Phosphorylation Status of Regulatory Proteins and Functional Characteristics in Myocardium of Dilated Cardiomyopathy of Syrian Hamsters

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Abstract: To understand the pathophysiology of hereditary cardiomyopathy, we measured the phosphorylation status of regulatory proteins, troponin I (TnI), troponin T (TnT), myosin light chain 2 (MLC2), and myosin-binding protein C (MyBP-C), and the Ca²⁺-dependence of tension development and ATPase activity in skinned right ventricular trabeculae obtained from cardiomyopathic (TO-2 strain, *n* = 8) and control (F1B strain, *n* = 8) hamsters. The Ca²⁺ sensitivities of tension development and ATPase activity (mean ± SD) were significantly (*P* < 0.0001) higher in the TO-2 strain (pCa₅₀ 5.64 ± 0.04 in tension and 5.65 ± 0.04 in ATPase activity) than in the F1B strain (pCa₅₀ 5.48 ± 0.03 in tension and 5.51 ± 0.03 in ATPase activity). No significant differences in their maximum values were observed between TO-2

(40.8 ± 7.4 mN/mm² in tension and 0.52 ± 0.15 μmol//s in ATP consumption) and F1B (42.3 ± 8.5 mN/mm² in tension and 0.58 ± 0.41 μmol//s in ATP consumption) preparations, indicating that the tension cost (ATPase activity/tension development) in TO-2 was quite similar to that in F1B. The phosphorylation levels of MLC2 and TnI were significantly (*P* < 0.01) lower in TO-2 than in F1B. These results suggest that the increase in the Ca²⁺ sensitivities of tension development and the ATPase activity in TO-2 hearts result from the decreased basal level of TnI phosphorylation, and these features can be considered to produce the incomplete diastolic relaxation and partly improve the systolic function in TO-2 hearts.

Key words: dilated cardiomyopathy, phosphorylation status, tension development, ATPase activity, tension cost.

Dilated cardiomyopathy (DCM) is a primary heart muscle disease characterized by ventricular dilation and systolic dysfunction [1]. Bio TO-2 strain hamster with congenital DCM shows similar clinical backgrounds to human cases and thus has been considered to be a representative animal model of human hereditary dilated cardiomyopathy [2–7]. The gene defect in δ -sarcoglycan has been identified as being responsible for DCM in TO-2 hamster [8, 9] as well as in human [10]. To understand the pathogenesis of this animal model and the pathophysiology of heart failure, the hemodynamic characteristics have been analyzed at the organ level [2, 4]. The subcellular mechanism accounting for the myocyte dysfunction, however, remains to be characterized, though it is reported to very likely include the abnormalities of the sarcolemma, the sarcoplasmic reticulum, and the myofibrils [11–13]. Recently, we [7] studied the contractile function of myocytes isolated from the ventricles of 10- to 12-week-old TO-2 hamsters over a wide range of loading conditions and found that (1) in the unloaded condition, the shorten-

ing fraction and maximum shortening velocity were decreased in TO-2 myocytes as compared with normal control (F1B strain hamster) myocytes; (2) the peak force in the isometric condition and the external work in physiologically loaded conditions were also decreased. Being consistent with these contractile dysfunctions, the calcium transients measured by Indo-1 were found to reveal an elevated diastolic level, a decreased peak level, and a slower diastolic decay in TO-2 myocytes as compared with F1B myocytes. Taken together, these results strongly suggest that the contractile dysfunctions in a TO-2 heart are largely caused by the impairment of the sarcoplasmic reticulum calcium transport function. Supportive evidence has been reported in dilated cardiomyopathic hamsters (Bio 53.58), which are identical to TO-2 hamsters [12, 13]. However, the myofibrillar contribution to the contractile dysfunction in TO-2 heart remains to be studied.

We therefore analyzed the phosphorylation status of regulatory proteins, myosin light chain 2 (MLC2), troponin I (TnI), troponin T (TnT), and myosin-binding protein

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C (MyBP-C), and also the Ca^{2+} -dependence of tension development and ATPase activity in skinned right ventricular trabeculae obtained from TO-2 and F1B hamsters.

