Protein Kinase D Is a Novel Mediator of Cardiac Troponin I Phosphorylation and Regulates Myofilament Function

Robert S. Haworth,* Friederike Cuello,* Todd J. Herron, Gereon Franzen, Jonathan C. Kentish, Mathias Gautel, Metin Avkiran

Abstract—Protein kinase D (PKD) is a serine kinase whose myocardial substrates are unknown. Yeast 2-hybrid screening of a human cardiac library, using the PKD catalytic domain as bait, identified cardiac troponin I (cTnI), myosin-binding protein C (cMyBP-C), and telethonin as PKD-interacting proteins. In vitro phosphorylation assays revealed PKD-mediated phosphorylation of cTnI, cMyBP-C, and telethonin, as well as myomesin. Peptide mass fingerprint analysis of cTnI by liquid chromatography–coupled mass spectrometry indicated PKD-mediated phosphorylation of a peptide containing Ser22 and Ser23, the protein kinase A (PKA) targets. Ser22 and Ser23 were replaced by Ala, either singly (Ser22Ala or Ser23Ala) or jointly (Ser22/23Ala), and the troponin complex reconstituted in vitro, using wild-type or mutated cTnI together with wild-type cardiac troponin C and troponin T. PKD-mediated cTnI phosphorylation was reduced in complexes containing Ser22Ala or Ser23Ala cTnI and completely abolished in the complex containing Ser22/23Ala cTnI, indicating that Ser22 and Ser23 are both targeted by PKD. Furthermore, troponin complex containing wild-type cTnI was phosphorylated with similar kinetics and stoichiometry (≈2 mol phosphate/mol cTnI) by both PKD and PKA. To determine the functional impact of PKD-mediated phosphorylation, Ca$^{2+}$ sensitivity of tension development was studied in a rat skinned ventricular myocyte preparation. PKD-mediated phosphorylation did not affect maximal tension but produced a significant rightward shift of the tension–$p_{Ca}$ relationship, indicating reduced myofilament Ca$^{2+}$ sensitivity. At submaximal Ca$^{2+}$ activation, PKD-mediated phosphorylation also accelerated isometric crossbridge cycling kinetics. Our data suggest that PKD is a novel mediator of cTnI phosphorylation at the PKA sites and may contribute to the regulation of myofilament function. (Circ Res. 2004;95:1091-1099.)

Key Words: protein kinase D | cardiac troponin I | protein phosphorylation | contractile function | calcium sensitivity | crossbridge cycling kinetics

Protein kinase D (PKD), whose human homologue was originally named protein kinase C (PKC) μ, is a serine kinase that was discovered in 1994.1,2 PKD consists of an N-terminal regulatory domain (which contains 2 cysteine-rich, zinc finger-like motifs and a pleckstrin homology domain) and a C-terminal catalytic domain (Figure 1). Its structural and enzymatic properties distinguish PKD from established PKC isoforms.3). PKD does not phosphorylate several PKC substrates1,4 and, relative to PKC isoforms, it has been classified into a distinct branch (the CAMK superfamily) of the kinase complement of the human genome.5 Nevertheless, as with classical and novel PKC isoforms, the tandem repeat of cysteine-rich motifs within the N-terminal regulatory domain of PKD bind phorbol esters with high affinity,1,4 and PKD has been shown to be activated in vitro by diacylglycerol and phorbol esters in the presence of phosphatidylserine.4 More recently, a second mechanism of PKD activation has been identified, which involves phosphorylation of PKD via a PKC-dependent pathway.6 It appears, therefore, that PKD can act either in parallel with or downstream of PKC.

Since the discovery of the archetypal kinase,1,2 the biological functions of PKD and its newer isoforms (PKD2 and PKD3/PKCνα) have been undergoing intense investigation, with proposed roles in processes as diverse as the control of cell growth and survival and Golgi organization and function, in various cell types.8). In our laboratory, we have obtained evidence that PKD regulates the activity of the Na’/H’ exchanger isoform 1 in COS7 and A10 fibroblasts.9 Nevertheless, very little information is currently available on the regulation and role(s) of PKD in myocardial cells. Previously, we have shown that PKD is expressed in rat ventricular myocytes, where its activity is stimulated by G protein-coupled receptors in a PKC-mediated manner,11 raising the possibility that increased PKD activity may modulate myocardial responses to PKC-activating stimuli. In the present

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study, we performed a yeast 2-hybrid (Y2H) screen of a human cardiac library using the PKD catalytic domain as bait to identify myocardial proteins that represent potential PKD substrates. Subsequently, we obtained evidence that 3 myofilament proteins highlighted by the Y2H assay as PKD-interacting proteins, namely the inhibitory subunit of cardiac troponin (cTnI), cardiac myosin-binding protein C (cMyBP-C), and telethonin, as well as the M band protein myomesin, were substrates for PKD-mediated phosphorylation in vitro. We focused on PKD-mediated phosphorylation of cTnI and identified the pertinent phosphorylation sites by peptide mass spectrometric analysis and site-directed mutagenesis. And we determined the functional consequences of PKD-mediated myofilament phosphorylation in chemically permeabilized (“skinned”) rat ventricular myocyte fragments.

