An exciting and developing concept in cellular biology is the idea that elements of the cytoskeleton not only form an internal super structure of the cell, but also participate in cell signaling. Cytoskeletal proteins are likely to transmit force generated by the molecular motors of the sarcomere to sensors that modify cellular activity as diverse as nuclear transcription and ion channel conductance. Moreover, cytoskeletal elements may serve as scaffolding for molecular signaling cascades, providing sites for localization and anchoring of signaling molecules. In the experiments reported in this article, we have tested these ideas by using a transgenic mouse model in which the function of a strategically located cytoskeletal element, actin capping protein, has been modified.

Actin capping proteins (CPs) are heterodimers composed of an α- and β-subunit (CP-β), and exist as an isoform population that displays tissue- and subcellular-specific patterns of localization. Vertebrate cells express three α-subunit isoforms, each encoded by a separate gene, and three β-isofoms, produced by alternate splicing of messenger RNA from one gene. Although CP-β1 and CP-β2 bind actin with nearly identical affinities and kinetics in vitro, each has a distinct function that cannot be substituted for by the other in cardiac myocytes. The subcellular distribution of the α-subunits is unknown. However, in cardiac myocytes actin capping protein composed of the β,β-subunit is confined to the Z-line, whereas β,β-containing actin capping protein localizes to the intercalated disc and cell periphery. The function of CP-β1 is to cap the barbed ends of the thin filaments and anchor them to the Z-line, a role that is critical for normal muscle development. CP-β1 assembles at Z-lines before the formation of actin filaments in developing myotubes. Disruption of actin–CP-β1 interaction impairs myofibrillogenesis and produces gross myofibrillar disarray. Although the importance of CP-β1 in the development of normal muscle architecture is well established, its role in muscle function has not been investigated. Transgenic mice in which the levels of CP-β1 are specifically and significantly dimin-
lished in the heart develop cardiac hypertrophy and die between 7 and 26 days postnatal. The high lethality of this model indicates that CP-β1 is critical for normal cardiac performance. Thus, our first aim was to understand how the sarcomeric disruption associated with the decrease in CP-β1 alters myofilament function.

Disrupting the interaction of CP-β1 with the Z-line may affect signaling through the protein kinase C (PKC) cascade by interrupting binding of PKC with its Z-line–associated anchoring proteins. PKC is a family of serine-threonine kinases that translocate to isoform-specific, subcellular locations on activation. Anchoring proteins, termed receptors for activated C-kinase (RACK), fix active PKC near its target to facilitate phosphorylation. Modulation of the interaction between PKC-isozymes and their respective RACKs alters the effectiveness of PKC. On activation, PKC-ε translocates and binds to cardiac myofilaments.

Our results indicate that the downregulation of CP-β1, or associated changes in CP-β2 or tropomodulin, increases myofilament Ca2+ sensitivity, without affecting maximum tension or actomyosin MgATPase activity. Moreover, we found that the reduction in CP-β1 abolished PKC-dependent regulation of myofilament function. Despite the absence of functional changes after PKC activation, PKC-dependent phosphorylation of tropinin I was observed in myocardium deficient in CP-β1, indicating an uncoupling of myofilament phosphorylation and functional control.

Materials and Methods

Generation of a Mouse Model

Single actin capping protein subunits are unstable and nonfunctioning. Overexpression of one isoform replaces the endogenous isoform. To decrease cardiac CP-β1, we generated a mouse model in which CP-β1 was overexpressed, lowering the level of the Z-line–associated CP-β1 in the functional α/β heterodimer. Previous work has shown that this approach reduces the amount of CP-β1 associated with the Z-line without causing CP-β1 to localize to the Z-discs. Transgenic mice that are deficient in the Z-line–associated CP-β1 are henceforth referred to as “CapZ mice.” CapZ mice were homozygous for the transgene allele.

Isolation of Papillary Fiber Bundles

Hearts were excised from 3- to 6-month-old mice that had been anesthetized with ether. Hearts were rinsed free of blood in ice-cold saline (0.9% NaCl), and left ventricular papillary muscle bundles quickly dissected. Some papillary muscle bundles were treated with receptor agonists/antagonists, whereas others were transferred to a dish containing ice-cold high relaxing solution (HR) and cut into fiber bundles approximately 100 μm in diameter. Triton X-100 (1% v/v final concentration; Sigma) was added to permeabilize the fiber bundles.