MATERIALS AND METHODS

Preparation. Skinned right ventricular trabeculae were prepared from 10- to 12-week-old male F1B and TO-2 hamsters (109.4 ± 8.5 g and 94.3 ± 10.0 g in weight, respectively), treated in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiology Science* published by the Physiological Society of Japan, as described previously [14].

Experimental solutions. Solution compositions were calculated using the computer program, as in our earlier study [14]. The relaxing solution contained (mmol/l) EGTA 5.9; CaEGTA 0.1; MgATP 6.3; Mg-propionate 1.2; phosphate (Pi) 1; Na-propionate 27; K-propionate 49; phosphoenol pyruvate (PEP) 15; nicotinamide adenine dinucleotide (NADH) 0.75; pyruvate kinase (PK) 100 U/ml; lactate dehydrogenase (LDH) 140 U/ml; NaN_3 10; diadenosine pentaphosphate (A_2P_5) 0.2; and MOPS 10 (pH 7.0 at 22°C). NaN_3 and A_2P_5 were used to inhibit the mitochondrial ATPase and adenylate kinase activities, respectively. The preactivating solution had the same composition as that of the relaxing solution, except that the EGTA concentration was reduced to 0.1 mmol/l. The activating solutions with the various Ca^{2+} concentrations were prepared by mixing the relaxing solution (pCa 8.0) and a solution (pCa 4.6), in which EGTA in the relaxing solution was replaced by equimolar amounts of CaEGTA. In all solutions, total Na concentration was maintained at 78 mM; the MgATP concentration was maintained at 5 mM; both the Pi and Mg^{2+} concentrations were maintained at 1 mM; and the ionic strength was adjusted to 200 mM with K-propionate.

Experimental apparatus of mechanoenergetic measurements. The preparation was mounted horizontally in an experimental chamber (volume 40 μl) between stainless steel hooks connected to a tension transducer (AE 801, SensoNor, Horten, Norway) and a servocontrolled piezoelectric translator (PZT) (E-662, Physik Instrumente, Germany), with a fast-setting glue (collodion) via the loop of silk strand at either end of the preparation. Tension, length, and ATPase activity (NADH absorbance signal, see below) were recorded with an A/D converter (Powerlab/8SP, ADInstruments, Australia) connected to a PC (Mebius, SHARP, Japan). The experimental chamber had quartz windows to allow the transmission of near-ultraviolet light for the measurement of NADH absorbance. The NADH absorbance signal was measured with a dual wave length spectrophotometer (JASCO Co., Tokyo, Japan). The solutions in the chamber were constantly stirred with a small magnet during the experiments. The temperature of the solutions was kept at 22°C with an accuracy of

0.1°C by circulating temperature-controlled water through a brass block beneath the experimental chamber. Sarcomere length was measured by a light diffraction with He-Ne laser (model GLG 5350, NEC, Tokyo, Japan).

Measurement of ATPase activity. The ATPase activity of the preparation was measured by the method utilizing two enzymatic reactions [14]. One reaction is $\text{ADP} + \text{PEP} \rightarrow \text{ATP} + \text{pyruvate}$, catalyzed by PK, and the other reaction is $\text{pyruvate} + \text{NADH} \rightarrow \text{lactate} + \text{NAD}$, catalyzed by LDH. The ATPase activity associated with the Ca^{2+} -activated tension development was obtained by subtracting the NADH absorbance change of the resting preparation from that of the Ca^{2+} -activated preparation.

Determination of phosphorylation status of contractile proteins. After the measurements of tension development and ATPase activity, proteins were extracted from the individual preparations with 15 μl of SDS sample buffer (75 mM Tris-HCl [pH 6.8], 3% [w/v] SDS, 5% [v/v] 2-mercaptoethanol, 30% [v/v] glycerol, and 0.02% [w/v] bromophenol blue), and the whole quantity (15 μl) of the extract was applied to 1D-PAGE on 10%–20% gradient polyacrylamide gels. Phosphorylated proteins were detected by Pro-Q Diamond stain according to the protocol of the supplier (Molecular Probes, Eugene, OR). Briefly, the gels were fixed in 10% acetic acid/50% methanol and stained with Pro-Q Diamond (1.5 h). The gels were destained and scanned using a laser-scanning instrument (FLA-3000G, Fuji Photo Film, Tokyo, Japan) with a 532 nm laser for excitation source and a 580 nm long-pass emission filter. Subsequently, the gels were stained with SYPRO Ruby (Molecular Probes) overnight to visualize total proteins. They were destained with 10% methanol/7% acetic acid for 30 min and scanned on the laser-scanning instrument (FLA-3000G) using a 473 nm laser for excitation source and a 580 nm long-pass emission filter. After measuring the fluorescent intensities of the band for myosin light chain 2 (MLC2), troponin I (TnI), troponin T (TnT), and myosin-binding protein C (MyBP-C) by using a densitometric analysis (Science Lab 99 Image Gauge, Fuji Photo Film), we calculated the ratio of Pro-Q Diamond dye to SYPRO Ruby dye signal intensities (Pro-Q/SYPRO) for each band to normalize the phosphorylation level to the total amount of protein.

