

Cardiac Actin Capping Protein Reduction and Protein Kinase C Inhibition Maintain Myofilament Function During Cardioplegic Arrest

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Key Words

CapZ • Cardioplegia • Protein kinase C • Actomyosin MgATPase • Heart

Abstract

Background: Heart transplantation is associated with cold, cardioplegic arrest that impairs myocardial function. Protein Kinase C (PKC) suppression of myofilaments may contribute to this dysfunction. CapZ-deficient cardiac myofilaments are unresponsive to PKC. We hypothesized that myofilaments from CapZ-deficient transgenic hearts are resistant to cardioplegic dysfunction and that PKC inhibition improves function. **Methods:** Heart function was assessed using a Langendorff apparatus. Myofilaments isolated from murine hearts were assessed with an actomyosin MgATPase assay and protein phosphorylation gels. PKC activation was examined by immunoblotting. **Results:** Wildtype hearts showed impaired function after cardioplegic arrest. CapZ-deficient transgenic mouse hearts performed significantly better after 1 h cardioplegia than wildtype hearts, but not after 4 h cardioplegic arrest. Wildtype

myofilaments had depressed activation at 1 and 4 h cardioplegic arrest, as demonstrated by reduced actomyosin MgATPase activity. CapZ-deficient myofilaments showed no reduced actomyosin MgATPase activity at either time. Troponin I (TnI) phosphorylation increased by approximately 20% at 1 and 4 h in wildtype mice. Myosin binding protein C (MyBP-C), and troponin T (TnT) phosphorylation increased by less than 10% at 1 h, and tended to rise at 4 h. Myofilament protein phosphorylation was largely unchanged in CapZ-deficient hearts at 1 h, but MyBP-C tended to be dephosphorylated at 4 h cardioplegic arrest. Myofilament-associated PKC- α , - β II, - δ , and - ϵ increased at 1 and 4 h cardioplegia in wildtype hearts, whereas only PKC- α increased in transgenic myofilaments at 1 h. PKC inhibition abolished the cardioplegic-dependent changes in actomyosin MgATPase activity and TnI phosphorylation of wildtype myofilaments. **Conclusions:** We demonstrate a direct link between PKC activation and myofilament dysfunction associated with cold, cardioplegic arrest. Moreover, we show for the first time a cardioprotective benefit of decreased cardiac CapZ.

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Introduction

Heart transplantations are increasingly common, with the rising rates of cardiovascular disease and improvements in surgical interventions which have increased the potential patient population. These procedures necessitate a period of cardiac ischaemic risk, primarily during organ transportation. Ischaemic damage is typically mitigated with cold crystalloid cardioplegia and storage in a protective solution. Despite the well recognized benefit of cold cardioplegic solutions, cardiac dysfunction resulting from ischaemia during transportation remains a significant problem [1-3].

Cardiac myofilaments serve as the central contractile apparatus of the heart. Alterations in their function, either through genetic changes or post-translational modifications, significantly influences myocardial performance [4-6]. It has been noted that cold crystalloid cardioplegia is associated with impaired myofilament activation, and that this depressed contractility may underlie the reduction in myocardial function that characterizes hearts that have been subjected to cold cardioplegic arrest [7]. The intracellular mechanisms responsible for cardioplegic myofilament dysfunction have not been determined, although it has been hypothesized that the impairment is a product of cardioplegia itself, and not a reperfusion injury [7].

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases that mediate a diverse range of activities in virtually all cell types. In the heart PKC is a paradoxical molecular messenger, mediating both the myocardial benefits of preconditioning [8] and the dysfunctional pathways that drive heart failure [9]. The discrepant outcomes of PKC activation may be due to the specific isoforms involved, their subcellular localization, time of activation, or some combination of all of these factors. During cold crystalloid cardioplegia several PKC isoforms translocate to a myofilament-rich isolate [10]. It has been hypothesized that the changes in PKC activity occurring with cold cardioplegic arrest might be a causative factor in the myofilament dysfunction of the same condition, but this idea has not been tested.

PKC translocates to a number of subcellular compartments upon activation to interact with its substrate proteins. In myocardium, several isoforms associate at or near Z-discs, which may serve as a subcellular signaling station [11]. We have found that decreasing the Z-disc actin capping protein (CapZ) reduces the ability of cardiac myofilaments to respond to PKC activation

[12, 13]. Given the contradictory relationship between PKC and cardiac myofilaments, it is uncertain whether a decline in cardiac CapZ protein levels is a beneficial or detrimental change in the heart. Using CapZ-deficient transgenic mice and pharmacologic antagonism of PKC, we tested the hypothesis that PKC-translocation to cardiac myofilaments during cold cardioplegic arrest impairs myofilament activation, and that disruption of this pathway prevents cardioplegic myofilament dysfunction.

Materials and Methods

Isolated Heart Function

Hearts were excised from C57/Bl6 female mice (4 to 6 months old) euthanized with carbon dioxide. The aorta was dissected free of tissue in ice-cold Krebs-Henseleit containing 5 IU/mL heparin. Krebs-Henseleit Buffer consisted of 118.5 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2 mM NaH₂PO₄, 26.6 mM NaHCO₃, 10 mM Glucose, and 0.2 mM CaCl₂. Hearts were then mounted on a Langendorff apparatus and perfused at 80 mmHg with oxygenated Krebs-Henseleit warmed to 37°C. A balloon attached to a pressure transducer was inserted into the left ventricle via the left atrium to assess myocardial function. Hearts were paced at ~450 bpm and perfused for 30 min. After cardioplegic arrest, some hearts were reperfused for 30 min under the same conditions outlined above to assess post-cardioplegic recovery.

