Myofibrillar Calcium Sensitivity of Isometric Tension Is Increased in Human Dilated diomyopathies

Role of Altered β-adrenergically Mediated Protein Phosphorylation

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Abstract

To examine the role of alterations in myofibrillar function in human dilated cardiomyopathies, we determined isometric tension–calcium relations in permeabilized myocyte-sized myofibrillar preparations ($n = 16$) obtained from left ventricular biopsies from nine patients with dilated cardiomyopathy (DCM) during cardiac transplantation or left ventricular assist device implantation. Similar preparations ($n = 10$) were obtained from six normal hearts used for cardiac transplantation. Passive and maximal Ca^{2+}-activated tensions were similar for the two groups. However, the calcium sensitivity of isometric tension was increased in DCM compared to nonfailing preparations ($[Ca^{2+}]_{50} = 2.46 \pm 0.49$ μM vs 3.24 ± 0.51 μM, $P < 0.001$). In vitro treatment with the catalytic subunit of protein kinase A (PKA) decreased calcium sensitivity of tension to a greater degree in failing than in normal preparations. Further, isometric tension–calcium relations in failing and normal myofibrillar preparations were similar after PKA treatment. These findings suggest that the increased calcium sensitivity of isometric tension in DCM may be due at least in part to a reduction of the β-adrenergically mediated (PKA-dependent) phosphorylation of myofibrillar regulatory proteins such as troponin I and/or C-protein. *(J. Clin. Invest.* 1996. 98:167–176.) **Key words: dilated cardiomyopathy, human • calcium sensitivity of isometric tension • protein phosphorylation**

Introduction

The subcellular mechanisms accounting for the myocyte dysfunction occurring in dilated cardiomyopathies (DCM)[1] remain incompletely characterized, but likely include abnormalities of the sarcolemma, the sarcoplasmic reticulum, and the myofibrils. Shortening and tension development in myofibrils ultimately determine cardiac performance, and myofibrillar protein composition is altered in chronic human heart failure (recently reviewed in reference 1). In addition, myofibrillar ATPase activity is significantly reduced in human heart failure (2). However, studies of myofibrillar mechanical function in this disorder have yielded conflicting results. For instance, both unchanged (3–5) and increased (6) myofibrillar calcium sensitivity of isometric tension have been observed in failing human myocardium.

The purpose of the present study was to determine isometric cardiac myofibrillar mechanical function in human dilated cardiomyopathies, using recently developed techniques not previously employed in studies of human myocardium. Ventricular biopsies were obtained from failing and normal donor hearts at the time of cardiac transplantation or left ventricular assist device implantation. The tissue was immediately frozen to prevent ischemic injury and to preserve myofibrillar protein phosphorylation status. Using myocyte-sized myofibrillar preparations from the previously frozen biopsies, we measured isometric tension over a range of calcium concentrations. Sarcomere length was monitored both in passive preparations and after calcium activation; preparations demonstrating significant shortening of central sarcomeres during maximal activation were rejected. Observed changes in myofibrillar calcium sensitivity of tension in failing hearts were correlated with the content and isoform distribution of several important myofilament-regulatory proteins. Because both the density and activity of the sarcolemmal β-adrenergic receptor/adenylate cyclase complex are reduced in human dilated cardiomyopathies, the role of β-adrenergically mediated protein phosphorylation in modulating isometric myofibrillar function in failing myocardium was also examined.

Methods

Patients. Transmural left ventricular biopsies (~ 1–3 g) were obtained from the hearts of nine patients with New York Heart Association Class III or IV heart failure at the time of cardiac transplantation ($n = 8$) or left ventricular assist device implantation ($n = 1$). Biopsies were obtained before cross clamping the aorta and performing the cardiectomy, and the tissue was immediately frozen in liquid nitrogen. Left ventricular endomyocardial biopsies (~ 0.01–0.03 g) were obtained from six hearts used as donors for cardiac transplantation. These biopsies were obtained by the transplant surgeon under direct visualization through the mitral valve apparatus, immediately after cold cardioplegic arrest (University of Wisconsin solution, Madison, WI) and explantation of the heart. This tissue was also immediately frozen in liquid nitrogen. All biopsies were stored at −70°C for later study. Tissue specimens were obtained as approved by the Human Subjects Investigational Review Board at the University of Wisconsin Medical School.

Clinical characteristics of the subjects are provided in Table I. Diagnoses included idiopathic dilated cardiomyopathy (ID DCM, $n = 4$), ischemic dilated cardiomyopathy (ISC DCM, $n = 4$), and end-stage

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1. *Abbreviations used in this paper:* DCM, dilated cardiomyopathies; ID DCM, idiopathic DCM; ISC DCM, ischemic DCM; MLC, myosin light chain; MV DCM, end-stage mitral valve disease; PKA, protein kinase A.

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Table I. Clinical Characteristics of Patients and Heart Donors

		Echocardiography*				
	Age and Sex	LA	LV	FS	Inotropic support	Dose
	yr	*mm*	*mm*	*%*		*μg/kg per min*
Patients						
Diagnosis						
ID DCM	48 M	51	80/73	9	None	
ID DCM	49 M	54	79/68	14	Dobutamine	2.0
					Milrinone	0.6
ID DCM	56 F	50	84/71	15	None	
ID DCM	38 M	29	71/66	7	None	
ISC DCM	47 M	42	57/46	18	Dobutamine	2.0
					Milrinone	0.5
ISC DCM	62 M	48	67/60	10	Milrinone	0.4
ISC DCM	58 M	61	68/52	24	None	
ISC DCM	59 M	39	77/63	19	None	
MV DCM	52 M	51	90/85	5	Milrinone	0.6
Donors						
Cause of death						
SAH	51 F	–	–	–	None	
CHI	29 F	–	–	–	None	
CHI	14 F	–	–	–	None	
CHI	14 F	–	–	–	Dopamine	15.0
CHI	35 M	–	–	–	Dopamine	5.0
CHI	19 M	–	–	–	Dopamine	5.0
CHI‡	24 F	–	–	–	Dopamine	7.0

