Genetic manipulation of calcium-handling proteins in cardiac myocytes. I. Experimental studies

Pierre Coutu1 and Joseph M. Metzger2

Departments of 1Biomedical Engineering and 2Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan

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Coutu, Pierre, and Joseph M. Metzger. Genetic manipulation of calcium-handling proteins in cardiac myocytes. I. Experimental studies. Am J Physiol Heart Circ Physiol 288: H601–H612, 2005. First published August 26, 2004; doi:10.1152/ajpheart.00424.2004.—Two genetic experimental approaches, de novo expression of parvalbumin (Parv) and overexpression of sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2a), have been shown to increase relaxation rates in myocardial tissue. However, the relative effect of Parv and SERCA2a on systolic function and on β-adrenergic responsiveness at varied pacing rates is unknown. We used gene transfer in isolated rat adult cardiac myocytes to gain a fuller understanding of Parv/SERCA2a function. As demonstrated previously, when Parv is expressed in elevated concentration (>0.1 mM), the transduced myocytes showed a reduction in sarcomere-shortening amplitude: 129 ± 17, 81 ± 8, and 149 ± 14 nm for control, Parv, and SERCA2a, respectively. At physiological temperature, shortening amplitude responses of Parv and SERCA2a myocytes to the β-adrenergic agonist isoproterenol (Iso) were not statistically different from that of control myocytes. However, in SERCA2a myocytes, in which baseline was slightly elevated and the Iso-stimulated value was slightly lower, the increase in shortening was slightly less than in Parv or control myocytes: 108 ± 14, 169 ± 39, and 34 ± 12% for control, Parv, and SERCA2a, respectively. In another test set, Parv myocytes had the strongest early postrest potentiation among all groups studied (rest time = 2–10 s), and SERCA2a myocytes were the least sensitive to variations in stimulation rhythm. To replicate the deficient Ca2+ removal observed in heart failure, we used 150 nM thapsigargin. Under these conditions, control myocytes exhibited slowed relaxation, whereas Parv myocytes retained their rapid kinetics, showing that Parv is still able to control relaxation, even when SERCA2a function is impaired.

parvalbumin; sarco(endo)plasmic reticulum calcium-adenosinetriphosphatase 2a; diastolic dysfunction; gene transfer; thapsigargin; postrest potentiation

IMPAIRED CALCIUM HANDLING plays a crucial role in heart failure (21, 31). In a normal heart, on sarcolemmal depolarization, a small amount of Ca2+ enters the myocytes and binds to the ryanodine receptor (RyR) in the terminal cisternae of the sarcoplasmic reticulum (SR) (3). This binding of Ca2+ to RyR allows a massive release of Ca2+ from the SR into the cytoplasm, which leads to mechanical contraction through interaction of Ca2+ with the myofilaments. Relaxation follows removal of Ca2+ from the cytoplasm (3). Three main proteins are involved in the control of Ca2+ removal in cardiac myocytes: Na+/Ca2+ exchanger (NCX), sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2a), and phospholamban (PLB). SERCA2a is responsible for translocation of Ca2+ from the cytoplasm to the SR. In most species, this is the dominant Ca2+ removal mechanism: ~97% in rat and ~70% in human (2). PLB acts as an inhibitor of SERCA2a. On PLB phosphorylation (e.g., via β-adrenergic stimulation), PLB dissociates from SERCA2a, leaving the pumps with an increased affinity for Ca2+ (5). Finally, NCX represents the main Ca2+ removal route for Ca2+ leaving the myocyte. Altered expression of these Ca2+-handling proteins has been associated with heart failure (31). There is some debate on the magnitude and direction of the change in expression of these Ca2+-handling proteins in heart failure (21, 24, 32). However, a decrease in SERCA2a-to-PLB and SERCA2a-to-NCX ratios appears to be a common feature of the failing myocardium (1, 16, 26, 35).

Recently, two strategies featuring genetic manipulation have been proposed to restore Ca2+-handling function in myocytes from the failing heart (30): 1) restoration of the SERCA2a-to-PLB and/or SERCA2a-to-NCX ratio or 2) introduction of parvalbumin (Parv), a specialized Ca2+ buffer (10). The SERCA2a-to-PLB ratio has been manipulated by overexpression of SERCA2a (20) and expression of antisense PLB (6) and in PLB-knockout mice (27). These approaches have restored Ca2+-handling function in a number of animal models of heart failure (13, 14, 17, 29, 37, 39). However, in recent studies, increasing the SERCA2a-to-PLB ratio did not rescue the failing heart in all animal models (38, 41). Paradoxically, in human studies, individuals expressing a nonfunctional mutated form of PLB (infinite SERCA2a-to-PLB ratio) have a high risk of developing cardiomyopathy early in adult life (8, 19, 40).