Materials and Methods

Purified human cTnI and protein kinase A (PKA) catalytic subunit were from Sigma-Aldrich. Recombinant human cTnI, cardiac troponin C (cTnC), and troponin T (TnT), expressed and purified as described,12 were kind gifts from Douglas G. Ward and Ian Trayer (University of Birmingham). Recombinant telethonin, cMyBP-C fragment C0-C2, and myomesin fragments My2–8 and My9–13 were expressed and purified as described.13–15 Recombinant PKD catalytic domain expressed in Sf21 insect cells was a kind gift from Harold Jefferies and Peter J. Parker (Cancer Research UK). Anti-catalytic domain expressed in Sf21 insect cells was a kind gift from Douglas G. Ward and Ian Trayer (University of Birmingham). Recombinant telethonin, cMyBP-C, and telethonin C (cTnC), and the reconstituted troponin complex were incubated with active PKD or the PKA catalytic subunit and 32P-ATP for up to 60 minutes at 37°C. Proteins were subsequently resolved on 9% or 12% acrylamide gels. For peptide mass fingerprint analysis, recombinant human cTnI was phosphorylated by active PKD in the presence of nonradiolabeled ATP.

Kinetics and Stoichiometry of Phosphorylation

Reconstituted troponin complexes were incubated with active PKD or PKA catalytic subunit and 32P-ATP for 0 to 60 minutes at 37°C. Proteins were then resolved on 12% acrylamide gels and subjected to autoradiography. For determination of stoichiometry, the troponin complex containing wild-type cTnI was incubated with active PKD or PKA catalytic subunit and 32P-ATP for 60 minutes at 37°C in the presence (100 μmol/L) or absence of Ca2+. After stopping the reaction, aliquots were spotted onto P81 paper, washed in H3PO4, and radiolabel was incorporation determined.

Peptide Mass Fingerprint Analysis

Recombinant human cTnI phosphorylated by active PKD was subjected to tryptic digestion and analysis by liquid chromatography–coupled mass spectrometry.

Immunoblot Analysis

Immunoblot analysis was performed as described previously,11 using specific antibodies for total or phosphorylated cTnI, myomesin, cMyBP-C, and telethonin.

Functional Studies in Skinned Myocyte Fragments

Myocyte fragments were clamped to a sensitive force transducer and a high-speed length controller at either end, as described previously.19–21 All mechanical experiments were performed at 18°C, with sarcomere length set to ±2.0 μm in relaxing solution. Skinned myocytes were activated in solutions (pH 7.10) containing a Ca2+ concentration ranging between 10−9 (pCa 9.0; relaxing solution) and 10−4.5 mol/L (pCa 4.5; maximal Ca2+ activating solution). Isometric crossbridge cycling kinetics were assessed by a release–restretch maneuver after Ca2+–activated tension had reached a steady-state (using a method described by Brenner22 and adapted to cardiac muscle23) and the rate of tension redevelopment (krest) was quantified. An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Identification of PKD-Interacting Cardiac Proteins

To identify PKD-interacting proteins, we performed a Y2H screen of a human cardiac library using the catalytically inactive Lys618Asn mutant of the PKD catalytic domain as bait. We elected to use this mutant, in preference to the wild-type equivalent, to promote a sustained interaction between the PKD catalytic domain and potential substrate proteins, which may otherwise dissociate on substrate phosphorylation. The Y2H screen revealed significant interactions between the PKD catalytic domain and the sarcromeric proteins cTnI, cMyBP-C, and telethonin.

PKD-Mediated Phosphorylation of Cardiac Myofilament Proteins

The regulatory domain of PKD exerts an auto-inhibitory effect on kinase activity, such that its deletion renders the PKD catalytic domain constitutively active.1,24 To study PKD-mediated phosphorylation of sarcromeric proteins, ventricular myofilibrillar preparations from adult rat hearts were

Figure 1. Key features of PKD. Cys1 and Cys2 indicate the cysteine-rich zinc finger-like domains that can bind phorbol esters and diacylglycerol, and PH domain indicates the pleckstrin homology domain that exerts an auto-inhibitory effect. Ser744 and Ser748 are PKC phosphorylation sites in the PKD activation loop and Ser916 is an autophosphorylation site. The recombinant PKD fragments that were used as bait in the yeast 2-hybrid screen (Y2H bait) and in vitro phosphorylation assays (active PKD) are also illustrated.
exposed to recombinant PKD catalytic domain (“active PKD” in Figure 1) in the presence of 32P-ATP in an in vitro phosphorylation assay. As illustrated in Figure 2A, this assay revealed PKD-mediated phosphorylation of several proteins, which migrated at ~20, 30, 150, and 190 kDa, in parallel with autophosphorylation of active PKD (50 kDa). On the basis of the Y2H findings and apparent molecular mass, we speculated that the 20, 30, and 150 kDa substrates might be telethonin, cTnI, and cMyBP-C, respectively. Immunoblot analysis revealed that each of these proteins was present in the myofibrillar preparation and migrated at the same molecular mass as each of the pertinent PKD substrates (Figure 2A). The myofibrillar preparation also contained the M band protein myomesin, a known phosphoprotein,15 which migrated at the same molecular mass as the 190-kDa PKD substrate (Figure 2A). To determine whether these proteins are phosphorylated by PKD, we used recombinant proteins comprising myomesin fragments My2-8 and My9-13, cMyBP-C fragment C0-C2, full-length cTnI, and full-length telethonin as substrates in an in vitro kinase assay. As illustrated in Figure 2B, myomesin fragment My2–8, cMyBP-C fragment C0-C2, cTnI, and telethonin, each of which contains at least one known phospho-acceptor site that conforms to the minimum PKD recognition motif of ArgXaaXaaSer, were all phosphorylated by PKD. In contrast, no phosphorylation of myomesin fragment My9-13 could be detected (data not shown); notably, the proposed C-terminal phospho-acceptor region of myomesin,25 which is contained in the My9-13 fragment, does not contain a PKD recognition motif. On the basis of these data, we identified myomesin (N-terminal domain), cMyBP-C (N-terminal domain), cTnI, and telethonin as putative PKD substrates. Subsequently, we focused on PKD-mediated phosphorylation of cTnI, because phosphorylation of this protein is known to have an important role in the regulation of myofilament function.26