Cardioplegia

After assessment of myocardial function, hearts were arrested with 10 mL ice-cold St. Thomas cardioplegic solution and maintained at 4°C for up to 4 h. St. Thomas cardioplegic solution contained 110 mM NaCl, 16 mM KCl, 1.2 mM CaCl₂, 16 mM MgCl₂, 1% NaHCO₃, pH 7.6 at 4°C. In some studies St. Thomas cardioplegic solution was supplemented with chelerythrine chloride (10 µM) to block PKC activation [14].

Isolation of Myofilaments

Cardiac muscle was rinsed free of cardioplegic solution and then homogenized in ice-cold Intracellular Buffer. Intracellular Buffer contained 60 mM KCl, 30 mM Imidazole (pH 7.0), 2 mM MgCl₂, 0.01 mM leupeptin, 0.1 mM PMSF, 0.2 mM benzamidine. Myofilaments were isolated as described previously [15]. Briefly, the homogenate was centrifuged at 14,000g for 15 min at 4°C. Pellets were dissolved in Skinning Buffer and left on ice for 45 min before being centrifuged at 1,100g for 15 min at 4°C. Skinning Buffer contained 10 mM EGTA (pH 7.0), 8.2 mM MgCl₂, 14.4 mM KCl, 60 mM Imidazole (pH 7.0), 5.5 mM Na₂ATP, 12 mM Creatine Phosphate, 10 U/mL Creatine Phosphokinase, 0.01 mM leupeptin, 0.1 mM PMSF, 0.2 mM benzamidine, and 1% Triton X-100. Resulting pellets were washed 3 times in ice-cold Intracellular Buffer and purified myofilaments stored on ice for up to 2 h or frozen for gel electrophoresis.

Fig. 1. Left ventricular function of wildtype and CapZ-deficient transgenic mouse hearts. Hearts were excised and mounted on a Langendorff apparatus for retrograde perfusion. A pressure transducer was inserted into the left ventricle to assess myocardial contractility. Pressures were stable over 30 min and no differences were seen between wildtype and CapZ-deficient transgenic mouse hearts in any parameter prior to cardioplegic storage. Sample traces of left ventricular pressures of A. wildtype and B. CapZ-deficient transgenic hearts are shown. Data were collected before cardioplegic arrest and during reperfusion after 1 and 4 h cardioplegic storage. Left ventricular developed pressure as a percent of pre-cardioplegic levels for wildtype and CapZ-deficient transgenic hearts following C. 1 h and D. 4 h cardioplegic storage are plotted against reperfusion time. Left ventricular developed pressure was significantly higher in the CapZ-deficient mouse hearts after 1 h cardioplegic storage, whereas values were similar to wildtype hearts after 4 h cardioplegic arrest.

Actomyosin MgATPase

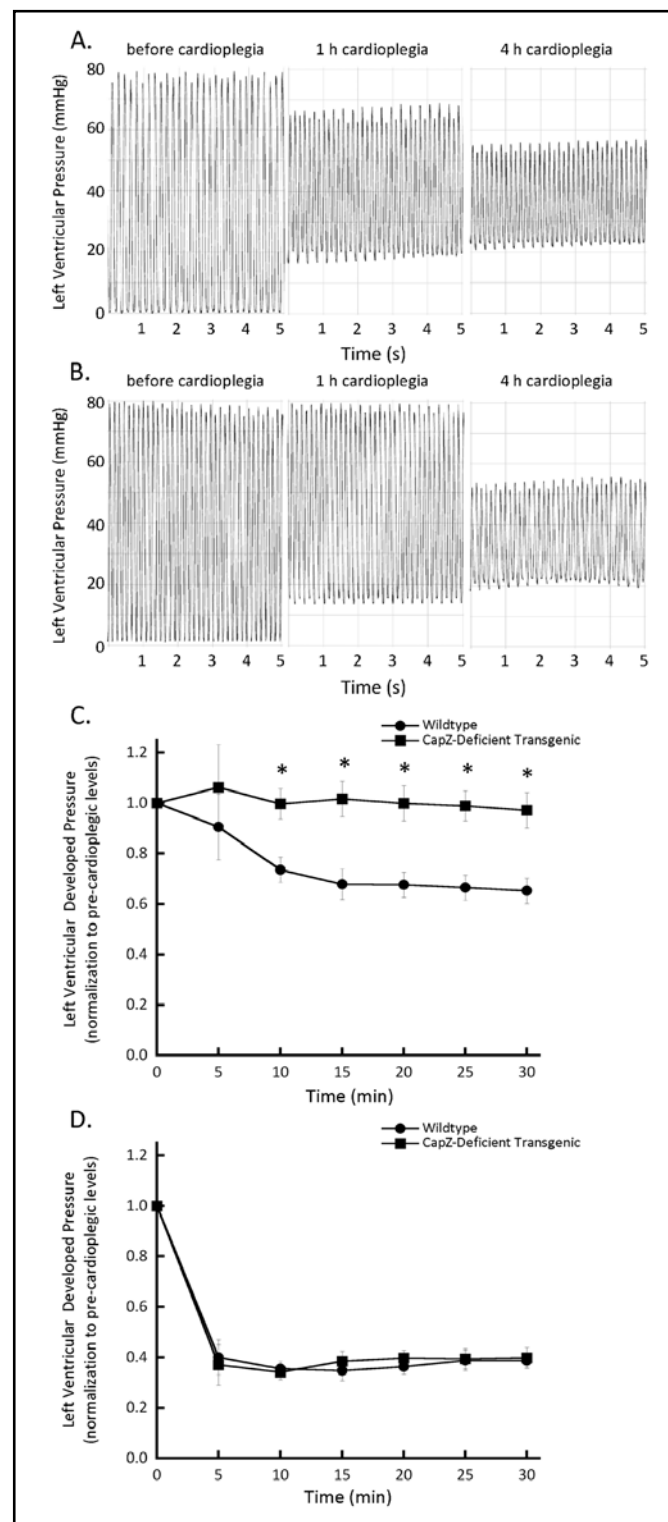
Actomyosin MgATPase activity was measured by spectrophotometry using a modified Carter assay. Purified myofilaments (50 μ g) were incubated in reaction buffers containing varying levels of free calcium created using combinations of Activating and Relaxing buffers. Activating buffer contained 23.5 mM KCl, 5 mM MgCl₂, 3.2 mM ATP, 2 mM EGTA, 20 mM Imidazole, and 2.2 mM CaCl₂, 0.1 mM PMSF, 0.01 mM leupeptin, 0.2 mM benzamidine, pH 7.0. Relaxing buffer contained 26 mM KCl, 5.1 mM MgCl₂, 3.2 mM ATP, 2 mM EGTA, 20 mM Imidazole, 4.9 μ M CaCl₂, 0.1 mM PMSF, 0.01 mM leupeptin, 0.2 mM benzamidine, pH 7.0. Free calcium was calculated using the programme from Patton et al. [16]. Myofilaments were incubated in reaction buffers for 5 min at 32°C and reactions quenched with ice-cold 10% trichloroacetic acid. The amount of inorganic phosphate produced was measured by adding an equal volume of 0.5% FeSO₄ and 0.5% ammonium molybdate in 0.5 M H₂SO₄, and reading the absorbance at 630 nm.

