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Protein Kinase D Selectively Targets Cardiac Troponin I and Regulates Myofilament Ca^{2+} Sensitivity in Ventricular Myocytes

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Abstract—Protein kinase D (PKD) is a serine/threonine kinase with emerging myocardial functions; in skinned adult rat ventricular myocytes (ARVMs), recombinant PKD catalytic domain phosphorylates cardiac troponin I at Ser22/Ser23 and reduces myofilament Ca^{2+} sensitivity. We used adenoviral gene transfer to determine the effects of full-length PKD on protein phosphorylation, sarcomere shortening and $[Ca^{2+}]_i$ transients in intact ARVMs. In myocytes transduced to express wild-type PKD, the heterologously expressed enzyme was activated by endothelin 1 (ET1) (5 nmol/L), as reflected by PKD phosphorylation at Ser744/Ser748 (PKC phosphorylation sites) and Ser916 (autophosphorylation site). The ET1-induced increase in cellular PKD activity was accompanied by increased cardiac troponin I phosphorylation at Ser22/Ser23; this measured approximately 60% of that induced by isoproterenol (10 nmol/L), which activates cAMP-dependent protein kinase (PKA) but not PKD. Phosphorylation of other PKA targets, such as phospholamban at Ser16, phospholemman at Ser68 and cardiac myosin-binding protein C at Ser282, was unaltered. Furthermore, heterologous PKD expression had no effect on isoproterenol-induced phosphorylation of these proteins, or on isoproterenol-induced increases in sarcomere shortening and relaxation rate and $[Ca^{2+}]_i$ transient amplitude. In contrast, heterologous PKD expression suppressed the positive inotropic effect of ET1 seen in control cells, without altering ET1-induced increases in relaxation rate and $[Ca^{2+}]_i$ transient amplitude. Complementary experiments in "skinned" myocytes confirmed reduced myofilament Ca^{2+} sensitivity by ET1-induced activation of heterologously expressed PKD. We conclude that increased myocardial PKD activity induces cardiac troponin I phosphorylation at Ser22/Ser23 and reduces myofilament Ca^{2+} sensitivity, suggesting that altered PKD activity in disease may impact on contractile function. (***Circ Res*. 2007;100:864-873.**)

Key Words: protein kinase D ■ cardiac troponin I ■ protein phosphorylation ■ contractile function ■ calcium sensitivity

Protein kinase D (PKD), previously referred to as protein kinase C (PKC) μ, is a serine/threonine kinase that consists of an N-terminal regulatory domain (containing 2 cysteine-rich, zinc finger-like motifs and a pleckstrin homology domain) and a C-terminal catalytic domain.[1,2] Its structural and enzymatic properties distinguish PKD from established PKC isoforms (see review by Rozengurt et al[3]). Indeed, PKD does not phosphorylate several PKC substrates[1,4] and has been classified into the calcium/calmodulin-dependent protein kinase superfamily of the kinase complement of the human genome.[5] Nevertheless, as with classical and novel PKC isoforms, the N-terminal regulatory domain of PKD binds phorbol esters with high affinity,[1,4] and PKD has been shown to be activated in vitro by diacylglycerol (DAG) and phorbol esters, in the presence of phosphatidylserine.[4] More recently, a second mechanism of PKD activation, involving PKD phosphorylation via a PKC-dependent pathway,[6] has been identified. Thus, 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[7] and PKD3/PKCν[8]) have been under 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.[3,9] In our laboratory, we have obtained evidence that PKD regulates the activity of the Na^+/H^+ exchanger isoform 1 in COS7 fibroblasts and A10 vascular smooth muscle cells.[10] Nevertheless, only limited information is currently available on the regulation and role(s) of PKD in myocardial cells. In this regard, we have shown previously

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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-activation stimuli. Indeed, there is now evidence that, in neonatal rat ventricular myocytes (NRVMs) exposed to neurohormonal stimuli, PKD plays a key role in the phosphorylation of class II histone deacetylase (HDAC) isoforms, in particular HDAC5, and thereby in the transcriptional regulation of hypertrophic gene expression.[12] In our own recent work, we have screened a human cardiac library in a yeast 2-hybrid (Y2H) assay, using the PKD catalytic domain as bait, and identified several myocardial proteins as potential PKD substrates.[13] This work also (1) revealed that the inhibitory subunit of cardiac troponin (cTnI) and cardiac myosin-binding protein C (cMyBP-C) not only interact with PKD in the Y2H assay but are also substrates for PKD in vitro; (2) identified Ser22 and Ser23 (the "protein kinase A [PKA] sites") as the PKD-mediated phosphorylation sites in cTnI; (3) showed that exposure of chemically permeabilized ("skinned") rat ventricular myocyte fragments to recombinant PKD catalytic domain induces cTnI dual phosphorylation at Ser22/Ser23, reduces myofilament Ca^{2+} sensitivity, and accelerates isometric cross-bridge cycle kinetics.[13] To explore the relevance of these findings to the intact myocyte, we have now used adenoviral gene transfer to determine the role of full-length PKD in regulating cTnI phosphorylation and contractile function in adult rat ventricular myocytes (ARVMs).

Materials and Methods

Detailed methodology is provided in the online data supplement, available at http://circres.ahajournals.org. Key techniques involved adaptations of previously published protocols, including those for the isolation, culture and adenoviral infection of adult rat ventricular myocytes,[14,15] generation of adenoviral vectors,[16] in vitro phosphorylation assays and immunoblot analysis,[13] phosphoprotein staining,[17] measurement of $[Ca^{2+}]_i$ transients and sarcomere shortening in intact myocytes,[18] and assessment of myofilament Ca^{2+} sensitivity in skinned myocytes.[19]

Results

Heterologous PKD Expression in ARVMs

To permit the heterologous expression of full-length PKD in ARVMs, we constructed the adenoviral vector AdV:PKDwt/EGFP, which expresses wild-type mouse PKD and enhanced green fluorescent protein (EGFP) driven by separate cytomegalovirus promoters. As illustrated in Figure 1A, infection of ARVMs with AdV:PKDwt/EGFP produced dose-dependent PKD expression. At a multiplicity of infection (moi) of 50 plaque-forming units (PFU) per cell, there was a robust increase in PKD expression and the transduced cells could be readily identified by EGFP fluorescence (Figure 1A), for single-cell assessment of their contractile phenotype (see later). Heterologously expressed PKD was activated by exposure to endothelin 1 (ET1) (5 nmol/L) in a time-dependent manner, as reflected by increased dual phosphorylation of Ser744/Ser748 (PKC phosphorylation sites within the PKD activation loop[20]) and phosphorylation of Ser916 (PKD autophosphorylation site[21]), thus achieving the desired objective of increased cellular PKD activity relative to control cells infected with AdV:EGFP (Figure 1B).