Identification of PKD Phosphorylation Sites in cTnI

To identify the cTnI residues that are targeted by PKD, we subjected recombinant human cTnI to peptide mass fingerprint analysis by liquid chromatography–coupled mass spectrometry, after a 30-minute phosphorylation in vitro by active PKD and tryptic digestion. This approach achieved 78% coverage of the cTnI sequence and, as illustrated in Figure 3A, identified peptide fragments containing Ser22 and Ser23 (commonly referred to as the PKA sites) as the phospho-acceptor regions. Both monophosphorylated and bisphosphorylated peptides were detected (with phosphorylation-induced miscleavage, as described27) and tandem mass spectrometry fragmentation of the latter resulted in the loss of two phosphate groups (Figure 3A). Ser198 was identified as an additional phosphorylation site (Figure 3A).

To confirm that Ser22 and Ser23 are targeted by PKD, we then performed in vitro phosphorylation assays using active PKD (or, for comparison, the PKA catalytic subunit) and recombinant human cTnI, the latter as either the wild-type protein or a mutated form with replacement of both Ser residues by nonphosphorylatable Ala (Ser22/23Ala). When the phosphorylation status of wild-type cTnI was assessed by immunoblot analysis, using a phosphospecific antibody that recognizes the phosphorylated (pSer22/23) protein, there was a significant increase in the signal in response to PKD and PKA, indicating that both enzymes do target Ser22 and Ser23 (Figure 3B). As also shown in Figure 3B, no signal was detected by this antibody when the Ser22/23Ala mutant was used as substrate, regardless of the presence of PKD or PKA, confirming the specificity of the antibody for pSer22/23 cTnI. When in vitro phosphorylation was

Figure 2. Autoradiograms illustrating the phosphorylation by PKD of (A) cardiac myofilament proteins and (B) recombinant myomesin fragment My2–8, cMyBP-C fragment C0-C2, cTnI, and telethonin in in vitro phosphorylation assays. The immunoblots (A) show that myomesin (6% acrylamide gel; 1:1000 antibody dilution), cMyBP-C (6%; 1:10,000), cTnI (12%; 1:1000), and telethonin (12%; 1:1000) are present in the cardiac myofilament protein preparation and comigrate with the 190-, 150-, 30-, and 20-kDa substrates, respectively. The Coomassie-stained gels in the lower part of (B) indicate protein loading. Con indicates control (no kinase).
performed in the presence of ^32P-ATP and detected by autoradiography, wild-type cTnI protein was phosphorylated by both PKD and PKA (Figure 3B). As expected, the Ser22/23Ala mutation markedly attenuated but did not abolish PKA-mediated phosphorylation of cTnI (Figure 3B). PKD-mediated phosphorylation of cTnI was also only attenuated by the double mutation (Figure 3B), which is consistent with the existence of an additional site at Ser198.

To identify the cTnI site(s) that are accessible for PKD-mediated phosphorylation within the troponin complex, we next reconstituted the complex in vitro, using recombinant wild-type or mutated (Ser22Ala, Ser23Ala, or Ser22/23Ala) cTnI together with recombinant wild-type cTnC and TnT. Figure 4A confirms that wild-type cTnI and each of the cTnI mutants could be incorporated into the troponin complex, because an identical high-molecular-mass complex was apparent in a nondenaturing gel, regardless of the identity of the cTnI constituent. In vitro phosphorylation of the complexes by PKD and PKA in the presence of ^32P-ATP and subsequent SDS-PAGE and autoradiography revealed partial attenuation of cTnI phosphorylation by each of the single (Ser22Ala or Ser23Ala) mutations and complete abolition of such phosphorylation by the double (Ser22/23Ala) mutation (Figure 4B). Interestingly, the Ser23Ala mutation...
had a greater impact than the Ser22Ala mutation on cTnI phosphorylation by either PKD or PKA (Figure 4B). This is consistent with our detection of the monophosphorylated peptide RSSNYR by liquid chromatography–coupled mass spectrometry, because this cleavage pattern has been shown to arise when only Ser23 is phosphorylated. These data indicate that PKD, like PKA, targets both Ser22 and Ser23 in cTnI, and that Ser23 is the favored phosphorylation site. In the troponin complex, Ser198 in cTnI is not accessible for phosphorylation by PKD and is therefore unlikely to be of physiological relevance.