Protein Phosphorylation

Myofilament proteins (40 μ g) were separated on 12% sodium-dodecyl sulfate-polyacrylamide gels. Gels were fixed in 50% methanol/10% acetic acid at room temperature overnight and phosphorylated proteins stained with Pro-Q Diamond (Molecular Probes, Eugene, OR, U.S.A.) according to the manufacturer's instructions. Imaging was done using a Typhoon gel scanner (GE Healthcare, Baie Quebec) and analysis done using Image J software. Protein loading was assessed by Coomassie stain.

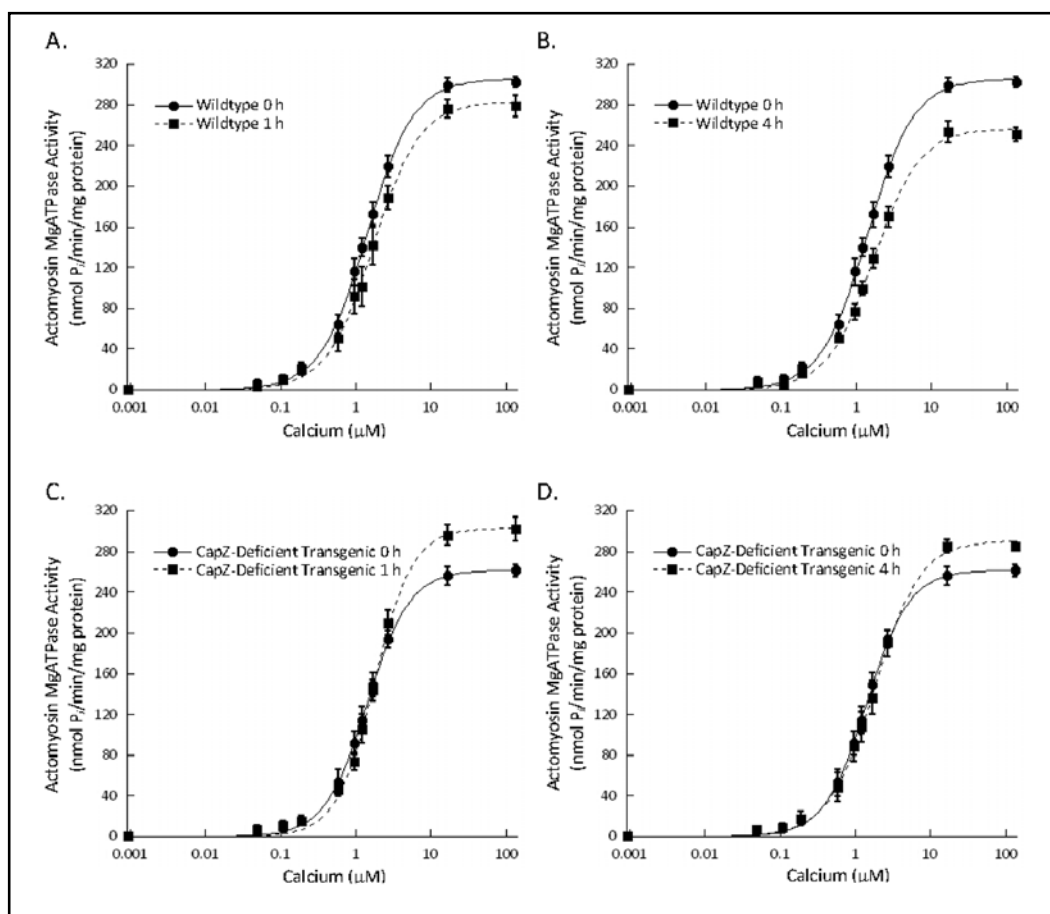
Immunoblotting

Myofilament proteins (40-75 μ g) were resolved by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% separating gel for PKC immunoblots, or 12% for troponin immunoblots. Proteins were transferred to nitrocellulose membranes and probed with antibodies for PKC- α , PKC- β II, PKC- δ , and PKC- ϵ (BD Biosciences, Mississauga, Ontario, Canada and Upstate, Mississauga, Ontario, Canada), or troponin I and troponin T (Santa Cruz Biotechnology Inc., Santa



Cruz, CA, USA). Secondary antibodies were conjugated to HRP (Sigma-Aldrich, Oakville, Ontario, Canada). Primary antibodies were used at 1:1000 dilution and secondary antibodies at 1:5000 dilution. Equal protein loading was determined by re-probing blots with anti-actin antibody (1:25000; Millipore, Billerica, MA, USA). Bands were detected using Western Lightning (PerkinElmer Life and Analytical Sciences, Woodbridge, Ontario, Canada) and analysis of band density was done using Image J software (NIH, Bethesda, MD, USA).