Experimental protocols. The preparation was first equilibrated for 5–10 min in the relaxing solution. Sarcomere length was adjusted to 2.2 μm at which the resting tension was about 10% of the maximum active tension in the TO-2 and F1B preparations. At first, the stabilized preparation was activated twice with the maximum activating solution (pCa 4.6) in the following sequential way of solution changes: relaxing solution, preactivating solution, maximum activating solution, and relaxing solution. This was to learn the amount of tension deterioration of the preparation. If the isometric tension of the second activation was greater than 90% of the first and the resting

tension was returned to the initial level, the preparation was retained for the current experiments. It was then activated to contract isometrically with various Ca^{2+} concentrations over a range from pCa 8.0 to pCa 4.6.

Data analysis and statistical evaluation. Either tension or ATPase activity measured at submaximal pCa was expressed as a fraction of the respective maximum value. The individual relative pCa-tension and pCa-ATPase activity relations were characterized by Hill plot analysis, as described previously [14]. All data are presented as mean $\pm$ SD. The statistical differences in the parameters of tension and ATPase activity between F1B and TO-2 preparations were determined by two-way ANOVA and those in the phosphorylation levels by unpaired *t*-test. *P* values <0.05 were taken as indicating significant differences.

RESULTS

Ca^{2+} -dependence of isometric tension and ATPase activity

Figure 1 shows the typical results of Ca^{2+} -activated isometric tension (lower trace) and ATPase activity (upper trace) simultaneously obtained from an F1B preparation. After the amount of tension deterioration of the preparation was confirmed to be less than 10% by the second application of pCa 4.6 maximum activating solution (see MATERIALS AND METHODS), the series of records was taken. As can be seen in Fig. 1, the ATPase activity, indicated as the slope representing the decrease of NADH, attained a steady value during the steady level of isometric tension for each pCa value used. The steady isometric ten-

Fig. 1. Typical results of Ca^{2+} -activated isometric tension (lower trace) and NADH absorbance changes (upper trace) in F1B preparation.

sion and ATPase activity values (relative to the respective maximum value) were plotted against pCa values and were well fitted to the Hill equation (Fig. 2). Average fit parameters (pCa_{50} , the Ca^{2+} concentration required for half maximal value and Hill *n*, Hill coefficient) obtained individually from 8 F1B and 8 TO-2 preparations are shown in Table 1. The Ca^{2+} sensitivities of tension development and ATPase activity are seen to be significantly ($P < 0.0001$) higher in TO-2 than in F1B preparations with no difference in Hill *n* between the two. However, no significant difference in their maximum values was observed between TO-2 (40.8 ± 7.4 mN/mm² in tension and 0.52 ± 0.15 $\mu\text{mol}/\text{l}/\text{s}$ in ATP consumption) and F1B (42.3 ± 8.5

Fig. 2. Average Ca^{2+} sensitivity of tension (a) and ATPase activity (b) in the F1B (open circles) and TO-2 (closed circles) preparations. Average (mean $\pm$ SD, $n = 8$) tension and ATPase activity values (relative to the respective maximum value) were plotted against pCa values, and the data were fitted to the Hill equation, as indicated by solid lines. Average fit parameters obtained individually from 8 preparations are shown in Table 1.

Table 1. Average Hill fit parameters in F1B and TO-2 preparations.

