Enhanced calcium mobilization in rat ventricular myocytes during the onset of pressure overload-induced hypertrophy

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Carvalho, Beatriz M. R., Rosana A. Bassani, Kleber G. Franchini, and José W. M. Bassani. Enhanced calcium mobilization in rat ventricular myocytes during the onset of pressure overload-induced hypertrophy. Am J Physiol Heart Circ Physiol 291: H1803–H1813, 2006.—Early cardiovascular changes evoked by pressure overload (PO) may reveal adaptive strategies that allow immediate survival to the increased hemodynamic load. In this study, systolic and diastolic Ca2+ cycling was analyzed in left ventricular rat myocytes before (day 2, PO-2d group) and after (day 7, PO-7d group) development of hypertrophy subsequent to aortic constriction, as well as in myocytes from time-matched sham-operated rats (sham group). Ca2+ transient amplitude was significantly augmented in the PO-2d group. In the PO-7d group, intracellular Ca2+ concentration ([Ca2+]i) was reduced during diastole, and mechanical twitch relaxation (but not [Ca2+]i, decline) was slowed. In PO groups, fractional sarcoplasmic reticulum (SR) Ca2+ release at a twitch, SR Ca2+ content, SR Ca2+ loss during diastole, and SR-dependent integrated Ca2+ flux during twitch relaxation were significantly greater than in sham-operated groups, whereas the relaxation-associated Ca2+ flux carried by the Na+/Ca2+ exchanger was not significantly changed. In the PO-7d group, mRNA levels of cardiac isoforms of SR Ca2+-ATPase (SERCA2a), phospholamban, calsequestrin, ryanodine receptor, and NCX were not significantly altered, but the SERCA2a-to-phospholamban ratio was increased 2.5-fold. Moreover, greater sensitivity to the inotropic effects of the β-adrenoceptor agonist isoproterenol was observed in the PO-7d group. The results indicate enhanced Ca2+ cycling between SR and cytosol early after PO imposition, even before hypertrophy development. Increase in SR Ca2+ uptake may contribute to enhancement of excitation-contraction coupling (augmented SR Ca2+ content and release) and protection against arrhythmogenesis due to buildup of [Ca2+]i during diastole.

Sustained increase in cardiac workload leads to ventricular hypertrophic growth, characterized by an increase in cardiac-myocyte volume (3). Although compensated hypertrophy may represent an adaptive response that allows the heart to meet the circulatory demand, in the long term, it is usually associated with high morbidity and mortality, often evolving to heart failure, in which altered cell Ca2+ cycling usually accompanies deterioration of contractile function (15, 16, 29, 62).

Sarcoplasmic reticulum (SR) function is of paramount importance for Ca2+ cycling and mechanical function in mammalian cardiac myocytes, not only because most of contractile-activating Ca2+ is released from this organelle, but also because Ca2+ sequestration by the SR Ca2+-ATPase is the main pathway for cytosolic Ca2+ removal, which allows proper relaxation and diastolic function. It is estimated that Ca2+ transport between SR and cytosol comprises 70–90% of total Ca2+ cycling during each beat, whereas most of the remaining Ca2+ fluxes are transsarcolemmal [influx and efflux mainly via L-type Ca2+ channels and Na+/Ca2+ exchanger (NCX), respectively] (6, 16).

A common finding in most studies of animal and human hypertrophied, failing myocardium is depression of the SR contribution to excitation-contraction coupling (ECC) and relaxation due to intrinsic changes in SR function or other mechanisms, such as SR Ca2+ unloading by increased NCX activity (14, 16, 18, 29, 44, 45, 47, 57, 61). Experimental manipulations to increase SR Ca2+-ATPase activity have been successful at rescuing ventricular performance in animal models of heart failure (18, 38, 39 but see Ref. 31), whereas experimental impairment of SR function can precipitate heart failure under pressure overload (PO) (50). On the other hand, up- and downregulation of the NCX and SR Ca2+ pump gene expression, respectively, in association with a trend toward decreased ability of the SR to release and/or take up Ca2+, were reported in nonfailing, chronically hypertrophied myocardium, even though Ca2+ transients, contractions, and ventricular performance in vivo were not markedly depressed (2, 22, 35, 36, 57).

Although much is known about the changes in myocyte Ca2+ homeostasis during late stages of PO-induced ventricular hypertrophy and transition to heart failure, little information is available on the acute responses to hemodynamic overload. Some authors reported a change in gene expression of proteins involved in SR Ca2+ transport and augmented SR Ca2+ uptake in ventricular subcellular preparations studied a few days after aortic constriction (2, 4, 42, 43). However, to our knowledge, Ca2+ balance during the onset of PO-induced hypertrophy has not been directly investigated in intact myocytes. A better understanding of early adjustments to overload and the underlying cell mechanisms is important, inasmuch as it may reveal aspects of the adaptive strategy involved in the acute physiological response of the heart to the hemodynamic perturbation. Insight into such a strategy may be valuable in the development of approaches to prevent and treat heart failure.