Another strategy has employed the delayed Ca2+-buffering properties of Parv to increase the relaxation properties in cardiac muscle in vitro (11, 43) and in vivo (42). The mechanism by which Parv increases relaxation performance in cardiac myocytes has been described elsewhere (10). Briefly, Parv is an 11-kDa protein naturally found in fast-twitch skeletal muscles and in the brain, but not in the heart (15). Parv functions as a delayed Ca2+ buffer and has been shown to be associated with increased relaxation rates in skeletal muscle (23). The Parv molecule has two binding sites with high affinity for Ca2+ (~10^8 M^-1) and moderate affinity for Mg2+ (~10^6 M^-1) (34). Because in myocytes the level of free Mg2+ is several orders of magnitude larger than the level of intracellular free Ca2+ at rest, a large portion of Parv is bound to Mg2+ at rest. When the Ca2+ level transiently rises in the myocyte, Mg2+ dissociates from Parv, and Ca2+ subsequently binds (10). Over an optimal Parv concentration range, the delayed Ca2+-buffering capacity of Parv is evident during the initial
phase of Ca\(^{2+}\) decay (increasing diastolic function), with no or little impact during the rising phase of the Ca\(^{2+}\) transient, thus preserving systolic function (11). As was the case for the first strategy (modification of the SERCA2a-to-PLB ratio), Parv also improved relaxation kinetics in animal models of diastolic dysfunction (9, 42, 43).

In this study, we investigated the effect of modifications in the SERCA2a-to-PLB ratio via SERCA2a overexpression and expression of Parv on cardiac myocyte relaxation performance. The overall objective was to gain mechanistic insights into Ca\(^{2+}\)-handling properties and to understand the consequences of SERCA2a and Parv gene transfer in important aspects of cardiac myocyte mechanical function. Accordingly, we used an experimental approach of gene transfer of Parv or SERCA2a in isolated rat adult cardiac myocytes (present study) combined with a theoretical analysis using mathematical modeling (12).

Our aims in this experimental study were threefold. Our first aim was to determine whether the increase in relaxation rate caused by Parv-delayed Ca\(^{2+}\)-buffering action could be maintained when the SERCA2a removal mechanism was impaired, as observed in human heart failure. As a mimetic of altered SERCA2a function in heart failure, we used a submaximal dose of thapsigargin (TG, 150 nM), a chemical compound that specifically inhibits SERCA2a (25, 45). Under these conditions, we tested the hypothesis that although TG in control myocytes would cause a significant slowing of relaxation, the rapid relaxation kinetics observed in Parv-transduced myocytes would be unaltered by TG.

Our second aim was to determine the effects of Parv expression and SERCA2a overexpression in the presence of \(\beta\)-adrenergic stimulation. In normal myocardium, \(\beta\)-adrenergic stimulation markedly enhances heart pump performance. In heart failure, \(\beta\)-adrenergic stimulation responsiveness is reduced (2). It is therefore of interest to investigate whether genetic manipulations in Ca\(^{2+}\)-handling proteins would affect the \(\beta\)-adrenergic response in myocytes.

Our third aim focused on postrest potentiation (PRP) tests in Parv- and SERCA2a-transduced myocytes. PRP is defined as the contractile response, relative to a steady state, obtained after a resting period of given duration (2). The nature of the PRP response is believed to be mainly dictated by the ability of the SR to reload Ca\(^{2+}\) and by the refractory properties of the myocytes (2). Although frequency response has been tested in Parv-transduced (11) and SERCA2a-overexpressing (37) myocytes, PRP offers additional insights into the interplay between modified Ca\(^{2+}\) removal and SR Ca\(^{2+}\)-reloading properties.

**METHODS**

**Adult cardiac myocyte isolation, gene transfer, and primary culture.** Cardiac myocytes were isolated from adult female Sprague-Dawley rats (200 g), and gene transfer was performed as described previously (11, 44). Briefly, myocytes were plated and incubated for 2 h with no virus (control) or adenovirus containing the Lac-Z gene [200–500 multiplicity of infection (MOI)], the human \(\alpha\)-Parv gene (500 MOI), or the human SERCA2a gene (200 MOI). These doses have been shown to achieve maximal (95–100%) efficiency of gene transfer (11, 43). The myocytes used for functional studies were transferred to stimulating chambers 18 h after gene transfer. The myocytes were then subjected to electrical stimulation (0.5-Hz frequency) sufficient to elicit contraction in 10–20% of the myocytes and were kept in M199+ solution (Sigma) supplemented with 10 mM glutathione, 26.2 mM sodium bicarbonate, 0.02% bovine serum albumin, and 50 U/ml penicillin-streptomycin, with pH adjusted to 7.4, as described previously (11).

The procedures used in this study were in agreement with the guidelines of the Internal Review Board of the University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Health Care, and the animal care use program conforms to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23).

**Western blots.** Each sample (~40,000 myocytes) was collected from two coverslips 3 days after gene transfer and stored at ~80°C in SDS-PAGE buffer (11). The following primary antibodies were used for Western blotting: 5c5 (Sigma; 1:5,000 dilution) for actin, Parv19 (Sigma; 1:1,000 dilution) for Parv, MA3-922 (ABR; 1:1,000 dilution) for PLB, and MA3-919 (ABR; 1:1,000 dilution) for exogenous SERCA2A (MA3-919 does not recognize endogenous rat SERCA2a). Goat anti-mouse conjugated to horseradish peroxidase secondary antibody (catalog no. A-9917, Sigma; 1:1,000 dilution) was used as secondary antibody. Antibody C-20 (Santa Cruz Biotechnology; 1:100) combined with donkey anti-goat Ig-horseradish peroxidase (Santa Cruz Biotechnology; 1:500 dilution) was used to detect simultaneously the endogenous (rat) and exogenous (human via gene transfer) SERCA2a by enhanced chemiluminescence. In all cases, actin was used to normalize for loading (11).