Isometric Tension and Actomyosin MgATPase Activity

Measurement of isometric tension and actomyosin MgATPase activity was done according to the methods of de Tombe and Stienen.

PKC Immunoblotting

Ventricular strips were dissected from hearts and treated with receptor agonists/antagonists. After agonists/antagonist treatment, ventricular tissue was homogenized in ice-cold HR containing 1% Triton X-100 v/v and myofibrils isolated according to a modified procedure from Huang et al. Sample loading buffer was added and the samples boiled for 5 minutes.

Samples were resolved on sodium-dodecyl sulfate-polyacrylamide gels (SDS-PAGE) using 4% stacking and 8% resolving gels. Proteins were transferred to nitrocellulose membranes and probed with monoclonal antibodies for PKC-ε (1:1000), PKC-β (1:1000), PKC-α (1:1000) (Santa Cruz Biotechnology), or PKC-β (1:500) (BD Transduction Laboratories). Density of PKC bands was determined using NIH Image software.

Back-Phosphorylation of Myofilament Proteins

Back-phosphorylation was done using a modified protocol from Karczewski et al.1 Ventricular strips were dissected from hearts and treated with receptor agonists/antagonists. Back-phosphorylation was done using recombinant PKC-ε made according to the procedure of Medkova and Cho. Reactions were carried out for 75 minutes at 30°C, which was sufficient for maximum back-phosphorylation (data not shown). Proteins were visualized by Coomassie staining to ensure equal loading of gels and exposed overnight in a phosphor screen. Band density of all agonist/antagonist treatment lanes were divided by control density to give a drug-to-control ratio for each isolation.

Tropomodulin and Actin Protein Levels

Control samples from back-phosphorylation studies were used to determine tropomodulin and actin protein levels. Actin was visualized by Coomassie staining and tropomodulin by immunoblotting with an anti-tropomodulin antibody (Dr M.A. Sussman, The Children’s Hospital and Research Foundation, Cincinnati, Ohio). Band density was measured with NIH Image software.

Agonist/Antagonist Treatment

Left ventricular papillary fibers or ventricular strips were placed in oxygenated (95% O2/5% CO2) Krebs-Henseleit solution at room temperature. Fibers and ventricular strips were treated with 10 μmol/L phenylephrine plus 1 μmol/L propranolol (α-adrenergic receptor/PKC activation), 100 nmol/L endothelin-1 (endothelial type A (ETₐ) receptor/PKC activation), or were untreated (control) for 5 minutes. To inhibit PKC activation, some preparations were pre-treated with chelerythrine chloride (2 μmol/L). After treatment, papillary fiber bundles were permeabilized (1% Triton X-100 v/v final concentration).

Solutions

High relaxing (HR) solution contained 10 mmol/L EGTA, 25 μmol/L CaCl₂, 20 mmol/L MOPS, 50 mmol/L KCl, 6.8 mmol/L MgCl₂, 12 mmol/L phosphocreatine, 5 mmol/L Na₂ATP, 5 mg/mL leupeptin, 12.5 mg/mL pepstatin, and 250 mmol/L PMSF, pH 7.0. Relaxing solution contained 8.37 mmol/L MgCl₂, 5.80 mmol/L Na₂ATP, 20 mmol/L EGTA, and 42.5 mmol/L potassium propionate, pH 7.1. Preactivating solution contained 7.78 mmol/L MgCl₂, 5.80 mmol/L Na₂ATP, 0.50 mmol/L EGTA, 19.5 mmol/L HDTA, and 43.6 mmol/L potassium propionate, pH 7.1. Activating solution contained 7.63 mmol/L MgCl₂, 5.87 mmol/L Na₂ATP, 20 mmol/L Ca2⁺-EGTA, and 43.6 mmol/L potassium propionate, pH 7.1. Relaxing, preactivating, and activating solutions also contained 900 μmol/L NADH, 100 mmol/L N,N,N,N-tetra(2-hydroxyethyl)-2-aminoethylanzenesulfonic acid, 5 mmol/L sodium azide, 10 mmol/L phospho(enol)pyruvic acid, 4 mmol/L EGTA, and 4 mmol/L potassium propionate, pH 7.1.
pyruvate, 4 mg/mL pyruvate kinase (500 U/mg), 0.24 mg/mL lactate dehydrogenase (870 U/mg), 10 μmol/L oligomycin B, 200 μmol/L P²P₅-di(adenosine-5’)-pentaphosphate, and 100 μmol/L leupeptin. Sample loading buffer contained 20% glycerol, 2% SDS, 0.025 mg/mL bromophenol blue, 7.1 mmol/L -mercaptoethanol, and 125 mmol/L Tris-HCl, pH 6.8.