The aim of this study was to examine systolic and diastolic function in isolated rat left ventricular myocytes 1 wk after PO
imposition by aortic constriction. Cells were studied 2 days after aortic banding, when, despite the PO, ventricular hypertrophy is still absent, and on the 7th day, after stable hypertrophic growth has been attained (51). Our findings indicate enhanced SR-cytosol Ca²⁺ cycling, even before development of hypertrophy, which may be important for cardiac acute adaptation to the increased circulatory load, as well for signalling involved in development of hypertrophy.

**METHODS**

*Induction of sustained PO.* Male Wistar rats (8–12 wk-old) were subjected to constriction of the transverse aorta under anesthesia with ketamine and diazepam (66 and 0.44 mg/kg, respectively, ip), as described elsewhere (59). A clip (0.9-mm aperture) was placed between the brachiocephalic trunk and the left common carotid artery branches. Except for the latter step, the surgical procedure for sham-operated rats was the same. PO and sham-operated rats were studied 2 days (PO-2d and Sh-2d, respectively) and 7 days (PO-7d and Sh-7d, respectively) after surgery, when femoral and carotid arteries were cannulated for blood pressure measurement under pentobarbital sodium anesthesia (50 mg/kg ip). Then the animals were euthanized by pentobarbital overdose, and the hearts were rapidly removed for further experiments. All procedures and care of the rats were approved by the Institutional Committee for Ethics in Animal Research (Proc. N 1070-1).

**RT-PCR.** After the left ventricles were homogenized in TRIZol, total RNA was isolated and precipitated with isopropanol. cDNA was synthesized in 6-µg aliquots of mRNA with the Superscript II preamplification system (Invitrogen, São Paulo, Brazil), as previously described (40). Taq DNA polymerase was used for cDNA amplification in the presence of specific primers for the cardiac isoforms of SR Ca²⁺-ATPase (SERCA2a; M. Porter and A. M. Samarel, personal communication), phospholamban (PLB) (27), ryanodine receptor (RyR2) (17), calsequestrin (CSQ) (49), sarcoplasmic reticulum NCX (NCX1) (48), and β-actin (40). For each cDNA species, the number of amplification cycles was that necessary for 50% saturation, as determined in preliminary assays. mRNA levels were normalized to that of β-actin mRNA [which is not affected in this PO model (40)] determined in the same assay.

*Isolated ventricular myocytes.* The myocytes were enzymatically dissociated as described elsewhere (8). Cells from at least four different hearts were used for each experimental protocol. Myocytes were perfused with modified Tyrode solution at 23°C and field stimulated at 0.5 Hz (unless otherwise stated) with 5-ms voltage pulses.

Unloaded cell shortening was measured with a video edge detector. Cells were loaded with the fluorescent Ca²⁺ indicator indo 1-AM (5 µM; Molecular Probes, Eugene, OR) for 15 min. Indo 1 was excited at 360 nm, and intracellular free Ca²⁺ concentration ([Ca²⁺]i) was estimated from the ratio of emission at 410 nm to emission at 485 nm, which was converted to [Ca²⁺], use of calibration parameters determined in vivo, as previously described (8). The time course of relaxation and [Ca²⁺], decline was evaluated by the respective halftime values (t½-s and t½-c). The SR Ca²⁺ content was estimated as the peak increase in cytosolic total Ca²⁺ concentration ([Ca²⁺]t) evoked by 10 mM caffeine in 0 Na⁺-0 Ca²⁺ solution (11), which yields estimates comparable to those obtained by integration of the extracellular Na⁺-dependent inward current evoked by millimolar caffeine (28). Passive Ca²⁺-buffering parameters previously determined in ventricular rat myocytes (13) and measured [Ca²⁺], were used for [Ca²⁺]t estimation, with the assumption that intracellular indo 1 concentration was 20 µM. The fraction of the SR Ca²⁺ content released during a twitch (fractional Ca²⁺ release) was directly estimated according to Bassani et al. (5, 7).

Briefly, after determination of steady-state SR Ca²⁺ content at 0.5 Hz, electrical stimulation was resumed for 5 min to reload the SR, and the cell was incubated with 5 µM thapsigargin (Calbiochem, San Diego, CA) in 0 Na⁺-0 Ca²⁺ solution for 90 s to irreversibly block the SR Ca²⁺-ATPase. Then Tyrode solution was switched on, a single twitch was evoked, and the remaining SR Ca²⁺ load was determined. The fractional release thus corresponds to the amount of Ca²⁺ lost from the SR during the twitch as a percentage of the steady-state SR Ca²⁺ load. Data from cells in which thapsigargin failed to completely abolish further SR Ca²⁺ reloading were discarded.