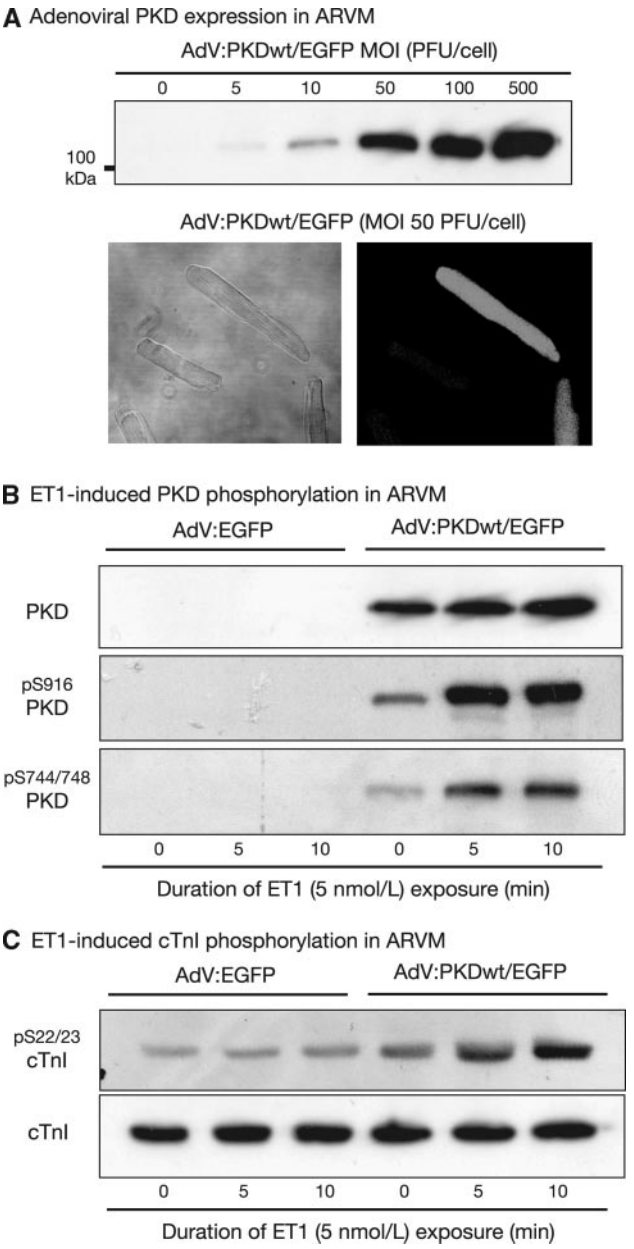


Figure 1. A, Expression of wild-type mouse PKD (PKDwt) in ARVMs infected with AdV:PKDwt/EGFP at a moi of 5 to 500 PFU/cell. PKD expression was determined 18 to 24 hours after infection by immunoblotting with an antibody that recognizes total PKD. Bottom, Identification of infected ARVMs by EGFP fluorescence, after infection at a moi of 50 PFU/cell. B, PKD expression and ET1-induced PKD phosphorylation at Ser916 and Ser744/Ser748 in ARVMs infected with AdV:EGFP or AdV:PKDwt/EGFP at a moi of 50 PFU/cell. Immunoblots are representative of 3 independent experiments. C, cTnI expression and ET1-induced cTnI phosphorylation at Ser22/Ser23 in ARVMs infected with AdV:EGFP or AdV:PKDwt/EGFP at a moi of 50 PFU/cell. Immunoblots are representative of 6 independent experiments.

Effect of Heterologous PKD Expression on Protein Phosphorylation

To determine the impact of increased cellular PKD activity on cTnI phosphorylation in intact ARVMs, we performed

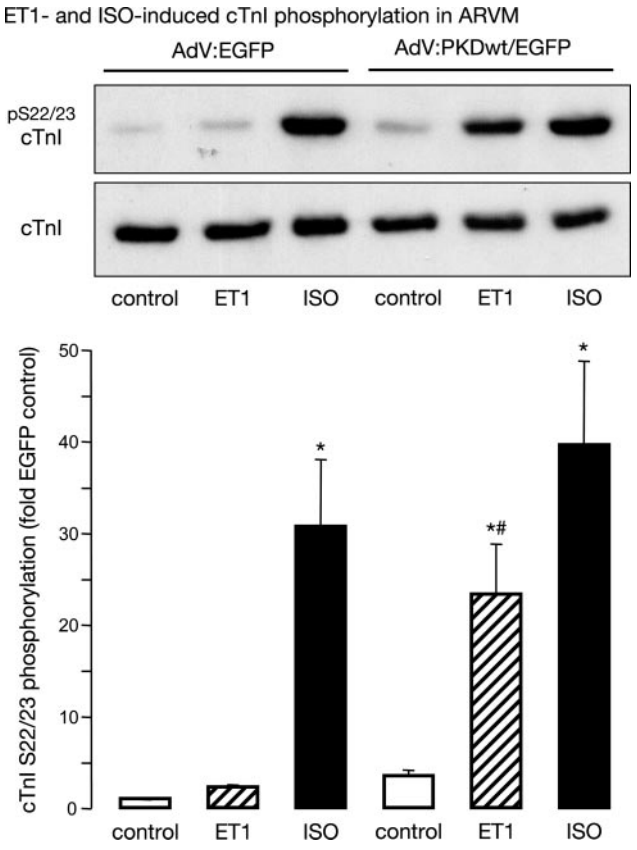


Figure 2. cTnI expression and agonist-induced cTnI phosphorylation at Ser22/Ser23 in ARVMs infected with AdV:EGFP or AdV:PKDwt/EGFP at a moi of 50 PFU/cell. Cells were exposed to ET1 (5 nmol/L) or ISO (10 nmol/L) for 10 minutes. Immunoblots are representative of 7 independent experiments, and the bar chart shows quantitative data on cTnI phosphorylation from those experiments. *$P<0.05$ vs control group infected with same adenovirus; #$P<0.05$ vs corresponding group infected with AdV:EGFP.

immunoblot analysis with a phospho-specific antibody that recognizes cTnI following its dual phosphorylation at Ser22/Ser23.[13] In ARVMs infected with AdV:PKDwt/EGFP, increased cellular PKD activity in response to ET1 (Figure 1B) was paralleled by increased cTnI phosphorylation at Ser22/Ser23 (Figure 1C). To estimate the extent and thus the likely functional impact of the increased cTnI phosphorylation seen in ARVMs infected with AdV:PKDwt/EGFP, we also examined the cTnI phosphorylation response to β-adrenoceptor (βAR) stimulation by isoproterenol (ISO) (10 nmol/L), which activates PKA but not PKD.[11] As illustrated in Figure 2, in control cells infected with AdV:EGFP, ET1 induced a small increase in cTnI phosphorylation at Ser22/Ser23, whereas ISO induced a large and significant increase in the same parameter. Infection with AdV:PKDwt/EGFP slightly elevated basal cTnI phosphorylation at Ser22/Ser23, relative to cells infected with AdV:EGFP; notably, in these cells with heterologous PKD expression, ET1 induced a marked and significant increase in cTnI phosphorylation at Ser22/Ser23, and this response measured approximately 60% of that induced by ISO (Figure 2). Heterologous PKD expression did not significantly affect the cTnI phosphorylation response to ISO. These data indicate that the increase in ET1-induced cTnI phosphorylation that is seen in ARVMs infected with AdV:PKDwt/EGFP is of a magnitude that is likely to have a significant functional impact.

