Contractile Properties and Ca\textsuperscript{2+} Release Activity of the Sarcoplasmic Reticulum in Dilated Cardiomyopathy

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Background. We performed a comparative study on Ca\textsuperscript{2+} release activity of the sarcoplasmic reticulum and calcium sensitivity of contractile apparatus of chemically skinned myocardial fibers obtained from four nonfailing human hearts and 13 excised hearts from patients with idiopathic dilated cardiomyopathy.

Methods and Results. Ca\textsuperscript{2+} sensitivity of contractile apparatus was studied by following the isometric tension developed by chemically skinned myocardial fibers challenged with solutions of decreasing pCa. Ca\textsuperscript{2+} release from sarcoplasmic reticulum was monitored indirectly by measurement of the isometric tension developed by skinned fibers challenged with caffeine. We observed no significant difference of Ca\textsuperscript{2+} sensitivity and cooperativity between normal myocardium (pCa\textsubscript{50} = 6.00 ± 0.05; Hill coefficient, n\textsubscript{Hill} = 2.07 ± 0.10) and dilated cardiomyopathy (pCa\textsubscript{50} = 6.03 ± 0.07; n\textsubscript{Hill} = 2.72 ± 0.30) when the fibers were stretched to 130% of the resting length. We also found that both in normal myocardium and dilated cardiomyopathy, stretching to 150% of the resting length increased the Ca\textsuperscript{2+} sensitivity of the contractile system; pCa\textsubscript{50} = 6.21 ± 0.01 and 6.13 ± 0.04 in normal and dilated cardiomyopathy, respectively, whereas in dilated cardiomyopathy there was a decrease of Hill coefficient with stretching that was not observed in the control group. The caffeine threshold in idiopathic dilated cardiomyopathy was markedly increased compared with the control group, 1.94 ± 0.27 mmol/l and 0.29 ± 0.04 mmol/l caffeine, respectively, whereas there were no significant differences in the extent and rate of caffeine-induced Ca\textsuperscript{2+} release.

Conclusions. These results indicate that in idiopathic dilated cardiomyopathy there is no alteration of contractile and regulatory proteins; on the contrary, the gating mechanism of the Ca\textsuperscript{2+} release channel of sarcoplasmic reticulum is abnormal, suggesting a possible involvement of the excitation-contraction coupling in the pathogenesis of this disease. It should also be taken into account, however, that the increased caffeine threshold in dilated cardiomyopathy would be a result of the enhanced resistance to the skinning procedure secondary to the modification of lipid species and/or content in sarcoplasmic reticulum membrane. (Circulation 1992;85:518–525)

Dilated cardiomyopathy (DCM) is defined as a heart muscle disease of unknown origin characterized by a deficient contractility of ventricular myocardium with dilatation of the ventricles and inadequate biventricular hypertrophy, reduced cardiac output and ejection fraction, and increased systemic vascular resistance. Several theories have been proposed to explain the etiology of the disease. Potential roles for viral infection, in particular for coxsackievirus B, autoimmune damage of myocardium, and myocardial damage by ethanol have been suggested. In addition, a selective downregulation of \(\beta\textsuperscript{1}\)-receptor subpopulation and an increase of \(G\textsubscript{i}\) protein have been reported. In summary, it seems likely that DCM is an end-stage condition resulting from various diseases of the myocardium.

The pathogenesis of the contractile failure in DCM is also unknown. Possible mechanisms could involve the contractile and regulatory proteins, the mechanism of excitation-contraction coupling, or both. In this study, we measured the pCa/tension...
relation and Ca\textsuperscript{2+} release from sarcoplasmic reticulum of chemically skinned cardiac fibers from patients with idiopathic DCM. We show here that in DCM there is a reduction of the release of Ca\textsuperscript{2+} from sarcoplasmic reticulum, whereas there are no alterations of Ca\textsuperscript{2+} sensitivity of contractile proteins.