Fig. 2. Cardiac myofilament actomyosin MgATPase activity following cold, cardioplegic storage. Actomyosin MgATPase activity decreased at calcium concentrations $> 1 \mu\text{M}$ in wildtype cardiac myofilaments after 1 (A) and 4 h (B) cardioplegic arrest. Actomyosin MgATPase activity increased at high calcium concentrations in CapZ-deficient cardiac myofilaments at 1 (C) and 4 h (D), but did not show any significant changes at free calcium $< 10 \mu\text{M}$. Data points are means \pm SEM. $N=10$ for 0 h; 8 for 1 h; and 6 for 4 h. Equal numbers were included for wildtype and CapZ-deficient transgenic mouse hearts.



Animal Care

Mice were handled according to the guidelines of the Animal Care Committee at the University of Guelph.

Statistical Analysis

All values are presented as mean \pm SEM. Statistical analysis was done using a one-way ANOVA and a post-hoc Dunnett's t-test for actomyosin ATPase data. Fisher's Least Significant Difference was used for immunoblot and phosphorylation analysis. Values of $P < 0.05$ were the criteria for statistical significance.

Results

Langendorff Perfused Hearts

Hearts were perfused for 30 min to assess myocardial function, prior to cardioplegic arrest. No differences were detected between the wildtype and CapZ-deficient transgenic mouse hearts in any parameter examined (Fig. 1). After cardioplegic arrest hearts were reperfused and left ventricular parameters assessed. Functional recovery was determined by comparing post-cardioplegic values with the average value obtained over the 30 min pre-cardioplegic period. Wildtype mouse hearts showed a decline in function

after 1 and 4 h cardioplegia (Fig. 1). By contrast, CapZ-deficient transgenic mouse hearts performed significantly better at 1 h than their wildtype counterparts. After 4 h cardioplegic arrest the functional advantage enjoyed by the CapZ-deficient transgenic mice was lost.

Myofilaments from CapZ-deficient transgenic mice show no depressed function during prolonged cardioplegia

Myofilaments were isolated after varying times of cold, cardioplegic arrest and function assessed using an actomyosin MgATPase activity assay. Activation at saturating levels of calcium was higher in wildtype myofilaments as compared to CapZ-deficient myofilaments at time 0, as we have reported previously [15]. At 1 and 4 h of cardioplegic arrest, actomyosin MgATPase activity decreased in wildtype hearts at free calcium levels greater than $1 \mu\text{M}$ (Fig. 2). By contrast CapZ-deficient myofilaments showed an increase in maximum actomyosin MgATPase activity at 1 and 4 h, with no significant difference in submaximal calcium activation. These data show that reducing CapZ levels buffers against myofilament inhibition during cold cardioplegic arrest.

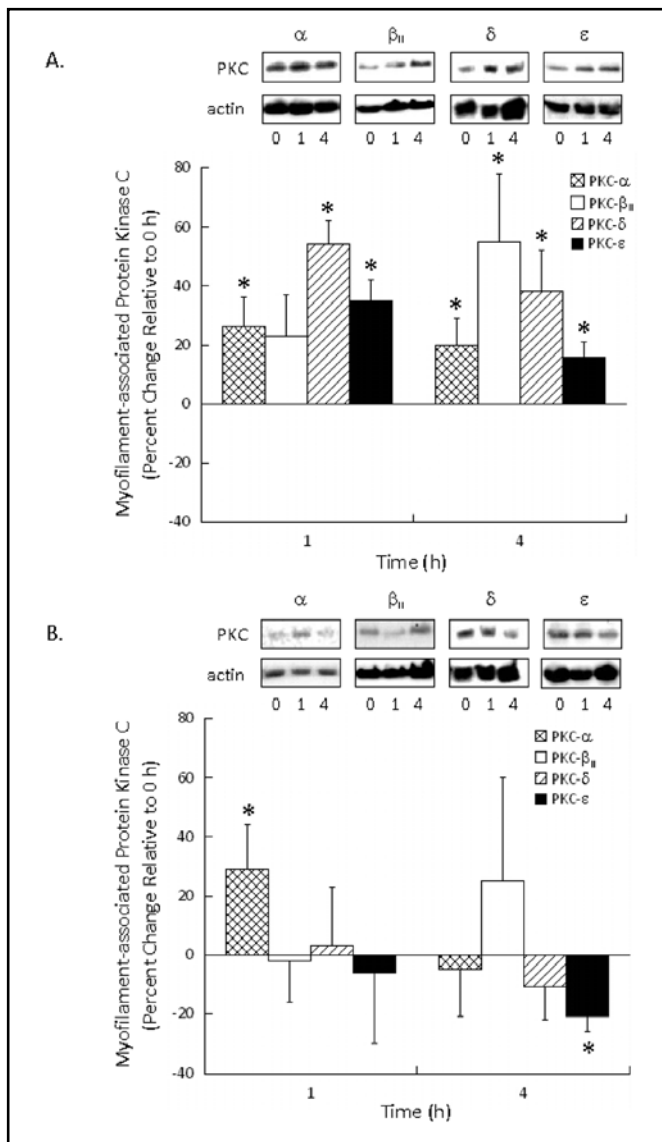


Fig. 3. Myofilament-associated Protein Kinase C (PKC) levels during cold, cardioplegic storage. Cardiac myofilaments isolated from wildtype (A) and CapZ-deficient transgenic mouse hearts (B) were probed with isoform-specific PKC antibodies. Representative blots for each PKC-isoform and the corresponding anti-actin blot for protein loading are shown above the summary graphs. Summary graphs show the percent change compared to 0 h. Values are mean \pm SEM. $N=7$ for PKC- α , δ , and ϵ , and $N=4$ for PKC- β II. Equal samples were collected from both wildtype and CapZ-deficient transgenic hearts. * $P < 0.05$ vs. 0 h of respective group (wildtype or transgenic).

