p21-Activated Kinase Increases the Calcium Sensitivity of Rat Triton-Skinned Cardiac Muscle Fiber Bundles via a Mechanism Potentially Involving Novel Phosphorylation of Troponin I

Nina Buscemi, D. Brian Foster, Irina Neverova, Jennifer E. Van Eyk

Abstract—Phosphorylation of myofilament proteins by kinases such as cAMP-dependent protein kinase and protein kinase C has been shown to lead to altered thin-filament protein-protein interactions and modulation of cardiac function in vitro. In the present study, we report that a small GTPase-dependent kinase, p21-activated kinase (PAK), increases the calcium sensitivity of Triton-skinned cardiac muscle fiber bundles. Constitutively active PAK3 caused an average 1.25-fold (25.0±6.0%, n=6) increase in force at pCa 5.75, 1.44-fold (44.0±7.78%, n=6) at pCa 6.25, and 2.41-fold (141.2±23.7%, n=4) at pCa 6.5, representing a change in pCa50 value of approximately 0.25. Constitutively active PAK3 produced no change in force under conditions of relaxation (pCa 8.0) or maximal contraction (pCa 4.5). Furthermore, an inactive, kinase-dead form of PAK3 failed to produce any change in force development at any pCa value. The myofilament proteins phosphorylated by PAK3, at pCa 6.5, are desmin, troponin T, troponin I, and an unidentified 70-kDa protein. Importantly, cardiac troponin I was found to be phosphorylated at serine 149 of human cardiac troponin I, representing a novel phosphorylation site. These findings suggest a novel mechanism of modulating the calcium sensitivity of cardiac muscle contraction. (Circ Res. 2002;91:509-516.)

Key Words: p21-activated kinase ■ calcium ■ cardiac ■ troponin I ■ phosphorylation

Signaling pathways of the heart form the basis of complex regulation of cardiac muscle contraction. Recently, pathways involving monomeric G proteins such as Rac1 and Cdc42, members of the Rho subfamily of Ras-related low molecular weight GTPases, have been the subject of intense research.1–2 One of the key effectors of Rac1 and Cdc42 is p21-activated kinase (PAK).3 Although some Rac1/Cdc42 effects are PAK-independent, PAK likely mediates at least some of the effects of these GTPases.4 PAK is a serine/threonine protein kinase that is widely expressed in mammalian tissues5 and at least three isoforms of PAK exist in the heart (PAK1, PAK2, and PAK3).5–8 PAK isoymes consist of two domains: an N-terminal regulatory domain and a C-terminal catalytic domain that is highly conserved across isoforms and species. Binding of Rac1/Cdc42 to the N-terminal regulatory domain of PAK is proposed to cause a conformational change in the kinase that relieves autoinhibition of the C-terminal domain. The release of inhibition of the catalytic domain of PAK allows for autophosphorylation at several sites and full activation of the kinase. In addition to monomeric G-protein activation, PAK may be activated by lipids such as sphingosine and phosphatidic acid, and PAK2, but not PAK1 or PAK3, may be activated by caspase-mediated cleavage. The role of PAK has been studied mainly in the context of nonmuscle cells, where it is involved in cytoskeletal reorganization, regulation of transcription, and apoptosis. Although the in vivo substrates of PAK remain elusive, PAK-mediated activation of the various subfamilies of the mitogen-activated protein kinase (MAPK) cascades is thought to be involved in the transcriptional effects of the kinase.9–14 However, PAK has been shown to cause calcium-independent contraction of Triton-skinned smooth muscle fiber bundles via phosphorylation of the contractile proteins desmin and caldesmon.15

Recently, the role of Rac1 and PAK in cardiac muscle function has begun to be explored. In a study by Clerk and Sugden,16 neonatal ventricular cardiac myocytes exposed to hyperosmotic shock showed a rapid and pronounced (approximately 2-fold) increase in PAK1 activation. In a similar study,17 PAK activity increased by approximately 1.5-fold, with peak activity occurring within 5 to 15 minutes after either hypoxia alone or hypoxia followed by reoxygenation. A transgenic mouse model of myocardial-specific expression of constitutively active Rac1 has supported a role for Rac and PAK in cardiac hypertrophy.18 These mice developed either lethal dilated cardiomyopathy or cardiac hypertrophy. In both phenotypes, PAK was found to translocate from the cytosolic to the cytoskeletal fraction, the latter also containing...
myofibrils, suggesting a role for PAK in cytoskeletal and possibly myofibrillar changes associated with cardiac hypertrophy.

In the present study, we show that PAK increases the calcium sensitivity of cardiac muscle contraction via a mechanism involving phosphorylation of several key myofilament proteins. These findings suggest a novel mechanism of modulating the calcium sensitivity of cardiac muscle contraction.

Materials and Methods
Preparation of Glutathione S-Transferase Mammalian PAK3 (GST-mPAK3) and GST-mPAK3K297R Proteins

Both constitutively active GST-mPAK3 and GST-mPAK3K297R mutant recombinant proteins were used in these studies.19 The mutation of lysine 297 in the ATP binding site of PAK is sufficient to render GST-mPAK3K297R unable to phosphorylate myelin basic protein, in vitro.19 The GST-mPAK3 fusion proteins were expressed in Escherichia coli and, when tested together, were simultaneously expressed, purified, and dialyzed against the same buffers. Both recombinant kinases were purified on a glutathione-Sepharose affinity column (Amersham Pharmacia Biotech), concentrated on a Centriprep 50 column (Amicon), and dialyzed against 10 mmol/L imidazole, pH 7.0. To ensure consistency, activities of the various constitutively active GST-mPAK3 preparations were standardized by their ability to phosphorylate myelin basic protein, in vitro.

Skinned Muscle Fiber Experiments

Rats were used in compliance with the Animals for Research Act (Province of Ontario), the Canadian Council on Animal Care, and the NIH (publication No. 85-23, revised 1985). Animals were supplied by Charles River Canada. Cardiac fiber bundles were generated from the trabeculae of adult Sprague-Dawley rats, as described in Strauss and colleagues,20,21 and skinned with 10% Triton X-100. Cardiac muscle fiber bundles (~0.1 mm in diameter) were mounted on an AME 801 (SensoNor) force transducer and adjusted to a sarcomere length of 2.2×10^{-2} mm, by laser diffraction, before analysis. Relaxing solutions consisted of 10 mmol/L (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) tetraethylammonium chloride, 20 mmol/L 3-(N-morpholino)propanesulfonic acid (MOPS), 50 mmol/L KCl, 6.8 mmol/L MgCl₂, 10 mmol/L creatine phosphate, 5.2 mmol/L ATP, 0.05 mmol/L phenylmethylsulfonylfluoride (PMSF), 3.6 μmol/L pepstatin, 2.3 μmol/L leupeptin, and 4 U/mL creatine phosphokinase (one unit will transfer 1 μmol of phosphate from phosphocreatine to ADP per minute at pH 7.4 at 30°C), buffered at pH 7.0. Contracting solution consisted of relaxing solution supplemented with additional CaCl₂, to produce solutions of varying pCa. The solutions used to bathe the fibers contained either GST-mPAK3 (~2 μg/mL or 13 μmol/L), GST-mPAK3K297R (~2 μg/mL or 13 μmol/L), or an equivalent volume of 10 mmol/L imidazole (PAK buffer) used in the dialysis of PAK. Skinned fiber experiments were conducted at 25°C. A Student’s t test was used to assess differences in force development in the absence and presence of GST-mPAK3 at a given pCa value. A difference with an associated probability value of less than 0.05 was considered statistically significant.

GST-mPAK3 Phosphorylation in Skinned Muscle Fiber Bundles

Skinned rat cardiac fiber bundles were mounted on U-shaped metal pins. Specifically, the ends of the fiber bundles were attached to the vertical portions of the pins with the use of an adhesive consisting of nitrocellulose solubilized in acetone.22 Fiber bundles were then incubated at 25°C under the various conditions used in the skinned fiber bundle assays described above, except, when required, assay buffers contained 1 mmol/L [γ³²P]-ATP (0.25 mCi/mL) instead of 7.2 mmol/L ATP. After 15 minutes of incubation in contracting solution of pCa 6.5 containing GST-mPAK3, fibers were submerged in ice-cold 15% trichloroacetic acid and 2 mmol/L inorganic phosphate followed by ice-cold acetone. Fibers were stored at −20°C until analysis. The phosphorylation reaction was carried out at pCa 6.5 because this was the calcium concentration at which PAK exerted the greatest effect on force development of skinned muscle fiber bundles.

Samples of radioactively labeled proteins from skinned fibers (4 skinned fibers/lane) were resolved by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on triplicate gels. One gel was stained with Coomassie Blue dye to confirm equal loading, and the others were transferred to nitrocellulose membranes. A number of autoradiographs were obtained by exposure of the nitrocellulose membranes to X-ray film (X-Omat Blue XB-1) for various periods of time and this procedure was followed by Western blotting of nitrocellulose membranes. Alignment of autoradiographs and Western blots was accomplished by pin-holing the nitrocellulose and autoradiography film with [γ³²P]-ATP and with the use of autoradiography labeling tape applied to nitrocellulose membranes as well as with the use of molecular weight standards, as previously described.15

Western Blot Analysis

SDS-PAGE and Western blot analysis were carried out as described previously.22 Western blot analysis of radiolabeled skinned fibers was carried out using DE-U-10 (Sigma), 4D-11 (Biodesign), 5C5 (Sigma) and C5 (Research Diagnostics) antibodies for the detection of desmin, cardiac troponin T (cTnT), sarcomeric actin, and cardiac troponin I (cTnI), respectively. Western blot analysis was also carried out on frozen left ventricular myocardial tissue samples from rat and swine, which were homogenized in 20 mmol/L Tris-Cl, 1× SDS-sample buffer (New England Biolabs), 2.3 μmol/L leupeptin, 1.5 μmol/L pepstatin A, and 0.25 mmol/L PMSF. PK3 and Rac1 were identified using anti-PAK3 (N-19) antibody (Santa Cruz; specific for the N-terminal 19-amino acid residues of mouse fibroblast mPAK3) and anti-Rac1 antibody (Transduction Laboratories, specific for residues 14–167 of human Rac1), respectively.

Protein and Peptides

Peptides were synthesized at the University of Alberta (Edmonton, Canada) or Queen’s University (Kingston, Canada), by the method of Triplet et al.23 Recombinant human cardiac TnI (hTnI), human cardiac TnT (hTnT), and human cardiac troponin C (hTnC) were obtained from Spectral Diagnostics (Toronto, Canada), and bovine cardiac troponin (hTn) was purified according to the method of Van Eyk et al.15 Isolation and purification of rabbit skeletal myosin and production of myosin subfragment 1 (myosin S1) and monomeric skeletal actin, as well as polymerization of actin were carried out as described previously.15 Purification of α/β skeletal tropomyosin (TM), the subsequent isolation of the α subunit, and the dimerization of α/α TM were carried out as described previously.24 Reconstitution of the troponin (Tn) complex from hTnC, hTnT, and hTnI was carried out as outlined previously.15 The complex showed calcium-dependent regulation of acto-S1-TM ATPase activity, demonstrating that it was in a functionally correct conformation. The Tn complex consisted of a 1:1:1 mol ratio of hTnI, hTnC, and hTnT, and the Tn:actin:myosin S1:TMMol Mol ratio was 1:7:2:2 as determined by quantification by reverse-phase high-performance liquid chromatography (HPLC) and 12% SDS PAGE.15 Protein and peptide concentrations were determined by amino acid analysis or by Lowry assay.15

In Vitro Phosphorylation Experiments

In vitro phosphorylation of cTnI synthetic peptides, hTnI, hTnC, and a synthetic peptide of skeletal actin residues 1–28 (actin 1–28) by GST-mPAK3, was carried out at 25°C in 20 mmol/L Tris-Cl, pH 7.5, 10 mmol/L MgCl₂, 1 mmol/L dithiothreitol (DTT), and 1 mmol/L [γ³²P]-ATP (5×10⁵ cpmmol). It is noteworthy that there is ~93%
amino acid sequence homology between rat and human isoforms of cTnI, permitting direct comparisons between isoforms. Aliquots of the reaction mixture were analyzed for protein phosphorylation using Whatman P81 paper and scintillation counting, as well as Western blotting and autoradiography of 12.5% SDS-PAGE and by dot blotting. In vitro autophosphorylation of GST-mPAK3 was carried out as described above except that the substrate was replaced with the equivalent volume of buffer in the reaction mixture. For dot blot analysis, the equivalent of 5 μg of total protein of the various γ²P-labeled substrates from GST-mPAK3 in vitro phosphorylations was applied to P81 paper, which was washed extensively with H₂PO₄, then dried with ethanol and acetone before autoradiography.

In Vitro Phosphorylation of Recombinant Human cTnT
In vitro phosphorylation of hcTnT by GST-mPAK3 was carried out at 30°C for 1 hour, under various salt conditions (either 50, 150, or 250 mmol/L NaCl), in 20 mmol/L Tris-Cl, pH 8.0, 5 mmol/L MgCl₂, 1 mmol/L DTT, and 0.5 mmol/L [γ²P]-ATP (5×10⁶ cpm/nmol). Aliquots of the reaction mixture were obtained at 2, 4, 10, 20, 40, and 60 minutes and analyzed by 15% SDS-PAGE to produce a Coomassie Blue-stained gel and a corresponding autoradiograph.

Mass Spectrometry
PAK phosphorylated or unphosphorylated hcTnT and hcTnT were digested with sequence-grade modified trypsin (Promega), mixed with 50 mmol/L α-cyano-4-hydroxy-trans-cinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid, spotted on a stainless steel 100-well mass spectrometry plate, and air-dried. Samples were analyzed using a Voyager DE-Pro matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems) operated in the delayed extraction/reflection mode ionization time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems) operated in the delayed extraction/reflection mode. Five spectra (50 to 100 laser shots/spectrum) were averaged using an accelerating voltage of 20 kV, grid voltage setting of 72%, and a 50-ns delay. Each spectrum was analyzed using a Voyager DE-Pro matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems) operated in the delayed extraction/reflection mode. Five spectra (50 to 100 laser shots/spectrum) were averaged using an accelerating voltage of 20 kV, grid voltage setting of 72%, and a 50-ns delay. Five spectra (50 to 100 laser shots/spectrum) were averaged using an accelerating voltage of 20 kV, grid voltage setting of 72%, and a 50-ns delay. Five spectra (50 to 100 laser shots/spectrum) were averaged using an accelerating voltage of 20 kV, grid voltage setting of 72%, and a 50-ns delay. Five spectra (50 to 100 laser shots/spectrum) were averaged using an accelerating voltage of 20 kV, grid voltage setting of 72%, and a 50-ns delay.

Results
Western Blot Analysis Establishes the Presence of Both Rac and PAK in Cardiac Muscle
The existence of the Rac/PAK signaling pathway in cardiac muscle of both rodent and large mammalian species was confirmed by Western blot analysis, which revealed the presence of Rac1 and PAK3 in rat and swine myocardium. An antibody raised against the N-terminal 19-amino acid residues of mouse fibroblast mPAK3 reacted with a protein of approximately 40 kDa, and adult rat (Figure 1B) myocardium, and an antibody raised against the N-terminal 19-amino acid residues of mouse fibroblast mPAK3 (right), respectively. Note that each lane of each Western blot represents tissue from a unique animal. B, Western blot of 12.5% SDS-PAGE analysis of adult rat left ventricular myocardium using the same mPAK3 antibody as in panel A. Experimental procedures are outlined in detail in Materials and Methods.

Skinned Muscle Fiber Experiments
Recombinant constitutively active GST-mPAK3 increased the calcium sensitivity of Triton-skinned adult rat cardiac muscle fiber bundles, as shown in the representative force measurement tracing of Figure 2B. GST-mPAK3 caused a leftward shift of the force-pCa curve with a decrease in pCa⁰ of approximately 0.25 (Figure 2A). Specifically, GST-mPAK3 caused a 1.25-fold (25.0±6.0%, n=6) increase in force at pCa 5.75, 1.44-fold (44.0±7.78%, n=6) at pCa 6.25, and 2.41-fold (141.2±23.7%, n=4) at pCa 6.5 (Figure 2A) while not producing any change under conditions of relaxation (pCa 8.0) or maximal contraction (pCa 4.5) (Figure 2A). Furthermore, recombinant inactive GST-mPAK3 caused a 78% decrease in force at pCa 6.5, the calcium concentration at which GST-mPAK3 produced the greatest increase in force development. GST-mPAK3 phosphorylated proteins of approximate molecular masses of 70, 50, 40, and 30 kDa (Figures 3A and 3B, autoradiographs). Western blot analysis of 15% SDS-PAGE identified three of these proteins as desmin (~50 kDa), cTnT (~40 kDa), and cTnI (~30 kDa) (Figures 3A and 3B, compare autoradiographs and corresponding Western blots). Because of the proximity of bands representing actin and cTnT on a 15% SDS-PAGE gel, Western blot analysis with anti-sarcomeric actin antibody was carried out to eliminate the possibility that the 40-kDa protein was actin. By this method, actin was shown not to be a substrate for GST-mPAK3 in the fibers (Figure 3B, Western blot). In addition, actin failed to become phosphorylated by GST-mPAK3 either in isolation (Figure 5B) or within the context of a biochemically reconstituted system (Figure 6B) in vitro.

GSTM-mPAK3–Mediated Phosphorylation in Skinned Muscle Fiber Bundles
Protein substrates for GST-mPAK3 in the cardiac skinned muscle fiber bundles were labeled with γ²P, at pCa 6.5, the calcium concentration at which GST-mPAK3 produced the greatest increase in force development. GST-mPAK3 phosphorylated proteins of approximate molecular masses of 70, 50, 40, and 30 kDa (Figures 3A and 3B, autoradiographs). Western blot analysis of 15% SDS-PAGE identified three of these proteins as desmin (~50 kDa), cTnT (~40 kDa), and cTnI (~30 kDa) (Figures 3A and 3B, compare autoradiographs and corresponding Western blots). Because of the proximity of bands representing actin and cTnT on a 15% SDS-PAGE gel, Western blot analysis with anti-sarcomeric actin antibody was carried out to eliminate the possibility that the 40-kDa protein was actin. By this method, actin was shown not to be a substrate for GST-mPAK3 in the fibers (Figure 3B, Western blot). In addition, actin failed to become phosphorylated by GST-mPAK3 either in isolation (Figure 5B) or within the context of a biochemically reconstituted system (Figure 6B) in vitro.

GSTM-mPAK3–Mediated Phosphorylation of cTnI and cTnT In Vitro
GSTM-mPAK3 phosphorylates recombinant hcTnI to approx-
imately 1 mol of phosphate/mol of TnI (Figures 4A and 5A, middle panel), with phosphorylation producing a change in mobility of the substrate by 12.5% SDS-PAGE (Figure 5A, left panel). This stoichiometry of phosphorylation was evident under various salt conditions (data not shown), and phosphorylation of hcTnI by GST-mPAK3 also occurred within the context of a biochemically reconstituted system (Figure 6B). To determine the exact site of hcTnI phosphorylation by GST-mPAK3, peptide mass fingerprinting (using trypsin) of both the unphosphorylated and phosphorylated substrate was carried out using MALDI-TOF mass spectrometry. By this method, the phosphorylation site was determined to reside within the peptide fragment hcTnI 148–161, suggesting that serine 149 of hcTnI is the amino acid residue phosphorylated by GST-mPAK3, in vitro (Figure 4B).

To confirm the site of hcTnI phosphorylation by GST-mPAK3, a series of in vitro phosphorylation reactions involving GST-mPAK3 and synthetic peptides of select regions of hcTnI were carried out. GST-mPAK3 phosphorylated a synthetic peptide of hcTnI 128–180 (Figure 5A, right panel) and hcTnI 146–162 (A160T) (Figure 5B, lane 6). However, GST-mPAK3 failed to phosphorylate appreciably recombinant hcTnI 1–99 and the synthetic peptides hcTnI 136–147 and hcTnI 137–147 (T142P) (Figure 5B, lanes 2, 3, and 4, respectively), in vitro. Taken together, these results confirm that recombinant hcTnI is phosphorylated at serine 149, by GST-mPAK3, in vitro (Figure 5C).

cTnT
A similar in vitro analysis was carried out on recombinant hcTnT. Importantly, we observed a positive linear relationship between the extent of phosphorylation of recombinant hcTnT by GST-mPAK3 and the NaCl concentration of the incubation solution. Specifically, we found a 3-fold increase in the extent of recombinant hcTnT phosphorylation under conditions of 250 mmol/L NaCl compared with 50 mmol/L NaCl (data not shown). At high salt concentration, the stoichiometry of hcTnT phosphorylation by GST-mPAK3 approached 0.5 mol phosphate/mol hcTnT (data not shown). It is noteworthy that optimal conditions for GST-mPAK3–