**Methods**

**Tissue Procurements**

Samples of the left ventricular myocardium were obtained from the explanted hearts of 13 patients, all male, aged 1–59 years (mean, 36.9±5.76), with class IV idiopathic DCM undergoing orthotopic cardiac transplantation. The diagnosis of DCM was made according to established criteria.\textsuperscript{1} All patients had undergone complete hemodynamic evaluation. They all had normal coronary arteries and no evidence of valvular or congenital heart disease, and the average cardiac catheterization values obtained before transplantation were: right atrial mean pressure, 10.71±3.05 mm Hg; pulmonary wedge mean pressure, 30.33±4.37 mm Hg; and cardiac index, 2.03±0.25 l/min/m\textsuperscript{2}. All the patients were on therapy with various combinations of digitals, diuretics, vasodilators, and antiarrhythmics.

As controls, samples of the right ventricle were obtained from four patients with coronary heart disease undergoing coronary artery bypass graft (CABG) surgery. All patients suffered from angina pectoris with no precedent myocardial infarction. Coronary angiography demonstrated involvement of the left coronary artery; the right coronary artery was not involved, and the cardiac catheterization values were in the normal range. Preliminary experiments showed that in rabbits, right and left ventricular myocardium were comparable with regard to the function of both sarcoplasmic reticulum and contractile and regulatory proteins. Thus, it is possible to compare results obtained from samples of left ventricular myocardium affected by DCM with right normal ventricular myocardium. Biopsies of normal myocardium were taken during the CABG surgery before the aorta was cross-clamped on the right ventricle and were immediately immersed in ice-cold 0.9% NaCl solution. Hearts affected with DCM were excised after infusion of cold cardioplegia solution into the roots of the cross-clamped aortas and immediately immersed in ice-cold 0.9% NaCl solution. The dimensions of each biopsy were about 2×5 mm.

**Preparation of Skinned Fibers**

Chemically skinned fibers were prepared as previously reported.\textsuperscript{10} Briefly, immediately after collection, each biopsy was attached to a wooden stick at the resting length and chemically skinned by incubation at 0–4°C for 24 hours in 10 ml of skinning solution (170 mmol/l K\textsuperscript{+}, 2.5 mmol/l Mg\textsuperscript{2+}, 2.5 mmol/l ATP, 5 mmol/l EGTA, 10 mmol/l imidazole, pH 7.0; all cations were salts of propionate). The skinning solution was changed at hours 1, 4, 6, 16, and 23. The biopsies were then stored at −20°C in the same solution supplemented with 50% glycerol until used (1–2 weeks).

**Calcium Sensitivity of Contractile Apparatus**

Single bundles were isolated with the help of a dissecting microscope, transferred into a chamber containing 1.0 ml of relaxing solution (170 mmol/l K\textsuperscript{+}, 2.5 mmol/l Mg\textsuperscript{2+}, 5 mmol/l ATP, 5 mmol/l EGTA, 10 mmol/l imidazole, pH 7.0), and inserted between two clamps, one of which was attached to a tension transducer. The fibers were stretched to 130% of the resting length. All the experiments were carried out at room temperature (20–23°C).

Calcium sensitivity was measured by following the isometric tension developed by the fibers rinsed in solutions of decreasing pCa (170 mmol/l K\textsuperscript{+}, 2.5 mmol/l Mg\textsuperscript{2+}, 5 mmol/l ATP, 5 mmol/l EGTA, pH 7.0, and Ca\textsuperscript{2+} from 0.8 mmol/l [pCa 7.0] to 4.78 mmol/l [pCa 5.0]). Free Ca\textsuperscript{2+} and Mg\textsuperscript{2+} concentrations were calculated according to the method of Orentlicher et al.\textsuperscript{11} After tension attainment plateau, fibers were released by the addition of 10 mmol/l EGTA and then incubated with the solutions of successively decreasing pCa. The experiments were carried out on fibers stretched to 130% of the resting length. In seven biopsies of DCM and four normal biopsies, we repeated the experiments with the fibers stretched to 150% of the resting length. The isometric tension, P\textsubscript{o}, developed at a given pCa, was normalized to the maximum tension, P\textsubscript{max}, developed at pCa 5.0.

