Protein Kinase A (PKA)-Dependent Troponin-I Phosphorylation and PKA Regulatory Subunits Are Decreased in Human Dilated Cardiomyopathy

Daniel R. Zakhary, BS; Christine S. Moravec, PhD; Robert W. Stewart, MD; Meredith Bond, PhD

**Background**—Most studies indicate that failing human hearts have greater baseline myofibrillar Ca\(^{2+}\) sensitivity of tension development than nonfailing hearts. Phosphorylation of cardiac troponin I (TnI) by cAMP-dependent protein kinase (PKA) decreases the affinity of the troponin complex for Ca\(^{2+}\), thus altering the Ca\(^{2+}\) sensitivity of force production. We tested the hypothesis that PKA-dependent TnI phosphorylation is altered in the failing human heart and investigated changes in PKA regulatory subunits as a potential mechanism.

**Methods and Results**—Using in vitro back-phosphorylation with \(\gamma\text{-}^{32}\text{P}\)ATP, we demonstrated a significant (\(P<0.05\)) ≈25% reduction in baseline PKA-dependent TnI phosphorylation in human hearts with dilated cardiomyopathy (DCM) compared with nonfailing (NF) human hearts. There was no significant difference in cAMP content or maximal PKA activity between DCM and NF hearts, but expression of the regulatory subunits of PKA-I (RI) and PKA-II (RII) was significantly decreased in DCM versus NF hearts (RI by ≈40%, \(P<0.05\); RII by ≈30%, \(P<0.01\)).

**Conclusions**—PKA activity is regulated at the substrate level through interactions of PKA regulatory subunits with A-kinase anchoring proteins. The reduced baseline PKA-dependent phosphorylation of TnI in DCM may be due to decreased expression of RI and RII and consequently reduced anchoring of PKA holoenzyme. These findings provide new evidence of deficiencies in downstream regulation of the β-adrenergic pathway in the failing human heart and may account for increased baseline myofibrillar Ca\(^{2+}\) sensitivity.

**Key Words:** cardiomyopathy • troponin • enzymes • proteins • receptors, adrenergic, beta

It has been estimated that the prevalence of congestive heart failure in the United States is as high as 3 million individuals, with 700 000 new cases diagnosed annually.\(^1\) Approximately half of these congestive heart failure patients are diagnosed with nonischemic dilated cardiomyopathy (DCM), yet the underlying causes are unknown. Several reports indicate that tension development by cardiac myofibrils is more sensitive to calcium in failing relative to nonfailing (NF) human hearts.\(^2,3\) In addition, because protein kinase A (PKA)-dependent phosphorylation of cardiac troponin I (TnI) controls myofibrillar sensitivity to calcium,\(^4\) TnI phosphorylation may be altered in failing hearts.\(^5,5,6\)

Although it is generally accepted that β-adrenergic receptor density is reduced in the failing human myocardium,\(^7\) evidence for the existence of defects distal to the receptor/adenylyl cyclase complex is rapidly accumulating. For example, even after stimulation by positive inotropic agents that act beyond the receptor, such as dibutyryl-cAMP and cAMP-phosphodiesterase inhibitors, the decreased positive inotropic response in failing hearts is still observed.\(^8\) PKA activity is regulated at the substrate level by PKA binding to A-kinase anchoring proteins (AKAPs) through interactions with the regulatory subunit (R).\(^9\) Two classes of the R subunit exist, RI and RII, which form type I and type II PKA, respectively. PKA-I is primarily cytosolic, whereas up to 75% of PKA-II is compartmentalized by binding to AKAPs.\(^9\) RI may also bind to AKAPs.\(^10\) Recently, this laboratory has demonstrated that AKAPI00 and RII colocalize in adult cardiac myocytes.\(^11\) The potential for local regulation of PKA activity implies that changes in PKA substrate phosphorylation are not necessarily due to changes in receptor-effector coupling. The purpose of the present study was to investigate whether PKA-dependent TnI phosphorylation is decreased in human DCM and to test the hypothesis that alterations in RI or RII may contribute to the differences observed.

