observation. So CGRP1–37-induced reduction of Akt activation would be expected to enhance calcium influx through slow calcium channel and the subsequent calcium-induced calcium-release mechanism. This does not preclude other mechanism being involved in the enhancement of intracellular calcium dynamics. Furthermore, these cellular CGRP1–37 effects are in concert with improved ventricular contractility with higher ESPVR and EF. Nonetheless, elevated basal intracellular calcium is also associated with reduced compliance (greater stiffness of the ventricle), as indicated by the PV-loop relationship with CGRP1–37.

Conclusion

This multilevel study shows that CGRP1–37 has positive inotropic but negative lusitropic effects on the heart. These findings were consistent in the sarcomere, cardiomyocyte, and LV in vivo and in vitro. The CGRP1–37 effects were enhanced by estrogen and progesterone and partly mediated by the PI3K/Akt signaling pathway. The CGRP1–37-dependent improvement in contractility led to an increase in the EF, despite a decrease in cardiac output. These effects were accompanied by an improvement in ESPVR, with a reduction in LV compliance. The inotropic effects of CGRP1–37 described herein were independent of the known vasodilatory effect of CGRP1–37, since in vivo whole heart measurements were corroborated with the in vitro cardiomyocytes/sarcomere load-independent measurements.

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