**Ca\textsuperscript{2+} Release From Sarcoplasmic Reticulum**

Ca\textsuperscript{2+} release was monitored indirectly by following tension development.\textsuperscript{12} Fibers were allowed to accumulate Ca\textsuperscript{2+} into the sarcoplasmic reticulum by incubating in a pCa 7.0 solution (170 mmol/l K\textsuperscript{+}, 2.5 mmol/l Mg\textsuperscript{2+}, 5 mmol/l ATP, 5 mmol/l EGTA, 10 mmol/l imidazole, pH 7.0, 0.8 mmol/l Ca\textsuperscript{2+}). After loading, fibers were rinsed in wash solution (same as relaxing solution but without EGTA; free Mg\textsuperscript{2+} was 0.1 mmol/l\textsuperscript{13–15}) and challenged with 20 mmol/l caffeine (an agonist of channels of sarcoplasmic reticulum\textsuperscript{13–17}). At this concentration of caffeine, all the sarcoplasmic reticulum Ca\textsuperscript{2+} is released.\textsuperscript{18} After bundles were rinsed in relaxing solution, the sarcoplasmic reticulum was reloaded with Ca\textsuperscript{2+} and then challenged with stepwise-increasing concentrations of caffeine (from 0.1 mmol/l caffeine) until development of tension was measured (caffeine threshold). Caffeine threshold concentration was defined as the lowest caffeine concentration that induced a tension, P\textsubscript{o}, greater than 10% of P\textsubscript{max} (tension in the presence of 20 mmol/l caffeine). We also measured the rate of rise in tension at caffeine threshold concentration and at 20 mmol/l caffeine concentration. At the end of the experiment, fibers were rinsed in a pCa 5.0 solution (170 mmol/l K\textsuperscript{+}, 2.5 mmol/l Mg\textsuperscript{2+}, 5 mmol/l ATP, 10 mmol/l imidazole, pH 7.0, 4.78
mmol/l Ca$^{2+}$) to record the maximum Ca$^{2+}$-activated tension and maximum rate of rise in tension.

**Calcium Uptake**

Calcium uptake of two normal and two DCM biopsies was measured by incubating skinned fibers for 10 minutes in 2.0 ml of a solution containing "Ca" buffered at pCa 7.0 (specific activity, 50×10$^3$ cpm/nmol Ca$^{2+}$). After incubation, the fibers were rinsed for 5 minutes in a modified wash solution containing 0.25 mmol/l EGTA, 10 mmol/l MgCl$_2$, and 50 μmol/l ruthenium red, and the fibers were then solubilized by incubating overnight in 100 μl of 1 N NaOH. Radioactivity was measured by liquid scintillation spectrometry.

Protein concentration was determined with the method of Lowry et al,$^{19}$ using bovine serum albumin as standard.

**Statistical Analysis**

Results were expressed as mean±SEM. Statistical significance was performed by Student's t test. A value of p<0.05 was considered significant.

The Hill coefficient (n) and K (the Ca$^{2+}$ concentration giving half-maximal activation) were calculated by fitting the data to the equation

$$ Y = \frac{(Ca^{2+})}{(Ca^{2+})^n + K^n} $$

by the least-squares method, where Y is the normalized P/P$_{0}$ tension. The Hill coefficient describes the degree of cooperativity of many steps between calcium binding to troponin C and tension development. Therefore, it does not represent a quantitative measurement of the number of calcium binding sites to the myofilaments. Maximal tension was the tension developed at pCa 5.0 normalized to the cross-sectional area of myocardial fibers (mN/mm$^2$).

**Materials**

Caffeine, ruthenium red, ATP, imidazole, and EGTA were purchased from Sigma Chemical Co., St. Louis, Mo. All chemicals were analytical grade.

**Results**

**Calcium Sensitivity and Cooperativity of Contractile Apparatus**

To analyze the Ca$^{2+}$ affinity of regulatory proteins in DCM, we studied the dependence of isometric tension of human cardiac skinned fibers on free Ca$^{2+}$ concentration. Figure 1 shows a typical experiment of isometric pCa/tension measurement. Incubation of cardiac fibers with increasing concentrations of Ca$^{2+}$ caused an increase in the isometric tension.