Relaxation-associated Ca²⁺ fluxes carried by the SR Ca²⁺ pump, NCX, and the slow transporters (sarcemmal Ca²⁺-ATPase plus mitochondrial Ca²⁺ uniporter) were calculated as described elsewhere (6, 9) from the [Ca²⁺], decline phase of different types of transients: 1) a caffeine-evoked transient in 0 Na⁺-0 Ca²⁺ solution (SR Ca²⁺ accumulation and NCX were inhibited), 2) an electrically evoked twitch after thapsigargin treatment (SR uptake was inhibited), and 3) a control twitch (all transporters were functional). Total Ca²⁺ flux (time derivative of [Ca²⁺],) was considered the sum of the fluxes carried by the transporters that were functional at each type of transient. Empirical kinetic parameters of these transporters were estimated for each cell, with the flux mediated by a given transporter considered to be equal to Vmax/[1 + (Km/[Ca²⁺],)]n, where Vmax is the maximal velocity of transport, Km is the [Ca²⁺], at which velocity is half-maximal, and n is the Hill coefficient. In this model, we assume that Ca²⁺ transport by each system depends only on its kinetic parameters and [Ca²⁺]. The estimated Ca²⁺ flux carried by each pathway was integrated over 1 s after the peak of the twitch Ca²⁺ transient and expressed as micromolar (i.e., micromoles of Ca²⁺ per liter of nonmitochondrial cell water).

To investigate diastolic SR Ca²⁺ loss, the amplitude of caffeine-evoked contractures in 0 Na⁺-0 Ca²⁺ solution was taken as an index of the SR Ca²⁺ content before (steady state) and after prolonged diastole (postrest). A steady-state contracture was evoked immediately after interruption of stimulation at 0.5 Hz for 5 min. After caffeine washout followed by electrical stimulation for 5 min, the cells were rest in Ca²⁺-free solution for 3 min (11) and then exposed again to caffeine.

Concentration-effect curves to isoproterenol (Iso) were determined at 0.5-Hz stimulation during simultaneous measurement of cell shortening and [Ca²⁺]. After stabilization of the inotropic response at each Iso concentration, the cells were rested for 1 min, when the frequency of spontaneous contractions was recorded. The maximal responses to the agonist and the negative logarithm of the molar Iso concentration that evoked half-maximal responses (pD₂) were obtained by nonlinear curve fitting.

**Solutions.** The composition of Tyrode solution was as follows (mM): 140 NaCl, 6 KCl, 1.5 MgCl₂, 1 CaCl₂, 5 HEPES, and 11 glucose (pH 7.4 at 23°C). In the 0 Na⁺-0 Ca²⁺ solution, LiCl and EGTA replaced NaCl and CaCl₂, respectively. Iso-HCl stock solution contained 1 mM ascorbic acid. Thapsigargin and indo 1 were dissolved in DMSO. Unless otherwise indicated, all reagents were obtained from Sigma Chemical (St. Louis, MO).

**Data analysis.** Values are means ± SE, unless otherwise indicated. Most comparisons were made by two-way analysis of variance. When, in addition to the type of surgery and postsurgical period, a third factor was involved (i.e., extracellular [Ca²⁺] or stimulation rate), a three-way analysis of variance followed by Bonferroni’s test was used. mRNA data were compared by the Mann-Whitney U test. Percent contributions of transporters to relaxation were transformed to arcsin√p (where p is the fractional percentage) for normal distribution before statistical analysis, then means and limits of the 95% confidence interval were converted back to percentages. Differences were considered statistically significant at P < 0.05. Prism 4.0 (Graphpad Software, San Diego, CA) was used for nonlinear curve fitting and most of the statistical tests.
RESULTS

Data in Table 1 show that aortic stenosis significantly increased (P < 0.001) diastolic and systolic arterial pressures upstream from the clamp (carotid artery), whereas pressure downstream from the clamp (femoral artery) was comparable to carotid pressure in sham-operated rats. The systolic pressure gradient (≈40 mmHg) was stable until 7 days after surgery. Heart rate was similar in all groups.

Left ventricular hypertrophy, identified as a significant increase in left ventricular wall mass relative to the right ventricle wall and whole body mass, was absent on the second day, but evident on the seventh day after aortic constriction (Table 1), when a significant increase in myocyte width (P = 0.019 for surgery-time interaction), but not length, was observed, as typical of PO (3). With the assumption that myocyte thickness was unchanged, volume was estimated as 30% greater in PO-7d than in Sh-7d myocytes, which could account for most of the left ventricular growth.