Myofilament-associated Protein Kinase C is altered during prolonged cardioplegia

Cardiac myofilaments were isolated from wildtype murine hearts and probed with anti-PKC antibodies. PKC translocation to the particulate elements of cardiac myocytes is accepted as an indicator of PKC activation

[17]. PKC- α , - β II, - δ , and - ϵ were all detected at 0 h (Fig. 3A). At 1 h post-cardioplegic arrest PKC- α , - δ , and - ϵ were significantly increased, while PKC- β II tended towards increased levels. By 4 h all measured isoforms were significantly increased in the myofilament fraction. These data demonstrate that cold cardioplegic arrest increases the association of several PKC isoforms with cardiac myofilaments.

Increased myofilament-associated Protein Kinase C during cardioplegia is attenuated in CapZ-deficient transgenic mouse hearts

Myofilament preparations were isolated from CapZ-deficient transgenic hearts at 1 or 4 h cardioplegia and probed with PKC isoform specific antibodies to identify those PKC isozymes activated by cardioplegic arrest. PKC- α , - β II, - δ , and - ϵ were all detected at 0 h (Fig. 3B). Unlike myofilaments from wildtype hearts which showed increased myofilament-associated PKC, at 1 h post-cardioplegic arrest only myofilament-associated PKC- α was elevated. At 4 h PKC- δ trended towards decreased levels while PKC- ϵ was significantly reduced. These results show that a downregulation of the Z-disc protein CapZ inhibits the cardioplegic-dependent increase in myofilament-associated PKC isoforms seen in wildtype samples.

Myofilament protein phosphorylation is increased in wildtype hearts during cardioplegia, but decreased in CapZ-deficient transgenic hearts

Troponin I phosphorylation was increased above pre-cardioplegic levels by $18 \pm 4\%$ at 1 h of cardioplegic arrest in wildtype hearts (Fig. 4). Myosin binding protein-C and troponin T phosphorylation levels increased by $10 \pm 4\%$ and $6 \pm 3\%$ at 1 h. At 4 h myosin binding protein C and troponin T phosphorylation increased to $16 \pm 9\%$ and $10 \pm 4\%$ respectively, while troponin I phosphorylation did not appreciably differ from the levels at 1 h. By contrast there were no detectable increases in myofilament protein phosphorylation in CapZ-deficient transgenic mice at 1 h of cardioplegic arrest. At 4 h post-arrest, myosin binding protein C showed a decline in phosphorylation levels to $14 \pm 5\%$ below pre-cardioplegic levels.

Protein Kinase C antagonism affects myofilament changes of cardioplegia in wildtype but not CapZ-deficient transgenic hearts

To determine if PKC mediates any or all of the effects on cardiac myofilaments during cardioplegia, we

Fig. 4. Myofilament protein phosphorylation changes during cold, cardioplegic storage. Myofilaments from wildtype (A and C) and CapZ-deficient transgenic (B and D) hearts were examined for phosphorylation changes during cold, cardioplegic storage. Phosphorylation gels (top gels) are presented in panels (A) and (B) showing typical changes in myofilament protein phosphorylation. Coomassie staining (bottom gels) was done to ensure equal loading across lanes. Graphs summarizing the collected data are shown in panels (C) and (D). Summary graphs are means \pm SEM. N=13 hearts for all time points. *P< 0.05 vs. 0 h of respective group (wildtype or transgenic).