To estimate Parv concentration, the transduced cardiac myocyte samples were compared, after loading normalization, with samples collected from rat superior vastus lateralis (SVL) muscles. A Parv concentration of 0.4 mM was used for the SVL-positive control samples (18).

We defined SERCA2a overexpression as the ratio of transduced SERCA2a to the endogenous level of SERCA2a. To quantify SERCA2a overexpression, we used two different methods. First, we used the antibody C-20, which detects the endogenous rat SERCA2a and the genetically transduced human SERCA2a. To calculate the amount of overexpression of human SERCA2a, we subtracted the amount of endogenous SERCA2a expressed in rat control myocytes from the total amount of SERCA2a expressed in the transduced myocytes and then normalized to the level of endogenous SERCA2a (see Eq. 1).

Second, we used the antibody MA3-919, which detects the transduced human SERCA2a, but not the endogenous rat SERCA2a. We were able to quantify the overexpression, because 1) this antibody also detects rabbit SERCA2a, and 2) the Ca\(^{2+}\) uptake rate in rat myocytes is 2.5 times that in rabbit myocytes and SERCA2a affinity for Ca\(^{2+}\) is the same in both species (25). Assuming that the difference in function is due to a difference in the number of pumps, we made the following assumptions: 1) the antibody affinity for SERCA2a is the same across the different species used, and 2) the amount of endogenous SERCA2a in transduced myocytes remains the same as in the control myocytes.

**Eq. 1**

\[
\frac{\text{SERCA2a}_{\text{rat\_transduced}}}{\text{SERCA2a}_{\text{rat\_endogenous}}} = \frac{\text{SERCA2a}_{\text{rat\_transduced}} - \text{SERCA2a}_{\text{rat\_endogenous}}}{\text{SERCA2a}_{\text{rat\_transduced}} + \text{SERCA2a}_{\text{rat\_endogenous}}} \]

**Eq. 2**

\[
\text{SERCA2a}_{\text{rat\_transduced}} = \text{SERCA2a}_{\text{rat\_endogenous}} + \text{SERCA2a}_{\text{rat\_transduced}} \]

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over expression = SERCA2a\textsubscript{rat transduced} \quad SERCA2a\textsubscript{rat endogenous} \quad (2)

\[ = \frac{\text{SERCA2a} \text{rat transduced}}{2.5 \times \text{SERCA2a rabbit endogenous}} \]

**RESULTS**

**Gene transfer.** Gene transfer to isolated rat adult cardiac myocytes in primary culture has been shown to be a highly efficient and synchronized means of acute genetic engineering (11, 28). Typically, >95% of the adult cardiac myocytes express the delivered gene product (44). In this study, all experiments were conducted 3 days after gene transfer to yield a robust expression of Parv (11) and SERCA2a (Fig. 1). Figure 1 shows representative Western blots, and, as expected, Parv was present only in the Parv-transduced myocytes. The level of expression was 34.5 ± 11.4% (n = 11) of the Parv level in SVL muscle samples. With the assumption of 0.4 mM Parv in SVL (18), this leads to an estimated 0.138 ± 0.046 mM in the transduced cardiac samples. Two different antibodies were used to detect SERCA2a. The first (anti-SERCA2a#1 in Fig. 1, antibody C-20) detected endogenous SERCA2a and human SERCA2a obtained through gene transfer. Although a small increase in total SERCA2a expression could be seen in some Western blot results, it did not reach statistical significance. However, with the use of the second antibody (anti-SERCA2a#2, antibody MA3-919), it was possible to uniquely detect the human SERCA2a expressed by gene transfer. As described in METHODS, we used non-transduced rabbit cardiac myocytes to quantify the level of exogenous SERCA2a in rat myocytes.

Three independent sets of experimental data were collected: TG, Iso, and PRP. For the first data set (TG), the stimulation-and-measurement protocol was optimized from results obtained in pilot studies (unpublished data), in which we found that 150 nM TG produced a nearly maximal inhibition effect, the drug effect was stable with 7–15 min of incubation, and continuous pacing empties the SR content, annihilating contractions. The protocol is as follows: myocytes were loaded for 5 min with Ca\textsuperscript{2+} fluorescence indicator [5 \mu M fura 2-AM with 0.01% Pluronic F-127 (both from Molecular Probes)], and after washout, an additional 5 min were allowed for deesterification. The myocytes were then stimulated for 1 min at 0.2 Hz before the baseline measurement was made (also at 0.2 Hz). Then the myocytes were treated with 150 nM TG in the quiescent state (no stimulation) for 8 min. At the end of the TG incubation period, the myocytes were again stimulated for 1 min at 0.2 Hz before measurements were made. TG (Sigma) was diluted to 500 \mu M in DMSO as stock solution. The stock solution was diluted in M199+ solution. Measurements were made at two external Ca\textsuperscript{2+} concentrations: 1.8 and 5.0 mM.

The same protocol was used for the second set of data (Iso), except for the external Ca\textsuperscript{2+} concentration (fixed at 1.8 mM), dose (10 nM Iso), and incubation time (1–2 min). The dose was selected to produce a strong inotropic effect with a minimal number of fibrillating myocytes, and the incubation time was the minimal amount of time required for the myocytes to stabilize for temperature. In addition to measurements at 37°C, measurements at room temperature (22 ± 2°C) were performed to emphasize the Iso effect on relaxation kinetics.

For the final data set (PRP), the myocytes were loaded with fura 2-AM as described above. The experimental protocol (see Fig. 3A) follows this sequence: conditioning train of 10 pulses (interpulse duration = 1 s), 2 min of rest, another train of 10 prepulses (interpulse duration = 1 s), a variable rest period of duration T, and a train of 10 postpulses (interpulse duration = 1 s). The ratio of postpulse 1 to the average of the last three prepulses (prepulses 8–10) was used to characterize PRP.

**Statistical analysis.** All statistical analysis was performed using GraphPad Prism software. For experiments where myocytes were studied as populations, one-way ANOVA was used to determine any significant differences among multiple groups of data. When significant differences were detected, a Newman-Keuls multiple comparison test was performed to find which groups were different from each other (P < 0.05). For experiments in which individual myocytes were used as their own baseline, a two-tailed paired t-test was used. Values are means ± SE.
between these two methods is unclear. Because the second method clearly indicates that the overexpressed SERCA2a is present only in the transduced myocytes and that a strong phenotype is present in these myocytes (see Figs. 2–8), it is possible that a difference in affinity for the endogenous rat SERCA2a and exogenous human SERCA2a by antibody C-20 could help explain our findings. The two methods used for quantifying SERCA2a overexpression give values of 15% and 46%. Because antibody MA3-919 clearly shows overexpression of human SERCA2a and given some uncertainties regarding the exact avidity of antibody C-20 between human and rat SERCA2a, we interpret the reason for the accelerated relaxation kinetics of SERCA2a myocytes to be due to 15–46% overexpression of SERCA2a. No significant changes were detected in the PLB level among the different groups of myocytes.

Three independent sets of functional measurements were collected: PRP measurements, single twitches with and without TG, and single twitches with and without Iso. To evaluate the functional efficiency of the gene transfer of Parv and SERCA2a, we used the baseline data of one of these data sets (0 nM TG and 1.8 mM extracellular Ca2+; Table 1, Fig. 2). In Fig. 2A, representative traces of normalized sarcomere shortening/relengething highlight the ability of Parv and SERCA2a to increase relaxation kinetics. Ca2+ fluorescence was measured simultaneously using the Ca2+ indicator fura 2-AM. In both myocyte groups, the early part of the Ca2+ fluorescence transient decay was accelerated compared with control, but only SERCA2a-transduced myocytes were able to maintain this acceleration during the late phase of the Ca2+ fluorescence decay. A summary of the data is presented in Fig. 2, C and D. As shown previously (11), on day 3 after gene transfer, sarcomere-shortening amplitude was reduced in Parv-transduced myocytes (Fig. 2C). This phenomenon has been attributed to some Ca2+ buffering during the rising phase of the Ca2+ transient by Parv (11) and is reduced when Parv is expressed in lower concentration. SERCA2a showed a tendency for higher shortening amplitude than control myocytes, but this did not reach statistical significance. Taken together, these data show that, under the conditions used in the present

Table 1. Ca2+ fluorescence and sarcomere-shortening measurements for TG data set

<table>
<thead>
<tr>
<th>Control</th>
<th>Parv</th>
<th>SERCA2a</th>
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<tbody>
<tr>
<td>0 nM TG</td>
<td>11006</td>
<td>11006</td>
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<tr>
<td>150 nM TG</td>
<td>11006</td>
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<td>RR, %</td>
<td>11006</td>
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<tr>
<td>Lac-Z</td>
<td>11006</td>
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</tr>
<tr>
<td>Parv</td>
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<tr>
<td>SERCA2a</td>
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Values are means ± SE of total number of myocytes [for 0 nM thapsigargin (TG), the number of myocytes that survived 150 nM TG treatment for sarcomere-shortening and all fluorescence measurements (1st n value), and the number of myocytes for which 150 nM TG sarcomere-shortening amplitude was ≥25% of its baseline (2nd n value) used for all kinetics sarcomere-shortening data. Parv, parvalbumin; SERCA2a, sarco(endo)plasmic reticulum Ca2+-ATPase; RR, relative ratio [average of (TG data/baseline)] × 100, where each myocyte is its own control; Ampl, amplitude; –d/dt, return velocity; Tp, time to peak; Tp,1/4, Tp,1/2, and Tp,3/4, times from peak to 1/4, 1/2, and 3/4 relengthening; TpCa, time to fluorescence peak; Tp,1/4d, Tp,1/2d, and Tp,3/4d, time from stimulus to 1/4, 1/2, and 3/4 decay.

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study. Parv- and SERCA2a-transduced myocytes showed similar acceleration of relaxation, with Parv having a tendency to reduce amplitude of contraction when expressed at high levels.