Figure 2 shows that the averaged curves representative of the isometric pCa/tension relations of ventricular skinned fibers obtained from 13 patients with DCM and from four patients with normal myocardium are sigmoidal. The sigmoidal morphology of the curves stands for the existence of cooperativity between the regulatory mechanisms. Ca$^{2+}$ sensitivity was expressed as pCa$_{50}$ that is, the Ca$^{2+}$ concentration (expressed as −log[Ca$^{2+}$]) that gives half maximal tension. Ca$^{2+}$ sensitivity in DCM (pCa$_{50}$=6.03±0.07) was similar to that obtained in control myocardium (pCa$_{50}$=6.00±0.05) (Table 1), with no statistically significant difference. The cooperativity indexes (Hill coefficient, n) of normal myocardium and DCM were also similar; n$_{Hill}$ was 2.07±0.13 in normal myocardium and 2.72±0.30 in DCM, with no statistical difference (Table 1). Maximal tension developed at pCa 5.0 was similar in DCM and in control myocardium (46.9±7.0 and 40.2±3.8 mN/mm$^2$, respectively). Also, the rate of rise of isometric tension developed at pCa 5.0 was similar.

**FIGURE 1.** Graphs of Ca$^{2+}$ sensitivity of myocardial skinned fibers. A bundle of chemically skinned fibers in relaxing solution was positioned between two clamps, one of which was attached to a tension transducer. Fibers were then incubated in solutions with decreasing pCa. After tension attained plateau, 10 mmol/l EGTA was added each time to the medium to relax the fibers before they were incubated in the next pCa solution.

**FIGURE 2.** Graph of Ca$^{2+}$ dependence of the isometric tension of normal and cardiomyopathic fibers. Sixteen bundles from four normal ventricles and 47 bundles from 13 dilated-cardiomyopathy ventricles were incubated with increasing concentrations of Ca$^{2+}$ as described in "Methods." Ca$^{2+}$ concentration is expressed as −log(Ca$^{2+}$)=pCa. Tension, P, is normalized to the maximum tension (P$_{0}$) developed at pCa 5.0. Each curve is the average of normal and cardiomyopathic patients. Error bars represent ±SEM values. □, Normal group; ■, dilated cardiomyopathy.
Caffeine Sensitivity of Normal and Dilated Cardiomyopathy

Sarcoplasmic reticulum Ca\(^{2+}\) release activity was studied by following the effects of caffeine. Caffeine is a well-known activator of the Ca\(^{2+}\) release channel of terminal cisternae of the sarcoplasmic reticulum.\(^{16,17,21-25}\) It is also known that caffeine activates the Ca\(^{2+}\)-induced Ca\(^{2+}\) release, the physiological process involved in excitation–contraction coupling in the heart.\(^{18,21,22,26}\) The release of Ca\(^{2+}\) is usually transient and is dependent on the Ca\(^{2+}\) load in the sarcoplasmic reticulum and on the concentrations of Mg\(^{2+}\), Ca\(^{2+}\), and ATP in the medium.\(^{15,16,26}\) The effects of caffeine on Ca\(^{2+}\) release from the sarcoplasmic reticulum of skinned fibers of four normal and 13 DCM hearts is summarized in Figures 4 and 5 and Table 3. After the sarcoplasmic reticulum is loaded with Ca\(^{2+}\), the addition of caffeine at concentrations higher than a definite threshold causes a tension development (Figure 4).

The caffeine threshold was higher in DCM than in the normal group. The mean caffeine threshold was 0.29±0.03 mmol/l in normal human ventricle and 1.94±0.27 mmol/l in DCM (\(p<0.01\)) (Table 3).
Figure 4. Graphs show caffeine sensitivity of sarcoplasmic reticulum. Fibers were incubated at pCa 7.0 for 30 seconds, rinsed in wash (W) solution, and then challenged with 20 mmol/l caffeine to measure maximum tension (P0). Thereafter, fibers were incubated again at pCa 7.0 for 30 seconds, rinsed in wash solution, and challenged with increasing concentrations of caffeine until tension was recorded. Caffeine threshold was defined as the lowest concentration of caffeine that induced tension development, P, larger than 10% of P0. At the end of the experiment, fibers were rinsed in a pCa 5.0 solution, and tension and rate of rise in tension were measured again. Panel A: normal; panel B: dilated cardiomyopathy. R, relaxing solution.

Figure 5. Distribution of caffeine threshold concentrations in 15 bundles from four normal ventricles and 42 bundles from 13 dilated cardiomyopathy ventricles. Caffeine threshold was measured as described in the legend of Figure 4. □, Normal group; ■, dilated cardiomyopathy.