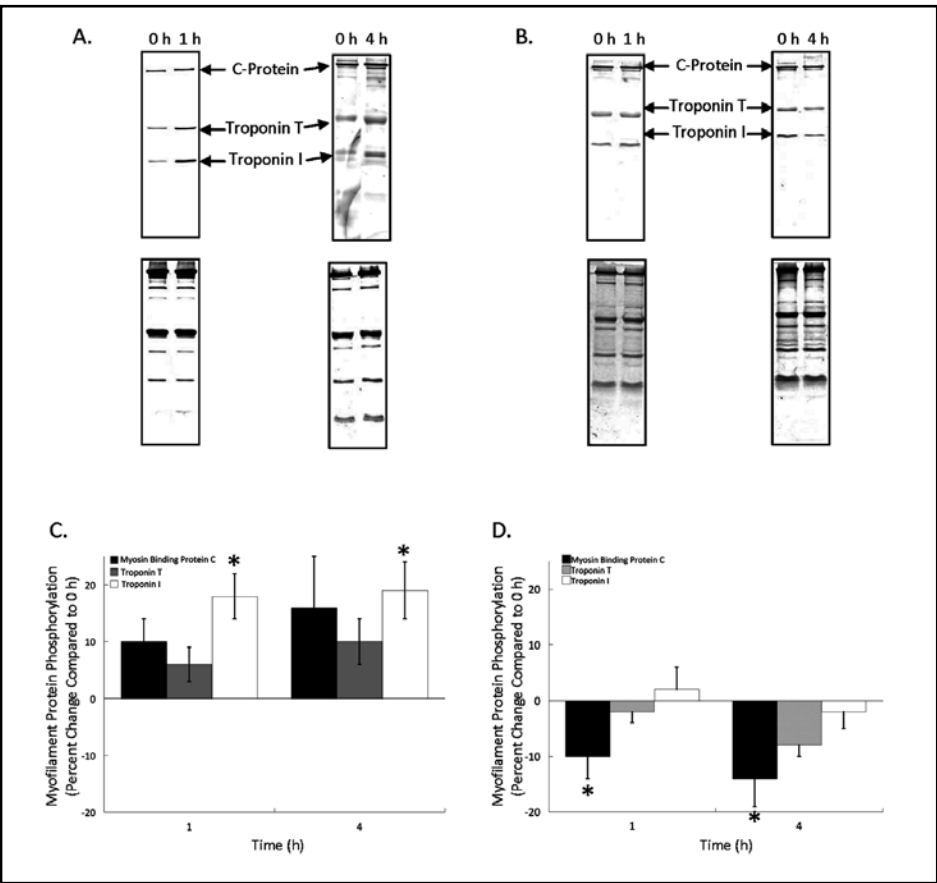
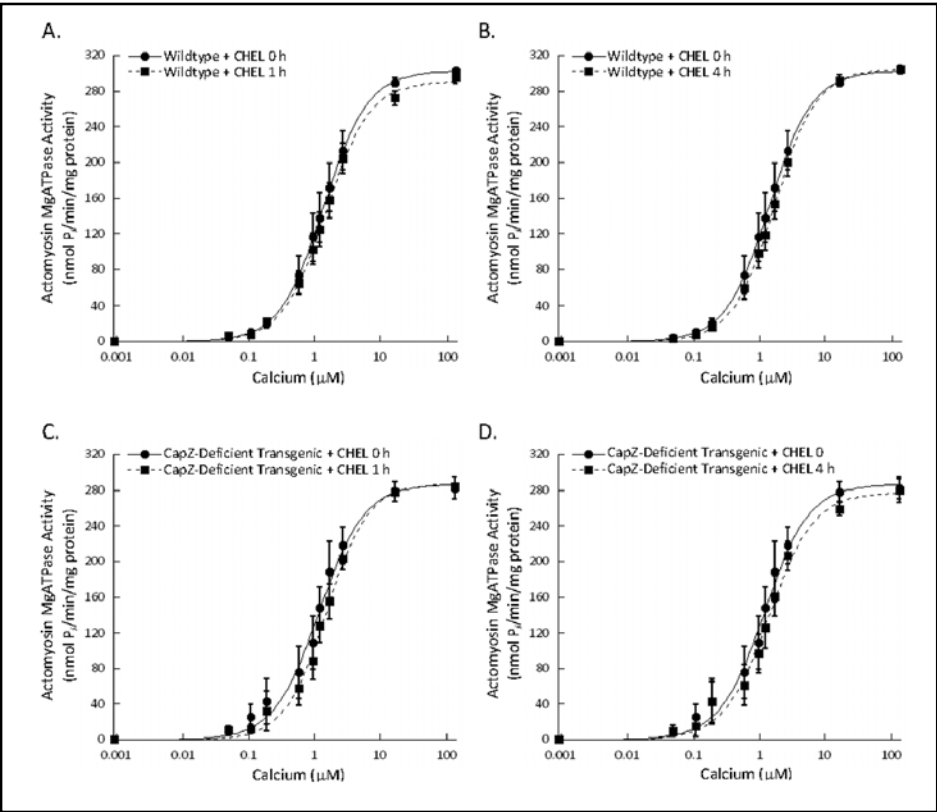


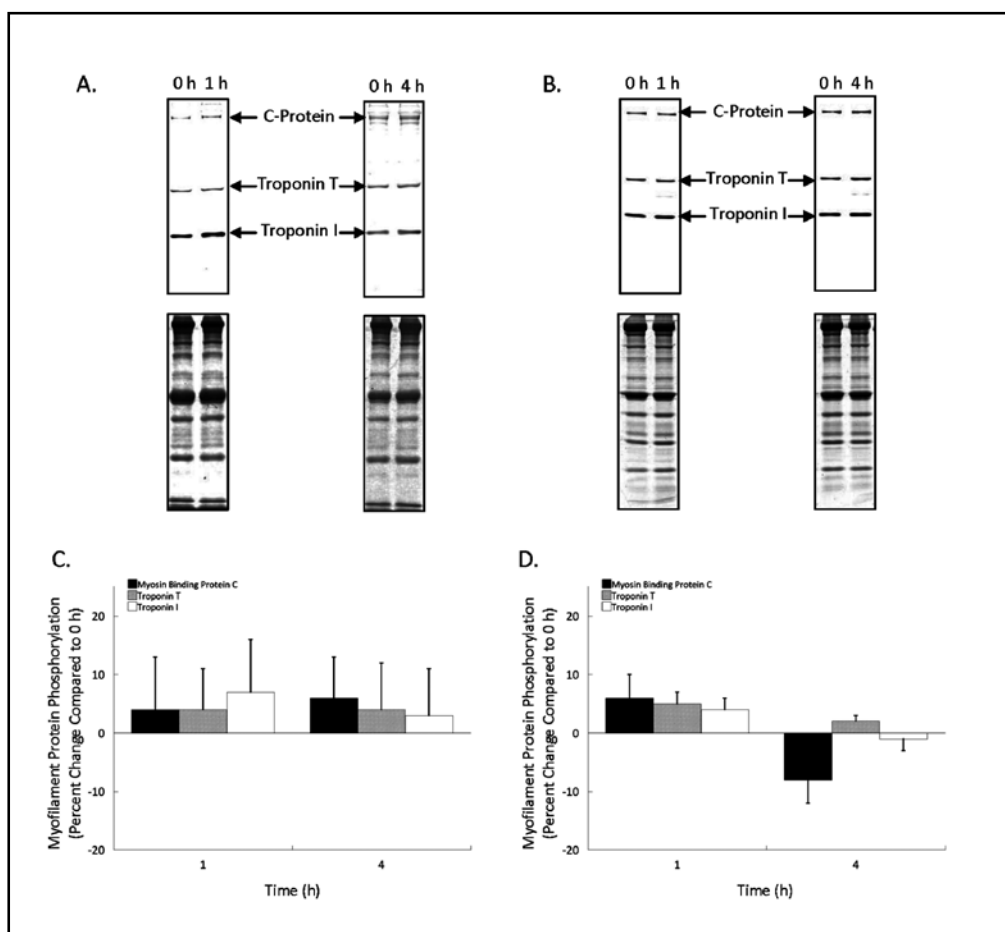
Fig. 5. Effects of cold, cardioplegic arrest on cardiac myofilament actomyosin MgATPase activity following Protein Kinase C inhibition. Protein Kinase C inhibition with chelerythrine chloride (10 μ M) abolished cardioplegic-dependent changes in actomyosin MgATPase activity. Wildtype actomyosin MgATPase activity was not different from control (0 h) after 1 (A) or 4 h (B) cold, cardioplegic arrest when hearts were stored in a buffer containing chelerythrine chloride. Actomyosin MgATPase activity of CapZ-deficient transgenic cardiac myofilaments was also unaltered following 1 (C) or 4 h (D) cold, cardioplegic arrest when chelerythrine chloride was included in the storage buffer. Data points are mean \pm SEM. N=5 for 0 h and 1 h; and 6 for 4 h. Equal numbers were included for wildtype and CapZ-deficient transgenic mouse hearts.