**Methods**

**Human Myocardial Tissue**

Failing human cardiac muscle was obtained from explanted hearts of cardiac transplant recipients at the Cleveland Clinic Foundation. NF hearts were obtained from explanted hearts of cardiac transplant recipients at the Cleveland Clinic Foundation who had died of noncardiac causes.
human heart tissue was obtained from unmatched organ donors through Life Banc of Northeast Ohio. All failing hearts had left ventricular ejection fractions <25% (15±2%), and all NF hearts had ejection fractions >60% (65±3%) (Table 1). Mean age was 48±4 years for patients with DCM and 42±8 years for NF patients. Effects of age or sex (DCM, 4 men and 4 women; NF, 5 men and 2 women) were therefore excluded. Seven of 8 patients in heart failure had been treated with digoxin and 6 of 8 with amiodarone. All NF patients were on inotropic support (dopamine or norepinephrine). All hearts were arrested and transported in ice-cold, oxygenated cardioplegic solution (in mmol/L): NaCl 147.16, MgCl2 16.00, KCl 20.0, NaHCO3 10.0, CaCl2 2.25, pH 7.4. Once in the laboratory, tissue was flash-frozen in liquid N2 and stored at −80°C. This protocol was approved by the Cleveland Clinic Institutional Review Board.

Preparation of Cardiac Myofibrils

Samples of cardiac left ventricular free wall (~5 g) from patients with end-stage (New York Heart Association class IV) idiopathic DCM and from NF donor hearts were homogenized in 5 vol of MOPS buffer (10 mmol/L, pH 7.4, 4°C) containing sucrose (290 mmol/L), NaN3 (3 mmol/L), DTT (1 mmol/L), NaF (20 mmol/L), pepstatin A (1 μmol/L), leupeptin (1 μmol/L), and PMSF (0.8 mmol/L) by use of three 20-second bursts and 40-second cooling intervals, position 6, with a Polytron homogenizer. This yielded total heart homogenate (THH). An equivalent volume of extraction buffer, 50 mmol/L Tris (pH 7.5) containing Triton X-100 (0.1%), NaF (20 mmol/L), DTT (0.05 mmol/L), MgCl2 (0.5 mmol/L), EDTA (0.125 mmol/L), antipain (5 μg/mL), leupeptin (10 μg/mL), pepstatin A (5 μg/mL), and PMSF (43 μg/mL), was added to the THH, and the mixture was centrifuged at 10 000g for 5 minutes at 4°C. The detergent-solubilized supernatant was set aside, and the pellet was resuspended in an equivalent volume of extraction buffer and washed twice again. The resultant myofibrillar fraction was resuspended in Ca2+-free extraction buffer and stored at −20°C.

Determination of TnI Concentration by ELISA

Equal concentrations of myofibrillar proteins were denatured in 8 mol/L urea, then coated onto 96-well microplates (50 μL/well in quadruplicate) in coating buffer (45.3 mmol/L NaHCO3, 18.2 mmol/L Na2CO3, pH 9.6) overnight at 4°C. The plates were blocked with PBS (pH 7.2) containing 3% BSA and 0.05% Tween 20 for 1 hour at 37°C. Bound TnI was detected with an anti-cardiac TnI monoclonal antibody (1:1000) (gift of S. Schiaffino, University of Padua, Padua, Italy) for 2 hours at 37°C, followed by incubation with goat anti-mouse IgG alkaline phosphatase (AP)-linked secondary antibody (1:5000) for 1 hour at 37°C. Absorbance was monitored with p-nitrophenyl phosphate (1 mg/mL in 11.8% diethanolamine, pH 9.6) at 405 nm. A standard curve of OD405 versus concentration of purified recombinant human cardiac TnI (gift of I. Trayer, University of Birmingham, Birmingham, UK) was generated to allow determination of absolute Tnl concentrations. Tnl protein was not different between DCM and NF hearts (Table 2).