Table 3. Caffeine Sensitivity of Sarcoplasmic Reticulum in Normal and Dilated Cardiomyopathy

<table>
<thead>
<tr>
<th></th>
<th>Normal human right ventricle</th>
<th>Dilated cardiomyopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine threshold mmol/l</td>
<td>0.29±0.03</td>
<td>1.94±0.27*</td>
</tr>
<tr>
<td>P/P0</td>
<td>0.20±0.02</td>
<td>0.18±0.01</td>
</tr>
<tr>
<td>P0/X0</td>
<td>0.71±0.06</td>
<td>0.65±0.07</td>
</tr>
<tr>
<td>V/V0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At threshold</td>
<td>0.08±0.03</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>At 20 mmol/l caffeine</td>
<td>0.36±0.07</td>
<td>0.32±0.04</td>
</tr>
</tbody>
</table>

Data represent mean±SEM of 15 fibers from four normal right ventricles and 42 fibers from 13 ventricles with dilated cardiomyopathy. Experiments were carried out as described in Figure 1. P, tension and rate of rise in tension at caffeine threshold concentration, respectively; P0, tension evoked by 20 mmol/l caffeine; V0, rate of rise in tension developed by incubating at pCa 5.0; X0, tension evoked by incubating at pCa 5.0. *p<0.005.

The fibers of DCM had a caffeine threshold higher than 0.50 mmol/l.

A second parameter of comparison of caffeine-induced Ca2+ release from the sarcoplasmic reticulum was related to the isometric tension developed by skinned fibers at threshold concentration of caffeine (P) normalized to the maximum tension evoked by 20 mmol/l caffeine (P0), which releases almost all the Ca2+ accumulated in sarcoplasmic reticulum. P/P0 is, therefore, an indirect measure of the extent of caffeine-induced Ca2+ release at the threshold. Table 3 shows that P/P0 was 0.18±0.01 in DCM and 0.20±0.02 in normal myocardium, with no statistical difference.

A third parameter studied was the rate of rise in tension elicited by caffeine at threshold concentration and at 20 mmol/l, which is assumed to be related to the rate of Ca2+ release from the sarcoplasmic reticulum.12 The values were normalized to the rate of rise in tension measured at pCa 5.0, when the rate of rise in tension is a function only of the diffusion rate of the externally applied Ca2+ and when the contractile machinery is fully activated by Ca2+. Table 3 shows that the rate of rise in tension—that is, the rate of Ca2+ release from the sarcoplasmic reticulum—induced by threshold concentrations of caffeine was 0.08±0.03 and 0.06±0.01 in the normal group and DCM, respectively, with no statistical difference, whereas at 20 mmol/l caffeine, it was 0.36±0.07 and 0.32±0.04 in the normal group and DCM, respectively. In summary, these results indicate that the sarcoplasmic reticulum Ca2+ efflux channel is less sensitive to caffeine in DCM than in the normal control group. On the other hand, there were no differences in extent and rate of caffeine-induced Ca2+ release between DCM and the normal control group.

Because caffeine-induced calcium release seems to be dependent on the Ca2+ loading by the sarcoplasmic reticulum,15,16,26 it is possible that the increased caffeine threshold in DCM could be a result of a lower Ca2+ loading of sarcoplasmic reticulum. This possibility was tested by measuring tension devel-
oped by skinned fibers at 20 mmol/l caffeine (P_0) normalized to that measured at pCa^{2+} 5.0 (X_0) when the contractile machinery is fully activated by external Ca^{2+}. Since 20 mmol/l caffeine releases all Ca^{2+} accumulated in the sarcoplasmic reticulum, P_0/X_0 is an index of Ca^{2+} accumulated in the sarcoplasmic reticulum. Table 3 indicates that P_0/X_0 was 0.71±0.06 in normal human ventricle and 0.65±0.07 in DCM, with no statistical difference. This conclusion is supported by a direct measure of maximum Ca^{2+} uptake by sarcoplasmic reticulum. This was done on two biopsies from normal human ventricle and two biopsies from DCM. The maximum Ca^{2+} uptake at pCa 7.0 was 1.81 and 2.68 nmol Ca^{2+}/mg fiber protein in the normal group and DCM, respectively. In conclusion, the results suggest that differences in sarcoplasmic reticulum Ca^{2+} loading cannot account for the altered caffeine threshold of DCM.