Figure 2. Constitutively active GST-mPAK3 increases calcium sensitivity (ΔpCa50 ≈ 0.25) of Triton X-100–skinned adult rat cardiac muscle fiber bundles. A, Relative force/pCa curves of skinned cardiac fiber bundles contracted in the presence of PAK buffer, in the absence (-) and presence (+) of constitutively active GST-mPAK3. Each data point represents an average of analysis of several (n = 4 to 6) Triton X-100–skinned cardiac fiber bundles subjected to a given experimental condition. B, Typical isometric force tracing of skinned cardiac fiber bundles subjected to constitutively active GST-mPAK3 at pCa 6.25. Fibers were contracted and relaxed in the presence of PAK buffer (in the absence of GST-mPAK3) before incubation with GST-mPAK3 at pCa 6.25. Experimental procedures are outlined in detail in Materials and Methods. *Statistically significant difference.

Figure 3. GST-mPAK3 phosphorylates desmin, troponin T, troponin I, and an unidentified 70-kDa protein in the skinned cardiac muscle fiber bundles. The protein substrates of GST-mPAK3 in the skinned muscle fibers were identified by autoradiography and Western blot analysis. Skinned cardiac muscle fiber bundles were incubated at pCa 6.5 in the presence of [γ-32P]ATP in the absence of GST-mPAK3 (PAK buffer, lane 1) or in the presence of GST-mPAK3 (GST-mPAK3, lane 2). A and B, Autoradiographs of short (A) and longer (B) exposures of 15% SDS-PAGE analysis of skinned cardiac muscle fiber bundles, from which corresponding Western blots were generated using anti-desmin (A), anti-troponin T (B), anti-actin (B), and anti-troponin I (B) antibodies. C, Corresponding Coomassie Blue–stained gel confirming equal loading of approximately 3 to 4 radiolabeled skinned cardiac muscle fiber bundles per lane. Experimental procedures are outlined in detail in Materials and Methods.
mediated phosphorylation of hcTnT in vitro were never achieved and this was due to the fact that hcTnT was only soluble at high salt concentrations and GST-mPAK3 became less effective under these conditions (data not shown). However, under all conditions tested, there was a progressive, time-dependent increase in GST-mPAK3–mediated phosphorylation of recombinant hcTnT in vitro (Figure 6A, autoradiograph, lanes 1 through 6). Indeed, when recombinant hcTnT was analyzed in the context of a biochemically reconstituted system, there was, again, a progressive, time-dependent increase in phosphorylation of this protein in the presence of GST-mPAK3 (Figure 6B, autoradiograph, lanes 1 through 6).
besides PKA and PKC, may be involved in modulation of muscle contraction and imply that other cellular kinases, mechanism of modulating the calcium sensitivity of cardiac activated ATPase activity of the contractile apparatus, in vitro.

Conclusions

The findings outlined in this report suggest a novel mechanism of modulating the calcium sensitivity of cardiac muscle contraction and imply that other cellular kinases, besides PKA and PKC, may be involved in modulation of myofilament calcium responsiveness of cardiac muscle via modification of Tn. Specifically, we show that constitutively active GST-mPAK3 increases the calcium sensitivity of Triton-skinned cardiac muscle fiber bundles ($p_{Ca_{50}} \approx 0.25$) via a mechanism involving phosphorylation of four myofilament proteins, namely, desmin, Tnl, TnT, and an unidentified 70-kDa protein. In the present study, we speculate on how these phosphorylation events may be involved in a mechanism of calcium sensitization of cardiac muscle contraction.