### Figure 4
Experiments with troponin complexes reconstituted in vitro, using recombinant cTnI (as the wild-type protein [WT] or a mutated form with Ala replacement of Ser22 and Ser23, either singly [S22A, S23A] or jointly [S22/23A]) together with recombinant wild-type cTnC and TnT. A, Coomassie-stained nondenaturating gel, illustrating comparable incorporation of WT and mutated cTnI into the complexes. B, Autoradiogram obtained after the complexes were phosphorylated by PKD or PKA in the presence of $^{32}$P-ATP and proteins resolved by 12% SDS/PAGE. The Coomasie stained gel in the lower part of (B) indicates protein loading. Con indicates control (no kinase).

### Time-course of cTnI phosphorylation in troponin complex
Experiments with troponin complexes reconstituted in vitro, using recombinant cTnI (as the wild-type protein [WT] or a mutated form with Ala replacement of either Ser22 [S22A] or Ser23 [S23A], together with recombinant wild-type cTnC and TnT. The complexes were phosphorylated by PKD or PKA in the presence of $^{32}$P-ATP and proteins resolved by 12% SDS/PAGE. Top panel shows a representative Coomassie-stained denaturing gel to illustrate protein loading. Autoradiograms in the bottom panel illustrate the time course of phosphorylation of wild-type and mutant cTnI in the relevant complexes.

### Kinetics and Stoichiometry of PKD-Mediated cTnI Phosphorylation
Having identified Ser22 and Ser23 as the principal PKD phosphorylation sites in cTnI, we next determined the kinetics and stoichiometry of such phosphorylation by PKD relative to PKA. Reconstituted troponin complexes containing wild-type or mutated cTnI were subjected to in vitro phosphorylation by PKD or PKA for 0 to 60 minutes in the presence of $^{32}$P-ATP and phosphate incorporation into cTnI detected by autoradiography. As illustrated in Figure 5, phosphorylation of the complexes containing wild-type cTnI or the Ser22Ala mutant by either PKD or PKA occurred rapidly and reached saturation within 5 to 10 minutes. In contrast, when the troponin complex containing Ser23Ala cTnI was used as substrate, the phosphorylation reactions exhibited slower kinetics and required 15 to 30 minutes to reach saturation (Figure 5). These data are consistent with Ser23 being the preferred substrate for both PKD and PKA. To determine the relative stoichiometry of phosphorylation by PKD versus PKA, and to determine the potential impact of Ca$^{2+}$ on each reaction, the troponin complex containing wild-type cTnI was subjected to in vitro phosphorylation...
by PKD or PKA for 60 minutes in the presence of $^{32}$P-ATP and in the presence (100 μmol/L) or absence of Ca$^{2+}$. In the presence of Ca$^{2+}$, PKD and PKA phosphorylated wild-type cTnI to $\approx 2.3 \pm 0.1$ and $2.2 \pm 0.1$ mol phosphate/mol cTnI, respectively. In the absence of Ca$^{2+}$, these figures were reduced to $\approx 1.5 \pm 0.1$ and $1.7 \pm 0.1$ mol phosphate/mol cTnI, respectively. We conclude from these data that the kinetics, stoichiometry, and Ca$^{2+}$ sensitivity of PKD-mediated phosphorylation of cTnI are similar to those of PKA-mediated phosphorylation.

**Functional Consequences of PKD-Mediated Myofilament Phosphorylation**

Because PKA-mediated phosphorylation of Ser22 and Ser23 in cTnI is associated with reduced Ca$^{2+}$ sensitivity of myofilaments,28,29 we next determined whether PKD-mediated phosphorylation produces such a response in skinned myocytes from the adult rat left ventricle. There was a significant increase in the phosphorylation of Ser22 and Ser23 in cTnI after a 30-minute exposure of the skinned myocyte preparation to active PKD, as revealed by immunoblot analysis (Figure 6A). Among the myocyte fragments that were used for functional assessment (Figure 6B), there was no difference between the control and PKD-treated groups in fragment size, sarcomere length, or the maximal tension at $p_{Ca}$ 4.50 (Table). However, PKD-mediated phosphorylation significantly reduced the Ca$^{2+}$ sensitivity of myofilaments (Figure

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<th>Dimensions and Maximal Tension of Myocyte Fragments Used for Functional Measurements</th>
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All values mean ± SEM (n=5).
mediated phosphorylation of cardiac myofilaments has a functional impact on both the Ca$^{2+}$ sensitivity of tension development and the crossbridge cycling kinetics.