*M-mode dimensions from *trans*-thoracic echocardiograms obtained before cardiac transplantation or left ventricular assist device implantation. While *trans*-thoracic echocardiograms were obtained in all donors before organ procurement, and ventricles were judged to be of normal size and function by a cardiologist, research-quality M-mode traces are not available. LA, left atrium dimension; LV, left ventricular end-diastolic dimension/end-systolic dimension; FS, left ventricular fractional shortening; MV DCM, end-stage mitral regurgitation and dilated cardiomyopathy; SAH, subarachnoid hemorrhage; CHI, closed head injury. ‡Biopsy used for SDS-PAGE only.

mitral valve disease (MV DCM, $n = 1$). Four patients (two ISC DCM, one ID DCM, and one MV DCM) were hospitalized and received chronic intravenous inotropic therapy before transplantation. One patient (ID DCM) received chronic oral β-adrenergic blocker therapy (Carvedilol, Smithkline Beecham Pharmaceuticals, Philadelphia, PA) before transplantation. All nine patients were also being treated with oral digoxin, diuretics, and angiotensin-converting enzyme inhibitors. Three of the six donors required brief intravenous vasopressor (dopamine) support before procurement; however, normal left ventricular function was documented by transthoracic echocardiography in all six before selection for organ donation. Because the smaller size of the endomyocardial biopsies obtained from the donor organs precluded biochemical analyses in addition to mechanical studies in all cases, an additional biopsy obtained from a seventh donor organ was used for biochemical studies only.

Myocyte-sized myofibrillar preparations. Ventricular biopsies were thawed in 5–15 ml of preparation solution (below), and immediately mechanically disrupted using a tissue homogenizer for 15 s at low speed (12,500 rpm). The resulting suspensions of small clumps of myocytes, single-myocyte–sized preparations and cell fragments were spun at 165 *g* for 120 s and were resuspended in 10 ml of preparation solution containing 0.3% Triton X-100 (Pierce Chemical Co., Rockford, IL) for 6 min at room temperature to remove adherent sarcolemma and sarcoplasmic reticulum. Permeabilized myocyte preparations were twice again centrifuged and suspended in preparation solution, and were then stored on ice for use within 8 h.

Solutions. Preparation solution contained (mM): NaF 20 (to inhibit endogenous phosphatases), ATP 4, free Mg^{2+} 1, imidazole 7, EGTA 7, creatine phosphate 7, and leupeptin (0.5 mg/ml). Relaxing and activating solutions contained (mM): ATP 4, free Mg^{2+} 1, imidazole, EGTA 7, and creatine phosphate 7. Ionic strength in preparation, relaxing, and activating solutions was adjusted to 180 mM with KCl; pH was adjusted to 7.0 at 15°C. A computer program was used to determine the concentration of metals and metal ligands (7), and calcium concentrations were varied from 10^{-9} M (relaxing and preparation solutions) to $10^{-4.5}$ M (maximally activating solution). Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Experimental apparatus. The experimental apparatus has been previously described (8) and consisted of a stainless steel plate with two glass-bottomed solution troughs (100 μl) mounted on the mechanical stage of an inverted microscope (Carl Zeiss, Inc., Thornwood, NY). Single cell–sized myofibrillar fragments were selected on the basis of size (100–150 μm long by 20–30 μm diameter) and uniformity of striation pattern, and were attached via borosilicate glass micropipettes (tip diameters of 5–10 μm) to a force transducer (403; Cambridge Technology, Inc., Cambridge, MA; sensitivity 20 mV/mg, resolution of < 50 μg, resonant frequency of 600 Hz) and a piezoelectric translator (Physik Instrumente GmbH & Co., Waldbronn, Germany) using a small drop of silicone adhesive (Dow-Corning, Midland, MI). After curing for 45 min, the permeabilized myocyte preparations were tightly adherent to the micropipettes, forming a low compliance attachment.

Force and preparation length (piezoelectric translator position) during experiments were recorded on a storage oscilloscope (NIC-310; Nicolet Instrument Corp., Madison WI) and saved on magnetic disk for later analysis. Sarcomere length was monitored by photomi-

croscopy (magnification 1,690) via a CCD camera (WV-B1600; Panasonic) and recorded on videotape (HR-s6600u super-VHS videorecorder; JVC, Salt Lake City, UT), and was set at 2.30–2.35 μm in relaxing solution. Sarcomere length and striation uniformity was verified after maximal calcium activation, and preparations with significant internal shortening (> 0.10 μm/sarcomere) during maximal activation were rejected. Approximately 40% of all preparations were rejected because of excessive compliance due to a suboptimal attachment.

After completion of the experiment, the permeabilized myocyte preparation was detached from one micropipette in relaxing solution. This micropipette was then used to bend the preparation upward 90°; the microscope was focused on the midportion of the preparation and cross-sectional area was calculated from the measured width and depth assuming an elliptical geometry.

Isometric tension-calcium measurements. Tension–calcium relations were determined at 15°C. Active tension was calculated as the difference between the total tension developed at any given calcium concentration and passive tension measured in relaxing solution. Tension was first determined at maximal Ca^{2+} activation, and maximal Ca^{2+}-activated tension was redetermined after every four or five submaximal activations. Active tensions were normalized assuming a linear decrease in maximal tension with each activation, but the cell was rejected if maximal tension declined by > 25% over the course of the experiment.

Calcium sensitivity of tension was determined for each preparation by least squares regression using the Hill transformation to linearize the sigmoidal tension–calcium relations (9): $\log [P_{rel}/(1 - P_{rel})] = n (\log [Ca^{2+}] + \log k)$. P_{rel} is active tension as a fraction of maximal Ca^{2+}-activated tension, n is the Hill coefficient, and k equals the calcium concentration at which tension is half-maximal ($[Ca]_{50}$). For purposes of displaying the data, mean tension–calcium curves were fitted to the sigmoidal Hill equation: $P_{rel} = [Ca^{2+}]^n/([Ca^{2+}]^n + [Ca]_{50})$.

Protein kinase A treatment. A subset of myofibrillar preparations were incubated at room temperature (22–23°C) for 40 min in relaxing solution containing the catalytic subunit of porcine cardiac protein kinase A (3.0 μg/ml; Sigma Chemical Co.) and 6 mM dithiothreitol to phosphorylate the protein kinase A–dependent sites of myofibrillar proteins. The protocol has been shown (using [γ-^{32}P]ATP autoradiography) to maximally phosphorylate rat cardiac troponin I and C-protein in vitro, and reduces myofibrillar calcium sensitivity of isometric tension in permeabilized rat myocytes to an extent similar to that observed after exposure of intact rat myocytes to a high concentration of isoproterenol (10). Isometric tension–calcium relations were determined before and after treatment with protein kinase A.