treated mouse hearts with the PKC inhibitor chelerythrine chloride during cardioplegic arrest. Chelerythrine

chloride had no effect on actomyosin MgATPase activity by itself (data not shown). In wildtype hearts the

Fig. 6. Impact of Protein Kinase C inhibition on cardioplegic-dependent changes in myofilament protein phosphorylation. Hearts were stored in cold St. Thomas cardioplegic solution supplemented with the Protein Kinase C inhibitor chelerythrine chloride (10 μ M). Myofilament proteins from wildtype (A and C) and CapZ-deficient transgenic (B and D) hearts were resolved by SDS-PAGE and examined for changes in phosphorylation. Representative phosphorylation gels (top gels) are presented in panels (A) and (B). Coomassie stained gels (bottom gels) are included to show equal loading across lanes. Graphs summarizing the collected data are shown in panels (C) and (D). Summary graphs are means \pm SEM. N=5 hearts for all time points. * P <0.05 vs. 0 h of respective group (wildtype or transgenic).



changes in maximum actomyosin MgATPase activity were abolished with PKC inhibition (Fig. 5). In CapZ-deficient transgenic myofilaments the increase in actomyosin MgATPase activity at high levels of free calcium was abolished.

PKC inhibition reduced or abolished the cardioplegic-dependent changes in myofilament protein phosphorylation in wildtype hearts (Fig. 6). Troponin I phosphorylation was reduced from 18% and 19% at 1 and 4 h cardioplegic, to 1% and 3% in hearts treated with chelerythrine chloride. Both myosin binding protein-C and troponin T showed small but consistent reductions in phosphorylation with PKC inhibition. CapZ-deficient myofilaments showed no significant changes in phosphorylation levels following chelerythrine chloride treatment.

Discussion

Myocardial dysfunction after prolonged, cold, cardioplegic arrest is a significant threat to post-transplant organ viability. Changes in cardiac myofilament func-

tion have been proposed to contribute to impaired myocardial performance following cardioplegic arrest, but the intracellular mechanisms responsible for these alterations have not been definitively identified. This study shows for the first time how the activation of PKC affects myofilament function and phosphorylation during cold cardioplegia. Moreover, we show that inhibition of PKC is sufficient to buffer myofilament functional changes for up to 4 h of cardioplegic storage. Finally, this is the first report showing that changes in the cardiac Z-disc protein CapZ are able to confer a protective benefit under stressful conditions.

Myocardial ischaemia time in heart transplants is increasing at a rate of 6 min/year through the combination of a prolonged surgical phase and transportation time [2]. Between 1995 and 2003 mean cardiac ischaemia times increased to 220 min, at the same time that 30 day survival rates declined from 91% to 84% [2]. These mean ischaemia times are approaching the limit of viability for cardiac tissue which is estimated at 4 h [18]. Our results show that contractile function of cardiac myofilaments decreases significantly by 4 h, a finding that is in agreement with the proposed limits for tissue viability. How-

ever, in myofilaments that are deficient in CapZ protein, function is relatively maintained at 4 h, suggesting a protective effect of the protein deficiency.

At the level of the whole heart a similar advantage is seen in CapZ-deficient transgenic mouse hearts, as myocardial contractility is maintained after 1 h cardioplegic arrest, whereas wildtype hearts show a significant decline in function. This cardioprotective effect is limited, however, as CapZ-deficient transgenic mice do not show any differences in myocardial contractility after 4 h of cardioplegic arrest. Cold cardioplegic arrest induces myocardial functional changes that are reflected in altered myofilament function [7]. It has been hypothesized that myocardial dysfunction following cardioplegic arrest may be due to myofilament protein proteolysis. Our data show that by 4 h there are no detectable changes in troponin T or I, which are in agreement with the findings of Powers et al. [7]. Together these findings indicate that cold cardioplegic arrest for a period of time that is similar to that experienced in heart transplantation causes no significant degradation of troponin T or I.

PKC is a family of serine/threonine kinases whose inhibitory effects on cardiac myofilaments are well known [19]. Sodha et al. [20] showed a movement of PKC- ϵ and - δ to cardiac Z-discs during cardioplegia in human samples. We have previously shown that this activation profile is associated with an inhibition of actomyosin MgATPase activity in the rat heart [8]. In the current study we demonstrate an increase in myofilament-associated PKC and found that inhibition of PKC abolished the decline in myofilament function during cold cardioplegic arrest. Moreover, the CapZ-deficient myofilaments that we have earlier reported to be resistant to PKC-dependent inhibition [12, 13], failed to exhibit a decline in activation. These data support the hypothesis that PKC activation during cold, cardioplegic arrest impairs myofilament activation.

The ubiquitous nature of PKC makes global, pharmacological inhibition a non-viable option for prolonging organ survival [21]. Even if an inhibitor is included in the cardioplegic solution after harvest and during transportation, its impact on non-myocyte cells of the heart could produce unexpected and unwanted side-effects. The observation that PKC inhibits myofilament activation and likely contributes to post-transplantation myocardial depression indicates that specifically targeting the functional interaction between PKC and cardiac myofilaments is a preferred approach. We show here that decreasing the sarcomeric protein CapZ prevents PKC-dependent inhibition of cardiac myofilaments. These data are in agree-

ment with our earlier work showing that CapZ is a key element in the transduction of the PKC signal to the myofilaments [12, 13]. Given the localization and confinement of CapZ to the myofilament compartment of cardiac myocytes, this protein offers a specific target for the blockade of PKC-dependent regulation of cardiac myofilaments, without the widespread inhibition offered by pharmacological agents.

During cold cardioplegic arrest we observed a decrease in actomyosin MgATPase activity and a trend towards decreasing calcium sensitivity. These data are in agreement with Spinale et al. [22] who found that during a 2h period of simulated cardioplegia, isolated myocytes saw a 40% decline in shortening velocity. However, both Powers et al. [7] and Fogelson et al. [23] failed to detect any reduction in maximum actomyosin MgATPase activity. This discrepancy may be due to the differences in the methods for cold storage. In our work we arrested hearts with ice-cold St. Thomas cardioplegic solution and maintained the hearts on ice for up to 4 h. By contrast Fogelson et al. [23] and Powers et al. [7] injected fresh cardioplegic solution every 15 min. The replacement of cardioplegic solution may remove elements that contribute to myofilament dysfunction. This suggestion is supported by the work of others who have found an improvement in organ survival with the continuous infusion of cold preservation fluid [24]. Despite the recognized benefits associated with continuous cold perfusion, this procedure presents a number of obstacles that makes its clinical use currently impractical [18].

Covalent modification of cardiac myofilament proteins through the addition or removal of phosphate groups is a well known regulatory mechanism. Recent work by Kirk et al has shown that increases in myofilament protein phosphorylation of less than 10% can profoundly impact myofilament and myocardial function [25]. This study is the first to examine changes in myofilament protein phosphorylation during cold-cardioplegic arrest. We observed time-dependent increases in the phosphorylation of several myofilament proteins that were attenuated by PKC inhibition. These data indicate that some phosphorylation changes associated with cardioplegic arrest are due to PKC activation, but the persistent Troponin I phosphorylation in particular suggests additional, PKC-independent pathways are simultaneously activated. The abolishment of the cardioplegic changes in actomyosin MgATPase activity without a full normalization of myofilament protein phosphorylation is consistent with the work of Sumandea et al. [26] who have shown that the phosphorylation of some

amino acids within myofilament proteins can yield no detectable changes in function.

Myofilaments from CapZ-deficient transgenic mouse hearts demonstrate a tendency towards decreased myofilament protein phosphorylation during cardioplegic storage, an effect that is apparently PKC-dependent as it is largely blocked by chelerythrine chloride. Our previous work has shown that CapZ-deficient myofilaments are resistant to PKC regulation [12, 13], but we have not addressed the possibility that PKC may still affect other signaling molecules that in turn control myofilament function. While the effects of protein phosphatases on cardiac myofilaments are relatively less understood than protein kinase signaling, it is known that PKC interacts directly or indirectly with both type 1 (PP1) and 2A (PP2A) protein phosphatases [27, 28]. Together these protein phosphatases account for 90% of the phosphatase activity in cardiac myocytes, and therefore represent the most probable candidates in the cascade involved here. PP1 has been shown to increase actomyosin MgATPase activity in both wildtype and CapZ-deficient myofilaments [15]. This effect would be consistent with the sustained actomyosin MgATPase activity seen in the CapZ-deficient transgenic myofilaments. The effects of PP2A on cross-bridge cycling have not yet been examined.

Cardiac transplantation necessitates a prolonged period of ischemia during organ harvest, transportation, and implantation. Ischaemic damage is typically mitigated

through cold crystalloid cardioplegia and storage in a protective solution. Despite the well recognized benefit of cold cardioplegic solutions, cardiac dysfunction resulting from transplantation-related ischaemia remains a significant problem [3, 29]. With increasing rates of organ transplantation, advances in organ storage techniques will improve patient outcomes and enlarge the geographical areas of transplantation centres. We note for the first time maintenance of myofilament function during cold cardioplegic arrest through the blockade of PKC-dependent myofilament inhibition. The data presented here put forward the Z-disc protein CapZ as a potential therapeutic target in cardiac transplantation. Together these findings shed light on the underlying intracellular mechanisms that contribute to myocardial dysfunction in the post-transplant heart, and offer insight into some potential strategies for the management of this disorder.

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