Back-Phosphorylation

Fractions enriched in myofibrils from failing and NF hearts were back-phosphorylated according to the method of Karczewski et al.12

**TABLE 1. Clinical Characteristics of Patients in Study**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>LVEF, %</th>
<th>Age, y</th>
<th>Sex</th>
<th>Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DCM</td>
<td>15–20</td>
<td>62</td>
<td>F</td>
<td>DIG, DOBU, ENAL</td>
</tr>
<tr>
<td>2</td>
<td>DCM</td>
<td>15</td>
<td>61</td>
<td>M</td>
<td>DIG, CAP, AMI</td>
</tr>
<tr>
<td>3</td>
<td>DCM</td>
<td>11</td>
<td>45</td>
<td>F</td>
<td>DIG, CAP, AMI</td>
</tr>
<tr>
<td>4</td>
<td>DCM</td>
<td>10</td>
<td>30</td>
<td>M</td>
<td>DIG, DOBU, ENAL</td>
</tr>
<tr>
<td>5</td>
<td>DCM</td>
<td>10–15</td>
<td>49</td>
<td>F</td>
<td>DIG, AMI</td>
</tr>
<tr>
<td>6</td>
<td>DCM</td>
<td>&lt;20</td>
<td>41</td>
<td>M</td>
<td>DIG, DOBU, AMI</td>
</tr>
<tr>
<td>7</td>
<td>DCM</td>
<td>20–25</td>
<td>55</td>
<td>M</td>
<td>DOBU, THYR, CAP, AMI</td>
</tr>
<tr>
<td>8</td>
<td>DCM</td>
<td>13</td>
<td>40</td>
<td>F</td>
<td>DIG, DOBU, CAP, AMI</td>
</tr>
<tr>
<td>9</td>
<td>NF</td>
<td>67</td>
<td>26</td>
<td>M</td>
<td>DOPA</td>
</tr>
<tr>
<td>10</td>
<td>NF</td>
<td>N/A</td>
<td>59</td>
<td>M</td>
<td>DOPA, MET*</td>
</tr>
<tr>
<td>11</td>
<td>NF</td>
<td>60</td>
<td>16</td>
<td>F</td>
<td>NE, THYR</td>
</tr>
<tr>
<td>12</td>
<td>NF</td>
<td>70</td>
<td>60</td>
<td>F</td>
<td>DOPA, LAB, NIMO</td>
</tr>
<tr>
<td>13</td>
<td>NF</td>
<td>65</td>
<td>20</td>
<td>M</td>
<td>NE, THYR, VASO</td>
</tr>
<tr>
<td>14</td>
<td>NF</td>
<td>55</td>
<td>64</td>
<td>M</td>
<td>DOPA, DOBU, THYR, VASO</td>
</tr>
<tr>
<td>15</td>
<td>NF</td>
<td>72</td>
<td>48</td>
<td>M</td>
<td>DOPA, VASO</td>
</tr>
</tbody>
</table>

DIG indicates digoxin; DOBU, dobutamine; ENAL, enalapril; CAP, captopril; AMI, amiodarone; THYR, l-thyroxine; DOPA, dopamine; MET, metoprolol; NE, norepinephrine; LAB, labetalol; NIMO, nimodipine; and VASO, vasopressin.

*Indicates chronic medications for donors; other medications given to donors in emergency room or intensive care unit.