Protein electrophoresis and Western blotting. An aliquot of the myofibrillar suspension from each experiment was centrifuged at 165 *g* for 120 s, and the pellet was resuspended in 100–200 μl of SDS sample buffer (11), then stored at −70°C for later analysis. Relative content of thick and thin filament regulatory proteins were determined by separating myofibrillar proteins using SDS-12% polyacrylamide gel electrophoresis, as previously described (11). Gels were silver stained, and relative myofibrillar regulatory protein content was determined using an imaging densitometer (GS-670; Bio Rad Laboratories, Hercules, CA) and commercially available software (Molecular Analyst; Bio Rad Laboratories).

To identify isoforms of troponin T, SDS-polyacrylamide gel electrophoresis was first used to separate myofibrillar proteins of human cardiomyopathic and donor hearts. The conditions used were identical to those described above except that the polyacrylamide concentration was 16%. After polyacrylamide gel electrophoresis (SE-260 Mighty-Small II; Hoefer Scientific Instruments, San Francisco, CA), proteins were electrophoretically transferred (TE-77 Semi-Phor; Hoefer Scientific Instruments) to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and reacted with monoclonal anti-skeletal troponin T antibody (JLT-12; Sigma Chemical Co.), which recognizes a conserved epitope in cardiac muscle isoforms (12). Immune complexes were detected on the blots with horseradish peroxidase–linked goat anti–mouse immunoglobulin (Bio Rad Laboratories) and visualized on photographic film using enhanced chemiluminescence (ECL; Amersham Life Science, Arlington Heights, IL). Exposure time of chemiluminescence-enhanced blots to photographic film was varied to maximize detection of very low levels of troponin T isoforms. After development of the photographic film, the troponin bands were scanned using the imaging densitometer and software as above.

Statistical methods. Results are presented as means±SD. Characteristics of permeabilized myocyte preparations such as dimensions, passive and maximal calcium-activated forces, and protein composition were compared using unpaired Student's *t* tests. Tension–calcium relations were linearized using the Hill transformation (9), and compared using multiple linear regression analysis as an alternative to analysis of covariance, with a dummy variable coding for the presence or absence of heart failure (13). Differences were accepted as significant for $P < 0.05$. Statistical tests were performed using commercially available software (SYSTAT, Inc., Evanston, IL).

Results

Isometric tension–calcium relations. Tension–calcium relations were determined in 16 myocyte-sized myofibrillar preparations obtained from left ventricular biopsies from patients with DCM and in 10 preparations obtained from normal hearts. Preparations obtained from cardiomyopathic hearts were significantly larger in size than those from the normal, donor hearts (Table II). This observation applied to both the ischemic and idiopathic subsets of myopathic preparations,

Table II. Characteristics of Permeabilized, Single-Myocyte–sized Myofibrillar Preparations for Donor Ventricles and Dilated Cardiomyopathic Ventricles

	Length	CSA	$P_{passive}$	P_{active}	$SL_{passive}$	SL_{active}
	μm	*$mm^2 \times 10^{-5}$*	*mN/mm^2*	*mN/mm^2*	*μm*	*μm*
Donors (n = 10 preparations)	86.7±17.7	1.96±0.96	5.0±2.7	29.7±9.3	2.33±0.02	2.27±0.05
DCM (n = 16 preparations)	118.9±30.2*	3.60±1.34*	3.6±1.5	25.1±9.1	2.30±0.03	2.27±0.03
Idiopathic DCM (n = 7 preparations)	119.5±29.8*	3.67±1.49‡	3.9±2.0	28.2±8.7	2.29±0.04	2.26±0.03
Ischemic DCM (n = 7 preparations)	115.5±32.0*	3.53±1.30‡	3.1±0.9	22.1±8.9	2.31±0.02	2.26±0.04

CSA indicates cross-sectional area; $P_{passive}$, passive tension; P_{active}, maximal Ca^{2+}-activated tension; $SL_{passive}$, passive sarcomere length; SL_{active}, sarcomere length after maximal Ca^{2+} activation; and DCM, dilated cardiomyopathy. Values are mean±SD. *$P < 0.01$ vs donors; ‡$P < 0.02$ vs donors.