To date, most investigative effort in phosphorylation-mediated regulation of cTnI function has focused on the actions of PKA and PKC. Evidence from studies in a variety of systems, ranging from reconstituted myofilament proteins to cultured myocytes or transgenic animals with heterologous expression of modified cTnI proteins, indicates that PKA-mediated functional effects (eg, decreased myofilament Ca$^{2+}$ sensitivity) are mediated principally through the phosphorylation of Ser22/23 (equivalent to Ser23/24, including the initiating Met, in the rodent sequence) within the unique N-terminal extension of cTnI.36 In contrast, PKC-mediated effects (eg, decreased maximum actomyosin ATPase activity or maximum tension) are mediated mainly by phosphorylation of Ser43/45 (rodent sequence) in a neighboring domain,26 with phosphorylation of Thr144 also likely to contribute.31 Recent evidence suggests that p21-activated kinase may also target cTnI and lead to phosphorylation of Ser149.32 Our data indicate that PKD targets specifically the “PKA sites” at Ser22/23, within the sequence ArgArgArgSerSer that is conserved in cTnI from multiple species. Notably, the targeted Ser residues within cTnI display the minimum recognition motif of ArgXaaXaaSer in common with 2 known physiological substrates of PKD, namely Ser919 in the neuronal protein Kidins22033 and Ser351 in the Ras effector RIN1,34 as well as with the synthetic peptide Syntide-2, which is phosphorylated by PKD with high efficiency.1 Thus, cTnI is likely to represent a physiological substrate for PKD in myocardium, particularly because active PKD phosphorylated Ser22/23 in cTnI not only when the substrate protein was used in isolation (Figure 2B and 3B) but also when it was incorporated within the troponin complex (Figure 4 and 5) or the myofilament lattice (Figures 2A and 6A). In the light of recent evidence that phosphomimetic substitution of Ser22/23 in cTnI modulates systolic and diastolic function of mouse hearts in vivo,35 PKD warrants attention as a novel mechanism through which phosphorylation of these residues may be regulated under physiological and/or pathophysiological conditions.

To obtain an indication of the functional consequences of PKD-mediated myofilament protein phosphorylation, we examined the effects of exposure to active PKD in a skinned myocyte preparation. On the whole, our new data mirror earlier observations on the functional consequences of PKA-mediated myofilament protein phosphorylation. First, we have shown that PKD-mediated phosphorylation significantly reduces myofilament Ca$^{2+}$ sensitivity, lowering the pCa$m_{50}$ by $\approx-0.22$ U (Figure 6D). PKA-mediated phosphorylation has been shown previously to produce comparable effects in similar skinned muscle preparations.36,37 Second, we have shown that at submaximal pCa, PKD-mediated phosphorylation accelerates isometric crossbridge cycling kinetics, with a significant increase in $k_w$ (Figure 7B). Again, previous studies in skinned muscle preparations from mouse37,38 and rat39 hearts have demonstrated accelerated crossbridge cycling kinetics in response to PKA-mediated phosphorylation. Furthermore, in intact rat trabeculae, PKA-mediated acceleration of crossbridge cycling kinetics has been shown to contribute to the enhancement of twitch relaxation and peak power output by $\beta$-adrenoceptor stimulation.40 PKA-

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**Discussion**

Covalent modification of cTnI by kinase-mediated phosphorylation is an important mechanism in the regulation of thin filament function and thereby the cardiac contractile phenotype.26 Furthermore, altered phosphorylation of cTnI and other myofilament proteins may contribute causally to cardiac dysfunction in the transition from compensated hypertrophy to heart failure.30 In this context, the present work shows, for the first time to our knowledge, that PKD interacts with and directly phosphorylates a number of myofilament proteins, including cTnI at Ser22 and Ser23, and that PKD-

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**Figure 7.** Effects of PKD-mediated phosphorylation on isometric crossbridge cycling kinetics in skinned myocytes. A, Representative recordings of tension redevelopment after a release–restretch protocol, under control conditions, and after PKD-mediated phosphorylation, obtained from multiple experiments like those illustrated in (A) (n=5 per group; *P<0.05). The data were obtained at pCa values that produced $\approx-40\%$ maximal tension.

6C), resulting in a rightward shift of the tension–pCa relationship (Figure 6D). Thus, pCa at 50% maximal tension ($pCa_{50}$) was 5.72±0.04 in the control group and 5.50±0.03 in the PKD-treated group (n=5; P<0.05). Also, at $\approx-40\%$ maximal tension, PKD-mediated phosphorylation accelerated isometric crossbridge cycling kinetics (Figure 7), such that $k_w$ measured 0.98±0.14 s$^{-1}$ in the control group and 2.13±0.28 s$^{-1}$ in the PKD-treated group (n=5, P<0.05).
mediated acceleration of crossbridge cycling kinetics most likely arises from the phosphorylation of Ser22/23 in cTnI, because the replacement of cTnI with the slow skeletal isoform (ssTnI), which lacks the N-terminal domain that contains these residues, abolished this effect in mouse trabeculae.38 At present, we cannot preclude the possibility that phosphorylation of other putative sarcomeric substrates (cMyBP-C, myomesin, and telethonin) may also contribute to the functional effects of active PKD in the skinned myocyte preparation. Furthermore, the stoichiometry and targets of PKD-mediated phosphorylation in cMyBP-C, myomesin, and telethonin remain to be determined. Notwithstanding, it is clear from our data that PKD activation represents a novel pathway through which functionally important changes may be brought about by the phosphorylation of cTnI (at Ser22/23) and possibly the other myofilament proteins.