Enhanced Ca\(^{2+}\) mobilization during ECC. Figure 1 depicts representative twitch Ca\(^{2+}\) transients and contractions recorded

<table>
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<th>Table 1. Pressure overload and LV hypertrophy</th>
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<td>Cell length, µm*</td>
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Values are means ± SE; n, number of animals or hearts. PO-2d and PO-7d, 2 and 7 days after aortic banding; Sh-2d and Sh-7d, time-matched sham-operated rats; LV, left ventricle wall mass; RV, right ventricular wall mass; SCAP and DCAP, systolic and diastolic pressure in carotid artery; SFAP and DFAP, systolic and diastolic pressure in femoral artery; Sgrad, trans-stenoses pressure gradient (SCAP-SFAP); HR, heart rate; BW, body wt. *Measured in 20 cells/heart. †P < 0.01 for surgery only; ‡P < 0.05 for surgery-time interaction (2-way ANOVA).

Fig. 1. Twitch in myocytes from pressure-overloaded (PO) ventricles. A: representative steady-state Ca\(^{2+}\) transients [intracellular Ca\(^{2+}\) concentration, ([Ca\(^{2+}\)]\text{i})] at 0.5 Hz in rat ventricular myocytes at days 2 and 7 after aortic constriction (PO-2d and PO-7d) or sham surgery (Sh-2d and Sh-7d). B: superimposed traces of cell shortening [percentage of resting cell length (RCL)]. C: superimposed Ca\(^{2+}\) transients in PO and Sh myocytes. Dashed lines, traces from Sh groups.

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from PO and sham myocytes at steady-state stimulation (0.5 Hz). Mean contraction and \( \text{Ca}^{2+} \) transient amplitude (\( \Delta [\text{Ca}^{2+}]_i \)), as well as \( t_{0.5-R} \) and \( t_{0.5-Ca} \), are shown in Table 2. Although differences in peak shortening did not attain statistical significance, \( \Delta [\text{Ca}^{2+}]_i \) was increased by 20% in PO-2d (\( P < 0.01 \) for surgery vs. time interaction), but not in PO-7d, cells. A significant interaction (\( P < 0.01 \)) was also observed for diastolic \( [\text{Ca}^{2+}]_i \), which was decreased in hypertrophied myocytes, but not in PO-2d cells.

Greater \( t_{0.5-R} \) values (Table 2, Fig. 1B) were observed only in hypertrophied cells (\( P < 0.001 \), surgery-time interaction), even though \( t_{0.5-Ca} \) was unaffected, as seen in hypertrophied myocytes from spontaneously hypertensive rats (SHR) (54). Thus prolonged relaxation in PO-7d cells was probably due to changes in the \( \text{Ca}^{2+} \)-myofilament interaction and/or cell mechanical properties [e.g., upregulation of \( \beta \)-myosin heavy chain and greater cytoskeletal stiffness and viscosity caused by increased microtubule density (24, 58)], rather than slower cytosolic \( \text{Ca}^{2+} \) removal.

The PO vs. sham differences observed in \( \text{Ca}^{2+} \) transients at 0.5 Hz were maintained over a broad range of stimulation rates (0.1–2 Hz). Although diastolic \( [\text{Ca}^{2+}]_i \), increased with frequency (\( P < 0.001 \), Fig. 2A), \( \text{Ca}^{2+} \) transient amplitude showed a small, but significant (\( P < 0.05 \)), rate-dependent increase that was more evident in PO-2d cells (\( P < 0.01 \); Fig. 2B). The \( [\text{Ca}^{2+}]_i \) decline during relaxation was accelerated by increasing rate (\( P < 0.001 \)), as typical of rodent myocytes and possibly due to enhancement of SR \( \text{Ca}^{2+} \) uptake associated with \( \text{Ca}^{2+} \)/calmodulin-dependent protein kinase II (CaMKII) activity (34, 62). This response was similar in all groups (Fig. 2C). The differences between groups during changes in stimulation rate were maintained when extracellular \( [\text{Ca}^{2+}]_o \) \( ([\text{Ca}^{2+}]_o) \) was decreased to 0.5 mM. Additionally, the positive inotropic effect of increasing \( [\text{Ca}^{2+}]_o \), from 0.5 to 2 mM, was comparable among groups (not shown).

Fractional SR \( \text{Ca}^{2+} \) release during a twitch was enhanced in PO compared with sham cells (\( P < 0.01 \) for surgery only; Fig. 3A), independently of the postsurgery period (\( P = 0.462 \) for surgery-time interaction). A similar pattern of change was observed for the SR \( \text{Ca}^{2+} \) content, which was increased in PO cells (\( P < 0.01 \) for surgery only, \( P = 0.848 \) for surgery-time interaction; Fig. 3B). Thus, considering both changes, one can conclude that the amount of \( \text{Ca}^{2+} \) released from the SR during a twitch is markedly increased in the early phases of hypertrophy development.