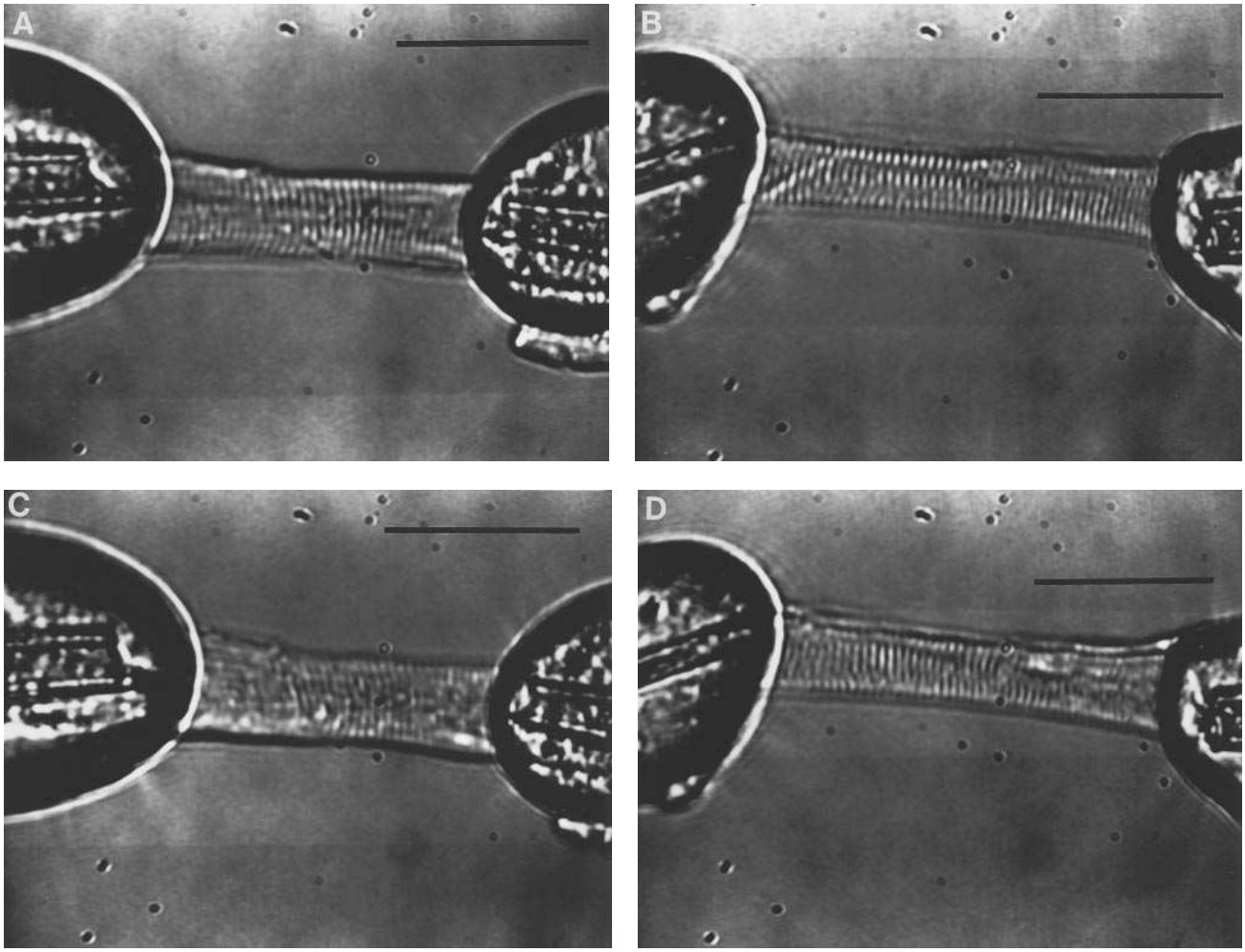

Figure 1. Photomicrographs of representative myocyte-sized myofibrillar preparations from a donor ventricle (*A* and *C*) and a cardiomyopathic ventricle (*B* and *D*). Preparations are shown in relaxing solution ($[Ca^{2+}] = 10^{-9}$ M, *A* and *B*) and maximally activating solution ($[Ca^{2+}] = 10^{-4.5}$ M, *C* and *D*), and demonstrate preserved striation resolution and minimal shortening of central sarcomeres with maximal activation. Scale bars equal 50 μm.

and likely reflects the myocyte hypertrophy previously described in dilated cardiomyopathies (14). However, passive tensions (normalized to cross-sectional area) were similar in the failing preparations and the normal donor preparations. Likewise, there was no difference in the maximal tension-generating capability of the failing and normal preparations. Minimal shortening of central sarcomeres was observed during maximal calcium activation (mean of 0.04 μm/sarcomere for all preparations, or ~ 2%), demonstrating that the attachment of the preparation to the experimental apparatus had low compliance and that tension–calcium relations were determined under essentially isometric conditions. Photomicrographs of representative failing and normal preparations in both relaxing ($[Ca^{2+}] = 10^{-9}$ M) and maximally activating solutions ($[Ca^{2+}] = 10^{-4.5}$ M) show preserved sarcomere pattern resolution and uniformity, and minimal internal shortening after maximal activation (Fig. 1).

Mean isometric tension–calcium relations for the 16 cardiomyopathic preparations and 10 preparations from normal donor ventricles are provided in Fig. 2, and demonstrate a significant increase in myofibrillar calcium sensitivity of isometric tension in dilated cardiomyopathies. The calcium concentration yielding half-maximal tension ($[Ca^{2+}]_{50}$) was significantly less in the failing myocardium (2.46±0.49 μM) than in normal preparations (3.24±0.51 μM, $P < 0.001$, Table III). This difference in calcium sensitivity of isometric tension was also significant when the mean $[Ca^{2+}]_{50}$ for each patient was compared (2.50±0.42 μM, $n = 6$ for donors, 3.18±0.48 μM, $n = 9$ for heart failure patients, $P = 0.012$ by *t* test). As shown in Table III, calcium sensitivity of isometric tension was greatest in myofibrillar preparations from patients with idiopathic dilated cardiomyopathy ($[Ca^{2+}]_{50}$ = 2.11±0.52 μM, $n = 7$), although preparations from the ischemic dilated cardiomyopathy group also demonstrated significantly greater calcium sensitivity of isometric tension (2.78±0.18 μM) than normal myocardium ($P < 0.001$). There was no significant difference in the Hill coefficients of the individual isometric tension–calcium relations (Table III), suggesting that the cooperativity of isometric tension development was similar in all three groups.

Role of protein kinase A (PKA)–mediated myofilament phosphorylation. β-adrenergic stimulation has been shown to decrease myofibrillar calcium sensitivity of tension in normal myocardium, an effect mediated by protein kinase A–dependent phosphorylation of myofilament proteins (troponin I and/

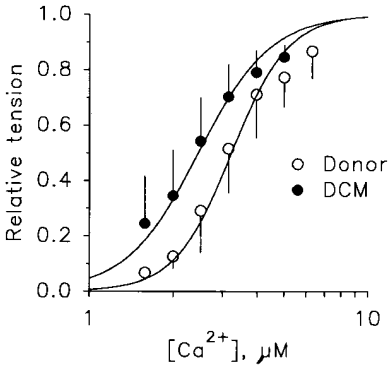

Figure 2. Mean tension–calcium relations for the donor ($n = 10$) and dilated cardiomyopathic myocyte-sized myofibrillar preparations (DCM, $n = 16$). Tensions are normalized to the maximal Ca^{2+}-activated tension for each preparation. The calcium concentration producing half-maximal tension ($[Ca^{2+}]_{50}$) was less in cardiomyopathic preparations (2.46±0.49 μM) than in donor preparations (3.24± 0.51 μM, $P < 0.001$), demonstrating an increase in myofibrillar calcium sensitivity of isometric tension with heart failure.