**PRP.** PRP is the measurement of recovery or decay from the steady state of the contractile force (or shortening) after a quiescent period (2). The protocol used in this study is presented in Fig. 3A (bottom; see METHODS). Although all control and Parv myocytes and most of the SERCA2a myocytes exhibited a flat or decreasing prepulse sequence, some of the SERCA2a myocytes (2 of 10) behaved very differently. Although these myocytes appeared normal (rod shape and good striation pattern), they showed a very strong increasing pattern (Fig. 3A). It was difficult to quantitatively assess the portion of the myocytes behaving “atypically,” but they have been noticed in almost every individual experiment involved in this study. Because of this “atypical” response, these traces have been separated from the “typical” traces in all reported results. Figure 3B shows the PRP of the sarcomere-shortening amplitude. The results are plotted as the ratio of postpulse 1 to the prepulse steady state (average of the last 3 prepulses) as a percentage. Parv myocytes had the most potentiation, control and “normal” SERCA2a myocytes had a similar trend, but to a lesser extent, and “abnormal” (SE not shown; n = 2) SERCA2a had a negative potentiation at long rest period.
function of the duration of the rest period (logarithmic scale): 100% indicates no potentiation, and >100% indicates a positive potentiation response. Control and typical SERCA2a-overexpressing myocytes show a small increase at a shorter rest period (2–10 s), with values increasing to 178.6 ± 11.6% and 154.0 ± 35.5% at 120 s, respectively. Parv myocytes showed a very high potentiation at 120 s (206.7 ± 20.2%). Part of this potentiation is due to the fact that Parv myocytes started from a lower baseline than controls. However, even at 2 s, Parv myocytes showed significant potentiation (124.5 ± 6.4%). For completeness, SERCA2a myocytes with atypical responses are also shown. These atypical SERCA2a myocytes show a flat and elevated potentiation (at low T, i.e., 2–10 s), followed by a negative potentiation at longer rest intervals (25–120 s). The kinetics, as measured by the time from stimulus to one-half relengthening, followed a trend similar to the amplitude, but to a lesser extent (Fig. 3C).

Another way to analyze these data is to observe how the 10 prepulses vary from the first to the last (Fig. 4). This is equivalent to examining how the myocytes transition to a steady state after a long rest period. All the prepulse trains were obtained after 120 s of rest. For each myocyte, six sets of measurements were obtained and averaged. In Fig. 4A, representative traces show the difference in control myocytes between pulse 1 and pulse 9 for Ca2+ fluorescence and sarcomere shortening. In both cases, the amplitude reduction was accompanied, to a lesser extent, by a decrease in $T_{S/2r}$ or $T_{S/2d}$. The summaries of the sarcomere-shortening amplitude and $T_{S/2r}$ are presented in Fig. 4, B and C, respectively. The amplitude curves for the control and SERCA2a myocytes showed a progressive decrease from pulse 1, with SERCA2a myocytes showing a flatter response. In contrast, Parv-treated myocytes showed an abrupt decline from pulse 1 to pulse 2 and then stabilized for the subsequent pulses with a smaller decline. Similar trends were observed in $T_{S/2r}$. When the change in $T_{S/2r}$ was plotted against the change in amplitude (100% = no change from prepulse 1), a strong and similar correlation was found in all groups (Fig. 4D). The magnitude of change in amplitude was larger than the magnitude of change in kinetics, and SERCA2a myocyte data were clustered in the region of minimal change (~80–100% ampl), whereas Parv myocytes were clustered around the other end of the trend lines (~40–60% ampl). In summary, the PRP data suggest that although expression of Parv and overexpression of SERCA2a accelerate relaxation similarly, they modify the ability of the myocyte to handle changes in pacing rhythm differently. The quantitative analysis of these PRP data is complex and well suited for mathematical modeling interpretation (12).

Inotropic and lusitropic response. To address how Parv- and SERCA2a-transduced myocytes respond to β-adrenergic stimulation, the myocytes were treated with 10 nM Iso (Table 2, Fig. 5). Figure 5A shows representative sarcomere-shortening traces for control, Parv, and SERCA2a myocytes at 37°C with and without Iso. Parv and control myocytes showed comparable increases in amplitude, whereas SERCA2a showed a blunted increase. The summary in Fig. 5B confirms these results. Indeed, SERCA2a myocytes started with a baseline amplitude slightly (but not significantly) more elevated than control. In the presence of 10 nM Iso, the SERCA2a shortening amplitude did not reach the level obtained in control myocytes;

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**Fig. 4.** Analysis of PRP prepulses. Sets of 10 prepulses were measured after 120 s of rest. For amplitude data, individual prepulses were used, whereas beat 1 and averages of beats 2–4, 5–7, and 8–10 were used for kinetics data. A: representative Ca2+ fluorescence and sarcomere-shortening traces of prepulse 1 and pulse 9 normalized to prepulse 1. B and C: summary of prepulse data for sarcomere-shortening amplitude and $T_{S/2r}$. Values are means ± SE; n = 8–9. D: correlation between changes in kinetics (y-axis) and amplitude (x-axis) sarcomere-shortening prepulse data, with each point normalized to corresponding prepulse 1 value.
however, they showed a significant increase of 33.7 ± 11.8%. In contrast, the Parv myocytes started with a reduced baseline amplitude and reached an amplitude slightly more elevated in the presence of Iso. The Ca\(^{2+}\) fluorescence data (Fig. 5D) showed faster \(T_{1/2d}\) for baseline value in Parv and SERCA2a than in control myocytes. However, 10 nM Iso did not significantly change these values in Parv and SERCA2a myocytes, although it had a significant effect on the \(Lac-Z\) and control myocytes, reducing \(T_{1/2d}\) to Parv and SERCA2a values. Although a similar trend was seen in sarcomere-shortening kinetics (\(T_{p_{1/4d}}\); Fig. 5C), the magnitude of the change due to Iso was small and significant only for the \(Lac-Z\) group.

To further investigate positive lusitropic effects (i.e., increase in relaxation rate) of de novo expression of Parv and overexpression of SERCA2a, we repeated the same experiments at room temperature (22 ± 2°C; Table 2, Fig. 6). Figure 6A shows two representative control myocyte shortening responses to 10 nM Iso. When normalized to the same amplitude, the myocytes at 37°C showed slight changes in relaxation parameters, whereas at 22°C there was a marked acceleration of the relaxation kinetics. There was a smaller increase in the amplitude response among the different myocyte groups at 22°C than at 37°C. The change in \(T_{p_{1/4d}}\) kinetics was more pronounced at 22°C for control and \(Lac-Z\) myocytes in the presence of 10 nM Iso (Fig. 6C). At 37°C (Fig. 5C), the Parv and SERCA2a myocytes did not increase their already rapid relaxation rates in the presence of Iso. The Ca\(^{2+}\) fluorescence kinetic data showed similar effects.

We also examined another strategy known to create positive inotropy by elevating external Ca\(^{2+}\) concentration (from 1.8 to 5.0 mM). The results are presented in Fig. 7 and were obtained from data presented in Table 1 (data at 1.8 and 5.0 mM Ca\(^{2+}\) were obtained from another set of myocytes). SERCA2a shortening amplitude was blunted compared with the other groups (Fig. 7A), whereas Parv and SERCA2a myocytes retained their faster kinetics with elevated external Ca\(^{2+}\) (Fig. 7B). The results show that Parv myocytes and, to a lesser extent, SERCA2a myocytes respond positively to inotropic interventions (Iso or elevated Ca\(^{2+}\)), but their already rapid kinetics could not be further accelerated in the presence of a lusitropic agent (Iso).

**Chemical model of diastolic dysfunction.** We used a submaximal dose of TG to chemically mimic diastolic dysfunction as observed in heart failure. We also found that, in addition to causing diastolic dysfunction, TG also reduced sarcomere-
shortening amplitude when used in rat cardiac myocytes. A submaximal dose of 150 nM TG was used to create diastolic dysfunction and to minimize contractile amplitude reduction. In addition to measurements at 1.8 mM (normal M199 solution), we also obtained measurements at 5.0 mM external Ca²⁺ to increase the sarcomere-shortening amplitude in the presence of TG to improve the signal-to-noise ratio. Results are presented in Table 1 and Fig. 8.

For all conditions taken together, >91% (106 of 116) of the myocytes survived the TG treatment (Table 1). Although all surviving myocytes were used to calculate shortening amplitude and Ca²⁺ fluorescence data, only those that retained ≥25% of the shortening amplitude of the pre-TG treatment (73%, or 77 of 106) were kept for sarcomere-shortening kinetic analysis, because low signal-to-noise ratio (or even the absence of contraction) makes evaluation of the kinetic parameters difficult (or impossible).

Figure 8A shows representative traces of normalized Ca²⁺ fluorescence measurements with and without TG. The magnitude of change from baseline in the Ca²⁺ fluorescence kinetic measurements at low and elevated external Ca²⁺ is summarized in Fig. 8, B and D, respectively. $T_s/2d$ was increased by 30.2 ± 12.1% and 72.4 ± 20.4% at 1.8 and 5.0 mM external Ca²⁺, respectively, in control myocytes, whereas the increase was 62.4 ± 13.4% and 45.2 ± 8.2%, respectively, in SERCA2a myocytes. On the other hand, Parv myocytes...

Fig. 6. Effect of 10 nM Iso at 22°C. A: representative normalized shortening traces for control myocytes showing stronger effect of Iso on relaxation kinetics at 22°C. B–D: summary of sarcomere-shortening amplitude, $T_p/2r$, and Ca²⁺ fluorescence $T_s/2d$ in response to 10 nM Iso. Values are means ± SE; $n = 8–9$, except in D, where $n = 4$ for SERCA2a. *Significantly different from baseline, $P < 0.05$. #Significantly different from control myocytes under the same conditions, $P < 0.05$. Legend in C also applies to B and D.
showed an increase of only 9.1 ± 6.1% and 22.2 ± 12.1%, respectively. When a two-tailed paired t-test was conducted, only Parv myocytes did not show a significant increase in $T_{\text{p}_{1/4d}}$ and $T_{\text{p}_{1/4d}}$ (at 1.8 and 5.0 mM external $\text{Ca}^{2+}$). The average attenuation of sarcomere-shortening amplitude was similar in all groups under all conditions and was 40.4–55.5% (Table 1). At low external $\text{Ca}^{2+}$, only the late relaxation kinetic parameter $T_{\text{p}_{1/4d}}$ showed significant slowing in control myocytes (Fig. 8C). At elevated $\text{Ca}^{2+}$, in control myocytes the relative increase from baseline in all measured kinetic parameters ($T_{\text{p}_{1/4r}}, T_{\text{p}_{1/2r}}, T_{\text{p}_{3/4r}},$ and $T_{\text{p}_{1/4d}}$) was statistically significant (Fig. 8E). Again, only Parv myocytes were able to preserve their kinetic properties in the presence of TG in all sarcomere-shortening kinetic parameters. The data suggest that Parv could have a protective role in maintaining relaxation properties under conditions where the main $\text{Ca}^{2+}$ sequestration mechanism is partially impaired, as seen in heart failure.

**DISCUSSION**

We have reported a direct comparison between the effects of Parv expression de novo and SERCA2a overexpression in isolated rodent cardiac myocytes under a variety of experimental conditions. Using unloaded sarcomere shortening and $\text{Ca}^{2+}$ fluorescence, we found that 1) Parv- and SERCA2a-transduced myocytes have similar effects on shortening kinetics, 2) SERCA2a myocytes better preserve contractile amplitude than Parv myocytes (at high levels of expression), and 3) although the shortening amplitude in the presence of 10 nM Iso is similar in all groups at physiological temperature, the increase in shortening amplitude of Parv myocytes in response to $\beta$-adrenergic stimulation was comparable to that of control myocytes, whereas the response of SERCA2a myocytes was reduced. In addition, we showed that Parv corrects diastolic dysfunction in a chemical model where only the SR $\text{Ca}^{2+}$-ATPase is impaired.

**TG as a chemical model of diastolic dysfunction.** TG is derived from the plant *Thapsia garganica* (45), has a very high affinity for SERCA2a ($K_i < 1$ nM) (25), and is a very specific inhibitor of SERCA2a. In this study, the intent was to mimic diastolic dysfunction by using a submaximal dose of TG to create a partial inhibition of SERCA2a function, a feature often seen in heart failure (see the introduction).

A number of cellular changes occur during heart failure. Indeed, in addition to the impairment observed in $\text{Ca}^{2+}$ removal, myosin heavy chain isoform changes from $\alpha$ to $\beta$ (4), several $K^+$ currents (e.g., $I_{\text{so}}$ and $I_{\text{sl}}$) are downregulated (32), and the increase in $\text{Ca}^{2+}$ diastolic level activates calmodulin-dependent signaling events, which can lead to hypertrophy (7, 33). Isolation of the effect specific to $\text{Ca}^{2+}$ removal deficiency in this context becomes difficult. Even more difficult is the task of understanding the basic effects of the addition or modification of $\text{Ca}^{2+}$-handling proteins to this complex pathophysiological system. The use of TG to mimic diastolic dysfunction allows the specific isolation of impaired SR $\text{Ca}^{2+}$ uptake to directly study the biophysical effects of adding a delayed $\text{Ca}^{2+}$ buffer such as Parv. However, this model is not suitable for use in studying the effect of overexpressing SERCA2a in diastolic dysfunction, because TG would also affect the newly expressed $\text{Ca}^{2+}$ pumps.

One consequence of using TG in rodent cardiac myocytes to mimic diastolic dysfunction is the progressive emptying of the SR. This also creates systolic dysfunction. This situation can be corrected by using TG combined with some inotropic strategy. In this study, we used elevated external $\text{Ca}^{2+}$ to partially improve the sarcomere-shortening amplitude in the presence of TG. A direct comparison between data without TG at 1.8 mM external $\text{Ca}^{2+}$ and data with 150 nM TG at 5.0 mM external $\text{Ca}^{2+}$ reveals a strong diastolic dysfunction with almost no diminution in systolic function (Table 1). Other strategies, such as an augmentation of $\text{Ca}^{2+}$ cellular entry via an increase in action potential duration or a reduced $\text{Ca}^{2+}$ cellular exit via a reduced NCX function, could produce similar results (12).

**TG does not affect Parv-mediated rapid relaxation.** In the presence of 150 nM TG, control (and Lac-Z-transduced) myocytes exhibited slower sarcomere-relengthening kinetics, especially in the late phase of the cycle. However, when Parv myocytes were treated with the same TG dose, no significant changes were observed (Table 1, Fig. 8). The sarcomere-shortening behavior of these two groups in the presence of TG could be explained better by first analyzing their $\text{Ca}^{2+}$ transient properties.

$\text{Ca}^{2+}$ fluorescence data showed that all phases of the $\text{Ca}^{2+}$ transient decay were slowed in control myocytes when TG was applied. In contrast, Parv-transduced myocytes were able to maintain their already rapid kinetics in the presence of TG, at least in the first 50–60% of the decay. The basis for this result is hypothesized as follows. In rat control myocytes, SERCA2a...
pumps are responsible for almost all Ca\(^{2+}\) sequestration (2) and, therefore, drive the entire shape of the Ca\(^{2+}\) transient decay. When these pumps become partially inhibited by TG, the entire decay is slowed. However, with Parv, the early part of the decay is mostly dominated by the delayed buffering of Ca\(^{2+}\) by Parv. In this case, inhibition of SERCA2a has little influence on this part of the response. In the late part of the Ca\(^{2+}\) fluorescence decay, preservation of the rapid kinetics in Parv myocytes was reduced. In the absence of TG, it has been shown that Parv slowly releases its bound Ca\(^{2+}\) in the myoplasm during the late decay phase in myocytes (10). The rate of this reaction is dictated by a combination of the SERCA2a sequestration properties and the Ca\(^{2+}\) off-rate from Parv. When TG is added to the Parv-transduced myocytes, the Ca\(^{2+}\) off-rate is unaffected but the SERCA2a uptake rate is diminished, resulting in an even slower late phase of Parv-transduced myocytes. However, this late phase appears to be below the threshold required to create mechanical contraction, because it has not been observed in any of the sarcomere-shortening measurements presented in this study.

On the basis of this model of diastolic dysfunction, we can speculate that, in addition to correction of diastolic function, as seen previously in some animal models (42, 43), Parv could potentially offer some degree of protection against downregulation of Ca\(^{2+}\) pumps in vivo, as seen in human heart failure (1, 16).

Parv and SERCA2a: \(\beta\)-adrenergic response and systolic function. Although de novo expression of Parv and overexpression of SERCA2a operate through very distinct mechanisms, both increase relaxation rates to a similar extent during single contractions. However, their response differs in at least two important aspects: responsiveness to \(\beta\)-adrenergic stimulation and management of contractile amplitude.

In this study, Parv-transduced myocytes showed a reduction in sarcomere-shortening amplitude compared with control myocytes. In a previous study (11), we found that Parv buffers most of its Ca\(^{2+}\) in the early phase of the Ca\(^{2+}\) transient decay. However, when Parv is expressed at higher concentrations, it can also affect the rising phase of the Ca\(^{2+}\) transient, resulting in reduced Ca\(^{2+}\) transient and sarcomere-shortening amplitudes (11). In the same study, we demonstrated that there was an optimal range of Parv concentration for which Parv increases relaxation rates without affecting contractile amplitude (11). This phenomenon occurs at \(\sim 0.01-0.1 \text{ mM}\) Parv (or at \(\sim 2.5\text{ days after gene transfer}\) for rat cardiac myocytes in vitro. In the present study, we focused on Parv concentrations above this optimal range (\(\sim 0.14\text{ mM}, 3.0\text{ days after gene transfer}\)) to have a greater effect on relaxation kinetics. In comparison,
under the conditions used in this study, SERCA2a-transduced myocytes showed only a tendency to increase shortening amplitude, without reaching statistical significance. Other studies have shown no change or an increase in amplitude, depending on the species studied and experimental conditions (6, 20). In particular, the increase in amplitude seems to be more apparent at higher stimulation frequency (6). In this study, our use of a low stimulus frequency (0.2 Hz) might explain this relatively small increase in sarcomere-shortening amplitude in SERCA2a-overexpressing myocytes. Pharmaceutical treatments that have negative inotropic effects on the myocardium, such as β-blockers, have been shown to help patients with heart failure (22, 36). It is therefore possible that, in addition to its positive impact on relaxation rate, Parv attenuation effects on contractile function may also be beneficial. Interestingly, not only did Szatkowski et al. (42) not find any reductions in systolic function in the Parv-transduced rat heart in vivo, they reported an increase in pressure development after gene transfer. Clearly, more research is required to determine the optimal inotropy/lusitropy strategy in vivo.

As mentioned above, the β-adrenergic response is critical in managing the wide range of pumping capacity of the heart. In this study, we examined the dynamic range of shortening amplitude when the Parv- and SERCA2a-transduced myocytes were treated with 10 nM Iso. Parv-transduced and control myocytes showed a comparable increase in sarcomere-shortening amplitude, whereas in SERCA2a myocytes the amplitude increased only moderately (Table 2). Part of the explanation for this limited increase in SERCA2a-transduced myocytes is the slightly more elevated baseline amplitude than in control myocytes. However, at 10 nM Iso, there was a tendency for SERCA2a myocytes to have lower shortening amplitude than controls, but it did not reach statistical significance. A possible explanation for this phenomenon is that the level of SERCA2a in overexpressing myocytes is increased without any change in PLB content (6, 20), most likely leading to a larger portion of the new pumps uncoupled to PLB and, therefore, less sensitive to Iso. A possible limitation of the present study is that measurements were made under unloaded conditions and that mechanical load could affect the baseline properties and the responsiveness to β-adrenergic stimulation.

In conclusion, we used TG as a chemical means to mimic cellular diastolic dysfunction and found that Parv myocytes retained their rapid relaxation properties. This study also provides, for the first time, a detailed characterization of rat cardiac myocytes transduced with Parv under a variety of conditions: β-adrenergic stimulation, elevated external Ca\(^{2+}\), lowered temperature, and PRP tests. A direct comparison with SERCA2a overexpression was performed, highlighting the strengths and possible limitations of each approach; SERCA2a overexpression has a reduced range of modulation by β-adrenergic stimulation, whereas Parv de novo expression is more susceptible to decrease in contractile function, at least in vitro.

This study, combined with our mathematical analysis (12), helps clarify the effects of adding new Ca\(^{2+}\)-handling proteins in normal and diseased cardiac myocytes. It remains to be determined whether the results and conclusions found in vitro in rodent myocytes will translate to the organ level of larger mammals, including humans.

REFERENCES


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CA\textsuperscript{2+} HANDLING IN MYOCYTES. I. EXPERIMENTS