**GST-mPAK3–Mediated Phosphorylation of cTnI**

GST-mPAK3–induced calcium sensitization of cardiac muscle contraction may involve phosphorylation of Ser149 of cTnI (Figures 4 and 5), which is located within the second TnC binding domain of TnI (cTnI 148–163). This region of TnI forms a calcium-sensitive interaction with the N-terminal regulatory domain of TnC. Specifically, in the presence of elevated intracellular calcium, the second TnC binding domain of TnI binds the N-domain of TnC, resulting in preferential binding of the inhibitory region of TnI (cTnI 128–147) with the C-domain of TnC instead of actin-TM. The release of TnI inhibitory region (cTnI 136–147) binding to actin-TM facilitates crossbridge attachment and, ultimately, contraction. Introduction of a negatively charged phosphate group into the second TnC binding domain of cTnI may result in greater calcium affinity of cTnC via (1) increased affinity of the second cTnC binding domain of cTnI (cTnI 147–163) with cTnC and/or (2) a reduction in the calcium off-rate of cTnC. Interestingly, the calcium-sensitizing agent bepridil, a small hydrophobic molecule, appears to exert its effects on cardiac muscle by altering the second TnC binding domain of cTnI (cTnI 147–163).

**GST-mPAK3–Mediated Phosphorylation of Desmin**

Desmin was found to be a substrate for GST-mPAK3 in the permeabilized skinned fiber system (Figure 3A), which has also been shown in other systems. In vitro characterization of desmin phosphorylation by PAK1 reveals that PAK1 phosphorylates mainly serine residues of the head region of desmin (residues 1–88), to a stoichiometry of 2.5 mol phosphate/mol desmin. It is known that sarcomere length and interfilament lattice spacing are intimately linked to calcium sensitivity and ATPase activity of muscle fiber strips. Thus, modification of these structural properties of the myofilament, with GST-mPAK3–mediated phosphorylation of desmin, may be part of a mechanism of PAK-induced calcium sensitization of cardiac muscle fiber contraction.

**GST-mPAK3–Mediated Phosphorylation of cTnT**

TnT was phosphorylated by GST-mPAK3 in the permeabilized cardiac fibers (Figure 3B), in isolation (Figure 6A) and within the context of a biochemically reconstituted system (Figure 6B) and may, therefore, be involved in a mechanism of PAK-induced calcium sensitization of force. Identification of amino acid residues phosphorylated by GST-mPAK3, in
vitro, as determined by MALDI-TOF mass spectrometry, revealed that hcTnT is phosphorylated in both the N- and C-terminal domains.

A correlation was found between pCa_{50} of rabbit myocardium and the expression of cTnT isoforms, which tend to vary in the N-terminal region. In addition, reconstitution of rabbit permeabilized cardiac muscle fibers with C-terminal mutated or truncated forms of TnT resulted in altered force development characteristics of muscle fibers. Thus, both the N- and C-terminal regions of cTnT are important for determining calcium sensitivity of the myofilament, and modification of these regions may result in altered myofilament calcium responsiveness.

Interestingly, we observed that the ratio of the stoichiometry of TnT to TnI phosphorylation by GST-mPAK3 was reversed in the biochemically reconstituted system compared with the permeabilized system, as assessed by autoradiography of SDS-PAGE (compare Figures 3B and 6B). This is the first study, to date, in which kinase substrates were identified in skinned cardiac fiber bundles and phosphorylation levels compared with corresponding isolated/purified components; therefore, the degree to which these results should be similar is unclear. However, such a finding has precedence in a similar study of skinned smooth muscle fiber bundles. This finding may be explained by fundamental structural differences between these model systems. Specifically, the permeabilized muscle fiber system is highly ordered, with an established sarcomere and interfilament lattice spacing that includes desmin and the 70-kDa protein substrate of GST-mPAK3. In contrast, these structural characteristics are either modified or absent in the reconstituted system. Thus, each system may produce a unique configuration of protein conformations that promotes or inhibits specific interactions between GST-mPAK3 and the other proteins of the system.

To recapitulate, we report a kinase-induced calcium sensitization of cardiac muscle contraction potentially involving a novel phosphorylation of cTnI. Under normal conditions of the heart, PKA may be involved in balancing the effects of calcium-desensitizing kinases, such as PKA and PKC, so as to fine-tune the myofilament response to calcium. In support of such an interaction, it has been demonstrated that PKA negatively regulates PKA in nonadherent cells, inhibiting MAPK activation, in vivo. Under pathological conditions of the heart, such as heart failure, it is possible that PKA acts to increase force production via two mechanisms, namely, increased calcium sensitivity of the myofilibr apparatus and morphological alterations of the cytoskeleton so as to promote protective remodeling of the heart. However, the role of PKA in normal and pathological functioning of the heart still remains to be explored.

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