or C-protein) (10, 15, 16). Because the density and activity of the β-adrenergic receptor/adenylate cyclase complex is reduced in human dilated cardiomyopathies (17–19), it is plausible that reductions in PKA-dependent phosphorylation of myofilament proteins account for the observed increase in calcium sensitivity of tension. To test this hypothesis, a subset of permeabilized myocyte preparations were exposed in vitro to the catalytic subunit of protein kinase A. Isometric tension–calcium relations were determined before and immediately after PKA treatment, and the subsequent decrease in calcium sensitivity of isometric tension was interpreted as a physiologic measure of basal PKA-dependent phosphorylation (i.e., a greater decrease in calcium sensitivity of tension after PKA treatment implies reduced basal phosphorylation, while a smaller decrease in calcium sensitivity of tension after PKA treatment suggests greater basal phosphorylation) (8). Calcium sensitivity of isometric tension was significantly reduced after PKA treatment both in normal donor preparations and cardiomyopathic preparations (Table IV, Fig. 3). However, the shift in $[Ca^{2+}]_{50}$ after PKA exposure was significantly greater in the cardiomyopathic preparations (1.70±0.78 vs 0.52±0.61 μM for the control preparations, $P = 0.005$). Further, tension–pCa relations did not differ significantly between the two groups after treatment with PKA. These data suggest that basal β-adrenergically mediated (PKA-dependent) phosphorylation of myofibrillar proteins is reduced in dilated cardiomyopathies, and that this difference in phosphorylation state may at least in part explain the observed differences in calcium sensitivity of tension.

Because of the apparent role of β-adrenergically mediated phosphorylation of myofibrillar proteins in determining calcium sensitivity of isometric tension in human heart failure, we also examined the effects of intravenous inotropic therapy (synthetic catecholamines and/or phosphodiesterase inhibitors) on our results. Not surprisingly, calcium sensitivity of isometric tension was greater in the cardiomyopathic patients who were not receiving intravenous inotropes before heart replacement ($[Ca^{2+}]_{50}$ = 2.31±0.55 μM, $n = 10$ preparations) than in those patients who received intravenous inotropes (2.72±0.24 μM, $n = 6$ preparations, $P < 0.001$). In both groups, calcium sensitivity of isometric tension was significantly greater than that observed in the donor preparations. No significant effect of inotropic therapy on calcium sensitivity of tension was seen in the donor preparations, although there was a trend towards increased calcium sensitivity of isometric tension in preparations from hearts not exposed to dopamine before organ procurement.

Table III. Calcium Sensitivity of Tension and Hill Coefficients of Individual Tension [Ca^{2+}] Relations

	$[Ca^{2+}]_{50}$	n_H	r^2
	μm		
Donors ($n = 10$ preparations)	3.24±0.51	4.29±1.26	0.97±0.02
DCM ($n = 16$ preparations)	2.46±0.49*	3.36±0.99	0.97±0.02
Idiopathic DCM ($n = 7$ preparations)	2.11±0.52*	2.87±0.87	0.98±0.03
Ischemic DCM ($n = 7$ preparations)	2.78±0.18*‡	3.32±0.46	0.98±0.01

$[Ca^{2+}]_{50}$ indicates $[Ca^{2+}]$ at which tension is half maximal; n_H, Hill coefficient; r^2, squared correlation coefficient from Hill transformation; and DCM, dilated cardiomyopathy. Values are mean±SD. *$P < 0.001$ vs donors (multivariate linear regression); ‡$P < 0.001$ vs idiopathic DCM (multivariate linear regression).

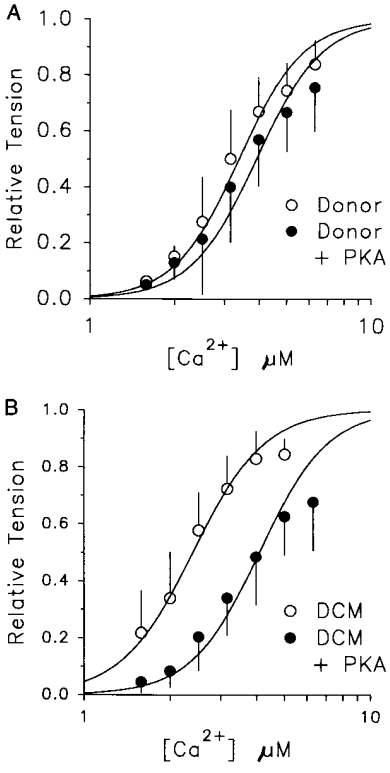

Figure 3. The effect in vitro treatment of myocyte-sized myofibrillar preparations ($n = 7$) from donor ventricles with the catalytic subunit of protein kinase A (*PKA*) is shown above. Myofibrillar phosphorylation with PKA resulted in a significant decrease in the calcium sensitivity of isometric tension ($P < 0.001$). PKA treatment also reduced calcium sensitivity of isometric tension in preparations from dilated cardiomyopathic ventricles as shown below (DCM, $n = 12$ preparations, $P < 0.001$). However, in vitro phosphorylation of myofibrillar proteins with PKA resulted in a greater decrease in calcium sensitivity of tension in DCM relative to donor preparations, and there was no significant difference in tension–calcium relations in the two groups after PKA exposure. These data suggest that the basal level of PKA-dependent phosphorylation of myofibrillar proteins is reduced in human dilated cardiomyopathic ventricles, and that this difference in PKA-dependent phosphorylation may account for the baseline differences in calcium sensitivity of isometric tension.

Table IV. Decrease in Calcium Sensitivity of Isometric Tension after Treatment with the Catalytic Subunit of Protein Kinase A

	P_{active}	$[Ca^{2+}]_{50}$	n_H	r^2	$\Delta[Ca^{2+}]_{50}$
	mN/mm²	*μM*			*μM*
Donors					
(*n* = 7 preparations)					
Baseline	27.9±9.2	3.39±0.51	3.79±0.99	0.97±0.02	
+ PKA	28.0±10.9	3.98±1.09*	3.72±0.67	0.97±0.02	0.59±0.74
DCM					
(*n* = 12 preparations)					
Baseline	24.3±9.1	2.40±0.48	3.47±1.15	0.97±0.02	
+ PKA	25.2±6.2	4.10±0.82*	3.65±0.95	0.98±0.03	1.70±0.78‡

P_{active} indicates maximal Ca^{2+}-activated tension; $[Ca^{2+}]_{50}$, $[Ca^{2+}]$ at which tension is half maximal; n_H, Hill coefficient; r^2, squared correlation coefficient from Hill transformation; and $\Delta[Ca^{2+}]_{50}$ is the change in $[Ca^{2+}]_{50}$ after treatment with PKA. Values are mean±SD. *$P < 0.001$ vs baseline (multivariate linear regression). ‡$P < 0.01$ vs donors (*t* test).