**Increased SR \( \text{Ca}^{2+} \) uptake during relaxation.** Available evidence indicates that, at steady-state activity, \( \text{Ca}^{2+} \) fluxes during ECC and relaxation are fairly balanced across the sarcolemma and the SR membrane (16, 60). Thus one would expect that enhanced systolic SR \( \text{Ca}^{2+} \) release in PO myocytes would be accompanied by increased SR \( \text{Ca}^{2+} \) uptake during relaxation. To investigate this possibility, we estimated the individual \( \text{Ca}^{2+} \) fluxes and the relative contributions of the SR and NCX to the total integrated \( \text{Ca}^{2+} \) flux associated with twitch relaxation. SR-mediated \( \text{Ca}^{2+} \) flux was increased in PO compared with sham myocytes (\( P = 0.044 \) for surgery only, \( P = 0.782 \) for surgery-time interaction), whereas neither the NCX-mediated flux nor the relative contributions of SR and NCX to cytosolic \( \text{Ca}^{2+} \) removal was significantly changed (Table 2, Fig. 3C). \( \text{Ca}^{2+} \) mobilization during a twitch (estimated as total integrated flux in Table 2, i.e., sum of fluxes

### Table 2. \( \text{Ca}^{2+} \) transient, contraction, and \( \text{Ca}^{2+} \) fluxes during a twitch

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<td>( \text{Ca}^{2+} ) flux, ( \mu \text{M} )</td>
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<td>Relative contribution to relaxation, %</td>
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Values are means ± SE or means with 95% confidence intervals (in parentheses). RCL, resting cell length; \( \Delta [\text{Ca}^{2+}]_o \), \( \text{Ca}^{2+} \) transient amplitude, i.e., peak – diastolic intracellular \( \text{Ca}^{2+} \) concentration (\( [\text{Ca}^{2+}]_i \)); \( t_{0.5-R} \) and \( t_{0.5-Ca} \) half times for relaxation and \( [\text{Ca}^{2+}]_i \), decline, respectively; SR, sarcoplasmic reticulum; NCX, \( \text{Na}^+/\text{Ca}^{2+} \) exchanger. *\( P < 0.05 \) for surgery; †\( P < 0.01 \) for surgery-time interaction (2-way ANOVA).

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mRNA ratio was ~2.5-fold greater in PO-7d than in Sh-7d cells (P < 0.05; Fig. 4), which is consistent with the functional data that indicate increased SR Ca^{2+} uptake.

*Increased diastolic SR Ca^{2+} loss.* Increased SR Ca^{2+} load leads to greater SR Ca^{2+} release during systole (7, 52, 60) and diastole (10, 53). Our present results point out a greater amount of Ca^{2+} stored in the SR and released during systole in myocytes from PO rats. To ascertain whether diastolic SR Ca^{2+} release was also affected, we analyzed SR Ca^{2+} depletion at rest, during which cells were perfused with Ca^{2+}-free medium. In this condition, stimulation of NCX-mediated Ca^{2+} efflux unmasks the time-dependent SR Ca^{2+} loss in the rat ventricle, which is otherwise not apparent because of reuptake of the leaked Ca^{2+} (11). SR Ca^{2+} loss was assessed by the ratio of the amplitude of postrest to steady-state caffeine contractures. The analysis of variance revealed that this ratio was lower in PO than in sham cells (P = 0.01 for surgery alone, P = 0.151 for surgery-time interaction; Fig. 5, A and B), which indicates greater diastolic SR Ca^{2+} leak.

Because Ca^{2+} overload and greater diastolic SR Ca^{2+} release may underlie spontaneous activity in the myocardium (10, 16, 37), we also analyzed the rate of spontaneous contractions at different [Ca^{2+}]_o. As shown in Fig. 5C, spontaneous activity during rest was enhanced by an increase in [Ca^{2+}]_o (P < 0.001), and, independently of the influence of [Ca^{2+}]_o, the rate of spontaneous contractions was significantly greater in PO than in sham cells (P < 0.01 for surgery alone, P = 0.525 for surgery-time interaction).

*Increased inotropic sensitivity to β-adrenoceptor stimulation.* Plasma catecholamine levels rise soon after the onset of hypertrophy in aorta-constricted rats (1), and myocardial responsiveness to β-adrenoceptor activation may be altered at this early stage. Concentration-effect curves to Iso inotropic responses were not significantly altered. When Iso facilitation of spontaneous activity during rest was examined, a significant increase in maximal response was observed in the PO compared with the sham myocytes (P = 0.038 for surgery alone, P = 0.357 for surgery-time interaction; Fig. 6C). The pH_{2} values for this response were comparable in all groups (P = 0.624 for surgery; Table 3).