We next explored whether heterologous expression of PKD affects the phosphorylation status of other cellular proteins, particularly those that are known to mediate myocardial responses to PKA activation. Initially, we performed immunoblot analysis with a phospho-PKA substrate antibody that recognizes specifically the phospho-motif ArgXaaXaaSer/Thr that is common to many PKA target sites. As shown in Figure 3A, in control cells infected with AdV:EGFP, exposure to ISO induced phosphorylation of proteins migrating at ≈30, ≈25, ≈15, and <10 kDa, whereas ET1 stimulation induced phosphorylation only of the proteins migrating at ≈30 and ≈15 kDa. Importantly, both ISO and ET1 also induced qualitatively and quantitatively similar responses in ARVMs infected with AdV:PKDwt/EGFP (Figure 3A). The phosphoproteins migrating at ≈25, ≈15, and <10 kDa were tentatively identified as the phospholamban (PLB) pentamer, phospholemman (PLM), and the PLB monomer, respectively, on the basis of the molecular masses of the proteins and known phosphorylation sites; protein identities were subsequently confirmed through the use of specific antibodies that recognize pSer16 PLB and pSer68 PLM (data not shown).

The pattern of phosphorylation displayed by the ≈30-kDa protein detected by the phospho-PKA substrate antibody (Figure 3A) differed markedly from that noted with the pSer22/Ser23 cTnI antibody (Figure 2), raising the possibility that the phospho-PKA substrate antibody might recognize a different phosphoprotein with a similar molecular mass to cTnI or distinct phosphorylated epitope(s) within cTnI. We therefore determined the affinity of the relevant antibodies for recombinant cTnI, in wild-type form or with replacement of one or both of the Ser22/Ser23 residues with nonphosphorylatable Ala (ie, Ser22Ala, Ser23Ala, Ser22/23Ala). The data confirmed the specificity of the pSer22/Ser23 antibody for wild-type cTnI phosphorylated at both of the pertinent sites (Figure 3B). In contrast, the phospho-PKA substrate antibody exhibited affinity for PKA-targeted pSer/Thr sites other than the dually phosphorylated pSer22/Ser23 motif (because wild-type, Ser22Ala, Ser23Ala, and Ser22/23Ala cTnI each showed enhanced detection following PKA phosphorylation). The phospho-PKA substrate antibody also appeared to recognize monophosphorylated pSer22, because Ser23Ala cTnI showed enhanced detection following PKD or PKA phosphorylation (Figure 3B); this moiety is unlikely to exist in nature, because of the ordered occupation of the pertinent phospho-acceptor sites in wild-type cTnI.[22]

We have shown previously that βAR stimulation does not activate PKD in NRVMs,[11] and a similar finding in ARVMs would explain the lack of effect of heterologous PKD expression on ISO-induced responses in the present study (Figures 2 and 3A). To address this issue, we examined the dual phosphorylation of the PKD activation loop at Ser744/Ser748 in response to ISO and ET1. As shown in Figure 3C, in ARVMs with heterologous PKD expression, robust PKD phosphorylation at Ser744/Ser748 was detected in response to ET1 but not to ISO, confirming that βAR stimulation does not activate PKD in this cell type.

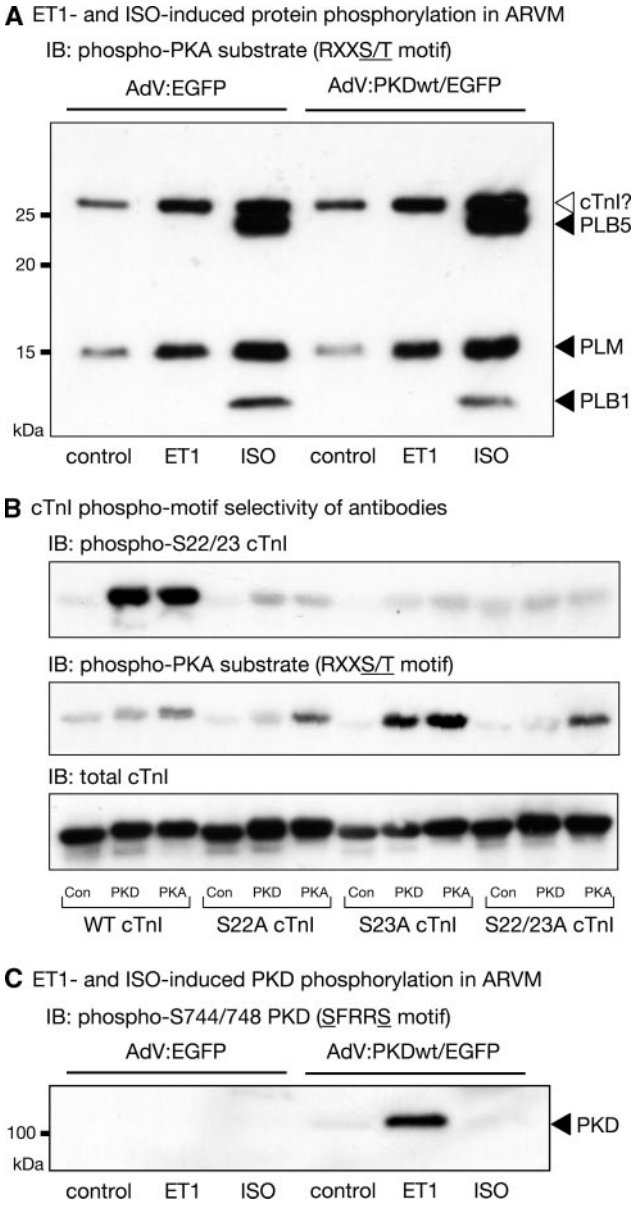


Figure 3. A, Protein phosphorylation profile detected by the phospho-PKA substrate antibody. ARVMs were infected with AdV:EGFP or AdV:PKDwt/EGFP at a moi of 50 PFU/cell and exposed to ET1 (5 nmol/L) or ISO (10 nmol/L) for 10 minutes. PLB5 indicates phospholamban pentamer; PLB1, phospholamban monomer. Immunoblot is representative of 5 independent experiments. B, Selectivity of phospho-PKA substrate and phospho-cTnI (pSer22/Ser23) antibodies for recombinant wild-type (WT) or mutated (Ser22Ala, Ser23Ala, Ser22/23Ala) cTnI following in vitro phosphorylation by the PKA catalytic subunit or recombinant PKD catalytic domain. Protein loading is illustrated by the total cTnI content of samples. Immunoblots (IB) are representative of 2 independent experiments. C, Protein phosphorylation profile detected by the phospho-PKD (pSer744/Ser748) antibody. ARVMs were infected with AdV:EGFP or AdV:PKDwt/EGFP at a moi of 50 PFU/cell and exposed to ET1 (5 nmol/L) or ISO (10 nmol/L) for 10 minutes. Immunoblot is representative of 4 independent experiments.