		F1B	TO-2
Isometric tension	pCa ₅₀	5.48 ± 0.03	5.64 ± 0.04*
	Hill <i>n</i>	2.81 ± 0.25	2.92 ± 0.31
ATPase activity	pCa ₅₀	5.51 ± 0.03	5.65 ± 0.04*
	Hill <i>n</i>	2.78 ± 0.22	2.87 ± 0.29

pCa₅₀ indicates the Ca²⁺ concentration required for half maximal value. Hill *n* indicates Hill coefficient. Values are mean ± SD, *n* = 8. **P* < 0.0001 by two-way ANOVA.

Fig. 3. Typical SDS-PAGE patterns (10%–20% gradient gel) for F1B (lanes 1 and 3) and TO-2 (lanes 2 and 4) preparations. The gel was stained with Pro-Q Diamond (lanes 1 and 2) specifically for phosphorylated proteins and subsequently stained with SYPRO Ruby (lanes 3 and 4) for total proteins.

mN/mm² in tension and 0.58 ± 0.41 μmol//s in ATP consumption) preparations, indicating that the tension cost (ATPase activity/tension development) in TO-2 preparations was quite similar to that in F1B preparations. The tension cost at submaximum activation was also almost the same between two preparations, though the data were not shown.

Phosphorylation status of contractile proteins

Figure 3 shows typical patterns of SDS-PAGE (10%–20% gradient gel) of skinned-trabeculae obtained from F1B (lanes 1 and 3) and TO-2 (lanes 2 and 4) hamsters. The gels were stained with Pro-Q Diamond (lanes 1 and 2) specific for phosphorylated proteins and subsequently with SYPRO Ruby (lanes 3 and 4) for total proteins. The Pro-Q Diamond stained gel shows prominent bands of phosphorylated proteins corresponding to myosin light chain 2 (MLC2), troponin I (TnI), troponin T (TnT), and myosin-binding protein C (MyBP-C), while the SYPRO

Fig. 4. Bar graph showing the relative phosphorylation levels of MLC2, TnI, TnT, and MyBP-C in the F1B (white columns) and the TO-2 (black columns) preparations. Each column and vertical bar represents the mean ± SD of eight preparations. The averaged mean value for each contractile protein in TO-2 preparations is normalized to the respective mean value in F1B preparations. **P* < 0.01 by unpaired *t*-test.

Ruby stained gel shows the protein bands of MLC2, TnI, TnT, actin, and MyBP-C. After measuring the fluorescent intensities of protein bands for MLC2, TnI, TnT, and MyBP-C, we calculated the intensity ratio of Pro-Q Diamond dye to SYPRO Ruby dye (Pro-Q/SYPRO) for each protein to normalize the phosphorylation level relative to each corresponding protein's content.

To assess the possible degradation of these regulatory proteins in TO-2 as compared with F1B hearts, we also calculated the band intensity ratio of SYPRO Ruby stained gels for MLC2, TnI, TnT, and MyBP-C relative to the corresponding actin in F1B and TO-2 preparations. We found no significant difference in the band intensity ratio (mean ± SD, *n* = 8) between F1B and TO-2 preparations (MLC2/actin = 0.16 ± 0.03 in F1B and 0.17 ± 0.03 in TO-2; likewise, TnI/actin = 0.074 ± 0.011 vs. 0.072 ± 0.015 , TnT/actin = 0.097 ± 0.016 vs. 0.108 ± 0.014 , MyBP-C/actin = 0.074 ± 0.015 vs. 0.070 ± 0.011), showing no apparent degradation of these regulatory proteins in TO-2 as compared with F1B hearts.

Figure 4 shows the phosphorylation levels of MLC2, TnI, TnT, and MyBP-C in TO-2 preparations relative to those of the respective protein in F1B preparations. Each column and vertical bar represents the mean ± SD of 8 preparations. As clearly seen in Fig. 3, the phosphorylation levels of MLC2 and TnI were significantly (*P* < 0.01) lower in TO-2 than in F1B preparations. Although the average values of TnT and MyBP-C phosphorylation levels were lower in TO-2 than in F1B preparations, no significant differences in their phosphorylation levels were observed between the two preparations.

DISCUSSION

In the present study, we found that (1) the Ca²⁺ sensitivities of tension development and ATPase activity were

higher in TO-2 than in F1B cardiac trabeculae (Table 1), and that (2) the phosphorylation levels of MLC2 and TnI were lower in TO-2 than in F1B cardiac trabeculae (Fig. 4). These results strongly suggest that the increased Ca^{2+} sensitivities of tension and ATPase activity in TO-2 cardiac trabeculae are largely due to the lower phosphorylation level of TnI [15–19], since the lower phosphorylation of MLC2 is reported to decrease the Ca^{2+} sensitivity of tension development [20–22]. The data similar to the present results have been reported in human dilated cardiomyopathies [18] and in end-stage human failing myocardium [23]. Velden *et al.* [23] observed that as compared with nonfailing myocardium, the Ca^{2+} sensitivity of tension development (pCa_{50}) was significantly higher in failing myocardium with an increased percentage of dephosphorylated TnI and an inverse correlation between pCa_{50} and a percentage of phosphorylated MLC2. Further, protein kinase A (PKA) was reported to decrease Ca^{2+} sensitivity to a large extent because of the greater changes in TnI phosphorylation level in failing than in nonfailing myocytes.

No difference in the phosphorylation level of TnT between nonfailing and failing hearts was also reported to be observed, as in the present and recent [19] studies. The lower phosphorylation levels of MLC2 and TnI in TO-2 heart as compared with those of F1B heart (Fig. 4) therefore seem to be due to the decreased PKA activity and/or the increased protein phosphatase activity [18, 22]. Actually, an expression of cardiac phosphatases has been reported to increase in patients with end-stage heart failure [24]. Neuman *et al.* [24] reported a 2.5-fold increase in phosphatase activity in the membrane fraction of failing heart, which was linked to decreased levels of phosphatase inhibitor-1 and decreased phosphatase inhibitor-1 phosphorylation by PKA, both of which lead to higher phosphatase activity [25, 26]. Furthermore, a reduction in the number of β -adrenoceptors and an increased activity of inhibitory G protein that couple in an inhibitory fashion to adenylyl cyclase activity have been reported to be involved in end-stage human heart failure [27–29]. In accordance with these findings, the β -adrenoceptor-mediated increase in cAMP levels is known to be smaller in patients with failing than in those with nonfailing hearts [30]. Feldman *et al.* [31] also observed a decrease in isoprenaline-mediated cAMP formation, resulting from a disturbed function of the Gs-protein in 100-day-old TO-2 hamster hearts. Thus the same seems to be true in TO-2 heart as in end-stage human heart failure.

Since the cross-bridge cycling rate, which is reflected as the tension cost [32], has been reported to be slower at lower TnI [14, 33] and MLC2 [34] phosphorylation level, the tension cost of a TO-2 heart should be lower than that of an F1B heart. No difference in the tension cost between the present two preparations imply that the cross-bridge cycling rate of the TO-2 heart decreased by lower TnI and

MLC2 phosphorylation might be increased nearly to that of the F1B heart by some factors. One factor might be the TnI phosphorylation at protein kinase C (PKC) sites, which has been reported to change the cross-bridge cycling rate opposing to the TnI phosphorylation at PKA sites [35]. The other possible factor might be related to the gene defect in δ -sarcoglycan in TO-2 hearts, which increases the cross-bridge cycling rate through unknown mechanisms. Quite recently, the primary effect of the PKA phosphorylation of TnI has been reported to be related only to the reduced Ca^{2+} sensitivity of tension, and not to the cross-bridge kinetics [36]. If this is so in the present preparations, the different TnI phosphorylation level between TO-2 and F1B preparations might not relate to their tension cost.

In our earlier study [7], the calcium transients measured by Indo-1 was found to reveal elevated diastolic levels, decreased peak levels, and slower diastolic decay in TO-2 myocytes as compared with F1B myocytes. From these results together with the previous similar findings [12, 13], the contractile dysfunctions in TO-2 heart have been considered to be caused mostly by the impairment of the sarcoplasmic reticulum calcium transport function. The present results, the increase in Ca^{2+} sensitivities of tension development and the ATPase activity associated with the decreased basal level of TnI phosphorylation, can thus, be considered to produce the incomplete diastolic relaxation and partly improve the systolic function in TO-2 hearts.

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