Statistical Analysis
All values are presented as mean±SEM, and values of P<0.05 were the criteria for statistical significance. Data from agonist/antagonist-treated fibers were analyzed using a 1-way ANOVA and post hoc Dunnett’s t test. Basal characteristics were analyzed with a Student’s t test.

Animal Care
All animals were handled in accordance with the guidelines of the Animal Care Committee at the University of Illinois, Chicago.

Results
Mechanoenergetic Characteristics of Left Ventricular Papillary Fiber Bundles From Transgenic Mice With Decreased Expression of CP-β₁
To determine if a decreased level of CP-β₁ leads to altered cardiac function, we examined the effects of downregulated CP-β₁ expression on Ca²⁺ activation of the myofilaments and the ratio of force generation to actomyosin MgATPase rate. Data in Figure 1 compare the relations between (A) isometric tension and (B) actomyosin MgATPase activity and free [Ca²⁺] for wild-type controls and transgenic myofilaments. Compared with wild-type, papillary fiber bundles from CapZ mice demonstrated a significant increase in Ca²⁺ sensitivity for both parameters, as is evidenced by the leftward shift in the curves and decreased EC₅₀ values (Table 1). There were no significant changes in the slope (ie, Hill coefficient) of either curve. Isometric tension and actomyosin MgATPase activity at maximally activating Ca²⁺ concentration were not different between wild-type and CapZ papillary fiber bundles. Tension cost, the relation between the rate of ATP consumption and level of steady-state tension development, was unaffected with the reduction of CP-β₁ (Table 1).

Effects of CP-β₁ Reduction on PKC-Mediated Changes in Myofilament Activity
In a second set of experiments, we tested whether the alterations associated with a reduction in CP-β₁ modified the cellular response to activation of the PKC pathway. Our approach was to activate PKC in wild-type and CapZ mice.

TABLE 1. Basal Mechanical and Energetic Characteristics of Left Ventricular Papillary Fiber Bundles From Transgenic Mice Deficient in Cardiac CP-β₁ (CapZ) and Wild-Type Control

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type</th>
<th>CapZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isometric tension, mN/mm²</td>
<td>40.91±0.52</td>
<td>40.09±0.23</td>
</tr>
<tr>
<td>Maximum</td>
<td>352.99±14.11</td>
<td>386.12±2.47</td>
</tr>
<tr>
<td>Actomyosin MgATPase activity, pmol/s per mm²</td>
<td>0.93±0.05</td>
<td>0.80±0.03*</td>
</tr>
<tr>
<td>Isometric tension</td>
<td>0.87±0.03</td>
<td>0.75±0.03*</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>5.24±0.39</td>
<td>5.61±0.44</td>
</tr>
<tr>
<td>Tension cost</td>
<td>5.56±0.68</td>
<td>4.05±0.60</td>
</tr>
<tr>
<td>No. of fiber bundles (No. of hearts)</td>
<td>9.06±0.49</td>
<td>9.11±0.31</td>
</tr>
</tbody>
</table>

*P<0.05 as compared to wild-type controls.
and to examine the effects on myofilament activation and ATP consumption. Stimulation of PKC-coupled $\alpha$-adrenergic or ET$\alpha$ receptors decreased maximum isometric tension and actomyosin MgATPase activity in wild-type fiber bundles, without altering myofilament Ca$^{2+}$ sensitivity (Figure 2; Table 2). Tension cost was not affected by any agonist/antagonist treatment in wild-type fiber bundles (Figure 3).

In contrast to the case with wild-type controls, PKC activation did not alter activity in fiber bundles from CapZ mouse hearts (Figure 4). Neither maximum isometric tension nor maximum actomyosin MgATPase activity was reduced with $\alpha$-adrenergic or ET$\alpha$-receptor activation (Table 2). Tension cost was not affected by any of the agonist/antagonist treatment in CapZ mouse fiber bundles (Table 2).