Our previous work has indicated that in cardiac myocytes,11 as in other cell types,41 (patho)physiologically relevant neurohumoral stimuli induce PKD activation in a PKC-mediated manner. On the basis of the present data, the potential contribution of PKD-mediated myofilament protein phosphorylation to myocardial contractile responses to PKC-activating stimuli needs to be considered. At present, direct investigation of the role(s) of PKD in mediating such responses is hindered by the lack of selective pharmacological inhibitors of PKD.42 Nevertheless, our present data may allow alternative interpretations of some pertinent data from the literature. For example, in a recent study, the replacement of native cTnI in mouse myocardium by a Ser22/23Ala mutant postulated the ability of PKC activation by phorbol 12-myristate 13-acetate or endothelin-1 (ET-1) to increase myocardial Ca2+ sensitivity.43 Furthermore, in response to phorbol 12-myristate 13-acetate or ET-1, a more substantial reduction in maximal actomyosin ATPase activity was seen in myofibrillar preparations from mice expressing Ser22/23Ala cTnI, relative to equivalent preparations from wild-type animals.43 These data indicate that in response to PKC activation, the functional consequences of PKD-mediated phosphorylation of Ser34/45 (and possibly Thr144) in cTnI are partially opposed by the concurrent phosphorylation of Ser22/23.44 The latter may be mediated by PKD, which is itself activated by PKC-activated phosphorylation of Ser744/748 in its activation loop.5 Another pertinent recent report by Westfall and Borton44 showed that relaxation of adult rat ventricular myocytes in primary culture was accelerated by ET-1 and that this response was abrogated by pretreatment with pharmacological PKC inhibitors and in cells in which cTnI was replaced by ssTnI, after adenoviral gene transfer. The possibility again exists that PKD-mediated phosphorylation of Ser22/23 in cTnI, downstream of PKC activation, contributed to the ET-1–induced acceleration of relaxation that was observed,44 particularly in the light of our present data on the effects of active PKD on myofilament Ca2+ sensitivity and crossbridge cycling kinetics, both of which would be expected to accelerate relaxation.

In conclusion, our study provides the first evidence to our knowledge that PKD-mediated phosphorylation of cTnI and other sarcomeric proteins may represent a novel signaling mechanism in the regulation of myofilament function. Interestingly, myomesin and telethonin have been implicated in myofilament assembly and turnover, but not in contraction; therefore, it is possible that PKD may play regulatory roles in these processes, in parallel with or in addition to other kinase pathways. Confirmation of the physiological role(s) of PKD, particularly in response to PKC-activating stimuli like ET-1, awaits the fruition of our ongoing efforts to develop novel molecular approaches that will allow the specific alteration of PKD activity in myocardium.

Acknowledgments

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Protein kinase D is a novel mediator of cardiac troponin I phosphorylation and regulates myofilament function

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EXPANDED MATERIALS AND METHODS:

Materials:
Purified human cTnI and protein kinase A (PKA) catalytic subunit were from Sigma-Aldrich Company Ltd. Expression vectors for human cTnI, cardiac troponin C (cTnC) and troponin T (TnT) and corresponding recombinant proteins, expressed and purified as described previously,1 were kind gifts from Douglas G. Ward and Ian Trayer (University of Birmingham). Recombinant telethonin, cMyBP-C fragment C0-C2 (N-terminal 3 immunoglobulin-I domains and intervening regions) and myomesin fragments My2-8 (N-terminal 2 immunoglobulin-cII and 5 fibronectin type III domains and intervening regions) and My9-13 (C-terminal 5 immunoglobulin-cII domains and intervening regions) were expressed and purified as described previously.2-4 Recombinant PKD catalytic domain expressed in Sf21 insect cells was a kind gift from Harold Jefferies and Peter J. Parker (Cancer Research UK). Rabbit polyclonal antibodies against total and phosphorylated (pSer22/23) cTnI were from Cell Signaling Technology. Rabbit polyclonal antibodies against cMyBP-C and telethonin, and rat polyclonal antibody against myomesin have been described previously.5-7 In some experiments,
adult male Wistar rats (approximately 250 g, B&K Universal Ltd) were used, in accordance with institutional and national regulatory requirements.

**Y2H screen of human cardiac library:**

The Matchmaker Y2H system was used as recommended by the manufacturer (Clontech). Briefly, the cDNA encoding aa 575-918 of PKD containing a Lys618Asn mutation (i.e. kinase-inactive catalytic domain; “Y2H bait” in Figure 1) was amplified by PCR and ligated into the bait vector pGBK7. The construct was transformed into *Saccharomyces cerevisiae* AH109 and yeast colonies able to grow on tryptophan-deficient media were tested for the expression of the bait protein, using an antibody directed at the PKD C-terminus. A single colony was selected for subsequent library screening. A human cardiac cDNA library (cDNA pooled from the hearts of 3 male Caucasians, aged 28-47 y), fused to the activating domain (aa 768-881) of GAL4 in plasmid pACT2, was titred by serial dilution. Yeast already containing the PKD bait were transformed with the library, and spread on selective plates lacking tryptophan (rescued by the bait vector), leucine (rescued by the library vector) and adenosine (interaction of the bait with a library protein drives the expression of ADE2, which permits growth in the absence of adenosine). This produced 4.4 x 10⁴ colonies per µg of library DNA. Library plasmids were recovered from individual yeast colonies after growth on ampicillin (selective for the library vector but not the bait vector) and, following transformation into *Escherichia coli* DH5α, up to 5 bacterial colonies per yeast DNA sample were tested for the presence of multiple library plasmids in yeast colonies by restriction digest mapping of the recovered plasmids. To test for the presence of false positive clones, recovered library plasmids were transformed back into AH109, together with either pGBK7 or the PKD bait plasmid. Growth was tested on nutrient-deficient plates to confirm the requirement for both library and bait proteins. Transformants able to grow in the absence of the PKD bait were eliminated from further study. Colonies that were able to grow on the appropriate selective media and additionally gave a positive β-galactosidase test indicated an interaction between PKD and a library protein. The selected
plasmids were sequenced using a primer based on the GAL4 activation domain and analyzed by reference to the GenBank public database, to identify individual PKD-interacting proteins.