**TABLE 2. Results**

<table>
<thead>
<tr>
<th></th>
<th>NF Hearts</th>
<th>DCM Hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnl protein levels, μg Tnl/mg total protein</td>
<td>1.99±0.17 (n=7)</td>
<td>2.02±0.19 (n=6)</td>
</tr>
<tr>
<td>Maximal Tnl phosphorylation, mol P/mol Tnl</td>
<td>1.7±0.2 (n=6)</td>
<td>1.8±0.4 (n=7)</td>
</tr>
<tr>
<td>Baseline PLB phosphorylation, normalized score</td>
<td>1.0±0.1 (n=6)</td>
<td>1.1±0.1 (n=7)</td>
</tr>
<tr>
<td>cAMP levels, pmol cAMP/mg protein</td>
<td>26.4±3.1 (n=5)</td>
<td>21.8±4.1 (n=9)</td>
</tr>
<tr>
<td>PKA activity, pmol · mg−1 · min−1</td>
<td>4543±313 (n=6)</td>
<td>3945±430 (n=8)</td>
</tr>
</tbody>
</table>
Briefly, proteins were incubated in 50 mmol/L MOPS (pH 7.0) containing 20 mmol/L NaF, 1 mmol/L DTT, 10 mmol/mL MgCl₂, [γ-32P]ATP (200 μCi/reaction, reaction volume 40 μL), and the catalytic subunit of cAMP-dependent protein kinase (PKAcat, 250 U/mL) for 45 minutes at 30°C. To ensure that the reaction reached completion and that kinase activity was not depleted, the myofibrillar fraction was incubated for 45 minutes with excess PKAcat plus NaF to block endogenous phosphatases. Phosphorylation was terminated by boiling for 5 minutes in gel-loading buffer (50 mmol/L Tris-HCl, pH 6.8, 1% SDS, 1.5% DTT, 5% glycerol, 0.05% bromophenol blue). Control samples were incubated without PKAcat or with PKAcat plus protein kinase inhibitor PKI1-26. Proteins were then separated by 12.5% SDS-PAGE. Gels were stained with Coomassie blue, destained, dried at 80°C, exposed to storage phosphor screens, and developed overnight. Protein loading was normalized to actin, as quantified by scanning densitometry. The radioactivity corresponding to the bands was quantified by volume analysis of background-corrected pixel intensities by use of a StormImager in phosphor image software. The measured phosphorylation increased linearly with increasing protein concentration (0.5 to 5.0 mg/mL). To calculate phosphorylation stoichiometry, [γ-32P]ATP standards of known specific activity (from 4.2×10⁶ to 4.2×10⁷ μCi/μL) prepared from the same aliquot as the phosphorylation assay were spotted onto nitrocellulose and developed with the gels. This generated a standard curve from which specific activity and moles of 32P were calculated.

Maximal PKA-dependent phosphorylation was determined by pretreatment with AP to remove bound phosphate, then treatment with PKAcat and [γ-32P]ATP to rephosphorylate only PKA sites. Briefly, samples were preincubated in 25 mmol/L Tris (pH 7.8), 25 mmol/mL KCl, 10 mmol/mL MgCl₂, 0.5 mmol/L DTT for 10 minutes at 30°C. Dephosphorylation was initiated by addition of AP (1:100 enzyme:protein) and allowed to proceed for 20 minutes at 35°C. In these experiments, NaF was omitted, then added after dephosphorylation to terminate the reaction. Maximal phosphate incorporation was achieved after 20 minutes of AP pretreatment. These conditions resulted in a statistically significant increase in 32P incorporation after rephosphorylation in the AP-treated compared with untreated myofibrils from every heart studied (P<0.001). 32P incorporation into proteins, as measured by back-phosphorylation, is inversely related to the extent of phosphorylation of the proteins in vivo. Thus, bands on the autoradiogram that were very intense in the AP-treated lanes but of low intensity in the untreated lanes represent proteins highly phosphorylated in vivo.

**cAMP Assay**

Supernatant fractions were prepared from total homogenates of DCM and NF hearts, as described above. Proteins were precipitated with 0.1% TCA and centrifuged for 30 minutes. The supernatants were extracted 4 times with 5 vol of water-saturated ether, then dried under vacuum. cAMP content was determined by use of a 125I-cAMP double-antibody radioimmunoassay kit. Acid precipitates from the samples were dissolved in NaOH, and protein content was determined by a bichinchoninic acid protein assay.

**PKA Activity**

Phosphorylation was monitored with a Gibco PKA assay system with kemptide as substrate. Supernatant fractions were incubated in 50 mmol/L Tris (pH 7.5) containing 10 mmol/L MgCl₂, 0.25 mg/mL BSA, [γ-32P]ATP (100 μCi/mL, 1 to 2×10⁶ dpm/nmol; 4 nmol/assay tube) with 50 μmol/L/kemptide at 30°C for 10 minutes. The reaction was quenched by spotting the sample mixture onto phosphocellulose discs followed by a 1% (vol/vol) phosphoric acid wash. 32P incorporation was measured by liquid scintillation counting. Total PKA activity (PKA-I and PKA-II) was measured with a maximally effective concentration of cAMP (10 μmol/L) in the reaction buffer. Samples were run in duplicate, counts were averaged, and protein concentration was determined. Activities were corrected for nonspecific 32P incorporation by subtracting the cpm of paired reactions containing PKI1-26 and 1 μmol/L [γ-32P]ATP. Background counts (without sample) represented <0.1% of total counts.