Protein electrophoresis and Western blotting. Typical silver-stained 12% polyacrylamide gels of electrophoretically separated myofibrillar proteins are shown in Fig. 4. Previous studies have reported reductions in myofibrillar content of the thick filament proteins myosin light chain-1 (MLC-1, or essential light chain) (20) and myosin light chain-2 (MLC-2, or regulatory light chain) (21) in diseased human myocardium; either abnormality could potentially alter calcium sensitivity of tension. However, we observed no difference in MLC-1 content (normalized to α-actinin content, to correct for small variations in the total protein load of each sample) in failing myocardium (0.81±0.15, $n = 8$) compared to the normal donor hearts (1.03±0.22, $n = 4$). Likewise, MLC-2 content, also normalized to α-actinin content, was similar in the two groups (0.44±0.13, DCM, $n = 8$ vs 0.56±0.07, donor, $n = 4$). As has been previously reported (22), C-protein content (a thick filament accessory protein) was similar in failing and normal myocardium.

We did not detect a difference in troponin C, I, or T content between cardiomyopathic and donor ventricles, although the low staining intensity of these thin filament regulatory proteins was likely outside the linear range for silver staining in some samples. Examination of troponin T isoform distribution using Western blots did reveal expression of a lower molecular weight isoform in most (7/8) of the failing hearts (Fig. 5). This isoform, which appears to be identical to the fetal isoform TnT_4 previously identified in failing human myocardium (23, 24), represented 12.2±7.7% of total troponin T in failing preparations. The fetal isoform was not observed in normal hearts ($n = 5$). There was no difference in the fraction of the fetal isoform of troponin T between the ischemic and idiopathic groups. Further, we observed no correlation between the fraction of fetal troponin T and myofibrillar calcium sensitivity of isometric tension ($r^2 = 0.08$). A preliminary study from our laboratory, using these same specimens, reported no expression of fetal isoforms of troponin T in failing and normal human myocardium (25). Further refinements of our electrophoretic technique (specifically, increasing the polyacrylamide concentration of the gels and the running time of the electrophoretic separation, and employing a sensitive chemiluminescence system to visualize immune complexes on the Western blots) facilitated detection of low levels of the lower molecular weight isoform of TnT.

Discussion

Myofibrillar calcium sensitivity of isometric tension. The present study demonstrates an increase in myofibrillar calcium sensitivity of tension in failing human myocardium, likely due at least in part to a reduction in β-adrenergically mediated phosphorylation of thin filament (troponin I) and/or thick filament (C-protein) regulatory proteins. Previous studies of calcium sensitivity of tension in human heart failure, using permeabilized multicellular cardiac preparations, have yielded conflicting results. Schwinger et al. (6) reported increased calcium sensitivity of tension in permeabilized papillary muscle strips obtained from cardiomyopathic human hearts. Using a similar preparation, D'Agnolo et al. (5) observed no difference in myofibrillar calcium sensitivity of tension between failing and normal human myocardium. Gwathmey and Hajjar also observed no difference in myofibrillar calcium sensitivity of tension between cardiomyopathic and normal myocardium, in studies employing both permeabilized and intact tetanized ventricular trabeculae (3, 4). In agreement with these previous studies (3–6), we found no difference in either passive or maximally calcium-activated tensions between failing and normal preparations.

The methods employed in the present study offer several methodologic advantages over the previous work. First, it was not certain in earlier studies that myofibrillar tension–calcium relations were determined under truly isometric conditions since sarcomere lengths were not monitored. Shortening of central sarcomeres during calcium activation, as a consequence of end compliance associated with attachment of myocardium to the experimental apparatus, is common in studies employing multicellular cardiac preparations (26, 27). This is a critical consideration since sarcomere length is a major determinant of both maximal tension and the calcium sensitivity of tension in cardiac muscle (26). We carefully monitored sarcomere lengths both in passive preparations and after maximal activation; preparations demonstrating significant compliance were rejected and tension relations were determined under essentially isometric conditions (mean of ~ 2% shortening of central sarcomeres during maximal activation). Ischemia, incurred during heart procurement and transportation from the operating room to the research laboratory, is another factor potentially affecting previous results. In addition, activation of

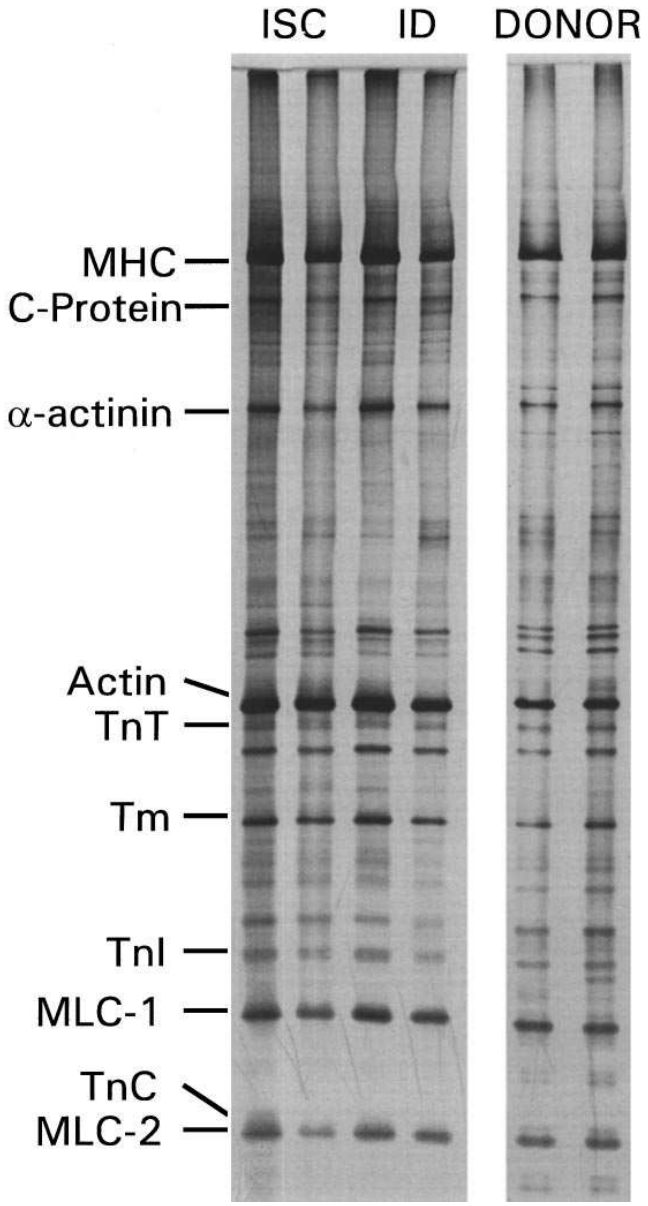

Figure 4. Representative silver stained SDS-12% polyacrylamide gels of electrophoretically separated myofibrillar proteins obtained from ventricular biopsies from patients with ischemic (*ISC*) and idiopathic (*ID*) dilated cardiomyopathies, and from donor ventricles used for cardiac transplantation. Densitometric analysis of the cardiomyopathic preparations ($n = 8$) and donor preparations ($n = 4$) demonstrated no difference in the content of thick filament regulatory proteins myosin light chain-1 (*MLC-1*), myosin light chain-2 (*MLC-2*), or C-protein. Other protein bands identified include myosin heavy chain (*MHC*), α-actinin, tropomyosin (*Tm*), and troponins T (*TnT*), I (*TnI*), and C (*TnC*).

phosphatases and kinases during organ procurement and tissue transportation, by altering the phosphorylation state of myofibrillar regulatory proteins, may also have contributed to the differing conclusions regarding calcium sensitivity. By rapidly freezing ventricular biopsies, storing at −70°C until the time of study, and then immediately disrupting the thawed myocardium in a preparation solution containing both protease and phosphatase inhibitors, we minimized ischemic injury to the permeabilized preparations and preserved the in situ phosphorylation status of myofilament regulatory proteins.

Altered β-adrenergically mediated myofibrillar protein phosphorylation. Our observation that calcium sensitivity of tension in failing preparations decreases to a greater extent after in vitro treatment with the catalytic subunit of protein kinase A compared to those from normal donor hearts, suggests a reduced basal level of PKA-dependent phosphorylation of myofilament regulatory proteins with heart failure. This result, and the observation that the calcium sensitivity of tension did not differ between the two groups after exposure to PKA, also suggests that altered PKA-dependent myofibrillar protein phosphorylation can at least in part explain the increased myofibrillar calcium sensitivity of tension observed in failing myocardium. Recent studies suggest that troponin I phosphorylation, rather than phosphorylation of PKA-dependent sites on C-protein, regulates myofibrillar tension at submaximally activating levels of calcium (16).

Altered PKA-dependent phosphorylation of troponin I in heart failure may be a consequence of alterations in the β-adrenergic receptor/adenylate cyclase complex. The difference in myofibrillar calcium sensitivity of tension observed in preparations from patients with idiopathic and ischemic cardiomyopathies is intriguing, and perhaps relates to differences in how the β-adrenergic receptor/adenylate cyclase complex is altered in the two conditions. Idiopathic dilated cardiomyopathy, in which we observed a greater myofibrillar calcium sensitivity of tension, is characterized by a large decrease in β_1-adrenergic receptor density, mild uncoupling of β_2-receptors from adenylate cyclase, and an increase in the activity of the inhibitory guanine nucleotide–binding protein G_i (19). In contrast, less downregulation of the β_1 receptor is seen in ventricles from patients with ischemic dilated cardiomyopathy, while adenylate cyclase activity is apparently less well coupled to β-adrenergic stimulation (19).

Alternative explanations for the increased calcium sensitivity of tension must be considered, because the maximal phosphorylation of PKA-dependent sites on myofilament regulatory proteins may minimize the effects of other biochemical alterations of myofibrils accompanying heart failure. We did not observe previously reported reductions in the content of myosin light chain-1 (20) or -2 (21) in ventricular biopsies from patients with dilated cardiomyopathies. We did observe expression of a fetal isoform of the thin filament regulatory protein troponin T in most failing ventricles, consistent with the previous reports by Anderson et al. (23, 24). The expression of this lower molecular weight isoform (TnT_4, originally referred to by Anderson et al. as TnT_2) in failing human left ventricles was correlated with reduced peak myofibrillar ATPase activity (23). Its effects on calcium sensitivity of tension are uncertain. Increased expression of a higher molecular weight fetal isoform of troponin T (TnT_2) has been correlated with increased calcium sensitivity of isometric tension in neonatal rabbit hearts (28). We did not observe expression of TnT_2, and also observed no correlation between TnT_4 expression and calcium sensitivity of tension in failing ventricles. In contrast to our observations, Solaro et al. found protein expression of TnT_4 in only 1 of 10 failing ventricles (28). Likewise, Mesnard et al. did not observe mRNA for this or other fetal isoforms of TnT in failing myocardium using RNase protection assays (29). The reasons for these conflicting observations regarding troponin T isoform distribution in failing human myocardium are not

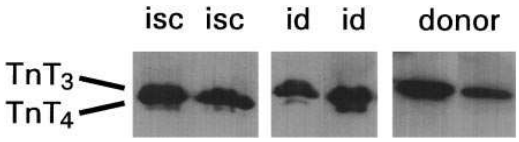

Figure 5. Western blots using a monoclonal anti–rabbit skeletal troponin T antibody (JLT-12) are shown above. Myofibrillar proteins from ventricular biopsies from patients with ischemic (*isc*) and idiopathic (*id*) dilated cardiomyopathies, and from donor ventricles used for cardiac transplantation, were first separated using SDS-16% polyacrylamide gel electrophoresis. Expression of a lower molecular weight isoform (TnT_4) of troponin T was evident in most (7/8) failing preparations, as is demonstrated in these examples. The adult isoform (TnT_3) of troponin T is also identified. In contrast, the fetal isoform of troponin T (TnT_4) was not detected in donor hearts (0/5).

clear. Alterations in the isoform distribution of the other subunits of the troponin complex are not likely in dilated cardiomyopathies. Troponin I isoform expression is developmentally regulated in the human ventricle; however the fetal isoform of this protein has not been observed in dilated cardiomyopathy (30). There is a unique cardiac isoform of troponin C (31).

Effects of protein kinases other than PKA could also affect myofibrillar calcium sensitivity of tension in human dilated cardiomyopathies. Both troponin T and I can be phosphorylated by protein kinase C (the latter at sites distinct from those phosphorylated by PKA) (32, 33). Myosin light chain 2 is phosphorylated by a specific Ca^{2+}-calmodulin–dependent protein kinase (34), and also by protein kinase C (35). Activation of protein kinase C by phorbol esters has been reported to decrease maximal calcium-activated tension and the calcium sensitivity of tension in failing and normal human myocardium (4), whereas phosphorylation of MLC-2 by myosin light chain kinase results in an increase in calcium sensitivity of tension in permeabilized skeletal muscle (36). The activity of these kinases in human heart failure has not been determined.

Physiologic implications. Given that maximal calcium-activated tension was unchanged and calcium sensitivity of tension was increased in myofibrillar preparations from failing ventricles, our results support the concept that alterations in excitation–contraction coupling contribute to the systolic contractile failure in human dilated cardiomyopathies. Unfortunately, studies directly examining intracellular calcium transients in failing and normal human myocardium have not yielded consistent results. While prolongation of the systolic calcium transient has been uniformly observed in failing myocardium, the peak of this transient has been reported to be reduced (in enzymatically dissociated, mechanically unloaded myocytes, using the fluorescent calcium indicator fura-2, reference 37) or similar (in isometrically contracting trabeculae loaded with aqueorin, reference 38) to those observed in normal human myocardium. This discrepancy may be a result of the different fluorescent indicators used, differences relating to unloaded vs isometric contractions (39), temperature and stimulation frequency (40), or other unknown factors.

It should also be emphasized that the present study examined only steady state, isometric myofibrillar mechanical function, and alterations of the dynamic mechanical function of myofibrils could contribute to systolic dysfunction in human heart failure. The rate of formation of tension-generating cross-bridges may be sufficiently slow in myocardium to limit the extent of tension development during isometric (isovolumic) contraction (41), and reductions in shortening velocities under physiologic loading conditions could directly decrease myofibrillar power output. Indeed, reduced unloaded velocity of shortening has been reported in permeabilized trabeculae from failing human ventricles (42), although this study was likely limited by some of the same methodological considerations discussed above regarding isometric myofibrillar function in human heart failure (43).

Finally, increased myofibrillar calcium sensitivity of tension may have direct deleterious effects on diastolic function in heart failure. Zhang et al. (44) recently observed that treatment of permeabilized cardiac trabeculae with protein kinase A increased the rate of tension decay induced by pulsed UV photolysis of diazo-2, a "caged" calcium chelator. To the extent that myofibrillar processes limit active relaxation (45), reduced PKA-dependent phosphorylation may contribute to the prolongation of twitch duration and reductions in relaxation rates previously described in failing human myocardium (37, 38).

Potential limitations. Because cardiomyopathic preparations were obtained from older and predominately male patients, while donor hearts were obtained from younger and predominately female patients, we cannot exclude an effect of age or sex on our results. In fact, a relationship between age and cross-bridge force-time integral has been previously demonstrated in studies of normal human myocardium (46). In our study, we did not observe a significant correlation between age and calcium sensitivity of isometric tension (using age as a covariate, with the presence or absence of heart failure, in the multiple regression analysis of linearized tension–calcium relations).

Donor preparations were obtained from endomyocardial biopsies obtained from hearts cardiopleged with University of Wisconsin solution, while failing preparations were obtained from transmural biopsies obtained from the beating heart just before cardiectomy. We cannot exclude a transmural difference in calcium sensitivity of isometric tension, or an effect of cardioplegic agent on myofibrillar protein phosphorylation. However, we have not observed transmural differences or an effect of University of Wisconsin solution on myofibrillar calcium sensitivity of isometric tension in porcine myocardium (data not shown).

Since brain death may be associated with catecholamine release, the significance of our findings to failing and nonfailing human myocardium under physiologic conditions may be questioned. This limitation, like the others cited above, is inherent in studies of failing human myocardium and is difficult to address experimentally. However, the alterations in myofibrillar calcium sensitivity of isometric tension and PKA-dependent myofibrillar protein phosphorylation reported here are qualitatively similar to those we have previously observed in a canine model of dilated cardiomyopathy produced by chronic rapid pacing (8). In this study, an increase in myofibrillar calcium sensitivity of isometric tension was observed with heart failure even when control dogs were pretreated with high dose β-adrenergic blockade to minimize the effects of surgery-related catecholamine release. In the present study, we found increased calcium sensitivity of isometric tension in preparations from patients who received chronic β-adrenergic agonists before transplantation, relative to preparations from brain-dead donors. These later observations support our hypothesis that alterations in isometric myofibrillar mechanical function are a consequence of heart failure per se.

The use of myocyte-sized myofibrillar preparations obtained from rapidly frozen ventricular biopsies provided several advantages over previous studies of failing human myocardium, as discussed above. However, this preparation has several potential limitations. First, maximal calcium-activated tensions are both less and more variable than those reported in some studies using permeabilized cardiac trabeculae (21, 39, 45). However, the mean maximal tensions and corresponding standard deviations observed in the present study are comparable to those previously reported for mechanically disrupted and permeabilized rat (47), porcine (48) and canine (8) myocardium and for enzymatically digested and detergent-permeabilized rat cardiac myocytes (10). Our values also compare favorably to maximal tensions previously reported in human myocardium (3–6, 25, 38). Second, myofibrillar calcium sensitivity of tension may be altered by permeabilization of the sarcolemma (49, 50). However, control and myopathic tissues were obtained and treated in a nearly identical manner in the present study, and a differential effect of the preparation process is unlikely. Finally, myofibrillar mechanical function was examined at a single sarcomere length, under steady state, isometric conditions. Alterations in the length dependence of tension has recently been described in human heart failure (6), and myofibrils shorten considerably under physiologic loads in situ.

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