Our previous work has suggested that myofilament proteins other than cTnI, such as cMyBP-C, may also be substrates for PKD.[13] We therefore prepared myofilament protein samples from ARVMs following the pertinent interventions and explored their phosphorylation status by multiple approaches. Immunoblot analysis of myofilament protein samples prepared from ARVMs that had been infected with AdV:EGFP or AdV:PKDwt/EGFP and exposed to ISO or ET1 revealed a pattern of cTnI dual phosphorylation resembling that seen in whole-cell extracts (Figure 4A, top panel versus Figure 2), confirming that myofilament phosphorylation was not disrupted during sample preparation. For a global assessment of protein phosphorylation, myofilament protein samples were separated by SDS-PAGE and stained using the phosphoprotein stain ProQ Diamond.[17] Counterstaining for total protein was performed using SYPRO Ruby. Two protein bands migrating at 150 and 30 kDa (marked with arrows in Figure 4A, bottom) showed increased phosphorylation on exposure to ET1 or ISO. Heterologous PKD expression had little effect on phosphorylation of the upper band but markedly enhanced ET1-induced phosphorylation of the lower band (Figure 4A, bottom). Thus, ET1 had no significant effect on the phosphorylation status of the 30-kDa protein in control cells infected with AdV:EGFP, but significantly increased this (by an average of 17.4±0.9%) in cells infected with AdV:PKDwt/EGFP. Both the 150- and the 30-kDa bands were excised, subjected to in-gel tryptic digestion and analyzed by tandem mass spectrometry. Tandem mass spectrometric spectra were searched against the Swiss–Prot database using the Sequest algorithm, which identified the 150- and 30-kDa proteins as cMyBP-C and cTnI, respectively (see the online data supplement). In complementary experiments, we also explored cMyBP-C phosphorylation by immunoblot analysis of myofilament protein samples, using a phospho-specific antibody that recognizes cMyBP-C phosphorylated at Ser282.[23] This analysis revealed an ISO-induced increase in cMyBP-C phosphorylation, regardless of the cellular PKD expression level (Figure 4B). Furthermore, there was only a small increase in cMyBP-C phosphorylation in response to ET1 and this effect was unaltered by heterologous PKD expression (Figure 4B).

Taken together, the complementary series of experiments described above indicate that increased PKD activity in intact ARVMs has a remarkably selective impact on cTnI phosphorylation at Ser22/Ser23.

ET1- and ISO-Induced Protein Phosphorylation in NRVMs

We have shown previously that NRVMs exhibit greater PKD expression than ARVMs[11]; we therefore explored whether native PKD might mediate an increase in cTnI phosphorylation in NRVMs, in response to ET1. In these cells, ET1 induced a marked increase in cTnI phosphorylation at Ser22/Ser23, with a greater increase observed in response to ISO (Figure 5A). A marked increase in PLB phosphorylation also occurred in response to ISO, but not ET1, whereas PLM phosphorylation was high in the basal state and increased only slightly in response to either agonist (Figure 5A). To explore the role of native PKD in the ET1-induced response, we constructed an adenoviral vector to downregulate PKD expression by RNA interference (RNAi). Adenoviral expression of short-hairpin RNA (shRNA) targeted at rat PKD1 achieved selective downregulation of PKD protein expression, which declined to ≈15% by 48 hours after infection, without affecting the expression of classical or novel PKC

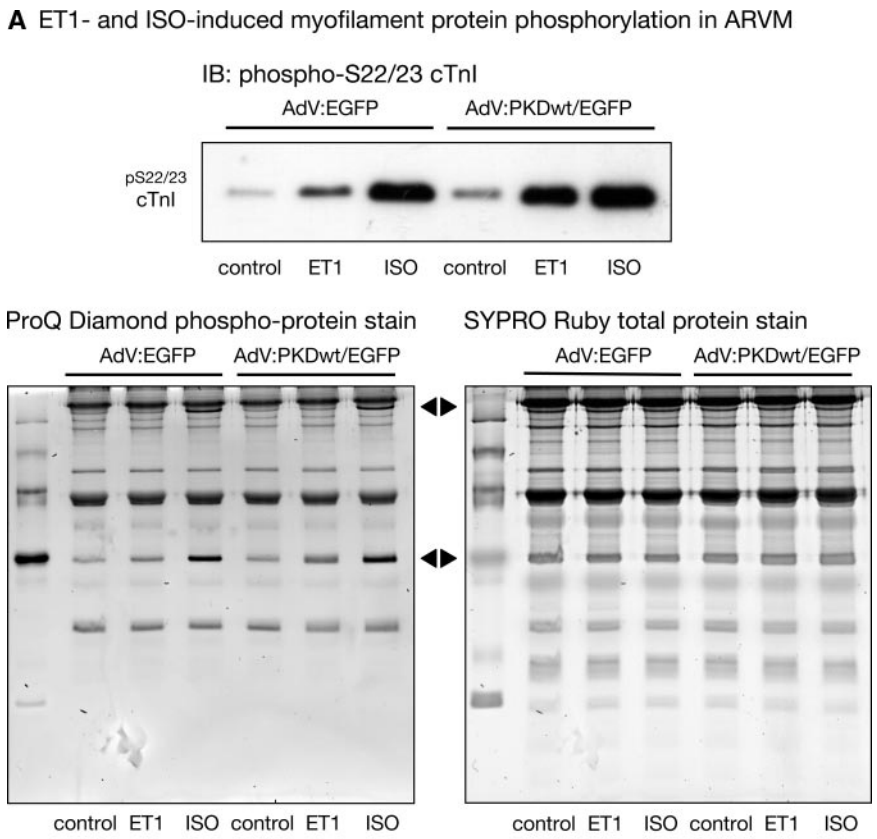
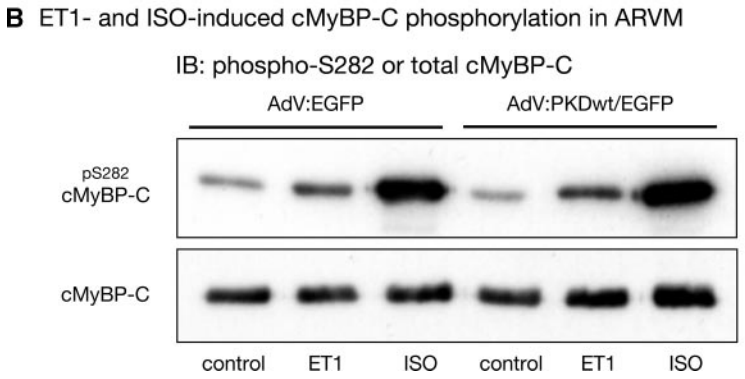


Figure 4. cTnI and cMyBP-C phosphorylation in ARVMs infected with AdV:EGFP or AdV:PKDwt/EGFP at a moi of 50 PFU/cell and exposed to ET1 (5 nmol/L) or ISO (10 nmol/L) for 10 minutes. Protein phosphorylation was assessed by immunoblot (IB) analysis using antibodies against phospho-cTnI (pSer22/Ser23) (A, top) or phospho-cMyBP-C (pSer282) (B), or by phosphoprotein staining with ProQ Diamond, with subsequent total protein staining with SYPRO Ruby to confirm equal loading (A, bottom). The images are representative of 4 independent experiments.



isoforms (Figure 5B). The ISO-induced increase in cTnI phosphorylation was unaffected by PKD downregulation (Figure 5C), which is consistent with the inability of βAR stimulation to activate PKD in NRVMs.[11] In contrast, PKD downregulation significantly attenuated ET1-induced cTnI phosphorylation (Figure 5C), indicating that this response occurred predominantly through native PKD activity.