“Skinned” myocyte preparation:
Rats were anesthetized with pentobarbitone sodium (60 mg/kg i.p.) and treated with labetalol (10 mg/kg i.v.), to minimize adrenergic activation of myofilament regulatory signaling pathways during subsequent surgery for heart excision. Heparin (100 U i.v.) was administered 15 min after labetalol injection and the heart excised and immersed in ice-cold relaxing solution, containing 10 mmol/L EGTA, 100 mmol/L BES, 55 mmol/L potassium propionate, 5 mmol/L ATP, 10 mmol/L creatine phosphate, 1 mmol/L free Mg$^{2+}$, 10 µmol/L leupeptin, 1 µmol/L E64, 0.5 mmol/L AEBSF and 1 mmol/L dithiothreitol (total ionic strength 200 mmol/L; pH 7.1). Skinned left ventricular cardiac myocyte fragments were then prepared as previously described. In brief, the myocardial tissue was homogenized in a Waring blender (~5 s in ice cold relaxing solution) and, following centrifugation (1400 rpm, 1 min), the myocyte pellet was re-suspended in Triton X-100 (1% v/v in relaxing solution) for 10 min, to disrupt lipid membranes. Following 2 further cycles of similar centrifugation and re-suspension, the myocyte pellet was washed, re-suspended and kept in ice-cold relaxing solution, until used for in vitro phosphorylation assays or mechanical measurements.

Reconstitution of the troponin complex:
The troponin complex was reconstituted in vitro, using recombinant wild-type or mutated cTnI together with recombinant wild-type cTnC and TnT, as described by Ward et al. In brief, cTnI (wild-type or Ser22Ala, Ser23Ala or Ser22/23Ala mutant) was mixed with TnT and cTnC, in a 1:2:2 molar ratio (to ensure the absence of free cTnI), in solubilization buffer containing 6 mol/L urea, 1 mol/L NaCl, 1 mmol/L CaCl$_2$ and 25 mmol/L MOPS/NaOH (pH 7.0). This mixture was then dialyzed for 48 h against 5 L of a buffer containing 300 mmol/L KCl, 5 mmol/L MgCl$_2$, 1 mmol/L dithiothreitol, 1 mmol/L CaCl$_2$ and 20 mmol/L MOPS/KOH (pH 7.0), with the dialysis-buffer exchanged after the first 24 h. The protein concentration of solutions containing the troponin
complex was determined by a Bradford protein assay and 5 µg of each complex was subjected to non-denaturing PAGE (for confirmation of complex formation) or used in *in vitro* phosphorylation assays.

**In vitro phosphorylation assays:**

*Cardiac myofibrils:* Skinned myocyte preparations were resuspended in kinase assay buffer (400 nmol/L calyculin A, 1 mmol/L dithiothreitol, 15 mmol/L MgCl₂ and 30 mmol/L Tris; pH 7.4) and aliquots containing approximately 200 µg myofibrillar protein were incubated for 30 min at 30 °C in the absence or presence of active PKD (350 ng), together with 100 µmol/L \(^{32}\)P-ATP. Proteins were resolved on 12 % acrylamide gels, which were subsequently dried and subjected to autoradiography. For functional studies, skinned myocyte preparations were phosphorylated by a 30-min incubation at room temperature in relaxing solution containing active PKD (7 ng/µL).

*Recombinant proteins:* Recombinant cTnI (200 pmol), cMyBP-C fragment C0-C2 (150 pmol), myomesin fragments My2-8 and My9-13 (40 pmol), telethonin (200 pmol) and the reconstituted troponin complex (5 µg) were incubated with active PKD (35 ng) and 100 µmol/L \(^{32}\)P-ATP in kinase assay buffer for up to 60 min at 37 °C. Proteins were subsequently resolved on 9 or 12 % acrylamide gels. Where indicated, the PKA catalytic subunit (5 U) was used, instead of active PKD. For peptide mass fingerprint analysis by liquid chromatography-coupled mass spectrometry (LC-MS), recombinant cTnI was phosphorylated by active PKD as above, but in the presence of non-radiolabeled ATP (1 mmol/L).