**Immunoblot Analysis**

Samples from DCM and NF human hearts were first adjusted for total protein. Protein loading was then normalized to actin and quantified by scanning densitometry of the Coomassie-stained gels. Proteins were denatured for 5 minutes at 95°C in 50 mmol/L Tris-HCl, pH 6.8, 1% SDS, 1.5% DTT, 5% glycerol, 0.05% bromophenol blue; separated by 10% SDS-PAGE at 125 V, 90 mA for 1 hour; then transferred to nitrocellulose membranes at 100 V, 320 mA, for 1 hour 45 minutes. The membranes were blocked in 3% fish gelatin (wt/vol) in Tris-buffered saline containing 0.1% Tween-20 (TBST). After two 10-minute washes in TBST, the membranes were incubated overnight with a monoclonal anti-RI antibody (Transduction Laboratories) or polyclonal anti-RII antibody (Santa Cruz Biotechnology). An AP-linked anti-mouse or anti-rabbit IgG was the secondary antibody, and the membranes were developed with Vistra ECF substrate. Fluorescence corresponding to the bands was quantified by StormImager analysis.

**Statistical Analysis**

Paired and unpaired t tests were used to evaluate the effect of treatments on individual samples and to compare differences between DCM and NF, respectively. Differences at P<0.05 were considered significant. Results are expressed as mean±SEM.

**Results**

TnI in each fraction from the myofibrillar extraction was identified as the phosphorylated protein running at the same position (28 kDa) as purified recombinant TnI (Figure 1). Phospholamban (PLB) was identified as the phosphorylated protein running at 8 kDa. Identity of both proteins was confirmed by Western blot analysis. Although TnI was present in the supernatant after detergent extraction, the suspension of the pellet after the third extraction (myofibrillar fraction) was enriched in TnI relative to other fractions. This fraction also contained PLB. The purpose of this extraction was not to obtain a pure myofibrillar preparation but rather to obtain a fraction enriched for TnI. This fraction was free of other phosphorylated proteins in the regions of TnI and PLB and thus provided the best signal-to-noise ratio for measurement.

**PKA-Dependent Phosphorylation**

To determine whether altered phosphorylation of TnI may contribute to the increased Ca²⁺ sensitivity observed in the failing human heart, we measured stoichiometry of PKA-
mediated TnI phosphorylation by back-phosphorylation. Other techniques, such as estimating protein phosphorylation levels based on band-shifting in SDS gels, are less quantitative and measure only total nonspecific phosphate incorporation. Back-phosphorylation has the significant advantage of allowing measurement of phosphorylation catalyzed specifically by PKA. In the presence of PKI(5–24), no phosphate incorporation could be detected, further substantiating the specificity of this method.

We first determined whether differences in maximal phosphorylation of TnI and PLB existed in DCM versus NF hearts. Figure 2 shows a representative autoradiogram of phosphorylated myofibrillar proteins from DCM and NF hearts before and after AP treatment. Pretreatment with AP resulted in a significant increase (P<0.001) in PKA\textsubscript{cat}-catalyzed $^{32}$P incorporation into all NF and DCM hearts studied. Left margin: molecular mass (kDa).

![Figure 2](image_url)

**Figure 2.** Autoradiogram showing effects of AP on TnI and PLB phosphorylation in a representative myofibrillar sample from DCM and NF hearts. Pretreatment with AP resulted in a significant increase ($P<0.001$) in PKA\textsubscript{cat}-catalyzed $^{32}$P incorporation into all NF and DCM hearts studied. Left margin: molecular mass (kDa).

protein levels of RI and RII in DCM and NF hearts by immunoblot analysis. Because it is not known which PKA isoform is primarily responsible for TnI phosphorylation in vivo, we measured protein levels of both RI and RII. RI showed a single major band at 48 kDa (Figure 4A), and RII showed a single major band at 55 kDa (Figure 5A). RI was significantly decreased (by $\approx40\%$; $P<0.001$) in DCM versus NF hearts (Figure 4B). RII was also significantly decreased (by $\approx30\%$; $P<0.05$) in DCM versus NF hearts (Figure 5B).

**Figure 3.** Phosphorylation of TnI by PKA. Data are expressed as percent of in vivo PKA-dependent TnI phosphorylation (maximal stoichiometry–back-phosphorylation/maximal stoichiometry×100). NF=62.4±3.9% (solid bar, n=6 hearts) and DCM=46.1±6.2% (open bar, n=6 hearts) (*$P<0.05$). Bars represent mean±SEM.

**Figure 4.** A, Immunodetection of RI in human cardiac tissue. Arrow indicates position of RI (48 kDa). Supernatant fractions from NF (n=6; N) and DCM (n=7; D) hearts were loaded onto 10% polyacrylamide gels in alternating order (each lane represents a different heart). Fifty micrograms of supernatant protein loaded/lane was in the linear range of the corresponding fluorescence intensity (not shown). B, Vertical point plot comparing RI levels between NF and DCM hearts. Protein content, normalized to actin, is expressed relative to NF (all values were divided by NF mean). RI=0.59±0.05, a decrease of 40% ($P<0.001$) in DCM compared with NF hearts. Bar represents mean.

**Figure 5.** A, Immunodetection of RII in human cardiac tissue. Arrow indicates position of RII (55 kDa). Supernatant fractions from NF (n=6; N) and DCM (n=7; D) hearts were loaded onto 10% polyacrylamide gels in alternating order (each lane represents a different heart). Fifty micrograms of supernatant protein loaded/lane was in the linear range of the corresponding fluorescence intensity (not shown). B, Vertical point plot comparing RII levels between NF and DCM hearts. Protein content, normalized to actin, is expressed relative to NF (all values were divided by NF mean). RII=0.59±0.05, a decrease of 30% ($P<0.05$) in DCM versus NF hearts. Bar represents mean.

**Discussion**

The main findings in this study are that baseline TnI phosphorylation is diminished in myocardial samples from pa-
tients with end-stage DCM and that PKA-RI and RII protein levels are decreased in these same hearts. Decreased TnI phosphorylation would lead to an increase in the Ca\(^{2+}\) affinity of troponin C (TnC) and subsequent decreased off-rate of Ca\(^{2+}\) from TnC. This results in an increased Ca\(^{2+}\) sensitivity of force development. Decreased baseline PKA phosphorylation of TnI in the failing human heart is consistent with our previous observations in spontaneously hypertensive rat (SHR). In the 76-week-old SHR, a model of decompensated cardiac hypertrophy and precursor to heart failure, baseline TnI phosphorylation was significantly reduced versus Wistar-Kyoto (WKY) controls. This was associated with increased Ca\(^{2+}\) sensitivity of actomyosin ATPase activity. Although PKA-dependent TnI phosphorylation under baseline conditions in the 76-week-old SHR was decreased, the \(\beta\)-adrenergic pathway was not attenuated because maximal TnI phosphorylation similar to WKY controls was attained.

Similarly, in the present study, we observed that maximal PKA activity was not different between failing and NF human hearts. Thus, despite decreased \(\beta\)-receptor density in SHR and human hearts, our results indicate that there is sufficient reserve in these pathways that downstream components can still be activated to levels comparable to controls. Interestingly, the 26-week-old SHR, which demonstrate compensated hypertrophy, did not show differences in basal TnI phosphorylation. Therefore, these findings, together with the current results, suggest that altered PKA-dependent TnI phosphorylation may be involved in development or progression of heart failure.

There is considerable opportunity for regulation of PKA activity at sites distal to cAMP production or breakdown. Early studies established that several pools of cAMP exist in the cardiomyocyte, implying that only a small proportion of total cAMP is directly involved in activation of specific PKA-dependent pathways. There is now increasing evidence for local regulation of PKA activity by binding of PKA to AKAPs through interactions with its regulatory subunits. PKA anchoring can effectively increase local concentrations of the enzyme, thus permitting selective substrate phosphorylation. For example, we found that PKA-mediated phosphorylation of PLB was not decreased in the same failing human hearts in which TnI phosphorylation was decreased. This is consistent with our SHR results, in which changes in TnI phosphorylation were not coupled to changes in PLB phosphorylation. Furthermore, Bohm et al found no difference in PLB phosphorylation in failing human hearts, although total cAMP was decreased. This suggests compartmentalization of PKA in human hearts, which may be disrupted in heart failure.