Effect of Heterologous PKD Expression on the $[Ca^{2+}]_i$ Transient and Sarcomere Shortening

To determine the impact of heterologous PKD expression on the fundamental components of the myocardial excitation–contraction coupling process, we monitored the $[Ca^{2+}]_i$ transient and sarcomere shortening in single, electrically stimulated ARVMs (which were selected on the basis of EGFP fluorescence) before and after exposure to ISO or ET1. Figure 6 illustrates typical responses in control cells infected with AdV:EGFP. As expected, ISO induced a rapid and large increase in sarcomere shortening (twitch amplitude) and accelerated myocyte relaxation, with these positive inotropic and lusitropic effects accompanied by similarly rapid and large increases in $[Ca^{2+}]_i$ transient amplitude and its rate of decay (Figure 6A). ISO also produced a small reduction in diastolic sarcomere length, which declined from 1.83 ± 0.01 to 1.79 ± 0.01 μm over 10 minutes ($n=13$, $P<0.05$). Relative to ISO, ET1 induced qualitatively similar but slower and markedly smaller effects on twitch amplitude and relaxation and $[Ca^{2+}]_i$ transient amplitude but a negligible effect on the rate of decay of the latter (Figure 6B). ET1 did not affect diastolic sarcomere length significantly (1.83 ± 0.01 μm at baseline and 1.82 ± 0.01 μm after 10 minutes, $n=11$). Figure 7 shows quantitative data on the functional effects of ISO and ET1 in ARVMs infected with AdV:EGFP or AdV:PKDwt/EGFP. ISO induced significant changes of similar magnitude

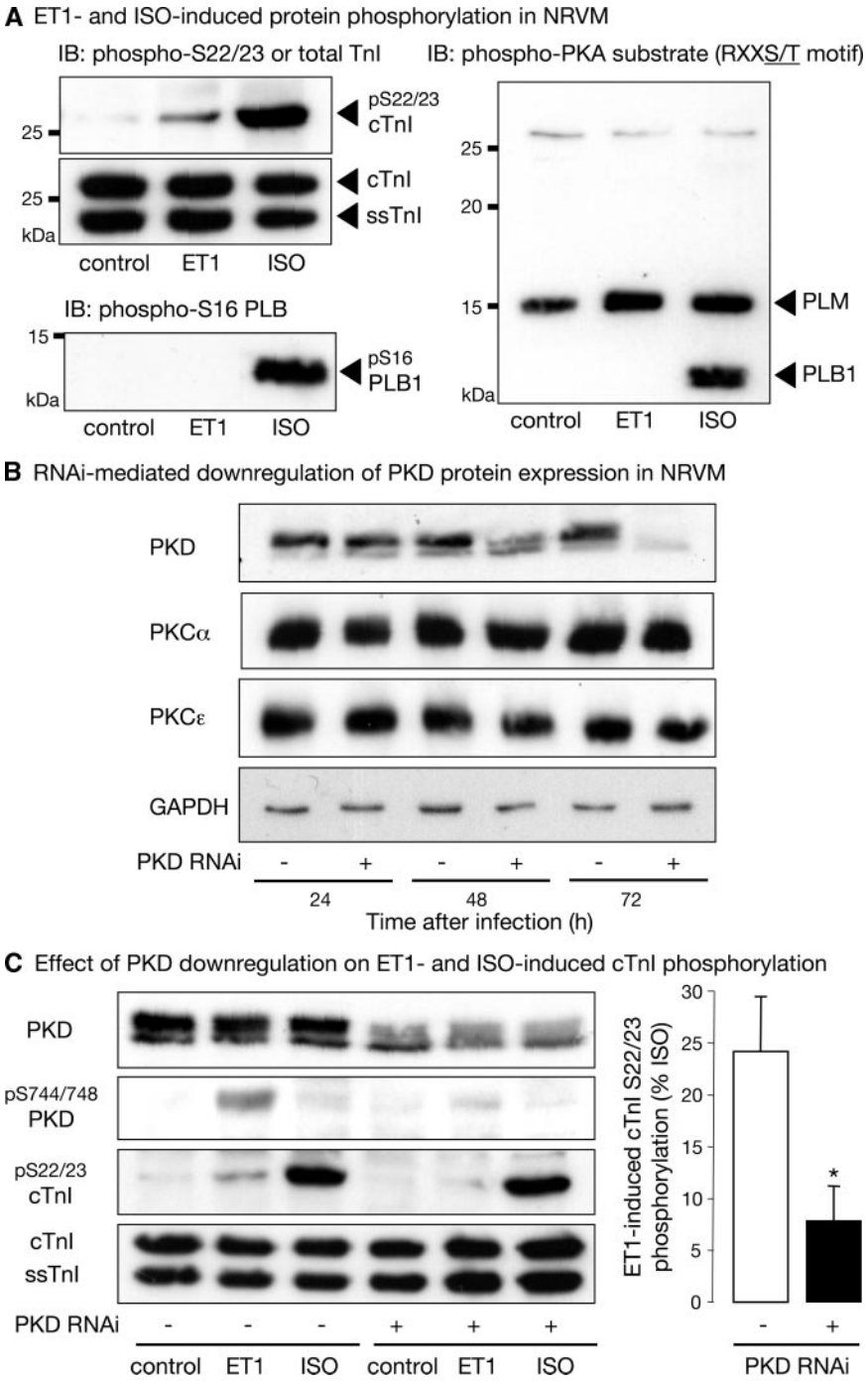


Figure 5. A, Protein phosphorylation in NRVMs exposed to ET1 (5 nmol/L) or ISO (10 nmol/L) for 10 minutes. Protein phosphorylation was assessed by immunoblot (IB) analysis using phospho-cTnI (pSer22/Ser23), phospho-PLB (pSer16), or phospho-PKA substrate antibodies, as indicated. B, Selective PKD downregulation in NRVMs by RNAi, through adenoviral shRNA expression. PKD, PKCα, PKCε, and GAPDH protein expression was determined by immunoblot analysis 24 to 72 hours after infection with adenovirus encoding either EGFP shRNA (−) or PKD shRNA (+). C, Impact of PKD downregulation in NRVMs on cTnI phosphorylation following exposure to ET1 (5 nmol/L) or ISO (10 nmol/L) for 10 minutes. Protein expression and phosphorylation were assessed by immunoblot analysis 48 to 72 hours after infection with adenovirus encoding either EGFP shRNA (−) or PKD shRNA (+). The quantitative data are from 6 independent experiments. *$P<0.05$ vs EGFP shRNA.

in sarcomere shortening and relaxation rate (Figure 7A) and in $[Ca^{2+}]_i$ transient amplitude and its rate of decay (Figure 7B) in both groups of ARVMs, regardless of the cellular PKD content. In contrast, whereas ET1 induced a significant increase in sarcomere shortening in control cells infected with AdV:EGFP, this positive inotropic response was abolished in cells infected with AdV:PKDwt/EGFP (Figure 7A). This difference in the inotropic response to ET1 occurred in the absence of any significant difference in ET1-induced increases in myocyte relaxation rate (Figure 7A) and, notably, $[Ca^{2+}]_i$ transient amplitude (Figure 7B). These data suggest that, in intact ARVMs, heterologously expressed PKD reduces myofilament Ca^{2+} sensitivity on ET1-induced activation. Furthermore, heterologous PKD expression appears to have no nonspecific effects on excitation–contraction coupling or its modulation by neurohormonal stimuli that do not activate PKD, because there was no significant difference in ISO-induced changes in twitch and $[Ca^{2+}]_i$ transient dynamics between ARVMs infected with AdV:EGFP and those infected with AdV:PKDwt/EGFP.

Effect of Heterologous PKD Expression on Myofilament Ca^{2+} Sensitivity

To determine the impact of increased cellular PKD activity on myofilament Ca^{2+} sensitivity directly, we measured sar-

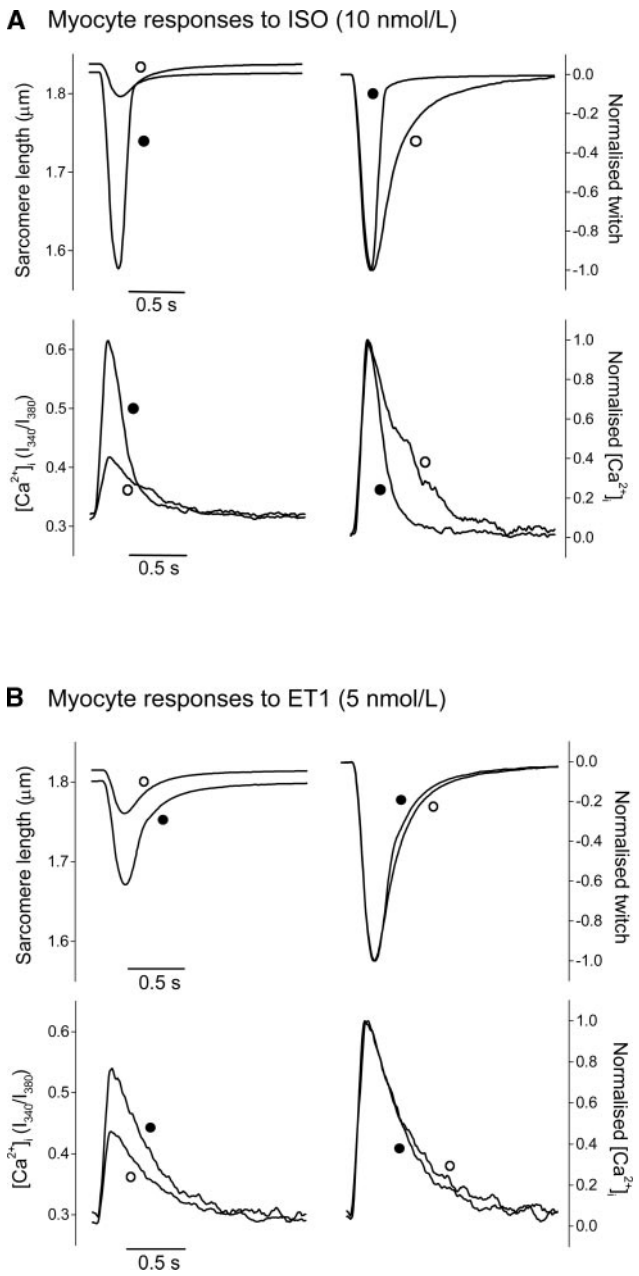


Figure 6. Illustrative changes in sarcomere shortening/relaxation and $[Ca^{2+}]_i$ transients in intact ARVMs infected with AdV:EGFP at a moi of 50 PFU/cell, during exposure to ISO (10 nmol/L) (A) or ET1 (5 nmol/L) (B). Left panels show representative signal-averaged twitches and $[Ca^{2+}]_i$ transients in absolute units; right panels, signal-averaged twitches and $[Ca^{2+}]_i$ transients normalized relative to the peak (to highlight differences in temporal characteristics). The signal-averaged data were obtained just before (open circles) and 10 minutes after (solid circles) the start of exposure to ISO or ET1.

comere shortening in skinned ARVMs during exposure to various activating $[Ca^{2+}]$. In cells infected with AdV:EGFP or AdV:PKDwt/EGFP and skinned immediately after exposure to ISO or ET1, the pattern of cTnI dual phosphorylation resembled that seen in intact ARVMs (Figure 8A versus Figure 2), again confirming that the chemical permeabilization protocol used did not disrupt myofilament protein phosphorylation. As expected, stepwise increases in superfusate $[Ca^{2+}]$ induced progressively greater reductions in the sarcomere length of skinned myocytes (Figure 8B), allowing the direct assessment of myofilament Ca^{2+} sensitivity. Figure 8C illustrates the pertinent quantitative data, in the form of sarcomere length *v* pCa relationships. ISO induced a significant rightward shift of the sarcomere length *v* pCa relationship, indicating a reduced myofilament Ca^{2+} sensitivity,[19] with no apparent difference in the ISO-induced response between cells infected with AdV:EGFP and those infected with AdV:PKDwt/EGFP (Figure 8C, top panel). In cells infected with AdV:EGFP, ET1 induced no significant change in the sarcomere length *v* pCa relationship, which is consistent with previous findings in skinned myocyte preparations (see the online data supplement). In contrast, in cells with heterologous PKD expression, exposure to ET1 induced a significant rightward shift of the sarcomere length *v* pCa relationship, indicating reduced myofilament Ca^{2+} sensitivity (Figure 8C, bottom). These data provide direct evidence that the ET1-induced increase in cellular PKD activity in ARVMs with heterologous PKD expression leads to reduced myofilament Ca^{2+} sensitivity, in parallel with increased cTnI phosphorylation.

Discussion

It is well established that cTnI phosphorylation is an important mechanism in the acute regulation of sarcomeric thin filament function and thereby myocardial contraction, particularly in response to neurohormonal stimuli.[24] Furthermore, sustained alterations in the phosphorylation of cTnI and other myofilament proteins are likely to contribute causally to cardiac dysfunction in the transition from compensated hypertrophy to heart failure.[25] Although most investigative effort in phosphorylation-mediated regulation of cTnI function has to date focused on the actions of PKA and PKC,[24] other signaling mechanisms are also likely to be involved.[26] In this context, we have recently reported that PKD interacts with and can directly phosphorylate in vitro a number of myofilament proteins, targeting cTnI specifically at Ser22/Ser23.[13] We have also shown that exposure of skinned myocytes to the constitutively active PKD catalytic domain induces cTnI phosphorylation at Ser22/Ser23 and reduces the Ca^{2+} sensitivity of tension development.[13] In the present investigation, we determined the role of full-length PKD, with functional regulatory domains that determine enzyme activity and localization in response to a variety of (patho) physiologically relevant stimuli,[3] as a regulator of cTnI phosphorylation and myofilament Ca^{2+} sensitivity in intact ventricular myocytes. Our new data provide further support for the hypothesis that PKD-mediated phosphorylation of cTnI (and possibly other PKD substrates with phosphorylation that is not detectable by the techniques used here) represents a novel signaling mechanism in the regulation of myocyte contractile function, principally through altered myofilament Ca^{2+} sensitivity.

In our earlier work in skinned myocytes exposed to the constitutively-active PKD catalytic domain,[13] PKD-mediated myofilament phosphorylation accelerated isometric cross-bridge cycle kinetics, in addition to reducing myofilament Ca^{2+} sensitivity. In mouse ventricular muscle,

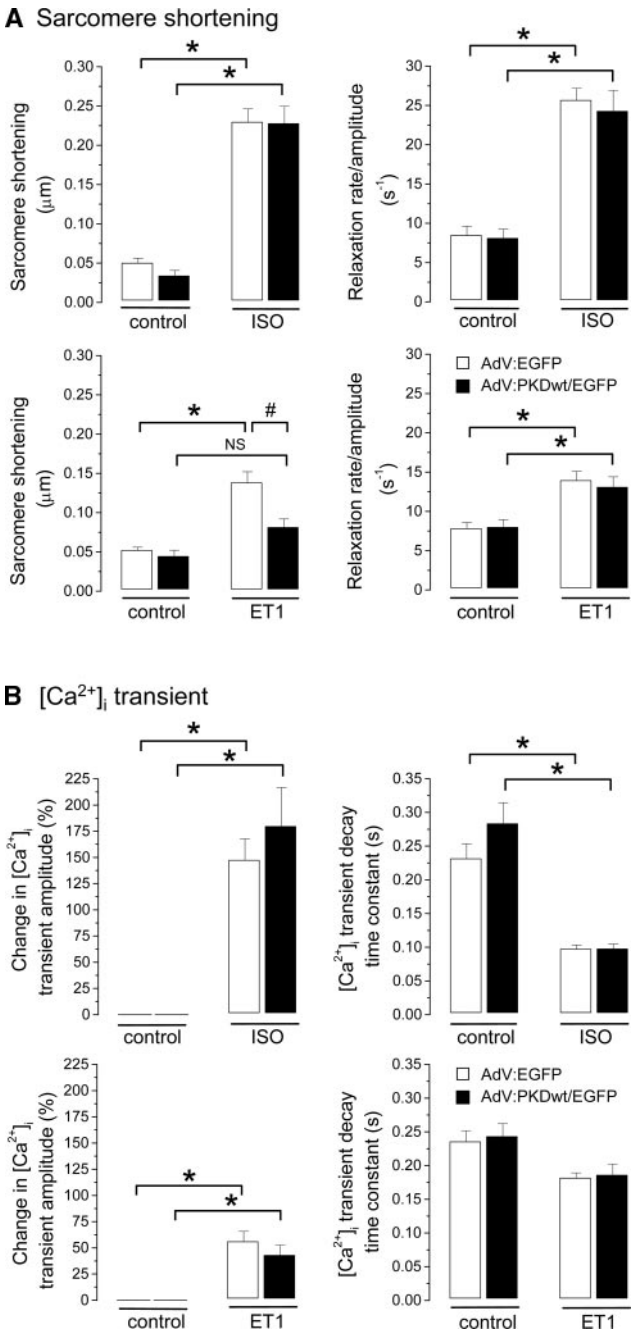


Figure 7. Quantitative data on changes in sarcomere shortening/relaxation (A) and $[Ca^{2+}]_i$ transients (B) in ARVMs infected with AdV:EGFP (open bars) or AdV:PKDwt/EGFP (solid bars) at a moi of 50 PFU/cell, before (control) and after a 10-minute exposure to ISO (10 nmol/L) or ET1 (5 nmol/L). A, left panels, Sarcomere shortening (diastolic minus systolic sarcomere length). A, right panels, Twitch relaxation rate normalized to twitch amplitude. B, left panels, Change in $[Ca^{2+}]_i$ transient amplitude (systolic minus diastolic fluorescence signal). B, right panels, $[Ca^{2+}]_i$ transient decay time constant. *$P<0.05$ vs control group infected with same adenovirus; #$P<0.05$ vs corresponding group infected with AdV:EGFP (n=10 to 13 cells per group).

cTnI phosphorylation within its unique N-terminal domain (which contains Ser22/Ser23) has been causally associated with PKA-mediated acceleration of cross-bridge cycle kinetics and thereby myofibrillar relaxation rate.[27] On this basis, any PKD-mediated acceleration of cross-bridge cycle kinetics in intact ARVMs might be expected to enhance twitch relaxation during ET1 stimulation of cells with heterologous PKD expression. In the present study, we found that the relaxation rate of control ARVMs infected with AdV:EGFP was accelerated by ET1 stimulation (Figure 7A), which is consistent with an earlier report in a similar cell system.[28] However, heterologous PKD expression had no effect on this response (Figure 7A), despite significantly increased cellular PKD activity (Figure 1) and cTnI phosphorylation (Figure 2). A likely explanation for this observation is that, even in control ARVMs without heterologous PKD expression, ET1 increases the cross-bridge cycle rate to such an extent that the relaxation rate becomes limited by the rate of decay of the $[Ca^{2+}]_i$ transient. Thus, in the absence of any effect on the rate of decay of the $[Ca^{2+}]_i$ transient (Figure 7B), a further increase in the cross-bridge cycle rate arising from increased PKD activity does not elicit an additional increase in the relaxation rate.