**Kinetics and stoichiometry of phosphorylation:**

For determination of the time course of phosphorylation, reconstituted troponin complexes (5 µg) were incubated with active PKD (35 ng) or PKA catalytic subunit (5 U) and 100 µmol/L \(^{32}\)P-ATP in kinase assay buffer for 0-60 min at 37 °C. The proteins were then resolved on 12-15 % acrylamide gels, dried and subjected to autoradiography. For determination of stoichiometry, the troponin
complex containing wild-type cTnI was incubated with active PKD (35 ng) or PKA catalytic subunit (5 U) and 100 µmol/L $^{32}$P-ATP in kinase assay buffer for 60 min at 37 °C. The reaction was carried out either in the presence (100 µmol/L) or absence (added 5 mmol/L EGTA and 10 mmol/L MgAc) of Ca$^{2+}$. The reaction was stopped by the addition of H$_3$PO$_4$ (62.5 mmol/L final concentration), and aliquots were spotted onto Whatman P81 phosphocellulose filter paper. Unincorporated radiolabel was removed by extensive washing in H$_3$PO$_4$ (75 mmol/L) and, after the papers were air-dried, radiolabel incorporation was determined by Cerenkov counting.

**Peptide mass fingerprint analysis:**

NH$_4$HCO$_3$ (16.7 mmol/L final concentration) and CaCl$_2$ (100 µmol/L final concentration) were added to aliquots of *in vitro* phosphorylation products, containing approximately 60 µg of recombinant cTnI. After heat denaturation of cTnI for 90 s at 56 °C, tryptic digestion was carried out overnight at 37 °C, essentially as described. For peptide mass fingerprint analysis of the tryptic digestion products, 50 µL of the solution was analyzed by LC-MS, using an HPLC system (Series 1100, Agilent Technologies) coupled to an electrospray ionization mass spectrometer (Finnigan LCQ Advantage MAX, Thermo Electron Corp.). For HPLC separation of the peptides, a CC 250/4 Nucleosil 100-5 C18 Nautilus column (Macherey-Nagel GmbH) was used, with a linear gradient over 60 min of 0-65 % solution B in solution A (solution A, 0.1 % formic acid in water; solution B, 0.1 % formic acid in acetonitrile). Mass spectra were analyzed using Finnigan Xcalibur software (Thermo Electron Corp.) and the peptide masses assigned, as previously described, using ExPASy software.

**Immunoblot analysis:**

Samples (40 µg myofibrillar protein or 0.2 µg recombinant protein) in Laemmli buffer were subjected to SDS-PAGE on 6 or 12 % acrylamide gels and transferred to polyvinylidene difluoride membranes. Non-specific binding sites were blocked by incubation of the membranes in 10 % non-fat milk in Tris-buffered saline containing 0.1 % Tween-20. The membranes were then probed for
the presence of total or phosphorylated cTnI, myomesin, cMyBP-C or telethonin, using specific antibodies (see Materials). Bound antibody was labeled with horseradish peroxidase-conjugated anti-rabbit or anti-rat secondary antibody, as appropriate, and the antibody complexes detected using the Amersham ECL system. To confirm equal protein loading, membranes were stained with Coomassie brilliant blue.

Functional studies in skinned myocyte fragments:

For mechanical measurements, myocyte fragments (see Figure 6B) were clamped to a sensitive force transducer (Model 403A, 200 mV/mg, Cambridge Technology, Inc.) and a high-speed length controller (Model 308B, Aurora Scientific, Inc.) at either end, as described previously.\(^8,10\) The rest of the experimental apparatus was as described previously,\(^8,11\) with minor modification. All mechanical experiments were performed at 18 °C. Sarcomere length was acquired (240 Hz CCD camera) and analyzed using commercial software (IonOptix Corp.). The sarcomere length was set to ~2.0 µm in relaxing solution for all functional measurements. Solution changes were made using a fast stepper motor attached to 2 parallel capillary tubes (Model SF-77B Perfusion Fast-Step, Warner Instrument Corp.). pClamp software (Axon Instruments) was used to trigger the stepper motor for solution changes or to impose rapid length changes on the myocyte fragment, for assessment of crossbridge cycling kinetics (see below). Force and length signals were recorded on a computer using a 12-bit analog/digital board, sampling at 2 kHz. Skinned myocytes were activated in solutions (pH 7.10) containing a Ca\(^{2+}\) concentration ranging between 10\(^{-9}\) (pCa 9.0; relaxing solution, composition as given above) and 10\(^{-4.5}\) mol/L (pCa 4.5; maximal Ca\(^{2+}\) activating solution), to measure the Ca\(^{2+}\) sensitivity of myofilaments.

Isometric crossbridge cycling kinetics were assessed by performing a release-restretch maneuver after Ca\(^{2+}\)-activated tension had reached a steady-state, using a method that was first described by Brenner\(^{12}\) and subsequently adapted to investigate isometric crossbridge cycling kinetics in cardiac muscle.\(^{13}\) The rate of tension redevelopment (\(k_o\)) was measured after forcible detachment of crossbridges during Ca\(^{2+}\) activation, by a rapid release and restretch of the muscle.
Briefly, the myocyte length was decreased by 20% in 1 ms, held for a 15-ms period, and then stretched back to the original length in 1 ms, following which tension redevelopment was recorded. The myocyte was subsequently relaxed. This maneuver was first performed in maximal Ca\(^{2+}\) activating solution (pCa 4.5) and then in a series of submaximal [Ca\(^{2+}\)] solutions, ending with a repeat in maximal Ca\(^{2+}\) activating solution. Force redevelopment following the release-restretch protocol was fitted with a single-exponential curve, using Clampfit software (Axon Instruments).
REFERENCES:


