Cellular basis for contractile dysfunction in the diaphragm from a rabbit infarct model of heart failure

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CHRONIC HEART FAILURE (CHF) is a syndrome in which myocardial contractile dysfunction results in a constellation of secondary changes in other organ systems. Exertional fatigue and breathlessness are perhaps the most common and debilitating symptoms experienced by patients with heart failure, but their etiology remains controversial (15).

The conventional explanation for orthopnea, exertional dyspnea, and fatigue is that the neuroendocrine response to left ventricular dysfunction results in sodium and water retention that increases left atrial pressure (to maintain cardiac output through the Starling mechanism). Along with this increase in left atrial pressure, there is a parallel rise in pulmonary venous pressure that predisposes to pulmonary congestion and dyspnea (2). It is apparent, however, that dyspnea is not simply related to pulmonary venous congestion. A variety of factors contribute to this symptom, e.g., afferent neuronal activity arising from medullary and peripheral chemoreceptors, respiratory muscles, and the lungs can all contribute to the perception of respiratory activity.

A variety of molecular and histological adaptations have been described in locomotor muscle (13) and the diaphragm during the development of CHF. Reduced mRNA, protein levels, and activity of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) (7, 14) and an increased proportion of type I muscle fibers (5), perhaps due to disproportionate atrophy of type II over type I muscle fibers (9), have all been observed in the diaphragm. The functional consequences of these molecular and histological adaptations are poorly described at a cellular level. Several studies suggest that the diaphragm is weak in patients with CHF, but the etiology and clinical relevance of such weakness remains unclear (4, 8, 10). In addition, despite the shift toward a fatigue-resistant muscle fiber profile, there is evidence to suggest that the susceptibility to fatigue is increased (21, 25).

The aim of this study was to compare changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and isometric force generated during direct phrenic nerve stimulation of hemidiaphragm preparations isolated from coronary artery-ligated rabbits with sham-operated controls. The results indicate that there is pronounced contractile dysfunction intrinsic to the diaphragm, the severity of which is related to the extent of myocardial dysfunction.

METHODS

Coronary ligation. Male New Zealand White rabbits (2.5–3.0 kg) were premedicated with intramuscular Hypnorm [0.3 mg/kg fluanisone (10 mg/ml); fentanyl citrate (0.315 mg/ml)] and further sedated with intravenous midazolam (0.25–0.5 mg/kg) to allow endotracheal intubation. The animal was ventilated (0.3–0.4 l min\(^{-1}\) kg\(^{-1}\)) and anesthesia was maintained with an inhaled mixture of equal concentrations of nitrous oxide and oxygen plus 1% halothane at a flow rate of 2 l/min. The heart was exposed through a left thoracotomy, the pericardium opened, and the left marginal artery ligated at the midpoint between the atrioventricular groove and the cardiac apex. Animals were treated with intravenous quinidine (15 mg/kg) 5 min before ligation of the coronary artery to reduce the incidence of ventricular arrhythmias. Postoperative analgesia was administered (0.04 mg/kg buprenorphine) at the end of the surgical procedure and again 10–12 h after the procedure. Ligated animals developed CHF over the next 8 wk. Sham-operated animals acted as controls and were treated in a similar fashion as the ligated group but without coronary artery ligation.
Echocardiography. Echocardiography was performed 1 wk before the in vitro experimental studies using a 7.5 MHz microconvex probe with a Medison sonograph (SonOAce 8800). Rabbit was sedated with 0.3 mg/kg Hypnorm, and a small area of the anterior chest wall was shaved to allow a satisfactory echo window. The animal was placed in the left lateral position and a parasternal long-axis view was obtained to assess left atrial dimension and left ventricular end-diastolic dimension in the M mode. Ejection fraction (EF) was assessed in the short-axis view at a level just below the mitral valve leaflets. The endocardial border was marked, using captured end-diastolic and end-systolic frames, the enclosed area computed, and EF calculated as the percentage area reduction with systole.

Tissue preparation and tension measurement. Rabbits were euthanized with an intravenous injection of 0.5 ml/kg Euthatal (pentobarbital sodium 200 mg/ml) mixed with 500 IU of heparin. The thoracic cavity was opened and the left hemidiaphragm was removed by cutting along the intercostal spaces and sternum above and below the diaphragm (leaving the phrenic nerve, artery, and adjacent ribs attached). The hemidiaphragm was released by two cuts to converge on the central tendon at the level of the aortic hiatus and was connected to a force transducer (FT03, Grass Instruments). The central tendon of the hemidiaphragm was connected to a force transducer (FT03, Grass Instruments) and the adjacent ribs were pinned to the bottom of the perfusion chamber to allow isometric force to be measured. The length of the diaphragm was progressively increased until maximum tension was obtained (at a constant stimulus frequency) and subsequent protocols were undertaken at this length.

Fluorescence measurement system. Fluorescence from the caudal aspect of the left hemidiaphragm was measured using a custom-built apparatus (12). Illumination was provided by a Nikon 75 W xenon lamp filtered at 360 ± 10 nm and focused via a 385-nm long-pass dichroic mirror onto a 3-mm-diameter liquid light guide (Ultrafine Technology) held in contact with the hemidiaphragm. Emitted light from the preparation was collected by the same light guide, and light with wavelengths >385 nm passed through the dichroic mirror and subsequently split by a second dichroic. Light with wavelengths >460 nm passed through the second dichroic onto a photomultiplier (Thorn EMI) fitted with an optical band-pass filter centered on 495 ± 20 nm. The remaining light was reflected onto a second photomultiplier with a band-pass filter centered on 405 ± 35 nm. The outputs of the photomultipliers were passed to an electronic ratio circuit to obtain an effective continuous signal of fluorescence intensity ratio (F405/F495). This analog ratio signal, the fluorescence intensity of the individual wavelengths, and the electrical signal representing isometric tension were displayed continuously on a Gould TA-11 chart recorder and stored on videotape using a CRC VR-100B digital recorder (Instrutech).

Indo 1 loading. An indo 1/AM (Molecular Probes, Ohio) stock solution was initially prepared by dissolving 1 mg of indo 1/AM in 1 ml of DMSO/10% (wt/vol) Pluronic acid F-127 solution. This was added to 100 ml of Ringer solution to make up a final concentration of 10 µM indo 1/AM after an initial equilibration period of 30 min with normal Ringer solution, the hemidiaphragm was perfused with the indo 1/AM-containing solution at 25°C for a period of 30 min. At the end of this period, the hemidiaphragm was perfused with normal Ringer solution, and the temperature was increased to 37°C to wash out theindicator from the extracellular space and allow intracellular breakdown of indo 1/AM to the Ca2+-sensitive form. The amplitude of the tension transient was significantly reduced during the loading period, either because of the low temperature (25°C) or the high concentration of DMSO (1%), but returned to preloading levels when the temperature returned to 37°C, indicating that there was no significant buffering of intracellular Ca2+ by indo 1.

Measurements of the ratio of the intrinsic fluorescence of the muscle before loading with indo 1 showed no phasic changes during tetanic stimulation, suggesting that this particular optical arrangement did not generate significant movement artifact in the fluorescence signal from the contracting muscle below the light guide, i.e., although small changes in fluorescence were observed in the individual wavelengths’ signals during tetanic stimulation, the movement artifact did not affect the ratio signal because the transients were equivalent on individual wavelengths. After the loading procedure, the fluorescence of the muscle surface had increased by approximately two- to threefold at both wavelengths. The value of the indo 1 fluorescence ratio signal contained a significant phasic component, as shown in Fig. 1, i.e., the fluorescence ratio increased rapidly to a peak and fell gradually to a steady level over the period of stimulation, and represents the time course of the changes of [Ca2+]i within the skeletal muscle. As described above, the fluorescence ratio was recorded from the muscle surface using a 3-mm-diameter light guide. The signal, therefore, represents the average [Ca2+]i signal from cells within an area of ~7 mm2. The depth of muscle from which recordings were made is unknown, but previously published work using this technique on rabbit myocardium suggests that the maximum depth of tissue contributing to the signal was 0.5–0.7 mm (12) and is consistent with results described by other studies (17, 20). Indo 1 fluorescence ratio can be directly related to the [Ca2+]i using an intracellular calibration curve for the dye. Conventional methods for intracellular calibration of the dye to Ca2+ could not be easily performed, however, in the multicellular skeletal muscle preparation. Furthermore, the fluorescence ratio measured at rest and during peak tetanic stimulation in different preparations can vary over a significant range, reflecting variable tissue absorbance of the emitted indo 1 fluorescence (12). The fluorescence signals were expressed, therefore, as values relative to the maximum tetanic indo 1 fluorescence for the series of stimulation frequencies. These measurements prevent comparisons of resting and peak tetanic [Ca2+]i values, but the time course of the transient changes in signal and the relative changes give information on the underlying causes of contractile dysfunction observed in the coronary ligation model.

Although indo 1 is regarded as a relatively high-affinity Ca2+-chelator, it has been used previously to measure peak tetanic [Ca2+]i in skeletal muscle fibers (22). It is possible that indo 1 fluorescence would saturate at the peak [Ca2+]i obtained during high-frequency tetanic stimulation, but because we can identify fluctuations in tetanic [Ca2+]i that correlate with the stimulation frequency, this is unlikely. In addition, fura 2 has also been used to measure tetanic [Ca2+]i in skeletal muscle fibers, and this probe has a higher affinity for Ca2+ (3).

Phrenic nerve stimulation protocols. Figure 1 demonstrates the experimental records obtained with the optical arrangement described here. The phrenic nerve was stimulated by a computer-controlled isolated stimulator (Model DS2, Digitimer). The nerve was stimulated at a constant 0.02 ms pulse
duration and the pulse amplitude was adjusted to 50% over threshold. The relationship between tetanic [Ca\textsuperscript{2+}], tetanic isometric force, and frequency of stimulation was established by applying 10 pulses at a specified frequency over the range 10–100 Hz. Frequency of stimulation was altered by increasing the cycle duration for the 10 pulses from 10 to 100 ms (so that the total duration for the 10 Hz stimulation was 1 s compared with 0.1 s at 100 Hz). Frequency of stimulation was tested in random order and showed no evidence of hysteresis or fatigue during these stimulation protocols. (Fatigue was excluded by comparing the amplitude of the isometric tension response to 10 stimuli at 40 Hz every 30 s before and after the protocol to establish the force-frequency relationship.)

Signal measurement and analysis. The peak tetanic indo 1 fluorescence ratio and isometric force was identified during each tetanic stimulation, and the average of four successive records at each stimulation frequency was recorded. All data are expressed as means ± SD, unless otherwise stated, and comparison between groups of data were made with Student’s t-test (with Bonferroni correction and paired where appropriate). A two-tailed P value of less than 0.05 was considered significant.

We have expressed tetanic force generated relative to the cross-sectional area of the diaphragm (measured at the costal-phrenic margin to allow a direct comparison between the experimental groups). The hemidiaphragm is a “double wedge-shaped muscle” (being both broad and thick (~8 × 1 cm) at the costal-phrenic margin but narrow and thin (~3 × 0.1 cm) at the central tendon) and thus a cross-sectional area measured at the costal-phrenic margin will represent a significant overestimate of the muscle mass being activated. The forces reported here are, therefore, lower than would be expected for similar isolated skeletal muscle fibers. To allow comparison of the force-frequency relationship in the experimental groups, the average tension measurements were normalized with respect to the maximum tetanic force achieved during the phrenic nerve stimulation protocols (typically around 30 Hz stimulation frequency in tissue from coronary-ligated animals but 100 Hz in controls).

**RESULTS**

Characterization of the coronary ligation model. The degree of left ventricular dysfunction resulting from coronary artery ligation was quantified by echocardiographic analysis of a reduced ejection fraction, increased left atrial dimension, and increased left ventricular end-diastolic dimension (results are summarized in Table 1). This shows a significant reduction in ejection fraction 8 wk after coronary ligation and an increase in left atrial and ventricular chamber sizes.

Table 1. Echocardiographic characterisation of the animal model

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 8)</th>
<th>Ligation (n = 6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAD, mm</td>
<td>11.6 ± 1.9</td>
<td>15.6 ± 1.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>14.4 ± 0.8</td>
<td>18.6 ± 1.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>EF, %</td>
<td>72.3 ± 2.6</td>
<td>46.5 ± 3.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>198 ± 23</td>
<td>220 ± 32</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Values are means ± SD. Transthoracic echocardiographic assessment of left heart morphology and systolic function was undertaken. LAD, left atrial dimension; LVEDD, left ventricular end-diastolic dimension; EF, ejection fraction (%area reduction in systole); HR, heart rate in beats per minute (bpm).
diaphragm was significantly increased after coronary ligation (2.89 ± 1.26 vs. 8.28 ± 1.55 N/mm², P < 0.05, n = 6 and 8, respectively). The maximum isometric force, however, generated by the diaphragm from coronary-ligated animals was not significantly different from sham-operated controls (18.04 ± 1.62 vs. 14.28 ± 1.81 N/mm², P > 0.2, n = 6 and 8, respectively). Maximum tetanic force was generated at 100 Hz stimulation frequency in all tissue from control animals but ranged from 16.6 to 33.3 Hz (mode 25 Hz) in tissue from coronary-ligated animals.

Force-frequency and [Ca²⁺]-frequency relationship after coronary ligation. Figure 2 shows the [Ca²⁺]-frequency and force-frequency relationships obtained in perfused, phrenic stimulated, hemidiaphragm preparations isolated from coronary-ligated rabbits and controls. Decreasing the tetanic stimulation frequency from 100 to 25 Hz caused a decrease in both developed force and [Ca²⁺] in tissue isolated from sham-operated controls (e.g., peak tetanic [Ca²⁺] was reduced from 100.0 ± 4.0 to 82.1 ± 3.4% of maximum, P < 0.001, n = 8). In contrast, decreasing tetanic stimulation from 100 to 25 Hz caused an increase in force and [Ca²⁺] in tissue isolated from rabbits subjected to coronary ligation (e.g., peak tetanic [Ca²⁺] was increased from 62.4 ± 4.4 to 100.0 ± 3.2% of maximum, P < 0.001, n = 6). Combined data for six ligated animals and eight controls are shown in Fig. 3. These data demonstrate that both the force-frequency and [Ca²⁺]-frequency relationships are shifted by coronary ligation such that peak tetanic force and [Ca²⁺] are achieved at around 25 Hz after coronary ligation, compared with 100 Hz in control preparations. A similar relationship was observed when the muscle was stimulated directly (see Fig. 3A). Examining the [Ca²⁺] records on an extended time base reveals that the responses were tetanic at all frequencies in both groups.

Effects of coronary ligation on the rate of decay of the tetanic Ca²⁺ transient. The rate constant for the decay in [Ca²⁺] was compared in sham-operated and coronary-ligated rabbits by fitting a single decay function to the falling phase of the intracellular Ca²⁺ response to tetanic stimulation. At both 10 and 100 Hz stimulation frequency, the rate constant for the decay in [Ca²⁺] was lower in preparations from ligated animals than controls (i.e., at 10 Hz 80.7 ± 17.4 vs. 21.6 ± 17.0 s⁻¹, P <
Effects of cyclopiazonic acid on force-frequency and \([\text{Ca}^{2+}]\)-frequency relationships. The previous results suggest that the contractile dysfunction within the diaphragm after coronary artery ligation is due to an inability of the muscle to increase \([\text{Ca}^{2+}]\). The sarcoplasmic reticulum is the major source of \([\text{Ca}^{2+}]\) during tetanic stimulation of skeletal muscle, and previous studies have observed a decrease in SERCA activity in the diaphragm during the development of heart failure. It is likely that decreased activity of this pump will account for the altered contractile function observed in this study.

The shift in both the force-frequency and \([\text{Ca}^{2+}]\)-frequency relationships after coronary ligation was modeled pharmacologically by the application of cyclopiazonic acid (a SERCA inhibitor). Figure 4 demonstrates the effect of 1 and 10 \(\mu\)M cyclopiazonic acid on the \([\text{Ca}^{2+}]\)-frequency (A) and force-frequency (B) relationships on control hemidiaphragm preparations (0.1 \(\mu\)M cyclopiazonic acid was without effect and has been omitted for clarity). These data demonstrate a leftward shift in the force-frequency and \([\text{Ca}^{2+}]\)-frequency relationships in the presence of 1 \(\mu\)M cyclopiazonic acid that is similar to the shift observed after coronary ligation. In the presence of 10 \(\mu\)M cyclopiazonic acid, both the force-frequency and \([\text{Ca}^{2+}]\)-frequency relationships were reversed (i.e., the ascending limb of these relationships was lost).

**DISCUSSION**

The results presented here demonstrate that the relationship between stimulation frequency, tetanic \([\text{Ca}^{2+}]\), and tetanic force is altered in hemidiaphragm preparations from a rabbit chronic myocardial infarction model. The mean rate constant for the decay in \([\text{Ca}^{2+}]\) was lower in preparations from ligated animals,
suggesting that the ability to regulate [Ca\(^{2+}\)] is altered in this muscle. Application of the SERCA inhibitor cyclosporine to control tissue modeled the shift in the relationship between stimulation frequency and both tetanic [Ca\(^{2+}\)], and force that was induced by coronary ligation.

Respiratory and skeletal muscle function in CHF. It has been suggested that the dyspnea observed in heart failure patients may be related to abnormal respiratory muscle function, such as diminished strength, endurance, or both. Skeletal muscle strength is reduced in animal models of CHF (13) and a similar weakness has been described in patients (11). The most consistent finding in locomotor muscle during the development of CHF is a reduced proportion of slow-twitch fibers (5) and atrophy of fast-twitch fibers (9). The diaphragm adapts differently, however, with an increase in the proportion of slow-twitch fibers representing an apparent shift toward fatigue resistance (19). Despite this, however, there is evidence of diaphragm dysfunction in both animal models and patients with CHF. In patients, maximum voluntary ventilation and maximum sustainable ventilatory capacity are both reduced, and the normal increase in inspiratory duty cycle (i.e., time on inspiration divided by time per breath) at maximum sustainable ventilatory capacity is absent (8). These findings of reduced airflow are similar to that observed with obstruction of airways (25). In addition, Evans et al. (4) reported that the transdiaphragmatic pressure during a voluntary sniff is reduced (i.e., voluntary power output is reduced).

During normal ventilation, the frequency of motor-neuron firing is less than 40 Hz but can be increased to 100 Hz to produce Pdi\(_{\text{max}}\) (maximum pressure difference across the diaphragm) (16). In this study, the peak of the relationship between stimulation frequency and force was shifted toward frequencies expected during normal “resting” ventilation after coronary ligation. Furthermore, force of contraction fell precipitously at higher stimulation frequencies. Thus, during ventilatory stimulation, e.g., when there is a marked increase in resistance of airways or an increase in the chemical drive for ventilation, the altered force-frequency relationship will paradoxically reduce the capacity of the diaphragm to generate the appropriate pressure of airways in CHF. These data are supported by the observations of Supinski et al. (18) where a reduction in tetanic force in response to 100 Hz of stimulation was found in a rapid pacing model of CHF (at 100 Hz stimulation in this study, there is an ~50% reduction in tetanic force). The fall in peak tetanic force was paralleled by a reduced amplitude of the Ca\(^{2+}\) transient, suggesting that altered Ca\(^{2+}\) release from the sarcoplasmic reticulum contributes to mechanical dysfunction. In addition, the altered molecular and histological profiles of the muscle will also influence the functional response of the diaphragm in CHF. The switch toward a fatigue-resistant muscle fiber profile in the diaphragm during the development of CHF would tend to reduce the force-producing ability of the diaphragm due to the relatively low contribution of slow-twitch fibers to the force-generating capacity of the muscle (16) (i.e., slow-twitch fibers produce less force for the same cross-sectional area compared with fast-twitch fibers). It should be noted that in the absence of a steady-state equilibrium between ambient [Ca\(^{2+}\)] and Ca\(^{2+}\) binding to regulatory proteins, no meaningful judgment regarding the force-[Ca\(^{2+}\)] relationship can be made from the data presented here.

Regulation of [Ca\(^{2+}\)]. This study demonstrates that the mean rate constant for [Ca\(^{2+}\)] removal was reduced in preparations from ligated animals. The rate of [Ca\(^{2+}\)] decay during the relaxation phase after a tetanic stimulation has previously been shown to display two clear time constants in single locomotor muscle fibers at low temperatures (6, 24). The first of these time constants is thought to depend upon 1) the permeability of the ryanodine receptor to Ca\(^{2+}\), 2) the rate at which parvalbumin binds Ca\(^{2+}\), and 3) the rate of Ca\(^{2+}\) uptake into the sarcoplasmic reticulum. The second rate constant is thought to depend uniquely on the activity of the SERCA. In this study, however, the rate of decay in [Ca\(^{2+}\)] showed only one exponential component. The reason for this apparent discrepancy is unknown, but significant differences between this and previous studies (6, 24) may form the basis for this inconsistency. Unlike previous studies, the rate constants calculated here were obtained in multicellular preparations and the signals from many individual fibers were averaged to provide the final signal. Thus any small differences in the phase of the decline of [Ca\(^{2+}\)], between fibers would tend to obscure multiphase time courses. Moreover, the indo 1 signal was not deconvoluted to [Ca\(^{2+}\)] and is therefore a nonlinear measure of [Ca\(^{2+}\)]. This feature would tend to disguise the multiple phases of [Ca\(^{2+}\)], decay. Finally, the measurements were made at 37°C in this study, and this may also prevent distinct phases of [Ca\(^{2+}\)], decay from being observed.

The removal of Ca\(^{2+}\) from the myoplasm in striated muscle is complex and can involve a number of processes (depending upon the cell type). In addition to the activity of SERCA, the Ca\(^{2+}\) can be transported out of the cell by Na\(^{+}/Ca\(^{2+}\) exchange and the sarcomemmal Ca\(^{2+}\)-ATPase or into intracellular organelles such as the mitochondria. The rate of removal of myoplasmic Ca\(^{2+}\) can be affected by the peak [Ca\(^{2+}\)], attained in the muscle, e.g., increasing tetanic [Ca\(^{2+}\)], will increase SERCA activity, and rate constants for the removal of Ca\(^{2+}\) are often normalized to peak [Ca\(^{2+}\)]. In this study, although the indo 1 fluorescence ratio was not calibrated and prevented direct comparison of the resting and peak [Ca\(^{2+}\)], the reduced rate of Ca\(^{2+}\) removal from the myoplasm does not appear to be due to changes in peak [Ca\(^{2+}\)]. Figure 3 and the associated text demonstrate two important points that support this assertion: 1) the peak tetanic force generated by preparations from control and coronary-ligated animals are not significantly different, and 2) the force-frequency and [Ca\(^{2+}\)]-frequency relationships are the same. Assuming no extraordinary change in myofilament Ca\(^{2+}\) sensitivity or peak force development, the lower tetanic force...
generated during 100 Hz stimulation in preparations from coronary-ligated animals would be consistent with a lower tetanic \([\text{Ca}^{2+}]\), that could contribute to the slower rate constant. At 10 Hz stimulation, however, tetanic force (and presumably \([\text{Ca}^{2+}]\)) is higher in the diaphragm from coronary-ligated animals, but the rate of removal of myoplasmic \([\text{Ca}^{2+}]\) remains lower. These data suggest that some other factor contributes to the slowed removal of \([\text{Ca}^{2+}]\).

The use of indo 1/AM in tissue with a high mitochondrial content like the diaphragm can be problematic due to accumulation of the dye in the mitochondrial compartment. As a result, the myoplasmic fluorescent signal may be contaminated by a mitochondrial \([\text{Ca}^{2+}]\) signal. However, because mitochondria have a relatively low affinity for myoplasmic \([\text{Ca}^{2+}]\), and the rate of exchange with this compartment is slow (12, 23), it is unlikely that this factor is a significant influence on the \([\text{Ca}^{2+}]\) signals reported in this study.

The levels of mRNA encoding for SERCA isoforms are reduced in CHF, and there is a concomitant reduction in SERCA1 protein levels and SERCA activity (14). A reduced SERCA activity could explain both the reduced rate constant for intracellular \([\text{Ca}^{2+}]\) removal and the altered \([\text{Ca}^{2+}]\)-frequency relationship observed during low-frequency stimulation in the tissue from coronary-ligated animals. When SERCA activity is reduced, the \([\text{Ca}^{2+}]\) released by a single stimulus will be taken up into the sarcoplasmic reticulum more slowly, and subsequent stimuli will summate and tetanize more easily. Such potentiation of tetanic \([\text{Ca}^{2+}]\) has been observed previously in the presence of the SERCA inhibitor 2,5-di-(tert-butyl)-1,4-benzohydroquinone in skeletal muscle fibers (23). Inhibiting SERCA activity should also potentiate tetanic \([\text{Ca}^{2+}]\), during high-frequency stimulation (23), whereas we have observed reduced tetanic \([\text{Ca}^{2+}]\) in preparations from coronary-ligated animals (and in control preparations exposed to cyclopiazonic acid). This reduced \([\text{Ca}^{2+}]\), did not reflect an inability of the preparation to tetanize (examining individual responses on a faster time base reveals that \([\text{Ca}^{2+}]\), was tetanic). Moreover, a similar \([\text{Ca}^{2+}]\)-frequency relationship was obtained by direct muscle stimulation in these preparations and indicates that the reduced \([\text{Ca}^{2+}]\) does not result from a deficit in transmitter release.

Our observation that inhibiting SERCA activity with cyclopiazonic acid during high-frequency tetanic stimulation does not potentiate tetanic \([\text{Ca}^{2+}]\), suggests that the regulation of \([\text{Ca}^{2+}]\) is different in the diaphragm and locomotor skeletal muscle. One possible difference could be in the \(\text{Na}^+/\text{Ca}^{2+}\) exchange that mediates both net \(\text{Ca}^{2+}\) entry and release from a variety of cell types (see Ref. 1 for review). Although the \(\text{Na}^+/\text{Ca}^{2+}\) exchange has been extensively investigated in the myocardium, its role in regulating myoplasmic \([\text{Ca}^{2+}]\) in skeletal muscle is poorly described. It has been suggested, however, that the physiological role of the \(\text{Na}^+/\text{Ca}^{2+}\) exchange could vary in different forms of skeletal muscle (1). Further studies are required to determine the mechanism by which peak tetanic \([\text{Ca}^{2+}]\) is reduced during SERCA inhibition at high-frequency stimulation. We have demonstrated that the response of the diaphragm to chronic coronary ligation is similar to pharmacological inhibition of SERCA. Thus the reduced protein levels and activity of SERCA described previously in the diaphragm during the development of CHF (14) could be responsible for the altered force-frequency and \([\text{Ca}^{2+}]\)-frequency relationships observed in the coronary artery ligation model.

Implications for alterations in the force-frequency and \([\text{Ca}^{2+}]\)-frequency relationships. In this study, the crossover between the apparently beneficial and detrimental effects of the altered force-frequency relationship occurred below the frequency for \(P_{\text{di, max}}\) in vivo. This observation could account for the apparent contradiction in studies that have assessed diaphragm function in CHF patients. In general, diaphragm function will have been assessed in such studies without accounting for the pattern of motorneuron stimulation under the specific circumstances of the experiment. Two apparently similar studies could, therefore, result in a normal or augmented pressure of Airways in one (via potentiation of \([\text{Ca}^{2+}]\) release and force generation at low-stimulation frequencies) or a reduced pressure of Airways in the other (due to diminished levels of \([\text{Ca}^{2+}]\) release at higher stimulation frequencies). The data presented here are consistent with the hypothesis that diaphragmatic strength is decreased in CHF (whether this is due to a switch toward a fatigue-resistant muscle fiber profile, compromised regulation of \([\text{Ca}^{2+}]\), or a combination of both). The altered force-frequency relationship is an important finding that must be considered when interpreting in vivo fatigue protocols while studying respiratory function in patients with chronic heart failure.

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REFERENCES