In the human heart, an RII binding protein (AKAP100) has been detected by Northern blot analysis. Recent work from this laboratory showed AKAP100 localization to the T-tubule/junctional sarcoplasmic reticulum region in rat cardiomyocytes. Confocal images showed that RII colocalizes with AKAP100. It is estimated that the distance from the T-tubule/junctional sarcoplasmic reticulum region to TnI in the A-band overlap zone of the myofibrils (500 to 700 nm) is probably sufficient for effective diffusion of C from the sites of AKAP-tethered PKA. However, this hypothesis remains to be tested.

We report, for the first time, decreased RI and RII protein in DCM hearts compared with NF control hearts. This could result from regulated proteolysis of R or down-regulation of R transcription. Future experiments will address this question. Decreased R implies decreased PKA holoenzyme (R\(\_2\)C\(\_2\)) targeting and therefore decreased local pools of C. This may contribute to the decreased baseline TnI phosphorylation observed in the dilated hearts.

There are potential limitations of this study concerning use of failing and NF human hearts. Because brain death may be associated with massive catecholamine release, NF hearts would presumably have been “treated” differently from DCM and so may not represent the ideal control. In addition, donor hearts were given inotropic support (dopamine or norepinephrine). Elevated circulating catecholamines are believed to trigger downregulation of \(\beta\)-receptors; however, myocardial \(\beta\)-receptor downregulation has been shown to take several weeks or months to develop. For example, in dogs, several weeks of high-dose norepinephrine causes a decreased mechanical response to isoproterenol but does not involve decreased \(\beta\)-receptor density. Nevertheless, \(\beta\)-receptor levels are not our principal focus in the present study, given the importance of downstream regulation of the \(\beta\)-pathway.

These limitations are inherent in any study of failing human myocardium and are difficult to address experimentally. However, animal models of experimental heart failure and hypertrophy do provide qualitatively similar results. In the dog model of cardiomyopathy, Ca\(^{2+}\) sensitivity of isometric tension was increased, as was also found in failing human hearts. In the 76-week-old SHR, we demonstrated that decreased TnI phosphorylation was associated with...
increased Ca$^{2+}$ sensitivity. This suggests that the functional differences and the altered PKA-dependent TnI phosphorylation between DCM and NF hearts reflect changes that occur during heart failure instead of alterations in the NF human myocardium. Together, these results suggest that the differences observed are a consequence of heart failure per se and not the result of life support or trauma.

In summary, our findings suggest that alterations in the β-adrenergic pathway, distal to cAMP and unrelated to downregulation of the β-receptor/adenylate cyclase complex, may contribute to the decreased β-adrenergic response observed in failing human hearts. In other words, rather than changes at the level of the receptor, altered RI or RII levels may ultimately explain the decreased phosphorylation of TnI, although not necessarily other PKA targets, in the failing heart.

Acknowledgments
This work was supported by NHLBI grants HL-56256 (to Dr Bond) and HL-49929 (to Dr Moravec) and by an AHA Established Investigator Award and Grant-in-Aid to Dr Moravec. We thank Dr Norman Ratliff of the Department of Pathology, Cleveland Clinic Foundation, and Life Banc of Northeast Ohio for providing human tissue. We also thank Drs Frank Brozovich, Tom Egelhoff, and J.P. Jin for helpful discussions, and Steve Schomisch and Mike Trentanelli for expert technical assistance.

References
Protein Kinase A (PKA)-Dependent Troponin-I Phosphorylation and PKA Regulatory Subunits Are Decreased in Human Dilated Cardiomyopathy
Daniel R. Zakhary, Christine S. Moravec, Robert W. Stewart and Meredith Bond

*Circulation*. 1999;99:505-510
doi: 10.1161/01.CIR.99.4.505

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/99/4/505

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/