**DISCUSSION**

The main finding of the present study was enhanced SR-cytosol Ca^{2+} cycling in rat left ventricular myocytes before and during development of ventricular hypertrophy. Therefore, the increase in Ca^{2+} cycling between SR and cytosol during ECC and relaxation demonstrates enhanced SR function before and during development of ventricular hypertrophy.

The functional changes in Ca^{2+} cycling in hypertrophied myocytes were not accompanied by a significant alteration in NCX1, RyR2, CSQ, PLB, and SERCA2a mRNA levels relative to β-actin mRNA, although there was a trend toward an increase in SERCA2a and NCX mRNA abundance in PO-7d cells (0.10 > P > 0.05). Unchanged message levels of SERCA2 and RyR 5 days after aortic banding have also been reported by Anger et al. (2). However, the SERCA2a-to-PLB carried by SR, NCX, and slow transporters) was greater in PO cells (P = 0.031 for surgery only, P = 0.715 for surgery-time interaction). The SR Ca^{2+}-free effluxes during ECC and relaxation demonstrate enhanced SR function before and during development of ventricular hypertrophy.
tracellular Ca\(^{2+}\) cycling was also described in hypertrophied myocytes from nonfailing SHR hearts (54). The presence of a similar change during the onset of left ventricular hypertrophy induced by stepwise imposition of PO suggests that enhanced ventricular Ca\(^{2+}\) cycling might be a response to increased hemodynamic load, rather than a characteristic of that particular genetic model of hypertension.

Our results show enhanced ECC during the first week after aortic constriction. Inotropic and lusitropic responses to changes in stimulation rate and [Ca\(^{2+}\)]\(_{o}\) in cells from PO and sham groups were qualitatively comparable, which indicates preservation of contractile reserve, in contrast to the blunted response to rapid pacing in failing, hypertrophied myocardium (30) and apparently reduced ECC efficiency at low [Ca\(^{2+}\)]\(_{o}\) in chronic, compensated, PO-induced hypertrophy (36).

Before development of hypertrophy (PO-2d group), twitch Ca\(^{2+}\) transient amplitude was greater, apparently because of markedly increased fractional SR Ca\(^{2+}\) release. Higher peak [Ca\(^{2+}\)]\(_{i}\) in this group would favor SR Ca\(^{2+}\) uptake during relaxation as a result of a substrate concentration-dependent increase in enzyme velocity. Accordingly, the estimated Ca\(^{2+}\) flux mediated by the SR Ca\(^{2+}\)-ATPase during relaxation was also greater, which demonstrates enhanced SR-cytosol Ca\(^{2+}\) cycling. Despite the indication of greater Ca\(^{2+}\) fluxes between SR and cytosol also in PO-7d myocytes, twitch Ca\(^{2+}\) transient amplitude was not increased. Stronger Ca\(^{2+}\) buffering due to greater abundance of myofibrillar proteins (3) would not be a likely explanation, inasmuch as changes in Ca\(^{2+}\) buffering capacity do not seem to accompany ventricular hypertrophy (44). Nevertheless, systolic [Ca\(^{2+}\)]\(_{i}\) variation might be attenuated because of the small, but significant, decrease in diastolic [Ca\(^{2+}\)]\(_{i}\) in PO-7d cells. Lower [Ca\(^{2+}\)]\(_{i}\) implies that less Ca\(^{2+}\) would be bound to buffers during diastole, and the greater availability of Ca\(^{2+}\) binding sites might increase the efficiency of Ca\(^{2+}\) buffering during systole, even if buffering capacity remained unchanged. On the basis of Ca\(^{2+}\) buffering data previously obtained in ventricular rat myocytes (13), we estimate that the 15% decrease in diastolic [Ca\(^{2+}\)]\(_{i}\) in PO-7d cells would allow additional buffering of \(\sim 7-10\) \(\mu\)M total Ca\(^{2+}\) during systole without a change in the free Ca\(^{2+}\) transient amplitude. Additionally, enhanced SR Ca\(^{2+}\) uptake might also
estimates of Ca^{2+} efflux via NCX with the present approach have shown agreement with direct measurement of Ca^{2+} influx by Ca^{2+} current integration (6, 16, 21). Thus it is likely that an increase in SR Ca^{2+} content is the main mechanism responsible for enhanced fractional SR Ca^{2+} release in PO myocytes. Greater SR Ca^{2+} loading in other models of ventricular hypertrophy has been attributed to an increase in NCX-mediated Ca^{2+} influx during the action potential (19, 55), rather than enhanced SR function. In our model, however, experimental findings did not suggest major changes in total Ca^{2+} influx and/or NCX function.