The present work provides strong evidence that the principal impact of increased cellular PKD activity on the fundamental excitation–contraction coupling process in adult ventricular myocardium is a reduction in myofilament Ca^{2+} sensitivity. The concomitant increase that occurs in cTnI phosphorylation at Ser22/Ser23 is likely to be a causal mechanism underlying this response, on the basis of the known impact of such phosphorylation on myofilament Ca^{2+} sensitivity.[24] In this regard, the phosphorylation status of cMyBP-C, another putative PKD target[13] with phosphorylation that has been suggested to contribute to the PKA-mediated reduction in myofilament Ca^{2+} sensitivity,[29] was unaltered by increased cellular PKD activity (Figure 4). Indeed, the protein phosphorylation fingerprint of increased cellular PKD activity seems to be quite distinct from that of increased cellular PKA activity, with no apparent effect of the former on the phosphorylation status of cMyBP-C, PLB, or PLM. Thus, the functional consequences of the activation of these pathways would be expected to be different, as indeed was the case in the present study. Nevertheless, our observations in unloaded ARVMs require validation in more complex systems, because loading conditions are likely to influence the functional impact of cTnI phosphorylation.[30]

We have shown previously that PKD expression is developmentally regulated, such that fetal and neonatal ventricular myocardium have a greater PKD content relative to adult ventricular myocardium.[11] Consistent with this, our present data indicate that native PKD in NRVMs mediates the increase in cTnI phosphorylation that arises from ET1 stimulation (Figure 5). With regard to the potential role of native PKD in adult myocardium, recent observations raise the possibility that PKD-mediated myocardial regulatory mechanisms may assume greater significance in pathological settings. In this context, Harrison et al have recently reported a significant increase in PKD expression and activity in the myocardium of spontaneously hypertensive heart failure rats, with such myocardial PKD activation further exaggerated by thoracic aortic

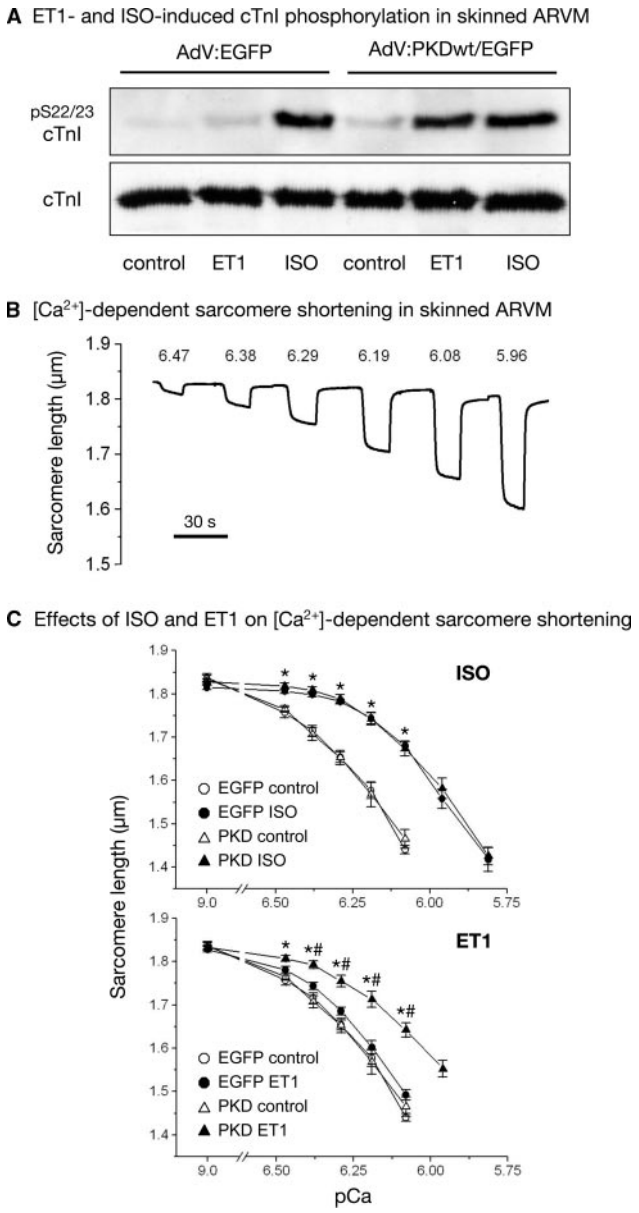


Figure 8. cTnI phosphorylation at Ser22/Ser23 and myofilament Ca^{2+} sensitivity in ARVMs infected with AdV:EGFP or AdV:PKDwt/EGFP at a moi of 50 PFU/cell and "skinned" following a 10-minute exposure to ISO (10 nmol/L) or ET1 (5 nmol/L). A, cTnI expression and cTnI phosphorylation at Ser22/Ser23. Immunoblots are representative of 5 independent experiments. B, Illustrative recording of sarcomere length during transient exposures of a skinned ARVMs to progressively greater activating $[Ca^{2+}]$. Numbers above the trace indicate activating solution pCa; during the interspersed intervals, superfusion was with relaxing solution. C, Sarcomere length *v* pCa relationships in ARVMs infected with AdV:EGFP (circles) or AdV:PKDwt/EGFP (triangles) and exposed to vehicle control (open symbols) or ISO or ET1 (solid symbols), as indicated. *$P<0.05$ vs control group infected with same adenovirus; #$P<0.05$ vs corresponding group infected with AdV:EGFP (n=12 to 15 cells per group).

banding.[31] There is now also preliminary evidence of increased PKD expression and activity in failing human and rabbit hearts.[32] The recent observations that cardiac-specific expression of a constitutively active PKD mutant in transgenic mice leads to ventricular chamber dilation and wall thinning and a marked deterioration of contractile function[31] suggest that increased PKD activity might be a causal, rather than merely a coincidental factor in adverse myocardial remodeling. Although the molecular mechanisms of PKD-mediated effects on myocyte and chamber remodeling are likely to involve HDAC phosphorylation and nuclear export,[12,31–33] our data suggest that direct effects on myofilament protein phosphorylation and Ca^{2+} sensitivity might contribute to contractile dysfunction. Of direct relevance to our work, Pieske et al have shown an attenuated inotropic response to ET1 in failing human myocardium,[34] which might arise from an increased PKD activity in this setting.[32] Indeed, in the present study, we found that the positive inotropic effect of ET1 was abolished by heterologous PKD expression (Figure 7A). In view of the evolving concepts regarding sarcomeric regulatory mechanisms in the context of heart failure,[35] PKD appears to be well positioned, both structurally[36] and functionally,[13] to play an important modulatory role.

In conclusion, the present work and other recent reports in the literature suggest that PKD may be a novel regulator of myocardial structure and function, most likely through its effects on the transcriptional regulation of key growth-regulatory genes (as suggested by the pioneering work of Olson and McKinsey and colleagues[12,31]) and/or by direct effects on myofilament proteins (as suggested by our previous[13] and current work). The myriad of proposed actions of PKD in fundamental cellular processes[3,9] suggest that developmental considerations might limit the viability and utility of conventional global gene-targeting approaches in deciphering the roles of native PKD in healthy and stressed myocardium. Such progress now requires the development of more sophisticated genetic models and selective pharmacological agents targeted at this hitherto underexplored myocardial signaling pathway.

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Disclosures

None.

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