**Discussion**

DCM is characterized by myocardial contractile failure. Many etiopathogenetic hypotheses have been proposed: the viral, immune, and alcoholic. This variety of etiopathogenetic hypotheses suggests that DCM may represent the final stage of various myocardial diseases. The contractile failure may be caused by changes in the contractile apparatus, changes in sarcoplasmic reticulum Ca^{2+} uptake and release, or both. Our results indicate that in DCM, there is an alteration of Ca^{2+} release from sarcoplasmic reticulum, whereas contractile activity seems to be unaffected.

**Calcium Sensitivity of Contractile Apparatus**

We were unable to detect any significant differences in either Ca^{2+} sensitivity, expressed as pCa_{50}, or degree of cooperativity, expressed as the Hill coefficient, n, in ventricular skinned fibers obtained from hearts with DCM compared with the normal group. In addition, the maximal tension and the rate of rise in tension, normalized to the cross-sectional area, were similar in normal and DCM fibers. This may indicate that the mechanisms of modulation of the contractile process in DCM are spared. These results are in good agreement with previous results on skinned fibers and on papillary muscles, showing that force development at different external Ca^{2+} concentrations was not significantly diminished in DCM. This suggests that, at least in vitro, myocardium from cardiomyopathic hearts can generate a normal contractile force.

Furthermore, we also examined the possibility that the myocardial contractile failure could result from an alteration of the relation between force of contraction and diastolic volume (Frank-Starling's law). The cellular basis for Starling's law is still not clear. It has been shown that stretching causes an increase in Ca^{2+} sensitivity of skinned fibers, as demonstrated by the shift to the left of the pCa/tension relation. Recent results show that troponin C is an important factor in producing stretch-related changes of Ca^{2+} sensitivity of cardiac muscle. Our results show that in DCM, the effect of stretching on the pCa/tension relation is comparable to the one seen in the normal myocardium, demonstrating that there are no major impairments of the mechanism of modulation of Starling's law.

**Caffeine Sensitivity of Sarcoplasmic Reticulum**

Our results show that in DCM, the sensitivity to caffeine of sarcoplasmic reticulum is lower than that of normal cardiac fibers, because higher concentrations of caffeine are required to release Ca^{2+}. Caffeine causes Ca^{2+} release from the sarcoplasmic reticulum by direct interaction with the sarcoplasmic reticulum Ca^{2+} release channel. There are two possible explanations for the lower caffeine sensitivity: 1) a decreased Ca^{2+} loading of sarcoplasmic reticulum and 2) a decreased caffeine affinity of the Ca^{2+} release channel.
tion of lipid species and/or content in sarcoplasmic reticulum membrane.

On the other hand, we did not find differences between DCM and the normal group with regard to the rate and extent of Ca²⁺ release induced by caffeine either at threshold concentrations or at 20 mmol/l caffeine. These findings suggest that in DCM, there are no changes in the number and conductance of caffeine-sensitive channels of sarcoplasmic reticulum.

These results may have physiological significance, considering that caffeine activates the Ca²⁺-induced Ca²⁺ release mechanism.³⁹ Caffeine activates the Ca²⁺-induced Ca²⁺ release by interacting directly with the Ca²⁺ release channel regulatory sites of the sarcoplasmic reticulum Ca²⁺-release channel, increasing their affinity for Ca²⁺. Since Ca²⁺ and caffeine are thought to activate the same mechanism—the Ca²⁺-induced Ca²⁺ release—and our results suggest that in DCM, higher free Ca²⁺ concentrations are required for maximum activation of the sarcoplasmic reticulum Ca²⁺ release channel.

In conclusion, our results demonstrate that in DCM, the Ca²⁺ sensitivity of the contractile apparatus is unaffected, whereas the gating mechanism of the sarcoplasmic reticulum Ca²⁺ release channel is abnormal, suggesting a possible involvement of the excitation–contraction coupling in the pathogenesis of this disease.

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