Additional RyR regulation by endogenous signaling molecules might further contribute to the increase in fractional SR Ca^{2+} release in PO cells. Interestingly, larger Ca^{2+} transient and spark amplitude, without concomitant changes in L-type Ca^{2+} current and SR Ca^{2+} content, were observed in myocytes from SHR with compensated hypertrophy and attributed to greater efficacy of the trigger signal to elicit SR Ca^{2+} release (54). Increased sensitivity to Ca^{2+}-releasing agonists in RyR isolated from PO hearts has also been described (32). Activation of Ca^{2+}-calmodulin-dependent enzymes has been considered an important step in hypertrophy signaling (34, 62). Ventricular CaMKII δ-isofrom expression and activity are increased as soon as 1 day after aortic banding and remain high for 7 days after banding (20, 62). This enzyme has been shown to positively modulate Ca^{2+} release from the SR (34, 61, 62). It is tempting to speculate whether the CaMKII enzyme would play a role in the stimulation of SR Ca^{2+} release in the early phase of PO.

Increased SERCA2 expression and in vitro activity have been described during the onset of mild hypertrophy due to aortic stenosis (4, 42, 43). These changes were implicated in the enhancement of left ventricular function, which may accompany the development of compensated hypertrophy (41–43). Here we present experimental evidence of greater SR Ca^{2+} uptake in intact PO-7d cells, namely, increased SR-mediated Ca^{2+} transport during relaxation, despite unchanged Ca^{2+} transient amplitude. The SERCA-to-PLB ratio has been considered the main determinant of SR Ca^{2+} release from the SR (34, 61, 62). It is tempting to speculate whether the CaMKII enzyme would play a role in the stimulation of SR Ca^{2+} release in the early phase of PO.

In our study, supersensitivity to Iso was observed 1 wk after aortic ligation, when increases in plasma norepinephrine concentration and ventricular epinephrine content have been reported (1, 56). Inasmuch as supersensitivity to Iso and epinephrine due to augmented efficiency of β2-adrenoceptor signaling was reported during adaptation to stress (12), it is...
possible that a similar change might account for the greater β-adrenergic responsiveness in hypertrophied myocytes. Although further studies are necessary to investigate this possibility, the present findings indicate increased inotropic reserve at the onset of hypertrophy, which is in sharp contrast to the impairment of β-adrenergic cascade signaling in the failing heart (25). Spontaneous contractions are thought to arise from greater diastolic Ca\(^{2+}\) release from an overloaded SR, which may give rise to delayed afterdepolarizations and arrhythmia due to the generation of depolarizing membrane current by electrogenic Ca\(^{2+}\) extrusion via NCX (10, 37, 45). The higher rate of spontaneous contractions and SR Ca\(^{2+}\) loss during rest in both PO groups may be associated with a greater propensity to arrhythmia during the PO acute phase, especially under conditions that favor Ca\(^{2+}\) overload, such as increased \([\text{Ca}^{2+}]_o\) and β-adrenoceptor stimulation. In the case of Iso-induced enhancement of spontaneous activity, an increase in the apparent efficacy (but not potency) of the agonist was observed in both PO groups, probably due to greater diastolic SR Ca\(^{2+}\) leak. However, in the PO-7d group, in which the pD\(_2\) values for Iso inotropic effects were increased, the ratio of the con-

Table 3. Inotropic response to isoproterenol

<table>
<thead>
<tr>
<th></th>
<th>Sh-2d (n = 10)</th>
<th>PO-2d (n = 9)</th>
<th>Sh-7d (n = 8)</th>
<th>PO-7d (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shortening Max, %RCL</td>
<td>8.77±1.08</td>
<td>9.49±1.48</td>
<td>11.50±1.14</td>
<td>9.09±1.79</td>
</tr>
<tr>
<td>pD(_2) (\Delta[\text{Ca}^{2+}]_i)</td>
<td>8.843±0.137</td>
<td>8.745±0.059</td>
<td>8.780±0.069</td>
<td>9.281±0.164†</td>
</tr>
<tr>
<td>Max, μM</td>
<td>0.750±0.095</td>
<td>0.535±0.087</td>
<td>0.478±0.079</td>
<td>0.625±0.151</td>
</tr>
<tr>
<td>pD(_2)</td>
<td>8.483±0.098</td>
<td>8.490±0.055</td>
<td>8.542±0.100</td>
<td>9.154±0.213†</td>
</tr>
<tr>
<td>Spontaneous activity Max, SC/min</td>
<td>8.8±1.5</td>
<td>11.2±1.8*</td>
<td>6.7±0.6*</td>
<td>12.2±3.2*</td>
</tr>
<tr>
<td>pD(_2)</td>
<td>7.990±0.142</td>
<td>7.923±0.159</td>
<td>8.110±0.097</td>
<td>7.965±0.215</td>
</tr>
</tbody>
</table>

Values are means ± SE. SC, spontaneous contraction; pD\(_2\), negative logarithm of molar isoproterenol concentration that evokes half-maximal response; max, maximal response. *P < 0.05 for surgery; †P < 0.05 for surgery-time interaction (2-way ANOVA).
centration required for 50% of maximal proarrhythmic and inotropic effects to that required for inotropic effects was markedly greater than in the other groups (~18 vs. 4). As a result, considerable enhancement of contractility might be achieved at levels of β-adrenergic mediators that are not high enough to be arrhythmogenic.

It is conceivable that enhanced diastolic SR Ca\(^{2+}\) loss and systolic fractional release may stem from common mechanisms, such as increased SR Ca\(^{2+}\) content (7, 10, 53) and, possibly, RyR modulation by other signaling molecules, such as CaMKII, which may stimulate diastolic and systolic SR Ca\(^{2+}\) release (61, 62). In addition to SR Ca\(^{2+}\) overload, enhanced NCX function can be a predisposing factor for myocardial spontaneous activity. Accordingly, enhanced susceptibility to arrhythmia has been attributed to SR Ca\(^{2+}\) overload and/or augmented arrhythmogenic currents in experimental models of ventricular hypertrophy and heart failure in which NCX function is upregulated (37, 45, 55). In the present study, however, there was no indication of significant NCX upregulation. This, associated with enhanced SR Ca\(^{2+}\) uptake, may attenuate the potentially arrhythmogenic effects of increased diastolic SR Ca\(^{2+}\) release (23).

The present study presents some limitations, such as the measurement of unloaded cell shortening vs. developed force under mechanical load and the temperature at which the experiments were carried out (23°C), which precluded the use of physiological stimulation rates. However, Puglisi et al. (46) demonstrated that the relative balance among Ca\(^{2+}\) transporters in ventricular myocytes is similar at 25°C and 35°C, although Ca\(^{2+}\) transients and contractions differ in amplitude and time course. Another limitation is that mRNA levels were used to investigate gene expression of proteins involved in Ca\(^{2+}\) transport. It has been shown that changes in mRNA levels may or may not be paralleled by changes in abundance and/or function of the respective protein (33, 36). Nevertheless, the functional changes observed in the PO-7d group (i.e., greater contribution of SR Ca\(^{2+}\) uptake during twitch relaxation and lower diastolic [Ca\(^{2+}\)]) in which a greater SERCA2a-to-PLB mRNA ratio was detected, are in agreement with parallelism of message and protein levels.

In summary, our results show that aortic ligation evokes a myocardial response characterized by enhanced SR-cytosolic Ca\(^{2+}\) cycling, even before the onset of hypertrophy. This very early response may be important for maintenance of cardiac output and also may be implicated in the activation of Ca\(^{2+}\)-dependent pathways involved in development of hypertrophic growth (14–16, 34, 62), although at the cost of greater propensity to arrhythmia because of augmented diastolic SR Ca\(^{2+}\) release. These changes are likely to be exacerbated in vivo because of stretch-dependent effects on Ca\(^{2+}\) homeostasis exerted by the high wall stress. At the onset of hypertrophy, greater SR Ca\(^{2+}\) uptake (possibly due to higher SERCA2a-to-PLB expression ratio) may help preserve systolic and diastolic functions by maintaining the SR Ca\(^{2+}\) load at a high level and decreasing diastolic [Ca\(^{2+}\)], respectively, while diminishing net SR Ca\(^{2+}\) loss. At this stage, the inotropic response to sympathetic mediators is likely to be enhanced because of greater cell sensitivity to β-adrenoceptor stimulation. This scenario is the opposite of that reported in heart failure, when SR Ca\(^{2+}\) pump activity and SR Ca\(^{2+}\) content are depressed, net diastolic SR Ca\(^{2+}\) loss is augmented, and β-adrenergic responses are attenuated (14, 16, 18, 25, 29, 30, 44, 45, 57, 61). Some of these alterations are present during chronic, compensated hypertrophy (25, 35, 56, 57). Thus it would be plausible to assume that the early changes in Ca\(^{2+}\) homeostasis evoked by PO might represent acute adaptive adjustments of myocardial function to cope with the circulatory overload. However, this short-term adaptive response may eventually degenerate into maladaptive changes and deterioration of cardiac function if the circulatory stress persists, because an increase in Ca\(^{2+}\) mobilization may precipitate cardiovascular decompensation in some models of hypertrophy (14, 54, 55). It remains to be established whether chronically enhanced Ca\(^{2+}\) cycling might contribute to further development of maladaptation.

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