### TABLE 2. Effects of Receptor Agonists/Antagonists on Mechanical and Energetic Properties of Left Ventricular Papillary Fiber Bundles From Transgenic Mice Deficient in CP-1 (CapZ) and Wild-Type Control

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type</th>
<th>CapZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PHE+PRO</td>
</tr>
<tr>
<td>Isometric tension, mN/mm$^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>42.02±0.82</td>
<td>31.03±0.56*†</td>
</tr>
<tr>
<td>Actomyosin MgATPase activity, pmol/s per mm$^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>339.65±23.97</td>
<td>249.37±20.36*†</td>
</tr>
<tr>
<td>Tension cost</td>
<td>8.30±0.35</td>
<td>8.76±0.74</td>
</tr>
<tr>
<td>No. of fiber bundles (No. of hearts)</td>
<td>10 (7)</td>
<td>5 (4)</td>
</tr>
</tbody>
</table>

PHE+PRO indicates phenylephrine plus propranolol ($\alpha$-adrenergic receptor activation); ET-1, endothelin-1 (ET$\alpha$ receptor activation).

*P<0.05 as compared to wild-type controls. †P<0.05 as compared to CapZ mice.
Immunoblot Analysis of PKC Activation

To determine if the translocation of PKC isoforms to the myofilaments was affected by CP-β1 downregulation, we examined the myofilament association of PKC-α, -β, -δ, and -ε after agonist/antagonist treatment by Western blot analysis (Figure 5). There was an increase in PKC-ε protein levels in the myofilament fraction after β-adrenergic and ETα-receptor stimulation of wild-type myocardium. By contrast, the same agonists/antagonists decreased PKC-ε protein levels in the myofilament fraction of CapZ murine myocardium. Both β-adrenergic and ETα-receptor activation decreased myofilament-associated PKC-β in wild-type ventricular tissue. Myofilament-associated PKC-β was undetectable in 4 out of 6 control CapZ mouse hearts. Endothelin increased the amount of PKC-δ protein in the myofilament fraction of wild-type hearts, whereas phenylephrine plus propranolol had no effect. Myofilament-associated PKC-β was decreased in transgenic hearts after α-adrenergic or ETα-receptor activation. α-Adrenergic and ETα-receptor stimulation decreased the amount of PKC-α in the myofilament fraction of both transgenic and wild-type ventricles.

Phosphorylation of Myofilament Proteins

Phosphorylation levels of myofilament proteins were determined using a back-phosphorylation assay. As such, lighter bands reflected relatively low incorporation of 32P and indicate higher levels of phosphorylation. α-Adrenergic and ETα-receptor stimulation increased the phosphorylation of troponin I in both wild-type and transgenic hearts. Endothelin also increased myosin-binding C-protein and troponin T phosphorylation.
phosphorylation in CapZ mouse hearts. The PKC inhibitor chelerythrine chloride abolished endothelin and α-adrenergic receptor–dependent changes in myofilament protein phosphorylation of both wild-type and transgenic mice without altering basal phosphorylation levels (data not shown). Basal phosphorylation levels of myosin binding C-protein, troponin T, and troponin I were higher in CapZ control myocardium as compared with wild-type controls.

**Actin and Tropomodulin Protein Levels**

Actin protein levels were not different between wild-type and CapZ hearts (data not shown). The expression of tropomodulin was 15±6% higher in CapZ transgenic myocardium, compared with wild-type (data not shown). Because tropomodulin binds the pointed ends of both cytoskeletal and sarcomeric actin filaments, we are uncertain if these results reflect changes in the capping of cytoskeletal or sarcomeric actin or both.

**Discussion**

Our results provide the first evidence that a reduction in the expression of CP-β1 increases myofilament Ca2+ sensitivity and disrupts PKC-dependent myofilament regulation. These data provide new insights into the functional significance of CP-β1 and support the hypothesis that a localized region at the thin filament–Z-line interface is critical for the transmission of signals in the PKC pathway.

Previous studies2,5 reported that reduced CP-β1 disrupts sarcomere assembly, leading to cardiac hypertrophy and juvenile lethality. Given the severe nature of this defect some functional impairment would be expected to be manifest along with the architectural disarray. The present study, however, found no overtly detrimental mechanical or energetic characteristics in CapZ mice. One explanation for the apparent discrepancy between the structural and functional data may be the different founder lines used in each study. Hart and Cooper2 characterized two transgenic lines (TG-wt β1 No. 2 and TG-wt β1 No. 3) in which CP-β1 was decreased by 37% and 64%, respectively, as compared to wild-type levels. By contrast, our study used mice derived from a line (TG-wt β1 No. 1) in which CP-β1 levels were decreased by 7%. The line with moderate overexpression produced a less severe phenotype and modest functional changes. Evidence in support of this hypothesis includes the relative viability of CapZ hearts (data not shown). The expression of tropomodulin–adrenergic or ET A receptor activation in wild-type preparations decreased both maximum tension and maximum actomyosin MgATPase activity with no change in maximum isometric tension or actomyosin MgATPase activity.21 The mechanism underlying the increase in myofilament Ca2+ sensitivity has not been definitively identified. One possible explanation involves the disruption of PKC-dependent myofilament regulation in these transgenic mice. The inability of PKC to effectively control myofilament activation may remove a regulator of Ca2+ sensitivity, thereby allowing the myofilaments to function with increased sensitivity to Ca2+.

In our present study, the stimulation of α-adrenergic or endothelial receptors did not alter myofilament Ca2+ sensitivity. Others have found changes in myofilament Ca2+ sensitivity after PKC activation.19,20 One explanation for these discrepancies may be the presence of multiple PKC isoforms in cardiac muscle. The stimulation of membrane receptors can activate a variety of PKC isoforms, each one of which may induce distinct effects. Even when identical PKC agonists are used, variations in treatment time can lead to different PKC isoform activation profiles with assorted temporal patterns.21

An important and novel finding of our study was that stimulation of PKC-coupled α-adrenergic or ET A receptors in CapZ mice had no effect on myofilament activity. In contrast, α-adrenergic or ET A receptor activation in wild-type preparations decreased both maximum tension and maximum actomyosin MgATPase activity.22 Mochly-Rosen22 has proposed that anchoring proteins for PKC (called RACKs) are essential for the mediation of PKC-dependent effects. On activation, PKC-ε translocates to the myofilaments, producing a pattern consistent with Z-line binding.9–11 Downregulation of CP-β1 disrupts normal Z-line formation, resulting in shortened or even absent Z-lines.2 Without normal Z-line structure, RACK binding to Z-lines or Z-line–associated proteins may be disrupted, diminishing the ability of PKC to translocate near myofilament substrates. We demonstrate here for the first time an important role for cardiac actin capping protein in the association of PKC with cardiac myofilaments.

Whereas the downregulation of cardiac actin capping protein did not prevent the association of PKC-ε, -α, or -δ with the myofilament subcellular fraction, it did correlate with alterations in the amount of activated PKC-ε that translocated to the myofilament fraction, as well as a reduction in basal myofilament-associated PKC-β levels. The reason for the disappearance of PKC-ε from the myofilament fraction on activation of α-adrenergic or ET A receptors is unknown. One possibility is that the downregulation of CP-β1...
C-protein in both wild-type and transgenic myocardium after the phosphorylation of troponin I and myosin binding on cardiac Z-discs is unknown. 34,35 The addition of the second two possible mechanisms that may account for this phenomenon levels and function has not been reported before. We suggest uncoupling between changes in myofilament phosphorylation activation, whereas transgenic myocardium demonstrated no such, a PKA-dependent increase in troponin I phosphorylation might precede any functional changes if the phosphorylation of serine 23 was significantly slower. Winegrad and colleagues have published a series of studies in which they propose a similar importance of ordered phosphorylation for the full compliment of residues required for functional regulation.

Intracellular signaling through PKC is a vital mechanism of myocardial regulation. Activation of PKC results in a wide range of effects, including compensated and decompensated myocardial hypertrophy,37,38 as well as the cardioprotection of preconditioning.39,40 Given the key role PKC occupies in the heart, manipulation of PKC activation might serve as a useful target for the management of myocardial dysfunction. However, the diverse nature of PKC requires that such treatment be specifically directed in order to minimize unintended effects. The precise management of PKC activity necessitates a full understanding of how activated PKC interacts with specific substrates. The results of this study demonstrate a crucial role for the Z-line–associated actin capping protein in the transduction of PKC signals to the myofilaments. These novel findings suggest that the Z-line–associated actin capping protein might serve as a useful target in the management of myofilament function by PKC.

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References


Actin Capping Protein: An Essential Element in Protein Kinase Signaling to the Myofilaments

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